

DNA-based Prenatal Diagnosis of Epidermolytic Palmoplantar Keratoderma in Two Pregnancies at Risk in One large Pedigree

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Background: Epidermolytic palmoplantar keratoderma (EPPK) is an autosomal dominant disease of cornification which presents as severe thickening of the palms and soles with prominent epidermolytic hyperkeratosis pathologically. Recent studies have shown that EPPK is caused by mutations in the keratin 9 (K9) gene which is expressed essentially only in the palms and soles. Previously, We have reported that patients in one large pedigree of EPPK have an R162W substitution in the K9 protein. In this pedigree, two women whose husbands are both EPPK patients had become pregnant.

Objective: Since both women were concerned about this genetic disorder, we have performed prenatal diagnosis by biopsy analysis of chorionic villi tissue.

Methods: Chorionic villi biopsies were performed at 12 weeks gestation. Since the skin lesions are strictly confined to the palms and soles of the babies, the prenatal diagnosis of EPPK by ultrastructural analysis of fetal skin biopsy or amniotic fluid cells is highly problematic. Polymerase chain reaction amplification of specific allele (PASA) assay and direct DNA sequencing analyses were performed whether the fetuses carried mutant allele of K9 gene.

Results: PASA assay and direct DNA sequencing analyses showed that one fetus was normal, but the other fetus carried the abnormal allele. Subsequently, the mother of the unaffected fetus delivered a normal child, but the mother of the affected fetus terminated the pregnancy.

Conclusion: We describe the analysis of the K9 mutation in the two fetuses at risk for EPPK. We believe that this is the first report of prenatal diagnosis for EPPK. But, we have to think about the ethical problems before we decide to perform the prenatal diagnosis of any kind of skin diseases.

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Epidermolytic palmoplantar keratoderma (EPPK) is one of the two autosomal dominantly inherited palmoplantar keratodermas which are characterized by severe thickening of the palms and soles. These are life-long conditions which usually manifest during the first week or month after birth. EPPK is characterized histologically by epidermolytic hyperkeratosis in the spinous and granular layers of the epidermis and ultrastructurally by abnormal keratin intermediate filament networks and the tonofilament clumping. The other autosomal dominantly inherited disorder is non-

epidermolytic PPK (NEPPK) which is also characterized by the thickening of the palms and soles, but does not have the epidermolytic changes of the epidermis. Since Voerner¹ first described the EPPK in 1901, several cases of familial inheritance of this disorder, as well as sporadic cases, have been reported in the literature^{2,3}.

Keratin intermediate filaments (KIF) constitute the major differentiation product of epidermal cells, and eventually constitute about 85% of the total protein in terminally differentiated cells⁴. To date, about 40 different keratin genes have been identified. Unlike the other IF proteins, KIF formation requires at least one acidic keratin (type I IF; pI 4.5-6.0, size from 40 to 65 kDa) and one neutral-basic keratin (type II IF; pI 6.5-8.5, size from 50-70 kDa). Each stage of keratinocyte differentiation can be characterized by the expression of specific type I /type II pairs that form obligate heteropolymers: for example, keratins 5 and 14 (K5/K14) are expressed in the basal layer; and K1/10 are expressed in the spinous layer^{4,5}. Keratin 9 (K9), a 64 kDa type I keratin, is almost exclusively expressed in suprabasal layer of the epidermis of palms and soles⁶. While it does not have a specific type II expression partner, it presumably forms a heteropolymer with K1. Based on these expression properties, the K9 gene was proposed as a candidate gene for EPPK. Indeed, the likely causative EPPK gene was mapped to the type I keratin gene cluster on chromosome 17q12-21⁷, and subsequently, three mutations in exon 1 of the K9 gene of EPPK patients were identified⁸. Since then, several different point mutations of the K9 gene have been reported from European, Japanese, and Korean families with EPPK⁹⁻¹⁶.

Prenatal diagnosis of inherited skin diseases, especially epidermolysis bullosa (EB) and epidermolytic hyperkeratosis (EHK), has been performed by ultrastructural analyses of fetal skin biopsies or amniotic fluid cells¹⁷. However, more recent molecular biology and molecular genetic techniques have made it possible to perform more reliable and unambiguous prenatal diagnoses of genetic diseases of cornification in affected families.

Previously, we reported a mutation resulting in an R162W substitution in the K9 gene product in affected members of a large pedigree of EPPK¹⁶. More recently, two family members had become pregnant. Here we report the prenatal diagnosis of two fetuses at risk for EPPK using direct sequencing of chorionic villi samples

at 12 weeks gestation.

MATERIALS AND METHODS

Family

The pedigree of this family is shown in Fig. 1. The clinical, histopathological and mutational analysis of the K9 gene from this pedigree have been reported¹⁶. More recently, two healthy pregnant women (III-9 and III-4) whose husbands have EPPK disease wanted prenatal diagnosis of their fetuses. The first child of III-9 was free of disease (IV-2), but that of III-4 was affected (IV-1). Thus transvaginal chorionic villi sampling under ultrasound guidance was performed at 12 weeks of gestation with informed consent. Fresh blood samples were also obtained from both affected and unaffected family members.

Extraction of Genomic DNA

Fetal DNA was isolated from the chorionic villi samples. Briefly, the samples were disrupted by incubating at room temperature for 5 hours with 300 μ l of lysis buffer containing 8M urea, 0.3M NaCl, 10mM Tris-HCl (pH 7.4), 10mM EDTA (pH 8.0) and 2% SDS. Then genomic DNA was extracted with an equal volume of phenol-chloroform-isoamyl alcohol mix (25:24:1), and DNA from aqueous phase was precipitated with final ammonium acetate concentration of 2M and two volumes of absolute ethanol. Precipitated DNA was resuspended in 50 μ l of 10mM Tris-HCl (pH 7.4), and 1mM EDTA (pH 8.0) solution. DNA concentrations and quality were assessed by agarose gel electrophoresis. Genomic DNAs from other family members were extracted by standard procedures described previously¹⁶.

PCR amplification for specific alleles (PASA) assay

For the PASA assay, two sets of K9 specific primers were designed to amplify the wildtype and mutant alleles of K9 gene. The sequence of two (-) primers (antisense primer) (KSwt(-) and KSmt(-)) differ only at 3' extreme end, i.e., the wildtype primer KSwt(-) has G at the 3' end (5' -TCC AAG TAA GAG GCC AGC CG-3'), while mutant primer KSmt(-) has A instead of G at the 3' end (5' -TCC AAG TAA GAG GCC ACG CA-3'). The primer la(+) (5' -ACT CCT ATC ACT GGT GCA ACC C-3') was used as (+) primer (sense primer) for both the wildtype and mutant alleles.

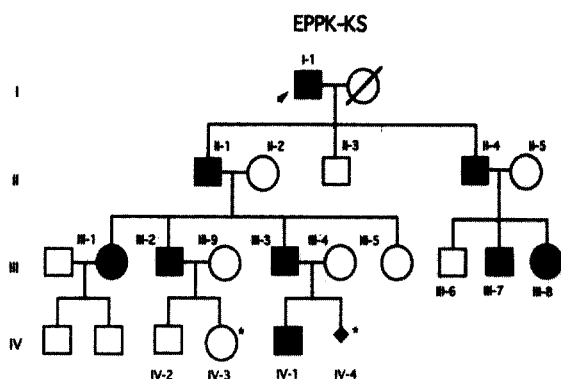


Fig. 1. Pedigree of the EPPK-KS. Arrow indicates the proband, and asterisks indicate fetuses who were examined by prenatal diagnosis.

Fig. 3. DNA sequences. The affected father (III-3) and the fetus (IV-4) have the C to T change which results in an R162W substitution.

Fig. 2. A PASA assay using primers specific for either the normal K9 gene sequence (3' C), or the mutant sequence (3' T). (a) unaffected fetus; DNAs from only the affected father (III-2) yielded a PCR amplified band of 545 bp with both the mutant (M) and wildtype (W) primers. (b) affected fetus; DNAs from the both affected father (III-3) and the fetus (IV-4) yielded an amplified band with both mutant and wildtype primer sets.

Amplification of the K9 gene from both wildtype and mutant genomic DNAs was performed as previously described¹⁶.

Direct PCR amplification of the K9 gene and DNA sequencing procedures

Oligonucleotide primers, the conditions of PCR for amplifying K9 exons and direct DNA sequencing of the PCR products have been described previously¹⁶. In this reaction single-stranded binding protein (bacteriophage T7 gene 32 product; US Biochemicals) was added into sequencing reaction to prevent strand reassociation. The primers used for amplifying exon 1 of K9 were;

1a(+), 5' -ACT CCT ATC ACT GGT GCA ACC C-3' ; BK11B(+), 5' -TTG GCT ACA GCT ACG GCG GAG GAT-3' ; and BK21(-), 5' -TGA GAT CAT CAA TAG TGT TAT AAT-3' . Primers 1a(+)/BK21(-) were used for the first PCR amplification reaction, and the primers BK11B(+)/BK21(-) were used for the second reamplification reaction. The latter primer BK11B(+) was used for DNA sequencing.

RESULTS

PCR amplification of specific alleles (PASA) assay

Previously, we identified a large pedigree of EPPK, and identified by DNA sequencing analyses a mutation in exon 1 of the K9 gene of all affected family members, which results in the substitution R162W (R10W of the 1A rod domain segment) of the K9 protein. Since the normal or mutated DNA sequences do not contain an available restriction enzyme site, we performed the PASA assay to confirm the mutation. The PASA assay did not require direct DNA sequencing for detection of the identified mutation site, and likewise, it was possible to unambiguously exclude the occurrence of the same mutation in the normal population. The wildtype specific primers yielded a product with both the affected and unaffected family members. However, only the mutant specific K9 primers generated a product in all affected family members (Fig. 2). In this way, we could predict that the fetus IV-3 whose parents are III-2 (affected) and III-9 (unaffected) did not carry the disease, but fetus IV-4 parented by III-3 (affected) and III-4 (unaffected) was affected.

Direct DNA sequencing analysis of K9 gene

To confirm the results of PASA assay, we analyzed DNA sequences of both sets of parents and the respective fetuses. As expected, the fetus IV-3 did not have the mutation, but the second fetus IV-4 did carry the mutation. The mutation is a C-to-T base substitution in exon 1 of the K9 gene (Fig. 3). This mutation results in an amino acid change of tryptophan instead of wildtype arginine in codon 162 of position 10 of the 1A rod domain segment of the K9 protein (R162W, CGG to TGG).

Pregnancy outcome

The PASA assay and DNA sequencing data indicated unambiguously that fetus IV-3 should be genotypically normal. Accordingly, the pregnancy was maintained to term, and a healthy female infant was delivered uneventfully at the 38th week of gestation. However, based on the prenatal diagnosis of the likely disease involvement, the mother of fetus IV-4 terminated the fetus at the local clinic, which was decided by herself.

DISCUSSION

EPPK is a life-long disease of cornification inherited in an autosomal-dominant mode. Since Reis et al⁸, first reported that point mutations of K9 gene underlie the cause of EPPK, a total of nine different mutations have

been identified to date⁹⁻¹⁶. All are located in sequences encoding the 1A domain segment of the K9 gene, and most involve a highly conserved arginine residue at position 10 of the 1A rod domain segment. Indeed, about half of all mutations in all type I keratin genes involve the similarly located residue (K14 in EB simplex, K10 in EHK, K9 in EPPK, and K16,17 in pachyonychia congenita)^{4,18,19}, presumably due to a susceptibility for mutations within the CpG dinucleotide²⁰.

Up to about five years ago, prenatal diagnoses of hereditary skin diseases had been performed by ultrastructural analyses of fetal skin biopsies or on amniotic fluid cells. However, this method had a few problems: fetal skin biopsy has a risk for abortion; it requires the procedure to be performed at >18 weeks of gestation; and the analysis of amniotic cells is not reliable enough to exclude the possibility of a false-negative diagnosis. Moreover, for a disease like EPPK which involves only the palms and soles and which presumably manifests early in the third trimester, it is impossible to perform analyses on fetal skin or amniotic fluid cells.

Recently, however, the discoveries of underlying gene mutations by use of modern molecular biological techniques have allowed a fetus with the risk of an inherited genetic diseases to be diagnosed prenatally during the first trimester of pregnancy. Further advantages of DNA-based prenatal diagnoses include: the simplicity of the procedure, e.g. DNA extraction, PCR, and DNA sequencing or other methods such as enzyme digestion or PASA assay if the mutation(s) in affected individuals of the family are already known; the results are available within 24-48 hours; and these methods can afford safe and unambiguous diagnoses. Indeed, numerous successful prenatal diagnoses on chorionic villi biopsies of different types of skin diseases have been performed, such as: EHK at 15 weeks gestation^{21,22}; Herlitz junctional EB²³⁻²⁵ and recessive dystrophic EB^{26,27} at about 10 weeks; lamellar ichthyosis²⁸ at 13 weeks; and pachyonychia congenita type 1²⁹ at 10 weeks. Our present study is the first report of successful prenatal diagnosis of fetuses at risk for EPPK.

Here, we describe the analysis of the K9 mutation in two fetuses at risk for EPPK. Prenatal diagnosis is the only method that we can do for the families who have genetic diseases, because gene therapy is not possible in the near future. We have to think about the ethical

problem before we decide to perform the prenatal diagnosis of the genetic skin diseases. All the prenatal diagnosis of any methods have been performed on the severe skin diseases or life-threatening diseases. Many people think that EPPK is confined to the palms and soles, and is clinically too mild to be accepted as an indication of the prenatal diagnosis. But, how about the patients or the families? They have suffered from social and psychological disadvantages, especially in the developing countries like us. Even though to decide or define the severe skin diseases is very problematic and subjective, it is time for us to think about this sensitive matter deeply.

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