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PAX6 Mutational Status Determines Aniridia-Associated Keratopathy Phenotype

Congenital aniridia (Online Mendelian Inheritance in Man identifier, 106210) is a rare, severely visually impairing disease caused principally by heterozygous mutation in the paired box 6 (*PAX6*) gene that orchestrates normal ocular development.[1] The disease results in underdevelopment or abnormal development of eye structures including the cornea, leading to a bilateral and progressive limbal stem cell insufficiency and conjunctivalization of the cornea called aniridia-associated keratopathy (AAK). However, clinical manifestation of AAK, rate of progression, and prognosis can vary widely across individuals, precluding the development of general guidelines for treatment. Congenital aniridia can result from any of more than 400 unique mutations in the *PAX6* gene that may lead to a spectrum of clinical phenotypes.[2] Aniridia-associated keratopathy phenotype can vary from a fully transparent cornea to a thick, opaque, vascularized pannus at any stage of life. As clinical genetic analysis becomes more sophisticated and widespread, the clinical consequence of various *PAX6* mutations requires more detailed attention. However, to date, genotype−phenotype studies in aniridia describe the entire eye,[3,4] providing only general assessment of corneal opacity. Accordingly, we performed detailed clinical characterization of AAK phenotype across a range of ages and in parallel documented *PAX6* mutational status to determine how genotype influences the clinical phenotype of AAK. Adult and pediatric patients with clinically diagnosed congenital aniridia included in a patient registry maintained at the Saarland University Medical Center, Department of Ophthalmology, Homburg/Saar, Germany, were identified. Patients provided blood samples for analysis at centers in Germany specialized in clinical genetics analysis and counselling. Genetic datasets were harmonized to match with the entries of the Leiden Open Variation Database (https://www.lovd.nl/) referenced to GenBank sequence number NM_000280.4 from the National Center for Biotechnology Information. Mutations were checked against ClinVAR, Leiden Open Variation Database, and EXAC databases and a Google search. If the mutation was not found, it was regarded as novel. Clinical examinations were conducted at the Department of Ophthalmology, Saarland University Medical Center, and consisted of slit-lamp biomicroscopy (Haag-Streit, Koeniz, Switzerland) with digital photography to perform detailed grading of AAK, Cochet-Bonnet esthesiometry (Luneau Technology, Pont-de-l'Arche, France), anterior segment swept-source OCT (Casia2; Tomey GmbH, Nürnberg, Germany) for corneal thickness measurement, and laser scanning in vivo confocal microscopy (Heidelberg Retina Tomograph 3 with Rostock Corneal Module; Heidelberg Engineering, Heidelberg, Germany) to determine central corneal epithelial phenotype and subbasal nerve density. A detailed clinical phenotypic assessment of AAK was performed for all eyes to assign an AAK grade from 0 to 4, as shown in Figure S1 (available at www.aaojournal.org). Statistical regression analysis was performed using IBM SPSS Statistics version 25 (IBM Corporation, Armonk, NY).

The collection of clinical and genetic data for this study was approved by the ethics committee of the Medical Association of Saarland (protocol no., 144/15). Written informed consent to participate was obtained from all aniridia patients (or from one or both parents of children younger than 18 years with aniridia) following the tenets of the Declaration of Helsinki. Forty-six patients in the cohort were examined bilaterally (92 eyes). The mean ± standard deviation cohort age was 23.0±17.9 years and included 23 children (50%) younger than 18 years. Demographic, genetic, and phenotypic data are presented in Table 1, including 9 novel *PAX6* mutations not reported previously. Five patients (11.1%) showed non-*PAX6* aniridia, that is, without detectable mutation in coding regions of *PAX6* or other genes, based on either whole exome sequencing (3 patients 4, 5, and 10 years of age) or by multiplex ligation-dependent probe amplification analysis (2 patients 26 and 34 years of age). In the 2 latter cases, no *PAX6* coding mutation was evident, but a heterozygous deletion of flanking genes was found, with deletion of *ELP4* and *DCDC1* in the first patient and deletion of *ELP4*, *DCDC1*, *DNAJC24*, and *IMMP1L* in the second patient.

Notably, AAK grade and corneal phenotype worsened with increasing degree of *PAX6* mutation (i.e., no *PAX6* coding mutation, amino acid substitution, single exon, multiple exon, and chromosomal *PAX6* gene deletion). Linear regression analysis indicated that AAK grade was associated strongly with type of *PAX6* mutation ($P < 0.001$) when adjusted for age and gender. For the entire cohort, AAK was age dependent ($\beta = 0.02$; $P = 0.001$), but the age dependence was significant only for premature termination codon (PTC) and C-terminal extension mutations ($\beta = 0.02$; $P = 0.004$), with other *PAX6* mutation types being age independent (nonprogressive). Relative to non-*PAX6* coding mutations, missense mutation resulted in an AAK grade increase of 0.97 ($\beta = 0.97$; 95% confidence interval, 0.08−1.85; $P = 0.03$), PTC and C-terminal extension mutations resulted in an AAK grade increase of 1.59 ($\beta = 1.59$; 95% confidence interval, 0.90−2.28; $P < 0.001$), and chromosomal mutations resulted in an AAK grade increase of 2.69 ($\beta = 2.69$; 95% confidence interval, 1.80−3.57; $P < 0.001$).

In patients with non-*PAX6* aniridia, AAK was mild with a transparent cornea, relatively preserved visual acuity, near normal subbasal nerve density, moderately reduced ocular surface sensitivity, and moderately increased central corneal thickness. Missense mutations resulting in amino acid substitution (5 patients [11.1%]) resulted in a generally milder form of AAK that was not progressive, with comparatively good vision, modestly reduced sensitivity and subbasal nerve density, and moderately increased central corneal thickness. Patients with PTC mutations in *PAX6* inducing nonsense-

Table 1. Molecular Cytogenetic and Clinical AAK Phenotype in the Aniridia Cohort

Mutation Type	Subject No.	Family No.	Age (yrs)	Gender	Functional Consequence	Exon/Intron	DNA Change	Protein Change	Aniridia-Associated Keratopathy Grade (Right Eye/Left Eye)	Distance-Corrected Visual Acuity Snellen	Sensitivity (mm)	CNFL (mm/mm2)	Epithelial Phenotype	Central Corneal Thickness (μm)	LOVD PMID entries
Non-PAX6 coding	1		4	M	Unknown, no WAGR	—	—	—	0/0	50/40	45/40	—/12.7	—/co	591/600	—
	2		5	F	Unknown, no WAGR	—	—	—	1/1	80/80	30/35	—/—	—/—	578/582	—
	3		10	F	Unknown, no WAGR	—	—	—	1/0	100/80	50/50	20.9/11.1	Co/co	594/589	—
	4		26	F	Gene deletion	—	ELP4, DCDC1	—	1/1	50/50	55/55	22.8/21.1	Co/co	624/601	—
	5		34	M	Gene deletion	—	ELP4, DCDC1, DNAJC24, IMMP1L	—	1/0	67/67	30/30	11.1/17.1	Co/co	639/641	—
Missense	6		4	M	36 amino acid substitution	Intron 6	c.357+1G>A	p.84_119del	1/2	167/167	45/30	12.9/4.2	Co/mix	613/648	8
	7		10	M	36 amino acid substitution	Intron 6	c.357+2dupT	p.84_119del	1/2	100/100	35/30	14.9/20.8	Co/co	654/658	3
	8		20	F	1 amino acid substitution	Exon 5	c.80A>C	p.Gln27Pro	1/1	67/100	55/60	8.9/7.4	Co/co	579/601	This study
	9		36	F	1 amino acid substitution	Exon 6	c.266A>C	p.Gln89Pro	3/3	200/200	20/30	0/—	Conj/mix	599/610	This study
	10		38	F	1 amino acid substitution	Exon 5	c.86T>G	p.Ile29Ser	1/2	240/240	35/25	3.6/5.6	Mix/mix	694/572	This study
PTC	11	1	1	M	NMD inducing	Exon 5	c.112delC	p.Arg38Glyfs*16	-/0	160/160	—/—	—/—	—/—	518/535	9
	12	1	9	M	NMD inducing	Exon 5	c.112delC	p.Arg38Glyfs*16	1/1	80/67	45/35	—/—	—/—	627/631	—
	13		4	M	NMD inducing	Exon 7	c.365C>A	p.Ser122*	2/2	180/360	40/40	—/—	Co/co	591/597	3
	14		4	M	NMD inducing	Exon 7	c.386_387delAC	p.Asn129Thrfs*3	2/2	330/400	30/30	—/—	—/—	631/641	This study
	15		6	F	NMD inducing	Intron 10	c.916+1G>C	p.(Val256Phefs*59)	2/2	125/125	25/35	—/—	—/—	579/571	2
	16		7	F	NMD inducing	Exon 7	c.392delC	p.Ala131ValFs*16	2/2	400/400	30/25	14.7/7.9	Co/mix	594/545	This study
	17		8	M	NMD inducing	Exon 5	c.109delG	p.Ala37Profs*17	1/1	400/200	25/35	15.8/15.0	Co/co	613/628	3
	18		8	F	NMD inducing	Exon 8	c.584_585delAAinsCAG	p.Glu195Alafs*5	2/1	5000/400	20/15	—/—	—/—	548/625	This study
	19	2	11	M	NMD inducing	Exon 7	c.391_392delGC	p.Arg131*	2/2	100/160	25/20	6.2/8.1	Co/co	572/574	This study
	20	2	12	M	NMD inducing	Exon 7	c.391_392delGC	p.Arg131*	2/3	250/250	30/30	—/—	Co/mix	573/509	—
	21	2	46	F	NMD inducing	Exon 7	c.391_392delGC	p.Arg131*	Enucleated/3	—/80	—/25	—/0	—/conj	-/517	—
	22	3	11	M	NMD inducing	Exon 5	c.140A>G	p.Gln47Arg	2/2	1000/400	10/10	0/0	Conj/conj	985/580	2
	23	3	42	F	NMD inducing	Exon 5	c.140A>G	p.Gln47Arg	3/Kpro	1000/LP	35/—	0/-	Conj/—	670/-	—
	24		13	M	NMD inducing	Exon 8	c.607C>T	p.Arg203*	2/2	200/200	30/30	16.1/19.4	Co/co	627/-	27
	25		20	F	NMD inducing	Exon 9	c.718C>T	p.Arg240*	4/4	400/1000	30/15	0/0	Conj/conj	806/-	35
	26		28	F	NMD inducing	Exon 9	c.718C>T	p.Arg240*	2/2	80/125	45/35	5.2/7.6	Co/co	636/634	35
	27		28	F	NMD inducing	Exon 5	c.112_116del	p.Arg38ValFs*16	3/3	750/750	20/10	0/0	Conj/conj	657/596	1
	28	4	34	M	NMD inducing	Exon 10	c.829C>T	p.Gln277*	3/3	1000/400	25/20	0/0	Conj/conj	595/521	2
	29	4	64	F	NMD inducing	Exon 10	c.829C>T	p.Gln277*	3/3	1000/LP	—/5	0/0	Conj/conj	873/1026	—
	30		36	M	NMD inducing	Exon 5	c.76delC	p.Arg26Glyfs*4	2/3	80/240	40/15	5.9/0	Mix/conj	665/692	This study
	31		42	F	NMD inducing	Intron 5	c.141+1G>A	unknown	4/4	400/2000	20/30	0/0	Conj/conj	821/890	3
	32		46	F	NMD inducing	Exon 5	c.130C>T	p.Arg44*	1/2	800/1000	30/20	17.2/9.0	Mix/mix	599/602	2
	33		54	M	NMD inducing	Exon 8	c.551delG	p.Gly184Glufs*23	2/2	200/160	25/20	0.6/6.2	Mix/mix	616/727	0
	34		57	M	NMD inducing	Exon 9	c.764A>G	p.Gln255Arg	3/2	200/100	15/20	0/10.7	Conj/mix	681/-	1
	35		16	F	Multiple exon deletion	Exon 11–15 + ELP4 exon 9		—	3/enucleated	12500/—	5/—	0/—	Conj/—	737/-	—
	36		52	F	Multiple exon deletion	Exon 5–6 + 15-base insertion		—	Transp/transp	330/330	10/5	0/0	Conj/conj	817/741	—
CTE	37		8	M	Run-on mutation	Exon 13	c.1268A>T	p.*423Leuext*15	2/2	400/400	55/50	—/—	—/—	686/697	6
	38		18	F	Run-on mutation	Exon 13	c.1268_1269delinsGT	p.*423Cysext*15	2/2	100/100	30/20	6.5/5.5	Mix/co	621/618	This study
	39		18	M	Run-on mutation	Exon 13	c.1268A>T	p.*423Leuext*15	2/2	400/200	30/55	9.5/15.6	Co/co	655/629	6
	40		51	F	Run-on mutation	Exon 13	c.1268A>T	p.*423Leuext*15	3/3	400/1000	25/10	0/0	Conj/conj	714/-	6
Chromosomal	41		4	F	WAGR, gene deletion	PAX6, ELP4, DCDC1, FSHB, RCN1, WT1, HIPK3, LMO2, EHF, CB44			2/2	333/167	30/30	3.1/0	Mix/mix	635/620	—
	42		11	M	WAGR, gene deletion	PAX6, ELP4, WT1, del(11)(p13p13)			4/4	2400/2400	25/25	—/—	—/—	1071/894	—
	43		11	M	PAX6, no WAGR	PAX6, del(11)(p13p13)			4/4	240/4000	20/20	—/—	—/—	1172/915	—
	44		14	F	WAGR, gene deletion	PAX6, WT1, del(p13-ter)			4/3	1000/12500	5/10	0/0	Conj/conj	1134/651	—
	45		20	F	WAGR, gene deletion	PAX6, WT1, del(11)(p11.2p13)			3/2	LP/400	55/25	—/0	—/conj	670/623	—
Unknown	46		55	F	—	—	—	—	3/4	1400/5000	0/25	0/0	Conj/conj	1034/996	—

— = indicates no information available or not relevant; CCT = central corneal thickness; CNFL = corneal nerve fiber length density of subbasal nerves, epithelial phenotype; Co = corneal cells; Conj = conjunctival cells; CTE = c-terminal extension; DCVA = distance-corrected visual acuity measured in Snellen units of feet (20/); Kpro = keratoprosthesis; LOVD = Leiden Open Variation Database (number of unique entries with associated publications); LP = light perception; Mix = mixed corneal/conjunctival cells; NMD = nonsense-mediated decay; PAX 6 = paired box gene 6; PMID = PubMed identification; PTC = premature termination codon; transp = corneal transplant; WAGR = Wilms' tumor, aniridia, genitourinary anomalies, mental retardation. Values separated by a forward slash (/) indicate values for each eye for the given parameter. DCVA was not be evaluated in cases of Kpro. "This study" indicates novel mutation found in a subject or family.

mediated mRNA decay comprised most cases (26 patients [57.7%]) in the present cohort and exhibited a classical AAK that typically is mild in childhood and progresses to a central corneal fibrovascular pannus after 20 years of age,[5] with associated loss of corneal sensitivity, nerves, visual acuity, limbal niche function, and elevated central corneal thickness resulting from the pannus. Of the PTC cases, 2 patients showed *PAX6* mutations spanning more than 1 exon, resulting in a more severe phenotype; 1 patient had AAK grade 4 and multiple (failed) corneal transplantations bilaterally, whereas the other had undergone an enucleation of one eye (because of severe pain and buphthalmos without light perception) and AAK grade 3 in the other eye. Those with C-terminal extension mutations (4 patients [8.9%]) showed a similar classical progressive phenotype as those with PTC mutations. Those with chromosomal deletion of the *PAX6* gene with additional deletions of 1 or more flanking genes (such as *WT1*, *FSHB*, *DCDC1*, *ELP4*, *RCN1*, *HIPK3*, *LM02*, *EHF*, and *CB44*) showed a severe, early aggressive AAK phenotype (5 patients [11.1%]), with 80% of these patients exhibiting Wilms' tumor, aniridia, genitourinary anomalies, mental retardation (WAGR) syndrome and all demonstrating poor vision, thick opaque pannus, severe nerve deficit, and poor corneal sensitivity at a relatively young age (4−20 years). Finally, the central corneal subbasal epithelial plexus of all patients in the cohort regardless of age or genetic mutation status contained a large population of dendritic cells of activated phenotype not normally present in the healthy, noninflamed cornea.[6]

Mutational correlation to detailed AAK morphologic features is rare, and therefore, clinical genetic and imaging studies are recommended in aniridia cohorts. Additionally, longitudinal natural progression studies are warranted to document AAK progression in the same individuals over a time span of several decades. The present findings indicate that AAK is not homogeneous. Although all cases of congenital aniridia have a minimal keratopathy (reduced touch sensitivity, increased corneal thickness, elevated dendritic cell invasion), further clinical and genetic classification of AAK into noncoding, mild, classical progressive, and early aggressive subtypes is recommended, as summarized in Table S1 (available at www.aaojournal.org). From a clinical management perspective, the AAK subtypes could be considered as separate disease entities, thereby facilitating treatment decisions[7] and patient stratification for future clinical studies and trials.

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