A New Keratin 2e Mutation in Ichthyosis Bullosa of Siemens

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Ichthyosis bullosa of Siemens (IBS) is a rare autosomal dominant skin condition with features similar to epidermolytic hyperkeratosis (EH). Clinical symptoms are characterized by mild hyperkeratosis with an acral distribution. Histology shows epidermolysis of upper spinous and granular cells, whereas ultrastructurally, tonofilaments form perinuclear aggregates. IBS has been linked to the type II keratin cluster on chromosome 12q, and K2e mutations have recently been identified in IBS patients. We have studied genomic DNA from two IBS families and in both cases heterozygous point mutations were found in the 2B helical domain of K2e. One family had an established mutation in codon 493 (E493K), whereas the other had an unreported mutation in the adjacent codon (E494K). Both mutations were confirmed by allele-specific PCR. These data reinforce the hypothesis that mutations in the TYRKLLEGEE motif of the 2B helix are deleterious to keratin filament network integrity and provide further evidence for the involvement of K2e mutations in IBS. ***Key words: intermediate filament/genodermatosis/epidermolysis/sequence. J Invest Dermatol 108:354–356, 1997***

IBS, a rare autosomal dominant hereditary skin disease (Siemens, 1937; Traupe *et al*, 1986), is present from birth, has an acral distribution (Mills and Marks, 1993), and is similar to epidermolytic hyperkeratosis (EH) [also known as bullous ichthyosiform erythroderma (BIE)] but less severe. Patients present with hyperkeratosis of the flexural surfaces of the arms and legs, which can assume a lichenified appearance. This skin bruises easily with blister formation after mild mechanical trauma. The condition recedes with time and by middle age is limited to mild hyperkeratosis of the flexures. The histopathology is characterized by thickening of the stratum corneum (again less marked than in EH/BIE) and epidermolysis of upper spinous and granular cells, producing vacuolar degeneration of the tissue below the stratum corneum. Ultrastructural studies reveal aggregation of tonofilaments into perinuclear shells and cytoplasmic bundles in keratinocytes of the upper epidermis.

Keratins comprise two distinct classes of intermediate filament (IF) proteins (Moll *et al*, 1982): type I acidic keratins (K9-K20) and type II basic-neutral keratins (K1-K8). They are obligate heteropolymers and maintain structural integrity of the epidermis by forming a three-dimensional tonofilament network (Coulombe and Fuchs, 1990; Steinert, 1993). This functional constraint has led to evolutionary conservation of both the primary structure and type-specific co-expression of these important structural proteins (Steinert and Roop, 1988). In human epidermis, K1 and K10 expression is suprabasal and replaces K5 and K14 as cells differentiate and leave the basal layer (Bowden *et al*, 1987). K2e is expressed later in differentiation as cells approach the granular layer (Collin *et al*, 1992) and is particularly prominent in palmar-plantar epidermis and skin around the knee, ankle, wrist, and elbow. Cytogenetic studies have linked the K2e gene to the type II keratin gene cluster on chromosome 12q (Steijlen *et al*, 1994) and mutations of the K2e gene have been found in IBS patients (Rothnagel *et al*, 1994; McLean *et al* 1994b; Kremer *et al*, 1994). The aim of this study was to characterize keratin mutations in two IBS families, and we now report two heterozygous point mutations of K2e in adjacent codons (E493K and E494K).

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Abbreviations: EH, epidermolytic hyperkeratosis; IBS, ichthyosis bullosa of Siemens.

MATERIALS AND METHODS

Histopathology and Ultrastructure Biopsies (4 mm) of affected skin were formalin fixed and processed for histology, and 5-μm sections examined by light microscopy after staining with hematoxylin and eosin. Part of each biopsy was also fixed in glutaraldehyde (3 h at 4°C), treated with 2% buffered osmium, and embedded in araldite. Ultrathin sections (60–90 nm) were stained with uranyl acetate–lead citrate and examined with a Philips CM12 electron microscope.

Genomic DNA Isolation and K2e Gene Amplification Genomic DNA was extracted from venous blood samples of affected and unaffected family members and normal controls (Nucleon II Kit, Scotlab Ltd., Strathclyde, UK). The helical encoding portions of the K2e gene were amplified by polymerase chain reaction (PCR). All reactions used "hot-start" (7 min, 94°C), 30 cycles (94°C, 30 s; 60°C, 50 s; 72°C, 3 min) and a 15-min extension (72°C). Exon 1 primers (HK2p1, 5′- CTTTCCTCCCT-GGACAAAGGCATC-3′; HK2p2RB, 5′-GGGGCGGGTGCCAACAT-TCATTTG-3′) amplified DNA encoding the 1A helix whereas exon 7 primers (HK2p4, 5′-AAGAATGTGCAAGATGCCATCGCA-3′; HK2p6RB, 5′-AGTCACATTGCTGCTGAGGTCTCC-3′) amplified DNA encoding the 2B helix.

PCR Fragment Analysis, Purification, and Sequencing Aliquots (4 μl) of the K2e gene fragments were analyzed on 1.5% agarose gels, and the remainder were purified on spin columns (Wizard PCR Preps, Promega Ltd., Southampton, UK). The biotinylated single-stranded DNA was isolated using streptavidin magnetic particles (Dynal) and sequenced by the

oligonucleotide primed dideoxy chain termination method (Amersham-USB Sequenase, Amersham International, Slough, UK). Three K2e sequencing primers were used (HK2p3, 5′-TACCCTGGTGGCATCCAC-3′; HK2p5, 5′-GAGCAGCGTGGGGAGCAT-3′; HK2p14R 5′-TCGTC-AGGAGGGCC-TGCC-3′). After sequencing by the standard protocol with [^{35}S]dATP (Amersham SJ1304, specific activity >3,000 Ci/mole), the samples were analyzed on 6% polyacrylamide denaturing gels (Gibco-BRL, Life Technologies, Paisley, UK), dried, and exposed to x-ray film (Kodak XAR-2).

Allele-Specific PCR Both K2e mutations were confirmed by allele-specific PCR involving high-stringency amplification with primers to either the normal (HK2p9RN, 5′-GTGCCCTCACCTGCACTCCTC-3′) or mutant allele (HK2p10RM, 5′-GTGCCCTCACCTGCACTCCTT-3′ for E493K and HK2p12RM, 5′-TTCCCAGTGCCCTCACCTGCACTT-3′ for E494K) together with a common upstream primer (HK2p4, as above). PCR fragments were analyzed on high-definition agarose gels (3% NuSieve plus 1% Agarose) and stained with ethidium bromide.

RESULTS

Five individuals from two unrelated IBS families (IBS-1 and IBS-2) have been studied. The IBS-1 index case (KW) was a teenage girl (13 y old) with an uncharacteristic form of IBS termed acral epidermolytic hyperkeratosis (Mills and Marks, 1993). She presented initially at 6 mo of age with mild erythema and fragile scaly skin at the ankles, lower leg, knees, wrists, forearm, and elbows, but the skin elsewhere was normal. Her father, uncle, and great aunt showed the same clinical appearance but had improved with age. The IBS-2 index case (HW) was a 3-y-old boy who also presented with fragile thickened skin in an acral distribution. He had an affected mother and an unaffected father. Skin biopsies from affected individuals of both families showed marked hyperkeratosis together with prominent vacuolar degeneration of the upper spinous and granular cells (data not shown). Typical ultrastructural alterations of these keratinocytes were observed in both families, perinuclear condensation and cytoplasmic aggregation of keratin filaments being a prominent feature (data not shown).

Direct sequencing of the K2e gene showed that exon 1, which encodes the 1A helix, was normal in all affected individuals (data not shown), but both families had point mutations at the distal end of the 2B helix of K2e. Affected members of family IBS-1 had a heterozygous G to A transition in codon 493 **(Fig 1)**, which altered a highly conserved glutamic acid residue (position 117 of the 2B helix) to a lysine (E493K or 2B:E117K). Affected members of family IBS-2 also had a heterozygous G to A transition in the adjacent codon **(Fig 1)**, which also substituted lysine for a highly conserved glutamic acid (E494K or 2B:E118K). These mutations were found in all affected family members examined and were absent from unaffected members and normal controls. Both 1A and 2B helical sequences of K1 and K10 were normal in all individuals (data not shown).

The loss of the same *Mnl*I site was associated with both mutations but because this enzyme cleaves the DNA into several small fragments, the size alteration is difficult to detect. The mutations were therefore confirmed using allele-specific PCR with primers designed to detect the single base change that occurred in each mutation. Amplified fragments were obtained in both lanes for KW and GW (IBS-1 affected), which indicates the presence of both the mutant and normal alleles **(Fig 2*a*)**. These were of equal intensity, which is characteristic of a heterozygous point mutation. Fragments were present in only one lane for control samples (RT and MT), representing amplification of the normal allele. The allele-specific PCR for IBS-2 also showed signals in both lanes for MW and HW **(Fig 2*b*)**, indicating the presence of both mutant and normal alleles. The mutant allele signal was absent from MN (unaffected sister) and a control DNA sample (RT).

DISCUSSION

It is apparent both from our results and those recently published (Rothnagel *et al*, 1994; McLean *et al* 1994b; Kremer *et al*, 1994) that familial mutations in the K2e gene are causal in IBS. The histopathology and ultrastructure of the involved epidermis indicate that

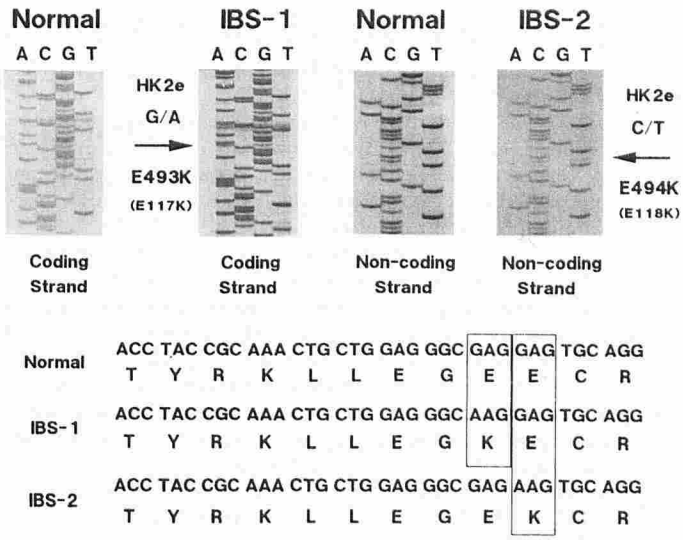

Figure 1. Sequencing of K2e (2B Helix) in both families (IBS-1 and IBS-2) and normal controls. Analysis on 6% denaturing acrylamide gels shows a G⇒A transition on the K2e coding strand in affected individuals (IBS-1) and produces a single-residue change (E493K). DNA from affected members of family IBS-2 have a C⇒T change on the non-coding strand (also G⇒A on coding strand), which alters the adjacent codon (E494K). The schematic below illustrates the deviation from the normal nucleotide sequence of K2e found in these two families (IBS-1 and IBS-2), which involves adjacent codons of the conserved "TYRKLLEGEE" motif at the distal end of the 2B helix.

the earliest cellular damage occurs at the precise point of K2e expression, providing unequivocal evidence for the association of K2e mutations with IBS. In addition, linkage of this condition to the type II keratin gene cluster on chromosome 12q (Steijlen *et al*, 1994) implicates the direct involvement of a keratin gene defect. As for most keratin mutations, however, the precise mechanism(s) by which the altered protein structure progresses to cellular degeneration and hyperkeratosis are not yet fully understood.

Both K2e gene mutations are located in exon 7, which encodes the 2B helix of the protein. One of the mutations (E493K or 2B:E117K) has been found in several IBS families (Rothnagel *et al*, 1994; McLean *et al*, 1994b; Kremer *et al*, 1994), and represents a

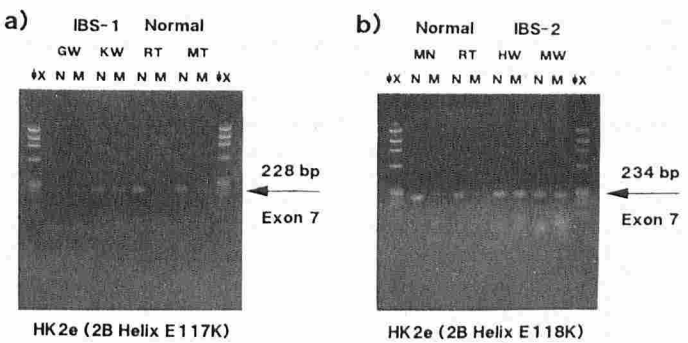

Figure 2. Allele-specific PCR of genomic DNA from each family (*a*, IBS-1 and *b*, IBS-2). PCR-generated DNA fragments were analyzed by agarose gel electrophoresis (3% NuSieve and 1% agarose). Affected IBS-1 family members (GW, KW) have a 228-bp fragment for both the normal (N) and mutant (M) alleles (left). Unrelated normal controls (RT, MT) have a fragment present only for the normal allele (N). The two affected members of the IBS-2 family (right) show the same pattern with 234-bp fragments for both the normal (N) and mutant (M) alleles but an absence of the mutant allele fragment in normal controls (MW, RT).

mutation "hotspot" for this particular keratin. We now report a mutation in the adjacent codon (E494K or 2B:E118K). Five of the seven different K2e mutations described are located in the 2B helix, whereas only two are found in the 1A helix. This is unusual, because the 1A helical domain is the predominant site for mutation in most other keratins. This atypical bias for mutational effects in K2e may reflect a different role for this keratin during filament formation. Whether these observations relate to structural functions or are coincidental cannot be determined at present.

Substitution of glutamic acid by lysine occurs at three locations (106, 117, and 118) in the 2B helix of K2e. This alteration is theoretically very disruptive to protein structure, as glutamic acid (E) is negatively charged whereas lysine (K) is positive. E117 and E118 are both highly conserved, but E117 appears particularly sensitive to alteration. Even substitution of this residue by aspartic acid (E117D), which only shortens the side chain by one carbon atom and is normally considered a conservative alteration, appears to be deleterious (Rothnagel *et al*, 1994). Mutations of conserved glutamic acid residues have also been described in other type II keratins, K1 (2B:E118Q) in an EH patient (Rothnagel *et al*, 1992) and K5 (2B:E115G) in a patient with epidermolysis bullosa simplex (EBS-DM; Hovnanian *et al*, 1993). In both cases, neutralization of the negative charge appears sufficient to disrupt filament structure. The residues of the termination peptide motif at the end of the 2B helix (TYRKLLEGEE) are highly conserved in all IF proteins (Wilson *et al*, 1992) and must therefore be essential to IF structure and function, making them intolerant to alteration by single point mutation.

The involvement of keratin mutations in genetic diseases of the epidermis and its appendages is becoming increasingly well understood. Several K5 and K14 mutations have been identified in patients with EBS (Coulombe *et al*, 1991; Lane *et al*, 1992; Letai *et al*, 1993; Stephens *et al*, 1993), and introduction of mutated K5 or K14 into transgenic mice produces an EBS-like phenotype (Vassar *et al*, 1991). Similarly, transgenic mice bearing K1 or K10 mutations have an EH-like phenotype (Fuchs *et al*, 1992). Transgenic mouse studies with mutated K2e have not yet been reported, but current evidence supports the view that helical domain mutations of K2e are causal in IBS.

It has been shown previously that different keratin mutations have the potential to produce a different phenotype in EBS (Letai *et al*, 1993). Also, K1 mutations in EH patients are associated with a clinical phenotype in which palmar–plantar regions are involved, yet these regions are usually spared in patients with K10 mutations. Furthermore, serine to proline and deletion mutations appear to be associated with a more severe EH phenotype (McLean *et al*, 1994a; Syder *et al*, 1994; Jones *et al*, 1996[1]). The location of the mutation within the helical domain and alteration of the amino acid both appear to be important in determining the disruption caused to the keratin filament network, but no correlation between mutations and clinical phenotype yet exists for IBS.

Recently, patients with linear epidermal naevi involving epidermolytic histopathology have had offspring with generalized EH (Paller *et al* 1994), and our genetic studies have shown that linear epidermal naevi cases can be genetically and phenotypically mosaic (Moss *et al*, 1995). It is therefore possible that cases of mosaic IBS also exist, the defective K2e gene being expressed in some but not all epidermal cells. Further studies are now required to establish whether a type I partner for K2e exists and whether mutations of such a keratin would produce an IBS phenotype. Finally, studies of a more basic nature are also required to assess whether K2e forms specialized heteropolymers with K1 and K10, which may explain why 2B helical mutations are particularly frequent in this keratin.

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