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BRIEF COMMUNICATION

Comprehensive screening for a complete set of Japanese-population-specific filaggrin gene mutations

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Keywords
allergic rhinitis; filaggrin; hay fever; mutation; real-time PCR.

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Abstract

Mutations in *FLG* coding profilaggrin cause ichthyosis vulgaris and are an important predisposing factor for atopic dermatitis. Until now, most case–control studies and population-based screenings have been performed only for prevalent mutations. In this study, we established a high-throughput *FLG* mutation detection system by real-time PCR with a set of two double-dye probes and conducted comprehensive screening for almost all of the Japanese-population-specific *FLG* mutations (ten *FLG* mutations). The present comprehensive screening for all ten *FLG* mutations provided a more precise prevalence rate for *FLG* mutations (11.1%, $n = 820$), which seemed high compared with data of previous reports based on screening for limited numbers of *FLG* mutations. Our comprehensive screening suggested that population-specific *FLG* mutations may be a significant predisposing factor for hay fever (odds ratio = 2.01 [95% CI: 1.027–3.936, $P < 0.05$]), although the sample sizes of this study were too small for reliable subphenotype analysis on the association between *FLG* mutations and hay fever in the eczema patients and the noneczema individuals, and it is not clear whether the association between *FLG* mutations and hay fever is due to the close association between *FLG* mutations and hay fever patients with eczema.

Mutations in *FLG*, the gene-coding profilaggrin/filaggrin, are an important predisposing factor for atopic dermatitis (AD) (1) and are significantly associated with asthma with AD, mainly in the European population (2–4). The prevalence of *FLG* mutations in AD patients seems to be increasing (5).

The presence of population-specific FLG mutations has been reported in both Europeans and Asians (1, 6, 7) and is a serious obstacle to *FLG* mutation screening in each population. We established a real-time polymerase chain reaction (PCR)-based rapid detection system for Japanese-population-specific *FLG* mutations and performed high-throughput *FLG* screening on 820 residents in a rural area of Japan.

Subjects and methods

The subjects were 820 residents (284 males and 536 females) aged 39–90 years in Yakumo, a rural town in Hokkaido, Japan (8) (Data S1 and Table S1). The participants were requested to answer a questionnaire on health and daily lifestyle at the occasion of the health checkup. Patients with hay fever were defined as individuals reported to have had frequent episodes of all three symptoms of watery eyes, running nose, and sneezing. Patients with asthma were defined as individuals reported to have had a history of asthma, which was diagnosed by physicians. This study was approved by the Ethics Review Committee of Nagoya University Graduate School of Medicine. Ten *FLG* mutations have been identified in the Japanese population, nine of them found by our group (7). We have already performed the sequencing of all the coding regions of *FLG* for more than 30 Japanese families with ichthyosis vulgaris, to identify Japanese-specific *FLG* mutations comprehensively. We expect that screening for these ten mutations can detect almost all Japanese *FLG* mutation carriers. Thus, the present *FLG* mutation screening addressed those ten *FLG* mutations (Table 1). Real-time PCR-based genotyping

Table 1 *FLG* genotypes in the present cross-sectional study

Genotype	p.Arg501X	c.3321delA	p.Ser1695X	p.Gln1701X	p.Ser2554X	p.Ser2889X	p.Ser3296X	p.Lys4022X	p.Q1790X	c.441-442delAG	Combined
AA	820 (1.000)	810 (0.988)	820 (1.000)	818 (0.998)	806 (0.983)	785 (0.957)	816 (0.995)	796 (0.971)	816 (0.995)	820 (1.000)	729
Aa	0 (0)	10 (0.012)	0 (0)	2 (0.002)	14 (0.017)	35 (0.043)	4 (0.005)	24 (0.029)	4 (0.005)	0 (0)	89
aa	0	0	0	0	0	0	0	0	0	0	2*
Total	820	820	820	820	820	820	820	820	820	820	820
Allele freq.(a) (%)	0.0	0.61	0.0	0.12	0.85	2.13	0.24	1.46	0.24	0.0	5.65
HWE† test (chi-square test, *P*-value)	n.a.	0.861	n.a.	0.972	0.805	0.532	0.944	0.671	0.944	n.a.	n.a.

In genotype data columns, numbers are actual measurement data and actual genotype rate in parentheses.
*These data mean compound heterozygote.
†Hardy–Weinberg equilibrium.

of the *FLG* mutations was performed with TaqMan probe genotyping assay (Data S1 and Table S1).

Results and discussion

Of the 820 participants, 89 individuals were heterozygous for one of the ten *FLG* mutations, and two individuals were compound heterozygous for two of the ten mutations. The distribution of genotypes is shown in Table 1. A total of 91 individuals were carriers of one or two of the ten *FLG* mutations. Thus, the mutant allele frequency was 0.057, and the carrier rate was 0.111 in the present study. To confirm the reliability of the present real-time PCR-based rapid detection system of *FLG* mutations, the accuracy of genotyping was confirmed by direct sequencing or PCR-restriction fragment length polymorphism (RFLP) of samples obtained from all carriers and selected noncarriers of the null mutations. As a result, the presence of *FLG* mutations was confirmed by direct sequencing or PCR-RFLP in all 93 alleles in which the mutations were detected by the real-time PCR-based system. We performed direct sequencing or PCR-RFLP on 220 alleles (11 individuals, ten mutations) in which no mutation was detected by the real-time PCR-based system, and we confirmed that no mutation was found in any of the studied alleles. These results clearly indicate that the present real-time PCR-based rapid detection system for the Japanese-population-specific *FLG* mutations is a highly reliable screening method for population-specific *FLG* mutations.

In Yakumo town, the prevalence rate of *FLG* mutations was found to be relatively high (11.1%) compared with the data of previous reports (2, 3). We do not know the exact reason, but one possibility is that we studied all the Japanese-population-specific FLG mutations that we have detected for the last 8 years in the Japanese population, whereas the prevalence rates of *FLG* mutations in European studies were mostly evaluated from the data of studies on only a few prevalent mutations, for example, screening only for R501X and 2282del4, or for the five prevalent mutations in the European population. If only the seven mutations reported in the previous study in 2009 (9) had been used in the present study, the prevalence rate of FLG mutations in Yakumo town would have been only 7.7%. In light of this, the higher prevalence rate of 11.1% in the present study might be a reasonable value. On the other hand, we cannot exclude the possibility that the present subjects have skewed genetic backgrounds regarding *FLG* mutations, because many Yakumo residents are descendants of immigrants who moved to Yakumo from a limited area of Nagoya city in central Japan.

According to the present data from a clinical questionnaire given to 816 Yakumo residents, 63 individuals reported having had frequent episodes of watery eyes, runny nose, and sneezing, and they were considered to be putative hay fever patients. Of the 91 individuals with *FLG* mutations, 12 individuals were putative hay fever patients (13.2%), and of the 725 individuals without *FLG* mutations, 51 were putative hay fever patients (7.0%). Thus, *FLG* mutations were significantly associated with putative hay fever (odds ratio = 2.01 [95% CI: 1.027–3.936, $P < 0.05$]; Table 2).

Table 2 Prevalence of history of asthma, watery eyes, and runny nose (n = 820) for the total group and the subgroups of individuals with the combined genotype (n = 91) and without the combined genotype (n = 729)

History	All individuals% (n)	Individuals with FLG mutations% (n)	Individuals without FLG mutations% (n)	OR	95% CI	P-value
Asthma	6.6 (54/815)	10 (9/90)	6.2 (45/725)	1.68	0.792–3.561	0.129
Watery eyes	20.8 (170/817)	22 (20/91)	20.7 (150/726)	1.08	0.638–1.833	0.431
Runny nose	25.9 (211/816)	24.2 (22/91)	26.1 (189/725)	0.90	0.544–1.502	0.403
Watery eyes and runny nose	9.1 (74/816)	14.3 (13/91)	8.4 (61/725)	1.81	0.954–3.451	0.056
Putative hay fever (watery eyes, runny nose, sneezing)	7.7 (63/816)	13.2 (12/91)	7 (51/725)	2.01	1.027–3.926	0.038

Concerning asthma, of the 90 individuals with *FLG* mutations, nine individuals had asthma history (10.0%), and of the 725 individuals without *FLG* mutations, 45 had asthma history (6.2%). Thus, asthma history was not significantly associated with *FLG* mutations (odds ratio = 1.68 [95% CI: 0.792–3.561, P = 0.129]; Table 2). This result, that is, nonsignificant association of general asthma with *FLG* mutations, is consistent with previous data by case–control study (4, 10). However, in the present study, we think that the association between the *FLG* mutations and asthma was maybe not significant, due to the reduced sample size and power.

Weidinger et al. (11) reported that, independent of eczema, *FLG* mutations confer a substantial risk for allergic rhinitis. Contrasting reports suggest that the significant association observed between *FLG* mutations and hay fever may be due to the close association between *FLG* mutations and patients with both hay fever and eczema (10, 12). As for asthma, the significant association between the *FLG* mutations and asthma was thought to be due to the close association between *FLG* mutations and asthma patients with eczema (10–12), although there is a report that suggests a significant association between *FLG* mutations and eczema-free asthma patients (13). In light of this, we tried to perform subphenotype analysis to clarify the connection between *FLG* mutations and hay fever/asthma in the eczema patients and the noneczema individuals. However, in these subphenotype analyses, the sample sizes were too small, and we were unable to obtain reliable data (Data S2). Thus, it is not clear whether the association between *FLG* mutations and hay fever in the present study is due to the close association between *FLG* mutations and hay fever patients with eczema.

A number of case–control studies have addressed the association of *FLG* mutations with asthma and hay fever (2–4, 10, 11). However, the number of studies among the general population is limited, and previous studies were performed only for prevalent *FLG* mutations in each population. In contrast, in the present study, we performed comprehensive screening for almost all the population-specific *FLG* mutations in the general population. In light of this, our present results are noteworthy when we discuss the significance of *FLG* mutations in the pathogenesis of hay fever.

Author contributions

MK, NH, WHIM, HS, and MA contributed study conception and design. MK, TN, NH, YO, OM, SS, HT, and NH contributed data acquisition and analysis. MK, NH, HS, and MA contributed data interpretation. MK and MA wrote the manuscript, and MK, TN, NH, HS, and MA revised it critically for important intellectual content. All authors approved the final version of the manuscript.

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Conflicts of interest

The authors declare no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. The sequence of assay probes/primers.

Data S1. Materials and methods.

Data S2. Results: subphenotype analysis.

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SHORT COMMUNICATION

Lamellar Ichthyosis Caused by a Previously Unreported Homozygous *ALOXE3* Mutation in East Asia

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Autosomal recessive congenital ichthyosis (ARCI) includes a wide range of ichthyosis phenotypes, including harlequin ichthyosis, lamellar ichthyosis (LI), congenital ichthyosiform erythroderma (CIE), and self-improving collodion ichthyosis (SICI) (1, 2). To date, 9 causative genes for ARCI have been identified (1, 2). *ALOXE3* is a causative gene in LI as well as CIE, and it encodes the eLOX-3 lipoxygenase, which is predominantly synthesised in the epidermis. ARCI caused by an *ALOXE3* mutation is very rare, with less than 30 families with the mutation reported in the literature. The previously reported cases with homozygous or compound heterozygous *ALOXE3* mutations were from Europe, North Africa, the Middle East, and South Asia (3–8). Here, we describe an LI patient with a previously unreported homozygous *ALOXE3* mutation in a consanguineous family from Japan and review ARCI cases with *ALOXE3* mutations.

CASE REPORT

The patient is a 58-year-old Japanese woman who presented with symptoms of ichthyosis since birth. Her parents were first cousins. She has 3 siblings, of which one has a similar ichthyosis phenotype (Fig. 1a). Ectropion was not reported at birth. She showed brown-to-gray scaling without erythroderma on her trunk and extremities (Fig. 1b). She did not show pal-

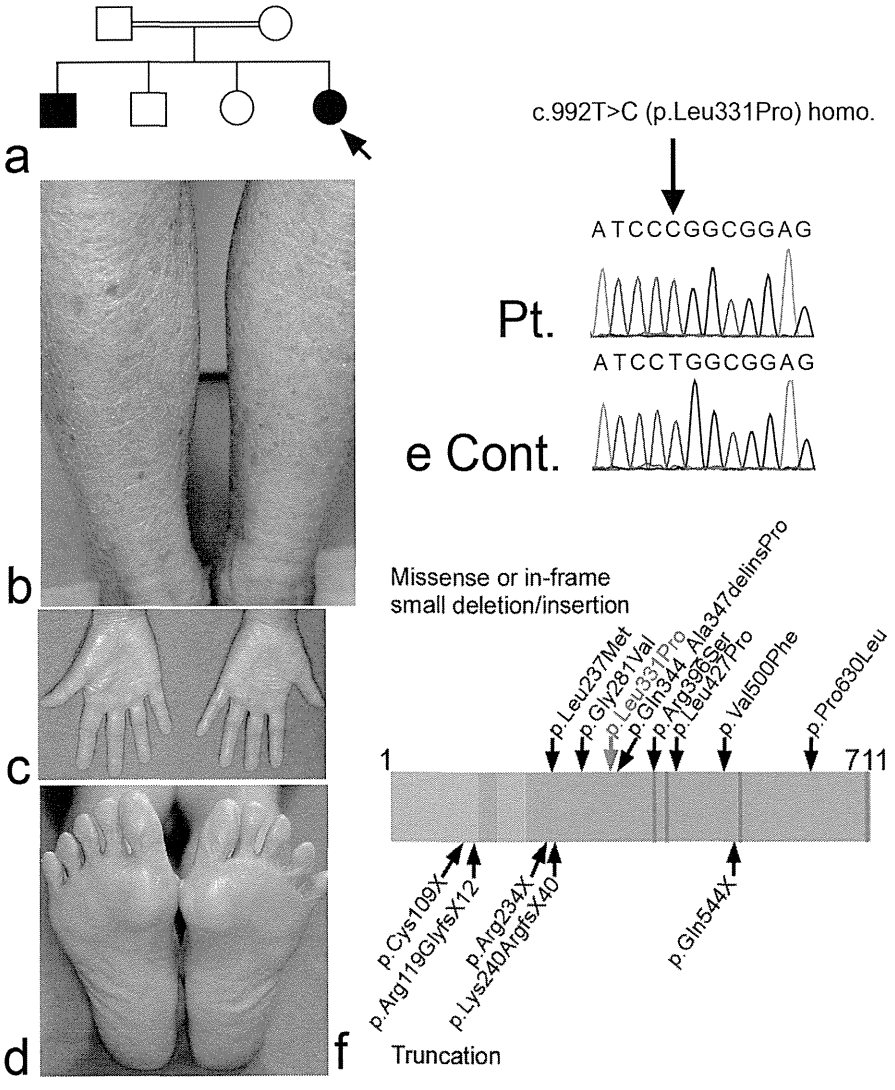

Fig. 1. Pedigree, clinical features, and *ALOXE3* sequence data of the patient; sequence alignments around the missense mutation; and a summary of known *ALOXE3* mutations. (a) Pedigree of the patient. (b) The patient showed brown-to-gray scaling bilaterally on the lower legs. (c) The patient did not show palmar keratosis. (d) The patient showed mild plantar hyperkeratosis. (e) Sequence data of *ALOXE3* in the patient with the mutation and a control without the mutation. The arrow indicates c.992T>C (homozygous). (f) The eLOX-3 protein domain structure and *ALOXE3* mutations from this study and the literature. The previously unreported missense mutation identified in this study, p.Leu331Pro, is shown in red. A blue box and a green box indicate the N-terminal β-barrel LH2 domain and an inserted specific extra domain, respectively. Pink boxes indicate C-terminal catalytic lipoxygenase domain from amino acid position 126. Putative iron ligands of the active sites are in red.

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mar keratosis or alopecia, but did show mild plantar keratosis during middle age (Fig. 1c, d).

Following ethical approval, informed written consent was obtained in compliance with the Declaration of Helsinki guidelines. The coding regions, including the exon-intron boundaries of *TGM1, ABCA12, ALOX12B*, and *ALOXE3*, were amplified from genomic DNA by PCR as described elsewhere (3). Direct sequencing of the patient's PCR products revealed that the patient had a homozygous *ALOXE3* mutation, c.992T>C (p.Leu331Pro) (gene accession number: NM_021628.2) (Fig. 1e). p.Leu331Pro was analysed using SIFT (http://sift.jcvi.org/) and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/). The SIFT score was 0.000 and PolyPhen-2 score was 1.000; both scores predicted that p.Leu331Pro had damaging effects. We found no mutation in the other 3 genes tested. c.992T>C was not detected in the 200 control alleles (100 control individuals, data not shown). Thus, the patient was diagnosed as having LI caused by the homozygous *ALOXE3* mutation.

DISCUSSION

All previously reported ARCI cases with *ALOXE3* mutations have been in families from Europe, North Africa, the Middle East, and South Asia (Fig. 1f, Table SI[1]). To our knowledge, the present patient is the first case with *ALOXE3* mutations in a family from East Asia. Our case suggests that *ALOXE3* mutations are possibly found in families worldwide. We reported more than 50 Japanese cases of ARCI that had *TGM1, ABCA12, ALOX12B*, or *CYP4F22* mutations (2, 9, 10). Although we do not have data indicating how often patients with ichthyosis are offered genetic testing in Japan, no other patients with *ALOXE3* mutations have been found to date. We hypothesise that the carrier rate of ichthyosis-causing *ALOXE3* mutations may be very low in Japan.

We reviewed 39 cases of ARCI from 29 families that had *ALOXE3* mutations, including the case described here (3–8) (Table SI[1]). Thirteen *ALOXE3* mutations have been reported (Fig. 1f). Truncation mutations, missense mutations, and an in-frame small deletion/insertion mutation have been reported. The truncation mutations include nonsense mutations, a deletion mutation resulting in a frame shift, and a splice site mutation. In 2 cases, *ALOXE3* mutations were identified only in one allele. In the literature (Table SI[1]), ARCI phenotypes caused by *ALOXE3* mutations were categorised as CIE, LI, and SICI. In 3 cases, clinical features were not described, and their ARCI phenotypes were unknown.

In conclusion, the present case clearly indicates that *ALOXE3* is a possible causative gene in East Asian ARCI patients.

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Whole-exome sequencing improves mutation detection in a diagnostic epidermolysis bullosa laboratory

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Conflicts of interest
None declared.

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Summary

Background Subtypes of inherited epidermolysis bullosa (EB) vary significantly in their clinical presentation and prognosis. Establishing an accurate diagnosis is important for genetic counselling and patient management. Current approaches in EB diagnostics involve skin biopsy for immunohistochemistry and transmission electron microscopy, and Sanger sequencing of candidate genes. Although informative in most cases, this approach can be expensive and laborious and may fail to identify pathogenic mutations in ~15% of cases.

Objectives Next-generation DNA sequencing (NGS) technologies offer a fast and efficient complementary diagnostic strategy, but the value of NGS in EB diagnostics has yet to be explored. The aim of this study was to undertake whole-exome sequencing (WES) in nine cases of EB in which established diagnostic methods failed to make a genetic diagnosis.

Methods Whole-exome capture was performed using genomic DNA from each case of EB, followed by massively parallel sequencing. Resulting reads were mapped to the human genome reference hg19. Potentially pathogenic mutations were subsequently confirmed by Sanger sequencing.

Results Analysis of WES data disclosed biallelic pathogenic mutations in each case, with all mutations occurring in known EB genes (*LAMB3*, *PLEC*, *FERMT1* and *COL7A1*). This study demonstrates that NGS can improve diagnostic sensitivity in EB compared with current laboratory practice.

Conclusions With appropriate diagnostic platforms and bioinformatics support, WES is likely to increase mutation detection in cases of EB and improve EB diagnostic services, although skin biopsy remains an important diagnostic investigation in current clinical practice.

What's already known about this topic?

- Skin microscopy and Sanger sequencing are useful techniques for the accurate diagnosis of specific subtypes of epidermolysis bullosa (EB).
- The specificity and sensitivity of these current diagnostic tools is good, although some cases of EB elude a precise laboratory diagnosis and the work involved is often time consuming, labour intensive and expensive.
- There is a need to refine and improve diagnostics for EB.

What does this study add?

- Whole-exome sequencing (WES) with bioinformatics support can identify mutations in cases of EB for which current diagnostic techniques fall short.
- WES has the potential to lessen the need for diagnostic skin biopsies in EB, and reduce laboratory costs.
- The adoption of WES into routine laboratory EB diagnostics requires a reduction in sample processing and data interrogation time to match current diagnostic tests for EB.

Epidermolysis bullosa (EB) constitutes a diverse group of genodermatoses characterized by trauma-induced skin fragility, blisters and erosions.[1] EB is currently divided into four main subtypes [EB simplex (EBS), junctional EB (JEB), dystrophic EB (DEB) and Kindler syndrome] based on the level of blister formation at or close to the dermoepidermal junction. The molecular pathology of EB now involves mutations in 18 genes.[1,2] Over the last 20 years, international consensus group meetings have been held, most recently in 2013,[1] to revise and update the diagnosis and classification of EB. New forms of EB have been added, some disease names have been changed, and recommendations have been made about the laboratory diagnosis of EB.[1]

Currently, the diagnosis of most cases of EB involves a skin biopsy. Typically, skin sections are stained with a panel of basement membrane zone antibodies and viewed by immunofluorescence microscopy (IFM). In many autosomal recessive forms of EB, the inherent loss-of-function mutations are likely to lead to a reduction or absence of immunolabelling for one particular protein, thus identifying the candidate gene for Sanger sequencing (SS).[3–5] However, in autosomal dominant forms of EB (and some autosomal recessive cases), IFM may not show clear differences from normal control skin, and further clues might be sought from transmission electron microscopy (TEM).[6] The overall objective is then to determine which gene(s) to investigate by SS. For the known EB genes, pairs of primers are designed to amplify individual exons and flanking introns. Polymerase chain reaction (PCR) products (typically 200–350 base pairs in size) are then individually examined by SS. PCR amplification protocols and primer pair sequences have been published for all known EB genes, and similar protocols and reagents have been adopted into laboratory practice for diagnosing EB throughout the world. Several of the EB genes contain numerous exons; for example, *COL7A1* (encoding type VII collagen, which is mutated in DEB) contains 118 exons, and > 70 PCR primer pairs are necessary for amplification of all exons and flanking introns.[7,8] To amplify all 18 genes implicated in the different forms of EB currently requires > 400 primer pairs for genomic DNA analysis, hence the almost inevitable need for candidate gene clues from skin biopsies.

In 2004, we established a national diagnostic service for EB in the U.K. (designated the Robin Eady National Diagnostic Epidermolysis Bullosa Laboratory, and based at St Thomas' Hospital, London, U.K.). The approach to diagnosis in cases of EB has involved IFM, TEM and SS, as outlined above, but in a number of cases (perhaps ~15% of > 1500 cases) these methods have failed to reveal any pathogenic mutations. Some of these cases may reflect erroneous clinical diagnoses by the referring clinicians, but undoubtedly technical limitations have also contributed to the sensitivity of current diagnostic methods.

One new technology that could potentially improve sensitivity in EB diagnostics is next-generation sequencing (NGS), in which the whole genome or a portion thereof is sequenced.[9,10] NGS has proven very useful for identifying novel genetic variants responsible for Mendelian disorders, including a new form of EBS.[11] However, although NGS is informative in a research setting, its diagnostic utility remains unclear.[12–14] To assess the potential impact of NGS on EB diagnostics, we undertook whole-exome sequencing (WES) and bioinformatic data analysis on nine autosomal recessive cases of EB in which current diagnostic strategies had failed to identify one or both of the pathogenic mutations.

Materials and methods

Cases for study

The nine cases with a clinical diagnosis of EB who were selected for study were all routine diagnostic cases referred to the National Diagnostic EB Laboratory between 2008 and 2013, for whom skin biopsy analysis and SS had failed to identify pathogenic mutations.

Whole-exome sequencing

Whole-exome capture was performed by in-solution hybridization using SureSelect All Exon 50 Mb Version 4.0 (Agilent, Santa Clara, CA, U.S.A.) followed by massively parallel sequencing with 100-bp paired-end reads on the HiSeq2000 platform (Illumina, San Diego, CA, U.S.A.). Resulting reads were mapped to the human genome reference hg19 using the Novoalign alignment tool (Novocraft Technologies Sdn Bhd, Selangor, Malaysia). Variant calling was undertaken at the

individual sample level with the SAMtools mpileup utility.[15] Resulting variant calls were filtered with the BCFtools utility,[15] filtered for a minimum coverage (calls with fewer than four reads filtered) and hard filtered for quality (variants with quality < 20 filtered from further analysis). This high-quality call set was then annotated with respect to the genes, and for consequences on protein sequence and/or splicing with the ANNOVAR tool.[16] Further annotation regarding previously reported observation of specific variants and estimated population frequencies was achieved through further rounds of ANNOVAR annotation against dbSNP137, population frequency estimates from the 1000 Genomes project (http://www.1000genomes.org/) and National Institutes of Health Heart, Lung and Blood Institute Grand Opportunity Exome Sequencing Project (https://esp.gs.washington.edu/drupal/), and ~1000 control exomes that have been processed through the same bioinformatics analysis pipeline. The exome data on all cases was deemed to be of very high quality. Over 8·6 gigabases of mappable sequence data were generated, such that > 90% of the coding bases of the exome defined by the GENCODE Project (http://www.gencodegenes.org/) were represented by at least 20 reads.

Results

Case 1: Generalized intermediate junctional epidermolysis bullosa with elusive mutation in *LAMB3*

A 58-year-old white man had a clinical diagnosis of generalized intermediate JEB (previously known as non-Herlitz JEB)[1] characterized by lifelong trauma-induced blistering and erosions, nail loss/dystrophy and some hair loss, along with two separate squamous cell carcinomas. Previous skin biopsy had shown a lamina lucida plane of cleavage by antigen mapping and markedly reduced-intensity immunolabelling for laminin-332. However, SS of *LAMA3*, *LAMB3* and *LAMC2*, and subsequently *COL17A1*, *ITGA6* and *ITGB4*, did not reveal any mutations. By WES, we identified 236 novel heterozygous and 20 novel homozygous variants in his genomic DNA (Table 1), including a homozygous 2-bp deletion mutation c.1587_1588delAG (p.Thr529Thrfs*6) in exon 13 of *LAMB3*, which was then verified by repeat SS. This deletion occurred within a ~4·7-Mbp block of homozygosity. The mutation has been reported previously and was shown to result in skipping of downstream exon 14,[17] which we were also able to verify by reverse-transcriptase PCR using RNA extracted from the patient's skin (data not shown). Typically, skipping of exon 14 would be out of frame, but with the addition of the 2-bp deletion in exon 13, the combined deletion is in frame. This case shows that WES can offer a more sensitive approach in identifying mutations in *LAMB3*, but in addition the findings question the current paradigm for genotype–phenotype correlation, as this mutation would usually be expected to result in severe generalized JEB (previously referred to as Herlitz JEB).[1] The WES findings also stress the need for further studies at the RNA and protein levels in some cases of EB.

Case 2: Mild acral blistering, nail dystrophy and hypotonia with a mutation in a different plectin isoform

An 18-year-old Pakistani man presented with mild blistering, developmental delay and hypotonia. A history of consanguinity supported autosomal recessive inheritance. IFM revealed a complete absence of plectin immunostaining, supporting biallelic loss-of-function mutations in *PLEC*. However, no mutation in *PLEC* was identified on SS. Given the proposed mode of inheritance and rarity of this phenotype, our hypothesis was that the disease-causing variant would be novel and homozygous. By WES we identified 454 heterozygous and 54 homozygous novel variants (Table 1). The filtered variant list generated after WES included a novel frameshift mutation (c.94dupG) in exon 1a of a plectin isoform not included in our current SS screening (transcript accession number NM_201384). We made new genomic primers to span this mutation and confirmed its presence on SS. The mutation identified by WES was located in a plectin isoform not previously thought to be expressed in skin and muscle; we have since modified our *PLEC* screening protocol accordingly.

Cases 3 and 4: Kindler syndrome with elusive *FERMT1* mutations

An 18-year-old Turkish man (case 3) had a history of trauma-induced blisters from early life with the development of some photosensitivity and poikiloderma in light-exposed sites during infancy and childhood. An unrelated case, an 8-year-old Indian boy (case 4), had acral blistering from the first week of life, as well as photosensitivity, palmoplantar skin thickening, poikiloderma and a urethral stricture/stenosis. SS of *FERMT1* (previously called *KIND1*) failed to reveal any mutations in either subject. However, in case 3, WES revealed 398 novel variants (393 heterozygous and five homozygous; Table 1), including compound heterozygous mutations in *FERMT1* (c.1811G>A, p.Trp604* and c.614G>A, p.Trp205*). The mutation p.Trp604* is novel, whereas p.Trp205* has been reported previously.[18] SS of *FERMT1* failed to reveal any mutations in case 4, but WES revealed 553 novel variants (541 heterozygous and 12 homozygous), which included compound known heterozygous donor splice-site mutations, c.1718 + 2T>C and c.384_385 + 2del4, in *FERMT1*.[19,20] All four mutations were confirmed in genomic DNA by repeat SS using new primers spanning the mutations. WES was therefore shown to be a sensitive means of identifying existing and new *FERMT1* mutations in Kindler syndrome.

Case 5: Clinically mild blistering with *DST-e* identified as incorrect candidate gene by immunofluorescence microscopy

A 21-year-old white man had experienced mild generalized skin fragility since early life; most of the blistering was acral. IFM showed a complete absence of immunostaining for the 230-kDa bullous pemphigoid antigen (BP230), but no other

Table 1 Whole-exome sequencing (WES) data and impact on epidermolysis bullosa (EB) diagnostics for the nine cases studied

Case no.	Clinical diagnosis	Gene	Mutation	Mutation type	PolyPhen-2: prediction, score[a]	SIFT: prediction, score[b]	Inheritance	Single-nucleotide substitutions in WES, n		Impact of WES
								Known	Novel (heterozygous/ homozygous)	
1	GIJEB	*LAMB3*	c.1587_1588del2, PTC	Frameshift			AR	23488	256 (236/20)	Identification of an atypical mutation in *LAMB3* with implications for revising genotype–phenotype correlation
2	EBS	*PLEC*	c.92_93insG to c.94dupG	Frameshift			AR	24257	508 (454/54)	Identification of a *PLEC* mutation in an isoform not thought to be expressed in skin and muscle with implications for expanding *PLEC* gene screening for diagnostic practice
3	Kindler	*FERMT1*	c.1811G>A, p.Trp604*	Nonsense			AR	24504	398 (393/5)	Improved detection of *FERMT1* mutations indicating current SS primers are suboptimal
			c.614G>A, p.Trp205*	Nonsense						
4	Kindler	*FERMT1*	c.1718 + 2T>C	Splice site			AR	24283	553 (541/12)	Improved detection of *FERMT1* mutations indicating current SS primers are suboptimal
			c.384_385 + 2del4	Splice site						
5	EBS/ RDEB	*COL7A1*	c.793C>T, p.Gln265*	Nonsense			AR	23649	184 (181/3)	Identification of mutations in a different, unsuspected EB gene providing a diagnosis that would otherwise have been clinically erroneous and missed in SS screening
			c.6005G>A, p.Arg2002His	Missense	Probably damaging, 1	Tolerated, 0·11				
6	Severe RDEB	*COL7A1*	c.904G>T, p.Glu302*	Nonsense			AR	24001	681 (657/24)	Improved detection of *COL7A1* mutations in exon 98 and 99 because of previous failure to amplify DNA due to a polymorphism within one of the PCR primers used for SS
			c.7505G>A, p.Gly2502Glu	Missense	Probably damaging, 0·999	Damaging, 0				
7	Mild RDEB	*COL7A1*	c.4027C>T, p.Arg1343*	Nonsense			AR	24260	217 (208/9)	Improved detection of *COL7A1* mutations indicating current SS primers are suboptimal
			c.8676G>A, p.Trp2892*	Nonsense						

(continued)

Table 1 (continued)

Case no.	Clinical diagnosis	Gene	Mutation	Mutation type	PolyPhen-2: prediction, score[a]	SIFT: prediction, score[b]	Inheritance	Single-nucleotide substitutions in WES, n		Impact of WES
								Known	Novel (heterozygous/homozygous)	
8	Severe RDEB	*COL7A1*	c.3630_3631insC, PTC c.520G>A, p.Gly174Arg	Frameshift Missense	Probably damaging, 1	Damaging, 0·002	AR	24250	638 (618/20)	Improved detection of *COL7A1* mutations indicating current SS primers are suboptimal
9	Mild RDEB	*COL7A1*	c.1732C>T, p.Arg578* c.2126T>C, p.Val709Ala	Nonsense Missense	Possibly damaging, 0·939	Damaging, 0	AR	24231	214 (207/7)	Improved detection of *COL7A1* mutations indicating current SS primers are suboptimal

GJEB, generalized intermediate junctional EB; EBS; EB simplex; RDEB, recessive dystrophic EB; PTC, premature termination codon; AR, autosomal recessive; SS, Sanger sequencing; PCR, polymerase chain reaction. [a]http://genetics.bwh.harvard.edu/pph2/; [b]http://sift.jcvi.org/.

major differences from control skin. However, SS of *DST-e*, which encodes BP230, failed to identify any pathogenic mutations. Surprisingly, among 181 heterozygous and three homozygous novel variants identified by WES (Table 1), we identified compound heterozygous mutations in a different basement membrane zone gene, *COL7A1*. The mutations were c.793C>T (p.Gln265*) and c.6005G>A (p.Arg2002His). These mutations in *COL7A1* were confirmed by SS, and both parents were shown to be respective carriers of one of the two mutations identified. Neither of these two mutations has been reported previously. Compound heterozygosity for this nonsense/missense combination of mutations in *COL7A1* would be expected to lead to recessive dystrophic EB (RDEB). In this case, WES revised the clinical diagnosis from EBS to RDEB, although why the IFM revealed an abnormality only in BP230 is not known.

Case 6: Missed *COL7A1* mutation due to polymorphism in the primer sequence

A newborn white British boy presented with generalized skin blistering. Skin biopsy revealed a complete absence of type VII collagen at the dermoepidermal junction, consistent with a diagnosis of severe generalized RDEB. Screening of *COL7A1* by SS revealed one heterozygous mutation in exon 7, c.904G>T (p.Glu302*), but no second mutation. However, by WES, among 657 heterozygous and 24 homozygous novel variants (Table 1), a second mutant *COL7A1* allele was identified: a heterozygous glycine substitution, c.7505G>A (p.Gly2502-Glu), in exon 99. Neither of these mutations has been reported previously, but both are typical of the type of gene pathology that is found in RDEB. The reason why the second mutation was missed by SS was a single-nucleotide polymorphism that we identified in intron 99–100, rs6781283, which is located within the reverse primer used to amplify this exon and flanking introns. This polymorphism is likely to have prevented primer annealing and, therefore, the deleterious allele would not have been amplified during PCR. New primers were designed and the heterozygous glycine substitution was subsequently detected by SS. This finding has important implications for the optimal design of working primers in gene amplification and SS.

Cases 7–9: Elusive second-allele *COL7A1* mutations in recessive dystrophic epidermolysis bullosa

A 27-year-old white British woman (case 7) with lifelong trauma-induced blistering and nail dystrophy had a clinical diagnosis of DEB, possibly localized RDEB or *de novo* dominant DEB, as no other family members were affected. Screening of *COL7A1* by SS revealed a known single heterozygous mutation, c.4027C>T (p.Arg1343*) in exon 34. This finding indicated a diagnosis of RDEB but no second mutation was found. However, WES identified 208 heterozygous and nine homozygous novel variants, which revealed a second heterozygous mutation, c.8676G>A (p.Trp2892*), in exon 117 of *COL7A1*. This

second mutation, which has not been reported previously, occurs close to the 3′ end of the gene and probably accounts for the relatively milder RDEB phenotype.

A 26-year-old Pakistani man (case 8) had clinical features of severe generalized RDEB, with marked trauma-induced skin fragility, hand contractures, neck scarring and oesophageal stenoses. SS of *COL7A1* revealed a single previously unreported heterozygous mutation c.3630_3631insC (p.Gln1211Profs*8) but no identifiable second loss of function mutation. However, WES disclosed 618 heterozygous and 20 homozygous novel variants (Table 1), including a second heterozygous mutation, c.520G>A (p.Gly174Arg) in exon 4 of *COL7A1*. This missense mutation within the noncollagenous domain of type VII collagen has been reported previously in RDEB and shown to cause aberrant splicing.[21]

A 61-year-old white British woman (case 9) had lifelong mild, predominantly acral blistering. Two siblings were similarly affected and all were thought to have a mild form of RDEB. SS of *COL7A1* revealed a single heterozygous mutation, c.1732C>T (p.Arg578*) in exon 13. The two siblings were also carriers of this mutation, which is known to be a common recurrent loss-of-function mutation in *COL7A1* in the U.K. and northern Europe.[22] However, no second mutation was identified by SS. In contrast, WES identified 207 novel heterozygous and seven homozygous variants, including a second mutant allele, c.2126T>C (p.Val709Ala), in exon 16 of *COL7A1*, which was also present in both siblings but not in the exome sequences from > 900 ethnically matched controls. This mutation is likely to cause subtle disruption to the function of type VII collagen, given the mild phenotype in this family. The WES data thus expand genotype–phenotype correlation by implicating a further nonglycine missense mutation in the pathophysiology of RDEB.

Discussion

In these nine cases of EB, WES proved to be highly informative in identifying the pathogenic mutations. In six of these cases (1, 3, 4, 7, 8, 9), the mutations were missed by initial SS – not because of human error, but rather due to the well-known variable peak heights associated with current SS chemical labelling of DNA, which can lead to imprecision in interpreting sequence traces. Review of the original sequence traces indicated either subtle changes in peak heights or equivocal findings, although repeat SS with close attention to the nucleotides implicated by WES did reveal the presence of the mutations. In case 5, it is uncertain why the IFM showed a lack of BP230 immunostaining when the pathogenic mutations were in type VII collagen – but this highlighted the potential fallibility of using IFM to determine the candidate gene for SS in cases of EB, even though this approach has proved to be highly informative, particularly in severe recessive forms of EB. In this case, the relatively mild clinical features were thought to be consistent with other cases that have demonstrated pathogenic mutations in *DST-e*,[23] and thus the combination of clinicopathological information unfortunately led to erroneous sequencing of *DST-e*. Only the finding of *COL7A1* mutations using WES provided the true molecular pathology and helped to correct the clinical diagnosis from autosomal recessive EBS to mild RDEB. However, for the other two cases, WES was fundamentally important in identifying the molecular basis of the EB.

In case 2, the clinicopathological evidence pointed to plectin pathology, but SS did not disclose any mutations in *PLEC*, at least using established sequencing protocols and primers. However, WES identified a recessive homozygous mutation in a different isoform of *PLEC*. We subsequently used reverse-transcriptase PCR to confirm that this isoform was indeed expressed in skin and muscle (data not shown), and we thereafter modified our PCR approach for SS of *PLEC* for screening future cases. In case 6, the failure of SS lay in the fact that there was a nonpathogenic polymorphism within one of the PCR primers that led to failure of amplification of that *COL7A1* allele, which in this case happened to contain one of the pathogenic mutations. We have previously encountered this failure to amplify one allele for exon 23 of *LAMB3* in cases of JEB, which led to a redesign of the genomic DNA PCR primers,[24] and our new findings for *COL7A1* have also prompted us to redesign primers for exons 98 and 99 and flanking introns. The primers we recommend are: 5′-CGTATGTCTTACTCCA CAGC (intron 97) and 5′-ACCCTTTAGTCCTGCACTC (intron 99). Given that the primer pair we were using was the same as that initially suggested in a widely cited *COL7A1* amplification protocol,[7] our data should encourage others involved in *COL7A1* mutation analysis by SS to review their choice of PCR primers for these particular exons.

Our study is not a direct comparison between our existing diagnostic approach for EB and NGS. Rather, this analysis was meant as a first step in determining whether WES might improve diagnostic sensitivity in EB, in being able to identify mutations that have proved elusive using current SS approaches. Unequivocally, WES is helpful in that regard,[25] although certain types of mutations may also be difficult or impossible to detect by WES, for example in noncoding regions.

From a practical perspective, the introduction of NGS into routine EB diagnostics requires further considerations of cost, facilities, staff and time to report. In our institution (King's College London), current costs of WES (including bioinformatics analysis) are ~£900 (2014 prices), which compares favourably with SS of EB genes. Of note, it costs a similar amount just to sequence the *COL7A1* gene alone. Moreover, the cost of WES is likely to decrease further over the next few years, thus providing a strong economic argument for adoption of NGS into EB diagnostics. However, the challenge lies in data interrogation and the bioinformatics analyses required to scrutinize the sequence variants and to determine causality. For EB diagnostics this would mean a realignment of technical wet lab skills (IFM and TEM) in favour of computer database and *in silico* work. The biggest challenge lies in the time it takes to process and analyse a case. In EB diagnostics a rapid diagnosis is often very important to optimize clinical management, particularly in neonates with fragile skin. The current

approach using skin biopsy assessment followed by SS of candidate genes (implicated by IFM and/or TEM) allows for possible diagnoses to be made within 2–3 days. In contrast, the quickest time in which WES could be completed (at present) would be a minimum of 5 days. Therefore, being able to reduce the time it takes to make a diagnosis using WES will be fundamental to its application in clinical service. New platforms to enable this are in development, but only when more rapid sample analysis is feasible in a diagnostic lab setting can one really begin to think about changing diagnostic practice. For now, skin biopsy remains an integral part of current EB diagnostics.

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RESEARCH ARTICLE Open Access

Anti-PM/Scl antibodies are found in Japanese patients with various systemic autoimmune conditions besides myositis and scleroderma

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Abstract

Introduction: Anti-PM/Scl antibodies are associated with polymyositis (PM)/systemic scleroderma (SSc) overlap syndromes and are also found in other systemic autoimmune diseases. Although anti-PM/Scl reactivity is found in 3-11% of PM or SSc patients and in approximately 25% of PM/SSc overlap patients, previous large studies of Japanese patients with scleroderma reported that anti-PM/Scl are not found in Japanese patients at all. The PM/Scl autoantigen complex comprises 11–16 different polypeptides; ELISA with PM1-α peptide, which is a major epitope of the PM/Scl complex, has frequently been used for the detection of these antibodies in recent studies. However, no ELISA kit is commercially available in Japan.

Methods: In this study, we developed an immunoassay for measuring antibodies against recombinant PM/Scl-100 and PM/Scl-75 polypeptides, which are the two major targets of the complex, and we investigated their presence in 600 Japanese patients with various systemic autoimmune conditions. Immunoprecipitation analysis using the recombinants in addition to traditional radiolabeled cell extracts were also applied to ELISA-positive sera.

Results: In ELISA, 11 patients were positive for anti-PM/Scl-100 antibodies and 7 of these 11 patients were also positive for anti-PM/Scl-75 antibodies. Immunoprecipitation analysis using the recombinants in addition to traditional radiolabeled cell extracts confirmed that 9 out of these 11 patients immunoprecipitated the typical sets of PM/Scl proteins. In total, 4/16 (25%) undifferentiated connective tissue disease (UCTD) patients, 3/126 (2.4%) dermatomyositis patients, 1/223 (0.4%) SSc patients, 1/88 (1.1%) Sjögren's syndrome patients, 0/123 patients with systemic lupus erythematosus, 0/17 patients with overlap syndrome and 0/7 patients with PM were judged to be positive for anti-PM/Scl antibodies.

Conclusions: This is the first report of Japanese autoimmune patients with anti-PM/Scl antibodies. In Japanese patients, anti-PM/Scl antibodies are only very rarely found, and they are not always specific for dermatomyositis (DM) or SSc; they are also present in various autoimmune conditions with the highest prevalence being in UCTD. All anti-PM/Scl-positive DM cases are complicated with interstitial lung disease and/or cancer, while no life-threatening involvement was found in other anti-PM/Scl-positive cases. Further studies on larger cohorts are necessary to define the clinical significance of anti-PM/Scl antibodies in autoimmune diseases.

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Introduction

A characteristic feature of patients with systemic autoimmune diseases is the presence of autoantibodies in their sera that target intracellular components [1]. Some of these autoantibodies are useful diagnostic markers for various systemic autoimmune diseases [1-3]. Some autoantibodies have great diversity in their prevalence among different races and countries [4-6].

Anti-PM/Scl antibodies, first described as 'anti-PM-1' in 1977, were found in patients with overlap syndrome of polymyositis (PM) and scleroderma (Scl) [7]. Anti-PM/Scl antibodies produce a homogenous nucleolar pattern in indirect immunofluorescence (IIF) staining and recognize the PM/Scl complex, which is the human counterpart of the yeast exosome and consists of 11 to 16 polypeptides [8]. Most anti-PM/Scl antibodies recognize two components, PM/Scl-100 and PM/Scl-75 [9-11], and are found mostly in patients with overlap syndrome (OL) of PM and systemic scleroderma (SSc) (approximately 25%) [12], as well as in PM or SSc patients (3% to 13%) [13]; however, they are rarely found in other diseases, such as Sjögren's syndrome (SS) [14]. For the detection of anti-PM/Scl antibodies, several techniques have been utilized: double immunodiffusion, immunoprecipitation (IPP), enzyme-linked immunosorbent assay (ELISA) and line immunoassay (LIA) [15]. ELISA using the PM-1α synthetic peptide, a major epitope of PM/Scl-100 composed of an alpha helical structure located at amino acid 231 to 245 of PM/Scl-100 [16], was used in a recent multicenter study that elucidated the diagnostic and prognostic relevance of anti-PM/Scl antibodies in SSc clinics [17]. Unfortunately, this ELISA kit is not available in Japan.

The frequencies of some autoantibodies vary by ethnicity. For example, in a U.S. SSc cohort, in African-American patients, anti-U3-RNP (fibrillarin) antibodies were found in 30% of patients; meanwhile anti-Th/To antibodies were found in only 4% [4]. In white patients, however, anti-Th/To antibodies were found in 9%, whereas anti-U3-RNP antibodies were found in only 3% [4]. Another example is that anti-RNA polymerase III antibodies were less prevalent in French patients than in U.S. patients [7]. Although anti-PM/Scl antibodies are found in certain populations of patients in Western countries, as stated above, clinical studies on Japanese autoimmune patients to detect these antibodies have not been reported. Surprisingly, in two large SSc cohorts from two Japanese centers, no anti-PM/Scl-positive patients were found among 272 and 316 patients, respectively [18].

We recently developed a method that allows for the rapid conversion of cDNAs to a chemiluminescent ELISA to detect autoantibodies in human sera [19]. In this study, we constructed an ELISA for measuring anti-PM/Scl-100 and also anti-PM/Scl-75 antibodies, in order to screen these antibodies in 600 patients with various autoimmune conditions from a single center in Japan, and we investigated their clinical significance in Japanese patients.

Methods

Serum samples

Serum samples were collected from 600 Japanese patients, consisting of 223 with SSc, 126 with dermatomyositis (DM), 123 with systemic lupus erythematosus (SLE), 88 with SS, 17 with OL, 7 with PM and 16 with undifferentiated connective tissue disease (UCTD), between 1994 and 2014 at Nagoya University Hospital. SSc was diagnosed according to the classification of the American College of Rheumatology (ACR) [20] or the ACR/European League Against Rheumatism (EULAR) 2013 classification criteria [21]. Of the SSc patients, 185 were classified as diffuse cutaneous and 85 as limited cutaneous, according to the criteria of LeRoy and colleagues [22]. The DM patients (76 with adult DM, 12 with juvenile DM (JDM) and 38 with clinically amyopathic DM (CADM)) and PM patients fulfilled Bohan and Peter's criteria [23], except for CADM, which was defined by Sontheimer's criteria [24]. SLE was diagnosed by the ACR criteria for SLE [25]. SS was diagnosed based on Japanese diagnostic criteria [26]. OL, including 11 patients with PM + SSc, was diagnosed as cases that fulfilled the criteria for two systemic autoimmune diseases. UCTD was diagnosed according to the preliminary classification criteria proposed by Mosaca and colleagues [27]. Interstitial lung disease (ILD) was diagnosed by chest radiograph or chest computed tomography (CT) scan. Clinical information was collected retrospectively by reviewing their medical charts. Our cohort consisted of newly diagnosed incipient patients, except for a few patients with juvenile DM. As for patients with UCTD, serum samples were collected at the first visit. These patients were confirmed, by follow-up with doctors, as not fulfilling the criteria for defined CTD for at least three years from the beginning of symptoms according to the criteria of UCTD [27]. As control samples, serum samples from 72 healthy volunteers were also used. This study was conducted with the approval of the ethics committees of the Nagoya University Graduate School of Medicine and the Kyoto University Graduate School of Medical Science. All patients gave written consent to participate in the study.

Recombinant antigens for ELISA and immunoprecipitation

The full-length cDNA clones of PM/Scl-100 (product No. FXC03779) and PM/Scl-75 (product No. FXC22044) were purchased from Flexi® ORF Clone (Promega, Madison, WI, USA). Biotinylated recombinant proteins were produced from the cDNA, using the T7 Quick Coupled Transcription/Translation System (Promega) according to our published protocol [28]. In short, 800 μl transcription

and translation (TnT) Quick Master Mix, 20 μl 1 mM methionine, 30 μl transcend biotin-lysyl-tRNA, 120 μl water and 30 μl DNA (1 μg/μl) were mixed and then incubated at 30°C for 60 minutes.

ELISA

Antibodies against PM/Scl-100 and PM/Scl-75 were tested by antigen-capture ELISA according to our published protocols [19]. Briefly, a 96-well Nunc™ Immobilizer™ Streptavidin Plate (Thermo Scientific Nunc, Roskilde, Denmark) was incubated with 1 μl/well of *in vitro* TnT reaction mixture including biotinylated recombinant protein. Wells were then incubated with 1:1000 diluted sera and probed with anti-human immunoglobulin G (IgG) antibody conjugated with horseradish peroxidase (HRP) (Dako, Glostrup, Denmark) (1:30,000 dilution). After incubation with SuperSignal® ELISA Femto Maximum Sensitivity Substrate (Thermo Scientific Pierce, Rockford, IL, USA), 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 with the background subtracted was used for data analysis. The RLU of the samples was converted into units using a standard curve created by a prototype positive serum. As a standard, the high-titer anti-PM/Scl-100 (patient A in Figure 1) or anti-PM/Scl-75 (patient E in Figure 1) antibody-positive sera diluted 1:5 serially, starting from 1:500, was run. Units correlated with the titers of antibodies: 1:500 dilution, 625 units; 1:2,500, 125 units; 1:12,500, 25 units; 1:62,500, 5 units; 1:312,500, 1 unit; 1:1,562,500, 0.2 units. The cutoff values (4.4 units for anti-PM/Scl-100 antibody and 2.1 units for anti-PM/Scl-75 antibody) were determined as the mean of the units obtained from 36 control sera from healthy volunteers + 5 standard deviations (SD).

Immunoprecipitation

IPP was performed using TnT products as previously described [28] and using radiolabeled extracts of HeLa cells [29]. Prototype sera containing anti-PM/Scl, anti-MDA5, anti-TIF1γ or anti-Mi-2 antibodies from TM's laboratory were also used.

Laboratory tests and serological assay

Sera that were positive for anti-PM/Scl by ELISA were analyzed with an IIF laboratory kit using HEp-2 cells (Fluoro HEPANA Test; MBL, Nagoya, Japan) [30]. The samples were also screened by ELISA for antibodies against CCP, SS-A, SS-B, U1-RNP, Sm, CENP-B, ribosomal P, aminoacyl tRNA synthetase (ARS) and ds-DNA with commercial kits (MBL, Nagoya, Japan). This anti-SS-A kit detects only anti-SS-A/Ro60 and not anti-SS-A/Ro52/TRIM21.

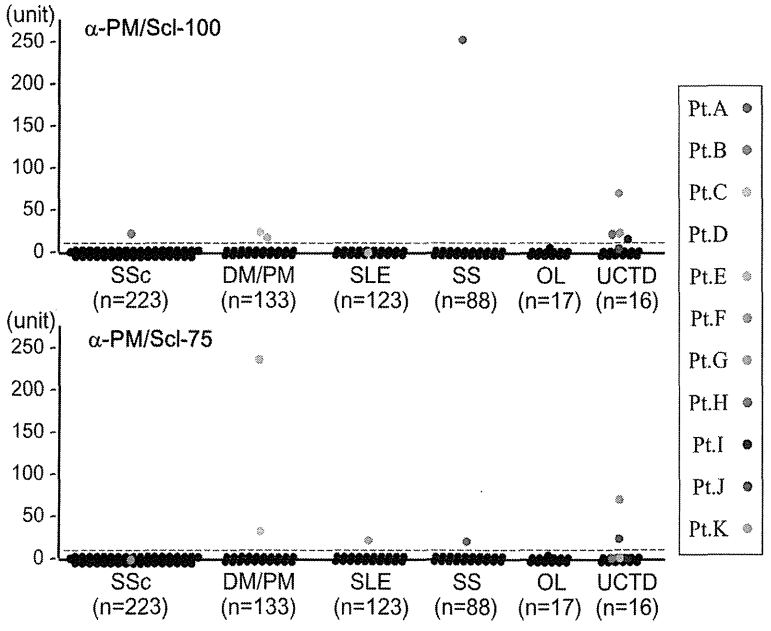

Figure 1 Qualitative measurement of anti-PM/Scl antibodies in ELISA. ELISA units of anti-PM/Scl-100 and anti-PM/Scl-75 antibodies are shown for a total of 600 serum samples from patients with various diseases. The antibody units are calculated from relative luminescence units using a standard curve obtained from serial concentrations of serum samples: patient A's serum for anti-PM/Scl-100 ELISA and patient E's serum for anti-PM/Scl-75 ELISA. The broken line indicates the cutoff value, which is the mean value of 36 healthy controls + 5 standard deviations. DM, dermatomyositis; OL, overlap syndrome; PM, polymyositis; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome; SSc, systemic scleroderma; UCTD, undifferntiated connective tissue disease.

Statistical analyses

Data were statistically evaluated using SPSS Statistics (IBM, Tokyo, Japan). Fisher exact probability tests were used for comparison of frequencies. Mann–Whitney U tests were used for comparison of ELISA units. *P* values of less than 0.05 were considered significant.

Results

Measurement of anti-PM/Scl antibodies by ELISA

For the screening of anti-PM/Scl antibodies in large numbers of serum samples, we developed an ELISA system that uses biotinylated recombinant PM/Scl-100 and PM/Scl-75. We screened a total of 600 serum samples obtained from patients with various systemic autoimmune diseases and an additional 36 serum samples from healthy volunteers for both antibodies. Based on the cutoff levels at 5 SDs above the mean value, nine (1.5%) and seven (1.2%) patients were positive for anti-PM/Scl-100 and anti-PM/Scl-75 antibodies, respectively (Figure 1). Five patients (A, C, D, E and F) had both antibodies, four (B, G, H and I) had only anti-PM/Scl-100 antibodies, and two (J and K) had only anti-PM/Scl-75 antibodies. When the cutoff was set at 3 SDs above the mean value, one sample from a patient (L mentioned in Figure 2) with overlap syndrome was just below the cutoff for both antibodies. Subsequently, serum samples from these 12 patients were used for immunoprecipitation to confirm whether they were truly positive for the anti-PM/Scl antibodies. An additional 36 samples from healthy volunteers showed levels below the cutoff for both antibodies.

Immunoprecipitation using recombinant PM/Scl protein and radiolabeled cellular protein

After the initial screening by ELISA, we investigated antibodies against PM/Scl in sera from 11 anti-PM/Scl-100 and/or anti-PM/Scl-75-positive patients and 1 equivocal patient for their ability to immunoprecipitate biotinylated recombinant PM/Scl-100 and PM/Scl-75 and radiolabeled cellular PM/Scl. All nine anti-PM/Scl-100-positive sera in ELISA immunoprecipitated biotinylated recombinant PM/Scl-100, whereas five of the seven anti-PM/Scl-75-positive sera in ELISA immunoprecipitated biotinylated recombinant PM/Scl-75 (Figure 2, TnT-IPP). Sera that were anti-PM/Scl-75-positive in ELISA but -negative in IPP (J and K) were negative for anti-PM/Scl-100 antibodies in ELISA and IPP. Serum of patient L with equivocal ranges in both ELISAs immunoprecipitated neither recombinant PM/Scl-100 nor PM/Scl-75 (data not shown).

To determine whether the positive sera in ELISA immunoprecipitate the PM/Scl complex, we applied conventional IPP using radiolabeled HeLa cell extract (Figure 2, HeLa-IPP). All nine sera (patients A to I) that had reacted with the recombinant PM/Scl-100 also immunoprecipitated a cellular 100-kDa protein. Eight of these sera also immunoprecipitated a 75-kDa protein,

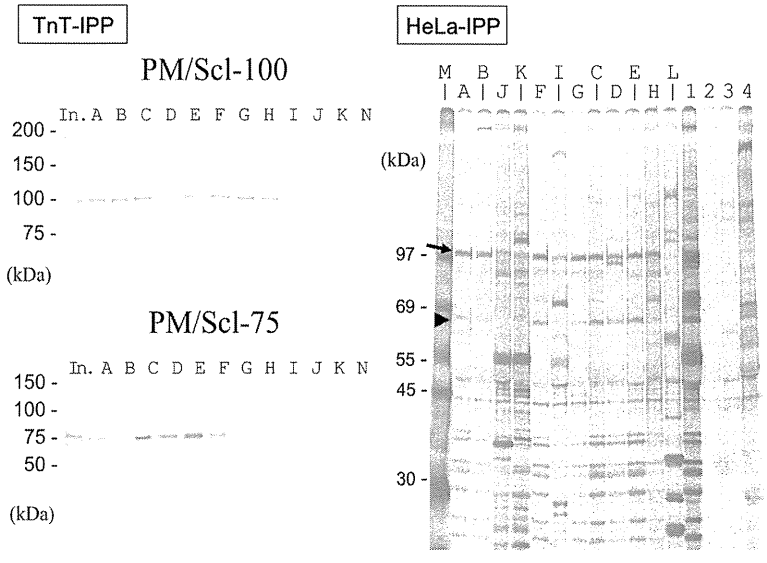

Figure 2 Detection of anti-PM/Scl antibodies in immuoprecipitation analysis. TnT-IPP: immunoprecipitation of biotinylated recombinant PM/Scl-100 and PM/Scl-75. Recombinant proteins were subjected to 4% to 20% SDS-PAGE and analyzed by immunoblotting with streptavidin-alkaline phosphatase and substrate. In., the input was half the dose for immunoprecipitation. Lanes A to K correspond to the anti-PM-Scl-100 and/or −75-positive patients shown in Figure 1. Lane N: healthy control serum. HeLa-IPP: immunoprecipitation analysis using radiolabeled HeLa cell extracts. Lanes A to L correspond to the patients shown in Figure 1 and Table 1. Lanes A to K correspond to anti-PM-Scl-100 and/or −75-positive patients shown in Figure 1. Lane M: [Methyl-^{14}C] methylated protein MW markers (PerkinElmer Japan, Yokohama, Japan). Lane L: anti-U1-RNP-positive serum with equivocal titers for both antibodies in ELISA. Lanes 1 to 4 show the reference sera; lane 1, anti-PM/Scl-positive serum; lane 2, anti-MDA5-positive serum; lane 3, anti-TIF1-γ-positive serum; lane 4, anti-Mi-2-positive serum. Arrow and arrowhead corrspond to the PM/Scl-100 and PM/Scl-75 antigens, respectively. IPP, immunoprecipitation; TnT, *in vitro* translation and transcription product.

but one another (patient I) did not. Two sera that were positive only for anti-PM/Scl-75 in ELISA (patients J and K) immunoprecipitated neither the 100-kDa nor the 75-kDa protein. The serum from an overlap syndrome patient (L) with an equivocal level of both anti-PM/Scl-100/75 antibodies in ELISA was negative in IPP. According to these results, eight sera (patients A to H) were judged to be positive for anti-PM/Scl antibodies, as was one other serum (patient I), which reacted to recombinant PM/Scl-100 and which immunoprecipitated a 100-kDa cellular protein.

Indirect immunofluorescence staining patterns of anti-PM/Scl-100 and/or anti-PM/Scl-75-positive sera

In IIF analysis, the eight sera (patients A to H) that immunoprecipitated the 100-kDa and 75-kDa proteins showed nucleolar patterns (Table 1). The serum (patient I) that only immunoprecipitated the 100-kDa protein showed a speckled pattern without nucleolar staining. Two sera (patients J and K) that immunoprecipitated neither the 100-kDa nor the 75-kDa protein, also showed no nucleolar patterns.

Clinical and laboratory data for anti-PM/Scl-positive patients

The nine patients with anti-PM/Scl were four with UCTD, three with DM (including one with CADM), one with limited cutaneous SSc and one with SS. The clinical features of these patients are summarized in Table 1. The prevalence of anti-PM/Scl in UCTD (25%) is significantly higher than that of DM (2.4%, $P = 0.0032$), SSc (0.5%, $P = 0.000066$), SS (1.2%, $P = 0.0018$), SLE (0%, $P = 0.00012$), OL (0%, $P = 0.045$) and healthy control (0%, $P = 0.0067$). Although the numbers of examined sera are very small, no patients with anti-PM/Scl antibodies are found among patients with PM or OL. Four patients with UCTD are clinically heterogeneous; two are suspected of having SLE, one of having SS and one of having rheumatoid arthritis (RA). All but one are young adult women. No common clinical features, including Raynaud's phenomenon and abnormal nail-fold capillaries, are present among these four patients.

Of the 126 DM patients, there are 8 anti-nucleolar antibody (ANoA)-positive patients, of whom 3 patients, all men, had anti-PM/Scl antibodies. Of the 123 anti-PM/Scl-negative DM patients, only 32 are men ($P = 0.020$). These three patients were complicated with ILD. The clinical manifestations of ILD for these three patients were improved by oral prednisolone and immunosuppressive agent therapy, and their ILD did not have a fatal outcome. Additionally, the complication of internal malignancy (mesopharynx and prostate) was also recognized in two patients three years before or after the disease onset. ILD and internal malignancy are

Table 1 Connective tissue disease manifestations of anti-PM/Scl-100-ELISA- and/or anti-PM/Scl-75-ELISA-positive patients

Patient	Age in years	Sex M/F	Diagnosis	IIF pattern[a], titer	ELISA PM/Scl-100/ PM/Scl-75	TnT-IPP PM/Scl-100/ PM/Scl-75	HeLa-IPP PM/Scl-100/ PM/Scl-75	other auto-antibodies	clinical features
A	52	F	SS	nucleolar, 1:2560	+ / +	+ / +	+ / +		dry eye, dry mouth
B	62	F	lSSc	nucleolar, 1:320	+ / -	+ / -	+ / +		Raynaud's ph, sclerodactyly
C	54	M	CADM	nucleolar, 1:640 diffuse, 1:80	+ / +	+ / +	+ / +		ILD, Gottron papules, mechanic's hands
D	69	M	DM	nucleolar, 1:640 diffuse, 1:80	+ / +	+ / +	+ / +		ILD, Gottron sign, mechanic's hands, V-neck sign, dysphagia, pharyngeal Ca
E	67	M	DM	nucleolar, 1:1280	+ / +	+ / +	+ / +		ILD, Gottron sign, Heliotrope rash muscle weakness, prostate Ca
F	73	F	UCTD	nucleolar, 1:640	+ / +	+ / +	+ / +		ILD, dry eye, dry mouth
G	33	F	UCTD	nucleolar, 1:640 diffuse, 1:80	+ / -	+ / -	+ / +		morning stiffness, polyarthralgia
H	31	F	UCTD	nucleolar, 1:160 speckled, 1:80	+ / -	+ / -	+ / +		polyarthralgia, photosensitivity
I	31	F	UCTD	speckled, 1:80	+ / -	+ / -	+ / -		oral ulcer, photosensitivity
J	24	F	UCTD	diffuse, 1:640 cytoplasmic, 1:160	- / +	- / -	- / -	SS-A	dry eye, dry mouth
K	23	F	SLE	diffuse, 1:320 cytoplasmic, 1:80	- / +	- / -	- / -	SS-A ribosomal P	polyarthralgia, malar rash, photosensitivity, leukopenia

[a] 'diffuse' and 'speckled' in the IIF pattern, respectively, refer to nuclear diffuse and nuclear speckled patterns. CADM, clinically amyopathic DM; Ca, carcinoma; DM, dermatomyositis; ILD, interstitial lung disease; lSSc, limited cutaneous SSc; ph, phenomenon; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome; SSc, systemic scleroderma; UCTD, undifferentiated connective tissue disease.

more frequent in anti-PM/Scl-positive DM patients than in anti-PM/Scl-negative DM patients, but not significantly ($P = 0.060$ and $P = 0.072$, respectively).

Besides the three DM patients with ILD, one patient who had UCTD was also complicated with ILD. Although the anti-PM/Scl-100 ELISA units of these four patients with ILD were not higher than those of five anti-PM/Scl-positive patients without ILD (mean 26.7 versus 62.0), anti-PM/Scl-75 titers of the four patients with ILD were significantly higher than those of five patients without ILD (mean 86.0 unit versus 0.96 unit, $P = 0.027$ by Mann–Whitney U test).

Discussion

The anti-PM/Scl antibody is a well-known ANoA and a serological marker of OL and other systemic autoimmune diseases such as SSc, PM and DM alone [15]. This antibody is common in the West. For example, it

Table 2 Frequencies of anti-PM/Scl antibodies in disease subsets

Frequencies of anti-PM/Scl antibodies in disease subsets						
Study	Marguerie	Mahler	Rozman	Hanke		Maes
Reference	[13]	[39]	[40]	[41]		[42]
Year	1992	2005	2008	2009		2010
Country	UK	Various	Europe	Germany		Belgium
Anti-PM/Scl detection	CIE	PM1-α ELISA	LIA	LIA		PM1-α ELISA
Patient selection and numbers of patients	1689 SLE	205 SSc	625 SSc	280 SSc[b]		70 SSc
	879 SSc[a]	114 SLE		88 RA		66 SLE
	256 PM or DM	40 PM		72 SLE		35 SS
		40 PM/SSc		49 SS		24 RA
						23 DM
						13 PM
						11 MCTD
Anti-PM/Scl-positive patients	27 PM (or DM)/SSc	22 PM/SSc (55%)	1 PM (7.7%)	Anti-PM/Scl-75	Anti-PM/Scl-100	3 SSc (4.3%)
	4 SSc	27 SSc (13%)	18 SSc (2.9%)	29 SSc (10%)	20 SSc (7.1%)	
	1 PM	3 PM (7.5%)	1 DM (1.7%)	3 RA (3.4%)	3 SLE (4.2%)	
				1 SLE (1.4%)	1 SS (2.0%)	
Study	Mierau	Koschik	Mehra	D'Aoust	Kazi	Muro
Reference	[31]	[57]	[58]	[17]	[18]	The present study
Year	2011	2012	2013	2014	2014	
Country	Germany	USA	Australia	Canada	Japan	Japan
Anti-PM/Scl detection	ID	ID	LIA	PM1-α ELISA	IIP	ELISA, IPP
Patient selection and numbers of patients	863 SSc	2425 SSc	528 SSc	763 SSc	Kanazawa cohort	223 SSc
					316 SSc	126 DM
					Keio cohort	123 SLE
					272 SSc	88 SS
						17 overlap
						16 UCTD
						7 PM
Anti-PM/Scl-positive patients	42 SSc (4.9%)	75 SSc (3.1%)	Anti-PM/Scl-75	55 SSc (7.2%)	0	4 UCTD (25%)
			66 SSc (12.5%)			3 DM (2.4%)
			Anti-PM/Scl-100			1 SS (1.1%)
			26 SSc (4.9%)			1 SSc (0.4%)

[a]Since the numbers of myositis overlap patients were not given, the frequencies of the antibodies in disease subsets were not calculated; [b]51 overlap and 16 undifferentiated connective tissue disease patients were included.

CIE, counter immunoelectrophoresis; DM, dermatomyositis; ID, immunodiffusion; IPP, immunoprecipitation; LIA, line immunoassay; MCTD, mixed connective tissue disease; PM, polymyositis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome; SSc, systemic scleroderma; UCTD, undifferentiated connective tissue disease.

was the third most-found, followed by anti-centromere and anti-topoisomerase I antibodies, in a large cohort of SSc patients in Germany [31]. However, large studies of Japanese patients with SSc showed this antibody to be absent [32,33], and a recent study noted that 0/588 Japanese patients with SSc had anti-PM/Scl antibodies [18]. Large-cohort studies using sera from more than 200 connective tissue disease patients in the literature are summarized in Table 2, although some studies with mostly overlapped patients are omitted. Anti-PM/Scl antibodies are strongly linked to HLA-DRB1*0301 [34], which is very rarely found in Japanese, with a prevalence of only 0.14%, according to an online database [35] (HLA Laboratory, Kyoto, Japan); however, the contribution of this finding remains unknown. Since we had found three patients to have strong ANoA in IIF analysis during our recent studies on myositis-specific or associated autoantibodies [36-38], we aimed to investigate anti-PM/Scl antibodies in our large cohort of systemic autoimmune disease.

Although LIA for anti-PM/Scl-75 and −100 antibodies and PM1-α ELISA have often been used recently [16,31,39-43], the latter is not available in Japan and the former is not cost-effective, costing around 13,000 yen/sample (Cosmic Corporation, Tokyo, Japan). For our in-house ELISA, the anti-PM/Scl-75 assay was found to be inferior to the anti-PM/Scl-100 assay both in sensitivity and specificity, according to the results of protein-IPP, which is widely accepted as a reference method for detecting several markers for SSc and PM/DM. Originally, most PM/Scl-positive sera have been shown to contain anti-PM/Scl-100 and about 50% to 60% of the sera have been shown to react with PM/Scl-75 [11,12,44,45]. Raijmakers and colleagues showed that PM/Scl-75 contains a previously unidentified N-terminal region that is important for the antigenicity of the protein [46]. This longer form, named PM/Scl-75c, was as reactive as PM/Scl-100 to sera from PM/SSc overlap patients in ELISA (28% and 25%, respectively) [46]. Subsequently, Hanke and colleagues showed the prevalence of anti-PM/Scl-75c to be higher than that of anti-PM/Scl-100 (10.4% versus 7.1%) in LIA using sera from 280 SSc patients [41]. There are several possible explanations for the lower prevalence of anti-PM/Scl-75 than anti-PM/Scl-100 in this study. The cDNA in this study, PM/Scl-75c-β, has a 17 amino acid insertion at the C-terminus which could introduce conformational changes in epitope [47]. In the study of Hanke and colleagues, recombinant PM/Scl-75 was expressed by a baculovirus [41]. The discrepancies might also be due to racial differences or clinical backgrounds. In a validation study by Jaskowski and colleagues, the anti-PM/Scl-100 LIA had better agreement for the detection of anti-PM/Scl with IPP as the reference method than with PM/Scl-75 LIA and PM1-α ELISA [48].

In this study, eight of nine anti-PM/Scl antibody-positive sera exhibited nucleolar staining in IIF analysis. Some studies have shown that anti-PM/Scl-positive sera do not always demonstrate a nucleolar staining pattern in IIF [16,17,47,49]. Interestingly, one ANoA-negative serum with anti-PM/Scl reacted with PM/Scl-100 but not with PM/Scl-75. Intramolecular epitope spreading from the initial response against PM/Scl-100 to a successive response by other exosomal components has been recognized, as have many other autoantibody responses [50]. Figure 3 shows a four-way Venn diagram depicting the overlap between anti-PM/Scl-100 by TnT-IPP, anti-PM/Scl-75 by TnT-IPP, anti-PM/Scl by cellular IPP and anti-nucleolar pattern by IIF. ANoA-positive anti-PM/Scl antibodies all immunoprecipitated both 100- and 75-kDa proteins in HeLa-IPP, whereas only one ANoA-negative anti-PM/Scl antibody (patient I) immunoprecipitated only a 100-kDa protein in HeLa-IPP. Since sera from Patient I immunoprecipitated several other polypeptides, the nuclear speckled staining of this patient in IIF may correspond to antibodies against these proteins. Moreover, anti-PM1α reactivity has been reported in apparently ANA-negative samples [16,17,49]. Although future studies are necessary to address whether monospecific anti-PM/Scl-100 antibodies show nucleolar staining in IIF, we can conclude that IIF is not a sensitive immunoassay for the detection or screening of anti-PM/Scl antibodies.

The findings of anti-PM/Scl antibodies in UCTD patients are of clinical importance. The classification criteria of UCTD are not well established [51]. Since our UCTD patients were not diagnosed with definite

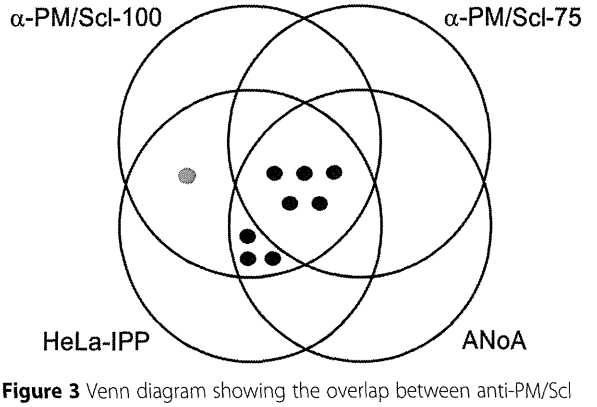

Figure 3 Venn diagram showing the overlap between anti-PM/Scl antibodies by different assays. Anti-PM/Scl-100 and anti-PM/Scl-75 circles show the antibody-positive patiens determined by immunoprecipitation with the corresponding recombinant protein. The HeLa-IPP circle shows anti-PM/Scl-positive patients determined by immunoprecipitation with HeLa cellular protein. Black dots show the patients with both anti-75 kDa and anti-100 kDa antibodies, and the gray dot shows the patient with only anti-75 kDa antibody in HeLa-IPP. The ANoA circle shows the patients demonstrating anti-nucleolar patterns as observed by indirect immunofluorecence.