Proteomic analysis for the identification of serum diagnostic markers for joint hypermobility syndrome

ATSUSHI WATANABE[1,2], KAZUMI SATOH[3], TOMOKO MANIWA[3] and KEN-ICHI MATSUMOTO[3]

[1]Division of Clinical Genetics, Nippon Medical School Hospital; [2]Department of Biochemistry and Molecular Biology, Nippon Medical School, Tokyo 113-8603; [3]Department of Biosignaling and Radioisotope Experiment, Interdisciplinary Center for Science Research, Organization for Research, Shimane University, Izumo, Shimane 693-8501, Japan

Received August 20, 2015; Accepted December 15, 2015

DOI: 10.3892/ijmm.2015.2437

Abstract. Joint hypermobility syndrome (JHS) (also termed Ehlers-Danlos syndrome, hypermobility type) is a heritable connective tissue disorder which is characterized by generalized joint hypermobility, chronic pain, dizziness, fatigue, and minor skin changes. However, it has yet to be determined in patients with JHS whether specific genetic factors are involved in the risk of developing the disorder. Therefore, interventions have been limited to symptomatic treatments, and biomarkers for diagnosis and therapy have not yet been identified. In the present study, to identify potential serum biomarkers for JHS, we examined proteins with differential levels in sera from patients with JHS and in sera from control individuals using isobaric tags for relative and absolute quantitation (iTRAQ) labeling in combination with nano LC-MALDI-TOF/TOF-MS/MS followed by ProteinPilot analysis. In the sera of patients with JHS, a total of 106 proteins with differential levels were identified, and they were further narrowed down to 6 proteins ($p<0.05$, patient vs. control). Of the 6 proteins, proteins involved in the complement system including complement C1r subcomponent (C1R), vitronectin (VTN), complement component C9 (C9), and C4b-binding protein alpha chain (C4BPA) were identified as increased proteins in sera from patients with JHS compared with those in sera from controls. We confirmed increased levels of C1R and VTN in sera from patients with JHS by western blot analyses. The results indicate the possibility of a locally occurring inflammatory process in patients with JHS.

Correspondence to: Professor Ken-ichi Matsumoto, Department of Biosignaling and Radioisotope Experiment, Interdisciplinary Center for Science Research, Organization for Research, Shimane University, 89-1 Enya-cho, Izumo, Shimane 693-8501, Japan
E-mail: matumoto@med.shimane-u.ac.jp

Key words: joint hypermobility syndrome, Ehlers-Danlos syndrome hypermobility type, complement system, proteomics, isobaric tags for relative and absolute quantitation

Introduction

Joint hypermobility syndrome (JHS) is a heritable connective tissue disorder characterized by generalized joint hypermobility (excessive range of movement in the joints), complications due to joint instability, minor skin changes, and chronic mild to severe pain (1-3). Severe complaints such as back pain, myalgias/myofascial pain, dysmenorrhea and fatigue have also been reported in patients with JHS (3). Patients with JHS often fall into social isolation, emotional distress and depression (3). Collective opinion is that JHS and Ehlers-Danlos syndrome, hypermobility type (EDS-HT) are not mutually exclusive and are in fact clinically overlapping disorders (4).

Zweers *et al* (5) reported that haploinsufficiency of a non-collagenous extracellular matrix (ECM) molecule, tenascin-X (TNX), is associated with JHS/EDS-HT. Of the 20 heterozygous individuals who exhibited haploinsufficiency of TNX regardless of clinical symptoms, 9 (45%) of the individuals presented with generalized joint hypermobility, recurring joint dislocations and chronic joint pain, as seen in patients with JHS/EDS-HT, whereas 6 (7.5%) of 80 patients with EDS-HT exhibited haploinsufficiency of TNX with significantly reduced serum TNX levels (5). These findings indicated that haploinsufficiency of TNX is present in but a small subset of patients with JHS/EDS-HT. Therefore, the causative gene in the majority of individuals with JHS remains unknown.

Joint hypermobility is measured by the Beighton nine-point scale (6). JHS can be defined by the Brighton criteria; the major criteria for this classification include a Beighton score of 4/9 or greater and arthralgia for more than 3 months in more than 4 joints, and the diagnosis is also based on minor criteria including joint dislocation, soft tissue lesions, and abnormal skin (7). However, at present there is no precise biomarker or reliable diagnostic test for JHS.

The development of proteomic analysis has assisted in comparisons of comprehensive differentially expressed proteins under pathological conditions and controls (8,9). Researchers have successfully applied quantitative proteome analyses using isobaric tags for relative and absolute quantitation (iTRAQ) technology (10), followed by nano liquid chromatography (nano LC)-matrix-assisted laser desorption ionization (MALDI)-time of flight (TOF/TOF)-tandem mass spectrometry (MS/MS) to reveal differentially expressed

proteins in lesion tissues and sera of patients with cardiovascular diseases such as aortic aneurysms and aortic valve stenosis. These analyses are valuable, as they elucidated the molecular mechanisms underlying these diseases and/or identified disease biomarkers in both tissue samples (11,12) and serum samples (13). However, to the best of our knowledge, proteomic analysis in the field of JHS had not been carried out prior to this research.

In the present study, in order to elucidate the distinct molecular alteration that will lead to the discovery of novel biomarkers in sera of patients with JHS, serum proteins with differential levels in patients with JHS compared with those in healthy individuals were investigated with iTRAQ labeling followed by nano LC-MALDI-TOF/TOF-MS/MS.

Patients and methods

Collection of blood samples from patients with JHS and controls. Blood samples were collected after approval from the Ethics Committee of Nippon Medical School (Tokyo, Japan). Blood samples from patients with JHS and healthy control individuals who came to Nippon Medical School Hospital were collected. The patients and healthy individuals provided written informed consent. Inclusion criteria for this study were based on JHS diagnostic criteria (14,15), modified by our experience to include the following: i) generalized joint hypermobility (Beighton score >4), ii) chronic joint pain, and iii) recurring joint dislocations. Clinical profiles of 18 patients with JHS (patient no. 1, 3, 4, 7, 10, 12, 14, 22, 29, 31, 34, 37, 40, 45, 51, 53, 68 and 73) [all were female, aged between 13 and 64 years (average age was 30.7 years)] in this study are shown in Table I. Blood samples were centrifuged at 1,500 x g for 15 min at 4˚C and the plasma layer was removed.

The blood samples for proteomic analyses and examination of correlations of iTRAQ ratios with western blot ratios were collected from 6 patients with JHS (patient no. 1, 4, 10, 12, 14 and 22) (average age, 25.2 years) and 6 control healthy individuals (no. 11, 13, 25, 36, 52, and 60) (all females; average age was 42 years) (Table II). In addition, other blood samples for western blot analyses were collected from 12 patients with JHS (patient no. 3, 7, 29, 31, 34, 37, 40, 45, 51, 53, 68 and 73) (average age, 33.9 years) and 7 control healthy individuals (no. 9, 21, 27, 35, 44, 55 and 57) (all females; average age, 51.3 years) (Table II). For control serum mixtures used in proteomic analyses and western blot analyses, sera mixed with equal amounts of 6 and 13 control healthy individuals were used, respectively.

Immunodepletion of abundant serum proteins. Fifty microliters of serum was first processed with an albumin and immunoglobulin (IgG) depletion SpinTrap column according to the manufacturer's instructions (GE Healthcare, Buckinghamshire, UK) and also according to our previous study (13) to remove the two major serum proteins, albumin and IgG. Thereafter, the serum samples were equilibrated with 50 mM triethylammonium bicarbonate (TEAB; Sigma, Tokyo, Japan) using Spin-X UF concentrators (Corning, Tokyo, Japan). Protein concentration was then determined using a bicinchoninic acid (BCA) protein assay reagent (Thermo Fisher Scientific, Rockford, IL, USA).

Trypsin digestion, iTRAQ labeling, strong cation exchange (SCX) chromatography, and nano LC. Sample preparation was performed according to the manufacturer's instructions (AB Sciex, Foster City, CA, USA) and according to our previous study (13). In order to investigate the differentially expressed serum proteins between a mixed sample of control healthy individuals and each sample taken from patients with JHS, 100 μg of the mixed sample consisting of sera of 6 control healthy individuals and an equivalent amount of each sample from the 6 patients with JHS were denatured by sodium dodecyl sulfate (SDS) and reduced by tris-(2-carboxyethyl) phosphine (TCEP), followed by cysteine alkylation with methylmethanethiosulfonate (MMTS). Subsequently, each sample was digested by trypsin (AB Sciex). Each digest was labeled with a different iTRAQ tag by an iTRAQ reagent multiplex kit (AB Sciex). iTRAQ label 114 or 115 was used for labeling the control mixed sample, whereas iTRAQ label 116 or 117 was used for each of the samples from patients with JHS. The labeled control mixed sample and sample from patients with JHS were combined. Subsequently, the combined sample was fractionated into 6 fractions by SCX chromatography according to the manufacturer's instructions (AB Sciex). Subsequently, each fraction was desalted by a Sep-Pak C18 cartridge according to the manufacturer's instructions (Waters, Milford, MA, USA). Further fractionation of each fraction from SCX chromatography to 171 spots was undertaken while mixing directly with a matrix [4 mg/ml alpha-cyano-4-hydroxycinnamic acid (CHCA); Wako, Osaka, Japan] with a DiNa nanoLC system (KYA Technologies, Tokyo, Japan) according to the manufacturer's instructions and our previous study (13). Subsequently, the spots were placed on an Opti-TOF LC/MALDI 384 target plate (AB Sciex) using a DiNa MaP fraction collector (KYA Technologies). Similarly, as a control in order to examine differentially expressed serum proteins between the mixed sample of control healthy individuals and each sample of the control individuals, the same experiments were also undertaken using these samples.

MALDI-TOF/TOF MS/MS analysis. The spots were analyzed on a 5800 MALDI-TOF/TOF MS/MS analyzer with TOF/TOF Series software (version 4.0; AB Sciex) to obtain MS and MS/MS data according to the manufacturer's instructions. The peptide data obtained from 5800 MALDI TOF/TOF MS/MS were analyzed with ProteinPilot™ 3.0 software using the Paragon protein database search algorithm (AB Sciex) (16). Search results were filtered for a global false discovery rate (FDR) of 5%, employing a decoy search strategy which utilized a reverse database constructed by AB Sciex (version 20081216, 40,978 entries). In the present study, the statistical analyses for iTRAQ were undertaken using ProteinPilot software (AB Sciex).

Panther classification analysis. In the present study, the Panther system (version 9.0) (http://www.pantherdb.org/) was used for protein classification analyses, as previously described (17). The statistical analyses of protein classification were performed with a statistical overrepresentation test using Panther software. The annotations of proteins were acquired from the UniProt database (http://www.uniprot.org/) and mentioned based on our knowledge.

Table I. Clinical profiles of patients with JHS in this study.

Clinical profile	No. (%) (n=18)
Family history of JHS	9 (50)
Joint dislocations (any)	18 (100)
Shoulders	16 (89)
Fingers	15 (83)
Knees	10 (56)
Wrists	7 (39)
Elbows	6 (33)
Ankles	6 (33)
Pain (any)	18 (100)
Joint pain	17 (94)
Back pain	16 (89)
Muscle pain	14 (78)
Skin manifestation (any)	7 (39)
Skin hyperextensibility	7 (39)
Fragile skin	3 (17)
Chronic fatigue	18 (100)
Bruising easily	12 (67)
Postural hypotension	18 (100)
Headache	14 (78)
Temporomandibular disorder	13 (72)
Recurrent caries	9 (50)
Gum fragility	11 (61)
Temporomandibular joint hypermobility	8 (44)
Gastritis	7 (39)
Abdominal pain	5 (28)
Chronic diarrhea	7 (39)

JHS, joint hypermobility syndrome.

Table II. Sera used for iTRAQ labeling followed by nano LC-MALDI-TOF/TOF-MS/MS proteomic analyses and western blot analyses.

Sera used for proteomic analyses		
JHS patient no.	Gender	Age (years)
1	F	22
4	F	34
10	F	16
12	F	19
14	F	32
22	F	28
Control individual no.		
11	F	45
13	F	53
25	F	44
36	F	16
52	F	44
60	F	50

Sera used for western blot analyses		
JHS patient no.	Gender	Age (years)
3	F	42
7	F	27
29	F	25
31	F	64
34	F	57
37	F	13
40	F	31
45	F	26
51	F	25
53	F	22
68	F	42
73	F	33
Control individual no.		
9	F	61
21	F	58
27	F	50
35	F	49
44	F	45
55	F	52
57	F	44

iTRAQ, isobaric tags for relative and absolute quantitation; JHS, joint hypermobility syndrome.

Western blot analysis. Western blot analyses were completed as described in our previous study (18). Briefly, albumin/IgG-immunodepleted serum samples and crude sera for western blot analyses of vitronectin (VTN) and complement C1r subcomponent (C1R) were electrophoresed through SDS-polyacrylamide gel (SDS-PAGE), and the proteins were then transferred onto Hybond ECL nitrocellulose membranes (GE Healthcare Japan, Hino, Japan). The amounts of albumin/IgG-immunodepleted sera (μg) and crude sera (μl) used for each analysis were as follows: VTN (10 μg and 0.75 μl) and C1R (10 μg and 0.5 μl), respectively. The membranes were reacted with a rabbit polyclonal anti-VTN antibody (dilution 5,000) and rabbit polyclonal anti-C1R antibody (dilution 2,500) (both from GeneTex, Irvine, CA, USA). The membranes were then reacted with donkey IRDye 680-conjugated anti-rabbit IgG (H+L) (dilution 5,000; LI-COR, Lincoln, NE, USA), followed by visualization using the infrared imaging system Odyssey (LI-COR). The intensity of each band that reacted with a corresponding antibody was measured for densitometric analysis of each protein level. To confirm equal levels of proteins per lane, nonspecific proteins were stained with Coomassie Brilliant Blue (CBB).

Statistical analysis. Data from triplicate experiments were subjected to unpaired Student's t-test for statistical significance. A

Table III. Proteins with differential levels in patients with JHS and in healthy control individuals.

Unused ProtScore[a]	Coverage[b] (%)	Peptides[c] (95%)	UniProt no.	Gene symbol	Protein name	iTRAQ ratio[d] average ± SE	P-value[e]	Molecular function
3.4	12.8	3	P00736	C1R	Complement C1r subcomponent	1.40±0.11	0.0100	Complement
211.3	42.8	133	P04114	APOB	Apolipoprotein B-100	1.26±0.05	0.0195	Ligand for LDL receptor
19.8	28.7	12	P04004	VTN	Vitronectin	1.23±0.06	0.0344	Cell adhesion molecule
6.0	16.3	4	P02748	C9	Complement component C9	1.15±0.12	0.0402	Complement
10.6	22.8	6	P04003	C4BPA	C4b-binding protein α chain	1.10±0.09	0.0372	Complement
6.2	52.4	4	P02766	TTR	Transthyretin	1.06±0.10	0.0171	Thyroxine and retinol-binding protein

[a]A score of protein confidence (ProtScore) for a detected protein that is calculated from the peptide confidence from spectra that are not already 'used' by higher scoring proteins in the experiments; [b]the percentage of matching amino acids from identified peptides; [c]the number of distinct peptides with at least 95% confidence in the experiments; [d]iTRAQ ratio of JHS patient sera compared with those of the serum mixture from 6 control individuals; [e]statistical analysis of iTRAQ ratio of JHS patient sera/control serum mixture compared with that of control sera/control serum mixture ($p<0.05$, JHS patient group vs. control group). iTRAQ, isobaric tag for relative and absolute quantitation; JHS, joint hypermobility syndrome.

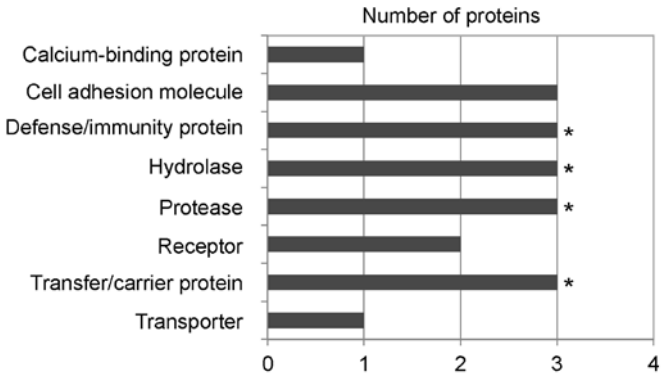

Figure 1. Panther protein class analysis of the 6 proteins with differential levels in sera from patients with joint hypermobility syndrome (JHS) and control individuals. Asterisks indicate a statistically significant difference ($p<0.05$).

p-value <0.05 was considered to indicate a statistically significant difference. Results are expressed as the means ± standard error (SE). The correlation coefficient between the iTRAQ value and western blot value was calculated by CORREL function in Excel 2010 (Microsoft, Redmond, WA, USA).

Results

Proteomic analyses of serum proteins with differential levels in patients with JHS and in healthy control individuals. Protein levels in serum from each of the patients with JHS were compared with mixed sera of the 6 healthy control individuals using iTRAQ labeling coupled with nano LC-MALDI-TOF/TOF-MS/MS followed by ProteinPilot analysis. Relative quantitation by ProteinPilot analysis was based on statistical analysis, as previously described (16). We set global FDR to 5%. The average iTRAQ ratios of peptides in sera of patients with JHS and those in sera of mixed control individuals were calculated. Aside from albumin and Ig family members, a total of 106 differential level proteins found in the sera of 6 patients with JHS were identified in at least one patient's serum compared with those in the mixture of the sera from 6 control individuals (data not shown). Of these proteins, 64 differential level proteins were identified as being in common with the sera of at least 4 patients, with an unused ProtScore of ≥3.4 (99.96% confidence) (data not shown). Subsequently, we examined the differential levels of the 64 identified proteins in the 6 healthy control individuals. Finally, 6 of the 64 proteins were identified as proteins with significantly different expression level in patient sera than in the sera of control individuals ($p<0.05$, JHS patient group vs. control group). In Table III, the six proteins [complement C1r subcomponent (C1R), apolipoprotein B-100 (APOB), VTN, complement component C9 (C9), C4b-binding protein alpha chain (C4BPA), and transthyretin (TTR)] are listed in order of iTRAQ ratios.

Classfication analyses of the six identified proteins. We examined the functional classification of each of the six identified proteins in sera of patients with JHS with Panther protein class analyses and Panther software, which sorts the proteins into respective classes based on their biological functions (Fig. 1). Defense/immunity proteins ($p=0.00018$) including complement components (C1R, C9 and C4BPA), hydrolase/protease including serine protease ($p=0.0073$) (C1R, C9 and C4BPA), and transfer/carrier proteins ($p=0.028$) (C9, C4BPA and TTR) were found to be statistically significant. VTN had previously been shown to be involved in the complement system: it acts as a potent inhibitor of complement activation by forming an inactive terminal complement complex, C5b-9 (19). Therefore, we suggest that at least four proteins (C1R, VTN, C9 and C4BPA) of the six identified proteins participate in the complement system.

Confirmation of iTRAQ ratios by western blot analysis. To validate the accuracy of quantitative results for the differentially expressed proteins that were identified, we focused on VTN and C1R due to their high iTRAQ ratios among the six proteins, as shown in Table III, and the participation of both proteins in

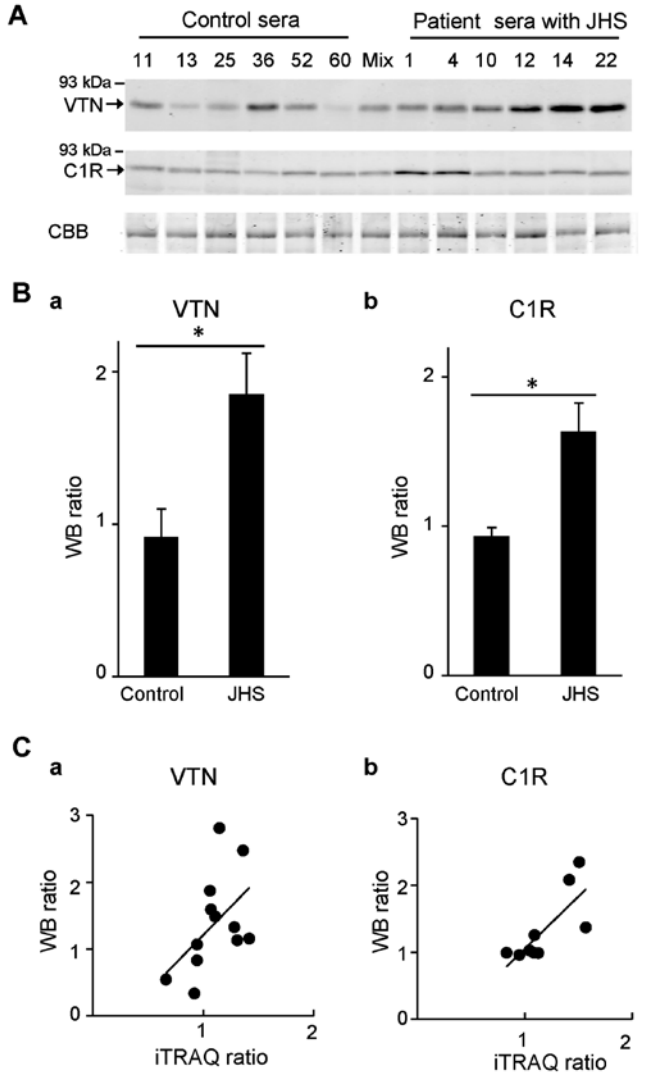

Figure 2. Confirmation of isobaric tags for relative and absolute quantitation (iTRAQ) ratios by western blot analyses. (A) Western blot analyses were performed with an anti-vitronectin (VTN) antibody and an anti-complement C1r subcomponent (C1R) antibody in the albumin/IgG-immunodepleted sera of 6 control individuals and 6 patients which were used for proteomic analyses. Mix indicates the mixture with equal amounts of the 6 control samples. Representative images are shown. To confirm equal levels of proteins per lane (5 μg), non-specific proteins stained with Coomassie Brilliant Blue (CBB) are shown in the lowest panel. The intensity of each band that reacted with the VTN antibody and C1R antibody indicated by an arrow was measured. (B) Ratios of levels of (a) VTN and (b) C1R in control mixed samples (left column) and samples from patients with joint hypermobility syndrome (JHS) (right column) compared with those in the control mixture (1.0), as determined by band intensity. The means ± SE of triplicate experiments were calculated, and statistical analysis was performed using the unpaired Student's t-test. $^{*}p<0.05$. (C) Correlation between iTRAQ and western blot ratios of each control and JHS sample compared to the control mixed samples for levels of (a) VTN (n=12) and (b) C1R (n=9).

the complement system. We quantified again VTN and C1R with an iTRAQ quantitative ratio by western blot analyses and investigated the correlations between iTRAQ ratios and relative quantitative ratios by western blot analyses (Fig. 2). The levels of VTN (iTRAQ ratio=1.23) and C1R (iTRAQ ratio=1.40) in albumin/IgG-immunodepleted serum samples of each of the 6 patients with JHS and the 6 control individuals used for iTRAQ analyses were examined by western blot analyses (Fig. 2A). Subsequently, the ratios of levels of VTN and C1R in these samples compared with those in the mixed control samples (1.0) were determined by examining band intensity, and the mean value of each group, as shown in Fig. 2B. We noted that relative quantitative ratios of the samples of patients with JHS compared to the mixed control samples were 1.85-fold for VTN and 1.63-fold for C1R. In addition, the correlations between iTRAQ ratios and western blot ratios of each JHS and control sample compared to the mixed 6 control samples were investigated (Fig. 2C). Both proteins demonstrated a tendency for positive correlation (VTN, n=12, r=0.51, p=0.094; C1R, n=9, r=0.77, p=0.015). These results indicated that iTRAQ ratios are almost consistent with quantitative results obtained by western blot analyses.

Verification of increased levels of VTN and C1R in patients with JHS compared with other sera. To further confirm the increased levels of VTN and C1R in sera of patients with JHS compared with those of control individuals, we performed western blot analyses using sera of 12 other patients with JHS and compared them with sera of 7 other control individuals, in addition to the sera of each of the 6 patients with JHS and control individuals which were used for the proteomic analyses. As a result, we confirmed increased levels of VTN and C1R in the sera of patients with JHS compared with those from the control individuals by western blot analyses (Fig. 3A). The ratios of levels of VTN and C1R in the sera of 18 patients with JHS compared with those in the mixed sera of the 13 control individuals (1.0) were measured by the intensity of each band, and mean ratios of the two groups were 1.68- and 1.11-fold, respectively (Fig. 3B). These results confirmed the increased levels of VTN and C1R in sera of other patients with JHS compared with those of control individuals.

Discussion

The molecular defect underlying JHS/EDS-HT remains largely unknown, and *TNX* has been identified as a gene in only 5-10% of patients with JHS/EDS-HT (5). As for serum disease markers for JHS, to the best of our knowledge, no attempt has yet been made to identify markers. In the present study, using iTRAQ labeling followed by nano LC-MALDI-TOF/TOF-MS/MS analysis, we determined the proteomic profiles of proteins with differential levels in sera of patients with JHS and in control individuals. Consequently, we identified six proteins, including four proteins involved in the complement system as proteins with differential levels in sera from patients with JHS and control individuals.

The complement system is not only involved in host defense recognition and the elimination of potentially harmful exogenous and endogenous microbial pathogens but also in different forms of acute and choronic inflammatory diseases such as sepsis and rheumatic disease. Previous research has revealed a fascinating interplay between the complement system and inflammatory network (20). C3a and C5a are known to be crucially involved in inflammatory responses. Activation of the complement system has been shown to be related to the pathogenesis of inflammatory arthritis and articular injury (21). However, the possible involvement of the complement system in the development of JHS remained unclear. In the present study, four proteins, C1R, VTN, C9 and

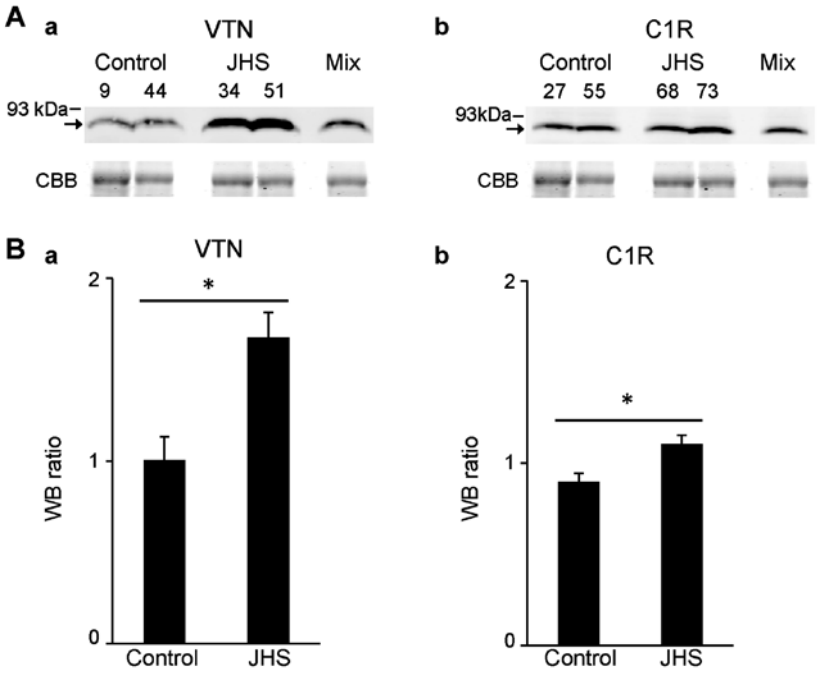

Figure 3. Verification of increased levels of vitronectin (VTN) and complement C1r subcomponent (C1R) in patients with joint hypermobility syndrome (JHS). (A) Western blot analyses with (a) anti-VTN antibody and (b) anti-C1R antibody in sera from 13 control individuals and 18 patients with JHS. Representative images of each of two control individuals [nos. 9 and 44 for VTN in (a) and nos. 27 and 55 for C1R in (b)] and those of two patients with JHS [nos. 34 and 51 for VTN in (a) and nos. 68 and 73 for C1R in (b)] as well as a mixture (Mix) of the sera from 13 control individual are shown. To confirm equal levels of proteins per lane (0.5 μl), non-specific proteins stained with Coomassie Brilliant Blue (CBB) are shown. The intensity of each band in the sera of 18 patients with JHS and the sera of 13 control individuals that reacted with each antibody was measured. (B) The intensity of each band in the sera of18 patients with JHS and of 13 control individuals compared with that in the mixture of sera from 13 control individual (1.0) was determined for ratios of levels of (a) VTN and (b) C1R. The means ± SE of triplicate experiments were calculated, and statistical analysis was performed using the unpaired Student's t-test. $^{*}p<0.05$.

C4BPA, were identified as proteins with differential levels in sera of patients with JHS and control individuals. Inflammation associated with joint instability, degenerative joint disease, and chronic pain is common in patients with JHS. C1R, which is a component of C1, is the serine protease responsible for intrinsic activation of the C1 complex of complement. It has been previously reported that C1R is synthesized and secreted in tissue and primary cell cultures of synovia from patients with rheumatoid arthritis, indicating the involvement of C1R in the inflammatory process in rheumatoid arthritis (22). VTN is a plasma multifunctional glycoprotein which is implicated in cell migration, blood coagulation, fibrinogenesis, the inflammatory process, and also membrane attack complex (MAC) formation. VTN binds directly to the C5b-7 complex and C9, with distinct binding sites on VTN (23). VTN also acts as an inhibitor of the cytolytic reactions of MAC. Notably, VTN and C9 have been identified as the differential level proteins in the sera of patients with JHS in the present study. It has previously been reported that C9 deposition was noted in the synovial vasculature of patients with acute arthritis and rheumatoid arthritis (24). In addition, it should be noted that C4BP is a potent circulating soluble inhibitor of the classical and lectin pathways of the complement system (25). C4BPA binds to C4b, and this interaction inhibits the complement activation pathway by reducing the formation and stability of C4bC2b (C3 convertase). Intriguingly, it has been shown that C4BP lacking the β-chain ($\alpha_7\beta_0$ isoform) induced a semimature and anti-inflammatory state in dendric cells activated by a pro-inflammatory stimulus (26).

Certain components of the local inflammatory response such as cytokines, neuropeptides and complement-related proteins, particularly C3a and C5a, are known to play roles in pain (27,28). Local injection of C5a and C3a elicited nociception, and C5a and C3a activate and sensitize cutaneous nociceptors (29). These results indicated that C5a and C3a are involved in pain. Patients with JHS exhibit chronic pain, distinct from that associated with acute dislocations (3). The changes in expression levels of some complement factors in patients with JHS compared with those in control individuals may contribute to such symptoms of chronic pain.

The six identified proteins include APOB and TTR as well as the four complement-related proteins. Plasma concentration of APOB is known to be a good marker of cardiovascular risk (30). APOB is a ligand for the low-density lipoprotein (LDL) receptor that participates in cholesterol transport to peripheral tissues and its accumulation in the arterial wall. Increased levels of apolipoprotein A-I (APOA1) and APOB have been observed in patients with osteoarthritis (31). TTR functions as a carrier for thyroxin and retinol-binding protein (RBP). Wilson (32) has reported that exome analysis of a patient with EDS classical type (type I) revealed three heterozygous mutations in TTR, fibrillin 1 (FBN1), and voltage-gated calcium channel subunit alpha Cav2.1 (CACNA1A) genes.

Our quantitative proteomic strategy employed in the present study allowed for the identification of six potential biomarkers of JHS, four of which are proteins involved in regulation of the complement system associated with inflammation and chronic pain. Our results provide valuable information on

the underlying mechanisms of JHS and will contribute to the establishment of a method for early diagnosis of JHS and to the development of pharmacological therapies.

In conclusion, to the best of our knowledge, this is the first comprehensive study on differentially expressed proteins in the sera of patients with JHS and control individuals using an approach involving iTRAQ labelling. Proteomic characterization of the sera of patients with JHS revealed increased levels of four proteins involved in the regulation of the complement system. Future work should focus on deciphering the role of each of the 6 potential biomarker proteins identified in the pathogenesis of JHS/EDS-HT.

Acknowledgements

This study was supported by the Ministry of Health Labour and Welfare for Health Labour Sciences Research Grant (Research on Measures for Intractable Diseases) grant no. 2011-Nanchi-Ippan-110 to A.W. and the Japan Society for the Promotion of Science (JSPS) KAKENHI grant nos. 26462296 to K.M.

References

1. Grahame R: Joint hypermobility and genetic collagen disorders: are they related? Arch Dis Child 80: 188-191, 1999.
2. Malfait F, Hakim AJ, De Paepe A and Grahame R: The genetic basis of the joint hypermobility syndromes. Rheumatology (Oxford) 45: 502-507, 2006.
3. Castori M, Morlino S, Celletti C, Ghibellini G, Bruschini M, Grammatico P, Blundo C and Camerota F: Re-writing the natural history of pain and related symptoms in the joint hypermobility syndrome/Ehlers-Danlos syndrome, hypermobility type. Am J Med Genet A 161A: 2989-3004, 2013.
4. Tinkle BT, Bird HA, Grahame R, Lavallee M, Levy HP and Sillence D: The lack of clinical distinction between the hypermobility type of Ehlers-Danlos syndrome and the joint hypermobility syndrome (a.k.a. hypermobility syndrome). Am J Med Genet A 149A: 2368-2370, 2009.
5. Zweers MC, Bristow J, Steijlen PM, Dean WB, Hamel BC, Otero M, Kucharekova M, Boezeman JB and Schalkwijk J: Haploinsufficiency of TNXB is associated with hypermobility type of Ehlers-Danlos syndrome. Am J Hum Genet 73: 214-217, 2003.
6. Beighton P: Hypermobility scoring. Br J Rheumatol 27: 163, 1988.
7. Grahame R, Bird HA and Child A: The revised (Brighton 1998) criteria for the diagnosis of benign joint hypermobility syndrome (BJHS). J Rheumatol 27: 1777-1779, 2000.
8. Sutton CW, Rustogi N, Gurkan C, Scally A, Loizidou MA, Hadjisavvas A and Kyriacou K: Quantitative proteomic profiling of matched normal and tumor breast tissues. J Proteome Res 9: 3891-3902, 2010.
9. Bijian K, Mlynarek AM, Balys RL, Jie S, Xu Y, Hier MP, Black MJ, Di Falco MR, LaBoissiere S and Alaoui-Jamali MA: Serum proteomic approach for the identification of serum biomarkers contributed by oral squamous cell carcinoma and host tissue microenvironment. J Proteome Res 8: 2173-2185, 2009.
10. Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, *et al*: Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. Mol Cell Proteomics 3: 1154-1169, 2004.
11. Matsumoto K, Satoh K, Maniwa T, Araki A, Maruyama R and Oda T: Noticeable decreased expression of tenascin-X in calcific aortic valves. Connect Tissue Res 53: 460-468, 2012.
12. Matsumoto K, Satoh K, Maniwa T, Tanaka T, Okunishi H and Oda T: Proteomic comparison between abdominal and thoracic aortic aneurysms. Int J Mol Med 33: 1035-1047, 2014.
13. Satoh K, Maniwa T, Oda T and Matsumoto K: Proteomic profiling for the identification of serum diagnostic biomarkers for abdominal and thoracic aortic aneurysms. Proteome Sci 11: 27, 2013.
14. Beighton P, De Paepe A, Steinmann B, Tsipouras P and Wenstrup RJ: Ehlers-Danlos syndromes: revised nosology, Villefranche, 1997. Ehlers-Danlos National Foundation (USA) and Ehlers-Danlos Support Group (UK). Am J Med Genet 77: 31-37, 1998.
15. Levy HP: Ehlers-Danlos Syndrome, Hypermobility Type. In: GeneReviews (Internet). Pagon RA, Adam MP, Ardinger HH, *et al* (eds). Last Update: September 13, 2012; University of Washington, Seattle, 1993-2015.
16. Shilov IV, Seymour SL, Patel AA, Loboda A, Tang WH, Keating SP, Hunter CL, Nuwaysir LM and Schaeffer DA: The Paragon Algorithm, a next generation search engine that uses sequence temperature values and feature probabilities to identify peptides from tandem mass spectra. Mol Cell Proteomics 6: 1638-1655, 2007.
17. Mi H, Muruganujan A and Thomas PD: PANTHER in 2013: modeling the evolution of gene function, and other gene attributes, in the context of phylogenetic trees. Nucleic Acids Res 41: D377-D386, 2013.
18. Nakamura Y, Takayama N, Minamitani T, Ikuta T, Ariga H and Matsumoto K: Primary structure, genomic organization and expression of the major secretory protein of murine epididymis, ME1. Gene 251: 55-62, 2000.
19. Podack ER and Müller-Eberhard HJ: Isolation of human S-protein, an inhibitor of the membrane attack complex of complement. J Biol Chem 254: 9808-9814, 1979.
20. Markiewski MM and Lambris JD: The role of complement in inflammatory diseases from behind the scenes into the spotlight. Am J Pathol 171: 715-727, 2007.
21. Mizuno M: A review of current knowledge of the complement system and the therapeutic opportunities in inflammatory arthritis. Curr Med Chem 13: 1707-1717, 2006.
22. Breitner S, Störkel S, Reichel W and Loos M: Complement components C1q, C1r/C1s, and C1INH in rheumatoid arthritis. Correlation of in situ hybridization and northern blot results with function and protein concentration in synovium and primary cell cultures. Arthritis Rheum 38: 492-498, 1995.
23. Milis L, Morris CA, Sheehan MC, Charlesworth JA and Pussell BA: Vitronectin-mediated inhibition of complement: evidence for different binding sites for C5b-7 and C9. Clin Exp Immunol 92: 114-119, 1993.
24. Konttinen YT, Ceponis A, Meri S, Vuorikoski A, Kortekangas P, Sorsa T, Sukura A and Santavirta S: Complement in acute and chronic arthritides: assessment of C3c, C9, and protectin (CD59) in synovial membrane. Ann Rheum Dis 55: 888-894, 1996.
25. Blom AM, Villoutreix BO and Dahlbäck B: Complement inhibitor C4b-binding protein-friend or foe in the innate immune system? Mol Immunol 40: 1333-1346, 2004.
26. Olivar R, Luque A, Naranjo-Gómez M, Quer J, García de Frutos P, Borràs FE, Rodríguez de Córdoba S, Blom AM and Aran JM: The $\alpha 7\beta 0$ isoform of the complement regulator C4b-binding protein induces a semimature, anti-inflammatory state in dendritic cells. J Immunol 190: 2857-2872, 2013.
27. Coutaux A, Adam F, Willer JC and Le Bars D: Hyperalgesia and allodynia: peripheral mechanisms. Joint Bone Spine 72: 359-371, 2005.
28. Jang JH, Liang D, Kido K, Sun Y, Clark DJ and Brennan TJ: Increased local concentration of complement C5a contributes to incisional pain in mice. J Neuroinflammation 8: 80, 2011.
29. Jang JH, Clark JD, Li X, Yorek MS, Usachev YM and Brennan TJ: Nociceptive sensitization by complement C5a and C3a in mouse. Pain 148: 343-352, 2010.
30. Thompson A and Danesh J: Associations between apolipoprotein B, apolipoprotein AI, the apolipoprotein B/AI ratio and coronary heart disease: a literature-based meta-analysis of prospective studies. J Intern Med 259: 481-492, 2006.
31. Sánchez-Enríquez S, Torres-Carrillo NM, Vázquez-Del Mercado M, Salgado-Goytia L, Rangel-Villalobos H and Muñoz-Valle JF: Increase levels of apo-A1 and apo B are associated in knee osteoarthritis: lack of association with VEGF-460 T/C and +405 C/G polymorphisms. Rheumatol Int 29: 63-68, 2008.
32. Wilson GN: Exome analysis of connective tissue dysplasia: death and rebirth of clinical genetics? Am J Med Genet A 164A: 1209-1212, 2014.