Scarcity of mutations detected in families with X linked hypohidrotic ectodermal dysplasia: diagnostic implications

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Abstract

Indirect molecular diagnosis of X linked hypohidrotic ectodermal dysplasia (XLHED), a congenital disorder of hair, teeth, and eccrine sweat glands, has been possible by linkage analysis. Direct mutation detection would enable carrier detection in female relatives of sporadic cases, as well as help distinguish XLHED from the rarer, clinically indistinguishable, autosomal recessive disorder ARHED. Recently, a candidate gene for XLHED has been identified. Genomic DNA from 162 affected males and 21 females, who were either obligate carriers or had manifestations of the disorder, were screened by SSCP analysis. A subset of the patients had been previously screened for large genomic deletions and had limited screening of a single exon by SSCP analysis. The two known exons were amplified using flanking primers. Approximately 7% of patients, all males, had putative mutations identified within exon 1, but no variants were found within exon 2. Ten different putative mutations and four probable polymorphisms were identified. Both of the known exons were sequenced in 10 patients who had no detectable SSCP changes, but no additional mutations were found. No correlation between phenotype and genotype was evident between either affected subjects or subjects with or without detectable mutations. The results of the study indicate that only a small minority of affected males can be diagnosed by direct mutation analysis, and that the remainder of the patients are likely to have mutations in as yet unidentified exons of the EDA gene. Linkage analysis, in informative situations, therefore remains the only practical diagnostic option available.

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Hypohidrotic (anhidrotic) ectodermal dysplasia (EDA), an X linked disorder, is the commonest of the ectodermal dysplasias, and displays abnormal development of teeth, hair, and eccrine sweat glands, resulting in either absent or malformed structures.¹ The disorder, if unrecognised, causes significant morbidity and mortality during infancy and early childhood because of the neurological sequelae of

hyperthermia. Female carriers have variable clinical involvement, ranging from no clinical manifestations to significant degrees of hypodontia and hypotrichosis.² The variability is likely to be the result of X inactivation (lyonisation), which significantly complicates carrier detection based on physical findings alone.³

Indirect molecular genetic diagnosis, using flanking polymorphic markers, has assisted in carrier identification in informative families.³ However, since there is a significant incidence of new mutations in the EDA gene, a large number of cases are sporadic, with either a single affected male or a single female with hypodontia.⁴ In families with a single affected male and no clear signs of the disorder in the mother, one cannot differentiate if the affected male is the result of a new mutation or whether the mother is a non-manifesting carrier. When females are seen with hypodontia and have no affected males in the family, it is impossible to differentiate if they are carriers of EDA or if they have the much more common form of hypodontia (5-10% of population), inherited as an autosomal dominant trait.⁴⁻⁷ In addition, it is now apparent that there is an autosomal recessive form of hypohidrotic ectodermal dysplasia (ARHED) that is clinically indistinguishable from the X linked disorder (XLHED), except that males and females are equally affected.⁸ Although ARHED is less common than XLHED, families with affected subjects in only a single sibship may be affected with ARHED. In addition, severely affected females may have a balanced X;autosome translocation or have XLHED with markedly skewed X inactivation. The ability directly to diagnose mutations in the EDA gene would greatly improve our diagnostic capabilities.

Recently, a candidate gene for the disorder was identified by a positional cloning strategy. The EDA gene structure consists of two exons, separated by a 200 kb intron, which code for a putative protein of 135 amino acids. The function of the protein is unknown, with the amino acid sequence showing no obvious homology to other proteins, but containing one possible transmembrane domain. SSCP analysis of only a single exon was performed on genomic DNA from 118 unrelated affected males, and plausible mutations were identified in eight (6.8%) patients. Since more than 90% of affected males did not have a detectable mutation within exon 1, we sought to identify mutations by SSCP analysis in exon 2 of the EDA gene, not previously studied in the original patients.

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In addition, both exons were analysed in 65 new unrelated subjects, including 21 females who were either obligate carriers or had manifestations of the disorder. The EDA gene was completely sequenced on a sample of people who showed no SSCP changes, to rule out the presence of a common mutation not detectable by the SSCP methodology used.

Methods

PATIENTS AND FAMILIES

A total of 183 affected subjects from unrelated families was analysed; 118 of them, all males, had been previously analysed by SSCP analysis for variants in exon 1 alone or by Southern analysis for large genomic deletions.⁹⁻¹⁰ These patients were reanalysed for mutations within exon 2. Sixty-five additional unrelated subjects, 44 males and 21 females, were analysed for mutations in both exons. Affected members of these families were clinically characterised from medical records and pedigrees provided primarily by clinicians from the US and the UK. All subjects who participated provided consent through an IRB approved protocol. There was a total of 162 males and 21 females in the study. Males had typical features of X linked hypohidrotic ectodermal dysplasia, including hypodontia, hypotrichosis, and hypohidrosis.¹ Females were either obligate carriers or had significant manifestations of the disorder. In families with both affected males and obligate or manifesting females, males were selected for study when possible. Forty-one of the families had been previously studied as part of earlier linkage studies and data were consistent with linkage to the Xq12-q13.1 EDA locus.^{1-11, 12}

MOLECULAR TECHNIQUES

SSCP analysis

The entire exon 1 with flanking segments was amplified by PCR using previously published primers.⁹ The 20 µl PCR mix contained 1 unit of AmpliTaq polymerase (Perkin-Elmer), 1 × AmpliTaq buffer, 0.3 mmol/l dNTPs (Pharmacia Biotech), 1.5 mmol/l MgCl₂, 10% DMSO, 50 ng of genomic template DNA, and 6 pmol of each primer. Reactions were heated to 94°C for 45 seconds, 65°C for 45 seconds, and 72°C for 45 seconds, for 35 cycles. The 686 bp PCR products were cleaved with *Pst*I and *Taq*I (New England Biolabs) to generate fragments of 135, 147, 159, and 245 base pairs.

Primers for exon 2 were designed from the flanking genomic sequence. PCR was performed in a 20 µl volume with a final concentration of 1 × PCR buffer (Perkin Elmer), 3 mmol/l MgCl₂, 0.2 mmol/l dNTP, 0.5 mmol/l forward primer (5'-TGGCTTCTCTAGTTAGGTTGG-3'), 0.5 mmol/l reverse primer (5'-CATCTCAAATTTTCCTTCTGGG-3'), 1 unit *Taq* DNA polymerase (Perkin Elmer), and 50 ng genomic DNA. Reactions were heated to 95°C for two minutes 30 seconds, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension step at 72°C for five minutes. The PCR product was 372 bp. A total of 5 µl of the

PCR product was digested in a final volume of 10 µl with 5 U of *Tru*9I, generating fragments of 84, 151, and 157 base pairs.

Digests of the PCR products were denatured at 95°C for 10 minutes, chilled on ice, and electrophoresed on a 0.5 × Hydrolink MDE gel (J T Baker Inc) at 1 watt/cm for three hours at room temperature. For samples analysed in the US, the DNA was stained by immersing the gel in 0.1% silver nitrate for 10 minutes, washing briefly in water, then soaking the gel in a solution of 1.5% sodium hydroxide, 0.01% sodium borohydride, and 0.15% formaldehyde. For the samples analysed in the UK, DNA from the gel was visualised by transfer onto a nylon membrane with subsequent hybridisation to radiolabelled exon 1 or 2 PCR products. The 686 bp PCR product, encompassing all of exons 1 and 35 and 51 bp of flanking sequence, was amplified from patients with abnormally migrating SSCP bands. This same product and one including 372 bp of exon 2 (32 and 38 bp of flanking sequence) were amplified from 10 subjects without any detectable changes. The complete DNA sequence was obtained from both exons, using a combination of flanking primers (see above) and internal primers (5'-GGTGACTGGTGATGGGGCTGTC-3' and 5'-AGTTGCGCTCGGAGTTG-3'). Sequencing was performed using ABI end terminator chemistry on a 373A automated sequencer. To determine if any variants detected represent polymorphisms, allele specific oligonucleotide (ASO) hybridisation was performed.¹³ Seventeen base oligomers designed to detect each of the variant sequences identified within the US patient cohort, as well as the corresponding wild type sequences, were hybridised against a panel of 100 X chromosomes from unaffected subjects. Variants identified among the UK patients were examined by SSCP analysis of a panel of 70 normal X chromosomes.

Results

Seven subjects were identified with variants within exon 1 among the 65 new families studied. No SSCP variants were identified within exon 2, in either the 118 affected subjects from our previous study or among the new families. Sequencing of exon 1 and its flanking regions from genomic DNA of the subjects with SSCP variants identified sequence changes in all of the subjects (table 1). Two of the variants were the result of single base pair changes in the 5' untranslated region (UTR) (228G→A, 187G→T). The 228G→A variant generates an in frame ATG start codon five amino acids upstream of the anticipated start of the EDA protein. However, this alternate start site lacks the Kozak sequence seen flanking the predicted start methionine. The 228G→A variant was also detected in one unaffected control in this study, as well as in the original genomic clone containing exon 1 of the EDA gene. These data suggest that 228G→A is a non-disease causing polymorphism. The 187G→T variant is not anticipated to alter the protein sequence, but might have an effect on gene expression. However, ASO hybridisation to genomic DNA from

Table 1 EDA sequence variants detected in 92 families with hypohidrotic ectodermal dysplasia

Family number	Origin	Nucleotide change	Predicted effect	Reference
Likely polymorphisms				
ED1075	USA	187G→T	5' UTR	This study
ED7	UK	187G→T	5' UTR	9
ED1013	USA	227G→A	5' UTR	This study
Putative mutations				
ED1166	USA	287insC	FS at 16	9
ED12	UK	363insC	FS at 41	9
ED1180	USA	366C→T	Q23ter	This study
ED19	UK	423T→C	Y61H	9
		180G→A	5' UTR	
ED-D14	UK	427delT	FS at 62	This study
ED1129	USA	429G→A	E63K	This study
ED73	UK	448G→T	R69L	9
ED1113	USA	448G→T	R69L	9
ED1108	USA	494delT	FS at 85	This study
ED1024	USA	494delT	FS at 85	9
ED52	UK	494delT	FS at 85	9
ED46	UK	594-595delCC,	FS at 118	This study
		593A→G		
ED1013	USA	636C→T	Q132ter	9

50 control females detected one with the 187G→T variant. This suggests that it also is not a causative mutation, but rather a polymorphic variant occurring with a low frequency in the population. The 187G→T variant was detected in a previously published EDA family, in whom no other changes were detected.⁹

Five variants were felt to be plausible disease causing mutations (table 1). A nonsense mutation (366C→T) in ED1180 is predicted to truncate the protein at residue 23. A missense mutation in the amino-terminal hydrophilic region of the EDA protein is predicted by one variant (429G→A) and changes a negatively charged amino acid (glutamic acid) to a positively charged one (lysine). Three variants (427delT, 494delT, 594-5del) were detected and predict frameshift mutations at residues 62, 85, and 118, respectively. The 494delT variant had been identified previously in two families.⁹ Haplotype analysis of the three families with 494delT, using polymorphic markers that closely flank the EDA locus,¹² indicate that this deletion arose independently in each family (data not shown). The 594-595delCC has an associated base change, 593A→G, which alone would be a silent mutation. All five putative mutations identified in the study were not detected among the panels of unaffected subjects.

Genomic DNA from 10 affected males with normal SSCP results was sequenced for both exons 1 and 2, including the flanking genomic regions, but no sequence variants were identified. No phenotypic differences were evident between subjects with or without identifiable mutations. In addition, there were no phenotypic differences between the subject with a putative missense mutation, the four subjects with either frameshift or nonsense mutations, or the four previously published males with

large genomic deletions of either exon 1 or 2. Thus, it is likely that all of the mutations involve a loss of protein function.

Discussion

Combining the results of this study with the previous one showed that 8% (13/162) of the males had putative disease causing point mutations detected within exon 1, while none was found among the 21 females studied (table 2). The low rate of detection of mutations and the small sample size of females does not allow conclusions as to the significance of this difference. Since females have a lesser degree of clinical manifestations, there may be a greater chance that some may have been misclassified as carriers of XLHED. If one excludes the variants in the 5' UTR as likely polymorphisms, the detection rate for mutations within exon 1 in this study was 5/65 (7.7%), compared to the 8/118 (6.8%) detected in our previous study.⁹ No mutations, or even polymorphic variants, were detected in exon 2 among the 183 patients studied. This exon contains only three amino acids of the putative protein and a 193 bp 3' untranslated region. Haplotype analysis of the two recurrent mutations (494delT and 448G→T) indicates that the mutations in each person are likely to have arisen independently.

There are several possible explanations for the low rate of mutation detection within the EDA gene. The first is the existence of extensive non-allelic genetic heterogeneity, including the autosomal recessive form of the disorder. However, the majority of families studied had multiple affected generations consistent with X linked inheritance. In addition, only two mutations (4.8%) were detected among a subset of 41 families which had been previously shown to be linked to the EDA locus, with the analysis showing no evidence for significant non-allelic genetic heterogeneity (<5%).¹¹ Thus, it is unlikely that non-allelic genetic heterogeneity accounts for a major portion of the missing mutations.

Another explanation for the low rate of mutation detection is the possible insensitivity of the SSCP assay. This technique is reported to detect some 80-100% of all mutations depending on the conditions used and it is likely to miss some mutations.^{11 14 15} To address the sensitivity limitations, the complete gene sequences of 10 affected males with normal SSCP results were determined. No mutations were identified in these subjects, suggesting that a majority of the mutations had not been missed because of technical limitations, and that there were no common mutations that escaped detection by SSCP analysis. Another possibility for the lack of detectable mutations would be a common genomic rearrangement, such as an inversion of one of the exons, as seen in factor VIII deficiency.¹⁶ Such a change would not be detected by either PCR analysis or routine Southern analysis, but would require pulse field gel analysis. Another theoretical possibility is that there are unidentified mutations in a regulatory region of the gene.

Table 2 Mutations detected within the EDA gene

	Deletions of exon 1 or 2	Point mutations exon 1	Point mutations exon 2	Unknown
Affected males	4 (2.3%)	13 (7.8%)	0	150 (89.9%)
Obligate carrier or manifesting females	0	0	0	21 (100%)
Total	4 (2.1%)	13 (6.9%)	0	171 (91%)

However, the most likely possibility is that the majority of the EDA mutations reside in exons not represented in the original cDNA clone. Previous northern analysis using exon 1 as a probe on various fetal and adult tissues indicated RNA species of 6.0 kb, 4.5 kb, 2.0 kb, and <1 kb.⁹ If the 6 kb form of the transcript is critical for normal development, then the 0.8 kb cDNA analysed would represent only 13% of the gene. Identification of alternatively spliced products, and their DNA sequences, should increase the yield of mutations detected by direct analysis. For the present, direct mutation analysis is not a practical diagnostic tool and indirect linkage based analysis must still be relied upon. Depending on the ultimate improvement in the rate of mutation detection, direct mutation analysis may replace linkage analysis, or the two techniques may be used in combination depending on the individual situation.

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