Missense Mutations in the PAX6 Gene in Aniridia

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PURPOSE. Aniridia is caused by a mutation of the PAX6 gene. Haploinsufficiency of the gene product is thought to result in the aniridia phenotype, because most mutations thus far detected have been large deletions encompassing the entire gene and nonsense, frameshift, or splice errors that result in premature translational termination on one of the alleles. Only two missense mutations have been detected in aniridia pedigrees, each of which occurs in its paired domain or homeodomain. In this study, four novel missense mutations were found in three aniridia pedigrees.

METHODS. Polymerase chain reaction-single-strand conformation polymorphism analysis and sequencing of the PAX6 gene were performed using genomic DNA of three aniridia pedigrees and more than 100 healthy control subjects.

RESULTS. Three mutations occurred in the N-terminal subdomain of the paired domain, namely N17S, I29V, and R44Q, the first two of which were detected on the same allele of one patient. The other mutation (Q178H) was in the linking portion of the paired domain and homeodomain.

CONCLUSIONS. These missense mutations give rise to haploinsufficiency by another route, because the missense mutations presented here resulted in an aniridia phenotype indistinguishable from that caused by a heterozygous deletion of the entire PAX6 gene. (Invest Ophthalmol Vis Sci. 1998; 39:2524–2528)

The Pax gene consists of a family of developmental control genes; nine members have been isolated in vertebrates since paired originally was identified as a segmentation gene in Drosophila melanogaster.1,2 The encoded proteins are transcriptional regulators with DNA binding through a conserved domain consisting of 128 amino acids (paired box).3 Some Pax genes share another conserved domain, homeobox, which also provides DNA binding.4,5 The PAX6 gene has been isolated as a candidate gene for aniridia by positional cloning based on an overlapping region of chromosomal deletions at 11p13 that are observed in some patients with aniridia, especially in those with Wilms' tumor, genitourinary abnormalities, and mental retardation (known as the WAGR syndrome).6 In addition to large deletions encompassing the whole gene, 100 mutations have been detected in patients with sporadic and autosomal dominant familial aniridia7–12 and are summarized in the database at http://www.hgu.mrc.ac.uk/Softdata/PAX6/. Most of these mutations cause translational termination by nonsense and frameshift mutations and by splice errors. Because the mutation occurs on one of the alleles, haploinsufficiency of the gene product has been suggested to cause the aniridia phenotype.13 Phenotypes of heterozygous and homozygous mutants of the small eye (Sey) locus, where a mouse homologue of the PAX6 gene is located, also support this hypothesis.14–16 In addition, two missense mutations in the paired domain and homeodomain have been found in aniridia pedigrees.10,17 Other mutations of the PAX6 gene have been shown to develop various manifestations of ocular morphogenesis. Missense mutations in the paired domain have been detected in Peters' anomaly18 and in isolated foveal hypoplasia.19 A missense mutation in the proline-serine-threonine activation (PST) domain has been found in an anterior segment anomaly,20 and a nonsense mutation in the PST domain causes corneal dystrophy.21 Thus, the PAX6 gene seems to play a role in a variety of processes during ocular morphogenesis, and the phenotype-genotype correlation must be carefully examined to elucidate the functions of PAX6. We report four novel missense mutations detected in patients with aniridia that will provide useful information for studies of the PAX6 gene.

MATERIALS AND METHODS

DNA Samples

These studies were conducted in accordance with the World Medical Association Declaration of Helsinki. Our use of human subjects was conducted with patients' informed consent, approved by the National Children's Hospital Experimental Review Board, and deemed exempt from human subject regulations. We analyzed PAX6 mutations in eight families (autosomal dominant trait) and in 26 sporadic patients with aniridia. After obtaining informed consent from all patients, blood samples from 1 patient with familial aniridia and 3 patients with sporadic aniridia were collected from peripheral veins into lithium-heparin tubes. Unaffected individuals who were immediate family members also were examined. Genomic DNA was prepared from isolated leukocytes using a standard phenol–chloroform procedure.22 The DNA samples from healthy control subjects have been described pre...
viously.22 Chromosomal analysis was also performed. High-resolution G-banded chromosomes (750 bands) were obtained from phytohemagglutinin synchronized blood lymphocyte culture for 72 hours. Twenty metaphases were analyzed.

Polymerase Chain Reaction–Single-Strand Conformation Polymorphism Assay and Sequencing

Polymerase chain reaction (PCR) primers used for amplification of 14 exons of PAX6 were synthesized using a DNA/RNA synthesizer (model 392; Applied Biosystems, Urayasu, Japan) as described in a previously published report.22 The PCR conditions we used were essentially the same as in our previous report and were modified as follows. The annealing temperature was adjusted to 55°C for exons 5a and 13 and to 60°C for all others, and Mg++ concentration was 1.5 mM. Single-strand conformation polymorphism (SSCP) analyses were carried out using automated mini-gel electrophoresis coupled with silver staining (Phastsystem; Pharmacia, Little Chalfont, UK).22 Because DNA fragments generated by PCR for exon 13 were large, the products were digested into two fragments with HindIII before being subjected to electrophoresis. SSCP analyses also were performed after radiolabeling with α-32P-dATP concentrations of glycerol and running temperatures. Nucleotide sequences were determined after cloning on pUC18 using a Sequenase version 2 kit (Amersham, Cleveland, OH) with PCR primers or universal primers in pUC18. After 10 independent subclones had been analyzed in each case, positive was scored when at least four subclones showed the same mutation. The mutations were also confirmed by direct sequencing from the genomic DNA.

CASE REPORTS

In the following three pedigrees with aniridia, missense mutations of the PAX6 gene were identified.

Patient 1 was a 9-year-old girl with visual impairment and nystagmus. She had bilateral total aniridia, cataract, and foveal hypoplasia with visual acuities of 0.1 and 0.1 in the right and left eyes. She had no systemic abnormalities, was of normal size and intelligence for her age, and had a normal karyotype (46,XX). Her parents and siblings had no ocular abnormalities.

Patient 2 was a 21-year-old man with visual impairment and nystagmus. He had bilateral total aniridia, cataract, and foveal hypoplasia with visual acuities of 0.1 and 0.1 in the right and left eyes. He had no systemic abnormalities, was of normal size and intelligence for his age, and had a normal karyotype (46,XY). His parents and siblings had no ocular abnormalities.

Patient 3 was a 6-year-old boy with visual impairment and nystagmus. He had bilateral total aniridia, cataract, and optic nerve hypoplasia with visual acuities of 0.05 and 0.1 in the right and left eyes, respectively. He had no systemic abnormalities, was of normal size and intelligence for his age, and had a normal karyotype (46,XY). His 35-year-old mother also had bilateral total aniridia and a cataract with a visual acuity of 0.02 in her left eye. Her right eye had no light perception vision because of a vitreous hemorrhage of unknown origin. The fundus of her right eye had optic nerve hypoplasia; the fundus of her left eye was not visualized because of hazy media. She was otherwise normal. No other members of the immediate family had ocular abnormalities.

RESULTS

Chromosomal Anomalies and PAX6 Mutations

In 34 pedigrees with aniridia, chromosomal analysis demonstrated deletions at 11p13 in one respective allele of 3 sporadic patients. Heterozygous mutations of 6 nonsenses, 4 frameshifts (3 insertions and 1 deletion of a nucleotide), and 3 splice junction errors were found in 3 families and 8 sporadic patients. One nonsense mutation and 1 splice error were present on the same allele of 1 sporadic patient. These truncation mutations will be reported elsewhere with correlation to their phenotypes. Four missense mutations were also found in 1 family and in 2 sporadic patients described above.

Missense Mutations

We analyzed genomic DNA isolated from leukocytes of the family members. Genomic DNA covering each of 14 exons was amplified by PCR and then subjected to SSCP analysis. Abnormal patterns in exon 5 were found in patients 1 and 2 and in exon 8 of patient 3 and his affected mother but not in unaffected members of the immediate families or in more than 100 healthy control subjects. The SSCP pattern indicated a heterozygous mutation. Sequencing analyses revealed the following mutations on one allele in each patient; no other changes were detected after careful analysis of PCR products from patients' genomic DNA. Patient 1 had two nucleotide substitutions and insertion of a short oligonucleotide on the same allele; nucleotide substitutions from A to G occurred at the 467th and 502nd positions of its cDNA form (in this study, the numbers of the nucleotide and amino acid were based on the sequence of GenBank Accession No. M93650) that caused amino acid substitutions N17S and I29V, respectively. The mutated allele had an additional insertion of 12 nucleotides in intron 5, and 10 nucleotides after the splice donor site of exon 5 (Fig. 1). Patient 2 had a nucleotide substitution from G to A at the 548th position, which resulted in R44Q (Fig. 2). The affected individuals of family 3 had a nucleotide substitution from G to T at the 951st position, which resulted in Q178H (Fig. 3).

DISCUSSION

We detected four novel missense mutations in three pedigrees with aniridia, three of which occurred in the paired domain of the PAX6 gene. The paired domain, through which the Pax protein binds DNA and functions as a transcriptional regulator, consists of the N-terminal subdomain and the C-terminal subdomain.1,2,23,24 The N-terminal subdomain, which contains two β turns and three α helices, is well conserved among the Pax family members, whereas amino acids of the C-terminal subdomain, which contains three α helices, are considerably diversified. The N-terminal subdomain is in extensive contact with minor and major DNA grooves. Most missense mutations thus far detected in the Pax family genes occur in this region (R256G in PAX6 in Peters' anomaly,18 five mutations in PAX3 in Waardenburg's syndrome,25–27 and splitoch-delayed (Sp') mice,28 and one mutation of Pax7 in a murine skeleton anom-
FIGURE 1. Mutation analysis of the PAX6 gene for patient 1. Sequencing of the normal and mutant alleles of the patient identified two A to G nucleotide substitutions at the 467th and 502nd positions in exon 5, which resulted in N17S and I29V, respectively. The same allele also had an insertion of 12 nucleotides at the +10 nucleotide position after the 3' splice site of exon 5.

Three of the four missense mutations identified in our patients with aniridia occurred in the N-terminal subdomain of the paired domain. N17S is in the second β turn, I29V in the first α helix, and R44Q in the second α helix. The isoleucine residue at 29 and arginine at 44 are conserved throughout all Pax family members identified to date, and the same can be said of asparagine at 17, which only changes to glycine in Drosophila ey (GenBank Accession No. K79492). The crystal structure study demonstrated that asparagine at 17 is in contact with the sugar phosphate backbone of DNA and with the base of the minor groove and that arginine at 44 also is in contact with the sugar phosphate backbone of DNA. Thus, mutations at these positions may abolish or alter normal DNA binding.

Mutations detected in patient 1 provide an interesting phenomenon. In addition to the two missense mutations described above, an insertion of 12 nucleotides occurred on the same allele. Multiple mutations within a small region are very interesting considering the mechanisms of the generating mutations; however, the insertion itself may not affect PAX6 function because it occurs in intron.

The haploinsufficiency theory of the aniridia phenotype seems to be consistent, but interpretation of PAX6 missense mutations with respect to their function (in the direction of...
phenotypic ocular manifestations) is still controversial. Recently, interesting data have been garnered from an in vivo transcriptional assay and a quantitative electrophoretic mobility shift assay using the mutations previously detected in Peters’ anomaly (R26G in the N-terminal subdomain) and in aniridia (I87R in the C-terminal subdomain). The R26G-mutated protein failed to bind to a subset of the consensus sequences for the PAX6 binding but still kept binding to another set, and even transactivated some promoters. The I87R mutant lost DNA binding to all the consensus sequences tested. The findings seem to be consistent with the haploinsufficiency theory for aniridia in the case of I87R and with a hypomorphomorphic change in Peters’ anomaly by R26G. However, it is still unclear how a mutation in the C-terminal subdomain affects DNA binding through the N-terminal subdomain, because the two subdomains are believed to have distinct DNA-binding abilities and to act independently. Another experimental analysis postulated autoregulation in DNA binding through the paired domain. The isolated N-terminal- and C-terminal subdomains do possess distinct DNA binding. However, when both subdomains are linked as in the PAX6 protein, they negatively regulate the other’s function, that is, some mutations in the N-terminal subdomain not only lose a function controlled by the N-terminal subdomain but also gain a function controlled by the C-terminal subdomain and vice versa. The role of the other mutation, Q178H, that was positioned in the linking portion of the paired domain and homeodomain is also controversial. Functional analyses are under way using PAX6 constructs with mutations detected in this study.

An expression pattern of the PAX6 gene indicates the multiple function of the gene. The gene expresses first in the optic vesicle, subsequently in the eye vesicle, in the lens placode invaginating from the surface ectoderm, in the differentiating retina, and, finally, in the cornea. Peters’ anomaly occurs at an early gestational age of 4 to 5 weeks when the lens separates from the surface ectoderm and mesenchymal cells invade the anterior space; however, aniridia occurs at 8 to 10 weeks when mesenchymal cells and the anterior rim of the optic cup differentiate to the iris. Foveal hypoplasia that often is associated with aniridia occurs at a late stage, because the fovea starts to develop at 30 weeks’ gestation and is complete at 4 months after birth. The same master control gene and isoforms probably are used repeatedly in the morphogenesis of various ocular tissues. It is established that aniridia affects the entire eye, whereas Peters’ anomaly is restricted to the anterior segment and foveal hypoplasia to the posterior fundus. Because numerous ocular tissues differentiate with multiple correlation during development, various phenotypes occur as a result of mutations of the same gene.

References


35. Walther C, Gruss P. Pax6, a murine paired box gene, is expressed in the developing CNS. Development. 1991;113:1435-1449.
