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A Novel Mutation in the Endothelin B Receptor Gene in a Moroccan Family with Shah-Waardenburg Syndrome

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Abstract
Waardenburg syndrome (WS) is a neurocristopathy disorder combining sensorineural deafness and pigmentary abnormalities. The presence of additional signs defines the 4 subtypes. WS type IV, also called Shah-Waardenburg syndrome (SWS), is characterized by the association with congenital aganglionic megacolon (Hirschsprung disease). To date, 3 causative genes have been related to this congenital disorder. Mutations in the *EDNRB* and *EDN3* genes are responsible for the autosomal recessive form of SWS, whereas *SOX10* mutations are inherited in an autosomal dominant manner. We report here the case of a 3-month-old Morrocan girl with WS type IV, born to consanguineous parents. The patient had 3 cousins who died in infancy with the same symptoms. Molecular analysis by Sanger sequencing revealed the presence of a novel homozygous missense mutation c.1133A>G (p.Asn378Ser) in the *EDNRB* gene. The proband's parents as well as the parents of the deceased cousins are heterozygous carriers of this likely pathogenic mutation. This molecular diagnosis allows us to provide genetic counseling to the family and eventually propose prenatal diagnosis to prevent recurrence of the disease in subsequent pregnancies.

Waardenburg syndrome (WS) is a rare neurocristopathy resulting from an abnormal migration or differentiation of neural crest cells during embryonic development. It is a clinically and genetically heterogeneous disorder with 4 subtypes. WS type IV (WS4), also called Shah-Waardenburg syndrome (SWS, OMIM 277580), is characterized by the presence of Hirschsprung disease (HSCR2, OMIM 600155) in addition to the common features of WS (deafness and pigmentary abnormalities). The endothelin B receptor gene (*EDNRB,* OMIM 131244) and the gene encoding its ligand, endothelin 3 (*EDN3,* OMIM 131242), were the first genes described to be associated with SWS [Baynash et al., 1994; Hosoda et al., 1994; Puffenberger et al., 1994], and their mutations are most frequently inherited in an autosomal recessive manner. They are involved in the endothelin signaling pathway which has an important role in the development of neural crest-derived cell lineages. Concerning the

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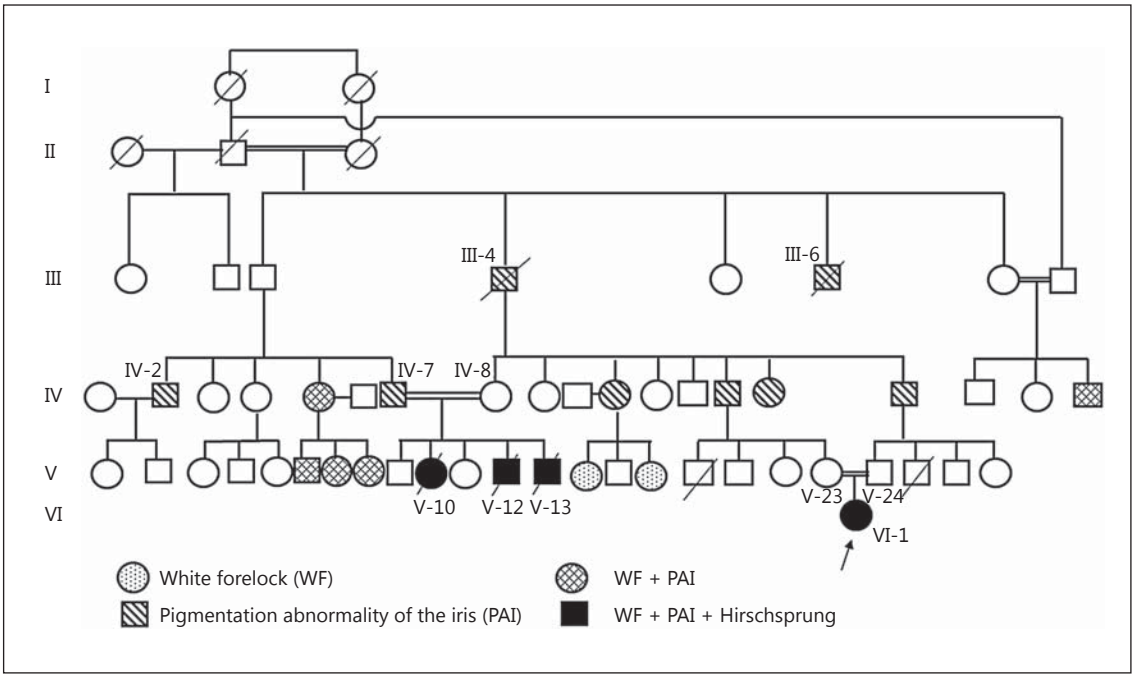

Fig. 1. Pedigree of the patient's family.

EDNRB gene, several mutations have been reported; these are mostly private mutations located throughout the protein [Pingault et al., 2010]. The *SOX10* gene (OMIM 602229) was also recognized as a SWS gene, responsible for the autosomal dominant form [Pingault et al., 1998]. It encodes the SRY-box 10 transcription factor involved in neural crest development. Mutations in this gene are also associated with a severe phenotype: PCWH syndrome (peripheral demyelinating neuropathy, central dysmyelinating leucodystrophy, Waardenburg syndrome, Hirschsprung disease) [Inoue et al., 1999, 2004; Pingault et al., 2000; Touraine et al., 2000].

Here, we describe a Morrocan patient with typical features of SWS who carries a novel mutation in the *EDNRB* gene.

Case Report

The propositus (VI-1) is the first child of healthy Moroccan consanguineous parents. She had 3 cousins who deceased at an early age with Hirschsprung disease and pigmentary anomalies (V-10, V-12 and V-13) (fig. 1). Eight members of the family have pigmentation abnormalities of the iris (III-4, III-6, IV-2, IV-7, IV-11, IV-14, IV-15, IV-16), 2 relatives have white forelocks (V-14, V-16) and 5 have a combination of both features (IV-5, IV-19, V-6, V-7, V-8) (fig. 1). Pregnancy and delivery were normal, and the child was born at term with normal physical measurements at birth. There was a failure to pass meconium within the first 48 h. Hirschsprung disease was confirmed and a colostomy was performed.

At the clinical examination, the proband was 3 months old with no dysmorphic features. The measurements were within the normal range: weight 5,200 g (30th percentile), height 59 cm (50th percentile) and OFC 38 cm (10th percentile). She had a frontal white forelock, an iris pigmentation abnormality with hypoplastic blue eyes and hypopigmented areas on the right forearm and the right lower limb. Both of the parents were normal on clinical examination.

The auditory evoked potential recorded at the age of 6 months objectified a bilateral profound sensorineural hearing loss requiring cochlear implant. At ophthalmological examination, the proband showed a depigmented retina. The temporal bone computed tomography revealed a subtotal filling of the hypotympanum and mesotympanum dishing in the external auditory meatus, thick appearance of the tympanic membrane respecting the wall of the stall and partial filling of the other mastoid. The cerebral MRI was normal. Based on these clinical findings, WS type IV was suspected. Informed consent was obtained from the proband's parents prior to genetic testing. Peripheral blood samples were collected from the affected child and her parents. DNA was extracted by standard methods.

To search for point mutations, the coding exons of *EDN3, EDNRB* and *SOX10* genes were analyzed by direct sequencing of the PCR products using the Sanger method. Quantitative multiplex fluorescent (QMF) PCR was used to detect rearrangements and copy number variants. *SOX10* was analyzed as previously described [Pingault et al., 2013], while the *EDN3* and *EDNRB* genes were amplified using the primers listed in table 1.

Table 1. Sequence of oligonucleotide primers used to amplify *EDN3* and *EDNRB* genes for sequencing and for QMF-PCR in a multiplex reaction with FAM-labeled reverse or forward primers

Gene and exons	Forward sequence	Reverse sequence
For sequencing		
EDN3 ex1	GGTGGTGCAGAAGCCAGAAA	TCCCCCAGGCGTCTTCACGA
EDN3 ex2	AGACATTTTGCTTgCTCCACC	GGGCAGGCTCTGGGCTAACT
EDN3 ex3	GTTCTCGCTCCACACCCTTG	ATCCTACACCCTCCTTTGAG
EDN3 ex4	GCCTGAGACGCAGTCCTTG	TGCCCCCAGAAACGGTCCAC
EDN3 ex5	CAATCAGGGAACAGGCTGGA	TAAGTGGGGACTCTTTGGGT
EDNRB ex1	AGCGTGGATACTGGCGAAGA	CTTTTAGGAGGGGCAGAACC
Additional internal primers (ex1)	GAGGCTTCCCGCCTGACAGG	TGGCACGGGGGAGGGGAGAT
EDNRB ex2+ex3	AAGTGATACAATTCAGAGGGCA	CACAGTCCTTGGATCTATACTC
EDNRB ex4	AACACATTGTCTTAGAGAACTGA	GAAGTGGAACCGAAGTGACTA
EDNRB ex5	TCACTTCGGTTCCACTTCAC	CTCTCAACAGGACCTCAGAT
EDNRB ex6	AGACAGAGACAGGCAGAGAA	TGGCTGACTAGGATTTATAGG
EDNRB ex7	AAAGTCAGAACCCTGGAGAG	CTTTCACGACGAGGCTTTCTT
For QMF-PCR with FAM-labeled reverse primers		
EDN3 ex1	CAAGCGGCCGTCCTCCTGGT	FAM-GTCCCCCGCCCTGGGTCCTT
EDN3 ex2	TCTGCACACTCAGCTTAGGA	FAM-CGGGAGCCACGTTCCTCACC
EDN3 ex3	GTTCTCGCTCCACACCCTTG	FAM-CTGGCCTTGCCGAGGGTTGA
EDN3 ex4	GCCTGAGACGCAGTCCTTGG	FAM-CTGGACCAGACCAGATGCCA
EDN3 ex5	CTACAGAGCTACACTTTCAT	FAM-GAACTGTGTGTGAGCAATGA
EDNRB ex1	GAGACAGGACGGCAGGATCT	FAM-ATCTCCCCGTCTCCAACCAG
EDNRB ex2	AAGTGATACAATTCAGAGGGCA	FAM-TTCTAAGTAACATGGAAAACAA
EDNRB ex3	ATGCCAGCTTAAAATACAATTC	FAM-GGCAAGAGCAGAAAGGAAAA
EDNRB ex4	GTTTAACATTTGTTATATAAGATTTT	FAM-TATAAATTCAACCACGAGTTATC
EDNRB ex5	GAGCCATCTTTTAAGGGTCA	FAM-CTGAGTGGCATTTATTTACAAA
EDNRB ex6	GTTAGCAAAGACGAGTGATA	FAM-GATGTAATAAAAGGGAAACTA
EDNRB ex7	AAAGTCAGAACCCTGGAGAG	FAM-TTTAATGACTTCGGTCCAATA
Internal controls for QMF-PCR with FAM-labeled forward primers		
F9	FAM-AAATGATGCTGTTACTGTCTA	GAAGTTTCAGATACAGATTTTC
DSCR1	FAM-GCGACGAGGACGCATTCCAA	GTCCTTGTGCGATCACCACA

This led to the identification of a homozygous nucleotide substitution within exon 6 of the *EDNRB* gene, c.1133A>G (Chromosome 13:77899920), as the sole putative causative variation. This variation predicts the replacement of asparagine by a serine at amino acid position 378 of the protein (p.Asn378Ser), which is located in the 7th helical transmembrane domain. The parents carried the same variant in a heterozygous state (fig. 2). Samples of the deceased cousins were not available, so we tested their parents who were shown to be also heterozygous for this mutation. The variant was not found in the 1000 Genomes database (http://www.1000genomes.org/), nor in the Exome Variant Server (http://evs.gs.washington.edu/EVS/). Finally, we excluded the possibility that it is a frequent polymorphism in Morocco by screening 200 Morrocan control chromosomes for this nucleotide substitution by real-time PCR (StepOne™ Real-Time PCR System) using TaqMan® probes.

In silico Analysis

Effects of the sequence variation were predicted by using the PROVEAN [Choi et al., 2012], PolyPhen-2 [Adzhubei et al., 2010] and SIFT [Ng and Henikoff, 2001] web-based platforms. PolyPhen-2 suggested that the Asn378Ser mutation was 'probably damaging', the SIFT algorithm and PROVEAN considered the mutation as damaging and deleterious, respectively. The Grantham matrix score for Asn→Ser is 65 [Grantham, 1974].

Discussion

SWS is defined by the association of pigmentary abnormalities involving skin, eyes and/or hair, sensorineural deafness and Hirschsprung disease [Shah et al., 1981]. Its prevalence is low, but not precisely defined (1/40,000 for WS as a whole), and about 50 cases have been reported in Europe (http://www.orpha.net/orphacom/cahiers/docs/GB/Prevalence_of_rare_diseases_by_alphabetical_list.pdf). This congenital developmental disorder is clini-

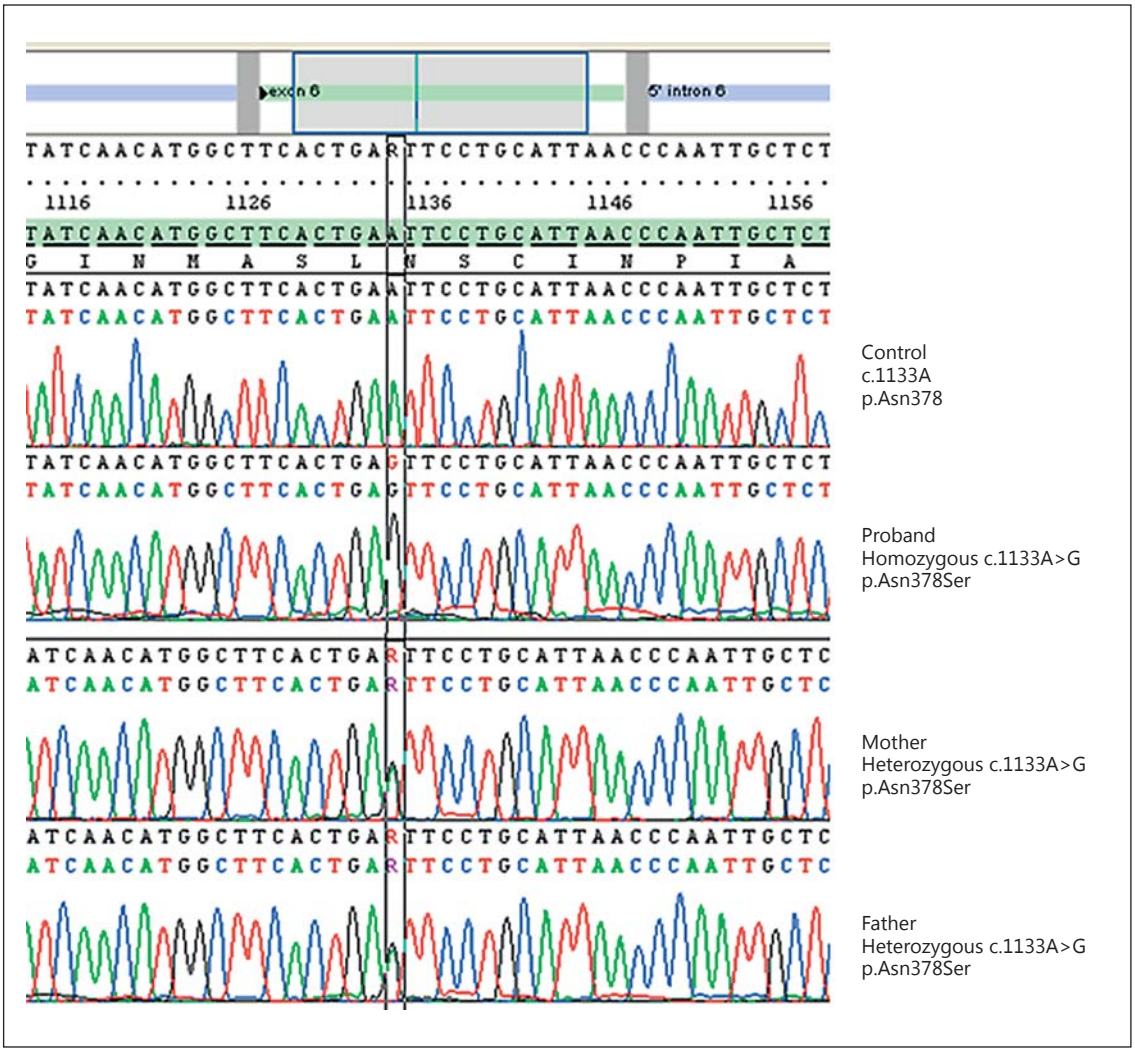

Fig. 2. Electropherogram of the patient and her parents showing the mutation in the *EDNRB* gene.

cally and genetically heterogeneous. It is caused by either homozygous or heterozygous mutations in 1 of the 3 genes: *EDN3*, *EDNRB* and *SOX10.* All are implicated in the proliferation, migration and differentiation of neural crest cells. However, 15–35% of SWS cases remain unexplained at the molecular level, suggesting the involvement of other genes [Pingault et al., 2010]. Mutations in *EDNRB* or *EDN3* genes are found in 20–30% of SWS patients.

The *EDNRB* gene, located in 13q22.3, spans 24 kb and comprises 7 exons. Each intron occurs near the border of the putative transmembrane domain in the coding region [Arai et al., 1993]. Until December 2013, 24 *EDNRB* mutations were reported to be associated with SWS (http://grenada.lumc.nl/LOVD2/WS), and they are located throughout the protein. About half of them are missense mutations, the rest being truncating mutations or full-gene deletions. In the present report, we identified a homozygous variant within exon 6 of the *EDNRB* gene where the adenine was replaced by guanine (c.1133A>G). This change leads to the substitution of the asparagine-378 residue with a serine residue, and it is located in the 7th transmembrane domain of the endothelin B receptor. Asparagine 378 is fully conserved between species, as well as between type A and type B endothelin receptors (fig. 3). This amino acid substitution does not change the charge or polarity, but replaces a hydroxyl by an amine group. As very few functional tests have been

Fig. 3. ClustalW alignment of the amino acid sequence of EDNRB in different species (upper panel) as well as with EDNRA in humans (lower panel). The transmembrane domain is in bold letters, and the non-conserved residues are highlighted in grey. The p.Asn378 is framed.

performed so far on this protein, it is difficult to predict whether this specific change destabilizes the transmembrane domain or impairs its function. According to the few references, a discussion on this subject would be very speculative for this mutation. The combined observation of its absence in 200 Moroccan control chromosomes and of its linkage with the disease in a distinct branch of the family strongly suggests that this missense change is the causative mutation in this family.

For the *EDNRB* (as well as *EDN3*) gene mutations, there is no obvious phenotype-genotype correlation. Most mutations are private with intra- and interfamilial variability [Pingault et al., 2010]. Individuals carrying homozygous mutations have SWS, while individuals with heterozygous mutations in the same family may either be asymptomatic or have some features of the disease. In the present report, the family members with pigmentary disturbances are likely heterozygous, as it is the case for the cousins' father (IV-7) and for several obligate carriers of the mutation (IV-5, IV-11). Then, the disease in this family appears as semi-dominant (mild phenotype with incomplete penetrance in heterozygotes, severe disease in homozygotes). The influence of modifier genes has been proposed to explain the phenotypic variability in WS, and may be more sensitive or visible in heterozygous individuals than in homozygotes in this family.

The diagnosis of SWS is mainly clinical. Molecular diagnosis is especially important for genetic counseling which should be adapted to the mode of transmission associated with the detected mutation. There is neither available data about the frequency of WS in Morocco nor in the Arab world. The prevalence of the autosomal recessive forms might be higher in Morocco than in developed countries, which can be explained by the high rate of consanguinity (15.25%) [Jaouad et al., 2009]. Parental consanguinity may increase the risk for SWS, and genetic counseling for families with such patients is important to prevent consanguineous marriages and consequently reduce the occurrence of SWS in these families.

In addition to SWS, mutations in the *EDNRB* gene are involved in susceptibility to Hirschsprung disease (mostly heterozygous). A homozygous mutation has also been reported in the ABCD syndrome with partial albinism, black lock, cell migration disorder of the neurocytes of the gut, and deafness. This autosomal recessive neural crest syndrome has a clinical phenotype strongly overlapping with SWS, suggesting that ABCD syndrome is not a separate entity, but an expression of SWS (the main difference being the extent of the depigmentation) [Verheij et al., 2002].

Heterozygous proximal 13q deletions (including *EDNRB*) have also been reported [Tüysüz et al., 2009]. In addition to common features seen in SWS, the 13q deletion syndrome includes hypertelorism and epicanthus with developmental delay.

Prenatal molecular diagnosis can be offered if the disease-causing mutation has been identified in the family. Given the clinical variability even within families, this testing is rarely requested, but it could be proposed

for severe cases with homozygous forms of *EDN3* and *EDNRB* mutations in which the prognosis is not always favorable.

To the best of our knowledge, among the cases in the Arab world, only 1 Lebanese family with SWS has been studied with molecular techniques and was found to carry a novel mutation in the *EDNRB* gene [Haddad et al., 2011]. We believe that the report of mutations in the genes involved in SWS is important and will help in the further delineation of the clinical and molecular spectrum in order to define genotype-phenotype correlations.

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