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Cutaneous Biology

EEC (*E*ctrodactyly, *E*ctodermal dysplasia, *C*lefting) syndrome: heterozygous mutation in the *p63* gene (R279H) and DNA-based prenatal diagnosis

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Summary

Background Germline mis-sense mutations in the DNA-binding domain of the *p63* gene have recently been established as the molecular basis for the autosomal dominant EEC (*E*ctrodactyly, *E*ctodermal dysplasia, *C*lefting) syndrome.

Objectives To examine genomic DNA from a 36-year-old woman, her 58-year-old father and her 11-year-old son, all with the EEC syndrome, to determine the inherent *p63* mutation and, after genetic counselling, to use knowledge of the mutation to undertake a first-trimester DNA-based prenatal diagnosis in a subsequent pregnancy.

Methods Fetal DNA was extracted from chorionic villi and used to amplify exon 7 of *p63* containing the potential mutation. Direct sequencing and restriction endonuclease digestion (loss of *Aci*I site on mutant allele) were used for DNA-based prenatal diagnosis.

Results We identified a heterozygous arginine to histidine *p63* mutation, R279H, in all three affected individuals. Prenatal diagnosis demonstrated a homozygous wild-type sequence predicting an unaffected child: a healthy boy was subsequently born at full-term.

Conclusions These data expand the *p63* gene mutation database and provide the first example of a DNA-based prenatal test in this ectodermal dysplasia syndrome.

Key words: EEC syndrome, genodermatosis, *p63* gene mutation, prenatal diagnosis

EEC (*E*ctrodactyly, *E*ctodermal dysplasia, *C*lefting) syndrome (OMIM 604292) is an autosomal dominant ectodermal dysplasia syndrome that produces ectrodactyly, limb defects with facial clefting and also affects the skin, hair, nails and teeth. Additional features may include lacrimal duct abnormalities, urinary tract problems, conductive hearing loss, facial dysmorphism, chronic respiratory infections and developmental delay. Most cases of the disorder have been mapped to 3q27 and pathogenic mutations have recently been identified in the *p63* gene.[1,2] Most of these mutations comprise heterozygous mis-sense mutations in the DNA-binding domain of *p63*. In this study, we examined genomic DNA from a three-generation family with the EEC syndrome to determine the inherent *p63* mutation. Knowledge of the mutation was also used to undertake DNA-based prenatal diagnosis in an at-risk pregnancy.

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Materials and methods

Patient details

The proband was a 36-year-old woman with the EEC syndrome. Specifically, she had features of ectrodactyly affecting all four limbs as well as abnormalities of her hair and nails. Hypodontia with delayed eruption of several teeth and enamel dysplasia were noted, as were developmental anomalies affecting the lacrimal ducts and urogenital tract. Facial clefting was not a feature. Using a scoring system for the EEC syndrome established by Roelfsema and Cobben,[3] she had a combined score of 12. Her 58-year-old father also had similar features of ectodermal dysplasia, but limb abnormalities were restricted to the feet (Fig. 1). Although he also had developmental anomalies in his lacrimal ducts, his urogenital tract was unaffected. He had no facial clefting and his EEC syndrome score was 9. The proband's 11-year-old son was born with bilateral upper and lower limb ectrodactyly and bilateral cleft lip and palate. He had partial anodontia and accelerated dental caries. He also had developmental anomalies of the lacrimal drainage system and urogenital tract. His EEC syndrome score was 16. Thus, there was evidence for intrafamilial variability in the severity of the physical signs in the affected individuals, as reflected in the overall EEC syndrome scores. No other family members were known to be affected.

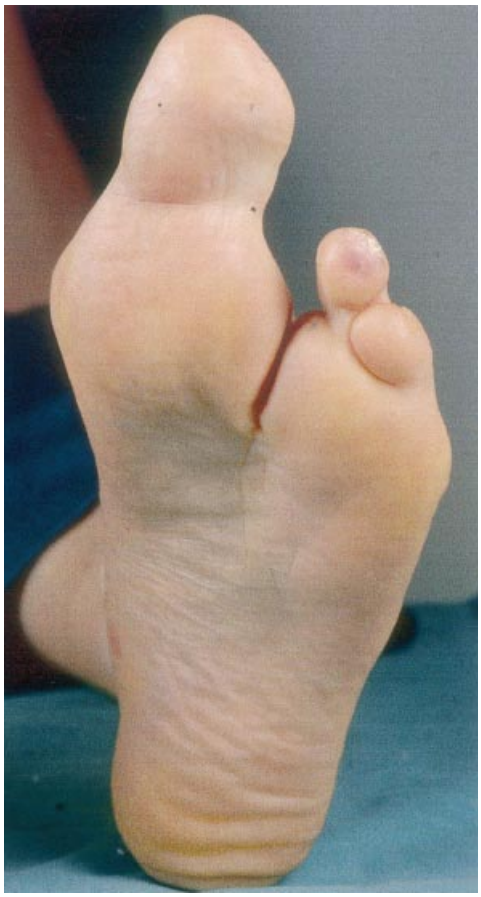

Figure 1. Clinical features of the 58-year-old individual with EEC syndrome showing ectrodactyly and a split-foot abnormality. His overall EEC syndrome score was 9.[3]

Mutation analysis

After informed consent, genomic DNA was isolated from peripheral blood leucocytes and used as a template for amplification of *p63* DNA-binding domain (exons 4–8) genomic sequences (GenBank AF124531–4), as described elsewhere.[1] Specifically, to amplify exon 7, the following flanking intronic primers were used: sense primer 5′-GGGAAGAACTGAGAAGGAACAAC-3′; anti-sense primer 5′-CAGCCACGATTTCACTTTGCC-3′. For polymerase chain reaction (PCR) amplification, 250 ng of genomic DNA was used as template in an amplification buffer containing 6·25 pmol of the primers, 37·5 nmol $MgCl_2$, 5 mmol of each nucleotide and 2·5 U *Taq* polymerase (Perkin-Elmer, Warrington, U.K.) in a 50-μL total volume reaction in an Omni-Gene thermal cycler (Hybaid, Basingstoke, U.K.). The amplification conditions were 94 °C for 5 min; then 94 °C for 45 s, 55 °C for 45 s, 72 °C for 45 s, for 40 cycles. The size of the expected PCR product was 253 bp. PCR products were purified using Qiaquick spin columns and sequenced directly using Big Dye labelling on an ABI 310 genetic analyser (Perkin Elmer). Sequence variants (see Results) were verified using restriction endonuclease digestion according to the manufacturer's instructions (New England BioLabs, Hitchin, U.K.).

DNA-based prenatal diagnosis

Prenatal diagnosis had not been offered for the proband's first pregnancy. Genetic counselling in a subsequent at-risk pregnancy had to consider the known intrafamilial variability that can occur in the EEC syndrome. This family was particularly concerned not to have a more severely affected child. The parents were also worried that ultrasound screening would not be able to detect specific changes associated with the EEC syndrome until at least the 14th week of gestation. Therefore, after extensive counselling, knowledge of the mutation was used to undertake DNA-based prenatal

diagnosis. The family understood that the presence of the pathogenic mutation would not give an accurate indication of phenotypic severity. DNA was extracted from a chorionic villus biopsy performed at 11 weeks' gestation using standard proteinase K digestion and salt/ethanol precipitation. DNA was amplified using the *p63* exon 7 primers as described above. To exclude maternal contamination, fetal DNA was also amplified for two other previously determined independent markers of paternal DNA (D3S2 and D3F22 microsatellites). The *p63* exon 7 PCR products were sequenced directly and also digested with the restriction endonuclease *Aci*I (see Results) followed by electrophoresis on a 2·5% agarose gel.

Results

p63 gene analysis

Direct sequencing of the *p63* PCR products from the three affected family members identified a heterozygous G → A substitution at nucleotide 835 in exon 7 of *p63* (based on nucleotide numbering of the p63α isotype with the A of the methionine start codon as 1). This transition converts an arginine residue (CGC) to histidine (CAC) and is designated R279H (Fig. 2). The mutation results in the loss of a solitary cut site for the endonuclease *Aci*I (C/CGC). Control PCR products were digested into fragments of 140 and 113 bp, while presence of the mutation led to an undigested product of 253 bp (Fig. 2). This heterozygous amino acid substitution was identified in amplified DNA from the three affected individuals with the EEC syndrome, but was not seen in 50 control individuals. This particular mutation, R279H, has been described previously in four other unrelated families with the EEC syndrome.[4] No other potentially pathogenic sequence variants were identified in the other exons or flanking intronic sequences of *p63*. Thus, the molecular basis of the EEC syndrome in this family is a heterozygous mutation R279H in exon 7 of *p63*.

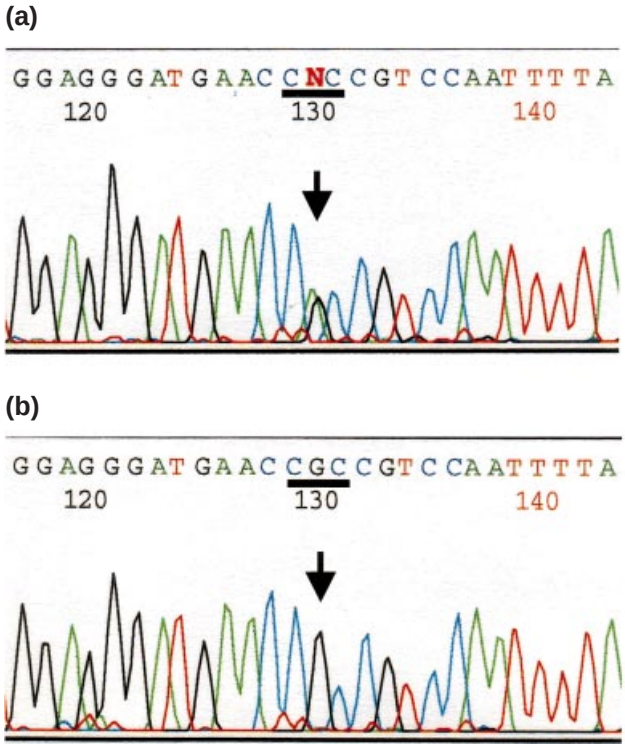

Figure 2. Automated DNA sequencing of *p63* exon 7 and flanking introns. In DNA from the affected individuals with EEC syndrome (a) there is a heterozygous G → A transition that converts an arginine residue (CGC) to histidine (CAC). The mutation is designated R279H. In control DNA (b) there is only a 'G' peak at this nucleotide position.

Prenatal diagnosis

p63 exon 7 PCR products from amplified fetal DNA along with the corresponding PCR products from the mother and previously affected child and control DNA were digested with *Aci*I. Agarose gel electrophoresis (Fig. 3) showed that the control PCR products were

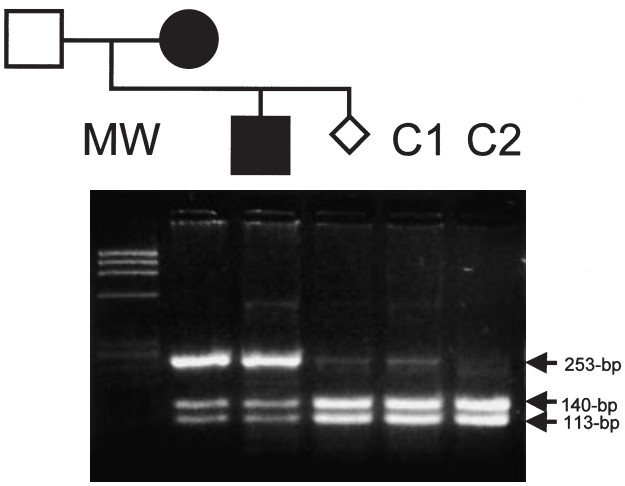

Figure 3. Restriction endonuclease digestion with *Aci*I and prenatal diagnosis. The mutation R279H results in loss of a cut site for *Aci*I. In control DNA (lanes C1 and C2), the polymerase chain reaction (PCR) product is completely digested into fragments of 140 and 113 bp. By contrast, there is an additional undigested band of 253 bp in DNA from the affected mother and child (lanes 1 and 2). The fetal PCR products are digested similarly to the control DNA, indicating that the fetus has inherited two wild-type *p63* alleles with respect to this mutation and is therefore predicted to be clinically unaffected with EEC syndrome.

completely digested into fragments of 140 and 113 bp. By contrast, in the digested PCR products from the mother and previously affected child there was an additional undigested band of 253 bp, consistent with heterozygosity for the mutation R279H. The fetal PCR products were completely digested into the 140- and 113-bp fragments as in the control DNA. This suggests that the fetus had inherited two genotypically normal *p63* alleles with respect to this particular mutation, and was therefore clinically unaffected with the EEC syndrome. Direct sequencing of fetal DNA revealed homozygous wild-type sequence only with no evidence of the pathogenic G → A transition at nucleotide 835. Karyotype analysis revealed a 46XY male fetus. An unaffected male child was subsequently born at full-term.

Discussion

The *p63* gene is related to the tumour suppressor gene *p53*, although whether *p63* also acts as a tumour suppressor, or as a regulator of *p53*, or just constitutes an evolutionary family member with similar or different functions, has not yet been fully established.[5,6] However, clues to the function of *p63* have recently been derived from knockout mouse studies that have demonstrated its key role in regenerative proliferation in limb, craniofacial and epithelial development.[6,7] Specifically, epithelia fail to develop because of an inability to maintain regenerative stem cells.[7] Therefore, *p63* appears to contribute to the control of the balance of proliferation and differentiation in epithelial cells. Mice that are −/− for *p63* show some phenotypic similarities to human ectodermal dysplasia syndromes and in 1999 genetic linkage was established between the *p63* locus on 3q27 and a number of related conditions, including the EEC syndrome.[1] Furthermore, germline heterozygous mis-sense mutations were reported in eight patients with the EEC syndrome.[1] Most of these mutations occurred *de novo* and were located within a specific domain of *p63*, the DNA-binding domain. The mutation R279H detected in our affected family has been detected in other individuals with the EEC syndrome, as have the mutations R304W and R304Q.[1,2,4,8] Interestingly, these two particular arginine amino acids correspond to the two residues in *p53* (R248 and R273) that are most frequently mutated in human tumours.[9] However, no definite association between the EEC syndrome and epithelial malignancy has yet been determined.[10] Further mutations in *p63* have also been described in other ectodermal dysplasia syndromes. Indeed, some cases of split hand–foot syndrome (OMIM 183600) have also been shown to result from mutations in the *p63* DNA-binding domain.[4,8] These findings, along with the intrafamilial variability that is known to occur in the EEC syndrome (as in this family), highlight the clinical diversity arising from similar or identical changes in *p63* DNA and emphasize the importance of other interacting genetic or environmental factors in determining the precise clinical manifestations. However, some genotype–phenotype correlation for *p63* mutations has been established. Notably, in AEC (*A*nkyloblepharon, *E*ctodermal dysplasia, *C*lefting) or Hay–Wells syndrome (OMIM 106260), heterozygous mis-sense mutations are clustered in a different part of *p63*, the sterile-α-motif or SAM domain.[11] There is no equivalent region of the gene in *p53* and the precise function of the SAM domain is currently unknown. Nevertheless, specific molecular pathological findings now exist for the EEC and AEC syndromes.

In the absence of any specific treatment for disabling or disfiguring genodermatoses, the development of DNA-based prenatal diagnosis represents one of the major translational benefits of molecular research into single-gene disorders. Whether families use prenatal diagnosis remains a matter of personal choice after thorough discussion and genetic counselling. Molecular prenatal analysis provides an earlier result for prospective parents. This enables them to make a more informed choice. In this case, knowledge that the fetus was unaffected gave them early reassurance and the confidence to continue the pregnancy. Such testing procedures have become established in pregnancies at risk for recurrence of severe forms of epidermolysis bullosa and other inherited skin disorders.[12] This report represents the first example of DNA-based testing for the EEC syndrome.

Acknowledgments

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