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QUARTERLY

Mutation in the regulatory region of the *EDA* gene coincides with the symptoms of anhidrotic ectodermal dysplasia*°

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We have investigated a fragment of the regulatory region of the *EDA* gene in a patient with clinical symptoms of anhidrotic ectodermal dysplasia (EDA), whose DNA sequence of exon 1 was normal. The single-strand conformation polymorphism (SSCP) analysis of PCR-amplified fragments of the regulatory region of the *EDA* gene suggested a mutation localized within the fragment extending from nucleotide -571 to -182 upstream of the 5′ end of the cDNA. Sequence analysis of this fragment documented an additional adenine in position -452, located 32 nucleotides upstream from the response element HK-1, a target for transcription factor LEF-1, involved in the differentiation of tissues of ectodermal and mesodermal origin. We postulate that this mutation might interfere with the transcription process of the *EDA* gene and might be responsible, at least in part, for the clinical symptoms of anhidrotic ectodermal dysplasia.

Anhidrotic ectodermal dysplasia (MIM 305100) [1], also known as Christ-Siemens-Touraine disease, is a rare X-linked and recessively inherited disorder, resulting from abnormalities of ectodermal-mesodermal interaction during embryonic life [2]. Medical examination permits the diagnosis of this syndrome on the basis of characteristic symptoms: anodontia or oligodontia with conical shape of teeth; hypohidrosis with concomitant hyperthermia, caused by a lack of sweat glands, and hypotrichosis (sparse hair) [3].

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Abbreviations: EDA, ectodermal dysplasia anhidrotic; SSCP, single strand conformation polymorphism.

The hypothetical gene responsible for the symptoms of this illness has recently been cloned [4]. This gene known as the *EDA* gene (*ectodermal dysplasia anhidrotic*) is localised 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 localised within exon 1, while three C-terminal amino acids are encoded by exon 2 [4].

Three translocations: t(X;1), [5], t(X;12), [6] and t(X;9), [7] which interrupt the coding sequence were reported in affected females, and submicroscopic deletions of the *EDA* gene were found in five males [4]. To date only exon 1 of the *EDA* gene was examined for mutations in a group of 118 patients with clinical symptoms of anhidrotic ectodermal dysplasia [4]. However, expected mutations in exon 1 have been found in only nine patients, suggesting localisation of the defect beyond exon 1 that is in exon 2 or in the regulatory region of the *EDA* gene.

During extensive search for defects within the *EDA* gene in a group of ten patients with the classical symptoms of anhidrotic ectodermal dysplasia we have not found any mutations within the DNA sequence of exon 1. Therefore, we have decided to examine a fragment of the regulatory region of the *EDA* gene in a patient with the typical symptoms of anhidrotic ectodermal dysplasia whose DNA sequence of exon 1 was normal.

MATERIALS AND METHODS

Patient A.P., a 10 year old male with anhidrotic ectodermal dysplasia was treated at the Department of Dermatology of the Medical University in Gdańsk. The diagnosis was based on three main clinical symptoms: oligodontia, hypohidrosis and hypotrichosis. Peripheral blood was withdrawn from the cubic vein and DNA was extracted from white blood cells [8].

Exon 1 of the *EDA* gene was amplified in two fragments by PCR using two pairs of primers [4]. The first fragment (529 bp) was amplified using the forward primer EDA1-f 5′-GTC GGC CGG GAC CTC CTC-3′ and the reverse primer EDA1-r 5′-TGC CAG AGG TGC CAG GGGT-3′. The second fragment (368 bp) was amplified using the forward primer EDA2-f 5′-GAG CGA GGG AGC CAG GGCT-3′ and the reverse primer EDA2-r 5´-GCC GCC GCC CCT ACT AGG-3′. The two fragments overlapped by approximately 200 nucleotides.

Next we amplified a sequence of the regulatory region of the *EDA* gene extending up to -571 bp upstream of the 5′ end of the cDNA using two pairs of primers designed by us. The first fragment (372 bp), overlapping exon 1, was amplified using the forward primer RR2-f 5′-GTT AAA CTG GGG CGG AGC-3′ and the reverse primer RR2-r 5′-CGT TCC TGC GCG ACA GTG-3′, whereas the second fragment (389 bp) localized upstream of the first fragment, was amplified using the forward primer RR1-f 5′-GGA GTG AGG CTG CTT GAG-3′ and the reverse primer RR1-r 5′-CTT GCC CCG TAC CAG TTG-3′, to yield a 389 bp fragment. The two fragments overlapped by approximately 60 nucleotides.

Single-strand conformation polymorphism (SSCP) analysis [9] was used to screen for mutations in the two fragments of exon 1 and the two fragments of the regulatory region of the *EDA* gene. The electrophoretic separation of the amplified fragments was conducted in 5% polyacrylamide gel at 21°C under the following conditions: 100 V, 7 mA, 1 W, 18 h. After electrophoresis the gels were silver-stained using Silver Staining Kit (Promega, U.S.A.).

In order to confirm the changes indicated by the SSCP analysis, direct sequencing of the amplified products of exon 1 and the fragments of the regulatory region of the *EDA* gene were performed [10] with the use of the following primers: the forward primer for exon 1 (EDA1-f) 5′-GTC GGC CGG GAC CTC CTC-3′, the forward primer for exon 1 (EDA2-f) 5′-GAG CGA GGG AGC CAG GGCT-3′, and

the forward primer for the regulatory region (RR1-f) 5′-GGA GTG AGG CTG CTT GAG-3′ and the reverse primer for the regulatory region (RR2-r) 5′-CGT TCC TGC GCG ACA GTG-3′. Sequencing reactions were conducted at the optimal annealing temperature with the use of ^{35}S-labeled dATP and the fmol Sequencing System (Promega, U.S.A.). Electrophoretic separation was carried out in a 6% polyacrylamide gel containing 8 M urea, at 1500 V, 40 mA and 30 W.

RESULTS

In a patient showing the basic clinical symptoms of anhidrotic ectodermal dysplasia, exon 1 and a fragment of the regulatory region of the *EDA* gene extending up to -571 bp upstream of the 5′ end of the cDNA, were analysed. The length of the amplification products of the two fragments of exon 1 and the two fragments of the regulatory region of the *EDA* gene were not appreciably different from the same products obtained from a healthy individual (control), suggesting that in this patient there are no major insertions or deletions within the amplified DNA fragments (Fig. 1).

To search for mutations, the PCR products were analysed by the use of the single-strand conformation polymorphism technique (SSCP), and no conformation polymorphism was evidenced in the fragments of exon 1. However, electrophoretic mobility of the PCR product of the fragment of the regulatory region of the *EDA* gene, extending from nucleotide -571 to -182 upstream of the 5′ end of the cDNA, differed from that of a healthy individual, indicating polymorphism of a single strand DNA within the analysed fragment (Fig. 2). The other fragment did not exhibit any difference in electrophoretic mobility as compared with the same fragment from the healthy individual (not shown).

Sequence analysis of PCR products of both fragments of exon 1, revealed no mutations (not shown), while the sequencing of one of the fragments of the regulatory region of the *EDA* gene extending from nucleotide -571 to -182 showed an insertion of adenine at -452 (Fig. 3), whereas no mutations were found within a fragment -245 to +127 (not shown). Thus far, in the group of investigated patients with anhidrotic ectodermal dysplasia, as well as in the healthy individuals, we did not find any signs of genetic polymorphism within this region (not shown).

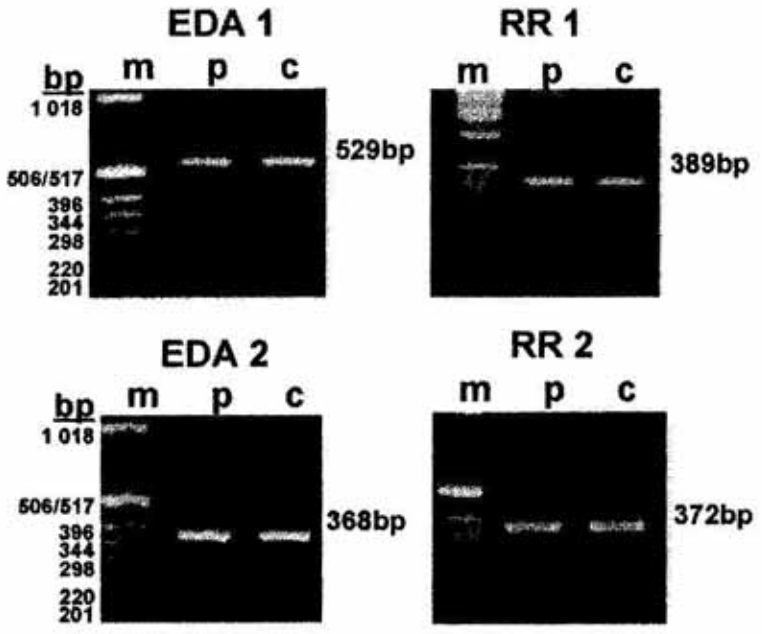


Figure 1. Electrophoretic analysis of the amplified fragments of exon 1 and the regulatory region of the *EDA* gene.

A 529 bp fragment (EDA 1) covers 5′ end of exon 1, whereas a 368bp fragment (EDA 2) covers 3′ end of exon 1. Both fragments partially overlap. A 389 bp fragment (RR 1) covers 5′ end extending from nucleotide -571 to -182, while a 372 bp fragment (RR 2), extending from nucleotide -245 to +127, covers the 3′ end of the regulatory region. Both fragments partially overlap; p, patient; c, control (healthy individual); m, molecular size markers.

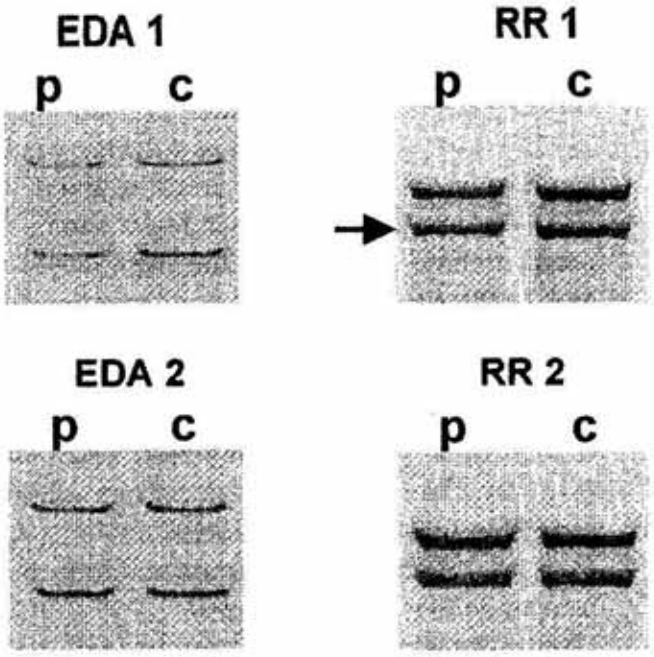
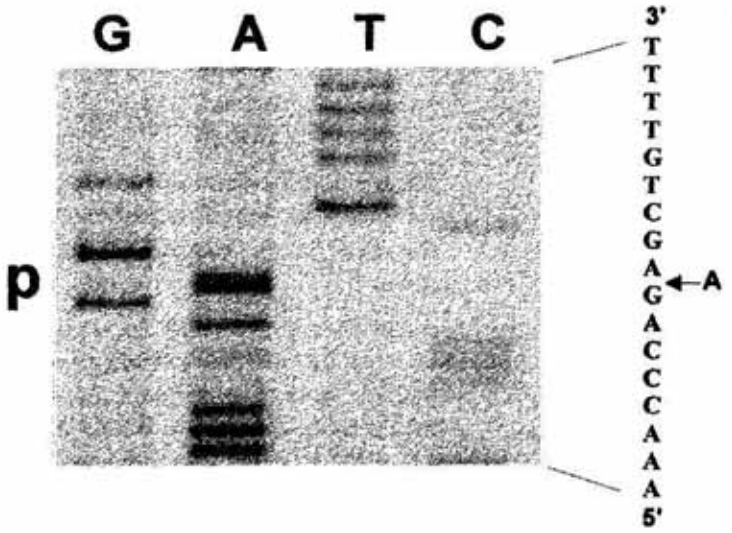


Figure 2. Single-strand conformation polymorphism (SSCP) analysis of the amplified fragments of exon 1 and the regulatory region of the *EDA* gene

The fragments of exon 1 and the regulatory region of the *EDA* gene described in legend to Fig. 1 were subjected to SSCP analysis as described in Materials and Methods. Arrow points to a strand exhibiting abnormal mobility; p, patient; c, control (healthy individual).



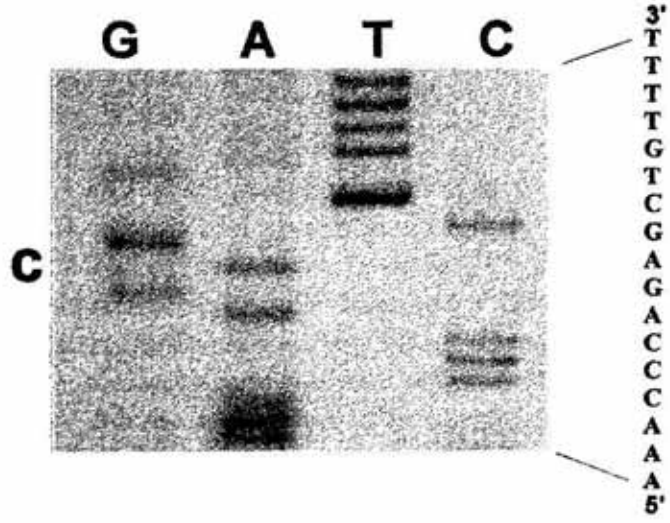


Figure 3. Sequence analysis of a −571 to −182 fragment (RR1) of the regulatory region of the *EDA* gene.

The figure shows part of the sequence harbouring the mutation; p, patient; c, control (healthy individual). Arrow points to adenine substitution at −452.

DISCUSSION

Kere *et al.* [4], have examined a group of 118 carefully selected patients with the diagnosis of anhidrotic ectodermal dysplasia, and found mutations in exon 1 in only nine patients. This exon covers almost the entire coding region of the *EDA* gene. Exon 2 was not examined for mutations. However, it would be surprising if in the remaining 109 patients mutations were localized in exon 2, encoding only three C-terminal amino acids of the putative protein product of the gene [11]. Therefore it would be reasonable to speculate that in those patients the *EDA* gene was not expressed. However, to date no reports on the impaired expression of this gene have been published and no mutations within the regulatory regions of the *EDA* gene have been described.

In our patient, who showed typical symptoms of anhidrotic ectodermal dysplasia, no expected mutations were found in exon 1 of the *EDA* gene. Therefore we have decided to investigate in detail the regulatory region of this gene. The SSCP analysis of the 389 bp fragment extending from -571 to -182 revealed polymorphism of single strand DNA (Fig. 2), suggesting possible mutation(s). The sequence analysis (Fig. 3) showed an insertion of adenine in position -452. Since the insertion of adenine was the only change in the DNA sequence found in this region, we have assumed that this change in nucleotide sequence was due rather to a mutation and did not represent genetic polymorphism. The mutation was located 32 bp upstream from the sequence 5'-CTTTGAAGA-3', known as the response element HK-1 [12]. This sequence motif has been found in the regulatory regions of all keratin genes expressed in human keratinocytes and hair follicles [13] and serves as a target for the transcription factor LEF-1 [14]. It has been demonstrated that the knock-out mice, devoid of the LEF-1 gene, developed structural abnormalities of teeth as well as of hair follicles, pointing to a role of the HK-1 motif in the development of skin appendages [15]. Since the mutation found within the regulatory region of the *EDA* gene in our patient was located in the vicinity of the HK-1 motif, this mutation might affect the binding of the transcription factor LEF-1 to this sequence and thus impair the expression of the *EDA* gene.

It should be established whether the mutation described herein, or other mutations present within or in close proximity to the numerous response elements found in the regulatory region of this gene, could be responsible for the mispaired transcription of the *EDA* gene. This would require extensive sequence analysis of the regulatory region, its cloning into an expression vector in front of a reporter gene and the expression of the reporter gene in eukaryotic cells.

Further work is in progress to screen the regulatory region of the *EDA* gene for possible mutations in patients with anhidrotic ectodermal dysplasia to study the role of these sequences for the expression in *EDA* gene.

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