Keratin 13 point mutation underlies the hereditary mucosal epithelia disorder white sponge nevus

Gabriela Richard[1], Vincenzo De Laurenzi[1,2], Biagio Didona[3], Sherri J. Bale[1] & John G. Compton[1]

Although pathogenic keratin mutations have been well characterized in inherited epidermal disorders, analogous defects in keratins expressed in non-epidermal epithelia have yet to be described. White sponge nevus (WSN) is a rare autosomal dominant disorder of non-cornifying squamous epithelial differentiation that presents clinically as bilateral white, soft, thick plaques of the oral mucosa. Less frequently the mucous membranes of the nose, esophagus, genitalia and rectum are involved[1,2]. Histopathological features, including epithelial thickening, parakeratosis, extensive vacuolization of the suprabasal keratinocytes[2] and compact aggregates of keratin intermediate filaments (KIF) in the upper spinous layers[3,4], resemble those found in epidermal disorders due to keratin defects. We analysed a multigenerational family with WSN and found cosegregation of the disease with the keratin gene cluster on chromosome 17. We identified a missense mutation in one allele of keratin 13 that leads to proline substitution for a conserved leucine. The mutation occurred within the conserved 1A region of the helical rod domain, which is critical for KIF stability and is the site of most pathogenic keratin mutations. This mutation enlarges the spectrum of keratins with disease-causing defects to include mucosally expressed keratin 13, and extends the known keratin diseases to disorders of non-cornifying stratified squamous epithelia.

The keratin family of over 20 proteins is expressed in tissue-specific patterns and forms the KIF cytoskeleton of epithelial cells. Sequence similarities identify type I and type II keratin subclasses; their genes are generally clustered in two loci on chromosomes 12 (type II) and 17 (type I). KIF are assembled from heterodimeric subunits comprised of a type I and a type II protein (for example, keratin 10/ keratin 1 or keratin 14/ keratin 5). Keratins have highly homologous central α-helical rod domains flanked by variable size amino and carboxyl terminal domains (Fig. 1) (ref. 5). Over 60 different single-amino acid substitutions in epidermal keratins have been reported in genodermatoses, with two-thirds of the mutations affecting the highly conserved sequences at the boundaries of the helical rod-domain (the 1A and 2B regions)[6]. Evidently normal KIF structure and function is critically dependent on these regions of either keratin in the heterodimers.

WSN shares clinical, histological and ultrastructural features with other inherited leukokeratoses, including pachyonychia congenita type Jadassohn-Lewandowsky (PC1). Although PC1 also features palmar-plantar and nail hyperkeratosis, the mucosal lesions are virtually identical to those in WSN[7]. Mutations in keratins 16 and 6 have recently been associated with PC1 (refs 8, 9). Indeed, KIF aggregation and vacuolization of suprabasal keratinocytes in WSN and PC1 are similar to structural abnormalities seen in other hyperkeratotic epidermal disorders that are known to be caused by keratin defects[6]. These include epidermolytic hyperkeratosis (EH) with underlying defects in keratins 1 and 10, ichthyosis bullosa of Siemens caused by mutations of keratin 2e, and epidermolytic palmar-plantar hyperkeratosis (EPPK) resulting from mutations in keratin 9. The distribution of clinical features in these diseases generally corresponds to the expression pattern of the defective proteins. In WSN, the restriction of lesions to mucosal epithelia and the suprabasal cell histopathology parallels the tissue-specific expression of keratins 4 and 13 in the differentiating cell layers[10,11].

We ascertained an Italian multigenerational family with WSN (Fig. 2), and performed genetic linkage analysis to test if WSN in this family localized to either of the two known keratin gene clusters on chromosomes 12q13 and 17q21. Recombination was observed between WSN and *D12S96*, *D12S368* and *D12S390*,

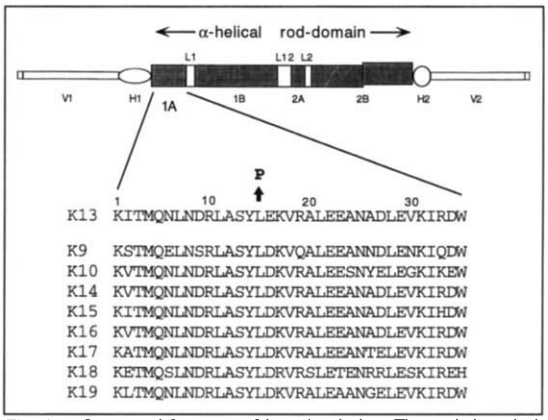

Fig. 1 *a*, Structural features of keratin chains. The rod domain is comprised of α–helical segments 1A, 1B, 2A and 2B (*shaded boxes*), interrupted by non-helical linker regions L1, L12 and L2. Flanking the rod domain are homology regions H1 and H2 (the latter in type II keratins only), and variable terminal domains. *b*, The amino acid sequence of the 1A helical region is highly conserved throughout the type I keratins. The arrow indicates the position of the K13 mutation Leu15Pro identified in WSN. Sequences from the Swiss Protein Database, accession numbers P13646 (keratin 13), P35527 (keratin 9), P13645 (keratin 10), P02533 (keratin 14), P19012 (keratin 15), P30654 (keratin 16), Q04695 (keratin 17), P05783 (keratin 18), P08727 (keratin 19).

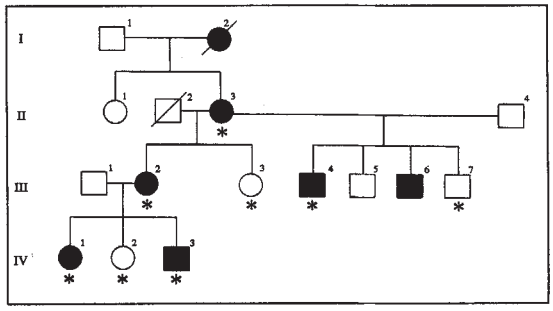

Fig. 2 Pedigree of a family with WSN showing the disease status (open symbols are unaffected; filled symbols are affected individuals). Individuals from whom DNA material was obtained are indicated by an asterisk.

[1]*Laboratory of Skin Biology, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, Maryland 20892-2757, USA*
[2]*Istituto Dermopatico Dell' Immacolata (IDI-IRCCS), Biochemistry Laboratory at Department of Experimental Medicine, University Tor Vergata, Rome 00167, Italy*
[3]*Istituto Dermopatico Dell' Immacolata (IDI-IRCCS), 1ª divisione, Rome 00167, Italy*

Correspondence should be addressed to J.G.C.

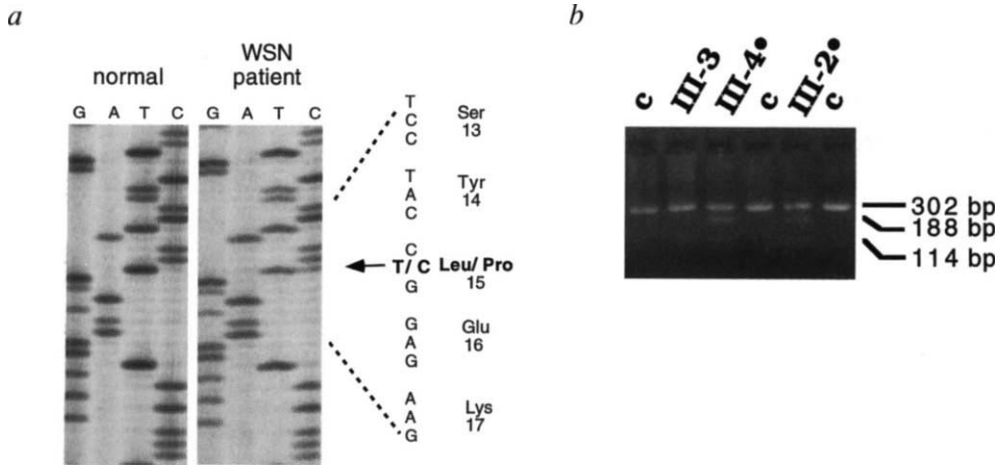

Fig. 3 *a*, Direct sequencing analysis of exon 1 of the keratin 13 gene. T→C transition, which alters the normal leucine codon to a proline codon, affecting one allele in a WSN patient is indicated. *b*, Analysis of the Leu15Pro mutation by *MspI* digestion of PCR-amplified DNA. Cleavage of half of the 302-bp fragment into 188-bp and 114-bp fragments indicates the presence of the heterozygous mutation in patients with WSN (•). An unaffected family member and several normal controls (c) are uncut. Numerals refer to the pedigree in Fig. 2.

markers found within the keratin gene cluster[12]. This excluded keratin 4 and other type II keratins as candidate genes for WSN in this family. In contrast, we observed complete cosegregation between WSN and *D17S579*, *D17S800* and *D17S932* (Z_{max} up to 1.7), markers tightly linked to the type I keratin locus on chromosome 17q21 (ref. 13). This result is consistent with a mutation in a type I keratin in WSN, with keratin 13 as the predominant candidate.

Although there are pseudogenes or functional isoforms of some type I keratins[14], no evidence exists for multiple K13 genes[15]. Therefore, direct mutation analysis of keratin 13 from genomic DNA of WSN patients and unaffected individuals was performed. As shown in Fig. 3*a*, the sequence ladder in affecteds revealed both T and C at one base position, indicating different codons in the two keratin 13 alleles (CCG for Pro and the normal CTG for Leu) at residue position 15 of the 1A subdomain of keratin 13 (designated Leu15Pro). The remainder of the DNA sequence coding for the central rod domain of keratin 13 exhibited no other differences. The presence of this heterozygous mutation was unequivocally demonstrated by restriction endonuclease digestion. Substitution of C for T creates a unique *Msp*I recognition site which results in cleavage of amplified DNA upon *Msp*I digestion (Fig. 3*b*). Using this assay, the mutation was shown to cosegregate with WSN in the family tested. Moreover, we excluded the hypothesis that this change in keratin 13 is a common polymorphism in the general population since it was not detected by *Msp*I cleavage in 116 alleles in unaffected, unrelated Caucasian individuals.

The Leu15Pro mutation in keratin 13 is strongly implicated as the proximal cause of WSN. Dominant mutations have been reported in the analogous residue of keratin 9 in EPPK (Leu15Ser)[16], of keratin 10 in EH (also Leu15Ser)[17], and of keratin 16 for PC1 (also Leu15Pro)[8]. As seen in Fig. 1, the sequence of the 1A region of type I keratins is very highly conserved, and leucine is invariably present at residue 15. The importance of the conserved 1A sequence motif has been demonstrated *in vitro* where the introduction of single-residue alterations produced defective KIF structures in assembly studies[18], and also diminished the interaction between mutated 1A peptides and normal KIF in a disaggregation assay[19]. In the latter study, the rate of disaggregation caused by peptides bearing the non-conservative substitutions Leu15Phe, Leu15Ser and Leu15Glu was significantly reduced.

Previously identified mutations in the epidermal keratins suggest that it is their deleterious effects on structure that undermines KIF function. If correct, then mutation of identical or analogous residues in other keratins should cause similar structural disturbances and, potentially, have pathogenic consequences for tissues in which they are found. As we have described, this expectation has been fulfilled in the case of keratin 13 (and for its type II partner keratin 4, see the accompanying report[20]), in which a missense mutation causes defects in differentiation in non-cornifying mucosal epithelia and WSN phenotype.

Methods

Patients and biological material. A four generation Italian family was clinically and histologically diagnosed as segregating white sponge nevus. Biopsies from oral plaques were obtained from individual III–2 and fixed for histopathological examination by light microscopy. For DNA analysis, buccal swabs were obtained from 5 affected and 3 unaffected family members (Fig. 2) and stored at 4 °C[21]. Six individuals showed bilateral, white, spongy plaques of the oral cavity affecting mainly the buccal mucosa, floor of the mouth and the ventral tongue. In patient III–4 additional involvement of the vestibular mucosa of the lips was noted. Patient III–2 also had lesions of the vaginal and rectal mucous membranes. Light microscopic examination of biopsy material from patient III–4 revealed marked acanthosis of the epithelium covered by a thick layer of parakeratotic cells. Extensive foci of enlarged, vacuolated keratinocytes with small eccentric nuclei were apparent.

Linkage analysis. WSN was modelled as a fully penetrant autosomal dominant disease. Marker allele frequencies were taken from the Genbank database. Computations were performed using the LINKAGE package of analysis programs on a VAX workstation[22].

DNA marker analysis. Microsatellite markers were analysed from buccal DNA (3 ul) prepared as described[21] using primers obtained from the Genome Data Base. PCR amplifications to incorporate ^{32}P-dCTP (3000 Ci/mmol) were performed in 20 ul reactions as previously described using 35 cycles at 94 °C for 30 s and 55 °C for 30 s[23]. Amplified products were separated on 6% denaturing polyacrylamide gels and analysed by autoradiography of dried gels exposed overnight at –70 °C.

Keratin 13 DNA amplification and sequencing. Primers for amplification of the coding region for the rod domain of the keratin 13 gene were designed from the published cDNA sequence (Genbank M27908) (ref. 24). Since intron sequences for the keratin 13 gene were unavailable, primers derived from cDNA sequences were designed to amplify exon-containing fragments for direct sequence analysis. A 302-bp fragment of exon 1 containing residues 57 to 158, including all of the 1A region of the rod domain, was amplified using the primer pair hK13-3(+) (5'–GCTGATAGTGGCTTTGGAGGTGGCTATGG–3') and hK13-2(–) (5'–TTGTAGTAGGGGCTGTAGTCCCGCTCAGG–3'). These primers have high Tm values, enabling efficient amplification of this GC-rich fragment in standard buffer (AmpliTaq, Perkin Elmer Cetus) by cycling 95 °C for 4 min and 35 cycles of 94 °C for 1 min, 62 °C for 1 min, 72 °C for 1.5 min. A variety of other fragments encompassing the remaining exons coding for the central rod domain of K13 were also sequenced (primer sequences provided upon request). Likely exon-intron boundaries were avoided based on the highly conserved organization of type I keratin genes[25]. In general, primary PCR products were reamplified using the same primers. DNA sequence was obtained from gel-purified double-stranded templates by PCR cycle-sequencing using the *fmol* DNA Sequencing System (Promega). Sequencing primers for exon 1 included hK13-2(–) and hK13-4(+) (5'–ACTGGCAATGAGAAGATCACCATGC–3'), which were end-labelled with ^{33}P (3000 Ci/mmol).

Restriction endonuclease cleavage analysis. The T to C transition in exon 1 of keratin 13 creates a unique *Msp*I cleavage sequence. Digestions were performed on gel-purified DNA fragments (302 bp, see above) amplified by PCR from genomic DNA with the recommended reaction conditions (New England Biolabs). Products were analysed by electrophoresis in 3% NuSieve (3:1) agarose (FMC Corp.).

Acknowledgments

We thank the family for their willingness to participate in this study, Y. Whyte for expert technical assistance, G. Melino for valuable discussion and G. Poy for his invaluable services in oligonucleotide synthesis. This work was supported in part by the Deutsche Forschungsgemeinschaft.

Received 17 August; accepted 5 October 1995.

1. Jorgenson, R.J. & Levin, L.S. White sponge nevus. *Arch. Dermatol.* **117**, 73–76 (1981).
2. Metz, J. & Metz, G. Der Naevus spongiosus albus mucosae-Übersicht und eigene Beobachtungen. *Z. Hautkr.* **54**, 604–612 (1979).
3. Frithiof, L. & Bánóczy, J. White sponge nevus (leukoedema exfoliativum mucosae oris): Ultrastructural observations. *Oral Surg.* **41**, 607–622 (1976).
4. Morris, R., Gansler, T.S., Rudisill, M.T. & Neville, B. White sponge nevus. Diagnosis by light microscopic and ultrastructural cytology. *Acta Cytol.* **32**, 357–361 (1988).
5. Parry, D.A.D. & Steinert, P.M. *Intermediate filament structure.* (R.G. Landes Company, Austin, 1995).
6. McLean, W.H.I. & Lane, B. Intermediate filaments in disease. *Curr. Opin. Cell Biol.* **7**, 118–125 (1995).
7. Anneroth, G. Isacsson, G., Lagerholm, B., Lindvall, A. & Thyresson, N. Pachyonychia congenita. A clinical, histological and microradiographic study with special reference to oral manifestations. *Acta Derm. Venereol. (Stockh)* **55**, 387 (1975).
8. McLean, W.H.I. *et al.* Keratin 16 and keratin 17 mutations cause pachyonychia congenita. *Nature Genet* **9**, 273–278 (1995).
9. Bowden, P.E., Haley, J.L., Kansky, A., Rothnagel, J.A., Jones, D.O. & Turner, R.J. Mutation of a type II keratin gene (K6a) in pachyonychia congenita. *Nature Genet.* **10**, 363–365 (1995).
10. Moll R., Franke, W.W. & Schiller, D.L. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell* **31**, 11–24 (1982).
11. Lindberg, K. & Rheinwald, J.G. Three distinct keratinocyte subtypes identified in human oral epithelium by their patterns of keratin expression in culture and in xenografts. *Differentiation* **45**, 230–241 (1990).
12. Yoon, S.J. *et al.* Organization of the human keratin type II gene cluster at 12q13. *Genomics* **24**, 502–508 (1994).
13. Albertsen, H.M. *et al.* A physical map and candidate genes in the *BRCA1* region on chromosome 17q12–q21. *Nature Genet.* **7**, 472–479 (1994).
14. Rosenberg, M., RayChaudhury, A, Shows, T.B., Le Beau, M.M. & Fuchs, E. A group of type I keratin genes on human chromosome 17: Characterization and expression. *Molec. cell. Biol.* **8**, 722–736 (1988).
15. Romano, V. *et al.* Chromosomal mapping of human cytokeratin 13 gene (KRT13). *Genomics* **14**, 495–497 (1992).
16. Rothnagel, J.A. *et al.* Mutations in the 1A domain of keratin 9 in patients with epidermolytic palmoplantar keratoderma. *J. invest. Dermatol.* **104**, 430–433 (1995).
17. Rothnagel, J.A. *et al.* Mutations in the rod domains of keratins 1 and 10 in epidermolytic hyperkeratosis. *Science* **257**, 1128–1130 (1990).
18. Letai, A. *et al.* Disease severity correlates with position of keratin point mutations in patients with epidermolysis bullosa simplex. *Proc. natn. Acad. Sci. U.S.A.* **90**, 3197–3201 (1993).
19. Steinert, P.M., Yang, J.M., Bale, S.J. & Compton, J.G. Concurrence between the molecular overlap regions in keratin intermediate filaments and the locations of keratin mutations in genodermatoses. *Biochem. biophys. Res. Commun.* **197**, 840–848 (1993).
20. Rugg, E.L. *et al.* A mutation in the mucosal keratin K4 is associated with oral white sponge nevus. *Nature Genet.* **11**, 450–452.
21. Richards, B. *et al.* Multiplex PCR amplification from the CFTR gene using DNA prepared from buccal brushes/swabs. *Hum. molec. Genet.* **2**, 159–163 (1993).
22. Ott, J. *Analysis of human genetic linkage.* (Johns Hopkins University Press, Baltimore, 1985).
23. Richard, G. *et al.* Fine mapping of the Darier disease locus on chromosome 12q. *J. invest. Dermatol.* **103**, 665–668 (1994).
24. Mischke, D., Wachter, E., Hochstrasser, K., Wild, A.G. & Schulz, P. The N-, but not the C-terminal domains of human keratins 13 and 15 are closely related. *Nucl. Acids Res.* **17**, 7984 (1989).
25. Steinert, P.M. & Roop, D.R. The structure, complexity and evolution of intermediate filament genes. In *Cellular and Molecular Biology of Intermediate Filaments* (eds. Goldman, R.D. & Steinert, P.M.) 353–367(Plenum Press, New York, 1990).