Detection of de novo mutations and analysis of their origin in families with X linked hypohidrotic ectodermal dysplasia

J Zonana, M Jones, A Clarke, J Gault, B Muller, N S T Thomas

Abstract

Hypohidrotic ectodermal dysplasia (EDA) has been localised to the q12–q13.1 region of the X chromosome by both physical and genetic mapping methods. Although linkage analysis using closely linked flanking markers can clarify the carrier status for many females at risk for the disorder, knowledge of the origin of the mutation in instances of possible de novo mutation is critical for accurate genetic counselling of families. Two methods have been used to confirm de novo mutation in families with EDA and to trace their origin. Direct detection of three de novo molecular deletions, one arising during oogenesis and the other two during spermatogenesis, was achieved by Southern analyses using cosmids isolated from the EDA region as probes. Seven de novo mutations arising during spermatogenesis, and two possible de novo mutations during oogenesis, were identified by an analysis of the cosegregation of the disorder with polymorphic markers closely linked to and flanking the EDA locus. The confirmation and analysis of the origin of the 10 de novo mutations greatly assisted genetic counselling in these families. The apparent 3·5:1 excess of male to female origin of mutation in families studied with unidentified types of mutation is similar to other studies of X linked disorders, and suggests that the majority of these mutations may involve single base pair substitutions.

(*J Med Genet* 1994;31:287–292)

Department of Molecular and Medical Genetics, L-103, Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, Oregon 97201, USA
J Zonana
M Jones
J Gault

Institute of Medical Genetics, University of Wales College of Medicine, Cardiff, UK
A Clarke
N S T Thomas

Department of Paediatric Genetics, University of Munich, Germany
B Muller

Correspondence to
Dr Zonana.

Received 8 September 1993
Revised version accepted for publication 22 November 1993

Hypohidrotic ectodermal dysplasia (EDA), a disorder involving abnormal morphogenesis of teeth, hair, and eccrine sweat glands, has been localised to the q12–q13.1 region of the X chromosome by genetic and physical mapping methods.[1–5] Linkage analysis using closely linked flanking markers, in combination with physical examination, can modify the carrier risk for many females at risk for EDA.[2 6] Although fully penetrant in males, clear physical manifestations of the disorder are present in only about two thirds of carriers, and reliance on more subtle subclinical signs, such as sweat pore counts, can be misleading.[7 8] The identification of de novo mutations and knowledge of their origin is critical for proper genetic counselling in many families. For example, clinically unaffected mothers of sporadic cases may have inherited the gene from their mother, be a non-manifesting carrier owing to a new germline mutation in one of the maternal grandparents, or be a non-carrier with mutation arising during oogenesis. We sought to confirm apparent de novo mutations and detect their origin in families with EDA by both direct and indirect methods of molecular analysis. The first method used screened for detectable rearrangements of genomic DNA by Southern analysis with cosmids, previously localised to the EDA region, as probes.[3] The second method analysed the segregation of the disorder in families with polymorphic marker loci closely flanking the EDA locus.

Methods and materials

DETECTION OF DNA REARRANGEMENTS

A panel of 80 unrelated families with EDA was screened for molecular rearrangements by Southern analysis. The proband in each family was a male with classical signs of EDA, and the panel is identical to the one previously screened for deletions at polymorphic loci closely linked to the EDA locus.[4] DNA samples were extracted, digested with *Eco*RI, electrophoresed, and transferred to nylon membranes by previously described techniques.[9]

Four separate cosmid contigs from an X chromosome cosmid library,[10] containing 26 individual cosmids, had been previously identified to be either within or contiguous to the EDA locus (fig 1).[3] A single anchor cosmid from cosmid contigs A, B, and C had been previously hybridised to the patient panel, but no cosmids from the most distal contig group D had been used.[3] Cosmid ICRFc104C11.138 from group D, and ICRFc104A09.80 from group B, were used as radiolabelled probes and hybridised to Southern blots of DNA from the patient panel. Lλ5c4, the distal end clone of a YAC (4757) that was used to identify the cosmid contigs in the EDA region, was also used as a probe.[3] The use of cosmids as probes for hybridisation to human genomic DNA was accomplished by previously described methods.[11]

ANALYSIS OF FLANKING POLYMORPHIC MARKERS

Families were selected for study of the cosegregation of EDA and flanking polymorphic markers, if they contained a mother who was either an obligate carrier, or a clearly manifesting carrier based on the congenital absence of

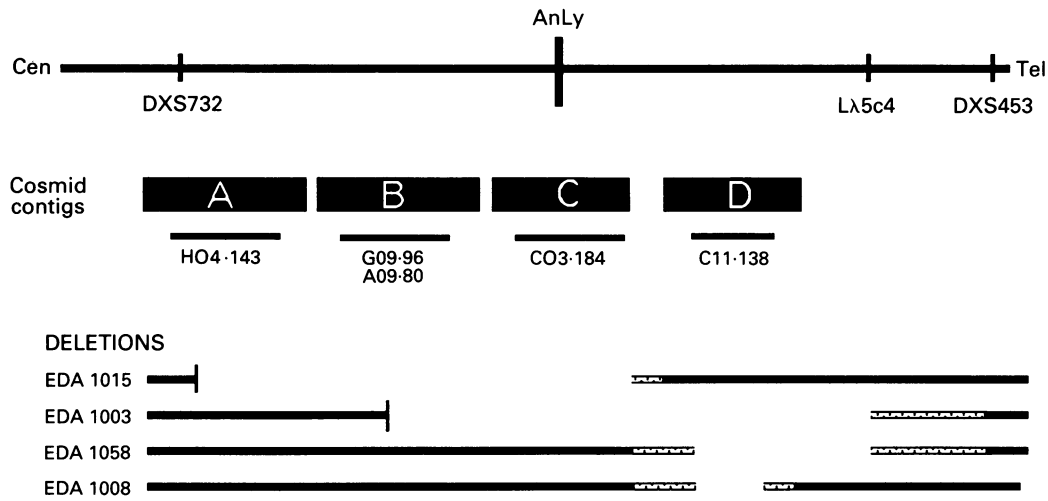

Figure 1 Location of genomic clones isolated from the EDA region and microdeletions in four unrelated male patients with EDA. AnLy represents the X chromosome breakpoint in a female with EDA and a balanced X autosome translocation.[3] Lλ54 is the distal end clone of a YAC used to identify the cosmid contigs from the EDA regions.[3]

two or more of her permanent teeth,[12] with at least one affected child. In addition, her parents had to be clinically unaffected with only the single affected offspring, have no other family history of the disorder, and be available for study. Thus, if the disorder segregated with the haplotype of flanking markers on the X chromosome of the maternal grandfather, the mutation would be proven to be de novo since the disorder is completely penetrant in males.[13] The mutation would most likely have occurred during spermatogenesis, although postzygotic mutation in the mother cannot be excluded. If the disorder segregated with the haplotype of the maternal grandmother's X chromosome, the mutation would either have occurred de novo during oogenesis, or the grandmother would be a non-manifesting carrier. Since the latter two possibilities cannot be readily distinguished, the ratio of male to female origin of de novo mutations in this study represents a minimal estimate.

Blood samples were obtained from the probands and appropriate family members for DNA preparation and analysis. The segregation of alleles at seven polymorphic loci from the Xq11–q21.1 region were studied to identify informative markers flanking the EDA locus (table).

Results

IDENTIFICATION OF FAMILIES WITH DE NOVO MOLECULAR DELETIONS

Family EDA 1003

Subject III·1 (fig 2A) had in a previous study been shown to be deleted for cosmids ICRFc104G09.96 and ICRFc104C03.184 from cosmid contigs B and C respectively, but the end points of the deletion were not identified and other family members were not studied.[3] Additional probes were used and the proband was shown to be deleted (no hybridisation signal) when Lλ5c4 and cosmid ICRFc104C11.138 were used as probes (fig 1). Hybridisation with cosmid ICRFc104A09.80 from contig group B identified the proximal end point of the deletion. There was absence of all seven normal sized *Eco*RI fragments but a unique 5·6 kb junctional fragment was present (fig 3). DNA digested separately with *Hin*dIII and *Bgl*II, blotted and hybridised to cosmid ICRFc104A09.80, showed absence of all normal sized fragments and the presence of unique junctional fragments of 16 and 5·9 kb respectively. These unique sized fragments were not observed upon hybridisation of cosmid ICRFc104A09.80 to genomic DNA from over 100 unrelated X chromosomes. Subject III·3, his affected brother, also has a molecular deletion, and their obligate carrier mother (II·2) displayed the expected junctional fragment. However, neither of the maternal grandparents (I·1 and I·2) nor the proband's sister (III·2) were deleted.

Informative markers flanking the EDA locus

Locus	*Location*	*θmax (Zmax)*	*Reference*
AR	Xq11.2–q12	—	14
PGK1P1	Xq11.2–q12	0·046 (13·04)	2
DXS339	Xq11.2–q13	0·000 (28·19)	2
DXS732	Xq12–q13.1	0·000 (21·20)	2
DXS453	Xq12–q13.1	0·009 (24·33)	2
DXS441	Xq13.2–q13.3	—	15
DXS72	Xq21.1	0.033 (9·77)	2

The proband (III·1), a 10 year old male, and his 4 year old brother, have classical findings of EDA, including marked hypodontia, hypotrichosis, and significant hypohidrosis. Both boys had normal growth and development and no signs of any other significant disability or disorder. Their mother (II·2), an obligate carrier of EDA, has obvious physical manifestations of the disorder, with congenital absence of 13 permanent teeth, a patchy distribution of sweating over her body, and noticeable hypotrichosis of her scalp hair. No other family member has signs or symptoms of EDA.

Analysis of the segregation of alleles at the polymorphic loci flanking the EDA locus showed the mother (II·2) to be informative for the flanking loci DXS339 and DXS441. The haplotype of flanking markers segregating with the disorder is that of the maternal grandfather (fig 2A). Therefore, the mutation probably

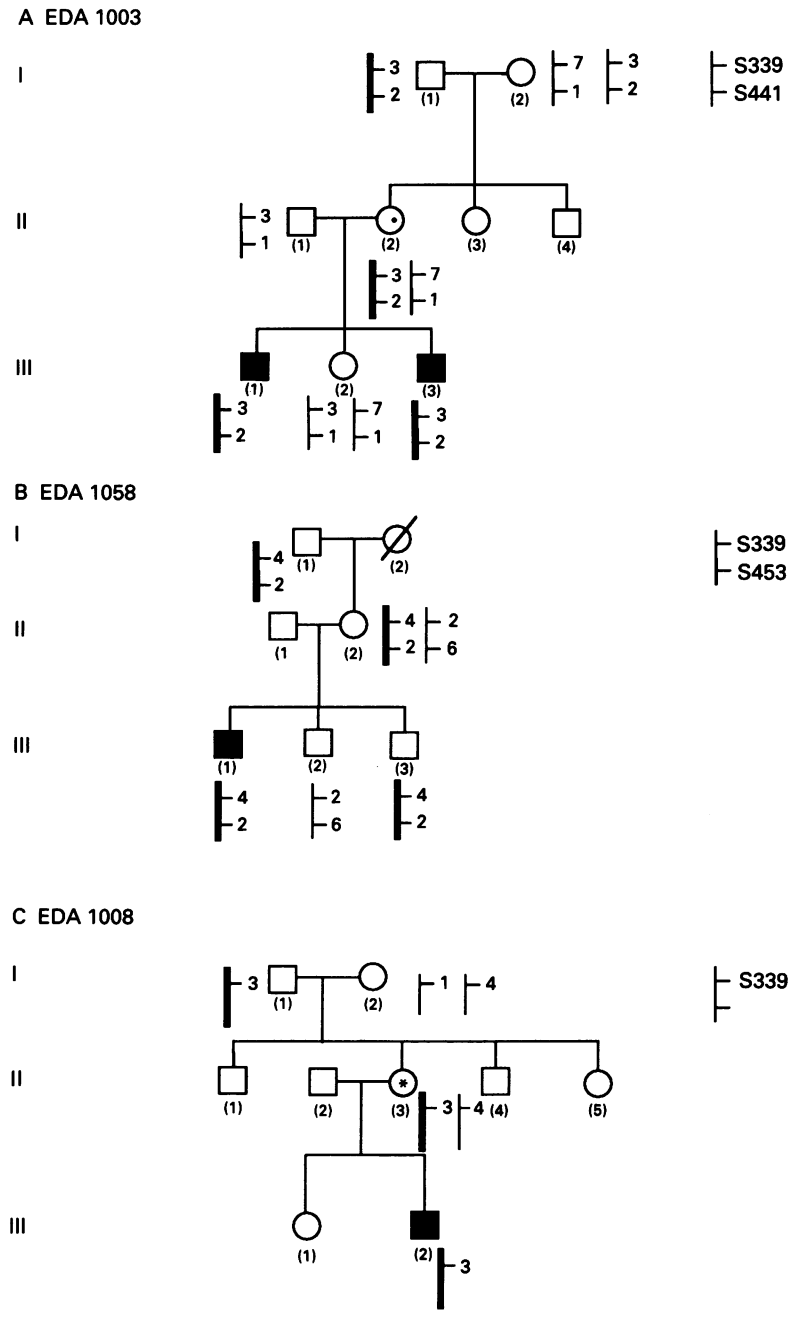

Figure 2A–C Pedigrees of families with de novo molecular deletions with haplotypes of linked polymorphic loci. Black square denotes affected males; dot in circle denotes obligate carrier female; asterisk in circle denotes manifesting carrier female.

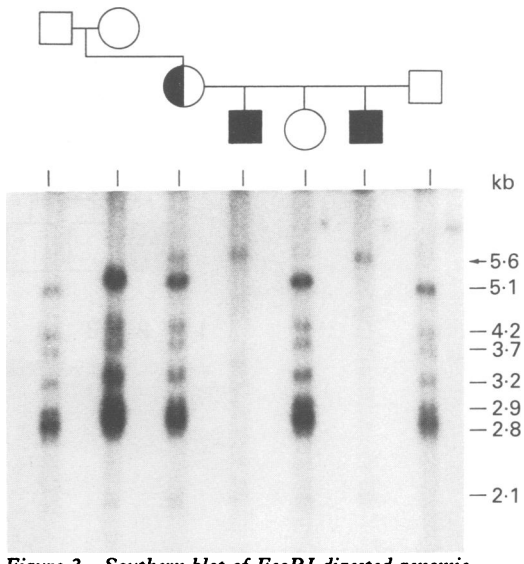

Figure 3 Southern blot of EcoRI digested genomic DNA from EDA family 1003, probed with cosmid ICRFc104A09.80. Lanes: (1) I·1, (2) I·2, (3) II·2, (4) III·1, (5) III·3, (6) III·2, (7) II·1. Note the 5·6 kb junctional fragment in the affected sons and their mother in lanes 3, 4, and 6.

occurred during spermatogenesis, although a postzygotic mutation in the mother cannot be excluded. As expected, the proband's sister, who did not display the junctional fragment, inherited the haplotype of the maternal grandmother. Barring gonadal mosaicism in the grandfather, the findings eliminate possible carrier status for six female relatives in the family.

Family EDA 1058

Except for a single normal sized fragment, no hybridisation signal was seen when cosmid ICRFc104C11.138 from contig D was used as a probe on hybridisation with genomic DNA restricted with *Eco*RI and *Hin*dIII in subject III·1 from family EDA 1058 (figs 2B, 4). He was not deleted for any of the cosmids from contig groups A–C, but showed no signal when Lλ5c4 was used as a probe. The proband (III·1), a 6 year old male, had only two permanent teeth, inability to sweat, fine white scalp hair, and bilateral absent nipples. His mother (II·2) had a normal physical examination, and no family history suggestive of EDA. She had been evaluated after the birth of the proband, and a sweat pore count of her palms was suggested to be "moderately reduced" at 12 to 14/cm. During a subsequent pregnancy, she was studied and found to be informative for the DXS339 and DXS453 loci closely flanking the EDA locus. Her male fetus (III·2) was shown to have the maternal grandmother's haplotype, rather than the maternal grandfather's haplotype carried by the affected proband (fig 2B) and, as expected, proved to be unaffected. During her most recent pregnancy, another male fetus was detected with the same haplotype as the proband. She was counselled that he would be affected if she was a carrier, but this could not be determined. She continued the pregnancy and shortly after delivery of this infant the molecular deletion was detected in the proband. Neither brother (III·2 and III·3) was deleted upon hybridisation with cosmid ICRFc104C11.138, indicating that the proband's deletion arose either during oogenesis in subject II·2, or she was a gonadal mosaic for the mutation (fig 4).

Family EDA 1008

The proband (III·1) is an 11 year old male with six secondary teeth, sparse, fine scalp hair, and marked hypohidrosis (fig 2C). His mother (II·2) is a manifesting carrier with conical shaped teeth, absent bilateral maxillary incisors, and patchy sweating. The maternal grandparents are clinically unaffected, and there is no other family history of EDA. Analysis of the proband's DNA shows him to be

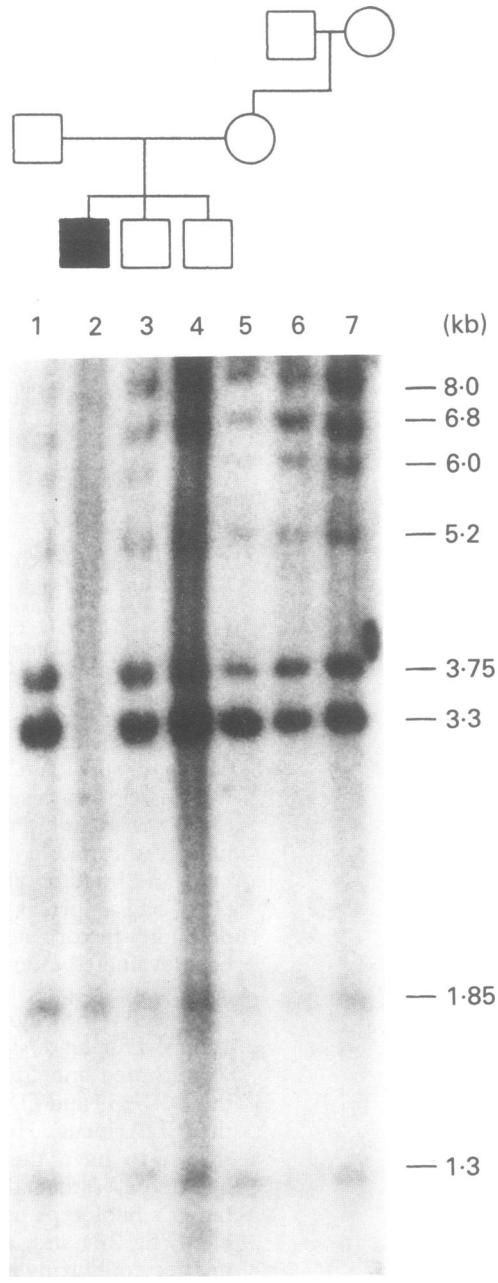

Figure 4 Southern blot and HindIII digested genomic DNA from EDA family 1058, probed with cosmid ICRFc104C11.138. Lanes: (1) II·1, (2) III·1, (3) III·2, (4) III·3, (5) II·2, (6) I·1, (7) I·2. Note the deletion of all but one normal fragment in subject III·1 in lane 2, but normal pattern in subject III·3 in lane 3.

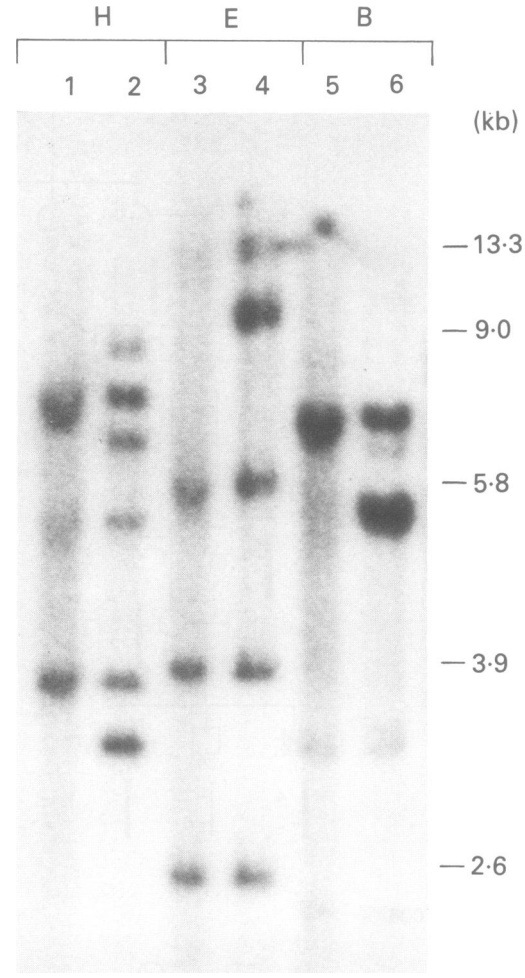

Figure 5 Southern blot of HindIII, EcoRI, and BglII digested genomic DNA from the proband (III·2) from EDA family 1008 (lanes 1, 3, 5) and a control (lanes 2, 4, 6), probed with cosmid ICRFc104C11.138. Note the absence of multiple normal sized fragments with each digest in lanes 1, 3, and 5.

missing multiple fragments on hybridisation of cosmid ICRFc104C11.138 to DNA digested separately with several restriction enzymes (fig 5). He was not deleted for any of the cosmids used as probes from contig groups A–C, nor for the YAC end clone Lλ5c4. Analysis of polymorphic loci flanking the EDA locus showed that the proband had inherited his allele at the DXS339 locus from the maternal grandfather. Since the proband's mother is clearly affected, and no recombinants have been observed to date between the DSX339 and EDA loci, the mutation probably arose during spermatogenesis in the maternal grandfather. Neither he nor the maternal grandmother showed evidence of the deletion upon hybridisation with cosmid ICRFc104C11.138 (data not shown).

IDENTIFICATION OF DE NOVO MUTATIONS BY HAPLOTYPE ANALYSIS

Nine families with unidentified mutations had a pedigree structure potentially informative for analysis of possible de novo mutations with relevant samples available (fig 6). Informative flanking markers were present in all nine families. In seven of the families studied, the haplotype segregating with the disorder was inherited from the maternal grandfather, and in two cases from the maternal grandmother.

In the seven families where the mutation was present on the maternal grandfather's haplotype, the mutation had to have arisen either during spermatogenesis or postzygotically in the mother, since the disorder shows complete penetrance in males. In either case, the carrier risk of the maternal grandmother and great aunts of the proband is eliminated. The risk to the maternal aunts and their female offspring is also eliminated, barring gonadal mosaicism in the maternal grandfather. In the two families where the disorder segregated with the haplotype of the maternal grandmother (EDA 1010 and 1108), it cannot be

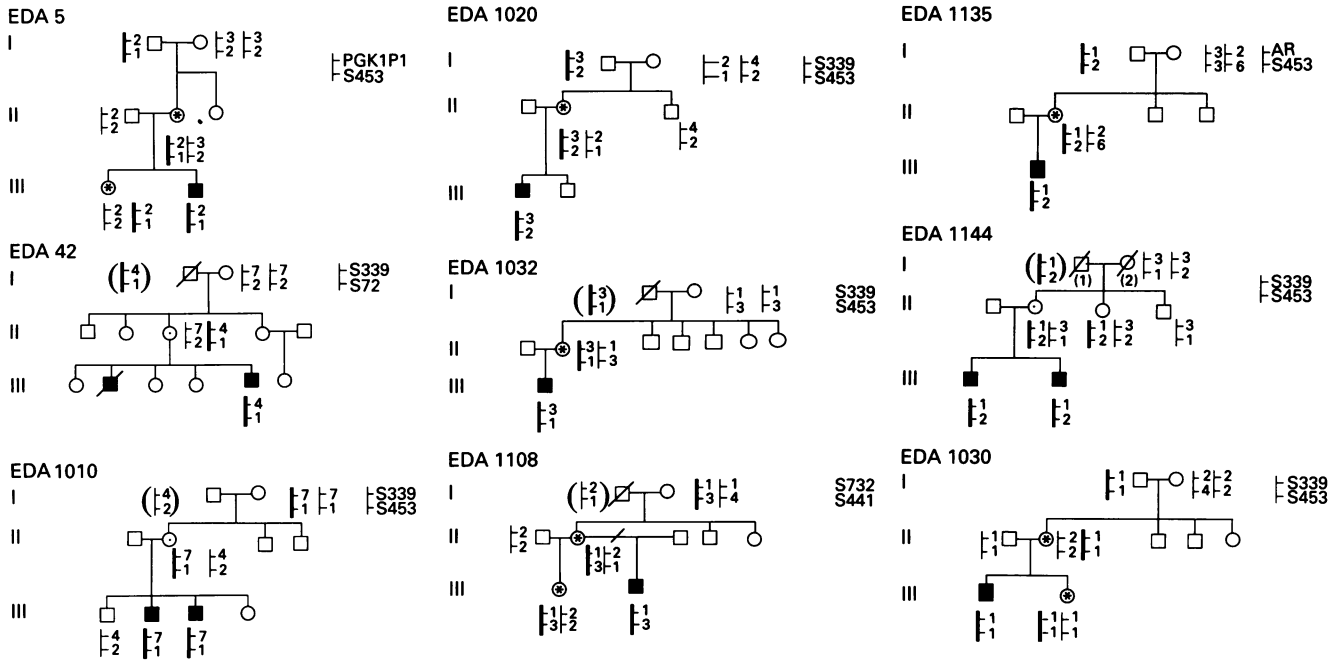

Figure 6 Pedigrees of EDA families with undefined mutations and possible de novo mutations, with haplotypes at polymorphic marker loci flanking the EDA locus.

ruled out that the mutations were not de novo, but inherited from a non-manifesting grandmother (fig 6). If DNA samples were available for analysis from the unaffected male offspring of these women, one might be able to distinguish between these two possibilities.

Discussion

We have been able to identify de novo mutations in 10 families with EDA, with three of them having detectable de novo deletions. One other family (EDA 1015) with a molecular deletion had been previously detected among the families in our panel, but the origin of the mutation could not be determined since the deletion was present in all three generations studied.[4] The proximal breakpoint of this deletion was localised within cosmid contig A (ICRFc104HO41.43), with a distal breakpoint located proximal to cosmid ICRFc104C11.138 from contig group D (fig 1). The clinical phenotype of the affected males in all four families with molecular deletions was no different from other males with EDA. Identification of the de novo nature of the molecular deletions was extremely helpful in counselling these families, and detection of four separate molecular deletions should aid in the positional cloning of the EDA locus. To date, 5% of the 80 families studied have detectable molecular deletions using cosmids localised to the EDA region as probes. Further deletions may be identified in the remaining families, including the seven with apparent de novo mutations, as additional genomic clones and cDNA from the EDA region are isolated.

Although the present sample size is small, the 3·5:1 ratio (95% CI of 0·8–14·8 to 1) of male to female origin of mutations in the families with yet to be defined mutations is consistent with the results of studies of other X linked loci, such as factors VIII and IX, and ZFX.[16–19] Sex differences in the origin of mutation appears to depend on the nature of the mutation, with single base substitutions in general, and transitions at the CpG dinucleotide in particular, having a male preponderance, while deletion cases display a sex ratio closer to unity.[14] This indicates that the majority of the unidentified mutations in the EDA gene may be the result of single base pair substitutions. The male to female ratio of origin of mutation in this study is a minimal estimate since in the cases where the disorder segregated with the X chromosome of the maternal grandmother, the mutation may not be de novo. Gonadal and somatic mosaicism in males and females can complicate both origin of mutation studies and genetic counselling,[20 21] but such mosaicism in EDA has yet to be documented.

The ability to prove that a mutation was de novo eliminated the risk of being a carrier for most female relatives in the 10 families. Our analyses indicate the need for caution in the interpretation of carrier status based on subclinical tests such as sweat pore counts.[7 8] Several of the females suspected of being carriers based on such tests were found not to be at risk after molecular analysis. The apparent excess of mutations during spermatogenesis rather than oogenesis, if confirmed by additional family studies, would significantly increase the risk of mothers of sporadic cases being carriers. Hopefully, direct detection of both deletions and single base pair substitutions will be possible in most families once the EDA gene is identified.

The authors would like to thank P Zimmerman and T K Hyatt for their assistance in preparation of the illustrations and J Chelly and T Monaco for the X chromosome cosmids used as probes. This work was supported by the following organisations: PHS RO1 AR40741 from the National Institute of Arthritis, Musculoskeletal and Skin Diseases (JZ), National Foundation for Ectodermal Dysplasias (JZ), and The Wellcome Trust (AC,NSTT).

1 Clarke A, Phillips D, Brown R, Harper P. Clinical aspects of X-linked hypohidrotic ectodermal dysplasia. *Arch Dis Child* 1987;**62**:989–96.
2 Zonana J, Jones M, Browne D, *et al.* High resolution mapping of the X-linked hypohidrotic ectodermal dysplasia locus. *Am J Hum Genet* 1992;**51**:1036–46.
3 Thomas NST, Chelly J, Zonana J, *et al.* Characterization of molecular DNA rearrangements within the Xq12–q13.1 region, in three patients with X-linked hypohidrotic ectodermal dysplasia (EDA). *Hum Mol Genet* 1993;**2**:1679–86.
4 Zonana J, Gault J, Davies KP, *et al.* Detection of a molecular deletion at the DXS732 locus in a patient with X-linked hypohidrotic ectodermal dysplasia (EDA), with the identification of a unique junctional fragment. *Am J Hum Genet* 1993;**52**:78–84.
5 Kere J, Grzeschik KH, Limon J, Gremaud M, Schlessinger D, de la Chapelle A. Anhidrotic ectodermal dysplasia gene region cloned in yeast artificial chromosomes. *Genomics* 1993;**16**:305–10.
6 Zonana J, Sarfarazi M, Thomas NST, Clarke A, Marymee K, Harper PS. Improved definition of carrier status in X-linked hypohidrotic ectodermal dysplasia by use of restriction fragment length polymorphism based linkage analysis. *J Pediatr* 1989;**114**:392–9.
7 Berg D, Weingold DH, Abson KG, Olsen EA. Sweating in the ectodermal dysplasia syndromes. *Arch Dermatol* 1990;**126**:1075–79.
8 Clarke A, Burn J. Sweat testing to identify female carriers of X-linked hypohidrotic ectodermal dysplasia. *J Med Genet* 1991;**28**:330–3.
9 Zonana J, Clarke A, Sarfarazi M, *et al.* X-linked hypohidrotic ectodermal dysplasia: localization within the region Xq11–21.1 by linkage analysis and implications for carrier detection and prenatal diagnosis. *Am J Hum Genet* 1988;**43**:75–85.
10 Nizetic D, Zehetner G, Monaco A, Gellen L, Young B, Lehrach H. Construction, arraying, and high-density screening of large insert libraries of human chromosomes X and 21: their potential use as reference libraries. *Proc Natl Acad Sci USA* 1991;**88**:3233–7.
11 Litt M, White R. A highly polymorphic locus in human DNA revealed by cosmid-derived probes. *Proc Natl Acad Sci USA* 1985;**82**:6206–10.
12 Crawford PJM, Aldred MJ, Clarke A. Clinical and radiographic dental findings in X-linked hypohidrotic ectodermal dysplasia. *J Med Genet* 1991;**28**:181–5.
13 Muller CR, Grimm T. Estimation of the male to female ratio of mutation rates from the segregation of X-chromosomal DNA haplotypes in Duchenne muscular dystrophy families. *Hum Genet* 1986;**74**:181–3.
14 Edwards A, Civitello A, Hammond HA, Caskey CT. DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. *Am J Hum Genet* 1991;**49**:746–56.
15 Ram KT, Barker DF, Puck JM. Dinucleotide repeat polymorphism at the DXS441 locus. *Nucleic Acids Res* 1992;**20**:1428.
16 Ketterling R, Vielhaber E, Bottema CDK, *et al.* Germ-line origins of mutation in families with hemophilia B: the sex ratio varies with the type of mutation. *Am J Hum Genet* 1993;**52**:152–66.
17 Kling S, Ljung R, Sjorin E, *et al.* Origin of mutation in sporadic cases of haemophilia-B. *Eur J Haematol* 1992;**48**:142–5.
18 Rosendaal FR, Brocker-Vriends AHJT, van Houwelingen JC, *et al.* Sex ratio of the mutation frequencies in haemophilia A: estimation and meta-analysis. *Hum Genet* 1990;**86**:139–46.
19 Shimmin LC, Hung-Jung Chang B, Li WH. Male-driven evolution of DNA sequences. *Nature* 1993;**362**:745–7.
20 Grimm T, Muller B, Muller CR, Janka M. Theoretical considerations on germline mosaicism in Duchenne muscular dystrophy. *J Med Genet* 1990;**27**:683–7.
21 Milewicz DM, Witz A, Smith AC, Manchester DK, Waldstein G, Byers P. Parental somatic and germ-line mosaicism for a multiexon deletion with unusual endpoints in a type III collagen (COL3A1) allele produces Ehlers–Danlos syndrome type IV in the heterozygous offspring. *Am J Hum Genet* 1993;**53**:62–70.