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Review article

Is the recently discovered *EDA* gene associated with anhidrotic ectodermal dysplasia?

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Abstract. The evidence from literature strongly suggests that Christ-Siemens-Touraine (CST) syndrome is associated with mutations of the newly discovered *EDA* gene. The gene is situated on the long arm of the X chromosome (Xq12.2-q13.1) and contains two exons separated by a 200 kbp intron. The 5'-untranslated region and most of the coding sequence are localized in exon 1, while three C-terminal amino acids are encoded by exon 2. The coding sequence was interrupted by translocations in three affected females: t(X;1), t(X;12), t(X;9), and submicroscopic deletions of the *EDA* gene were found in five males with CST syndrome, and point mutations were discovered in exon 1 in nine other patients. Northern blot analysis and in situ hybridization studies revealed that the *EDA* gene was expressed in the foetus, and postnatally in a specific type of skin cell and that the expression was limited to cells of ectodermal origin. A predicted protein product of the *EDA* gene contains 135 to 140 amino acids, organized in three distinct domains and may belong to class II transmembrane receptors.

Key words: *EDA* gene, ectodermal dysplasia anhidrotic, linkage analysis, X chromosome, YAC clones.

Introduction

Ectodermal dysplasia is a rare genetically determined disorder resulting from abnormalities of ectodermal-mesodermal interaction during embryonic life. This disorder was first reported by THURMAN in 1848 and in 1875 DARWIN

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noticed that the disease is transmitted by females to their male offspring. Ectodermal dysplasia comprises approximately 150 related genetic diseases (FREIRE-MAIA, PINHEIRO 1994), the most common form being ectodermal dysplasia anhidrotic (EDA) (KERE et al. 1996), also called Christ-Siemens-Touraine syndrome (CST) (MIM 305100; MCKUSICK 1994). Medical examination permits the diagnosis of CTS syndrome on the basis of characteristic symptoms: anodontia or oligodontia with conical shape of teeth; hypohidrosis with concomitant hyperthermia, caused by lack of sweat glands, and hypotrichosis (sparse hair). In the affected boys, the phenotype is characterized by a special facial appearance, in particular: prominent lips, a depressed nasal bridge (saddle nose), periorbital wrinkling and pigmentation. Hair is very fine and sparse, eyelashes are short and eyebrows are lacking. Moreover, dryness of the mucous membranes may lead to atrophic rhinitis, pharyngitis, chronic laryngitis and otitis. These recurrent infections substantially increase the risk of death in infancy and cause height retardation, weight loss and anaemia. In the affected boys, photophobia related to a reduction in lacrimal gland function is commonly noticed. Some boys present with pigeon chest, cryptorchism or moderate mental retardation.

Invariably, in females some clinical features of the disease can be identified. In female carriers, hair is thin and fragile and often some teeth are missing or are conical in shape. Sweat glands may be scarce and abnormally distributed. The anhidrotic ectodermal dysplasia is X-linked and recessively inherited (KERE et al. 1996). Last year a candidate for the *EDA* (ectodermal dysplasia anhidrotic) gene was identified. Although the localization and structure of the candidate *EDA* gene has been elucidated, the function of the protein product of the gene and its role in the development of skin appendages remains unknown. To date, the literature of the subject has not been extensively reviewed.

The purpose of this report is to review and discuss recent literature concerning the genetic and molecular background of CST syndrome and to present the probable implications of the laboratory findings in understanding the process of the development of skin appendages.

Genetic mapping of the *EDA* gene

Segregation patterns indicate X-linked inheritance

Inspection of pedigrees of the affected individuals has shown that women carrying CST syndrome deliver male offspring with clinical symptoms of

the disease and healthy female offspring in a 1:1 ratio (GILGENKRANTZ et al. 1989). Affected males are hemizygous for X-linked genes and develop full clinical symptoms, whereas female carriers often present some subclinical features. The different intensity of phenotypic expression of the symptoms can be explained by random inactivation of one of the X chromosomes in a process called lyonisation (HAPPLE, FLOSCH 1985), which takes place early in embryonic development. Because of lyonisation, X-linked traits are variably expressed in women who are heterozygous for the X-linked genes. These findings imply recessive X-linked inheritance of CST syndrome.

Chromosome abnormalities were useful in mapping the *EDA* gene

So far, profound changes in chromosome structure were detected only in three cases of CST syndrome, by analysis of banding pattern in metaphase chromosomes. Initial observations of the affected girl with translocation t(X;9) (described by COHEN et al. 1972), allowed them to draw the conclusion that the *EDA* gene is situated at Xq12 (GERALD, BROWN 1974). A second case of de novo translocation t(X;12) in the affected female was reported by TURLEAU et al. (1989) and LIMON et al. (1991) described de novo translocation (X;1)(q13.1;p36.33) in a 2-year-old girl with typical clinical features of CST. These three translocations: t(X;9), t(X;12) and t(X;1) in the affected females reported to date confirmed X-linked inheritance of CST syndrome inferred from the analysis of pedigrees. Cytogenetic studies had shown that in all reported cases of translocations, the breakpoints were situated in the same region (Xq13.1), suggesting localization of the *EDA* gene.

Linkage analysis allowed mapping of the *EDA* gene

The position of the *EDA* gene on the X-chromosome, in relation to other genes, was established by recombination mapping, which assessed the approximate distance between different known loci and the *EDA* gene. MACDERMOT et al. (1986), who analysed 11 families with CST syndrome, calculated a lod score of 2.66 at a recombination fraction of 0.006 for linkage between the *EDA* gene and locus *DXYS1*. In the same year KOLVRAA et al. (1986) found a linkage between locus *DXS146* and the Xp-Xq21 region. The suggested linkage to locus *DXYS1* was confirmed by CLARKE et al. (1987), who in addition established the linkage of *EDA* gene to loci *DXS14* and *DXS3*. These findings indicated that the *EDA* gene is located in the pericentromeric region of the X chromosome, between *DXYS1* on the long arm and *DXS14* on the short arm, probably

on proximal Xq. One year later ZONANA et al. (1988), by the use of linkage analysis suggested that the *EDA* gene was located on Xq12-q13. HANAUER et al. (1988) concluded that locus *DXS159* was the nearest marker of the *EDA* region (Xq11-q12) and KRUSE et al. (1989), based on multipoint linkage analysis, reasoned that the *EDA* gene was most likely located at a distance of 10 cM distal to locus *DXS1*, resting between loci *DXS1* and *DXSY1*.

Application of a probe derived from the mouse *Tabby* (*Ta*) gene

A highly conserved DNA probe originating from the murine locus *DXCrc169*, tightly linked to the *Ta* gene (putative mouse homologue of the *EDA* gene) was hybridized to human genomic DNA and a new locus *DXS732* was identified (BLECHER 1986). Next, the DNA of 80 unrelated affected males were screened for deletions at seven genetic loci (*DXS159*, *PGKP1*, *DXS339*, *DXS732*, *DXS348*, *DXS453* and *PGK1*) located within the Xq12-q13 region. The mouse genomic probe corresponding to the *Ta* gene identified a single patient with a deletion of the *DXS732* locus. This suggested that locus *DXS732*, at least in part, lies within the region of the *EDA* gene (Fig. 3). However, no deletions in the remaining six loci were found in other patients.

Molecular cloning of the *EDA* gene

Somatic cell hybrids help to establish the breakpoint regions

Precise localization of the breakpoints and its relation to the *EDA* gene, was established by the use of cell hybrids (AnLyRAG and RCAR), prepared by the fusion of cells derived from patient t(X;9) (AnLy line), and the lymphoblastoid cell line derived from patient t(X;12), with mouse cells and Chinese hamster cells. This procedure allowed the allocation of a region, spanning the two breakpoints, that hybridized successively with four probes (*DXS153*, *DXS224*, *DXS132* and *DXS469*). Molecular analysis of the two translocations indicated that the breakpoint derived from t(X;9) was located telomeric of the breakpoint from t(X;12). Therefore it was concluded that this region was contig to the *EDA* gene (PLOUGASTEL et al. 1992) (Fig. 1).

The next step was to compare the DNA from the two affected females with translocations (KERE et al.1993). The first somatic cell hybrid line (AKRAG9) was constructed by fusing fibroblasts from the patient with t(X;1), with rodent

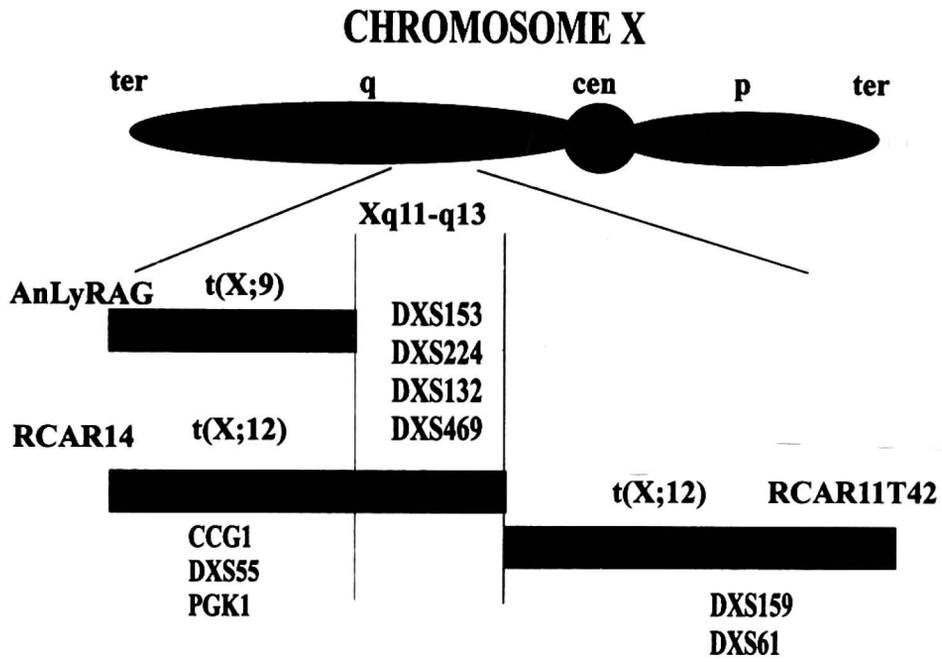

Fig. 1. The use of cell hybrids to identify the region between the individual breakpoints in translocations t(X;12) and t(X;9)

AnLy RAG, RCAR14 and RCAR11T42 designate cell hybrids containing der (9), (12) and (X), respectively. Hybridization of nine probes identified three subregions in the breakpoint area indicating two different breakpoints of chromosome X

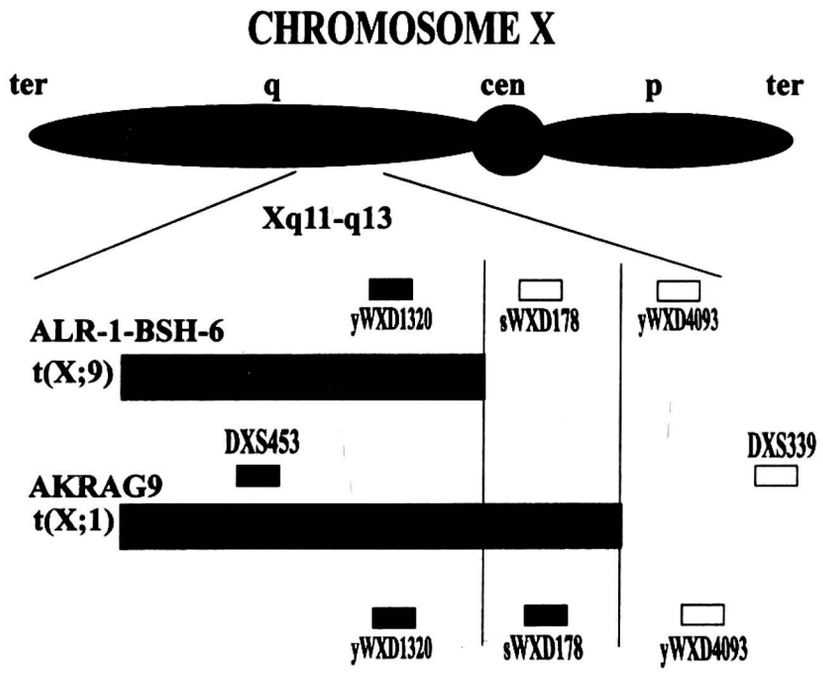

Fig. 2. The use of cell hybrids to identify the region between the individual breakpoints in translocations t(X;9) and t(X;1)

ALR-1-BSH-6 and AKRAG9 designate cell hybrids containing der (9) and (1), respectively. Shadowed boxes indicate hybridization with the probes, open boxes no hybridization. Hybridization of five probes identified two different breakpoints of chromosome X and an intragenic marker *sWXD178*

cells. The second cell hybrid line (AnLyRAG) was derived from the patient with t(X;9). In both patients the breakpoints were located between loci *DXS339* and *DXS453*.

Yeast artificial chromosome clones (YACs) covering regions of two translocation breakpoints facilitated cloning of the *EDA* gene

To construct a physical map of the *EDA* region, YAC clones were isolated from three genomic libraries. This was accomplished by PCR, using primers specific for the two loci (*DXS453* and *DXS339*), adjacent to the breakpoints in patients t(X;1) (LIMON et al. 1991) and t(X;9) (MACDERMOT, HULTEN 1990) (Fig. 2). Since no YACs covering the entire *EDA* region were found, the chromosome walking technique was applied, and the region between the adjacent loci was cloned in YACs. Comparison of the arrangement of the YAC clones in relation to the breakpoints, allowed the identification of the nearest flanking loci, and to localize the previously described locus *WXD178* (KERE et al. 1992) between these breakpoints (KERE et al. 1993) (Fig. 2).

Application of cosmid clones permitted the location of the *EDA* region

To identify homologous YAC clones THOMAS et al. (1993) used a DNA probe specific for locus *DXS453* (WEBER et al. 1990), and a probe, recognising the newly discovered locus *DXS732* (ZONANA et al. 1993). The *DXS732*-spe-

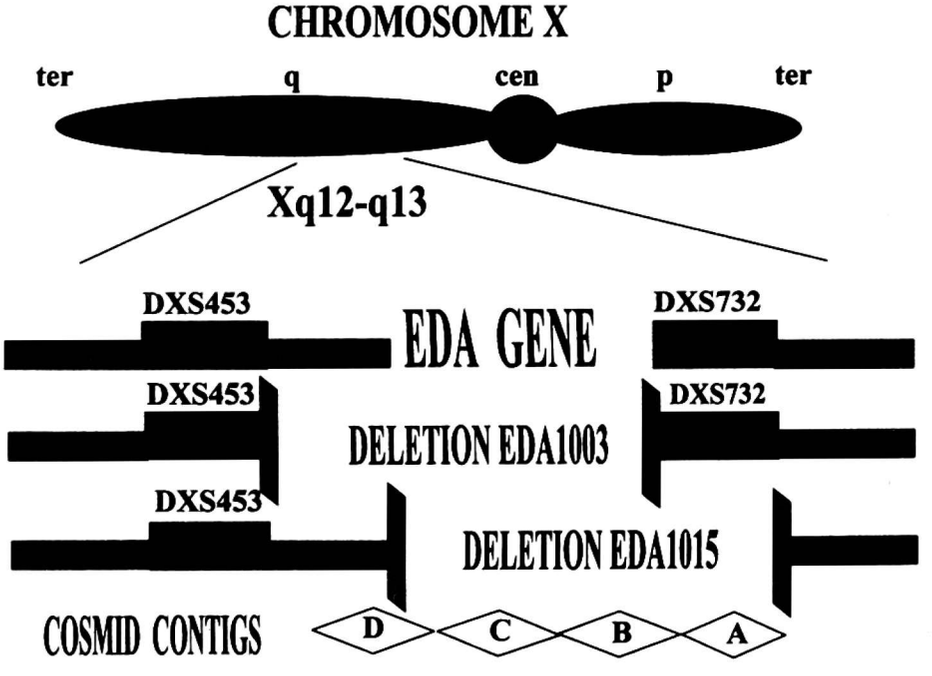

Fig. 3. Physical map of the *EDA* region based on approximate localization of the four cosmid contigs in relation to the deletions found in patients EDA1015 and EDA1003 *DXS453* and *DXS732* designate adjacent loci telomeric and centromeric to the *EDA* gene, respectively. A through D designate cosmid contigs spanning loci *DXS453* and *DXS732*

cific YACs identified 36 cosmid clones in the human X chromosome cosmid library, and permitted the arrangement of subsets of these clones into four contigs A, B, C and D (THOMAS et al. 1993). With the use of one of the cosmid clones of contig C, potential junction-fragments were identified in several restriction digests of DNA of AnLy hybrid t(X;9). This was subsequently confirmed by fluorescence in situ hybridization (FISH) analysis which revealed hybridization of the same cosmid clone to both chromosome X and chromosome 9.

Application of the same probe for screening genomic DNA samples from 80 unrelated affected males (THOMAS et al. 1993) confirmed the previously described deletion in the EDA 1015 patient (ZONANA et al. 1993) and permitted detection of a new deletion in one more patient EDA 1003. Characterization of these two deletions allowed the localization of the *EDA* gene within the region situated telomeric of locus *DXS732*, and centromeric of locus *DXS453*, i.e., within the region cloned in the four cosmid contigs (Fig. 3).

Blot analysis

Within the region of the *EDA* gene cloned in YACs, five CpG islands were discovered using several rare-cutter restriction enzymes (Not I, EagI, BssHII and SacII). The regions spanning the CpG islands 1 through 3 were subcloned in phage. A 1 kbp fragment of one of the subclones covering the third CpG

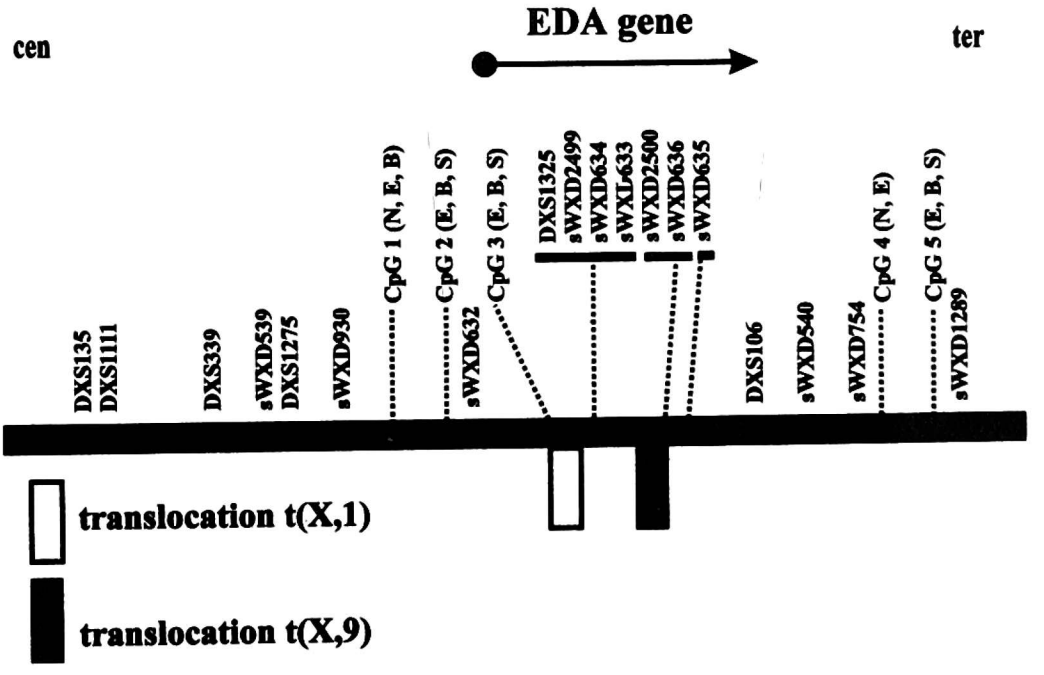

Fig. 4. Localization of the *EDA* region with respect to marker sequences (according to SRIVASTAVA et al. 1996, modified)

CpG designates CG-rich islands recognised by rare cutter restriction enzymes (B-BssH II; E-Eag I; N-Not I; Sac II). Open box designates t(X;1), shadowed box t(X;9). Arrow indicate the *EDA* region known loci and their approximate arrangement in the *EDA* region is shown in the upper part of the figure

island hybridized with RNA (Northern blot) giving a prominent signal of 6 kb in foetal tissues, and a weak signal in adult tissues. This suggested that a gene associated with the third CpG island was developmentally regulated (Fig. 4).

	Hybridization with probe 1 fragments			Hybridization with probe 2 fragments		
Template DNA	2,6kb	7,0kb	9,0kb	2,6kb	7,0kb	9,0kb
X chromosome	□	■	□	□	■	□
AK patient	□	■	■	■	■	□
AK hybrid	□	□	□	■	□	□

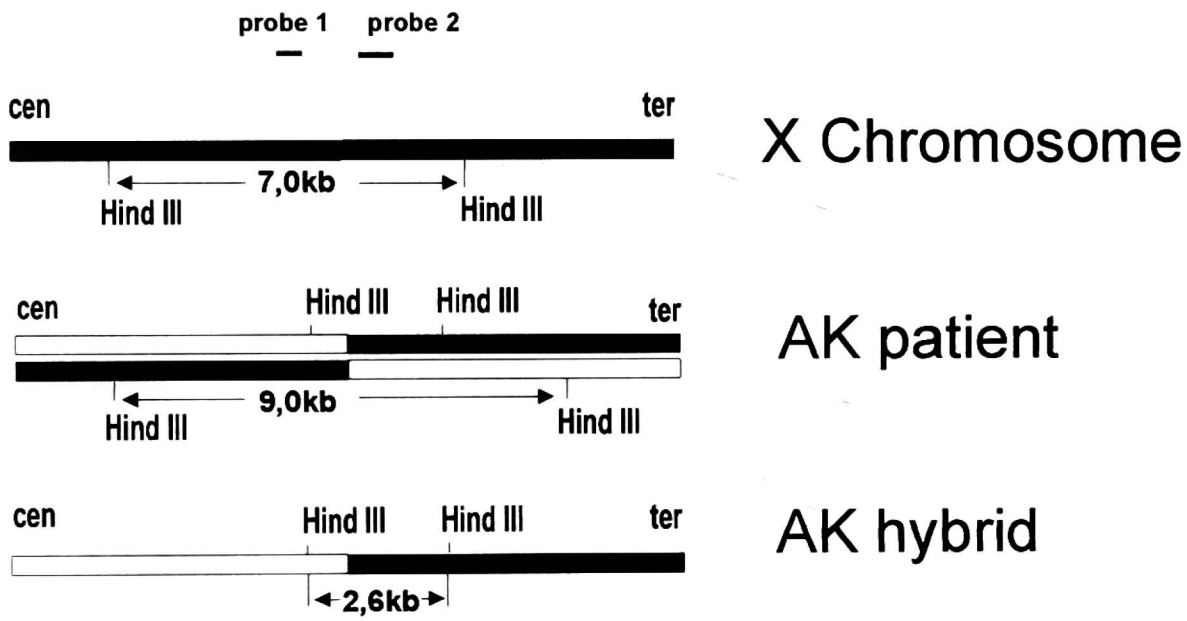

Fig. 5. Southern blot analysis of t(X;1) in AK patien and der (X) AK hybrid (according to SRIVASTAVA et al. 1996, modified)

Hind III fragment of CpG island 3 was hybridized with two probes derived from λ clone. Closed squares represent hybridization signal, open squares – no hybridization. Probes 1 and 2 indicate localization of the breakpoint of X chromosome

The same probe, used in Southern analysis revealed a 7 kbp Hind III fragment in human genomic DNA and the X chromosome. Whereas in patient AK t(X;1) two fragments: one of 7 kbp and the other of 9 kbp were identified. In addition, no hybridization was evident with the DNA from the AK hybrid. This suggested that the probe binds centromeric of the breakpoint. The results were substantiated by finding that another probe, derived from the same clone, but covering the region telomeric of the previous probe, recognized a 9 kbp fragment of the AK hybrid, and an additional fragment of 2.6 kbp, which presumably contained a piece of chromosome 1 (SRIVASTAVA et al. 1996) (Fig. 5).

Identification of the *EDA* gene

The recently reported results of positional cloning of the *EDA* gene permitted the identication of the structure and mutations in the *EDA* gene. A genomic subclone, containing the segment of the X chromosome comprising the third CpG island, was sequenced. The sequence contained two possible open reading frames (ORFs) and ten GC-box promoter elements. Primers designed for the putative ORFs were used to screen a sweat gland cDNA library and an identical cDNA clone was found. This cDNA was used as a hybridization probe to screen the YAC *EDA* clones. It revealed that the genomic clone, a putative *EDA* gene, contained two exons, the first mapped to the third CpG island, and the second approximately 200 kbp telomeric of the CpG island (KERE et al. 1996). The 5'-untranslated region and most of the coding region were located in exon 1, whereas three C-terminal amino acids were encoded by exon 2 (Fig. 6).

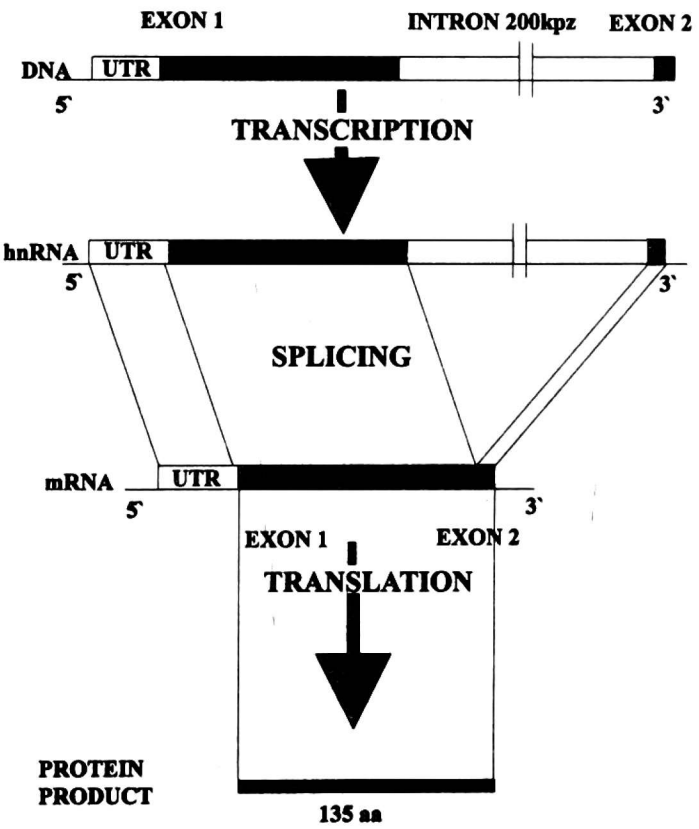

Fig. 6. Schematic representation of the *EDA* gene structure and expression

Studies of *EDA* gene expression

It has been demonstrated that the genomic clone corresponding to the *EDA* cDNA, hybridized at high stringency conditions to a 6 kb mRNA species in foetal brain, lung, liver and kidney. In the adult tissues the expression was much lower except for the kidney, and the hybridization signal was also found

in the heart, skeletal muscle and pancreas. At low stringency multiple mRNA species of 6.0, 4.5, 2.5, 2.0 and less than 1 kb were detected in the tissue extracts (KERE et al. 1996).

By in situ hybridization with [^{35}S]-labelled cRNA probes, specific mRNA was found in the epidermis, epithelial cells in the upper and lower hair follicle, sebaceous glands and occassionally in eccrine sweat glands, but not in the dermal papilla, dermis and smooth muscle (KERE et al. 1996).

These results suggested that the expression of the *EDA* gene was highly specific and was limited to cells of ectodermal origin and that the mRNA encoding the putative protein product of the gene was absent from the mesenchymal cells of the skin.

The putative protein product of the *EDA* gene

Sequence analysis of the cDNA, identical to the genomic sequence, predicted a 135 amino acid protein, or if an alternative in-frame start site was used, a five amino acid longer product.

The hydrophobicity profile revealed three distinct domains in the protein: a hydrophylic, N-terminal domain, 39 or 44 amino acids long, a hydrophobic domain, consisting of 22 amino acids, and a C-terminal, hydrophylic domain, containing 74 amino acids.

The structure of the predicted protein suggested that it might belong to class II transmembrane receptors that have the N-terminal domain located within the cytosol, and the C-terminal domain projecting outward (KERE et al. 1996).

Newly discovered mutations in the *EDA* gene

Genomic DNA samples from 118 unrelated patients were analysed for mutations in exon 1, encoding the putative transmembrane protein, excluding the last three amino acids encoded by exon 2. In nine patients new mutations were detected. In four of them, one-base insertions or deletions and in five of them, single-base substitutions were detected (Table 1). In the remaining 109 patients no mutations in exon 1 were found. In these patients exon 2 was not analysed for possible mutations. None of the changes were detected in 160 non-EDA patients with other defects localized on the X chromosome.

A possible role of the protein product of the *EDA* gene in the differentiation of skin appendages

The expression of the *EDA* gene in foetal tissues and its highly specific pattern of expression in skin appendages suggests that the protein product

Table 1. Mutations in the *EDA* gene discovered to date (according to KERE et al. 1996, modified)

Family number	Nucleotide change	Predicted effect on protein product
ED19	180G > A	None (change in 5'UTR*)
ED7	187G > T	None (change in 5'UTR*)
ED1166	287insC	Frameshift at residue 16
ED12	363insC	Frameshift at residue 41
ED19	423T > C	Y61H
ED73	448G > T	R69L
ED1113	448G > T	R69L
ED52	494delT	Frameshift at residue 85
ED1024	494delT	Frameshift at residue 85
ED1013	636C > T	Q132ter

* untranslated region

of the gene may play an important role in the development of these structures. However, to date the regulation and ontogeny of the expression of this gene has not been investigated.

It has been shown that the regulatory region of the gene contains a response element 5'-CTTTGAAGA-3, known as the HK-1 motif (ROGERS, POWELL 1993) found in the regulatory regions of all keratin genes published so far. In addition, the same motif was reported to bind lymphoid enhancer factor LEF-1 (TRAVIS et al. 1991) expressed in human keratinocytes and hair follicles (ZHOU et al. 1995). Moreover, the knock-out mice, devoid of the LEF-1 gene, developed structural abnormalities of teeth and hair follicles (van GENDEREN et al. 1994). This suggests that the response element HK-1, found in the regulatory region of the *EDA* gene, and an enhancer factor LEF-1 which binds to this motif, may play a role in the expression of the *EDA* gene and hence the development of skin appendages.

The role of the *EDA* gene in the process of differentiation of ectodermal structures was further elucidated by the investigations in *Tabby* (*Ta*) mice (BLECHER 1986). These animals provide a model to study the differentiation of these structures since they exhibit dental, hair and sweat gland abnormalities, resembling those found in CST. These abnormalities are accompanied by the mutations in the *Ta* gene located on the mouse X chromosome between the sites whose positions correspond to the position of the *EDA* gene in humans (BROCKDORFF et al. 1990, ZONANA et al. 1993).

It has been noticed that the phenotypic abnormalities found in the *Ta* mice can be partly reversed by injecting new-born mice with epidermal growth factor (EGF), which suggests that the protein product of the *Ta* gene may either interact with EGF or with the EGF signaling pathway in the differentiation of skin appendages. So far however, no direct evidence for the role of EGF in the development of skin appendages in humans has been reported.

Concluding remarks

Despite strong evidence for the association of the *EDA* gene with CST syndrome several questions still remain to be answered. It is surprising that in exon 1, encoding almost the complete sequence of the protein product of the gene, mutations were found only in nine out of 118 patients. It seems unrealistic to assume that in the remaining 109 patients all mutations are localized in exon 2 of the *EDA* gene, encoding only three C-terminal amino acids of the putative transmembrane protein. It will be equally unjustified to believe that most of the mutations are localized in the regulatory region of the gene. It also remains to be answered whether a deletion which removed a segment less than 9 kbp long located telomeric of exon 2, detected in one of the patients, is associated with the symptoms of CST syndrome.

In conclusion, the answer to the question of whether the recently discovered *EDA* gene is associated with the CST syndrome is affirmative. However, the discovery of the *EDA* gene provided even more questions than answers. It remains to be established whether the *EDA* gene is the only gene responsible for the abnormalities found in the CST syndrome. Since a mutation found beyond the region of the *EDA* gene in the affected individual may suggest that in fact only a fragment of the *EDA* gene has been discovered. In addition, the breakpoint in translocation t(X;9) mapped around 100 kbp from exon 1 (i.e., in the middle of the large intron). In theory this should not affect the expression of protein product albeit three amino acids shorter. Moreover, several hybridizing transcripts obtained by Northern blot analysis may indicate alternatively spliced or alternative transcript containing additional exons located telomeric of exon 2. This is the explanation as to why only ten mutations in the *EDA* gene have been detected in 118 patients with the CST syndrome.

The question of the role of the protein product of the *EDA* gene, a putative type II receptor, should also be addressed. The structure of the receptor is unusual for two reasons: a small size and a short transmembrane domain. In addition, no ligands to these types of receptors were described. These yet unsolved problems indicate the prospect of future investigations.

The elegant studies presented in this review had clearly demonstrated how the use of different techniques applied to achieve the goal of cloning of *EDA* gene complement each other, since the discovery of the gene with the aid of the sophisticated methods of molecular genetics could not have been achieved without adopting the classical techniques of clinical genetics, cytogenetics, linkage analysis, construction of cell hybrids and an animal model of the disease.

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