Original Research Article

Overexpression of Sema3E and Sema5A in pilocytic astrocytoma

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Abstract: We have previously found that Sema5A, a member of the semaphorin gene family, is up-regulated in pediatric pilocytic astrocytomas (PA) at the mRNA level by microarray analysis and real-time RT-PCR. By further analysis of the expression level of all 17 semaphorin genes in the microarray dataset, we found that Sema3A and Sema3E are the only two semaphorin genes that are highly up-regulated in pilocytic astrocytomas. In this study, the up-regulation of Sema3E was further confirmed by real-time RT-PCR. Furthermore, the over-expression of both Sema3E and Sema5A proteins were also confirmed by Western blot analysis and immunohistochemistry. Since pilocytic astrocytoma is characterized by extensive vasculature, co-immunofluorescent staining of both CD31 and Sema3E (or Sema5A) was performed. The result showed that a higher expression of Sema3E was around the CD31 positive endothelial cells. Based on semaphorin’s function in angiogenesis and a higher expression of Sema3E and Sema5A around endothelial cells in these PA samples, these genes could be potential biological markers and anti-angiogenesis therapeutic targets for pilocytic astrocytomas.

Keywords: Sema3E; Sema5A; astrocytoma; vasculature


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Introduction

The incidence of brain tumors is 3.5 per 100,000 in children under 15 years of age[1]. Gliomas represent about one-third of all pediatric central nervous system tumors and pilocytic astrocytomas account for roughly half of these pediatric glial tumors. Pediatric pilocytic astrocytoma has previously been referred to as juvenile pilocytic astrocytoma (JPA) but pilocytic astrocytoma (PA) is more commonly used now to describe both pediatric and rare adult cases[2]. This low-grade tumor is generally slow growing with a low potential for tissue infiltration[3]. However, high microvascular densities have been observed in PA[4]. Despite these generally encouraging biological characteristics, treatment of these tumors are dependent on many factors including tumor location and extent of surgical resection. These tumors most commonly arise in the cerebellum (~85%), optic chiasm, hypothalamus and deep cerebral midline structures[5]. For tumors that are treated by complete surgical resection, the overall survival rate of 95.8% at 10 years has been reported[6]. However, the prognosis for PA is dependent on the extent of surgical removal. For tumors located in the deep midline area of the brain, approximately one-third of these tumors are not completely resected[7]. Reasons for incomplete surgical removal are mainly based on the deep location and the risk of damaging important functions (eye and body movement, cardiac function, consciousness) of this region of the brain. About 50% of incompletely resected tumors will progress and most of the
Expression profiling data previously done showed an overall high expression of two members of the semaphorin family, Sema3E and Sema5A in PA. However, only the mRNA expression of Sema5A was confirmed by real-time RT-PCR in our previous study. The semaphorin family comprises a group of secretory and membrane-anchored proteins, characterized by a 500 amino acid sema domain. Broadly, their roles include axon guidance and regulating neural network, cell migration and angiogenesis. Class 5 semaphorin family expression has been seen in the nervous system and has been associated with neural development. This transmembrane protein functions as inhibitors of retinal growth and navigation in the presence of guidance molecules. The function of class 3 semaphorin has been found to be associated with repulsive axon guidance molecules. Generally, this class binds to neuropilins as ligand-binding receptor and plexins as signal-transducing components. However, Sema3E has been reported to bind to plexinD1 directly instead of neuropilin-1 and is involved in vascular patterning. Activation of the Sema3E-Plexin D1 signaling can also drive human cancer cell invasiveness and metastasis. Overexpression of Sema3E has also been shown to enhance cancer cell growth and is associated with poor pancreatic cancer patient survival.

The primary goal of this study is to validate the overexpression of these two proteins in PA, and to determine the histological expression of these proteins in reference to the endothelial cells since PA is characterized by extensive vasculature.

Materials and methods

Tissue

Brain tissue was provided by the Texas Children’s Hospital, Baylor College of Medicine and The University of Texas M.D. Anderson Cancer Center from patients undergoing tumor resection or other related neurosurgical procedures. These tissues were collected with approved IRB protocols at both institutions and patients’ consent. The normal cerebellar tissues were provided by the Harvard Brain Tissue Resource Center, which is supported in part by PHS grant number R24 MH068855. Sections were prepared from tissues fixed and embedded in paraffin and other residual tissue samples were snap-frozen in liquid nitrogen and stored at -80°C until use. RNA was isolated using Trizol reagent (Invitrogen) followed by DNase I treatment and a RNeasy spin column (Qiagen) was used for clean-up. Absorbance was measured at A260/A280 to ensure RNA quality and purity.

Expression profiling

In our previous study, we have analyzed a total of 24 tissue samples (21 PA, 3 normal cerebellar samples) using Affymetrix U133A microarray and the data has been deposited at NCBI Gene Expression Omnibus with accession number GSE12907. The expression level of 17 semaphorin genes in these tissue samples were extracted from this dataset. The average expression level of each of these genes in the 21 pediatric PA samples were calculated and compared to the average of the corresponding expression level in 3 normal cerebellar samples.

Antibodies

SEMA3E and SEMA5A antibodies were raised by immunizing rabbits with an oligopeptide. Anti-SEMA3E: Ac-CLRSEPETHLPRHTLDS-OH. Anti-SEMA5A: Ac-QKELVGVARNYLEFRQLEDCL-amide. Affinity-purified anti-serum was used in all western blot and immunohistochemistry experiments. The specificity of the antibodies was tested with sera from pre-immunized rabbit as negative control.

Protein extraction and Western blot

Frozen PA samples were homogenized with TRIzol reagent (Invitrogen). After DNA and RNA were extracted, protein was precipitated with acetone, guanidine hydrochloride and ethanol from the phenol-ethanol supernatant. Protein pellets were vacuum dried and resuspended. Protein extracts were separated in a SDS-polyacrylamide gel and blotted onto a PVDF membrane. Membrane was blocked and incubated with anti-SEMA3E or SEMA5A antibody overnight. Membrane was placed in secondary antibody for one hour and detected by chemifluorescence using ECF Western Blotting Reagents (GE Healthcare).
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Quantitative real-time RT-PCR

Briefly, one microgram of RNA from each sample was used in a 100 µl reaction to generate cDNA using High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). PCR primers used were designed by Primer Express Version 2.0 (Applied Biosystems) software. The primer pairs for Sema3E are 5'-GGGAAAGATGCGGGTGAA-3' and 5' TATAGTGATGAAAAACCCGAACA-3'. A volume of 0.5 µl of cDNA was used in a 20-µl PCR reaction containing the appropriate primers and 1x SYBR Green PCR master mix (PE Applied Biosystems). Each sample was run in triplicate, and each PCR experiment included three no-template negative control wells. The comparative Ct method (PE Applied Biosystems) was used to determine the relative ratio of gene expression for each gene corrected using 18S rRNA and referenced to normal cerebellum RNA (Ambion).

Immunohistochemistry

Sections were deparaffinized and rehydrated. Sections were placed in pre-warmed Reveal (Biocare) at 121°C for 3 min, 95°C for 1 min and cooled back to room temperature for antigen retrieval. Sections were then blocked (Background Sniper reagent; Biocare Medical) and incubated with anti-human Sema3E or anti-human Sema5A polyclonal antibody overnight at 4°C. A 5 micron section was placed in 4% paraformaldehyde then permeabilized in 0.1% Triton-X for 15 min. Sections were then stained with goat anti-rabbit Cy3-conjugated IgG (Perkin Elmer; 1:400 dilution). Anti-human CD31 monoclonal antibody was used to stain endothelial cells and then detected with goat anti-mouse Cy5-conjugated IgG (Perkin Elmer; 1:50 dilution) for one hour. Cell nuclei were stained with SlowFade Gold anti-fade reagent containing blue-fluorescent nuclear counterstain DAPI (Invitrogen).

Results

The expression values of 17 semaphorin genes were extracted from the expression profile of 21 pediatric PA samples and 3 normal cerebellar samples (deposited at NCBI Gene Expression Omnibus with accession number GSE 12907). The ratio between the average of the expression level of 17 semaphorin genes in PA samples and 3 normal cerebella samples were calculated (Figure 1). Figure 1 shows that Sema3E and Sema5A were highly up-regulated in PA samples in comparison to normal cerebella.
The fold increases for Sema3E and Sema5A were more than 6-fold and 7-fold respectively.

Previously, we have confirmed the up-regulation of Sema5A by quantitative real-time RT-PCR. Here, quantitative real-time RT-PCR was used to confirm up-regulation of Sema3E. Using one of the normal cerebellar samples (NcAb) as reference, the fold increase of Sema3E varied from 1.5-fold to more than 29-fold (Table 1). However, PA705, which was derived from the spinal cord, did not show any up-regulation of Sema3E.

Protein expression of Sema5A and Sema3E were evaluated by both western blot and immunostaining. In consonance with gene expression, higher levels of Sema5A and Sema3E protein expression were detected in comparison to normal cerebella tissues (Figure 2). The specificity of anti-CD31 and anti-Sema5A were tested on PA paraffin sections (Figure 3). To test the co-localization of CD31 and Sema5A (or Sema3E), co-immunofluorescent staining was performed (Figure 4 and Figure 5). Merging of CD31-positive and Sema3E-positive images showed overlapping regions, specifically high expression of Sema3E was seen where CD31 expression was also high (Figure 4). On the other hand, Sema5A expression, in comparison to Sema3E, was not localized to any specific area; rather expression was seen in the majority of the PA tissue. However, the expression of Sema5A was as robust as Sema3E (Figure 5).

### Discussion

PA, in general, has a very favorable prognosis with almost 100% 5-year survival rates after surgical resection and PA rarely undergoes malignant transformation. However, inoperable tumors will need chemotherapy and/or radiotherapy as the first line of treatment. For tumors that are incompletely resected, adjuvant therapy will subsequently be needed. Current chemotherapy and radiotherapy can have significant adverse effect. Thus, new targeted therapies with minimal toxicity will be desirable. Since the MAPK pathway appears to be activated either through NF-1 lost or the presence of gene fusion between KIAA1549 and BRAF genes or other mutations in PA, several phase 1 and 2 clinical trials targeting the MAPK pathway for PA are ongoing (NCT01089101, NCT02285439). Recent data suggests that Selumetinib (a MEK inhibitor) may be effective and safe for the treatment of recurrent or refractory PA harboring BRAF V600E or BRAF-KIA1549 fusion. The identification of up-regulation of Sema3E in this study may be related to the activation of MAPK in PA. A recent study of treating SEM3E-overexpressing pancreatic cancer cells with a MEK inhibitor (trametinib) resulted in a significantly number of cells in G1 phase than cells without SEM3E overexpression. The result suggests that MAPK pathway could be one of the mechanisms involved in the proliferation of SEM3E-overexpressing pancreatic cancer cells. If so, SEM3E could be a potential biomarker to predict MEK inhibitor sensitivity.

Here we have demonstrated that Sema3E and Sema5A are highly expressed in pediatric PA tissue at both mRNA and protein level. More interestingly, a higher expression of Sema3E protein was associated with CD31 positive endothelial cells. Higher expression of Sema3E on endothelial cells in PA tissue could suggest its role in angiogenesis. Although PA is characterized by high vascularity, factors involved in angiogenesis have not been previously elucidated.

<table>
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<tr>
<th>Tissue</th>
<th>SEMA3E</th>
<th>SEMA5A</th>
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<tbody>
<tr>
<td>Normal Cerebellar Tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>739 Nc</td>
<td>0.05 ± 0.59</td>
<td>1.92 ± 0.07</td>
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<tr>
<td>321 Nc</td>
<td>0.35 ± 0.55</td>
<td>3.08 ± 0.55</td>
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<tr>
<td>NcAb</td>
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<tr>
<td>PA Tissue</td>
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<tr>
<td>110T</td>
<td>4.02 ± 0.06</td>
<td>49.64 ± 0.36</td>
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Figure 2. Western Blot of Sema3E and Sema 5A. Nc, normal cerebellar samples; PA, pilocytic astrocytoma samples. The protein Sema3E and 5A expression in the blots were quantified with ImageJ software and normalized to the corresponding Beta-tubulin expression. The normalized expression was used to calculate the fold change in each of the PA samples in comparison to the normal cerebellar sample 5601C.

Figure 3. Immunostaining of CD31 and Sema3E in PA samples (original magnification 100X). Higher expression of Sema3E around large blood vessel.
**Figure 4.** Immunofluorescence staining of CD31 and Sema3E in three independent PA samples. First column shows CD31 expression (green) with cell nuclei stained with DAPI (blue). Second column shows Sema3E (red) with DAPI. The last column shows merged images of CD31 and Sema3E.

**Figure 5.** Co-immunofluorescent staining of CD31 and Sema5A in three independent PA samples. First column shows CD31 expression (green) with cell nuclei stained with DAPI (blue). Second column shows Sema5A (red) with DAPI. The last column shows merged images of CD31 and Sema5A.
Tumor angiogenesis is essential for tumor growth and PA is also characterized by high microvessel density counts than anaplastic astrocytoma and has wider vessels than glioblastoma. A Phase II clinical trial of Bevacizumab (an anti-angiogenesis agent targeting vascular endothelial growth factor, VEGF) plus irinotecan has been tested in 35 recurrent pediatric low-grade glioma patients including 16 PA. This regimen appeared to produce sustained disease control in some children with recurrent low-grade glioma including one recurrent thalamic PA. Previous study has shown that Sema3E can promote endothelial cell migration and tumor growth by angiogenesis. In addition, Sema3E can also suppress tumor cell death triggered by the plexin D1 dependence receptor in metastatic breast cancers and promote pancreatic cancer cell growth. Thus, targeting the Sema3E-Plexin D1 axis should be explored as a novel anti-angiogenesis therapy strategy for recurrent PA.

High level of Sema5A expression in PA might suggest its role in tumor development. Studies have shown high expression of Sema5A during development of the embryo. Inactivation of this gene has also been shown to cause defective cranial vascular system and early embryonic death. Secreted Sema5A has been shown to increase endothelial cell proliferation. Sema5A has also been studied in stages of development, where it has been found that the expression is highest during the peak of embryonic development, following which the expression then decreases gradually.

Based on the known function of members of the semaphorin family and the expression patterns of Sema3E and Sema5A in PA, we believe that Sema3E and Sema5A may be involved in blood vessel formation in PA and therefore contributing to angiogenesis. Sema3E and Sema5A could serve as potential therapeutic targets for treatment of PA. These observations could provide insight into potential functions associated in tumor progression. Further studies that include disrupting the expression of both genes and observing changes in vascular patterns, proliferation and migration are warranted.

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**Conflict of interest**

The authors declare no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

**References**


