Ichthyosis Bullosa of Siemens—A Disease Involving Keratin 2e

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Ichthyosis bullosa of Siemens (IBS) is a congenital bullous ichthyosis without erythroderma. In contrast to bullous congenital ichthyosiform erythroderma (BCIE), there is a relatively mild involvement of the skin and epidermolytic hyperkeratosis (EHK) is restricted to the upper suprabasal layers of the epidermis. Tonofilament aggregation was observed by EM in suprabasal cells from affected patients in the two families under study, indicative of a keratin abnormality. Keratin 2e is a differentiation specific type II keratin expressed suprabasally in the epidermis. Part of the K2e gene was amplified by polymerase chain reaction using genomic DNA from affected and unaffected individuals from two IBS families. Direct sequencing of polymerase chain reaction products revealed a point mutation in the highly conserved helix termination motif, producing the protein sequence change LLEGEE—LLEGKE. This mutation was found in all affected members of a five-generation kindred and also in a sporadic case in a second unrelated family. No mutation was seen in unaffected individuals. The mutation destroys a MnlI restriction site, which allowed exclusion of the mutation from a population of 50 unaffected unrelated individuals by restriction fragment analysis of K2e PCR products. This is the sixth keratin gene found to be involved in an inherited epidermal disorder. Keywords: epidermolytic hyperkeratosis (EHK)/intermediate filaments/keratin mutation/K2e/epidermis/differentiation. [Invest Dermatol 103:277–281, 1994]
Polymerase Chain Reaction (PCR) A 1.5-kb fragment (approximately) of the K2e gene was amplified from genomic DNA using primers K2-5 (5’ GCA GTG TAA GAA TGT GCA AGA TG 3’, + strand) and K2-6 (5’ CAG TCA CAT TGC TGC TGA GG 3’, – strand). Primers were designed from the cDNA sequence [8] using the program PRIMER version 0.5 [13] and, due to the high degree of conservation between K2e and K1, primers were checked against a K1/K2e DNA alignment for K2e specificity. Due to the proximity of the helix termination sequence to the predicted position of intron VII, this intron is included in the K2-5/K2-6 fragment. Intron VII is about 1.25 kb in size. In PCR, was performed in 100-μl reaction volumes with standard buffer with 1 mM MgCl2 and 10% dimethylsulfoxide and 1 U of AmpliTag polymerase (Perkin-Elmer-Cetus, CA). After an initial incubation at 94°C for 5 min, PCR was carried out for 30 cycles consisting of 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min on an Ommagn Temperature Cycler (Hybaid, UK).

Cycle Sequencing PCR fragments were resolved on 1.5% low melting point agarose gels and excised bands purified using Gelase (Epicentre, Madison, WI). Approximately 50 fmol of purified template DNA was sequenced by the following program: 20 cycles consisting of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min; followed by 10 cycles consisting of 94°C for 30 s, 72°C for 1 min. Sequencing ladders were resolved on standard 6% polyacrylamide denaturing gels.

Mutation Screening by Restriction Fragment Length Polymorphism (RFLP) The mutation found in both families destroys a recognition site for the restriction enzyme MnlI. Due to the close proximity of intron VII, it was necessary to design a primer for screening purposes. This was carried out by cycle sequencing as above with primer K2-7 (above). For screening, a 163-bp fragment containing the mutation was amplified using primers K2-7 and K2-14 (5’ TTC CCA GTG CCC ACA CCT GGG GGA GG 3’, + strand). Cycle sequencing was performed using the following program: 20 cycles consisting of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min; followed by 10 cycles consisting of 94°C for 30 s, 72°C for 1 min. Sequencing ladders were resolved on standard 6% polyacrylamide denaturing gels.

RESULTS

IBS Is Clinically Distinct from BCIE The proband in family 1 is a 10-year-old girl (Fig 1) who was born with normal skin. She has never been erythrodemic. Blisters developed within a few months of age, mainly localized to the hands and knees, precipitated by physical trauma. From the age of 6 months, keratotic lichenification was noted over the extensor aspects of the elbows and knees, the arms, superficial erosion of skin was seen, a phenomenon known as mosaering or moulting phenomenon. Nails, hair, and oral mucosa were normal. There was no nail-patellar keratoderm. Other members of the family are affected in five consecutive generations. The proband in family 2 has a phenotype indistinguishable from those affected in family 1. No other family members are affected.

Outer Suprabasal Cells in IBS Contain Abnormal Tonofilaments The microscopic findings were essentially the same in all biopsy samples. Light microscopy showed hyperkeratosis with poorly compacted cornified cells; slight or moderate acanthosis and papillomatosis; and a minimal inflammatory cell infiltrate in the superficial dermis. Epidermal cells from the mid-spinous layer up to and including the granular layer appeared enlarged with a pale edematous or vacuolated cytoplasm. Disintegration of the epidermal cells in the mid-spinous layer up to and including the granular layer appeared enlarged with a pale edematous or vacuolated cytoplasm. Disintegration of the epidermal cells in the mid-spinous layer up to and including the granular layer appeared enlarged with a pale edematous or vacuolated cytoplasm.

Figure 1. IBS is inherited in an autosomal dominant fashion. Pedigrees of IBS families under study. Arrows, probands in each family. Family 1 shows clear autosomal dominant Mendelian inheritance; family 2 is a sporadic case. Individuals are numbered by standard genetics nomenclature; *, those which took part in this study.

mis associated with cell lysis was not apparent. These altered cells could occur focally in groups rather than diffusely throughout the upper epidermis. Keratohyalin granules were often small and indistinct.

In some sections, the stratum corneum had become detached just above its normal junction with the granular layer. Electron microscopy revealed a structurally normal basal and lower suprabasal layer. Bundling or clumping of the tonofilaments was evident in the mid-spinous layer and became more marked in the upper spinous and lower granular layer (Fig 2A). The filament clumps tended to be dispersed more in the cell periphery than around the nuclei; much of the cytoplasm around the cells was dematous by light microscopy was filament free and contained polyribosomes, mitochondria and, in the granular layer, lamellar granules. Keratohyalin was associated with the clumps and also occurred as small round granules associated with polyribosomes (Fig 2A). In normal spinous or granular epidermal keratinocytes the tonofilaments were often found in loose bundles (Fig 2B) but never formed the tight clumps as seen in IBS skin.

IBS Patients Are Heterozygous for a K2e Mutation A 1.5-kb fragment of the K2e gene was amplified from genomic DNA samples using primers K2-5 and K2-6. This fragment contains all of exon 7, which encodes part of the 2B domain and the helix termination peptide of K2e, intron VII, and a small portion of exon 8 encoding the V2 domain. 256 bp of this PCR fragment are coding sequence and so the deduced size of intron VII is about 1.25 kb, about twice the size of the equivalent intron in K1. However, the position of the intron was determined by direct sequencing of the PCR product and is completely conserved, in keeping with the other type II keratins [14]. Intron splice sites were found to conform to the reported consensus sequences [15].

Gel-purified PCR products derived from affected and unaffected individuals from IBS families 1 and 2 were directly sequenced using an internal primer K2-7, located in exon 7. In five unaffected individuals analyzed from families 1 and 2 and in four normal, unrelated individuals, the coding sequence was found to be identical to the published cDNA sequence, with no polymorphisms or silent mutations observed. However, all affected individuals analyzed in both IBS families (five in total) were found to be heterozygous for a G to A transition at nucleotide 1510 of the cDNA sequence (G1510A) as shown in Fig 2A. This produces a change of glutamic acid to lysine in codon 493 (E493K) in the highly conserved LLEGEE helix termination motif and thus is likely to be highly detrimental to keratin filament assembly and/or functional integrity (Fig 2B). This mutation is analogous to those reported in K5 causing EBS-DM [16] and...
in K1 causing BCIE [17] thus demonstrating once again the sensitivity of this motif to pathologic mutation in keratin molecules.

The mutation produces a Muli restriction fragment length polymorphism that was used to design a PCR-based screening test. Digestion of [32P]-labeled PCR products amplified from normal individuals using primers K2-7 and K2-14 results in a 34-bp band. All affected members of both families were found to have an additional 40-bp band (Fig 4). This test was used to exclude the mutation from 50 normal unrelated control samples indicating that this mutation is not a common polymorphism.

Members of family 2 were genotyped with a number of highly polymorphic markers (data not shown). Normal Mendelian inheritance with no evidence of non-paternity was observed; therefore, it is likely that this sporadic case represents a new mutation.

**DISCUSSION**

**The Observed K2e Mutation May Be a Common Cause of IBS** Here we describe (with Kremer et al [18]) mutations in K2e that are associated with IBS, a pathologic condition affecting the outer layers of the epidermis. The base change observed in two unrelated incidences is consistent with a methylated CpG deamination mutation of the 5-methyl-cytosine on the antisense strand at position 1510. This results in a CG to CA transition in the sense strand. Methylated CpG sequences have a higher mutation rate than other dinucleotides, taking the obvious bias for deleterious mutations into account [19]. Here, IBS family 1 has affected members in five generations; however, the affected member of IBS family 2 represents a new mutation causing this disease, the parents having been shown to have a normal K2e sequence. Kremer et al [18] describe two further unrelated Dutch families with identical mutations. The occurrence of the same mutation in four apparently unrelated families including one sporadic case indicates that this is likely to be a common mutation causing IBS.

**IBS Ultrastructural Changes Are Consistent with Phenotype** At the electronmicroscopic level, IBS is a disorder characterized by filament aggregation in the upper suprabasal cells leading to
hyperkeratosis and shedding of the stratum corneum. There are a number of ultrastructural features that are distinct from BCIE. Firstly, frank lysis of epidermal cells is not a prominent feature. Secondly, tonofilament clumps were not seen in the lower epidermal layers and also tended to be distributed more peripherally in the cells. The clinically superficial erosions or “moultng” probably results from separation of the stratum corneum at a low level. This could be due to abnormal structure of horny cells as reported in BCIE [20].

Figure 3. DNA Sequencing for Families 1 and 2. a) IBS individuals are heterozygous for a G to A mutation in K2e. Excerpts from DNA sequencing gels showing K2e sequence derived from IBS affected and unaffected individuals in both families 1 and 2. IBS individuals are shown to be heterozygous for a G to A transition corresponding to nucleotide 1510 of the cDNA sequence (arrows). b) The IBS mutation in both families results in an E to K transition. DNA sequence and deduced protein sequence in the region of the reported mutation showing the loss of an MnlI restriction site (GGAG) in the mutant allele. The LLEGEE motif is most highly conserved within the various intermediate filament proteins.
These ultrastructural differences between IBS and BCIE are in agreement with the differences in expression between K1/K10 and K2e suprabasally [8].

The observed ultrastructural changes result in the observed clinical phenotype of the disease—very superficial epidermolytic hyperkeratosis, with scaling confined to the outermost layers of the skin. The disease tends to preferentially affect certain body sites—the elbows and knees where the epidermis is subject to a lot of flexion and/or abrasion. Further work is required, using specific antibodies or in situ hybridization, to determine the expression of K2e at these different body sites.

The Observed IBS Mutations Cause Keratin Filament Aggregation IBS falls into a category of diseases where grossly abnormal filaments are seen by EM. The other examples where tonofilament clumping is evident are EBS-DM, BCIE, and EPPK. The unifying feature of all four of these diseases is the location of mutation in the keratin protein, within the highly conserved ends of the rod domain. In EBS-WC and EBS-K, the milder EBS variants, mutations in K5 and K14 are found in locations other than the helix initiation and termination peptides (reviewed in [10]). There is a body of evidence that indicates that the rod end sequences are important in production of normal 10-nm filaments—transfection experiments [21,22], transgenics [23], and now disease causing mutations in 6 keratins [10].

The question arises as to why these motifs are so sensitive to sequence alteration. Cross-linking studies have been interpreted as suggesting that there is a 1A/2B overlap involved in higher-order assembly of intermediate filaments [24]. The manner of such an interaction is as yet unknown although studies using synthetic peptides of desmin rod ends has shown that these tend to self-aggregate into filamentous structures that contain beta-sheet conformation [25]. The region where mutations produce filament aggregation is slowly being defined by the discovery of more keratin mutations. If the rod ends are really involved in overlap interactions between dimers or tetramers, these interactions would explain why mutations in these parts of the protein are severe in their effects. However, the evidence for this is largely circumstantial at present and awaits direct experimental proof.