Co-expression of plexin-B1 and Met in human breast and ovary tumours enhances the risk of progression

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Abstract. *Background*: Plex-B1, the receptor of Sema4D, has been implicated in tumour growth, angiogenesis and metastasis. The binding of Sema4D to Plex-B1 can trigger the activation of Met tyrosine kinase, thereby promoting cell dissociation and invasive growth. We tested the hypothesis that the expression of Plex-B1, either alone or in association with Met, can be of predictive value for tumour progression.

Methods: The expression and distribution of Plex-B1 and Met were investigated by immunohistochemistry and immunofluorescence in 50 human neoplasias originating in the breast and ovary, and correlated with clinical–pathological data at diagnosis.

Results: Plex-B1 and Met were individually expressed in 14% and in 24% of the tumours, respectively. Plex-B1 and Met were co-expressed in 24/50 cases (48%), and in the majority of these (83%) Met was tyrosine phosphorylated. The expression of Plex-B1 or Met alone showed no significant correlation with tumour aggressiveness, whereas advanced stage tumours (III–IV) frequently showed Plex-B1–Met double-positive (9/13). Tumours co-expressing Plex-B1 and Met were characterised by worse grading and higher incidence of lymph node metastases. Out of 22 tumours with lymph node metastases, as many as 19 were Plex-B1 and Met double-positive ($p = 0.0008$), and 17 expressed phosphorylated Met ($p = 0.002$).

Conclusion: Plex-B1 assumes a predictive value for unfavourable outcome when co-expressed with Met.

Keywords: Plexin-B1, Met, CD100, tumour prognosis, lymph node metastases

Abbreviations

FITC   fluorescein isothiocynate;
Plex-B1   Plexin-B1;
Sema4D   Semaphorin 4D.

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1. Introduction

Plexins constitute a family of widely expressed trans-membrane receptors for the semaphorins (see [42]). Semaphorins are secreted, trans-membrane or glycosylphosphatidylinositol(GPI)-anchored proteins grouped in eight subclasses [35], that play a fundamental role in the regulation of axon guidance, cell migration in organ development and vascular morphogenesis [24,25,43]. Moreover, emerging evidence indicates a major role of semaphorins in cancer progression, tumour angiogenesis and immune response [29]. The extracellular portion of plexins shares structural homol-

ogy with their ligands (the semaphorins), as well as with Met and Ron, two cell surface tyrosine kinases belonging to the family of scatter factor receptors [1, 46]. The cytoplasmic region of plexins, however, has no homology with the tyrosine kinase domain of Met and Met-like proteins, but comprises a unique domain interacting with monomeric GTPases of Rho and Ras families [16,30]. Plex-B1, the high-affinity receptor of Semaphorin 4D (Sema4D; CD100) [42], has gained the attention of oncologists because of its association in receptor complexes with Met and other oncogenic tyrosine kinase receptors, and its potential involvement in tumour invasive growth and metastasis [6,10,19,40, 46]. The Sema4D-Plex-B1 complex has further been shown to regulate angiogenesis [5,11] and to trigger survival and proliferation of B lymphocytes in chronic lymphocytic leukaemia [20]. On Sema4D ligation, the biological responses mediated by Plex-B1 through Met trans-activation and tyrosine phosphorylation appear of crucial relevance in the invasive/metastatic process, in consideration of the involvement of Met in cell proliferation, motility, neo-angiogenesis and invasion of the extracellular matrix [8,19]. The molecular pathways through which Met controls tumour progression have been widely studied *in vitro* [9,33]. Met over-expression has been reported in several types of human cancers [13,14,21]. Moreover, the expression of Met and/or of its cognate ligand Scatter Factor 1 (also known as HGF, hepatocyte growth factor) has been shown to be a prognostic indicator in patients bearing mammary carcinomas [12,15]. To date, there is only a limited knowledge about the expression of Plex-B1 in neoplastic tissues and their biological significance in tumour progression. The present study was designed to assess the potential value of Plex-B1 expression as a marker of tumour progression, either alone or in conjunction with that of the associated receptor Met. Moreover, we aimed at evaluating whether interactions between Plex-B1 and Met, both known to participate in the processes of cell migration/invasion, may associate with an increased risk of metastatic dissemination. To this end, a series of 50 malignant epithelial tumours (35 breast and 15 ovary) were analysed by immunohistochemistry and immunofluorescence. Met expression was previously demonstrated in this group of neoplasias, and its relevance for tumour aggressiveness has been reported [44]. We thus correlated the expression levels of Plex-B1 and its co-expression with Met in tumour samples with the clinical–pathological parameters and the presence of metastases. We found that the simultaneous expression of Plex-B1 and Met

identifies a subset of tumours particularly aggressive, presenting at diagnosis with a higher histologic grade of malignancy and lymph node metastases. The present data strongly support the relevance of Plex-B1 and Met co-expression as a predictive marker of cancer progression, and suggest that evaluating their expression in human cancers may provide valuable diagnostic and prognostic information.

2. Materials and methods

2.1. Patients and tissues

The study included 50 cases of malignant epithelial tumours from breast (35 cases) and ovary (15 cases) selected from the files of the Laboratory of Pathology, Amedeo Avogadro University (Novara, Italy) in a restricted period (2000–2003) and homogeneously treated by standard surgery and chemo-radiotherapy.

Clinical and pathological classification, which include histologic type and grade and pathologic stage, was done according to the TNM classification criteria of the UICC [39]. These data are reported in Table 1. For breast tumours, data on hormone receptors expression were also considered. All the 35 breast tumours were diagnosed as infiltrating carcinoma; the histologic subtype was ductal in 25 cases and lobular in the other 10. Histological grading was determined according to Elston and Ellis [17]. In ovarian tumours, histologic subtypes were serous adenocarcinoma (11 cases), endometrioid carcinoma (2 cases) and mucinous adenocarcinoma (2 cases); most tumours showed a multicystic growth pattern. Histological grading was formulated according to Silverberg [37].

Representative tissue sections of non-neoplastic counterparts for each case were also included.

Immunohistochemistry

The following antibodies were used on paraffin sections: rabbit polyclonal antiserum against Plex-B1 EC-3 [18]; monoclonal antibody against Met (DQ-13) [34]; monoclonal anti-phosphoMet (Abcam, Cambridge, UK). Standard immunocytochemistry procedure was used [47]. Briefly, the sections were deparaffinized, incubated in 10 mM citrate buffer pH 6.0 and microwaved at 750 W (2×5 min). After incubation with the primary antibody, immunostaining was performed using Dako Citomation Envision plus system labelled polymer HRP anti-rabbit (for polyclonal antibody) or anti-mouse (for monoclonal antibodies) (DAKO, Glastrup, Denmark), using diaminobenzi-

Table 1A

Cases included in this study. List of cases of breast carcinomas indicating histologic type and grading, pathologic stage, lymph node metastasis (LN) and positivity to oestrogen (ER) and progesterone (PR) receptors

Site	Stage	Grading	Histological type	LN	ER	PR	Plex-B1	Met
Breast	I	3	Ductal infiltrating carcinoma	+	+	−	2	3
Breast	I	3	Lobular infiltrating carcinoma	+	+	+	3	3
Breast	II	3	Ductal infiltrating carcinoma	+	−	−	3	2
Breast	II	3	Ductal infiltrating carcinoma	+	+	+	0	3
Breast	II	3	Ductal infiltrating carcinoma	+	−	−	2	3
Breast	II	3	Ductal infiltrating carcinoma	+	+	+	2	3
Breast	I	3	Ductal infiltrating carcinoma	+	+	−	1	3
Breast	I	3	Ductal infiltrating carcinoma	+	−	+	3	2
Breast	II	3	Ductal infiltrating carcinoma	+	+	+	3	2
Breast	I	3	Ductal infiltrating carcinoma	+	+	+	3	2
Breast	I	3	Ductal infiltrating carcinoma	+	−	−	2	2
Breast	II	3	Ductal infiltrating carcinoma	+	+	+	1	1
Breast	IV	3	Lobular infiltrating carcinoma	+	+	−	2	3
Breast	II	3	Ductal infiltrating carcinoma	+	+	+	3	3
Breast	I	3	Ductal infiltrating carcinoma	+	+	+	0	0
Breast	II	3	Ductal infiltrating carcinoma	+	nd	nd	2	3
Breast	II	3	Ductal infiltrating carcinoma	−	−	−	0	3
Breast	IV	3	Ductal infiltrating carcinoma	+	+	−	2	2
Breast	I	3	Ductal infiltrating carcinoma	+	+	−	0	2
Breast	II	3	Lobular infiltrating carcinoma	+	+	+	3	3
Breast	IV	3	Ductal infiltrating carcinoma	+	+	+	3	2
Breast	I	2	Lobular infiltrating carcinoma	−	+	−	1	0
Breast	I	1	Ductal infiltrating carcinoma	−	+	−	2	0
Breast	I	2	Lobular infiltrating carcinoma	−	+	+	1	0
Breast	I	3	Ductal infiltrating carcinoma	−	+	+	0	2
Breast	I	2	Ductal infiltrating carcinoma	−	+	+	0	3
Breast	II	3	Ductal infiltrating carcinoma	−	+	+	0	0
Breast	II	3	Ductal infiltrating carcinoma	−	−	−	0	2
Breast	I	2	Ductal infiltrating carcinoma	−	+	+	2	2
Breast	I	3	Ductal infiltrating carcinoma	−	+	+	3	0
Breast	I	2	Lobular infiltrating carcinoma	−	−	−	0	3
Breast	II	2	Lobular infiltrating carcinoma	−	−	−	0	2
Breast	II	2	Lobular infiltrating carcinoma	−	−	−	0	0
Breast	I	2	Lobular infiltrating carcinoma	−	+	+	0	3
Breast	II	2	Lobular infiltrating carcinoma	−	+	+	0	1

dine 3,3-tetrachloride as chromogen. Negative controls omitted the primary antibody. The positivity of each antibody was separately scored by two qualified pathologists (GV and SK) similarly to that previously reported [47]. Briefly, in both normal and neoplastic tissues staining intensity was defined as 0 (absent), 1 (weak), 2 (weak to strong) and 3 (strong). The proportion score was a continuous variable from 0% to 100% of stained cells. A hybrid-score (H-score) was calculated as the product of intensity score and proportion score, with a possible range of results from 0

to 300. The results below 20 were considered as negative reaction. The interobserver differences were below 10%, providing a good reproducibility. Images were captured using a microscope (Leitz Diaplan, Germany) equipped with a digital camera.

Immunofluorescence

Formalin-fixed paraffin-embedded tissue sections were cut from all cases of tumours. Sections were mounted on glass slides, deparaffinized in xylene, rehydrated in graded alcohol and finally washed in water. Prior to the incubation with antibodies, the sections

Table 1B

List of cases of ovary carcinomas indicating histologic type and grading, pathologic stage, lymph node metastasis (LN). Positivity for Plex-B1 and Met is also indicated

Site	Stage	Grading	Histological type	LN	Plex-B1	Met
Ovary	III	3	Serous cystadenocarcinoma	−	2	0
Ovary	I	2	Serous cystadenocarcinoma	−	0	2
Ovary	III	2	Serous cystadenocarcinoma	+	3	3
Ovary	III	2	Endometrioid adenocarcinoma	−	2	0
Ovary	III	3	Serous cystadenocarcinoma	−	3	3
Ovary	III	3	Serous cystadenocarcinoma	−	3	3
Ovary	III	3	Serous cystadenocarcinoma	−	3	3
Ovary	III	3	Serous cystadenocarcinoma	−	0	0
Ovary	III	3	Serous cystadenocarcinoma	−	0	2
Ovary	I	*	Serous cystadenocarcinoma	−	0	0
Ovary	III	3	Serous cystadenocarcinoma	−	1	2
Ovary	III	3	Serous cystadenocarcinoma	+	2	1
Ovary	I	1	Mucinous adenocarcinoma	−	0	0
Ovary	I	1	Endometrioid adenocarcinoma	−	2	0
Ovary	I	2	Mucinous adenocarcinoma	−	0	0

Note: * One data lacking.

were subjected to a heat-induced antigen-demasking reaction. Immunofluorescence was performed using a standard procedure [47]. Sections were incubated with the primary antibody (1:100 in PBS supplemented with 0.1% Triton X-100 and 4% Foetal Calf Serum, for 16 h in a humid chamber at 4°C) and then with appropriate secondary antibodies (diluted 1:200 as above, for 1 h at room temperature in a humid chamber) conjugated with Texas Red or FITC as indicated. The latter were purchased from Sigma Aldrich Corp. (St. Louis, MO, USA). At each step, the excess of unbound antibody was removed by two washes with PBS. As negative control, the sections were incubated with the secondary antibody alone. Stained sections were mounted with Slow-FAD (Light AntiFADE kit, Molecular Probes, Invitrogen Corp.) and observed under a laser confocal immunofluorescence microscope (Leica DMIREZ, Leica Microsystem, Heidelberg, Germany). Each biopsy was tested at least three times. At least four fields per section were imaged by two independent investigators (GN and CI). Fluorescence stained cover-slips were imaged using a Leica Fluorescent Microscopy DMI600 equipped with a digital camera. Representative images are shown.

2.2. SDS-page and western blotting

Liquid nitrogen quick-frozen tumour samples were homogenized with Retsch MM301 ball mill, then lysed with extraction buffer [2] containing 1% Triton X100, 4 mM Na_3VO_4, 1 mM PMSF, 200 μg/ml leupeptin, 40 μg/ml aprotinin, 20 μg/ml pepstatin, 400 μg/ml soybean trypsin inhibitor STI. Cellular debris was removed by centrifugation (14,000g, 30 min), supernatants were collected and proteins concentration was quantified by BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Equal amount of proteins for each sample (approx. 100 micrograms) were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blocked in PBS, 5% bovine serum albumine. The filter was incubated with anti-Plex-B1 IC-2 rabbit polyclonal antiserum [2] and anti-Vinculin monoclonal antibody (Sigma Aldrich Corp.), followed by the appropriate peroxidase-conjugated secondary antibody (Amersham Biosciences, UK). Final detection was done by enhanced chemiluminescence (Amersham Biosciences, UK).

2.3. Statistical analysis

Data were statistically analysed with the XL-Statistics program (Excel workbooks for statistical data analysis 1997–2006). To correlate the various antigen parameters (expression of Plex-B1, Met and phospho-Met) with clinical–pathological data (pathologic stage, histologic grade and presence of metastases), the following statistic tests were performed: Chi-square, Fisher's test and Odd-Ratio. A value of $p < 0.05$ was considered to indicate a significant correlation.

The analysis of the data has been carried out keeping into careful consideration the recommendations of NCI-EORTC [27].

3. Results

3.1. Expression of Plex-B1 in normal and tumour tissues

To test the potential significance of Plex-B1 as a marker of tumour progression, we first evaluated its expression by immunohistochemical analysis in normal and neoplastic tissues. The detected protein was usually localized in a restricted cytoplasmic area close to the cell membrane. No nuclear positivity was observed. In the normal breast, cells of the inner layer of the lobules and ducts were positive (Fig. 1); the intralobular stroma was negative, as well as the epidermis. In normal ovary, no Plex-B1 immunostaining was found in epithelial structures, whereas stromal cells were faintly positive in some cases. Usually, in all normal tissues a number of blood vessels were positive. Amongst non-epithelial tissues, Plex-B1 was detected in lymphoid cells, predominantly in tumour infiltrating lymphocytes. Normal epithelial tissues were only sporadically Plex-B1 positive.

In contrast, 31/50 cases of malignant tumours revealed expression of Plex-B1 (Table 1). In detail, most of ovarian tumours (9/15), and of these 7/11 serous adenocarcinomas, showed positivity in neoplastic cells (Table 2A and Fig. 2). Amongst mammary neoplasias, both ductal (17/25) (Fig. 3A) and lobular (5/10) (Fig. 3B) infiltrating carcinomas were Plex-B1-positive (Table 2A). Intraductal component was also positive (Fig. 3C and D). Notably, positivity was found in tumours of all histologic types.

Table 2

Tables summarize the positivity for Plex-B1, Met and phospho-Met found in the whole series of biopsies examined. Data demonstrate that Met is more frequently phosphorylated when co-expressed with Plex-B1

Table 2A

The positivity for either Plex-B1 and Met, alone or together, is indicated for each separate histotype

Tumour site	Histological type	Plex-B1 alone	Met alone	Plex-B1 Met double-positive	Plex-B1 Met double-negative	Total
Ovary	Serous cystadenocarcinoma	1	2	6	2	11
	Endometrioid adenocarcinoma	2	0	0	0	2
	Mucinous adenocarcinoma	0	0	0	2	2
Breast	Ductal infiltrating carcinoma	2	6	15	2	25
	Lobular infiltrating carcinoma	2	4	3	1	10

Table 2B

The proportion of cases positive for phospho-Met in relation to the expression of Plex-B1 is indicated

Tumour site	Histological type	P-Met in Plex-B1/Met double-positive	P-Met in Met alone
Ovary	Serous cystadenocarcinoma	5/6	1/2
	Endometrioid adenocarcinoma	0	0
	Mucinous adenocarcinoma	0	0
Breast	Ductal infiltrating carcinoma	13/15	3/6
	Lobular infiltrating carcinoma	2/3	1/4

Table 2C

Statistics applied to the presence of phospho-Met as related to the expression of Plex-B1

Total tumours	P-Met		Total
	+	−	
Plex-B1+Met+	20	4	24
Met alone	5	7	12
Total	25	11	36

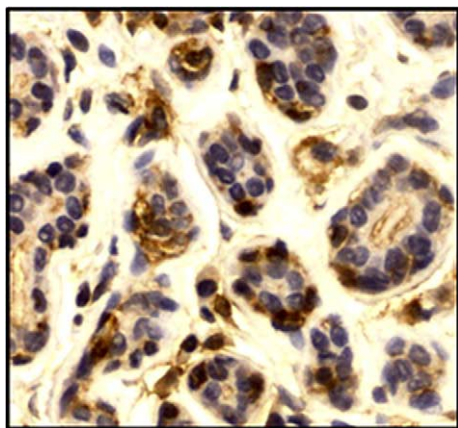*Notes*: Chi-square = 4.7, DF = 1, $p = 0.03$, O.R. = 7.

Fig. 1. Plex-B1 is displayed by a number of normal structures. In mammary lobules, cells of the inner layer are positive (original magnification: 220×).
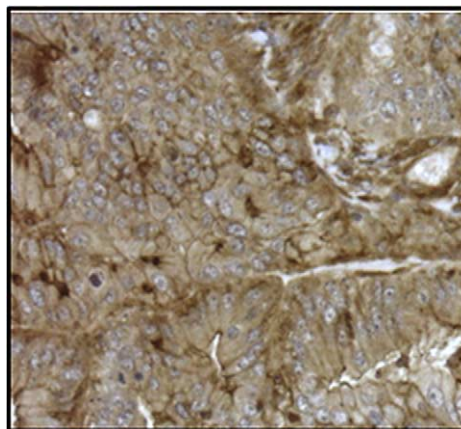

Fig. 2. Plex-B1 positivity was displayed by 7/11 ovarian serous adenocarcinomas and 2/2 endometrioid adenocarcinomas. The image shows the positivity in an ovarian endometrioid adenocarcinoma (original magnification: 220×).
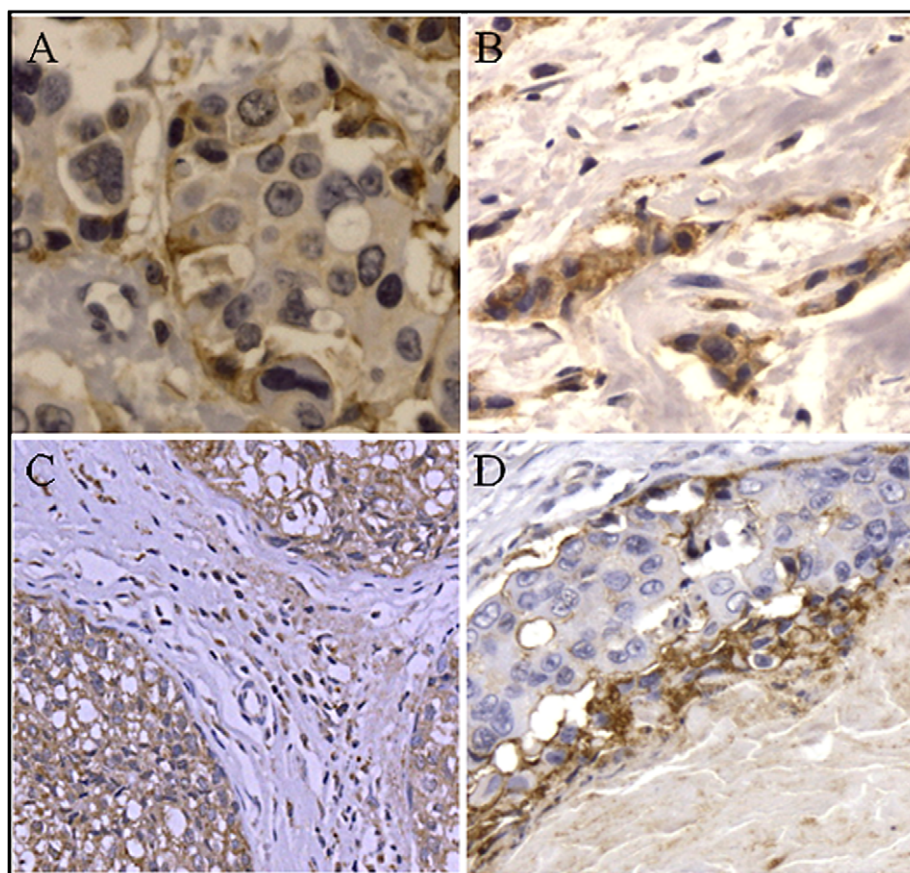

Fig. 3. Plex-B1 positivity was displayed by 31/50 cases of tumour, and was localized mainly in a restricted cytoplasmic area close to the cell membrane. The majority of the breast carcinomas were positive. Panel A: positivity in ductal infiltrating carcinoma of the breast (magnification: 440×); panel B: positivity in lobular infiltrating carcinoma of the breast (magnification: 220×); panels C and D: positivity in intraductal fields of breast carcinomas (magnification: 220× in panel C and 440 in panel D).

In order to validate the specificity of Plex-B1 expression data, we analyzed in parallel by immunofluorescence and Western blotting a subset of ovarian carcinoma samples. An extract of normal ovary, previously found to be consistently negative for Plex-B1 expression in immunostaining, was included as negative control for immunoblotting. As positive control, the homogenates of NIH3T3 fibroblasts transfected with either the empty vector or a plasmid carrying the Plex-B1 cDNA were included. Data shown in Fig. 4 (panel A, immunofluorescence; panel B, immunoblotting) confirmed high expression of Plex-B1 in two cases of ovarian carcinoma (cases no. 448 and 555), intermediate-low expression in the others, and barely detectable levels in normal ovary tissue.
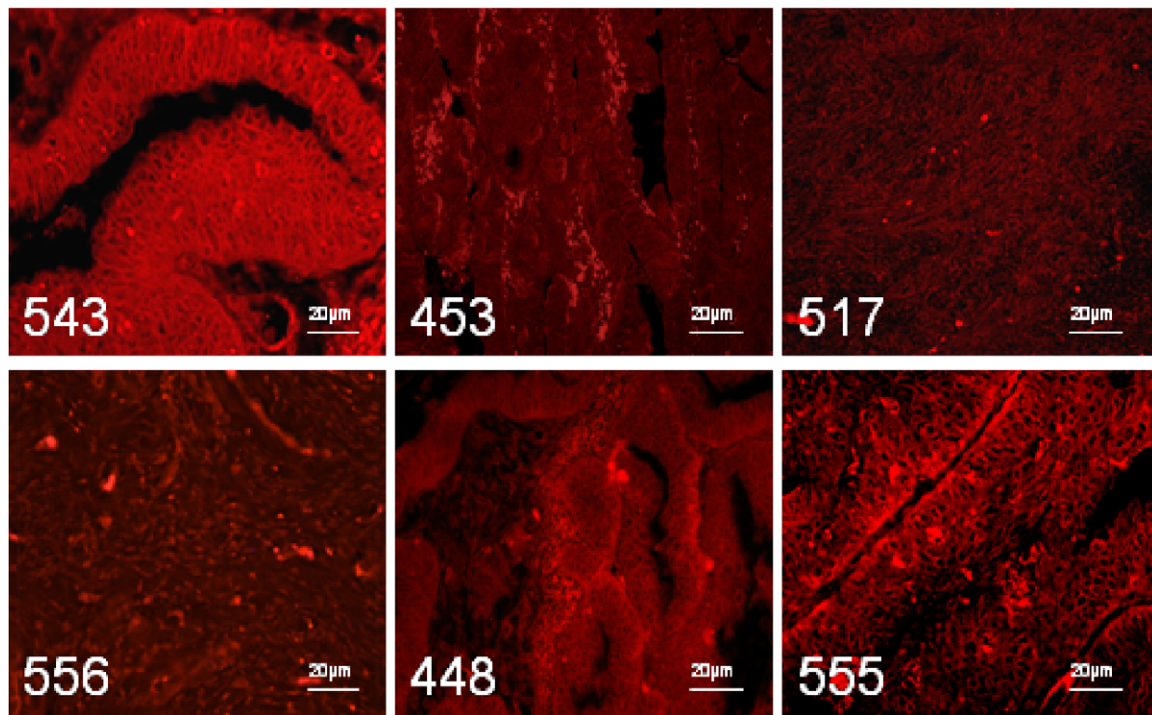
(A)
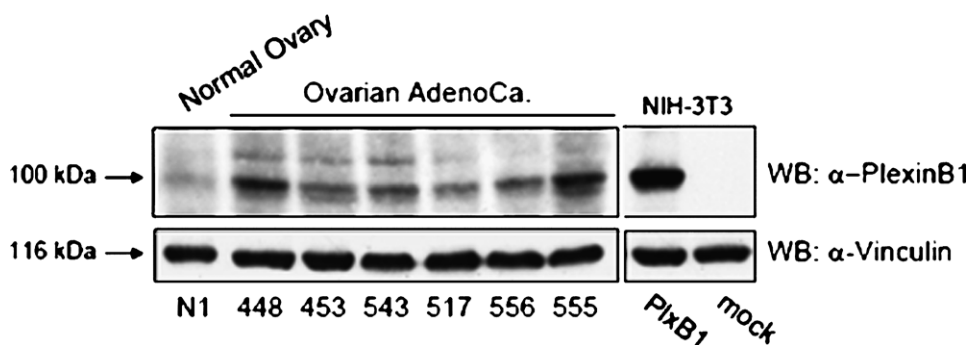
(B)

Fig. 4. The specificity of Plex-B1 reaction was validated by parallel analysis of six cases of ovarian carcinomas using Western blot (shown in panel B) and immunofluorescence staining of corresponding paraffin sections (shown in panel A; magnification: bar = 20 μm). Protein lysates of NIH-3T3 cells transduced to over express human Plex-B1 were used as positive controls for immunoblotting. Homogenate from normal ovary tissue was also included in the immunoblotting analysis. The following histotypes of ovary adenocarcinomas are represented: 543 – serous; 453 – endometrioid; 517 – serous; 556 – serous; 448 – serous; 555 – endometrioid.

3.2. Co-expression of Plex-B1, Met and phospho-Met in tumour tissues

In vitro studies demonstrated that Plex-B1 couples with Met tyrosine kinase receptor to form a complex that triggers the invasive growth program [19]. We therefore investigated the expression of Met in the whole series of tumour samples: a high expression of Met was observed in most cases of mammary carcinomas (28/35) and ovarian adenocarcinomas (8/15) (Table 2A and Fig. 5). Based on our immunohistochemical analysis, 24 cases of human tumours (18/35 mammary carcinomas and 6/15 ovarian adenocarcinomas) expressed both Plex-B1 and Met (Table 2A). To determine whether both molecules were expressed in the same cells, we performed double-fluorescence immunostainings for the two proteins. This analysis revealed that although not every tumour cell co-expressed both molecules, in a relevant proportion of the neoplastic tissue the cells were positive for both Plex-B1 and Met (Fig. 6A). As the complex Plex-B1–Met has been shown to signal via tyrosine phosphorylation of Met [11,19], to determine whether co-expression with Plex-B1 indeed led to Met activation, we immunostained tumour sections with a phosphospecific antibody directed against activated Met. We found that a large majority of neoplastic tissues that were positive for both Plex-B1 and Met displayed phospho-Met positivity (20/24, i.e. 83.3%) (Table 2B). More in detail, phospho-Met was revealed in 15/18 and in 5/6 Plex-B1–Met double-positive cases of breast and ovary cancer, respectively. By contrast, when Met was expressed in the absence of Plex-B1 it was frequently found in the non-phosphorylated inactive form. These differences were statistically significant ($p = 0.03$) and strongly suggest that the co-expression with Plex-B1 favours Met activation in tumours (Table 2C). Moreover, double-fluorescence immunostaining demonstrated that Plex-B1 and P-Met were both expressed on the same cells (Fig. 6B).

3.3. Statistical correlation of Plex-B1 and Met expression with clinical–pathological data

The results above indicated that Plex-B1 and Met are expressed, either individually or in association, in a large majority of samples examined and, when co-expressed in the same tumour, Met was found frequently tyrosine phosphorylated. We hypothesized that the latter mechanism could underlie a particularly aggressive behaviour of tumour cells. We therefore checked whether a statistically significant correlation exists between the clinical–pathological parameters that define tumour malignancy (pathologic staging at diagnosis, histological grading of the tumours, and presence of metastases) and the expression pattern of Plex-B1, Met and phospho-Met for each case. Of the tumours considered in the present study, 22 presented with lymph node metastases, 16 were of low (1, 2) and 33 were of high (3) histologic grade, 37 were diagnosed at stage I–II and 13 at stage III–IV (Table 1). Statistics was applied separately to ovary and breast cancers (Table 3A). Single positivity for either Plex-B1 or Met was not significantly associated with advanced stage, high histological grading or presence of metastases. In contrast, these parameters of clinical progression were significantly poorer in tumours positive for both Plex-B1 and Met, compared to those expressing either one of the two molecules alone. In particular, double-positivity for Plex-B1 and Met was strongly associated to a worse histological grading in 5/6 ovary ($p = 0.04$) and in 17/18 breast ($p = 0.001$) tumours (Table 3B). Also, 21 out of 25 (84%) tumours, and more in detail 4/6 ovary and 17/19 breast, expressing P-Met were of the highest histologic grade (Table 3C). This association was statistically significant for breast tumours ($p = 0.01$). On the whole, the correlation between Plex-B1–Met co-expression and pathologic stage was not statistically significant (Table 4). Still, it is to note that the tumours diagnosed at the III–IV stage positive for Plex-B1 more frequently (6/8 ovary and 3/3 breast) co-expressed Met. Similarly, the phosphorylation status of Met was not correlated with the pathologic stage at diagnosis, either in the case of ovary and of breast cancers (Table 4C). Finally and more importantly, double-positivity for Plex-B1 and Met as well as P-Met strongly associated to the presence of lymph node metastases ($p = 0.0008$ and $p = 0.002$, respectively). In detail, of 22 tumours (2 ovary plus 20 breast) with lymph node metastases, 19 were Plex-B1–Met double-positive and of these 17 expressed P-Met (Table 5). This association was statistically significant also when breast cancer was considered separately (15/20 lymph nodes positive tumours expressed P-Met; $p = 0.01$). Of note, no correlation was found between Plex-B1 positivity alone or in association with Met and hormonal receptor status in breast carcinomas (Table 6).

4. Discussion

Plex-B1 has been shown to control several functions in response to its specific ligand Sema4D, including
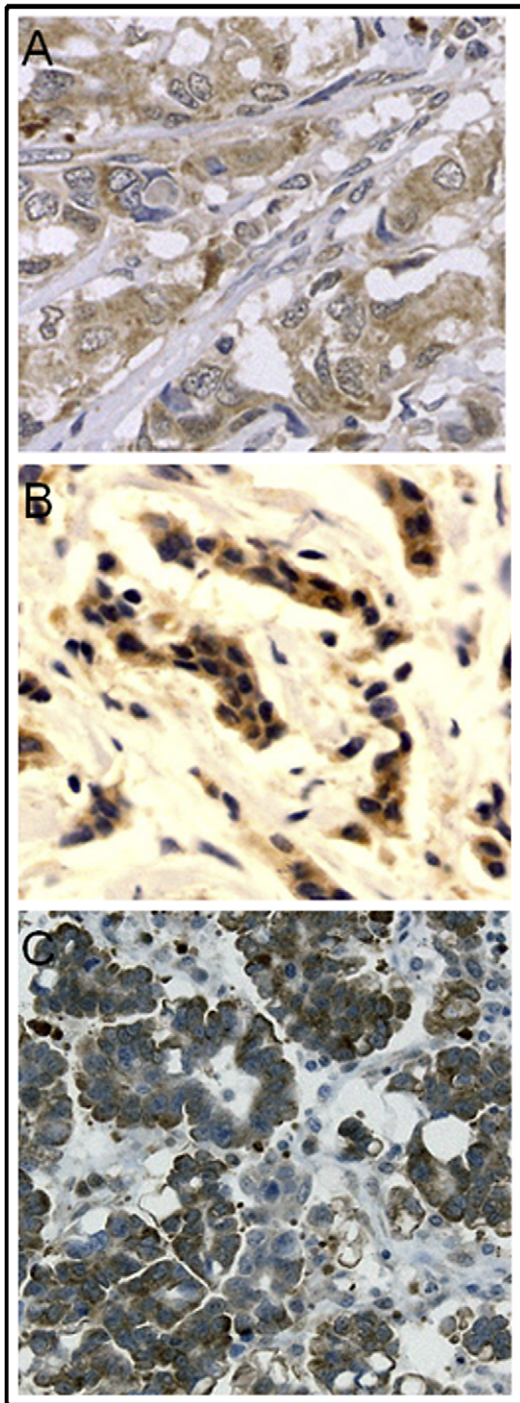
Fig. 5. Immunohistochemistry staining of Met. Most of tumour cases (36/50) showed Met positivity in the cytoplasms of the neoplastic cells. Panel A: positivity in a ductal infiltrating carcinoma of the breast (original magnification: 440×); panel B: positivity in a lobular infiltrating carcinoma of the breast (original magnification: 220×); panel C: positivity in an ovarian serous adenocarcinoma (original magnification: 440×).
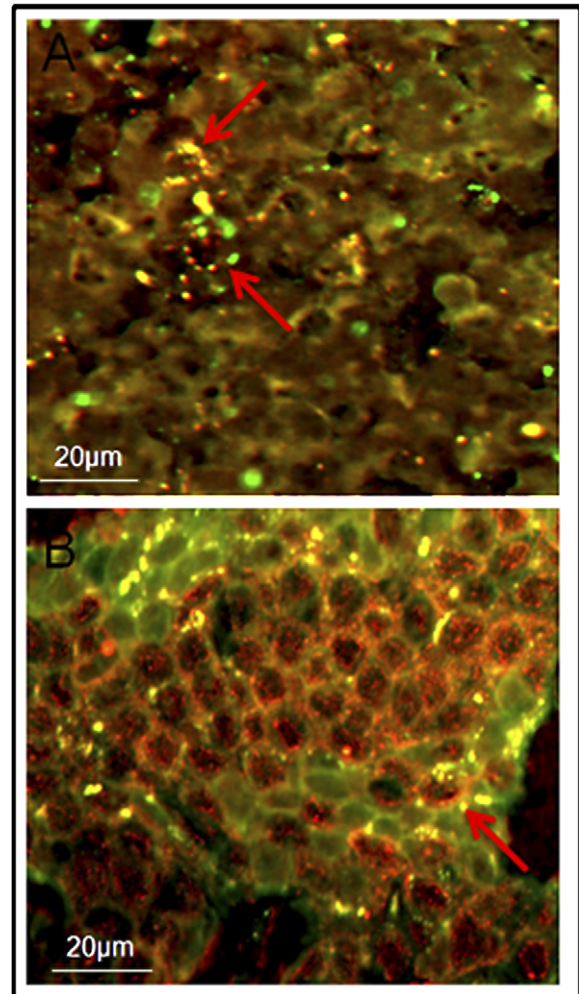

Fig. 6. In 24/50 cases Plex-B1 and Met were simultaneously expressed in the neoplastic cells, and these cases revealed a statistically more aggressive clinical outcome. In most of these cases, Met was phosphorylated. Panel A: ductal infiltrating carcinoma of the breast showing a double expression (yellow positivity, arrows) of the two molecules (plex-B1 in green and Met in red fluorescence) (original magnification: bar = 20 μm); panel B: in ovarian serous adenocarcinoma, neoplastic cells are positive for Plex-B1 (green) and P-Met (red). Co-localization is identified by yellow positivity (arrows) (original magnification: bar = 20 μm).

tumour cell migration/invasion and angiogenesis. Notably, Sema4D is abundantly present in a number of tumours, such as head and neck squamous cell carcinomas, lung, breast, colon and prostate carcinomas [7], both in cancer cells and in tumour-infiltrating stromal cells [36]. Notably, it was found that Sema4D can mediate divergent functions in different cells, due to interaction with alternative receptor complexes, comprising Plex-B1 in association with tyrosine kinase re-

Table 3A

Data on the correlation between the expression of Plex-B1, Met and P-Met and their combination with the histologic grade in ovary and breast cancers separately

	Histologic grade		Total
	Grade 1, 2	Grade 3	
Ovary			
Met alone	1	1	2
Plex-B1 alone	2	1	3
Plex-B1+Met+	1	5	6
Plex-B1−Met−	2	1	3*
Breast			
Met alone	5	5	10
Plex-B1 alone	3	1	4
Plex-B1+Met+	1	17	18
Plex-B1−Met−	1	2	3

Note: *One data lacking.

Table 3B

Statistics was applied to ovary and breast cancers to see whether Plex-B1–Met double-positivity correlated significantly with histological grading

	Histologic grade		Total
	Grade 1, 2	Grade 3	
Ovary*			
Plex-B1 alone	2	1	3
Plex-B1+Met+	1	5	6
Breast**			
Plex-B1 alone	3	1	4
Plex-B1+Met+	1	17	18

Notes: *Chi-square = 4.4, DF = 1, $p = 0.04$; **Chi-square = 10.6, DF = 1, $p = 0.001$.

Table 3C

Data indicate that, both in ovary and breast cancers, double-positivity for Plexin-B1 and Met significantly associated with a worse grading. Statistics demonstrates that phospho-Met also correlates significantly with a worse grading in breast cancers, not in ovary cancers

	Histologic grade		Total
P-Met	Grade 1, 2	Grade 3	
Ovary*			
Negative	4	4	8***
Positive	2	4	6
Total	6	8	14
Breast**			
Negative	8	8	16
Positive	2	17	19
Total	10	25	35

Notes: *Chi-square = 0.4, DF = 1, $p = 0.53$; **Chi-square = 6.6, DF = 1, $p = 0.01$; *** one data lacking.

Table 4A

Data on the correlation between the expression of Plex-B1, Met and P-Met and their combination with the pathologic stage in ovary and breast cancers separately

	Pathologic stage		Total
	I–II	III–IV	
Ovary			
Met alone	1	1	2
Plex-B1 alone	1	2	3
Plex-B1+Met+	0	6	6
Plex-B1−Met−	3	1	4
Breast			
Met alone	10	0	10
Plex-B1 alone	4	0	4
Plex-B1+Met+	15	3	18
Plex-B1−Met−	3	0	3

ceptors. In particular, the trans-activation of Met induced by Sema4D in tumour cells co-expressing Plex-B1 and Met has been shown in experimental models to mediate the invasive growth program and it was potentially correlated with increased metastasis [19], while in other cellular contexts Sema4D signalling could inhibit tumour cell migration [4,41]. Intriguingly, contrasting data indicated either reduced or increased expression of Plex-B1 in advanced metastatic tumours with poor prognosis [3,48], leaving unanswered the question whether this receptor should actually be considered as a marker of tumour progression.

Our study shows that Plex-B1 is highly significantly expressed in a large fraction of tumours originating in the breast and in the ovary, while it is physiologically present only in selected normal structures (e.g., epithelial inner cells of breast ducts and lobules). More-over, we found Plex-B1 expression in tumour infiltrating lymphocytes, in keeping with previous data indicating a functional role of Sema4D in these cells [5], and in tumour blood vessels, consistent with previous evidence that Sema4D controls tumour angiogenesis [7]. Importantly, our data indicate that Plex-B1 expression "per se" does not correlate with the presence of metastases: by contrast, we found a strict correlation between co-expression of both Plex-B1 and Met in tumour samples and clinical–pathological parameters of aggressiveness, mainly metastatic diffusion to lymph nodes. In the subgroup of breast carcinomas ($n = 35$), half of the cases showed positivity for both Plex-B1

Table 4B

Statistics was applied to ovary and breast cancers to see whether Plex-B1–Met double-positivity correlated significantly with the pathologic stage

	Pathologic stage		Total
	I–II	III–IV	
Ovary*			
Plex-B1 alone	1	2	3
Plex-B1+Met+	0	6	6
Breast**			
Plex-B1 alone	4	0	4
Plex-B1+Met+	15	3	18

Notes: *Chi-square = 2.25, DF = 1, $p = 0.13$; **Chi-square = 0.8, DF = 1, $p = 0.37$.

Table 4C

Data indicate that, both in ovary and breast cancers, double-positivity for Plexin-B1 and Met was not significantly associated with any stage at presentation. Statistics demonstrates that phospho-Met also was not significantly correlated with the pathological staging, both in ovary and in breast cancers

	Pathologic stage		Total
P-Met	I–II	III–IV	
Ovary*			
Negative	4	5	9
Positive	1	5	6
Total	5	10	15
Breast**			
Negative	15	1	16
Positive	17	2	19
Total	32	3	35

Notes: *Chi-square = 1.25, DF = 1, $p = 0.26$; **Chi-square = 0.2, DF = 1, $p = 0.6$.

Table 5A

Data on the correlation between the expression of Plex-B1, Met and P-Met and their combination with lymph nodes metastases in the whole series of tumour esamine. Statistics demonstrates that Plex-B1–Met double-positivity significantly correlates with the presence of lymph node metastases ($p = 0.0008$)

	Lymph node metastases		Total
	−	+	
Met alone	10	2	12
Plex-B1 alone	7	0	7
Plex-B1+Met+	5	19	24
Plex-B1−Met−	6	1	7
Plex-B1 alone*	7	0	7
Plex-B1+Met+*	5	19	24

Notes: *Chi-square = 11, DF = 1, $p = 0.0008$.

Table 5B

Statistics demonstrates that phospho-Met also correlates significantly with lymp nodes positivity in the whole series of tumours ($p = 0.002$). Phospho-Met was shown to correlate significantly with lymp nodes positivity also when considered only for breast cancers ($p = 0.01$) (lower panel)

	Lymph node metastases		Total
P-Met	Negative	Positive	
Total tumours*			
Negative	20	5	25
Positive	8	17	25
Total	28	22	50
Only breast**			
Negative	11	5	16
Positive	4	15	19
Total	15	20	35

Notes: *Chi-square = 9.8, DF = 1, $p = 0.002$; **Chi-square = 6.2, DF = 1, $p = 0.01$.

and Met and virtually all of these (17 out of 18) had developed metastases in axillary lymph nodes, while metastases were rarely seen in absence of Plex-B1–Met co-expression (3 cases out of 17). Unlike that recently reported by Rody et al. [32], in our study Plex-B1-negative cases are not characterised by high histologic grade or metastatic behaviour.

In addition, Plex-B1-positive cases not co-expressing Met were not associated with metastases, and were mostly classified in stage I or II. These findings are consistent with the hypothesis that Plex-B1 expression *per se* is not responsible for controlling the "metastatic phenotype", but rather this receptor must cooperate with Met to trigger invasive growth and tumour progression. Notably, the fact that nearly 70%

of the tumours diagnosed at stage III or IV is positive for both Plex-B1 and Met suggests that the expression of Met is acquired at a later step of tumour progression compared to Plex-B1, or that its expression precipitates cancer progression. In addition, the presence of P-Met statistically correlated with the risk of progression in terms of lymph node metastases at diagnosis, further supporting the activation of the Met signalling pathway associated with expression of Plex-B1 during tumour progression. The observation that both Plex-B1–Met double-positivity and phospho-Met are

Table 6

Data on the correlation between the expression of Plex-B1, Met and P-Met and their combination with sexual hormone receptors status in breast cancers. Statistics demonstrates that Plex-B1–Met double-positivity does not correlate either with the estrogen receptor (ER) or the progesterone receptor (PR)

	ER*		Total
	Positive	Negative	
Plex-B1 alone	4	0	4
Plex-B1/Met double-positive	13	4	17
Total	17	4	21
	PR**		
Plex-B1 alone	2	2	4
Plex-B1/Met double-positive	10	7	17
Total	12	9	21

Notes: *Chi-square $= 0.16$, DF $= 1$, $p = 0.3$; **Chi-square $= 0.1$, DF $= 1$, $p = 0.7$.

strongly associated with the metastatic phenotype of breast cancers is of clinical relevance. In fact, the majority of affected women that are lymph node negative receive an adjuvant systemic treatment, despite this is not recommended in patients considered at low risk on the basis of tumour grade and oestrogen receptor status. It has recently been reported that the expression of the oncosuppressor and autophagy regulator PTEN (phosphatase deleted on chromosome ten) predicts a favourable clinical outcome in lymph node negative breast cancer patients [23]. Our study provides additional prognostic markers that might help to identify the patients that would need an adjuvant systemic treatment.

The functional interaction between Plex-B1 and Met has been well studied *in vitro*. These molecules can form receptor complexes on the cell surface, and the binding of Sema4D/CD100 to Plex-B1 induces the tyrosine kinase activity of the associated Met receptor [19]. Met and Ron receptors have been shown to interact with each of the three members of class B Plexins, even in the absence of the ligand [10,31], and to induce tyrosine kinase activation and the invasive growth response [14,15]. The interaction between Plex-B1 and Met is reminiscent of that described for the complex Met/$\alpha6\beta4$, mediating the activation of signalling pathways required for invasion and metastasis [31,45]. In this case, however, Met gets tyrosine phosphorylated not only in response to its cognate ligand HGF, but also in response to Sema4D if Plex-B1 receptor is co-expressed in tumour cells [19]. Consistent with data reported previously, we found that most cases undergoing a metastatic progression were positive for tyrosine-phosphorylated Met, which is an activated form of the receptor [14,15,33]. Tyrosine phosphorylation of Met, in turn, is known to be crucial for intracellular signalling, since it mediates the interaction with several transducer molecules involved in cell dissociation, protection from apoptosis and tumour progression. Notably, Met activation is more evident in cells of primary and metastatic tumours than in the correspondent precancerous lesions [26]. Moreover, phospho-Met positivity seems to be preferentially found in neoplastic cells located at the invasive front of the tumour [26]. Furthermore, Met activation is known to trigger VEGF (vascular endothelial growth factor) expression in tumour cells [31], an observation that we confirmed by using three-colour immunofluorescence in tumours expressing PlexB1 and Met (data not shown). Although the molecular mechanisms elicited by Plex-B1–Met signalling complex await full elucidation, our data indicate that the co-expression of these markers in tumour samples is a negative prognostic indicator, commonly associated with metastatic progression.

In conclusion the present study demonstrate that: (1) Plex-B1, the receptor of Sema4D, is highly expressed in a number of malignant tumour cells of breast and ovarian carcinomas; (2) the tumours co-expressing Plex-B1 and Met show a clinical progression significantly worse than those expressing any of the two molecules alone, thus suggesting that the co-expression of these receptors is a reliable phenotypic marker of invasive growth.

Acknowledgements

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