Three novel mutations of the *PAX6* gene in Japanese aniridia patients

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Abstract Mutations in the *PAX6* gene of Japanese aniridia patients were analyzed. Four types of mutations including one known (474delC) and three novel (786_787ins10,

678_688del11 and 572_575delAATCins14) were found in six patients from four families. A patient with the mutation 572_575delAATCins14 also manifested VATER association. This is the first case of aniridia accompanied by VATER association. All of mutations found in this study are frameshift type, resulting in premature termination of translation. The database for *PAX6* gene mutation has been made using a graphical data display system *MutationView* (<http://mutview.dmb.med.keio.ac.jp/>).

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Introduction

Aniridia is a panocular autosomal dominant disorder in which the development of the iris, cornea, lens, angle, and retina is disturbed. In the general population, aniridia occurs at a frequency of 1 in 50,000–100,000 (Elsas et al. 1997). Aniridia is often caused by mutations of the *PAX6* gene, located on the band p13 of human chromosome 11 (Ton et al. 1991). The *PAX6* gene encodes a transcription regulator that recognizes target genes through the paired domain and is involved in ocular morphogenesis. Numerous mutations have been detected in patients with aniridia (Prosser et al. 1998) and the Human *PAX6* Mutation Database (<http://www.pax6.hgu.mrc.ac.uk/>) collected approximately 300 cases with 200 different mutations. Of these, 26 cases with 19 different types of mutations are from Japanese patients (Azuma et al. 1996, 1999, 2003; Sonoda et al. 2000; Yasuda et al. 2002). Here, we report additional mutations of the *PAX6* gene in Japanese aniridia patients, including three novel types of mutations.

Materials and methods

Patients

Six patients analyzed in this study were members of four unrelated families (Table 1; Supplemental Fig. 1). Patient AN011 and his mother AN012 are from family 1, in which no other patients were found with aniridia or related disorders including generation I. It is notable that their aniridia phenotype was mild. Patient AN021 is from family 2, which includes ten patients for four generations. Patient AN031 is from family 3, and is apparently a sporadic case of aniridia. Two members of generation I and III had color blindness. Patients AN041 and AN042 are from family 4. AN041 developed anal atresia, hydronephrosis, right radial dysplasia and loss of the right thumb. Consequently, AN041 was diagnosed as VATER association (Quan and Smith 1972) with aniridia. However, patient AN042, a younger brother of AN041, showed only aniridia. Three members of generation I and II in family 4 showed strabismus, whereas the mother of AN041 and AN042 was apparently normal.

DNA samples

The molecular genetic study was approved by the Institutional Review Board for Human Genetic and Genomic Research of Hamamatsu University School of Medicine, and the procedures conformed to the tenets of the Declaration of Helsinki. High molecular weight genomic DNA was isolated from peripheral blood leukocytes. Informed consent was obtained from the patients and non-patient members of their families.

Direct DNA sequencing

The genomic regions corresponding to the 13 exons of the *PAX6* gene were amplified with PCR using the primers previously described (Glaster et al. 1992). The reaction condition was as follows: initial denaturation at 94°C for 10 min, 30 cycles of denaturation at 94°C for 30 s,

annealing at 60°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. The PCR products were separated on 2% agarose gel electrophoresis and visualized by ethidium bromide staining. Agarose gel blocks containing amplified products were excised and DNAs were purified using QIAquick Gel Extraction Kit (Qiagen). The purified PCR products were sequenced on an Applied Biosystems model 3100 automated sequencer (Perkin-Elmer Applied Biosystems Division, CA) with primers that were used for PCR amplification.

Cloning and sequencing of each allele

In the cases where some sequence abnormality was detected by direct sequencing, two alleles were cloned to examine their sequences separately unless otherwise described. The purified PCR products for direct sequencing were ligated to the 3.9-kb pDrive vector (Qiagen) according to the manufacturer's instructions. The construct was transformed into TOP10 *E. coli* cells, and plated on 50 µg/ml kanamycin, 80 µg/ml X-gal containing LB agar plates. Plasmids were isolated from each colony of the transformed cells by the alkaline lysis method. Sequencing was performed using M13 universal primers.

Results

Mutation analysis

We carried out a mutation analysis of the *PAX6* gene in six patients with aniridia from four families. Four types of mutations including three novel ones were found (Fig. 1; Supplemental Fig. 2). All of these mutations were heterozygous in patients. A summary of the patient diagnoses and mutation types are listed in Table 1.

Patients AN011 (son) and AN012 (mother) had a mutation 474delC in exon 5 (Supplemental Fig. 2A), which results in frameshift and premature termination of translation within the paired domain of *PAX6* protein. The mutation was not detected in his unaffected father

Table 1 Mutations in the *PAX6* gene of aniridia patients analyzed in this study

Patient ID	Remark	Exon	Domain	Type	Nucleotide change	Consequence	Phenotype
AN011	Son of AN012	5	Paired	Deletion	474delC	Frameshift	Aniridia
AN012	Mother of AN011	5	Paired	Deletion	474delC	Frameshift	Aniridia
AN021		7	Link	Insertion	786_787ins10	Frameshift	Aniridia
AN031		6	Paired	Deletion	678_688del11	Frameshift	Aniridia
AN041	Sister of AN042	6	Paired	Indel	572_575delAATCins14	Frameshift	Aniridia, VATER association
AN042	Younger brother of AN041	6	Paired	Indel	572_575delAATCins14	Frameshift	Aniridia

These mutations are being submitted to The Human *PAX6* Mutation Database of MRC, UK

Fig. 1 *PAX6* gene mutation analysis of case AN042. **A** A trace pattern of direct sequencing of the patient DNA. A genomic region including exon 6 was amplified with PCR and subjected to direct sequencing. The sequence in the *top line* is an output of the automatic sequencer. Since the region with mutation showed mixed pattern of mutated and normal allele (**A**), *curves* corresponding to normal sequence (**B**) was manually removed using an image-editing software Photoshop (Adobe) to visualize only the mutated sequence. **B** Normal

sequence of the tested region. The four bases with *single underline* indicate the deleted part of the mutated allele (**C**). **C** A trace pattern of the mutant allele generated by eliminating normal sequence. *Gray letters* show nucleotide sequence determined by manual reading. The 14 bases with *double underline* indicate the inserted sequence in the mutant allele. *Dotted lines* show a common sequence between normal and mutant alleles. A *wavy line* indicates a sequence with a quasi-palindromic structure

(Supplemental Fig. 1). This type of mutation was previously reported and included in the Human *PAX6* Mutation Database. The aniridia symptom of these patients was mild.

Patient AN021 revealed a novel mutation 786_787ins10, ten-base (CGCAGACGGC) insertion in the sequence between Nt785 and Nt786 (Supplemental Fig. 2B). The inserted ten-base sequence is an exact duplicate of the sequence between Nt776 and Nt785. This mutation results in frameshift and premature termination of translation in the link domain of *PAX6* protein.

Patient AN031 of family 3 with apparently sporadic aniridia showed another novel mutation 678_688del11 (Supplemental Fig. 2C), which also causes frameshift and premature termination in the paired domain of *PAX6* protein. While no other aniridia patients were found in this family (Supplemental Fig. 1), the mother and great-grandfather of AN031 had color blindness. However, the relation between *PAX6* mutation and color blindness is unclear.

Patient AN041 and her younger brother AN042 revealed another novel mutation, 572_575delAATCins14 (Fig. 1), which results in frameshift and premature termination in the paired domain of *PAX6* protein. The patient AN042 presented only aniridia, whereas the patient AN041 was accompanied by VATER association and their mother appeared normal. As a pathogenic mutation for VATER association, A–G point mutation at nucleotide position

3,243 of mitochondrial DNA was previously described (Quan and Smith 1972; Damian et al. 1996). However, such point mutation was not detected in the patient AN041 (data not shown). Strabismus was seen in several members of family 4, and it may be related to the same *PAX6* mutation.

Database construction

To support understanding of the relationship between *PAX6* gene mutation and clinical phenotype, we made a database using the *MutationView* system (<http://mutview.dmb.med.keio.ac.jp/>) (Minoshima et al. 2001). A typical screen shot of the *MutationView* displays cDNA structure (exon 5–exon 13) and protein domains (Supplemental Fig. 3). The height of the bar represents the family number of each mutation that appeared in the literature and the Human *PAX6* Mutation Database at MRC. Since novel mutations found in exons 5, 6 and 7 are hard to see in the screen shot, they were highlighted in B.

Discussion

We have described the *PAX6* mutations including three novel types in Japanese aniridia patients. All the analyzed cases revealed deletion, insertion or indel mutation with

frameshift, resulting in premature termination of translation. Previously, several reports were made regarding Japanese *PAX6* mutations mainly for non-typical aniridia or other disease cases, in which missense mutations were predominantly associated (Azuma et al. 1996, 1999, 2003; Sonoda et al. 2000; Yasuda et al. 2002). Two of these, V7D (exon 5a) and S363P, were associated with Peters anomaly, but not aniridia (Azuma et al. 1999, 2003). R125C mutation, first reported from Japan, caused isolated foveal hypoplasia (Azuma et al. 1996). Sonoda et al. reported P118R mutation, which is associated with congenital nystagmus, but not aniridia (Sonoda et al. 2000). Another report by Yasuda et al. described three types of mutations (590ins7, 790del4 and R203X) in five Japanese aniridia patients, all of which are frameshift or nonsense mutations, resulting in premature termination in the paired or link domain of *PAX6* protein (Yasuda et al. 2002). Our present results confirmed a general tendency that frameshift/nonsense mutations of *PAX6* are frequently associated with aniridia, whereas missense mutations are often found in non-aniridia cases (van Heyningen et al. 2002).

Of three novel mutations, the mutation 572_575delA-ATCins14 found in the patients AN041 and AN042 is unique and interesting. The 7-base sequence (GGTAGTA) of the 11-base insertion is exactly same as the sequence 579–585, while the 11-base insertion surrounded by those 7-base sequences shows a quasi-palindromic structure (Fig. 1c). These unique sequence characteristics may have some relation to generating unusual type of mutation.

We have reported an aniridia case AN041 accompanied by VATER association (Quan and Smith 1972; Damian et al. 1996). To our knowledge, this is the first report of an aniridia case accompanied by VATER association.

The area affected with VATER association is generally derived from the mesoderm or endoderm, while aniridia is a disorder of the iris that is in the lineage of the ectoderm. Since the *PAX6* gene is generally known to function in differentiating neuronal cells derived from the ectoderm, it is understandable that aniridia is caused by *PAX6* mutation. However, we do not know whether VATER association is caused by the detected *PAX6* mutation or not.

The database *MutationView* has various functions including graphical display of all mutations in the genomic and cDNA sequences and protein amino acid sequence with functional domain, frequency of each mutation, or their change by mutation, and functions to classify mutations such as disease symptom and inheritance pattern (Supplemental Fig. 3). This database should be useful to

overview *PAX6* mutations and genotype–phenotype correlation (Minoshima et al. 2001).

In conclusion, this study added three novel types of mutations that are associated with the development of aniridia.

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