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ISSN 2544-1361 (online); ISSN 2544-2406
doi: 10.15584/ejcem.2020.2.3

http://www.ejcem.ur.edu.pl/en/
European Journal of Clinical and Experimental Medicine
Eur J Clin Exp Med 2020; 18 (2): 93–100

ORIGINAL PAPER

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Genetic study of a family with affected members with Waardenburg syndrome type 4 without Hirschsprung disease

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ABSTRACT

Introduction. Waardenburg syndrome (WS) is an autosomally inherited disorder with the most common state compounding pigmentary abnormality and sensorineural deafness. The rarest type of the disease is WS4 with the general characteristic discriminated from other types by the attendance of Hirschsprung disease (HD). Among the several genes, one of the causative genes in WS4 is endothelin 3 (*EDN3*) with both autosomal recessive and dominant inheritance.

Aim. The intention of the present study is to report a pathogenic mutation as the genetic cause of WS in an Iranian family with four patients without any segregation criteria for the type of the disease.

Material and methods. In order to detect of causing gene or genes related to the disease, Whole exome sequencing (WES) technique in proband's sample was done. To confirm the detected mutation in proband and some family members with or without the disease direct sequencing of *END3* gene was performed using Sanger method.

Results. Pedigree analysis suggested segregation of WS as an autosomal recessive trait in the family. WES analysis suggested a gene (*EDN3*) related to WS type 4B. DNA sequencing confirmed a pathogenic missense mutation c.293C>T, p.T98M in *EDN3* gene in all of the four patients.

Conclusion. Determination of WS can usually be missed owing to the lack of some attributes in every sufferer and also conventional clinical variance, in spite of several affected members in a single family. So, Genetic counseling is pivotal for families with multiple members influenced. We detected c.293C>T, p.T98K mutation in *EDN3* gene as a pathogenic variant which has been known as a likely pathogenic state in the American College of Medical Genetics and Genomics (ACMG) guidelines, despite one prior report. It will be helpful in genetic diagnosis of affected persons and increases the mutation spectrum of *EDN3* gene.

Keywords. *EDN3* gene, Waardenburg syndrome, WS4

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Participation of co-authors: A – Author of the concept and objectives of paper; B – collection of data; C – implementation of research; D – elaborate, analysis and interpretation of data; E – statistical analysis; F – preparation of a manuscript; G – working out the literature; H – obtaining funds

Received: 19.02.2020 | Accepted: 12.04.2020
Publication date: June 2020

Moghadam MS, Rayat S, Morovvati S. *Genetic study of a family with affected members with Waardenburg syndrome type 4 without Hirschsprung disease*. Eur J Clin Exp Med. 2020;18(2):93–100. doi: 10.15584/ejcem.2020.2.3

Introduction

Waardenburg syndrome (WS) is an infrequent autosomally inherited disorder with varying circumstance and a prevalence of 1 in 40,000 resulting from the nonexistence of melanocytes in the skin and the striavascularis of the cochlea.[1] Indeed, the anomalous proliferation, survival, migration, and distinction of pluri potent neural crest cells of the neural tube in the course of embryogenesis has been ascertained as the reason of the clinically disparate attributes in particular the absence of melanocytes of the skin and inner ear.[2] In general, WS is a sort of syndromic hearing loss which explicates 2-5% of the patients with congenital deafness and 0.9-2.8% of the deafness patients.[1] WS described by achromia (lack of typical pigmentation), such as depigmented speckles of the skin and hair (white forelock or white hairs and patches in other sites on the body), iris melanocytic malformation, bright blue eyes or Heterochromia irides, and sensorineural hearing loss (SNHL), most often non advancing, varying from slight to profound. Therefore, WS have been observed as a disease with variable penetrance.[3] WS is the most common state compounding pigmentary abnormality and sensorineural deafness. In the most of cases, bilateral ear affection turns up although in scarce cases it occurred unilateral.[4] Based on the further symptoms, it has been categorized into four subtypes, including WS1 (OMIM: 193500) with dystopia canthorum, WS2 (OMIM: 193510) without dystopia canthorum, WS3 (OMIM: 148820) with dystopia canthorum and extra musculoskeletal abnormalities, and WS4 (OMIM: 277580) with added Hirschsprung's disease (HD) (OMIM: 142623) or chronic intestinal pseudo-obstruction.[5] Among which WS1 and WS2 are the most common and WS4 is the rarest type with an incidence of <1/1,000,000 live born infant. WS4 (furthermore, known as Waardenburg-Shah Syndrome) with general characteristic including hereditary sensorineural hearing loss, Heterochromia of the eyes, white forelock, melanocytic deficiencies of the hair and skin is discriminated from other types by the attendance of HD.[6] Owing to the expression of various genes have been occurring the discrepancy in the medical appearance of the disease. So far, at the molecular status, six pathogenic genes with variable rates of occurrence are implicated; Paired box3 (*PAX3*) is connected with types 1 and 3, melanocyte inducing transcription factor *(MITF)* and snail family transcriptional repressor2 (*SNAI2*) with type 2, *EDN3* and endothelin receptor type B (*EDNRB*) with type 4, and SRY-box transcription factor10 (*SOX10*) gene with types 2 and 4. All six genes are involved in a complex interaction related to the function of melanocytes (pigment-producing cells). Dysregulation of them results in abnormal development of neural crest cells; whereby, changes in the pigmentation of the ears, iris, hair, and skin.[7] Based on the genetic causes, WS4 is classified into three subtypes: 4A, 4B induced by *EDNRB* and *EDN3* gene mutations with hereditary an autosomal recessive manner, individuals carrying homozygous mutations manifesting WS4 and those with heterozygous mutations in either gene presenting with isolated HD, and 4C with mutation in the *SOX10* gene that is inherited in an autosomal dominant manner. In truth, these genes are engaged in the formation and development of numerous kinds of cells, and are crucial constituents of a signaling cascade that controls the progression of melanocytes and the enteric nervous system.[8] Pathogenic variations in any of these genes disrupt ordinary development of melanocytes, altering pigmentation of skin, hair, eyes, and the normal functioning of the inner ear result in sensorineural hearing deterioration.[9] Endothelins (*EDN*) are a family of three active peptides *EDN-1*, *EDN-2*, and *EDN-3* that deed as ligands and exert their effects by binding to a G-protein heptahelical receptor known as endothelin receptors (*EDNR*) involved in the development of neural crest cells, which engenders the enteric nervous system and melanocytes. Two sorts of *EDNR* are determined; *EDNRB* which binds all three peptides and *EDNRA* which selectively binds *EDN1*. The significance of *EDN3*/*EDNRB* ligand/receptor interplay for the development of two varied cell lineages, melanocytes and enteric neurons, originate from the neural crest is well acknowledged.[10] Endothelin mRNAs are first translated into preproendothelin, which undergoes two step enzymatic cleavages, proteolytic cleavage of the prepro-endothelin by furin enzyme to release pro-endothelin and then converted into mature active by endothelin-converting enzyme (*ECE-1*) that produces the mature active endothelin peptide. This small peptide with 21 amino acidsis stabilized through the formation of two disulfide bonds between cysteine residues four contains cysteines involved in two disulphide bonds. [11]

Aim

The intention of the present study is to report a pathogenic mutation as the genetic cause of WS in an Iranian family with four patients without any segregation criteria for the type of the disease.

Material and methods

Clinical characteristics and family history

Two asymptomatic parents were referred to our Genetic laboratory for genetic counseling and diagnosis of their affected child, the first son of consanguineous healthy parents, who was a 2-year-old symptomatic Iranian boy with a profound congenital hearing loss, which was treated successfully with a cochlear implant, blue-colored irises, and other pigmentary deficiency; for instance, pigmentation on his face. His parents were healthy with typical hearing, eyes, and skin. Based on

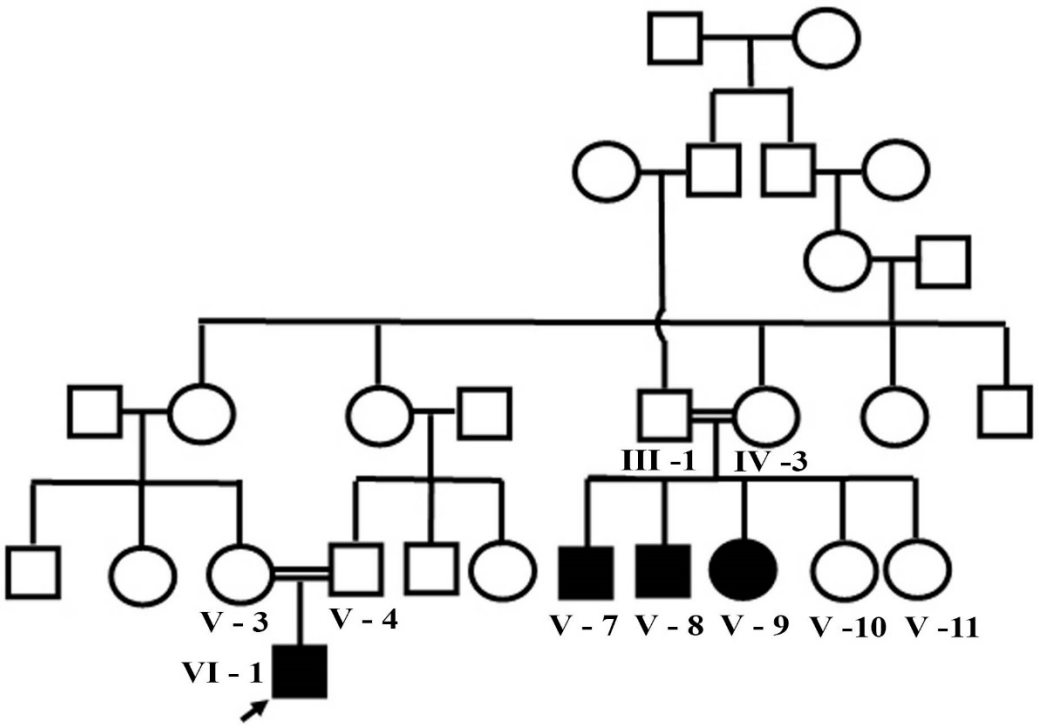


Fig. 1. Pedigree chart of the family with WS4. The black and unfilled shapes represent affected and unaffected family members, respectively; squares represent males and circles represent females; The black arrow indicates the proband (VI-1); V-3 and V-4 are the proband's parents; V-7, V-8 ,and V-9 another affected persons in the family in fifth-generation; V-10 and V11 unaffected sisters in 5th generation

the clinical/Para-clinical investigations the referring physician suggested WS as a possible diagnosis for him without any discriminate criteria for the type of the disease. According to the family pedigree, the affected child was born from a first cosines marriage. Also, there were multiple cases with the same presentations in the pedigree in the 5th generation of this family; two affected males and one female with exact same clinical symptoms compared to the proband, such as hearing impairment (Figure 1).

Each of these affected individuals was referred to a relevant physician for monitoring of clinical manifestations and other further investigations. After confirming the indications of the disease in all patients including a 25-year-old women with depigmented patches on her skin, white forelock, bright blue eyes and two 35 and 40 year-old man with equivalent symptoms (Figure 2); addition to, premature graying of the hair observed since birth. Relied on radiological examinations, none of the members in this family in particular the patients presented any colonic aganglionosis and similarly in accordance with the discriminator criteria of diverse types of WS, so four patients did not accord with the any especial type.

In general, the parents of the proband and the three other patients further their healthy sisters were participated in the study and whole blood samples were gained for next molecular investigations. Written informed consent was obtained from all of the individuals contributed in this genetic research.

Mutational screening

WES (Whole exome sequencing)

In order to detect of causing gene or genes related to the disease, WES technique in the proband's sample was done. For this purpose Genomic DNA was extracted from whole blood of him using standard protocols. Human whole exome enrichment was performed using Twist Human Core Exome Kit and the library was sequenced on the Illumina platform with a raw coverage of 199X and mean on-target coverage of 63X. Nearly all exons and flanking 10bp were detected and analyzed. Detected variations include single point mutations, and small insertions or deletions (indels) within 20bp.

PCR and Sanger sequencing

For corroboration of the detected variant in the proband and the inspection of it as a possible cause of the disease in other affected persons in the family, the revealed mutation was screened in parents of the proband; in addition, three affected persons and their two unaffected sisters by directly Sanger sequencing method. Genomic DNA was extracted from blood samples using a commercial kit (**GeneAll Exgen**$^{e\text{TM}}$), according to the manufacturer's protocol. For PCR amplification and direct sequencing of the detected gene two oligonucleotide primer

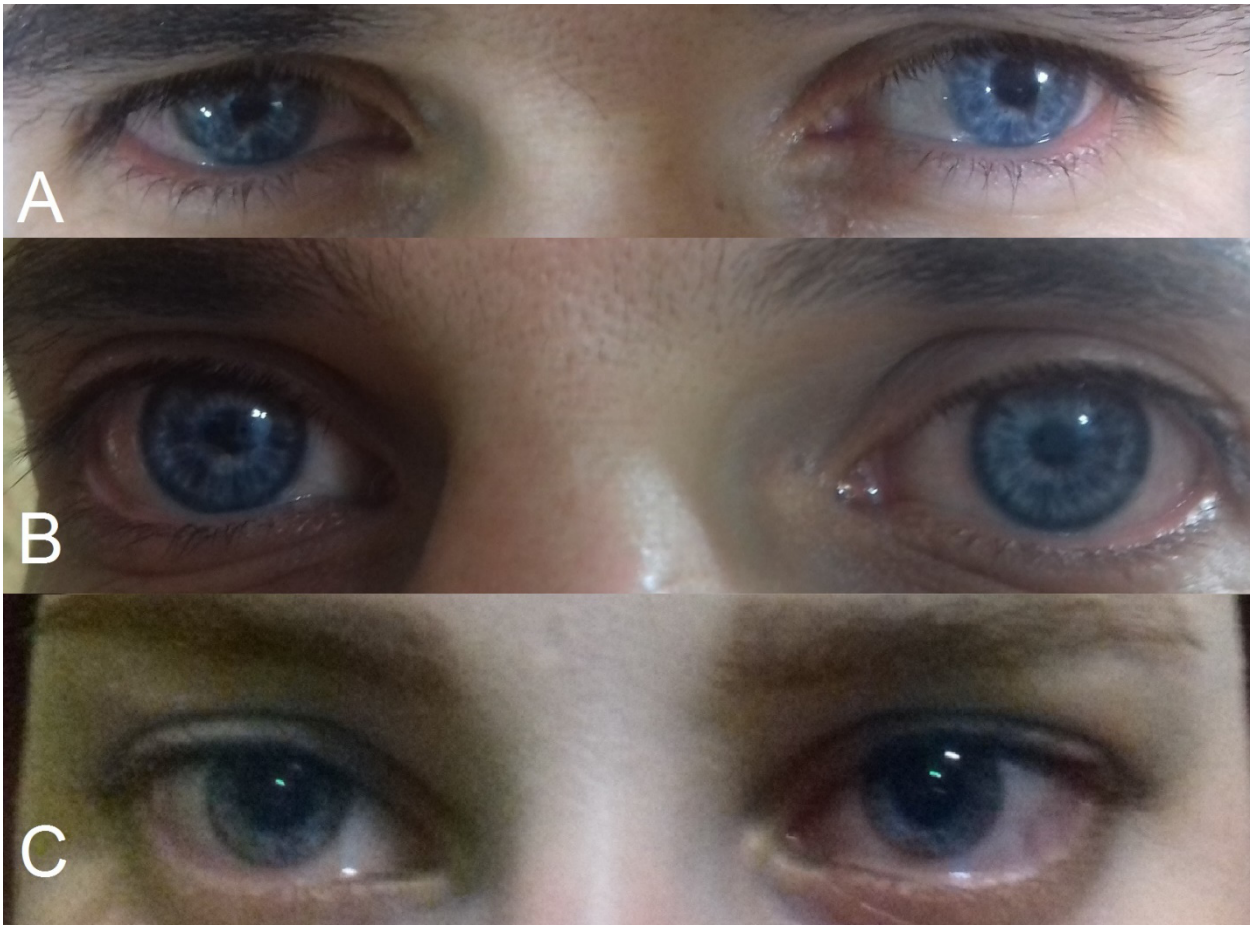


Fig. 2. Clinical features of the affected individuals with bilateral hearing loss and also bright blue eyes as the clearest characteristics. A, B, and C: Partial facial photograph of affected individuals V-7, V-8, and V-9

pairs were designed, including forward 5-'ATAGCTTG-GAAACTTTTCAGAAACTG-3' and Reverse5'-TG-GTCAGTTCACTTCATTCAGAG-3'. The amplification reaction was set in a total volume of 25 µl; containing 12.5µl PCR master mix (Yekta Tajhiz Azma, Iran), 1µl of each described primers, 8.5µl H2O, and 2µl DNA samples. The reaction was performed in initial denaturation at 95°C for 5 min, 35 cycles at 95°C for 30 sec, 60°C for 1 min and 72°C for 90 sec, and final extension step of 72°C was conducted for 7 min on a thermal cycler (Veriti, Applied Biosystems, USA). The PCR products were analyzed on 2% agarose gel before sequencing. The products sequenced on ABI 3500 Genetic analyzer. Subsequently, the similarities between the obtained sequences and the reference sequence were evaluated using the Nucleotide-Nucleotide BLAST (blastn) tool. Miscellaneous lines of in silico computational analysis (Mutation Taster, CADD, Poly phen, Varsome, SIFT, etc.) were made use to augur pathogenicity of the variation.

Results

WES was used to scrutinize all genes known to be responsible for hearing loss, including the WS pathogenic genes *PAX3*, *SOX10*, *MITF*, *EDNRB*, *EDN3*, and *SNAI2*, owing to the proband's clinical manifestations. Analysis of exome data, showed three variants as possible candidates that may explain the clinical history of the proband (Table1).

The detected heterozygous missense variant in tenascin C (*TNC*) gene has not been previously reported for its pathogenicity. Prediction of computational tools was conflicting. MutationTaster, CADD, and SIFT supported the deleterious effect of the variant on the gene or gene product(s), while Poly Phen has predicted it as tolerated/benign. The variant is absent in population databases (ExAC, 1000G, and our local database). Based on ACMG guidelines, this variant has been classified as a Variant of Uncertain Significance (VUS).

The detected heterozygous missense variant in tectorin alpha (*TECTA*) gene has not been previously reported for its pathogenicity. Prediction of computational tools was conflicting. MutationTaster and CADD has supported the deleterious effect of the variant on the gene or gene product(s), while SIFT and Poly Phen has predicted it as tolerated/benign. The variant has very low frequency in population databases (ExAC, 1000G, and our local database). Based on ACMG guidelines, this variant has been classified as a VUS.

Table 1. All data were derived from UCSC Genome Browser. Het: Heterozygous, Hom: Homozygous, OMIM number: Five-digit number assigned to each phenotype in Online Mendelian Inheritance in Man (OMIM) database. AR: Autosomal recessive, AD: Autosomal dominant, VUS: Variant of Uncertain Significance. Based on American College of Medical Genetics and Genomics (ACMG) standards and guidelines for the interpretation of sequence variants [1]

Gene / Transcript (RefSeq)	Variant Location	Variant	Chromosome Position	Zygosity[1]	Related Phenotypes	OMIM number	Inheritance Pattern	Variant[1] Classification
EDN3 NM_207034.3	Exon 2	c.293C>T p.T98M	Chr20: 57,876,705	Hom	Waardenburg syndrome-type 4B	613265	AR/ AD	Likely pathogenic
					Congenitalcentral hypoventilation syndrome	209880	AD	
TNC NM_002160.4	Exon 2	c.346G>A p.A116T	Chr9: 117,852,952	Het	Autosomal dominant deafness-56	615629	AD	VUS
TECTA NM_005422.2	Exon 7	c.1682G>T p.G561V	Chr11: 120,996,489	Het	Autosomal dominant deafness-8/12	601543	AD	VUS
					Autosomal recessive deafness-21	603629	AR	

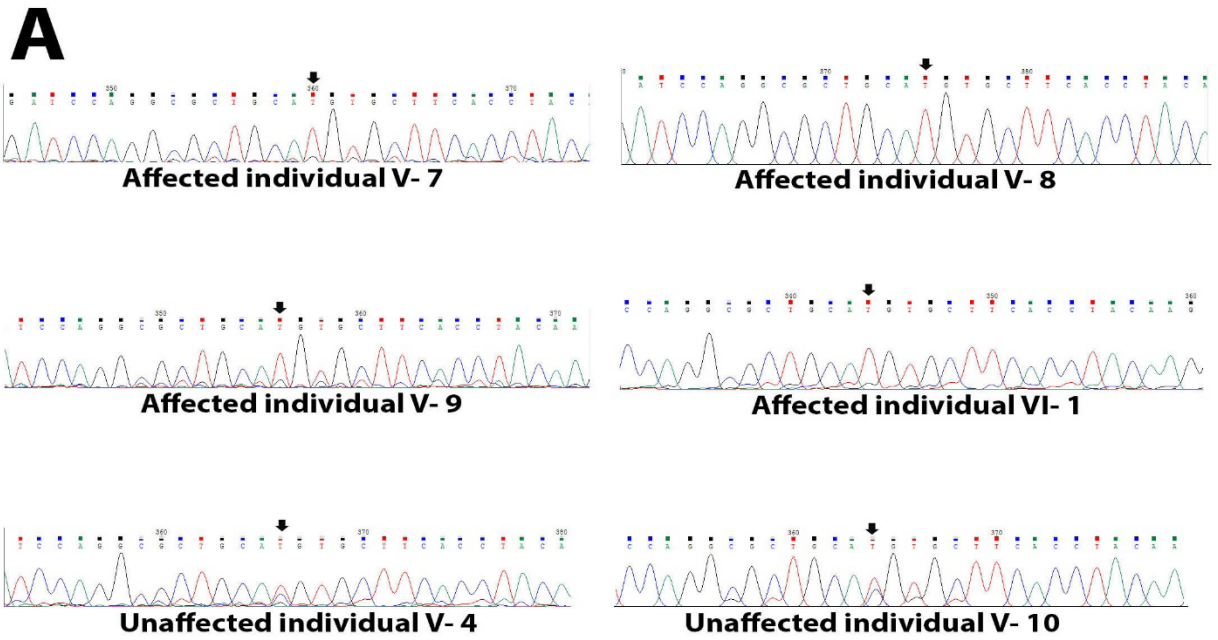
The detected homozygous missense variant in *EDN3* gene has been reported in Human Gene Mutation Database (HGMD) as a pathogenic variant. Moreover, the HGMD has reported another missense variant at this amino acid position (c.293C>A, p.T98K) as a pathogenic variant. Multiple lines of in silico computational analysis (MutationTaster, CADD, etc.) have been supported the deleterious effect of the variant on the gene or gene product(s). The variant is absent in population databases (ExAC, 1000G, and our local database). Based on ACMG guidelines, this variant has been classified as a likely pathogenic variant.

On the basis of the heterozygous status of detected variant in *TECTA* gene, this variant could not be explained the proband's deafness phenotype which had an autosomal recessive state. However, the possibility of large deletion/duplications and existence of a second causative variant in untested regions (introns, UTRs, etc.) of this gene could not be ruled out. On the other hand, *TNC* and *TECTA* genes were associated with autosomal dominant conditions, but analysis of detected variant in *EDN3* gene rejected the role of them resulting in their autosomal dominant conditions.

The same mutation, c.293C>T (p.T98M), on *EDN3* gene was detected in a homozygous state in the proband and three other affected members of the family, as a result of Sanger sequencing. The similar mutation, c.293C>T (p.T98M), on *EDN3* gene was detected in a heterozygous state in patients' Parents and also one sister of affected members in 5th generation of the family. Another sister was normal without any mutation in this variant of the gene (Figure 3).

Discussion

We are reporting a mutation in *EDN3* gene (causing gene in WS4) carried in the homozygous state by three men and one woman in a family. The observable assessing of the family suggested the WS disease as an autosomal recessive trait (based on family pedigree) and the molecular results revealed a likely pathogenic (Based on ACMG guidelines) homozygous substitution mutation, c.293C>T in exon 2, changing codon position 98 from threonine to methionine (p.T98M) in the proband (VI-1). It was possible to assume this variant as the causative variant in the proband and confirm diagnosis, but due to the likely pathogenic classification of the variant further examination (such as checking other members of the family) confirmed pathogenic nature of this variant. It could be used in definitive diagnosis. Furthermore, Genetic counseling and investigating the detected variant in *EDN3* gene in patients' parents and other members of his family/pedigree, especially affected members, were done. The affected family members (V-7, V-8, and V-9) were also homozygous for the mutation. Both parents of the proband (V-3/V-4) and other patients (III-1/IV-3) were heterozygous. Inspections in two normal siblings of three patients in fifth-generation (V-10, V11) showed the heterozygous variant in V-10 and wild-type allele in another sister. The c.293C>T change in *EDN3* gene were reported previously in an affected boy in an Indian family as a causing mutation of the WS4. The results of this research revealed a novel homozygous substitution mutation in *EDN3*. According to the Mutation Taster, the mutation was predicted to be disease causing with a p-value (probability) of 0.52. They also used two other in silico methods, PolyPhen-2 and SIFT, to see the effect of this mutation on the protein function. The effect of this mutation on *EDN3* was predicted to be probably damaging and intolerant with a score of zero (score ranges from 0 to 1, where 0 is damaging and 1 is neutral) and a score of 1 (score ranges from 0 to a positive number, where 0 is neutral, and a high positive num-



B

GCAGAAAGTATGCAAAATGCATGCAAGTTTTCAACGGGTGCAGATAGCTTGCAAGCTTTTCACAGAGTGT
GCAGATAGCTTGGAAACTTTTCAGAAACTGCGCAGAGCTTTGGAAACTTTGCAGACATTTTGCTTGCTCC
ACCCCCCTCCTCAGGTGTTTGGGGGTGGCGGGTGTTGAGGGGTCTGCACACTCAGCTTAGGAGCCCCTCA
ATCTGCCTTCTGCAGGATTCGTGCCTTGCTCCCAGTCTGGGGATGCTGGCAGGCGCGGCGTGTCCCAGGC
CCCCACTGCAGCCAGATCTGAGGGGGACTGTGAAGAGACTGTGGCTGGCCCTGGCGAGGAGACTGTGGCT
GGCCCTGGCGAGGGGACTGTGGCCCCGACAGCACTGCAGGGTCCAAGCCCTGGAAGCCCTGGGCAGGAGC
AGGCGGCCGAGGGGGCCCCTGAGCACCACCGATCCAGGCGCTGCACGTGCTTCACCTACAAGGACAAGGA
GTGTGTCTACTATTGCCACCTGGACATCATTTGGATCAACACTCCCGAGTAAGTCAGCCTTTTGTGGTGA
GGAACGTGGCTCCCGGACCAGGCCCACATCTGCTCATTCCCAGGAGGACCTCACTCCACCCCAGCCCCGC
TCAGTTAGCCCAGAGCCTGCCCTGGCACAGCCTTTAGCTCTTGCTGGGGCCTCTTGCCAGCCCAGGTCTC
TTCTGCAGGTCAGGGGTGTGACCCACATCCCCCCTGAGGGCCTGGCCCAAGAGAAACTCAATCCTGAGTT
TGATCCTGTGGAAGGAGGAGCCCTCCCTCTGAATGAAGTGAACTGACCAGGGGGATTAGATACCTGAGAT
GTCTCTCTGAGCCTCTTTTCTGAGATAGAGAAAGCCCCGAAAGTTTATCTCATTCCCCAGGGGCCAAGGG

Fig. 3. Mutation screening of the EDN3 gene in the family. A: Sequencing chromatograms of persons from the family; the homozygous change C>T in four affected individuals V-7, V-8, V9, and VI-1; the heterozygous change in two unaffected individual V-4 and V-10. Arrows indicate the position of the mutation. B: Sequencing of exon 2 of the EDN3 gene; Highlight shows primer designed for this region

ber is damaging) by SIFT and PolyPhen-2 analysis, respectively.[12] We are reporting second family with four affected persons with same mutation in this position of the *EDN3* gene. Our findings in multiple lines of in silico computational analysis (MutationTaster, CADD, etc.) have been supported the deleterious effect of the variant on the gene or gene product(s). But yet Based on ACMG guidelines, this variant has been classified as a likely pathogenic variant.

This family included four affected persons who have presented WS malformation. Genetic analysis confirmed *EDN3* mutation which is a causing gene in WS4; none of the patents had HD clinical manifestations, the hallmark of WS4, however, with other atypical clinical picture of the disease (deafness, pigment anomalies). And also five heterozygous persons in this family had no each of criteria effect resulting in the disease. The consequence of *EDN3/EDNRB* interaction for accurate development of neural crest-derived melanocytes and enteric neurons is well acknowledged.[13,14] Indeed, the genes connected with WS4 encode proteins that are indispensable components of a signaling cascade that supervises the progression of melanocytes and the enteric nervous system.[18] Pigmentation anomaly, aganglionic megacolon, and cochlear disorder in mouse models with homozygous mutations in the *EDNRB* or *EDN3* genes was reported in previous studies.[15] Heterozygous *EDN3* mutations have been known in patients with HD and homozygous *EDN3* mutations in patients with WS4. In one of the WS4 families heterozygous members for the *EDN3* gene C159F mutation had one or more WS characteristics, but without megacolon. This was contradictory with recessive inheritance of WS and with dominant mode of HD transmission.[11] Differentiation in the chronological arrangement and sites of distinctive subcategory appearance of cells originated from the neural crest could somewhat interpret the variable manifestations associated with *EDN3* and *EDNRB* mutation. Generally, variable penetrance and phenotypic variability are frequent in neurocristopathies.[16, 17] This could be explained by environmental factors, multigenic inheritance, modifier genes or by accidently incidents acting on cell differentiation in early embryogenesis.[18]It is worth mentioning, a heterozygous *EDN3* mutation in a severe case of Waardenburg-Hirschsprung disease confirms the difficulty in predicting the phenotypic manifestations of *EDN3* mutations and complicates genetic counselling.[15] On the other hand, a similar situation in mutations related to *SOX10* was observed. Most of these mutations generate premature stop codons and cause WS4 with or without neurological manifestations. The severity of the phenotype depends on the mutant mRNA's ability of escaping the nonsense mediated mRNA decay (NMD) pathway.[19, 20]

Conclusion

Determination of WS can commonly be missed because of all attributes are not found in every sufferer and clinical variance is conventional, in spite of several affected members of a single family. So, genetic counseling is pivotal for families with multiple members influenced. However, primitive diagnosis of the disease is based on the recognition of the clinical pictures, for the reasons mentioned it is normally confirmed by identification of a mutation in one of the disease-causing genes. Accordingly, its diagnosis has always been a challenging task for practitioners due to its scarcity and nonspecific presentation. Hence, further molecular scrutinizations are compulsory to attain a definite conclusion.

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