Table 2 *Primers and restriction endonucleases for mutation analysis*

Mutation	Primer	Sequence	Restriction endonuclease
425A→G (exon 3)	Sense	5′-GGCCAGAAGAGATCCTGAGT-3′	*Sty*I
	Antisense	5′-CTGACCTGTCACTCCTGCTC-3′	
R578X (exon 13)	Sense	5′-CCTTCTCACTCTGCGTCCCT-3′	*Xho*I
	Antisense	5′-AACCAGGACGAGAGTGAGGC-3′	
5818delC (exon 70)	Sense	5′-TGAGTGCQGATGTTGGGTAG-3′	*Msp*I
	Antisense	5′-AGCAAGAGGTCAGAGGAGCG-3′	
G2043R (exon 73)	Sense	5′-CCCGTGGAGTGGGGTGTAGC-3′	*Sma*I
	Antisense	5′-TGCAGGAAACAAGAAAATGG-3′	
6573+1G→C (exon81/intron81)	Sense	5′-CAAGTGAGGCCCAGATTGAG-3′	*Msp*I
	Antisense	5′-GGCATGGACACAGCTTGAA-3′	
7786delG (exon 104)	Sense	5′-CGGGCTCGTTGTATTCTAAG-3′	*Bst*EII
	Antisense	5′-CAAAAQCTACCACACTGGT-3′	
R2814X (exon 114)	Sense	5′-CCCTCTGCCTGTGTGTCTCT-3′	*Pml*I
	Antisense	5′-CTGCATTCATGGACACCCAT-3′	
E2857X (exon 116)	Sense	5′-ACAGTGGAAATCAGTGCTGC-3′	*Mae*I
	Antisense	5′-AGGGTTTGTGGGAATCAGAG-3′	

Mutation analysis

Mutation analysis was conducted as follows. Genomic DNA obtained from blood samples was used as a template for polymerase chain reaction (PCR) amplification of individual exons and their flanking regions in the COL7A1 gene, as described elsewhere [5]. The primer sets to amplify exon 3, exon 13, exon 70, exon 73, exon 81/intron 81, exon 104, exon 114, and exon 116 are shown in Table 2. To examine the presence or absence of eight recurrent mutations, the PCR products were subjected to digestion with restriction endonuclease *Sty*I [10], *Xho*I [14], *Sma*I [15], *Bst*EII, *Pml*I [16], *Msp*I [18], and *Mae*I [18] (Table 2). The genomic DNAs from British patients with the respective recurrent mutation were also used as positive controls. All digested products were analyzed by 2% agarose gel electrophoresis.

Results

Analysis of the British recurrent mutations R578X, 7786delG and R2814X

The PCR products of exons 13, 104 and 114 were digested by the restriction endonucleases *Xho*I, *Bst*EII, and *Pml*I, respectively. In all 42 patients with DEB, the results of the restriction digestion showed the normal pattern (Fig. 1). The mutation R578X in exon 13 abolished an *Xho*I site. An individual heterozygous for the mutation as a positive control demonstrated the presence of 299-, 187-, and 112-bp bands. In contrast, the undigested 299-bp PCR product was not detected in 42 patients with DEB (Fig. 1a). The mutation 7786delG in exon 104 resulted in loss of a cutting site for the restriction endonuclease *Bst*EII. While an individual heterozygous for the mutation as a positive control demonstrated the presence of 512-, 420-, and 93-bp bands, the PCR products from 42 patients with DEB showed complete digestion of the 513-bp product (Fig. 1b). The mutation R2814X in exon 114 led to loss of a cutting site for the restriction endonuclease *Pml*I. An individual heterozygous for the mutation as a positive control demonstrated the presence of 418-, 298-, and 120-bp bands. In contrast, the PCR products from 42 patients with DEB showed complete digestion of the 418-bp PCR product (Fig. 1c). None of the mutations R578X, 7786delG or R2814X was detected in any of the 42 patients with DEB (Table 1).

Analysis of the worldwide recurrent mutations 425A→G and G2043R

The mutation 425A→G in exon 3 resulted in loss of a cutting site for the restriction endonuclease *Sty*I. An individual heterozygous for the mutation as a positive control demonstrated the presence of 422-, 213-, and 209-bp bands. Although the mutation 425A→G was identified in a French patient with RDEB (Table 1, no. 36), the 422-bp PCR product was not detected in the other 41 Asian patients with DEB, indicating the absence of the mutation 425A→G (Fig. 2a). The mutation G2043R in exon 73 resulted in loss of an *Sma*I restriction site which was used to assess the presence or absence of this mutation in the patients with DEB. The 469-bp PCR product with the G2043R mutation was digested into 309- and 160-bp fragments, whereas the normal PCR product was further digested into 264-, 160-, and 45-bp fragments. In the DNA of the positive control and two patients with DDEB, a 9-year-old Japanese girl (Table 1, no. 11) and a 1-year-old Chinese girl (Table 1, no. 12), an undigested band of 309-bp was present, indicating the presence of the mutation G2043R (Fig. 2b). In the other 40 patients with DEB, the 469-bp PCR product was digested into 264-, 160- and 45-bp fragments, indicating the absence of the mutation G2043R.

Analysis of the Japanese recurrent mutations 5818delC, 6573+1G→C and E2857X

The 5818delC mutation destroys a restriction mutation site for *Msp*I, whereas the mutation 6573+1G→C creates a new

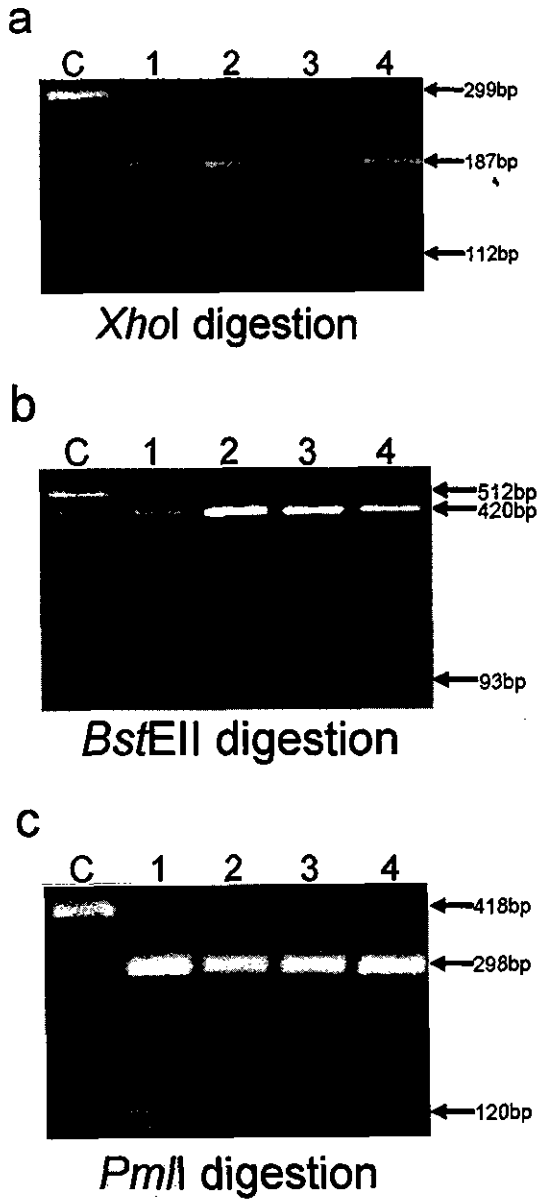

Fig. 1a–c Results of the restriction endonuclease digestion for British recurrent mutations. **a** The mutation R578X in exon 13 abolishes an *Xho*I site. An individual heterozygous for the mutation as a positive control demonstrated the presence of 299-, 187-, and 112-bp bands (*C*). In contrast, the 299-bp undigested PCR product was not detected in any of the 42 patients with DEB (*lanes 1–4*, not all the data are shown), indicating the absence of the R578X mutation. **b** The mutation 7786delG results in loss of a cutting site for the restriction endonuclease *Bst*EII. Restriction digestion of the PCR product of exon 104 in an individual heterozygous for the mutation as a positive control demonstrated the presence of 512-, 420-, and 93-bp bands (*C*). In contrast, the PCR products in all of the 42 patients with DEB showed complete digestion of the 513-bp product to 420-bp and 93-bp fragments (*lanes 1–4*, not all the data are shown), indicating the absence of the mutation 7786delG. **c** The mutation R2814X results in loss of a cutting site for the restriction endonuclease *Pml*I. Restriction digestion of the PCR product of exon 114 in an individual heterozygous for the mutation as a positive control demonstrated the presence of 418-, 298-, and 120-bp bands (C). In contrast, the PCR products in all of the 42 patients with DEB showed complete digestion of the 418-bp PCR product to 298-bp and 120-bp fragments (*lane 1–4*, not all the data are shown), indicating the absence of the R2814X mutation

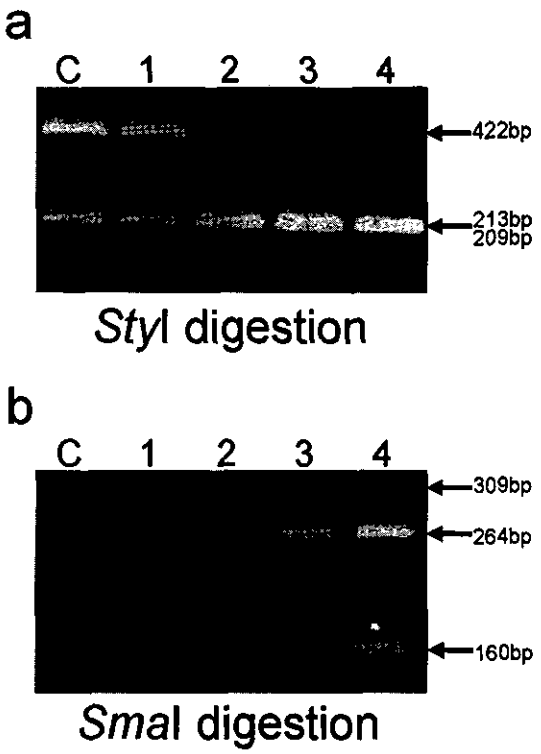

Fig. 2a, b Results of the restriction endonuclease digestion for worldwide recurrent mutations. **a** The mutation 425A→G in exon 3 results in loss of a cutting site for the restriction endonuclease *Sty*I. An individual heterozygous for the mutation as a positive control demonstrated the presence of 422-, 213-, and 209-bp bands (*C*). In a French patient with DEB, the mutation 425A→G was identified (*lane 1*). In contrast, the 422-bp PCR product was not detected in the other 41 patients with DEB (*lanes 2–4*, not all the data are shown), indicating the absence of the mutation 425A→G. **b** The mutation G2043R in exon 73 resulted in loss of an *Sma*I restriction site which was used to assess the presence or absence of this mutation in the patients with DEB. In 40 patients with DEB, the 469-bp PCR product was digested into 264-, 160- and 45-bp fragments by *Sma*I endonuclease, indicating the absence of the mutation G2043R (*lanes 2–4*, not all the data are shown). While in the DNA of the positive control and two patients with DEB, an additional undigested band of 309-bp was present (*C* and *lane 1*, only the data of the Japanese proband are shown), indicating the presence of the mutation G2043R

restriction enzyme site for *Msp*I. The E2857X mutation destroys a restriction enzyme site for *Mae*I. Screening of Japanese DEB patients by *Msp*I and *Mae*I digestion revealed the presence of these recurrent mutations in 13 alleles of 11 Japanese patients of DEB as shown in Table 1. In 2 of the 11 Japanese patients, both paternal and maternal mutations were identified as recurrent mutations.

Discussion

In this study, we demonstrated the absence of the recurrent mutations R578X, 7786delG, and R2814X in all 42 patients with DEB, and detected the mutations 425A→G and G2043R. The mutation 425A→G was found in a French patient with RDEB. The mutation G2043R was found in

Japanese and Chinese patients with DDEB. The Japanese recurrent mutations 5818delC, 6573+1G→C, and E2857X were detected in 13 alleles of 11 Japanese patients with DEB.

It is interesting that some recurrent mutations seem to be exclusive to specific ethnic groups; for example, R578X, 7786delG, and R2814X in British patients with DEB and 5818delC, 6573+1G→C, E2857X in Japanese patients with DEB. Other examples of recurrent mutations that seem to be exclusive to a specific human ethnic group are 4783-1G→A, 497insA, and G1664A found only in Italian patients with DEB [12]. On the other hand, the mutations 425A→G and G2043R have been identified with no ethnic specificity, although there are to date not so many reports of the mutation 425A→G. In general, recurrent mutations can arise in two ways [19]. The first is propagation of a common ancestral allele. In this way, the mutant gene arose from a common source. This is the basis of the recurrent mutations in particular ethnic groups, such as the mutations R578X, 7786delG and R2814X. The absence of the mutations R578X, 7786delG and R2814X in our present series indicates that there is racial heterogeneity in the observed frequency of these mutations within COL7A1 between British DEB patients and DEB patients in other parts of the world, and further supports the notion that these mutations may arise by propagation of a common ancestral allele. The mutations 5818delC, 6573+1G→C, and E2857X are recurrent mutations restricted to Japanese DEB patients [18], which were not identified in other ethnic groups in the present study, and which have not been reported previously in other ethnic groups. These are another example of recurrent mutations in a particular ethnic group.

Recurrent mutations can also arise at a position on a gene that is vulnerable to mutations. In this way, the patients are not distantly related and the mutation has arisen at a "hotspot" such as the mutation R125C in the KRT14 gene encoding keratin 14 found in patients with Dowling-Meara type of EB simplex [1]. Identification of the mutation 425A→G in a French patient and G2043R in Japanese and Chinese patients in our study confirms that these mutations can be found with no relationship to ethnicity and can be regarded as a worldwide mutational hotspot of COL7A1. Among the previously reported ten patients with the G2043R mutation, in seven the mutation has been reported as a de novo mutation. This fact further suggests that the G2043R mutation arises in the part of the gene that is vulnerable to mutations.

Our results also indicate that screening for the mutations 425A→G and G2043R, irrespective of ethnic differences, and the ethnic-specific mutations corresponding to a patient's ethnicity may also be efficient in mutational searching of COL7A1 in patients with DEB. In conclusion, we confirmed that recurrent mutations can be classified into two types, one limited to a specific ethnic group, and the other found throughout the world.

Acknowledgements The authors thank Dr. John A. McGrath for providing genomic DNA with the respective recurrent mutation and Ms. Yuriko Okuno for her technical assistance with the mutation analysis. This work was supported by Grants-in-Aid for Scientific Research (nos. 05404036 and 07457191) from the Ministry of Education, Science, Sports, and Culture of Japan and by Keio Gijuku Academic Development Funds to Hiroshi Shimizu.

References

1. Anton-Lamprecht I, Gedde-Dahl T Jr (2002) Epidermolysis bullosa. In: Rimoin DL, Connor JM, Pyeritz RE, Korf BR (eds) Emery and Rimoin's principles and practice of medical genetics, 4th edn. Churchill Livingstone, London, pp 3810–3897
2. Bruckner-Tuderman L (1999) Hereditary skin diseases of anchoring fibrils. J Dermatol Sci 20:122–133
3. Christiano AM, Hoffman GG, Chung-Honet LC, Lee S, Cheng W, Uitto J, Greenspan DS (1994) Structural organization of the human type VII collagen gene (COL7A1), comprised of more exons than any previously characterized gene. Genomics 21: 169–179
4. Christiano AM, Morricone A, Paradisi M, Angelo C, Mazzanti C, Cavalieri R, Uitto J (1995) A glycine-to-arginine substitution in the triple-helical domain of type VII collagen in a family with dominant dystrophic epidermolysis bullosa. J Invest Dermatol 104:438–440
5. Christiano AM, Suga Y, Greenspan DS, Ogawa H, Uitto J (1997) Strategy for identification of sequence variants in COL7A1, and a novel 2-bp deletion mutation in recessive dystrophic epidermolysis bullosa. Hum Mutat 10:408–414
6. Cserhalmi-Friedman PB, Kárpáti S, Horvath A, Christiano AM (1997) Identification of the glycine-to-arginine substitution G2043R in type VII collagen in a family with dominant dystrophic epidermolysis bullosa from Hungary. Exp Dermatol 6:303–307
7. Cserhalmi-Friedman PB, Kárpáti S, Horvath A, Christiano AM (1997) Identification of a glycine substitution and a splice site mutation in the type VII collagen gene in a proband with mitis recessive dystrophic epidermolysis bullosa. Arch Dermatol Res 289:640–645
8. Dunnill MGS, Richards AJ, Milana G, Mollica F, Eady RAJ, Pope FM (1994) A novel homozygous point mutation in the collagen VII gene (COL7A1) in two cousins with recessive dystrophic epidermolysis bullosa. Hum Mol Genet 3:1693–1694
9. Fine JD, Eady RAJ, Bauer EA, Briggaman RA, Bruckner-Tuderman L, Christiano AM, Heggerty A, Hintner H, Jonkman MF, McGrath JA, McGuire J, Moshell A, Shimizu H, Tadini G, Uitto J (2000) Revised classification system for inherited epidermolysis bullosa: report of the second international consensus meeting on diagnosis and classification of epidermolysis bullosa. J Am Acad Dermatol 42:1051–1066
10. Gardella R, Belletti L, Zoppi N, Marini D, Barlati S, Colombi M (1996) Identification of two splicing mutations in the collagen type VII gene (COL7A1) of a patient affected by the localisata variant of recessive dystrophic epidermolysis bullosa. Am J Hum Genet 59:292–300
11. Gardella R, Zoppi N, Ferraboli S, Marini D, Tadini G, Barlati S, Colombi M (1999) Three homozygous PTC mutations in the collagen type VII gene of patients affected by recessive dystrophic epidermolysis bullosa: analysis of transcript levels in dermal fibroblasts. Hum Mutat 13:439–452
12. Gardella R, Castiglia D, Posteraro P, Bernardini S, Zoppi N, Paradisi M, Tadini G, Barlati S, McGrath JA, Zambruno G, Colombi M (2002) Genotype-phenotype correlation in Italian patients with dystrophic epidermolysis bullosa. J Invest Dermatol 119:1456–1462
13. Järvikallio A, Pulkkinen L, Uitto J (1997) Molecular basis of dystrophic epidermolysis bullosa: mutations in the type VII collagen gene (COL7A1). Hum Mutat 10:338–347
14. Mellerio JE, Dunnill MG, Allison W, Ashton GH, Christiano AM, Uitto J, Eady RA, McGrath JA (1997) Recurrent mutations in the type VII collagen gene (COL7A1) in patients with recessive dystrophic epidermolysis bullosa. J Invest Dermatol 109:246–249

15. Mellerio JE, Salas-Alanis JC, Talamantes ML, Horn H, Tidman MJ, Ashton GHS, Eady RAJ, McGrath JA (1998) A recurrent glycine substitution mutation, G2043R, in the type VII collagen gene (COL7A1) in dominant dystrophic epidermolysis bullosa. Br J Dermatol 139:730–737
16. Mohammedi R, Mellerio JE, Ashton GH, Eady RAJ, McGrath JA (1999) A recurrent COL7A1 mutation, R2814X, in British patients with recessive dystrophic epidermolysis bullosa. Clin Exp Dermatol 24:37–39
17. Rouan F, Pulkkinen L, Jonkman MF, Bauer JW, Cserhalmi-Friedman PB, Christiano AM, Uitto J (1998) Novel and de novo glycine substitution mutations in the type VII collagen gene (COL7A1) in dystrophic epidermolysis bullosa: implications for genetic counseling. J Invest Dermatol 111:1210–1213
18. Tamai K, Murai T, Mayama M, Kon A, Nomura K, Sawamura D, Hanada K, Hashimoto I, Shimizu H, Masunaga T, Nishikawa T, Mitsuhashi Y, Ishida-Yamamoto A, Ikeda S, Ogawa H, McGrath JA, Pulkkinen L, Uitto J, the Japanese Collaborative Study Group on Epidermolysis Bullosa (1999) Recurrent COL7A1 mutations in Japanese patients with dystrophic epidermolysis bullosa: positional effects of premature termination codon mutations on clinical severity. J Invest Dermatol 112: 991–993
19. Uitto J, Pulkkinen L, Christiano AM (1999) The molecular basis of the dystrophic form of epidermolysis bullosa. In: Fine JD, Bauer EA, McGuire J, Moshell A (eds) Epidermolysis bullosa. John Hopkins University Press, Baltimore, pp 326–350
20. Wessagowit V, Ashton GHS, Mohammedi R, Salas-Alanis JC, Denyer JE, Mellerio JE, Eady RAJ, McGrath JA (2001) Three cases of de novo dominant dystrophic epidermolysis bullosa associated with the mutation G2043R in COL7A1. Clin Exp Dermatol 26:97–99
21. Whittock NV, Ashton GHS, Mohammedi R, Mellerio JE, Mathew CG, Abbs SJ, Eady RAJ, McGrath JA (1999) Comparative mutation detection screening of the type VII collagen gene (COL7A1) using the protein truncation test, fluorescent chemical cleavage of mismatch, and conformation sensitive gel electrophoresis. J Invest Dermatol 113:673–686
22. Winberg J-O, Hammami-Hauasli N, Nilssen O, Anton-Lamprecht I, Naylor SL, Kerbacher K, Zimmermann M, Krajci P, Gedde-Dahl T Jr, Bruckner-Tuderman L (1997) Modulation of disease severity of dystrophic epidermolysis bullosa by a splice site mutation in combination with a missense mutation in the COL7A1 gene. Hum Mol Genet 6:1125–1135

Journal of Dermatological Science (2004) 34, 195–200

ELSEVIER

Journal of Dermatological SCIENCE

www.intl.elsevierhealth.com/journals/jods

The G2028R glycine substitution mutation in *COL7A1* leads to marked inter-familiar clinical heterogeneity in dominant dystrophic epidermolysis bullosa

Hiroyuki Nakamura[a], Daisuke Sawamura[a], Maki Goto[a], Kazuko C. Sato-Matsumura[a], Jeffrey LaDuca[b], Julia Yu-Yun Lee[c], Takuji Masunaga[d], Hiroshi Shimizu[a,*]

[a] *Department of Dermatology, Hokkaido University Graduate School of Medicine, North 15 West 7, Kita-ku, Sapporo 060-8638, Japan*
[b] *Department of Dermatology, University of Rochester, NY, USA*
[c] *Department of Dermatology, National Cheng-Kung University, Tainan, Taiwan*
[d] *Department of Dermatology, Keio University, Fujisawa Kanagawa, Japan*

Received 22 December 2003; received in revised form 2 February 2004; accepted 6 February 2004

KEYWORDS
Type VII collagen;
Nail dystrophy;
EB pruriginosa

Summary ***Background:*** Glycine substitution mutations in *COL7A1* not only cause dominant dystrophic epidermolysis bullosa (DDEB), but can also be silent mutations which lead to recessive dystrophic epidermolysis bullosa (RDEB) in combination with additional mutations in the other allele. ***Objective:*** In this study, we have examined a large American Caucasian pedigree in which 10 family members from four generations presented with simple toenail dystrophy without skin fragility in autosomal dominant manner. ***Method:*** We sequenced *COL7A1* of this pedigree. ***Results:*** Mutational analysis indeed detected a heterozygous G-to-A transition at nucleotide position 6082 leading to G2028R in all the affected members. Surprisingly, mutation database revealed that this G2028R mutation had been previously identified in two distinct Asian families with DDEB showing apparent skin fragility and blister formation. One case was a 17-month-old Chinese female with classical phenotype of DDEB and the other was a 27-year-old Japanese female with typical epidermolysis bullosa (EB) pruriginosa. To better understand the molecular mechanisms of this marked inter-familiar clinical heterogeneity, we examined the entire sequence of all the exons and exon–intron borders as well as the promoter region of *COL7A1* in all the three families. Sequence results demonstrated no significant nucleotide difference in *COL7A1* among the three pedigrees. ***Conclusion:*** This paper has demonstrated for the first time that identical *COL7A1* glycine substitutions can cause remarkably heterogeneous clinical phenotypes extending from simple toe nail dystrophy without skin fragility to typical DDEB

Abbreviations: EB, epidermolysis bullosa; DDEB, dominant dystrophic epidermolysis bullosa; RDEB, recessive epidermolysis bullosa

* Corresponding author. Tel.: +81-11-706-7387; fax: +81-11-706-7820.
E-mail address: shimizu@med.hokudai.ac.jp (H. Shimizu).

doi:10.1016/j.jdermsci.2004.02.005

and EB pruriginosa. In addition, the fact of inter-familiar, not intra-familiar clinical heterogeneity associated with G2028R suggest that the other molecular mechanisms not controlled by *COL7A1* coding sequence might be responsible for the clinical heterogeneity.

1. Introduction

Epidermolysis bullosa (EB) is a group of cutaneous hereditary mechano-bullous disorders that can be classified into three major categories, the simplex, the junctional, and the dystrophic forms, on the basis of the level of tissue separation within the basement membrane [1]. Dystrophic EB (DEB) is clinically characterized by mucocutaneous blistering in response to minor trauma, followed by scarring and nail dystrophy, and patients' exhibit tissue separation beneath the lamina densa at the level of the anchoring fibrils within the upper papillary dermis. It occurs in either autosomal dominant (DDEB) or recessive (RDEB) fashions, each form having different specific clinical presentation and severity [1].

Both DDEB and RDEB are caused by mutations in the *COL7A1* gene encoding type VII collagen, the major component of anchoring fibrils [1,2]. The most severe subtype of RDEB, Hallopeau–Siemens type, shows a lack of expression of collagen VII, and results from combinations of premature termination codon (PTC) mutations [3]. The non-Hallopeau–Siemens type with the presence of some type VII collagen expression is generally due to a combination of PTC with mis-sense mutations including glycine substitutions, an in-frame deletion or a splice site mutation [3,4]. Glycine substitution in one allele has been found in many patients with DDEB while a few patients showed in-frame deletion mutations [5]. Thus, glycine substitution mutations can cause dominantly or recessively inherited forms of DEB. However, recent reports have shown presence of some glycine substitutions, which lead to only nail dystrophy but not skin blistering when combined with a normal allele and, when compound heterozygous with another *COL7A1* mutation, these mutations lead to RDEB [6,7].

In this study, we reported a large American pedigree with familial nail dystrophy and we have detected a pathogenic glycine substitution G2028R in the *COL7A1* gene. Furthermore, we analyzed the entire genes of unrelated two families with clinically different DDEB phenotypes but having the same G2028R mutation.

2. Materials and methods

2.1. Patient details

Family A: The proband (III-3, Fig. 1) was a 42-year-old American Caucasian female who was a fraternal twin and who had presented with only toenail dystrophy since childhood. Although, as an adult, she could get blisters on her fingers with marked trauma, the blisters have never been a significant problem. There were 10 affected individuals with toenail dystrophy in fourth generation (Fig. 1). Her parents were not consanguineous. The proband and her affected child (III-3 and IV-3) were available for examination and their big toenails were markedly dystrophic (Fig. 1). There were not any other clinical findings in the proband.

Family B: These details have already been reported by Lee et al. [8]. The clinical features of the proband, a 17-month-old Chinese female, were recurrent vesicles on the knees, shins, and acral regions since birth, followed by scarring and milia. In

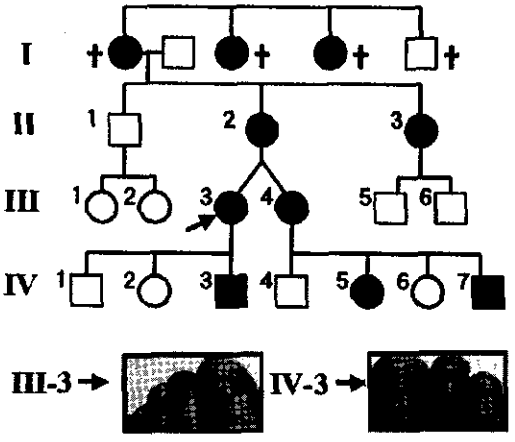

Fig. 1 The pedigree and clinical manifestation of family A. There are 10 individuals with nail dystrophy (closed circles and squares) throughout four generations. Open circles and squares indicate unaffected family members. Marked toenail dystrophy without any other signs of DEB was seen in the proband and her children.

addition, a marked atrophy was noted in the big toenails and on the ring finger nail, but not any mucosal lesion. All members of her family were clinically normal [8]. The parents were non-consanguineous. Electron microscopy showed poorly formed anchoring fibrils beneath the lamina densa.

Family C: These details already have been reported by Murata et al. [9]. The proband, a 27-year-old Japanese female, was characterized by intensely itchy prurigo-like papules and milia predominantly on the pretibial area from the age of 20, which was consistent with EB pruriginosa. Dystrophic changes were seen in some of her fingernails and toenails. There were no abnormalities in her mucous membranes, hair or teeth. The presence of similar clinical features in her family indicated an autosomal dominant inheritance [9]. Electron microscopy of the skin of the proband showed normal-appearing but slightly reduced the number of anchoring fibrils [9].

2.2. PCR amplification and heteroduplex analysis

Total genomic DNA was extracted from peripheral blood lymphocytes, and then subjected to mutation screening. *COL7A1* segments including all 118 exons, all exon–intron borders and the promoter region were amplified by PCR using pairs of oligonucleotide primers synthesized on the basis of intronic sequences according to the report by Christiano, et al. [10] (GenBank numbers L02870, L23982). Specifically, the following primers were used to amplify the 286 bp product containing exon 73 and flanking intronic sequences: Sense primer, 5′-GGGTGTAGCTGTACAGCCAC-3′, anti-sense primer, 5′-CCCTCTTCCCTCACTCTCCT-3′.

For PCR amplification, approximately 200 ng of genomic DNA, 40 pmol of each primer, 0.5 mM $MgCl_2$, 20 μmol of each dNTP and 1.25 U of Taq polymerase were used in a total volume of 50 μl. The amplification conditions were 94 °C for 5 min, followed by 40 cycles of 94 °C for 45 s, 55 °C for 45 s and 72 °C for 45 s, and extension at 72 °C for 10 min in GeneAmp PCR System 9700 (Applied Biosystems). The PCR products were examined on 2% agarose gel and 4–8 μl of the samples were prepared for heteroduplex analysis using conformation-sensitive gel electrophoresis (CSGE) [11]. Staining with ethidium bromide was used to visualize the heteroduplexes. The PCR products demonstrating shifted bands were subjected to automated nucleotide sequencing in an ABI 310 genetic analyzer (Perkin-Elmer, Warrington, UK), when we could not detect any significant shifted bands.

2.3. Verification of the mutations

Since the G-to-A transition at nucleotide 6082 within exon 73 results in the loss of an enzyme restriction site for *Sma*I (New England Biolabs Inc., Beverly, MA, USA), the 286 bp PCR products generated with primers indicated above were subjected to *Sma*I digestion according to the manufacturer's recommendation. The digestion products were analyzed on 2% agarose gels, using ethidium bromide to visualize the bands.

2.4. *COL7A1* sequence among families A, B and C

To compare *COL7A1* sequences among families A, B and C, we obtained genomic DNA from the probands of families A, B and C, and have examined the entire sequences of all the exons, exon–intron borders and promoter in the *COL7A1* gene.

3. Results

3.1. Detection of *COL7A1* mutation in family A

CSGE of the PCR products of exon 73 sequences of *COL7A1* revealed a distinct heteroduplex pattern in all available affected individuals of the family A, whereas all available unaffected family members showed a homoduplex band only, similar to that noted in an unrelated healthy control (data not shown).

Direct DNA sequencing of the products from the proband showed a heterozygous G-to-A transition at nucleotide 6082 within the exon 73 of the proband (Fig. 2a). The substitution converted a glycine (GGG) to arginine (AGG), designated as G2028R. This mutation was confirmed by the loss of a *Sma*I restriction site. Digestion of the 286 bp PCR products with *Sma*I revealed three bands 148, 93, and 45 bp in the unaffected members and the normal control, whereas the affected members showed another 193 bp band, indicating that other affected members had the same mutation as the proband (Fig. 2b).

3.2. Comparison of *COL7A1* sequence among families A, B, and C

Search for previous reports about *COL7A1* mutation revealed that the same G2028R mutation was found in two distinct families B and C as mentioned above. The one proband of family B was a

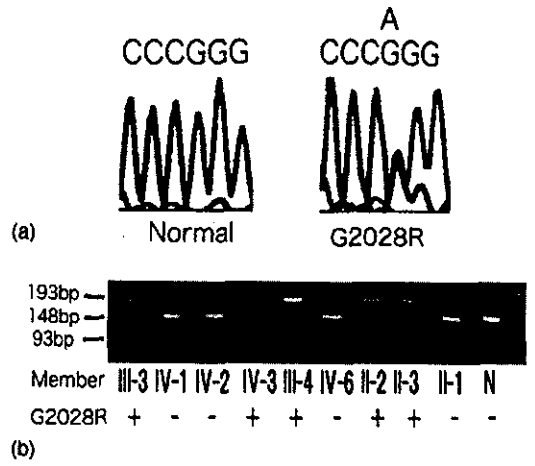

Fig. 2 Detection of the G2028 mutation in *COL7A1*. (a) Nucleotide sequencing of the PCR products disclosed a heterozygous G-to-A transition at nucleotide position 6082 in exon 73 in the proband. This nucleotide transition led to the glycine substitution G2028R. The mutation was recognized in all the other affected individuals as well as the proband, but not in unaffected members of the family. (b) The mutation G2028R causes the loss of a restriction site for *Sma*I endonuclease. The 266 bp PCR products of the unaffected individuals (VI-2, VI-1, IV-6, and II-1) and a normal control (N) were digested to 148, 93, and 45 bp fragments, whereas the additional 193 bp fragment is seen in those of affected individuals (III-3, IV-3, III-4, II-2, and II-3). The 45 bp bands are too short to be seen on the gel.

17-month-old Chinese female whose clinical phenotype was common type of DDEB with skin fragility [8], and the other proband in family C was a 27-year-old Japanese female with clinical features of EB pruriginosa which was inherited as autosomal dominant pattern [9]. To address a mechanism of this marked inter-familiar clinical heterogeneity among these three pedigrees in molecular level, we have obtained genomic DNA from these two probands and have examined the entire sequences of all the exons and exon–intron borders in the *COL7A1* gene. Several polymorphisms were seen in their genes, but we found neither nucleotide change that substituted amino acid, nor nucleotide change that induced aberrant splicing.

Furthermore, we suspected the mutation in the promoter area of the *COL7A1* gene might be associated with marked clinical heterogeneity in the three pedigrees. To examine this possibility, mutation detection was performed adjacent to the promoter area (from −730 to +75) of the gene, which was responsible for the regulation of gene expression [12]. However, there was neither mutation nor difference among the sequences of the region in the present three families.

4. Discussion

Collagens are a family of closely related, although genetically distinct, extracellular matrix proteins. Each collagen consists of three polypeptide chains, chains, which contain a characteristic repeating "collagenous" triplet amino acid sequence -Gly-Xaa-Yaa-, where Xaa and Yaa denote amino acids other than glycine. A glycine substitution mutation generates a change in the glycine residue within the -Gly-Xaa-Yaa- repeat to another residue. Replacement of a glycine residue may distort the conformation of the triple helix, because each of the glycine residues is located in a sterically restricted position in the center of the triple helix. Both the position within the triple helix and the nature of the substituting residue are thought to be important for phenotypic outcome.

Almost 100 pathogenic glycine substitution mutations have been identified in *COL7A1* in unrelated DEB patients, thus far (Fig. 3). Approximately 60 of these glycine substitution mutations in COL7A1 can cause a dominantly inherited DEB phenotype through dominant negative interference, and are concentrated in the amino acid residues ranging from 2000 to 2080. Also about 30 silent glycine substitution mutations have been described, and are scattered along the entire collagenous domain. No abnormal phenotype is found in individuals heterozygous for one of these mutations in association with a wild-type allele [7,13]. However, if such a mis-sense mutation is inherited on both alleles or in association with a PTC or splice site mutation on the other allele, the clinical consequence may be mild, moderate or severe form of recessive DEB, depending on the nature of the second mutation [14,15].

EB pruriginosa, a rare clinical phenotype of DEB, is characterized by severe pruritus, lichenoid or nodular prurigo-like lesions and violaceous linear scarring, and occasional trauma-induced blistering that mainly affected the shins [16]. Most patients with this rare phenotype show an autosomal dominant inheritance pattern [16] and several glycine substitution mutations G1791E, G2028R, G2034R, G2037E, G2396S, G2242R, G2239R, and G2713R have been reported [9,17,18] (Fig. 4). Furthermore, recent reports have shown presence of some glycine substitutions G1595R, G*1776*A, G1815R, G2251E, and G2287R can cause dominantly inherited nail dystrophy [6,7,19,20].

In this study, we have performed mutation analysis on a large American pedigree with familial nail dystrophy in the dominantly inherited fashion and detected a glycine substitution mutation, G2028R. Although the mutation has been already reported in two other unrelated cases [8,9], the clinical fea-

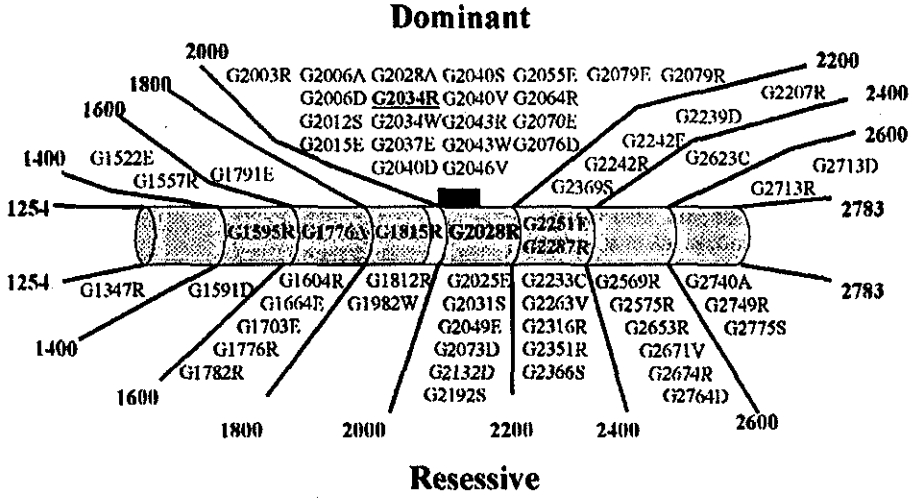

Fig. 3 Database of glycine substitution mutations in *COL7A1*. The collagenous domain of type VII collagen extends from amino acids 1254–2783. Glycine substitution mutations (black characters) can cause a dominant DEB phenotype through dominant negative interference, and are concentrated in the area between amino acid residues from 2000 to 2080 (red bar), in which the G2028R mutation is located. Silent glycine substitution mutations with recessive DEB (brown characters) have been described, and are scattered over the entire collagenous domain. The majority of mutations causing EB pruriginosa (blue characters) show an autosomal dominant inheritance pattern. G2034R mutations (underlined) can cause dominant DEB and EB pruriginosa. Some glycine substitution mutations G1595R, G1776A, G1815R, G2251E, and G2287R (green characters) can cause dominantly inherited nail dystrophy alone without skin fragility.

tures of the present American family was markedly different from those in the other families with an ordinary phenotype of DDEB and EB pruriginosa. A wide diversity of clinical severity can be demonstrated in dominant DEB even if the same mutation is detected in each case, and the mutation G2034R [18,21,22,23] has been reported to generate both DDEB and EB pruriginosa phenotypes. However, there has been no report showing that the same *COL7A1* glycine substitution can be the cause of three different dominantly-inherited subtypes, including common DDEB, EB pruriginosa and familial nail dystrophy without skin fragility.

In other autosomal dominant genodermatosis, EB simplex, we recently confirm that the clinical heterogeneity within the family was due to the presence of second mutation of the causative keratin 5 gene. We demonstrated that one mutation was clinically silent when combined with the wild type allele but aggravated the clinical severity of EBS caused by another dominant mutations on the other allele. To address a possibility that another nucleotide changes in *COL7A1* are responsible for this marked clinical heterogeneity among these families, we have compared the *COL7A1* sequences in all exons, all exon–intron borders and in the promoter region in three probands. We found no significant nucleotide change except several polymorphisms that did not lead to the substitution of any amino acids. *COL7A1* mutations are the cause of rare instances of familial nail dystrophy without any history of blistering as the main clinical feature [6,7,19,20]. It is generally thought that, if a dominant *COL7A1* glycine substitution mutation is not at a critical sequence, a minimal phenotypical severity, such as nail dystrophy, may arise in the DEB patient [19]. However, since the G2028R mutation resides at an important DDEB hotspot between from amino acid residues 2000–2080 (Fig. 3), this hypothesis was not proposed.

In this paper, we have first demonstrated that the same *COL7A1* glycine substitution can cause a remarkable range of inter-familiar clinical heterogeneity, including nail dystrophy, typical DDEB, and EB pruriginosa. Furthermore, no other apparent nucleotide change in *COL7A1* sequence in the unrelated three families suggests the likelihood that other additional molecular mechanisms, not involving *COL7A1* coding sequence, could affect the regulation of production and organization of anchoring fibrils, and might be responsible for this clinical heterogeneity.

Acknowledgements

The authors wish to thank James R. McMillan for his critical reading and comments on this manuscript.

This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan, (13357008, 15390337, 15390336), Lydia O'Leary Memorial Foundation and by grants from the Ministry of Health of Japan (H13- Specific Disease-02 to HS).

References

[1] Fine JD, Eady RA, Bauer EA, et al. Revised classification system for inherited epidermolysis bullosa: report of the Second International Consensus Meeting on diagnosis and classification of epidermolysis bullosa. J Am Acad Dermatol 2000;42:1051–66.

[2] Uitto J, Pulkkinen L. Molecular genetics of heritable blistering disorders. Arch Dermatol 2001;137:1458–61.

[3] Uitto J, Hovnanian A, Christiano AM. Premature termination codon mutations in the type VII collagen gene (*COL7A1*) underlie severe recessive dystrophic epidermolysis bullosa. Proc Assoc Am Phys 1995;107:245–52.

[4] Shimizu H, McGrath JA, Christiano AM, Nishikawa T, Uitto J. Molecular basis of recessive dystrophic epidermolysis bullosa: genotype/phenotype correlation in a case of moderate clinical severity. J Invest Dermatol 1996;106:119–24.

[5] Christiano AM, Morricone A, Paradisi M, et al. A glycine-to-arginine substitution in the triple-helical domain of type VII collagen in a family with dominant dystrophic epidermolysis bullosa. J Invest Dermatol 1995;104:438–40.

[6] Hammami-Hauasli N, Raghunath M, Kuster W, Bruckner-Tuderman L. Transient bullous dermolysis of the newborn associated with compound heterozygousity for recessive and dominant *COL7A1* mutations. J Invest Dermatol 1998;111:1214–9.

[7] Shimizu H, Hammami-Hauasli N, Hatta N, Nishikawa T, Bruckner-Tuderman L. Compound heterozygousity for silent and dominant glycine substitution mutations in *COL7A1* leads to a marked transient intracytoplasmic retention of procollagen VII and a moderately severe dystrophic epidermolysis bullosa phenotype. J Invest Dermatol 1999;113:419–21.

[8] Lee JYY, Li C, Chao SC, Pulkkinen L, Uitto J. A de novo glycine substitution mutation in the collagenous domain of *COL7A1* in dominant dystrophic epidermolysis bullosa. Arch Dermatol Res 2000;292:159–63.

[9] Murata T, Masunaga T, Shimizu H, et al. Glycine substitution mutations by different amino acids in the same codon of *COL7A1* lead to heterogenous clinical phenotypes of dominant dystrophic epidermolysis bullosa. Arch Dermatol Res 2000;292:477–81.

[10] Christiano AM, Hoffman GG, Zhang X, et al. Strategy for identification of sequence variants in *COL7A1*, and a novel 2 bp deletion mutation in recessive dystrophic epidermolysis bullosa. Hum Mutat 1997;10:408–14.

[11] Ganguly A, Rock MJ, Prockop DJ. Conformation-sensitive gel electrophoresis for rapid detection of single-base differences in double-stranded PCR products and DNA fragments: evidence for solvent-induced bends in DNA heteroduplexes. Proc Natl Acad Sci USA 1993;90:10325–9.

[12] Vindevoghel L, Lechleider RJ, Kon A, et al. SMAD3/4-dependent transcriptional activation of the human type VII collagen gene (*COL7A1*) promoter by transforming growth factor beta. Proc Natl Acad Sci USA 1998;95:14769–74.

[13] Christiano AM, McGrath JA, Tan KC, Uitto J. Glycine substitutions in the triple-helical region of type VII collagen result in a spectrum of dystrophic epidermolysis bullosa phenotypes and patterns of inheritance. Am J Hum Genet 1996;58:671–81.

[14] Cserhalmi-Friedman PB, Karpati S, Horvath A, Christiano AM. Identification of the glycine-to-arginine substitution G2043R in type VII collagen in a family with dominant dystrophic epidermolysis bullosa from Hungary. Exp Dermatol 1997;6:303–7.

[15] Winberg JO, Hammami-Hauasli N, Nilssen O, et al. Modulation of disease severity of dystrophic epidermolysis bullosa by a splice site mutation in combination with a missense mutation in the *COL7A1* gene. Hum Mol Genet 1997;6:1125–35.

[16] McGrath JA, Schofield OMV, Eady RAJ. Epidermolysis bullosa pruriginosa: dystrophic epidermolysis bullosa with distinctive clinicopathological features. Br J Dermatol 1994;130:617–25.

[17] Mellerio JE, Ashton GH, Mohammedi R, et al. Allelic heterogeneity of dominant and recessive *COL7A1* mutations underlying epidermolysis bullosa pruriginosa. J Invest Dermatol 1999;112:984–7.

[18] Chen X, Li G, Zhu X. Study on *COL7A1* gene mutation in an epidermolysis bullosa pruriginosa family. Zhonghua Yi Xue Za Zhi 2000;80:869–71.

[19] Dharma B, Moss C, McGrath JA, Mellerio JE, Ilchyshyn A. Dominant dystrophic epidermolysis bullosa presenting as familial nail dystrophy. Clin Exp Dermatol 2001;26:93–6.

[20] Sato-Matsumura KC, Yasukawa K, Tomita Y, Shimizu H. Toenail dystrophy with *COL7A1* glycine substitution mutations segregates as an autosomal dominant trait in 2 families with dystrophic epidermolysis bullosa. Arch Dermatol 2002;138:269–71.

[21] Hammami-Hauasli N, Schumann H, Raghunath M, et al. Some, but not all, glycine substitution mutations in *COL7A1* result in intracellular accumulation of collagen VII, loss of anchoring fibrils, and skin blistering. J Biol Chem 1998;273:19228–192334.

[22] Kon A, Nomura K, Pulkkinen L, Sawamura D, Hashimoto I, Uitto J. Novel glycine substitution mutations in *COL7A1* reveal that the Pasini and Cockayne–Touraine variants of dominant dystrophic epidermolysis bullosa are allelic. J Invest Dermatol 1997;109:684–7.

[23] Wessagowit V, Ashton GH, Mohammedi R, et al. Three cases of de novo dominant dystrophic epidermolysis bullosa associated with the G2043R in *COL7A1*. Clin Exp Dermatol 2001;26:97–9.

British Journal of Dermatology 2004; **150**: 652–657. DOI: 10.1111/j.0007-0963.2004.05868.x

Cutaneous Biology

Mutational analysis of the *ATP2A2* gene in two Darier disease families with intrafamilial variability

T.ONOZUKA, D.SAWAMURA, K.YOKOTA AND H.SHIMIZU

Department of Dermatology, Hokkaido University Graduate School of Medicine, N15 W7, Sapporo, 060-8638, Japan

Accepted for publication 28 September 2003

Summary

Background Darier disease (DD), an autosomal dominant genodermatosis characterized by warty papules and plaques over seborrhoeic areas, is caused by mutations in the *ATP2A2* gene, which encodes the sarco/endoplasmic reticulum Ca^{2+} ATPase type 2 isoform (SERCA2). While markedly different clinical severity within DD-affected family members is known, the pathomechanism has not been elucidated.

Objectives Based on the hypothesis that multiple *ATP2A2* mutations might contribute to the pathomechanism, we have analysed two DD families in which the clinical severity differs markedly within a single pedigree, and, as controls, eight DD families without differing clinical severity.

Methods All the exons and intron–exon borders of *ATP2A2* were directly sequenced from the genomic DNA extracted from all the subjects.

Results We identified the heterozygous mutations, G233R in pedigree 1 and C318R in pedigree 2, respectively, whereas no other *ATP2A2* mutations in any of severely affected individuals were found. In eight DD pedigrees as control, we have found M1V, N39D, L180R, A838P and 2170 insertion G in each of five pedigrees, but no mutation was found in three DD pedigrees.

Conclusions Our results together with previous data indicate that the distribution of mutations is scattered over the entire *ATP2A2* without any, as yet, discernible 'hotspots'. The mutations in pedigrees 1 and 2 with intrafamiliar clinical differences occurred around the Ca^{2+}-binding sites on SERCA2, which might be associated with differences in clinical severity. These variations in *ATP2A2* mutations alone cannot account for the clinical heterogeneity within DD pedigrees.

Key words: *ATP2A2*, Darier disease, mutation, SERCA2

Darier disease (DD, keratosis follicularis; OMIM 124200) is an autosomal dominant genodermatosis exhibiting various clinical features, including warty papules and plaques on the mid trunk, flexures, scalp and forehead. The palms and soles show punctate or filiform keratoses, or minute pits. Sunlight, heat and sweating often exacerbate these skin lesions. Neuropsychiatric disorders have been reported in DD, including mental retardation, schizophrenia, bipolar disorders and epilepsy. The prevalence of the disease has been estimated at 1 : 36 000,[1] 1 : 55 000[2] and 1 : 100 000;[3] the age of onset is usually within the second decade, and penetrance in adults is high.[1]

The causes of DD are defects in the *ATP2A2* gene on chromosome 12q23-24.1, which encodes the sarco/endoplasmic reticulum Ca^{2+} ATPase type 2 isoform (SERCA2).[2] SERCA2 is a calcium pump in the sarcoplasmic reticulum that utilizes the chemical energy from ATP, and has two isoforms, SERCA2a and SERCA2b, that differ in their carboxy-terminals and have distinct tissue-specific expression patterns.[4] SERCA2a is primarily located in the heart and slow-twitch skeletal muscle, whereas SERCA2b is present in the smooth muscle, epidermis and adnexal structures.[2,3,5] Despite the previous *ATP2A2* mutation data in DD, the precise correlations between DD genotype and

Correspondence: Hiroshi Shimizu MD.
E-mail: j.fungi@ijk.com

phenotype, or the position of 'hotspot' mutations, have not yet been established.

Many DD families demonstrate considerable phenotypic variation within each pedigree. In another autosomal dominant genodermatosis, epidermolysis bullosa simplex (EBS), we recently confirmed that the clinical heterogeneity within patients in the same family pedigree was due to the presence of a second causative mutation in keratin 5. We demonstrated that one mutation was clinically silent when combined with the wild-type allele but aggravated the EBS clinical severity caused by the other mutation.

The precise molecular mechanisms of the clinical heterogeneity within DD families have not yet been clarified, and there has been no mutational analysis that has focused on this theme. There is a possibility that other nucleotide changes apart from the causative dominant mutation in the *ATP2A2* result in the clinical differences within a DD family.

Materials and methods

Patients with Darier disease

We enrolled patients from 10 unrelated Japanese pedigrees with DD who were diagnosed by dermatologists, based on the clinical and histopathological examination. Diagnostic clinical features were classified as mild, moderate and severe, following the criteria of Ringpfeil *et al.*[6]

Two Darier disease pedigrees with different clinical severity. Pedigree 1 (Fig. 1A) included a 77-year-old man (case 1-1) and his 50-year-old son (case 1-2). The father had shown slight hyperkeratotic papules on his scalp since the age of 30, and the condition gradually spread to almost his entire body. By the age of 77, he had developed mild clinical features including pigmentation and postinflammatory scars on the trunk without mental problems (Fig. 1B). His son, however, showed a much more severe clinical phenotype compared with the father, including verrucous papules and plaques on the scalp, face and trunk since his mid-teen years (Fig. 1C).

Pedigree 2 comprised eight DD patients from three generations (Fig. 2A) and included a 17-year-old girl (case 2-1) and her 13-year-old sister (case 2-2). Case 2-1 presented with more severe clinical features including hyperkeratotic papules and plaques over seborrhoeic areas and extremities since 4 years of age (Fig. 2B). Pitting keratotic papules were present on her palms. Nail involvement, including longitudinal streaks

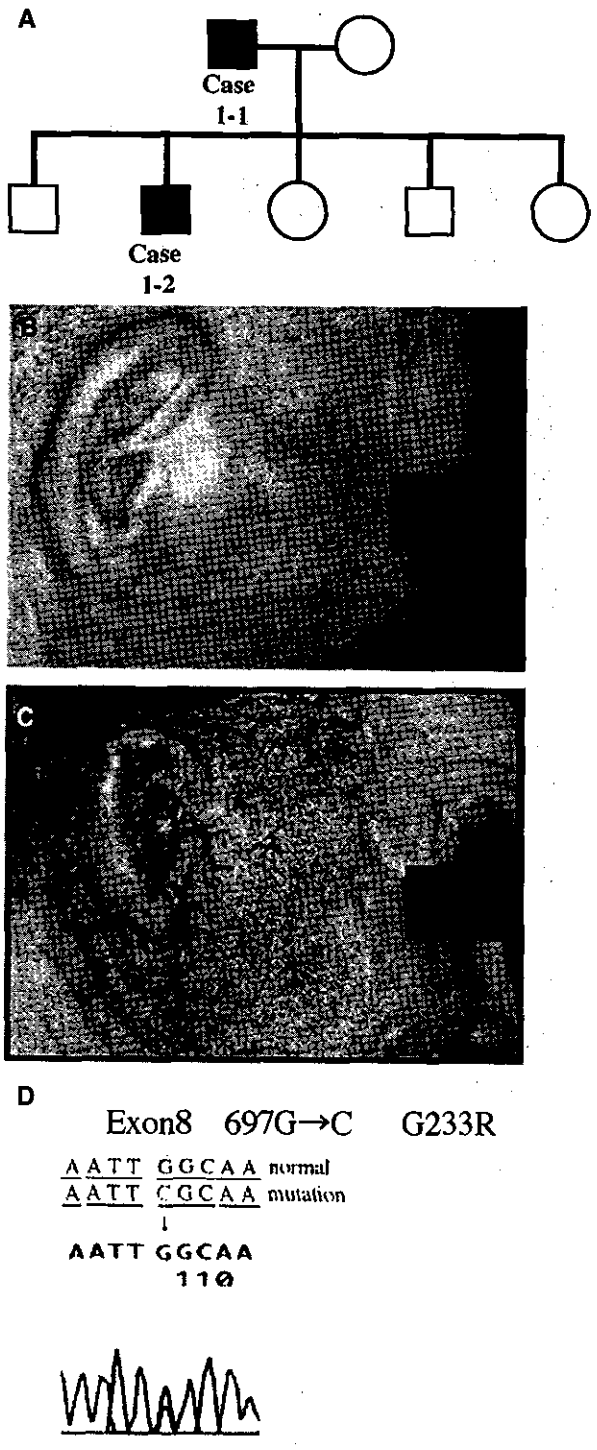

Figure 1. (A) Family tree of pedigree 1. (B) Clinical presentation of case 1-1, the father in pedigree 1. Small brown papules on the face. (C) Case 1-2 (the son in pedigree 1). Severe verrucous plaques on the face. (D) Sequence analysis of the *ATP2A2* gene in pedigree 1. The transversion 697G → C base substitution resulted in a missense mutation in exon 8.

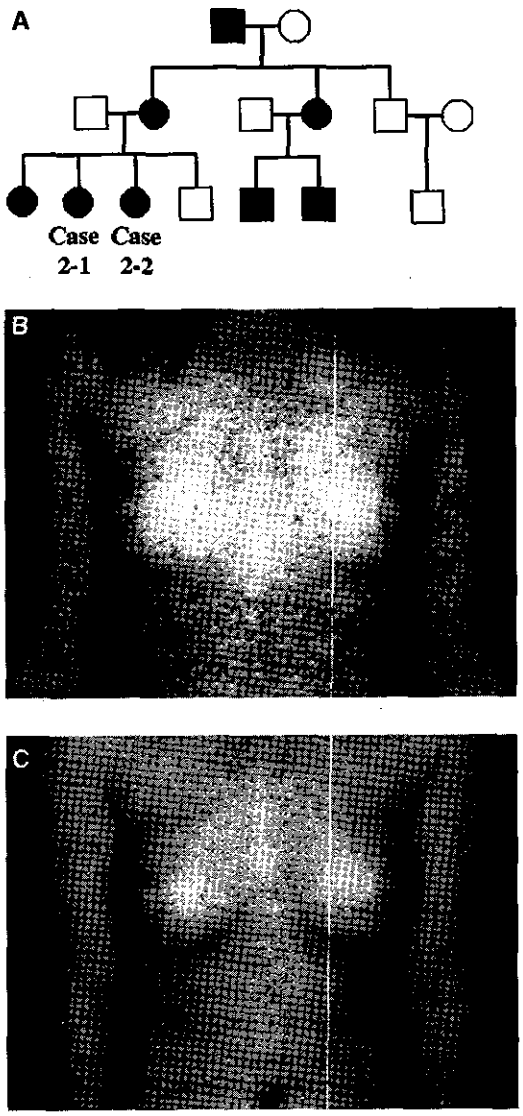

Figure 2. (A) Family tree of pedigree 2. (B) Severe phenotype of this Darier disease patient (case 2-1 in pedigree 2) with brown verrucous papules over the seborrhoeic area. On the extremities and face, hyperkeratotic papules were noticed. (C) Case 2-2, the younger sister of case 3 who exhibits only a few brown papules on the trunk. (D) The sequence analysis of the *ATP2A2* in pedigree 2 shows the transition G952T → C in one allele of exon 8.

and notches, were present on all her fingers. Mental disease has not been detected. Interestingly, her sister, case 2-2, had only a mild clinical phenotype with scattered mild keratotic papules on her forehead, neck and trunk since 12 years of age (Fig. 2C).

Pedigree 3 comprised three DD patients with approximately similar clinical severity whose detailed clinical features were previously reported.[7] A 54-year-old mother (case 3-1) manifested with hyperkeratotic papules on her face, neck and trunk since her teen years. Her 33-year-old daughter (case 3-2) and 28-year-old daughter (case 3-3) showed similar hyperkeratotic papules and plaques on the face, scalp and trunk since they were early teenagers.

Seven sporadic cases. Case 4, a 40-year-old man, had widespread papules and hyperkeratotic plaques on the trunk and extremities, especially on both shins. Case 5, a 13-year-old girl, had hyperkeratotic papules and plaques over seborrhoeic areas and her extremities since 11 years of age. Case 6, a 33-year-old woman, had developed DD 2 years ago. Brown-coloured verrucous papules were scattered on her neck, axillae and extremities. Case 7, a 48-year-old man, had developed DD 4 years previously. Disseminated verrucous nodules with pruritus were noted on the chest, trunk and extremities. Case 8, a 37-year-old man, had hyperkeratotic papules on the face and trunk that had developed 6 years previously. Case 9, a 24-year-old woman, had milia-sized hyperkeratotic papules on the neck, axillae, and chest for only 4 years. Case 10, a 57-year-old man, had disseminated brown papules on seborrhoeic areas of skin. He had developed DD during early childhood.

Mutation analysis of the ATP2A2 gene

Genomic DNA was extracted from peripheral blood lymphocytes and each of the 21 exons of the *ATP2A2* gene were amplified by polymerase chain reaction (PCR). The primers were synthesized according to the previous report.[5] PCR reactions were carried out in a 38-μL volume containing 1 × PCR buffer (Takara, Kyoto, Japan), 2·5 mmol L^{-1} magnesium chloride, 1·25 mmol L^{-1} dNTPs (PE Applied Biosystems, Foster City, CA, U.S.A.), 200 ng genomic DNA, 40 pmol of

each primer and 1·25 unit AmpliTaq polymerase (PE Applied Biosystems). Each reaction began with an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at the optimally determined annealing temperature (50–68 °C) and 30 s at 72 °C. Finally, the reaction was completed with 5 min at 72 °C. All the PCR products were examined on 2% agarose gels and purified by QIAquick PCR Purification Kit (Qiagen, Chatsworth, CA, U.S.A.). Direct sequencing was performed using an ABI PRISM 3100 genetic analyser (ABI Advanced Biotechnologies, Columbia, MD, U.S.A.), and all identified mutations were confirmed by restriction enzyme digestion.

Results

We screened all 21 exons and intron–exon borders of *ATP2A2* using genomic DNA obtained from 10 DD pedigrees. In case 1-1 and case 1-2 in pedigree 1, we found a mutation 697G → C on exon 8, resulting in G233R (glycine → arginine) (Fig. 1d). In case 2-1 and case 2-2 in pedigree 2, we detected a mutation 952T → C which induced C318R (cytosine → arginine) on exon 8 (Fig. 2d).

As the result of screening of *ATP2A2* in the other pedigrees, we identified single mutations, A838P (all of pedigree 3), M1V (case 4), N39D (case 5), L180R (case 6), and 2170 insertion G (case 7). We found no mutations in cases 8–10. Collectively, we identified heterozygous mutations in seven out of 10 pedigrees, including six missense mutations (M1V, N39D, L180R, G233R, C318R, and A838P) and one frameshift insertion (2170 insertion G), and three of which were novel (L180R, G233R, and 2170 insertion G) (Table 1). We found no other mutations within the remaining DD pedigrees. These novel mutations were not detected in over 50 Japanese control individuals who showed neither skin nor neuropsychiatric disorders.

Discussion

The dysfunction of one *ATP2A2* allele in encoding SERCA2 causes DD. Since Sakuntabhai *et al.*[2] reported that mutations in the *ATP2A2* cause DD, a total of 113 different *ATP2A2* mutations in DD patients have been identified.[5–11] While it is known that DD patients have marked variations in their clinical phenotype even within a family,[1,5,11] the molecular mechanisms of this clinical heterogeneity within a family have not yet been clarified. We recently highlighted clinical variations within an EBS family in which a second mutation on a different allele in the causative keratin gene was identified. In addition to the dominant mutation (E170K) detected in all the affected family members in this EBS family, a second silent mutation (E418K) was found only in the cases showing marked clinical severity. We demonstrated that E418K was clinically silent when combined with the wild-type allele but aggravated the clinical severity of the EBS when combined with E170K on the other allele.[12]

Based on our previous findings, we hypothesized that a similar mechanism might occur in DD. We therefore searched for multiple nucleotide changes in *ATP2A2* in our more severely affected individuals from these families. In spite of an extensive search including all exons and intron–exon borders of *ATP2A2*, we failed to find any other mutations apart from the one common dominant mutation. These results indicate that the intrafamilial clinical heterogeneity in DD cannot be explained in these cases by the presence of multiple mutations in *ATP2A2*.

Table 1. The mutations of *ATP2A2* gene in 10 pedigrees of Darier disease

Case/ pedigree	Location	Nucleotide	Mutation	Consequence	Protein domain	Clinical severity[6]	Verification method
1	Exon 8	697G → C	G233R*	Missense	β-strand	Mild and severe	*Eco*RI
2	Exon 8	952T → C	C318R	Missense	S4	Mild and severe	*Pvu*II
3	Exon 16	2512G → C	A838P	Missense	M7	Severe	*Bsl*I
4	Exon 1	1A → G	M1V	Missense	Start codon	Severe	*Nco*I
5	Exon 1	115A → G	N39D	Missense	Upstream stalk	Moderate	*Age*I
6	Exon 6	539T → G	L180R*	Missense	β-strand	Moderate	*Fsp*I
7	Exon 15	TGC → TGGC	2170insG*	Frameshift	Hinge	Moderate	*Msp*I
8	ND	ND	ND	ND	ND	Moderate	
9	ND	ND	ND	ND	ND	Mild	
10	ND	ND	ND	ND	ND	Moderate	

*Novel mutation. ND, Not determined.

Genotype–phenotype correlations in DD have not yet been clearly demonstrated. In this study, we have identified G233R and C318R in pedigrees 1 and 2, respectively. G233 is located in the N-terminal of the β-strand domain, which comprises the A (actuator or anchor) domain; together with M1-P42 and E125-G233, they form the three cytoplasmic domains of SERCA2.[13] However, the influence of an amino acid substitution within this region on the function of SERCA2 is unknown, because the role of the A domain itself is still obscure. C318R was reported in cases with a severe DD phenotype by Sakuntabhai *et al.*[11] This codon is located in the helix M4 that composes a part of the Ca^{2+}-binding site.

SERCA, including SERCA2, has two Ca^{2+}-binding sites. Site I of SERCA is located in the space between the M5 and M6 helices with a contribution from the distal position of M8, and site II is formed by helix M4. Zhang *et al.*[14] reported that the mutations affecting site I in SERCA1a are sufficient completely to inhibit binding of both Ca^{2+} molecules, but mutations against site II only reduce the enzyme activity by 46% of wild-type levels. It is possible to predict that C318R either completely or partially inactivates the functions of the M4 helix, because the structure of SERCA2 is similar to SERCA1. However, even a reduction by half in the Ca^{2+} transport activity of one *ATP2A2* allele might induce severe clinical manifestations in DD patients. The mutations G233R and C318R therefore might produce these clinical differences because of their locations near the Ca^{2+}-binding sites.

In pedigree 3 the DD patients showed similar clinical severity, and the mutation A838P that was detected may induce some decisive changes in the structure of the M7 helix because of the characteristic that proline (P) changes the α-helix structure.[15] The helix M7 is grouped with helix M8-M10, whose functions are unknown. Previous cases with A838P were associated with petit mal epilepsy and severe infected lesions on their lower legs, but there were no descriptions of these patients' clinical phenotypes.[5] Our cases demonstrated no complications. In the sporadic cases, we found M1V, N39D, L180R, and 2170insG. M1V is a mutation involving the start codon, and has previously been reported in one very severe DD case and two cases with flexural involvement.[5] Ringpfeil *et al.*[6] reported a case with N39D with a moderate phenotype that was associated with violent behaviour. In our study, the patient had a moderate phenotype but was without violent behaviour. N39 is located in an upstream stalk region, but the function of this region is unknown. L180R is also a novel missense mutation and is located in the β-strand domain. 2170insG causes a novel frameshift mutation and premature stop codon (TAA) that occurs downstream of the mutation.

In this study, we could not find any correlation between genotype and phenotype in DD. These results together with previous DD mutation data indicate that mutations are distributed throughout the entire *ATP2A2* without hot spot or high frequency mutations. Interestingly, the mutations G233R and C318R found in pedigree 1 and pedigree 2 with intrafamiliar clinical difference occurred around the Ca^{2+}-binding sites (Fig. 3). Several mutations out of 113 different mutations have been shown to cause a moderate to mild or severe clinical phenotype, but only two mutations have been found to cause a shift in intrafamilial severity from mild to severe. These two mutations, 91ins57 and S920Y, were not located in or near the Ca^{2+}-binding sites, but occurred upstream of the S1 region and the M8–M9 loop, respectively, and were clearly different from our cases. Sakuntabhai *et al.*[11] suggested that compensatory mechanisms might include increased expression of the normal *ATP2A2* allele and/or compensation by other SERCA pumps expressed in the epidermis such as *SERCA1* and *SERCA2*, because similar mutations can lead to very different DD phenotype severities.

In conclusion, our study reveals that the distribution of DD mutations is scattered over the entire *ATP2A2*

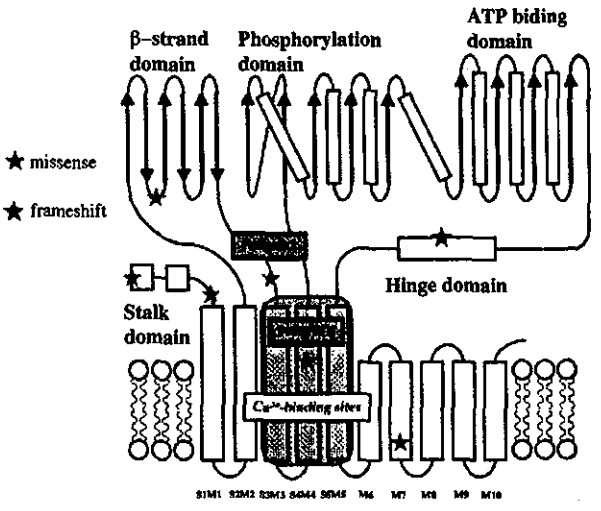

Figure 3. The diagrammatic scheme of the SERCA2 molecule and the position of mutations in our study.[16] The mutations G233R and C318R found in pedigree 1 and pedigree 2 with intrafamiliar clinical difference occurred around the Ca^{2+}-binding sites.

gene without any discernible 'hotspots', and that variations in *ATP2A2* mutations alone cannot account for the clinical heterogeneity within DD families.

Acknowledgments

The authors thank Maki Goto for technical assistance and James R. McMillan for critical reading of and comments concerning this manuscript.

References

1 Munro CS. The phenotype of Darier's disease: penetrance and expressivities in adult and children. *Br J Dermatol* 1992; **127**: 126–30.
2 Sakuntabhai A, Ruiz-Perez V, Carter S *et al.* Mutations in ATP2A2, encoding a Ca2+ pump, cause Darier disease. *Nat Genet* 1999; **21**: 271–7.
3 Missiaen L, Wuytack F, Raeymaekers L *et al.* Ca2+ extrusion across plasma membrane and Ca2+ uptake by intracellular stores. *Pharmacol Ther* 1991; **50**: 191–232.
4 Lytton J, MacLennan DH. Molecular cloning of cDNAs from human kidney coding for two alternatively spliced products of the cardiac Ca2+-ATPase gene. *J Biol Chem* 1988; **263**: 15024–31.
5 Ruiz-Perez VL, Carter SA, Healy E *et al. ATP2A2* mutations in Darier's disease: variant cutaneous phenotypes are associated with missense mutations, but neuropsychiatric features are independent of mutation class. *Hum Mol Genet* 1999; **8**: 1621–30.
6 Ringpfeil F, Raus A, DiGiovanna JJ *et al.* Darier disease—novel mutations in ATP2A2 and genotype–phenotype correlation. *Exp Dermatol* 2001; **10**: 19–27.
7 Inada M, Shimizu H, Yamada S *et al.* Three cases of Darier's disease in a family showing marked heterogeneous clinical severity. *Dermatology* 1999; **198**: 167–70.
8 Takahashi H, Atsuta Y, Sato K *et al.* Novel mutations of *ATP2A2* gene in Japanese patients of Darier's disease. *J Dermatol Sci* 2001; **26**: 169–72.
9 Yang Y, Li G, Bu D *et al.* Novel point mutations of the *ATP2A2* gene in two Chinese families with Darier disease. *J Invest Dermatol* 2001; **116**: 482–3.
10 Chao SC, Yang MH, Lee JY. Mutation analysis of the *ATP2A2* gene in Taiwanese patients with Darier's disease. *Br J Dermatol* 2002; **146**: 958–63.
11 Sakuntabhai A, Burge S, Monk S *et al.* Spectrum of novel *ATP2A2* mutations in patients with Darier's disease. *Hum Mol Genet* 1999; **8**: 1611–9.
12 Yasukawa K, Sawamura D, McMillan JR *et al.* Dominant and recessive compound heterozygous mutations in epidermolysis bullosa simplex demonstrate the role of the stutter region in keratin intermediate filament assembly. *J Biol Chem* 2002; **277**: 23670–4.
13 Toyoshima C, Nakasako M, Nomura H *et al.* Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 a resolution. *Nature* 2000; **405**: 647–55.
14 Zhang Z, Lewis D, Strock C *et al.* Detailed characterization of the cooperative mechanism of Ca(2+) binding and catalytic activation in the Ca(2+) transport (SERCA) ATPase. *Biochemistry* 2000; **39**: 8758–67.
15 Branden C, Tooze J. Motifs of protein structure. In: *Introduction to Protein Structure.* (C.I. Branden, J. Tooze, eds) 2nd edn. New York: Garland Science Publishing, Taylor & Francis Group; 1999; 16–18.
16 MacLennan DH, Rice WJ, Green NM. The mechanism of Ca2+ transport by sarco(endo)plasmic reticulum Ca2+-ATPases. *J Biol Chem* 1997; **272**: 28815–8.

Journal of Dermatological Science (2004) 34, 234–236

Journal of Dermatological SCIENCE

www.intl.elsevierhealth.com/journals/jods

LETTER TO THE EDITOR

In vivo transfer of TGF-α and β genes to keratinocytes

Sir

Cytokines, which are produced by various kinds of cells, possess multiple biological properties and are now being used clinically for treatment of many diseases. Since recent studies have shown that keratinocytes can be a significant source for many of these cytokines, introduction of cytokine genes to keratinocytes has great potential for treating intractable skin diseases. Intradermal injection of naked plasmid with the gene can induce expression of the gene product in keratinocytes in vivo [1]. We introduced cytokine genes to keratinocytes using the naked DNA method, and found their biological effect on the local site [2] as well as on the systemic body [3]. Recently we developed a modified naked DNA method [4], in which intradermally injected DNA bound to nuclear protein, high mobility group 1 (HMG-1). HMG-1 enhanced transfer of DNA from the cytoplasm into the nucleus. Actually injection of β-galactosidase expression vector with HMG-1 induced 2–3 times higher activity than that without HMG-1 as control [4]. In this study, we selected the representative cytokines TGF-α, and β produced by keratinocytes, introduced these genes by this highly efficient method, and examined transgene expression and their biological effects on the local skin.

For construction of human TGF-α and β expression vectors, we first amplified the cytokine cDNA from oligo-dT-primed keratinocyte cDNA using PCR with specific primers. The primers were synthesized based on the human cDNA sequences of TGF-α (GenBank accession no: NM 003236), β1 (NM 000660) and β2 (NM 003238) contained restriction enzyme sites at the 5′ and 3′ ends for subcloning. Primers for TGF-α are 5′-CGCAAGCTTAAAATGGTCCCCTCGGCTGGA-3′ and 5′-CGCGAATTCTCAGACCACTGTTTCTGAGTG-3′; primers for TGF-β1 are 5′-ATAAAGCTTCCCATGCCGCCCTCCGGGCTG-3′ and 5′-ATAGAATTCTCAGCTGCACTTGCAGGAGCG-3′; primers for TGF-β2 are 5′-CCCAAGCTTAAAATGCACTACTGTGTGCTG-3′ and 5′-CCCGAATTCTTAGCTGCATTTGCAAGACTT-3′. After sequence analysis of PCR fragments, they were digested with restriction enzymes and subcloned into pCY4B, which derived a robust expression in keratinocytes [5]. These expression vectors were designated phTGFa, phTGFb1 and phTGFb2.

Hirosaki hairless rats were used for experiment. Plasmid was incubated with HMG-1 for 30 min at 4 °C and the HMG-1 to DNA weight ratio was 1:10 [4]. After the binding reaction, the mixture was diluted with phosphate-buffered saline to a DNA concentration of 0.2 μg/μl, and 50 μl was injected into the subepidermal dermis. Plasmid pCY4B was used as a control. The biopsy specimens were taken to examine mRNA and protein expression, and histological changes [2,3]. For mRNA expression, oligo-dT-primed keratinocyte cDNA was synthesized from keratinocyte RNA and PCR was performed with above specific primers. RNA samples untreated with the enzyme were used as negative controls. The concentration of human TGF-β1 and β2 in keratinocytes was measured with ELISA kits (R&D Systems, Minneapolis, MN). For histological analysis, the skin specimens were taken 96 h after introduction.

RT-PCR from the keratinocyte samples showed the expected 503, 1197 and 1266-bp bands for TGFα, TGF-β1 and TGF-β2, respectively (Fig. 1A). We could not find any bands in samples untreated with reverse transcriptase and in samples from pCY4B treated site. We also examined the time course of TGF-β1 and β2 expression in transfected keratinocytes using ELISA. The results showed that both levels reached a maximum at 24 h and then decreased (Fig. 1B). Previous our study showed that cytokine gene transduction using the naked DNA method induced the similar amount of transgenic cytokines in keratinocytes in vivo [2,3]. Intradermal injection of HMG-1-bound DNA was indicated to provide proper expression of the transferred genes.

Next, we examined the histological changes 96 h after the injection of phTGFa. Hematoxylin–eosin staining revealed epidermal hypertrophy with thickening of the spinous and granular layers (Fig. 2b), indicating that the TGF-α plasmid induced keratinocyte proliferation in vivo. In vitro studies revealed that TGF-α enhanced keratinocyte proliferation [6], and a transgenic mouse study showed that TGF-α induced epidermal thickening

0923-1811/$30.00
doi:10.1016/j.jdermsci.2004.01.005

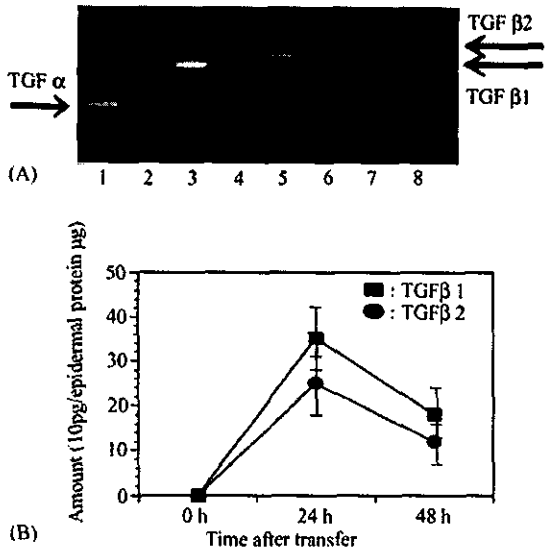

Fig. 1 Detection of human TGF-α, β1, and β2 expression after injection of each expression vector. (A) Plasmids phTGFa, phTGFb1 and phTGFb2 with HMG-1 were injected into the skin. Total epidermal RNA was isolated from the treated area 24 h after transfer. Oligo-dT-primed keratinocyte cDNA was synthesized with reverse transcriptase (RT), and PCR was performed with appropriate primers (lanes 1, 3 and 5). RNA samples without RT were used as negative controls (lanes 2, 4 and 6). Plasmid pCY4B was also used as control vector and PCR amplification was performed with primers for TGFα (lanes 7 and 8). (1) TGFα; (2) TGFα(RT-); (3) TGFβ1; (4) TGFβ1(RT-); (5) TGFβ2; (6) TGFβ2(RT-); (7) pCY4B; (8) pCY4B(RT-). (B) Plasmids phTGFb1 and phTGFb2 with HMG-1 were injected into the skin. The epidermal sheet was prepared from the treated area 24 and 48 h after transfer. The levels of TGFβ1 and β2 were determined by ELISA. Each values shown represents the mean ± S.D. of the three individual samples.

in vivo [7]. Furthermore, topical application of viral vectors yielded in vivo transfer of TGF-α to keratinocytes and caused epidermal thickening in treated skin [8]. We introduced a TGF-α expression vector using the HMG-1-DNA injection method and obtained a similar result, indicating that the proteins synthesized from the genes introduced by this method. Since there are many intractable skin diseases showing epidermal atrophy, local injection of TGF-α plasmid may be useful for treatment of these skin diseases in the future.

TGF-β is a growth factor that has pleiotropic effects on a wide range of target cells. The TGF-β family comprises TGF-β1, β2, and β3 and the biological activities of these molecules are known to be very similar. It is well known that TGF-β induces the synthesis of ECM proteins including collagens and fibronectin, and inhibits the synthesis of ECM-degrading enzymes [9]. In vitro studies demonstrated that TGF-β enhanced differentiation of keratinocytes (i.e. enhancement of keratinization and suppression of growth). Transgenic mice where K14 promoter drove TGF-β1 gene showed hyperkeratosis, hypogranulosis, and epidermal thinning [10]. When we transfected the TGF-β gene into keratinocytes using the HMG-1-DNA injection method, we found not only hyperkeratosis and hypogranulosis, but also epidermal thickening. Since there was no clear difference in the histological changes between TGF-β1 and β2, we only showed the results of TGFβ1. TGF-β also activates collagen synthesis from fibroblasts [9]. Although dermal thickening was not observed in the TGF-β transgenic mice [10], we observed dermal thickening in skin where the TGF-β gene was introduced by the HMG-1-DNA injection method. Our previous results showed that

Fig. 2 Histological changes after introduction of expression plasmids of TGFα and β1. Plasmids pCY4b (control) (a and d); phTGFa (b and e); and phTGFb1 (c and f) with HMG-1 were injected into the skin and the treated sites were biopsied 96 h after injection. Magnification: (a–c) 100×, scale bar 30μm; (d–f) 10×, scale bar 80 μm.

the cassette used in this study increased gene expression 25–50 times over the keratin promoter cassettes which were used widely in transgenic mouse experiments [5]. We postulated that the combination of this cassette and the HMG-1-DNA injection method provided high level expression of TGF-β in the epidermis, which affected fibroblasts and induced dermal thickening. However, dermal cells, including fibroblasts, endothelial cells, and leukocytes, were activated by transgenic TGF-β and may release factors that cause epidermal thickening. Epidermal thickening is often accompanied by dermal infiltration of leukocytes and fibroblastic tumors in the area of dermatopathology. Further research needs to clarify this finding.

In this study, we utilized HMG-1-DNA injection method for introducing TGF genes to keratinocytes and found biological effect in treated skin. This suggests that keratinocyte gene therapy using these cytokine genes may be applicable to skin diseases with epidermal and dermal changes, which are corrected by overexpression of TGF-α and TGF-β.

References

[1] Hengge UR, Chan EF, Foster RA, Walker PS, Vogel JC. Cytokine gene expression in epidermis with biological effects following injection of naked DNA. Nat Genet 1995;10:161–6.

[2] Sawamura D, Meng X, Ina S, Ina S, Sato M, Tamai K, et al. Induction of keratinocyte proliferation and lymphocyte infiltration by in vivo introduction of the IL-6 gene into keratinocytes and possibility of keratinocyte gene therapy for inflammatory skin diseases using IL-6 mutant genes. J Immunol 1998;161:5633–9.

[3] Meng X, Sawamura D, Tamai K, Hanada K, Ishida H, Hashimoto I. Keratinocyte gene therapy for systemic diseases: Circulating interleukin-10 released from gene-transferred keratinocytes inhibited contact hypersensitivity at distant areas of the skin. J Clin Invest 1998;101:1462–7.

[4] Ina S, Sawamura D, Meng X, Tamai K, Hanada K, Hashimoto I. In vivo gene transfer method in keratinocyte gene therapy: intradermal injection of DNA complexed with high mobility group-1 protein in rats. Acta Derm Venereol 2000;80:10–3.

[5] Sawamura D, Meng X, Ina S, Nakano H, Tamai K, Nomura K, et al. Promoter/enhancer cassettes for keratinocyte gene therapy. J Invest Dermatol 1999;112:828–30.

[6] Coffey RJ, Derynck R, Wilcox JN, Bringman TS, Goustin AS, Moses HL, et al. Production and auto–induction of transforming growth factor-alpha in human keratinocyte. Nature 1987;328:817–20.

[7] Vassar R, Fuchs E. Transgenic mice provide new insights into the role of TGF-α during epidermal development and differentiation. Genes Dev 1991;5:714–27.

[8] Lu B, Federoff HJ, Wang Y, Goldsmith LA, Scott G. Topical application of viral vectors for epidermal gene transfer. J Invest Dermatol 1997;108:803–8.

[9] Border WA, Noble NA. Transforming growth factor beta in tissue fibrosis. N Engl J Med 1994;331:1286–92.

[10] Sellheyer K, Bickenbach JR, Rothnagel JA, Budman D, Longley MA, Krieg T, et al. Inhibition of skin development by over expression of transforming growth factor β1 in the epidermis of transgenic mice. Proc Natl Acad Sci USA 1993;90:5237–41.

Daisuke Sawamura*
Maki Goto
Masashi Akiyama
Hiroshi Shimizu
Department of Dermatology
Hokkaido Graduate University School of Medicine
N15 W7, Sapporo 060-8638, Japan

Shinsuke Ina
Division of Dermatology
Sennpoku General Hospital
Omagari 014-0027, Japan
*Corresponding author. Tel.: +81-11-716-1161
fax: +81-11-706-7820
E-mail address: smartdai@med.hokudai.ac.jp
(D. Sawamura)

9 January 2004

Taylor & Francis
healthsciences

Mediators of Inflammation, 13(4), 293–295 (August 2004)

Induction of macrophage migration inhibitory factor precedes the onset of acute tonsillitis

Tadamichi Shimizu[1,CA], Hironori Niizeki[2*], Osamu Takeuchi[3], Yuichiro Yamasaki[2], Nobuko Inamoto[4], Yukihiro Kusunoki[5], Kunihiko Kobayashi[6], Riichiro Abe[1], Hiroshi Shimizu[1], Takeji Nishikawa[7], Kazuhiro Hashiguchi[8] and Jun Nishihira[9]

[1]Department of Dermatology, [6]Department of Pediatrics and [9]Department of Molecular Biochemistry, Hokkaido University Graduate School of Medicine, Sapporo, Japan; [2]Department of Dermatology and Clinical Research Institute, National Tokyo Medical Center, Tokyo, Japan; [3]Division of Basic Research, Kitasato Institute Hospital, Tokyo, Japan; [4]Department of Dermatology and [5]Department of Otolaryngology, Kitasato Institute Hospital, Tokyo, Japan; [7]Department of Dermatology, Keio University School of Medicine, Tokyo, Japan and [8]Department of Pediatrics, Teine Keijinkai Hospital, Sapporo, Japan

[CA]Corresponding Author
Tel: +81 11 716 1161, ext. 5962
Fax: +81 11 706 7820
E-mail: michiki@med.hokudai.ac.jp

We investigated the serum macrophage migration inhibitory factor (MIF) levels of palmoplantar pustulosis patients, before and after the tonsillar provocation test. Higher serum MIF levels of palmoplantar pustulosis patients were decreased after the tonsillar provocation test ($n=29$). To confirm these phenomena, two patients with acute tonsillitis had their changes in body temperature, C-reactive protein (CRP) and serum MIF levels examined during the course of their illness. Surprisingly, increased MIF preceded fever and CRP elevation, and MIF subsequently decreased at the onset of fever and CRP elevation. Since MIF is an initiator of other proinflammatory cytokines, we suggest that the induction of MIF may precede other inflammatory conditions.

Key words: Migration inhibitory factor, Focal infection, Tumor necrosis factor alpha, Tonsillitis

Macrophage migration inhibitory factor (MIF) is an important cytokine in the innate immune system and plays an important role in the control of such inflammatory responses.[1] Once released, MIF induces the expression of proinflammatory mediators by macrophages and activates T cells, thereby promoting inflammatory and immune responses.[2] Elevated concentrations of MIF have been detected in sera from patients with a variety of inflammatory disorders such as atopic dermatitis.[3]

We investigated serum levels of MIF in patients with palmoplantar pustulosis (PPP) and compared them before and after the tonsillar provocation test (TPT) in each patient. PPP is a common chronic skin disease characterized by recurrent sterile pustules and usually also by erythematous, scaly skin on the palms and soles. It has been reported that PPP is closely related to focal tonsil infections, and that tonsillectomy is often effective in curing this condition.[4] The TPT is a well-established laboratory procedure to evaluate whether the tonsils participate in the etiology of inflammatory skin diseases such as PPP and psoriasis.[4] Twenty-nine patients with PPP (10 males and 19 females; aged 19–78 years, mean age 46.3±1.3 years) and 135 healthy volunteers (47 males and 88 females; aged 22–73 years, mean age 47.6±0.82 years) were enrolled in the present study. None of the patients had been treated with systemic immunosuppressants before the TPT. Controls did not have any acute or chronic illness at the time of the study. All participants gave informed consent. The TPT was performed by indirect ultramicrowave irradiation (Micro thermy ME-320; OG Giken, Okayama, Japan) as described previously.[4] Blood samples were collected before and 1 h after TPT. Serum MIF was measured by enzyme-linked immu-

* Present address: Department of Dermatology, Nara Medical University, 840 Shijocho, Kashihara, Nara 634-8522, Japan.

ISSN 0962-9351 print/ISSN 1466-1861 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/09629350400003175