# Identification of Plexin D1as a tumor biomarker in brain, breast and thyroid cancers

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**Abstract:** The expression patterns of Plexin D1 in endothelial cells during developmental angiogenesis and in the vascular coating of specific tumors empahsize this protein as a potentially powerful tool as a diagnostic tumour biomarker in different types of cancer. Various tumour samples (n= 31) (11 brain, 8 breast, 12 thyroid) were compared to 33 control samples for the expression of Plexin D1 using immunohistochemical analysis. Plexin D1 was detected in most of the endothelial cells of the various cancer samples [brain cancer (63.6%), breast cancer (66.7%) and thyroid cancer (75%)], yet no expression could be detected in endothelial cells of normal tissues. Our results verify the role of Plexin D1 in brain, breast and thyroid cancer associated angiogenesis. Regarding the implications of Plexin D1 and its associations with cancer angiogenesis it might be a potential cancer biomarker providing that further studies confirm the present preliminary findings.
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## 1. Introduction

An imbalance in the process of angiogenesis contributes to the pathogenecity of numerous diseases including cancers, cardiovascular disease, blindness, arthritis, complications of AIDS, diabetes and Alzheimer's disease **(Yancopoulos *et al.*, 2000)**. Once a tumour reaches a certain size of 1–2 mm in diameter, diffusion is no longer adequate to supply the cells with oxygen and it requires a blood supply in order to grow larger and migrate **(Zetter, 1998).** Tumour associated endothelial cells secrete pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and platelet-derived endothelial cell growth factor (PD-ECGF) **(Cantu De Leon *et al.*, 2003)**. Several reports have suggested that semaphorins and their receptors can act as antiangiogenic and/or antitumorigenic. Semaphorins are known to modulate the migratory and growth characteristics of cultured endothelial cells by competing with VEGF for neuropilin-containing receptors **(Basile *et al.*, 2005)**. This could be attributable to the cross-interactions between different subfamilies of semaphorins and Plexins, which show differential binding affinity and specificity, thereby triggering different sets of signaling events and functions. A second level of regulation is attained by coupling Plexins and/or neuropilins to various coreceptors expressed in a cell- or tissue-specific manner **(Law and Lee, 2012)**.

Semaphorins are known to activate Plexins via the binding of neuropilins (Npn) such as Npn-1 to form a multicomponent complex **(Takahashi *et al.*, 1999)**. Interestingly Npn-1 is known to interact with specific isoforms of VEGF in order to mediate heterophilic cell adhesion **(Fujisawa *et al.*, 1997, Shimizu *et al.*, 2000)**. In the vascular system, Plexin D1 is the main receptor for semaphorin 3E (Sema3E) which may regulates angiogenesis and vascular pathfinding *in vivo* through binding directly or indirectly to PlexinD1 on endothelial cells **(Gu *et al.*, 2005)**. PlexinD1 also function as a receptor for other ligands during brain development **(Gu *et al.*, 2005)**.

While mouse embryogenesis studies showed that plexin D1 is expressed on neuronal and endothelial cells **(van der Zwaag *et al.*, 2002)**, it was later confirmed that endothelial cells loose Plexin D1 expression during development **(Carmeliet and Tessier-Lavigne, 2005)**. *In situ* hybridization studies showed that endothelial cells regain expression of this protein during tumor angiogenesis in a mouse model of brain metastasis **(Roodink *et al.*, 2005)**.

Tissue-specific gene inactivation of the semaphorins and their receptors has been investigated as a potential targeted therapy for various models of cancer. Notably high levels of plexin D1 have been detected in a variety of tumour types **(Roodink *et al.*, 2008)** which is consistent with studies that have shown high levels of semaphorin 3E associated with tumour invasion and metastasis **(Christensen *et al.*,**

**1998)**. Since Plexin D1 is uniquely expressed in both tumour vasculature and tumour cells, it has been proposed as a potential candidate for targeted cancer therapy **(Roodink *et al.*, 2005)**. To examine whether plexin D1 might be clinically useful as a diagnostic biomarker in solid tumors, this study investigates Plexin D1 expression in human tumors of different origins including brain, breast and thyroid tumour and compared to non-tumor related tissues by immunohistochemistry. The expression of Plexin D1was specifically investigated in the vascular tissue of normal and malignant tissues.

## 2. Material and Methods

**Patient section:**

Brain, breast and thyroid tumor samples were retrieved from the archival files of the departments of Pathology, King Khalid hospital, Riyadh, Saudi Arabia, from 2004 to 2006. IRB approval was obtained. The specimens were surgical biopsies and resections. The clinical history and final pathological diagnostic report were reviewed for each patient.

**Tissue samples:**

The role of Plexin D1 as a potential biomarker for different types of cancer, brain, breast and thyroid, was investigated using the samples explained below. Eleven brain cancer samples were classified histopathologically according to WHO grading as follows; 3 medulloblastoma of grade IV, 3 pilocytic astrocytoma (all of them were low grade, grade I), 2 astrocytoma (both of them were grade III), 2 glioblastoma (both of them were grade IV), 1 glioma with anaplastic ependymoma high grade (IV). Nine breast cancer samples were classified histopathologically according to Scarff-Bloom-Richardson grading system (SBR); 8 ductal cell carcinoma (4 of them were grade II and 4 were grade III) and 1 infiltrative ductal carcinoma, grade III. Twelve thyroid tumor samples were classified according to invasiveness 7 classical papillary thyroid carcinoma (4 of them were encapsulated and 3 were invasive), 3 follicular cell carcinoma (1 of them was widely invasive and 2 invasive), 1 follicular adenoma and 1 follicular variant of papillary thyroid carcinoma with no vascular, extracapsular or lymphovascular invasion. Thirty three normal tissue sections were investigated for Plexin D1 expression as a control.

Four brain cancer samples were tissues conducted from female patients and 7 were conducted from male patients, all samples for breast cancer and thyroid cancer were conducted from females. The age of most patients ranged between 30 and 59 except 5 patients with brain cancer aged between 1-14 years. Tumour size was less than or equal to 1 $cm^3$ in 5 of the brain tumor samples, 1 of breast tumor and 3 of thyroid tumor samples and it was more than 1 $cm^3$ in 6 of brain tumor samples, 8 of breast tumor samples and 9 of thyroid tumor samples.

**Immunohistochemistry:**

Slides were deparaffinized in three changes of xylene for 5 min each. They were hydrated in decreasing concentrations of ethanol and rinsed in PBS. A hydrophobic barrier created around the section using an Immerge pent pen (Dako, Cambridgeshire, UK). Antigen retrieval was performed by immersing the slides in 0.01 M citrate buffer pH 6.0 and heating for 2-3 minutes microwaving at 100% power followed by 10-30 minutes at 20-30% power using an 800-900 Watt maximum capacity microwave oven. Endogenous peroxidase was quenched with 3% $H_20_2$ for 6 min at room temperature. Slides were incubated overnight at 4'C with a **1:200** dilution of goat plexin D1 antibody. A biotin-streptavidin detection system was employed with diaminobenzidine (DAB) as the chromogen. Slides were washed twice with PBS and incubated with the linking reagent (biotinylated Donkey antigoat) for 1 hour at room temperature. After rinsing in PBS, the slides were incubated with the peroxidase-conjugated streptavidin label for 20 min. The sections were again rinsed with PBS and incubated with DAB for 10 min in the dark. After chromogen development, slides were washed in two changes of water for **8** min each and counterstained with 0.2% methyl green (MD Supplies, UK) in sodium acetate buffer, pH 4.0. The sections were then dehydrated, cleared in xylene, and mounted with DPX mounting medium (Raymond A. Lamb Laboratory supplies, UK).

**Microscopical Analysis:**

Two investigators independently evaluated Plexin D1 staining under a light microscope at a magnification of 10X and 40X. Five images of representative areas were acquired for each specimen.

## 3. Results

Our results showed that there was no expression could be detected in the endothelial cells lining blood vessels of normal brain, breast and thyroid tissues (Figures 1, 2, 3). Most significant was the observation that positive staining was observed in the endothelial cells lining the blood vessels in the majority of the investigated types of cancer [brain cancer (63.6%), breast cancer (66.7%) and thyroid cancer (75%)] as shown in figures 1, 2, 3.

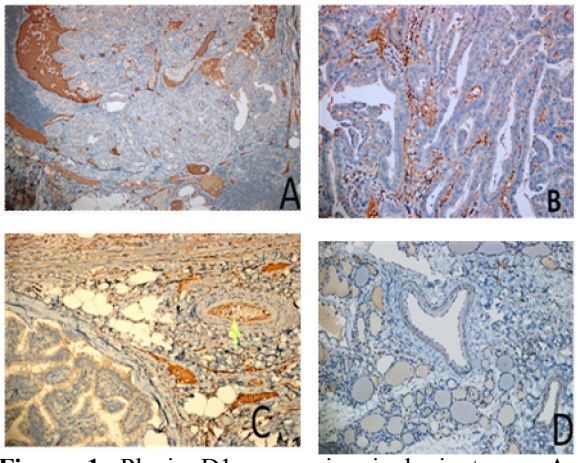


**Figure 1,** Plexin D1 expression in brain tumor A: Astrocytoma (400x) BV endothelium are positive for Plexin D1. B: Glioblastoma (400X) multiforme small capillaries endothelium are positive for Plexin D1. C: Medulloblastoma (200X). endothelium are positive for P Plexin D1. D: Control healthy brain tissue (100X) negative for Plexin D1.

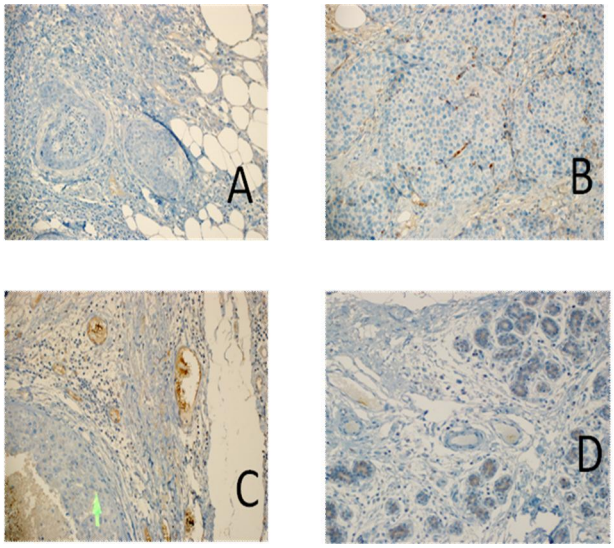


**Figure 2,** Plexin D1 expression in breast cancer tissues. A: Duct cell carcinoma(100X), breast with week staining at endothelium. B: Small blood vessel proliferation in between neoplastic cells (100X) which are positive for Plexin D1. C: In situ duct cell carcinoma (100X) arrow indicates the nearby blood vessel positive for Plexin D1 in the endothelium. D: Control normal healthy (100X) breast tissue Endothelium are negative for Plexin D1.

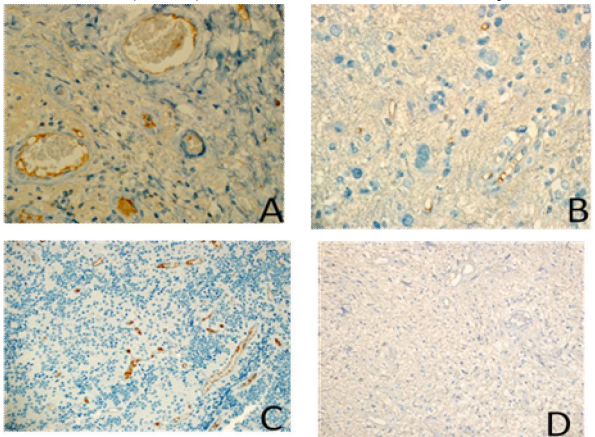


**Figure 3,** Plexin D1 expression in Thyroid cancer A: Lymph node metastatic thyroid carcinoma (40X) with positive staining for Plexin D1 in the endothelium. B: Papillary thyroid carcinoma (200X), positive endothelium within the papillary stalk blood vessel. C: Papillary carcinoma (200X) with +ve endothelium lining the blood vessel surrounding the neoplasm. D: Control healthy thyroid tissue (200X) The endothelium is negative for Plexin D1.

**Table 1. Comparison between cases and control according to Plexin in brain tumor, breast cancer and thyroid cancer**

| | Cases | | | | |
|---|---|---|---|---|---|
| | No. | % | No. | Control | |
| **Brain tumor** | | | | | |
| -ve | 4 | 36.4 | 11 | 100.0 | 0.004* |
| +ve | 7 | 63.6 | 0 | 0.0 | |
| **Breast cancer** | | | | | |
| -ve | 3 | 33.3 | 11 | 100.0 | 0.002* |
| +ve | 6 | 66.7 | 0 | 0.0 | |
| **Thyroid cancer** | | | | | |
| -ve | 3 | 25.0 | 11 | 100.0 | <0.001* |
| +ve | 9 | 75.0 | 0 | 0.0 | |

FEp : p value for Fisher Exact test
* : Statistically significant at $p \leq 0.05$

Eleven brain tumour tissues were investigated for Plexin D1 expression, microscopical analysis showed that 7 of the examined tissues were positive for Plexin D1 expression while the remaining 4 cases showed no expression for the protein (Figure 1, Table 1). Plexin D1 expression was investigated in 9 breast tumour tissues, microscopical analysis showed that 6 of the examined tissues was highly positive for Plexin D1 expression while the remaining 3 cases showed no expression for the protein. (Figure 2, Table 1) Twelve thyroid tumour tissues were investigated for Plexin D1 expression, microscopical analysis showed that 9 of the examined tissues were highly positive for Plexin D1 while 3 cases showed negative expression for this protein. (Figure 3, Table 1). The statistical analysis showed that the level of Plexin D1expression in brain, breast and thyroid cancers was significantly higher ($P$= 0.004, = 0.002, <0.001 respectively) as compared to control samples (Table 1).

Statistical analysis of Plexin D1 expression in different tissues showed that there was no significant association between sexuality and the

expression of this protein in brain tissue, while this was not applicable for the studied case of breast and thyroid cancers as they were all females. The expression level of this protein was studied in different grades and ages of the three types of cancer, statistical analysis showed no significant difference of the expression levels (Tables 2, 3, 4). Fisher exact test was used to study the significant difference of the expression level of Plexin D1 investigated in the three types of tumor samples of different sizes, our data showed that Plexin D1 expression is significantly different (FE*p*= 0.005) in thyroid tumor samples of size >1cm$^3$ than the smaller sizes (table 4).

**Table 2. Relation between Plexin with sex, age, tumor size and grades in brain tumor**

| | Plexin | | | | Test of sig. |
|---|---|---|---|---|---|
| | -ve | | +ve | | |
| | No. | % | No. | % | |
| **Sex** | | | | | |
| Male | 3 | 75.0 | 4 | 57.1 | FEp = 1.000 |
| Female | 1 | 25.0 | 3 | 42.9 | |
| **Age** | | | | | |
| ≤ 40 | 1 | 25.0 | 6 | 85.7 | FEp = 0.088 |
| > 40 | 3 | 75.0 | 1 | 14.3 | |
| **Tumor size** | | | | | |
| ≤ 1 cm$^3$ | 2 | 33.3 | 3 | 42.9 | FEp = 1.000 |
| > 1 cm$^3$ | 2 | 66.7 | 4 | 57.1 | |
| **Grades** | | | | | |
| Low | 1 | 25.0 | 2 | 28.6 | FEp = 0.194 |
| High | 3 | 75.0 | 5 | 71.4 | |
| I | 1 | 25.0 | 2 | 28.6 | |
| II | 0 | 0.0 | 0 | 0 | MCp = 0.337 |
| III | 0 | 0.0 | 2 | 28.6 | |
| IV | 3 | 75.0 | 3 | 42.9 | |

FEp : *p* value for Fisher Exact test
MCp: *p* value for Monte Carlo test

**Table 3. Relation between Plexin with sex, age, tumor size and grades in breast cancer**

| | Plexin | | | | FE*p* |
|---|---|---|---|---|---|
| | -ve | | +ve | | |
| | No. | % | No. | % | |
| **Sex** | | | | | |
| Male | 0 | 0.0 | 0 | 0.0 | - |
| Female | 3 | 100.0 | 6 | 100.0 | |
| **Age** | | | | | |
| ≤ 40 | 1 | 33.3 | 2 | 33.3 | FEp = 1.000 |
| > 40 | 2 | 66.7 | 4 | 66.7 | |
| **Tumor size** | | | | | |
| ≤ 1 cm$^3$ | 0 | 0.0 | 1 | 16.7 | FEp = 1.000 |
| > 1 cm$^3$ | 3 | 100.0 | 5 | 83.3 | |
| **Grades** | | | | | |
| Low | 1 | 33.3 | 3 | 50.0 | FEp = 1.000 |
| High | 2 | 66.7 | 3 | 50.0 | |
| I | 0 | 0.0 | 0 | 0.0 | |
| II | 1 | 33.3 | 3 | 50.0 | MCp = 1.000 |
| III | 2 | 66.7 | 3 | 50.0 | |
| IV | 0 | 0.0 | 0 | 0.0 | |

FE*p* : *p* value for Fisher Exact test
MC*p*: *p* value for Monte Carlo test

**Table 4. Relation between Plexin with sex, age, tumor size, invasiveness, metastasis and types of cancer in thyroid tumor**

| | Plexin | | | | Test of sig. |
|---|---|---|---|---|---|
| | -ve | | +ve | | |
| | No. | % | No. | % | |
| **Sex** | | | | | |
| Male | 0 | 0.0 | 0 | 0.0 | - |

| | | | | | |
|---|---|---|---|---|---|
| Female | 3 | 100.0 | 9 | 100.0 | |
| **Age** | | | | | |
| ≤ 40 | 1 | 33.3 | 5 | 55.6 | FEp = 1.000 |
| > 40 | 2 | 66.7 | 4 | 44.4 | |
| **Tumor size** | | | | | |
| ≤ 1 $cm^3$ | 3 | 100.0 | 0 | 0.0 | FEp = 0.005* |
| > 1 $cm^3$ | 0 | 0.0 | 9 | 100.0 | |
| **Invasivness** | | | | | |
| Encapsulated | 3 | 100.0 | 3 | 33.3 | |
| invasive | 0 | 0.0 | 5 | 55.6 | MCp = 0.248 |
| Widely invasive | 0 | 0.0 | 1 | 11.1 | |
| **Metastasis** | | | | | |
| No | 3 | 100.0 | 5 | 55.6 | FEp = 0.491 |
| Yes | 0 | 0.0 | 4 | 44.4 | |
| **Types of cancer** | | | | | |
| Papillary | 3 | 100.0 | 5 | 55.6 | |
| Follicular | 0 | 0.0 | 3 | 33.3 | MCp = 0.619 |
| Follicular variant of papillary | 0 | 0.0 | 1 | 11.1 | |

FE*p* : *p* value for Fisher Exact test
MC*p*: *p* value for Monte Carlo test
* : Statistically significant at $p \leq 0.05$

## 4. Discussions

Investigating Plexin D1 as a tumor biomarker reflect the multiple biological processes that could be involved in angiogenesis associated with solid tumors. This study investigated the association of this biomarker with brain, breast and thyroid angiogenesis using immunohistochemical analysis. Our results demonstrated that Plexin D1 was positively associated with angiogenesis in brain cancer (63.6%), breast cancer (66.7%) and thyroid cancer (75%). Using immunohistochemistry, Plexin D1 was detected in the endothelium of most breast, brain and thyroid tumours and was up-regulated compared with its expression in normal tissues. Statistical analysis results revealed that there is no correlation between Plexin D1 expression with age, sex and different cancer grades. Expression of this protein in tumors of size more and less than $1cm^3$ showed insignificant difference except in thyroid cancer where Plexin D1 expression was significantly higher in tumour size > $1cm^3$.

Previously published data on the expression of Sema3E and its receptor in human tumors do not univocally establish a correlation with tumor progression. For instance, some reports have shown that high Sema3E levels in mammary carcinomas **(Christensen *et al.*, 2005)**, while others have found an elevated expression in high- versus low-grade glioblastomas **(Kotliarov *et al.*, 2006, Sanchez-Carbayo *et al.*, 2006)**.

Our study confirmed that Plexin D1 could be considered as a leading candidate and an endogenous mediator of tumour angiogenesis. Consistent with our result others has reported that Plexin D1 is expressed in the endothelium of a variety of different tumors **(Roodink *et al.*, 2009)** and blocking this receptor may inhibit the growth of a number of tumors.

Expression studies showed that plexin D1 is not absolutely tumor specific. Low levels of the Plexin D1 transcript can be found in adult heart, liver, and testis **(van der Zwaag *et al.*, 2002)**.

The role of Plexins as regulators of migration through Semaphorin/plexin interactions has been proven to prompt communication of multiple intracellular signaling pathways including small GTPases, integrin, Mitogen-activated protein kinases **(**MAPK) and Phosphoinositide-dependent kinase-1/Protein Kinase Bl/Glycogen synthase kinase-3 (PI3K/Akt/GSK-3β) axes, all of which are events that are critically involved in formation of filopodia, lammelipodia integrin to bind the extracellular matrix (ECM), thus causing Focad adhesion disassembly and endothelial cell detachment and cellular migration **(Kruger *et al.*, 2005, Tran *et al.*, 2007, Zhou *et al.*, 2008)** . It is suggested that, Plexin-D1 recruits yet another receptor VEGFR2 upon Sema3E binding and then activates PI3K to promote cortical axon attraction and outgrowth.

Anti-Plexin-D1 therapy may prune and normalize tumor vasculature, and decrease the number of circulating endothelial cells and progenitor cells. Various angiogenic approaches to treat cancer are already in clinical trials (Antonarakis and Carducci 2012). Most interventions involve the delivery of inhibitors VEGF to the malignant tissue, Also manipulating Plexin D1 inhibitor alone or in the combination with inhibitors of VEGF and neuropilins, offers an attractive model for future investigation.

Our data show that Plexin D1 expression is found almost exclusively in tumour-associated blood vessels, and not in the blood vessels of normal tissues.

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