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ORIGINAL ARTICLE

Phenotypic diversity and genetic complexity of *PAX3*-related Waardenburg syndrome

Puneeth H. Somashekar[1] | Priyanka Upadhyai[1] | Dhanya L. Narayanan[1] | Nutan Kamath[2] | Shruti Bajaj[3] | Katta M. Girisha[1] | Anju Shukla[1]

[1]Department of Medical Genetics, Kasturba Medical College, Manipal, Manipal Academy of Higher Education, Manipal, India

[2]Department of Pediatrics, Kasturba Medical College, Mangalore, Manipal Academy of Higher Education, Manipal, India

[3]Suchak Hospital, Mumbai, India

Correspondence
Anju Shukla, Department of Medical Genetics, Kasturba Medical College, Manipal, Manipal, Academy of Higher Education, Manipal 576104, India.
Email: anju.shukla@manipal.edu

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Abstract

Waardenburg syndrome subtypes 1 and 3 are caused by pathogenic variants in *PAX3*. We investigated 12 individuals from four unrelated families clinically diagnosed with Waardenburg syndrome type 1/3. Novel pathogenic variants identified in *PAX3* included single nucleotide variants (c.166C>T, c.829C>T), a 2-base pair deletion (c.366_367delAA) and a multi-exonic deletion. Two novel variants, c.166C>T and c.829C>T and a previously reported variant, c.256A>T in *PAX3* were evaluated for their nuclear localization and ability to activate *MITF* promoter. The coexistence of two subtypes of Waardenburg syndrome with pathogenic variants in *PAX3* and *EDNRB* was seen in one of the affected individuals. Multiple genetic diagnoses of Waardenburg syndrome type 3 and autosomal recessive deafness 1A was identified in an individual. We also review the phenotypic and genomic spectrum of individuals with *PAX3*-related Waardenburg syndrome reported in the literature.

KEYWORDS

EDNRB, multiple genetic diagnoses, *PAX3*, phenotypic variability, Waardenburg syndrome

1 | INTRODUCTION

Waardenburg syndrome (WS) is a clinically and genetically heterogeneous disorder with an estimated prevalence of 1 in 42,000 (Nayak & Isaacson, 2003; Pingault et al., 2010; Read & Newton, 1997; Song et al., 2016). The distinctive phenotypic features include congenital sensorineural hearing loss (SNHL), hypopigmentation of hair, eyes, and skin with or without other associated features like telecanthus, wide nasal bridge, hypoplastic nasal alae, white eyelashes/eyebrows, and Hirschsprung disease (HD) in different subtypes. Based on the presence or absence of additional symptoms, it is classified into four subtypes (WS1-4). Pathogenic variants in six genes, *PAX3*, *MITF*, *SOX10*, *SNAI2*, *EDNRB*, and *EDN3* are known to cause different subtypes of WS (Pingault et al., 2010; Read & Newton, 1997).

WS1 (MIM# 193500) is characterized by congenital SNHL (47–53%), heterochromia iridis (15–30%), telecanthus (W-index >1.95), and white forelock (43–48%). Approximately 52–100% have high nasal roots and 63–73% have medial eyebrow flare (Milunsky, 1993; Song et al., 2016). Spina bifida and neural tube defects are less frequently reported (Hart & Miriyala, 2017). Heterozygous variants in *PAX3* are reported to cause WS1. They occur either as de novo variants or are inherited in an autosomal dominant mode with intra-familial phenotypic variability or non-penetrance (Bocángel et al., 2018; Minami et al., 2019; Pingault et al., 2010).

WS3 (MIM# 148820) shares most features of WS1. In addition, individuals with WS3 may have upper limb abnormalities like contractures of the joints, hypoplasia of the bones of the upper limbs, and wrists, syndactyly, brachydactyly, and finger contractures (Klein & Opitz, 1983; Pingault et al., 2010). Both bi-allelic and heterozygous variants in *PAX3* are reported to cause WS3 (Mousty et al., 2015; Pingault et al., 2010; Saberi et al., 2018). Here, we report four Indian families with phenotypic diversity and genetic complexity observed in *PAX3*-related WS.

2 | MATERIALS AND METHODS

2.1 | Editorial policies and ethical considerations

The study was approved by the Institutional Ethics Committee. Written informed consent was obtained for the use of photographs and research findings.

2.2 | Subjects

Twelve individuals from four families clinically diagnosed to have WS1/3 based on Waardenburg consortium diagnostic criteria were recruited in the study (Farrer et al., 1992).

2.3 | Genetic analysis

DNA was isolated from EDTA blood of the probands and available family members by the standard phenol-chloroform method. Sanger sequencing of *PAX3* and exome sequencing (ES) was performed as previously described (Somashekar et al., 2019). Sanger sequencing of *GJB2* was performed using primers listed in Table S2. Copy number variation (CNV) analysis from exome data and validation by quantitative PCR (qPCR) were performed as previously described (Somashekar et al., 2019). The relative exon copy number was calculated by the expression $2 \times 2^{-\Delta\Delta Ct}$ and is approximately two for a diploid sample and one for heterozygous deletion. Primers specific to the target region, exon 1 of *PAX3* (NM_181459.3), and *CFTR* (NM_000492.4) primers used as an internal control were designed using NCBI Primer-BLAST. Primers are listed in Table S3.

2.4 | Expression and reporter constructs

The reporter vector, pGL3-MITF-Luc, and expression vector, pECE-PAX3 containing *PAX3* cDNA were kindly provided by Dr. Nadege Bondurand (Bondurand et al., 2000). Full-length *PAX3* cDNA from pECE-PAX3 was PCR amplified and subcloned into pcDNA3.1-3xFLAG vector at HindIII and EcoRI restriction sites to generate pcDNA3.1-3xFLAG-PAX3. Primers are listed in Table S4. The *PAX3* (NM_181459.3) variants identified in this study, c.166C>T p.Arg56Cys (PAX3_166), c.829C>T p.Gln277Ter (PAX3_829) and a previously reported variant, c.256A>T p.Ile86Phe (PAX3_256) were introduced into the pcDNA3.1-3xFLAG-PAX3 expression vector by site-directed mutagenesis (SDM) using PCR (Somashekar et al., 2019). Primers used for SDM are listed in Table S5. PCR amplicons were treated with DpnI (NEB, cat# R0176S), ligated by T4 DNA ligase (NEB, cat# M0202T), and transformed into ultra-competent *E. coli*, DH10β. The constructs generated were purified by PureLink HiPure Plasmid Miniprep Kit (Invitrogen, cat# K210002) and verified by Sanger sequencing.

2.5 | Cell culture and luciferase reporter assay

HEK293T cells were grown in DMEM, high glucose (Gibco, cat# 11965118) supplemented with 10% fetal bovine serum (HiMedia, cat# RM9955) and Penicillin–Streptomycin (HiMedia cat# A001). Cells were seeded at 5×10^4 cells/well in a 24-well plate 24 hr before transfection and were co-transfected with 100 ng expression vector (pCMV-FLAG-PAX3 containing wild type or mutant *PAX3*), 100 ng reporter plasmid, (pGL3-MITF-Luc) and 50 ng pRL-CMV vector (pRL Renilla luciferase control reporter vector, Promega, cat.E2261) using Lipofectamine 3000 reagent (Invitrogen, cat# L3000001). Forty-eight hours post-transfection, cells were washed with 1X phosphate-buffered saline (PBS) and lysed with 1X Reporter Lysis Buffer (Promega, cat# E3971). Firefly luciferase and renilla luciferase activity were measured using the Dual-Glo® Luciferase Assay System (Promega, cat# E2920) according to the manufacturer's protocol.

2.6 | Immunofluorescence

HEK293T cells were seeded on glass coverslips placed in a 24-well plate at 5×10^4 cells/well. After 24 hr, cells were transfected with 200 ng of the expression vector, pCMV-FLAG-PAX3 containing wild type or mutant *PAX3*. Forty-eight hours post-transfection, cells were washed with 1X PBS (3X) and fixed using 4% paraformaldehyde for 15 min at room temperature (RT). Cells were permeabilized by using 0.2% Triton X-100 for 10 min at RT. 5% normal goat serum (NGS) was used as blocking solution for 1 hr at RT, followed by overnight incubation with the following primary antibodies, anti-FLAG (Sigma, cat# F3165, 1:2,000 dilution) at 4°C. Subsequently, cells were washed with 1XPBS (3X) and blocked with blocking solution described above for 1 hr at RT followed by incubating with the secondary antibody, Alexa fluor 488 (Invitrogen, cat# A11029, 1:500 dilution) for 2 hr at RT in the dark. This was followed by washing cells with 1× PBS (3×) and incubating with 4,6-diamino-2-phenylindole (DAPI, Invitrogen, cat# D1306) for 3 min. Post DAPI staining cells were washed with 1× PBS (3×) and cells were mounted on a glass slide with Prolong Diamond without DAPI (Invitrogen, cat# P36961). Imaging was done with a Zeiss Axio Vert fluorescent microscope with a 63× oil immersion objective.

3 | RESULTS

3.1 | Phenotypes and genotypes

Family 1: Three-years-old boy (proband, P1) presented to the clinic with complaints of speech delay and hypopigmentation of hair and iris since birth. His weight was 13 kg (normal), height was 91 cm (normal), head circumference was 50.5 cm (normal). On examination, bright blue iridis, white forelock, hypopigmented eyelashes, telecanthus, hypopigmented patches on forehead, chest, and upper limbs were noted (Figure 1a2). Brainstem Evoked Response Audiometry (BERA)

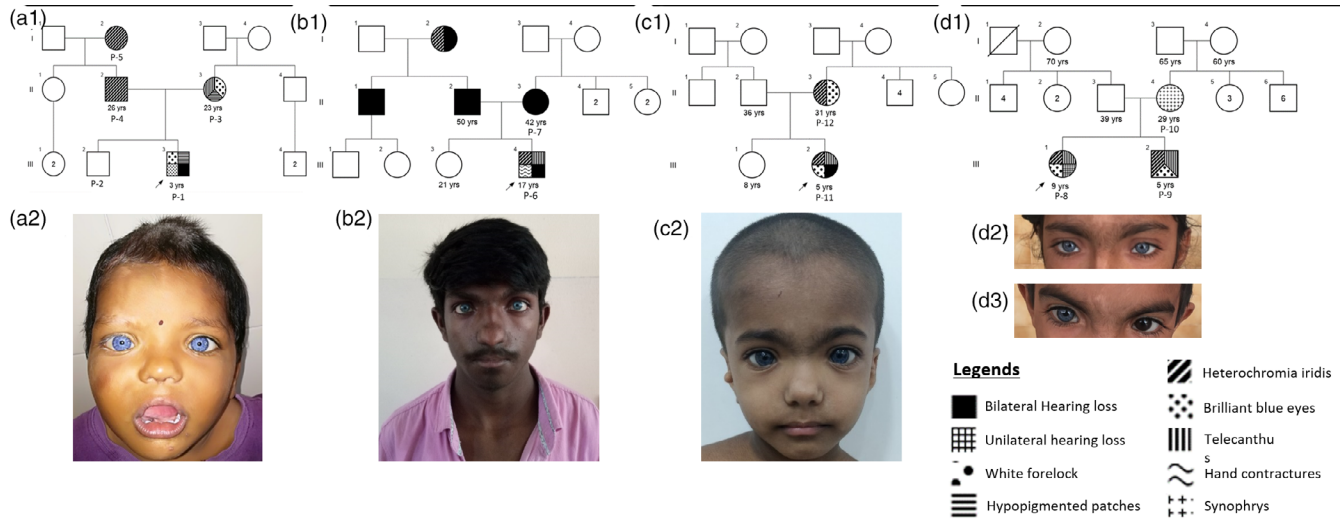

FIGURE 1 (a1) Pedigree of Family 1 and (a2) facial photograph of P1; (b1) Pedigree of Family 2 and (b2) facial photograph of P6; (c1) Pedigree of Family 4 and (c2) facial photograph of P11 (I); (d1) Pedigree of Family 3 and (d2 and d3) facial photographs of P8 and P9 [Color figure can be viewed at wileyonlinelibrary.com]

was suggestive of bilateral severe to profound SNHL. His mother (P3) was noted to have telecanthus, white forelock, and white patches on the skin. His father (P4) and paternal grandmother (P5) had heterochromia iridis without any other manifestations. Proband's elder sibling (P2) was clinically normal (Figure 1a1).

Sanger sequencing of *PAX3* revealed a novel missense variant, c.166C>T in heterozygous state in the proband. On segregation, this variant was seen in his affected mother and unaffected sibling but was absent in father and paternal grandmother who were variably affected. Hence, we performed ES in the proband and identified a novel pathogenic variant, c.1047delC in *EDNRB* in the heterozygous state. This variant was inherited from his father and paternal grandmother.

Family 2: Seventeen-years-old male (proband, P6) presented with white forelock, telecanthus, synophrys, heterochromia iridis, dysmorphic ears, and bilateral hand contractures (Figure 1b2). Pure tone audiometry (PTA) revealed severe bilateral SNHL. His parents were nonconsanguineously married and had isolated congenital hearing loss (Figure 1b1).

Sanger sequencing of *PAX3* revealed a novel nonsense variant, c.829C>T in heterozygous state in the proband. However, this variant was not present in his mother (P7) with congenital hearing loss. Sequencing of *GJB2* carried out for the proband and mother revealed a known pathogenic variant, c.71G>A p.(Trp24Ter) in *GJB2* in a homozygous state in both. Segregation could not be completed due to the nonavailability of the father's sample.

Family 3: Nine-years-old girl (proband, P8) presented with heterochromia iridis and skin hypopigmentation. Her weight was 22.5 kg (−2 to −3 *SD*), height was 137 cm (normal) and head circumference was 50.5 cm (normal). On examination, white forelock, telecanthus (W-index: 2.079), heterochromia iridis, synophrys, wide nasal bridge, underdeveloped nasal alae, and hypopigmented patches on the forehead and left forearm were noted (Figure 1d2). PTA was suggestive of moderate to severe unilateral hearing loss. Her five-years-old sibling (P9), was noted to have white forelock, telecanthus, heterochromia iridis, synophrys, wide nasal bridge, and underdeveloped nasal alae (Figure 1d3). Her mother (P10) was noted to have synophrys and her father was clinically asymptomatic. Hearing evaluation could not be done for the sibling, mother, and father (Figure 1d1).

ES for P8 did not reveal any clinically relevant single nucleotide variations, small deletions/duplications, or indels. CNV analysis from ES data revealed multi exonic heterozygous deletion (chr2:223,158,887–223,169,113) spanning exons 1–4 of *PAX3* and *CCDC140* in the proband. To confirm the deletion, qPCR was performed on the genomic DNA of the proband, sibling, parents, and unrelated control for the putative target region (exon 1 of *PAX3*). Values of amplicons in the proband, affected sibling and mother corresponded to single-copy number indicative of heterozygous deletion, while values of amplicons in father were comparable to that of the diploid unrelated control sample. Thus, the deletion in the proband and her sibling was inherited from their mother (Figure S1).

Family 4: Five-years-old girl (proband, P11) presented with complaints of hearing loss and absent speech. Her weight was 10.6 kg (−3.65 *SD*), height was 92 cm (−3 *SD*), head circumference was 44 cm (−4 *SD*). On examination, telecanthus (W-index: 2.586), synophrys, brilliant blue iridis, hypoplastic nasal alae, few hypopigmented hairs, and bilateral preauricular tags were noted (Figure 1c2). The hearing evaluation was suggestive of profound bilateral hearing loss. Her mother (P12) was noted to have heterochromia iridis and white forelock (Figure 1c1).

Sanger sequencing of *PAX3* revealed a novel two base pair deletion, c.366_367delAA in exon 3 in heterozygous state in the proband and her mother. Clinical and genetic findings of all four families are summarized in Table S1.

3.2 | Transcriptional activity of wild type and mutant protein

PAX3 regulates the expression of *MITF* by binding to its regulatory region (Galibert, Yavuzer, Dexter, & Goding, 1999). Transcriptional activities of wild type and mutant *PAX3* constructs (c.829C>T, c.256A>T, and c.166C>T) were determined by luciferase reporter assays. For this, the *MITF* promoter containing luciferase vector (pGL3-MITF-Luc) was co-transfected with empty vector, wild type (PAX3_WT), and mutant *PAX3* (PAX3_829, PAX3_256, and PAX3_166) expression vectors.

The variant, c.829C>T (PAX3_829) identified in Family 2 showed a significant decrease in the transactivation capability of the mutant protein. However, the other two variants, c.166C>T (PAX3_166) identified in Family 1 and a previously reported variant, c.256A>T (PAX3_256) did not show a significant alteration in the transactivation capabilities of the mutant proteins (Figure 2).

3.3 | Nuclear localization of wild type and mutant protein

PAX3, being a transcription factor is known to localize to the nucleus for transactivation of target genes by binding to their regulatory regions. We assessed the subcellular localization of PAX3 wild type (PAX3_WT) and PAX3 mutants (PAX3_829, PAX3_256, and PAX3_166) using immunofluorescence. As expected, PAX3_WT protein localized to the nucleus. However, all mutant PAX3 proteins were distributed throughout the nucleus, and the cytoplasm (Figure 3).

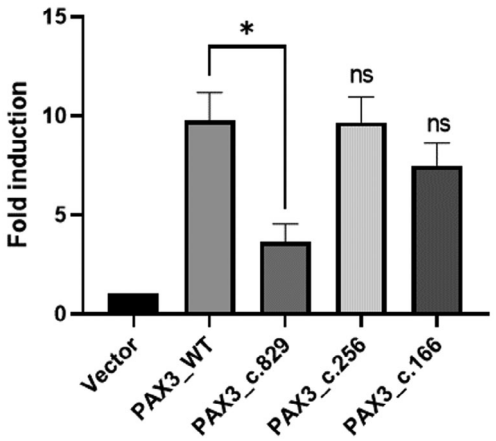

FIGURE 2 Transcriptional activities of wild type PAX3 and its mutant PAX3 determined by luciferase assays. Firefly luciferase activity was normalized by measuring Renilla luciferase activity. Reporter gene activity is shown as luciferase fold induction relative to the empty vector. Results are the mean ± SEM of at three to five different experiments, each performed in triplicates (*$p < .05$, One-way ANOVA followed by Tukey multiple comparison test)

4 | DISCUSSION

WS is a neurocristopathy resulting from abnormal survival, proliferation, differentiation, and migration of neural crest-derived melanocytes (Vega-Lopez, Cerrizuela, Tribulo, & Aybar, 2018). The phenomenon of extreme phenotypic variability necessitates the careful evaluation of families affected with WS. *PAX3* related WS is the most common and well-described disorder among various subtypes (Pingault et al., 2010; Song et al., 2016). The use of genomic techniques for evaluation is providing several new insights into phenotypes, genetic complexities, and genotype–phenotype correlations in WS.

4.1 | Genomic and phenotypic spectrum of *PAX3* related WS

Individuals with WS1 show features of congenital SNHL, heterochromia iridis, telecanthus, and white forelock. WS3 individuals have upper limb abnormalities in addition to the WS1 phenotype (Pingault et al., 2010). Intra and interfamilial phenotypic variability is frequently observed in both WS1 and WS3 and is seen across all types of genetic variants in *PAX3*, from SNVs to partial/complete gene deletion. *PAX3* related WS is highly penetrant and most individuals exhibit at least one subtle/minor clinical feature of WS. Out of the reported 198 families with WS due to pathogenic variant in *PAX3*, only two families (1.01%) having the variants, c.238C>G and c.808C>G in *PAX3* showed nonpenetrance (Chen et al., 2010; Jalilian, Tabatabaiefar, Farhadi, Bahrami, & Noori-Daloii, 2015; Matsunaga, Mutai, Namba, Morita, & Masuda, 2013; Milunsky, 1993; Mousty et al., 2015; Niu et al., 2018; Pingault et al., 2010; Saberi et al., 2018; Tassabehji et al., 1995; Tekin, Bodurtha, Nance, & Pandya, 2001; Wildhardt et al., 2013). In our study, we observed significant intrafamilial phenotypic variability in Families 3 and 4. Nonpenetrance was noted in one individual (P2) of Family 1.

To date, 172 pathogenic variants in *PAX3* have been reported. Among them, 60 are missense variants, 24 nonsense variants, 17 splicing variants, 41 small deletions, 12 small insertions, 3 indels, and 15 are large deletions (HGMD® Professional 2020.2, date of accession 17th July 2020) (Stenson et al., 2003). The majority of the variants occur in the exons 2–6, especially in the exons 2 and 6 which encode for paired domain and homeodomain of PAX3 protein respectively (Pingault et al., 2010). To this spectrum, we add two single nucleotide variants (c.166C>T and c.829C>T) located in the exon 2 and 6 respectively, a small deletion (c.366_367delAA) in the exon 3 and a large deletion encompassing multiple exons of *PAX3*.

The more severe phenotype of WS3 has been hypothesized to be due to either bi-allelic variants in *PAX3*, heterozygous variants with a probable dominant-negative mechanism, or an unknown modifier (Tekin et al., 2001). It has been noted that few variants in heterozygous state cause WS1 and in homozygous state cause WS3 (Wollnik et al., 2003; Zlotogora, Lerer, Bar-David, Ergaz, & Abeliovich, 1995). The pathogenic variant observed in the individual with WS3 in our cohort was a heterozygous loss-of-function variant. No other

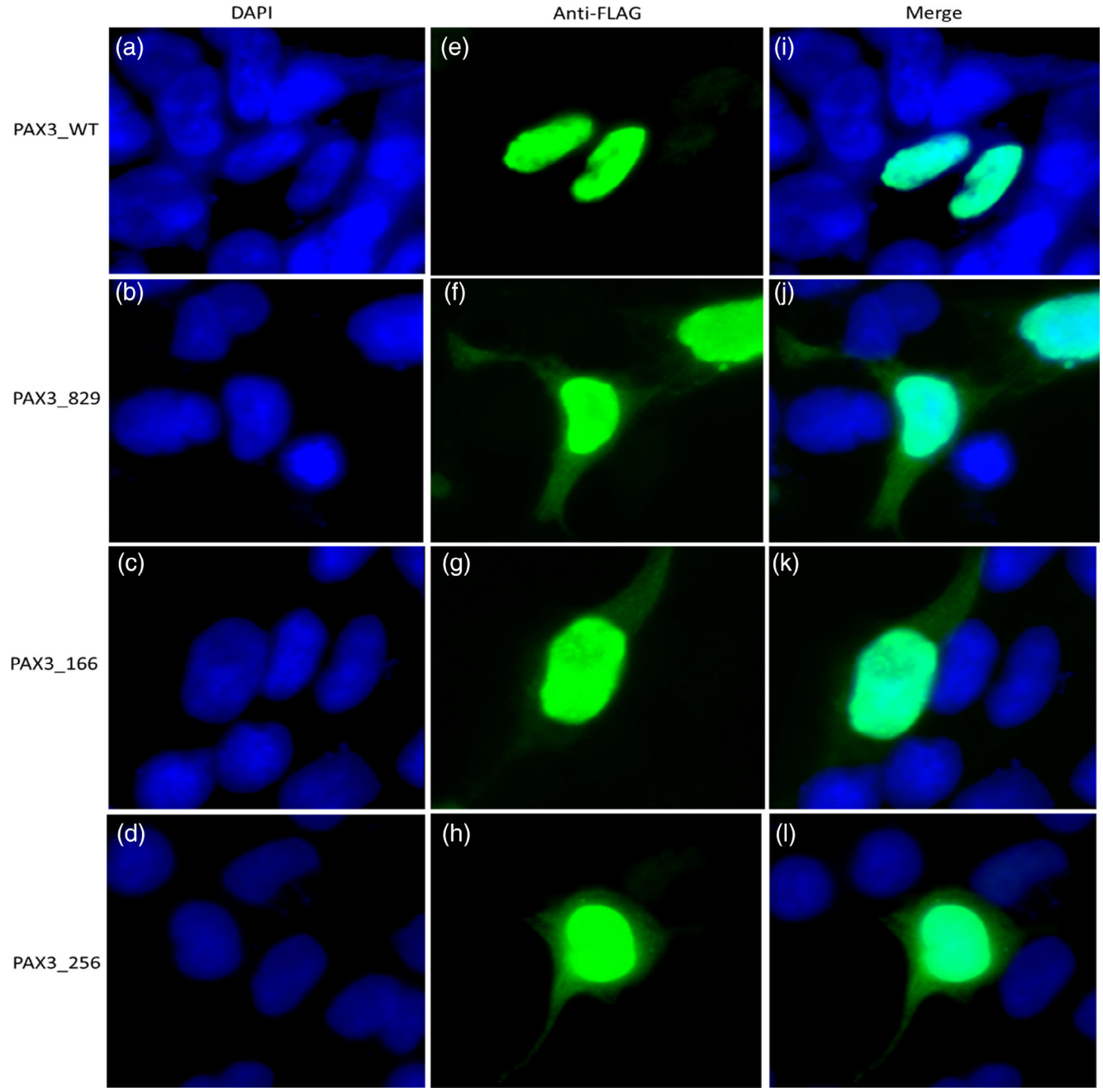

FIGURE 3 Subcellular localization of PAX3 protein in HEK293T cells. Wild type, PAX3_WT (e) and mutants, PAX3_829, PAX3_256 and PAX3_166 (f–h) are shown in green. Nuclei were stained with DAPI shown in blue (a–d). Merged images (i–l). PAX3_WT localizes to the nucleus, whereas mutants are distributed both in the nucleus and the cytoplasm [Color figure can be viewed at wileyonlinelibrary.com]

significant variants in *PAX3* or any other genes were identified in him, highlighting the lack of a clear genotype–phenotype correlation for *PAX3* related WS.

4.2 | Co-existing two genotypes of WS in a single individual

Pathogenic variants in *PAX3* and *EDNRB* are known to cause WS1 and WS4, respectively. Individuals with WS caused due to *PAX3* or *EDNRB* variants have common clinical findings of congenital SNHL, heterochromia iridis, and hypopigmentation of hair and skin. Telecanthus is specific to *PAX3* related WS and HD is known to occur in most cases of WS due to *EDNRB* pathogenic variants. Recently, *EDNRB* has been reported to cause WS2, that is, WS without HD (Issa et al., 2017). The proband (P1) of Family 1, clinically diagnosed with WS1 was found to have pathogenic variants in *PAX3* (c.166C > T) and *EDNRB* (c.1047delC). His family members (P3, P4, P5) having either *PAX3* or *EDNRB* variants showed subtle clinical features when compared to P1. P2 despite having pathogenic variants in *PAX3* was nonpenetrant.

However, detailed clinical evaluation could not be carried out for him. None of the individuals with the pathogenic variant in *EDNRB* had HD. This is the first report with the coexistence of *PAX3* and *EDNRB* genotypes in a single individual with WS. Previously, an individual with two coexisting WS genotypes has been reported with pathogenic variants in *PAX3* and *MITF*. He had WS with more severe pigmentory abnormalities than that noted in parents carrying one pathogenic variant each (Yang et al., 2013).

4.3 | Multiple genetic diagnoses

P6 was clinically diagnosed with WS3 and we identified a novel pathogenic variant, c.829C>T in *PAX3* in the heterozygous state. However, this variant was not present in his mother (P7) with congenital hearing loss. Since *GJB2* accounts for approximately 50% for all hearing loss cases, sequencing of *GJB2* was carried out in mother and proband. A known pathogenic variant, c.71G>A in a homozygous state was observed in both of them (Angeli, 2008; Snoeckx et al., 2005). Since the WS3 phenotype is inclusive of the DFNB1A phenotype, multiple diagnoses in the proband would not have been detected if testing of the parent would not have been carried out. Phenotypic variability and nonpenetrance could not be estimated as the samples of the father and other affected members of the family were not available for testing. Previously, multiple genetic diagnosis of WS-albinism and WS-DFNB1A have been reported, exemplifying this process (Morell et al., 1997; Yan et al., 2011).

4.4 | Functional validation of variants in *PAX3*

The DNA binding domains of PAX3 protein, paired domain and homeodomain are encoded by exons 2–4 and exons 5–6 respectively. They bind to the major groove of DNA and palindromic DNA sequences as homo or heterodimer respectively (Apuzzo & Gros, 2007; Corry et al., 2010; Wilson, Sheng, Lecuit, Dostatni, & Desplan, 1993). Nuclear localization signal (NLS) is present at the N-terminal of the homeodomain (Corry, Hendzel, & Underhill, 2008). Recently, a study identified a novel NLS was in the paired domains of the in PAX6 protein, which is structurally similar to PAX3 protein (Tabata, Koinui, Ogura, Nishihara, & Yamamoto, 2018). Functional consequences of the two novel variants, c.166C>T (PAX3_166) in exon 2 and c.829C>T (PAX3_829) in exon 6 of *PAX3* identified in this study and a previously reported variant, c.256A>T (PAX3_256) in exon 2 of *PAX3* were studied by luciferase reporter and immunofluorescence assays. The variant, c.829C>T (PAX3_829) significantly diminished PAX3 transactivation ability and showed increased cytoplasmic retention compared to the wild-type protein (Figures 2 and 3). However, this variant might trigger nonsense-mediated decay in vivo. Since we were unable to perform NMD assay for it this cannot be confirmed. In the event that this protein is indeed undergoing NMD, due to overexpression in our in vitro system its levels were likely in excess of that efficiently processed by the NMD machinery. Hence, through our experiments, a diminished PAX3 transactivation ability could be validated for this variant. The other two variants, c.166C>T (PAX3_166) and c.256A>T (PAX3_256) did not affect PAX3 activity. However, they showed elevated cytoplasmic distribution reflecting defective nuclear translocation of the mutant proteins (Figures 2 and 3).

In conclusion, our study further delineates the complexities of *PAX3*-related WS syndrome and adds to the spectrum of phenotypic and genetic diversity of WS.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Puneeth H. Somashekar collected the data, performed the experiments and drafted the article. Anju Shukla conceptualized and supervised the research, acquired funding, performed clinical evaluation and reviewed the article. Priyanka Upadhyai supervised experiments, reviewed and revised the article. Katta Mohan Girisha aided conception, clinical evaluation and provided critical inputs for article. Dhanya Lakshmi Narayanan, Nutan Kamath, Shruti Bajaj provided clinical information and critical reviews for the article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from corresponding author upon reasonable request.

ORCID

Katta M. Girisha https://orcid.org/0000-0002-0139-8239
Anju Shukla https://orcid.org/0000-0003-2471-4094

REFERENCES

Angeli, S. I. (2008). Phenotype/genotype correlations in a DFNB1 cohort with ethnical diversity. *The Laryngoscope*, *118*(11), 2014–2023. https://doi.org/10.1097/MLG.0b013e31817fb7ad

Apuzzo, S., & Gros, P. (2007). Cooperative interactions between the two DNA binding domains of Pax3: Helix 2 of the paired domain is in the proximity of the amino terminus of the homeodomain. *Biochemistry*, *46*(11), 2984–2993. https://doi.org/10.1021/bi062107q

Bocángel, M. A. P., Melo, U. S., Alves, L. U., Pardono, E., Lourenço, N. C. V., Marcolino, H. V. C., … Mingroni-Netto, R. C. (2018). Waardenburg syndrome: Novel mutations in a large Brazilian sample. *European Journal of Medical Genetics*, *61*(6), 348–354. https://doi.org/10.1016/j.ejmg.2018.01.012

Bondurand, N., Pingault, V., Goerich, D. E., Lemort, N., Sock, E., Caignec, C. L., … Goossens, M. (2000). Interaction among SOX10, PAX3 and MITF, three genes altered in Waardenburg syndrome. *Human Molecular Genetics*, *9*(13), 1907–1917. https://doi.org/10.1093/hmg/9.13.1907

Chen, H., Jiang, L., Xie, Z., Mei, L., He, C., Hu, Z., … Feng, Y. (2010). Novel mutations of PAX3, MITF, and SOX10 genes in Chinese patients with type I or type II Waardenburg syndrome. *Biochemical and Biophysical Research Communications*, *397*(1), 70–74. https://doi.org/10.1016/j.bbrc.2010.05.066

Corry, G. N., Hendzel, M. J., & Underhill, D. A. (2008). Subnuclear localization and mobility are key indicators of PAX3 dysfunction in Waardenburg syndrome. *Human Molecular Genetics*, *17*(12), 1825–1837. https://doi.org/10.1093/hmg/ddn076

Corry, G. N., Raghuram, N., Missiaen, K. K., Hu, N., Hendzel, M. J., & Underhill, D. A. (2010). The PAX3 paired domain and Homeodomain function as a single binding module in vivo to regulate subnuclear localization and mobility by a mechanism that requires base-specific recognition. *Journal of Molecular Biology*, *402*(1), 178–193. https://doi.org/10.1016/j.jmb.2010.07.016

Farrer, L. A., Grundfast, K. M., Amos, J., Arnos, K. S., Asher, J. H., Beighton, P., … Friedman, T. B. (1992). Waardenburg syndrome (WS) type I is caused by defects at multiple loci, one of which is near ALPP on chromosome 2: First report of the WS consortium. *American Journal of Human Genetics*, *50*(5), 902–913.

Galibert, M. D., Yavuzer, U., Dexter, T. J., & Goding, C. R. (1999). Pax3 and regulation of the melanocyte-specific tyrosinase-related protein-1 promoter. *The Journal of Biological Chemistry*, *274*(38), 26894–26900. https://doi.org/10.1074/jbc.274.38.26894

Hart, J., & Miriyala, K. (2017). Neural tube defects in Waardenburg syndrome: A case report and review of the literature. *American Journal of Medical Genetics. Part A*, *173*(9), 2472–2477. https://doi.org/10.1002/ajmg.a.38325

Issa, S., Bondurand, N., Faubert, E., Poisson, S., Lecerf, L., Nitschke, P., … Pingault, V. (2017). EDNRB mutations cause Waardenburg syndrome type II in the heterozygous state. *Human Mutation*, *38*(5), 581–593. https://doi.org/10.1002/humu.23206

Jalilian, N., Tabatabaiefar, M. A., Farhadi, M., Bahrami, T., & Noori-Daloii, M. R. (2015). A novel mutation in the PAX3 gene causes Waardenburg syndrome type I in an Iranian family. *International Journal of Pediatric Otorhinolaryngology*, *79*(10), 1736–1740. https://doi.org/10.1016/j.ijporl.2015.07.039

Klein, D., & Opitz, J. M. (1983). Historical background and evidence for dominant inheritance of the Klein-Waardenburg syndrome (type III). *American Journal of Medical Genetics*, *14*(2), 231–239. https://doi.org/10.1002/ajmg.1320140205

Matsunaga, T., Mutai, H., Namba, K., Morita, N., & Masuda, S. (2013). Genetic analysis of PAX3 for diagnosis of Waardenburg syndrome type I. *Acta Oto-Laryngologica*, *133*(4), 345–351. https://doi.org/10.3109/00016489.2012.744470

Milunsky, J. M. (1993). Waardenburg Syndrome Type I. In M. P. Adam, H. H. Ardinger, R. A. Pagon, S. E. Wallace, L. J. Bean, K. Stephens, & A. Amemiya (Eds.), *GeneReviews®*. Seattle (WA): University of Washington, Seattle. Retrieved from http://www.ncbi.nlm.nih.gov/books/NBK1531/

Minami, S. B., Nara, K., Mutai, H., Morimoto, N., Sakamoto, H., Takiguchi, T., … Matsunaga, T. (2019). A clinical and genetic study of 16 Japanese families with Waardenburg syndrome. *Gene*, *704*, 86–90. https://doi.org/10.1016/j.gene.2019.04.023

Morell, R., Spritz, R. A., Ho, L., Pierpont, J., Guo, W., Friedman, T. B., & Asher, J. H. (1997). Yan. *Human Molecular Genetics*, *6*(5), 659–664. https://doi.org/10.1093/hmg/6.5.659

Mousty, E., Issa, S., Grosjean, F., Col, J.-Y., Khau Van Kien, P., Perez, M.-J., … Pingault, V. (2015). A homozygous PAX3 mutation leading to severe presentation of Waardenburg syndrome with a prenatal diagnosis. *Prenatal Diagnosis*, *35*(13), 1379–1381. https://doi.org/10.1002/pd.4703

Nayak, C. S., & Isaacson, G. (2003). Worldwide distribution of Waardenburg syndrome. *The Annals of Otology, Rhinology, and Laryngology*, *112*(9 Pt 1), 817–820. https://doi.org/10.1177/000348940311200913

Niu, Z., Li, J., Tang, F., Sun, J., Wang, X., Jiang, L., … He, C. (2018). Identification and functional analysis of a novel mutation in the PAX3 gene associated with Waardenburg syndrome type I. *Gene*, *642*, 362–366. https://doi.org/10.1016/j.gene.2017.11.035

Pingault, V., Ente, D., Dastot-Le Moal, F., Goossens, M., Marlin, S., & Bondurand, N. (2010). Review and update of mutations causing Waardenburg syndrome. *Human Mutation*, *31*(4), 391–406. https://doi.org/10.1002/humu.21211

Read, A. P., & Newton, V. E. (1997). Waardenburg syndrome. *Journal of Medical Genetics*, *34*(8), 656–665. https://doi.org/10.1136/jmg.34.8.656

Saberi, M., Golchehre, Z., Salmani, H., Karamzade, A., Tabatabaie, S. Z., & Keramatipour, M. (2018). First report of Klein-Waardenburg syndrome in Iran and a novel pathogenic splice site variant in PAX3 gene. *International Journal of Pediatric Otorhinolaryngology*, *113*, 229–233. https://doi.org/10.1016/j.ijporl.2018.08.009

Snoeckx, R. L., Huygen, P. L. M., Feldmann, D., Marlin, S., Denoyelle, F., Waligora, J., … Van Camp, G. (2005). GJB2 mutations and degree of hearing loss: A multicenter study. *American Journal of Human Genetics*, *77*(6), 945–957. https://doi.org/10.1086/497996

Somashekar, P. H., Girisha, K. M., Nampoothiri, S., Gowrishankar, K., Devi, R. R., Gupta, N., … Shukla, A. (2019). Locus and allelic heterogeneity and phenotypic variability in Waardenburg syndrome. *Clinical Genetics*, *95*(3), 398–402. https://doi.org/10.1111/cge.13468

Song, J., Feng, Y., Acke, F. R., Coucke, P., Vleminckx, K., & Dhooge, I. J. (2016). Hearing loss in Waardenburg syndrome: A systematic review: Hearing loss in Waardenburg syndrome. *Clinical Genetics*, *89*(4), 416–425. https://doi.org/10.1111/cge.12631

Stenson, P. D., Ball, E. V., Mort, M., Phillips, A. D., Shiel, J. A., Thomas, N. S. T., … Cooper, D. N. (2003). Human gene mutation database (HGMD®): 2003 update. *Human Mutation*, *21*(6), 577–581. https://doi.org/10.1002/humu.10212

Tabata, H., Koinui, A., Ogura, A., Nishihara, D., & Yamamoto, H. (2018). A novel nuclear localization signal spans the linker of the two DNA-binding subdomains in the conserved paired domain of Pax6. *Genes & Genetic Systems*, *93*(2), 75–81. https://doi.org/10.1266/ggs.17-00057

Tassabehji, M., Newton, V. E., Liu, X. Z., Brady, A., Donnai, D., Krajewska-Walasek, M., … Reardon, W. (1995). The mutational spectrum in Waardenburg syndrome. *Human Molecular Genetics*, *4*(11), 2131–2137. https://doi.org/10.1093/hmg/4.11.2131

Tekin, M., Bodurtha, J. N., Nance, W. E., & Pandya, A. (2001). Waardenburg syndrome type 3 (Klein-Waardenburg syndrome) segregating with a heterozygous deletion in the paired box domain of PAX3: A simple variant or a true syndrome? *Clinical Genetics*, *60*(4), 301–304. https://doi.org/10.1034/j.1399-0004.2001.600408.x

Vega-Lopez, G. A., Cerrizuela, S., Tribulo, C., & Aybar, M. J. (2018). Neurocristopathies: New insights 150 years after the neural crest discovery. *Developmental Biology*, *444*(Suppl. 1), S110–S143. https://doi.org/10.1016/j.ydbio.2018.05.013

Wildhardt, G., Zirn, B., Graul-Neumann, L. M., Wechtenbruch, J., Suckfüll, M., Buske, A., … Steinberger, D. (2013). Spectrum of novel mutations found in Waardenburg syndrome types 1 and 2: Implications for molecular genetic diagnostics. *BMJ Open*, *3*(3), e001917. https://doi.org/10.1136/bmjopen-2012-001917

Wilson, D., Sheng, G., Lecuit, T., Dostatni, N., & Desplan, C. (1993). Cooperative dimerization of paired class homeo domains on DNA. *Genes & Development*, *7*(11), 2120–2134. https://doi.org/10.1101/gad.7.11.2120

Wollnik, B., Tukel, T., Uyguner, O., Ghanbari, A., Kayserili, H., Emiroglu, M., & Yuksel-Apak, M. (2003). Homozygous and heterozygous inheritance of PAX3 mutations causes different types of

Waardenburg syndrome. *American Journal of Medical Genetics. Part A*, *122A*(1), 42–45. https://doi.org/10.1002/ajmg.a.20260

Yan, X., Zhang, T., Wang, Z., Jiang, Y., Chen, Y., Wang, H., … Li, H. (2011). A novel mutation in the MITF may be digenic with GJB2 mutations in a large Chinese family of Waardenburg syndrome type II. *Journal of Genetics and Genomics = Yi Chuan Xue Bao*, *38*(12), 585–591. https://doi.org/10.1016/j.jgg.2011.11.003

Yang, S., Dai, P., Liu, X., Kang, D., Zhang, X., Yang, W., … Yuan, H. (2013). Genetic and phenotypic heterogeneity in Chinese patients with Waardenburg syndrome type II. *PLoS One*, *8*(10), e77149. https://doi.org/10.1371/journal.pone.0077149

Zlotogora, J., Lerer, I., Bar-David, S., Ergaz, Z., & Abeliovich, D. (1995). Homozygosity for Waardenburg syndrome. *American Journal of Human Genetics*, *56*(5), 1173–1178.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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