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ORIGINAL PAPER

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Increased expression of podoplanin in malignant astrocytic tumors as a novel molecular marker of malignant progression

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Abstract Podoplanin (aggrus) is a mucin-like transmembrane sialoglycoprotein that is expressed on lymphatic endothelial cells. Podoplanin is putatively involved in cancer cell migration, invasion, metastasis, and malignant progression and may be involved in platelet aggregation. Previously, we showed upregulated expression of podoplanin in central nervous system (CNS) germinomas, but not in non-germinomatous germ cell tumors, except for parts of immature teratomas in limited numbers. However, little information exists about its role in CNS astrocytic tumors. In this study, 188 astrocytic tumors (30 diffuse astrocytomas, 43 anaplastic astrocytomas, and 115 glioblastomas) were investigated using immunohistochemistry with an anti-podoplanin antibody, YM-1. In 11 of 43 anaplastic astrocytomas (25.6%) and in 54 of 115 glioblastomas (47.0%), podoplanin was expressed on the surface of anaplastic astrocytoma cells and glioblastoma cells, especially around necrotic areas and proliferating endothelial cells. However, the surrounding brain parenchyma was not stained by YM-1. On the other hand, podoplanin expression was not observed in diffuse astrocytoma (0/30: 0%). Furthermore, we investigated the expression of podoplanin using quantitative real-time PCR and Western blot analysis in 54 frozen astrocytic tumors (6 diffuse astrocytomas, 14 anaplastic astrocytomas, and 34 glioblastomas). Podoplanin mRNA and protein expression were markedly higher in glioblastomas than in anaplastic astrocytomas. These data suggest that podoplanin expression might be associated with malignancy of astrocytic tumors.

Keywords Podoplanin · Astrocytoma · Glioblastoma · YM-1

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Introduction

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 (AA; WHO Grade III) and glioblastomas (GBM; WHO Grade IV) [11]. Among them, GBMs are the most frequent and most malignant type of astrocytic tumor. Despite advances in surgical techniques, radiation therapy, and adjuvant chemotherapy, their prognosis remains poor: the median survival time for patients with GBMs is only 1 year [2]. Glioblastoma may occur de novo or may result from progression of low-grade astrocytomas [4]. Molecular mechanisms of tumorigenesis and malignant progression are associated with the inactivation of tumor suppressor genes such as p53-Rb pathway or the overexpression of oncogenes such as epidermal growth factor receptor [10]. However, the mechanisms of tumorigenesis and progression of astrocytic tumors have not been resolved. Identification of genes that are expressed differentially in high-grade astrocytomas, low-grade tumors, or normal brain tissues is important to elucidate the molecular mechanisms of tumorigenesis and to develop novel therapeutic strategies.

Podoplanin was reported to be expressed in lymphatic endothelium and in tumor-associated lymphangiogenesis; also, podoplanin deficiency resulted in congenital lymphedema and impaired lymphatic vascular patterning [16]. Furthermore, expression of podoplanin has been

shown to be upregulated in skin squamous cell carcinoma [12], lung squamous cell carcinoma [9], malignant mesothelioma [14], Kaposi's sarcoma, angiosarcoma [1], hemangioblastoma [15], testicular seminoma [7, 8], and dysgerminoma [17].

We have recently shown that podoplanin is overexpressed in CNS germinomas, but not in non-germinomatous germ cell tumors, except in a limited number of immature teratomas with partial positive reactivity [13]. In adult non-neoplastic CNS, podoplanin was evident in the subependymal areas, the leptomeninges, choroid plexus, ependyma, and Purkinje cells [15, 17]. However, podoplanin expression in CNS astrocytic tumors has not been studied intensively. In this study, we investigated podoplanin expression in 188 astrocytic tumors.

Materials and methods

Tissue samples

Tumor specimens were obtained during surgery from eight patients with diffuse astrocytomas, 14 patients with anaplastic astrocytomas, and 34 patients with glioblastomas. Informed consent had been obtained previously from patients or their guardians. The tumor specimens were routinely fixed in 10% buffered formalin for 18–20 h at room temperature and processed to paraffin. Sections (5 μm thick) were cut and attached to poly-L-lysine-coated glass slides. Hematoxylin-eosin was used for routine staining. Tissue microarrays of 132 astrocytic tumors (22 diffuse astrocytomas, 29 anaplastic astrocytomas, and 81 glioblastomas) were purchased from Cybrdi, Inc. (Frederick, MD). The histology of these tissue samples was confirmed by experienced neuropathologists.

Immunohistochemical analysis

Specimens were deparaffinized, rehydrated, and incubated first with YM-1 (1/100 diluted; Medical Biological Laboratories Co., Ltd, Nagoya, Japan) at room temperature for 1 h, then with biotin-conjugated secondary anti-rat IgG antibody (DakoCytomation, 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 (DakoCytomation) for 3 min. Podoplanin 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

Tissues were solubilized 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). They were then electrophoresed under reducing conditions on 10–20% polyacrylamide gels (Daiichi Pure Chemicals Co., Ltd, Tokyo, Japan). The separated proteins were transferred to a nitrocellulose membrane. After blocking with 4% skim milk in PBS, the membrane was incubated with YM-1 (1/500 diluted) or anti-β-actin antibody (1 μg/ml; Sigma Chemical Co., St. Louis, MO), and then with peroxidase-conjugated secondary antibodies (1/1,000 diluted; Amersham Pharmacia Biotech UK Ltd, Buckinghamshire, UK). The proteins were subsequently developed for 3 min using ECL reagents (Amersham Pharmacia Biotech) using X-Omat AR film (Eastman Kodak Co.).

Quantitative real-time PCR

Total RNAs were prepared from frozen sections that have been obtained from astrocytic tumor patients, employing an RNeasy mini prep kit (Qiagen, Inc., Hilden, Germany). The initial cDNA strand was synthesized using SuperScript III transcriptase (Invitrogen Co., Carlsbad, CA) by priming nine random oligomers and an oligo-dT primer according to the manufacturer's instructions. We performed PCR using oligonucleotides: human podoplanin sense (5′-GGAAGGTGTCAGCT CTGCTC-3′) and human podoplanin antisense (5′-CG CCTTCCAAACCTGTAGTC-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, 72°C for 10 s. Subsequently, a melting curve program was applied with continuous fluorescence measurement. A standard curve for podoplanin templates was generated through serial dilution of PCR products (1×10^8–1×10^2 copies/μl). The expression level of podoplanin was normalized by total RNA weights. The statistical significance of podoplanin mRNA expression in astrocytic tumor tissues was determined using paired *t* tests.

Results

Immunohistochemical staining for podoplanin in malignant astrocytic tumors

The cellular distribution of podoplanin in astrocytic tumors was examined immunohistochemically using anti-podoplanin antibody, YM-1, which can strongly recognize podoplanin [6, 13]. In this study, we used 56 surgical tissues (8 diffuse astrocytomas, 14 anaplastic astrocytomas, and 34 glioblastomas). Podoplanin immunoreactivity was detected in 5 of 14 (35.7%) anaplastic astrocytomas and in 18 of 34 (52.9%) glioblastomas; staining was graded as +++ in 16 glioblastomas and as ++ in two glioblastoma cases. We also stained other astrocytic tumors of tissue microarrays. Podoplanin was detected in 6 of 29 (21%) anaplastic astrocytomas and in 36 of 81 (44%) glioblastomas. In all, 11 of 43 anaplastic

astrocytomas (25.6%) and 54 of 115 (47.0%) glioblastomas were stained using YM-1 (χ^2, $P<0.05$; Table 1). Representative staining for podoplanin in glioblastoma samples is shown in Fig. 1. Immunostaining for podoplanin demonstrated predominantly cell surface patterns in glioblastoma cells (Fig. 1). In anaplastic astrocytoma, the tumor cell surface was stained using YM-1 (Fig. 1c, d). In glioblastomas, podoplanin-positive tumor cells were prominent around microvascular proliferations (Fig. 1e, f) and necrotic tissues (Fig. 1g). Proliferating endothelial cells were negative for podoplanin (Fig. 1e, f). Podoplanin was detected strongly in the plasma membrane of highly anaplastic multinucleated giant cells (Fig. 1h). In non-neoplastic areas of the brain (Fig. 1a) and in diffuse astrocytoma (Fig. 1b), podoplanin immunostaining was absent.

Podoplanin expression in malignant astrocytic tumors using Western blot analysis

To confirm immunohistochemical findings from astrocytic tumors, lysates of frozen tumor specimens from 54 patients (6 diffuse astrocytomas, 14 anaplastic astrocytomas, and 34 glioblastomas) were analyzed using Western blotting. As shown in Fig. 2, an antibody to podoplanin, YM-1, detected about 36-kDa proteins in extracts of malignant astrocytomas. Using YM-1, 6 of 14 anaplastic astrocytomas (42.8%) and 22 of 34 glioblastomas (64.7%) showed strong labeling (χ^2, $P<0.05$), while all diffuse astrocytomas were negative. Podoplanin expression detected by Western blot analysis was closely correlated with the results of immunohistochemistry.

Differential expression of podoplanin mRNA in astrocytic tumors

To quantify the expression of podoplanin mRNA in human astrocytic tumors of different grades, we performed quantitative real-time PCR analyses of astrocytic tumors from 54 patients (6 diffuse astrocytomas, 14 anaplastic astrocytomas, and 34 glioblastomas). The relative podoplanin mRNA expression levels of each tumor grade are shown in Fig. 3. Average copies of podoplanin mRNA/μg total RNA in diffuse astrocytomas (Grade II), anaplastic astrocytomas (Grade III), and glioblastomas (Grade IV) were 21.7±30.6, 16.1±35.4, and 411.2±511.7, respectively. Podoplanin transcript levels were significantly higher in glioblastomas than those in diffuse astrocytomas, anaplastic astrocytomas, or non-neoplastic human brain tissues ($P<0.01$).

Discussion

Immunohistochemical, Western blot, and real-time PCR analyses demonstrate that the expressions of podoplanin mRNA and protein are correlated with the malignant progression from anaplastic astrocytoma (Grade III) to glioblastomas (Grade IV). Of particular interest, 47.0% of highly invasive glioblastomas express podoplanin, whereas 25.6% of anaplastic astrocytomas and 0% of the less invasive diffuse astrocytomas (Grade II) express podoplanin by immunohistochemical staining on surgically resected and microarray tissues (Table 1). On the other hand, 6 of 14 anaplastic astrocytomas (42.8%) and 22 of 34 glioblastomas (64.7%) were strongly labeled by Western blot analyses using YM-1 (Fig. 2). Of all 188 astrocytic tumors analyzed immunohistochemically, 132 cases were derived from tissue microarrays whose tissue spots are small and, therefore, the percentage of podoplanin-positive tumors might have been underestimated. Furthermore, YM-1 detected podoplanin strongly by Western blot analysis, as described previously [6]. For these reasons, podoplanin-positive ratios in immunohistochemical analysis are inferred to be smaller than those of Western blot analysis, although the expression of podoplanin detected by immunohistochemistry was closely correlated with that by Western blot or real-time PCR analyses. The distribution of podoplanin-positive tumor cells was prominent around necrotic tissue and proliferating endothelial cells in glioblastomas (Fig. 1). Normal brain tissue surrounding the tumor bed was negative for podoplanin. Therefore, podoplanin expression was correlated with high tumor grades and aggressive histological behavior.

The biological functions of podoplanin remain largely unknown. In vascular endothelial cells, overexpression of T1α/podoplanin induces elongated cell extensions and considerably increases cell adhesion, migration, and tube

Table 1 Results of podoplanin immunostaining in 188 patients with astrocytic tumors

Tumor type	No. of cases	Podoplanin immunostaining				Positive rate (%)
		+++	++	+	–	
Diffuse astrocytoma	30	0	0	0	30	0
Surgical resection samples	8	0	0	0	8	0
Tissue microarray	22	0	0	0	22	0
Anaplastic astrocytoma	43	6	3	2	32	25.6
Surgical resection samples	14	3	1	1	9	35.7
Tissue microarray	29	3	2	1	23	20.7
Glioblastoma	115	39	10	5	61	47
Surgical resection samples	34	16	2	0	16	52.9
Tissue microarray	81	23	8	5	45	44

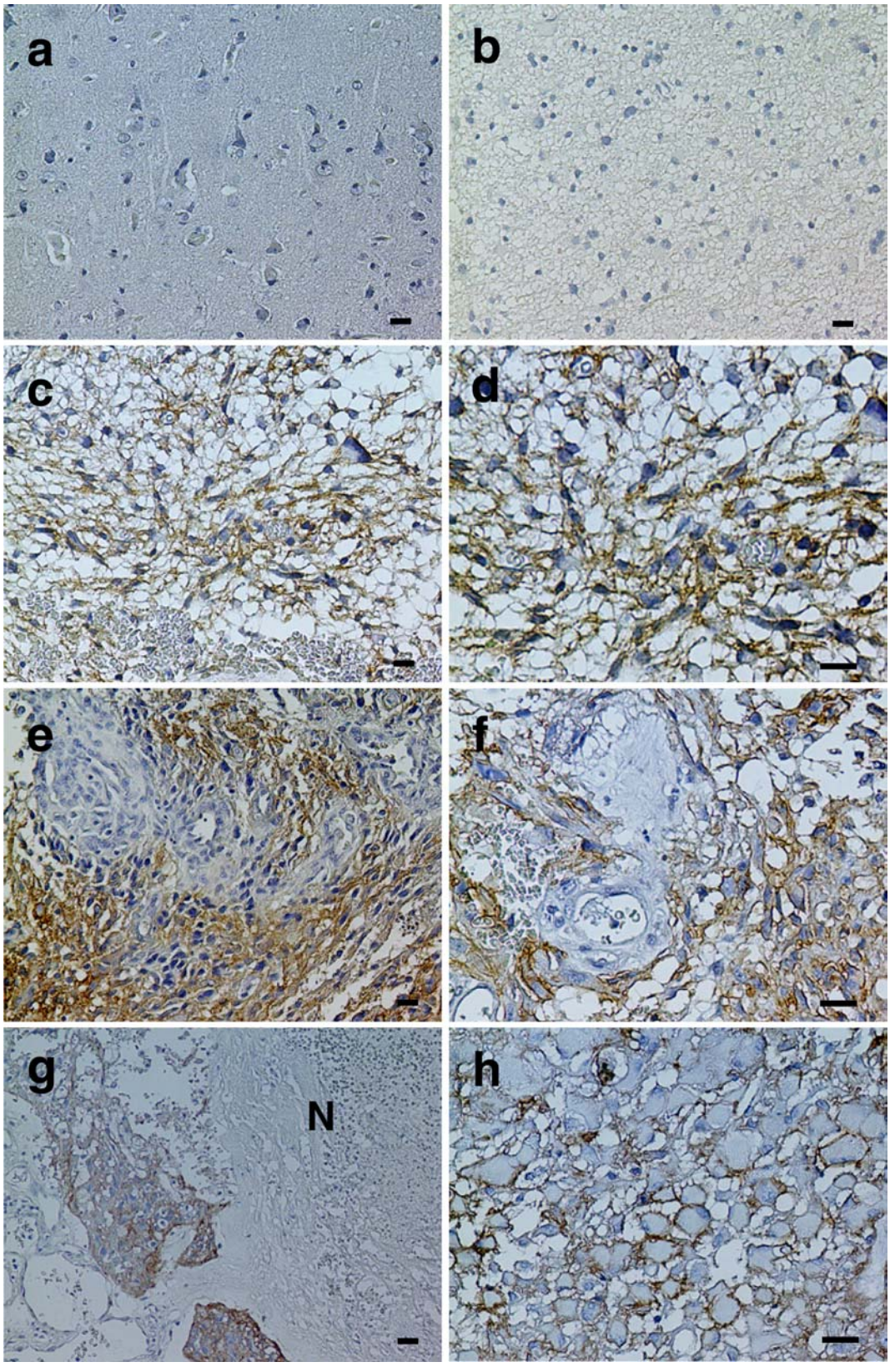

Fig. 1 Immunohistochemical detection of podoplanin in astrocytic tumors. No staining is apparent in a normal brain (**a**, ×200) and in diffuse astrocytoma (**b**, ×200). In anaplastic astrocytoma, the tumor cell surface was stained positively (**c**, ×200; **d**, ×400). Accentuated staining is visible around an area of microvascular proliferation in glioblastoma (**e**, ×200; **f**, ×400). Podoplanin immunostaining of glioblastoma cells at the necrotic area (*N*) (**g**, ×200) and in the plasma membrane of highly anaplastic multinucleated giant cells (**h**, ×400). *Bar* = 10 μm

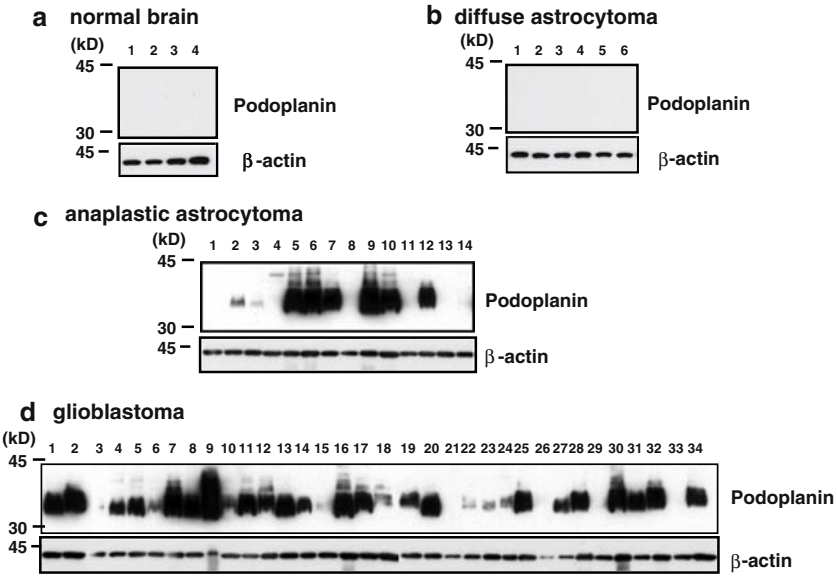

Fig. 2 Western blot analyses of podoplanin expression in astrocytic tumors. Tissues from normal brain (**a**), diffuse astrocytomas (**b**), anaplastic astrocytomas (**c**), and glioblastomas (**d**) were solubilized and immunoblotted using anti-human podoplanin monoclonal antibody YM-1 (*upper panel*) or anti-β-actin antibody (*lower panel*)

formation by promoting the rearrangement of the actin cytoskeleton [16]. PA2.26/podoplanin was identified as a cell surface protein induced in epidermal carcinogenesis and skin remodeling [18, 19]. Expression of PA2.26/podoplanin in pre-malignant keratinocytes induces a fully transformed and metastatic phenotype. Furthermore, human PA2.26/podoplanin has been found in the invasive front of oral squamous cell carcinomas, consistent with a role in tumor cell migration and invasion [12]. Moreover, a monoclonal antibody against gp44/aggrus/podoplanin inhibits pulmonary metastasis of a highly metastatic clone of mouse colon adenocarcinoma in vivo [21, 22]. In this study, we showed upregulated expression of podoplanin in CNS malignant astrocytic tumors. Recently, Shibahara et al. [20] also reported podoplanin expression in subsets of CNS tumors. However, the results obtained so far showed only associations between podoplanin expression and malignancy of astrocytic tumors, while its direct biological function in malignant astrocytomas remains to be established.

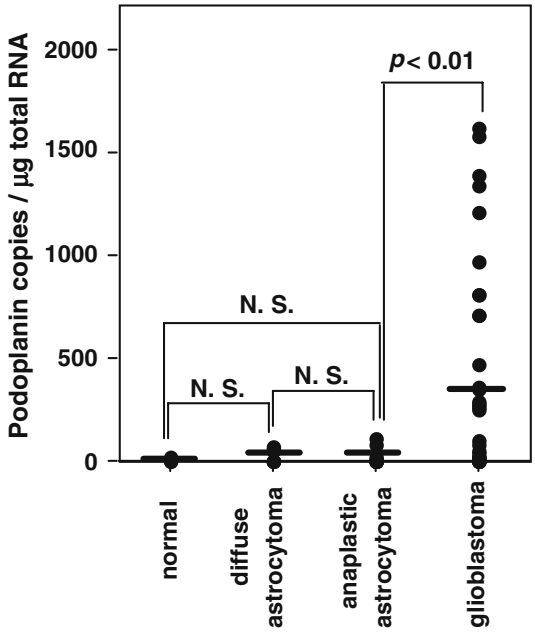

Fig. 3 Quantitative real-time PCR analysis of podoplanin transcripts in astrocytic tumors. First-strand cDNA samples derived from astrocytic tumor tissues of 54 patients (6 diffuse astrocytomas, 14 anaplastic astrocytomas, and 34 glioblastomas) and four normal brain tissues were used as real-time PCR templates. The respective expression levels of podoplanin were normalized to μg or total RNA, as described in Materials and methods

PA2.26/podoplanin was co-localized with ezrin, radixin, moesin family proteins, which are concentrated in cell surface projections, where they link the actin cytoskeleton to plasma membrane proteins [18]. Consistent with the association of podoplanin with ezrin, the latter's immunoreactivity is also associated with increasing malignancy of astrocytic tumors [3, 23]. The combination of podoplanin and ezrin might thus represent a possible tool for grading of astrocytic tumors.

Platelets play an important role in hemostasis and thrombosis and are also involved in tissue repair and tumor metastasis [5]. Glioblastoma is differentiated from low-grade astrocytomas based on the histological presence of tumor necrosis and associated microvascular proliferation [11]. Large necroses are attributable to insufficient blood supply and thrombosed tumor vessels are often observed. We speculate that the local platelet aggregation and thrombus formation might be increased by podoplanin-expressing malignant astrocytic tumor cells, resulting in tumor vessel obstruction and subsequent necrosis. Indeed, our unpublished results suggest that podoplanin expressed by glioblastoma cells induces platelet aggregation in vitro (data not shown).

In conclusion, podoplanin expression was markedly higher in glioblastomas than in anaplastic astrocytomas. Furthermore, podoplanin expression was not observed in diffuse astrocytoma. It will be intriguing to investigate the functional basis of the association between podoplanin expression and malignant progression of astrocytomas.

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