·**Basic Research**·

Comprehensive analysis of genetic variations in strictly–defined Leber congenital amaurosis with whole–exome sequencing in Chinese

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Abstract

• **AIM: To make a comprehensive analysis of the potential pathogenic genes related with Leber congenital amaurosis (LCA) in Chinese.**

• **METHODS: LCA subjects and their families were retrospectively collected from 2013 to 2015. Firstly, whole–exome sequencing was performed in patients who had underwent gene mutation screening with nothing found, and then homozygous sites was selected, candidate sites were annotated, and pathogenic analysis was conducted using softwares including Sorting Tolerant from Intolerant (SIFT), Polyphen –2, Mutation assessor, Condel, and Functional Analysis through Hidden Markov Models (FATHMM). Furthermore, Gene Ontology function and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses of pathogenic genes were performed followed by co –segregation analysis using Fisher exact Test. Sanger sequencing was used to validate single –nucleotide variations (SNVs). Expanded verification was performed in the rest patients.**

• **RESULTS: Totally 51 LCA families with 53 patients and 24 family members were recruited. A total of 104 SNVs (66 LCA –related genes and 15 co –segregated genes) were submitted for expand verification. The frequencies of homozygous mutation of *KRT12* and *CYP1A1* were simultaneously observed in 3 families. Enrichment analysis showed that the potential pathogenic genes were mainly enriched in functions related to cell adhesion, biological adhesion, retinoid metabolic process, and eye development biological adhesion. Additionally, *WFS1* and *STAU2* had the highest homozygous frequencies.**

• **CONCLUSION: LCA is a highly heterogeneous disease. Mutations in *KRT12*, *CYP1A1*, *WFS1*, and *STAU2* may be involved in the development of LCA.**

INTRODUCTION

Leber congenital amaurosis (LCA) is a rare inherited dystrophy of the retina, which is characterized by severe loss of retinal and visual functions early in life with progressive degeneration of the cellular structure of the retina [1]. LCA patients usually have poor visual function and non-detectable or subnormal electroretinogram (ERG), and are often accompanied by several complications such as nystagmus, photophobia, and keratoconus [1]. Visual acuity is rarely better than 20/400 [2]. Presently, this disease affects approximately one in 80 000 of the population[3]. About 20% of children with LCA attend blind schools, accounting for about 5% of all retinal dystrophies [4-6]. However, the molecular mechanisms underlying this disease are so complex to be fully understood.

LCA is a heterogeneous and autosomal recessive disease due to the abnormal development of photoreceptor cells[1]. Broad expression variability is observed in patients with LCA, and the mechanisms of LCA disease are involved with disruptions in phototransduction (*AIPL1*, *GUCY2D*), retinoid cycle (*RDH12*, *LRAT*, *RPE65*), photoreceptor development and structure (*CRX*, *CRB1*), transport across the photoreceptor connecting cilium (*TULP1*, *PRGRIP1*, *CEP290*, *LCA5*), and other ERG functions (*IMPDH1*, *MERTK*, *RD3*)[1]. Moreover, it has been well demonstrated that mutations in a single LCA gene can lead to varied clinical phenotypes, and more than 60% LCA is caused by numerous mutations in these genes[7]. However, these known genes share no specific regions that can be used as the genetic markers for most LCA cases, and few clinical features are specific to individual genetic abnormalities. Furthermore, genetic cause for 30%-50% patients suffering from LCA is still unclear, besides some other candidate genes have not been identified.

Whole-exome sequencing presents a broad molecular background of disease, and can be used to distinguish new candidate genes. Based on homozygosity mapping, whole-exome sequencing has been successfully used to identify mutations in LCA-related genes [8-10]. In the present study, we aimed to make a comprehensive analysis of the potential pathogenic genes related with LCA in Chinese. Briefly, whole-exome sequencing was used to screen gene variants in parents and offsprings of LCA pedigrees who had not ever been identified mutations in known LCA genes. Then, the identified pathogenic variants were firstly verified *via* Sanger sequencing, and then were further verified in another 41 patients. Specially, this study only investigated the homozygous mutations, while was not involved in compound heterozygous mutations and gene modifications. In addition, in order to exclude patients with early onset retinitis pigmentosa or other syndromic diseases that shared same phenotypes with LCA, clinical samples were restricted to those who were <1 year old and had typical LCA phenotypes.

SUBJECTS AND METHODS

Subjects This was a retrospective study conducted at Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (Shanghai, China). From May 2013 to November 2015, all subjects with LCA were collected. They were performed fundus screening and ERG using RetCamⅡ (Massie Research Laboratorles, Inc., USA). All patients were recognized suitable if they met the following criteria: 1) poor eyesight (without fixation, pendular nystagmus, or the ability to follow light or subject) at birth or within one year of age; 2) patients with extinguished ERG results. The following cases were excluded: patients suffered from other congenital eye disease (retinopathy of prematurity, congenital glaucoma, or familial exudative vitreoretinopathy) or systemic hypoplasia (hearing, vestibular function, teeth, bone, muscle tension, intelligence, liver, kidney, or blood sugar). The legal guardians of all patients were provided with informed consent, and the protocol was approved by the ethics committee of Xinhua Hospital.

Whole−exome Sequencing All participants were subjected to whole-exome sequencing. Briefly, blood samples were collected and the genome DNA was isolated using the Qiagen blood genomic DNA extraction kit (Qiagen, Valencia, CA, USA) according to the instructions. The DNA samples were ligated to paired-end adapters and amplified by polymerase chain-reaction assay. Exome hybridization was performed in all samples based on Ion Torrent platform and the whole exons sequencing was performed by using Ion Torrent™ semiconductor sequencing system (Life Technologies, USA).

Data Analysis Considering that LCA is usually an autosomal recessive genetic disease, the homozygous sites were mainly selected as reliable mutation sites. If a site was recessive homozygous in the immediate family, it would be excluded. Then, the candidate sites were annotated based on the single-nucleotide polymorphism database (dbSNP) database, HapMap project, 1000 genomes project, and exome sequencing project using SeattleSeq SNP annotation and variant effect predictor in Ensemble database. After excluding synonymous mutations and mutations in intron area or untranslated region (UTR), the mutations with biological functions were remained, including non-synonymous mutation, frameshift mutation, splice site variants, and termination codon mutations. Subsequently, pathogenic analysis of the mutations with biological functions was carried out using softwares (SIFT, Polyphen-2, Mutation assessor, Condel, FATHMM). Furthermore, Gene Ontology (GO) function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of pathogenic genes were performed using database for annotation, visualization and integration discovery (DAVID). $P<0.05$ was set as significant cut-off value for GO and KEGG pathway analysis. Moreover, sight-related pathogenic genes were identified based on pathways which were enriched by the known LCA-related genes, including pathways of eye development, retinoidmetabolic process, sensory perception of light stimulus, visual perception, vitamin A biosynthetic process, and photoreceptor cell maintenance.

Co−segregation Analysis The disease information of pathogenic genes was obtained from DAVID, and the expression information was extracted from BioGPS database which collected expression information from 84 human tissues by using Affymetrix U133A arrays. These pathogenic genes in both LCA patients and normal subjects were analyzed using Fisher's exact test. The non-empty hypothesis was that the frequency of single-nucleotide variations (SNVs) was higher in LCA patients compared with normal subjects ($P \leqslant 0.05$), which was in order to verify whether genotype mutations were consistent with the phenotypes of LCA or not.

Sanger Sequencing Sanger sequencing was used to evaluate SNVs in each subject. All segments were amplified through standard polymerase chain reaction (PCR), and primers were designed using Primer Premier 5.0. The sequencing results were analyzed through Chromas 2.2 software, and sequence alignment was performed to exclude false positive sites.

Expand Verification SNVs were further verified using targeted next-generation sequencing in the other 41 subjects. In brief, after preparation of sequencing template with Ion PGM template OT2 200 kit (Life Technologies, Gaithersburg, MD, USA), sequencing was performed using Ion proton 200 sequencing kit (Life Technologies, Carlsbad, USA) based on the Ion Ponton™ system.

RESULTS

Pedigrees Totally, ten families (family 1, 4, 7, 10, 11, 13, 14,

Table 1 Clinical characteristics of the 11 LCA patients from 10 families

Patient ID	Sex	Visual acuity (right/left)	Nystagmus	Fundus change	Vascular changes	Consan-guineous
1-3	M	LP/LP	No	Pigmentary deposit	Normal	No
4-3	F	HM/HM	No	Leopard-like	Attenuated	Yes
7-3	M	HM/HM	No	Salt-pepper	Attenuated	No
10-3	F	LP/LP	No	Salt-pepper	Attenuated	No
11-3	M	LP/LP	Yes	Normal	Normal	No
13-3	F	FC/HM	Yes	Bone spicule	Attenuated	Yes
14-3	F	0.02/0.03	No	Salt-pepper	Attenuated	No
15-3	F	0.1/0.1	No	Nummular	Attenuated	No
24-3	M	LP/HM	No	Salt-pepper	Normal	Yes
37-3	F	0.03/0.02	No	Fishnet latticed	Normal	No
37-4	M	LP/HM	No	Fishnet latticed	Normal	No

LP: Light perception; NLP: No light perception; FC: Finger counting; HM: Hand movement.
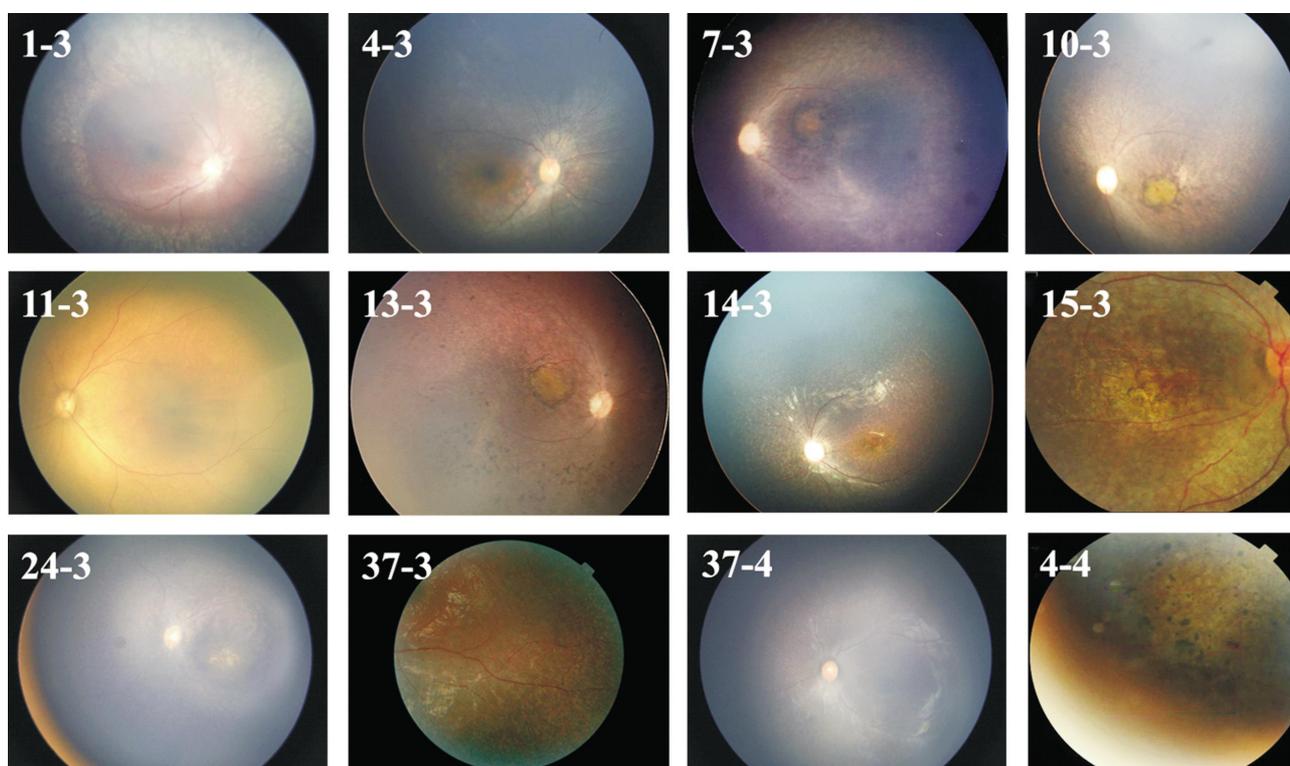
Figure 1 Color retinal photographs of the 11 LCA subjects and one suspected member 4–4.

15, 24 and 37) were recruited in the whole-exome sequencing, among which family 37 included two LCA patients (patient identifiers: 37-3, 37-4). The clinical characteristics of these patients were listed in Table 1. One member of family 4 (4-4, the aunt of 4-3) had typical clinical symptoms of LCA (Figure 1) but the age of onset was unclear. Specially, the previously known LCA-associated gene mutations were not detected in 10 families in our hospital or other hospitals. Additionally, the photographs of retina color for all the 11 LCA patients were exhibited in Figure 1.

Functional and Pathway Enrichment Analyses of Pathogenic Genes GO functions and KEGG pathways enriched by the potential pathogenic genes showed that the potential pathogenic genes were mainly enriched in functions related to cell adhesion, biological adhesion, retinoid metabolic process, and eye development biological adhesion. For instance, the pathogenic genes in sample 13-3 were significantly enriched in the functions associated with cell adhesion and biological adhesion. The pathogenic genes in sample 7-3 were mainly enriched in pathways of viral myocarditis, olfactory transduction, and autoimmune thyroid disease.

Leker Congenital Amaurosis–related Potential Pathogenic Gene Based on the sight-related pathways enriched by previously known LCA-related genes, 68 LCA-related potential pathogenic genes were identified, and their occurrence frequencies in the 11 samples were calculated.

Among these pathogenic genes, mutations in cytochrome P450, family 1, subfamily A, polypeptide 1 (*CYP1A1*) and eyes shut homologue (*EYS*) were simultaneously found in

four families, while *EYS* was not co-segregated. The mutation (amino acid Ile was converted to Val) in *CYP1A1* was observed in three families (sample 1-3, 4-3 and 10-3). Moreover, another two co-segregated genes were found in three families (sample 7-3, 13-3 and 24-3), including solute carrier family 22, member 16 (*SLC22A16*: amino acid His was converted to Arg) and keratin 12 (*KRT12*: amino acid Pro was converted to Ser). *SLC22A16* was not detected in retinal tissue.

Sanger Sequencing Fifty percent of the potential LCA-related mutations (82) were randomly selected for further validation within the 11 samples. The results revealed that the accuracy of mutation detection was 72%.

Expand Verification A total of 104 SNVs (66 sight-related genes and 15 co-segregated genes) were submitted to expand verification in another 41 samples. The frequencies of homozygous mutation for *KRT12* and *CYP1A1* were respectively 0.333 and 0.118 in total 52 samples. In addition, wolfram syndrome 1 (*WFS1*) and staufen double-stranded RNA binding protein 2 (*STAU2*) had the highest homozygous frequencies (0.941 and 0.922, respectively).

DISCUSSION

LCA is usually considered as the most severe and earliest dystrophy of the retina, which causes childhood blindness[11]. Half blindness in children is caused by genetic alterations, and some special retinal appearance and longitudinal varies in visual function seem to be gene-specific [12]. At present, Online Mendelian Inheritance In Man (OMIM) have recognized 18 types of LCA. At least 22 LCA associated genes, such as *RPE65, AIPL1, NMNAT1,* and *LCA5* have been identified by linkage analysis with microsatellite markers or identity-by descent mapping or the candidate gene method [13]. In the present study, a total of 104 novel SNVs (66 sight-related genes and 15 co-segregated genes) were identified. After co-segregation analysis and verification, *CYP1A1*and *KRT12* were simultaneously found in three families, and *WFS1* and *STAU2* had the highest homozygous frequencies. Interestingly, the four genes in our study have not been discussed before, which may be because they are specific to Chinese population.

Reportedly, *CYP* genes are involved in the process of organism response to environmental challenges, and directly interfere with the embryo development [14]. Deleterious mutation of *CYP1B1* causes human primary congenital glaucoma *via* disrupting the development of trabecular meshwork [15]. *CYP1A1*, an extrahepatic phase I metabolizing enzyme, is usually suppressed under physiologic conditions and its expression is regulated by AHR (aryl hydrocarbon receptor) signaling. The up-regulation and polymorphisms of *CYP1A1* have been found to be associated with cell proliferation in some cancers [16]. Moreover, *CYP1A1* is up-regulated in the retina by intraperitoneal injection of doxin, which finally induces abnormal vascularization in the

eye [17]. Dong *et al*[18] demonstrated that mutants in *CYP1A1* (Arg34Asp and Lys39Ile) could abolish the mitochondrial targeting signal. However, to our knowledge, there is hardly any study about the role of *CYP1A1* in LCA. In the present study, *CYP1A1* mutations (Ile433Val, Ile434Val, and Ile462Val) were found in sample 1-3, 4-3 and 10-3, which had a phenotype of thinner vascular. What's more, GO annotation suggested that *CYP1A1* was mainly involved in functions related to retinoid metabolic process and eye development. Therefore, we speculated that *CYP1A1* mutation might be related to the process of LCA.

Keratin proteins are divided into type I (*KRT9-21*) and type II (*KRT1-8*) based on their sizes and isoelectric points, both of which are specifically and predominantly expressed in corneal epithelial cells and function as obligate heterodimers [19]. *KRT12* is located on chromosome 17q12, and several mutations in *KRT12* can cause Meesmann corneal epithelial dystrophy [20]. These mutations are located either in the highly conserved α-helix-initiation motif of domain 1A or the α-helix-termination motif of domain 2B, which play essential roles in the assembly of keratin filament [19,21]. Besides, altered expression of keratin is also found in human retinal pigment epithelial cells [22]. In the current study, a missense mutation (Pro15Ser) of *KRT12* was identified in LCA samples. Additionally, function annotation suggested that *KRT12* was involved in functions including sensory perception and visual perception. Therefore, mutation in *KRT12* may be associated with LCA.

In addition, our result showed that *WFS1* and *STAU2* had the highest homozygous frequencies. *WFS1* and *STAU2* are expressed in the central nervous system. *WFS1* is selectively expressed in neurons in different brain areas. Kawano *et al*[23] have suggested that the mRNA and protein of *WFS1* are detected in retina especially in amacrine and Müller cells, playing a physiological role in normal visual system. Moreover, *WFS1* mutation has an effect on the survival of retinal ganglion cells and subsequently results in anterograde atrophy of retinal axons [24]. *STAU2*, a double-strand mRNA binding protein, has been reported to be associated with spinocerebellar ataxia, retinal development, and eye morphogenesis[25]. Cockburn *et al* [26] have found that ectopic expression of *STAU2* increases eye size, and silencing of *STAU2* results in a significantly reduced right/left eye diameter ratio in embryos. Combining with previous studies, we speculated that *WFS1* and *STAU2* mutations might have important effects on the development of LCA.

For the treatment of LCA, gene therapy has been reported to be safe and effective. In one form of LCA, patients bear a mutation in the *RPE65* gene. In clinical trials, *PRE65* -targeted therapy has been suggested to be a successful treatment for LCA through gene augmentation therapy. Importantly, this therapeutic method can maintain the vision function of LCA patients for more than three years [27].

Additionally, the therapeutic efficiencies of *GUCY2D* and *RPGRIP1* on LCA have been analyzed in LCA animal models [28-29]. In the present study, the newly detected genes might be involved in vision-related pathways, therefore, it may be worthy to carry out more *in vivo* and *vitro* studies with larger samples to confirm their specific mechanism and further therapy efficiency.

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