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De novo deletions in the paired domain of *PAX6* in south Indian aniridic patients

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Abstract We analyzed the sequence variation in the coding exons of *PAX6* in eight unrelated south Indian pedigrees with one aniridic individual in each family. Mutations were detected by PCR, SSCP and allele-specific cloning followed by sequencing. Here we report two de novo deletion mutations, c.537delA (codon 59) and c.728del14 (codon 122), in the paired domain of *PAX6*. These deletions probably represent null or hypomorphic alleles consistent with *PAX6* haploinsufficiency as the underlying genetic factor of aniridia.

Keywords *PAX6* · Aniridia · Allele-specific clones · PCR-SSCP

Introduction

Aniridia is a panocular human eye malformation caused by heterozygous null mutations within the paired box transcription factor gene *PAX6* (Ton et al. 1991; Glaser et al. 1992). Loss-of-function mutations in *PAX6* are responsible for several naturally occurring ocular phenotypes characterized by iris hypoplasia, cataract and

corneal opacity in association with other ocular disorders like congenital ptosis, nystagmus, strabismus, and glaucoma (Nelson et al. 1984; Glaser et al. 1992, 1994; Hanson et al. 1993). The gene has both homeobox and paired box motifs characteristic of transcriptional regulators and has multiple roles in eye development and maintenance. Aniridia is most simply explained by the reduction in *PAX6* activity at the cellular level: most described mutations are predicted to truncate the polypeptides through nonsense-mediated mRNA decay (van Heyningen and Williamson 2002).

In this report, we describe two novel paired box deletions in the *PAX6* gene of aniridic patients out of eight pedigrees studied in south India. These deletions result in premature truncation of the polypeptide.

Methods

Clinical description

A total of eight pedigrees with affected probands and 80 controls without any history of eye disease were recruited for this study with informed consent. The Institutional Review Board and Ethics Committee of the Aravind Eye Hospital approved the study and tenets of the Declaration of Helsinki on Human trials were strictly adhered to.

Genomic DNA isolation

DNA was isolated from lymphocytes by the salting out method (Miller et al. 1988) and suspended in TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8).

PCR-SSCP

Amplification was carried out in a 20- μ l reaction containing 100 ng of genomic DNA, 0.5 U of Taq, 2 mM $MgCl_2$, 200 μ M dNTPs and 100 pM of published primers (Glaser et al. 1992) and a touch down PCR

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program was performed with initial denaturation at 95°C for 9 min followed by 35 cycles of 95°C for 30 s to 62°C (−0.3°C/cycle) for 30 s to 72°C for 2 min. The amplified products were denatured and electrophoresed on a 10% polyacrylamide gel.

TA cloning

PCR products of the mutant were cloned into pGEMT vector and sequence confirmation performed, as previously described (Neethirajan et al. 2003). Allele-specific PCR with repeated sequencing of different clones was performed since direct sequencing of genomic PCR gave superimposed signals. The clones with mutant alleles were selected by performing PCR-SSCP for plasmid DNA from the transformed colonies in comparison with genomic DNA (Fig. 2a).

Restriction digestion

The PCR products of exon 7 of normal and mutant alleles were digested with 2 U of *NheI* and analyzed on 2% agarose gel to reconfirm the presence of the variation, which was identified by sequencing.

Results

SSCP analysis of the *PAX6* gene in cases affected by aniridia showed mobility shift compared with wild type (Fig. 1a,d). The allele-specific clones, which showed the same pattern as that of genomic DNA with the mutant allele in the polyacrylamide gel, help us to pick up the exact clone for sequencing (Fig. 2a). The plasmid-based sequencing revealed two novel mutations in the *PAX6* gene c.537delA in exon 6 (Fig. 1b,c) of proband from

family AJA and c.728del14 in exon 7 (Fig. 1e,f) of proband from family MAH. These mutations were completely absent in the normal controls screened. The novel mutant sequences were deposited in GenBank (accession no. AY485787, AY485788). The 14-bp deletion in exon 7 (c.728del14) resulted in the loss of *NheI* restriction site (Fig. 2b) and cosegregated with the disease-causing phenotype.

Discussion

Deletion of single and multiple base-pair changes found in these two cases of sporadic aniridia were within the paired box of the *PAX6* gene and have not previously been reported in the Human *PAX6* Allelic Variant Database (<http://pax6.hgu.mrc.ac.uk>). The proband with c.537delA showed corneal pannus with corneal haze and associated upward subluxation of clear lens associated with aniridia. The other proband with c.728del14 had corneal edema and upward subluxation of cataractous lens with total aniridia. Family relationships have not been confirmed by genetic fingerprinting, but we assume that both mutations are sporadic. The deletion (c.537delA) is predicted to disrupt codon 59 (arginine) causing a frame-shift mutation that leads to a stop codon within the paired box of *PAX6* (Fig. 2c). The amino acid sequence of the c.728del14 mutant polypeptide is altered from the serine residue (Fig. 2c).

Fourteen base-pair deletions are rare events in the *PAX6* gene. Previously, other studies reported a 14-bp deletion, c.709del14, and a 6-bp deletion, c.719del6, in the paired box of *PAX6* correlating with the pathogenicity of aniridia (Vincent et al. 2002; Wolf et al. 1998). Both mutations resulted in termination of the polypeptide chain in the paired domain and hence truncation of the *PAX6* protein.

Fig. 1 SSCP analysis of family AJA and MAH and the electropherogram of normal and mutant alleles. **a** AJA pedigree and the SSCP gel showing the mobility shift (arrow) in the mutant, which was not seen in unaffected family members. **b** Electropherogram of unaffected controls of exon 6. **c** Electropherogram of affected patient showing a single adenosine residue deletion (arrow). **d** MAH pedigree and the SSCP gel showing the unusual banding pattern of the affected mutant (arrows). **e** Electropherogram of exon 7 of the unaffected parent of MAH family. **f** Electropherogram of the affected patient in family MAH showing the deletion of 14 bp (box in normal)

Fig. 2 Allele-specific clones and restriction digestion of DNA from families AJA and MAH. **a** Gel analysis of mutant clones picked up using plasmid-based PCR-SSCP showing the same pattern with that of genomic DNA with altered allele in AJA and MAH families. *G* genomic DNA used in PCR, *N* normal allele, *M* mutant allele from the clones. **b** *NheI*-digested PCR product of exon 7 in family MAH. *M* 100 bp marker; normal control digested with *NheI* enzyme, which shows 174 bp and 79 bp (lanes 1, 3, 4); mutant PCR product, which lost the *NheI* restriction site and remains intact with 253 bp (lane 2); undigested PCR product (lane 5). **c** (1) The normal amino acid sequence (top) compared with mutant amino acid sequence of exon 6 in proband of family AJA. *Box*: the conserved amino acid sequence. *Arrow* indicates the stop codons. (2) Graphical representation of the *PAX6* gene and the *arrow* shows the location of *paired box* deletions. (3) The comparison of normal and mutant amino acid sequence of exon 7 in the proband of family MAH and the *arrow* indicates the stop codons

In summary, novel deletions within the paired box region of *PAX6* were observed in two families out of the eight south Indian pedigrees. The deletions are predicted to lead to premature polypeptide chain termination with disruption of DNA binding activity, consistent with the conventional aniridia phenotype being caused by haploinsufficiency at the *PAX6* locus. It is likely that the mutant alleles produce polypeptides that are subjected to nonsense-mediated decay and are therefore null (Byers 2002; Neethirajan et al. 2003). In contrast, we could not detect any mutations in six affected families, where mutations might have occurred in one of the *cis* regulatory regions.

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