



**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 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 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]

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5 The exact description of the mutations responsible for the WS type is of significant  
6 importance in genetic counseling of WS patients and their families. Whole-gene sequencing  
7 enables the discovery of point mutations and small alterations in the gene, but cannot  
8 reliably detect whole-exon or whole-gene copy number changes, which have been reported  
9 for many genes resulting in a specific genetic disorder. In recent years, MLPA (multiplex  
10 ligation-dependent probe amplification) has become a widespread method in molecular  
11 genetic diagnostics to detect or exclude copy number changes in targeted genes.  
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21 We applied both whole-gene sequencing and MLPA analysis for the detection of point  
22 mutations and copy number variations in the genes *PAX3* and *MITF*, and describe 19  
23 mutations, of which 16 have not been previously reported for patients with features of WS.  
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## 32 **MATERIALS AND METHODS**

### 33 **Patients**

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35 19 caucasian patients with the clinical phenotype of WS were included in our study. Clinical  
36 data were collected for patients and their affected family members.  
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### 41 **Molecular genetic analysis**

42 All 19 patients were analysed for point mutations and copy number changes in the genes  
43 *PAX3* and *MITF*. Genomic DNA was prepared from white blood cells using a standard  
44 procedure. All 10 exons of *PAX3* and all 9 exons of *MITF* were PCR-amplified and directly  
45 sequenced. Sequence variant numbering was based on the transcript ENST00000392069  
46 for *PAX3* and ENST00000314557 for *MITF*. Nucleotide numbering used the A of the ATG  
47 translation initiation site as nucleotide +1. The nomenclature of the alterations was adopted  
48 according to the guidelines of the human variation society (<http://www.hgvs.org/>). MLPA  
49 (multiplex ligation-dependent probe amplification) analysis was carried out in order to detect  
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3 or exclude deletions and duplications of the genes *PAX3* and *MITF*. For this purpose, a mix  
4 of probes was hybridized to template DNA. The probes, which contained a common  
5 sequence tail, were ligated, and subsequently amplified by PCR. Products were separated  
6 electrophoretically and analyzed by means of sizing and quantification. The Software  
7 Sequence Pilot (JSI Medical Systems, Kippenheim, Germany) was used for evaluation.  
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## 13 14 15 **RESULTS**

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17 Molecular genetic analysis of the genes *PAX3* and *MITF* for 19 unrelated index patients with  
18 the clinical phenotype of WS revealed 16 novel and three previously published heterozygous  
19 mutations (table 1). 14 mutations occurred in the *PAX3* gene: one small insertion, two  
20 missense mutations, four nonsense mutations, two small deletions, one splice-site mutation,  
21 and four large deletions comprising at least one exon. Five mutations were detected in the  
22 *MITF* gene: one missense mutation, one nonsense mutation, one large deletion and one  
23 large duplication. In addition, the combination of a *MITF* missense mutation with a small  
24 deletion was found in family 11 (table 1). Both mutations were proven to be located on the  
25 same chromosome.  
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37 Of specific interest, six of the 19 mutations (32 %) were not detectable by direct sequencing.  
38 These were five large deletions and one duplication that could only be detected by MLPA  
39 analysis. In ten of 19 index patients, parents were available for genetic analysis. Seven  
40 mutations were familial (i.e. shown to be inherited from one parent), whereas three  
41 mutations (one mutation in *PAX3* and two in *MITF*) occurred *de novo*. 16 of 19 mutations  
42 detected in *PAX3* and *MITF* represented novel mutations. These mutations and all  
43 previously published mutations are summarized in figure 1 (*PAX3* gene) and figure 2 (*MITF*  
44 gene).  
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55 All index patients with *PAX3* mutations had the typical phenotype of Waardenburg syndrome  
56 type 1 including dystopia canthorum and at least one of the following criteria: heterochromia  
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Wildhardt et al. Spectrum of novel mutations found in Waardenburg syndrome type 1 and type 2: Implications for molecular genetic diagnostics

index patient	age	gene	mutation description				transmission mode	dystopia canthorum	heterochromia 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)



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3 iridis, hearing loss, and additional features such as white forelock, craniofacial dysmorphism  
4 including high nasal bridge, synophrys, eyebrow flaring, or anomalies of skin pigmentation  
5 (table 1, figure 3). Clinical information was available for 10 of 12 patients with *PAX3*  
6 mutations and all of these presented hearing. No differences in phenotype were noted  
7 between patients with *PAX3* point mutations and *PAX3* deletions.  
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15 Patients with *MITF* mutations had the clinical phenotype of Waardenburg syndrome type 2,  
16 i.e. heterochromia irides and/or hearing loss without dystopia canthorum (figure 4; clinical  
17 data not available for one patient). Patient 13, who was found to carry a *de novo* missense  
18 mutation in *MITF*, did not present with heterochromia irides, but with blue eyes and  
19 hypoplasia of iris stroma.  
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## 29 DISCUSSION

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31 Molecular genetic analyses of the *PAX3* and *MITF* gene revealed 16 novel and three  
32 previously known mutations in patients with the clinical phenotype of Waardenburg  
33 syndrome. Of these, 14 mutations occurred in *PAX3*, and 5 mutations were found in *MITF*.  
34 The spectrum of mutations includes nonsense, missense and splice site mutations,  
35 insertions, as well as deletions and duplications.  
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43 Most of the novel mutations in *PAX3* are localized in exons 2 to 6 and hence influence  
44 functionally relevant domains (table 1, figure 1). This predominant localization in exons 2 to 6  
45 has previously been described.[18] Twelve of the 14 *PAX3* mutations are truncating  
46 mutations including larger deletions. Deletions comprised two whole gene deletions and two  
47 intragenic deletions of exon 7 and exons 8-9, respectively. The two missense mutations are  
48 localized in the N-terminal part of the paired domain, which mediates intensive DNA contact.  
49 There is no correlation between the mutation type (missense, nonsense, deletion) and the  
50 severity of the phenotype. Therefore, loss of protein function seems to be the disease  
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3 causing mechanism for WS1. All patients with *PAX3* mutations had phenotypes of WS1  
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5 (table 1, figure 3).  
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9 Five patients had mutations in the *MITF* gene (table 1, figure 2). Four of these represented  
10 novel mutations. Clinical information was available in four patients. Three patients had  
11 features corresponding to the WS2 phenotype (figure 4). Interestingly, one of these (patient  
12 11) was the first WS2 patient who was found to carry two *MITF* mutations (missense  
13 mutation and small deletion) within the same gene copy. Since both mutations are novel, it  
14 remains to be clarified whether one or the combination of both mutations leads to the WS2  
15 phenotype.  
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25 One patient with a *de novo* missense mutation c.650G>T (p.Arg217Ile) in the *MITF* gene did  
26 not present with heterochromia irides, but with bilateral blue irides and hypoplasia of iris  
27 stroma (patient 13 in table 1). Interestingly, *MITF* mutations affecting amino acid position 217,  
28 which is located in the basic domain of MITF, were also described in patients with Tietz  
29 syndrome (TS, MIM #103500). Compared to WS2, Tietz syndrome is characterized by a  
30 more severe phenotype with generalized hypopigmentation and complete hearing loss.[19]  
31 Instead of heterochromia irides, which is typical for Waardenburg syndrome type 1 and 2,  
32 patients with Tietz syndrome present with bilateral blue irides. To date, only three patients  
33 with Tietz syndrome and mutation in the *MITF* gene have been described.[20-22] Two of  
34 these with typical Tietz syndrome had a mutation altering amino acid position 217 (3-bp in-  
35 frame deletion Arg217). Compared to these patients, our patient with a *de novo* missense  
36 mutation at the same amino acid position has a less severe phenotype with bilateral hearing  
37 loss, a white forelock and blue irides with hypoplasia of iris stroma. These clinical features  
38 are part of WS2, while blue eyes with hypoplastic iris stroma may correspond to Tietz  
39 syndrome. Apparently, a missense mutation at amino acid position 217 leads to milder or  
40 intermediate phenotype than a 3-bp in-frame deletion at the same position. Therefore, both  
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3 WS2 and TS most probably correspond to a common clinical spectrum that is influenced by  
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5 mutation type and position.  
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9 In summary, the molecular genetic analyses of the *PAX3* and *MITF* genes are important  
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11 diagnostic steps to explain the molecular cause of clinical features of Waardenburg  
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13 syndrome and facilitate genetic counseling of affected patients and their families. For six of  
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15 19 patients with suspected WS and previously genetically unconfirmed diagnosis, we found  
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17 either large deletions (four patients with deletions in *PAX3* and one patient with deletion in  
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19 *MITF*) or duplications (one patient, *MITF* gene). This indicates that deletion/duplication  
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21 screening is indispensable for a successful molecular genetic diagnostics of WS. As a  
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23 genetic diagnostic strategy it is thus recommended to perform both sequence analysis and  
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25 copy number analysis of the *PAX3* and *MITF* genes in all patients with clinical features of  
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27 Waardenburg syndrome.  
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### 30 31 **Competing Interests**

32  
33 There are no competing interests.  
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35

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37  
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39

### 40 **Contributorship Statement**

41  
42 GW and DS responsible for study concept and design and acquisition of genetic data and  
43  
44 interpretation. BZ, LGN, JW, MS, AB, ABo, CK, SV, GSW, MG and OB collected the  
45  
46 clinical data. GW, BZ and DS wrote the manuscript. All authors have critically revised the  
47  
48 paper.  
49

### 50 51 **Data Sharing Statement**

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53 There is no additional data available  
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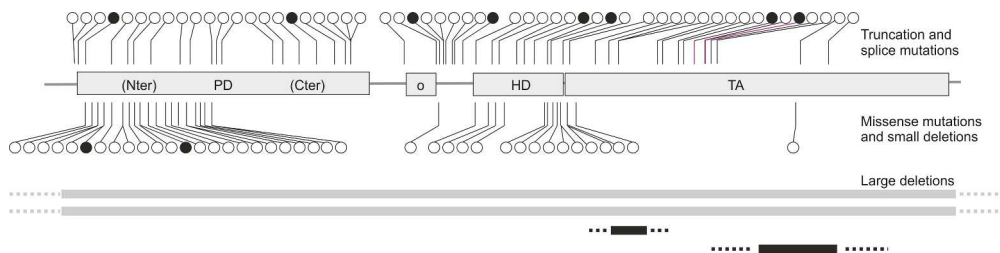


Figure 1: Survey of *PAX3* mutations detected in patients with Waardenburg syndrome. Black circles = mutations detected in this study; black bars = copy number variations detected in this study; white circles and grey bars = previously published mutations and deletions. PD = paired domain; o = octapeptide;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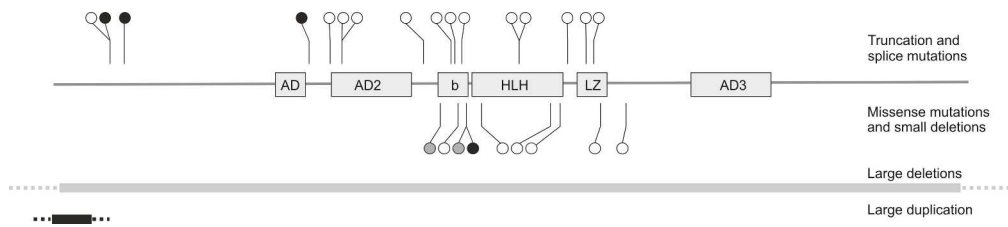


Figure 2: Survey of *MITF* mutations detected in patients with Waardenburg and Tietz syndrome. Black circles and bars = mutations and copy number variations detected in this study; white circles and grey bars = previously published mutations and deletions; grey circles = *MITF* mutations associated with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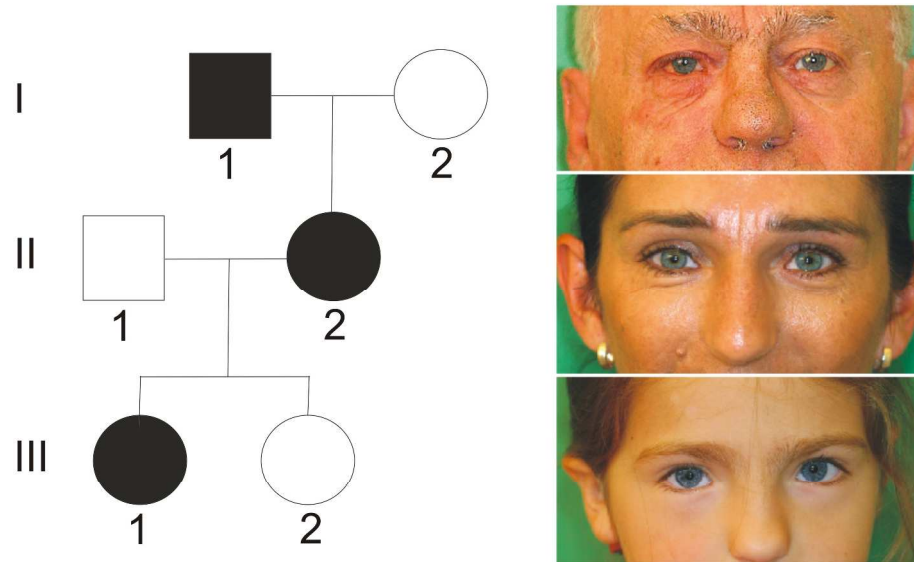


Figure 3: Pedigree of index patient 2 with *PAX3* mutation. The girl presented with clinical features of Waardenburg syndrome type 1 (see table 1): dystopia canthorum, high nasal bridge, synophrys, eyebrow flaring, skin depigmentation and bilateral hearing loss. Her mother and maternal grandfather had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

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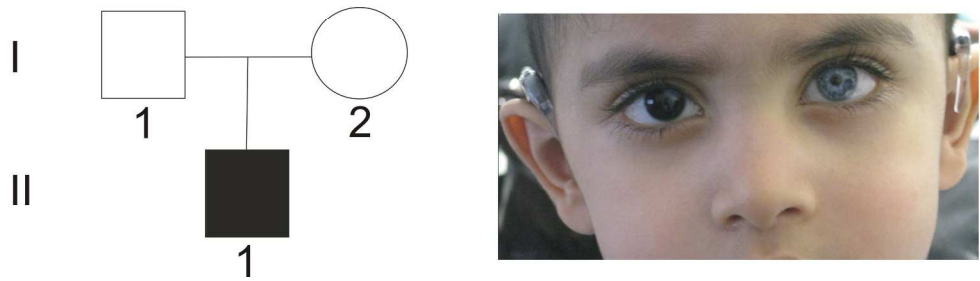


Figure 4: Sporadic case (patient 19 in table 1) of Waardenburg syndrome type 2 with *MITF* mutation. The boy presented with heterochromia irides and bilateral hearing loss, but did not show dystopia canthorum.  
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**SPECTRUM OF NOVEL MUTATIONS FOUND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

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3 correspond to a clinical spectrum that is influenced by *MITF* mutation type and  
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## 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

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3 associated with WS2 is SOX10. [16] In addition, mutations in *SOX10*, *EDN3* and *EDNRB*  
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5 were found in WS4.[17]  
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9 The exact description of the mutations responsible for the WS type is of significant  
10 importance in genetic counseling of WS patients and their families. Whole-gene sequencing  
11 enables the discovery of point mutations and small alterations in the gene, but cannot  
12 reliably detect whole-exon or whole-gene copy number changes, which have been reported  
13 for many genes resulting in a specific genetic disorder. In recent years, MLPA (multiplex  
14 ligation-dependent probe amplification) has become a widespread method in molecular  
15 genetic diagnostics to detect or exclude copy number changes in targeted genes.  
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25 We applied both whole-gene sequencing and MLPA analysis for the detection of point  
26 mutations and copy number variations in the genes *PAX3* and *MITF*, and describe 19  
27 mutations, of which 15 have not been previously reported for patients with features of WS.  
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## 36 MATERIALS AND METHODS

### 37 Patients

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39 19 Caucasian patients with the clinical phenotype of WS were included in our study. Clinical  
40 data were collected for patients and their affected family members.  
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### 45 Molecular genetic analysis

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

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27 Molecular genetic analysis of the genes *PAX3* and *MITF* for 19 unrelated index patients with  
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29 the clinical phenotype of WS revealed 15 novel and four previously published heterozygous  
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31 mutations (table 1). 14 mutations occurred in the *PAX3* gene: one small insertion, two  
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33 missense mutations, four nonsense mutations, two small deletions, one splice-site mutation,  
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35 and four large deletions comprising at least one exon. Five mutations were detected in the  
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37 *MITF* gene: one missense mutation, one nonsense mutation, one large deletion and one  
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39 large duplication. In addition, the combination of a *MITF* missense mutation with a small  
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41 deletion was found in family 11 (table 1). Both mutations were proven to be located on the  
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43 same chromosome by segregation analysis. The paternal grandfather, a brother, two sisters,  
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45 a niece and a nephew with Waardenburg syndrome were all proven to be carrier of these  
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47 two mutations.  
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50 Of specific interest, six of the 19 mutations (32 %) were not detectable by direct sequencing.  
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52 These were five large deletions and one duplication that could only be detected by MLPA  
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54 analysis. In ten of 19 index patients, parents were available for genetic analysis. Seven  
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56 mutations were familial (i.e. shown to be inherited from one parent that presented signs and  
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58 symptoms of WS), whereas three mutations (one mutation in *PAX3* and two in *MITF*)  
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3 occurred *de novo*. 15 of 19 mutations detected in *PAX3* and *MITF* represented novel  
4 mutations. These mutations and all previously published mutations are summarized in figure  
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7 1 (*PAX3* gene) and figure 2 (*MITF* gene).  
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11 All index patients with *PAX3* mutations had the typical phenotype of Waardenburg syndrome  
12 type 1 including dystopia canthorum and at least one of the following criteria: heterochromia  
13 iridis, hearing loss, and additional features such as white forelock, craniofacial dysmorphism  
14 including high nasal bridge, synophrys, eyebrow flaring, or anomalies of skin pigmentation  
15 (table 1, figure 3). Detailed clinical information was available for 13 of 14 patients with *PAX3*  
16 mutations and 11 of these presented hearing loss. No differences in phenotype were noted  
17 between patients with *PAX3* point mutations and *PAX3* deletions.  
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27 Patients with *MITF* mutations had the clinical phenotype of Waardenburg syndrome type 2,  
28 i.e. heterochromia irides and/or hearing loss without dystopia canthorum (figure 4). Patient  
29 13, who was found to carry a *de novo* missense mutation in *MITF*, did not present with  
30 heterochromia irides, but with blue eyes and hypoplasia of iris stroma.  
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## 40 DISCUSSION

41 Molecular genetic analyses of the *PAX3* and *MITF* gene revealed 15 novel and four  
42 previously known mutations in patients with the clinical phenotype of Waardenburg  
43 syndrome. Of these, 14 mutations occurred in *PAX3*, and 5 mutations were found in *MITF*.  
44 The spectrum of mutations includes nonsense, missense and splice site mutations,  
45 insertions, as well as deletions and duplications.  
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54 Most of the novel mutations in *PAX3* are localized in exons 2 to 6 and hence influence  
55 functionally relevant domains (table 1, figure 1). This predominant localization in exons 2 to 6  
56 has previously been described.[18] Twelve of the 14 *PAX3* mutations are truncating  
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3 mutations including large deletions. Deletions comprised two whole gene deletions and two  
4 intragenic deletions of exon 7 and exons 8 - 9, respectively. The two missense mutations are  
5 localized in the N-terminal part of the paired domain, which mediates intensive DNA contact.  
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7 There is no correlation between the mutation type (missense, nonsense, deletion) and the  
8 severity of the phenotype. Therefore, loss of protein function respectively haploinsufficiency  
9 seems to be the disease causing mechanism for WS1. All patients with *PAX3* mutations had  
10 phenotypes of WS1 (table 1, figure 3).  
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19 Five patients had mutations in the *MITF* gene (table 1, figure 2). Four of these represented  
20 novel mutations. Detailed clinical information was available in four patients. Three patients  
21 had features corresponding to the WS2 phenotype (figure 4). Interestingly, one of these  
22 (patient 11) was the first WS2 patient who was found to carry two *MITF* mutations (missense  
23 mutation and small deletion) within the same gene copy. Since both mutations are novel, it  
24 remains to be clarified whether one or the combination of both mutations leads to the WS2  
25 phenotype.  
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35 One patient with a *de novo* missense mutation c.650G>T (p.Arg217Ile) in the *MITF* gene did  
36 not present with heterochromia irides, but with bilateral blue irides and hypoplasia of iris  
37 stroma (patient 13 in table 1). Interestingly, *MITF* mutations affecting amino acid position 217,  
38 which is located in the basic domain of MITF, were also described in patients with Tietz  
39 syndrome (TS, MIM #103500). Compared to WS2, Tietz syndrome is characterized by a  
40 more severe phenotype with generalized hypopigmentation and complete hearing loss.[19]  
41 Instead of heterochromia irides, which is typical for Waardenburg syndrome type 1 and 2,  
42 patients with Tietz syndrome present with bilateral blue irides. To date, only three patients  
43 with Tietz syndrome and mutation in the *MITF* gene have been described.[20-22] Two of  
44 these with typical Tietz syndrome had a mutation altering amino acid position 217 (3-bp in-  
45 frame deletion Arg217). Compared to these patients, our patient with a *de novo* missense  
46 mutation at the same amino acid position has a less severe phenotype with bilateral hearing  
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3 loss, a white forelock and blue irides with hypoplasia of iris stroma. These clinical features  
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5 are part of WS2, while blue eyes with hypoplastic iris stroma may correspond to Tietz  
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7 syndrome. Apparently, a missense mutation at amino acid position 217 leads to milder or  
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9 intermediate phenotype than a 3-bp in-frame deletion at the same position. Therefore, both  
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11 WS2 and Tietz syndrome most probably correspond to a common clinical spectrum that is  
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13 influenced by mutation type and position.

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17 In summary, the molecular genetic analyses of the *PAX3* and *MITF* genes are important  
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19 diagnostic steps to explain the molecular cause of clinical features of Waardenburg  
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21 syndrome and facilitate genetic counseling of affected patients and their families. For six of  
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23 19 patients with suspected WS and previously genetically unconfirmed diagnosis, we found  
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25 either large deletions (four patients with deletions in *PAX3* and one patient with deletion in  
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27 *MITF*) or duplications (one patient, *MITF* gene). This indicates that deletion/duplication  
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29 screening is indispensable for a successful molecular genetic diagnostics of WS. As a  
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31 genetic diagnostic strategy it is thus recommended to perform both sequence analysis and  
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33 copy number analysis of the *PAX3* and *MITF* genes in all patients with clinical features of  
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35 Waardenburg syndrome.  
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index patient	age	gender	gene	mutation description				transmission mode	dystopia canthorum	heterochromia 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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## SPECTRUM OF NOVEL MUTATIONS IN WAARDENBURG SYNDROME TYPE 1 AND TYPE 2: IMPLICATIONS FOR MOLECULAR GENETIC DIAGNOSTICS

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## 7 ABSTRACT

8 **Objectives:** To date, mutations in the genes *PAX3* and *MITF* have been described in  
9  
10 Waardenburg syndrome (WS), which is clinically characterized by congenital hearing loss,  
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12 pigmentation anomalies and [depending on the subtype of the disease, presence or absence](#)  
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14 [of craniofacial dysmorphism](#). Our study intended to determine the frequency of mutations  
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16 and deletions in these genes, to assess the clinical phenotype in detail and to identify  
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18 rational priorities for molecular genetic diagnostics procedures.

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

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

31 **Results:** 16 novel and ~~four~~ previously published heterozygous mutations in *PAX3* and  
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33 *MITF* were identified. Of these, six were large deletions or duplications, that were only  
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35 detectable by copy number analysis. All patients with *PAX3* mutations had the typical  
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37 phenotype of WS with dystopia canthorum (WS1), whereas patients with *MITF* gene  
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39 mutations presented without dystopia canthorum (WS2). In addition, one patient with  
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41 bilateral hearing loss and blue eyes with iris stroma dysplasia had a *de novo* missense  
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43 mutation (p.Arg217Ile) in *MITF*. *MITF* 3-bp deletions at amino acid position 217 have  
44  
45 previously been described in patients with Tietz syndrome, a clinical entity with hearing loss  
46  
47 and generalized hypopigmentation.

48 **Conclusion:** Based on these findings, we conclude that sequencing and copy number  
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50 analysis of both *PAX3* and *MITF* have to be recommended in the routine molecular  
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52 diagnostic setting for WS patients with and without dystopia canthorum. [Furthermore, our](#)  
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54 [genotype-/phenotype analyses indicate that WS without dystopia \(WS2\) and TS](#)  
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7 correspond to a clinical spectrum that is influenced by *MITF* mutation type and  
8 position.  
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Widhardt et al. Spectrum of novel mutations found in Waardenburg syndrome type 1 and type 2: Implications for molecular genetic diagnostics

## 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](#)

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6 [mutations associated with ~~additional candidate gene for~~ WS2 is SOX10 and SNAI2](#). [16];

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8 In addition, mutations in *SOX10*, *EDN3* and *EDNRB* were found in WS4. [17]

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11 The exact description of the mutations responsible for the WS type is of significant  
12 importance in genetic counseling of WS patients and their families. Whole-gene sequencing  
13 enables the discovery of point mutations and small alterations in the gene, but cannot  
14 reliably detect whole-exon or whole-gene copy number changes, which have been reported  
15 for many genes resulting in a specific genetic disorder. In recent years, MLPA (multiplex  
16 ligation-dependent probe amplification) has become a widespread method in molecular  
17 genetic diagnostics to detect or exclude copy number changes in targeted genes.  
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21 We applied both whole-gene sequencing and MLPA analysis for the detection of point  
22 mutations and copy number variations in the genes *PAX3* and *MITF*, and describe 19  
23 mutations, of which 156 have not been previously reported for patients with features of WS.  
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## 26 27 28 29 30 31 32 33 34 35 **MATERIALS AND METHODS**

### 36 37 **Patients**

38 19 eCaucasian patients with the clinical phenotype of WS were included in our study. Clinical  
39 data were collected for patients and their affected family members.  
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### 43 44 **Molecular genetic analysis**

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

## 27 28 RESULTS

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30 Molecular genetic analysis of the genes *PAX3* and *MITF* for 19 unrelated index patients with  
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32 the clinical phenotype of WS revealed 156 novel and fourthree previously published  
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34 heterozygous mutations (table 1). 14 mutations occurred in the *PAX3* gene: one small  
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36 insertion, two missense mutations, four nonsense mutations, two small deletions, one splice-  
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38 site mutation, and four large deletions comprising at least one exon. Five mutations were  
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40 detected in the *MITF* gene: one missense mutation, one nonsense mutation, one large  
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42 deletion and one large duplication. In addition, the combination of a *MITF* missense mutation  
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44 with a small deletion was found in family 11 (table 1). Both mutations were proven to be  
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46 located on the same chromosome by segregation analysis. The paternal grandfather, a  
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48 brother, two sisters, a niece and a nephew with Waardenburg syndrome are were all proven  
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50 to be carriers of these two mutations.

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52 Of specific interest, six of the 19 mutations (32 %) were not detectable by direct sequencing.  
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54 These were five large deletions and one duplication that could only be detected by MLPA  
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56 analysis. In ten of 19 index patients, parents were available for genetic analysis. Seven  
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7 mutations were familial (i.e. shown to be inherited from one parent [that presented signs and](#)  
8 [symptoms of WS](#)), whereas three mutations (one mutation in *PAX3* and two in *MITF*)  
9 occurred *de novo*. 165 of 19 mutations detected in *PAX3* and *MITF* represented novel  
10 mutations. These mutations and all previously published mutations are summarized in figure  
11 1 (*PAX3* gene) and figure 2 (*MITF* gene).  
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17 All index patients with *PAX3* mutations had the typical phenotype of Waardenburg syndrome  
18 type 1 including dystopia canthorum and at least one of the following criteria: heterochromia  
19 iridis, hearing loss, and additional features such as white forelock, craniofacial dysmorphism  
20 including high nasal bridge, synophrys, eyebrow flaring, or anomalies of skin pigmentation  
21 (table 1, figure 3). [Detailed cClinical](#) information was available for 103 of 142 patients with  
22 *PAX3* mutations and 11a# of these presented hearing [loss](#). No differences in phenotype  
23 were noted between patients with *PAX3* point mutations and *PAX3* deletions.  
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31 Patients with *MITF* mutations had the clinical phenotype of Waardenburg syndrome type 2,  
32 i.e. heterochromia irides and/or hearing loss without dystopia canthorum (figure 4: ~~clinical~~  
33 ~~data not available for one patient~~). Patient 13, who was found to carry a *de novo* missense  
34 mutation in *MITF*, did not present with heterochromia irides, but with blue eyes and  
35 hypoplasia of iris stroma.  
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#### 44 DISCUSSION

45 Molecular genetic analyses of the *PAX3* and *MITF* gene revealed 156 novel and ~~threefour~~  
46 previously known mutations in patients with the clinical phenotype of Waardenburg  
47 syndrome. Of these, 14 mutations occurred in *PAX3*, and 5 mutations were found in *MITF*.  
48 The spectrum of mutations includes nonsense, missense and splice site mutations,  
49 insertions, as well as deletions and duplications.  
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6 Most of the novel mutations in *PAX3* are localized in exons 2 to 6 and hence influence  
7 functionally relevant domains (table 1, figure 1). This predominant localization in exons 2 to 6  
8 has previously been described.[18] Twelve of the 14 *PAX3* mutations are truncating  
9 mutations including large deletions. Deletions comprised two whole gene deletions and two  
10 intragenic deletions of exon 7 and exons 8-9, respectively. The two missense mutations are  
11 localized in the N-terminal part of the paired domain, which mediates intensive DNA contact.  
12 There is no correlation between the mutation type (missense, nonsense, deletion) and the  
13 severity of the phenotype. Therefore, loss of protein function respectively haploinsufficiency  
14 seems to be the disease causing mechanism for WS1. All patients with *PAX3* mutations had  
15 phenotypes of WS1 (table 1, figure 3).  
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26 Five patients had mutations in the *MITF* gene (table 1, figure 2). Four of these represented  
27 novel mutations. Detailed clinical information was available in four patients. Three patients  
28 had features corresponding to the WS2 phenotype (figure 4). Interestingly, one of these  
29 (patient 11) was the first WS2 patient who was found to carry two *MITF* mutations (missense  
30 mutation and small deletion) within the same gene copy. Since both mutations are novel, it  
31 remains to be clarified whether one or the combination of both mutations leads to the WS2  
32 phenotype.  
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40 One patient with a *de novo* missense mutation c.650G>T (p.Arg217Ile) in the *MITF* gene did  
41 not present with heterochromia irides, but with bilateral blue irides and hypoplasia of iris  
42 stroma (patient 13 in table 1). Interestingly, *MITF* mutations affecting amino acid position 217,  
43 which is located in the basic domain of MITF, were also described in patients with Tietz  
44 syndrome (TS, MIM #103500). Compared to WS2, Tietz syndrome is characterized by a  
45 more severe phenotype with generalized hypopigmentation and complete hearing loss.[19]  
46 Instead of heterochromia irides, which is typical for Waardenburg syndrome type 1 and 2,  
47 patients with Tietz syndrome present with bilateral blue irides. To date, only three patients  
48 with Tietz syndrome and mutation in the *MITF* gene have been described.[20-22] Two of  
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6 these with typical Tietz syndrome had a mutation altering amino acid position 217 (3-bp in-  
7 frame deletion Arg217). Compared to these patients, our patient with a *de novo* missense  
8 mutation at the same amino acid position has a less severe phenotype with bilateral hearing  
9 loss, a white forelock and blue irides with hypoplasia of iris stroma. These clinical features  
10 are part of WS2, while blue eyes with hypoplastic iris stroma may correspond to Tietz  
11 syndrome. Apparently, a missense mutation at amino acid position 217 leads to milder or  
12 intermediate phenotype than a 3-bp in-frame deletion at the same position. Therefore, both  
13 WS2 and ~~TS~~ Tietz syndrome most probably correspond to a common clinical spectrum that  
14 is influenced by mutation type and position.  
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24 In summary, the molecular genetic analyses of the *PAX3* and *MITF* genes are important  
25 diagnostic steps to explain the molecular cause of clinical features of Waardenburg  
26 syndrome and facilitate genetic counseling of affected patients and their families. For six of  
27 19 patients with suspected WS and previously genetically unconfirmed diagnosis, we found  
28 either large deletions (four patients with deletions in *PAX3* and one patient with deletion in  
29 *MITF*) or duplications (one patient, *MITF* gene). This indicates that deletion/duplication  
30 screening is indispensable for a successful molecular genetic diagnostics of WS. As a  
31 genetic diagnostic strategy it is thus recommended to perform both sequence analysis and  
32 copy number analysis of the *PAX3* and *MITF* genes in all patients with clinical features of  
33 Waardenburg syndrome.  
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Wildhardt et al. Spectrum of novel mutations found in Waardenburg syndrome type 1 and type 2: Implications for molecular genetic diagnostics

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.

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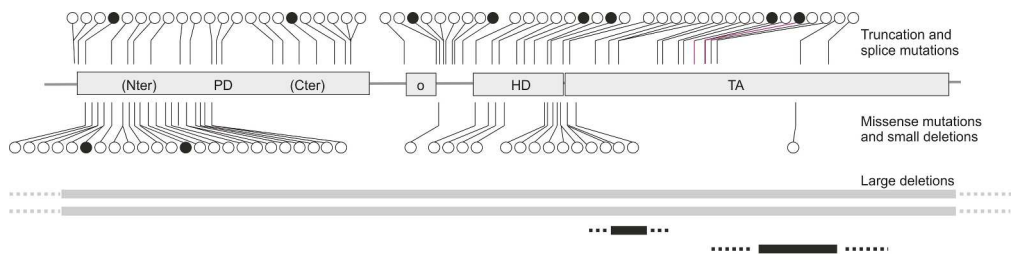


Figure 1: Survey of *PAX3* mutations detected in patients with Waardenburg syndrome. Black circles = mutations detected in this study; black bars = copy number variations detected in this study; white circles and grey bars = previously published mutations and deletions. PD = paired domain; o = octapeptide;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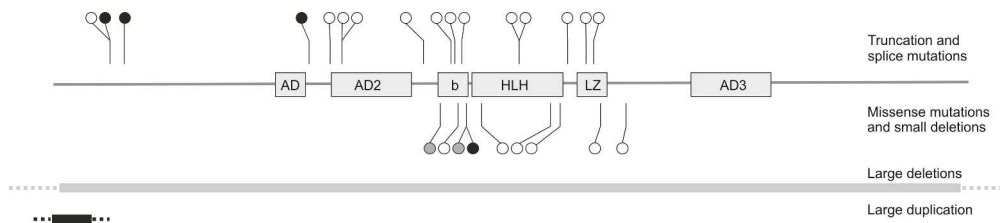


Figure 2: Survey of *MITF* mutations detected in patients with Waardenburg and Tietz syndrome. Black circles and bars = mutations and copy number variations detected in this study; white circles and grey bars = previously published mutations and deletions; grey circles = *MITF* mutations associated with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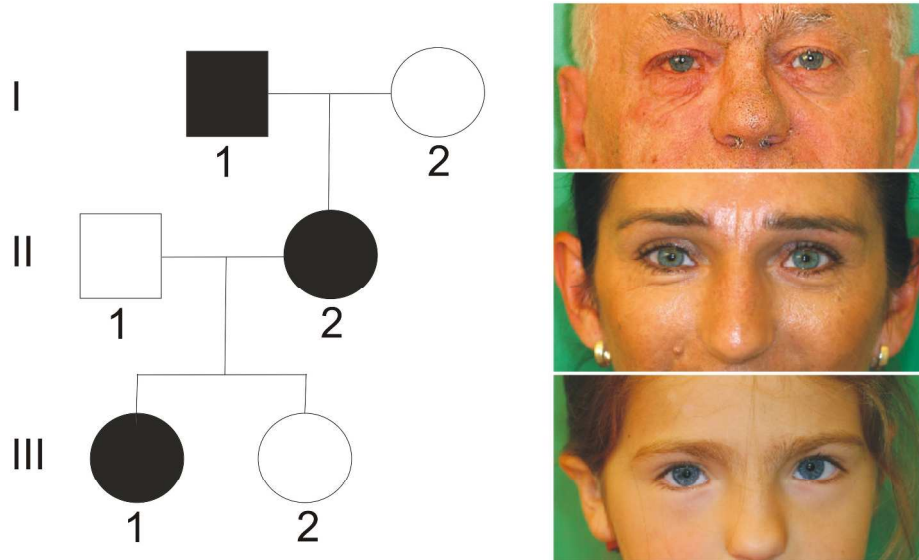


Figure 3: Pedigree of index patient 2 with *PAX3* mutation. The girl presented with clinical features of Waardenburg syndrome type 1 (see table 1): dystopia canthorum, high nasal bridge, synophrys, eyebrow flaring, skin depigmentation and bilateral hearing loss. Her mother and maternal grandfather had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

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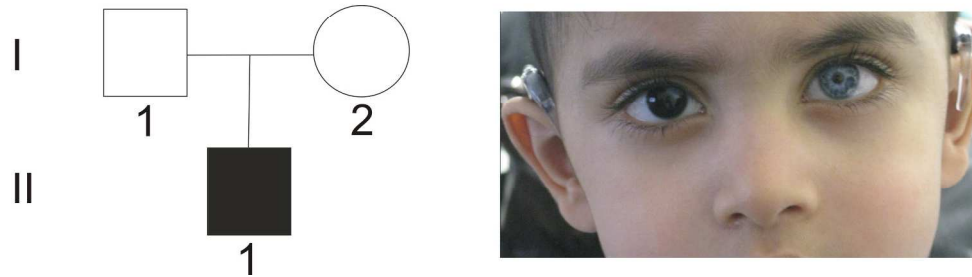


Figure 4: Sporadic case (patient 19 in table 1) of Waardenburg syndrome type 2 with *MITF* mutation. The boy presented with heterochromia irides and bilateral hearing loss, but did not show dystopia canthorum.  
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**SPECTRUM OF NOVEL MUTATIONS FOUND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

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3 associated with WS2 is SOX10. [16] In addition, mutations in *SOX10*, *EDN3* and *EDNRB*  
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5 were found in WS4.[17]  
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9 The exact description of the mutations responsible for the WS type is of significant  
10 importance in genetic counseling of WS patients and their families. Whole-gene sequencing  
11 enables the discovery of point mutations and small alterations in the gene, but cannot  
12 reliably detect whole-exon or whole-gene copy number changes, which have been reported  
13 for many genes resulting in a specific genetic disorder. In recent years, MLPA (multiplex  
14 ligation-dependent probe amplification) has become a widespread method in molecular  
15 genetic diagnostics to detect or exclude copy number changes in targeted genes.  
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25 We applied both whole-gene sequencing and MLPA analysis for the detection of point  
26 mutations and copy number variations in the genes *PAX3* and *MITF*, and describe 19  
27 mutations, of which 15 have not been previously reported for patients with features of WS.  
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## 35 MATERIALS AND METHODS

### 36 Patients

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38 19 Caucasian patients with the clinical phenotype of WS were included in our study. Clinical  
39 data were collected for patients and their affected family members.  
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### 45 Molecular genetic analysis

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

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27 Molecular genetic analysis of the genes *PAX3* and *MITF* for 19 unrelated index patients with  
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29 the clinical phenotype of WS revealed 15 novel and four previously published heterozygous  
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31 mutations (table 1). 14 mutations occurred in the *PAX3* gene: one small insertion, two  
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33 missense mutations, four nonsense mutations, two small deletions, one splice-site mutation,  
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35 and four large deletions comprising at least one exon. Five mutations were detected in the  
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37 *MITF* gene: one missense mutation, one nonsense mutation, one large deletion and one  
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39 large duplication. In addition, the combination of a *MITF* missense mutation with a small  
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41 deletion was found in family 11 (table 1). Both mutations were proven to be located on the  
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43 same chromosome by segregation analysis. The paternal grandfather, a brother, two sisters,  
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45 a niece and a nephew with Waardenburg syndrome were all proven to be carrier of these  
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47 two mutations.  
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50 Of specific interest, six of the 19 mutations (32 %) were not detectable by direct sequencing.  
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52 These were five large deletions and one duplication that could only be detected by MLPA  
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54 analysis. In ten of 19 index patients, parents were available for genetic analysis. Seven  
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56 mutations were familial (i.e. shown to be inherited from one parent that presented signs and  
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58 symptoms of WS), whereas three mutations (one mutation in *PAX3* and two in *MITF*)  
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3 occurred *de novo*. 15 of 19 mutations detected in *PAX3* and *MITF* represented novel  
4 mutations. The positions of these mutations and all previously published mutations are  
5 summarized in figure 1 (*PAX3* gene) and figure 2 (*MITF* gene).  
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11 All index patients with *PAX3* mutations had the typical phenotype of Waardenburg syndrome  
12 type 1 including dystopia canthorum and at least one of the following criteria: heterochromia  
13 iridis, hearing loss, and additional features such as white forelock, craniofacial dysmorphism  
14 including high nasal bridge, synophrys, eyebrow flaring, or anomalies of skin pigmentation  
15 (table 1, figure 3). Detailed clinical information was available for 13 of 14 patients with *PAX3*  
16 mutations and 11 of these presented hearing loss. No differences in phenotype were noted  
17 between patients with *PAX3* point mutations and *PAX3* deletions.  
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27 Patients with *MITF* mutations had the clinical phenotype of Waardenburg syndrome type 2,  
28 i.e. heterochromia irides and/or hearing loss without dystopia canthorum (figure 4). Patient  
29 13, who was found to carry a *de novo* missense mutation in *MITF*, did not present with  
30 heterochromia irides, but with blue eyes and hypoplasia of iris stroma.  
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## 40 DISCUSSION

41 Molecular genetic analyses of the *PAX3* and *MITF* gene revealed 15 novel and four  
42 previously known mutations in patients with the clinical phenotype of Waardenburg  
43 syndrome. Of these, 14 mutations occurred in *PAX3*, and 5 mutations were found in *MITF*.  
44 The spectrum of mutations includes nonsense, missense and splice site mutations,  
45 insertions, as well as deletions and duplications.  
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54 Most of the novel mutations in *PAX3* are localized in exons 2 to 6 and hence influence  
55 functionally relevant domains (table 1, figure 1). This predominant localization in exons 2 to 6  
56 has previously been described.[18] Twelve of the 14 *PAX3* mutations are truncating  
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3 mutations including large deletions. Deletions comprised two whole gene deletions and two  
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5 intragenic deletions of exon 7 and exons 8 - 9, respectively. The two missense mutations are  
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7 localized in the N-terminal part of the paired domain, which mediates intensive DNA contact.  
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9 There is no correlation between the mutation type (missense, nonsense, deletion) and the  
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11 severity of the phenotype. Therefore, loss of protein function leading to haploinsufficiency  
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13 seems to be the disease causing mechanism for WS1. All patients with *PAX3* mutations had  
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15 phenotypes of WS1 (table 1, figure 3).  
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19 Five patients had mutations in the *MITF* gene (table 1, figure 2). Four of these represented  
20  
21 novel mutations. Detailed clinical information was available in four patients. Three patients  
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23 had features corresponding to the WS2 phenotype (figure 4). Interestingly, one of these  
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25 (patient 11) was the first WS2 patient who was found to carry two *MITF* mutations (missense  
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27 mutation and small deletion) within the same gene copy. Since both mutations are novel, it  
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29 remains to be clarified whether one or the combination of both mutations leads to the WS2  
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31 phenotype.  
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35 One patient with a *de novo* missense mutation c.650G>T (p.Arg217Ile) in the *MITF* gene did  
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37 not present with heterochromia irides, but with bilateral blue irides and hypoplasia of iris  
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39 stroma (patient 13 in table 1). Interestingly, *MITF* mutations affecting amino acid position 217,  
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41 which is located in the basic domain of MITF, were also described in patients with Tietz  
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43 syndrome (TS, MIM #103500). Compared to WS2, Tietz syndrome is characterized by a  
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45 more severe phenotype with generalized hypopigmentation and complete hearing loss.[19]  
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47 Instead of heterochromia irides, which is typical for Waardenburg syndrome type 1 and 2,  
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49 patients with Tietz syndrome present with bilateral blue irides. To date, only three patients  
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51 with Tietz syndrome and mutation in the *MITF* gene have been described.[20-22] Two of  
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53 these with typical Tietz syndrome had a mutation altering amino acid position 217 (3-bp in-  
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55 frame deletion Arg217). Compared to these patients, our patient with a *de novo* missense  
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57 mutation at the same amino acid position has a less severe phenotype with bilateral hearing  
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3 loss, a white forelock and blue irides with hypoplasia of iris stroma. These clinical features  
4 are part of WS2, while blue eyes with hypoplastic iris stroma may correspond to Tietz  
5 syndrome. Apparently, a missense mutation at amino acid position 217 leads to milder or  
6 intermediate phenotype than a 3-bp in-frame deletion at the same position. Therefore, both  
7 WS2 and Tietz syndrome most probably correspond to a common clinical spectrum that is  
8 influenced by mutation type and position.  
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17 In summary, the molecular genetic analyses of the *PAX3* and *MITF* genes are important  
18 diagnostic steps to explain the molecular cause of clinical features of Waardenburg  
19 syndrome and facilitate genetic counseling of affected patients and their families. For six of  
20 19 patients with suspected WS and previously genetically unconfirmed diagnosis, we found  
21 either large deletions (four patients with deletions in *PAX3* and one patient with deletion in  
22 *MITF*) or duplications (one patient, *MITF* gene). This indicates that deletion/duplication  
23 screening is indispensable for a successful molecular genetic diagnostics of WS. As a  
24 genetic diagnostic strategy it is thus recommended to perform both sequence analysis and  
25 copy number analysis of the *PAX3* and *MITF* genes in all patients with clinical features of  
26 Waardenburg syndrome.  
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Wildhardt et al. Spectrum of novel mutations found in Waardenburg syndrome type 1 and type 2: Implications for molecular genetic diagnostics

index patient	age	gender	gene	mutation description				transmission mode	dystopia canthorum	heterochromia 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.

Wildhardt et al. Spectrum of novel mutations found in Waardenburg syndrome type 1 and type 2: Implications for molecular genetic diagnostics

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

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**Word count:** ~~1646~~-1727 (Abstract: ~~298~~313 words)

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Wildhardt et al. Spectrum of novel mutations found in Waardenburg syndrome type 1 and type 2: Implications for molecular genetic diagnostics

## 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~~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.

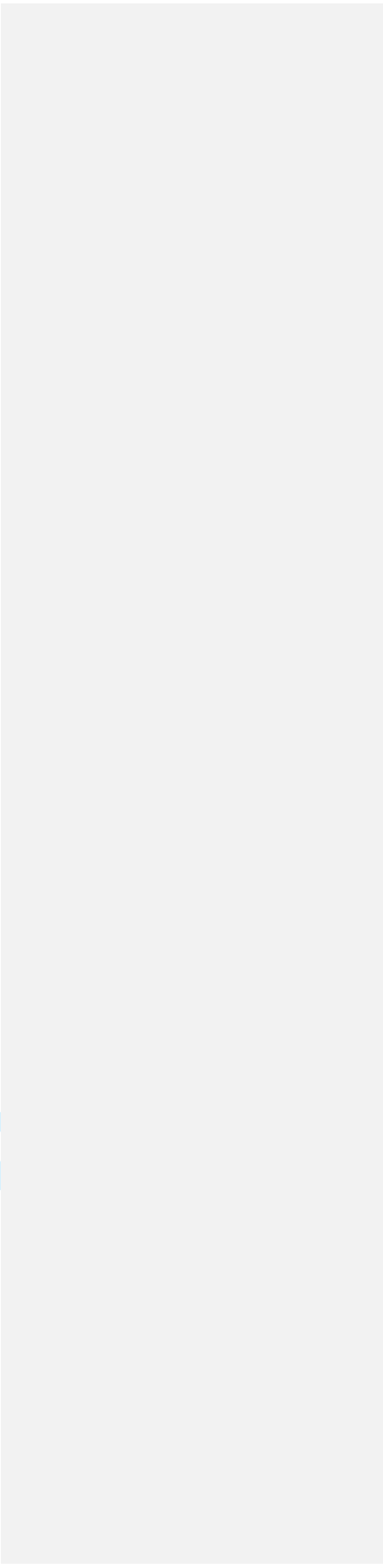
**Conclusion:** Based on these findings, we conclude that sequencing and copy number analysis of both *PAX3* and *MITF* have to be recommended in the routine molecular diagnostic setting for WS patients with and without dystopia canthorum. [Furthermore, our genotype-/phenotype analyses indicate that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



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## 7 INTRODUCTION

8 Waardenburg syndrome (WS) is an auditory-pigmentary syndrome that occurs with a  
9 frequency of 1 in 40,000.[1] WS has been classified into four main phenotypes: Type 1  
10 (WS1) is characterized by congenital sensorineural hearing loss, heterochromia irides,  
11 partial hypopigmentation of the hair including premature graying, and lateral displacement of  
12 the inner ocular canthi (dystopia canthorum). Type 2 (WS2) is distinguished from WS1 by  
13 the absence of dystopia canthorum. WS3 or Klein-Waardenburg syndrome is similar to WS1,  
14 but includes upper limb abnormalities. WS4 or Waardenburg-Shah syndrome has features of  
15 Hirschsprung disease in addition to WS2.  
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24 Waardenburg syndrome is genetically heterogeneous. Point mutations in the *PAX3* gene are  
25 described to be the most frequent cause of WS1 and WS3.[2-4] *PAX3* is a member of the  
26 mammalian *PAX* gene family and encodes a DNA binding transcription factor expressed in  
27 neural crest cells.[5] It plays an important role for the migration and differentiation of  
28 melanocytes, which originate from the embryonic neural crest. The *PAX3* gene is structurally  
29 defined by the presence of a highly conserved 128 amino acid DNA-binding domain, known  
30 as the paired domain, and a second DNA-binding domain, the homeodomain.[5, 6] *PAX3*  
31 mutations associated with WS1 include substitutions of conserved amino acids in the paired  
32 domain or the homeodomain of the protein, splice-site mutations, nonsense mutations, and  
33 insertions or deletions leading to frame shifts.[7, 8] All previously published *PAX3* mutations  
34 in WS1 are heterozygous, whereas both heterozygous and homozygous *PAX3* mutations  
35 have been described in the allelic disease WS3.[8-13]  
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47 Heterozygous mutations in the *MITF* gene are one of the major category of molecular  
48 causes of WS 2.[14, 15] The *MITF* gene encodes a transcription factor with a basic helix-  
49 loop-helix leucine zipper motif. Proteins with this kind of motif form homo- or heterodimers by  
50 their HLH-zip regions and bind DNA with their basic domain. Another gene that is in case of  
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6 [mutations associated with ~~additional candidate gene for~~ WS2 is SOX10 and SNAI2](#). [16];

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8 In addition, mutations in *SOX10*, *EDN3* and *EDNRB* were found in WS4. [17]

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11 The exact description of the mutations responsible for the WS type is of significant  
12 importance in genetic counseling of WS patients and their families. Whole-gene sequencing  
13 enables the discovery of point mutations and small alterations in the gene, but cannot  
14 reliably detect whole-exon or whole-gene copy number changes, which have been reported  
15 for many genes resulting in a specific genetic disorder. In recent years, MLPA (multiplex  
16 ligation-dependent probe amplification) has become a widespread method in molecular  
17 genetic diagnostics to detect or exclude copy number changes in targeted genes.  
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21 We applied both whole-gene sequencing and MLPA analysis for the detection of point  
22 mutations and copy number variations in the genes *PAX3* and *MITF*, and describe 19  
23 mutations, of which 156 have not been previously reported for patients with features of WS.  
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## 26 27 28 29 30 31 32 33 34 35 **MATERIALS AND METHODS**

### 36 37 **Patients**

38 19 eCaucasian patients with the clinical phenotype of WS were included in our study. Clinical  
39 data were collected for patients and their affected family members.  
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### 43 44 **Molecular genetic analysis**

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

## 27 28 RESULTS

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30 Molecular genetic analysis of the genes *PAX3* and *MITF* for 19 unrelated index patients with  
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32 the clinical phenotype of WS revealed 156 novel and fourthree previously published  
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34 heterozygous mutations (table 1). 14 mutations occurred in the *PAX3* gene: one small  
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36 insertion, two missense mutations, four nonsense mutations, two small deletions, one splice-  
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38 site mutation, and four large deletions comprising at least one exon. Five mutations were  
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40 detected in the *MITF* gene: one missense mutation, one nonsense mutation, one large  
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42 deletion and one large duplication. In addition, the combination of a *MITF* missense mutation  
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44 with a small deletion was found in family 11 (table 1). Both mutations were proven to be  
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46 located on the same chromosome by segregation analysis. The paternal grandfather, a  
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48 brother, two sisters, a niece and a nephew with Waardenburg syndrome are were all proven  
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50 to be carriers of these two mutations.

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52 Of specific interest, six of the 19 mutations (32 %) were not detectable by direct sequencing.  
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54 These were five large deletions and one duplication that could only be detected by MLPA  
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56 analysis. In ten of 19 index patients, parents were available for genetic analysis. Seven  
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7 mutations were familial (i.e. shown to be inherited from one parent [that presented signs and](#)  
8 [symptoms of WS](#)), whereas three mutations (one mutation in *PAX3* and two in *MITF*)  
9 occurred *de novo*. ~~165~~ of 19 mutations detected in *PAX3* and *MITF* represented novel  
10 mutations. [The positions of](#) ~~†~~ these mutations and all previously published mutations are  
11 summarized in figure 1 (*PAX3* gene) and figure 2 (*MITF* gene).  
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17 All index patients with *PAX3* mutations had the typical phenotype of Waardenburg syndrome  
18 type 1 including dystopia canthorum and at least one of the following criteria: heterochromia  
19 iridis, hearing loss, and additional features such as white forelock, craniofacial dysmorphism  
20 including high nasal bridge, synophrys, eyebrow flaring, or anomalies of skin pigmentation  
21 (table 1, figure 3). [Detailed c](#)linical information was available for ~~103~~ of ~~142~~ patients with  
22 *PAX3* mutations and ~~11a~~ of these presented hearing [loss](#). No differences in phenotype  
23 were noted between patients with *PAX3* point mutations and *PAX3* deletions.  
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31 Patients with *MITF* mutations had the clinical phenotype of Waardenburg syndrome type 2,  
32 i.e. heterochromia irides and/or hearing loss without dystopia canthorum (figure 4: ~~clinical~~  
33 ~~data not available for one patient~~). Patient 13, who was found to carry a *de novo* missense  
34 mutation in *MITF*, did not present with heterochromia irides, but with blue eyes and  
35 hypoplasia of iris stroma.  
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#### 44 DISCUSSION

45 Molecular genetic analyses of the *PAX3* and *MITF* gene revealed ~~156~~ novel and ~~threefour~~  
46 previously known mutations in patients with the clinical phenotype of Waardenburg  
47 syndrome. Of these, 14 mutations occurred in *PAX3*, and 5 mutations were found in *MITF*.  
48 The spectrum of mutations includes nonsense, missense and splice site mutations,  
49 insertions, as well as deletions and duplications.  
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7 Most of the novel mutations in *PAX3* are localized in exons 2 to 6 and hence influence  
8 functionally relevant domains (table 1, figure 1). This predominant localization in exons 2 to 6  
9 has previously been described.[18] Twelve of the 14 *PAX3* mutations are truncating  
10 mutations including large deletions. Deletions comprised two whole gene deletions and two  
11 intragenic deletions of exon 7 and exons 8-9, respectively. The two missense mutations are  
12 localized in the N-terminal part of the paired domain, which mediates intensive DNA contact.  
13 There is no correlation between the mutation type (missense, nonsense, deletion) and the  
14 severity of the phenotype. ~~Therefore, loss of protein function leading to haploinsufficiency  
15 seems to be the disease causing mechanism for WS1. Therefore, loss of protein function  
16 seems to be the disease-causing mechanism for WS1.~~ All patients with *PAX3* mutations had  
17 phenotypes of WS1 (table 1, figure 3).  
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27 Five patients had mutations in the *MITF* gene (table 1, figure 2). Four of these represented  
28 novel mutations. ~~Detailed c~~linical information was available in four patients. Three patients  
29 had features corresponding to the WS2 phenotype (figure 4). Interestingly, one of these  
30 (patient 11) was the first WS2 patient who was found to carry two *MITF* mutations (missense  
31 mutation and small deletion) within the same gene copy. Since both mutations are novel, it  
32 remains to be clarified whether one or the combination of both mutations leads to the WS2  
33 phenotype.  
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42 One patient with a *de novo* missense mutation c.650G>T (p.Arg217Ile) in the *MITF* gene did  
43 not present with heterochromia irides, but with bilateral blue irides and hypoplasia of iris  
44 stroma (patient 13 in table 1). Interestingly, *MITF* mutations affecting amino acid position 217,  
45 which is located in the basic domain of MITF, were also described in patients with Tietz  
46 syndrome (TS, MIM #103500). Compared to WS2, Tietz syndrome is characterized by a  
47 more severe phenotype with generalized hypopigmentation and complete hearing loss.[19]  
48 Instead of heterochromia irides, which is typical for Waardenburg syndrome type 1 and 2,  
49 patients with Tietz syndrome present with bilateral blue irides. To date, only three patients  
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6 with Tietz syndrome and mutation in the *MITF* gene have been described.[20-22] Two of  
7 these with typical Tietz syndrome had a mutation altering amino acid position 217 (3-bp in-  
8 frame deletion Arg217). Compared to these patients, our patient with a *de novo* missense  
9 mutation at the same amino acid position has a less severe phenotype with bilateral hearing  
10 loss, a white forelock and blue irides with hypoplasia of iris stroma. These clinical features  
11 are part of WS2, while blue eyes with hypoplastic iris stroma may correspond to Tietz  
12 syndrome. Apparently, a missense mutation at amino acid position 217 leads to milder or  
13 intermediate phenotype than a 3-bp in-frame deletion at the same position. Therefore, both  
14 WS2 and ~~TS~~ Tietz syndrome most probably correspond to a common clinical spectrum that  
15 is influenced by mutation type and position.  
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26 In summary, the molecular genetic analyses of the *PAX3* and *MITF* genes are important  
27 diagnostic steps to explain the molecular cause of clinical features of Waardenburg  
28 syndrome and facilitate genetic counseling of affected patients and their families. For six of  
29 19 patients with suspected WS and previously genetically unconfirmed diagnosis, we found  
30 either large deletions (four patients with deletions in *PAX3* and one patient with deletion in  
31 *MITF*) or duplications (one patient, *MITF* gene). This indicates that deletion/duplication  
32 screening is indispensable for a successful molecular genetic diagnostics of WS. As a  
33 genetic diagnostic strategy it is thus recommended to perform both sequence analysis and  
34 copy number analysis of the *PAX3* and *MITF* genes in all patients with clinical features of  
35 Waardenburg syndrome.  
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Wildhardt et al. Spectrum of novel mutations found in Waardenburg syndrome type 1 and type 2: Implications for molecular genetic diagnostics

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+6delT]	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.

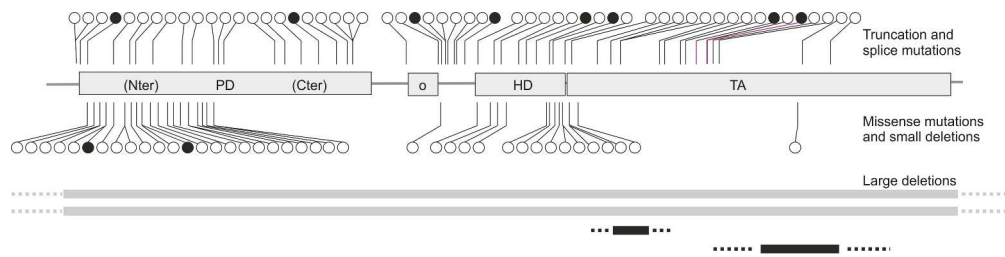


Figure 1: Survey of *PAX3* mutations detected in patients with Waardenburg syndrome. Black circles = mutations detected in this study; black bars = copy number variations detected in this study; white circles and grey bars = previously published mutations and deletions. PD = paired domain; o = octapeptide;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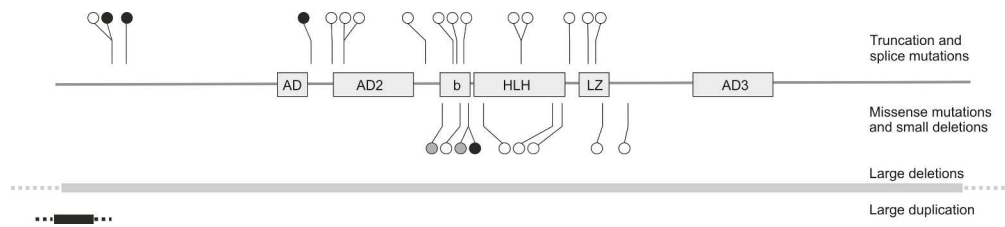


Figure 2: Survey of *MITF* mutations detected in patients with Waardenburg and Tietz syndrome. Black circles and bars = mutations and copy number variations detected in this study; white circles and grey bars = previously published mutations and deletions; grey circles = *MITF* mutations associated with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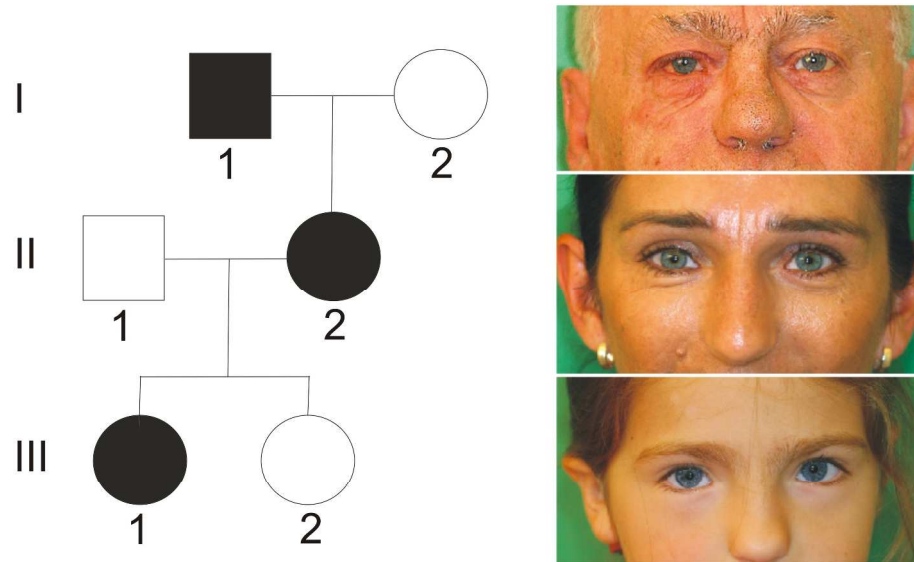


Figure 3: Pedigree of index patient 2 with *PAX3* mutation. The girl presented with clinical features of Waardenburg syndrome type 1 (see table 1): dystopia canthorum, high nasal bridge, synophrys, eyebrow flaring, skin depigmentation and bilateral hearing loss. Her mother and maternal grandfather had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

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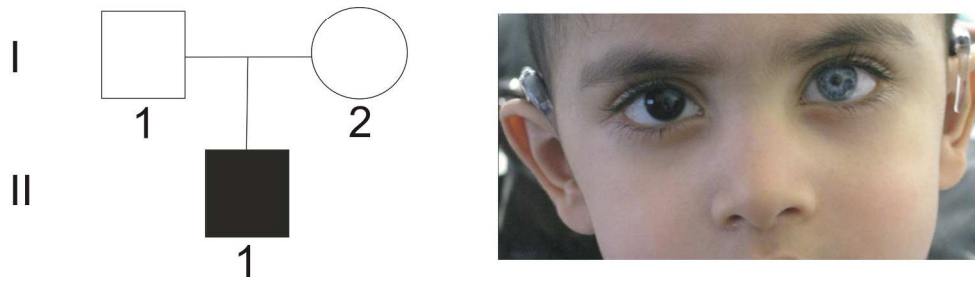


Figure 4: Sporadic case (patient 19 in table 1) of Waardenburg syndrome type 2 with *MITF* mutation. The boy presented with heterochromia irides and bilateral hearing loss, but did not show dystopia canthorum.  
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**SPECTRUM OF NOVEL MUTATIONS FOUND IN  
WAARDENBURG SYNDROME TYPE 1 AND TYPE 2:  
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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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**Word count:** 1727 (Abstract: 313 words)

## 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

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

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3 associated with WS2 is *SOX10*. [16] In addition, mutations in *SOX10*, *EDN3* and *EDNRB*  
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5 were found in WS4.[17]  
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9 The exact description of the mutations responsible for the WS type is of significant  
10 importance in genetic counseling of WS patients and their families. Whole-gene sequencing  
11 enables the discovery of point mutations and small alterations in the gene, but cannot  
12 reliably detect whole-exon or whole-gene copy number changes, which have been reported  
13 for many genes resulting in a specific genetic disorder. In recent years, MLPA (multiplex  
14 ligation-dependent probe amplification) has become a widespread method in molecular  
15 genetic diagnostics to detect or exclude copy number changes in targeted genes.  
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25 We applied both whole-gene sequencing and MLPA analysis for the detection of point  
26 mutations and copy number variations in the genes *PAX3* and *MITF*, and describe 19  
27 mutations, of which 15 have not been previously reported for patients with features of WS.  
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## 35 MATERIALS AND METHODS

### 36 Patients

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

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

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35 Molecular genetic analysis of the genes *PAX3* and *MITF* for 19 unrelated index patients with  
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37 the clinical phenotype of WS revealed 15 novel and four previously published heterozygous  
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39 mutations (table 1). 14 mutations occurred in the *PAX3* gene: one small insertion, two  
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41 missense mutations, four nonsense mutations, two small deletions, one splice-site mutation,  
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43 and four large deletions comprising at least one exon. Five mutations were detected in the  
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45 *MITF* gene: one missense mutation, one nonsense mutation, one large deletion and one  
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47 large duplication. In addition, the combination of a *MITF* missense mutation with a small  
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49 deletion was found in family 11 (table 1). Both mutations were proven to be located on the  
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51 same chromosome by segregation analysis. The paternal grandfather, a brother, two sisters,  
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53 a niece and a nephew with Waardenburg syndrome were all proven to be carrier of these  
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55 two mutations.  
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3 Of specific interest, six of the 19 mutations (32 %) were not detectable by direct sequencing.  
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5 These were five large deletions and one duplication that could only be detected by MLPA  
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7 analysis. In ten of 19 index patients, parents were available for genetic analysis. Seven  
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9 mutations were familial (i.e. shown to be inherited from one parent that presented signs and  
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11 symptoms of WS), whereas three mutations (one mutation in *PAX3* and two in *MITF*)  
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13 occurred *de novo*. 15 of 19 mutations detected in *PAX3* and *MITF* represented novel  
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15 mutations. The positions of these mutations and all previously published mutations are  
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17 summarized in figure 1 (*PAX3* gene) and figure 2 (*MITF* gene).  
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21 All index patients with *PAX3* mutations had the typical phenotype of Waardenburg syndrome  
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23 type 1 including dystopia canthorum and at least one of the following criteria: heterochromia  
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25 iridis, hearing loss, and additional features such as white forelock, craniofacial dysmorphism  
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27 including high nasal bridge, synophrys, eyebrow flaring, or anomalies of skin pigmentation  
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29 (table 1, figure 3). Detailed clinical information was available for 13 of 14 patients with *PAX3*  
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31 mutations and 11 of these presented hearing loss. No differences in phenotype were noted  
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33 between patients with *PAX3* point mutations and *PAX3* deletions.  
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37 Patients with *MITF* mutations had the clinical phenotype of Waardenburg syndrome type 2,  
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39 i.e. heterochromia irides and/or hearing loss without dystopia canthorum (figure 4). Patient  
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41 13, who was found to carry a *de novo* missense mutation in *MITF*, did not present with  
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43 heterochromia irides, but with blue eyes and hypoplasia of iris stroma.  
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## 46 47 48 49 **DISCUSSION**

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51 Molecular genetic analyses of the *PAX3* and *MITF* gene revealed 15 novel and four  
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53 previously known mutations in patients with the clinical phenotype of Waardenburg  
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55 syndrome. Of these, 14 mutations occurred in *PAX3*, and 5 mutations were found in *MITF*.  
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3 The spectrum of mutations includes nonsense, missense and splice site mutations,  
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5 insertions, as well as deletions and duplications.  
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9 Most of the novel mutations in *PAX3* are localized in exons 2 to 6 and hence influence  
10 functionally relevant domains (table 1, figure 1). This predominant localization in exons 2 to 6  
11 has previously been described.[18] Twelve of the 14 *PAX3* mutations are truncating  
12 mutations including large deletions. Deletions comprised two whole gene deletions and two  
13 intragenic deletions of exon 7 and exons 8 - 9, respectively. The two missense mutations are  
14 localized in the N-terminal part of the paired domain, which mediates intensive DNA contact.  
15 There is no correlation between the mutation type (missense, nonsense, deletion) and the  
16 severity of the phenotype. Therefore, loss of protein function leading to haploinsufficiency  
17 seems to be the disease causing mechanism for WS1. All patients with *PAX3* mutations had  
18 phenotypes of WS1 (table 1, figure 3).  
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31 Five patients had mutations in the *MITF* gene (table 1, figure 2). Four of these represented  
32 novel mutations. Detailed clinical information was available in four patients. Three patients  
33 had features corresponding to the WS2 phenotype (figure 4). Interestingly, one of these  
34 (patient 11) was the first WS2 patient who was found to carry two *MITF* mutations (missense  
35 mutation and small deletion) within the same gene copy. Since both mutations are novel, it  
36 remains to be clarified whether one or the combination of both mutations leads to the WS2  
37 phenotype.  
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48 One patient with a *de novo* missense mutation c.650G>T (p.Arg217Ile) in the *MITF* gene did  
49 not present with heterochromia irides, but with bilateral blue irides and hypoplasia of iris  
50 stroma (patient 13 in table 1). Interestingly, *MITF* mutations affecting amino acid position 217,  
51 which is located in the basic domain of MITF, were also described in patients with Tietz  
52 syndrome (TS, MIM #103500). Compared to WS2, Tietz syndrome is characterized by a  
53 more severe phenotype with generalized hypopigmentation and complete hearing loss.[19]  
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3 Instead of heterochromia irides, which is typical for Waardenburg syndrome type 1 and 2,  
4 patients with Tietz syndrome present with bilateral blue irides. To date, only three patients  
5 with Tietz syndrome and mutation in the *MITF* gene have been described.[20-22] Two of  
6 these with typical Tietz syndrome had a mutation altering amino acid position 217 (3-bp in-  
7 frame deletion Arg217). Compared to these patients, our patient with a *de novo* missense  
8 mutation at the same amino acid position has a less severe phenotype with bilateral hearing  
9 loss, a white forelock and blue irides with hypoplasia of iris stroma. These clinical features  
10 are part of WS2, while blue eyes with hypoplastic iris stroma may correspond to Tietz  
11 syndrome. Apparently, a missense mutation at amino acid position 217 leads to milder or  
12 intermediate phenotype than a 3-bp in-frame deletion at the same position. Therefore, both  
13 WS2 and Tietz syndrome most probably correspond to a common clinical spectrum that is  
14 influenced by mutation type and position.  
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29 In summary, the molecular genetic analyses of the *PAX3* and *MITF* genes are important  
30 diagnostic steps to explain the molecular cause of clinical features of Waardenburg  
31 syndrome and facilitate genetic counseling of affected patients and their families. For six of  
32 19 patients with suspected WS and previously genetically unconfirmed diagnosis, we found  
33 either large deletions (four patients with deletions in *PAX3* and one patient with deletion in  
34 *MITF*) or duplications (one patient, *MITF* gene). This indicates that deletion/duplication  
35 screening is indispensable for a successful molecular genetic diagnostics of WS. As a  
36 genetic diagnostic strategy it is thus recommended to perform both sequence analysis and  
37 copy number analysis of the *PAX3* and *MITF* genes in all patients with clinical features of  
38 Waardenburg syndrome.  
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**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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Wildhardt et al. Spectrum of novel mutations found in Waardenburg syndrome type 1 and type 2: Implications for molecular genetic diagnostics

index patient	age	gender	gene	mutation description				transmission mode	dystopia canthorum	heterochromia 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; 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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**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.

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## 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

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3 associated with WS2 is SOX10. [16] In addition, mutations in *SOX10*, *EDN3* and *EDNRB*  
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5 were found in WS4.[17]  
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9 The exact description of the mutations responsible for the WS type is of significant  
10 importance in genetic counseling of WS patients and their families. Whole-gene sequencing  
11 enables the discovery of point mutations and small alterations in the gene, but cannot  
12 reliably detect whole-exon or whole-gene copy number changes, which have been reported  
13 for many genes resulting in a specific genetic disorder. In recent years, MLPA (multiplex  
14 ligation-dependent probe amplification) has become a widespread method in molecular  
15 genetic diagnostics to detect or exclude copy number changes in targeted genes.  
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25 We applied both whole-gene sequencing and MLPA analysis for the detection of point  
26 mutations and copy number variations in the genes *PAX3* and *MITF*, and describe 19  
27 mutations, of which 15 have not been previously reported for patients with features of WS.  
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## 35 MATERIALS AND METHODS

### 36 Patients

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39 ~~19 Caucasian patients with the clinical phenotype of WS were included in our study. Clinical~~  
40 ~~data were collected for patients and their affected family members. For 119 patients~~  
41 ~~molecular genetic analyses were requested due to the clinical suspicion of Waardenburg~~  
42 ~~syndrome. From this cohort, for 15 Caucasian patients we have identified previously not~~  
43 ~~described mutations in *PAX3* or *MITF*. Furthermore for four Caucasian patients already~~  
44 ~~published alterations were found in one of these genes. Subsequently more detailed clinical~~  
45 ~~data were collected from these individuals as well as from their clinically affected family~~  
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### 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,

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

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7 Of specific interest, six of the 19 mutations (32 %) were not detectable by direct sequencing.  
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9 These were five large deletions and one duplication that could only be detected by MLPA  
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11 analysis. In ten of 19 index patients, parents were available for genetic analysis. Seven  
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13 mutations were familial (i.e. shown to be inherited from one parent that presented signs and  
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15 symptoms of WS), whereas three mutations (one mutation in *PAX3* and two in *MITF*)  
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17 occurred *de novo*. 15 of 19 mutations detected in *PAX3* and *MITF* represented novel  
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19 mutations. The positions of these mutations and all previously published mutations are  
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21 summarized in figure 1 (*PAX3* gene) and figure 2 (*MITF* gene).  
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25 All index patients with *PAX3* mutations had the typical phenotype of Waardenburg syndrome  
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27 type 1 including dystopia canthorum and at least one of the following criteria: heterochromia  
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29 iridis, hearing loss, and additional features such as white forelock, craniofacial dysmorphism  
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31 including high nasal bridge, synophrys, eyebrow flaring, or anomalies of skin pigmentation  
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33 (table 1, figure 3). Detailed clinical information was available for 13 of 14 patients with *PAX3*  
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35 mutations and 11 of these presented hearing loss. No differences in phenotype were noted  
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37 between patients with *PAX3* point mutations and *PAX3* deletions.  
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41 Patients with *MITF* mutations had the clinical phenotype of Waardenburg syndrome type 2,  
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43 i.e. heterochromia irides and/or hearing loss without dystopia canthorum (figure 4). Patient  
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45 13, who was found to carry a *de novo* missense mutation in *MITF*, did not present with  
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47 heterochromia irides, but with blue eyes and hypoplasia of iris stroma.  
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## 50 51 52 53 **DISCUSSION**

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55 Molecular genetic analyses of the *PAX3* and *MITF* gene revealed 15 novel and four  
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57 previously known mutations in patients with the clinical phenotype of Waardenburg  
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3 syndrome. Of these, 14 mutations occurred in *PAX3*, and 5 mutations were found in *MITF*.  
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5 The spectrum of mutations includes nonsense, missense and splice site mutations,  
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7 insertions, as well as deletions and duplications.  
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11 Most of the novel mutations in *PAX3* are localized in exons 2 to 6 and hence influence  
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13 functionally relevant domains (table 1, figure 1). This predominant localization in exons 2 to 6  
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15 has previously been described.[18] Twelve of the 14 *PAX3* mutations are truncating  
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17 mutations including large deletions. Deletions comprised two whole gene deletions and two  
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19 intragenic deletions of exon 7 and exons 8 - 9, respectively. The two missense mutations are  
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21 localized in the N-terminal part of the paired domain, which mediates intensive DNA contact.  
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23 There is no correlation between the mutation type (missense, nonsense, deletion) and the  
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25 severity of the phenotype. Therefore, loss of protein function leading to haploinsufficiency  
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27 seems to be the disease causing mechanism for WS1. All patients with *PAX3* mutations had  
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29 phenotypes of WS1 (table 1, figure 3).  
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33 Five patients had mutations in the *MITF* gene (table 1, figure 2). Four of these represented  
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35 novel mutations. Detailed clinical information was available in four patients. Three patients  
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37 had features corresponding to the WS2 phenotype (figure 4). Interestingly, one of these  
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39 (patient 11) was the first WS2 patient who was found to carry two *MITF* mutations (missense  
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41 mutation and small deletion) within the same gene copy. Since both mutations are novel, it  
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43 remains to be clarified whether one or the combination of both mutations leads to the WS2  
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45 phenotype.  
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49 One patient with a *de novo* missense mutation c.650G>T (p.Arg217Ile) in the *MITF* gene did  
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51 not present with heterochromia irides, but with bilateral blue irides and hypoplasia of iris  
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53 stroma (patient 13 in table 1). Interestingly, *MITF* mutations affecting amino acid position 217,  
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55 which is located in the basic domain of MITF, were also described in patients with Tietz  
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57 syndrome (TS, MIM #103500). Compared to WS2, Tietz syndrome is characterized by a  
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3 more severe phenotype with generalized hypopigmentation and complete hearing loss.[19]  
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5 Instead of heterochromia irides, which is typical for Waardenburg syndrome type 1 and 2,  
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7 patients with Tietz syndrome present with bilateral blue irides. To date, only three patients  
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9 with Tietz syndrome and mutation in the *MITF* gene have been described.[20-22] Two of  
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11 these with typical Tietz syndrome had a mutation altering amino acid position 217 (3-bp in-  
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13 frame deletion Arg217). Compared to these patients, our patient with a *de novo* missense  
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15 mutation at the same amino acid position has a less severe phenotype with bilateral hearing  
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17 loss, a white forelock and blue irides with hypoplasia of iris stroma. These clinical features  
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19 are part of WS2, while blue eyes with hypoplastic iris stroma may correspond to Tietz  
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21 syndrome. Apparently, a missense mutation at amino acid position 217 leads to milder or  
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23 intermediate phenotype than a 3-bp in-frame deletion at the same position. Therefore, both  
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25 WS2 and Tietz syndrome most probably correspond to a common clinical spectrum that is  
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27 influenced by mutation type and position.  
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31 In summary, the molecular genetic analyses of the *PAX3* and *MITF* genes are important  
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33 diagnostic steps to explain the molecular cause of clinical features of Waardenburg  
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35 syndrome and facilitate genetic counseling of affected patients and their families. For six of  
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37 19 patients with suspected WS and previously genetically unconfirmed diagnosis, we found  
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39 either large deletions (four patients with deletions in *PAX3* and one patient with deletion in  
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41 *MITF*) or duplications (one patient, *MITF* gene). This indicates that deletion/duplication  
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43 screening is indispensable for a successful molecular genetic diagnostics of WS. As a  
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45 genetic diagnostic strategy it is thus recommended to perform both sequence analysis and  
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47 copy number analysis of the *PAX3* and *MITF* genes in all patients with clinical features of  
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49 Waardenburg syndrome.  
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Wildhardt et al. Spectrum of novel mutations found in Waardenburg syndrome type 1 and type 2: Implications for molecular genetic diagnostics

index patient	age	gender	gene	mutation description				transmission mode	dystopia canthorum	heterochromia 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; n.a. = not available). "De novo" = under consideration of provided information concerning kinship, no paternity testing was performed.

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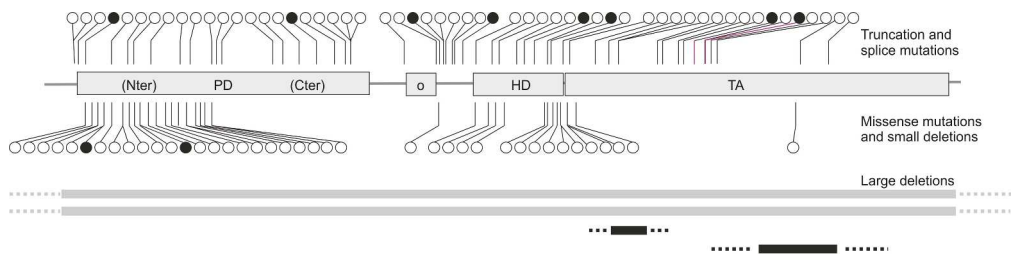


Figure 1: Survey of *PAX3* mutations detected in patients with Waardenburg syndrome. Black circles = mutations detected in this study; black bars = copy number variations detected in this study; white circles and grey bars = previously published mutations and deletions. PD = paired domain; o = octapeptide;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

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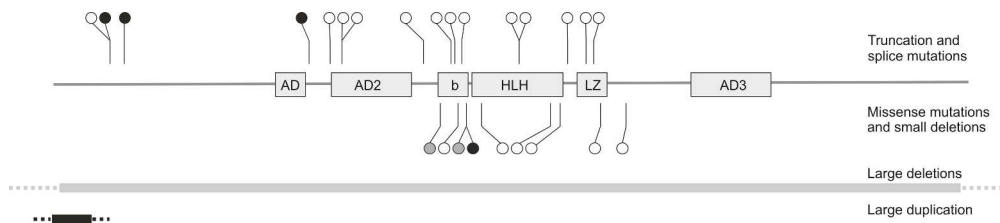


Figure 2: Survey of *MITF* mutations detected in patients with Waardenburg and Tietz syndrome. Black circles and bars = mutations and copy number variations detected in this study; white circles and grey bars = previously published mutations and deletions; grey circles = *MITF* mutations associated with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

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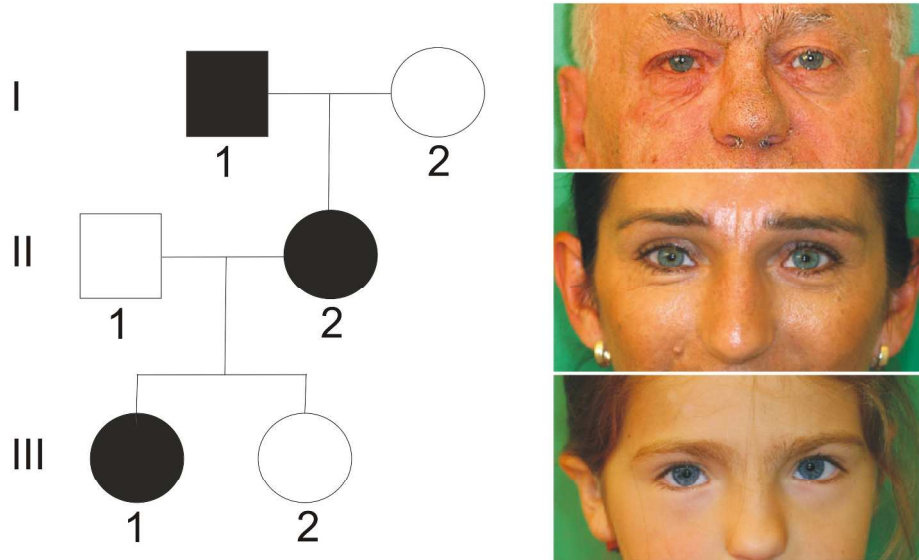


Figure 3: Pedigree of index patient 2 with *PAX3* mutation. The girl presented with clinical features of Waardenburg syndrome type 1 (see table 1): dystopia canthorum, high nasal bridge, synophrys, eyebrow flaring, skin depigmentation and bilateral hearing loss. Her mother and maternal grandfather had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

301x172mm (300 x 300 DPI)

view only

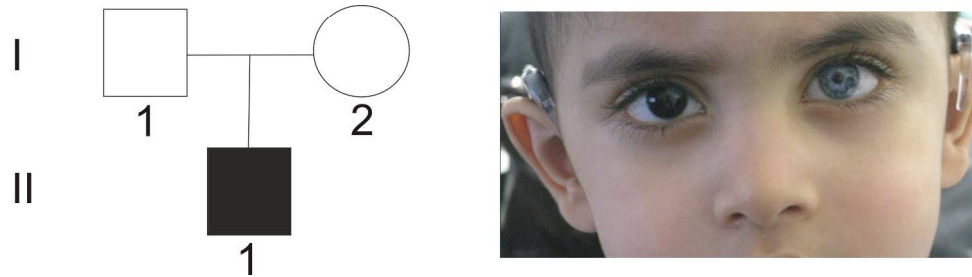


Figure 4: Sporadic case (patient 19 in table 1) of Waardenburg syndrome type 2 with *MITF* mutation. The boy presented with heterochromia irides and bilateral hearing loss, but did not show dystopia canthorum.  
226x77mm (300 x 300 DPI)

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