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A 556 kb deletion in the downstream region of the *PAX6* gene causes familial aniridia and other eye anomalies in a Chinese family

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Purpose: The paired box gene 6 (*PAX6*) on human chromosome 11p13 is an essential transcription factor for eye formation in animals. Mutations in *PAX6* can lead to varieties of autosomal-dominant ocular malformations with aniridia as the major clinical signs. Known genetic alterations causing haplo-insufficiency of *PAX6* include nonsense mutations, frame-shift mutations, splicing errors, or genomic deletions. The purpose of this study was to identify genetic defects as the underlying cause of familial aniridia in a large Chinese family.

Methods: All exons of *PAX6* in the proband were sequenced by the Sanger sequencing technique. The genome of the proband was evaluated by a microarray-based comparative genomic hybridization (aCGH). Quantitative real-time PCR was applied to verify the abnormal aCGH findings in the proband and to test five other family members.

Results: There were no detectable pathogenic mutations in the exons of *PAX6* in the proband. The aCGH analysis showed two copies of *PAX6* but revealed a 566 kb hemizygous deletion of chromosome 11p13, including four annotated genes doublecortin domain containing 1 (*DCDC1),* DnaJ homolog subfamily C member 24 (*DNAJC24*), IMP1 inner mitochondrial membrane (*IMMP1L*), and elongation factor protein 4 (*ELP4*) downstream of *PAX6*. Quantitative real-time PCR verified the deletion in the proband and further identified the deletion in a blind fashion in four affected family members but not in the one with a normal phenotype.

Conclusions: The 566 kb hemizygous deletion of chromosome 11p13 downstream of *PAX6* should be the cause of the familial aniridia in this Chinese family, although two copies of *PAX6* are intact. aCGH evaluation should be applied if there is a negative result for the mutation detection of *PAX6* in patients with aniridia.

Aniridia (OMIM 106210) is a congenital eye disorder characterized by the complete or partial absence of the iris and iris hypoplasia. Eight-five percent of individuals with aniridia inherit this disorder as an autosomal-dominant trait, 13% occur as part of the autosomal-dominant WAGR syndrome (Wilms' tumor, aniridia, genitourinary abnormalities, and mental retardation), and the remaining 2% occur as part of other disorders, including Peters' anomaly and Gillespie's syndrome, in either autosomal-dominant or autosomal-recessive inheritances [1-3].

The paired box gene 6 (*PAX6*) is located on chromosome 11p13 and contains 14 exons encoding a protein (PAX6) with 422 amino acids. PAX6 is a transcriptional factor controlling development of a diversity of organs and tissues (forebrain, pancreas, and ocular tissues, including corneal epithelium, lens, and retina) by recognizing specific DNA sequences of its downstream target genes [4]. Nonsense mutations or deletions of the *PAX6* gene primarily cause aniridia due to its haplo-insufficiency [5], while missense mutations of this gene are associated with a diversity of eye abnormalities through gain-of-function of the mutated protein, such as Peters' anomaly, corneal dystrophy and opacification, congenital cataracts, glaucoma, and foveal hypoplasia [2,6,7]. Whereas mutations or intragenic deletions of *PAX6* represent the major causes of aniridia, genomic rearrangements involving the downstream region of *PAX6* were identified in patients with aniridia although both copies of *PAX6* are intact in these patients [8-13].

In the present study, we report a genomic microdeletion in the downstream region of *PAX6* in a large Chinese family with familial aniridia and other eye anomalies, using microarray-based comparative genomic hybridization (aCGH) techniques. To our knowledge this is the first case found in an Asian population and is one of few similar cases with this kind of genetic mechanism [9,11,12].

METHODS

Patients: A five-generation Chinese family with familial aniridia was recruited from Heilongjiang province, northeastern China. There were 15 affected individuals in this family from which five affected members (II:2, III:4, III:6, III:

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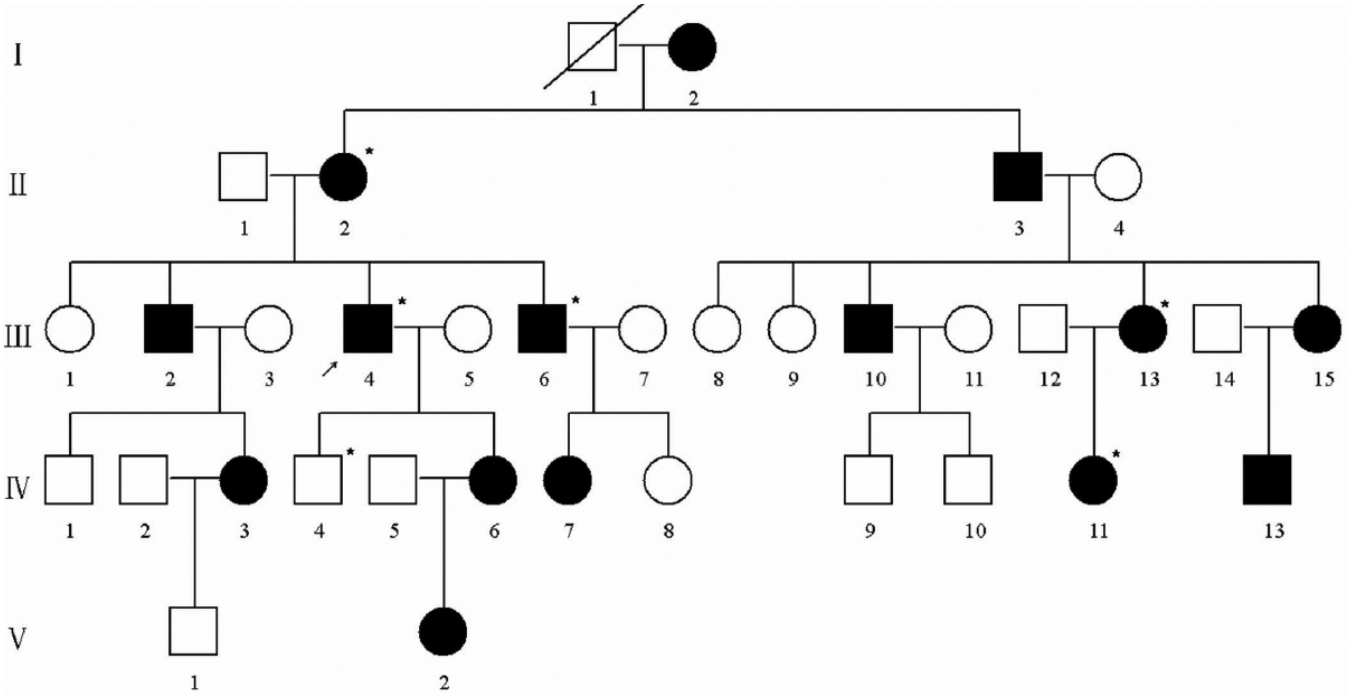

Figure 1. Pedigree of the family in this study. Squares and circles indicated males and females respectively. The symbols in black represent the affected members. The asterisks indicate those subjects who participated in this study. The arrow indicates the proband. The square with a line indicated a deceased individual.

Table 1. Primer information.

Primer sets	Primers sequences	Amplified region	Amplicon size	Target genes
Target primers	Forward: aatgtttcggcctacgatgggagt Reverse: tttagcacccacttacccttccca	chr11:31586811–31586956	146 bp	*FOXC2*
Target primers	Forward: tcataaacactgcagccagcctct Reverse: tcccaacactgcagagaccttgaa	chr11:31781362–31781507	146 bp	*PAX6*
Reference primers	Forward: aggtgttctgctgctgagatggaa Reverse: tccctttgtgcccagatgaagaga	chrX:13693097–13693233	137 bp	*OFD1*

13, and IV:11) and one unaffected individual (IV:4) participated in this study (Figure 1). Ocular tests for these six members included visual acuity of naked eyes and corrected visual acuity, which were recorded using the logarithm of the minimum angle of resolution E chart (Precision Vision, Villa Park, IL), slit-lamp biomicroscopy, measurement of intraocular pressure by applanation tonometry, and gonioscopic evaluation of the anterior chamber angle. Systemic evaluation was performed in the six subjects in this study to exclude WAGR syndrome (Wilms' tumor, aniridia, genitourinary abnormalities, and mental retardation), Axenfeld–Rieger syndrome, iridocorneal endothelial syndromes, sclerocornea, and Peter's anomaly. Informed consents were obtained from the six individuals in this study. Five affected patients and one family member with normal phenotype were further investigated using molecular techniques. The research protocol for this study was approved by the Harbin Medical University Ethics Committee (Harbin, China).

Experiments: All exons of *PAX6* in the proband (III:4) were amplified and sequenced with the ABI BigDye Terminator Cycle Sequencing kit v3.1, (ABI Applied Biosystems, Foster City, CA) according to the described protocol [14]. The superimposed mutant PCR products were subcloned into pGEM-T vector (Promega, Madison, WI) and sequenced to identify the mutation. The genomic DNA from the proband was analyzed by aCGH using the Agilent Human Genome Microarray Kit 244K (Agilent Technologies, Santa Clara, CA) based on the published procedures [15]. The digested test DNA and reference DNA were labeled with cyanine (Cy)3-deoxyuridine triphosphate (dUTP) or Cy5-dUTP, respectively. Following purification, the appropriate Cy3-labeled test DNA and Cy5-labeled reference DNA were mixed together and combined with 2× Hybridization buffer (Agilent), 10× blocking agent (Agilent), and Human Cot-1

DNA (Invitrogen, Carlsbad, CA). The hybridization mixture was slowly dispensed to a microarray chip and assembled with an Agilent SureHyb chamber. After washing, all microarray slides were scanned on an Agilent Microarray Scanner G2565BA with 5-µm resolution. Captured images were transformed to data with Feature Extraction Software, version 9.5 (Agilent), and then imported into Agilent CGH Analytics 3.2.5 software for analysis. Quantitative real-time PCR (qPCR) was performed to verify the abnormal aCGH findings in the proband and to test other family members, according to published guidelines [16]. Individual optimization qPCRs were performed in a 20 µl volume including 25 ng template DNA, 200 nM of each primer, and 1× Premix-Choice with ROX reference dye in an initial denaturation of 93 °C for 10min, followed by 35 cycles of 93 °C for 1 min and 61 °C for 1 min, and 72 °C for 1 min. The three sets of primers for qPCR analysis (Table 1) include primers targeted to the elongation factor protein 4 *(ELP4)* gene within the deleted genomic region, primers within the *PAX6* gene, and reference primers within the Oral-facial-digital syndrome 1 protein *(OFD1)* gene on the human X chromosome. Both *ELP4* and *PAX6* were assayed as test genes compared with *OFD1*, and both *ELP4* and *PAX6* were assayed as test/controls.

RESULTS

Clinical findings: The clinical findings are summarized in Table 2. The proband (III:4), was diagnosed with bilateral iris coloboma, optic atrophy of the left eye, right corneal opacity, and strabismus. Because of glaucoma, the proband had undergone cyclocryotherapy on the left eye in 2002. Other recurrent symptoms in the other affected participants include optic atrophy, retinitis pigmentosa, cataract, subluxation of lens, and dysplasia of trabecular meshwork, fovea, and optic nerve (Table 2). Poor vision is due to foveal or optic nerve hypoplasia, cataract, glaucoma and amblyopia. No visible autistic problems or intellectual disabilities were identified in these affected individuals.

Sequencing results for PAX6: All exons of *PAX6* from the six participants of the family were amplified and sequenced using standard techniques. No intragenic point mutation or deletion could be identified (data not shown).

Microarray-based comparative genomic hybridization findings in the proband: The aCGH analysis detected 35 copy number variations (CNVs) in the proband's genome (Table 3). One of them is a 566 kb hemizygous deletion of chromosome 11p13 (chr:31,074,403–31,640,263) with approximately a 123 kb distance from the 3′ end of *PAX6* (chr11:31,762,916–31,796,085) according to HG18 (NCBI 36, March 2006; Figure 2. This deletion contains four annotated genes: doublecortin domain containing 1 (*DCDC1*), DnaJ homolog subfamily C member 24 (*DNAJC24*), IMP1 inner mitochondrial membrane (*IMMP1L*), and elongation factor protein 4 (*ELP4*). The remaining 34 CNVs were considered to be benign because these CNVs were identified in healthy individuals documented in the Database of Genomic Variation or in recent publications [17,18].

Quantitative real-time PCR assays in the proband and other family members: The 566 kb hemizygous deletion of chromosome 11p13 in the proband was verified by qPCR using primers targeted to *ELP* within the deleted region, which showed one threshold cycle difference between the patient and reference DNA samples for the amplification of the test gene (Figure 3). Two copies of *PAX6* were confirmed by qPCR, consistent with the aCGH results in this individual (data not shown). We used the qPCR method for testing the other five family members in a blind fashion, four affected family members with eye anomalies, and one healthy individual. All four patients were confirmed to carry the deletion, including *ELP*, but not *PAX6*. The phenotypic normal individual showed two copies for both *ELP* and *PAX6* (data not shown).

DISCUSSION

The major finding in this Chinese family with familial aniridia is the presence of a 566 kb heterozygous deletion containing four annotated genes: *DCDC1, DNAJC24, IMMP1L,* and *ELP4*. The proximal breakpoint of this deletion is approximately 123 kb from the 3′ end of *PAX6*. We postulate that this 566 kb heterozygous deletion is the underlying cause of the familial aniridia and acts by disrupting the transcription in one of the two PAX6 alleles, even though the two copies of *PAX6* were intact in all individuals investigated in this study.

Our finding provides further evidence of the existence of the remote 3′ regulatory elements in the downstream region of *PAX6* controlling the expression of this gene, if disrupted, leading to aniridia and other eye anomalies. To our knowledge, this is the first case found in Asian patients with aniridia and one of few similar cases with this kind of genetic mechanism.

Our postulation is based on following evidence: 1) The heterozygous deletion segregated with aniridia in the five affected individuals but not in the unaffected individual, while the exons and splicing regions of *PAX6* are apparently free of mutations. 2) Several publications reported similar observations in patients with aniridia, but the chromosomal breakpoint from the 3′ end of *PAX6* and the fragment of deletion were different. For example, two aniridia pedigrees have been characterized in which the disease segregates with chromosomal re-arrangements that involve 11p13 but do not disrupt the *PAX6* gene since the chromosomal breakpoint is at least 85 kb away from the 3′ end of *PAX6* [8]. Two aniridia pedigrees have also been described in which deletion in the *ELP4* gene region, not involving *PAX6*, was present in all subjects with aniridia but not in the investigated normal

Table 2. The clinical findings of individuals in this study.

Individual	Age	Sex	Naked visual acuity		IOP (mm Hg)		Clinical findings	Eye surgery	Corrected visual acuity with patients' own spectacles		Anterior chamber angle	
			Right	Left	Right	Left			Right	Left	Right	Left
II:2	74	female	HM/10cm	HM/10cm	17	15	bilateral optic atrophy; iris coloboma; retinitis pigmentosa; pannus and aphakia of both eyes	cataract surgery	HM/10cm	HM/10cm	dysplasia of trabecular meshwork and closure of the trabecular meshwork by residual iris stump	dysplasia of trabecula r meshwork and closure of the trabecular meshwork by residual iris stump
III:4	46	male	NLP	FC/20cm	15	22	optic atrophy of left eye;	cyclocryotheraphy on the left eye; bilateral iris coloboma, corneal opacity and strabismus of right eye	NLP	0.2	Cannot been seen clearly	the rudimentary stump of iris and the angle covered by the iris stump
III:6	43	male	0.04	0.02	22	19	bilateral cataract, iris coloboma and subluxation of lens;	none	0.5	0.4	the rudimentary stump of iris and the angle covered by the iris stump	the rudimentary stump of iris and the angle covered by the iris stump
III:13	38	female	0.1	0.1	18	15	bilateral aniridia; amblyopia;	none	0.6	0.6	dysplasia of trabecular meshwork	dysplasia of trabecular meshwork
IV:4	22	male	1.0	1.0	16	16	normal	none	1.0	1.0	open	open
IV:11	14	female	0.08	0.1	14	15	bilateral aniridia; amblyopia;	none	0.6	0.6	the rudimentary stump of iris and the angle covered by the iris stump	the rudimentary stump of iris and the angle covered by the iris stump

Notes: IOP: intraocular pressure; HM: hand movement; NLP: no light perception; FC: finger count.

Table 3. Total CNVs identified in the genome of this individual.

Number	Chromosome	Cytoband	Start	Stop	Size	Duplication	Deletion	Gene names
1	chr1	q21.3	150822873	150848709	25836	2.044245	0	*LCE3C*
2	chr3	p12.3	75895820	75958100	62280	0	−0.682351	*ZNF717*
3	chr3	q26.1	164038717	164101976	63259	0	−0.921074	
4	chr3	q29	196943180	196950295	7115	0	−0.784749	*MUC20, MUC20*
5	chr4	q13.2	69057535	69166014	108479	0	−4.405964	*UGT2B17*
6	chr4	q28.3	131700605	131865354	164749	0	−0.598187	
7	chr5	p15.33	802318	878490	76172	0.667749	0	*ZDHHC11*
8	chr5	q13.2	69741118	70422497	681379	0	−1.028287	*GTF2H2C, GTF2H2B, GTF2H2D, SERF1A, SERF1A, SERF1B, SMN1, SMN2, SMN1, SMN2, NAIP, GTF2H2B, GTF2H2C, GTF2H2, GTF2H2D, OCLN, LOC647859*
9	chr6	p22.1	29201691	29260945	59254	0	−1.115529	*OR2J2*
10	chr6	p21.32	32567182	32660290	93108	0	−2.059653	*HLA-DRB5, HLA-DRB6, HLA-DRB1*
11	chr6	p21.32	32595202	32605500	10298	0	−4.758382	*HLA-DRB5*
12	chr6	p12.1	55468168	55501199	33031	0	−0.627628	*HMGCLL1*
13	chr7	p22.3	140013	225040	85027	0.843971	0	
14	chr7	q21.2	92484285	92530356	46071	0	−0.870801	
15	chr7	q32.3 - q33	132382438	132457449	75011	0	−1.214264	*CHCHD3*
16	chr7	q34	141413152	141438704	25552	1.057004	0	*MGAM*
17	chr7	q34	142158954	142171818	12864	0	−2.550414	*TRY6*
18	chr8	p11.23 - p11.22	39356395	39505456	149061	1.698241	0	*ADAM5P, ADAM3A*
19	chr11	p13	31074403	31640263	565860	0	−1.081172	*DCDC1, DNAJC24, IMMP1L, ELP4*
20	chr11	q11	55124530	55195190	70660	0	−0.554403	*OR4C11, OR4P4, OR4S2, OR4C6*
21	chr12	p13.31	9528390	9585356	56966	1.893183	0	
22	chr14	q32.33	105602556	105630289	27733	0	−3.599436	
23	chr14	q32.33	105962105	105985658	23553	0.992122	0	
24	chr15	q11.2	18809804	18884636	74832	0.536094	0	
25	chr15	q11.2	19845742	20080135	234393	0.435061	0	*LOC727924, OR4M2, OR4N4, LOC650137*
26	chr16	p13.3	2638381	2672274	33893	0.722366	0	
27	chr16	p13.11	14955977	15023221	67244	0	−0.94928	*PDXDC1*
28	chr16	q12.2	54354443	54380034	25591	0	−0.650633	*CES4*
29	chr16	q22.1	68706040	68754434	48394	0	−0.495966	*PDPR*
30	chr17	q21.31 - q21.32	41553026	42049740	496714	0	−0.347977	*KIAA1267, LRRC37A, ARL17, ARL17, LRRC37A2, ARL17P1, ARL17P1, ARL17, NSF*
31	chr17	q25.3	77422498	77503885	81387	0.905508	0	*ARHGDIA, THOC4, ANAPC11, ANAPC11, NPB, PCYT2, SIRT7, MAFG, MAFG, LOC92659, PYCR1, PYCR1, MYADML2, NOTUM*
32	chr19	p12	20422376	20493601	71225	0	−0.809494	
33	chr20	p13	1516766	1539355	22589	0	−1.330169	*SIRPB1, SIRPB1*
34	chr22	q12.3	35217849	35225862	8013	0.921708	0	*FOXRED2, FOXRED2*
35	chr22	q13.1	37688858	37715585	26727	0	−0.689394	*APOBEC3A, APOBEC3B*

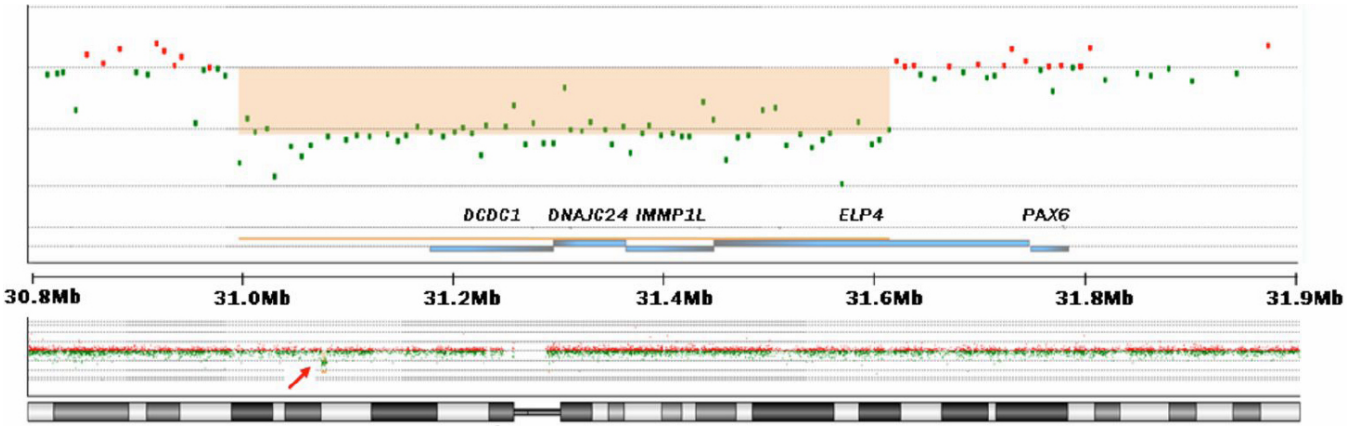

Figure 2. The 556 kb genomic deletion of chromosome 11p13. This deletion harbors four annotated genes, *DCDC1, DNAJC24, IMMP1L*, and *ELP4*.

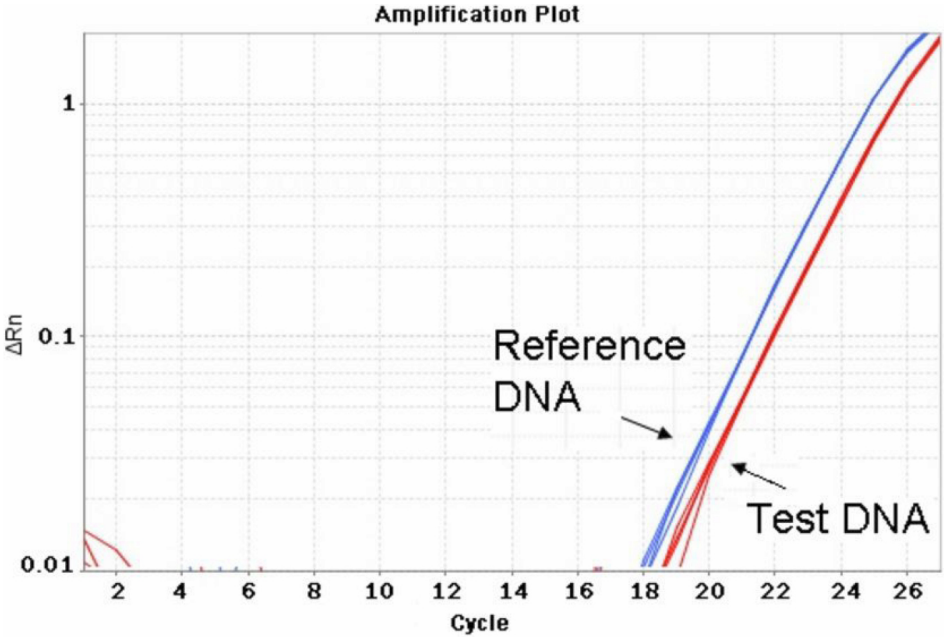

Figure 3. qPCR analysis result. An example of the qPCR amplification plot showed a single copy of the test gene, *ELP4*, in the affected individuals compared to the two copies of this gene in the reference DNA.

relatives [12]. A 1.3 Mb deletion (including seven annotated genes: metallophosphoesterase domain containing 2 (*MPPED2*), doublecortin domain containing 5 (*DCDC5*), *DCDC1*,*DNAJC24, IMMP1L,* zinc finger CLS domain containing 3 (*DPH4*), and *ELP4*) has been characterized that starts 35 kb from the 3′ end of *PAX6* in a patient with aniridia, autism, and mental retardation [11]. Recently a ~406 kb heterozygous genomic deletion containing four annotated genes (*DCDC1, DNAJC24, IMMP1L,* and *ELP4*) was found in patients with aniridia [13]; apparently the gene contents in this deletion are the same as the 566 kb heterozygous deletion in the family we report here. 3) Functional studies in both human cells and animal models confirmed the existence of remote 3′ regulatory elements in the downstream region of *PAX6*. Deletions in this region have been shown to abolish PAX6 expression and cause aniridia and other eye anomalies due to loss of enhancers and a downstream regulatory region [9,19,20].

Little information is known about the four annotated genes within the 566 kb deletion in this study. It is also unknown whether these genes are involved in any of the phenotypic features found in these individuals carrying the deletion. The *DCDC1* gene encodes a member of the doublecortin family, which is highly expressed in testis and fetal brain [21]. *DNAJC24* is one of several enzymes involved in synthesis of diphthamide, which is a unique posttranslationally modified histidine found only in translation elongation factor 2 [22]. *IMMP1L* encodes a peptidase similar to mitochondrial inner membrane peptidase (IMP1), which is one of the catalytic subunits of the IMP complex proteolytically removing the mitochondrial targeting presequence of nuclear-encoded proteins [23]. *ELP4* encodes

a component of the six subunit elongator complex, a histone acetyltransferase complex that associates directly with RNA polymerase II during transcriptional elongation. Two recent reports indicate that *ELP4* is possibly associated with the centrotemporal sharp wave electroencephalogram (EEG) trait in rolandic epilepsy and speech sound disorder [24,25].

Submicroscopic copy number variations may play a role in human diseases either by loss of gene expression regulatory elements or by disrupting coding sequences. As well as the point mutations in *PAX6* exons, copy number variation should be investigated in the flanking regions of *PAX6*. We suggest that patients, such as the subjects reported here, should be investigated using high resolution aCGH techniques in a clinical setting if sequencing analyses for *PAX6* in patients with aniridia is negative.

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