A Novel Insertional Mutation in Loricrin in Vohwinkel's Keratoderma

Dermot K.B. Armstrong, Kevin E. McKenna,* and Anne E. Hughes

Division of Molecular Medicine, Queen's University Belfast, Belfast, Northern Ireland, U.K.; *Dermatology Department, Craigavon Area Hospital, Craigavon, Northern Ireland, U.K.

A mutation in the gene encoding loricrin has recently been reported in a subset of patients with Vohwinkel's Keratoderma manifesting an associated ichthyosiform dermatosis. We have studied a further kindred with this clinical phenotype. Microsatellite marker analysis was consistent with linkage to chromosome 1q21 and direct sequencing of loricrin identified a heterozygous mutation with an insertion of a T residue at codon 209. This mutation is predicted to produce a mutant protein with a frameshift of its terminal 107 amino acids and to be 22 amino acids longer than the wild-type protein due to a delayed termination codon. The only previously reported mutation is a G insertion producing a frameshift after codon 231. The novel mutation we report is likely to have a similar functional effect on cornified envelope formation, with disturbance of transglutaminase-mediated cross-linking of envelope components, and serves to confirm the predicted role of insertional mutations in Vohwinkel's Keratoderma associated with ichthyosis. ***Key words: bipartite nuclear localization signals/cornified envelope/ichthyosis. J Invest Dermatol 111:702–704, 1998***

During terminal differentiation of keratinocytes the cornified envelope (CE) is deposited on the inner surface of the plasma membrane in a highly complex, multistage process involving the sequential deposition of multiple interacting components, including involucrin, loricrin, and small proline-rich proteins, amongst others (Hohl, 1990; Steinert and Marekov, 1995). Many of the genes encoding essential components of this process are localized to chromosome 1q21, a region designated as the epidermal differentiation complex (EDC) (Marenholz *et al*, 1996; Mischke *et al*, 1996). Despite evidence of abnormalities of the CE in ichthyoses and other keratinizing disorders, the first genetic disease to be attributed to an abnormality in CE formation was autosomal recessive lamellar ichthyosis in which a subgroup of patients were shown to have mutations in the transglutaminase 1 gene (Huber *et al*, 1995; Russel *et al*, 1995). More recently, linkage was demonstrated to the EDC and subsequently a mutation in the gene encoding loricrin was detected in a family with Keratoderma Hereditaria Mutilans (Vohwinkel's Keratoderma) (Maestrini *et al*, 1996). This represents the first human genetic disease to be directly attributed to a defect in a structural component of the CE, and offers novel insights into the potential pathogenic mechanisms underlying the disorder. An identical mutation has since been described in a further kindred with this disorder, although the possibility of common ancestry could not be excluded in this report (Korge *et al*, 1997). Vohwinkel's Keratoderma (VK) is a rare dominantly inherited disorder characterized by (i) diffuse palmoplantar keratoderma with a honeycomb appearance, (ii) variable degrees of constricting bands encircling the digits (pseudoainhum), and (iii) starfish-shaped keratoses and/or warty papules (Vohwinkel, 1929). Other clinical features that have been reported in a variable proportion of cases include alopecia, high tone deafness, myopathy, spastic paraplegia, and an associated generalized ichthyosiform dermatosis (Wirz, 1930; Grschebin, 1936; Drummond, 1939; Gibbs and Frank, 1966; Reddy and Gupta, 1983; Camisa and Rosssana, 1984; Camisa *et al*, 1988). Interestingly, the mutation in loricrin previously reported in this disorder was demonstrated in two families with the clinical phenotype manifesting an associated ichthyosiform dermatosis (Maestrini *et al*, 1996; Korge *et al*, 1997). A further family studied with VK and associated deafness but lacking ichthyosis failed to show linkage to the EDC and had no underlying loricrin mutation (Korge *et al*, 1997). Thus, there is evidence of both clinical and genetic heterogeneity in this disorder.

To elucidate further the underlying pathogenesis of this disease we have studied a further kindred with VK in whom an associated ichthyosiform dermatosis was present and we report a novel mutation in the gene encoding loricrin.

MATERIALS AND METHODS

Patients A family with VK associated with ichthyosiform dermatosis was identified and all available family members were examined. Biopsies of affected palmar skin and skin demonstrating ichthyosiform features were obtained for light microscopy.

Genotyping and linkage analysis Genomic DNA was obtained from peripheral white blood cells from all family members. Microsatellite markers were polymerase chain reaction (PCR) amplified using $\gamma[^{32}P]$-labeled primers, analyzed on standard 6% sequencing gels, and visualized by autoradiography. Two point lod scores were calculated using the MLINK program of FASTLINK assuming autosomal dominant inheritance with complete penetrance and a zero mutation rate.

Mutation analysis The terminal one-third of the loricrin gene coding sequence was PCR amplified from genomic DNA of affected and unaffected family members. This region included the site of the previously reported mutation (Maestrini *et al*, 1996; Korge *et al*, 1997). Primers were designed from

Manuscript received February 19, 1998; revised April 6, 1998; accepted for publication June 5, 1998.

Reprint requests to: Dr. D.K.B. Armstrong, Department of Medical Genetics, A Floor, Belfast City Hospital, Lisburn Road, Belfast BT9 7AB, Northern Ireland, U.K.

Abbreviations: CE, cornified envelope; EDC, epidermal differentiation cluster; VK, Vohwinkel's Keratoderma.

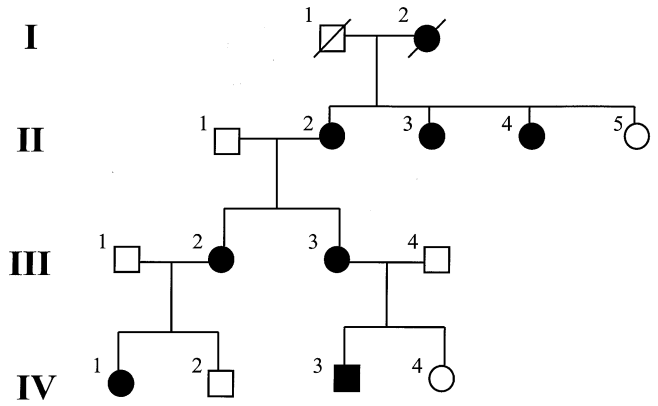

Figure 1. Pedigree of family with VK.

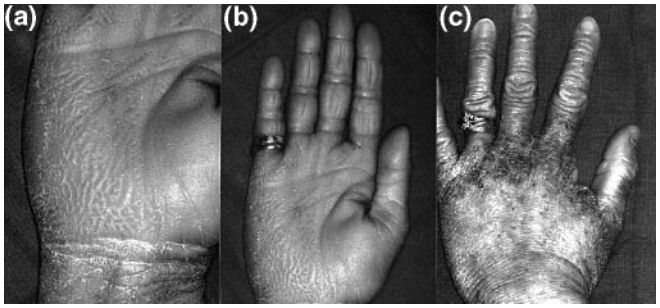

Figure 2. Clinical findings in VK. (*a*) Honeycomb keratoderma; (*b*) mild pseudo-ainhum; (*c*) thickening of skin over knuckles with prominent skin markings and associated hyperpigmentation.

the published sequence (Genbank M94077): 5′-TACGGAGGCGTCT-CTAGCGG-3′ (sense), 5′-CCTGCTTCTGCTGGGTCTGG-3′ (anti-sense). PCR was performed with Amplitaq Gold and 0.4 micromolar primer concentrations (96°C for 12 min followed by 35 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and then 72°C for 10 min). PCR products were purified using a Wizard Kit (Promega Southampton, UK) and sequenced with both primers using the ABI PRISM Ready Reaction system (Perkin Elmer, Warrington, U.K.). Sequencing products were analyzed on an ABI 377 automated sequencer. The mutation detected destroys an Nla IV restriction site in the mutant allele. To confirm the mutation in affected family members and exclude it in unaffected family members and normal controls, PCR products generated with the above primers were digested at 37°C for 3 h with Nla IV (New England Biolabs, Herts, U.K.) and analyzed on 3% agarose gel.

RESULTS

Clinical features The family consisted of 15 individuals with eight affected members (**Fig 1**). The clinical phenotype corresponded closely to the variant described by Camisa and Rossana (1984). The palmoplantar hyperkeratosis was diffuse with a honeycomb appearance and was noted within a few weeks of birth (**Fig 2*a***). An erythematous hyperkeratosis extended from the palms on to the flexor aspects of the wrists and had a discrete proximal edge at this site. Digital constrictions were present in all but two individuals, but were mild in all cases (**Fig 2*b***). There was no evidence of digital circulatory impairment or auto-amputation. All individuals had hyperkeratosis with prominent skin markings over the knuckles; in one individual there was notable hyperpigmentation associated with this (**Fig 2*c***). All affected patients had an associated generalized nonerythematous ichthyosiform dermatosis, present from birth, which was most prominent over the extensor aspects of the limbs with marked hyperkeratosis over the elbows and knees. There was no evidence of hearing impairment, alopecia, or muscle weakness and the nails, hair, and teeth were normal.

Light microscopy Histopathology of palmar skin and an area of ichthyosis showed similar features with moderate epidermal hyperplasia, marked thickening of the granular layer (7–8 layers), and hyperkeratosis with retention of rounded nuclei. The initial biopsies were performed

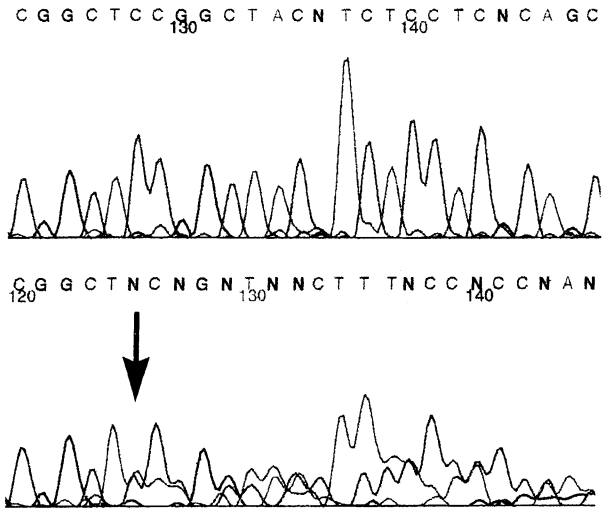

Figure 3. Identification of heterozygous insertion at codon 209 of loricrin in affected individual. Automated sequencing of coding strands of loricrin around the T insertion (*arrow*) at codon 209 in normal (top) and affected (bottom) individuals.

in a nonteaching center and subsequent requests for a further biopsy for electron microscopy were declined by the index case and her family.

Linkage to EDC at 1q21 Linkage analysis showed complete cosegregation of the disease phenotype with the markers D1S306 and D1S1664, which map to the EDC (data not shown). The maximum lod score of 2.41 at $\theta = 0$ with both markers was consistent with linkage to the EDC but not conclusive of this.

Mutation in loricrin PCR product of appropriate size was amplified from the terminal third of the gene encoding loricrin from genomic DNA of affected and unaffected family members. Direct sequencing of the purified product demonstrated a heterozygous mutation with insertion of a T residue at codon 209 in all five affected family members sequenced and in none of five unaffected members tested (**Fig 3**). The mutation was confirmed by restriction digestion of PCR product of all affected and unaffected family members with NlaIV. The insertion in the mutant allele results in a loss of a NlaIV cutting site with the production of a 168 bp fragment, whereas digestion of the normal allele produces a 150 bp fragment. The mutation was also excluded from 100 normal control alleles.

DISCUSSION

VK is the first human genetic disease to be attributed to an abnormality of a structural component of the CE. To date a single mutation has been described in two families with a heterozygous G residue insertion at an area of six consecutive G residues at codons 230–231 of loricrin. It should be noted that both families had an associated ichthyosiform dermatosis (Maestrini *et al*, 1996; Korge *et al*, 1997). We report a second insertional mutation upstream in the loricrin gene with insertion of a T residue at codon 209 in a further family with this variant of VK. This mutation is predicted to produce a mutant protein with a frameshift of its terminal 107 amino acids and to be 22 amino acids longer than the normal wild-type protein due to a delayed termination codon. The functional consequences of this mutation are predicted to be essentially similar to those of the previously described mutation. Loricrin consists of four glycine loop domains that are interspersed and flanked by glutamine- and glutamine/lysine-rich regions (Hohl *et al*, 1991). The glycine loop domains are predicted to be highly flexible and may function to impart flexibility and extensibility to the CE and epidermis. In addition, it is believed that the glycine loops of loricrin interact with similar motifs in the V1 and V2 keratin domains (Hohl *et al*, 1991; Steinert *et al*, 1991). The glutamine- and glutamine/lysine-rich regions, however, function as critical substrates for transglutaminase-mediated Nε-(γ-glutamyl)lysine isodipeptide cross-linking of CE components (Hohl *et al*, 1991; Candi *et al*, 1995; Steinert and Marekov,

```
                ↓
WT          GSGYVSSQQVTQTSCAPQPSYGGGS
Mut         GFRLRLLAAGHSDLVRAPAELRRGV          232

WT          SGGGGSGGSGCFSSGGGGGSSGCGG
Mut         VRRRRQRRKRLLLQRRGRRELRLRR          257

WT          GSSGIGSGCIISGGGSVCGGGSSGG
Mut         RLLRDWQRLHHQWRGLRLRRWFLWR          282

WT          GGGGSSVGGSGSGKGVPICHQTQQK
Mut         RRRRLLRGWLREWQGRPDLPPDPAE          307

WT          QAPTWPSK
Mut         AGAYLAVQIDPPGYHGGEGVGGVFQ          332

WT
Mut         GHRWA                              337
```

Figure 4. Predicted amino acid sequence of mutant loricrin at 3′ end. Comparison of mutant (Mut) and wild-type (WT) predicted amino acid sequence after T insertion at codon 209 (*arrow*). The presence of a potential nuclear targeting motif is underlined.

1995). The presence of such isodipeptide cross-links in addition to disulfide bonds between constituent proteins accounts for the highly insoluble nature of the CE. Although studies indicate that almost every lysine and glutamine residue of loricrin can be identified in cross-links, the residues Gln^{215}, Gln^{216}, and Lys^{315} appear to be particularly functionally prominent in this process (Candi *et al*, 1995; Steinert *et al*, 1995). The novel mutation we report results in the replacement of the fourth glycine loop domain and the C-terminal glutamine/lysine-rich domain of the normal protein with an arginine- and leucine-rich region in the mutant peptide. This is likely to affect the function of the mutant peptide both by altering its flexibility and by disrupting transglutaminase-mediated cross-linking of the peptide to other loricrin molecules and to other components of the CE.

A recent report has also suggested that such an insertional mutation in loricrin will result in the introduction of potential nuclear targeting motifs in the arginine-rich sequences of the mutant C-terminal peptide that are lacking in the normal protein (Korge *et al*, 1997). Indeed the presence of such sequences may explain the presence of intranuclear accumulations of loricrin in the granular cell layer that has been previously well reported in this disorder (Maestrini *et al*, 1996; Korge *et al*, 1997). Unfortunately, electron microscopic studies are not available for this kindred. Bipartite nuclear localization signals comprise a pair of basic amino acids, a spacer of any 10 amino acids, and then a cluster in which three of the next five amino acids are basic (Dingwall and Laskey, 1991; Nigg, 1997). The length of the amino acid spacer, however, may not be critical (Dingwell and Laskey, 1991). The previously reported G insertion resulting in a frameshift after codon 231 was noted to give rise to four bipartite nuclear localization signals with a 10 amino acid spacer and a potential fifth signal with a 16 amino acid spacer (Korge *et al*, 1997). Although the novel mutation we report has no further bipartite nuclear localization signals as defined by a 10 amino acid spacer compared with the previously reported mutation, a further potential sequence with a 9 amino acid spacer begins at codon 229 in the mutant allele (**Fig 4**). Loricrin monomers are known to cross-link to each other by disulfide bonds. The intranuclear granules in this disorder have been shown to contain normal loricrin in addition to the mutant protein, and it has been suggested that complexing of the normal protein by the mutant type and subsequent sequestration in the nuclear granules may produce relative loricrin deficiency and compound further the defective CE formation (Korge *et al*, 1997).

In conclusion, we report a second insertional mutation in loricrin in a family with VK associated with ichthyosis likely to have similar functional consequences to the previously reported mutation. The possible presence of other types of loricrin mutation in ichthyotic disorders or keratodermas deserves further study.

DKBA is grateful for support from the British Skin Foundation and the Royal Hospitals Trust, Belfast.

REFERENCES

Camisa C, Rosssana C: Variant of keratoderma hereditaria mutilans (Vohwinkel's Syndrome). *Arch Dermatol* 120:1323–1328, 1984

Camisa C, Hessel A, Rossana C, Parks A: Autosomal dominant keratoderma, ichthyosiform dermatosis and elevated beta-glucuronidase. *Dermatologica* 177:341–347, 1988

Candi E, Melino G, Mei G, Tarcsa E, Chung S, Marekov LN, Steinert PM: Biochemical, structural, and transglutaminase substrate properties of human loricrin, the major epidermal cornified cell envelope protein. *J Biol Chem* 270:26382–26390, 1995

Dingwall C, Laskey RA: Nuclear targeting sequences – a consensus? *Trends Biochem Sci* 16:478–481, 1991

Drummond M: A case of unusual skin disease. *Irish J Med Sci* 8:85–86, 1939

Gibbs RC, Frank S: Keratoma hereditaria mutilans (Vohwinkel). *Arch Dermatol* 94:619–625, 1966

Grschebin S: Observations on ainhum: does it exist as an independent disease? *Urol Cutan Rev* 40:98–102, 1936

Hohl D: Cornified cell envelope. *Dermatologica* 180:201–211, 1990

Hohl D, Mehrel T, Lichti U, Turner ML, Roop DR, Steinert PM: Characterisation of human loricrin. Structure and function of a new class of epidermal cell envelope proteins. *J Biol Chem* 266:6626–6636, 1991

Huber M, Rettler I, Bernasconi K, *et al*: Mutations of keratinocyte transglutaminase in lamellar ichthyosis. *Science* 267:525–528, 1995

Korge BP, Ishida-Yamamoto A, Punter C, *et al*: Loricrin mutation in Vohwinkel's keratoderma is unique to the variant with ichthyosis. *J Invest Dermatol* 109:604–610, 1997

Maestrini E, Monaco AP, McGrath JA, *et al*: A molecular defect in loricrin, the major component of the cornified envelope, underlies Vohwinkel's syndrome. *Nature Genet* 13:70–77, 1996

Marenholz I, Volz A, Ziegler A, Davies A, Ragoussis I, Korge BP, Mischke D: Genetic analysis of the epidermal differentiation complex (EDC) on human chromosome 1q21: chromosomal orientation, new markers, and a 6-Mb YAC contig. *Genomics* 37:295–302, 1996

Mischke D, Korge BP, Marenholz I, Volz A, Ziegler A: Genes encoding structural proteins of epidermal cornification and S100 calcium-binding proteins form a gene complex ("epidermal differentiation complex") on human chromosome 1q21. *J Invest Dermatol* 106:989–992, 1996

Nigg E: Nucleocytoplasmic transport: signals, mechanisms and regulation. *Nature* 386:779–787, 1997

Reddy BSN, Gupta SK: Mutilating keratoderma of Vohwinkel. *Int J Dermatol* 20:530–533, 1983

Russel IL, DiGiovanna JJ, Rogers GR, Hashem N, Steinert PM, Compton JG, Bale SJ: Mutations in the gene for transglutaminase 1 in autosomal recessive lamellar ichthyosis. *Nature Genet* 9:279–283, 1995

Steinert PM, Marekov LN: The proteins elafin, filaggrin, keratin intermediate filaments, loricrin, and small proline-rich proteins 1 and 2 are isodipeptide cross-linked components of the human epidermal cornified cell envelope. *J Biol Chem* 270:17702–17711, 1995

Steinert PM, Mack JW, Korge BP, Gan SQ, Haynes SR, Steven AC: Glycine loops in proteins: their occurrence in certain intermediate filament chains, loricrins and single-stranded RNA binding proteins. *Int J Biol Macromol* 13:130–139, 1991

Vohwinkel KH: Keratoma hereditarium mutilans. *Arch Dermatol Syphil* 158:354–364, 1929

Wirz F: Keratoma hereditarium mutilans. *Arch Dermatol Syphil* 159:311–312, 1930