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ARTICLE

Genotype–phenotype correlation of *PAX6* gene mutations in aniridia

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The objective of this study was to investigate the genotype–phenotype correlation of the *PAX6* gene in aniridia. We clinically examined 5 families and 16 sporadic patients with aniridia. We performed chromosomal analysis and PCR analysis of the *PAX6* gene using patient genomic DNA. Chromosomal analysis demonstrated deletions at 11p13 in one allele in four sporadic patients. Seven nonsense mutations, two frameshifts (two insertions), four splice junction errors and two missense mutations were found, and all were heterozygous. The iris phenotype ranged from total to normal in each patient, and the characteristic phenotypes, including cataract, glaucoma or optic nerve hypoplasia, varied widely even among members of the same family. Foveal hypoplasia was detected in all patients except for one. No obvious genotype–phenotype correlation was identified; however, the aniridia phenotype between the two eyes in each patient was quite similar in all patients. Because PAX6 regulates numerous downstream genes and its expression is regulated by several factors during eye development, the aniridia phenotype may be complex even in family members. However, because *PAX6* regulation, resulting from both paternal and maternal alleles associated with *PAX6*, is considered to be roughly similar in both eyes of each patient, the aniridia phenotype may be similar in both eyes of each patient.

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INTRODUCTION

It is well known that aniridia is caused by mutation of the *PAX6* gene.[1,2] The *PAX6* gene codes a transcriptional regulator and is a master control gene in the development of ocular tissues in invertebrates.[3] This is evident from the ectopic formation of eyes on the wings, legs and antennae in *Drosophila* upon targeted expression of the *eyeless* gene, a homolog of the human *PAX6*.[4] Furthermore, an expression pattern of the *Pax6* gene also indicates the multiple functions of the gene in mammals.[5] *In situ* hybridization for *murine Pax6* demonstrated its expression in the developing central nervous system as well as in ocular tissues derived from the ectoderm and neuroectoderm, that is, the corneal epithelium, lens and retina.[6] These findings can explain various phenotypic manifestations in the entire eye of aniridia patients, including corneal opacity, absence of the iris, glaucoma, cataract and hypoplasia of the fovea and optic nerve. These findings also present the possibility that mutations of the *PAX6* gene cause a variety of developmental anomalies of ocular tissues. In addition to aniridia, *PAX6* mutations have been reported in Peters' anomaly,[7] corneal dystrophy[8] and foveal hypoplasia.[9]

The *PAX6* gene was isolated as a candidate aniridia gene by positional cloning from an overlapping region of chromosomal deletions at 11p13, which are observed in some aniridia patients, especially in those with Wilms' tumor, genitourinary abnormalities and mental retardation (WAGR syndrome).[1] In addition to large deletions encompassing the whole gene, more than 330 mutations have been detected in autosomal dominant and sporadic aniridia patients.[10] Because most of these mutations are nonsense, frameshifts or splicing errors that result in premature translational termination on one of the alleles,[11] haploinsufficiency of the gene,[12] or the presence of one amorphic allele, has been suggested to cause the aniridia phenotype.[13] We recently identified five additional *PAX6* missense mutations in four pedigrees.[14] These findings indicate that hypomorphic alleles that significantly disturb an important motif also cause the aniridia phenotype. While different genotypic mutations cause different phenotypes of developmental anomalies, the same *PAX6* mutation sometimes resulted in a variety of aniridia phenotypes that ranged from normal-sized irises to the complete absence of the iris in one pedigree,[15] which indicated that one genotype produces different phenotypes of aniridia. Thus, the genotype–phenotype correlation of *PAX6* mutations in aniridia is still controversial. In the present study, we investigated the correlation between the genotype of amorphic or hypomorphic *PAX6* mutations and the phenotype of aniridia.

MATERIALS AND METHODS

Patients and clinical data

The study included five families (autosomal dominant trait) and 16 sporadic patients with aniridia. Visual acuity was examined by Landordt's ring procedure, and examination of ocular anterior and posterior segments was performed with a slit-lamp biomicroscope and a binocular indirect ophthalmoscope. Glaucoma was diagnosed by measurement of intraocular pressure, examination of the iridocorneal angle, a slit-lamp biomicroscope, and visual field measurement if available. Full-field electroretinographies (ERGs) were recorded in six patients (Patient no. 12, 19 and 21–24) according to the International Society for Clinical Electrophysiology of Vision (ISCEV) standards. Some examinations in infants were performed under general anesthesia when necessary.

Optic nerve hypoplasia was diagnosed when the ratio of DM (the distance from disc to macular) to DD (the mean disc diameter) was greater than 3.2 and the disc showed a typical double ring sign, simultaneously. The pediatrician also evaluated systemic and neurological findings, and screening for the presence of Wilms' tumor was performed by echography.

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Table 1. Mutations and phenotype of each patient

Patient no.	*Family no.*	*Inheritance*	*Exon/Intron*	*Nucleotide change*	*Mutation*	*Amino acid change*	*Pax6 domain*	*Likely NMD*	*Age*	*Sex*	*Aniridia (R/L)*	*CO*	*Cataract*	*Glaucoma*	*FH*	*ONH*	*BCVA*	*WT*	*MR*	*Other remarks*
1		Sporadic	Exon5	c.50A>G	Missense	p.Asn17Ser	PD	No	9	F	Total/total	−/−	+/+	−/−	+/+	−/−	0.1/0.1	−	−	−
2		Sporadic	Exon5	c.131G>A	Missense	p.Arg44Gln	PD	No	21	M	Total/total	−/−	+/+	−/−	+/+	−/−	0.1/0.1	−	−	−
3		Sporadic	Exon5	c.79C>T	Nonsense	p.Gln27X	PD	Yes	9	M	Total/total	−/−	+/+	−/−	+/+	−/−	0.2/0.2	−	−	−
			Intron6	IVS6+1G>A	Splice error	NA	PD	Yes												
4	1	Familial	Exon5	c.109_110insG	Frameshift	p.Ala37fs	PD	Yes	31	F	Total/total	+/+	+/+	−/−	+/+	−/−	0.1/0.1	−	−	−
5	1		Exon5	c.109_110insG	Frameshift	p.Ala37fs	PD	Yes	10	M	Total/total	+/+	+/+	+/+	+/+	−/−	0.1/0.1	−	−	−
6	1		Exon5	c.109_110insG	Frameshift	p.Ala37fs	PD	Yes	2	M	Total/total	−/−	+/+	−/−	+/+	−/−	ND	−	−	−
7	2	Familial	Intron5	IVS5+1G>T	Splice error	NA	PD	Yes	36	F	Total/total	−/−	+/+	+/+	+/+	−/−	0.1/0.1	−	−	Hepatoblastoma
8	2		Intron5	IVS5+1G>T	Splice error	NA	PD	Yes	1	M	Total/total	+/+	+/+	+/+	+/+	−/−	ND	−	+	−
9	3	Familial	Intron5	IVS5+2T>G	Splice error	NA	PD	Yes	64	M	Total/total	−/−	+/+	−/−	+/+	−/−	0.1/0.1	−	−	−
10	3		Intron5	IVS5+2T>G	Splice error	NA	PD	Yes	38	M	Total/total	−/−	+/+	−/−	+/+	−/−	0.1/0.1	−	−	−
11	3		Intron5	IVS5+2T>G	Splice error	NA	PD	Yes	30	M	Partial/ partial	−/−	−/−	−/−	−/−	−/−	0.6/0.6	−	−	−
12		Sporadic	Exon6	c.307C>T	Nonsense	p.Arg103X	PD	Yes	13	M	Total/total	−/−	+/+	−/−	+/+	−/−	0.15/0.15	−	−	−
13		Sporadic	Exon8	c.551_552insG	Frameshift	p.Glu184fs	LNK	Yes	4	F	Total/total	−/−	+/+	−/−	+/+	−/−	ND	−	−	−
14		Sporadic	Exon8	c.607C>T	Nonsense	p.Arg203X	LNK	Yes	11	M	Total/total	−/−	+/+	−/−	+/+	−/−	0. 1/ 0.1	−	−	−
15		Sporadic	Exon8	c.607C>T	Nonsense	p.Arg203X	LNK	Yes	9	F	Partial/ partial	−/−	+/+	−/−	+/+	−/−	ND	−	−	−
16		Sporadic	Exon9	c.718C>T	Nonsense	p.Arg240X	HD	Yes	27	F	Total/total	−/−	+/+	−/−	+/+	−/−	0.3/0.3	−	−	−
17	4	Familial	Exon9	c.718C>T	Nonsense	p.Arg240X	HD	Yes	29	M	Total/total	−/−	+/+	−/−	+/+	+/+	0.08/0.06	−	−	−
18	4		Exon9	c.718C>T	Nonsense	p.Arg240X	HD	Yes	5	F	Total/total	−/−	+/+	−/−	+/+	−/−	0.1/0.1	−	−	−
19		Sporadic	Exon10	c.802G>T	Nonsense	p.Glu268X	PST	Yes	6	F	Total/total	−/−	−/−	−/−	+/+	−/−	0.2/0.15	−	−	−
20		Sporadic	Exon10	c.829C>T	Nonsense	p.Gln277X	PST	Yes	13	F	Total/total	−/−	+/+	−/−	+/+	−/−	0.1/0.1	−	−	−
21		Sporadic	Exon11	c.949C>T	Nonsense	p.Arg317X	PST	Yes	10	M	Total/total	−/−	−/−	+/+	+/+	−/−	0.03/0.1	−	−	−
22		Sporadic	Intron11	IVS11+1G>T	Splice error	NA	PST	Yes	4	M	Total/total	−/−	+/+	−/−	+/+	−/−	0.15/ 0.15	−	−	−
23	5	Familial	Intron11	IVS11+1G>T	Splice error	NA	PST	Yes	7	M	Total/total	−/−	+/+	−/−	+/+	−/−	0.2/0.3	−	−	−
24	5		Intron11	IVS11+1G>T	Splice error	NA	PST	Yes	36	M	Normal/ normal	−/−	+/+	−/−	+/+	−/−	0.2/0.3	−	−	−

Abbreviations: BCVA, best corrected visual acuity; CO, Corneal opacity; F, female; FH, foveal hypoplasia; HD, homeodomain; L, left; LNK, linker region; M, male; MR, mental retardation; N/A, not applicable; ND, not detected; NMD, nonsense-mediated decay; ONH, optic nerve hypoplasia; PD, paired domain; PST, proline/serine/threonine-rich region; R, right; WT, Wilms' tumor. Patients list shows correlation between genotype and phenotype. Family no. 2 and no. 3 are supposed to have splice error at intron 5, while no obvious similarity of clinical phenotype is identified. Sporadic cases of no.14 and no.15 have c. 607C>T mutation, but exhibit different iris phenotype, partial and normal iris. Sporadic case no. 16 and family no. 4 exhibit same genotype, c. 718C>T, while visual acuity shows variety. In addition, only case no. 17 exhibits optic nerve hypoplasia. Meanwhile, phenotype of two eyes of the same patient is almost completely similar.

Table 2. Chromosomal abnormalities and phenotype of each patient

Patient no.	*Inheritance*	*Chromosomal anomaly*	*Age*	*Sex*	*Aniridia (R/L)*	*Corneal opacity*	*Cataract*	*Glaucoma*	*FH*	*ONH*	*BCVA*	*Wilms' tumor*	*MR*	*Other remarks*
25	Sporadic	del(11) (p12p14.2)	1	F	Total/total	−/−	+/+ (cyst)	−/−	+/+	−/−	ND	−	ND	−
26	Sporadic	del(11) (p13p14)	3	M	Total/total	−/−	+/+	−/−	+/+	−/−	0.06/0.08	−	−	−
27	Sporadic	del(11) (p11.2p14.2)	3	F	Total/total	+/+	+/+	+/+	+/+	−/−	ND	−	+	Polydactylia
28	Sporadic	del(11) (p12p14)	8	F	Total/total	+/+	+/+	+/+	+/+	−/−	0.1/0.1	+	+	−

Abbreviations: BCVA, best corrected visual acuity; F, female; FH, foveal hypoplasia; L, left; M, male; MR, mental retardation; ONH, optic nerve hypoplasia; R, right. A chromosomal deletion including 11p13 was detected in four sporadic patients. Almost all eyes exhibit completely hypoplastic iris and fovea, and have cataracts. Mental retardation is observed in patients with cloudy cornea and glaucoma. Wilms' tumor and polydactylia is identified in one patient each. Those two patients are clinically diagnosed as WAGR syndrome.

Chromosomal and genetic analysis

These studies were conducted in accordance with the Declaration of Helsinki. Our use of human subjects was conducted with patients' informed consent, and approved by the Ethics Committee of National Center for Child Health and Development (Approval no. 518). Blood samples of the patients and family members were collected from the peripheral veins. High-resolution G-banded chromosomes were obtained from phytohemagglutinin -synchronized blood lymphocyte culture after 72 h. Twenty metaphases were analyzed. Genomic DNA was prepared from isolated leukocytes using a standard procedure.[14] For mutation analysis, the extracted DNA was amplified using PCR primer sets (Supplementary Table S1) for 13 exons of *PAX6* (NCBI; M93650; http://www.ncbi.nlm.nih.gov/nuccore/189632/) under the PCR conditions previously described.[16] The PCR product was purified and directly sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI 3100 autosequencer (Applied Biosystems, MA, USA), as previously described.[17] The sequences were compared with the reference in the database at the National Center for Biotechnology Information (NCBI; NC_000011.9; http://www.ncbi.nlm.nih.gov/nuccore/NC_000011.9).

RESULTS

Clinical findings

The lists of clinical phenotypes associated with mutations and chromosomal defects are summarized in Tables 1 and 2, respectively. Regarding the aniridia phenotype, the iris was totally absent in 50 eyes (89%), partially absent in 4 eyes (7%) and had an almost normal appearance in 2 eyes (4%). Corneal opacity was observed in 10 eyes (18%), congenital or developmental cataract in 50 eyes (89%), glaucoma in 12 eyes (21%), foveal hypoplasia in 54 eyes (96%) and optic nerve hypoplasia in 2 eyes (4%). Visual acuity ranged from 0.06 to 0.6 but was not greater than 0.3 in any of the eyes except for both eyes of Patient no. 11 without foveal hypoplasia. Wilms' tumor, hepatoblastoma and polydactylia were observed in one patient each, and mental retardation was observed in three patients. A series of ERGs was obtained from six patients (Patient no. 12, 20–24) (Figure 1). Mild to moderate abnormal ERGs were detected in five patients, but ERGs were within the normal limit in Patient no. 19. Patient no. 12, who had a c.307C>T mutation in the paired domain (PD), exhibited abnormal maximum and cone ERG with a mild reduction of amplitude. Patient no. 19, who had a c.802G>T mutation in the proline/serine/threonine-rich region (PST), exhibited a normal ERG response. Patient no. 21, who had a c.949C>T mutation in PST, exhibited subnormal cone ERGs with a mild reduction of cone and 30 Hz flicker amplitude. Patient no. 22, who had an IVS11+1G>T mutation, exhibited nearly negative ERG in maximum response, accompanied by a mild reduction in cone ERGs. Patient no. 23, who was a familial case (family no. 5) and had the same mutation as Patient no. 22, only exhibited a reduced b-wave of maximum response with subnormal Rod ERG. Patient no. 24, the father of Patient no. 23, exhibited a relatively severe phenotype with delay and reduced amplitude throughout the ERGs, except for a subnormal Rod response.

PAX6 mutations

Seven nonsense mutations located in exons 5–11 of the *PAX6* gene, including two frameshifts (two insertions), four splicing errors and two missense mutations, were found in five families and 12 sporadic patients. Among those mutations, eight mutations were associated with PD, two with the linking region, two with the homeodomain and four with PST. Of these nonsense mutations, frameshifts and splicing errors, the transcripts of mRNA were likely degraded by nonsense-mediated decay.[18] Patient no. 3 had two mutations: a nonsense mutation (p.Q27X) in exon 5 and a splicing error in intron 6. These mutations were not found in his parents or siblings (data not shown). Novel mutations, including c.79C>T (p.Gln27X), c.109_110insG (p.Ala2GlyfsX19), IVS5+1G>T, IVS5+2T>G, c.551_552insG (p.Glu9ArgfsX15), c.802G>T (p.Glu268X) and IVS11+1G>T, were identified in the current

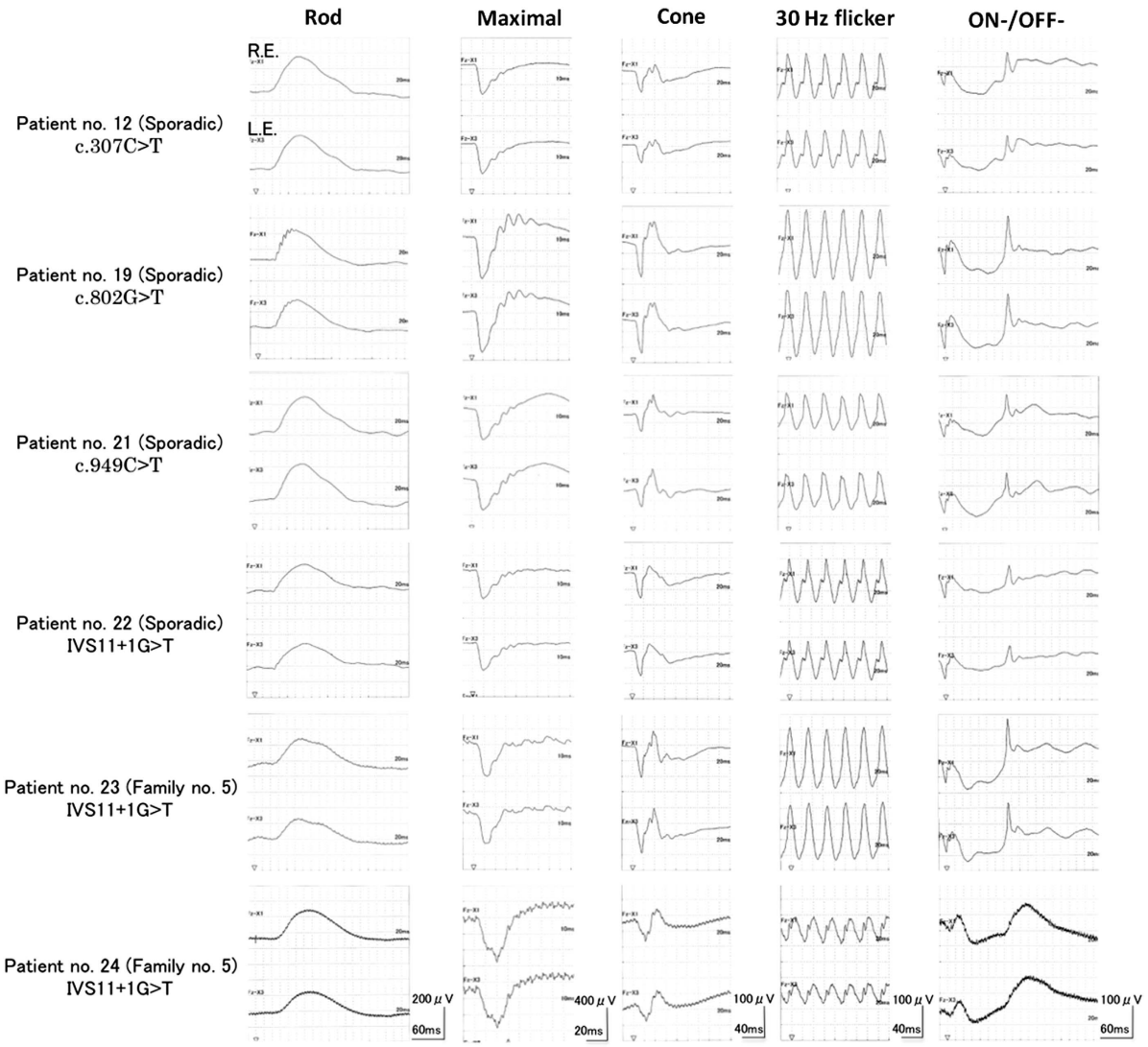

Figure 1. Electroretinographies of the patients (Patient no. 12, 20–24). Patient no. 12 exhibited a mild reduction of maximum and cone ERG. Patient no. 19 exhibited almost normal ERGs. Patient no. 21 exhibited subnormal cone ERGs. Patient no. 22 exhibited a reduced maximum response with amplitude reduction of b-wave, and ON-/OFF ERG were abnormally reduced. Patient no. 23 exhibited a mild reduction of maximum response. Patient no. 24 exhibited a relatively severe abnormality in maximum, cone, 30 Hz flicker and ON-/OFF ERG with both reduction of amplitude and delayed response. R.E., right eye; L.E., left eye. Calibrations are presented in the bottom of the respective ERGs. ERGs, electroretinographies.

study. As previously reported, the two missense mutations c.131G>A (p.Arg44Gln) and c.50A>G (p.Asn17Ser) were found in two sporadic patients.

Genotype–phenotype correlation

Although *PAX6* mutations were found throughout the region from exon 5 to 11, where the PD to PST was coded, no clear correlation was identified between phenotype and genotype. Moreover, even in members of the same family who had the same mutation, the phenotype of aniridia was partially different: the appearances of corneal opacity and glaucoma were variable in family no. 1; the appearance of corneal opacity was variable in family no. 2; the appearances of iris remnant, cataract and foveal hypoplasia were variable in family no. 3; and the appearance of optic nerve hypoplasia was variable in family no. 4. ERG also showed variation even in Patient no. 22–24 who had the same IVS11+1G>T mutation. However, all ocular findings were almost completely symmetrical in both eyes of each patient.

Chromosomal anomalies

A chromosomal deletion including 11p13 was detected in four sporadic patients. In all of the affected eyes, one of which also had posterior lenticular cyst, the iris and fovea were totally absent, and cataracts were observed. Patients with severe phenotype accompanied with cloudy cornea and glaucoma showed mental retardation, and one of the patients was affected by Wilms' tumor, while the other had polydactylia. Those two patients were clinically considered to have WAGR syndrome before chromosomal analysis.

DISCUSSION

The present study demonstrated some novel genotypes of the *PAX6* gene in aniridia. Patient no. 3 had two mutations, a nonsense mutation (p.Q27X) in exon 5 and a splicing error in exon 6. It is well known that *PAX6* mutations' effects are dose-dependent,[19] and the abnormalities depend on the severity of the mutation of *PAX6*. In mutants of the small eye (*Sey*) locus, where the murine homolog of the *PAX6* gene is located,[20] the inactivation of both alleles causes the

eyes not to develop and results in severe craniofacial disorders,[21] whereas inactivation of one allele results in the development of small eyes and mild central nervous system disorders, the latter of which are detectable only at the embryonic stage. In humans, compound heterozygotes are usually embryonic lethal, but two cases have survived with no or small eyes and central nervous system disorders.[19,22] In the present study, Patient no. 3 exhibited only aniridia; thus, the two mutations may be present in one allele and the former one (p.Q27X) first terminates the PAX6 protein.

Three chromosomal abnormalities of 11p13 and seven nonsense mutations, two frameshifts and four splicing errors of the *PAX6* gene were found in our patients. Although some mutations in the PST region are reported to cause run-on translation into the 3′UTR, which results in a dominant negative mutation and a severe aniridia phenotype,[23,24] all mutations in the current study were limited in location from exon 5 to exon 11 and likely induced haploinsufficiency caused by nonsense mediated decay. The other two missense mutations in the N-terminal subdomain of the paired domain had already been identified in Japanese patients,[14] which confirmed that significant disturbance of an important motif by the hypomorphic allele also causes the aniridia phenotype.

In our aniridia patients, an obvious genotype–phenotype correlation was not observed. Although haploinsufficiency of the *PAX6* gene causes aniridia, which is associated with characteristic phenotypes including iris hypoplasia, foveal hypoplasia, cloudy cornea, glaucoma and cataract, the variety of the phenotype was not totally correlated with the genotype. In the current study, even among the same family members, the aniridia phenotype varied widely. Notably, in family no. 5, the father (Patient no. 24) exhibited less severe iris hypoplasia than his son (Patient no. 23); however, the ERGs demonstrated a more severely abnormal phenotype than the son, which also suggested that the phenotype was variable even in patients carrying the same mutation. In a spatio-temporal manner, Pax6 regulates various downstream targets, including Ngn2, Lews X, Wnt7b and δ-crystallin,[25] and Pax6 is regulated by at least six enhancer regions, three promoters, including P0, P1 and Pα,[26] and sometimes by Pax6 itself. In addition, co-activators, such as Sox2 and Maf,[27] and co-repressors, such as Smad3[28] and Nkx6.1,[29] also have vital roles in the expression of downstream targets of Pax6. This complex gene expression associated with *PAX6*, which is regulated by both paternal and maternal alleles, may result in phenotypic variation even in cases with the same *PAX6* genotype. Simultaneously, both of the eyes of each patient, which have the same *PAX6* genotype and associated regulatory mechanisms, may exhibit almost identical phenotypes.

COMPETING INTERESTS

The authors declare no conflict of interest.

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