Association of Waardenburg syndrome with a new mutation in the *PAX3* gene: A case report and literature review

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Abstract. The present case report focuses on Waardenburg syndrome (WS), which occurs with a frequency of 1/40,000 and is manifested by neurosensory deafness, pigmentation deficiencies of the skin, hair and iris of the eye, and various tissue defects originating from the neural crest. WS is a genetically heterogeneous disease classified into four clinical and genetic phenotypes. In addition, it is caused by mutations in the following genes: Paired box 3 (*PAX3)*, Endothelin 3, Endothelin Receptor Type B, Melanocyte Inducing Transcription Factor, Snail Family Transcriptional Repressor 2 and SRY-Box Transcription Factor 10. The present study focuses mainly on the structure and function of the *pax3* gene and describes the case of a 33-year-old male, in whom a thorough clinical evaluation of physical characteristics and a detailed patient and family history was performed prior to concluding that the patient had WS. To confirm the diagnosis, genetic counseling was recommended and molecular genetic testing using next generation sequencing (NGS) methods was suggested. NGS revealed a novel c.209G>A (p.Cys70Tyr) mutation in the *PAX3* gene, suggesting that this method is beneficial for disease screening, genetic diagnosis and counseling. The purpose of the present report is to assess the novel *PAX3* gene mutation and its association with WS by analyzing the data from the case and the existing international literature, and by presenting further documented clinical cases. The present study is important due to the discovery of a new mutation, which may contribute to the understanding of the correlation between genotypes and phenotypes, aiding the field of genetic counseling. In addition, the present research study may aid the identification of potential diagnostic and therapeutic approaches for WS.

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Introduction

Waardenburg syndrome (WS) consists of a group of four genetic disorders (WS1, OMIM#193500; WS2, OMIM#193510; WS3, OMIM#148820 and WS4, OMIM#277580; www.omim.org), which were initially described in 1951 by the Dutch ophthalmologist and geneticist Petrus Johannes Waardenburg, after whom WS was named. The prevalence varies throughout the world. A general estimate of the syndrome is ~1 case/42,000 individuals worldwide. The types of the syndrome are characterized by variable features in affected individuals, including hearing loss (HL), ophthalmological abnormalities and pigmentation disorders, as well as facial dysmorphism (1).

WS is caused by mutations in various genes that affect the function of nerve cells in embryonic development. The majority of the types are caused by autosomal dominant mutations. Autosomal recessive mutations are few and rare. In the majority of the cases, the affected individual inherits WS from a parent with one of the dominant forms of the condition. A low percentage of cases result from spontaneous new mutations (*de novo*) in the gene, where there is no family history of the condition. WS is usually caused by mutations in the genes paired box 3 (*PAX3*), Endothelin 3 *(EDN3)*, Endothelin Receptor Type B *(EDNRB)*, Melanocyte Inducing Transcription Factor *(MITF)*, Snail Family Transcriptional Repressor 2 *(SNAI2)*, SRY-Box Transcription Factor 10 *(SOX10)* (2). Mutations in the *pax3* gene are associated with WS1 and WS3, which are caused in the majority of the cases by heterozygous pathogenic mutations, while WS3 has been reported as a result of homozygous pathogenic mutations with more severe manifestations (3,4). WS2 usually occurs from pathogenic heterozygous mutations in the *MITF*, *SOX10* and *EDNRB* genes (2,5), and in the *KITLG* gene in heterozygous and homozygous forms (6,7). Overall, ~50% of WS4 cases are caused by heterozygous pathogenic mutations in *sox10*, whereas 20-30% of the cases are caused by pathogenic

mutations in *EDNRB* and *EDN3*, with dominant/recessive forms of inheritance (8-10).

In humans the chromosomal locus of the *PAX3* gene is Chr2q36.1, from 222.199.888 to 222.298.996 bp in a 100-kb region (Ensembl assembly release GRCh38.p10). The *PAX3* gene includes 10 exons; more specifically exons 2, 3 and 4 encode the paired box (PB), 5 and 6 encode the conserved octopeptide and homeodomain (HD), and 7 and 8 encode the activation region (11). At the mRNA level, at least 10 different pax3 isoforms have been detected as a consequence of alternative splicing and processing. The longest isoform appears to be pax3e, consisting of 10 exons, which encodes a 505-amino acid protein (12). The expression of the protein occurs during the formation of the central nervous system, skeletal muscle, neural crest and derivatives. The pax3 protein operates as a regulator of target gene expression affecting differentiation, survival, migration, motility and proliferation in these lineages (13). The pax3 protein is a transcription factor, which consists of the two following functional domains: An N-terminal DNA-binding region that includes the PB, octapeptide and HD, and a C-terminal transcription activation region that includes a proline, serine and threonine-rich site.

The diagnosis of WS can often be difficult due to the wide range of symptoms and genetic variants. The condition can be diagnosed before/at birth, in early childhood or in certain cases at an older age with a comprehensive clinical evaluation, identification of clinical features, a detailed patient and family history, and several specialized genetic tests. A detailed medical evaluation and genetic testing are necessary to determine the presence of the condition and select appropriate supportive and symptomatic treatments (14).

The present study describes the case of a 33-year-old male with clinical features compatible with WS undergoing genomic testing by next generation sequencing (NGS). NGS testing identified a novel c.209G>A (p.Cys70Tyr) mutation in the *PAX3* gene, which was likely responsible for the observed pathogenesis of the syndrome. The aim of the present study is to investigate the association between the syndrome and mutations in the *PAX3* gene. The existing international literature is analyzed and the documented clinical cases are presented to provide an improved understanding of the role of the *PAX3* gene in the pathogenesis of the syndrome. The present study is important as it identifies a novel mutation that may contribute to more extensive genetic counseling and improved diagnosis and management of WS.

Case report

The present study is a case report of a 33-year-old male, who after a detailed clinical evaluation, identification of characteristic physical findings, and analysis of a detailed patient and family history, was found to have clinical features compatible with Waardenburg syndrome (WS). In particular, the patient had undergone regular hearing tests, which revealed bilateral neurosensory HL bordering on practical deafness and a hearing impairment rate of >67%. This clinical presentation was established during infancy and oral speech disorders had since occurred. In addition, following a medical examination, it was confirmed that the patient had skin pigmentation disorders, including leukodermia, manifested by white patches of skin in the abdominal area and pigmentary deficiencies of the hair, namely a white lock of hair in the front-center of the head (Fig. 1). Although these features had been evident since infancy, at the age of 33 the patient attended Access To Genome, Genetics Laboratory (Athens, Greece) in October 2023 to confirm the diagnosis of WS. Admission was not required and the patient was not hospitalized.

According to the family history it was noted that both of the patient's parents exhibited normal hearing. The patient's brother had a hearing test as a child and the results were normal, with no concerns regarding hearing results up to the completion of the present study. The brother's hair started to turn grey when he was 33 years old and a white lock of hair in the front-center of the head was present for as long as could be remembered. Additional information indicated that the paternal grandmother also had patches of white hair on the head. Furthermore, the uncle on the father's side had normal hearing and white hair since puberty (covering the entire head); the uncle was ischemic since the age of 25 years and developed colonic diverticula. Finally, no other family history of sensorineural HL, kidney problems, cancer or iris heterochromia was reported. The clinicians, based on the clinical examination and the comprehensive history of the patient and the patient's family, assumed that the patient presented symptoms compatible with WS.

The patient and the family members were fully informed regarding the nature, purpose and procedures of the study; they received adequate information and had the opportunity to ask questions and receive clarification. Subsequently, they provided written consent to participate in the study.

Molecular genetic screening of the patient. To confirm the diagnosis, genetic counseling was recommended and molecular genetic testing of the patient was suggested. Initially peripheral blood was collected from the patient using standard phlebotomy procedures with EDTA anticoagulant tubes. Genomic DNA was extracted from whole blood using the bead-based method MagAttract HMW DNA Kit (cat. no. 67563; Qiagen, Inc.). DNA quantity was measured fluorometrically with the Qubit dsDNA HS Assay Kit (cat. no. Q32854; Thermo Fisher Scientific, Inc.) and a Qubit Fluorometer. Qualified genomic DNA was randomly fragmented by non-contact, isothermal sonochemistry using the Covaris LE220-plus (Covaris, LLC). Fragment ends were end-repaired, A-tailed, and ligated to sequencing adapters, IDT for Illumina TruSeq DNA UD Indexes (cat. no. 20022370; Illumina Inc.). Size selection was performed with a bead-based clean-up method, AMPure XP (cat. no. A63880; Beckman Coulter, Inc.). Libraries were PCR-amplified according to manufacturer recommendations and enriched by hybridization-based capture using the Twist Clinical Exome workflow with a custom Waardenburg Syndrome Panel. The custom target set captured *EDN3*, *EDNRB*, *KIT*, *KITLG*, *MITF*, *PAX3*, *SNAI2* and *SOX10* genes, including all protein-coding exons, exon-intron boundaries (±20 bp) and selected non-coding deep intronic variants. Library quality was assessed by verifying insert size distribution and absence of adapter dimers, using the Agilent 2100 Bioanalyzer with the High Sensitivity DNA Kit (cat. no. 5067-4626; Agilent Technologies, Inc.). Library concentrations were determined by qPCR, with the KAPA

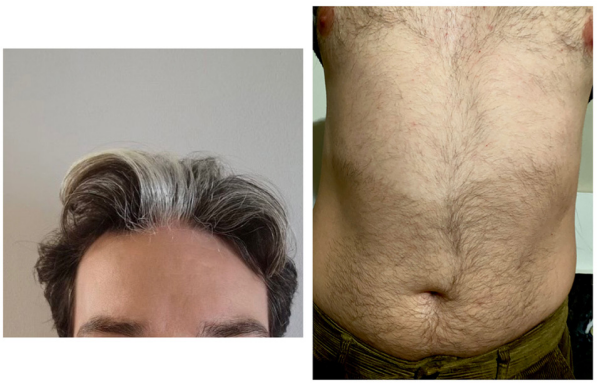

Figure 1. Clinical images of the case. Photographic features of the patient's appearance showing area pigmentary deficiencies of the hair, namely a white lock of hair in the front-center of the head and white patches of skin in the abdomen.

Library Quantification Kit for Illumina (cat. no. KK4824; Roche Diagnostics GmbH) on a real-time PCR platform. Final libraries were normalized to 1.5-2.0 pM prior to sequencing. Sequencing was performed on an Illumina NovaSeq™ 6000 using paired-end 150-bp sequencing-by-synthesis chemistry with the Twist Clinical Exome workflow. The reagent kit used was the NovaSeq 6000 SP 300-cycle kit (cat. no. 20040326; Illumina, Inc.). In-process reference samples and control libraries were included for process monitoring and quality control. The Waardenburg Syndrome Panel achieved 100% of targeted bases at ≥20x coverage with a median depth of 282x, calculated from MQ0-filtered aligned reads. Sequencing runs met established sensitivity and specificity thresholds. Bioinformatics and quality control were performed as follows. Raw sequencing data (BCL files) were converted to FASTQ using Illumina bcl2fastq v2.20 (Illumina, Inc.). Reads were aligned to the human reference genome GRCh37/hg19 using Burrows-Wheeler Aligner (BWA-MEM) v0.7.17 (https://github.com/lh3/bwa/releases/tag/v0.7.17). Post-alignment processing included duplicate marking, local realignment around indels and base quality score recalibration performed with the Sentieon implementation of GATK algorithms. Single-nucleotide variants and small insertions/deletions were called per standard pipeline parameters. Variants were annotated using VcfAnno (https://github.com/brentp/vcfanno) and Ensembl Variant Effect Predictor (https://www.ensembl.org/info/docs/tools/vep/index.html), referencing public databases including gnomAD (https://gnomad.broadinstitute.org/), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) and HGMD (https://www.hgmd.cf.ac.uk/). Sample-level quality control included assessments for contamination, sex concordance and sample mix-ups. Coverage metrics such as median depth and percentage of targeted bases at ≥20x were computed across all intervals to verify adequate performance. Exonic copy-number variants were detected using CNVkit (https://cnvkit.readthedocs.io/) in conjunction with an in-house depth-of-coverage-based pipeline. Expected sequencing depth for target regions was modeled using other samples sequenced in the same batch as references, and GC-content bias was corrected during analysis. Copy-Number Variants (CNVs) detected by NGS were confirmed by quantitative PCR or digital PCR. Variants not meeting stringent NGS quality metrics, such as those with low quality scores, difficult genomic regions or complex sequence contexts, as well as selected variants of uncertain significance, were confirmed by bi-directional Sanger sequencing. CNVs involving fewer than 10 exons (heterozygous) or three exons (homozygous/hemizygous), or novel events, were confirmed orthogonally unless previously validated by the laboratory. Variant classification followed the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) 2015 guidelines (15). Clinical interpretation incorporated the patient's phenotype, segregation and clinical context, curated literature, and all sequencing and quality control data. Findings were reviewed by a multidisciplinary team prior to reporting.

Molecular testing of the patient revealed the presence of a novel likely pathogenic nucleotide variant, c.209G>A (p.Cys70Tyr), in the PAX3 gene in heterozygosity. This missense mutation results in the substitution of guanine (G) by adenine (A) at nucleotide position 209, leading to an amino acid change from cysteine (Cys) to tyrosine (Tyr) at position 70 of the PAX3 protein.

Molecular genetic screening of the family. Based on the patient's family history and the fact that WS can be inherited, it was considered appropriate to perform molecular analysis to detect the aforementioned nucleotide change in first-degree relatives. Therefore, genomic DNA was extracted from venous blood samples obtained from the patient's mother, father, and brother, and subsequently analyzed by PCR and Sanger sequencing. Targeted amplification of the *PAX3* gene segment harboring the c.209G>A (p.Cys70Tyr) variant was performed by PCR. PCR amplification was carried out using the primers PAX3_F (5'-CGCCTTTACGCACCT TCACAA-3') and PAX3_R (5'-GAGTCCGATGTCGAG CAGTTT-3'), designed to amplify the region containing the variant. The primers had melting temperatures of 60-61˚C, and the PCR program RYR1-59 was applied. Each reaction was prepared in a final volume of 50 μl, containing 10 μl of 5X PCR buffer (final concentration 1X), 3 μl $MgCl_2$ (25 mM stock; final concentration 1.5 mM), 4 μl dNTP mix (2.5 mM stock; final concentration 0.2 mM), 0.25 μl forward primer (100 μM stock; final concentration 1.0 μM), 0.25 μl reverse primer (100 μM stock; final concentration 1.0 μM), 0.2 μl Taq DNA polymerase (5 U/μl; final concentration 0.04 U/μl), 5 μl genomic DNA and nuclease-free water to reach the final volume. Thermal cycling conditions consisted of an initial denaturation at 95˚C for 5 min, followed by 35 cycles of denaturation at 95˚C for 30 sec, annealing at 60˚C for 30 sec and extension at 72˚C for 30 sec, and then a final extension at 72˚C for 5 min. PCR products were then held at 4˚C until further analysis. Amplicons were evaluated by capillary electrophoresis on the ABI 3500 Genetic Analyzer (Thermo Fisher Scientific) to confirm product quality. Direct Sanger sequencing of the PCR products was subsequently performed on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). Variant detection and segregation analysis in the family were carried out by aligning the obtained sequences to the PAX3 reference sequence NM_181457.4 (https://www.ncbi.nlm.nih.gov/nuccore/NM_181457.4) using Chromas

Table I. Molecular testing results.

Gene	Nucleotide change	Amino acid change	Results	Genotype	Inheritance pattern	Clinical interpretation
PAX3 (NM_181457.4)	c.209G>A	p.Cys70Tyr	Missense variant	Heterozygous	Autosomal dominant/ recessive	Likely pathogenic

PAX3, paired box 3; Cys, cysteine; Tyr, tyrosine.

software v2.6.6 (Technelysium Pty. Ltd.; https://technelysium.com.au/wp/chromas/).

Molecular genetic testing of the family indicated that the patient's father and brother were heterozygous for the same nucleotide change c.209G>A (p.Cys70Tyr) in the *PAX3* gene, while the mother was found to be negative for the presence of this mutation (Table I). The outcome for the patient was genetic counselling only.

Discussion

Mutations in the *PAX3* gene are associated with the clinical features of WS1 and with craniofacial deafness-hand syndrome (CDHS) following an autosomal dominant inheritance pattern, and with WS3 following an autosomal dominant or recessive inheritance pattern (OMIM#: 606597, 122880, 193500 and 148820) (16). There is also evidence that a fusion between the *PAX3* and *FKHR* (foxo1a) genes is associated with alveolar rhabdomyosarcoma (OMIM#: 606597 and 268220). This variant, however, has not, to the best of the authors knowledge, been reported to be associated with clinical conditions. However, another variant at this gene position, p.Cys70Arg, has been associated with WS, suggesting that a change at this position adversely affects protein structure and/or function and is potentially pathogenic (17). In general, this variant has not been reported in the Broad Institute dataset (https://www.broadinstitute.org/datasets) (individuals without severe disease with childhood onset). The physicochemical difference between Cys and Tyr, as measured by the distance 'Grantham score', is 194. This variant has a high score and is predicted to be harmful. This score is considered a 'radical' variation, indicating that Cys and Tyr have significantly different physicochemical properties (18,19). Amino acid conservation analysis indicates that the wild-type amino acid, Cys70, is fully conserved in all 99 vertebrates examined, raising the possibility that a change at this position would not be tolerated and could adversely affect protein structure and/or function. In addition, a previous study indicated that a similar mutation affected a highly conserved Cys residue in the paired region, which was predicted to be involved in DNA binding (20). In addition, this c.209G>A (p.Cys70Tyr) nucleotide change is not recorded in the Genome Aggregation Database (gnomAD) (https://gnomad.broadinstitute.org) and is not reported in the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/) nor has it been described to date in patients with pax3-related diseases. This nucleotide change is located in a gene region where several point missense pathogenic mutations are concentrated and is expected to have a deleterious effect on protein structure. According to the ACMG/AMP guidelines (15), it is classified in the category of likely pathogenic findings.

According to Jalilian *et al* (21), the *PAX3* gene contains ~100 mutations that have been reported and only few are repeatable. These mutations appear to be missense in 38%, minor deletions in 20%, nonsense in 15%, large deletions in 11%, splicing in 8% and minor insertions in 8%. According to Pingault *et al* (2), the majority of these mutations are found from exon 2 (with the highest frequency) to 6, altering the structure of PB or HD and therefore affecting the DNA binding site. According to Baldwin *et al* (11), Carey *et al* (22) and Jalilian *et al* (21), a low number of mutations are identified in exons 1, 7 and 8, comprising variations that affect the activation site. However, no mutations have been found in exons 9 and 10. According to Hoth *et al* (23) and Tassabehji *et al* (24), 80% of WS1 cases are heterozygous mutations. In contrast to these observations, WS3 cases have been found to be either heterozygous or homozygous. Furthermore, partial or total deletion or even minor mutations of *PAX3* and adjacent genes are frequently observed in WS3 cases. In addition, Wang *et al* (25) and Jang *et al* (26) detected a *de novo* mutation in WS1 in a Chinese and a Korean population, respectively. Furthermore, Chen *et al* (27) detected germline mosaicism in a rare case of two siblings with WS1. In addition, mutations in the *PAX3* gene are also found in CDHS, which is an autosomal dominant condition classified as an allelic variant of WS and characterized by craniofacial abnormalities and neurosensory deafness (28). Asher *et al* (29) detected the mutation p.Asn47Lys in exon 2 of *PAX3* in a family with three affected members.

In addition, it is worth noting that the genotype-phenotype correlations in *PAX3* are not well documented, except for the pathogenic variants p.Asn47His in WS3 (23) and p.Asn47Lys in CDHS (29). Baldwin *et al* (11) and Tassabehji *et al* (24) concluded no connection among the type and location of the mutation and the phenotype severity. DeStefano *et al* (30) demonstrated that the presence of pigmentation abnormalities in individuals with WS1 was more strongly associated with pathogenic *PAX3* variants that delete the HD than with missense or deletions of pathogenic variants that include PB. Furthermore, no genotype and phenotype association has been found for HL in WS1. According to Milunsky *et al* (31) no discernible difference has been noted in the severity between whole or partial gene deletions and the clinical spectrum reported for small endogenous *PAX3* pathogenic variants. Finally, Yang *et al* (32) described a case of a combined phenotype of WS type 1 and 2, where one parent exhibited WS1 as a result of a heterozygous pathogenic variant in *PAX3* at the intron 4 splicing site. The other parent exhibited WS2 as a

result of a heterozygous pathogenic variant in the *MITF* gene. As a result, the child was heterozygous for both pathogenic variants. In addition, the patient appeared to have significantly more pigmentation abnormalities than both parents, indicating an additive impact of those two gene variations.

Future research and clinical trials for WS should focus on several promising areas, such as understanding the genetic basis of the syndrome, developing new diagnostic methods and exploring potential treatments. Firstly, conducting genetic research is important to understand the genetic mechanisms behind WS. Studies focusing on the functional characterization of novel mutations are essential, as they involve studying the way by which these genetic changes affect protein function and contribute to the symptoms of WS. In addition, it is important to analyze the relationships between specific genetic mutations and clinical expression of the syndrome. Secondly, researchers should aim to improve diagnostic accuracy and personalize treatment plans. The use of NGS can aid the identification and recording of novel etiological variants in patients, enriching international databases. This approach aids the identification of specific genetic mutations responsible for the disease. Finally, therapeutic approaches should consider possible biological therapies that could target the underlying genetic causes of WS. This includes gene therapy and other molecular therapies that could correct or mitigate the effects of mutations. Although assistive technologies, such as hearing aids, cochlear implants or other hearing aids can alleviate HL symptoms, there is currently no cure for WS. Recently, significant progress has been made in preclinical research on hereditary HL in animal models, including gene transfer and stem cell replacement therapy. The review article by Huang *et al* (33) highlights the current understanding of the pathogenic mechanisms and potential biological treatments for HL in WS, and provides strategies and directions for the implementation of WS biological therapies and possible issues in the future. Therefore, these areas of research are essential for advancing the understanding of the syndrome and developing more effective treatments.

Overall, WS exhibits several different types with certain differences in symptoms, which may differ between patients of the same type. The two features that are common to all types of the syndrome are congenital sensory HL to a certain degree and pigmentation abnormalities. The diagnosis of the syndrome can often be difficult due to its variable symptoms and genetic variations. A thorough medical evaluation and genetic testing are important to determine the presence of the condition. WS has no cure and is a difficult condition to manage, requiring a multi-scientific team to address all complications. The present study described the case of a 33-year-old male with clinical features and a family history compatible with WS. The patient and first-degree relatives were submitted to genetic testing by NGS and it was found that the patient, father and brother were heterozygous for the novel nucleotide change c.209G>A (p.Cys70Tyr) in the *PAX3* gene, while the mother was negative for the presence of this mutation. The mutation is classified as a likely pathogenic finding and is likely to be responsible for the observed pathogenicity of the syndrome. The discovery and reporting of new mutations associated with the syndrome contributes to the understanding of the association between genotypes and phenotypes, which aids the genetic counseling and diagnosis. In addition, the present study demonstrated that NGS is a useful approach for the diagnosis of congenital diseases and is beneficial for disease screening, genetic diagnosis and counseling.

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Availability of data and materials

The data generated in the present study may be found in the European Nucleotide Archive at EMBL-EBI under accession number (PRJEB89662) or at the following URL: https://www.ebi.ac.uk/ena/browser/view/PRJEB89662.

Authors' contributions

JZ designed the study, contributed to the analysis and interpretation of the data, and drafted, revised and submitted the manuscript. EP, AP, SS, OV and GKP contributed to the design of the study and to the analysis and interpretation of the data, and revised the manuscript critically for important intellectual content. ES and EM participated in the study design, analyzed the laboratory data, and provided technical expertise for the laboratory techniques and results. JZ and EM confirm the authenticity of all raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present case report was prepared in accordance with institutional policies. The patient and their family provided consent to participate in the study.

Patient consent for publication

Written informed consent was obtained from the patient and their family for the publication of any patient data or associated images.

Competing interests

The authors declare that they have no competing interests.

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