Original article

Title: A recurrent missense mutation in the *EDAR* gene causes severe autosomal recessive hypohidrotic ectodermal dysplasia in two consanguineous Kashmiri families

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Running title: A RECURRENT MUTATION OF *EDAR* CAUSING HYPOHIDROTIC ECTODERMAL DYSPLASIA

Abstract

Background: Hypohidrotic ectodermal dysplasia (HED) is a rare congenital disorder arising from the abnormal development of ectoderm derived structures including skin, hair, nails, teeth and glands. These patients have sparse hair on the whole body including scalp and hypoplastic teeth. They have no resistance to heat due to abnormal sweat glands. A total number of four genes namely ectodysplasin A (*EDA*), ectodysplasin A receptor (*EDAR*), EDAR-associated death domain protein (*EDARADD*) and Wnt family member 10A (*WNT10A*) are known to be involved in the etiology of HED.

Methods: In the present study we have ascertained two consanguineous Kashmiri families (A &B) with autosomal recessive form of HED. By using whole exome sequencing and different bioinformatics tools we detected a recurrent mutation causing severe HED.

Results: We identified an already known rare homozygous missense (NM_022336 c.1300T>C; p.W434R; MAF 0.00007) variant in exon 12 of *EDAR* gene. This variant segregated with homozygous form in all patients, and their obligate carriers were heterozygous. A panel of >100

unrelated ethnically matched controls was screened, and the mutation was not identified outside the families. Furthermore, the candidate variant is predicted to be damaging by all in-silico software with CADD score of 25.5 this indicates that the variant is among the top 1% of the deleterious variants in the human genome.

Conclusion: Identification of the same homozygous mutation segregating with disease in two different families supports the gene's important role in the development of the disorder and it may contribute to novel approaches, prenatal diagnosis and genetic counseling of families with *EDAR* related disorders.

Key words: *EDAR*, Mutation, Hypohidrotic ectodermal dysplasia, Kashmiri Families

Introduction

Ectodermal dysplasia (EDs) is a term used to describe a large group of clinically and congenitally heterogeneous disorders characterized by developmental failure in two or more ectodermal structures involving alterations in hair, teeth, nails, or sweat glands. This group of disorders decreases the quality of life of patients [1-2]. There are other ectodermal structures that could be involved in ED, such as mammary glands, thyroid glands, thymus anterior pituitary, adrenal medulla, central nervous system, melanocytes, external ear, lacrimal gland and duct, conjunctiva, cornea, and meibomian glands [2]. The incidence of ED is ~7 cases per 10,000 live births [3].

More than 200 types of ED have been described [4] but the most common phenotype is the anhidrotic or hypohidrotic ectodermal dysplasia (HED, OMIM 305100). It is well-characterized ED subtype and a genetically heterogeneous disorder. The disease exhibits specific developmental abnormalities in the structures of ectodermal origin, hair, teeth, nails and eccrine glands [5-7].

HED can be inherited in an X-linked, autosomal dominant or recessive manner and four genes namely ectodysplasin A (*EDA*), ectodysplasin A receptor (*EDAR*), EDAR-associated death domain protein (*EDARADD*) and Wnt family member 10A (*WNT10A*) account for 90% of HED cases [8-9].

EDAR is a NF-kB-activating member of the tumor necrosis factor (TNF) receptor family that is required for the development of hair, teeth and other ectodermal derivatives. It is a type I transmembrane protein containing a cysteine-rich domain in the extracellular region as well as a potential death domain in its intracellular region. *EDAR* is activated by *EDA-A1* and uses *EDARADD* as an adaptor to activate the NF-kB signaling pathway, which contributes to ectodermal morphogenesis. This linear pathway explains the clinical homogeneity among HED patients displaying different modes of inheritance and the genetic heterogeneity of the disorder [10].

In this study, we performed clinical and genetic investigation on two consanguineous Kashmiri families containing a total number of fourteen patients with apparently autosomal recessive form of HED and identified a recurrent homozygous missense mutation (p.W434R) in exon 12 of *EDAR* gene segregating with the disease in both families.

Patients and methods

Subjects

Two consanguineous Kashmiri families A and B (Fig. 1) with autosomal recessive HED were investigated from two different villages of district Neelum Azad Jammu & Kashmir. Prior to the start of the study, approval was obtained from the University of Azad Jammu and Kashmir Institutional Review Board (a committee for ethics and research). An informed written consent was obtained from the affected and unaffected members of both families participated in the study. The family members rarely marry outside the community; consequently, consanguineous unions are common. Peripheral blood was collected from all the available affected and normal individuals. Phenol chloroform method was used for the extraction of genomic DNA for the further analysis.

Whole exome sequencing

We selected a total of 2 affected individuals (V-5 and VI-12) from family A (Fig.1A) and three affected individuals (V-2, V-5 and V-8) from family B (Fig. 1B), for whole exome sequencing. Target enrichment and library preparations were performed using SeqCap EZ Exome v3 kit (Roche Nimblegen). Libraries were barcoded and paired end (2x151 bp) sequencing was

performed on Illumina HiSeq 4000 platform. On an average each individual was sequenced to a mean depth of 51X and, ~94% of the target region covered by 10 or more reads.

NGS data processing and variant calling

Reads were mapped using BWA MEM algorithm (https://arxiv.org/abs/1303.3997) and variants were called using the GATK v2 Unified Genotyper and hard filtered following the recommended guidelines by GATK 'Best practices for variant calling v3. VCF files were annotated with ANNOVAR [11].

Variant prioritization

Since 90% of the disease is known to be caused by four genes *(EDA, EDAR, EDARADD* and *WNT10*) we first focused the analysis on the four genes only. With an assumption, causal variant(s) in these families are a very rare in general population and protein sequence altering, we followed following prioritization steps. Initially all the non-coding and synonymous variants were removed and in the next step all the variants were filtered and validated according to minor allele frequency (MAF) >0.001 in the Single Nucleotide Polymorphism database (dbSNP) , 1000 Genome Project, Exome Variant Server (EVS), Exome Aggregation Consortium (ExAC), genome aggregation database (gnomAD), ESP5400, ESP6500AA, ESP6500EA, gadexome and gadexome. Subsequently we focused only on homozygous variants shared among the affected individuals in the respective families.

Sanger sequencing

Sanger sequencing was performed to validate the candidate variant and its segregation in both families. Briefly primers were designed using Primer Z software [12] and amplified the variant encompassing region using PCR and bi-directionally sequenced using Big Dye termination kit V3.0 (Amersham Biosciences) and the ABI 3730 DNA analyzer (Applied Biosystem). The same oligonucleotide primers that were used for PCR amplification were also used in sequencing reactions. Alignments and sequence comparisons were carried out using the Bio Edit (V.7.2) software. We used the following primer sequences for the segregation analysis of candidate mutation in EDAR by polymerase chain reaction and Sanger sequencing**: Forward**: GTTGACCTTCTATTGACTGTG ; **Reverse:** CTGGAGCAGGGTGTCTGC

In-silico functional characterization of the prioritized variant

In-silico functional characterization of the prioritized variant was done by using these different tools: SIFT, Polyphen2, Mutation Taster, Mutation Assessor, FATHMM, VEST3, PROVEAN and CADD [13-17].

Results

Clinical report

Affected individuals from both families presented here have the characteristic clinical features of HED, including sparse hair, absent eyebrows and eyelashes, missing teeth, diminished sweating, dry and thin skin, prominent lips and saddle-shaped nose (Fig. 1 C-F). However, all the affected individuals have normal growth and development. Heterozygous carriers were clinically indistinguishable from unaffected normal individuals of the families.

Molecular findings

There were 71,632 variants identified in the initially analyzed variant call file of WES data. Out of these, 22 variants were present in those genes which had been previously known for autosomal recessive hypohidrotic ectodermal dysplasias (*EDA, EDAR, EDARADD* and *WNT10A*). They included 11 missense 10 synonymous and one splicing. After removing all the synonymous and common variants with (MAF, >0.01) we found only 5 variants in three genes *(EDAR, EDA and WNT10A)* (Table 1). Out of these five only one rare (MAF 0.00007) homozygous missense mutation (NM_022336 c.1300T>C; p.W434R) was shared in all the affected individuals of the respective families. The variant was further confirmed as a homozygous change with Sanger sequencing (Fig.1 G-H). Segregation analysis revealed that the mutation was homozygous in all affected siblings, heterozygous in parents and carrier siblings, and absent in other unaffected members of the both families. A panel of >100 unrelated ethnically matched controls was screened the mutation was not identified outside the families. Furthermore, the selected variant is predicted to be damaging by all in-silico software with CADD score of 25.5 this indicates that the variant is among the top 1% of the deleterious variants in the human genome.

Discussion

Hypohidrotic ectodermal dysplasia is characterized by varying degrees of abnormalities of teeth (anodontia or hypodontia), sparse hair (hypotrichosis or alopecia) and absent or reduced sweating (anhidrosis or hypohidrosis) that may lead to life-threatening hyperthermia. Four genes (*EDA1, EDAR, EDARADD,* and *WNT10A*) account for 90% of hypohidrotic/anhidrotic ectodermal dysplasia cases [1,8]. The phenotypes associated with mutations in genes *EDA1, EDAR,* and *EDARADD* are consistent and indistinguishable however, clinical expression of mutations in WNT10A gene is highly variable [18]. In the present study we investigated two unrelated Kashmiri families affected with HED. The clinical findings of the affected individuals are similar to those previously reported [19-22] including defective development of hair, teeth and eccrine sweat glands. By using WES followed by Sanger sequencing, we found a rare homozygous missense (c.1300T>C; p.W434R; MAF 0.00007) variant in exon 12 of *EDAR* gene. The same variant was previously reported in a Pakistani family by Shimomura et al however, the families under this study have no connection with Pakistani family having same mutation, and the amino acid residue 434Trp in the *EDAR* protein is evolutionally highly conserved from frog to human [23].

The *EDAR* gene is located on chromosome 2q11-q13 and contains 12 exons, encodes a protein which is a member of the NF-κB signaling pathway [24]. *EDAR* is expressed during early development and has a crucial role in the development of ectoderm derived structures such as the hair, teeth, glands, scales and nails [25-26]. During the development of ectodermally derived organs, *EDA-A1* is cleaved by a furin-like enzyme at the furin consensus recognition site, which leads to formation of the soluble extracellular molecule and activates *EDAR* through the interaction between the TNF homology domain of *EDA-A1* and the ligand binding domain of *EDAR* [27]. *EDAR* interacts with *EDARADD* via their death domains to activate the NF-kB signaling pathway therefore, mutations in any of these three pathway components result in identical phenotypic characteristics among patients [28].

In conclusion, the current study further supports the previous study by Shimomura et al in a Pakistani family. The study expands the catalogue of *EDAR* mutations causing HED. Since the various forms of HED are clinically indistinguishable, genetic testing is required to facilitate screening, prenatal diagnosis and risk assessment in affected families.

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Conflicts of interest

None

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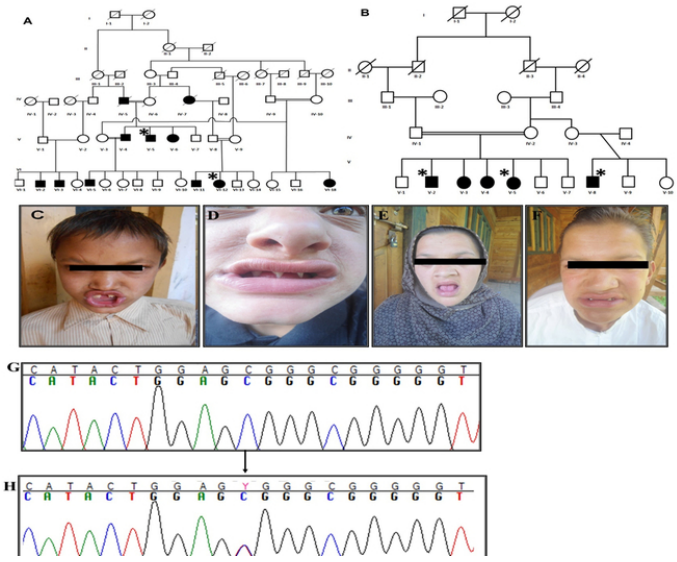
Figure legends

Figure 1: A and **B** indicate the pedigree of the both families; filled symbols indicate the affected individuals while unfilled represent the normal individuals of the families. * Indicate the individuals used for whole exom sequencing; **C-E** clinical presentation of the affected individuals of both families; **G**. Chromatogram of homozygous affected individuals**. H**. Chromatogram of the heterozygous career individual**.**

Table 1. Prioritization of variants in candidate genes (*EDA, EDAR, EDARADD, WNT10*) in both families

	Number of variants
Number of coding variants	71632
Number of variants in candidate genes already reported for HED (EDA, EDAR, EDARADD and WNT10)	22
Number of variants after removal of synonymous variants	12
Number of variants after removing the common variants with MAF(<0.01)	5
Number of variants shared among all the exom sequenced affected individuals in the respective families	1



Legend

338x190mm (72 x 72 DPI)