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Defect in dermatan sulfate in urine of patients with Ehlers-Danlos syndrome caused by a CHST14/D4ST1 deficiency

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Running title: Urinary dermatan sulfate in Ehlers-Danlos syndrome

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Abstract

Purpose: Dermatan sulfate (DS) plays a number of roles in a wide range of biological activities such as cell signaling and tissue morphogenesis through interactions with various extracellular matrix proteins including collagen. Mutations in the carbohydrate sulfotransferase 14 gene *(CHST14)* encoding CHST14/dermatan 4-*O*-sulfotransferase-1 (D4ST1), which is responsible for the biosynthesis of DS, cause a recently delineated form of Ehlers-Danlos syndrome (EDS, musculocontractural type 1), an autosomal recessive connective tissue disorder characterized by congenital malformations (specific craniofacial features, and congenital multiple contractures) and progressive fragility-related complications (skin hyperextensibility, bruisability, and fragility with atrophic scars; recurrent dislocations; progressive talipes or spinal deformities; and large subcutaneous hematomas). In an attempt to develop a diagnostic screening method for the various types of EDS, the amount of DS in the urine of patients was analyzed.

Methods: Urinary DS was quantified by an anion-exchange chromatography after treatment with DS-specific degrading enzyme.

Results: DS was not detected in the urine of patients with homo- or compound heterozygous mutations in *CHST14*. These results suggest that the quantification of DS in urine is applicable to an initial diagnosis of DS-defective EDS.

Conclusions: This is the first study to perform a urinary disaccharide compositional analysis of chondroitin sulfate (CS)/DS chains in patients with EDS caused by a CHST14/D4ST1 deficiency, and demonstrated the absence of DS chains. This result suggests systemic DS depletion in this disorder, and also proposes the usefulness of a urinary disaccharide compositional analysis of CS/DS chains as a non-invasive screening method for this disorder.

1. Introduction

Dermatan sulfate (DS) is a linear polysaccharide that is covalently attached to specific core proteins forming DS-proteoglycans (DS-PGs), which are widely expressed at cell surfaces and extracellular matrices [1]. DS is abundantly distributed in the skin, cartilage, and aorta, and consists of alternating disaccharide units of L-iduronic acid (IdoUA) and *N*-acetyl-D-galactosamine (GalNAc) with 50-200 repeats [2]. DS chains are irregularly modified by sulfation at the hydroxy groups of C-2 on IdoUA and the C-4 positions of GalNAc residues, respectively, and are involved in the regulation of a number of biological functions such as the assembly of extracellular matrices, signal transduction, wound healing, and anticoagulation through interactions with growth factors [3, 4].

The biosynthesis of repeating disaccharide regions of DS chains is initiated by the formation of chondroitin as a precursor backbone, which is composed of alternating D-glucuronic acid (GlcUA) and GalNAc. Chondroitin is synthesized on the carbohydrate-protein linkage region tetrasaccharide sequence (GlcUA-galactose-galactose-xylose) attached to the Ser residues of specific core proteins by various glycosyltransferases. DS-epimerase (DSE) then converts GlcUA into IdoUA by epimerizing the C-5 position of GlcUA residues after the formation of the chondroitin backbone [5]. Dermatan chains are subsequently matured by sulfation reactions catalyzed by dermatan 4-*O*-sulfotransferase-1 (D4ST1), also named carbohydrate sulfotransferase 14 (CHST14), and uronosyl 2-*O*-sulfotransferase, which transfer a sulfate group from the sulfate donor 3'-phosphoadenosine 5'-phosphosulfate to the C-4 position of GalNAc and C-2 position of IdoUA residues, respectively [6-8].

Ehlers-Danlos syndrome (EDS) is a heterogenous group of heritable connective tissue disorders characterized by skin hyperextensibility, joint hypermobility, and tissue fragility, and has been classified into six major types: the classical type (MIM#130000), hypermobility type (MIM#130020), vascular type (MIM#130050), kyphoscoliosis type (MIM#225400), arthrochalasia type (MIM#130060), and dermatospraxis type (MIM#225410) [9, 10]. The

dominant negative effects of a haploinsufficiency in mutant procollagen α-chain genes or deficiency in collagen-processing enzymes have been identified as the basis for these types of EDS [9]. Additional forms of EDS have also been identified in association with molecular and biochemical abnormalities [10, 11]. EDS caused by biallelic mutations in *CHST14* was recently identified in three independently reported conditions: a rare type of arthrogryposis syndrome, "adducted thumb-clubfoot syndrome" [12], a specific type of EDS, "EDS, Kosho Type" [13, 14], and a subset of kyphoscoliosis-type EDS without a lysyl hydroxylase deficiency, "musculocontractural EDS" [15]. All these conditions are now concluded to be a single clinical entity, with the proposed names "D4ST1-deficient EDS (DDEDS)" [16], "EDS caused by a CHST14/D4ST1 deficiency" [11], or "EDS, musculocontractural type 1 (EDSMC1) (MIM#601776) in order to distinguish a subsequently identified form of EDS caused by biallelic loss-of-function mutations in *DSE*, which is registered as "EDS, musculocontractural type 2 (EDSMC2)" (#615539) [17, 18]. To date, 40 patients from 27 families have been reported to have a CHST14/D4ST1 deficiency, manifesting multiple congenital malformations (craniofacial characteristics, multiple congenital contractures, and visceral or ophthalmological malformations) and progressive multisystem fragility-related complications (skin hyperextensibility, bruisability, and fragility with atrophic scars; recurrent dislocations; progressive talipes or spinal deformities; pneumothorax or pneumohemothorax; large subcutaneous hematomas; and diverticular perforation) [10-15, 19-26].

Sulfotransferase activity toward dermatan in the skin fibroblasts of affected EDS patients with *CHST14* mutations was previously shown to be significantly lower in a patient (p.P281L/p.Y293C) (6.7%) and in another patient (p.P281L/p.P281L) (14.5%) than in each age- and sex-matched control [14]. A disaccharide compositional analysis of chondroitin sulfate (CS)/DS chains using the affected skin fibroblasts of these two patients showed a negligible amount of DS and excess amount of CS [14], presumably due to an impaired 4-*O*-sulfation lock and subsequent back-epimerization from IdoUA to GlcUA because of a

CHST14/D4ST1 deficiency [12, 14, 18]. Decorin is a major DS-PG in the skin and plays an important role in the assembly of collagen fibrils, possibly through an electrostatic interaction between decorin glycosaminoglycan (GAG) chains and adjacent collagen fibrils [27). CS, but not DS dissacharides have been detected in the GAG chain of decorin from affected skin fibroblasts, while DS disaccharides (approximately 95%) have mainly been found in the GAG chain of decorin from control skin fibroblasts [14, 18]. Transmission electron microscopy revealed that collagen fibrils in affected skin specimens were dispersed in the reticular dermis, in contrast to the regularly and tightly assembled collagen fibrils observed in the controls [14]. Each collagen fibril in the affected skin specimens was smooth and round, and did not vary in size or shape, similar to that of the controls [14]. These findings indicate that skin fragility in patients with a CHST14/D4ST1 deficiency is caused by the impaired assembly of collagen fibrils through the replacement of a DS chain with a CS chain of decorin, which may alter the electrostatic binding of decorin to collagen fibrils [10, 11, 14].

We herein present our results on the first attempt to perform a disaccharide compositional analysis on CS/DS chains in the urine samples of patients with the disorder, which demonstrate the systemic effects of a CHST14/D4ST1 deficiency and also indicate the potential of this analysis as a non-invasive screening method for this disorder.

2. Materials and methods

2.1. Patient materials

Urine samples were obtained from seven previously reported patients with EDS caused by a CHST14/D4ST1 deficiency [13, 19, 20, 25], 15 healthy subjects (aged 6 months and 3−43 years old; 10 females, 5 males), and the parents (42 and 37 years old) and unaffected sisters (3 and 11 years old) of patients #D7 and D8 (Table 1).

2.2. Materials

Six unsaturated standard disaccharides derived from CS, chondroitinase ABC (EC 4.2.2.20) from *Proteus vulgaris*, chondroitinase AC-I (EC 4.2.2.5) from *Flavobacterium heparinum*, and chondroitinase AC-II (EC 4.2.2.5) from *Arthrobacter auresens* were purchased from Seikagaku Biobusiness Corp. (Tokyo, Japan). Chondroitinase B (EC 4.2.2.19) from *F. heparinum* was from IBEX Technologies (Montreal, Canada). All other chemicals and reagents were of the highest quality available.

2.3. Quantification of urinary DS

The disaccharide compositions of the CS and DS chains in urine samples were assessed as described previously [28]. Briefly, urine samples were concentrated and desalted using Amicon Ultra-0.5 (10 k) centrifugal filter units (Millipore, Billerica, MA). After an aliquot was individually digested with chondroitinase ABC, a mixture of chondroitinase AC-I and AC-II, or chondroitinase B, each digest was labeled with 2AB, and excess 2AB reagents were removed by extraction with chloroform. The 2AB-labeled digest was analyzed by anion-exchange HPLC on a PA-G silica column (4.6 x 150 mm, YMC Co., Kyoto, Japan) using isocratic conditions with 16 mM of NaH_2PO_4 for the first 10 min followed by a linear gradient from 16 to 530 mM NaH_2PO_4 at room temperature over a 60-min period at a flow rate of 0.5 ml/min. The eluates were monitored using a fluorometric detector with excitation and emission wavelengths of 330 and 420 nm, respectively. The identification and quantification of the resulting disaccharides were achieved by comparisons with the elution positions of CS- or DS-derived authentic unsaturated disaccharides. The amounts of CS and DS were normalized by urine creatinine levels, which were measured using the kit, LabAssay™ Creatinine (Wako, Osaka, Japan). This study was approved by the local Ethics Committees of Meijo University (Nagoya, Japan), Hokkaido University (Sapporo, Japan), and Shinshu University (Matsumoto, Japan).

3. Results

The 4-*O*-sulfated disaccharide unit, ΔHexUA-GalNAc(4-*O*-sulfate), in which ΔHexUA stands for 4,5-unsaturated hexuronic acid, in CS/DS was predominantly detected in the urine samples of healthy controls and EDS patients (Fig. 1 and Table 2). The non-sulfated and 6-*O*-sulfated disaccharide units, ΔHexUA-GalNAc and ΔHexUA-GalNAc(6-*O*-sulfate), respectively, were also detected (Table 2). Furthermore, a small proportion of disulfated disaccharide units including ΔHexUA(2-*O*-sulfate)-GalNAc(4-*O*-sulfate), ΔHexUA(2-*O*-sulfate)-GalNAc(6-*O*-sulfate), and ΔHexUA-GalNAc(4-*O*-, 6-*O*-sulfate) was found (Table 2). The total amount of CS/DS in urine is known to vary depending on sex and age [29]. An average value for the total amount of CS/DS disaccharides from healthy subjects (#N6 and N7), who were approximately 30- and 20-year-old males, respectively, was 7.5 nmol/mg creatinine (Table 2). In contrast, an average value of 14.0 nmol/mg creatinine was detected in the urine of healthy subjects #N3 and N4 (29-year-old females). The average of total CS/DS disaccharides from 3-year-old female controls (#N9 and N10) was 72.2 nmol/mg creatinine (Table 2). Thus, in order to compare urinary CS and DS between a healthy control and patient, sex- and age-matched controls are required.

The amount of CS/DS (6.2 nmol/mg creatinine) was markedly lower in the urine sample of patient #D2 than in those of control samples #N3 and N4 (16.4 and 9.6 nmol /mg creatinine, respectively) based on the results of chondroitinase ABC digestion (Table 2 and Fig. 1). The amount of CS/DS in other patients, except for #D3 (p.P281L/p.P281L), was also reduced (Table 2 and Fig. 2). However, the amount of CS/DS in urine samples from patient #D3 was slightly higher than that of the corresponding control (Fig. 2).

The amount of CS disaccharides generated by digestion with chondroitinase AC (Table 3 and Fig. 2B) was similar to that of CS/DS disaccharides obtained by digestion with chondroitinase ABC (Table 2 and Fig. 2A). The concentration of CS disaccharides in the urine of patient #D2 (p.P281L/p.P281L) was 5.6 nmol/mg creatinine (Table 3). On the other

hand, those of the corresponding healthy controls, #N3 and N4, were 18.1 and 8.8 nmol/mg creatinine, respectively (Table 3). Thus, the amount of CS in urine samples was slightly lower in the patient than in the healthy controls.

DS disaccharide was not detected in the urine of any patient, but was present in the urine of healthy controls (0.2~1.2 nmol/mg creatinine) (Table 4).

The amounts of urinary CS and DS in family members of EDS patients #D7 and #D8 were also measured (Supplemental Table S1). The amounts of CS disaccharides from the father (P281L/+) and mother (F209S/+) of patients #D7 and D8 (F209S/P281L) were 3.3 and 7.0 nmol/mg creatinine, respectively (Supplemental Table S1). The amounts of urinary CS from the sisters, who have no phenotypes with EDS, of the patients were 22.8 and 47.8 nmol/mg creatinine, respectively (Supplemental Table S1). On the other hand, the amounts of DS disaccharides were 0.2 ~ 1.6 nmol/mg creatinine in the father, mother, and sisters of patients #D7 and D8 (Supplemental Table S1). The amounts of CS and DS from family members of patients were similar to those from respective sex-matched and closely age-matched normal subjects (Tables 3 and 4). Heterozygous carriers for *CHST14* mutations, with no phenotypic features of the disorder, showed a similar level of DS to healthy subjects with no genotypic information available.

4. Discussion

In the present study in which a disaccharide compositional analysis was performed on urinary CS and DS chains in patients with EDS caused by a CHST14/D4ST1 deficiency, DS chains were not detected in any patient, which was significantly different from age- and sex-matched healthy controls. These results suggest general DS depletion in this disorder; previous findings only showed DS depletion in skin fibroblasts, and not in urine [12, 14, 18, 24]. These results also indicate the usefulness of a urinary disaccharide compositionnal analysis of CS/DS chains as a non-invasive screening method for this disorder.

In the cultured skin fibroblasts of patients with EDS caused by a CHST14/D4ST1 deficiency, the DS side chain on DS-PG, decorin, was found to be replaced by CS, resulting in the disruption of the assembly of collagen fibrils [14, 18]. The loss of decorin in mice was previously reported to cause abnormal collagen fibrogenesis and skin fragility [30], and affected the binding of fibroblast growth factor-7 (FGF7) and FGF2 to keratinocytes [31]. Thus, post-translational modifications induced in decorin by DS play an important role in collagen fibril assembly and FGF signaling. Several frameshift mutations in the decorin gene (*DCN*), predicted to result in the loss of C-terminal amino acids, were found to cause congenital stromal corneal dystrophy, an autosomal dominant eye disorder characterized by diffuse bilateral corneal clouding with flake-like whitish opacities throughout the stroma [32]. No patients with EDS caused by a CHST14/D4ST1 deficiency exhibited corneal dystrophy, while no patients with congenital stromal corneal dystrophy caused by *DCN* mutations had EDS-like systemic features. This result suggests that the decorin core protein, rather than the DS side chain on decorin-PG is important for the formation of corneal collagen fibrils [32]. On the other hand, spondyloepimetaphyseal dysplasia, which is characterized by anomalies in the spine and epiphyses and metaphyses of long bones, resulting in a short stature and osteoarthritic changes in joints, has been reported to be caused by mutations in *BGN* encoding biglycan [33]. Biglycan is a DS-PG that is involved in skeletal growth and bone formation through signaling pathways including transforming growth factor-β, bone morphogenetic protein 4, and Wnt [34, 35, 36]. Patients with EDS caused by a CHST14/D4ST1 deficiency typically have congenital and progressive skeletal abnormalities [12-15, 18-25], suggesting that alterations in DS side chains on biglycan and other DS-PG(s) affect bone development in EDS patients.

EDSMC2 caused by a loss-of-function mutation in *DSE* has been reported in three patients from two families [17, 18]. Patients showed characteristic facial features, congenital contractures in the thumbs and feet, hypermobility of the finger, elbow, and knee joints, and a

susceptibility to atrophic scarring of the skin [17]. The enzymatic activity of DSE in fibroblasts from these patients was significantly weaker than that of healthy subjects [17]. In addition, the amount of DS from the fibroblasts of these patients was less than that in the control [17]. It may be difficult at present, with no urinary DS data available on patients with DSE deficiency, to identify which of the genes, *CHST14* or *DSE*, are associated with DS-defective EDS by measuring urinary DS.

The major characteristics of the kyphoscoliosis type of EDS are severe muscle hypotonia, generalized joint laxity, and scoliosis [37]. This type of EDS is caused by mutations in *PLOD1* encoding lysyl hydroxylase 1 (procollagen-lysine 2-oxoglutarate 5-dioxygenase 1), which hydroxylates lysyl residues on procollagen α-chains. The ratio of urinary lysyl pyridinoline to hydroxylysyl pyridinoline in these patients is abnormally high [38]. D4ST1-defective EDS has been classified as the kyphoscoliosis type of EDS without a lysyl hydroxylase deficiency (EDS-type VIB), based on similarities to the characteristic facial and skeletal features of the kyphoscoliosis type of EDS (EDS-type VIA) [13, 19]. Thus, the quantification of urinary DS is also a useful diagnostic test for identifing the kyphoscoliosis type of EDS with lysyl hydroxylase or D4ST1 deficiencies.

Chst14/D4st1-deficient mice have smaller body weights, kinked tails, and more fragile skin and are also less fertile than the wild type [39]. In addition, the impaired proliferation of neural stem cells, reduced neurogenesis, and altered subpopulations of radial glial cells have been demonstrated in *Chst14/D4st1*-deficient mice [40]. These phenotypes are partially consistent with those of patients with EDS caused by a CHST14/D4ST1 deficiency. However, the amount of the DS chain in *Chst14*$^{-/-}$*/D4st1*$^{-/-}$ mice was not reported in detail. An analysis of urinary DS in the knockout mice may support our results.

5. Conclusion

In conclusion, this is the first study to perform a urinary disaccharide compositional

analysis on CS/DS chains in patients with EDS caused by a CHST14/D4ST1 deficiency, and demonstrate the absence of DS chains. This result suggests systemic DS depletion in this disorder, and also proposes the usefulness of a urinary disaccharide compositional analysis of CS/DS chains as a non-invasive screening method for this disorder.

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Conflict of Interests

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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Abbreviations

CHST14, carbohydrate sulfotransferase 14; C4ST, chondroitin 4-*O*-sulfotransferase; CS,

chondroitin sulfate; D4ST, dermatan 4-*O*-sulfotransferase; DS, dermatan sulfate; DSE, dermatan sulfate epimerase; GAG, glycosaminoglycan; GalNAc, *N*-acetyl-D-galactosamine; GlcUA, D-glucuronic acid; IdoUA, L-iduronic acid; PG, proteoglycan.

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Figure legends

Fig. 1. HPLC profiles of digests of CS and DS prepared from urine samples following treatments with three kinds of chondroitinases.

CS and DS in the urine of a healthy subject, #N3 (*A, C, E*) and EDS patient, #D2 (*B, D, F*) were digested with chondroitinase ABC (*A, B*), a mixture of chondroitinases AC-I and AC-II (*C, D*), and chondroitinase B (*E, F*) into disaccharides for analyses of CS and DS together, CS alone, and DS alone, respectively. Each digest was labeled with 2AB, and 2AB-labeled CS/DS disaccharides were separated by anion-exchange HPLC on an amine-bound silica PA-G column using a linear gradient of NaH_2PO_4, as indicated by the *dashed line*. The elution positions of authentic 2-AB-labeled CS disaccharides are indicated by the numbered arrows: 1, ΔHexUA-GalNAc; 2, ΔHexUA-GalNAc(6S); 3, ΔHexUA-GalNAc(4S); 4, ΔHexUA(2S)-GalNAc(6S); 5, ΔHexUA(2S)-GalNAc(4S); 6, ΔHexUA-GalNAc(4S,6S). The longitudinal axis of chromatograms in panels E and F are magnified (4-fold).

Fig. 2. Comparison of CS and DS amounts in healthy controls and EDS patients.

The amount of CS/DS (*A*), CS (*B*), or DS (*C*) disaccharides in the urine of EDS patients is depicted in the bar graphs based on Tables 2-4. Sex- and age-matched urine from healthy control subjects (*shaded bars*) was utilized for comparisons (*black bars*). The error bars indicate standard errors.

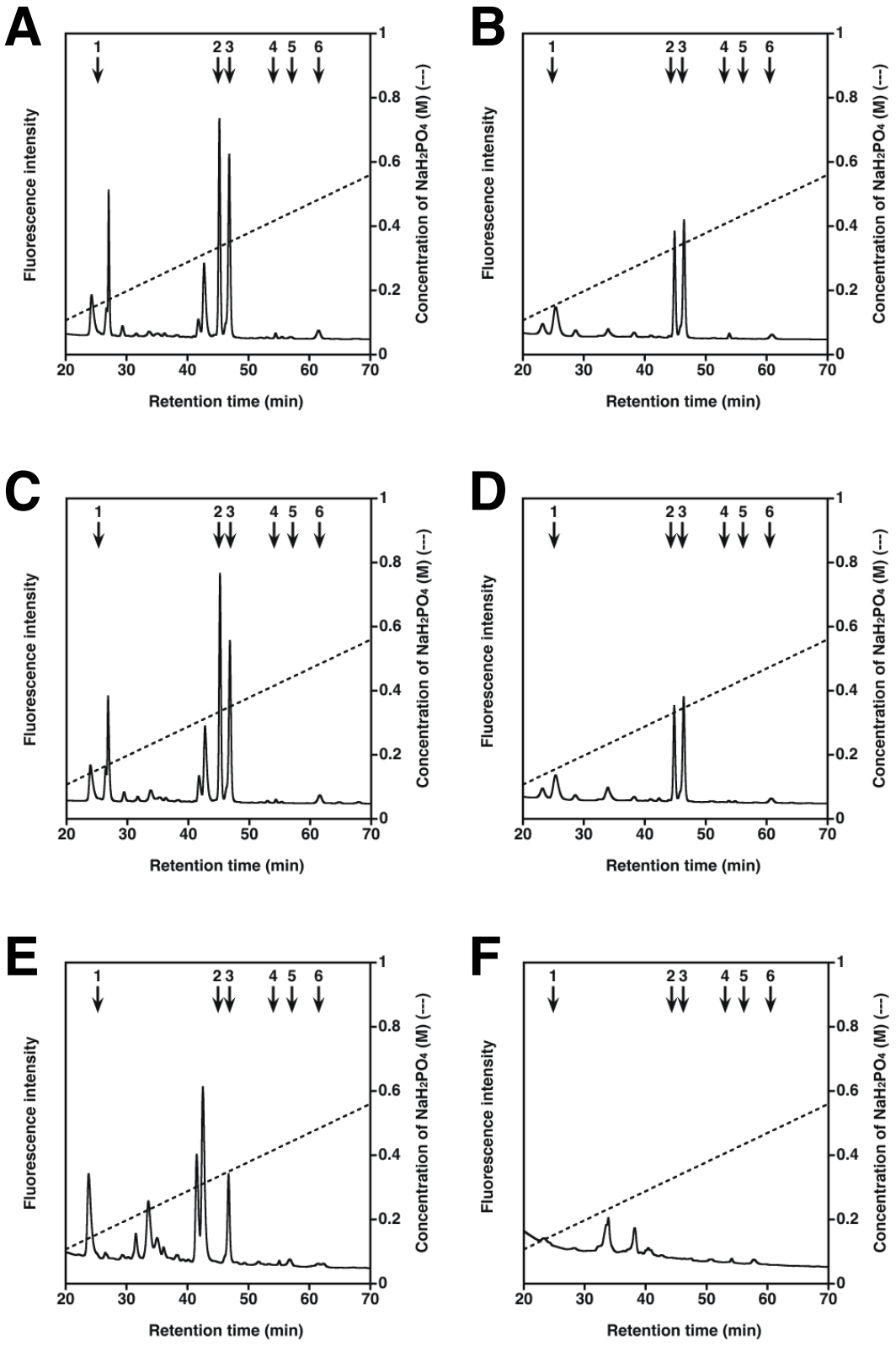


Fig. 1 **(Mizumoto *et al.*)**

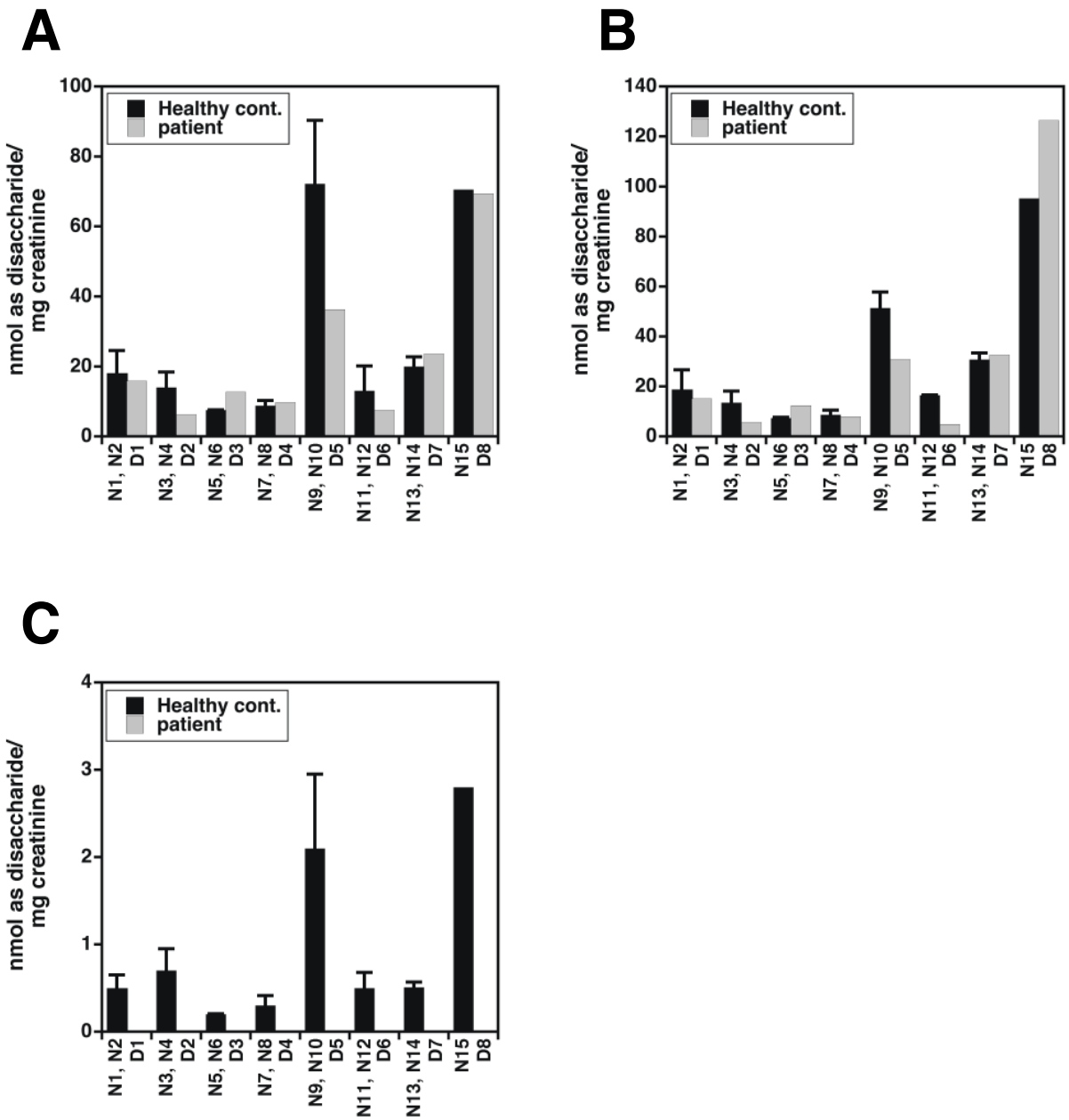


Fig. 2 **(Mizumoto *et al.*)**

Table 1. Urine preparations from healthy subjects and patients with a CHST14/D4ST1 deficiency.

No.	Sex	Age	*CHST14* mutations	Others
D1	F	11y	P281L/Y293C	Patient 1 in Ref. 17
D2	F	29y	P281L/P281L	Patient 2 in Ref. 17
D3	M	32y	P281L/P281L	Patient 3 in Ref. 17
D4	F	20y	P281L/C289S	Patient 5 in Ref. 17
D5	F	4y	P281L/Y293C	Patient 6 in Ref. 17
D6	F	41y	F209S/P281L	Ref. 25
D7	M	10y	F209S/P281L	Patient 2 in Ref. 20
D8	M	3m	F209S/P281L	Brother of patient D7
Father	M	42y	P281L/WT	Father of D7 & D8
Mother	F	37y	F209S/WT	Mother of D7 & D8
Sister #1	F	11y	WT/WT	Sister #1 of D7 & D8
Sister #2	F	3y	P281L/WT	Sister #2 of D7 & D8
N1	F	12y	N.E.	Normal subject
N2	F	10y	N.E.	Normal subject
N3	F	29y	N.E.	Normal subject
N4	F	29y	N.E.	Normal subject
N5	M	30y	N.E.	Normal subject
N6	M	31y	N.E.	Normal subject
N7	F	18y	N.E.	Normal subject
N8	F	21y	N.E.	Normal subject
N9	F	3y	N.E.	Normal subject
N10	F	3y	N.E.	Normal subject
N11	F	39y	N.E.	Normal subject
N12	F	43y	N.E.	Normal subject
N13	M	10y	N.E.	Normal subject
N14	M	11y	N.E.	Normal subject
N15	M	6m	N.E.	Normal subject

WT, wild-type.

N.E., not examined (no features of EDS caused by a CHST14/D4ST1 deficiency).

Table 2. Disaccharide composition of CS and DS chains in urine of healthy subjects and EDS patients.

Urine samples were individually digested with chondroitinase ABC for the analysis of CS and DS together, and each digest was treated with 2-AB to label the yielded CS/DS-derived disaccharides, which were analyzed by anion-exchange HPLC (Fig. 1). The amount of resultant disaccharides in each sample was calculated based on the peak area in each chromatogram.

	Normal #N1	Normal #N2	Normal #N3	Normal #N4	Normal #N5	Normal #N6	Normal #N7	Normal #N8
	nmol/mg creatinine (*mol%*)							
ΔO[a]	2.8 (24.3)	4.2 (17.0)	3.4 (18.3)	2.8 (28.9)	2.8 (37.1)	2.8 (37.2)	2.6 (25.1)	2.2 (29.8)
ΔC	3.8 (32.9)	7.7 (31.4)	7.0 (38.3)	3.1 (31.6)	2.1 (27.9)	2.5 (34.0)	3.5 (34.2)	2.2 (29.3)
ΔA	4.7 (40.0)	12.1 (49.3)	7.3 (39.7)	3.5 (36.5)	2.5 (32.8)	2.0 (26.8)	3.8 (37.5)	2.8 (38.4)
ΔD	0.09 (0.8)	0.2 (0.8)	0.2 (0.9)	0.05 (0.5)	0.04 (0.5)	0.05 (0.7)	0.07 (0.6)	0.04 (0.6)
ΔB	0.01 (0.1)	0.04 (0.2)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
ΔE	0.2 (1.8)	0.3 (1.3)	0.5 (2.9)	0.2 (2.4)	0.1 (1.6)	0.1 (1.3)	0.3 (2.6)	0.1 (2.0)
ΔT	N.D.[b]	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Total	11.6 (100)	24.5 (100)	18.4 (100)	9.6 (100)	7.5 (100)	7.5 (100)	10.3 (100)	7.3 (100)

	Normal #N9	Normal #N10	Normal #N11	Normal #N12	Normal #N13	Normal #N14	Normal #N15
	nmol/mg creatinine (*mol%*)						
ΔO	11.7 (21.7)	11.1 (12.3)	4.0 (19.8)	2.0 (33.5)	N.D.	N.D.	N.D.
ΔC	12.4 (23.0)	21.7 (24.1)	7.2 (35.7)	1.7 (28.5)	3.4 (19.9)	3.9 (17.0)	11.2 (16.0)
ΔA	28.6 (52.9)	53.3 (59.0)	7.8 (39.0)	2.0 (34.9)	13.8 (80.1)	18.9 (83.0)	58.0 (82.2)
ΔD	0.2 (0.3)	0.6 (0.7)	N.D.	N.D.	N.D.	N.D.	0.7 (1.0)
ΔB	0.3 (0.5)	1.5 (1.6)	0.4 (1.8)	N.D.	N.D.	N.D.	N.D.
ΔE	0.9 (1.6)	2.1 (2.3)	0.7 (3.6)	0.2 (3.0)	N.D	N.D	0.6 (0.8)
ΔT	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Total	54.1 (100)	90.3 (100)	20.1 (100)	5.9 (100)	17.3 (100)	22.7 (100)	70.5 (100)

	Patient #D1	Patient #D2	Patient #D3	Patient #D4	Patient #D5	Patient #D6	Patient #D7	Patient #D8
	nmol/mg creatinine (*mol%*)							
ΔO	2.9 (18.3)	1.5 (24.5)	2.4 (19.0)	1.6 (16.3)	5.9 (16.2)	2.0 (26.7)	N.D.	N.D.
ΔC	5.4 (33.9)	2.3 (36.9)	5.2 (40.4)	5.1 (52.5)	11.3 (31.1)	2.0 (26.8)	4.9 (20.6)	9/0 (13.0)
ΔA	7.1 (44.3)	2.1 (34.2)	4.6 (35.6)	2.6 (27.0)	18.1 (50.0)	3.3 (43.8)	18.7 (79.4)	59.1 (85.1)
ΔD	0.2 (1.4)	0.1 (1.4)	0.2 (1.5)	0.2 (2.1)	0.5 (1.3)	N.D.	N.D.	0.8 (1.2)
ΔB	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
ΔE	0.3 (1.8)	0.2 (3.0)	0.5 (3.5)	0.2 (2.1)	0.5 (1.4)	0.2 (2.7)	N.D.	0.5 (0.7)
ΔT	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Total	15.9 (100)	6.2 (100)	12.7 (100)	9.7 (100)	36.3 (100)	7.5 (100)	23.6 (100)	69.4 (100)

a, ΔO,ΔC, ΔA, ΔD, ΔB, ΔE, and ΔT represent the unsaturated disaccharides, ΔHexUA-GalNAc, ΔHexUA-GalNAc(6S), ΔHexUA-GalNAc(4S), ΔHexUA(2S)-GalNAc(6S), ΔHexUA(2S)-GalNAc(4S), ΔHexUA-GalNAc(4S,6S), and ΔHexUA(2S)-GalNAc(4S,6S), respectively. ΔHexUA, GalNAc, 2S, 4S, and 6S stand for 4,5-unsaturated hexuronic acid, *N*-acetyl-D-galactosamine, 2-*O*-, 4-*O*-, and 6-*O*-sulfate, respectively.

b, not detected (<0.01 nmol/mg creatinine).

Table 3. Disaccharide composition of CS chains in urine of healthy subjects and EDS patients.

Urine samples were individually digested with a mixture of chondroitinases AC-I and AC-II for the analysis of CS only, and each digest was treated with 2-AB to label the yielded CS-derived disaccharides, which were analyzed by anion-exchange HPLC (Fig. 1). The amount of resultant disaccharides in each sample was calculated based on the peak area in each chromatogram.

	Normal #N1	Normal #N2	Normal #N3	Normal #N4	Normal #N5	Normal #N6	Normal #N7	Normal #N8
	nmol/mg creatinine (*mol%*)							
ΔO	1.8 (16.1)	4.4 (16.7)	3.3 (18.4)	2.6 (29.4)	2.8 (36.8)	2.6 (37.2)	2.6 (25.2)	2.1 (30.9)
ΔC	4.1 (37.3)	8.5 (31.7)	7.6 (41.8)	2.9 (32.8)	2.2 (28.5)	2.5 (35.7)	3.6 (34.3)	2.1 (31.4)
ΔA	4.8 (44.1)	13.3 (49.9)	6.6 (36.4)	3.1 (35.1)	2.5 (32.8)	1.8 (25.5)	3.9 (37.4)	2.4 (35.7)
ΔD	0.06 (0.5)	0.1 (0.5)	0.1 (0.5)	0.03 (0.3)	0.03 (0.3)	0.03 (0.5)	0.04 (0.4)	0.02 (0.3)
ΔB	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
ΔE	0.2 (2.0)	0.3 (1.2)	0.5 (2.9)	0.2 (2.4)	0.1 (1.5)	0.08 (1.1)	0.3 (2.7)	0.1 (1.7)
ΔT	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Total	11.0 (100)	26.6 (100)	18.1 (100)	8.8 (100)	7.6(100)	7.0 (100)	10.4 (100)	6.7 (100)

	Normal #N9	Normal #N10	Normal #N11	Normal #N12	Normal #N13	Normal #N14	Normal #N15
	nmol/mg creatinine (*mol%*)						
ΔO	13.3 (29.6)	15.9 (27.6)	6.9 (41.7)	2.5 (15.5)	N.D.	N.D.	N.D.
ΔC	12.4 (27.4)	17.0 (29.5)	6.5 (39.5)	9.5 (57.9)	7.4 (25.6)	8.1 (25.0)	21.0 (22.1)
ΔA	19.2 (42.7)	23.8 (41.2)	2.9 (17.7)	4.4 (26.6)	21.5 (74.4)	24.4 (75.0)	73.5 (77.2)
ΔD	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
ΔB	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
ΔE	0.1 (0.3)	1.0 (1.7)	0.2 (1.1)	N.D.	N.D.	N.D.	0.7 (0.7)
ΔT	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Total	45.0 (100)	57.7 (100)	16.5 (100)	16.4 (100)	28.8 (100)	32.5 (100)	95.2 (100)

	Patient #D1	Patient #D2	Patient #D3	Patient #D4	Patient #D5	Patient #D6	Patient #D7	Patient #D8
	nmol/mg creatinine (*mol%*)							
ΔO	2.7 (17.9)	1.3 (23.4)	2.3 (18.5)	1.2 (14.7)	4.7 (15.1)	1.6 (33.3)	N.D.	N.D.
ΔC	5.2 (34.5)	2.2 (39.2)	5.2 (41.9)	4.4 (54.9)	9.7 (31.4)	1.4 (28.2)	9.5 (29.1)	21.9 (17.3)
ΔA	6.8 (45.4)	1.9 (34.0)	4.4 (35.6)	2.2 (27.6)	15.9 (51.6)	1.4 (28.4)	23.1 (70.9)	103.2 (81.6)
ΔD	0.05 (0.3)	0.02 (0.4)	0.04 (0.4)	0.05 (0.6)	0.1 (0.4)	N.D.	N.D.	0.3 (0.3)
ΔB	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
ΔE	0.3 (1.8)	0.2 (3.0)	0.5 (3.6)	0.2 (2.1)	0.4 (1.4)	0.5 (10.1)	N.D.	1.0 (0.8)
ΔT	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Total	15.1 (100)	5.6 (100)	12.4 (100)	8.0 (100)	30.8 (100)	4.9 (100)	32.6 (100)	126.4 (100)

Table 4. Disaccharide composition of DS chains in urine of healthy subjects and EDS patients.

Urine samples were individually digested with chondroitinase B for the analysis of DS only, and each digest was treated with 2-AB to label the yielded DS-derived disaccharides, which were analyzed by anion-exchange HPLC (Fig. 1). The amount of resultant disaccharides in each sample was calculated based on the peak area in each chromatogram.

	Normal #N1	Normal #N2	Normal #N3	Normal #N4	Normal #N5	Normal #N6	Normal #N7	Normal #N8
	nmol/mg creatinine (*mol%*)							
ΔO	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
ΔC	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
ΔA	0.3 (100)	0.6 (100)	0.9 (100)	0.4 (100)	0.2 (95.9)	0.2 (100)	0.4 (92.3)	0.2 (100)
ΔD	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
ΔB	N.D.	N.D.	N.D.	N.D.	0.01 (4.2)	N.D.	0.03 (7.8)	N.D.
ΔE	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
ΔT	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Total	0.3 (100)	0.6 (100)	0.9 (100)	0.4 (100)	0.21 (100)	0.2 (100)	0.43 (100)	0.2 (100)

	Normal #N9	Normal #N10	Normal #N11	Normal #N12	Normal #N13	Normal #N14	Normal #N15
	nmol/mg creatinine (*mol%*)						
ΔO	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
ΔC	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
ΔA	1.1 (91.9)	2.6 (90.2)	0.6 (91.1)	0.2 (61.8)	0.4 (100)	0.6 (100)	2.3 (80.6)
ΔD	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
ΔB	0.1 (8.1)	0.3 (9.8)	0.06 (8.9)	0.1 (38.2)	N.D.	N.D.	0.5 (19.4)
ΔE	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
ΔT	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Total	1.2 (100)	2.9 (100)	0.66 (100)	0.3 (100)	0.4 (100)	0.6 (100)	2.8 (100)

	Patient #D1	Patient #D2	Patient #D3	Patient #D4	Patient #D5	Patient #D6	Patient #D7	Patient #D8
	nmol/mg creatinine (*mol%*)							
ΔO	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
ΔC	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
ΔA	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
ΔD	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
ΔB	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
ΔE	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
ΔT	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Total	—	—	—	—	—	—	—	—

Supplemental Table S1. Disaccharide composition of CS and DS chains in urine of family members of EDS patients.

Urine samples from the family of EDS patient #D7 were individually digested with chondroitinases ABC, AC, or B for analyses of CS/DS, CS, or DS, respectively, and each digest was treated with 2-AB to label the yielded CS/DS-derived disaccharides, which were analyzed by anion-exchange HPLC (data not shown). The amount of resultant disaccharides in each sample was calculated based on the peak area in each chromatogram.

CS/DS disaccharide analysis by chondroitinase ABC

	Father (42y)	Mother (37y)	Sister #1 (11y)	Sister #2 (3y)
	nmol/mg creatinine (*mol%*)			
ΔO	N.D.	N.D.	N.D.	N.D.
ΔC	0.4 (21.2)	0.7 (20.6)	1.9 (15.8)	3.8 (12.5)
ΔA	1.4 (78.8)	2.5 (73.5)	9.9 (81.0)	26.0 (85.4)
ΔD	N.D.	N.D.	0.1 (1.2)	0.3 (1.1)
ΔB	N.D.	N.D.	N.D.	N.D.
ΔE	N.D.	0.2 (5.9)	0.2 (2.0)	0.3 (1.0)
ΔT	N.D.	N.D.	N.D.	N.D.
Total CS	1.8 (100)	3.4 (100)	12.3 (100)	30.5 (100)

CS disaccharide analysis by chondroitinase AC

	Father (42y)	Mother (37y)	Sister #1 (11y)	Sister #2 (3y)
	nmol/mg creatinine (*mol%*)			
ΔO	N.D.	N.D.	N.D.	N.D.
ΔC	1.0 (29.8)	2.0 (28.4)	5.1 (22.4)	8.3 (17.3)
ΔA	2.3 (70.2)	4.7 (67.7)	17.3 (75.8)	39.1 (81.8)
ΔD	N.D.	N.D.	N.D.	N.D.
ΔB	N.D.	N.D.	N.D.	N.D.
ΔE	N.D.	0.3 (3.9)	0.4 (1.9)	0.9 (0.9)
ΔT	N.D.	N.D.	N.D.	N.D.
Total CS	3.3 (100)	7.0 (100)	22.8 (100)	47.8 (100)

DS disaccharide analysis by chondroitinase B

	Father (42y)	Mother (37y)	Sister #1 (11y)	Sister #2 (3y)
	nmol/mg creatinine (*mol%*)			
ΔO	N.D.	N.D.	N.D.	N.D.
ΔC	N.D.	N.D.	N.D.	N.D.
ΔA	0.2 (100)	0.5 (100)	0.7 (80.9)	1.3 (83.2)
ΔD	N.D.	N.D.	N.D.	N.D.
ΔB	N.D.	N.D.	0.2 (19.1)	0.3 (16.8)
ΔE	N.D.	N.D.	N.D.	N.D.
ΔT	N.D.	N.D.	N.D.	N.D.
Total DS	0.2 (100)	0.5 (100)	0.9 (100)	1.6 (100)

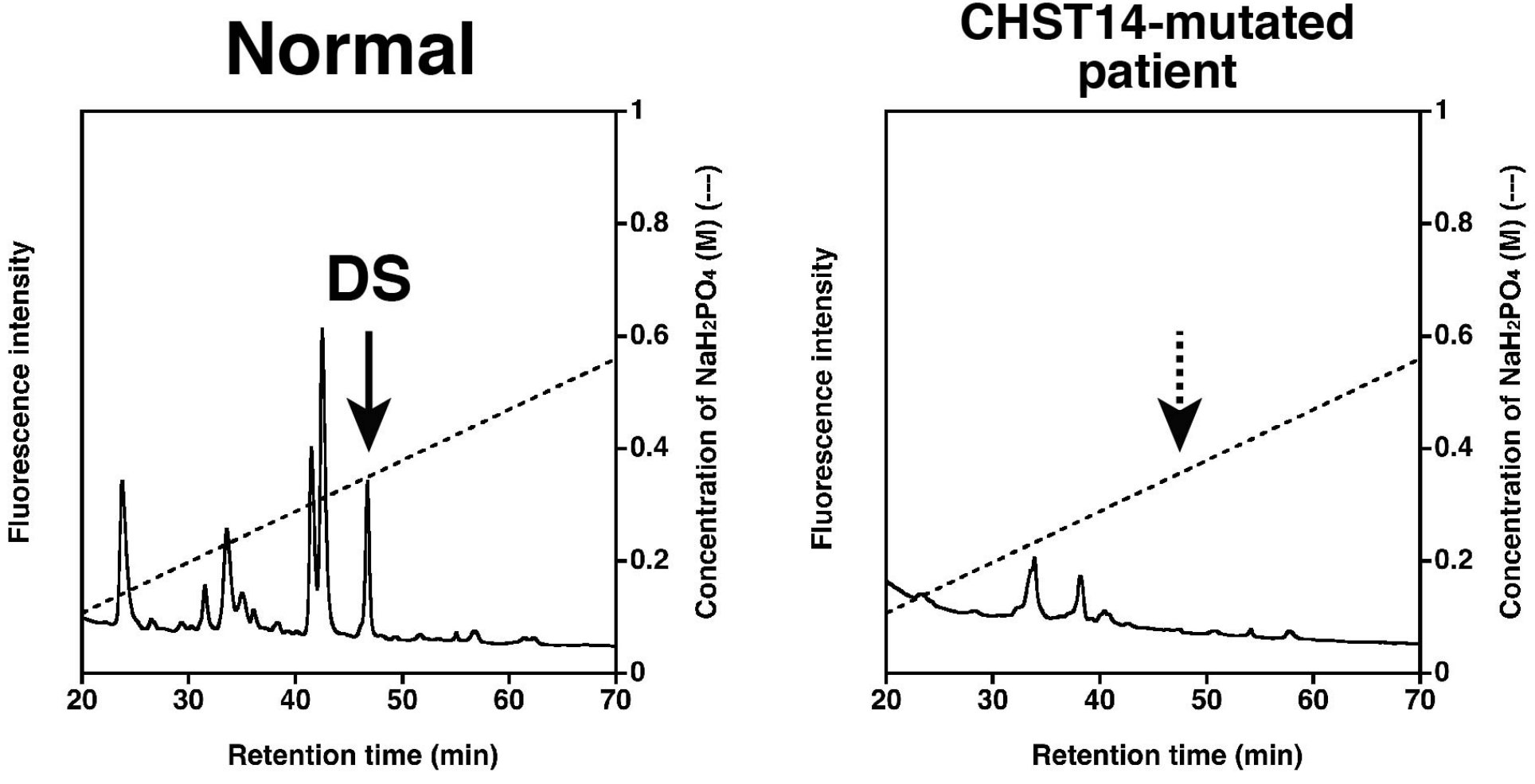
Highlight

(Mizumoto *et al.*, Defect in dermatan sulfate in urine of patients with Ehlers-Danlos syndrome caused by a CHST14/D4ST1 deficiency)

・CHST14/D4ST1 deficiency causes a specific type of Ehlers-Danlos syndrome (EDS)

・No urinary dermatan sulfate was detected in the EDS patients with CHST14 deficiency

・Measurement of urinary dermatan sulfate could be a non-invasive screening of the EDS with CHST14 deficiency


Normal
DS
Fluorescence intensity
Concentration of NaH2PO4 (M) (---)
Retention time (min)
20
30
40
50
60
70
0
0.2
0.4
0.6
0.8
1
CHST14-mutated patient
Fluorescence intensity
Concentration of NaH2PO4 (M) (---)
Retention time (min)