

## ORIGINAL ARTICLE

# Mutational spectrum in 101 patients with hypohidrotic ectodermal dysplasia and breakpoint mapping in independent cases of rare genomic rearrangements

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Hypohidrotic ectodermal dysplasia (HED), a rare and heterogeneous hereditary disorder, is characterized by deficient development of multiple ectodermal structures including hair, sweat glands and teeth. If caused by mutations in the genes *EDA*, *EDA1R* or *EDARADD*, phenotypes are often very similar as the result of a common signaling pathway. Single-nucleotide polymorphisms (SNPs) affecting any gene product in this pathway may cause inter- and intrafamilial variability. In a cohort of 124 HED patients, genotyping was attempted by Sanger sequencing of *EDA*, *EDA1R*, *EDARADD*, *TRAF6* and *EDA2R* and by multiplex ligation-dependent probe amplification (MLPA). Pathogenic mutations were detected in 101 subjects with HED, affecting *EDA*, *EDA1R* and *EDARADD* in 88%, 9% and 3% of the cases, respectively, and including 23 novel mutations. MLPA revealed exon copy-number variations in five unrelated HED families (two deletions and three duplications). In four of them, the genomic breakpoints could be localized. The *EDA1R* variant rs3827760 (p.Val370Ala), known to lessen HED-related symptoms, was found only in a single individual of Asian origin, but in none of the 123 European patients. Another SNP, rs1385699 (p.Arg57Lys) in *EDA2R*, however, appeared to have some impact on the hair phenotype of European subjects with *EDA* mutations.

*Journal of Human Genetics* (2016) 61, 891–897; doi:10.1038/jhg.2016.75; published online 16 June 2016

## INTRODUCTION

Ectodermal dysplasia (ED) is a large and heterogeneous group of congenital disorders affecting the normal development of ectoderm-derived structures such as hair, nails, teeth and eccrine glands.<sup>1,2</sup> Hypohidrotic ED (HED), the most common form of ED, is characterized by a triad of hypotrichosis, missing teeth and hypohidrosis, which can lead to life-threatening hyperthermia.<sup>3</sup> Furthermore, many patients with HED display some distinctive craniofacial characteristics including frontal bossing, sparse eyebrows, periorbital wrinkling and hyperpigmentation, prominent lips, protruding ears and peg-shaped teeth,<sup>4,5</sup> which may result in psychosocial problems.<sup>6,7</sup> Owing to the deficient development of eccrine glands, such as sebaceous, salivary, lacrimal, meibomian and submucous glands, many patients suffer from a very dry and eczematous skin, atrophic rhinitis, dry eyes and recurrent infections of the upper respiratory tract.<sup>4,8–11</sup> Affected females frequently report a reduced ability to breast-feed, referable to the malformation of mammary glands.<sup>12</sup> Breast development may be completely missing uni- or bilaterally.<sup>13,14</sup>

The highly conserved ectodysplasin A signaling pathway has a crucial role in the embryonic development of ectodermal structures. It starts with binding of ectodysplasin A1 (EDA1; encoded by the gene *EDA* on the X chromosome, MIM \*300451) to its receptor EDA1R (alternatively termed EDAR; encoded by *EDA1R* on chromosome 2, MIM \*604095), which leads to recruitment of the EDAR-associated

death domain adapter protein (EDARADD; encoded by *EDARADD* on chromosome 1, MIM \*606603). EDARADD interacts with a complex consisting of tumor necrosis factor receptor-associated factor 6 (encoded by *TRAF6* on chromosome 11, MIM \*602355), transforming growth factor-beta-activated kinase 1 (TAK1) and TAK1-binding protein 2. This results in activation of the inhibitor of nuclear factor kappa-B kinase (IKK) complex, comprising the IKK subunit alpha (IKK1), the IKK subunit beta (IKK2) and the nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) essential modulator (encoded by *NEMO* on the X chromosome, MIM \*300248). The active IKK complex induces phosphorylation of the I-κB complex (inhibitors of NF-κB alpha/beta) and its proteasomal degradation, which enables translocation of the transcription factor NF-κB into the nucleus and transcription of several target genes. One of them is *WNT10A* (MIM \*606268), mutations of which cause a partially overlapping phenotype of ED.<sup>15</sup> The second isoform of ectodysplasin A, EDA2, binds to the ectodysplasin A2 receptor (EDA2R, previously termed XEDAR; encoded by *EDA2R* on Xq12, MIM \*300276), that is assumed to lead to NF-κB activation similar to the 'classical' EDA1 pathway, although the exact role of EDA2R is not well understood.<sup>16–18</sup>

X-linked HED (MIM #305100), the most frequent subtype of HED, is caused by variations in *EDA*.<sup>19</sup> More than 200 different mutations have been published.<sup>20</sup> Most autosomal recessive (MIM #24900 and #614941) and autosomal dominant forms of HED (MIM #129490

and #614940) are due to mutations in *EDA1R*,<sup>21</sup> with more than 40 different mutations published, or in *EDARADD*,<sup>22</sup> with seven mutations known so far.<sup>14,20</sup> Single mutations in *EDA2R* and *TRAF6* have also been reported to cause HED.<sup>23,24</sup> Previous investigations of potential genotype–phenotype correlations revealed intra- and inter-familial variabilities, not solely explicable by the patients' mutations.<sup>25</sup> The single-nucleotide polymorphism (SNP) rs3827760 (c.1109T>C; p.Val370Ala) in *EDA1R*, a gain-of-function allele with a high frequency in the East Asian and Native American population, has been associated with increased hair thickness and shovel-shaped incisors<sup>26,27</sup> and was found to attenuate the severity of X-linked HED-related symptoms in a family of Asian origin.<sup>28</sup> The SNP rs1385699 (c.170G>A; p.Arg57Lys) in *EDA2R* with a high frequency in the European population (~70%), in contrast, has been associated with androgenetic alopecia.<sup>29</sup> The locally diverging allele frequencies of the closely related genes *EDA1R* and *EDA2R* may be the result of positive selection of certain phenotypes.<sup>30,31</sup> This study aimed at detecting disease-causing mutations and phenotypically relevant polymorphisms of candidate genes in a large cohort of European HED patients, including genomic characterization of rare copy-number variations.

## SUBJECTS AND METHODS

### Subjects

DNA samples from 68 male and 56 female patients with clinically evident HED (age range, 1–72 years) were investigated. All adults gave written informed consent to the use of their DNA for molecular analysis of HED-related genes and further genetic research; in the case of minors, parental consent was obtained.

### Standard mutation analysis

Specific primers covering the exons and intron–exon boundaries of *EDA*, *EDA1R*, *EDARADD*, *TRAF6* and *EDA2R* were designed using the online design and analysis tools ExonPrimer (<https://ihg.gsf.de/ihg/ExonPrimer.html>) and the *In silico* PCR tool from UCSC (<https://genome.ucsc.edu/>; specific primer sequences and thermal cycling conditions available upon request). Genomic DNA was extracted from blood samples using a commercial kit (QIAamp DNA Blood Mini Kit; Qiagen, Hilden, Germany), amplified by polymerase chain reaction (PCR) and analyzed by Sanger sequencing of individual genes. The Sequencher 4.8 software (Gene Codes Corporation, Ann Arbor, MI, USA; <https://www.genecodes.com>) was used for analysis of the electropherograms. All detected variants were assessed with the mutation prediction tools Mutation Taster (Charité, Berlin, Germany; Cardiff University, Cardiff, UK; <http://www.mutationtaster.org>) and NNSplice (Berkeley Drosophila Genome Project, Berkeley, CA, USA; [http://www.fruitfly.org/seq\\_tools/splice.html](http://www.fruitfly.org/seq_tools/splice.html)).

### Detection of exon copy-number aberrations by MLPA

Multiplex ligation-dependent probe amplification (MLPA) was performed with the commercial kit SALSA MLPA P183 *EDA-EDAR-EDARADD* probemix and SALSA MLPA EK1 reagent kit-Cy5 (MRC-Holland, Amsterdam, the Netherlands) according to the manufacturer's instructions. Fragment sizes were determined by capillary electrophoresis using a Beckman CEQ-8800 sequencer. Relative exon copy-numbers were calculated after normalization of the peak height value against the mean peak height value of a control group (at least two individuals of the same sex known to have normal gene dosage).

### Primer arrangement and long-range PCR in special cases

Detection of genomic breakpoints in patients with exon duplications required long-range PCR (LongAmp Taq DNA Polymerase, New England Biolabs GmbH, Frankfurt am Main, Germany), as the largest intronic region with a putative breakpoint had a size of more than 340 000 base pairs (bps). Assuming that the duplicated exons were in direct tandem orientation, we designed a series of forward primers binding within the intronic region after the last duplicated exon and a series of reverse primers binding to the intronic region preceding the first duplicated exon. In order to narrow down candidate regions of deletion breakpoints, we designed and combined a series of primer pairs

binding upstream and downstream of the deleted exons (specific primer sequences and thermal cycling conditions available upon request). For exact determination of the breakpoints within the fragments obtained, PCR products were sequenced as described above.

## RESULTS

### Mutational spectrum

Pathogenic mutations in *EDA*, *EDA1R* or *EDARADD* were detected in 101 patients, including 23 novel (Table 1) and 24 known (Table 2) disease-causing mutations. Most of them were found in *EDA* ( $n=42$ ), distributed over the regions encoding the intracellular domain, the furin cleavage site, the collagen-like domain and the tumor necrosis factor homology domain. Four novel mutations in *EDA1R* affected the extracellular and the death domain of the respective protein and one in *EDARADD* affected the region encoding the death domain. All variants absent from the Exome Aggregation Consortium control cohort have been included in the Leiden Open Variation Database ([http://databases.lovd.nl/shared/variants?search\\_owned\\_by\\_=%3D%22Sigrun%20Wohlfart%22](http://databases.lovd.nl/shared/variants?search_owned_by_=%3D%22Sigrun%20Wohlfart%22)).

### Genomic rearrangements affecting *EDA*

In all cases, where no disease-causing mutation could be identified in *EDA*, *EDA1R* or *EDARADD* by Sanger sequencing, MLPA analysis was performed to detect potential exon copy-number variations. This method revealed a hemizygous deletion of *EDA* exon 2 in one individual and hemizygous deletion of *EDA* exons 4–6 in another. Furthermore, three cases of hemizygous exon duplication within *EDA*, two affecting exon 2 and one affecting exons 3–8, became evident (Figure 1).

In order to confirm the results of this MLPA analysis with a non-quantitative method and for better understanding of the disease-causing recombination events, breakpoint mapping of the genomic rearrangements was performed. The recombination sites could be defined exactly in both cases of exon deletions and in the two cases with single-exon duplications, but not for the multiple-exon duplication. On the basis of our DNA-sequencing results, the deletion of exon 2 was designated as c.397–?\_502+?del (p.Met133AlafsX112), with a fragment of 91 bp, which may originate either from intron 1 or intron 2 because of their sequence homology at this position (Figure 2a). The two breakpoints are located within long interspersed nuclear elements, namely L1PA2 (intron 1) and L1PA3 (intron 2). The breakpoint region in case of the deletion of exons 4–6, c.527–3066\_793+1017del-ins8 (p.Lys177ValfsX17), additionally contains a small insertion of 8 bp (Figure 2b). One of the breakpoints lies closely to a microsatellite element consisting of a simple repeat of (CTCA) $n$  (intron 3). The two cases of exon 2 duplication leading to the same change of the amino-acid sequence (p.Gly168AspfsX10) were shown to be independent, as different breakpoints in the intronic regions were found. The first duplication, with one of the breakpoints situated within a region of low complexity because of its high frequency of adenine repeats (intron 1), was named c.397–5858\_502+3441dup (Figure 2c). The second one, named c.397–6070\_502+3112dup (Figure 2d), has its breakpoints within the in long interspersed nuclear elements L2a (intron 2) and L1PA3 (intron 1).

### Screening for the *EDA1R* variant rs3827760 (p.Val370Ala) and possible relevance of additional polymorphisms

The *EDA1R* variant rs3827760 was detected only in one of 124 individuals with clinically diagnosed HED. The hair phenotype (amount and color) of this male patient of Turkish (Asian) origin was rather normal for a Turkish boy.

**Table 1** The novel mutations detected in *EDA* and *EDA1R*

Gene	Mutation	Changes at the amino-acid level	Predicted effect	Number
<i>EDA</i>	c.64_71dup	p.Cys25AlafsX35	Truncated, dysfunctional protein (might also cause NMD)	4
<i>EDA</i>	c.397-5858_502+3441dup	Duplication of exon 2 (p.Gly168AspfsX10)	Shortened mRNA with premature termination codon	3
<i>EDA</i>	c.467_468del	p.Arg156GlnfsX2	Abolished furin cleavage; no functional protein	3
<i>EDA</i>	c.601G>T	p.Gly201X	Truncated, dysfunctional protein (might also cause NMD)	3
<i>EDA</i>	c.608C>T	p.Pro203Leu	Interrupted collagen helix, impaired multimerization	2
<i>EDA</i>	c.793G>T	p.Asp265Tyr/splice site modification	Altered splicing	2
<i>EDA</i>	c.935T>A	p.Ile312Asn	Impaired receptor binding	2
<i>EDA</i>	c.252del	p.Gly85AlafsX6	Truncated, dysfunctional protein (might also cause NMD)	1
<i>EDA</i>	c.376_379del	p.Asp126ProfsX10	Truncated, dysfunctional protein (might also cause NMD)	1
<i>EDA</i>	c.396+5G>A	Splice site modification	Altered splicing	1
<i>EDA</i>	c.397-6070_502+3112dup	Duplication of exon 2 (p.Gly168AspfsX10)	Shortened mRNA with premature termination codon	1
<i>EDA</i>	c.397-?_502+?del	Deletion of exon 2 (p.Met133AlafsX112)	Shortened mRNA with premature termination codon	1
<i>EDA</i>	no breakpoints detected	Duplication of exons 3–8	Unpredictable	1
<i>EDA</i>	c.527-3066_793+1017del-ins8	Deletion of exons 4–6 (p.Lys177ValfsX17)	Shortened mRNA with premature termination codon	1
<i>EDA</i>	c.542_577del	p.Gly180_Pro191del	Shortened collagen helix, impaired multimerization	1
<i>EDA</i>	c.707-13T>G	Splice site modification	Altered splicing	1
<i>EDA</i>	c.1009G>T	p.Glu337X	Truncated, dysfunctional protein (might also cause NMD)	1
<i>EDA</i>	c.1075A>T	p.Lys359X	Truncated, dysfunctional protein (might also cause NMD)	1
<i>EDA</i>	c.1112T>A	p.Ile371Asn	Impaired receptor binding	1
<i>EDA1R</i>	c.126del	p.Leu43CysfsX60	Truncated, dysfunctional protein (might also cause NMD)	3
<i>EDA1R</i>	c.486del	p.Ser163ArgfsX26	Truncated, dysfunctional protein (might also cause NMD)	3
<i>EDA1R</i>	c.1146_1149del	p.Leu383ArgfsX8	Truncated, dysfunctional protein (might also cause NMD)	2
<i>EDA1R</i>	c.1169del	p.Gly390AlafsX2	Truncated, dysfunctional protein (might also cause NMD)	1

Abbreviation: NMD, nonsense-mediated decay.

**Table 2** The previously described mutations detected in *EDA* and *EDARADD*

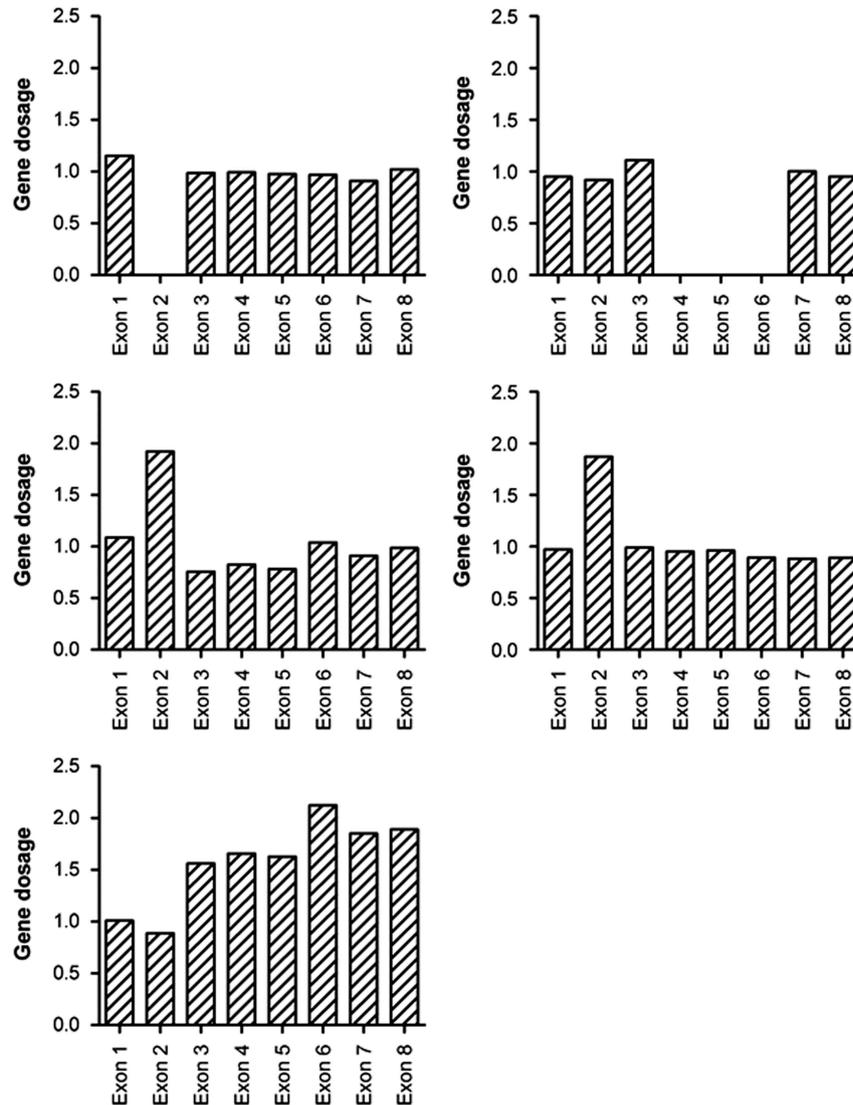
Gene	Mutation	Changes at the amino-acid level	Predicted effect	Number	Reference <sup>a</sup>
<i>EDA</i>	c.463C>T	p.Arg155Cys	Impaired furin cleavage	8	32
<i>EDA</i>	c.467G>A	p.Arg156His	Abolished furin cleavage	8	32
<i>EDA</i>	c.659_676del	p.Pro220_Pro225del	Shortened collagen helix, impaired multimerization	4	16
<i>EDA</i>	c.1133C>T	p.Thr378Met	Impaired receptor binding	4	33
<i>EDA</i>	c.502+1G>A	Splice site modification	Altered splicing	3	25
<i>EDA</i>	c.533_552del-ins5	p.Lys178_Pro184del-ins2	Shortened collagen helix, impaired multimerization	3	25
<i>EDA</i>	c.911A>G	p.Tyr304Cys	Impaired receptor binding	3	34
<i>EDA</i>	c.925-3C>G	Splice site modification	Altered splicing	3	35
<i>EDA</i>	c.1072C>T	p.Gln358X	Truncated, dysfunctional protein (might also cause NMD)	3	36
<i>EDA</i>	c.457C>T	p.Arg153Cys	Impaired furin cleavage	2	33
<i>EDA</i>	c.653G>A	p.Gly218Asp	Interrupted collagen helix, impaired multimerization	2	33
<i>EDA</i>	c.801A>G	Ser267Ser/splice site modification	Altered splicing	2	37
<i>EDA</i>	c.913_914ins2	p.Ser305IlefsX4	Truncated, dysfunctional protein (might also cause NMD)	2	38
<i>EDA</i>	c.1141G>C	p.Gly381Arg	Impaired receptor binding	2	39
<i>EDA</i>	c.466C>T	p.Arg156Cys	Abolished furin cleavage	1	32
<i>EDA</i>	c.467G>T	p.Arg156Leu	Abolished furin cleavage	1	11
<i>EDA</i>	c.546_581del	p.Asn185_Pro196del	Shortened collagen helix, impaired multimerization	1	32
<i>EDA</i>	c.572_589del	p.Pro191_Pro196del	Shortened collagen helix, impaired multimerization	1	33
<i>EDA</i>	c.574G>A	p.Gly192Arg	Interrupted collagen helix, impaired multimerization	1	40
<i>EDA</i>	c.648_665del	p.Pro220_Pro225del	Shortened collagen helix, impaired multimerization	1	36
<i>EDA</i>	c.784G>T	p.Val262Phe	Impaired receptor binding	1	41
<i>EDA</i>	c.1069C>T	p.Arg357Trp	Impaired receptor binding	1	42
<i>EDA</i>	c.1119G>A	p.Met373Ile	Impaired receptor binding	1	35
<i>EDARADD</i>	c.367G>A	p.Asp123Asn	Impaired ability to activate NF-κB signaling	3	14

Abbreviations: NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cell; NMD, nonsense-mediated decay.

<sup>a</sup>First report of the respective mutation.

We assessed various other SNPs that might be associated with phenotypic differences between HED patients (variants of intronic regions with no predicted influence on splicing excluded). In *EDA*, the

known polymorphism rs140642493 (c.458G>A; p.Arg153His) possibly affecting the furin cleavage site of the respective protein was detected only in a single individual. Many of our patients carried the



**Figure 1** Estimation of the *EDA* exon copy numbers by multiplex ligation-dependent probe amplification (MLPA) analysis in five patients with hypohidrotic ectodermal dysplasia (HED). The relative height of each individual probe peak, as compared with the relative probe peak height in various reference DNA samples, reflects the relative copy number of the corresponding exon (values significantly below 1.0 indicate a deletion and values significantly above indicate a duplication).

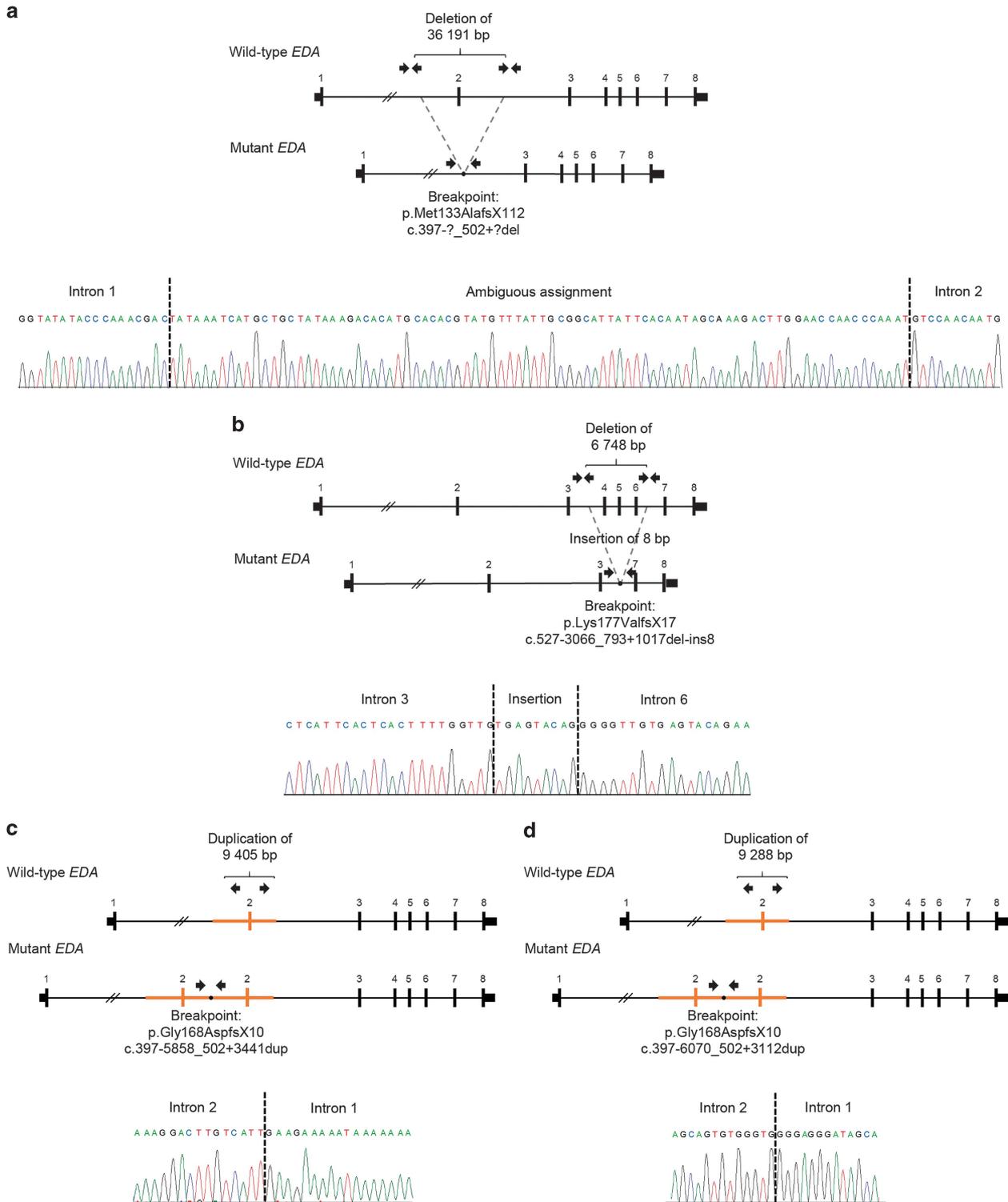
*EDAIR* variants rs260632 (c.750C>T) and rs12623957 (c.1056C>T), which seem to be irrelevant for the disease phenotype because they do not change the amino-acid sequence. Two polymorphisms of *EDARADD*, rs966365 (c.27G>A; p.Met9Ile) and rs604070 (c.369C>T), were found in our cohort, the latter of which without impact on the respective amino-acid sequence. Two more interesting polymorphisms, rs1385699 (c.170G>A; p.Arg57Lys) and rs1385698 (c.385A>G; p.Thr129Ala) of *EDA2R*, were detected in 86% and 100% of our male HED patients, respectively. Two patients without *EDA2R* rs1385699 had more and thicker hair than most other male subjects with HED.

## DISCUSSION

HED is a rare congenital disorder with high clinical variability. In this genetic study on a large cohort of HED patients, we detected 47 different disease-causing mutations, 23 of which have not yet been reported and therefore widen the mutational spectrum of HED. Their

distribution among *EDA*, *EDAIR* and *EDARADD* (88%, 9% and 3%, respectively) coincides with published data.<sup>20</sup> Most of the mutations are situated in evolutionarily conserved and functionally relevant domains. In conclusion, the intra- and intergenic distributions of these variants confirm the mutational hotspots known from the literature.

Most of our HED patients had missense, nonsense or splice site mutations, small deletions or duplications, except for five families with rare genomic rearrangements, namely exon deletions and duplications in *EDA*. To date, few cases of such exon deletions and only one case of exon duplication in *EDA* have been reported.<sup>13</sup> Genomic breakpoint mapping allowed us to confirm results of the MLPA analysis (a technique with an immanent risk of false-positive or -negative results) and to search for a common origin of variants in subjects with similar MLPA readouts. To our knowledge, this is the first report on breakpoints in *EDA* associated with exon duplication, whereas breakpoint characterization of an exon deletion in *EDA* has been



**Figure 2** Schematic illustration of *EDA* variants characterized by breakpoint mapping and sequencing chromatograms of the detected breakpoints. Primers used for (long-range) PCR are indicated by arrows, deleted parts by dotted lines. (a) The mutant form of *EDA* lacks 36 191 bp that normally include exon 2. (b) This *EDA* variant lacks 6748 bp that normally include exons 4–6. (c, d) Duplication of 9405 or 9288 bp (orange-colored) led to a second copy of exon 2 in the *EDA* variants.

published for a single individual.<sup>43</sup> At least one of the two breakpoints of each exon duplication or deletion was found within or very close to repetitive elements like in long interspersed nuclear elements or microsatellites. Such elements make up more than one-third of the

mammalian genome and are known to be frequently responsible for genomic rearrangements due to deficient replication.<sup>44,45</sup> Interestingly, neither our two cases of exon 2 duplication nor the case of exon 2 deletion and another one reported in the literature had any

breakpoints in common, which excludes the possibility of a common ancestor. Independent recombination events with repetitive sequence motifs as driving forces seem to be responsible for these exon copy-number aberrations. In case of the large duplication of exons 3–8, however, we failed to localize the genomic breakpoints exactly, although various primer combinations were tried. Not all duplications are in direct tandem orientation; they may also arise as an inversion or extragenic copy, which makes breakpoint mapping much more difficult. We suspect exon copy-number aberrations to be an underestimated cause of HED and recommend the routine performance of MLPA analysis in all laboratories offering molecular diagnosis of this disorder.

Although HED patients often have a quite similar appearance, deviations in the degree of severity are observed. Some genotype–phenotype correlations have been evident with respect to the number and function of sweat glands,<sup>41</sup> cutaneous signs and certain hair findings,<sup>25</sup> and also to the number of teeth.<sup>36</sup> Nevertheless, intrafamilial differences exist, which may be because of additional genetic factors, as already reported for the variant rs3827760 of *EDA1R* that seems to attenuate HED-related symptoms. In our cohort, HED patients with the *EDA2R* polymorphism rs1385699 appeared to have less and thinner hair compared with carriers of the wild-type allele, suggesting this variant to be an intensifier of HED-related hair issues. Nevertheless, further studies with larger numbers of participants are required to clarify whether there is a distinct association between the variant and phenotypes of hair and possibly other ectodermal structures. Furthermore, we suppose that more such gain-of-function alleles exist and that systematic mapping of all polymorphisms in genes relevant to ectodermal development will allow for clearer genotype–phenotype correlations.

Despite routine performance of MLPA analysis and screening for mutations in more than the usually investigated genes of the signaling pathway, we were unable to detect disease-causing mutations in a significant portion of our HED patients. Next-generation sequencing may offer better chances to establish a molecular diagnosis in such cases and may allow the detection of yet unknown variants in genes not considered so far.

Both a correct molecular diagnosis and more profound knowledge about genotype–phenotype correlations are required for valid assessments of the efficacy of a drug currently under investigation for use in newborn infants with HED ([www.clinicaltrials.gov](http://www.clinicaltrials.gov) NCT01775462 and NCT01992289).

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

We thank all patients and their families for participation in the study and Ida Allabauer for expert technical assistance. This work was funded partially by a grant from the German-Swiss-Austrian ectodermal dysplasia patient support group (to HS).

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