SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

RAD: 11-144859- -4 **FECHA**: 2013-11-18 14:07:44
DEP: 2020 DIRECCION DE NUEVAS CREACIONES **EVE:** 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II **FOLIOS**: 23
ACT: 519 PRESENTACION

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
JUAN CARLOS URIBE RESTREPO

Asunto: Radicación: 11-144859- -4
Trámite: 11
Evento: 378
Actuación: 519
Oficio: 21477
Folios: 23

Patente de Invención	**X**			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	**X**	Capítulo I	**X**	Capítulo II	
No. Solicitud Internacional	PCT/US2010/031719				
Fecha de Presentación Internacional	20/04/2010				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Radicado No 11-144859-00000-0000	26/Octubre/2011
Reivindicaciones:	Radicado No 11-144859-00000-0000	28/Septiembre/2010
Secuencias	Radicado No 11-144859-00000-0000	28/Septiembre/2010
Dibujos:	Radicado No 11-144859-00000-0000	28/Septiembre/2010
Prioridad:	US 61/170.980	20/Abril/2009

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capítulo primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada.

En el presente caso se estudiaran las reivindicaciones 1-18 (Radicado No 11-144859-00000-0000)

Título de la solicitud: “Anticuerpos específicos para cadherina-17”

El problema planteado en esta solicitud consiste en proporcionar anticuerpos monoclonales que se unen a cadherina-17 con alta afinidad, útiles para tratar diferentes tipo de cáncer incluido cáncer colon rectal.

Para resolver este problema técnico la solicitud en estudio proporciona anticuerpo específico para cadherina 17, ácido nucleico que codifica dicho anticuerpo, vectores de expresión, células huésped, métodos de detección y composiciones farmacéuticas que contienen anticuerpo en estudio.

4. Excepción a la Patentabilidad (Art 20 D 486 literal (d))

El objeto de las reivindicaciones 15-17 no es patentable de acuerdo con el Art citado.

5. De la solicitud de patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

1- La reivindicación 1 da lugar a un sinnúmero de combinaciones entre secuencias de regiones variable de la cadena ligera y pesada Se sugiere definirlo a partir de su estructura completa como lo es, indicando la secuencia aminoácida de las regiones variables de la cadena ligera y pesada ó las secuencias de los 6 CDRs de las cadenas pesada y ligera.
2- En la reivindicación 2 solo tienen soporte los anticuerpos cuyas secuencias son: SED ID NOs: 2, 6,15, 72, 48, 23, 29, 33, 61, 37, 27, 28, 29, 64, 40, 4, 8, 17, 25, 31, 33, 75, 51, 81, 55, 35, 1, 5, 14, 60, 36, 73, 49, 59, 71, 47, 34, 39, 50, 65, 42. Adicionalmente, es importante aclarar que un anticuerpo se define por su secuencia aminoácida de las regiones variables de la cadena ligera y pesada ó las secuencias de los 6 CDRs de las cadenas pesada y ligera.
3- Los inmunoconjugados de las reivindicaciones 7 y 8 carecen de soporte. Se sugiere incorporar en los ejemplos de la descripción para dar soporte. Esta modificación no supone ampliación puesto que las reivindicaciones iniciales se presentaron con la descripción (Art. 26 D 486).
4- El método de la reivindicación 13 no es claro ni conciso porque hay una repetición innecesaria de palabras lo cual hace difícil definir cuál es el objeto que se busca proteger. Se sugiere, entonces, definir la invención por sus características esenciales, estructurales o funcionales.
5- En la reivindicación 4 la referencia a fragmentos del anticuerpo no es aceptable, tampoco se encuentra soporte para obtención de los mismos. Se sugiere

definirlo a partir de su estructura completa como lo es, indicando la secuencia aminoácida de las regiones variables de la cadena ligera y pesada ó las secuencias de los 6 CDRs de las cadenas pesada y ligera.

6- Las reivindicaciones 5-6 no son claras porque mencionan aparentemente diferentes grupos de anticuerpos y nombres comerciales del mismo, que no dejan apreciar las características esenciales de la técnica.

7- La reivindicación 10 se refiere a un ácido nucleico caracterizado por codificar un anticuerpo, lo cual no es aceptable. Se sugiere definirlo a partir de la secuencia nucleótida y el número de la misma (SEQ ID NO)sin mencionar su estructura completa.

8- La reivindicación 11 se refiere a un vector solo por contener un ácido nucleico. Se sugiere indicar el tipo de vector y la secuencia recombinante que contiene.

6. Unidad de invención (Art 25 D 486)

La solicitud no cumple con el requisito de unidad de invención porque se refiere a 14 grupos inventivos. El concepto común inventivo referido como anticuerpos anticadherina se divulgados en el documento D1.

Los grupos inventivos están definidos por las siguientes características esenciales:

- Grupo I: Anticuerpo anti-cadherina 17 que comprende una región variable de cadena pesada SEQ ID N° 38 y una región variable de cadena liviana SEQ.ID. N° 49 respectivamente.
- Grupo II: Anticuerpo anti-cadherina 17 que comprende una región variable de cadena pesada SEQ ID N° 39 y una región variable de cadena liviana SEQ.ID. N° 50 respectivamente.
- Grupo III: Anticuerpo anti-cadherina 17 que comprende una región variable de cadena pesada SEQ ID N° 40 y una región variable de cadena liviana SEQ.ID. N° 51 respectivamente.
- Grupo IV: Anticuerpo anti-cadherina 17 que comprende una región variable de cadena pesada SEQ ID N° 35 y una región variable de cadena liviana SEQ.ID. N° 47 respectivamente.
- Grupo V: Anticuerpo anti-cadherina 17 que comprende una región variable de cadena pesada SEQ ID N° 36 y una región variable de cadena liviana SEQ.ID. N° 48 respectivamente.
- Grupo VI: Anticuerpo anti-cadherina 17 que comprende una región variable de cadena pesada SEQ ID N° 37 y una región variable de cadena liviana SEQ.ID. N° 48 respectivamente.
- Grupo VII: Anticuerpo anti-cadherina 17 que comprende una región variable de cadena pesada SEQ ID N° 41 y una región variable de cadena liviana SEQ.ID. N° 52 respectivamente.
- Grupo VIII: Anticuerpo anti-cadherina 17 que comprende una región variable de cadena pesada SEQ ID N° 42 y una región variable de cadena liviana SEQ.ID. N° 53 respectivamente.
- Grupo IX: Anticuerpo anti-cadherina 17 que comprende una región variable de cadena pesada SEQ ID N° 42 y una región variable de cadena liviana SEQ.ID. N° 53 respectivamente.
- Grupo X: Anticuerpo anti-cadherina 17 que comprende una región variable de cadena pesada SEQ ID N° 40 y una región variable de cadena liviana SEQ.ID. N° 55 .
- Grupo XI: Anticuerpo anti-cadherina 17 que comprende una región variable de cadena pesada SEQ ID N° 43 y una región variable de cadena liviana SEQ.ID. N° 56.

- Grupo XII Anticuerpo anti-cadherina 17 que comprende una región variable de cadena pesada SEQ ID N° 44 y una región variable de cadena liviana SEQ.ID. N° 55.
- Grupo XIII: Anticuerpo anti-cadherina 17 que comprende una región variable de cadena pesada SEQ ID N° 45 y una región variable de cadena liviana SEQ.ID. N° 57.
- Grupo XIV: Anticuerpo anti-cadherina 17 que comprende una región variable de cadena pesada SEQ ID N° 46 y una región variable de cadena liviana SEQ.ID. N° 58.

Se sugiere presentar las solicitudes divisionales que considere necesarias, o limitar la solicitud inicial a una sola invención, eliminando de ella las otras invenciones.

7. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de solicitud de prioridad : Abril 20 de 2019, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación*
D1	. Ming-Cheng Su. Modern Pathology. Vol:21, No11, (Pag.1379 – 1386)	Cadherin-17 is a useful diagnostic marker for adenocarcinomas of the digestivw system for adenocarcinomas of the digestive system"	2008
D2	Japanese Cancer Association, Tokio, JP. Cancer Science. Vol 94. No 5. Pág.(425-430)	"Expression of liver intestine cadherin and its possible interaction with galectin -3 in ductal adenocarcinoma of the pancreas"	10/marzo/2003

El documento D1[1] divulga anticuerpos anti cadherin-17. (Pág.1380. Col. Izq)

El documento D2[2]divulga Anticuerpo Anti-cadherina-17 (Pág. 425 Col. 5). También divulga que el isotipo es IGg1, células huésped, hibridoma seleccionado, producción de una anticuerpo monoclonal (Pág 425. Col. Der.).

Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 14 consiste en *"un método para formar anticuerpos anti-cadherina 17, que comprende los pasos de inmunizar un animal con un péptido de cadherina 17; recuperar ARNm de los linfocitos B de dicho animal; convertir dicho ARNm a ADNc; expresar dicho ADNc en fagos de manera que los anticuerpos anticadherina-17 codificados por dicho ADNc se presenten en la superficie de*

11 http://www.nature.com/modpathol/journal/v21/n11/pdf/modpathol2008107a.pdf
2 http://onlinelibrary.wiley.com/doi/10.1111/j.1349-7006.2003.tb01459.x/abstract

dichos fagos; seleccionar fagos que presentan anticuerpos anti-Cadherin 17; recuperar las moléculas de ácido nucleico de dichos fagos seleccionados que codifican dichas inmunoglobulinas anti-cadherina-17; expresar dichas moléculas de ácido nucleico recuperadas en una célula hospedera; y recuperar anticuerpos de dicha célula hospedera que se une a Cadherina -17".

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	**D2**
Método ´para formar anticuerpos anti caderina-17 que comprende:	Producción de anticuerpos monoclonales:
Inmunizar un animal con un péptido de de cadherina 17.	Inmunización de ratón (Pág.425-426 , Col.der))
recuperar ARNm de los linfocitos B de dicho animal	Recuperacion de ARNm (Pág.425-426)
recuperar las moléculas de ácido nucleico	recuperar las moléculas de ácido nucleico (Pág.425-426
expresar dichas moléculas de ácido nucléico recuperadas en una célula hospedera	expresar dichas moléculas de ácido nucléico recuperadas en una célula hospedera ratón (Pág.425-426)

Las características esenciales del método de la reivindicación 14 habían sido divulgadas previamente en el documento D1, el cual enseña, Producción de anticuerpos monoclonales: Inmunización de ratón, recuperación de ARNm recuperar las moléculas de ácido nucleico, expresar dichas moléculas de ácido nucleico recuperadas en una célula hospedera (Pág.425-426)

En consecuencia, la reivindicación 14 no es nueva o carece de novedad, según lo divulgado en el documento D1.

Nivel inventivo (Art18 D486)

Las reivindicaciones 1 y 18 consisten en "*un anticuerpo aislado que se une a un epitopo en cadherina -17 humana reconocido por un anticuerpo que comprende una región variable de cadena pesada que comprende una secuencia de aminoácidos expuesta en una SEQ ID NO: seleccionada del grupo que consiste de 38, 35, 36, 37, 39, 40, 41, 42, 43, 44, 45, y 46 y una región variable de cadena ligera que comprende una secuencia de aminoácidos expuesta en una SEQ ID NO. seleccionada del grupo que consiste de 49, 47, 48, 50, 51, 52, 53, 54, 55, 56, 57 y 58"*

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	**D1**	**D2**
Anticuerpo anticaderina 17 humana que comprende:	Anticuerpo Anti-cadherina-17	Anticuerpo Anti-cadherina-17

una región variable de cadena pesada que comprende una secuencia de aminoácidos expuesta en una SEQ ID NO: seleccionada del grupo que consiste de 38, 35, 36, 37, 39, 40, 41, 42, 43, 44, 45, y 46 y una región variable de cadena ligera que comprende una secuencia de aminoácidos expuesta en una SEQ ID NO. seleccionada del grupo que consiste de 49, 47, 48, 50, 51, 52, 53, 54, 55, 56, 57 y 58	No menciona-17	No menciona (Pág.425 , Col 5)

El documento D1 se considera el estado de la técnica cercano a la invención definida en la reivindicación 1 porque divulga anticuerpos anti cadherin-17 (Pág.1380. Col. Izq)

El documento D2 se considera el estado de la técnica cercano a la invención definida en las reivindicaciones 1 y 18 porque divulga Anticuerpo Anti-cadherina-17 (Pág. 425 Col. 5). También divulga que el isotipo es IGg1, células huésped, hibridoma seleccionado, producción de una anticuerpo monoclonal (Pág 425. Col. Der.).

La diferencia entre la invención, definida en las reivindicaciones 1 y 18 y anticuerpos de D1 consiste en la secuencia de aminoácidos expuesta en una SEQ ID NO: seleccionada del grupo que consiste de 38, 35, 36, 37, 39, 40, 41, 42, 43, 44, 45, y 46 y una región variable de cadena ligera que comprende una secuencia de aminoácidos expuesta en una SEQ ID NO. seleccionada del grupo que consiste de 49, 47, 48, 50, 51, 52, 53, 54, 55, 56, 57 y 58.

La diferencia entre la invención, definida en las reivindicaciones 1 y 18 y los anticuerpos de D2 consiste en la secuencia de aminoácidos expuesta en una SEQ ID NO: seleccionada del grupo que consiste de 38, 35, 36, 37, 39, 40, 41, 42, 43, 44, 45, y 46 y una región variable de cadena ligera que comprende una secuencia de aminoácidos expuesta en una SEQ ID NO. seleccionada del grupo que consiste de 49, 47, 48, 50, 51, 52, 53, 54, 55, 56, 57 y 58.

No se evidencia un efecto técnico inesperado porque anticuerpos anticadeirna -17 humana ya se encuentra divulgados en los documentos D1 y D2.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: “cómo proporcionar anticuerpos de unión a cadherina-17, anticuerpos conocidos en D1 para obtener anticuerpos con secuencia de aminoácidos alternativas.”.

Sin embargo, anticuerpos anticaderina 17 humana ya está divulgada en el documento D2 que se refiere a anticuerpo Anti-cadherina-17 (Pág. 425 Col. 5).

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría anticuerpos anticaderina 17 humana del documento D1 en el procedimiento divulgado en el documento D2 para llegar así al objeto de las reivindicaciones 1 y 18 de la solicitud. Por lo cual se consideran obvias.

Las reivindicaciones 2-8, 10-13, no mencionan características adicionales, por lo cual tampoco tienen nivel inventivo.

Conclusión:
Las reivindicaciones 1 – 8, 10 – 13 y 18 cumple el requisito de novedad (Art. 16), pero no cumple con el requisito de nivel inventivo (Art. 18).

La reivindicación 2 consiste en "*un anticuerpo aislado anticadherina 17 que comprende una región variable de cadena pesada y una región variable de cadena ligera seleccionado del grupo que consiste de: la secuencia de aminoácidos de la región variables de cadena pesada expuesta en la SEQ ID NO:38 y la secuencia de aminoácidos de la región variable de cadena ligera expuesta en la SEQ ID NO 49; la secuencia de aminoácidos de la región variable de cadena pesada expuesta en la SEQ ID NO 39…*"

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	**D1**	**D2**
Anticuerpo anticaderina 17 que comprende:	Anticuerpo Anti-cadherina-17	Anticuerpo Anti-cadherina-17
una región variable de cadena pesada que comprende una secuencia de aminoácidos expuesta en una SEQ ID NO: 38, 35, 36, 37, 39, 40, 41, 42, 43, 44, 45, y 46 y una región variable de cadena ligera que comprende una secuencia de aminoácidos expuesta en una SEQ ID NO. 49	No menciona-17	No menciona (Pág.425 , Col 5)

El documento D1 se considera el estado de la técnica cercano a la invención definida en la reivindicación 2 porque divulga anticuerpos anti cadherin-17.

El documento D2 se considera el estado de la técnica cercano a la invención definida en la reivindicación 2 porque divulga Anticuerpo Anti-cadherina-17 (Pág. 425 Col. 5). También divulga que el isotipo es IGg1, células huésped, hibridoma seleccionado, producción de una anticuerpo monoclonal (Pág 425. Col. Der.).

La diferencia entre la invención, definida en la reivindicación 2 y anticuerpos de D1 consiste en la secuencia de aminoácidos expuesta en una SEQ ID NO: seleccionada del grupo que consiste de 38, 35, 36, 37, 39, 40, 41, 42, 43, 44, 45, y 46 y una región variable de cadena ligera que comprende una secuencia de aminoácidos expuesta en una SEQ ID NO. seleccionada del grupo que consiste de 49, 47, 48, 50, 51, 52, 53, 54, 55, 56, 57 y 58.

La diferencia entre la invención, definida en las reivindicación 2 y los anticuerpos de D2 consiste en la secuencia de aminoácidos expuesta en una SEQ ID NO: seleccionada del grupo que consiste de 38, 35, 36, 37, 39, 40, 41, 42, 43, 44, 45, y 46 y una región variable de cadena ligera que comprende una secuencia de aminoácidos expuesta en una SEQ ID NO. seleccionada del grupo que consiste de 49, 47, 48, 50, 51, 52, 53, 54, 55, 56, 57 y 58.

No se evidencia un efecto técnico inesperado porque anticuerpos anticadeirna -17 humana ya se encuentra divulgados en los documentos D1 y D2.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: cómo proporcionar anticuerpos de unión a cadherina-17 alternativo a los anticuerpos conocidos en D1.

Sin embargo, anticuerpos anticaderina 17 humana ya está divulgada en el

documento D2 que se refiere a anticuerpo Anti-cadherina-17 (Pág. 425 Col. 5) y los anticuerpos definidos en la reivindicación 2 no presentan ningún efecto técnico inesperado.

En consecuencia, la persona del oficio normalmente versada en la materia, obtendría anticuerpos anticaderina 17 humana alternativos a los divulgados en los documentos D1 o D2 según lo revelado en estos mismos documentos para llegar así al objeto de las reivindicación 2 de la solicitud. Por lo cual se consideran obvias.

Conclusión:
La reivindicación 2 cumple el requisito de novedad (Art. 16), pero no cumple con el requisito de nivel inventivo (Art. 18).

8. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Item	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	.Ming-Cheng Su. Modern Pathology. Vol:21, No11, (Pag.1379 – 1386)	1-8, 10-13, 18	Nivel inventivo
D2	Japanese Cancer Association, Tokio, JP. Cancer Science. Vol 94. No 5. Pág.(425-430)	14	Novedad
D2	Japanese Cancer Association, Tokio, JP. Cancer Science. Vol 94. No 5. Pág.(425-430)	1 –11	Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.

JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2013-11-26

Elaboró: Edna Milena Bautista Rodriguez
Revisó: Jeny Paola Villate Becerra

Modern Pathology (2008) 21, 1379–1386

www.modernpathology.org

Cadherin-17 is a useful diagnostic marker for adenocarcinomas of the digestive system

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Cadherin-17, also called liver-intestine cadherin, is a calcium-dependent transmembrane glycoprotein that mediates cell–cell adhesion in intestinal epithelium. Expression of cadherin-17 was reported in gastric, pancreatic, and colorectal adenocarcinomas but not in other tumors. Whether cadherin-17 can be used as a marker for diagnosis of cancers is still unclear. In this study, we used immunohistochemical methods to stain cadherin-17 in tissue arrays containing most normal tissues and 518 carcinomas from many anatomic sites. Among normal tissues, the expression of cadherin-17 was limited to epithelial cells of small intestine and colon. Colorectal adenocarcinomas showed staining in 96% of cases and most of them had strong and diffuse staining. Gastric, pancreatic, and biliary adenocarcinomas showed diffuse or scattered staining in about 25–50% of cases. Fewer than 1% of carcinomas outside the digestive system were positive for cadherin-17. When a two-marker, Cadherin-17/cytokeratin 7, profile was used, 37 of 38 (97%) cadherin-17(+)/cytokeratin 7(−) tumors were colorectal adenocarcinomas; 49 of 56 (86%) cadherin-17(+)/cytokeratin 7(+) tumors were gastric, pancreatic, or biliary adenocarcinomas. Our results show that cadherin-17 is a useful immunohistochemical marker for diagnosis of adenocarcinomas of the digestive system.
Modern Pathology (2008) **21,** 1379–1386; doi:10.1038/modpathol.2008.107; published online 13 June 2008

Keywords: cadherin-17; immunohistochemistry; adenocarcinoma

Cancer of an unknown primary site is the seventh to eighth most frequently occurring cancer found in clinical practice.[1] The age-adjusted annual incidence per 100 000 population is 7–12 cases in the United States, 18 in Australia, and 6 in the Netherlands. It is the fourth commonest cause of cancer death in both men and women.[2] Because the optimal treatment of cancer is linked to the primary tumor site, failure to identify the primary site causes considerable problems during management of patients. Adenocarcinomas account for almost half of cancers of unknown primary site. The use of immunohistochemistry to detect specific antigens in tumor cells has led to significant improvement in the diagnosis of cancers. For example, prostate-specific antigen is used as a marker of prostatic cancer.[3] Thyroid transcription factor is a marker of pulmonary cancer.[4] However, a good marker for gastrointestinal (GI) tumors is still lacking.

Cadherin-17, also called liver-intestinal cadherin or human peptide transporter-1, is a member of the cadherin superfamily and is a Ca^{2+}-dependent cell–cell adhesion molecule.[5,6] Unlike the so-called classic cadherins, such as E-, N-, and P-cadherins, cadherin-17 has seven cadherin repeats instead of five within the extracellular domain and only 20 amino-acid residues in the cytoplasmic domain. The markedly short cytoplasmic domain lacks homology with other cadherins and the adhesive function of cadherin-17 is not dependent on association with other cytoplasmic proteins.[7] The subcellular distribution of cadherin-17 is also different from classic cadherins. In intestinal epithelial cells, E-cadherin is concentrated in adherens junctions whereas cadherin-17 is evenly distributed along the lateral contact area.[8]

The tissue distribution of cadherin-17 differs by species. In the rat, cadherin-17 is expressed in the liver and intestinal epithelial cells. In human and mouse, expression is limited to intestinal epithelial

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Table 1 Cadherin-17 immunohistochemical staining in normal tissues

No staining	
Adrenal cortex	Skeletal muscle
Bile duct epithelium	Skin
Blood vessel	Smooth muscle, uterus
Breast (duct and lobule)	Spleen
Cerebrum	Stomach, antrum
Cerebellum	Stomach, body
Endometrial epithelium	Testis, Leydig cells
Endometrial stroma	Testis seminiferous tubules
Esophagus, squamous epithelium	Thymus
Heart myocardium	Tonsil
Kidney, cortex	
Kidney medulla	4+ staining
Liver	Appendicular epithelium
Lung	Colonic epithelium
Lymph node	Small intestine epithelium
Pancreas acinar cells	
Pancreas islets cells	
Placenta	
Prostate	
Salivary gland, parotid	

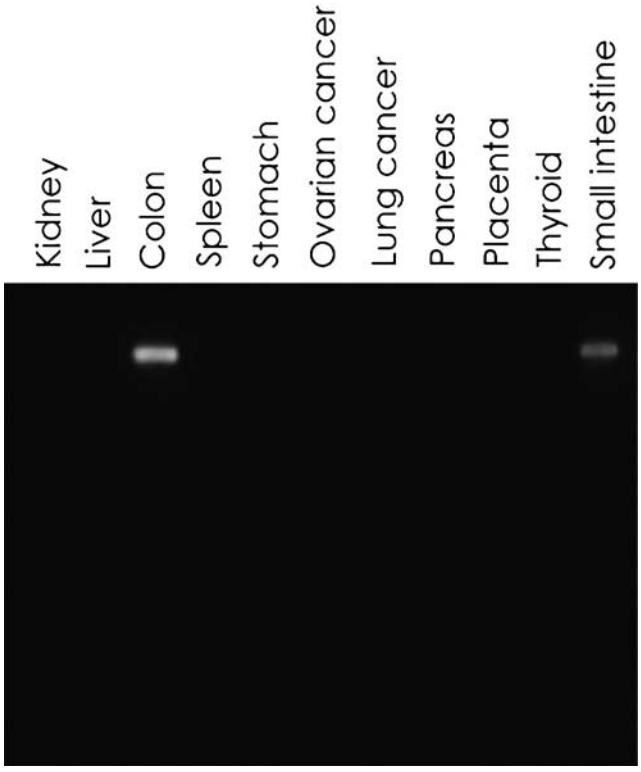

Figure 1 Western blot analysis of cadherin-17 protein in tissue lysates. Equal amounts of the protein (25 μg) were loaded in each lane. Among these tumors, only colon and small intestine tissue expressed cadherin-17 protein. Cadherin-17 is seen as a single band with a molecular weight of 110 kDa.

cells and is not found in the liver.[9] The limited distribution of cadherin-17 in normal tissues suggests that it may be a useful marker for identification of primary sites of tumors. It has been reported that cadherin-17 is expressed in colorectal,[10] gastric,[11,12] and pancreatic cancers.[13] Whether it is expressed in tumors in other organs is unknown.

Accordingly, in this study, we examined the expression of cadherin-17 in tumors derived from various body sites using tissue arrays. We found cadherin-17 was diffusely and strongly expressed in colorectal adenocarcinomas, scattered expressed in adenocarcinomas of the stomach, the pancreas and the bile duct, and virtually not expressed in tumors of other body sites. Our study indicates cadherin-17 may be a useful marker for identification of primary sites of metastatic adenocarcinomas.

Materials and methods

Tissue Specimens

Formalin-fixed, paraffin-embedded tissue blocks from 518 previously characterized primary carcinomas and 60 nontumorous tissues were obtained from the archives of the Department of Pathology, National Taiwan University Hospital. The study was conducted according to the guidelines of our institutional review board. These tumor cases included 44 breast cancers, 39 carcinomas of the uterine cervix, 33 cholangiocarcinomas, 47 colorectal cancers, 57 hepatocellular carcinomas, 50 lung cancers, 47 renal cell carcinomas, 89 ovarian cancers, 28 pancreatic cancers, 39 prostatic cancers, and 45 gastric cancers. The spectrum of nontumorous tissue examined is listed in Table 1.

Tissue Arrays

Targeted tissue areas were marked on H&E-stained sections. A 2 mm tissue core was removed for each case using a manual tissue array device (Beecher Instruments, Silver Spring, MD, USA) and inserted into a recipient paraffin block. The arrayed tissues were cut into 4-μm thick slices and placed on positively charged slides.

Western Blot Analysis

Western blots were carried out on the protein lysates of 11 representative normal and tumor tissues, including kidney, liver, colon, small intestine, stomach, spleen, thyroid, pancreas, placenta, and carcinomas from ovary and lung. Similar amounts of total protein from each lysate were separated by 8% Tris-glycine-SDS-polyacrylamide gels (Novex, San Diego, CA, USA), and then electroblotted to Millipore Immobilon-P

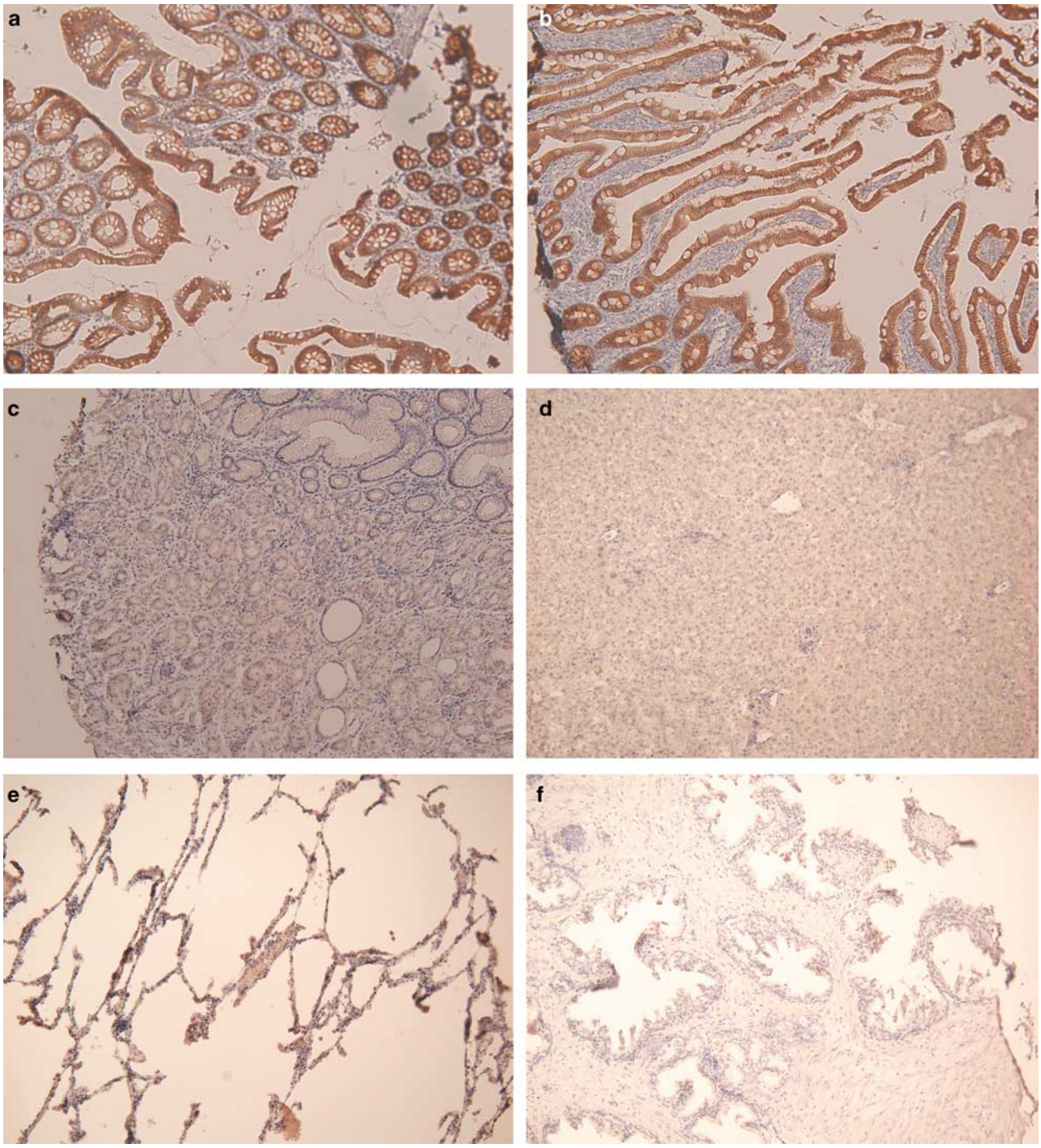

Figure 2 Immunohistochemical staining of cadherin-17 in normal tissue. Cadherin-17 was expressed in epithelial cells of colon (**a**) and small intestine (**b**), but not in gastric mucosa (**c**), liver (**d**), lung (**e**), or prostate (**f**).

polyvinylidene difluoride membranes. The membranes were probed with anti-cadherin-17 mouse monoclonal antibody, clone 1H3 (IgG1), at a concentration of 1 μg/ml (Abnova, Taipei, Taiwan) followed by a peroxidase-conjugated goat anti-mouse immunoglobulin (1:2500). Western blots were developed using chemiluminescence (Pierce, Rockford, IL, USA).

Immunohistochemical Staining

Tissue sections were dewaxed and rehydrated. Antigen retrieval was done by incubating slides in 0.01 M citric acid buffer (pH 6.0) at 100°C for 10 min. After blocking with 3% H_2O_2 and 5% fetal bovine serum, slides were allowed to react with a monoclonal antibody against cadherin-17 (clone 1H3,

Table 2 Immunohistochemical staining of cadherin-17, cytokeratin 7, cytokeratin 20, and CDX-2 in human cancers

	Cadherin-17					*Cytokeratin 7*					*Cytokeratin 20*					*CDX-2*				
	0	*1+*	*2+*	*3+*	*4+*	*0*	*1+*	*2+*	*3+*	*4+*	*0*	*1+*	*2+*	*3+*	*4+*	*0*	*1+*	*2+*	*3+*	*4+*
Breast cancer	44	0	0	0	0	3	1	0	1	38	41	0	0	1	0	46	0	0	0	0
Cervical carcinoma																				
Adenocarcinoma	6	0	0	0	0	0	2	0	0	4	6	0	1	0	0	7	0	0	0	0
Squamous cell carcinoma	33	0	0	0	0	7	3	1	1	21	35	0	0	0	0	36	0	0	0	0
Cholangiocarcinoma	24	5	0	1	3	0	1	2	2	24	25	2	2	0	7	30	3	1	1	1
Colorectal adenocarcinoma	2	0	3	4	38	39	5	1	0	0	8	2	5	5	28	4	0	1	1	47
Hepatocellular carcinoma	57	0	0	0	0	41	4	3	3	3	54	0	0	0	0	55	0	0	0	0
Lung																				
Adenocarcinoma	39	0	0	0	0	0	1	1	2	34	36	3	1	0	1	41	0	0	0	0
Squamous cell carcinoma	11	0	0	0	0	5	2	0	1	3	11	0	0	0	0	11	0	0	0	0
Kidney (renal cell carcinoma)																				
Conventional type	42	0	0	0	0	32	5	0	1	2	32	5	1	1	0	41	0	0	0	0
Chromophobe	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
Collecting duct	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0
Papillary	3	0	0	0	0	0	0	0	0	3	3	0	0	0	0	3	0	0	0	0
Ovary																				
Serous carcinoma	55	0	0	0	0	0	2	4	7	41	54	0	0	0	0	53	0	0	0	0
Mucinous carcinoma	34	0	0	0	0	1	2	2	2	27	14	10	2	2	6	25	4	3	1	1
Pancreas (adenocarcinoma)	12	7	5	3	1	0	1	1	1	23	15	9	1	0	0	25	1	0	0	2
Prostate (adenocarcinoma)	38	0	0	0	1	25	3	2	1	0	32	5	1	1	0	35	0	0	0	0
Stomach adenocarcinoma	20	8	5	8	4	3	5	5	4	26	42	1	0	0	1	16	8	6	5	12

Abnova), cytokeratin 7 (DakoCytomation, Glostrup, Denmark), cytokeratin 20 (DakoCytomation), or CDX-2 (Biogenix, San Ramon, CA, USA) at a dilution of 1:100 at 4°C overnight. Slides were then incubated with polymer-HRP reagent (BioGenex). Peroxidase activity was visualized with diaminobenzidine tetrahydroxychloride solution (BioGenex). Sections were counterstained with hematoxylin. Dark brown membranous staining was defined as positive and no staining was defined as negative. For negative controls, we replaced the primary antibody with 5% fetal bovine serum. Staining was scored as follows: 0 (no detectable staining); 1+ (<25% positive cells), 2+ (25–49%); 3+ (50–74%); and 4+ (>75%). In cells with positive staining, the staining was intense and uniform, so intensity was not factored into the scoring.

Results

We first determined the specificity of the anti-cadherin-17 antibody (clone 1H3) by western blot analysis. Cadherin-17 expression was detected exclusively in human lysates of small intestine and colon and not in lysates from other normal tissues and tumors (Figure 1). The antibody was specific to the cadherin-17 protein because a single band with apparent molecular weight of about 110 kDa was present in lysates of small intestine and colon. This band corresponded to the cadherin-17 protein. There was no detectable cross reactivity with other proteins as revealed in the western blot analysis.

Table 1 shows the distribution of cadherin-17 in normal tissues by immunohistochemistry on samples from tissue arrays. Strong diffuse membranous staining of cadherin-17 was seen in intestinal epithelium, including those samples taken from small intestine, appendix, and colorectal mucosa (Figures 2a and b). Cadherin-17 was expressed in the surface epithelium of duodenum but not in Brunner's gland. Cadherin-17 was not expressed in normal gastric mucosa (Figure 2c) but was seen in areas with intestinal metaplasia. No other tissue type had detectable cadherin-17 expression (Figures 2d–f).

Table 2 shows results of cadherin-17 immunohistochemical staining for 518 tumors from various body sites. Membranous staining of cadherin-17 was present in 45 (96%) of 47 colorectal cancers; 38 (81%) of the 47 showed diffuse staining (>75%) (Figure 3a). Cadherin-17 was also expressed in 25 (56%) of 45 gastric adenocarcinomas, 16 (57%) of 28 pancreatic adenocarcinomas, and 9 (27%) of 33 cholangiocarcinomas (Figures 3b–d). Most of the gastric, pancreatic, and biliary adenocarcinomas (42 of 50, 84%) showed a focal or scattered staining pattern. Cadherin-17 was rarely expressed in tumors of other body sites (Figures 3e and f). Of the 331 tumors examined, cadherin-17 was expressed in only 1 (0.3%), a prostatic adenocarcinoma.

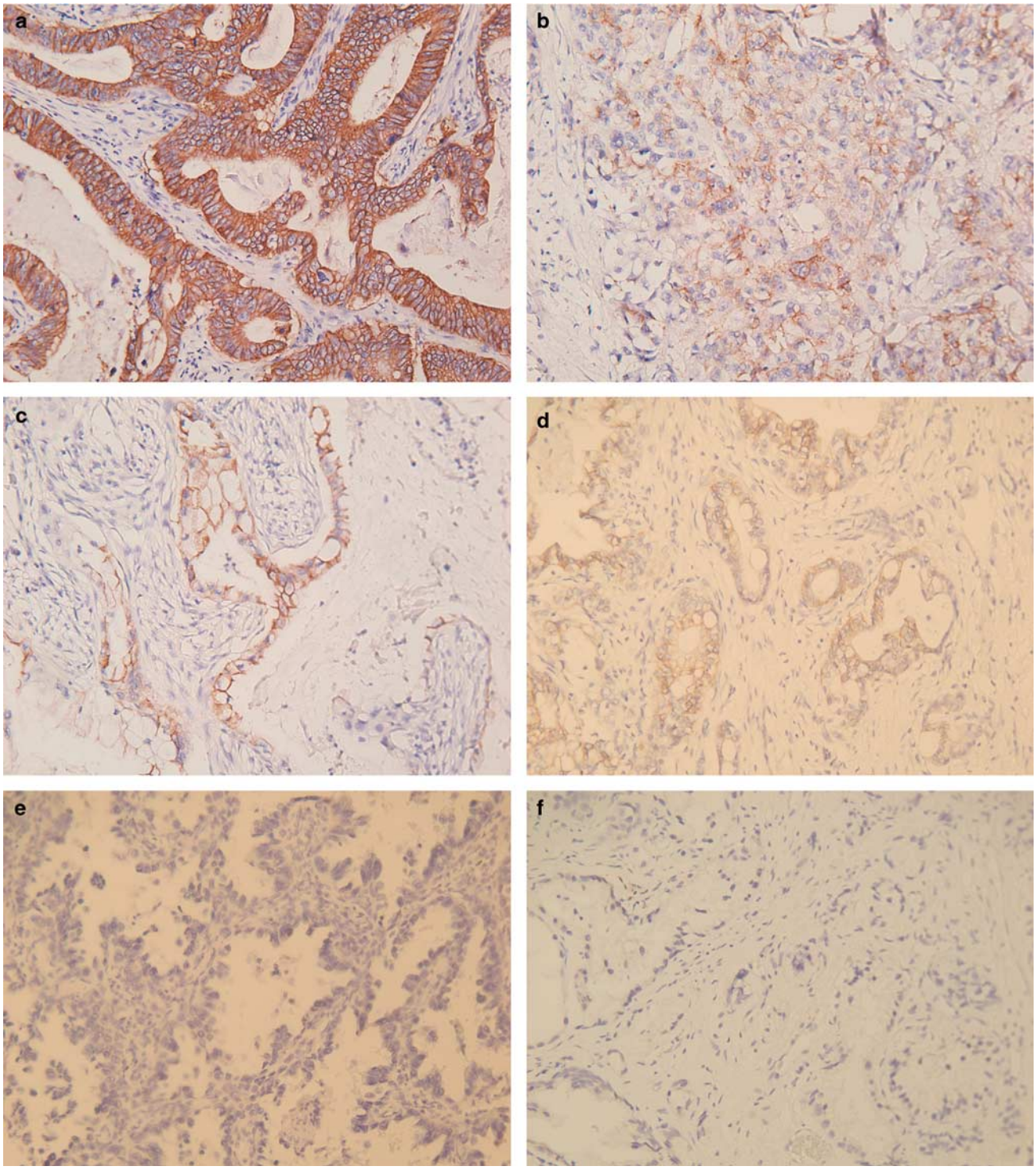

Figure 3 Immunohistochemical staining of cadherin-17 in tumor tissue. Cadherin 17 was expressed in colorectal adenocarcinoma (**a**), gastric adenocarcinoma (**b**), pancreatic ductal adenocarcinoma (**c**), and cholangiocarcinoma (**d**), but not in pulmonary (**e**) or prostatic adenocarcinoma (**f**).

To compare with current standard markers for the identification of GI adenocarcinomas, the same arrays were stained with cytokeratin 7, cytokeratin 20, and CDX2. Similar to cadherin-17, CDX2 showed diffuse staining in colorectal carcinomas, and more scattered staining patterns in gastric, pancreatic, and biliary carcinomas. The sensitivity of cadherin-17 was slightly better than CDX2 (colorectal, 96 *vs* 92%; gastric, 56 *vs* 66%; pancreatic, 57 *vs* 11%; biliary, 27 *vs* 16%). CDX2 was expressed in 26% of mucinous ovarian carcinomas, a common differential diagnosis for colorectal adenocarcinoma. In

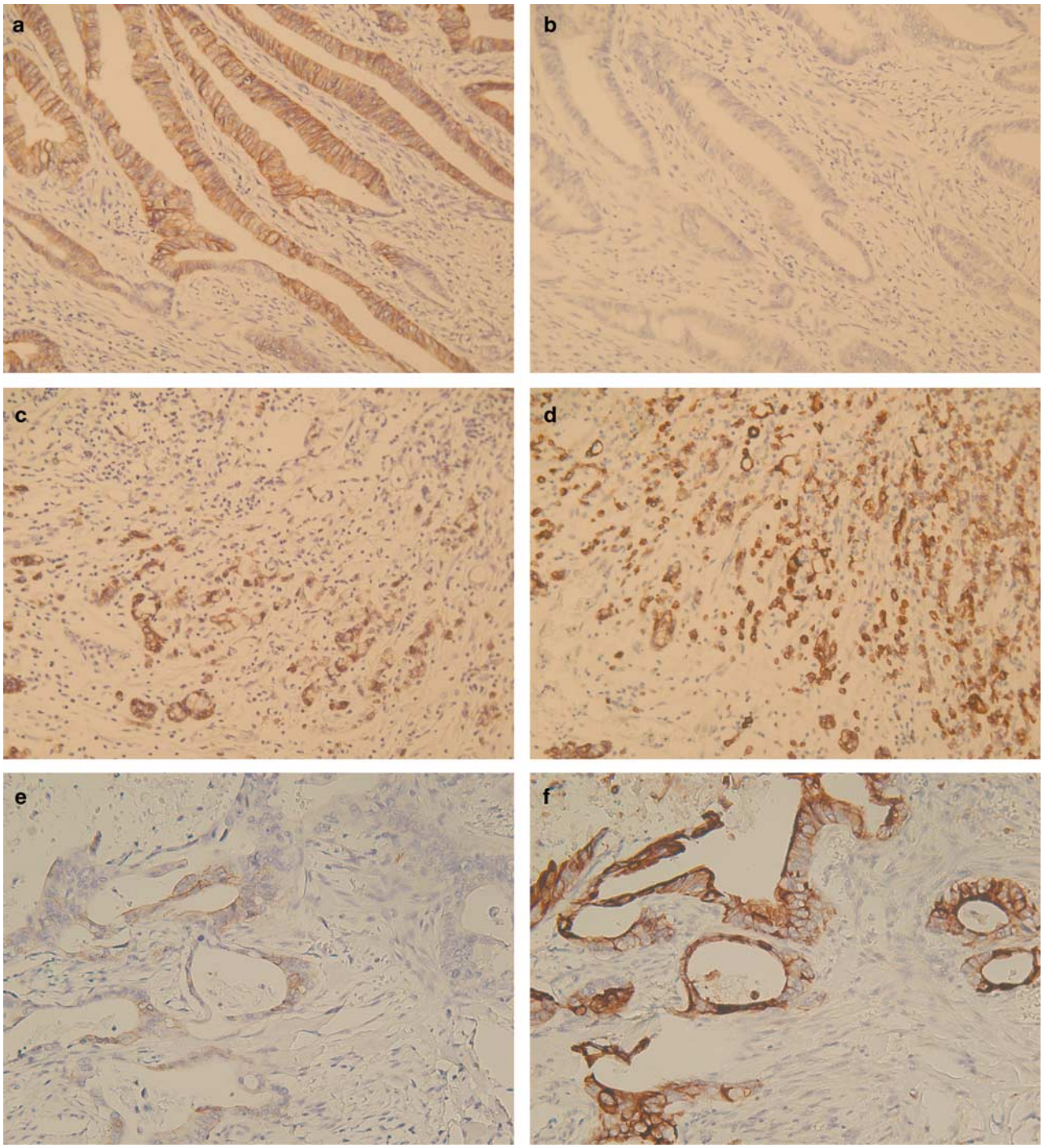

Figure 4 Expression of cadherin-17 and cytokeratin 7 in colorectal, gastric and pancreatic adenocarcinoma. (**a**, **b**) A colorectal adenocarcinoma positive for cadherin-17 (**a**) and negative for cytokeratin 7 (**b**). (**c**, **d**) A gastric adenocarcinoma positive for cadherin-17 (**c**) and cytokeratin 7 (**d**). (**e**, **f**) A pancreatic adenocarcinoma positive for cadherin-17 (**e**) and cytokeratin 7 (**f**).

contrast, none of the 34 mucinous ovarian carcinomas expressed cadherin-17. These results suggest cadherin-17 is a comparable, if not better marker, than CDX2. As in previous reports,[14] we found that cytokeratin 7 is frequently expressed in adenocarcinomas from most organs but not expressed in colorectal cancers. We reasoned that a cadherin-17/cytokeratin 7 two-marker profile may further differentiate colorectal cancers from those arising from upper GI organs. Immunostaining of cytokeratin 7 in our tissue array showed that 37 of 47 (79%) of colorectal adenocarcinomas were cadherin-17(+)/cytokeratin 7(−) (Table 3a; Figures 4a and b). Another 6 (13%) colorectal adenocarcinomas were cadherin-17(+)/cytokeratin 7(+). In total, 24 of 45 (53%) gastric adenocarcinomas, 16 of 28 (51.7%)

Table 3 Results of cadherin-17/cytokeratin 7 profile in colorectal cancer, pancreatic cancer, gastric cancer, and cholangiocarcinoma

a Colorectal cancer

Cytokeratin 7 \ Cadherin-17	0	1	2	3	4
0	2		2	4	31
1					5
2					1
3					
4					

b Pancreatic cancer

Cytokeratin 7 \ Cadherin-17	0	1	2	3	4
0					
1				1	
2				1	
3					1
4	11	7	4	1	

c Gastric cancer

Cytokerain 7 \ Cadherin-17	0	1	2	3	4
0	2			1	
1	3	1			1
2	1	3	1		
3	3			1	
4	11	4	3	6	2

d Cholangiocarcinoma

Cytokerain 7 \ Cadherin-17	0	1	2	3	4
0					
1					1
2	1	1			
3	2				
4	18	3		1	2

pancreatic adenocarcinomas, and 9 (27%) of 33 cholangiocarcinomas were cadherin-17(+)/cytokeratin 7(+) (Figures 4c–f). One gastric adenocarcinoma was cadherin-17(+)/cytokeratin 7(−) (Table 3b–d). The staining of cytokeratin 20 in tumors was heterogeneous and did not give information additional to that obtained from the other three markers.

Discussion

Adenocarcinoma of an unknown primary site is one of the most common clinical problems. The seven commonest primary sites are breast, colon, lung, ovary, pancreas, prostate, and stomach.[15] Because metastatic adenocarcinomas from different locations may have a similar microscopic appearance, identification of the primary site is difficult. A number of immunohistochemical markers were identified to assist the histologic diagnosis such as cytokeratin 7, cytokeratin 20, thyroid transcription factor 1, CDX2, prostate-specific antigen, and mesothelin.[3,4,15] However, a highly specific marker for GI adenocarcinoma is still lacking.

Cadherin-17 is an adhesion protein that is normally expressed only in intestinal epithelium. This highly specific localization makes cadherin-17 a potential tool for immunohistochemical diagnosis. Our results show that cadherin-17 expression is very common in colorectal adenocarcinomas and is also expressed in more than half of gastric and pancreatic adenocarcinomas, and in a small number of cholangiocarcinomas. Its expression is not limited to tumors with intestinal metaplasia. Furthermore, expression of cadherin-17 is very uncommon in carcinomas outside the GI tract. These observations make cadherin-17 a useful marker for histologic diagnosis of adenocarcinoma of unknown primary site.

To further differentiate tumors from the colorectal region from those derived from the upper GI tract, two approaches were used. Expression of cadherin-17 in colorectal adenocarcinoma was usually diffuse and strong whereas expression in adenocarcinomas of the upper GI tract was usually focal or scattered. The other approach we used was immunostaining of cytokeratin 7. Immunoreactivity of cytokeratin 7 was seen in adenocarcinomas from most body sites, which limits its clinical application. However, using cadherin-17/cytokeratin 7 two-marker profiles

greatly narrowed down the candidate primary site. In our data set, 37 of 38 (97.3%) cadherin-17(+)/cytokeratin 7(−) tumors were colorectal adenocarcinomas. The other one was a gastric adenocarcinoma. Of 56, 49 (86.0%) cadherin-17(+)/cytokeratin 7(+) tumors were gastric, pancreatic, or biliary adenocarcinomas, 6 were colorectal adenocarcinomas, and 1 was a prostatic adenocarcinoma. Hence, the cadherin-17/cytokeratin 7 profile can differentiate adenocarcinoma of upper and lower GI tract with high specificity.

In our tissue array, cadherin-17 was expressed in more than 95% of colorectal adenocarcinomas. One of the two cases immunonegative for cadherin-17 fulfilled the criteria of large cell minimally differentiated carcinomas of the colon,[16] a tumor characterized by loss of glandular differentiation and microsatellite instability. The other was an adenosquamous carcinoma. Cadherin-17 was only expressed in slightly over half of gastric and pancreatic adenocarcinomas and 27% of cholangiocarcinomas. These results indicate a cadherin-17(–) adenocarcinoma is very unlikely to be a colorectal adenocarcinoma, but the possibility of it arising from stomach, pancreas, and biliary tract cannot be completely ruled out.

The tissue samples used in our tissue array were obtained from the primary sites of the tumor. Whether cadherin-17 retained its utility in metastatic tumors remains to be clarified. In gastric cancer, cadherin-17 expression was significantly more frequent in advanced-stage cases,[11] but in pancreatic and colorectal cancer, cadherin-17 was more frequent in well-differentiated tumors[10,13] without lymph node metastasis. Further testing using biopsy specimens from additional metastatic cancers would be worthwhile.

Acknowledgement

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Expression of liver-intestine cadherin and its possible interaction with galectin-3 in ductal adenocarcinoma of the pancreas

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Liver-intestine (LI) cadherin represents a novel type of cadherin within the cadherin superfamily, and is distinguished from other cadherins by specific structural and functional features. Among normal tissues, LI-cadherin is known to be expressed in the intestinal mucosa, while its expression in cancerous tissue has not been investigated to date, except in gastric carcinoma. In the present study we investigated LI-cadherin expression immunohistochemically using our newly established monoclonal antibody in a large set (n=102) of tumor specimens from patients with ductal adenocarcinoma of the pancreas, and correlated the findings with the patients' survival. LI-cadherin expression was seen focally in normal pancreatic ducts. In carcinoma, well-differentiated carcinoma cases strongly expressed LI-cadherin, whereas less differentiated areas and poorly differentiated carcinoma cases expressed less or were negative. Kaplan-Meier analysis for all patients demonstrated that high LI-cadherin expression (>25% of cells stained positive) correlated with good survival (P<0.001). Cox regression analyses demonstrated that LI-cadherin expression was one of the strongest predictors of outcome, independent of all other variables, and low LI-cadherin expression correlated with tumor de-differentiation and advanced stage. Furthermore, galectin-3 was identified as being coimmunoprecipitated with LI-cadherin and this interaction was inhibited by lactose in a dose-dependent manner, but not by sucrose. Because galectin-3 has been observed to show a similar expression pattern to LI-cadherin in ductal adenocarcinoma of the pancreas, expression of LI-cadherin and this interaction could have some role in ductal adenocarcinoma of the pancreas. (Cancer Sci 2003; 94: 425–430)

Pancreatic carcinoma is one of the most lethal malignancies worldwide, and its incidence has been increasing in industrialized countries.[1, 2] Most patients with pancreatic carcinoma are diagnosed at an advanced stage because of the aggressiveness of this disease and the lack of early symptoms, and the 5-year survival rate is only 10–26% even after curative surgical resection.[3–5] A number of studies have been done to date to reveal the pathological and biological prognostic factors in pancreatic carcinoma,[6] but the precise reason for the biological aggressiveness of this disease has not yet been elucidated.

Cadherins are a family of Ca^{2+}-dependent homotypic cell-cell adhesion molecules that are involved in the maintenance of tissue structure and morphogenesis.[7, 8] It is known that all members of this family share common structural features, with some exceptions, having an amino-terminal extracellular region characterized by a unique domain called the cadherin repeat, a single transmembrane domain, and a cytoplasmic domain at the carboxyl terminus.[9] A large number of cadherin superfamily members have been identified to date, and are expressed in different tissues of a variety of multicellular organisms. There is increasing evidence that cadherin-mediated cell adhesion additionally plays a crucial role in carcinoma cell behavior.[10, 11]

LI-cadherin, which is also called human peptide transporter-1 (HPT-1), is a structurally unique member of the cadherin superfamily.[12, 13] Whereas the so-called classic cadherins, such as E-, N- and P-cadherin, have five cadherin repeats within the extracellular domain, LI-cadherin consists of seven cadherin repeats. LI-cadherin has only 20 amino acids in the cytoplasmic domain, although classic cadherins have a highly conserved cytoplasmic domain which consists of 150 to 160 amino acids. The expression of LI-cadherin differs by species. In the rat, LI-cadherin is expressed in the liver and intestinal epithelial cells, while in humans and the mouse, LI-cadherin is expressed in the intestinal epithelial cells, but not in the liver.[14] Although there is only one study with respect to expression of LI-cadherin in gastric carcinomas,[15] to the best of our knowledge the clinicopathologic significance of its expression in ductal adenocarcinoma of the pancreas has not previously been investigated.

LI-cadherin is also known to possess biological functions distinct from classic cadherin, in addition to the differences in its structure as described above, e.g., the adhesive function of LI-cadherin is independent of any interaction with cytoplasmic components, although E-cadherin lacking the cytoplasmic domain is known to be unable to mediate strong cell-cell adhesion.[16, 17] Indeed, no binding partners have been identified for LI-cadherin, to date.

In this study, we raised monoclonal antibodies (mAbs) against a pancreatic cancerous tissue and one of these antibodies was an anti-LI-cadherin antibody. Using this antibody, we initially immunohistochemically investigated LI-cadherin expression in tumor specimens from a large series of patients (n=102) with ductal adenocarcinoma of the pancreas, and attempted to determine correlations between the extent of its expression and clinico-pathological parameters. To better understand the biological function of LI-cadherin, we further explored the molecules which associate preferentially with LI-cadherin in pancreatic carcinoma cells, and one of these molecules was identified as galectin-3.

Materials and Methods

Production of monoclonal antibody. Male mice (C.B-17/Icr Crj-scid/scid) with severe combined immunodeficiency were immunized with a pancreatic carcinoma surgical specimen by a rejection method, and hybridomas were produced as described previously.[18] A hybridoma clone producing mAb, D-4 (IgG1, k), was selected on the basis of immunohistochemical reactivity with pancreatic carcinoma tissue.

Cell cultures and reagents. The human pancreatic carcinoma cell

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lines AsPC-1 and PANC-1 were obtained from the American Type Culture Collection (Rockville, MD). They were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37°C.

The goat polyclonal antibody against LI-cadherin (pAb anti-LI-cadherin) was purchased from Santa Cruz Biotechnology, Inc. (C-17; Santa Cruz, CA). MAb galectin-3 was obtained as described previously.[19] Normal mouse IgG and normal goat IgG were purchased from Sigma Chemical Co. (St. Louis, MO).

Immunoprecipitation and immunoblotting. For immunoprecipitation, cells were lysed using an ice-cold lysis buffer [150 m*M* NaCl, 1% Triton X-100, 10 m*M* Hepes (pH 7.4)] containing a protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany) on ice for 30 min. Lysates were cleared by centrifugation (15 000 *g*, 30 min) and the soluble fractions were precleared with 50 μl of protein-G Sepharose (Pharmacia Biotechnologies, Uppsala, Sweden) (50% slurry) for 2 h. After the addition of the indicated antibodies, samples were incubated overnight at 4°C. Immunocomplexes were precipitated by incubating the samples for 3 h with 50 μl of protein G Sepharose. Sepharose beads were washed 5 times with a lysis buffer and were heat-denatured in an equal volume of 2× sodium dodecyl sulfate (SDS) sample buffer containing 25 m*M* dithiothreitol (Sigma Chemical Co.). Samples were electrophoresed on SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). After blocking, the filters were reacted with primary antibodies followed by horseradish peroxidase-conjugated anti-mouse IgG+IgM (IBL, Fujioka) or anti-goat IgG (Santa Cruz Biotechnology, Inc.). Peroxidase-labeled bands were visualized using an enhanced chemiluminescence detection system (Amersham International, Buckinghamshire, UK) according to the manufacturer's instructions.

To ascertain the possible association of LI-cadherin with galectin-3, immunoprecipitation was carried by adding 2 μg of mAb D-4 in the presence of 0, 1, 10, 100 m*M* lactose or 100 m*M* sucrose at 4°C for 2 h. Then, immunoprecipitates were washed five times with a lysis buffer. Immunoprecipitates with SDS sample buffer were heat-denatured and processed for immunoblotting as described above.

Protein sequencing. Protein immunoprecipitated with mAb D-4 was subjected to 7.5% SDS-PAGE and subsequently transferred onto a PVDF membrane. Protein was revealed by Coomassie blue staining [25% methanol, 7.5% acetic acid, and 0.25% brilliant blue R-250 (Sigma Chemical Co.)] for 3 min and subsequent destaining (40% methanol, 10% acetic acid) until bands became visible. The 120-kDa band of the antigen was cut out and sequenced (TaKaRa Shuzo Co., Ltd., Shiga). The protein, which co-immunoprecipitated with mAb D-4 was visualized by SYPRO Ruby protein gel staining (Bio-Rad Laboratories, Hercules, CA) and its band was excised from the gel and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis (Promega Co., Ltd., Tokyo).

Patients and tissue specimens. Formalin-fixed, paraffin-embedded tumor specimens were obtained from a series of 102 patients with ductal adenocarcinoma of the pancreas, who had undergone surgical resection at the National Cancer Center Hospital, Japan, between 1990 and 1999. There were 62 men (61%) and 40 women (39%). The mean age was 62.0 years (range, 45 to 82 years). Eight patients underwent total pancreatectomy, 28 patients underwent distal pancreatectomy, and 66 patients underwent pancreaticoduodenectomy, of which 14 involved pylorus-preserving pancreaticoduodenectomy. Intraoperative radiotherapy was given in 77 cases. The specimens were classified by their TNM stage according to the Union Internationale Contre le Cancer (UICC),[20] and by the other clinico-pathological parameters (histologic differentiation, venous invasion, lymphatic invasion, and perineural invasion) according to the Japan Pancreas Society classification.[21] The median duration of follow-up was 654 days (range, 79 to 2685 days). Patients with pancreatic tumors of a special type, such as mucinous cystadenocarcinoma, intraductal papillary-mucinous adenocarcinoma, and adenosquamous carcinoma, were excluded. Three patients who died in the immediate postoperative period were also excluded.

Immunohistochemistry. Sections (5 μm thick) of formalin-fixed, paraffin-embedded tissues were deparaffinized with xylene, treated with 0.3% hydrogen peroxide in methanol, immersed in 10 m*M* citrate buffer (pH 6.0), heated to 120°C in an autoclave for 10 min, and then allowed to cool at room temperature for 30 min. The sections were preincubated in 2% normal porcine serum in phosphate-buffered saline, incubated with the purified mAb D-4 (1.2 μg/ml) at 4°C overnight, washed with phosphate-buffered saline, and incubated with the biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) for 30 min at room temperature. Subsequently, they were incubated with the avidin-biotin-peroxidase complex using a Vectastain ABC kit (Vector Laboratories) for 30 min. For visualization of the antigen, sections were immersed in 0.05% diaminobenzidine tetrahydrochloride solution containing 0.01% hydrogen peroxide, and counter-stained with hematoxylin. Sections of formalin-fixed, paraffin-embedded human intestinal epithelial cells known to express LI-cadherin were used as a positive control. Negative control staining of every specimen, which was performed using the same class of mouse immunoglobulin instead of the primary antibody, yielded negative results.

The LI-cadherin expression of the tumor was evaluated according to the proportion of positively stained cells. When ≥25% of the carcinoma cells were positively stained, the case was classified as positive (+), with other cases being classified as negative (−). All the hematoxylin and eosin-stained and immunohistochemistry slides were assessed by two independent observers who had no knowledge of the patients' clinical information. To confirm the quality of all sections, we immunohistochemically examined all samples using an antibody for cytokeratin (KL-1; Immunotech, Marseille, France), as described previously (data not shown).[19]

Statistical analysis. Statistical analyses were performed using the computer software Stat View-J 5.0 (Abacus Concepts, Berkeley, CA). Expression of LI-cadherin was assessed for associations with various clinico-pathological parameters using the χ^2 test. Survival rates were calculated by the Kaplan-Meier method. The difference between the survival curves was ana-

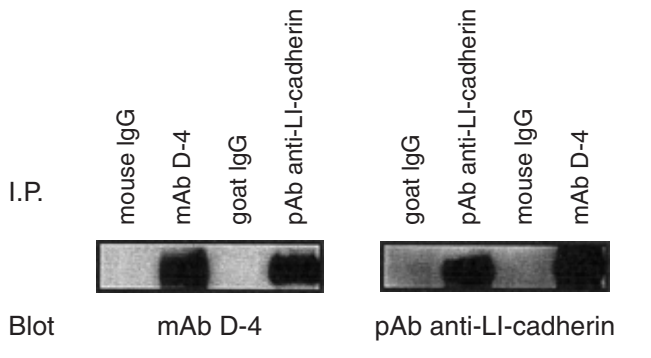

Fig. 1. MAb D-4 recognizes a 120-kDa protein, LI-cadherin. Lysates from AsPC-1 cells were immunoprecipitated with either mAb D-4 or pAb anti-LI-cadherin, resolved on 7.5% SDS-PAGE, and transferred to a membrane. Membranes were then probed with either pAb anti-LI-cadherin or mAb D-4 antibody. Immunoprecipitates with normal mouse and goat IgGs were used as controls for antibody reactivity.

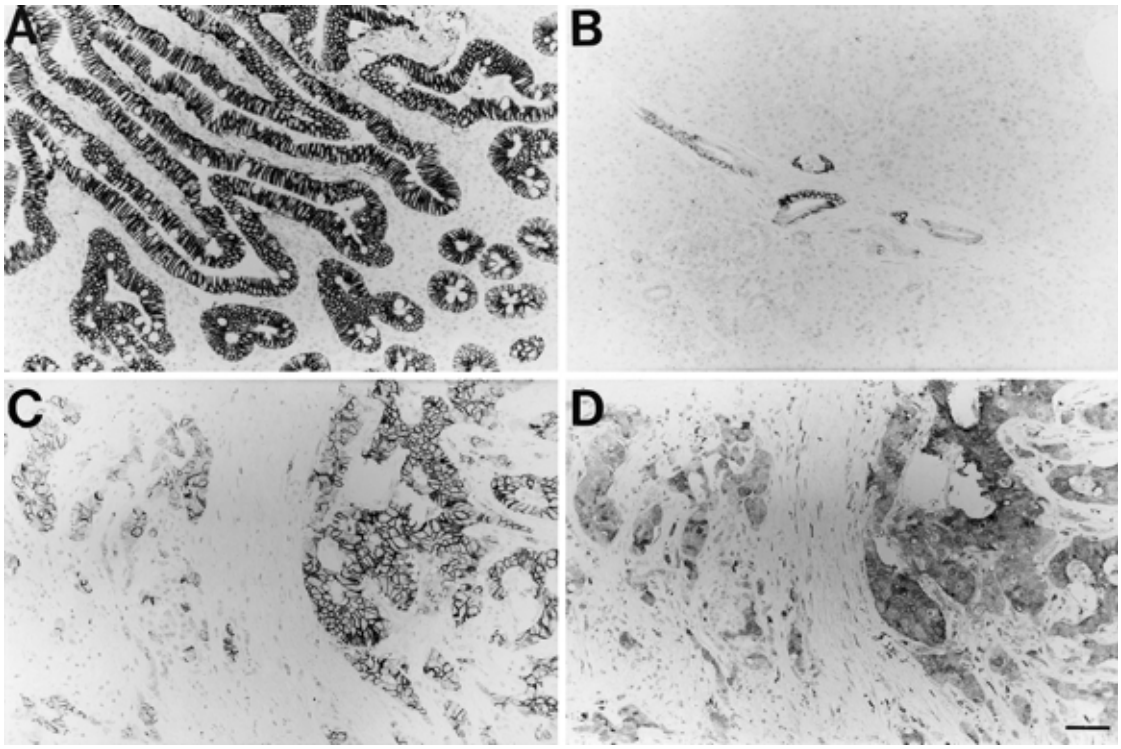

Fig. 2. Immunohistochemical expression of LI-cadherin and galectin-3 in normal and cancerous tissues. LI-cadherin is present strongly in the basolateral membrane in normal duodenal epithelial cells (A), and focally and weakly in normal pancreatic ducts (B). In pancreatic ductal adenocarcinoma (C and D: consecutive sections), LI-cadherin is expressed strongly in well-differentiated cancer nests, but less in invading poorly differentiated cancer nests (C). The expression pattern of galectin-3 (D) is observed to be similar to that of LI-cadherin. The scale bar indicates 100 μm.

lyzed by means of the log-rank test. To assess the correlation between survival time and multiple clinico-pathological parameters, multivariate analyses were performed using the Cox proportional hazards regression model. Differences were considered significant when the *P* value was less than 0.05.

Results

Characterization of mAb D-4. The mAb D-4 showed strong reactivity to pancreatic carcinoma tissues and also other types of carcinoma tissues, such as colon carcinoma and gastric carcinoma (data not shown). To purify the D-4 antigen, we performed immunoprecipitation experiments. We chose the pancreatic cancer cell line AsPC-1 as an antigen source because it strongly expressed the D-4 antigen. Precipitated antigen was separated on SDS-PAGE, blotted onto a PVDF membrane, and detected by Coomassie blue staining as a 120-kDa protein. Amino acid sequence analysis revealed the sequence IDHVTGEIFSVA. This amino acid sequence corresponds to the human LI-cadherin amino acid sequence at residues 612–623. AsPC-1 cell lysates were immunoprecipitated with pAb anti-LI-cadherin, and the resulting immunoprecipitates were reacted with mAb D-4 on immunoblots, and vice versa (Fig. 1). The mAb D-4 had the same immunoreactivity in the specimens of this study as did pAb anti-LI-cadherin (data not shown). These analyses showed clearly that mAb D-4 reacted specifically with LI-cadherin.

LI-cadherin expression in normal tissues and ductal adenocarcinoma of the pancreas. LI-cadherin immunoreactivity was observed in normal tissues and ductal adenocarcinoma of the pancreas. In agreement with previous reports,[13, 15] LI-cadherin expression was seen in normal intestinal epithelial cells (Fig. 2A) and pancreatic ducts (Fig. 2B); however, LI-cadherin expression of the latter was only seen focally and weakly in about half of the cases, as compared with the former. Also, in other normal tissues (brain, esophagus, stomach, lung, liver, kidney, adrenal gland and skin), LI-cadherin expression was not seen, as re-

Table 1. Relationship between LI-cadherin expression and clinicopathological parameters

	LI-cadherin expression		*P* value
	(+)	(–)	
Gender			
Male	23	40	
Female	15	24	0.84
Age (years)			
<65	19	33	
≥65	19	31	0.88
Tumor size (cm)			
<3.6	16	31	
≥3.6	22	33	0.54
Histologic differentiation			
Well	24	14	
Moderately, poorly	14	50	<0.001
Venous invasion			
v0, 1	25	38	
v2, 3	13	26	0.52
Lymphatic invasion			
ly0, 1	24	32	
ly2, 3	14	32	0.20
Perineural invasion			
ne0, 1	19	30	
ne2, 3	19	34	0.76
pTNM stage			
I, II, III	23	24	
IVa, IVb	15	40	0.02

ported previously (data not shown).[13, 15] Immunoreactivity was present in the basolateral plasma membrane, but not the apical membrane.

In carcinoma tissues, LI-cadherin expression was noted in 84 of 102 carcinoma cases (82%). Most of the cases of well-differentiated carcinoma with tight cell-cell adhesion expressed LI-cadherin strongly, but in the less differentiated areas, LI-cadherin expression seemed to be reduced (Fig. 2C). Expression of LI-cadherin was evaluated according to our criteria (see "Materials and Methods"). Thirty-eight cases (37%) were classified as positive, whereas the remaining 64 cases (63%) were classified as negative.

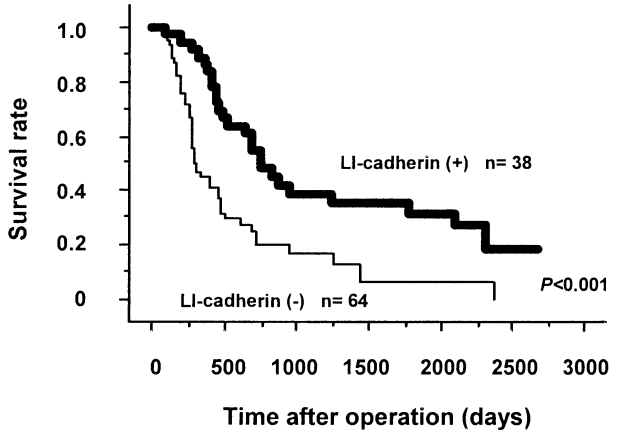

Fig. 3. Kaplan-Meier survival curves of 102 patients. The prognosis became significantly worse in the negative LI-cadherin expression group (thin line) compared with the positive LI-cadherin expression group (thick line; log-rank test, $P<0.001$).

Relationship of LI-cadherin expression with clinico-pathological parameters. Table 1 shows the relationship between LI-cadherin expression and clinico-pathological parameters. Among 38 well-differentiated carcinomas, 24 (63%) cases were positive, whereas 14 (22%) cases were positive among 64 moderately to poorly differentiated carcinomas. Similarly, among 47 cases in stage pI, pII, pIII, 23 (49%) were positive, whereas 15 (23%) cases were positive among 55 cases in stage pIVa, pIVb. Thus, the extent of LI-cadherin staining decreased with histologic dedifferentiation and advanced pTNM stage, and these findings were statistically significant ($P<0.001$ and $P=0.02$, respectively).

Prognostic significance of LI-cadherin expression. Kaplan-Meier survival curves demonstrated that patients whose tumors were LI-cadherin-positive had a significantly longer overall survival time than did those with LI-cadherin-negative tumors ($P<0.001$ by log-rank test, Fig. 3). In univariate analysis of overall survival, histologic differentiation ($P=0.01$; hazard ratio 1.87, 95% confidence interval (CI) 1.14–3.05), pTNM stage ($P<0.001$; hazard ratio 2.44, 95% CI 1.50–3.95) and the extent of LI-cadherin staining ($P<0.001$; hazard ratio 2.48, 95% CI 1.51–4.09) were all significant (Table 2). Multivariate analysis of overall survival revealed that the independent significant factors were the extent of LI-cadherin staining ($P=0.01$; hazard ratio 2.04, 95% CI 1.16–3.61) and the pTNM stage ($P=0.02$; hazard ratio 1.90, 95% CI 1.11–3.24) (Table 3).

Purification and identification of the 30-kDa protein coimmunoprecipitated with LI-cadherin. To explore the role of LI-cadherin in pancreatic carcinoma, we next attempted to isolate LI-cadherin-interacting proteins using immunoprecipitation experiments. A 30-kDa protein coimmunoprecipitated with mAb D-4 in AsPC-1 cells, but not in PANC-1 cells, a LI-cadherin negative cell line (Fig. 4). The purified 30-kDa protein was identified as ga-

Table 2. Univariate analysis of overall survival for LI-cadherin expression and clinico-pathological parameters

	Number of cases	Hazard ratio	95% CI	*P* value
Gender				
Male	63	1		
Female	39	1.07	0.66–1.72	0.79
Age (years)				
<65	52	1		
≥65	50	1.16	0.73–1.83	0.54
Tumor size (cm)				
<3.6	47	1		
≥3.6	55	1.20	0.76–1.90	0.43
Histologic differentiation				
Well	38	1		
Moderately, poorly	64	1.87	1.14–3.05	0.01
Venous invasion				
v0, 1	63	1		
v2, 3	39	1.60	1.00–2.57	0.05
Lymphatic invasion				
ly0, 1	56	1		
ly2, 3	46	1.49	0.94–2.36	0.09
Perineural invasion				
ne0, 1	49	1		
ne2, 3	53	1.20	0.75–1.91	0.45
pTNM stage				
I, II, III	47	1		
IVa, IVb	55	2.44	1.50–3.95	<0.001
LI-cadherin staining				
+	38	1		
–	64	2.48	1.51–4.09	<0.001

CI: confidence interval.

Table 3. Multivariate analysis of overall survival

	Number of cases	Hazard ratio	95% CI	*P* value
Gender				
Male	63	1		
Female	39	0.93	0.56–1.54	0.79
Age (years)				
<65	52	1		
≥65	50	1.29	0.80–2.08	0.30
Tumor size (cm)				
<3.6	47	1		
≥3.6	55	1.32	0.78–2.22	0.30
Histologic differentiation				
Well	38	1		
Moderately, poorly	64	1.34	0.76–2.35	0.31
Venous invasion				
v0, 1	63	1		
v2, 3	39	1.09	0.60–1.96	0.78
Lymphatic invasion				
ly0, 1	56	1		
ly2, 3	46	1.15	0.65–2.03	0.64
Perineural invasion				
ne0, 1	49	1		
ne2, 3	53	0.89	0.51–1.56	0.69
pTNM stage				
I, II, III	47	1		
IVa, IVb	55	1.90	1.11–3.24	0.02
LI-cadherin staining				
+	38	1		
–	64	2.04	1.16–3.61	0.01

CI: confidence interval.

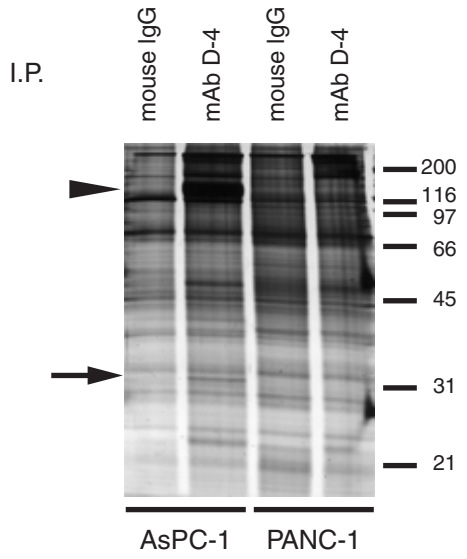

Fig. 4. Detection of protein coimmunoprecipitated with LI-cadherin. A nonreducing, silver-stained SDS-PAGE gel of protein immunoprecipitated with either normal mouse IgG or mAb D-4 is shown. A 30-kD protein (arrow) coimmunoprecipitated with mAb D-4 in AsPC-1 cells, but not in PANC-1 cells, a LI-cadherin-negative cell line. Molecular weight markers ($\times 10^{-3}$) are shown in the right margin. An arrowhead indicates the position of the LI-cadherin. These results are representative of three independent experiments.

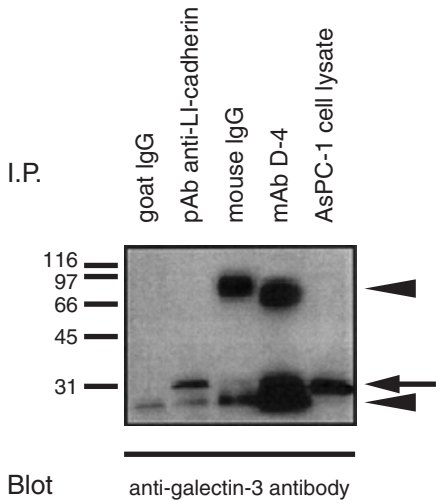

Fig. 5. Interaction of LI-cadherin with galectin-3. Galectin-3 was detected in LI-cadherin immune complexes precipitated either with pAb anti-LI-cadherin or mAb D-4, but not with control Abs. Molecular weight markers ($\times 10^{-3}$) are shown in the left margin. An arrow indicates the position of galectin-3. Arrowheads indicate the position of the immunoglobulin heavy and light chains. These results are representative of three independent experiments.

lectin-3 by LC-MS/MS which identified three tryptic peptides (MLITILGTVKPNANR, QSVFPFESGKPFK and VAVNDAHLLQYNHR). In fact, galectin-3 was detected in LI-cadherin immune complexes immunoprecipitated with pAb anti-LI-cadherin as well as mAb D-4 (Fig. 5). Moreover, using immunohistochemical experiments, we and others have revealed that galectin-3 was frequently expressed in well-differentiated carcinomas, but its expression was decreased in moderately and poorly differentiated carcinomas (Fig. 2D).[19, 22] This result indicates that the expression pattern of galectin-3 is very similar to that of LI-cadherin in ductal adenocarcinoma of the pancreas (Fig. 2C), and this interaction is likely not only *in vitro*, but also *in vivo*.

Galectin-3 binds to LI-cadherin in a carbohydrate-dependent manner. Next, we tested whether the interaction between LI-cadherin and galectin-3 is mediated by the carbohydrate recognition domain of galectin-3; thus, the effect of lactose, a competitive disaccharide of galectin-3 carbohydrate recognition domain, on galectin-3 binding to LI-cadherin was assayed. In the presence of lactose, binding of galectin-3 to LI-cadherin was inhibited in a dose-dependent manner, but in the presence of sucrose, a noncompetitive disaccharide, their binding was not inhibited (Fig. 6).

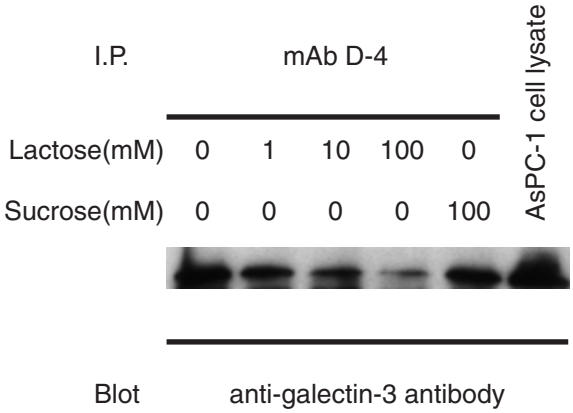

Fig. 6. LI-cadherin interacts with galectin-3 in a carbohydrate-dependent manner. Lysates from AsPC-1 cells were incubated with or without various concentrations of lactose (1, 10, 100 m*M*) and 100 m*M* sucrose, and immunoprecipitated with mAb D-4. Binding of galectin-3 to LI-cadherin was inhibited by lactose in a dose-dependent manner, but not by sucrose. These results are representative of three independent experiments.

Discussion

This is the first study to investigate the clinico-pathologic significance of LI-cadherin expression in ductal adenocarcinoma of the pancreas. Consistent with the findings of others on gastric carcinomas,[15] we observed that LI-cadherin expression was reduced in less differentiated areas and poorly differentiated carcinomas exhibiting invasive growth, compared with well-differentiated carcinomas in pancreatic carcinoma. This observation is also in good agreement with some reports about E-cadherin,[23, 24] and supports the previous reports that LI-cadherin is capable of mediating cell-cell adhesion as well as the classic cadherins.[12, 17] In normal pancreatic ducts, we observed that LI-cadherin was expressed focally and weakly in about half of the cases. On the other hand, E-cadherin was expressed uniformly in almost all the normal pancreatic ducts as reported previously (data not shown).[24] These observations suggest that in normal pancreatic ducts, LI-cadherin plays an auxiliary role in cell-cell adhesion.

We have found that LI-cadherin expression was associated with histologic differentiation and pTNM stage, which are important prognostic factors in pancreatic carcinoma.[6] Moreover, the survival analysis showed that the prognosis for survival was significantly poorer in the LI-cadherin-negative tumors compared with the LI-cadherin-positive tumors, in both univariate and multivariate analyses. These observations suggest that LI-cadherin expression is a good predictor for the prognosis of patients with ductal adenocarcinoma of the pancreas.

In the present study, we found that galectin-3 was coimmu-

noprecipitated with LI-cadherin. Galectins are a family of proteins defined by having at least one characteristic carbohydrate recognition domain with an affinity for β-galactosides, and sharing certain conserved sequence elements.[25–27] At least 10 galectins have now been identified in mammals, and galectin-3 has been the best characterized and studied among them. Galectin-3 is presumed to be involved in multiple biological processes, such as cell-cell and cell-matrix interactions, cell proliferation, differentiation, cell cycle regulation, angiogenesis, apoptosis resistance and metastasis.[28–35] In pancreatic carcinoma, we have previously demonstrated that decreased expression of galectin-3 was associated with an advanced stage, tumor de-differentiation.[19] In this study, we have found that the expression pattern of galectin-3 is very similar to that of LI-cadherin. Therefore, we speculate that LI-cadherin interacts with galectin-3 not only *in vitro*, but also *in vivo*, and this interaction may have some role in ductal adenocarcinoma of the pancreas.

Galectin-3 is known to interact with cell surface proteins, such as laminin, carcinoembryonic antigen, and α1β1 integrin via its carbohydrate recognition domain.[28–30] We have demonstrated that the interaction between LI-cadherin and galectin-3 is mediated by the carbohydrate recognition domain. Thus, we speculate that galectin-3 binds to LI-cadherin on the cell surface of pancreatic carcinoma. Further study is necessary to understand the cellular changes induced by this interaction between LI-cadherin and galectin-3.

In conclusion, we have shown in this study that the expression of LI-cadherin could be useful as a marker for predicting the outcome of patients with resectable ductal adenocarcinoma of the pancreas. LI-cadherin might participate in the early stage of this disease through interacting with galectin-3.

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