Molecular screening of the *LPCAT1* gene in patients with retinitis pigmentosa without defined mutations in known retinitis pigmentosa genes

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Abstract. Retinitis pigmentosa (RP) is an inherited retinopathy, which affects the photoreceptors in the retina. Lysophosphatidylcholine acyltransferase (LPCAT) is a critical phospholipid biosynthesis enzyme, which promotes the conversion of lysophosphatidylcholine into phosphatidylcholine in the remodeling pathway of PC biosynthesis. A previous study reported a homozygous insertion in the *LPCAT1* gene in mice exhibiting retinal degeneration (rd11). However, whether genetic mutations in *LPCAT1* predispose individuals to RP remains to be elucidated. Therefore, the aim of the present study was to investigate whether *LPCAT1* mutations exist in patients with RP. A total of 50 unrelated patients diagnosed with either a sporadic or recessive inheritance pattern of RP were recruited in the present study. All of the patients were comprehensively screened for genes associated with the predisposition of RP, and no pathogenic mutations were identified. Reverse transcription-polymerase chain reaction and Sanger sequencing were performed to investigate the coding regions and exon-intron boundaries of the *LPCAT1* gene in the recruited patients. In total, three genetic variations in the coding regions, which lead to amino acid changes, were identified. Although two of these mutations were predicted to be pathogenic, co-segregation analysis in the pedigrees excluded these as disease-causing mutations. In addition, the *LPCAT1* gene was screen in a panel of RP patients who exhibited no identifiable mutations in any of the known RP-associated genes. No disease-causing mutations in the *LPCAT1* gene were identified, indicating that *LPCAT1* either does not confer a genetic predisposition to RP, or that the incidence of mutations in *LPCAT1* is particularly rare in patients with RP.

Introduction

Retinitis pigmentosa (RP) is a classical inherited eye disorder, which predominantly involves damage to the function and structure of the rod and cone cell photoreceptors and the retina pigment epithelium (1). Individuals affected by RP usually experience night blindness from the early stage of RP, accompanied or followed by the loss of peripheral visual field (1). The typical signs usually present as bone spicule-like pigmentation deposits and a reduced or absent electroretinogram. There are several different genetic defects that could lead to the degeneration of cone or rod cells or to degeneration of the connections between these cells. To date, >70 genes have been identified and seven loci have been mapped for RP. The majority of these genes encode proteins, which are involved in a wide variety of cellular processes, including phototransduction, transcriptional regulation and membrane structure formation. Progress in gene discovery and mutation screening in individuals affected by RP and their families has contributed to personalized treatments for RP, which uses various novel treatment approaches, including stem cells, gene therapy and nutritional supplementation (2-4).

The *LPCAT1* gene is located on chromosome 5 and it encodes a 65 kDa protein, which contains three putative transmembrane domains (5). This enzyme, lysophosphatidylcholine acyltransferase 1 (LPCAT1), is involved in lysophosphatidylcholine (LPC) and lipid syntheses, which are important in the formation of biological membranes, endocytosis, signaling and neuroprotection. The outer segment of retinal photoreceptors contains stacks of membranous disks, which are filled with opsin, a light-absorbing protein in the visual transduction

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process (6). The loss of phosphatidylcholine (PC) can lead to the disruption of membrane structure and homeostasis maintenance, which can ultimately contribute to photoreceptor cell degeneration (6). In addition, *LPCAT1* has been identified to be involved in the non-inflammatory PAF remodeling pathway and in the progression of cancer, including like colorectal and prostate cancer (7-9).

A previous study by Friedman *et al* (6) identified a single nucleotide insertion (c.420_421insG) in exon 3 of the *LPCAT1* gene in rd11 mice, and identified a seven-nucleotide deletion (c.14-20delGCCGCGG) in exon 1 in mice of the B6-JR2845 strain, which leads to premature truncation of the *LPCAT1* protein. These two mouse strains present with typical symptoms of RP. Dai *et al* demonstrated that retinal function and structure can be rescued in rd11 mice using gene replacement therapy (10). This further supports the hypothesis that *LPCAT1* may be a possible candidate disease-causing gene of RP in humans. However, the specific genetic mutations in *LPCAT1*, which predispose individuals to RP remain to be elucidated. With the exception of *LPCAT1*, a number of genes (Table I) have been demonstrated to cause retinal degeneration in animal models, but not in human subjects (11-15).

There are three major patterns of inheritance in RP, including autosomal dominant (ad), autosomal recessive (ar) and X-linked inheritance. Of the total cases of RP, ~30% are adRP, 20% are arRP and 15% are X-linked RP (16). The remaining cases, at least 30%, are isolated cases, the patterns of which are difficult to distinguish as either recessive or dominant inheritance. Since the *LPCAT1* mutations, which have been identified in mice are homozygous, the present study hypothesized that the mode of inheritance of this gene is either recessive or sporadic. Therefore, the present study recruited a cohort of patients who were diagnosed with RP and exhibited a either one of these two types of inheritance patterns. In the present study, the *LPCAT1* gene was screened in 50 Chinese patients with RP, and mutations in all previously investigated genes were excluded based on a targeted exome-sequencing panel, suggesting the existence of novel causative genes.

Materials and methods

Patient recruitment. The present study was performed in accordance with the Declaration of Helsinki. The study was supported by the Ethics Committee of The Eye Hospital of Wenzhou Medical University (Division of Opthalmic Genetics, Wenzhou, China). Written informed consent was obtained from each patient prior to commencement of the investigation. All the patients were natives of China and the detailed family histories of the patients were collected. The majority of the participants were affected by isolated or simple cases of RP, without any obvious genetic predisposition. The diagnoses of RP were made based on the presence of symptoms, including night blindness and impaired visual acuity, observed typical fundus, reduced peripheral visual field and abnormal optical coherence tomography (OCT) results. The total number of patients enrolled in the present study was 50, which included 24 females and 26 males, aged between 5 and 65 years.

The 50 patients recruited were comprehensively screened for mutations in all the known RP genes through targeted exome sequencing using an Illumina HiSeq 2000 sequencer,

Table I. List of RD-associated genes in an animal model and human subjects (8-12).

Gene	Animal model		Human	
	Mutant	Phenotype	Mutant	Phenotype
LPCAT1	Fs/Fs	RD	NS	ND
ARL3	-/-	RD	NS	ND
TMEM218	-/-	RD	NS	ND
CRB2	-/-	RD	S	ND
CCDC66	-/-	RD	NS	ND
CCND1	-/-	RD	NS	ND

Fs, frameshift; -/-, knockout; RD, retinal degeneration; NS, not screened; S, screened; ND, not determined.

as described previously (17). This was performed with the intention of identifying genetic factors that may have predisposed these patients to RP.

DNA extraction. Total genomic DNA was extracted from the peripheral blood (3 ml) using a Tiangen DNA Extraction kit (Tiangen Biotech, Co., Ltd., Beijing, China) according to the manufacturer's instructions. DNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Gene screening and data analysis. The primers used in polymerase chain reaction (PCR) analysis were designed to detect mutations and polymorphisms in the entire coding region and exon-intron boundaries of the *LPCAT1* gene in the patients with RP. The PCR reactions were performed using a 50 μl reaction volume, which contained 100 ng genomic DNA, 2 pmol of each of the primers and 25 μl 2X Taq PCR Master Mix (Biotake, Beijing, China). The PCR process was performed using an ABI Veriti thermocycler (Applied Biosystems Life Technologies, Foster City, CA, USA). With the exception of exon 1, for which the denaturation temperature was set at 98˚C, all PCR reactions were performed for 32 cycles with a denaturation temperature set at 95˚C for 30 sec, an annealing temperature set 58˚C for 30 sec, an extension step at 72˚C for 40 sec and a final elongation step at 72˚C for 5 min. Sequence analyses were performed using a Mutation Surveyor software (Softgenetics, State College PA, USA) and the suspected variants were assessed using the polymorphism phenotype (PolyPhen-2; http://genetics.bwh.harvard.edu/pph2/) and Mutation Taster (http://www.mutationtaster.org/) tools to predict the pathogenicity. Detailed information on the PCR primers used are listed in Table II.

Gene expression of LPCAT1 in various tissues and the developmental retina. A three-month-old female C57BL/6 mouse was supplied by the Animal Resource Center at Wenzhou Medical University, where it was fed a standard chow diet and kept under standard conditions. Animal care and husbandry followed the Association for Research in Vision and Ophthalmology (ARVO) guidelines (http://www.arvo.org/

Table II. Primer sequences for the *LPCAT1* exons.

| Exon	Forward (5'-3')	Reverse (5'-3')
1	GGGAGGCGAGGCTTCCAG	CTCCCTGGCCCCAGCATC
2	TCAGCCTTGGTCAGCTGTG	CAGAAGGGAAGGACAGATGG
3	TGTTCCGGAGTCTCATGTTG	CACTCATTTCCAGCAAGTGG
4	CCTGGGGCATCTGGAGTC	TGAGTTCACGCACTCACTAGG
5	CGTACTGAATATGATCCCAGTGTC	TCTAAGAACCCCAGCACACAG
6	CTGGGTGTTTACGGTCAGGA	CTGTCCTGCCCTCTCCAG
7	CTGACTGACCCTGCCTCTTC	ACAATGGGCTGAACCTAACG
8	CGTGGGAGCGTTGACTG	CTGAGAAACGGAAAGATGGG
9	TGAAGACGGTTCTAATGGGC	ACATGCATGAAGCTGGTTCC
10	ATCGGCGGTTATTCTGGTG	TGCTCAAGGAAGAAGAACCA
11	TTCTTGAAAACTAGCTTGCTGC	TGGGTGGTTTTCCTTCTCTG
12	TCCTTCCAAGATTCCCTTTTC	TGGGCATTTTACAAACAACAG
13	CCACATGGAAGTTCGAGTCC	AGAACCTTCCTTCTCAGGGG
14	ACGATTCTAACCCTCCCTGG	CTGGAACTCGGGCTGAAGAC
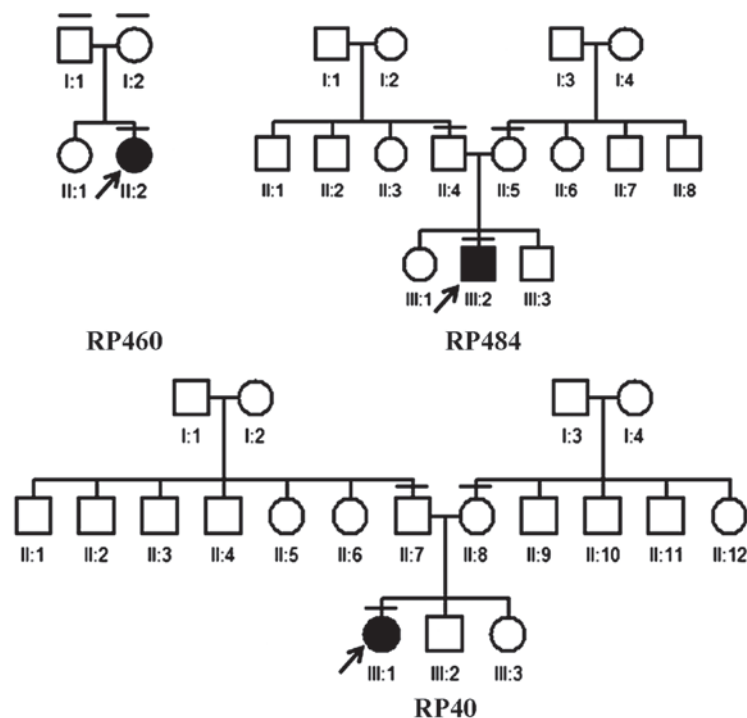
Figure 1. Pedigrees of the Chinese families recruited in the present study. Filled symbols represent patients affected by retinose pigmentosa, and the unfilled symbols indicate unaffected individuals. The bars over the symbols indicate the individuals recruited in the present study, and the circles and squares represent females and males, respectively.

Journals_and_Publications/Toolkit_for_Biomedical_Researchers_ Using_Laboratory_Animals/) and the mouse was sacrificed by cervical dislocation. Mouse total RNA was prepared from various tissues, including whole brain, retina, lens, sclera, cornea, spinal cord, heart, lung, pancreas, testis, epencephal, vascular and skeletal muscle, and spleen. Total RNA was extracted from different tissues of mouse, then cDNA was synthesized using for semi-quantitative PCR. Retinal RNA at various stages of development were also extracted using the Tiangen RNA Extraction kit (Tiangen). A 354 bp fragment

of *LPCAT1* was generated by reverse transcription using the Tiangen RT-PCR kit (Tiangen) with the following primers: Forward 5'-GACTCGCGAAGGAAGACAGT-3' and reverse 5'-CATGACACGCCTCACATTGC-3'. The RNA was also used as a template for PCR, using β-actin primers as a control.

Results

Phenotype determination. All patients recruited in the present study were diagnosed with either recessive or sporadic RP
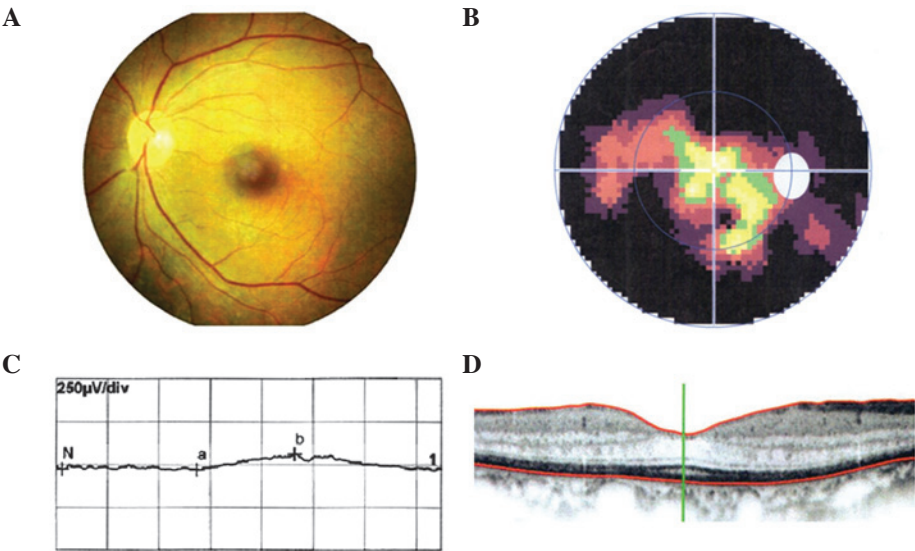
Figure 2. Representative clinical characteristics of the patient (RP40-III:1). (A) Images of the fundus show bone spicule-like pigmentation and retinal vascular attenuation. (B) Visual field assessment point locations indicate the loss of peripheral visual field. (C) Patients exhibited extinguished response to electroretinogram. N presented a given stimulus time point, and labels a and b present the location of a-wave and b-wave after a stimulus. The label 1 shows that this ERG curve was the result of the right eye. A div or unit was 100 ms on the x-axis, while 250 µv in y-axis. (D) Optical coherence tomographic images show severe thinning of the photoreceptor inner/outer segment. The upper red line was along inner side of retina nerve fiber layer, and the lower red line was along the outside of retinal pigment epithelium. The green line presents the location of the center area of the macular. This OCT image showed serious reduction of the retinal thickness, particularly the photoreceptor layer. It also shows a detachment was located at the base of the macular.
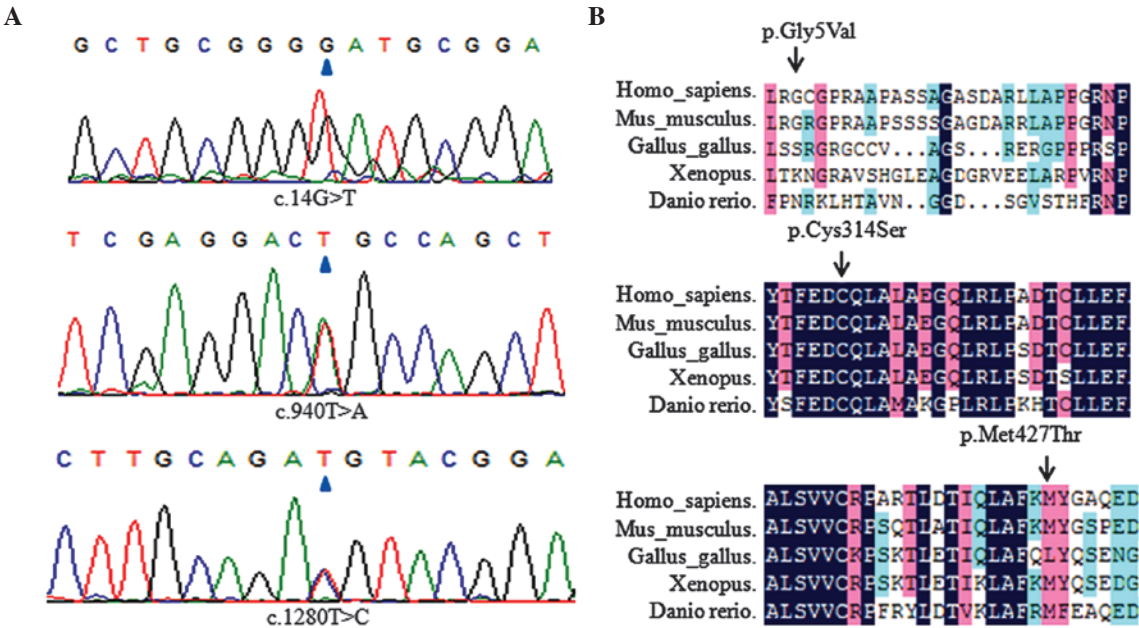

Figure 3. Mutations identified in the patients and subsequent alignment analysis. (A) Sanger sequencing results obtained from the affected individuals. (B) Amino acid alignment of the mutation sites.

(Fig. 1). The ages of disease onset varied substantially, between childhood and late adult life. All patients reported experiencing typical RP signs and symptoms, including night blindness, visual field constriction, visual impairment, bone spicule-like pigmentation, artery attenuation and waxy pallor of the optic nerve head in the fundi. Fig. 2 shows the representative clinical results of patient RP40-III:1, which was from the family RP40.

Mutation screening of LPCAT1. The results obtained from direct sequencing were analyzed, from which three heterozygous missense variants (p.Gly5Val, p.Met427Thr and p.Cys314Ser) and four synonymous variants (c.399G>A, c.645A>C, c.657C>A and c.1365C>T) were identified in the *LPCAT1* gene in the patients. Of these variants, two were predicted to be pathogenic, according to the results of the computational prediction performed using PolyPhen-2 and Mutation Taster.

Co-segregation analysis. For the three possible heterozygous missense mutations, their grade of conservation was analyzed

Table III. Summary of variants in patients with retinitis pigmentosa.

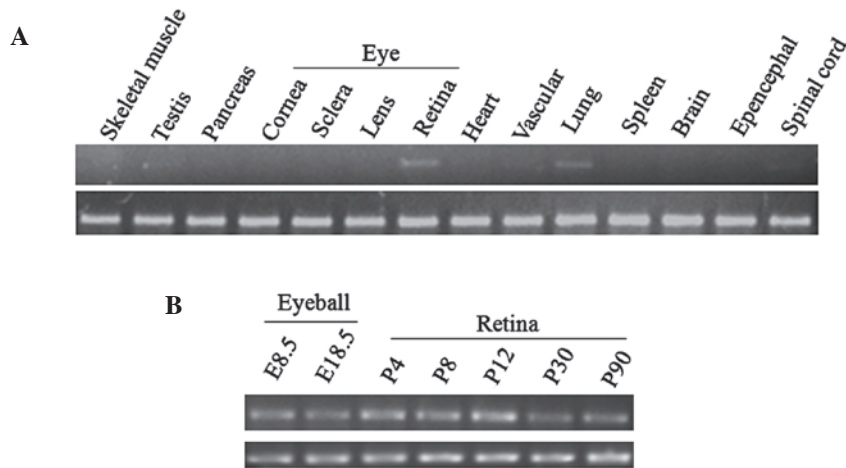| Exon	Mutation	Type	Amino acid	Frequency	Prediction[a]
1	c.14G>GT	Hetero	p.Gly5Val	7/50	Possibly damaging
3	c.399G>A	Homo	-	34/50	Benign
5	c.645A>AC	Hetero	-	20/50	Benign
5	c.657C>AC	Hetero	-	16/50	Benign
10	c.940T>AT	Hetero	p.Cys314Ser	12/50	Possibly damaging
13	c.1280T>CT	Hetero	p.Met427Thr	21/50	Benign
13	c.1365C>CT	Hetero	-	7/50	Benign

[a]Predicted using PolyPhen-2.

Figure 4. Expression analysis of *LPCAT1* in mice. (A) Expression of *LPCAT1* was observed in the retina, lung and spinal cord. (B) Expression of *LPCAT1* was expressed as early as stage E8.5 in retinal development, and its expression was sustained throughout the different stages of retinal development. Top row, *LPCAT1*; bottom row, β-actin.

and co-segregation analysis was performed in each pedigree (Fig. 3). The three heterozygous mutation sites in the parents of these patients were also screened. The results demonstrated that the mutations were inherited from either the paternal or the maternal allele. Since their parents did not suffer from RP, the heterozygous variants were not considered to predispose an individual to RP. Overall, no definite pathogenic variant was identified in the *LPCAT1* gene. In addition, the frequencies of these three single nucleotide polymorphisms were observed to be particularly high (>0.01)in the patients examined (Table III).

Tissue distribution of LPCAT1 in mice. The tissue distribution of *LPCAT1* was analyzed using RT-PCR. The highest expression level of *LPCAT1* was observed in the retina, followed by the lung and the spinal cord. Other tissues had particularly low expression levels of *LPCAT1* (Fig. 4). These results suggested that *LPCAT1* may be important in the development and function of the mammalian retina.

Discussion

LPCAT, which is the most important enzyme in membrane biogenesis and surfactant production, converts LPC to PC (6). Based on previous studies on *LPCAT1* in mice and

the availability of an animal model for human RP, the present study aimed to investigate whether *LPCAT1* is involved in the development of RP in a Chinese population (18).

In the present study, a total 50 patients diagnosed with RP were enrolled for investigation. These patients were previously screened for mutations in ~164 retinal-associated genes using established targeted exome sequencing technology. Based on the obtained family histories, the families selected for investigation in the present study were affected by RP exhibiting either recessive or sporadic inheritance patterns. All the coding regions and exon-flanking regions of the *LPCAT1* gene were sequenced in the participants and, with the exception of certain synonymous variants, three variants, which resulted in amino acid changes, were identified. However, none of these missense variants were determined as being pathogenic.

The results of the present study indicated that none of the *LPCAT1* variants identified were significantly associated with RP in the examined group of Chinese patients. The lack of correlation between the *LPCAT1* variants and patients with RP suggested that the possibility of these *LPCAT1* genetic variations attributing to the pathogenesis of RP was low. It is possible, however, that pathogenic mutations in this gene may exist outside of the coding exons and flanking intron splice sites. It is also possible that the pathogenic mutations, which

occur in *LPCAT1*, result in a form of retinal degeneration that was absent in the patients included in the present study.

In conclusion, the results of the present study suggested either that mutations in *LPCAT1* do not confer a genetic predisposition to RP, or that the incidence is rare in patients with RP. An increase in sample sizes may enable the screening of more patients with RP patients for pathogenic mutations in *LPCAT1*. In addition, additional genetic investigations in other ethnic populations are required to further elucidate the potential association between *LPCAT1* and RP.

Acknowledgements

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