Chondroitin sulfate synthase-1 expression is associated with malignant potential of soft tissue sarcomas with myxoid substance

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Summary

The glycosyltransferases chondroitin sulfate synthase-1 (CHSY1) and exostoses-like 3 (EXTL3) specifically function in biosynthesis of the glycans chondroitin sulfate (CS) and heparan sulfate (HS), respectively. Although these glycans play important roles in pathogenesis of various tumors, their significance in soft tissue sarcoma remains unknown. Here, we asked whether CHSY1 or EXTL3 expression correlates with malignant potential of soft tissue sarcomas with myxoid substance. To do so, we examined 40 samples representing specific types, including 12 cases of myxoid liposarcoma, 14 of myxofibrosarcoma (MFS), 12 of malignant peripheral nerve sheath tumor (MPNST), and 2 of low-grade fibromyxoid sarcoma. We performed immunohistochemistry with anti-CHSY1 and anti-EXTL3 antibodies and compared enzyme expression levels with tumor histological grade as assessed by the FNCLCC classification and with patient 5-year survival rate. CHSY1 and EXTL3 were expressed in 72.5% and 32.5% of all tumors, respectively. Notably, CHSY1 was strongly expressed in MFS and MPNST compared with other tumors and significantly associated with higher rather than lower grade tumors ($P < 0.01$). Strong CHSY1 expression was also significantly associated with poorer patient outcomes ($P = 0.031$). By contrast EXTL3 expression was not correlated with histological grade or patient prognosis. We conclude that CHSY1 expression is closely associated with malignant potential of soft tissue sarcomas with myxoid substance.

Keywords: Chondroitin sulfate; CHSY1; EXTL3; Heparan sulfate; Soft tissue sarcoma

1. Introduction

Soft tissue sarcoma is a malignant tumor of non-epithelial extraskeletal origin classified by specific, differentiated cell types [1]. Importantly, over half of all soft tissue sarcomas possess intermediate- to high-grade malignant potential, reflecting high metastatic capacity [2,3]. The five-year survival rate of patients with soft tissue sarcoma is estimated as ~50% [4]. Although the incidence of soft tissue sarcoma is ~1% of malignant tumors, its histopathological classification includes more than 50 different subtypes of the tumor [5]. In particular, in soft tissue sarcomas with myxoid substance, it is difficult to make a pathological diagnosis or determine malignant potential [6], because there are few tissue-specific markers available to identify tumor subtypes.

Glycosaminoglycan (GAG) is a sugar chain that binds core proteins such as aglycan and percan, thus forming proteoglycan [7]. GAG is present on both the cell surface and in the extracellular matrix, where it interacts with growth factors and extracellular matrix components and thus functions in cell adhesion, migration, differentiation, and morphogenesis [8-10]. The presence of GAG is also closely associated with tumor metastasis and inflammation [11,12].

GAG is composed of repeating disaccharide structures such as [4GlcAβ1-3GalNAcβ1] or [4GlcAβ1-4GlcNAcα1]; based on these structures GAG is broadly classified as chondroitin sulfate (CS) or heparan sulfate (HS), respectively. Chondroitin sulfate synthase-1 (CHSY1) is the key biosynthetic enzyme forming the repeating CS backbone disaccharide structure [4GlcAβ1-3GalNAcβ1], and polymerization of chondroitin chain by CHSY1 requires chondroitin polymerizing

factors, including CHPF and CHPF2 [13,14] (Fig. 1). By contrast, exostoses-like 3 (EXTL3) is a glycosyltransferase that adds GlcNAc to the common linker tetrasaccharide [GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-Ser] in both CS and HS synthesis or to nascent heparan sulfate structures with an α1,4-linkage, thus initiating synthesis and elongating HS [15,16] (Fig. 1). Expression levels of CS or genes required for CS biosynthesis reportedly correlate positively with poor prognosis of some malignant tumors [17-19]. However, the significance of GAG including HS or its related enzymes expressed in soft tissue sarcoma remains unknown.

In the present study, we examined CHSY1 and EXTL3 expression by immunohistochemistry in human soft tissue sarcomas with myxoid substance and then compared their expression with pathological grade of the tumors based on the Fédération Nationale des Centres de Lutte Contre le Cancer (FNCLCC) classification and 5-year survival rate of patients. Our results indicate that CHSY1 expression in tumor cells is closely correlated with the pathological grades and patients' outcome.

2. Materials and methods

2. 1. Patient samples

Formalin-fixed and paraffin-embedded tissue blocks of surgically resected primary soft tissue sarcomas with myxoid substance obtained at Shinshu University Hospital, Matsumoto, Japan, between January 2000 to December 2012 were retrieved from the pathology files of the Department of Laboratory Medicine of the same hospital. Samples represented 40 patients ranging in age from 10 to 94 years (median years, 57.2). Patients underwent wide margin resection and were followed up from 4 to 133 months (median months, 48.3). Samples included 12 cases of myxoid liposarcoma

(MLS), 14 of myxofibrosarcoma (MFS), 12 of malignant peripheral nerve sheath tumor with myxoid substance (MPNST), and 2 of low-grade fibromyxoid sarcoma (LGFMS). A profile of patients enrolled in this study is provided in Table 1. For each tumor subtype, representative tumors were selected for analysis based on Hematoxylin & Eosin (HE)-staining of tissue sections. All tissue specimens were fixed in 10% buffered formalin for 48 hours, embedded in paraffin, and then sectioned serially at 3-μm thickness. Clinicopathological data analyzed here were based on original pathology reports, including histological grade using the FNCLCC classification [6], and reviewed by three of the authors (TM, MF, and JN). The Ethics Committee of Shinshu University School of Medicine approved the protocol and use of human samples in this study (no. 438).

2. 2. Immunohistochemistry

Expression of CHSY1 and EXTL3 in soft tissue sarcomas with myxoid substance was examined by immunohistochemistry using rabbit polyclonal anti-CHSY1 (sc-292185; Santa Cruz Biotechnology, USA) and mouse monoclonal anti-EXTL3 (sc-271986; Santa Cruz Biotechnology, USA). Before immunostaining, antigen retrieval was carried out by microwaving samples in 10 mM Tris-HCL buffer (pH 8.0) containing 1 mM EDTA for 30 min. Tissue sections were incubated in 10% BSA in phosphate-buffered saline (PBS) for 10 minutes prior to primary antibody application. Samples were incubated with primary antibodies overnight at 4℃. Secondary antibodies were HRP-labeled anti-rabbit immunogulobulins (Dako, Carpinteria, CA, USA) for CHSY1 and Histofine for mouse (Nichirei, Tokyo, Japan) for EXTL3. Secondary antibody incubations were performed at 25℃ for 30 min. Peroxidase activity was visualized using diaminobenzidine-H_2O_2 solution. Controls, which were

assessed by omitting primary antibody, showed no specific staining.

2. 3. Evaluation

Expression levels of CHSY1 and EXTL3 were scored from 0 to 3 based on criteria established by Nakajima et al [20] with minor modification: 0 indicated <10% positively-stained cells; 1 indicated 10-29% positively-stained cells; 2 indicated 30-59% positively-stained cells; and 3 indicated 60% or more positively-stained cells. For each tissue subtype, we calculated means ± SE of expression to assess potential differences in expression level. Based on these expression levels, tumors whose scores were 0 or 1 were defined as “weak expression”, and those with scores of 2 or 3 were defined as “strong expression”. Those tumors were then examined for pathological FNCLCC grade and patient 5-year survival rate.

2. 4. Statistical analysis

Statistical analysis was carried out using the SPSS software package version 21 (IBM, Armonk, NY, USA). Differences in enzyme expression in tumor subtypes were evaluated by the Mann-Whitney U test. Differences in enzyme expression in each grade of tumors based on FNCLCC classification were evaluated using the χ-square test. Cancer-specific 5-year survival rates were analyzed using the Kaplan-Meier method, and significant differences between expression levels of enzymes and patient survival rates were analyzed using the Log-rank test. *P*-values less than 0.05 were considered statistically significant.

3. Results

3. 1. CHSY1 expression in soft tissue sarcomas with myxoid substance

CHSY1 and EXTL3 are localized in the Golgi apparatus and endoplasmic reticulum, respectively [21,22]; therefore, we evaluated only perinuclear dot-like staining for further evaluation. CHSY1 was expressed in 29 (72.5%) of 40 soft tissue sarcomas with myxoid substance. Representative Golgi-specific CHSY1 staining in tumor cells is shown in Fig. 2. The frequency of CHSY1 expression in each histological subtype was: 3 cases (25%) for MLS, 14 cases (100%) for MFS, 10 cases (83.3%) for MPNST, and 2 cases (100%) for LGFMS. Interestingly, in the case of MLS, CHSY1 was detected only in tumor cells exhibiting a round morphology (Fig. 2). Strong CHSY1 expression was seen in 71.4% of MFS and in all cases of MPNST (Fig. 2). Expression levels of each tumor subtype were 0.58 ± 0.31 for MLS, 2.07 ± 0.22 for MFS, 2.08 ± 0.31 for MPNST, and 2.0 ± 1.0 for LGFMS (Fig. 3). Finally, CHSY1 expression in MFS and MPNST was significantly higher than that in MLS.

3. 2. EXTL3 expression in soft tissue sarcomas with myxoid substance

EXTL3 was expressed in 13 (32.5%) of 40 soft tissue sarcomas with myxoid substance. Representative staining for EXTL3 in tumor cells is shown in Fig. 2. The frequency of EXTL3 expression in each histological subtype was: 0% in 12 MLS cases, 50% (7 cases) for MFS, 25% (3 cases) for MPNST, and 50% (1 case) for LGFMS. Notably, no samples from MLS patients expressed EXTL3. Expression levels for each tumor subtype were 0 for MLS, 0.64 ± 0.20 for MFS, 0.25 ± 0.13 for MPNST, and 0.5 ± 0.5 for LGFMS (Fig. 3). Tumor subtypes showed no statistically significant difference in expression.

3. 3. Comparison of CHSY1 and EXTL3 expression in subtypes of soft tissue sarcomas with myxoid substance

We next compared expression of CHSY1 and EXTL3 in each subtype of soft tissue sarcomas with myxoid substance. CHSY1 and EXTL3 showed a significant difference in expression in MFS and MPNST ($P < 0.01$) (Fig. 3). However, there were no significant differences in expression of CHSY1 and EXTL3 in MLS ($P = 0.07$).

3. 4. Correlation between histological grade and CHSY1 and EXTL3 expression in soft tissue sarcomas myxoid substance

We then compared CHSY1 and EXTL3 expression levels in tumor cells of soft tissue sarcomas with myxoid substance with pathological grade as assessed by FNCLCC classification. Relevant to CHSY1, the frequency of patients showing strong CHSY1 expression in tumors was 23.1% for grade 1, 82.4% for grade 2, and 90.0% for grade 3, indicating that CHSY1 expression is significantly higher in higher pathological grades of tumor ($P < 0.01$) (Fig. 4). On the other hand, we observed no statistically significant correlation of EXTL3 expression and tumor grade ($P = 0.519$).

3. 5. Correlation between patient survival and enzyme expression in soft tissue sarcoma myxoid substance

Finally, we compared CHSY1 and EXTL3 expression in tumor cells of soft tissue sarcoma myxoid substance with 5-year survival rate of patients based on Kaplan-Meier assessment. Five-year survival rate of patients with high CHSY1 expression was 26.9%, whereas 5-year survival rate of patients with weak CHSY1 expression was 81.8%, indicating that CHSY1 expression is significantly associated with poorer patient prognosis ($P = 0.031$) (Fig. 5). On the other hand, we observed no significant association between EXTL3 expression and patient 5-year survival rate ($P = 0.284$).

4. Discussion

In the present study, we revealed that CHSY1 expression in soft tissue sarcomas with myxoid substance is correlated with both pathological grade and clinical prognosis of patients. To our knowledge, this is the first report showing a relationship between clinicopathological variables and expression of GAG synthases in soft tissue sarcoma. Previously, it was reported that expression levels of CS, including highly sulfated forms, were correlated with clinical outcome in some tumors: Svensson et al. evaluated breast cancer tissues by immunohistochemistry using an anti-CS antibody and demonstrated that higher expression levels of CS were positively correlated with disease recurrence and poor patient survival [17]. Vallen et al. reported that intense expression of 4,6-disulfated CS (CS-E) in ovarian cancer were correlated with high tumor grade, elevated tumor marker CA125, and poor prognosis [18]. More recently, we reported that expression of *N*-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase, which produces highly sulfated CS-E, is correlated with clinical outcome of patients with astrocytoma, and that tumor cell motility is enhanced in the presence of CS-E's preferred ligands, midkine or pleiotrophin [19].

Here, among various enzymes involved in GAG synthesis, we chose CHSY1 and EXTL3 for evaluation, in part because CHSY1 exhibits the most potent enzyme activity in forming repeating disaccharide structure [4GlcAβ1-3GalNAcβ1] seen in CS among other enzymes, including CHSY3, CHPF, and CHPF2 [13,14]. Moreover, EXTL3 is a key enzyme functioning both in initiation and elongation of HS chains [15,16]. Importantly, we found that CHSY1 expression in soft tissue sarcoma with myxoid substance showed a significant positive correlation with pathological grade,

as judged by the FNCLCC classification, and with a lower cancer-specific 5-year survival rate of patients. Of note, CHSY1 was strongly expressed in rounded cells in all MLS tumors examined. It is known that the presence of round cells in this tumor is indicative of poor patient prognosis [24], suggesting that CHSY1 immunohistochemistry could be helpful in evaluating tumor cell aggressiveness.

Kalathas et al. has analyzed CS synthase expression in colon cancer using RT-PCR and shown that expression of these enzymes, including CHSY1, CHSY2, CHSY3, and CS glucuronyltransferase (CSGlcA-T), was higher in cancer patients than in healthy controls [25]. The same study also revealed that expression of both CHSY2 and CSGlcA-T increased as patient clinical stages advanced. CHSY1 possesses both glucuronyltransferase and galactosaminyltaranferase activities, thus playing a dual role in CS initiation and elongation [13,14(check)]. Because there were no reports of whether CHSY1 is correlated with a tumor's malignant potential, we focused on CHSY1 and confirmed that its expression is correlated with both pathological grade and survival of patients with soft tissue sarcoma with myxoid substance. The molecular mechanisms underlying CHSY1 regulation of tumor cells progression remain to be elucidated.

EXTL3 is an EXT family member that plays a dual role on initiation and elongation of HS chain biosynthesis [15,16]. EXTL3 is considered a putative tumor suppressor, as the gene that encodes it is located on chromosome 8p21, which shows high frequency "loss of heterozygosity" in various human cancers including colorectal cancer. Arai et al. have revealed that EXTL3 is mutated in 25% of colon cancer tumors, and EXTL3 inactivation is associated with colorectal cancer growth [26]. In

addition, Karibe et al. demonstrated that HS expression in mucinous colorectal cancer is suppressed by methylated EXTL3 [27]. The predominant expression of CHSY1 relative to EXTL3 in soft tissue sarcoma with myxoid substance shown here strongly suggests that CS rather than HS is a major GAG produced by these tumor cells, an observation to be addressed in future biochemical analysis. Recently, investigators showed that other enzymes such as EXTL2 function in initiation of HS synthesis [23]; thus further analysis of expression levels of other HS synthesis enzymes in soft tissue sarcoma is required.

In conclusion, we have revealed that high CHSY1 expression in soft tissue sarcoma with myxoid substance is associated with pathological grade and clinical prognosis. Thus, CHSY1 immunohistochemistry could be useful to predict the malignant potential of tumor cells of soft tissue sarcoma with myxoid substance.

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Figure Legends

Fig. 1 CHSY1 and EXTL3 function in CS and HS biosynthesis.

CHSY1 exhibits both GlcAT-II and GalNAc-II activities, thus forming the GlcA-GalNAc disaccharide structure specific for CS. On the other hand, in HS biosynthesis EXTL3 is involved in both initiation and elongation of HS backbone structure with its GlcNAcT-I and GlcNAcT-II activities, respectively. XylT; xylosyltransferase, GalT-I; β1,4-galactosyltransferase-I, GalT-II; β1,3-galactosayltransferase-II, GlcAT-I; β1,3-glucuronyltransferase-I, GalNAcT-I; *N*-acetylgalactosaminyltransferase-I, CHSY1; chondroitin synthase-1; EXTL3; exostoses like-3. Xyl, xylose; Gal, galactose; GlcA, glucuron acid; GalNAc, *N*-acetylgalactosamine: GlcNAc, *N*-acetylglucosamine.

Fig. 2 CHSY1 and EXTL3 expression in various subtypes of soft tissue sarcomas with myxoid substance. HE, Hematoxylin & Eosin staining; CHSY1, immunohistochemistry using anti-CHSY1; and EXTL3, immunohistochemistry using anti-EXTL3. Scale bar: 500μm.

Fig. 3 CHSY1 and EXTL3 expression in various subtypes of soft tissue sarcomas with myxoid substance. CHSY1 expression is significantly elevated in MFS and MPNST compared to MLS. The number of patients examined was 12 for MLS, 14 for MFS, 12 for MPNST, and 2 for LGFMS. $^{**}P < 0.01$ $^{*}P < 0.05$.

Fig. 4 CHSY1 and EXTL3 expression in various histopathological grades (G1 to G3) of soft tissue sarcomas with myxoid substance, as assessed by FNCLCC classification. CHSY1 expression becomes significantly higher as histopathological

grade advances, while a similar association is not observed in terms of EXTL3 expression. *$P < 0.05$

Fig. 5 Correlation of CHSY1 and EXTL3 expression with 5-year survival rate of patients with soft tissue sarcomas with myxoid substance. Patients with tumor cells strongly positive for CHSY1 show significantly poorer outcomes than do patients whose tumor cells are weakly positive for CHSY1 ($P = 0.031$) (A). There is no significant association between patient survival rate and EXTL3 expression ($P = 0.284$) (B).

Table 1 Profiles of patients with soft tissue sarcomas analyzed in this study

	n	Gender (M, F)	Median age (range)
Soft tissue sarcoma	40	21,19	57.2 (10-94)
Histopathology[a]			
MLS	12	5, 7	54.1 (37-84)
MFS	14	11, 3	68.6 (48-86)
MPNST	12	4, 8	54.6 (23-94)
LGFMS	2	1, 1	11.0 (10, 12)
FNCLCC grading scale[b]			
Grade 1	13	7, 6	57.8 (37-84)
Grade 2	17	11, 6	62.6 (10-94)
Grade 3	10	3, 7	51.3 (28-86)

[a] MLS, myxoid liposarcoma; MFS, myxofibrosarcoma; MPNST, malignant peripheral nerve sheath tumor; LGFMS, low-grade fibromyxoid sarcoma.

[b] FNCLCC, Fédération Nationale des Centres de Lutte Contre le Cancer.