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, frameshift 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 [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:
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).

**Table 1. Primer information.**

| Primer sets        | Primers sequences                  | Amplified region       | Amplicon size | Target genes |
|--------------------|------------------------------------|------------------------|---------------|--------------|
| Target primers     | Forward: aatgttctgctacagatggagt    | chr11:31586811–31586956 | 146 bp        | FOXC2        |
|                    | Reverse: tttagacaccaacctacccctccca |                        |               |              |
| Target primers     | Forward: ttcraacagcagccgcctct      | chr11:31781362–31781507 | 146 bp        | PAX6         |
|                    | Reverse: tcccaacactgacgacggtgaa    |                        |               |              |
| Reference primers  | Forward: aggtttcgcttggtgaggaga     | chrX:13693097–13693233 | 137 bp        | OFD1         |
|                    | Reverse: tcccttggccagatgagaga      |                        |               |              |

**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 (Cy3)-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.
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 10 min, 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 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 (chr1:31,762,916–31,796,085) 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
| Individual | Age | Sex | Corrected visual acuity with patients’ own spectacles | Anterior chamber angle |
|------------|-----|-----|----------------------------------------------------|------------------------|
|            |     |     | Right | Left | Right | Left | Right | Left |
| II:2       | 74  | female | HM/10cm | HM/10cm | 17 | 15 | bilateral optic atrophy; iris coloboma; retinitis pigmentosa; panus and aphakia of both eyes | cataract surgery | HM/10cm | HM/10cm |
| 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 |
| 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 |
| III:13     | 38  | female | 0.1 | 0.1 | 18 | 15 | bilateral aniridia; amblyopia | none | 0.6 | 0.6 | dysplasia of trabecular meshwork |
| IV:4       | 22  | male | 1.0 | 1.0 | 16 | 16 | normal bilateral aniridia; amblyopia | none | 1.0 | 1.0 | open |
| IV:11      | 14  | female | 0.08 | 0.1 | 14 | 15 | none | none | 0.6 | 0.6 | open |

Notes: IOP: intraocular pressure; HM: hand movement; NLP: no light perception; FC: finger count.
| 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    | 7042249     | 681379     | 0           | -1.028287 |                                                                            |
| 9      | chr6       | p22.1    | 29201691    | 29260945    | 59254      | 0           | -1.115529 |                                                                            |
| 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 |                                                                            |
| 12     | chr6       | p12.1    | 55468168    | 55501199    | 33031      | 0           | -0.627628 |                                                                            |
| 13     | chr7       | q22.3    | 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, IMPM1L, 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   | 105985685   | 23553      | 0.992122    | 0        |                                                                            |
| 24     | chr15      | q11.2    | 18809804    | 18884636    | 74832      | 0.536094    | 0        |                                                                            |
| 25     | chr15      | q11.2    | 19845742    | 20081035    | 23493      | 0.435061    | 0        | LOC72924, 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 |                                                                            |
| 29     | chr16      | q22.1    | 68706040    | 68754434    | 48394      | 0           | -0.49566 |                                                                            |
| 30     | chr17      | q21.31   | 41553026    | 42049740    | 496714     | 0           | -0.347977 | KIA12167, LRRC37A, ARL17, ARL17, LRRC37A2, ARL17P1, ARL17P1, ARL17, NSF |
| 31     | chr17      | q25.3    | 77422498    | 77503885    | 81387      | 0.905508    | 0        |                                                                            |
| 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    | 26277      | 0           | -0.689394 | APOBEC3A, APOBEC3B                                                          |
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 (IMP), 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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