ORIGINAL ARTICLE

Sweating ability and genotype in individuals with X-linked hypohidrotic ectodermal dysplasia

Holm Schneider,[1] Johanna Hammersen,[1] Sabine Preisler-Adams,[2] Kenneth Huttner,[3] Wolfgang Rascher,[1] Axel Bohring[2]

[1]German Competence Centre for Children with Ectodermal Dysplasias, Department of Pediatrics, University of Erlangen-Nürnberg, Erlangen, Germany
[2]Institute of Human Genetics, University of Münster, Münster, Germany
[3]Edimer Pharmaceuticals Inc, Cambridge, Massachusetts, USA

Correspondence to
Professor Dr Holm Schneider, Children's Hospital, University of Erlangen-Nürnberg, Loschgestr. 15, Erlangen 91054, Germany; holm.schneider@uk-erlangen.de

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ABSTRACT

Background X-linked hypohidrotic ectodermal dysplasia (XLHED), the most common type of ectodermal dysplasia, is caused by *EDA* gene mutations. Reduced sweating contributes substantially to XLHED associated morbidity and mortality. To characterise the genotype—phenotype relationship, sweat gland function was assessed non-invasively in XLHED patients and healthy controls.

Subjects and methods In 36 genotyped XLHED patients and 29 control subjects aged 0—57 years, pilocarpine-induced sweat volume, palmar sweat pore density, and palmar skin conductance before and after stimulation were determined.

Results Among 31 XLHED males, 14 had neither detectable sweat pores nor inducible sweating, 10 showed a few sweat pores but absent sweating, and 7 produced reduced sweat volumes (1—11 μl) as compared with controls (38—93 μl). Two of the low sweating XLHED subjects had normal sweat pore counts. In all 5 heterozygous females, some sweat was detected, but generally less than in female controls. Basal and stimulated skin conductance readings were reduced in 23 of 24 non-sweating, but only in 3 of 12 low-sweating XLHED subjects. There was no correlation between sweat production and number of missing teeth.

Conclusions In contrast to prior reports on non-genotyped hypohidrotic ectodermal dysplasia populations, this study confirmed a consistent, quantifiable defect of sweat gland function in male XLHED subjects as a disease biomarker. Among 26 different *EDA* genotypes, specific mutations were shown to be consistently associated with anhidrosis, implying that systematic mapping of *EDA* mutations together with the analysis of objective clinical data may help to distinguish functionally crucial mutations from those allowing residual activity of the gene product.

INTRODUCTION

X-linked hypohidrotic ectodermal dysplasia (XLHED (MIM 305100)), the most common type of ectodermal dysplasia, is characterised by severe hypohidrosis, hypoplasia of sweat, sebaceous, submucous, meibomian and mammary glands, sparse hair and eyebrows, and oligodontia. The disease is caused by *EDA* (MIM 300451) gene mutations. Reduced sweating is assumed to be a major contributor to XLHED-associated morbidity and mortality.[1]

The *EDA* gene spans approximately 425 kb of genomic sequence on the human X chromosome and encodes ectodysplasin A, a trimeric type II transmembrane protein belonging to the tumour necrosis factor (TNF) superfamily of ligands. Eight transcriptional variants due to alternative splicing are known. The longest transcript consists of exons 1a, 3a, and 4—9 (nomenclature according to Bayés *et al*[2] and Monreal *et al*[3]), encoding the 391 amino acids of the ectodysplasin A1 isoform (EDA-A1), lack of which has been demonstrated to underly XLHED. EDA-A1 comprises a small N-terminal intracellular, a transmembrane, and a large C-terminal extracellular domain with a furine cleavage site, a collagen-like domain consisting of 19 Gly-X-Y repeats with a single interruption, and a TNF homology domain. To be able to bind to its receptor EDAR, EDA-A1 has to be released by proteolytic processing at the furin recognition site as a soluble homotrimerised protein consisting of the collagen-like and TNF homology domains. Further multimerisation through the collagen-like region has been shown[4] and seems to be important for protein function.

The phenotype of individuals affected by XLHED shows interfamilial as well as intrafamilial variability, and no clear genotype—phenotype correlation has been observed so far.[5–8] However, at least six *EDA* missense mutations causing a specific phenotype of non-syndromic selective tooth agenesis have been described.[4 9–13] Thus, one may hypothesise that the occurrence or severity of particular XLHED-related symptoms might correlate with the *EDA* genotype. Our present study, therefore, aimed at establishing quantitative skin assessment techniques to evaluate a genotype—phenotype relationship with respect to sweating ability.

SUBJECTS AND METHODS

Thirty-one male XLHED patients aged from 0 to 57 years and 24 healthy, age-matched male controls with no family history of XLHED or negative testing for an *EDA* mutation, as well as five heterozygous females and five healthy female controls, were studied. All adults gave written informed consent to participate; in the case of minors, parental consent and child assent in >7-year-olds was obtained. The study (http://clinicaltrials.gov/ NCT01109290) was approved by an independent institutional ethics committee and conducted according to national regulations and GCP/ICH (Good Clinical Practice/International Conference on Harmonisation) guidelines.

XLHED patients were included only if pathogenic *EDA* mutations had been detected and liquid

intake on the day of the study had been normal. Criteria for exclusion were acute febrile illness, implantable electronic devices, pregnancy or breastfeeding, and hypersensitivity to self-adhesive electrodes. Subjects were studied either at a German–Austrian bi-national family conference in Germany in May 2010 or during an appointment at the Competence Centre for Children with Ectodermal Dysplasias of the University Hospital Erlangen between May and August 2010. Subjects ED-Ch1 and ED-AS4, EDCh2 and ED-AS5, and ED-Ch11 and ED-AS7, respectively, have documented familiar relationships.

Medical and dental history was taken using a specific questionnaire, interviews, and orthodontic records of permanent dentition. Parameters determined included palmar sweat pore density in an area of 16 mm^2, sweat volume produced in a 30 min period subsequent to stimulation with pilocarpine using the Wescor 3700 device (Wescor Biomedical Systems, Logan, USA), and palmar skin conductance, an indirect measure of skin hydration,[14] taken with the Med-Storm 1001 device (Med-Storm, Oslo, Norway). The examiners were blinded to the genotype of the participant.

First, standard sweat collection was conducted at the forearm site, as an even surface area of 57 mm^2 was needed. Maximum volume that could be collected in the Wescor device was 93 μl (estimated by weight). Then, sweat pore density was determined on graphite prints of the palm, which had been enlarged 16-fold, and calculated per cm^2. Finally, palmar skin conductance measurements were recorded at baseline and following capillary blood sampling.

For genotyping, DNA was extracted from peripheral blood or buccal swabs (OmniSwab, Whatman International Ltd., UK) using standard procedures or following manufacturer's instructions, respectively. PCR was performed with specific primer pairs for exons 1a, 3a, and 4–9 selected with Primer3 and checked with MFOLD and SNPCheck. The PCR products were treated with ExoSAP-IT (USB Corporation, Cleveland, Ohio, USA) according to the manufacturer's instructions, and sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Carlsbad, California, USA) and a 3730 DNA Analyser (Applied Biosystems). Screening for larger deletions/duplications was done by multiplex ligation-dependent probe amplification (MLPA; MRC-Holland_P183, Netherlands) according to the manufacturer's instructions. To validate that any previously unreported nucleotide substitutions are real mutations, at least 100 healthy, anonymised, population-matched males were tested for each alteration by *EDA* sequence analysis.

Group comparisons, both between affected and control subjects and between adult and paediatric subsets, were done by Mann–Whitney and exact Fisher's tests using GraphPadPrism software version 4.0 for Windows (GraphPad Software, Inc, La Jolla, California, USA) or SPSS software version 17.0 for Windows (SPSS Inc).

RESULTS

Assessment and quantification of sweat gland function

As perspiration may change with puberty, which had also been observed in one of our previous studies on ectodermal dysplasia,[15] the XLHED cohort and the controls were divided into groups of children and postpubertal individuals (adults) with a cut-off of 16 years.

Individual data for each subject tested are shown in tables 1 and 2. As expected, control children had a significantly higher density of sweat pores than control adults ($p<0.001$), which is explained by the smaller body surface area of the children. However, control adults produced larger sweat volumes than control children ($p=0.0014$). When stimulated by pilocarpine, sweat glands of children did not equal the function of adult sweat glands, but seem to reach maturity with puberty.

One of the XLHED patients, ED-AS1, reported that despite being unable to sweat as a child, he had realised some sweating for the first time during puberty with further improvement thereafter and got to know a few regions of his body, including the margins of both palms, where he can sweat. However, XLHED children and adults did not differ in sweat volume, basal or stimulated skin conductance. Therefore, age groups were pooled where appropriate for further comparisons of XLHED and control subjects.

Control subjects had 455 sweat pores/cm^2 on average (range 294–900), which varied by age as expected, and a mean sweat volume of 72 μl following stimulation (range 29–93 μl). There was a clear demarcation between the XLHED males and controls across all measurements of sweat gland function ($p<0.0001$), that was less consistent in the females studied (table 3). However, in seven of 31 male adults and all female adults with XLHED some sweat was detectable by the Wescor assay. Dermatoglyphic investigation of the graphite prints revealed the complete absence or a significant lack of sweat pores and partial ridge dissociation in the 24 male XLHED patients who were unable to sweat (figure 1). Palmar skin conductance measurements in these 24 males typically showed both low baseline values and the absence of conductance increase (equivalent to absence of sweat gland function) following capillary blood sampling, whereas in control subjects a transient rise in palmar skin conductance indicated pain-inducible sweat gland function (figure 1). Among XLHED males (tables 1 and 2), 14/31 had neither detectable sweat pores nor inducible sweating, 15/31 showed a few sweat pores (6–113 cm^2) correlating with absence of sweat or low sweat volumes (up to 11.0 μl), and 2/31 had normal sweat pore counts but notably reduced pilocarpine-stimulated sweat volumes. In general, the number of sweat pores at the palm did not correlate with the ability to sweat. Although no adult male XLHED subject had more than 10 permanent teeth, there was also no correlation between the number of missing teeth and sweat production.

Basal skin conductance, an indirect measure of skin hydration, was reduced (<20.0 μS) in 23 of 24 non-sweating as well as in two of seven low-sweating XLHED males and in one of five XLHED females, but none of the controls. In healthy subjects, baseline values were lowest in the neonate and two adults known to have dry skin. Similarly, stimulated skin conductance following blood sampling was reduced (<7.0 μS) in 23 of 24 non-sweating XLHED males, two of seven with decreased sweating, one of five XLHED females, but only one of 28 controls. Typically, a relatively broad peak was observed within 2 s after the needle stick associated with capillary blood draw, the maximum of which was taken for the calculation of conductance increase. However, the pain threshold differed remarkably among individuals. The largest increase was observed in the control children, whereas athletic, tall men showed the weakest reaction. Despite large interindividual differences, the skin conductance measurements were reproducible and well tolerated by both age groups. This non-invasive technology, validated for use in newborns,[16] provided evidence of lack of sweat gland function even in a neonate (subject ED-Ch11) with a known family history of XLHED.

Genotype–phenotype correlations

Sequence analysis of the *EDA* gene in our XLHED cohort revealed a total of 26 different *EDA* mutations, 11 of which are

Table 1 *EDA* mutations and clinical symptoms in male children with X-linked hypohidrotic ectodermal dysplasia (XLHED) and age matched controls

Code	Age (years)	Body height (cm)	Weight (kg)	Resting heart rate (/min)	*EDA* mutation according to GenBank AF040628.1*	Amino acid substitution or deletion	Predicted effect of the mutation	Sweat volume, forearm (μl)†	Number of sweat pores, palm‡	Basal skin conductance at the palm (μS)	Conductance increase after stimulation (μS)
Male XLHED children, unable to sweat											
ED-Ch1	15	172	58	62	c.659_676del	p.P220_P225del	Shortened collagen helix	0.0	6	5.7	0.0
ED-Ch2	2	92	12	102	c.871G→A	p.G291R	Altered protein folding	0.0	0	16.0	0.0
ED-Ch3	8	125	27	80	**c.671G→T**	**p.G224V**	Interrupted collagen helix structure	0.0	0	12.0	0.0
ED-Ch4	7	123	25	85	c.871G→A	p.G291R	Altered protein folding	0.0	0	14.0	0.1
ED-Ch5	9	135	26.5	74	**c.831delC**	**p.T278LfsX2**	Truncated protein	0.0	6	7.5	0.0
ED-Ch6	9	137	24	84	c.463C→T	p.R155C	Abolished furin cleavage	0.0	0	6.3	0.0
ED-Ch7	3	95	13	104	c.467G→A	p.R156H	Abolished furin cleavage	0.0	0	9.0	0.0
ED-Ch8	14	170	50	73	c.467G→A	p.R156H	Abolished furin cleavage	0.0	0	6.9	0.0
ED-Ch9	12	143	28	78	**c.643G→A**	**p.G215R**	Interrupted collagen helix structure	0.0	6	4.0	0.0
ED-Ch10	0.5	65	6.9	118	c.911A→G	p.Y304C	—	0.0	75	2.4	0.0
ED-Ch11	0	51	3.1	148	**c.45_49del**	**p.P17GfsX81**	Truncated protein	0.0	0	4.7	0.06
Average	7.23	—	—	—	—	—	—	0.00	8.45	8.05	0.01
SD	5.28	—	—	—	—	—	—	0.00	22.24	4.30	0.03
Male XLHED children, able to sweat											
ED-ChS1	12	170	55	64	**c.826C→T**	**p.R276C**	—	5.0	356	41.0	44.0
ED-ChS2	11	135	25	74	c.206G→T	p.R69L		6.0	112	116.0	23.0
ED-ChS3	5	104	17	96	**c.896G→C**	**p.G299A**		1.5	12	19.0	0.8
ED-ChS4	9	143	33	73	**c.896G→C**	**p.G299A**		1.0	31	54.0	10.0
ED-ChS5	6	120	20.5	92	**c.527G→T**		Aberrant splicing, in-frame deletion	11.0	62	34.0	10.0
Average	8.60	—	—	—	—	—	—	4.90	104.2	52.80	17.56
SD	2.73	—	—	—	—	—	—	3.61	131.3	33.56	14.99
Controls (male children)											
C-Ch1	5	120	21	96	None	—	—	49.0	669	112.0	38.0
C-Ch2	14	170	55	74	None	—	—	55.0	575	29.0	24.0
C-Ch3	0	54	4.2	147	None	—	—	52.0	681	20.0	18.0
C-Ch4	5	125	23	90	None	—	—	56.0	606	40.0	95.0
C-Ch5	12	158	52	67	None	—	—	74.0	494	57.0	52.0
C-Ch6	12	150	50	84	None	—	—	38.0	481	65.0	54.0
C-Ch7	15	174	67	61	None	—	—	93.0	387	51.0	17.0
C-Ch8	0.5	73	8.1	123	None	—	—	64.0	900	32.0	14.0
C-Ch9	5	120	21.5	82	None	—	—	44.0	544	57.0	51.0
C-Ch10	3	104	17	86	None	—	—	48.0	369	46.0	37.0
C-Ch11	7	130	26.5	101	None	—	—	76.0	600	47.0	41.0
Average	7.14	—	—	—	—	—	—	59.00	573.3	50.55	40.10
SD	5.31	—	—	—	—	—	—	16.30	148.8	24.40	23.33

*Novel mutations in bold type.
†Upper detection limit=93 μl.
‡Within a 1 cm^2 area of the palm.

known from the literature[2–4 7 8 17–19] while 15 have not yet been described (tables 1–3). None of the missense or splice site mutations was present in a control group of 100 healthy males.

The 25 mutations detected in male XLHED subjects (figure 2) included 15 mutations expected to cause pronounced impairment of ectodysplasin A synthesis or release. All patients carrying gross deletions (exon 1, exon 3 or exons 4–9), small out-of-frame deletions (Pro17GlyfsX81, Thr278LeufsX2) or a nonsense mutation (W274X) were anhidrotic. Four *EDA* mutations altering a splice acceptor (c.527G→T, c.707-2A→G, c.707-2A→T) or splice donor site (c.793+1G→C) were identified, three of which also led to a severe phenotype without any sweating ability (subjects ED-A6, ED-A8, ED-A10). In addition, two small in-frame deletions detected in exon 5 (P220_P225del) and exon 9 (N372_S374del) were associated with anhidrosis. Among the 12 missense mutations found in this study, three are known to abolish furin cleavage (R153C, R155C, R156H). These mutations were discovered in non-sweating patients only.

The mutation G291R provided an unexpected example for clear genotype–phenotype correlation; all subjects with this mutation were unable to sweat under test conditions. Moreover, anhidrosis was caused by the mutation G2C within the intracellular domain of ectodysplasin A, by mutations G215R and G224V affecting its collagen triple helix, and by mutation Y304C within the TNF homology domain (figure 2).

On the other hand, all individuals with the missense mutations V262F, R276C or G299R, which are also situated in the TNF homology domain (figure 2), showed some residual sweating ability. Another missense mutation outside of a known functional domain (R69L) was associated with moderately reduced sweat gland function and a mild phenotype in general (short stature and low body weight, but dark hair, normal teeth, and a face indistinguishable from healthy children).

Interestingly, a small in-frame deletion in exon 5 (N185_P196del) was detected in subject ED-AS1 who was able to produce some sweat.

Table 2 *EDA* mutations and clinical symptoms in male adults with X-linked hypohidrotic ectodermal dysplasia (XLHED) and age matched controls

Code	Age (years)	Body height (cm)	Weight (kg)	Resting heart rate (/min)	*EDA* mutation according to GenBank AF040628.1*	Amino acid substitution or deletion	Predicted effect of the mutation	Sweat volume, forearm (μl)†	Number of sweat pores, palm‡	Basal skin conductance at the palm (μS)	Conductance increase after stimulation (μS)	Number of missing adult teeth§
Male XLHED adults, unable to sweat												
ED-A1	33	169	73	68	c.457C→T	p.R153C	Abolished furin cleavage	0.0	12	13.0	10.0	18
ED-A2	57	182	100	70	c.821G→A	p.W274X	Truncated protein	0.0	19	9.8	0.1	22
ED-A3	33	176	74	72	**c.4G→T**	**p.G2C**		0.0	56	28.0	6.0	21
ED-A4	43	174	75	77	c.457C→T	p.R153C	Abolished furin cleavage	0.0	0	9.1	0.7	19
ED-A5	44	176	85	80	c.457C→T	p.R153C	Abolished furin cleavage	0.0	56	14.0	0.2	24
ED-A6	18	180	74	70	**c.707-2A→G**	–	Aberrant splicing	0.0	6	5.4	0.0	22
ED-A7	36	174	97	74	**Exon4-9del**	–	Truncated protein	0.0	0	8.8	0.0	23
ED-A8	36	184	83	63	**c.707-2A→T**	–	Aberrant splicing	0.0	0	6.9	0.0	23
ED-A9	29	171	56	71	Exon3del	–	Truncated protein	0.0	37	5.5	0.0	25
ED-A10	33	170	60	76	**c.793+1G→C**	–	Aberrant splicing	0.0	0	8.6	0.0	24
ED-A11	43	178	107	80	Exon1del	–	No protein produced	0.0	0	3.0	0.0	26
ED-A12	56	170	75	77	c.871G→A	p.G291R	Altered protein folding	0.0	0	2.7	0.0	23
ED-A13	17	190	67	68	**c.1116_1124del**	**p.N372_S374del**	–	0.0	0	4.2	0.0	26
Average	36.77	176.5	78.92	72.77	–	–	–	0.00	14.31	9.15	1.31	22.77
SD	12.10	6.24	15.15	5.10	–	–	–	0.00	21.45	6.65	3.09	2.42
Male XLHED adults, able to sweat												
ED-AS1	35	175	59	71	c.553_588del	p.N185_P196del	Shortened collagen helix	2.0	400	46.0	13.0	28
ED-AS2	20	184	76	74	**c.784G→T**	**p.V262F**	–	1.0	94	16.3	1.8	18
Average	27.50	179.5	67.50	72.50	–	–	–	1.50	247.0	31.15	7.40	23.00
SD	10.61	6.36	12.02	2.12	–	–	–	0.71	216.4	21.00	7.92	7.07
Controls (male adults)												
C-A1	41	178	73	58	None	–	–	93.0	394	68.0	77.0	0
C-A2	56	178	93	69	None	–	–	93.0	312	23.0	22.0	0
C-A3	37	183	85	68	None	–	–	93.0	306	76.0	29.0	0
C-A4	39	185	86	65	None	–	–	90.0	506	42.0	23.0	0
C-A5	31	180	110	74	None	–	–	52.0	350	21.0	29.0	0
C-A6	34	183	82	72	None	–	–	93.0	294	29.5	18.0	0
C-A7	45	182	83.5	74	None	–	–	70.0	362	52.0	10.0	0
C-A8	26	184	85	68	None	–	–	93.0	400	56.0	10.0	0
C-A9	35	174	81	75	None	–	–	87.0	350	40.0	29.0	0
C-A10	48	188	78	80	None	–	–	93.0	319	29.0	5.0	0
C-A11	45	190	82	71	None	–	–	93.0	369	34.0	8.0	0
C-A12	23	170	67	72	None	–	–	93.0	425	41.0	32.0	0
C-A13	16	183	75	68	None	–	–	93.0	400	60.0	32.0	0
Average	36.62	181.4	83.12	70.31	–	–	–	87.38	368.2	43.96	24.92	0
SD	10.94	5.44	10.40	5.38	–	–	–	12.40	58.14	17.32	19.83	0

*Novel mutations in bold type.
†Upper detection limit=93 μl.
‡Within a 1 cm^2 area of the palm.
§Excluding third molars and extracted teeth.

Table 3 *EDA* mutations and clinical symptoms in female adults with X-linked hypohidrotic ectodermal dysplasia (XLHED) and healthy controls

Code	Age (years)	Body height (cm)	Weight (kg)	Heart rate (/min)	*EDA* mutation according to GenBank AF040628.1*	Amino acid substitution or deletion	Predicted effect of the mutation	Sweat volume, forearm (μl)	Number of sweat pores, palm†	Basal skin conductance at the palm (μS)	Conductance increase after stimulation (μS)	Number of missing adult teeth‡
Female XLHED adults												
ED-AS3	33	167	56	70	**c.449_456del**	**p.E150AfsX6**	Truncated protein	0.5	37	13.6	3.0	7
ED-AS4	41	158	70	68	c.659_676del	p.P220_P225del	Shortened collagen helix	11.0	362	47.0	27.0	2
ED-AS5	31	165	119	82	c.871G→A	p.G291R	Altered protein folding	43.0	219	38.0	10.0	13
ED-AS6	26	165	52	70	c.467G→A	p.R156H	Abolished furin cleavage	6.0	287	52.0	45.0	2
ED-AS7	30	164	54	80	**c.45_49del**	**p.P17GfsX81**	Truncated protein	3.0	300	34.0	7.5	6
Average	32.20	163.80	70.20	74.00	–	–	–	12.70	241.00	36.92	18.50	6.00
SD	5.54	3.42	28.18	6.48	–	–	–	17.38	124.84	14.85	17.38	4.53
Controls (female adults)												
C-A14	43	163	62	56	None	–	–	63.0	412	40.0	11.0	0
C-A15	29	160	51	72	None	–	–	61.0	350	38.0	17.0	0
C-A16	26	165	68	74	None	–	–	79.0	506	28.0	23.0	0
C-A17	43	167	68	68	None	–	–	73.0	375	20.5	7.1	0
C-A18	52	167	75	73	None	–	–	29.0	450	62.0	110.0	0
Average	38.60	164.40	64.80	68.60	–	–	–	61.00	418.60	37.70	33.62	0
SD	10.83	2.97	8.98	7.40	–	–	–	19.34	61.80	15.70	43.12	0

*Novel mutations in bold type.
†Within a 1 cm^2 area of the palm.
‡Excluding third molars and extracted teeth.

DISCUSSION

This study demonstrates that sweat collection following pilocarpine iontophoresis on the forearm can discriminate clearly between XLHED males and healthy controls. The sweat pore count also delineated affected males with high sensitivity (94% for children, 92% for adults), whereas skin conductance was less definitive as a diagnostic parameter. Such conductance measurements appear useful as a paediatric screening tool, but

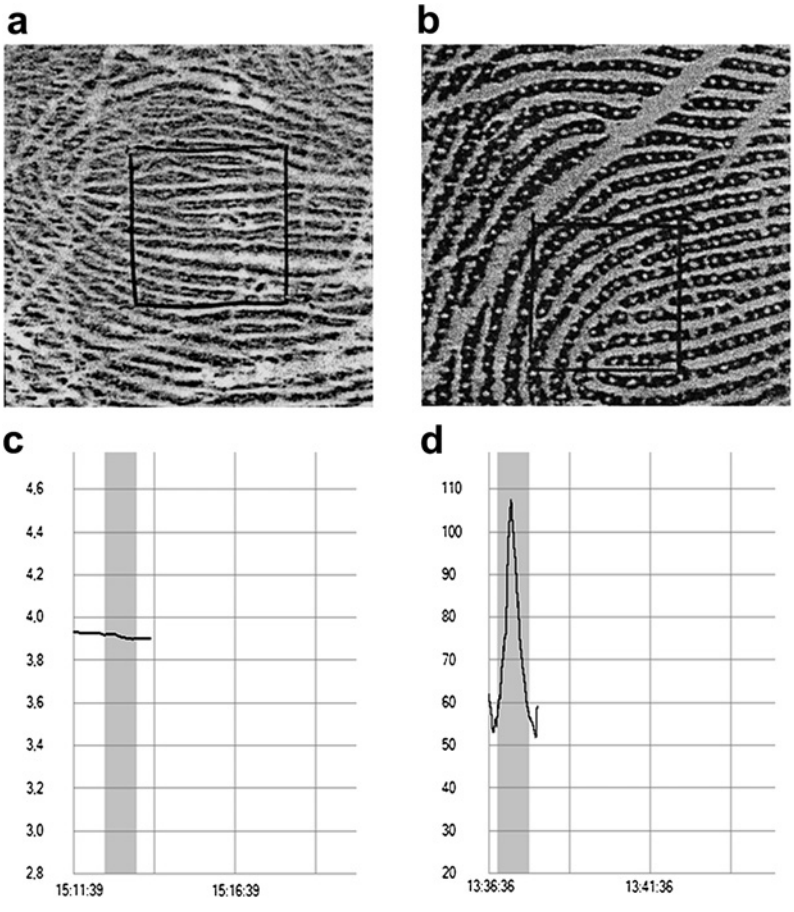

Figure 1 Examples of graphite prints and skin conductance measurements. Graphite prints of 16 mm^2 areas of the palm (outlined in black) from a boy with X-linked hypohidrotic ectodermal dysplasia (XLHED) (A) and a healthy control subject (B) were magnified 16-fold. Sweat pores can be recognised as circular white dots. Skin conductance measurements at the same site show significantly reduced baseline values and indicate absence of sweating after stimulation of the sympathetic nervous system in the XLHED patient (C). The time period of 60 s following such stimulation is indicated by grey colour. In the control subject, pain-induced sweating leads to a temporary rise in palmar skin conductance (D).

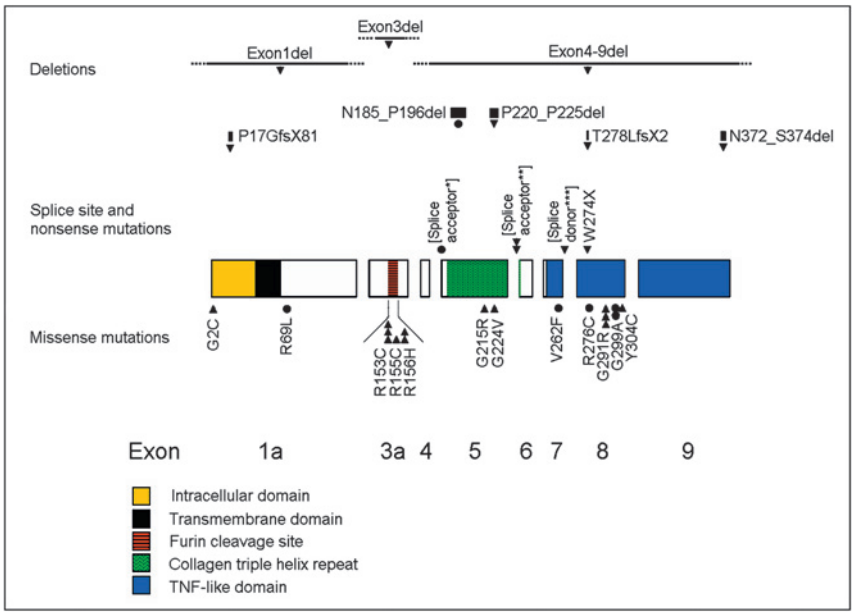

Figure 2 Distribution of *EDA* mutations among 31 male X-linked hypohidrotic ectodermal dysplasia (XLHED) patients. Numbers of triangles (▼▲) and circles (●) indicate numbers of patients with this mutation. Triangles are used for patients unable to sweat, circles for patients able to sweat. For exon deletions, exact breakpoints were not determined. The position and extension of smaller deletions is indicated by black bars. Predicted splice site mutations: *c.527G→T, **c.707-2A→G and A→T, ***c.793+1G→C.

have reduced sensitivity in detecting XLHED patients with residual sweat gland function.

Furthermore, the study identified deleterious mutations of the *EDA* gene resulting in most severe sweat gland dysfunction, whereas other mutations were found to be associated with residual sweating ability.

Sweating could not be induced in any male XLHED subject with an exon deletion, small out-of-frame deletion or nonsense mutation in *EDA*, for all of which premature termination of translation was predicted. Moreover, missense mutations affecting the furin recognition site of ectodysplasin A consistently resulted in anhidrosis. The altered protein sequence at Arg^{153}, Arg^{155} or Arg^{156} disrupts this recognition site and prevents proteolytic processing and release of the soluble ligand.[20] Two other functionally crucial missense mutations (Gly291Arg, Tyr304Cys) and an in-frame deletion (N372_S374del) were identified within the TNF homology domain. As the impact of various mutations on protein structure and receptor binding capacity has been shown in vitro to differ significantly,[4] it is noteworthy that all three subjects carrying the mutation Gly291Arg hemizygously were anhidrotic, suggesting that the affected amino acid may be essential for proper protein folding as already hypothesised.[4]

A severe phenotype without any sweating ability would also be expected in all four males with splice site mutations in exon 5 of *EDA* predicted to abolish the regular splice site using the BDGP splice site prediction tool (referenced at the end). Such mutations commonly result in an aberrant, non-functional protein due to out-of-frame exon skipping or use of alternative splice sites, respectively. However, only three of these four patients showed a severe phenotype. Surprisingly, subject ED-ChS5 who carries the putative splice site mutation c.527G→T was able to sweat. In this patient, two of the predicted alternative splice sites within exon 5, which codes mainly for the collagen-like domain, revealed a high acceptor score of 0.67 and 0.83 and would result in an in-frame deletion of three or 13 of the 19 Gly-X-Y repeats, respectively. Although this may explain the milder phenotype seen in this patient, prediction of phenotypic consequences of in-frame deletions within the collagen-like domain seems particularly difficult. FLAG-tagged recombinant EDA-A1 without the collagen domain (EDA-A1 E245) as well as a deletion mutant lacking four of the 19 Gly-X-Y repeats (EDA-A1 S160 Δ185–196) have been shown to bind to EDAR equally well in vitro, and the latter also did not affect multimerisation.[4] In line with this observation, patient ED-AS1 had a normal number of sweat pores and was able to produce some sweat. In contrast, multimerisation was not observed in vitro if the first half of the collagen domain was deleted (in frame), suggesting that certain mutations have specific impact on multimerisation.[4] This may provide an explanation for the different findings in subject ED-Ch1 with a small in-frame deletion within the collagen domain as well as for two of our non-sweating patients (ED-Ch3, ED-Ch9) who carry missense mutations altering glycine residues, which leads to an additional interruption of the Gly-X-Y repeats and might disturb protein tri- or multimerisation due to reduced solidity of the collagen helix.[3]

In patients with the missense mutations Val262Phe, Arg276Cys or Gly299Arg in the TNF homology domain, sweat gland function was affected only moderately. Although one of these mutations (Gly299Arg) is located within the most homologous motif of TNF ligands (Leu^{293}-Val^{309}),[21] a residual, probably tissue-specific receptor binding activity cannot be excluded and has already been demonstrated for other mutations within the TNF homology domain.[13]

The missense mutation Arg69Leu, situated outside of a known functional domain but close to the extracellular end of the transmembrane domain, led to a mild phenotype including some sweating ability which may, however, not be sufficient to preclude overheating. This mutation may reduce expression or translocation, and therefore dosage, of otherwise normal ligand, as proposed for two similar mutations (Arg65Gly,[22] Glu67Val[12]) associated with an oligosymptomatic phenotype (hypodontia).[13] However, the mutation Arg69Leu was also detected in patients with apparently typical XLHED,[4 7 19] making the prediction of its phenotypic consequences rather difficult.

In conclusion, we have shown that within the *EDA* gene gross deletions, splice site mutations expected to lead to exon skipping or use of alternative splice sites, nonsense mutations, missense mutations altering furin cleavage as well as mutations affecting specific amino acids of ectodysplasin-A (eg, glycine residues within the Gly-X-Y repeats or Gly^{291}) are associated with

Web resources

- The URLs for data presented herein are as follows:
  - BDGP splice site prediction: http://www.fruitfly.org/seq_tools/splice.html.
  - Genbank: http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide.
  - MFOLD: http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py?form=mfold.
  - Online Mendelian Inheritance in Man (OMIM): http://www.ncbi.nlm.nih.gov/Omim/.
  - Primer3: http://frodo.wi.mit.edu/primer3/input.htm.
  - SNPCheck: http://ngrl.man.ac.uk/SNPCheck/SNPCheck.html.

severely compromised sweat gland function, which is a major determinant of XLHED-associated morbidity and mortality. Genotype–phenotype correlation was seen across all measurements of sweat gland function, but, surprisingly, not with respect to the number of sweat glands (which can be determined very soon after birth) and the degree of oligodontia (diagnosable already by prenatal ultrasonography). This is important since such easily recognisable signs obviously do not allow prediction of the risk of hyperthermia and the associated morbidity and mortality in XLHED patients.

Our results imply that systematic mapping of *EDA* mutations together with the analysis of objective clinical data may help to distinguish functionally crucial mutations from those allowing residual, possibly tissue-specific activity of the EDA-A1 protein. Furthermore, these data might be useful to improve our understanding of ectodysplasin A function and signalling and to identify putative sites of interaction with other proteins.

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