GJB2 mutation p.Asp50Asn and may contribute the proband's phenotype. Nevertheless, the limited scope of this study (single case report) does not allow us to determine the clinical significance of p.Ser59Arg in K17, and the influence of other genetic and epigenetic factors cannot be excluded.

*Department of Dermatology, Hokkaido University Graduate School of Medicine, North 15 West 7, Sapporo 060-8638, Japan
†Department of Dermatology, University of Miyazaki Faculty of Medicine, Miyazaki, Japan
‡Department of Dermatology, Nagoya University Graduate School of Medicine, Nagoya, Japan
E-mail: natsuga@med.hokudai.ac.jp

K. NATSUGA*
S. SHINKUMA*
M. KANDA*
Y. SUZUKI*
N. CHOSA†
Y. NARITA†
M. SETOYAMA†
W. NISHIE*
M. AKIYAMA*‡
H. SHIMIZU*

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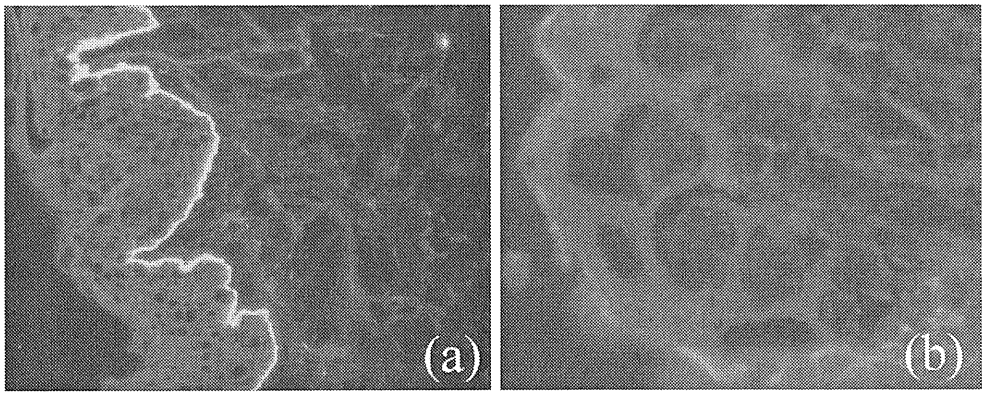

Fig. 2. Indirect immunofluoresence for collagen VII autoantibodies on normal skin (a) and collagen VII deficient skin (b) with serum from EBA patient, 200×.

We agree with the authors that more studies are indicated to determine the use of this test for monitoring disease activity in EBA patients. Similar studies in pemphigus patients with recombinant desmoglein 1 and 3 ELISA's reveal that the sera with identical titers of antibodies by IIF give variable results with ELISA [7]. Unless high titer sera are diluted, saturation of antibody–antigen reactions in ELISA may lead to false low positive ELISA index values to begin with. Such sera may not appear to show a decline in ELISA index values with treatment response [8]. We also have observed, in some pemphigus sera, that even though the IIF titers show a decline, ELISA index values still remain high. Therefore, we may have to use this ELISA with caution to monitor the disease.

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E. Eugene Bain[a], Raminder K. Grover[b,*], Richard W. Plunkett[b], Ernst H. Beutner[a,b]

[a]*Department of Dermatology, School of Medicine and Biomedical Sciences, University at Buffalo, State University of New York, Buffalo, NY 14203, USA*;

[b]*Beutner Laboratories and the Department of Microbiology and Immunology, School of Medicine and Biomedical Sciences, University at Buffalo, State University of New York, Buffalo, NY 14214, USA*

*Corresponding author at: 138 Farber Hall, Beutner Laboratories and the Departments of Microbiology and Immunology, School of Medicine and Biomedical Sciences, University at Buffalo, SUNY, Buffalo, NY 14215, USA. Tel.: +1 716 838 0549; fax: +1 716 838 0798
E-mail address: rgrover2@buffalo.edu (R.K. Grover)

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Letter to the Editor

CYP4F22 is highly expressed at the site and timing of onset of keratinization during skin development

Keywords:
Ichthyosis;
Keratinization;
Skin barrier

Autosomal recessive congenital ichthyoses (ARCI) include several subtypes: harlequin ichthyosis (HI), lamellar ichthyosis (LI) and congenital ichthyosiform erythroderma (CIE). To date, six causative genes have been identified in ARCI patients: *ABCA12*, *TGM1*, *NIPAL4*, *CYP4F22*, *ALOXE3* and *ALOX12B* [1]. The localization of transglutaminase 1, ABCA12 and 12R-lipoxygenase have been analyzed using samples from patients and model mice [1]. However, as for NIPAL4, CYP4F22, and lipoxygenase-3, neither localization nor function has been fully clarified yet. Herein, we investigate the expression pattern and localization of NIPAL4, CYP4F22 and lipoxygenase-3 in developing human epidermis and primary cultured normal human keratinocytes.

By quantitative reverse transcription (RT)-PCR analysis, at 10 and 14 weeks EGA, mRNA of *NIPAL4*, *CYP4F22* and *ALOXE3* was hardly expressed (Fig. 1A). The *CYP4F22* mRNA expression at 18

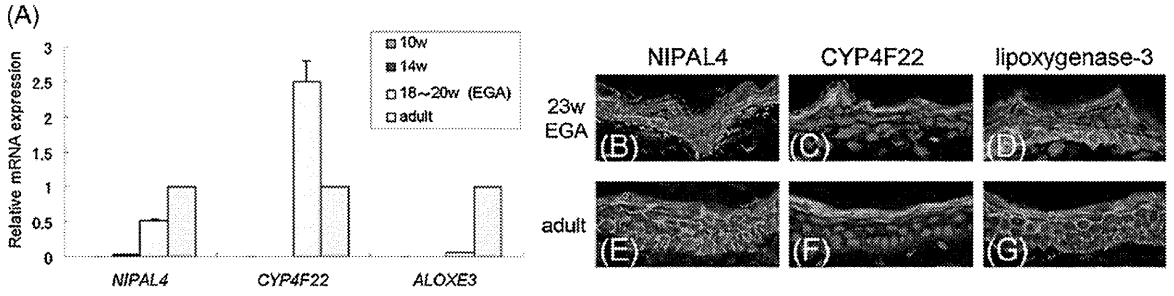

Fig. 1. NIPAL4, CYP4F22 and lipoxygenase-3 expression in developing human skin. (A) mRNA expression in developing human skin. The mRNA expression of *NIPAL4*, *CYP4F22* and *ALOXE3* in fetal human whole skin was studied by quantitative RT-PCR analysis, normalized by *GAPDH* [Applied Biosystems: Hs00398027_m1*, Hs00403446_m1*, Hs00222134_m1*, Hs03929097_ gl*]. At 10 and 14 weeks EGA, *NIPAL4*, *CYP4F22* and *ALOXE3* mRNA are hardly expressed. At 18–20 weeks EGA, the rate of *CYP4F22* mRNA expression is higher than in adult human whole skin ($n = 3$, mean ± SD). (B–G) Immunofluorescence staining of NIPAL4, CYP4F22 and lipoxygenase-3 in developing human skin. Fetal skin samples at 10–23 weeks EGA and adult skin samples were stained for NIPAL4 [Rabbit polyclonal anti-NIPAL4 antibody against a 16-amino acid sequence synthetic peptide (residues 445–461)], CYP4F22 [B01; Abnova, Taipei City, Taiwan], and lipoxygenase-3 [T-14; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.] (Supplementary Fig. S1). For the 23 weeks EGA sample and the adult skin, CYP4F22 (C and F) is expressed in the upper layer of the epidermis, mainly in the granular layers. NIPAL4 (B and E) and lipoxygenase-3 (D and G) are expressed at the cell periphery throughout the epidermis. NIPAL4 expression is seen evenly from the basal cell layer to the granular layers, although lipoxygenase-3 expression is slightly stronger towards the granular layers. NIPAL4, CYP4F22 and lipoxygenase-3 green (FITC), nuclear stain, red (PI solution) (original magnification 40×). Data are presented as representative of triplicate experiments.

and 20 weeks EGA was higher than that in adult human skin. At 18 and 20 weeks EGA, *NIPAL4* mRNA expression was approximately half of that in adult skin, and only a tiny amount of *ALOXE3* mRNA was expressed.

We investigated protein localization by immunofluorescence staining (Fig. 1B–G). For the 10 weeks EGA sample, NIPAL4, CYP4F22 and lipoxygenase-3 were not detected. A similar pattern was obtained for the 14 weeks EGA sample. For the 23 weeks EGA sample, CYP4F22 was expressed in the upper layer of epidermis, mainly in the granular layers, and NIPAL4 and lipoxygenase-3 were expressed at the cell periphery in the entire epidermis. Staining patterns of NIPAL4, CYP4F22 and lipoxygenase-3 in the adult skin were similar to those at 23 weeks EGA. Lipoxygenase-3 is usually considered to be a partner with 12R-LOX. 12R-LOX has been visualized at the cell periphery only in the upper epidermis [2]. In our results, lipoxygenase-3 was distributed at the cell periphery in the entire epidermis. Concerning to lipoxygenase-3 in the upper epidermis, lipoxygenase-3 is thought to work with 12R-LOX, although function of lipoxygenase-3 in the lower epidermis is unknown.

In cultured keratinocytes, RT-PCR analysis (Fig. 2A) and immunoblot analysis (Fig. 2B and C) confirmed that mRNA and protein expression of CYP4F22 were increased under the high Ca^{2+} condition (1.2 mmol/L for 48 h). In contrast, there was no significant increase in the mRNA or protein expression of NIPAL4 or ALOXE3 under the high Ca^{2+} condition.

The present study of the adult human epidermis clarified that NIPAL4 and lipoxygenase-3 were expressed at the cell periphery in the entire epidermis of adult human skin. CYP4F22 was expressed in the cytoplasm of keratinocytes in the upper layer of adult human epidermis, mainly in the granular layers. One previous report [3] noted that, inconsistent with our present observations, *NIPAL4* mRNA is highly expressed in the granular layers of the epidermis with *in situ* hybridization analysis. The cause of this discrepancy is unclear, but it might be due to difference in sensitivity between *in situ* hybridization and immunostaining.

We have demonstrated that the mRNAs of *NIPAL4*, *CYP4F22* and *ALOXE3* are not expressed in the early stages of fetal development, at 10 weeks EGA or at 14 weeks EGA. At 18 and 20 weeks EGA, *NIPAL4* mRNA expression was about half that in adult skin, although *ALOXE3* mRNA was only weakly expressed. Among the keratinization-associated genes, the mRNA expression pattern of *NIPAL4* is similar to that of *ABCA12*, and the pattern of *ALOXE3* resembles those of other keratinization-related molecules, such as *TGM1*, *LOR* and *KLK7* [4].

NIPAL4 encodes a putative transmembrane protein of 404 amino acids with a molecular weight of 44 kDa [6]. The NIPAL4 protein is highly expressed in the brain, lung and stomach, and in

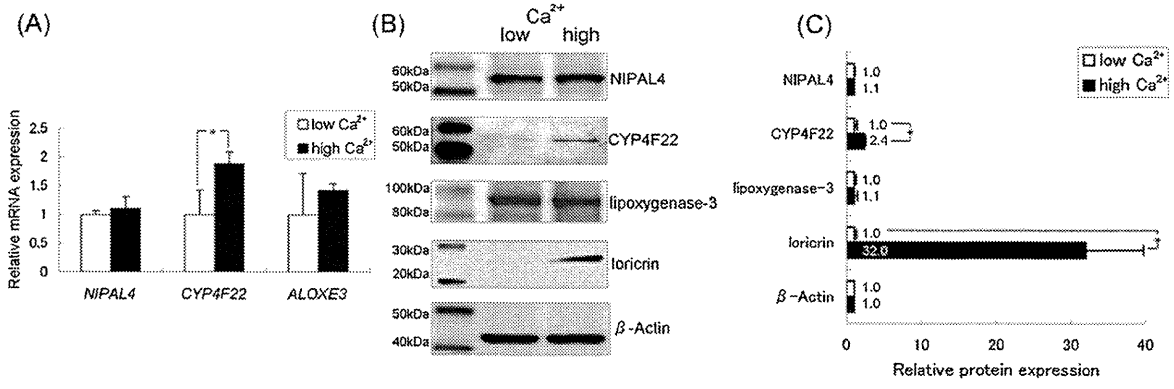

Fig. 2. mRNA and protein expression of NIPAL4, CYP4F22 and ALOXE3 in developing human skin and NHEK. (A) mRNA expression in NHEK. mRNA expression of *CYP4F22* is significantly higher in the NHEK under the high Ca^{2+} condition than in those under the low Ca^{2+} condition. There are no significant differences between the high and low Ca^{2+} conditions in terms of the mRNA expression of *NIPAL4* and *ALOXE3* ($n = 3$, mean ± SD, $^*p < 0.05$). (B) Protein expression assessed by Western blot analysis. The expression of CYP4F22 is higher in the NHEK raised under the high Ca^{2+} condition than in those raised under the low Ca^{2+} condition. However, neither NIPAL4 nor lipoxygenase-3 is increased under high Ca^{2+} condition. Anti-ALOXE3 antibody for immunoblotting: NBP1-32533; Novus Biologicals, LLC, U.S.A. (C) Quantitative analysis by Image J software revealed that the protein expression of CYP4F22 was significantly increased under the high Ca^{2+} condition. Data are presented as representative of triplicate experiments.

leukocytes and keratinocytes. The protein product of the *ALOXE3* gene, lipoxygenase-3, is thought to function as a hydroperoxide isomerase to generate epoxy alcohol [5]. CYP4F22 is a member of the cytochrome P450 family 4, subfamily F. The gene includes 12 coding exons and the cDNA spans 2.6 kb in length. All *CYP4F22* mutations reported to date are predicted to abolish the function of the encoded CYP protein and to compromise the 12(R)-lipoxygenase (hepoxilin) pathway.

Human epidermis contains 15S-lipoxygenase type 1, 12S-lipoxygenase and 12R-lipoxygenase [6]. Skin also contains cytochrome 450, and members of the CYP4 family with unknown epidermal function [3]. 12R-lipoxygenase has attracted great medical interest. 12R-lipoxygenase is expressed only in the epidermis and the tonsils [6,7] and is upregulated in psoriatic lesions [8]. It transforms 20:4n-6 to 12R-hydroperoxyeicosatetraenoic acid (12R-HPETE), which is important for the development of the water permeability barrier function in the epidermis [2]. 12R-LOX and eLOX3 play a crucial role in releasing ω-hydroxyceramide for construction of the corneocyte lipid envelope which is essential for intact skin barrier [9]. O-linoleoyl-ω-hydroxyceramide is oxygenated by the consecutive actions of 12R-LOX and eLOX3 and the products are covalently attached to protein *via* the free ω-hydroxyl of the ceramide, forming the corneocyte lipid envelope [9].

It is hypothesized that CYP4F22 may be linked to the 12R-lipoxygenase and lipoxygenase-3 pathway. Hydroxyeicosatetraenoic acids (HEETs) can be hydrolyzed to triols by epoxide hydrolases, and these products might be substrates of CYP4F members. Thus, it is possible that CYP4F22 might be involved in a downstream step in the 12R-lipoxygenase/lipoxygenase-3 pathway. CYP4F22 could be involved in the oxidation of 8R,11R,12R-HEET. However, from a systemic study of MS/MS spectra of HEETs derived from 12- and 15-HPETE, CYP4F22 did not appear to oxidize 8R,11R,12R-HEET [10]. Nilsson et al. [10] reported that recombinant CYP4F22 catalyzed the omega-3 hydroxylation of 20:4n-6; however, oxygenation of 8R,11R,12R-HEET was not detected. An additional function of CYP4F22 is to synthesize the omega-hydroxy fatty acids in the ceramide [10].

Our study revealed CYP4F22 to be highly expressed at the site and the onset of keratinization during skin development. From this it is speculated that CYP4F22 is involved in the metabolism of lipid substrates that are important to differentiation/keratinization of epidermal keratinocytes, at least during the fetal period. Further studies of the function of CYP4F22 would be needed to elucidate its function in development of the epidermis and keratinocytes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jdermsci.2011.12.006.

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Kaori Sasaki[a], Masashi Akiyama[a,b,*], Teruki Yanagi[a], Kaori Sakai[a], Yuki Miyamura[a], Megumi Sato[a], Hiroshi Shimizu[a]
[a]*Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan;*
[b]*Department of Dermatology, Nagoya University Graduate School of Medicine, Nagoya, Japan*

*Corresponding author at: Department of Dermatology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan.
Tel.: +81 52 744 2314
E-mail address: makiyama@med.nagoya-u.ac.jp (M. Akiyama)

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Novel adenosine triphosphate (ATP)-binding cassette, subfamily A, member 12 (*ABCA12*) mutations associated with congenital ichthyosiform erythroderma

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MADAM, Autosomal recessive congenital ichthyosis (ARCI) is a keratinization disorder, characterized by general desquamation. ARCI is a heterogeneous entity, including harlequin ichthyosis (HI, MIM 242500), lamellar ichthyosis type 2 (LI2, MIM 601277) and congenital ichthyosiform erythroderma (CIE, MIM 242100). The reported mutations in CIE include adenosine triphosphate (ATP)-binding cassette, subfamily A, member 12 (*ABCA12*),[1] transglutaminase 1 (*TGM1*),[2] lipoxygenase-3, 12(R)-lipoxygenase,[3] *NIPAL4*[4] and *CYP4F22*.[5] Mutations in *ABCA12* also result in LI2 and HI.[6,7] We report *ABCA12* mutations in four unrelated Japanese patients with CIE and identified five unreported and two recurrent mutations.

Patient 1 is a 3-year-old girl with generalized scales on erythroderma, ectropion, eclabium, severely deformed ears and alopecia (Fig. 1a–c). Her elder sister displayed similar symptoms and died after dehydration and infection. Patient 2 is a 9-year-old girl with generalized scales on an erythrodermic skin, mild ectropion, alopecia of the forehead and mild auricular malformation. Her younger sister died after severe skin symptoms and subsequent complications. Patient 3 is a 4-month-old boy, born as a collodion baby, with systemic whitish scales and generalized erythrodermic skin. There is no family history. Patient 4 is a 3-month-old boy, born as a collodion baby, with generalized whitish scales on a mild erythrodermic skin (Fig. 1d,e). Ectropion, eclabium and auricular malformation were not seen. There is no family history. Pathological findings of all patients revealed hyperkeratosis, mild acanthosis and perivascular lymphocytic infiltration.

We initially examined for *ABCA12* mutation, because *ABCA12* mutations have been found frequently in Japanese patients with CIE. For analysis of the *ABCA12* gene, polymerase chain reaction (PCR) fragments were amplified with 53 primer pairs, as previously reported.[6] We identified five unreported and two recurrent mutations (Table 1). Patient 1 had compound heterozygosity of missense/small deletion mutations [(p.Thr1575Pro)+(c.6031delG)]. Patients 2 and 3 had compound heterozygosity of missense/splice-site mutations [(p.Arg986Trp)+(c.5940–1G>C), (p.Asn1380Ser)+(c.5128+3A>G), respectively]. Patient 4 had compound heterozygosity

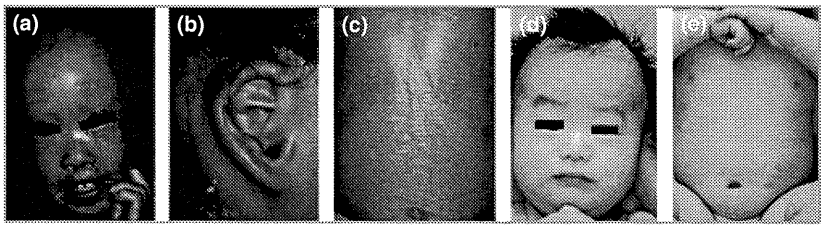

Fig 1. (a–c) Clinical features of patient 1. The whole body was covered with whitish scales on the erythrodermic skin. Ectropion, eclabium and alopecia of the forehead were seen. (d,e) Clinical features of patient 4. Whitish scales and generalized erythrodermic skin were seen.

Table 1 Summary of mutation analysis of *ABCA12* in the present study

Patient	Age, sex	Mutation	Maternal	Paternal
1	3 years, girl	Compound heterozygous	p.Thr1575Pro (c.4723A>C)	c.6031delG
2	9 years, girl	Compound heterozygous	p.Arg986Trp (c.2956C>T)	c.5940–1G>C
3	4 months, boy	Compound heterozygous	p.Asn1380Ser (c.4139A>G)	c.5128+3A>G
4	3 months, boy	Compound heterozygous	p.Thr1575Pro (c.4723A>C)	p.Gly1651Ser (c.4951G>A)

of missense mutations [(p.Thr1575Pro)+(p.Gly1651Ser)]. Each of the parents was a heterozygous carrier. Five mutations (p.Thr1575Pro, c.6031delG, p.Arg986Trp, c.5940–1G>C and c.5128+3A>G) have not been reported previously. Two recurrent mutations (p.Asn1380Ser and p.Gly1651Ser) have been reported previously in LI2.[6] These mutations were not found in 200 normal, unrelated Japanese alleles.

In cDNA from the skin of patient 2, reverse transcriptase-PCR (RT-PCR) across the c.5940–1G>C mutation site showed a single band of 526 bp. Subcloning and direct sequencing

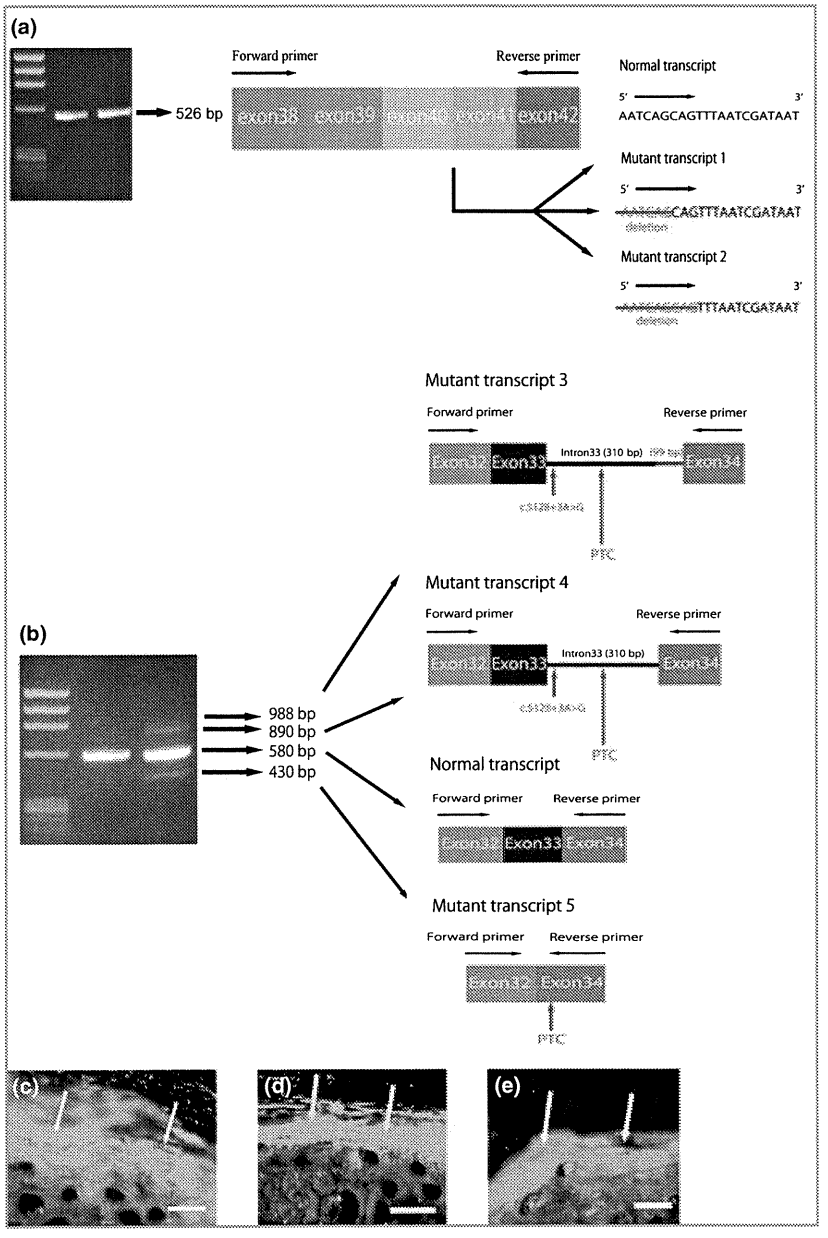

Fig 2. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of mRNA fragments around the splice-site mutations and immunofluorescent analysis. (a) In patient 2, RT-PCR, subcloning and direct sequencing through the exon 40–41 boundary revealed two mutant transcripts as well as a normal transcript. Mutant transcript 1 had lost 6-bp nucleotides from exon 41, which resulted in a 2-amino acid deletion (Ile1981_Ser1982del). Mutant transcript 2 had lost 9-bp nucleotides from exon 41, which resulted in a 3-amino acid deletion (Ile1981_Ser1983del). Both mutant transcripts were within-frame deletions. (b) In patient 3, three aberrant mutant transcripts, all of which led to a premature termination codon, were identified by RT-PCR, subcloning and direct sequencing through the exon 33–34 boundary. Mutant transcript 3 was 988 bp in length with the inclusion of 310 bp and another 99 bp of intron 33. Mutant transcript 4 was 890 bp in length with the inclusion of 310 bp of intron 33. Mutant transcript 5 had exon 33 skipping. (c–e) Immunofluorescent labelling of ABCA12 in the skin. (c,d) A dot-like pattern of ABCA12 staining was seen in the cytoplasm of keratinocytes in the upper epidermis in patient 1 (c) and patient 2 (d). (e) In the normal control epidermis, ABCA12 staining was relatively strong in the granular layers and seemed to be dominant at the cell periphery. Bar = 5 μm.

revealed two mutant transcripts with in-frame deletions (Fig. 2a). In cDNA from the skin of patient 3, RT-PCR across the c.5128+3A>G mutation site identified four bands of 988, 890, 580 and 430 bp, with a single 580-bp band in the control sample (Fig. 2b). Subcloning and direct sequencing revealed three aberrant mutant transcripts, all of which led to premature termination codons. Immunofluorescence using anti-ABCA12 antibody revealed a diffuse staining of ABCA12 in the granular layers of control skin (Fig. 2e) and of the non-ABCA12 form (TGM1) from patient CIE (data not shown), while a dot-like staining in the cytoplasm was observed in patients 1 and 2 (Fig. 2c,d).

ABCA12 is a membrane lipid transporter that functions in the lipid transport from the *trans*-Golgi network to lamellar granules.[8] *ABCA12* mutations result in heterogeneity, including LI2, HI and CIE.[1,6,7] LI2 is characterized by generalized scales without serious erythroderma, and caused by either homozygote or compound heterozygote for missense mutations within the first nucleotide-binding folds of *ABCA12*.[6] HI is the severest form of ARCI, characterized by generalized large, plate-like scales with ectropion, eclabium and flattened ears.[7] HI is usually caused by homozygous or compound heterozygous truncation mutations in *ABCA12*.[7] In contrast, CIE with *ABCA12* mutation clinically shows milder manifestations.[1] Thus far, 17 different mutations in *ABCA12* have been reported in 12 cases of CIE. Eleven of 12 cases have at least one missense mutation. Only three of 17 mutations (p.Asn1380Ser, p.Ile1494Thr and p.Arg1514His) were located in the first nucleotide-binding folds. Other mutations were located outside ABCA12 active transporter sites: two nucleotide-binding folds and 12 transmembrane domains. The mutation p.Thr1575Pro was identified in two unrelated patients with different clinical severity. Patient 1 with severer features had a heterozygous truncation mutation (c.6031delG) on another allele, while patient 4, with a milder phenotype, had another heterozygous missense mutation (p.Gly1651Ser). We suggest that the phenotypic variability in these two patients was caused by different mutations.

We identified two *ABCA12* splice-site mutations, which were not reported in CIE: c.5128+3A>G and c.5940–1G>C. RT-PCR analysis across the site of the c.5940–1G>C mutation in patient 2 revealed two mutant transcripts. These findings demonstrate expression of the in-frame shorter transcript lacking two or three amino acids due to this splice-site mutation, which may account for the mild phenotype. In contrast, RT-PCR analysis across the site of the c.5128+3A>G mutation in patient 3 revealed three aberrant mutant transcripts, all of which led to premature termination codons. Therefore, patient 3 had a compound heterozygosity for missense/truncated combinations of mutations.

Using high-throughput sequencing analyses, screening of all ARCI-related genes is currently possible, but the cost is still expensive.[9] Once this is overcome, the elucidation of the pathogenesis of ARCI will greatly progress in the near future.

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Department of Dermatology, Kurume University School of Medicine, and Institute of Cutaneous Cell Biology, 67 Asahimachi, Kurume, Fukuoka 830-0011, Japan — S. FUKUDA, T. HAMADA, N. ISHII, S. SAKAGUCHI

Department of Dermatology, Hokkaido University Graduate School of Medicine, Sapporo, 060-8638, Japan — K. SAKAI*, M. AKIYAMA†, H. SHIMIZU*

†*Department of Dermatology, Nagoya University Graduate School of Medicine, Nagoya, 466-8550, Japan* — K. MASUDA‡, K. IZU§

‡*Department of Dermatology, Kyoto Prefectural University of Medicine Graduate School of Medical Science, Kyoto, 62-8566, Japan* — K. TEYE, D. TSURUTA, T. KARASHIMA

§*Department of Dermatology, Kyushu Kosei-Nenkin Hospital, Kitakyushu, 806-8501, Japan* — T. NAKAMA, S. YASUMOTO

Correspondence: Takashi Hashimoto. — T. HASHIMOTO

E-mail: hashimot@med.kurume-u.ac.jp

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Iatrogenic androgenetic alopecia in a male phenotype 46XX true hermaphrodite

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MADAM, Androgenetic alopecia (AGA) is a term that describes the androgen-dependent and genetically determined nature of the disease.[1] However, although it is known that androgen replacement therapy can induce AGA, no report has previously been issued regarding the development of iatrogenic AGA in a hermaphrodite undergoing androgen therapy. Herein, we describe a unique case of a castrated male phenotype 46XX true hermaphrodite receiving exogenous androgen supplementation who developed male-type hair loss.

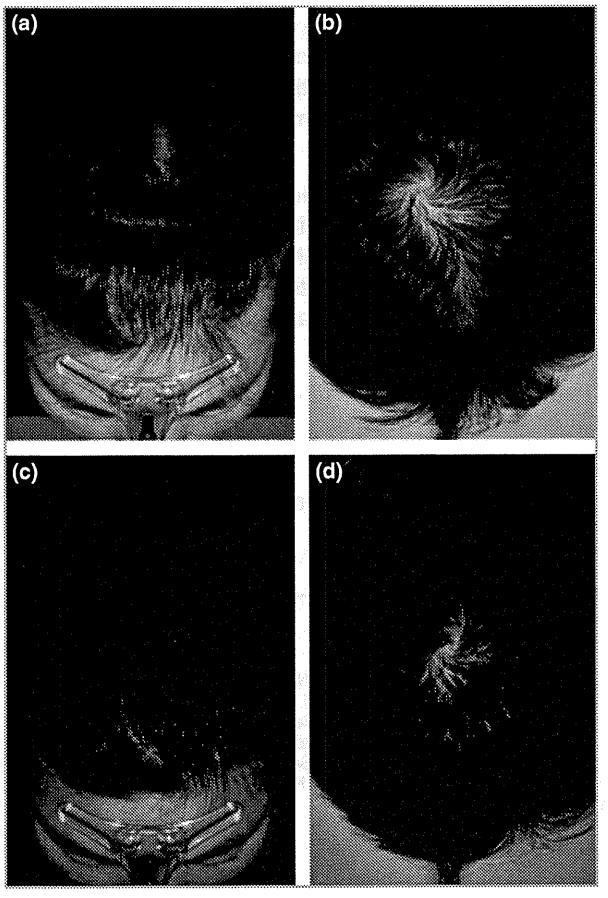

Fig 1. Iatrogenic androgenetic alopecia in a male phenotype 46XX true hermaphrodite showed a great improvement compared with baseline (a, b) after 4 months of finasteride treatment (c, d).

A 21-year-old male phenotype 46XX true hermaphrodite presented with a 3-year history of progressive hair loss. At the age of 16 years he was diagnosed as a 46XX true hermaphrodite with bilateral ovotestis, and subsequently underwent bilateral orchiectomy and testis prosthesis insertion. In addition, he was then given testosterone replacement therapy (testosterone enanthate, Jenasteron®; Jenapharm, Jena, Germany) for surgically induced andropausal status, which halted the development of secondary sexual characteristics. After 3 years of androgen therapy, progressive hair thinning developed on the scalp. Hair examination revealed nonscarring Norwood–Hamilton type III vertex alopecia with frontotemporal recession or BASP classification M1V2 alopecia (Fig. 1a, b).[2] Digital microscopy (Folliscope®; LeadM Corporation, Seoul, Korea) showed miniaturized hair shafts, and hair shaft size variation over the vertex scalp (Fig. 2). Serum testosterone, at the time, was 4·1 ng mL^{-1} (normal 2·7–10·7) and serum dehydroepiandrosterone sulphate was 1845 ng mL^{-1} (normal 800–5600). Under a diagnosis of iatrogenic androgen-induced alopecia, finasteride (1 mg daily) therapy was started. After 4 months of treatment, the hair loss stabilized and scalp hair regrowth was observed, despite the continuance of testosterone replacement therapy (Fig. 1c, d).

True hermaphroditism is an extremely rare disorder, which is defined as the coexistence of testicular and ovarian tissue in the same subject. The most frequent karyotype of true hermaphrodites is 46XX.[3] Gender assignments for hermaphrodites are made according to genetic, gonadal, social and psychologically determined sex, and the requests of patients and their relatives.[4] To be reared as male or female, surgical correction of ambiguous external genitalia, surgical removal of dysgenetic gonads, and sex hormone replacement for the surgically induced andropausal or menopausal state are required. The unwanted dermatological side-effects of testosterone replacement therapy include acne, excessive hair growth and male pattern baldness. As in our case, to be reared

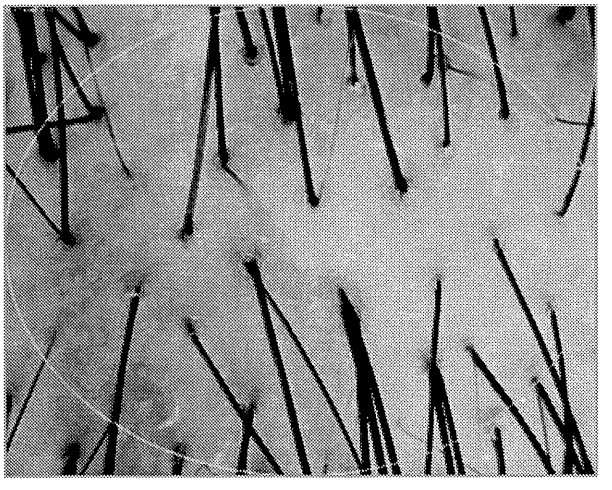

Fig 2. Photomicrograph showing miniaturized hair shafts, and variations in hair shaft size over the vertex scalp (original magnification ×50).

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Concise report

Investigation of prognostic factors for skin sclerosis and lung function in Japanese patients with early systemic sclerosis: a multicentre prospective observational study

Minoru Hasegawa[1], Yoshihide Asano[2], Hirahito Endo[3], Manabu Fujimoto[1], Daisuke Goto[4], Hironobu Ihn[5], Katsumi Inoue[6], Osamu Ishikawa[7], Yasushi Kawaguchi[8], Masataka Kuwana[9], Yoshinao Muro[10], Fumihide Ogawa[11], Tetsuo Sasaki[12], Hiroki Takahashi[13], Sumiaki Tanaka[14], Kazuhiko Takehara[1] and Shinichi Sato[2]

Abstract

Objective. To clarify the clinical course of SSc in Japanese patients with early-onset disease. It is well known that ethnic variations exist in the clinical features and severity of SSc. However, neither the clinical course nor prognostic factors have been thoroughly investigated in the Japanese population.

Methods. Ninety-three Japanese patients of early-onset SSc (disease duration: <3 years) with diffuse skin sclerosis and/or interstitial lung disease were registered in a multi-centre observational study. All patients had a physical examination with laboratory tests at their first visit and at each of the three subsequent years. Factors that could predict the severity of skin sclerosis and lung involvement were examined statistically by multiple regression analysis.

Results. Two patients died from SSc-related myocardial involvement and four patients died from other complications during the 3-year study. Among various clinical data assessed, the initial modified Rodnan total skin thickness score (MRSS) and maximal oral aperture were associated positively and negatively with MRSS at Year 3, respectively. Additionally, initial ESR tended to be associated with final MRSS. Pulmonary vital capacity (VC) in the third year was significantly associated with initial %VC. Furthermore, patients with anti-topo I antibody tended to show reduced %VC at Year 3.

Conclusions. Several possible prognostic factors for skin sclerosis and lung function were detected in Japanese patients with early SSc. Further longitudinal studies of larger populations will be needed to confirm these findings.

Key words: systemic sclerosis, scleroderma, prognostic factor, skin sclerosis, interstitial lung diseases, treatment

[1]Department of Dermatology, Kanazawa University Graduate School of Medical Science, Kanazawa, [2]Department of Dermatology, Faculty of Medicine, University of Tokyo, Tokyo, [3]Department of Internal Medicine (Omori), Toho University School of Medicine, Tokyo, [4]Department of Rheumatology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, [5]Department of Dermatology and Plastic Surgery, Faculty of Life Sciences, Kumamoto University, Kumamoto, [6]Division of Rehabilitation Science, Kanazawa University Graduate School of Medical Science, Kanazawa, [7]Department of Dermatology, Gunma University Graduate School of Medicine, Maebashi, [8]Institute of Rheumatology, Tokyo Women's Medical University, [9]Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, [10]Department of Dermatology, Nagoya University Graduate School of Medicine, Nagoya, [11]Department of Dermatology, Nagasaki University Graduate School of Biomedical Science, Nagasaki, [12]Department of Dermatology, Atami Hospital, International University of Health and Welfare, Atami, [13]First Department of Internal Medicine, Sapporo Medical University School of Medicine, Sapporo and [14]Department of Rheumatology and Infectious Diseases, Kitasato University School of Medicine, Sagamihara, Japan.

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Correspondence to: Minoru Hasegawa, Department of Dermatology, Kanazawa University Graduate School of Medical Science, 13-1 Takaramachi, Kanazawa, Ishikawa 920-8641, Japan.
E-mail: minoruha@derma.m.kanazawa-u.ac.jp

Introduction

SSc is a CTD characterized by tissue fibrosis in the skin and internal organs. Interstitial lung diseases (ILDs) develop in more than half of SSc patients and are one of the major SSc-related causes of death [1, 2]. The natural course of skin sclerosis and internal organ involvement and identification of prognostic factors have been extensively reported in Europe and the USA [3–6]. However, there are some racial differences in the clinical and laboratory features of SSc [7]. For example, the severity of skin sclerosis is modest in Japanese patients [8]. Furthermore, pulmonary arterial hypertension and renal crisis are rare in Japanese SSc patients [9]. Furthermore, racial differences are found in the distribution of SSc-related serum ANAs [10]. The frequency of anti-RNA polymerases antibody (Ab) is lower in the Japanese population than in US or European patient populations [9]. However, there have been no multiple-centre prospective studies concerning the clinical features of SSc in Japanese individuals.

In most patients, severe organ involvement occurs within the first 3 years of disease and skin sclerosis seldom progresses after 5 or 6 years [3, 11]. Therefore, predicting disease progression is particularly important for SSc patients at their first visit. In the present study, we aimed to determine if any initial clinical or laboratory features were associated with subsequent disease severity in Japanese SSc patients with a short disease duration of <3 years.

Materials and methods

Patients

Patients were grouped according to the degree of skin involvement, based on the classification system proposed by LeRoy *et al.* (dcSSc *vs* lcSSc) [12]. In this study, 93 Japanese patients with early SSc (disease duration: <3 years) who had dcSSc or ILD were registered at 12 major scleroderma centres in Japan (Atami Hospital, International University of Health and Welfare; Gunma University Hospital; Kanazawa University Hospital; Keio University Hospital; Kitasato University Hospital; Kumamoto University Hospital; Nagasaki University Hospital; Nagoya University Hospital; Sapporo Medical University Hospital; Tokyo University Hospital; Tokyo Women's Medical University Hospital; Tsukuba University Hospital).

Among these patients, two died from SSc-related myocardial involvement and four died from complications (ANCA-associated vasculitis, sepsis, thrombotic thrombocytopenic purpura and uterine cancer, respectively) during the 3-year study. Therefore, 87 patients (49 patients had dcSSc with ILD, 27 patients had dcSSc without ILD and 11 patients had lcSSc with ILD) were followed for 3 years. Sixty-four were females and 23 were males; the median (range) age was 50 (3–74) years. All patients fulfilled the criteria for SSc proposed by the ACR [13]. The median (range) disease duration (the period from the development of any symptoms excluding RP to our first assessment) of patients was 20 (1–35) months. With respect to ANA, 56 patients were positive for anti-topo I Ab and 7 patients were positive for ACA. Medical ethics committee of Kanazawa University approved the study. In addition, this study was approved by the ethics committees of International University of Health and Welfare, Gunma University, Keio University, Kitasato University, Kumamoto University, Nagasaki University, Nagoya University, Sapporo Medical University, Tokyo University, Tokyo Women's Medical University and Tsukuba University. Informed consent was obtained from all patients.

Clinical assessments

Patients had a physical examination and laboratory tests performed at their first visit and at each subsequent year for 3 years. The degree of skin involvement was determined according to the modified Rodnan total skin thickness score (MRSS), as described elsewhere [14]. Organ system involvement was defined as described previously [15] with some modifications: ILD = bibasilar interstitial fibrosis or ground-glass shadow on high-resolution CT (HRCT); pulmonary arterial hypertension (PAH) = clinical evidence of pulmonary hypertension and elevated right ventricular systolic pressure (>45 mmHg) documented by echocardiography in the absence of severe pulmonary interstitial fibrosis; oesophagus = apparent dysphasia, reflux symptoms or hypomotility shown by barium radiography; heart = pericarditis, congestive heart failure or arrhythmias requiring treatment; kidney = malignant hypertension and rapidly progressive renal failure unexplained by certain diseases other than SSc; joint = inflammatory polyarthralgias or arthritis; and muscle = proximal muscle weakness and elevated serum creatine kinase. An HAQ modified for Japanese patients [16], digital ulcer, pitting scar, maximal oral aperture (the maximum vertical length of opened mouth) and skin pigmentation/depigmentation were also evaluated. ESR and pulmonary function, including vital capacity (VC) and diffusion capacity for carbon monoxide (DL_{CO}) were also tested.

Statistical analysis

JMP Statistically Discovery Software (SAS institute, Cary, NC, USA) was used for analysis. Potential prognostic factors for the severity of skin sclerosis and lung function were statistically examined by multiple regression analysis. A $P < 0.05$ was considered to be statistically significant. All values are expressed as the median (range).

Results

The clinical course of SSc in Japanese patients

To provide a comprehensive evaluation of the clinical features of SSc in Japanese patients, we analysed clinical data as well as laboratory test results from 87 patients with short disease duration (Table 1). To assess the degree of skin involvement in patients, MRSS values were calculated; VC and DL_{CO} percentages were used to assess lung involvement. For the patient population as a whole, the median (range) MRSS decreased from 17 (2–42) to 12 (0–41) during the first year. The median (range) MRSS

was 12 (0–41) at the end of Year 2 and 10 (0–47) at the end Year 3. Median (range) values for %VC did not significantly change during the 3-year evaluation period: 95 (49–144) at first visit, 93 (26–137) at the end of the first year, 95 (49–144) at the end of the second year and 92 (51–137) at the end of the third year. Similarly, median values for % DL_{CO} did not significantly change during the 3 years.

The frequency of patients with ILD or PAH was stable during the evaluation period. Similarly, the number of patients with oesophageal or joint involvement, pitting scar or skin pigmentation/depigmentation did not vary significantly over time. The value of HAQ and maximal oral aperture did not significantly change during the course. The median (range) value of ESR was 18 (2–95) mm/h at the first visit, then it reduced to 16 (2–84), 13 (1–63) and 12 (0.5–122) mm/h, during the subsequent 3 years. Oral prednisolone (~20 mg/day) use was common, with 56 patients starting to take this drug after the first visit and 70 patients having taken it by the end of Year 3. Two patients developed renal crisis during the course of the study (data not shown). Patients with digital ulcer or heart or muscle involvement were rare during the course (fewer than 10 patients, data not shown).

Prognostic factors of the progress of skin sclerosis

Next, we evaluated clinical or laboratory factors presenting at first visit that could predict the severity of skin sclerosis of 3 years later. Investigated factors were as follows: age, gender, disease duration, anti-topo I Ab, ACA, MRSS at the first visit, %VC, % DL_{CO}, existence of each organ involvement (ILD, PAH, oesophagus, joint), pitting scar, skin pigmentation/depigmentation, HAQ, maximal oral aperture, ESR, CS treatment and cyclophosphamide treatment. Cases that have any missing data were excluded and thereby 80 patients were analysed. We performed multiple regression using stepwise way that specified the α-level for either adding or removing a regression as 0.20 (Table 2). As a result, the multiple regression equation predicting MRSS at the third year = 17.11 + 0.35 × MRSS at the first visit + −0.26 × maximal oral aperture + 0.042 × ESR ($R^2 = 0.63$, $P < 0.0001$). Thus, MRSS at the third year was significantly associated with MRSS at first visit ($P < 0.001$) and was negatively associated with initial maximal oral aperture at first visit ($P < 0.01$). Additionally, initial ESR tended to be associated with final MRSS ($P = 0.17$).

Prognostic factors of lung function

We similarly assessed the prognostic factors of impaired lung function to estimate ILD severity. Here, we used %VC as representative markers of lung function. Cases that have any missing data including %VC at the third year were excluded and thereby 58 patients were analysed. We performed multiple regression in a stepped manner that specified the α-level for either adding or removing a regression as 0.20 (Table 3). As a result, the multiple regression equation predicting %VC at the third

Table 2 Factors predicting MRSS at the third year determined by multiple regression analysis

	Estimate	Standard error	*P*-value
Intercept	17.11	4.88	<0.01
MRSS at the first visit	0.35	0.089	<0.001
Maximal oral aperture	−0.26	0.075	<0.01
ESR	0.042	0.043	0.17

The multiple regression equations predicting MRSS at the third year are as follows; MRSS at the third year = 17.11 + 0.35 × MRSS at the first visit + −0.26 × maximal oral aperture + 0.042 × ESR. R^2 (determination coefficient) = 0.63; Root mean square error = 4.73; $P < 0.0001$.

Table 1 The course of clinical and laboratory features in patients with SSc

	First visit	Year 1	Year 2	Year 3
MRSS	17 (2–42); *n* = 87	12 (0–41); *n* = 84	12 (0–41); *n* = 84	10 (0–47); *n* = 87
%VC	95 (49–144); *n* = 70	93 (26–137); *n* = 55	95 (49–144); *n* = 57	92 (51–137); *n* = 60
% DL_{CO}	70 (11–113); *n* = 70	68 (10–105); *n* = 55	69 (11–96); *n* = 57	68 (10–120); *n* = 60
ILD	54 (62); *n* = 87	47 (64); *n* = 73	47 (64); *n* = 73	46 (63); *n* = 73
PAH	9 (10); *n* = 87	9 (12); *n* = 76	8 (11); *n* = 72	11 (13); *n* = 84
Oesophagus	33 (37); *n* = 87	26 (34); *n* = 77	35 (48); *n* = 73	34 (40); *n* = 85
Joint	20 (23); *n* = 86	14 (18); *n* = 77	9 (12); *n* = 73	17 (20); *n* = 84
Pitting scar	27 (33); *n* = 87	29 (38); *n* = 76	35 (48); *n* = 73	33 (38); *n* = 86
Pigmentation/depigmentation	54 (62); *n* = 87	49 (64); *n* = 77	41 (57); *n* = 72	50 (60); *n* = 84
HAQ	0.08 (0–2); *n* = 83	0.125 (0–1.75); *n* = 74	0.25 (0–2.5); *n* = 73	0.125 (0–2.25); *n* = 83
Maximal oral aperture	45 (18–70); *n* = 87	45 (28–65); *n* = 75	46 (25–67); *n* = 72	45 (10–67); *n* = 83
ESR	18 (2–95); *n* = 80	16 (2–84); *n* = 61	13 (1–63); *n* = 52	12 (0.5–122); *n* = 57
CS	56 (64); *n* = 87	61 (82); *n* = 74	64 (86); *n* = 74	70 (80); *n* = 87
Cyclophosphamide	11 (13); *n* = 87	14 (19); *n* = 75	8 (12); *n* = 68	9 (10); *n* = 87

Values are represented as median (range) or as number of positive cases with percentage within parentheses, in total patients in whom those data are available.

TABLE 3 Factors predicting %VC at the third year determined by multiple regression analysis

	Estimate	Standard error	*P*-value
Intercept	10.94	8.54	0.20
%VC at the first visit	0.85	0.09	<0.0001
Anti-topo I Ab (+)	2.32	1.64	0.19

The multiple regression equations predicting %VC at the third year are as follows: %VC at the third year = 10.94 + 0.85 × %VC at the first visit + anti-topo I Ab ('+' → −2.32, '−' → 2.32). $R^2 = 0.70$; Root mean square error = 12.00; $P < 0.0001$.

year = 10.94 + 0.85 × %VC at the first visit + anti-topo I Ab ('+' → −2.32, '−' → 2.32) ($R^2 = 0.70$, $P < 0.0001$). Thus, %VC at the third year was significantly associated with the value of %VC at first visit ($P < 0.0001$). In addition, %VC at the third year tended to be lower in patients with anti-topoisomerase I Ab ($P = 0.19$).

Discussion

To our knowledge, this study is the first multiple-centre, longitudinal prospective study to investigate the clinical course of Japanese patients. For this study, 87 patients with early-onset SSc (<3 years) were followed over 3 years. Median MRSS was reduced 5 points during the first year, and continued to decrease through the third year. This trend was similar to that identified in our previous, single-centre prospective observational study of Japanese SSc patients [17]. Although the reason for the prominent first-year reduction in MRSS in our current study is unknown, our previous single-centre study [17] indicated that the dose of oral CS was related to the decrease of MRSS. However, in this multi-centre observational study we could not perform a similar analysis of prednisolone dose in patients at each centre. In addition, other therapies including cyclophosphamide were also used in a part of patients in our observational study. Previous large studies demonstrated that MRSS naturally reduced during the disease course and time was a significant predictor of MRSS [3-6]. Therefore, the effect of CS therapy for MRSS remains unclear from our data. Since it has been suggested that CS therapy can induce renal crisis, high doses of CSs have not been recommended for the treatment of SSc [18]. However, renal crisis is not as common in Japanese patients [9], and only two patients (one had been taking low-dose CS, whereas the other had not) developed renal crisis during the course of our study.

The main aim of this study was to define the prognostic factors of skin sclerosis and ILD. The multiple regression equation was defined to predict the MRSS at the third year among multiple factors presenting at the first visit. MRSS at the first visit was significantly correlated with MRSS at the third year in all patients. Maximal oral aperture was correlated inversely with MRSS in the third year. Thus, the current skin sclerosis likely reflects the extent of skin sclerosis of 3 years later independent of other organ's involvement or treatment. Additionally, ESR tended to be associated with final MRSS. The presence of autoantibodies such as anti-topo I Ab and ACA was not shown to have value as a prognostic indicator of MRSS. However, this may be due to population bias in our study, since most patients were positive for anti-topo I Ab and negative for ACA.

The current study revealed that %VC and $\%DL_{CO}$ remained nearly constant or slightly reduced during the 3-year period. Since patients with progressive ILD received immunosuppressive treatment, including cyclophosphamide therapy in the participating facilities, this may have affected the stabilization of lung function in our cases. The frequency of ILD detected by HRCT was not increased during the course of the study, indicating ILD is usually detected early in the disease course and rarely develops later. In consistent with generally stable course of %VC, %VC at their first visit highly associated with the %VC at the third year in all patients with or without treatment. Patients with anti-topo I Ab tended to show reduced %VC at the third year. Although these findings are not surprising, we first confirmed them in Japanese patients.

Our study has some limitations. The population is not large and the follow-up period is not long. This is an observational study and therefore the treatment is heterogeneous. In addition, other parameters including CRP could not be analysed due to the lack of data. We should also include disease activity variables [19] and disease severity scale [20] in our future study. Further longitudinal studies in a larger population will be needed to clarify the natural course and prognostic factors in Japanese SSc patients.

Rheumatology key messages

- Initial ESR tended to be associated with skin score at Year 3 in Japanese scleroderma patients.
- Japanese scleroderma patients with anti-topo I Ab tended to show reduced %VC at the third year.

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observations. First, cutaneous mosaicism has been demonstrated only in dermal fibroblasts or adnexal keratinocytes,[9] both cell types following different embryologic paths from that of melanocytes and both giving rise to nevi which always follow Blaschko's lines (exception: Becker's nevus). Second, melanocytic nevi, in which nevus cells most likely carry the genetic defect, never follow Blaschko's lines, the only exception seemingly being the recently framed "nevus lentiginosus linearis".[10] Thus, I suggest that the relative non-specificity of the syndromic associations of mosaic hypomelanosis and hypermelanosis (excluding McCune–Albright syndrome) might rely on the fact that melanocytes are just "innocent bystanders" of mosaic states affecting other cells and tissues.

Daniele TORCHIA
University of Florence, Florence, Italy

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Yellow nail syndrome: Nail change reflects disease severity

Dear Editor,

The triad of yellow nail syndrome (YNS) includes nail yellowing and thickening, lymphedema, and respiratory manifestations.[1] Although the pathogenesis of YNS is unknown, acquired lymphatic dysfunction and microvasculopathy with protein leakage have been thought to be the predominant mechanisms underlying the clinical manifestations.[1,2] YNS has been described in association with malignancies,[3,4] immunodeficiencies[4] and connective tissue diseases.[5] Herein, we report a case of YNS, in which the first manifestation was nail changes alone, then lymphedema and pleural effusion became prominent.

A 71-year-old man was referred to our department with a 5-year history of yellow discoloration of the fingernails and toenails. For 10 years, he had suffered recurrent episodes of chronic sinusitis and pneumonitis. From 3.5 years before his visit, general malaise, dry cough, exertional dyspnea and edema of both legs had presented. Edema of both legs had been improving due to the use of a diuretic. However, the nail changes remained.

When he visited our department, the fingernails and toenails all showed yellowish discoloration, slow growth, absent lunulae, increased curvature and thickening (Fig. 1). Fungal infection was ruled out by KOH examination of the nails. Neither fungus nor bacterium was cultured in the samples taken from the nails.

X-ray and computed tomography of the chest showed bilateral pleural effusions, predominantly in the right lung (Fig. 2).

Thoracentesis revealed light yellow fluid and exudative pleural effusion. No malignant cells were found. Culture of pleural fluid did not identify any bacterial infection. The common causes of transudates (cardiac failure, hepatic cirrhosis, nephropathy) and exudates (lymphoma, metastatic disease, connective tissue disease,

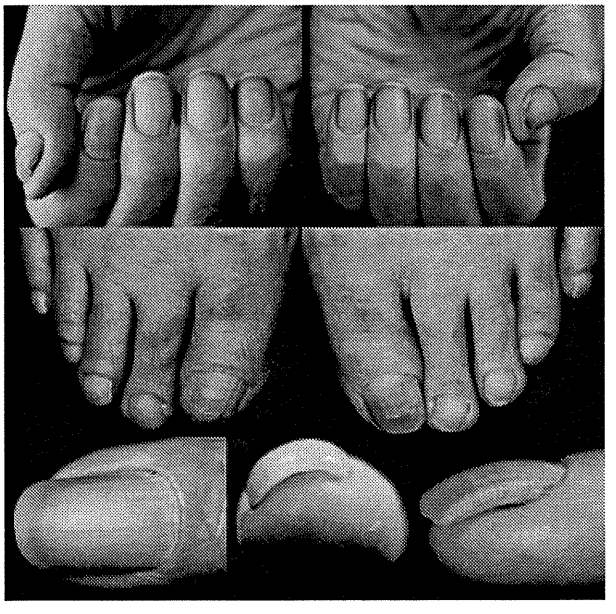

Figure 1. Clinical findings. The fingernails and toenails show yellowish discoloration, thickening, increased curvature and absent lunulae.

Correspondence: Riichiro Abe, M.D., Department of Dermatology, Hokkaido University Graduate School of Medicine, North 15 West 7, Kita-ku, Sapporo 060-8638, Japan. Email: aberi@med.hokudai.ac.jp

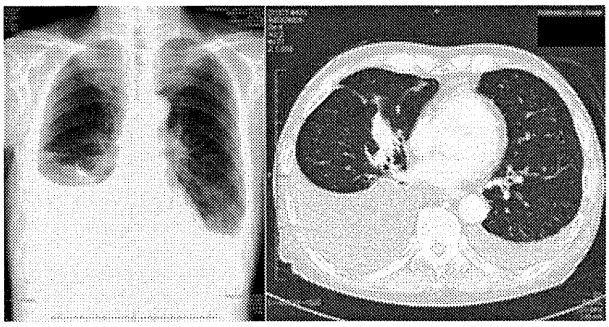

Figure 2. Bilateral pleural effusion. X-ray and computed tomography scans of the chest show bilateral pleural effusion.

infection) were excluded based on analysis of the pleural fluid. Diagnosis of YNS was made.

Yellow nail syndrome is characterized by the triad of yellow nails, lymphedema and respiratory manifestations. The presence at any given time of one of these three manifestations is sufficient to establish the diagnosis.[1] Characteristic nail features in YNS seem to be the most variable finding and the first to be recognized.[6] We questioned whether there are other manifestations suggesting YNS when we found yellow discoloration of the nails. The complete triad, which was seen in our case, is observed in only approximately 23.4% of YNS patients.[7]

Many conditions have been associated with YNS, particularly respiratory manifestations, such as bronchiectasis and recurrent lower respiratory tract infection, which are present in approximately half of the patients.[8] Other conditions with YNS include immunodeficiency states,[6] connective tissue diseases, and several malignancies, such as breast cancer[3] and lymphoproliferative disorders.[4] Rheumatoid arthritis is the autoimmune disease that is most commonly associated with YNS.[5]

The pathogenesis of the YNS manifestations is unknown. Recent studies have suggested that microvasculopathy with protein leakage may be more likely than functional lymphatic insufficiency as an explanation for the etiology of YNS.[8] The characteristic discoloration of the nails may be due to accumulation of lipofuscin, which is the product of fatty acid oxidation in the nail plate.[9] Another suggestion is that there are melanin particles in the nails, which become apparent when the nail matrix becomes inflamed.

Although there are no established effective treatments for the nail manifestations, partial or complete improvement occurs spontaneously in up to one-third of patients.[5] Some cases were reported to improve with better control of the respiratory manifestations.[6] Another paper reported that the nails returned to normal when a complicated tumor regressed.[3] Therefore, yellow nails may be an indicator of other coexistent manifestations of YNS or complications. In our case, worsening of the nail manifestations might be associated with the severity of lymphedema or pleural effusion.

The manifestations seen in YNS are not necessarily coincidental. When a patient clinically shows yellow nails, we should carefully consider YNS and conduct follow ups for any complications.

Inkin HAYASHI, Riichiro ABE, Teruki YANAGI, Yukiko ABE, Hiroshi SHIMIZU

Department of Dermatology, Hokkaido University Graduate School of Medicine, Hokkaido University, Sapporo, Japan

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Can end organ damage in scleroderma be predicted based on nail fold dermatoscopy findings?

Dear Editor,

Systemic scleroderma (SSc) is a connective tissue disease characterized by fibrosis and thickening of the skin. Decreased number of capillaries, dilated capillary loops and giant capillaries are frequently observed on nail fold examination.[1] Basillar pulmonary fibrosis, pulmonary arterial hypertension (PAH) and esophageal dysmotility are the most common comorbidities, and frequently cause SSc-related mortality.[2]

A total of 35 Turkish patients; 15 with diffuse cutaneous SSc (dcSSc) and 20 with limited cutaneous SSc (lcSSc), were

Correspondence: Sibel Dogan, M.D., Department of Dermatology, Faculty of Medicine, Hacettepe University, Sihhiye 06100, Ankara, Turkey. Email: sibel.dogan@hacettepe.edu.tr

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RESEARCH ARTICLE Open Access

Epidemiologic study of clinically amyopathic dermatomyositis and anti-melanoma differentiation-associated gene 5 antibodies in central Japan

Yoshinao Muro[1*], Kazumitsu Sugiura[1], Kei Hoshino[1,2], Masashi Akiyama[1] and Koji Tamakoshi[3]

Abstract

Introduction: Several reports have found the onset or activity of inflammatory myopathies to show spatial clustering and seasonal association. We recently detected autoantibodies against melanoma differentiation-associated gene 5 (MDA-5) in more than 20% of patients with dermatomyositis. Anti-MDA-5 antibodies were associated with the presence of rapidly progressive interstitial lung disease in clinically amyopathic dermatomyositis (CADM). The present study aims to assess the growing prevalence of CADM and the geographical incidence of anti-MDA-5-positive patients.

Methods: We reviewed medical charts and examined the presence of anti-MDA-5 antibodies in 95 patients, including 36 CADM patients. Sera were obtained from 1994 through 2011. Statistical analyses were performed to assess whether CADM development and the presence of anti-MDA-5 antibodies were associated with various parameters, including age at disease onset, season of onset, annual positivity, and population of resident city.

Results: Tertiles based on the year when the sera were collected showed increasing tendencies of CADM and anti-MDA-5-positive patients among all of the dermatomyositis patients. From 1994 to 2010, the relative prevalence of CADM and anti-MDA-5 antibody-positive patients significantly increased. Interestingly, the presence of anti-MDA-5 antibodies in 26 patients was inversely associated with the population of their city of residence.

Conclusions: This is the first study to examine the distribution of anti-MDA-5-positive dermatomyositis phenotypes in Japan. Regional differences in the incidences of these phenotypes would suggest that environmental factors contribute to the production of antibodies against MDA-5, which triggers innate antiviral responses.

Introduction

Idiopathic inflammatory myopathies are a heterogeneous group of autoimmune disorders that target the skeletal muscle and skin. Disease-related death is generally associated with malignancy and interstitial lung disease. The most frequent forms, polymyositis and dermatomyositis (DM), are thought to result from environmental exposure that leads to immune activation in genetically susceptible individuals. Several reports have found the onset or activity of inflammatory myopathies to show spatial clustering and seasonal association [1-5].

A subgroup of DM patients who have typical skin manifestations of DM but little evidence of myositis has been recognized as clinically amyopathic dermatomyositis (CADM) [6]. Although it is still undetermined whether CADM is a distinct clinical entity or just an early phase of classic DM, rapidly progressive interstitial lung disease (ILD) can occur in CADM patients, especially in East Asia [7]. This patient subset with CADM and rapidly progressive ILD has been shown to have specific autoantibodies, originally called anti-CADM-140 antibodies [8]. The target autoantigen is melanoma differentiation-associated gene 5 (MDA-5) [9-11], which

* Correspondence: ymuro@med.nagoya-u.ac.jp
[1]Division of Connective Tissue Disease and Autoimmunity, Department of Dermatology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan
Full list of author information is available at the end of the article

plays important roles in the innate immune system during RNA virus infections [12].

To better understand this subset of patients, it is important to examine the epidemiologic characteristics of CADM patients with anti-MDA-5 antibodies, whose outcome is often fatal. According to our clinical experiences, we have recently noticed that the prevalence of CADM patients with anti-MDA-5 antibodies seems to be growing, particularly in rural areas. We therefore examined the epidemiologic features of CADM and anti-MDA-5 antibodies in a single cohort of DM patients.

Materials and methods

Patients

We reviewed medical charts and examined the presence of anti-MDA-5 antibodies in 95 Japanese patients (one of them a half-Japanese, half-Filipino boy) with DM, including 36 patients with CADM, 15 patients with cancer-associated DM and 44 patients with classical DM, who were seen by or consulted the Department of Dermatology at Nagoya University Graduate School of Medicine from 1994 to 2011. These patients were diagnosed with DM or CADM based on the criteria of Bohan *et al.* [13] or Sontheimer [6], respectively. In general, CADM presents as typical skin lesions and amyopathy or hypomyopathy that lasts for more than 6 months. The CADM group included patients who developed fatal ILD within the first 6 months after disease onset. Since juvenile DM with rapidly progressive ILD and/or anti-MDA-5 antibodies has been reported in Japan [7,11,14], patients who manifested the disease at < 18 years of age were also included. Patients who were originally seen at other hospitals far outside our area and who then transferred to our hospital were excluded from the present study. Serum samples were obtained from all of the patients between 1 October 1994, the date when we began to build a serum bank of autoimmune rheumatic disease patients, and 30 June 2011. The population data on city of residence in 2010 were obtained from web data published by public offices in 25 cities, eight counties and one village.

The present study was approved by the Ethics Committee of Nagoya University Graduate School of Medicine. This study meets and is in compliance with all ethical standards in medicine. Informed consent including that for publication of the study was obtained from all patients according to the Declaration of Helsinki.

Immunoprecipitation

Anti-MDA-5 antibodies were screened by an immunoprecipitation assay using biotinylated recombinant MDA-5 produced from full-length MDA-5 cDNA using the TnT® T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI, USA) and the Transcend™ Colorimetric Non-Radioactive Translation Detection System (Promega), according to our published protocol [11]. This method was confirmed to produce consistent results based on a standard immunoprecipitation assay using ^{35}S-methionine-labeled cell extracts [11]. Serum samples from 82 patients were already characterized in our previous report [11]. All serum samples were stored at -70°C until the experiments.

Statistical analysis

The subjects were divided into tertiles based on year the sera were collected, age at collection, age at onset, or population of the city of residence, separately, to examine the associations between each of these factors and the development of CADM and the presence of anti-MDA-5 antibodies. The differences and linear trends across the tertiles were assessed using the chi-square test and the Cochran-Armitage trend test, respectively. SPSS version 17.0 for Windows (SPSS Japan Inc., Tokyo, Japan) was used to perform the statistical analysis. $P < 0.05$ was considered significant.

Results

Patient population

Between 1 October 1994 and 30 June 2011, sera from 95 patients with DM were collected. During 1994 sera were drawn from 24 patients, two-thirds of whom had been diagnosed with DM and treated by our department. The mean age at onset was 46.9 years (range: 1 to 80 years) and that at the time of sera collection was 50.2 years (range: 3 to 84 years). There were 67 (70.5%) female patients. Ten patients developed the disease under 18 years of age.

A review of the medical records indicated that 36 patients (28/36, 77.8% female; 5/36, 13.9% juvenile) had CADM. For these 36 patients, the mean age at onset was 44.9 years (range: 1 to 73 years) and that at the time of sera collection was 48.2 years (range: 3 to 84 years). Based on the immunoprecipitation assays, 26 patients (21/26, 80.8% female; 1/26, 3.8% juvenile) had anti-MDA-5 antibodies. For these 26 patients, the mean age at onset was 46.8 years (range: 11 to 66 years) and that at the time of sera collection was 48.2 years (range: 11 to 71 years). Twenty-five patients with anti-MDA-5 antibodies were diagnosed as CADM, and the remaining patient met the criteria for classical DM. All but one of our patients with anti-MDA-5 antibodies had ILD.

To grasp the overall trend, tertile analysis was conducted based on the number of cases for all patients with DM as well as for patients with CADM and those with anti-MDA-5 antibodies (Table 1). The mean ages at onset and at the time of sera collection did not significantly differ among the tertiles (data not shown), but

Table 1 Patient characteristics based on the presence of CADM or anti-MDA-5 antibodies

			CADM			α-MDA-5-positive		
Years of sera collection	Total number of DM patients (M:F)	Mean age at onset (range)	Number (%) of patients (M:F)	*P* value*	Mean age at onset (range)	Number (%) of patients (M:F)	*P* value**	Mean age at onset (range)
T1 (1994 to 1995)	32 (12:20)	47.5 (4 to 80)	6 (18.8%) (2:4)	*P* for difference = 0.012	45.5 (4 to 73)	2 (6.3%) (1:1%)	*P* for difference = 0.003	53 (43 to 63)
T2 (1996 to 2003)	30 (6:24)	50.1 (15 to 79)	12 (40.0%) (1:11)	*P* for trend = 0.003	50.7 (20 to 73)	10 (33.3%) (0:10)	*P* for trend = 0.001	48.9 (20 to 66)
T3 (2004 to 2011)	33 (10:23)	43.6 (1 to 73)	18 (54.5%) (5:13)		40.8 (1 to 69)	14 (42.4%) (4:10)		44.4 (11 to 58)

CADM, clinically amyopathic dermatomyositis; DM, dermatomyositis; M:F, male:female; MDA-5, melanoma differentiation-associated gene 5. *Prevalence of CADM in total DM. **Prevalence of anti-MDA-5 in total DM.

the proportions of CADM and anti-MDA-5-positive patients significantly increased from the first to the third periods of the study.

Annual prevalence of CADM and anti-MDA-5 antibodies

Since many of the patients whose sera were drawn in 1994 had been treated at our hospital, the above tertile analysis was partially biased. Before the sampling in 1994 there may have been some fatal cases of rapidly progressive ILD. Moreover, some CADM patients stopped seeing their doctors due to minor illness. In light of these possibilities, we analyzed only the 72 patients who manifested the disease after 1994, in order to investigate the growing trend of CADM and anti-MDA-5-positive patients (Figure 1). The relative prevalence of both CADM and anti-MDA-5-positive patients among all DM patients was found to have significantly increased (P = 0.029 and P = 0.044, respectively).

Geographical incidence of dermatomyositis patients with anti-MDA-5 antibodies

Our university hospital is in Nagoya (population 2.2 million), the biggest city in central Japan. To clarify the regional differences in a subgroup of patients, we compared the prevalence of CADM and anti-MDA-5-positive patients by tertiles based on the population of the patient's city of residence (Table 2), and we plotted the anti-MDA-5-positive patients on a map (Figure 2). Interestingly, CADM patients were less prevalent in

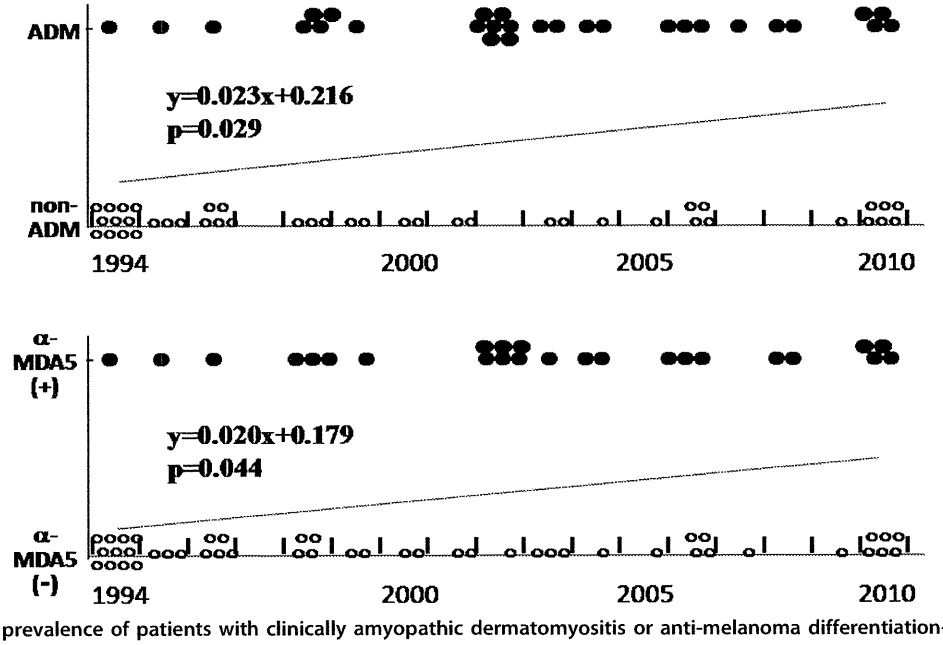

Figure 1 Annual prevalence of patients with clinically amyopathic dermatomyositis or anti-melanoma differentiation-associated gene 5 antibodies. The regression equation is shown, in which the year of disease onset is defined as 1994 = 1, 1995 = 2,..., 2010 = 17 on the *x* axis and the presence or absence of clinically amyopathic dermatomyositis (CADM) or anti-melanoma differentiation-associated gene 5 (anti-MDA-5) antibodies is defined as 1 and 0, respectively, on the *y* axis (*P* for linear trend).

Table 2 Population of the area of residence and the presence of CADM or anti-MDA-5 antibodies

			CADM			α-MDA-5-positive		
Population of area of residence (×1,000)	**Total number of DM patients (M:F)**	**Mean age at onset (range)**	**Number (%) of patients (M:F)**	***P* value***	**Mean age at onset (range)**	**Number (%) of patients (M:F)**	***P* value****	**Mean age at onset (range)**
T1 (0.5 to 108)	31 (4:27)	49.0 (4 to 70)	16 (51.6%) (1:15)	*P* for difference = 0.096	47.3 (4 to 69)	14 (45.2%) (1:13)	*P* for difference = 0.012	48.8 (20 to 66)
T2 (130 to 826)	26 (7:19)	44.0 (9 to 80)	10 (38.5%) (3:7)	*P* for trend = 0.031	40.0 (9 to 59)	7 (26.9%) (3:4)	*P* for trend = 0.003	39.9 (11 to 59)
T3 (2,200)	38 (17:21)	47.3 (1 to 79)	10 (26.3%) (4:6)		45.9 (1 to 73)	5 (13.2%) (1:4)		51.0 (39 to 63)

CADM, clinically amyopathic dermatomyositis; DM, dermatomyositis; M:F, male:female; MDA-5, melanoma differentiation-associated gene 5. *Prevalence of CADM in total DM. **Prevalence of anti-MDA-5 in total DM.

urban areas, but this association was only marginally significant, whereas there were significantly more anti-MDA-5-positive patients in rural areas than in urban ones. Areas northeast and far northwest of Nagoya contained particularly high numbers of patients with anti-MDA-5 antibodies: 10 patients in the northeast, and five patients in the northwest (Figure 2, circular dotted area). These areas had nine and six CADM patients in the northwest and northeast, respectively. All 15 patients with anti-MDA-5 antibodies were natives of the

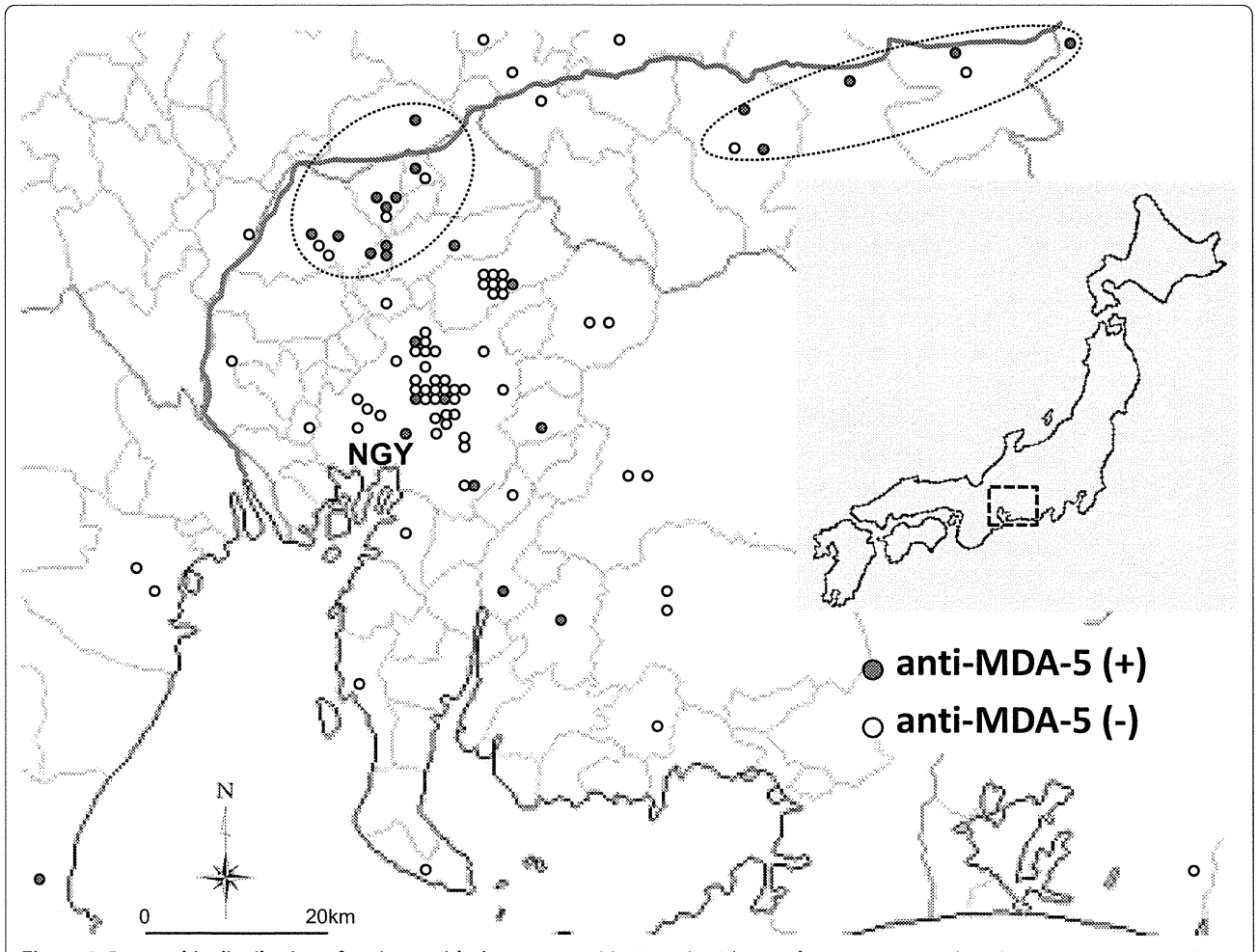

Figure 2 Geographic distribution of patients with dermatomyositis. A residential area of 95 patients was plotted. NGY, Nagoya city. Red and white circles show patients with and without anti-melanoma differentiation-associated gene 5 (anti-MDA-5) antibodies, respectively. Blue line shows the Kiso River, which is the biggest in the area.

area; five and four of these 15 patients had manifested the disease in 2002 and 2010, respectively. Notably, five of the six patients with anti-MDA-5 antibodies whose disease began in 2002 and all four of the patients with anti-MDA-5 antibodies whose disease began in 2010 were from these two areas (Figure 1).

Seasonal onset

The information on seasonality of disease onset was available for 78 patients, including 33 CADM and 25 anti-MDA-5-positive patients. There were no significant seasonal patterns of disease onset in the overall patient group or in the subgroups of male, female, CADM or anti-MDA-5-positive patients (data not shown). However, the incidence of anti-MDA-5 antibodies in areas with populations under 108×10^3, but not in areas with populations over 130×10^3, was the highest in autumn (onset in autumn in areas with populations under 108×10^3 vs. onset in autumn in areas with populations over 130×10^3 = 8/14 vs. 1/11, $P = 0.033$).

Discussion

A Japanese multicenter study confirmed recently that patients with anti-MDA-5 antibodies frequently have CADM with rapidly progressive ILD and a poor prognosis [15]. With increasing awareness of the CADM disease subtype, which was proposed by Sontheimer in the 1990s, we felt not only that the prevalence of CADM is increasing but also that more CADM patients with anti-MDA-5 antibodies have recently been coming from rural areas than from urban ones. To examine these matters statistically, we investigated the prevalence of CADM and anti-MDA-5 antibodies among all of the DM patients.

Because the present study was neither population based nor community based, it is difficult to say that the incidence of CADM is increasing. However, the frequency of anti-MDA-5 antibodies among all DM patients is increasing. Although this autoantibody was only recently characterized [8,9], our initial study found that the serum collected from one patient in 1994 was anti-MDA-5 antibody-positive. Contrary to the increasing prevalence of anti-MDA-5 antibodies, other types of autoantibodies appear to be decreasing. We also characterized the prevalence of anti-transcriptional intermediary factor-1γ antibodies among all patients examined in this study. These antibodies, however, which were detected in 12 patients, showed no significant epidemiological characteristics under the same analysis (data not shown). In our previous study using traditional immunoprecipitation experiments, we did not detect significant decreases in the prevalence of any specific autoantibodies [11]. There is little possibility that the long storage of the sera caused the autoantibodies to become less active, however, because various kinds of DM/polymyositis-specific autoantibodies were found in many of the sera that were drawn in 1994 and 1995 (two patients with anti-transcriptional intermediary factor 1γ, two patients with anti-MJ, two patients with anti-PL-7, one patient with anti-Jo-1, one patient with anti-EJ and one patient with anti-KS; our unpublished observations), along with the anti-MDA-5 antibodies found in the two other patients during this period.

MDA-5 detects some viruses, including picornaviruses, and is involved in the host defense response to infection. Antibodies to coxsackievirus B, a picornavirus, were previously reported to be prevalent in patients with juvenile DM [16]. Although we could not find an epidemiologic study on the environmental levels of picornavirus in our district, the seasonal distribution of viruses in the river water in Nara Prefecture, which is also in central Japan, has been examined [17]. The coxsackievirus B levels peaked there in the summer, and the virus continued to be detected in the autumn and winter. Interestingly, there was a marked increase in the prevalence of anti-MDA-5 antibodies in our study in areas northeast and northwest of Nagoya (Figure 2). These regions are on the Kiso River, which is the biggest river in our area (blue line in Figure 2). In these areas, there was also an accumulation of CADM. It is unlikely that sun exposure strongly contributed to the pathogenesis, because the 15 patients with CADM included only one outdoor worker.

The present study has several limitations because of the small number of study subjects. The time lag between the initial presentation of disease and the clinical assessment should be considered. The interval between disease onset and the time of sera collection in this study was not significantly different, however, between patients with and without CADM, between patients with and without anti-MDA-5 antibodies, or among the tertiles depicted in Table 1 (data not shown), suggesting that the patient follow-up periods did not differ by disease subtype. Since people in rural areas generally have reduced access to specialists, patients with severe illness, such as anti-MDA-5-positive patients, might be more prevalent in rural areas than in urban areas. Moreover, medical practices at a university hospital have an inherent referral bias.

Many reports have suggested that environmental factors play a role in the development of DM and the production of myositis-related autoantibodies (reviewed in [18]). No single factor, however, can explain that development and that production, and the possible growing prevalence of CADM and anti-MDA-5-positive patients. It seems difficult to identify environmental factors that possibly increase the annual prevalence of CADM and anti-MDA-5-positive patients, because patients could have several environmental exposures that have possible interrelationships with genetic risk factors. Various environmental