Genetic and Phenotypic Heterogeneity in Chinese Patients with Waardenburg Syndrome Type II

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Abstract

Waardenburg Syndrome (WS) is an autosomal-dominant disorder characterized by sensorineural hearing loss and pigmentary abnormalities of the eyes, hair, and skin. Microphthalmia-associated transcription factor (MITF) gene mutations account for about 15% of WS type II (WS2) cases. To date, fewer than 40 different MITF gene mutations have been identified in human WS2 patients, and few of these were of Chinese descent. In this study, we report clinical findings and mutation identification in the MITF gene of 20 Chinese WS2 patients from 14 families. A high level of clinical variability was identified. Sensorineural hearing loss (17/20, 85.0%) and heterochromia iridum (20/20, 100.0%) were the most commonly observed clinical features in Chinese WS2 patients. Five affected individuals (5/20, 25.0%) had numerous brown freckles on the face, trunk, and limb extremities. Mutation screening of the MITF gene identified five mutations: c.20A>T, c.647_649delGAA, c.649A>G, and c.763C>T. The total mutational frequency of the MITF gene was 21.4% (3/14), which is significantly higher than the 15.0% observed in the fair-skinned WS2 population. Our results indicate that MITF mutations are relatively common among Chinese WS2 patients.

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Introduction

Waardenburg syndrome (WS) is an autosomal-dominant disorder of neural crest cell differentiation, most commonly described in Western populations. Its main clinical manifestations include sensorineural hearing loss, pigmentary abnormalities of the eyes, hair, and skin (e.g., heterochromia iridum, white forelock, and patchy hypopigmented skin), and dystopia canthorum [1,2]. Four types of WS have been identified, depending on their clinical characteristics. WS type I (WS1; MIM 193500) and type II (WS2; MIM 193510) are distinguished by the presence or absence, respectively, of dystopia canthorum. The presence of limb abnormalities distinguishes type III (WS3; also called Klein-Waardenburg syndrome; MIM 148820) from WS1. Type IV (WS4; also called Shah-Waardenburg syndrome or Waardenburg-Hirschsprung disease; MIM 277580) is characterized by the presence of an aganglionic megacolon. Of these subtypes, WS1 and WS2 are the most common.

WS2 is well-defined phenotypically and is genetically heterogeneous; its hallmarks are sensorineural hearing loss and heterochromia iridum. Other abnormal pigmentation disturbances, including white forelock, early graying, and hypopigmented skin patches, are also manifested in relatively low proportions of WS2 patients [3]. Five subtypes of WS2 have been identified based on molecular findings. WS2A (MIM 193510) is caused by MITF mutations [4]. Mutations in SNAI2 (snail homolog of 2) gene are known to cause WS2D (MIM 608890) [5], and mutations in SOX10 (SRY (sex-determining region Y)-box10) gene are responsible for WS2E (MIM 611584) [6]. WS2B (MIM 600193) maps to chromosome 1p [7] and WS2C (MIM 606662) maps to chromosome 8p [8], but the causative genes for these have not yet been identified. To date, about 30% of WS2 cases can be explained at the molecular level. Some researchers have proposed that the MITF gene is responsible for approximately 15% of WS2 cases and the SOX10 gene for approximately another 15% [4,6]. A homozygous deletion in the SNAI2 gene was described in two unrelated WS2 patients [5], and a heterozygous endothelin receptor type-B (EDNRB) mutation has been found in three patients from one family [9]. However, no other patients have been identified to confirm these results, indicating that SNAI2 and EDNRB are not major causes of WS2 [4–9]. In this study, we conducted detailed analyses of the clinical manifestations and molecular bases of 20 Chinese WS2 patients from 14 families. Specifically, we examined three WS2-related genes: MITF, SOX10, and SNAI2.

Materials and Methods

Patients and DNA samples

The subjects for this study were recruited from the Otology Clinic at Chinese PLA General Hospital and deaf-mute schools from nine different regions in China. The study was approved by
Written informed consent was obtained from all adult subjects and guardians on behalf of the children prior to the clinical evaluation and blood sample collection. In total, 20 WS2 subjects from 14 unrelated families were assessed. The patients consisted of 13 males and seven females, ranging in age from 2–69 years. Among the 14 families, only families WS01, WS02, and WS03 had more than one patient (Fig. 1); the remaining 11 were sporadic cases. Blood samples were obtained from the 20 WS patients, four married-in-control family members, and the parents of the 11 sporadic cases. Additionally, blood samples were obtained from 200 region- and ethnicity-matched volunteers with normal hearing. DNA was extracted from peripheral blood leukocytes using a DNA extraction kit (Watson Biotechnologies Inc., Shanghai, China).

Clinical evaluation
Twenty WS2 patients were diagnosed according to the criteria proposed by the WS consortium [10]. A comprehensive clinical history and neurotological, ophthalmological, and dermatological examinations were performed on all subjects. The audiological and neurotological examination consisted of otoscopy, pure-tone audiometry, immittance testing, and auditory brain-stem response (ABR). Additional auditory steady-state response (ASSR) tests were performed for young individuals who did not respond well to the pure-tone audiometry test. The ophthalmological examination included visual acuity measurements, visual field examination, and fundus ophthalmoscopy. Special attention was given to the color of skin, hair, and iris as well as developmental defects such as dystopia canthorum and limb abnormalities. The degree of hearing loss was defined according to the pure-tone average (PTA), which was based on three frequencies (500, 1000, and 2000 Hz) as follows: normal, <26 dB HL; mild, 26–40 dB HL; moderate, 41–70 dB HL; severe, 71–90 dB HL; and profound, >90 dB HL.

Mutational analysis
All coding exons and 200 bp of the flanking intron regions of the WS2-related genes MITF-M isoform, SOX10, and SNAI2 were amplified in all patients by polymerase chain reaction (PCR) using specific primers (for details, see the File S1). All PCR amplifications were carried out using 40 ng of genomic DNA and 2 pmol of...
PCR conditions were 94°C for 4 min, then 30 cycles of denaturation at 94°C for 30 s, annealing at various temperatures for 30 s for the different primers, and extension at 72°C for 30 s, followed by a 7-min final extension at 72°C. PCR fragments were ethanol-purified and sequenced in both directions using the ABI BigDye Terminator Cycle Sequencing Kit (ver. 3.1; ABI Applied Biosystems, Foster City, CA), with the same primers used for PCR. The raw sequence data produced by the ABI Prism 3100 DNA sequencer were aligned with the wild-type sequence using the GeneTool program.

Bioinformatics Analysis
Phylogenetic conservation of missense mutations was analyzed by aligning the amino acid sequences from several species (retrieved from the Entrez protein database at NCBI) using the ClustalX 2.012 program [11]. In silico predictions of the putative functional effects of the missense mutations were conducted with the PMut (http://mmb2.pcb.ub.es:8080/PMut/) [12], Polyphen2 [13] (http://tiddlyspace.com/bags/icgc_public/tiddlers/PolyPhen2), and SIFT (http://sift.jcvi.org) software [14].

Results
Clinical findings
All 20 patients were diagnosed with WS2 based on their calculated W index (<1.85) and the absence of musculoskeletal anomalies and intestinal aganglionosis. Among the 20 WS2 cases, deafness and heterochromia iridum were the most frequent features, and 17 patients had sensorineural hearing impairment (17/20, 85.0%), which varied from moderate to profound. The age of deafness onset varied from congenital to 40 years. Additionally, younger patients frequently had congenital, profound, bilateral hearing loss (14/20, 70.0%).

All 20 affected individuals (20/20, 100.0%) had heterochromia iridum, including different-colored eyes in four cases and partial or segmental heterochromia of one or both eyes in eight cases. The characteristic brilliant blue iris, which is more often seen in WS1 cases, was observed in eight of the 20 WS2 patients. A white forelock was observed in two patients (2/20, 10.0%). Premature graying was not assessed due to the young age of most patients. Five affected individuals (5/20, 25.0%) had numerous brown freckles on the face, trunk, and limb extremities (Fig. 2). No patchy or generalized skin depigmentation was observed in any of the patients. Table 1 lists the clinical data of these 20 Chinese WS2 patients.

Identification of mutations
A heterozygous nonsense mutation, c.763C>T, in MITF exon 8 was identified in three affected members (II:7, III:21, and IV:15) of family WS01, resulting in a premature termination codon at 255 within the helix-loop-helix leucine zipper domain of the MITF protein (p. Arg255X). Unaffected married-in-control family members (II:8 and III:22) did not have the same mutation.

Three heterozygous missense mutations in MITF were identified in the WS2 patients. Heterozygous c.20A>G was identified in subjects II:1, III:2, and III:3 of family WS02 and resulted in the replacement of tyrosine by cysteine at codon 7 (p. Tyr7Cys). Unaffected married-in-control family member II:2 did not carry this mutation. Heterozygous c.649A>G (p. Arg217Gly) was found in family WS08. A nucleotide substitution, c.332C>T (p.
A heterozygous c.647_649delGAA deletion was identified in family WS04 and caused the deletion of one of a run of four arginines in the basic domain of the MITF protein. Although the DNA and protein sequences did not reveal which of the four arginines was deleted, we named this mutation p. Arg217del, based on the literature. The unaffected parents of family WS04 did not carry this mutation. None of these five MITF mutations was found in the 200 unrelated normal controls. Figure 3 presents the chromatograms of five mutations. We did not detect any mutations in the SOX10 or SNAI2 genes.

In silico analysis of missense substitutions

The p. Tyr7Cys and p. Ala111Val mutations fell outside of the important MITF domains, and were predicted to have a neutral effect by the PolyPhen-2, PMut, and SIFT software, which indicated ‘benign’, ‘neutral’, and ‘tolerated’, respectively. The missense substitution p. Arg217Gly fell within the MITF helix-loop-helix domain, and was predicted to have a pathogenic effect by the PolyPhen-2, PMut, and SIFT software, which indicated ‘probably damaging’, ‘pathological’, and ‘affect protein function’, respectively. We also examined the evolutionary conservation of the mutated residues and surrounding amino acids. This analysis revealed that arginine at position 217 in MITF is highly conserved in different species (Fig. 3).

**Discussion**

Sensorineural hearing loss is the most common feature of WS2, and is also the main reason motivating patients to visit a physician. Hageman and Delleman, who reviewed 159 cases in the literature, reported that deafness occurred in 57% of WS2 cases [15], and Liu et al. reported a rate of 77% (62/81) [3]. We found a similarly high rate of 85.0% (17/20) in these Chinese WS2 patients, and found that the severity of deafness varied within and between families, ranging from moderate to profound. Bilateral hearing loss (16/20, 80.0%) was more frequent than unilateral hearing loss (1/20, 5.0%). Age at onset varied from congenital to postlingual hearing loss, with congenital, bilateral profound hearing loss frequently observed in teenagers. Heterochromia iridum was observed in 100.0% (20/20) of the patients in our study, much higher than the rates reported by Liu et al. [3] in a British population (40/81, 49.4%) or by Hageman and Delleman (73/159, 46.0%) [15]. Heterochromia iridum included partial or segmental heterochromia of one or both eyes in eight cases (8/20, 40.0%), eyes of different color in four cases (4/20, 20.0%), and the characteristic brilliant blue iris in eight cases (8/20, 40.0%). Our results support the findings of Liu et al. [3]: the characteristic brilliant blue iris often seen in WS1 is not a rare clinical feature.

| Pedigree | Gender | Age (years) | Iris | Skin | W | Severity of HL |
|----------|--------|-------------|------|------|---|----------------|
|          |        | At Testing | At Onset |      |    | Left ear | Right ear |
| WS01     | II-7   | Male       | 58    | 10   | A | +   | 1.80   | profound  |
|          |        |            |       |      |    |         | moderate|
|          | III-21 | Male       | 30    | 10   | B | +   | 1.76   | profound  |
|          |        |            |       |      |    |         | normal  |
|          | IV-15  | Male       | 3     | Prelingual | C | -   | 1.45   | profound  |
|          |        |            |       |      |    |         | profound|
| WS02     | II-1   | Male       | 69    | 40   | B | -   | 1.84   | severe   |
|          |        |            |       |      |    |         | severe  |
|          | III-2  | Female     | 39    |     | B | +   | 1.82   | normal   |
|          |        |            |       |      |    |         | normal  |
|          | III-3  | Male       | 36    |     | B | +   | 1.76   | normal   |
|          |        |            |       |      |    |         | normal  |
| WS03     | II-4   | Female     | 36    |     | B | -   | 1.70   | normal   |
|          |        |            |       |      |    |         | normal  |
|          | III-2  | Female     | 12    | Prelingual |  | -   | 1.65   | profound  |
|          |        |            |       |      |    |         | profound|
|          | III-4  | Male       | 6     | Prelingual |  | -   | 1.63   | profound  |
|          |        |            |       |      |    |         | profound|
|          | WS04   | Female     | 16    | Prelingual |  | +   | 1.64   | profound  |
|          |        |            |       |      |    |         | profound|
|          | WS05   | Male       | 18    | Prelingual |  | -   | 1.76   | profound  |
|          |        |            |       |      |    |         | profound|
|          | WS06   | Male       | 14    | Prelingual |  | -   | 1.80   | profound  |
|          |        |            |       |      |    |         | profound|
|          | WS07   | Male       | 15    | Prelingual |  | -   | 1.74   | profound  |
|          |        |            |       |      |    |         | profound|
|          | WS08   | Male       | 4     | Prelingual |  | -   | 1.43   | profound  |
|          |        |            |       |      |    |         | profound|
|          | WS09   | Female     | 9     | Prelingual |  | -   | 1.41   | profound  |
|          |        |            |       |      |    |         | profound|
|          | WS10   | Male       | 2     | Prelingual |  | -   | 1.40   | profound  |
|          |        |            |       |      |    |         | profound|
|          | WS11   | Male       | 6     | Prelingual |  | -   | 1.75   | profound  |
|          |        |            |       |      |    |         | profound|
|          | WS12   | Female     | 7     | Prelingual |  | -   | 1.53   | profound  |
|          |        |            |       |      |    |         | profound|
|          | WS13   | Female     | 14    | Prelingual |  | -   | 1.86   | profound  |
|          |        |            |       |      |    |         | profound|
|          | WS14   | Male       | 3     | Prelingual |  | -   | 1.60   | profound  |
|          |        |            |       |      |    |         | profound|

A, complete heterochromia iridis; B, partial or segmental heterochromia iridis; C, brilliant blue iris; Skin, numerous brown freckles on the face, trunk, and limb extremities; W, W index; HL, hearing loss; +, sign present; -, sign absent.

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manifestation of heterochromia iridum in WS2. Skin pigmentary abnormalities were less frequent (5/20, 25.0%), and generally appeared as numerous brown freckles on the face, trunk, and limb extremities. Chen et al. reported 13 Chinese WS2 cases, five of whom manifested very similar skin pigmentary abnormalities [16]. Instead of patchy, depigmented skin, as seen in most Western cases, numerous brown freckles on the skin might be a more common phenotype of skin pigmentary abnormalities in Chinese WS2 patients.

MITF (microphthalmia-associated transcription factor) protein is a member of the helix-loop-helix leucine zipper (b-HLH-Zip) transcription factor family and is the key transcription factor for melanocyte development [17]. Through binding specific DNA sequences, MITF also regulates the transcription of several key melanocytic genes, including tyrosinase (TYR), tyrosinase-related protein 1 (TRP1), and TRP2 (also known as DCT) [18,19]. To date, fewer than 80 MITF and mitf mutations have been identified in human and mouse alleles [MITF homepage-LOVD-Leiden Open Variation Database, <http://grenada.lumc.nl/LOVD2/WS/>; Mouse Genome Database, <http://www.informatics.jax.org>]. Among these, fewer than 40 MITF mutations have been identified in a number of human WS2 and Tietz syndrome families, and most were private mutations, except for c.33+1G>A, c.640C>T (p. Arg214X), c.775C>T (p. Arg259X), and c.649_651delAGA (p. Arg217del). Several human mutations are identical to mouse mitf alleles, including p. Arg217del (mi allele in mouse), p. Arg216Lys (miore allele in mouse), and p. Ile224Ser (mienu122 allele in mouse). The majority of these mutations are located in exons 7 and 8 of the MITF gene; these encode the b-HLH-Zip domain. The b-HLH-Zip domain makes sequence-specific DNA contacts with the basic region of the domain and mediates the homo- and heterodimeric interactions necessary for
DNA binding. Interruption of the b-HLH-Zip domain binding decreases the ability of the mutant MITF protein to bind to the CATGTT core DNA sequence in the human tyrosinase promoter [20].

In this study, we identified five MITF variations: p. Tyr7Cys, p. Ala111Val, p. Arg217Gly, p. Arg217del, and p. Arg255X. Missense mutations, p. Tyr7Cys and p. Ala111Val, are more likely to have neutral effects, while p. Arg217Gly, p. Arg217del and p. Arg255X appear to be more likely to have disease-causing effects. The total mutational frequency of the MITF gene in our study was 21.4% (3/14), higher than the rate of 15% reported in other studies. The total mutational frequency in our study may be partly due to bias associated with the small number of samples we tested. A larger cohort of WS2 patients should be recruited for future studies.

In the literature, we found three de novo MITF gene mutations in 14 Chinese WS2 cases for which only exons and flanking splicing sites had been sequenced [16,21]. Thus, we propose creating a MITF mutation database for the Chinese population. Table 2 summarizes the MITF gene mutations identified in Chinese WS2 patients.

The p. Arg255X mutation appears to result in a truncated MITF protein, lacking the 3' end of the helix-loop-helix domain, the whole leucine zipper domain, and the third transactivation domain. Mutant MITF proteins are thought to have defects in homo- or heterodimerization and DNA binding through their basic regions, as well as DNA binding with related transfer factors domain. Mutant MITF proteins are thought to have defects in the basic region, and the third transactivation domain. Mutant MITF proteins are thought to have defects in the homo- or heterodimerization and DNA binding through their basic regions, as well as DNA binding with related transfer factors domain.

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**Table 2.** Summary of MITF Gene Mutations Identified in Chinese WS2 Patients.

| No. | Nucleotide change | Amino acid change | Exon | References |
|-----|------------------|------------------|------|------------|
| 1   | c.20A>G*         | p. Tyr7Cys*      | 1    | This study |
| 2   | c.332C>T*        | p. Ala111Val*    | 3    | This study |
| 3   | c.575delC*       | P. Thr192LysfsX20* | 6    | [16] |
| 4   | c.639delA*       | p. Glu213AspfsX8* | 7    | [21] |
| 5   | c.647_649delGAA  | p. Arg217del     | 7    | This study |
| 6   | c.648_650delAAAG | p. Arg217del     | 7    | [16] |
| 7   | c.649A>G*        | p. Arg217Gly*    | 7    | This study |
| 8   | c.650G>T*        | p. Arg217Asn*    | 7    | [16] |
| 9   | c.763C>T*        | p. Arg255X*      | 8    | This study |

a. Description of the mutations is based on the GenBank reference sequence for the M isoform of the MITF gene: NM_000248.3.

b. Amino acid numbering is based on GenBank Reference Sequence: NP_000239.1.

* mutations first described in a Chinese population.

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**Conclusions**

In this study, we conducted clinical evaluations and mutation identification of the MITF gene in 20 Chinese WS2 patients from 14 families. The molecular basis of 78.6% of WS2 cases remains unclear, and no correlation appeared between WS2 phenotype and genotype. Based on the high rates of MITF mutation in our Chinese WS2 patients, a MITF mutation database for the Chinese population should be created. Future research should examine more WS2 cases, employ new techniques to identify new mutations in the known genes and identify other possibly related genes, and focus on functional studies using animal models.

**Supporting Information**

**File S1**

(DOC)

**Acknowledgments**

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