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Investigation of a *PAX6* gene mutation in a Malaysian family with congenital aniridia

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ABSTRACT. Mutations in the *PAX6* gene that cause aniridia have been identified in various ethnicities but not in the Malaysian population. Therefore, the objective of this study was to investigate the *PAX6* mutation in a Malaysian family with congenital aniridia. In this study, a complete ophthalmic examination was performed on a Dusun ethnic family with aniridia. Genomic DNA was extracted from the peripheral blood of the subjects and screened for the *PAX6* gene mutation using polymerase chain reaction amplification high-resolution melting curve analysis (PCR-HRM) followed by confirmation via direct DNA sequencing. A heterozygous G deletion (c.857delG) in exon 7 causing a frame shift in *PAX6* was identified in all affected family members. Genotype-phenotype correlation analysis revealed congenital cataract and all affected family members showed a similar spectrum of aniridia with no phenotypic variability but with differences in severity that

were age dependent. In summary, by using a PCR-HRM approach, this study is the first to report a *PAX6* mutation in a Malaysian family. This mutation is the cause of the aniridia spectra observed in this family and of congenital cataract.

Key words: Aniridia; *Pax6*; Mutation; Malaysian; PCR-HRM; Sequencing

INTRODUCTION

Aniridia is a panocular disorder characterized by iris hypoplasia and other anterior and posterior eye defects, leading to severe visual impairment. It is caused by mutations in the *PAX6* gene that encodes a transcription factor regulating processes during ocular morphogenesis and forebrain development (Georgala et al., 2011). The *PAX6* gene is 26 kilobases (kb) long, located on chromosome 11p13, and contains 14 exons. The PAX6 transcription factor recognizes its target genes via a paired domain (PD) and homeodomain (HD) at the PAX6 N-terminus (Mishra et al., 2002). Most mutations in *PAX6* have been detected by single-strand conformation polymorphism (SSCP) methods or by direct sequencing of the *PAX6* exons. To date, more than 700 mutations have been identified in *PAX6*, which are catalogued in the *PAX6* mutation database and which are associated with a wide spectrum of aniridia in different populations (http://lsdb.hgu.mrc.ac.uk/home.php?select_db=PAX6).

In this study, we applied an approach that is relatively rapid to identify mutations in *PAX6* using polymerase chain reaction (PCR) amplification high-resolution melting curve analysis and report the first case of a *PAX6* mutation identified in Malaysia.

MATERIAL AND METHODS

Subjects and DNA extraction

We investigated a Dusun ethnic family of 4 generations from Kota Belud, a remote area of Sabah, Malaysia. The Dusun tribe is indigenous to Sabah and of Austronesian ancestry. Eight of the family members were diagnosed with aniridia and were given complete ophthalmic evaluation. It is of note that the severity of the aniridia phenotype in this family was very variable, ranging from mild in a 2-month-old infant (P8) to severe in the oldest family member aged 70 (P1). The pedigree of the family is shown in Figure 1. Two non-affected members of the family and 10 unrelated healthy controls were also recruited for this study. Peripheral blood was collected from the subjects with informed consent, and genomic DNA was extracted using a similar conventional method as reported previously (Chua et al., 2009; Tan et al., 2010). This study was approved by The Ministry of Health Medical Research Ethics Committee, Malaysia (NMRR-09-482-4024).

Polymerase chain reaction high-resolution melting curve analysis (PCR-HRM) and sequencing

A wide screening for *PAX6* gene mutations was carried out using a PCR-HRM ap-

proach. Briefly, primers used for amplification of the 13 exons in *PAX6* gene were as previously reported (Glaser et al., 1992). These primers were checked using *in silico* PCR to ensure that they targeted the correct loci (Thong et al., 2011; Chua et al., 2011; Ng et al., 2012a). Next, conventional thermal cycler-based PCR for optimizing PCR conditions was carried out. The optimized conditions were used in a 7500 Fast Real-Time PCR system (Applied Biosystems, USA) for PCR-HRM analysis. The reaction consisted of 20 ng of genomic DNA in a final volume of 20 µl, which contained 0.3 µM of each primer and 10 µl of 2x MeltDoctor™ HRM Master Mix (Applied Biosystems). The PCR cycling program was as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing and elongation at 60°C for 1 min. Analysis of PCR melting curve dissociation was performed after the amplification in the same real-time PCR system. The melting curve program used was that of Ng et al. (2012b): denaturation at 95°C for 10 s, annealing at 60°C for 1 min, high-resolution melting at 95°C for 15 s, and final annealing at 60°C for 15 s. In this program, short PCR amplicons were allowed to denature and re-anneal before the high-resolution melting. The change in fluorescence signal due to the dissociation of DNA duplexes and the release of DNA-binding dye in the melting step were monitored in real time. The high-resolution melting curve profile was analyzed using HRM analysis software for Windows® version 2.0.1 with fluorescence normalization. Finally, direct DNA sequencing was also performed using an Applied Biosystems 3130 automated sequencer to confirm any mutations identified by the PCR-HRM method.

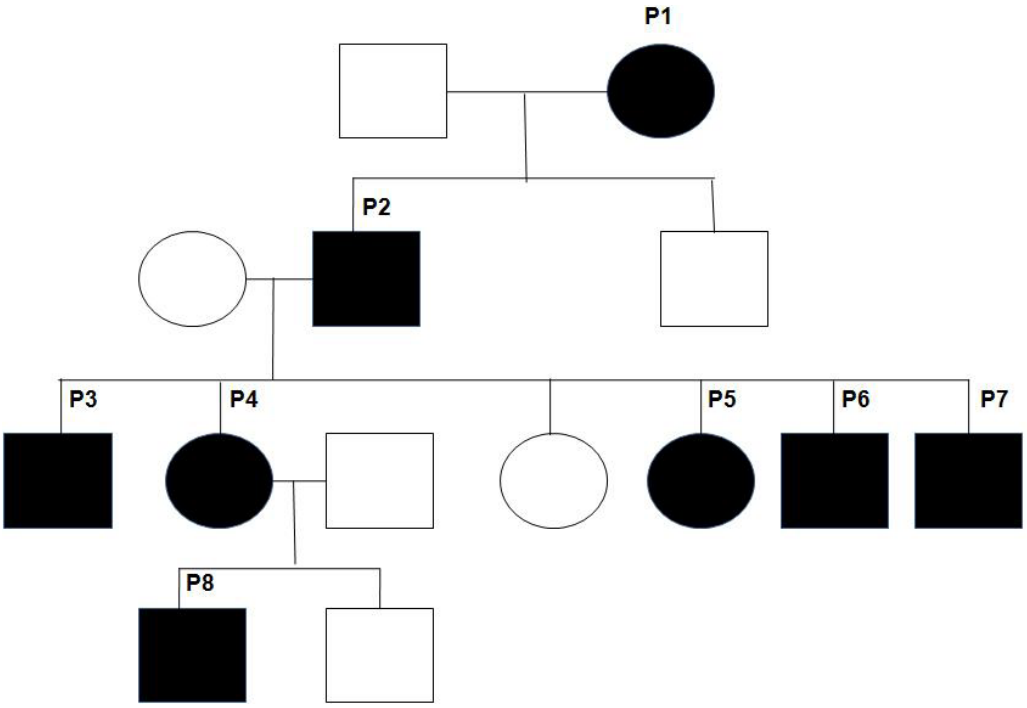

Figure 1. Pedigree of a Dusun family of four generations with aniridia. Filled symbols represent subjects with aniridia and open symbols represent normal subjects.

RESULTS

Real-time PCR with high-resolution melting curve analysis indicated differences in the shape of the melting curve of the PCR-amplified exon 7 of the *PAX6* gene in the aniridia patients but not in the controls (Figure 2). In addition, direct sequencing indicated a heterozygous G deletion (c.857delG) in the exon 7 of *PAX6* (Figure 3). This deletion generates a frame shift and a premature termination codon in the *PAX6* open reading frame, resulting in a transcript that is recognized by the nonsense-mediated mRNA decay system that prevents the accumulation of truncated proteins (Byers, 2002). We identified the same (c.857delG) deletion in all affected family members, indicating that this single mutation is the cause of the aniridia spectrums observed in this family. We also analyzed the genotype-phenotype correlation arising from the c.857delG mutation in *PAX6* gene. All family members diagnosed with bilateral aniridia displayed bilateral jerky eye movement, and this symptom manifested at birth because it was already observed in P8, the 2-month-old infant. P2, the father aged 55, had a Snellen acuity of less than 6/48 and required a low-vision aid. The infant's siblings, P3-P7 only required glasses to improve their vision. We were unable to determine the visual acuity in the 2-month-old infant with the same accuracy as in the adults. All patients also developed photophobia. Further examination revealed the presence of a bilateral horizontal nystagmus. Figure 4 shows the phenotypic expression of the *PAX6* gene with the identified mutation. All patients had clear microcorneas measuring only about 10 mm. In P1 and P2, the presence of bilateral cornea opacity and vascularization demonstrated that the severity of the disorder increases with the age of the affected individuals. Nevertheless, the whole lens and zonules were visible and subluxation was not observed in any patient. P2, who had been diagnosed with bilateral cataract, had undergone eye surgery 18 years before this study and wore aphakic glasses. All other affected family members had received no treatment at the time of this study.

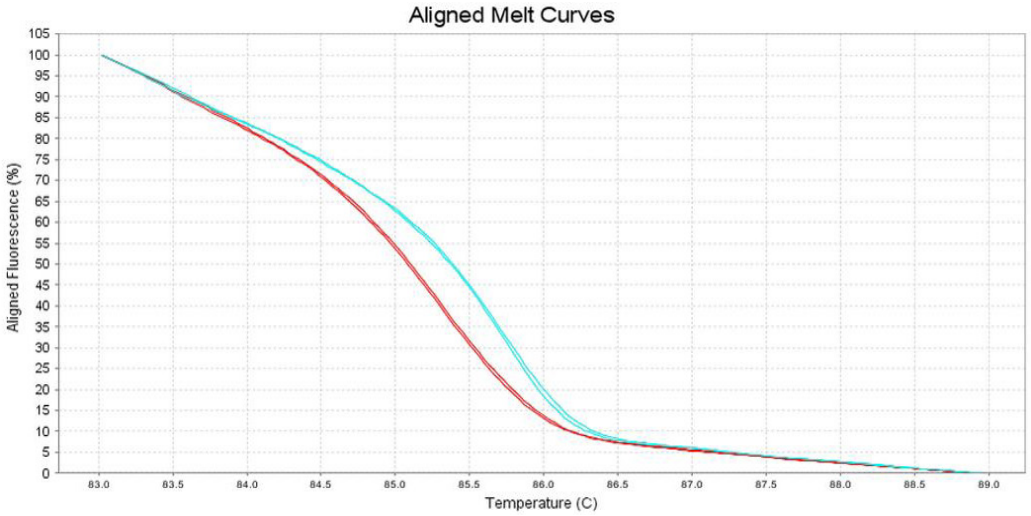

Figure 2. Melt curve analysis of *PAX6* mutation in patient samples. Normal (red) and mutated (blue) sequences of *PAX6* exon 7 are presented in aligned melt curves.

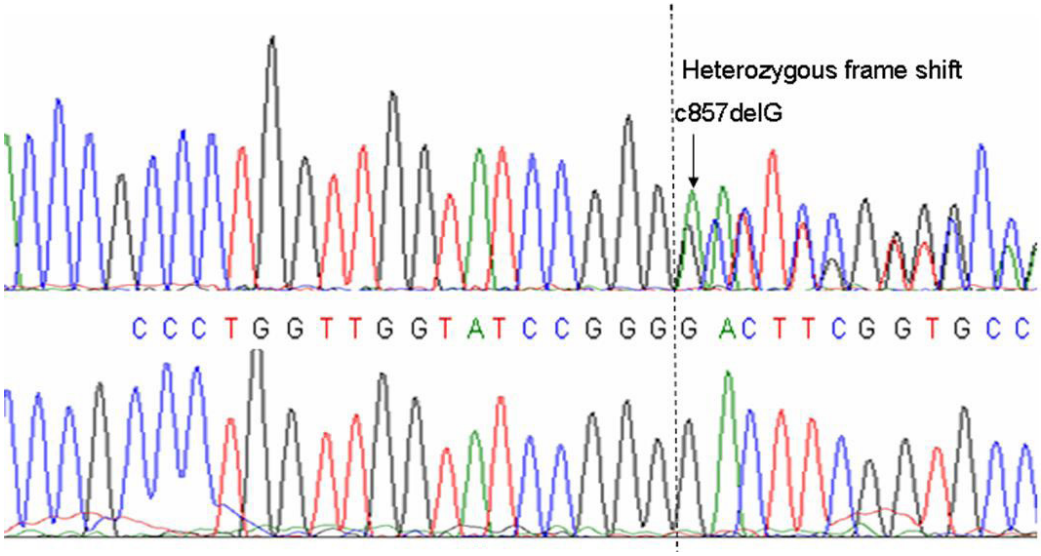

Figure 3. *PAX6* gene mutation analysis by direct sequencing of the PCR products of exon 7. A heterozygous deletion of a G residue at nucleotide 857 generating frame-shift in one of the DNA strand (i) compared to a normal sequence (ii). Arrow indicates the location of the deletion.

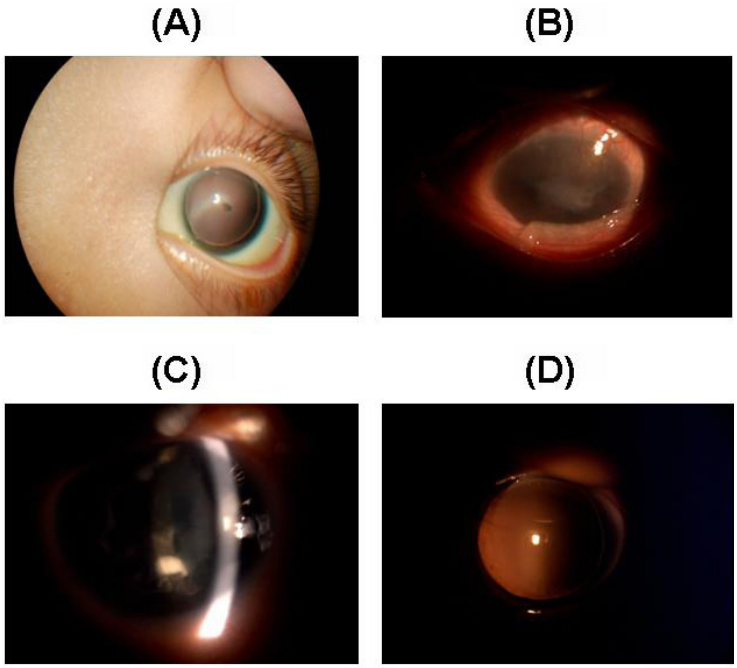

Figure 4. Examination of whole lens and zonules was visible (**A**) and no lens subluxation but cataractous in P6, P2, P5, and P7 (**B-D**) bilateral nystagmus with aniridia and cataractous lens.

DISCUSSION

Real-time PCR with high-resolution melting curve analysis is an approach for detecting genetic variants and mutations based on the theory of PCR amplicon melting (dissociation). Differences in T_m among the PCR amplicons from exon 7 of the *PAX6* gene resulted in melting curve difference that suggested the presence of a SNP heterozygote in this exon. The detection of the *PAX6* mutation in this case study is very useful for genetic counseling and prenatal diagnosis. This study showed that the congenital cataract phenotype observed in these individuals is associated with the frame-shift mutation identified in *PAX6*. In this disorder, the eye lens may develop cataracts that may have a significant impact on visual acuity by the 3rd decade of life as manifested in P1 and P2. Approx. 12% of the mutations recorded in the Human *PAX6* Mutation Database do not result in cataracts. Nevertheless, a similar frame-shift mutation in a Chinese family has been reported to generate the congenital cataract phenotype (Song et al., 2005). This phenotype had also been reported to be associated with missense mutations in exons 1-6 of *PAX6* at the region of its PD domain with subsequent variability in severity (Glaser et al., 1994; Gronskov et al., 1999; Cai et al., 2010). It is worth noting that all the family members affected by aniridia had satisfactory intraocular eye pressures. None of the patients developed glaucoma or foveal hypoplasia, indicating that the mutation reported here appears to inhibit the function of *PAX6* less than other reported *PAX6* mutations that allow normal optic nerve development. All affected members in this family harboring the c.857delG mutation exhibited a similar spectrum of aniridia with no phenotypic variability but with differences in severity. The variability in severity might be caused or influenced by other gene products belonging to diverse families of transcription factors or cotranscription factors such as PAX2, SOX, MITF, and others that might interact with PAX6 during cellular development (Kamachi et al. 2001, Planque et al., 2001).

In conclusion, to the best of our knowledge, this is the first analysis of an aniridia mutation using PCR-HRM analysis that overcomes the low rate of detection of *PAX6* mutations. This is also the first study reporting a frame-shift mutation (c.857delG) in exon 7 of the *PAX6* gene in a Dusun family and that describes the consequences of this mutation, which is often underrepresented in aniridia. We suggest that the c.857delG mutation plays a causative role in the phenotypes reported in this family. Although approximately 50% of aniridia cases reported include glaucoma and foveal hypoplasia, these phenotypes were not observed in the family examined in this study. The frame-shift mutation in *PAX6* generated a limited spectrum of aniridia and congenital cataract developed at different ages; therefore, the aniridia disorder requires lifelong management.

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