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Genetic analysis of PAX3 for diagnosis of Waardenburg syndrome type I

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Abstract

Conclusion: PAX3 genetic analysis increased the diagnostic accuracy for Waardenburg syndrome type I (WS1). Analysis of the three-dimensional (3D) structure of PAX3 helped verify the pathogenicity of a missense mutation, and multiple ligation-dependent probe amplification (MLPA) analysis of PAX3 increased the sensitivity of genetic diagnosis in patients with WS1. Objectives: Clinical diagnosis of WS1 is often difficult in individual patients with isolated, mild, or non-specific symptoms. The objective of the present study was to facilitate the accurate diagnosis of WS1 through genetic analysis of PAX3 and to expand the spectrum of known PAX3 mutations. Methods: In two Japanese families with WS1, we conducted a clinical evaluation of symptoms and genetic analysis, which involved direct sequencing, MLPA analysis, quantitative PCR of PAX3, and analysis of the predicted 3D structure of PAX3. The normal-hearing control group comprised 92 subjects who had normal hearing according to pure tone audiometry. Results: In one family, direct sequencing of PAX3 identified a heterozygous mutation, p.I59F. Analysis of PAX3 3D structures indicated that this mutation distorted the DNA-binding site of PAX3. In the other family, MLPA analysis and subsequent quantitative PCR detected a large, heterozygous deletion spanning 1759–2554 kb that eliminated 12–18 genes including a whole PAX3 gene.

Keywords: Mutation, MLPA, clinical diagnosis, hearing loss, dystopia canthorum, pigmentary disorder

Introduction

Waardenburg syndrome (WS) is a hereditary auditory pigmentary disorder that is responsible for 1–3% of congenital deafness cases [1]. WS is classified into four types based on symptoms other than the auditory and pigmentary disorder. Type I WS (WS1) includes dystopia canthorum, and this feature distinguishes WS1 from type II WS. Type III WS is similar to WS1 but is associated with musculoskeletal anomalies of the upper limbs. Type IV WS is similar to type I but is associated with Hirschsprung disease. Diagnostic criteria for WS1 have been proposed [2]. The clinical features of WS1 demonstrate incomplete penetrance and highly varied expression [3,4], which makes diagnosis in individual patients challenging. For example, WS1 patients may present only one isolated symptom. Diagnosis of high nasal root and medial eyebrow flare can be difficult when they are mild. Hearing loss and early graying are relatively common in the general population and are not specific to WS1. Thus, the accuracy of WS1 diagnosis needs to be improved by the use of additional diagnostic procedures.

It is reported that more than 90% of patients with WS1 harbor point mutations in PAX3 [5], and an additional 6% of WS1 patients harbor partial or complete PAX3 deletions [6]. This high frequency of PAX3 mutation in WS1 suggests that clinical diagnosis of WS1 could be facilitated by PAX3 genetic analysis. To date, more than 80 PAX3
mutations are reported to be associated with WS1 [5]. A de novo paracentric inversion on chromosome 2 in a Japanese child with WS1 provided a clue for identification of PAX3 in the distal part of chromosome 2 [7]. However, only a few PAX3 mutations including the chromosomal inversion have been reported in Japanese patients with WS1 since then [8,9].

In the present study, we conducted PAX3 genetic analysis to facilitate diagnosis of WS1 in two Japanese families. In one family, to verify the pathogenicity of analysis to facilitate diagnosis of WS1 in two Japanese children with WS1 provided a clue for identification of PAX3. In the other family, no mutations were identified by direct sequencing, so multiple ligation-dependent probe amplification (MLPA) analysis was used to search for large deletions in PAX3 and thereby increase the sensitivity of genetic diagnosis.

Material and methods

Patients and control subjects

Two Japanese families with WS1 were included in the study. The diagnosis of WS1 was based on criteria proposed by the Waardenburg Consortium [2]. The normal-hearing controls comprised 92 subjects who had normal hearing according to pure tone audiometry. This study was approved by the institutional ethics review board at the National Tokyo Medical Center. Written informed consent was obtained from all subjects included in the study or from their parents.

Clinical evaluation

A comprehensive clinical history was taken from subjects who were examined at our hospitals or from their parents. During physical examination, special attention was given to the color of the skin, hair, and iris, and to other anomalies such as dystopia canthorum, medial eyebrow flare, limb abnormalities, and Hirschsprung disease. After otoscopic examination, behavioral audiometric testing was performed. The test protocol was selected according to the developmental age of the subject (conditioned orientation response audiometry, play audiometry, or conventional audiometric testing, from 125 to 8000 Hz), and testing was performed using a diagnostic audiometer in a soundproof room. Auditory brainstem response (ABR) and otoacoustic emission were also evaluated in some subjects.

Direct sequencing

Genomic DNA from the subjects was extracted from peripheral blood leukocytes using the Gentra Puregene® Blood kit (QIAGEN, Hamburg, Germany). Mutation screening of PAX3 was performed by bidirectional sequencing of each exon (exons 1–11) together with the flanking intronic regions using an ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Primer sequences for PAX3 are listed in Table I. Mutation nomenclature is based on the genomic DNA sequence of [GenBank accession no. NG_011632.1], with the A of the translation initiation codon considered as +1. Nucleotide conservation between mammalian species was evaluated using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). PolyPhen-2 software (http://genetics.bwh.harvard.edu/pph2/) was used to predict the functional consequence(s) of each amino acid substitution.

MLPA

MLPA analysis was performed using an MLPA kit targeting PAX3, MITF, and SOX10 (SALSA MLPA Kit P186-B1, MRC-Holland, Amsterdam, The Netherlands) according to the manufacturer’s protocol. Exon-specific MLPA probes for exons 1–9 of PAX3 and control probes were hybridized to genomic DNA from the subjects and normal controls and ligated with fluorescently labeled primers. A PCR reaction was then performed to amplify the hybridized probes. The amplified probes were fractionated on an ABI3130xl Genetic Analyzer (Applied Biosystems) and the peak patterns were evaluated using GeneMapper (Applied Biosystems).

Real-time PCR

To determine the length of each deleted genomic region, 100 ng of genomic DNA from the subjects and a normal control were subjected to quantitative PCR (Prism 7000, Applied Biosystems) using Power SYBR® Green Master Mix (Life Technologies, Carlsbad, CA, USA) and 12 sets of primers designed to amplify sequence-tagged sites on chromosome 2 (GenBank accession nos: RH46518, RH30035, RH66441, GDB603632, 1988, RH24952, RH47422, RH65573, RH26526, RH35885, RH16314, and RH92249).

Homology modeling of the PAX3 paired domain

The DNA-binding site of the paired domain of PAX3 was modeled using SWISS-MODEL [10] with the crystal structure of the PAX5 paired domain-DNA complex (PDB ID: 1PDN_chain C) as the template because PAX3 and PAX5 are functionally and structurally similar [11]. The amino acid...
sequences of the PAX3 and PAX5 paired domains were 79% homologous. The predicted PAX3 structure and the p.I59F mutation structure were superimposed on the backbone atoms of the PAX5 paired domain-DNA complex and displayed using the extensible visualization system, UCSF Chimera [12].

**Results**

In family 1, the proband, a 9-month-old male, was the first child of unrelated Japanese parents. Abnormal responses were found upon newborn hearing screening in the left ear, and left hearing loss was diagnosed by ABR. On physical examination, dystopia canthorum was noted, with a W-index of 2.77. The patient’s mother also had dystopia canthorum, with a W-index of 2.68. She also had a history of early graying that started at age 16 years. She had not been diagnosed with WS1. According to the parents, 10 members of this family, including the proband and the mother, showed clinical features consistent with WS1 (Figure 1). ABR performed in the proband...
revealed normal hearing in the right ear and no responses to 105 dB click stimuli in the left ear. Computed tomography (CT) of the temporal bone showed normal structures in the inner, middle, and outer ears.

Genetic analysis of PAX3 was conducted in this family, and direct sequencing of PAX3 revealed a heterozygous mutation, c.175A>T, in the proband and his mother. This mutation resulted in a missense mutation, p.I59F (Figure 2A). The proband’s father did not harbor this mutation. p.I59F is located within exon 2 and is part of the paired domain of PAX3, which is a critical region for interaction between transcription factors and target DNA (Figure 2B). A multiple alignment of PAX3 orthologs at this region demonstrated that I59 was evolutionarily conserved among various species (Figure 2C). The p.I59F mutation was not identified in any of the 184 alleles from the normal control subjects. This mutation was predicted to be ‘probably damaging’ according to PolyPhen-2 software.

The predicted 3D structures of the paired domain of the PAX3-DNA complex indicated that the PAX3 paired domain binds to the corresponding DNA (white double helixes) via hydrogen bonds (pink lines) at the N-terminal of α-helix1 (H1), α-helix2 (H2), and α-helix3 (H3) (indicated in blue; Figure 3A). I59 is located in the middle of H1, H2, and H3 and is surrounded by hydrophobic residues (green) protruding from H1, H2, and H3. Because the van der Waals radius of phenylalanine (Figure 3C; white arrows) is larger than that of isoleucine (Figure 3B, white arrowheads), F59 repels the surrounding hydrophobic residues by van der Waals forces and increases the distance between F59 and the surrounding hydrophobic residues, resulting in structural distortion of the DNA-binding site of PAX3. Since this site is precisely shaped for maximal binding to the corresponding DNA, this mutation is likely to reduce the binding ability of the paired domain of PAX3 and cause WSI. A mutational search found the same mutation in another Japanese family [8].

In family 2, the proband, a female aged 4 years and 4 months, was the first child of unrelated Japanese parents. Abnormal responses were found upon newborn hearing screening in the right ear, and right hearing loss was diagnosed by ABR. On physical examination, dystopia canthorum, medial eyebrow flare, and a white forelock were noted. She was admitted to hospital suffering from ketotic hypoglycemia of unknown cause when aged 4 years. Her mother presented with heterochromia iridis, dystopia canthorum, and medial eyebrow flare, and a white forelock were noted. She was admitted to hospital suffering from ketotic hypoglycemia of unknown cause when aged 4 years. Her mother presented with heterochromia iridis, dystopia canthorum, and medial eyebrow flare, and a white forelock were noted. She was admitted to hospital suffering from ketotic hypoglycemia of unknown cause when aged 4 years. Her mother presented with heterochromia iridis, dystopia canthorum, and medial eyebrow flare, and a white forelock were noted. She was admitted to hospital suffering from ketotic hypoglycemia of unknown cause when aged 4 years. Her mother presented with heterochromia iridis, dystopia canthorum, and medial eyebrow flare, and a white forelock were noted. She was admitted to hospital suffering from ketotic hypoglycemia of unknown cause when aged 4 years. Her mother presented with heterochromia iridis, dystopia canthorum, and medial eyebrow flare, and a white forelock were noted. She was admitted to hospital suffering from ketotic hypoglycemia of unknown cause when aged 4 years. Her mother presented with heterochromia iridis, dystopia canthorum, and medial eyebrow flare, and a white forelock were noted. She was admitted to hospital suffering from ketotic hypoglycemia of unknown cause when aged 4 years. Her mother presented with heterochromia iridis, dystopia canthorum, and medial eyebrow flare, and a white forelock were noted.
and her father had never been diagnosed with WS1. Pure tone audiometry of the proband showed severe hearing loss in the right ear and normal hearing in the left ear. The results of ABR and distortion product otoacoustic emissions in the proband were compatible with those obtained for pure tone audiometry.

Because direct sequencing of PAX3 in the proband and her grandmother revealed no mutations, we conducted MLPA analysis to search for a large deletion of PAX3, and found that the copy number of all tested exons (exons 1–9) of PAX3 was half that of the number of other chromosomal regions in both subjects (Figure 5A). In control subjects, all tested exons of PAX3 showed the same copy number as the other chromosomal regions (Figure 5B). To determine the size of the deleted region, quantitative PCR was performed at 12 sequence-tagged sites on chromosome 2q36, which includes PAX3. In the proband, copy numbers at nine sites in the middle of the tested region (white arrows) were half that of those examined in normal controls, but the copy numbers at three of the sites near the 5' and 3' ends of the tested region (black arrows) were identical to those examined in normal controls (Figure 6). This result demonstrated that the chromosomal region spanning 1759–2554 kb at 2q36, which includes the whole PAX3 gene, was deleted in one of the alleles of the proband. The same results were detected in the grandmother. A search for the deleted region revealed that this region contained between 12 and 18 genes, including PAX3.

Discussion

The heterozygous missense mutation, p.I59F, was identified in family 1. The pathogenicity of a novel or rare missense mutation in the causative gene is not necessarily verified even when the mutation is absent from a large number of normal controls, when the residue is evolutionary conserved among different species, or if the mutation is associated with the phenotype within a family, because an identified
missense mutation may be a rare normal variant. Thus, the pathogenicity of such mutations needs to be verified by detection of the same mutation in multiple families with the same phenotype or by functional analysis. The functional consequences of a few PAX3 mutations have been tested and reduced DNA-binding properties have been reported [13–15]. The p.I59F mutation was reported in a Japanese family [8], but functional analysis has not been conducted. We analyzed the predicted 3D structures of the paired domain of the PAX3-DNA complex and showed that this mutation was likely to distort the structure of the DNA-binding site of PAX3 and lead to functional impairment. This result substantially supports the hypothesis that the p.I59F mutation is pathogenic, although it is based on a theoretical prediction rather than functional experiments.

In family 2, the distinct phenotypes of the proband, the proband’s mother, and the proband’s grandmother were congenital unilateral hearing loss, heterochromia iridis, and early graying, respectively. Because of these differences, they were not aware of the hereditary nature of the symptoms. Identification of the PAX3 mutation in the proband and the proband’s grandmother led to an accurate diagnosis of WS1 and facilitated understanding of the symptoms. In this family, direct sequencing of PAX3 did not detect any mutations, but MLPA analysis detected a large heterozygous deletion. Furthermore, quantitative PCR analysis revealed that the deleted region spanned 1759–2554 kb and included 12–18 genes. Large deletions of PAX3 in patients with WS1 have been reported in several families [6,16–18]. To our knowledge, however, this is the largest deletion identified in patients with WS1 and has, therefore, expanded the spectrum of PAX3 mutations. There is no reported correlation between the nature of the mutation (deleted vs truncated or missense) or

![Figure 5](image1.png)

**Figure 5.** Results of MLPA analysis of PAX3 in family 2. (A, B) Relative ratios of DNA quantity in each exon compared with that in the control region are shown for the proband (A) and control (B).

![Figure 6](image2.png)

**Figure 6.** Genetic map showing the estimated location of the PAX3 deletion together with the regions surrounding PAX3. Sites examined by quantitative PCR are indicated by arrows. Blank and white arrows indicate that the quantities of DNA at these sites are half or identical to the quantities of DNA at the corresponding sites in the control, respectively. The 5’ and 3’ ends of the deletion are located within the blue regions flanking the white region, designated as ‘deletion,’ and flanked by the green regions, designated as ‘no deletion.’ All genes mapped within this region, including PAX3, are shown in the lower map.
its location in *PAX3*, and the severity of the WS1 phenotype [19,20]. Similarly, no evidence of such a correlation was found in the data presented in this study.

In the present study, *PAX3* genetic diagnosis contributed to the accurate diagnosis of WS1. Such diagnosis could help provide genetic counseling to patients with isolated or few phenotypic symptoms, those with mild phenotypes or few first-degree relatives, or those who have yet to develop any symptoms. In addition, analysis of the predicted 3D structure of PAX3 facilitated the verification of pathogenicity of a missense mutation, and MLPA analysis increased the sensitivity of genetic diagnosis of WS1.

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**References**


