A novel mutation of PAX3 in a Chinese family with Waardenburg syndrome

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Purpose: The molecular characterization of 34 members of a Chinese family, with 22 members in four generations, affected with Waardenburg syndrome (WS1).

Methods: A detailed family history and clinical data were collected. A genome-wide scan by two-point linkage analysis using more than 400 microsatellite markers in combination with haplotype analysis was performed. Mutation screening was carried out in the candidate gene by sequencing of amplified products.

Results: A maximum two-point lod score of 6.53 at θ=0.00 was obtained with marker D2S2248. Haplotype analysis placed the WS1 locus to a 45.74 cM region between D2S117 and D2S206, in close proximity to the PAX3 gene on chromosome 2q35. Mutation screening in PAX3 identified a 701T>C mutation which converted a highly conserved Leu to Pro. This nucleotide alteration was neither seen in unaffected members of the family nor found in 50 unrelated control subjects.

Conclusions: The present study identified a novel 701T>C mutation in PAX3. The mutation observed in this family highlights the phenotypic heterogeneity of the disorder.

Waardenburg syndrome (WS) is an autosomal dominant disorder with an incidence of 1 in 40,000 that manifests with sensorineural deafness and pigmentation defects of the hair, skin, and iris [1]. Waardenburg syndrome is classified into four types depending on the presence or absence of additional symptoms. Waardenburg syndrome type 1 WS (WS1; OMIM 193500) and type 2 WS (WS2; OMIM 193510) are distinguished by the presence or absence of dystopia canthorum, respectively. Type 3 WS (Klein-Waardenburg syndrome, WS3; OMIM 148820) is distinguished by the presence or absence of limb abnormalities. Type 4 WS, referred to as Shah-Waardenburg syndrome or Waardenburg-Hirschsprung disease (WS4; OMIM 277580) is characterized by the presence of an aganglionic megacolon. WS1 and WS3 are due to mutations in the PAX3 gene [2-4], whereas some WS2 cases are associated with mutations in the microphthalmia-associated transcription factor (MITF) gene [5]. The WS4 phenotype can result from mutations in the endothelin-B receptor gene (EDNRB), and in the gene for its ligand, endothelin-3 (EDN3) [6-8], or in the SOX10 gene [9]. PAX3 is a member of the mammalian PAX gene family [10], which is structurally defined by the presence of a highly conserved 128 amino acid DNA-binding domain, known as the paired domain and initially identified in the paired segmentation gene of Drosophila [11]. In addition to the paired domain, PAX3 harbors a second DNA-binding domain, the homeodomain [10,12]. Since the original cloning of PAX3, a number of alternatively spliced PAX3 cDNAs have been reported. Included in this list of PAX3 splice variants is one in which there are two additional exons downstream of the originally published exon 8 preceded by an alternatively retained intron [13]. The formation of different isofroms in humans probably accounts for some phenotypic differences observed between humans and mice with dominant mutations in PAX3. For example, the deafness phenotype associated with Waardenburg syndrome might result from haploinsufficiency of the PAX3 isoform with exons 8, 9, and 10, but lacking introns 9 and 10. Mice with mutations in PAX3 are not deaf [14], perhaps because murine auditory system development is not dependent on this PAX3 isoform [15]. In the present study, we report the molecular analysis of PAX3 in a Chinese family with Waardenburg syndrome type 1, in which a novel missense T to C mutation in helix 1 of the homeodomain was identified. Even though PAX3 mutations have been reported in various racial groups, few of them are in helix 1 of the homeodomain.

METHODS
Family description: The proband, a five-year-old child, was diagnosed as having WS1 (Figure 1). Diagnosis of WS1 was made in accordance with the WS Consortium criteria [1]. The family history revealed 22 affected members in four genera-
tions (Figure 2). A detailed ophthalmological examination was performed on 34 members of the family which revealed 22 members who were bilaterally affected and 12 individuals (including 10 spouses) who were unaffected.

**Genotyping and linkage analysis:** Informed consent was obtained from each individual studied. This study was approved by the ethics review board of the Shanghai Jiaotong University, Bio-X research center, consistent with the provisions of the Declaration of Helsinki. Blood was drawn and DNA isolated by standard methods. A genome-wide search with more than 400 microsatellite markers (Genethon linkage map) was done on DNA samples of all 34 ophthalmologically examined individuals (22 affected and 12 unaffected, including 10 spouses). Microsatellites were amplified in single plex...
reactions by touchdown PCR (MJ-Research, Watertown, MA) using fluorescent labeled primers following standard methods. Polymerase chain reaction (PCR) products were pooled and denatured at 95 °C for 1 min and electrophoresed on 96-capillary automated DNA sequencers (MegaBACE 1000, Amersham, Freiburg, Germany). Genotyping was done using the Genetic Profiler version 1.5 (Amersham) software. Autosomal dominant inheritance with a disease gene frequency of 0.0001 and complete penetrance of the trait was assumed. Recombination frequencies were considered equal between males and females. Two-point linkage analysis was carried out with MLINK from the LINKAGE program package [16]. Autosomal dominant inheritance was assumed with a reduced penetrance of 98% and a phenocopy rate of 0.5%.

**Mutation analysis:** Nine pairs of primers were designed from intronic regions to amplify the coding regions and splice sites of PAX3 (Table 1). Genomic DNA from all members in the family and 50 unrelated control subjects were amplified. Amplification was carried out in 25 µl reactions containing 100 ng genomic DNA, 10 pmoles each of forward and reverse

<table>
<thead>
<tr>
<th>Exon</th>
<th>Sequence (5'-3')</th>
<th>Product size (bp)</th>
<th>Annealing temperature (°C)</th>
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</thead>
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<tr>
<td>1F</td>
<td>TCACCACAGGGGAGACTCA</td>
<td>472</td>
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<tr>
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<td>GAGGCCCTCCTTACCTTC</td>
<td>442</td>
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<td>TACGTGCTGCTGCTTTC</td>
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<tr>
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<td>TTACGACCTTCTCAACAACCT</td>
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<td>TCGGTCTGCCCCTTTCTAA</td>
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<td>5R</td>
<td>AGAGAGAGAGAGAGGAGAAGA</td>
<td>351</td>
<td>57</td>
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Primer sequences for amplification of exonic regions of PAX3.
primers, 200 µM dNTP, 10X PCR buffer, 1.5 mM MgCl₂, and 0.25 U Taq DNA polymerase (AmpliTaq Gold; ABI, Foster City, CA). Amplification conditions consisted of an initial denaturation step at 95 °C for 5 min, followed by 35 cycles consisting of a denaturation step at 95 °C for 45 s, an annealing step for 30 s at 57 °C, and an extension at 72 °C for 45 s, followed by a final extension step at 72 °C for 10 min. PCR products were purified using a PCR product purification kit (QIA quick; Qiagen, Valencia, CA). Purified PCR products were sequenced bidirectionally using the ABI BigDye™ Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (ABI) for a 10 µl final volume, containing 5.0 µl purified PCR product, 4.0 µl BigDye Terminator ready reaction mix and 3.2 pmols of primers. Cycling conditions were 95 °C for 2 min, 25 cycles at 95 °C for 30 s, 52 °C for 15 s, and 60 °C for 4 min. The sequencing reaction products were purified by the isopropanol precipitation method (ABI protocol), suspended in 10 µl of formamide (Hi-Di-Formamide; ABI), denatured at 95 °C for 5 min, and electrophoresed on an ABI 3100 Genetic Analyzer. Sequencing results were assembled and analyzed using the SeqMan II program of the Lasergene package (DNA STAR Inc., Madison, WI).

RESULTS

Phenotype analysis: Diagnostic criteria for WS proposed by the Waardenburg Consortium and the clinical phenotype of every affected subject are presented in Appendix 1. Our patients had all of these symptoms. Dystopia canthorum and more severe pigmentation disorders are typical. Every patient had dystopia canthorum, broad nasal root, and rounded nasal tip. The proband V:11 showed obvious ocular abnormalities, in particular dystopia canthorum and heterochromic irides (both, hypochromic blue). A relatively broad nasal root and upturned nasal tip were both obvious. Diagnosis of dystopia canthorum has now been standardized by the Waardenburg Consortium.

To calculate the W index:
1. Measure a (inner canthus), b (interpapillary), and c (outer canthal) distances in millimeters using a rigid ruler held against the face.
2. Calculate X=(2a-0.2119c-3.909)/c
3. Calculate Y=(2a-0.2479b-3.909)/b
4. Calculate W=X+Y+a/b [1].

WS type 1 is diagnosed if the W of subject is 1.95 or more [1]. In our subject; a=36 mm, b=53 mm, c=80 mm and W = 2.35. Her mother has; a=40 mm, b=61 mm, c=83 mm and W=2.36. Among the affected subjects lateral displacement of the inner canthi was evident in all, and distribution of best corrected visual acuity was as follows: seven with 0.1 to about 0.5, ten with 0.5 to about 1.0, five with 1.0 to about 1.2. No nystagmus or strabismus was detected, and fundus changes were all within normal limits. Refraction and axial length examination were unavailable. Hypopigmented patches of the skin were present in fourteen patients. Piebaldism was observed with circumscribed, sharply delimited amelanotic

![Figure 4](http://www.molvis.org/molvis/v12/a112/)

Figure 4. The locations and types of mutations uncovered in different patients with WS1 are shown under the diagram of the gene.

![Diagram](http://www.molvis.org/molvis/v12/a112/)

Figure 5. Schematic representation of the PAX3 homeodomain, together with structural features based on the three-dimensional structure of the homeodomain of the Drosophila Prd protein (α, α-helix) is shown [29]. The amino acid sequence for positions 1-61 of the Pax-3 homeodomain is shown, and invariant residues among this class of homeodomain [33] are identified below. The position and nature of the mutation introduced in Pax-3 is shown in red. The systematic nomenclature of the mutants with respect to their position within the homeodomain and the substitution involved are shown to the left.
macules on the upper chest, abdomen or back. Within the areas of hypomelanosis, there were normally pigmented and hyperpigmented macules of various sizes. Sensorineural deafness was present in IV:9 and VI:3. Detailed information on degree of hearing loss was unavailable. None of them had any limb defects, whereas a white forelock was present in six, and sapphire blue eyes were present in five. Confluent white eyebrows were present in IV:6 and VI:2, poliosis was present in VI:2.

**Linkage analysis:** In a genome-wide scan using more than 400 markers, we initially obtained positive two-point lod scores of 5.74 at θ=0.000 with marker D2S2382 in WS1 locus. Further analysis with more markers in this region on chromosome 2 gave a maximum lod score of 6.53 at θ=0.000 with marker D2S2248. Haplotype analysis placed the WS1 locus to a 45.74 cM region between D2S117 and D2S206, in close proximity to the PAX3 gene on chromosome 2q35.

Figure 6. View of the crystal structure of the homeodomain in PAX3. Three α-helixes are shown in red. The Leu (A) to Pro (B) mutation in helix 1 is shown in green. The sixteenth Leu residue in helix 1 is important in controlling how helix 1 packs against helix 3, stabilizing the folded structure. The program Swiss-PdbViewer was used [37].
Mutation analysis: Sequencing of PAX3 in two affected individuals (IV:13 and V:11) showed a heterozygous change T>C (Figure 3) in exon5, at position 701 from the translation start site. This position belongs to helix 1 of homeodomain in PAX3, and converts the sixteenth amino acid in this domain from Leu to Pro. All other affected family members tested also showed this nucleotide change. This alteration was neither seen in any unaffected member of the family nor found in 50 unrelated control subjects (data not shown).

DISCUSSION
Waardenburg syndrome type I (WS1) is an autosomal dominant disorder characterized by wide bridge of nose owing to lateral displacement of the inner canthus of each eye, pigmented disturbances such as a frontal white blaze of hair, heterochromia of irides, white eyelashes, leukoderma, and sensorineural deafness [1,17]. It is characterized clinically by dysopia canthorum (99%), broad nasal root (78-80%), confluent eyebrows (45%), heterochromia irides (25%), congenital sensorineural hearing loss (20%), a white forelock (17%), and piebaldism (12%) [18-21]. The syndrome shows variable clinical expression even within families, and at present it is not possible to predict the severity, even when a mutation is detected.

Mutations in PAX3 are responsible for most WS1. The interplay between PAX3, SOX10, and MITF during development may explain the molecular bases of the auditory-pigmentary abnormalities that are common to the various forms of Waardenburg syndrome. PAX3 is a member of the paired class homeodomain family of transcription factors, which are involved in various cell processes. It is expressed in the neural tube and developing brain, neural crest and their derivatives, the dermomyotome of the developing somites and the limb buds [22]. In addition to playing a role in MITF gene expression, this factor has been shown to regulate skeletal muscle formation [23,24]. TRP-1 promoter activity has been shown to be upregulated by PAX3 as well [25]. Another interesting “biological capacitor” model is also currently being debated [26,27].

Auditory-pigmentary syndromes are caused by the absence of melanocytes in skin, hair, eyes, or the stria vascularis of the cochlea [1]. In this family, congenital leucoderma was a generally minor syndrome, while only two of affected members showed hearing loss, which means that the 701T>C mutation may mostly interrupt melanocyte metabolism in skin. Two mechanisms have been proposed to explain the skin findings in piebaldism: a defect in the migration of melanoblasts from the neural crest to the ventral aspect of the skin during development or a failure of melanoblasts to survive or differentiate into melanocytes once they reach the ventral aspect of the skin [19]. Ultrastructural studies on the amelanotic skin show an absence of melanocytes. Pigmented areas show abnormalities of melanocytes and melanosomes [20].

It is still not clear what accounts for the reduced penetrance of deafness. Active melanocytes are normally present in the inner ear and they may serve a function during develop-
ment of this organ. An alternative explanation for the congenital hearing loss is the failure of nerve cells to migrate properly from the neural crest to the inner ear [19]. Stochastic events during development may be the factors that determine whether a person with a PAX3 mutation will be congenitally deaf or not. Alternatively, genetic background or nonrandom environmental factors, or both, may be significant [28].

Dystopia canthorum was detected in all affected members. Craniofacial abnormalities arise from poor development of the first and second visceral arches, which form the facial bones and ears at about the seventh week of embryonic development [21]. Unfortunately, there is no unifying concept or developmental mechanism to explain all the clinical findings in WS.

PAX3 encodes a paired domain and a homeodomain. The paired domain is structurally divided into two subdomains, each containing three α-helices that form a classical helix-turn-helix (HTH) motif. In addition, the NH2-terminal subdomain contains a β-hairpin motif and a type II β-turn that, together with the HTH motif, make important contributions to DNA binding [29]. The homeodomain is composed of three α-helices, the last two of which form an HTH motif [30,31]. The most conserved segment of the homeodomains known to date is helix 3, which makes extensive DNA contacts in the major groove, roughly parallel to it. It is important for sequence specificity of individual homeodomain [32]. Helix 1 and 2 are aligned in an antiparallel arrangement above the DNA, each spanning the major groove, nearly perpendicular to the local direction of the DNA backbones. Conserved residues in helix 1 and 3 play a major role in stabilizing the folded structure and in controlling how helix 1 packs against helix 3. This will be critical for DNA recognition because it affects the spatial relationship between contacts made by the NH2-terminal arm and contacts made by helix 3 [30]. The remaining DNA contacts, include helix 1, are made by a flexible NH2-terminal arm that contacts the DNA-phosphate backbone and minor groove [31]. Besides this, the homeodomain has distinct sequence preferences and shows a unique ability to dimerize cooperatively on target sequences containing the palindromic TAAT-(N)2/3-ATTAG motif [33]. Two known WS1 mutations in the homeodomain are located in helix 3 at position 47 (V47F) and 53 (R53G) [34]. Both residues are situated within the DNA major groove and make several phosphate and base-specific contacts that are important for docking of the homeodomain on DNA. Neither mutation affected protein stability or amounts, but both abrogate DNA binding to target DNA probes [35]. Furthermore, the loss of paired domain binding ability due to mutant R53G indicates that both domains are functionally interdependent and cooperate to achieve DNA-binding specificity [35].

To date, more than 50 mutations have been identified in PAX3, identified from the Human Gene Mutation Database at Cardiff, and the Harvard Medical School Center for Hereditary Deafness (Figure 4). Most of these mutations are located in the paired domain or helix 3 of homeodomain. The 701T>C mutation identified in our study was in the middle of helix 1, which lead to a Leu to Pro substitution on position 16 of homeodomain (Figure 5, Figure 6). Among 337 homeodomain sequences analyzed by Kappen et al. [36], more than 300 of them are Leu at position 16, which indicates that it represents one of the most highly conserved residues in the homeodomain superfamily (Figure 7). The substitution of conserved Leu in helix 1 is most likely disrupt the spatial relationship between contacts made by helix 1 and 3, and influences the stability of folded structure and DNA recognition. Most analyses on homeodomains before now were focused on DNA contacts made by residues within helix 3, while few studies about the intercontact of three helices have been reported. Our data provides a new clue to elucidate interaction between helices in homeodomain, and more work is needed to clarify crystal structure alternation made by this mutation.

In summary, our analysis of the Waardenburg syndrome family identified a novel missense mutation in homeodomain of PAX3, and this information is helpful for analysis of the interaction between three helices.

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REFERENCES


