Prenatal Diagnosis of Epidermolytic Hyperkeratosis by Direct Gene Sequencing

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Epidermolytic hyperkeratosis (bullous congenital ichthyosiform erythroderma) is an autosomal dominant skin disorder caused by defects in the suprabasal keratins. Recently, mutations in the keratins 1 and 10 have been identified in patients with this disease. In this study, direct gene sequencing was used to establish the prenatal diagnosis in 15-week gestation twins at risk for epidermolytic hyperkeratosis. Direct sequence analysis of genomic DNA from the affected father and from both chorionic villus samples revealed a tyrosine to asparagine mutation at position 14 within the highly conserved 1A alpha-helical segment of keratin 10. None of the unaffected family members that were analyzed exhibit this mutation nor have polymorphic variations been observed in the normal population at this position. This residue is invariant in all type I keratins sequenced to date and is also conserved in related intermediate filament proteins such as vimentin and lamin. Given this high degree of conservation it is probable that any mutation at this position is deleterious and will result in disease.

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Epidermolytic hyperkeratosis (EHK) is an autosomal dominant genodermatosis (number 11380 in McKusick [1]) manifested by erythroderma and widespread blistering at birth and the development of hyperkeratosis in later weeks [2]. Blistering is less common in older individuals but superficial erosions can occur after physical trauma, stress, or in some patients, on treatment with retinoids. Histopathology is characterized by a marked expansion of the granular and stratum corneum layers. Analysis of blistering skin shows cytolytic and intra-epidermal cleavage in the suprabasal layers [3]. The ultrastructural hallmark of EHK is the appearance of keratin filament aggregates and clumping, often with a perinuclear distribution, in the spinous and granular cells of these patients [4,5]. In fact, ultrastructural studies were the first to suggest an association between the disease and defects in the suprabasal keratins [4–6] and this view was later reinforced by immunohistochemical [7] and gene linkage [8–10] studies. Recently, we and others have described mutations in highly conserved regions of keratins 1 and 10 (K1 and K10) in patients with this disease [11–14]. All defects documented in EHK patients to date have involved point mutations resulting in amino acid substitutions within the H1 segment of K1 or within the specialized sequences at the ends of the rod domains of K1 and K10. These conserved regions are critical to normal filament function and assembly, and mutations in them are thought to weaken the integrity of these filaments, leading to collapse of the network and tonofilament clumping around the nucleus (reviewed in [15]). Interestingly, most of these mutations occur within a conserved sequence motif at the beginning of the rod domain of K10, with the arginine codon at position 10 being particularly vulnerable to mutation [14].

Prenatal diagnosis of EHK has been performed previously by ultrastructural analysis of fetal skin biopsies and on amniotic fluid cells [16–19]. The diagnosis is based on the finding of keratin filament aggregates in these cells. However, analysis of amniotic cells is not considered reliable enough to exclude the possibility of a false-negative diagnosis and ultrastructural analysis of fetal skin biopsies requires the sampling of more than one site, because tonofilament clumping may not be uniformly presented throughout the fetal epidermis [20]. Although in the normal fetus keratinization does not begin until the twenty-fourth week, the suprabasal keratins K1 and K10 are expressed as early as week 14 [21], giving this gestational age as the absolute lower limit for a diagnosis based on ultrastructure analysis. However, the earliest gestational age reported for a prenatal diagnosis of EHK is 19 weeks [20]. Here we report the prenatal diagnosis of twin fetuses at risk for EHK using direct gene sequencing of chorionic villus samples at 15 weeks gestation. In addition, the genetic defect causing EHK in this family is described.

**MATERIALS AND METHODS**

**Extraction of Nucleic Acids** DNA was extracted from 10 ml of whole blood by lysing cell membranes in an equal volume of 10 mM Tris-HCl (pH 7.5), 0.32 M sucrose, 5 mM MgCl2, and 1% Triton X100. Intact nuclei were collected by centrifugation and suspended in 1.5 ml 0.9% NaCl and 1.5 ml lysis buffer (Applied Biosystems, Foster City, CA), and incubated with proteinase K at a final concentration of 250 μg/ml at 37°C for 16 h. DNA was extracted from chorionic villus samples by incubating approximately 20 mg of tissue in 200 μl lysis buffer (10 mM Tris-HCl, pH 8.0, 2 mM ethylenediamine tetraacetic acid, 10 mM NaCl, 5% sodium dodecyl sulfate) and pancreatinase K to a final concentration of 250 μg/ml at 55°C for 16 h. All DNAs were purified using phenol/chloroform extraction and isopropanol precipitation [22]. The extracted DNA was dissolved in 10 mM Tris (pH 7.4), 1 mM ethylenediamine tetraacetic acid and stored at 4°C. Concentration and purity were assessed by spectrophotometry.

**PCR and Nucleotide Sequencing** The 1A region of K10 was amplified from genomic DNA using specific oligonucleotides (5’-GAGG-GATGGGGCCTTCTCRTTG-3’ and 5’-GCATAAGTGAACGC-CACATTGTGGC-3’) corresponding to bp 2080–2104 and to 2372–2395 of the published sequence [23]. The 5’ oligonucleotide was biotinylated to enable the purification of single stranded DNA for sequencing [24]. The sequencing primer used was 5’-GATTTGCTGTAGTACG-3’, corresponding to bp 2253–2270 of the published sequence [23]. The simulta-
neous amplification and sequencing of both the mutant and wild-type alleles enables the rapid identification of nucleotide changes in affected individuals. After PCR amplification, the DNA was captured onto streptavidin-coated magnetic beads via the biotinylated primer and denatured, and the resultant single-stranded DNA was used as a template in a manual DNA sequencing reaction [11].

RESULTS AND DISCUSSION

The proband, a 32-year-old man with EHK, first approached us for a prenatal diagnosis during the thirteenth week of his wife's second pregnancy. The proband was born to unaffected non-consanguineous parents and is the only member of his family with a dermatologic disorder (Fig 1). Diagnosis of EHK was made at birth and later confirmed by ultrastructural analysis.

By history, he suffered from extensive blistering as a neonate and development of erosions and inflammation with bacterial superinfection later as a child. Episodes of blistering gradually diminished with age but were replaced with widespread hyperkeratosis. He has responded well to retinoid treatment (etretinate 35 mg daily, orally) with a diminution of hyperkeratosis. External therapy consisted of 10% urea pura ointment. The couple has had one previous pregnancy resulting in a healthy unaffected boy, now 6 years old (individual III.1 in Fig 1).

To provide a prenatal diagnosis, we first had to ascertain the genetic defect of the father. Of the EHK patients that we have studied to date (total of 12 probands) ([11,14] and unpublished data), nearly 50% bear mutations within the "helix initiation" motif of K10, a further 25% bear mutations elsewhere in the rod domain of K10 or in K1 and in the other 25% of patients we have been unable to locate a mutation within the rod domains of these keratins. It is probable that these patients harbor mutations in one of the other suprabasal keratins such as K2e [25]. In our initial analysis we sequenced the regions in which all mutations causal for EHK have been documented including the H1,1A, and 2B segments of K1 and the 1A and 2B segments of both K1 and K10 from the proband. This analysis revealed a T to A transversion, resulting in a tyrosine (Y) to asparagine (N) substitution at position 14 of the K10 1A segment (Fig 2). No other substitutions were observed in either K1 or K10. To ensure that this mutation was not the result of a spurious alteration introduced by the polymerase chain reaction, each reaction was repeated and the products sequenced independently. Unaffected family members did not exhibit this base change in their DNA (Fig 3). Moreover, we have observed no sequence polymorphisms at this site in the DNA from 50 unrelated individuals, nor are there any reports of polymorphic variations of this residue in the literature.

The first 15 residues of the 1A segment are highly conserved among all intermediate filament types [26] and are thought to be involved in filament assembly through interactions with the TYRXXLEGGE motif at the end of the 2B segment of neighboring molecules [27]. The tyrosine to asparagine substitution at position 14 of this highly conserved region is therefore likely to affect the stability of these head-to-tail interactions, producing a weakened filament that is prone to collapse. Although there is as yet no functional data available, either from transgenic or tissue culture studies, confirming that this mutation is causal for this disease, the nature and position of the mutation suggests that it is so. This tyrosine is absolutely conserved in all human type 1 keratins sequenced to date as well as in keratins of other species and in the other intermediate filament proteins [12,26,28]. The high degree of conservation of this residue suggests that few, if any, changes at this position can be tolerated. Thus any mutations are likely to be deleterious and lead to disease, and on this basis we felt confident to proceed with the prenatal diagnosis.

The chorionic villus DNA samples were subsequently analyzed and both were found to contain the same T to A transversion as the proband (Fig 3) and a diagnosis of EHK was made. We were fortunate that the proband's mutation occurred within a highly conserved and well-characterized region of the gene. Had the proband's mutation occurred in a less-conserved region we would have been very circumspect in rendering a diagnosis in the absence of supportive functional data from in vitro and transgenic studies. As our data base increases for both mutations segregating with the disease, as well as polymorphic variation within the unaffected population, these determinations will be made with greater confidence. Although this is the first report of this substitution in K10 or in any other type 1 keratin, a tyrosine-to-aspartate substitution has been described at this position in another EHK proband.§


Figure 1. Pedigree of the EHK-M family. Men are represented by squares, women by circles, and the fetuses by diamonds. Solid symbols denote affected individuals.

Figure 2. Sequence analysis of the 1A segment of K10 from the EHK-M proband (individual II.2 in Fig 1). Normal, sequence obtained from an unaffected individual. Hetero, sequence obtained from the proband who is heterozygous for the mutant allele. The amino acid residues are numbered with respect to the beginning of the rod domain [11]. The proband bears a T to A transversion that results in a tyrosine (Y) to asparagine (N) substitution at residue 14 of the rod domain. This position corresponds to residue 160 in Rieger and Franke [23].
The autosomal dominant mode of inheritance of EHK, coupled with its virtually complete penetrance [14], permits prenatal diagnosis once the exact defect has been identified in the affected parent. In this case chorionic villus samples were analyzed at 15 weeks but the technique would work equally well at a gestational age as early as 8 weeks. Furthermore, the direct gene sequencing protocol can be used to screen in vitro fertilized embryos, from affected couples, prior to implantation [29]. Once screened, unaffected embryos can be selected for transfer to the uterus, thereby eliminating the need for risky surgical procedures for prenatal diagnosis later in the pregnancy or for the need to terminate an affected fetus.

**Note Added in Proof:** The prenatal diagnosis of EHK has since been confirmed by ultrastructural analysis of epidermal biopsies taken at 18 weeks gestational age (Dr. M. Megahed, Department of Dermatology, University of Düsseldorf, personal communication).

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**REFERENCES**


**FIRST ANNOUNCEMENT**

International Symposium on Inherited Epidermolysis Bullosa will be held April 25–26, 1994, sponsored by the Department of Dermatology and The Office of Continuing Medical Education, University of North Carolina School of Medicine and The National Epidermolysis Bullosa Registry (NEBR).

An intensive two day meeting is planned which will include lectures and panel discussions by leading authorities in the field. A poster session will also be held. Clinical topics include subclassification of EB phenotypes, demographic and epidemiologic findings from the NEBR and other cohorts, application of biostatistical modeling techniques as predictors of diagnosis and prognosis, carcinogenesis and EB, critical re-evaluation of laboratory diagnostic criteria, and multifaceted approaches to therapy. Basic research topics will include qualitative and morphometric ultrastructural changes within skin in selected EB phenotypes, altered antigenicity as a marker of disease, the role of enzymes in the pathogenesis of EB, and a mini-symposium on the molecular biology of EB.

For more detailed information and registration and abstract forms, contact Jo-David Fine, M.D., M.P.H., Department of Dermatology, The University of North Carolina at Chapel Hill, 137 NCMH, CB# 7600, Chapel Hill, NC 27514, (919) 966-3321, FAX (919) 966-3921.