

# Mutational analysis and genotype-phenotype correlations in southern Indian patients with sporadic and familial aniridia

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**Purpose:** Aniridia is a rare panocular disorder characterized by iris hypoplasia and other associated eye anomalies. Heterozygous null mutations in *paired box gene 6* (*PAX6*) are the major cause of the classic aniridia phenotype. This study aims to detect the mutational spectrum of *PAX6* and associated phenotypes in southern Indian patients with sporadic and familial aniridia.

**Methods:** Genomic DNA was isolated from peripheral blood from all participants. The coding regions and flanking intronic sequences of *PAX6* were screened with Sanger sequencing in 30 probands with aniridia. The identified variations were further evaluated in available family members and 150 healthy controls. The pathogenic potential of the mutations were assessed using bioinformatics tools.

**Results:** Thirteen different mutations were detected in eight sporadic and five familial cases. Eleven novel mutations, including five insertions (c.7\_10dupAACA, c.567dupC, c.704dupC, c.868dupA and c.753\_754insTA), two deletions (c.242delC and c.249delT), and four splicing variants (c.10+1G>A, c.141G>A, c.141+4A>G and c.764A>G) were identified in this study. Clinical findings of the patients revealed phenotypic heterogeneity with the same or different mutations.

**Conclusions:** This study reported 11 novel mutations and thus expanded the spectrum of *PAX6* mutations. Interestingly, all mutations reported in this study were truncations, which confirms the hypothesis that haploinsufficiency of *PAX6* causes the aniridia phenotype. Our observations revealed inter- and intrafamilial phenotypic variability with *PAX6* mutations. The common ocular findings associated with *PAX6* mutations were iris hypoplasia, nystagmus, and foveal hypoplasia reported in almost all cases, with cataract, glaucoma, and keratopathy reported in approximately 50% of the patients.

Aniridia (OMIM 106210) is a congenital panocular disorder characterized by complete or partial absence of the iris. In most cases, aniridia is typically accompanied by foveal hypoplasia with impaired visual acuity and nystagmus [1,2]. Other sight-threatening complications, which may or may not be present, include corneal abnormalities, cataract, lens subluxation, glaucoma, strabismus, and optic nerve hypoplasia [2]. Heterozygous mutations in *paired box gene 6* (*PAX6*, OMIM 607108) are primarily responsible for aniridia [3-8]. About two-thirds of aniridia cases are familial, in which they are transmitted as an autosomal dominant trait with complete penetrance and variable expressivity; the remainder are sporadic and result from de novo mutations [1,2]. The prevalence of aniridia in the general population is around 1 in 40,000–100,000 with no known predilection regarding race or gender [2]. Most of the aniridia cases are isolated but some can occur as a part of Wilms tumor, aniridia, genitourinary anomalies and mental retardation (WAGR) syndrome (OMIM

194072) caused by deletion of the 11p13 region encompassing *PAX6* and the Wilms tumor gene (*WT1*) [9].

*PAX6* encodes a highly conserved transcriptional regulator that plays a crucial role in morphogenesis of the eye, central nervous system, and pancreas [1,10]. The 22 kb genomic region of human *PAX6* contains 14 exons, including an alternatively spliced exon 5a. As a result, the *PAX6* locus encodes two isoforms: a 422 amino acid *PAX6* protein and an alternatively spliced 436 amino acid *PAX6* protein [10,11]. The *PAX6* protein contains two DNA-binding domains: a bipartite paired domain (PD) at the NH<sub>2</sub> terminal, a paired-type homeodomain (HD) separated by a glycine-rich linker region (LNK), and a proline-serine-threonine rich transactivation domain (PST) at the COOH terminus [10,11]. Although most mutations in *PAX6* are responsible for aniridia, some *PAX6* mutations are associated with other ocular anomalies including microcornea, microphthalmia, ocular coloboma, foveal hypoplasia, congenital cataract, keratitis, morning glory disc anomaly, Gillespie syndrome, Peter's anomaly, and optic nerve hypoplasia [3,12-17]. Typically, heterozygous truncating mutations in *PAX6* are predominantly associated with aniridia, while non-aniridia phenotypes are mainly due to missense mutations [12]. These missense mutations

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may change the degree and specificity of DNA binding and transcriptional regulation by the PAX6 protein to a varying extent, which results in phenotypic heterogeneity [12,13]. Human PAX6 mutations and polymorphisms are archived in the PAX6 Allelic Variant Database (Leiden Open Variation Database, LOVD). Presently, about 357 unique DNA variants have been reported in the PAX6 mutation database. Most changes in the PAX6 gene are caused by mutations that introduce premature termination codons (PTCs) into the PAX6 open reading frame (ORF). The mRNAs containing PTCs are degraded by the nonsense-mediated decay (NMD) process, which results in the loss of function of one copy of PAX6 [12,18].

Although PAX6 variations have been reported in southern Indian patients with aniridia, the mutational spectrum of PAX6 in this cohort has not been studied since most investigations were conducted using a small number of cases [5-8]. In this study, we evaluated the coding regions and flanking intronic sequences of PAX6 in 30 unrelated patients clinically diagnosed with aniridia. In addition, the identified mutations and the associated clinical phenotypes of the patients were evaluated using bioinformatics tools.

## METHODS

**Subject recruitment and clinical evaluation:** This study adhered to the ARVO statement on human subjects and was approved by the institutional review board of Aravind Eye Hospital, Madurai, India. The research followed the tenets of the Declaration of Helsinki. Written informed consent was obtained either from the study participants or from parents or legal guardians in the case of minor study subjects. Thirty unrelated probands clinically diagnosed with aniridia, their available family members, and 150 ethnically matched healthy controls were recruited for this study. Clinical diagnosis of aniridia was made after a comprehensive ocular examination. Detailed examinations included corneal inspection, refraction, best-corrected visual acuity (BCVA), slit-lamp biomicroscopy, gonioscopic evaluation of anterior chamber angle, measurement of intraocular pressure (IOP) with applanation tonometry, and dilated funduscopy.

**Mutation screening of PAX6:** Peripheral blood samples (3 ml) were collected from all study participants, and genomic DNA was extracted using the modified salt precipitation method [19]. For mutation identification in 30 probands with aniridia, the PAX6 gene was screened with direct DNA sequencing. All coding exons of PAX6 (exon 4–13) were amplified from genomic DNA with PCR. Primers for all coding exons and exon-intron boundaries of PAX6 were either designed by the Primer3 program or taken from a previous study [10]. PCR,

using gradient thermocycler (ASTEC, Fukuoka, Japan), was performed in a total volume of 20 µl, containing 1X PCR buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM MgCl<sub>2</sub>; and 0.001% gelatin), 200 µM of dNTPs (Medox Biotech India Pvt. Ltd, Chennai, India), 0.5 pmol of each primer, 100 ng of genomic DNA and 1 unit of Taq DNA polymerase (Sigma, Saint Louis, MO). Thermal cycling conditions were 5 min at 95 °C, followed by 34 cycles [45 s at 95 °C, 45 s at the annealing temperature of the primers (55 °C- 63 °C) and 45 s at 72 °C] and a final extension for 7 min at 72 °C. PCR products were gel (1.2% agarose) purified using EZ-10 spin-column DNA gel extraction kit (Bio Basic Inc., East Markham Ontario, Canada). Bidirectional sequencing was performed using Big Dye Terminator ready reaction mix and analyzed on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). The sequence analysis was performed using either Chromas (version 2.33; Technelysium Pty Ltd, South Brisbane, Australia) or DNA Baser (version 4.16.0) tools and compared with the NCBI reference sequence (NG\_008679.1). Genetic variants were named according to the Human Genomic Variation Society (HGVS) recommendations, and RefSeq ID: NM\_000280.3 was used for cDNA nucleotide numbering of PAX6. Of the families in which mutations were identified, cosegregation was tested by direct sequencing on all available family members to check the presence or absence of mutations and disease penetrance. Identified mutations were further evaluated by sequencing of 150 ethnically matched normal controls.

**In silico analysis:** The possible effect of the missense mutation was predicted by Sorting Intolerant From Tolerant (SIFT) [20], Polymorphism Phenotyping (PolyPhen-2) [21], and Mutation Taster [22]. The pathogenic potential of the splice-site mutations was predicted using MaxEntScan, NNSplice, GeneSplicer, and Human Splicing Finder (HSF) tools. These in silico predictions were done using Alamut Visual version 2.4 (Interactive Biosoftware, Rouen, France). The DNA binding residues of the PAX6 HD were predicted using the BindN tool for wild-type (WT) and mutant (containing p.Gln255Arg) sequences [23].

## RESULTS

Thirty unrelated patients with aniridia were recruited in this study: 18 males and 12 females. Among the 30 probands, 19 were sporadic, and 11 were familial cases. A total of 49 members from 30 families were clinically diagnosed with the aniridia phenotype, of whom 39 patients had total aniridia (no visible iris) while ten patients presented with partial aniridia (loss of some portion of the iris). The age range of the patients included in this study was from 1 to 65 years. A range of

developmental ocular defects such as nystagmus, foveal hypoplasia, cataract, glaucoma, keratopathy, microcornea, and coloboma were also observed by clinical evaluation of the patients in addition to iris anomaly.

**PAX6 mutational spectrum:** The screening of *PAX6* revealed 13 different heterozygous mutations in eight sporadic and five familial aniridia cases (Table 1). Each mutation cosegregated with the disease phenotype in the affected family with complete penetrance. A total of 19 members in 13 families with aniridia were identified as positive for *PAX6* mutations (Table 1). The clinical findings of the affected members confirmed with *PAX6* mutations are given in Table 2. Of the 13 mutations observed, 11 were novel, and two were known mutations. All identified mutations were predicted to cause loss of function of one copy of *PAX6*. Of the 13 mutations, five were duplications and insertions (38.5%), three were deletions (23.0%), and five were single nucleotide substitutions (38.5%). None of these mutations were detected in the 150 normal controls.

**Duplication/insertion mutations:** Five novel duplication/insertion mutations (c.7\_10dupAACA, c.567dupC, c.704dupC, c.868dupA, and c.753\_754insTA) were identified in one familial and four sporadic aniridia cases (Table 1, Figure 1). All five mutations were predicted to introduce PTCs into the *PAX6* ORF, leading to NMD of the mutant mRNA and failure of translation. The mutations c.7\_10dupAACA (p.Ser4Lysfs\*53), c.567dupC (p.Ile190Hisfs\*10), c.704dupC (p.Asp236Argfs\*16), and c.868dupA (p.Ser290Lysfs\*51) created a frameshift in the ORF and introduced PTCs into exon 6, exon 8, exon 9, and exon 11, respectively, whereas the c.753\_754insTA (p.Ala252\*) mutation introduced an immediate PTC by replacing the Ala252 codon with a stop codon. DNA analysis of the family members of the proband with the c.7\_10dupAACA mutation showed an identical mutation in the proband's father while the mother was normal for this change (Figure 1A).

**Deletion mutations:** One known (c.112delC) and two novel (c.242delC and c.249delT) deletions were identified in one sporadic (AN-87) and two familial (AN-107 and AN-120) aniridia cases (Table 1, Figure 2). All three mutations were identified in the highly conserved PD of *PAX6*, and were predicted to introduce PTCs into exon 6, leading to NMD of mutant mRNA. The c.112delC (p.Arg38Glyfs\*16) mutation has been reported (eight times) previously in various ethnic populations [3], but this is the first report in an Indian population (Figure 2A). Analysis of *PAX6* mRNA by Chen et al. [24] in a patient with the c.112delC mutation demonstrated 50% lower expression in the patient than in unaffected family members. This clearly suggests that the c.112delC mutation

in *PAX6* resulted in a transcript recognized by the NMD system leading to a half reduction of the full-length *PAX6* protein. The c.242delC mutation identified in family AN-107 was contributed by the aniridia-affected mother (Figure 2B) while the c.249delT mutation observed in family AN-120 was contributed by the aniridic father (Figure 2C).

**Substitution mutations:** Five single nucleotide substitutions, including one reported [3] nonsense mutation (c.607C>T, p.Arg203\*), one novel synonymous mutation (c.141G>A, p.Gln47Gln), one novel missense mutation (c.764A>G, p.Gln255Arg), and two novel intronic mutations (c.10+1G>A and c.141+4A>G), were identified in five unrelated families with aniridia (Table 1, Figure 3). In family AN-86 (Figure 3A), the sequence variation c.607C>T caused a premature stop at codon 203 (CGA>TGA). This variant has been previously reported (30 times) in several ethnic groups and is another example of the most commonly reported *PAX6* mutation [3].

Of the total 13 mutations observed, only a single missense mutation (p.Gln255Arg) was identified in this study. This mutation lies in the last codon of exon 9 in the highly conserved HD and makes a substitution at amino acid position 255 from glutamine to arginine. PolyPhen-2 analysis showed a score of 0.968, which implied that the mutation is probably damaging, and SIFT analysis predicted that the mutation is deleterious (score: 0.00, median: 3.68). Mutation Taster also predicted this mutation was disease causing (p value: 1.0). The DNA binding residues of the *PAX6* HD were predicted using the BindN tool for the WT and mutant sequences (containing p.Gln255Arg mutation) with a high threshold for sensitivity (90%). The glutamine residue at position 255 in the WT protein did not interact with the DNA as seen by the negative symbol (-) in the prediction output (Figure 4A). The prediction, when repeated with the mutant sequence, the arginine at position 255, showed binding to DNA, indicated by the positive symbol (+) in the prediction output and a confidence value of 9, on a scale of 0 to 9 (Figure 4B). The number of residues interacting with DNA increased from 14 in the WT DNA complex to 15 in the mutant DNA complex.

The possible effect of the mutations identified at exon-intron boundaries (c.10+1G>A, c.141G>A, c.141+4A>G, and c.764A>G) of the *PAX6* was evaluated with four splicing prediction tools (Table 3). As predicted by all four tools, the mutations c.10+1G>A, c.141G>A, c.141+4A>G, and c.764A>G markedly altered the strength of the donor splice sites and thus abolished the natural splicing site. The c.10+1G>A mutation was located in the donor splice site of intron 4, and this alteration is most likely to cause skipping of exon 4 (which contains the translation start site), which results in the possible failure of translation from its natural start site.

TABLE 1. SUMMARY OF *P4X6* MUTATIONS IDENTIFIED IN THE PRESENT STUDY.

| S. No. | Patient ID | Inheritance | Exon/Intron | Domain | Mutation†      | Type of mutation            | Effect on protein/ mRNA     |
|--------|------------|-------------|-------------|--------|----------------|-----------------------------|-----------------------------|
| 1      | AN-118-1   | AD          | Exon 4      | PD     | c.7_10dupAACA  | Duplication, PTC in exon 6  | p.Ser4Lysfs*53              |
| 2      | AN-118-2   | AD          | Exon 4      | PD     | c.7_10dupAACA  | Duplication, PTC in exon 6  | p.Ser4Lysfs*53              |
| 3      | AN-99-1    | AD          | Intron 4    | PD     | c.10+1G>A      | Substitution, splicing      | Splicing error              |
| 4      | AN-99-2    | AD          | Intron 4    | PD     | c.10+1G>A      | Substitution, splicing      | Splicing error              |
| 5      | AN-87-1    | Sporadic    | Exon 5      | PD     | c.112delC      | Deletion, PTC in exon 6     | p.Arg38Glyfs*16             |
| 6      | AM-68-1    | Sporadic    | Exon 5      | PD     | c.141G>A       | Substitution, splicing      | Splicing error              |
| 7      | AN-82-1    | Sporadic    | Intron 5    | PD     | c.141+4A>G     | Substitution, splicing      | Splicing error              |
| 8      | AN-107-1   | AD          | Exon 6      | PD     | c.242delC      | Deletion, PTC in exon 6     | p.Pro81Glnfs*4              |
| 9      | AN-107-2   | AD          | Exon 6      | PD     | c.242delC      | Deletion, PTC in exon 6     | p.Pro81Glnfs*4              |
| 10     | AN-120-1   | AD          | Exon 6      | PD     | c.249delT      | Deletion, immediate PTC     | p.Val84*                    |
| 11     | AN-120-2   | AD          | Exon 6      | PD     | c.249delT      | Deletion, immediate PTC     | p.Val84*                    |
| 12     | AN-105-1   | Sporadic    | Exon 8      | LNK    | c.567dupC      | Duplication, PTC in exon 8  | p.Ile190Hisfs*10            |
| 13     | AN-86-1    | Sporadic    | Exon 8      | LNK    | c.607C>T       | Nonsense, immediate PTC     | p.Arg203*                   |
| 14     | AN-85-1    | Sporadic    | Exon 9      | HD     | c.704dupC      | Duplication, PTC in exon 9  | p.Asp236Argfs*16            |
| 15     | AN-92-1    | Sporadic    | Exon 9      | HD     | c.753_754insTA | Insertion, immediate PTC    | p.Ala252*                   |
| 16     | AN-91-1    | AD          | Exon 9      | HD     | c.764A>G       | Missense, splicing          | p.Gln255Arg, splicing error |
| 17     | AN-91-2    | AD          | Exon 9      | HD     | c.764A>G       | Missense, splicing          | p.Gln255Arg, splicing error |
| 18     | AN-91-3    | AD          | Exon 9      | HD     | c.764A>G       | Missense, splicing          | p.Gln255Arg, splicing error |
| 19     | AN-104-1   | Sporadic    | Exon 10     | PST    | c.868dupA      | Duplication, PTC in exon 11 | p.Ser290Lysfs*51            |

†The numbering is based on the cDNA sequence (RefSeq ID: NM\_000280.3), with +1 corresponding to the A of the ATG translation initiation codon. **Abbreviations:** AD, autosomal dominant; PD, paired domain; LNK, linker region; HD, homeodomain; PST, transactivation domain

TABLE 2. REVIEW OF CLINICAL FINDINGS OF ANIRIDIA PATIENTS IDENTIFIED WITH PAX6 MUTATIONS.

| S.No. | Patient ID | Age/Sex | Best vision (RE & LE) | Nystagmus | Foveal hypoplasia | Cataract | Glaucoma | Keratopathy | Comments  |
|-------|------------|---------|-----------------------|-----------|-------------------|----------|----------|-------------|---|
| 1     | AN-118-1   | 5 y/M   | 6/36 & 6/36           | +         | +                 | -        | -        | -           | Microcornea, skeletal anomaly (cross over toe, misshapen thumb) |
| 2     | AN-118-2   | 39 y/M  | 6/24 & 6/24           | +         | +                 | +        | +        | -           | Partial aniridia, cataract surgery                              |
| 3     | AN-99-1    | 1.5 y/M | NA & NA               | +         | +                 | -        | -        | -           |   |
| 4     | AN-99-2    | 28 y/M  | 3/60 & 3/60           | +         | +                 | +        | +        | +           |   |
| 5     | AN-87-1    | 8 y/F   | 4/60 & 3/60           | +         | +                 | +        | -        | +           | Partial aniridia, mild ptosis                                   |
| 6     | AM-68-1    | 15 y/F  | CF & CF               | +         | +                 | +        | +        | +           |   |
| 7     | AN-82-1    | 5.5 y/M | 6/60 & 6/60           | +         | +                 | +        | +        | +           | Lens subluxation  |
| 8     | AN-107-1   | 6 y/M   | 5/60 & 5/60           | +         | +                 | -        | -        | -           |   |
| 9     | AN-107-2   | 34 y/F  | CF & 1/60             | +         | +                 | +        | +        | +           | Cataract surgery, trabeculectomy                                |
| 10    | AN-120-1   | 1.5 y/M | NA & NA               | +         | +                 | -        | -        | -           |   |
| 11    | AN-120-2   | 29 y/M  | PL & PL               | +         | +                 | +        | +        | -           | Optic nerve anomaly   |
| 12    | AN-105-1   | 8 y/F   | 6/60 & 5/60           | +         | +                 | -        | -        | +           |   |
| 13    | AN-86-1    | 24 y/F  | 2/60 & PL             | +         | +                 | +        | +        | +           |   |
| 14    | AN-85-1    | 21 y/M  | 1/60 & CF             | +         | +                 | +        | -        | -           | Lens subluxation  |
| 15    | AN-92-1    | 21 y/M  | 6/36 & 5/60           | +         | +                 | -        | +        | +           | Microcornea   |
| 16    | AN-91-1    | 37 y/M  | PL & PL               | +         | +                 | +        | -        | +           | Partial aniridia  |
| 17    | AN-91-2    | 5 y/M   | 6/24 & 6/24           | +         | +                 | +        | -        | -           |   |
| 18    | AN-91-3    | 65 y/F  | NA & NA               | +         | NA                | +        | +        | +           | Trabeculectomy  |
| 19    | AN-104-1   | 8 y/M   | 6/60 & 6/60           | +         | +                 | +        | -        | -           | Ptosis  |

Abbreviations: RE, right eye; LE, left eye; PL, perception of light; NA, Not available; CF, counting fingers.

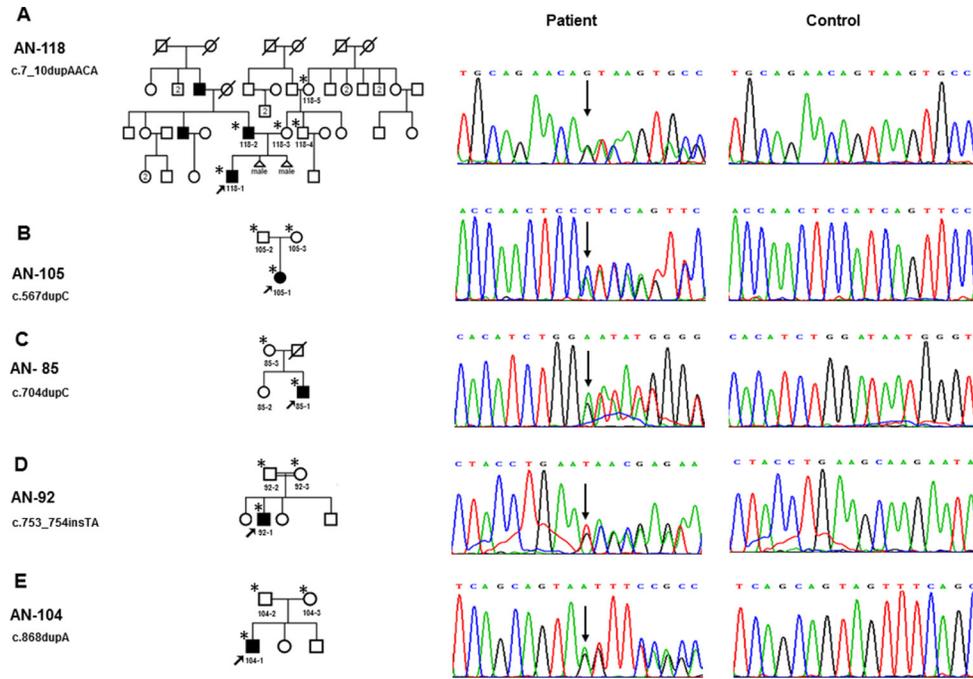


Figure 1. Duplication/insertion mutations detected in *PAX6*. Five novel duplication/insertion mutations were identified in five probands with aniridia from unrelated families. The mutations were named according to the nomenclature recommended by the Human Genomic Variation Society (HGVS). Pedigrees (left) are accompanied by mutant and normal control chromatograms (right) of *PAX6*. Arrows in pedigrees indicate the probands. The asterisks indicate the individuals whose DNA samples were available for genetic analysis. The exact mutations in chromatograms are indicated by the arrows.

**Genotype-phenotype correlation:** Cosegregation analysis in all familial cases demonstrated the autosomal dominant mode of inheritance of disease with complete penetrance. Clinical findings of the patients with identified mutations revealed phenotypic heterogeneity of the disease with the same or different mutations (Table 2). In this study, neither the location nor the nature of the mutation correlated with the observed phenotypic variability. Of the 19 patients with *PAX6* mutations, all had nystagmus, and 18 had foveal hypoplasia. Extensive phenotypic heterogeneity was observed

in cataract (13/19), glaucoma (9/19), keratopathy (10/19), microcornea (2/19), ptosis (2/19), lens subluxation (2/19), and optic nerve anomaly (1/19). In the family with the c.764A>G mutation, proband AN-91-1 showed complete aniridia, but the proband's mother and son showed partial aniridia with the same mutation. Proband AN-118-1 with the c.7\_10dupAACA mutation had complete aniridia, bilateral microcornea, and skeletal anomalies (cross toe and misshapen thumb), which were not detected in the proband's father (partial aniridia, cataract, glaucoma) with the same mutation (Table 1, Table

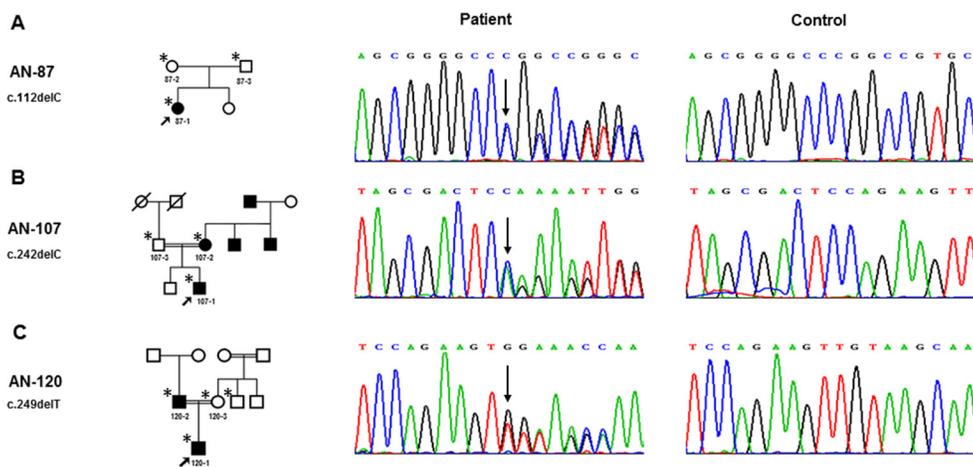


Figure 2. Deletion mutations detected in *PAX6*. One known (c.112delC) and two novel (c.242delC and c.249delT) deletions were identified in three unrelated families with aniridia. A–C: Pedigrees of the families as well as sequencing chromatograms from probands with aniridia, and corresponding sequences from normal controls. The asterisks indicate the individuals whose DNA samples were available for genetic analysis. The arrows in the chromatograms indicate the position of the deletion.

The asterisks indicate the individuals whose DNA samples were available for genetic analysis. The arrows in the chromatograms indicate the position of the deletion.



was predicted to cause abnormal splicing (Table 3). The role of synonymous mutations in aberrant splicing that results in a defective protein or NMD has been well established [25-27]. p.Gln255Arg (c.764A>G) was the only missense mutation identified in this study. This mutation was present at the exon 9–intron 9 junction and predicted to cause abnormal splicing and introduce a PTC in the *PAX6* ORF (Table 3). The protein, if at all generated from p.Gln255Arg mutant mRNA, will not function like a wild-type protein as predicted by the BindN tool (Figure 4). PolyPhen-2, SIFT, and Mutation Taster also predicted the mutant protein was pathogenic.

Seven mutations were identified in the PD (53.8%), two in the LNK (15.4%), three in the HD (23.1%), and one in the PST domain (7.7%). The mutations identified in this study were distributed all over the gene. However, fewer mutations were identified in the PST domain despite its length (152 amino acids) compared to the relatively shorter PD (128 amino acids). The PD was identified as the mutational hot spot in this study. Our previous studies [5-8,28] also reported approximately 50% mutations in the PD.

The NMD surveillance mechanism typically operates if a PTC is located 50–55 nucleotides 5' to the last exon-exon junction, and therefore, any PTC located within the last 44 codons of the *PAX6* ORF is predicted to escape the NMD mechanism [12,29,30]. Interestingly, all the mutations identified in this study are truncations and located before the last 44 codons of the ORF. Therefore, NMD surveillance is possibly the primary mechanism by which *PAX6* null alleles are generated. The mRNAs transcribed from a single functional copy of *PAX6* are unable to produce an adequate level of the PAX6 protein to initiate the transcription of its downstream target genes [31-33]. This loss of function of one copy (haploinsufficiency) observed in the PAX6 protein reduces protein levels below the required critical dose and, consequently, hinders normal eye development [33]. Therefore, the finding of this study is consistent with the hypothesis that haploinsufficiency

of *PAX6* is the main mechanism leading to the aniridia phenotype [5-8,34-37].

The clinical expression associated with aniridia demonstrated variable phenotypes. The main clinical findings associated with *PAX6* mutations in the present cohort were iris anomalies (100%), nystagmus (100%), foveal hypoplasia (94.7%), cataracts (68.4%), keratopathy (52.6%), and glaucoma (47.4%). In addition, microcornea, lens subluxation, ptosis, and optic nerve anomaly were identified in a few cases. This study did not show any phenotypic differences according to the location of the identified mutations. Phenotypic variability within the family and between the families has also been observed. The reason for variable expressivity among patients with aniridia with the same or different mutations is unclear. This study also reported a mild skeletal defect (cross toe and misshapen thumb) in a proband with aniridia (AN-118-1) with a *PAX6* mutation (c.7\_10dupACA). The proband's father was positive for the same mutation and presented the aniridia phenotype (Figure 1A). The proband's maternal family was negative for *PAX6* mutations but had a history of ocular defects (severe bilateral microphthalmia). There is no report of an association of *PAX6* mutations with skeletal anomalies. Therefore, these findings suggest that the proband's maternal family might have a mutation in a different gene, and the proband inherited mutations from both parents and presented with ocular and skeletal anomalies.

In the present study, the mutation detection rate was 43.3% (13/30), which is comparable to previous reports [36,38-40]. However, several other studies have also described higher mutation detection rates [35,41-43]. In 17 patients with aniridia, a *PAX6* mutation was not identified with the direct DNA sequencing approach. In the present study, we evaluated only protein-coding regions and intron-exon boundaries, and therefore, the mutations present in the regulatory regions [44] of *PAX6* were missed by our approach. Furthermore, different types of genetic aberrations have been reported [3] with the aniridia phenotype, and direct sequencing fails to detect all

TABLE 3. PREDICTED CONSEQUENCES OF MUTATIONS IDENTIFIED AT SPLICE JUNCTIONS.

| S.N | Mutation   | MaxEntScan [scale 0–12, WT:Mut (% diff)] | NNSPLICE [scale 0–1, WT:Mut (% diff)] | GeneSplicer [scale 0–15, WT:Mut (% diff)] | HSF [scale 0–100, WT:Mut (% diff)] |
|-----|------------|--|---------------------------------------|---|------------------------------------|
| 1   | c.10+1G>A  | 9.5:0 (–100%)                            | 1.0:0 (–100%)                         | 9.2:0 (–100%)                             | 83.7:0 (–100%)                     |
| 2   | c.141G>A   | 4.3:0 (–100%)                            | 0.32:0.005 (–98.5%)                   | 7.4:1.5 (–79.4%)                          | 84.3:73.8 (–12.5%)                 |
| 3   | c.141+4A>G | 4.3:1.1 (–75.0%)                         | 0.3:0.05 (–84.6%)                     | 7.4:2.2 (–69.6%)                          | 84.3:76.0 (–9.9%)                  |
| 4   | c.764A>G   | 10.1:5.4 (–45.9%)                        | 0.9:0.5 (–44.7%)                      | 4.2:0.5 (–87.2%)                          | 77.2:72.4 (–6.3%)                  |

MaxEntScan, maximum entropy modeling of short sequence motifs; NNSPLICE, neural network splice site analysis; HSF, human splicing finder; WT:Mut, the ratio of scores between wild-type and mutant alleles; % diff, the percentage difference between the wild-type and mutant allele score.

types of variations. Therefore, to identify all kinds of variations and to achieve the maximum detection rate, different molecular methods such as high-resolution comparative genomic hybridization (HR-CGH) arrays, fluorescence in situ hybridization (FISH), and multiplex ligation-dependent probe amplification (MLPA) should also be combined with the direct sequencing technique. Alternatively, mutations in other eye developmental genes may also contribute to the aniridia phenotype [2,45,46].

In summary, this study identified 11 novel mutations and thus significantly extends the number of mutations known for *PAX6*-related aniridia. All mutations detected in this cohort are truncations, which further supports the hypothesis of haploinsufficiency of *PAX6* in aniridia. Our cohort demonstrated considerable phenotypic heterogeneity with cataract, glaucoma, keratopathy, microcornea, lens subluxation, and ptosis. The variable expression observed in our cohort suggests that not only *PAX6* but also other unknown factors might influence the aniridia phenotype.

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