Received: 13 January 2020 | Revised: 17 February 2020 | Accepted: 24 February 2020

DOI: 10.1002/mgg3.1217

ORIGINAL ARTICLE

Molecular Genetics & Genomic Medicine Open Access WILEY

Two novel mutations of *PAX3* and *SOX10* were characterized as genetic causes of Waardenburg Syndrome

Yongbo Yu[1] | **Wei Liu**[2] | **Min Chen**[2] | **Yang Yang**[2] | **Yeran Yang**[1,3] | **Enyu Hong**[1] | **Jie Lu**[1,3] | **Jun Zheng**[2] | **Xin Ni**[2] | **Yongli Guo**[1,3] | **Jie Zhang**[2]

[1]Beijing Key Laboratory for Pediatric Diseases of Otolaryngology, Head and Neck Surgery, Beijing Pediatric Research Institute, Beijing Children's Hospital, Capital Medical University, National Center for Children's Health (NCCH), Beijing, China

[2]Department of Otolaryngology, Head and Neck Surgery, Beijing Children's Hospital, Capital Medical University, National Center for Children's Health (NCCH), Beijing, China

[3]Beijing Advanced Innovation Center for Big Data-Based Precision Medicine, Beihang University & Capital Medical University, Beijing, China

Correspondence

Yongli Guo, Beijing Key Laboratory for Pediatric Diseases of Otolaryngology, Head and Neck Surgery, Beijing Pediatric Research Institute, Beijing Children's Hospital, Capital Medical University, National Center for Children's Health (NCCH), Beijing, 100045, P.R. China.
Email: guoyongli@bch.com.cn

Jie Zhang, Department of Otolaryngology, Head and Neck Surgery, Beijing Children's Hospital, Capital Medical University, National Center for Children's Health (NCCH), Beijing, 100045, P.R. China.
Email: stzhangj@263.net

Funding information

Beijing Natural Science Foundation Program and Scientific Research Key Program of Beijing Municipal Commission of Education, Grant/Award Number: KZ201810025034; National Natural

Abstract

Background: The objective of this study was to investigate the genetic causes of two probands diagnosed as Waardenburg syndrome (WS type I and IV) from two unrelated Chinese families.

Methods: *PAX3* and *SOX10* were the main pathogenic genes for WS type I (WS I) and IV (WS IV), respectively; all coding exons of these genes were sequenced on the two probands and their family members. Luciferase reporter assay and co-immunoprecipitation (CO-IP) were conducted to verify potential functional outcomes of the novel mutations.

Results: The first proband is a 9 years old girl diagnosed with WS I. A novel *PAX3* heterozygous mutation of c.372-373delGA (p.N125fs) was identified, which results in a frameshift and truncation of *PAX3* protein. In family II, a 2 years old girl was diagnosed with WS IV, and Sanger sequencing revealed a de novo *SOX10* mutation of c.1114insTGGGGCCCCCACACTACACCGAC (p.Q372fs), a frameshift mutation that extends the amino acid chain of *SOX10* protein. Functional studies indicated that the novel mutation of *SOX10* had no effects on the interaction of *SOX10* and *PAX3*, but reduced transactivate capacity of melanocyte inducing transcription factor (*MITF*) promoter. Both *PAX3* and *SOX10* mutation-induced defects of *MITF* transcription might contribute to the WS pathogenesis.

Conclusion: We revealed a novel mutation in *PAX3* and a de novo mutation in *SOX10*, which might account for the underlying pathogenesis of WS. This study expands the database of both *PAX10* and *PAX3* mutations and improves our understanding of the causes of WS.

KEYWORDS

hearing loss, *PAX3*, *SOX10*, Waardenburg syndrome

Yongbo Yu and Wei Liu contribute equally.

Science Foundation of China, Grant/Award Number: 81702463; Beijing Advanced Innovation Center for Big Data-Based Precision Medicine, Beihang University & Capital Medical University, Beijing, Grant/Award Number: BHME- 201804

1 | INTRODUCTION

Waardenburg syndrome (WS) is a rare autosomal dominantly inherited disease, which is characterized by hearing loss and pigment alteration in hair, skin, and iris (Read & Newton, 1997). The incidence of WS is estimated 1/40 000; however, it accounts for 2% to 5% of all congenital hearing loss (Dourmishev, Dourmishev, Schwartz, & Janniger, 1999; Song et al., 2016; Zaman, Capper, & Baddoo, 2015), which is the most common clinical feature of WS. WS is divided into four subtypes (WS I-IV) depending on the presence or absence of additional symptoms such as dystopia canthorum, giant colon, and upper limb abnormalities. WS I (OMIM #193500) and WS II (OMIM #193510) are the most common types, while WS III (OMIM #148820) and WS IV (OMIM #277580) are rare (Read & Newton, 1997). WS is genetically different and six genes have been identified: paired box 3 (PAX3), SRY-box transcription factor 10 (*SOX10*), melanocyte inducing transcription factor (*MITF*), endothelin receptor type B (*EDNRB*), endothelin 3 (*EDN3*), and snail family transcriptional repressor 2 (*SNAI2*) (Otreba, Milinski, Buszman, Wrzesniok, & Beberok, 2013). *PAX3* is associated with WS I and WS III. *MITF* and *SNAI2* are found to be involved in the WS II. *SOX10*, *EDNRB*, and *EDN3* are responsible for WS II and WS IV (Pingault et al., 2010). All these genes play crucial roles in the formation and development of melanocytes.

WS with dystopia canthorum is diagnosed as WS I and *PAX3* is the most pathogenic gene. *PAX3* encodes the paired box 3 transcription factor and contributes to the development of the central nervous system, skeletal muscle, and melanocytes (Wildhardt et al., 2013). For WS IV, it is characterized by additional feature of megacolon syndrome. In humans, 45%-55% of WS IV cases are involved in the mutation of *SOX10*, which works in the development and differentiation of melanocytes (Bondurand et al., 2007). Up to date, dozens of mutations in *PAX3* and *SOX10* have been identified, including missense mutations, frameshift mutations, and insertions/deletions (Bocangel et al., 2018; Ma et al., 2016). Great efforts are still been made to discover novel mutations in recent studies (Hogan et al., 2019; Ma, Lin, et al., 2019; Ma, Zhang, et al., 2019). Functional analysis also reported that these mutants might induce loss of DNA-binding ability, failed to transactivate *MITF* promoter, and inhibited melanin synthesis (Wang et al., 2017; Zhang et al., 2012). Although mutations in *PAX3* and *SOX10* are the most common genetic cause of WS I and WS IV, respectively, mutational spectrum still needs to be extended to understand the genetic causes of WS pathogenesis.

In the present study, *PAX3* and *SOX10* were individually screened in two Chinese probands with WS I and WS IV, as well as their family members. A novel mutation in *PAX3* [c.372-373delGA (p.N125fs)] and a de novo mutation in *SOX10* [c.1114insTGGGGCCCCCACACTACACCGAC (p.Q372fs)] were identified by Sanger sequencing. The mutation of N125fs resulted in truncated *PAX3* while Q372fs caused structural extension of *SOX10*. To understand functional consequences of Q372fs-induced *SOX10* extension, transcriptional activity of *MITF* was detected by dual-luciferase reporter assay. The effects of mutant *SOX10* on the interaction between *SOX10* and *PAX3* were studied using co-immunoprecipitation assay.

2 | MATERIALS AND METHODS

2.1 | Patients

Two Chinese girls with severe-profound hearing loss and their family members were recruited. Photos and blood were collected before informed consent was signed. Ethics Committees of Beijing Children's Hospital approved our study. After medical history was described by their parents, the two girls got both physical and radiological examination. Auditory evaluations were conducted by play audiometry (PA), auditory steady-state response (ASSR), auditory brainstem response (ABR), and distortion product otoacoustic emission (DPOAE).

2.2 | Gene mutation analysis

Blood DNA was extracted from the probands and their family members using Blood DNA Kit (CWBIO, China). Polymerase chain reaction (PCR) was used to amplify fragments covering coding exons of *PAX3* (NM_181457.3) and *SOX10* (NM_006941.4) with specific primers (Table S1). PCR experiment was done as previously described (Yu et al., 2019). All PCR products were evaluated by 1% agarose gel electrophoresis and sequenced by Sanger sequencing (Applied Biosystems, USA). Gene mutation was identified by Sequencher software.

2.3 | Plasmids construction

To generate plasmids of pGV230-PAX3-HA and pGV362-*SOX10*-Flag, full-length cDNA of *PAX3* and *SOX10* were PCR amplified and subcloned into their corresponding vectors. For pGV362-*SOX10*-Q372fs-Flag construction, frameshift mutation (c.1114ins TGGGGCCCCCACACTACACCGAC) was synthesized and subcloned into the vector. To construct pGV238-*MITF* luciferase reporter, *MITF* promoter region (−1500 bp to +50 bp from transcription start site) was synthesized and subcloned into the luciferase vector. All constructs were verified by direct Sanger sequencing.

2.4 | Dual-luciferase reporter assay

Human melanoma A375 cell line was cultured in DMEM with 10% fetal bovine serum. Cells were seeded in 24-well plates for 24 hr and then transfected by *MITF* luciferase reporter together with *SOX10*-wt-Flag or *SOX10*-Q372fs-Flag using Lipofectamine 2000 (Life Technologies). Renilla luciferase reporter plasmid was co-transfected to normalize transfection efficiencies. Luciferase activity was finally measured (CLARIOstar, BMG labtech) using Dual-Luciferase Reporter Assay system (Promega).

2.5 | Co-immunoprecipitation (CO-IP) and western blotting

SOX10 interacts with *PAX3* to regulate *MITF* expression. To study the interaction between *SOX10* and *PAX3*, Co-IP was performed. Briefly, *PAX3*-HA plasmid was co-transfected with *SOX10*-wt-Flag or *SOX10*-Q372fs-Flag in A375 cells for 48 hr. Total proteins were extracted and concentration was determined. Equal amounts of proteins were incubated with anti-HA antibody (Abcam) overnight at 4°C. Commercial protein A agarose beads (Roche, Switzerland) were added and incubated for 4 hr at room temperature. The beads were washed, collected, and resolved repeatedly in chilled GUO HEPES buffer for six times. Finally, the beads were boiled for western blotting detection, according to standard protocol previously described (Yu et al., 2018). Protein bands were obtained by Odyssey CLx imaging system (LI-COR).

3 | RESULTS

3.1 | Clinical features and evaluation

These two girls were from two unrelated Chinese families. Both of them delayed in speech development and diagnosed

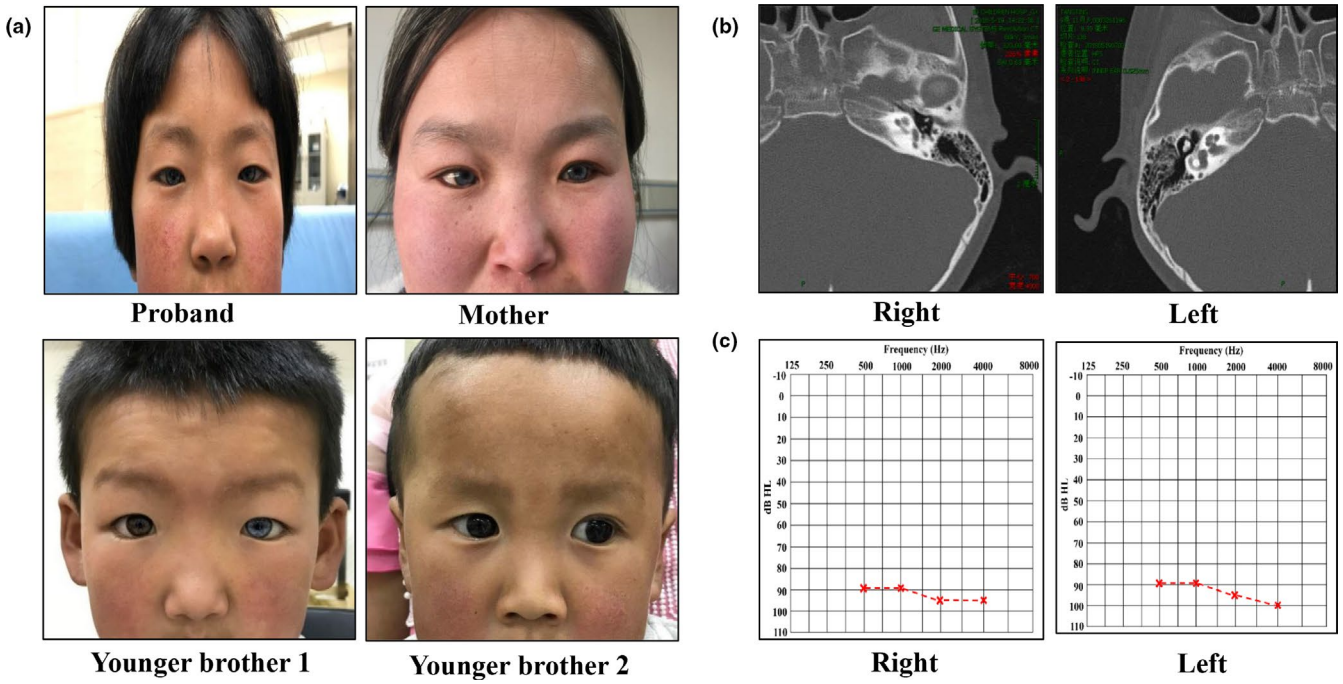

FIGURE 1 Clinical phenotypes in family diagnosed with WS I. (a) Iris heterochromia in the eyes, dystopia canthorum, and a pinch of white hair on forehead of the proband; Bright blue irides in eyes and unilateral severe hearing loss of the mother; Iris heterochromia in the left eye of one younger brother with normal hearing; Normal younger brother. (b) Temporal bone CT scan showed that the structure of the inner ear was normal. (c) Bilateral play audiometry (PA) detection. The *x*-axis indicates frequency in hertz (Hz) and the *y*-axis indicates hearing level in decibels (dB nHL)

as bilateral hearing loss. The first proband was 9 years old and diagnosed with WS I. As shown in Figure 1a, physical examination found iris heterochromia in eyes, a pinch of white hair on forehead, and dystopia canthorum. Symptoms of iris heterochromia in eyes and unilateral severe hearing loss were also found in her mother. For one of her younger brothers, iris heterochromia was in left eye with normal hearing. The other younger brother was normal. Temporal bone CT scan showed that the structure of the inner ear of the girl was normal (Figure 1b). However, she suffered from severe sensorineural hearing loss in both ears (Figure 1c). The second proband was 2 years old diagnosed with WS IV. Both her parents were normal. Physical examination found iris heterochromia in her right eye and her hair was gray (Figure 2a). She also suffered from Hirschsprung disease and got surgery when she was 2 months old. Both CT and MRI scan showed that the structure of the inner ear and cochlear nerve was normal (Figure 2b). Based on audiometry (PA) detection, her both ears got severe hearing loss (Figure 2c). Both probands have received unilateral cochlear implantation (CI) and were in our close follow-up.

3.2 | Identification of novel mutations in *PAX3* and *SOX10*

The pedigree chart of family I was described in Figure 3a. As shown in Figure 3b, novel heterozygous mutations in both intron and exon of *PAX3* were detected. Based on the fact that genotype was in accordance with clinical appearance, c.372-373delGA (p.N125fs) in exon 3 might be the pathogenic mutation. This mutation caused a stop codon at position of 143 amino acid and truncated *PAX3* (Figure 3c). The protein truncation retains most of the paired domain (PD) but deletes other domains (Figure 3d), which might be crucial for *PAX3* function.

In family II, a fragment of 23bp was inserted in exon 4 of *SOX10* at site of 1114bp (Figure 4a,b). It is a de novo mutation because c.1114insTGGGGCCCCCACACTACACCGAC (p.Q372fs) only occurred in the proband but not inherited from her parents. It caused a frameshift mutation of *SOX10* from position of 372 to 508 amino acid, which is 41 amino acids longer than wide-type *SOX10* (Figure 4c). Functionally, the structural extension might affect its binding with *PAX3*. The schematic image of *SOX10* protein and its mutant is described in Figure 4d.

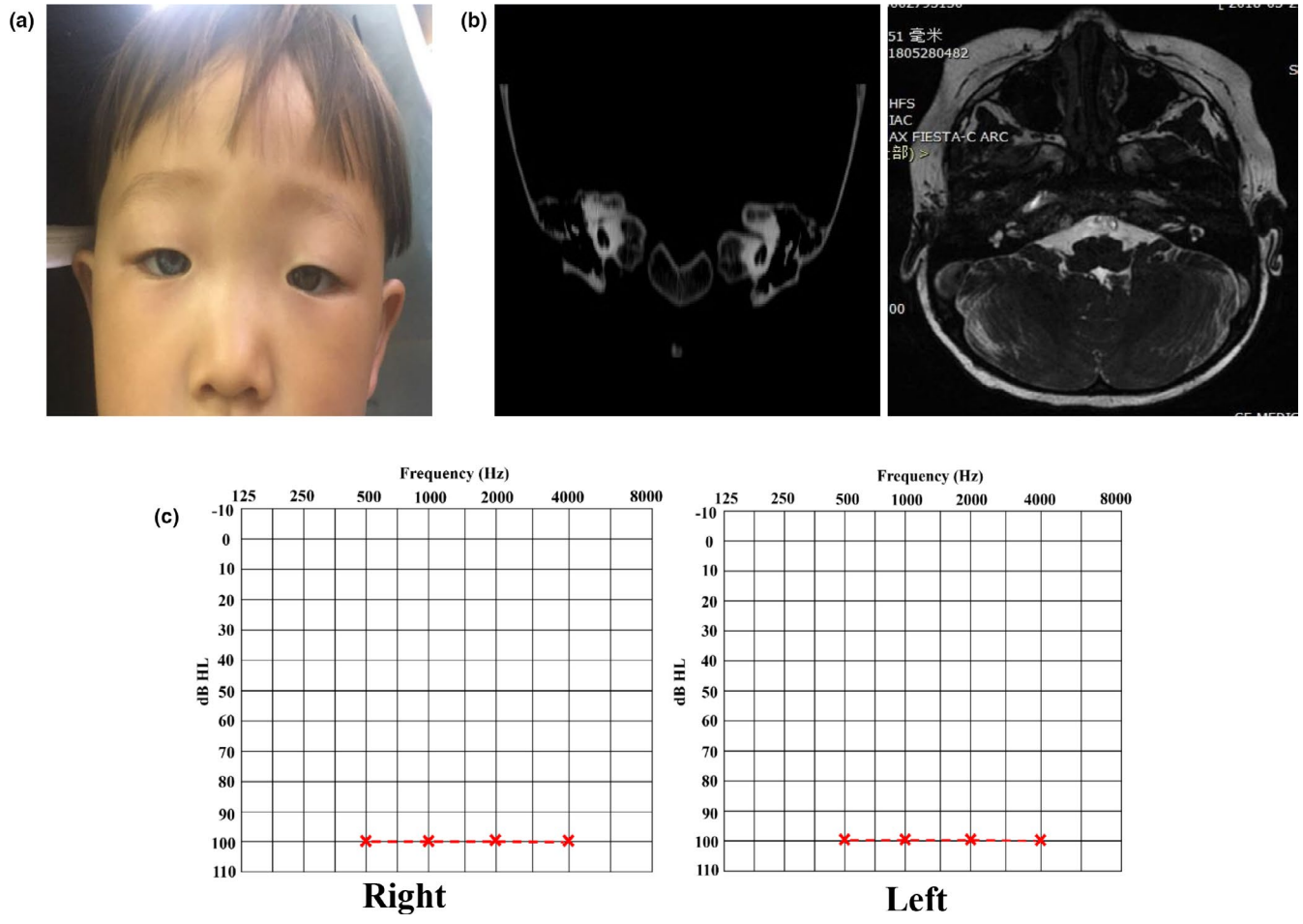

FIGURE 2 Clinical features of the proband diagnosed with WS IV. (a) Iris heterochromia in right eye and premature graying of the hair. (b) CT and MRI showed that the structure of the inner ear and cochlear nerve is normal. (c) Bilateral play audiometry (PA) detection

3.3 | The effect of mutant *SOX10* on *MITF* transcriptional activity

The role of *SOX10* in melanocyte is to regulate *MITF* expression together with *PAX3*. To identify whether Q372fs affects *SOX10* protein function, luciferase assay was performed. As shown in Figure 5a, wild-type *SOX10* induced *MITF* promoter activity by approximately fourfold than mutant *SOX10* in A375 cells. As a result, mutant *SOX10* loses its ability to activate *MITF* promoter.

3.4 | The effect of mutant *SOX10* on the interaction between *SOX10* and *PAX3*

We have demonstrated that mutant *SOX10* cannot activate *MITF* expression, but the molecular mechanism is not clear. Since *SOX10* interacts with *PAX3* to regulate *MITF* expression (Dai et al., 2019), the interaction changes might help in answering this issue. To investigate whether *SOX10* Q372fs affects the interaction between *SOX10* and *PAX3*, Co-IP assay was performed. As shown in Figure 5b, both wild-type *SOX10* and mutant *SOX10* can be co-immunoprecipitated when co-expressed with *PAX3* in A375 cells. This result suggested that Q372fs-induced *SOX10* protein extension did not affect its interaction with *PAX3*.

4 | DISCUSSION

Two patients from two unrelated families were diagnosed as WS I and WS IV, which is genetically associated with mutations in *PAX3* and *SOX10*, respectively. To investigate potential pathogenic causes, a genetic and functional analysis was performed. We identified a novel mutation of *PAX3* [c.372-373delGA (p.N125fs)] in proband I, which truncated *PAX3* and resulted in protein structure disruption. In proband II, the de novo mutation of *SOX10* [c.1114insTGGGGCCCC CACACTACACCGAC (p.Q372fs)] caused structural extension of *SOX10*. Further in vitro results demonstrated that the mutant *SOX10* inhibited transcriptional activity of *MITF* but not affected the interaction between *SOX10* and *PAX3*.

The first proband in family I was diagnosed as WS I, based on additional symptoms of dystopia canthorum and phenotypes

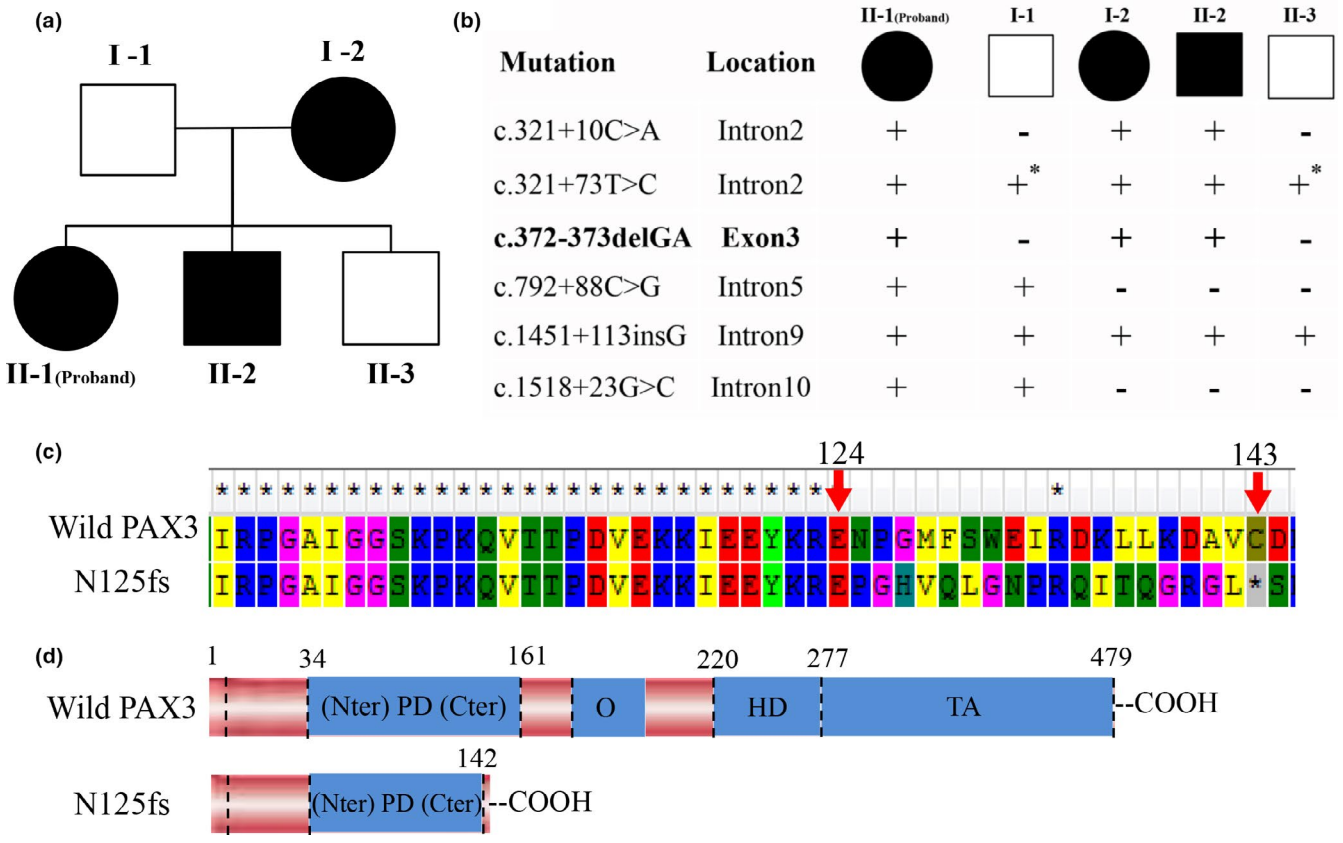

FIGURE 3 Pedigree map and *PAX3* (NM_181457.3) mutation detection in family I. (a) Pedigree map. Squares and circles denote males and females, respectively. (b) Mutations in exons and primer-covered introns of all family members. The "*" indicates homozygous mutation. (c) Protein alignment showed that c.372-373delGA (p.N125fs) induced a frameshift mutation, caused a stop codon at position of 143 amino acid, resulting in truncation of *PAX3*. (d) The putative schematic representation of *PAX3* protein and N125fs mutation, which lead to a large reduction in the *PAX3* protein from 479 amino acids to 142 amino acids

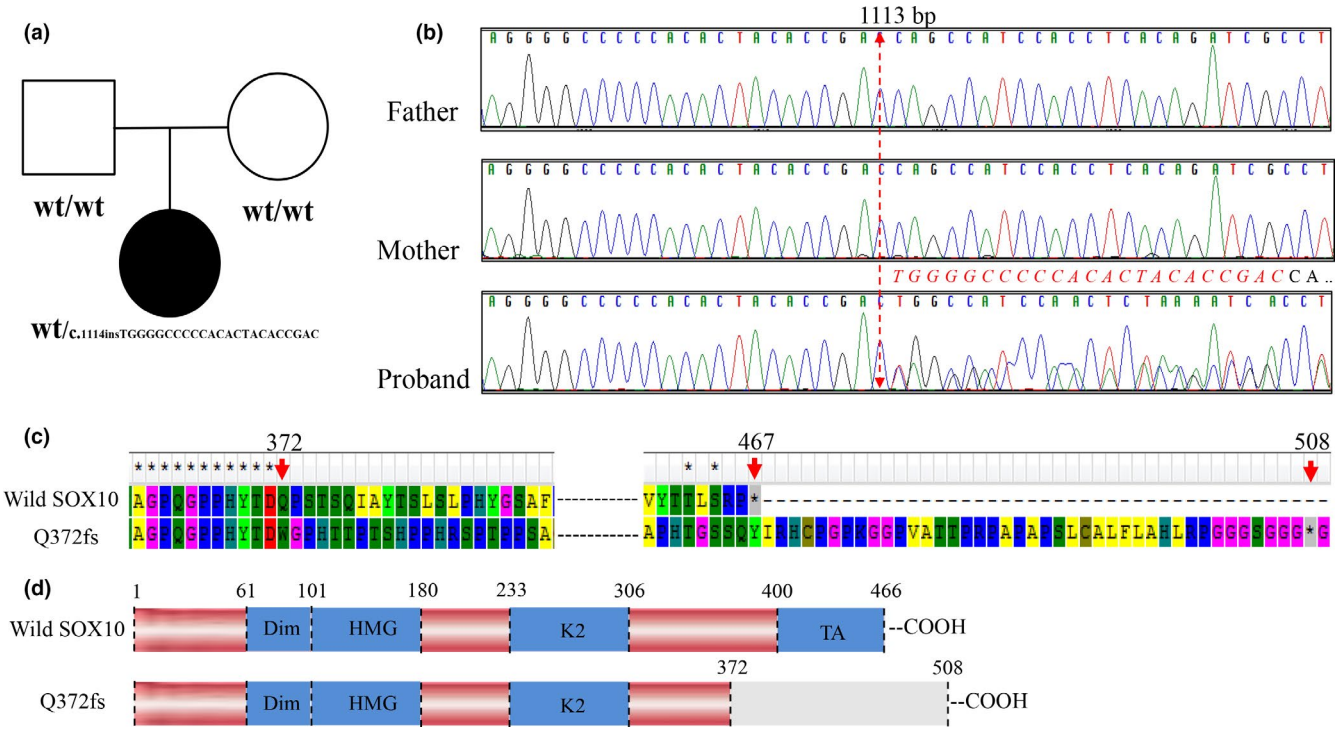

FIGURE 4 Pedigree map and *SOX10* (NM_006941.4) sequence in family II. (a) Pedigree map. Squares and circles denote males and females, respectively. (b) Sequence electropherograms showed that c.1114insTGGGGCCCCCACACTACACCGAC (p.Q372fs) was a de novo mutation, which was not inherited from parents. (c) Q372fs caused a frameshift mutation from position of 372 to 508 amino acid, which is 41 amino acids longer than wide-type *SOX10*. (d) The putative schematic representation of *SOX10* protein and the extended mutants.

in family members. Genetically, *PAX3* is the main causative gene of WS I, and 90% of WS I patients carry *PAX3* variants. Recently, many novel *PAX3* mutations were reported, such as c.91-95de1ACTCC, c.808C>G, and c.117C>A (Choi, Choi, & Lee, 2018; Li et al., 2019; Ma, Lin, et al., 2019). To verify the clinical diagnosis in proband I, genetic screening of *PAX3* was performed and c.372-373delGA (p.N125fs) was identified. This frameshift mutation changed *PAX3* structure and may result in loss of gene function. Up to March 2019, a total of 164 variants in *PAX3* have been reported in the Human Gene Mutation Database (HGMD), among which point mutations and deletions comprised more than 90% (Stenson et al., 2017). As no report of c.372-373delGA was found in the HGMD, we consider it is a novel mutation. This mutation detected in the proband and his brother came from their mother and was inherited autosomal dominantly.

PAX3 locates on chromosome 2q35, encoding a 479 amino acids protein, which is a transcriptional factor from the paired box (PAX) family (Boudjadi, Chatterjee, Sun, Vemu, & Barr, 2018). *PAX3* contributes to the migration and differentiation of melanocytes, which originate from the embryonic neural crest. In melanoblast, *PAX3* is associated with the expression of markers for melanocyte development, including *MITF* (Boudjadi et al., 2018; Dye, Medic, Ziman, & Coombe, 2013). Due to the fact that color change is a typical characteristic for WS, *PAX3* function in pigmentation defects of the hair, skin, and eye might account for the pathogenesis of WS I (Bocangel et al., 2018). Previous functional study has demonstrated that *PAX3* R270G mutation failed to activate *MITF* promoter but retained abilities of nuclear distribution and DNA-binding (Niu et al., 2018). However, another study reported that *PAX3* H80D can retain partial activity (Zhang et al., 2012). According to the guidelines from ACMG (American College of Medical Genetics and Genomics), one missense variant is known to be pathogenic in most cases. However, frameshift mutation was a null variant, which may disrupt gene function (Richards et al., 2015). Therefore, the new reported frameshift mutation of c.372-373delGA in *PAX3* might be the major molecular pathogenesis of WS I in this family.

With regard to the second proband in family II, a de novo mutation of c.1114insTGGGGCCCCCACACTACACCGAC (p.Q372fs) was found, which induced structural extension of *SOX10*. In humans, half of WS IV cases are associated with *SOX10* mutation and more than 160 variants have been reported in HGMD till March 2019 (Stenson et al., 2017). *SOX10* encodes a transcription factor and acts as a transcriptional activator to regulate *MITF* expression by forming a protein complex with *PAX3* (Kamachi, Cheah, & Kondoh, 1999; Seberg, Van Otterloo, & Cornell, 2017). Consistent with previous studies (Dai et al., 2019; Wang et al., 2017), our results demonstrated that Q372fs *SOX10* lost the ability to activate *MITF* expression. Generally, *SOX10* is localized in the nucleus and promotes target DNA transcription (Seberg et al., 2017).

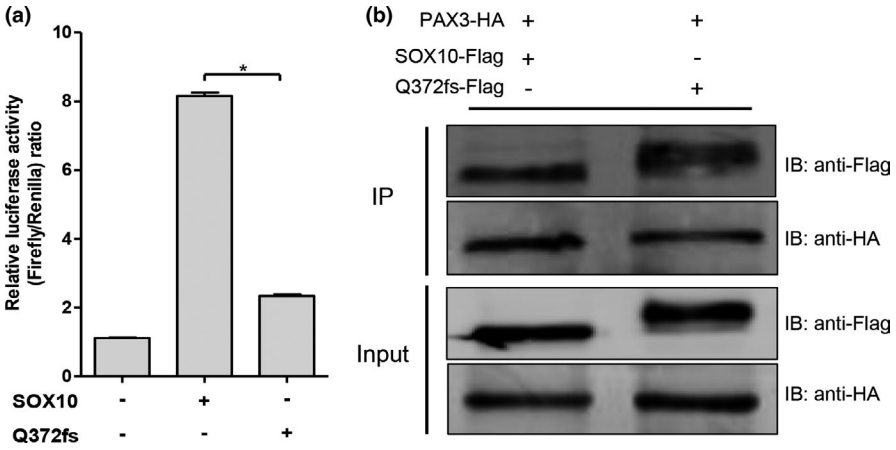

FIGURE 5 Functional analysis of *SOX10* Q372fs in A375 cells. (a) Transcriptional capacity of wide-type *SOX10* and its mutant detected by luciferase assays. (b) Protein–protein interaction between *PAX3* and wide-type/mutant *SOX10*, which is performed by Co-IP assay using anti-Flag and anti-HA antibodies. The "*" indicates $p < .05$

Functional study reported that *SOX10* mutation of p.L141P affected DNA or protein-binding capacity, and inhibited *MITF* expression by inducing aberrant cytoplasmic and nuclear localization (Wang et al., 2017). However, mutant *SOX10* was also reported to reduce *MITF* transcription but not affect nuclear localization and DNA-binding capacity (Dai et al., 2019). Therefore, *SOX10* function is not localization-dependent and Q372fs *SOX10*-induced suppression of MITF might be the genetic cause of WS IV.

Although we have demonstrated that mutant *SOX10* affected *MITF* expression, the molecular mechanism is not clear. Functionally, *SOX10* was reported to interact with *PAX3* to regulate *MITF* expression (Dai et al., 2019). To determine whether the reduced *MITF* transcription was caused by defects in the interaction between *PAX3* and *SOX10*, Co-IP was performed but the results showed that Q372fs *SOX10* did not affect the ability to interact with *PAX3*. Although the structure of *SOX10* is not fully characterized (Pingault et al., 1998), it structurally contains a DNA-binding HMG (high mobility group) domain, a dimerization region right upstream to the HMG, a conserved domain in the center, and a transactivation (TA) domain at the extreme C-terminus (Pingault et al., 2010). Herein, the frameshift mutation of Q372fs made *SOX10* protein extended in structure, destroying the TA domain but not affecting other domains. This might be the reason why Q372fs *SOX10* failed to transactivate *MITF*, but still can interact with *PAX3*.

In summary, we identified a novel mutation in *PAX3* and a de novo mutation in *SOX10* in two unrelated probands diagnosed with WS I and WS IV, respectively. In family I, the mutation of N125fs in *PAX3* leads to a frameshift mutation and truncates *PAX3* protein. In family Π, the de novo mutation of Q372fs caused structural extension of *SOX10*. Further in vitro results demonstrated that the Q372fs *SOX10* inhibited transcriptional activity of *MITF*, but not affected the interaction between *SOX10* and *PAX3*. Our finding is expected to expand the mutation spectra of *PAX3* and *SOX10*, which might be the genetic causes of WS pathogenesis.

ACKNOWLEDGMENTS

We are grateful to the family members for their participation in this study. This work was supported by Beijing Natural Science Foundation Program and Scientific Research Key Program of Beijing Municipal Commission of Education (KZ201810025034), the National Natural Science Foundation of China (No. 81702463), Beijing Advanced Innovation Center for Big Data-Based Precision Medicine, Beihang University & Capital Medical University, Beijing (BHME- 201804).

CONFLICT OF INTEREST

The authors declare they have no actual or potential competing financial interests.

ORCID

Yongbo Yu https://orcid.org/0000-0002-2717-4073

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Yu Y, Liu W, Chen M, et al. Two novel mutations of *PAX3* and *SOX10* were characterized as genetic causes of Waardenburg Syndrome. *Mol Genet Genomic Med*. 2020;8:e1217. https://doi.org/10.1002/mgg3.1217