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SPECTRUM OF NOVEL MUTATIONS FOUND IN WAARDENBURG SYNDROME TYPE 1 AND TYPE 2: IMPLICATIONS FOR MOLECULAR GENETIC DIAGNOSTICS

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SPECTRUM OF NOVEL MUTATIONS IN WAARDENBURG SYNDROME TYPE 1 AND TYPE 2: IMPLICATIONS FOR MOLECULAR GENETIC DIAGNOSTICS

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ABSTRACT

Objectives: To date, mutations in the genes *PAX3* and *MITF* have been described in Waardenburg syndrome (WS), which is clinically characterized by congenital hearing loss, pigmentation anomalies and craniofacial dysmorphism. Our study intended to determine the frequency of mutations and deletions in these genes, to assess the clinical phenotype in detail and to identify rational priorities for molecular genetic diagnostics procedures.

Design and patients: In a prospective analysis, 19 caucasian patients with typical features of Waardenburg syndrome underwent stepwise investigation of *PAX3* and *MITF*. When point mutations and small insertions/deletions were excluded by direct sequencing, copy number analysis by MLPA was performed to detect larger deletions and duplications. Clinical data and fotographs were collected to facilitate genotype-phenotype analyses.

Setting: All analyses were performed in a large german laboratory specialized in genetic diagnostics.

Results: 16 novel and three previously published heterozygous mutations in *PAX3* and *MITF* were identified. Of these, six were large deletions or duplications, that were only detectable by copy number analysis. All patients with *PAX3* mutations had the typical phenotype of WS with dystopia canthorum (WS1), whereas patients with *MITF* gene mutations presented without dystopia canthorum (WS2). In addition, one patient with bilateral hearing loss and blue eyes with iris stroma dysplasia had a *de novo* missense mutation (p.Arg217Ile) in *MITF*. *MITF* 3-bp deletions at amino acid position 217 have previously been described in patients with Tietz syndrome, a clinical entity with hearing loss and generalized hypopigmentation.

Conclusion: Based on these findings, we conclude that sequencing and copy number analysis of both *PAX3* and *MITF* have to be recommended in the routine molecular diagnostic setting for WS patients with and without dystopia canthorum. Furthermore WS without dystopia (WS2) and TS most probably correspond to a clinical spectrum that is influenced by *MITF* mutation type and position.

ARTICLE SUMMARY

Article Focus:

• To determine the frequency of point mutations and copy number variations in the genes PAX3 and MITF in patients with clinical features of Waardenburg syndrome (congenital hearing loss, pigmentation anomalies, craniofacial dysmorphism);

• To assess the clinical phenotype in detail;

• To identify rational priorities for molecular genetic diagnostics procedures in Waardenburg syndrome.

Key Messages:

• 16 novel and three previously published heterozygous mutations in PAX3 and MITF were identified; of these, one third were larger deletions and duplications, which can only be ascertained by copy number analysis in addition to direct sequencing.

• All patients with PAX3 mutations had the typical phenotype of WS with dystopia canthorum (WS1), whereas patients with MITF gene mutations presented without dystopia canthorum (WS2) or had an overlapping phenotype with Tietz syndrome (TS).

• Both sequence and copy number analysis of the genes PAX3 and MITF should be performed in WS patients with and without dystopia canthorum.

Strength and Limitations:

• Our study revealed a large proportion of novel mutations in PAX3 and MITF, provides a detailed genotype-phenotype correlation, and points to an overlap of Waardenburg and Tietz syndrome.

INTRODUCTION

Waardenburg syndrome (WS) is an auditory-pigmentary syndrome that occurs with a frequency of 1 in 40,000.[1] WS has been classified into four main phenotypes: Type 1 (WS1) is characterized by congenital sensorineural hearing loss, heterochromia irides, partial hypopigmentation of the hair including premature graying, and lateral displacement of the inner ocular canthi (dystopia canthorum). Type 2 (WS2) is distinguished from WS1 by the absence of dystopia canthorum. WS3 or Klein-Waardenburg syndrome is similar to WS1, but includes upper limb abnormalities. WS4 or Waardenburg-Shah syndrome has features of Hirschsprung disease in addition.

Waardenburg syndrome is genetically heterogeneous. Point mutations in the *PAX3* gene are described to be the most frequent cause of WS1 and WS3.[2-4] *PAX3* is a member of the mammalian *PAX* gene family and encodes a DNA binding transcription factor expressed in neural crest cells.[5] It plays an important role for the migration and differentiation of melanocytes, which originate from the embryonic neural crest. The *PAX3* gene is structurally defined by the presence of a highly conserved 128 amino acid DNA-binding domain, known as the paired domain, and a second DNA-binding domain, the homeodomain.[5, 6] *PAX3* mutations associated with WS1 include substitutions of conserved amino acids in the paired domain or the homeodomain of the protein, splice-site mutations, nonsense mutations, and insertions or deletions leading to frame shifts.[7, 8] All previously published *PAX3* mutations in WS1 are heterozygous, whereas both heterozygous and homozygous *PAX3* mutations have been described in the allelic disease WS3.[8-13]

Heterozygous mutations in the *MITF* gene are the major cause of WS 2.[14, 15] The *MITF* gene encodes a transcription factor with a basic helix-loop-helix leucine zipper motif. Proteins with this kind of motif form homo- or heterodimers by their HLH-zip regions and bind DNA with their basic domain. An additional candidate gene for WS2 is *SNAI2*.[16] In addition, mutations in *SOX10, EDN3* and *EDNRB* were found in WS4.[17]

The exact description of the mutations responsible for the WS type is of significant importance in genetic counseling of WS patients and their families. Whole-gene sequencing enables the discovery of point mutations and small alterations in the gene, but cannot reliably detect whole-exon or whole-gene copy number changes, which have been reported for many genes resulting in a specific genetic disorder. In recent years, MLPA (multiplex ligation-dependent probe amplification) has become a widespread method in molecular genetic diagnostics to detect or exclude copy number changes in targeted genes.

We applied both whole-gene sequencing and MLPA analysis for the detection of point mutations and copy number variations in the genes *PAX3* and *MITF,* and describe 19 mutations, of which 16 have not been previously reported for patients with features of WS.

MATERIALS AND METHODS

Patients

19 caucasian patients with the clinical phenotype of WS were included in our study. Clinical data were collected for patients and their affected family members.

Molecular genetic analysis

All 19 patients were analysed for point mutations and copy number changes in the genes *PAX3* and *MITF*. Genomic DNA was prepared from white blood cells using a standard procedure. All 10 exons of *PAX3* and all 9 exons of *MITF* were PCR-amplified and directly sequenced. Sequence variant numbering was based on the transcript ENST00000392069 for *PAX3* and ENST00000314557 for *MITF*. Nucleotide numbering used the A of the ATG translation initiation site as nucleotide +1. The nomenclature of the alterations was adopted according to the guidelines of the human variation society (http://www.hgvs.org/). MLPA (multiplex ligation-dependent probe amplification) analysis was carried out in order to detect

or exclude deletions and duplications of the genes *PAX3* and *MITF*. For this purpose, a mix of probes was hybridized to template DNA. The probes, which contained a common sequence tail, were ligated, and subsequently amplified by PCR. Products were separated electrophoretically and analyzed by means of sizing and quantification. The Software Sequence Pilot (JSI Medical Systems, Kippenheim, Germany) was used for evaluation.

RESULTS

Molecular genetic analysis of the genes *PAX3* and *MITF* for 19 unrelated index patients with the clinical phenotype of WS revealed 16 novel and three previously published heterozygous mutations (table 1). 14 mutations occurred in the *PAX3 gene:* one small insertion, two missense mutations, four nonsense mutations, two small deletions, one splice-site mutation, and four large deletions comprising at least one exon. Five mutations were detected in the *MITF* gene: one missense mutation, one nonsense mutation, one large deletion and one large duplication. In addition, the combination of a *MITF* missense mutation with a small deletion was found in family 11 (table 1). Both mutations were proven to be located on the same chromosome.

Of specific interest, six of the 19 mutations (32 %) were not detectable by direct sequencing. These were five large deletions and one duplication that could only be detected by MLPA analysis. In ten of 19 index patients, parents were available for genetic analysis. Seven mutations were familial (i.e. shown to be inherited from one parent), whereas three mutations (one mutation in *PAX3* and two in *MITF*) occurred *de novo*. 16 of 19 mutations detected in *PAX3* and *MITF* represented novel mutations. These mutations and all previously published mutations are summarized in figure 1 (*PAX3* gene) and figure 2 (*MITF* gene).

All index patients with *PAX3* mutations had the typical phenotype of Waardenburg syndrome type 1 including dystopia canthorum and at least one of the following criteria: heterochromia

index patient	age	gene	mutation description				transmission mode	dystopia canthorum	hetero-chromia iridis	hearing loss	other clinical symptoms	notes
			gene structure affected	nucleotide level	protein level	type						
1	6 1/2 y.	*PAX3*	exon 2	c.111dupC	p.Arg37fs	insertion	n.a.	+	+	(+)	synophrys, low frontal and nuchal hairline, skin hyperpigmentation anomalies	-
2	7 y.	*PAX3*	exon 2	c.143G>A	p.Gly48Asp	missense	maternal	+	-	+	high nasal bridge, synophrys, eyebrow flaring, skin depigmentation; affected family members with premature greying and dystopia canthorum	different amino acid change at same position described in [23]
3	3 y.	*PAX3*	exon 2	c.186G>A	p.Met62Ile	missense	paternal	+	+	++	-	different amino acid change at same position: described in [24]
4	34 y.	*PAX3*	exon 3	c.400C>T	p.Arg134X	nonsense	n.a.	+	+	-	-	-
5	6 mo.	*PAX3*	exon 5	c.589delT	p.Ser197fs	deletion	paternal	+	-	+	white forelock	-
6	3 mo.	*PAX3*	exon 5	c.655C>T	p.Gln219X	nonsense	paternal	n.a.	n.a.	n.a.	-	-
7	17 mo.	*PAX3*	exon 5	c.784C>T	p.Arg262X	nonsense	maternal	+	+	+	white forelock, synophrys, high nasal bridge, prognathia, hypopigmentation anomalies	described in [25]
8	1 wk.	*PAX3*	intron 5	c.793-1G>T	-	splice site	*de novo*	+	-	-	white forelock, craniofacial dysmorphism	-
9	17 mo.	*PAX3*	exon 6	c.946_956del	p.Thr315fs	deletion	n.a.	+	-	+	unilateral hearing loss	-
10	2 1/2 y.	*PAX3*	exon 6	c.955C>T	p.Gln319X	nonsense	n.a.	n.a.	n.a.	n.a.		-
11	n.a.	*MITF*	exon 1	c.28T>A / c.33+6del7	p.Tyr10Asn	missense / deletion	paternal	-	+	+	-	-
12	16 y.	*MITF*	exon 3	c.328C>T	p.Arg110X	nonsense	n.a.	-	+	+	-	-
13	7 mo.	*MITF*	exon 7	c.650G>T	p.Arg217Ile	missense	*de novo*	-	-	+	blue eyes with hypoplasia of iris stroma, white forelock	in-frame deletion at same amino acid position described in [20]
14	23 y.	*PAX3*	entire gene	c.(?_-61)_(1452-33_?)		deletion entire gene	paternal	+	-	+	pigmentation anomalies, unilateral hearing loss	described in [26]
15	42 y.	*PAX3*	exon 7	c.958+?_1174-?del		deletion exon 7	n.a.	+	-	+	pigmentation anomalies	-
16	6 y.	*PAX3*	exons 8-9	c.1173+?_(1452-33_?)del		deletion exons 8-9	n.a.	+	-	+	blue eyes, synophrys, medial eyebrow flaring	-
17	5 mo.	*PAX3*	entire gene	c.(?_-61)_(1452-33_?)		deletion entire gene	n.a.	+	+	+	unilateral hearing loss	described in [26]
18	7 y.	*MITF*	5´-UTR region	c.?_1-70453dup		duplication	n.a.	n.a.	n.a.	n.a.	-	-
19	3 y.	*MITF*	exons 1-9	c.1-70433_?_(988_?)del		deletion exons 1-9	*de novo*	-	+	+	bilateral hearing loss	-

Table 1: Genotype and phenotype of all Waardenburg syndrome index patients (wk = week; mo = month; y = years; n.a. = no information available; + mild; ++ severe)

iridis, hearing loss, and additional features such as white forelock, craniofacial dysmorphism including high nasal bridge, synophrys, eyebrow flaring, or anomalies of skin pigmentation (table 1, figure 3). Clinical information was available for 10 of 12 patients with *PAX3* mutations and all of these presented hearing. No differences in phenotype were noted between patients with *PAX3* point mutations and *PAX3* deletions.

Patients with *MITF* mutations had the clinical phenotype of Waardenburg syndrome type 2, i.e. heterochromia irides and/or hearing loss without dystopia canthorum (figure 4; clinical data not available for one patient). Patient 13, who was found to carry a *de novo* missense mutation in *MITF,* did not present with heterochromia irides, but with blue eyes and hypoplasia of iris stroma.

DISCUSSION

Molecular genetic analyses of the *PAX3* and *MITF* gene revealed 16 novel and three previously known mutations in patients with the clinical phenotype of Waardenburg syndrome. Of these, 14 mutations occurred in *PAX3*, and 5 mutations were found in *MITF*. The spectrum of mutations includes nonsense, missense and splice site mutations, insertions, as well as deletions and duplications.

Most of the novel mutations in *PAX3* are localized in exons 2 to 6 and hence influence functionally relevant domains (table 1, figure 1). This predominant localization in exons 2 to 6 has previously been described.[18] Twelve of the 14 *PAX3* mutations are truncating mutations including larger deletions. Deletions comprised two whole gene deletions and two intragenic deletions of exon 7 and exons 8-9, respectively. The two missense mutations are localized in the N-terminal part of the paired domain, which mediates intensive DNA contact. There is no correlation between the mutation type (missense, nonsense, deletion) and the severity of the phenotype. Therefore, loss of protein function seems to be the disease

causing mechanism for WS1. All patients with *PAX3* mutations had phenotypes of WS1 (table 1, figure 3).

Five patients had mutations in the *MITF* gene (table 1, figure 2). Four of these represented novel mutations. Clinical information was available in four patients. Three patients had features corresponding to the WS2 phenotype (figure 4). Interestingly, one of these (patient 11) was the first WS2 patient who was found to carry two *MITF* mutations (missense mutation and small deletion) within the same gene copy. Since both mutations are novel, it remains to be clarified whether one or the combination of both mutations leads to the WS2 phenotype.

One patient with a *de novo* missense mutation c.650G>T (p.Arg217Ile) in the *MITF* gene did not present with heterochromia irides, but with bilateral blue irides and hypoplasia of iris stroma (patient 13 in table 1). Interestingly, *MITF* mutations affecting amino acid position 217, which is located in the basic domain of MITF, were also described in patients with Tietz syndrome (TS, MIM #103500). Compared to WS2, Tietz syndrome is characterized by a more severe phenotype with generalized hypopigmentation and complete hearing loss.[19] Instead of heterochromia irides, which is typical for Waardenburg syndrome type 1 and 2, patients with Tietz syndrome present with bilateral blue irides. To date, only three patients with Tietz syndrome and mutation in the *MITF* gene have been described.[20-22] Two of these with typical Tietz syndrome had a mutation altering amino acid position 217 (3-bp in-frame deletion Arg217). Compared to these patients, our patient with a *de novo* missense mutation at the same amino acid position has a less severe phenotype with bilateral hearing loss, a white forelock and blue irides with hypoplasia of iris stroma. These clinical features are part of WS2, while blue eyes with hypoplastic iris stroma may correspond to Tietz syndrome. Apparently, a missense mutation at amino acid position 217 leads to milder or intermediate phenotype than a 3-bp in-frame deletion at the same position. Therefore, both

WS2 and TS most probably correspond to a common clinical spectrum that is influenced by mutation type and position.

In summary, the molecular genetic analyses of the *PAX3* and *MITF* genes are important diagnostic steps to explain the molecular cause of clinical features of Waardenburg syndrome and facilitate genetic counseling of affected patients and their families. For six of 19 patients with suspected WS and previously genetically unconfirmed diagnosis, we found either large deletions (four patients with deletions in *PAX3* and one patient with deletion in *MITF)* or duplications (one patient, *MITF* gene). This indicates that deletion/duplication screening is indispensable for a successful molecular genetic diagnostics of WS. As a genetic diagnostic strategy it is thus recommended to perform both sequence analysis and copy number analysis of the *PAX3* and *MITF* genes in all patients with clinical features of Waardenburg syndrome.

Competing Interests

There are no competing interests.

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Contributorship Statement

GW and DS responsible for study concept and design and acquisition of genetic data and interpretation. BZ, LGN, JW, MS, AB, ABo, CK, SV, GSW, MG and OB collected the clinical data. GW, BZ and DS wrote the manuscript. All authors have critically revised the paper.

Data Sharing Statement

There is no additional data available

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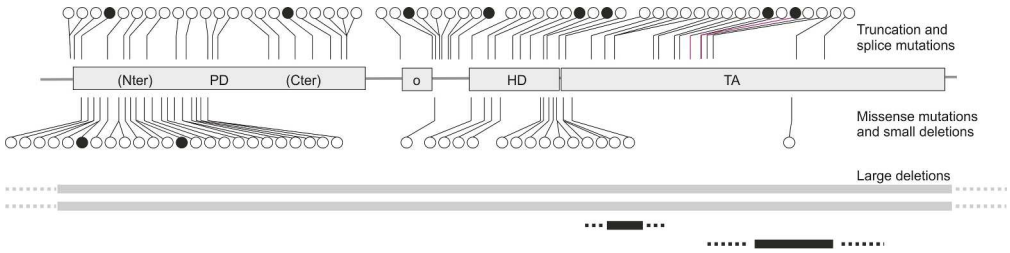

Figure 1: Survey of *PAX3* mutations detected in patients with Waardenburg syndrome. Black circles = mutations detected in this study; black bars = copy number variations detected in this study; white circles and grey bars = previously published mutations and deletions. PD = paired domain; o = octapeptide; HD = homeodomain; TA = transactivation domain.
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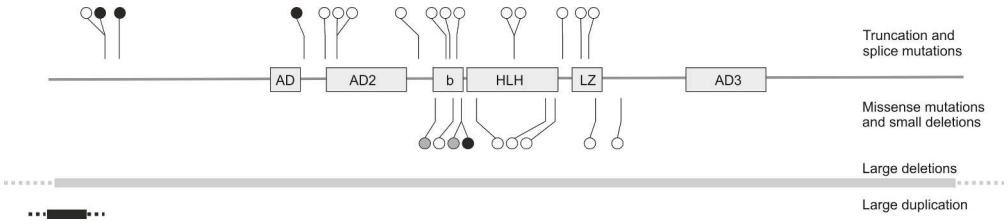

Figure 2: Survey of *MITF* mutations detected in patients with Waardenburg and Tietz syndrome. Black circles and bars = mutations and copy number variations detected in this study; white circles and grey bars = previously published mutations and deletions; grey circles = MITF mutations associated with Tietz syndrome. AD1-3 = (trans)activation domains; b = basic domain; HLH = helix-loop-helix domain; LZ = leucine zipper domain.
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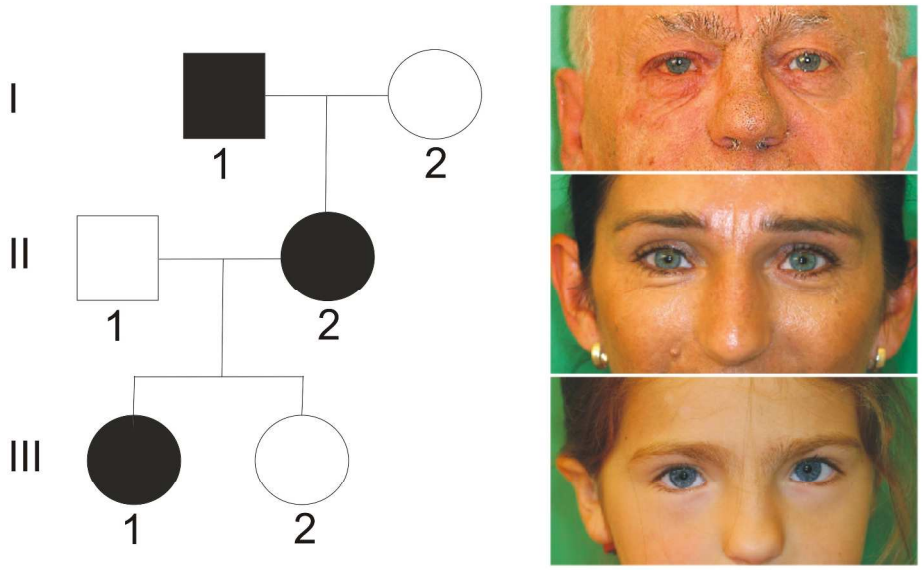

Figure 3: Pedigree of index patient 2 with *PAX3* mutation. The girl presented with clinical features of Waardenburg syndrome type 1 (see table 1): dystopia canthorum, high nasal bridge, synophrys, eyebrow flaring, skin depigmentation and bilateral hearing loss. Her mother and maternal grandfather had only premature graying.

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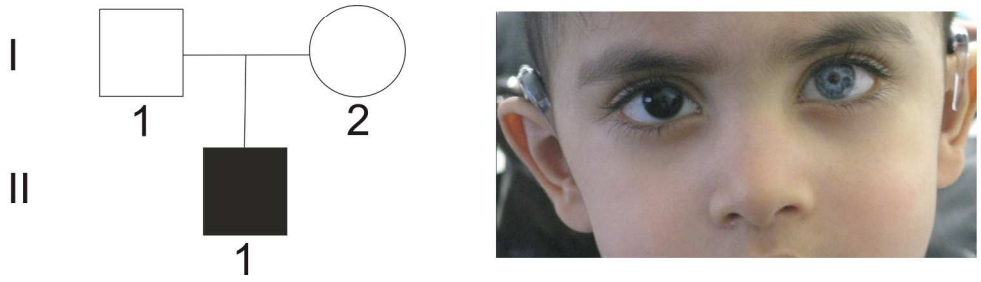

Figure 4: Sporadic case (patient 19 in table 1) of Waardenburg syndrome type 2 with *MITF* mutation. The boy presented with heterochromia irides and bilateral hearing loss, but did not show dystopia canthorum.
226x77mm (300 x 300 DPI)

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SPECTRUM OF NOVEL MUTATIONS IN WAARDENBURG SYNDROME TYPE 1 AND TYPE 2: IMPLICATIONS FOR MOLECULAR GENETIC DIAGNOSTICS

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Keywords: Waardenburg syndrome, Tietz syndrome, hearing loss, *PAX3* gene, *MITF* gene
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ABSTRACT

Objectives: To date, mutations in the genes *PAX3* and *MITF* have been described in Waardenburg syndrome (WS), which is clinically characterized by congenital hearing loss, pigmentation anomalies and depending on the subtype of the disease, presence or absence of craniofacial dysmorphism. Our study intended to determine the frequency of mutations and deletions in these genes, to assess the clinical phenotype in detail and to identify rational priorities for molecular genetic diagnostics procedures.

Design and patients: In a prospective analysis, 19 Caucasian patients with typical features of Waardenburg syndrome underwent stepwise investigation of *PAX3* and *MITF*. When point mutations and small insertions/deletions were excluded by direct sequencing, copy number analysis by MLPA was performed to detect larger deletions and duplications. Clinical data and photographs were collected to facilitate genotype-phenotype analyses.

Setting: All analyses were performed in a large German laboratory specialized in genetic diagnostics.

Results: 16 novel and four previously published heterozygous mutations in *PAX3* and *MITF* were identified. Of these, six were large deletions or duplications, that were only detectable by copy number analysis. All patients with *PAX3* mutations had the typical phenotype of WS with dystopia canthorum (WS1), whereas patients with *MITF* gene mutations presented without dystopia canthorum (WS2). In addition, one patient with bilateral hearing loss and blue eyes with iris stroma dysplasia had a *de novo* missense mutation (p.Arg217Ile) in *MITF*. *MITF* 3-bp deletions at amino acid position 217 have previously been described in patients with Tietz syndrome, a clinical entity with hearing loss and generalized hypopigmentation.

Conclusion: Based on these findings, we conclude that sequencing and copy number analysis of both *PAX3* and *MITF* have to be recommended in the routine molecular diagnostic setting for WS patients with and without dystopia canthorum. Furthermore, our genotype-/phenotype analyses indicate that WS without dystopia (WS2) and TS

correspond to a clinical spectrum that is influenced by *MITF* mutation type and position.

INTRODUCTION

Waardenburg syndrome (WS) is an auditory-pigmentary syndrome that occurs with a frequency of 1 in 40,000.[1] WS has been classified into four main phenotypes: Type 1 (WS1) is characterized by congenital sensorineural hearing loss, heterochromia irides, partial hypopigmentation of the hair including premature graying, and lateral displacement of the inner ocular canthi (dystopia canthorum). Type 2 (WS2) is distinguished from WS1 by the absence of dystopia canthorum. WS3 or Klein-Waardenburg syndrome is similar to WS1, but includes upper limb abnormalities. WS4 or Waardenburg-Shah syndrome has features of Hirschsprung disease in addition to WS2.

Waardenburg syndrome is genetically heterogeneous. Point mutations in the *PAX3* gene are described to be the most frequent cause of WS1 and WS3.[2-4] *PAX3* is a member of the mammalian *PAX* gene family and encodes a DNA binding transcription factor expressed in neural crest cells.[5] It plays an important role for the migration and differentiation of melanocytes, which originate from the embryonic neural crest. The *PAX3* gene is structurally defined by the presence of a highly conserved 128 amino acid DNA-binding domain, known as the paired domain, and a second DNA-binding domain, the homeodomain.[5, 6] *PAX3* mutations associated with WS1 include substitutions of conserved amino acids in the paired domain or the homeodomain of the protein, splice-site mutations, nonsense mutations, and insertions or deletions leading to frame shifts.[7, 8] All previously published *PAX3* mutations in WS1 are heterozygous, whereas both heterozygous and homozygous *PAX3* mutations have been described in the allelic disease WS3.[8-13]

Heterozygous mutations in the *MITF* gene are one category of molecular causes of WS 2.[14, 15] The *MITF* gene encodes a transcription factor with a basic helix-loop-helix leucine zipper motif. Proteins with this kind of motif form homo- or heterodimers by their HLH-zip regions and bind DNA with their basic domain. Another gene that is in case of mutations

associated with WS2 is SOX10. [16] In addition, mutations in *SOX10, EDN3* and *EDNRB* were found in WS4.[17]

The exact description of the mutations responsible for the WS type is of significant importance in genetic counseling of WS patients and their families. Whole-gene sequencing enables the discovery of point mutations and small alterations in the gene, but cannot reliably detect whole-exon or whole-gene copy number changes, which have been reported for many genes resulting in a specific genetic disorder. In recent years, MLPA (multiplex ligation-dependent probe amplification) has become a widespread method in molecular genetic diagnostics to detect or exclude copy number changes in targeted genes.

We applied both whole-gene sequencing and MLPA analysis for the detection of point mutations and copy number variations in the genes *PAX3* and *MITF,* and describe 19 mutations, of which 15 have not been previously reported for patients with features of WS.

MATERIALS AND METHODS

Patients

19 Caucasian patients with the clinical phenotype of WS were included in our study. Clinical data were collected for patients and their affected family members.

Molecular genetic analysis

All 19 patients were analysed for point mutations and copy number changes in the genes *PAX3* and *MITF*. Clinical information and specimens were obtained with informed consent in accordance with German law for genetic diagnostics. Genomic DNA was prepared from white blood cells using a standard procedure. All 10 exons of *PAX3* and all 9 exons of *MITF* were PCR-amplified and directly sequenced. Sequence variant numbering was based on the transcript ENST00000392069 for *PAX3* and ENST00000314557 for *MITF*. Nucleotide

numbering used the A of the ATG translation initiation site as nucleotide +1. The nomenclature of the alterations was adopted according to the guidelines of the human variation society (http://www.hgvs.org/). MLPA (multiplex ligation-dependent probe amplification) analysis was carried out in order to detect or exclude deletions and duplications of the genes *PAX3* and *MITF*. For this purpose MRC-Holland® SALSA MLPA P186 was used. The mix of probes was hybridized to template DNA. The probes, which contained a common sequence tail, were ligated, and subsequently amplified by PCR. Products were separated electrophoretically and the signals captured by a CCD camera. To evaluate quantity and size of the fragments the software Sequence Pilot (JSI Medical Systems, Kippenheim, Germany) was used..

RESULTS

Molecular genetic analysis of the genes *PAX3* and *MITF* for 19 unrelated index patients with the clinical phenotype of WS revealed 15 novel and four previously published heterozygous mutations (table 1). 14 mutations occurred in the *PAX3 gene:* one small insertion, two missense mutations, four nonsense mutations, two small deletions, one splice-site mutation, and four large deletions comprising at least one exon. Five mutations were detected in the *MITF* gene: one missense mutation, one nonsense mutation, one large deletion and one large duplication. In addition, the combination of a *MITF* missense mutation with a small deletion was found in family 11 (table 1). Both mutations were proven to be located on the same chromosome by segregation analysis. The paternal grandfather, a brother, two sisters, a niece and a nephew with Waardenburg syndrome were all proven to be carrier of these two mutations.

Of specific interest, six of the 19 mutations (32 %) were not detectable by direct sequencing. These were five large deletions and one duplication that could only be detected by MLPA analysis. In ten of 19 index patients, parents were available for genetic analysis. Seven mutations were familial (i.e. shown to be inherited from one parent that presented signs and symptoms of WS), whereas three mutations (one mutation in *PAX3* and two in *MITF*)

occurred *de novo*. 15 of 19 mutations detected in *PAX3* and *MITF* represented novel mutations. These mutations and all previously published mutations are summarized in figure 1 (*PAX3* gene) and figure 2 (*MITF* gene).

All index patients with *PAX3* mutations had the typical phenotype of Waardenburg syndrome type 1 including dystopia canthorum and at least one of the following criteria: heterochromia iridis, hearing loss, and additional features such as white forelock, craniofacial dysmorphism including high nasal bridge, synophrys, eyebrow flaring, or anomalies of skin pigmentation (table 1, figure 3). Detailed clinical information was available for 13 of 14 patients with *PAX3* mutations and 11 of these presented hearing loss. No differences in phenotype were noted between patients with *PAX3* point mutations and *PAX3* deletions.

Patients with *MITF* mutations had the clinical phenotype of Waardenburg syndrome type 2, i.e. heterochromia irides and/or hearing loss without dystopia canthorum (figure 4). Patient 13, who was found to carry a *de novo* missense mutation in *MITF,* did not present with heterochromia irides, but with blue eyes and hypoplasia of iris stroma.

DISCUSSION

Molecular genetic analyses of the *PAX3* and *MITF* gene revealed 15 novel and four previously known mutations in patients with the clinical phenotype of Waardenburg syndrome. Of these, 14 mutations occurred in *PAX3*, and 5 mutations were found in *MITF*. The spectrum of mutations includes nonsense, missense and splice site mutations, insertions, as well as deletions and duplications.

Most of the novel mutations in *PAX3* are localized in exons 2 to 6 and hence influence functionally relevant domains (table 1, figure 1). This predominant localization in exons 2 to 6 has previously been described.[18] Twelve of the 14 *PAX3* mutations are truncating

mutations including large deletions. Deletions comprised two whole gene deletions and two intragenic deletions of exon 7 and exons 8 - 9, respectively. The two missense mutations are localized in the N-terminal part of the paired domain, which mediates intensive DNA contact. There is no correlation between the mutation type (missense, nonsense, deletion) and the severity of the phenotype. Therefore, loss of protein function respectively haploinsufficiency seems to be the disease causing mechanism for WS1. All patients with *PAX3* mutations had phenotypes of WS1 (table 1, figure 3).

Five patients had mutations in the *MITF* gene (table 1, figure 2). Four of these represented novel mutations. Detailed clinical information was available in four patients. Three patients had features corresponding to the WS2 phenotype (figure 4). Interestingly, one of these (patient 11) was the first WS2 patient who was found to carry two *MITF* mutations (missense mutation and small deletion) within the same gene copy. Since both mutations are novel, it remains to be clarified whether one or the combination of both mutations leads to the WS2 phenotype.

One patient with a *de novo* missense mutation c.650G>T (p.Arg217Ile) in the *MITF* gene did not present with heterochromia irides, but with bilateral blue irides and hypoplasia of iris stroma (patient 13 in table 1). Interestingly, *MITF* mutations affecting amino acid position 217, which is located in the basic domain of MITF, were also described in patients with Tietz syndrome (TS, MIM #103500). Compared to WS2, Tietz syndrome is characterized by a more severe phenotype with generalized hypopigmentation and complete hearing loss.[19] Instead of heterochromia irides, which is typical for Waardenburg syndrome type 1 and 2, patients with Tietz syndrome present with bilateral blue irides. To date, only three patients with Tietz syndrome and mutation in the *MITF* gene have been described.[20-22] Two of these with typical Tietz syndrome had a mutation altering amino acid position 217 (3-bp in-frame deletion Arg217). Compared to these patients, our patient with a *de novo* missense mutation at the same amino acid position has a less severe phenotype with bilateral hearing

loss, a white forelock and blue irides with hypoplasia of iris stroma. These clinical features are part of WS2, while blue eyes with hypoplastic iris stroma may correspond to Tietz syndrome. Apparently, a missense mutation at amino acid position 217 leads to milder or intermediate phenotype than a 3-bp in-frame deletion at the same position. Therefore, both WS2 and Tietz syndrome most probably correspond to a common clinical spectrum that is influenced by mutation type and position.

In summary, the molecular genetic analyses of the *PAX3* and *MITF* genes are important diagnostic steps to explain the molecular cause of clinical features of Waardenburg syndrome and facilitate genetic counseling of affected patients and their families. For six of 19 patients with suspected WS and previously genetically unconfirmed diagnosis, we found either large deletions (four patients with deletions in *PAX3* and one patient with deletion in *MITF)* or duplications (one patient, *MITF* gene). This indicates that deletion/duplication screening is indispensable for a successful molecular genetic diagnostics of WS. As a genetic diagnostic strategy it is thus recommended to perform both sequence analysis and copy number analysis of the *PAX3* and *MITF* genes in all patients with clinical features of Waardenburg syndrome.

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index patient	age	gender	gene	mutation description				transmission mode	dystopia canthorum	hetero-chromia iridis	hearing loss	other clinical symptoms	notes
				gene structure affected	nucleotide level	protein level	type						
1	6 1/2 y.	m	*PAX3*	exon 2	c.111dupC	p.Arg37fs	insertion	n.a.	+	+	(+)	synophrys, low frontal and nuchal hairline, skin hyperpigmentation anomalies	-
2	7 y.	f	*PAX3*	exon 2	c.143G>A	p.Gly48Asp	missense	maternal	+	-	+	high nasal bridge, synophrys, eyebrow flaring, skin depigmentation; affected family members with premature greying and dystopia canthorum	different amino acid change at same position described in [23]
3	3 y.	f	*PAX3*	exon 2	c.186G>A	p.Met62Ile	missense	paternal	+	+	++	-	different amino acid change at same position: described in [24]
4	34 y.	m	*PAX3*	exon 3	c.400C>T	p.Arg134X	nonsense	n.a.	+	+	-	-	-
5	6 mo.	m	*PAX3*	exon 5	c.589delT	p.Ser197fs	deletion	paternal	+	-	+	white forelock	-
6	3 mo.	m	*PAX3*	exon 5	c.655C>T	p.Gln219X	nonsense	paternal	§	§	§	clinical designation WS1	-
7	17 mo.	m	*PAX3*	exon 5	c.784C>T	p.Arg262X	nonsense	maternal	+	+	+	white forelock, synophrys, high nasal bridge, prognathia, hypopigmentation anomalies	described in [25]
8	1 wk.	m	*PAX3*	intron 5	c.793-1G>T	-	splice site	*de novo*	+	-	-	white forelock, craniofacial dysmorphism	-
9	17 mo.	m	*PAX3*	exon 6	c.946_956del	p.Thr315fs	deletion	n.a.	+	-	+	unilateral hearing loss	-
10	2 1/2 y.	m	*PAX3*	exon 6	c.955C>T	p.Gln319X	nonsense	n.a.	+	+	++	high nasal bridge, mild skin depigmentation	-
11	n.a.	m	*MITF*	exon 1	c.28T>A / c.33+6del7	p.Tyr10Asn	missense / deletion	paternal	-	+	+	-	-
12	16 y.	m	*MITF*	exon 3	c.328C>T	p.Arg110X	nonsense	n.a.	-	+	+	-	-
13	7 mo.	m	*MITF*	exon 7	c.650G>T	p.Arg217Ile	missense	*de novo*	-	-	+	blue eyes with hypoplasia of iris stroma, white forelock	described in [27]
14	23 y.	m	*PAX3*	entire gene	c.(?_-61)_(1452-33_?)		deletion entire gene	paternal	+	-	+	pigmentation anomalies, unilateral hearing loss	described in [26]
15	42 y.	m	*PAX3*	exon 7	c.958+?_1174-?del		deletion exon 7	n.a.	+	-	+	pigmentation anomalies	-
16	6 y.	f	*PAX3*	exons 8-9	c.1173+?_(1452-33_?)del		deletion exons 8-9	n.a.	+	-	+	blue eyes, synophrys, medial eyebrow flaring	-
17	5 mo.	f	*PAX3*	entire gene	c.(?_-61)_(1452-33_?)		deletion entire gene	n.a.	+	+	+	unilateral hearing loss	described in [26]
18	7 y.	m	*MITF*	5´-UTR region	c.?_1-70453dup		duplication	n.a.	§§	§§	§§	clinical designation WS2	-
19	3 y.	m	*MITF*	exons 1-9	c.1-70433_?_(988_?)del		deletion exons 1-9	*de novo*	-	+	+	bilateral hearing loss	-

Table 1: Genotype and phenotype of all Waardenburg syndrome index patients (wk = week; mo = month; y = years; m = male; f = female; § = clinical designation WS1, §§ = clinical designation WS2; (+) mild + moderate; ++ severe). *De novo* = under consideration of provided information concerning kinship.

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Conclusion: Based on these findings, we conclude that sequencing and copy number analysis of both *PAX3* and *MITF* have to be recommended in the routine molecular diagnostic setting for WS patients with and without dystopia canthorum. Furthermore, our genotype-/phenotype analyses indicate that WS without dystopia (WS2) and TS

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INTRODUCTION

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MATERIALS AND METHODS

Patients

19 ~~c~~Caucasian patients with the clinical phenotype of WS were included in our study. Clinical data were collected for patients and their affected family members.

Molecular genetic analysis

All 19 patients were analysed for point mutations and copy number changes in the genes *PAX3* and *MITF*. Clinical information and specimens were obtained with informed consent in accordance with German law for genetic ~~testing~~diagnostics. Genomic DNA was prepared from white blood cells using a standard procedure. All 10 exons of *PAX3* and all 9 exons of *MITF* were PCR-amplified and directly sequenced. Sequence variant numbering was based on the transcript ENST00000392069 for *PAX3* and ENST00000314557 for *MITF*. Nucleotide

numbering used the A of the ATG translation initiation site as nucleotide +1. The nomenclature of the alterations was adopted according to the guidelines of the human variation society (http://www.hgvs.org/). MLPA (multiplex ligation-dependent probe amplification) analysis was carried out in order to detect or exclude deletions and duplications of the genes *PAX3* and *MITF*. For this purpose MRC-Holland® SALSA MLPA P186 was used. The mix of probes was hybridized to template DNA. The probes, which contained a common sequence tail, were ligated, and subsequently amplified by PCR. Products were separated electrophoretically and the signals captured by a CCD camera. To evaluate quantity and size of the fragments the Software Sequence Pilot (JSI Medical Systems, Kippenheim, Germany) was used.

RESULTS

Molecular genetic analysis of the genes *PAX3* and *MITF* for 19 unrelated index patients with the clinical phenotype of WS revealed 15 novel and four previously published heterozygous mutations (table 1). 14 mutations occurred in the *PAX3 gene:* one small insertion, two missense mutations, four nonsense mutations, two small deletions, one splice-site mutation, and four large deletions comprising at least one exon. Five mutations were detected in the *MITF* gene: one missense mutation, one nonsense mutation, one large deletion and one large duplication. In addition, the combination of a *MITF* missense mutation with a small deletion was found in family 11 (table 1). Both mutations were proven to be located on the same chromosome by segregation analysis. The paternal grandfather, a brother, two sisters, a niece and a nephew with Waardenburg syndrome were all proven to be carriers of these two mutations.

Of specific interest, six of the 19 mutations (32 %) were not detectable by direct sequencing. These were five large deletions and one duplication that could only be detected by MLPA analysis. In ten of 19 index patients, parents were available for genetic analysis. Seven

mutations were familial (i.e. shown to be inherited from one parent that presented signs and symptoms of WS), whereas three mutations (one mutation in *PAX3* and two in *MITF*) occurred *de novo*. 16~~6~~5 of 19 mutations detected in *PAX3* and *MITF* represented novel mutations. These mutations and all previously published mutations are summarized in figure 1 (*PAX3* gene) and figure 2 (*MITF* gene).

All index patients with *PAX3* mutations had the typical phenotype of Waardenburg syndrome type 1 including dystopia canthorum and at least one of the following criteria: heterochromia iridis, hearing loss, and additional features such as white forelock, craniofacial dysmorphism including high nasal bridge, synophrys, eyebrow flaring, or anomalies of skin pigmentation (table 1, figure 3). Detailed c~~C~~linical information was available for 10~~0~~3 of 14~~2~~2 patients with *PAX3* mutations and 11~~all~~ of these presented hearing loss. No differences in phenotype were noted between patients with *PAX3* point mutations and *PAX3* deletions.

Patients with *MITF* mutations had the clinical phenotype of Waardenburg syndrome type 2, i.e. heterochromia irides and/or hearing loss without dystopia canthorum (figure 4~~; clinical data not available for one patient~~). Patient 13, who was found to carry a *de novo* missense mutation in *MITF,* did not present with heterochromia irides, but with blue eyes and hypoplasia of iris stroma.

DISCUSSION

Molecular genetic analyses of the *PAX3* and *MITF* gene revealed 15~~6~~ novel and ~~three~~four previously known mutations in patients with the clinical phenotype of Waardenburg syndrome. Of these, 14 mutations occurred in *PAX3*, and 5 mutations were found in *MITF*. The spectrum of mutations includes nonsense, missense and splice site mutations, insertions, as well as deletions and duplications.

Most of the novel mutations in *PAX3* are localized in exons 2 to 6 and hence influence functionally relevant domains (table 1, figure 1). This predominant localization in exons 2 to 6 has previously been described.[18] Twelve of the 14 *PAX3* mutations are truncating mutations including larger deletions. Deletions comprised two whole gene deletions and two intragenic deletions of exon 7 and exons 8 - 9, respectively. The two missense mutations are localized in the N-terminal part of the paired domain, which mediates intensive DNA contact. There is no correlation between the mutation type (missense, nonsense, deletion) and the severity of the phenotype. Therefore, loss of protein function respectively haploinsufficiency seems to be the disease causing mechanism for WS1. All patients with *PAX3* mutations had phenotypes of WS1 (table 1, figure 3).

Five patients had mutations in the *MITF* gene (table 1, figure 2). Four of these represented novel mutations. Detailed clinical information was available in four patients. Three patients had features corresponding to the WS2 phenotype (figure 4). Interestingly, one of these (patient 11) was the first WS2 patient who was found to carry two *MITF* mutations (missense mutation and small deletion) within the same gene copy. Since both mutations are novel, it remains to be clarified whether one or the combination of both mutations leads to the WS2 phenotype.

One patient with a *de novo* missense mutation c.650G>T (p.Arg217Ile) in the *MITF* gene did not present with heterochromia irides, but with bilateral blue irides and hypoplasia of iris stroma (patient 13 in table 1). Interestingly, *MITF* mutations affecting amino acid position 217, which is located in the basic domain of MITF, were also described in patients with Tietz syndrome (TS, MIM #103500). Compared to WS2, Tietz syndrome is characterized by a more severe phenotype with generalized hypopigmentation and complete hearing loss.[19] Instead of heterochromia irides, which is typical for Waardenburg syndrome type 1 and 2, patients with Tietz syndrome present with bilateral blue irides. To date, only three patients with Tietz syndrome and mutation in the *MITF* gene have been described.[20-22] Two of

these with typical Tietz syndrome had a mutation altering amino acid position 217 (3-bp in-frame deletion Arg217). Compared to these patients, our patient with a *de novo* missense mutation at the same amino acid position has a less severe phenotype with bilateral hearing loss, a white forelock and blue irides with hypoplasia of iris stroma. These clinical features are part of WS2, while blue eyes with hypoplastic iris stroma may correspond to Tietz syndrome. Apparently, a missense mutation at amino acid position 217 leads to milder or intermediate phenotype than a 3-bp in-frame deletion at the same position. Therefore, both WS2 and ~~TS~~ Tietz syndrome most probably correspond to a common clinical spectrum that is influenced by mutation type and position.

In summary, the molecular genetic analyses of the *PAX3* and *MITF* genes are important diagnostic steps to explain the molecular cause of clinical features of Waardenburg syndrome and facilitate genetic counseling of affected patients and their families. For six of 19 patients with suspected WS and previously genetically unconfirmed diagnosis, we found either large deletions (four patients with deletions in *PAX3* and one patient with deletion in *MITF)* or duplications (one patient, *MITF* gene). This indicates that deletion/duplication screening is indispensable for a successful molecular genetic diagnostics of WS. As a genetic diagnostic strategy it is thus recommended to perform both sequence analysis and copy number analysis of the *PAX3* and *MITF* genes in all patients with clinical features of Waardenburg syndrome.

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index patient	age	gender	gene	mutation description				transmission mode	dystopia canthorum	hetero-chromia iridis	hearing loss	other clinical symptoms	notes
				gene structure affected	nucleotide level	protein level	type						
1	6 1/2 y.	m	*PAX3*	exon 2	c.111dupC	p.Arg37fs	insertion	n.a.	+	+	(+)	synophrys, low frontal and nuchal hairline, skin hyperpigmentation anomalies	-
2	7 y.	f	*PAX3*	exon 2	c.143G>A	p.Gly48Asp	missense	maternal	+	-	+	high nasal bridge, synophrys, eyebrow flaring, skin depigmentation; affected family members with premature greying and dystopia canthorum	different amino acid change at same position described in [23]
3	3 y.	f	*PAX3*	exon 2	c.186G>A	p.Met62Ile	missense	paternal	+	+	++	-	different amino acid change at same position: described in [24]
4	34 y.	m	*PAX3*	exon 3	c.400C>T	p.Arg134X	nonsense	n.a.	+	+	-	-	-
5	6 mo.	m	*PAX3*	exon 5	c.589delT	p.Ser197fs	deletion	paternal	+	-	+	white forelock	-
6	3 mo.	m	*PAX3*	exon 5	c.655C>T	p.Gln219X	nonsense	paternal	§	§	§	clinical designation WS1	-
7	17 mo.	m	*PAX3*	exon 5	c.784C>T	p.Arg262X	nonsense	maternal	+	+	+	white forelock, synophrys, high nasal bridge, prognathia, hypopigmentation anomalies	described in [25]
8	1 wk.	m	*PAX3*	intron 5	c.793-1G>T	-	splice site	*de novo*	+	-	-	white forelock, craniofacial dysmorphism	-
9	17 mo.	m	*PAX3*	exon 6	c.946_956del	p.Thr315fs	deletion	n.a.	+	-	+	unilateral hearing loss	-
10	2 1/2 y.	m	*PAX3*	exon 6	c.955C>T	p.Gln319X	nonsense	n.a.	+	+	++	high nasal bridge, mild skin depigmentation	-
11	n.a.	m	*MITF*	exon 1	c.28T>A / c.33+6del7	p.Tyr10Asn	missense / deletion	paternal	-	+	+	-	-
12	16 y.	m	*MITF*	exon 3	c.328C>T	p.Arg110X	nonsense	n.a.	-	+	+	-	-
13	7 mo.	m	*MITF*	exon 7	c.650G>T	p.Arg217Ile	missense	*de novo*	-	-	+	blue eyes with hypoplasia of iris stroma, white forelock	described in [27]
14	23 y.	m	*PAX3*	entire gene	c.(?_-61)_(1452-33_?)		deletion entire gene	paternal	+	-	+	pigmentation anomalies, unilateral hearing loss	described in [26]
15	42 y.	m	*PAX3*	exon 7	c.958+?_1174-?del		deletion exon 7	n.a.	+	-	+	pigmentation anomalies	-
16	6 y.	f	*PAX3*	exons 8-9	c.1173+?_(1452-33_?)del		deletion exons 8-9	n.a.	+	-	+	blue eyes, synophrys, medial eyebrow flaring	-
17	5 mo.	f	*PAX3*	entire gene	c.(?_-61)_(1452-33_?)		deletion entire gene	n.a.	+	+	+	unilateral hearing loss	described in [26]
18	7 y.	m	*MITF*	5´-UTR region	c.?_1-70453dup		duplication	n.a.	§§	§§	§§	clinical designation WS2	-
19	3 y.	m	*MITF*	exons 1-9	c.1-70433_?_(988_?)del		deletion exons 1-9	*de novo*	-	+	+	bilateral hearing loss	-

Table 1: Genotype and phenotype of all Waardenburg syndrome index patients (wk = week; mo = month; y = years; m = male; f = female; § = clinical designation WS1, §§ = clinical designation WS2; (+) mild + moderate ~~mild~~; ++ severe). *De novo* = under consideration of provided information concerning kinship.

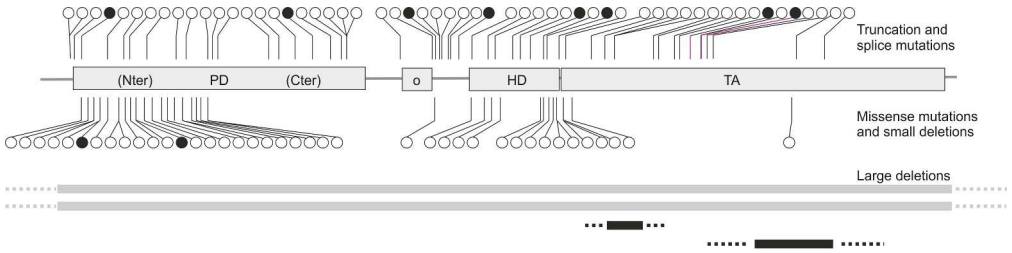

Figure 1: Survey of *PAX3* mutations detected in patients with Waardenburg syndrome. Black circles = mutations detected in this study; black bars = copy number variations detected in this study; white circles and grey bars = previously published mutations and deletions. PD = paired domain; o = octapeptide; HD = homeodomain; TA = transactivation domain.
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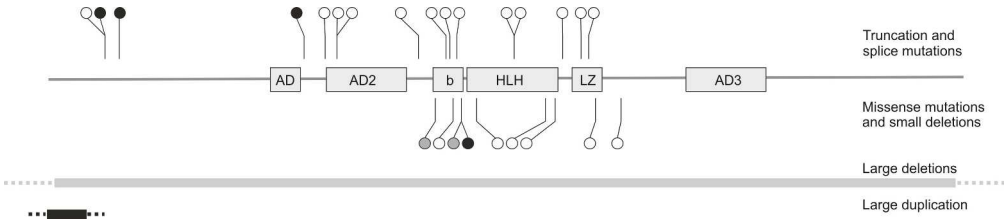

Figure 2: Survey of *MITF* mutations detected in patients with Waardenburg and Tietz syndrome. Black circles and bars = mutations and copy number variations detected in this study; white circles and grey bars = previously published mutations and deletions; grey circles = MITF mutations associated with Tietz syndrome. AD1-3 = (trans)activation domains; b = basic domain; HLH = helix-loop-helix domain; LZ = leucine zipper domain.
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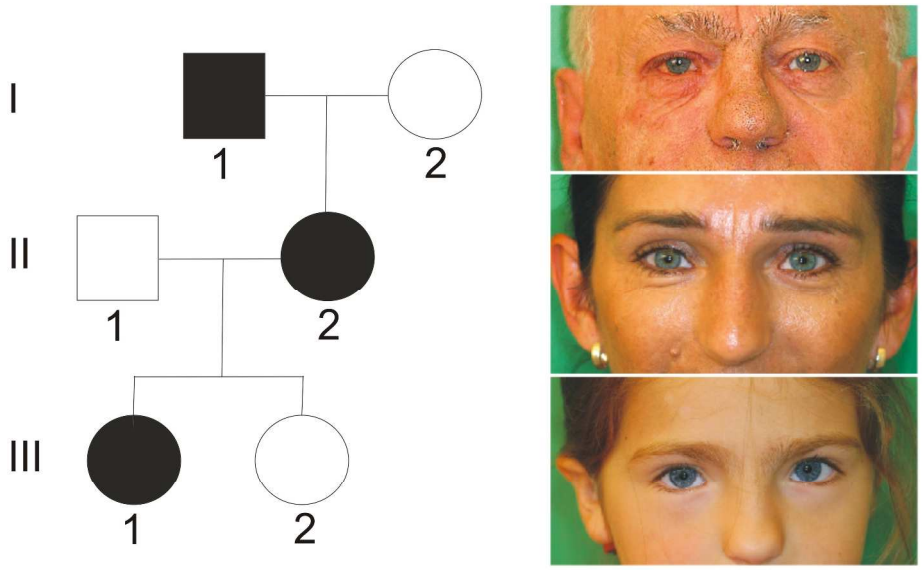

Figure 3: Pedigree of index patient 2 with *PAX3* mutation. The girl presented with clinical features of Waardenburg syndrome type 1 (see table 1): dystopia canthorum, high nasal bridge, synophrys, eyebrow flaring, skin depigmentation and bilateral hearing loss. Her mother and maternal grandfather had only premature graying.

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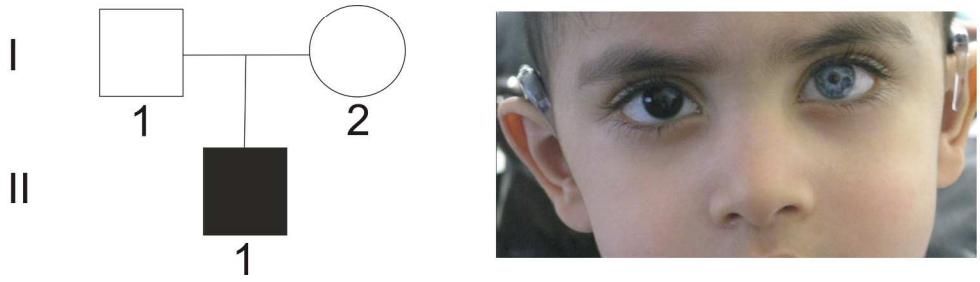

Figure 4: Sporadic case (patient 19 in table 1) of Waardenburg syndrome type 2 with *MITF* mutation. The boy presented with heterochromia irides and bilateral hearing loss, but did not show dystopia canthorum.
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SPECTRUM OF NOVEL MUTATIONS FOUND IN WAARDENBURG SYNDROME TYPE 1 AND TYPE 2: IMPLICATIONS FOR MOLECULAR GENETIC DIAGNOSTICS

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SPECTRUM OF NOVEL MUTATIONS IN WAARDENBURG SYNDROME TYPE 1 AND TYPE 2: IMPLICATIONS FOR MOLECULAR GENETIC DIAGNOSTICS

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Keywords: Waardenburg syndrome, Tietz syndrome, hearing loss, *PAX3* gene, *MITF* gene
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ABSTRACT

Objectives: To date, mutations in the genes *PAX3* and *MITF* have been described in Waardenburg syndrome (WS), which is clinically characterized by congenital hearing loss, pigmentation anomalies and depending on the subtype of the disease, presence or absence of craniofacial dysmorphism. Our study intended to determine the frequency of mutations and deletions in these genes, to assess the clinical phenotype in detail and to identify rational priorities for molecular genetic diagnostics procedures.

Design and patients: In a prospective analysis, 19 Caucasian patients with typical features of Waardenburg syndrome underwent stepwise investigation of *PAX3* and *MITF*. When point mutations and small insertions/deletions were excluded by direct sequencing, copy number analysis by MLPA was performed to detect larger deletions and duplications. Clinical data and photographs were collected to facilitate genotype-phenotype analyses.

Setting: All analyses were performed in a large German laboratory specialized in genetic diagnostics.

Results: 16 novel and four previously published heterozygous mutations in *PAX3* and *MITF* were identified. Of these, six were large deletions or duplications, that were only detectable by copy number analysis. All patients with *PAX3* mutations had the typical phenotype of WS with dystopia canthorum (WS1), whereas patients with *MITF* gene mutations presented without dystopia canthorum (WS2). In addition, one patient with bilateral hearing loss and blue eyes with iris stroma dysplasia had a *de novo* missense mutation (p.Arg217Ile) in *MITF*. *MITF* 3-bp deletions at amino acid position 217 have previously been described in patients with Tietz syndrome, a clinical entity with hearing loss and generalized hypopigmentation.

Conclusion: Based on these findings, we conclude that sequencing and copy number analysis of both *PAX3* and *MITF* have to be recommended in the routine molecular diagnostic setting for WS patients with and without dystopia canthorum. Furthermore, our genotype-/phenotype analyses indicate that WS without dystopia (WS2) and TS correspond to a clinical spectrum that is influenced by MITF mutation type and position.

INTRODUCTION

Waardenburg syndrome (WS) is an auditory-pigmentary syndrome that occurs with a frequency of 1 in 40,000.[1] WS has been classified into four main phenotypes: Type 1 (WS1) is characterized by congenital sensorineural hearing loss, heterochromia irides, partial hypopigmentation of the hair including premature graying, and lateral displacement of the inner ocular canthi (dystopia canthorum). Type 2 (WS2) is distinguished from WS1 by the absence of dystopia canthorum. WS3 or Klein-Waardenburg syndrome is similar to WS1, but includes upper limb abnormalities. WS4 or Waardenburg-Shah syndrome has features of Hirschsprung disease in addition to WS2.

Waardenburg syndrome is genetically heterogeneous. Point mutations in the *PAX3* gene are described to be the most frequent cause of WS1 and WS3.[2-4] *PAX3* is a member of the mammalian *PAX* gene family and encodes a DNA binding transcription factor expressed in neural crest cells.[5] It plays an important role for the migration and differentiation of melanocytes, which originate from the embryonic neural crest. The *PAX3* gene is structurally defined by the presence of a highly conserved 128 amino acid DNA-binding domain, known as the paired domain, and a second DNA-binding domain, the homeodomain.[5, 6] *PAX3* mutations associated with WS1 include substitutions of conserved amino acids in the paired domain or the homeodomain of the protein, splice-site mutations, nonsense mutations, and insertions or deletions leading to frame shifts.[7, 8] All previously published *PAX3* mutations in WS1 are heterozygous, whereas both heterozygous and homozygous *PAX3* mutations have been described in the allelic disease WS3.[8-13]

Heterozygous mutations in the *MITF* gene are one category of molecular causes of WS 2.[14, 15] The *MITF* gene encodes a transcription factor with a basic helix-loop-helix leucine zipper motif. Proteins with this kind of motif form homo- or heterodimers by their HLH-zip regions and bind DNA with their basic domain. Another gene that is in case of mutations

associated with WS2 is SOX10. [16] In addition, mutations in *SOX10, EDN3* and *EDNRB* were found in WS4.[17]

The exact description of the mutations responsible for the WS type is of significant importance in genetic counseling of WS patients and their families. Whole-gene sequencing enables the discovery of point mutations and small alterations in the gene, but cannot reliably detect whole-exon or whole-gene copy number changes, which have been reported for many genes resulting in a specific genetic disorder. In recent years, MLPA (multiplex ligation-dependent probe amplification) has become a widespread method in molecular genetic diagnostics to detect or exclude copy number changes in targeted genes.

We applied both whole-gene sequencing and MLPA analysis for the detection of point mutations and copy number variations in the genes *PAX3* and *MITF,* and describe 19 mutations, of which 15 have not been previously reported for patients with features of WS.

MATERIALS AND METHODS

Patients

19 Caucasian patients with the clinical phenotype of WS were included in our study. Clinical data were collected for patients and their affected family members.

Molecular genetic analysis

All 19 patients were analysed for point mutations and copy number changes in the genes *PAX3* and *MITF*. Clinical information and specimens were obtained with informed consent in accordance with German law for genetic diagnostics. Genomic DNA was prepared from white blood cells using a standard procedure. All 10 exons of *PAX3* and all 9 exons of *MITF* were PCR-amplified and directly sequenced. Sequence variant numbering was based on the transcript ENST00000392069 for *PAX3* and ENST00000314557 for *MITF*. Nucleotide

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RESULTS

Molecular genetic analysis of the genes *PAX3* and *MITF* for 19 unrelated index patients with the clinical phenotype of WS revealed 15 novel and four previously published heterozygous mutations (table 1). 14 mutations occurred in the *PAX3 gene:* one small insertion, two missense mutations, four nonsense mutations, two small deletions, one splice-site mutation, and four large deletions comprising at least one exon. Five mutations were detected in the *MITF* gene: one missense mutation, one nonsense mutation, one large deletion and one large duplication. In addition, the combination of a *MITF* missense mutation with a small deletion was found in family 11 (table 1). Both mutations were proven to be located on the same chromosome by segregation analysis. The paternal grandfather, a brother, two sisters, a niece and a nephew with Waardenburg syndrome were all proven to be carrier of these two mutations.

Of specific interest, six of the 19 mutations (32 %) were not detectable by direct sequencing. These were five large deletions and one duplication that could only be detected by MLPA analysis. In ten of 19 index patients, parents were available for genetic analysis. Seven mutations were familial (i.e. shown to be inherited from one parent that presented signs and symptoms of WS), whereas three mutations (one mutation in *PAX3* and two in *MITF*)

occurred *de novo*. 15 of 19 mutations detected in *PAX3* and *MITF* represented novel mutations. The positions of these mutations and all previously published mutations are summarized in figure 1 (*PAX3* gene) and figure 2 (*MITF* gene).

All index patients with *PAX3* mutations had the typical phenotype of Waardenburg syndrome type 1 including dystopia canthorum and at least one of the following criteria: heterochromia iridis, hearing loss, and additional features such as white forelock, craniofacial dysmorphism including high nasal bridge, synophrys, eyebrow flaring, or anomalies of skin pigmentation (table 1, figure 3). Detailed clinical information was available for 13 of 14 patients with *PAX3* mutations and 11 of these presented hearing loss. No differences in phenotype were noted between patients with *PAX3* point mutations and *PAX3* deletions.

Patients with *MITF* mutations had the clinical phenotype of Waardenburg syndrome type 2, i.e. heterochromia irides and/or hearing loss without dystopia canthorum (figure 4). Patient 13, who was found to carry a *de novo* missense mutation in *MITF,* did not present with heterochromia irides, but with blue eyes and hypoplasia of iris stroma.

DISCUSSION

Molecular genetic analyses of the *PAX3* and *MITF* gene revealed 15 novel and four previously known mutations in patients with the clinical phenotype of Waardenburg syndrome. Of these, 14 mutations occurred in *PAX3*, and 5 mutations were found in *MITF*. The spectrum of mutations includes nonsense, missense and splice site mutations, insertions, as well as deletions and duplications.

Most of the novel mutations in *PAX3* are localized in exons 2 to 6 and hence influence functionally relevant domains (table 1, figure 1). This predominant localization in exons 2 to 6 has previously been described.[18] Twelve of the 14 *PAX3* mutations are truncating

mutations including large deletions. Deletions comprised two whole gene deletions and two intragenic deletions of exon 7 and exons 8 - 9, respectively. The two missense mutations are localized in the N-terminal part of the paired domain, which mediates intensive DNA contact. There is no correlation between the mutation type (missense, nonsense, deletion) and the severity of the phenotype. Therefore, loss of protein function leading to haploinsufficiency seems to be the disease causing mechanism for WS1. All patients with *PAX3* mutations had phenotypes of WS1 (table 1, figure 3).

Five patients had mutations in the *MITF* gene (table 1, figure 2). Four of these represented novel mutations. Detailed clinical information was available in four patients. Three patients had features corresponding to the WS2 phenotype (figure 4). Interestingly, one of these (patient 11) was the first WS2 patient who was found to carry two *MITF* mutations (missense mutation and small deletion) within the same gene copy. Since both mutations are novel, it remains to be clarified whether one or the combination of both mutations leads to the WS2 phenotype.

One patient with a *de novo* missense mutation c.650G>T (p.Arg217Ile) in the *MITF* gene did not present with heterochromia irides, but with bilateral blue irides and hypoplasia of iris stroma (patient 13 in table 1). Interestingly, *MITF* mutations affecting amino acid position 217, which is located in the basic domain of MITF, were also described in patients with Tietz syndrome (TS, MIM #103500). Compared to WS2, Tietz syndrome is characterized by a more severe phenotype with generalized hypopigmentation and complete hearing loss.[19] Instead of heterochromia irides, which is typical for Waardenburg syndrome type 1 and 2, patients with Tietz syndrome present with bilateral blue irides. To date, only three patients with Tietz syndrome and mutation in the *MITF* gene have been described.[20-22] Two of these with typical Tietz syndrome had a mutation altering amino acid position 217 (3-bp in-frame deletion Arg217). Compared to these patients, our patient with a *de novo* missense mutation at the same amino acid position has a less severe phenotype with bilateral hearing

loss, a white forelock and blue irides with hypoplasia of iris stroma. These clinical features are part of WS2, while blue eyes with hypoplastic iris stroma may correspond to Tietz syndrome. Apparently, a missense mutation at amino acid position 217 leads to milder or intermediate phenotype than a 3-bp in-frame deletion at the same position. Therefore, both WS2 and Tietz syndrome most probably correspond to a common clinical spectrum that is influenced by mutation type and position.

In summary, the molecular genetic analyses of the *PAX3* and *MITF* genes are important diagnostic steps to explain the molecular cause of clinical features of Waardenburg syndrome and facilitate genetic counseling of affected patients and their families. For six of 19 patients with suspected WS and previously genetically unconfirmed diagnosis, we found either large deletions (four patients with deletions in *PAX3* and one patient with deletion in *MITF)* or duplications (one patient, *MITF* gene). This indicates that deletion/duplication screening is indispensable for a successful molecular genetic diagnostics of WS. As a genetic diagnostic strategy it is thus recommended to perform both sequence analysis and copy number analysis of the *PAX3* and *MITF* genes in all patients with clinical features of Waardenburg syndrome.

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index patient	age	gender	gene	mutation description				transmission mode	dystopia canthorum	hetero-chromia iridis	hearing loss	other clinical symptoms	notes
				gene structure affected	nucleotide level	protein level	type						
1	6 1/2 y.	m	*PAX3*	exon 2	c.111dupC	p.Val38Argfs*76	insertion	n.a.	+	+	(+)	synophrys, low frontal and nuchal hairline, skin hyperpigmentation anomalies	-
2	7 y.	f	*PAX3*	exon 2	c.143G>A	p.Gly48Asp	missense	maternal	+	-	+	high nasal bridge, synophrys, eyebrow flaring, skin depigmentation; affected family members with premature greying and dystopia canthorum	different amino acid change at same position described in [23]
3	3 y.	f	*PAX3*	exon 2	c.186G>A	p.Met62Ile	missense	paternal	+	+	++	-	different amino acid change at same position: described in [24]
4	34 y.	m	*PAX3*	exon 3	c.400C>T	p.Arg134X	nonsense	n.a.	+	+	-	-	-
5	6 mo.	m	*PAX3*	exon 5	c.589delT	p.Ser197fs	deletion	paternal	+	-	+	white forelock	-
6	3 mo.	m	*PAX3*	exon 5	c.655C>T	p.Gln219X	nonsense	paternal	+	+	-	high nasal bridge	-
7	17 mo.	m	*PAX3*	exon 5	c.784C>T	p.Arg262X	nonsense	maternal	+	+	+	white forelock, synophrys, high nasal bridge, prognathia, hypopigmentation anomalies	described in [25]
8	1 wk.	m	*PAX3*	intron 5	c.793-1G>T	-	splice site	*de novo*	+	-	-	white forelock, craniofacial dysmorphism	-
9	17 mo.	m	*PAX3*	exon 6	c.946_956del	p.Thr315fs	deletion	n.a.	+	-	+	unilateral hearing loss	-
10	2 1/2 y.	m	*PAX3*	exon 6	c.955C>T	p.Gln319X	nonsense	n.a.	+	+	++	high nasal bridge, mild skin depigmentation	-
11	n.a.	m	*MITF*	exon 1	[c.28T>A;c.33+6del7]	p.Tyr10Asn	missense / deletion	paternal	-	+	+	-	-
12	16 y.	m	*MITF*	exon 3	c.328C>T	p.Arg110X	nonsense	n.a.	-	+	+	-	-
13	7 mo.	m	*MITF*	exon 7	c.650G>T	p.Arg217Ile	missense	*de novo*	-	-	+	blue eyes with hypoplasia of iris stroma, white forelock	described in [27]
14	23 y.	m	*PAX3*	entire gene	c.(?_-61)_(1452-33_?)		deletion entire gene	paternal	+	-	+	pigmentation anomalies, unilateral hearing loss	described in [26]
15	42 y.	m	*PAX3*	exon 7	c.958+?_1174-?del		deletion exon 7	n.a.	+	-	+	pigmentation anomalies	-
16	6 y.	f	*PAX3*	exons 8-9	c.1173+?_(1452-33_?)del		deletion exons 8-9	n.a.	+	-	+	blue eyes, synophrys, medial eyebrow flaring	-
17	5 mo.	f	*PAX3*	entire gene	c.(?_-61)_(1452-33_?)		deletion entire gene	n.a.	+	+	+	unilateral hearing loss	described in [26]
18	7 y.	m	*MITF*	5´-UTR region	c.?_1-70453dup		duplication	n.a.	-	-	(+)	-	-
19	3 y.	m	*MITF*	exons 1-9	c.1-70433_?_(988_?)del		deletion exons 1-9	*de novo*	-	+	+	bilateral hearing loss	-

Table 1: Genotype and phenotype of all Waardenburg syndrome index patients (wk = week; mo = month; y = years; m = male; f = female; (+) mild + moderate; ++ severe). "*De novo"* = under consideration of provided information concerning kinship, no paternity testing was performed.

SPECTRUM OF NOVEL MUTATIONS IN WAARDENBURG SYNDROME TYPE 1 AND TYPE 2: IMPLICATIONS FOR MOLECULAR GENETIC DIAGNOSTICS

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ABSTRACT

Objectives: To date, mutations in the genes *PAX3* and *MITF* have been described in Waardenburg syndrome (WS), which is clinically characterized by congenital hearing loss, pigmentation anomalies and depending on the subtype of the disease, presence or absence of craniofacial dysmorphism. Our study intended to determine the frequency of mutations and deletions in these genes, to assess the clinical phenotype in detail and to identify rational priorities for molecular genetic diagnostics procedures.

Design and patients: In a prospective analysis, 19 Ccaucasian patients with typical features of Waardenburg syndrome underwent stepwise investigation of *PAX3* and *MITF*. When point mutations and small insertions/deletions were excluded by direct sequencing, copy number analysis by MLPA was performed to detect larger deletions and duplications. Clinical data and fphotographs were collected to facilitate genotype-phenotype analyses.

Setting: All analyses were performed in a large Ggerman laboratory specialized in genetic diagnostics.

Results: 16 novel and fourthree previously published heterozygous mutations in *PAX3* and *MITF* were identified. Of these, six were large deletions or duplications, that were only detectable by copy number analysis. All patients with *PAX3* mutations had the typical phenotype of WS with dystopia canthorum (WS1), whereas patients with *MITF* gene mutations presented without dystopia canthorum (WS2). In addition, one patient with bilateral hearing loss and blue eyes with iris stroma dysplasia had a *de novo* missense mutation (p.Arg217Ile) in *MITF*. *MITF* 3-bp deletions at amino acid position 217 have previously been described in patients with Tietz syndrome, a clinical entity with hearing loss and generalized hypopigmentation.

Conclusion: Based on these findings, we conclude that sequencing and copy number analysis of both *PAX3* and *MITF* have to be recommended in the routine molecular diagnostic setting for WS patients with and without dystopia canthorum. Furthermore, our genotype-/phenotype analyses indicate that WS without dystopia (WS2) and TS correspond to a clinical spectrum that is influenced by MITF mutation type and position.

INTRODUCTION

Waardenburg syndrome (WS) is an auditory-pigmentary syndrome that occurs with a frequency of 1 in 40,000.[1] WS has been classified into four main phenotypes: Type 1 (WS1) is characterized by congenital sensorineural hearing loss, heterochromia irides, partial hypopigmentation of the hair including premature graying, and lateral displacement of the inner ocular canthi (dystopia canthorum). Type 2 (WS2) is distinguished from WS1 by the absence of dystopia canthorum. WS3 or Klein-Waardenburg syndrome is similar to WS1, but includes upper limb abnormalities. WS4 or Waardenburg-Shah syndrome has features of Hirschsprung disease in addition to WS2.

Waardenburg syndrome is genetically heterogeneous. Point mutations in the *PAX3* gene are described to be the most frequent cause of WS1 and WS3.[2-4] *PAX3* is a member of the mammalian *PAX* gene family and encodes a DNA binding transcription factor expressed in neural crest cells.[5] It plays an important role for the migration and differentiation of melanocytes, which originate from the embryonic neural crest. The *PAX3* gene is structurally defined by the presence of a highly conserved 128 amino acid DNA-binding domain, known as the paired domain, and a second DNA-binding domain, the homeodomain.[5, 6] *PAX3* mutations associated with WS1 include substitutions of conserved amino acids in the paired domain or the homeodomain of the protein, splice-site mutations, nonsense mutations, and insertions or deletions leading to frame shifts.[7, 8] All previously published *PAX3* mutations in WS1 are heterozygous, whereas both heterozygous and homozygous *PAX3* mutations have been described in the allelic disease WS3.[8-13]

Heterozygous mutations in the *MITF* gene are one ~~of the major~~ category of molecular causes of WS 2.[14, 15] The *MITF* gene encodes a transcription factor with a basic helix-loop-helix leucine zipper motif. Proteins with this kind of motif form homo- or heterodimers by their HLH-zip regions and bind DNA with their basic domain. Another gene that is in case of

mutations associated with ~~additional candidate gene for~~ WS2 is SOX10~~, and *SNAI2*~~. [16]~~.~~ In addition, mutations in *SOX10, EDN3* and *EDNRB* were found in WS4.[17]

The exact description of the mutations responsible for the WS type is of significant importance in genetic counseling of WS patients and their families. Whole-gene sequencing enables the discovery of point mutations and small alterations in the gene, but cannot reliably detect whole-exon or whole-gene copy number changes, which have been reported for many genes resulting in a specific genetic disorder. In recent years, MLPA (multiplex ligation-dependent probe amplification) has become a widespread method in molecular genetic diagnostics to detect or exclude copy number changes in targeted genes.

We applied both whole-gene sequencing and MLPA analysis for the detection of point mutations and copy number variations in the genes *PAX3* and *MITF,* and describe 19 mutations, of which 15~~6~~ have not been previously reported for patients with features of WS.

MATERIALS AND METHODS

Patients

19 ~~c~~Caucasian patients with the clinical phenotype of WS were included in our study. Clinical data were collected for patients and their affected family members.

Molecular genetic analysis

All 19 patients were analysed for point mutations and copy number changes in the genes *PAX3* and *MITF*. Clinical information and specimens were obtained with informed consent in accordance with German law for genetic ~~testing~~diagnostics. Genomic DNA was prepared from white blood cells using a standard procedure. All 10 exons of *PAX3* and all 9 exons of *MITF* were PCR-amplified and directly sequenced. Sequence variant numbering was based on the transcript ENST00000392069 for *PAX3* and ENST00000314557 for *MITF*. Nucleotide

numbering used the A of the ATG translation initiation site as nucleotide +1. The nomenclature of the alterations was adopted according to the guidelines of the human variation society (http://www.hgvs.org/). MLPA (multiplex ligation-dependent probe amplification) analysis was carried out in order to detect or exclude deletions and duplications of the genes *PAX3* and *MITF*. For this purpose MRC-Holland® SALSA MLPA P186 was used.~~,~~ The~~a~~ mix of probes was hybridized to template DNA. The probes, which contained a common sequence tail, were ligated, and subsequently amplified by PCR. Products were separated electrophoretically ~~, and analyzed by means of sizing and quantification~~and the signals captured by a CCD camera. To evaluate quantity and size of the fragments ~~T~~the s~~S~~oftware Sequence Pilot (JSI Medical Systems, Kippenheim, Germany) was used~~, for evaluation~~.

RESULTS

Molecular genetic analysis of the genes *PAX3* and *MITF* for 19 unrelated index patients with the clinical phenotype of WS revealed 15~~6~~ novel and four~~three~~ previously published heterozygous mutations (table 1). 14 mutations occurred in the *PAX3 gene:* one small insertion, two missense mutations, four nonsense mutations, two small deletions, one splice-site mutation, and four large deletions comprising at least one exon. Five mutations were detected in the *MITF* gene: one missense mutation, one nonsense mutation, one large deletion and one large duplication. In addition, the combination of a *MITF* missense mutation with a small deletion was found in family 11 (table 1). Both mutations were proven to be located on the same chromosome by segregation analysis. The paternal grandfather, a brother, two sisters, ~~,~~a niece and a nephew with Waardenburg syndrome ~~are~~ were all proven to be carrier~~s~~ of these two mutations.

Of specific interest, six of the 19 mutations (32 %) were not detectable by direct sequencing. These were five large deletions and one duplication that could only be detected by MLPA analysis. In ten of 19 index patients, parents were available for genetic analysis. Seven

mutations were familial (i.e. shown to be inherited from one parent that presented signs and symptoms of WS), whereas three mutations (one mutation in *PAX3* and two in *MITF*) occurred *de novo*. 16~~6~~5 of 19 mutations detected in *PAX3* and *MITF* represented novel mutations. The positions of ~~T~~these mutations and all previously published mutations are summarized in figure 1 (*PAX3* gene) and figure 2 (*MITF* gene).

All index patients with *PAX3* mutations had the typical phenotype of Waardenburg syndrome type 1 including dystopia canthorum and at least one of the following criteria: heterochromia iridis, hearing loss, and additional features such as white forelock, craniofacial dysmorphism including high nasal bridge, synophrys, eyebrow flaring, or anomalies of skin pigmentation (table 1, figure 3). Detailed c~~C~~linical information was available for 10~~0~~3 of 14~~2~~2 patients with *PAX3* mutations and 11~~all~~ of these presented hearing loss. No differences in phenotype were noted between patients with *PAX3* point mutations and *PAX3* deletions.

Patients with *MITF* mutations had the clinical phenotype of Waardenburg syndrome type 2, i.e. heterochromia irides and/or hearing loss without dystopia canthorum (figure 4~~; clinical data not available for one patient~~). Patient 13, who was found to carry a *de novo* missense mutation in *MITF,* did not present with heterochromia irides, but with blue eyes and hypoplasia of iris stroma.

DISCUSSION

Molecular genetic analyses of the *PAX3* and *MITF* gene revealed 15~~6~~ novel and ~~three~~four previously known mutations in patients with the clinical phenotype of Waardenburg syndrome. Of these, 14 mutations occurred in *PAX3*, and 5 mutations were found in *MITF*. The spectrum of mutations includes nonsense, missense and splice site mutations, insertions, as well as deletions and duplications.

Most of the novel mutations in *PAX3* are localized in exons 2 to 6 and hence influence functionally relevant domains (table 1, figure 1). This predominant localization in exons 2 to 6 has previously been described.[18] Twelve of the 14 *PAX3* mutations are truncating mutations including large~~r~~ deletions. Deletions comprised two whole gene deletions and two intragenic deletions of exon 7 and exons 8 - 9, respectively. The two missense mutations are localized in the N-terminal part of the paired domain, which mediates intensive DNA contact. There is no correlation between the mutation type (missense, nonsense, deletion) and the severity of the phenotype. Therefore, loss of protein function leading to haploinsufficiency seems to be the disease causing mechanism for WS1. ~~Therefore, loss of protein function seems to be the disease causing mechanism for WS1.~~ All patients with *PAX3* mutations had phenotypes of WS1 (table 1, figure 3).

Five patients had mutations in the *MITF* gene (table 1, figure 2). Four of these represented novel mutations. Detailed c~~C~~linical information was available in four patients. Three patients had features corresponding to the WS2 phenotype (figure 4). Interestingly, one of these (patient 11) was the first WS2 patient who was found to carry two *MITF* mutations (missense mutation and small deletion) within the same gene copy. Since both mutations are novel, it remains to be clarified whether one or the combination of both mutations leads to the WS2 phenotype.

One patient with a *de novo* missense mutation c.650G>T (p.Arg217Ile) in the *MITF* gene did not present with heterochromia irides, but with bilateral blue irides and hypoplasia of iris stroma (patient 13 in table 1). Interestingly, *MITF* mutations affecting amino acid position 217, which is located in the basic domain of MITF, were also described in patients with Tietz syndrome (TS, MIM #103500). Compared to WS2, Tietz syndrome is characterized by a more severe phenotype with generalized hypopigmentation and complete hearing loss.[19] Instead of heterochromia irides, which is typical for Waardenburg syndrome type 1 and 2, patients with Tietz syndrome present with bilateral blue irides. To date, only three patients

with Tietz syndrome and mutation in the *MITF* gene have been described.[20-22] Two of these with typical Tietz syndrome had a mutation altering amino acid position 217 (3-bp in-frame deletion Arg217). Compared to these patients, our patient with a *de novo* missense mutation at the same amino acid position has a less severe phenotype with bilateral hearing loss, a white forelock and blue irides with hypoplasia of iris stroma. These clinical features are part of WS2, while blue eyes with hypoplastic iris stroma may correspond to Tietz syndrome. Apparently, a missense mutation at amino acid position 217 leads to milder or intermediate phenotype than a 3-bp in-frame deletion at the same position. Therefore, both WS2 and ~~TS~~ Tietz syndrome most probably correspond to a common clinical spectrum that is influenced by mutation type and position.

In summary, the molecular genetic analyses of the *PAX3* and *MITF* genes are important diagnostic steps to explain the molecular cause of clinical features of Waardenburg syndrome and facilitate genetic counseling of affected patients and their families. For six of 19 patients with suspected WS and previously genetically unconfirmed diagnosis, we found either large deletions (four patients with deletions in *PAX3* and one patient with deletion in *MITF)* or duplications (one patient, *MITF* gene). This indicates that deletion/duplication screening is indispensable for a successful molecular genetic diagnostics of WS. As a genetic diagnostic strategy it is thus recommended to perform both sequence analysis and copy number analysis of the *PAX3* and *MITF* genes in all patients with clinical features of Waardenburg syndrome.

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index patient	age	gender	gene	mutation description				transmission mode	dystopia canthorum	hetero-chromia iridis	hearing loss	other clinical symptoms	notes
				gene structure affected	nucleotide level	protein level	type						
1	6 1/2 y.	m	*PAX3*	exon 2	c.111dupC	p.Val38Argfs*76	insertion	n.a.	+	+	(+)	synophrys, low frontal and nuchal hairline, skin hyperpigmentation anomalies	-
2	7 y.	f	*PAX3*	exon 2	c.143G>A	p.Gly48Asp	missense	maternal	+	-	+	high nasal bridge, synophrys, eyebrow flaring, skin depigmentation; affected family members with premature greying and dystopia canthorum	different amino acid change at same position described in [23]
3	3 y.	f	*PAX3*	exon 2	c.186G>A	p.Met62Ile	missense	paternal	+	+	++	-	different amino acid change at same position: described in [24]
4	34 y.	m	*PAX3*	exon 3	c.400C>T	p.Arg134X	nonsense	n.a.	+	+	-	-	-
5	6 mo.	m	*PAX3*	exon 5	c.589delT	p.Ser197fs	deletion	paternal	+	-	+	white forelock	-
6	3 mo.	m	*PAX3*	exon 5	c.655C>T	p.Gln219X	nonsense	paternal	+	+	-	high nasal bridge	-
7	17 mo.	m	*PAX3*	exon 5	c.784C>T	p.Arg262X	nonsense	maternal	+	+	+	white forelock, synophrys, high nasal bridge, prognathia, hypopigmentation anomalies	described in [25]
8	1 wk.	m	*PAX3*	intron 5	c.793-1G>T	-	splice site	*de novo*	+	-	-	white forelock, craniofacial dysmorphism	-
9	17 mo.	m	*PAX3*	exon 6	c.946_956del	p.Thr315fs	deletion	n.a.	+	-	+	unilateral hearing loss	-
10	2 1/2 y.	m	*PAX3*	exon 6	c.955C>T	p.Gln319X	nonsense	n.a.	+	+	++	high nasal bridge, mild skin depigmentation	-
11	n.a.	m	*MITF*	exon 1	[c.28T>A;c.33+6del7]	p.Tyr10Asn	missense / deletion	paternal	-	+	+	-	-
12	16 y.	m	*MITF*	exon 3	c.328C>T	p.Arg110X	nonsense	n.a.	-	+	+	-	-
13	7 mo.	m	*MITF*	exon 7	c.650G>T	p.Arg217Ile	missense	*de novo*	-	-	+	blue eyes with hypoplasia of iris stroma, white forelock	described in [27]
14	23 y.	m	*PAX3*	entire gene	c.(?_-61)_(1452-33_?)		deletion entire gene	paternal	+	-	+	pigmentation anomalies, unilateral hearing loss	described in [26]
15	42 y.	m	*PAX3*	exon 7	c.958+?_1174-?del		deletion exon 7	n.a.	+	-	+	pigmentation anomalies	-
16	6 y.	f	*PAX3*	exons 8-9	c.1173+?_(1452-33_?)del		deletion exons 8-9	n.a.	+	-	+	blue eyes, synophrys, medial eyebrow flaring	-
17	5 mo.	f	*PAX3*	entire gene	c.(?_-61)_(1452-33_?)		deletion entire gene	n.a.	+	+	+	unilateral hearing loss	described in [26]
18	7 y.	m	*MITF*	5´-UTR region	c.?_1-70453dup		duplication	n.a.	-	-	(+)	-	-
19	3 y.	m	*MITF*	exons 1-9	c.1-70433_?_(988_?)del		deletion exons 1-9	*de novo*	-	+	+	bilateral hearing loss	-

Table 1: Genotype and phenotype of all Waardenburg syndrome index patients (wk = week; mo = month; y = years; m = male; f = female; § ~~= clinical designation WS1, §§clinical designation~~ (+) mild + moderate ~~mild~~; ++ severe). *"De novo"* = under consideration of provided information concerning kinship, no paternity testing was performed.

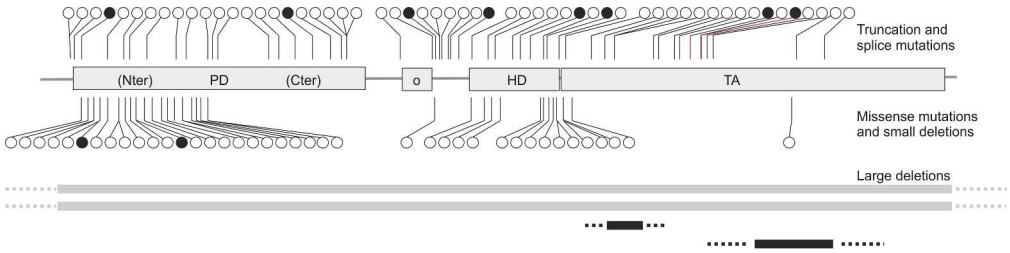

Figure 1: Survey of *PAX3* mutations detected in patients with Waardenburg syndrome. Black circles = mutations detected in this study; black bars = copy number variations detected in this study; white circles and grey bars = previously published mutations and deletions. PD = paired domain; o = octapeptide; HD = homeodomain; TA = transactivation domain.
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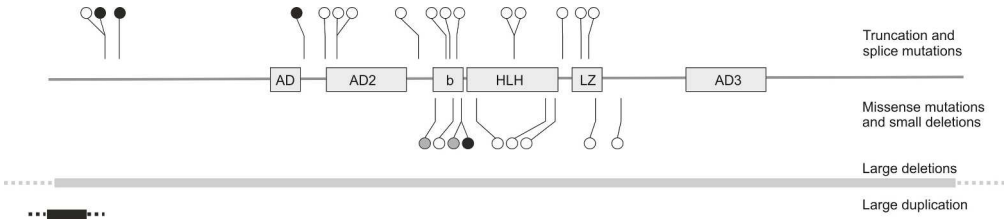

Figure 2: Survey of *MITF* mutations detected in patients with Waardenburg and Tietz syndrome. Black circles and bars = mutations and copy number variations detected in this study; white circles and grey bars = previously published mutations and deletions; grey circles = MITF mutations associated with Tietz syndrome. AD1-3 = (trans)activation domains; b = basic domain; HLH = helix-loop-helix domain; LZ = leucine zipper domain.
271x57mm (300 x 300 DPI)

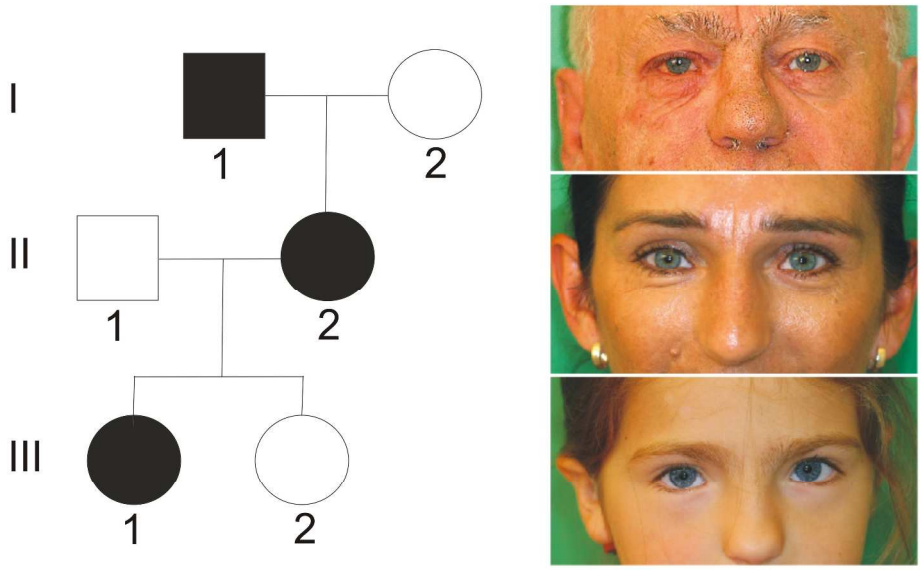

Figure 3: Pedigree of index patient 2 with *PAX3* mutation. The girl presented with clinical features of Waardenburg syndrome type 1 (see table 1): dystopia canthorum, high nasal bridge, synophrys, eyebrow flaring, skin depigmentation and bilateral hearing loss. Her mother and maternal grandfather had only premature graying.

301x172mm (300 x 300 DPI)

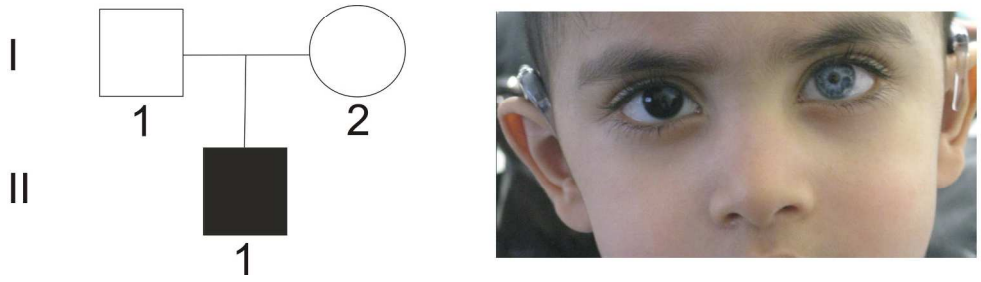

Figure 4: Sporadic case (patient 19 in table 1) of Waardenburg syndrome type 2 with *MITF* mutation. The boy presented with heterochromia irides and bilateral hearing loss, but did not show dystopia canthorum.
226x77mm (300 x 300 DPI)

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SPECTRUM OF NOVEL MUTATIONS FOUND IN WAARDENBURG SYNDROME TYPE 1 AND TYPE 2: IMPLICATIONS FOR MOLECULAR GENETIC DIAGNOSTICS

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SPECTRUM OF NOVEL MUTATIONS FOUND IN WAARDENBURG SYNDROME TYPE 1 AND TYPE 2: IMPLICATIONS FOR MOLECULAR GENETIC DIAGNOSTICS

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ABSTRACT

Objectives: To date, mutations in the genes *PAX3* and *MITF* have been described in Waardenburg syndrome (WS), which is clinically characterized by congenital hearing loss, pigmentation anomalies and depending on the subtype of the disease, presence or absence of craniofacial dysmorphism. Our study intended to determine the frequency of mutations and deletions in these genes, to assess the clinical phenotype in detail and to identify rational priorities for molecular genetic diagnostics procedures.

Design and patients: In a prospective analysis, 19 Caucasian patients with typical features of Waardenburg syndrome underwent stepwise investigation of *PAX3* and *MITF*. When point mutations and small insertions/deletions were excluded by direct sequencing, copy number analysis by MLPA was performed to detect larger deletions and duplications. Clinical data and photographs were collected to facilitate genotype-phenotype analyses.

Setting: All analyses were performed in a large German laboratory specialized in genetic diagnostics.

Results: 15 novel and four previously published heterozygous mutations in *PAX3* and *MITF* were identified. Of these, six were large deletions or duplications, that were only detectable by copy number analysis. All patients with *PAX3* mutations had the typical phenotype of WS with dystopia canthorum (WS1), whereas patients with *MITF* gene mutations presented without dystopia canthorum (WS2). In addition, one patient with bilateral hearing loss and blue eyes with iris stroma dysplasia had a *de novo* missense mutation (p.Arg217Ile) in *MITF*. *MITF* 3-bp deletions at amino acid position 217 have previously been described in patients with Tietz syndrome, a clinical entity with hearing loss and generalized hypopigmentation.

Conclusion: Based on these findings, we conclude that sequencing and copy number analysis of both *PAX3* and *MITF* have to be recommended in the routine molecular diagnostic setting for WS patients with and without dystopia canthorum. Furthermore, our

genotype-/phenotype analyses indicate that WS without dystopia (WS2) and TS correspond to a clinical spectrum that is influenced by MITF mutation type and position.

INTRODUCTION

Waardenburg syndrome (WS) is an auditory-pigmentary syndrome that occurs with a frequency of 1 in 40,000.[1] WS has been classified into four main phenotypes: Type 1 (WS1) is characterized by congenital sensorineural hearing loss, heterochromia irides, partial hypopigmentation of the hair including premature graying, and lateral displacement of the inner ocular canthi (dystopia canthorum). Type 2 (WS2) is distinguished from WS1 by the absence of dystopia canthorum. WS3 or Klein-Waardenburg syndrome is similar to WS1, but includes upper limb abnormalities. WS4 or Waardenburg-Shah syndrome has features of Hirschsprung disease in addition to WS2.

Waardenburg syndrome is genetically heterogeneous. Point mutations in the *PAX3* gene are described to be the most frequent cause of WS1 and WS3.[2-4] *PAX3* is a member of the mammalian *PAX* gene family and encodes a DNA binding transcription factor expressed in neural crest cells.[5] It plays an important role for the migration and differentiation of melanocytes, which originate from the embryonic neural crest. The *PAX3* gene is structurally defined by the presence of a highly conserved 128 amino acid DNA-binding domain, known as the paired domain, and a second DNA-binding domain, the homeodomain.[5, 6] *PAX3* mutations associated with WS1 include substitutions of conserved amino acids in the paired domain or the homeodomain of the protein, splice-site mutations, nonsense mutations, and insertions or deletions leading to frame shifts.[7, 8] All previously published *PAX3* mutations in WS1 are heterozygous, whereas both heterozygous and homozygous *PAX3* mutations have been described in the allelic disease WS3.[8-13]

Heterozygous mutations in the *MITF* gene are one category of molecular causes of WS 2.[14, 15] The *MITF* gene encodes a transcription factor with a basic helix-loop-helix leucine zipper motif. Proteins with this kind of motif form homo- or heterodimers by their HLH-zip regions and bind DNA with their basic domain. Another gene that is in case of mutations

associated with WS2 is SOX10. [16] In addition, mutations in *SOX10, EDN3* and *EDNRB* were found in WS4.[17]

The exact description of the mutations responsible for the WS type is of significant importance in genetic counseling of WS patients and their families. Whole-gene sequencing enables the discovery of point mutations and small alterations in the gene, but cannot reliably detect whole-exon or whole-gene copy number changes, which have been reported for many genes resulting in a specific genetic disorder. In recent years, MLPA (multiplex ligation-dependent probe amplification) has become a widespread method in molecular genetic diagnostics to detect or exclude copy number changes in targeted genes.

We applied both whole-gene sequencing and MLPA analysis for the detection of point mutations and copy number variations in the genes *PAX3* and *MITF,* and describe 19 mutations, of which 15 have not been previously reported for patients with features of WS.

MATERIALS AND METHODS

Patients

For 119 patients molecular genetic analyses were requested due to the clinical suspicion of Waardenburg syndrome. From this cohort, for 15 Caucasian patients we have identified previously not described mutations in *PAX3* or *MITF*. Furthermore for four Caucasian patients already published alterations were found in one of these genes. Subsequently more detailed clinical data were collected from these individuals as well as from their clinically affected family members.

Molecular genetic analysis

All 19 patients were analysed for point mutations and copy number changes in the genes *PAX3* and *MITF*. Clinical information and specimens were obtained with informed consent in

accordance with German law for genetic diagnostics. Genomic DNA was prepared from white blood cells using a standard procedure. All 10 exons of *PAX3* and all 9 exons of *MITF* were PCR-amplified and directly sequenced. Sequence variant numbering was based on the transcript ENST00000392069 for *PAX3* and ENST00000314557 for *MITF*. Nucleotide numbering used the A of the ATG translation initiation site as nucleotide +1. The nomenclature of the alterations was adopted according to the guidelines of the human variation society (http://www.hgvs.org/). MLPA (multiplex ligation-dependent probe amplification) analysis was carried out in order to detect or exclude deletions and duplications of the genes *PAX3* and *MITF*. For this purpose MRC-Holland® SALSA MLPA P186 was used. The mix of probes was hybridized to template DNA. The probes, which contained a common sequence tail, were ligated, and subsequently amplified by PCR. Products were separated electrophoretically and the signals captured by a CCD camera. To evaluate quantity and size of the fragments the software Sequence Pilot (JSI Medical Systems, Kippenheim, Germany) was used.

RESULTS

Molecular genetic analysis of the genes *PAX3* and *MITF* for 19 unrelated index patients with the clinical phenotype of WS revealed 15 novel and four previously published heterozygous mutations (table 1). 14 mutations occurred in the *PAX3 gene:* one small insertion, two missense mutations, four nonsense mutations, two small deletions, one splice-site mutation, and four large deletions comprising at least one exon. Five mutations were detected in the *MITF* gene: one missense mutation, one nonsense mutation, one large deletion and one large duplication. In addition, the combination of a *MITF* missense mutation with a small deletion was found in family 11 (table 1). Both mutations were proven to be located on the same chromosome by segregation analysis. The paternal grandfather, a brother, two sisters, a niece and a nephew with Waardenburg syndrome were all proven to be carrier of these two mutations.

Of specific interest, six of the 19 mutations (32 %) were not detectable by direct sequencing. These were five large deletions and one duplication that could only be detected by MLPA analysis. In ten of 19 index patients, parents were available for genetic analysis. Seven mutations were familial (i.e. shown to be inherited from one parent that presented signs and symptoms of WS), whereas three mutations (one mutation in *PAX3* and two in *MITF*) occurred *de novo*. 15 of 19 mutations detected in *PAX3* and *MITF* represented novel mutations. The positions of these mutations and all previously published mutations are summarized in figure 1 (*PAX3* gene) and figure 2 (*MITF* gene).

All index patients with *PAX3* mutations had the typical phenotype of Waardenburg syndrome type 1 including dystopia canthorum and at least one of the following criteria: heterochromia iridis, hearing loss, and additional features such as white forelock, craniofacial dysmorphism including high nasal bridge, synophrys, eyebrow flaring, or anomalies of skin pigmentation (table 1, figure 3). Detailed clinical information was available for 13 of 14 patients with *PAX3* mutations and 11 of these presented hearing loss. No differences in phenotype were noted between patients with *PAX3* point mutations and *PAX3* deletions.

Patients with *MITF* mutations had the clinical phenotype of Waardenburg syndrome type 2, i.e. heterochromia irides and/or hearing loss without dystopia canthorum (figure 4). Patient 13, who was found to carry a *de novo* missense mutation in *MITF,* did not present with heterochromia irides, but with blue eyes and hypoplasia of iris stroma.

DISCUSSION

Molecular genetic analyses of the *PAX3* and *MITF* gene revealed 15 novel and four previously known mutations in patients with the clinical phenotype of Waardenburg syndrome. Of these, 14 mutations occurred in *PAX3*, and 5 mutations were found in *MITF*.

The spectrum of mutations includes nonsense, missense and splice site mutations, insertions, as well as deletions and duplications.

Most of the novel mutations in *PAX3* are localized in exons 2 to 6 and hence influence functionally relevant domains (table 1, figure 1). This predominant localization in exons 2 to 6 has previously been described.[18] Twelve of the 14 *PAX3* mutations are truncating mutations including large deletions. Deletions comprised two whole gene deletions and two intragenic deletions of exon 7 and exons 8 - 9, respectively. The two missense mutations are localized in the N-terminal part of the paired domain, which mediates intensive DNA contact. There is no correlation between the mutation type (missense, nonsense, deletion) and the severity of the phenotype. Therefore, loss of protein function leading to haploinsufficiency seems to be the disease causing mechanism for WS1. All patients with *PAX3* mutations had phenotypes of WS1 (table 1, figure 3).

Five patients had mutations in the *MITF* gene (table 1, figure 2). Four of these represented novel mutations. Detailed clinical information was available in four patients. Three patients had features corresponding to the WS2 phenotype (figure 4). Interestingly, one of these (patient 11) was the first WS2 patient who was found to carry two *MITF* mutations (missense mutation and small deletion) within the same gene copy. Since both mutations are novel, it remains to be clarified whether one or the combination of both mutations leads to the WS2 phenotype.

One patient with a *de novo* missense mutation c.650G>T (p.Arg217Ile) in the *MITF* gene did not present with heterochromia irides, but with bilateral blue irides and hypoplasia of iris stroma (patient 13 in table 1). Interestingly, *MITF* mutations affecting amino acid position 217, which is located in the basic domain of MITF, were also described in patients with Tietz syndrome (TS, MIM #103500). Compared to WS2, Tietz syndrome is characterized by a more severe phenotype with generalized hypopigmentation and complete hearing loss.[19]

Instead of heterochromia irides, which is typical for Waardenburg syndrome type 1 and 2, patients with Tietz syndrome present with bilateral blue irides. To date, only three patients with Tietz syndrome and mutation in the *MITF* gene have been described.[20-22] Two of these with typical Tietz syndrome had a mutation altering amino acid position 217 (3-bp in-frame deletion Arg217). Compared to these patients, our patient with a *de novo* missense mutation at the same amino acid position has a less severe phenotype with bilateral hearing loss, a white forelock and blue irides with hypoplasia of iris stroma. These clinical features are part of WS2, while blue eyes with hypoplastic iris stroma may correspond to Tietz syndrome. Apparently, a missense mutation at amino acid position 217 leads to milder or intermediate phenotype than a 3-bp in-frame deletion at the same position. Therefore, both WS2 and Tietz syndrome most probably correspond to a common clinical spectrum that is influenced by mutation type and position.

In summary, the molecular genetic analyses of the *PAX3* and *MITF* genes are important diagnostic steps to explain the molecular cause of clinical features of Waardenburg syndrome and facilitate genetic counseling of affected patients and their families. For six of 19 patients with suspected WS and previously genetically unconfirmed diagnosis, we found either large deletions (four patients with deletions in *PAX3* and one patient with deletion in *MITF)* or duplications (one patient, *MITF* gene). This indicates that deletion/duplication screening is indispensable for a successful molecular genetic diagnostics of WS. As a genetic diagnostic strategy it is thus recommended to perform both sequence analysis and copy number analysis of the *PAX3* and *MITF* genes in all patients with clinical features of Waardenburg syndrome.

CONTRIBUTORSHIP

GW and DS responsible for study concept and design and acquisition of genetic data and interpretation. BZ, LGN, JW, MS, AB, ABo, CK, SV, GSW, MG and OB collected the clinical data. GW, BZ and DS wrote the manuscript. All authors have critically revised the paper.

DATA SHARING

No additional data available.

COMPETING INTERESTS

None

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None

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index patient	age	gender	gene	mutation description				transmission mode	dystopia canthorum	hetero-chromia iridis	hearing loss	other clinical symptoms	notes
				gene structure affected	nucleotide level	protein level	type						
1	6 1/2 y.	m	*PAX3*	exon 2	c.111dupC	p.Val38Argfs*76	insertion	n.a.	+	+	(+)	synophrys, low frontal and nuchal hairline, skin hyperpigmentation anomalies	-
2	7 y.	f	*PAX3*	exon 2	c.143G>A	p.Gly48Asp	missense	maternal	+	-	+	high nasal bridge, synophrys, eyebrow flaring, skin depigmentation; affected family members with premature greying and dystopia canthorum	different amino acid change at same position described in [23]
3	3 y.	f	*PAX3*	exon 2	c.186G>A	p.Met62Ile	missense	paternal	+	+	++	-	different amino acid change at same position: described in [24]
4	34 y.	m	*PAX3*	exon 3	c.400C>T	p.Arg134X	nonsense	n.a.	+	+	-	-	-
5	6 mo.	m	*PAX3*	exon 5	c.589delT	p.Ser197fs	deletion	paternal	+	-	+	white forelock	-
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7	17 mo.	m	*PAX3*	exon 5	c.784C>T	p.Arg262X	nonsense	maternal	+	+	+	white forelock, synophrys, high nasal bridge, prognathia, hypopigmentation anomalies	described in [25]
8	1 wk.	m	*PAX3*	intron 5	c.793-1G>T	-	splice site	*de novo*	+	-	-	white forelock, craniofacial dysmorphism	-
9	17 mo.	m	*PAX3*	exon 6	c.946_956del	p.Thr315fs	deletion	n.a.	+	-	+	unilateral hearing loss	-
10	2 1/2 y.	m	*PAX3*	exon 6	c.955C>T	p.Gln319X	nonsense	n.a.	+	+	++	high nasal bridge, mild skin depigmentation	-
11	n.a.	m	*MITF*	exon 1	[c.28T>A;c.33+6del7]	p.Tyr10Asn	missense / deletion	paternal	-	+	+	-	-
12	16 y.	m	*MITF*	exon 3	c.328C>T	p.Arg110X	nonsense	n.a.	-	+	+	-	-
13	7 mo.	m	*MITF*	exon 7	c.650G>T	p.Arg217Ile	missense	*de novo*	-	-	+	blue eyes with hypoplasia of iris stroma, white forelock	described in [27]
14	23 y.	m	*PAX3*	entire gene	c.(?_-61)_(1452-33_?)		deletion entire gene	paternal	+	-	+	pigmentation anomalies, unilateral hearing loss	described in [26]
15	42 y.	m	*PAX3*	exon 7	c.958+?_1174-?del		deletion exon 7	n.a.	+	-	+	pigmentation anomalies	-
16	6 y.	f	*PAX3*	exons 8-9	c.1173+?_(1452-33_?)del		deletion exons 8-9	n.a.	+	-	+	blue eyes, synophrys, medial eyebrow flaring	-
17	5 mo.	f	*PAX3*	entire gene	c.(?_-61)_(1452-33_?)		deletion entire gene	n.a.	+	+	+	unilateral hearing loss	described in [26]
18	7 y.	m	*MITF*	5´-UTR region	c.?_1-70453dup		duplication	n.a.	-	-	(+)	-	-
19	3 y.	m	*MITF*	exons 1-9	c.1-70433_?_(988_?)del		deletion exons 1-9	*de novo*	-	+	+	bilateral hearing loss	-

Table 1: Genotype and phenotype of all Waardenburg syndrome index patients (wk = week; mo = month; y = years; m = male; f = female; (+) mild + moderate; ++ severe; n.a. = not available). "*De novo"* = under consideration of provided information concerning kinship, no paternity testing was performed.

SPECTRUM OF NOVEL MUTATIONS IN WAARDENBURG SYNDROME TYPE 1 AND TYPE 2: IMPLICATIONS FOR MOLECULAR GENETIC DIAGNOSTICS

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Keywords: Waardenburg syndrome, Tietz syndrome, hearing loss, *PAX3* gene, *MITF* gene
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ABSTRACT

Objectives: To date, mutations in the genes *PAX3* and *MITF* have been described in Waardenburg syndrome (WS), which is clinically characterized by congenital hearing loss, pigmentation anomalies and depending on the subtype of the disease, presence or absence of craniofacial dysmorphism. Our study intended to determine the frequency of mutations and deletions in these genes, to assess the clinical phenotype in detail and to identify rational priorities for molecular genetic diagnostics procedures.

Design and patients: In a prospective analysis, 19 Caucasian patients with typical features of Waardenburg syndrome underwent stepwise investigation of *PAX3* and *MITF*. When point mutations and small insertions/deletions were excluded by direct sequencing, copy number analysis by MLPA was performed to detect larger deletions and duplications. Clinical data and photographs were collected to facilitate genotype-phenotype analyses.

Setting: All analyses were performed in a large German laboratory specialized in genetic diagnostics.

Results: 156 novel and four previously published heterozygous mutations in *PAX3* and *MITF* were identified. Of these, six were large deletions or duplications, that were only detectable by copy number analysis. All patients with *PAX3* mutations had the typical phenotype of WS with dystopia canthorum (WS1), whereas patients with *MITF* gene mutations presented without dystopia canthorum (WS2). In addition, one patient with bilateral hearing loss and blue eyes with iris stroma dysplasia had a *de novo* missense mutation (p.Arg217Ile) in *MITF*. *MITF* 3-bp deletions at amino acid position 217 have previously been described in patients with Tietz syndrome, a clinical entity with hearing loss and generalized hypopigmentation.

Conclusion: Based on these findings, we conclude that sequencing and copy number analysis of both *PAX3* and *MITF* have to be recommended in the routine molecular diagnostic setting for WS patients with and without dystopia canthorum. Furthermore, our genotype-/phenotype analyses indicate that WS without dystopia (WS2) and TS correspond to a clinical spectrum that is influenced by MITF mutation type and position.

INTRODUCTION

Waardenburg syndrome (WS) is an auditory-pigmentary syndrome that occurs with a frequency of 1 in 40,000.[1] WS has been classified into four main phenotypes: Type 1 (WS1) is characterized by congenital sensorineural hearing loss, heterochromia irides, partial hypopigmentation of the hair including premature graying, and lateral displacement of the inner ocular canthi (dystopia canthorum). Type 2 (WS2) is distinguished from WS1 by the absence of dystopia canthorum. WS3 or Klein-Waardenburg syndrome is similar to WS1, but includes upper limb abnormalities. WS4 or Waardenburg-Shah syndrome has features of Hirschsprung disease in addition to WS2.

Waardenburg syndrome is genetically heterogeneous. Point mutations in the *PAX3* gene are described to be the most frequent cause of WS1 and WS3.[2-4] *PAX3* is a member of the mammalian *PAX* gene family and encodes a DNA binding transcription factor expressed in neural crest cells.[5] It plays an important role for the migration and differentiation of melanocytes, which originate from the embryonic neural crest. The *PAX3* gene is structurally defined by the presence of a highly conserved 128 amino acid DNA-binding domain, known as the paired domain, and a second DNA-binding domain, the homeodomain.[5, 6] *PAX3* mutations associated with WS1 include substitutions of conserved amino acids in the paired domain or the homeodomain of the protein, splice-site mutations, nonsense mutations, and insertions or deletions leading to frame shifts.[7, 8] All previously published *PAX3* mutations in WS1 are heterozygous, whereas both heterozygous and homozygous *PAX3* mutations have been described in the allelic disease WS3.[8-13]

Heterozygous mutations in the *MITF* gene are one category of molecular causes of WS 2.[14, 15] The *MITF* gene encodes a transcription factor with a basic helix-loop-helix leucine zipper motif. Proteins with this kind of motif form homo- or heterodimers by their HLH-zip regions and bind DNA with their basic domain. Another gene that is in case of mutations

associated with WS2 is SOX10. [16] In addition, mutations in *SOX10, EDN3* and *EDNRB* were found in WS4.[17]

The exact description of the mutations responsible for the WS type is of significant importance in genetic counseling of WS patients and their families. Whole-gene sequencing enables the discovery of point mutations and small alterations in the gene, but cannot reliably detect whole-exon or whole-gene copy number changes, which have been reported for many genes resulting in a specific genetic disorder. In recent years, MLPA (multiplex ligation-dependent probe amplification) has become a widespread method in molecular genetic diagnostics to detect or exclude copy number changes in targeted genes.

We applied both whole-gene sequencing and MLPA analysis for the detection of point mutations and copy number variations in the genes *PAX3* and *MITF,* and describe 19 mutations, of which 15 have not been previously reported for patients with features of WS.

MATERIALS AND METHODS

Patients

~~19 Caucasian patients with the clinical phenotype of WS were included in our study. Clinical data were collected for patients and their affected family members.~~ For 119 patients molecular genetic analyses were requested due to the clinical suspicion of Waardenburg syndrome. From this cohort, for 15 Caucasian patients we have identified previously not described mutations in *PAX3* or *MITF*. Furthermore for four Caucasian patients already published alterations were found in one of these genes. Subsequently more detailed clinical data were collected from these individuals as well as from their clinically affected family members.

Molecular genetic analysis

All 19 patients were analysed for point mutations and copy number changes in the genes *PAX3* and *MITF*. Clinical information and specimens were obtained with informed consent in accordance with German law for genetic diagnostics. Genomic DNA was prepared from white blood cells using a standard procedure. All 10 exons of *PAX3* and all 9 exons of *MITF* were PCR-amplified and directly sequenced. Sequence variant numbering was based on the transcript ENST00000392069 for *PAX3* and ENST00000314557 for *MITF*. Nucleotide numbering used the A of the ATG translation initiation site as nucleotide +1. The nomenclature of the alterations was adopted according to the guidelines of the human variation society (http://www.hgvs.org/). MLPA (multiplex ligation-dependent probe amplification) analysis was carried out in order to detect or exclude deletions and duplications of the genes *PAX3* and *MITF*. For this purpose MRC-Holland® SALSA MLPA P186 was used. The mix of probes was hybridized to template DNA. The probes, which contained a common sequence tail, were ligated, and subsequently amplified by PCR. Products were separated electrophoretically and the signals captured by a CCD camera. To evaluate quantity and size of the fragments the software Sequence Pilot (JSI Medical Systems, Kippenheim, Germany) was used.

RESULTS

Molecular genetic analysis of the genes *PAX3* and *MITF* for 19 unrelated index patients with the clinical phenotype of WS revealed 15 novel and four previously published heterozygous mutations (table 1). 14 mutations occurred in the *PAX3 gene:* one small insertion, two missense mutations, four nonsense mutations, two small deletions, one splice-site mutation, and four large deletions comprising at least one exon. Five mutations were detected in the *MITF* gene: one missense mutation, one nonsense mutation, one large deletion and one large duplication. In addition, the combination of a *MITF* missense mutation with a small deletion was found in family 11 (table 1). Both mutations were proven to be located on the same chromosome by segregation analysis. The paternal grandfather, a brother, two sisters,

a niece and a nephew with Waardenburg syndrome were all proven to be carrier of these two mutations.

Of specific interest, six of the 19 mutations (32 %) were not detectable by direct sequencing. These were five large deletions and one duplication that could only be detected by MLPA analysis. In ten of 19 index patients, parents were available for genetic analysis. Seven mutations were familial (i.e. shown to be inherited from one parent that presented signs and symptoms of WS), whereas three mutations (one mutation in *PAX3* and two in *MITF*) occurred *de novo*. 15 of 19 mutations detected in *PAX3* and *MITF* represented novel mutations. The positions of these mutations and all previously published mutations are summarized in figure 1 (*PAX3* gene) and figure 2 (*MITF* gene).

All index patients with *PAX3* mutations had the typical phenotype of Waardenburg syndrome type 1 including dystopia canthorum and at least one of the following criteria: heterochromia iridis, hearing loss, and additional features such as white forelock, craniofacial dysmorphism including high nasal bridge, synophrys, eyebrow flaring, or anomalies of skin pigmentation (table 1, figure 3). Detailed clinical information was available for 13 of 14 patients with *PAX3* mutations and 11 of these presented hearing loss. No differences in phenotype were noted between patients with *PAX3* point mutations and *PAX3* deletions.

Patients with *MITF* mutations had the clinical phenotype of Waardenburg syndrome type 2, i.e. heterochromia irides and/or hearing loss without dystopia canthorum (figure 4). Patient 13, who was found to carry a *de novo* missense mutation in *MITF,* did not present with heterochromia irides, but with blue eyes and hypoplasia of iris stroma.

DISCUSSION

Molecular genetic analyses of the *PAX3* and *MITF* gene revealed 15 novel and four previously known mutations in patients with the clinical phenotype of Waardenburg

syndrome. Of these, 14 mutations occurred in *PAX3*, and 5 mutations were found in *MITF*. The spectrum of mutations includes nonsense, missense and splice site mutations, insertions, as well as deletions and duplications.

Most of the novel mutations in *PAX3* are localized in exons 2 to 6 and hence influence functionally relevant domains (table 1, figure 1). This predominant localization in exons 2 to 6 has previously been described.[18] Twelve of the 14 *PAX3* mutations are truncating mutations including large deletions. Deletions comprised two whole gene deletions and two intragenic deletions of exon 7 and exons 8 - 9, respectively. The two missense mutations are localized in the N-terminal part of the paired domain, which mediates intensive DNA contact. There is no correlation between the mutation type (missense, nonsense, deletion) and the severity of the phenotype. Therefore, loss of protein function leading to haploinsufficiency seems to be the disease causing mechanism for WS1. All patients with *PAX3* mutations had phenotypes of WS1 (table 1, figure 3).

Five patients had mutations in the *MITF* gene (table 1, figure 2). Four of these represented novel mutations. Detailed clinical information was available in four patients. Three patients had features corresponding to the WS2 phenotype (figure 4). Interestingly, one of these (patient 11) was the first WS2 patient who was found to carry two *MITF* mutations (missense mutation and small deletion) within the same gene copy. Since both mutations are novel, it remains to be clarified whether one or the combination of both mutations leads to the WS2 phenotype.

One patient with a *de novo* missense mutation c.650G>T (p.Arg217Ile) in the *MITF* gene did not present with heterochromia irides, but with bilateral blue irides and hypoplasia of iris stroma (patient 13 in table 1). Interestingly, *MITF* mutations affecting amino acid position 217, which is located in the basic domain of MITF, were also described in patients with Tietz syndrome (TS, MIM #103500). Compared to WS2, Tietz syndrome is characterized by a

more severe phenotype with generalized hypopigmentation and complete hearing loss.[19] Instead of heterochromia irides, which is typical for Waardenburg syndrome type 1 and 2, patients with Tietz syndrome present with bilateral blue irides. To date, only three patients with Tietz syndrome and mutation in the *MITF* gene have been described.[20-22] Two of these with typical Tietz syndrome had a mutation altering amino acid position 217 (3-bp in-frame deletion Arg217). Compared to these patients, our patient with a *de novo* missense mutation at the same amino acid position has a less severe phenotype with bilateral hearing loss, a white forelock and blue irides with hypoplasia of iris stroma. These clinical features are part of WS2, while blue eyes with hypoplastic iris stroma may correspond to Tietz syndrome. Apparently, a missense mutation at amino acid position 217 leads to milder or intermediate phenotype than a 3-bp in-frame deletion at the same position. Therefore, both WS2 and Tietz syndrome most probably correspond to a common clinical spectrum that is influenced by mutation type and position.

In summary, the molecular genetic analyses of the *PAX3* and *MITF* genes are important diagnostic steps to explain the molecular cause of clinical features of Waardenburg syndrome and facilitate genetic counseling of affected patients and their families. For six of 19 patients with suspected WS and previously genetically unconfirmed diagnosis, we found either large deletions (four patients with deletions in *PAX3* and one patient with deletion in *MITF)* or duplications (one patient, *MITF* gene). This indicates that deletion/duplication screening is indispensable for a successful molecular genetic diagnostics of WS. As a genetic diagnostic strategy it is thus recommended to perform both sequence analysis and copy number analysis of the *PAX3* and *MITF* genes in all patients with clinical features of Waardenburg syndrome.

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2	7 y.	f	*PAX3*	exon 2	c.143G>A	p.Gly48Asp	missense	maternal	+	-	+	high nasal bridge, synophrys, eyebrow flaring, skin depigmentation; affected family members with premature greying and dystopia canthorum	different amino acid change at same position described in [23]
3	3 y.	f	*PAX3*	exon 2	c.186G>A	p.Met62Ile	missense	paternal	+	+	++	-	different amino acid change at same position: described in [24]
4	34 y.	m	*PAX3*	exon 3	c.400C>T	p.Arg134X	nonsense	n.a.	+	+	-	-	-
5	6 mo.	m	*PAX3*	exon 5	c.589delT	p.Ser197fs	deletion	paternal	+	-	+	white forelock	-
6	3 mo.	m	*PAX3*	exon 5	c.655C>T	p.Gln219X	nonsense	paternal	+	+	-	high nasal bridge	-
7	17 mo.	m	*PAX3*	exon 5	c.784C>T	p.Arg262X	nonsense	maternal	+	+	+	white forelock, synophrys, high nasal bridge, prognathia, hypopigmentation anomalies	described in [25]
8	1 wk.	m	*PAX3*	intron 5	c.793-1G>T	-	splice site	*de novo*	+	-	-	white forelock, craniofacial dysmorphism	-
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10	2 1/2 y.	m	*PAX3*	exon 6	c.955C>T	p.Gln319X	nonsense	n.a.	+	+	++	high nasal bridge, mild skin depigmentation	-
11	n.a.	m	*MITF*	exon 1	[c.28T>A;c.33+6del7]	p.Tyr10Asn	missense / deletion	paternal	-	+	+	-	-
12	16 y.	m	*MITF*	exon 3	c.328C>T	p.Arg110X	nonsense	n.a.	-	+	+	-	-
13	7 mo.	m	*MITF*	exon 7	c.650G>T	p.Arg217Ile	missense	*de novo*	-	-	+	blue eyes with hypoplasia of iris stroma, white forelock	described in [27]
14	23 y.	m	*PAX3*	entire gene	c.(?_-61)_(1452-33_?)		deletion entire gene	paternal	+	-	+	pigmentation anomalies, unilateral hearing loss	described in [26]
15	42 y.	m	*PAX3*	exon 7	c.958+?_1174-?del		deletion exon 7	n.a.	+	-	+	pigmentation anomalies	-
16	6 y.	f	*PAX3*	exons 8-9	c.1173+?_(1452-33_?)del		deletion exons 8-9	n.a.	+	-	+	blue eyes, synophrys, medial eyebrow flaring	-
17	5 mo.	f	*PAX3*	entire gene	c.(?_-61)_(1452-33_?)		deletion entire gene	n.a.	+	+	+	unilateral hearing loss	described in [26]
18	7 y.	m	*MITF*	5´-UTR region	c.?_1-70453dup		duplication	n.a.	-	-	(+)	-	-
19	3 y.	m	*MITF*	exons 1-9	c.1-70433_?_(988_?)del		deletion exons 1-9	*de novo*	-	+	+	bilateral hearing loss	-

Table 1: Genotype and phenotype of all Waardenburg syndrome index patients (wk = week; mo = month; y = years; m = male; f = female; (+) mild + moderate; ++ severe; n.a. = not available). "*De novo*" = under consideration of provided information concerning kinship, no paternity testing was performed.

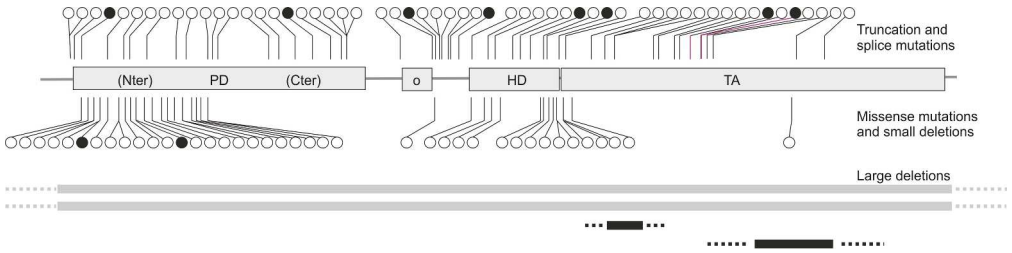

Figure 1: Survey of *PAX3* mutations detected in patients with Waardenburg syndrome. Black circles = mutations detected in this study; black bars = copy number variations detected in this study; white circles and grey bars = previously published mutations and deletions. PD = paired domain; o = octapeptide; HD = homeodomain; TA = transactivation domain.
287x68mm (300 x 300 DPI)

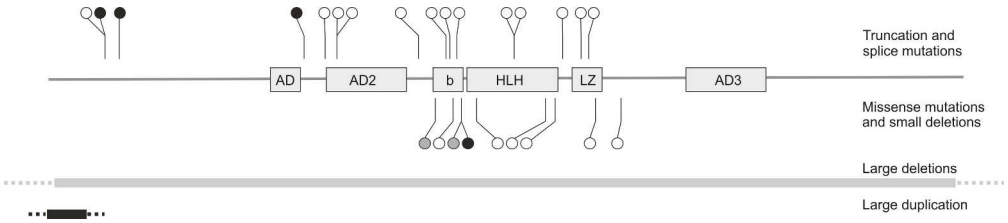

Figure 2: Survey of *MITF* mutations detected in patients with Waardenburg and Tietz syndrome. Black circles and bars = mutations and copy number variations detected in this study; white circles and grey bars = previously published mutations and deletions; grey circles = MITF mutations associated with Tietz syndrome. AD1-3 = (trans)activation domains; b = basic domain; HLH = helix-loop-helix domain; LZ = leucine zipper domain.
271x57mm (300 x 300 DPI)

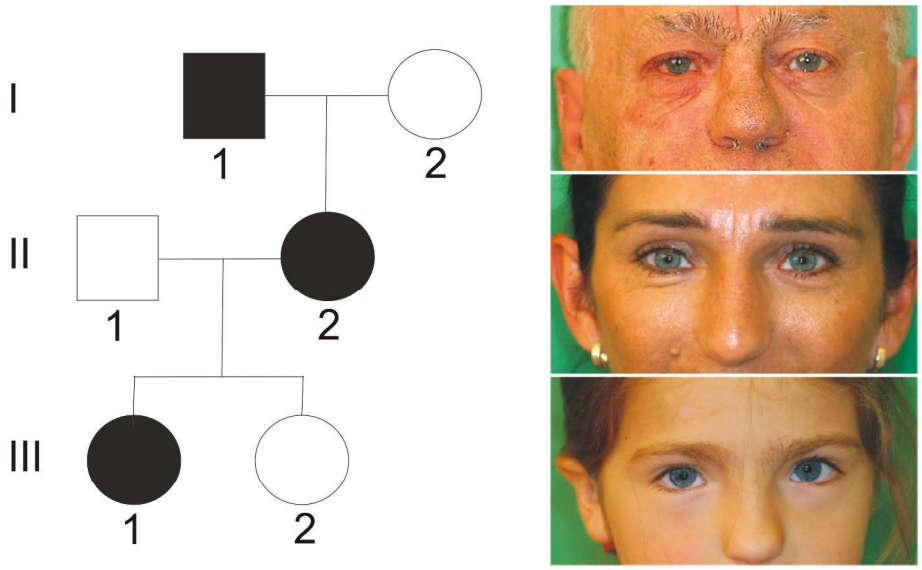

Figure 3: Pedigree of index patient 2 with *PAX3* mutation. The girl presented with clinical features of Waardenburg syndrome type 1 (see table 1): dystopia canthorum, high nasal bridge, synophrys, eyebrow flaring, skin depigmentation and bilateral hearing loss. Her mother and maternal grandfather had only premature graying.

301x172mm (300 x 300 DPI)

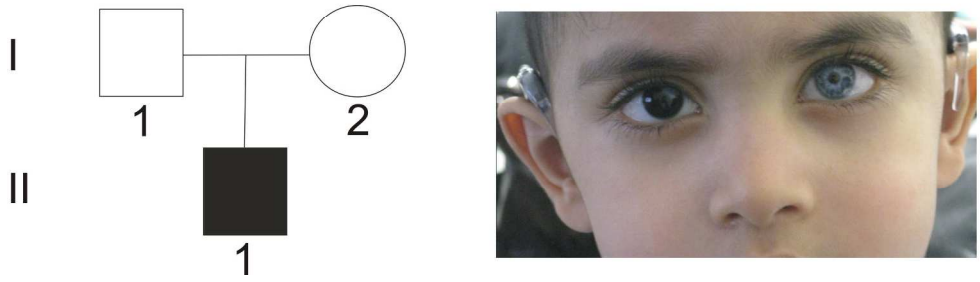

Figure 4: Sporadic case (patient 19 in table 1) of Waardenburg syndrome type 2 with *MITF* mutation. The boy presented with heterochromia irides and bilateral hearing loss, but did not show dystopia canthorum.
226x77mm (300 x 300 DPI)