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Increased expression of highly sulfated keratan sulfate synthesized in malignant astrocytic tumors

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ABSTRACT

Keratan sulfate (KS) proteoglycans are expressed on a subpopulation of microglia in normal adult brain. We previously showed the up-regulated expression of KS in one of glioblastoma cell lines using anti-KS antibody (5D4). However, it has not been clarified whether KS is expressed in brain tumors and is involved in their malignancy. In this study, 54 astrocytic tumors were investigated about KS-expression using Western-blot with 5D4. In six of 14 anaplastic astrocytomas (43%) and 23 of 34 glioblastomas (68%), KS was detected by 5D4. KS was hardly detected by 5D4 in diffuse astrocytoma, suggesting that KS-expression is significantly expressed in malignant astrocytic tumors. In immunohistochemistry, KS is highly expressed in cell surface of malignant astrocytic tumors. Taken together, KS might be associated with the malignancy of astrocytic tumors, and be useful for a prognostic factor of astrocytic tumors.

Keratan sulfate (KS) proteoglycans (KSPGs) consist of different core proteoglycans that are individually glycosylated to varying degrees with keratan sulfate chains [1]. Theses keratan sulfates are known to be inhibitory to axonal growth. During development, sulfated keratans are temporally and spatially located in areas in which restricted axonal growth occurs. In the adult, KS is also found on a subpopulation of microglia throughout the brain; however, KS was not detected on astrocyte or oligodendrocyte [2]. KS consists of a linear polymer of *N*-acetyllactosamine, Galβ1-4GlcNAcβ1-3, which is sulfated at the C-6 positions of galactose (Gal) and *N*-acetylglucosamine (GlcNAc) [1]. Elongation of the carbohydrate backbone of KS chain is catalyzed by enzymes of two glycosyltransferase families: β1,3-*N*-acetylglucosaminyltransferase (β3GnT) and β1,4-galactosyltransferase (β4GalT). Sulfation of the chain is catalyzed by two carbohydrate sulfotransferases. Recent reports have described enzymes which are involved in KS synthesis [3–9]. Keratan sulfate Gal-6-sulfotransferase (KSGal6ST) is for sulfation of Gal. In addition, *N*-acetylglucosamine-6-*O*-sulfotransferase (GlcNAc6ST)-1 and GlcNAc6ST-5 (also known as CGn6ST) are responsible for sulfation of *N*-acetylglucosamine.

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) [10]. Despite advances in surgical techniques, radiation therapy, and adjuvant chemotherapy, their prognoses remain poor: the median survival time for patients with GBMs is only one year [11]. Glioblastoma may occur de novo or may result from progression of low-grade astrocytomas [12]. 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 (EGFR) [13]. However, the mechanisms of tumorigenesis and progression of astrocytic tumors have not been resolved completely. Identification of glycogenes or glycans 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.

In our recent study, we investigated the KS expression in glioblastoma cell lines using a monoclonal anti-KS antibody (5D4) and revealed KS is highly expressed in one of glioblastoma cell lines, LN229 [14]. Furthermore, we investigated the relationship

Abbreviations: KS, keratan sulfate; KSPGs, KS proteoglycans; Gal, galactose; GlcNAc, *N*-acetylglucosamine; KSGal6ST, keratan sulfate Gal-6-sulfotransferase; GlcNAc6ST, *N*-acetylglucosamine-6-*O*-sulfotransferase; β3GnT, β1,3-*N*-acetylglucosaminyltransferase; β4GalT, β1,4-galactosyltransferase.

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between expression of KS and glycogenes that are involved in KS synthesis using quantitative real-time PCR, and showed clearly that LN229 expresses a high level of KSGal6ST. However, it has not been clarified whether KS is expressed in astrocytic tumors and is involved in their malignancy. In this study, we investigated the KS-expression in astrocytic tumors of various malignancies using Western-blot and immunohistochemistry with 5D4, and discussed whether KS-expression is associated with the malignancy of 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 34 patients with glioblastomas. Informed consent had been obtained previously from patients or their guardians. Tissue microarrays of astrocytic tumors were purchased from Cybrdi, Inc. (Frederick, MD). The histology of these tissue samples was confirmed by experienced neuropathologists.

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) including protease inhibitor solution. Samples of the supernatant fraction were collected after centrifuging at 15,000*g* 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, the membrane was incubated with 5D4 (1/5000 dilution; Seikagaku Corp., Tokyo, Japan) or anti-β-actin antibody (1/5000 dilution; Sigma, St. Louis, MO), and subsequently with peroxidase-conjugated 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.).

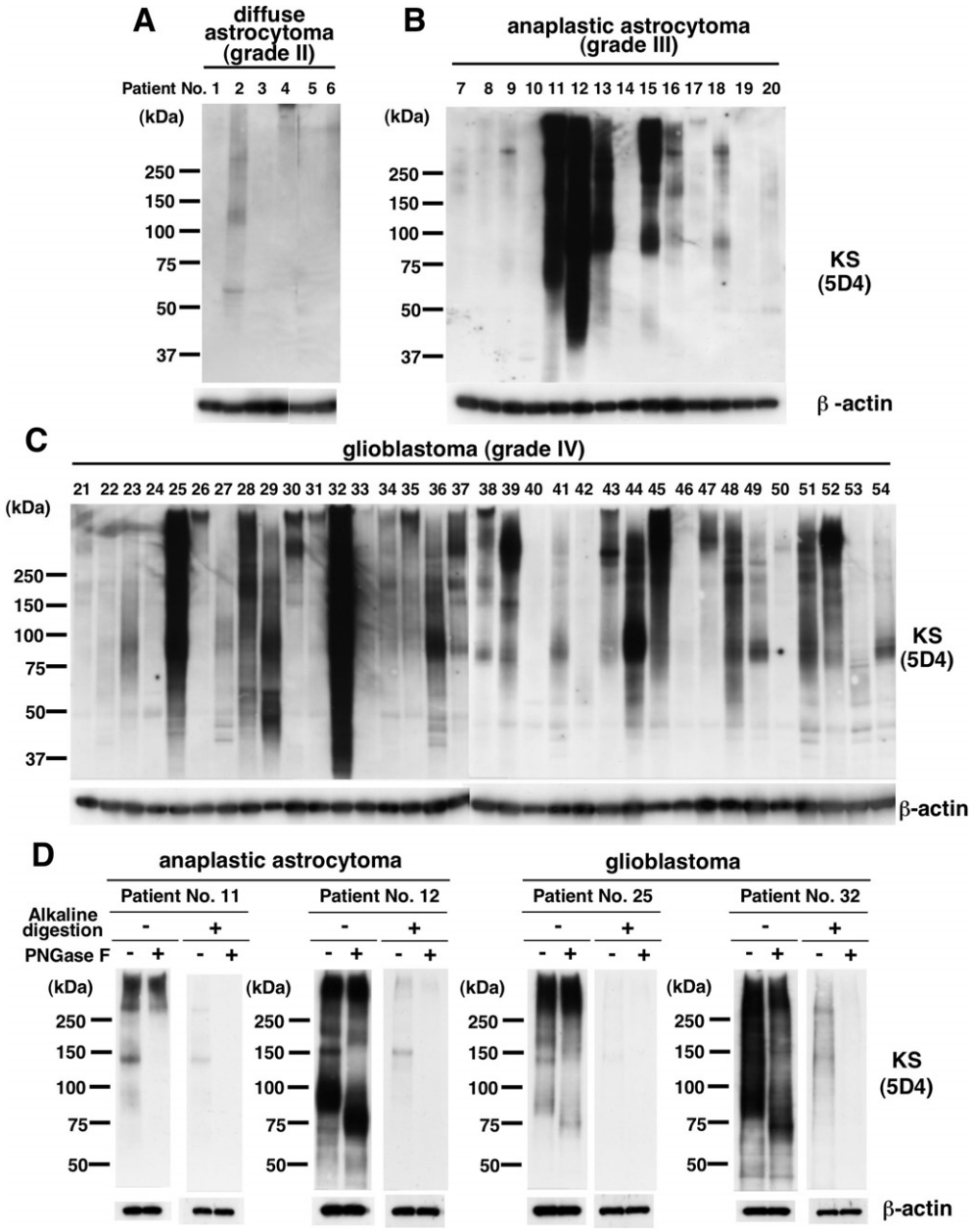

Fig. 1. Analysis of KS-expression in astrocytic tumors. Tissues from diffuse astrocytomas (A: lanes 1–6), anaplastic astrocytomas (B: lanes 7–20), and glioblastomas (C: lanes 21–54) were solubilized and immunoblotted using 5D4 (upper panel) or anti-β-actin antibody (lower panel). (D) The tissue lysates (patient Nos. 11, 12, 25, and 32) after treatment with PNGase F were electrophoresed and transferred onto PVDF membranes. The membranes were treated with PBS (left panel) or 0.05 M NaOH (right panel), and immunoblotted with 5D4 or anti-β-actin.

Deglycosylation experiments. The tissue lysate was treated PNGase F (Takara Bio Inc., Shiga, Japan; 0.1 mU/μg of protein) for 24 h at 37 °C according to the manufacturer's instructions. Alkaline digestion of *O*-glycans on the Western-blot was performed as described [14]. Briefly, transferred membrane of tissue lysate was incubated with 0.05 N NaOH for 16 h at 40 °C, prior to probing with antibody.

Immunohistochemical analysis. Specimens were deparaffinized, rehydrated, and incubated first with 5D4 (1/1000 dilution) at room temperature for 1 h, then with biotin-conjugated secondary anti-mouse 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%; +++, >50%.

Quantitative real-time PCR analysis. Total RNAs were prepared from 53 astrocytic tumors (seven diffuse astrocytomas, 14 anaplastic astrocytomas, and 32 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 nine random oligomers and an oligo-dT primer according to the manufacturer's instructions. Real-time PCR was carried out on a LightCycler 480 Instrument with 96-well unit (Roche, Mannheim, Germany), using the LightCycler 480 SYBR Green I Master (Roche). Sets of primers were designed online with Primer3 (http://frodo.wi.mit.edu). The PCR conditions were 95 °C for 5 min (1 cycle), followed by 45 cycles of 95 °C for 10 s, 65 °C for 20 s, 72 °C for 20 s. Subsequently, a melting curve program was applied with continuous fluorescence measurement. Standard curves for each glycogene and β-actin template were generated by serial dilution of the PCR products (1×10^8 copies/μl to 1×10^2 copies/μl). The expression levels of glycogenes were normalized by the total RNA.

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

Table 1
Results of keratan sulfate immunostaining in 78 patients with astrocytic tumors

Tumor type	No. of cases	KS immunostaining				Positive rate
		+++	++	+	–	
Diffuse astrocytoma	20	0	0	0	20	0.0%
Tissue microarray	20	0	0	0	20	0.0%
Anaplastic astrocytoma	27	3	2	2	20	25.9%
Surgical resection samples	3	1	0	0	2	33.3%
Tissue microarray	24	2	2	2	18	25.0%
Glioblastoma	31	5	6	8	12	61.3%
Surgical resection samples	13	3	1	6	3	76.9%
Tissue microarray	18	2	5	2	9	50.0%

Results

Analysis of KS expression in astrocytic tumors

Lysates of frozen tumor specimens from 54 patients (six diffuse astrocytomas, 14 anaplastic astrocytomas, and 34 glioblastomas) were analyzed using Western-blot analysis with monoclonal anti-KS antibody, 5D4, which recognize highly sulfated KS [15]. As shown in Fig. 1, 5D4 detected highly sulfated KS in extracts of anaplastic astrocytoma (Grade III) and glioblastoma (Grade IV). One of six diffuse astrocytomas (17%), six of 14 anaplastic astrocytomas (43%), and 23 of 34 glioblastomas (68%) were detected by 5D4: two of 14 anaplastic astrocytomas (14%) and four of 34 glioblastomas (12%) were detected strongly; two of 14 anaplastic astrocytomas (14%) and 10 of 34 glioblastomas (29%) were detected moderately. Only one of diffuse astrocytomas (17%) was de-

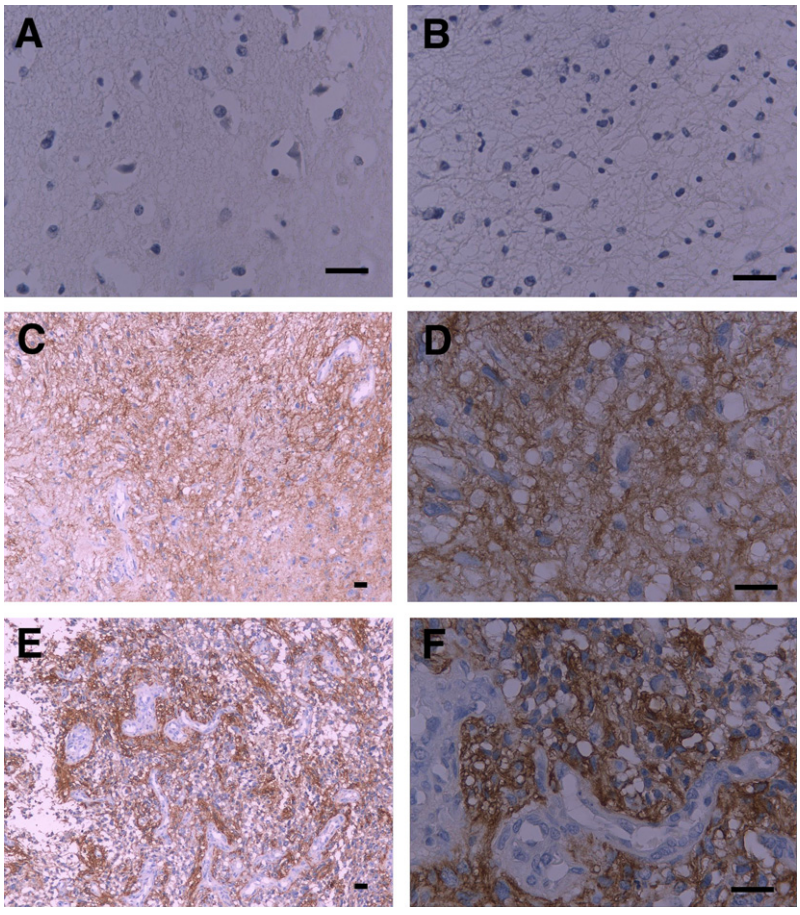

Fig. 2. Immunohistochemical detection of KS-containing proteoglycans in astrocytic tumors. No staining is apparent in a normal brain (A: 400×) and in diffuse astrocytoma (B: 400×). In anaplastic astrocytoma (patient No. 13), the tumor cell surface was stained positively (C: 100×, D: 400×). Accentuated staining is visible around an area of microvascular proliferation in glioblastoma (patient No. 32; E: 100x, F: 400x). Bar 10 μm.

tected weakly by 5D4. To determine whether 5D4-positive tissues express *N*-linked or *O*-linked KS, the tissue lysate was digested with PNGase F, then analyzed by Western-blot using 5D4. As depicted in Fig. 1D, the PNGase F treatment reduced some 5D4-positive signals. However, most signals were still observed. The PNGase F-undigested signals disappeared almost completely by sequential alkaline hydrolysis treatment to remove *O*-glycans (Fig. 1D), indicating that 5D4-positive tissues express both *N*-linked and *O*-linked KS.

Immunohistochemical staining for KS in malignant astrocytic tumors

The cellular distribution of KS in astrocytic tumors was examined immunohistochemically using 5D4. We used 62 tissues (20 diffuse astrocytomas, 24 anaplastic astrocytomas, and 18 glioblastomas) of tissue microarrays and 16 surgical tissues (three anaplastic astrocytomas and 13 glioblastomas) in immunohistochemistry. KS immunoreactivity was detected in seven of 27 (26%) anaplastic astrocytomas and in 19 of 31 (61%) glioblastomas; staining was graded as +++ in five glioblastoma and as ++ in six glioblastoma cases (Table 1). KS was not detected in diffuse astrocytomas. Representative staining for KS in astrocytic tumor samples is shown in Fig. 2. Immunostaining for KS demonstrated predominantly cell surface patterns. In anaplastic astrocytoma, the tumor cell surface was stained using 5D4, and the regional tendency was not observed (Fig. 2C and D). In glioblastomas, KS-positive tumor cells were prominent around microvascular proliferations (Fig. 2E and F). Proliferating endothelial cells were negative for KS (Fig. 2F). In non-neoplastic areas of the brain (Fig. 2A) and in diffuse astrocytoma (Fig. 2B), KS immunostaining was absent.

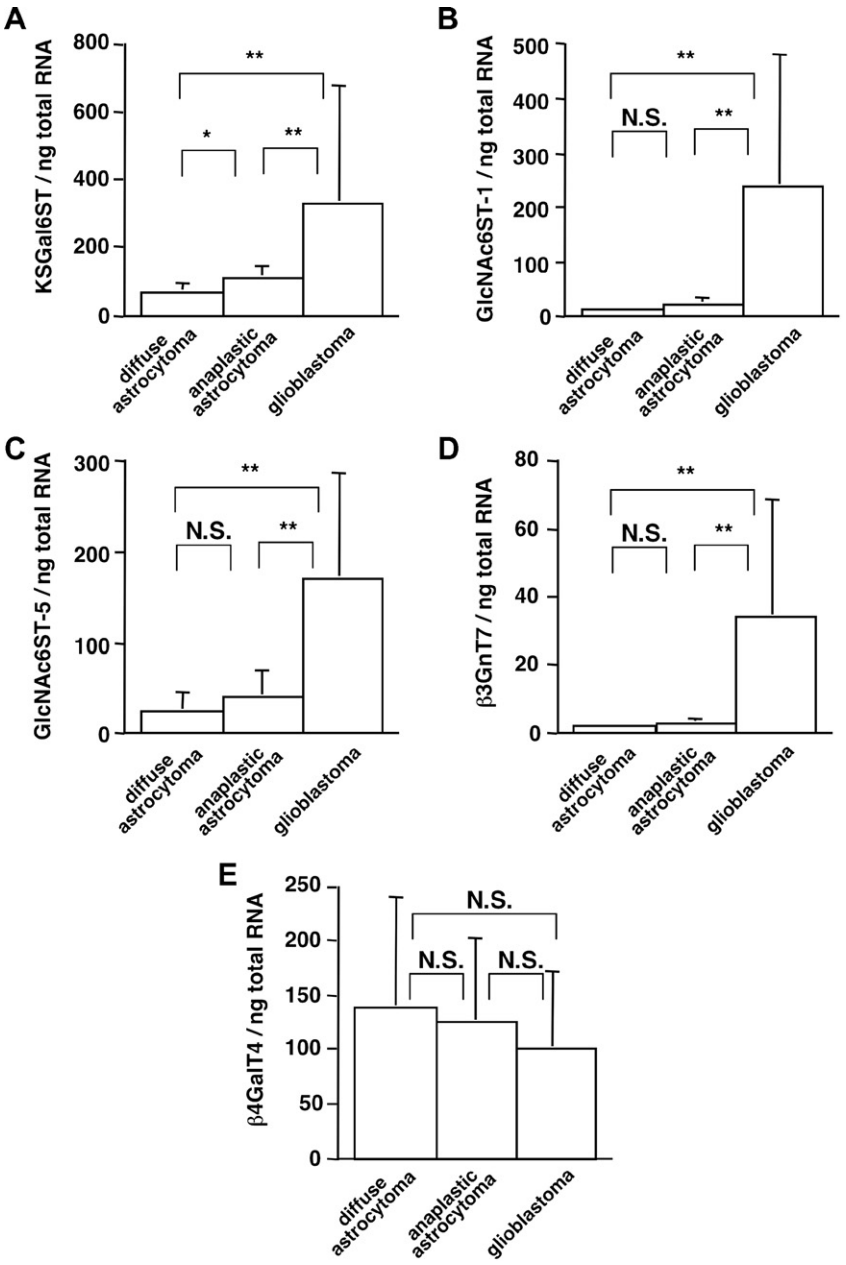

Fig. 3. Quantitative real-time PCR analysis of glycogenes for KS synthesis. The transcript levels for (A) KSGal6ST, (B) GlcNAc6ST-1, (C) GlcNAc6ST-5, (D) β3GnT7, and (E) β4GalT4 genes in 53 astrocytic tumors (seven diffuse astrocytomas, 14 anaplastic astrocytomas, and 32 glioblastomas) were measured using real-time PCR. Values normalized to the level of total RNA are presented. $^{**}p < 0.01$, $^{*}p < 0.05$.

Comparison of expression patterns of five genes involved in KS synthesis in astrocytic tumors

We performed quantitative real-time PCR analysis to compare the respective expression levels of five genes (KSGal6ST, GlcNAc6ST-1/-5, β3GnT7, and β4GalT4) involved in KS synthesis in 53 astrocytic tumors (seven diffuse astrocytomas, 14 anaplastic astrocytomas, and 32 glioblastomas). As shown in Fig. 3, the expression of KSGal6ST, GlcNAc6ST-1/-5, and β3GnT7 gradually increased associated with tumor malignancy. Comparing anaplastic astrocytoma with glioblastoma, the expression of KSGal6ST, GlcNAc6ST-1/-5, and β3GnT7 in glioblastoma is significantly higher than in anaplastic astrocytoma ($p < 0.01$). In comparison of diffuse astrocytoma with anaplastic astrocytoma, the expression of GlcNAc6ST-1 in anaplastic astrocytoma is significantly higher than in diffuse astrocytoma ($p < 0.05$). The expression of β4GalT4 has no significant difference among different grades of astrocytic tumors.

Discussion

Keratan sulfate (KS) is a glycosaminoglycan that is formed through the elongation of *N*-glycans or *O*-glycans attached covalently to scaffold proteins [1,16]. KS proteoglycans (KSPGs) are found in the extracellular matrix or cell surface in numerous tissues, predominantly in those of the cornea, cartilage, and brain. In the normal adult brain, KS expression is restricted to a subpopulation of microglia [2]. KS expression responds to embryonic development, physiological variations, and wound healing [1,16,17]. However, it has not been investigated whether KS is expressed in brain tumors and is further involved in malignant progression of brain tumors, especially astrocytic tumors.

In this study, lysates of frozen tumor specimens were analyzed using Western-blot analysis with monoclonal anti-KS antibody, 5D4 (Fig. 1). As a result, one of six diffuse astrocytomas (Grade II; 17%) and 29 of 48 high-grade astrocytic tumors (Grade III and IV; 60%) were detected by 5D4, indicating that highly sulfated KS-expression might be associated with its malignancy. Of high-grade astrocytic tumors, six of 14 anaplastic astrocytomas (43%) and 23 of 34 glioblastomas (68%) were detected by 5D4 ($p < 0.05$); therefore, there is significant difference about KS-expression between anaplastic astrocytomas and glioblastomas. We also investigated the KS expression in immunohistochemistry, and also showed the significant difference about KS expression between anaplastic astrocytomas (26%) and glioblastoma (61%; $p < 0.05$). We previously investigated the expression of podoplanin, which is associated with malignant progression of astrocytic tumors [18,19]. Podoplanin was expressed on the cell surface in 27% of anaplastic astrocytomas and 47% of glioblastomas using immunohistochemistry with anti-podoplanin antibody. On the other hand, podoplanin expression was not observed in diffuse astrocytomas. Furthermore, podoplanin expression is associated with poor prognosis of glioblastoma patients. Interestingly, KS-expression is highly associated with podoplanin expression in diffuse astrocytomas and anaplastic astrocytomas (data not shown), also suggesting that KS-expression might be involved in malignancy of astrocytic tumors.

In our recent study, we investigated how KS expression is regulated in glioblastoma cell lines [14]. We performed quantitative real-time PCR analysis in 10 glioblastoma cell lines to compare the respective expression levels of five glycogenes (KSGal6ST, GlcNAc6ST-1/5, β3GnT7, and β4GalT4), which are involved in KS synthesis; LN229 cells expressed high level of these five glycogenes, especially KSGal6ST. Knockdown of KSGal6ST in LN229 using siRNAs for KSGal6ST considerably reduced the 5D4-reactivity shown by Western-blot analysis. KSGal6ST-transfected LN464 cells express a higher level of highly sulfated KS than control LN464. In this study, we also performed quantitative real-time PCR analysis to compare the respective expression levels of five glycogenes involved in KS synthesis in 53 astrocytic tumors. Of five glycogenes, the expression of KSGal6ST, GlcNAc6ST-1/-5, and β3GnT7 gradually increased associated with tumor malignancy (Fig. 3). Interestingly, the expression of KSGal6ST, GlcNAc6ST-1/-5, and β3GnT7 in glioblastoma is significantly higher than in anaplastic astrocytoma. Moreover, the expression of GlcNAc6ST-1 in anaplastic astrocytoma is significantly higher than in diffuse astrocytoma, which is coincident with the high level of expression of KSGal6ST in LN229 glioblastoma cells [14]. These results suggest that glycogenes involved in KS synthesis increased in the malignant progression of astrocytic tumors, leading to the up-regulation of highly sulfated KS.

In summary, we revealed that KS significantly expressed in malignant astrocytic tumors, not in low-grade astrocytic tumors using Western-blot. In immunohistochemistry, KS is highly expressed in cell surface of tumor cells. Taken together, KS might be associated with the malignancy of astrocytic tumors, and be useful for a prognostic factor of astrocytic tumors. Further study is necessary to investigate how KS is involved in the invasion of astrocytic tumors or their malignant progression.

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