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We hope that making available the relevant information on Pachyonychia Congenita will be a means of furthering research to find effective therapies and a cure for PC.

## A Novel Point Mutation in the Keratin 17 Gene in a Japanese Case of Pachyonychia Congenita Type 2

To the Editor:

Pachyonychia congenita type 2 (PC-2; MIM#167210), also known as Jackson-Lawler type (Jackson and Lawler, 1951), is a rare autosomal dominantly inherited disease characterized by hyper-trophic nail dystrophy, focal keratoderma, multiple pilosebaceous cysts, natal teeth, and hair abnormalities (McKusick, 2001). Heterozygous missense mutations of the keratin 17 (K17) gene have been identified in PC-2 (McLean *et al*, 1995). A mutation in the keratin 6b gene, which encodes K6b, the expression partner of K17, has also been reported in PC-2 (Smith *et al*, 1998). By sequencing genomic DNA from our PC-2 patient, we found a novel heterozygous mutation (G→A transition at nucleotide 452, 452G>A) in the helix initiation motif of the K17 gene resulting in a predicted substitution of valine (GTG) for methionine (ATG) at codon 102, V102M.

A 19-y-old Japanese man visited our university clinic in March 2000. All of his nails had become thick 2 mo after birth. He had multiple normally colored papules and cysts on his scalp and axilla since he was 5 y old, and had hyperkeratosis with blister formation on his soles since he was 10 y old. At 16, the cysts on his scalp and axilla became painful and were repeatedly infected. His father, mother, and younger brother are healthy.

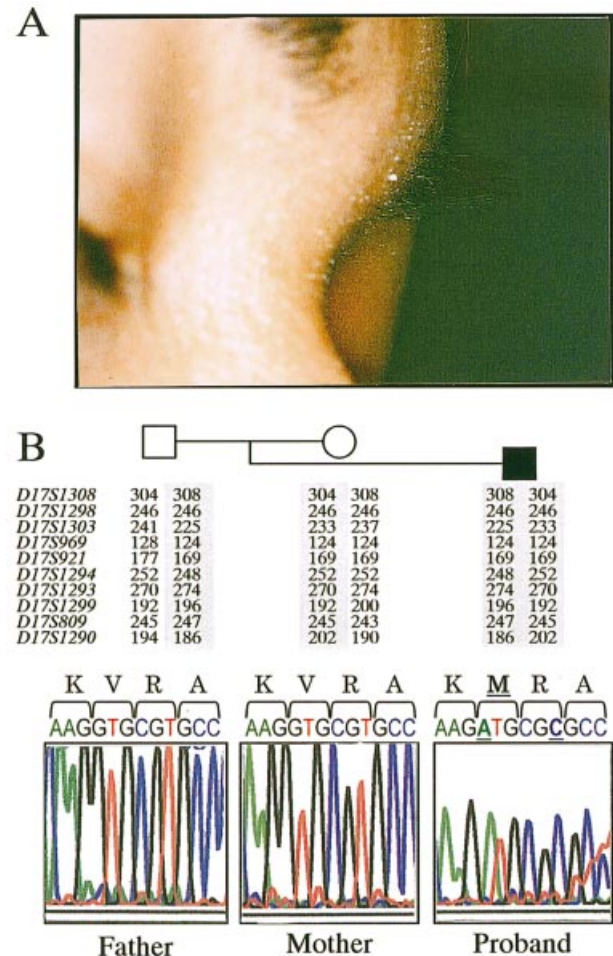
Physical examination showed that he had many cysts and nodules all over his body, pachyonychia on all his fingers and toes, *pili torti* (twisted hair), and plantar hyperkeratosis with bullae. His eyebrows stand erect (Fig 1A). Histologic examination of a subcutaneous cyst showed that the cyst wall was composed of several layers of epithelial cells accompanied by sebaceous gland lobules. Therefore, a diagnosis of steatocystoma was made.

After informed consent, genomic DNA was extracted from a sample of his peripheral blood lymphocytes by a standard method (Qiagen, Hilden, Germany). Genomic DNA from 50 normal healthy Japanese people was used as a control. Exon 1 of the K17 gene was amplified by polymerase chain reaction (PCR) with specific primers for the K17 gene as described previously (McLean *et al*, 1995). Nested PCR was initially performed using primers K17p8 (5'-GCC TAT AAA GGA AGC GGG C-3') and K17p10 (5'-CTC CTT TCT GCC TCC TCC T-3') and then primers K17p3 (5'-TAT GGC AGC AGC TTT GGG-3') and K17p4 (5'-GGT ACC AGT CAC GGA TCT TCA-3'). Amplification conditions were 95°C for 1 min, followed by 35 cycles of 95°C for 40 s, 58°C for 40 s, and 72°C for 1 min, and a final extension at 72°C for 3 min. PCR products (157 bp) were purified using a QIA quick PCR purification kit (Qiagen), and were sequenced on an Applied Biosystem 310 automated sequencer using an ABI PRISM fluorescent dye terminator system (Perkin Elmer, Foster City, CA).

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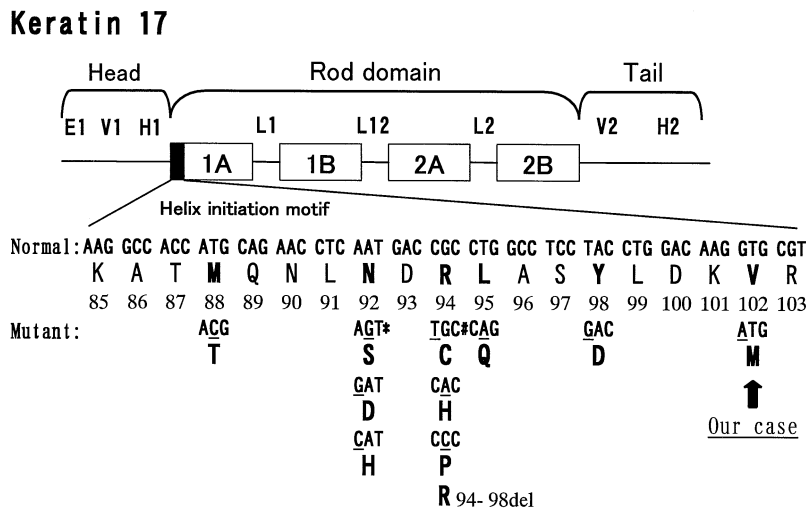
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Abbreviation: PC-2, pachyonychia congenita type 2.



**Figure 1. Pedigree, haplotype, and detection of a K17 gene mutation in a family with PC-2.** (A) Photograph of the patient's upright eyebrows (left eyebrow). (B) Pedigree of the patient's family with haplotypes for 10 genetic markers on chromosome 17. Analyses of the haplotypes and sequences are shown below the corresponding symbols in the pedigree. Black symbol represents the patient. The marker alleles are shown in base pairs. The patient has a heterozygous missense mutation at nucleotide 452 (452G>A) resulting in V102M and a sequence change at nucleotide 457 resulting in R103R. These two nucleotide changes were detected in a mutant allele by sequencing five of 12 clones. The other seven clones had normal sequences. His father and mother had no mutations.

Direct sequencing of DNA from the patient revealed a point mutation, 452G>A. This transition results in the substitution of a valine for a methionine (V102M). Another sequence change was found (T→C transition at nucleotide 457, 457T>C) but this transition does not change the predicted amino acid. To confirm whether these two mutations exist on one allele simultaneously, we subcloned the PCR products into the pCR2.1-TOPO TA-cloning



**Figure 2. Schema of K17 protein structure and summary of reported K17 gene mutations in PC-2.** Keratin has helix domains (1A, 1B, 2A, 2B) separated by linker domains (L1, L12, L2). The DNA and deduced amino acid sequence of the 1A domain are shown. Mutations in 16 cases of pachyonychia congenita type 2 and steatocystoma multiplex described previously are shown. All mutations were detected in one family, N92S mutation indicated by an asterisk in six cases, and R94C indicated by # in two families. Single-letter abbreviations for the amino acid residues: A, Ala; C, Cys; D, Asp; H, His; K, Lys; L, Leu; M, Met; N, Asn; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; Y, Tyr.

vector (Invitrogen, Carlsbad, CA) and sequenced them. Five clones out of 12 were mutants, and the two nucleotide changes were on the mutant allele (**Fig 1B**). The mutations 452G>A and 457T>C were on the same allele. These mutations were not detected in the genomic DNA from his parents (**Fig 1B**). Because the sequence change, 457T>C, created an *HhaI* restriction enzyme site in the mutant allele, we could distinguish a mutant allele (104 and 53 bp, or 106 and 51 bp fragments) from a normal allele (157 bp) by *HhaI* digestion of the 157 bp PCR products. The patient had both mutant and normal alleles, whereas his father and mother and the 50 controls had only the normal allele (data not shown). In addition, by direct sequencing we confirmed that the 452G>A mutation was not present in 50 normal Japanese individuals (data not shown). To exclude the possibility of nonpaternity, we did microsatellite analysis using 10 genetic markers on chromosome 17 (**Fig 1B**). The keratin 17 gene is located on chromosome 17 between D17S1299 and D17S809. Amplification conditions were 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 55°C, 57°C, or 58°C for 1 min, and 72°C for 2 min, and a final extension at 72°C for 5 min. PCR products were analyzed on Gene Scan 3.1 using an ABI PRISM fluorescent dye terminator system (Perkin Elmer). The analysis of haplotypes showed that the patient was descended from his parents, suggesting that he had the *de novo* mutations as a heterozygote (**Fig 1B**).

Keratins are heterodimeric proteins that form the intermediate filament cytoskeleton of epithelial cells. They are expressed in specific epithelial tissues as specific type I/type II keratin pairs. Most of the pathogenic mutations in human keratin diseases occur in the helix initiation or termination motifs. The K17 type I keratin, which has a tissue-specific and differentiation-specific expression pattern, is expressed in the nail bed, hair follicle, and sebaceous gland (Trojanovsky *et al*, 1989; Pantelevey *et al*, 1997; Smith *et al*, 1998; McGowan and Coulombe, 2000). We identified a novel missense mutation (V102M) in the 1A domain of K17 in a Japanese patient with PC-2. To date, 10 mutations in K17 have been described in 16 families with either PC-2 or steatocystoma multiplex (McLean *et al*, 1995; Covello *et al*, 1998; Fujimoto *et al*, 1998; Celebi *et al*, 1999; Smith *et al*, 1997, 2001). All of them were heterozygous missense mutations in the helix initiation 1A domain of K17 (**Fig 2**). This area is highly conserved and even subtle amino acid changes are thought to disrupt intermediate filament formation and assembly. A 423A>G mutation was found in six families, and a 428C>T mutation in two families. Of the 16

families, six were sporadic cases like ours, in which all of the point mutations occurred at nucleotide 423. In our case, two *de novo* sequence changes occurred at nucleotides 452 and 457 simultaneously. This probably happened during gametogenesis or before pronuclear fusion in the zygote, because the parents had no mutations in their somatic cells. This region of nucleotides may be fragile and easily damaged by some environmental factors.

It is of interest that the site of expression of the K17 gene and the clinical phenotype of PC-2 are highly associated. In the murine hair follicle, K17 protein is expressed not only in the outer root sheath but also with polarity in a certain part of the matrix (McGowan and Coulombe, 2000). It was suggested that disruption of its polarization in the matrix of the hair follicle alters the orientation of the hair follicle against the skin surface (McGowan and Coulombe, 2000), resulting in the upright eyebrows found in our patient (**Fig 1A**). Localization of abnormal K17 in the medulla of hair, nail matrix, and the luminal cell layers of sebaceous glands (the sebaceous duct epithelium) may cause twisted coarse hair, thick and hypertrophic nail deformity, and multiple dermal cysts with sebaceous lobules. Thus, abnormal K17 may lead to hyperproliferation rather than differentiation. Although the changes in the three-dimensional structure and the function of abnormal K17 protein produced by point mutations, especially within the helix initiation motif, are unknown, it is possible that K17 plays an important role in the morphology and maintenance of nails, facial hair, and pilo-sebaceous glands. Further study of the overexpression and/or disruption of the K17 gene will facilitate our understanding of the precise function of the K17 protein.

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## EB Simplex Superficialis Resulting from a Mutation in the Type VII Collagen Gene

To the Editor:

Epidermolysis bullosa simplex (EBS) is an inherited blistering disease characterized by intraepidermal cleavage (Gedde-Dahl and Anton-Lamprecht, 1990; Fine *et al*, 1991). A very rare subset of EBS, termed “EBS superficialis” (EBSS), has been described in two families by Fine *et al* (1989). Skin biopsy of these patients shows clefts of variable size just beneath the level of the stratum corneum, which can be completely separated from the rest of the epidermis in some cases. In two of the patients reported, there are also some clefts in the lower one-third of the epidermis.

Together with this unusual clinical picture, most of the patients show atrophic scarring, milia, nail dystrophy, and blistering involving the oral cavity. After the first description of EBSS in two unrelated families, no other cases have been reported, emphasizing the rareness of these findings.

Up to now, mutations in the genes *KRT5*, *KRT14*, and *PLEC1* have been described underlying different subsets of EBS. None of them, however, is a good candidate for the superficial lesions observed in EBSS. *KRT5* and *KRT14* are expressed in basal keratinocytes, whereas mutations in the *PLEC1* gene are associated with EBS together with muscular dystrophy (Smith *et al*, 1996). In the absence of a candidate gene for EBSS, we performed a genome-wide screen in one of the pedigrees previously described (Fine *et al*, 1989). The second family was not included in the study, as only the proband showed evidence of blister formation, suggesting a sporadic mutation. Briefly, the affected individuals studied here belong to a five-generation pedigree with an autosomal dominant pattern of inheritance (Fig 1a). In affected family members, variably sized clefts were noted just beneath the level of the stratum corneum in each biopsy specimen (Fine *et al*, 1989). In some, clefts were subcorneal; in others, lower intraepidermal. In none of the affected individuals was sub-lamina densa cleavage noted, nor was any diminution of type VII collagen staining noted using the anti-type VII antibody LH 7:2 (Fine *et al*, 1989).

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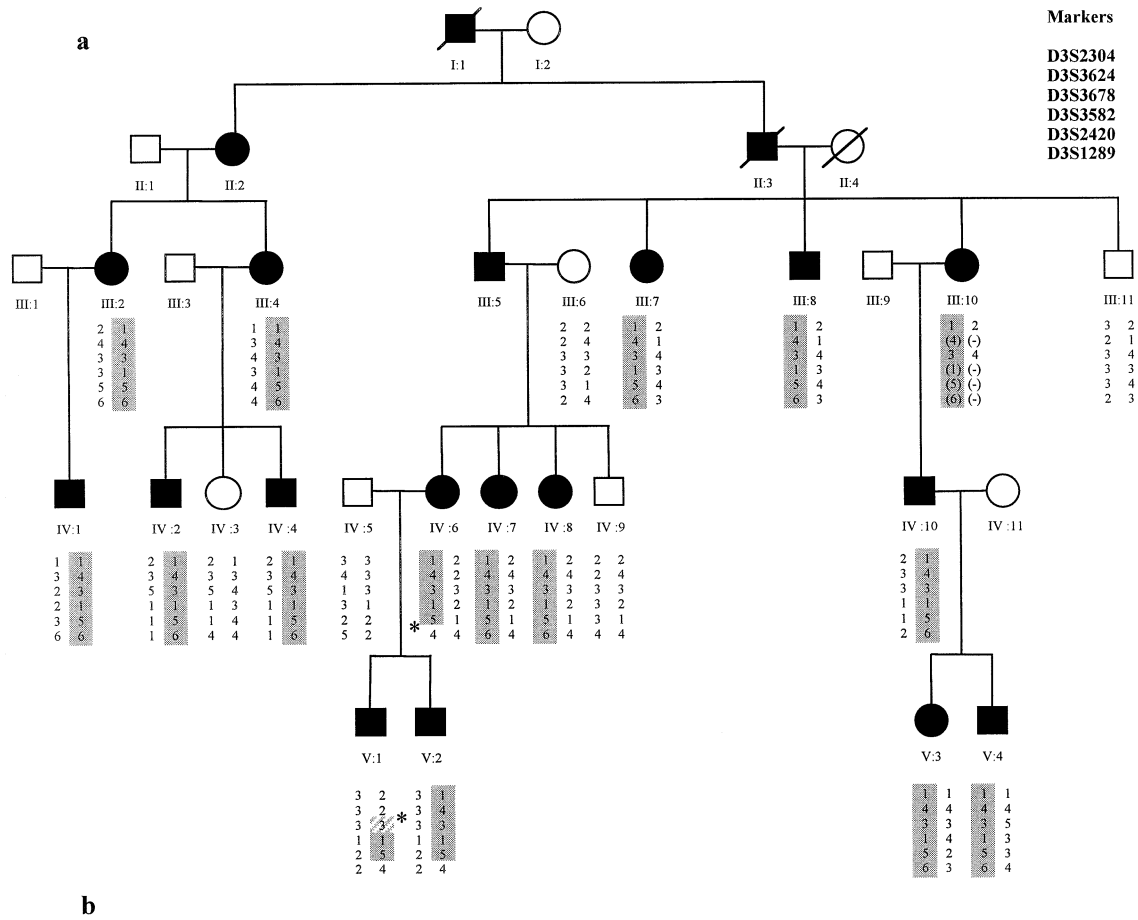
Abbreviations: DEB, dystrophic epidermolysis bullosa; DDEB, dominant forms of DEB; EBS, epidermolysis bullosa simplex; EBSS, epidermolysis bullosa simplex superficialis.

A genome-wide screen was performed using a panel of 324 microsatellite markers, with an average marker spacing of 10 cM and a semiautomated fluorescence-based genotyping system (Aita *et al*, 1999). Two-point linkage analyses were carried out using the MLINK program of the FASTLINK suite of programs (Lathrop *et al*, 1984; Cottingham *et al*, 1993; Schaffer *et al*, 1994). A disease allele frequency of 0.001 and an autosomal dominant mode of inheritance with complete penetrance were assumed. The marker allele frequencies were estimated from observed and reconstructed genotypes of founders within the pedigree. To avoid computation errors due to observed allele frequencies of 0.0, alleles for all markers were re-coded using the RECODE program (Weeks, 2000). Multipoint analyses and reconstruction of pedigrees were carried out using the SIMWALK program version 2.6 (Sobel and Lange, 1996).

The results of the initial genome-wide scan revealed three chromosomal regions with a maximum two-point LOD score greater than 1.4, on chromosomes 3 ( $Z_{\max} = 1.62$ ), 8 ( $Z_{\max} = 1.80$ ), and 10 ( $Z_{\max} = 1.40$ ). Haplotype and multipoint analyses of additional markers allowed us to exclude the regions on chromosomes 8 and 10, and to more decisively establish the linkage to chromosome 3.

A total of 28 additional markers were used for the fine-mapping of the EBSS locus. Maximum two-point LOD scores of 4.11 and 3.77 at  $\theta = 0.0$  were obtained for markers D3S2420 and D3S3582, respectively. Multipoint linkage analysis showed a maximum LOD score of 5.96 for marker D3S2420 (Fig 1b). Finally, analysis of the reconstructed haplotypes confirmed the linkage results and placed the disease locus within a 2.94 cM region on chromosome 3, flanked by markers D3S3624 and D3S1289 (Fig 1a).

According to the different maps derived from the Human Genome Project (Human Genome Project Working Draft; National Center for Biotechnology Information; Ensembl Genome Server; GeneMap'99), the region flanked by markers D3S3624 and D3S1289 spans approximately 10 Mb of genomic DNA on 3p21. Coincidentally, the *COL7A1* gene, in which mutations are responsible for DEB (Uitto *et al*, 1999; Fine *et al* 2000), lies within this genetic interval. Type VII collagen is the main constituent of the anchoring fibrils and at the microscopic level, DEB is characterized by skin cleavage beneath the lamina densa. The morphologic defect is a reduced number or complete absence of the anchoring fibrils in the dominant and recessive forms of DEB, respectively (Tidman and Eady, 1985). In DEB skin, blisters appear just beneath an intact lamina densa. In contrast, the family we studied and reported by Fine *et al* (1989) showed blister formation just beneath the stratum corneum. On the basis of the differences in phenotype between



**Figure 1. Haplotype and multipoint analysis in the pedigree with EBSS.** (a) Pedigree with EBSS and haplotypes for microsatellite markers on chromosome 3. The disease-associated haplotype is shaded. Asterisks indicate the key recombination events. Non-informative marker is cross-hatched. Genotypes in parenthesis have been inferred; (b) multipoint LOD scores for family with EBSS. Markers used are listed on the X-axis. The map positions were computed by SIMWALK 2.6. The order and distance between the markers were deduced from the genetic map at the Center for Medical Genetics, Marshfield Medical Research Foundation (Broman *et al*, 1998).

these two subtypes of EB, the *COL7A1* gene was not considered to be a candidate gene for EBSS. Nevertheless, to unequivocally rule out this possibility, we performed heteroduplex analysis and direct sequencing of the coding region.

Quite unexpectedly, we identified a heterozygous transition in exon 73, 6100G→A, leading to the amino acid change G2034R

(**Table I**). Exon 73 codes for a 67-amino-acid collagenous polypeptide sequence preceded by the 39-amino-acid noncollagenous segment (Christiano *et al*, 1994a). The substitution of a glycine residue within the collagenous domain of the molecule, characterized by the repeating Gly-X-Y sequence, is the major class of pathogenic mutations in the dominant forms of DEB (DDEB)

**Table I. Mutations involving residue G2034 in the COL7A1 gene**

Mutation	Type of EB	Inheritance	Reference
G2034R	EBSS	AD	This study
	DEB-CT <sup>a</sup>	AD	Kon <i>et al</i> (1997)
	DEB-generalisata	AD	Hammami-Hauasli <i>et al</i> (1998); Mecklenbeck <i>et al</i> (1999)
	DEB <sup>b</sup>	AD	Mecklenbeck <i>et al</i> (1999)
G2034W	DEB-mild	AD	Rouan <i>et al</i> (1998)
	DEB-localisata	AD	Mecklenbeck <i>et al</i> (1999)
	DEB-localisata	AD	Mecklenbeck <i>et al</i> (1999)

<sup>a</sup>DDEB-CT: Cockayne-Touraine variant of DDEB.

<sup>b</sup>The subtype of EB has not been specified.

(Christiano *et al*, 1994b, 1995; Burgeson *et al*, 1995). Moreover, mutations involving the glycine residue at position 2034 have been previously reported (Kon *et al*, 1997; Hammami-Hauasli *et al*, 1998; Rouan *et al*, 1998; Mecklenbeck *et al*, 1999) (Table I). The very same amino acid substitution has been described in three families with different forms of DDEB: the "Cockayne-Touraine" variant of DDEB (Kon *et al*, 1997), DDEB "generalisata" (Hammami-Hauasli *et al*, 1998; Mecklenbeck *et al*, 1999), and an unspecified subtype of DEB (Mecklenbeck *et al*, 1999). In addition, a different amino acid substitution affecting the same residue, G2034W, has also been reported. Rouan *et al* (1998) identified this second amino acid change in a family with a mild form of DDEB, mainly involving the hands, feet, and mouth. In another study, Mecklenbeck *et al* (1999) described the same mutation, G2034W, in two families with the so-called DDEB "localisata" (Table I). In these families, a clear DDEB phenotype has been reported, and these types of glycine substitution mutations are the most prevalent in DDEB.

In light of our results, we believe that the clinical phenotype in the EBSS kindred studied here actually represents a case of DDEB, rather than a unique subset of EBS. The molecular data suggest that the subcorneal cleavage observed in different members of this kindred would likely not be pathogenic or contribute to the disease process. As the proband from the second family reported in the original work (Fine *et al*, 1989) was not available for this study, these findings do not fully exclude the possibility that rare forms of EBS having superficial skin cleavage may also exist. Although it is true that the family studied here does indeed have several clinical findings that are commonly associated with DEB, data from the National EB Registry have also shown that at least 10%–25% of all EBS patients have one or more of these "dystrophic" features as well, making the diagnosis based on clinical phenotypes sometimes imprecise. Genetic studies such as the one presented here become an invaluable tool to clarify the true molecular basis of a disease like EBSS, where the clinical features cannot be used to unequivocally classify a particular phenotype. Collectively, these findings allow us to reclassify a previously uncharacterized form of EB as another clinical variant of DDEB.

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## Analysis of ATP2C1 Gene Mutation in 10 Unrelated Japanese Families with Hailey–Hailey Disease

To the Editor:

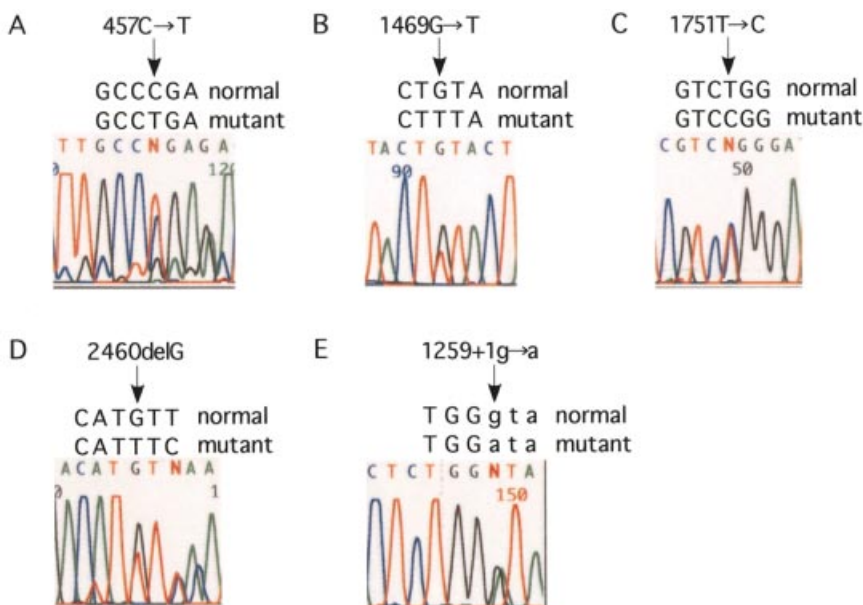
Hailey–Hailey disease (HHD) is an autosomal dominant chronic blistering disease, which is histologically characterized by keratinocyte acantholysis and epidermal cleft formation (Burge, 1992). The disease typically presents in middle age as crusted erosions or circinate plaques in sites exposed to friction such as the neck, axillae, groin, and perineum. Recently, the gene ATP2C1 has been identified as defective in HHD. It has been suggested that HHD is caused by a haploinsufficiency of this new  $\text{Ca}^{2+}$ -ATPase (Hu *et al*, 2000; Sudbrak *et al*, 2000). So far 33 different mutations in the ATP2C1 gene from 35 unrelated HHD families have been identified in the literature. Mutations in 42 cases have yet to be identified out of a total of 77 so far analyzed. In this study, we screened the genomic DNA of the ATP2C1 gene in 10 unrelated Japanese families with HHD, and found five mutations including four novel mutations of ATP2C1.

Ten genetically unrelated Japanese patients with HHD were analyzed in this study (Table I). There were nine males and one female. They have had no family history of HHD or other skin diseases.

Genomic DNA was extracted from peripheral blood leukocytes using a standard procedure. Pairs of primers spanning all 28 exons and flanking intronic splice sites of the ATP2C1 gene were used to amplify the genomic DNA. Aliquots of the PCR products were analyzed on 2% agarose gel electrophoresis, and 8  $\mu\text{l}$  of the samples were used for conformation sensitive gel electrophoresis (CSGE) analysis (Ganguly *et al*, 1993). Subsequently, PCR products containing the heteroduplexes were subject to direct sequencing by an ABI automated sequencer (PE Applied Biosystems, Foster City, CA). Alternatively, in samples where heteroduplexes were not detected by CSGE analysis, all the PCR products were subject to direct sequencing.

CSGE analysis of PCR fragments detected three abnormal shifts in electrophoretic mobility. Sequencing of these PCR products revealed three different mutations in three cases (Table I). One 457C→T base substitution resulted in a nonsense mutation in exon 7 of case 1 (Fig 1A). This mutation was verified by a DdeI digestion. This was identical to a mutation as reported by Hu *et al* (2000). Also detected was a C490F missense mutation in exon 17 resulting from a 1469G→T substitution in case 2 (Fig 1B). As no enzymatic verification could test this mutation, we performed direct sequencing of exon 17 in 50 normal control subjects to verify this mutation. The mutation 2460delG created a shift in the reading frame and resulted in a downstream PTC at exon 25 in case 10 (Fig 1D). In the other seven cases, we conducted direct sequencing of the entire coding sequence of the ATP2C1 gene, because we could not detect any heteroduplex bands. One L584P missense mutation was found in case 3 (Fig 1C), and was confirmed by Nci I digestion. A splice site mutation occurred in case 10 at the site of a guanine of a conserved GT nucleotide within the donor splice site of intron 12 (Fig 1E). This mutation would alter the correct splicing of exon 12 and result in PTC at eight nucleotides downstream from the donor splice site, although the precise transcription product is unknown as mRNA was unavailable from this case. We were unable to detect any nucleotide changes in five cases despite direct sequencing of the entire genomic coding sequence of the ATP2C1 gene (Table I).

We have analyzed ATP2C1 mutations in 10 genetically unrelated Japanese families with HHD showing typical clinical and histopathologic features of this condition. We have found five attributable mutations including four novel mutations comprising two missense mutations (C490F and L584P), one deletion (2460delG), and one splice-site mutation (1259+1g→a). We could not determine any ATP2C1 mutations in five of the 10 HHD families after direct sequencing of the entire coding region of ATP2C1 genomic DNA, including the exon–intron



**Figure 1. Mutation analysis in HHD families.** (A) Mutation in case 1. A 457C→T base substitution resulted in a nonsense mutation in exon 7. (B) Mutation in case 2. A 1469G→T substitution resulted in a C490F missense mutation in exon 17. (C) Mutation in case 3. A 1751T→C substitution resulted in a L584P missense mutation in exon 19. (D) Mutation in case 4. A 2460delG created a shift in the reading frame and resulted in a downstream PTC in exon 25. (E) Mutation in case 5. A 1259+1g→a splice site mutation resulted in skipping exon 12.

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**Table I. ATP2C1 mutations in patients with HHD**

Patient	Age/sex	Age of onset	Mutation <sup>a</sup>	Location of mutation	Nucleotide change <sup>b</sup>	Consequence
1	55/M	51	R153X	exon 7	457C→T	nonsense
2	54/M	38	C490F	exon 17	1469G→T	missense
3	70/M	40	L584P	exon 19	1751T→C	missense
4	58/M	40	2460delG	exon 25	CATGTT→CATTT	frameshift (PTC+5 aa)
5	62/F	55	1259+1g→a	intron 12	TGGgta→TGGata	skip exon 12 (PTC)
6	45/M	30	ND			
7	45/M	20	ND			
8	33/M	27	ND			
9	44/M	30	ND			
10	63/M	62	ND			

<sup>a</sup>Numbering of the amino acids refers to the peptide sequence.

<sup>b</sup>Numbering of the nucleotides refers to ATP2C1 cDNA sequence, with the first nucleotide of ATG initiation codon as 1. Bases in the exons are denoted by uppercase letters and bases in the introns by lowercase letters. PTC+n aa indicates that the premature termination codon is "n" amino acids downstream of the mutation. X, stop codon; ND, "not detected"; PTC, premature termination codon.

boundaries. All of the cases analyzed have typical features of HHD suggesting several possible causes of this discrepancy. First, we cannot detect large deletions spanning the entire coding region of ATP2C1 with our detection system. Second, we cannot detect intronic mutations, mutations in the promoter regions, or mutations in the 3'-untranslated region. The fact that Hu *et al* (2000) detected only 21 mutations out of a possible 61 HHD cases using the same primer sets supports our findings.

Among the five families in which we could determine mutations, those with missense mutations (cases 2 and 3) and the one with a nonsense mutation in exon 25 (case 4) were predicted to produce abnormal ATP2C1 protein. These three cases showed early clinical symptoms (before the age of 40) compared with those with nonsense mutations in the 5' proximal exons (cases 1 and 5). In cases 1 and 5, severely reduced amounts of ATP2C1 protein are expected to be found because of "nonsense-mediated mRNA decay" (Frischmeyer and Dietz, 1999; Hentze and Kulozik, 1999). mRNA that has a nonsense mutation at the 5' proximal region would result in breakage because of the mechanism that is called "nonsense-mediated mRNA decay", and no abnormal truncated protein would be translated. On the other hand, mRNA that has a missense mutation or a nonsense mutation close to the end of the gene could be translated, and abnormal protein could interfere the action of normal ATP2C1 protein. Although HHD has been considered to be the result of haploinsufficiency of the ATP2C1 gene, the dominant negative effects of abnormal ATP2C1 protein might also contribute to the disease phenotype. Our data provide a significant addition to the HHD mutation

database and will contribute further to the understanding of HHD genotype/phenotype correlations and to the pathogenesis of this disease.

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## Unusual Pemphigus Phenotype in the Presence of a Dsg1 and Dsg3 Autoantibody Profile

To the Editor:

Pemphigus foliaceus (PF) and pemphigus vulgaris (PV) are two autoimmune blistering diseases presenting with distinct but related antigenic specificity and histopathologic features (Amagai, 1995; Stanley, 1995; Suter *et al*, 1998; Anhalt and Diaz, 2001). In both

diseases, the target antigens are transmembrane components of desmosomal junctions that mediate strong intercellular adhesion between keratinocytes (Green and Gaudry, 2000). In PF, the autoantibodies target desmoglein (Dsg) 1, and in PV Dsg3 (Amagai, 1995; Stanley, 1995; Suter *et al*, 1998; Anhalt *et al*, 2001). Whereas the autoantibodies bind to all sites within stratified squamous epithelia where the antigen is expressed, blister formation is restricted to specific locations. In PF, split formation occurs in the subcorneal zone of the epidermis where Dsg1 is present without Dsg3, and in PV blisters form between basal and suprabasal layers of initially mucous membranes where Dsg3 is present without Dsg1

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(Shirakata *et al*, 1998; Mahoney *et al*, 1999). These observations led to the prevailing model that in pemphigus the unaffected Dsg isoform can compensate for the autoantibody-targeted Dsg, and that consequently blisters only form in zones with exclusive expression of the targeted Dsg (Shirakata *et al*, 1998; Mahoney *et al*, 1999; Wu *et al*, 2000). Based on the histopathologic and serologic features, the two pemphigus forms are thus clearly distinct. Nevertheless, rare cases have been reported where histopathologic changes of PV have shifted to PF lesions and *vice versa* (Iwatsuki *et al*, 1991; Kawana *et al*, 1994; Chorzelski *et al*, 1995; Chang *et al*, 1997; Ishii *et al*, 2000; Komai *et al*, 2001; Sami *et al*, 2001). These shifts are usually associated with a change in the profile of Dsg1 and Dsg3 antibodies. Even rarer cases have been published where PF and PV coexist histopathologically without shifting towards one or the other form (Chorzelski *et al*, 1995; Komai *et al*, 2001). In all these latter cases with coexisting PF and PV, the concurrent lesions on skin and mucous membranes correlated with antibodies against Dsg1 and Dsg3 as well as acantholysis in both subcorneal and suprabasilar localization.

We here report another unusual variant of these rare pemphigus cases exhibiting coexisting PF and PV. In contrast to the previous cases (Chorzelski *et al*, 1995; Komai *et al*, 2001), our patient never developed oral lesions, despite skin lesions concomitantly in suprabasilar and subcorneal localization and antibodies against both Dsg1 and Dsg3.

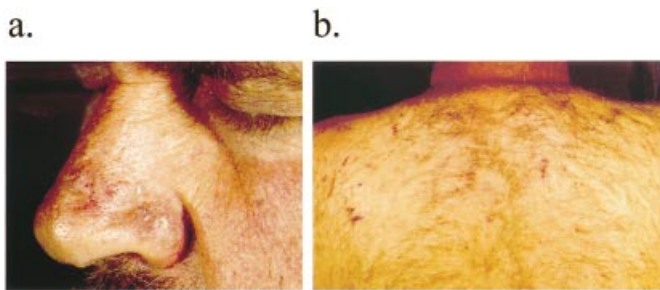
Without a history of previous skin disease and in the absence of any medication, a 51-y-old male developed itching scales and crusts on the scalp in spring 1998. Six months later multifocal, pruritic

erythematous lesions appeared on the rest of the integument, without loss of well being. The patient presented at our outpatient clinic 1 y after onset of the skin lesions. Initially, clinical nosological assignment was difficult. The lesions on the scalp were reminiscent of extensive seborrheic dermatitis, whereas plaque-like lesions on the nose resembled discoid lupus erythematosus or granulomatous dermatosis (Fig 1a). The multifocal lesions on the trunk and extremities, partly covered with hemorrhagic crusts, were reminiscent of eczema (Fig 1b), and a slightly elevated titer of IgE pointed to possible atopic diathesis. Mucous membranes were not affected.

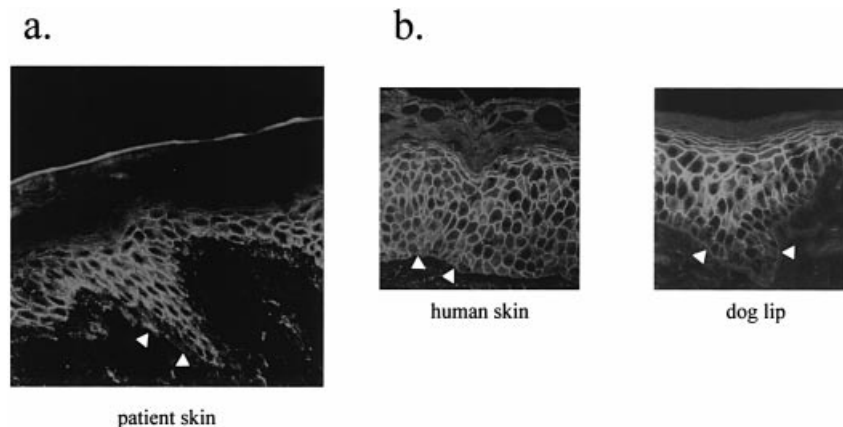
The work-up based on the differential diagnosis listed above was negative for antinuclear antibodies (ANA, dsDNA, and SS-A). A biopsy taken from the back showed an unspecific, superficial perivascular and spongiotic dermatitis, and one taken from the nose a pigmented solar keratosis. Direct immunofluorescence of the biopsy from the nose demonstrated C3 (data not shown) and granular IgG deposits (Fig 2a) along keratinocyte membranes.

The staining pattern for IgG gradually increased in intensity from the *stratum basale* to the *stratum granulosum*. Subsequent indirect immunofluorescence on both human skin and dog lip exhibited intercellular antiepidermal antibodies at a titer of 1:512 with a similarly graded pattern (Fig 2b). This pattern was particularly visible on dog lip where no Dsg1 is expressed in the basal cell layer (Müller *et al*, unpublished observation). In the meantime, the patient developed a stable blister on his left ankle measuring 4 × 3 cm. A biopsy thereof showed a mid-epidermal cleft (data not shown), whereas further biopsies from the scalp revealed epidermal (Fig 3a) and infundibular acantholysis concomitantly located subcorneally as well as suprabasally. This cleft formation typical of PV and PF correlated with antibodies to both Dsg1 and Dsg3, as demonstrated by western blot analysis and an enzyme-linked immunosorbent assay (ELISA) using recombinant proteins (Figs 3b, c; Amagai *et al*, 1999).

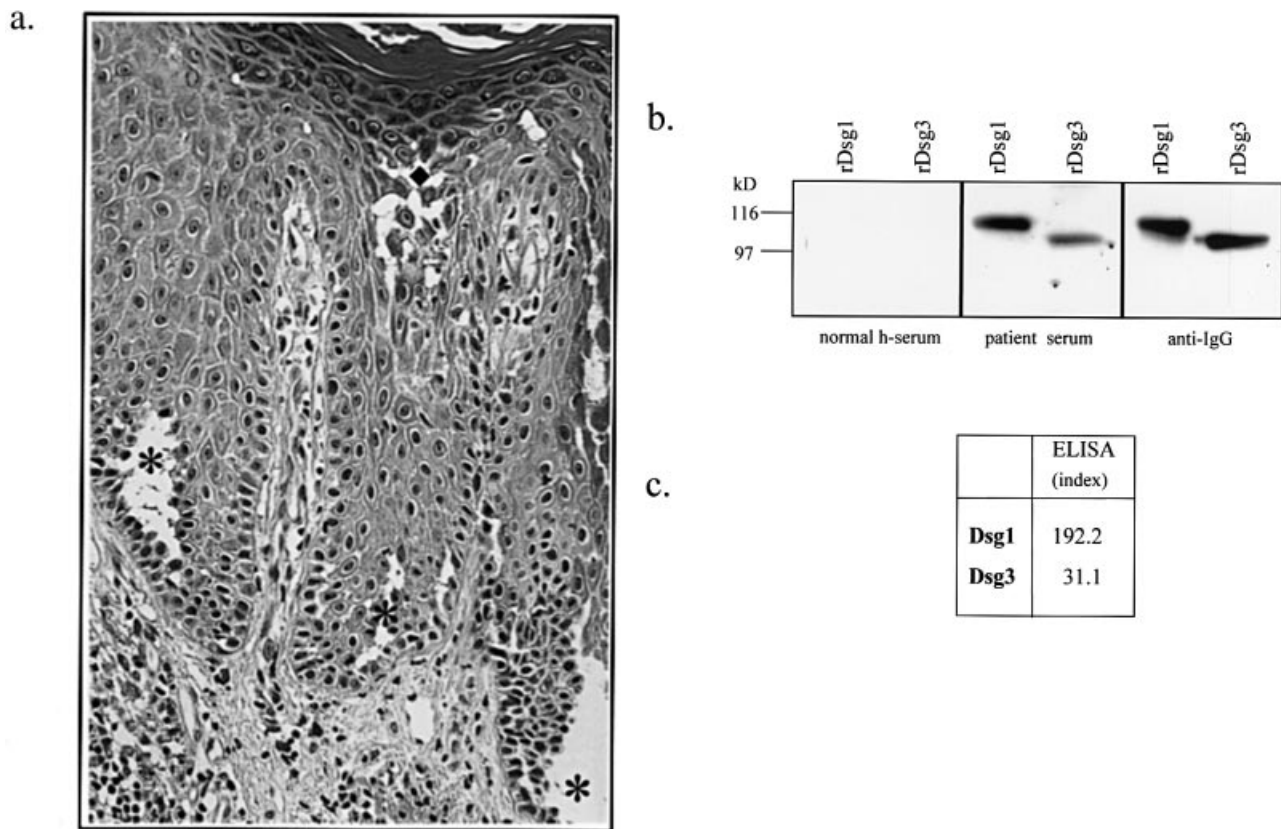
Dsg3 antibodies were present in the serum of the patient at a lower titer than antibodies to Dsg1, consistent with the graded staining pattern seen in the direct and indirect immunofluorescence analyzes (Figs 2a, b). The presence of Dsg1 and Dsg3 antibodies of the IgG4 isotype (revealed by western blot analysis), to which the wide majority of the pathogenic pemphigus antibodies belong (Rock *et al*, 1989; Bhol *et al*, 1995), was consistent with blister formation in a subcorneal and suprabasilar location of the skin. Systemic corticosteroids (initially 1.5 mg prednisone per kg per d) rapidly induced remission and correlated with little remaining intraepidermal IgG deposits as seen after 1 y by direct immunofluorescence.



**Figure 1. Macroscopic view of lesions.** (a) Plaque-like lesions on the nose resembled discoid lupus erythematosus or granulomatous dermatosis. (b) Multifocal lesions on the upper trunk partly covered with hemorrhagic crusts were reminiscent of eczema.



**Figure 2. Direct and indirect immunofluorescence.** (a) A cryosection of a biopsy from the patient's noninflamed peri-lesional skin of the nose was stained with FITC-labeled antihuman antibodies. Note that the granular aspect of IgG deposits likely results from clustering of the antigenic target through cross-linking by the bivalent antibodies (de Bruin *et al*, submitted), which has been described in lesional and perilesional skin of pemphigus patients (Burge *et al*, 1993; Hashimoto *et al*, 1995; Iwatsuki *et al*, 1999). (b) Cryosections of human skin and dog lip taken from healthy donors were incubated with the patient serum in a dilution of 1:512 and revealed with antihuman antibodies. Note that the staining pattern is stronger in the superficial layers of the dog lip than in the deep epithelium where no Dsg1 is expressed (Müller *et al*, unpublished observation). Arrowheads point to the basement membrane zone.

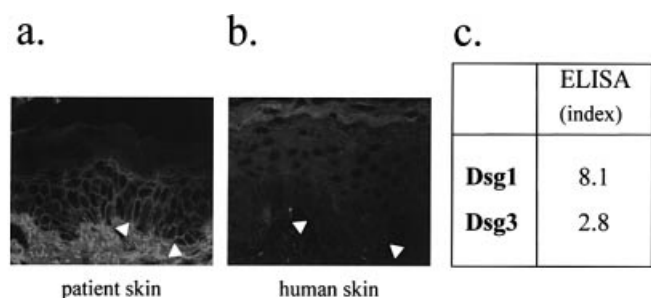


**Figure 3. PV and PF phenotype.** (a) Haematoxylin and eosinophil staining of a section of paraffin-embedded tissue originating from the scalp of the patient. The diamond indicates a blister in PF location and stars those in a PV location. (b) 2.5  $\mu$ l and 5  $\mu$ l, respectively, of supernatant from baculovirus-infected insect cells producing the extracellular domain of Dsg1 (rDsg1) or Dsg3 (rDsg3) in fusion with the constant region of human IgG1 (Fc; Amagai *et al*, 1994), were loaded in triplicates and subjected to western blot analysis. One blot was incubated with normal human serum (*left panel*) and the second with the patient serum (*middle panel*), both in a dilution of 1:100. The secondary antibody was in either case antihuman IgG4 coupled to alkaline phosphatase (HP6025, Southern Biotechnology Associates). As a loading control the last blot (*right panel*) was incubated with antihuman IgG coupled to AP, which binds to the Fc region of the fusion proteins. Antibody binding was revealed with Immuno-Star Substrate Pack. (c) An ELISA with the patient's serum was performed on recombinant Dsg1 and Dsg3 as described previously (Amagai *et al*, 1999). An index above 20 is considered as specific.

fluorescence (**Fig 4a**), and no detectable serum titer as determined by indirect immunofluorescence analysis (**Fig 4b**) and ELISA (**Fig 4c**). No recurrences have been observed within 2 y. In conclusion, the findings of epidermal blisters in a PF as well as PV location in the presence of Dsg 1 and Dsg 3 antibodies, supported by the fact that their clearance correlated with remission, provided compelling evidence that either antibody was pathogenic, the Dsg1 antibodies but also the Dsg3 antibodies, despite the lack of mucosal membrane involvement.

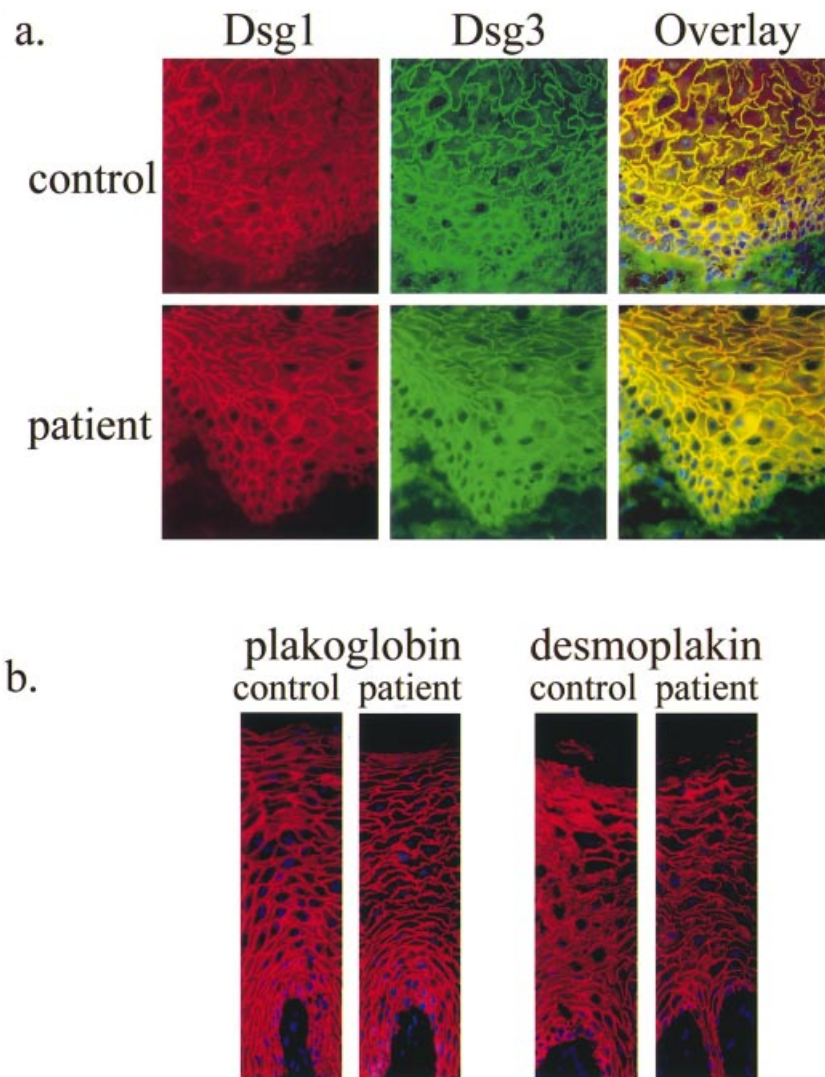
To directly address at the time point of our initial diagnosis whether the mucosa of the patient remained unaffected because of an unusual expression of Dsg1 in the deep layers of oral epithelium, we performed a double labeling immunofluorescence study with antibodies to Dsg1 and Dsg3 using the patient's oral mucosa and the mucosa of a control donor (**Fig 5a**). The distribution of Dsg1 and Dsg3, including desmoplakin, which was used as control for the presence of intact desmosomes (**Fig 5b**), did not differ and was found to correspond with that reported in the literature (Shirakata *et al*, 1998). This indicated that the absence of lesion formation in the patient's oral mucosa was not a consequence of an abnormal Dsg1 expression by basal cells, but had to rely on a different mechanism. Furthermore, even if some Dsg1 protein was to be expressed that remained undetected by the immunofluorescence analysis, lesions formation would nevertheless be expected, as pathogenic Dsg1 antibodies are present in the serum of the patient.

Recently another patient has been described, presenting with autoantibody deposits in the deep and upper epidermis, the sites



**Figure 4. Immunopathology and ELISA 1 y after remission.** (a) Cryosections from the scalp of the patient were tested by direct immunofluorescence as described in **Fig 2a**, except that the photographic exposure was prolonged to illustrate a faint anti-IgG staining (as seen by the substantial back-ground in the dermis). Arrowheads point to the basement membrane zone (also in (b)). (b) Indirect immunofluorescence was performed as described in **Fig 2b** at a dilution of the patients serum of 1:10. The nuclei were counter-stained with DAPI. (c) The same serum as in (b) was tested by ELISA (Amagai *et al*, 1999). The titer between 10 and 20 is considered as gray zone, and below 10 as nonspecific.

of Dsg3 and Dsg1 expression, respectively (Izumi *et al*, 1998). Despite presumable presence of Dsg3 and Dsg1 antibodies (the antigenic specificity of the patient's serum was not identified on recombinant Dsg1 and Dsg3), no mucosal involvement was observed. Similarly, eight patients have recently been reported



**Figure 5. Expression of desmosomal components in the mucosa of the patient.** (a) Cryosections from the patient and a healthy donor were incubated concomitantly with anti-Dsg1 (red; DG 3.10; recognizes also Dsg2) and anti-Dsg3 (green; RDI, Flanders, NJ) antibodies. After incubation with the relevant secondary antibody, the tissue was fixed with 2% paraformaldehyde and analyzed. (b) Cryosections were fixed with 2% paraformaldehyde and permeabilized with 0.5% Triton at room temperature for 15 min prior to incubation with plakoglobin-specific antibodies (PG 5.10; a kind gift of P. Wheelock, Nebryka Medical Center, Oyaha) or antibodies to desmoplakin (multiepitope cocktail, Progen, Germany). For each antibody in (a) and (b) experimental settings and photographic exposures were held constant between the sections from the patient and the healthy donor.

presenting with a mixed antibody profile and no mucosal involvement (Sami *et al*, 2001). Even though both of these reports indicated that antibodies to Dsg1 and Dsg3 can be present without lesions on mucous membranes, the pathogenicity of the Dsg3 antibodies in these patients is questionable because no suprabasal acantholysis has been demonstrated. The situation was different in a dog with PV who developed blisters in the deep layers of the epidermis but not in mucous membranes (Olivry *et al*, 1992). Although it can presently not be excluded that the pathogenesis in man and dog differ to some extent, this latter case appears consistent with our finding that the presence of pathogenic Dsg3 antibodies, which are able to induce skin lesions in a PV localization, does not invariably imply mucosal involvement.

What could account for lack of blister formation in the mucosa despite the presence of pathogenic Dsg3 antibodies? It could be argued that the titer of circulating Dsg3 antibodies in our patient was too low to cause blisters in the mucosa but was sufficient for blister formation in the basal layers of the epidermis. In these layers, the presence of pathogenic Dsg1

antibodies may exacerbate the effect of pathogenic Dsg3 antibodies, similarly as described by Mahoney *et al* (1999). This enhancement would not occur in the mucosa as Dsg1 antibodies only bind to the basal layer of the skin and not to that of mucous membranes. Nonetheless, a lower Dsg3 antibody titer than the one measured in our study was found to cause skin blisters and to also induce mucous membrane lesions in another patient (Komai *et al*, 2001). Alternatively, we and others have recently shown that lesion formation also depends on the activation of intracellular signaling pathways (Kitajima *et al*, 1999; Caldelari *et al*, 2001). Our studies demonstrated that plakoglobin, a component of the desmosomal plaque, is essential in this process and that its absence can abolish responsiveness. Similarly as in plakoglobin null mutant cells (Caldelari *et al*, 2001), the pathogenic response mechanisms may be impaired in the mucosa of some patients, protecting them from lesion development. As our patient had a normal distribution pattern of plakoglobin as compared with a healthy donor (Fig 5b), it might be the lack of another, yet to be defined element of the pathogenic signaling pathway that enables cells in the oral

mucosa of rare patients to resist pathogenic Dsg3 antibodies. Such a mechanism would also explain the finding in the dog patient (described above) with PV and no apparent PF lesions (Olivry *et al*, 1992). Even though this explanation is still speculative, in-depth analysis of such unusual cases will provide us with further insight into the pathophysiology of pemphigus. Furthermore, the elucidation of a mechanism leading to "resistance" to pathogenic antibodies would represent a potent tool to develop the novel therapeutic strategies pemphigus patients need.

In conclusion, we report a patient with features of both PF and PV, including skin blisters in suprabasilar as well as a subcorneal localization, but without mucosal involvement. This represents, to the best of our knowledge, the first example in which concomitant PF and PV lesions are depicted within the same location and more importantly, the first example in humans that provides evidence for the presence of circulating pathogenic Dsg3 antibodies despite lack of mucosal involvement. Even though such cases are probably very rare, they will hopefully provide the key for a better understanding of the pathophysiology of pemphigus.

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## Increased Macrophage Migration Inhibitory Factor (MIF) in the Sera of Patients with Extensive Alopecia Areata

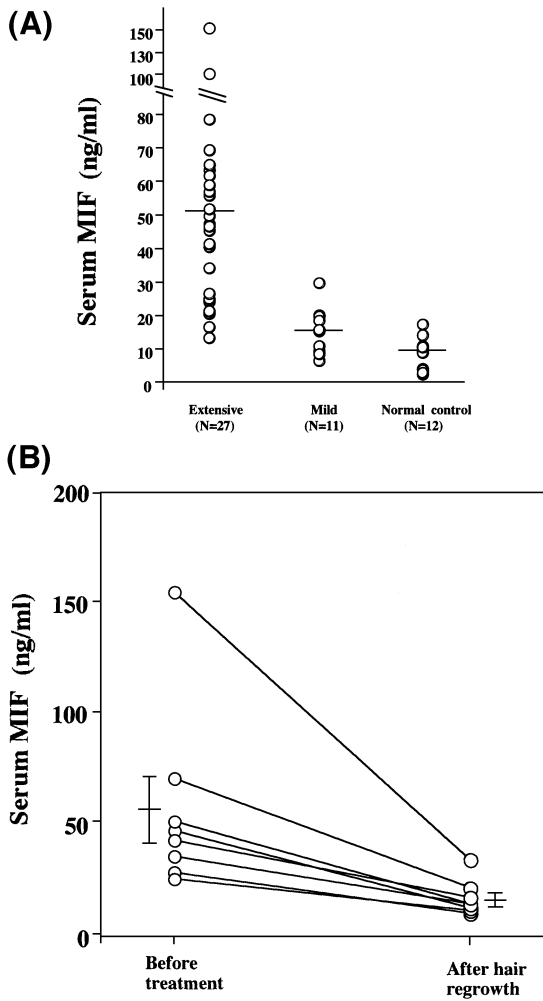
To the Editor:

The pathogenesis of alopecia areata is still uncertain. The immune system has been implicated in the pathogenesis of alopecia areata

and certain immunomodulatory cytokines play an important role in this disease. The contribution of cytokines thought to be involved in the pathogenesis of extensive alopecia areata has been studied. Several lines of clinical and experimental data point towards cytokines such as interleukin (IL)-1 and tumor necrosis factor (TNF)- $\alpha$ , which may be crucial inducers of hair loss in alopecia areata. For example, IL-1 has been shown to inhibit hair growth *in vitro* and may be one of the factors triggering the arrest of hair growth *in vivo* (Harmon and Nevins, 1993). TNF- $\alpha$  also inhibits

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**Figure 1. Serum MIF contents of alopecia areata patients.** (A) Serum MIF concentration in alopecia areata patients and healthy individuals. ELISA measured the serum level of MIF for patients with extensive alopecia areata ( $n = 27$ ), patients with mild alopecia areata ( $n = 11$ ), or healthy individuals ( $n = 12$ ). Serum MIF levels were significantly increased in extensive alopecia areata compared with mild alopecia areata or healthy controls ( $p < 0.001$ ). (B) Serum MIF levels before (>50% bald area of the scalp) and after hair regrowth (100% hair regrowth of the scalp; 6 mo to 2 y, mean duration  $12.5 \pm 2.1$  mo) ( $n = 8$ ). The mean serum MIF concentration before treatment was  $55.8 \pm 14.9$  ng per ml, whereas that after the hair regrowth was  $15.7 \pm 2.8$  ng per ml ( $p < 0.01$ ).

hair follicle growth *in vitro* (Philpott *et al*, 1996). Thus, IL-1 and TNF- $\alpha$  may play a role in the pathophysiology of inflammatory hair loss in alopecia areata.

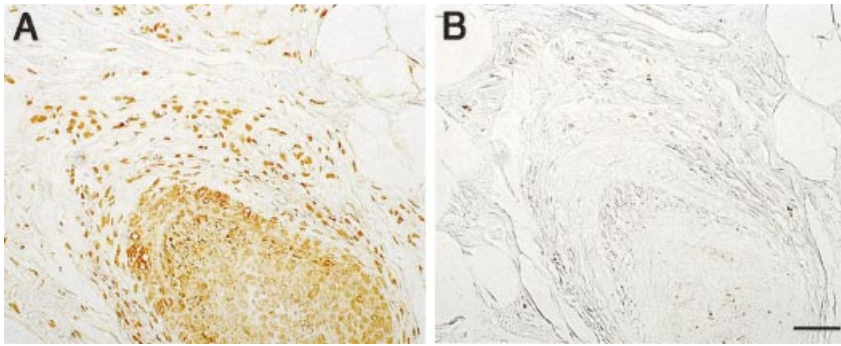
MIF is the first lymphokine reported to prevent the random migration of macrophages (Bloom and Bennett, 1966). A recent finding demonstrated that MIF functions as an initiator of inflammation and the immune response by the regulation of a number of proinflammatory cytokines, including TNF- $\alpha$  and IL-1 (Calandra *et al*, 1994). In human inflammatory diseases, MIF has a regulatory role in acute respiratory distress syndrome, asthma, and rheumatoid arthritis. In skin diseases, we have reported that MIF production by peripheral blood mononuclear cells was markedly upregulated in patients with atopic dermatitis and that increased serum MIF levels were observed (Shimizu *et al*, 1999). We postulated that MIF might play a key role in the pathogenesis of extensive alopecia areata. In this study, we analyzed the serum MIF concentration in patients with alopecia areata and normal healthy individuals.

The study group composed of 27 patients with extensive alopecia areata (with >50% bald area of the scalp; aged from 13 to 32 y, mean age  $21.3 \pm 1.3$  y; nine males and 18 females; the duration of their alopecia areata was between 6 mo and 11 y, mean 3.8 y), 11 patients with mild alopecia areata (1–3 patchy hair loss lesions with < 10% bald area of the scalp and with an inactive condition; aged from 11 to 36 y, mean age  $24.9 \pm 1.6$  y; two males and nine females; the duration of their alopecia areata was between 2 mo and 3 y, mean 1.2 y), and 12 normal healthy individuals (aged from 18 to 45 y, mean age  $29.3 \pm 1.8$  y; four males and eight females). None of the patients was having topical immunotherapy, systemic, or topical steroids therapy at the time of the study. Eight patients with extensive alopecia areata showed successful hair regrowth (100% hair regrowth) when treated with topical sensitizer (squaric acid dibutylester or diphencyprone) and the serum was obtained at least 1 mo after the final topical therapy. The serum level of MIF was measured by enzyme-linked immunosorbent assay (ELISA) as described previously (Shimizu *et al*, 1999). MIF levels were compared using the Student's *t* test ( $p < 0.05$ ). Five patients' scalp biopsy specimens with an extensive alopecia areata were immunohistochemically examined for MIF immunoreactivity. Sections were stained using an avidin-biotin-peroxidase complex procedure using a Vector ABC kit according to the manufacturer's protocol (Shimizu *et al*, 1996).

The mean serum MIF concentration in extensive alopecia areata patients ( $n = 27$ ) was  $50.6 \pm 5.7$  ng per ml (mean  $\pm$  SE), whereas that of mild alopecia areata ( $n = 11$ ) or healthy individuals ( $n = 12$ ) was  $15.1 \pm 2.1$  ng per ml or  $8.9 \pm 1.3$  ng per ml, respectively ( $p < 0.001$ ) (Fig 1A). In eight extensive alopecia areata patients serum MIF was also examined before (>50% bald area of the scalp) and after hair regrowth (100% hair regrowth of the scalp). The mean serum MIF concentration before treatment was  $55.8 \pm 14.9$  ng per ml, whereas that after the treatment was  $15.7 \pm 2.8$  ng per ml ( $p < 0.01$ ) (Fig 1B). Immunohistochemical studies with an anti-MIF antibody were positive in perifollicular-infiltrated lymphocytes of telogen hair follicles in patients with extensive alopecia areata (Fig 2).

The results presented here demonstrate that the mean levels of MIF in the sera were significantly elevated in patients with extensive alopecia areata. Alopecia areata is considered to be a T cell-mediated autoimmune disease involving the hair follicle, which is characterized by perifollicular infiltration by activated T cells (Bodemer *et al*, 2000). Although the function of these T cells in the pathogenesis is still unknown, cytokines released from T cells are important mediators leading to hair loss in alopecia areata. It is speculated that MIF in inflammatory diseases may be produced by multiple cellular sources such as activated T lymphocytes and monocytes. On the basis of our immunohistochemical results, we speculate that activated T cells might be a potential source of serum MIF. MIF is known to stimulate the production of proinflammatory cytokines such as IL-1 and TNF- $\alpha$  by macrophage and vice versa (Bacher *et al*, 1996). From the data available to date, together with these results, we believe that a positive feedback loop may be the cause of the inflammatory interaction between IL-1, TNF- $\alpha$ , and MIF in this disease. It is known that the proinflammatory mediators IL-1 and TNF- $\alpha$  are potent inhibitors of hair follicle cell proliferation with a concomitant inhibition of hair growth (Philpott *et al*, 1996). Therefore, these inflammatory cytokines may be implicated in the induction or continuation of damage of hair follicles and MIF may play an important part in the pathophysiology of inflammatory hair loss conditions such as alopecia areata.

Whereas alopecia areata is a common disease, treatment of its extensive form is difficult and its outcome is not easily predicted. Recent work demonstrated that anti-MIF antibodies have a potent therapeutic action in the severe inflammatory condition such as murine hepatitis (Kobayashi *et al*, 1999). We assume that the elevated serum levels of MIF may reflect the inflammatory symptoms in extensive alopecia areata and that control of MIF production may have important therapeutic implications.



**Figure 2. Immunohistochemical analysis of MIF expression in an extensive alopecia areata scalp biopsy.** Tissue specimens were stained with a Vector ABC staining kit using polyclonal antihuman MIF antibody. (A) Intense MIF staining was observed in the perifollicular-infiltrated lymphocytes of telogen hair follicles. Hair follicle MIF staining was also apparent. (B) No specific positive staining was observed using the tissue sample stained with preimmune rabbit IgG. Scale bar: 50  $\mu$ m.

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## Evidence for Extensive Locus Heterogeneity in Naxos Disease

To the Editor:

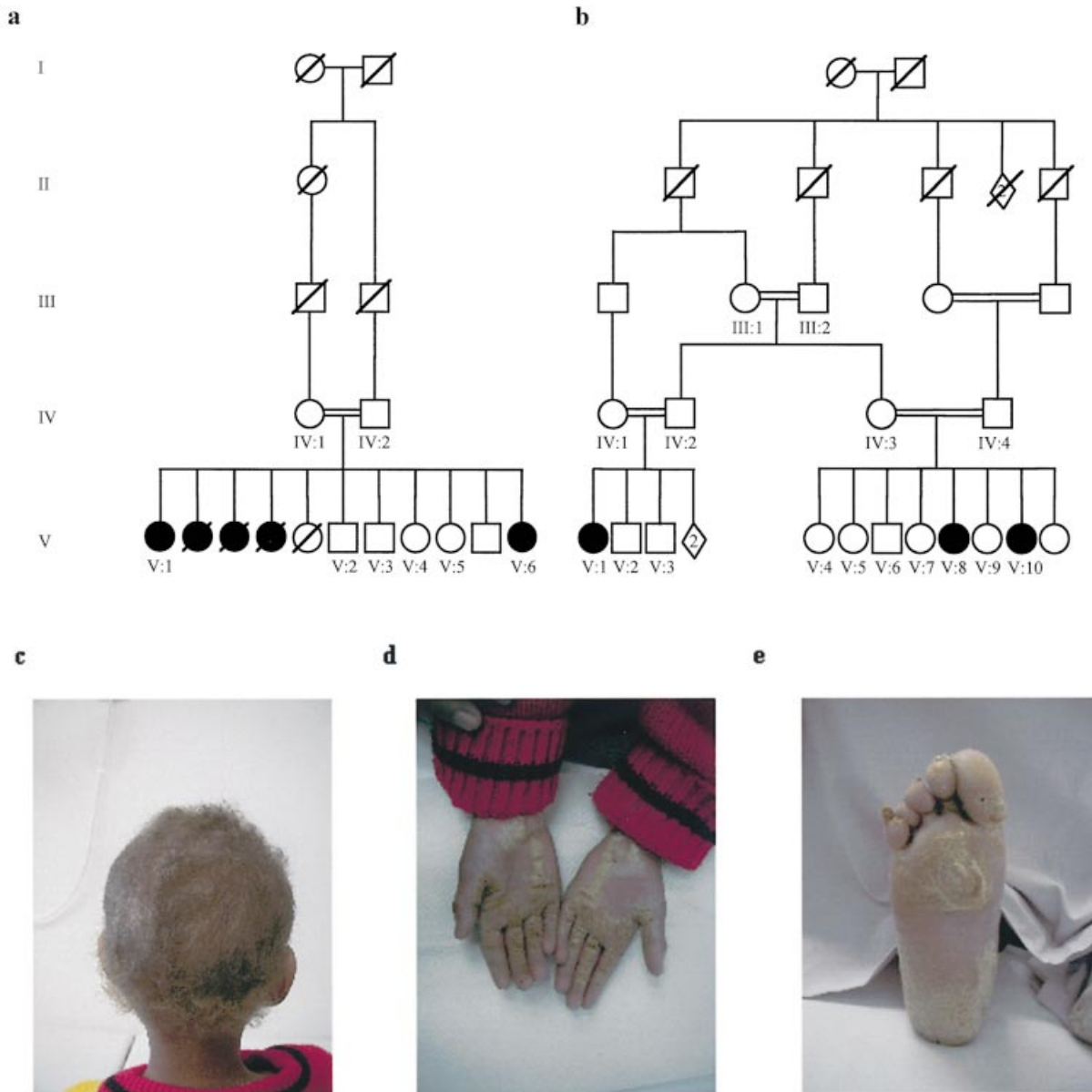
Naxos disease is a rare autosomal recessive disease that consists of an associated triad of woolly hair, thickened palms and soles (keratoderma), and heart involvement. The hair phenotype is unique, characterized by congenital woolly, curly, rough, and light colored scalp hair and sparse eyebrows. The nonepidermolytic keratoderma appears during the first years of life and involves mainly pressure areas in the palms and soles. The heart manifestations appear during the teenage years and are severe and progressive and may end with arrhythmia and premature sudden death. The disease was originally described in individuals from the Greek Island Naxos by Protonotarios *et al* in 1986 and by Barker *et al* in 1998. In 1998, Carvajal-Huerta reported patients from Guayaquil, Ecuador with a similar autosomal recessive triad, with combined epidermolytic palmoplantar keratoderma with woolly hair and dilated cardiomyo-

pathy, and described the skin manifestations. Another family with the autosomal dominant association of nonepidermolytic palmoplantar keratoderma, woolly hair, and dilated right ventricle was reported by Tosti *et al* (1994) in an Italian family. Recently, Coonar *et al* (1998) mapped the gene for the Greek families (Naxos disease) to 17q21, and a mutation in the plakoglobin gene was identified as responsible for the disease in the Greek families (McKoy *et al*, 2000). In the Ecuadorian family, a mutation in the desmoplakin gene was found to be responsible for the disease (Norgett *et al*, 2000). In this study, we report the clinical findings in two new Arab families with Naxos disease originating from villages near Jerusalem. Importantly, we have excluded both plakoglobin and desmoplakin as the candidate genes in these families. Furthermore, we have analyzed several other regions harboring candidate genes of interest, and found no evidence for linkage.

The pedigree structures of the two families are shown in **Fig 1(a, b)**. The history and clinical examination of patients from families A and B included the congenital appearance of woolly, curly, rough, light colored scalp hair with sparse eyebrows, axillary, and pubic hair (**Fig 1c**). Skin involvement included palmoplantar keratoderma (**Figs 1d, e**) starting around age 3, as well as follicular keratosis on extensor arms, shins, back, and cheeks, lichenoid papules mainly on the lower shins and psoriasiform keratosis. A plantar skin biopsy

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**Figure 1. Pedigrees and clinical presentation of Naxos families A(a) and B(b).** (a, b) Pedigrees of the two Naxos disease families from Israel. Filled circles and squares represent affected females and males, respectively. A diagonal line through a symbol indicates a deceased individual. Double lines are indicative of consanguinity. (c–e) Clinical presentation of the Naxos disease phenotype. Note in (c) the woolly and light colored, sparse hair typical of affected patients. (d, e) The presence of marked palmoplantar hyperkeratosis is also a hallmark of this phenotype.

taken from affected individuals in these families showed two types of keratoderma, specifically, epidermolytic in family A and non-epidermolytic in family B. Furthermore, hair plucked from the scalp of members of the two families revealed in affected members plenty of trauma-related hair shaft abnormalities, including longitudinal and oblique fractures, tapered hairs, trichorrhexis nodosa like lesions, pseudomonilethrix, twisted and corkscrew-like hair, without pili torti. Hairs were of different diameters and some of them were curly. Electrocardiogram and echocardiogram were performed, following the history of a sudden death at age 18 in one affected family member (from family A) and complaints of dizziness, syncope, and chest pain in other members. The electrocardiogram demonstrated tachycardia in the young members and different types of arrhythmia, including ventricular premature beats, couplets, triplets, and nonsustained VT in older members. In two members (family A, V-1, V-6) the echocardiogram showed right ventricular dysplasia with right ventricular dilatation and

decreased function of the right and left ventricles. These clinical observations provided the final diagnosis of arrhythmogenic right ventricular cardiomyopathy (ARVC).

Since all the affected members descended from consanguineous couples, we hypothesized that the impaired gene must have arisen from the same ancestral mutation in both alleles. Thus, heterozygosity for the candidate gene/region was considered sufficient for exclusion of genetic linkage. Although both families originated from the same geographic region in Israel, suggesting they might be related, we elected to evaluate each candidate locus allowing for genetic heterogeneity. First, we screened for mutations in the human desmoplakin (NM\_004415) and plakoglobin (AJ249711) genes (Franke *et al*, 1989; Virata *et al*, 1992). Sequence analysis of each exon and splice junctions of the desmoplakin and plakoglobin genes (Whitlock *et al*, 1999, 2000) from affected individuals revealed no mutation in both genes compared with unrelated unaffected individuals. Furthermore, we identified several heterozygous

**Table I. Candidate genes excluded from linkage to Naxos disease**

Gene/loci	Chromosome location	Markers <sup>a</sup>	Distance (cM)
Plakophilin 1	1q32-q44	D1S2622	214.08
		D1S477	215.17
		D1S2686	216.46
Plakophilin 4	2q23-q31	D2S2241	156.92
		D2S321	157.55
		D2S284	161.81
		D6S477	9.18
Desmoplakin	6p24.3	D6S470	18.22
		D6S2434	25.08
		D11S1765	61.78
Desmoyokin	11q13.1	D11S1883	65.05
Type II keratin	12q13.13	D12S1661	63.89
		D12S390	67.63
		D12S398	68.16
Plakophilin 2	12p13	D12S1053	51.99
		GATA63D01	53.64
		GATA123B12	55.50
		D17S800	62.01
Type I keratin	17q21.2	D17S1789	63.09
Plakoglobin	17q21.1	D17S800	62.01
		D17S1789	63.09
Desmocollins/desmogleins	18q12.1	D18S819	52.86
		D18S847	56.71
		GATA173A03	62.29

<sup>a</sup>PCR primers for the microsatellite markers were designed according to the sequences on the Genome Database and the Cooperative Human Linkage Center (<http://www.gdb.org>; <http://lpg.nci.nih.gov/CHLC>).

sequence changes in the introns of affected individuals, supporting the exclusion of these genes (data not shown).

Second, on the basis of the role of both desmoplakin and plakoglobin in cell adhesion, several other genes coding for components of the desmosomes or proteins involved in different aspects of cell adhesion were considered as candidate genes for the disease in our families. These included the genes coding for type I and type II keratins on chromosomes 17 and 12, respectively (Romano *et al*, 1988; Yoon *et al*, 1994; Aberle *et al*, 1995; Coonar *et al*, 1998), desmoyokin on 11q13.1 (Courseaux *et al*, 1996), and the desmocollin/desmoglein cluster on 18q12.1 (Arnemann *et al*, 1991). We also analyzed markers for plakophilin 1 at 1q32 (Cowley *et al*, 1997), plakophilin 2 at 12p13 (Bonne *et al*, 1998), and plakophilin 4 (or p0071) at 2q23-q31 (Hatzfeld and Nachtsheim, 1996; Bonne *et al*, 1998). As the gene structures were not available for each of these genes, we performed cosegregation and homozygosity studies using microsatellite markers covering these loci (Table I). As a result, none of the regions analyzed showed cosegregation with the disease trait. Moreover, heterozygosity in the affected members could be used to exclude the regions on chromosomes 12, 17, and 18, harboring the keratins and desmocollin/desmoglein gene clusters, respectively, as well as for the chromosomal regions containing plakophilin 1, 2, and 4 as specified in Table I.

These findings emphasize the heterogeneous genetic basis of Naxos disease, which appears to segregate in a dominant manner as well as the autosomal recessive forms (Tosti *et al*, 1994). Our future work will be directed toward the evaluation of additional desmosomal proteins, as well as conducting a whole genome scan to find regions homozygous by descent, and identify a novel causative gene(s) underlying Naxos disease.

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## The Use of Histamine in Cancer Immunotherapy

To the Editor:

We read with interest the article by Hegyesi *et al* (2001) that reports on the expression of histidine decarboxylase (HDC) in human melanoma cells *in vivo* by western blotting and immunostaining on tissue specimens from patients with primary melanoma ( $n = 20$ ), melanoma metastases ( $n = 5$ ), nevi ( $n = 3$ ), and normal skin ( $n = 10$ ). Furthermore, the report demonstrated that a HDC-specific antisense oligonucleotide (ASO) suppressed melanoma cell proliferation in two cell lines *in vitro* compared with a nonsense 18-mer ASO. The authors speculate that endogenous histamine may act as an autocrine growth factor, influencing cell proliferation via H2 histamine receptors (Burtin *et al*, 1988; Bolton *et al*, 2000), or that melanoma growth is indirectly favored by locally produced histamine that suppresses IFN- $\gamma$  production of surrounding immune cells and therefore contributes to immune escape of melanoma cells. Consequently, the authors argue, not only traditional antihistaminics such as H2 histamine antagonists but also ASO targeting HDC may soon become candidates for use in melanoma treatment.

As some experimental findings as well as current clinical strategies are completely opposed, we would like to comment on some of the reported statements of Hegyesi *et al* (2001).

First of all, we believe that the methodology employed to determine HDC (e.g., western blot) is not adequate to draw any firm conclusions as it is long known that many tumors, including melanoma, are surrounded by significantly larger numbers of mast cells containing histamine and HDC compared with nevi and normal skin (Schadendorf *et al*, 1995). Furthermore, mast cell counts are highly dependent on anatomical sites (Eady *et al*, 1979; Cowen *et al*, 1979). Neither anatomical location nor the number of mast cells is taken into account when tissues specimens are analyzed by western blotting, thus leading to false hypotheses. Nevertheless, the suppression of melanoma cell proliferation in two cell lines *in vitro* is convincing. It cannot, however, be ruled out that other genes critically involved in proliferation are affected which also contribute to growth retardation. Our own data demonstrate that histamine within a concentration range of 1–1000  $\mu\text{M}$  had no influence on melanoma cell proliferation *in vitro* (D.S. and C.S., unpublished results).

Instead of using antihistaminics (as was suggested by these authors), histamine was introduced into the treatment of patients with metastatic melanoma with some success in combination with interleukin-2 (IL-2) (Hellstrand *et al*, 1994, 2000; Hauschild *et al*, 2001; Agarwala *et al*, 2002). Although the exact basis of action is presently unclear, preclinical data suggest that histamine protects T cells and natural killer cells against oxidative stress (Hansson *et al*, 1999) and leads to synergistic activation of lymphocytes *in vitro*. Furthermore, histamine and IL-2 synergize in activating such effects (Hellstrand *et al*, 2000). In pilot studies (Hellstrand *et al*, 1994, summarized in Hellstrand *et al*, 2000) as well as in a prospective, randomized multicenter study (Agarwala *et al*, 2002), it became clear that histamine dihydrochloride (Ceplene; previously known as Maxamine) exhibits unexpected clinical benefits in patients with metastatic melanoma of the liver (Hauschild *et al*, 2001; Agarwala *et al*, 2002), thus achieving for the first time significant survival advantage for metastatic melanoma patients using a combination of histamine and IL-2 in comparison with patients treated with IL-2 only.

In conclusion, as histamine and HDC have been detected in other cells besides melanoma cells, differentiating analytical methods have to be chosen appropriately. Conclusion and suggestions regarding possible clinical benefits have to be drawn with utmost caution. Whether histamine dihydrochloride in combination with IL-2 is really of benefit in a subset of melanoma patients is currently being tested in a confirmatory prospective, randomized, international multicenter trial in patients with liver metastases. Results can be expected in 2–3 y.

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## Reply

To the Editor:

Earlier and recent data (cited by the letter of Dr. Schadendorf) about the combined application of exogenously added histamine and interleukin (IL) seem to indicate a promising approach in melanoma therapy. The effect is likely based on an earlier view (Hellstrand *et al*, 2001) that histamine suppresses the inhibitory action of macrophages on natural killer cells.

Our and others' recent and earlier data suggest five things:

1. Regardless of the sometimes debated presence of mast cells in melanoma tissue, there is endogenous expression of histidine decarboxylase (HDC) mRNA (thus presence of HDC is proved not only by western blots, but also by HDC mRNA being visualized by *in situ* hybridization) and immunoreactive histamine within melanoma cells (Haak-Fredescho *et al*, 2000). This fact and the amount of HDC is not in direct relation with poor prognosis of melanoma; however, suppression of melanoma cell growth with HDC-specific antisense oligonucleotides suggests that the involvement of locally produced histamine is hardly questionable.

2. Histamine receptor (HR) 1 agonists directly decrease, whereas HR2 agonists directly increase proliferation of melanoma cells (Reynolds *et al*, 1996; Lazar-Molnar *et al*, submitted), thus actual and local ratio between HR1 and HR2 on melanoma cells seems to be critical for the net outcome of the action of endogenously produced histamine.

3. HR2 antagonist cimetidine, particularly if combined with a tamoxifen derivative N,N-diethyl-2-[4-(phenylmethyl)phenoxy]-ethanamine-HCl (DPPE) (Brandes *et al*, 1991), strongly decreases tumor mass and increases survival of immunodeficient SCID xenotransplanted with human melanoma cell line (Szincsak *et al*, in press).

4. There are abundant clinical data on the beneficial effect of HR2 antagonists on carcinoma (Nielsen, 1996; Bolton *et al*, 2000).

5. Concerning the interactions with local immune response, reciprocal inhibitory interactions are found between histamine and interferon  $\gamma$  (Horvath *et al*, 1999; Heninger *et al*, 2000). Furthermore, histamine and locally produced IL-6 are mutually stimulatory (Lazar-Molnar *et al*, 2000; Lazar-Molnar *et al*, submitted). This fact further emphasizes the role of local histamine, as IL-6 (partially generated by the melanoma cells themselves) is also one of the multiple growth factors for melanoma depending on the metastatic potential of the tumor. Interestingly, IL-6 influences the local expression pattern of histamine receptors, this fact provides a further factor in the autocrine and paracrine effect of histamine on melanoma cells.

In conclusion, impressive data suggest that therapeutically administered histamine potentiates the effect of IL-2, as an adjuvant or by another, not yet discovered mechanism. Nevertheless, this is not in controversy with our view that locally produced histamine acting on paracrine or autocrine way influence tumor growth on a receptor (i.e., signal pathway)-dependent manner and endogenously produced and acting histamine shifts local T cell response toward Th2 polarization.

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