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DNA based prenatal testing for the skin blistering disorder epidermolysis bullosa simplex

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Epidermolysis bullosa simplex (EBS) is a skin fragility disorder in which mild physical trauma leads to blistering. The phenotype of the disorder is variable, from relatively mild affecting only the hands and/or feet, to very severe with widespread blistering. For the severest forms of EBS there is a demand for prenatal diagnosis which until now has involved a fetal skin biopsy in the second trimester. The identification of mutations in the genes encoding keratins K5 and K14 as the cause of EBS opens up the possibility of much earlier diagnosis of the disease. We report here four cases in which prenatal testing was performed. In three of the cases the genetic lesions were known at the start of the pregnancy, requiring the identification of the causative mutation prior to testing fetal DNA. In two of the four cases novel mutations were identified in K14 and in the two remaining families, a previously identified type of mutation was found. Fetal DNA, obtained by chorionic villus sampling or amniocentesis, was analysed for the identified mutations. Three of the DNA samples were found to be normal; a mutant K14 allele was identified in the fourth case and the pregnancy was terminated. These results demonstrate the feasibility of DNA-based prenatal testing for EBS in families where causative mutations can be found. Copyright © 2000 John Wiley & Sons, Ltd.

KEY WORDS: prenatal diagnosis; epidermolysis bullosa simplex; keratin 14; mutation detection

INTRODUCTION

Epidermolysis bullosa simplex (EBS) is one of a number of genodermatoses caused by mutations in keratin genes (Corden and McLean, 1996). The phenotype of EBS is variable but in its severest form it is extremely debilitating and may be life-threatening, particularly in early childhood. In families with a history of the disease there is a demand for prenatal diagnosis but until recently this was only possible by ultrastructural analysis of fetal skin biopsies taken at around 18 weeks' gestation (Eady, 1992). The identification of mutations in keratins K5 and K14 as the cause of the majority, if not all, EBS cases offers the prospect for diagnosis of the disorder during the first trimester of pregnancy.

EBS is characterized by fragility of the basal keratinocytes of the epidermis which causes them to rupture upon mild physical trauma and leads to the formation of fluid-filled blisters. Clinically, EBS is divided into three main subtypes based on the severity, distribution and age of onset of blistering. Weber–Cockayne EBS is the mildest and commonest form of the disease: blisters are usually confined to the hands and/or feet and often do not occur until well into childhood. Koebner EBS is more severe: blistering is more generalized and onset is generally earlier than for the Weber–Cockayne form. The severest form of the disease is Dowling–Meara EBS. This is an autosomal dominant disorder characterized by widespread hornified blisters which appear at or shortly after birth. Dowling–Meara EBS can be distinguished ultrastructurally from other forms of the disease by the presence of electron dense keratin aggregates in the cytoplasm of the basal keratinocytes.

The keratin target genes of EBS are members of the multigene family of intermediate filament proteins (IFs) which form a fibrous cytoskeleton in the cytoplasm of cells. The keratin IFs are divided into two groups, type I and type II proteins, based on their size and charge characteristics. Keratins are obligate heteropolymers, requiring equal amounts of type I and type II proteins for filament formation. They share a common structure with other members of the intermediate filament protein family comprising of a long central, predominantly z-helical rod domain flanked by a non-helical head and tail. Highly conserved motifs at the beginning (helix initiation motif) and end (helix termination motif) of the rod domain are important for filament assembly and mutations in these regions lead to filament abnormalities (Hatzfeld...
and Weber, 1991; Letal et al., 1992). These helix boundary motifs are the most common sites of mutations in EBS and other keratin disorders (Corden and McLean, 1996).

Keratin expression is strikingly tissue-specific, and dependent on the differentiation state of the cell. K5 and K14 are expressed by the basal keratinocytes of the epidermis and it is mutations in these proteins that are the cause of EBS (Bonifas et al., 1991; Coulombe et al., 1991; Lane et al., 1992). More than 40 different pathogenic mutations have now been identified in K5 and K14 and there is growing evidence of a correlation between the position of the mutation and disease severity (Corden and McLean, 1996; Corden et al., 1998; Galligan et al., 1998; Irvine et al., 1997a, b; Muller et al., 1998, 1999; Nomura et al., 1996; Rugg et al., 1999; Sasaki et al., 1999; Sorensen et al., 1999; Stephens et al., 1997). In addition, a number of non-pathogenic polymorphisms have also been reported in K5 and K14 (Wanner et al., 1993; Sorensen et al., 1999). Mutations identified to date in individuals affected by the Dowling-Meara form of EBS are confined to the conserved helix initiation motifs of K5 and K14 and the helix termination motif of K5. Of these mutations 50% occur in the helix initiation motif of K14. Although no mutations have yet been reported in the helix termination motif of K14, in vitro studies predict that mutations in this region are likely to result in a similar phenotype (Hatzfeld and Weber, 1991). The clustering of mutations has enabled us to develop protocols for rapidly identifying pathogenic mutations in patients affected by the severest forms of EBS and offer prenatal diagnosis of the disease.

We report here prenatal testing for EBS in four families affected by the disorder. In three cases the fetuses were found to be normal but in the fourth case a keratin abnormality was detected.

METHODS

DNA and tissue samples

Blood samples were obtained from Family A: II.1, II.2, III.1; Family B: I.1, I.2, II.1; Family C: I.1, I.2, II.1; Family D: II.1, II.2, II.3, II.4, III.1, III.2, III.3 (see Figure 1) and DNA was extracted from peripheral lymphocytes. Fetal DNA was extracted from cells recovered from amniotic fluid (A.III.2 and D.IV.1) or from chorionic villi (B.II.2 and C.II.2). Skin biopsies were taken from A.III.1, B.II.1.

Electron microscopy and immunochemistry

Electron microscopy and immunochemistry were carried out as previously described (Bergman et al., 1997). Antibodies used were a broad spectrum keratin (pankeratin antibody; Diagnostics Product Corporation, USA), Col 94 (collagen IV; Accurate Chemical and Scientific Corp., Westbury, NY, USA), GB3 (laminin-5; Accurate Chemical and Scientific Corp., Westbury, NY, USA) and KL2 (keratin-19; Accurate Chemical and Scientific Corp., Westbury, NY, USA). The primers were specific for the following regions: 5' CGC ATG TCT CTG ACA CCT CAG 3' (sense); 5' GTA GAG CAG CTT CGG TAC TCC ATC 3' (antisense). The PCR products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics, Ltd., Lewes, UK) and sequenced directly on an ABI PRISM® 373 or 377 DNA sequencer using the

Figure 1—Pedigree of the four families analyzed in this report. Open symbols are unaffected or not known to be affected; filled symbols are affected; crossed symbols are deceased; ○ indicates unknown sex; shaded ○ indicates fetuses on which the prenatal testing was performed.

Westbury, NY, USA) and collagen VII (LH7.2; Chemicon International, Inc., Temecula, CA, USA).

PCR amplification and sequencing of target regions

PCR was used to amplify the regions encoding the helix initiation and termination motifs of K5 and K14. 0.1 to 1 μg of DNA was incubated in the presence of 200 μM dNTP, 10% DMSO, 2 mM MgCl2, 5 μg/ml oligonucleotide primers and a buffer comprising of 60 mM tris-HCl, pH 9.5, 15 mM (NH4)2SO4. Incubations were for 3 min at 94°C, followed by 30 cycles of 30 s at 94°C, 1 min, at 55°C, and 2 min at 72°C and a final incubation of 5 min at 72°C. The following primers were used to specifically amplify each region: K5 helix initiation motif: 5' GGT GGT GCG TTT GGG CTC GGT 3' (sense), 5' CTC TTT GCC ATT TCT TTC AGA CCC 3' (antisense); K14 helix initiation motif: 5' TAC CCG AGC ACC TTC TCT TC 3' (sense), 5' TGC TGG AGA ACA AGT AGC TGC 3' (antisense); K14 helix termination motif: 5' CAG TAT TCA GGC CTA AGG AAC A 3' (sense), 5' GGA AGA GGT GGG AAG AGC AC 3' (antisense). These K14 primers exclude the K14 pseudogene. The helix termination motif of K5 was amplified in a similar manner except the buffer was replaced by a buffer containing 50 mM KCl, 10 mM tris-HCl, pH 8.3, 0.1% Triton X-100 and the temperature of the annealing step was increased to 60°C. The primers were as follows: 5' CGC ATG TCT CTG ACA CCT CAG 3' (sense); 5' GTA GAG CAG CTT CGC TAT TCC ATC 3' (antisense).

The PCR products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics, Ltd., Lewes, UK) and sequenced directly on an ABI PRISM® 373 or 377 DNA sequencer using the
following primers. K5 helix initiation motif: 5’ CTC TTT GGC ATT TAT TTC AGA CCC 3’ (antisense); K5 helix termination motif: 5’ GCT GGC CTA GCT GGA GGA GCC 3’ (sense); K14 helix initiation motif: 5’ GGG GGA GCC TAT GGG TTG GGG 3’ (sense); K14 helix termination motif: 5’ ACG GAG CCC CTA GCC AAC 3’ (antisense).

**Confirmation of K14: Tyr<sub>413</sub>His mutation**

A new mutation, K14: Tyr<sub>413</sub>His, was found in the helix termination motif of K14. The mutation was confirmed by polymerase chain reaction amplification of specific alleles (PASA) analysis (Sommer et al., 1992). A 751 fragment of K14 DNA was amplified using the forward primer 5’ CAG TAT TCA GGC CTA AGG AAC 3’ and either 5’ CGC CCT CCA GCA GGC GGC GGT A 3’ (wild type) or 5’ CGC CCT CCA GCA GGC GGC GGT C 3’ (mutant). The incubations were carried out in the presence of 200 μM dNTP, 1 mM MgCl2, 50 mM KCl, 10 mM tris.HCl, pH 8.3, 0.1% triton X-100. The reaction was performed in HotStart reaction tubes (Molecular Bio-Products Inc., San Diego, CA, USA) and the cycling parameters were 2 min at 94°C, followed by 30 cycles of 30s at 94°C, 1 min at 65°C and 1 min at 72°C. Products were analysed on a 1.5% agarose gel.

**RESULTS**

**Clinical and histopathological observations**

Over the last three years, four families with a history of EBS have requested prenatal testing for the disorder (Figure 1). In three of the cases the pregnancy was already well established and the causative mutation unknown.

Family A is of Jewish origin. Two members of the family were affected by blistering. The affected child (A.III.1) presented with generalized blisters and erosions at birth which healed with almost no scarring except for some dystrophy of the nails. There was a marked improvement in blistering around puberty and now blisters mainly occur in summer and are confined to the hands and feet. Microscopic analysis showed cleavage occurred through basal cells and keratin clumps were present (Figure 2a). Collagen VII and laminin 5 staining were normal.

Family B is also of Jewish origin. The father is of Jewish-Syrian background and the mother is of Jewish-Iranian ancestry. There was no history of blistering in other family members. The affected child was born with blisters on various areas of his skin but predominantly on acral areas. The child continues to blister severely at his extremities and has a tendency to herpetiform blisters on his body. He has some hyperkeratosis on his soles and two toes show some nail dystrophy. He has no milia formation and there is no evidence of oral involvement. A diagnosis of EBS was made on the basis of clinical, histological and electron microscopical examinations. Immunohistochemical staining of formalin-fixed, paraffin-embedded sections with the 'pankeratin' antibody showed positive staining of both the floor and the roof of a blister indicating a separation through the keratinocytes. Collagen IV staining was confined to the dermal floor. Frozen sections stained with antibodies against laminin 5 and collagen VII showed normal linear staining along the basement membrane under the

![Figure 2](image-url)
blisters. Ultrastructural analysis showed the basal lamina on the dermal floor of the blister along with attached hemidesmosomes and cytoplasmic remnants of the lower epidermal keratinocytes confirming the intra-epidermal split characteristic of EBS. Keratin aggregates were seen in some keratinocytes confirming Dowling–Meara EBS (Figure 2b).

Family C was of Thai origin and had one EBS affected child who was clinically diagnosed as being affected by the Dowling–Meara variant of the disease. There was no previous history of the disease in this family.

A fourth family (family D) of Jewish-Ashkenazi origin has been affected by EBS for at least three generations with an autosomal dominant pattern of inheritance. The proband (D.III.2) suffered as a child from recurrent blistering of his feet, hands and body at any site of slight friction. His skin condition has improved significantly with age. The diagnosis of Koeberner EBS was made by clinical examination, electron microscopic analysis and immunocytochemistry of the proband. There was no evidence of keratin filament aggregation in the keratinocytes of this patient.

Identification of mutations and prenatal testing

The majority of pathogenic keratin mutations occur in the conserved helix boundary motifs (Corden and McLean, 1995) and therefore these regions of K5 and K14 were targeted for analysis. DNA was obtained from affected and unaffected members of all four families. PCR was used to specifically amplify the regions encoding the helix initiation and termination motifs of K5 and K14 from genomic DNA and the products were sequenced directly. Fetal DNA was obtained from chorionic villus samples taken at 11 weeks gestation (B and C) and by amniocentesis at 16 weeks’ gestation (A and D).

Affected individuals in families A and C were all found to be heterozygous for a C-to-T transition in codon 125 which results in the change of arginine to cysteine. This mutation destroys an Aci I restriction endonuclease site and this was used to confirm the mutation; (Stephens et al., 1993). Both fetal DNA samples were negative for this mutation (results not shown).

A novel mutation was identified in the affected child of family B. A heterozygous T-to-C transition was found at codon 415 (Figure 3a, b) which results in the change of tyrosine to histidine in the helix termination peptide of keratin 14 (Figure 3c). The mutation was confirmed by PASA analysis (Figure 4) and was not found in either parent or in more than 50 unrelated samples. The fetal DNA was also found to be normal.

A different novel mutation was identified in family D. A G-to-C transversion was found in the region encoding helix 1A of K14 which results in the change of the arginine at position 134 to proline (Figure 3d, e).

All affected family members analysed were heterozygous for this mutation. To exclude the possibility that this mutation was a common polymorphism, DNA from 50 unaffected, unrelated individuals was analysed by direct sequencing of this region of the K14 gene. The identified mutation was not present in any of the control samples. In this case, chorionic villus sampling failed to yield fetal DNA for testing and amniocentesis was performed at 16 weeks. The fetus was found to carry the pathogenic mutation and the mother elected to terminate the pregnancy.

In the three cases in which the fetal DNA was found to be normal the pregnancies continued to full term and the babies were normal.

DISCUSSION

EBS is a debilitating disorder which can cause considerable physical and emotional suffering. Prenatal diagnosis for EBS has been available for a number of years by ultrastructural analysis of fetal skin biopsies taken during the second trimester of pregnancy (Eady, 1992). The identification of keratin genes 5 and 14 as the site of mutations leading to EBS has permitted the development of DNA-based analysis as an alternative, minimally invasive, procedure which can be offered during the first trimester of pregnancy.

The keratin genes, K5 and K14 harbour the pathogenic mutations leading to EBS and more than 40 mutations have so far been identified. No direct test is therefore available for the genetic diagnosis of EBS and for each family, mutation analysis must be carried out. The majority of the pathogenic mutations are clustered in the helix initiation motifs of K5 and 14 and the helix termination motif of K5. The clustering of the mutations has permitted the development of a rapid protocol for mutation analysis of families with EBS. Using these rapid protocols the disease-specific mutations were identified in two families with a history of EBS and in two other families in which the disease was apparently de novo event. In three of the cases pregnancy was established prior to a request for prenatal testing and the pathogenic mutations unknown. Despite these pressures imposed we were able to identify causative keratin mutations and screen the fetal DNA for the sequence alterations. The fetal DNA samples were found to be normal in three of the tests but in the fourth case, the fetus was found to carry the pathogenic mutation and the pregnancy was terminated.

Clinically EBS is divided into three groups based on the distribution and severity of the blistering; however within this classification there is a spectrum of overlapping phenotypes (Horn and Tidman, 2000). Dowling–Meara EBS is the severest form of the disease and it is families with this form of EBS who are the most likely to seek prenatal testing. Koeberner EBS is generally considered milder than Dowling–Meara EBS; however, it can still be an extremely painful and debilitating disorder, particularly during...
Figure 3—Chromatograms showing the DNA sequence obtained for part of helix 2B of K14 from (a) an unaffected (II.2) and (b) an affected (II.1) member of family B. Similar chromatograms are shown for part of helix 1A of K14 for DNA from (c) an unaffected (II.1) and (d) an affected (III.2) member of family D. The corresponding DNA and amino acid sequences for the normal (top) and mutant (bottom) alleles of helix 2B and helix 1A are shown in e and f respectively.

Figure 4—A 1.5% agarose gel showing the results of PASA analysis of DNA from B.I.1 (unaffected), B.II.1 (affected) and H2O. Lanes 1, 3 and 5 are the PCR products using primers to the normal allele and lanes 2, 4 and 6 are the same PCR reaction but in this case the antisense primer is specific for the mutant allele.

Considering prenatal diagnosis, that each case is assessed on its own merit.

In families B and C, who had no previous history of EBS, the identification of the pathogenic mutation in the affected children permitted testing of the parents to confirm the \textit{de novo} nature of the disease in these families. Testing of the parents confirmed that they did not carry the mutation, therefore the affected children were likely to be due to a germ line mutation in one of the parents. The relative risk to future pregnancies was therefore very low but parental concern was alleviated by the availability of prenatal diagnosis.

Two of the cases reported here had novel keratin mutations. In family B, the mutation, K14: Tyr415 His, resulted in keratin filament aggregation and severe generalized blistering. The mutation lies in the highly conserved helix termination motif (Geisler and Weber, 1983) and the mutated tyrosine is invariant in all intermediate filament proteins sequenced to date (Quinlan \textit{et al.}, 1995). This is the first report of a mutation in this region of K14 although pathogenic mutations have been found in the helix termination...
motif of another type I keratin, K10 and in the equivalent region of a number of type II keratins (see Corden and McLean, 1996). This motif is clearly critical for normal filament assembly and function, as demonstrated by several studies. For example, transfection of a C-terminal truncated K14 missing part of the conserved motif into keratin expressing cell lines results in disruption of the endogenous network (Albers and Fuchs, 1987). Similar experiments with neurofilament proteins NF-L and NF-M resulted in disruption of endogenous vimentin and NF-L networks (Wong and Cleveland, 1990). Hatzfeld and Weber (1991) made a number of substitutions of conserved residues in the helix termination motifs of K8 and K18, including changing this conserved tyrosine residue for alanine. All the mutant keratins caused the formation of large electron dense aggregates in vitro polymerization studies (Hatzfeld and Weber, 1991). The identification of a pathogenic mutation in the helix termination motif of K14 supports a role for this region in normal filament assembly and that mutations in this motif lead to gross keratin cytoskeleton disruption.

A second novel mutation, Arg114Pro, was identified in helix 1A of K14, in affected members of family D. This mutation caused severe widespread blistering; however, no filament aggregates characteristic of Dowling–Meara EBS were detected and therefore the disorder was classified as the Koebner variant. Although, proline residues are often disruptive in α-helices, Letal and colleagues showed that whilst the introduction of proline residues into the conserved rod domains of K14 caused keratin filament disruption, similar substitutions at more internal sites were without any observable effect (Letal et al., 1992). Our results suggest that although some proline substitutions may result in normal-looking keratin filaments, the cytoskeleton formed by these filaments may be defective and cause cellular fragility.

The same mutation, K14: Arg112Cys, was identified in the two remaining families. Mutations in codon 125 of K14 account for more than 50% of mutations identified in Dowling–Meara EBS and of these the majority result in the change of arginine to cysteine. The majority of mutations in other type I keratins also occur at this site and thus this codon, in type I keratins, is by far the most common site for pathogenic mutations (Corden and McLean, 1996).

Mutations causing the severest forms of EBS are clustered in the helix initiation or termination motifs of either K5 or K14 and this has enabled us to develop protocols for rapidly identifying keratin mutations in the majority of patients affected by this disorder. DNA based prenatal diagnosis can now be carried out for EBS and in the majority of cases can be performed towards the end of the first trimester.

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