Mutation spectrum of PAX6 in Chinese patients with aniridia

Xiaohui Zhang, Panfeng Wang, Shiqiang Li, Xueshan Xiao, Xiangming Guo, Qingjiong Zhang

State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou 510060, China

Purpose: To identify mutations in the paired box 6 (PAX6) gene of 33 probands with aniridia and to reveal the mutational spectrum in the Chinese population.

Methods: Unrelated probands with aniridia from 27 newly selected families and six previously analyzed families participated in this study. The coding regions of PAX6 in the 27 new families were analyzed using cycle sequencing. Families that lacked detectable variations based on sequencing (14 new and six previously analyzed) were further analyzed using multiplex ligation-dependent probe amplification (MLPA).

Results: Fifteen mutations were identified in 16 of the 33 families: c.[65_94del30; 99_105dup7], c.101_102insA, c.177delIG, c.238_239insGCCGA, c.1033–42_1033–26del17insG, c.1A>G, c.120C>A, c.718C>T, c.949C>T, c.1062C>A, c.1183G>A, c.1268A>T, and three gross deletions involving exons 1–14, exons 8–14, and exons 9–14. The first five mutations were novel and the c.1268A>T mutation was present in two families. Phenotypic variations were observed between families and between different affected patients within the families.

Conclusions: The PAX6 mutation spectrum in Chinese aniridia patients is comparable to that reported in other ethnic groups. Further studies of the 17 families with no detected mutations may provide additional information to improve the understanding of the molecular genetics of aniridia.
exons for each patient were sequenced using the ABI BigDye Terminator v3.1 Cycle Sequencing Kit (ABI Applied Biosystems, Foster City, CA) and the ABI 3100 Genetic Analyzer (ABI Applied Biosystems) according to the manufacturer’s recommendations. Sequencing results from patients’ sequences and PAX6 consensus sequences from the National Center for Biotechnology Information (NCBI) human genome database (NC_000011.9) were imported into the SeqManII program of the Lasergene package (DNAStar Inc., Madison, WI) and aligned to identify variations. Each mutation was confirmed by bidirectional sequencing. Mutation descriptions followed the nomenclature recommended by the Human Genomic Variation Society (HGVS) [28].

**MLPA analysis:** For patients who were determined not to have a PAX6 mutation based on sequencing analysis, MLPA was used to detect deletions of part or all of PAX6, according to the manufacturer’s instructions (SALSA MLPA Kits P219-B1 PAX6; MRC-Holland bv, Amsterdam, the Netherlands) [10]. Briefly, 100 ng DNA samples were denatured for 5 min at 98 °C and then cooled to 25 °C. Probes were mixed and hybridized with DNA samples at 60 °C overnight and then reacted with ligase 65 at 54 °C for 15 min, followed by 5 min at 98 °C and then held at 4 °C. Finally, all probes and sample ligations were amplified by PCR using specific carboxyfluorescein (FAM) labeled PCR primers. PCR products were separated by electrophoresis using the ABI PRISM 3100 Analyzer. Data analysis was performed using GeneMarker V1.5 software. A peak area between 0.7 and 1.3 times was considered normal; however, peak areas below 0.7 represent deletions and those above 1.3 represent duplications.

**RESULTS**

Sequencing of the 14 exons of PAX6 of the 27 probands revealed 12 mutations in 13 patients, including five novel deletion/insertion mutations and seven known point mutations, as follows: c.[65_94del30; 99_105dup7]

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**Table 1. Primers used for amplification and sequencing of PAX6.**

| Primer ID     | Sequence (5′-3′) | Product length (bp) | Annealing Temperature (°C) |
|---------------|------------------|---------------------|--------------------------|
| Extra-E1-F*   | GAGCTGTGCCCAACTCTAGC | 399                | 56                       |
| Extra E1-R    | TCCATCTTTTGATGCTTACTT | 396                | 56                       |
| Exon1F        | GGAGAGGGAGCATCCAT | 318                | 56                       |
| Exon1R        | TCCGGAAGAGAGACAGAGA | 467                | 56                       |
| Exon2F        | ACACACCTGAGCCATCAACCA | 396                | 56                       |
| Exon2R        | CTCTGCTGGAAACTCTTCTTCT | 318                | 56                       |
| Exon3F        | AGAGAGCCCATACGGATATG | 318                | 56                       |
| Exon3R        | CCCAATCTGTCCCTCTCACACA | 318                | 56                       |
| Exon4F        | TGCAGCTGCCGCCAGGATTAC | 144                | 66                       |
| Exon4R        | GCACCCCGAGCGGGAAGTC | 301                | 61                       |
| Exon5F        | TCCCTCTCTCTCCTCTACACT | 301                | 61                       |
| Exon5R        | GGGTCCATAATTAGATCAT | 301                | 61                       |
| Exon6–7F#     | GCTCTCTACAGTAAAGTTCTC | 457                | 61                       |
| Exon6–7R      | AGGAGAGAGACATTTTGCGTTA | 457                | 61                       |
| Exon8F        | GATTTTGAGGGTCATCATCAAT | 212                | 65                       |
| Exon8R        | ATATGGGAGACTGCCTGATGAT | 212                | 65                       |
| Exon9F        | TTTGTTGAGGGCTGCAGGA | 339                | 58                       |
| Exon9R        | TTCTCTCTAGGAAGATGTCGC | 339                | 58                       |
| Exon10F       | GTAGTCTGCGCCAAATATGG | 206                | 62                       |
| Exon10R       | GTACTCTGTAACAGACACACT | 206                | 62                       |
| Exon11–12F*   | GGCCTCAGCTAGTACAGCAGT | 500                | 62                       |
| Exon11–12R    | TGCAGACACAGCCAAATGAGG | 500                | 62                       |
| Exon13F       | GCTGTGATGTATGTCTTCTCA | 245                | 62                       |
| Exon13R       | AAGAGAGATCCCTGCTTG | 245                | 62                       |
| Exon14F       | CATGTCTGTTTCTC指引AAGGG | 202                | 61                       |
| Exon14R       | CCATAGTCACTGACTGAATTACAC | 202                | 61                       |

*Exon numbers are based on the current version of gene structure and NM_001604.4, where the original exon 5a is numbered as exon 6. *This extra exon is based on another transcript variant NM_001127612.1, which is not present in the transcript variant of NM_001604.4.
### Table 2. Clinical data and PAX6 mutations in the 16 probands.

| ID    | Age (years) | Gender | Inheritance | Visual acuity | Clinical manifestations | Mutations detected in PAX6 |
|-------|-------------|--------|-------------|---------------|-------------------------|---------------------------|
| QT183 | 8           | M      | sporadic    | 0.1; 0.1      | normal                  | E5 c.120C>A               |
| QT314 | 3/12        | F      | sporadic    | NA            | normal                  | E9 c.718C>T                |
| QT322 | 12          | M      | AD          | 0.1; 0.1      | normal                  | E5 c.65_94del30.c.99_105dup7 |
| QT346 | 3           | M      | sporadic    | NA            | inferior leucoma        | E12 c.1183G>A              |
| QT350 | 4           | M      | sporadic    | 0.1; 0.1      | normal                  | E9–14 del                 |
| QT374 | 7           | M      | sporadic    | 0.1; 0.1      | normal                  | E10 c.949C>T               |
| QT462 | 4           | F      | sporadic    | NA            | normal                  | E8–14 del                 |
| QT464 | 19          | M      | AD          | 0.2; 0.2      | normal                  | E5 c.101_102insA           |
| QT467 | 3           | F      | AD          | NA            | normal                  | E1–14 del                 |
| QT468 | 8           | M      | AD          | 0.2; 0.2      | normal                  | E6 c.238_239dupGCGA        |
| QT517 | 2/12        | M      | sporadic    | NA            | normal                  | E6 c.177delG               |
| QT522 | 18          | F      | AD          | 0.1; 0.1      | microcornea              | E4 c.1A>G                 |
| QT527 | 17          | F      | AD          | 0.2; 0.3      | normal                  | E12 c.1033–42_1033–26del17insG |
| QT602 | 14          | M      | AD          | 0.1; 0.1      | normal                  | E12 c.1062C>A              |
| QT609 | 5           | F      | AD          | 0.1; 0.2      | normal                  | E13 c.1268A>T             |
| QT634 | 7           | F      | AD          | 0.1; 0.1      | normal                  | E13 c.1268A>T             |

Note: # Nystagmus and foveal hypoplasia were present in all patients with PAX6 mutations. AD: Autosomal dominant. NA: Not available. *Her father had the same mutation but had complete aniridia.
Figure 1. Frameshift mutations detected in PAX6. Five novel deletion/insertion mutations were identified in five probands with aniridia from unrelated families. Pedigrees (left) are accompanied with sequence chromatography (right). Arrows indicate the probands. R represents reverse sequence.

DISCUSSION

The mutation frequency of PAX6 in Chinese aniridia patients is similar to that in Caucasian aniridia patients. In this study, PAX6 mutations were identified in 16 of the 33 families tested. When the results of this study are combined with those of our previous study [22], the PAX6 mutations have been identified in 21 of 38 unrelated patients using cycle sequencing and MLPA. Of the 21 patients, mutations in 18 patients were identified by analyzing PAX6 coding regions using direct sequencing, and mutations in 3 patients were detected using MLPA. For PAX6 mutations in Chinese aniridia patients, the overall mutation detection rates detected with cycle sequencing, MLPA, or both were 47% (18/38), 8% (3/38), and 55% (21/38), respectively. In a similar study of Caucasian aniridia patients [10], PAX6 mutations were detected in 49% (34/70), 11% (8/70), and 60% (42/70) patients with cycle sequencing, MLPA, or both, respectively. Several other studies have detected aniridia-associated PAX6 mutations in 30% (9/30) of Mexican patients [29], 56% of Indian patients [30], 67% (4/6) of Thai patients [31], 38%–58% (3/8–14/24) of German patients [32,33], 50% (2/4) of Japanese patients.
Figure 2. Point mutations detected in PAX6. One missense and six nonsense mutations in PAX6 were found in eight probands with aniridia from unrelated families. From left to the right, the columns represent pedigrees, sequencing results from probands with aniridia, and corresponding sequences from normal controls.
Figure 3. PAX6 mutations detected by MLPA. Three gross deletions were involved in exons 9–14, 8–14, and 1–14, respectively. Black arrows indicate the exons with deletions, in which each peak area is below 0.7 compared to internal controls.
[34], 79% (30/38) of Danish patients [35], and 83%–94% (10/12–67/71) of British patients [9,36]. The detection of these mutations was based solely on sequence analysis in most studies, but chromosomal analysis was additionally performed in a few studies. These reports demonstrate that while PAX6 mutations were prevalent, they were not detected in all patients with aniridia. One reason that may account for this is the possibility that small variations outside the exons, such as intronic regions [10], may not be detectable by the techniques used to analyze PAX6. Furthermore, frequent chromosomal rearrangements have been described in aniridia patients previously [37], which may not be detectable by cycle sequencing and MLPA. It is also possible that there are mutations in other genes which contribute to aniridia given that mutations in FOXC1 are associated with aniridia [17,18] and that no PAX6 mutations were detected in aniridia patients with preserved visual function [19].

The spectrum of PAX6 mutations in aniridia is similar within both Chinese and Caucasian patient cohorts. The majority of PAX6 mutations reported so far would lead to truncation of encoded proteins (such as nonsense, splicing, insertion, or deletion mutation) and only about 2%–11.7% are missense mutations [38,39]. In one review [38], 257 aniridia-associated mutations were classified as nonsense mutations (38.9%), splice mutations (13.2%), frame-shifting insertions or deletions (25.3%), in-frame insertions or deletions (6.2%), missense mutations (11.7%), and run-on mutations (4.7%). For the 21 mutations in the Chinese patients analyzed in the present study, these percentages are 33.3%, 14.3%, 19.0%, zero, 9.5%, and 9.5%, with an additional 14.3% being gross deletions of the PAX6 gene. In addition, seven point mutations detected in this study are known mutations, suggesting common mutations. Of the seven, the p.R240X and p.R317X mutations involving CpG dinucleotides are the most common nonsense mutations in PAX6. The p.C40X mutation was detected in one patient in this study and another patient in our previous study [22]. The run-on mutation, X423LeuextX*15, was detected in two unrelated Chinese patients.

In this study, we detected five novel small insertion/deletion mutations, seven known point mutations, and three known gross deletions in 33 unrelated aniridia patients. In this and one of our previous studies [22], the PAX6 gene was analyzed by cycle sequencing and MLPA in 38 unrelated aniridia patients. However, PAX6 mutations were only detected in 55% (21/38) patients. Further studies of the 17 families without PAX6 mutations may elucidate the molecular basis of aniridia in these families.

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