Introduction

Leber congenital amaurosis (LCA) is the most common genetic cause of congenital visual impairment in children and infants, and is characterized by a severe dystrophy of the retina. LCA affects around 1 in 80,000 of the population. Visual function of LCA patients is usually poor and often accompanied by nystagmus, sluggish or near-absent pupillary responses, photophobia, high hyperopia, and keratoconus. There are 17 genes, including the \textit{RPE65} gene, known to cause LCA, and mutations in these genes account for at least half of the LCA cases. Mutation of the \textit{RPE65} gene may be associated with LCA type 2 (LCA2), which causes night blindness. \textit{RPE65} contains 14 coding exons and encodes a protein of 65,000 expressing specifically and abundantly in the retinal pigment epithelium (RPE), which is involved in the production of 11-cis retinal and visual pigment regeneration.\cite{1,2} Clinical trials using \textit{RPE65} as the only targeting molecule for LCA gene therapy are progressing rapidly recently. According to a research by Morimura \textit{et al}.,\cite{3} mutations of the \textit{RPE65} gene account for 16\% of the cases of LCA. In the case of LCA2, though some patients may experience transient improvement in vision, they eventually progress to a complete vision loss.\cite{4,5}

While LCA has been identified as a major cause of congenital visual impairment, the prevalence of the disease varies across different geographical origins.\cite{6,7} The purpose of this study was to analyze \textit{RPE65} mutation in Chinese patients with LCA, which may provide useful information for gene therapy of this disease in China.
**Methods**

The study was conducted in accordance with the Declaration of Helsinki and approved by the local ethics committee of Peking University Third Hospital (No. 2012093). Informed written consent was obtained from all patients prior to their enrollment in this study.

Clinical data and 4-ml blood samples were collected from patients with LCA. The patients underwent complete physical and ophthalmic examinations. To identify causative mutations, genomic DNA was extracted from peripheral blood cells according to standard protocol (Roche Diagnostics Corporation, Indianapolis, USA). Then, all the exons and exon-intron boundaries of RPE65 were amplified using the standard polymerase chain reaction (PCR) buffer system with primers [Table 1].

PCR reactions were each performed in a 10 μl volume containing 1.5 mmol/L MgCl₂, 0.4 mmol/L of each primer, 200 μmol/L dNTPs, 1 U Taq DNA polymerase (Takara, Japan), and 10–20 ng template DNA. Amplification was performed with an initial denaturation for 3 min at 95°C, followed by 30 cycles of denaturation at 95°C for 1 min; we then annealed at 55°C for 1 min with extension at 72°C for 1 min, and a final extension at 72°C for 3 min.

PCR products were purified using a PCR product purification kit (Qiagen, CA). Purified PCR products were sequenced using the BigDye Terminator Cycle Sequencing v3.1 kit (Applied Biosystems, CA, USA). Then, 10 ng of template DNA was added in each reaction followed by a temperature program which included 25 cycles of denaturation at 97°C for 30 s, annealing at 50°C for 15 s, and an extension at 60°C for 4 min. All samples were analyzed in an ABI Prism 310 Genetic Analyzer (Applied Biosystems, CA, USA).

The RPE65 cDNA reference sequence with GenBank accession No. NC_000001.10 was used (National Center for Biotechnological Information, Bethesda, Md; available at: http://www.ncbi.nlm.nih.gov).

We predicted the protein structure via the threading approach. Both protein sequences were searched against PDB database to select the most similar templates along with sequence-structured alignment. Given the candidate templates and target-template alignments, a modeler was used to build candidate models for each corresponding template.

**Results**

Totally 52 sporadic LCA patients were recruited. All patients have early severe visual deficits in childhood with their visual acuity <20/400. Sequencing of the 14 coding exons of RPE65 identified a mutation in exon 11 [c.1174 A > C, Figure 1a] in one patient, which resulted in substitution of threonine by proline (T392P). The mutation was not found in other patients and 100 ethnic unrelated and unaffected normal controls [Figure 1b].

The mutation led to a significant change in the RPE65 protein’s structure. For each model, we observed difficulties in obtaining the most stable tertiary structure of the side chain structures of each amino acid [Figure 2].

The RPE65 mutation patient was a 23-year-old male without a family history of LCA. The disease appeared when he was 17 years and his vision decreased to 0.01 gradually. Pendular nystagmus and deep-set eyes were found in this patient, who was extremely sensitive to light. The results of fundus examination displayed a salt-and-pepper appearance with minimal attenuated retinal vessels, and many whitish punctuate lesions in the midperipheral retina [Figure 3]. Extinguished electroretinogram was observed [Figure 4].

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**Table 1: Primers used to amplify the exons of RPE65**

| Primer name | Sequence | Melting temperature (°C) | Product size (bp) |
|-------------|----------|--------------------------|-------------------|
| RPE65_E1_F  | aagcaactcgttgtcccct  | 60.11                    | 308               |
| RPE65_E1_R  | ttccccccacaaaaatcaag  | 59.77                    |                   |
| RPE65_E2_F  | ggagtgaacagctggagcc  | 60.00                    | 324               |
| RPE65_E2_R  | aaaccaactgatcctctcc  | 60.31                    |                   |
| RPE65_E3_F  | caactgccagctctatgagga | 59.14                    | 410               |
| RPE65_E3_R  | actggcccaggtacattgtg  | 60.83                    |                   |
| RPE65_E4/5_F| tttgattgtgacctgtgagga| 58.63                    | 367               |
| RPE65_E4/5_R| catttgagctgtaggtggt  | 59.93                    |                   |
| RPE65_E6_F  | agaggagtggatgtaggggagca| 57.62                   | 402               |
| RPE65_E6_R  | ataggagtggatgtaggggagca| 60.67                   |                   |
| RPE65_E7/8/9_F| tcaaaatgtgtttctttgcct| 57.41                    | 900               |
| RPE65_E7/8/9_R| ttgactcatacctgctctctgt| 60.00                   |                   |
| RPE65_E10_F | agcagtttcttcttgtgagga| 60.69                    | 379               |
| RPE65_E10_R | gcctatttaaaaacccctttcctgtgc  | 59.55                    |                   |
| RPE65_E11/12/13_F| tctctgacatggcataaa  | 59.12                    | 826               |
| RPE65_E11/12/13_R| ggtctggtttaggtattagggga| 59.41                   |                   |
| RPE65_E14_F | tcaggttctaggtttcttacatttg  | 57.75                    | 499               |
| RPE65_E14_R | ggctggtctcagaggaag  | 59.99                    |                   |

RPE: Retinal pigment epithelium.
**DISCUSSION**

LCA accounts for at least 5% of all retinal dystrophies and is one of the main causes of blindness in children.[8,9] Missense mutations in RPE65 were identified in a patient with LCA2 using the candidate gene scanning approach.[10] Since the initial report, a wide range of RPE65 mutations associated with LCA had been identified.[5,11,12] The RPE65 protein has an essential role in maintaining retinal function and photoreceptor viability, and mutations in this protein affect the essential pathways involved in the processing and metabolism of Vitamin A and retinoid cycling between the RPE and photoreceptors.[13]

Young patients with RPE65 mutations display a foveal cone loss along with shortened inner and outer segments of the remaining cones. Maeda et al.[14] suggested that chronic lack of chromophore might lead to a progressive loss of cones in mice and humans, and that therapy for LCA patients could be geared toward early adequate delivery of chromophore to cone photoreceptors. RPE65 was the
first candidate for gene therapy of this disorder. Most patients in RPE65 gene therapy exhibited some extent of improvement in visual function without obvious adverse effects.\(^3\)\(^{-18}\)

It has been reported that 133 RPE65 mutations are associated with LCA (HGMD), with the frequency of RPE65 mutation ranging from 6% to 21%.\(^3\)\(^{-15}\) In this study, however, we identified a novel mutation in the 11th exon of RPE65 (c.1174 A > C), resulting in the substitution of threonine by proline at codon 392 (T392P) in one LCA patient. This novel homozygous missense mutation in RPE65 was found to be responsible for causing LCA. But in this study cohort of Chinese patients with LCA, only one of the 52 patients recruited was identified to be carrying RPE65 mutation – a frequency which is much lower than that found in LCA patients in Northwest Europe and the United States.\(^7\) This indicates that RPE65 mutations may not play a major role in LCA patients in China. However, while estimating the RPE65 mutation frequency in LCA patients in China may provide useful information for gene therapy of this disease, the LCA patients’ cohort in our study may not have been sufficient to estimate an accurate RPE65 mutation frequency in our LCA patients given that only 1 out of 52 patients carried mutation in RPE65. This necessitates further studies with a larger cohort to enhance better understanding of the role of RPE65 mutations in LCA patients in China.

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Conflicts of interest
There are no conflicts of interest.

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