Case Report/Series

Mutation of *PAX3* and *MITF* genes in a family with type 1 Waardenburg syndrome: a case series

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ABSTRACT

Waardenburg syndrome (WS) is a rare genetical disorder, characterized with pigmentary abnormalities of the eyes, skin, hair, dystopia canthorum, and sensorineural deafness. In Majene, West Sulawesi, 12 members of a 4-generation family presented manifestations of WS. We examined the presence of mutations in 5 family members with type 1 WS and the other 5 normal phenotype family members to identify mutations of *PAX3* and *MITF* genes. Ophthalmic examination and peripheral blood test were done. Conventional polymerase chain reaction and direct Sanger sequencing were then performed to detect the mutation. 26 mutations of *PAX3* gene were only identified in patients with major and minor criteria, including 7 missense mutations (substitutions) and 2 insertions in exons 1, 2, and 6, as well as 17 intronic changes in intron 8. No mutations were detected in *MITF* gene.

KEYWORDS genes, Waardenburg syndrome

Waardenburg syndrome (WS) is a rare genetic disorder of the neural crest, characterized by pigment abnormalities in the eyes, skin, and hair, dystopia canthorum, and sensorineural deafness.[1,2] WS is divided into four subtypes based on specific clinical signs. Type 1 WS (WS1) is characterized by the presence of dystopia canthorum, whereas type 2 WS usually presents with hearing loss without dystopia canthorum. In type 3 WS, dystopia canthorum is observed, along with musculoskeletal abnormalities in the arms and hands. In type 4 WS, the patient also has Hirschsprung disease, in which intestinal blockage and severe constipation may happen.[3,4]

The major, minor, and rare diagnostic features of WS1 were defined based on the WS1 consortium. The major diagnostic features include pigmentary disorders of the iris, hair hypopigmentation, congenital sensorineural deafness, dystopia canthorum, and having a first-degree relative with WS1. Minor diagnostic features include synophrys or medical eyebrow flare, congenital leukoderma, hypoplasia of the alae nasi, broad and high nasal roots, and premature graying of the hair (<30 years of age). An individual must meet either (a) at least two of the major criteria or (b) one of the major criteria and two of the minor criteria (not including the rare criteria, such as Hirschsprung disease, Sprengel anomaly, spina bifida, cleft lip and/or palate, limb defects, congenital heart abnormalities, abnormalities of vestibular function, broad square jaw, and low anterior hairline) to be diagnosed with WS1.[5,6]

At the molecular level, WS1 can be influenced by six genes, namely paired box 3 transcription factor (PAX3), endothelin-3, SRY-box transcription factor

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10, microphthalmia-associated transcription factor (MITF), endothelin receptor type B, and snail homolog 2.[5] Each of which has interfamilial and intrafamilial variabilities.[3] These genes are responsible for the establishment and development of specific cell types, such as melanocytes. Abnormalities caused by defects in these cells contribute to the clinical signs of WS1. This disorder is caused by the absence of melanocytes from hair, skin, eyes, and/or cochlear stria vascularis.[4] Furthermore, any pigmentary changes in the eye do not only affect the iris but also the choroid.[7,8] The affected choroid areas are slightly thinner compared to normal tissues but without lipofuscin abnormalities.[8]

In Majene, West Sulawesi, Indonesia, 12 members of the same family were diagnosed with WS1. To our knowledge, there have been no studies presenting data about genetic mutations of this rare genetic disease in Indonesia. Therefore, this case study aimed to investigate the presence and type of mutations in *PAX3* and *MITF* genes and compare them with normal family members. We hope this will provide insight into the genetic factors influencing WS1.

CASE REPORT

Twelve members of a family across four generations presented with manifestations of WS (Figure 1). Among them, three had bright blue irides in both eyes, and four had heterochromia. Of the members with bright blue irides, two had sensorineural hearing loss and poliosis accompanied by a broad nasal bridge. Three other patients exhibited only hypertelorism and poliosis. One family member passed away prior to this case study, and one other was not present during the assessment. All the participants had normal visual acuity. No history of consanguineous marriage was reported.

The presence of genetic mutations was examined in five of the family members with WS1, including the proband and the proband's sister, brother, niece, and grandson. Five unaffected family members with normal phenotypes were also examined.

The diagnosis of WS1 was confirmed by ophthalmological and otorhinolaryngological examinations. Normal participants were family members without the WS1 phenotype. The blood samples were examined at the Hasanuddin University Medical Research Center (HUMRC) laboratory, Makassar, Indonesia.

Cases

The procedures were performed following Declaration of Helsinki and approved by the Ethics Committee of Universitas Hasanuddin (No: 644/UN4.6.4.5.31/PP36/2021). Written and signed informed consent from the patients or the parents was obtained before the examinations done.

Of the five patients with WS1, three exhibited major criteria for WS1, and two exhibited minor criteria (Figure 2). The proband (case 1) had blue irides, congenital leukoderma, and poliosis. Other family members with WS1 had varied signs of WS1, such as blue irides, congenital leukoderma, poliosis, premature hair graying, and congenital sensorineural deafness.

In this study, we identified 74 variations in *PAX3* expression in patients with WS1. Of these, 53 were found in normal family members. Twenty-one mutations of the *PAX3* gene were only identified in patients with major and minor criteria, including 11 missense mutations (substitutions), 1 nonsense mutation, 2 insertion mutations in exons 6 and 10, and 7 intronic changes in introns 2 and 9. An example of a *PAX3* heterozygous mutation in exon 10 is shown in Figure 3. Five variations of the *PAX3* gene are known,

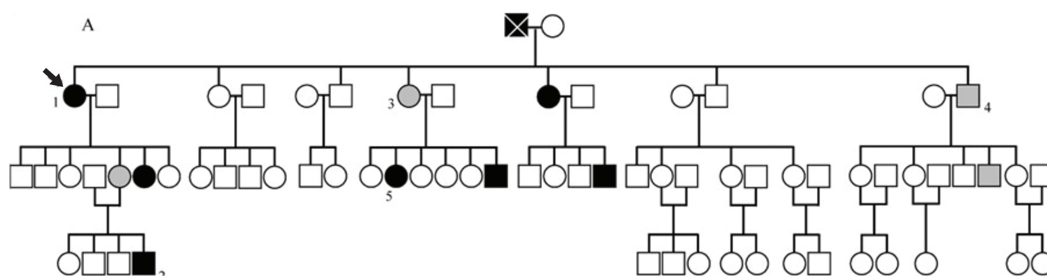

Figure 1. Pedigree of the family with type 1 Waardenburg syndrome (WS1) cases. Square represented male, and round represented female. Number showed orders of cases. Cross symbol represented deceased person. Arrow showed the proband (case no. 1). Solid black represented person with major criteria, while solid gray represented those with minor criteria

Figure 2. Five patients with Waardenburg syndrome (WS)

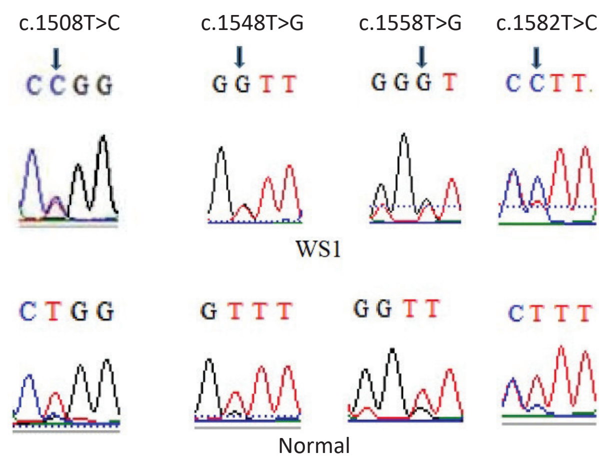

Figure 3. Sequencing result of PAX3 heterozygous mutations in exon 10. PAX3=paired box 3 transcription factor; WS1=type 1 Waardenburg syndrome

all of which were also found in family members. Mutations of the *PAX3* gene that were found only in patients with WS1 are shown in Table 1. Variations in the *MITF* gene, which were also observed in healthy individuals, are presented in Table 2.

Sample collection and DNA extraction

A total of 5 ml of peripheral blood was collected into standard EDTA tubes and stored at −20°C until DNA extraction. Blood samples were analyzed at the HUMRC laboratory. DNA was isolated from peripheral blood leukocytes using a gSYNC DNA Extraction Kit (Geneaid Biotech Ltd, Taiwan) according to the manufacturer's protocol.[9] DNA quality was evaluated by 2% agarose gel electrophoresis at 100 V (Bio-Rad, USA); the DNA quantity was not calculated.

Polymerase chain reaction (PCR) and sequencing

Mutational screening of whole exons from the *PAX3* and *MITF* genes was performed using direct Sanger sequencing. The primer sequences used are listed in Table 3. A total volume of 50 µl of DNA was amplified in a PCR DNA thermal cycler (Bio-Rad). The reaction components were 1 µl forward and 1 µl reverse *PAX3* and *MITF* gene primers (Table 3), 25 µl KAPA2G fast enzyme, 18 µl nuclease-free water, and 5 µl DNA template. The PCR conditions were as follows: denaturation at 95°C for 15 min, then cycles of 94°C for 1 min, annealing at 55°C for 30 sec, and extension at 72°C for 1.5 min, followed by 72°C for 10 min for final extension, and then 12°C for 30 min. Sequencing was performed and examined using the BioEdit Sequence Alignment Editor version 7.0.5.1 (Hall, Tom; North Carolina State University, USA). The results and data from GenBank of the National Center for Biotechnology Information GRCh37 database were compared using a basic local alignment search tool. Additional information regarding mutations in the *PAX3* and *MITF* genes was obtained from The Human Gene Mutation Database and the Leiden Open Variation Database.

DISCUSSION

In this study, all five patients with WS1 had dystopia canthorum. The other most common signs were congenital leukoderma (80%), pigmentary iris disorder (60%), and poliosis (60%). Two patients had premature hair graying, and one had congenital sensorineural deafness. Unfortunately, we could not examine the remaining six family members with WS1.

Most genetic mutations were found in exon 10. The most common PAX3 protein is formed by a stop codon in exon 8.[4,10] Differential splicing before the exon 8 stop codon and within exons 9 and 10 will produce a different C-terminal end of the protein.[11] Although the significance of these changes is unknown, they may have functional abilities analogous to the most common isoform.[3,4]

We found one mutation in exon 1 and six mutations in exon 2 of the *PAX3* gene. Mutations in exon 2 have also been commonly reported in previous studies.[3,12,13] Approximately 95% of *PAX3* mutations were found in the DNA-binding domains. The highest number of pathogenic variants were found in exon 2 of the paired domain, followed by exons 5 and 6, which encode the homeodomain.[3] Missense mutations in

Table 1. *PAX3* gene mutations in patients with WS1

Patient ID	Exon/intron no	g. position	c. position	Protein change	Type of mutation
WS 1	Intron 2	g.7077T>C	c.321+73T>C	-	Intronic
WS 5	Intron 2	g.7078T>C	c.321+74T>C	-	Intronic
WS 5	Exon 6	g.82613InsC	c.811InsC*	-	Insertion
WS 5	Exon 6	g.82664InsC	c.862InsC*	-	Insertion
WS 1	Intron 9	g.102699A>C	c.1452-43A>C	-	Intronic
WS 1	Intron 9	g.102701T>A	c.1452-41T>A	-	Intronic
WS 1	Intron 9	g.102709A>C	c.1452-33A>C	-	Intronic
WS 1	Intron 9	g.102720A>G	c.1452-22A>G	-	Intronic
WS 1	Intron 9	g.102724A>G	c.1452-18A>G	-	Intronic
WS 1	Exon 10	g.102764A>C	c.1474A>C*	His>Pro	Missense
WS 1	Exon 10	g.102779A>C	c.1489A>C*	Gln>Pro	Missense
WS 1	Exon 10	g.102781T>G	c.1491T>G*	Trp>Gly	Missense
WS 1	Exon 10	g.102798T>C	c.1508T>C*	Pro>Thr	Missense
WS 1	Exon 10	g.102820A>C	c.1530A>C*	Asn>His	Missense
WS 1	Exon 10	g.102823A>C	c.1533A>C*	Ile>Leu	Missense
WS 1	Exon 10	g.102838T>G	c.1548T>G*	Phe>Val	Missense
WS 1	Exon 10	g.102844A>C	c.1554A>C*	Met>Leu	Missense
WS 1	Exon 10	g.102848T>G	c.1558T>G*	Val>Gly	Missense
WS 1	Exon 10	g.102855T>A	c.1565T>A*	Tyr>Stop	Nonsense
WS 1	Exon 10	g.102862A>C	c.1572A>C*	Ile>Leu	Missense
WS 1	Exon 10	g.102872T>C	c.1582T>C*	Leu>Pro	Missense

*Novel mutation
Based on *PAX3* gene transcript 205 ensembl Asia
ID=identity; *PAX3*=paired box 3 transcription factor; WS1=type 1 Waardenburg syndrome

Table 2. *MITF* gene variations in patients with WS1

Patient ID	Exon/intron no	g. position
WS 1	Promoter	g. 1554&1555 AT>TA
WS 3	Promoter	g. 1554&1555 AT>TA
WS 5	Promoter	g. 1554&1555 AT>TA
WS 6	Promoter	g. 1554&1555 AT>TA
WS 10	Promoter	g. 1554&1555 AT>TA

ID=identity; *MITF*=microphthalmia-associated transcription factor; WS1=type 1 Waardenburg syndrome

the *PAX3* gene cause amino acid changes from glycine to glutamic acid in exon 1, and valine to alanine and threonine to serine in exon 2. Most human genetic mutations are caused by single-nucleotide changes.[14,15] Amino acid changes caused by mutations in the DNA-binding domain may alter the structure of the paired DNA-binding sites, causing functional impairment. Those possessing a mutation in *PAX3* develop several phenotypes, including abnormalities of the central nervous system, eye, and nose, and pigmentation abnormalities affecting the skin, hair, and otic pigment cells that are important for normal hearing, as found in WS1.[16,17]

In the present study, most of the mutations in exons were missense mutations (71.43%). Pingault et al[3] identified trimmed variations in approximately half of the mutations, while the remaining half were missense mutations in the *PAX3* gene. Other studies have reported a low amount of recurrency among approximately 100 sequence changes in the *PAX3* gene.[3,18] Previously reported mutations include missense mutations (38%), small deletions (20%), nonsense mutations (15%), gross deletions (11%), small insertions (8%), and splicing mutations (8%).[19] Among these variants, seven intronic mutations were identified in the present study. Only a small number of bases in introns are functional targets for pathogenic mutations;[20] however, point mutations deep within introns may alter normal splicing,

Table 3. Primer sequences used in PCR

Exons no	Length (bp)	Forward primers (5'-3')	Reverse primers (5'-3')	Annealing temp (°C)
PAX3				
1	788	GATGGGAAGAGAAAGTGGTC	TGCAGAAAGGAAATCGAGTA	62
2	503	CCGATGTCGAGCAGTTTCAG	CGCACCTTCACAAACCTCAG	64
3	420	TGGGATGTGTTCTGGTCTG	TCCCAATAGCTGAGATCGA	60
4	383	CTGGAGAAGGATGAGGATGT	CGTCAGATCACCAATGTCAG	62
5	508	TACGGATTGGTTAGACTTGT	AACAATATGCATCCCTAGTAA	57
6	445	CAACACAGAAGGCAGAGA	ATAGGTACGTTCAGGACAA	57
7	586	TGTGCAGAGATAGGTGTGAC	TTTGATGAAGCCAGTAGGA	57
8	480	TCTCCTGGACAGCTCTTTAA	GGCATGTGTGGCTTAATCT	57
9 & 10	580	GGTCAGCTCCAGGATCATAT	GCAAATGGAATGTTCTAGCT	60
MITF				
1		GGATACCTTGTTTATAGTACCTTC	AAAAGAGCAGATTTATACTTATTG	
2		TCTGAAACTCACAAATAACAGCGC	TATTCAACAGACAAGTTATTTAGC	
3		CCATCAGCTTTGTGTGAACAGGTC	TTTCAGGAAGGTGTGATCCACCAC	
4		AACTAAAGACCATTATTGCTTTGG	AGAAAAGAACCCTGGAAACACCTC	
5		ATAAATCCTAGAGTAGGATATAGG	ACTTTGTCTTATCAGGAAATGGAC	
6		TCAAGTCAAATAAGCTTCTGTATG	GTAGGAATCAACTCTCCTCTACAG	
7		GTGCTAAATGCATACATGGCACGT	TTAGGAATAGAACCAAAGGGAGAG	
8		TTCATTGAGCCTCAAATCCTAAAG	CTGTTTCTACTGTCTTGAAGTCGG	
9		AGTCCTCTGTGCTCGTCCTATTTC	AAGCTAAAGTCTGTGGTGAATTC	

bp=base pair; *MITF*= microphthalmia-associated transcription factor; *PAX3*= paired box 3 transcription factor; PCR=polymerase chain reaction

transcription regulators, and non-coding RNA genes.[21,22] Additional analysis, such as minigene assays, are needed to confirm the pathogenicity of intronic mutations.[23,24]

In the present study, no mutations of the *MITF* gene were detected in patients with WS1. *MITF* gene mutations occur in approximately 15% of WS2 cases.[3,4] Studies have indicated that WS2 tends to be associated with clinical signs influenced by MITF gene mutations.[25] The absence of *MITF* gene mutations in this study might be because all patients were classified as having WS1. The limitations of this study included having a relatively small sample size and incomplete family mapping. Moreover, additional analyses, such as minigene assays, were not performed. In conclusion, when observing genetic mutations in related WS1 patients, 26 mutations in the *PAX3* gene were found, including 7 missense mutations and 2 insertions in exons 1, 2, and 6, as well as 17 intronic changes in intron 8; no mutations were found in the *MITF* gene. The finding has implications for genetic counseling and risk prediction for the families with this genetic disorder.

Conflict of Interest

The authors affirm no conflict of interest in this study.

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