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Pemphigus foliaceus associated with oesophageal cancer

Editor

Pemphigus vulgaris (PV) and pemphigus foliaceus (PF) are the two major subtypes of pemphigus. [1] In PV patients, mucous membranes including the oesophageal mucosa are frequently affected target tissues.[2] In contrast, patients with PF seldom exhibit mucous membrane involvement because desmoglein (Dsg) 1, the major target of PF autoantibodies, is expressed at a much lower level than Dsg3 throughout squamous mucosal epithelia. [3] Here, we report a patient with PF, who had esophageal symptoms including swallowing difficulty. Oesophageal cancer was later found.

A 68-year-old man visited our hospital with erythema, erosion and crust on his trunk (Fig. 1a). No mucosal lesion was found. A skin biopsy specimen taken from his back showed acantholysis and blisters within the upper epidermis (Fig. 1b). Direct immunofluorescence (DIF) studies demonstrated immunoglobulin G (IgG) and C3 depositions in the intercellular space of keratinocytes. Enzyme-linked immunosorbent assay (MBL, Nagoya, Japan) of his serum revealed positive IgG antibody to Dsg1 (index value = 580.0; cut-off value = 20), while IgG antibody to Dsg3 was negative. We diagnosed his skin lesions as typical PF. The patient was treated with oral prednisolone at an initial dosage of 0.6 mg/kg/day and showed a good response.

As the patient had complained of swallowing difficulty at his initial visit, oesophageal endoscopy was performed and revealed multiple erosions in his oesophageal mucosa located between 25 and 36 cm distant from the upper incisors (Fig. 1c). A biopsy specimen from the oesophageal lesion showed atypical keratinocytes, which was diagnosed as squamous cell carcinoma (SCC). While DIF studies on the surrounding normal oesophageal mucosa showed IgG and C3 depositions within the intercellular spaces of the epithelium, there were few IgG deposits in the specimen from the erosive lesion. Video-assisted transthoracic esophagectomy with 3-field lymph node dissection was performed under the diagnosis of SCC (T_1, N_0, M_0; stage I; Fig. 1d). Nine months after the operation, there has been no recurrence of PF or oesophageal cancer and enzyme-linked immunosorbent assay for Dsg1 has remained negative. Oral prednisolone has been stopped.

Oesophageal endoscopic examination is a useful diagnostic tool prior to starting therapy in PV patients.[4] Conversely, oesophageal endoscopy is rarely performed on PF patients because esophageal involvement is rare in PF. In the present case, because of his oesophageal dysphagia, we conducted upper endoscopy and were able to identify these oesophageal lesions. We thoroughly

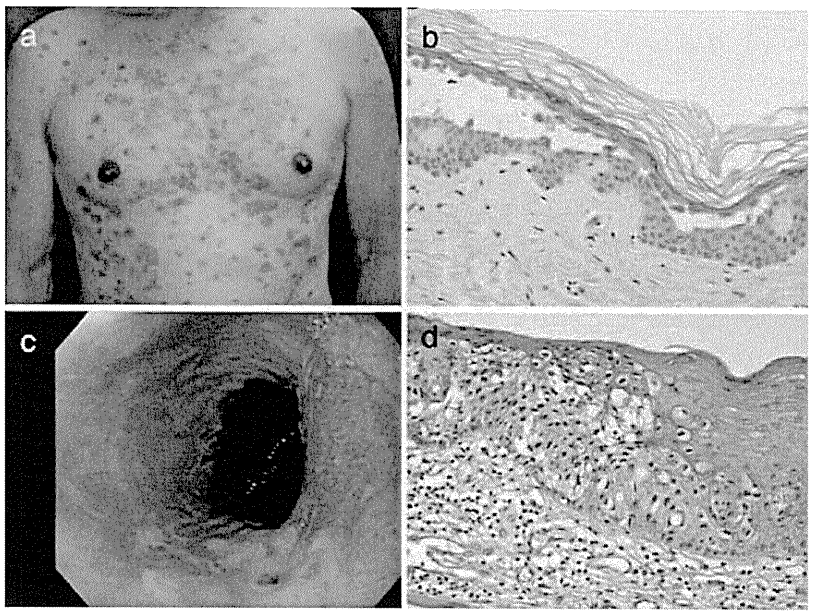

Figure 1 Erythema, erosion and crust were scattered on the patient's chest and abdomen (a). Skin biopsy specimens obtained from the lesion on his back showed acantholysis and blistering in the upper epidermis (b). Endoscopic examination revealed multiple erosions in the oesophageal mucosa at sites 25–36 cm distant from the upper incisors (c). Samples taken from the oesophageal lesion showed that abnormal keratinocytes with individual cell keratinization and disturbance in cellular arrangement were found within the mucous and submucosal layers, which was diagnosed as SCC (d). Haematoxylin–eosin stain, original magnifications (b, d: ×200).

examined the biopsy specimen and eventually diagnosed oesophageal carcinoma.

As far as we know, this is the first reported case of PF associated with oesophageal cancer. There have been several reports concerning the incidence of neoplasms in patients with pemphigus.[5] In PF, it was reported that the frequency of malignancies was higher than that in PV.[6] Types of associated neoplasms in pemphigus are variable. A pathogenic role of SCC is not established in the development of pemphigus.[7]

In non-lesional oesophageal mucosal epithelium, DIF studies showed IgG and C3 depositions in the intercellular space, as could be predicted from the known fact that both Dsg3 and Dsg1 are expressed in mucosal epithelium in the oesophagus. Whereas, in cells of SCC, the expression of Dsg1 is markedly reduced or absent,[8,9] in our case, there was some reduced level of deposition in the tumour cells of oesophageal cancer compared with the skin or normal oesophageal membrane.

We first reported a case of PF associated with oesophageal cancer. This suggests the importance of upper endoscopic examination not only on PV but also on another skin disorders including PF if they have any oesophageal symptoms.

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Successful treatment of recalcitrant relapsing polychondritis with monoclonal antibodies

Editor

Relapsing polychondritis (RP)[1,2] is a rare, potentially life-threatening multisystem disorder characterized by inflammation of cartilaginous tissues. Treatment with corticosteroids is often effective but limited by side effects. Methotrexate, dapsone, azathioprine and cyclophosphamide have been used with less reliable success.

Targeted therapies with monoclonal antibodies have expanded the treatment of immunological diseases. Infliximab [3,4] and adalimumab are directed against tumour necrosis factor- α (TNF-α) and rituximab against CD20. [5] Few case reports about successful treatment of RP with infliximab can be found,[6–9] while adalimumab and rituximab have not been reported in this context before. We would like to present our experience with these monoclonal antibodies in three patients with RP.

A 56-year-old woman presented with swelling of the right ear (Fig. 1), arthralgia, scleritis, hypakusis and dyspnoea. Computer tomography of her lungs showed an increase of inflammatory tissue around the trachea (Fig. 2). Antibodies against collagen type II were detectable. Despite intensive immunosuppressive measures, the disease progressed. Thus, we started infliximab. Already 1 day after the first infusion, she felt remarkable improvement, and after 2 weeks, she was free of symptoms. Although the effect of infliximab has been decreasing over time, she still achieves almost unrestricted daily activity.

A 43-year-old woman suffered from swelling of the right ear, urticaria, arthralgia and dyspnoea. She had been treated with

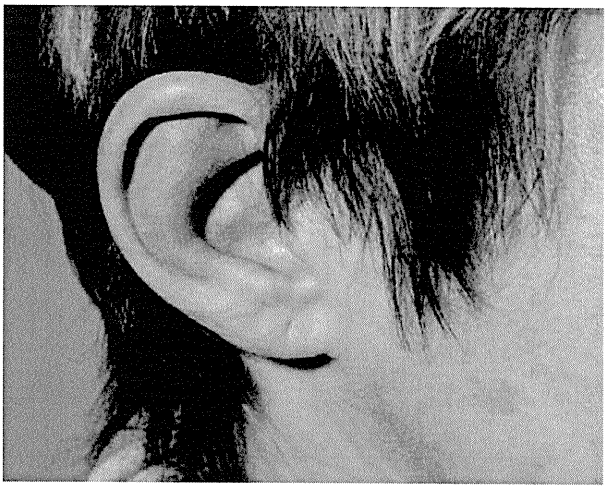

Figure 1 Redness and swelling of the right ear sparing the lobule.

Secondary syphilis mimicking warts in an HIV-positive patient

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LETTER

Secondary syphilis mimicking warts in an HIV-positive patient

A 35-year-old Japanese man visited our hospital with a 2-year history of skin lesions affecting his palms and soles. The palmar lesions comprised diffuse papillomatous hyperkeratotic and macerative reddish skin eruptions (fig 1A). In addition, he had warty-like plaques of 4.5 cm in diameter on his right sole (fig 1B). Upon his initial visit, he did not report any other symptoms such as chills or malaise. These unusual and severe hyperkeratotic lesions did not fit any typical known skin disease such as verruca, Reiter's syndrome and so on.

Skin biopsy specimens of the hyperkeratotic lesions on the palm and sole showed marked exophytic papillomatous acanthosis with a few koilocytotic cells. In the upper dermis, a dense inflammatory infiltrate containing large numbers of plasma cells was present.

Clinical and histopathological findings led us to suspect syphilis. The serological test showed high reactivity of the rapid plasma reagin test for syphilis at a titre of 1 : 1024 and a positive *Treponema pallidum* haemagglutination test (2450TU). Around the same time, numerous spirochaetes were detected from his swollen tonsils with Indian ink staining of the smear.

In addition, we suspected that he was in an immunodeficient state because of his severe clinical features of syphilis, therefore we performed a supplementary study. In the following results, a viral serological test revealed that he had detectable antibodies to HIV-1 (HIV viral load 11 000 copies/ml). The CD4 cell count (479 cells/mm^3) was slightly decreased.

After 3 weeks of penicillin antibiotic treatment, his skin eruptions and swollen tonsils had dramatically improved (fig 1C, D). Furthermore, we could not detect any evidence suggesting human papillomavirus infection using a method that can detect a broad range of DNA from multiple human papillomavirus types in both the palm and sole lesions.[1] Finally, we diagnosed his hyperkeratotic skin lesions and enlarged tonsils as secondary syphilis because of the good response to the antibiotic treatment and pathological and serological findings. Furthermore, we suspected his immunodeficiency from the atypical skin eruptions and reached a diagnosis of HIV infection.

Infections with unusual clinical features are frequently observed in patients with HIV.[2] In recent years, some cases of syphilis in HIV patients with various manifestations and a rapidly progressive course have been reported, which have led to the hypothesis that HIV superinfection modifies the clinical presentation and disease course of syphilis.[2 3]

Secondary syphilis has various clinical forms, such as macular syphilide, papular syphilide, pustular ulcerative syphilide and syphilitic alopecia.[4] In papular syphilide, there are several subtypes including syphilitic psoriasis and condyloma latum, which may present as slightly hyperkeratotic lesions. It has been reported that a very small number of syphilis patients manifest severe palmoplantar keratoderma such as that seen in Reiter's syndrome.[5 6]

As this patient exhibited such significant and atypical clinical features, we were able to diagnose HIV infection. This case emphasises the importance of suspecting and checking for HIV infection after a rare clinical presentation of secondary syphilis, such as these severe wart-like hyperkeratotic lesions.

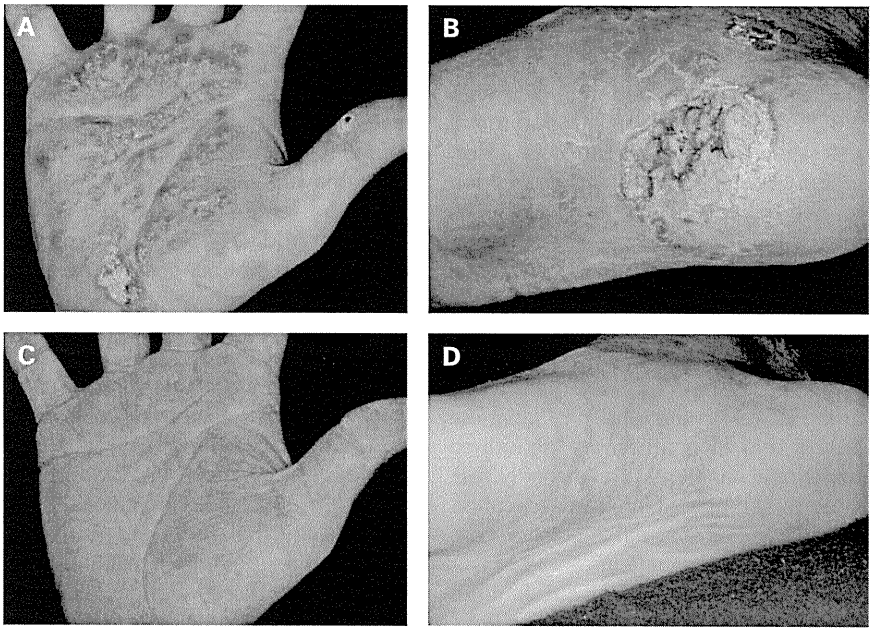

Figure 1 Skin changes on the right palm (A) and sole (B) on the initial visit. After penicillin antibiotic treatment, the skin lesions had completely disappeared (C, D).

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ABCA12 Is a Major Causative Gene for Non-Bullous Congenital Ichthyosiform Erythroderma

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TO THE EDITOR

Non-bullous congenital ichthyosiform erythroderma (NBCIE) and lamellar ichthyosis (LI) are both heterogeneous genetic disorders, and several causative molecules, including ABCA12 and transglutaminase-1 (TGase1), have been identified (Huber *et al.*, 1995; Russell *et al.*, 1995; Jobard *et al.*, 2002; Lefévre *et al.*, 2003; Akiyama, 2006; Lefèvre *et al.*, 2004, 2006). Patients with NBCIE exhibit fine, whitish scales on a background of erythematous skin over the whole body. Conversely, LI is clinically characterized by large, thick, and dark scales over the entire body without serious background erythroderma.

ABCA12 missense mutations leading to defects in the adenosine triphosphate-binding cassette were reported in LI cases (Lefévre *et al.*, 2003), and *ABCA12* truncation mutations were reported in patients with harlequin ichthyosis (Akiyama *et al.*, 2005; Kelsell *et al.*, 2005). Recently, we also identified *ABCA12* missense mutations in a small number of NBCIE cases (Natsuga *et al.*, 2007; Akiyama *et al.*, 2008).

Since the first identification of TGase1 gene (*TGM1*) mutations in LI (Huber *et al.*, 1995; Russell *et al.*, 1995), more than 50 *TGM1* mutations have been reported in LI. In addition, *TGM1* mutations were reported in a subset of patients with NBCIE (Laiho *et al.*, 1997; Akiyama *et al.*, 2001a; Becker *et al.*, 2003).

A number of reports concluded that there was no clear correlation between clinical differentiation of NBCIE and LI and the genetic heterogeneity underlying these autosomal recessive congenital ichthyoses (Hennies *et al.*, 1998; Richard, 2004). The clinical subgroups of NBCIE and LI were not always carefully and consistently recorded in publications, and a number of genetic studies were conducted including both NBCIE and LI patients as autosomal recessive congenital ichthyosis cases, without detailed clinical information for the patients. Thus, uncertainty has often surrounded reports of NBCIE and LI patients for whom there have been accounts of published mutations, as well as their references to previous publications.

To assess the frequency of *ABCA12* and *TGM1* mutations in Japanese NBCIE and LI families, we performed mutational analyses in eight NBCIE families and nine LI families as a cohort of Japanese autosomal recessive congenital ichthyosis patients.

The eight unrelated NBCIE families and nine independent LI families were seen at Hokkaido University Hospital or were seen by other clinicians within Japan. All the patients included in the study showed a typical phenotype of either NBCIE or LI. Only one patient, LI1, had an apparent history as a collodion baby at birth. Fully informed consent was obtained from the participants or their legal guardians. The study had been evaluated and approved by the Ethical Committee at Hokkaido University Graduate School of Medicine and was conducted according to the Declaration of Helsinki Principles.

TGM1 and *ABCA12* mutations have been considered major causative factors of autosomal recessive congenital ichthyosis in Japanese patients (Akiyama *et al.*, 2003, 2008), but several other causative molecules are also known (Akiyama and Shimizu, 2008). Therefore, the entire coding regions of *ABCA12* and *TGM1,* including the exon/intron boundaries, were sequenced using genomic DNA samples from patients and their family members. One hundred normal alleles from unrelated, healthy Japanese individuals were sequenced as normal controls.

Among eight independent NBCIE families (one patient in each family) (Table 1), *ABCA12* mutations were detected in five independent NBCIE families (one patient in each family), even though direct sequence analysis revealed no *TGM1* mutation in the NBCIE families. A total of six *ABCA12* mutations were identified, and four of them—p.Trp1235Ser in the extracellular domain between the fifth and the sixth transmembrane domains, p.Pro1798Leu in the extracellular domain close to the seventh transmembrane domain, p.Thr1980Lys in the

Abbreviations: LI, lamellar ichthyosis; NBCIE, non-bullous congenital ichthyosiform erythroderma; SNP, single-nucleotide polymorphism; TGase1, transglutaminase-1

Table 1. *ABCA12* or *TGM1* mutations in NBCIE and LI patients included in this study and our previous reports

Patient no.	Age	Sex	*ABCA12* mutation	*TGM1* mutation	References
NBCIE1	0	F	p.[Pro1798Leu]+[?]	(–)	This study
NBCIE2	1	F	p.[Thr1980Lys]+[?]	(–)	This study
NBCIE3	2	M	(–)	(–)	This study
NBCIE4	2	F	(–)	(–)	This study
NBCIE5	6	M	p.[Arg1950X]+[Trp1235Ser]	(–)	This study
NBCIE6	9	F	(–)	(–)	This study
NBCIE7	11	F	p.[Arg2482X]+[?]	(–)	This study
NBCIE8	52	M	p.[Arg1514His]+[=]	(–)	This study
NBCIE9	0	M	(–)	p.[Arg389His]+c.[2111delA]	Akiyama *et al.* (2001a)
NBCIE10	37	F	p.[Thr345Pro]+[=]	(–)	Natsuga *et al.* (2007)
NBCIE11	42	M	p.[Ile1494Thr]+[?]	(–)	Natsuga *et al.* (2007)
NBCIE12	2	M	p.[Gly1136Asp]+[Gln1669X]	(–)	Akiyama *et al.* (2008)
LI1	0	M	(–)	p.[Arg307Trp]+[=]	This study
LI2	0	F	(–)	p.[Arg307Trp]+c.[842_846delCACAGinsTAA]	This study
LI3	0	M	(–)	p.[Arg143Cys]+[Arg307Trp]	This study
LI4	0	F	(–)	p.[Arg286Gln]+[Gly291Ser]	This study
LI5	0	F	(–)	p.[Arg155Trp]+[?]	This study
LI6	6	F	(–)	(–)	This study
LI7	20	F	(–)	p.[Asn125Thr]+[=]	This study
LI8	21	F	(–)	p.[Arg155Trp]+[?]	This study
LI9	72	F	(–)	(–)	This study
LI10	56	F	(–)	p.[Leu205Gln]+[Arg307Trp]	Akiyama *et al.* (2001b)
LI11	33	F	(–)	c.[371delA]+[=]	Akiyama *et al.* (2003)

LI, lamellar ichthyosis; NBCIE, non-bullous congenital ichthyosiform erythroderma.
(–) No mutation detected. Previously unidentified mutations are underlined.

extracellular domain close to the eighth transmembrane domain, and p.Arg2482X in the second adenosine triphosphate-binding cassette—were previously unidentified mutations. All the *ABCA12* mutations in NBCIE were missense mutations, and *ABCA12* truncation mutations were not found in any of the NBCIE families.

We identified *TGM1* mutations in seven of the nine Japanese LI families in this study (Table 1). Seven mutations were identified: c.919C>T (p.Arg307Trp) in three families, p.Arg155Trp in two families, and p.Arg143Cys, p.Arg286Gln, p.Arg291Ser, c.845_849delinsTAA, and p.Asn125Thr in one family each. Three of these mutations—c.842_846delinsTTA and p.Gly291Ser in the catalytic core domain and p.Arg155trp in the β-sandwich domain—were previously unidentified.

In the present study, only one of the presumed two mutant alleles was detected in NBCIE and LI patients (in five of the 12 probands). NBCIE and LI are autosomal recessive diseases, and we assume that the other unidentified mutations in these patients are heterozygous exon-deletion mutations or mutations in the promoter regions or within introns, which cannot be detected by conventional sequencing of the coding regions. In fact, heterozygous exon-deletion mutations of *ABCA12,* which were not detected by direct sequencing, were reported in harlequin ichthyosis patients (Thomas *et al.*, 2006). To confirm this hypothesis, immunofluorescence studies for ABCA12 and glucosylceramide were performed as described earlier (Akiyama *et al.*, 2006) in skin samples from two NBCIE patients, NBCIE1 and NBCIE2, both with a single identified *ABCA12* mutated allele. The results demonstrated an intense ABCA12 staining within the granular layer cells of normal epidermis that was absent in the epidermis of both patients (Figure S1). Immunofluorescent staining showed that glucosylceramide was sparsely distributed in the upper layers of NBCIE patients' epidermis, compared with a more restricted, but intense, distribution in the granular layers of normal skin (Figure S1). If the ABCA12 transporter activity had been completely deficient in the epidermis, the patients would have shown a harlequin ichthyosis phenotype, but they had an NBCIE phenotype. The immunofluorescence stainings were not sufficiently quantitative, and, on the basis of the present results, we cannot exclude the possibility that ABCA12 activity remained in the patients' epidermis. An *in situ* TGase activity assay was performed using monodansylcadaverine as a substrate as previously described (Akiyama *et al.*, 2001b) in skin samples from two LI patients: LI1, harboring two identified *TGM1* mutations, and LI5, with only one identified *TGM1* mutation. Under pH 7.4-buffered conditions, the detection of TGase 1 activity (Raghunath *et al.*, 1998) demonstrated remarkably reduced membrane-associated labeling in both patients' epidermis compared with that in control epidermis (Figure S2). These results confirmed that defects in either ABCA12 or TGase1 underlie NBCIE1, NBCIE2, and LI5 diseases, in which *ABCA12* or

TGM1 mutations were identified in only one allele.

In addition to the NBCIE and LI cases reported here, we reviewed previously performed mutational research on four NBCIE cases (Akiyama *et al.*, 2001a, 2008; Natsuga *et al.*, 2007) and two LI cases (Akiyama *et al.*, 2001b, 2003) (Table 1). Therefore, the total number of Japanese NBCIE and LI families we examined were 12 NBCIE and 11 LI families. These diseases are extremely rare; their frequency in the Japanese population is 1 in 300,000–500,000 live births, and the number of live births in Japan per year is approximately 1,090,000 (1,089,745 in 2007). It is thus estimated that only two or three individuals with NBCIE or LI are born each year in Japan. In this context, the 23 families in our cohort can be expected to represent most of the newborn patients for a decade in Japan and therefore to be a significant sample of the Japanese NBCIE and LI populations. Interestingly, *ABCA12* mutations were frequently seen in Japanese NBCIE patients but were not detected in any of the 11 LI families. Thus, *ABCA12* appears to be a more important gene underlying NBCIE than was previously thought, at least in the Japanese population.

In our cohorts, *TGM1* mutations were detected in 9 of 11 LI families and in only 1 of 12 NBCIE families. In the literature to date, only seven NBCIE *TGM1* mutations have been reported in unrelated families (Laiho *et al.*, 1997; Akiyama *et al.*, 2001a; Becker *et al.*, 2003). From the present data, we suggest that the frequency of *TGM1* mutations as a primary cause of NBCIE is relatively low, at least in the Japanese population.

In the present study, the LI patient mutation c.919C>T (p.Arg307Trp) was found in three LI families out of seven families with *TGM1* mutations. In addition, c.919C>T (p.Arg307Trp) had previously been reported in three Japanese LI families (Yang *et al.*, 2001; Akiyama *et al.*, 2001b; Muramatsu *et al.*, 2004). Thus, we now know that c.919C>T (p.Arg307Trp) is the first common mutation to be found in the Japanese LI patient population. Among all the reported Japanese LI or NBCIE families with *TGM1* mutations, including families in the present study (a total of 14 families), the c.919C>T (p.Arg307Trp) mutation was found in six families. We screened more than 200 unrelated Japanese individuals as controls, but failed to find the p.Arg307Trp allele. Thus, although we could not determine its exact allele frequency, it is thought to be very low.

The TGM1 mutation p.Arg307Trp, which is common in the Japanese population, was identified in only one Korean LI patient (Yang *et al.*, 2001) in all the studied populations outside Japan; this might be a common LI mutation only in the Japanese population. To investigate whether a specific haplotype was associated with the p.Arg307Trp mutation, we analyzed five single-nucleotide polymorphisms (SNPs) within the *TGM1* gene in the four families harboring this mutation: three families from the present study and a previously reported family (Akiyama *et al.*, 2001b). In all four families, p.Arg307Trp mutant alleles had a C nucleotide at SNP rs2229463 (a T-to-C SNP; C-allele frequency = 46.7%), a T nucleotide at SNP rs2256989 (a C-to-T SNP; T-allele frequency = 44.4%), a G nucleotide at SNP rs8193033 (an A-to-G SNP; G-allele frequency = 57.8%), a G nucleotide at SNP rs8193032 (a T-to-G SNP; G-allele frequency = 35.6%), and a G nucleotide at SNP rs3814813 (a C-to-G SNP; G-allele frequency = 45.6%). We demonstrated that all the *TGM1* mutant alleles associated with p.Arg307Trp shared the same five SNP nucleotides, suggesting that the mutation had a single origin. Thus, the high frequency of the mutation is thought to be due to a founder effect.

Summarizing the present results, *ABCA12* mutations were frequently found in NBCIE families (in five of eight families), but not in LI families. In contrast, *TGM1* mutations were frequently detected in LI families (in seven of nine families), but were rare in NBCIE families. *TGM1* mutations in Japanese LI patients showed a founder effect. In conclusion, the present results suggest that *ABCA12* and *TGM1* are the major causative genes in NBCIE and LI, respectively, at least in the Japanese population, although this situation would clearly be different from that in Europe. Thus, when we speculate about candidate causative genes, it may be useful to make a distinct diagnosis of LI or NBCIE from the salient clinical features in autosomal recessive congenital ichthyosis patients in the Japanese population.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure S1. Double immunolabeling of ABCA12 and glucosylceramide in the epidermis.

Figure S2. Reduced *in situ* TGase 1 activity in the patients' epidermis.

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Pathogenic Anti-Desmoglein MAbs Show Variable ELISA Activity because of Preferential Binding of Mature versus Proprotein Isoforms of Desmoglein 3

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TO THE EDITOR

The desmosomal cadherins desmoglein (DSG) 3 and DSG1 are targets of autoantibodies in the potentially fatal blistering disease, pemphigus vulgaris (PV) (Stanley and Amagai, 2006). DSGs are synthesized as preproproteins, which are processed first in the endoplasmic reticulum to remove the signal sequence and subsequently by the Golgi proprotein convertases to remove the propeptide before transport to the cell surface. The cadherin propeptide is thought to modulate the conformation of the extracellular domains to prevent intracellular aggregation because of interaction with other cadherins within the secretory pathway. Propeptide cleavage occurs upstream of the conserved tryptophan residue at position 2, which is responsible for cadherin strand dimer formation, suggesting that propeptide removal may unmask residues important in intermolecular adhesion. The proprotein convertase furin processes recombinant DSGs in baculoviral overexpression systems (Posthaus *et al.*, 2003), which are widely used for pemphigus research and clinical diagnostic purposes. Commercial DSG ELISA kits use baculovirally produced recombinant DSG antigen (Ag) and have been shown to be a sensitive and specific diagnostic tool for pemphigus (Ishii *et al.*, 1997).

Abbreviations: Ag, antigen; DSG3, desmoglein 3; PV, pemphigus vulgaris

Collagen XVII Participates in Keratinocyte Adhesion to Collagen IV, and in p38MAPK-Dependent Migration and Cell Signaling

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Collagen XVII (COL17) participates in keratinocyte adhesion and possibly migration, as COL17 defects disrupt keratinocyte-basal lamina adhesion and underlie the disease non-Herlitz junctional epidermolysis bullosa. Using small interference RNA (siRNA) to knock down COL17 expression in HaCaT cells, we assessed cell characteristics, including adhesion, migration, and signaling. Control and siRNA-transfected keratinocytes showed no difference in adhesion on plastic dishes after incubation for 8 hours in serum-free keratinocyte-growth medium; however, when grown on collagen IV alone or BD matrigel (containing collagen IV and laminin isoforms), COL17-deficient cells showed significantly reduced adhesion compared with controls ($P<0.01$), and mitogen-activated protein kinase (MAPK)/ERK kinase (MEK)1/2 and MAPK showed reduced phosphorylation. Furthermore, COL17-deficient HaCaT cells plated on plastic exhibited reduced motility that was p38MAPK-dependent (after addition of the p38MAPK inhibitor SB203580). Together, these results suggest that COL17 has significantly wider signaling roles than were previously thought, including the involvement of COL17 in keratinocyte adhesion to collagen IV, in p38MAPK-dependent cell migration, and multiple cell signaling events pertaining to MEK1/2 phosphorylation.

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INTRODUCTION

Collagen XVII (formerly known as BPAG2 or BP180) (COL17) is a transmembrane protein that plays a critical role in linking the cytoskeleton and the extracellular environment (Shimizu *et al.*, 1989; Franzke *et al.*, 2005). It is also an autoantigen in bullous pemphigoid, a blistering skin disease (Jablonska *et al.*, 1958; Sams, 1970; Shimizu *et al.*, 1989). Mutations in the human COL17 gene, *COL17A1*, lead to COL17 protein deficiency, reduced keratinocyte-basement membrane adhesion, and reductions in the size of hemidesmosome (HD) plaques, involved in epidermal adhesion (McMillan *et al.*, 1998) (Zillikens and Giudice, 1999). These defects lead to non-Herlitz junctional epidermolysis bullosa, an autosomal recessive blistering disease with a variable clinical phenotype largely dependent on mutation severity (McGrath *et al.*, 1995, 1996; Bauer and Lanschuetzer, 2003).

Epidermal keratinocytes expressing defective COL17 show altered basement-membrane adhesion, increased skin separation (Nakamura *et al.*, 2006; Nishie *et al.*, 2007), and increased migration rates (Tasanen *et al.*, 2004). COL17-knockout mice (Nishie *et al.*, 2007) show a similar phenotype to that of nHJEB patients, including multiple erosions and hair defects and premature loss of hair (McGrath *et al.*, 1995, 1996; Bauer and Lanschuetzer, 2003).

Regulation of keratinocyte adhesion and migration likely involves COL17 collagenous ectodomain shedding because of cleavage close to the plasma membrane of keratinocytes and malignant epithelial cells (Franzke *et al.*, 2002, 2004; Labrousse *et al.*, 2002; Zimina *et al.*, 2005, 2007). The shed ectodomain is thought to regulate attachment by inducing cell detachment, profoundly affecting cell adhesion and subsequent signaling, thereby increasing motility, and disrupting differentiation, and it is already known to be involved in autoimmune disease development (Schumann *et al.*, 2000).

The process of cell migration over the extracellular matrix plays a critical role not only in maintaining epidermal homeostasis but also in promoting angiogenesis, and it is involved in inflammation, embryonic development (Martin and Parkhurst, 2004), wound repair (Friedl, 2004; Friedl *et al.*, 2004), and tumor metastasis (Braiman-Wiksman *et al.*,

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Abbreviations: COL17, collagen XVII; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; HD, hemidesmosome; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PBS, phosphate-buffered saline; TCP, tissue culture plastic

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2007; Raja *et al.*, 2007). Central to this process, several papers have reported that activation of the mitogen-activated protein kinase (MAPK) pathway leads to transcriptional control of genes important for cell proliferation and differentiation (Zhang *et al.*, 2004; Deng *et al.*, 2006; Choma *et al.*, 2007). However, both growth factor receptors and integrins can also induce multiple signaling events leading to MAPK activity and the rapid induction of cell migration, suggesting that MAPK can lead to direct activation of the intracellular motility machinery independent of *de novo* gene transcription. (Pearson *et al.*, 2001; Stoll *et al.*, 2003; Deng *et al.*, 2006; Fitsialos *et al.*, 2007).

In this study, we analyzed the precise mechanism(s) whereby COL17 modulates keratinocyte migration under various physiological and pathological situations to gain a better understanding of the general role of COL17 in the regulation of keratinocyte adhesion, signaling activation, and p38MAPK-dependent migration.

RESULTS

Establishment of COL17-knockdown clones

In an effort to determine whether expression of COL17 in HaCaT cells can affect cell characteristics such as cell motility and morphology, we used RNA interference approaches to knock down COL17 expression. First, HaCaT cells were transfected using lipofectamine with the plasmid-based vector pSilencer 3.0-hygro, specific to human COL17 or to green fluorescent protein (GFP), and clones were selected using 0.4 mg/ml hygromycin. To confirm the extent of COL17-expression knockdown in subcloned cell lines (si17-4 and -6; N. C., respectively *vs* controls), total RNA and protein were harvested and analyzed by RT-PCR and western blotting. COL17 gene expression studied by RT-PCR (Figure 1a) showed a marked reduction in the relative ratio of COL17 expression to the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene internal control. These data show an approximate fourfold reduction in COL17 message expression in comparison with the control cells. Western blotting data showed similar reductions in both mRNA and protein expression. The blotting results, shown in Figure 1b, showed a significantly reduced level of COL17 in cells that had been transfected with the two vectors expressing short hairpin RNA against COL17 (pSi-COL17). The levels were significantly lower than those in wild type or control short hairpin RNA (pSi-GFP) transfected-HaCaT cells, without any detectable change in β-actin expression. Densitometry scanning to quantify the western blots revealed the degree of protein expression to be about 70% of control COL17 protein levels, whereas the siGFP-treated cells failed to show any significant change in COL17 expression. All cell lines showed no changes in cell viability compared with wild-type HaCaT cells (data not shown).

COL17-knockdown HaCaT cells show reduced motility but no change in adhesion

To study the role of COL17 in the migration of cells plated onto uncoated plastic dishes, cells were incubated in serum-free keratinocyte-growth medium at 37 °C for 8 hours

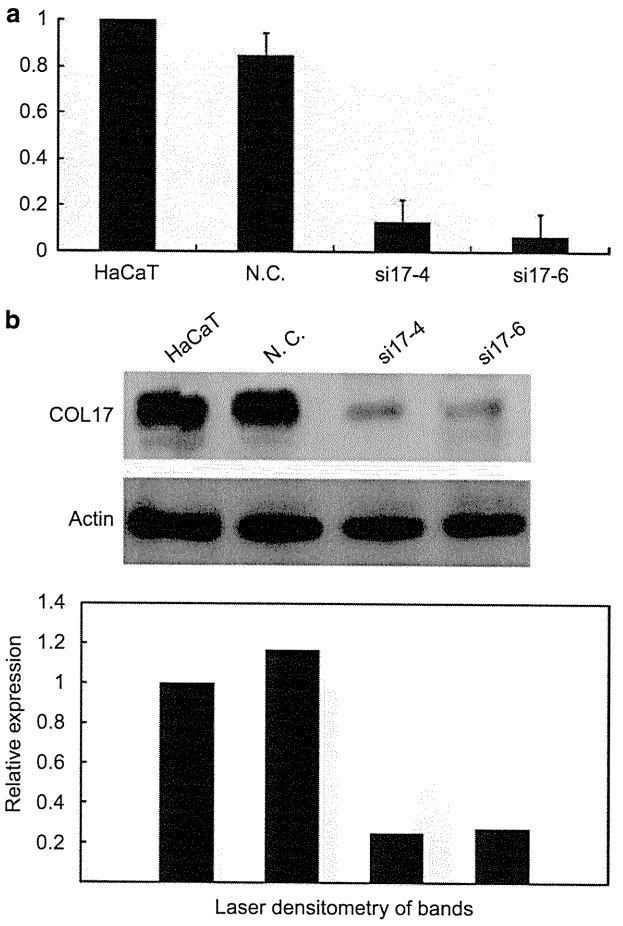

Figure 1. Expression of COL17 in HaCaT cell lines and COL17-knockdown clones by RT-PCR and immunoblot analysis. (**a**) RT-PCR studies of reference RT-RNA samples validated decreased expression of COL17 (by approximately fourfold) in si17-4 and si17-6 cell lines compared with normal and mock-transfected controls. HaCaT: parental cells; N. C.: GFP siRNA vector-transfected HaCaT cells; si17-4 and -6: COL17 siRNA-expressed clones. Three experiments were performed in duplicate, and values represent the mean + SE. (**b**) Confirmation of stable expression of COL17 in the HaCaT cell line. The expression of actin was monitored to ensure equivalent loading and protein transfer.

(calcium concentration at 0.2 or 1.44 mM). Subconfluent cells were seeded, and after 2 hours the distance (in μm) migrated by the cells was measured using ImageJ software (Figure 2a). Compared with COL17-knockdown clones, HaCaT cells and negative-control cell line cells migrated approximately 2- to 2.5-fold further during the ensuing 12-hour period (Figure 2b). The addition of keratinocyte growth factor to the medium increased the migration rates in control HaCaT cells and COL17-knockdown cells (Figure 2b). These findings suggest that untreated or control GFP-transfected HaCaT cells are more motile than COL17-knockdown cells when plated on uncoated tissue culture plastic (TCP) dishes. We then compared the adhesion of HaCaT cells to COL17-knockdown siRNA-treated HaCaT cells in a short-term adhesion assay on uncoated dishes. The adhesion of siRNA-treated HaCaT cells was equivalent to that of HaCaT cells on uncoated dishes

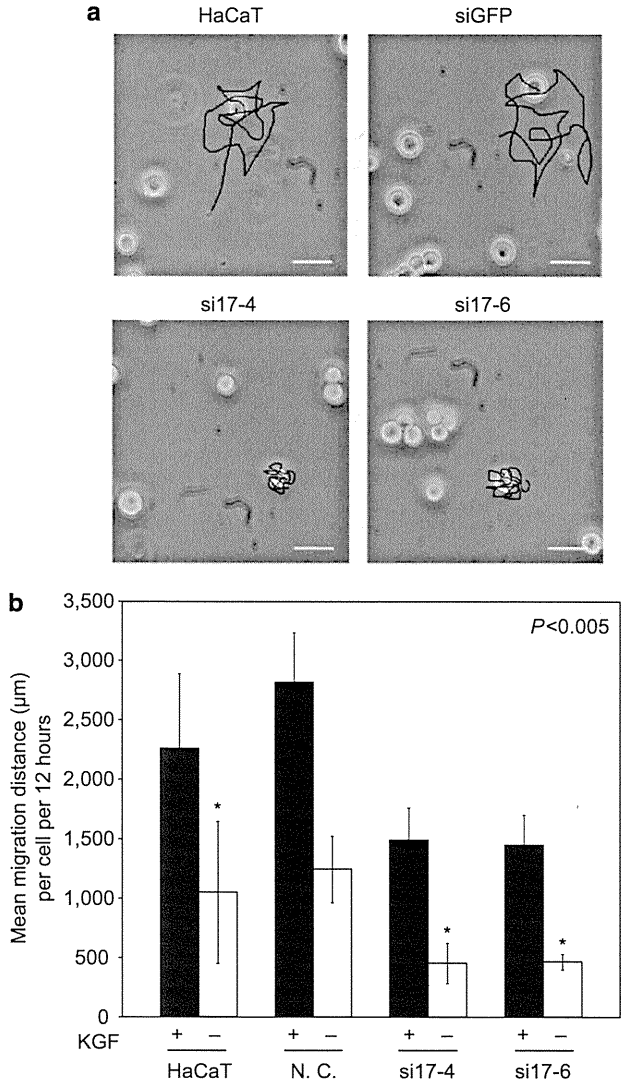

Figure 2. Cell migration of control and COL17 knockdown HaCaT-derived cell lines. (**a**) Representative cell tracks of control and siRNA-treated cells, scale bar: 100 μm. The distance (in μm) migrated by the cells was measured using ImageJ software. (**b**) Assessment of total cell migration distance over 12 hours showed that control and GFP-transfected HaCaT cell lines both showed high migration rates, but the two COL17-knockdown cell lines showed dramatically reduced rates of cell migration (by more than twofold) on uncoated tissue culture plastic. KGF was added into HaCaT cells and COL17-knockdown cells as positive control for assessing migration potential. Three experiments were performed in duplicate, and values represent the mean + SE.

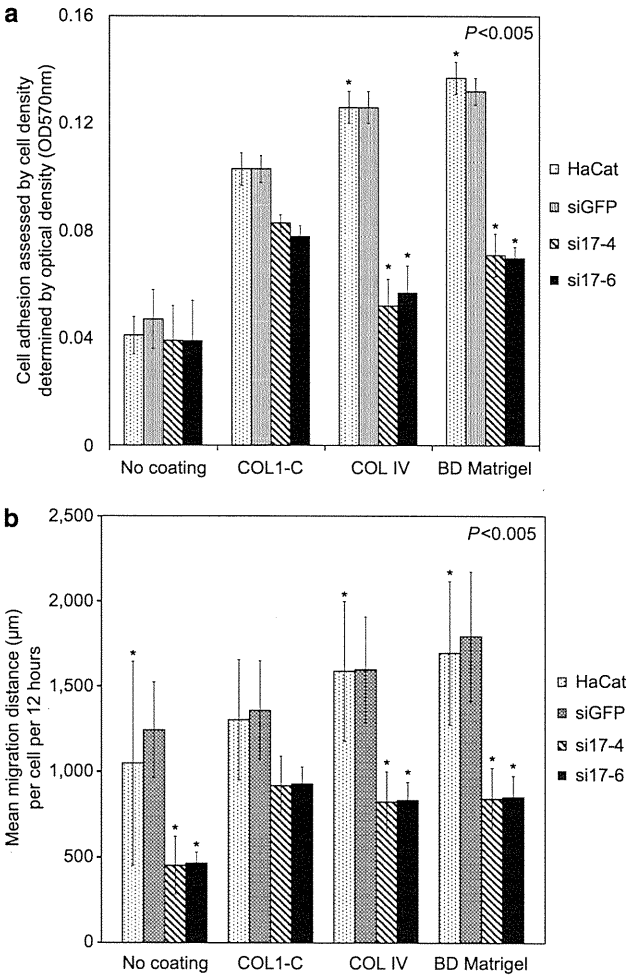

Figure 3. Cell adhesion and migration assays on collagen I, IV or BD-matrigel coated dishes. (**a**) The results showed no differences in adhesion in the control and COL17-deficient cells on uncoated tissue culture plastic, but significant reductions in the number of COL17 deficient cells attached ($P<0.005$) on collagen IV and BD-Matrigel substrate. Collagen I-coated dishes showed only minor and statistically insignificant changes in adhesive cell number between control and COL17-deficient cells. (**b**) Cell migration distance was also checked under the same conditions as adhesion. Similar results were obtained with cells on uncoated plastic dishes with COL17-knockdown cells showing reduced migration rates on all three substrates compared with controls. Migration of COL17-knockdown cells showed slightly larger reductions compared with controls when grown on collagen IV and BD-Matrigel substrate. Three experiments were performed in duplicate, and values represent the mean + SE.

(Figure 3a). Thus, COL17 is involved in regulating normal migration of HaCaT cells on uncoated TCP dishes, but is not involved in HaCaT cell attachment to uncoated TCP. To analyze the role of COL17 in adhesion, cells were plated onto dishes coated with different proteins, collagen types I, IV, and BD-Matrigel. The adhesion of COL17 knockdown HaCaT cells was significantly reduced ($P<0.005$) compared with that of control HaCaT cells on collagen IV and BD-Matrigel-coated dishes (BD-Matrigel comprises both collagen IV and multiple laminin isoform chains) (Figure 3a). Furthermore, the extent to which adhesion was reduced was roughly equivalent in both collagen and Matrigel-coated dishes. We surmise that COL17 is important in cell adhesion to collagen IV, and a similar effect can be seen with the collagen IV present in the BD-Matrigel-coated dishes. However, COL17-depleted cells showed only marginally weaker binding to collagen I and is therefore likely to be less important in cultured cell adhesion to collagen I. The migration of these cells on different substrates was also investigated. Similar to the results shown on uncoated TCP dishes, HaCaT cells and GFP-transfected

control cells migrated approximately twofold farther compared with COL17-knockdown clones over 12 hours when plated on collagen IV or Matrigel-coated dishes (Figure 3b). In addition, COL17-knockdown HaCaT cells plated on collagen I coated dishes showed marginal reductions in motility, although the difference was not statistically significant.

Activation of MAPK in HaCaT cells

Earlier reports have implicated the involvement of MAPK activity in cell motility (Stoll *et al.*, 2003; Choma *et al.*, 2007; Fitsialos *et al.*, 2007). We therefore used siRNA-transfected HaCaT cells to investigate the role of MAPK in COL17-regulated cell motility. The activation of MAPK was measured by antiphosphotyrosine immunoblotting. Compared with the untreated HaCaT cells cultured in keratinocyte-growth medium, siRNA-treated COL17-knockdown HaCaT cells showed reduced MAPK/ERK kinase (MEK) 1/2 activity (Figure 4). It is known that activated MEK1/2 can activate p38MAPK (Slack-Davis *et al.*, 2003; Manohar *et al.*, 2004; Deng *et al.*, 2006), and this is thought to be important in the regulation of keratinocyte migration. We therefore examined whether siRNA treatment downregulates p38MAPK activity in HaCaT keratinocytes (Figure 4). siRNA-induced COL17 knockdown reduced p38MAPK activity in HaCaT cells. In contrast, the total amount of both MEK 1/2 and p38MAPK was not changed by siRNA-induced COL17 knockdown. These results indicate that COL17 knockdown reduced MAPK activity, possibly resulting in reduced HaCaT cell migration.

MAPK inhibitors inhibit COL17-regulated cell migration but not adhesion

To further analyze the role of p38MAPK activation in the control of keratinocyte migration, we next investigated whether inhibition of the p38MAPK pathways could prevent

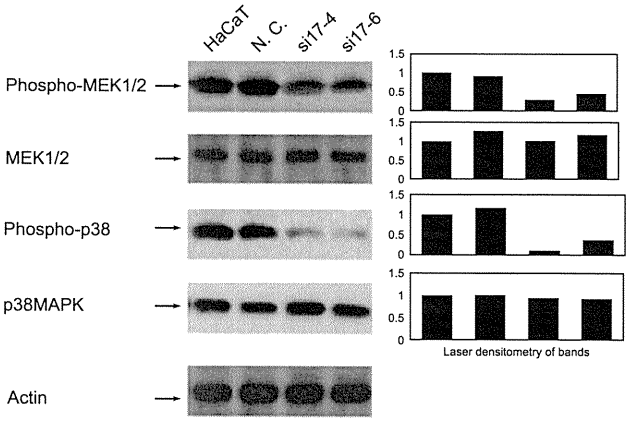

Figure 4. Laser densitometry analysis of protein immunoblots from control and COL17-deficient HaCaT-derived cell lines. Laser densitometry showed that both phospho-MEK1/2 and phospho-p38 MAPK showed reduced immunostaining in COL17-deficient cell lines. These results showed that MEK1/2 and MAP kinase activation is greater in untreated HaCaT cells than in siRNA COL17-knockdown HaCaT cells. We observed no change in MEK1/2 and p38MAPK, and there was normal staining of equal intensity for the unphosphorylated forms of MEK1/2 and p38MAPK. The expression of actin was used as an internal standard loading and protein transfer control.

the regulation of cell migration by COL17. Pretreatment of cells with the p38MAPK inhibitor SB 203580 blocked cell migration in a dose-dependent manner and also blocked the activation of p38MAP (Figure 5a). The 10 μM dose was selected as the lowest optimal dose of SB 203580 to be used in these inhibition of migration experiments. Pretreatment of cells with 10 μM SB 203580 inhibited cell migration by approximately 60% compared with untreated cells (Figure 5b). Under identical conditions, adhesion was again measured and the p38 inhibitor SB 203580 failed to show any effect on cell adhesion (Figure 5c). These data show that MAPK-inhibitors inhibit COL17-dependent cell migration but not adhesion, and therefore p38MAPK activation is important for migration but not adhesion in keratinocyte culture systems.

DISCUSSION

COL17 is a HD component that is involved in HD-attachment plaque stability and in providing basal-keratinocyte adhesion to the underlying epidermal basement membrane and extracellular matrix (McGrath *et al.*, 1995; McMillan *et al.*, 1998; Nishie *et al.*, 2007). Defects in keratinocyte COL17 expression have marked consequences on cell behavior, as earlier papers have reported that COL17 deficiency induces a migratory phenotype (Tasanen *et al.*, 2004; Zimina *et al.*, 2005). *In vivo*, HDs are associated with stable keratinocyte anchorage, and conversely, HD disassembly is a prerequisite for keratinocyte migration (De Luca *et al.*, 1994; Poumay *et al.*, 1994; Raja *et al.*, 2007). Thus, the integration of COL17 in the keratinocyte attachment complex represents an important step in the sequence of events regulating robust keratinocyte adhesion, limiting migration, and our results show that siRNA COL17 knockdown affects cell migration and adhesion on collagen IV and Matrigel substrates. Interestingly, siRNA COL17 knockdown of HaCaT cells on TCP showed a reduced migratory phenotype that contradicts other studies. Tasanen *et al.* reported that keratinocytes with COL17 null mutations showed increased cell migration compared with wild-type cells (Tasanen *et al.*, 2004). However, they studied human junctional epidermolysis bullosa patient keratinocytes with COL17 null mutations and used laminin 332 (laminin 5)-coated substrates that affect keratinocyte adhesion in a different manner, and thus explain the different findings. BD-Matrigel contains mouse laminin isoforms (including laminin 111, formerly laminin 1, and likely several other isoforms) and siRNA COL17-depleted HaCaT cells exhibited reduced adhesion, which may allow for the more motile environment shown in earlier reports. The involvement of COL17 in cell migration has been shown earlier(Tasanen *et al.*, 2004; Parikka *et al.*, 2006; Huilaja *et al.*, 2007). An optimum level of adhesion strength is generally thought to be required for cell migration, suggesting that markedly weak adhesion may also impair proper cell traction. Similarly, excessive adhesion can also inhibit motility, and therefore precise and correct control of cell dysadhesion is required for optimal migration rates. The role of COL17 in the stabilization of epithelial cells on various matrices *in vitro* provides an explanation for the lack of

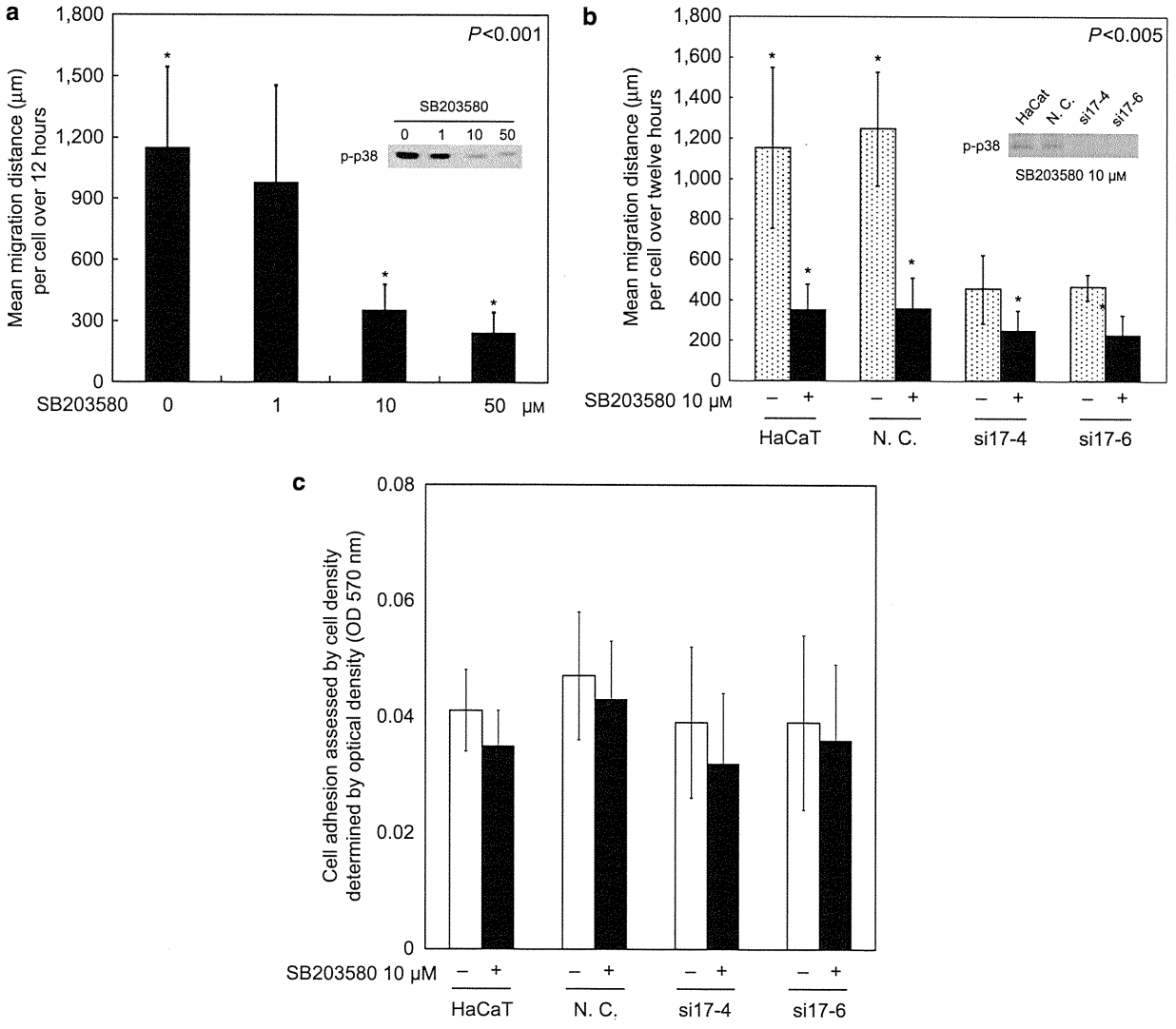

Figure 5. SB 203580 inhibits COL17-regulated cell migration but not adhesion. Inhibition of keratinocyte migration by the MAPK inhibitor SB 203580 (**a**) is dose-dependent, (**b**) but does not rely on normal COL17 levels of expression. Dose response of MAPK inhibitor SB 203580 shows a significant reduction in keratinocyte motility at levels above 10 μM (this value was then used in subsequent experiments to determine the role of MAPK in COL17-deficient migration suppression). Both COL17-deficient HaCaT cells and MAPK inhibition using SB 203580 suppressed keratinocyte migration to a similar extent, but (**b**) without any synergistic effect, and (**c**) this inhibitor did not affect adhesion in any cell line. Results of western blotting, which show phosphorylation status of p38 in each condition, three experiments were performed in duplicate, and values represent the mean + SE.

keratinocyte adhesion to the dermal-epidermal junction in COL17-deficient nHJEB patients. Indeed, patients lacking COL17 do exhibit a relatively mild blistering phenotype because of their lack of robust adhesion to the basal lamina extracellular matrix components that likely include the collagens (particularly collagen IV), $\alpha 6\beta 4$ integrin, or laminin 332. COL17 is expressed in the upper part of the outer root sheath hair follicle keratinocytes (Messenger *et al.*, 1991; Joubeh *et al.*, 2003) and in ameloblast cells involved in tooth formation, consistent with nHJEB patients exhibiting associated hair and tooth defects. Cell adhesion and migration are generally thought to be critical for the maintenance of the keratinocyte hair follicle bulge population and interfollicular regions, and thus loss of COL17 and subsequent effects on cell adhesion, migration, and signaling during the hair cycle and differentiation in these regions explain the hair loss and skin thinning observed in nHJEB COL17-deficient patients.

Cell migration requires a complex coordinated interaction of proteins and signaling events that control important cell motility events. Our results show that COL17 siRNA knockdown affects cell migration through the p38MAPK-signaling pathway. The MAPK pathway influences many cellular processes, including cell division, gene transcription, and stress responses. Many papers have reported the role of MAPK in cell migration, acting on cytoskeletal components (Osmanagic-Myers *et al.*, 2006; Pullikuth and Catling, 2007). MAPK signaling events have been shown to be triggered by a number of different growth factors, cytokines, and integrins,

which influence specific cell migration events. Cellular transformation by H-Ras or c-Src is also associated with increased MAPK activity and enhanced cell proliferation, and migration in neoplasia. The MAPK pathways are involved not only in cell migration but also in aspects of cell adhesion (integrin attachment initiates downstream signaling). In this report, we provide evidence that p38MAPK signaling can regulate cell migration by directly affecting the migratory machinery. Blocking p38MAPK activity with a selective inhibitor resulted in the loss of cell migration, with no obvious effect on cell adhesion. Our results suggest that COL17 may be involved in p38MAPK-signaling pathways during cell migration, but that it is not a prerequisite for *in vitro* adhesion, suggesting mutually exclusive p38MAPK-signaling pathways involving COL17 adhesion and migration. The precise relationship between COL17 and p38MAPK in cell migration is not yet clear: whether it is a direct interaction or acts through other secondary factors. The MAPK pathway cross talks with many different signaling pathways to regulate cellular activities and likewise other signaling molecules can influence upstream and downstream targets of the p38MAPK pathway, which allows fine control of specific cellular activities. With ongoing investigation, the interactions of p38MAPK and COL17 with other signaling pathways can be clarified.

A further important finding was that cells with normal levels of COL17 showed higher rates of adhesion to collagen IV or BD-Matrigel (comprising mouse laminin isoforms and collagen IV) than to collagen I or plastic in comparison. From our data, we hypothesize that COL17 may play a specific role in collagen IV adhesion, albeit with weaker association in comparison to laminin 332-integrin $\alpha 6\beta 4$ adhesive interaction. Such interactions fit with the clinically milder phenotype observed in non-Herlitz junctional epidermolysis bullosa human patients and mouse models.

Taken together, our data suggest that keratinocyte adhesion and migration are differentially regulated. This is the case as many different adherent cell types do not migrate without prior specific cytokine or growth factor stimulation. Earlier studies have shown that MAPK activity is critical for transcriptional gene events leading to cell proliferation and differentiation, which may explain how COL17 and MAPK activation can independently influence cell movement on different extracellular matrices during tissue remodeling, as well as tumor cell invasion. Our findings suggest that COL17 is important in keratinocyte adhesion and in relaying signals from the extracellular matrix to the internal signaling apparatus during cell migration.

MATERIALS AND METHODS

Cell culture and establishment of stable cell lines

The HaCaT cell line (Boukamp *et al.*, 1990), a spontaneously transformed but non-malignant human keratinocyte cell line, was used in this study. The cells were cultured at 37 °C in a 5% CO_2 humidified atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (Gibco BRL, Gaithersburg, MD). HaCaT cells were passaged overnight at a concentration of 5×10^4/ml to 1×10^5/ml, in 6-well plates under standard conditions. Fifteen-minutes before transfection, the medium was changed to OPTI-MEM (Gibco Invitrogen, Carlsbad, CA) quiescent medium (without fetal calf serum). Transfections were carried out using Lipofectamine transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Four hours after transfection, medium containing 10% fetal calf serum was added. Two stable monoclonal lines from the single cell of siRNA expression vector transfectants (si17-4, si17-6) were established after medium treatment with 0.3 mg/ml hygromycin (Wako, Osaka, Japan) for 7 days, using limiting dilution methods. The relative reduction in COL17 expression by siRNA was analyzed by immunoblotting cell culture extracts and RT-PCR.

RNA interference (I)

We designed small interference siRNA nucleotides to knock down COL17 expression as described in the manufacturer's technical information (Ambion, Austin, TX). A set of 19-mer oligonucleotides (AAGTATTGCTGTCAAGCCGTG), corresponding to 4,642 nucleotides downstream of the transcription start site, was selected. We confirmed that the selected oligonucleotide sets failed to show homology to any other genes by BLAST searching and that they would not therefore interfere with other genes. The oligonucleotides were synthesized and column purified. The 19-mer sense siRNA sequence and antisense siRNA sequences were linked with a nine nucleotide spacer (TTCAAGAGA) loop. Six T bases and 6 A bases were added as a termination signal to the 3′ end of the forward oligonucleotides and the 5′ end of the reverse oligonucleotides, respectively. Five nucleotides corresponding to the Bam HI (GATCC) and Hind III (AGCTT) restriction sites were then added to the 5′ end of the forward oligonucleotides and the 3′ end of the reverse oligonucleotides, respectively. Forward and reverse oligonucleotides were incubated in annealing buffer (100 mM K-acetate, 30 mM HEPESKOH (pH 7.4), and 2 mM Mg-acetate) for 3 minutes at 90 °C, followed by incubation for 1 hour at 37 °C. The annealed DNA fragment was ligated with the linearized pSilencer 3.1-H1 hygro siRNA plasmid expression vector (Ambion) at the Bam HI and Hind III sites, and a COL17 siRNA vector (pSi-COL17) was thus constructed. A negative-control siRNA vector (pSi-GFP) that targeted using an unrelated (non-specific) GFP cDNA sequence 5′-GGTTATGTACAGGAACGCA-3′ that had no matches to any human gene was also prepared under similar conditions.

RNA extraction and quantification using RT-PCR

Total RNA was extracted from HaCaT cells using TRIzol (Invitrogen, Burlington, ON, Canada). RNA was dissolved in 30 DEPC-H_2O and immediately stored at −70 °C. The concentration and purity of RNA were evaluated by measuring the absorbance at 260 nm, and by calculating the ratio of absorbance at 260–280 nm using a UV spectrophotometer (Ultrospec-3000 spectrophotometer, Pharmacia Biotech, UK). RT-PCR analysis for COL17 was performed on RNA extracts using ABI prism 7500 (Applied Biosystems, Foster City, CA) and the 5′exonuclease assay (TaqMan technology). The cDNA was used for RT-PCR performed in 96-well optical reaction plates with cDNA equivalent to 30 ng RNA in a reaction of 25 μl containing 1X Taqman Universal Master Mix, 900 nM of specific forward and reverse primer for COL17. Controls included RNA subjected to RT-PCR without reverse transcriptase and PCR with water replacing cDNA template. The data were normalized using glyceraldehyde-3-

phosphate dehydrogenase mRNA expression levels as an internal standard, and converted into fold change on the basis of a doubling of PCR product in each PCR cycle, according to the manufacturer's guidelines described earlier.

Analysis of migration assay

The effects of COL17 on cell migration were studied using 3 cm plastic coated dishes. HaCaT cells were incubated in serum-free keratinocyte growth medium (Cambrex, Walkersville, MD) at 37 °C for 8 hours. HaCaT cells in logarithmic-growth phase were detached using trypsin–EDTA. In all, 3,000 cells were seeded in 3 cm TCP dishes and further cultured at 37 °C in a 5% CO_2 humidified atmosphere in serum-free DMEM. Migrating cells were photographed every 5 minutes using time-lapse video (Olympus DP70, Tokyo, Japan) from 2–14 hours after plating. The distance migrated by 40 cells over 12 hours was later measured using ImageJ software (McMillan *et al.*, 2007). To analyze the migration on dishes coated with different proteins, 50 µl cell matrix type I (2.4 mg/ml), type IV collagens (2.4 mg/ml) (Nitta Gelatin, Osaka, Japan), and 50 µl BD Matrigel (10.0–12.0 mg/ml) (BD Bioscience, San Jose, CA) were coated on petri dishes according to the manufacturer's protocol. After drying, multiwell tissue culture plates were washed in serum-free DMEM and then used immediately for cell migration assays. For MAPK inhibition experiments, a p38MAPK specific inhibitor (SB 203580) (Hornby, ON, Canada) was purchased from Calbiochem and used at a final concentration of 10 µM after dose optimization, and was added to serum-free medium. At the same time, 0.5 nM keratinocyte growth factor (NIBSC, Hertfordshire, UK) was used as positive control for migration assays (Ceccarelli *et al.*, 2007).

Cell adhesion assays

To analyze adhesion, 96-well plates were used. Wells were rinsed with phosphate-buffered saline (PBS) and blocked with 0.1% BSA in PBS for 30 minutes before use. HaCaT cells in serum-free DMEM containing 0.1% BSA were seeded at a concentration of 5×10^4 cells/well. After 1 hour at 37 °C, cells were rinsed twice with PBS, fixed for 10 minutes at room temperature in 70% ethanol, rinsed again with PBS and stained in 0.1% crystal violet (Tokyo Chemical Industry, Tokyo, Japan), and kept in water for 30 minutes at room temperature. After staining, cells were rinsed 3 times with water, air dried, and solubilized in 1% SDS in PBS, and the OD color read with an ELISA-plate reader (Mithras LB 940, Berthold Inc., Tokyo, Japan) at 570 nm. A blank value corresponding to BSA-coated wells was automatically subtracted. To analyze adhesion on dishes coated with different proteins, cell matrix type I and type IV collagens, BD-Matrigel was coated to the dishes using the same method as described above. After drying, multiwell tissue culture plates were washed in serum-free DMEM, then immediately used for cell adhesion assays as described above.

Activation of MEK1/2 and p38MAPK

Cells were incubated in serum-free keratinocyte-growth medium at 37 °C for 8 hours. Cells were solubilized in SDS-sample buffer (40 mM Tris-HCl, pH 7.4, 5% 2ME, 2% SDS, 0.05% bromphenol blue), and the cell extracts were subjected to western immunoblotting analyses using either anti-phospho-MEK1/2 antibody (166F8) or anti-phospho-p38MAPK antibody (12F8), which selectively recognizes the activated forms of MEK1/2 (phosphorylated Ser 221) or p38MAPKs (phosphorylated Thr180/Tyr182), respectively. To detect MEK1/2 or p38MAPKs, anti-MEK1/2 antibody or anti-p38MAPK antibody was used. All of these antibodies were purchased from Cell Signaling (Danvers, MA). Actin was used as the loading control to account for equal protein loading for each blot lane. For these experiments, equal amounts of cell extract (> 50 mg of total proteins) were resolved on an SDS polyacrylamide gel, transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Tokyo, Japan), and immunoblotted with corresponding antibodies. The results were visualized by a horseradish-peroxidase-conjugated secondary antibody.

Immunoblotting analysis

Total cell cultures were extracted using lysis buffer as described earlier. Cell lysates were analyzed by SDS-polyacrylamide gel electrophoresis and blotted as described earlier, using goat anti-COL17 (*N*-18) polyclonal antibody (Santa Cruz, CA), anti-MEK1/2 monoclonal antibody, and anti-phospho-MEK1/2, anti-p38MAPK and phospho-p38MAPK (Cell Signaling, Danvers, MA), and anti-β-actin monoclonal antibody (Chemicon, Temecula, CA). The bound primary antibodies on membranes were incubated with peroxidase-conjugated anti-mouse IgG + M (Jackson ImmunoResearch Lab., West Grove, PA) or anti-goat IgG (R&D Systems, Inc., Minneapolis, MN) and detected by enhanced chemiluminesence western blotting detection reagents (Amersham Biosciences, Amersham, UK). Band images were detected by an LAS 1000 mini system (Fuji Film, Kanagawa, Japan).

Statistical analysis

The data shown represent mean values of at least three different experiments, expressed as mean ± SE. Student's *t*-test was used to compare the data, and a *P*-value of <0.05 was considered to be statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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Gene Corner

A novel *PTPN11* missense mutation in a patient with LEOPARD syndrome

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LEOPARD syndrome (LS) is an autosomal dominant, multiple congenital anomaly syndrome so named because it is characterized by multiple lentigines, electrocardiographic conduction abnormalities, ocular hypertelorism, pulmonic stenosis, abnormal genitalia, retardation of growth and sensorineural deafness.[1] We report a Japanese boy with LS harbouring the novel PTPN11 (protein-tyrosine-phosphatase nonreceptor type 11) gene mutation c.1381G>T (p.Ala461Ser).

An 8-year-old boy had lentigines at birth which gradually increased in number with age. Physical examination revealed multiple light or dark brown lentigines of various sizes scattered over the whole body, including the face (Fig. 1a–c). He also had pigeon breast (Fig. 1d) and cryptorchidism. There was no sensorineural deafness, ocular hypertelorism, growth retardation or cardiology abnormalities including electrocardiographic conduction defect (Fig. 1e–g). A diagnosis of LS was made based on multiple lentigines, skeletal anomalies and genitourinary abnormalities.

The patient's parents gave their written informed consent as the patient's legal guardians. Direct sequencing of the entire coding regions, exon 2 to exon 15, of PTPN11 (GenBank accession number NT123456) revealed a single nucleotide substitution at codon 461 in exon 12 on one allele of PTPN11 (TGC to TTC; alanine to serine; p.Ala461Ser) (Fig. 2a). The

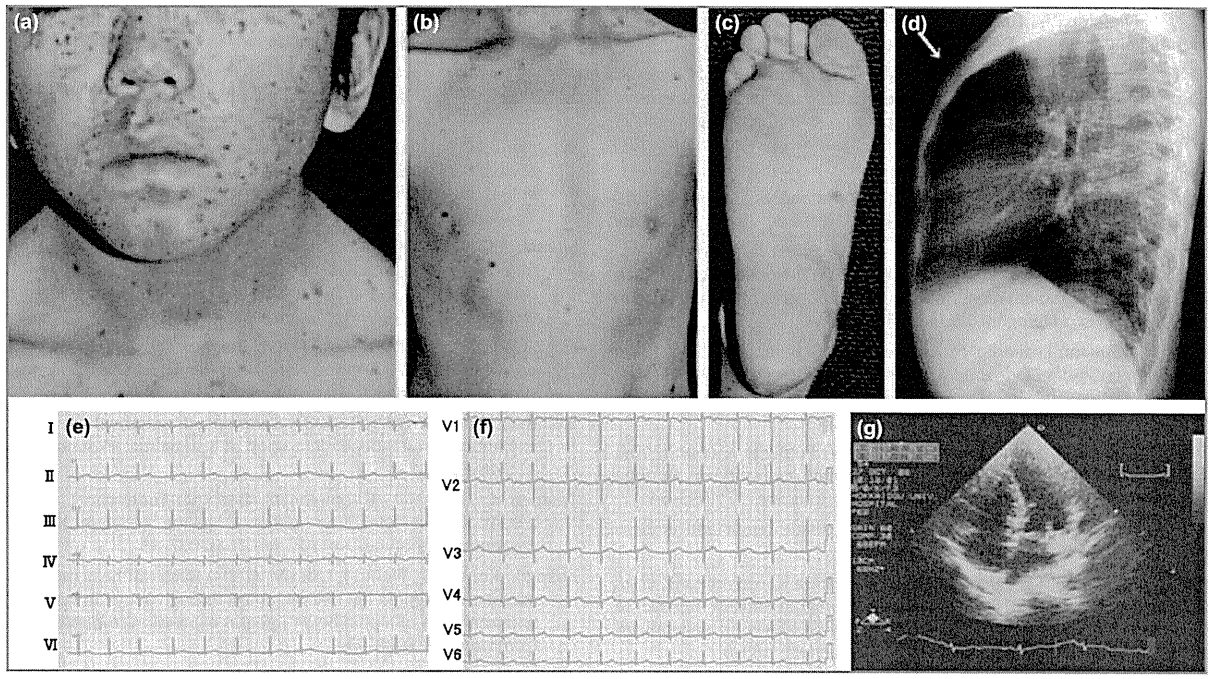

Fig 1. Clinical features of the patient. (a) Multiple light or dark brown lentigines of varied sizes scattered on the face. (b) Pigeon breast and various-sized lentigines are seen on the chest. (c) Lentigines are also observed on the sole. (d) Lateral chest radiograph demonstrates forward protrusion of the breastbone (arrow). (e, f) Electrocardiogram showing a normal sinus rhythm without any conduction defect: PQ duration 0·12 s (normal atrial ventricular conduction delay), QRS axis 90, QRS duration 0·08 s (normal QRS axis, no ventricular conduction disorders), no left ventricular hypertrophy or right ventricular hypertrophy pattern on precordial lead. (g) An apical four chamber view of echocardiogram revealing a normal heart structure and a balanced atrial and ventricular size: normal left ventricular size and wall thickness (end-diastolic left ventricular dimension 37·3 mm, 97% of normal, end-diastolic left ventricular posterior wall thickness 5·8 mm, 99% of normal), normal left ventricular pump function (left ventricular fractional shortening, 0·39), no right ventricular outflow tract stenosis or pulmonary stenosis.

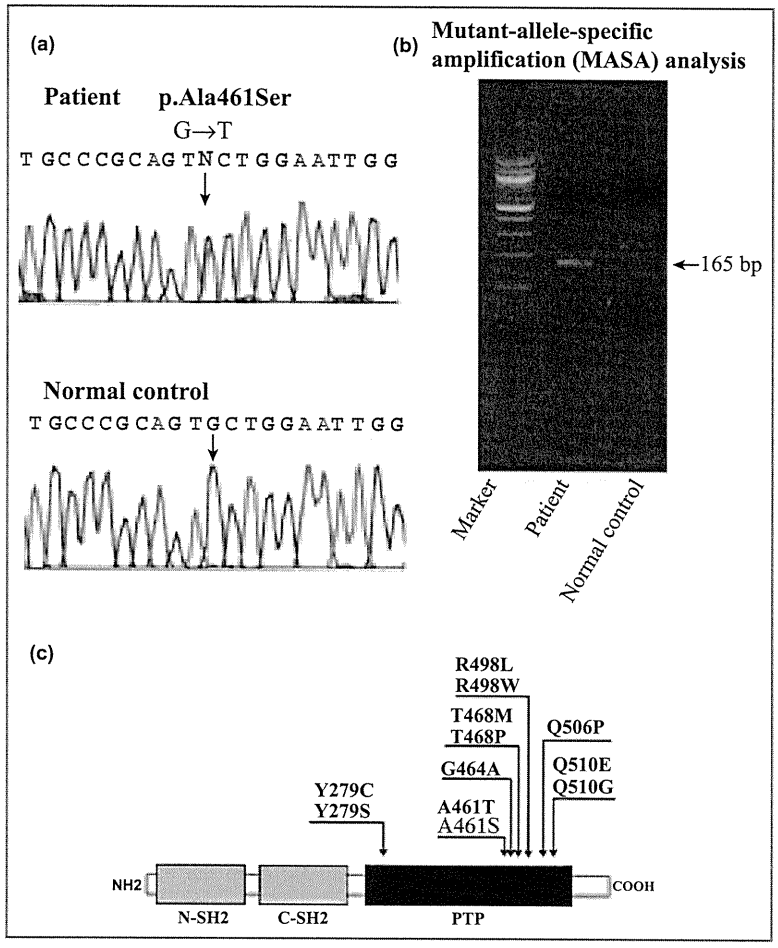

Fig 2. PTPN11 mutation analysis. (a) Direct sequencing of polymerase chain reaction (PCR) products from the patient and a normal control. Heterozygous c.1381G>T (p.Ala461Ser) mutation is found in exon 12 of PTPN11 of the patient, but not of a normal individual. (b) Mutant allele-specific amplification analysis. The amplification band from the mutant alleles is detected as a 165-bp fragment only in the PCR product from the genomic DNA sample of the patient, and not in the PCR product from the control DNA samples, confirming the presence of the mutation p.Ala461Ser in the patient. (c) Summary of PTPN11 mutations reported in LEOPARD syndrome. Note the mutations cluster at the PTP enzyme active site. The mutation found in the present study is p.Ala461Ser (red characters).

oligonucleotide primers and polymerase chain reaction (PCR) conditions were as previously described.[2] No other mutation was found in any part of the coding region or at any of the intron/exon borders of PTPN11. Mutant allele-specific amplification analysis (MASA) was performed with mutant allele-specific primers carrying the substitution of two bases at the 3′-end (mutant allele-specific primers: forward, TTGTCCTTC-TGCCCGCAGCT; reverse, CCCAGACTGTTTTCGTGAGCAC) and a 165-bp fragment derived from the mutant allele was amplified from the patient's genomic DNA (Fig. 2b). MASA showed no PCR product band from the control DNA samples (Fig. 2b). The mutation p.Ala461Ser was not found by sequence analysis in 200 alleles from 100 healthy unrelated Japanese individuals, so it was unlikely to be a polymorphism (data not shown).

The long-term prognosis of patients with LS is usually favourable. General growth, cardiology and hearing assessments were planned annually until adulthood for our patient.

PTPN11 is the major causative gene of LS, and patients with LS with PTPN11 mutations show various LS phenotypes.[3] PTPN11 encodes an SRC homology 2 (SH2) domain-containing protein-tyrosine-phosphatase (SHP-2) protein characterized by two SH2 domains and one protein-tyrosine-phosphatase (PTP) domain. The two SH2 domains interact with the PTP domain, keeping it folded and inactive. Upon binding of an appropriate SH2-binding protein (several growth factor receptors and docking proteins, e.g. GAB family members), this closed structure is opened, allowing a substrate to access the PTP enzyme active site. SHP-2 functions as a cytoplasmic signal transducer downstream of multiple receptors for growth factors, cytokines and hormones.

Mutations in the PTPN11 gene are associated with LS and Noonan syndrome (NS).[3,4] Most NS mutations disrupt key connections between the N-SH2 and PTP domains, resulting in biochemically and biologically 'active mutants' of SHP-2. In contrast, all 11 known LS mutation sites are confined to the seven residues predicted to affect catalytic activity of the PTP domain, in exons 7, 12 and 13 (Fig. 2c),[5] and LS mutations are thought to result in open, catalytically impaired forms of SHP-2.[6,7]

Among the PTPN11 mutations underlying LS, pTyr279Cys in exon 7 and p.Thr468Met in exon 12 are the prevalent mutations. It was reported that 65% of patients with LS harbour pTyr279Cys or p.Thr468Met.[5] As for genotype/phenotype correlations in patients with PTPN11 mutations, an association between exon 7 and exon 12 mutations and hypertrophic

cardiomyopathy, and an association between exon 8 mutations and pulmonary valve stenosis have been established.[5] The present patient with LS harboured p.Ala461Ser in exon 12 and showed multiple lentigines, pigeon breast and cryptorchidism, but he did not have electrocardiographic conduction defect, sensorineural deafness, ocular hypertelorism or growth retardation. Alanine-461 is in a highly conserved catalytic PTP-loop region (454–467) that contacts tyrosine-279.[8] Alanine is a hydrophobic nonpolar amino acid. In contrast, serine is a hydrophilic, polar and neutral amino acid. p.Ala461Ser might contort the catalytic site and interfere with substrate phosphotyrosine binding. It is noteworthy that another mutation at the identical codon of SHP-2, p.Ala461Thr, was reported in two other patients with LS. The two patients showed more severe clinical features than the present case, and the bulkier threonyl substitution at alanine-461 is expected to contort the catalytic site and interfere with substrate phosphotyrosine binding.[8] One of these patients with LS had multiple lentigines, hypertelorism, short stature, cardiovascular lesions, sensorineural deafness and abnormal genitalia.[9] Interestingly, the other had Noonan-like/multiple giant cell lesion syndrome with a complex phenotype that progressed over the years from NS to LS and presented cyst-like lesions of the bones.[10] The present case further confirms that mutations in an identical residue can lead to different phenotypes in LS.

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