·Basic Research·

Novel mutations in *PDE6B* causing human retinitis pigmentosa

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Abstract

• AIM: To identify the genetic defects of a Chinese patient with sporadic retinitis pigmentosa (RP).

• METHODS: Ophthalmologic examinations were performed on the sporadic RP patient, 144 genes associated with retinal diseases were scanned with capture next generation sequencing (CNGS) approach. Two heterozygous mutations in *PDE6B* were confirmed in the pedigree by Sanger sequencing subsequently. The carrier frequency of *PDE6B* mutations of reported *PDE6B* mutations based on the available two public exome databases (1000 Genomes Project and ESP6500 Genomes Project) and one in-house exome database was investigated.

• RESULTS: We identified compound heterozygosity of two novel nonsense mutations c.1133G>A (p.W378X) and c.2395C>T (p.R799X) in *PDE6B*, one reported causative gene for RP. Neither of the two mutations in our study was presented in three exome databases. Two mutations (p.R74C and p.T604I) in *PDE6B* have relatively high frequencies in the ESP6500 and in-house databases, respectively, while no common dominant mutation in each of the database or across all databases.

• CONCLUSION: We demonstrates that compound heterozygosity of two novel nonsense mutations in *PDE6B* could lead to RP. These results collectively point to enormous potential of next-generation sequencing in determining the genetic etiology of RP and how various mutations in *PDE6B* contribute to the genetic heterogeneity of RP.

• **KEYWORDS:** compound heterozygosity; retinitis pigmentosa; mutation; capture next generation sequencing; *PDE6B*

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INTRODUCTION

Retinitis pigmentosa (RP) is one of the leading causes of incurable blindness in the world and its prevalence is estimated at approximately 1 in 3000 to 1 in 5000 individuals [1]. It is typically characterized by initial symptoms of night blindness, with onset in adolescence or early adulthood, loss of peripheral vision and, as the disease progresses, loss of central vision leading to complete blindness or severe visual impairment. It is a clinically and genetically heterogeneous disorder [1-2]. Clinically, the age-at-onset of symptoms is highly variable and ranges from childhood to mid-adulthood. Genetically, so far, more than 190 genes have been identified as the cause of one or another form of inherited retinal disease. More than 60 genes were known as the cause of non-syndromic RP [1-3]. Syndromic forms of RP are heterogeneous: mutations in 13 genes cause Usher syndrome and 17 genes are associated with Bardet-Biedl syndrome. In addition to genetic heterogeneity, different diseases may be caused by mutations in the same gene, symptoms of different diseases may overlap, and there is extensive variation in clinical expression even among individuals sharing the same mutation in the same gene [1]. It makes RP as one of the most complicate single gene inherited diseases. It is inherited in autosomal [autosomal dominant (AD) and autosomal recessive (AR)], sex-linked (x-Link RP, XLRP) and mitochondrial modes of inheritance. Among the modes of inheritance in RP, AR inheritance appears to be more common than other form, accounting for approximately a third or more of all cases of RP[1].

The goal of the present study was to identify the causative mutations of the RP patient. We studied the using of Capture Next Generation Sequencing (CNGS) for genetic screening of a sporadic patient with RP, followed by Sanger sequencing and segregation analysis. Meanwhile, we sought to investigate the carrier frequency of the reported causative mutations based on the available two public exome databases (1000 Genomes Project and ESP6500 Genomes Project) and one in-house exome database.

SUBJECTS AND METHODS

Subjects This study abode by the treaty of the Declaration of Helsinki and was ratified by the Ethics Committee of the Eye Hospital of Wenzhou Medical University. All participating individuals signed the written informed consent. A five milliliter venous blood sample of the patient and his parents was drawn into an ethylenediaminetetraaceticacid (EDTA) sample tube. Genomic DNA was extracted from peripheral blood leukocytes using standard phenol/chloroform extraction protocols.

Identification of the Disease Mutation As previously described, 144 genes that associated with retinal diseases, such as *PDE6B*, were selected by a gene capture strategy by the GenCap custom enrichment kit (MyGenostics)[4]. In brief, taking advantage of the Solexa QA, cutadapt (http://code.google.com/p/cutadapt/), BWA (http://bio-bwa.sourceforge.net/bwa.shtml), SOAP aligner (http://soap.genomics.org.cn/) and GATK (https://www.broadinstitute.org/gatk/) programs to retrieve and align, identifying SNPs or insertions and deletions (InDels). We used the exome-assistant program (http://122.228.158.106/exomeassistant) to note SNPs and InDels. SIFT, PolyPhen2_HDIV, PolyPhen2_HVAR and likelihood ratio test (LRT)[5] were used to predict the impact of an amino acid substitution on protein function in order to evaluate and determine pathogenicity[4].

As for mutations, according to journal guidelines (www.hgvs.org/mutnomen), the number of nucleotide reflects the number of cDNA with +1 that corresponds to the A of the ATG initiation codon of translation in the reference sequence. And the initiation codon is codon 1.

Frequency and Evaluation of *PDE6B* Mutations We searched the PubMed database with keywords: "*PDE6B* AND mutations" (http://www.ncbi.nlm.nih.gov/pubmed/?term=PDE6B+AND+Mutation), then collected all the reported causative mutations in the human *PDE6B* gene. The corresponding frequencies of DNA mutations identified in the 1000 Genomes Project, ESP6500 Genomes Project and the in-house databases with 1402 samples from Chinese population.

RESULTS

Clinical Data The patient was a 32-year-old male. He provided a history of being suspect for RP in early childhood due to profound nyctalopia. When examined at age 32, his decimal best-corrected visual acuity (BCVA) was 0.4. Fundus photographs and optical coherence tomography (OCT) images revealed widespread macular atrophy, retinal vascular attenuation, optic disc pallor, intraretinal bone spicule pigmentation and overall thinning of the outer retinal layer (Figure 1). There is no family history. In summary, the diagnosis of this patient is sporadic RP according to the clinical manifestations.

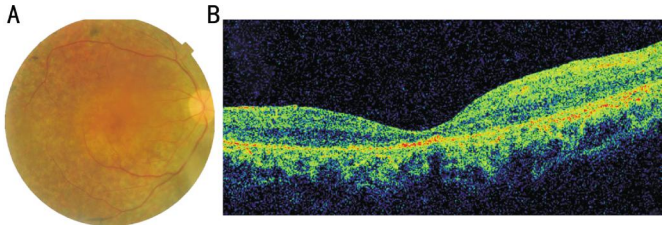

Figure 1 Fundus photograph and OCT image of the patient (OD) A: Color fundus photographs of the patient revealed widespread macular atrophy, narrow vessels, optic disc pallor and early bone spicule pigment; B: OCT of the patient showed that retinal thickness was significantly decreased, and inner segment/outer segment (IS/OS) are not visualized in some part of the retina.

Capture Next Generation Sequencing and Mutation Identification We used a CNGS platform for the screening of all the 144 genes which associated with retinal diseases. We obtained a mean coverage of 164X of the sequencing depth by manual checking, and the depth is sufficient to ensure that all the exons of disease genes have been well covered. Here we showed one (*PDE6B*) of disease genes coverage. Results of primary bioinformatic analysis showed 13 heterozygous variants in 11 different genes, and 7 variants seemed to be deleterious proved by three prediction tools (SIFT, PolyPhen2_HDIV and PolyPhen2_HVAR). Then two novel compound nonsense mutations (p.W378X, p.R799X) were confirmed by Sanger sequencing, both of which encode a truncated *PDE6B* protein. The parents of the patient were non-symptomatic and were heterozygous carriers of the corresponding mutations. We performed multiple sequence alignments (Figure 2) and found that the nonsense mutations of *PDE6B* were located in a phylogenetically conserved region (Figure 3). Moreover, it has been predicted to be deleterious using prediction tools (Table 1).

Frequency and Evaluation of *PDE6B* Mutations There were 28 reported mutations found in the literature. Among these mutations, only three (p.R74C, p.T604I and p.H620fs) of them were observed in one of the databases (Table 2). Mutations p.R74C and p.T604I have relatively high frequencies of 0.000385 and 0.0024, in the ESP6500 and in-house databases, respectively. We found that the mutational hot spot residue glycine 323 is critical because p.G323C and p.G323A were identified in two distinct families (Table 2). Four nonsense mutations (p.C270X,

Table 1 DNA variants of *PDE6B* in RP pateint

Exon	DNA change	Protein change	Mutation type	Novel	SIFT	Polyphen2_HDIV	PolyPhen2_HVAR	LRT
9	c.1133G>A	p.W378X	Nonsense	Yes	N/A	N/A	N/A	P
21	c.2395C>T	p.R799X	Nonsense	Yes	N/A	N/A	N/A	P

N/A: No answer; P: Pathological.

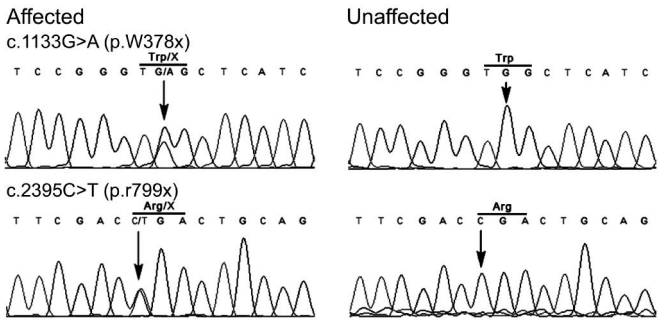

Figure 2 DNA sequence chromatograms DNA sequence chromatograms of the patient with RP and non-related control. The heterozygous peaks of the mutations are pointed out by gray arrows.

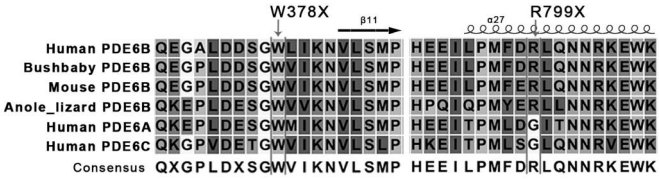

Figure 3 Multiple –sequence alignment of p.W378X and p. R799X Structure-based sequence multiple-sequence alignment of PDE6B different species and human PDE6A, PDE6C revealed that two mutations (p.W378X and p.R799X) from RP patient were located within a conserved region. Secondary structure elements are indicated above.

p.Q298X, p.Q534X, p.K706X) identified in the literature were not present in the databases. These data provides additional evidence to show that RP is a genetically heterogeneous disease, because there is no common dominant mutation in each of the populations or across all populations.

Reported *PDE6B* Mutations and Their Associated Phenotypes Various *PDE6B* mutations have been reported to cause retinitis autosomal recessive retinitis pigmentosa (ARRP) and congenital stationary night blindness (CSNB), which include p.H258N, p.L228I, p.C270X, p.Q298X, p. P387L, p.L527P, p.R531X, p.I535N, p.R552Q, p.H557Y, p. G576D, p.E640fs, p.D600N, p.H620fs, p.L699R, p.K706X, p.W807R and two splicing mutations c.469-1G>T and c. 2193+1G>T [6-20]. In this study, we summarized these mutations and their associated phenotypes from 18 families. Patients from 6 unrelated families were reported carrying two compound heterozygosity mutations. The clinical phenotypes in these cases with ARRP has early childhood onset of night vision loss, bone spicule pigment, attenuated retinal vessels, elevated dark adapted threshold, and generally well-preserved visual acuity until late stages. Forty-five point five

Table 2 Frequency of reported causative mutations in *PDE6B*

Exon	DNA change	Protein change	1000G	ESP6500	In-house
1	c.220C>T	p.R74C	<0.0004	0.000385	<0.0007
2	c.469-1G>T	Splicing	<0.0004	<0.000005	<0.0007
4	c.810C>A	p.C270X	<0.0004	<0.000005	<0.0007
5	c.892C>T	p.Q298X	<0.0004	<0.000005	<0.0007
5	c.922G>A	p.G308S	<0.0004	<0.000005	<0.0007
6	c.967G>T	p.G323C	<0.0004	<0.000005	<0.0007
6	c.968G>C	p.G323A	<0.0004	<0.000005	<0.0007
9	c.1160C>T	p.P387L	<0.0004	<0.000005	<0.0007
10	c.1328A>C	p.N443T	<0.0004	<0.000005	<0.0007
11	c.1417delC	p.L473fs	<0.0004	<0.000005	<0.0007
12	c.1487delC	p.P496fs	<0.0004	<0.000005	<0.0007
12	c.1580T>C	p.L527P	<0.0004	<0.000005	<0.0007
12	c.1600C>T	p.Q534X	<0.0004	<0.000005	<0.0007
12	c.1604T>A	p.I535N	<0.0004	<0.000005	<0.0007
13	c.1655G>A	p.R552Q	<0.0004	<0.000005	<0.0007
13	c.1669C>T	p.H557Y	<0.0004	<0.000005	<0.0007
13	c.1688T>A	p.F563Y	<0.0004	<0.000005	<0.0007
14	c.1727G>A	p.G576D	<0.0004	<0.000005	<0.0007
14	c.1798G>A	p.D600N	<0.0004	<0.000005	<0.0007
14	c.1811C>T	p.T604I	<0.0004	<0.000005	0.0024
15	c.1860delC	p.H620fs	<0.0004	0.00008	<0.0007
16	c.1976T>C	p.L659P	<0.0004	<0.000005	<0.0007
17	c.2096T>G	p.L699R	<0.0004	<0.000005	<0.0007
17	c.2116_2118 del	p.706_706del	<0.0004	<0.000005	<0.0007
17	c.2116A>T	p.K706X	<0.0004	<0.000005	<0.0007
18	c.2131G>A	p.A711T	<0.0004	<0.000005	<0.0007
21	c.2419T>A	p.W807R	<0.0004	<0.000005	<0.0007
22	c.2560C>G	p.L854V	<0.0004	<0.000005	<0.0007

1000G: http://www.1000genomes.org/; ESP6500: http://evs.gs.washington.edu/EVS/; In-house: In-house exome database.

percent (10/22) of these patients from 6 families had cataracts. The clinical manifestations of the present patients were generally consistent with the literatures. There appears to be no particularly unique phenotype associated with compound heterozygosity of nonsense mutations, or with mutations affecting the catalytic domain of *PDE6B*.

DISCUSSION

The comprehensive diagnosis of RP is made challenging by the large number of disease genes and alleles associated with RP. Significant progress has been made in determining the molecular causes of RP based on the next generation sequencing technique. In our study, we used CNGS method to scan 144 genes associated with retinal diseases. Genetic examination revealed two novel nonsense heterozygous

mutations c.1133G>A (p.W378X) and c.2395C>T (p.R799X) in *PDE6B*. The two novel nonsense mutations in this study were predicted as likely to be disease-causing mutations as both of them will lead to truncated proteins.

Mutations in *PDE6B* (NM_000283) have been linked to humans autosomal recessive RP (arRP) [6-7]. The human *PDE6B* gene has been mapped to chromosome 4p16.3, spans approximately 45 kb, and has 22 exons, encoding a protein of 854 amino acid residues, named phosphodiesterase 6B. Which is the β-subunit of rod phosphodiesterase 6 (PDE6) [21]. PDE6 regulates the cytoplasmic level of cyclic guanosine monophosphate (cGMP) in the photoreceptors. Activated by light stimulation, PDE6 could reduce the level of cGMP, which leads to closure of the cGMP-gated Na+ and Ca++ channels then to hyperpolarize the rod plasma membrane [22]. A dysfunction of the β-subunit of PDE6 results in a high concentration of cGMP and Ca++ in rod photoreceptors, and it promote apoptosis of the rod photoreceptors[23].

PDE6B mutations are associated with one of the earliest onset and most aggressive forms of RP[6-9]. Mice homozygous for the rd mutation display hereditary retinal degeneration and have been considered a model for human retinitis pigmentosa. Adeno-associated virus (AAV) -mediated gene therapy had been reported available to prevent retinal degeneration in rd10 mice containing a recessive PDEbeta mutation [24]. Since there are no effective treatments for this disease, so far, it is important to identify the underlying genetic defects. It would be helpful for prenatal diagnosis, and the selection of patients for clinical trials, for example, virus-based gene therapy for patients with *PDE6B* mutations, to restore visual function[25-26].

The CNGS strategy has been reported to be a fast, effective, and reliable tool to detect known and novel mutations in AD RP patients [27]. Approximately 40% of ARRP families also could be successfully molecular diagnosed by CNGS [28]. Our data also clearly support that CNGS is a useful approach to identify the mutation of RP patients, even in sporadic cases because of its ability to detect variants in *PDE6B* with full coverage. However, for CNGS clinical screening to become a widely accepted stand-alone *PDE6B* mutation screening tool, more studies need to be done, especially, to assign pathogenicity status to newly discovered or rare variants.

In our recent study, we showed missing coverage of some exons in the *PROM1* gene from CNGS based molecular diagnosis of putative Stargardt disease[4]. It has been reported that with deep sequencing, missing coverage of some regions is common [29]. So before searching for the disease-associated mutations, it is necessary to check the coverage of the targeted sequence, even if no mutations were found in the missing coverage region. We also showed, in the present study, that *PDE6B* has been well covered. Missing coverage issue may be treated case by case.

We noticed that two novel nonsense compound mutations in our study are still not present in databases. This highlights that the present mutational spectra from these databases may still need to be enlarged and also reveals that *PDE6B* gene contributes to the genetic heterogeneity of RP disease. In order to increase the rate of RP mutation detection and to correctly evaluate the residual risk of being a RP carrier after molecular analysis, it is essential that genetic tests are designed based on the characteristic mutation frequency profile for a given population and that the sensitivity of mutation detection assays is as high as possible [30-31]. However, so far, no data are available to define the carrier frequency of *PDE6B* mutations. To address this, one approach is to collect a large number of DNA samples in different populations and sequence the whole *PDE6B* coding sequence with classical Sanger sequencing. However, this is a labor intensive approach [30]. Public and in-house exome databases from large population samples would be expected to uncover many variants, some of them known to be benign, some rare, some not yet described [12]. Several studies have been performed to analyze carrier frequency in RP or even being into question the previously reported mutations [32-33]. Therefore, here we sought to take advantage of public and in-house exome databases to address it. We found 28 reported mutations in the literature. Only three (p.R74C, p. T604I and p.H620fs) reported mutations were observed in one of the databases (Table 2). This study provides additional evidence to show that RP is a genetically heterogeneous disease, because there is no common dominant mutation in present databases. While, we still can't clearly define the carrier frequency with each reported mutations due to relative small capacity of the exome databases even we have in-house databases with 1402 samples from Chinese population.

We took advantage of online prediction tools to predict the impact of amino acid substitutions on *PDE6B* protein function. The prediction results strongly suggested that all the reported mutations except p.L854V are potentially pathogenic mutations, although p.L854V is found in patients[34]. Additional studies may be required before excluding it as a "disease-associated" mutation.

Token together, here we demonstrated a successful case of using next generation sequencing to identify compound heterozygosity of two novel nonsense *PDE6B* mutations in a Chinese patient with sporadic retinitis pigmentosa, which points to enormous potential of next-generation sequencing in determining the genetic etiology of RP, and also we showed how various mutations in *PDE6B* contribute to the genetic heterogeneity of RP.

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