Note

Measurement of Serum Tenascin-X in Joint Hypermobility Syndrome Patients

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Joint hypermobility syndrome (JHS) (also termed hypermobility type Ehlers–Danlos syndrome, hEDS) is a heritable connective tissue disorder that is characterized by generalized joint hypermobility, chronic pain, fatigue, and minor skin changes. Initially, it was reported that there is a small subset of patients with JHS/hEDS who have haploinsufficiency of tenascin-X (TNX). However, the relationship between *TNXB* and JHS/hEDS has not been reported at all afterwards. EDS was reclassified into thirteen types in 2017, and the causative gene of JHS/hEDS remained to be identified. Therefore, in this study in order to determine whether JHS/hEDS can be diagnosed by the concentrations of serum form of TNX (sTNX), we measured the concentrations of sTNX in 17 JHS/hEDS patients. The sTNX concentrations in half of the JHS/hEDS patients were significantly lower than those in healthy individuals. No mutations, insertions or deletions were detected in the TNX exon sequence of the JHS/hEDS patients except for one in patient. That patient has a heterozygous mutation. A correlation between sTNX concentration and mutation of the *TNXB* genomic sequence was not found in the JHS/hEDS patients. These results indicate that the decrease in sTNX concentration could be used as a risk factor for JHS/hEDS.

Key words joint hypermobility syndrome; Ehlers–Danlos syndrome; tenascin-X

INTRODUCTION

Joint hypermobility syndrome (JHS) is a hereditary connective tissue disorder mainly characterized by generalized joint hypermobility, complications of joint instability, chronic pain, and minor skin changes.^{1,2)} Severe complaints such as back pain, myalgia/myofascial pain, dysmenorrhea and fatigue have also been reported in patients with JHS. Since the characteristics of JHS and hypermobility type Ehlers–Danlos syndrome (hEDS) significantly overlap, it is clinically very difficult to distinguish JHS from hEDS.³⁾ Complete deficiency of tenascin-X (TNX) causes an autosomal recessive classic type of EDS that is characterized by joint hypermobility, hyperelastic skin, and easy bruising without atrophic scarring.⁴⁾ TNX, an extracellular protein, plays essential roles in fibrillogenesis,⁵⁾ epithelial-to-mesenchymal transition,⁶⁾ and wound healing.⁷⁾

In 1997, EDS was classified into six types including hEDS.⁸⁾ Initially, Zweers *et al.* reported that haploinsufficiency of *TNXB* was associated with JHS/hEDS in a small subset of the patients.⁹⁾ However, the relationship between *TNXB* and JHS/hEDS has not been reported at all afterwards. Therefore, since the genetic basis of JHS/hEDS has been largely unknown and other new causative genes of EDS have been also identified, EDS was reclassified into thirteen types in 2017.¹⁰⁾ According to this, TNX-deficient type of EDS was reclassified as classical-like EDS (cEDS).¹⁰⁾ On the other hand, the causative gene of JHS/hEDS remained to be identified.¹⁰⁾

Clinical diagnostic criteria are helpful to determine JHS/hEDS. The most commonly used diagnostic criteria for JHS/hEDS were proposed by Beighton *et al.*⁸⁾; the major criteria include a Beighton score of 4/9 and arthralgia for more than 3 months in more than 4 joints, and the diagnosis is also based on minor criteria including a history of joint dislocation, soft tissue lesions, and skin hyperextensibility or scarring.¹¹⁾ However, there are no specific molecular tests for evaluation of JHS/hEDS.

Recently, we have developed a quantification method for the serum form of TNX (sTNX) using nano-liquid chromatography tandem mass spectrometry (LC/MS/MS) and reported that this method is useful for diagnosis of cEDS.¹²⁾ In this study, to determine whether JHS/hEDS can be diagnosed by sTNX concentration in sera, we measured sTNX concentrations from 17 JHS/hEDS patients by nano-LC/MS/MS and analyzed the whole exome sequences of the patients.

MATERIALS AND METHODS

Collection of Blood Samples from Patients with JHS/hEDS and Control Healthy Individuals Approval was obtained from the Ethics Committees of Nippon Medical School (Tokyo, Japan) and Shimane University School of Medicine (Izumo, Japan). Patients and healthy individuals were required to give informed consent to be included in this study. Blood samples were collected from patients with JHS/hEDS and healthy control individuals who came to Nippon Medical

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Table 1. sTNX Concentrations in Sera from Patients with JHS/hEDS

JHS/hEDS patient No.	1	3	4	7	10	12	14	22	29	31	34	40	45	51	53	68	73
JHS/hEDS family No.	7	1	5	8	6	9	10	11	12	10	8	14	13	15	16	18	19
Pedigree of proband	Proband	Affected mother of JHS/hEDS 2	Proband	Proband	Proband	Proband	Proband	Proband	Proband	Affected mother of JHS/hEDS 14	Affected mother of JHS/hEDS 7	Proband	Proband	Proband	Proband	Proband	Proband
Age	22	42	34	27	16	19	32	28	25	64	57	31	26	25	22	42	33
sTNX concentration (ng/mL)	95 ± 10	72 ± 5	76 ± 6	71 ± 7	131 ± 1	87 ± 5	75 ± 11	138 ± 12	100 ± 8	80 ± 5	76 ± 8	97 ± 10	97 ± 6	80 ± 1	87 ± 3	83 ± 6	83 ± 8
Mean ± S.E.																	
Student's <i>t</i> -test	n.s.	##	##	#	#	n.s.	#	n.s.	n.s.	##	#	n.s.	n.s.	##	#	#	n.s.
Ratio	0.86	0.66	0.7	0.65	1.19	0.8	0.68	1.26	0.92	0.73	0.69	0.88	0.88	0.73	0.8	0.76	0.75
MLPA	—	N. P.	—	—	—	—	—	—	—	N. P.	N. P.	—	—	—	—	—	—
WES	—	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—

The sTNX concentrations in sera from JHS/hEDS patients were measured at least two times by nano-LC/MS/MS. Data are mean ± standard error (S.E.). The mean sTNX concentration in sera from healthy individuals was 110 ± 6 (ng/mL, Mean ± S.E., $n = 3$). Statistical analysis was performed as compared with control sTNX concentration using Student's *t*-test. [#] $p < 0.05$, ^{##} $p < 0.01$, n.s.: not significant. Relative values of sTNX concentration in sera from JHS/hEDS patients against control sTNX concentration measured by nano-LC/MS/MS were calculated. *a*) Heterozygous nonsense mutation [NM_019105.7:c.4957>T (p.Arg1653*)] was inherited from unaffected mother. Minor allele frequency: 0.0002 (1000 genome project), 0.0002724 (4/14686, South Asian individuals in Exome aggregation consortium), in-house Japanese 0.00 (0/574). MLPA, Multiplex ligation-dependent probe amplification; N, P.; not performed; WES, Whole exome sequencing; +, positive; —, negative.

Fig. 1. Correlation between Results of Nano-LC/MS/MS and Western Blot Analyses

Relative value of sTNX concentration measured by nano-LC/MS/MS was plotted against relative intensity of sTNX determined by Western Blot analysis. Western blot analysis was performed using JHS/hEDS sera of which the relative value obtained by LC/MS/MS analysis was less than 0.8.

School Hospital. Inclusion criteria for this study were based on JHS/hEDS diagnostic criteria,¹⁰ *i.e.*, generalized joint hypermobility (Beighton score >4). Profiles of 17 patients with JHS/hEDS (patient no. 1, 3, 4, 7, 10, 12, 14, 22, 29, 31, 34, 40, 45, 51, 53, 68 and 73) [all females, aged between 16 and 64 years (average age of 32.1 years)] were described previously.¹³ Profiles of 50 healthy control individuals were as follows: all of the healthy control individuals were females aged between 25 and 71 years (average age of 55.1 years). The blood samples were centrifuged at $1500 \times g$ for 15 min at 4°C and serum (JHS/hEDS patients) or plasma (healthy control individuals) was collected, frozen immediately, and kept at -80°C until use. A mixed plasma sample of healthy control individuals was obtained by adding an equal volume of each plasma sample from the healthy control individuals.

Nano-LC/MS/MS To determine sTNX concentration, nano-LC/MS/MS analysis was carried out as described in our previous paper.¹² The peptide AVAVSGLDPAK was used for the quantification of sTNX.¹²

Western Blot Analysis To examine relative intensity of sTNX, Western blot analysis was performed as described in our previous paper.¹²

Genomic Analysis Whole exome sequencing (WES) was performed to identify *TNXB* pathogenic mutations. Genomic DNA was partitioned using the SureSelect Human All Exon v4 Kit (Agilent Technologies, Santa Clara, CA, U.S.A.) and sequenced by Illumina HiSeq2000 (Illumina, San Diego, CA, U.S.A.) as previously described.¹⁴ Candidate variants of *TNXB* were selected by excluding synonymous variants present in more than five of our in-house database of 574 Japanese individuals or in an allele frequency of ≥ 0.01 in the Exome Aggregation Consortium (ExAC, <http://exac.broadinstitute.org/>) database. We evaluated the pathogenicity of candidate missense variants using in-silico prediction tools, SIFT (<http://sift.bii.a-star.edu.sg/>), Polyphen2 (<http://genetics.bwh.harvard.edu/pph2/>), and MutationTaster (<http://www.mutationtaster.org/>).

In order to detect deletions or duplications of *TNXB*, we performed multiplex ligation-dependent probe amplification (MLPA, P155, MRC-Holland, Amsterdam, the Netherlands),

according to the manufacturer's instruction. Electrophoresis was conducted by using a 3130xl or 3500xl genetic analyzer (Thermo Fisher Scientific, Waltham, MA, U.S.A.) and the data were analyzed with GeneMapper software (Thermo Fisher Scientific).

Statistical Analysis Data were subjected to unpaired Student's *t*-test for statistical analysis. A *p*-value <0.05 was considered to indicate a statistically significant difference. Results are expressed as means $\pm$ standard error (S.E.). The correlation coefficient between the results obtained by the LC/MS/MS and Western blot analyses was calculated by the CORREL function in Excel 2010 (Microsoft, Redmond, WA, U.S.A.).

RESULTS AND DISCUSSION

To determine whether JHS/hEDS can be diagnosed by sTNX concentration, we measured the concentrations of sTNX in healthy individuals and 17 patients with JHS/hEDS using a quantitative method by nano-LC/MS/MS as described previously.¹² The criteria for this study were based on JHS/hEDS diagnostic criteria,¹⁰ *i.e.*, generalized joint hypermobility (Beighton score >4). The mean sTNX concentration in sera from healthy individuals was 110 ng/mL (Table 1). This value was almost the same as that in our previous study.¹² No age difference in serum TNX levels was found in control healthy individuals. The mean sTNX concentration in serum samples from half of the JHS/hEDS patients (patient no. 3, 4, 7, 14, 31, 34, 51, 53, 68) was significantly lower than that in serum samples from healthy individuals as shown in Table 1. We also analyzed the relative abundance of sTNX in sera of JHS/hEDS patients by Western blot analysis compared with that of control healthy individuals. The results obtained by nano-LC/MS/MS analysis were consistent with those obtained by Western blot analysis (Fig. 1).

Subsequently, to examine whether mutations of *TNXB* are indeed present in JHS/hEDS patients who have a significantly low sTNX concentration, we analyzed the whole exome sequences in all JHS/hEDS patients. However, no mutations, insertions or deletions in *TNXB* were detected in the JHS/hEDS patients in this study except for one in patient. The patient (no. 12) has a heterozygous nonsense mutation resulting in a premature stop codon at the position of 1653 (Table 1). However, the sTNX concentration in the patient was not statistically different from that in healthy individuals. Thus, an association between sTNX concentration and mutation of the *TNXB* genomic sequence was not found in the JHS/hEDS patients.

Previously, Zweers *et al.* reported that mean sTNX concentration in 80 patients with hEDS (approx. 90% female) is not different from that in control individuals, but six of these patients (7.5% [all female]) showed reduced sTNX levels (65%).⁹ However, they did not show *TNXB* genomic sequences in these patients. In the present study, we demonstrated that mean sTNX concentration in the 17 patients (90 ng/mL) is not different from that in control individuals (110 ng/mL) and the sTNX concentrations of two (patient nos. 3 and 7) in the 17 patients (11.8%) showed 72 and 71 ng/mL, respectively, which are approx. 65% of the control sTNX concentration, although they did not have any mutations, insertions or deletions in *TNXB*. Therefore, in JHS/hEDS patients our results concerning to the frequency of patients who show reduced sTNX concentration (approx. 65%) seem to be almost the same as those

of Zweers *et al.*⁹⁾

At present, the causative genes of JHS/hEDS remain unclear. Recent transcriptome analysis using fibroblasts of patients with JHS/hEDS showed increased expression of inflammatory proteins.¹⁵⁾ We also reported that levels of proteins involved in the complement system including complement C1r subcomponent, complement component C9, vitronectin, and C4b-binding protein alpha chain were increased in the sera of JHS/hEDS patients used in the present study compared with those in sera from controls.¹³⁾ These results indicate the possibility that the genes involved in inflammation are candidate genes for the progression of JHS/hEDS.

In conclusion, we found out that sTNX concentrations in half of the 17 JHS/hEDS patients were significantly lower than those in healthy individuals and there were no mutations, insertions or deletions in *TNXB* except for one patient. At present, the reason for reduction in sTNX concentration without mutations of *TNXB* is not clear, but the expression of TNX might be affected by epigenetic changes that occur in the JHS/hEDS patients. Therefore, the results indicate that measurement of the sTNX concentration in patients with JHS/hEDS is beneficial and the decrease in sTNX concentration could be used as a risk factor for JHS/hEDS. To verify these possibility, further sample collection from the JHS/hEDS patients and analyses are necessary.

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Conflict of Interest The authors declare no conflict of interest.

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