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Original article

Development of an ELISA for detection of autoantibodies to nuclear matrix protein 2

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Abstract

Objectives. Autoantibodies characterizing certain forms of inflammatory myopathy, which are myositis-specific autoantibodies, are useful in the diagnosis and prediction of prognosis in DM/PM. This study aimed to identify a subset of DM patients who have anti-nuclear matrix protein 2 (anti-NXP-2) antibodies by using biotinylated recombinant proteins, and to clarify the clinical features of DM patients with these antibodies.

Methods. Sera from 170 Japanese patients with CTDs including 106 with DM, 8 with PM, 21 with SLE, 20 with SSc, 15 with myositis overlap syndrome and 20 healthy controls were screened for anti-NXP-2 antibodies by our novel ELISAs. Positive sera were further examined by immunoprecipitation.

Results. Sera from 6 of the 170 patients with CTDs were confirmed to be positive for anti-NXP-2 antibodies. These six positives were from female patients, with five of the six sera being from adult DM patients and only one of the six being from 1 of the 12 JDM patients. All these patients had myositis. None of the anti-NXP-2-positive patients had interstitial lung disease, but one patient was complicated with ovarian cancer.

Conclusion. Our newly developed ELISA is applicable for the measurement of anti-NXP-2 antibodies. The results show that anti-NXP-2 antibodies, which have been characterized in JDM, exist in adult DM patients. Further studies using large populations are necessary to elucidate the characteristic clinical features and the prognosis of patients with anti-NXP-2 antibodies, especially for adult patients.

Key words: anti-MJ antibody, anti-NXP-2 antibody, dermatomyositis, ELISA, myositis-specific autoantibody.

BASIC

Introduction

The idiopathic inflammatory myopathies (IIMs) are a group of acquired, systemic autoimmune diseases that include PM, DM and inclusion body myopathies [1, 2]. Several myositis-specific autoantibodies (MSAs) that are associated with certain clinical forms of IIMs have been identified, and they are useful tools for predicting the prognosis of IIMs. For example, anti-melanoma differentiation-associated protein 5 (MDA5)-antibody-positive patients demonstrate rapid progressive interstitial lung disease (ILD) and anti-transcriptional intermediary factor 1 (TIF1)-γ-antibody-positive patients are often complicated with cancer [3]. Recently anti-nuclear matrix protein 2 (anti-NXP-2, also called anti-MJ) antibodies that react to a 140-kDa polypeptide have been described in cohorts of JDM patients in the UK and Argentina [4].

Anti-NXP-2 antibodies were reported to be associated with calcinosis, although the antibodies were investigated only in cohorts of juvenile patients [4–6]. The prevalences of anti-NXP-2 antibodies in adult patients with CTDs have been unknown. We hypothesized that anti-NXP-2 antibodies could be detected not only in JDM patients, but also in adult DM cases, and that these antibodies could be a new serological marker for a certain disease subset of adult DM. To investigate the presence of anti-NXP-2 antibodies in sera from patients with various CTDs, this study used our newly developed ELISAs and immunoprecipitation (IPP) with biotinylated recombinant protein. We clarified the prevalence of the antibodies against NXP-2 in adult DM and examined clinical features associated with anti-NXP-2 antibodies.

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Materials and methods

Patients and sera

From the serum bank of the Department of Dermatology, Nagoya University Hospital, we used sera from 170 Japanese patients with CTDs. The CTD cohort consisted of 106 patients with DM [including 12 with JDM, 38 with clinically amyopathic DM (ADM) and 14 with cancer-associated DM], 8 with PM, 21 with SLE, 20 with SSc and 15 with myositis overlap syndrome (Table 1). The group of patients with myositis overlap syndrome consisted of seven patients with overlapping CTDs and eight with MCTD. Serum samples were collected at the time of diagnosis or before aggressive therapy. Twenty healthy Japanese individuals were also assessed as normal controls. All the DM patients, except those clinically ADM, and all the PM patients fulfilled Bohan and Peter's criteria [7, 8]. All the clinically ADM patients fulfilled Sontheimer's criteria [9]. The clinically ADM group included patients who had developed ILD within 6 months after disease onset. Patients were classified as having JDM if they were <16 years at the onset of DM according to the criteria of Bohan and Peter [10]. Patients were classified as cancer-associated DM if the internal malignancy was diagnosed within 3 years (before or after) of the DM diagnosis based on previous studies [11]. SLE was diagnosed by the ACR criteria for SLE [12]. SSc was diagnosed according to the SSc diagnostic criteria [13] established by the Ministry of Health, Labour and Welfare of Japan, which were modified from the ACR criteria [14]. MCTD was diagnosed according to the MCTD diagnostic criteria [15]. Patients were classified as having myositis in overlap with another CTD if they met published criteria for the CTD. The clinical records were retrospectively reviewed and the following information was recorded: demographic data including gender, age at onset, diagnosis, date of serum sampling and clinical features including the presence of cutaneous signs of DM (heliotrope rash or Gottron's papules), RP or calcinosis. Elevated creatine kinase (CK) was defined by elevation of CK above the normal range in clinical laboratory tests. Patients were diagnosed as having ILD, which was defined by fibrosis on chest radiographs or chest CT scans. Internal malignancy was investigated according to the results of whole-body CT, endoscopy, colonoscopy, gynaecological examination and breast cancer screening. The ages at the time of sera collection and gender ratios of each clinical group are summarized in Table 1. In the juvenile-onset patients with DM, two patients were originally seen at other hospitals far outside our area but then transferred to our hospital. Their intervals between disease onset and serum sampling were 26 and 28 years. All the patients and healthy individuals in the present study gave fully informed consent for participation, including provision of serum samples. This study was approved by the Ethics Committee of Nagoya University Graduate School of Medicine and conducted in accordance with the Declaration of Helsinki.

ELISAs

Specific binding of serum antibodies to recombinant NXP-2 was analysed using direct solid-phase ELISA. This method was based on our previous protocol, which quantitatively measured the antibodies against MDA-5 [16]. Instead of a conventional optical system for ELISA, this study used a microplate luminometer to increase the

TABLE 1 Patient groups and anti-NXP-2 antibody frequencies

Clinical group	Age[a], range, years	Age[a], mean (S.D.), years	Gender, M:F	Total, *n*	α-NXP-2, *n* (%)
Total DM	3–84	51 (18)	33:73	106	6 (5.7)
Clinically ADM	3–84	48 (19)	9:29	38	0
Cancer-associated DM	48–80	66 (10)	6:8	14	1 (7.1)
Classical DM	16–80	49 (17)	18:36	54	5 (9.3)
Adult DM	19–84	55 (14)	26:68	94	5 (5.3)
Clinically ADM	23–84	54 (13)	5:27	32	0
Cancer-associated DM	48–80	66 (10)	6:8	14	1 (7.1)*
Classical DM	19–80	53 (15)	15:33	48	4 (8.3)*,**
JDM	3–32	18 (9)	7:5	12	1 (8.3)
Clinically ADM	3–32	16 (12)	4:2	6	0
Classical DM	16–27	20 (5)	3:3	6	1 (17)
Total other CTDs	18–75	45 (13)	6:58	64	0
PM	32–70	59 (13)	1:7	8	0
SLE	18–57	38 (11)	3:18	21	0
SSc	26–55	45 (8)	0:20	20	0
Overlap syndrome	24–75	49 (16)	2:13	15	0

[a]Age at the time of sera collection. In JDM, two patients were originally seen at other hospitals far outside our hospital. Their intervals between disease onset and serum sampling were 26 and 28 years. *$P < 0.05$ in classical adult DM and cancer-associated DM (5/62) *vs* other CTDs (0/64) by Fisher's exact test ($P = 0.0265$). **$P < 0.05$ in classical adult DM (4/48) *vs* other CTDs (0/64) by Fisher's exact test ($P = 0.0313$). M: male; F: female.

sensitivity, thereby reducing the required amount of biotinylated recombinant protein for the assays. The full-length NXP-2 cDNA clone was purchased from Kazusa DNA Research Institute (Chiba, Japan) and its DNA sequences were confirmed to be identical to GenBank accession number D50926. Biotinylated recombinant NXP-2 was produced from the cDNA, using the TnT T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI, USA) according to our protocol [3]. Nunc Immobilizer Streptavidin Plates (Thermo Scientific Nunc, Roskilde, Denmark) to which streptavidin was covalently coupled via a spacer were pre-washed three times with PBS containing 0.05% Tween-20 (T-PBS) and were coated with biotinylated recombinant NXP-2 diluted with T-PBS (50 μl/well) and incubated for 1 h at room temperature with gentle agitation. After three washes with T-PBS, the wells were blocked with 200 μl of a blocking buffer of 0.5% BSA (Wako, Osaka, Japan) in T-PBS for 1 h. Uncoated wells were used to measure the background levels for each sample. Diluted sample sera with blocking buffer (50 μl/well) were incubated for 1 h at room temperature, followed by incubation with anti-human IgG antibody conjugated with HRP (Dako, Glostrup, Denmark) as a secondary antibody (50 μl/well) at 1:30 000 dilution after five washes. After incubation for 1 h at room temperature, the plates were washed five times and incubated with SuperSignal ELISA Femto Maximum Sensitivity Substrate (Thermo Scientific Pierce, Rockford, IL, USA) (50 μl/well) as the substrate according to the manufacturer's protocol. Then, the relative luminescence unit (RLU) was determined using the GloMax-Multi Detection System (Promega). Each serum sample was tested in duplicate, and the mean RLU subtracted background was used for data analysis. A standard curve was obtained from serial concentrations of a serum sample containing a high titre of the anti-NXP-2 antibody. The cut-off level was set at 17.0 U, based on 5 S.D. above the mean value obtained from 20 healthy control sera.

Detection of anti-NXP-2 antibodies using IPP

IPP was performed using transcription and translation (TnT) products as previously described [17–20], with minor modifications. Briefly, 10 μl of patient sera was mixed and incubated with 20 μl of a 50% slurry of Protein G Sepharose 4 Fast Flow (GE Healthcare, Buckinghamshire, UK) and 270 μl IPP buffer (PBS containing 1% Nonidet P-40) at 4°C for 1 h. Sepharose beads were mixed and incubated with 270 μl binding buffer (IPP buffer containing 0.5% BSA) and 10 μl of the TnT products, which was pre-cleared using the sepharose beads, at 4°C for 1 h. The beads were washed five times with IPP buffer, suspended in Laemmli sample buffer and the IgG-bound proteins retained by the sepharose beads were electrophoresed on 7.5% SDS-PAGE gel. The proteins were electrophoretically transferred onto Immobilon-P transfer membranes (Millipore Corporation, Billerica, MA, USA) and the biotinylated proteins were subsequently detected with Western Blue Substrate (Promega).

IIF

IIF was performed by standard methods [21] using HEp-2 cells (Fluoro HEPANA Test; MBL, Nagoya, Japan).

Statistical analysis

The frequency of antibodies between each CTD and control was analysed using Fisher's exact test. The associations between clinical features and antibodies in DM were analysed using Fisher's exact test or unpaired Student's *t*-test. SPSS version 17.0 for Windows (SPSS Japan, Tokyo, Japan) was used to perform the statistical analysis. $P < 0.05$ was considered statistically significant.

Results

Establishment of ELISA with biotinylated recombinant NXP-2

For the screening of anti-NXP-2 antibodies in large numbers of serum samples, we established an ELISA system that uses biotinylated recombinant NXP-2. In the present study, by using a luminometer, we succeeded in decreasing the required amount of TnT product for the assay from 10 μl/well in the previous system to 1 μl/well in our newly developed system, based on the results of serial dilution experiments (data not shown). We screened a total of 170 serum samples obtained from patients with various CTDs. Based on the cut-off level (17.0) at 5 S.D. above the mean value, six patients with DM and one patient with PM were classified into the anti-NXP-2-positive group (Fig. 1). When the cut-off was set at 3 S.D. above the mean value (9.7), one sample from a patient with SLE was just beneath the cut-off (9.2). Subsequently serum samples from these eight patients were used for IPP to confirm whether they were truly positive for the anti-NXP-2 antibodies.

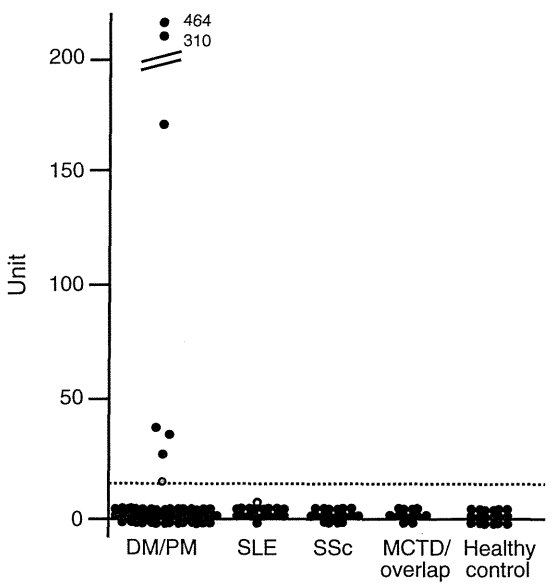
Detection of anti-NXP-2 antibodies using IPP

After the initial screening by ELISA we investigated antibodies against NXP-2 in sera from 7 anti-NXP-2-positive patients, 1 equivocal patient and 24 anti-NXP-2-negative patients for their ability to immunoprecipitate biotinylated recombinant NXP-2. Six of the seven anti-NXP-2-positive sera in ELISA showed a distinct protein band with a molecular weight of 140 kDa in IPP assays (Fig. 2, lanes 1–6). All were from patients with DM, whereas the serum from the one patient with PM did not react to the recombinant (Fig. 2, lane 7). The serum from an SLE patient with an equivocal level of anti-NXP-2 antibodies in ELISA was negative in IPP (Fig. 2, lane 8). Furthermore, anti-NXP-2 antibodies were not detected in normal control sera as well as 24 anti-NXP-2-negative sera in ELISA by IPP assays (representative negative sera, Fig. 2, lane 9).

Clinical and serological features of DM patients with anti-NXP-2 antibodies

Of the 106 DM sera, 6 (5.7%) had anti-NXP-2 antibodies (Table 1). In contrast, anti-NXP-2 antibodies were not detected in any serum from patients with other CTDs.

FIG. 1 ELISA using biotinylated recombinant NXP-2 protein. Measurement of anti-NXP-2 antibodies in 170 serum samples from patients with various CTDs or 20 healthy control subjects. We used the 1 μl/well of TnT mixture and the diluted patient serum samples at 1:1000 for measuring all samples (closed circles). The antibody units were calculated from the RLU using a standard curve obtained from serial concentrations of a serum sample containing a high titre of the anti-NXP-2 antibody. Broken line indicates the cut-off value (17.0 U). Six samples from patients with DM, one sample from a patient with PM (grey circle, just around the cut-off value) and one sample from a patient with SLE (open circle, under the mean values of 20 healthy controls + 3 S.D.) were introduced to IPP assays with biotinylated recombinant proteins (see Fig. 2).



Anti-NXP-2 antibodies were found at a higher frequency in DM patients than in patients with other CTDs (6/106 *vs* 0/64, $P = 0.0844$). Concerning JDM, only 1 (8.3%) of the 12 JDM patients had serum that reacted with NXP-2. The patient with anti-NXP-2 antibodies had no findings associated with calcinosis. As for adult DM, sera from 5 (5.3%) of 94 patients were positive. In adult cancer-associated DM, serum from 1 (7.1%) of 14 patients was positive for anti-NXP-2 antibodies. This patient had ovarian cancer that resulted in a fatal outcome. Adult patients with classical DM had anti-NXP-2 antibodies with a significantly higher frequency than patients with other CTDs (4/48 *vs* 0/64, $P = 0.0313$). Even if adult patients with cancer-associated DM were added to the classical adult DM group, a significant difference would still remain between adult patients with DM and patients with other CTDs (5/62 *vs* 0/64, $P = 0.0265$). According to these results, anti-NXP-2 antibodies were detected not only in JDM, but also in cancer-associated adult DM and classical adult DM. No patients with any other CTD, clinically ADM or healthy individuals had anti-NXP-2 antibodies.

FIG. 2 IPP of biotinylated recombinant NXP-2 with patient's sera. The input lane contains half the dose (5 μl) of biotinylated recombinant NXP-2 protein that was used for the IPP assay. The band in this lane that migrated at around 140 kDa corresponds to NXP-2. Lanes 1–6 contain the NXP-2 immunoprecipitated by the sera of patients that were anti-NXP-2-positive in ELISA. Lane 7 is the serum that was just above the cut-off level in ELISA. Lane 8 is the equivocal serum in ELISA. Lane 9 is serum from a healthy control. We arranged the samples from lanes 1–9 according to the levels of ELISA units. The asterisk denotes biotinylated NXP-2.

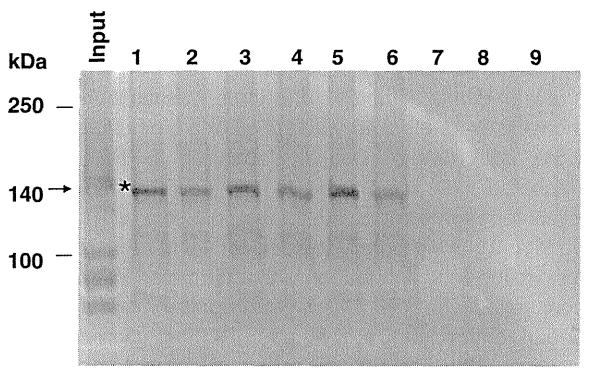


Table 2 compares the clinical features between anti-NXP-2-positive patients and anti-NXP-2-negative patients. There was little significant difference in gender, age at onset, diagnosis or prevalence of DM-specific skin symptoms (heliotrope rash and Gottron's papules or sign) and RP. All six of the anti-NXP-2-positive patients had myopathy, but none of them were complicated with ILD. The frequency of calcinosis was relatively higher in the positive group, although the difference was not significant.

Table 3 summarizes the serological information of the six patients with anti-NXP-2 antibodies in the present study. IIF revealed diverse nuclear staining patterns with various titres in all the anti-NXP-2-positive sera. The IIF titres did not correspond well to the ELISA units.

Discussion

There is increasing evidence that MSAs are associated with clinical subsets of DM/PM. Several MSAs have shown positive polypeptide bands ~140–160 kDa on SDS-PAGE by the traditional assay of IPP with cell extracts, including anti-NXP-2, anti-Mi-2, anti-MDA-5, anti-TIF1-γ and anti-OJ antibodies. It is difficult to differentiate these antibodies by molecular weight. We used biotinylated recombinant proteins and successfully detected anti-MDA-5 and anti-TIF1-γ antibodies in our IPP study [3]. However, there are technical limits to handling large numbers of serum samples efficiently with IPP. Thus we needed to establish an ELISA system. In our previous study, taking advantage of the high affinity between biotin

and avidin, we used streptavidin-coated ELISA plates for simultaneous purification and immobilization of the biotinylated protein for the detection of anti-MDA-5 antibodies [16]. However, improving the sensitivity of the ELISA was required, because this ELISA system was not suitable for measuring large numbers of serum samples. The TnT system cannot produce a practical amount of recombinant proteins compared with the *Escherichia coli* expression system. In this study, we established highly sensitive ELISA with TnT products by using a luminometer.

This study examined the specificity of anti-NXP-2 antibodies in Japanese patients with DM. Anti-NXP-2 antibodies were finally detected in sera of 6 of 170 patients with CTDs by ELISA and IPP. We conclude that only the DM patients who screened positive by ELISA were confirmed to have anti-NXP-2 antibodies. The results of ELISA and IPP in this study with biotinylated recombinant NXP-2 were inconsistent with previously reported findings of anti-NXP-2 antibodies being detected in JDM patients, especially patients with calcinosis in Argentina and the UK [4–6], Espada *et al.* [5] reported that 25% of JDM patients were found to have anti-NXP-2 (anti-MJ) antibodies and that anti-NXP-2-positive juvenile patients demonstrated severe disease characterized by muscle atrophy with contractures. Some of them also had large-joint arthritis, dysphagia, cutaneous vasculitis and calcinosis universalis. Gunawardena *et al.* [6] showed that anti-140-kDa protein (NXP-2) antibodies were significantly associated with the presence of calcinosis when compared with the overall juvenile myositis cohort. In our study, only 1 (8.3%) of 12 JDM patients were anti-NXP-2 positive. The prevalence of other MSAs in our JDM patients was 42%, i.e. five patients with anti-TIF1-γ antibodies (data not shown). The prevalence of anti-NXP-2 antibodies in JDM was low in our study, but this may depend on the fact that some patients had long periods (26 and 28 years) between onset and serum sampling. In contrast to adult DM, in which calcification is relatively uncommon, it is estimated that 20–40% of JDM patients have calcification [22, 23]. In our study, 2 of the 12 JDM patients were complicated with calcinosis. Both patients were anti-TIF1-γantibody positive (data not shown).

Few reports have addressed the correlation between adult-onset DM and calcinosis. In our study, 4 (4.3%) of 94 adult DM patients had calcinosis. Among adult DM patients with calcinosis, only one patient (Patient 3 in Table 3) was detected as having anti-NXP-2 antibodies. She had additional anti-centromere antibodies (ACAs) and multiple cutaneous calcinosis. Although ACAs are known to be associated with calcinosis, it is not clear whether the

TABLE 2 Clinical features of anti-NXP-2-positive and -negative DM patients

Feature	Anti-NXP-2 antibodies	
	Positive ($n=6$)	Negative ($n=100$)
Gender, male:female	0:6	33:67
Age at onset, mean (range), years	50.7 (16–73)	50.9 (1–80)
Age at diagnosis, mean (range), years	51.3 (16–74)	51.5 (3–84)
Type of skin lesion		
Heliotrope rash, *n* (%)	4 (67)	52 (52)
Gottron's papules or sign, *n* (%)	4 (67)	68 (68)
RP, *n* (%)	1 (17)	11 (11)
Calcinosis, *n* (%)	1 (17)	5 (5)
Elevation of CK, *n* (%)	6 (100)	74 (74)
ILD, *n* (%)	0 (0)*	41 (41)
JDM, *n* (%)	1 (17)	11 (11)
Internal malignancy, *n* (%)	1 (17)	13 (13)

*$P=0.0796$ by Fisher's exact test.

TABLE 3 Laboratory data for the anti-NXP-2-positive patients

Patient no.	Age, years	Gender, M/F	Diagnosis	Calcinosis	ILD	Cancer	IIF		ELISA units[a]	Other antibodies
							Pattern	Titre		
1	19	F	Classical DM	–	–	–	Spe. Nuc. Dots.	160 40	4644	
2	68	F	Cancer-associated DM	–	–	+ Ovary	Diffuse Nuc. Dots.	640 640	3104	
3	74	F	Classical DM	+	–	–	Spe. Dis. spe.	2560 2560	1731	ACA
4	76	F	Classical DM	–	–	–	Diffuse Nuc. Dots.	80 320	367	
5	16	F	JDM	–	–	–	Diffuse Nuc. mem. Mit. spi.	80 80 80	326	
6	66	F	Classical DM	–	–	–	Spe.	160	255	

[a]ELISA units of anti-NXP-2 antibodies. Spe.: speckled; Nuc. dots.: nuclear dots; Dis. spe.: discrete speckled; Nuc. mem.: nuclear membrane; Mit. spi.: mitotic spindle.

concomitant presence of the two kinds of antibodies was coincidental and which antibody was more pathogenic of calcinosis. Six patients with anti-NXP-2 antibodies presented no common characteristic clinical features other than myositis. Their myositis was moderate and their CK levels were not elevated by >500 U/l. We noticed that none had ILD, which sometimes occurs in adult DM. One (Patient 2 in Table 3) of the six patients had cancer. She was found to have ovarian cancer at the same time as being diagnosed with DM. Anti-TIF1-γ antibodies, which had been associated with cancer in our previous study [3], were not detected in her serum. The present findings suggest that DM patients who are anti-NXP-2 positive do not always have benign forms of DM, at least not adult patients, even when their myositis is moderate. One of the few published studies on anti-NXP-2 antibodies in adult DM patients is a preliminary report [24]. They reported that the frequency and clinical associations of anti-NXP-2 autoantibodies varied between adults and juveniles.

By IIF, the staining patterns by anti-NXP-2-positive sera were mainly divided into speckled or diffuse. NXP-2, also called MORC3 (microrchidia family CW-type zinc finger 3), is a MORC-family protein that is characterized by three conserved domains consisting of RNA-binding, nuclear matrix-binding and coiled-coil domains [25]. They are structurally separated and they may have important roles in diverse nuclear functions, including regulation of transcription, maintenance of nuclear architecture and RNA metabolism. NXP-2 is a nuclear matrix protein that is shown as speckled-like staining in IIF. The nuclear staining of NXP-2 becomes diffuse after nuclear matrix treatment with RNase [25]. Nuclear domains, which are demonstrated as nuclear dots by anti-MORC3 antibodies in IIF [26], were not clearly detected by patients' sera. The differences among IIF patterns in anti-NXP-2-positive patients might have resulted from other concomitant antibodies. It is also possible that altered conformational structures of NXP-2 affect the accessibility of antibodies to antigenic epitopes. In any case, IIF patterns are not useful for screening anti-NXP-2 antibodies.

The previously described DM-specific antigens, Mi-2 [27] and TIF1-γ [28] are nuclear proteins that are also involved in transcriptional regulation. It is notable that antibodies to small ubiquitin-like modifier (SUMO) activating enzyme (SAE), which is involved in a post-transcriptional modification called sumoylation, have been recently described in adult DM [29]. NXP-2 was reported to be a target of SAE and to have a possible role in SUMO-mediated transcriptional repression [30]. Mi-2 is also thought to be directly involved in mediating SUMO-dependent repression [31]. TIF1-γ is considered to act as a repressor of TGF-β superfamily-induced transcription via its E3 ubiquitin ligase activity [32]. The SUMO pathway may play a potential role in the pathogenic mechanisms of DM.

In summary, our newly developed ELISA is applicable for the measurement of anti-NXP-2 antibodies. Our present study revealed that anti-NXP-2 antibodies were observed not only in JDM but also in adult DM. Adult DM patients with anti-NXP-2 antibodies had uniformly moderate myositis without ILD, even though this serological group featured no major demographic differences with respect to the other adult DM patients in our study. To elucidate the characteristic clinical features and the prognosis of patients with anti-NXP-2 antibodies, a much larger cohort is needed, especially for adult patients.

Rheumatology key messages

- A newly established ELISA for biotinylated recombinant proteins is useful for detecting anti-NXP-2 antibodies.
- Anti-NXP-2 antibodies are detected not only in JDM, but also in adult DM.

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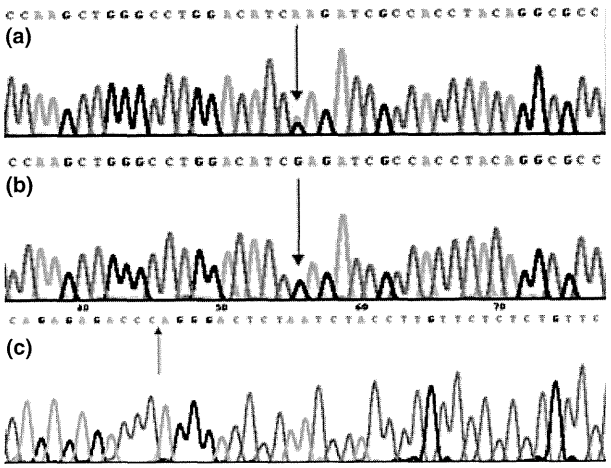

Figure 2. (a) Heterozygous missense mutation c.1204G>A. This mutation predicts the amino acid change glutamic acid to lysine at codon 402 (p.E402K). (b) Sequence of the normal subjects. (c) Single nucleotide deletion polymorphism from both 50 controls and patients. Partial normal sequence of the *KRT86* gene in GeneBank is as follows: agagagacccaagggactctaatctaccttgttctctctgttc. Red a base ccc indicates the deletion site.

this speculation needs more pedigree reports and studies to prove or disprove.

As far as we know, co-existence of congenital monilethrix and UAA in some members of a Chinese pedigree has not been reported to date.

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Four novel *ADAR1* gene mutations in patients with dyschromatosis symmetrica hereditaria

Dear Editor,

Dyschromatosis symmetrica hereditaria (DSH; Mendelian Inheritance in Man no. 127400) is a highly penetrant autosomal dominant skin disease and is characterized by a mixture of hyper- and hypopigmented macules on the dorsal aspects of the hand and foot. This disorder commonly develops during infancy or early childhood.[1]

Previous work has shown that a heterozygous mutation of the adenosine deaminase acting on RNA1 gene (*ADAR1* or *DSRAD*) caused DSH in four Japanese DSH families.[2] Subsequently, 115 mutations in the *ADAR1* gene (GenBank accession no. NM_001111.3, http://www.ncbi.nlm.nih.gov/genbank/) have been reported in Japanese, Taiwanese and Chinese patients with DSH. The ADAR1 protein catalyzes the deamination of adenosine to inosine in double-stranded RNA.[3] This creates alternative splice sites or alterations of the codon that lead to functional changes in the target substrate(s). However, the target gene(s) for ADAR1 in the skin as well as the mechanisms by which mutations in *ADAR1* cause DSH remain unknown. In this study, we performed mutational analysis of the *ADAR1* gene in three Japanese and a Chinese Canadian with DSH.

The analysis was performed as follows. Genomic DNA was extracted from peripheral blood with a QIAamp DNA blood maxi kit (QIAGEN, Valencia, CA, USA) and was used as a template for mutational screening with a polymerase chain reaction (PCR)-based single-strand conformation polymorphism (SSCP)/heteroduplex (HD) analysis.[4] Standard PCR amplification procedures were employed with high fidelity polymerase, Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA) and an annealing temperature of 62°C for all primers.[5] SSCP gel with glycerol concentrations of 7.5% was used. PCR products showing aberrant patterns on SSCP were re-amplified and sequenced directly to identify a mutation. In patients without any mutation detected by the SSCP/HD method, all of their PCR products were directly sequenced to identify mutations. Informed consent and blood samples of patients were obtained under protocols approved by the Ethics Committee of Nagoya University School of Medicine. Three Japanese patients in this study were not related to each other.

We identified four novel heterozygous *ADAR1* mutations including one splice mutation (IVS9-1G>T) and three frame-shift mutations (p.Lys1167fsX1178, p.Asn398fsX401 and p.Asp1147fsX1152) (Table 1).

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Table 1. Four mutations of the *ADAR1* gene in the patient with dyschromatosis symmetrica hereditaria

Patient no.	Patient			Mutation		
	Race	Onset	Complication	Nucleotide change†	Amino acid change	Position
1	Japanese	3 years old	Healthy	c.3499del A	p.Lys1167fsX1178	Exon 15
2	Japanese	2 years old	Healthy	c.1192-3 del AA	p.Asn398fsX401	Exon 2
3	Chinese Canadian	At birth	Healthy	c.3440insG	p.Asp1147fsX1152	Exon 14
4	Japanese	6 years old	Febrile seizure at 2 year-old	c.IVS9-1 G>T	p.Arg921_Tyr924del	IVS9

†GenBank accession no. NM_001111.3. Position 1 is A of the translation initiation codon.

The three frame-shift mutations code a different amino acid sequence and eventually make a new stop codon. In a previous report,[6] the ADAR1 mutations p.Gln513X and p.Cys519fs, which lead to premature termination codon (PTC) and to nonsense-mediated mRNA decay (NMD), were reported to result in ADAR1 haploinsufficiency. Generally, PTC could initiate NMD as long as the stop codons are located more than 50–55 nucleotides upstream of the 3′-most splice site.[7] We speculate that p.Asn398fsX401 on exon 2 of 15 exons in the present patient would also lead to haploinsufficiency of ADAR1 enzyme activity. The other two frame-shift mutations made PTC in exon 15. Thus, these mutations would not result in NMD, but made aberrant proteins.

On the other hand, the site of the novel splice mutation (IVS9-1G>T) was the conserved sequence on splice accepter site on intron 9 (Fig. 1a). On the *ADAR1* gene of a DSH patient some splice mutations have been reported.[8] So far, only one investigation on aberrant splicing of the *ADAR1* gene by the splice mutation has been reported.[5] We investigated aberrant splicing of the mutation IVS9-1G>T. Total RNA was extracted from whole blood of patient 4 and a healthy control.

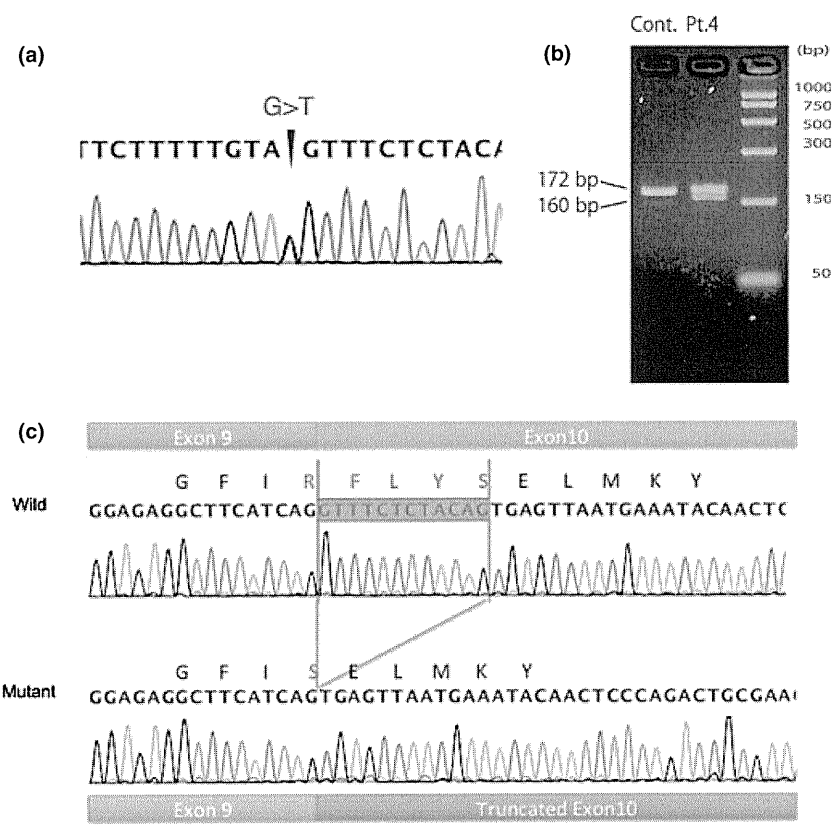

Figure 1. Molecular analysis of splicing mutation on *ADAR1* of patient 4. (a) Sequence analysis of boundary between exons 9 and 10 on *ADAR1* genomic DNA from patient 4 reveals G to T transversion, IVS9-1G>T. (b) Agarose gel electrophoresis of reverse transcription polymerase chain reaction (RT–PCR) products of boundary between exons 9 and 10 on *ADAR1* gene. The RT–PCR product from the patient 4 shows 172-bp (normally spliced fragment) and 160-bp (aberrantly spliced fragment) bands, although a control specimen exhibits only a 172-bp band. (c) Sequence analysis of boundary between exons 9 and 10 on *ADAR1* cDNA. In the mutant cDNA, the aberrant splicing results in 12-bp deletion.

Reverse transcription PCR was done for amplification of the 172-bp fragment at the boundary between exon 9 and 10 on mRNA from whole blood of the patient. Extra and normal bands, 172 bp (normal product) and 160 bp (aberrantly splicing product), were confirmed in mRNA from the patient (Fig. 1b). These were separated by agarose gel extraction and subcloned each with a TOPO TA Cloning kit for Sequencing (Invitrogen). DNA sequencing of subcloned PCR products showed the truncation of the 12-bp nucleotides on the 5′-side of exon 10 (Fig. 1c). Subsequently, the deletion of four amino acids (p.Arg921-Tyr924del) was in the deaminase domain and should produce the aberrant protein of ADAR1.

All patients phenotypically presented typical macules on the dorsal aspects of the hands and feet, lower arms and lower legs. No patients had complications but patient 4 had a febrile seizure at 2 years old. The mutations reported in the published work including the present report are scattered through the entire gene and there is no hotspot for the *ADAR1* gene mutations. However, in more than 100 mutations on *ADAR1*, all missense mutations except for p.Arg26Lys were in the adenosine–deaminase domain. Thus, this domain is thought to be essential for the biochemical function of ADAR1. There is no apparent genotype/phenotype correlation in *ADAR1* mutations. Even in one family, the identical mutation was reported to lead to different phenotypes.[5] The reason why a low activity of ADAR1 induces the peculiar skin lesions localized on the extremities is still unknown. We speculate that, when melanoblasts migrate from the neural crest to the skin during development, insufficient ADAR1 enzyme activity might affect differentiation of melanoblasts to melanocytes and the melanoblasts located distant from the neural crest might be most seriously affected during migration.[9]

In conclusion, we reported four novel mutations in the *ADAR1* genes of DSH patients. The accumulation of the patients with genetic information may provide new insight into the pathogenesis of DSH.

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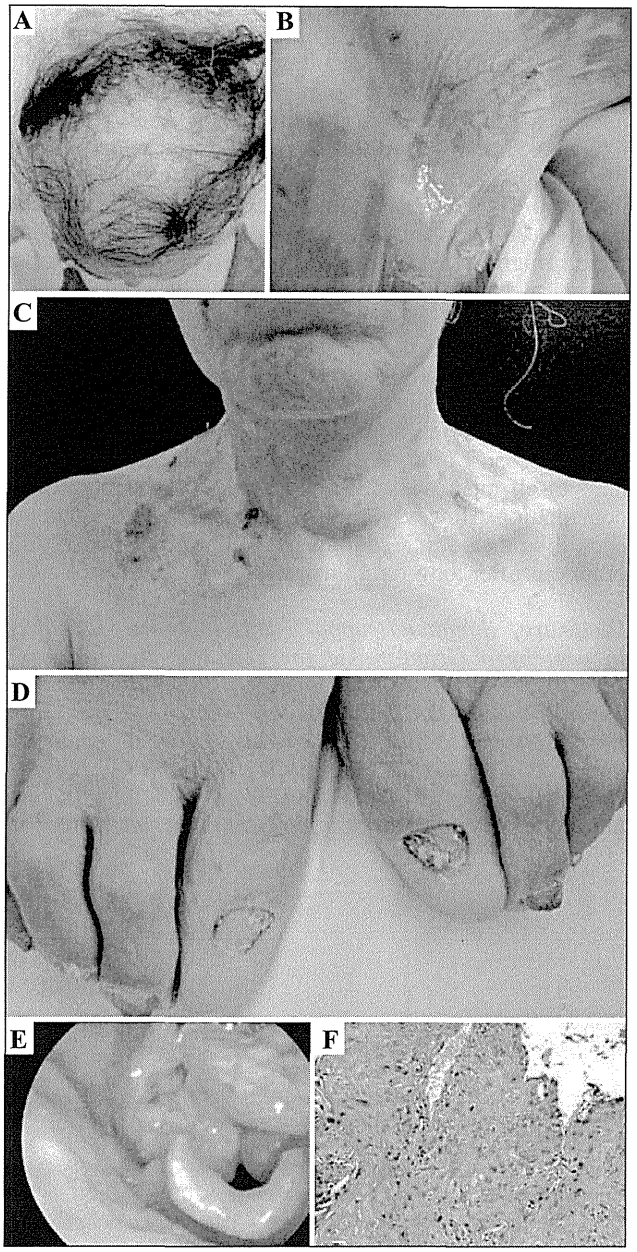

Figure 1. A) Cicatricial alopecia on the scalp; **B)** Erosions, bulla and atrophic, hypopigmented scars in the axillary region; **C)** Atrophic, hypopigmented scars at the neck; **D)** Dystrophic changes in the nails; **E)** Endoscopic image of the tumor; **F)** Atypical cell groups consistant with squamous cell carcinoma (hematoxylin-eosin stain, magnification ×100).

cutaneous lesions [4]. The etiology of esophageal carcinoma is related to exposure of the esophageal mucosa to noxious or toxic stimuli. Smoking and chronic alcohol exposures are the most common etiological factors for SCC. Vitamin and nutritional defects have been recognized as contributing factors. The epithelial lining of the esophagus in junctional EB and DEB is subject to chronic inflammation and damage. This may increase the risk of epithelial metaplasia and malignancy. Additionally, esophageal strictures cause vitamin, iron, and nutritional deficiencies in DEB. Recently, abnormalities in the p53 and p16 tumor suppressor genes in RDEB-associated SCC have been demonstrated. All these factors could play roles in the development of esophageal SCC in DEB [6].

Because of the serious complications of EB, patients with dysphagia should be followed by a multidisciplinary team for life-threatening risks. ■

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Drug eruption due to sodium picosulfate

A 62-year-old man who had been suffering from severe constipation presented with a 2-day history of pruritic papules and vesicules on the trunk and extremities. He had been prescribed sodium picosulfate, sennoside, pantethine and magnesium oxide sporadically over the course of 10 years. Medical examination revealed tense vesicles and blisters on the extremities and palms *(figures 1A-B)*. Vital signs were within normal ranges. There were no symptoms of fever, lymphadenopathy, mucous or systemic involvement throughout the disease course. The laboratory result was negative for herpes simplex virus antibodies, varicella zoster virus IgG was 3.6 (normal range <2.0), and Epstein-Barr virus antibodies indicated previous infection.

A skin biopsy from a tense blister on the left arm revealed a subepidermal blister with eosinophilic infiltration *(figures 1C-D)*. Direct immunofluorescence labeling of the lesional skin sections showed no IgG, IgM, IgA or C3 deposition at the basement membrane zone. Circulating anti-BP180 autoantibody was negative by ELISA. Antinuclear antibody was negative. Patch tests were negative for all the medicines prescribed (see above). A lymphocyte stimulation test (LST) was positive for sodium picosulfate twice; the stimulation index was 208% (normal <180%) at Day 5 and 187% at Day 33, however this LST was negative with three healthy controls. In contrast, sennoside, pantethine and magnesium oxide were all negative at Days 5 and 33. After discontinuation of all laxatives including sodium picosulfate, the patient's eruptions subsided remarkably without therapy. Thus, the case was diagnosed as sodium picosulfate-induced bullous eruption. The patient has recently taken all the laxatives except for sodium picosulfate, but he has not suffered from a drug eruption.

Sodium picosulfate is a popular laxative. It is not digested in the stomach or the small intestine; it is hydrolysated in the large intestine and transformed to active diphenole compounds. These active compounds stimulate intestinal motility and prevent water absorption, resulting in relief from constipation. Most sodium picosulfate compounds are excreted in the feces. When the usual dosage is taken, only a tiny portion is absorbed, and this is glucuronidated in the liver and excreted in urine and bile. Thus, in general, the side effects of sodium picosulfate are limited to abdominal pain and nausea.

To our knowledge, there has only been one other report of sodium picosulfate-induced drug eruptions: a fixed drug eruption reported in the Japanese literature [1]. The eruptions in that case appeared after 5 months of sodium picosulfate intake. Our patient had a ten-year history of sodium picosulfate intake. These cases suggest that long-term intake of sodium picosulfate can induce eruptions.

The pathomechanism of such eruptions is uncertain. Sennoside, another laxative, is also scarcely absorbed in the intestine, similar to sodium picosulfate, and sennoside has been reported to lead to drug eruptions after long-term intake [2-4]. We presume that drug eruptions induced by laxatives are caused by delayed T-cell hypersensitivity, which might explain why the skin eruptions occur after long-term intake. For drug eruptions to develop, it might take a long time for T cells to become sensitized or for the drug or reactive metabolites to achieve sufficient distribution. Further studies are needed to fully understand the mechanisms.

In conclusion, sodium picosulfate is generally believed to be safe, because allergic reactions are so rare. Thus, patients tend to self-medicate with it frequently and persistently for constipation. However, the present case suggests that we should be aware that sodium picosulfate can induce drug eruptions after long-term intake. ■

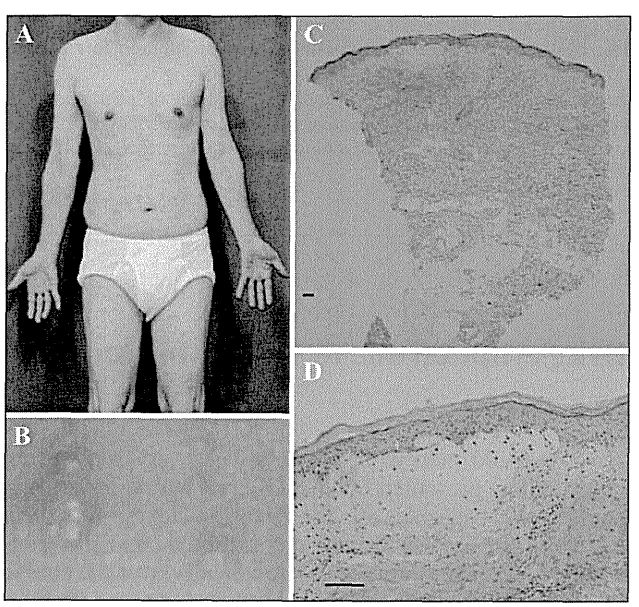

Figure 1. Skin manifestations and pathological tissue of the patient.
A) Widespread papulo-vesicular eruption in a 62-year-old man. **B)** Vesicles on the left arm. **C)** Skin biopsy specimen from the left arm showing subepidermal blister formation. **D)** There is mixed eosinophilic and lymphocytic infiltration in the bulla. (C-D: Hematoxylin and eosin stain; Bar=100 μm).

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The syndrome of inappropriate antidiuretic hormone secretion (SIADH) associated with metastatic malignant melanoma

The syndrome of inappropriate antidiuretic hormone secretion (SIADH) is a state where excess vasopressin is secreted from hypophysis in conditions when sufficient water is sustained in the body [1]. Although SIADH occurs in

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Concise report

Clinical features of anti-TIF1-α antibody-positive dermatomyositis patients are closely associated with coexistent dermatomyositis-specific autoantibodies and anti-TIF1-γ or anti-Mi-2 autoantibodies

Yoshinao Muro[1], Asuka Ishikawa[1], Kazumitsu Sugiura[1] and Masashi Akiyama[1]

Abstract

Objective. Myositis-specific autoantibodies (MSAs), which characterize certain forms of inflammatory myopathy, are useful in the diagnosis and prediction of prognosis in DM/PM. Anti-transcriptional intermediary factor 1-α (TIF1-α) antibodies were recently reported to be associated with cancer-associated DM in conjunction with anti-TIF1-γ antibodies. This study aimed to identify a subset of DM patients who have anti-TIF1-α antibodies by using biotinylated recombinant proteins and to clarify the clinical and other serological features of DM patients with these antibodies.

Methods. Sera from 202 Japanese patients with CTDs, including 108 with DM and 20 healthy controls, were screened for anti-TIF1-α antibodies by our novel ELISAs. Positive sera were further examined by immunoprecipitation and also investigated for the detection of anti-TIF1-γ and anti-Mi-2 antibodies.

Results. Sera from 12 patients with DM were confirmed to be positive for anti-TIF1-α antibodies. None of the patients with other CTDs and none of the healthy controls had the antibodies. Seven anti-TIF1-α-positive patients simultaneously had anti-TIF1-γ antibodies and the other five had anti-Mi-2 antibodies, both of which are well known to be MSAs. These double-positive patients with anti-TIF1-α and anti-γ antibodies included three JDM and two cancer-associated adult DM patients, whereas all the double-positive patients with anti-TIF1-α and anti-Mi-2 antibodies were classical adult DM.

Conclusion. Although MSAs have been regarded as mutually exclusive, anti-Mi-2 antibody-positive patients simultaneously have anti-TIF1-α antibodies. Anti-Mi-2 antibody-positive patients are associated with classical DM without cancer even with the simultaneous presence of anti-TIF1-α antibodies.

Key words: autoantibodies, dermatomyositis, Mi-2, TIF1-α, TIF1-γ.

SCIENCE

Introduction

The idiopathic inflammatory myopathies (IIMs) are a group of systemic autoimmune diseases that include PM, DM and inclusion body myopathies [1]. Several myositis-specific autoantibodies (MSAs) are associated with certain clinical forms of IIMs, and they are useful tools for predicting the prognosis. For example, anti-MDA5-antibody-positive patients demonstrate rapid progressive interstitial lung disease (ILD) and anti-transcriptional intermediary factor 1-γ (TIF1-γ) antibody-positive patients are often complicated with cancer [2]. Very recently, anti-p155/140 antibodies, which are serological markers of cancer-associated DM [3, 4], were analysed by Fujimoto *et al.* [5]. They determined that p140 is identical to TIF1-α, whereas p155 is known as TIF1-γ [6]. Their study showed that TIF1-β was also targeted in DM patients, but infrequently, although anti-TIF1-γ antibodies alone were frequently detected. Interestingly, anti-TIF1-α antibodies were always associated with anti-TIF1-γ antibodies.

MSAs have been regarded as mutually exclusive [1, 7]. Fujimoto *et al.* [5] showed that autoantibodies against the

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TIF1 protein family occurred in various combinations, often in patients with cancer-associated DM. However, they concentrated on analysing anti-p155/140-positive sera in a large cohort to find autoantibodies against the TIF1 protein family. In this study, we investigated sera from patients with various CTDs for the presence of anti-TIF1-α antibodies by using our developed ELISAs and immunoprecipitation (IPP) with biotinylated recombinant protein. We confirmed that anti-TIF1-α antibodies are specific to DM and are often associated with anti-TIF1-γ antibodies. Surprisingly, the other anti-TIF1-α antibody-positive sera also had anti-Mi-2 antibodies.

Materials and methods

Patients and sera

From the serum bank of the Department of Dermatology, Nagoya University Hospital, we used sera from 202 Japanese patients with CTDs. They consisted of 108 patients with DM [including 13 with JDM, 38 with clinically amyopathic DM (ADM) and 15 with cancer-associated DM], 9 with PM, 24 with SLE, 20 with SSc, 26 with SS and 15 with myositis overlap syndrome (Table 1). Sera from 21 cancer patients that were used in our previous study [2] were also analysed. Twenty healthy individuals were assessed as normal controls. All the DM patients except those with clinical ADM and all the PM patients fulfilled Bohan and Peter's criteria [8, 9]. All the clinically ADM patients fulfilled Sontheimer's criteria [10]. The clinically ADM group included patients who had developed ILD within 6 months after disease onset. Patients were classified as having JDM if they were aged <16 years at the onset of DM [11] and as cancer-associated DM if internal malignancy was diagnosed within 3 years (before or after) of the DM diagnosis [12]. The criteria of other CTDs were based on the established criteria for these diseases used in our previous studies [2, 13]. The ages at disease onset and gender ratios of each clinical group are summarized in Table 1. All the patients and healthy individuals gave fully informed consent for participation. This study was approved by the Ethics Committee of Nagoya University Graduate School of Medicine and conducted in accordance with the Declaration of Helsinki.

ELISAs

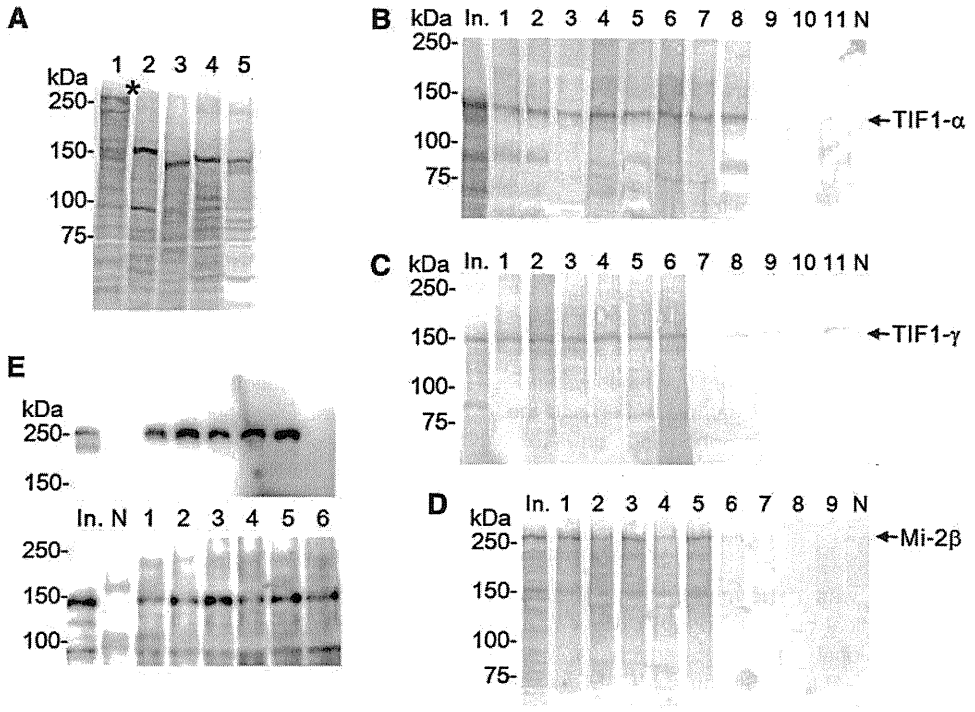
Specific binding of serum antibodies to recombinant TIF1-α or Mi-2β was analysed using our recently established sensitive ELISA [14]. This method was based on our previous protocol, which quantitatively measured the antibodies against MDA-5 [15]. Instead of a conventional optical system, this ELISA uses a microplate luminometer for increased sensitivity, thereby reducing the amount of recombinant protein required for the assays. The full-length TIF1-α cDNA clone was purchased from Kazusa DNA Research Institute (Chiba, Japan). The full-length Mi-2β cDNA clone [16] was a kind gift

Table 1 Patient groups and anti-TIF1-α antibody frequencies

Clinical group	Age at onset, mean (range), years	Gender, M:F	Total, *n*	Anti-TIF1-α (*n* = 12)		Anti-TIF1-α ELISA unit[a], mean (range)
				Anti-TIF1-γ	Anti-Mi-2	
Total DM	47 (1–80)	33:75	108	7	5	41.9 (−12.8 to 472.5)
Clinically ADM	45 (1–73)	9:29	38	5	5	42.1 (−4.3 to 366.4)
Cancer-associated DM	65 (48–80)	6:9	15	2	0	45.8 (−6.3 to 279.0)
Classical DM	44 (1–80)	18:37	55	0	0	40.7 (−12.8 to 472.5)
Adult DM	52 (19–80)	26:69	95	4	5	38.3 (−12.8 to 472.5)
Clinically ADM	51 (20–73)	5:27	32	2	0	23.7 (−4.3 to 268.4)
Cancer-associated DM	65 (48–80)	6:9	15	2	0	45.8 (−6.3 to 279.0)
Classical DM	49 (19–80)	15:33	48	0	5	45.8 (−12.8 to 472.5)
Juvenile DM	9 (1–16)	7:6	13	3	0	68.0 (13.8 to 366.4)
Clinically ADM	6 (1–11)	4:2	6	3	0	140.0 (13.8 to 366.4)
Classical DM	11 (1–16)	3:4	7	0	0	6.2 (−6.4 to 16.3)
Total of other CTDs	42 (15–81)	11:83	94	0		15.2 (−30.5 to 63.2)
PM	52 (32–67)	1:8	9	0		27.9 (13.1 to 63.2)
SLE	30 (15–51)	4:20	24	0		11.1 (−30.5 to 43.7)
SSc	40 (22–55)	0:20	20	0		16.1 (−23.2 to 58.2)
Overlap syndrome	49 (23–69)	2:13	15	0		16.9 (7.8 to 62.5)
SS	47 (21–81)	4:22	26	0		13.0 (10.2 to 39.5)
Cancer	58 (48–78)[b]	5:15[b]	21	0[c]		Not done

[a]The cut-off level is 121.3 and 83.0 U based on 5 S.D.s and 3 S.D.s above the mean value obtained from 20 healthy control sera, respectively. No patient had an ELISA in the range of 83.0–121.3 U. M:F = male:female. [b]Information on the age and gender of one patient was not available. [c]All sera from cancer patients were examined for anti-TIF1-α antibodies by IPP.

FIG. 1 Anti-TIF1-α antibodies coexist with anti-TIF1-γ or anti-Mi-2 antibodies.



(**A**) Biotinylated recombinant proteins used in our laboratory were subjected to 7.5% SDS–PAGE and analysed by immunoblotting. Lane 1: Mi-2β; lane 2: TIF1-γ; lane 3: TIF1-α; lane 4: NXP-2; lane 5: MDA-5. Mi-2β was shown at the larger size of 250 kDa, probably due to the addition of N-terminal haemagglutinin tag and C-terminal V6His tag indicated by an asterisk [16]. (**B**) IPP of recombinant TIF1-α. In.: the input was a full dose (10 μl) of biotinylated TIF1-α protein that was used for the IPP assay. Lanes 1–8 contain the TIF1-α immunoprecipitated by the sera of different DM patients. In lanes 9–11, anti-TIF1-γ-positive sera from DM patients, which immunoprecipitated TIF1-γ, did not immunoprecipitate TIF1-α. N: normal control. (**C**) IPP of recombinant TIF1-γ. In.: the input was half the dose (5 μl) of the biotinylated TIF1-γ protein used for the IPP assay. Lanes 1–6 contain the TIF1-γ immunoprecipitated by the sera of different DM patients. In lanes 7–11, anti-TIF1-α-positive and anti-Mi-2-positive sera from DM patients, which immunoprecipitated TIF1-α and Mi-2β, did not immunoprecipitate TIF1-γ. N: normal control. (**D**) IPP of recombinant Mi-2β. In.: the input was half the dose (5 μl) of the biotinylated Mi-2β protein used for the IPP assay. Lanes 1–5 contain the Mi-2β immunoprecipitated by the sera of different DM patients. In lanes 6–9, anti-TIF1-α/γ-positive sera from DM patients, which immunoprecipitated TIF1-α and TIF1-γ, did not immunoprecipitate Mi-2β. N: normal control. (**E**) Immunoprecipitates from cell extracts with anti-TIF1-α-positive sera were probed with anti-Mi-2α MoAb (upper panel) and with anti-TIF1-α PolyAb (lower panel). Lane In. contains a half dose of the input of K562 cell extracts. Lane N: normal control; lanes 1–5: anti-TIF1-α/Mi-2-positive sera; lane 6, anti-TIF1-α/TIF1-γ-positive serum.

from Drs Kato and Takahashi at Nagoya University. Biotinylated recombinant protein was produced from the cDNA, using the transcription and translation (TnT) T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI, USA) according to our protocol (Fig. 1A) [2, 15]. Nunc Immobilizer Streptavidin Plates (Thermo Scientific Nunc, Roskilde, Denmark) were pre-washed three times with PBS containing 0.05% Tween-20 (T-PBS) and were coated with 1 μl of TnT product diluted with T-PBS (50 μl/well) and incubated for 1 h at room temperature. After three washes with T-PBS, the wells were blocked with 200 μl of a blocking buffer of 0.5% BSA (Wako, Osaka, Japan) in T-PBS for 1 h. Uncoated wells were used to measure the background levels for each sample. Sample sera diluted with blocking buffer (50 μl/well) were incubated for 1 h at room temperature, followed by incubation with anti-human IgG antibody conjugated with horseradish peroxidase (HRP) (Dako, Glostrup, Denmark) (50 μl/well) at 1 : 30 000 dilution. After incubation for 1 h at room temperature, the plates were washed and incubated with SuperSignal ELISA Femto Maximum Sensitivity Substrate (Thermo Scientific Pierce, Rockford, IL, USA) (50 μl/well) as the substrate. Relative luminescence unit (RLU) number was then determined using the GloMax-Multi Detection System (Promega). Each serum sample was tested in duplicate, and the mean RLU subtracted background was used for data analysis.

A standard curve was obtained from serial concentrations of a serum sample containing a high titre of the autoantibody against each antigen.

Detection of autoantibodies using IPP

IPP was performed using TnT products of TIF1-α, Mi-2β or TIF1-γ as previously described [2]. Briefly, 10 μl of patient sera was mixed and incubated with 20 μl of a 50% slurry of Protein G Sepharose (GE Healthcare, Buckinghamshire, UK) and 270 μl IPP buffer (PBS containing 1% Nonidet P-40) at 4°C for 1 h. Sepharose beads were mixed and incubated with 270 μl binding buffer (IPP buffer containing 0.5% BSA) and 10 μl of the TnT products, which was pre-cleared using the sepharose beads, at 4°C for 1 h. The beads were washed five times with IPP buffer and suspended in Laemmli sample buffer, and the IgG-bound proteins were electrophoresed on 7.5% SDS–PAGE gel. Immunoblot was performed as previously described [2].

IPP–western blotting

IPP–western blotting was performed using K562 cell extracts without chemical cross-linking [17]. Monoclonal anti-Mi-2α and polyclonal anti-TIF1-α antibodies were purchased from Sigma-Aldrich (St Louis, MO, USA) and MBL (Nagoya, Japan), respectively. HRP-conjugated anti-mouse immunoglobulin and anti-rabbit IgG antibodies were purchased from DAKO (Glostrup, Denmark).

Statistical analysis

The frequency of antibodies between disease groups was analysed using chi-square test with Yates' correction. $P < 0.05$ was considered statistically significant.

Results

ELISA and IPP with biotinylated recombinant TIF1-α

For the screening of anti-TIF1-α antibodies in a large number of serum samples we used an ELISA system with biotinylated recombinant TIF1-α. We examined a total of 202 serum samples from patients with various CTDs. When the cut-off level was set at 121.3 U, based on 5 S.D.s above the mean value obtained from 20 healthy control sera, the 12 sera from patients with DM were positive for anti-TIF1-α antibodies, whereas none of the sera from patients with other CTDs was positive for those antibodies (12 positive sera/108 total DM sera *vs* 0/94 total CTD sera, $P = 0.0012$) (Table 1). Even based on the cut-off level (83.0) at 3 S.D.s above the mean value, the results were not different.

After the initial screening by ELISA, we investigated antibodies against TIF1-α in sera from 12 anti-TIF1-α ELISA-positive patients and several anti-TIF1-α ELISA-negative patients in order to confirm their ability to immunoprecipitate biotinylated recombinant TIF1-α. All 12 anti-TIF1-α-positive sera in ELISA showed a distinct protein band with a molecular weight of 140 kDa in IPP (Fig. 1B). Twelve anti-TIF1-α-negative sera in ELISA and six healthy control sera did not immunoprecipitate the recombinant. Moreover, none of the 21 serum samples from cancer patients immunoprecipitated (data not shown).

Coexistence of anti-TIF1-α and anti-TIF1-γ antibodies

According to the previous report [5], anti-TIF1-α antibodies always coexist with anti-TIF1-γ antibodies. Our anti-TIF1-α-positive sera were examined for anti-TIF1-γ antibodies by IPP (Fig. 1C). Unexpectedly, only 7 of the 12 sera were also positive for anti-TIF1-γ antibodies. Since IPP for anti-TIF1-γ antibodies had been performed on 81 sera from DM patients in our previous study [2], we examined the antibodies by IPP for the remaining 27 sera. We found a total of 16 sera with anti-TIF1-γ antibodies among the 108 patients with DM. Summarizing the results, in the present DM cohort of 108 patients, 12 and 16 patients had anti-TIF1-α antibodies and anti-TIF1-γ antibodies, respectively. Only 7 patients had both anti-TIF1-α antibodies and anti-TIF1-γ antibodies (anti-TIF1-α/γ-positive), and 87 patients had neither anti-TIF1-α antibodies nor anti-TIF1-γ antibodies.

Coexistence of anti-TIF1-α and anti-Mi-2 antibodies

As mentioned above, we found five anti-TIF1-α-positive, anti-TIF1-γ-negative sera. Previously, two of them had been examined and identified as positive for anti-Mi-2 antibodies by IPP with cell extract (data not shown). Thus, in the present study, we investigated anti-Mi-2 antibodies by IPP with the recombinant Mi-2β in the 12 anti-TIF1-α-positive sera (Fig. 1D). All five of the anti-TIF1-α-positive, anti-TIF1-γ-negative sera were positive for anti-Mi-2 antibodies, whereas none of the seven anti-TIF1-α/γ-positive sera was positive for anti-Mi-2 antibodies. In addition, we investigated anti-Mi-2 antibodies for all the sera from the present DM cohort by ELISA with recombinant Mi-2β, although we detected no additional anti-Mi-2-positive sera except for the five sera described above (data not shown). The IPP–western blotting results showed that each protein precipitated by the five sera reacted to mAb against Mi-2α (Fig. 1E, upper panel) and to polyclonal antibody against TIF1-α (Fig. 1E, lower panel). These sera were confirmed to have anti-Mi-2 antibodies, which, in general, react to both Mi-2α and Mi-2β isoforms [7] and to have anti-TIF1-α antibodies.

Clinical features of DM patients with anti-TIF1-α antibodies

Of the 12 anti-TIF1-α-positive patients, 5 patients with anti-Mi-2 antibodies were classical DM without cancer/ILD, a subset of DM that is associated with anti-Mi-2 antibodies [1, 7]. In contrast, the seven patients with anti-TIF1-α/γ antibodies consisted of three patients with JDM, two with adult ADM and two with cancer-associated DM (Table 1). Demographic data of nine adult patients with anti-TIF1-α antibodies, including gender (male: female = 2 : 7) and age at onset of adult DM [mean (S.D.) = 60.0 (13.5)], and clinical features, including the presence of cutaneous signs of DM (heliotrope rash or Gottron's

papules), RP and elevated creatine kinase, were not statistically different compared with anti-TIF1-α-negative patients (data not shown). However, the presence of ILD in anti-TIF1-α-positive patients was significantly less than that for anti-TIF1-α-negative patients (0/9 *vs* 40/86, $P = 0.0098$).

We investigated other MSA or myositis-associated autoantibodies, e.g. anti-MDA5, -MJ -PL-7, -PL-12, -EJ and -KS (by IPP with TnT product), -Jo-1, -SS-A and -U1-RNP (by commercial ELISA kits) in the 12 anti-TIF1-α antibody-positive patients. Only two patients had concomitant anti-SS-A antibodies, and no other autoantibodies were found.

Discussion

Recently, Fujimoto *et al.* [5] reported that the TIF1 protein family of TIF1-α, -β and -γ is an autoimmune target in DM, especially in cancer-associated DM and JDM patients. Our study clarified that anti-TIF1-α antibodies were not specific markers of a certain subset of DM and that clinical features in the antibody-positive patients were influenced by the other coexistent antibodies against TIF1-γ or Mi-2. The most surprising result in our study is that all classical DM patients with anti-Mi-2 antibodies simultaneously carried anti-TIF1-α antibodies in our cohort, because MSAs have been generally regarded as mutually exclusive [1, 7]. Another new finding in this study was that anti-TIF1-α antibodies were found only in patients with DM and not in those with other CTDs or cancer. The previous report [5] investigated the prevalence of the autoantibodies only in DM patients.

The different result in our study from the previous study is that anti-TIF1-α/γ-positive sera (7/108) are seen less than anti-TIF1-γ-positive, anti-TIF1-α-negative sera (9/108). Fujimoto *et al.* [5] showed that anti-TIF1-α/γ-positive sera ($n = 52$) were more numerous than anti-TIF1-γ-positive, anti-TIFI-α-negative sera ($n = 25$). This discrepancy might be caused by the difference of experimental methods. They evaluated anti-TIF1-α/γ-positive sera using conventional IPP assays, whereas our assays use original recombinant proteins that we have developed. The stringency of our IPP buffer was much higher than that of their methods (the present methods, 1% NP-40 and 150 mM NaCl *vs* their methods, 0.1% NP-40 and 50 mM NaCl). They noted the possibility that TIF1-γ IPP would be caused by anti-TIF1-α antibodies that have cross-reactivity to a TIF1-γ sequence. Our assay may have failed to find such cross-reactive antibodies with low avidity.

Some sera from myositis patients have shown several positive polypeptide bands around 140–160 kDa on SDS-PAGE by IPP, corresponding to anti-NXP-2, -MDA5, -TIF1-γ and -OJ antibodies [18]. Although anti-Mi-2 antibodies mainly target Mi-2α/β of ~240 kDa, bands immunoprecipitated by the antibodies included polypeptides ~140 kDa [18, 19]. The Mi-2/NuRD complex is involved in multiple transcriptional regulatory processes and contains many components [20]. Although TIF1-β was reported to interact with Mi-2α [21], there have been no reports of the NuRD complex containing TIF1-α.

Rheumatology key messages

- Anti-TIF1-α antibodies are detected in DM patients with anti-TIF1-γ or anti-Mi-2 antibodies.
- Anti-Mi-2 antibodies are associated with classical DM even when coexistent with anti-TIF1-α antibodies.

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modifier genes, like *ALAS2* and others, can explain the homozygous, asymptomatic individual with porphyrin levels 10 times the upper limit of normal in the report by Ged *et al.*

Simultaneous occurrence of thalassaemia and CEP due to a single *trans*-acting mutation has been previously reported.[10] We herein report the occurrence of thalassaemia trait and CEP due to two independent mutations. Thalassaemia is endemic to our region and the co-occurrence is probably a coincidence; however, it allows us to examine the existence of any interaction. Despite reported exceptions, the S47P generally displays a severe phenotype, as is the case in our patient. Our patient is also heterozygous for a severe β-chain mutation and an interaction cannot be ruled out. Thalassaemia might increase the severity of the phenotype by increasing the demand for haem products through ineffective erythropoiesis. This remains unknown, but it is a theory that merits consideration.

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A novel *IL36RN/IL1F5* homozygous nonsense mutation, p.Arg10X, in a Japanese patient with adult-onset generalized pustular psoriasis

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Madam, Generalized pustular psoriasis (GPP) is a rare but severe form of psoriasis that is sometimes life-threatening. It is characterized by sudden, repeated episodes of high-grade fever, generalized rash and disseminated pustules. The pathogenesis is unclear except for familial GPP, whose cause was recently identified as homozygous or compound heterozygous mutations in the *IL36RN* gene, also known as *IL1F5*, encoding the interleukin (IL)-36 receptor antagonist (IL36RN).[1,2]

IL36RN is primarily expressed in the skin,[3] and is an antagonist of three cytokines that belong to the IL-1 family: IL-36α, IL-36β and IL-36γ, which are also known as IL-1F6, IL-1F8 and IL-1F9, respectively.[4,5] These cytokines activate several proinflammatory signalling pathways, such as the nuclear factor-κB and mitogen-activated protein kinase pathways.[6,7]

We have followed a Japanese male patient with GPP; *IL36RN* mutation analysis revealed the previously unreported homozygote nonsense mutation p.Arg10X.

A 68-year-old man presented with recurrent episodes of localized sterile pustules with erythema, but without scaly erythematous plaques, on the extremities (Fig. 1a,b) and the trunk, but not on the palmoplantar areas. He had been suffering from similar eruptions since the age of 34 years. On one occasion he showed widespread generalized pustules accompanied by high fever and elevation of circulating c-reactive protein to 30 mg dL^{-1}, which were triggered by infection, and he was hospitalized. A skin biopsy from a pustular eruption on the trunk revealed a spongiform pustule of Kogoj in the epidermis (Fig. 1c), which is consistent with GPP. He was diagnosed with

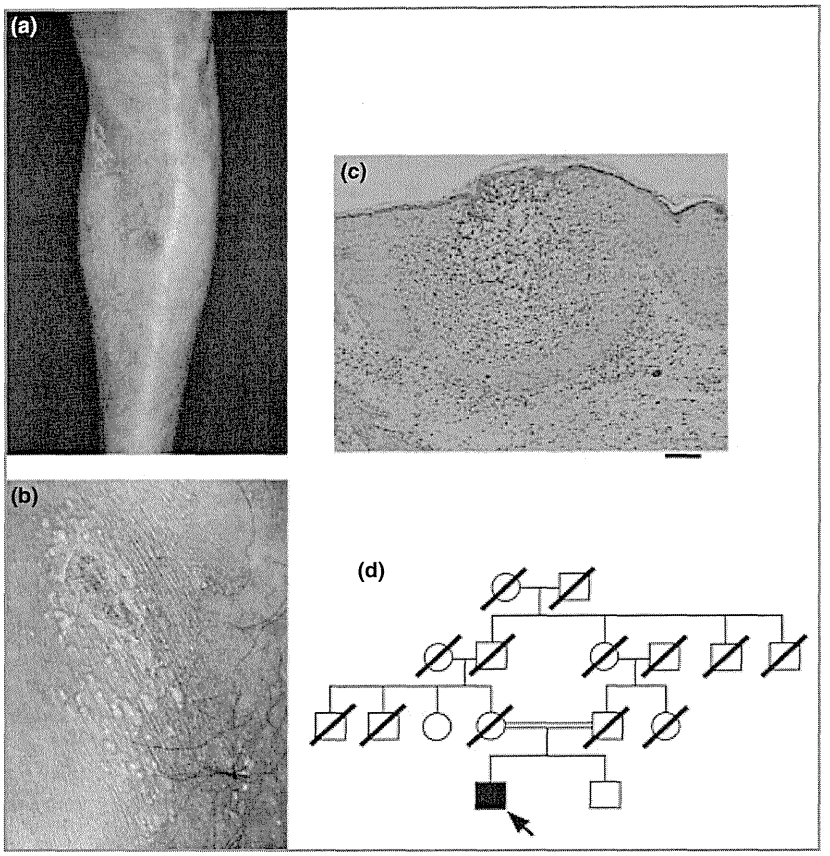

Fig 1. Skin manifestation, histopathology of the skin lesion and pedigree of the patient. (a, b) Pustular erythema on the internal left knee of the patient. (c) Spongiosis of Kogoj and acanthosis were observed in the epidermis of the pustular erythema lesion in the trunk upon the patient's admission to hospital. Scale bar = 100 μm. (d) Pedigree of the patient.

GPP unassociated with psoriasis vulgaris (PV) or palmoplantar pustulosis (PPP). There was no apparent family history of skin disorders, although his parents are first cousins (Fig. 1d).

The ethics committee of Nagoya University approved the study, which was conducted according to the Declaration of Helsinki principles. The participants gave written informed consent. The coding region of *IL36RN* (GenBank accession no. 26525) was amplified from genomic DNA by polymerase chain reaction (PCR), as described previously.[1] Direct sequencing of the patient's PCR products revealed that the patient was homozygous for the previously unreported nonsense mutation of p.Arg10X (c.28C>T) in *IL36RN* (Fig. 2a). C at nucleotide position 28 is two bases upstream from the C′ end of exon 2 (the exon 2–intron 2 boundary) of *IL36RN*. *In silico* analysis using the splicing donor score algorithm[8] was conducted to predict whether this mutation would lead to aberrant or normal splicing; the findings suggested that this mutation results in normal splicing (data not shown).

Immunohistochemistry with rabbit polyclonal anti-IL1F5 antibody (R&D Systems Inc., Minneapolis, MN, U.S.A.) showed almost no expression of IL36RN in the patient's epidermal lesion but strong IL36RN expression in a positive control of psoriatic epidermis (Fig. 2b,c), as reported previously.[9] Thus, it was apparent that the IL36RN protein was almost absent in the patient.

Very recently, IL36RN mutations were reported as causative genetic defects in GPP cases in Tunisian and European populations.[1,2] In these reports, only three missense mutations were identified in IL36RN, i.e. pLeu27Pro in the Tunisian population,[1] and p.Arg48Trp and p.Ser113Leu in the European population.[2] pLeu27Pro and p.Ser113Leu were thought to be very prevalent mutations in the respective (Tunisian and European) populations.

We report for the first time a patient with GPP with an *IL36RN* mutation in an Asian population, and we note that the mutation differs from those prevalent in the Tunisian and European populations. In addition, the present mutation is the first documented nonsense mutation of IL36RN. It is nearly a null mutation of IL36RN, and its abolition or extreme reduction of the protein expression of IL36RN was confirmed in the patient's skin. Thus, the present case bolsters the argument that IL36RN functional deficiency really contributes to GPP.

It is interesting that the disease onset of the present case was the rather late age of 34 years, although the present case was homozygous for the *IL36RN* loss-of-function mutation and had no apparent IL36RN protein. In previous reports, most GPP cases with *IL36RN* mutations have been children, although they included three young adults (disease onset in their 20s). The only exceptional case in previous reports was a patient whose age of onset was 51 years.[2] The present case suggests that even when onset is not until middle age, we cannot exclude the possibility of underlying IL36RN mutations as causative genetic defects.