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Señora Jefe de la
DIVISION DE NUEVAS CREACIONES
Superintendencia de Industria y Comercio
E. S. D.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 05-091402- -00003-0000

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Evento : 379

Actuación : 538

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Asunto : Solicitud de Patente para **ANTICUERPO CONTRA
ANTIGENO ESPECIFICO DE TUMOR COMO
OBJETIVO**
Solicitante : **SANKYO COMPANY, LIMITED**
Clase : A61K 031/7088, A61K 039/395, A61K 048/000,
Publicada en : La Gaceta No. 578 Del 31 de julio de 2007
Fecha de Solicitud : 9 de septiembre de 2005

1. Yo, Emilio FERRERO, identificado como aparece al pie de mi firma, actuando como apoderado en el asunto de la referencia, atentamente, solicito se efectúe el examen de patentabilidad de la solicitud en referencia presentada de acuerdo con el Capítulo II del PCT y según lo establecido en el artículo 44 de la Decisión 486 de la Comisión de la Comunidad Andina. Para tal efecto presento junto con este escrito el recibo por valor de \$210.000.00, que corresponde a la tasa fijada según lo dispuesto en la resolución No. 44372 de 26 de diciembre de 2007.

2. Ruego atentamente a la Señora Jefe se sirva ordenar que se anexe este recibo para que se efectúe dicho examen y se continúe con el trámite de la solicitud.

Señora Jefe,

EMILIO FERRERO

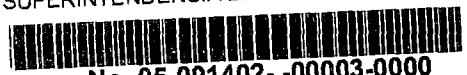
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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800176089-2



No. 05-091402- -00003-0000

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RECIBO OFICIAL DE CAJA : 08 - 220
FECHA : ENERO 2 DE 2008

***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
ELIER ABOGADOS	CONSIGNACION	BANCO DE BOGOTA	062754387	2808637	02/01/2008	42,278,000.00

***** CONCEPTO *****

CANT.	RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-09	OTROS SERVICIOS DE PROPIEDAD I	
		998 VALOR COMPLEMENTO TARIFA A#0 ANTR	8,000.00
		TOTAL :	\$ 8,000.00

SON: OCHO MIL PESOS

RESPONSABLE : _____

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

Colo 01579-841-019-5

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800176089-2

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 FECHA : DICIEMBRE 10 DE 2007

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

SECRETARIA DE INDUSTRIA Y COMERCIO

Act. 536 PATENTE Y
 Act. 536 PETICION

Act. 2420 INDECRACIONE
 Dec. 379 FASENACIONALII
 Folio: 3

***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
CAVE ABGADOS	CONSIGNACION	BANCO POPULAR	050-00110-6	49377610	10/12/2007	44,065,000.00

***** CONCEPTO *****

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1	50005-01-01 SOLICITUDES	114 PTC CAP-II EXAMEN DE PATENTIBILID	202,000.00
		TOTAL :	\$ 202,000.00

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DIVISIÓN DE NUEVAS CREACIONES

RADICACIÓN EXPEDIENTE No. 05-91402

EL EXTRACTO DE LA PRESENTE SOLICITUD DE **PATENTE DE INVENCION**

FUE PUBLICADO EN LA GACETA DE PROPIEDAD INDUSTRIAL No **578** DE

FECHA **31 DE JULIO DE 2007** EL TÉRMINO HÁBIL SEÑALADO POR LA LEY

EXPIRA EL **26 DE OCTUBRE DE 2007**

NOTA: Dentro del plazo de los seis (6) meses siguientes a partir de la fecha de publicación en la gaceta de Propiedad Industrial, deberá solicitar y cancelar el examen de patentabilidad so pena de que la solicitud caiga en abandono, de acuerdo con lo establecido en el Artículo 44 de la Decisión 486/2000.

SE PRESENTARON OPOSICIONES POR PARTE DE TERCEROS.

SI _____

NO X

Regulatory role of tetraspanin CD9 in tumor-endothelial cell interaction during transendothelial invasion of melanoma cells

Natividad Longo, María Yáñez-Mó, María Mittelbrunn, Gonzalo de la Rosa, María-Luisa Muñoz, Francisco Sánchez-Madrid, and Paloma Sánchez-Mateos

Heterotypic interaction among tumor cells (TCs) and endothelial cells (ECs) may play a critical role during the vascular dissemination of neoplastic cells and during pathologic angiogenesis in tumors. To identify molecules involved in these processes, the distribution of vascular junctional proteins was first studied by immunofluorescence at sites of heterologous intercellular contact using TC-EC mosaic monolayers grown on 2-dimensional collagen. Several members of the tetraspanin superfamily, including CD9,

CD81, and CD151, were found to localize at the TC-EC contact area. The localization of tetraspanins to the TC-EC heterologous contact area was also observed during the active transmigration of TCs across EC monolayers grown onto 3-dimensional collagen matrices. Dynamic studies by time-lapse immunofluorescence confocal microscopy showed an active redistribution of endothelial CD9 to points of melanoma insertion. Anti-CD9 monoclonal antibodies were found to specifically inhibit the transendothelial migra-

tion of melanoma cells; the inhibitory effect was likely caused by a strengthening of CD9-mediated heterotypic interactions of TCs to the EC monolayer. These data support a novel mechanism of tetraspanin-mediated regulation of TC transcellular migration independent of TC motility and growth during metastasis and a role for these molecules in the formation of TC-EC mosaic monolayers during tumor angiogenesis. (Blood. 2001;98:3717-3726)

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Introduction

The dissemination of cells from a primary tumor by invasion and metastasis represents the hallmark of malignancy.¹ Invasion is the local dissemination of neoplastic tissue, whereas metastasis is the distant dissemination to secondary places of growth involving the transport of neoplastic cells through the fluid spaces of the body (blood, lymph, cerebrospinal, or peritoneal fluid).² Although tumor cell (TC) migration through the extracellular matrix (ECM) is required for invasion, success in metastasis requires migration through the ECM and across host cell layers (transcellular migration).³ Because most cancer cells reach distant sites by dissemination through blood or lymphatic circulation, transendothelial migration of TCs is a crucial event in vascular metastasis formation. In addition, 2 recent works in human melanoma⁴ and colon carcinoma⁵ suggesting that tumor vessels are "mosaic" lined by endothelial cells (ECs) and malignant TCs extend the role of TC-EC interactions to the process of tumor angiogenesis. Thus, TC transendothelial invasion and molecules involved in TC-EC interactions could contribute to the formation of new blood vessels in tumors.

Tetraspanins comprise a numerous group of proteins that have 4 putative transmembrane domains and that have been implicated in the regulation of cell development, proliferation, activation, and motility.^{6,7} It has been suggested that tetraspanins may provide a

bridge between $\beta 1$ integrins and other proteins to generate functional complexes that regulate cell-cell and cell-ECM interactions.⁸ The expression of many members of the tetraspanin superfamily is altered in different types of human cancer.⁹ For the most part, tetraspanins become down-regulated in metastatic tumors,¹⁰⁻¹³ though CD151 has a positive effect in cell metastasis.¹⁴ Transfection of tetraspanins CD9, CD63, and CD82 reduces metastasis in vivo,¹⁵⁻¹⁷ and this effect has been related to the suppression of tumor cell growth and motility.

A large array of adhesion molecules has been described as mediating the adhesion and migration of TCs through ECM and in the initial attachment to EC monolayers, including integrins, selectins, cadherins, immunoglobulins, and proteoglycans.^{18,19} However, little is known about the mechanisms that regulate the passage of TCs through endothelial junctions. Earlier studies²⁰⁻²² suggest that TCs disseminate by breaching the vasculature wall, and they point to several tumor-derived factors to induce EC retraction. In contrast, recent work²³ indicates that the establishment of heterologous gap junctions between melanoma and ECs may contribute to in vivo metastasis formation. Furthermore, heterologous cell-cell interactions between TCs and other host cell layers can take place during TC dissemination through visceral cavities, and the cellular and molecular pathways implicated could be related to those acting

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N.L. and M.Y.-M. contributed equally to this paper.

The online version of the article contains supplemental videos.

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was seen at the bottom of the EC-junction depression induced by the attached TCs, without a significant disruption of the neighboring EC monolayer (Figure 1C, right panel). At 16 hours, some TCs displayed a spread morphology and were observed under the plane of the silver-stained vascular junctions (Figure 1B, panel iii). Continuous lines of silver stain were observed overlying the TCs that appeared to have completed their passage through the monolayer to the abluminal surface (Figure 1B, panels iii-iv), indicating that EC cell-cell junctions were re-established after TC passage.

Tetraspanins CD9, CD81, and CD151 localize at the heterotypic TC-EC intercellular junctions

To identify EC molecules involved in the interaction with TCs, mAbs against several vascular junction proteins were studied for their ability to stain the TC-EC heterotypic contact areas by immunofluorescence. To study these interactions, a mosaic monolayer of TCs and ECs was prepared by seeding fluorescently labeled TCs over a confluent EC monolayer grown onto cross-linked gelatin. After 16 hours of coculture, the A375 cells were observed inserted between the ECs, forming a mosaic TC-EC monolayer. VE-cadherin staining completely disappeared from the edges of the ECs in contact with the TCs (Figure 2Ai), as previously described.³⁴ Similarly, a lack of staining at sites of TC-EC contact was observed for β -catenin (Figure 2Aii), a cytoplasmic protein that links cadherins with the actin cytoskeleton, and for PECAM-1 (Figure 2Aiii), a receptor involved in the

transmigration of leukocytes. The tight junction protein ZO-1 was localized at EC-EC and TC-TC junctions, but it was absent from heterologous TC-EC contacts (Figure 2Aiv). Interestingly, the tetraspanin superfamily members (TM4SF) CD9, CD81, and CD151, which have been previously described to localize at the lateral junctions of endothelial and epithelial cells,^{6,30} were concentrated at TC-EC contact areas and at EC-EC junctions (Figure 2B, panels i-iii). The α_3 integrin that associates with members of the TM4SF³⁰ was also observed to be partially redistributed to the TC-EC contact area (Figure 2Biv). In addition, the intensity of the fluorescence signal in the images was quantified along lines traced over TC-EC contact regions and over homotypic (EC-EC or TC-TC) junctions (Figure 2, histograms). Although cadherin/ β -catenin, ZO-1, and PECAM-1 showed a sharp increment in the intensity of the fluorescence signal only at homotypic junctions, the quantification of the stainings of CD9, CD81, CD151, and α_3 integrin peaked at sites of TC-EC contact and at EC-EC contacts, indicating that these molecules are indeed concentrated at heterotypic intercellular contact regions. The concentration of CD9, CD81, and CD151 was only observed at sites of intercellular contact (heterologous or homologous), and no accumulation was observed in the periphery of TC or EC when cell-cell contact was lost (data not shown).

The distribution of the tetraspanins CD9, CD81, and CD151 was also studied during the active transmigration of TCs across ECs grown on 3-dimensional collagen matrix and analyzed by laser scanning confocal microscopy. TCs above the endothelium (yet to

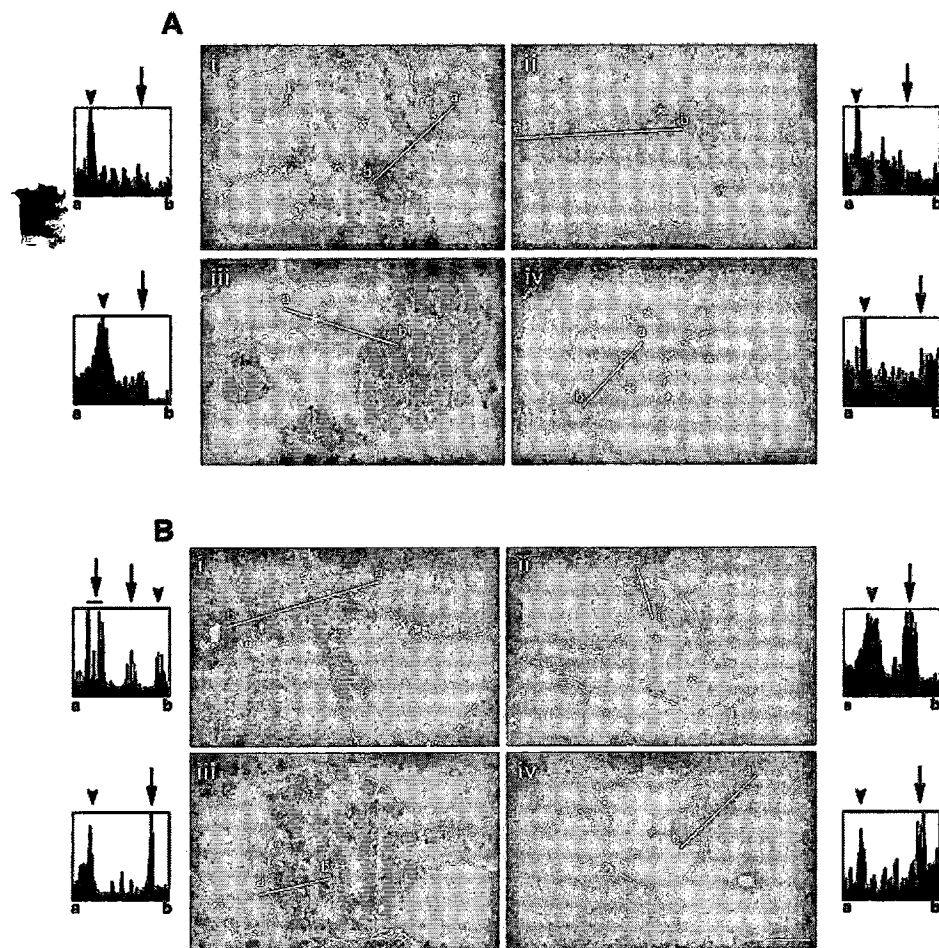


Figure 2. Distribution of junctional proteins in TC-EC monolayers. BCECF-labeled A375 cells were added to confluent ECs grown onto cross-linked gelatin and were allowed to spread for 16 hours. The mosaic TC-EC monolayer was fixed and stained in red fluorescence for (A) EC junctional proteins—(i) VE-cadherin, (ii) β -catenin, (iii) PECAM-1, and (iv) ZO-1—or for (B) some members of the tetraspanin family (i) CD9, (ii) CD81, (iii) CD151, or (iv) the associated α_3 integrin. The locations of melanoma cells are indicated (*) (originally labeled in green fluorescence). Bars, 15 μ m. Histograms show the fluorescence intensity quantification along the white line traced on the immunofluorescence images; a and b correspond to start and end points, respectively. TC-EC heterotypic junctions are indicated by arrows, and EC-EC and TC-TC homotypic junctions are indicated by arrowheads in the fluorescence histograms.

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Eukaryotic Expression Cloning with an Antimetastatic Monoclonal Antibody ^{D3} Identifies a Tetraspanin (PETA-3/CD151) as an Effector of Human Tumor Cell Migration and Metastasis¹

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ABSTRACT

A monoclonal antibody (mAb), 50-6, generated by subtractive immunization, was found to specifically inhibit *in vivo* metastasis of a human epidermoid carcinoma cell line, HEP-3. The cDNA of the cognate antigen of mAb 50-6 was isolated by a modified eukaryotic expression cloning protocol from a HEP-3 library. Sequence analysis identified the antigen as PETA-3/CD151, a recently described member of the tetraspanin family of proteins. The cloned antigen was also recognized by a previously described antimetastatic antibody, mAb 1A5. Inhibition of HEP-3 metastasis by the mAbs could not be attributed to any effect of the antibodies on tumor cell growth *in vitro* or *in vivo*. Rather, the antibodies appeared to inhibit an early step in the formation of metastatic foci. In a chemotaxis assay, HEP-3 migration was blocked by both antibodies. HeLa cells transfected with and overexpressing PETA-3/CD151 were more migratory than control transfectants expressing little CD151. The increase in HeLa migration was inhibitable by both mAb 50-6 and mAb 1A5. PETA-3 appears not to be involved in cell attachment because adhesion did not correlate with levels of PETA-3 expression and was unaffected by mAb 50-6 or mAb 1A5. The ability of PETA-3 to mediate cell migration suggests a mechanism by which this protein may influence metastasis. These data identify PETA-3/CD151 as the first member of the tetraspanin family to be linked as a positive effector of metastasis.

INTRODUCTION

Metastasis is a complex, multistep cascade of cellular events including migration of tumor cells through the surrounding stroma, entry into the circulatory system, and finally arrest, extravasation, and growth at a distant secondary site (reviewed in Refs. 1-6). Given the complexity of the metastatic process, it is not surprising that a number of proteins have been associated with tumor cell dissemination including transcription factors, signaling proteins, adhesion molecules, proteases, motility factors, and others (1-6). Although nuclear, cytoplasmic, and secreted proteins have been associated with metastatic potential, tumor cell dissemination is executed via the physical interactions of the cancer cell surface with various host tissue elements. Not only is the cell membrane the interface at which cell-cell and cell-substrate contacts are made, it is a portal through which external signals must pass. Activation of cell surface receptors, by mutation or by ligand binding, initiates intracellular signaling cascades that influence expression of genes that promote the malignant phenotype (2, 3, 6). Proteases expressed on or bound to the cell membrane mediate degradation of tissue barriers. Integrins mediate tumor cell motility and transmit environmental cues by virtue of their

interactions with different matrix proteins. Our efforts to identify novel metastasis-associated antigens have, therefore, focused on the tumor cell surface. As our model system, we have used a highly metastatic human epidermoid carcinoma cell line, HEP-3, which disseminates to host lung tissue. The distinct characteristics and behavior patterns of this tumor cell line have been described by Ossowski in a series of papers published between 1980 and 1998 (7-9). These cells are very aggressive and readily give rise to metastasizing tumors in both the chicken embryo (10-12) and in the nude mouse model (13, 14). As such, these cells should possess distinct surface antigens that are functionally involved in mediating tumor cell dissemination.

Brooks *et al.* (11) generated several mAbs⁶ against HEP-3 cell surface proteins using an approach termed subtractive immunization. Their protocol allowed them to produce mAbs with no preconceived notion as to the identity or function of the targeted antigen. Two of the antibodies, DM12-4 and 1A5, inhibited spontaneous HEP-3 metastasis in the chicken embryo metastasis assay by 86 and 90%, respectively. Neither antibody affected primary tumor growth on the chorioallantoic membrane or tumor cell growth *in vitro*, indicating that the mAbs specifically blocked metastatic behavior. The identification of the antigens recognized by the mAbs was not reported or was unknown.

In the present study, another monoclonal antibody generated by subtractive immunization, mAb 50-6, was used to clone and characterize a cell surface, metastasis-associated antigen expressed on HEP-3 cells. This antibody inhibits both spontaneous and experimental HEP-3 metastasis. Eukaryotic expression cloning of the antigen identifies it as PETA-3/CD151, a member of the tetraspanin family of proteins. We show that PETA-3/CD151 appears to be required at an early step in the formation of metastatic foci. Furthermore, this protein mediates tumor cell migration but does not appear to affect cell adhesion to various purified matrix proteins. The work described herein identifies PETA-3/CD151 as the first member of the tetraspanin family to be linked as a positive effector of metastasis.

MATERIALS AND METHODS

Cell Lines and Hybridomas. Human breast adenocarcinoma cells (MDA-MB-231), human cervical carcinoma (HeLa), human fibrosarcoma (HT1080), and monkey kidney cells (COS-7) were obtained from the American Type Culture Collection (Rockville, MD). Metastatic human epidermoid carcinoma cells (HEP-3) were obtained from solid tumors serially passaged on the CAMs of chicken embryos (10, 11). All cells were maintained as monolayer cultures in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FBS (HyClone, Logan, UT), sodium pyruvate, penicillin/streptomycin, and nonessential amino acids (Life Technologies, Inc.; growth medium). Cultures were grown in a humidified atmosphere of 5% CO₂ at 37°C.

Hybridomas producing mAb 50-6 and mAb 1A5 were generated by subtractive immunization (11). Cultures of each hybridoma were maintained in one part DMEM, one part Hybridoma SFM (Life Technologies, Inc.), supplemented with 2.5% alpha calf serum (HyClone), sodium pyruvate, penicillin/

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⁶ The abbreviations used are: mAb, monoclonal antibody; CAM, chorioallantoic membrane; FBS, fetal bovine serum; FN, fibronectin; LN, laminin; TM4SF, transmembrane 4 superfamily; VN, vitronectin.

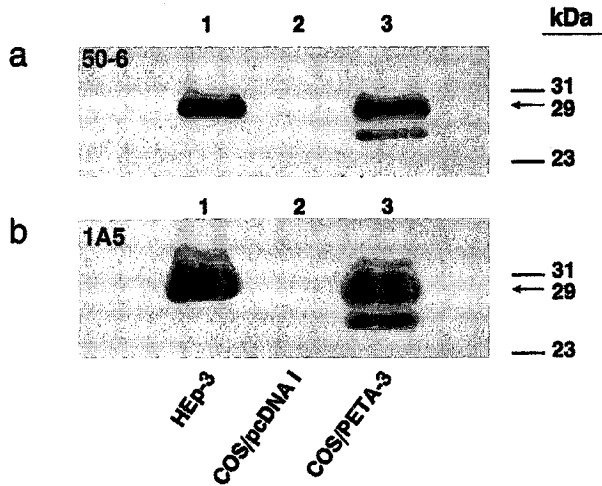


Fig. 4. PETA-3/CD151 is recognized by mAb 50-6 and mAb 1A5. COS-7 cells were transiently transfected with the cloned PETA-3/CD151 cDNA or vector (pcDNA 1) alone. Lysates of HEP-3 cells and the transfected COS cells were resolved by SDS-PAGE and analyzed by Western blotting. *a*, Western blot of HEP-3 cell lysate (10 μ g; Lane 1) and lysates from control (COS/pcDNA 1; 20 μ g; Lane 2) and PETA-3-transfectants (COS/PETA-3; 20 μ g; Lane 3) probed with mAb 50-6. *b*, Western blot of HEP-3 cell lysate (0.5 μ g; Lane 1) and lysates from control (COS/pcDNA 1; Lane 2; 2.5 μ g) and PETA-3-transfectants (COS/PETA-3; Lane 3; 2.5 μ g) probed with mAb 1A5. In both panels, the signals were visualized with a peroxidase-conjugated goat anti-mouse IgG by chemiluminescence. Arrow, PETA-3, M_r 29,000. A M_r 25,000 signal is also seen in COS cell lysates probed with both mAb 50-6 and mAb 1A5. Right, molecular weight markers.

mAb 50-6 Inhibits Spontaneous and Experimental Metastasis of HEP-3 Cells. To determine whether the M_r 29,000 cell surface protein was involved in HEP-3 dissemination, the effect of mAb 50-6 on spontaneous metastasis was measured using an *in vivo* model, the chicken embryo metastasis assay (11, 20). In the spontaneous metastasis assays, purified antibody (mAb 50-6 or normal mouse IgG) was inoculated i.v. 24 h after metastatic HEP-3 cells were implanted onto prepared CAMs, and the extent of tumor cell dissemination to the lungs was measured (see "Materials and Methods"). As shown in Table 1, spontaneous metastasis was reduced by 74% in embryos that received mAb 50-6. The effect of mAb 50-6 on experimental metastasis also was tested by coinoculating the tumor cells with the antibody directly into the circulatory system. In these experiments, dissemination to the embryonic lungs was inhibited by 57% when compared with controls. The antimetastatic properties of mAb 50-6 cannot be attributed to a cytostatic or cytotoxic effect, because this antibody had no effect on proliferation of HEP-3 cells *in vitro* (Fig. 2), nor did it result in a decrease in the size of the primary tumor on the CAM (Table 1). These results indicate that mAb 50-6 specifically blocks a step(s) in the metastatic cascade.

Eukaryotic Expression Cloning Identifies the M_r 29,000 Metastasis-associated Antigen as PETA-3/CD151. The antibody inhibition studies demonstrated a functional role for the M_r 29,000 protein in mediating metastasis. To identify the protein, mAb 50-6 was used in a eukaryotic expression cloning strategy to isolate the cDNA from a full-

length HEP-3 cDNA library. COS cells were subjected to several rounds of transfection and immunoselection with mAb 50-6. In the final round of the cloning protocol, immunopositive cells showing a strong cell surface signal were selected for isolation of cDNA clones because these cells would more likely harbor full-length inserts (see "Materials and Methods"). Indeed, a clone that directed the synthesis of a cell surface antigen recognized by mAb 50-6 was isolated and sequenced. The open reading frame of this clone encodes a core protein having a predicted molecular mass of 27.8 kDa. There are four distinct hydrophobic domains, and a single *N*-glycosylation site in a large hydrophilic region that separates the third and fourth hydrophobic domains. Comparison of the sequence of this clone with the National Center for Biotechnology Information database identified the metastasis-associated antigen as PETA-3/CD151, a recently described Member of the tetraspanin family of proteins, which is also known as the TM4SF. The coding sequence was identical to that reported by Fitter *et al.* (21). The cell surface distribution of PETA-3 on HEP-3 cells and COS cells transfected with the cloned cDNA was analyzed by immunocytochemistry. On HEP-3 cells incubated with mAb 50-6, there was a strong staining pattern over the entire surface of the cell membrane, including filopodia extending from the cell body (Fig. 3*a*). The same cell surface staining pattern was seen on COS cells transiently transfected with PETA-3 cDNA (Fig. 3*c*). No signal was observed on HEP-3 or PETA-3-transfected COS cells incubated with normal mouse IgG (Fig. 3, *b* and *d*) or on control COS transfectants incubated with mAb 50-6 (Fig. 3*e*). Western blot analysis of the cell lysates from PETA-3-transfected COS cells also demonstrated that the encoded protein comigrates with the M_r 29,000 antigen expressed on HEP-3 cells (Fig. 4*a*).

Brooks *et al.* (11) had described an antimetastatic mAb, mAb 1A5, which also recognizes a M_r 29,000 HEP-3 cell surface antigen. To determine whether this mAb also recognizes PETA, mAb 1A5 was used to probe Western blots of COS cells transiently transfected with the PETA-3/CD151 cDNA (Fig. 4*b*). No signal was observed in lysates from control transfectants (Lane 2); however, mAb 1A5 recognized PETA-3/CD151 expressed by the COS cells (Lane 3). A minor M_r 25,000 band, immunostained with mAbs 50-6 and 1A5, was also evident in lysates of transfected COS cells (Fig. 4) and in HEP-3 lysates (seen upon prolonged exposure of the Western blot; data not shown). This is likely the nonglycosylated form of the protein, because cultivation of HEP-3 cells with tunicamycin or treatment of HEP-3 cell lysates with *N*-glycanase generates a M_r 25,000 band that is immunoreactive with mAb 1A5 (data not shown). This is smaller than the predicted mass of 27.8 kDa, and may be due to the hydrophobic nature of the protein and/or the presence of disulfide bonds resulting from 15 cysteine residues. These results demonstrate that both mAb 50-6 and mAb 1A5 recognize PETA-3 and suggest that the epitope(s) is resident in the protein core.

PETA-3/CD151 Mediates an Early Event in the Formation of Metastatic Foci. To examine the possible mechanisms by which PETA-3/CD151 effects metastasis, a time course study of inhibition of experimental metastasis was carried out. As shown in Table 2,

Table 2 Time course of inhibition of HEP-3 experimental metastasis

Purified mAb 1A5 (180–200 μ g) was inoculated i.v. into 11-day-old chicken embryos either 2 h before or 2, 6, 10, or 20 h after inoculation of 2×10^4 HEP-3 cells. As a control, normal mouse IgG was inoculated 2 h before the HEP-3 cells. The inoculated embryos were incubated for an additional 6 days, at which time the embryonic lungs were removed, finely minced, and transferred to the CAMs of a second set of embryos, which were incubated for an additional 7 days. The extent of lung metastasis was measured by determining the amount of human urokinase-type plasminogen activator activity, which has been shown to be a quantitative reflection of the numbers of human cells in the lung (8–10).

Antibody	Time of antibody inoculation	No. of embryos	Lung metastasis (huPA in lung)		% inhibition
			mean $\pm$ SEM		
Normal mouse IgG	-2 h	47	1522 $\pm$ 54		0
mAb 1A5	-2 h	36	295 $\pm$ 60		81
mAb 1A5	+6 h	21	401 $\pm$ 36		74
mAb 1A5	+10 h	8	1471 $\pm$ 36		3
mAb 1A5	+20 h	20	1408 $\pm$ 67		7



D1

Expressed sequence tag analysis of human retina for the NEIBank Project: Retbindin, an abundant, novel retinal cDNA and alternative splicing of other retina-preferred gene transcripts

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¹Section on Molecular Structure and Function, National Eye Institute, National Institutes of Health, Bethesda, MD; ²Departments of Ophthalmology and Neurobiology & Genetics, University of Maryland School of Medicine, Baltimore, MD; ³NIH Intramural Sequencing Center, Gaithersburg, MD

Purpose: Expressed sequence tag (EST) analysis was performed on un-normalized, unamplified cDNA libraries constructed from adult human retina to examine the expression profile of the tissue and to contribute resources for functional genomics studies.

Methods: Two size fractionated cDNA libraries (designated *hd* and *he*) were constructed from human retina RNA. Clones were randomly selected for sequencing and analyzed using the bioinformatics program GRIST (GRouping and Identification of Sequence Tags). PCR, Northern blotting and other techniques have been used to examine selected novel transcripts.

Results: After informatics analysis, 2200 retina cDNAs yield 1254 unique clusters, potentially representing individual genes. Opsin is the most abundant transcript and other retina transcripts are prominently represented. One abundant cluster of cDNAs encodes retbindin, a novel, retina preferred transcript which has sequence similarity to riboflavin binding proteins and whose gene is on chromosome 19. Variant transcripts of known retina genes are also observed, including an alternative exon in the coding sequence of the transcription factor NRL and a skipped coding sequence exon in the phosphodiesterase γ subunit (PDE6G).

Conclusions: The new retina cDNA libraries compare favorably in quality with those already represented in public databases. They are rich in retina specific sequences and include abundant cDNAs for a novel protein, retbindin. The function of retbindin remains to be determined, but it is a candidate for flavinoid or carotenoid binding. Analysis of multiple clones for highly expressed retina genes reveals several alternative splice variants in both coding and noncoding sequences which may have functional significance. The validated set of retina cDNAs will contribute to a nonredundant set for microarray construction.

The retina is the tissue of the eye where light begins its transformation into sight. Specialized photoreceptor cells transduce photons into nerve impulses that are organized through complex layers of nerve cells and transmitted on to the perception centers of the brain. Not surprisingly, the retina has a large complement of tissue specific proteins and mutations in the genes for many of these proteins have been identified in a wide variety of inherited eye defects [1,2]. The retina also has much in common with other neural tissues, particularly those related to hearing, so that conditions like Usher's syndrome affect both hearing and sight through mutations in genes common to ear and eye [3-7].

Expressed sequence tag (EST) analysis of retina has already produced thousands of sequences in dbEST and these have been instrumental in identifying several disease loci. An important example of this was the discovery of the gene that

is defective in Stargardt's disease, an inherited macular degeneration [8]. In addition to their value in gene discovery, ESTs are an essential resource for functional genomics studies, particularly for the construction of cDNA microarrays to compare gene expression levels in disease, aging and development [9].

Although many retinal ESTs have been sequenced (see compilation at Unigene), not all are available as DNA for further studies. The public databases also contain many sequences of poor quality or of contaminant origin. In addition, many of the libraries that have been used for EST analysis are amplified or subtracted [10]. These manipulations have the potential to reduce the content of certain clones, such as those that grow poorly in bacterial hosts. Most EST projects also concentrate on 3' reads. Since this biases data towards 3' untranslated regions (UTR) where introns are very uncommon, this reduces the chances of observing novel splice forms that would give rise to variant proteins with structural and functional differences.

For these reasons and to obtain clones for construction of a human eye cDNA microarray, we constructed unamplified cDNA libraries from dissected, post mortem human retina as

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part of the NEIBank project [11]. Here we describe initial analysis of over 2200 5' sequence reads from this effort. The data reveal novel, or previously unidentified, genes and numerous splice variants with potential functional significance. All sequences are available through NEIBank.

METHODS

Tissue and RNA preparation: Human post-mortem eye tissues were obtained under University of Maryland School of Medicine IRB exemptions SB-019701 and SB-129901. Neural retina tissue was dissected from two 80 year old white male donors with no observed eye disease. Total RNA was extracted using RNeasy (Tel-Test Inc., Friendswood, TX). 100 µg of total RNA was used for library construction. PolyA RNA was prepared using an oligo(dT) cellulose affinity column [12].

cDNA Library Construction: A directionally cloned cDNA library in the pSPORT1 vector (Life Technologies, Rockville MD) was constructed at Bioserve Biotechnology (Laurel, MD). General details of library construction for NEIBank cDNA libraries are described elsewhere [13]. For this library, cDNA inserts were cloned into the NotI/SalI sites of the vector. In this case, as an additional measure to remove small contaminant fragments, the cDNA was run over a Sephacryl S-500 HR resin column designed to fractionate cDNA >500 bp (Life Technologies). The columns were run in TEN buffer: 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 25 mM NaCl. Sub-libraries (designated *hd* and *he* for sequencing purposes) were made from the first two 35 µl fractions containing cDNA.

TABLE 1. MOST ABUNDANT CLONES FROM COMBINED PUBLIC HUMAN RETINA EST COLLECTIONS

Rank	Name	#
1	GAPDH	159
2	elongation factor 1-α	112
3	λ Phage Vector DNA	102
4	collagen, type I, α 1	99
5	phosphodiesterase 6G (PDE6G)	98
6	rhodopsin	96
7	glutathione peroxidase 3	95
8	cyclin I	80
9	transferrin	75
10	Mitochondrion	67
11	α-transducin	59
12	amyloid β (A4) precursor-like 1	58
13	recoverin	50
14	elongation factor 1 γ	46
15	α-tubulin	46
16	S-antigen (arrestin)	44
17	aldolase A	42
18	amyloid β (A4) precursor-like 2	39
19	enolase 2, (γ, neuronal)	37
20	CD74 antigen	37
21	G protein, β polypeptide 1	34
22	hsp86	33
23	aldolase C	33
24	phosphoglycerate mutase 1	31
25	collagen, type I, α 2	31

The most abundant clones from the 30,000 human retina ESTs listed in dbEST. ESTs are ranked according to their abundance (#). Clones labelled in red are non-mRNA contaminants.

cDNA Sequencing and Bioinformatics: Methods for sequencing and bioinformatics analysis are described in detail elsewhere [14]. Briefly, randomly picked clones were sequenced at the NIH Intramural Sequencing Center (NISC). Clones were sequenced only from the 5' end. A specially developed software tool, GRIST (GRouping and Identification of Sequence Tags) was used to analyze the data and assemble the results in web page format. Clusters of sequences were aligned using SeqMan II (DNASTar, Madison, WI) to examine alternative transcripts. Sequences were also searched through genome resources at the National Center for Biotechnology Information (NCBI), the Human Genome Project, and the Celera Genomics Group. Protein motifs were searched using GenomeNet, the NCBI, and the Swiss Institute of Bioinformatics.

Polymerase Chain Reaction (PCR) Methods: PCR was used to validate alternative splice forms, to obtain probes for hybridization, and to complete sequences. For template, a sample of the complete cDNA library representing at least one million primary clones was amplified and plasmid was isolated using reagents from QIAGEN (Valencia, CA). Fragments were amplified using either Taq (Roche, Indianapolis, IN) or Elongase (Life Technologies, Gaithersburg, MD) polymerase systems and following manufacturer's protocols.

Northern Blot: Multi-tissue northern blots were purchased from Clontech (Palo Alto CA). Northern blots for rhesus monkey (*Macaca mulatta*) eye tissues were prepared as described previously [15]. A cDNA for human retbindin protein was identified from the retina library. The insert was excised and labelled using a prime-it II kit (Stratagene systems, La Jolla, CA) and ³²P-labelled dCTP.

Northern blots were prehybridized in Hybrisol II (Oncor, Gaithersburg, MD) for 4 h, followed by hybridization with

TABLE 2. THE "TOP 30" OF NEIBANK RETINA ESTS

Rank	Description	GenBank	UniGene	Chromosome	#
1	rhodopsin	NM_000539	247565	3q21-q24	52
2	glutathione peroxidase 3	D00632	172153	8q23	33
3	elongation factor 1-α	AK026650	181165	6q14	26
4	phosphodiesterase 6G (PDE6G)	NM_002602	1857	17q25	25
5	ferritin, heavy	BC000857	62954	11q13	25
6	GAD6H	AF261085	169476	12p13	21
7	clusterin (apolipoprotein J)	M64722	75106	8p21-p12	19
8	creatine kinase, brain	BC001130	173724	14q32	18
9	transferrin	NM_002793	284176	11	17
10	S-antigen (arrestin)	NM_000541	32721	2q37.1	13
11	tubulin, β 2	AK026167	251653	?	11
12	ribosomal protein, large, P0	BC000087	73742	12	10
13	ribosomal protein L3 (RPL3)	NM_000967	119598	22q12.1-q12.3	10
14	glutamine synthetase	S70290	170171	1q31	9
15	prosapin	D00422	78575	10q21-q22	9
16	ribosomal protein S3A	BC000204	77039	4q31.2-q31.3	9
17	α-transducin	AC002077	51147	3p21	9
18	neural retina leucine zipper (NRL)	XM_007297	89606	14q11.1-q11.2	9
19	ROM1	L07894	281564	11q13	9
20	vimentin	NM_005833	2064	10p13	9
21	recoverin	NM_002903	80539	17p13.1	8
22	ribosomal protein L13a	BC000514	119122	19q13.3	8
23	aldolase A	M11560	273415	16q22-q24	8
24	elongation factor-1 γ	Z11531	2186	11	8
25	Retbindin	AY028917	21162	19p13	8
26	unc119 (C.elegans) homolog	NM_005148	81728	17q11.2	7
27	prostaglandin D2 synthase	XM_005431	8272	9q34.2-q34.3	7
28	HSP-70 1A (HSPA1A)	NM_005345	8997	6p21.3	7
29	ATPase, Na ⁺ /K ⁺ transporting, α3	NM_008878	274371	18q11.2	7
30	triosephosphate isomerase	XM_006972	83848	12p13	7

All ESTs represented by at least seven clones in the combined *hd/he* dataset. This list is an edited detail from the output of the clustering procedure GRIST. The most abundant novel transcript, retbindin, is shown in red. Representative GenBank entries are shown, along with the current Unigene cluster, chromosomal location and the number of ESTs identified.

**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES**

Abogado
Emilio Ferrero

ASUNTO	Radicación	05-91402
	Trámite	002
	Evento	001
	Actuación	430
	Oficio	
	Folios	011

06446

Patente de Invención	☒	Patente de Modelo de Utilidad	☐
Vía Nacional	☐		
Vía PCT (fase nacional)	☒	Cap. I	☐
		Cap. II	☒
No. Sol. Internacional		PCT/JP2004/003048	

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 le comunica:

1. Documentos estudiados

<u>Descripción:</u>	Folios: 68 a 209 como se radicó inicialmente.
<u>Reivindicaciones:</u>	Folios: 210 a 215 como se radicó inicialmente.
<u>Figuras:</u>	Folios: 217 a 237 como se radicó inicialmente.
<u>Prioridad:</u>	10/03/2003, Japón, JP 2003-063648

2. Objeto de la invención

Título de la solicitud: "Anticuerpo contra antígeno específico de tumor como objetivo".

El objeto de la presente solicitud de patente de invención consiste en un método para detectar cáncer mediante el uso de un oncogen, a un método para clasificar un compuesto activo útil para tratar y/o prevenir el cáncer y la composición farmacéutica derivada.

El problema planteado en esta solicitud es suministrar un anticuerpo monoclonal útil para detectar otros tipos de tumores y que sea específico para detectar una proteína asociada a tumores.

Para solucionar este problema técnico la solicitud en estudio proporciona un anticuerpo que se une a la proteína oculospanina humana, la cual se expresa en células tumorales, con actividad citotóxica contra estas células.

IPC: C07K 16/32.

3. De la solicitud de Patente

3.1. Descripción (artículo 28 D. 486)

A lo largo de todo el capítulo descriptivo no existe suficiencia en la información sobre el anticuerpo anti-oculospanina humana que se desea proteger. Aunque se suministra información sobre la proteína oculospanina, que en este caso es la molécula de unión del anticuerpo, no hay información relacionada con la estructura misma del anticuerpo. No se suministran ni secuencias aminoácidas del anticuerpo completo ni segmentos CDR del mismo.

De esta manera, la descripción presentada no cumple con los requisitos establecidos en el art. 28 de la Decisión 486.

3.2. Reivindicaciones (artículo 30 D 486)

El capítulo reivindicatorio presentado carece de concisión, claridad y sustento por cuánto:

- 1) La reivindicación 1 que trata sobre un anticuerpo es caracterizada por la molécula de unión del mismo, pero no por la estructura del anticuerpo;
- 2) En el mismo sentido, la reivindicación 2 menciona el anticuerpo, caracterizándolo por las secuencia aminoácidas precisas de la molécula a la cual se une, pero no de su propia estructura;
- 3) Las reivindicaciones relacionadas con el equipo de detección para cáncer (reivindicaciones 20-23) y de la composición farmacéutica (reivindicaciones 24-27) tienen como característica esencial el anticuerpo de la invención, pero el mismo no es caracterizado por su estructura lo cual no permite definirlo y hace que estas reivindicaciones carezcan de sustento.

De esta manera, el capítulo reivindicatorio presentado no cumple con los requisitos establecidos en el art. 30 de la Decisión 486.

4. Determinación del Estado de la Técnica

La fecha que se ha tenido en cuenta para determinar el Estado de la Técnica es:

Fecha de presentación de la solicitud prioritaria 10/03/2003.

Teniendo en cuenta la fecha indicada se realizó la búsqueda en el estado de la técnica de documentos relacionados con la solicitud encontrándose los siguientes documentos más relevantes:

Nº	Documento	Título	Fecha de publicación
D1	Wistow, <i>et.al.</i> Molecular Vision 2002; 8:196-204	"Expressed sequence tag analysis of human retina for the NEIBank Project: Retbindin, an abundant, novel retinal cDNA and alternative splicing of other retina-preferred gene transcripts"	15/06/2002
D2	Longo, <i>et.al.</i> BLOOD, VOL 98 (13): 3717-3726	"Regulatory role of tetraspanin CD9 in tumor-endothelial cell interaction during transendothelial invasion of melanoma cells"	12/2001
D3	Testa, <i>et.al.</i> CANCER RESEARCH 59, 3812-3820,	"Eukaryotic Expression Cloning with an Antimetastatic Monoclonal Antibody Identifies a Tetraspanin (PETA-3/CD151) as an Effector of Human Tumor Cell Migration and Metastasis"	01/08/1999

5. **Análisis de patentabilidad** (artículo 14 D486)

5.1. Nivel inventivo (artículo 18 D486)

La reivindicación 1 y dependientes reclaman un anticuerpo caracterizado porque se une específicamente a oculospanina humana y tiene actividad citotóxica contra una célula que expresa esa proteína.

La reivindicación 2 define la molécula a la que se une el anticuerpo (la oculospanina) por la secuencia de aminoácidos representada por la SEQ ID N° 2 y/o N° 4.

En el estado del arte, había sido divulgada la secuencia nucleótida y aminoácida específica de la oculospanina, también conocida como Retbindin, una proteína expresada en el pigmento retinal humano y ubicada en el cromosoma 19 (ver D1, resumen y tabla 2). Esta proteína es reconocida como una tetraspanina novedosa.

Con esta información, el experto en la materia tenía la información precisa de la estructura de esta proteína. La diferencia entre D1 y la solicitud en estudio, es que esta última a partir de la información de la secuencia aminoácida de la oculospanina provee un anticuerpo que se podría unir a ella y tener actividad citotóxica.

Sin embargo, la información que requería el experto sobre la utilidad que tiene esta proteína para inhibir la adherencia e interconexión de las células tumorales y epiteliales fue divulgada anteriormente por varios documentos, tales como el de Longo, *et.al.* (D2) y Testa, *et.al* (D3), los cuales mencionan la interacción entre las células tumorales y las células endoteliales que promueven las proteínas de la superfamilia de las tetraspinas.

Específicamente, el documento D2, menciona la actividad de la proteína CD9, una proteína de la familia de las tetraspinas, la cual interactúa durante la adherencia de las células tumorales de tipo melanoma, en la invasión transendotelial (ver resumen; introducción pág 3717 y pág 3720). También se divulgan anticuerpos y los métodos utilizados para identificar la presencia de las proteínas tetraspaninas en esta interacción con células cancerosas, indicando su utilidad para inhibir la formación de tumores.

En este documento adicionalmente se mencionan otras proteínas de la tetraspaninas, tales como las CD81 y CD151 que igualmente actúan en la interacción entre estos dos tipos de células (ver pág 3720).

Vale la pena resaltar que la proteína CD9 además de hacer parte de la misma familia de las tetraspaninas, tiene una similitud u homología del 42% con la secuencia de la oculospanina dada en la solicitud. Pese a que esta similitud en primera instancia no parece muy alta, la diversidad de esta familia de proteínas explica la divergencia y su alineamiento (entre las proteínas CD9 y oculospanina) muestra que tienen dominios altamente conservados que explicarían su similitud funcional.

El documento D3 por su parte, divulga la actividad de la proteína CD151 otra proteína de la familia de las tetraspaninas, como un efector de la migración de las células tumorales y metástasis. En este documento se comprueba la presencia de esta proteína en la línea de carcinoma epidermoide humano usando el anticuerpo monoclonal 50-6, en las técnicas convencionales del campo técnico (ver D3 resumen). Adicionalmente, se probó la inhibición de la metástasis usando este mismo anticuerpo anti-CD151 (ver pág 3816, columna izq).

Esta proteína tetraspanina CD151 tiene una similitud u homología del 42% con la oculospanina de la solicitud. Tal aparente baja homología muestra del alineamiento de estas dos proteínas tetraspaninas que presentan varios dominios altamente conservados.

Como se observa, del estado del arte era conocida la actividad anti-tumoral que podrían tener los anticuerpos dirigidos a diferentes tetraspaninas, especialmente contra células tumorales de melanoma o epidermiales. Por tanto, a partir de la secuencia aminoácida de la proteína oculospanina divulgada por D1, un experto en la materia habría construido un anticuerpo y sabría que el mismo tendría una actividad citotóxica dirigida a células tumorales, especialmente células tumorales epidermiales.

De esta manera, el anticuerpo reclamado se deriva evidentemente del estado de la técnica, específicamente de la combinación de D1 y D2 ó de la combinación de D1 y D3. Por lo tanto carece de actividad inventiva e incumple con el artículo 18 de la D486.

6. Conclusión:

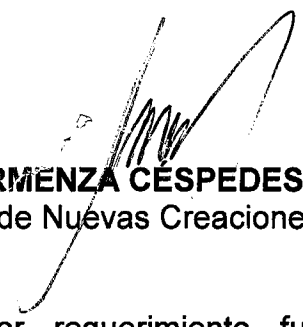
Los requisitos de patentabilidad de la presente solicitud se encuentran afectados así:

N°	Documento N°	Reivindicaciones afectadas	Requisito que afecta
D1	Wistow, <i>et.al.</i> Molecular Vision 2002; 8:196-204	1-27	Nivel inventivo
D2	Longo, <i>et.al.</i> BLOOD, VOL 98 (13): 3717-3726	1-27	Nivel inventivo
D3	Testa, <i>et.al.</i> CANCER RESEARCH 59, 3812-3820,	1-27	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 60 días contados a partir 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.

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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 N° 10 de 2001.


ALIX CARMENZA CÉSPEDES DE VERGEL
Directora de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha
18 MAYO 2010,

CONCEPTO TÉCNICO No. 1482	EXAMINADOR TÉCNICO Código No.202052
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