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ORIGINAL ARTICLE

Diversity of humoral responses to the centromere proteins among HCV-related chronic liver disease, PBC and AIH patients

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Summary
Background: Anticentromere antibodies (ACAs) have been observed in patients with autoimmune hepatitis (AIH) and hepatitis C virus (HCV)-related chronic liver disease (CLD-C) as well as those with primary biliary cirrhosis (PBC). However, little is known about the differences in immune responses to the centromere proteins among these liver diseases.
Objective: By synthesizing recombinant proteins consisting of the N- and C-termini of major centromere proteins, we investigated the humoral responses against them in each disease.
Results: Eight of the 754 (1%) patients with CLD-C, 14 of the 57 (25%) patients with PBC and six of the 38 (16%) patients with AIH were seropositive for ACAs. There were no significant differences in ACA titers determined by an indirect immunofluorescent method among the groups of patients with CLD-C, PBC and AIH. However, the analysis of immunoreactivities against each recombinant protein revealed that the titers of IgG-subclass autoantibodies against the C-terminus of centromere protein (CENP)-B were significantly higher in the CLD-C patients than in the AIH patients. Likewise, the titers of IgM-subclass autoantibodies against the N-terminus of CENP-A were significantly higher in the PBC group than in the CLD-C group. The ACA-positive patients who developed liver cirrhosis had significantly higher titers of the IgA-subclass autoantibodies against the C-terminus of CENP-C than those who did not.

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Conclusion: These findings suggest that immunoreactivities against the fragments of centromere proteins show distinct patterns among CLD-C, PBC and AIH and that the determination of immunoreactivities against the centromere proteins may be useful for the prediction of disease progression.

Introduction

Anticentromere antibodies (ACAs) were identified by Moroi et al. as a serological hallmark specific to CREST (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia) syndrome [1]. ACAs seem to recognize the centromere region of mitotic chromosomes and discrete speckles within interphase nuclei [2]. Centromeres are located in the regions in which microtubules of the spindle apparatus attach to chromosomes. The autoantigens of centromeres have been well characterized as proteins or polypeptide components tightly bound to the centromeric DNA of the chromosome [1]. Previous studies demonstrated that ACAs reacted with multiple centromere proteins (CENPs), three of which are regarded as major autoantigens of ACA: CENP-A (17 kD), CENP-B (80 kD) and CENP-C (140 kD) [3]. CENP-A was identified as a protein with high homology to histone H3 [4]. CENP-B has a DNA-binding domain which contains a helix-loop-helix DNA-binding motif at the N-terminus [5,6]. CENP-C is the main component of the kinetochore and seems to play an indispensable role in kinetochore function during mitosis and meiosis [7,8].

It is well established that ACAs are complementary serological markers of primary biliary cirrhosis (PBC) [9], because ACAs are often present in the sera of patients with PBC who have low titers of antimitochondrial antibodies (AMAs) or show seronegativity for AMA [10]. ACAs appeared to be a favorable prognostic serological hallmark for PBC patients, who had milder liver damage than PBC patients without ACAs [10]. In contrast, Nakamura et al. elucidated that PBC patients with ACAs often proceeded to portal hypertension [11].

Earnshaw et al. documented that the C-terminal domain of CENP-B was the most commonly recognized by ACAs [12]. Antibodies to CENP-B have thus been applied to the detection of ACAs using an enzyme-linked immunosorbent assay (ELISA) method [13]. However, autoantibodies against CENP-A are also detectable in ACA-positive sera by an ELISA technique [14]. There are a few interesting articles showing that ACAs were likely to react against CENP-A and CENP-B simultaneously in patients with scleroderma [15,16]. We previously revealed that ACAs were restricted to the N-terminus of CENP-A, whereas the C-terminal domain of CENP-A, which has homology to histone H3, turned out not to be antigenic [17]. The efficacy of using antibodies to CENP-C in patients with Sjögren's syndrome who simultaneously react with Ro and/or La has also been shown [18].

ACAs usually react with CENP-B in patients with PBC [10], autoimmune hepatitis (AIH) [19] or even in patients with hepatitis C virus (HCV)-related chronic liver disease (CLD-C) [20]. However, little has been known about whether humoral responses to other centromere antigens are evoked in patients with PBC, AIH or CLD-C. In addition, a distinct recognition of ACAs has been shown between patients with Sjögren's syndrome and those with limited scleroderma [21]. The primary purposes of this study were to analyze the autoimmune responses to the centromere proteins, including CENP-A, CENP-B and CENP-C, in patients with PBC, AIH and CLD-C in order to determine the humoral reactivities against these centromere autoantigens among these diseases, and to establish whether or not the reactivities against these centromere proteins predict the patients' prognosis.

Patients and methods

Study population

We enrolled 754 patients with CLD-C, 57 patients with PBC and 38 patients with type 1 AIH, who were admitted to the Hospital of the Kagawa University School of Medicine during the 10-year span from 2003 to 2013. This clinical study was approved by the Ethics Committee of the Kagawa University School of Medicine. Fully informed consent was obtained from each individual.

The clinical diagnosis of type 1 AIH was made on the basis of the scoring system proposed by the International Autoimmune Hepatitis Group [22]. All 38 of the study's AIH patients fulfilled the criteria for ''definite'' AIH. PBC was diagnosed using the internationally accepted criteria [23]. The clinical diagnosis of CLD-C was based on the emergence of HCV RNA in the patients' sera and histological or biochemical characteristics consistent with chronic hepatitis or liver cirrhosis. We investigated the patients' age at entry, gender, the prevalence of liver cirrhosis and the prevalence of concurrent CREST syndrome in the enrolled patients.

Laboratory assessments

Seropositivity for ACA was defined as a discrete speckled pattern on HEp-2 cells by an indirect immunofluorescence method at 1:40 or higher titers. AMAs were determined by an indirect immunofluorescent method. We considered 1:20 or higher titers as seropositive for AMA. Immunoglobulin A (IgA), immunoglobulin M (IgM) and immunoglobulin G (IgG) were also assessed in the ACA-positive patients.

Humoral responses to recombinant centromere protein fragments

Full-length CENP-A, -B, and -C cDNAs were kind gift from Dr. K. Yoda of Nagoya University. The N-termini of CENP-A (CENP-A-Nt, covering amino acids 1-49), CENP-B (CENP-B-Nt, covering amino acids 1-74), and CENP-C (CENP-C-Nt, covering amino acids 74-244) were subcloned into a pET28a vector to express recombinant protein with the 6-amino-acid-histidine tag. The C-termini of CENP-B (CENP-B-Ct, covering amino acids 535-599) and CENP-C (CENP-C-Ct, covering amino acids 811-943) were also subcloned into a pET28a vector using polymerase chain reaction (PCR) amplification as well as the above three cDNAs. These five regions are representative autoepitopes of ACAs according to our published data [6,8,17,24,25].

These five types of inserts were verified by sequencing. The centromere proteins were induced according to the manufacturer's protocol. The histidine-tagged recombinant proteins were purified using Ni Sepharose 6 Fast Flow (GE Healthcare, Buckinghamshire, UK) under denaturing conditions according to the manufacturer's protocol [26]. The ELISA was performed as described with a slight modification [27]. Briefly, the wells of microplates (MediSorp; Nunc, Roskilde, Denmark) were coated with purified recombinant proteins (CENP-A-Nt, 0.63 μg/well; CENP-B-Nt, 0.8 μg/well; CENP-B-Ct, 0.1 μg/well; CENP-C-Nt, 0.033 μg/well; CENP-C-Ct, 0.32 μg/well).

After blocking with (phosphate buffered saline with 0.05% Tween 20 [T-PBS] containing 5% skim milk, the wells were incubated with 1:100-diluted sera or serially diluted standard sera with high-titer antibodies against each recombinant in the Assay Diluent of the ELISA kits (Medical & Biological Laboratories, Nagoya, Japan) for 1 hour at room temperature. After five washes with T-PBS, the wells were incubated with 1:1000-diluted anti-human IgG, IgM, or IgA antibody-conjugated with HRP (DAKO, Glostrup, Denmark) in T-PBS, and then washed and developed. 1-Step ABTS (Pierce, Rockford, IL) was used as a substrate solution according to the manufacturer's protocol. Each serum sample from patients was tested in duplicate, and the mean optimal density (OD) at 405 nm was used for data analysis.

The corrected OD value, calculated by subtracting the background level from the OD value of each serum, was converted into ELISA unit using the SkanIt Software 2.5.1 program (Thermo Fisher Scientific, Waltham, MA). The high-titer antibody-positive serum diluted 1:5 serially, starting from 1:100, was run as a standard. Units generally correlate with the titers of antibodies: 1:100 dilution, 625 units; 1:500, 125 units; 1:2500, 25 units; 1:12,500, five units; 1:62,500, 1 unit; 1:312,500, 0.2 units. Each cut-off value of the autoantibody titer was determined as the mean of the units obtained from 26 control sera + 5 standard deviations (SD).

Histological assessments

Liver tissue specimens were obtained by liver biopsy under the guidance of ultrasound, using 16-gauge needles. The tissue samples were fixed in 10% formalin and embedded in paraffin. The tissue sections were stained with hematoxylin and eosin. The severity of fibrosis and necroinflammation in the liver were evaluated in accordance with the histological activity index (HAI) scores designed by Knodell et al. [28]. The histological evaluation of PBC was performed using Scheuer's classification system [29].

Statistical analysis

The data are represented as the mean ± SD. The Mann-Whitney *U* test and the Bonferroni/Dunn method were applied for the comparisons of two groups and three or more groups, respectively. $P < 0.05$ was considered statistically significant.

Results

Clinical characteristics of the enrolled patients

Twenty-eight patients were found to be seropositive for ACA: eight of the 754 (1%) patients with CLD-C, 14 of the 57 (25%) patients with PBC and six of the 38 (16%) patients with AIH had ACA. Nine of the 14 (64%) ACA-positive patients with PBC had AMAs, whereas none of the ACA-positive patients with CLD-C or AIH did.

The clinical characteristics of the 28 ACA-positive patients are shown in Table 1. The age at entry in patients with CLD-C was slightly higher than that in patients with PBC, although the difference was not significant. All of the ACA-positive patients were female except for one patient with AIH. There were no differences in the prevalence of liver cirrhosis among the groups of patients with CLD-C, PBC and AIH. The prevalence of concurrent CREST syndrome was almost equivalent among the three groups. The concurrent

Table 1 Clinical characteristics of the ACA-positive patients.

	CLD-C ($n = 8$)	PBC ($n = 14$)	AIH ($n = 6$)	*P*-values
Age (y.o.)	65.9 ± 11.3	55.8 ± 13.0	54.8 ± 8.8	N.S.
Gender (male/female)	(0/8)	(0/14)	(1/5)	N.S.
Prevalence of liver cirrhosis (%)	3 (38%)	2 (14%)	2 (33%)	N.S.
Prevalence of concurrent CREST syndrome (%)	2 (25%)	5 (36%)	2 (33%)	N.S.

CLD-C: HCV-related chronic liver disease; PBC: primary biliary cirrhosis; AIH: autoimmune hepatitis, N.S.: not significant. Data represent numbers. Parentheses represent frequencies.

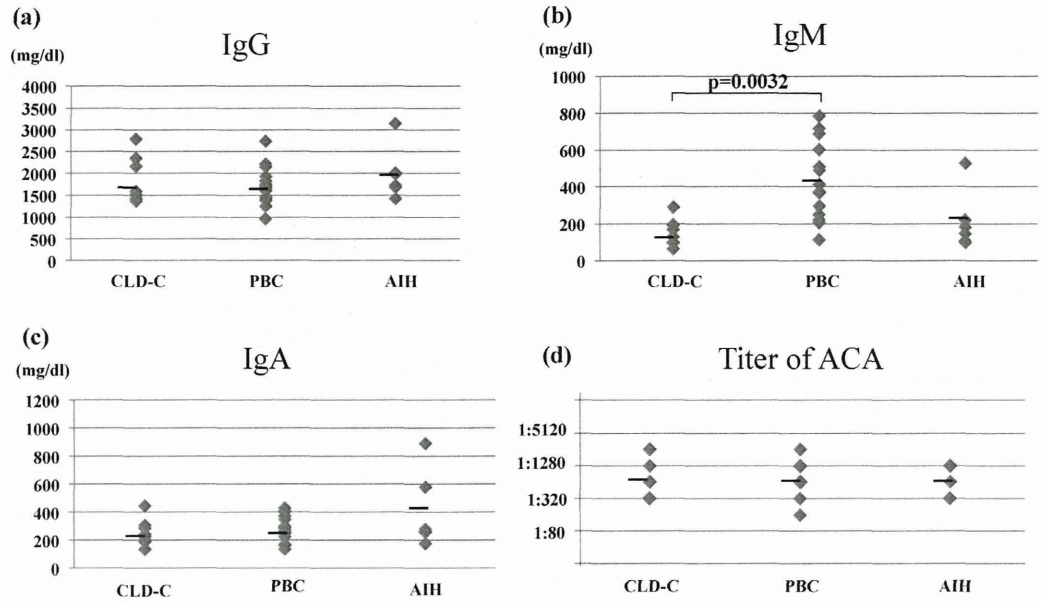

Figure 1 Distribution of serum (a) IgG, (b) IgM and (c) IgA level and (d) ACA titer in each group. ACA-seropositive PBC patients had significantly higher IgM levels than ACA-seropositive CLD-C patients with ACAs. Horizontal bar represents the average value in each group. CLD-C: HCV-related chronic liver disease; PBC: primary biliary cirrhosis; AIH: autoimmune hepatitis; ACA: anticentromere antibody.

CREST syndrome in the patients with ACAs was the incomplete type.

Comparison of laboratory data

The immunoglobulin subclass levels were compared between the groups of patients with CLD-C, PBC or AIH seropositive for ACA. As shown in Fig. 1a, the serum IgG levels were almost equivalent among the three groups. However, the patients with PBC seropositive for ACAs had significantly higher serum IgM levels than those with CLD-C seropositive for ACAs (431 ± 209 mg/dL vs. 179 ± 82 mg/dL, $P = 0.0032$, Fig. 1b). The ACA-positive AIH patients had a trend toward higher serum IgA levels (408 ± 275 mg/dL in AIH vs. 255 ± 95 mg/dL in CLD-C, 287 ± 95 mg/dL in PBC), although the difference was not significant (Fig. 1c).

The titers of ACAs by the indirect immunofluorescent method using HEp-2 cells, ranging from 1:160 to 1:2560, were almost equivalent among the three groups (Fig. 1d).

Humoral responses to the fragments of centromere proteins

The immunoreactivities against the recombinant centromere proteins were analyzed using the sera of ACA-positive patients. Fig. 2 shows the humoral responses of the IgG subclass to the N- and C-terminal domains of CENP-A, CENP-B and CENP-C in the groups of CLD-C, PBC and AIH patients seropositive for ACA. All sera seropositive for ACAs reacted with CENP-A-Nt and CENP-B-Ct (Fig. 2a and c). The titers of IgG subclass autoantibodies against CENP-B-Ct in the patients with CLD-C were significantly higher than those in the patients with AIH (465 ± 239 units vs. 210 ± 237 units, $P = 0.0397$, Fig. 2c).

As shown in Fig. 3a, the PBC patients had significantly higher titers of IgM-subclass autoantibodies against CENP-A-Nt than the CLD-C patients (293 ± 266 units vs. 65 ± 94 units, $P = 0.0312$), and a higher positive rate for this type of autoantibody than the patients of the other two groups (71% in the PBC group vs 38% in the CLD-C group and 33% in the AIH group). All ACA-positive patients had the IgM subclass of autoantibodies against CENP-C-Ct (Fig. 3c). None of the enrolled patients had IgM class autoantibodies against CENP-B-Nt or CENP-B-Ct.

There were no significant differences in the titers of IgA-subclass autoantibodies against CENP-A-Nt, CENP-C-Nt, and CENP-C-Ct among the three groups (Fig. 4a–c). No humoral responses of IgA-subclass to CENP-B-Nt or CENP-B-Ct were found in the enrolled patients.

Relationship between the reactivities against the fragments of centromere proteins and the development of liver cirrhosis or the concurrent CREST syndrome

As shown in Table 1, three patients with CLD-C, two patients with PBC and two patients with AIH developed liver cirrhosis. These seven patients had mild to moderate esophageal varices, reflecting portal hypertension. We compared the titers of autoantibodies against the fragments of centromere proteins between the patients with and without liver cirrhosis (Table 2a). The ACA-positive patients with liver cirrhosis had significantly higher titers of the IgA subclass of autoantibodies against CENP-C-Ct (454 ± 225 units vs. 237 ± 205 units, $P = 0.0460$) than those without liver cirrhosis.

Table 2b summarizes the relationship between autoantibodies against centromere protein fragments and concurrent CREST syndrome. There were significantly high

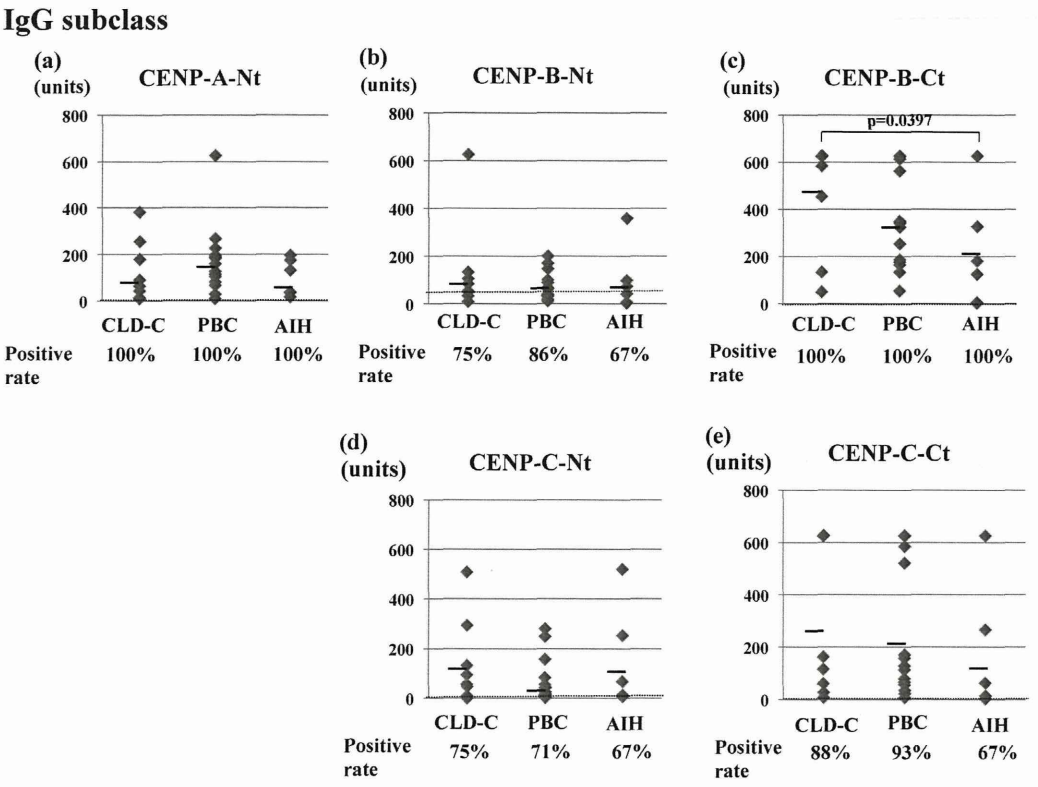

Figure 2 Titers of IgG-subclass autoantibodies against the N- and C-terminal domains of centromere antigens in each group. The CLD-C patients had significantly higher titers of IgG subclass autoantibodies against CENP-B-Ct than the AIH patients. Horizontal bar represents the average titer in each group. Broken line represents a cut-off value for each autoantibody. CLD-C: HCV-related chronic liver disease; PBC: primary biliary cirrhosis; AIH: autoimmune hepatitis; Nt: N-terminus; Ct: C-terminus.

titers of the IgG type of autoantibodies against CENP-C - Ct in the ACA-positive patients with CREST syndrome than those without CREST syndrome (388 ± 259 units vs. 154 ± 216 units, $P = 0.0453$).

Discussion

The results of the present study demonstrated a stronger response of the IgG subclass autoantibodies against CENP-B-Ct in patients with CLD-C, and stronger immunoreactivities of the IgM subclass autoantibodies against CENP-A-Nt in patients with PBC, suggesting that the diversity of humoral responses against the fragments of centromere proteins was shown among the patients with CLD-C, PBC and AIH. We found that the titers of ACAs determined by the indirect immunofluorescent technique on HEp-2 cells were almost equivalent in the patients with CLD-C, PBC and AIH. However, a diversity of humoral responses against the

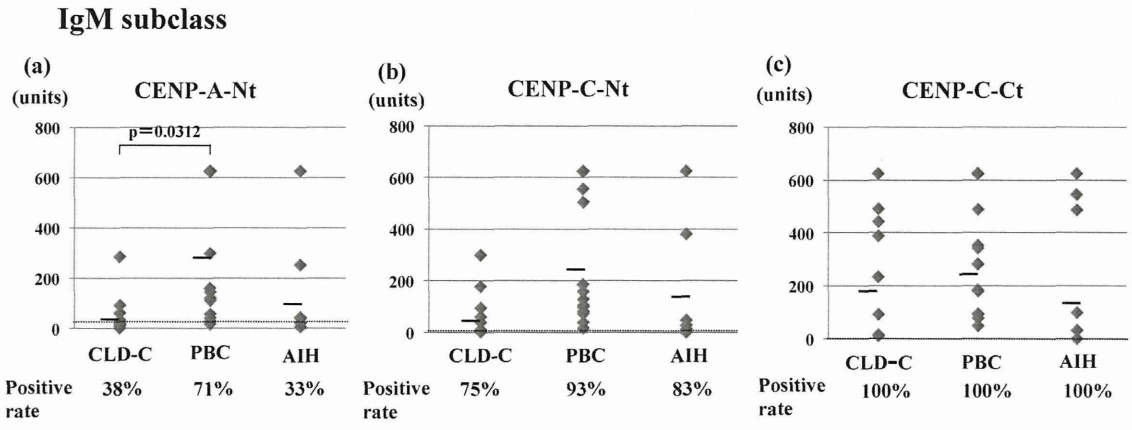

Figure 3 Titers of IgM-subclass autoantibodies against the N- and C-terminal domains of centromere antigens in each group. The PBC patients had significantly high titers of anti-CENP-A-Nt than the CLD-C patients. The positive rate for anti-CENP-A-Nt in the group of PBC patients was higher than those in the other groups. Horizontal bar represents the average titer in each group. Broken line represents a cut-off value for each autoantibody. CLD-C: HCV-related chronic liver disease; PBC: primary biliary cirrhosis; AIH: autoimmune hepatitis; Nt: N-terminus; Ct: C-terminus.

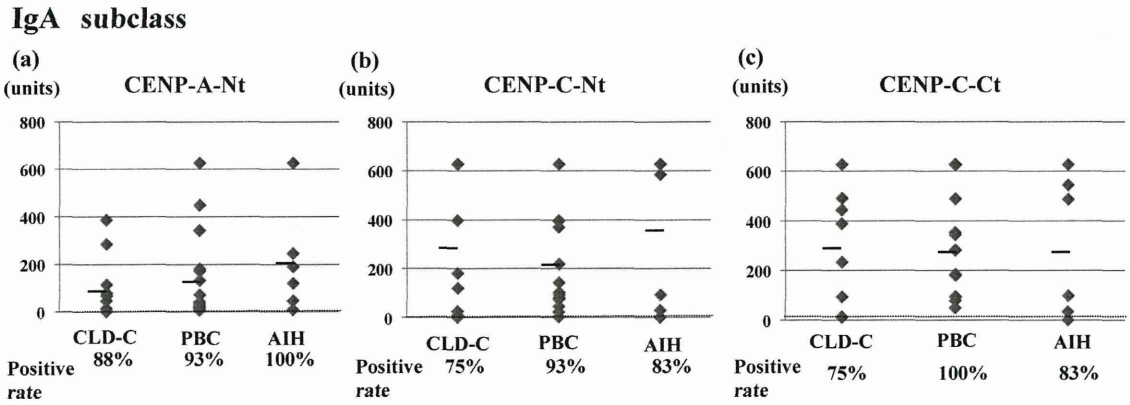

Figure 4 Titers of IgA-subclass autoantibodies against the N- and C-terminal domains of centromere antigens in each group. Horizontal bar represents the average value in each group. Broken line represents a cut-off value for each autoantibody. CLD-C: HCV-related chronic liver disease; PBC: primary biliary cirrhosis; AIH: autoimmune hepatitis; Nt: N-terminus; Ct: C-terminus.

Table 2a Relationship between autoantibodies against centromere fragments and the development of liver cirrhosis.

	Development of liver cirrhosis ($n=7$)	Without development of liver cirrhosis ($n=21$)	*P*-value
IgG			
Anti-CENP-A-Nt	119 ± 103	151 ± 142	N.S.
Anti-CENP-B-Nt	143 ± 109	79 ± 131	N.S.
Anti-CENP-B-Ct	470 ± 247	306 ± 212	N.S.
Anti-CENP-C-Nt	213 ± 219	72 ± 95	N.S.
Anti-CENP-C-Ct	388 ± 285	176 ± 221	N.S.
IgM			
Anti-CENP-A-Nt	197 ± 292	202 ± 107	N.S.
Anti-CENP-C-Nt	296 ± 241	168 ± 219	N.S.
Anti-CENP-C-Ct	149 ± 168	256 ± 218	N.S.
IgA			
Anti-CENP-A-Nt	131 ± 137	164 ± 195	N.S.
Anti-CENP-C-Nt	421 ± 270	175 ± 194	N.S.
Anti-CENP-C-Ct	454 ± 225	237 ± 205	0.0460

Nt: N-terminal; Ct: C-terminal; N.S.: not significant.

Table 2b Relationship between autoantibodies against centromere fragments and concurrent CREST syndrome.

	Concurrent CREST syndrome ($n=9$)	Without CREST syndrome ($n=19$)	*P*-value
IgG			
Anti-CENP-A-Nt	186 ± 184	123 ± 99	N.S.
Anti-CENP-B-Nt	149 ± 187	70 ± 81	N.S.
Anti-CENP-B-Ct	423 ± 238	311 ± 221	N.S.
Anti-CENP-C-Nt	153 ± 182	86 ± 135	N.S.
Anti-CENP-C-Ct	388 ± 259	154 ± 216	0.0453
IgM			
Anti-CENP-A-Nt	244 ± 288	180 ± 219	N.S.
Anti-CENP-C-Nt	278 ± 259	163 ± 208	N.S.
Anti-CENP-C-Ct	159 ± 87	262 ± 242	N.S.
IgA			
Anti-CENP-A-Nt	151 ± 215	158 ± 168	N.S.
Anti-CENP-C-Nt	280 ± 234	215 ± 241	N.S.
Anti-CENP-C-Ct	309 ± 181	283 ± 250	N.S.

Nt: N-terminus; Ct: C-terminus; N.S.: not significant.

N- and C-terminus of each centromere protein was observed among these three diseases. Therefore, it is worth analyzing the autoimmune responses to the N- and C-termini of these centromere proteins in patients with CLD-C, PBC and AIH.

Our findings indicated that the IgA type of autoantibodies against CENP-C-Ct is associated with the development of liver cirrhosis (portal hypertension). Tomita et al. showed that serum IgA levels were elevated in patients with a more advanced stage of nonalcoholic steatohepatitis [30], implying a correlation between the serum IgA level and hepatic fibrosis. ACA-positive PBC patients were previously considered to have a favorable prognosis [10], but an article revealed that ACA-positive PBC patients often proceeded to portal hypertension [11]. The diversity of humoral responses against centromere proteins shown in the present study may account for the discrepancy described above.

All of the enrolled patients seropositive for ACAs did not necessarily have the symptoms of CREST syndrome. We, thus, attempted to identify the autoantibodies against the fragments of centromere proteins closely associated with CREST syndrome. Our results revealed the relationship between the IgG type of autoantibodies against CENP-C-Ct and concurrent CREST syndrome in the ACA-positive patients. However, a previous report noted that the autoantibodies to CENP-A seemed to be associated with Raynaud's phenomenon in patients with CREST syndrome [31].

Our previous study revealed that the autoantigenic domain of CENP-A was not the C-terminus but the N-terminus [17]. We also elucidated that the HCV core antigen had molecular similarity with the N-terminus of CENP-A [17]. It has been well recognized that the sequence homology between the HCV core antigen and cytochrome p450 2D6 (CYP2D6) is likely to trigger the production of antibodies to liver-kidney microsome type 1 (anti-LKM1) [32]. Nonetheless, the humoral responses against CENP-A-Nt in the patients with CLD-C were not much stronger than those in the patients with PBC or AIH, indicating that the molecular mimicry between the HCV core antigen and CENP-A did not contribute to the humoral response to CENP-A-Nt. We also observed that a lack in the response of the IgM and IgA subclasses of autoantibodies against CENP-B in ACA-positive patients including those with CREST syndrome [24].

The present results also revealed that ACAs simultaneously recognized CENP-B and CENP-A and/or CENP-C as the target antigens in most of the enrolled patients with CLD-C, PBC and AIH, suggesting that the ACAs in those patients were frequently cross-reactive between CENP-B and CENP-A and/or CENP-C [3]. It was previously shown that ACAs were simultaneously directed against CENP-A and CENP-B in patients with scleroderma, whereas the ACAs in most patients with Sjögren's syndrome or systemic lupus erythematosus (SLE) were primarily directed against either CENP-A or CENP-B [15]. It is of interest that all sera of the enrolled ACA-positive patients responded to CENP-A-Nt, CENP-B-Ct in the IgG subclass and CENP-C-Ct in the IgM class. Shoji et al. revealed that all sera positive for ACAs reacted with CENP-A [33].

ACAs are commonly found in the sera of patients with PBC [9,10], but the emergence of ACA is uncommon in the sera of patients with AIH [19] or CLD-C [20]. It is thus difficult to obtain a sufficient number of serum samples seropositive for ACAs in patients with autoimmune liver disease at a single institution for the analysis of the humoral response against the centromere antigens. This is a limitation in the present study. Therefore, a multi-center research study will be required to perform more refined analyses of the disease-specific humoral response against each fragment of centromere protein.

In conclusion, a diversity of humoral responses against the N-terminal and C-terminal domains of recombinant centromere proteins was shown among patients with CLD-C, PBC and AIH. Some autoantibodies against these fragments of the centromere proteins may turn out to be complementary serological hallmarks for the development of liver cirrhosis or concurrent CREST syndrome.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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SHORT COMMUNICATION

TRPS1 Haploinsufficiency Results in Increased *STAT3* and *SOX9* mRNA Expression in Hair Follicles in Trichorhinophalangeal Syndrome

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Trichorhinophalangeal syndrome types I and III (TRPS1, OMIM 190350; TRPS3, OMIM 190351) are rare hereditary diseases with autosomal dominant inheritance (1, 2). The first case was reported in 1966 (3). In 2000 the *TRPS1* gene was identified as one of its causative genes and mapped to chromosomal region 8q24.1 (1).

These syndromes have characteristic sparse and slow-growing hair, craniofacial abnormalities, such as bulbous pear-shaped nose, and skeletal abnormalities (3–5). We report here the effects of TRPS1 protein deficiency in a case of TRPS1.

CASE REPORT

A 26-year-old Japanese woman was referred to us for sparse scalp hair from birth. Physical examination revealed characteristic symptoms of TRPS1 (Fig. 1a–c). Her serum creatinine level was 0.71 mg/dl (normal range <0.70 mg/dl). Radiologically, cone-shaped epiphyses were found at the phalanges (Fig. S1[1]). She had never had growth hormone treatment. She was 158 cm tall, roughly the mean height of Japanese females, and had no growth retardation. None of her family members showed hypotrichosis or skeletal abnormalities. The patient was suspected of having TRPS, and a *TRPS1* mutation search was performed. The ethics committee of Nagoya University approved the studies described below, which were conducted according to the principles of the Declaration of Helsinki. The participants gave written informed consent. Direct sequencing of the entire coding regions and exon-intron boundaries of *TRPS1* revealed the patient to be heterozygous for the previously unreported nonsense mutation c.2191G>T in *TRPS1*, resulting in an immediate stop codon (p.Glu732X) (Fig. 1d). This mutation was not found in 100 healthy Japanese control individuals. No other mutation was found in the *TRPS1* gene of the patient. TRPS III has missense mutations specifically in the GATA DNA-binding zinc finger of the TRPS1 protein, located in the region, the amino acid positions 896–920. TRPS III shows similar clinical features to TRPS I, except that TRPS III presents more severe brachydactyly and growth retardation (2). From the clinical features and causative gene mutation, we diagnosed the patient as TRPSI. In our patient, we analysed mRNA levels of TRPS1 and TRPS1-related molecules expressed

[1]http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1948

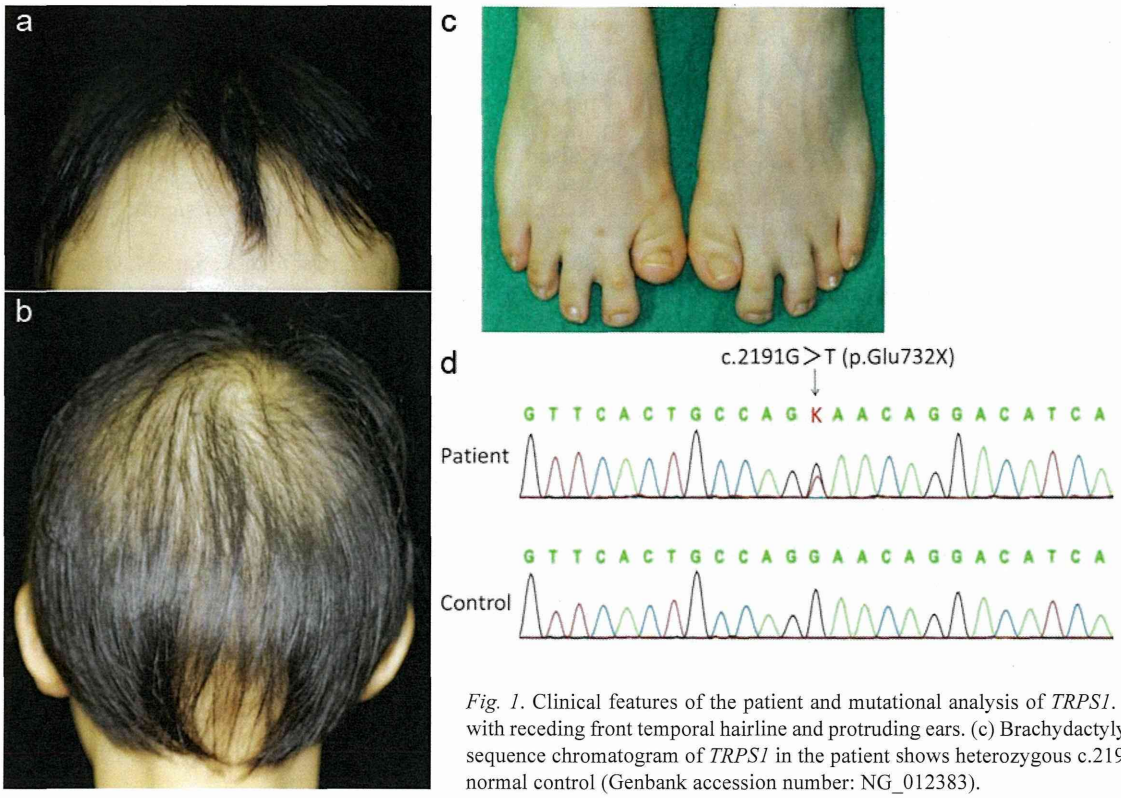

Fig. 1. Clinical features of the patient and mutational analysis of *TRPS1*. (a, b) Sparse scalp hair with receding front temporal hairline and protruding ears. (c) Brachydactyly of the big toes. (d) The sequence chromatogram of *TRPS1* in the patient shows heterozygous c.2191G>T compared with a normal control (Genbank accession number: NG_012383).

by cutaneous epithelial cells using total RNA samples extracted from plucked hairs of the patient and 7 normal control individuals. *TRPS1* was down-regulated and *STAT3*, *SOX9* and *CTNNB1* were up-regulated in plucked hairs from the patient compared with those in normal controls (Fig. S2 a, b[1]). *FGF5*, *TGFB1*, *STAT6* and *STAT1* were not up-regulated (Fig. S2c[1]).

DISCUSSION

The *TRPS1* gene encodes a zinc-finger transcription factor TRPS1 protein composed of 1,281 amino acids with 9 putative zinc-finger motifs (1). The *IKAROS*-like sequence consists of the last 2 zinc-finger motifs (motifs 8 and 9) and mediates the transcription repressive function. The 7th zinc-finger motif binds to the GATA consensus *cis* element and also mediates repressive activity (6). For example, Trps1 is assumed to be a regulator of chondrocyte proliferation and survival via the control of Stat3 expression (7). Indeed, up-regulated expression of STAT3 was observed in the outer root sheath of hair follicles in a TRPS1 patient with a TRPS1 mutation by immunohistochemistry (8). The Sox9 gene is known to regulate the proliferation and survival of hair follicle stem cells. Trps1 also regulates epithelial proliferation in the developing hair follicle via its control of Sox9 expression by the binding GATA sequence (9). β-catenin drives a hair shaft formation signal and is a key component of canonical Wnt signalling. Recent evidence suggests that the Wnt/β-catenin pathway cross-talks with STAT3 signalling to regulate the survival of retinal pigment epithelial cells (10). In mice model, Trps1is also reported to interact with 2 histone deacetylases, Hdac1 and Hdac4, thereby increasing their activity. Loss of Trps1 results in histone H3 lysine 9 (H3K9) hyperacetylation, which is maintained during mitosis (11).

We confirmed that mRNA decay occurred in *TRPS1* in plucked hairs. The GATA-type zinc-finger is resident at position 896–920. Thus, it is thought that transcription repressive function would be lost and haploinsufficiency would occur. We assumed that TRPS1 would also directly repress STAT3 and SOX9 activity by binding the GATA sequence in human hair follicles as in the mice model. The mutant TRPS1 in the present case was unable to repress *STAT3* and *SOX9* mRNA expression. The present results show that the elevation of CTNNB1 in mRNA level indicated that crosstalk of STAT3 and Wnt/β-catenin would also occur in hair follicles. Thus, TRPS1 would also repress Wnt/β-catenin signalling by repressing STAT3. In light of this, we think that the present TRPS1 loss-of-function mutation leads to the up-regulation of Sox9 and STAT3 and that the increased STAT3 signal results in the activation of hair shaft formation signalling by Wnt/β-catenin and the depletion of progenitor cells for the hair follicle epithelium. This depletion might be associated with the hypotrichosis phenotype in TRPS1. We speculate that FGF-5 and TGF-β1, which are degradation period shift signals in hair cycle, STAT6 and STAT1 were not directly influenced by TRPS1. Furthermore, loss of TRPS1 function may result in H3K9 hyperacetylation, which is maintained during mitosis (11) and supposedly lead to hypotrichosis.

In conclusion, we clearly demonstrate for the first time that haploinsufficiency of TRPS1 leads to the up-regulation of STAT3 and SOX9, and that activated STAT3 and β-catenin signalling might be associated with the hypotrichosis phenotype in TRPS1 (Fig. S2d[1]). Our data give us further insight into the function of TRPS1 in hair signalling and into the pathomechanisms of TRPS.

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