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A novel *PAX6* gene mutation in an Indian aniridia patient

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Purpose: A mutation in the *PAX6* gene is thought to be the genetic cause of aniridia. Here we search for *PAX6* gene mutations in Indian aniridia patients.
Methods: We amplified the coding exons of the *PAX6* gene from the genomic DNA of 15 unrelated aniridia patients using polymerase chain reaction technology. We then performed single-strand conformation polymorphism analysis and heteroduplex analysis to search for sequence variants.
Results: Sequencing of shifted bands in two patients revealed *PAX6* gene mutations. One of these was a novel mutation, 1180insA, located in exon 10 at the start of the PST domain. The other mutation, 1080C->T (R240X), located in exon 9 within the homeodomain, and is another example of the most commonly reported *PAX6* mutation.
Conclusions: Although *PAX6* gene mutations and polymorphisms have been reported from various ethnic groups, we report for the first time the identification of *PAX6* gene mutations in Indian aniridia patients.

Aniridia is a severe panocular disorder that results in partial or complete absence of the iris. Other ocular complications include glaucoma, cataract, and optic nerve hypoplasia [1]. Aniridia can occur by itself, in which case it shows an autosomal dominant inheritance, or as part of the WAGR syndrome (Wilm's tumor, aniridia, genitourinary abnormalities, and mental retardation) [1,2]. In the general population aniridia occurs at a frequency of 1 in 50,000 to 100,000.

The gene responsible for aniridia is *PAX6*, [3-5]. It is located on chromosome 11p13 [3]. The gene consists of 14 exons with the initiation codon in exon 4 and the termination codon in exon 13, the most 3' exon [5]. It encodes a transcriptional regulator with two DNA binding domains, a paired domain of 128 amino acids, and a paired-type homeodomain of 61 amino acids. These domains are separated by a linker segment of 79 amino acids, followed by a proline, serine, and threonine-rich (PST) domain of 152 amino acids [3,5-7]. An alternative splice event, inclusive of exon 5a, results in the insertion of 14 amino acids into the paired domain, and increases the total length of the protein from 422 amino acids (-5a form) to 436 amino acids (+5a form) [5]. The 14 amino acid insertion changes the DNA binding properties of the paired domain [8]. Both splice variants are essential for normal eye development [8-10].

Aniridia is caused by loss of function of one copy of the *PAX6* gene, which may occur by deletion of the entire gene [2,3], or by intragenic mutation [4-6,11,12]. Pathological intragenic mutations are scattered throughout the gene. *PAX6* mutations and polymorphisms have been identified independently by many groups and are archived in the PAX6 Allelic Variant Database [13]. Of 209 pathological mutations in the coding region of *PAX6*, nonsense mutations are the most common single class, accounting for 35% of the total. Frame-shifting insertions and deletions account for 26% and splice mutations make up 17%. Missense mutations account for 19% and termination mutations (where the normal stop codon is mutated so that translation continues into the 3' untranslated region) account for 2%. In-frame deletions and insertions are rare, accounting for just 1% of the total. Inactivation of both *PAX6* alleles causes a lethal phenotype of anophthalmia and severe brain defects [14].

In addition to mutations in a growing number of aniridia patients, heterozygous *PAX6* mutations have also been reported in a spectrum of variant phenotypes including Peter's anomaly, keratitis, isolated foveal hypoplasia, and ectopia pupillae [6,9,15].

Here we report mutation analysis of the *PAX6* gene in two Indian aniridia patients. We detected a novel mutation in the PST region and a previously described nonsense mutation in the homeobox. *PAX6* mutations have been identified in various ethnic groups but this is the first report of mutations in the Indian population. Our study supports the hypothesis that premature translational termination mutations in the *PAX6* gene result in haploinsufficiency and generate the aniridia phenotype.

METHODS

Patients: All studies were conducted in accordance with the tenets of the Declaration of Helsinki. Ophthalmologists recruited fifteen unrelated aniridia patients with their family members and age-matched controls (with informed consent from each individual). The patients and family members were evaluated by a glaucoma specialist and individuals with congenital anomalies other than aniridia (for instance, Axenfeld-

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Rieger syndrome, iridocorneal endothelial syndromes, sclerocornea, Peters' anomaly) were excluded by a careful clinical ocular evaluation and history. The ocular associations and relevant clinical data of the patients with *PAX6* gene mutations are shown in Table 1. Ophthalmic examinations included slit lamp biomicroscopy, measurement of intraocular pressure (IOP) by applanation tonometry, gonioscopic evaluation of the anterior chamber angle, and perimetry by automated field analyzer, where appropriate. Clinical manisfestations recorded included elevated IOP, micro-, or megalocornea, corneal opacification and peripheral corneal pannus and degeneration, partial or total absence of iris, lens abnormalities including cataracts and subluxation, evidence of glaucomatous disc and/or optic nerve head damage, and macular hypoplasia. Systemic evaluation was performed to exclude associated anomalies like Wilms' tumour, urogenital anomalies, and mental retardation in all subjects included in the study.

DNA preparation: Blood was collected in EDTA coated tubes. Total genomic DNA was isolated from the blood by the salt precipitation method [16]. Genomic DNA was suspended in TE buffer, pH 8, and stored at -20 °C.

PCR: The polymerase chain reaction was used to amplify exons 4-13 (the coding exons) of *PAX6* from genomic DNA using the primers previously described [5]. Each 20 µl reaction contained 100 ng of genomic DNA, 0.5 U of *Taq*, 2 mM $MgCl_2$, 200 mM dNTPs and 100 pM of primers. Modified thermocycling conditions were performed for exon 9 and exon 10. These are 94 °C for 9 min (94 °C for 1 min, 58 °C for 2 min, and 72 °C for 2 min, 1 s/cycle) for 35 cycles, and 72 °C for 10 min.

SSCP and heteroduplex analysis: PCR products were analysed by combined single-strand conformation polymorphism (SSCP) analysis and heteroduplex analysis (HDA) on 10% non-denaturing polyacrylamide gels [17,18]. The amplified products were mixed with loading solution (98% formamide, 10 mM NaOH, 20 mM EDTA, 0.25 % bromophenol blue and xylene cyanol), denatured at 98 °C for 3-5 min and immediately placed in ice. Polyacrylamide gels were prerun for 15 min then the samples were separated by electrophoresis on a DNA sequencing gel apparatus at 800 V for 7 h. All the probands were checked on gels both with and without 2.5% glycerol. Gels were silver stained by the following protocol, gels were soaked in fixing solution (40% methanol, 35% formaline, 25% water) for 10 min followed by 1 min in 0.02% $Na_2S_2O_3$, then the gels were washed two times water and soaked in 0.1% $AgNO_3$ for 10 min, followed by a quick rinse in water. Finally the gels were immersed in developing solution until bands appeared. The reactions were stopped with 2.3 M citric acid.

Sequencing: Exons having altered mobility were re-amplified and purified using QIAGEN columns. The PCR products were sequenced directly on an Applied Biosystems (ABI, Rotkreuz, Switzerland) model 377 automated sequencer. The direct sequencing of some PCR products gave superimposed peaks because of the presence of both normal and mutant alleles. With these samples, the PCR product was cloned and then sequenced.

TA cloning: PCR products were ligated to the 3.85 kB pDrive vector (QIAGEN Gmbh, Hilden Germany) according to the manufacturer's instructions. The construct was transformed into DH5α *E.coli* cells and plated on 100 µg/ml ampicillin, 80 µg/ml X-gal, and 1 mM IPTG on LB Agar plates. Plasmids were isolated from the transformed cells by the alkaline lysis method and digested with *Eco*R I to confirm the presence of the PCR product. Sequencing was performed using T7 universal primers. To make this strategy more efficient, a second round of SSCP analysis directly from the bacterial clones was carried out to allow the clear selection of allele-specific clones for sequencing [19].

Table 1. Clinical data and phenotype of subjects with *PAX6* mutations

Patient	Status	Sex	Exon	DNA Outcome	Codon	Domain	Macular hypoplasia		Predicted outcome of the mutation
23-1	sporadic	M	9	c.1080 C>T	R240	HD	-	-	Nonsense Arginine to stop codon
14-1	sporadic	M	10	c.1180insA	N273	PST	+	+	Frame shift by insertion of a nucleotide at position 1180 in exon 10 results in premature termination

Table summarising clinical data and phenotypes of subjects with *PAX6* mutations. Both patients had bilateral cataracts, bilateral glaucoma, nystagmus, and aniridia. The term HD refers to Homeodomain and PST refers to proline serine and threonine region. The letter R refers to Arginine and N refers to Asparagine.

RESULTS

We looked for mutations in the *PAX6* gene by amplifying exons 4-12 using previously published PCR primers [5] in 25 individuals, among them 15 aniridia patients and 10 normal controls. Information about the inheritance status and clinical feature of the Indian aniridia patients found to carry a *PAX6* mutation is summarized in Table 1.

Detection of mutations by SSCP gel electrophoresis was based on the assumption that a single base mismatch can produce changes in the secondary conformation of single-stranded DNA that alters the mobility of the strand during non-denaturing gel electrophoresis. During SSCP, a significant proportion of the single-stranded DNA re-anneals to form duplexes. If a base change is present, heteroduplexes will form, which may also be detected by non-denaturing electrophoresis. Heteroduplexes generally show greater mobility and appear in the lower part of the gel [17,18].

Patient 23-1 had sporadic bilateral anridia with nystagmus and cataract (Figure 1). Heteroduplex analysis of the *PAX6*-derived exon 9 PCR product in this patient revealed a shifted band. Direct sequencing and sequence analysis of this PCR product revealed the presence of a heterozygous mutation in the *PAX6* homeodomain of this patient (Figure 2). The C->T transition at nucleotide 1,080 (systematic name 1080C->T) replaces the arginine at codon 240 (CGA) by a stop codon

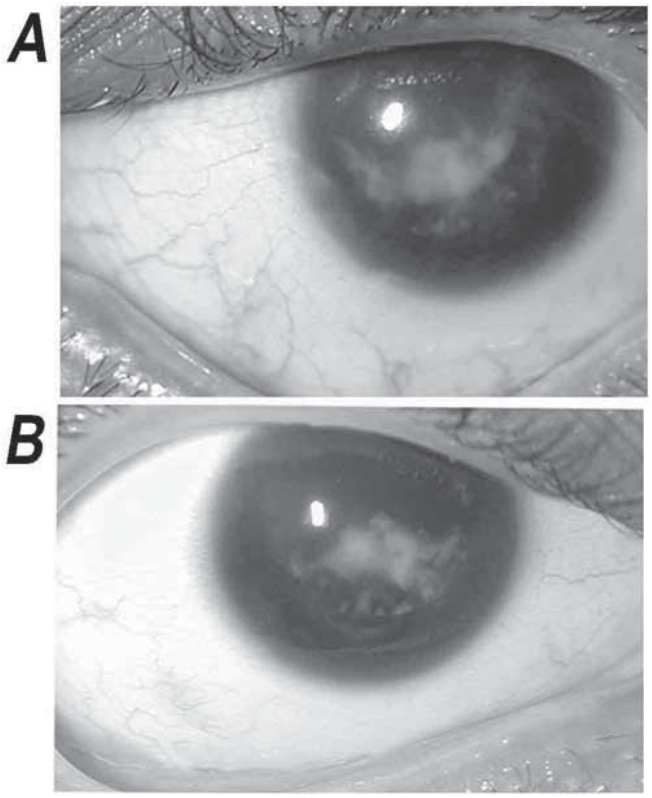

Figure 1. Photographic demonstration of aniridia expression. Phenotype of 14 year old male proband (23-1) shows bilateral aniridia with nystagmus and cataract. **A**: Left eye. **B**: Right eye.

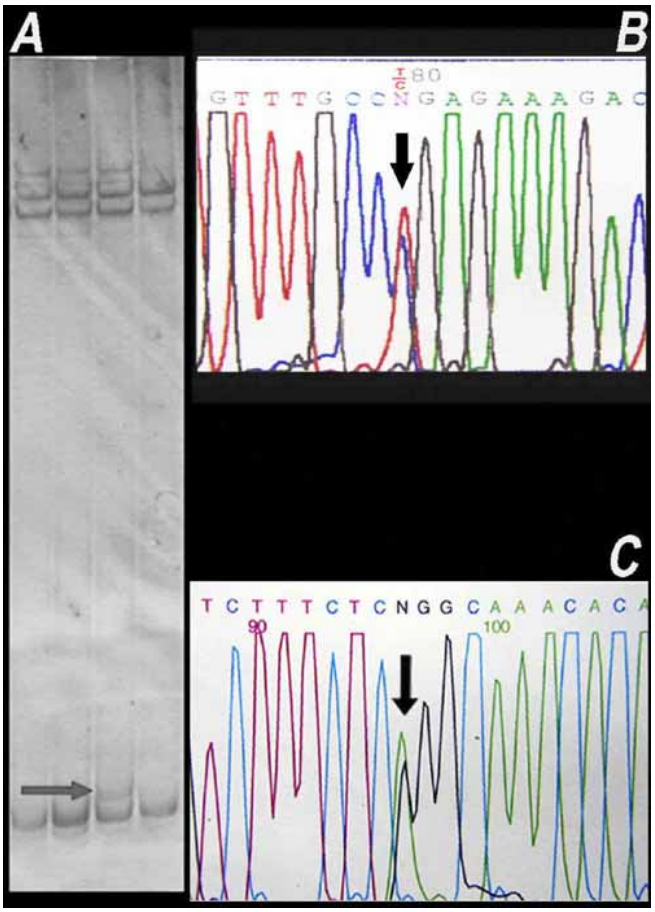

Figure 2. Detection of nonsense mutation (1080C->T) in proband 23-1. **A**: SSCP gel for exon 9 showing the mobility shift (arrow). Electropherogram of forward (**B**) and reverse (**C**) sequence of *PAX6* exon 9 derived from proband 23-1 showing the heterozygous mutation (arrow).

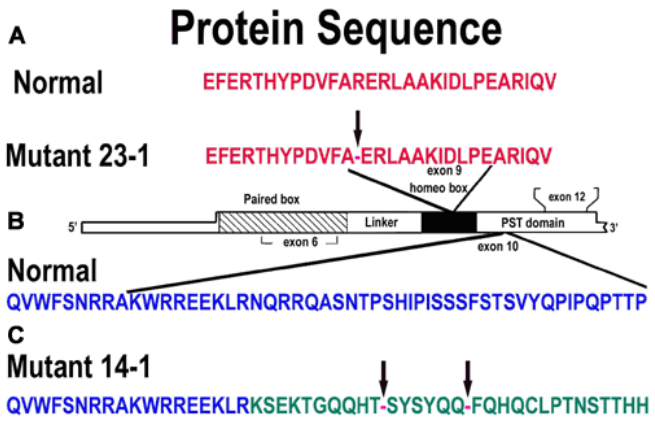

Figure 3. Comparison of normal and mutant protein sequences of the *PAX6* gene. **A**: *PAX6* exon 9 derived from proband 23-1 shows the premature truncation of protein with stop codon (arrow). **B**: Graphical representation of the *PAX6* gene showing the paired box, homeo box, glycine rich region, and PST domain. **C**: *PAX6* exon 10 derived proband 14-1 shows the frameshift mutation which leads to premature truncation of protein by a stop codon (arrow).

(TGA), which results in a premature truncation of the *PAX6* protein in the homeodomain (Figure 3). This is the most commonly reported mutation in the *PAX6* gene and occurs at a so-called mutation hotspot [12]. This mutation has been identified in various ethnic groups but this is the first report of the mutation in patients with aniridia from the Indian population. The mutation was submitted to Genbank (AF548390), and to the PAX6 Allelic Variant Database.

Patient 14-1 has sporadic bilateral aniridia and ectopia lentis with cataract (Figure 4). Heteroduplex analysis of the exon 10 PCR product revealed a shifted band (Figure 5). However, upon direct sequencing, superimposed peaks were observed on the sequencing trace and it was difficult to deduce the identity of the mutation (Data not shown).

To separate the two alleles and avoid the superimposed signals, the exon 10 PCR product was cloned into a T-tailed vector and individual recombinants were then sequenced. This revealed that the mutant allele had an insertion of a single base (adenosine) at position 1,180, codon 273 (systematic name 1180insA; Figure 5). This mutation, which has not previously been reported in any population, leads to a frame shift and is predicted to result in premature truncation of the protein (Figure 3). This mutated sequence was also submitted to Genbank (AY208677), and to the PAX6 Allelic Variant Database.

DISCUSSION

We have described the first *PAX6* mutations in Indian aniridia patients. The two mutations, 1180insA and 1080C->T, fall into the most common categories of known *PAX6* mutations, frameshift mutations and nonsense mutations. These account for 26% and 35% respectively of all pathological *PAX6* mutations PAX6 Allelic Variant Database.

Both mutations are predicted to result in the introduction of a premature termination codon and therefore might be predicted to trigger degradation of the mutant mRNA by RNA surveillance [20]. RNA surveillance is a powerful mechanism by which newly synthesized mRNAs are monitored for errors that would result in premature termination of translation [21]. Error-containing mRNAs are degraded so that truncated proteins, which might interfere with the function of the normal protein, or have other toxic effects in the cell, are not made.

The fact that the classical aniridia phenotype can result from deletion of the entire gene, or from a mutation anywhere within the coding region of the gene, is strong evidence that anirida-associated intragenic mutations are functionally null [2,20]. Although there is no specific evidence as yet that RNA surveillance acts on mutant *PAX6* mRNAs, it is clearly a mechanism by which nonsense, splice and frameshift mutations could generate null alleles no matter where they occur in the gene [21].

It is interesting to note that many of the variant (not classical aniridia) eye phenotypes that are caused by *PAX6* mutations have been found in association with missense mutations [6,9,15]. Missense mutations would not be detected by RNA surveillance and would be predicted to generate a full-length protein with a single amino acid substitution. Such proteins

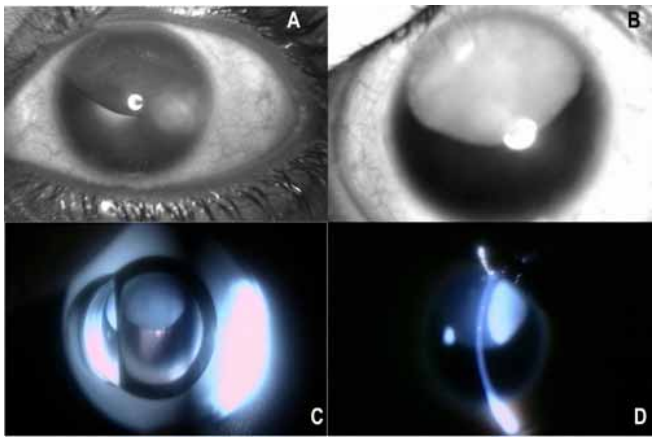

Figure 4. Clinical anterior segment photographs of patient 14-1. Left (**A**) and right (**B**) eyes with total aniridia and ectopia lentis with cataract. Goniophotographs reveal the anterior chamber angle of the proband's eye with rudimentary iris process confirming the phenotype (aniridia). **C**: Left eye. **D**: Right eye.

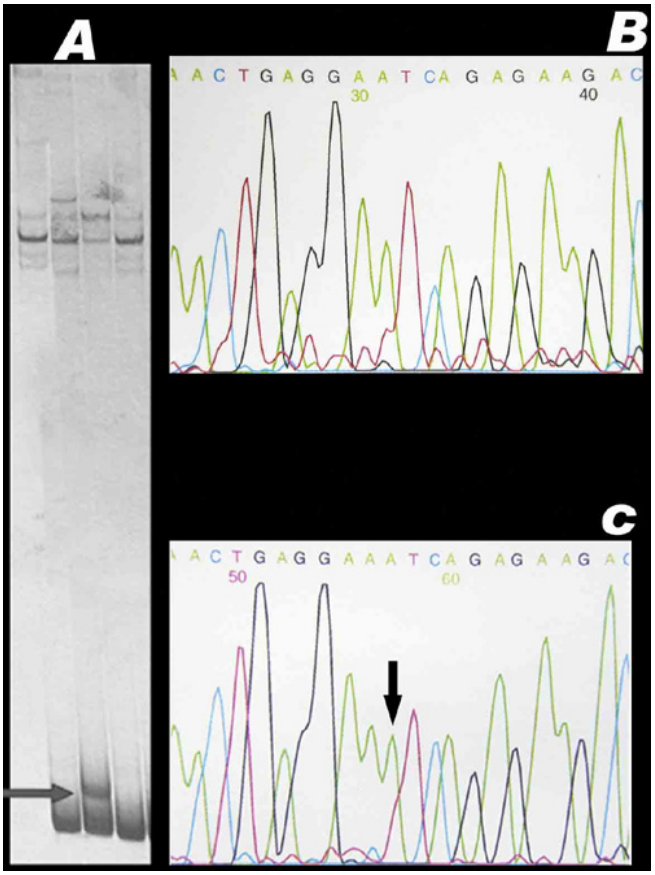

Figure 5. Detection of frameshift mutation (1180insA) in proband 14-1. **A**: Silver stained SSCP gel for exon 10 shows the mobility shift (arrow). **B**: Electropherogram shows the normal sequence in control sample. **C**: Electropherogram shows the addition of an adenine nucleotide (arrow) in *PAX6* exon 10 sequences derived from proband 14-1. The mutation results in a frameshift mutation at position 1180.

could display partial loss-of-function, or even gain of function, which in turn could account for their association with phenotypes that are distinct from classical aniridia [22].

In summary, this is the first report of mutations in the *PAX6* gene of aniridia patients from the Indian population. Our report adds one novel mutation to the existing spectrum of mutations that cause the aniridia phenotype.

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