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Two nonsense mutations of *PAX6* in two Japanese aniridia families: case report and review of the literature

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PURPOSE. *To identify PAX6 mutations in patients from four Japanese families with aniridia.*
METHODS. *Polymerase chain reaction (PCR)-single stand conformational polymorphism (SSCP) analysis (SSCA) was performed in probands of the families, and restriction analysis using MaeIII or AvaI was carried out in other affected family members.*
RESULTS. *PCR-SSCA demonstrated in the proband from one family an extra-band in the PCR product for PAX6 exon 8. Base sequence analysis revealed that the patient is a heterozygote for a C to T transition mutation at codon 203. DNAs from the patient and another affected member in the same family were cut with MaeIII into two fragments, while non-affected members in the family showed only one MaeIII fragment, the result confirmed the mutation. In another family, PCR-SSCA revealed an extra-band in the PCR product for exon 9. Sequencing detected a C→T substitution at codon 240 in the patient, the mutation resulted in loss of an AvaI site. AvaI cleavage analysis confirmed the mutation in the patient. The two transition mutations observed in the two families also predict the conversion of arginine to a stop codon (R203X and R240X, respectively) around the homeodomain (HD), leading to the truncation of the PAX6 protein within its glycine-rich region. No abnormal SSCP bands or abnormal restriction fragments were detected in patients from the other two families.*
CONCLUSIONS. *The two mutations sites identified in the two families, one at codon 203 and the other at codon 240, are those most frequently observed among 118 previously reported PAX6 mutations. This indicates that the two mutations are two hot-spots in the gene. (Eur J Ophthalmol 2000; 10: 167-72)*

KEY WORDS. *Aniridia, Hot-spot of mutation, PAX6, Transition mutation*

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INTRODUCTION

Aniridia is a heritable eye anomaly (MIM 106200) (1), affecting not only the iris, but also the cornea, anterior chamber and its angle, lens, retina, and optic nerve (2). Aniridia can be diagnosed at birth and has almost the same prevalence (1/64,000 - 1/96,000 birth) in different populations. About two thirds of cases are familial, and the disease is inherited in an autosomal dominant fashion with almost complete penetrance but variable expressivity (3). The human paired-box gene (*PAX6*) responsible for aniridia, the human ortholog to *Pax6* for the small-eye mutant and the Drosophila eyeless gene (4-6), was isolated using positional cloning strategy as a candidate gene for aniridia or WAGR syndrome from a chromosome deletion at region 11p13 (7, 8). *PAX6* is transcribed as a 2.7-kb mRNA and encodes 422 amino acids that contain at least four functional domains: paired domain (PD), homeodomain

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(HD), link domain (LNK), and proline-serine-threonine rich domain (PST) (9).

PAX6/*Pax6* is one of nine members of the *PAX*/*Pax* gene family in the human/mouse (10), and its gene product acts as a transcriptional regulator. *In situ* hybridization study of the mouse *Pax6* showed that it is expressed in the developing neuroectoderm and subsequently in the developing eye and nose tissues, as well as in the forebrain, hindbrain and neural tube (4, 6, 11). *PAX6*/*Pax6* is thus considered a master control gene for eye development.

PAX6 mutations have been identified in many aniridia patients from various ethnic groups (12), as well as in patients with Peters' anomaly (13), autosomal dominant keratitis (14), congenital cataract and corneal dystrophy (11), and familial isolated foveal hypoplasia (15). *PAX6* mutations involved in the various eye diseases suggest a genotype-phenotype correlation (11).

This report describes mutation analysis in four Japanese families with aniridia.

MATERIALS AND METHODS

Family 1

Patient 1 (III-5, Fig. 1a) was a 54 year-old Japanese man. Ophthalmologic examinations revealed horizontal nystagmus, bilateral, peripheral and superficial corneal opacification at the epithelial and subepithelial levels, absence of the palisades of Vogt, bilateral iris hypoplasia, cataract, and no macular reflex. Intraocular pressure was normal bilaterally. His visual acuity was 20/200 in both eyes. His 13-year-old daughter (Patient 2, IV-6) had similar ocular abnormalities, but another daughter (IV-5) was phenotypically normal. There were six other relatives who had or were said to have aniridia. The condition in the family was consistent with an autosomal dominant inheritance with complete penetrance.

Family 2

Patient 2 (II-I, Fig. 1b), a 21-year-old Japanese man, was a sporadic case. Ophthalmologic abnormalities included horizontal nystagmus, bilateral corneal opacification similar to Patient 1, absence of palisades of Vogt, bilateral iris hypoplasia, and congenital cataract without glaucoma or optic nerve hypoplasia. The macular reflex was absent and intraocular pressure was normal bilaterally. His visual acuity was 20/400 in both eyes.

Family 3

Patient 3 (II-4, Fig. 1c), a 69-year-old Japanese man, had peripheral corneal opacification at the epithelial-subepithelial level, absence of palisades of Vogt, bilateral iris hypoplasia and cataract, and had had bilateral cataract surgery at age 55 years. The macular reflex was absent. Intraocular pressure had been high bilaterally, but was well controlled by drug treatment. There was no optic nerve hypoplasia. His left visual acuity was 20/100 and right acuity 20/50. A 33-year-old daughter (III-2), a 29-year-old son (III-3) and a one-year-old granddaughter (IV-1) all had similar ocular abnormalities.

Family 4

Patient 4 (II-5, Fig. 1d) was a 51-year-old Japanese man, who had a history of left retinal detachment. He had received surgical treatment but the retina was not reattached, leaving phthisis bulbi in his left eye. He had had right cataract surgery at 43 years of age. Ophthalmologic examination revealed horizontal nystagmus, bilateral corneal opacification, absence of palisades of Vogt, bilateral iris hypoplasia, and cataract. The macular reflex was absent, and intraocular pressure was normal. His right visual acuity was 20/100 and null in the left eye. His 21-year-old son (IV-1) had similar ocular abnormalities.

DNA studies

We studied five aniridia patients (Nos 1-5) and several non-affected members of these families. After obtaining informed consent, genomic DNA was extracted from their peripheral blood leukocytes. The DNA was amplified for 33 cycles by PCR using 13 sets of oligonucleotides as primers for exons 1-8 and 10-13 of *PAX6*, according to the method of Glaser and Walton (9). In addition, a sense/antisense primer set to amplify exon 9 was designed as 5' TTACTCTTTCAGAGTT TGAG/TCGGTACCTGTATTCTTGCT-3'. PCR conditions for exons 1-4, 6-8, 10-11 and 13 were as follows: ini-

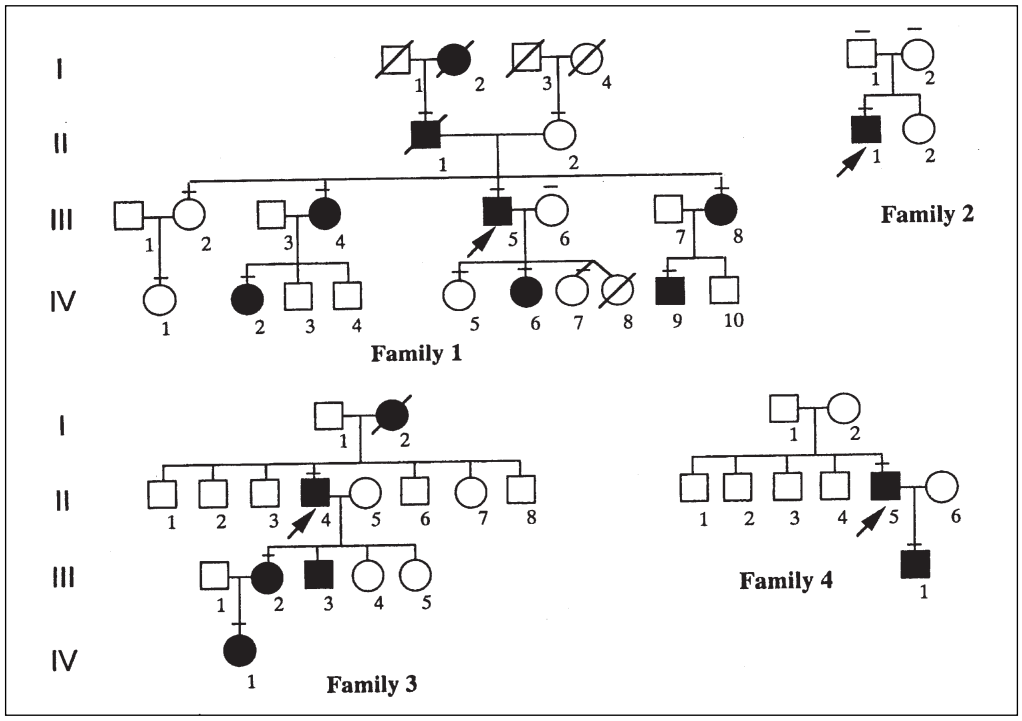

Fig. 1 - *Pedigrees of Families 1, 2, 3, and 4. Short bars above individual symbols indicate those examined by us.*

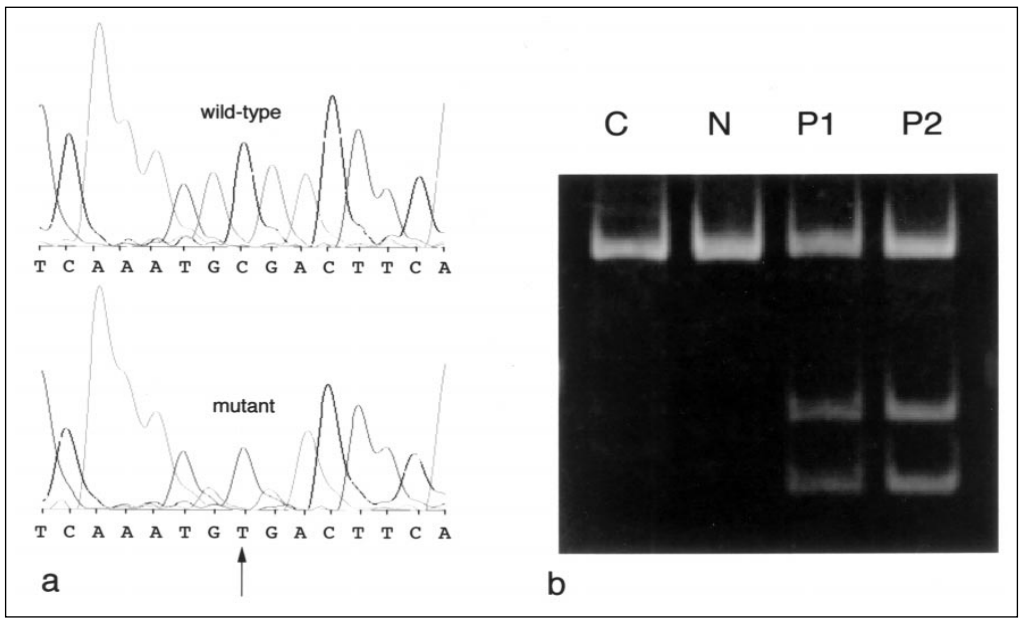

Fig. 2 - *A C-to-T substitution at codon 203 of PAX6 in Patient 1 identified by sequence analysis (**a**), and three Mae*III *fragments in Patients 1 and 2 detected by enzyme cleavage analysis (**b**). Arrow indicates the substitution. C, N, P1, P2 are a normal control and a non-affected person, Patients 1 and 2 from Family 1, respectively.*

tial denaturation at 95°C for 5 min, further denaturation at 95°C for 1 min, annealing at 62°C for 2 min, initial extension at 72°C for 2 min and further extension for 7 min. The conditions for other exons were the same except that annealing was done at 64°C for exons 5 and 12, and at 55°C for exons 5a and 9.

SSCP analysis (SSCA) was carried out according to the method of Orita et al (16). Briefly, PCR products were denatured to single strands, electrophoresed with non-denaturing polyacrylamide gel and autoradiographed. In parallel, the PCR products were subcloned into pGEM-T vector (Promega, USA), and sequenced with Cy5 Sequencing Kit (Pharmacia Biotech, Sweden) and Automated Laser Fluorescent Se-

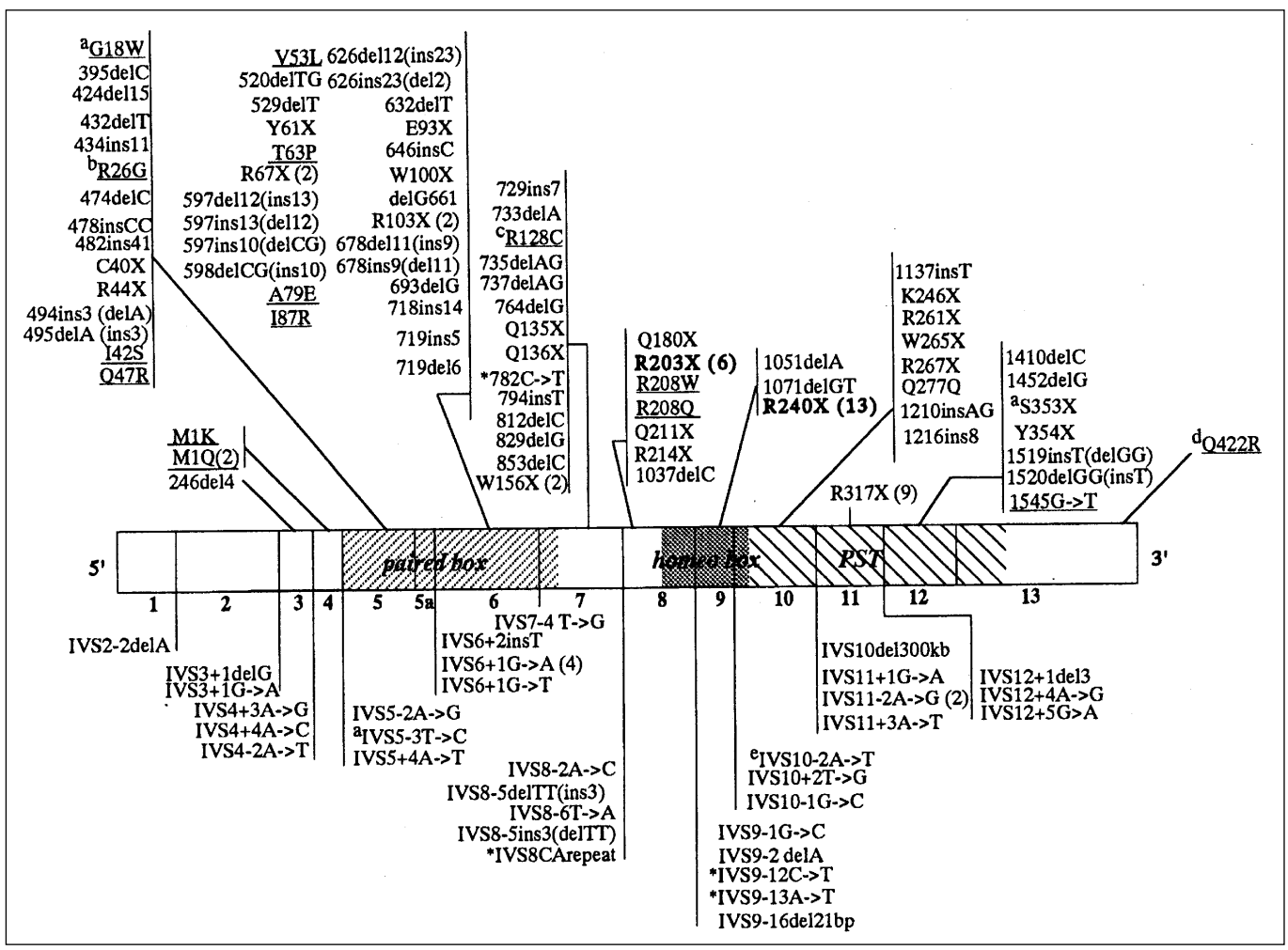

Fig. 3 - *Reported PAX6 mutations and disorders. Mutations above and below the bar for PAX6 are those observed in exons and introns, respectively. Numbers in parentheses are cases reported. Mutations in bold letters and underlined depict those identified in the present study and missense mutations, respectively. Superscripts indicate a) congenital cataract, b) Peters' anomaly, c) isolated foveal hypoplasia, d) anterior segment anomalies, and e) autosomal dominant keratitis. Mutations with asterisks are those not leading to abnormality.*

quencer, ALFexpress (Pharmacia Biotech). In addition, to determine whether patients 1, 3, 4 and 5 had the reported mutations, the PCR products were digested with five restriction enzymes, *Mae*III, *Hae*II, *Dde*I, *Ava*I and *Hph*I. The digests were electrophoresed on 8% polyacrylamide gel, and the gel was stained with ethidium bromide for visualization.

RESULTS

SSCA for *PAX6*-exon 8 revealed an extra band on the autoradiogram of Patient 1 (III-5, Fig. 1a) in Family 1, and a similar fragment was observed in Patient 2 (IV-6, Fig. 1a) suggesting they had mutations in exon 8 (data not shown). Sequence analysis of DNA from Patient 1 showed a C-to-T transition at codon 203 (Fig. 2), resulting in the conversion of arginine to a stop codon (R203X). This nonsense mutation predicts the truncation of the *PAX6* protein within the glycine-rich region (Fig. 3) and creates a *Mae*III restriction site in the DNA.

The restriction enzyme cleavage study in Family 1 indicated that Patients 1 and 2 both had two *Mae*III-cut DNA fragments in addition to an uncut fragment, while the non-affected individual (IV-5 Fig. 1a) had only one undigested *Mae*III fragment (Fig. 2). Thus, Patients 1

and 2 were heterozygotes for the mutation.

In Family 4 (Patient 5, II-5 in Fig. 1d), SSCA for *PAX6*-exon 9 revealed an aberrant fragment, resulting from a C-to-T substitution at codon 240. By this nonsense mutation (R240X), the DNA was expected to lose an *Ava*I site, and an enzyme cleavage study detected an undigested *Ava*I-fragment in the patient. Thus Patient 5 is a heterozygote for the mutation. No aberrant SSCP fragments were found in Patients 3 and 4, and restriction analyses using *Mae*III, *Ava*I or other enzymes also gave normal results in these patients.

DISCUSSION

Of the four aniridia families analyzed in the present study, two were found to have C-to-T substitutions in *PAX6*: one at codon 203 and the other at codon 240. The same mutations have been reported in respectively 5 and 12 Caucasian families (17, 18). Among the 151 *PAX6* mutations so far known (1), including the two in our families, single-base substitutions account for 62%, while deletions and insertions are less frequent: 38% (Fig. 3). The substitutions include far more transitions than transversions. It is of interest that many of these mutations occur at around the PD (37.1%), HD (19.2%) and PST (16.6%), but a few unique coding mutations are reported in more 5'-coding regions (exons 1-4). One explanation for the frequent mutations in HD may be the presence of a hypermutable CpG dinucleotide in codon 240. Generally, a C-to-T transition is frequent, because when cytosine is methylated, the resulting 5-methylcytosine may be converted to thymine through deamination, and the DNA repair system is not involved in this step. Therefore the CGA triplets at codons 203 and 240 observed in our families are hot spots of mutation.

Almost all the 151 patients reported were heterozygotes for a *PAX6* mutation, and no homozygotes have been described, indicating that all the mutations lead to loss (or reduction) of function of *PAX6*, and in homozygous individuals this may be lethal. This argument is supported by the lethality of the mouse homozygote, which had anophthalmos and severe CNS defects (11). A compound heterozygote was only reported by Glaser et al (11), where a child of a father with bilateral cataracts had very severe ocular, craniofacial and CNS malformations. The majority of the 151 patients had nonsense mutations, but a few (16 patients) had missense mutations, and four were reported not to have any eye abnormalities (Fig. 3) (1, 19). The phenotype in four of the 16 patients with missense mutations was Peters' anomaly, congenital cataract, familial isolated foveal hypoplasia, or anterior segment anomalies. Thus, missense mutations tend to lead to milder manifestations. However, since the eight missense mutations lay at various domains in PAX6 (Fig. 3), we cannot find a concrete phenotype-genotype correlation in this subgroup.

Tang and co-workers compared the functions of two missense mutations: one was conversion of arginine to glycine at codon 26 (R26G) which caused Peters' anomaly, and the other a change from isoleucine to arginine at codon 87 (I87R) that caused aniridia (20). As a result, the R26G mutant failed to bind a subset of PD binding sites but did bind other sites and transactivated promoters containing these sites, whereas the I87R lost the ability to bind DNA at these sites (20). Thus, their data supported the theory that aniridia is mainly caused by haplo-insufficiency of PAX6 (21), whereas Peters' anomaly results from an allele that is defective but still keeps its activity to a certain degree.

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Abbreviations

CNS: Central nervous system
PAX6: Paired box homeotic gene 6
PCR: Polymerase chain reaction
SSCP: Single-stranded conformational polymorphism
WAGR: Wilms tumor, aniridia and genitourinary abnormalities and mental retardation

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