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Podocalyxin expression in malignant astrocytic tumors

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ABSTRACT

Podocalyxin is an anti-adhesive mucin-like transmembrane sialoglycoprotein that has been implicated in the development of aggressive forms of cancer. Podocalyxin is also known as keratan sulfate (KS) proteoglycan. Recently, we revealed that highly sulfated KS or another mucin-like transmembrane sialoglycoprotein podoplanin/aggrus is upregulated in malignant astrocytic tumors. The aim of this study is to examine the relationship between podocalyxin expression and malignant progression of astrocytic tumors. In this study, 51 astrocytic tumors were investigated for podocalyxin expression using immunohistochemistry, Western blot analysis, and quantitative real-time PCR. Immunohistochemistry detected podocalyxin on the surface of tumor cells in six of 14 anaplastic astrocytomas (42.9%) and in 17 of 31 glioblastomas (54.8%), especially around proliferating endothelial cells. In diffuse astrocytoma, podocalyxin expression was observed only in vascular endothelial cells. Podocalyxin might be associated with the malignant progression of astrocytic tumors, and be a useful prognostic marker for astrocytic tumors.

Astrocytic tumors are the most common tumors of the central nervous system (CNS) and are categorized into diffuse astrocytomas (World Health Organization (WHO) Grade II), anaplastic astrocytomas (WHO Grade III), and glioblastomas (WHO Grade IV) [1]. Glioblastoma may occur de novo or may result from progression of low-grade astrocytomas [2]. Molecular mechanisms of malignant progression are associated with inactivation of tumor suppressor genes such as p53-Rb pathway or overexpression of oncogenes such as epidermal growth factor receptor (EGFR) [3]. However, the mechanisms of malignant progression of astrocytic tumors have not been resolved completely. Identification of genes that are expressed differentially in high-grade or low-grade astrocytomas is important to elucidate the molecular mechanisms of malignant progression and to develop novel therapeutic strategies.

Podocalyxin is a type I transmembrane sialoglycoprotein, which belongs to the CD34 family. Podocalyxin is expressed on the surface of various normal cells, including kidney podocytes, vascular endothelial cells, hematopoietic stem cells, and platelets [4–7]. The physiological function of podocalyxin is as an anti-adhesion molecule that maintains open filtration pathways between neighboring podocyte foot processes through the charge-repulsive effects of its large, highly sialylated and sulfated extracellular domain [8]. In canine kidney (MDCK) cells, podocalyxin overexpression leads to the inhibition of cell–cell interaction as shown by decreased cell–cell adhesion, decreased tight junction-dependent transepithelial resistance, and redistribution of cell junction proteins [9]. Decreased cell–cell interaction is a prominent feature of cancer cells that display a metastatic phenotype, suggesting a possible role for anti-adhesive molecules, such as podocalyxin, in cancer progression. The role of podocalyxin in cancer remains unclear, although its expression has been reported in breast, liver, pancreas, kidney, prostate, testis, and blood cell cancers [10–16]. However, podocalyxin has been implicated in the development of aggressive forms of cancer. Increased podocalyxin protein expression is correlated with poor outcome in breast carcinomas, and is implicated in more aggressive forms of prostate cancer [10,14]. Furthermore, podocalyxin variants were found to be associated with both the risk of prostate cancer and prostate tumor aggressiveness. Recently, podocalyxin was reported to increase the aggressive phenotype of breast and prostate cancer in vitro through its interaction with ezrin [17]. Thus, podocalyxin is a candidate for playing a critical role in cancer aggressiveness and malignancy. Podocalyxin is also reported to be useful to differentiate pancreatic ductal adenocarcinomas from adenocarcinomas of the biliary and gastrointestinal tracts [12].

Recently, we showed that the expression of highly sulfated keratan sulfate (KS) recognized by 5D4 antibody is increased in

Abbreviations: KS, keratan sulfate; CNS, central nervous system; WHO, World Health Organization.

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parallel with increasing malignancy of astrocytic tumors [18,19]. KS expression is induced by high expression of five glycogenes involved in KS synthesis. However, in addition to high expression levels of glycogenes involved in KS synthesis, core proteins of KS proteoglycan also might contribute to the high expression of KS. Podocalyxin was recently identified as a KS proteoglycan [20]. In this study, 51 astrocytic tumors (six diffuse astrocytomas, 14 anaplastic astrocytomas, and 31 glioblastomas) were investigated using immunohistochemistry and Western blot with an anti-podocalyxin antibody. Furthermore, we investigated the podocalyxin transcript levels using quantitative real-time PCR in 51 frozen astrocytic tumors.

Materials and methods

Tissue samples. Tumor specimens were obtained during surgery from six patients with diffuse astrocytomas, 14 patients with anaplastic astrocytomas, and 31 patients with glioblastomas [19,21]. Informed consent had been obtained previously from patients or their guardians. The histology of these tissue samples was confirmed by experienced neuropathologists.

Immunohistochemical analysis. Specimens were deparaffinized, rehydrated and incubated first with goat anti-human podocalyxin (2 μg/ml) at 4 °C for 18 h, then with biotin-conjugated secondary anti-goat IgG antibody (Dako, Glostrup, Denmark) for 1 h, and finally with peroxidase-conjugated biotin-streptavidin complex (Vectastain ABC Kit; Vector Laboratories Inc., Burlingame, CA) for 1 h. Color was developed using 3, 3-diaminobenzidine tetrahydrochloride tablet sets (Dako) for 3 min. KS expression was assessed semi-quantitatively from the percentage of tumor cells with cytoplasmic/membrane staining: 0, no staining; +, <10%; ++, 10–50%; and +++, >50%.

Western blot analysis. The tissues were lysed with lysis buffer (25 mM Tris (pH 7.4), 50 mM NaCl, 0.5% Na deoxycholate, 2% Nonidet P-40, 0.2% SDS, 1 mM phenylmethylsulfonyl fluoride, and 50 mg/ml aprotinin) [19,21]. Samples of the supernatant fraction were collected after centrifuging at 15,000g for 30 min. Four micrograms of the proteins were electrophoresed under reducing conditions on 10% polyacrylamide gel (Atto Bioscience, Tokyo, Japan). The separated proteins were transferred to a PVDF membrane. After blocking with 3% skim milk in PBS with 0.05% Tween 20, the membrane was incubated with goat anti-human podocalyxin (0.1 μg/ml; R&D Systems, Minneapolis, MN) or anti-β-actin antibody (1/5000 dilution; Sigma, St. Louis, MO), and subsequently with peroxidase-conjugated anti-goat or anti-mouse antibodies (1/5000 dilution; Bio-Rad Laboratories Inc., Hercules, CA). It was then developed for 1 min with ECL reagents (Amersham Pharmacia Biotech Inc.) using Amersham Hyperfilm ECL (Amersham Pharmacia Biotech Inc.).

Quantitative real-time PCR analysis. Total RNAs were prepared from 51 astrocytic tumors (six diffuse astrocytomas, 14 anaplastic astrocytomas, and 31 glioblastomas) using an RNeasy mini prep kit (Qiagen Inc., Hilden, Germany). The initial cDNA strand was synthesized using SuperScript III transcriptase (Invitrogen Corp., Carlsbad, CA) by priming an oligo-dT primer according to the manufacturer's instructions. We performed PCR using oligonucleotides: human podocalyxin sense (5′-acaggaaacaccctctgtgc-3′) and human podocalyxin antisense (5′-gaaggtggctttgactgctc-3′). Real-time PCR was carried out using the QuantiTect SYBR Green PCR (Qiagen Inc.). The PCR conditions were 95 °C for 15 min (1 cycle), followed by 40 cycles of 94 °C for 15 s, 53 °C for 20 s, and 72 °C for 10 s. Subsequently, a melting curve program was applied with continuous fluorescence measurement. A standard curve for podocalyxin templates was generated through serial dilution of PCR products (1×10^8 to 1×10^2 copies/ μl). The expression level of podocalyxin was normalized by total RNA weights. The statistical significance of podocalyxin mRNA expression in astrocytic tumor tissues was determined using paired *t* tests.

Statistical analyses. Results are expressed as the mean ± standard deviation. Student's *t*-test was used to determine significance among the groups. A value of $p < 0.05$ was considered significant.

Results

Immunohistochemical staining for podocalyxin in malignant astrocytic tumors

The cellular distribution of podocalyxin in astrocytic tumors was examined immunohistochemically using goat anti-human podocalyxin polyclonal antibody. This polyclonal antibody was produced in goats immunized with recombinant human podocalyxin extracellular domain (23–425 a.a.), and purified using podocalyxin affinity chromatography. In many recent studies, this antibody was applied to the immunohistochemistry, Western blot, and immunoprecipitation, indicating that this antibody is specific to human podocalyxin [20,22,23]. Herein, we used 51 surgical tissue samples (six diffuse astrocytomas: Grade II, 14 anaplastic astrocytomas: Grade III, and 31 glioblastomas: Grade IV) for immunohistochemistry. Podocalyxin immunoreactivity was detected in six of 14 (42.9%) anaplastic astrocytomas and in 17 of 31 (54.8%) glioblastomas; staining was graded as +++ in seven glioblastoma and as ++ in three glioblastoma cases (Table 1). Podocalyxin was not detected on tumor cell surfaces in diffuse astrocytomas, yet was observed in vascular endothelial cells in these specimen (Fig. 1A and B). Representative staining for podocalyxin in astrocytic tumor samples is shown in Fig. 1. Immunostaining for podocalyxin demonstrated predominantly cell-surface patterns. In anaplastic astrocytoma, the tumor cell surface was stained using anti-podocalyxin (Fig. 1C and D). In glioblastomas, podocalyxin-positive tumor cells were prominent around microvascular proliferations (Fig. 1E and F). Proliferating endothelial cells were also positive for podocalyxin (Fig. 1F).

Analysis of podocalyxin expression using Western blot in astrocytic tumors

To confirm the podocalyxin expression in astrocytic tumors, lysates of frozen tumor specimens from 51 patients were analyzed using Western blot analysis with anti-podocalyxin antibody. As shown in Fig. 2, podocalyxin was highly detected in extracts of anaplastic astrocytoma and glioblastoma. Samples of one of 14 anaplastic astrocytomas (7.1%) and seven of 31 glioblastomas (22.6%) showed relatively high levels of podocalyxin, while expression in five of 14 anaplastic astrocytomas (35.7%) and 11 of 31 glioblastomas (35.5%) was more moderate. The other astrocytic tumors including diffuse astrocytic tumors produced weak immunoreactive bands, since podocalyxin is expressed in all vascular endothelial cells (Fig. 1).

Table 1
Results of podocalyxin immunostaining in 51 patients with astrocytic tumors

Tumor type	No. of cases	Podocalyxin				Positive rate
		+++	++	+	–	
Diffuse astrocytoma (grade II)	6	0	0	0	6	0%
Anaplastic astrocytoma (grade III)	14	0	1	5	8	42.9%
Glioblastoma (grade IV)	31	7	3	7	14	54.8%

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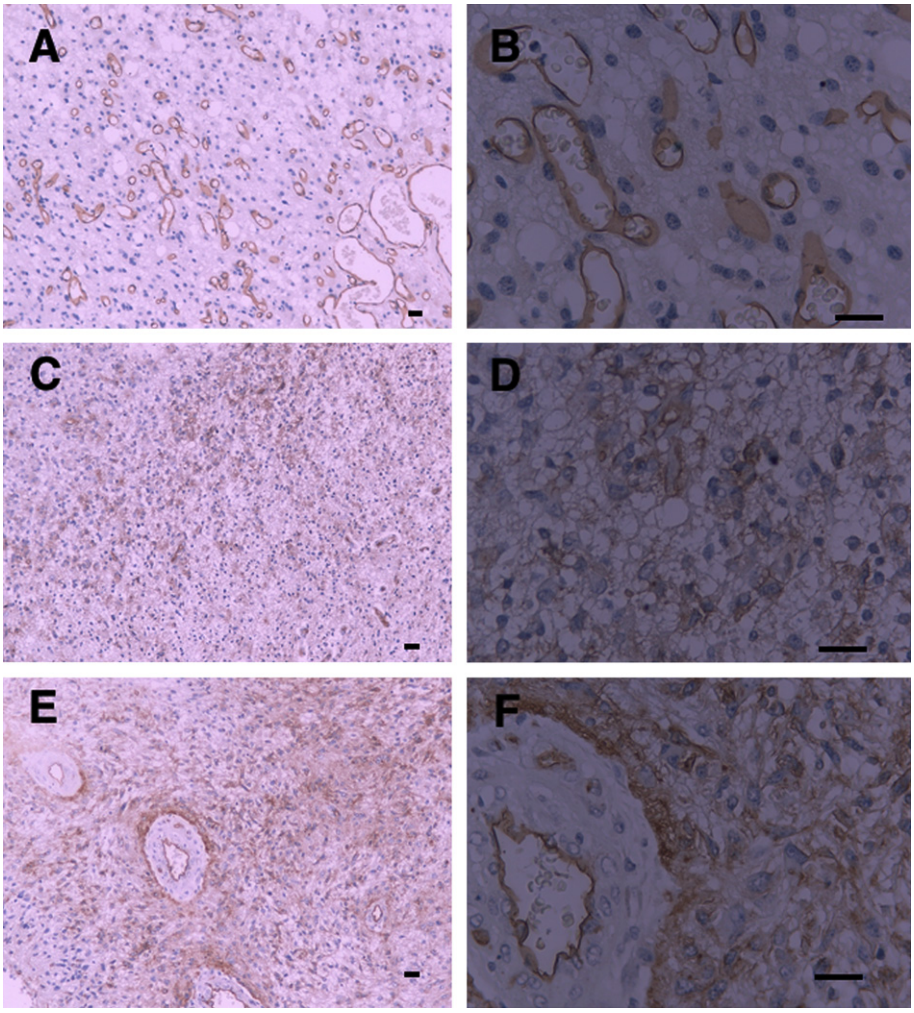

Fig. 1. Immunohistochemical detection of podocalyxin in astrocytic tumors. Podocalyxin was not detected on tumor cell surfaces in diffuse astrocytomas; however, podocalyxin staining was observed in vascular endothelial cells, although as shown in this figure few endothelial cells are usually detected in diffuse astrocytomas (A: 100×, B: 400×). In anaplastic astrocytoma, the tumor cell surface was stained positively (C: 100×, D: 400×). Accentuated staining is visible around an area of microvascular proliferation in glioblastoma (E: 100×, F: 400×). Bar, 10 μm.

Differential expression of the podocalyxin mRNA in astrocytic tumors

To quantify the expression of podocalyxin mRNA in human astrocytic tumors of different grades, we performed quantitative real-time PCR analyses of astrocytic tumors from 51 patients. The relative podocalyxin mRNA expression levels of each tumor grade are shown in Fig. 3. Average copy numbers of podocalyxin mRNA/μg total RNA in diffuse astrocytomas, anaplastic astrocytomas, and glioblastomas were 535 ± 289, 918 ± 595, and 3670 ± 2916, respectively. Podocalyxin transcript levels were significantly higher in glioblastomas than in diffuse astrocytomas or anaplastic astrocytomas ($p < 0.01$).

Discussion

Podocalyxin expression has been reported to increase the aggressive phenotype of breast and prostate cancer, and be correlated with their poor prognosis [10,14,17]. Recently, we showed that highly sulfated keratan sulfate detected by 5D4 antibody is upregulated in accordance with malignancy of astrocytic tumors [19]. We speculated that expression of core proteins of KS proteoglycan is upregulated in malignant astrocytic tumors. Podocalyxin was recently identified as a keratan sulfate (KS) proteoglycan: podocalyxin has the keratan sulfate antigens TRA-1-60 and TRA-1-81, which are also known as human pluripotent stem cell markers, in embryonal carcinoma [20]. Furthermore, podocalyxin expression correlates with tumor aggressiveness or malignancy; therefore, we herein investigated the podocalyxin expression in brain tumors, focusing on astrocytic tumors of different grades.

In this study, we first investigated the podocalyxin expression by immunohistochemistry (Fig. 1), and showed significantly different podocalyxin expression between anaplastic astrocytomas (Grade III; 42.9%) and glioblastoma (Grade IV; 54.8%; $p < 0.05$). Using Western blot analysis and real-time PCR, we confirmed this result (Figs. 2 and 3). Taken together, these results indicate that podocalyxin expression might be associated with the malignant progression of astrocytic tumors. We previously investigated the expression of podoplanin, another mucin-like sialoglycoprotein, which is associated with malignant progression of astrocytic tumors [21,24,25]. Podoplanin was detected on the cell surface in 27% of anaplastic astrocytomas and 47% of glioblastomas. In contrast, podoplanin expression was not observed in diffuse astrocytomas. Because both podocalyxin and podoplanin are associated with the malignancy of astrocytic tumors, we investigated whether podocalyxin expression is correlated with podoplanin; however, we found no correlation between the expression levels of the two sialoglycoproteins (data not shown). We might be able to predict the malignancy of astrocytic tumors if we appropriately combine

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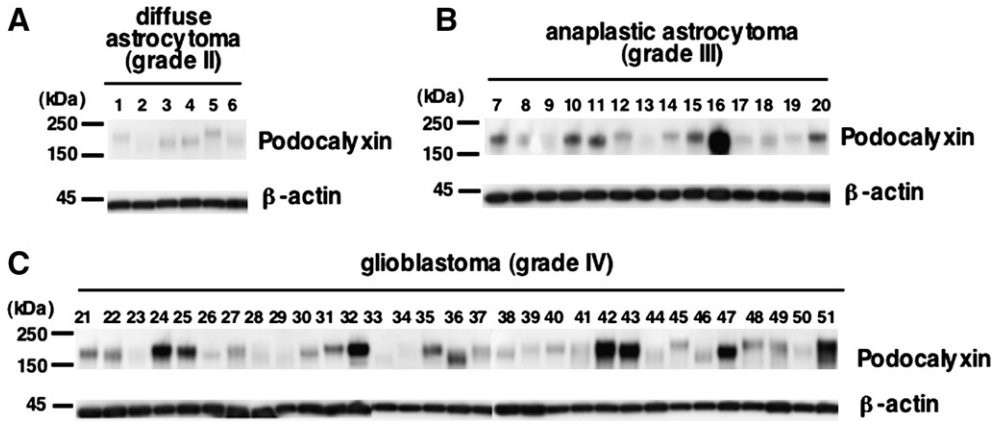

Fig. 2. Western blot analysis of podocalyxin expression in astrocytic tumors. Tissues from diffuse astrocytomas (A: lane 1–6), anaplastic astrocytomas (B: lane 7–20), and glioblastomas (C: lane 21–51) were solubilized, and 4 μg of the proteins were electrophoresed under reducing conditions on 10% polyacrylamide gel. The separated proteins were transferred to a PVDF membrane. After blocking with 3% skim milk in PBS, the membrane was incubated with anti-podocalyxin (upper panel) or anti-β-actin antibody (lower panel).

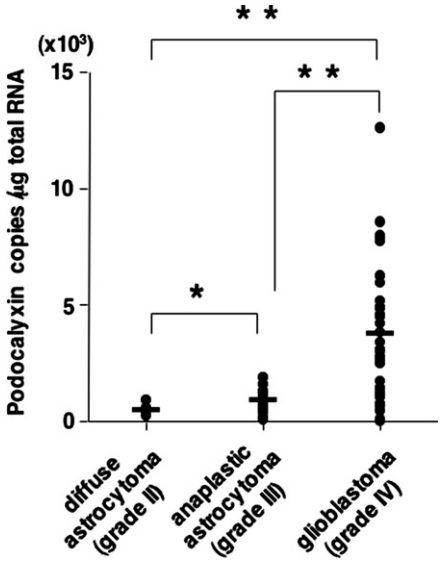

Fig. 3. Quantitative real-time PCR analysis of podocalyxin in astrocytic tumors. The transcript levels for a podocalyxin gene in 51 astrocytic tumors (six diffuse astrocytomas, 14 anaplastic astrocytomas, and 31 glioblastomas) were measured using real-time PCR. Values normalized to the level of total RNA are presented. $^{**}p < 0.01$, $^{*}p < 0.05$.

the expression level of these several molecules. Further study is needed to clarify the pathophysiological function of podocalyxin in astrocytic tumors, such as invasiveness or angiogenesis, using more clinical samples.

In summary, we investigated the expression of podocalyxin in 51 astrocytic tumors using immunohistochemistry, Western blot, and real-time PCR analyses. Podocalyxin expression was not observed in diffuse astrocytoma except in vascular endothelial cells. Furthermore, podocalyxin mRNA and protein expression were markedly higher in glioblastomas than in anaplastic astrocytomas. These data suggest that podocalyxin expression is associated with malignancy of astrocytic tumors, and might be useful as a prognostic factor.

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