Industria y Comercio
SUPERINTENDENCIA

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
No. 14-187582- -00004-0000
Fecha: 2015-03-10 16:30:11 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 457 PROCEDIPPH Folios: 209

DIRECCIÓN DE NUEVAS CREACIONES
Procedimiento acelerado de exámenes de patente (PPH)

1	**NÚMERO DE SOLICITUD ANTE LA SIC**	**14 187582**
2	**OFICINA DE PRIMERA PRESENTACIÓN (OPP)**	**ESTADOS UNIDOS**
3	**NÚMERO(S) DE SOLICITUD O PUBLICACIÓN DE LA(S) SOLICITUD(ES) (OPP):**	1. 13/752,199 2. 3.

4 ☐ **REPRESENTANTE LEGAL** ✕ **APODERADO**

APELLIDOS	NOMBRES	IDENTIFICACIÓN
CASTELLANOS ABONDANO	**MARGARITA**	**C.C . 39'779.654 T.P . 70819**

DIRECCIÓN	**CALLE 94A No. 7A-09**	No. TELÉFONO	**7560770**
CIUDAD	**BOGOTÁ**	CORREO ELECTRÓNICO	**sic@castellanosyco.co**
PAÍS	**COLOMBIA**	No. RADICACIÓN O PROTOCOLO DE PODER GENERAL	

5 CONDICIONES PARA ACCEDER A ESTE PROCESO:

5.1. DATOS DE PRIORIDAD ✕ SI ☐ NO

(33) PAÍS DE ORIGEN	CÓDIGO PAÍS	(31) NÚMERO	(32) FECHA (AAAA/MM/DD)
1. **ESTADOS UNIDOS**	US	61/593,106	2012/01/31
2. **ESTADOS UNIDOS**	US	61/672,679	2012/07/17
3. **ESTADOS UNIDOS**	US	61/713,734	2012/10/15

5.2. DATOS SOLICITUD INTERNACIONAL PCT ✕SI ☐ NO

Solicitud Internacional No.	PCT/US2013/023409	**Fecha**	28/01/2013
Publicación Internacional No.	WO2013/116144	**Fecha**	08/08/2013

El solicitante presenta una copia de la(s) reivindicación(es) patentable(s) u otorgable(s) de la solicitud de la Oficina de Primera Presentación que profirió el examen de patentabilidad, junto con una traducción al castellano. ✕ SI ☐ NO

REQUISITOS PARA EL RECONOCIMIENTO DE LOS RESULTADOS DE EXÁMENES DE PATENTABILIDAD EFECTUADOS A SOLICITUDES EXTRANJERAS

Para que la Superintendencia de Industria y Comercio pueda reconocer los resultados de exámenes de patentabilidad producidos por otras Oficinas de Propiedad Industrial se debe cumplir con las siguientes condiciones:

(a) Al momento de solicitar el examen de patentabilidad, si el solicitante pide que se reconozcan exámenes de patentabilidad de otras oficinas extranjeras, deberá aportar junto con el pago de la tasa correspondiente, los siguientes anexos:

(i) Copia de las resoluciones, fallos o decisiones que han sido relevantes para la determinación de la patentabilidad de la(s) solicitud(es) en la Oficina que haya proferido el examen de patentabilidad que contienen reivindicaciones patentables u otorgables que son la base de la petición, y si es del caso, su traducción al castellano. ✕ SI ☐ NO

(ii) Copia de todas las reivindicaciones determinadas como patentables u otorgables en la Oficina que profirió el examen de patentabilidad y, si es del caso, su traducción al castellano. ✕ SI ☐ NO

(iii) Copia de los antecedentes, información o documentos citados por el examinador de la Oficina de Primera Presentación del examen de patentabilidad, en las resoluciones, fallos o decisiones correspondientes, incluyendo la literatura no patente. Los documentos de patente deberán presentarse siempre y cuando no estén disponibles para la Superintendencia de Industria y Comercio mediante el simple acceso a bases de datos gratuitas. ✕ SI ☐ NO

(iv) Una tabla de correspondencias de las reivindicaciones que han sido determinadas como patentables u otorgables en la solicitud de la Oficina que haya proferido el examen de patentabilidad. ✕ SI

7 FIRMA DEL SOLICITANTE, DEL APODERADO O DEL REPRESENTANTE LEGAL
Junto a cada firma, indicar el nombre del firmante y su calidad (si tal calidad no es obvia al leer el petitorio)

10 MAR. 2015

6	TABLA DE CORRESPONDENCIA DE REIVINDICACIONES	
Reivindicaciones en la solicitud en Colombia	Reivindicaciones otorgables en la solicitud internacional	Explicación sobre la suficiente correspondencia ó Comentarios que explican la correspondencia
1.	1.	Se presenta según la reivindicación otorgada para Estados Unidos.
2.	2.	Se presenta según la reivindicación otorgada para Estados Unidos.
3.	3.	Se presenta según la reivindicación otorgada para Estados Unidos.
4.	4.	Se presenta según la reivindicación otorgada para Estados Unidos.
5.	5.	Se presenta según la reivindicación otorgada para Estados Unidos.
6.	6.	Se presenta según la reivindicación otorgada para Estados Unidos.
7.	7.	Se presenta según la reivindicación otorgada para Estados Unidos.
8.	8.	Se presenta según la reivindicación otorgada para Estados Unidos.
9.	9.	Se presenta según la reivindicación otorgada para Estados Unidos.
10.	10.	Se presenta según la reivindicación otorgada para Estados Unidos.
11.	11.	Se presenta según la reivindicación otorgada para Estados Unidos.
12.	12.	Se presenta según la reivindicación otorgada para Estados Unidos.
13.	13.	Se presenta según la reivindicación otorgada para Estados Unidos.
14.	14.	Se presenta según la reivindicación otorgada para Estados Unidos.
15.	15.	Se presenta según la reivindicación otorgada para Estados Unidos.
16.	16.	Se presenta según la reivindicación otorgada para Estados Unidos.
17.	17.	Se presenta según la reivindicación otorgada para Estados Unidos.
18.	18.	Se presenta según la reivindicación otorgada para Estados Unidos.
19.	19.	Se presenta según la reivindicación otorgada para Estados Unidos.
20.	20.	Se presenta según la reivindicación otorgada para Estados Unidos.
21.	21.	Se presenta según la reivindicación otorgada para Estados Unidos.
22.	22.	Se presenta según la reivindicación otorgada para Estados Unidos.

B
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UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

101365 7590 12/26/2013
Finnegan/Genomic Health
901 New York Avenue, NW
Washington, DC 20001

EXAMINER	
BRUSCA, JOHN S	
ART UNIT	PAPER NUMBER
1631	

DATE MAILED: 12/26/2013

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/752,199	01/28/2013	Steve Shak	09042.0008-00000	1702

TITLE OF INVENTION: Gene Expression Profile Algorithm and Test for Determining Prognosis of Prostate Cancer

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1780	$300	$0	$2080	03/26/2014

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

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PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), to: Mail **Mail Stop ISSUE FEE**
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
or Fax (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

101365 7590 12/26/2013
Finnegan/Genomic Health
901 New York Avenue, NW
Washington, DC 20001

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission
I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/752,199	01/28/2013	Steve Shak	09042.0008-00000	1702

TITLE OF INVENTION: Gene Expression Profile Algorithm and Test for Determining Prognosis of Prostate Cancer

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	$1780	$300	$0	$2080	03/26/2014

EXAMINER	ART UNIT	CLASS-SUBCLASS
BRUSCA, JOHN S	1631	702-020000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).
☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.
1 _____
2 _____
3

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☐ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:
☐ Issue Fee
☐ Publication Fee (No small entity discount permitted)
☐ Advance Order - # of Copies _____

4b. Payment of Fee(s): **(Please first reapply any previously paid issue fee shown above)**
☐ A check is enclosed.
☐ Payment by credit card. Form PTO-2038 is attached.
☐ The Director is hereby authorized to charge the required fee(s), any deficiency, or credits any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)
☐ Applicant certifying micro entity status. See 37 CFR 1.29
NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
☐ Applicant asserting small entity status. See 37 CFR 1.27
NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
☐ Applicant changing to regular undiscounted fee status.
NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____

PTOL-85 Part B (10-13) Approved for use through 10/31/2013. OMB 0651-0033 U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/752,199	01/28/2013	Steve Shak	09042.0008-00000	1702

101365 7590 12/26/2013
Finnegan/Genomic Health
901 New York Avenue, NW
Washington, DC 20001

EXAMINER	
BRUSCA, JOHN S	

ART UNIT	PAPER NUMBER
1631	

DATE MAILED: 12/26/2013

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)
(application filed on or after May 29, 2000)

The Patent Term Adjustment to date is 0 day(s). If the issue fee is paid on the date that is three months after the mailing date of this notice and the patent issues on the Tuesday before the date that is 28 weeks (six and a half months) after the mailing date of this notice, the Patent Term Adjustment will be 0 day(s).

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (http://pair.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notices of Allowance and Fee(s) Due mailed between October 1, 2013 and December 31, 2013

(Addendum to PTOL-85)

If the "Notice of Allowance and Fee(s) Due" has a mailing date on or after October 1, 2013 and before January 1, 2014, the following information is applicable to this application.

If the issue fee is being timely paid on or after January 1, 2014, the amount due is the issue fee and publication fee in effect January 1, 2014. On January 1, 2014, the issue fees set forth in 37 CFR 1.18 decrease significantly and the publication fee set forth in 37 CFR 1.18(d)(1) decreases to $0.

If an issue fee or publication fee has been previously paid in this application, applicant is not entitled to a refund of the difference between the amount paid and the amount in effect on January 1, 2014.

Notice of Allowability	Application No. 13/752,199	Applicant(s) SHAK ET AL.	
	Examiner JOHN S. BRUSCA	Art Unit 1631	AIA (First Inventor to File) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *the amendment received 07 October 2013*.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on_____.
2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.
3. ☒ The allowed claim(s) is/are *21-42*. As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov .
4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   Certified copies:

   a) ☐ All b) ☐ Some *c) ☐ None of the:
   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).
6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)
2. ☒ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date See Continuation Sheet
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____ .
5. ☒ Examiner's Amendment/Comment
6. ☐ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/JOHN S BRUSCA/
Primary Examiner, Art Unit 1631

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Continuation Sheet (PTOL-37) **Application No. 13/752,199**

Continuation of Attachment(s) 2. Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date: 06/20/2013, 10/07/2013.

The present application is being examined under the pre-AIA first to invent provisions.

EXAMINER'S AMENDMENT

1. An examiner's amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 CFR 1.312. To ensure consideration of such an amendment, it MUST be submitted no later than the payment of the issue fee.

Authorization for this examiner's amendment was given in a telephone interview with Kelly McClellan on 11 December 2013.

The application has been amended as follows:

In claim 21, line 3, the term "determining" has been deleted and --measuring-- has been substituted therefor.

2. Any inquiry concerning this communication or earlier communications from the examiner should be directed to JOHN S. BRUSCA whose telephone number is (571)272-0714. The examiner can normally be reached on M-F 8:30 AM - 5:00 PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Marjorie A. Moran can be reached on 571-272-0720. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/JOHN S BRUSCA/
Primary Examiner, Art Unit 1631

jsb

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OFICINA DE PATENTES Y MARCAS REGISTRADAS DE ESTADOS UNIDOS

DEPARTAMENTO DE COMERCIO DE ESTADOS UNIDOS
Oficina de Patentes y Marcas Registradas de Estados Unidos
Dirección: COMISIONADO DE PATENTES
P.O. Box 1450
Box 1450, Alexandria, Virginia 22313-1450 -
www.uspto.gov

NOTIFICACIÓN DE APROBACIÓN Y TARIFAS POR PAGAR

101365 7590
26/12/2013
Finnegan/Genomic Health
901 New York Avenue, NW
Washington, DC 20001

EVALUADOR	
BRUSCA, JOHN S	
UNIDAD DE ARTE	DOCUMENTO
NÚMERO 1631	

FECHA DE ENVÍO 26/12/2013

SOLICITUD No.	FECHA DE SOLICITUD	NOMBRE DEL PRIMER INVENTOR	Código de abogado No.:	CONFIRMACIÓN No.
131752,199	28/01/2013	Steve Shak	09042.0008-00000	1702

TÍTULO DE LA INVENCIÓN: Algoritmo para Perfil de Expresión Genética y Prueba para Determinar el Pronóstico del Cáncer de Próstata

TIPO SOLICITUD	ESTATUS ENTIDAD	EXPEDICIÓN	PUBLICACIÓN	PAGADA	TOTAL	VENCE
no provisional	SIN DESCUENTO	$1780	$300	$0	$2080	03/26/2014

LA SOLICITUD REFERENCIADA ARRIBA HA SIDO EVALUADA Y SE AUTORIZA SU EXPEDICIÓN COMO PATENTE. EL JUZGAMIENTO DE LOS MÉRITOS HA CONCLUIDO. ESTA NOTICIA DE APROBACIÓN NO ES UNA CONCESIÓN DE DERECHOS DE PATENTE. ESTA SOLICITUD ESTÁ SUJETA A SER RETIRADA POR INICIATIVA DEL FUNCIONARIO O PETICIÓN DEL SOLICITANTE. VER 37 CFR 1.313 Y MPEP 1308.

LAS TARIFAS DE EXPEDICIÓN Y PUBLICACIÓN (SI SE REQUIRIERAN) DEBEN PAGARSE EN LOS TRES MESES SIGUIENTES A PARTIR DE LA FECHA EN EL MATASELLOS DE ESTA NOTIFICACIÓN O ESTA SOLICITUD SE CONSIDERARÁ COMO ABANDONADA. ESTE PERIODO ESTATUTARIO NO PUEDE SER EXTENDIDO. VER 35 U.S.C. 151. LA TARIFA DE EXPEDICIÓN INDICADA NO REFLEJA NINGÚN CRÉDITO POR TARIFAS DE EXPEDICIÓN PREVIAMENTE PAGADAS PARA ESTA SOLICITUD. SI UNA TARIFA DE EXPEDICIÓN HUBIERA SIDO PREVIAMENTE PAGADA PARA ESTA SOLICITUD (COMO SE MUESTRA A CONTINUACIÓN), EL RETORNO DE LA PARTE B DE ESTE FORMULARIO SERÁ CONSIDERADO COMO UNA SOLICITUD PARA QUE LA TARIFA DE EXPEDICIÓN PREVIAMENTE PAGADA SEA APLICADA A LA TARIFA DE EXPEDICIÓN ACTUAL.

CÓMO RESPONDER A ESTA NOTIFICACIÓN

I. Revise el ESTATUS DE LA ENTIDAD que se muestra abajo. Si el ESTATUS DE LA ENTIDAD aparece como PEQUEÑO o MICRO, verifique si el derecho a dicho estatus de entidad está todavía vigente.

Si el ESTATUS DE LA ENTIDAD es el mismo indicado arriba, pague el TOTAL DE LAS TARIFAS que se indica continuación.

Si el ESTATUS DE LA ENTIDAD ha cambiado con respecto al que se muestra arriba, en la PARTE B – TARIFA(S) DE TRANSMISIÓN, complete la sección número 5 titulada "Cambio en el Estatus de la Entidad (con respecto al estatus indicado arriba)".

Para la finalidad de esta noticia, las tarifas para entidades pequeñas son 1/2 de las tarifas sin descontar, y las tarifas de las entidades micro son 1/2 de las tarifas para entidades pequeñas.

II. La PARTE B – TARIFA(S) DE TRANSMISIÓN, o su equivalente, debe ser diligenciada y devuelta a la Oficina de Patentes y Marcas Registradas de Estados Unidos con su TARIFA DE EXPEDICIÓN y su TARIFA DE PUBLICACIÓN (si se requiere). Si usted está autorizando el pago de las tarifas desde su cuenta de ahorros, debe diligenciar la sección "4b" o Parte B - Tarifas de transmisión y debe enviar una copia adicional del formulario. Si se presenta un equivalente de la Parte B, se debe hacer la expresa solicitud de reaplicar una tarifa pagada con anterioridad y pueden ocurrir demoras en el proceso debido a la dificultad de reconocer el documento como un equivalente de la Parte B.

III. Todas las comunicaciones referentes a esta solicitud deben dar el número de la solicitud. Por favor dirija todas las comunicaciones previas a la expedición a la parada del correo TARIFA DE EXPEDICIÓN, a menos que se le indique lo contrario.

PARA TENER EN CUENTA: Patentes de utilidad expedidas o solicitudes presentadas antes de diciembre 12 de 1980 pueden requerir el pago de tarifas de mantenimiento. Es la responsabilidad del beneficiario de la patente garantizar el pago oportuno de las tarifas de mantenimiento.

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PARTE B – TARIFAS DE TRANSMISIÓN

Diligencie y envíe este formulario junto con las tarifas aplicables a: Correo: Parada del correo TARIFA DE EXPEDICIÓN Comisionado para Patentes, P.O. Box 1450, Box 1450, Alexandria, Virginia 22313-1450

o Fax: (571) -273-2885

INSTRUCCIONES: Este formulario debe usarse para transmitir la TARIFA DE EXPEDICIÓN y la TARIFA DE PUBLICACIÓN (si se requiere). Los bloques 1 al 5 debe diligenciarse cuando sea apropiado. Toda la correspondencia adicional, incluyendo la Patente, órdenes de avance y notificación y las tarifas de mantenimiento, debe ser enviada por correo a la dirección de correspondencia indicada a menos que se haga en ella alguna corrección o se dé una instrucción diferente en el Bloque 1, mediante (a) las especificación de una nueva dirección de correspondencia; y/o (b) la indicación de una "DIRECCIÓN DE TARIFA" diferente para las notificaciones de tarifa de mantenimiento.

Nota: Un certificado de envío por correo puede ser usado únicamente para envíos nacionales de las Tarifas de Transmisión. Este certificado no puede usarse para ningún otro documento adjunto. Todos los documentos adicionales, tales como una cesión o un plano técnico, deben tener su propio certificado de envío por correo o transmisión.

DIRECCIÓN DE CORRESPONDENCIA VIGENTE adjunta (Nota: Use el Bloque 1 para el cambio de dirección)

101365 7590 26/12/2013
Finnegan/Genomic Health
901 New York Avenue, NW
Washington, DC 20001

Certificado de envío por correo o transmisión

Por la presente certifico que esta Tarifa de Transmisión está siendo depositada en el Servicio Postal de Estados Unidos con importe pagado suficiente para envío por correo de primera clase en un sobre dirigido a la parada de correo TARIFA DE EXPEDICIÓN, cuya dirección aparece arriba, o es un facsímil transmitido a la Oficina de Patentes y Marcas Registradas de Estados Unidos (571) 273-2885, en la fecha indicada abajo.

(Nombre del depositante)
(Firma)
(Fecha)

SOLICITUD No.	FECHA DE SOLICITUD	NOMBRE DEL PRIMER INVENTOR	Código de abogado No.:	CONFIRMACIÓN No.
131752,199	28/01/2013	Steve Shak	09042,0008-00000	1702

TÍTULO DE LA INVENCIÓN: Algoritmo para Perfil de Expresión Genética y Prueba para Determinar el Pronóstico del Cáncer de Próstata

TIPO SOLICITUD	ESTATUS ENTIDAD	EXPEDICIÓN	PUBLICACIÓN	PAGADA	TOTAL	VENCE
no provisional	SIN DESCUENTO	$1780	$300	$0	$2080	26/03/2014
EVALUADOR		UNIDAD DE ARTE	CLASE-SUBCLASE			
BRUSCA, JOHN S		1631	702-020000			

1. Cambio de dirección de correspondencia o indicación de "Dirección de Tarifa" (37 CFR 1.363).
__ Cambio de dirección de correspondencia (o formulario de cambio de dirección de correspondencia PTO/SB/122) adjunto.
__ Indicación de "Tarifa de Dirección" (o indicación de "Tarifa de dirección" formulario PTO/SB/47; Rev 03-02 o más reciente) adjunto. Se requiere el uso de un Número de Cliente.

2. Para imprimir en la página de portada de la patente, enumere
(1) Los nombres de hasta 3 abogados o agentes de patentes registrados o, como alternativa,
(2) El nombre de una firma (que tenga como miembro un abogado o agente registrado) y los nombres de hasta 2 abogados o agentes de patentes

3. NOMBRE Y RESIDENCIA DEL CESIONARIO A IMPRIMIRSE EN LA PATENTE (use letra de molde o escriba a máquina)

FAVOR TENER EN CUENTA: A menos que el cesionario se identifique a continuación, en la patente no aparecerán datos del cesionario. Si se identifica un cesionario a continuación, el documento se ha presentado con el propósito de ser registrado, como está provisto en 7 CFR 3.11. El diligenciamiento de esta forma NO sustituye el diligenciamiento de una cesión.

(A) NOMBRE DEL CESIONARIO

(B) RESIDENCIA: (CIUDAD y ESTADO O PAÍS)

Por favor marque la categoría o categorías de cesionario adecuadas (no se imprimirán en la patente): __ Individuo __ Corporación u otro grupo privado __ Gobierno

4a. Las siguientes tarifas están pagas:
__ Tarifa de expedición
__ Tarifa de publicación (No se permite el descuento para entidades pequeñas)
__ Pre orden - # de copias

4b. Pago de las tarifas: (Por favor aplique primero cualquier tarifa de expedición pagada previamente)
__ Se adjunta un cheque
__ Pago con tarjeta de crédito. Se adjunta el formulario PTO-2038
__ Por la presente se autoriza al Director a debitar las tarifas requeridas o cualquier faltante, o a acreditar cualquier pago en exceso, en la cuenta de ahorros (adjunte una copia extra de este formulario).

5. Cambio en el estatus de la entidad (con respecto al estatus indicado arriba)
__ Certificación de estatus de entidad Micro por parte del solicitante Ver 37 CFR 1.29.
__ El solicitante declara estatus de entidad pequeña Ver 37 CFR 1.27.
__ El solicitante cambia a estatus de tarifa normal sin descuento.

NOTA: En ausencia de una certificación válida de Estatus de entidad micro (ver los formularios PTO/SB/15A y 15B), el pago de la tarifa de expedición por la cantidad para entidades micro no será aceptado a riesgo de abandonar la solicitud.
NOTA: Si la solicitud fue hecha previamente bajo el estatus de entidad micro, marcar esta caja será tomado como una notificación de pérdida del derecho a estatus de entidad micro.
NOTA: Marcar esta caja se tomará como una notificación de pérdida del derecho a estatus de entidad pequeña o micro, según sea aplicable.

NOTA: Este formulario debe estar firmado de acuerdo con 37 CFR 1.31 y 1.33. Ver 37 CFR 1.4 para los requerimientos y certificaciones de firma.

Firma autorizada _____ Fecha _____

Nombre impreso o mecanografiado _____ Registro No. _____

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OFICINA DE PATENTES Y MARCAS REGISTRADAS DE ESTADOS UNIDOS

DEPARTAMENTO DE COMERCIO DE ESTADOS UNIDOS
Oficina de Patentes y Marcas Registradas de Estados Unidos
Dirección: COMISIONADO DE PATENTES
P.O. Box 1450
Box 1450, Alexandria, Virginia 22313-1450 -
WWW.USPTO.GOV

SOLICITUD No.	FECHA DE SOLICITUD	NOMBRE DEL PRIMER INVENTOR	CÓDIGO DE ABOGADO NO.:	CONFIRMACIÓN No.
131752,199	28/01/2013	Steve Shak	09042.0008-00000	1702

EVALUADOR
BRUSCA, JOHN S

UNIDAD DE ARTE	DOCUMENTO
NÚMERO 1631	

101365 7590 26/12/2013

Finnegan/Genomic Health 901 New York Avenue, NW Washington, DC 20001

FECHA DE ENVÍO 26/12/2013

Determinación de ajuste de término de patente bajo el U.S.C 154 (b)
(solicitud presentada antes de o el 29 de mayo de 2000)

El ajuste de término en la patente a la fecha es de 0 días. Si la tarifa de expedición se paga tres meses después de la fecha del matasellos de esta notificación y la patente se expide el martes antes de transcurridas las 28 semanas (seis meses y medio) después de la fecha de envío de esta notificación el ajuste de término de la patente será de 0 días.

Si se presentó una Solicitud de Juzgamiento Continuado (CPA, por sus siglas en inglés) con la solicitud arriba identificada, la fecha de presentación que determina el ajuste del término de la patente es la fecha de presentación del CPA más reciente.

El solicitante puede obtener información más detallada si accede al sitio web sistema de Información de solicitudes de patente (PAIR, por sus siglas en inglés) http://pair.uspto.gov

Cualquier pregunta referente a Extensión del término de patente debe enviarse directamente a la Oficina de Administración Legal de Patentes en el tel. (571)-272-7702. Las preguntas relacionadas con los pagos de las tarifas de expedición y publicación deben dirigirse al Centro de Servicio al Cliente de la Oficina de Publicación de Patentes en los tel. 1-(888)-786-0101 o (571)-272-4200.

Aprobación de la Oficina de Administración y Presupuesto (OMB) y Declaración de carga probatoria bajo la Ley de reducción de papeleo para PTOL-85 Part B

La Ley de reducción de papeleo (PRA) de 1995 requiere que las agencias federales obtengan la aprobación de la Oficina de Administración y Presupuesto (OMB) previo a hacer casi cualquier requerimiento de información a la ciudadanía. Cuando la OMB aprueba la solicitud de una agencia para recolectar información de la ciudadanía, la OMB (i) otorga un número OMB de control y la fecha de expiración del documento usado para recolectar la información y (ii) requiere que la agencia informe a la ciudadanía acerca de la importancia legal del número de control OMB, de acuerdo con 5 CFR 1320.5(b).

La información recolectada en PTOL-85 Part B se requiere para 37 CFR 1.311. La información se requiere para obtener o retener un beneficio para el público que vaya a presentar (y para el proceso de la Oficina de Patentes de Estados Unidos) una solicitud. La confidencialidad está regida por 35 U.S.C. 122 y 37 CFR 1.14. Se estima que esta recolección de información requiere de 2 horas para el diligenciamiento, preparación y envío del formulario de solicitud completo a la Oficina de Patentes de Estados Unidos El tiempo variará dependiendo de cada caso individual. Cualquier comentario con respecto al tiempo requerido por usted para llenar este formulario y/o sugerencias para reducir esta exigencia debe enviarse al Director de Información, Oficina de Patentes y Marcas Registradas de los Estados Unidos, Departamento de Comercio de Estados Unidos, P.O Box 1450, ALEXANDRIA, Virginia 22313-1450. NO ENVÍE PAGOS DE TARIFAS NI FORMULARIOS DILIGENCIADOS A ESTA DIRECCIÓN. ENVÍELOS A: Comisionado de Patentes, P.O Box
1450, ALEXANDRIA, Virginia 22313-1450. Bajo la Ley de reducción de papeleo de 1995, ninguna persona está obligada a responder a una solicitud de información a menos que esta exhiba un número OMB de control válido.

Declaración bajo la Ley de privacidad

La Ley de privacidad de 1974 (P.L. 93-579) requiere que usted proporciones cierta información adicional a la forma adjunta relacionada con la solicitud de patente o patente. De acuerdo con lo anterior, en cumplimiento de las disposiciones de la Ley, por favor tenga en cuenta que: (1) la regulación que rige la recolección de esta información es 35 U.S.C. 2(b)(2); (2) proporcionar la información es voluntario; y (3) la finalidad principal para la que se usa la información en la Oficina de Patentes y Marcas Registradas de Estados Unidos es procesar y/o examinar su solicitud relacionada con una solicitud de patente o patente. Si usted no proporciona la información solicitada, es posible que la Oficina de Patentes y Marcas Registradas de Estados Unidos no pueda procesar y/o examinar su solicitud, lo que puede causar la terminación de los trámites, el abandono de la solicitud o el vencimiento de la patente.

La información que usted proporcione en este formulario estará sujeta a los siguientes usos de rutina:

1. La información en este formulario será tratada confidencialmente hasta donde lo permitan la Ley de libertad de información (5 U.S.C. 552) y la Ley de privacidad (5 U.S.C 552a). Los registros en este sistema pueden ser divulgados al Departamento de Justicia para determinar si la divulgación de estos registros se requiere bajo la Ley de libertad de información.
2. Un registro de este sistema puede divulgarse en el proceso de presentar evidencia a una corte, magistrado o tribunal administrativo, incluyendo la divulgación al abogado de la parte contraria en el transcurso de negociaciones de conciliación.
3. Un registro de este sistema puede divulgarse, como un uso rutinario, a un miembro del Congreso que haga un requerimiento en relación con un individuo que aparezca en el registro, cuando el individuo haya solicitado ayuda del miembro con respecto al asunto del registro.
4. Un registro de este sistema puede divulgarse, como un uso rutinario, a un contratista de la agencia que necesite la información con el fin de ejecutar un contrato. Quienes reciban la información estarán obligados a cumplir con los requisitos de la Ley de privacidad de 1974, como se enmendó, en cumplimiento de 5 U.S.C. 552a(m).
5. Un registro en este sistema en relación con una Solicitud internacional presentada bajo el Tratado de Cooperación en materia de Patentes puede divulgarse, como un uso rutinario, al Comité Internacional de la Organización Mundial de Propiedad Intelectual, en cumplimiento del Tratado de Cooperación en materia de Patentes.
6. Un registro de este sistema puede divulgarse, como un uso rutinario, a otra agencia federal con la finalidad de revisión de seguridad nacional (35 U.S.C. 181) para revisión en cumplimiento de la Ley de energía atómica (42 U.S.C. 218(c)).
7. Un registro de este sistema puede divulgarse, como un uso rutinario, al administrador, Servicios Generales, o su designado, durante una inspección de registros llevada a cabo por la Administración de Servicios Generales (GSA) como parte de las responsabilidades de la agencia para recomendar mejoras en el manejo, prácticas y programas de registro, por la autoridad que le confieren 4 U.S.C. 2904 y 2906. Tal divulgación deberá hacerse de acuerdo con las regulaciones de la GSA pertinentes a la inspección de registros con este propósito, y de cualquier otra regulación relevante (ej. del Departamento de Comercio o GSA). Tal divulgación no será utilizada para tomar determinaciones con respecto a individuos.
8. Un registro de este sistema puede divulgarse, como un uso rutinario, a la ciudadanía después de la publicación de la solicitud en cumplimiento con 35 U.S.C. 122(b) o la expedición de una patente en cumplimiento de 35 U.S.C. 151. Adicionalmente, un registro de este sistema puede divulgarse, con sujeción a 37 CFR 1.14, como un uso rutinario, a la ciudadanía si el registro fue incluido en una solicitud que se abandonó o cuyo tramité se finalizó y dicha solicitud es referenciada en cualquiera, una solicitud publicada o una solicitud abierta a inspección pública o una patente expedida.
9. Un registro de este sistema puede divulgarse a un ente acusador federal, estatal o local, si la Oficina de Patentes y Marcas Registradas de Estados Unidos se entera de una violación o una violación potencial de la ley o las regulaciones.

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Notificación de aprobación y tarifas por pagar enviada por correo entre Octubre 1 de 2013 y Diciembre 31 de 2013

(Anexo a PTOL-85)

Si la fecha de envío por correo de la Noticia de aprobación y tarifas por pagar es octubre 1 de 2013 o posterior, y es antes de enero 1 de 2014, la siguiente información se aplica a esta solicitud.

Si la tarifa de expedición se paga oportunamente en enero 1 de 2014 o después, la cantidad a pagar es la tarifa de expedición y la tarifa de publicación vigentes en enero 1 de 2014. En enero 1 de 2014, las tarifas de expedición establecidas en 37 CFR 1.18 disminuyen significativamente y la tarifa de publicación establecida en 37 CFR 1.18(d)(l) disminuye a $0.

Si una tarifa de expedición o publicación ha sido pagada con anterioridad para esta solicitud, el solicitante no recibirá un reembolso de la diferencia entre la cantidad pagada y la cantidad en efecto en enero 1 de 2014.

Notificación de aptitud para la aprobación	Solicitud No. 13/752,199	Solicitantes SHAK ET	
	Evaluador JOHN S. BRUSCA	Unidad de Arte 1631	Estatus AIA (Primer inventor en presentarse) N-

-- La FECHA DE ENVÍO de esta comunicación aparece en la hoja de portada con la dirección correspondiente.

Siendo todas las reivindicaciones aprobables, EL JUZGAMIENTO DE LOS MÉRITOS ESTÁ (O PERMANECE) CERRADO para esta solicitud. Si no se incluye con la presente (o se ha enviado previamente), una Noticia de Aprobación (PTOL-85) u otra comunicación adecuada se enviará por correo oportunamente. ESTA NOTICIA DE APROBACIÓN NO ES UNA CONCESIÓN DE DERECHOS DE PATENTE. ESTA SOLICITUD ESTÁ SUJETA A SER RETIRADA POR INICIATIVA DEL FUNCIONARIO O PETICIÓN DEL SOLICITANTE. Ver 37 CFR 1.313 y MPEP 1308.

1. X Esta comunicación responde a *las modificaciones recibidas el 7 de octubre de 2013.*

   __ Una declaración/declaración extrajuicio bajo 37 CFR 1.130(b) fue presentada el

2. __ El solicitante eligió en respuesta a un requerimiento de restricción establecido durante la entrevista el ; el requerimiento de restricción y la escogencia han sido incorporados en esta decisión.

3. X Las reivindicaciones permitidas son *21-42.* Como resultado de las reivindicaciones permitidas, usted puede ser elegible para beneficiarse del programa Procedimiento Acelerado de Juzgamiento de Patentes en una oficina de propiedad intelectual participante con la correspondiente solicitud. Para más información, por favor ver 1tl;Q://v1r Nw.us2to.gov/gatents/init_e/ents/Qgh/indexjs.Q o envíe sus preguntas aPPHfeedback@uS(Qto.aov .

4. __ Se admite una reivindicación para prioridad en el extranjero bajo 35 U.S.C. § 119(a)-(d) o (f).

   Copias autenticadas:

   a) __ Todas b) Algunas *c) __ Ninguna de las:

   1. __ Copias autenticadas de los documentos de prioridad han sido recibidas.
   2. __ Copias autenticadas de los documentos de prioridad han sido recibidas en la Solicitud No.
   3. __ Se han recibido copias de las copias autenticadas de los documentos de prioridad en esta solicitud en fase nacional enviadas por el Comité Internacional (PCT Rule 17.2(a)).

   * No se han recibido copias autenticadas:

El solicitante dispone de TRES MESES A PARTIR DE LA FECHA DE ENVÍO de esta notificación para presentar una respuesta que cumpla con los requisitos mencionados abajo. El no cumplir oportunamente con este requisito resultará en el ABANDONO de esta solicitud. ESTE PERIODO DE TRES MESES NO ES PRORROGABLE.

5. __ Deben enviarse ILUSTRACIONES CORREGIDAS (en la forma de "hojas de remplazo").

   __ Incluir cambios requeridos en el Comentario o Modificación del Evaluador o en la Decisión de la Oficina del Documento No./Fecha de envío

Elementos para la identificación, tales como el número de solicitud (ver 37 CFR 1.84(c)) deben escribirse en el frente de los dibujos (no en el respaldo) en cada hoja. Las hojas de reemplazo deben titularse de este modo en el encabezado de acuerdo con 37 CFR 1.121(d).

6. __ Debe enviarse el DEPÓSITO DE y/o INFORMACIÓN acerca del depósito de MATERIAL BIOLÓGICO. Note el comentario adjunto del examinador con respecto al REQUERIMIENTO PARA EL DEPÓSITO DE MATERIAL BIOLÓGICO.

Adendas

1. __ Notificación de referencias citadas (PT0-892)
2. X Declaraciones de divulgación de información (PTO/SB/08), la aprobación Documento No./Fecha de envío Ver Hoja de continuación
3. __ Comentario del examinador con respecto al requerimiento de depósito de material biológico
4. __ Resumen de la entrevista (PT0-413), Documento No./Fecha de envío
5. X /Modificaciones/Comentario del examinador
6. __ Declaraciones del examinador sobre las razones de
7. __ Otro

/JOHN S. BRUSCA/
Evaluador primario, Unidad de Arte 1631

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Continuación de Adendas 2. Declaraciones de divulgación de información (PTO/SB/08), Documento No./Fecha de envío: 20/06/2013, 07/10/2013.

La presente solicitud se examina bajo las provisiones anteriores a la Ley América inventa.

MODIFICACIONES DEL EXAMINADOR

1. A continuación se presenta una modificación del examinador al registro. En caso de que los cambios y/o adiciones sean inaceptables para el solicitante, puede presentarse una modificación tal como se establece en 37 CFR

1.312. Para asegurar que dicha modificación sea considerada, DEBE ser enviada antes o al momento de pagar la tarifa de expedición.

La autorización para las modificaciones de este evaluador se dio durante una entrevista telefónica con Kelly McClellan el 11 de diciembre de 2013.

La solicitud ha sido enmendada así:

En la reivindicación 21, línea 3, el término "determinación" ha sido eliminado y se ha sustituido con --medición-- de ahí en adelante.

2. Cualquier consulta en relación con esta u comunicaciones anteriores del evaluador debe dirigirse a JOHN S. BRUSCA, cuyo número de teléfono es (571) 272- 0714. El evaluador por lo general se encuentra disponible de lunes a viernes de 8:30 am a 5:00 pm.

Si los intentos de comunicarse con el evaluador por teléfono son infructuosos, la supervisora del evaluador, Marjorie A. Moran, puede localizarse en el teléfono 571-272-0720. El número de fax de la organización donde se asignó esta solicitud o trámite es 571-273-8300.

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/JOHN S BRUSCA/
Evaluador primario, Unidad de Arte 1631

jsb

Copia Patente otorgada 21



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(12) **United States Patent**
Shak et al.

(10) **Patent No.: US 8,725,426 B2**
(45) **Date of Patent: May 13, 2014**

(54) **GENE EXPRESSION PROFILE ALGORITHM AND TEST FOR DETERMINING PROGNOSIS OF PROSTATE CANCER**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **13/752,199**

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G06F 19/00 (2011.01)
C12Q 1/68 (2006.01)

(52) **U.S. Cl.**
USPC **702/20**; 435/6.14

(58) **Field of Classification Search**
None
See application file for complete search history.

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Primary Examiner - John S Brusca

(74) *Attorney, Agent, or Firm* — Finnegan, Henderson, Farabow, Garrett & Dunner, L.L.P.

(57) **ABSTRACT**

The present invention provides algorithm-based molecular assays that involve measurement of expression levels of genes, or their co-expressed genes, from a biological sample obtained from a prostate cancer patient. The genes may be grouped into functional gene subsets for calculating a quantitative score useful to predict a likelihood of a clinical outcome for a prostate cancer patient.

22 Claims, 7 Drawing Sheets

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Reivindicaciones otorgadas 23

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What is claimed is:

1. A method of predicting a likelihood of a clinical outcome for a patient with prostate cancer, comprising:

(a) measuring levels of RNA transcripts of the following genes: BGN, COL1A1, SFRP4, FLNC, GSN, TPM2, GSTM2, FAM13C, KLK2, AZGP1, SRD5A2, and TPX2, in a biological sample containing cancer cells obtained from the patient;

(b) normalizing the level of the RNA transcripts to obtain normalized gene expression levels;

(c) calculating a quantitative score for the patient, wherein the quantitative score (QS) is calculated as follows:

$QS = 0.735 \times ECM$ (Stromal Response) group score

$-0.368 \times$Migration (Cellular Organization) group score

$-0.352 \times$PSA (Androgen) group score

$+0.095 \times$Proliferation ($TPX2$) score,

wherein

ECM (Stromal Response) group score $= 0.527 \times BGN + 0.457 \times COL1A1 + 0.156 \times SFRP4$

Migration (Cellular Organization) group score=0.163×*FLNC*+0.504×*GSN*+0.421×*TPM2*+0.394×*GSTM2*

PSA (Androgen) group score=0.634×*FAM13C*+1.079×*KLK2*+0.642×*AZGP1*+0.997×*SRD5A2* Threshold score

Proliferation (*TPX2*) score=*TPX2* Threshold score,

wherein

$$SRD5A2 \text{ Threshold Score} = \begin{cases} 5.5 & \text{if } SRD5A2 < 5.5 \\ SRD5A2 & \text{otherwise} \end{cases} \text{ and}$$

$$TPX \text{ Threshold Score} = \begin{cases} 5.0 & \text{if } TPX2 < 5.0 \\ TPX2 & \text{otherwise,} \end{cases} \text{ and}$$

wherein the gene symbols in the equations represent the normalized gene expression levels of the RNA transcripts of the respective genes; and

(d) predicting the likelihood of a clinical outcome for the patient based on the quantitative score.

2. The method of claim **1**, wherein the normalized expression levels of BGN, COL1A1, SFRP4, FLNC, GSN, TPM2, GSTM2, FAM13C, KLK2, AZGP1, SRD5A2, and TPX2 are normalized relative to the expression levels of ARF1, ATP5E, CLTC, GPS1, and PGK1.

3. The method of claim **1**, wherein an increase in the quantitative score correlates with an increased likelihood of a negative clinical outcome.

4. The method of claim **1**, wherein the clinical outcome is upgrading of prostate cancer.

5. The method of claim **1**, wherein the clinical outcome is upstaging of prostate cancer.

6. The method of claim **1**, wherein the clinical outcome is recurrence of prostate cancer.

7. The method of claim **1**, wherein the clinical outcome is non-organ-confined disease.

8. The method of claim **1**, wherein the clinical outcome is adverse pathology.

9. The method of claim **1**, wherein determination of the level of the RNA transcripts includes quantitative polymerase chain reaction.

10. The method of claim **1**, further comprising creating a report summarizing the likelihood of a clinical outcome.

11. The method of claim **1**, wherein the biological sample containing cancer cells is fixed, paraffin-embedded, or fresh, or frozen.

12. The method of claim **1**, further comprising scaling the quantitative score as follows:

Quantitative Score (scaled)

$$\begin{cases} 0 & \text{if } 13.4\times(QS+10.5)<0 \\ 13.4\times(QS+10.5) & \text{if } 0\le 13.4\times(QS+10.5)\le 100 \\ 100 & \text{if } 13.4\times(QS+10.5)>100. \end{cases}$$

13. The method of claim **12**, wherein the normalized expression levels of BGN, COL1A1, SFRP4, FLNC, GSN, TPM2, GSTM2, FAM13C, KLK2, AZGP1, SRD5A2, and TPX2 are normalized relative to the expression levels of ARF1, ATP5E, CLTC, GPS1, and PGK1.

14. The method of claim **12**, wherein an increase in the quantitative score correlates with an increased likelihood of a negative clinical outcome.

15. The method of claim **12**, wherein the clinical outcome is upgrading of prostate cancer.

16. The method of claim **12**, wherein the clinical outcome is upstaging of prostate cancer.

17. The method of claim **12**, wherein the clinical outcome is recurrence of prostate cancer.

18. The method of claim **12**, wherein the clinical outcome is non-organ-confined disease.

19. The method of claim **12**, wherein the clinical outcome is adverse pathology.

20. The method of claim **12**, wherein determination of the level of the RNA transcripts includes quantitative polymerase chain reaction.

21. The method of claim **12**, further comprising creating a report summarizing the likelihood of a clinical outcome.

22. The method of claim **12**, wherein the biological sample containing cancer cells is fixed, paraffin-embedded, or fresh, or frozen.

* * * * *

REIVINDICACIONES OTORGADAS EN ESTADOS UNIDOS

1. A method of predicting a likelihood of a clinical outcome for a patient with prostate cancer, comprising:

(a) measuring levels of RNA transcripts of the following genes: BGN, COL1A1, SFRP4, FLNC, GSN, TPM2, GSTM2, FAM13C, KLK2, AZGP1, SRD5A2, and TPX2, in a biological sample containing cancer cells obtained from the patient;

(b) normalizing the level of the RNA transcripts to obtain normalized gene expression levels;

(c) calculating a quantitative score for the patient, wherein the quantitative score (QS) is calculated as follows:

QS = 0.735 x ECM (Stromal Response) group score
-0.368 x Migration (Cellular Organization) group score
-0.352 x PSA (Androgen) group score
+0.095 x Proliferation (TPX2) score,

wherein

ECM (Stromal Response) group score = 0.527 x BGN + 0.457 x COL1A1 + 0.156 x SFRP4

Migration (Cellular Organization) group score= 0.163 x FLNC + 0.504 x GSN + 0.421 x TPM2 + 0.394 x GSTM2

PSA (Androgen) group score = 0.634 x FAM13C + 1.079 x KLK2 + 0.642 x AZGP1 + 0.997 x SRD5A2 Threshold score

Proliferation (TPX2) score = TPX2 Threshold score,

wherein

$$\text{SRD5A2 Threshold Score} = \begin{cases} 5.5 & \text{if SRD5A2} < 5.5 \\ \text{SRD5A2} & \text{otherwise} \end{cases} \text{ and}$$

$$\text{TPX Threshold Score} = \begin{cases} 5.0 & \text{if TPX2} < 5.0 \\ \text{TPX2} & \text{otherwise} \end{cases}, \text{ and}$$

wherein the gene symbols in the equations represent the normalized gene expression levels of the RNA transcripts of the respective genes; and

(d) predicting the likelihood of a clinical outcome for the patient based on the quantitative score.

2. The method of claim 1, wherein the normalized expression levels of BGN, COL1A1, SFRP4, FLNC, GSN, TPM2, GSTM2, FAM13C, KLK2, AZGP1, SRD5A2, and TPX2 are normalized relative to the expression levels of ARF1, ATP5E, CLTC, GPS1, and PGK1.

3. The method of claim 1, wherein an increase in the quantitative score correlates with an increased likelihood of a negative clinical outcome.

4. The method of claim 1, wherein the clinical outcome is upgrading of prostate cancer.

5. The method of claim 1, wherein the clinical outcome is upstaging of prostate cancer.

6. The method of claim 1, wherein the clinical outcome is recurrence of prostate cancer.

7. The method of claim 1, wherein the clinical outcome is non-organ-confined disease.

8. The method of claim 1, wherein the clinical outcome is adverse pathology.

9. The method of claim 1, wherein determination of the level of the RNA transcripts includes quantitative polymerase chain reaction.

10. The method of claim 1, further comprising creating a report summarizing the likelihood of a clinical outcome.

11. The method of claim 1, wherein the biological sample containing cancer cells is fixed, paraffin-embedded, or fresh, or frozen.

12. The method of claim 1, further comprising scaling the quantitative score as follows:

$$\text{Quantitative Score (scaled)} \begin{cases} 0 & \text{if } 13.4 \times (QS+10.5) < 0 \\ 13.4 \times (QS+10.5) & \text{if } 0 \leq 13.4 \times (QS+10.5) \leq \\ 100 & \text{if } 13.4 \times (QS+10.5) > 100. \end{cases}$$

13. The method of claim 12, wherein the normalized expression levels of BGN, COL1A1, SFRP4, FLNC, GSN, TPM2, GSTM2, FAM13C, KLK2, AZGP1, SRD5A2, and TPX2 are normalized relative to the expression levels of ARF1, ATP5E, CLTC, GPS1, and PGK1.

14. The method of claim 12, wherein an increase in the quantitative score correlates with an increased likelihood of a negative clinical outcome.

15. The method of claim 12, wherein the clinical outcome is upgrading of prostate cancer.

16. The method of claim 12, wherein the clinical outcome is upstaging of prostate cancer.

17. The method of claim 12, wherein the clinical outcome is recurrence of prostate cancer.

18. The method of claim 12, wherein the clinical outcome is non-organ-confined disease.

19. The method of claim 12, wherein the clinical outcome is adverse pathology.

20. The method of claim 12, wherein determination of the level of the RNA transcripts includes quantitative polymerase chain reaction.

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21. The method of claim 12, further comprising creating a report summarizing the likelihood of a clinical outcome.

22. The method of claim 12, wherein the biological sample containing cancer cells is fixed, paraffin-embedded, or fresh, or frozen.

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REIVINDICACIONES APROBADAS EN ESTADOS UNIDOS – GHI-052US (13/752,199)

1. Un método para predecir una probabilidad de un resultado clínico para un paciente con cáncer de próstata que comprende:

(a) la medición de los niveles de secuencias de ARN transcritas de los siguientes genes: BGN, COL1A1, SFRP4, FLNC, GSN, TPM2, GSTM2, FAM13C, KLK2, AZGP1, SRD5A2 y TPX2, en una muestra biológica que contiene células cancerosas obtenidas del paciente;

(b) la normalización del nivel de secuencias de ARN transcritas para obtener niveles normalizados de expresión de genes;

(c) el cálculo de un puntaje cuantitativo para el paciente, puntaje cuantitativo (QS) que se calcula así:

QS = 0.735 x Puntaje del grupo ECM (Respuesta estromal)
-0.368 x Puntaje del grupo migración (Organización celular)
-0.352 x Puntaje del grupo PSA (Andrógeno)
+0,095 x Puntaje de proliferación (TPX2),

en donde

Puntaje del grupo ECM (Respuesta estromal) = 0,527 x BGN + 0.457 x COL1A1 + 0.156 x SFRP4

Puntaje del grupo migración (Organización celular) = 0.163 x FLNC + 0.504 x GSN + 0.421 x TPM2 + 0.394 x GSTM2

Puntaje del grupo PSA (Andrógeno) = 0.634 x FAM13C + 1.079 x KLK2 + 0.642 x AZGP1 + 0.997 x Puntaje de umbral SRD5A2

Puntaje de proliferación (TPX2) = Puntaje de umbral TPX2,

en donde

$$\text{Puntaje de umbral SRD5A2} = \begin{cases} 5.5 & \text{si SRD5A2} < 5.5 \\ \text{SRD5A2} & \text{de otro modo} \end{cases} \text{ y}$$

$$\text{Puntaje de umbral TPX} = \begin{cases} 5.0 & \text{si TPX2} < 5.0 \\ \text{TPX2} & \text{de otro modo} \end{cases}, \text{ y}$$

en donde los símbolos de genes en las ecuaciones representan los niveles de expresión genética normalizados de secuencias de ARN transcritas de los respectivos genes; y

(d) la predicción de la probabilidad de un resultado clínico para el paciente basado en el puntaje cuantitativo.

2. El método de la reivindicación 1, en donde los niveles de expresión normalizados de BGN, COL1A1, SFRP4, FLNC, GSN, TPM2, GSTM2, FAM13C, KLK2, AZGP1, SRD5A2 y TPX2 se normalizan con respecto a los niveles de expresión de ARF1, ATP5E, CLTC, GPS1 y PGK1.

3. El método de la reivindicación 1, en donde un incremento en el puntaje cuantitativo se correlaciona con un incremento en la probabilidad de un resultado clínico negativo.

4. El método de la reivindicación 1, en donde el resultado clínico es un acenso del cáncer de próstata,

5. El método de la reivindicación 1, en donde el resultado clínico es un descenso del cáncer de próstata,

6. El método de la reivindicación 1, en donde el resultado clínico es la recurrencia del cáncer de próstata,

7. El método de la reivindicación 1, en donde el resultado clínico es la enfermedad no confinada al órgano.

8. El método de la reivindicación 1, en donde el resultado clínico es una patología adversa.

9. El método de la reivindicación 1, en donde la determinación del nivel de secuencias de ARN transcritas incluye una reacción en cadena de la polimerasa cuantitativa.

10. El método de la reivindicación 1, que además comprende la creación de un reporte que resume la probabilidad de un resultado clínico.

11. El método de la reivindicación 1, en donde la muestra biológica que contiene células cancerosas se fija, se embebe en parafina, o es fresca o congelada.

12. El método de la reivindicación 1, que además comprende la siguiente escala de puntajes cuantitativos:

$$\text{Puntaje cuantitativo (escala)} \begin{cases} 0 & \text{if } 13.4 \times (QS+10.5) < 0 \\ 13.4 \times (QS+10.5) & \text{if } 0 \leq 13.4 \times (QS+10.5) \leq \\ 100 & \text{if } 13.4 \times (QS+10.5) > 100. \end{cases}$$

13. El método de la reivindicación 12, en donde los niveles de expresión normalizados de BGN, COL1A1, SFRP4, FLNC, GSN, TPM2, GSTM2, FAM13C, KLK2, AZGP1, SRD5A2 y TPX2 se normalizan con respecto a los niveles de expresión de ARF1, ATP5E, CLTC, GPS1 y PGK1.

14. El método de la reivindicación 12, en donde un incremento en el puntaje cuantitativo se correlaciona con un incremento en la probabilidad de un resultado clínico negativo.

15. El método de la reivindicación 12, en donde el resultado clínico es un acenso del cáncer de próstata,

16. El método de la reivindicación 12, en donde el resultado clínico es un descenso del cáncer de próstata,

17. El método de la reivindicación 12, en donde el resultado clínico es la recurrencia del cáncer de próstata,

18. El método de la reivindicación 12, en donde el resultado clínico es la enfermedad no confinada al órgano.

19. El método de la reivindicación 12, en donde el resultado clínico es una patología adversa.

20. El método de la reivindicación 12, en donde la determinación del nivel del nivel de secuencias de ARN transcritas incluye una reacción en cadena de la polimerasa cuantitativa.

21. El método de la reivindicación 12, que además comprende la creación de un reporte que resume la probabilidad de un resultado clínico.

22. El método de la reivindicación 12, en donde la muestra biológica que contiene células cancerosas se fija, se embebe en parafina, o es fresca o congelada.

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Listado de Referencias

33

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 1

Complete if Known	
Application Number	13/752,199
Filing Date	January 28, 2013
First Named Inventor	Steve Shak
Art Unit	1634
Examiner Name	Steven C. Pohnert
Attorney Docket Number	09042.0008-00000

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)		Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
/JSB/		US- 20120028264		02-02-2012	Shak et al.	
/JSB/		US- 20110265197		10-27-2011	Depinho et al.	
/JSB/		US- 20110153534		06-23-2011	Chudin et al.	
/JSB/		US- 20110123990		05-26-2011	Baker et al.	
/JSB/		US- 20080254481		10-16-2008	Love et al.	
/JSB/		US- 20070048738		03-01-2007	Donkena et al.	

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3] Number[4] Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]

NON PATENT LITERATURE DOCUMENTS

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/JSB/		PCT Application No. PCT/US13/023409 PCT Search Report and Written Opinion dated June 7, 2013 (12 pages).	
/JSB/		TAIOLI et al., "Multi-Institutional Prostate Cancer Study of Genetic Susceptibility in Populations of African Descent," Carcinogenesis 32:1361-1365 (2011).	

Examiner Signature	/John S. Brusca/ (12/10/2013)	Date Considered	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet	1	of	7

Complete if Known	
Application Number	13/752,199
Filing Date	January 28, 2013
First Named Inventor	Steve SHAK
Art Unit	1634
Examiner Name	Steven C. Pohnert
Attorney Docket No: GHI-052/US	

U.S.PATENTS

Examiner Initial*	Cite No[1]	Patent Number	Kind Code[2]	Issue Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		6692916		2004-02-17	BEVILACQUA et al.	
		6960439		2005-11-01	BEVILACQUA et al.	
		6964850		2005-11-15	BEVILACQUA et al.	
		7695913		2010-04-13	COWENS et al.	
		7957909		2011-06-07	BEVILACQUA et al.	
		8067178		2011-11-29	BAKER et al.	
		8114597		2012-02-14	LIEW	

U.S.PATENT APPLICATION PUBLICATIONS

Examiner Initial*	Cite No[1]	Publication Number	Kind Code[2]	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		20030087818	A1	2003-05-08	JIANG et al.	
		20030148410		2003-08-07	BERGER et al.	
		20030198970	A1	2003-10-23	ROBERTS	
		20030219771	A1	2003-11-27	BEVILACQUA et al.	
		20030229455	A1	2003-12-11	BEVILACQUA et al.	
		20040053317		2004-03-18	GLINSKII	
		20040133352	A1	2004-07-08	BEVILACQUA et al.	
		20050048542	A1	2005-03-03	BAKER et al.	
		20050191673		2005-09-01	SCHLEGEL et al.	
		20050260646		2005-11-24	BAKER et al.	
		20060281122		2006-12-14	BRYANT et al.	

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Examiner Name	Steven C. Pohnert
Attorney Docket No: GHI-052/US	

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		20070099209	A1	2007-05-03	CLARKE et al.	
		20070105133	A1	2007-05-10	CLARKE et al.	
		20070212702		2007-09-13	TOMLINS et al.	
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		20110039269		2011-02-17	COWENS et al.	
		20110039271		2011-02-17	COWENS et al.	
		20110059447		2011-03-10	LIEW	
		20110097759		2011-04-28	COWENS et al.	
		20110111421		2011-05-12	COWENS et al.	
		20110171633		2011-07-14	COWENS et al.	

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Attorney Docket No: GHI-052/US	

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		2011236903	A1	2011-09-29	McCLELLAND et al.	
		20120040842		2012-02-16	BAKER et al.	
		20120136583		2012-05-31	LAZAR et al.	
		20120171688		2012-07-05	COWENS et al.	

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		0231209	WO	A2	2002-04-18	US Gov Health & Human Serv et al.		☐
		03050243	WO	A2	2003-06-19	Millennium Pharm Inc.		☐
		2005012875	WO	A2	2005-02-10	Squibb Bristol Myers Co et al.		☐
		2005076005	WO	A2	2005-08-18	Univ Wien Med et al.		☐
		2005117943	WO	A2	2005-12-15	Vertex Pharma et al.		☐
		2006028655	WO	A2	2006-03-16	Millennium Pharm Inc. et al.		☐
		2006124836	WO	A1	2006-11-23	Univ Duke et al.		☐
		2007070621	WO	A2	2007-06-21	Childrens Medical Center et al.		☐
		2007072225	WO	A2	2007-06-28	Medical Prognosis Inst et al.		☐

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				First Named Inventor	Steve SHAK
(Use as many sheets as necessary)				**Art Unit**	1634
				Examiner Name	Steven C. Pohnert
Sheet	4	**of**	7	**Attorney Docket No: GHI-052/US**	

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/JSB/		2007082099	WO	A2	2007-07-19	Genomic Health Inc. et al.		☐
/JSB/		2009143603	WO	A1	2009-12-03	Genomedx Biosciences Inc et al.		☐
/JSB/		2010009337	WO	A2	2010-01-21	Dana Farber et al.		
/JSB/		2010080933	WO	A1	2010-07-05	Myriad Genetics Inc. et al.		
/JSB/		199964626	WO	A2	1999-12-16	Genostic Pharma Ltd. et al.		☐
/JSB/		199964627	WO	A2	1999-12-16	Genostic Pharma Ltd. et al.		☐

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/JSB/		ANDERS M., et al., "Microarray Meta-analysis Defines Global Angiogenesis-related Gene Expression Signatures in Human Carcinomas," Molecular Carcinogenesis, 2011. Wiley Periodicals	☐
/JSB/		CLARKE R.A., et al., "Markers for Detection of Prostate Cancer," Cancers, 2010, Vol. 2, pp. 1125-1154.	☐
/JSB/		International Search Report and Written Opinion for Application No. PCT/US11/45253, mailed on 27 February 2012, 13 pages.	☐

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				Examiner Name	Steven C. Pohnert
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/JSB/		NISHIDATE T., et al., "Genome-wide Gene-expression Profiles of Breast-cancer Cells Purified with Laser Microbeam Microdissection: Identification of Genes Associated with Progression and Metastasis," International Journal of Oncology, 2004, Vol. 25 (4), pp. 797-819.	☐
/JSB/		PETERS D., et al., "Genome-wide Transcriptional Analysis of Carboplatin Response in Chemosensitive and Chemoresistant Ovarian Cancer Cells," Molecular Cancer Therapeutics, 2005, Vol. 4 (10), pp. 1605-1616.	☐
/JSB/		SHEN R., et al., "Prognostic Meta-signature of Breast Cancer Developed by Two-stage Mixture Modeling of Microarray Data," BMC Genomics, 2004, Vol. 5 (1), pp. 94.	☐
/JSB/		TRUE L., et al., "A Molecular Correlate to the Gleason Grading System for Prostate Adenocarcinoma," Proceedings of the National Academy of Sciences, 2006, Vol. 103 (29), pp. 10991-10996.	☐
/JSB/		TURASHVILI G., et al., "Novel Markers for Differentiation of Lobular and Ductal Invasive Breast Carcinomas by Laser Microdissection and Microarray Analysis," BMC Cancer, 2007, Vol. 7, pp. 55.	☐
/JSB/		VAARALA M., "DIFFERENTIAL GENE EXPRESSION IN PROSTATE CANCER," Biocenter oulu, 2000. Dissertation University of Oulu	☐

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DECLARACIÓN DE DIVULGACIÓN DE INFORMACIÓN POR EL SOLICITANTE

(Use tantas hojas como sea necesario)

Hoja 1 de 1

Completar si se conoce	
Número de solicitud	(13) 752 199
Fecha de solicitud	Enero 28 de 2013
NOMBRE DEL PRIMER INVENTOR	Steve Shak
Unidad de arte	1634
Nombre de evaluador	Steven C. Pohnert
Código del abogado	09042.0008-00000

DOCUMENTOS DE PATENTES DE ESTADOS UNIDOS

Iniciales del evaluad	Cita No 1	Número del documento / Número-Código de clase 2 (si se conoce)		Fecha de publicación MM-DD-YYYY	Nombre del patentador o solicitante del documento citado	Páginas, columnas, líneas, donde aparecen pasajes o figuras relevantes
/JSB/		US- 20120028264		02-02-2012	Shak et al.	
/JSB/		US- 20110265197		10-27-2011	Depinho et al.	
/JSB/		US- 20110153534		06-23-2011	Chudin et al.	
/JSB/		US- 20110123990		05-26-2011	Baker et al.	
/JSB/		US- 20080254481		10-16-2008	Lave et al.	
/JSB/		US- 20070048738		03-01-2007	Donkena et al.	

DOCUMENTOS DE PATENTE EXTRANJEROS

Iniciales del evaluad or	Cita No 1	Documentos de patente extranjeros / Código país[3] Número[4] Código clase[5] (si se conoce)	Fecha de publicación MM-DD-YYYY	Nombre del patentador o solicitante del documento citado	Páginas, columnas, líneas, donde aparecen pasajes o figuras relevantes	T[6]

DOCUMENTOS NO RELACIONADOS CON PATENTES

Iniciales del evaluador	Cita No. 1	Incluya el nombre del autor (en MAYÚSCULAS), título del artículo (cuando sea apropiado), título de la publicación (libro, magazín, revista, periódico, simposio, catálogo, etc.), fecha, páginas, número del volumen, editor, ciudad y/o país en el que se publicó.	T[6]
/JSB/		Solicitud TCP No. PCT/US13/023409 PCT Reporte de búsqueda y Opinión escrita fechada el 7 de junio de 2013 (12 páginas).	
/JSB/		TAIOLI etal., "Multi-Institutional Prostate Cancer Study of Genetic Susceptibility in Populations of African Descent," Carcinogenesis 32: 1361-1365 (2011).	

Firma del examinador: John S. Brusca 12/10/2013 Fecha considerado

*EVALUADOR: Escriba sus iniciales si la referencia fue considerada, sin importar si la citación cumple con MPEP 609. Cruce la citación con una línea si no cumple y no se ha considerado. Incluya una copia de este formulario con el siguiente comunicado para el solicitante. 1Número único de designación de la solicitud del solicitante (opcional). 2Ver códigos de clases de la Oficina de Patentes y Marcas Registradas de Estados Unidos en www.uspto.gov o MPEP 3Ingrese la oficina que emitió el documento, mediante el código de dos letras (Estándar 3 de OMPI) 4Para documentos de patente japoneses, la indicación del año de reinado del Emperador debe anteceder el número de serie del documento de patente. 5Tipo de documentos según los símbolos apropiados, de ser posible tal como se indican en el documento Estándar 16 de OMPI. 6El solicitante debe marcar aquí si se adjunta traducción en inglés.

Esta recolección de información es requerida por 37 CFR 1.97 y 1.98. La información se requiere para obtener o retener un beneficio para el público que vaya a presentar (y para el proceso de la Oficina de Patentes de Estados Unidos) una solicitud. La confidencialidad está regida por 35 U.S.C. 122 y 37 CFR 1.14. Se estima que esta recolección de información requiere de 2 horas para el diligenciamiento, preparación y envío del formulario de solicitud completo a la Oficina de Patentes de Estados Unidos El tiempo variará dependiendo de cada caso individual. Cualquier comentario con respecto al tiempo requerido por usted para llenar este formulario y/o sugerencias para reducir esta exigencia debe enviarse al Director de Información, Oficina de Patentes y Marcas Registradas de los Estados Unidos, Departamento de Comercio de Estados Unidos, P.O 1450, ALEXANDRIA, Virginia 22313-1450. NO ENVÍE PAGOS DE TARIFAS NI FORMULARIOS DILIGENCIADOS A ESTA DIRECCIÓN. ENVIAR A: Comisionado de Patentes, P.O 1450, ALEXANDRIA, Virginia 22313-1450.

Si necesita ayuda para diligenciar este formulario llame al 1-800-PTO-9199 y escoja la opción 2.

Fecha de recibo:
10/07/2013

Basado en el formulario PTO/SB/08a; SB/08b
Oficina de Patentes y Marcas Registradas de Estados Unidos.
DEPARTAMENTO DE COMERCIO

Bajo la Ley de reducción de papeleo de 1995, ninguna persona está obligada a responder a una solicitud de información a menos que esta exhiba un número OMB de control válido.

Sustituye el formulario 1449/PTO

DECLARACIÓN DE DIVULGACIÓN DE INFORMACIÓN POR EL SOLICITANTE

	Completar si se conoce
Número de solicitud	13/752,199
Fecha de solicitud	Enero 28 de 2013
Nombre del primer	Steve SHAK
Unidad de arte	1634
Nombre del evaluador	Steven C. Pohnert
Código de abogado No.: GHI-052/US	

Hoja 1 de 7

PATENTES DE ESTADOS UNIDOS

Inicial del evaluador*	Cita No [1]	Número de patente	Código de clase [2]	Fecha de expedición	Nombre del patentador o solicitante del documento citado	Páginas, columnas, líneas en las cuales aparecen pasajes o figuras relevantes
		6692916		17/02/2004	BEVILACQUA et al.	
		6960439		01/11/2005	BEVILACQUA et al.	
		6964850		15/11/2005	BEVILACQUA et al.	
		7695913		13/04/2010	COWENS et al.	
		7957909		2011-06-07	BEVILACQUA et al.	
		8067178		2011-11-29	BAKER et al.	
		8114597		14/02/2012	LIEW	

SOLICITUD DE PATENTE EEUU PUBLICACIONES

Inicial del evaluador*	Cita No [1]	Número de publicación	Código de clase [2]	Fecha de publicación	Nombre del patentador o solicitante del documento citado	Páginas, columnas, líneas en las cuales aparecen pasajes o figuras relevantes
		20030087818	A1	08/05/2003	JIANG et al.	
		20030148410		07/08/2003	BERGER et al.	
		20030198970	A1	23/10/2003	ROBERTS	
		20030219771	A1	27/11/2003	BEVILACQUA et al.	
		20030229455	A1	11/12/2003	BEVILACQUA et al.	
		20040053317		18/03/2004	GLINSKII	
		20040133352	A1	08/07/2004	BEVILACQUA et al.	
		20050048542	A1	03/03/2005	BAKER et al.	
		20050191673		01/09/2005	SCHLEGEL et al.	
		20050260646		24/11/2005	BAKER et al.	
		20060281122		14/12/2006	BRYANT et al.	

TODAS LAS REFERENCIAS CONSIDERADAS, EXCEPTO CUANDO APARECEN TACHADAS /JSB/ (12/10/2013)

FIRMA DEL EVALUADOR **FECHA CONSIDERADO**

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Fecha de recibo:
07/10/2013

Basado en el formulario PTO/SB/08a; SB/08b
Oficina de Patentes y Marcas Registradas de Estados Unidos.
DEPARTAMENTO DE COMERCIO

Bajo la Ley de reducción de papeleo de 1995, ninguna persona está obligada a responder a una solicitud de información a menos que esta exhiba un número OMB de control válido.

Sustituye la forma 1449/PTO	*Completar si se conoce*	
DECLARACIÓN DE DIVULGACIÓN DE INFORMACIÓN POR EL SOLICITANTE	**Número de solicitud**	13752.199
	FECHA DE SOLICITUD	Enero 28 de 2013
	Nombre del primer	Steve SHAK
	Unidad de arte	1634
	Nombre del evaluador	Steven C. Pohnert
Hoja 2 de 7	**Código de abogado No.: GHI-052/US**	

SOLICITUD DE PATENTE EEUU PUBLICACIONES

Inicial del evaluador*	Cita No [1]	Número de publicación	Código de clase[2]	Fecha de publicación	Nombre del patentador o solicitante del documento citado	Páginas, columnas, líneas en las cuales aparecen pasajes o figuras relevantes
		20070099209	A1	03/05/2007	BRYANT et al.	
		20070105133	A1	01/05/2007	BRYANT et al.	
		20070212702		13/09/2007	TOMLINS et al.	
		20070275398		29/11/2007	KIEFER et al.	
		20080015448	A1	17/01/2008	KEELY et al.	
		20080085519	A1	01/04/2008	GABRIN et al.	
		20090023149		22/01/2009	KNUDSEN	
		20090233279		17/09/2009	GLINSKII	
		20090258795		15/10/2009	COWENS et al.	
		20090298082		03/12/2009	KLEE et al.	
		20090305277	A1	01/12/2009	BAKER et al.	
		20090170075	A1	02/07/2009	PETROVICS et al.	
		2009298082	A1	03/12/2009	KLEE et al.	
		20100124745		20/05/2010	LIEW	
		20100291573		18/11/2010	COWENS et al.	
		2010297657	A1	25/11/2010	CHINNAIYAN	
		20110039269		17/02/2011	COWENS et al.	
		20110039271		17/02/2011	COWENS et al.	
		20110059447		01/03/2011	LIEW	
		20110097759		28/04/2011	COWENS et al.	
		20110111421		12/05/2011	COWENS et al.	
		20110171633		14/07/2011	COWENS et al.	

TODAS LAS REFERENCIAS CONSIDERADAS EXCEPTO CUANDO APARECEN TACHADAS

FIRMA DEL EVALUADOR **FECHA CONSIDERADO**

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DEPARTAMENTO DE COMERCIO DE ESTADOS UNIDOS

Bajo la Ley de reducción de papeleo de 1995, ninguna persona está obligada a responder a una solicitud de información a menos que esta exhiba un número OMB de control válido.

Sustituye el formulario 1449/PTO	*Completar si se conoce*	
DECLARACIÓN DE DIVULGACIÓN DE INFORMACIÓN POR EL SOLICITANTE	**Número de solicitud**	13752.199
	FECHA DE SOLICITUD	Enero 28 de 2013
	Nombre del primer	Steve SHAK
	UNIDAD DE ARTE	1634
	Nombre del evaluador	Steven C. Pohnert
Hoja 3 de 7	**Código de abogado No.: GHI-052/US**	

SOLICITUD DE PATENTE EEUU PUBLICACIONES

Inicial del evaluador*	Cita No [1]	Número de publicación	Código de clase[2]	Fecha de publicación	Nombre del patentador o solicitante del documento citado	Páginas, columnas, líneas en las cuales aparecen pasajes o figuras relevantes
		2011236903	A1	29/09/2011	McCLELLAND et al.	
		20120040842		16/02/2012	BAKER et al.	
		20120136583		31/05/2012	LAZAR et al.	
		20120171688		05/07/2012	COWENS et al.	

DOCUMENTOS DE PATENTE EXTRANJEROS

Inicial del evaluador*	Cita No [1]	Número de publicación	Código de clase[2]	Fecha de publicación	Nombre del patentador o solicitante del documento citado	Páginas, columnas, líneas en las cuales aparecen pasajes o figuras relevantes	Inicial del evaluador *	T[6]
		0231209	WO	A2	18/04/2002	Servicios Humanos y de Salud del Gobierno de Estados Unidos		—
		03050243	WO	A2	19/06/2003	Millennium Pharm Inc.		—
		2005012875	WO	A2	01/02/2005	Squibb Bristol Myers Co et al.		—
		2005076005	WO	A2	18/08/2005	Univ Wien Med et al.		—
		2005117943	WO	A2	15/12/2005	Vertex Pharma et al.		—
		2006028655	WO	A2	16/03/2006	Millennium Pharm Inc. et al.		—
		2006124836	WO	A1	23/11/2006	Univ Duke et al.		—
		2007070621	WO	A2	21/06/2007	Childrens Medical Center et al.		—
		2007072225	WO	A2	28/06/2007	Medical Prognosis Inst et al.		—

TODAS LAS REFERENCIAS CONSIDERADAS, EXCEPTO CUANDO APARECEN TACHADAS /JSB/ (12/10/2013)

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~~AB~~

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Fecha de recibo:
07/10/2013

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Oficina de Patentes y Marcas Registradas de Estados Unidos.
DEPARTAMENTO DE COMERCIO

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Sustituye el formulario 1449/PTO	*Completar si se conoce*	
DECLARACIÓN DE DIVULGACIÓN DE INFORMACIÓN POR EL SOLICITANTE	**Número de solicitud**	13752.199
	FECHA DE SOLICITUD	Enero 28 de 2013
	Nombre del primer	Steve SHAK
	Unidad de arte	1634
	Nombre del evaluador	Steven C. Pohnert
Hoja 4 de 7	**Código de abogado No.: GHI-052/US**	

DOCUMENTOS DE PATENTE EXTRANJEROS

Inicial del evaluador*	Cita No [1]	Número de publicación	Código de clase[2]	Fecha de publicación	Nombre del patentador o solicitante del documento citado	Páginas, columnas, líneas en las cuales aparecen pasajes o figuras relevantes	Inicial del evaluador *	T[6]
/JSB/		2007082099	WO	A2	19/07/2007	Genomic Health Inc. et al.		—
/JSB/		2009143603	WO	A1	03/12/2009	Genomedx Biosciences Inc et al.		—
/JSB/		2010009337	WO	A2	21/01/2010	Dana Farber et al.		
/JSB/		2010080933	WO	A1	05/07/2010	Myriad Genetics Inc. et al.		
/JSB/		199964626	WO	A2	16/12/1999	Genostic Pharma Ud. et al.		—
/JSB/		199964627	WO	A2	16/12/1999	Genostic Pharma Ud. et al.		—

DOCUMENTOS NO RELACIONADOS CON PATENTES

Iniciales del evaluador*	Cita No 1	Incluya el nombre del autor (en MAYÚSCULAS), título del artículo (cuando sea apropiado), título del publicación (libro, magazín, revista, periódico, simposio, catálogo, etc.), fecha, páginas, número del volumen, editor, ciudad y/o país en el que se publicó.	T6
/JSB/		ANDERS M., et al., "Microarray Meta-analysis Defines Global Angiogenesis-related Gene Expression Signatures in Human Carcinomas," Molecular Carcinogenesis, 2011. Wiley Periodicals	—
/JSB/		CLARKE R.A., et al., "Markers for Detection of Prostate Cancer," Cancers, 201 O, Vol. 2, pp. 1125-1154.	—
/JSB/		Reporte de Búsqueda Internacional y a la Opinión Escrita No. PCT/US11/45253, enviado el 27 de febrero de 2012, 13 páginas.	—

FIRMA DEL EVALUADOR **FECHA CONSIDERADO**

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07/10/2013

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DEPARTAMENTO DE COMERCIO DE ESTADOS UNIDOS

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Sustituye el formulario 1449/PTO	*Completar si se conoce*	
DECLARACIÓN DE DIVULGACIÓN DE INFORMACIÓN POR EL SOLICITANTE	**Número de solicitud**	13752,199
	Fecha de solicitud	Enero 28 de 2013
	Nombre del primer	Steve SHAK
	Unidad de arte	1634
	Nombre del evaluador	Steven C. Pohnert
Hoja 5 de 7	**Código de abogado No.: GHI-052/US**	

DOCUMENTOS NO RELACIONADOS CON PATENTES

Iniciales del evaluador*	Cita No 1	Incluya el nombre del autor (en MAYÚSCULAS), título del artículo (cuando sea apropiado), título del publicación (libro, magazin, revista, periódico, simposio, catálogo, etc.), fecha, páginas, número del volumen, editor, ciudad y/o país en el que se publicó.	T6
/JSB/		NISHIDATE T., et al., "Genome-wide Gene-expression Profiles of Breast-cancer Cells Purified with Laser Microbeam Microdissection: Identification of Genes Associated with Progression and Metastasis," International Journal of Oncology, 2004, Vol. 25 (4), pp. 797-819.	—
/JSB/		PETERS D., et al., "Genome-wide Transcriptional Analysis of Carboplatin Response in Chemosensitive and Chemoresistant Ovarian Cancer Cells," Molecular Cancer Therapeutics, 2005, Vol. 4 (10), pp. 1605-1616.	—
/JSB/		SHEN R., et al., "Prognostic Meta-signature of Breast Cancer Developed by Two-stage Mixture Modeling of Microarray Data," BMC Genomics, 2004, Vol. 5 (1), pp. 94.	—
/JSB/		TRUE L., et al., "A Molecular Correlate to the Gleason Grading System for Prostate Adenocarcinoma," Proceedings of the National Academy of Sciences, 2006, Vol. 103 (29), pp. 10991-10996.	—
/JSB/		TURASHVILI G., et al., "Novel Markers for Differentiation of Lobular and Ductal Invasive Breast Carcinomas by Laser Microdissection and Microarray Analysis," BMC Cancer, 2007, Vol. 7, pp. 55.	—
/JSB/		VAARALA M., "DIFFERENTIAL GENE EXPRESSION IN PROSTATE CANCER," Biocenter Oulu, 2000. Disertación Universidad de Oulu	—

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Microarray Meta-Analysis Defines Global Angiogenesis-Related Gene Expression Signatures in Human Carcinomas

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Angiogenesis is a prerequisite for progression of cancers. The number of genes linked to angiogenesis suggests the existence of complex gene-networks, which remain to be elucidated. To identify angiogenesis genes deregulated in carcinomas, we performed a meta-profiling analysis of published gene expression microarray studies. Own microarray and quantitative RT-PCR data were obtained from a colorectal carcinoma cohort. Applying highly stringent inclusion criteria, 15 cancer array studies were suitable for our analysis. These studies provided 789 tumor specimens and 190 samples of healthy tissues yielding a total of approx. 1,000,000 gene expression measurements. Meta-analysis on the expression of 480 angiogenesis-related genes in 10 cancer types identified a characteristic, entity-independent "global" cancer expression signature of 25 angiogenesis-related genes showing high frequency down-regulation when compared to corresponding healthy tissues. Furthermore, we characterized 25 genes displaying frequent up-regulation, yet less often than the 25 down-regulated genes. Comparative inter-study cross-validation revealed that both signatures discriminate cancers from healthy tissues with high accuracy in independent test sets. Moreover, own microarray data of colorectal carcinomas confirmed the specific and sensitive discriminating potential of both signatures. These results were validated by quantitative RT-PCR for eight genes displaying the highest differences in the microarray analysis. Our study for the first time defines global gene expression signatures linked to angiogenesis in carcinomas. Our findings suggest that gene down-regulation may represent a central aspect of tumor angiogenesis.
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Key words: angiogenesis; cancer; meta-analysis; gene expression; microarray

INTRODUCTION

Angiogenesis, the formation of new blood vessels from endothelial precursors, is a prerequisite for growth and progression of solid malignancies and is driven by a disturbed balance between pro- and anti-angiogenic factors [1–3]. Angiogenic factors are produced and released from endothelial, stromal, and/or cancer cells as well as blood components, while their receptors are mainly found on vascular endothelial cells (reviewed in Refs. [4–7]). Recently, it became clear that basic mechanisms of hemangiogenesis may also apply to the lymphatic system, and that tumor lymphangiogenesis is regulated by the interaction of lymphangiogenic factors with their receptors on lymphatic endothelial cells [8]. The clinical relevance of angiogenesis in cancer pathobiology has further been highlighted by the finding that the expression of angiogenic genes such as vascular endothelial growth factor A (VEGF-A), vascular endothelial growth factor receptor 2 (VEGFR-2), and basic fibroblast growth factor (bFGF) correlates with poor prognosis in cancer patients [9–11]. Moreover, the finding that targeting of angiogenic molecules such as VEGF-A provides effective therapeutic options for cancer patients further confirmed the clinical relevance of angiogenesis [12,13].

The increasing number of genes that have been linked to angiogenesis in recent years suggests the existence of complex gene-networks. In order to identify such networks employment of high throughput approaches, for example, DNA microarray technology, enabling the parallel analysis of multiple genes, is crucially needed. The use of

Abbreviations: VEGF, vascular endothelial growth factor; SAM, significance analysis of microarrays; TGFBR, transforming growth factor, beta receptor.

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microarray techniques has already led to a major progress in the understanding of compound tumor-related mechanisms, identifying gene expression profiles associated with neoplastic transformation, cancer progression, and/or clinical outcomes [14,15]. Recently, new bioinformatic approaches, such as meta-analysis of microarray data, have been suggested to substantially improve the gain of information derived from microarray experiments. Hereby, gene expression data from individual studies are summarized and analyzed by statistical algorithms in order to identify common, "higher-level", transcriptional profiles [14,16–18]. Particularly the inclusion of large sample numbers promises to detect differential expression of genes that may not be found in individual studies and to minimize the rate of false positive findings [19]. Making use of meta-analysis techniques, gene expression signatures underlying malignant transformation, as well as progression in both individual cancers and across various malignancies ("global profiles") have been identified [17,19–23]. Despite these advantages microarray technology has rarely been used to investigate angiogenesis in human cancers [24–26]. Moreover, the clinical relevance of these reports has been hampered by the fact that only small numbers of patients were assessed and/or that control samples representing healthy control samples were not included.

In order to gain more detailed insights into molecular networks contributing to cancer angiogenesis, we employed the advantages of the meta-array approach investigating publicly available cancer microarray data sets.

MATERIALS AND METHODS

Selection of Angiogenesis Genes

The PubMed® database was screened for angiogenesis related genes using the following criteria: (a) differential expression during angiogenesis (b) functional impact on proliferation, differentiation, and/or migration of endothelial cells in vitro and/or in vivo. Search terms included: "angiogenesis", "vascularization", "endothelium", "endothelial cells", "blood or lymphatic vessels", "tumor associated angiogenesis". Selected genes were assigned a unique identification code (Unigene Cluster ID) using the SOURCE® data base (http://source.stanford.edu/cgi-bin/source/sourceSearch).

Microarray Data Collection

Using the PubMed® database, we searched for studies investigating gene expression profiles in solid human cancers by cDNA or oligonucleotide microarray technology. Search terms comprehended: "gene expression profiling", "cDNA microarray", "oligonucleotide microarray", "cancer", "carcinoma", and "neoplasm". Publications providing gene expression measurements in malignant tissues of epithelial origin ($n \geq 10$ samples) and corresponding healthy tissues ($n > 3$ samples) were selected. Data sets were downloaded from the Stanford microarray database (genome-www5.stanford.edu), Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo), and supplementary information of the published manuscripts. Subsequently data sets were normalized using "Quantile normalization" according to Bolstad et al. [27] when normalized data was not available and imported into Microsoft Excel®. All genes were assigned an individual Unigene cluster ID as described above. Tissue samples within each study were assigned "1" (healthy) and "2" (cancer) for further statistical analysis; data from cell lines were excluded.

Data Analysis "Cancer Related Angiogenesis"

Expression data of 535 angiogenesis-related genes were extracted from the individual microarray data sets using Microsoft Access®. To determine differential gene expression in carcinomas compared to corresponding healthy control tissues the "significance analysis of microarrays" (SAM, version 2.11) approach was applied to each study individually [28]. Hereby, we computed a statistic score indicating the strength of relationship between the expression level of each gene and cancer. The lowest false discovery rate at which differential gene expression was called significant, the "q-value", was set to $q < 5\%$ in all data sets. Genes needed to have at least three measurements for cancer and healthy specimens each within an individual data sets to be included in this analysis, otherwise they were considered "not present" for this respective study.

Meta-Profiling "Cancer Related Angiogenesis"

To identify entity-independent "global" gene expression signatures, we determined the number of data sets containing significant regulation in tumor versus healthy tissues of each individual gene as determined by "SAM". Subsequently, all genes were ranked according to this frequency. To further confirm the validity of the results obtained by this approach, we evaluated the 100 top ranking genes regarding their power to correctly classify malignant versus healthy tissues in microarray data sets that were not included into the meta-array analysis [29,30]. Employing a "leave one out" method for cross validation (Alyuda Forecaster XL®, Alyuda, Cupertino, CA) all but one sample were used to create a neuronal network. Based on this network, the class ("healthy" or "cancer") of the one sample that had been left out initially was predicted. All steps were repeated until every sample had been left out once. During this procedure increasing numbers of genes, beginning at the top of the list with 1, 5, 10, 25, 50, 75, up to 100 were

analyzed. Furthermore, we applied this method exclusively to the 25 genes that displayed highest frequency regarding either up- or down-regulation. To calculate the percentage of classifications by chance, we applied the "leave one out" approach to an additional set of 25 genes showing no significant expression changes in our initial "SAM" analysis to the same test sets [29,30].

Gene Ontology

Angiogenesis meta-signatures were classified to gene ontology annotations using the Onto-express™ database (http://vortex.cs.wayne.edu/ontoexpress/) according to their molecular function as well as biological process and cellular components to which they were linked in *"Homo sapiens"*.

Patients and Sample Collection for Colorectal Cancer Study

Frozen tumor tissue samples from 47 colorectal cancer patients (UICC I-IV) and 7 healthy tissue samples were obtained from the Robert-Rössle-Klinik, Charité Berlin. Primary tumor and adjacent normal tissues were collected at the time of surgery following internal protocols. Histopathological examination following hematoxilin/eosin staining of tissue sections ensured that only tumor samples containing at least 60% of non-necrotic tumor epithelium were used for further analysis. Uniform follow-up data were accessed from the Tumorbank of the Robert-Rössle-Klinik, Charité Berlin.

RNA Isolation

Total RNA was extracted from the tissue samples using RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA quantity was measured using the NanoDrop Spectrophotometer (PEQLAB Biotechnologie, Erlangen, Germany). Only high quality RNA samples according to Bioanalyzer analysis (Agilent, Santa Clara, CA) were used for further processing.

Labeling and Microarray Hybridization

Total RNA was converted into Cy3-labeled cRNA using the Low RNA Input Fluorescent Linear Amp Kit and hybridized onto 4x44K-Whole Human Genome Chips using the In-Situ Hybridization Kit Plus according to the manufacturer's protocol for 17 h at 65°C (Agilent, Santa Clara, CA). Microarrays were scanned by using standard protocols and a G2565AA Microarray Scanner (Agilent, Santa Clara, CA). Data were obtained using Feature Extraction 8.0 (Agilent, Santa Clara, CA).

Chip Data Analysis

All data were imported into GeneSpring software (Agilent, Santa Clara, CA) and normalized per chip to the 50th percentile and per gene to the median after setting measurements <5.0–5.0. Supervised hierarchical clustering was performed using the 25 most often down/and up-regulated angiogenesis related genes, respectively. Differential expression analysis was performed and multiple testing was controlled by adjusting all *P*-values using the Benjamini–Hochberg method.

Statistical Analysis

Leave-one-out cross-validation was performed with GeneSpring (Agilent, Santa Clara, CA) using the "Support Vector Machines" algorithm and different Kernel Functions (Radial Basis (Gaussian), Polynomial Dot (order 1), Polynomial Dot (order 2), Polynomial Dot (order 3) as well as Diagonal Scaling Factors from 0 to 5. The mean was calculated considering the results from all combinations.

qRT-PCR

Total RNA (2.5 μg) of was reverse transcribed into cDNA by M-MLV reverse transcriptase with random hexamers. qRT-PCR was performed according to the manufacturers protocols, using the TaqMan instrument TaqMAN 7900 (Applied Biosystems, Foster City, CA) and the pre-designed TaqMan Assays-on-demand Hs01081598_m1 (INHBA), Hs01568063_m1 (THBS2), Hs00959010_m1 (SPP1), Hs00171022_m1 (CXCL12), Hs01547656_m1 (IGF1), Hs00162844_m1 (TNA), Hs00153350_m1 (BCL2) und Hs00230918_m1 (BTEB1).

RESULTS

Primary Data Analysis

Our search for angiogenesis-related genes identified 535 candidates matching the criteria defined in the Materials and Methods Section. Evaluation of 509 cancer microarray studies identified 39 studies matching our search criteria; for 15 out of these 39 reports raw gene expression data were publicly available [31–45]. The data sets of these studies provided measurements on 789 cancer specimens as well as 190 samples of corresponding healthy tissues (prostate, lung, stomach, colon, pancreas, liver, bladder, kidney, ovarian, and mamma). Fourhundred eighty of the initially selected 535 angiogenesis genes were present on the microarray chips used in these studies, ranging from a minimum of 188 genes [41] up to a maximum of 425 genes analyzed [39] (Table 1).

Meta-signature "Cancer Related Angiogenesis"

Following separate SAM analysis of the individual data sets (Table 2), results were pooled and underwent meta-analysis according to the frequencies of significant gene regulation within the studies. The most prominent finding of this analysis was that gene down-regulation occurred predominantly. Hereby, 3 genes displayed highest frequency of down-regulation in 9 out of 15 studies. In

Table 1. Eligible Studies Used for the Meta-Analysis

Entity	Author	Patients *n*		Genes
		Cancer	Normal	
Prostate	Welsh et al.	23	9	415
Prostate	Dhanasekaran et al.	18	8*	206
Prostate	Luo et al.	16	9	272
Prostate	LaTulippe et al.	23	3	415
Lung	Virtanenet al.	43	6	248
Lung	Garber et al.	67	5	371
Lung	Bhattacharjee et al.	186	17	413
Stomach	Leung et al.	90	22	425
Colon	Nottermann et al.	18	18	188
Pancreas	Iacobuzio Donahue et al.	17	5	400
Liver	Chen et al.	102	74	375
Bladder	Dyrskot et al.	40	4	241
Kidney	Higgins et al.	41	3	317
Ovarian	Welsh et al.	27	3	329
Mamma	Sorlie et al.	78	4	293

All studies include publicly available raw data sets.
The table shows number of patients from whom the malignant and non-transformed control tissues where derived from, as well as the numbers of angiogenesis related genes investigated in the individual studies.
*Benign prostate hyperplasia.

addition, a profile representing angiogenesis genes up-regulated in cancers was identified. However, the highest frequency of regulation did not exceed a maximum of 2 genes being up-regulated in 6 out of the 15 data sets (Figure 1).

Comparative Inter-Study Cross Validation

To assess the validity of the cancer spanning expression signatures, we evaluated 100 genes displaying the highest frequency of differential regulation across all data sets regarding their power to correctly classify malignant and non-malignant tissues in independent microarray data sets of lung cancer ($n = 86$, non-malignant lung tissue: $n = 10$ [29]) and prostate cancer ($n = 62$, non-malignant lung tissue: $n = 40$ [30]). Using the 25 most frequently regulated genes, most accurate discrimination between cancer ($n = 148$) and corresponding

Table 2. Number of Differentially Up- and Down-Regulated Angiogenesis Related Genes in Cancer Compared to Non-Transformed Control Tissue Samples

Author	Differential expression/genes (*n*)		% of present genes
	Up	Down	
Welsh et al.[a]	13	121	30
Dhanasekaran et al.	—	60	29
Luo et al.	10	86	35
LaTulippeet al.	—	88	21
Virtanen et al.	—	58	23
Garber et al.	58	74	36
Bhattacharjee et al.	239	121	87
Leung et al.	244	86	78
Nottermannet al.	26	36	33
Iacobuzio Donahue et al.	87	27	29
Chen et al.	114	216	88
Dyrskot et al.	—	61	25
Higgins et al.	13	4	5
Welsh et al.[b]	4	45	15
Sorlie et al.	6	38	15

Results are based on a SAMs applied to each individual study.
[a]Prostate.
[b]Ovarian.



Figure 1. "Meta-Array" of top ranking 25 most frequent down-regulated (upper panel) and up-regulated genes in cancer compared to corresponding non-transformed tissues (red = significantly regulated; green = present, but not regulated in the respective study; white = not present on the microarray chip, n = number of included studies; genes are sorted by frequency of regulation).

healthy samples ($n = 50$) was achieved: 94.5% of all cases were classified correctly with 14% false positive and 2.7% false negative results. The number of 25 genes proved optimal for this analysis, while both less and more genes increased the false positive and false negative classification rates, as determined gradually for 1 up to 100 genes (Figure 2). Our analyses gained a significantly higher percentage of "correct" class predictions compared to the same approach based on 25 genes displaying no regulation between healthy and malignant samples in the initial "SAM" analysis (67.2%; $P < 0.0001$). As this signature contained exclusively down-regulated genes, we investigated a separate group of the 25 most frequently up-regulated genes yielding a correct classification of 84.8% of the cases. This classification rate was significantly higher than by chance, determined by 25 non-regulated genes, yet worse than the classification rate of the 25 down-regulated genes (see above; $P = 0.0043$).

Molecular Analysis of Angiogenesis Gene Expression Signatures in Colorectal Cancer

In order to further verify our findings by an independent validation method, we performed a

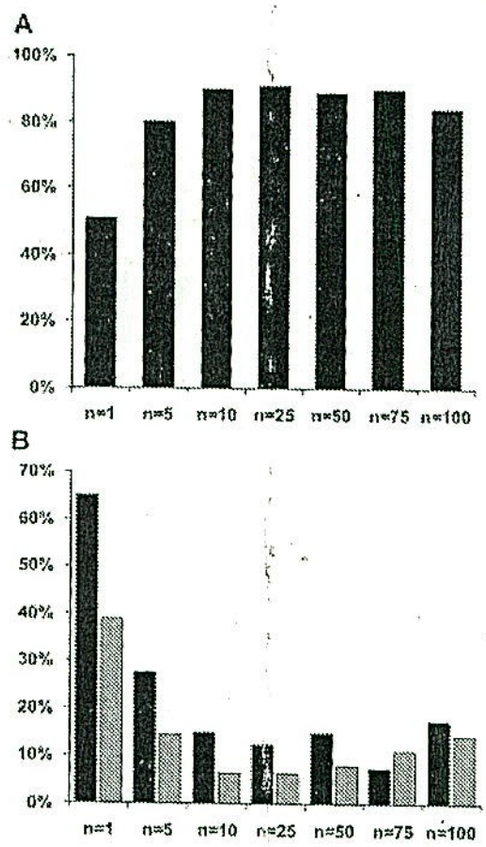


Figure 2. Percentage of correctly classified malignant and non-malignant tissues (A) and of false positive (black bars) and false negative (grey bars) results (B). Classification was performed based on an increasing number of the top ranking genes applying a "leave one out" method for cross validation to an independent microarray data set [30].

whole genome microarray study on 47 colorectal cancer and 7 normal tissue samples. These tissue samples were obtained from the Tumorbank of the Robert-Rössle-Klinik, Charité, Berlin providing known, uniform and high tissue quality, as well as consistent patient follow-up data. Only samples containing a minimum of 60% of non-necrotic tumor epithelium were used for our cDNA microarray, which is publically available (www.ncbi.nlm.nih.gov/geo; GSE31905). Supervised clustering of the acquired gene expression data, based on either the 25 most frequently down- or up-regulated genes identified by meta-analysis respectively yielded a clear distinction between tumor and normal tissues (Figure 3, Table 3). Furthermore, we observed a formation of subgroups within the tumor-collective especially when clustering based on the signature of down-regulated genes. However, no significant correlation between these subclusters and standard clinical or histopathological parameters (UICC stage, T, N, M, G, L, V, disease-free time, recurrence, survival time, gender or age) was found.

These results were further substantiated by leave-one-out cross-validation using the "Support Vector Machines" algorithm and different Kernel Functions as well as different diagonal scaling factors. Hereby, a mean predictive power of 98% (94–100%; 2% false negative results) was achieved for the down-regulated signature and a mean predictive power of 99% (96–100%; 1% false negative results) for the up-regulated angiogenesis genes. Subsequent quantitative RT-PCR analysis for 8 representative genes displaying the highest differences in the microarray analysis performed on all normal samples and 45 of the tumor samples used for microarray analysis confirmed our microarray results (Table 4).

Gene Ontology Classification

In order to identify functionally related gene-subgroups within the identified meta-signatures, both were classified according to their gene ontology annotation using the Ontoexpress[19] database. For "molecular function", we identified within both signatures genes linked to protein binding and receptor activity. Transferase- and hydrolase activity, as well as nucleid acid- and ATP binding occurred preferentially in the signature of down-regulated genes, whereas calcium ion and heparin binding were assigned to up-regulated genes. Regarding the "biological process", genes within both signatures were linked to cell adhesion, signal transduction, and cell–cell signaling, while anti-apoptotic genes were preferentially found to be down-regulated. On the other hand, cell matrix and apoptosis associated genes were found exclusively among up-regulated genes. Furthermore, our analyses revealed that genes within down- as well as up-regulated metaprofiles become translated into proteins that are preferentially localized at the plasmamembrane and the extracellular matrix.

DISCUSSION

Here, we describe for the first time global, entity-independent expression signatures of differentially regulated angiogenesis genes in human cancers relative to healthy controls. Given that these signatures were defined by analyzing 10 different cancer entities these reflect with high likelihood genes contributing to cancer angiogenesis. For a number of genes, we describe for the first time a potential in cancer-related angiogenesis whereas other genes previously assigned to tumor angiogenesis do not show frequent transcriptional regulation in cancer.

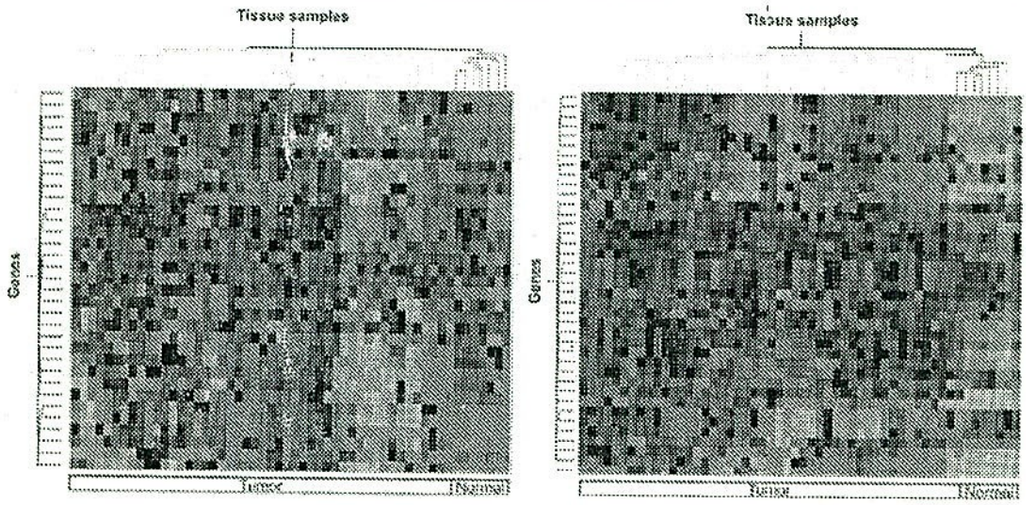


Figure 3. The application of the 25 most frequently down-regulated (left panel) and up-regulated (right panel) angiogenesis-related genes to a data-set of 47 colorectal cancer samples and 7 normal control tissues results in a clear discrimination between tumor (left side) and normal mucosa (right). Each row represents a gene and each column a sample. Red indicates up-regulation, green down-regulation and black no change. Please note: genes are displayed in random order. Fold changes and *P*-values are given in Table 3.

Table 3. Fold Changes and *P*-Values for the 25 Most Often Down- (left) and Up-Regulated (right) Angiogenesis-Related Genes in Differential Expression Between Tumor and Normal Colorectal Tissues Obtained by Microarray Hybridization

	Down			Up	
Gene name	Fold change	*P*-value	Gene name	Fold change	*P*-value
PDGFRA	0.631	1.86E-04	GRP58	1.23	9.55E-01
SPARCL1	0.72	1.65E-02	INHBA	63.07	1.91E-11
ANG	0.391	1.53E-04	F2R	1.12	7.34E-01
AOC3	0.56	1.65E-02	SCARB1	2.94	1.03E-03
ENPP2	0.526	4.56E-03	POSTN	2.19	1.32E-03
TGFBR3	1.166	6.01E-01	COL1A2	4.03	8.07E-03
FGFR2	0.333	1.01E-03	CALR	1.63	1.27E-04
PPAP2B	0.59	8.80E-03	IFNGR	0.79	1.53E-02
FGF7	0.451	2.26E-02	CSPG2	2.85	3.16E-04
CAV1	1.432	4.91 E-02	THBS2	21.04	4.84E-14
CXCL12	0.125	4.60E-10	PLAU	4.36	3.56E-04
RBPMS	1.834	2.17E-04	M11S1	4.03	2.70E-04
BTEB1	0.333	1.13E-07	ANAPC5	1.41	5.79E-04
TIMP3	1.681	3.98E-03	THY1	2.00	1.65E-08
SERPING1	0.995	9.73E-01	MMP11	8.00	3.40E-13
TGFBR2	1.144	5.61 E-02	NT5E	0.79	3.06E-01
TIEG	1.331	1.10E-01	MMP17	0.19	6.46E-05
TCF8	1.138	6.01E-01	THBS3	1.15	1.99E-01
CCL2	0.377	2.65E-02	CBFB	1.61	1.88E-04
EDG1	0.992	9.73E-01	SLC7A5	9.64	1.28E-02
IGF1	0.161	4.02E-10	ERBB3	3.17	2.61 E-04
PPARG	0.748	4.44E-02	BGN	8.22	3.74E-06
CD36	0.182	1.61E-08	SPP1	11.40	1.79E-10
BCL2	0.203	2.61E-13	PGF	1.60	1.27E-04
TNA	0.167	1.86E-07	COL5A2	4.23	1.53E-02

Genes are sorted according to the results of the meta-array.

Table 4. The Results of qRT-PCR (TaqMan) Performed for 8 Genes Displaying the Highest Differences in the Microarray Analysis (Top 5 = Down-Regulated Genes; bottom 3 = Up-Regulated Genes) on 7 Normal and 45 of the 47 Tumor Samples Confirm the Microarray Data

Transcript	Fold change	Range
CXCL12	0.19	0.16–0.23
BTEB1	0.37	0.34–0.40
IGF1	0.22	0.19–0.25
BCL2	0.29	0.23–0.37
TNA	0.26	0.20–0.34
INHBA	6.85	6.05–7.76
THBS2	5.32	4.29–6.60
SPP1	7.32	5.93–9.04

Our cancer spanning, "global" gene signatures comprehend 25 down- and up-regulated genes, respectively, classifying cancer and healthy tissue samples at high accuracy in independent tests sets. A selection of 25 genes proved to be optimal in our approach. Our findings could be further confirmed by supervised clustering of gene expression data generated in our own laboratory, derived from colorectal cancer and respective normal tissue samples. Quantitative RT-PCR substantiated the differential expressions obtained from our microarray experiment. These findings suggest that the gene signatures identified by our meta-array are closely linked to cancer angiogenesis.

Despite, the various biological functions that genes within the metasignature have been assigned all have been linked to angiogenesis, the central inclusion criteria of our study. Therefore, the following conclusions upon its impact on cancer neovascularization may be made: (1) The genes contained within these meta-signatures can be assigned to various biological functions including cell adhesion [e.g., CXCL12, AOC3], signal transduction [e.g., transforming growth factor, beta receptor III (TGFBR3), FGF7], cell–cell signaling [e.g., CCL2], anti-apoptosis [e.g., BCL2], lipid metabolism [e.g., PPARG, CD36], and inflammatory response [e.g., AOC3, CCL2]. These observations support the current concept that tumor angiogenesis comprises a complex biological process clearly reaching beyond endothelial proliferation. (2) Our findings of frequent and parallel gene down- and up-regulation underline the hypothesis that cancers display a major disturbance compared to "resting" angiogenesis gene networks [1,3,46]. (3) Our data suggest that down-regulation of angiogenesis genes appears to be the more prominent feature. To the best of our knowledge, the current study, marks the first description of this phenomenon, whereas previous reports of gene down-regulation have been limited to single angiogenesis-related genes in individual cancer types [e.g., transforming growth factor-β (TGFB) and TGFBR2 [47,48]]. Our observations suggest that extensive down-regulation of anti-angiogenic genes represents a crucial prerequisite of cancer angiogenesis and loss of pro-angiogenic genes, for example, those that facilitate vessel integrity under healthy conditions, contributes to the formation of immature vasculature characteristic to cancers. In line with this hypothesis, we do find for example, PDGFRA and SPARC-like 1 that potentially facilitate anti-angiogenic roles: PDGFRA mediates the inhibitory effects of platelet-derived growth factor on angiogenic properties of fibroblast growth factor [49]. Therefore, a down-regulation of PDGFRA might lead to disinhibition of FGF-triggered angiogenesis. SPARC-like 1 inhibits the attachment and spreading of endothelial cells, is a negative regulator of cell growth and is therefore seen as a potential tumor suppressor [50–52]. The down-regulation of pro-angiogenic genes, for example, RNASE4 that has been shown to be involved in adhesion and tube formation of endothelial cells, underlines our hypothesis that hereby the development of immature, irregular vessels is promoted as suggested above [53,54]. (4) Upon further confirmation, these data suggest that re-expression of anti-angiogenesis genes may offer a therapeutic approach, applicable in a broad, cancer spanning manner.

Interestingly, some genes previously assigned crucial functions in angiogenesis did not reveal frequent transcriptional regulation in our meta-analysis. VEGF-A for instance, which was previously found to be up-regulated in various cancers [55], was significantly up-regulated in 3 data sets, while VEGF-B and VEGF-C were found to be significantly up-regulated in 2 out of 15 studies only. Other molecules that have been assigned angiogenic functions have also been found rarely regulated in our analysis such as FGF, HPA, Ang2, Il-8, and MMP-2. Given the known impact of these genes in cancer angiogenesis as demonstrated previously, our observations suggest that complex, additional mechanism besides transcriptional regulation contribute to the role of these genes to angiogenesis.

Taken together our results reveal the existence of angiogenesis related gene expression signatures in cancers. Hereby, it appears that loss of gene expression marks a central feature of angiogenesis in solid cancers. Based on these findings it may be speculated that gene down-regulation contributes substantially to the imbalance of angiogenesis genes in cancers, potentially contributing to the formation of immature vessels. Interestingly, we found genes to be regulated in cancers that have rarely been studied under malignant conditions before. In contrast, other genes that have been assigned central functions in cancer angiogenesis displayed infrequently transcriptional

regulation. Upon confirmation of our findings, these may lead to the development of new strategies in cancer therapy.

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Review

Markers for Detection of Prostate Cancer

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Abstract: Early detection of prostate cancer is problematic, not just because of uncertainly whether a diagnosis will benefit an individual patient, but also as a result of the imprecise and invasive nature of establishing a diagnosis by biopsy. Despite its low sensitivity and specificity for identifying patients harbouring prostate cancer, serum prostate specific antigen (PSA) has become established as the most reliable and widely-used diagnostic marker for this condition. In its wake, many other markers have been described and evaluated. This review focuses on the supporting evidence for the most prominent of these for detection and also for predicting outcome in prostate cancer.

Keywords: prostate cancer; biomarker; detection

1. Introduction

Prostate cancer (PCa) is now the most commonly diagnosed internal cancer in the western world and the second most lethal male malignancy. Prior to clinical availability of a blood test for prostate specific antigen (PSA) in the mid 1980s, most PCa patients were diagnosed with more advanced disease.

Advances in surgical and radiation therapies now result in cure for a proportion of patients who would not have benefitted from combination PSA/digital rectal examination (DRE)-based detection in the past. However, from a public health perspective, indiscriminate PSA population screening cannot be justified because of the low sensitivity and specificity of this blood test in identifying cancer, amongst other concerns [1]. Furthermore, there is prevailing uncertainty on the basis of the PSA blood test as to who will benefit from treatment with curative intent [2–5]. Recognition of these shortcomings has focussed research into markers to detect the presence of PCa and, more importantly, to try to discriminate between those patients harbouring indolent as opposed to aggressive disease including those for whom any form of local therapy will not prevent subsequent declaration of metastases.

There is a clinical imperative in being able to identify those who do and do not have PCa since, apart from the obvious advantage from detecting and treating the condition at the earliest stage possible, the diagnostic process itself is not without its problems. Suspicion of the presence of PCa, alerted as a result of an abnormal serum PSA and/or and abnormal DRE, requires an invasive procedure to provide histological confirmation with biopsies (usually $\geq$12) almost always obtained via imaging with a transrectal ultrasound (TRUS) probe to ensure spatial positioning of biopsy needles.

The majority of men who have prostatic biopsies do not have PCa diagnosed but continue to have raised serum PSA levels with ongoing concerns of a possible undetected PCa due to the imprecise nature of both the PSA test and TRUS biopsies. US Medicare-SEER analyses 1993 to 2001 indicated that, among men whose first recorded biopsy did not detect cancer, the likelihood of undergoing subsequent biopsies was 11.6% at 1 year and 38% at 5 years [6]. In addition, although the dreaded complication of life-threatening sepsis is fortunately uncommon but reported to be increasing [7], ~50% experience lesser symptoms after TRUS biopsy so diagnosing PCa remains problematic for many reasons. Consequently, it is not surprising that the need to be able to detect prostate cancer by a simple and reliable approach which can be repeated easily over time, is a diagnostic priority in the western world.

2. Scope of the Review

The strengths and weaknesses of PSA in the detection of PCa have been examined and published extensively and, since the topic has been reviewed very recently by Roobol *et al.* [8], who comprehensively examined the relevant medical literature with respect to the risk of developing PCa, this dissertation will not re-visit this marker. These authors reported that, despite its limitations, published evidence indicates that total serum PSA is the single most significant clinically-used predictive factor for identifying men at increased risk for PCa. Total PSA was superior to percentage free PSA, PSA velocity and Human kallikrein 2 (hK2), the most studied kallikrein protein after PSA (hK3) itself. A suspicious DRE, a family history of PCa, the presence of high grade prostatic intraepithelial neoplasia (HGPIN) or atypical small acinar proliferation (ASAP) and black ethnicity

also were reported to be important predictive factors. For men of screening age (50 to 70 years) a serum PSA of greater than 1.5 ng/mL has been found to indicate a greater than average risk up to 8 years (7.5-times greater risk *vs.* 1.5 ng/mL or less) for developing PCa [8]. In terms of diagnosing established PCa, PSA cut-offs are useful with the likelihood of men harbouring PCa greater the higher the level of serum PSA. However, it is important to note that PSA is a continuous variable and that a proportion of patients with low serum PSA levels <4 ng/mL have PCa of clinical significance [9].

Histological interpretation too has advanced with the Gleason scoring criteria agreed upon at the WHO ISUP consensus meeting in 2005 [10,11] being more useful clinically than those criteria with Gleason grading used previously. Much has been published in the Uro-pathology literature in relation to histological nuances in addition to describing a range of tissue biomarkers for PCa characterisation. These include apoptotic factors such as p53 and Bcl-2, the androgen receptor (AR), signal transduction factors within the EGF receptor family, cell cycle regulators exemplified by c-Myc, p16, p27, pRb and Ki67, cell adhesion and cohesion factors and factors involved in neo-angiogenesis, such as vascular endothelial growth factor (VEGF), VEGF receptors and nitric oxide [12,13]

Histopathology will not be addressed further nor will imaging adjuncts to biopsy. However, in interpreting the value of diagnostic biomarkers from biological fluids, which is the subject of this review, accurate histology and appropriate targeting with biopsy needles are essential. It is particularly important to be aware that the diagnostic biopsy reference on which biological fluid biomarkers are judged is deficient, varying with sites targeted and numbers of biopsies obtained, ranging in one study from 31.7% for sextant biopsies to 38.7%, 41.5%, and 42.5% for 12, 18 and 21 cores respectively in a study of 1000 patients [14].

Accurately determining the presence of disease is surprisingly difficult. Autopsy studies permit exhaustive and comprehensive scrutiny of the prostate histologically but are flawed in terms of clinical relevance for epidemiological reasons. True incidence derived from more epidemiologically-vetted clinical trials is almost non-existent given the rarity of complete cross-sectional 'end of study biopsies'. Sakr found in autopsy studies that, for the third through to the eighth decades of life, the incidence of PCa in a cohort of 1051 subjects was 7, 23, 39, 44, 65 and 72% [15].

Haas *et al.* [16] performed 18 core biopsies on autopsy prostates from 164 men who had no history of PCa. They reported that six-core biopsies were taken from each of the mid peripheral zone (MPZ), the lateral peripheral zone (LPZ), and the central zone (CZ). PCa was present in 47 (29%) prostates. Of the 47 cancers detected, 20 were clinically significant according to histologic criteria. Biopsies from the CZ did not detect any cancer that was not present in biopsies from either the MPZ or LPZ. The sensitivities of biopsies from the MPZ for clinically significant and insignificant cancer were 55% (95% CI = 32% to 77%) and 11%, respectively, compared with 80% and 33% for those from the MPZ and LPZ combined. In a single institutional study, Djavan *et al.* [17] found in an assessment of repeat biopsies that PCa was present in 22% (231/1051), 10% (83/820), 5% (36/737) and 5% (4/94) of first, second, third and fourth biopsies. Together with others, these studies illustrate the fact that the reference by which the sensitivity and specificity of biomarkers are judged is well short of perfect and this deficiency also should be borne in mind in assessing the predictive values of potential biomarkers.

3. Sources for Biomarker Analyses

A number of tissue/fluid sources and approaches have been employed in the quest to develop a non-invasive diagnostic test for PCa. These range from analysing tears [18] to changes in the odour of urine detected by dogs [19] but, in terms of commonly studied sources, prostatic fluid and blood have received most attention. Patient acceptance of venesection for taking samples of blood (for analysis as whole blood or serum) and providing urine is extremely high and laboratory staff are so conversant with the properties of both that they do not warrant detailed discussion in this manuscript.

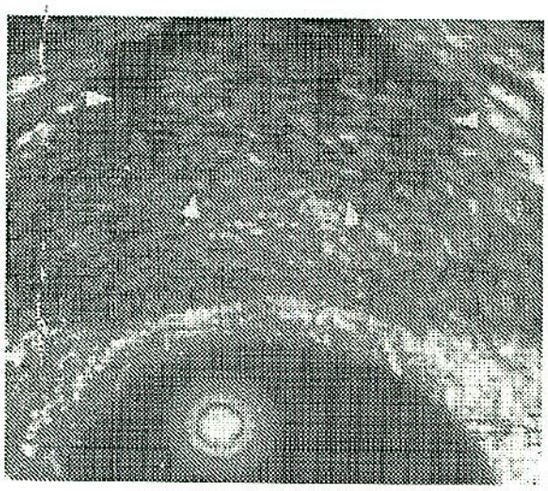
However, the use of prostatic fluid is not nearly as well appreciated. It is logical that fluid and/or cells obtained as directly as possible from the PCa itself should have differences in greatest abundance to those from normal prostates. Prostatic fluid may be obtained either as post-prostatic massage urine or ejaculate and/or post-ejaculate urine.

3.1. Post-Prostatic Massage Urine

Since DRE by itself dislodges too few retrievable prostatic cells, most studies using prostatic fluid have obtained specimens for analysis by prostatic massage [20–29] which is recommended to involve 3 sweeps per lobe, optimally depressing the prostate by 0.5–1 cm in a milking action to ensure sufficient numbers of prostatic cells are obtained [30]. Micturition following massage then washes prostatic fluid and cells dislodged into the prostatic urethra to the external meatus for collection.

Although there is a low likelihood of testicular cells or sperm being collected with this approach, one limitation is that only the MPZ is directly targeted for massaging with the anterior LPZ missed completely. In addition to being unpleasant, a further potential problem with vigorous prostatic massage is dislodging cancer cells into the systemic circulation with possible adverse consequences in terms of facilitating metastatic spread although evidence at 5 years does not support accelerated cancer evolution judged as a result of blood seeding cancer cells during operative intervention [31].

Figure 1. Ultrasound probe in rectum illustrating the relative relationship of rectum and prostate: white arrows indicate the transition zone (TZ).



3.2. Ejaculate

Prostatic fluid with disaggregated cells in ejaculate is expelled during orgasm following contraction of prostatic smooth muscle, distributed widely throughout the gland. In addition to the prostatic component, ejaculate contains contributions from the testes, epididymi and seminal vesicles and there is a tendency for a reduction in ejaculate volume with ageing. Certainly, following resection of the bladder neck with transurethral resection of prostate (TURP), there is retrograde emission so that seminal fluid mixes with urine to be expelled subsequently during micturition.

Much has been made of a reduced capability of older men to be able to perform but evidence in the medical literature [32,33] is to the contrary. Our experience has been that when the request is optional and not directly related to management outcome, a proportion of patients, although capable, will elect not to comply in producing a specimen in private and returning it promptly to the laboratory but, if there is a diagnostic imperative such as a sperm analysis, the response is quite different. Therefore, using ejaculate as a preferred body fluid for PCa testing is considered a practical option.

Impotence does not exclude an ability to ejaculate although firm tumescence certainly helps with this process. It is notable that erectile dysfunction is now well-recognized as a sentinel event for underlying cardiovascular disease and has been reported to be a predictor for the combined outcome of acute myocardial infarction, stroke and sudden death [34]. Therefore, an inability to ejaculate may translate as a surrogate for identifying a subset of patients for whom a diagnosis of PCa is less likely to affect life expectancy in the context of what is a relatively slowly progressing malignancy in the majority of cases.

4. Identifying Discriminating Markers

Although the cause of PCa continues to remain elusive, family histories indicate a strong inherited genetic predisposition in both the risk of developing PCa and the likelihood of a poor outcome should PCa be present. Not only is a family history of PCa with early age of onset and numbers of first degree relatives especially relevant in identifying predisposition [35], but a family history of breast cancer is also emerging as important especially in relation to BRCA2 mutations. Certainly, BRCA2 mutations are reported to be uncommon in sporadic PCa, estimated as <1% in US patients and 2.3% in UK patients [36] and also not common in hereditary PCa [37]. However, men who do develop PCa and harbour a BRACA2 mutation are more likely to have aggressive tumours and tend to develop these malignancies at an earlier age than other patients [36,38].

Genome-wide association studies (GWAS) indicate genetic heterogeneity for the onset of disease with numerous low risk loci described along with two notable high-risk loci at 8q24 and 7q31. The linked loci on 8q24 are located immediately downstream of the MYC gene that is upregulated in PCa [39,40] and linkage at 7q31 in African American men is in close proximity to the PODXL gene [41] which has been mutated in men with aggressive forms of the disease [42]. It is noteworthy that a combination of a family history of PCa together with genetic SNP variations increases the risk association significantly. In addition to genetic heterogeneity there is also a high likelihood that PCa genes/alleles act cooperatively in the aetiopathogenesis of the disease supporting the notion that it is unlikely that any one biomarker alone is likely to be conclusive in detecting and predicting outcome.

The Practical consortium recently reported the conclusion of their successive GWAS studies. They evaluated over 500,000 alleles (SNPs) in 3 separate cohorts of cases/controls (up to 30,000 men [43]. The consortium eventually identified alleles in 7 genomic regions (encompassing 11 genes) that were linked to PCa diagnosis. The allele penetrance ranged from 6–50% of the population and each allele altered PCa risk by 0.35–1.89 (odds ratio).

Chromosomal aberrations are very common in prostate tumours [44] and may act together in the progression of the disease. For example, the loss of both the PTEN gene on chromosome 10q and a recurrent 30Mb deletion on 21q, that leads to the fusion of TMPRSS2 and ERG (see section on ETS fusions), are common aberrations which appear to act together in disease progression [45]. Gain and amplification of the MYC gene on 8q24 [44,46] is of great interest especially given the strong GWAS linkage established immediately downstream of the gene [39,40].

5. Assay Approaches & Most Promising Markers

In reaching the current position with respect to identifying markers of detection, RNA profiling using microarray-based technologies has been particularly useful in tracking changes in gene expression during tumorigenesis. To date, the two most prominent candidate RNA biomarkers are the PCA3 gene and TMPRSS2 fusion transcripts. These, together with other genetic and non-genetic markers, are discussed below with respect to their likelihood in differentiating cancer from non-cancer and identifying indolent as opposed to aggressive and life-threatening tumours.

Given the many markers reported in the scientific literature, we have had to follow the practice employed in other reviews of selecting a short list of those which are considered to be most relevant and most promising.

5.1. Markers of Detection versus *Prognosis*

Arguably far more important than a diagnosis of PCa is the true nature of the condition in an individual patient. Although, without doubt, timely intervention does save lives, it is indisputable that many men who have treatment with curative intent for PCa are unlikely to benefit in terms of survival [47–49]. However, all these patients are at-risk of not inconsiderable side effects from intervention including previously unrecognised problems afflicting both them and, indirectly, their loved ones [50–53]. Contemporarily, the large majority of patients are diagnosed with PCa as a result of PSA testing and ~30% are stratified to low-risk disease with another ~25% already having occult metastases which declare themselves subsequently [54,55]. Because of the disparity between TRUS biopsy findings and those of whole-gland histology, there is a tendency for many urologists and patients to err on the side of intervention and to proceed to treatment with curative intent, accepting that a significant proportion of patients will have treatment that will not affect survival or benefit their well-being.

In order to identify the minority of patients stratified as having low-risk PCa following biopsy but whose disease is likely to progress, Klotz initiated an active surveillance protocol [55] undertaking a period of very intense 2-year monitoring of these men with PSA testing, DRE and at least yearly TRUS biopsies to unearth the ~25% of men who actually have more aggressive disease not evident from initial TRUS biopsies [55]. This approach has been emulated by colleagues in the UK, US and Europe and a randomised study has been undertaken to examine this strategy further, in the

Surveillance Therapy Against Radical Treatment (START) trial. Klotz has the largest and most mature study of active surveillance and has reported an 85% overall survival and 99% disease specific survival with a median follow-up of 8 years (range 2–11 years) [55]. Clearly there is an overwhelming need for a prognostic profile that would, non-invasively, permit accurate stratifying of PCa patients.

A distinction between detection, prediction and prognosis is not clear for many of the markers reported with some having the potential to fulfil both roles. Identification of potential markers has been based largely on histological expression of candidates but, interestingly, it is common for a disparity to exist between tissue expression and detection in various biofluids [56]. Furthermore, although many of the biomarkers identified have been at a protein level or produce protein(s)/peptides, there are many such as microRNAs, non-coding RNAs and metabolic products not directly associated with proteins or peptides contributing to the pot-pourri of potentially useful biomarkers for identifying the presence and nature of PCa non-invasively.

6. Comments on Selected Genetic Markers

A selection of biomarkers is listed in tables 1 & 2 (below). For the multivariate analysis, AIC-based backward selection was used to drop insignificant terms.

Table 1. Post-prostate massage-urine biomarkers for the detection of prostate cancer from Laxman *et al.* [26].

Variable	Coefficient	*P*
Univariate logistic regression analysis		
GOLPH2	0.4444	0.0002
SPINK1	0.25	0.0002
PCA3	0.187	0.001
TMPRSS2:ERG	0.609	0.034
ERG	0.043	0.166
TFF3	0.11	0.189
PSA (serum)	0.0151	0.376
AMACR	0.049	0.45
Multivariate logistic regression analysis		
SPINK1	0.308	7.41E-05
PCA3	0.191	0.003
GOLPH2	0.372	0.004
TMPRSS2:ERG	0.924	0.006

Table 2. Potential urine markers for monitoring prostate cancer, modified from Jamaspishvili *et al.* [57].

Symbol	Description	Type of marker				Ref.	Body Fluid
		DNA	RNA	Protein	Metabolite		
8-OhdG	8- Hydroxydeoxyguanosine	+			+	[58]	U
ANXA3	Annexin A3			+		[59–62]	PD
BHUAE	Basic human urinary arginine amidase			+		[63]	U

Table 2. *Cont.*

F3	Coagulation factor III (thromboplastin, tissue factor)		+		[64]	U
GSTP1	Glutathione S-transferase P 1	+			[65–69]	PM
LOH	Loss of heterozygosity e.g., loss of PTEN	+			[70,71]	PM
MCM5	Minichromosome maintenance complex component 5		+		[72]	U
MMP9	Matrix metalloproteinases 9		+		[73–75]	U
PIP	Prostatic inhibin-like peptide		+		[76]	U
PSA	Urinary prostate specific antigen		+		[77]	U
S100A9	S100 calcium binding protein A9 (alias calgranulin B)		+		[78]	PM
SAR	Sarcosine			+	[79]	PD
SRD5A2	Steroid 5-alpha-reductase type 2		+		[80]	U
TERT	Telomerase reverse transcriptase	+			[81–83]	PM
TMSB15A	Thymosin beta 15a		+		[84]	U
VEGF	Vascular endothelial growth factor		+		[85,86]	U

Key: PM~ post-prostate-massage urine, PD ~ post digital examination urine, U~ voided urine.

6.1. PCA3

PCA3, previously known as *DD-3*, is a non-coding gene first reported by Bussemakers *et al.* [87]. mRNA of the PCA3 gene is highly over-expressed (median 66-fold) in >95% of PCa tissue compared with normal or benign prostatic tissue of the same patients [20,87]. PCA3 has been assayed from urine following prostatic massage in 11 separate clinical studies totalling 2737 men from Western countries [21–24,28,30,88–93] with an overall sensitivity of 69% and specificity of 70% for men with PCa.

The role of PCA3 in clinical practice as a commercially-available test remains uncertain with most advocates indicating a place in patients who have already had TRUS biopsies with a negative result for cancer but in whom PCa remains suspected. Another application may be as an adjunct to repeat DREs, serum PSA estimations and prostatic biopsies in following men diagnosed with low-risk PCa who have elected to be monitored carefully in active surveillance protocols rather than have treatment with curative intent. However, more recently, a number of investigators have combined their PCA3 findings with those from other markers to try to incrementally improve detection rates since it is clear that no one marker by itself is adequate for detecting all cases of PCa.

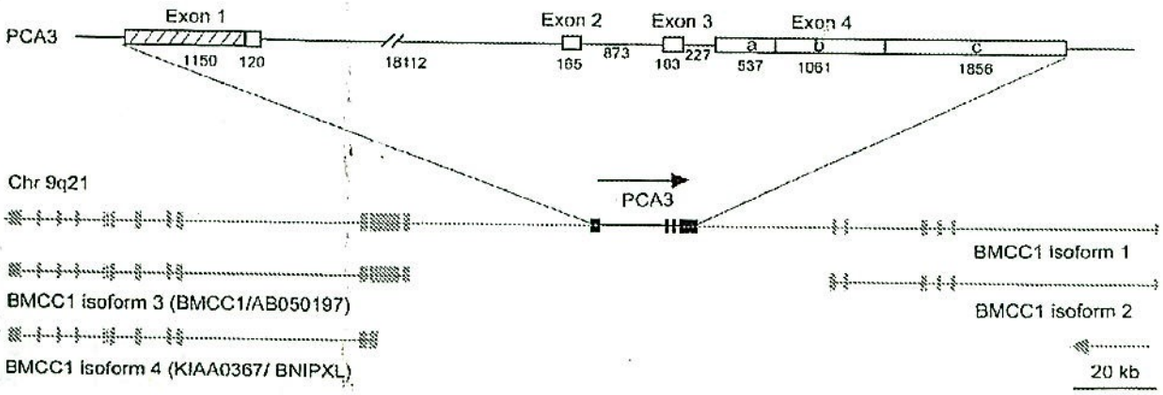
6.2. PCA3 Redefined

Although upregulation of *PCA3* was first described in PCa specimens in 1999 [87], it was another 10 years before the complete structure of the gene was resolved [94] with description of new start sites, additional exons and a range of novel alternatively-spliced transcripts (Figure 2), some of which are more highly enriched in PCa and metastases, expression not normally being seen outside the prostate. *PCA3* is embedded in an anti-sense orientation within an intron of another much larger gene, *BMCC1-1*. In contrast to *PCA3*, a weakly conserved non-coding gene, *BMCC1-1* has a conserved protein domain with an established role in Rho-signalling, cellular transformation and metastasis [94,95].

PCA3 expression is androgen responsive with alternatively spliced *PCA3* mRNA transcripts differentially upregulated in up to 95% of PCas and metastases [94–96]. *PCA3* mRNA levels have been detected in primary and metastatic PCa tissue specimens at up to 43 and 110 times the levels expressed in normal prostate tissue, respectively, compared with up to a 3 fold increase in benign prostatic hyperplasia (BPH) [94]. By combining *PCA3* with 2 other biomarkers (*i.e.*, *PSMA* and *hepsin*) 100% of tissue samples (55 in total) were classified as either PCa or BPH [97]. The recent discovery of novel *PCA3* transcripts, inclusive of exons 2a and 2b, that are more highly enriched in PCa and metastases and the identification of complementary biomarkers [26,97] tantalises investigators who seek further improvements in PCa detection and characterisation through a non-invasive molecular approach.

PCA3 is expressed at high levels in metastatic PCa but, empirical data do not support an association between *PCA3* upregulation and clinical stage, Gleason scores, tumour volume, pathological stage or cancer progression [93,98]. Nevertheless, the genomic inter-relationship between *PCA3* and the different *BMCC1* isoforms [94] may not be coincidental and investigations are underway for clues to the association between *BMCC1* regulation and PCa initiation and progression [94].

Figure 2. Redefined PCA3 gene.



The truncated form of *PCA3* described by Bussemakers *et al.* [87] was extended by Clarke *et al.* [94] using a thorough 5' and 3' RACE analysis of the mature message and DNA and RT-PCR sequencing in their description of the complete *PCA3* gene with:

- Exon 1 over 10 times longer than previously reported
- 4 new transcription start sites

- 4 polyadenylation sites
- 2 new differentially spliced exons
- PCA3 embedded in intron 6 of the *BMCC1-1* gene

6.3. ETS Gene Fusions

ETS gene fusion provides a highly specific biomarker assay for the detection and prognosis of PCa. In 2005, microarray analyses identified two E26 avian erythroblastosis virus (ETS) family transcription factors, ETS related gene (ERG) and ETS variant gene 1 (ETV1), as potentially important PCa biomarkers [99]. Aberrant expression of these ETS genes was found coincident with gene fusion between the 5′ untranslated region of androgen-regulated transmembrane protease, serine 2 (TMPRSS2) gene and the ETS genes *ERG* (chromosome 21q22.2), *ETV1* (7p21.2), *ETV4* (17q21) or *ETV5* (3q28) or one of ~20 other gene fusion variants [100–103]. ERG and *ETV1* rearrangement and overexpression has been reported to occur in the majority (50–60%) of PCas with the most common variant being a recombination between exon 1 of *TMPRSS2* and exon 4 of *ERG*, designated T1/E4 [104–108] represented in ~85% of all reported fusion cases [101,109]. Identification of ETS fusions in the 'post-prostatic massage urines' from men with PCa using RT-PCR has a sensitivity of 37%, a specificity of 93%, a negative predictive value of 36% and positive predictive value of 94% [25,110,111].

ETS gene fusions have an important role in PCa development and prognosis. *ERG* and *ETV1* show mutually exclusive overexpression in PCa tissues, suggesting a redundant function in PCa development. In addition, ERG and ETV1 gene fusions have been detected in only a limited number of BPH and High-Grade Prostate Intra-epithelial Neoplasia (HGPIN) lesions, albeit in the absence of ETS upregulation [112,113]. In one cohort *TMPRSS2–ERG* T1/E4 fusion associated upregulation of ERG was found to be highly prognostic of disease recurrence [114] independent of grade, stage and PSA level [105]. ETS fusions are strongly linked with survival when associated with loss of PTEN ($P < 0.001$) [115] and have been identified in 48% of men who died of castrate-resistant disease [116]. Fusion-related upregulation of ERG is increased by oestrogens and the incidence and prognostic significance of these gene fusions may vary with cohort race/ethnicity and different techniques of detection [108,115,117–121]. Electrophoresis after RT-PCR using fusion specific primers followed by Southern transfer and probing with fusion specific probes appears to be a more rigorous method of detection [110].

7. Other Markers of Detection

7.1. Early Prostate Cancer Antigen

Leman *et al.* [122] reported results on a serum biomarker called early prostate cancer antigen (EPCA) using an antibody assay against the EPCA-2.22 epitope. The study involved 385 men and reported a 92% specificity for healthy men and men with benign prostatic hyperplasia and a 94% sensitivity for overall PCa detection. In addition, the authors indicated that EPCA-2.22 was highly accurate in differentiating between localized and extracapsular disease [122]. More recently, the same group reported on a second epitope of EPCA, designated EPCA-2.19, which they found provided almost identical results [123]. Despite the promising nature of these findings, more details are required

including the results from multicentre studies. As a result of challenges to the work regarding EPCA-2, the place of this candidate as a PCa marker is currently uncertain [124].

7.2. GOLPH2

GOLPH2/GP73, elevated in PCa tissues, is detectable in post-prostatic massage urine from PCa patients. GOLPH2 immunihistochemical staining indicates a perinuclear Golgi-type pattern that is more intense in PCa glands compared with normal glands ($P < 0.001$) [125]. In a large study by Kristiansen *et al.* [125], upregulation of GOLPH2 protein was reported in 567 of 614 tumours (92.3%) and AMACR in 583 of 614 tumours (95%) (correlation coefficient 0.113, $P = 0.005$). Importantly, GOLPH2 immunohistochemical analysis indicates a lower level of intratumoral heterogeneity (25 *vs.* 45%). Further, GOLPH2 upregulation was detected in 26 of 31 (84%) AMACR-negative PCa cases. Using PCR analysis, Laxman *et al.* [26] demonstrated increased GOLPH2 levels in post prostatic massage urines as a significant predictor of PCa when multiplexed with PCA3 and SPINK1 [26].

7.3. SPINK1

SPINK1 (also referred to as TATI) is a biomarker for PCa that can be detected in prostatic massage urine. SPINK1, a trypsin inhibitor secreted from pancreatic acinar cells, is thought to function in the prevention of trypsin-catalyzed premature activation of zymogens within the pancreas and the pancreatic duct. Mutations of this gene are associated with hereditary pancreatitis and tropical calcific pancreatitis [127,128]. SPINK1 is also overexpressed in other cancers, and an elevated serum level is an independent prognostic indicator in many of these, as reviewed by Paju and Paju [129,130]. Tomlins *et al.* [126] found SPINK1 expression to be an outlier (using a meta-COPA outlier meta-analysis) exclusively in a subset (10%) of ETS rearrangement-negative cancers and only in 6% of ETS negative PCa ductal adenocarcinoma variants [131]. Laxman *et al.* [26] showed that a multiplexed qPCR assay including SPINK1 on sedimented urine from patients presenting for prostate biopsy or prostatectomy outperformed serum PSA or *PCA3* alone. SPINK1 expression in urine is also an independent predictor of biochemical recurrence after resection [26,126]. The aggressive 22RV1 prostate cancer cell line expresses SPINK1 and SPINK1 knockdown attenuates 22RV1 invasion, suggesting a functional role in ETS rearrangement-negative prostate cancers [126].

7.4. α-Methylacyl Coenzyme A Racemase (AMACR)

AMACR is a very commonly used immunohistochemical marker for PCa which can also be detected in the urine of PCa patients [26,57,125]. AMACR, also known as P504S, is involved in β-oxidation of branched-chain fatty acids and fatty acid derivates. AMACR is consistently upregulated at both the mRNA and protein levels in prostate tissue [57], however, its usefulness as a biomarker in urine is controversial [26,132,133]. Western blot analysis for AMACR was used on voided urine after TRUS and biopsy, showing a 100% sensitivity and 58% specificity for PCa detection in one group of patients with negative biopsy findings [133].

8. Specific Prognostic Markers

As the second step in the non-invasive detection process, prognosticating is less well established with the possible exception of tissue characterisation using markers, with the presumption that the biopsied samples are representative of each gland's malignancy status. Furthermore, although a number of markers have been reported to indicate aggressive and lethal PCa, there are considerably fewer indicators of low-risk PCa which is of particular relevance if an active surveillance or close observation clinical approach is to be pursued.

8.1. AZGP1 & hCAP-D3

Zinc-alpha2-glycoprotein (AZGP1) is present in high concentration in human seminal plasma and considered to be a soluble homologue of MHC-I [135]. Hale *et al.* [136] reported that Anti-AZGP1 monoclonal antibodies reacted strongly with normal prostatic epithelium but not with other components of prostate or seminal vesicles and that 35 of 48 PCas also reacted with anti-AZGP1 antibodies. However, it was notable that high-grade tumours expressed significantly less AZGP1 than moderate-grade tumours. In addition, men with AZGP1-producing PCas had elevated levels of serum AZGP1 relative to normal age- and race-matched controls ($P < 0.02$). More recently, Bondar *et al.* [137] have developed a serum bio-assay for AZGP1 [137].

Henshall *et al.* [138] reported on the highly predictive capacity of AZGP1 expression in radical prostatectomy (RP) specimens. More recently, Lapointe *et al.* [139] reported a combination of immunohistology for AZGP1 and RNA *in situ* hybridisation for hCAP-D3 expression in tissues from 225 RP specimens which distinguished even more clearly those patients whose tumours would recur and those whose would not ($p = 0.0002$). Loss of both hCAP-D3 and AZGP1 expression was associated with the worst outcomes whereas expression of both markers indicated a very low recurrence rate raising the potential for identifying those patients better suited to an active surveillance protocol than an active treatment approach with curative intent.

8.2. Prostatic Acid Phosphatase (PAcP)

Prior to the introduction of serum PSA, PAcP was used widely to indicate advanced PCa, but fell into disuse. However, a number of publications have promoted a renewed role for this enzyme as a prognostic indicator in early disease. Moul *et al.* [140] reported on 295 patients who underwent RP and compared the value of pre-treatment serum PSA and PAcP. The Kaplan-Meier disease-free survival rate at 4 years was 78.8% for men with a PAcP < 3 ng/mL and 38.8% for those with PAcP ≥ 3 ng/mL, which was significant overall ($p < 0.001$) and, when pre-treatment PSA was <10 ng/mL ($p = 0.047$), ≥10 ng/mL ($p = 0.012$). They concluded that PAcP testing added prognostic information to pre-treatment PSA values and that PAcP was an independent predictor of recurrence [140].

More recently, Han *et al.* [141] reviewed pre-operative PAcP levels in 1681 men who proceeded to RP and confirmed that PAcP was an independent predictor of tumour recurrence ($p < 0.001$). Most recently Fang *et al.* [142] examined case histories of 193 patients with clinically localized PCa, a Gleason score ≥7 and/or a PSA level of ≥10 ng/mL treated with ^{103}Pd brachytherapy and supplemental external beam radiotherapy (EBRT) between 1992 and 1996. The 10-year cause-specific survival

(CSS) rate for patients with a pre-treatment PAcP level < 1.5, 1.5–2.4 and ≥2.5 U/L was 93%, 87%, and 75%, respectively (P = 0.013). The 10-year CSS rate for patients with a PSA level < 10, 10–20, and >20 ng/mL was 92%, 76%, and 83%, respectively (P = 0.393). On Cox multivariate regression analysis, PAcP (hazard ratio 1.31, P < 0.0001) and Gleason score (hazard ratio 2.37, P = 0.0007) were associated with CSS. PSA was not predictive of CSS (P = 0.393) but PAcP was a stronger predictor of CSS than PSA or Gleason score these men with higher risk PCa.

9. Multiple Markers

In a recent study of 423 consecutive patients treated with radical prostatectomy for clinically localized PCa, pre-operative plasma levels of Endoglin, interleukin-6 (IL-6), interleukin-6 soluble receptor (IL-6sR), transforming growth factor-beta1 (TGF-beta1), urokinase plasminogen activator (uPA), urokinase plasminogen inhibitor-1 (PAI-1), urokinase plasminogen receptor (uPAR), vascular cell adhesion molecule-1 (VCAM1), and VEGF were measured using commercially available enzyme immunoassays. Plasma IL-6 (P = 0.03), IL-6sR (P < 0.001), TGF-beta1 (P = 0.005), and V-CAM1 (P = 0.01) achieved independent predictor status after adjusting for the effects of standard post-operative features. After stepwise backward variable elimination, a model relying on RP Gleason sum, IL-6sR, TGF-beta1, VCAM1, and uPA improved the predictive accuracy of the standard post-operative nomogram model by a modest 4%. The long-term follow-up of these patients will be of particular interest [143].

9.1. Annexin A3 (ANXA3)

ANXA3, a recently identified PCa biomarker, has an inverse relationship to PCa progression and can be detected in the urine of PCa patients [144]. ANXA3 belongs to a family of calcium and phospholipid binding proteins that are implicated in cell differentiation and migration, immunomodulation, bone formation and mineralization in PCa metastasis [145]. ANXA3 has an inverse relationship to cancer and the immunhistochemical staining in prostatic tissue correlates with disease progression, Gleason score and malignancy [144]. The presence of ANXA3 in urinary exosomes and prostasomes might be the reason for its remarkable stability in urine [144,147]. ANXA3 has been quantified by western blot in the urine samples of patients with negative DRE findings and low total PSA (2–10 ng/mL^{-1}), which is the clinically relevant group facing the biopsy dilemma. Combined readouts of PSA and urinary ANXA3 gave the best results with the Area Under the Receiver Operating Curve (AUROC) of 0.82 for a total PSA range of 2–6 ng/mL^{-1}, 0.83 for a total PSA range of 4–10 ng/mL^{-1} and 0.81 in all patients [147].

10. MicroRNA Profiling

MicroRNAs (miRs) are small non-coding RNA molecules with a length of 21–25 nucleotides. They are mostly located within inter-genic chromosomal regions and can be found as solitary or clustered gene units. MiRs post-transcriptionally regulate gene expression by binding to complementary sequences within the 3'UTR of mRNAs. In most cases annealing leads to a modest down regulation of gene expression, although a few upregulating miRs have been described [148]. The first 6 to 8 bases of

a miR direct its targeting and so each species may bind up to thousands of mRNAs. Very recently, researchers have demonstrated that not only can miRs indirectly disable gene function by associating with mRNAs, they can silence genes directly by adding methlyl groups and it has been proposed that epigenetic silencing by DNA methylation depends on the ratio of miRNA to its target RNA [149].

Profiling of PCa cell lines and specimens has been performed for miR expression. The first dedicated report by Porkka *et al.* [150] found expression of many species and that their expression varied with malignancy and with the cell's androgen receptor status. Follow up reports have confirmed this finding and extended the profiling of prostate-specific miRs [151]. Evidence to date suggests that many miRs with altered expression in PCa are generic to malignancy. This includes over expression of miR-21 (which is known to suppress members of the p53 network [152,153], over expression of miRs-15a and 15b, and loss of miRs 145/34c/221 and 222. The expression of these miRs appears to be androgen dependent (in part) and results from chromosomal and epigenetic alterations. Few studies have assessed the translational use of these miRs [154,155].

11. Metabonomics/Metabolomics

Metabonomics/metalolomics involves the study of metabolites in a biological sample on a global scale, with the aim of understanding its metabolic profile and correlating it with a biological, physiological or clinical state. In oncology the identification of metabolites that have been observed to be different, and characteristically so in PCa compared with non-malignant prostates, has the potential to lead to the discovery of biomarkers that are useful for diagnosis and prognosis as well as for monitoring effectiveness of therapy. It has been known for many years that citrate and its preferred prostatic cation zinc are reduced in PCa epithelial cells, reflected in gland luminal contents and prostatic fluid whereas levels of choline and spermine are increased. However, unlike choline, spermine is decreased in prostatitis, this condition causing an elevation in serum PSA which can be a confounder in the diagnosis of PCa using serum PSA as the sole biomarker [156–161].

A variety of mass spectroscopic techniques is available to provide analytical information on small molecular metabolites, proteins and other molecules. They include tandem mass spectrometry (MS/MS) [162], electron ionisation (EI), electrospray, liquid secondary ionisation mass spectroscopy (LSIMS) [163], collision-induced dissociation tandem mass spectrometry (CID/MS/MS) [164] and fast atom bombardment mass spectrometry (FAB-MS) [165].

However, nuclear magnetic resonance spectroscopy (NMR or MRS) [166] is the method most commonly reported. Although studies have examined the prostate by *in vivo* MRS using specially designed endo-rectal coils for this purpose, this approach has decreased in recent times with an emerging interest in perfoming the MRS analyses on prostatic fluid itself or on biopsies of prostatic tissue. A number of recent reviews cover the topic in detail [166–168], thus this section focuses on a number of topical contributions and on the most recent developments.

Using MRS, Lynch and Nicholson [169] examined prostatic fluid from 26 patients (10 BPH, 4 PCas and 12 controls) by prostatic massage and from ejaculate from 11 men with vasal aplasia. They reported significantly lower citrate: spermine ratios in PCa ($p < 0.02$) and that findings from ejaculate paralleled those obtained following prostatic massage. Also using MRS a difference in citrate levels between seminal fluid from 3 patients with PCa compared with specimens from non-PCa donors, was

demonstrated by Averna *et al.* [170] who subsequently confirmed this in a study of 61 participants, of whom 16 without and 21 with PCa donated seminal fluid, and 17 without and 7 with PCa donated expressed prostatic secretions. Mean citrate levels compared with those from controls were 2.7-fold lower in patients with PCa for semen (132.2 ± 30.1 *vs.* 48.0 ± 7.9 mM, $p < 0.05$) and expressed prostatic secretions [171]. Similarly, Serkova *et al.* [172] reported that the concentrations of citrate, spermine and myo-inositol in human expressed prostatic secretions are age-independent markers of PCa. The median concentrations of all three metabolites were significantly decreased in PCa subjects ($n = 52$) when compared with healthy controls ($n = 26$), with citrate decreasing from 349 mM to 114 mM ($p < 0.0001$), spermine decreasing from 57 mM to 27 mM ($p < 0.002$) and myo-inositol decreasing from 21 mM to 7 mM ($p < 0.0001$). These three markers were highly predictive of PCa with AUROC values of 0.89 for citrate, 0.87 for myo-inositol, and 0.79 for spermine. In addition, some other metabolites such as pyroglutamate and uracil [173,174] continue to emerge as potential biomarkers to indicate the presence of PCa and its prognosis.

In recent years a number of reports have emerged that use high-resolution magic angle spinning (HR-MAS) proton NMR spectroscopy to analyse the metabolite content in intact human prostate tissue samples obtained via biopsy or following surgery. As HR-MAS MRS is non-destructive, NMR and histopathological data can in principle be obtained for the same sample [175,176]. For example, the concentration of citrate ($r = 0.763$, $p < 0.001$) and spermine ($r = 0.604$, $p < 0.018$), measured by HR-MAS MRS, has been shown to correlate with the volume percentage of normal, healthy prostatic epithelial cells assessed histologically on the same samples [175]. This is physiologically significant, as spermine is a proposed endogeneous inhibitor to PCa growth, and the findings mirror solution MRS results [173], as well as other HR-MAS changes reflecting a decrease in spermine and other polyamines in PCa.

In two contributions, Swanson *et al.* [176,177] observed different metabolite profiles in healthy glandular and stromal tissue compared with PCa in post-surgical prostate samples using HR-MAS MRS. Healthy glandular tissue contained significantly higher levels of citrate (43.1 ± 21.2 mmol/kg) and polyamines (PA) (18.5 ± 15.6 mmol/kg), and lower levels of the choline-containing compounds choline (3.52 ± 1.44 mmol/kg), phosphocholine (PC) and glycerophosphocholine (GPC) ($[\text{t-choline}]_{\text{mean}} = 7.06 \pm 2.36$ mmol/kg) than PCa tissue ($[\text{Citrate}]_{\text{mean}} = 19.6 \pm 12.7$ mmol/kg, $p < 0.01$; $[\text{PA}]_{\text{mean}} = 5.28 \pm 5.44$ mmol/kg, $p < 0.01$; $[\text{t-choline}]_{\text{mean}} = 13.8 \pm 7.4$ mmol/kg, $p < 0.01$). Healthy stromal tissue contained lower levels of choline compounds ($[\text{t-choline}]_{\text{mean}} = 7.04 \pm 3.10$ mmol/kg, $p < 0.01$) than PCa, but was similarly low in citrate ($[\text{Citrate}]_{\text{mean}} = 16.1 \pm 5.6$ mmol/kg) and polyamines ($[\text{PA}]_{\text{mean}} = 3.15 \pm 1.81$ mmol/kg) [177]. In addition, levels of taurine, myo-inositol and scyllo-inositol were higher in PCa than either healthy tissue component [176]. Remarkably, larger increases in choline and decreases in citrate and polyamines ($p = 0.05$) correlated with more aggressive cancers [176]. Using an improved quantitation method by combining HR-MAS MRS with T_1 and T_2 relaxation times optimisation and internal concentration standards, Swanson *et al.* [177] confirmed their earlier observations [176]. In addition, they observed elevated concentrations of lactate (69.8 ± 27.1 mmol/kg, $p < 0.01$) and alanine (12.6 ± 6.8 mmol/kg, $p < 0.01$) in PCa compared with healthy glandular or stromal tissue [177]. Elevated levels of lactate (1.59 ± 0.61 mmol/kg, $p < 0.0001$) and alanine (0.26 ± 0.07 mmol/kg, $p < 0.0001$) in PCa versus healthy samples ($[\text{lactate}]_{\text{mean}} = 0.61 \pm 0.28$ mmol/kg; $[\text{Ala}]_{\text{mean}} = 0.14 \pm 0.06$ mmol/kg) were also recently confirmed by combining HR-MAS MRS with the

electronic reference to access *in vivo* concentrations (ERETIC) method [178] as external concentration standard on TRUS-guided biopsy samples [179]. Finally, HR-MAS MRS can be combined with the use of ^{13}C-labeled substrates as probes to characterise the various metabolic pathways involved in PCa in greater detail [180,181].

As new biomarkers for PCa are discovered, it is highly likely that newer and more sensitive methods of detection promise to reveal even more markers in this technology-driven discipline. For example a recent study of metabolite profiles in urine, blood plasma and surgical tissue samples of benign, PCa and metastatic PCa, characterised levels of a large number of metabolites by LC-MS and GC-MS, and found that sarcosine levels were increased in PCa ($p = 0.0004$ in urine sediments, and $p = 0.0025$ in urine supernatants) and the increase correlated with the invasiveness of the cancer [181]. Indeed, the mere addition of exogenous sarcosine caused benign prostate epithelial cells to assume an invasive phenotype.

In the same vein, a combination of different analytical techniques with different strengths that complement each other is likely make the process of biomarker discovery more robust. The successful route to future PCa biomarker discovery will involve a combination of different techniques and hinges on the integration of information gained from different tissues (urine, EPF, plasma, biopsies) different analytical techniques (MRS and MS) and even different hierarchical levels (genomics, proteomics and metabolomics).

12. Conclusions

It is expected that, when accurate markers for detection become established in routine clinical practice, those patients with normal marker profiles will be able to be spared TRUS biopsies with the accompanying potential for untoward effects. If these men are considered to be at-risk of developing PCa subsequently, non-invasive testing for abnormal marker profiles will be able to be repeated, as appropriate. By contrast, an abnormal detection marker profile indicating the presence of tumour would select patients for prognostic profiling and prostatic biopsies. A favourable prognostic profile would increase confidence in pursuance of an active surveillance or close observation management strategy. On the other hand, a most unfavourable prognostic profile would indicate a very high likelihood that the disease is no longer localised and is expected to be already micro-metastatic. Thus, the advent of reliable markers for detection and prognosis promises to facilitate tailored management to individuals with levels of confidence not currently available. At this point in time, it is evident that no one marker by itself is able to accurately detect all cancers with 100% sensitivity and specificity, let alone reliably predict outcome, so there is a move to include several markers to improve detection rates. Currently, this usually involves total serum PSA, amongst others, with the most commonly used molecular marker PCA3, testing for which is available commercially.

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PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To:
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PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT AND THE WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY, OR THE DECLARATION

(PCT Rule 44.1)

Applicant's or agent's file reference GHDX-046WO	Date of mailing *(day/month/year)* 27 FEB 2012 FOR FURTHER ACTION See paragraphs 1 and 4 below
International application No. PCT/US 11/45253	International filing date *(day/month/year)* 25 July 2011 (25.07.2011)
Applicant GENOMIC HEALTH, INC.	

1. ☒ The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.

   Filing of amendments and statement under Article 19:
   The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

   When? The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.

   Where? Directly to the International Bureau of WIPO, 34 chemin des Colombettes 1211 Geneva 20, Switzerland, Facsimile No.: +41 22 338 82 70

   For more detailed instructions, see *PCT Applicant's Guide*, International Phase, paragraphs 9.004 – 9.011.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.

3. ☐ With regard to any protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

   ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with any request to forward the texts of both the protest and the decision thereon to the designated Offices.

   ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Reminders**

   The applicant may submit comments on an informal basis on the written opinion of the International Searching Authority to the International Bureau. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established. Following the expiration of 30 months from the priority date, these comments will also be made available to the public.

   Shortly after the expiration of 18 months from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau before the completion of the technical preparations for international publication (Rules 90*bis*.1 and 90*bis*.3).

   Within 19 months from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later); otherwise, the applicant must, within 20 months from the priority date, perform the prescribed acts for entry into the national phase before those designated Offices.

   In respect of other designated Offices, the time limit of 30 months (or later) will apply even if no demand is filed within 19 months.

   For details about the applicable time limits, Office by Office, see www.wipo.int/pct/en/texts/time_limits.html and the *PCT Applicant's Guide*, National Chapters.

Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer Lee W. Young PCT Helpdesk: 571-272-4300 Telephone No. PCT OSP: 571-272-7774

Form PCT/ISA/220 (July 2010)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference GHDX-046WO	FOR FURTHER ACTION	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
International application No. PCT/US 11/45253	International filing date *(day/month/year)* 25 July 2011 (25.07.2011)	(Earliest) Priority Date *(day/month/year)* 27 July 2010 (27.07.2010)
Applicant GENOMIC HEALTH, INC.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 5 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of:

   ☒ the international application in the language in which it was filed.

   ☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

   b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).

   c. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. ☐ **Certain claims were found unsearchable** (see Box No. II).

3. ☒ **Unity of invention is lacking** (see Box No. III).

4. With regard to the **title**,

   ☒ the text is approved as submitted by the applicant.

   ☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

   ☒ the text is approved as submitted by the applicant.

   ☐ the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. With regard to the **drawings**,

   a. the figure of the drawings to be published with the abstract is Figure No. 1

   ☒ as suggested by the applicant.

   ☐ as selected by this Authority, because the applicant failed to suggest a figure.

   ☐ as selected by this Authority, because this figure better characterizes the invention.

   b. ☐ none of the figures is to be published with the abstract.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 11/45253

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

----------Continued in Supplemental Box----------

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-17

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 11/45253

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68; G01N 33/574 (2011.01)
USPC - 435/6.11, 6.12, 6.14, 7.23
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - C12Q 1/68; G01N 33/574 (2011.01)
USPC - 435/6.11, 6.12, 6.14, 7.23

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/6.1, 7.1
(Text Search)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST - DB=PGPB,USPT,USOC,EPAB,JPAB; PLUR=YES; OP=ADJ; Google Scholar
search terms: prostate cancer, cancer, cancer$, tumor, tumor$, prostate, recurrence, reocurence, reoccurrence, recur$, expression, panel, profile, express$, gene, marker, biomarker, TMPRSS2, fusion, fused, erg, translocation, interva, ALCAM, apoe, cdc20, il1b, il11,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2009/0298082 A1 (KLEE et al.) 03 December 2009 (03.12.2009) para [0009]; [0010]; [0012]; [0023]; [0024]; [0027]; [0028]; [0031]; [0034]; [0036]; [0055]; [0056]; [0061]; [0062]; [0075]; tables 1-3.	1-5, 11-14 and 16-17 --- 6-10 and 15
Y	US 2009/0233279 A1 (GLINSKII) 17 September 2009 (17.09.2009) para [0016]; [0017]; [0023]; [0033]; [0095]; [0123]; [0131], [0140].	6-7, 10
Y	US 2007/0212702 A1 (TOMLINS et al.) 13 September 2007 (13.09.2007) para [0006], [0094], [0194], [0197], [0411], [0418].	8-9 and 15

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 February 2012 (17.02.2012)	27 FEB 2012
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 11/45253

continuation of Box No III: Observations where unity of invention is lacking

Group I: Claims 1-17, drawn to a method for determining a likelihood of cancer recurrence in a patient with prostate cancer, comprising: measuring an expression level of at least one gene in a biological sample comprising prostate tissue obtained from the patient, wherein the at least one gene comprises a gene from Tables 3A, 3B, 4A, 4B, 5A, 5B, 6A, 6B, 7 A, 7B, 8A, 8B, 10A, or 10B, or genes that coexpress with the at least one gene; predicting a likelihood of cancer recurrence for the patient; wherein an expression level of any gene in Tables 3A, 4A, 5A, 6A, 7 A, 8A, and 10A is positively associated with an increased risk of recurrence, and wherein an expression level of any gene in Tables 3B, 4B, 5B, 6B, 7B 8B, and 10B is negatively associated with a increased risk of recurrence; or a method for determining a likelihood of upgrading or upstaging in a patient with prostate cancer, comprising: measuring an expression level of at least one gene in a biological sample comprising prostate tissue obtained from the patient, wherein the at least one gene comprises a gene from Table 13A or 13B, or genes that co-express with the at least one gene; wherein an expression level of any gene in Tables 13A is positively associated with an increased risk of upgrading/upstaging, and wherein an expression level of any gene in Table 13B is negatively associated with a increased risk of upgrading/upstaging.

Group II: Claims 18-20, drawn to a method for determining a likelihood of cancer recurrence in a patient with prostate cancer, comprising: measuring an expression level of at least one microRNA in a biological sample comprising prostate tissue obtained from the patient, wherein the at least one microRNA is a microRNA selected from hsa-miR-93; hsa-miR-106b; hsa-miR-2l; hsa-miR-449a; hsa-miR- 182; hsa-miR-27a; hsa-miR-103; hsa-miR-14l; hsa-miR-92a; hsa-miR-22; hsa-miR-29b; hsamiR-210; hsa-miR-331; hsa-miR-19l; hsa-miR-425; hsa-miR-200c; hsa-miR-30e-5p; hsamiR- 133a; hsa-miR-30a; hsa-miR-222; hsa-miR-l; hsa-miR-145; hsa-miR-486-5p; hsa-miR- 19b; hsa-miR-205; hsa-miR-3l; hsa-miR-155; hsa-miR-206; hsa-miR-99a; and hsa-miRl46b-5p; and normalizing said expression level to obtain a normalized expression level; wherein a normalized expression level ofhsa-miR-93; hsa-miR-106b; hsa-miR-2l; hsa-miR-449a; hsa-miR-182; hsa-miR-27a; hsa-miR-103; hsa-miR-14l; hsa-miR-92a; hsamiR- 22; hsa-miR-29b; hsa-miR-21O; hsa-miR-33l; hsa-miR-19l; hsa-miR-425; and hsamiR- 200c is positively associated with an increased risk of recurrence; and wherein a normalized expression level ofhsa-miR-30e-5p; hsa-miR-133a; hsa-miR- 30a; hsa-miR-222; hsa-miR-l; hsa-miR-145; hsa-miR-486-5p; hsa-miR-19b; hsa-miR-205; hsa-miR-3l; hsa-miR-155; hsa-miR-206; hsa-miR-99a; and hsa-miR-146b-5p is negatively associated with an increased risk of recurrence.

The inventions listed as Groups I-II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Group I does not include the inventive concept of measuring an expression level of at least one microRNA in a biological sample comprising prostate tissue obtained from the patient, wherein the at least one microRNA is a microRNA selected from hsa-miR-93; hsa-miR-106b; hsa-miR-2l; hsa-miR-449a; hsa-miR- 182; hsa-miR-27a; hsa-miR-103; hsa-miR-14l; hsa-miR-92a; hsa-miR-22; hsa-miR-29b; hsamiR- 210; hsa-miR-331; hsa-miR-19l; hsa-miR-425; hsa-miR-200c; hsa-miR-30e-5p; hsamiR- 133a; hsa-miR-30a; hsa-miR-222; hsa-miR-l; hsa-miR-145; hsa-miR-486-5p; hsa-miR- 19b; hsa-miR-205; hsa-miR-3l; hsa-miR-155; hsa-miR-206; hsa-miR-99a; and hsa-miRl46b- 5p; and normalizing said expression level to obtain a normalized expression level; wherein a normalized expression level ofhsa-miR-93; hsa-miR-106b; hsa-miR-2l; hsa-miR-449a; hsa-miR-182; hsa-miR-27a; hsa-miR-103; hsa-miR-14l; hsa-miR-92a; hsamiR- 22; hsa-miR-29b; hsa-miR-21O; hsa-miR-33l; hsa-miR-19l; hsa-miR-425; and hsamiR- 200c is positively associated with an increased risk of recurrence; and wherein a normalized expression level ofhsa-miR-30e-5p; hsa-miR-133a; hsa-miR- 30a; hsa-miR-222; hsa-miR-l; hsa-miR-145; hsa-miR-486-5p; hsa-miR-19b; hsa-miR-205; hsa-miR-3l; hsa-miR-155; hsa-miR-206; hsa-miR-99a; and hsa-miR-146b-5p is negatively associated with an increased risk of recurrence, as required by Group II.

Group II does not include the inventive concept of measuring an expression level of at least one gene in a biological sample comprising prostate tissue obtained from the patient, wherein the at least one gene comprises a gene from Tables 3A, 3B, 4A, 4B, 5A, 5B, 6A, 6B, 7 A, 7B, 8A, 8B, 10A, or 10B, or genes that coexpress with the at least one gene; predicting a likelihood of cancer recurrence for the patient; wherein an expression level of any gene in Tables 3A, 4A, 5A, 6A, 7 A, 8A, and 10A is positively associated with an increased risk of recurrence, and wherein an expression level of any gene in Tables 3B, 4B, 5B, 6B, 7B 8B, and 10B is negatively associated with a increased risk of recurrence; or measuring an expression level of at least one gene in a biological sample comprising prostate tissue obtained from the patient, wherein the at least one gene comprises a gene from Table 13A or 13B, or genes that co-express with the at least one gene; wherein an expression level of any gene in Tables 13A is positively associated with an increased risk of upgrading/upstaging, and wherein an expression level of any gene in Table 13B is negatively associated with a increased risk of upgrading/upstaging, as required by Group I.

----------continued on next blank page----------

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 11/45253

continuation of previous page

Groups I-II share the technical feature of a method for determining a likelihood of cancer recurrence in a patient with prostate cancer, comprising measure an expression level of at least one nucleic acid I a biological sample; predicting a likelihood of cancer recurrence for the patient, wherein an expression level of a specified nucleic acid is positively associated with an increased risk of recurrence; and wherein an expression level of a second specified nucleic acid is negative associated with increased risk of recurrence. However, this shared technical feature does not represent a contribution over the prior art, specifically, US 2009/0298082 A1 to Klee et al. (hereinafter 'Klee') teaches a method for determining a likelihood of cancer recurrence in a patient with prostate cancer (abstract; para [0009], [0034]-'After therapy for prostate cancer many men develop a rising PSA. Such men may develop a local or metastatic recurrence that warrants further therapy. However many men will have no evidence of disease progression other than the rising PSA and will have a good outcome. A case-control design, incorporating test and validation cohorts, was used to test the association of gene expression results with outcome after PSA progression. Using arrays optimized for paraffin-embedded tissue RNAs, a gene expression model significantly associated with systemic progression after PSA progression was developed. The model also predicted prostate cancer death
(in men with systemic progression) and systemic progression beyond 5 years (in PSA controls) with hazard ratios 2.5 and 4.7, respectively (log-rank p-values of 0.0007 and 0.0005). The measurement of gene expression pattern may be useful for determining which
men may benefit from additional therapy after PSA recurrence.'), comprising:
--measuring an expression level of at least one gene in a biological sample comprising prostate tissue obtained from the patient, wherein the at least one gene comprises a gene from Tables 3A, 3B, 4A, 4B, 5A, 5B, 6A, 6B, 7 A, 7B, 8A, 8B, 10A, or 10B (table 2-RAD21, SRD5A2, TOP2A; Fig 1; para [0023]-[0024]);
--predicting a likelihood of cancer recurrence for the patient (para [0009], [0023], [0034]);
---wherein an expression level of any gene in Tables 3A, 4A, 5A, 6A, 7 A, 8A, and 10A is positively associated with an increased risk of recurrence (table 2; fig 1 ? RAD21 and TOP2A; para [0023], [0034], [0062]), and
----wherein an expression level of any gene in Tables 3B, 4B, 5B, 6B, 7B 8B, and 10B is negatively associated with a increased risk of recurrence (table 2; fig 1-SRD5A2; para [0023], [0034], [0062]).
As said share technical feature was known at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the groups.

Groups I-II therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.

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PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To: CAROL L. FRANCIS
BOZICEVIC, FIELD & FRANCIS LLP
1900 UNIVERSITY AVENUE
SUITE 200
EAST PALO ALTO, CA 94303

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing *(day/month/year)*	27 FEB 2012

Applicant's or agent's file reference	FOR FURTHER ACTION
GHDX-046WO	See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US 11/45253	25 July 2011 (25.07.2011)	27 July 2010 (27.07.2010)

International Patent Classification (IPC) or both national classification and IPC
IPC(8) - C12Q 1/68; G01N 33/574 (2011.01)
USPC - 435/6.11, 6.12, 6.14, 7.23

Applicant GENOMIC HEALTH, INC.

1. This opinion contains indications relating to the following items:

- [X] Box No. I Basis of the opinion
- [ ] Box No. II Priority
- [ ] Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- [X] Box No. IV Lack of unity of invention
- [X] Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- [ ] Box No. VI Certain documents cited
- [ ] Box No. VII Certain defects in the international application
- [ ] Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1 *bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to th IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Fom PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/US	Date of completion of this opinion	Authorized officer:
Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	17 February 2012 (17.02.2012)	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/237 (cover sheet) (July 2011)

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 11/45253

Box No. 1 Basis of this opinion

1. With regard to the language, this opinion has been established on the basis of:

   ☒ the international application in the language in which it was filed.

   ☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2. ☐ This opinion has been established taking into account the rectification of an obvious mistake authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a))

3. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this opinion has been established on the basis of a sequence listing filed or furnished:

   a. (means)

      ☐ on paper

      ☐ in electronic form

   b. (time)

      ☐ in the international application as filed

      ☐ together with the international application in electronic form

      ☐ subsequently to this Authority for the purposes of search

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 11/45253

Box No. IV Lack of unity of invention

1. ☒ In response to the invitation (Form PCT/ISA/206) to pay additional fees the applicant has, within the applicable time limit:

   ☐ paid additional fees

   ☐ paid additional fees under protest and, where applicable, the protest fee

   ☐ paid additional fees under protest but the applicable protest fee was not paid

   ☒ not paid additional fees

2. ☐ This Authority found that the requirement of unity of invention is not complied with and chose not to invite the applicant to pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rule 13.1, 13.2 and 13.3 is

   ☐ complied with

   ☒ not complied with for the following reasons:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-17, drawn to a method for determining a likelihood of cancer recurrence in a patient with prostate cancer, comprising: measuring an expression level of at least one gene in a biological sample comprising prostate tissue obtained from the patient, wherein the at least one gene comprises a gene from Tables 3A, 3B, 4A, 4B, 5A, 5B, 6A, 6B, 7 A, 7B, 8A, 8B, 10A, or 10B, or genes that coexpress with the at least one gene; predicting a likelihood of cancer recurrence for the patient; wherein an expression level of any gene in Tables 3A, 4A, 5A, 6A, 7 A, 8A, and 10A is positively associated with an increased risk of recurrence, and wherein an expression level of any gene in Tables 3B, 4B, 5B, 6B, 7B 8B, and 10B is negatively associated with a increased risk of recurrence; or a method for determining a likelihood of upgrading or upstaging in a patient with prostate cancer, comprising: measuring an expression level of at least one gene in a biological sample comprising prostate tissue obtained from the patient, wherein the at least one gene comprises a gene from Table 13A or 13B, or genes that co-express with the at least one gene; wherein an expression level of any gene in Tables 13A is positively associated with an increased risk of upgrading/upstaging, and wherein an expression level of any gene in Table 13B is negatively associated with a increased risk of upgrading/upstaging.

Group II: Claims 18-20, drawn to a method for determining a likelihood of cancer recurrence in a patient with prostate cancer, comprising: measuring an expression level of at least one microRNA in a biological sample comprising prostate tissue obtained from the patient, wherein the at least one microRNA is a microRNA selected from hsa-miR-93; hsa-miR-106b; hsa-miR-2l; hsa-miR-449a; hsa-miR- 182; hsa-miR-27a; hsa-miR-103; hsa-miR-14l; hsa-miR-92a; hsa-miR-22; hsa-miR-29b; hsamiR-210; hsa-miR-331; hsa-miR-19l; hsa-miR-425; hsa-miR-200c; hsa-miR-30c-5p; hsamiR- 133a; hsa-miR-30a; hsa-miR-222; hsa-miR-l; hsa-miR-145; hsa-miR-486-5p; hsa-miR- 19b; hsa-miR-205; hsa-miR-3l; hsa-miR-155; hsa-miR-206; hsa-miR-99a; and hsa-miR146b- 5p; and normalizing said expression love' to obtain a normalized expression level; wherein a normalized expression level ofhsa-miR-93; hsa-miR-106b; hsa-miR-2l; hsa-miR-449a; hsa-miR-182; hsa-miR-27a; hsa-miR-103; hsa-miR-14l; hsa-miR-92a; hsamiR- 22; hsa-miR-29b; hsa-miR-210; hsa-miR-33l; hsa-miR-19l; hsa-miR-425; and hsamiR- 200c is positively associated with an increased risk of recurrence; and wherein a normalized expression level ofhsa-miR-30a-5p; hsa-miR-133a; hsa-miR- 30a; hsa-miR-222; hsa-miR-l; hsa-miR-145; hsa-miR-486-5p; hsa-miR-19b; hsa-miR-205; hsa-miR-3l; hsa-miR-155; hsa-miR-206; hsa-miR-99a; and hsa-miR-146b-5p is negatively associated with an increased risk of recurrence.

The inventions listed as Groups I-II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

----------continued in next supplemental box----------

4. Consequently, this opinion has been established in respect of the following parts of the international application:

   ☐ all parts

   ☒ the parts relating to claims Nos. 1-17

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 11/45253

Box No. V **Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Claims	6-10, 14-16	YES
	Claims	1-5, 11-13 and 17	NO
Inventive step (IS)	Claims	None	YES
	Claims	1-17	NO
Industrial applicability (IA)	Claims	1-17	YES
	Claims	None	NO

2. Citations and explanations:

Claims 1-5, 11-13 and 17 lack novelty under PCT Article 33(2) as being anticipated by US 2009/0298082 A1 to Klee et al. (hereinafter 'Klee').

Regarding claim 1, Klee teaches a method for determining a likelihood of cancer recurrence in a patient with prostate cancer (abstract; para [0009], [0010]-'prognosing the human as later developing systemic disease or as not later developing systemic disease based on at least the expression profile score', 'The method can be performed at the time of the retropubic radial prostatectomy', [0034]-'After therapy for prostate cancer many men develop a rising PSA. Such men may develop a local or metastatic recurrence that warrants further therapy. However many men will have no evidence of disease progression other than the rising PSA and will have a good outcome. A case-control design, incorporating test and validation cohorts, was used to test the association of gene expression results with outcome after PSA progression. Using arrays optimized for paraffin-embedded tissue RNAs, a gene expression model significantly associated with systemic progression after PSA progression was developed. The model also predicted prostate cancer death (in men with systemic progression) and systemic progression beyond 5 years (in PSA controls) with hazard ratios 2.5 and 4.7, respectively (log-rank p-values of 0.0007 and 0.0005). The measurement of gene expression pattern may be useful for determining which men may benefit from additional therapy after PSA recurrence.'), comprising:
--measuring an expression level of at least one gene in a biological sample comprising prostate tissue obtained from the patient, wherein the at least one gene comprises a gene from Tables 3A, 3B, 4A, 4B, 5A, 5B, 6A, 6B, 7A, 7B, 8A, 8B, 10A, or 10B (table 2-RAD21, SRD5A2, TOP2A; Fig 1; para [0023]-[0024])
--predicting a likelihood of cancer recurrence for the patient (para [0009], [0023], [0034]);
---wherein an expression level of any gene in Tables 3A, 4A, 5A, 6A, 7 A, 8A, and 10A is positively associated with an increased risk of recurrence (table 2, 3; fig 1-RAD21 and TOP2A; para [0023], [0034], [0062]), and
---wherein an expression level of any gene in Tables 3B, 4B, 5B, 6B, 7B 8B, and 10B is negatively associated with a increased risk of recurrence (table 2; fig 1-SRD5A2; para [0023], [0034], [0062]).

Regarding claim 2, Klee teaches said expression level is measured using an RNA transcript of the at least one gene (para [0010]-'mRNA expression levels').

Regarding claim 3, Klee teaches said expression is measured using an oligonucleotide associated with the at least one gene (para [0010] and [0075]-'Oligonucleotide probe sets').

Regarding claim 4, Klee teaches normalizing said expression level to obtain a normalized expression level (para [0024], [0049]-'Expression of each gene was measured (the number of cycles required to achieve a threshold, or Ct) in triplicate and then normalized relative to the set of four reference genes.').

Regarding claim 5, Klee teaches generating a report based on the Recurrence Score (RS) (para [0010]-' expression profile score', and [0030]-[0031] - 'the levels of biomarkers (e.g., an expression level score) can be used in combination with one or more other factors to assess a prostate cancer patient. For example, expression level scores can be used in combination with the clinical stage, the serum PSA level, and/or the Gleason score of the prostate cancer to determine, at the time of PSA reoccurrence or at the time of retropubic radial prostatectomy, whether or not a man/al will later develop systemic disease.', 'A professional can be assisted by (1) determining the level of one or more than one biomarker in a sample, and (2) communicating information about that level to that professional').

Regarding claim 11, Klee teaches the biological sample comprises prostate tumor tissue with the primary Gleason pattern for said prostate tumor (para [0056] and Table 1A).

Regarding claim 12, Klee teaches the biological samples comprises prostate tumor tissue with the highest Gleason pattern for said prostate tumor (para [0036] and [0055]-[0056] and Table 1A - 'Gleason 8-10 tumors').

Regarding claim 13, Klee teaches the biological sample is prostate tumor tissue (para [0024] and [0056]).

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IV.3: Lack of unity of invention

Group I does not include the inventive concept of measuring an expression level of at least one microRNA in a biological sample comprising prostate tissue obtained from the patient, wherein the at least one microRNA is a microRNA selected from hsa-miR-93; hsa-miR-106b; hsa-miR-2l; hsa-miR-449a; hsa-miR- 182; hsa-miR-27a; hsa-miR-103; hsa-miR-14l; hsa-miR-92a; hsa-miR-22; hsa-miR-29b; hsamiR- 210; hsa-miR-331; hsa-miR-19l; hsa-miR-425; hsa-miR-200c; hsa-miR-30c-5p; hsamiR- 133a; hsa-miR-30a; hsa-miR-222; hsa-miR-l; hsa-miR-145; hsa-miR-486-5p;'hsa-miR- 19b; hsa-miR-205; hsa-miR-3l; hsa-miR-155; hsa-miR-206; hsa-miR-99a; and hsa-miRl46b- 5p; and normalizing said expression level to obtain a normalized expression level; wherein a normalized expression level ofhsa-miR-93; hsa-miR-106b; hsa-miR-2l; hsa-miR 449a; hsa-miR-182; hsa-miR-27a; hsa-miR-103; hsa-miR-14l; hsa-miR-92a; hsamiR- 22; hsa -miR-29b; hsa-miR-21O; hsa-miR-33l; hsa-miR-19l; hsa-miR-425; and hsamiR- 200c is positively associated with an increased risk of recurrence; and wherein a normalized expression level ofhsa-miR-30c-5p; hsa-miR-133a; hsa-miR- 30a; hsa-miR-222; hsa-miR-l; hsa-miR -145; hsa-miR-486-5p; hsa-miR-19b; hsa-miR-205; hsa-miR-3l; hsa-miR-155; hsa-miR-206; hsa-miR-99a; and hsa-miR-146b-5p is negatively associated with an increased risk of recurrence, as required by Group II.

Group II does not include the inventive concept of measuring an expression level of at least one gene in a biological sample comprising prostate tissue obtained from the patient, wherein the at least one gene comprises a gene from Tables 3A, 3B, 4A, 4B, 5A, 5B, 6A, 6B, 7 A, 7B, 8A, 8B, 10A, or 10B, or genes that coexpress with the at least one gene; predicting a likelihood of cancer recurrence for the patient; wherein an expression level of any gene in Tables 3A, 4A, 5A, 6A, 7 A, 8A, and 10A is positively associated with an increased risk of recurrence, and wherein an expression level of any gene in Tables 3B, 4B, 5B, 6B, 7B 8B, and 10B is negatively associated with a increased risk of recurrence; or measuring an expression level of at least one gene in a biological sample comprising prostate tissue obtained from the patient, wherein the at least one gene comprises a gene from Table 13A or 13B, or genes that co-express with the at least one gene; wherein an expression level of any gene in Tables 13A is positively associated with an increased risk of upgrading/upstaging, and wherein an expression level of any gene in Table 13B is negatively associated with a increased risk of upgrading/upstaging, as required by Group I.

Groups I-II share the technical feature of a method for determining a likelihood of cancer recurrence in a patient with prostate cancer, comprising measure an expression level of at least one nucleic acid I a biological sample; predicting a likelihood of cancer recurrence for the patient, wherein an expression level of a specified nucleic acid is positively associated with an increased risk of recurrence; and wherein an expression level of a second specified nucleic acid is negative associated with increased risk of recurrence. However, this shared technical feature does not represent a contribution over the prior art, specifically, US 2009/0298082 A1 to Klee et al. (hereinafter 'Klee') teaches a method for determining a likelihood of cancer recurrence in a patient with prostate cancer (abstract; para [0009], [0034]-'After therapy for prostate cancer many men develop a rising PSA. Such men may develop a local or metastatic recurrence that warrants further therapy. However many men will have no evidence of disease progression other than the rising PSA and will have a good outcome. A case-control design, incorporating test and validation cohorts, was used to test the association of gene expression results with outcome after PSA progression. Using arrays optimized for paraffin-embedded tissue RNAs, a gene expression model significantly associated with systemic progression after PSA progression was developed. The model also predicted prostate cancer death (in men with systemic progression) and systemic progression beyond 5 years (in PSA controls) with hazard ratios 2.5 and 4.7, respectively (log-rank p-values of 0.0007 and 0.0005). The measurement of gene expression pattern may be useful for determining which men may benefit from additional therapy after PSA recurrence.'), comprising:
--measuring an expression level of at least one gene in a biological sample comprising prostate tissue obtained from the patient, wherein the at least one gene comprises a gene from Tables 3A, 3B, 4A, 4B, 5A, 5B, 6A, 6B, 7 A, 7B, 8A, 8B, 10A, or 10B (table 2-RAD21, SRD5A2, TOP2A; Fig 1; para [0023]-[0024]);
--predicting a likelihood of cancer recurrence for the patient (para [0009], [0023], [0034]);
---wherein an expression level of any gene in Tables 3A, 4A, 5A, 6A, 7 A, 8A, and 10A is positively associated with an increased risk of recurrence (table 2; fig 1 . RAD21 and TOP2A; para [0023], [0034], [0062]), and
---wherein an expression level of any gene in Tables 3B, 4B, 5B, 6B, 7B 8B, and 10B is negatively associated with a increased risk of recurrence (table 2; fig 1-SRD5A2; para [0023], [0034], [0062]).
As said share technical feature was known at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the groups.

Groups I-II therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.

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Supplemental Box

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citations and explanations from box V 2:.

Regarding claim 17, Klee teaches a method for determining a likelihood of upgrading or upstaging in a patient with prostate cancer (para [0023]-'which patients, at the time of retropubic radial prostatectomy, will later develop systemic disease, and (3) which patients, at the time of systemic disease, will later die from prostate cancer'), comprising:
--measuring an expression level of at least one gene in a biological sample comprising prostate tissue obtained from the patient (para [0023]-[[0024]), wherein the at least one gene comprises a gene from Table 13A (fig 1-TOP2A; para [0010] . BUB1, para [0062] and Table 2, TOP2A, TPX2), or 13B (para [0062] and Table 2 . SRD5A2 and AZGP1), or genes that co-express with the at least one gene;
--wherein an expression level of any gene in Tables 13A is positively associated with an increased risk of upgrading/upstaging (fig 1 TOP2A; para [0012], [0023], [0034], [0062] and Table 3 -BUB1), and
--wherein an expression level of any gene in Table 13B is negatively associated with a increased risk of upgrading/upstaging (table 2; fig 1 -SRD5A2; para [0023], [0034], [0062]).

Claims 14 and 16 lack an inventive step under PCT Article 33(3) as being obvious over Klee.

Regarding claim 14, Klee does not expressly disclose that the biological sample is non-tumor prostate tissue, however, this would have been obvious to one of ordinary skill in the art, because it was common practice in the art to also measure relevant gene expression in tissues neighboring the tumor tissue so as to assess the possibility of residual tumor cells remaining in the subject, because the presence of said residual tumor cells were commonly known in the art to be a major cause of cancer recurrence.

Regarding claim 16, Klee discloses that the at least one gene comprises a gene from Tables 10A or 10B (fig 1-SRD5A2; para [0061]; Table 2 - SRD5A2).
Klee does not expressly disclose that teaches the biological sample is non-tumor prostate tissue, however, this would have been obvious to one of ordinary skill in the art, because it was common practice in the art to also measure relevant gene expression in tissues neighboring the tumor tissue so as to assess the possibility of residual tumor cells remaining the subject, wherein said residual tumor cells were commonly known in the art to be a major cause of cancer recurrence.

Claims 6, 7 and 10 lack an inventive step under PCT Article 33(3) as being obvious over Klee, as above, in view of US 2009/0233279 A1 to Glinskii et al. (hereinafter 'Glinskii').

Regarding claim 6, Klee teaches the report comprises an estimate of recurrence risk (para [0010] and [0030]-[0031]-'In determining, ...at the time of retropubic radial prostatectomy, whether or not a mammal will later develop systemic disease or in determining, at the time of systemic disease, whether or not a mammal will later die of prostate cancer').
Klee does not specifically teach said estimate is based on clinical recurrence-free interval (cRFI). However, Klee does teach the estimate of recurrence risk is based on various factors including clinical measurements (para [0030]).
Further Glinskii teaches a method for determining a likelihood of cancer recurrence in a patient with prostate cancer (para [0016]-[0017] and [0023]), wherein the estimate of recurrence risk is based on clinical recurrence-free interval (cRFI) (para [0123]) (para [0095]-'clinical end-point disease-free interval (DFI)', [0131], [0140]-' relapse-free'). It would have been obvious to one of ordinary skill in the art to have performed a method for determining a likelihood of cancer recurrence in a patient with prostate cancer as claimed, wherein said estimate is based on clinical recurrence-free interval (cRFI), in view of Klee teaching determining an estimate of recurrence risk of prostate cancer using various factors including clinical and biochemical measurements (para [0030]), and in light of Glinskii teaching determining the estimate of recurrence risk of prostate cancer (para [0017]), based on factors including clinical recurrence-free interval (cRFI) (para [0123]), since a skilled artisan would have readily appreciated use of determining factors including cRFI as taught by Glinskii applied to the methods to determine recurrence risk of prostate cancer taught by Klee, to improve the accuracy of risk diagnosis by increasing the number of predicting factors.

Regarding claim 7, Klee does not expressly disclose that the RS is based on a biochemical recurrence-free interval (bRFI). However, Klee discloses estimating recurrence risk based on various factors including biochemical measurement.
Further Glinskii discloses a method for determining a risk of cancer recurrence in a patient with prostate cancer (para [0016]-[0017] and [0023]), wherein the estimate of recurrence risk is based on a biochemical recurrence free interval (bRFI) (para [0095]-'The end point for survival analysis in prostate cancer was the biochemical recurrence defined by serum PSA increase after therapy. Disease-free interval (DFI) was defined as the time period between the date of radical prostatectomy (RP) and the date of PSA relapse (recurrence group) or date of last follow-up (non-recurrence group)'), based on this disclosure, one of ordinary skill in the art would have found it obvious to incorporate said bRFI into the method disclosed by Klee so as to achieve a more accurate prognosis by incorporating various different parameters.

Regarding claim 10, Klee does not specifically teach the patient has early-stage prostate cancer. Glinskii teaches a method for determining a likelihood of cancer recurrence in a patient with prostate cancer (para [0016]-[0017] and [0023]), wherein the patient has early-stage prostate cancer (para [0033] and [0140]). It would have been obvious to one of ordinary skill in the art to have performed a method for determining a likelihood of cancer recurrence in a patient with prostate cancer as claimed, wherein the patient has early-stage prostate cancer, in view of Klee teaching a method for determining a likelihood of cancer recurrence in a patient with prostate cancer in patients having various stages of cancer (para [0055] and Table 1), and in view of Glinskii teaching a method for determining a likelihood of cancer recurrence in a patient with prostate cancer (para [0016] and [0023]), wherein the patient has early-stage prostate cancer (para [0033] and [0140]), since a skilled artisan would have readily appreciated application of the Klee methods to patients having early stage cancer, in order to determine risk at an early stage, thus preventing possibly negative treatment options for those at low risk and increasing survival likelihood in patients at higher risk.

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Supplemental Box

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Claims 8-9 and 15 lack an inventive step under PCT Article 33(3) as being obvious over Klee, as above, in view of US 2007/0212702 A1 to Tomlins et al. (hereinafter "Tomlins").

Regarding claim 8-9, Klee does not expressly disclose that the biological sample has a positive TMPRSS2 fusion status or a negative TMPRSS2 fusion status. However, Tomlins discloses that TMPRSS2 fusion is common in prostate cancer (para [0006], [0094], [0418]) and that a subject with a prostate tumor that has a positive TMPRSS2 fusion status has a much worse prognosis than a subject with a negative TMPRSS2 fusion status (para [0194], [0197], [0411]), based on this disclosure, one of ordinary skill in the art would have found it obvious that both a sample having a positive TMPRSS2 status and a sample having a negative TMPRSS2 status could be used in the method disclosed by Klee to further determine the likelihood of recurrence in order to achieve a more accurate and complete prognosis.

Regarding claim 15, Klee does not specifically teach classifying the patient as TMPRSS2 fusion positive or negative, wherein an expression level of any gene in Table 9A is associated with a positive TMPRSS2 fusion status, and wherein an expression level of any gene in Table 9B is associated with a negative TMPRSS2 fusion status. However, Klee teaches that
--an expression level of a gene in Table 9A is positively associated within increased risk of prostate cancer systemic progression (table 3-SLC44A1; para [0063]); and
--an expression level of a gene in Table 9B is negatively associated with increased risk of prostate cancer systemic progression (fig 1-SRD5A2; table 2; para [0010], [0023]-[0024])
Further Tomlins discloses that TMPRSS2 fusion is common in prostate cancer (para [0006], [0094], [0418]) and that a subject with a prostate tumor that has a positive TMPRSS2 fusion status has a much worse prognosis than a subject with a negative TMPRSS2 fusion status (para [0194], [0197], [0411]), based on this disclosure, one of ordinary skill in the art would have found it obvious classify the patient as TMPRSS2 fusion positive or negative, and to associate the expression level of one or more gene in Table 9 which is s positively associated within increased risk of prostate cancer systemic progression disclosed by Klee with positive TMPRSS2 fusion status which indicates a worse prognosis than negative TMPRSS2 fusion status as taught by Tomlins; and to associate the expression level of one or more gene in table 9B which is negatively associated with increased risk of prostate cancer systemic progression as taught by Klee with negative TMPRSS2 fusion status which indicates a better prognosis as shown by Tomlin, so as to further stratify the prostate cancer using a combination of multiple parameters in order to achieve a more acurate prognosis.

Claims 1-17 have industrial applicability as defined by PCT Article 33(4) because the subject matter can be made or used in the industry.

INTERNATIONAL JOURNAL OF ONCOLOGY 25: 797-819, 2004

Genome-wide gene-expression profiles of breast-cancer cells purified with laser microbeam microdissection: Identification of genes associated with progression and metastasis

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Abstract. Breast carcinoma is a complex disease characterized by accumulation of multiple genetic alterations, and the understanding of the molecular basis of mammary tumorigenesis is still incomplete. In this study we analyzed gene-expression profiles of 81 surgical specimens of 12 ductal carcinoma *in situ* (DCIS) and 69 invasive ductal carcinoma (IDC). After applying laser-microbeam micro-dissection to all samples we achieved 98-99% pure populations of breast cancer cells, and of normal breast epithelial cells used as controls. A cDNA-microarray analysis of 23,040 genes in these samples and a subsequent unsupervised hierarchical clustering distinguished two tumor groups, mainly in terms of estrogen-receptor (ER) status. We then undertook a supervised analysis and identified 325 genes that were commonly either up- or down-regulated in both pathologically discrete stages (DCIS and IDC), indicating that these genes might play important roles in malignant transformation of breast ductal cells. In addition, we searched invasion-associated gene candidates whose expression was altered in IDC, but not in DCIS, and identified 24 up-regulated genes and 41 down-regulated genes. Furthermore, we identified 34 genes that were expressed differently in tumors from patients with lymph node metastasis as opposed to no metastasis. On that basis we developed a scoring system that correlated well with the metastatic status. Tumors from all of the 37 test patients with lymph-node metastasis yielded positive scores by our definition, whereas 38 of the 40 tumors (95%) without lymph node metastasis had negative scores. Our data should provide useful information for identifying predictive markers for invasion or metastasis, and suggest potential target molecules for treatment of breast cancers.

Introduction

Breast cancer is a complex disease characterized by accumulation of genetic and epigenetic changes in a large number of genes. A multi-step model of mammary carcinogenesis, i.e., transformation of normal cells through stages of atypical ductal hyperplasia, ductal carcinoma *in situ* (DCIS), and invasive ductal carcinoma (IDC), is similar to the stages of carcinogenesis in other tissues, but the precise molecular mechanisms driving breast cancer remain unknown. Obviously, molecular factors leading to development of primary breast cancer, its progression, and its metastasis would be likely targets for development of better tools for prevention and treatment of this disease.

Gene-expression profiles obtained by cDNA microarray analysis can provide a considerable amount of information for characterizing the nature of individual cancers; the promise of such information lies in its potential for improving clinical strategies for treating neoplastic diseases through development of novel drugs (1). With that goal in mind, we have been analyzing expression profiles of tumors from various tissues using cDNA microarrays (2-8). This technology has fueled reports indicating that thousands of genes can show distinct patterns of expression in different types of breast cancer (9-15). However, although those studies have highlighted candidate genes that might be useful as diagnostic markers, data based on expression of mRNAs isolated from tumor masses might not exactly reflect changes during the course of mammary carcinogenesis because: i) tumor masses are generally mixtures

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Key words: breast cancer, invasive ductal carcinoma, ductal carcinoma *in situ*, laser microbeam microdissection, cDNA microarray

Table I. Clinicopathological features of patients examined.

ID No.	Age in operation	Menopausal status	T	N	M	Stage	Histological type	ER	PgR
MMK010003	51	pre	2	1	0	2	a3	+	+
MMK010004	47	pre	2	1	0	2	a1	+	+
MMK010005	44	pre	2	0	0	2	a1	+	+
MMK010013	45	pre	2	1	0	2	a1	-	-
MMK010016	44	pre	2	0	0	2	a2	-	-
MMK010025	46	pre	2	0	0	2	a1	+	+
MMK010031	29	pre	2	2	0	3	a3	-	-
MMK010037	62	post	0	0	0	0	1a	+	+
MMK010042	47	pre	2	1	0	2	a3	+	+
MMK010086	42	pre	2	0	0	2	a1	+	+
MMK010102	51	pre	2	1	0	3	a2	+	+
MMK010110	39	pre	2	0	0	2	a1	-	-
MMK010129	52	pre	2	2	0	3	a1	-	-
MMK010135	41	pre	2	0	0	2	a1	+	+
MMK010138	38	pre	2	0	0	2	a1	+	+
MMK010145	51	pre	2	1	0	2	a3	+	+
MMK010147	49	pre	2	1	0	2	a1	+	+
MMK010149	35	pre	2	0	0	2	a3	-	-
MMK010175	38	pre	2	0	0	2	a3	+	+
MMK010178	51	pre	0	0	0	0	1a	+	+
MMK010207	40	pre	2	0	0	2	a1	+	+
MMK010214	42	pre	2	1	0	2	a1	-	-
MMK010247	48	pre	2	1	0	2	a2	-	-
MMK010252	52	pre	2	1	0	2	a2	-	-
MMK010255	47	pre	2	0	0	2	a2	-	-
MMK010302	46	pre	2	1	0	2	a2	-	-
MMK010304	48	pre	2	1	0	2	a3	+	+
MMK010326	53	post	0	0	0	0	1a	-	-
MMK010327	43	pre	2	1	0	2	a1	+	+
MMK010341	42	pre	2	1	0	2	a1	+	+
MMK010370	46	pre	2	1	0	2	a3	+	+
MMK010397	38	pre	2	1	0	2	a3	+	+
MMK010411	46	pre	2	0	0	2	a1	+	+
MMK010431	50	pre	2	0	0	2	a3	-	-
MMK010435	49	pre	2	1	0	2	a3	+	+
MMK010453	49	pre	2	1	0	2	a3	+	+
MMK010471	42	pre	2	1	0	2	a1	-	-
MMK010473	40	pre	2	1	0	2	a2	-	-
MMK010478	38	pre	2	2	0	3	a2	+	+
MMK010491	46	pre	2	0	0	2	a3	+	+
MMK010497	44	pre	0	0	0	0	1a	-	+
MMK010500	45	pre	2	0	0	2	a1	+	+
MMK010502	51	pre	2	0	0	2	a2	-	-
MMK010508	51	pre	2	1	0	2	a2	-	-
MMK010521	21	pre	2	0	0	2	a1	-	-
MMK010552	49	pre	2	0	0	2	a2	-	-
MMK010554	51	pre	2	0	0	2	a3	+	+
MMK010571	45	pre	2	1	1	4	a3	+	+
MMK010591	40	pre	0	0	0	0	1a	-	+
MMK010613	37	pre	0	0	0	0	1a	-	+
MMK010623	39	pre	2	1	0	2	a1	+	+
MMK010624	39	pre	2	1	0	2	a1	+	+
MMK010626	48	pre	2	0	0	2	a1	-	-
MMK010631	41	pre	2	0	0	2	a1	+	+
MMK010640	35	pre	0	0	0	0	1a	+	+
MMK010644	47	pre	2	2	0	2	a3	+	+
MMK010646	37	pre	2	1	0	2	a3	+	+
MMK010660	46	pre	2	0	0	2	a1	-	-
MMK010671	45	pre	2	0	0	2	a1	-	-
MMK010679	68	post	0	0	0	0	1a	+	+
MMK010680	58	post	0	0	0	0	1a	-	+
MMK010709	33	pre	2	0	0	2	a3	-	-
MMK010711	51	pre	0	0	0	0	1a	-	+
MMK010724	40	pre	2	1	0	2	a3	+	+

Table I. Continued.

ID No.	Age in operation	Menopausal status	T	N	M	Stage	Histological type	ER	PgR
MMK010744	41	pre	0	0	0	0	1a	+	+
MMK010758	40	pre	2	1	0	2	a1	+	+
MMK010760	42	pre	2	0	0	2	a1	+	+
MMK010762	50	pre	2	1	0	2	a3	+	+
MMK010769	33	pre	2	0	0	2	a2	-	-
MMK010772	45	pre	2	1	0	2	a3	-	-
MMK010779	46	pre	2	1	0	2	a2	-	-
MMK010780	31	pre	2	0	0	2	a2	-	-
MMK010781	44	pre	2	0	0	2	a3	+	+
MMK010794	52	pre	2	1	0	2	a3	+	+
MMK010818	51	pre	2	0	0	2	a1	+	+
MMK010835	42	pre	0	0	0	0	1a	+	+
MMK010846	47	pre	2	0	0	2	a1	+	+
MMK010858	42	pre	2	1	0	2	a3	+	+
MMK010864	52	pre	2	1	0	2	a1	-	-
MMK010869	45	pre	2	0	0	2	a1	-	-
MMK010903	47	pre	2	0	0	2	a1	+	+

All information were judged according to the Japanese Breast Cancer Society. T, tumor stage; N, lymph node metastasis staus; M, distant metastasis. Histological type: 1a, ductal carcinoma *in situ*; a1, papillotubular carcinoma (well-differentiated); a2, solid tubular carcinoma (moderately-differentiated); a3, scirrhous carcinoma (poorly-differentiated).

of various cell populations such as inflammatory cells, stroma cells, and fibroblasts in addition to cancer cells and ii) the proportions of each cell type vary significantly from one individual to another (the proportions of cancer cells in a given breast tumor being from 20 to 90%). Therefore, most of microarray data published previously are likely to have been influenced significantly by cellular heterogeneity among tumor preparations except a few reports (16,17). Furthermore, since breast cancers originate from epithelial cells, normal breast tissue used as a control to determine which genes might have changed during mammary carcinogenesis will contain a large proportion of adipocytes, further complicating the interpretation of results.

To investigate differences in gene-expression patterns between breast-cancer cells and their tissue of origin, we purified populations of breast-cancer cells and normal breast epithelial ductal cells by means of laser-microbeam microdissection (LMM) before analyzing the expression profiles of 81 breast tumors, including 12 DCIS and 69 IDC, using a cDNA microarray representing 23,040 genes. The data from these experiments not only should provide important information about breast tumorigenesis, but should be valuable for identifying candidate genes whose products might serve as diagnostic markers and/or as molecular targets for treatment of breast cancer.

Materials and methods

Patients and tissue samples. Primary breast cancers were obtained with informed consent from 81 patients (12 ductal carcinomas *in situ* and 69 T2-stage IDC 2-5 cm in diameter) (T2), with a median age of 45 (range 21-68 years), who were treated at the Department of Breast Surgery, Cancer Institute Hospital, Tokyo. Clinical information was obtained from medical records and each tumor was diagnosed by pathologists according to histopathological sub-type and grade. Tumor tissues were also evaluated according to the Japanese Cancer Society classification; clinical stage was judged according to the Japanese Breast Cancer Society's TNM classification. Expression of estrogen and progesterone receptors was determined by EIA; ER was judged as negative when it was <13 fmol/mg protein. A mixture of RNAs isolated from normal breast ductal cells of 15 pre-menopausal breast-cancer patients served as a normal control.

Tissue samples and LMM. Clinical and pathological information for each patient is summarized in Table I. Samples were embedded in TissueTek OCT medium (Sakura, Tokyo, Japan) and stored at -80˚C until use. Frozen specimens were serially sectioned in 8-µm slices with a cryostat and stained with hematoxylin and eosin to define the analyzed regions. We purified cancer and normal ductal cells which were clearly observed by a pathologist using the EZ Cut LMM System (SL Microtest GmbH, Germany) following the manufacturer's protocol, with several modifications; we handled the cancer tissues carefully to minimize the effects of tissue collection and storage. We electrophoresed total RNAs extracted from residual tissue of each case on a denaturing agarose gel, to confirm quality by the presence of ribosomal RNA bands.

RNA extraction and T7-based amplification. Total RNA was extracted from each population of microdissected cells into 350 µl of RLT lysis buffer (Qiagen, Valencia, CA). The extracted RNA was treated for 30 min at room temperature with 30 units of DNase I (Qiagen). After inactivation of DNase I at 70˚C for 10 min, RNAs were purified with an RNeasy Mini Kit (Qiagen) according to the manufacturer's procedures. All of the DNase I-treated RNA was subjected to

T7-based amplification using the Ampliscribe T7-Transcription Kit (Epicentre Technologies, Madison, MI). Anti-sense RNA amplified by this method accurately reflects the proportions in the original RNA source (18,19), as we have confirmed by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) experiments (20). Two cycles of amplification yielded 28.8-329.4 μg of amplified RNAs (aRNAs) for each sample. On the other hand, amplification of RNAs in microdissected normal cells from 15 pre-menopausal patients or 12 post-menopausal patients except samples on Table I yielded totals of 2240.2 and 2023.8 μg respectively. Then 2.5-μg aliquots of aRNA from each preparation of breast-cancer cells and normal breast ductal cells were reverse-transcribed in the presence of Cy5-dCTP and Cy3-dCTP (Amersham Biosciences, Buckinghamshire, UK), respectively.

cDNA microarrays and acquisition of data. We established a 'genome-wide' cDNA microarray system containing 23,040 cDNAs selected from the UniGene database (build #131) of the National Center for Biotechnology Information (NCBI). cDNA microarray slides were prepared as described previously (21), except that the PCR products were spotted in duplicate on type-7 glass slides (Amersham Biosciences) using a Lucidea Array Spotter (Amersham Biosciences); 9,216 genes were spotted in duplicate on a single slide. We prepared two and half sets of slides (total 23,040 genes), on each of which the same 52 housekeeping genes and two kinds of negative-control genes were spotted as well.

Hybridization and washing were performed according to protocols described previously (21). Signal intensities of Cy3 and Cy5 from the 23,040 spots were quantified and analyzed by substituting backgrounds, using ArrayVision software (Imaging Research Inc., St. Catharines, Ontario, Canada). Subsequently the fluorescent intensities of Cy5 (tumor) and Cy3 (control) for each target spot were adjusted so that the mean Cy5/Cy3 ratio of 52 housekeeping genes on the array was equal to one. Because data derived from low signal intensities are less reliable, we determined a cut-off value on each slide as described previously (20) and excluded genes from further analysis when both Cy3 and Cy5 dyes yielded signal intensities lower than the cut-off. For other genes we calculated the Cy5/Cy3 ratio using the raw data of each sample.

Calculation of contamination percentage. Perilipin (*PLIN*) and fatty acid binding protein 4 (*FABP4*) are expressed exclusively in adipose tissue and mammary gland, as had been determined by their expression profiles in 29 normal human tissues on a cDNA microarray (21). These genes were used to evaluate the proportion of adipocytes present in populations of microdissected normal breast ductal epithelial cells. Amplified RNAs derived from poly A$^+$ RNA isolated from normal whole-mammary gland tissues (BD Clontech, San Diego, CA) and from microdissected normal breast ductal epithelial cells were reverse-transcribed in the presence of Cy5-dCTP or Cy3-dCTP, respectively. After hybridization on microarray slides, we calculated the Cy5/Cy3 ratio as described above. The average of each ratio was decided by the result using mammary gland tissues and microdissected normal breast ductal cells from pre-menopausal and post-menopausal patients.

Since *MUC1* is significantly over-expressed in breast cancers, we measured expression of this gene to evaluate the proportion of breast-cancer cells in each population of whole breast-cancer or microdissected breast-cancer cells. Each aRNA of whole breast-cancer tissues (cases 10453W and 10397W), and of microdissected breast-cancer cells (cases 10397 and 10453) were labeled with Cy5-dCTP, whereas a mixture of microdissected normal breast ductal cells from pre-menopausal patients was labeled with Cy3-dCTP. We calculated the Cy5/Cy3 ratios as relative expression ratios, as follows: expression levels of *MUC1* were calculated as the ratio of *MUC1* in whole breast-cancer tissues to a mixture of microdissected normal breast ductal cells, and as the ratio of *MUC1* in microdissected breast cancer cells to a mixture of microdissected normal breast ductal cells.

Cluster analysis according to gene-expression profiles. We applied an unsupervised hierarchical clustering method to both genes and tumors. To obtain reproducible clusters for classification of 102 samples, we selected 710 genes for which valid data were obtained in 80% of the experiments, and whose expression ratios varied by standard deviations of more than 1.1. The analysis was performed using web-available software (Cluster and TreeView) made by Dr M. Eisen (http://genome-www5.stanford.edu/MicroArray/SMD/restech.html). Prior to application of the clustering algorithm, we log-transformed the fluorescence ratio for each spot and then median-centered the data for each sample to remove experimental biases and used average linkage.

Identification of up- or down-regulated genes in DCIS versus IDC. The relative expression ratio of each gene (Cy5/Cy3 intensity ratio) was classified into one of four categories: a) up-regulated (expression ratio >3.0); b) down-regulated (expression ratio <0.33); c) unchanged (expression ratio between 0.33 and 3.0); and d) not expressed (or slight expression but under the cut-off level for detection). We used these categories to detect a set of genes for which changes in the expression ratios were common among samples. To detect candidate genes that were commonly up- or down-regulated in each group, the overall expression patterns of 23,040 genes were first screened to select genes with expression ratios of >3.0 or <0.33 that were present in >50% of the groups categorized.

Semi-quantitative RT-PCR. We selected five up-regulated genes and confirmed their expression levels by semi-quantitative RT-PCR experiments. A 1-μg aliquot of aRNA from each sample was reverse-transcribed for single-stranded cDNAs using random primer and Superscript II (Invitrogen, Carlsbad, CA). Each cDNA mixture was diluted for subsequent PCR amplification with the primer sets shown in Table II. Expression of *GAPDH* served as an internal control because *GAPDH* expression levels were consistent throughout all samples through the cDNA microarray data. PCR reactions were optimized for the number of cycles to ensure product intensity at the linear phase of amplification.

Identification of genes associated with histological status, ER status and lymph node metastasis of breast cancers. We

Table II. Primer sequences for semi-quantitative RT-PCR experiments.

Accession No.	Symbol	Forward primer	Reverse primer
AI261804	EST	5'-CTGTTCTGGCTTCGTTATGTTCT-3'	5'-AGAAAATACGGTCCTCTTGTTGC-3'
AA205444	*AP1S2*	5'-CACTGTAATGCACGACATTTGA-3'	5'-GTTACAGCTTAGCACAAGGCATC-3'
AA167194	LOC253782	5'-ACCTCTGAGTTTGATTTCCCAA-3'	5'-CGAGGCTTGTAACAATCTACTGG-3'
AA676987	EST	5'-GAAACTGTACGGGGGTTAAAGAG-3'	5'-CATCAATGTGGTGAGTGACATCT-3'
H22566	*DACH*	5'-AAGCCCTTGGAACAGAACATACT-3'	5'-CAGTAAACGTGGTTCTCACATTG-3'
NM_002046	*GAPDH*	5'-CGACCACTTTGTCAAGCTCA-3'	5'-GGTTGAGCACAGGGTACTTTATT-3'

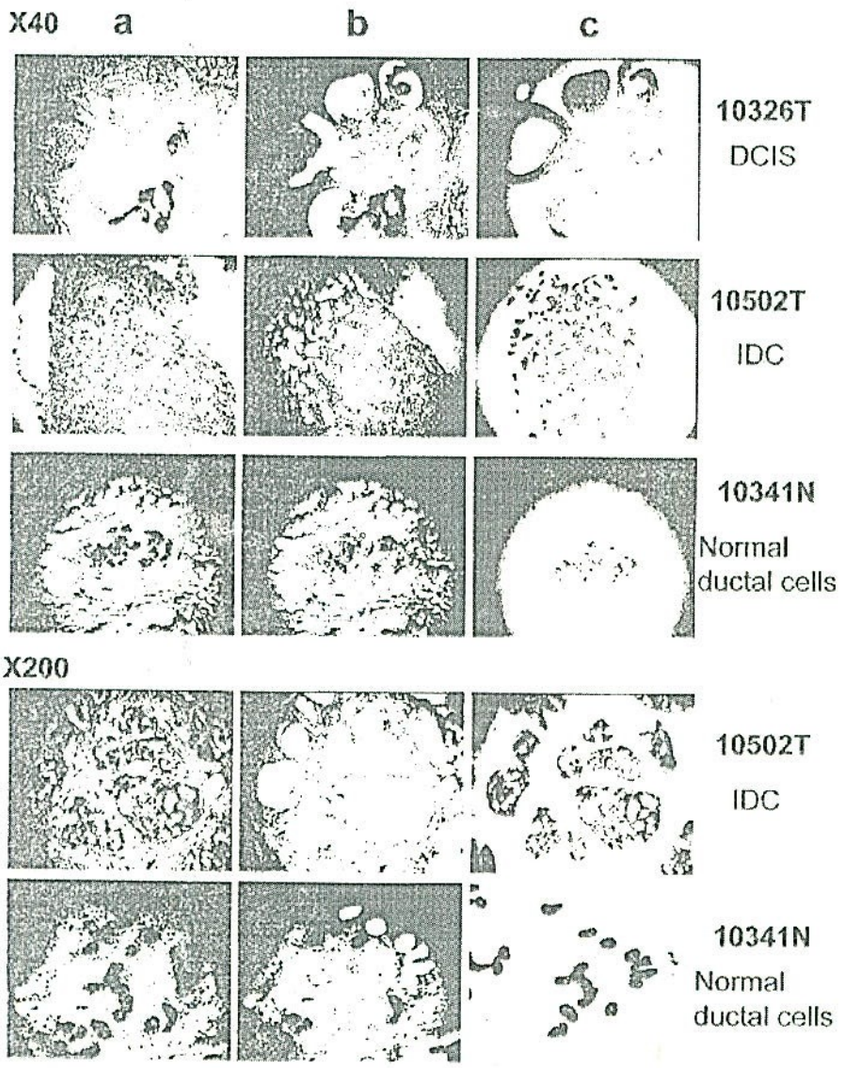
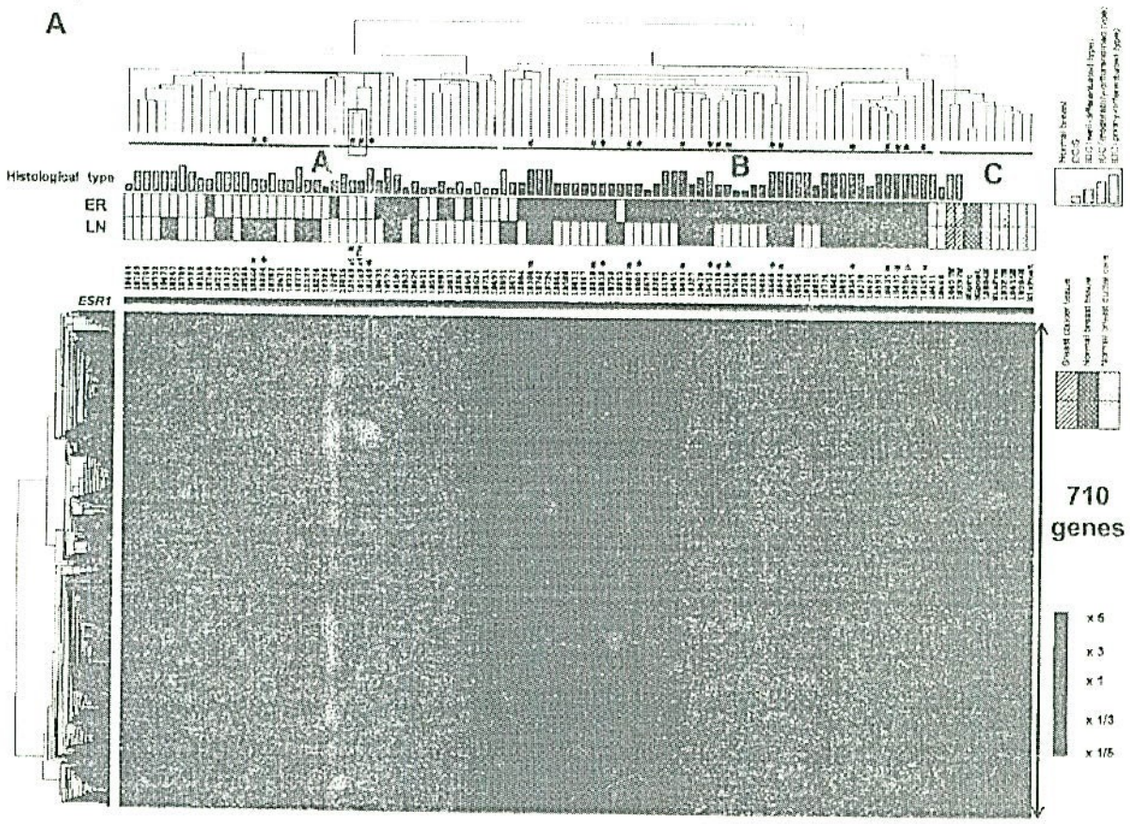
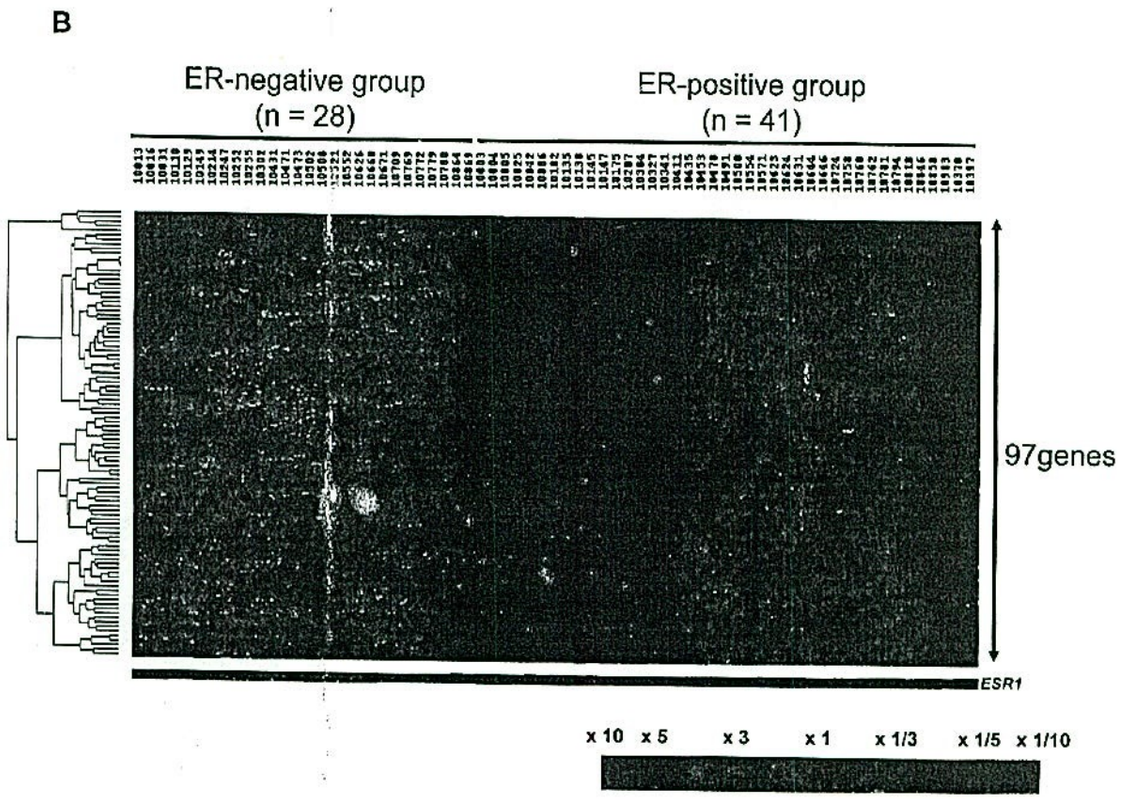


Figure 1. Purification of cancer cells from DCIS (10326T) and IDC (10502T), and normal breast ductal epithelial cells (10341N), by means of laser microbeam microdissection (LMM). Images of pre-microdissected (lane a), post-microdissected (lane b), and microdissected cells (lane c) are shown after staining with hematoxylin and eosin.

selected discriminating genes using two criteria: a) signal intensities higher than the cut-off level in at least 70% (ER status) or 50% (histological status or lymph-node metastasis) of the cases, and b) $|Med_i - Med_j| > 1$ (ER status) or 0.5 (histopathological status and lymph node metastasis) of the cases, where Med indicates the median derived from log-transformed


A
Histological type
ER
LN
A
B
C
ESR1
710
genes
x 6
x 3
x 1
x 1/3
x 1/5


B
ER-negative group
(n = 28)
ER-positive group
(n = 41)
97genes
ESR1
x 10
x 5
x 3
x 1
x 1/3
x 1/5
x 1/10

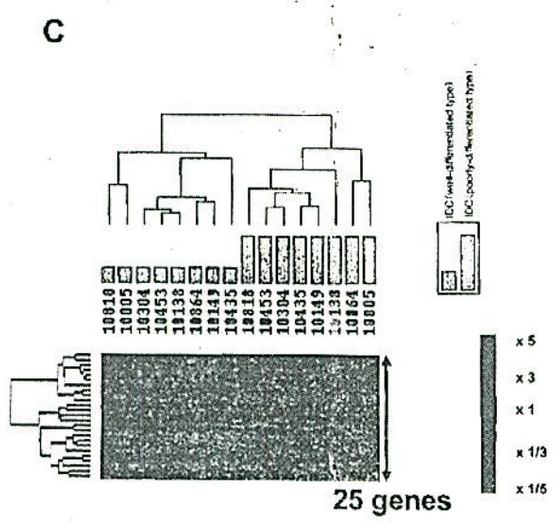


Figure 2. Unsupervised two-dimensional hierarchical clustering analysis of 102 samples on the basis of expression of 710 genes. A, Each horizontal row represents a single gene, and each vertical column corresponds to a breast-cancer patient. In each well a red or green color indicates a transcript level respectively above or below the median for that gene across all samples. Asterisks indicate the major histological type, and a sharp sign the minor histological type in cases where two lesions from the same patient were analyzed. A red outline in the dendrogram indicates one duplicated case. Black, unchanged expression. ER, ER status measured by EIA; LN, lymph-node metastasis status; *ESR1*, expression profiles of *ESR1* in this microarray. Mgpre, mammary gland tissue/pre-menopausal microdissected normal ductal cells; Mgpost, mammary gland tissue/post-menopausal microdissected normal ductal cells; mixPre, pre-menopausal microdissected normal ductal cells/pre-menopausal microdissected normal ductal cells; mixPost, post-menopausal microdissected normal ductal cells/pre-menopausal microdissected normal ductal cells. The average of each ratio was decided by the result when we used mammary-gland tissue and microdissected normal breast ductal cells from pre-menopausal and post-menopausal patients. B, Supervised hierarchical clustering analysis of 97 genes selected by a random-permutation test. Each vertical column corresponds to 1 of 41 ER-positive or 28 ER-negative cases selected from among pre-menopausal patients. Each horizontal row represents 1 of the 97 genes that were clustered in different branches according to similarities in relative expression ratios. Genes in the lower main branch were expressed at a relatively high level in tumors with *ESR1* expression. Those in the upper branch were expressed at relatively high levels in *ESR1*-negative tumors. C, Supervised clustering analysis using 25 genes that showed differential expression within pairs of well- (a1) or poorly-differentiated (a3) invasive ductal cancer cells micro-dissected from breast-cancers of eight patients.

relative expression ratios in node-positive or node-negative cases. Next, we applied a random-permutation test to identify genes that were expressed differently between one group (group A) and another (group B). Mean (μ) and standard (σ) deviations were calculated from the log-transformed relative expression ratios of each gene in group A (r) and group B (n) cases. A discrimination score (DS) for each gene was defined as follows: $DS = (\mu_r - \mu_n)/(\sigma_r + \sigma_n)$. We carried out permutation tests to estimate the ability of individual genes to distinguish between groups A and B; samples were randomly permutated between the two classes 10,000 times. Since the DS dataset of each gene showed a normal distribution, we calculated a p-value for the user-defined grouping (22).

Calculation of prediction score for lymph node metastasis. We calculated prediction scores according to procedures described previously (22). Each gene (gi) votes for either lymph node-negative or node-positive depending on whether the expression level (xi) in the sample is closer to the mean expression level of node-negative or -positive in reference samples. The magnitude of the vote (vi) reflects the deviation of the expression level in the sample from the average of the two classes: $V_i = |x_i - (\mu_r + \mu_n)/2|$. We summed the votes to obtain total votes for the node-negative (Vr) and node-positive (Vn), and calculated PS values as follows: PS = (Vr - Vn)/(Vr + Vn) x 100-15.8, reflecting the margin of victory in the direction of either node-negative or node-positive. PS values range from -120 to 120; a higher absolute value of PS reflects a stronger prediction.

Evaluation of classification and leave-one-out test. We calculated the classification score (CS) using prediction scores of lymph node-negatives (PSr) and node-positives (PSn) in each gene set as follows: $CS = (\mu_{PSr} - \mu_{PSn})/(\sigma_{PSr} + \sigma_{PSn})$. A larger value of CS indicates better separation of the two groups by the prediction-scoring system. For the leave-one-out test, one sample is withheld, the permutation p-value and mean expression levels are calculated using remaining samples, and the class of the withheld sample is subsequently evaluated by calculating its prediction score. We repeated this procedure for each of the 20 samples.

Results

Purification of breast cancer cells and normal ductal epithelial cells by LMM. Because breast tumors contain mixtures of different cell types, any differences observed in expression profiles using mRNAs from tumor masses are influenced to a large extent by the proportions of various cell types present. Moreover, since the great majority of cells in normal breast tissue are adipocytes, we considered whole-breast tissue to be unsuitable for analyzing genetic alterations associated with mammary carcinogenesis. Hence, to obtain precise gene-expression profiles during the course of breast-cancer development and progression, we purified both cancerous cells and normal ductal epithelial cells (for control) by LMM. Fig. 1 shows representative examples of DCIS, IDC, and normal ductal epithelium before and after microdissection from clinical specimens.

We estimated the proportion of adipocytes in the micro-dissected population of normal breast ductal epithelial cells by measuring the signal intensities of two genes, perilipin (*PLIN*) and fatty acid-binding protein 4 (*FABP4*), which are expressed highly and specifically in adipose tissues (21). When we compared the signal intensities of those genes in whole mammary-gland tissue with signals of the micro-dissected normal breast ductal epithelial cells, the average ratio of signal intensities of the two genes were approximately 99.4%, an indication that the proportion of adipocytes in the microdissected normal breast ductal cells was only about 0.6% (section Calculation of contamination percentage in Materials and methods).

We also analyzed the expression levels of mucin 1 (*MUC1*), which shown to be significantly over-expressed in breast-cancer cells (23,24). The Cy5/Cy3 ratios of this gene in two

Table III. Genes with altered expression between ER-positive and ER-negative tumors.

Accession No.	Symbol	Gene name	p-value	ER-positive
AW949747	*GATA3*	GATA binding protein 3	3.2E-20	+
BE868254	ESTs	ESTs	2.2E-14	+
AF037335	*CA12*	Carbonic anhydrase XII	1.6E-13	+
BF724977	*ASB13*	Ankyrin repeat and SOCS box-containing 13	8.5E-13	+
NM_004636	*SEMA3B*	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B	9.7E-13	+
NM_000125	*ESR1*	Estrogen receptor 1	1.2E-12	+
M73554	*CCND1*	Cyclin D1 (PRAD1: parathyroid adenomatosis 1)	3.9E-12	+
NM_005544	*IRS1*	Insulin receptor substrate 1	4.4E-12	+
M14745	*BCL2*	B-cell CLL/lymphoma 2	5.1E-12	+
BE826171	*BCMP11*	Breast cancer membrane protein 11	2.8E-11	+
AI087270	*SIAH2*	Seven in absentia homolog 2 (*Drosophila*)	2.8E-11	+
L07033	*HMGCL*	3-Hydroxymethyl-3-methylglutaryl-Coenzyme A lyase (hydroxymethylglutaricaciduria)	2.8E-11	+
AB014523	*ULK2*	Unc-51-like kinase 2 (*C. elegans*)	4.0E-11	+
AL137588	EST	Hypothetical protein DKFZp434K1210	5.2E-11	+
AL137566	EST	*Homo sapiens* mRNA; cDNA DKFZp586G0321	5.4E-11	+
AF038421	*GFRA1*	GDNF family receptor α 1	8.4E-11	+
AI194045	*FE65L2*	FE65-like protein 2	9.2E-11	+
BG163478	ESTs	ESTs, weakly similar to BAI1_HUMAN brain-specific angiogenesis inhibitor 1 precursor (*H. sapiens*)	1.1E-10	+
M31627	*XBP1*	X-box binding protein 1	1.1E-10	+
AA156269	EST	*Homo sapiens*, clone IMAGE:4794107, mRNA	1.3E-10	+
NM_006763	*BTG2*	BTG family, member 2	1.9E-10	+
AW504052	*SEC15L*	SEC15 (*S. cerevisiae*)-like	2.1E-10	+
NM_005400	*PRKCE*	Protein kinase C ε	2.3E-10	+
AI628151	*XBP1*	X-box binding protein 1	2.7E-10	+
AF043045	*FLNB*	Filamin B, ß (actin binding protein 278)	3.5E-10	+
U31383	*GNG10*	Guanine nucleotide binding protein (G protein), γ 10	4.6E-10	+
L10333	*RTN1*	Reticulon 1	5.6E-10	+
AK025099	*SIGIRR*	Single Ig IL-1R-related molecule	6.2E-10	+
AL039253	*LIV-1*	LIV-1 protein, estrogen regulated	7.4E-10	+
AW949662	KIAA0239	KIAA0239 protein	8.0E-10	+
D13629	*KTN1*	Kinectin 1 (kinesin receptor)	1.5E-09	+
NM_000165	*GJA1*	Gap junction protein, α 1, 43 kDa (connexin 43)	1.5E-09	+
AA533079	*C1orf21*	Chromosome 1 open reading frame 21	1.8E-09	+
AF251056	*CAPS2*	Calcyphosphine 2	1.9E-09	+
AF061016	*UGDH*	UDP-glucose dehydrogenase	2.0E-09	+
U92544	*MAGED2*	Melanoma antigen, family D, 2	2.1E-09	+
BE617536	*RPLI3A*	Ribosomal protein L13a	2.4E-09	+
AK024102	*MYST1*	MYST histone acetyltransferase 1	2.5E-09	+
BF212902	EST	*Homo sapiens* mRNA; cDNA DKFZp564F053	2.8E-09	+
AK025480	FLJ21827	Hypothetical protein FLJ21827	3.0E-09	+
AI376713	ESTs	ESTs, weakly similar to hypothetical protein FLJ20378 (*H. sapiens*)	3.6E-09	+
AI028483	ESTs	ESTs	3.8E-09	+
AK022249	EST	*Homo sapiens* cDNA FLJ12187 fis, clone MAMMA1000831	4.2E-09	+
AI568527	EST	*Homo sapiens* cDNA FLJ34849 fis, clone NT2NE2011687	5.0E-09	+
AL133074	*TP53INP1*	Tumor protein p53 inducible nuclear protein 1	5.3E-09	+
AF022116	*PRKAB1*	Protein kinase, AMP-activated, ß 1 non-catalytic subunit	6.1E-09	+
AF007170	*C1orf34*	Chromosome 1 open reading frame 34	9.7E-09	+
AF042081	*SH3BGRL*	SH3 domain binding glutamic acid-rich protein like	1.2E-08	+
AK027813	MGC10744	Hypothetical protein MGC10744	1.4E-08	+
M57609	*GLI3*	GLI-Kruppel family member GLI3 (Greig cephalopolysyndactyly syndrome)	1.7E-08	+
AL359600	EST	*Homo sapiens* mRNA; cDNA DKFZp547C136	1.9E-08	+
BQ006049	*TIMP1*	Tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collagenase inhibitor)	2.1E-08	+
AF111849	*HELO1*	Homolog of yeast long chain polyunsaturated fatty acid elongation enzyme 2	2.2E-08	+
AL157499	*RAB5EP*	Rabaptin-5	2.2E-08	+
AK023199	EST	*Homo sapiens* cDNA FLJ13137 fis, clone NT2RP3003150	2.5E-08	+
J05176	*SERPINA3*	Serine (or cysteine) proteinase inhibitor, clade A (α-1 antiproteinase, antitrypsin), member 3	3.2E-08	+
AA028101	KIAA0303	KIAA0303 protein	3.3E-08	+

Table III. Continued.

Accession No.	Symbol	Gene name	p-value	ER-positive
AI300588	*MAP2K4*	Mitogen-activated protein kinase kinase 4	4.1E-08	+
AA682861	ESTs	ESTs, moderately similar to hypothetical protein FLJ20378 (*Homo sapiens*)	4.6E-08	+
M26393	*ACADS*	Acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain	5.4E-08	+
NM_001609	*ACADSB*	Acyl-Coenzyme A dehydrogenase, short/branched chain	5.5E-08	+
U91543	*CHD3*	Chromodomain helicase DNA binding protein 3	5.7E-08	+
AK023813	FLJ10081	Hypothetical protein FLJ10081	6.0E-08	+
BF111711	FLJ20727	Hypothetical protein FLJ20727	7.0E-08	+
AL049987	EST	*Homo sapiens* mRNA; cDNA DKFZp564F112	7.2E-08	+
AW081894	EST	EST	8.2E-08	+
AK000350	FLJ20343	Hypothetical protein FLJ20343	1.1E-07	+
AA418493	*DPP7*	Dipeptidylpeptidase 7	1.1E-07	+
BE674061	*PIN4*	Protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting, 4 (parvulin)	1.2E-07	+
AB011155	*DLG5*	Discs, large (*Drosophila*) homolog 5	1.2E-07	+
L15203	*TFF3*	Trefoil factor 3 (intestinal)	1.4E-07	+
NM_001552	*IGFBP4*	Insulin-like growth factor binding protein 4	1.4E-07	+
M57230	*IL6ST*	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	1.5E-07	+
N92706	EST	*Homo sapiens* cDNA FLJ38461 fis, clone FEBRA2020977	1.5E-07	+
M30704	*AREG*	Amphiregulin (schwannoma-derived growth factor)	1.8E-07	+
AB004066	*BHLHB2*	Basic helix-loop-helix domain containing, class B, 2	2.2E-07	+
M15518	*PLAT*	Plasminogen activator, tissue	2.3E-07	+
BM697477	*Shrml*	Shroom-related protein	2.4E-07	+
R45979	*CELSR1*	Cadherin, EGF LAG seven-pass G-type receptor 1 (flamingo homolog, *Drosophila*)	3.0E-07	+
AL049365	EST	*Homo sapiens* mRNA; cDNA DKFZp586A0618	6.5E-07	+
NM_003225	*TFF1*	Trefoil factor 1 (breast cancer, estrogen-inducible sequence expressed in)	7.1E-07	+
AI733356	EST	*Homo sapiens* cDNA FLJ31746 fis, clone NT2R12007334	7.8E-07	+
AF078853	KIAA1243	KIAA1243 protein	8.2E-07	+
N30179	*PLAB*	Prostate differentiation factor	1.0E-06	+
BG026429	*SFRS2*	Splicing factor, arginine/serine-rich 2	2.4E-06	-
AU149272	ESTs	ESTs	2.5E-06	+
J03827	*NSEP1*	Nuclease sensitive element binding protein 1	3.0E-06	-
AJ276469	*C20orf35*	Chromosome 20 open reading frame 35	3.4E-06	+
AW295100	LOC201562	Hypothetical protein LOC201562	3.9E-06	-
J03817	*GSTM1*	Glutathione S-transferase M1	4.8E-06	+
AF288571	*LEF1*	Lymphoid enhancer-binding factor 1	5.1E-06	+
AF069301	*PECI*	Peroxisomal D3, D2-enoyl-CoA isomerase	5.3E-06	+
AA621665	EST	EST	6.7E-06	-
AI739486	ESTs	ESTs	8.0E-06	+
X81438	*AMPH*	Amphiphysin (Stiff-Man syndrome with breast cancer 128 kDa autoantigen)	8.7E-06	+
U89606	*PDXK*	Pyridoxal (pyridoxine, vitamin B6) kinase	8.8E-06	-
NM_017555	*EGLN2*	Egl nine homolog 2 (*C. elegans*)	9.2E-06	+

ER-positive, + indicates the genes that relatively over-expressed in the ER-positive group as compared with the ER-negative group; - indicates the genes that relatively under-expressed in the ER-positive group as compared with the ER-negative group.

populations of microdissected breast cancer cells (cases 10397 and 10453) were 18.2 and 17.0, whereas the ratios in the corresponding whole-tumor cell populations were 12.5 and 5.25 (69 and 31%) respectively. These results suggested that Cy5/Cy3 ratios of genes that appear to be up-regulated in whole-tumor preparations are likely to be underestimated due to contamination by a large proportion of non-cancerous cells.

Unsupervised clustering analysis of gene-expression profiles. We first applied an unsupervised two-dimensional hierarchical clustering algorithm to group genes on the basis of similarities in expression patterns, using the data from 102 experiments corresponding to 92 clinical breast cancer specimens (two distinct histological lesions were examined in ten cases and one case was analyzed in duplicate), 2 breast cancer tissues without microdissection, 6 microdissected normal-breast ductal cells, and 2 whole mammary-gland tissues without microdissection. Reproducible clusters were obtained using the expression data for 710 genes (Materials and methods); the 102 samples were clustered into two major groups and one minor group (groups A, B and C) on the basis of their expression profiles (Fig. 2A). Profiles from the single

Table IV. Genes with altered expression between well- or poorly-differentiated cancer.

Accession No.	Symbol	Gene name	p-value	Poorly
AF053712	*TNFSF11*	Tumor necrosis factor (ligand) superfamily, member 11	1.2E-06	+
BF973404	LOC201725	Hypothetical protein LOC201725	3.2E-05	+
AV752313	*KPNA6*	Karyopherin α 6 (importin α 7)	1.1E-04	+
AK026898	*FOXP1*	Forkhead box P1	7.4E-04	+
AA148107	*ITGA5*	Integrin, α 5 (fibronectin receptor, α polypeptide)	7.9E-04	+
AK001067	*NFAT5*	Nuclear factor of activated T-cells 5, tonicity-responsive	8.2E-04	+
AB007919	KIAA0450	KIAA0450 gene product	1.8E-03	+
BG026429	*SFRS2*	Splicing factor, arginine/serine-rich 2	2.0E-03	-
M87770	*FGFR2*	Fibroblast growth factor receptor 2 (bacteria-expressed kinase, keratinocyte growth factor receptor, craniofacial dysostosis 1, Crouzon syndrome, Pfeiffer syndrome, Jackson-Weiss syndrome)	2.1E-03	-
L02785	*SLC26A3*	Solute carrier family 26, member 3	2.7E-03	-
BF037402	EST	*Homo sapiens*, clone MGC:17296 IMAGE:3460701, mRNA, complete cds	2.8E-03	-
L12350	*THBS2*	Thrombospondin 2	2.8E-03	-
N36875	EST	*Homo sapiens*, clone IMAGE:4994678, mRNA	3.8E-03	-
AL135342	EST	ESTs, weakly similar to neuronal thread protein (*Homo sapiens*)	4.3E-03	-
AL049426	*SDC3*	Syndecan 3 (N-syndecan)	4.5E-03	-
AW961424	KIAA1870	KIAA1870 protein	5.2E-03	-
AA523117	*DC-TM4F2*	Tetraspanin similar to TM4SF9	5.5E-03	-
Z11531	*EEF1G*	Eukaryotic translation elongation factor 1 γ	6.1E-03	-
AI423028	*SMARCD3*	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 3	6.8E-03	-
AB002391	*MN7*	D15F37 (pseudogene)	7.1E-03	+
D32050	*AARS*	Alanyl-tRNA synthetase	7.2E-03	+
BE876949	*RAB7*	RAB7, member RAS oncogene family	7.9E-03	+
AW291083	EST	ESTs	8.0E-03	-
AI568910	EST	ESTs	8.2E-03	-
AK023480	*SRP72*	Signal recognition particle 72 kDa	8.7E-03	-

Poorly: + indicates the genes that relatively over-expressed in the poorly-differentiated cases as compared with the well-differentiated cases; - indicates the genes that relatively under-expressed in the poorly-differentiated cases as compared with the well-differentiated cases.

duplicated case (case 10149a1/10149a1T) were clustered next to each other with the shortest branch in breast cancer cases, supporting the reproducibility and reliability of our microarray data. We investigated possible association of this classification with clinical parameters such as estrogen receptor (ER) status, histological type, or lymph node (LN) status. Only the ER status of the cancer cells showed some association: of the 55 tumors diagnosed as ER-positive by enzyme immunoassay (EIA), 45 were classified into the same cluster (group B) of the tumor dendrogram. These results indicated that the most significant factor influencing expression patterns in breast cancers is ER status. Microdissected normal breast ductal cells (cases 10004N, 10327N, 10435N, and 10304N) and cancer tissues without microdissection (cases 10453W and 10397W) constituted a separate group (group C) that included one microdissected tumor case (case 10660), an indication that our data derived from purified breast-cancer and normal breast ductal cells were more precise in terms of breast cancer-specific expression profiles than the profiles reported by others (9-15).

Identification of gene clusters associated with clinical parameters. To detect genes that might reflect the ER status of breast cancers, we carried out a supervised clustering analysis of 41 ER-positive and 28 ER-negative tumors of the IDC type. All of the tumors we examined were from pre menopausal patients. We listed 97 genes that were able to distinguish the ER-positive and negative cases with permutation p-value <0.0001 (Materials and methods) (Fig. 2B and Table III). Among them, 92 were relatively over-expressed and the other 5 were under-expressed in the ER-positive group as compared with the ER-negative group. Genes that were over-expressed in the ER-positive group included GATA-binding protein 3 (*GATA3*), trefoil factor 3 (*TFF3*), cyclin D1 (*CCND1*), MAPKK homolog (*MAP2K4*), tissue inhibitor of metalloprotease 1 (*TIMP1*), insulin receptor substrate 1 (*IRS1*), and GLI-Kruppel family member GLI3 (*GLI3*). Those genes were already known to be associated with ER-positive status (10,15). On our list, estrogen receptor (*ESR1*) was rank-ordered no. 6 on the basis of magnitude of p-value (Table III), and the classification of *ESR1* by its expression profiles correlated closely with ER status in tumors measured by EIA (Fig. 2B, bottom panel) in most cases.

As Fig. 2A shows, histopathological types judged by microscopy were not clearly discerned by an unsupervised classification according to gene-expression patterns. However, in 7 of the 10 cases in which samples of different histological type were obtained from the same patient (cases 10864, 10149,

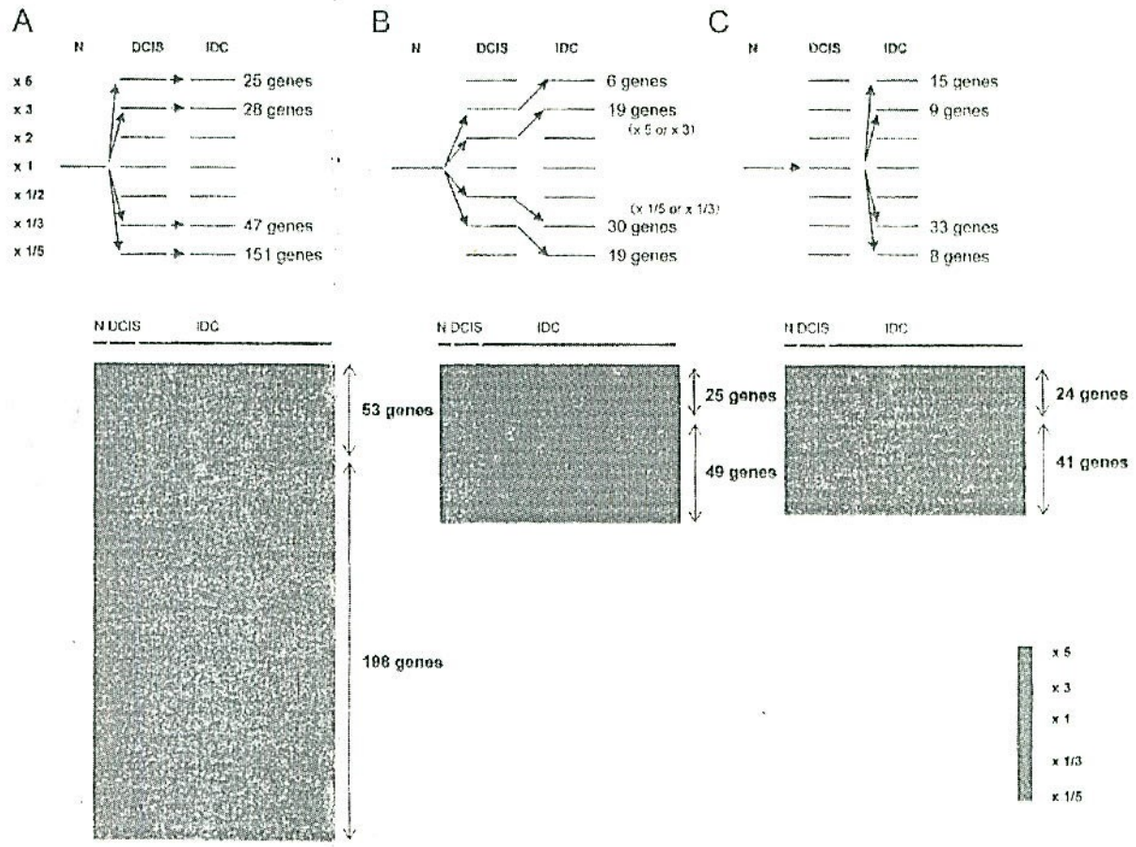


Figure 3. Genes showing altered expression in breast cancers. A, 251 genes that were commonly up- or down-regulated in both DCIS and IDC. B, 74 genes whose expression was further elevated or decreased during transition from DCIS to IDC. C, 65 genes that were up- or down-regulated specifically in IDC. N, normal breast ductal cells.

10818, 10138, 10005, 10646 and 10435) the 2 samples were clustered more closely to each other in one group than to their respective types from other patients. This finding suggess that two lesions prepared from a single breast specimen could show similar expression patterns even when they are histologically distinct (IDC).

We subsequently carried out random-permutation test to search for genes that would reflect histological differences (i.e., well- or poorly-differentiated lesions), using eight pairs of microdissected IDC samples. As shown in Fig. 2C, a supervised clustering analysis using 25 genes that showed differential expression clearly separated the two histologically-distinct groups of ductal-cancer cells, an indication that certain of those genes might be associated with invasiveness of breast cancer (Table IV). Among them, nuclear factor of activated T-cells 5 (*NFAT5*), and integrin α 5 (*ITGA5*), whose possible roles in invasion and cell growth had been reported previously (25,26).

Identification of genes commonly up- or down-regulated in DCIS and IDC. Gene-expression profiles in 77 pre-menopausal breast tumors (8 DCIS and 69 IDC) identified 78 genes that were commonly up-regulated >3-fold in tumors over normal breast ductal cells, and 247 genes whose expression was down-regulated to <1/3 of normal (Fig. 3A and B, Tables V and VI). Among the genes commonly up-regulated in both DCIS and IDC were neuropeptide Y receptor Y1 (*NYRY1*) and mucin 1 (*MUC1*), already known to be over-expressed in breast cancers (23,24,27), whereas among the commonly down-regulated genes we documented transforming growth factor ß receptor II (*TGFBR2*) and early growth response 2 (*EGR2*), both of which also show dysfunction in various cancers (28,29). In particular, as Fig. 3B shows, expression levels of 25 genes were further increased (19 >3-fold; 6 >5-fold) and levels of 49 genes were further decreased (30 <3-fold; 19 <5-fold) during malignant progression from DCIS to IDC. The 25 up-regulated genes included forkhead box M1 (*FOXM1*), whose over-expression stimulates expression of cyclin B1 (*CCNB1*) (30). Suppression of tumorigenicity 5 (*ST5*) and schwannomin-interacting protein 1 (*SCHIP1*), both of them known to function as tumor suppressors (31,32), were among the genes showing decreased expression as malignancy progressed.

We then attempted to identify genes whose expression was altered in IDC but not in DCIS, and identified 24 up-regulated genes and 41 down-regulated genes (Fig. 3C, Table VII). The

Table V. Genes commonly up- and down-regulated in DCIS or IDC.

Up-regulated genes		
Accession No.	Symbol	Gene name
D90041	*NAT1*	N-acetyltransferase 1 (arylamine N-acetyltransferase)
M13755	*G1P2*	Interferon, α-inducible protein (clone IFI-15K)
D88308	*SLC27A2*	Solute carrier family 27 (fatty acid transporter), member 2
AW235061	*SLC1A1*	Solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1
K02215	*AGT*	Angiotensinogen [serine (or cysteine) proteinase inhibitor, clade A (α-1 antiproteinase, antitrypsin), member 8]
AB032261	*SCD*	Stearoyl-CoA desaturase (δ-9-desaturase)
NM_000909	*NPY1R*	Neuropeptide Y receptor Y1
AF017790	*HEC*	Highly expressed in cancer, rich in leucine heptad repeats
NM_007019	*UBE2C*	Ubiquitin-conjugating enzyme E2C
AF065388	*TSPAN-1*	Tetraspan 1
N70334	*DUSP10*	Dual specificity phosphatase 10
AA621719	*SMC4L1*	SMC4 structural maintenance of chromosomes 4-like 1 (yeast)
AA676987	ESTs	ESTs
AK001402	*C10orf3*	Chromosome 10 open reading frame 3
AW949747	*GATA3*	GATA binding protein 3
AK001472	*ANLN*	Anillin, actin binding protein (scraps homolog, *Drosophila*)
AA789233	*COL1A1*	Collagen, type I, α 1
AF070632	EST	*Homo sapiens* clone 24405 mRNA sequence
H04544	*NPY1R*	Neuropeptide Y receptor Y1
AI015982	*CDCA1*	Cell division cycle associated 1
NM_003979	*RAI3*	Retinoic acid induced 3
BF516445	*CLIC6*	Chloride intracellular channel 6
AI361654	EST	EST
AI077540	EST	*Homo sapiens* cDNA FLJ38379 fis, clone FEBRA2002986
AI261804	EST	*Homo sapiens* MSTP020 (MST020) mRNA, complete cds
AK026559	*TPM3*	Tropomyosin 3
J03473	*ADPRT*	ADP-ribosyltransferase [NAD+; poly(ADP-ribose) polymerase]
NM_000187	*HGD*	Homogentisate 1,2-dioxygenase (homogentisate oxidase)
L43964	*PSEN2*	Presenilin 2 (Alzheimer disease 4)
J05581	*MUC1*	Mucin 1, transmembrane
AA602499	*GLCCI1*	Glucocorticoid induced transcript 1
U37707	*MPP3*	Membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3)
AB030905	*CBX3*	Chromobox homolog 3 (HP1 γ homolog, *Drosophila*)
AL138409	EST	*Homo sapiens* mRNA; cDNA DKFZp313L231
AV756928	*SEC61G*	Sec61 γ
AI205684	*HSPA2*	Heat shock 70 kDa protein 2
BE739464	*ARL6IP*	ADP-ribosylation factor-like 6 interacting protein
AI081356	LOC253782	Hypothetical protein LOC253782
AA167194	LOC253782	Hypothetical protein LOC253782
M90516	*GFPT1*	Glutamine-fructose-6-phosphate transaminase 1
AL133074	*TP53INP1*	Tumor protein p53 inducible nuclear protein 1
AL137257	EST	*Homo sapiens*, clone IMAGE:5296692, mRNA
AK025240	LOC223082	LOC223082
AJ007042	*WHSC1*	Wolf-Hirschhorn syndrome candidate 1
U42068	*GRP58*	Glucose regulated protein, 58 kDa
AJ132592	*ZNF281*	Zinc finger protein 281
W93638	ESTs	ESTs
AW977394	*C9orf12*	Chromosome 9 open reading frame 12
AI347925	*HSPB1*	Heat shock 27 kDa protein 1
AK026587	*NET-6*	Transmembrane 4 superfamily member tetraspan NET-6
AI264621	*LASS2*	LAG1 longevity assurance homolog 2 (*S. cerevisiae*)
AA767828	FLJ10980	Hypothetical protein FLJ10980
AU142881	FLJ10702	Hypothetical protein FLJ10702

Down-regulated genes		
Accession No.	Symbol	Gene name
X52186	*ITGB4*	Integrin, ß 4
NM_006297	*XRCC1*	X-ray repair complementing defective repair in Chinese hamster cells 1
X73460	*RPL3*	Ribosomal protein L3
NM_001436	*FBL*	Fibrillarin
X59373	*HOXD10*	Homeo box D10
J04208	*IMPDH2*	IMP (inosine monophosphate) dehydrogenase 2
L24203	*TRIM29*	Tripartite motif-containing 29
L10340	*EEF1A2*	Eukaryotic translation elongation factor 1 α 2

Table V. Continued.

Down-regulated genes		
Accession No.	Symbol	Gene name
J04621	*SDC2*	Syndecan 2 (heparan sulfate proteoglycan 1, cell surface-associated, fibroglycan)
L08424	*ASCL1*	Achaete-scute complex-like 1 (*Drosophila*)
AI376713	EST	ESTs, weakly similar to hypothetical protein FLJ20378 (*Homo sapiens*)
AK026966	EST	*Homo sapiens* cDNA: FLJ23313 fis, clone HEP11919
NM_001050	*SSTR2*	Somatostatin receptor 2
AA632025	ESTs	ESTs
N22918	FLJ32332	Hypothetical protein FLJ32332
AF272043	*ITM2C*	Integral membrane protein 2C
M58459	*RPS4Y*	Ribosomal protein S4, Y-linked
AI133697	EST	*Homo sapiens*, clone MGC:16362 IMAGE:3927795, mRNA, complete cds
AA780301	*CTSF*	Cathepsin F
M92843	*ZFP36*	Zinc finger protein 36, C3H type, homolog (mouse)
AA570186	EST	Human full-length cDNA 5-PRIME end of clone CS0DK007YB08 of HeLa cells of *H. sapiens*
R56906	EST	EST
AF208860	*TNFRSF21*	Tumor necrosis factor receptor superfamily, member 21
AK025216	*TAZ*	Transcriptional co-activator with PDZ-binding motif (TAZ)
AA758394	*PTPN1*	Protein tyrosine phosphatase, non-receptor type 1
AA628530	*ISYNA1*	Myo-inositol 1-phosphate synthase A1
AF161416	*IRS2*	Insulin receptor substrate 2
AL045916	ESTs	ESTs
AW340972	EST	*Homo sapiens* cDNA: FLJ22864 fis, clone KAT02164
AI189414	RNPC2	RNA-binding region (RNP1, RRM) containing 2
AV705636	EIF3S6IP	Eukaryotic translation initiation factor 3, subunit 6 interacting protein
U28977	CASP4	Caspase 4, apoptosis-related cysteine protease
AV708528	MSCP	Mitochondrial solute carrier protein
AA022956	FLJ12750	Hypothetical protein FLJ12750
AI928443	EST	*Homo sapiens* cDNA FLJ38855 fis, clone MESAN2010681
U14966	*RPL5*	Ribosomal protein L5
AI857997	*TPBG*	Trophoblast glycoprotein
BF697545	*MGP*	Matrix Gla protein
AW575754	FLJ35564	Hypothetical protein FLJ35564
AI352534	*CAV1*	Caveolin 1, caveolae protein, 22 kDa
NM_001985	*ETFB*	Electron-transfer-flavoprotein, ß polypeptide
AI743134	*SERPINE2*	Serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2
AW444709	*CD47*	CD47 antigen (Rh-related antigen, integrin-associated signal transducer)
BF688910	*COPEB*	Core promoter element binding protein
AI818579	EST	*Homo sapiens*, clone IMAGE:3625286, mRNA, partial cds
S95936	*TF*	Transferrin
AF074393	*RPS6KA5*	Ribosomal protein S6 kinase, 90 kDa, polypeptide 5
NM_000591	*CD14*	CD14 antigen
AK027181	*IBA2*	Ionized calcium binding adapter molecule 2
X73079	*PIGR*	Polymeric immunoglobulin receptor
NM_001343	*DAB2*	Disabled homolog 2, mitogen-responsive phosphoprotein (*Drosophila*)
M31452	*C4BPA*	Complement component 4 binding protein, α
X07696	*KRT15*	Keratin 15
AF016004	*GPM6B*	Glycoprotein M6B
NM_004078	*CSRP1*	Cysteine and glycine-rich protein 1
L36645	*EPHA4*	EphA4
D78011	*DPYS*	Dihydropyrimidinase
W60630	*JAM3*	Junctional adhesion molecule 3
AW956111	*D4S234E*	DNA segment on chromosome 4 (unique) 234 expressed sequence
AF035752	*CAV2*	Caveolin 2
D37766	*LAMB3*	Laminin, ß 3
U66406	*EFNB3*	Ephrin-B3
X52001	*EDN3*	Endothelin 3
NM_000856	*GUCY1A3*	Guanylate cyclase 1, soluble, α 3
U60115	*FHL1*	Four and a half LIM domains 1
D14520	*KLF5*	Kruppel-like factor 5 (intestinal)
M99487	*FOLH1*	Folate hydrolase (prostate-specific membrane antigen) 1
U09873	*FSCN1*	Fascin homolog 1, actin-bundling protein (*Strongylocentrotus purpuratus*)
AF017418	*MEIS2*	Meis1, myeloid ecotropic viral integration site 1 homolog 2 (mouse)
AF038540	*RTN2*	Reticulon 2
AF049884	*ARGBP2*	Arg/Abl-interacting protein ArgBP2
NM_001122	*ADFP*	Adipose differentiation-related protein
Y09926	*MASP2*	Mannan-binding lectin serine protease 2
M58297	*ZNF42*	Zinc finger protein 42 (myeloid-specific retinoic acid-responsive)

Table V. Continued.

Down-regulated genes		
Accession No.	Symbol	Gene name
AF035811	*PNUTL2*	Peanut-like 2 (*Drosophila*)
L22214	*ADORA1*	Adenosine A1 receptor
AF177775	*CES1*	Carboxylesterase 1 (monocyte/macrophage serine esterase 1)
U07643	*LTF*	Lactotransferrin
S76474	*NTRK2*	Neurotrophic tyrosine kinase, receptor, type 2
BE299605	*MRAS*	Muscle RAS oncogene homolog
NM_006225	*PLCD1*	Phospholipase C, δ 1
NM_005036	*PPARA*	Peroxisome proliferative activated receptor, α
M22324	*ANPEP*	Alanyl (membrane) aminopeptidase (aminopeptidase N, aminopeptidase M, microsomal aminopeptidase, CD13, p150)
BE877416	*TGFBR2*	Transforming growth factor, ß receptor II (70/80 kDa)
BE561244	*RPL18A*	Ribosomal protein L18a
AL048962	EST	*Homo sapiens*, clone IMAGE:4243767, mRNA
L08895	*MEF2C*	MADS box transcription enhancer factor 2, polypeptide C (myocyte enhancer factor 2C)
U48707	*PPP1R1A*	Protein phosphatase 1, regulatory (inhibitor) subunit 1A
X56134	*RPLP2*	Ribosomal protein, large P2
D84239	*FCGBP*	Fc fragment of IgG binding protein
AK026181	*PHLDA1*	Pleckstrin homology-like domain, family A, member 1
K01144	*CD74*	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)
U25138	*KCNMB1*	Potassium large conductance calcium-activated channel, subfamily M, ß member 1
X85337	*MYLK*	Myosin, light polypeptide kinase
D83597	*LY64*	Lymphocyte antigen 64 homolog, radioprotective 105 kDa (mouse)
NM_004024	*ATF3*	Activating transcription factor 3
BF126636	*SAA1*	Serum amyloid A1
D13789	*MGAT3*	Mannosyl (ß-1,4-)-glycoprotein ß-1,4-N-acetylglucosaminyltransferase
L41142	*STAT5A*	Signal transducer and activator of transcription 5A
AB040969	KIAA1536	hIAA1536 protein
NM_002153	*HSD17B2*	Hydroxysteroid (17-ß) dehydrogenase 2
AV646610	*ID4*	Inhibitor of DNA binding 4, dominant negative helix-loop-helix protein
X03663	*CSF1R*	Colony stimulating factor 1 receptor, formerly McDonough feline sarcoma viral (v-fms) oncogene homolog
U47025	*PYGB*	Phosphorylase, glycogen; brain
M81349	*SAA4*	Serum amyloid A4, constitutive
AI264201	*EGR2*	Early growth response 2 (Krox-20 homolog, *Drosophila*)
U18018	*ETV1*	ets variant gene 1 (ETA enhancer binding protein, E1AF)
NM_004350	*RUNX3*	Runt-related transcription factor 3
BF337516	*CRYAB*	Crystallin, α B
AF027208	*PROML1*	Prominin-like 1 (mouse)
D17408	*CNN1*	Calponin 1, basic, smooth muscle
NM_004010	*DMD*	Dystrophin (muscular dystrophy, Duchenne and Becker types)
BF183952	*CSTA*	Cystatin A (stefin A)
M16445	*CD2*	CD2 antigen (p50), sheep red blood cell receptor
AF055015	*EYA2*	Eyes absent homolog 2 (*Drosophila*)
AI745624	*ELL2*	ELL-related RNA polymerase II, elongation factor
AK025329	DKFZP566H073	DKFZP566H073 protein
BE745465	*KLK5*	Kallikrein 5
AK024578	DKFZp761F241	Hypothetical protein DKFZp761F241
AI870306	*IRX1*	Iroquois homeobox protein 1
H37853	*C9orf19*	Chromosome 9 open reading frame 19
BF000047	EST	*Homo sapiens* full length insert cDNA clone ZA79C08
AF126780	*RetSDR2*	Retinal short-chain dehydrogenase/reductase 2
AI700341	EST	ESTs, weakly similar to hypothetical protein FLJ20489 (*Homo sapiens*)
M87770	*FGFR2*	Fibroblast growth factor receptor 2 (bacteria-expressed kinase, keratinocyte growth factor receptor, craniofacial dysostosis 1, Crouzon syndrome, Pfeiffer syndrome, Jackson-Weiss syndrome)
AA452368	FLJ30046	Hypothetical protein FLJ30046
NM_021200	*PLEKHB1*	Pleckstrin homology domain containing, family B (evectins) member 1
AK026343	*hIAN2*	Human immune associated nucleotide 2
AF251040	*C5orf6*	Chromosome 5 open reading frame 6
M87507	*CASP1*	Caspase 1, apoptosis-related cysteine protease (interleukin 1, ß, convertase)
M97675	*ROR1*	Receptor tyrosine kinase-like orphan receptor 1
NM_020549	*CHAT*	Choline acetyltransferase
X00457	*HLA-DPA1*	Major histocompatibility complex, class II, DP α 1
W72411	*TP73L*	Tumor protein p73-like
AI769569	EST	ESTs

Table V. Continued.

Down-regulated genes		
Accession No.	Symbol	Gene name
K02765	*C3*	Complement component 3
AW971490	FLJ14906	Hypothetical protein FLJ14906
AF077044	*RPAC2*	Likely ortholog of mouse RNA polymerase I-3 (16 kDa subunit)
H70803	KIAA0790	KIAA0790 protein
AL050367	LOC221061	Hypothetical protein LOC221061
AK001643	FLJ10781	Hypothetical protein FLJ10781
AW182273	EST	*Homo sapiens* cDNA FLJ31517 fis, clone NT2RI2000007
W67951	EST	Human S6 A-5 mRNA expressed in chromosome 6-suppressed melanoma cells
AL117605	EST	*Homo sapiens* mRNA; cDNA DKFZp564N1063
AI376418	EST	*Homo sapiens* cDNA FLJ35169 fis, clone PLACE6012908
AA683373	EST	EST
AK022877	EST	*Homo sapiens* cDNA FLJ12815 fis, clone NT2RP2002546
NM_002258	*KLRB1*	Killer cell lectin-like receptor subfamily B, member 1
M69225	*BPAG1*	Bullous pemphigoid antigen 1, 230/240 kDa
AW299572	*EIIZF*	Early hematopoietic zinc finger
BE044467	*ARL7*	ADP-ribosylation factor-like 7
AA938297	FLJ20716	Hypothetical protein FLJ20716
AA706316	*ZD52F10*	Hypothetical gene ZD52F10
AI827230	*APCDD1*	Adenomatosis polyposis coli down-regulated 1
AK000251	FLJ20244	Hypothetical protein FLJ20244
N62352	KIAA1573	KIAA1573 protein
H53164	*ICSBP1*	Interferon consensus sequence binding protein 1
BE394824	*WFDC2*	WAP four-disulfide core domain 2
AL117462	*ZFP385*	Likely ortholog of mouse zinc finger protein 385
NM_003186	*TAGLN*	Transgelin
U58514	*CHI3L2*	Chitinase 3-like 2
AB026125	*ART-4*	ART-4 protein
AL080059	KIAA1750	KIAA1750 protein
AA747005	*SDCCAG43*	Serologically defined colon cancer antigen 43
NM_005928	*MFGES*	Milk fat globule-EGF factor 8 protein
D62470	*NRXN3*	Neurexin 3
N29574	*RAGD*	Rag D protein
K02276	*MYC*	v-myc myelocytomatosis viral oncogene homolog (avian)
D78611	*MEST*	Mesoderm specific transcript homolog (mouse)
NM_022003	*FXYD6*	FXYD domain containing ion transport regulator 6
BF508973	*RPL13*	Ribosomal protein L13
NM_001615	*ACTG2*	Actin, γ 2, smooth muscle, enteric
R41532	EST	ESTs, weakly similar to POL2_MOUSE retrovirus-related POL polyprotein (Contains: Reverse transcriptase; Endonuclease) (M. musculus)
AA142875	EST	ESTs
U03688	*CYP1B1*	Cytochrome P450, family 1, subfamily B, polypeptide 1
W94363	EST	*Homo sapiens* full length insert cDNA clone ZE12G01
W44613	*HSJ001348*	cDNA for differentially expressed CO16 gene
AL118812	EST	*Homo sapiens* mRNA; cDNA DKFZp761G1111
D56064	*MAP2*	Microtubule-associated protein 2
BF966838	KIAA2028	Similar to PH (pleckstrin homology) domain
AI338625	*FJX1*	Four jointed box 1 (*Drosophila*)
AI263022	EST	ESTs
AL050107	*TAZ*	Transcriptional co-activator with PDZ-binding motif (TAZ)
AI056364	FLJ14855	Hypothetical protein FLJ14855
AI351898	*DRCTNNB1A*	Down-regulated by Ctnnb1, a
AV700003	*ARL6IP2*	ADP-ribosylation-like factor 6 interacting protein 2
NM_000700	*ANXA1*	Annexin A1
M81141	*HLA-DQB1*	Major histocompatibility complex, class II, DQ β 1
AI598227	FLJ23091	Hypothetical protein FLJ23091
BG034740	*ROPN1*	Ropporin, rhophilin associated protein 1
AB011175	*TBC1D4*	TBC1 domain family, member 4
AK024449	*PP2135*	PP2135 protein
AW978770	EST	Hypothetical protein DKFZp566A1524
AI821113	EST	*Homo sapiens* cDNA FLJ36327 fis, clone THYMU2005748
AI057450	*SLC13A2*	Solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 2
X86693	*SPARCL1*	SPARC-like 1 (mast9, hevin)
AI224952	*FLJ40906*	Hypothetical protein FLJ40906
D13639	*CCND2*	Cyclin D2

Table VI. Genes with elevated or decreased expression from DCIS to IDC.

Genes with elevated expression from DCIS to IDC		
Accession No.	Symbol	Gene name
U74612	*FOXM1*	Forkhead box M1
U63743	*KIF2C*	Kinesin family member 2C
D88532	*PIK3R3*	Phosphoinositide-3-kinase, regulatory subunit, polypeptide 3 (p55, γ)
NM_005532	*IFI27*	Interferon, α-inducible protein 27
D14657	KIAA0101	KIAA0101 gene product
AF030186	*GPC4*	Glypican 4
Z11566	*STMN1*	Stathmin 1/oncoprotein 18
U90914	*CPD*	Carboxypeptidase D
NM_002534	*OAS1*	2′,5′-oligoadenylate synthetase 1, 40/46 kDa
S67310	*BF*	B-factor, properdin
AA192445	*TMEPAI*	Transmembrane, prostate androgen induced RNA
AB003103	*PSMD12*	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 12
BE878057	DKFZP564K0822	Hypothetical protein DKFZp564K0822
AB003698	*CDC7L1*	CDC7 cell division cycle 7-like 1 (*S. cerevisiae*)
M91670	*E2-EPF*	Ubiquitin carrier protein
AK023414	FLJ13352	Hypothetical protein FLJ13352
L09235	*ATP6V1A1*	ATPase, H+ transporting, lysosomal 70 kDa, V1 subunit A, isoform 1
AF007152	*ABHD3*	Abhydrolase domain containing 3
U33632	*KCNK1*	Potassium channel, subfamily K, member 1
AA621719	*SMC4L1*	SMC4 structural maintenance of chromosomes 4-like 1 (yeast)
AF176228	*DNMT3B*	DNA (cytosine-5-)-methyltransferase 3 ß
H22566	*DACH*	Dachshund homolog (*Drosophila*)
AI185804	*FN1*	Fibronectin 1
AI189477	*IDH2*	Isocitrate dehydrogenase 2 (NADP+), mitochondrial
AA205444	*AP1S2*	Adaptor-related protein complex 1, σ 2 subunit
Genes with decreased expression from DCIS to IDC		
Accession No.	Symbol	Gene name
AF070609	*SLC1A3*	Solute carrier family 1 (glial high affinity glutamate transporter), member 3
U85267	*DSCR1*	Down syndrome critical region gene 1
NM_005397	*PODXL*	Podocalyxin-like
D13811	*AMT*	Aminomethyltransferase (glycine cleavage system protein T)
X53586	*ITGA6*	Integrin α 6
L13288	*VIPR1*	Vasoactive intestinal peptide receptor 1
M12125	*TPM2*	Tropomyosin 2 (ß)
M65066	*PRKAR1B*	Protein kinase, cAMP-dependent, regulatory, type I, ß
AJ001183	*SOX10*	SRY (sex determining region Y)-box 10
AW241712	*MXI1*	MAX interacting protein 1
AL160111	KIAA1649	KIAA1649 protein
X93920	*DUSP6*	Dual specificity phosphatase 6
AF132734	*SEC8*	Secretory protein SEC8
AI133467	EST	ESTs
D88153	*HYA22*	HYA22 protein
AF014404	*PTE1*	Peroxisomal acyl-CoA thioesterase
BE907755	*C16orf5*	Chromosome 16 open reading frame 5
AA135341	*GCN5L2*	GCN5 general control of amino-acid synthesis 5-like 2 (yeast)
AL110126	EST	*Homo sapiens* mRNA; cDNA DKFZp564H1916
BE254330	EST	*Homo sapiens* mRNA; cDNA DKFZp564D016
BE264353	*RBP1*	Retinol binding protein 1, cellular
W75991	EST	*Homo sapiens*, clone IMAGE:4249217, mRNA
AF091434	*PDGFC*	Platelet derived growth factor C
W67577	*CD74*	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)
NM_002996	*CX3CL1*	Chemokine (C-X3-C motif) ligand 1
AA024459	EST	ESTs
NM_000163	*GHR*	Growth hormone receptor
AA858162	*NCAG1*	NCAG1
BE327623	EST	ESTs, weakly similar to hypothetical protein FLJ20234 (*Homo sapiens*)
BE671156	*MAPRE2*	Microtubule-associated protein, RP/EB family, member 2
D12614	*LTA*	Lymphotoxin α (TNF superfamily, member 1)
L13720	MGC5560	Hypothetical protein MGC5560
U15131	*ST5*	Suppression of tumorigenicity 5
Y00711	*LDHB*	Lactate dehydrogenase B

Table VI. Continued.

Genes with decreased expression from DCIS to IDC		
Accession No.	Symbol	Gene name
AI651212	EST	*Homo sapiens* cDNA FLJ31125 fis, clone IMR32200819
M31159	*IGFBP3*	Insulin-like growth factor binding protein 3
NM_014447	*HSU52521*	Arfaptin 1
AB011089	*TRIM2*	Tripartite motif-containing 2
BF969355	*PDK4*	Pyruvate dehydrogenase kinase, isoenzyme 4
AK025950	KIAA1695	Hypothetical protein FLJ22297
D86961	LHFPL2	Lipoma HMGIC fusion partner-like 2
AK025953	EST	*Homo sapiens* cDNA: FLJ22300 fis, clone HRC04759
AJ223812	*CALD1*	Caldesmon 1
R40594	EST	*Homo sapiens* cDNA: FLJ22845 fis, clone KAIA5195
AF145713	*SCHIP1*	Schwannomin interacting protein 1
AK024966	FLJ21313	Hypothetical protein FLJ21313
NM_005596	*NFIB*	Nuclear factor I/B
NM_001613	*ACTA2*	Actin, α 2, smooth muscle, aorta
H03641	*FAM13A1*	Family with sequence similarity 13, member A1

up-regulated genes included *ERBB2*, *CCNB1* and *BUB1B* (*BUB1* budding uninhibited by benzimidazoles-1 homolog ß), known to be involved in development or invasion of cancers in a variety of tissues (33-35). Among the down-regulated genes, we have reported that *AXUD1* (*AXIN1* up-regulated-1), which is frequently down-regulated in lung, liver, colon and kidney cancers (36), might be involved also in invasion of cancer cells.

Verification of selected genes by semi-quantitative RT-PCR. To confirm the reliability of the expression data obtained by cDNA microarray analysis, we performed semi-quantitative RT-PCR experiments for three genes (Accession Nos. AI261804, AA205444 and AA167194) that were highly up-regulated in informative tumors of the well-differentiated type, and two (Accession Nos. AA676987 and H22566) that were highly up-regulated in informative tumors of the poorly-differentiated type. The RT-PCR results accorded well with those of the microarray analysis in most of the tested cases (Fig. 4).

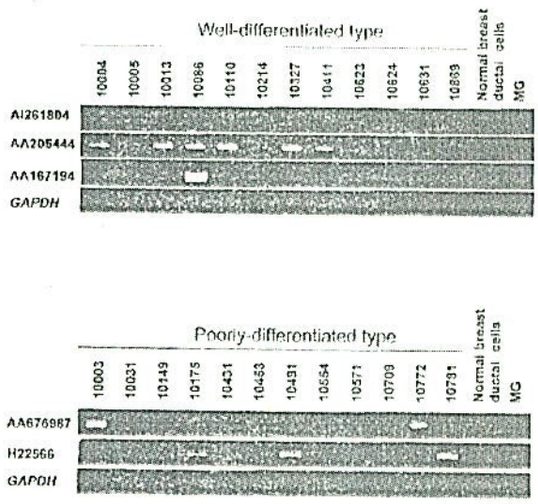


Figure 4. Semi-quantitative RT-PCR validation of over-expressed genes, showing expression of 3 genes in 12 well-differentiated tumors (a1) (upper panel) and 2 genes in 12 poorly-differentiated tumors (a31 (lower panel), along with *GAPDH* as an internal control. The results of these semi-quantitative RT-PCR experiments corresponded to signals on the microarray. Normal breast ductal cells were prepared as a mixture of normal ductal epithelial cells from 15 pre-menopausal patients. MG indicates whole human mammary gland.

Development of a scoring method for prediction of lymph-node metastasis. Metastasis to axillary lymph nodes is the most important prognostic factor for patients with breast cancer (37). To develop an equation for achieving a scoring parameter to predict metastasis to axial lymph nodes, we performed a random-permutation test to identify genes that were differentially expressed in 20 node-positive cases and 20 node-negative cases of IDC. Following criteria described in Materials and methods, we first selected the 93 discriminating genes that showed permutation p-value <0.001 between the two groups. Using the leave-one-out cross validation method, we selected 34 genes showing the smallest p-value that could provide the best separation of node-positive from node-negative cases (Table VIII). As shown in Fig. 5A, a hierarchical clustering analysis using these 34 genes clearly classified all 40 breast cancer cases into either of the two groups according to lymph node status. This set of 34 genes included some key factors whose potential roles in metastasis had been reported previously. On that basis we attempted to construct a prediction-scoring system that could clearly distinguish node-positive cases from node-negative cases through the expression profiles of this set of 34 genes (Materials and methods).

To clarify the system further, we calculated prediction scores for 17 node-positive and 20 node-negative cases that had not been used in the original procedure for selecting discriminating genes. We chose a score of zero as a borderline between node-positive and node-negative groups (Fig. 5B). On the basis of this definition, all 17 cases with lymph node metastasis were judged to have positive scores. The 20 cases without lymph node metastasis were given negative scores

Table VII. Genes commonly up- or down-regulated in IDC.

Up-regulated genes		
Accession No.	Symbol	Gene name
X14420	*COL3A1*	Collagen, type III, α 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)
AF044588	*PRC1*	Protein regulator of cytokinesis 1
AF161499	*HSPC150*	HSPC150 protein similar to ubiquitin-conjugating enzyme
AA789233	*COL1A1*	Collagen, type I, α 1
U16306	*CSPG2*	Chondroitin sulfate proteoglycan 2 (versican)
NM_004425	*ECM1*	Extracellular matrix protein 1
NM_006855	*KDELR3*	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 3
AI972071	*CCNB1*	Cyclin B1
AF237709	*TOPK*	T-LAK cell-originated protein kinase
BE747327	*HIST1H1C*	Histone 1, H1c
J03464	*COL1A2*	Collagen, type I, α 2
AI080640	*AGR2*	Anterior gradient 2 homolog (*Xenopus laevis*)
AA971042	*RHPN1*	Rhophilin, Rho GTPase binding protein 1
AI419398	MGC33662	Hypothetical protein MGC33662
AI149552	EST	ESTs, moderately similar to ERB2_HUMAN Receptor protein-tyrosine kinase erbB-2 precursor (p185erbB2) (NEU proto-oncogene) (c-erbB-2) (tyrosine kinase-type cell surface receptor HER2) (MLN 19) (*H. sapiens*)
D14874	*ADM*	Adrenomedullin
X03674	*G6PD*	Glucose-6-phosphate dehydrogenase
NM_002358	*MAD2L1*	MAD2 mitotic arrest deficient-like 1 (yeast)
BF214508	*CYCS*	Cytochrome c, somatic
BG030536	*TOP2A*	Topoisomerase (DNA) II α 170 kDa
X57766	*MMP11*	Matrix metalloproteinase 11 (stromelysin 3)
AA029900	*SULF1*	Sulfatase 1
AF053306	*BUB1B*	BUB1 budding uninhibited by benzimidazoles 1 homolog ß (yeast)
AF074002	*LGALS8*	Lectin, galactoside-binding, soluble 8 (galectin 8)

Down-regulated genes		
Accession No.	Symbol	Gene name
NM_004484	*GPC3*	Glypican 3
NM_006219	*PIK3CB*	Phosphoinositide-3-kinase, catalytic, ß polypeptide
BE793000	*RBP1*	Retinol binding protein 1, cellular
AL117565	*AXUD1*	AXIN1 up-regulated 1
BF055342	*ZNF6*	Zinc finger protein 6 (CMPX1)
U03688	*CYP1B1*	Cytochrome P450, family 1, subfamily B, polypeptide 1
AF038193	EST	*Homo sapiens*, clone IMAGE:3610040, mRNA
X72760	*LAMB2*	Laminin, ß 2 (laminin S)
J03817	*GSTM1*	Glutathione S-transferase M1
M69226	*MAOA*	Monoamine oxidase A
BF690180	*WASF2*	WAS protein family, member 2
AL133600	*STAM2*	Signal transducing adaptor molecule (SH3 domain and ITAM motif) 2
AF215981	*GPR2*	G protein-coupled receptor 2
BG149764	EST	*Homo sapiens*, clone IMAGE:5286091, mRNA, partial cds
AF067800	*CLECSF6*	C-type (calcium dependent, carbohydrate-recognition domain) lectin, superfamily member 6
AA713487	*PIK3R1*	Phosphoinositide-3-kinase, regulatory subunit, polypeptide 1 (p85 α)
AA828505	*FBXW7*	F-box and WD-40 domain protein 7 (archipelago homolog, *Drosophila*)
AK021865	*CKIP-1*	CK2 interacting protein 1; HQ0024c protein
AK001605	FLJ10743	Hypothetical protein FLJ10743
AI041186	*HSPC182*	HSPC182 protein
AA873363	*ADH8*	Alcohol dehydrogenase 8
NM_013409	FST	Follistatin
AK000322	FLJ20315	Hypothetical protein FLJ20315
AB020637	KIAA0830	KIAA0830 protein
AA872040	*INHBB*	Inhibin, ß B (activin AB ß polypeptide)
NM_004430	*EGR3*	Early growth response 3
D59989	EST	ESTs
D78013	*DPYSL2*	Dihydropyrimidinase-like 2
AI081821	EST	*Homo sapiens* mRNA; cDNA DKFZp313M0417
AA309603	KIAA1430	KIAA1430 protein
NM_004107	*FCGRT*	Fc fragment of IgG, receptor, transporter, α
AW268719	EST	*Homo sapiens* cDNA FLJ32438 fis, clone SKMUS2001402
BF446578	LOC221002	CG4853 gene product
BG054844	*ARHE*	Ras homolog gene family, member E

Table VII. Continued.

Down-regulated genes		
Accession No.	Symbol	Gene name
AF054987	*ALDOC*	Aldolase C, fructose-bisphosphate
AI052390	FLJ20071	Dymeclin
NM_004530	*MMP2*	Matrix metalloproteinase 2 (gelatinase A, 72 kDa gelatinase, 72 kDa type IV collagenase)
AF054999	*EPB41L2*	Erythrocyte membrane protein band 4.1-like 2
AU151591	*NAV2*	Neuron navigator 2
AA447744	EST	ESTs
R61253	*ST6GalII*	β-galactoside α-2,6-sialyltransferase II

Table VIII. Genes with altered expression between lymph node-positive and node-negative tumors.

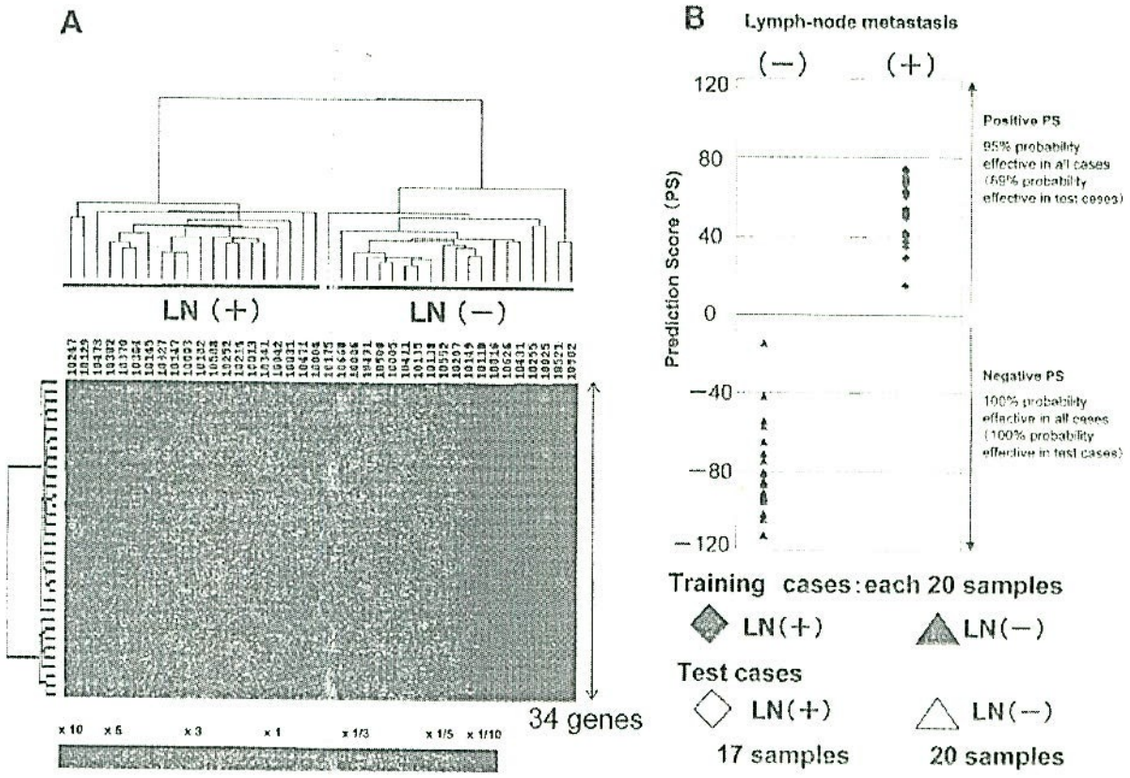
Accession No.	Symbol	Gene name	p-value	LN
BF686125	*UBA52*	Ubiquitin A-52 residue ribosomal protein fusion product 1	8.1E-09	-
AA634090	EST	*Homo sapiens*, similar to heterogeneous nuclear ribonucleoprotein A1, clone IMAGE:2900557, mRNA	1.4E-07	-
L00692	*CEACAM3*	Carcinoembryonic antigen-related cell adhesion molecule 3	4.2E-07	-
AW954403	*VAMP3*	Vesicle-associated membrane protein 3 (cellubrevin)	2.2E-06	+
AA865619	*C21orf97*	Chromosome 21 open reading frame 97	2.6E-06	-
W74502	MGC11257	Hypothetical protein MGC11257	2.4E-05	+
NM_002094	*GSPT1*	G1 to S phase transition 1	2.7E-05	+
T55178	KIAA1040	KIAA1040 protein	3.2E-05	-
L36983	*DNM2*	Dynamin 2	4.1E-05	+
Z21507	*EEF1D*	Eukaryotic translation elongation factor 1 δ (guanine nucleotide exchange protein)	5.2E-05	-
AI581728	*CFL1*	Cofilin 1 (non-muscle)	8.0E-05	+
NM_001293	*CLNS1A*	Chloride channel, nucleotide-sensitive, 1A	9.0E-05	+
BF680847	*SENP2*	Sentrin-specific protease	9.0E-05	+
AF100743	*NDUFS3*	NADH dehydrogenase (ubiquinone) Fe-S protein 3, 30 kDa (NADH-coenzyme Q reductase)	9.8E-05	+
NM_004960	*FUS*	Fusion, derived from t(12;16) malignant liposarcoma	9.8E-05	-
AK023975	*NOP5/NOP58*	Nucleolar protein NOP5/NOP58	1.3E-04	+
AF083245	*PSMD13*	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 13	1.5E-04	+
AA129776	*SUOX*	Sulfite oxidase	1.8E-04	+
U55766	*HRB2*	HIV-1 rev binding protein 2	2.0E-04	+
BF526092	LOC154467	Hypothetical protein BC003515	2.1E-04	+
BF677579	*THTPA*	Thiamine triphosphatase	2.3E-04	+
X98260	*ZRF1*	Zuotin related factor 1	2.3E-04	+
BE440010	LOC51255	Hypothetical protein LOC51255	2.7E-04	+
AF007165	*DEAF1*	Deformed epidermal autoregulatory factor 1 (*Drosophila*)	2.7E-04	+
X78687	*NEU1*	Sialidase 1 (lysosomal sialidase)	3.0E-04	+
AW965200	EST	*Homo sapiens*, clone IMAGE:5286019, mRNA	3.1E-04	-
AK023240	*UGCGL1*	UDP-glucose ceramide glucosyltransferase-like 1	3.1E-04	+
M95712	*BRAF*	v-raf murine sarcoma viral oncogene homolog B1	3.7E-04	+
L38995	*TUFM*	Tu translation elongation factor, mitochondrial	3.9E-04	+
AW014268	FLJ10726	Hypothetical protein FLJ10726	4.2E-04	+
D49547	*DNAJB1*	DnaJ (Hsp40) homolog, subfmaily B, member 1	4.4E-04	+
BE466450	*AP4S1*	Adaptor-related protein complex 4, σ 1 subunit	4.5E-04	+
AB007944	KIAA0475	KIAA0475 gene product	4.9E-04	-
AF034091	*MRPL40*	Mitochondrial ribosomal protein L40	5.1E-04	+

LN: + indicates the genes that relatively over-expressed in the LN-positive cases as compared with the LN-negative cases; - indicates the genes that relatively under-expressed in the LN-positive cases as compared with the LN-negative cases.

with two exceptions; thus 35 (95%) of the 37 cases were placed correctly according to their lymph node status (Fig. 5C, upper panel). This result indicates that our system can predict LN status with a higher probability than any other reported approach has been able to achieve.

Discussion

Breast cancer is a multifactorial disease that develops as a result of interactions of various genetic, environmental, and hormonal factors. Although distinct pathological stages of



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All Cases		LN status (+)	LN status (−)	Total
PS	Positive	37	2	39
	Negative	0	38	38
Total		37	40	77

P<0.001 (χ^2 test for independence)

Test Cases		LN status (+)	LN status (−)	Total
PS	Positive	17	2	19
	Negative	0	18	18
Total		17	20	37

P<0.001 (χ^2 test for independence)

Figure 5. A, Two-dimensional hierarchical clustering analysis based on the expression levels of 34 genes selected by a random-permutation test. Each vertical column corresponds to 1 of 20 node-positive or 20 node-negative cases. Each horizontal row represents 1 of the 34 selected genes. Genes in the upper main branch were preferentially expressed in cases with lymph node metastasis; those in the lower branch were more highly expressed in lymph node negative cases. B, Strengths of genes for separating cases with lymph node-metastasis from cases without metastasis. The 20 sky-blue squares are lymph node-positive test cases and the 17 yellow triangles represent lymph node-negative test cases that had not been used for establishing prediction scores. C, Correlation between the prediction score for metastasis and clinical information after surgery.

breast cancer have been described, the molecular basis of differences among these stages is largely unknown. Genome-wide expression analysis using microdissected-purified populations of cancerous breast cells and their originating ductal epithelial cells makes it possible to identify genes that are involved in mammary carcinogenesis (16,17). These data can be useful for molecular classification, treatment, and prediction of outcomes in breast cancers as in a variety of other tumors.

Adipocytes account for >90% of the cells in the mammary gland; the normal breast ductal epithelial cells from which breast cancer originates correspond to a very small proportion of mammary tissue. Hence, analysis of gene-expression profiles using whole-tumor mass versus normal whole mammary gland is significantly influenced by the proportion of each cell type in the particular tissues examined; proportional differences of cancer cells, adipocytes, fibroblasts, and inflammatory cells can significantly affect for searching genes that are specifically involved in breast carcinogenesis and generate false-positive or false-negative data. Taking these points into consideration, we applied an LMM system to purify as much as possible the populations of cancerous and normal epithelial cells obtained from surgical specimens (3,8) (Fig. 1). To evaluate the purity of microdissected cell populations we incorporated *PLIN* and *FABP4*, which are expressed abundantly in adipose tissue, in our gene-expression profiling (21).

By applying the microdissection procedure carefully, we were able to minimize the proportion of contaminating adipo-

cytes to as little as 0.6% in preparations of normal breast ductal epithelial cells. To evaluate the specificity of our profiles of breast-cancer cells further, we examined expression levels of *MUC1*, a gene already known to be significantly up-regulated in breast cancers (23,24). We compared the Cy5/Cy3 ratios of that gene in experiments for which we used whole breast cancer tissues from two patients with ratios achieved using microdissected breast cancer cells. As a result, its expression levels in whole breast cancer tissues were underestimated, being only 69 and 31% of their respective microdissected counterparts isolated from the same individuals. The results suggest that our microarray data derived from microdissected tumors and normal breast ductal epithelial cells would be likely to show expression profiles precisely associated with the carcinogenic step, and to clarify mechanisms leading to breast cancer.

We analyzed potential associations between expression profiles and clinical parameters such as ER status, histological type, and LN status. An unsupervised classification analysis divided tumors into two major groups (groups A and B) and one minor group (group C); 82% of the primary breast tumors belonging to group B were ER-positive, whereas 87% of the tumors in group A were ER-negative as measured by EIA (Fig. 2A), indicating that the major factor influencing the expression patterns of breast-cancer cells is the ER status. To reveal genes that were differentially expressed between the ER-positive and ER-negative groups, we carried out random permutation tests and identified 97 genes whose expression was significantly different; 41 of the 97 genes had also been selected in the unsupervised clustering analysis, including *ESR1* and genes such as *BCL-2*, *CCND1*, and *LIV-1* that were previously reported to be associated with ER status (38-40). Estrogens are important regulators of growth and differentiation of the normal mammary gland but also contribute to development and progression of breast carcinomas (37).

A random-permutation test identified 25 genes that were differentially expressed between well- and poorly-differentiated lesions isolated as separate samples from each of eight IDC patients (Fig. 2C). Among those 25 genes, thrombospondin 2 (*THBS2*), an extracellular matrix protein, appears to play a role in cell adhesion and cell migration (41). Integrin α 5 (*ITGA5*) is essential to cell invasion (26); and nuclear factor of activated T-cells 5 (*NFAT5*), which is expressed in invasive human ductal breast carcinomas, is associated with invasiveness of colon carcinomas as well (25). These genes might be associated with histological differences among breast cancers.

The 78 elements we found to be up-regulated in DCIS and IDC included genes encoding transcriptional factors and proteins involved in the signal transduction pathway and in the cell cycle (Tables V and VI). We assume that some of them could play important roles in the mammary carcinogenesis. The up-regulated genes included *FOXM1* and *CCNB1*, which are also up-regulated in various other tumor types. Over-expression of *FOXM1* stimulates expression of *CCNB1*, a cell cycle-control protein that is required for passage through G2 and mitosis (30). *CCNB1* was more highly up-regulated in IDC than in DCIS in our experiments, suggesting that over-expression of these two proteins might be important for transition from DCIS to IDC. Although the functions of most of the other genes on this list are unknown at present, functional analyses in future may shed light on the invasiveness of breast cancer cells.

Finally, we focused on genes that might be involved in lymph node metastasis, a critical clinical step in tumor progression and one of the major factors determining prognosis of patients (37). Our discovery of a subset of 34 genes that showed positive associations to lymph node metastasis should contribute to an understanding of the precise biophysical events involved, and might suggest molecular markers for metastasis. For example, among those 34 genes *FUS* [fusion, derived from t(12;16) in malignant liposarcoma] was down-regulated in breast tumors with lymph node metastasis. Since wild-type *FUS* is reported to be important for genomic stability (42), down-regulation of this gene in our metastasis-positive group might have led to genomic instability of cells and consequent progression. Although our prediction scoring system for lymph node metastasis using the expression profiles of these 34 genes has potential for directing the selection of treatment methods and for improvement of quality of life for breast cancer patients, verification in a larger number of cases will be essential before the system can be applied in clinical practice.

Cancer therapies directed at specific molecular alterations observed in cancer cells, such as trastuzumab (Herceptin), have been validated in clinical settings (43,44). This drug is more effective and better tolerated than traditional anti-cancer agents, because it targets malignant cells very selectively and improves survival of cancer patients. Cancer treatments in the near future are expected to follow this model, combining conventional drugs with molecular-targeting agents that can attack tumor cells that have specific characteristics. The information provided here should help to identify novel tumor markers that may be present at abnormal levels in blood or nipple aspirates of a woman who has breast cancer, and prove useful for identifying molecular targets of novel drugs to treat breast cancers.

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Genome-wide transcriptional analysis of carboplatin response in chemosensitive and chemoresistant ovarian cancer cells

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Abstract

We have recently described an *ex vivo* chemoresponse assay for determining chemosensitivity in primary cultures of human tumors. In this study, we have extended these experiments in an effort to correlate chemoresponse data with gene expression patterns at the level of transcription. Primary cultures of cells derived from ovarian carcinomas of individual patients ($n = 6$) were characterized using the ChemoFx assay and classified as either carboplatin sensitive ($n = 3$) or resistant ($n = 3$). Three representative cultures of cells from each individual tumor were then subjected to Affymetrix gene chip analysis ($n = 18$) using U95A human gene chip arrays. Data were analyzed using the dCHIP software package. We identified a significant number of genes whose expression patterns were altered between carboplatin chemosensitive and chemoresistant cells, in normal culture conditions and in the presence of carboplatin for either 2 or 72 hours. Among these differentially expressed genes, we found a significant proportion to be associated with apoptosis, cell-cell communication, cell adhesion, DNA repair, and cell proliferation. In general, the molecular phenotype displayed by chemoresistant cells was reflective of an extended life span in culture in the presence of carboplatin and the genes that define this phenotype are potential biomarkers for the prognostic management of ovarian cancer patients. [Mol Cancer Ther 2005;4(10):1605–16]

Introduction

Gene expression profiling at the level of transcription by oligonucleotide microarray analysis is a powerful molecular tool for the discovery of genes that are involved in a variety of disease processes, including cancer. Patterns of global gene expression can reveal the molecular pathways relevant to the disease process and identify potential new therapeutic targets. The use of this technology for the molecular classification of cancer was recently shown with the identification of an expression profile that was predictive of patient outcome for B-cell lymphoma (1). In addition, this study showed that histologically similar tumors can be differentiated based on their gene expression profiles. Ultimately, these unique patterns of gene expression may be used as guidelines to direct different modes of therapy.

Although it is widely recognized that patients with the same histologic stage and grade of cancer respond to therapies differently, few clinical tests can predict individual patient responses. The next great challenge will be to use the power of post-genomic technology, including microarray analyses, to correlate gene expression patterns with individual patient responses to clinical therapies. The major objective of the current study was to determine whether predictive gene expression patterns can be identified that correlate with the results of an *ex vivo* chemoresponse assay done on primary cultures of cells derived from ovarian carcinomas of individual patients. Specifically, we used Affymetrix gene chip microarrays to analyze gene expression patterns in 18 cell cultures that were functionally characterized as either chemoresponsive or chemosensitive to carboplatin *in vitro*.

Materials and Methods

Patients

Tumors were submitted to Precision Therapeutics, Inc. (Pittsburgh, PA) for assessment of chemoresponsiveness to oncologist-selected chemotherapeutic agents. They were tested using the ChemoFx phenotypic chemoresponse assay that assesses tumor cell viability in the presence of a range of drug concentrations (see below; Table 1).

Primary Cell Culture and the ChemoFx Assay

Precision Therapeutics' ChemoFx assay involves the isolation, short-term growth, and drug dosage treatment of epithelial cells derived from solid tumors (2, 3). At the time of surgical "debulking," pieces of solid tumor are obtained by the surgeon or pathologist and placed in tissue culture transport medium for overnight shipment to the laboratories of Precision Therapeutics. Upon arrival, the tumor is minced into small pieces and placed with cell culture medium into multiple small flasks for cell outgrowth. Over time, cells move out of the tumor pieces and form a monolayer on the bottom of the flask. Once enough cells have migrated out of the *ex vivo* explant pieces, they are then trypsinized and reseeded into

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Table 1. Patient profiles

Patient no.	Tumor type	Tumor site	Cell kill (%)*	ChemoFx response†
1	PSA	Liver	66	S
2	PSA	Ovary	37	S
3	PSA	Ovary	53	S
4	PSA	Ovary	8	R
5	PSA	Ovary	3	R
6	PSA	Omentum	9	R

Abbreviations: PSA, papillary serous adenocarcinoma; S, sensitive; R, resistant.
*% Cell kill at the highest drug dose (1,000 mmol/L).
†*In vitro* response to carboplatin.

microtiter plates for either the ChemoFx assay (described below) or for immunohistochemistry analysis for the identification of malignant epithelial cells, as previously described (2).

For the ChemoFx assay, ~350 cells in 10 μL of medium are seeded into 60-well microtiter plates and allowed to attach and grow for 24 hours. After 24 hours in culture, 10 μL of six doses of 2× drug are dissolved in HBSS and added to each well for 2 hours at 37°C. For the present study, carboplatin was added in concentrations of 20, 50, 100, 200, 500, and 1,000 mmol/L. Plates are then rinsed rapidly with HBSS four times to remove any drug residue, and then fresh medium is added for 72 hours. After the 72 hours of recovery period, the medium and any nonadherent dead cells are rinsed off with HBSS, and the remaining cells are fixed for 5 minutes in 95% ethanol containing the DNA intercalating blue fluorescent dye, 4′,6-diamidino-2-phenylindole. The number of cells per well was then quantitated by counting fluorescent nuclei using an operator-controlled, computer-assisted image analysis system (Zeiss, Hertfordshire, United Kingdom) customized for Precision Therapeutics. A complete dose-response curve was generated for each drug evaluated. The data are presented graphically as the cytotoxic index. The cytotoxic index (% kill) is defined as 1 − [no. of cells in treated wells / no. of cells in control wells] × 100. For each drug dose, the cytotoxic index was calculated using two columns of untreated cells in the plate as the negative control. Drug responses were scored from 0 to 5, with the score determined by the number of drug doses where the cytotoxic index was ≥35%. For the purposes of the present study, a drug response score of 0 to 1 was considered resistant and a score of 3 to 5 was considered sensitive.

Affymetrix Gene Expression Analysis

For oligonucleotide analysis using the Affymetrix platform, RNA extraction, reverse transcription, cRNA preparation, and chip hybridization were done according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). The full protocol can be found at http://www.affymetrix.com/support/technical/manual/expression_manual.affx. In brief, total RNA was extracted from cultured cells using the Trizol (Invitrogen, Carlsbad, CA) and RNeasy (Qiagen, Valencia, CA) protocols. Twenty micrograms of purified RNA were then used as a template for double-stranded cDNA synthesis primed using a T7-$(dT)_{24}$ oligonucleotide. Double-stranded cDNA was then used as a template for biotin-labeled cRNA preparation using T7 RNA polymerase. Resulting cRNA was fragmented and hybridized to Affymetrix GeneChip Human Genome U95 version 2 (HG-U95A) oligonucleotide microarrays.

Affymetrix data were analyzed using the dCHIP software package (4). The 18 DAT files generated by the Affymetrix Microarray Suite version 5.0 were converted into DCP files using dCHIP,[3] as described previously by Li and Wong (4). The DCP files were normalized, and raw gene expression data generated. Normalization was carried out according to the Invariant Set Normalization method as described by Li and Wong (5). The resulting gene list was then filtered such that only genes whose expression levels satisfied the following criteria were included: 0.5 < SD/mean >10, gene called P (present) in 20% of samples, and expression level >20 in >50% of samples. Global comparisons of data derived from resistant and sensitive cells was done as follows (where E = experimental samples and B = baseline samples): $E_{mean}/B_{mean} > 2$ or $B_{mean}/E_{mean} > 2$; $E_{mean} - B_{mean} > 100$ or $B_{mean} - E_{mean} > 100$; and P for testing $E_{mean} = B_{mean} < 0.03$. The t statistic was computed as $(mean_1 - mean_2) / \sqrt{[SE(mean_1)_2 + SE(mean_2)_2]}$ and its P computed based on the t distribution, and the degree of freedom is set according to the Welch-modified two-sample t test. The clustering algorithm used is as follows: the distance between two genes is defined as $1 - r$, where r is the Pearson correlation coefficient between the standardized expression values (make mean = 0 and SD = 1) of the two genes across the samples used.[4] Full data sets are available [accession no. GSE1926 (Gene Expression Omnibus series)].[5]

Results

The experiments done in this study were designed to (a) identify genes whose expressions were altered in primary cultures of carboplatin-sensitive versus carboplatin-resistant ovarian carcinoma cells; (b) to determine the differences in genomic response of chemosensitive versus resistant cells to 2 or 72 hours of carboplatin exposure *in vitro*; and (c) to analyze the temporal genomic response to carboplatin in either chemosensitive or resistant cells independently of each other. Affymetrix data sets were obtained for three representative cultures of cells from each of six individual tumors (18 arrays), of which, based on data from the ChemoFx assay (Table 1), three were resistant and three were sensitive to carboplatin. Data were analyzed using the dCHIP package (4) as described in Material and Methods.

[3] http://www.dCHIP.org
[4] http://biosun1.harvard.edu/complab/dchip/clustering.htm
[5] http://www.ncbi.nlm.nih.gov/projects/geo/

Carboplatin-Sensitive Ovarian Tumor Cells Display a Distinct Molecular Phenotype at the Level of Transcription Relative to Resistant Cells

We first sought to identify genes whose altered expressions correlated with chemosensitivity or resistance. We therefore compared expression profiles of sensitive and resistant cells in culture without *in vitro* exposure to carboplatin. The underlying hypothesis for this experiment was that fundamental differences in molecular phenotype exist at the mRNA level between ovarian tumor cells before

Table 2. Genes whose expressions (> 2-fold) are altered between carboplatin-sensitive and carboplatin-resistant cells ($n = 3$)

Gene	Symbol	Accession no.	Function	Fold change (R/S)	*P*
Proteoglycan 1, secretory granule	*PRG1*	X17042	Apoptosis	>−20	0.02932
Armadillo repeat protein	*ALEX2*	AB011084		>−20	0.012058
C-type (calcium-dependent, carbohydrate recognition domain) lectin, superfamily member 2 (activation induced)	*CLECSF2*	X96719	Cell-cell adhesion	>−20	0.014593
MD-2 protein	*MD-2 (Ly96)*	AB018549	Inflammation	>−20	0.001373
Dihydropyrimidinase like 3	*DPYSL3*	D78014	Signal transduction	>−20	0.029675
Cadherin 2, type 1, N-cadherin (neuronal)	*CDH2*	M34064	Cell-cell adhesion	−14.08	0.013468
Fc fragment of IgG, receptor, transporter, α	*FCGRT*	U12255	Immune response	−13.22	0.010178
Zinc finger protein 238	*ZNF238*	AJ223321	DNA binding	−9.37	0.009983
Neuron navigator 3	*NAV1*	AB023155	Nucleotide binding	−7.62	0.02058
Hypothetical protein BC001096	—	W87466	—	−7.58	0.002457
KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 3	*KDELR3*	AL035081	Protein transport	−7.23	0.007759
ALL1-fused gene from chromosome 1q	*AF1Q*	U16954	Unknown	−6.34	0.012183
Small nuclear ribonucleoprotein polypeptide N	*SNRPN*	U41303	RNA metabolism	−6.01	0.009959
γ-Aminobutyric acid(A) receptor-associated protein like 1	*GABARAPL1*	W28281	Unknown	−5.9	0.015469
Expressed sequence tags, weakly similar to hypothetical protein FLJ22184 (*H. sapiens*)	—	AL046940	—	−5.5	0.028947
Fc fragment of IgG, receptor, transporter, α	*FCGRT*	U12255	Immune response	−5.23	0.001166
Serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	*SERPINE2*	AI743134	Heparin binding	−5.07	0.019903
Adipose differentiation–related protein	*ADFP*	X97324	Lipid metabolism	−4.89	0.002709
Melanoma antigen, family D, 1	*MAGED1*	W26633	Apoptosis	−4.87	0.00796
Major vault protein	*MVP*	X79882	Protein transport	−4.36	0.000862
RecQ protein like (DNA helicase Q1 like)	*RECQL*	D37984	DNA repair	−4.27	0.017178
Retinitis pigmentosa 2 (X-linked recessive)	*RP2*	AJ007590	Protein folding	−4.23	0.005434
SEC24-related gene family, member D (*Saccharomyces cerevisiae*)	*SEC24D*	AB018298	Protein transport	−3.95	0.020784
Lectin, galactoside-binding, soluble, 1 (galectin 1)	*LGALS1*	AI535946	Cell-cell adhesion	−3.8	0.018688
Myosin X	*MYO10*	AB018342	Cytoskelatal	−3.45	0.013408
KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2	*KDELR3*	M88458	Protein transport	3.09	0.015031
Nucleobindin 2	*NUCB2*	X76732	DNA binding	−3.08	0.017437
Activin A receptor, type I	*ACVR1*	Z22534	Transforming growth factor-B1 signaling	−2.81	0.004938
Small nuclear ribonucleoprotein polypeptide F	*SNRPF*	AI032612	RNA metabolism	2.59	0.017056
Fatty acid synthase	*FASN*	U29344	Lipid metabolism	2.74	0.006118
KIAA0116 protein	—	D29958	—	3.15	0.014451
D00591/FEATURE = exons 7-14/DEFINITION = HUMRCC1 *H. sapiens* RCC1 gene, exons 1-14, complete cds	—	D00591	—	3.16	0.00635
Hypothetical protein BC008326	—	AI057614	—	3.2	0.003631
Hypothetical protein FLJ11220	—	AL050064	—	3.26	0.001465
BCL2-associated athanogene	*BAG1*	Z35491	Antiapoptosis	4.55	0.003866
DC12 protein	*DC12*	AF035296	Unknown	4.77	0.021593
Peroxisome proliferator-activated receptor γ	*PPARG*	L40904	Signal transduction	6.79	0.02744
Ephrin A-1	*EFNA1*	M57730	Cell-cell signaling	>20	0.067722*

Abbreviations: R, resistant; S, sensitive.
*Differentially expressed genes that did not reach significance but which were clearly altered.

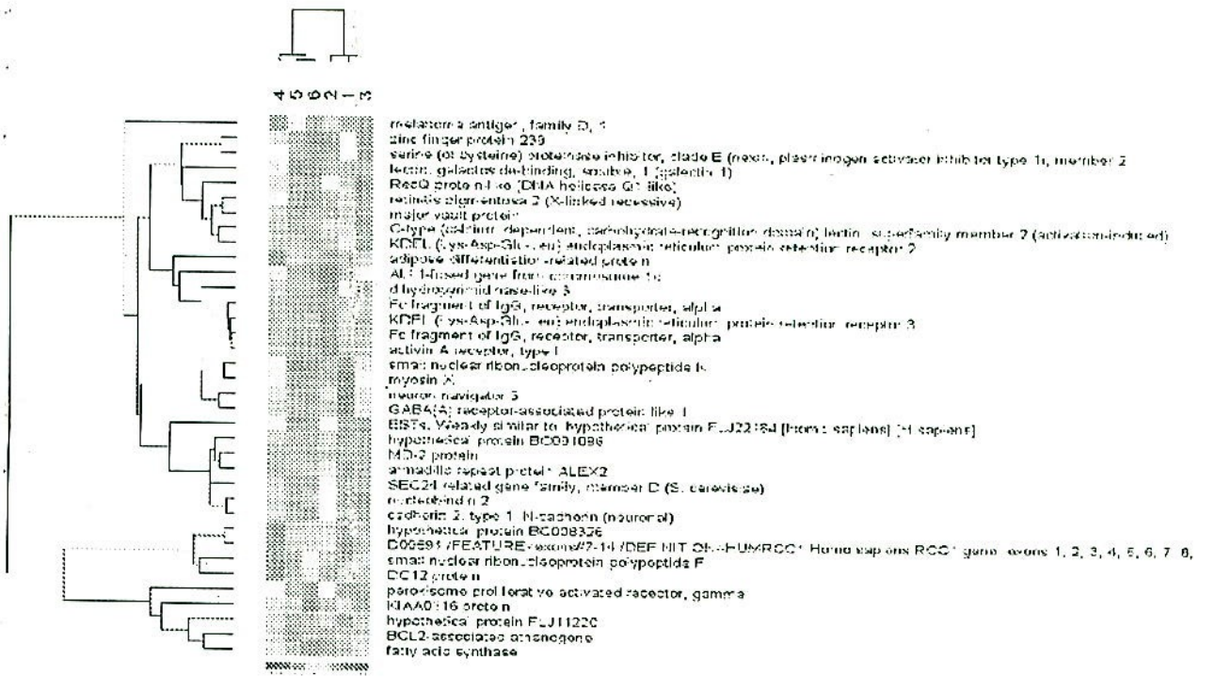


Figure 1. Two-fold expression changes at $t_i = 0$, sensitive versus resistant (see Table 2). Carboplatin sensitive and carboplatin resistant as determined by ChemoFx assay. Numbers refer to patient ID as in Table 1. Red and blue boxes indicate up-regulation and down-regulation, respectively.

treatment *ex vivo* with carboplatin and are potential prognostic markers for the clinical identification of drug-sensitive and drug-resistant tumors. Genes consistently altered by ≥2-fold differences between chemosensitive and resistant cells are shown in Table 2 and Fig. 1.

Interestingly, a number of the genes whose expressions are altered between sensitive and resistant cells play a role in apoptosis. For example, the expression of BCL2-associated athanogene (*BAG1*) is elevated in the chemoresistant cells relative to the chemosensitive cells. *BAG1* encodes a protein with antiapoptotic function. In keeping with this antiapoptotic phenotype, the proapoptotic factors adipose differentiation–related protein (*ADFP*) and melanoma antigen family D1 (*MAGED1*) are elevated in chemosensitive versus chemoresistant cells. Similarly, proteoglycan 1 (*PRG1*), which has been shown to trigger apoptosis in HeLa cells (6), is very highly expressed in chemosensitive cells relative to resistant cells. Chemosensitive cells also expressed higher levels of galectin 1 (*LGALS1*), c-type lectin superfamily member 2 (*CLECSF2*), RecQ protein like (*RECQL*), activin A receptor (*ACVR1*), and armadillo repeat protein (*ALEX2*). The possible significance of these observations is discussed below.

A number of uncharacterized genes are differentially expressed between chemosensitive and resistant cells without *in vitro* exposure to carboplatin. These include expressed sequence tags, similar to hypothetical protein FLJ22184, hypothetical protein BC001096, hypothetical protein FLJ11220, and KIAA0116 protein and expressed sequence tags similar to T00636 hypothetical protein F21856. These may represent entirely novel markers for chemoresistance in ovarian cancer.

Furthermore, there are a number of characterized genes whose altered expressions are difficult to interpret in the context of chemoresistance. These include MD-2 protein (*LY96*), *deafness autosomal dominant 5*, endoplasmic reticulum protein retention receptors 2 and 3 (*KDEL2* and *KDEL3*), γ-aminobutyric acid(A) receptor–associated protein like 1 (*GABARAPL1*), small nuclear ribonucleoprotein polypeptide N (*SNRPN*), SEC24-related gene family member D (*SEC24D*), retinitis pigmentosa 2 (*RP2*), and nucleobindin 2 (*NUCB2*).

Transcriptional Response to Carboplatin Is Altered in Chemoresistant versus Chemosensitive Ovarian Tumor Cells

We next sought to identify genes whose expression levels were altered by exposure to carboplatin *ex vivo* and specifically to define the differences in this response, if any, between chemoresistant and chemosensitive cultured ovarian tumor cells. We hypothesized that the molecular response to drug exposure would be specific to each cell type and that the differences between chemosensitive and resistant cells might define potential markers for use in predictive assays for disease prognosis. Chemoresistant and sensitive cells, as defined by the ChemoFx assay (Table 1), were exposed in culture to carboplatin for 2 or

Table 3. Genes whose expressions (> 2-fold) are altered between carboplatin-sensitive and carboplatin-resistant cells following either 2 h (A) or 72 h (B) exposure to carboplatin *in vitro* ($n = 3$)

Gene	Symbol	Accession no.	Function	Fold change (R/S)	*P*
A. Two-hour exposure to carboplatin					
Proteoglycan 1, secretory granule	*PRG1*	X17042	Apoptosis	>−20	0.02932
Armadillo repeat protein	*ALEX2*	AB011084		>−20	0.046459*
C-type (calcium-dependent, carbohydrate recognition domain) lectin, superfamily member 2 (activation induced)	*CLECSF2*	X96719	Cell-cell adhesion	> 20	0.03044*
Dihydropyrimidinase-like 3	*DPYSL3*	D78014	Signal transduction	>−20	0.023379
MD-2 protein	*MD-2 (Ly96)*	AB018549	Inflammation	−17.97	0.011945
Zinc finger protein 238	*ZNF238*	AJ223321	DNA binding	−12.25	0.004093
IFNγ-inducible protein 16	*IFI16*	M63838	DNA binding	−11.11	0.012357
Fc fragment of IgG, receptor, transporter, α	*FCGRT*	U12255	Immune response	−10.45	0.009317
KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 3	*KDELR3*	AL035081	Protein transport	−7.78	0.028435
Neuron navigator 3	*NAV1*	AB023155	Nucleotide binding	−6.93	0.004931
Small nuclear ribonucleoprotein polypeptide N	*SNRPN*	U41303	RNA Metabolism	−6.91	0.007716
Hypothetical protein BC001096	—	W87466	—	−6.77	0.005009
ALL1-fused gene from chromosome 1q	*AF1Q*	U16954	Unknown	−6.66	0.019783
Expressed sequence tags, weakly similar to hypothetical protein FLJ22184 (*H. sapiens*)	—	AI.046940	—	−5.74	0.027368
Serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	*SERPINE2*	AI743134	Heparin binding	−5.65	0.019291
RecQ protein like (DNA helicase Q1-like)	*RECQL1*	D37984	DNA repair	−4.74	0.005347
Major vault protein	*MVP*	X79882	Protein transport	−4.72	0.012515
Adipose differentiation–related protein	*ADFP*	X97324	Lipid metabolism	−4.71	0.005218
Fc fragment of IgG, receptor, transporter, α	*FCGRT*	U12255	Immune response	−4.61	0.002221
Melanoma antigen, family D, 1	*MAGED1*	W26633	Apoptosis	−4.53	0.010493
SEC24-related gene family, member D (*S. cerevisiae*)	*SEC24D*	AB018298	Protein transport	−4.3	0.006707
Lectin, galactoside-binding, soluble, 1 (galectin 1)	*LGALS1*	AI535946	Cell-cell adhesion	3.93	0.012395
Retinitis pigmentosa 2 (X-linked recessive)	*RP2*	AJ007590	Protein folding	−3.4	0.015383
HLA class II region–expressed gene KE4	*SLC39A7*	AL031228	Ion transport	−3.3	0.005645
Myosin X	*MYO10*	AB018342	Cytoskeletal	−3.13	0.019424
Leukotriene A4 hydrolase	*LTA4H*	J03459	Inflammation	2.63	0.029435
Regulator of chromosome condensation 1	*RCC1*	X12654	Cell cycle	2.69	0.003837
Small nuclear ribonucleoprotein polypeptide F	*SNRPF*	AI032612	RNA metabolism	2.8	0.025743
Chloride channel, nucleotide-sensitive, 1A	*CLNS1A*	X91788	Ion transport	2.8	0.01432
REST corepressor	*RCOR1*	D31888	DNA binding	2.81	0.001903
Programmed cell death 2	*PDCD2*	S78085	Apoptosis	2.89	0.004035
Metastasis-associated 1	*MTA1*	U35113	DNA binding	3.22	0.021499
D00591/FEATURE = exons 7-14/DEFINITION = HUMRCC1 *H. sapiens* RCC1 gene, exons 1-14, complete cds	—	D00591	—	3.26	0.010929
DKFZP564C186 protein	—	AL050019	—	3.37	0.003486
Hypothetical protein BC008326	—	AI057614	—	3.37	0.007721
MAX-interacting protein 1	*MX1*	L07648	DNA binding	4.19	0.021369
BCL2-associated athanogene	*BAG1*	Z35491	Antiapoptosis	4.81	0.028184
DC12 protein	*DC12*	AF035296	Unknown	4.91	0.015992
KIAA0601 protein	—	AB011173	—	5	0.018079
Ephrin A-1	*EFNA1*	M57730	Cell-cell signaling	>20	0.06189*
B. Seventy-two-hour exposure to carboplatin					
Armadillo repeat protein	*ALEX2*	AB011084	Unknown	>−20	0.03187*
Dihydropyrimidinase-like 3	*DPYSL3*	D78014	Signal transduction	>−20	0.003027*
Proteoglycan 1, secretory granule	*PRG1*	X17042	Apoptosis	>−20	0.068708*

Abbreviations: R, resistant; S, sensitive.
*Differentially expressed genes that did not reach significance but which were clearly altered.

(*Continued on the following page*)

Table 3. Genes whose expressions (>2-fold) are altered between carboplatin-sensitive and carboplatin-resistant cells following either 2 h (A) or 72 h (B) exposure to carboplatin *in vitro* (n = 3) (Cont'd)

Gene	Symbol	Accession no.	Function	Fold change (R/S)	P
C-type (calcium-dependent, carbohydrate recognition domain) lectin, superfamily member 2 (activation induced)	*CLECSF2*	X96719	Cell-cell adhesion	>−20	0.017664
MD-2 protein	*MD-2 (Ly96)*	AB018549	Inflammation	>−20	0.005349
Fc fragment of IgG, receptor, transporter, α	*FCGRT*	U12255	Immune response	−14.94	0.025556
Sarcospan (Kras oncogene–associated gene)	*SSPN*	N21470	Cell-cell adhesion	−12.65	0.001181
IFNγ-inducible protein 16	*IFI16*	M63838	DNA binding	7.69	0.006012
Fc fragment of IgG, receptor, transporter, α	*FCGRT*	U12255	Immune response	−6.61	0.02581
Adipose differentiation–related protein	*ADFP*	X97324	Lipid metabolism	−5.65	0.002593
Glutathione peroxidase 1	*GPX1*	X13710	Stress response	−5.16	0.027208
ALL1-fused gene from chromosome 1q	*AF1Q*	U16954	Unknown	−4.84	0.019144
H. sapiens mRNA; cDNA DKFZp564A026 (from clone DKFZp564A026)	—	AL050367	—	−4.57	0.015384
RecQ protein like (DNA helicase Q1-like)	*RECQL1*	L36140	DNA repair	−4.42	0.018595
CLIP-170-related protein	*CLIPR-59*	N99340	Unknown	−4.29	0.003918
Aldehyde dehydrogenase 1 family, member A3	*ALDH1A3*	U07919	Lipid metabolism	−4.24	0.013915
H. sapiens unknown mRNA	—	AI674208	—	−4.12	0.022238
Hypothetical protein BC001096	—	W87466	—	−4.05	0.017738
Serine/threonine kinase 17a (apoptosis inducing)	*STK17A*	AI961743	Apoptosis	−3.6	0.004848
CGI-65 protein	*NDUFAF1*	AI566877	Mitochondrial electron transport	−3.34	0.014449
BET1 homologue (*S. cerevisiae*)	*BET1*	AF007551	Protein transport	−3	0.003188
CGI-100 protein	*TMED5*	AL080084	Protein transport	−2.7	0.003929
H. sapiens clone 24416 mRNA sequence	—	AF052159	—	2.67	0.001003
KIAA1014 protein	—	AB023231	—	2.74	0.022371
CCCTC-binding factor (zinc finger protein)	*CTCF*	U25435	DNA binding	2.77	0.009499
Hypothetical protein MGC5576	—	AI525633	—	2.83	0.004847
L-3-hydroxyacyl-coenzyme A dehydrogenase, short chain	*HADHSC*	X96752	Lipid metabolism	2.83	0.003823
REST corepressor	*RCOR1*	D31888	DNA binding	2.99	0.001522
Chromobox homologue 1 (HP1β homologue *Drosophila*)	*CBX1*	U35451	Chromatin assembly	3.11	0.029683
Espressed sequence tags	—	AI827895	—	3.27	0.014036
Fatty acid synthase	*FASN*	U29344	Lipid metabolism	3.34	0.001912
Uracil-DNA glycosylase	*UNG*	Y09008	DNA repair	3.4	0.00155
Cyclin F	*CCNF*	Z36714	Cell cycle	3.42	0.024405
MCM4 minichromosome maintenance-deficient 4 (*S. cerevisiae*)	*MCM4*	X74794	DNA repair	3.46	0.003692
Vaccinia-related kinase 1	*VRK1*	AB000449	Protein Modification	3.68	0.00879
KIAA0101 gene product	—	D14657	—	3.68	0.009866
Rad2	*RAD2*	HG4074-HT4344	DNA repair	3.69	0.015096
Replication factor C (activator 1) 3, 38 kDa	*RFC4*	L07541	DNA replication	3.73	0.004902
KIAA0186 gene product	—	D80008	—	3.8	0.010346
MCM2 minichromosome maintenance-deficient 2, mitotin (*S. cerevisiae*)	*MCM2*	D21063	DNA replication	3.87	0.015244
H2A histone family, member X	*H2AX*	X14850	Chromosome organization	3.89	0.000189
Paired basic amino acid cleaving system 4	*PCSK6*	M80482	Cell-cell signaling	4.1	0.003952
Flap structure–specific endonuclease 1	*FEN1*	AC004770	DNA /Repair	4.13	0.017241
Hypothetical protein FLJ10719	—	W74442	—	4.21	0.023813
Desmoplakin (DPI and DPII)	*DSP*	AL031058	Cell-cell adhesion	4.28	0.011781
Adenosine deaminase, RNA-specific, B1 (RED1 homologue rat)	*ADARB1*	U76421	RNA metabolism	4.4	0.008376
CDC45 cell division cycle 45 like (*S. cerevisiae*)	*CDC45L*	AJ223728	DNA replication	4.75	0.02302
DC12 protein	*DC12*	AF035296	Unknown	5.22	0.005536

(*Continued on the following page*)

Table 3. Genes whose expressions (>2-fold) are altered between carboplatin-sensitive and carboplatin-resistant cells following either 2 h (A) or 72 h (B) exposure to carboplatin *in vitro* (n = 3) (Cont'd)

Gene	Symbol	Accession no.	Function	Fold change (R/S)	P
v-myb myeloblastosis viral oncogene homologue (avian) like 2	*MYBL2*	X13293	Antiapoptosis	7.26	0.028861
KIAA1028 protein	—	AB028951	—	7.43	0.026059
KIAA0906 protein	—	AB020713	—	8.08	0.006995
Ephrin-A1	*EFNA1*	M57730	Cell-cell signaling	>20	0.000447

72 hours. Genes whose expression levels were altered following 2 hours of exposure to carboplatin are shown in Table 3A and Fig. 2A. These data are strikingly similar to those obtained when chemoresistant and chemosensitive cells were compared in the absence of carboplatin exposure (Table 2). Specifically, of the genes identified as being differentially expressed between sensitive and resistant cells in the absence of carboplatin (Table 2), 79% were also altered after 2 hours of exposure to carboplatin. This likely indicates that relatively few alterat ons in gene expression occur within the first 2 hours following the onset of carboplatin exposure and therefore provides a convenient external control for the experimental data summarized in Table 2. Notably, a number of genes that were altered between chemoresistant and chemosensitive cells in the absence of carboplatin (Table 2) were also altered after 2 hours (and 72 hours) of carboplatin exposure. These include *PRG1*, *ALEX2*, *MD2*, ephrin A-1 (*EFNA1*), and *RECQL1*. Those genes whose altered expression was not common to the unexposed and 2-hour carboplatin-exposed cells are generally poorly characterized, and/or information relating to their potential involvement in chemosensitivity/resistance is limited. They included REST corepressor (*RCOR1*); leukotrine A4 hydrolase (*LTA4H*); HLA class II region–expressed gene KE4 (*KE4*); chloride channel, nucleotide-sensitive 1A (*CLNS1A*); programmed cell death 2 (*PCD2*); MAX-interacting protein (*MXI1*); and IFNγ-inducible protein 16 (*IFI16*). One exception is metastasis-associated 1 (*MTA1*), which is discussed below.

In contrast, we found that following 72 hours of exposure to carboplatin (Table 3B; Fig. 2B), differentially expressed genes had only moderate overlap with those identified after 2 hours of exposure or no exposure, indicating a marked response at the level of transcription to 72 hours of carboplatin exposure. Specifically, of the 37 genes whose expressions were altered between chemosensitive and resistant cells in the absence of carboplatin, only 15 (28%) overlapped with the 53 differentially expressed genes identified after 72 hours of carboplatin exposure. This suggests that the transcriptional response to carboplatin overrides many of the transcriptional differences that define the cells in their carboplatin-free baseline state.

We found marked differences in gene expression patterns between the chemosensitive and resistant cells as shown in Table 3B and Fig. 2B. For example, a number of genes of functional significance in the context of drug response were elevated in resistant versus sensitive cells. These genes were either associated with cell proliferation, including CDC45 cell division cycle 45 like (*CDC45L*), cyclin F (*CCNF*), or DNA replication and repair, including replication factor C 4 (*activator 1*) 38-kDa (*RFC4*), minichromosome maintenance-deficient 2 (*MCM2*), *MCM4*, uracil-DNA glycosylase (*UNG*), flap structure–specific endonuclease 1 (*FEN1*; also *RAD2*). Also elevated in resistant cells was v-myb myeloblastosis viral oncogene homologue (avian) like 2 (*MYBL2*), which encodes a protein with antiapoptotic activity. In keeping with the apoptosis-resistant phenotype of chemoresistant cells, we also found that serine/threonine kinase 17a (*STK17A*) was reduced in chemoresistant cells. STK17A encodes a protein with proapoptotic function (7).

We also identified H2A histone family member X (*H2AX*), which is involved in the maintenance of genome integrity (see below), as being elevated in resistant cells, as well as chromobox homologue 1 (*HP1*). The elevated expression of HP1 in chemoresistant cells is notable, because loss of function of HP1 has been shown to cause cell death (8), and the down-regulation of HP1α expression is associated with the metastatic phenotype in breast cancer (9).

Notable genes whose expressions were elevated in chemosensitive cells included glutathione peroxidase 1 (*GPX1*) and *IFI16*, both of which have been previously implicated in chemoresistance and apoptosis, respectively. The significance of these observations is discussed below.

Finally, a number of uncharacterized genes were found to be differentially expressed by 72 hours of carboplatin exposure. These included *KIAA0101* gene product, hypothetical protein *BC001096*, *Homo sapiens* clone *24416*, *CGI-100* protein, and *CGI65* protein.

Characterization of the Temporal Response to Carboplatin in either Chemosensitive or Resistant Cells

We also searched our data for genes whose expressions were independently altered in a temporal fashion in either chemosensitive or resistant cells by exposure to carboplatin. Using this approach with a 2-fold expression change cutoff, we found that in the chemosensitive cells, only 19 and 11 genes were altered when (a) t = 0 hour was compared with t = 72 hours and (b) t = 2 hours was compared with t = 72 hours, respectively, for the sensitive cells (Table 4A). Furthermore, only two and three genes

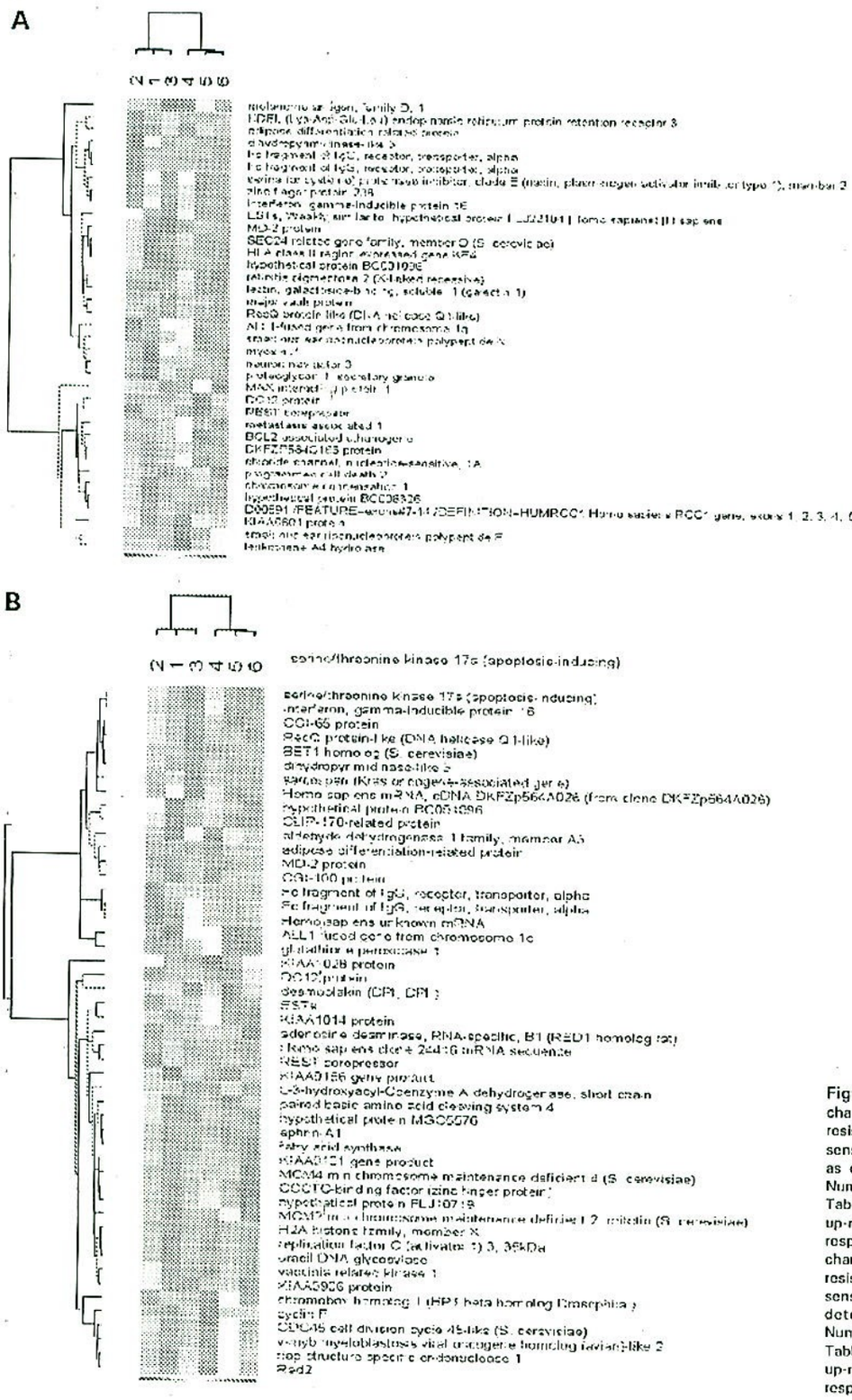


Figure 2. **A,** 2-fold expression changes at $t = 2$, sensitive versus resistant (see Table 3A). Carboplatin sensitive and carboplatin resistant as determined by ChemoFx assay. Numbers refer to patient ID as in Table 1. Red and blue boxes indicate up-regulation and down-regulation, respectively. **B,** 2-fold expression changes at $t = 72$, sensitive versus resistant (see Table 3B). Carboplatin sensitive and carboplatin resistant as determined by ChemoFx assay. Numbers refer to patient ID as in Table 1. Red and blue boxes indicate up-regulation and down-regulation, respectively.

were altered when (a) $t = 0$ hour was compared with $t = 72$ hours and (b) $t = 2$ hours was compared with $t = 72$ hours, respectively, for the resistant cells (Table 4B). Not surprisingly, we found considerable overlap between the $t = 0$ hour versus $t = 72$ hours and $t = 2$ hours and $t = 72$ hours comparisons, which is consistent with the previous finding that few expression changes are evident when chemosensitive and resistant cells were directly compared after 2 hours of carboplatin exposure (Table 3A). Interestingly, we found very few statistically significant gene expression

Table 4. Genes whose expressions are temporally altered in (A) chemosensitive and (B) chemoresistant cells following carboplatin exposure *in vitro* .

0 h versus 72 h	*P*	2 h versus 72 h	*P*	Gene	Symbol	Function	Accession no.	Locus link
A. Chemosensitive cells								
↓72 h	0.022345	↓72 h	0.018408	CDC6 cell division cycle 6 homologue (*S. cerevisiae*)	*CDC6*	Cell cycle	U77949	990
↓72 h	0.025498	—	—	Inhibin, β A (activin A, activin AB α polypeptide)	*INHBB*	Cell cycle	J03634	3624
↓72 h	0.01845	↓72 h	0.033219	Kinetochore associated 2	*KNTC1*	Cell cycle/ DNA repair	AF017790	10403
↓72 h	0.019354	↓72 h	0.024426	Flap structure–specific endonuclease 1	*FEN1*	DNA repair	AC004770	2237
↓72 h	0.032797	—	—	Ribonucleotide reductase M2 polypeptide	*RRM2*	DNA replication	X59618	6241
↓72 h	0.032727	↓72 h	0.033662	MCM7 minichromosome maintenance-deficient 7 (*S. cerevisiae*)	*MCM7*	DNA repair	D55716	4176
↓72 h	0.021449	↓72 h	0.02774	Rad2	*RAD2*	DNA repair		
↓72 h	0.016836	↓72 h	0.032576	Ubiquitin-specific protease 1	*USP1*	Ubiquination cycle	AB014458	7398
↓72 h	0.016794	↓72 h	0.027252	H4 histone family, member G	*HIST1H4C*	Chromatin Organization	AA255502	8364
↓72 h	0.039039	↓72 h	0.040177	Replication factor C (activator 1) 4, 37 kDa	*RFC4*	DNA replication	M87339	5984
↓72 h	0.035747	—	—	MCM4 minichromosome maintenance-deficient 4 (*S. cerevisiae*)	*MCM4*	DNA repair	X74794	4173
↓72 h	0.02134	—	—	Sprouty homologue 2 (*Drosophila*)	*SPRY2*	Cell-cell signaling	AF039843	10253
↓72 h	0.024581	—	—	Fusion, derived from t(12;16) malignant liposarcoma	*FUS*	DNA binding/ unknown	S62140	2521
↓72 h	0.033626	—	—	MAD, mothers against decapentaplegic homologue 7 (*Drosophila*)	*SMAD7*	DNA binding transforming growth factor B1 signaling	AF010193	4092
↓72 h	0.021064	—	—	Gelsolin (amyloidosis, Finnish type)	*GSN*	Cytoskeletal	X04412	2934
↓72 h	0.03123	—	—	H2A histone family, member L	*HIST1HH2AH*	Chromatin organization	U90551	8334
↓72 h	0.015597	↓72 h	0.02281	Chemokine (C-C motif) ligand 2	*CCL2*	Inflammation/ cell-cell adhesion	M28225	6347
↓72 h	0.022596	↓72 h	0.022538	p8 protein (candidate of metastasis 1)	*P8*	Apoptosis	AI557295	26471
↓72 h	0.009538	—	—	Chemokine (C-C motif) ligand 2	*CCL2*	Inflammation/ cell-cell adhesion	M26683	6347
B. Chemoresistant cells								
↓72 h	0.037631	↓72 h	0.041482	Inhibitor of DNA binding 2, dominant-negative helix-loop-helix protein	*ID1*	DNA binding/ development	D13891	3398
—	—	↓72 h	0.029638	HIF-1 responsive RTP801 (DNA damage–inducible transcript 4)	*DDIT4*	DNA damage response	AA522530	54541
↓72 h	0.028745	↓72 h	0.028122	Cyclin G2	*CCNG2*	Cell cycle	U47414	901

changes following 2 or 72 hours of exposure to carboplatin in chemoresistant cells. This suggests that the transcriptional response to carboplatin is subtle in these cells and this may reflect the chemoresistant nature of their behavior in culture in the presence of the drug.

Strikingly, a large number of the genes altered by exposure to carboplatin in chemosensitive cells are involved in DNA replication and maintenance, and these are reduced by carboplatin exposure at the mRNA level. These genes include, for example, *MCM7*, *MCM4*, *RFC4* (37 kDa), kinetochore-associated 2 (*KNTC2*), *FEN1*, and ribonucleotide reductase M2 polypeptide (*RRM2*). Genes whose expressions were elevated in chemosensitive cells in response to carboplatin have a variety of functions but include H2A histone family member L (*HIST1H2AC*), chemokine (C-C motif) ligand 2 (*CCL2*), Sprouty 2 (*SPRY2*), Gelsolin (*GSN*), and p8 protein (*P8*).

Discussion

The data presented here are the result of a preliminary investigation into the molecular phenotype of patient-derived ovarian tumor cells in the context of sensitivity or resistance to carboplatin. We have identified a number of differentially expressed genes that define transcriptional differences between chemosensitive and resistant cells and temporal responses to carboplatin expressed in an *ex vivo* setting. These genes represent a variety of functional classes but are biased towards those that encode proteins involved in apoptosis, cell-cell communication, cell anchorage, DNA repair, and chromosome stability. These findings are consistent with increased survival of resistant cells in the presence of carboplatin in culture as defined by the ChemoFx assay.

One feature of our data was the finding that chemosensitive cells in the absence of carboplatin have a transcriptional profile that can be associated with a proapoptotic phenotype compared with chemoresistant cells. BAG-1, for example, was elevated in chemoresistant relative to chemosensitive cells. BAG-1 has been shown to inhibit apoptosis (10, 11), and its overexpression in resistant cells is consistent with the ChemoFx data (Table 1). Significantly, it has recently been suggested that BAG-1 up-regulation is associated with the acquisition of paclitaxel resistance (12). PRG1, which was highly expressed in the chemosensitive cells at all time points, has been shown to trigger apoptosis in HeLa cells (6) and to mediate the clearance of apoptotic cells by dendritic cells *in vivo*. Similarly, *ADFP*, which is a peroxisome proliferator–activated receptor γ target gene (13), has been shown to be involved in apoptotic processes in epithelial cells derived from cervical carcinomas (14), whereas MAGED1 is involved in the p75 neurotrophin receptor–mediated programmed cell death pathway (15). The increased expression in chemosensitive cells of LGALS1 is perhaps not surprising, given that it encodes a protein with antiproliferative function that may be associated with apoptosis in cancer cells (16). ALEX2, which is also elevated in chemosensitive cells, may play a role in tumor suppression (17). In general, these markers, which were elevated in chemosensitive cells, confer proapoptotic traits that contribute to defining the molecular phenotype underlying the ChemoFx data presented in Table 1.

Our data also highlight a number of other genes that are involved in DNA repair and maintenance of chromosome stability. For example, RECQL is a DNA helicase that acts to restore productive DNA replication following S-phase arrest and hence prevent subsequent genomic instability. RECQL expression was found to be elevated in chemosensitive cells, which is consistent with the previously documented observation that loss of DNA repair function has been correlated with resistance to a variety of DNA-damaging agents, including many anticancer drugs (18). Interestingly, chemosensitive cells were also found to overexpress ACVR1. Activin signaling may play a significant role in cell proliferation and ovarian tumor development. Furthermore, several ovarian cancer cell lines produce activin *in vitro*, and exogenous activin induced their proliferation (19–22). Paradoxically, the gene encoding the major vault protein is overexpressed in chemosensitive versus resistant cells. The major vault protein has been associated with drug resistance in cancer, and its overexpression has been suggested as a marker for chemoresistance in ovarian cancer (23). However, its use in this context has recently been called into question (24).

Our analysis of the transcriptional response to carboplatin at 2 hours revealed that very few robust alterations in gene expression occur during the short duration of this exposure. This likely reflects the fact that the temporal response to drug stimulus requires >2 hours to be manifested at the transcriptional level. Those genes whose expressions are altered following 2 hours of carboplatin exposure are generally poorly characterized. Exception include MTA1, overexpression of which has been associated with metastasis in ovarian cancer (25), and PCD2, which is involved in apoptosis (26).

The effects of carboplatin were much more pronounced after 72 hours and revealed alterations in the expression of a variety of genes. These include those encoding either proteins involved in cell proliferation (*CDC45L* and *CCNF*) or DNA replication and repair (*RFC4*, *MCM2*, *MCM4*, *UNG*, and *FEN1/RAD2*), which not surprisingly were coordinately elevated in chemoresistant cells. Also altered was the gene (*DSP*) that encodes the cell-cell adhesion protein desmoplakin. Desmoplakin is an intercellular junction protein in vertebrate epithelial cells that is able to form both membrane anchorage sites for intermediate-size filaments and interact with the plasma membrane of adjacent cells to mediate stable intercellular adhesion. Interestingly, a reduction in the DSP-encoded protein has been observed in drug-resistant breast cancer cell lines (27), and a reduction in expression of the related protein γ-catenin has been observed in cisplatin-resistant adenocarcinoma cell lines (28), although this was not apparent in our data.

The glycosylphosphatidylinositol-anchored ligand EFNA1 preferentially binds to the receptor tyrosine kinase

EFNA2, and EFNA1 expression was elevated in chemoresistant versus chemosensitive cells in the absence of carboplatin and after 2 and 72 hours of carboplatin exposure. It has recently been shown, in a colon carcinoma cell line, that activation of EFNA2 by EFNA1 may trigger an indirect growth signal via phosphorylation of E-cadherin and β-catenin by overcoming contact inhibition (29). Similarly elevated in chemoresistant cells is FASN, which is almost universally up-regulated in human cancers and is thought to be involved in the development, maintenance, and enhancement of the malignant phenotype. FASN protein inhibition induces programmed cell death in human breast cancer cells (30). These observations are consistent with our own data and suggest that a relative reduction in FASN expression in chemosensitive cells may be involved in their apoptosis. Paradoxically, however, FASN expression has also been shown to induce apoptosis in a prostate carcinoma cell line (31).

GPX1 is one of the most important antioxidant enzymes in humans. It has been reported that the protein encoded by GPX1 is protective against CD95-induced apoptosis in cultured breast cancer cells, and that GPX1 overexpression increases resistance to toxic challenges. Furthermore, GPX1 executes intracellular inactivation of the carboplatin relative cisplatin, and detoxification of reactive oxygen species is a mechanism for cisplatin resistance (32). Paradoxically, GPX1 is elevated in carboplatin-sensitive cells in our data, suggesting that GPX1 activity is not an essential component of carboplatin resistance in ovarian cancer cells. Clearly, our observation that GPX1 expression is increased in chemosensitive versus chemoresistant ovarian cancer cells requires further investigation in the context of the previous data.

One gene of particular interest that we found to be up-regulated in chemoresistant versus chemosensitive cells is *H2AX*. This encodes a core component of chromatin that is phosphorylated in chromatin-flanking DNA double-strand breaks. It has recently been shown in mice that H2AX helps prevent aberrant repair of both programmed and general DNA breakage and thereby functions as a dosage-dependent suppressor of genomic instability and tumor formation (33). Similarly, Celeste et al. (34) recently showed that loss of a single *H2AX* allele compromises genomic integrity and enhances the susceptibility to cancer in the absence of p53. They report that H2AX acts as a "genomic caretaker" in protection against tumorigenesis. Although this evidence is not directly related to chemoresistance, it can plausibly be interpreted to suggest that H2AX overexpression leads to improved maintenance of genomic integrity in the face of carboplatin exposure.

The observation that IFI16 is overexpressed in chemosensitive cells is significant, because the *IFI16* gene product has recently been shown to negatively regulate p21/waf1, resulting in p53-dependent cell cycle arrest (35). Our finding that IFI16 mRNA is elevated in carboplatin-sensitive ovarian tumor cells suggests a possible mechanism whereby drug resistance is conferred, in part, via a reduction in IFI16 expression. For example, cisplatin increases p21/waf1 protein levels (36). If carboplatin has the same effect, elevated IFI16 could potentially counteract p21/waf1 induction, resulting in reduced apoptosis. Interestingly, low p21/waf1 protein levels have recently been shown to sensitize tumor cells, albeit not ovarian tumor cells, to apoptosis (37). However, p21/waf1 overexpression has been found to result in apoptosis in ovarian cancer cell lines (38), which would not support this hypothesis.

In keeping with the above results, and consistent with an association between cellular survival and chemoresistance, analysis of the temporal response to carboplatin in only the chemosensitive cells revealed a coordinated alteration in the expression levels of genes that are involved in DNA replication and/or maintenance. For example, FEN1, whose expression is reduced by carboplatin, is a member of the XPG/RAD2 endonuclease family and is one of 10 proteins essential for cell-free DNA replication. It processes the 5′ ends of Okazaki fragments in lagging strand DNA synthesis and is involved in DNA repair (39). Similarly down-regulated is RFC4, which is involved in the elongation of primed DNA templates by DNA polymerase δ and DNA polymerase epsilon (39) and KNTC2, which is one of several proteins involved in spindle checkpoint signaling. This surveillance mechanism assures correct segregation of chromosomes during cell division by detecting unaligned chromosomes and causing prometaphase arrest until the proper bipolar attachment of chromosomes is achieved. Depletion of KNTC2 has been shown to result in catastrophic mitotic exit (40), which is consistent with the expected cellular response to a cytotoxic compound such as carboplatin. Also down-regulated by carboplatin in sensitive cells was CDC6, which is essential for the initiation of DNA replication. The protein encoded by CDC6 functions as a regulator at the early stages of DNA replication and is rapidly destroyed in cells undergoing apoptosis (41). Finally, RRM2 overexpression is associated with gemcitabine chemoresistance in pancreatic adenocarcinoma cells; in addition, that suppression of RRM2 expression by small interfering RNA enhanced gemcitabine-induced cytotoxicity *in vitro* has been shown to result in markedly suppressed tumor growth, increased tumor apoptosis, and inhibition of metastasis (42). These observations are consistent with our finding that RRM2 is down-regulated by carboplatin in chemosensitive cells.

In summary, we present preliminary data that defines the transcriptome of cultured ovarian tumor cells that display either carboplatin sensitivity or resistance in an *ex vivo* assay of cell survival. Our data suggest that there is a comprehensive and coordinated alteration in gene expression that defines an apoptosis-resistant phenotype in chemoresistant cells. The genes identified in the present study have potential as markers of ovarian tumor chemosensitivity, both in the presence (and absence) of *ex vivo* carboplatin treatment. Although this is a preliminary investigation into the molecular phenotype of carboplatin resistance in ovarian cancer, it provides a platform of comprehensive and robust gene expression data that will form the basis of future investigation by ourselves and others.

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Prognostic meta-signature of breast cancer developed by two-stage mixture modeling of microarray data

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Abstract

Background: An increasing number of studies have profiled tumor specimens using distinct microarray platforms and analysis techniques. With the accumulating amount of microarray data, one of the most intriguing yet challenging tasks is to develop robust statistical models to integrate the findings.

Results: By applying a two-stage Bayesian mixture modeling strategy, we were able to assimilate and analyze four independent microarray studies to derive an inter-study validated "meta-signature" associated with breast cancer prognosis. Combining multiple studies (n = 305 samples) on a common probability scale, we developed a 90-gene meta-signature, which strongly associated with survival in breast cancer patients. Given the set of independent studies using different microarray platforms which included spotted cDNAs, Affymetrix GeneChip, and inkjet oligonucleotides, the individually identified classifiers yielded gene sets predictive of survival in each study cohort. The study-specific gene signatures, however, had minimal overlap with each other, and performed poorly in pairwise cross-validation. The meta-signature, on the other hand, accommodated such heterogeneity and achieved comparable or better prognostic performance when compared with the individual signatures. Further by comparing to a global standardization method, the mixture model based data transformation demonstrated superior properties for data integration and provided solid basis for building classifiers at the second stage. Functional annotation revealed that genes involved in cell cycle and signal transduction activities were over-represented in the meta-signature.

Conclusion: The mixture modeling approach unifies disparate gene expression data on a common probability scale allowing for robust, inter-study validated prognostic signatures to be obtained. With the emerging utility of microarrays for cancer prognosis, it will be important to establish paradigms to meta-analyze disparate gene expression data for prognostic signatures of potential clinical use.

Introduction

DNA microarray analysis has been shown to be a powerful tool in various aspects of cancer research [1]. With the increasing availability of published microarray data sets,

there is a tremendous need to develop approaches for validating and integrating results across multiple studies. A major concern in the meta-analysis of DNA microarrays is the lack of a single standard experimental platform for data generation. Expression profiling data based on different technologies can vary significantly in measurement scale and variation structure. It poses a great challenge to compare and integrate results across independent microarray studies. In a recent study of diffuse large B cell lymphoma (DLBCL), Wright et al. [2] sought to bridge two different microarray platforms by validating findings from a cDNA lymphochip microarray using an independent dataset generated using Affymetrix oligonucleotide arrays. Although the idea of training and testing classifiers is frequently used for discriminant analysis, this application to distinct expression array platforms is less common.

More systematic approaches have been proposed for integration of findings from multiple studies using different array technologies. Rhodes et al. [3] have proposed methods to summarize significance levels of a gene in discriminating cancer versus normal samples across multiple gene profiling studies. By ranking the q-values [4] from sets of combinations, a cohort of genes from the four studies was identified to be abnormally expressed in prostate cancer. Choi et al. [5] suggested combining effect size using a hierarchical model, where the estimated effect size in individual studies follows a normal distribution with mean zero and between study variance τ^2. The effect size was defined to be the difference between the tumor and normal sample means divided by pooled standard deviation. From a Bayesian perspective, Wang et al. [6] used data from one study to generate a prior distribution of the differences in logarithm of gene expression between diseased and normal groups, and subsequent microarray studies updated the parameter values of the prior. Assuming a normal error distribution, the differences were then combined to form a posterior mean. Although phrased using different model frameworks, these methods are similar in the spirit of combining the standardized differences between two sample means across multiple studies. It has been shown, however, that the overlap between significant gene detection on different array platforms is only moderate due to low comparability of independent data sets [7]. The large variability brought in by microarray datasets using different platforms is expected to affect the sensitivity and specificity of summary statistics constructed in various ways across studies. Given the inherent differences of the microarray techniques, heterogeneity of the sample populations, and low comparability of the independently generated data sets, meta-analysis of microarrays remains a difficult task.

A recent study proposed a Bayesian mixture model based transformation of DNA mi-croarray data with potential features applicable to meta-analysis of microarray studies [8]. The basic idea is to estimate the probability of over-, under- or baseline expression for gene sample combinations given the observed expression measurements. With data-driven estimation of these quantities, one can translate the raw expression measurement into a probability of differential expression. As a result, *poe* (i.e., probability of expression) was introduced as a new scale and used in the context of molecular classification [8]. The platform-free property of this scale, however, motivated us to incorporate *poe* in a framework to meta-analyze microarray data. Several desirable features of using *poe* as a new expression scale include the following: 1. *poe* provides a scaleless measure and thereby facilitates data integration across microarray platforms; 2. *poe* is a model-based transformation with direct biological implications in the context of gene expression data, as it is estimated based on a method that adopts an underlying mixture distribution that accommodates over-, under-, and unchanged expression categories; 3. *poe* unmasks differential expression patterns in microarray data by offsetting the influence of extreme expression values [9]; 4. Data integration based on *poe* allows merging of samples on the unified scale rather than using gene-specific summaries.

In recent publications of breast cancer microarray studies, several groups have explored the hypothesis that the capacity to metastasize is intrinsic to the tumor and therefore can be revealed by gene expression pattern. Four independent studies have correlated gene expression profiles generated from distinct DNA microarray platforms to breast cancer prognosis [10-13]. Among the four, Sorlie et al. [10] and Sotiriou et al. [12], both cDNA microarray studies, applied unsupervised clustering and identified several breast cancer subtypes characterized by differential expression of a cohort of genes. Further, they correlated the tumor subtypes derived from the expression profile with survival outcome and in both cases found that, as expected, the ERBB2+ subtype correlated with shorter survival times. On the other hand, van't Veer et al. [11], an inkjet oligonucleotide array study, and Huang et al. [13], an Affymetrix GeneChip study, have built classification models based on gene expression profiles to predict 5-year or 3-year recurrence status. In all four studies, however, the authors explored a common hypothesis that molecular profiles were able to provide a more accurate prediction of patient survival compared with clinical/pathological parameters. These studies therefore provided an excellent basis for developing a meta-analysis of microarrays with regard to disease prognosis.

In this proof-of-concept study, we propose a two-stage meta-analysis of microarrays based on *poe*. We applied our method to the aforementioned breast cancer DNA microarray data sets. With the strength of the *poe* transfor-

mation and data integration, our goal was to develop an inter-study validated meta-signature that predicts relapse-free survival in breast cancer patients with improved statistical power and reliability.

Results

Development of the two-stage Bayesian mixture modeling approach for the meta-analysis of microarray data

Figure 1 outlines the two-stage Bayesian mixture modeling strategy. The idea is to build a scale that can be combined across different microarray platforms, and therefore allows simultaneous examination of independent data sets. The stage 1 of the analysis involves data-driven estimation of posterior probability of differential expression, namely *poe*. The Bayesian hierarchical model employed for estimation borrows strength across genes by assuming further distributions for the gene-specific parameters (see Methods). For data integration purposes, we focused on a common set of 2,555 genes that were profiled in each of the four studies. Although the cost for compiling common genes is a loss of potential predictive features, it is not unreasonable to assume, given the analogous hypothesis explored in each study, that the common set represents the most relevant genes of interest for breast cancer prognosis. The resulting values of *poe* represent signed probability of differential expression for gene j in sample i, and thus provide a unified measure across studies. Further, the transformation improves contrast in each data set by removing the influence of extreme expression values. In stage 2, the expression profile of tumor samples from multiple studies were combined on the *poe* scale to generate a meta-cohort. The benefit of data integration using *poe* is twofold. First, it improves power of statistical analysis by increasing the sample size. Such integration of independent data sets renders sensitivity to those small yet consistent expression changes for certain genes. Second, it reduces the chance of false positive features due to artifacts from a single study, and allows reliable findings across studies. In this paper, we integrated four breast cancer microarray data sets of distinct platforms (Table 1), and developed a prognostic meta-signature for disease recurrence.

Building a gene expression meta-signature for breast cancer prognosis

In the second stage of the analysis, We assessed the performance of the genes found using the meta-analysis methods based on classification accuracy. A complication is that while most methods of classification deal with data from two populations, the response with which we wish to build classifiers to predict is time to breast cancer recurrence. While the ideal data would have information on time to recurrence on all subjects (potentially censored), not all studies have the time to recurrence information available and instead provide data on recurrence within a certain time interval (e.g., recurrence within five years versus no recurrence within five years). To deal with this issue, we utilized a dichotomization where a bad outcome is recurrence during followup and a good outcome is remaining recurrence-free for at least three years. The additional constraint for the good outcome group is to reduce potential bias introduced by short censoring due to insufficient length of follow-up. This is particularly relevant in cross-study analysis, given the heterogeneity in patient recruitment criteria and study designs. Accordingly, of the combined meta-cohort (n = 305) of breast cancer patients, 48.9% were in the poor outcome group, whereas 51.1% in the good outcome group. The sample sizes for each study are shown in Table 1.

Each gene was then associated with the recurrence status by a logistic regression within a leave-one-out cross validation scheme, and rank-ordered by the significance level of the coefficient. As a result, 23 genes held up as significant predictor of recurrence ($P \leq 0.001$) in all cross-validation steps, representing a cohort of essential genes strongly associated with breast cancer recurrence. By random chance, there would be on average 2.5 genes to be found significant at $P \leq 0.001$ in a set of 2,555. By finding 23 genes with a $P \leq 0.001$, it is clear that there are much more predictive features than would be expected by random chance.

To identify a prognostic meta-signature, we define a risk index (RI) as a linear combination of the *poe* profile and the coefficient estimates from the univariate logistic regression for each gene j. Large positive values of RI indicate high risk of failure, whereas large negative values of RI indicate low risk of failure. Classification of sample i to the risk groups is then based on the i^{th} leave-one-out risk index. The classifier is $C(X) = I\{RI_i > c\}$, with c being the empirical quantiles of the risk indices. The number of genes in a classifier is treated as a parameter and optimized to minimize the prediction error rates. More details on building a classifier at the second stage are described in the Methods section.

The 90-gene expression meta-signature predicts clinical outcome in breast cancer patients

By minimizing the misclassification error, we obtained a 90 gene meta-signature that reliably predicts outcome in the meta-cohort. This meta-signature classified 122 patients into a high risk group, where 84 (69%) of them had a recurrence. On the other hand, the signature classified 183 patients into a low risk group, where 118 (64%) of them did not recur by the end of the followup. By cross-tabulating the risk groups predicted by the meta-signature and the actual recurrence status, we obtained an estimated odds ratio of 4.0 (95% CI: 2.5–6.5, $P < 0.0001$). In spite

Goals: Inter-study validation of microarray data to obtain prognostic profiles.

Strategy: Two-stage Bayesian mixture modeling.

Stage1

Collect microarray data from studies that address similar questions.

↓

Obtain the set of genes that are present on each independent array data, take base 2 logarithm and apply proper normalization on each data set.

↓

Fit mixture models through a Markov Chain Monte Carlo (MCMC) sampling algorithm on each independent raw data matrix and obtain $poe(\hat{p}^*)$ matrices by Bayes rule:

$$\underset{(-\infty,\infty)}{\log_2(Raw)} \begin{bmatrix} 5.8 & 4.2 & . & . \\ -1.6 & -3.1 & . & . \\ . & . & . & . \\ . & . & . & . \end{bmatrix} \rightarrow \underset{(-1,1)}{poe} \begin{bmatrix} 0.8 & 0.6 & . & . \\ -0.1 & -0.2 & . & . \\ . & . & . & . \\ . & . & . & . \end{bmatrix}$$

↓

Stage2

Combine data on *poe* scale.

↓

Build prognostic signature on combined data matrix.

(Can be generalized to any type of microarray data analyses such as discriminant analysis, gene screening, class identification and prediction, etc.)

Figure 1
Meta-analysis of microarray data using a two-stage mixture model approach.

Table 1: Breast cancer gene expression data sets used in the prognostic meta-analysis. Bad outcome (Y = 1) is defined as recurrence during follow-up, and good outcome (Y = 0) is defined as remaining recurrence-free for at least three years.

Authors	Array platform	Number of array elements	Sample size (n)	Good outcome (n_0)	Bad outcome (n_1)
Sorlie *et al.*	Spotted cDNA	8102	58	23	35
van't Veer *et al.*	Inkjet oligonucleotide	25000	78	44	34
Sotiriou *et al.*	Spotted cDNA	7650	98	53	45
Huang *et al.*	Affymetrix chip	12625	71	36	35

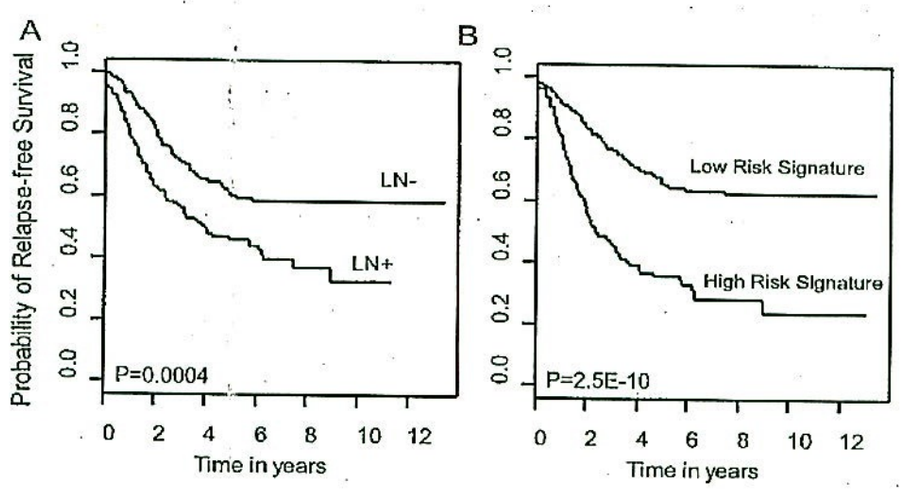
of the heterogeneity of the combined patient population, the meta-signature predicted the odds of recurrence for a patient showing a high risk signature as four times of the odds of recurrence for a patient showing a low risk signature. Several studies have implicated that the lymph node status is one of the principal clinical factors to classify patients in relation to the risk of relapse of breast cancer [14-16]. Although there have been controversial findings with regard to its predictive values in breast cancer survival outcome, we have shown in the meta-cohort that the nodal status is a significant risk factor of recurrence. The estimated odds of recurrence for node-positive patients is two times higher than the odds of recurrence for node-negative patients (95% CI:1.3–3.2, P = 0.002) in the combined samples.

Kaplan-Meier analysis provides further evidence that the meta-signature was a significant prognostic index of breast cancer recurrence in the meta-cohort (Figure 2). The estimated three-year survival rate was 76.0%(± 3.2%) for low risk signature and 45.9%(± 4.5%) for high risk signature. Nodal status, on the other hand, was less discriminative at the three-year time point with an estimated survival rate of 71.7%(± 3.7%) for lymph node negative patients and 56.2%(± 4.0) for lymph node positive patients. Node-negative patients, although generally considered to be at low risk of recurrence, are heterogeneous in disease progression. About one third of node-negative patients develop local recurrence [17]. Many studies have therefore explored the potential of using molecular biomarkers to further differentiate patient survival outcome in nodal negative cohort [18-21]. As shown in Figure 2C and 2D, the meta-signature further differentiated 48 (31.6%) of the LN- patients to be at higher risk of recurrence during followup ($P < 0.0001$). Similarly for nodal positive patients, a cohort thought to be at high risk of recurrence, the meta-signature identified 79 (51.6%) of the LN+ patients to have, in fact, lower recurrence risk over time ($P < 0.0001$, Figure 2D). In contrast, nodal status failed to maintain its predictive power after controlling for the meta-signature risk groups ($P = 0.05$ and 0.12 in low risk signature and high risk signature group respectively). A multivariate logistic regression model suggested that the meta-signature is an independent predictor of the recurrent status with respect to nodal status in the meta-cohort (OR = 3.7(2.3–6.1), $P < 0.0001$).

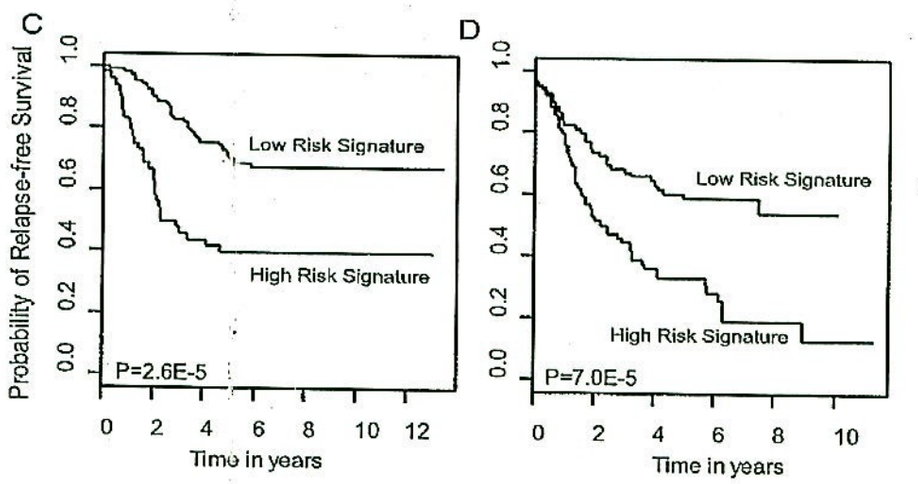
Comparison of the meta-signature to the study-specific signatures

To comprehend the potential gains of such two-stage meta analysis over individual analysis in each single study cohort, we constructed study-wise gene expression signatures using the same method. By minimizing the misclassification errors, we obtained a signature consisting 10, 60, 100, and 130 genes for Sorlie, van't Veer, Sotiriou, and Huang study cohort respectively (Additional file 5). The results of the classifiers are summarized in Table 2. In fact, not only did the size of the study-specific signatures vary significantly, but the elements of the signatures had very little overlap. At most two genes appeared in more than one signature among the four. In addition, signature identified in one study tended to have poor performance in other studies. Table 3 lists the estimated odds ratios for disease outcome and risk groups predicted by a gene expression signature. An individual signature identified in one study cohort demonstrated considerable shrinkage in the odds ratio estimates and non-significant 95% confidence intervals in the validation studies, indicating significantly reduced discriminative power in the testing cohorts. Kaplan-Meier analysis provided further evidence that the study-specific signatures performed poorly in pairwise cross-validations (Additional file 6).

Meta-analysis accounts for such heterogeneity of the individual signatures in two ways. First its overlap with the study-specific signatures ranged from 3–40%. The excluded genes are likely to be cohort-specific findings that can not be replicated. Second, the meta-signature recruited 41 genes not previously picked by any of the single cohort signature, likely representing predictive features with small but consistent effects previously masked in single studies. When examining the performances of the gene signatures, the meta-signature showed a comparable or better performance compared with the individually optimized signatures both in the odds ratio estimates (Bottom row of Table 3) and in Kaplan-Meier analysis (Figure 3). This shows that the meta-signature can serve as a common breast cancer recurrence index that is able to predict



	All (n=305)			All (n=305)	
	Recurred	Censored		Recurred	Censored
Lymph node negative	61	91	Low risk signature	65	118
Lymph node positive	88	65	High risk signature	84	38



	LN- cohort (n=152)			LN+ cohort (n=153)	
	Recurred	Censored		Recurred	Censored
Low risk signature	32	72	Low risk signature	33	46
High risk signature	29	19	High risk signature	55	19

Figure 2
The 90-gene meta-signature displayed greater performance than nodal status in predicting relapse-free survival in breast cancer, and it further predicts survival outcome in nodal status sub-cohorts. (A) Lymph node status correlates with survival outcome ($P = 0.0004$). (B) The meta-signature correlates with survival outcome ($P = 2 \times 10^{-10}$). (C) The meta-signature differentiates risk groups in nodal negative patients ($P = 2.6 \times 10^{-5}$). (D) The meta-signature predicts risk groups in nodal positive patients ($P = 7.0 \times 10^{-5}$).

Table 2: Comparisons of the number of genes (Size), the number of elements overlap with the meta-signature (overlap), and the prediction error rates for the signatures identified in individual study cohort and in the meta-cohort.

	Sorlie	van't Veer	Sotiriou	Huang	Meta-cohort
Size	10	60	90	140	90
Overlap	4	14	19	6	–
Prediction error rate	0.28	0.29	0.35	0.18	0.33

Table 3: Comparison of the performances of the individual signatures and the meta-signature in each single study cohort. Table lists odds ratios (95% confidence interval) comparing the odds of actual recurrence for those being classified as high risk to the odds of recurrence for those being classified as low risk of recurrence by each signature.

Signature	Cohort			
	Sorlie (n = 58)	van't Veer (n = 78)	Sotiriou (n = 98)	Huang (n = 71)
Sorlie (D = 10)	**18.6 (5.0, 69.5)**	2.1 (0.8, 5.4)	2.3 (1.0, 5.3)	10.87 (3.5, 33.8)
van't Veer (D = 60)	3.1 (1.1, 9.2)	**10.6 (3.3, 33.9)**	4.1 (1.7, 9.7)	1.3 (0.5, 3.4)
Sotiriou (D = 100)	1.7 (0.6, 5.0)	3.5 (1.4, 8.9)	**7.8 (3.0, 20.1)**	1.5 (0.6, 3.7)
Huang (D = 130)	5.1 (1.6, 15.7)	2.3 (0.9, 5.6)	0.9 (0.4, 2.0)	**184.9 (30.1, 1137.2)**
Meta (D = 90)	**25.0 (4.2, 149.0)**	**4.1 (1.6, 10.6)**	**6.0 (2.5, 14.5)**	**5.8 (2.1, 16.5)**

D is the number of genes in a signature. n is the sample size for each cohort.

patient survival in heterogeneous sample populations. When a gene signature built in one study cohort performs differently in another, such meta analysis provides a solution to identify a cross-study validated expression signature that holds across independent samples.

Comparison of data integration based on poe transformation and simple linear rescaling

An alternative approach to integrating data across multiple datasets is to perform a study-wise global normalization. For one study, let $x_{ij}^{*} \equiv (x_{ij} - \bar{x})/s.d.(x_{ij})$ be the globally scaled expression value for gene j in sample i. Each study dataset is then standardized to have zero mean and unit standard deviation. The linearly rescaled values can also be used for data integration purposes in that expression values generated from different array platforms are standardized to a common scale.

Such an approach is less computationally challenging compared to the mixture model-based rescaling described in the previous sections. However, there are several advantages to the mixture model-based transformation. First, the method incorporates biological information into estimating the posterior probabilities of expression. The transformed values carry meaningful interpretations as signed probabilities of differential expression of a gene in a particular sample. Second, the underlying normal and uniform mixture distributions give equal density in the tails and is effective in reducing the influence of extreme expression values. And third, the Bayesian hierarchical modeling approach borrows strength across genes resulting in shrinkage-type estimators for a large correlated gene-specific parameter vector. This is a method in which the high dimensional gene expression data are denoised.

To study the benefit of data integration based on *poe* compared to that based on the linearly rescaled values, we compared the model performances based on data integration by these two methods. Figure 4A shows that with the *poe* transformation, misclassification rates steadily decreases as more genes are used in the classifier. Performance based on linearly rescaled data (Figure 4B), however, is unpredictable. Figure 4C and 4D uses a 90-gene meta-signature based on *poe* and based on the global standardization respectively in predicting survival. The signature based on *poe* is noticeably better than the signature based on global standardization in differentiating patients at low risk of recurrence from those at high risk of recurrence. Taken together, the *poe* transformation outperforms the linear rescaling method in combining multiple microarray data sets. The meta-signature identified based on *poe* values therefore offers more reliable prediction of recurrence-free survival in the meta-cohort.

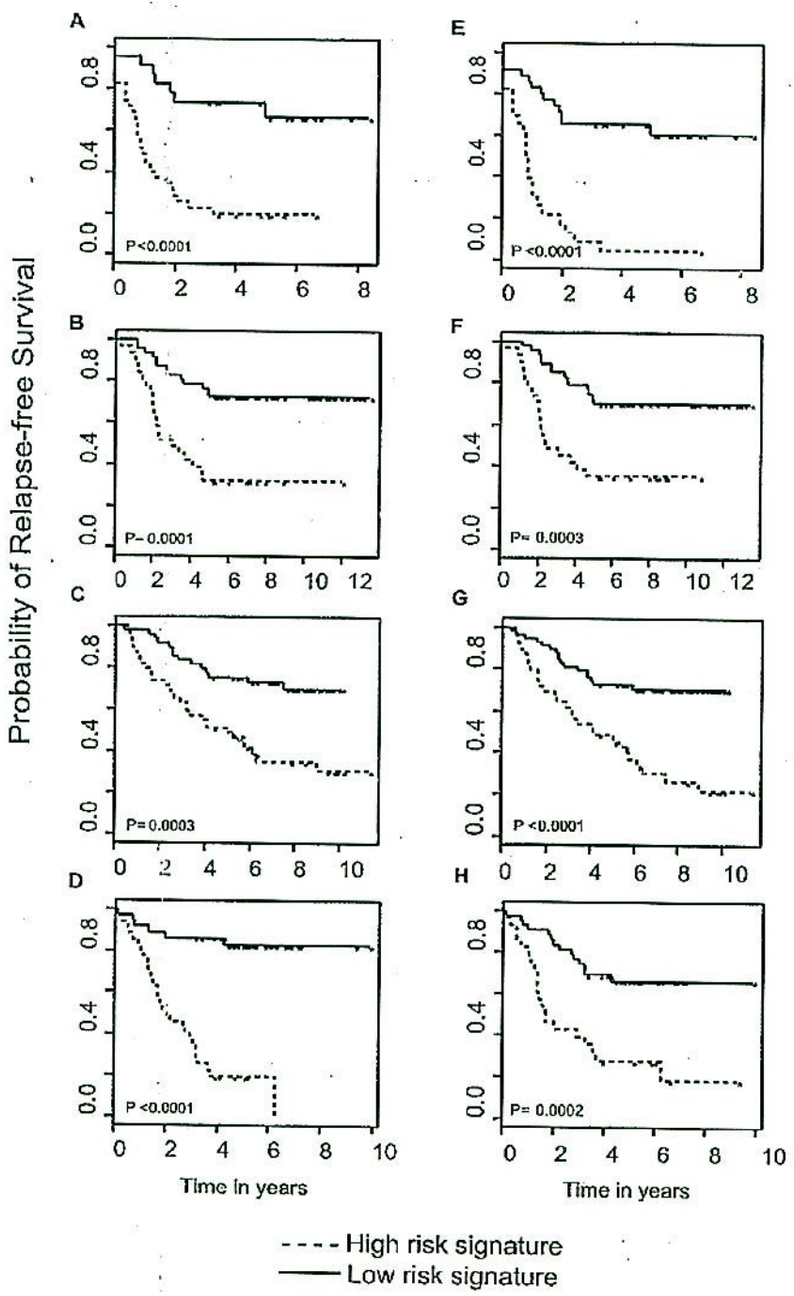


Figure 3
The 90-gene meta-signature achieves similar or better performance than the individually optimized signatures. A and E compare the Kaplan-Meier curves stratified by high versus low risk group predicted by the study-specific signature and by the meta-signature respectively in the Sorlie study cohort; B and F show similar comparison in the van't Veer study cohort; C and G show similar comparison in the Sotiriou study cohort; and D and H show comparison in the Huang study cohort.

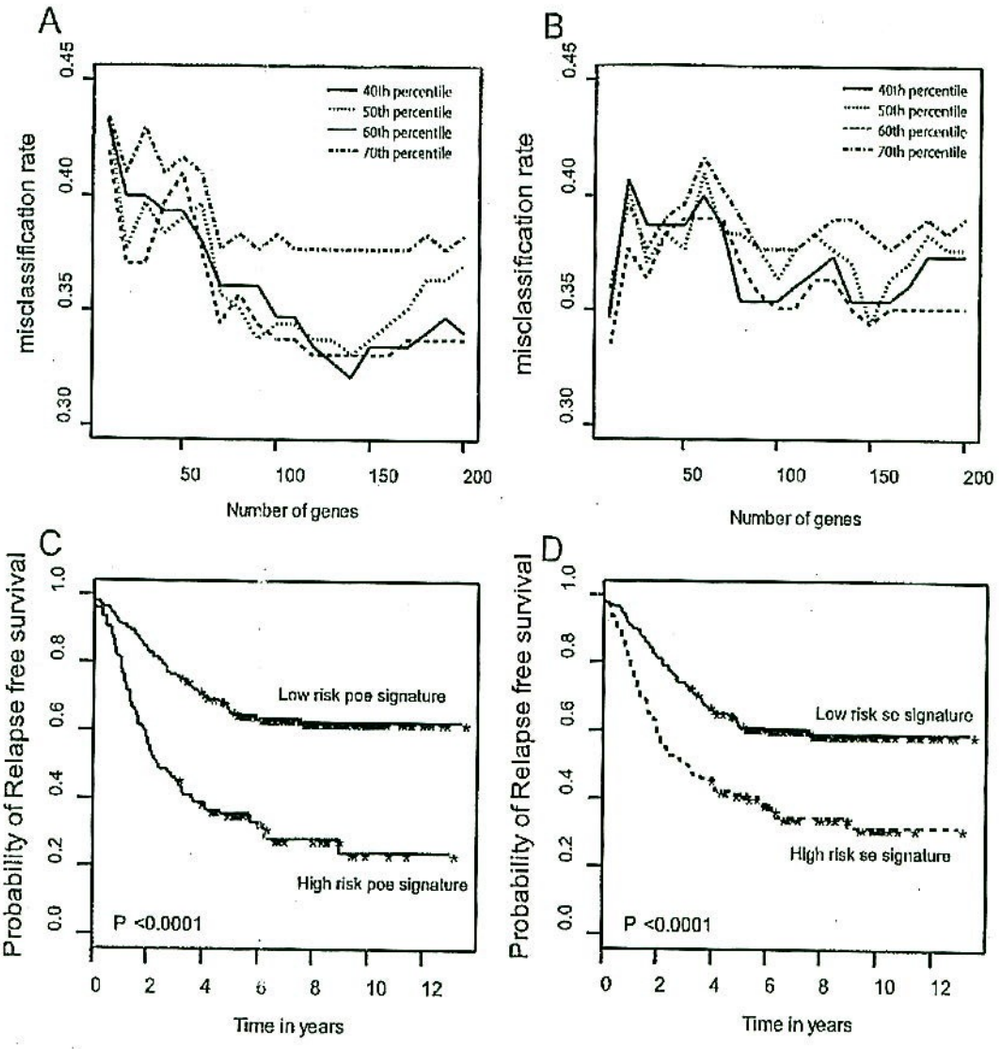


Figure 4
Comparison of model performances based on data integrated by poe transformation (A and C) and global standardization (B and D). A set of top 10 to 200 genes were used in a classifier to construct risk index and 40th to 70th percentiles of the cross-validated RIs were then used to dichotomize samples into a high risk or a low risk group. A. Misclassification rates based on poe transformation and B. based on global standardization. C. Performance of the 90-gene signature built on poe and D. built on global standardized data in differentiating patients at low risk of recurrence from those at high risk of recurrence.

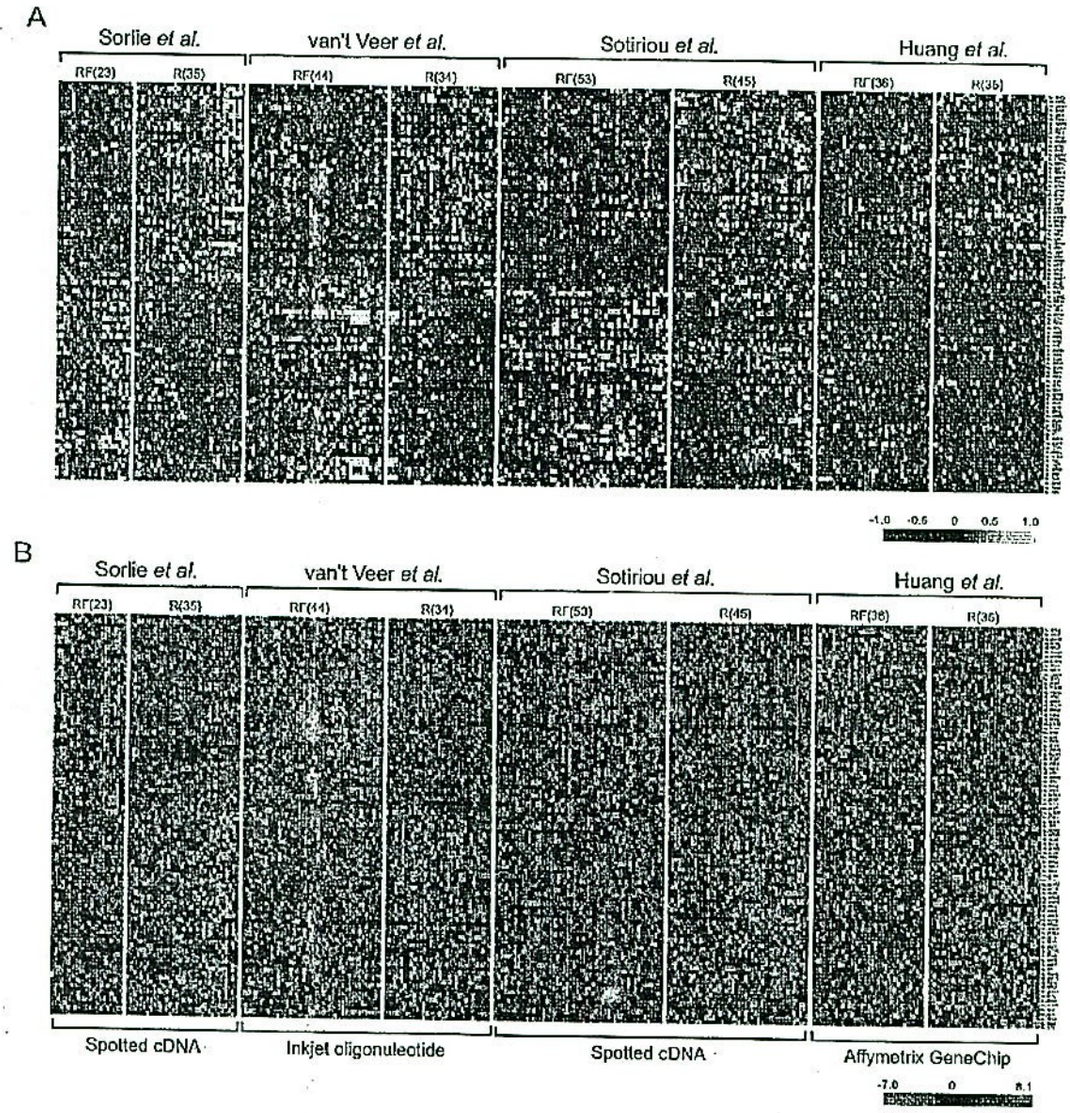


Figure 5
The 90 gene meta-signature displayed two distinct patterns of expression in breast cancer groups. (A) Heat map representation of differential expression probabilities for the 90 gene meta-signature across the combined samples. The top set of genes showed consistently high probability of over-expression (yellow) in the poor outcome group, and the bottom set of genes showed consistently high probability of down-regulation (blue) in the poor outcome group. (B) Heat map of log-transformed raw data. Individually generated heat maps of the raw measurements of gene expression confirmed the distinct expression patterns of the meta-signature from independent studies. Red represents up-regulation while green represents down-regulation. R (recurred) – poor outcome group; RF (recurrence-free) – good outcome group.

The meta-signature displays two distinct expression patterns

A heat map representation of the *poe* profile for the 90 gene meta-signature revealed two distinct patterns of differential expression (Figure 5A). Genes in the top half of the matrix displayed consistently high probability of over-expression (yellow) in the recurrent samples (R). On the other hand, genes in the bottom half displayed great probability of under-expression (blue) in the recurrent group. Individually generated heat maps of the raw data confirmed such distinct patterns at raw measurement levels (Figure 5B). Functional annotation revealed genes involved in many important biological processes such as cell cycle regulation (e.g., CDC28 protein kinase regulator subunit 2), cell adhesion (e.g., chemokine C-X3-C motif receptor 1), and apoptosis (e.g., secreted frizzled-related protein 4). A complete list of the meta-signature genes can be found in the Additional file 7. Some of the genes in the meta-signature were previously shown to correlated with breast cancer survival outcome. For example, Keyomarsi et al. [22] demonstrated the association of the cell cycle regulator cyclin E and death due to breast cancer.

Enriched functional classes in the meta-signature

To gain a better understanding of the processes related to disease recurrence, we examined whether a particular functionally defined biological process is enriched in the recurrence signature. Each of the ninety genes were mapped to Gene ontology (GO) terms and then grouped by functional classes. Based on the hypergeometric distribution, we calculated the significance of over-representation of a particular process in the signature. Figure 6 demonstrated the top seven enriched functional groups in the meta-signature, comparing the total proportion (out of 2310 annotated) and the signature proportion (out of 85 annotated) of genes in each group. Cell cycle regulation is the most highly over-represented category ($P = 0.001$). All genes under this category except BCL2 displayed increased expression level, reflecting elevated cell cycle activities. Signal transduction represents the largest functional class over-represented in the meta-signature. Genes involved in signalling pathways that regulate cell growth (VEGF, PPP2R5C), immune response (TRAF3), apoptosis (SFRP4), and other processes are found to constitute 15.7% of the meta-signature compared to the 9.7% in the entire gene set (the common set).

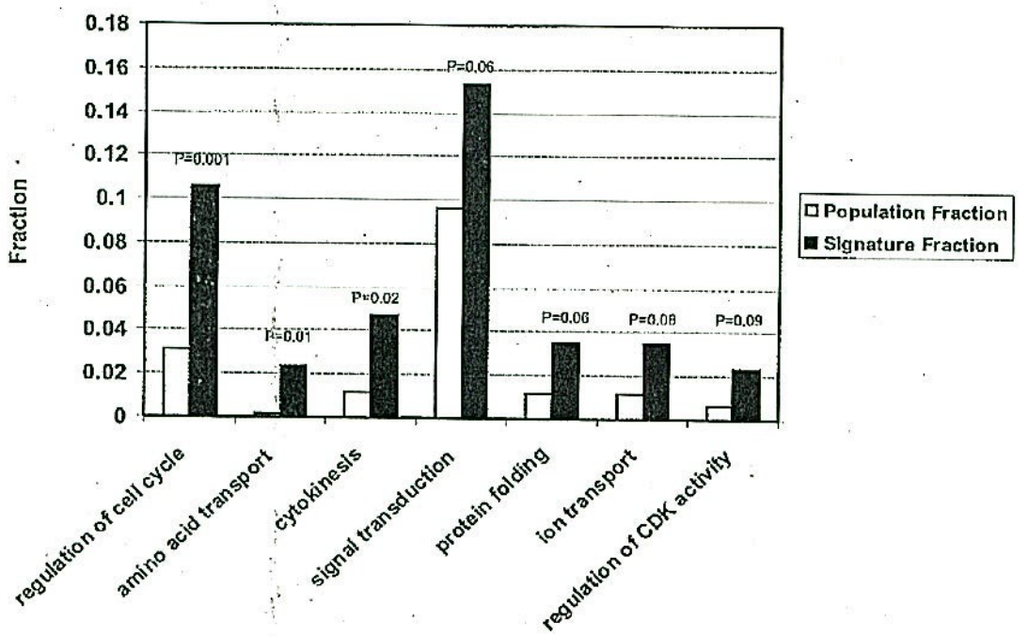
Discussion

Several important issues to consider when integrating microarray studies include use of different gene expression measurement scales, varying analytical power and reliability of the results for individual studies. To account for these issues, we proposed a two-stage mixture modeling strategy, the strength of which was built on the mixture model based transformation and the subsequent data integration on the *poe* scale. In particular, *poe* provides a unified platform-free scale, and simultaneously enhances the intrinsic contrast in the expression data. Furthermore, combining sample pools on the *poe* scale mitigates the influence of potential artifacts from a single study. The benefit of such data integration is reflected on two counts. One, integrated sample cohorts improve the reliability of the findings by guarding against false positive results from a single study. Two, it increases the statistical power to detect small consistent effects that can be otherwise masked by inadequacy of the sample size of an individual data set. By implementing this modeling approach, we were able to combine information from four microarray studies to build an inter-study validated meta-signature for predicting survival in breast cancer patients.

As described earlier, a common set of 2555 genes was used in this meta-analysis, as it is important to provide the same context for data-driven estimation of the posterior probabilities. Although we assume the common set comprises the most biologically relevant genes, the loss of potential predictive genes, however, may offset the statistical power of the analysis. For example, one of our recent studies has established the polycomb protein EZH2 to be an independent predictor of breast cancer survival outcome[23]. This gene was filtered out of the meta-analysis as one of the studies [12] did not profile EZH2. However, in each of the other three studies where EZH2 was profiled on the array, its expression level was found to correlate with survival (data not shown), which confirmed its role as a prognostic marker. Alternative approaches to allow genes profiled in some studies but not others is a topic for future research.

Functional annotation of the meta-signature revealed genes such as Cyclin E and BCL2, which were previously shown to be correlated with survival outcome in breast cancer [22,24]. A strength of the inter-study validated signature is the capability of recruiting genes which may not be significant in one study due to limiting sample size or artifacts of the experiments. In this sense, the meta-signature will be more stable and less subjective to variations in subsets of the samples. As a result, the predictive genes in a meta-signature may carry more reliable information about tumor progression and patient survival.

In conclusion, a distinction of the analysis presented here relative to those by other authors [3,6] is that we sought to find genes that were predictive of recurrence rather than predictive of diseased versus nondiseased status. Given the heterogeneity of the tumors with respect to treatment response and survival outcome, a prognostic prediction analysis is generally more difficult because it is a more complicated phenotype. Further, a prognostic signature (classifier) of failure risk trained in one cohort is often



Regulation of cell cycle	Amino acid transport	Cytokinesis	Signal transduction	Protein folding	Ion transport	Regulation of CDK activity
TFDP1	SLC3A2	PPP1CB	TXNRD1	FKBP1A	GABRD	CKS2
PCTK1	SLC12A5	CKS2	VEGF	CCT7	SLC21A3	CDKN3
VEGF		CCNE1	TRAF3	CCT4	SLC12A5	
CCT7		CCNC	GABRD			
MYBL2			PPP2R5C			
CCT4			SFRP4			
CCNE1			INSR			
CCNC			ADRA2A			
BCL2			SCAP1			
			GUCA1A			

Figure 6
Top seven over-represented functional classes in the meta-signature. Black bars represent proportion of genes associated with each of the GO terms among the meta-signature, and white bars represent the corresponding proportion among the total study population of 2555 genes. P-value represents the significance of over-representation based on a hypergeometric distribution, and is calculated as the probability of observing larger proportion of a particular functional group genes in the meta-signature than in the entire gene set. The meta-signature genes are listed under each functional class.

times difficult to validate in independent cohorts. The meta-analysis method presented here may potentially provide more powerful gene signatures that are predictive of prognosis because they are validated across multiple studies.

Methods

Data collection and preparation

The breast cancer microarray data sets were obtained at the author's websites from four recently published studies [10-13]. Each data were preprocessed, either by a lowess

normalization for two-channel microarray data [25] or a robust analysis for Affymetrix data [26]. We filtered for a common set of 2,555 genes from these four studies by Unigene Cluster IDs. Each data matrix of the 2,555 genes was then normalized by median centering and dividing by the standard deviation for each gene. Missing data were imputed by the k-nearest neighbors imputation algorithm [27].

Mixture modeling of microarray data

Each of the four raw data sets was treated as an expression matrix X with elements x_{ij}, where $i = 1, ..., m_k$, $j = 1, ..., n$ ($k = 1, .., 4$ and $n = 2{,}555$). The expression measurement x_{ij} can be the ratio of the two fluorescent dye hybridization intensities for the spotted cDNA arrays[10,12] and the Intjek oligonucleotide array [11], or averaged difference between the perfect match and mismatch probe hybridizations for the Affymetrix gene chip [13]. Let E be a latent class variable, and e_{ij} indicates over-, under- or normal expression for each entry of the R matrices. We have:

$$e_{ij} = \begin{cases} 1 & \text{gene } j \text{ is overexpressed in sample } i; \\ 0 & \text{gene } j \text{ is normaly expressed in sample } i; \\ -1 & \text{gene } j \text{ is underexpressed in sample } i. \end{cases}$$

The values of e_{ij} are latent and not directly observed from the data. We were interested in estimating the probabilities of e_{ij} being 1 or -1 given the observed raw expression x_{ij}, which were denoted as $p_{ij}^{+} = Pr(e_{ij} = 1 \mid x_{ij})$ and $p_{ij}^{-} = Pr(e_{ij} = -1 \mid x_{ij})$. Estimates of these latent quantities were obtained under a Bayesian mixture model setting. In particular, we assume the raw expression x_{ij} falls into one of the three expression categories. For each gene j, the expression then arises from a mixture of three distributions:

$(x_{ij} \mid e_{ij} = 1) \sim f_{1,j}(\cdot)$, $(x_{ij} \mid e_{ij} = 0) \sim f_{0,j}(\cdot)$, and $(x_{ij} \mid e_{ij} = -1) \sim f_{-1,j}(\cdot)$.

In the mixture, $f_{1,j}$, $f_{0,j}$ and $f_{-1,j}$ are the density functions of the following distributions:

$$U(\alpha_i + \mu_j, \alpha_i + \mu_j + \kappa_j^{1}), \quad N(\alpha_i + \mu_j, \sigma_j^2), \quad \text{and } U(-\kappa_j^{-1} + \alpha_i + \mu_j, \alpha_i + \mu_j)$$

respectively. Here, U refers to a uniform distribution and N refers to a normal distribution. $\alpha_i + \mu_j$ is both the mean of the normal distribution and the threshold point in the uniform distribution. μ_j is the gene effect and α_i is the sample effect. The κ_j^{+} and κ_j^{-} provide limits to the uniform distribution in the mixture, and are set to be at least $3\sigma_j$. $\pi_j^{+} = P(e_{ij} = 1)$ and $\pi_j^{-} = Pe_{ij} = -1)$ are the multinomial probabilities for e_{ij}. With the specifications of models, we can calculate the latent quantities by Bayes' rule:

$$p_{ij}^{+} = P(e_{ij} = 1 \mid x_{ij}) = \frac{\pi_j^{+} f_{1,j}(x_{ij})}{\pi_j^{+} f_{1,j}(x_{ij}) + \pi_j^{-} f_{-1,j}(x_{ij}) + (1 - \pi_j^{+} - \pi_j^{-}) f_{0,j}(x_{ij})} \quad (1)$$

$$p_{ij}^{-} = P(e_{ij} = -1 \mid x_{ij}) = \frac{\pi_j^{-} f_{-1,j}(x_{ij})}{\pi_j^{+} f_{1,j}(x_{ij}) + \pi_j^{-} f_{-1,j}(x_{ij}) + (1 - \pi_j^{+} - \pi_j^{-}) f_{0,j}(x_{ij})}. \quad (2)$$

By noting that the supports for the two uniform distributions are disjoint, the probabilities of differential expression are mutually exclusive with the forms:

$$(p^{+}, p^{-}) = \left(\frac{\pi^{+} / k^{+}}{\pi^{+} / k^{+} + (1 - \pi^{+} - \pi^{-}) / l_0}, \; 0 \right) \text{ or } (p^{+}, p^{-}) = \left(0, \frac{\pi^{-} / k^{-}}{\pi^{-} / k^{-} + (1 - \pi^{+} - \pi^{-}) / l_0} \right)$$

A one dimension measure can thus be constructed as $poe = p^{+} - p^{-}$. As a result, poe ranges from -1 to 1, and can be interpreted as the signed conditional probability of differential expression.

To borrow strength across genes, the estimation of the gene-specific parameters was formulated under a Bayesian hierarchical model setting. Given the large amount of parameters, prior distributions were specified to model the variation of the gene-specific parameter estimates, in particular,

$$\mu_j \sim N(0_\mu, \tau_\mu); \quad \kappa_j^{+} \sim \text{Exp}(0_\kappa^{+}); \quad logit(\pi_j^{1}) \sim N(\theta_\pi^{1}, \tau_\pi^{1});$$
$$\sigma_j^{-2} \sim \text{Gamma}(r, \lambda); \quad \kappa_j^{-} \sim \text{Exp}(\theta_\kappa^{-}); \quad logit(\pi_j^{-}) \sim N(\theta_\pi^{-}, \tau_\pi^{-}).$$

We followed the recommendations of Parmigiani et al. [8] in terms of the prior choices. A Metropolis-Hastings MCMC sampling algorithm was then implemented to approximate the posterior distributions of the parameters. Data augmentation started at a set of data-driven initiating parameter values. For example, trimmed means and variances across samples were used as the initial values for the parameters in the normal distribution of the mixture. Further details of the Bayesian hierarchical mixture model used here can be found in Parmigiani et al. [8].

Matrices of $poe(p^{*} = p^{+} - p^{-})$ were obtained for each of the five data sets (Additional files 1, 2, 3, 4).

Leave-one-out cross validation and risk index computation

For the combined sample pool of the breast cancer patients (the meta-cohort), we defined outcome groups as recurred during followup and remained relapse-free for at least 3 years. In particular, Let T_i be the event time for subject i, C_i be the censoring time for subject i, and $\delta_i = 1\{T_i$

$<C_i\}$ be the censoring indicator. Define a new outcome variable,

$$\gamma_i = \begin{cases} 1, & \delta_i = 1 \\ 0, & \delta_i = 0 \text{ and } C_i \geq t^*, \end{cases}$$

where t^* can be specified with clinical knowledge. We chose $t^* = 3$ years in this study. We then consider constructing classifiers using γ; note that $\gamma = 1$ corresponds to the poor outcome group and $\gamma = 0$ to the good outcome group. The sample sizes for each study are shown in Table 1.

Logistic regression was used to build a classifier for prognosis. For each gene j, we fit the following univariate logistic regression model using data from all studies:

$$\text{logit}\{Pr(\gamma_i = 1 | x_{ij}^*)\} = \eta_j + \beta_j x_{ij}^*,$$

where x^* is the rescaled value that allows data integration across multiple studies. The esti-mated values of β_j, $\hat{\beta}_j$, are then used to form a risk score using a variation of the compound covariate predictor method [28,29]; for a given set of covariate values $x_1, \ldots, x_D$, the risk index is given as $RI = \sum_{j=1}^{D} \hat{\beta}_j x_j$.

If we want to assess the performance of the classifier, we must deal with the issue of training and testing the model using the same data. An "honest" estimate of the prediction error rate is obtained using leave-one-out cross-validation. Define a risk index $RI_i = \sum_{j=1}^{D} \hat{\beta}_{j,-i} x_{ij}^*$, where $i = 1, \ldots, \sum_{i=1}^{K} m_k$, and $\hat{\beta}_{j,-i}$ is the effect estimate for gene j in the combined meta-cohort without the i^{th} sample. The risk index for sample i is a weighted linear combination of the expression profiles of the top D genes, where the ranking of the genes is based on their corresponding significance in the univariate logistic model fit. Classification of sample i to the risk groups is then based on the i^{th} leave-one-out risk index, i.e., $C(X^*) = I\{RI_i > c\}$ with c being the empirical quantiles (40^{th} - 70^{th}) of the RI's. The number of genes D in a classifier is treated as a parameter and optimized to minimize the prediction error rates.

The list of the top cumulative genes in the meta-signature was obtained by ranking all 2,555 genes by the number of times in the leave-one-out cross-validation steps that each one had a P-value from the univariate logistic regression less than 0.001.

Heat map display

We used the treeview software [30] to generate a heat map representation of the *poe* pro-files of the meta-signature. Yellow represents high probability of over-expression and blue represents high probability of under-expression. For heat maps of raw data matrices, we preprocessed the data by mean centering and then dividing by the standard deviation for each row. The means and the standard deviations used in the normalization were the relapse-free (RF) sample means and variances for each study data. The values for the recurrence (R) samples after standardizing then represented the number of standard deviations over or under the mean RF sample expression.

Authors' contributions

RS, DG, and AC designed the study. RS carried out the statistical analysis and prepared the manuscript. DG and AC supervised the analysis and participated in the manuscript preparation. All authors read and approved the final manuscript.

Additional material

Additional File 5

Plots of misclassification rates. The PDF file lists plots of misclassification error rates for classifiers identified in each individual study cohort and the meta-cohort.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-5-94-S5.PDF]

Additional File 6

Plots of Kaplan-Meier curves. The PDF file lists Kaplan Meier plots for study-wise cross-validation of the individually identified signatures. A gene signature was trained in one study cohort and used to validate in each of the other study cohorts as testing sets.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-5-94-S6.PDF]

Additional File 7

Meta-signature gene list. The excel file contains a list of Unigene ID, gene symbol, and full name of the 90 genes in the meta-signature.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-5-94-S7.xls]

Additional File 1

POE imputation of the Sorlie data. The excel file contains a table of imputed signed probability matrix transformed from the Sorlie et al. study data (2,555 times 58 in dimension).
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-5-94-S1.xls]

Additional File 2

POE imputation of the van't Veer data. The excel file contains a table of imputed signed probability matrix transformed from the van't Veer et al. study data (2,555 times 78 in dimension).
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-5-94-S2.xls]

Additional File 3

POE imputation of the Sotiriou data. The excel file contains a table of imputed signed probability matrix transformed from the Sotiriou et al. study data (2,555 times 98 in dimension).
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-5-94-S3.xls]

Additional File 4

POE imputation of the Huang data. The excel file contains a table of imputed signed probability matrix transformed from the Huang et al. study data (2,555 times 71 in dimension).
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-5-94-S4.xls]

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Prognostic meta-signature of breast cancer developed by two-stage mixture modeling of microarray data

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Abstract

Background: An increasing number of studies have profiled tumor specimens using distinct microarray platforms and analysis techniques. With the accumulating amount of microarray data, one of the most intriguing yet challenging tasks is to develop robust statistical models to integrate the findings.

Results: By applying a two-stage Bayesian mixture modeling strategy, we were able to assimilate and analyze four independent microarray studies to derive an inter-study validated "meta-signature" associated with breast cancer prognosis. Combining multiple studies (n = 305 samples) on a common probability scale, we developed a 90-gene meta-signature, which strongly associated with survival in breast cancer patients. Given the set of independent studies using different microarray platforms which included spotted cDNAs, Affymetrix GeneChip, and inkjet oligonucleotides, the individually identified classifiers yielded gene sets predictive of survival in each study cohort. The study-specific gene signatures, however, had minimal overlap with each other, and performed poorly in pairwise cross-validation. The meta-signature, on the other hand, accommodated such heterogeneity and achieved comparable or better prognostic performance when compared with the individual signatures. Further by comparing to a global standardization method, the mixture model based data transformation demonstrated superior properties for data integration and provided solid basis for building classifiers at the second stage. Functional annotation revealed that genes involved in cell cycle and signal transduction activities were over-represented in the meta-signature.

Conclusion: The mixture modeling approach unifies disparate gene expression data on a common probability scale allowing for robust, inter-study validated prognostic signatures to be obtained. With the emerging utility of microarrays for cancer prognosis, it will be important to establish paradigms to meta-analyze disparate gene expression data for prognostic signatures of potential clinical use.

Introduction

DNA microarray analysis has been shown to be a powerful tool in various aspects of cancer research [1]. With the increasing availability of published microarray data sets,

A molecular correlate to the Gleason grading system for prostate adenocarcinoma

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Adenocarcinomas of the prostate can be categorized into tumor grades based on the extent to which the cancers histologically resemble normal prostate glands. Because grades are surrogates of intrinsic tumor behavior, characterizing the molecular phenotype of grade is of potential clinical importance. To identify molecular alterations underlying prostate cancer grades, we used microdissection to obtain specific cohorts of cancer cells corresponding to the most common Gleason patterns (patterns 3, 4, and 5) from 29 radical prostatectomy samples. We paired each cancer sample with matched benign lumenal prostate epithelial cells and profiled transcript abundance levels by microarray analysis. We identified an 86-gene model capable of distinguishing low-grade (pattern 3) from high-grade (patterns 4 and 5) cancers. This model performed with 76% accuracy when applied to an independent set of 30 primary prostate carcinomas. Using tissue microarrays comprising >800 prostate samples, we confirmed a significant association between high levels of monoamine oxidase A expression and poorly differentiated cancers by immunohistochemistry. We also confirmed grade-associated levels of defender against death (DAD1) protein and HSD17β4 transcripts by immunohistochemistry and quantitative RT-PCR, respectively. The altered expression of these genes provides functional insights into grade-associated features of therapy resistance and tissue invasion. Furthermore, in identifying a profile of 86 genes that distinguish high- from low-grade carcinomas, we have generated a set of potential targets for modulating the development and progression of the lethal prostate cancer phenotype.

carcinoma | monoamine oxidase A | microarray | expression profile

The prognosis and choice of therapy for prostate cancer is based primarily on three parameters obtained at the time of diagnosis: clinical stage, serum prostate-specific antigen (PSA), and the Gleason score of the cancer (1). The Gleason grading system, which is based on microscopic tumor architecture, consists of five histological patterns that annotate cancers into categories exhibiting well differentiated (pattern 1) to poorly differentiated (pattern 5) features (2, 3). A number from 1 to 5 is assigned to the most prevalent pattern. A second number, also from 1 to 5, is assigned to the second most prevalent pattern. The Gleason grade, which is the sum of these two numbers, has a value between 2 and 10. In current practice, the vast majority of prostate cancers have a Gleason score of ≥6 (4, 5). Hence, tumors composed of patterns 3, 4, and/or 5 are considered clinically significant. The reporting of individual Gleason patterns is not a trivial distinction, because the amount of pattern 4 and the presence of any pattern 5 has been highly correlated with probability of cancer dissemination, response to therapy, disease outcome, patient-management decisions, and clinical-trial enrollment (6, 7). Numerous studies have demonstrated a direct correlation between Gleason score and clinical measurements of disease outcome, including death due to tumor within 15 years and likelihood of remaining free of biochemical evidence of disease recurrence after either definitive, potentially curative radical prostatectomy or radiation therapy (8). Although different Gleason patterns are histologically distinctive, the molecular features underlying these tumor phenotypes are not precisely defined. In this study, we sought to characterize the molecular profile of prostate carcinomas of specific Gleason patterns. Tumor cell transcript levels were used to develop a model capable of distinguishing the low-grade (pattern 3) from high-grade (patterns 4 and 5) tumors. We validated the predictive power of the model by using an independent set of primary prostate cancers and confirmed grade-associated differences in protein-expression levels using a third sample cohort. Together, these results identify both consistent and divergent features of the molecular framework that underlies the histological classification of cancer grade.

Results and Discussion

Gene-Expression Profiles Associated with Prostate Cancer Grades. To identify molecular alterations correlating with histological tumor grade, we used laser-capture microdissection to exclusively acquire the epithelial cell component of prostate carcinoma foci corresponding to individual Gleason pattern 3, 4, or 5 cancers. After linear amplification, gene-expression alterations in tumor cells were measured by cDNA microarray hybridizations in a head-to-head fashion against patient-matched microdissected benign secretory epithelial cells. A total of 121 benign and neoplastic samples from 59 radical prostatectomies and 30 prostate needle core biopsies contributed to the analysis.

To assess the generalized applicability and consistency of the methods, we first sought to identify consistent prostate cancer-associated transcript alterations irrespective of grade. Using microdissected epithelium from radical prostatectomy samples, we identified 736 genes with altered expression levels between benign and neoplastic epithelium (false discovery rate <0.01%). This cohort included several genes previously reported to be differentially expressed in prostate cancers, such as *hepsin*, *AMACR*, and *CAMKK2* (Fig. 1*A*) (9–12). These findings provided validation that our methods replicated results of earlier expression-profiling studies, despite using the very small sample quantities obtained by laser microdissection and subjecting the

Conflict of interest statement: No conflicts declared.

Abbreviations: IHC, immunohistochemical; MAOA, monoamine oxidase A; PAM, prediction analysis for microarrays; TMA, tissue microarray.

Data deposition: The microarray data sets reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database (accession no. GSE5132).

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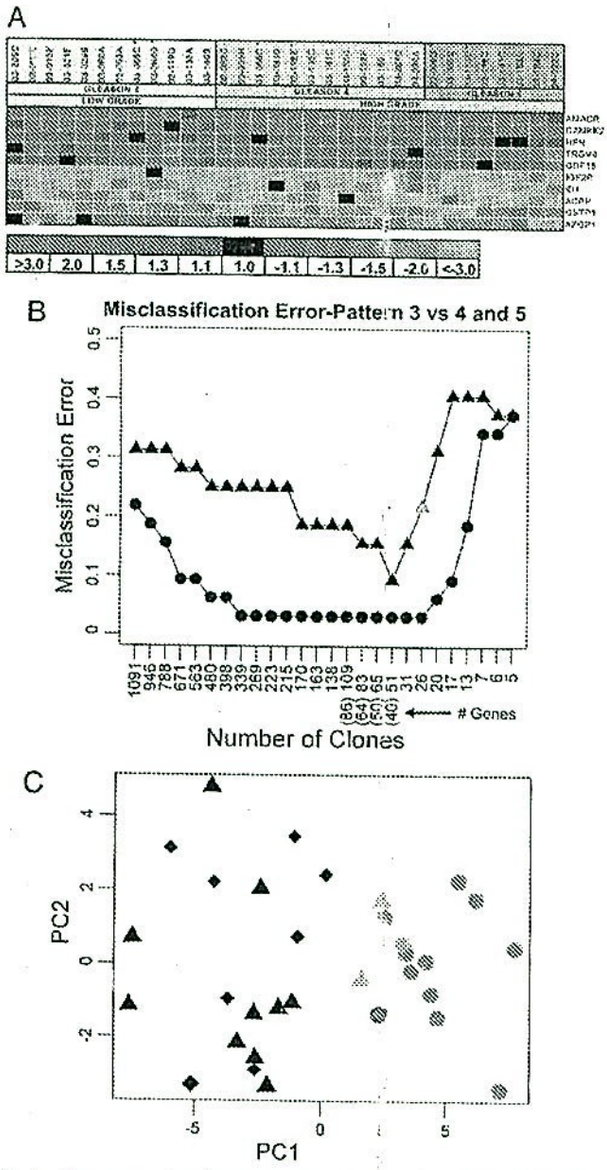


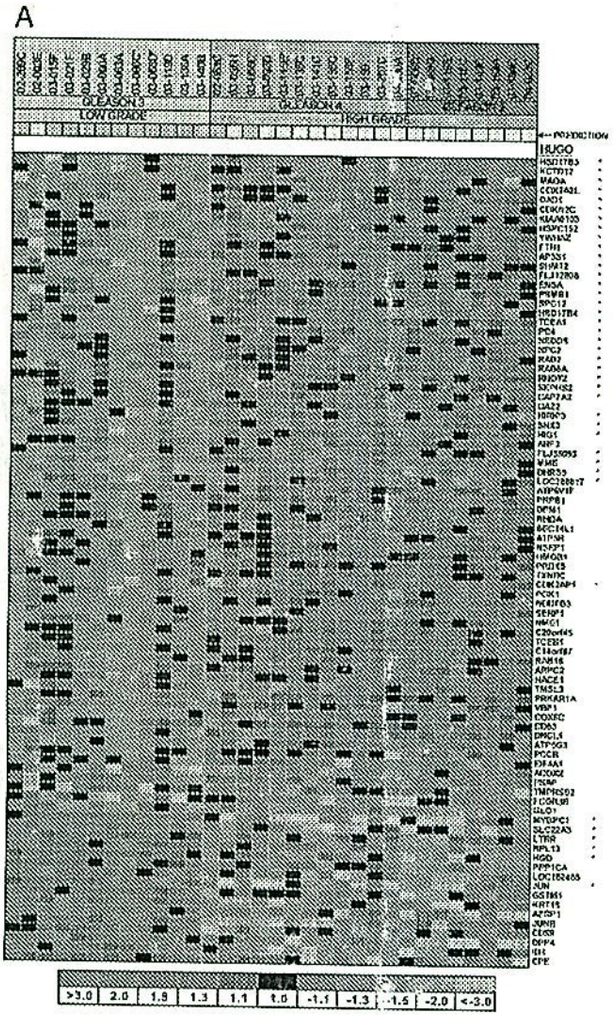
Fig. 1. Gene-expression changes associated with prostate cancer grade. (*A*) Genes differentially expressed between microdissected benign and neoplastic prostate epithelium were identified in a supervised analysis of transcript profiles of 32 matched samples from 29 individuals. A Statistical Analysis Of Microarrays (SAM) one-sample *t* test comparing cancerous epithelium to matched benign epithelium across all Gleason patterns identified 736 differentially expressed genes (false discovery rate ≤0.05%). Cancer-associated transcript alterations for several genes with previously described alterations in prostate carcinoma are shown. (*B*) Identification of gene-expression alterations predictive of Gleason pattern. PAM analysis of gene-expression profiles generated from microdissected Gleason pattern 3, 4, and 5 cancers. Circles represent the training error. Triangles represent the leave-one-out cross-validation error. The *x* axis shows the number of array clones (and corresponding unique genes) used for classification. (*C*) Principal-components analysis of grade-specific prostate cancers using the 40-gene (51-clone) Gleason pattern classifier. For each sample, the score on the first (*x* axis) and second (*y* axis) principal component is plotted. Gleason pattern 3 (circle) samples are generally distinct from Gleason pattern 4 and 5 sample space (triangle and diamond, respectively). Pattern 4 and 5 samples were intermixed, indicating a high degree of molecular similarity. Three misclassified samples (under cross-validation of the 40-gene model) between pattern 3 and pattern 4/5 cancers are denoted in blue.

RNA from these samples to amplification and transcript quantitation by microarray analysis.

To identify gene-expression alterations that associated with specific Gleason patterns, we used a supervised-learning approach, Prediction Analysis for Microarrays (PAM) that represents a modification of standard nearest-neighbor classification (13). Models comprising 40, 64, and 86 genes were able to partition Gleason 3 vs. Gleason 4 and 5 cancers with 90%, 84%, and 81% accuracy (under leave-one-out cross-validation) (Fig. 1*B*). We were unable to identify a cohort of genes that could distinguish between pattern 4 and 5 cancers with sufficiently high accuracy to be useful, suggesting a high degree of similarity between these cancer histologies or substantial molecular heterogeneity in one or both of these groups. Therefore, we grouped pattern 4 and 5 cancers together for subsequent comparisons. Principal-components analysis of the 40-gene model demonstrated segregation of cancers according to histological grade, with only one pattern 4/5 cancer misclassified as a pattern 3, and two pattern 3 cancers misclassified as pattern 4/5 (Figs. 1*C* and 2*A*). Rereview of the histology and the specific composition of cells acquired by laser-capture microdissection did not alter the histological classification of these samples. Although Gleason patterns can be reproducibly categorized (14), there are clear examples of heterogeneity in clinical behavior within each histological category (15). Further studies are required to determine whether molecular signatures within a specific cancer pattern exhibit prognostic power.

Performance Characteristics of the Gleason Grade Classifier. To determine the general applicability of the Gleason pattern classifier, we generated and analyzed an independent prostate cancer gene-expression data set derived from microdissected matched benign and neoplastic epithelium from 30 prostate needle biopsies. We measured expression alterations by microarray hybridization and assessed the models that provided the greatest discriminatory power in the original prostatectomy samples. The classifier comprised 86 genes (109 clones), including the 40 genes with 90% discriminatory power in the original radical prostatectomy cases performed with highest accuracy, producing a 76% overall correct classification rate ($P = 0.056$, Fisher's exact test) (Fig. 2*B*) on this independent sample set. Of the 12 cancers histologically called Gleason pattern 3, all but one was correctly classified. Of the cancers with a histological classification of 4 or 4 + 5, 6 of 11 were correctly identified (Fig. 2*B*). As expected, microdissected samples recognized to contain mixed grades of 3 + 4 or 4 + 3 cancer cells were divided between pattern 3 or pattern 4 molecular categories. These results suggest that pattern 3 cancers exhibit relatively consistent molecular alterations, whereas cancers with histological features of patterns 4 and 5 are more diverse and, in some cases, exhibit molecular features common to pattern 3 cancers.

We next attempted to compare our results with other reports correlating prostate tumor gene expression with grade. These comparisons were problematic, because all publicly available prostate cancer data sets generated expression profiles and classifiers based on Gleason-sum scores rather than individual patterns. For example, a given Gleason-sum score 8 cancer could be composed of patterns 5 + 3 or 4 + 4. Reports by Singh *et al.* (11) and LaPointe *et al.* (12) identified 29 and 42 genes, respectively, that statistically associated with Gleason-sum score. However, only three genes, *SPARC*, *BGN*, and *COL1A2*, were in common between these analyses. None of the genes comprising our classifier were present in the published grade-associated gene sets. An additional contributor to these discrepancies concerns the use of samples that, although enriched for tumor, also contain variable amounts of tumor-adjacent stroma, benign glands, and inflammatory cells. Alterations in the expression of genes in tumor-adjacent stroma is well recognized (16). In the



B

Observed	Predicted: Gleason 3	Predicted: Gleason 4 or 5
Gleason 3	11	1
Gleason 3+4	3	2
Gleason 4	5	5
Gleason 4+3	1	1
Gleason 5	0	1

Fig. 2. Genes associated with specific Gleason pattern prostate cancers. (*A*) Shown are the 86 genes exhibiting the greatest discriminatory power to partition cancer grades using an independent set of prostate cancer tissues. Genes are ordered according to *t* test score. The 40 genes used in the original PAM model are denoted by asterisks. (*B*) Application of the 86-gene Gleason classification model to an independent test set of 23 prostate cancers of singular pattern (e.g., 3 + 3) or exclusively high grade (4 + 4 or 4 + 5) resulted in an overall classification accuracy of 74%. The predicted pattern of 7 additional cancers of mixed low grade and high grade (3 + 4 and 4 + 3) varied between pattern 3 and pattern 4/5. The functional properties of grade-classifying genes are annotated in Table 1, which is published as supporting information on the PNAS web site.

context of these studies, the expression of *SPARC* has been shown to be up-regulated in the stroma adjacent to non-small-cell lung cancers (17) and pancreatic carcinomas (18) and may be similarly overexpressed in prostate cancer-adjacent stroma. A final source of discrepancies between studies involves technical variables, attributable, for example, to the use of different platforms (cDNA arrays vs. Affymetrix chips) and the necessity of amplifying RNA before hybridization.

Although the grade-discriminatory gene sets derived from our analyses and those of prior studies exhibited no concordance, we explored the ability of the Gleason grade classifier to partition prostate cancers in available data sets that were composed of singular grade combinations such as 3 + 3 and 4 + 4. Of 86 genes used in our classifier, 77 were present in the study by LaPointe *et al.*, with sufficient data to allow for grade-associated analyses. Unsupervised hierarchical clustering of samples using these 77 genes produced two major clusters generally partitioned according to grade. Of 26 tumors with Gleason 3 + 3 histology, 19 (73%) were correctly classified, as were 11 of 15 tumors (73%) with 4 + 4 or 4 + 5 Gleason patterns.

Characteristics and Confirmation of Gleason Grade-Associated Molecular Alterations. Several genes that we found to be associated with prostate cancer cell differentiation patterns have been linked to cancer grade or stage classifications. A study of breast carcinomas found a statistical relationship between increased expression of the antioxidant enzyme peroxiredoxin 5 (PRDX5) and larger tumor size, positive lymph node status, and shorter survival (19). Elevated levels of the transcription elongation factor TCEB1/Elongin C are associated with advanced androgen-independent prostate cancers (20), and changes in the subcellular localization of the membrane metalloendopeptidase MME/CD10 expression are linked to prostate cancer grade (21). Immunohistochemical analysis of the Akt-regulated transcriptional repressor NSEP/YB-1 demonstrates a strong positive correlation with Gleason grade (22). NSEP/YB-1 may influence cancer progression through multiple mechanisms that include enhancement of anchorage-independent cell growth and up-regulation of the *p*-glycoprotein multidrug-resistance protein (22, 23).

Many genes exhibiting altered expression in high- relative to low-grade prostate cancers possess characteristics supporting potential roles in cancer cell survival, invasion, or metastasis. Of the 86 genes we used for grade classification, 6 are located on chromosome 8. Of these, 4 map to 8q21–23, a region shown to be amplified in >40% of primary prostate cancers in association with higher cancer grades (24). The expression of 4 genes involved in aspects of sex hormone metabolism or regulation were increased in higher-grade cancers, including two members of the 17β-hydroxysteroid dehydrogenase (HSD) family, HSD17β3 and HSD17β4. The 3-α-hydroxysteroid dehydrogenase DHRS9 is capable of catalyzing the conversion of 3-α-androstanediol to dihydrotestosterone (25) and also contributes to the β-oxidation of fatty acids. The expression of HSD17β4, also known as D-bifunctional protein, was recently shown to be up-regulated in neoplastic prostate epithelium (26). We confirmed this result using quantitative RT-PCR analysis of microdissected prostate epithelium and further confirmed the grade-associated elevation of HSD17β4 transcripts found in the microarray-based analyses (see *Supporting Materials and Methods* and Fig. 4, which are published as supporting information on the PNAS web site). Together, these results suggest the emergence of mechanisms that favor intracrine utilization of alternative androgen precursors by tumor cells. Five members of the *ras* homologue gene family *RHOT2*, *RHOA*, *RAB2*, *RAB6A*, and *RAB18* were up-regulated in high-grade cancers. The *RAB18*-related gene, *RAB25*, was recently shown to influence the malignant potential of breast and ovarian cancers, suggesting that members of this gene family may be general mediators of epithelial cancer progression (27). The products of *RHOA*, *CAPZA2*, *HMGB1*, *NM23H1*, *CD63*, and Saposin C have all been

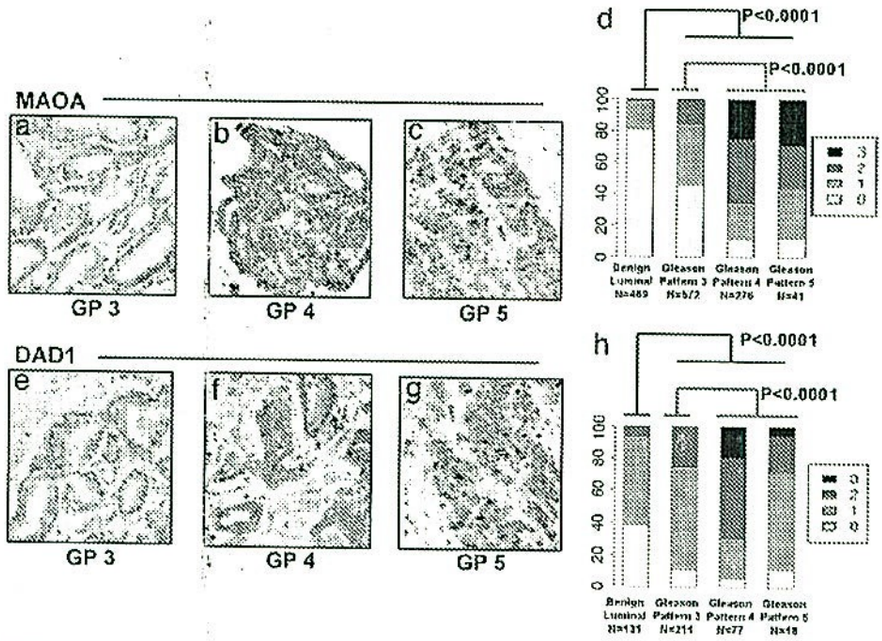


Fig. 3. IHC analysis of Gleason grade-associated gene expression. Representative immunohistochemical staining of Gleason pattern 3 (*a* and *e*), 4 (*b* and *f*), and 5 (*c* and *g*) prostate adenocarcinoma for MAOA (*a–c*) and DAD1 (*e–g*) expression. Magnifications are ×400. Summary of MAOA (1,358 scoreable samples) (*d*) and DAD1 (437 scoreable samples) (*d* and *h*) expression quantitation in tissue cores comprised of benign secretory epithelium and specific Gleason pattern adenocarcinomas.

shown to directly modulate tumor progression through enhanced cell motility, invasion, or metastasis.

Immunohistochemical (IHC) Analysis of Gleason Grade-Associated Protein Expression. In current clinical practice, diagnostic and prognostic cancer markers are most commonly assessed by using IHC quantitation. We sought to determine whether grade-associated alterations identified through transcript profiling would have corresponding changes in protein levels discernable by IHC. Although immunoperoxidase histochemistry is an imprecise method of protein quantification, it is the only technique currently available for assessing protein levels in small tissue samples and localizing expression to specific cell types. We focused on two pathways known to be associated with prostate cancer behavior: neuroendocrine effectors and apoptosis (28, 29–31). Of the 80 genes in the grade classifier with known biological functions, 5 are reported to modulate neuropeptide or amine metabolism: monoamine oxidase A (*MAOA*), *YWHAZ/14–3-3-ζ*, *OAZ2*, *CPE*, and *SLC22A3*. MAOA is encoded by a polymorphic gene located on the X chromosome that catalyzes the oxidative deamination of biogenic amines, such as catecholamines and indolamine transmitters, throughout the body. Gene variants conferring up to 50-fold differences in MAOA activity have been associated with a variety of neurological and psychosocial disorders, but few studies of MAOA in the context of cancer biology have been reported. The physiological functions of amine oxidases remain to be completely established, but amine metabolism is linked to essential cellular processes such as cell growth and differentiation (32). The byproducts of amine metabolism include H_2O_2 and hydroxyl radicals that may contribute to aspects of tumorigenesis, including redox-sensitive pathways such as MAP-kinase signaling (33).

To further characterize the association between *MAOA* expression and the differentiation state of prostate cancers, we measured MAOA protein levels by IHC on panels of tissue microarrays (TMAs) representing independent patient cohorts not evaluated in the initial analyses of transcript levels. A comparison of 469 benign and 889 cancerous samples demonstrated that MAOA protein expression was elevated in cancerous epithelium relative to benign secretory epithelium ($P < 0.0001$, proportional odds-regression analysis) (34) (Fig. 3 *a–d*), and MAOA expression was significantly elevated in Gleason 4 or 5 samples relative to Gleason 3 samples ($P < 0.0001$, proportional odds-regression analysis). The finding that high levels of expression of MAOA protein characterizes high-grade prostate carcinoma raises the possibility that patients on long-term MAOA inhibitors might have lower frequencies of high-grade prostate cancer if MAOA plays a mechanistic role in the development of high-grade prostate cancer.

Six genes functionally linked to the regulation of apoptosis were present in the cohort of grade-classifying genes. Of these, the gene encoding defender against death (DAD1) provides an intriguing link between apoptosis and the influence of tumor survival factors associated with perineural invasion (PNI). DAD1 is a downstream target of the NFkB survival pathway and exhibits an antiapoptotic function (35). *In vitro* studies modeling PNI-associated prostate cancer growth measured increased proliferation, reduced apoptosis, and elevated expression of NFkB and DAD1 in tumor cells located in proximity to ganglia and nerve tissue (36). We evaluated the expression of DAD1 protein by IHC in TMAs of formalin-fixed radical prostatectomy cores that, together, comprised 131 benign and 306 cancerous samples. High DAD1 expression was significantly associated with cancerous epithelium relative to benign secretory epithelium ($P < 0.0001$). In agreement with the transcript analyses, DAD1 protein levels also exhibited a strong association with Gleason pattern. Cancers of patterns 4 and 5 were more likely to stain intensely than low-grade cancer of pattern 3 ($P < 0.0001$) (Fig. 3 *e–h*).

Conclusions

In this study, we have identified a panel of molecular alterations that associate with the histological interpretation of prostate cancer grades. The panel performed with high accuracy across three independent panels of prostate adenocarcinomas processed and analyzed by using divergent techniques. Gleason patterns 4 and 5 cancers were virtually indistinguishable at the molecular level with our model. This finding is in accord with the clinical observation that Gleason patterns 4 and 5 tumors are associated with similar prognoses and, thus, are managed identically (37). If demonstrated to be mechanistically involved in cancer progression, the proteins encoded by grade-discriminating genes could serve as targets for pharmaceutical inhibition. Grade-discriminatory proteins may also have utility as serum markers for identifying high-grade prostate cancer. In this context, the association of MAOA expression with prostate cancer and grade is a finding that further implicates neuroendocrine features as mediators of prostate carcinogenesis. This finding has clinical importance, because the presence and extent of a neuroendocrine component in a prostate carcinoma correlates with tumor aggressiveness (37). The ready availability of monamine oxidase inhibitors offers an immediate opportunity to determine the clinical relevance of this finding.

Materials and Methods

Tissue Acquisition. All materials were acquired and used in conformity with Institutional Review Board-approved protocols at the University of Washington and Oregon Health & Science University. Two types of tissue were used for this study: fresh, nonfixed tissue as a source of RNA for laser microdissection and formalin-fixed, paraffin-embedded tissue for IHC studies. The nonfixed tissue consisted of frozen tissue blocks from radical prostatectomies and an independent sample set of frozen tissue blocks containing prostate needle core biopsies. Details of tissue handling are provided in *Supporting Materials and Methods*. Fixed tissue samples consisted of two types: blocks corresponding to the fresh tissue samples that were used for the laser microdissection preparations and TMAs.

Laser-Capture Microdissection (LCM) and RNA Preparation. Frozen sections (8 μm) were cut from optimal cutting temperature medium (OCT) blocks and immediately fixed in cold 95% ethanol. After brief (5–10 seconds) staining with hematoxylin using the HistoGene staining solution (Arcturus Engineering Mountain View, CA), the sections were dehydrated in 100% ethanol, followed by xylenes (per the manufacturer's protocol). Epithelial cells (≈5,000) from both histologically benign glands and cancer glands were separately laser-capture microdissected by using the Arcturus PixCell II instrument. Only one Gleason pattern was included in each laser-captured cancer sample. A total of 32 different Gleason patterns were captured from the 29 radical prostatectomy samples: 12 Gleason pattern 3, 12 Gleason pattern 4, and 8 Gleason pattern 5 samples. A total of 30 Gleason patterns were captured from the 30 needle core biopsy samples, with some samples comprising combinations of Gleason patterns. Matched benign epithelium was captured for each cancer sample, for a total of 121 samples. Digital photographs were taken of tissue sections before, during, and after LCM and assessed independently by two investigators to confirm the Gleason patterns of the laser-captured cells. RNA extraction and amplification were performed by using standard procedures described in *Supporting Materials and Methods*.

Microarray Hybridization, Data Acquisition, and Analysis. Prostate Expression Database cDNA microarrays were prepared on poly-L-lysine-coated glass microscope slides by using a robotic spotting tool as described in ref. 38. cDNA probes were made from 2 μg of amplified RNA and randomly labeled with either Cy3 or Cy5 dye to account for dye bias. Patient-matched normal and cancer probes were combined, filtered, and competitively hybridized to microarrays under a coverslip for 14 h at 63°C. Further details are provided in *Supporting Materials and Methods*.

Fluorescent array images were collected for both Cy3 and Cy5 emissions by using a GenePix 4000B fluorescent scanner (Axon Instruments, Foster City, CA). The image-intensity data were gridded and extracted by using GENEPIX PRO 4.1 software. The specifics of microarray data processing are provided in *Supporting Materials and Methods*. Microarray data sets from this study are deposited in the GEO respository under accession no. GSE5132.

To compare the overall expression patterns of all radical prostatectomy cancer samples to their patient-matched normal samples, the filtered log-ratio measurements were analyzed by using the Significance Analysis of Microarrays (SAM) procedure (39) (www-stat.stanford.edu/~tibs/SAM). In this analysis, a one-sample t test was used to determine which genes were significantly differentially expressed between cancer samples and their patient-matched normal samples. We call the set of significant genes the expression profile associated with prostate cancer.

To identify gene-expression alterations associated with specific Gleason patterns, we used Prediction Analysis for Microarrays (PAM) (13), a supervised classification method. Full technical details are provided on the PAM web site (www-stat.stanford.edu/~tibs/PAM). We divided the radical prostatectomy samples into two classes (Gleason pattern 3 and Gleason pattern 4 or 5) and applied PAM to identify several small gene cohorts that classified the samples with low error rates under leave-out-one cross-validation. To evaluate the predictive properties of these gene sets, we classified an independent sample set of Gleason pattern cancers composed of prostate needle core biopsy samples. An additional visual assessment of the degree to which our gene model partitioned the radical prostatectomy samples by Gleason pattern was undertaken by using principal-components analysis of the samples (40).

Quantitative (q)RT-PCR. cDNA was generated from 1 μg of aRNA by using 2 μg of random hexamers for priming reverse transcription by SuperScript II (200 units per reaction; Invitrogen). qRT-PCRs were done in triplicate, by using ≈5 ng of cDNA, 0.2 mM each primer, and SYBR green PCR master mix (Applied Biosystems) in a 20-μl reaction volume. Reactions were carried out and analyzed by using an Applied Biosystems 7900 sequence detector. Samples were normalized to the cycle threshold value obtained during the exponential amplification of RPLI3A. The expression level of HSD17B4 was calculated. Values were reported as the ratio of gene expression in neoplastic to normal epithelium. Additional details and primer sequences are provided in *Supporting Materials and Methods*.

TMAs. Eight TMAs were used for these studies. All samples in all arrays were provided in duplicate. Two arrays of predominantly primary prostate cancers (of 159 and 234 samples, respectively) have been described (41). Six arrays represented a range of Gleason grades, a mix of prostate cancer tissue of different biologic states [normal, atrophy, benign prostatic hyperplasia (BPH), prostatic intraepithelial neoplasia (PIN), primary prostate carcinoma, and metastatic prostate carcinoma], and a mix of different normal and neoplastic tissues. Altogether, 469 unique samples of benign prostate glands and 889 unique samples of primary prostate carcinoma (572 Gleason pattern 3, 276 Gleason pattern 4, and 41 Gleason pattern 5) were used for MAOA immunostaining. And 131 unique samples of benign prostate glands and 306 unique samples of primary prostate carcinoma (211 Gleason pattern 3, 77 Gleason pattern 4, and 18 Gleason pattern 5) were used for DAD1 immunostaining.

IHC. Antibodies recognizing MAOA (sc-20156; Santa Cruz Biotechnology) and DAD1 (sc-25557; Santa Cruz Biotechnology) were used to stain TMAs composed of benign and neoplastic prostate tissues. Specificity of labeling was confirmed by both omission of the primary antibody and immunostaining the sections with a primary antibody against an irrelevant antigen. Immunolocalization was done by using a three-step avidin–biotin–peroxidase method. The sections were counterstained with hematoxylin. Further details are provided in *Supporting Materials and Methods*.

IHC stains were evaluated by using the following categorical compositional scale: 0, no expression; 1, ≤5% of the cells express the antigen; 2, 5–20% of the cells express the antigen; and 3, 20–100% of cells express the antigen. The following cell types were evaluated: secretory and basal epithelia, high-grade PIN, and Gleason pattern 3, pattern 4, and pattern 5 tumor cells. When a section had several Gleason patterns, each pattern was scored.

To test for differences in the staining intensity of different cell types, we used a proportional-odds model and included the covariates Gleason grade and tissue source. The model was fit in SAS (SAS Institute, Cary, NC), implementing a generalized estimating-equations approach to account for multiple sections from the same patient. Further details of the analytical methods are provided in *Supporting Materials and Methods*.

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A molecular correlate to the Gleason grading system for prostate adenocarcinoma

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Adenocarcinomas of the prostate can be categorized into tumor grades based on the extent to which the cancers histologically resemble normal prostate glands. Because grades are surrogates of intrinsic tumor behavior, characterizing the molecular phenotype of grade is of potential clinical importance. To identify molecular alterations underlying prostate cancer grades, we used microdissection to obtain specific cohorts of cancer cells corresponding to the most common Gleason patterns (patterns 3, 4, and 5) from 29 radical prostatectomy samples. We paired each cancer sample with matched benign lumenal prostate epithelial cells and profiled transcript abundance levels by microarray analysis. We identified an 86-gene model capable of distinguishing low-grade (pattern 3) from high-grade (patterns 4 and 5) cancers. This model performed with 76% accuracy when applied to an independent set of 30 primary prostate carcinomas. Using tissue microarrays comprising >800 prostate samples, we confirmed a significant association between high levels of monoamine oxidase A expression and poorly differentiated cancers by immunohistochemistry. We also confirmed grade-associated levels of defender against death (DAD1) protein and HSD17β4 transcripts by immunohistochemistry and quantitative RT-PCR, respectively. The altered expression of these genes provides functional insights into grade-associated features of therapy resistance and tissue invasion. Furthermore, in identifying a profile of 86 genes that distinguish high- from low-grade carcinomas, we have generated a set of potential targets for modulating the development and progression of the lethal prostate cancer phenotype.

carcinoma | monoamine oxidase A | microarray | expression profile

The prognosis and choice of therapy for prostate cancer is based primarily on three parameters obtained at the time of diagnosis: clinical stage, serum prostate-specific antigen (PSA), and the Gleason score of the cancer (1). The Gleason grading system, which is based on microscopic tumor architecture, consists of five histological patterns that annotate cancers into categories exhibiting well differentiated (pattern 1) to poorly differentiated (pattern 5) features (2, 3). A number from 1 to 5 is assigned to the most prevalent pattern. A second number, also from 1 to 5, is assigned to the second most prevalent pattern. The Gleason grade, which is the sum of these two numbers, has a value between 2 and 10. In current practice, the vast majority of prostate cancers have a Gleason score of ≥6 (4, 5). Hence, tumors composed of patterns 3, 4, and/or 5 are considered clinically significant. The reporting of individual Gleason patterns is not a trivial distinction, because the amount of pattern 4 and the presence of any pattern 5 has been highly correlated with probability of cancer dissemination, response to therapy, disease outcome, patient-management decisions, and clinical-trial enrollment (6, 7). Numerous studies have demonstrated a direct correlation between Gleason score and clinical measurements of disease outcome, including death due to tumor within 15 years and likelihood of remaining free of biochemical evidence of disease recurrence after either definitive, potentially curative radical prostatectomy or radiation therapy (8). Although different Gleason patterns are histologically distinctive, the molecular features underlying these tumor phenotypes are not precisely defined. In this study, we sought to characterize the molecular profile of prostate carcinomas of specific Gleason patterns. Tumor cell transcript levels were used to develop a model capable of distinguishing the low-grade (pattern 3) from high-grade (patterns 4 and 5) tumors. We validated the predictive power of the model by using an independent set of primary prostate cancers and confirmed grade-associated differences in protein-expression levels using a third sample cohort. Together, these results identify both consistent and divergent features of the molecular framework that underlies the histological classification of cancer grade.

Results and Discussion

Gene-Expression Profiles Associated with Prostate Cancer Grades. To identify molecular alterations correlating with histological tumor grade, we used laser-capture microdissection to exclusively acquire the epithelial cell component of prostate carcinoma foci corresponding to individual Gleason pattern 3, 4, or 5 cancers. After linear amplification, gene-expression alterations in tumor cells were measured by cDNA microarray hybridizations in a head-to-head fashion against patient-matched microdissected benign secretory epithelial cells. A total of 121 benign and neoplastic samples from 59 radical prostatectomies and 30 prostate needle core biopsies contributed to the analysis.

To assess the generalized applicability and consistency of the methods, we first sought to identify consistent prostate cancer-associated transcript alterations irrespective of grade. Using microdissected epithelium from radical prostatectomy samples, we identified 736 genes with altered expression levels between benign and neoplastic epithelium (false discovery rate <0.01%). This cohort included several genes previously reported to be differentially expressed in prostate cancers, such as *hepsin*, *AMACR*, and *CAMKK2* (Fig. 1*A*) (9–12). These findings provided validation that our methods replicated results of earlier expression-profiling studies, despite using the very small sample quantities obtained by laser microdissection and subjecting the

Conflict of interest statement: No conflicts declared.

Abbreviations: IHC, immunohistochemical; MAOA, monoamine oxidase A; PAM, prediction analysis for microarrays; TMA, tissue microarray.

Data deposition: The microarray data sets reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database (accession no. GSE5132).

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Novel markers for differentiation of lobular and ductal invasive breast carcinomas by laser microdissection and microarray analysis

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Abstract

Background: Invasive ductal and lobular carcinomas (IDC and ILC) are the most common histological types of breast cancer. Clinical follow-up data and metastatic patterns suggest that the development and progression of these tumors are different. The aim of our study was to identify gene expression profiles of IDC and ILC in relation to normal breast epithelial cells.

Methods: We examined 30 samples (normal ductal and lobular cells from 10 patients, IDC cells from 5 patients, ILC cells from 5 patients) microdissected from cryosections of ten mastectomy specimens from postmenopausal patients. Fifty nanograms of total RNA were amplified and labeled by PCR and *in vitro* transcription. Samples were analysed upon Affymetrix U133 Plus 2.0 Arrays. The expression of seven differentially expressed genes (*CDH1, EMP1, DDR1, DVL1, KRT5, KRT6, KRT17*) was verified by immunohistochemistry on tissue microarrays. Expression of *ASPN* mRNA was validated by *in situ* hybridization on frozen sections, and *CTHRC1, ASPN* and *COL3A1* were tested by PCR.

Results: Using GCOS pairwise comparison algorithm and rank products we have identified 84 named genes common to ILC versus normal cell types, 74 named genes common to IDC versus normal cell types, 78 named genes differentially expressed between normal ductal and lobular cells, and 28 named genes between IDC and ILC. Genes distinguishing between IDC and ILC are involved in epithelial-mesenchymal transition, TGF-beta and Wnt signaling. These changes were present in both tumor types but appeared to be more prominent in ILC. Immunohistochemistry for several novel markers (EMP1, DVL1, DDR1) distinguished large sets of IDC from ILC.

Conclusion: IDC and ILC can be differentiated both at the gene and protein levels. In this study we report two candidate genes, asporin (*ASPN*) and collagen triple helix repeat containing 1 (*CTHRC1*) which might be significant in breast carcinogenesis. Besides E-cadherin, the proteins validated on tissue microarrays (EMP1, DVL1, DDR1) may represent novel immunohistochemical markers helpful in distinguishing between IDC and ILC. Further studies with larger sets of patients are needed to verify the gene expression profiles of various histological types of breast cancer in order to determine molecular subclassifications, prognosis and the optimum treatment strategies.

Background

Invasive ductal (IDC) and lobular carcinomas (ILC) are the most common histological types of breast cancer [1,2]. The terminology of ductal and lobular carcinomas is considered to be controversial as on purely anatomical grounds there is no justification for this nomenclature. Both carcinomas are derived from the terminal duct lobular unit (TDLU), and the differences in their morphology are likely to reflect the differences in mechanisms of carcinogenesis rather than the anatomical origin of the lesions. At presentation the clinical pathological parameters such as tumor site, size, grade and stage are similar for both tumor types [3], however, clinical follow-up data and the patterns of metastasis suggest that their development and progression are different [4,5]. Treatment for stage-matched tumors is similar [6], but ILCs are often resistant to neoadjuvant therapy [7]. Although patients with ILCs are older, have low grade tumor and less lymphatic invasion, they have no survival advantage compared with IDCs [8,9].

Expression profiling using microarrays is a powerful technology which enables the simultaneous study of the expression of thousands of genes and, in conjunction with laser capture microdissection, the high-throughput genetic analysis of morphologically distinct cell subpopulations within tumor tissue [10,11]. Microarray analysis has a number of applications, including tumor classification, molecular pathway modeling, functional genomics, and comparison of gene expression profiles between groups [12]. The study of gene expression in primary breast tumor tissues is complicated for two major reasons. First, breast cancer consists of many different cell types, including normal epithelial, stromal, adipose and endothelial cells. Second, tumor cells are morphologically and genetically diverse [13]. The recent development of laser capture microdissection has provided an opportunity to generate gene expression signatures from individual cell types [14-20].

Microarrays were used for analysis of breast tumor subclasses with clinical implication [21,22], for analysis of gene expression changes in single breast cancer cells from within the same tumor [14], for expression analysis of different gene families in breast cancer [23,24], and for analysis of gene expression in different cellular and tumor types [25-28]. ER status of the tumor was the most important discriminator of expression subtypes. Unsupervised hierarchical clustering segregated these tumors into two main clusters based on their basal (predominantly ER negative) and luminal (predominantly ER positive) characteristics [29]. Ductal breast cancer classes have been identified with aggressive phenotype and poor prognosis versus those with good prognosis [30-32]. Another study reported distinct expression patterns based on BRCA1 and BRCA2 status [33].

To date, few papers have been published on gene expression profiles of normal cell populations in the mammary gland [34]. Several studies suggest differences in expression profiles of IDC and ILC. Inactivating mutations of E-cadherin gene are very frequent in ILC [35]. However, the loss of E-cadherin expression was shown to be an independent prognostic marker for recurrence, especially in node-negative breast cancer patients, irrespective of the histological type [36]. Abnormal cytoplasmic and nuclear localization of p120, a member of the E-cadherin/catenin adhesion complex, is mediated by E-cadherin loss and occurs in the early stages of ILC [37]. Non-microdissected IDC and ILC tissues have also been used to study specific gene expression profiles of lobular and ductal tumors [38,39]. We aimed to identify genes differentially expressed between normal cell types (ductal and lobular), between tumor cells and normal cells as well as between tumor types (ductal no special type and lobular). Despite examining limited number of patients, our study is the first full genome analysis of microdissected ductal and lobular tumor and normal cells which allowed us to detect normal mammary epithelium- and cancer-specific genes.

Methods

Laser capture microdissection and RNA isolation

Altogether ten surgical specimens with either invasive ductal carcinoma NST (no special type) (n = 5) or invasive lobular carcinoma (n = 5) were investigated. This research protocol was approved by the ethics committee at Palacky University. Tumor and normal tissues from the same mammary gland were identified by an experienced pathologist, snap-frozen in liquid nitrogen and stored at -80°C for further analysis. For microdissection, frozen tissues were embedded in TissueTek medium and cut on standard cryostat (Leica CM1850, Leica Microsystems GmbH, Wetzlar, Germany). Frozen sections (7 μm) were immediately fixed in acetone, stained by hematoxylin and dehydrated in alcohol and xylene. All solutions were prepared using diethyl pyrocarbonate-treated water. RNase free instruments and RnaseZap (Sigma, St Louis, MO, USA) were used throughout.

At least 1000 infiltrating ductal or lobular tumor cells together with normal lobular and ductal cells were microdissected from cryosections using the Veritas™ Laser Capture Microdissection System (Arcturus Bioscience, Inc., Mountain View, CA, USA) according to standard protocols. When microdissecting normal cells, no attempt has been made to exclude basal or myoepithelial cells for two reasons. First, both ducts and lobules are atrophic in menopause, and no clearly defined layers of luminal and

myoepithelial cells are preserved. Second, it is impossible to exclude contamination with or entrapment of myoepithelial cells during the microdissection procedure. Caps with captured cells were directly placed in 100 µl lysis buffer (Qiagen, Hilden, Germany). Total cellular RNA was isolated (RNeasy® Micro Kit, Qiagen) according to manufacturer's recommendations and subsequently quantified on a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

RNA amplification, microarray target synthesis and hybridization

Fifty nanograms of total RNA were reverse transcribed and amplified by Microarray Target Amplification Kit (Roche diagnostics, Basel, Switzerland). In brief, total RNA (50 ng) was converted into cDNA using a modified oligo (dT) primer (TAS-T7 Oligo $(dT)_{24}$). A unique Target Amplification Sequence (TAS) with no homology to any known sequences in public databases generated the 3' anchor on the cDNA for subsequent PCR amplification. In order to include a 5' anchor sequence on the cDNA, the TAS-$(dN)_{10}$ primer was used for the initiation of the second strand cDNA synthesis. After purification using the Microarray Target Purification Kit (Roche), PCR was performed using the TAS primer and Expand PCR Enzyme Mix which is optimized for long (>1 kb) and unbiased PCR products. In order to ensure that messages were not amplified to saturation, the optimal number of PCR cycles was estimated by preliminary PCR and agarose electrophoresis of PCR products from cycles 21, 24, 27, 30 and 33. The optimal number of PCR cycles was set either to 27 cycles for patients 3, 4, 8, 9, and 10 or 29 cycles for patients 1, 2, 5, 6, and 7. All three populations from each patient were amplified by the same number of PCR cycles. If higher PCR cycling was needed for any cell population, new microdissection, RNA isolation, cDNA synthesis and PCR amplification were performed until the same PCR cycling was possible. After purification with the Microarray Target Purification Kit (Roche), the PCR products were labeled with biotin-14-CTP (Invitrogen, CarlsBad, CA, USA) and biotin-16-UTP (Roche) by *in vitro* transcription using Microarray RNA Target Synthesis Kit T7 (Roche). The labeled cRNA was purified using the Microarray Target Purification Kit (Roche), quantified by spectrophotometer and checked by agarose electrophoresis. The entire amplification and labeling process was monitored by GeneChip® Eukaryotic Poly-A RNA Control Kit (Affymetrix, Santa Clara, CA, USA) with exogenous positive controls which were spiked into the total RNA before cDNA synthesis. In all cases, 25 µg of each biotinylated cRNA preparation was fragmented, assessed by gel electrophoresis, and placed in hybridization cocktail containing biotinylated hybridization controls (GeneChip™ Eukaryotic Hybridization Control Kit, Affymetrix). Samples were first hybridized to Test3 Arrays for 16 hours, washed, stained using antibody-mediated signal amplification and scanned. After passing this quality control stage, the samples were hybridized onto the large Human Genome U133 Plus 2.0 Arrays (Affymetrix).

Microarray data analysis

Scanned images of microarray chips were analysed by the GCOS (GeneChip Operating Software) from Affymetrix with the default settings except that the target signal was set to 100. Differentially expressed genes between cell types were identified using the GCOS change algorithm and Rank Products (RP) [40] following RMA (Robust Multiarray Analysis) [41]. GCOS pairwise analysis was performed to compare gene expression levels among the normal lobular, normal ductal and tumor cells within individual patients and between lobular and ductal carcinoma cells of different patients. For each comparison between two cell types, the number of increase and decrease calls of each probe set was calculated using MS Excel and probe sets with the highest number of consistent changes among all patients were identified. Probe level quantile normalization [40] and robust multiarray analysis [41] on the raw. CEL files were performed using the Affymetrix package of the Bioconductor [42]. The cut-off value of percentage of false-positives for RP analysis was set to 10%. Gene lists were uploaded to DAVID (Database for Annotation, Visualization and Integrated Discovery) [43], and functional annotation was performed. Further informations on genes were obtained from public databases, such as Gene Cards [44] and NETAFFX Analysis Center [45]. Hierarchical clustering was performed using dChip software [46]. The data discussed in this publication have been deposited in NCBI Gene Expression Omnibus (GEO) [47], and are accessible through GEO Series accession number GSE5764.

Validation by immunohistochemistry

Seven differentially expressed genes were verified by immunohistochemical staining on tissue microarrays (TMA), which were constructed from 119 breast cancer cases and contained 278 cores of 2.0 mm diameter. They consisted of 80 ductal carcinomas, 29 lobular carcinomas, one tubular carcinoma, 3 medullary carcinomas, 2 tubular-lobular carcinomas, 2 mixed ductal-lobular carcinomas, one mucinous and one papillary carcinoma. The construction of the tissue microarrays was done using a tissue arrayer (Beecher Instruments, Inc., Sun Prairie, WI, USA) [48]. The paraffin sections from 22 additional breast cancer samples containing normal mammary gland structures were used for comparison.

TMA sections were stained using standard immunohistochemical methods. Monoclonal mouse antibodies against cytokeratin 17 (dilution 1:100, clone 2D4-1G9, Abnova, Taipei, Taiwan), cytokeratin 5/6 (dilution 1:100, clone

D5/16 B4, Dako, Denmark) and E-cadherin (dilution 1:50, clone NCH-38, Dako), polyclonal mouse antibodies against EMP1 (epithelial membrane antigen 1; dilution 1:100, clone 2D4-1G9, Abnova), DDR1 (discoidin domain receptor 1; dilution 1:100, Abnova) and DVL1 (human homolog of the Drosophila dishevelled gene; dilution 1:100, Abnova) were used. Microwave antigen retrieval was performed in citrate buffer (pH 6.0). For antigen visualization, the EnVision/HRP system and DAB+ (Dako) were used, slides were subsequently counterstained with hematoxylin, dehydrated and mounted in Canadian balsam. Immunohistochemical procedure was optimized by testing different antigen retrieval methods and negative controls. We regarded cells as immunoreactive when an obvious membranous or submembranous (E-cadherin, DDR1, EMP1) and cytoplasmic (cytokeratins, DVL1) staining was seen. Immunoreactivity was scored as follows: retained (++) when more than 50% of membrane/cytoplasm were strongly positive, reduced (+) when 10–50% of the membrane/cytoplasm were positive, and absent (-) when 0–10% of the membrane/cytoplasm were positive. Frequencies of positive and negative staining in tumor and normal tissues were compared by Fisher's exact test (Statistica 6.0, StatSoft, Prague, Czech Republic).

Validation by in situ hybridization

Asporin mRNA expression was verified by chromogenic *in situ* hybridization (CISH) on frozen sections. Fluorescein labeled oligonucleotide (5'AAG TTG GTG GTA AGC CTT TAG GAA CTG AGG-FAM) was designed to the junction of exons five and six of the asporin mRNA (Generi Biotech, Hradec Kralove, Czech Republic). Sections were mounted on Superfrost Plus slides and stored at -80°C until further staining. Before CISH, the sections were heated at 50°C for 2 min, and then dried at room temperature for 30 min. Tissues were delipidized in chloroform at room temperature for 5 min and dried to evaporate the chloroform. Block of endogenous peroxidase activity was followed by incubation of tissue sections in 10% paraformaldehyde for 7 min, then washed in PBS for 3 min and in 2× SSC (1× SSC contains 150 mM NaCl, 15 mM sodium citrate; pH 7.2) twice for 5 min. The sections were covered with 100 μl hybridization buffer (60% formamide, 5× SSC) containing 150 ng/ml of probe and incubated in moist chamber at 37°C for 22 hours. After the hybridization, unbound probe was washed from the sections at 37°C by 2× SSC for 5 min, then three times with 60% formamide in 0.2× SSC at 37°C for 5 min, and twice with 2× SSC at room temperature for 5 min. Then polyclonal rabbit anti-FITC/HRP (dilution 1:40, Dako) was applied at room temperature for 45 min, followed by washing in 2× SSC twice for 10 min. For visualization, DAB+ (Dako) was used, slides were subsequently counterstained with hematoxylin, dehydrated and mounted in Canadian balsam.

Validation by PCR

Products after PCR amplification (see RNA amplification, microarray target synthesis and hybridization) were used for verification of *CTHRC1*, *ASPN* and *COL3A1* gene expression using gene specific PCR. The primers were as follows: *CTHRC1* forward 5'ACA AGT GCC AAC CCA GAT AGC AAC, reverse 5'ATC GCA CTT CTT CTG TGG AAG GAC (20 cycles of 95°C, 59°C and 72°C 1 min each, product length 79 bp, kindly provided by Dr. Danuta Radzioch, McGill University, Montreal, Canada), *ASPN* forward 5'GTT CAG CTT GGG AAC TTT GGA ATG TAA, reverse 5'ACT GCA ATA GAT GCT TGT TTC TCT CAA CCC (20 cycles of 95°C, 58.5°C and 72°C 1 min each, product length 243 bp [modified from [49]], and *COL3A1* forward 5'TTG TCA ACC AGT GCA AGT GAC CGA C, reverse 5'TGG TGA GCA CAG TCA TTG CTC TGC A (20 cycles of 95°C, 59°C and 72°C 1 min each, product length 276 bp) [50]. PCR products were separated on 10% polyacrylamide gels, stained with ethidium bromide and captured using a gel documentation system (DIANA II with cooled CCD camera, Raytest, Germany). Band intensities, expressed as "OD (optical density) × cm", were assessed by Multi-Analyst densitometric software (Bio-Rad, USA). The results were correlated with fluorescence signals from the relevant Affymetrix probe sets by Spearman coefficient using Statistica 6.0 software (StatSoft, Czech Republic).

Results

Unsupervised clustering

The clinical and histopathological characteristics including lymph node status, tumor grade, expression of estrogen/progesterone receptors (ER/PgR), c-erbB-B2/HER2/neu and accompanying lesions of ten breast cancer patients are shown in Table 1. We microdissected normal and tumor cells from these cases which resulted in a total of 30 samples for gene expression profiling (10 normal ductal, 10 normal lobular, 5 tumor ductal and 5 tumor lobular). Unsupervised clustering of all samples was performed using all probe sets without filtering. No tumor types were grouped together but two large clusters were evident: one mainly consisted of tumor cells and the other cluster mainly of normal cells. This clearly suggests differences in global gene expression profiles of tumor and normal cells (Figure 1).

Genes differentially expressed between normal ductal and lobular cells

In this comparison 367 probe sets were identified by pairwise comparison, and 501 probe sets by Rank Products (RP). There were 82 probe sets (78 named genes) identified by both methods. In pairwise comparisons, the differentially expressed genes were found by selecting those with count of changes more than 6 (out of 10, either increase or decrease). Multiple cytokeratins (5, 7, 14, 15, 17) were expressed in both cells, but none of them was

Table 1: Clinical and histopathological characteristics of breast cancer patients involved in microarray analysis

No	Diagnosis	Grade[1]	AC	LN	ER[3]	PgR[3]	HER-2	Bcl-2	E-CD
1	IDC	G3	IDH	N_1	0	0	0	80%	>90%
2	IDC	G2	none	N_1	40%	0	0	80%	>90%
3	IDC	G2	none	N_0	10%	2%	0	70%	>90%
4	IDC	G3	none	N_0	90%	50%	2+ [2]	100%	>90%
5	IDC	G2	DCIS	N_0	90%	1%	0	95%	>90%
6	ILC	G2	LCIS	N_0	0	0	0	30%	0
7	ILC	G1	none	N_0	80%	80%	0	100%	0
8	ILC	G1	none	N_1	50%	0	0	80%	0
9	ILC	G2	LCIS	N_1	0	0	0	<5%	0
10	ILC	G1	none	N_0	50%	70%	0	80%	0

[1]Tumors were graded using the Nottingham combined histologic grading system.
[2]No gene amplification was detected by fluorescent *in situ* hybridization.
[3]A complete H-score was calculated by summing the products of the percentage cells stained at a given staining intensity (0–100) and the staining intensity (0–3).
AC, accompanying changes; DCIS, ductal carcinoma *in situ*; E-CD, E-cadherin; ER, estrogen receptor; IDC, invasive ductal carcinoma, IDH, intraductal hyperplasia; ILC, invasive lobular carcinoma; LCIS, lobular carcinoma *in situ*; LN, lymph node; PgR, progesterone receptor.

useful to separate two cell types. Genes encoding proteins involved in proteolysis and metabolism (*USP25, TMPRSS3, ACACB*), protein biosynthesis and modification (*PDE4DIP, LOXL1*), ion transport (*CLCN3, GABRP*) and protein kinase cascade (*MAP4K5*) as well as genes regulating transcription (*RFXAP, HSZFP36*) were upregulated in ductal cells. However, genes regulating cell growth, activation and motility (*TSPAN5*), genes encoding actin-binding proteins (*EHBP1*), small leucine-rich proteoglycan (*DCN*), and proteins with GTPase activity (*RHOBTB3, ARHGEF9*) were overexpressed in lobular cells (Table 2). Hierarchical clustering of normal cell types based on the 82 probe sets found by both RP and pairwise comparison showed that gene expression profiles of normal ductal and lobular cells from the same patients were similar, and they could not be well separated from each other (Figure 2).

Genes differentially expressed between tumor cell types and normal cells

A comparison of ductal carcinoma cells with normal ductal cells identified 1055 probe sets by pairwise analysis, 604 probe sets by RP and 326 probe sets by both methods. A comparison of ductal carcinoma cells with normal lobular cells identified 792 probe sets by pairwise analysis, 347 probe sets by RP and 171 probe sets by both methods. A comparison of lobular carcinoma cells with normal ductal cells identified 1022 probe sets by pairwise analysis, 350 probe sets by RP and 201 probe sets by both methods. A comparison of lobular carcinoma cells with normal lobular cells identified 983 probe sets by pairwise analysis, 344 probe sets by RP and 208 probe sets by both methods (see Additional file 1). In pairwise comparisons, the differentially expressed genes were identified by selecting those with count of changes more than 4 (out of 5, either increase or decrease).

Comparison between lobular carcinoma and normal cells

Combined pairwise analysis and RP have identified 106 differentially expressed probe sets (84 named genes) common to lobular carcinoma versus normal ductal and lobular cells. Of these probe sets, several genes encoding proteins involved in extracellular matrix (ECM)-tumor interaction and focal adhesion (*COL6A3, COL8A1, CTHRC1, THBS2, COMP*) were upregulated, whereas *ITGA2*gene with the same function was downregulated in tumor cells. *ASPN* (asporin) was one of the upregulated genes with the highest fold change. It encodes a protein with porin activity which belongs to a family of leucine-rich repeat (LRR) proteins associated with the cartilage matrix.

Of the differentially expressed genes involved in Wnt signaling, SFRP2 (secreted frizzled-related protein 2) and WISP1 (WNT1 inducible signaling pathway protein 1) were upregulated in lobular carcinomas. Several genes encoding proteins involved in actin, calcium and metal ion binding were downregulated (MYBPC1, DST, PIP, CA2), and some genes with the same function (BGN, ADAM12) were upregulated in lobular carcinomas. CDH1 (E-cadherin) was downregulated in lobular tumors accompanied by downregulation of ITGA2 (integrin, alpha 2) which it is a ligand of. Several other genes were differentially expressed between lobular carcinomas and normal cells. LYZ/LILRB1 was upregulated in lobular cancer cells. Other downregulated genes were as follows: CGNL1 is a cellular myosin that appears to play a role in cytokinesis, cell shape, and specialized functions such as secretion and capping; EHF encodes a protein that belongs to an ETS transcription factor subfamily characterized by epithelial-specific expression (ESEs). This protein may be involved in epithelial differentiation and carcinogenesis; CLIC6 encodes a member of the chloride

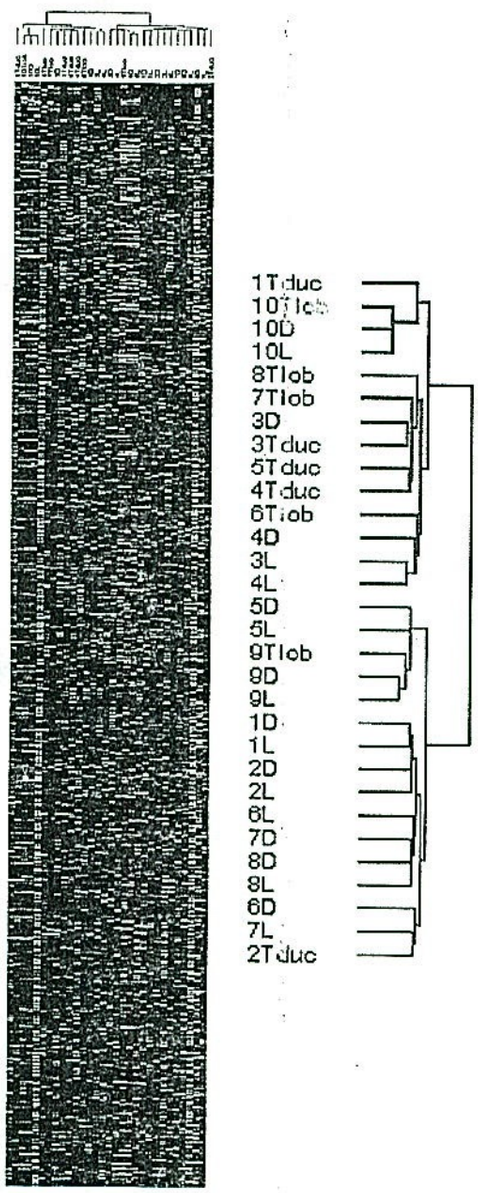


Figure 1
Unsupervised hierarchical clustering of all samples using all probe sets. No tumor types are grouped together, however, two large clusters are evident: one (blue) mainly consists of tumor cells and the other (black) mainly consists of normal cells. This suggests differences in global gene expression profiles of tumor and normal cells. 1–10, case numbers; D, ductal normal cells; L, lobular normal cells; Tduc, ductal tumor; Tlob, lobular tumor.

intracellular channel family of proteins and is involved in ion transport (Table 3).

Comparison between ductal carcinoma and normal cells

Combined pairwise analysis and RP have identified 90 differentially expressed probe sets (74 named genes) common to ductal carcinoma versus normal ductal and lobular cells. In particular, transcription regulators (ATF3, PIGR), genes encoding proteins with cytokine and growth factor activity (PTN, CX3CL1) and genes encoding proteins involved in ion transport and metabolism (ATP1A2, MMP7) were downregulated in ductal carcinomas when compared with normal cells (Table 3).

Comparison between both types of tumor cells and normal cells

Of 90 probe sets differentially expressed between ductal carcinoma and both normal cells, and 106 probe sets differentially expressed between lobular carcinoma and both normal cells, only 25 probe sets were common (Table 3). Hierarchical clustering based on those 25 probe sets showed that tumor samples were grouped together, however, gene expression profiles of normal cell types from cases 10 and 3 were different from other normal cells, and tumor cells from case 2 were different from other tumor cells (Figure 3).

Several upregulated genes (collagen type I, III, V, XI, fibronectin 1, versican) were related to tumor- ECM interactions and focal adhesion. However, another gene mediating focal adhesion, *MYLK*, was downregulated in tumor cells. This gene encodes a myosin light polypeptide kinase containing 1 fibronectin type-III domain. Genes encoding proteins involved in ion and electron transport (*CYB5R2*, *GABRP*), and genes encoding proteins with transcription factor and regulator activity (*ELF5*, *ID4*) were downregulated in both populations of tumor cells. They have also been implicated as regulators of cell proliferation, differentiation, and transformation. In addition, genes involved in cell differentiation and apoptosis (*PDZK1*) and genes encoding actin and actin-binding proteins (*CNN1*, *ACTA2*) were also downregulated in both types of tumor cells. Wnt signaling molecules were differentially expressed in our samples. Of these, *SFRP1* (secreted frizzled-related protein 1) was downregulated in tumor cells, and genes involved in calcium regulation pathway were not significantly changed. Structural constituents of cytoskeleton such as type I (14, 15, 17, 23) and type II (5) keratins were downregulated in tumor cells. Several other genes were also differentially expressed between tumor and normal cells. *PI15* (peptidase inhibitor 15) was downregulated, whereas *LRRC15* (leucine rich repeat containing 15) was upregulated in both tumor cells.

Table 2: Genes differentially expressed between normal lobular versus normal ductal cells by both rank products and pairwise analysis

Probe Set[1]	Gene Symbol	Gene Title	Rank products	Pairwise comparison
			Fold-change	Incr/Decr
Regulation of transcription				
229431_at	*RFXAP*↓	Regulatory factor X-associated protein	-3.1	1/6
229732_at	*HSZFP36*↓	ZFP-36 for a zinc finger protein	-3.0	0/6
207117_at	*H-plk*↑	Krueppel-related zinc finger protein	2.4	6/1
Protein biosynthesis and modification				
214130_s_at	*PDE4DIP*↓	Phosphodiesterase 4D interacting protein (myomegalin)	-3.2	1/6
232752_at	*— (LOXL1)* ↓	CDNA FLJ10302 fis, clone NT2RM2000042 (lysyl oxidase-like 1)	-1.4	1/6
Proteolysis and metabolism				
49452_at	*ACACB*↓	Acetyl Coenzyme A carboxylase beta	-18	0/8
220419_s_at	*USP25*↓	Ubiquitin specific peptidase 25	-2.9	1/6
220177_s_at	*TMPRSS3*↓	Transmembrane protease, serine 3	-2.8	1/6
Ion transport				
201735_s_at	*CLCN3*↓	Chloride channel 3	-2.8	2/6
205044_at	*GABRP*↓	Gamma-aminobutyric acid (GABA) A receptor, pi	-1.8	1/6
Actin binding				
212650_at	*EHBP1*↑	EH domain binding protein 1	2.6	6/0
GTPase activity				
202975_s_at	*RHOBTB3*↑	Rho-related BTB domain containing 3	2.5	7/1
203264_s_at	*ARHGEF9*↑	Cdc42 guanine nucleotide exchange factor (GEF) 9	2.6	6/0
Regulation of cell development, activation, growth and motility				
209890_at	*TSPAN5*↑	Tetraspanin 5	2.6	7/0
Small leucine-rich proteoglycan (slrp) family				
211896_s_at	*DCN*↑	Decorin	2.0	7/1
Protein kinase cascade				
211081_s_at	*MAP4K5*↓	Mitogen-activated protein kinase kinase kinase kinase 5	-2.3	1/7
Other				
206157_at	*PTX3*↓	Pentraxin-related gene, rapidly induced by IL-1 beta	-2.8	2/7
218431_at	*C14orf133*↓	Chromosome 14 open reading frame 133	-2.7	0/7
218514_at	*FLJ10587*↓	Hypothetical protein FLJ10587	-2.6	1/7
226760_at	*LOC203411*↑	Hypothetical protein LOC203411	3.0	6/2

↑ – upregulated in normal lobular cells; ↓ – downregulated in normal lobular cells

[1]Affymetrix probe set number; If there was more than one probe set for the particular gene found among top 20 changed genes (according to the fold change), the probe set with the highest fold change is used and number of other probe set is indicated.

Genes differentially expressed between ductal and lobular carcinomas

In this comparison 208 probe sets were identified by pairwise comparison, 122 probe sets by RP, and 32 probe sets (28 named genes) by both methods (Table 4). In pairwise comparisons, the differentially expressed genes were identified by selecting those with count of changes more than 19 (out of 25 inter-patient comparison, every ductal carcinoma against every lobular carcinoma, either increase or decrease). These tumors were well separated by hierarchical clustering based on all 325 probe sets identified by RP and/or pairwise analysis (Figure 4). The expression of genes encoding proteins involved in cell adhesion was changed. Although *CDH1* (E-cadherin), a classical member of the cadherin superfamily, was downregulated, *THBS4* encoding calcium-binding adhesive glycoprotein, thrombospondin-4, was upregulated in all lobular carcinomas. *DDR1* encoding receptor tyrosine kinase was overexpressed in ductal carcinomas.

The *DVL1* gene encoding protein involved in Wnt signaling and the leucine-rich repeat protein *ASPN* (asporin) were upregulated in lobular carcinomas. Ductal carcinomas showed upregulated genes which are involved in cell proliferation, signaling and cell cycle regulation, including *RHOU*, member of the Rho family of GTPases stimulating quiescent cells to reenter the cell cycle; *PCSK6* encoding a calcium-dependent serine endoprotease; *PRKCI* encoding calcium-independent and phospholipid-dependent protein kinase C; *PPP3CB* encoding protein phosphatase 3; and *CKS2* encoding a component of the CDC28 protein kinase. Epithelial membrane protein 1 (*EMP1*) was the only upregulated gene involved in cell growth and proliferation in lobular carcinomas. These changes were accompanied by the differential expression of transcription regulators. Majority of genes with this function were upregulated in ductal carcinomas such as *AHCTF1*, *IRAK1*, *NRIP1*, *ADNP*. Overexpressed genes in lobular carcinomas were the tumor suppressor *FOXP1* and another transcription regulator *MID1*.

The genes encoding proteins involved in ubiquitin-mediated proteolysis, such as *USP3*, *RKHD2* and *TTC3*, and nuclear components, such as *DTL*, *GLCC11*, *TTC14*, *FAM54A*, *HIST1H3B*, were upregulated in ductal carcinomas. Majority of genes encoding proteins with enzyme

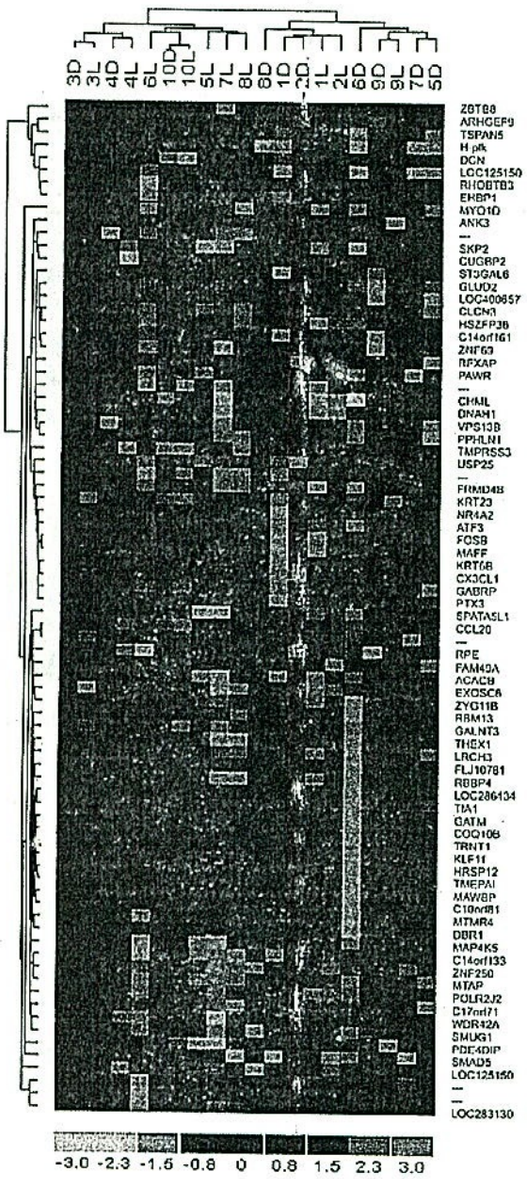


Figure 2
Hierarchical clustering of normal ductal and lobular cells based on 82 probe sets found by both rank products and pairwise analysis. Ductal and lobular cells from the same patients tend to cluster together, and they can not be well separated from each other. This suggests expected similarities of expression signatures in normal epithelial (ductal and lobular) cells of mammary gland tree. 1–10, case numbers; D, ductal normal cells; L, lobular normal cells.

activity or implicated in metabolism were also upregulated in ductal carcinomas (*STK4, SLC1A2, B3GALT3, OSBPL10, CRBN, CHML, YWHAB*).

Two lobular and three ductal carcinomas were estrogen receptor-negative, whereas three lobular and two ductal carcinomas were estrogen receptor-positive. Hierarchical clustering using all probe sets was performed to determine whether receptor-positive and receptor-negative tumors could be separated. The tumors of the same histological type showed similar gene expression profiles without differences in relation to ER status as well as to other clinical parameters such as nodal status, stage and the expression of other immunohistochemical markers (data not shown).

Validation by immunohistochemistry on tissue microarrays

Seven differentially expressed genes (*KRT5, KRT6* and *KRT17* between tumor and normal cells, *CDH1, EMP1, DDR1* and *DVL1* between lobular and ductal carcinomas) were verified by immunohistochemical detection of proteins on TMA slides comprising of cores from 119 cases. The clinical and histopathological characteristics of these patients are shown in Table 5. The reduced expression or absence of cytokeratins 5/6 and 17 (*KRT5, KRT6, KRT17*) was found in both tumor tissues in comparison to terminal duct lobular units in 22 normal mammary tissues ($p < 0.0001$) (Table 6 and Figure 5). In a majority of ducts and lobules including TDLU, these cytokeratins were expressed in both basal and luminal cells, verifying the previously described variability of the expression of basal cytokeratins and their relationship to the cellular origin [51]. Cytokeratin 5 and 17 have also been found in a subset of breast cancer and identified patients with poor clinical outcome [31].

E-cadherin (CDH1) has successfully separated ductal and lobular invasive carcinomas. It was absent in 93.3% of lobular tumors compared with only 15% of ductal tumors ($p < 0.0001$). Epithelial membrane protein 1 (EMP1), discoidin domain receptor 1 (DDR1) and human homolog of the Drosophila dishevelled gene (DVL1) were found by pairwise comparison analysis to be differentially expressed between lobular and ductal carcinomas. Immunohistochemistry confirmed higher expression of DVL1 and EMP1 in lobular carcinomas and of DDR1 in ductal carcinomas ($p < 0.0001$) (Table 7 and Figure 5). Of the special type carcinomas included on TMA slides, a papillary and two medullary carcinomas were positive for basal cytokeratins, one out of three medullary carcinomas was positive for EMP1 and E-cadherin, a ductal-lobular carcinoma was positive for DDR1, all other special type carcinomas were negative for these markers, and finally none were positive for DVL1 (data not shown).

Table 3: Genes differentially expressed between tumor versus normal cells by both rank products and pairwise analysis

Probe Set	Gene Symbol	Gene Title	Fold-change			
			Tduc		Tlob	
Lobular carcinoma vs normal cells (↑ – upregulated in Tlob, ↓ – downregulated in Tlob)			L	D	L	D
Extracellular matrix-receptor interaction and focal adhesion (epithelial-mesenchymal transition)						
227314_at	ITGA2↓	Integrin, alpha 2	n	n	-4.7	-6.2
201438_at	COL6A3↑	Collagen, type VI, alpha 3	n	n	4.5	7.7
203083_at	THBS2↑	Thrombospondin 2	n	n	6.5	6.5
205713_s_at	COMP↑	Cartilage oligomeric matrix protein	n	n	6.7	8.1
226237_at	COL8A1↑	Collagen, type VIII, alpha 1	n	n	8.4	5.9
225681_at	CTHRC1↑	Collagen triple helix repeat containing 1	n	n	18.5	48.4
219087_at	ASPN↑	Asporin	n	n	23.3	22.1
WNT signalling						
223121_s_at 223122_s_at	SFRP2↑	Secreted frizzled-related protein 2	n	n	5.8 3.9	7.4 7.1
229802_at	WISP1↑	WNT1 inducible signaling pathway protein 1	n	n	9.1	12.4
Actin, calcium and metal ion binding						
204455_at	DST↓	Dystonin	n	n	-22.2	-18.3
214087_s_at	MYBPC1↓	Myosin binding protein C, slow type	n	n	-10.1	-12.2
209301_at	CA2↓	Carbonic anhydrase II	n	n	-9.2	-8.7
206509_at	PIP↓	Prolactin-induced protein	n	n	-7.0	-6.3
201131_s_at	CDH1↓	Cadherin 1, type 1, E-cadherin	n	n	-6.2	-7.2
226777_at	ADAM12↑	ADAM metallopeptidase domain 12	n	n	5.7	5.3
213905_x_at	BGNISDCCAG↑	Biglycan/serologically defined colon cancer antigen 33	n	n	8.3	7.2
Other						
227742_at	CLIC6↓	Chloride intracellular channel 6	n	n	-9.3	-8.2
225817_at	CGNL1↓	Cingulin-like 1	n	n	-5.9	-7.8
219850_s_at	EHF↓	Ets homologous factor	n	n	-4.1	-6.3
213975_s_at	LYZ//LRB1↑	Lysozyme	n	n	10.8	7.3
Ductal carcinoma vs normal cells (↓ – upregulated in Tduc, ↓ – downregulated in Tduc)						
Regulation of transcription						
202672_s_at	ATF3↓	Activating transcription factor 3	-4.5	-7.7	n	n
226147_s_at	PIGR↓	Polymeric immunoglobulin receptor	-4.8	-10	n	n
Cytokine and growth factor activity						
209466_x_at 211737_x_at	PTN↓	Pleiotrophin	-5.8 -7.1	-8.3 -12.5	n	n
823_at	CX3CL1↓	Chemokine (C-X3-C motif) ligand 1	-3.6	-8.3	n	n
Ion transport and metabolism						
203296_s_at	ATP1A2↓	ATPase, Na+/K+ transporting, alpha 2	-8.2	-9.6	n	n
204259_at	MMP7↓	Matrix metallopeptidase 7	-4.5	-7.2	n	n

Table 3: Genes differentially expressed between tumor versus normal cells by both rank products and pairwise analysis *(Continued)*

212353_at	*SULF1*↓	Sulfatase 1	4.6	4.0	n	n
Other						
1553602_at	*LOC118430*↓	Small breast epithelial mucin	-7.1	-10.4	n	n
223623_at	*ECRG4*↓	Esophageal cancer related gene 4 protein	-6.6	-5.6	n	n
206548_at	*FLJ23556*↓	Hypothetical protein FLJ23556	-6.3	-11.2	n	n
218585_s_at	*DTL*↑	Denticleless homolog (Drosophila)	3.6	4.8	n	n

Tumor vs normal cells
(↑ – upregulated in tumor, ↓ – downregulated in tumor)

Extracellular matrix-receptor interaction and focal adhesion (epithelial-mesenchymal transition)						
224823_at	*MYLK*↓	Myosin, light polypeptide kinase	-3.5	-3.4	-5.9	-5.4
204620_s_at 221731_x_at	*CSPG2*↑	Chondroitin sulfate proteoglycan 2 (versican)	3.0 3.5	4.9 5.3	4.5 4.0	5.8 6.1
221729_at	*COL5A2*↑	Collagen, type V, alpha 2	3.5	4.4	9.3	11.3
211161_s_at	*COL3A1*↑	Collagen, type III, alpha 1	4.1	3.3	9.8	14.9
212464_s_at	*FN1*↑	Fibronectin 1	5.2	5.0	8.0	10.5
202311_s_at 202310_s_at	*COL1A1*↑	Collagen, type I, alpha 1	5.6 5.1	6.0 5.1	12.7 10.4	13.0 10.8
37892_at	*COL11A1*↑	Collagen, type XI, alpha 1	6.7	7.8	6.6	5.9
202404_s_at	*COL1A2*↑	Collagen, type I, alpha 2	6.8	10.1	30.5	18.3
Ion and electron transport						
205044_at	*GABRP*↓	Gamma-aminobutyric acid (GABA) A receptor, pi	-4.2	-8.7	-6.2	-8.6
220230_s_at	*CYB5R2*↓	Cytochrome b5 reductase 2	-3.6	-3.8	-4.3	-4.2
Regulation of transcription						
220625_s_at	*ELF5*↓	E74-like factor 5 (ets domain transcription factor)	-4.0	-4.8	-5.9	-8.4
209292_at	*ID4*↓	Inhibitor of DNA binding 4	-2.9	-5.1	-10.8	-4.5
Actin and actin-binding proteins						
203951_at	*CNN1*↓	Calponin 1, basic, smooth muscle	-6.7	-6.6	-6.9	-7.3
243140_at	*ACTA2*↓	Actin, alpha 2, smooth muscle, aorta	-4.0	-4.1	-8.7	-4.0
WNT signaling						
202037_s_at	*SFRP1*↓	Secreted frizzled-related protein 1	-7.1	-8.8	-8.5	-7.3
Intermediate filament proteins						
209351_at	*KRT14*↓	Keratin 14	-22.9	-31.0	-21.9	-24.0
205157_s_at	*KRT17*↓	Keratin 17	-17.7	-21.4	-9.9	-12.6
212730_at	*DMN*↓	Desmuslin	-10.0	-10.4	-7.2	-7.5
201820_at	*KRT5*↓	Keratin 5	-8.9	-12.7	-11.3	-12.1
Other						
229947_at	*PI15*↓	Peptidase inhibitor 15	-6.5	-7.5	-18.5	-15.0
205380_at	*PDZK1*↓	PDZ domain containing 1	-4.5	-5.8	-4.2	-6.4
213909_at	*LRRC15*↑	Leucine rich repeat containing 15	6.2	5.7	7.3	11.8

Tduc – ductal tumor; Tlob – lobular tumor; ◌ – normal ductal cells; L – normal lobular cells; n – not differentially expressed among top 20 genes.
[1]Affymetrix probe set number; If there was more than one probe set for the particular gene found among top 20 changed genes (according to the fold change), the probe set with the highest fold change is used and number of other probe set is indicated.

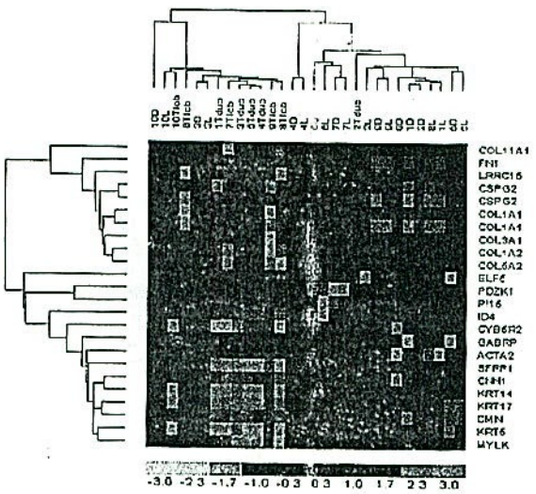


Figure 3
Hierarchical clustering of tumor and normal samples based on 25 probe sets common to ductal and lobular carcinoma versus normal cells identified by both rank products and pairwise analysis. Tumor samples are grouped together, however, gene expression profiles of normal cell types from cases 10 and 3 are different from those of other normal cells, and tumor cells from case 2 are different from those of other tumor cells. 1–10, case numbers; D, ductal normal cells; L, lobular normal cells; Tduc, ductal tumor; Tlob, lobular tumor.

Validation of asporin expression by in situ hybridization

Combined RP and pairwise comparison showed that *ASPN* was one of the most upregulated genes in lobular carcinomas, when compared with normal ductal (fold change 23.3) and lobular (fold change 22.1) cells as well as with ductal carcinomas (fold change 3.9). None of the available antibodies against asporin worked on paraffin sections, therefore we utilised chromogenic *in situ* hybridization to detect asporin mRNA in frozen sections. All five lobular carcinomas appeared positive, whereas five cases of ductal carcinomas were negative or weakly and focally positive which is in agreement with the microarray data (Figure 6).

Validation of CTHRC1, ASPN *and* COL3A1 *by PCR*

Besides asporin, *CTHRC1* was also upregulated in lobular cancer cells, when compared with normal ductal cells (fold change 48.4) and lobular cells (fold change 18.5), and *COL3A1* was upregulated in both IDC and ILC cells, when compared with normal ductal cells (fold change 3.3 and 14.9, respectively) and lobular cells (fold change 4.1 and 9.8, respectively). As both RNA and cDNA were fully utilized for amplification, we used PCR amplification products (see Methods) for validation by another PCR with specific primers. The results of semi-quantitative PCR correlated with the microarray data. Spearman coefficients were r = 0.85, r = 0.73 and r = 0.70 for *CTHRC1*, *ASPN* and *COL3A1*, respectively (all significant at $p < 0.0001$) (Figure 7).

Discussion

We examined 30 samples (microdissected tumor and normal ductal and lobular cells) from postmenopausal patients with lobular and ductal invasive breast carcinomas using Affymetrix arrays. Genes differentially expressed between the normal ductal and lobular cell types, which are less likely to be affected by fluctuating levels of female hormones as they were derived from postmenopausal women, are involved in ion transport and protein kinase cascade, protein biosynthesis and modification, proteolysis and metabolism, regulation of transcription and cell growth. However, hierarchical clustering of normal cell types based on the 82 probe sets identified by both RP and pairwise comparison showed that the gene expression profiles of normal ductal and lobular cells taken from the same patient are more similar to each other than ductal cells or lobular cells from different patients, and thus could not be well separated from each other. This may suggest expected similarities between expression signatures in normal epithelial (ductal and lobular) cells of the mammary gland. Since both IDC and ILC are believed to start in the terminal duct lobular unit (TDLU) of the breast [1,2], and these normal cell populations showed similar gene expression profiles, it is likely that the different morphological appearances of the two tumor types are mediated by differences in their mechanisms of carcinogenesis.

Combined pairwise comparison and RP analysis revealed that ductal and lobular carcinomas have a number of genes in common, however, they can be discriminated both at the gene and protein levels in our study. cDNA microarrays have previously been used to distinguish between IDC and ILC [38,39]. Unsupervised clustering of tumors failed to separate the two subtypes. There were 8 genes identified by MaxT permutation analysis using t tests, significance analysis for microarrays (SAM) and prediction analysis for microarrays (PAM) (E-cadherin, survivin, cathepsin B, TPI1, SPRY1, SCYA14, TFAP2B, and thrombospondin 4), and an additional 3 were identified by SAM and PAM (osteopontin, HLA-G, and CHC1) [38]. It has also been found that over half of ILCs differed from IDCs in global transcription programs, whereas the remaining ILCs closely resembled IDCs. Fifty two percent of the ILCs ("typical" ILCs) clustered together and displayed different gene expression profiles from the IDCs, whereas the other ILCs ("ductal-like" ILCs) were distributed between different IDC subtypes. Many of the differ-

Table 4: Genes differentially expressed in lobular versus ductal invasive carcinomas by rank products and/or pairwise analysis

Probe Set[1]	Gene Symbol	Gene Title	Rank products	Pairwise comparison
			Fold-change	Incr/Decr
Cell adhesion				
201131_s_at	*CDH1*↓	Cadherin 1, type 1, E-cadherin (epithelial)	-6.0	3/19
207169_x_a	*DDR1*↓	Discoidin domain receptor family, member 1	n	5/19
204776_at	*THBS4*↑	Thrombospondin 4	n	19/2
219087_at	*ASPN*↑	Asporin (LRR class 1)	3.9	23/0
Wnt signalling				
223168_at	*RHOU*↓	RAS homolog gene family, member U	n	2/20
203230_at	*DVL1*↑	Dishevelled, dsh homolog 1 (Drosophila)	n	20/0
Protein ubiquitination				
215392_at	*USP3*↓	Ubiquitin specific peptidase 3	-5.0	1/21
218247_s_at	*RKHD2*↓	Ring finger and KH domain containing 2	-4.6	0/19
1569472_s_at	*TTC3*↓	Tetratricopeptide repeat domain 3	-4.5	0/23
Regulation of transcription				
226115_at	*AHCTF1*↓	AT hook containing transcription factor 1	-4.0	0/22
201587_s_at	*IRAK1*↓	Interleukin-1 receptor-associated kinase 1	n	2/19
202599_s_at	*NRIP1*↓	Nuclear receptor interacting protein 1	n	0/19
201773_at	*ADNP*↓	Activity-dependent neuroprotector	n	0/20
203637_s_at	*MID1*↑	Midline 1 (Opitz/BBB syndrome)	4.0	20/0
235444_at	*FOXP1*↑	Forkhead box P1	n	19/4
Electron and protein transport				
204351_at	*S100P*↓	S100 calcium binding protein P	-5.3	0/19
225177_at	*RAB11FIP1*↓	RAB11 family interacting protein 1 (class I)	-3.5	2/21
204041_at	*MAOB*↑	Monoamine oxidase B	3.5	20/4
212902_at	*SEC24A*↑	SEC24 related gene family, member A	3.5	19/2
Cell cycle regulation, cell proliferation and signaling				
204170_s_at	*CKS2*↓	CDC28 protein kinase regulatory subunit 2	-4.8	2/20
207414_s_at	*PCSK6*↓	Proprotein convertase subtilisin/kexin type 6	n	4/19
209678_s_at	*PRKCI*↓	Protein kinase C, iota	n	0/22
209817_at	*PPP3CB*↓	Protein phosphatase 3, catalytic subunit, beta isoform (calcineurin A beta)	n	0/24
201324_at	*EMP1*↑	Epithelial membrane protein 1	n	21/0
Metabolism and enzyme activity				
205509_at	*CPB1*↓	Carboxypeptidase B1 (tissue)	-11.1	0/19
223746_at	*STK4*↓	Serine/threonine kinase 4	-4.5	2/20
225491_at	*SLC1A2*↓	Solute carrier family 1 (glial high affinity glutamate transporter), member 2	-4.1	0/20
211379_x_at	*B3GALT3*↓	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 3	-4.0	0/20
219073_s_at	*OSBPL10*↓	Oxysterol binding protein-like 10	-4.0	1/21
229366_at	*CRBN*↓	Cereblon	-3.6	0/20
226350_at	*CHML*↓	Choroideremia-like (Rab escort protein 2)	n	0/24
217717_s_at	*YWHAB*↓	Tyrosine 3-monooxygenase/tryptophan 5-mono-oxygenase activation protein, beta polypeptide	n	1/20
49452_at	*ACACB*↑	Acetyl-Coenzyme A carboxylase beta	n	19/0
220770_s_at	*LOC63920*↑	Transposon-derived Buster3 transposase-like	5.3	24/0
Actin and cytoskeletal protein binding				
211776_s_at	*EPB41L3*↓	Erythrocyte membrane protein band 4.1-like 3	-5.3	0/21
201341_at	*ENC1*↓	Ectodermal-neural cortex (with BTB-like domain)	n	19/3
Nuclear components				
218585_s_at	*DTL*↓	Denticleless homolog (Drosophila)	-4.6	1/19
225706_at	*GLCCI1*↓	Glucocorticoid induced transcript 1	-4.5	0/25
241863_x_at	*TTC14*↓	Tetratricopeptide repeat domain 14	-4.4	1/20
228069_at	*FAM54A*↓	Family with sequence similarity 54, member A	-3.8	0/20
208576_s_at	*HIST1H3B*↓	Histone 1, H3b	-3.3	1/19
Other				
1568838_at	---↓	Full-length cDNA clone CS0DF033YE17 of Fetal brain of Homo sapiens (human)	-5.0	1/20
226067_at	*C20orf114*↓	Chromosome 20 open reading frame 114	-4.9	0/19
1553979_at	---↓	Homo sapiens, clone IMAGE:3906992, mRNA	-4.1	0/19
230534_at	*MGC15634*↓	Hypothetical protein MGC15634	-3.6	2/20
226671_at	---↓	CDNA clone IMAGE:4797120	-3.8	2/20
219768_at	*VTCN1*↑	V-set domain containing T cell activation inhibitor 1	7.8	21/2
214657_s_at	*TncRNA*↑	Trophoblast-derived noncoding RNA	4.5	21/0
213004_at	*ANGPTL2*↑	Angiopoietin-like 2	n	19/4

↑ – upregulated in lobular carcinoma; ↓ – downregulated in lobular carcinoma; n – gene not found by rank products at the level of 10% of false positives, Incr/decr – increase or decrease in lobular versus ductal carcinoma by pairwise comparison.
[1]All 32 probe sets identified by combined pairwise comparison and rank products are included, also a number of genes found by pairwise comparison were validated by immunohistochemistry (DVL1, DDR1, EMP1), therefore, functional categories also include named genes identified by pairwise analysis.

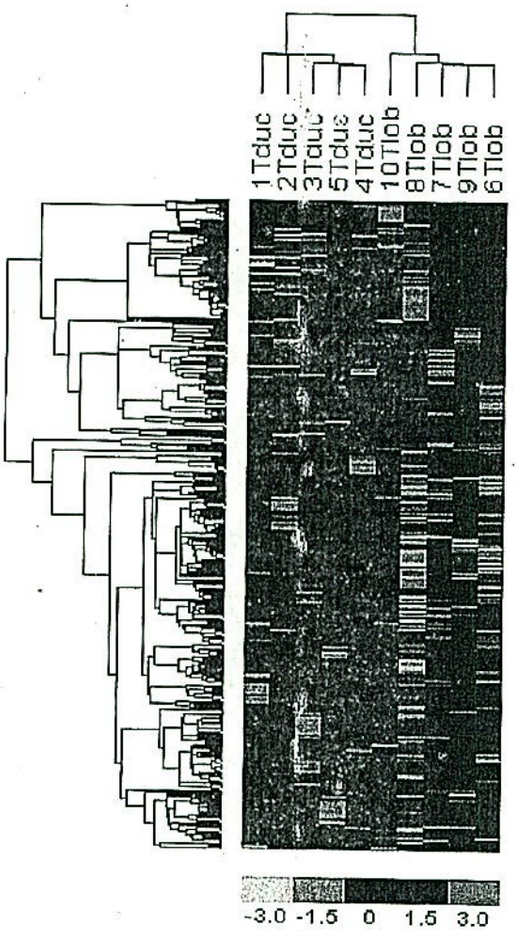


Figure 4
Hierarchical clustering of invasive ductal and lobular breast carcinomas based on 325 probe sets identified by rank products and/or pairwise analysis. Tumor types are well separated. 1–10, case numbers; Tduc, ductal tumor; Tlob, lobular tumor.

entially expressed genes encode for proteins involved in cell adhesion/motility, lipid/fatty acid transport and metabolism, immune/defense response, and electron transport. Many genes distinguishing between typical and ductal-like ILCs are involved in regulation of cell growth and immune response [39]. However, these two previous studies examined whole tumor tissues without microdissection, suggesting that expression of a number of genes could be related not only to tumor cells but also to other components of mammary tissue such as stromal, adipose, endothelial etc. Our study is the first full genome analysis of microdissected ductal and lobular tumor and normal cells reporting both normal mammary epithelium- and cancer-specific genes expression profiles.

Importantly, *CDH1* (E-cadherin gene) was downregulated in our lobular carcinomas, and immunohistochemistry confirmed this loss at the protein level within tumors. E-cadherin is considered to be the most important cell adhesion molecule in the mammary gland. It acts as a tumor suppressor inhibiting invasion and metastasis. Mutations of this gene are correlated with gastric, breast, colorectal, thyroid and ovarian cancer. During tumor progression, E-cadherin can be functionally inactivated or silenced by different mechanisms such as post-translational control, somatic mutations, downregulation of gene expression through promoter hypermethylation, histone deacetylation, and transcriptional repression [52,53]. The latter induces cellular responses leading to the conversion of epithelial cells into invasive mesenchymal-like cells with increased motility and invasiveness, and this process is called an epithelial-mesenchymal transition (EMT) [52]. To date, it is believed that lost, non-polar or cytoplasmic expression of E-cadherin protein and/or transcriptional repression of its mRNA are hallmarks of EMT in cancer progression [53-55]. It has also been shown that several proteins such as fibronectin and integrin αvβ6 [54], Ets, TGFβ, FGF-1,-2,-8, α-SMA, collagen type I, III and thrombospondins increase in abundance during EMT [56], conversely, amongst proteins that decrease in abundance are E-cadherin and cytokeratins [54]. According to our results, collagen type I and III, fibronectin and Ets domain transcription factor are upregulated and cytokeratins are downregulated in both tumor cell types. The expression of collagens and other mesenchyme-associated genes in microdissected breast cancer cells was also confirmed by Nishidate and co-workers [57]. In addition, pairwise comparisons revealed that thrombospondin 4 was upregulated only in lobular cancer cells, which agrees with the literature [38]. Thus we propose that the EMT plays a role in both tumor types but appears to be more important in lobular carcinomas. The EMT phenomenon seems to be promising because multiple molecules involved in EMT, such as receptor- and SRC-family tyrosine kinases, RAS and other small GTPases, can be envisioned as targets for anti-EMT therapy [58].

The tissue microenvironment, including the ECM-cell and cell-cell interactions, plays an important role in both normal mammary gland development and cancer. Neoplastic transformation of cells dramatically alters the synthesis of proteoglycans and other ECM proteins both in tumor and the surrounding matrix [59]. This can stimulate the growth and spread of tumor cells by decreasing the adhesive functions of the ECM [60]. Both tumor types examined show upregulated genes involved in tumor-ECM

Table 5: Clinical and histopathological characteristics of TMA cases

Features	IDC	ILC
G1	6 (7.5%)	15 (51.7%)
G2	57 (71.3%)	11 (37.9%)
G3	17 (21.2%)	3 (10.3%)
ER+	57 (71.3%)	19 (65.5%)
PgR+	50 (62.5%)	17 (58.6%)
HER-2+	12 (15%)	1 (3.4%)
Bcl-2+	13 (16.3%)	4 (13.8%)
N_0	56 (70%)	21 (72.4%)
N_1	16 (20%)	6 (20.7%)
N_2	8 (10%)	2 (6.9%)
Total number of cases	80	29

ER, estrogen receptor; G1-3, grade 1–3; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; N_{0-1}, lymph node status; PgR, progesterone receptor; TMA, tissue microarray.

interactions, cell adhesion and migration processes including metastasis. Expression of majority of the proteins encoded by these genes is related to TGFβ or Wnt signaling, and both the TGFβ and Wnt pathways may affect ECM.

Asporin is a cartilage extracellular protein that has been reported to be associated with knee and hip osteoarthritis. This leucine-rich repeat protein, was shown to interact with and inhibit TGFβ signaling which is thought to lead to insufficient quantities of aggrecan and type II collagen in osteoarthritis [61,62]. Asporin was more upregulated in our lobular carcinomas when compared with ductal tumors as well as with normal cell types. Overexpression of asporin mRNA in lobular carcinomas was then confirmed by chromogenic *in situ* hybridization and PCR. In support of our findings, upregulation of this gene has also been described in microdissected androgen-independent prostate cancer cells using Affymetrix Human Genome U133A GeneChips [63]. The authors did not discuss it and importantly, asporin has not been related to carcinogenesis to date.

We have found another candidate gene, collagen triple helix repeat containing 1 (*CTHRC1*), which was upregulated in ILC in comparison with normal cells, and its expression was also validated by PCR. Aberrant expression of *CTHRC1* has recently been reported in human solid tumors, including cancers of the gastrointestinal tract, lung, breast, thyroid, ovarian, cervix, liver, and the pancreas. It is associated with cancer tissue invasion and metastasis and potentially plays important functional roles in cancer progression, perhaps by increasing cancer cell migration [64]. TGFβ upregulates CTHRC1, versican, ADAM12, and downregulates SFRP1 and E-cadherin [65-67]. The loss of SFRP1 is known to be associated with breast cancer progression and poor prognosis in early stages [68], and a similar expression profile is seen in our study. *SFRP1* is downregulated and versican is upregulated in both tumor types. Furthermore, pairwise comparison identified other overexpressed genes in lobular carcinoma such as *SFRP2* and *ADAM12*. E-cadherin, which is downregulated in lobular cancer cells, can also be repressed by TGFβ-induced expression of transcription factor complexes [69]. TGFβ signaling inhibitors have been shown to prevent EMT, to inhibit mammary tumor viability and to block metastasis in various murine models [70]. According to these results, we propose that deregulated TGFβ signaling is likely to be more important in lobular carcinogenesis.

Wnt signaling molecules are also expressed in our samples in several comparisons. The Wnt proteins are small secreted glycoproteins which are involved in the self-renewal of stem cells and may be responsible for the maintenance of mature tissues [71]. On binding to Frizzled receptors, Wnts can activate canonical (β-catenin-dependent) and/or non-canonical (β-catenin-independ-

Table 6: Immunohistochemical staining of cytokeratins in tumor and normal tissues

Antibodies	Tumors		Normal tissues (n = 22)	Fisher's p value
Cytokeratin 5/6	Ductal carcinomas (n = 80)	9 (11.2%)	22 (100%)	<0.0001
	Lobular carcinomas (n = 29)	3 (10.3%)		<0.0001
Cytokeratin 17	Ductal carcinomas (n = 80)	8 (10%)	22 (100%)	<0.0001
	Lobular carcinomas (n = 29)	2 (6.9%)		<0.0001

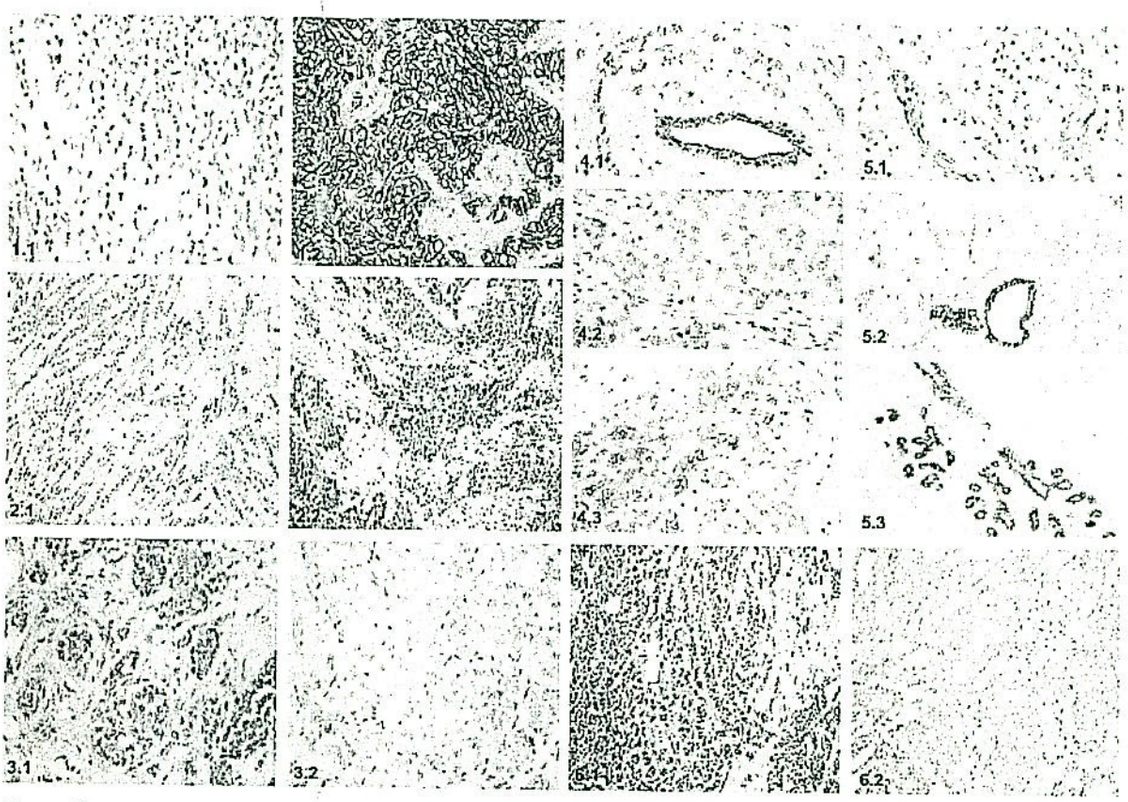


Figure 5

Representative immunohistochemical staining for the selected proteins: E-cadherin, DDR1, DVL1, cytokeratin 5/6, cytokeratin 17 and EMP1. 1.1. E-cadherin is negative in lobular carcinoma; 1.2. E-cadherin is positive in ductal carcinoma; 2.1. DDR1 is negative in lobular carcinoma; 2.2. DDR1 is positive in ductal carcinoma; 3.1. DVL1 is positive in lobular carcinoma; 3.2. DVL1 is negative in ductal carcinoma; 4.1. Cytokeratin 5/6 is negative in lobular carcinoma cells, but its expression is retained in normal ductal epithelial cells; 4.2. Cytokeratin 5/6 is negative in ductal carcinoma cells; 4.3. Duct lobular unit in normal mammary gland tissue is positive for cytokeratin 5/6; 5.1. Cytokeratin 17 is negative in lobular carcinoma cells, but its expression is retained in normal ductal epithelial cells; 5.2. Cytokeratin 17 is negative in ductal carcinoma cells, but its expression is retained in normal ductal epithelial cells; 5.3. Duct lobular unit in normal mammary gland tissue is positive for cytokeratin 17; 6.1. EMP1 is positive in lobular carcinoma; 6.2. EMP1 is negative in ductal carcinoma.

ent Wnt/planar cell polarity pathway and Wnt/Ca2+ pathway) Wnt signaling [72,73]. Downregulation of frizzled related proteins has been described in breast cancer [68]. Of these, *SFRP1* is downregulated in both types of our tumor cells, and genes involved in calcium regulation pathway are not significantly changed. The promoter of fibronectin 1 contains LEF/TCR-binding sites, making it a direct target of canonical Wnt signaling [59,74]. Fibronectin 1 is also upregulated in both tumor types. However, there is a difference in Wnt signaling between ILC and

Table 7: Immunohistochemistry differentiates ductal and lobular carcinomas

Antibodies	Ductal carcinomas (n = 80)	Lobular carcinomas (n = 29)	Fisher's *p* value
E-cadherin	68 (85%)	2 (6.7%)	<0.0001
DVL1	20 (25%)	28 (96.5%)	<0.0001
EMP1	13 (16.3%)	27 (93.1%)	<0.0001
DDR1	77 (96.2%)	4 (13.8%)	<0.0001

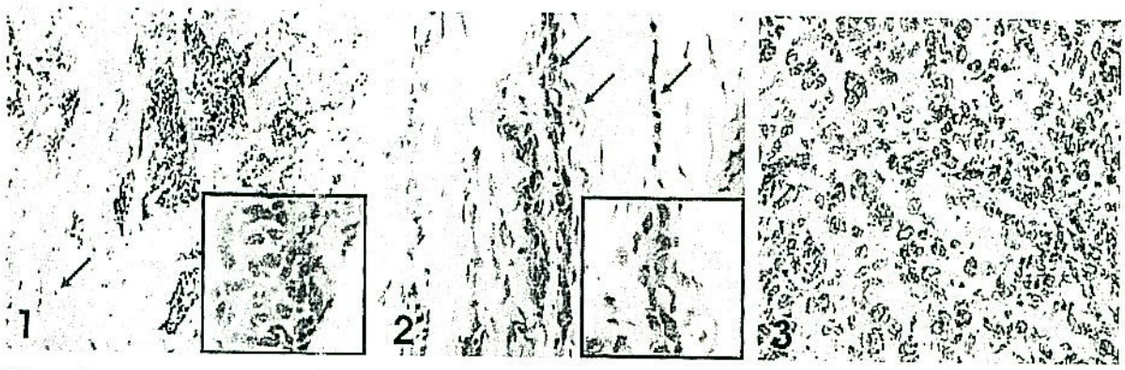


Figure 6
Asporin mRNA detection by chromogenic *in situ* hybridization. 1–2. Various magnifications (×100, ×400, ×400) of lobular carcinoma; red arrows, lobular tumor cells are positive; blue arrows, stromal cells are negative; 3. Ductal carcinoma is negative (×200).

IDC. *SULF1* (sulfatase 1) is upregulated and *MMP7* is downregulated only in IDCs. *MMP7* is a confirmed Wnt target [75] and it has been shown to be activated in both canonical [76,77] and non-canonical Wnt signaling [78]. Binding Wnt ligands to frizzled receptors is regulated by the 6-O sulfation-desulfation of cell surface heparan sulfates (HSs) by sulfatase 1. Sulfated HSs bind to Wnt ligand with high affinity and inhibit Wnt signaling. Sulfatase 1 removes 0–6 sulfates from HSs and reduces their binding to Wnt ligands which in turn allows the formation of functional Wnt-Frizzled complexes and thus promotes Wnt signaling [79,80]. Frizzled-related proteins also have heparin-binding domains that promote the formation of Wnt-Frizzled complexes [81]. Although *SULF1* is upregulated, *SFRP1* and *MMP7* are downregulated in ductal cancer cells, whereas *SFRP2* and other Wnt molecules, such as *DVL1* and *WISP1*, are upregulated in lobular cancer cells. This suggests that Wnt signaling is activated in ILC cells but not in IDC cells. Since mRNA level of β-catenin was not changed between normal and tumor cells, the expression of Wnt molecules appears to be β-catenin-independent, favoring the non-canonical Wnt signaling in ILC. There is evidence that Wnts acting through the non-canonical pathway can promote tumor progression [82,83] which may also be true in ILC.

In addition to Wnt molecules such as *DVL1*, pairwise comparison revealed that *EMP1*, gene encoding a tumor-associated membrane protein involved in cell-cell interactions and proliferation control [84], was upregulated, whereas *DDR1*, epithelial-specific receptor kinase capable of binding Wnt5 and regulating the adhesion of mammary cells [85,86], was downregulated in ILCs. Immunohistochemistry has also confirmed the same differential expression of these three proteins in IDC and ILC on tissue microarrays. Thus all the evidence suggests that the two tumor types can be distinguished both at the gene and protein levels. Specific changes in gene and protein expression are likely to reflect the differences in mechanisms of carcinogenesis as well as the specific histological and clinical characteristics of these tumors derived from the same anatomical compartment, TDLU.

Conclusion

Microdissection of normal and tumor cell types from the breast and full genome expression analysis by Affymetrix arrays allowed us to provide novel data on breast cancer. Invasive lobular and ductal breast carcinomas can be differentiated both at the gene and protein levels. Despite analyzing only thirty samples from ten patients, the results are in good accordance with previous literature [14,38,39,57,64]. Our data provide evidence for deregulated TGFβ and Wnt signaling accompanied by the overexpression of mesenchyme-associated genes like the collagens, asporin and others which might be occurring in conjunction with an altered EMT. We propose that deregulated TGFβ signaling and EMT phenomenon are involved in both tumor types, but they seem to be more important in lobular carcinomas which is in concordance with the loss of E-cadherin expression and their distinct morphology from ductal tumors. In this study we report two candidate genes, asporin (*ASPN*) and collagen triple helix repeat containing 1 (*CTHRC1*), which might be significant in mammary gland carcinogenesis and may also be important either in cancer diagnosis or therapy. Besides E-cadherin, the proteins validated on tissue microarrays by immunohistochemistry (EMP1, DVL1, DDR1) may represent novel tissue markers helpful in the differentiation of ductal and lobular cancers. Further studies with larger sets of patients are needed to verify the gene

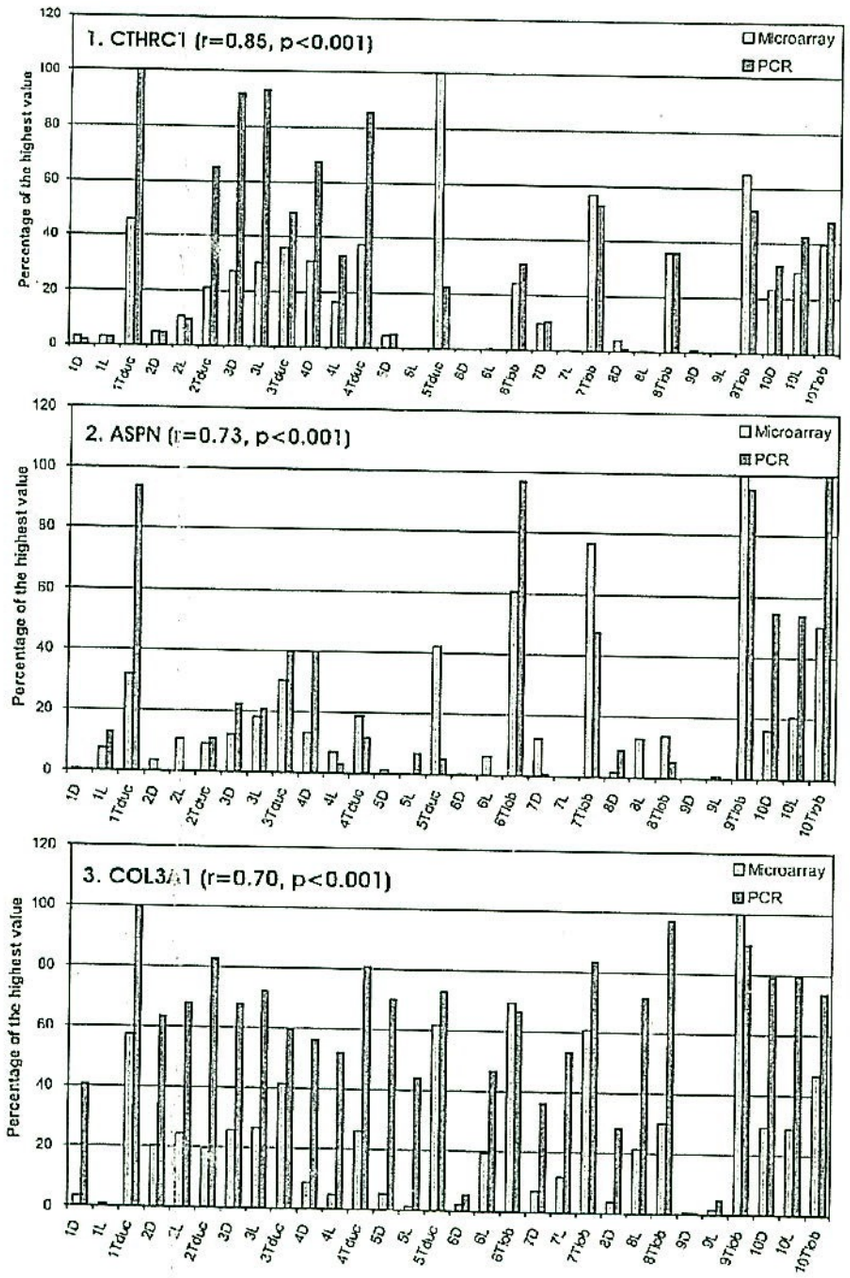


Figure 7
PCR validation of microarray results for *CTHRC1* (1), *ASPN* (2), and *COL3A1* (3). Fluorescence signals from Affymetrix probe sets (225681_at, 219087_at and 211161_s_at, respectively) and optical density of PCR bands (OD × cm) were transformed to a percentage of the highest value. 1–10, case numbers; Tduc, ductal tumor; Tlob, lobular tumor; D, normal ductal cells; L, normal lobular cells.

expression profiles of various histological types of breast cancer in order to determine molecular subclassifications, prognosis, and the optimum treatment strategies.

Abbreviations

ADAM12 = ADAM metallopeptidase domain 12; ASPN = Asporin; CK = Cytokeratin; CTHRC1 = Collagen triple helix repeat containing 1; DAVID = Database for Annotation, Visualization and Integrated Discovery; DDR1 = Discoidin domain receptor 1; DVL1 = Human homolog of the Drosophila dishevelled gene; ECM = Extracellular matrix; EMP1 = Epithelial membrane antigen 1; EMT = Epithelial-mesenchymal transition; ER = Estrogen receptor; GCOS = GeneChip Operating Software; IDC = Invasive ductal carcinoma; ILC = Invasive lobular carcinoma; ISH = *In situ* hybridization; IVT = *In vitro* transcription; PCR = Polymerase chain reaction; PgR = Progesterone receptor; RMA = Robust multiarray analysis; RNA = Ribonucleic acid; RP = Rank products analysis; SFRP1 = Secreted frizzled-related protein 1; TDLU = Terminal duct lobular unit; TGFβ = Transforming growth factor β ; TMA = Tissue microarray; WISP1 = WNT1 inducible signaling pathway protein 1.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

GT and JB contributed equally to this work. GT participated in collecting surgical specimens, cutting and evaluation of frozen sections, laser microdissection, immunohistochemistry, ISH, analysis of microarray results and drafting the manuscript. JB participated in the design of the study, collecting surgical specimens, RNA isolation, amplification and labeling, analysis of microarray results, ISH and PCR validation, drafting the manuscript. KB carried out the hybridization and scanning of the arrays and proofreading of the manuscript. WW performed data analysis and participated in drafting the manuscript. MD and MH were involved in laser microdissection. JEparticipated in collection and evaluation of frozen sections. JK participated in obtaining surgical specimens. EF and JS participated in tissue microarray construction. JS was involved in RNA isolation and PCR validation. PM participated in analysis of microarray results. ZK conceived of the study, participated in its design and coordination, as well as in collecting surgical specimens, evaluation of frozen sections and drafting the manuscript. All authors read and approved the final manuscript.

Additional material

Additional File 1

Supplementary tables. The data provided represent supplementary tables listing all differentially expressed probe sets found by both rank products and pairwise analysis between normal ductal and normal lobular cells, ductal carcinoma and normal ductal cells, ductal carcinoma and normal lobular cells, ductal carcinoma and lobular carcinoma cells, lobular carcinoma and normal ductal cells, and lobular carcinoma and normal lobular cells.

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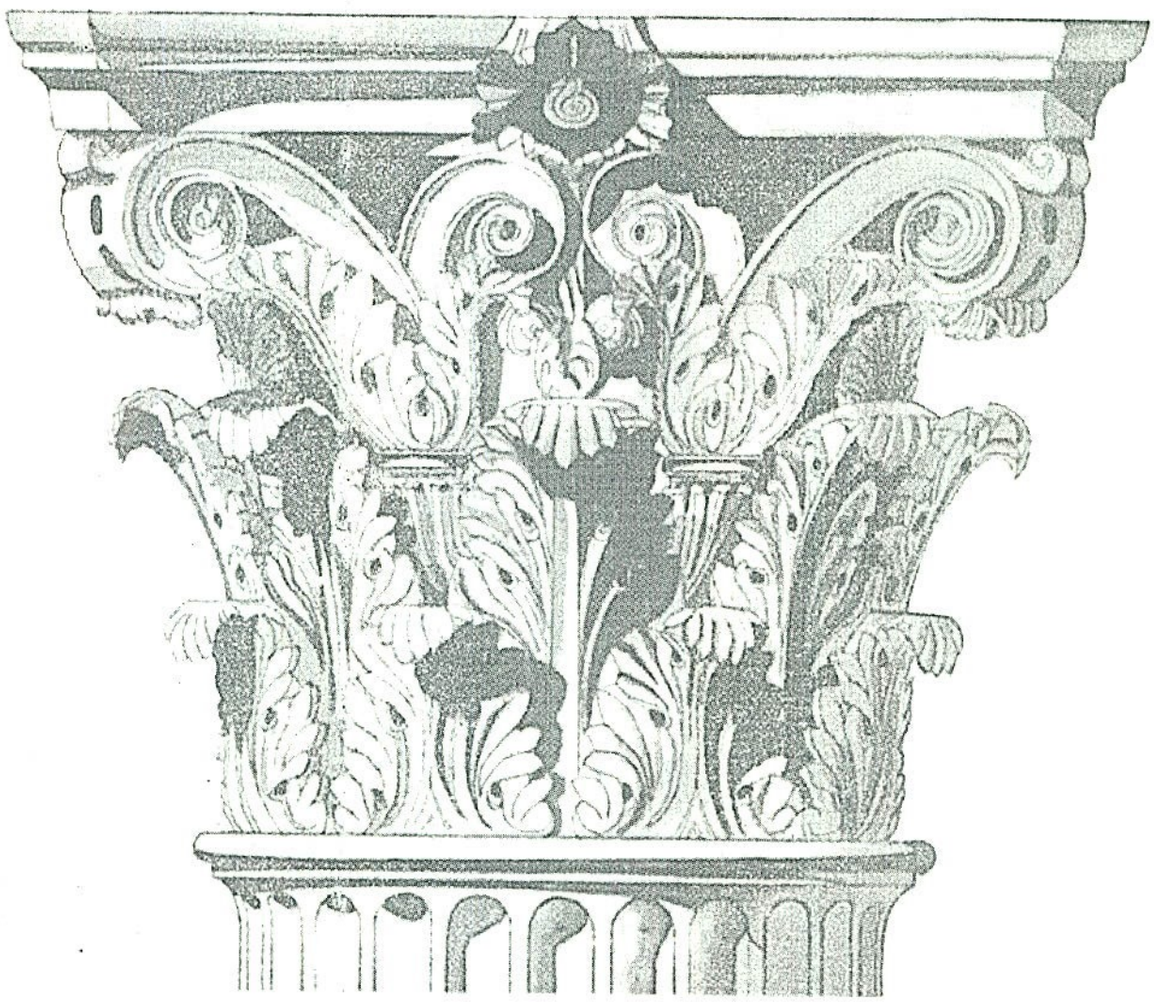
DIFFERENTIAL GENE EXPRESSION IN PROSTATE CANCER

Identification of genes expressed in prostate cancer, androgen-dependent and androgen-independent LNCaP cell lines, and characterization of TMPRSS2 expression

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Abstract

Prostate cancer is the most common solid tumor among men in Western industrialized countries. A major problem in prostate cancer treatment is the development of androgen-independence, as androgen-deprivation therapy is the basic therapy for the disease. Molecular mechanisms behind prostate cancer and androgen-independent growth development are poorly known.

In this study, subtractive hybridization was used for the generation of a cDNA library specific for prostate cancer. Analysis of the cDNA library revealed over-expression of several ribosomal proteins namely L4, L5, L7a, L23a, L30, L37, S14 and S18, in prostate cancer cell lines. Over-expression of L7a and L37 was also confirmed in prostate cancer tissue samples.

Further, cDNA array was used in order to examine differentially expressed genes in androgen-dependent and androgen-independent prostate cancer cell line LNCaP. Monoamine oxidase A, an Expressed Sequence Tag (EST) similar to rat P044, and EST AA412049 were highly over-expressed in androgen-dependent LNCaP cells. Tissue-type plasminogen activator, interferon-inducible protein p78 (MxB), an EST similar to galectin-1, follistatin, fatty acid-binding protein 5, EST AA609749, annexin I, the interferon-inducible gene 1-8U and phospholipase D1 were highly over-expressed in androgen-independent LNCaP cells. The EST similar to rat P044, the EST similar to galectin-1, follistatin, annexin I and the interferon-inducible gene 1-8U were also expressed in benign prostatic hyperplasia tissue. The Y-linked ribosomal protein S4, Mat-8, and EST AA307912 were highly expressed in benign prostatic hyperplasia tissue.

In situ hybridization of mouse embryos and adult mouse tissues revealed the expression of TMPRSS2 in the epithelium throughout the gastrointestinal, urogenital and respiratory tracts during development. In human multiple tissue RNA dot blot, the highest level of expression was detected in prostate, and lower levels in colon, stomach and salivary gland. TMPRSS2 transcript levels were significantly higher in prostate cancer tissue between benign and malignant epithelium of prostate cancer patients with untreated disease. Similarly, in poorly differentiated adenocarcinomas, expression in malignant tissue was significantly higher. Enzymatic mutation detection and direct sequencing of TMPRSS2 coding region revealed only one deletion in aggressive disease among 9 non-aggressive and 9 aggressive prostate cancer samples. No other mutations were found. Detected 7-base pair deletion leads to premature stop codon and disruption of serine protease substrate binding and catalytic active site.

We cloned several potential genes whose expression is changed during prostate cancer initiation or progression. These genes may serve as prostate cancer markers, and further studies are needed to clarify the expression of these proteins during the disease.

Keywords: gene expression profiling, prostatic neoplasms, messenger RNA, cultured tumor cells

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Oulu, November 2000

Markku Vaarala

5 Results

5.1 Characterization of prostate cancer-specific cDNA library (I)

Subtractive hybridization was used for the generation of a cDNA library specific for prostate cancer. One hundred clones were sequenced, 45 of which were clones containing artificial cloning vector fragments. Eight clones were cDNAs encoding ribosomal proteins (RP), namely L4, L5, L7a, L23a, L30, L37, S14 and S18. In slot blot analyses, expression of all these cDNAs was higher in at least one prostate cancer cell line (DU-145, LNCaP, PC-3) compared to BPH. RPL4 mRNA was significantly over-expressed only in androgen-dependent LNCaP cell line. RPL30 and RPL7a were significantly over-expressed in androgen-independent prostate cancer cell lines PC-3 and PC-3/DU-145, respectively. *In situ* hybridization was used for studying RPL7a and RPL37 expression in prostate tissue. Results indicate clearly elevated levels of RPL7a and RPL37 transcripts in prostate cancer tissue compared to benign prostatic tissue.

Among sequenced clones were several known, or lately published, genes to have functional significance in prostate carcinogenesis. In addition, there were some clones that were not identical to any published sequence. hPAP was present as two clones, as well as FAS apoptosis inhibitory molecule (Schneider *et al.* 1999). Seven clones encoding mitochondrial cytochrome proteins were detected. Na+/K+-ATPase β1 subunit, recently shown to be an androgen down-regulated gene in androgen responsive cell line and an over-expressed gene in androgen-independent cell lines (Blok *et al.* 1999), was also cloned. FHL2/DRAL cDNA found from this library was shortly identified as a novel tissue-specific co-activator of the androgen-receptor (Muller *et al.* 2000). However, all clones were screened in Northern blot and/or dot blot analyses, but we were not able to confirm differential expression in the above mentioned cases.

5.2 Differentially expressed genes in two LNCaP cell lines (II)

RNA isolated from androgen-dependent and androgen-independent LNCaP cell lines was used for cDNA array screening. The loss of PSA production in LNCaP cells was regarded as a marker of androgen-independent cell growth. The growth induction by androgen was lost simultaneously to PSA production (data not shown). Genes with most prominent differential expression were selected for further characterization by Northern blot and *in situ* hybridization. Genes were studied for their expression in androgen-dependent and androgen-independent LNCaP cell lines, PC-3 cell line and in BPH, and for some cases in DU-145 cell line, by Northern blotting.

Monoamine oxidase A, EST similar to rat P044, and EST AA412049 were highly over-expressed in androgen-dependent LNCaP cells. Tissue-type plasminogen activator, MxB, EST similar to galectin-1, follistatin, fatty acid-binding protein 5, EST AA609749, annexin I, the interferon-inducible gene 1-8U, and phospholipase D1 were highly over-expressed in androgen-independent LNCaP cells. The Y-linked ribosomal protein S4, Mat-8, and EST AA307912 were most prominently expressed in BPH tissue.

In situ hybridization analyses showed monoamine oxidase A, EST AA412049, and fatty acid-binding protein 5 transcripts in both benign and malignant epithelium of human prostate. EST similar to rat P044 was expressed at low level, but was detectable in both benign and malignant prostate tissue. EST AA781244 was expressed in BPH tissue and androgen-dependent LNCaP cell line, and both benign and malignant prostate epithelium. EST similar to galectin-1 was expressed in stroma of human prostate. Interferon-inducible gene 1-8U was expressed mainly in benign epithelium. In cancer tissue, expression was concentrated in some locations. Further, in some places expression was also detectable in stroma. 2'-5' oligoadenylate synthetase 2 was expressed in androgen-independent LNCaP cell line among DU-145, PC-3 cells and BPH tissue. It was expressed at a low level in prostate tissue, and expression was detected only in part of the prostate cancer samples analysed.

Some related genes were characteristic for different LNCaP cell lines. In androgen-dependent LNCaP cells, serine proteases PSA, KLK2, TMPRSS2 and kallikrein 1 were over-expressed. Interferon-inducible genes 2'-5' oligoadenylate synthetase 2, MxA, MxB, interferon-inducible protein 17, a 56-kDa protein, interferon-alpha-inducible protein 27, interferon-gamma-inducible protein 16 and the interferon-inducible gene 1-8U were over-expressed in androgen-independent LNCaP cell line. Differential expression for protein phosphatase 3 and myosin VI was seen in the array. They were, however, expressed at comparable levels in androgen-dependent and androgen-independent LNCaP cell lines.

As a summary, none of the selected differentially expressed genes were functional exclusively in distinctly pathological states of prostate or normal/BPH tissue.

5.3 Cloning and tissue distribution of mouse TMPRSS2 (III)

To study the tissue-distribution and ontogeny of TMPRSS2, mouse TMPRSS2 cDNA was cloned and *in situ* hybridization was used for expression analyses. RT-PCR and RACE

were used for cloning of the mouse TMPRSS2 cDNA. Analysis of cloned cDNA revealed 77.1% identity in coding region with human TMPRSS2. Predicted mouse TMPRSS2 protein contains 490 aa residues and shares 81.4% similarity and 77.3% identity with human TMPRSS2. The serine protease domain of mouse TMPRSS2 has conserved active site residues His294, Asp343 and Ser439. The Asp433 residue in the substrate binding site suggests that mouse TMPRSS2 will have trypsin-like proteolytic activity with cleavage after Lys or Arg residues. In Northern blot analysis, mouse TMPRSS2 was mainly expressed in prostate and kidney among the tissues studied (heart, brain, spleen, lung, liver, skeletal muscle, kidney, testis and prostate). A hybridization signal corresponding to a mRNA species of approximately 3.1 kb in size and another weakly hybridizing mRNA species of 1.85 kb were detected. *In situ* hybridization of mouse embryos from days 8 to 16 and adult mouse tissues revealed the expression of TMPRSS2 in the epithelium throughout the gastrointestinal, urogenital and respiratory tracts during development. In adult mouse tissues, expression of TMPRSS2 was highest in prostate luminal epithelial cells. Expression was evident in the epithelium of small intestine villi and intestinal glands. In mouse kidney, expression was located in epithelium covering the pelvis and, to a lower extent, in distal convoluted and collecting tubules. In lungs, airway epithelium expressed TMPRSS2, while alveolar epithelium did not. In addition, bile ducts in liver and epithelium of uterine tubes express mouse TMPRSS2. After completion of mouse TMPRSS2 studies, an identical cDNA clone was published (Jacquinet *et al.* 2000). Mouse TMPRSS2 (identified as epitheliasin) was located at chromosome 16q. The protein was expressed primarily in the apical surfaces of renal tubular and airway epithelial cells (Jacquinet *et al.* 2000).

5.4 Tissue distribution of human TMPRSS2 mRNA (III)

Human TMPRSS2 mRNA expression was studied in multiple tissue Dot blot (Human RNA Master Blot, Clontech). The highest level of expression was detected in prostate, and lower levels in colon, stomach and salivary gland. Some expression was further detected in mammary gland, pancreas, kidney, liver, small intestine, lung, trachea, fetal kidney and fetal lung.

5.5 *In situ* hybridization analysis of TMPRSS2 expression in human prostate (III, IV)

Because of conflicting localization of published human TMPRSS2 in prostate (Lin *et al.* 1999) and that seen in mouse prostate, *in situ* hybridization of human prostate samples was done. Human TMPRSS2 mRNA expression was detected in luminal epithelial cells of human prostate, identical to the localization of mouse TMPRSS2 in prostate.

Localization of expression in luminal epithelial cells was also confirmed by identifying basal epithelial cells by CK-HMW staining.

Because TMPRSS2 cDNA was differentially expressed in androgen-dependent and androgen-independent LNCaP cell lines, we examined the expression of TMPRSS2 mRNA in prostate tissue in order to reveal its potential significance for diagnostic purposes. Nine patients with BPH and 23 patients with adenocarcinoma of the prostate were selected. TMPRSS2 transcript levels were significantly higher in prostate cancer tissue between benign and malignant epithelium of prostate cancer patients with untreated (no chemical treatments for prostate diseases, or surgical castration) disease (paired differences mean 0.29 (95% CI 0.05-0.53), p=0.022). Similarly, in poorly differentiated adenocarcinomas, expression in malignant tissue was significantly higher (paired differences mean 0.53 (95% CI 0.15-0.90), p=0.014). Furthermore, in endocrine-treated samples (chemical or surgical castration, or anti-androgen) expression in benign areas was lower than in untreated samples (mean difference 0.25 (95% CI 0.08-0.42), p=0.007).

5.6 Mutational analysis of TMPRSS2 coding region (IV)

Because TMPRSS2 was over-expressed in prostate cancer, the coding region was examined for the presence of mutations. Enzymatic mutation detection and direct sequencing of TMPRSS2 coding region revealed only one deletion in aggressive disease among 9 non-aggressive and 9 aggressive prostate cancer samples. Additional 14 samples from clinically aggressive cases were used for direct sequencing of the same exon. No other mutations were found. The detected 7-bp deletion leads to premature stop codon and disruption of serine protease substrate binding and catalytic active site. Nucleotide 403 was replaced in all samples (C→G), and nucleotide 1527 change A→G was detected in all samples as well. Polymorphism detected is presented in Table 2.

Table 2. Polymorphism detected in TMPRSS2 coding region. Respective nucleotides in TMPRSS2 cDNA (Accession number U75329), affected nucleotide, prevalence of polymorphism in this material and possible amino acid change due to polymorphism are presented.

Nucleotides in U75329	nucleotide change	Prevalence	amino acid change
280-281	CA→TG	2/18	75 Thr→75Met
534	A→G	16/18	160Met→160Val
824	C→T	14/18	-
833	T→C	6/18	-
1211	G→T	3/18	-

6 Discussion

6.1 Ribosomal proteins over-expressed in prostate cancer

Several ribosomal proteins shown to be over-expressed in prostate cancer in this study have also been described in other contexts. RPL7a is DNA-damage inducible (Ben-Ishai *et al.* 1990). Further, the trk-2h oncogene encodes a chimeric phosphoprotein, which consists of the amino-terminal region of human RPL7a fused to the trk receptor tyrosine kinase domain (Ziemiecki *et al.* 1990). RPL4 and RPL30 inhibit their own translation (Wool 1996, and references therein). RPL5 interacts with MDM2, which in turn is able to bind to p53 and thus inhibit the cell cycle arrest and apoptosis inductive functions of p53 (Marechal *et al.* 1997). RPL23a mRNA is down-regulated during interferon-β-mediated growth inhibition. 37LRP/p40 protein has been identified as a ribosomal protein and a precursor of the 67-kDa laminin receptor, whose enhanced expression is associated with tumor invasion and metastatic potential. However, 37LRP/p40 is different from RP37L presented in this study. Over-expression of some ribosomal proteins have been reported at least in carcinomas of colon and breast, in addition to squamous cell and hepatocellular carcinomas (Naora & Naora 1999, and references therein). The elevated ribosomal protein encoding mRNA expression shown in this study was, to our knowledge, the first time this phenomenon was described in prostate-cancer.

Ribosome biogenesis is accurately tuned to the cellular need for protein synthesis and increased ribosome biogenesis is generally associated with increased proliferative activity. Because ribosome assembly and function is dependent on a stoichiometric balance of ribosomal proteins, a fall in the synthesis of one or more ribosomal proteins may conceivably reduce the efficiency of the translational apparatus, or even render it inoperative, and thereby signal the cell to self-destruct (reviewed by Naora & Naora 1999). There is increasing evidence of ribosomal proteins having functions other than translational apparatus, including cell growth and death regulation (reviewed by Wool *et al.* 1995, Wool 1996, Naora & Naora 1999, and Chen & Ioannou 1999). These findings suggest that induction of some ribosomal protein synthesis is merely occasionally associated with increased protein synthesis. For some ribosomal proteins, over-

expression is connected to the malignant transformation. Certain levels of ribosomal protein expression may be needed for survival of cells, but this threshold-level may vary in cells affected by different processes, such as transformation. Finally, the same ribosomal protein expression in different tissues or cells may function separately.

6.2 Differentially expressed genes in androgen-dependent and androgen-independent LNCaP cell lines

Genes over-expressed in androgen-dependent LNCaP cells included serine proteases PSA, KLK2, kallikrein 1 and TMPRSS2, which likely reflect androgen up-regulated genes in this LNCaP cell line, although kallikrein 1 is not known to be regulated by androgens. Transmembrane proteins TMPRSS2, Mat-8 (Schiemann *et al.* 1998) and nma (Degen *et al.* 1996) may be associated with adhesive properties in androgen-dependent LNCaP cells. Up-regulation of MxA and MxB genes in androgen-independent LNCaP cells, and simultaneous down-regulation of TMPRSS2 in the same cells indicates a distinct regulatory mechanism for TMPRSS2 down-regulation, as all these genes are located close to each other at chromosome 21q. This expression pattern is thus not likely be a consequence of loss of chromosomal material at chromosome 21 seen in androgen-independent LNCaP cell line variants (Hyytinen *et al.* 1997). Further, ETS2 whose expression was elevated in some prostate cancer samples (Liu *et al.* 1997), is located in the same area. ETS2 is translocated to chromosome 8 in the commonly found translocation in patients with acute myeloid leukemia with morphology M2 (AML-M2) (Sacchi *et al.* 1988). Taken together, these results indicate the potential significance of this area in a subset of progressive prostate cancers.

Over-expression of several interferon-inducible genes in androgen-independent LNCaP cells may be a consequence of the activation of interferon-activated pathways in those cells. An interesting speculative possibility for over-expression of interferon-inducible genes in androgen-independent LNCaP cells is the existence of viral organism activating prostate cancer or its progression, as seen with human papillomavirus promoted cervical carcinomas (Durst *et al.* 1987). Expression of 2'-5' oligoadenylate synthetase 2 and interferon-inducible gene 1-8U was varying in prostate tissue, as shown here. 2'-5' oligoadenylate synthetase polymerizes 2'-5' oligoadenylates from adenosine triphosphate. 2'-5' oligoadenylates in turn are responsible for RNaseL activation, which is needed for RNA degradation and antiviral activation. Due to its central role in RNA degradation, the oligoadenylate-RNaseL pathway is likely to play an important role in growth regulation and probably also in malignant transformation. Interferon-inducible gene 1-8U, a gene without known function, was recently shown to be over-expressed in ulcerative colitis-associated colon cancers (Hisamatsu *et al.* 1999). Further studies are needed in order to clarify the significance of this gene in prostate cancer.

The widely used prostate cancer model LNCaP (Horoszewicz *et al.* 1980, Horoszewicz *et al.* 1983), and androgen-independent variant of LNCaP (van Steenbrugge *et al.* 1991), have been utilized for differential gene expression studies, but the results are poorly applicable to clinical material, and also reproducibility in human tissue is

unsatisfactory (Chang *et al.* 1997, Kokontis *et al.* 1998, Stubbs *et al.* 1999, Chang *et al.* 1999, Blok *et al.* 1999). LNCaP cells have been raised from human prostate cancer lymph node metastasis. Therefore it is no wonder that results that are hardly reproducible in primary prostate cancer tissue are obtained. There is a lack of widely characterized prostate cancer cell lines representing organ-confined or non-metastasis-origin prostate cancer, and primary cultures (Clarke & Merchant 1980) are used for this purpose (Krill *et al.* 1997). Maintaining primary cell cultures is hard, and slow growth rate and transformation of the cells in the culture are substantial problems (Zwergel *et al.* 1998, Truschel *et al.* 1999). Xenograft models for prostate cancer (Navone *et al.* 1998) are one way of studying prostate cancer and its progression, but because they do not represent primary prostate cancer, similar weaknesses exist as in the case of cultured cells. To date, several xenograft models have been presented, *e.g.* CWR22 (Cheng *et al.* 1996b) and TabBO (Navone *et al.* 2000). The use of cell line models has been a must for effective gene expression studies in prostate cancer as they are reproducible and availability of material is not the limiting factor in the study. The utilization of sensitive methods, such as RT-PCR, may be used on a small scale, but screening of a great number of clones by RT-PCR is laborious and time-consuming, and reliable quantification as well as contaminations pose great problems. Generation of a pool of potential differentially expressed mRNAs can easily be done from prostate tissue samples, as shown here. The lack of an effective screening method for those clones has been a major problem in our studies. Some time ago a sophisticated method, tissue microarray, was described (Kononen *et al.* 1998) and used (Bubendorf *et al.* 1999). Tissue microarrays are definitely a method of choice for gene expression studies from now on, as single hybridization will give expression data for tens or hundreds of different specimens.

6.3 TMPRSS2

TMPRSS2 mRNA distribution in human tissues revealed expression in human epithelial tissues in respiratory, gastrointestinal and urogenital tracts. Expression was also seen in mammary gland and liver. Expression was thus seen mainly in secretory epithelial organs. In mouse liver, TMPRSS2 expression was localized in bile ducts and was, in other words, found in epithelial cells in mice as well. Further, TMPRSS2 protein is expressed primarily in the apical surfaces of renal tubular and airway epithelial cells (Jacquinet *et al.* 2000). There are at least two explanations for this distribution. First, TMPRSS2 function is associated with and needed in those secretory epithelial tissues. It could function either in association with secretory function or with the protection of epithelium. Second, TMPRSS2 may function mainly in prostate, and mRNA expression seen in other tissues is just leakage of the gene in the epithelium without functional significance in these tissues (as seen *e.g.* with imprinted genes of mouse embryonic stem cell lines (Szabo & Mann 1994)).

Cell surface proteases may serve as receptors for foreign organisms. Human aminopeptidase N (CD13) is a metalloprotease on intestinal, lung and kidney epithelial cell surfaces. It is a receptor for at least human coronavirus 229E (Yeager *et al.* 1992) and

human cytomegalovirus (Soderberg *et al.* 1993). Aminopeptidase N in animals also has a similar receptor function for influenza A virus (Kido *et al.* 1992) and transmissable gastroenteritis virus (Delmas *et al.* 1992). Protease-activated receptors (PARs) are self-activated by tethered peptide ligands that become exposed after extracellular aminoterminal cleavage by thrombin or trypsin (Dery *et al.* 1998). Protective function of trypsin mediated by PAR-2 has been suggested in pancreatitis, as PAR-2-associated ductal secretion may promote clearance of toxins and debris (Nguyen *et al.* 1999). Trypsin released from the airway epithelium can initiate powerful bronchoprotection in the airways by activation of epithelial PAR-2 (Cocks *et al.* 1999). Some proteases cleave PARs at areas other than the activating site to generate a receptor that is unresponsive to subsequent proteolytic activation (Dery *et al.* 1998, and references therein). Interestingly, PAR-2 is expressed in gastrointestinal tract, pancreas, kidney, liver, airway, prostate, ovary, and eye (Nystedt *et al.* 1995, Dery *et al.* 1998, and references therein), partly covering the expression distribution of TMPRSS2. TMPRSS2 could possibly activate PAR-2 in prostate and mediate specific protective signals there.

TMPRSS2 encodes transmembrane serine protease with intracellular, LDL receptor class A (LDLRA), scavenger-receptor cysteine rich (SRCR) and serine protease-like domains (Paoloni-Giacobino *et al.* 1997). Similar, structurally complicated proteins have been described lately (enterokinase (Kitamoto *et al.* 1994), human airway trypsin-like protease (HAT) (Yamaoka *et al.* 1998) and corin (Yan *et al.* 1999)). These type 2 transmembrane proteins may be synthesized as a membrane-bound precursor proteins translocated to the cell surface, processed to a soluble form, and released. It is unknown whether the membrane-bound TMPRSS2 is active on the cell surface. Activation of many of the proteases present in blood, pancreas, and other tissues goes through pro-enzyme proteolysis to an active serine protease (*e.g.* PSA (Takayama *et al.* 1997), trypsin and enterokinase (Kitamoto *et al.* 1994), and hepatocyte growth factor (Mizuno *et al.* 1994)). *Bacillus amyloliquefaciens* substilisin is synthesized as a membrane-associated precursor and released outside the cell after it is auto-catalytically converted to an active form (Power *et al.* 1986), and only mature substilisin has been detected extracellularly (Wells *et al.* 1983). Some proteases, exemplified by angiotensin-converting enzyme, convert peptides to their principal biological forms in the extracellular fluid and thereby mediate their function.

Recently, a serine protease PSA has been shown to have antiangiogenic activity (Fortier *et al.* 1999, Heidtmann *et al.* 1999). TMPRSS2 could also have activity for proteolysis of plasminogen to angiostatin (Gately *et al.* 1997), and down-regulation of TMPRSS2 may have a harmful effect for tumor growth-limiting angiogenesis inhibition.

TMPRSS2 is over-expressed in prostate cancer tissue. Compared to the results from LNCaP cell lines, results obtained from prostate tissue are somewhat conflicting. However, LNCaP cells are isolated from prostate cancer lymph node metastasis, and due to heterogeneity of prostate cancer, several clonal variants are likely to exist in prostate tumors. Down-regulation of TMPRSS2 expression may exist in some androgen-independent cancer clones similar to androgen-independent LNCaP cells. The TMPRSS2 coding region deletion detected in a sample from a prostate cancer patient with aggressive, chemotherapy-resistant prostate cancer is likely to have also functional significance, as it leads to the premature stop codon and disruption in substrate binding and catalytic triad of serine protease domain. This deletion potentially inactivates the

enzyme (Carter & Wells 1988), probably leading to a totally non-functional protein. In that way all serine protease domain functions speculated here may be reduced or lost. This deletion was detected only in 1 out of 32 prostate cancer samples, so it seems to be a rare event. The possible existence of this deletion in prostate cancer metastases remains to be solved.

6.4 Conclusions

Prostate cancer is the most common cancer among aging men. With occult carcinoma of the prostate, watchful waiting is useful action when the disease affects men over 70 years of age. The most widely used treatment for prostate cancer is androgen ablation therapy, which can be put into effect by orchiectomy or by medical castration using luteinizing hormone-releasing hormone (LHRH)-agonists with or without antiandrogens. However, a remarkable portion of diagnosed prostate cancers have already metastasized at the time of diagnosis, and, furthermore, the long-term response to endocrine treatment is often poor. For the moment, the mechanisms behind androgen-independence of prostate cancer are mainly unsolved. Differentially expressed genes in various types of prostate diseases may give clues for genes affecting the respective disease states. On the other hand, normal phenotype may contain cells with cancer genotype long before clinical cancer is detectable (Tsao *et al.* 2000).

This study shows, for the first time, over-expression of mRNAs for several ribosomal proteins in prostate cancer. This over-expression in prostate cancer is most likely to involve extraribosomal functions of these proteins. The use of androgen-dependent and androgen-independent LNCaP cell lines offers a fascinating possibility to study differential gene expression in a very limited cell population. But this is simultaneously a weakness, as heterogenous prostate cancer probably very seldom contains cancer cells with exclusively similar characteristics and hardly ever cells identical to LNCaP. The previously unknown differential expression of several cDNAs was shown in different prostate cancer cell lines. The genes examined using *in situ* hybridization revealed no striking differences between benign and malignant prostate tissues, but to further study their significance for androgen-dependence and metastasis, more detailed experiments need to be done. Based on their known functions, many of these genes are potential candidates for carcinogenesis. Serine protease TMPRSS2 has the potential to act on numerous processes, and its gene is thus an interesting candidate to be affected during malignant transformation. Based on its structure, TMPRSS2 could also be a serum marker for prostate diseases. Antibodies specific for TMPRSS2 are needed in order to study further its significance in prostatic diseases and their diagnosis.

In the near future, as soon as all the human genes have been sequenced and powerful techniques, such as cDNA microarrays and tissue microarrays, are effectively used for cancer gene examination, the focus of research in this field will shift to functional studies. At the present, it can be said that there is no single prostate cancer gene, but the development and progression of cancer is a combination of numerous genes affecting the disease. Furthermore, these changes in gene expression may, in the worst case, be

individually regulated and result in more or less unique disease characteristics for each person affected.

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