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Targeted Exome Capture and Sequencing Identifies Novel PRPF31 Mutations in Autosomal Dominant Retinitis Pigmentosa in Chinese Families

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Abstract

Objectives— To identify disease-causing mutations in two Chinese families with autosomal dominant retinitis pigmentosa.

Design—Prospective analysis

Patients— Two Chinese adRP families underwent genetic diagnosis. A specific Hereditary Eye Disease Enrichment Panel (HEDEP) basing on targeted exome capture technology was used to collect the protein coding regions of targeted 371 hereditary eye disease genes, and high throughput sequencing was done with Illumina HiSeq 2000 platform. The identified variants were confirmed with Sanger sequencing.

Setting— All experiment were performed and analyzed in a large laboratory specialized in genetic studies in Department of Ophthalmology, Peking University Third Hospital.

Results—Two novel mutations including one splice site mutation (Int10 c.1074-2 A>T; p.Y359SfsX29) and one insertion (c.824_825insA; p.Y275X) of PRPF31 were identified in these two Chinese adRP families. These two mutations segregated with the disease phenotype in their respective families.

Conclusions—Our findings broaden the spectrum of PRPF31 mutations causing adRP and the phenotypic spectrum of the disease in Chinese patient. The HEDEP basing on targeted exome capture technology is an efficient method for molecular diagnosis in adRP patients.

ARTICLE SUMMARY

Article focus: The focus of this study was the analysis of variations in PRPF31 gene not described previously, and their association with the clinical phenotype of autosomal dominant Retinitis Pigmentosa in Chinese families.

Key messages:

- We demonstrated that one novel splice site mutation (Int10 c.1074-2 A>T; p.Y359SfsX29) and one novel insertion (c.824_825insA; p.Y275X) of PRPF31 were the disease causing mutations in two Chinese adRP families respectively.
- A specific Hereditary Eye Disease Enrichment Panel (HEDEP) basing on targeted exome capture technology was used to collect the protein coding regions of targeted 371 hereditary eye disease genes in these two families. Sanger sequencing in this study confirmed the mutation detected with the HEDEP, which proved the sensitivity and specificity of the HEDEP for application in molecular diagnosis of inherited eye diseases.

Strengths and limitations of this study:

- The HEDEP basing on targeted exome capture technology is an efficient method for molecular diagnosis in adRP patients.
- Both mutations result in premature termination codons (PTC) before the last exon, thus insufficient functioning due to haploinsufficiency instead of aberrant function of the mutated

proteins seems to be the most probably reason in these two families. However no experiment was done to prove in this study.

INTRODUCTION

Retinitis pigmentosa (RP) is an inherited retinal degeneration that affects approximately one in 3500 individuals, with an estimated total of 1.5 million patients worldwide.[1-2] Typically, patients affected by RP first suffer from night blindness, most often during adolescence. Rod and cone photoreceptor cells start to degenerate from the mid periphery to the far periphery and the center of the retina, resulting in the so-called tunnel vision. Later in life, central vision is also lost, leading to legal or complete blindness.[3] Clinical hallmarks of RP include bone-spicule deposits, attenuated retinal blood vessels, optic disc pallor, visual field loss, and abnormal, diminished or non-recordable electroretinographic responses (ERG).

Autosomal-dominant RP (adRP) account for 20-25% of all RP patients.[4] To date, more than 24 genes (Best 1, C1QTNF5, CA4, CRX, FSCN2, GUCA1B, IMPDH1, KLHL7, NR2E3, NRL, PRPF3, PRPF31, PRPF6, PRPF8, PRPH2, RDH12, RHO, ROM1, RP1, RP9, RPE65, SEMA4A, SNRP200 and TOPORS) are reported to be associated with adRP (https://sph.uth.edu/retnet/disease.htm). Traditionally, patients from RP families are studied with linkage analysis, or gene-by-gene screening,[5-6] which is costly, requires substantial human resources, and is time-consuming, thus making molecular diagnosis difficult and complex. This has led to the development of mutation screens based on arrayed primer extension technology, which enable the simultaneous detection of multiple mutations from one individual.[7] However, this approach is limited by the fact that it only can detect sequence variants previously reported,

with novel mutations unreported.[8] It would be advantageous to screen for all the variants in the protein coding regions of targeted genes, one approach to achieve this is to develop a specific hereditary eye disease enrichment panel based on exome capture technology to re-sequence simultaneously all the exons from targeted genes.

The aim of this study was to describe the development of a specific Hereditary Eye Disease Enrichment Panel (HEDEP) and its application in molecular diagnosis of two Chinese families with adRP, and to characterize the phenotypic manifestation associated with the mutation.

MATERIALS AND METHODS

Study subjects and clinical evaluation

Two Chinese families of Han ethnicity with adRP were identified in Anhui and Hubei province respectively, and there was no history of other ocular or systemic abnormalities in the families. The family adRP-19 has sixteen affected individuals in four generations, fourteen individuals, including nine affected and five unaffected participated in the study (Figure 1A). The family adRP-61 has eighteen affected individuals in three generations, seventeen individuals, including nine affected and eight unaffected participated in the study (Figure 1B). Medical and ophthalmic histories were obtained, and ophthalmological examination was carried out. One hundred sporadic RP patients recruited in Department of Ophthalmology, Peking University Third Hospital were used for RPPF31 gene mutation screening. One hundred of general healthy individuals from the Chinese Han ethnic population were recruited to serve as controls. All procedures used in this study conformed to the tenets of the Declaration of Helsinki. All experiments involving DNA and RNA of the patients and their relatives were approved by Peking University Third Hospital Medical Ethics Committee. Informed consent was obtained from all participants.

Screening for Mutations

Blood samples were collected and genomic DNA was extracted by standard protocols (D2492 Blood DNA Maxi Kit, Omega Bio-Tek Inc, Norcross, GA). A specific Hereditary Eye Disease Enrichment Panel (HEDEP) basing on targeted exome capture technology was used to collect the

protein coding regions of targeted genes. This HEDEP was able to capture 371 hereditary eye disease genes, which covers 24 adRP associated genes (Best 1, C1QTNF5, CA4, CRX, FSCN2, GUCA1B, IMPDH1, KLHL7, NR2E3, NRL, PRPF3, PRPF31, PRPF6, PRPF8, PRPH2, RDH12, RHO, ROM1, RP1, RP9, RPE65, SEMA4A, SNRP200 and TOPORS). Fifty μg of genomic DNA from two patients (Ⅲ3 and Ⅳ14), one carrier (Ⅲ7) and one control (Ⅲ18) of family adRP-19, and three patients (Ⅲ13, Ⅲ30 and Ⅳ2) and one control (Ⅲ2) of family adRP-61 were used for targeted exome capture. The exon-enriched DNA libraries were then prepared for High throughput sequencing with Illumina HiSeq 2000 platform. The obtained mean exome coverage was more than 98%，with variants accuracy at more than 99%. In these two families, we only analyzed mutations occurred in 24 adRP related genes. The shared changes in the affected individuals but not in the normal control were identified. The changes were filtered against exome data from ethnic Han Chinese Beijing available in the 1000 Genomes Project (fttp://www.1000genome.org), and against the Han Chinese Beijing SNPs in the dbSNP131. Sanger sequencing was then used to validate the identified potential disease-causing variants. Splice-site variants were analyzed using the prediction program AUGUSTUS (http://bioinf.uni-greifswald.de/augustus/submission).

Mutation Validation

The shared variants in the affected individuals but not in the normal control were then confirmed by direct polymerase chain reaction (PCR)-product sequencing using Bigdye terminator v3.1 cycle sequencing kits (Applied Biosystems, Foster City, CA, USA) and analyzed on an ABI 3130XL

Genetic Analyzer. Sanger sequencing was used to determine whether the variant co-segregated with the disease phenotype in these two families. Primer pairs for individual exons were designed using the primer program (http://www.yeastgenome.org/cgi-bin/web-primer) (DNA reference number NG_009759). PCR primers, annealing temperatures, and amplimer-specific details are listed in the Table 1.

Table 1. PCR primers used for PRPF31 amplification

Primer	Forward	Reverse	Product
PRPF31 Exon 2&3	CTGGGGGAGAATCATCGCTC	AAGGCTCTGGAAAAGGCT	557
PRPF31 Exon 4	CGAGAGGGGGTAGGGATTTAGATAC	ACCTCGATCTGAGCTTGGGCTTAG	252
PRPF31 Exon 5	AAGAAGGGGACATGGGTGTTA	TCCTCTCCATCGTCTCCAGA	287
PRPF31 Exon 6-7	CAAGAGAGGTTCTCGAGCCTT	TTTCCCAAGGTCACAGTGTCA	589
PRPF31 Exon 8	AGCCCCCAGGCAGATTTACT	TCCTGAGTGCTACCGTCAGCT	350
PRPF31 Exon 9	TAGAGCCCAAGGGTGGAAA	TTGGTAGGACAGTGCTCGCT	333
PRPF31 Exon 10&11	GGCAGCATTAGGTGCTGATTT	GTCGCTTTGGGGCTGAAT	599
PRPF31 Exon 12&13	CAACTCTGAGCTCACAGAGCA	TCATCCTGGCCTTCTTCACA	632
PRPF31 Exon 14	CTGTCTCATGCCCACCAA	TGGACCTCTGTGTCCCTTCA	295

Isolation of total RNA and reverse transcription (RT)-PCR analysis

Total RNA was extracted from peripheral whole blood samples by standard protocols (R6814 Blood RNA Kit, Omega). Reverse transcription was performed with oligonucleotide primers using Superscript II reverse transcriptase according to manufacturer's protocol (Invitrogen Corporation, Grand Island, NY). Primers for RT-PCR were designed to amplify exon 7-12 of PRPF31 mRNA

(mRNA reference number NM_015629). The forward primer is 5'-GCCAAGATCATGGGTGTGG-3', and the reverse primer is 5'-TGCAGCGTCTTGGAGATCCT-3'. The RT-PCR products were further cloned into plasmid for sequence analysis.

RESULTS

Family	ID	Sex	Age(y)	Disease status	Onset age (y)	Visual acuity Unaided (corrected) R eye	L eye	Fundus features

Phenotype details

The clinical features of fourteen members of family adRP-19 and seventeen members of family adRP-61 who participated in this study are shown in Table 2. All the tested affected individuals except Ⅲ7 complained of night blindness and photophobia since childhood. Onset of the disease was noted to be ranging from 2 to 12 years of age. Fundus examination in the 38-year-old proband (Ⅳ14) of family adRP-19 showed bone spicules like pigmentation in the peripheral part of the retina, retinal arteriolar attenuation, and retinal pigment epithelium (RPE) degeneration (Figure 2A-B). Fundus examination in the 48-year-old proband (Ⅲ9) of family adRP-61 showed bone spicule like pigmentation in the mid-periphery retina, bilateral attenuation of retinal vessels, RPE degeneration and pale optic disc (Figure 2 C-D). The 68-year old asymptomatic carrier (Ⅲ7) of family adRP-19 did not complain of night blindness, and fundus examination showed no RP changes in both eyes. Visual acuity in majority of the patients declined quickly after 40 years old.

adRP-19	**Ⅱ:1**	F	82	Yes	Childhood	LP	LP	Cataract, can't see the fundus
	Ⅱ:3	F	80	Yes	8	LP	LP	Cataract, can't see the fundus
	Ⅲ:2	F	58	Yes	3	LP	LP	Cataract, can't see the fundus
	Ⅲ:3	M	54	Yes	3	0.1	0.1	Bilateral attenuation of retinal vessels, bone spicule pigments throughout the fundus, RPE degeneration, pale optic disc
	Ⅲ:6	M	46	No	-	1.5	1.5	No RP changes in both eyes
	Ⅲ:7	F	65	No	-	0.9	0.8	No RP changes in both eyes
	Ⅲ:18	M	68	No	-	1.2	1.2	No RP changes in both eyes
	Ⅳ:3	F	29	Yes	Childhood	1.0	1.0	Bone spicule pigments in peripheral part of the fundus
	Ⅳ:5	F	27	Yes	Childhood	1.0	1.0	Bone spicule pigments in peripheral part of the fundus
	Ⅳ:14	M	38	Yes	3	0.3	0.5	Bilateral attenuation of retinal vessels, bone spicule pigments in peripheral part of the fundus, RPE degeneration
	Ⅳ:15	F	34	No	-	1.5	1.5	No RP changes in both eyes
	Ⅳ:17	F	37	No	-	1.5	1.2	No RP changes in both eyes
	Ⅴ:3	M	16	Yes	2	1.2	1.2	Bone spicule pigments in peripheral part of the fundus
	Ⅴ:6	M	3	Yes	2	1.2	1.2	Bone spicule pigments in peripheral part of the fundus
adRP-61	**Ⅱ:9**	M	80	No	-	1.0	1.0	No RP changes in both eyes
	Ⅲ:1	F	63	Yes	12	0.4	0.5	Bone spicule pigments in peripheral part of the fundus
	Ⅲ:2	M	63	No	-	0.8	0.8	No RP changes in both eyes
	Ⅲ:3	M	61	No	-	1.0	1.0	No RP changes in both eyes
	Ⅲ:7	F	49	No	-	1.2	1.0	No RP changes in both eyes
	Ⅲ:9	F	48	Yes	Childhood	0.06	0.06	Bilateral attenuation of retinal vessels, bone spicule pigments throughout the fundus, RPE degeneration, pale optic disc
	Ⅲ:10	M	50	No	-	1.2	1.2	No RP changes in both eyes
	Ⅲ:12	M	40	Yes	Childhood	0.5	0.6	Bone spicule pigments in peripheral part of the fundus

	Ⅲ:13	M	38	Yes	Childhood	0.7	0.6	Bone spicule pigments in peripheral part of the fundus
	Ⅲ:14	M	45	No	-	1.0	1.0	No RP changes in both eyes
	Ⅲ:22	F	64	No	-	1.2	1.2	No RP changes in both eyes
	Ⅲ:25	M	50	Yes	Childhood	0.2	0.1	Bilateral attenuation of retinal vessels, bone spicule pigments throughout the fundus, RPE degeneration, pale optic disc
	Ⅲ:30	M	49	Yes	Childhood	0.1	0.06	Bilateral attenuation of retinal vessels, bone spicule pigments throughout the fundus, RPE degeneration, pale optic disc
	Ⅳ:2	M	32	Yes	Childhood	0.7	0.6	Bone spicule pigments in peripheral part of the fundus
	Ⅳ:5	F	35	Yes	Childhood	0.9	0.7	Bone spicule pigments in peripheral part of the fundus
	Ⅳ:8	F	28	No	-	1.5	1.5	No RP changes in both eyes
	Ⅳ:11	F	40	Yes	Childhood	0.5	0.6	Bone spicule pigments in peripheral part of the fundus

Table 2. Clinical data of the family members participating in the study

Identification of mutations in PRPF31

We selected four individuals in each family for targeted exome capture. We generated an average of 0.77Gb of sequence with 228× average coverage for each individual with paired 100bp reads. The generated sequence covered average 99.2% of the targeted bases with the accuracy of a variant call more than 99%, which is sufficient to pass the thresholds for calling SNPs and short insertions or deletions (indels). We filtered all the detected variants and found the potential disease causing mutations. Then we compared the shared variants in affected individuals with the ethnic Han Chinese Beijing available in the 1000 Genomes Project (fttp://www.1000genome.org), and against the Han Chinese Beijing SNPs in the dbSNP131. This left one splice site variation (Int10

c.1074-2 A>T) in family adRP-19 and one insertion (c.824_825insA; p.Y275X) in family adRP-61 respectively, which was shared among affected individuals but not in the normal control in each family. Sanger sequencing validation and segregation analysis was carried out, which demonstrated that these two variants (Figure 3) co-segregated with the disease phenotype in each family (Figure 1), but was absent in 100 matched normal controls.

We further carried out direct PCR sequencing of the PRPF31 exons in an additional 100 unrelated sporadic RP patients. All of these patients showed typical RP fundus features, including bilateral attenuation of retinal vessels, bone spicule pigments throughout the fundus, RPE degeneration and pale optic disc. No disease causing mutations were identified in these 100 sporadic patients.

Functional characterization of the Int10 c.1074-2 A>T mutation using RT-PCR

To determine whether the Int10 c.1074-2 A>T splicing mutation in family adRP-19 has any effect on mRNA splicing, we performed RT-PCR for PRPF31 using total RNA samples isolated from peripheral blood samples from two patients (Ⅳ3 and Ⅴ3) and one unaffected family member (Ⅳ15), and two normal controls not related to the family. The RT-PCR products were further cloned into plasmid for sequence analysis. It yielded a 590bp product from normal control samples as expected with normal splicing. One 590bp fragment and another 517bp product were amplified from the sample of affected individuals. Sequence analysis showed that the 517bp product skipped Exon11, resulting in frameshift i.e. p.Y359SfsX29 leading to premature

termination with 28 new amino acids downstream. The sequence comparison details between wild type and mutated is listed in the Supplementary Figure 1.

DISCUSSION

In this study we report the identification of two novel mutations i.e. one splice site mutation (Int10 c.1074-2 A>T; p.Y359SfsX29) and one insertion (c.824_825insA; p.Y275X) of PRPF31 in two Chinese adRP families. The identified splice site mutation (Int10 c.1074-2 A>T; p.Y359SfsX29) in family adRP-19 segregated in all the nine affected patients and one asymptomatic carrier, mother of an affected patient who manifest the disease from childhood. The identified insertion mutation (c.824_825insA; p.Y275X) in family adRP-61 segregated in all the nine affected patients, including one female who inherited the mutation from her 65-year old asymptomatic father. This is consistent with the incomplete penetrance of PRPF31 mutation.

The human PRPF31 gene contains 14 exons and encodes a 61kDa protein of 499 amino acids, the core component of the U4/U6 • U5 tri-snRNP complex which constitutes a major building block of the pre-mRNA processing spliceosome.[9] Although PRPF31 is ubiquitously expressed, patients with mutant PRPF31 alleles only show symptoms in the retina but not other organs.[10] At present, up to 50 mutations (including 15 splice defects, 10 missenses, 23 deletions and 2 insertions) in PRPF31 have been reported to be linked with adRP and sporadic RP cases.[5, 11-12] Splice defect have been reported in Int1,[13] Int 2,[14] Int 4,[14] Int 5,[15] Int 6,[16] Int 8,[14] Int 10,[17] Int 11 and Int 13.[10,18] Insertions have been reported in Exon 7 and Exon 8.[19] All the above splice defect and insertion mutations resulted in frameshift, leading to premature termination. Previous study from Rio Frio et al.[20] demonstrated that most PRPF31 mutations bearing premature termination codons (PTCs)

before the last exon behave as null allele, resulting in haploinsufficiency as their corresponding mRNA is degraded by nonsense-mediated mRNA decay (NMD). However, if the PTC produced by the mutation occurs in the last exon of a given gene, the mutant mRNA is insensitive to NMD and is thought to be translated into a truncated protein.[21] The splice site mutation (Int10 c.1074-2 A>T) in the present study caused the skipping of exon 11, resulting in frameshift, leading to premature termination with 28 new amino acids downstream (i.e. p.Y359SfsX29). The insertion mutation (c.824_825insA) in the present study happened in exon 8, resulting in the following amino acid changing from Thr to stop codon (p.Y275X). Since both mutations result in PTC before the last exon, thus insufficient functioning due to haploinsufficiency instead of aberrant function of the mutated proteins seems to be the most probably reason in these two families.

Several studies have been reported on the use of genotyping microarray for genetic diagnosis of retinal disease, such as Stargardt disease,[22] Leber congenital amaurosis,[23] Usher syndrome,[24] autosomal recessive RP,[25] and adRP.[7] Different from Arrayed Primer Extension technology used in most of previous microarray studies, the HEDEP in this study was based on target exon capture technology. HEDEP was able to capture 371 hereditary eye disease genes, which cover 53 RP associated genes, 7 Stargardt associated genes, 19 Leber congenital amaurosis associated genes, 11 Usher syndrome associated genes, 33 Cone and rod dystrophy associated genes, 13 Chorioretinal atrophy associated genes, 35 Microphthalmia associated genes, 46 Congenital cataract associated genes, 4 Glaucoma associated genes, 6 Familial exudative vitreoretinopathy associated genes, and 35 RP related syndrome associated genes etc. It is applicable to a broader inherited eye disease

genetic study. Sanger sequencing in this study confirmed the mutation previously detected with the HEDEP, which proved the sensitivity and specificity of the HEDEP for application in molecular diagnosis of inherited eye diseases.

We present a successful genetic diagnosis with a specific Hereditary Eye Disease Enrichment Panel (HEDEP), and identified two novel mutations i.e. one splice site mutation (Int10 c.1074-2 A>T; p.Y359SfsX29) and one insertion (c.824_825insA; p.Y275X) of PRPF31 in two Chinese adRP families with incomplete penetrance. Both mutations result in PTC before the last exon, thus insufficient functioning due to haploinsufficiency is the most probably reason in these two families. Our finding broad the spectrum of PRPF31 mutations causing adRP and the phenotypic spectrum of the disease in Chinese patient, which will be helpful for genetic consultation and genetic diagnosis in the future.

Supplementary Material

Please feter to Web version on PubMed Central for supplementary material.

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Author contributions

Conceived and designed the experiment: LP Yang; HR Zhang; GL Li and ZZ Ma.

Performed the experiments: LP Yang; XB Yin; LM Wu; NN Chen.

Analyzed the data: LP Yang; LM Wu; NN Chen.

Wrote the paper: LP Yang; ZZ Ma;

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FIGURE LEGENDS

Figure 1. Pedigrees of two Chinese adRP families with PRPF31 mutations and co-segregation in available family members. Filled symbols represent affected, unfilled unaffected, dotted asymptomatic carrier. Question marks indicate that it is not clear whether the individual is affected or not. Square signify male, circles females. Arrows mark the index patients. M refers to the mutant allele, and + means normal allele.

Figure 2. Fundus photographs of two probands with mutations in the PRPF31 gene. (A-B) Proband Ⅳ14 is from family adRP-19. (C-D) Proband Ⅲ9 is from family adRP-61. Typical retinitis pigmentosa changes can be seen.

Figure 3. Sequencing results of the PRPF31 mutations in the two families. (A) Family adRP-19 carried the mutation Int10 c.1074-2 A>T; p.Y359SfsX29. (B) Family adRP-61 carried the mutation c.824_825insA; p.Y275X.

APPENDICES

Supplementary Figure 1. The sequence comparison details between wild type and mutated is listed. RP19-2_134 from patient □3; RP19-2_146 from patient□3; RP19-2_136 from unaffected family member □15; RP19-2_145 and 147 from normal controls not related to the family.

Figure 1A—adRP 19

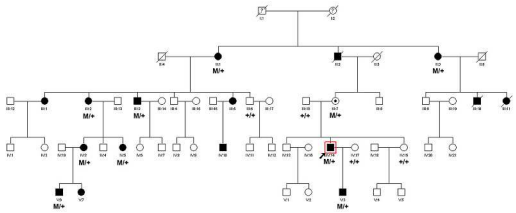
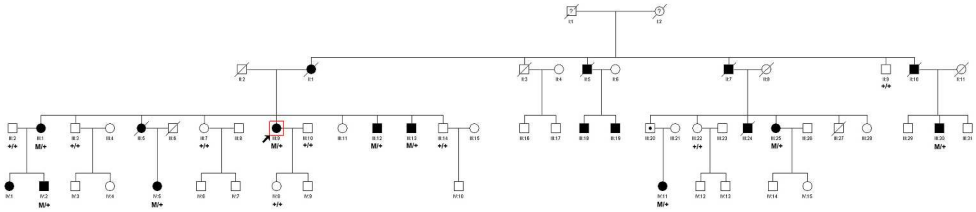


Figure 1B—adRP 61



Pedigrees of two Chinese adRP families with PRPF31 mutations and co-segregation in available family members. Filled symbols represent affected, unfilled unaffected, dotted asymptomatic carrier. Question marks indicate that it is not clear whether the individual is affected or not. Square signify male, circles females. Arrows mark the index patients. M refers to the mutant allele, and + means normal allele.
254x164mm (300 x 300 DPI)

Figure 2

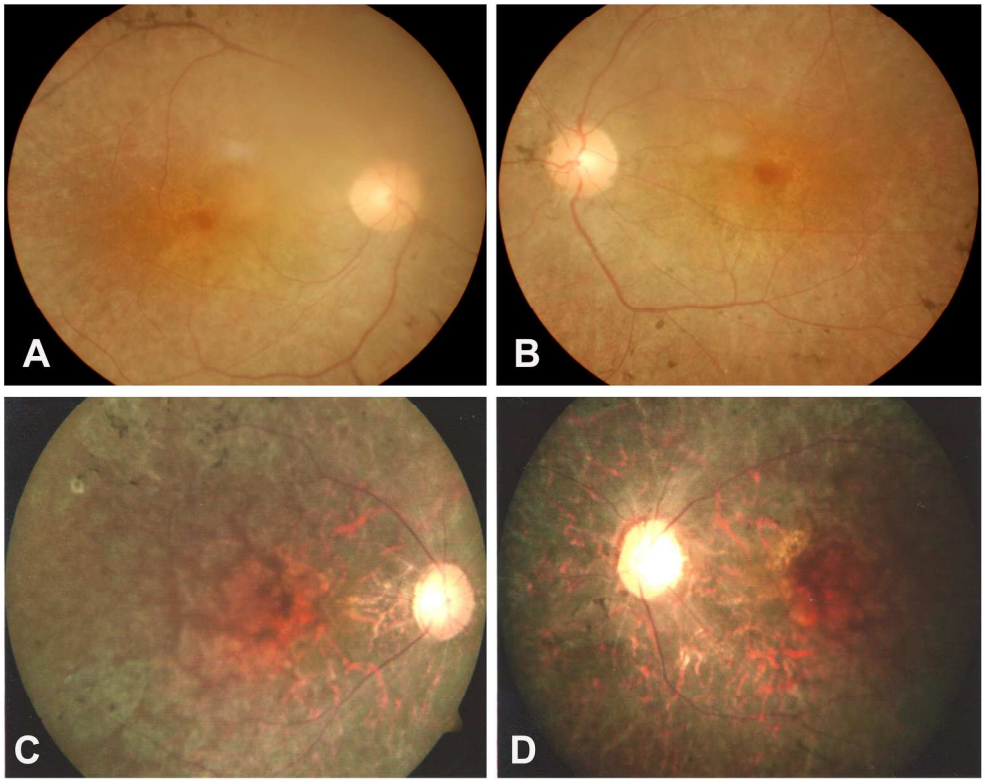
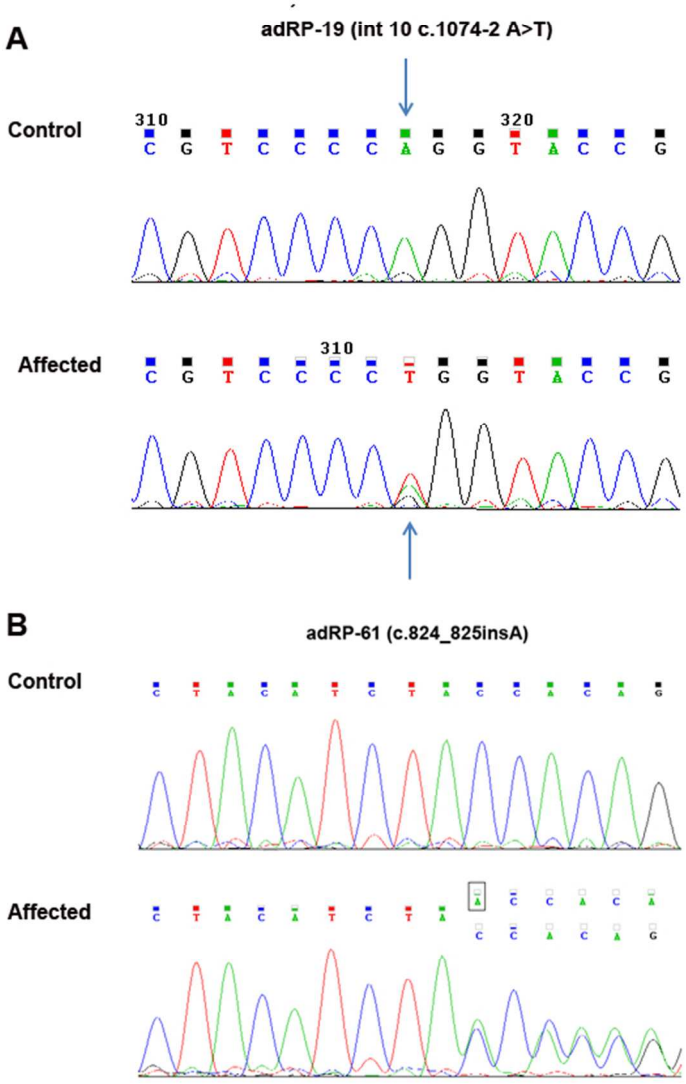
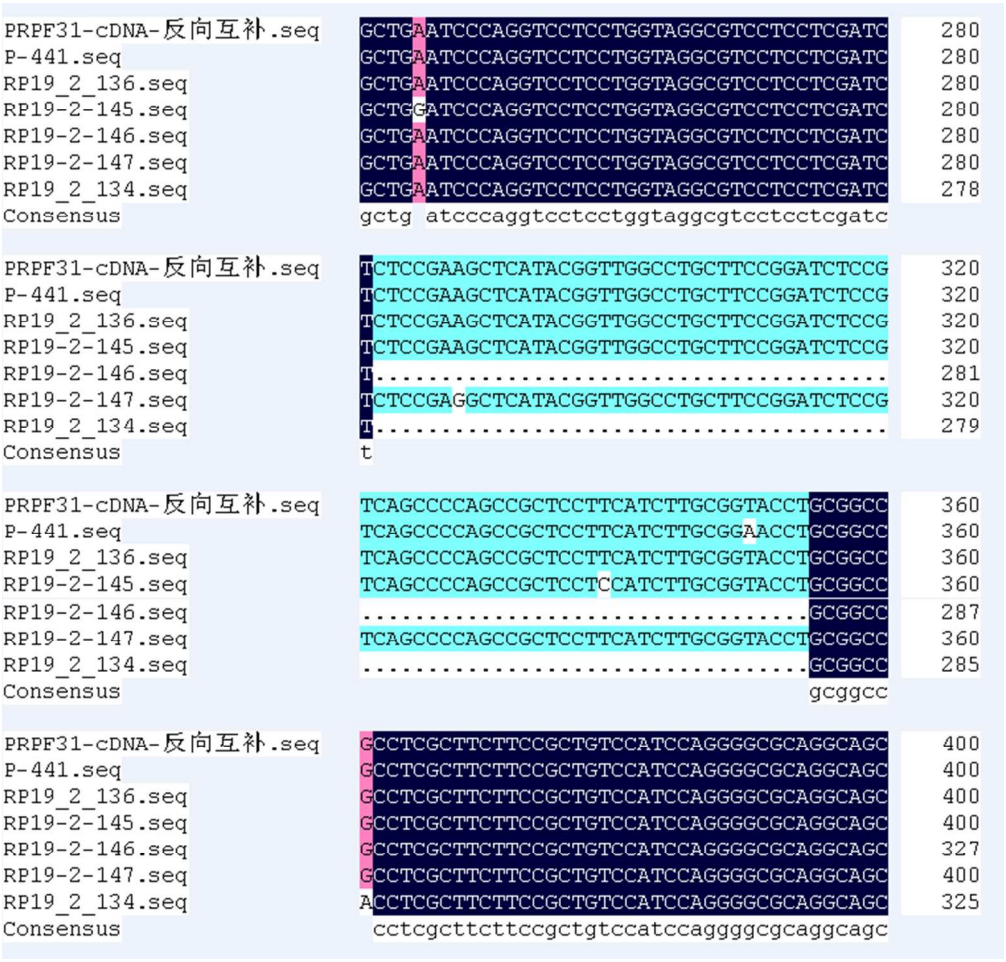


Figure 2. Fundus photographs of two probands with mutations in the PRPF31 gene. (A-B) Proband Ⅳ14 is from family adRP-19. (C-D) Proband Ⅲ9 is from family adRP-61. Typical retinitis pigmentosa changes can be seen.
220x192mm (300 x 300 DPI)

Figure 3



Sequencing results of the PRPF31 mutations in the two families. (A) Family adRP-19 carried the mutation Int10 c.1074-2 A>T; p.Y359SfsX29. (B) Family adRP-61 carried the mutation c.824_825insA; p.Y275X.
157x243mm (96 x 96 DPI)



The sequence comparison details between wild type and mutated is listed. RP19-2_134 from patient Ⅳ3; RP19-2_146 from patient Ⅴ3; RP19-2_136 from unaffected family member Ⅳ15; RP19-2_145 and 147 from normal controls not related to the family.

Targeted Exome Capture and Sequencing Identifies Novel PRPF31 Mutations in Autosomal Dominant Retinitis Pigmentosa in Chinese Families

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Targeted Exome Capture and Sequencing Identifies Novel PRPF31 Mutations in Autosomal Dominant Retinitis Pigmentosa in Chinese Families

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Word Count: 2313

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Patient consent: Obtained

Ethics approval: This study was conducted with the approval of Peking University Third Hospital Medical Ethics Committee (No. 2012093).

Abstract

Objectives— To identify disease-causing mutations in two Chinese families with autosomal dominant retinitis pigmentosa.

Design—Prospective analysis

Patients— Two Chinese adRP families underwent genetic diagnosis. A specific Hereditary Eye Disease Enrichment Panel (HEDEP) basing on targeted exome capture technology was used to collect the protein coding regions of targeted 371 hereditary eye disease genes, and high throughput sequencing was done with Illumina HiSeq 2000 platform. The identified variants were confirmed with Sanger sequencing.

Setting— All experiment were performed and analyzed in a large laboratory specialized in genetic studies in Department of Ophthalmology, Peking University Third Hospital.

Results—Two novel mutations including one splice site mutation (Int10 c.1074-2 A>T; p.Y359SfsX29) and one insertion (c.824_825insA; p.Y275X) of PRPF31 were identified in these two Chinese adRP families. These two mutations segregated with the disease phenotype in their respective families.

Conclusions—Our findings broaden the spectrum of PRPF31 mutations causing adRP and the phenotypic spectrum of the disease in Chinese patient. The HEDEP basing on targeted exome capture technology is an efficient method for molecular diagnosis in adRP patients.

ARTICLE SUMMARY

Article focus: The focus of this study was the analysis of variations in PRPF31 gene not described previously, and their association with the clinical phenotype of autosomal dominant Retinitis Pigmentosa in Chinese families.

Key messages:

- We demonstrated that one novel splice site mutation (Int10 c.1074-2 A>T; p.Y359SfsX29) and one novel insertion (c.824_825insA; p.Y275X) of PRPF31 were the disease causing mutations in two Chinese adRP families respectively.
- A specific Hereditary Eye Disease Enrichment Panel (HEDEP) basing on targeted exome capture technology was used to collect the protein coding regions of targeted 371 hereditary eye disease genes in these two families. Sanger sequencing in this study confirmed the mutation detected with the HEDEP, which proved the sensitivity and specificity of the HEDEP for application in molecular diagnosis of inherited eye diseases.

Strengths and limitations of this study:

- The HEDEP basing on targeted exome capture technology is an efficient method for molecular diagnosis in adRP patients.
- Both mutations result in premature termination codons (PTC) before the last exon, thus insufficient functioning due to haploinsufficiency instead of aberrant function of the mutated proteins seems to be the most probably reason in these two families. However no experiment was done to prove it in this study.

INTRODUCTION

Retinitis pigmentosa (RP) is an inherited retinal degeneration that affects approximately one in 3500 individuals with an estimated total of 1.5 million patient worldwide.[1-2] Typically patients affected by RP first suffer from night blindness, most often during adolescence. Rod and cone photoreceptor cells start to degenerate from the mid periphery to the far periphery and the center of the retina, resulting in the so-called tunnel vision. Later in life, central vision is also lost, leading to legal or complete blindness.[3] Clinical hallmarks of RP include bone-spicule deposits, attenuated retinal blood vessels, optic disc pallor, visual field loss, and abnormal, diminished or non-recordable electroretinographic responses (ERG).

Autosomal-dominant RP (adRP) account for 20-25% of all RP patients.[4] To date, more than 24 genes (Best 1, C1QTNF5, CA4, CRX, FSCN2, GUCA1B, IMPDH1, KLHL7, NR2E3, NRL, PRPF3, PRPF31, PRPF6, PRPF8, PRPH2, RDH12, RHO, ROM1, RP1, RP9, RPE65, SEMA4A, SNRP200 and TOPORS) are reported to be associated with adRP (https://sph.uth.edu/retnet/disease.htm). Traditionally, patients from RP families are studied with linkage analysis, or gene-by-gene screening,[5-6] which is costly, requires substantial human resources, and is time-consuming, thus making molecular diagnosis difficult and complex. This has led to the development of mutation screens based on arrayed primer extension technology, which enable the simultaneous detection of multiple mutations from one individual.[7] However, this approach is limited by the fact that it only can detect sequence variants previously reported, with novel mutations unreported.[8] It would be advantageous to screen for all the variants in the protein coding regions of targeted genes, one approach to achieve this is to develop a specific

hereditary eye disease enrichment panel based on exome capture technology to re-sequence simultaneously all the exons from targeted genes.

The aim of this study was to describe the development of a specific Hereditary Eye Disease Enrichment Panel (HEDEP) and its application in molecular diagnosis of two Chinese families with adRP, and to characterize the phenotypic manifestation associated with the mutation.

MATERIALS AND METHODS

Study subjects and clinical evaluation

Two Chinese families of Han ethnicity with adRP were identified in Anhui and Hubei province respectively, and there was no history of other ocular or systemic abnormalities in the families. The family adRP-19 has sixteen affected individuals in four generations, fourteen individuals, including nine affected and five unaffected participated in the study (Figure 1A). The family adRP-61 has eighteen affected individuals in three generations, seventeen individuals, including nine affected and eight unaffected participated in the study (Figure 1B). Medical and ophthalmic histories were obtained, and ophthalmological examination was carried out. One hundred sporadic RP patients recruited in Department of Ophthalmology, Peking University Third Hospital were used for RPPF31 gene mutation screening. One hundred of general healthy individuals from the Chinese Han ethnic population were recruited to serve as controls. All procedures used in this study conformed to the tenets of the Declaration of Helsinki. All experiments involving DNA and RNA of the patients and their relatives were approved by Peking University Third Hospital Medical Ethics Committee. Informed consent was obtained from all participants.

Screening for Mutations

Blood samples were collected and genomic DNA was extracted by standard protocols (D2492 Blood DNA Maxi Kit, Omega Bio-Tek Inc, Norcross, GA). A specific Hereditary Eye Disease Enrichment Panel (HEDEP) basing on targeted exome capture technology was used to collect the protein coding regions of targeted genes. This HEDEP was able to capture 371 hereditary eye disease genes, which covers 24 adRP associated genes (Best 1, C1QTNF5, CA4, CRX, FSCN2,

GUCA1B, IMPDH1, KLHL7, NR2E3, NRL, PRPF3, PRPF31, PRPF6, PRPF8, PRPH2, RDH12, RHO, ROM1, RP1, RP9, RPE65, SEMA4A, SNRP200 and TOPORS). Fifty μg of genomic DNA from two patients (Ⅲ3 and Ⅳ14), one carrier (Ⅲ7) and one control (Ⅲ18) of family adRP-19, and three patients (Ⅲ13, Ⅲ30 and Ⅳ2) and one control (Ⅲ2) of family adRP-61 were used for targeted exome capture. The exon-enriched DNA libraries were then prepared for High throughput sequencing with Illumina HiSeq 2000 platform. The obtained mean exome coverage was more than 98%，with variants accuracy at more than 99%. In these two families, we only analyzed mutations occurred in 24 adRP related genes. The shared changes in the affected individuals but not in the normal control were identified. The changes were filtered against exome data from ethnic Han Chinese Beijing available in the 1000 Genomes Project (fttp://www.1000genome.org), and against the Han Chinese Beijing SNPs in the dbSNP131. Sanger sequencing was then used to validate the identified potential disease-causing variants. Splice-site variants were analyzed using the prediction program AUGUSTUS (http://bioinf.uni-greifswald.de/augustus/submission).

Mutation Validation

The shared variants in the affected individuals but not in the normal control were then confirmed by direct polymerase chain reaction (PCR)-product sequencing using Bigdye terminator v3.1 cycle sequencing kits (Applied Biosystems, Foster City, CA, USA) and analyzed on an ABI 3130XL Genetic Analyzer. Sanger sequencing was used to determine whether the variant co-segregated with the disease phenotype in these two families. Primer pairs for individual exons were designed using the primer program (http://www.yeastgenome.org/cgi-bin/web-primer) (DNA reference number NG_009759). PCR primers, annealing temperatures, and amplimer-specific details are

listed in the Table 1.

Table 1. PCR primers used for PRPF31 amplification

Primer	Forward	Reverse	Product
PRPF31 Exon 2&3	CTGGGGGAGAATCATCGCTC	AAGGCTCTGGAAAAGGCT	557
PRPF31 Exon 4	CGAGAGGGGGTAGGGATTTAGATAC	ACCTCGATCTGAGCTTGGGCTTAG	252
PRPF31 Exon 5	AAGAAGGGGACATGGGTGTTA	TCCTCTCCATCGTCTCCAGA	287
PRPF31 Exon 6-7	CAAGAGAGGTTCTCGAGCCTT	TTTCCCAAGGTCACAGTGTCA	589
PRPF31 Exon 8	AGCCCCCAGGCAGATTTACT	TCCTGAGTGCTACCGTCAGCT	350
PRPF31 Exon 9	TAGAGCCCAAGGGTGGAAA	TTGGTAGGACAGTGCTCGCT	333
PRPF31 Exon 10&11	GGCAGCATTAGGTGCTGATTT	GTCGCTTTGGGGCTGAAT	599
PRPF31 Exon 12&13	CAACTCTGAGCTCACAGAGCA	TCATCCTGGCCTTCTTCACA	632
PRPF31 Exon 14	CTGTCTCATGCCCACCAA	TGGACCTCTGTGTCCCTTCA	295

Isolation of total RNA and reverse transcription (RT)-PCR analysis

Total RNA was extracted from peripheral whole blood samples by standard protocols (R6814 Blood RNA Kit, Omega). Reverse transcription was performed with oligonucleotide primers using Superscript II reverse transcriptase according to manufacturer's protocol (Invitrogen Corporation, Grand Island, NY). Primers for RT-PCR were designed to amplify exon 7-12 of PRPF31 mRNA (mRNA reference number NM_015629). The forward primer is 5'-GCCAAGATCATGGGTGTGG-3', and the reverse primer is 5'-TGCAGCGTCTTGGAGATCCT-3'. The RT-PCR products were further cloned into plasmid for sequence analysis.

RESULTS

Phenotype details

The clinical features of fourteen members of family adRP-19 and seventeen members of family adRP-61 who participated in this study are shown in Table 2. All the tested affected individuals except Ⅲ7 complained of night blindness and photophobia since childhood. Onset of the disease was noted to be ranging from 2 to 12 years of age. Fundus examination in the 38-year-old proband (Ⅳ14) of family adRP-19 showed bone spicule like pigmentation in the peripheral part of the retina, retinal arteriolar attenuation, and retinal pigment epithelium (RPE) degeneration (Figure 2A-B). Fundus examination in the 48-year-old proband (Ⅲ9) of family adRP-61 showed bone spicule like pigmentation in the mid-periphery retina, bilateral attenuation of retinal vessels, RPE degeneration and pale optic disc (Figure 2 C-D). The 68-year old asymptomatic carrier (Ⅲ7) of family adRP-19 did not complain of night blindness, and fundus examination showed no RP changes in both eyes. Visual acuity in majority of the patients declined quickly after 40 years old.

Table 2. Clinical data of the family members participating in the study

Family	ID	Sex	Age(y)	Disease status	Onset age (y)	Visual acuity Unaided (corrected) R eye	L eye	Fundus features
adRP-19	Ⅱ:1	F	82	Yes	Childhood	LP	LP	Cataract, can't see the fundus
	Ⅱ:3	F	80	Yes	8	LP	LP	Cataract, can't see the fundus
	Ⅲ:2	F	58	Yes	3	LP	LP	Cataract, can't see the fundus
	Ⅲ:3	M	54	Yes	3	0.1	0.1	Bilateral attenuation of retinal vessels, bone spicule pigments throughout the fundus, RPE degeneration, pale optic disc
	Ⅲ:6	M	46	No	-	1.5	1.5	No RP changes in both eyes
	Ⅲ:7	F	65	No	-	0.9	0.8	No RP changes in both eyes
	Ⅲ:18	M	68	No	-	1.2	1.2	No RP changes in both eyes
	Ⅳ:3	F	29	Yes	Childhood	1.0	1.0	Bone spicule pigments in peripheral part of the fundus
	Ⅳ:5	F	27	Yes	Childhood	1.0	1.0	Bone spicule pigments in peripheral part of the fundus
	Ⅳ:14	M	38	Yes	3	0.3	0.5	Bilateral attenuation of retinal vessels, bone spicule pigments in peripheral part of the fundus, RPE degeneration
	Ⅳ:15	F	34	No	-	1.5	1.5	No RP changes in both eyes
	Ⅳ:17	F	37	No	-	1.5	1.2	No RP changes in both eyes
	Ⅴ:3	M	16	Yes	2	1.2	1.2	Bone spicule pigments in peripheral part of the fundus
	Ⅴ:6	M	3	Yes	2	1.2	1.2	Bone spicule pigments in peripheral part of the fundus
adRP-61	Ⅱ:9	M	80	No	-	1.0	1.0	No RP changes in both eyes
	Ⅲ:1	F	63	Yes	12	0.4	0.5	Bone spicule pigments in peripheral part of the fundus
	Ⅲ:2	M	63	No	-	0.8	0.8	No RP changes in both eyes
	Ⅲ:3	M	61	No	-	1.0	1.0	No RP changes in both eyes
	Ⅲ:7	F	49	No	-	1.2	1.0	No RP changes in both eyes
	Ⅲ:9	F	48	Yes	Childhood	0.06	0.06	Bilateral attenuation of retinal vessels, bone spicule pigments throughout the fundus, RPE degeneration, pale optic disc
	Ⅲ:10	M	50	No	-	1.2	1.2	No RP changes in both eyes
	Ⅲ:12	M	40	Yes	Childhood	0.5	0.6	Bone spicule pigments in peripheral part of the fundus
	Ⅲ:13	M	38	Yes	Childhood	0.7	0.6	Bone spicule pigments in peripheral part of the fundus
	Ⅲ:14	M	45	No	-	1.0	1.0	No RP changes in both eyes
	Ⅲ:22	F	64	No	-	1.2	1.2	No RP changes in both eyes
	Ⅲ:25	M	50	Yes	Childhood	0.2	0.1	Bilateral attenuation of retinal vessels, bone spicule pigments throughout the fundus, RPE degeneration, pale optic disc
	Ⅲ:30	M	49	Yes	Childhood	0.1	0.06	Bilateral attenuation of retinal vessels, bone spicule pigments throughout the fundus, RPE degeneration, pale optic disc
	Ⅳ:2	M	32	Yes	Childhood	0.7	0.6	Bone spicule pigments in peripheral part of the fundus
	Ⅳ:5	F	35	Yes	Childhood	0.9	0.7	Bone spicule pigments in peripheral part of the fundus
	Ⅳ:8	F	28	No	-	1.5	1.5	No RP changes in both eyes
	Ⅳ:11	F	40	Yes	Childhood	0.5	0.6	Bone spicule pigments in peripheral part of the fundus

Identification of mutations in PRPF31

We selected four individuals in each family for targeted exome capture. We generated an average of 0.77Gb of sequence with 228× average coverage for each individual with paired 100bp reads. The generated sequence covered average 99.2% of the targeted bases with the accuracy of a variant call more than 99%, which is sufficient to pass the thresholds for calling SNPs and short insertions or deletions (indels). We filtered all the detected variants and found the potential disease causing mutations. Then we compared the shared variants in affected individuals with the ethnic Han Chinese Beijing available in the 1000 Genomes Project (fttp://www.1000genome.org), and against the Han Chinese Beijing SNPs in the dbSNP131. This left one splice site variation (Int10 c.1074-2 A>T) in family adRP-19 and one insertion (c.824_825insA; p.Y275X) in family adRP-61 respectively, which was shared among affected individuals but not in the normal control in each family. Sanger sequencing validation and segregation analysis was carried out, which demonstrated that these two variants (Figure 3) co-segregated with the disease phenotype in each family (Figure 1), but was absent in 100 matched normal controls.

We further carried out direct PCR sequencing of the PRPF31 exons in an additional 100 unrelated sporadic RP patients. All of these patients showed typical RP fundus features, including bilateral attenuation of retinal vessels, bone spicule pigments throughout the fundus, RPE degeneration and pale optic disc. No disease causing mutations were identified in these 100 sporadic patients.

Functional characterization of the Int10 c.1074-2 A>T mutation using RT-PCR

To determine whether the Int10 c.1074-2 A>T splicing mutation in family adRP-19 has any effect

on mRNA splicing, we performed RT-PCR for PRPF31 using total RNA samples isolated from peripheral blood samples from two patients (Ⅳ3 and Ⅴ3), one unaffected family member (Ⅳ15), and two normal controls not related to the family. The RT-PCR products were further cloned into plasmid for sequence analysis. It yielded a 590bp product from normal control samples as expected with normal splicing. One 590bp fragment and another 517bp product were amplified from the sample of affected individuals. Sequence analysis showed that the 517bp product skipped Exon11, resulting in frameshift i.e. p.Y359SfsX29 leading to premature termination with 28 new amino acids downstream. The sequence comparison details between wild type and mutated is listed in the Supplementary Figure 1.

DISCUSSION

In this study we reported the identification of two novel mutations i.e. one splice site mutation (Int10 c.1074-2 A>T; p.Y359SfsX29) and one insertion (c.824_825insA; p.Y275X) of PRPF31 in two Chinese adRP families. The identified splice site mutation (Int10 c.1074-2 A>T; p.Y359SfsX29) in family adRP-19 segregated in all the nine affected patients and one asymptomatic carrier, mother of an affected patient who manifest the disease from childhood. The identified insertion mutation (c.824_825insA; p.Y275X) in family adRP-61 segregated in all the nine affected patients, including one female who inherited the mutation from her 65-year old asymptomatic father. This is consistent with the incomplete penetrance of PRPF31 mutation.

The human PRPF31 gene contains 14 exons and encodes a 61kDa protein of 499 amino acids, the core component of the U4/U6 • U5 tri-snRNP complex which constitutes a major building block of the pre-mRNA processing spliceosome.[9] Although PRPF31 is ubiquitously expressed, patients with mutant PRPF31 alleles only show symptoms in the retina but not other organs.[10] At present, up to 50 mutations (including 15 splice defects, 10 missenses, 23 deletions and 2 insertions) in PRPF31 have been reported to be linked with adRP and sporadic RP cases.[5, 11-12] Splice defect have been reported in Int1,[13] Int 2,[14] Int 4,[14] Int 5,[15] Int 6,[16] Int 8,[14] Int 10,[17] Int 11 and Int 13.[10,18] Insertions have been reported in Exon 7 and Exon 8.[19] All the above splice defect and insertion mutations resulted in frameshift, leading to premature termination. Previous study from Rio Frio et al.[20] demonstrated that most PRPF31 mutations bearing premature termination codons (PTCs) before the last exon behave as null allele, resulting in haploinsufficiency as their corresponding mRNA is degraded by nonsense-mediated mRNA decay (NMD). However, if the PTC produced

by the mutation occurs in the last exon of a given gene, the mutant mRNA is insensitive to NMD and is thought to be translated into a truncated protein.[21] The splice site mutation (Int10 c.1074-2 A>T) in the present study caused the skipping of exon 11, resulting in frameshift, leading to premature termination with 28 new amino acids downstream (i.e. p.Y359SfsX29). The insertion mutation (c.824_825insA) in the present study happened in exon 8, resulting in the following amino acid changing from Thr to stop codon (p.Y275X). Since both mutations result in PTC before the last exon, thus insufficient functioning due to haploinsufficiency instead of aberrant function of the mutated proteins seems to be the most probably reason in these two families.

Several studies have been reported on the use of genotyping microarray for genetic diagnosis of retinal disease, such as Stargardt disease,[22] Leber congenital amaurosis,[23] Usher syndrome,[24] autosomal recessive RP,[25] and adRP.[7] Different from Arrayed Primer Extension technology used in most of previous microarray studies, the HEDEP in this study was based on target exon capture technology. HEDEP was able to capture 371 hereditary eye disease genes, which cover 53 RP associated genes, 7 Stargardt associated genes, 19 Leber congenital amaurosis associated genes, 11 Usher syndrome associated genes, 33 Cone and rod dystrophy associated genes, 13 Chorioretinal atrophy associated genes, 35 Microphthalmia associated genes, 46 Congenital cataract associated genes, 4 Glaucoma associated genes, 6 Familial exudative vitreoretinopathy associated genes, and 35 RP related syndrome associated genes etc. It is applicable to a broader inherited eye disease genetic study. Sanger sequencing in this study confirmed the mutation previously detected with the HEDEP, which proved the sensitivity and specificity of the HEDEP for application in molecular diagnosis of inherited eye diseases.

We presented a successful genetic diagnosis with a specific Hereditary Eye Disease Enrichment Panel (HEDEP), and identified two novel mutations i.e. one splice site mutation (Int10 c.1074-2 A>T; p.Y359SfsX29) and one insertion (c.824_825insA; p.Y275X) of PRPF31 in two Chinese adRP families with incomplete penetrance. Both mutations resulted in PTC before the last exon, thus insufficient functioning due to haploinsufficiency was the most probably reason in these two families. Our finding broaden the spectrum of PRPF31 mutations causing adRP and the phenotypic spectrum of the disease in Chinese patient, which will be helpful for genetic consultation and genetic diagnosis in the future.

Supplementary Material

Please feter to Web version on PubMed Central for supplementary material.

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Author contributions

Conceived and designed the experiment: LP Yang; HR Zhang; GL Li and ZZ Ma.

Performed the experiments: LP Yang; XB Yin; LM Wu; NN Chen.

Analyzed the data: LP Yang; LM Wu; NN Chen.

Wrote the paper: LP Yang; ZZ Ma;

Competing Interests

None

Data sharing

The original sequencing results is available on request.

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FIGURE LEGENDS

Figure 1. Pedigrees of two Chinese adRP families with PRPF31 mutations and co-segregation in available family members. Filled symbols represent affected, unfilled unaffected, dotted asymptomatic carrier. Question marks indicate that it is not clear whether the individual is affected or not. Square signify male, circles females. Arrows mark the index patients. M refers to the mutant allele, and + means normal allele.

Figure 2. Fundus photographs of two probands with mutations in the PRPF31 gene. (A-B) Proband Ⅳ14 is from family adRP-19. (C-D) Proband Ⅲ9 is from family adRP-61. Typical retinitis pigmentosa changes can be seen.

Figure 3. Sequencing results of the PRPF31 mutations in the two families. (A) Family adRP-19 carried the mutation Int10 c.1074-2 A>T; p.Y359SfsX29. (B) Family adRP-61 carried the mutation c.824_825insA; p.Y275X.

APPENDICES

Supplementary Figure 1. The sequence comparison details between wild type and mutated is listed. RP19-2_134 from patient □3; RP19-2_146 from patient□3; RP19-2_136 from unaffected family member □15; RP19-2_145 and 147 from normal controls not related to the family. It yielded a 590bp product from unaffected family member and control samples, One 590bp fragment and another 517bp product were amplified from the affected individuals.

Targeted Exome Capture and Sequencing Identifies Novel PRPF31 Mutations in Autosomal Dominant Retinitis Pigmentosa in Chinese Families

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Ethics approval: This study was conducted with the approval of Peking University Third Hospital Medical Ethics Committee (No. 2012093).

Abstract

Objectives— To identify disease-causing mutations in two Chinese families with autosomal dominant retinitis pigmentosa.

Design—Prospective analysis

Patients— Two Chinese adRP families underwent genetic diagnosis. A specific Hereditary Eye Disease Enrichment Panel (HEDEP) basing on targeted exome capture technology was used to collect the protein coding regions of targeted 371 hereditary eye disease genes, and high throughput sequencing was done with Illumina HiSeq 2000 platform. The identified variants were confirmed with Sanger sequencing.

Setting— All experiment were performed and analyzed in a large laboratory specialized in genetic studies in Department of Ophthalmology, Peking University Third Hospital.

Results—Two novel mutations including one splice site mutation (Int10 c.1074-2 A>T; p.Y359SfsX29) and one insertion (c.824_825insA; p.Y275X) of PRPF31 were identified in these two Chinese adRP families. These two mutations segregated with the disease phenotype in their respective families.

Conclusions—Our findings broaden the spectrum of PRPF31 mutations causing adRP and the phenotypic spectrum of the disease in Chinese patient. The HEDEP basing on targeted exome capture technology is an efficient method for molecular diagnosis in adRP patients.

ARTICLE SUMMARY

Article focus: The focus of this study was the analysis of variations in PRPF31 gene not described previously, and their association with the clinical phenotype of autosomal dominant Retinitis Pigmentosa in Chinese families.

Key messages:

- We demonstrated that one novel splice site mutation (Int10 c.1074-2 A>T; p.Y359SfsX29) and one novel insertion (c.824_825insA; p.Y275X) of PRPF31 were the disease causing mutations in two Chinese adRP families respectively.
- A specific Hereditary Eye Disease Enrichment Panel (HEDEP) basing on targeted exome capture technology was used to collect the protein coding regions of targeted 371 hereditary eye disease genes in these two families. Sanger sequencing in this study confirmed the mutation detected with the HEDEP, which proved the sensitivity and specificity of the HEDEP for application in molecular diagnosis of inherited eye diseases.

Strengths and limitations of this study:

- The HEDEP basing on targeted exome capture technology is an efficient method for molecular diagnosis in adRP patients.
- Both mutations result in premature termination codons (PTC) before the last exon, thus insufficient functioning due to haploinsufficiency instead of aberrant function of the mutated proteins seems to be the most probably reason in these two families. However no experiment was done to prove it in this study.

INTRODUCTION

Retinitis pigmentosa (RP) is an inherited retinal degeneration that affects approximately one in 3500 individuals with an estimated total of 1.5 million patient worldwide.[1-2] Typically patients affected by RP first suffer from night blindness, most often during adolescence. Rod and cone photoreceptor cells start to degenerate from the mid periphery to the far periphery and the center of the retina, resulting in the so-called tunnel vision. Later in life, central vision is also lost, leading to legal or complete blindness.[3] Clinical hallmarks of RP include bone-spicule deposits, attenuated retinal blood vessels, optic disc pallor, visual field loss, and abnormal, diminished or non-recordable electroretinographic responses (ERG).

Autosomal-dominant RP (adRP) account for 20-25% of all RP patients.[4] To date, more than 24 genes (Best 1, C1QTNF5, CA4, CRX, FSCN2, GUCA1B, IMPDH1, KLHL7, NR2E3, NRL, PRPF3, PRPF31, PRPF6, PRPF8, PRPH2, RDH12, RHO, ROM1, RP1, RP9, RPE65, SEMA4A, SNRP200 and TOPORS) are reported to be associated with adRP (https://sph.uth.edu/retnet/disease.htm). Traditionally, patients from RP families are studied with linkage analysis, or gene-by-gene screening,[5-6] which is costly, requires substantial human resources, and is time-consuming, thus making molecular diagnosis difficult and complex. This has led to the development of mutation screens based on arrayed primer extension technology, which enable the simultaneous detection of multiple mutations from one individual.[7] However, this approach is limited by the fact that it only can detect sequence variants previously reported, with novel mutations unreported.[8] It would be advantageous to screen for all the variants in the protein coding regions of targeted genes, one approach to achieve this is to develop a specific

hereditary eye disease enrichment panel based on exome capture technology to re-sequence simultaneously all the exons from targeted genes.

The aim of this study was to describe the development of a specific Hereditary Eye Disease Enrichment Panel (HEDEP) and its application in molecular diagnosis of two Chinese families with adRP, and to characterize the phenotypic manifestation associated with the mutation.

MATERIALS AND METHODS

Study subjects and clinical evaluation

Two Chinese families of Han ethnicity with adRP were identified in Anhui and Hubei province respectively, and there was no history of other ocular or systemic abnormalities in the families. The family adRP-19 has sixteen affected individuals in four generations, fourteen individuals, including nine affected and five unaffected participated in the study (Figure 1A). The family adRP-61 has eighteen affected individuals in three generations, seventeen individuals, including nine affected and eight unaffected participated in the study (Figure 1B). Medical and ophthalmic histories were obtained, and ophthalmological examination was carried out. One hundred sporadic RP patients recruited in Department of Ophthalmology, Peking University Third Hospital were used for RPPF31 gene mutation screening. One hundred of general healthy individuals from the Chinese Han ethnic population were recruited to serve as controls. All procedures used in this study conformed to the tenets of the Declaration of Helsinki. All experiments involving DNA and RNA of the patients and their relatives were approved by Peking University Third Hospital Medical Ethics Committee. Informed consent was obtained from all participants.

Screening for Mutations

Blood samples were collected and genomic DNA was extracted by standard protocols (D2492 Blood DNA Maxi Kit, Omega Bio-Tek Inc, Norcross, GA). A specific Hereditary Eye Disease Enrichment Panel (HEDEP) basing on targeted exome capture technology was used to collect the protein coding regions of targeted genes. This HEDEP was able to capture 371 hereditary eye disease genes, which covers 24 adRP associated genes (Best 1, C1QTNF5, CA4, CRX, FSCN2,

GUCA1B, IMPDH1, KLHL7, NR2E3, NRL, PRPF3, PRPF31, PRPF6, PRPF8, PRPH2, RDH12, RHO, ROM1, RP1, RP9, RPE65, SEMA4A, SNRP200 and TOPORS). Fifty μg of genomic DNA from two patients (Ⅲ3 and Ⅳ14), one carrier (Ⅲ7) and one control (Ⅲ18) of family adRP-19, and three patients (Ⅲ13, Ⅲ30 and Ⅳ2) and one control (Ⅲ2) of family adRP-61 were used for targeted exome capture. The exon-enriched DNA libraries were then prepared for High throughput sequencing with Illumina HiSeq 2000 platform. The obtained mean exome coverage was more than 98%，with variants accuracy at more than 99%. In these two families, we only analyzed mutations occurred in 24 adRP related genes. The shared changes in the affected individuals but not in the normal control were identified. The changes were filtered against exome data from ethnic Han Chinese Beijing available in the 1000 Genomes Project (fttp://www.1000genome.org), and against the Han Chinese Beijing SNPs in the dbSNP131. Sanger sequencing was then used to validate the identified potential disease-causing variants. Splice-site variants were analyzed using the prediction program AUGUSTUS (http://bioinf.uni-greifswald.de/augustus/submission).

Mutation Validation

The shared variants in the affected individuals but not in the normal control were then confirmed by direct polymerase chain reaction (PCR)-product sequencing using Bigdye terminator v3.1 cycle sequencing kits (Applied Biosystems, Foster City, CA, USA) and analyzed on an ABI 3130XL Genetic Analyzer. Sanger sequencing was used to determine whether the variant co-segregated with the disease phenotype in these two families. Primer pairs for individual exons were designed using the primer program (http://www.yeastgenome.org/cgi-bin/web-primer) (DNA reference number NG_009759). PCR primers, annealing temperatures, and amplimer-specific details are

listed in the Table 1.

Table 1. PCR primers used for PRPF31 amplification

Primer	Forward	Reverse	Product
PRPF31 Exon 2&3	CTGGGGGAGAATCATCGCTC	AAGGCTCTGGAAAAGGCT	557
PRPF31 Exon 4	CGAGAGGGGGTAGGGATTTAGATAC	ACCTCGATCTGAGCTTGGGCTTAG	252
PRPF31 Exon 5	AAGAAGGGGACATGGGTGTTA	TCCTCTCCATCGTCTCCAGA	287
PRPF31 Exon 6-7	CAAGAGAGGTTCTCGAGCCTT	TTTCCCAAGGTCACAGTGTCA	589
PRPF31 Exon 8	AGCCCCCAGGCAGATTTACT	TCCTGAGTGCTACCGTCAGCT	350
PRPF31 Exon 9	TAGAGCCCAAGGGTGGAAA	TTGGTAGGACAGTGCTCGCT	333
PRPF31 Exon 10&11	GGCAGCATTAGGTGCTGATTT	GTCGCTTTGGGGCTGAAT	599
PRPF31 Exon 12&13	CAACTCTGAGCTCACAGAGCA	TCATCCTGGCCTTCTTCACA	632
PRPF31 Exon 14	CTGTCTCATGCCCACCAA	TGGACCTCTGTGTCCCTTCA	295

Isolation of total RNA and reverse transcription (RT)-PCR analysis

Total RNA was extracted from peripheral whole blood samples by standard protocols (R6814 Blood RNA Kit, Omega). Reverse transcription was performed with oligonucleotide primers using Superscript II reverse transcriptase according to manufacturer's protocol (Invitrogen Corporation, Grand Island, NY). Primers for RT-PCR were designed to amplify exon 7-12 of PRPF31 mRNA (mRNA reference number NM_015629). The forward primer is 5'-GCCAAGATCATGGGTGTGG-3', and the reverse primer is 5'-TGCAGCGTCTTGGAGATCCT-3'. The RT-PCR products were further cloned into plasmid for sequence analysis.

RESULTS

Phenotype details

The clinical features of fourteen members of family adRP-19 and seventeen members of family adRP-61 who participated in this study are shown in Table 2. All the tested affected individuals except Ⅲ7 complained of night blindness and photophobia since childhood. Onset of the disease was noted to be ranging from 2 to 12 years of age. Fundus examination in the 38-year-old proband (Ⅳ14) of family adRP-19 showed bone spicule like pigmentation in the peripheral part of the retina, retinal arteriolar attenuation, and retinal pigment epithelium (RPE) degeneration (Figure 2A-B). Fundus examination in the 48-year-old proband (Ⅲ9) of family adRP-61 showed bone spicule like pigmentation in the mid-periphery retina, bilateral attenuation of retinal vessels, RPE degeneration and pale optic disc (Figure 2 C-D). The 68-year old asymptomatic carrier (Ⅲ7) of family adRP-19 did not complain of night blindness, and fundus examination showed no RP changes in both eyes. Visual acuity in majority of the patients declined quickly after 40 years old.

Table 2. Clinical data of the family members participating in the study

Family	ID	Sex	Age(y)	Disease status	Onset age (y)	Visual acuity Unaided (corrected) R eye	L eye	Fundus features
adRP-19	**Ⅱ:1**	F	82	Yes	Childhood	LP	LP	Cataract, can't see the fundus
	Ⅱ:3	F	80	Yes	8	LP	LP	Cataract, can't see the fundus
	Ⅲ:2	F	58	Yes	3	LP	LP	Cataract, can't see the fundus
	Ⅲ:3	M	54	Yes	3	0.1	0.1	Bilateral attenuation of retinal vessels, bone spicule pigments throughout the fundus, RPE degeneration, pale optic disc
	Ⅲ:6	M	46	No	-	1.5	1.5	No RP changes in both eyes
	Ⅲ:7	F	65	No	-	0.9	0.8	No RP changes in both eyes
	Ⅲ:18	M	68	No	-	1.2	1.2	No RP changes in both eyes
	Ⅳ:3	F	29	Yes	Childhood	1.0	1.0	Bone spicule pigments in peripheral part of the fundus
	Ⅳ:5	F	27	Yes	Childhood	1.0	1.0	Bone spicule pigments in peripheral part of the fundus
	Ⅳ:14	M	38	Yes	3	0.3	0.5	Bilateral attenuation of retinal vessels, bone spicule pigments in peripheral part of the fundus, RPE degeneration
	Ⅳ:15	F	34	No	-	1.5	1.5	No RP changes in both eyes
	Ⅳ:17	F	37	No	-	1.5	1.2	No RP changes in both eyes
	Ⅴ:3	M	16	Yes	2	1.2	1.2	Bone spicule pigments in peripheral part of the fundus
	Ⅴ:6	M	3	Yes	2	1.2	1.2	Bone spicule pigments in peripheral part of the fundus
adRP-61	**Ⅱ:9**	M	80	No	-	1.0	1.0	No RP changes in both eyes
	Ⅲ:1	F	63	Yes	12	0.4	0.5	Bone spicule pigments in peripheral part of the fundus
	Ⅲ:2	M	63	No	-	0.8	0.8	No RP changes in both eyes
	Ⅲ:3	M	61	No	-	1.0	1.0	No RP changes in both eyes
	Ⅲ:7	F	49	No	-	1.2	1.0	No RP changes in both eyes
	Ⅲ:9	F	48	Yes	Childhood	0.06	0.06	Bilateral attenuation of retinal vessels, bone spicule pigments throughout the fundus, RPE degeneration, pale optic disc
	Ⅲ:10	M	50	No	-	1.2	1.2	No RP changes in both eyes
	Ⅲ:12	M	40	Yes	Childhood	0.5	0.6	Bone spicule pigments in peripheral part of the fundus
	Ⅲ:13	M	38	Yes	Childhood	0.7	0.6	Bone spicule pigments in peripheral part of the fundus
	Ⅲ:14	M	45	No	-	1.0	1.0	No RP changes in both eyes
	Ⅲ:22	F	64	No	-	1.2	1.2	No RP changes in both eyes
	Ⅲ:25	M	50	Yes	Childhood	0.2	0.1	Bilateral attenuation of retinal vessels, bone spicule pigments throughout the fundus, RPE degeneration, pale optic disc
	Ⅲ:30	M	49	Yes	Childhood	0.1	0.06	Bilateral attenuation of retinal vessels, bone spicule pigments throughout the fundus, RPE degeneration, pale optic disc
	Ⅳ:2	M	32	Yes	Childhood	0.7	0.6	Bone spicule pigments in peripheral part of the fundus
	Ⅳ:5	F	35	Yes	Childhood	0.9	0.7	Bone spicule pigments in peripheral part of the fundus
	Ⅳ:8	F	28	No	-	1.5	1.5	No RP changes in both eyes
	Ⅳ:11	F	40	Yes	Childhood	0.5	0.6	Bone spicule pigments in peripheral part of the fundus

Identification of mutations in PRPF31

We selected four individuals in each family for targeted exome capture. We generated an average of 0.77Gb of sequence with 228× average coverage for each individual with paired 100bp reads. The generated sequence covered average 99.2% of the targeted bases with the accuracy of a variant call more than 99%, which is sufficient to pass the thresholds for calling SNPs and short insertions or deletions (indels). We filtered all the detected variants and found the potential disease causing mutations. Then we compared the shared variants in affected individuals with the ethnic Han Chinese Beijing available in the 1000 Genomes Project (fttp://www.1000genome.org), and against the Han Chinese Beijing SNPs in the dbSNP131. This left one splice site variation (Int10 c.1074-2 A>T) in family adRP-19 and one insertion (c.824_825insA; p.Y275X) in family adRP-61 respectively, which was shared among affected individuals but not in the normal control in each family. Sanger sequencing validation and segregation analysis was carried out, which demonstrated that these two variants (Figure 3) co-segregated with the disease phenotype in each family (Figure 1), but was absent in 100 matched normal controls.

We further carried out direct PCR sequencing of the PRPF31 exons in an additional 100 unrelated sporadic RP patients. All of these patients showed typical RP fundus features, including bilateral attenuation of retinal vessels, bone spicule pigments throughout the fundus, RPE degeneration and pale optic disc. No disease causing mutations were identified in these 100 sporadic patients.

Functional characterization of the Int10 c.1074-2 A>T mutation using RT-PCR

To determine whether the Int10 c.1074-2 A>T splicing mutation in family adRP-19 has any effect

on mRNA splicing, we performed RT-PCR for PRPF31 using total RNA samples isolated from peripheral blood samples from two patients (Ⅳ3 and Ⅴ3), one unaffected family member (Ⅳ15), and two normal controls not related to the family. The RT-PCR products were further cloned into plasmid for sequence analysis. It yielded a 590bp product from normal control samples as expected with normal splicing. One 590bp fragment and another 517bp product were amplified from the sample of affected individuals. Sequence analysis showed that the 517bp product skipped Exon11, resulting in frameshift i.e. p.Y359SfsX29 leading to premature termination with 28 new amino acids downstream. The sequence comparison details between wild type and mutated is listed in the Supplementary Figure 1.

DISCUSSION

In this study we reported the identification of two novel mutations i.e. one splice site mutation (Int10 c.1074-2 A>T; p.Y359SfsX29) and one insertion (c.824_825insA; p.Y275X) of PRPF31 in two Chinese adRP families. The identified splice site mutation (Int10 c.1074-2 A>T; p.Y359SfsX29) in family adRP-19 segregated in all the nine affected patients and one asymptomatic carrier, mother of an affected patient who manifest the disease from childhood. The identified insertion mutation (c.824_825insA; p.Y275X) in family adRP-61 segregated in all the nine affected patients, including one female who inherited the mutation from her 65-year old asymptomatic father. This is consistent with the incomplete penetrance of PRPF31 mutation.

The human PRPF31 gene contains 14 exons and encodes a 61kDa protein of 499 amino acids, the core component of the U4/U6 • U5 tri-snRNP complex which constitutes a major building block of the pre-mRNA processing spliceosome.[9] Although PRPF31 is ubiquitously expressed, patients with mutant PRPF31 alleles only show symptoms in the retina but not other organs.[10] At present, up to 50 mutations (including 15 splice defects, 10 missenses, 23 deletions and 2 insertions) in PRPF31 have been reported to be linked with adRP and sporadic RP cases.[5, 11-12] Splice defect have been reported in Int1,[13] Int 2,[14] Int 4,[14] Int 5,[15] Int 6,[16] Int 8,[14] Int 10,[17] Int 11 and Int 13.[10,18] Insertions have been reported in Exon 7 and Exon 8.[19] All the above splice defect and insertion mutations resulted in frameshift, leading to premature termination. Previous study from Rio Frio et al.[20] demonstrated that most PRPF31 mutations bearing premature termination codons (PTCs) before the last exon behave as null allele, resulting in haploinsufficiency as their corresponding mRNA is degraded by nonsense-mediated mRNA decay (NMD). However, if the PTC produced

by the mutation occurs in the last exon of a given gene, the mutant mRNA is insensitive to NMD and is thought to be translated into a truncated protein.[21] The splice site mutation (Int10 c.1074-2 A>T) in the present study caused the skipping of exon 11, resulting in frameshift, leading to premature termination with 28 new amino acids downstream (i.e. p.Y359SfsX29). The insertion mutation (c.824_825insA) in the present study happened in exon 8, resulting in the following amino acid changing from Thr to stop codon (p.Y275X). Since both mutations result in PTC before the last exon, thus insufficient functioning due to haploinsufficiency instead of aberrant function of the mutated proteins seems to be the most probably reason in these two families.

Several studies have been reported on the use of genotyping microarray for genetic diagnosis of retinal disease, such as Stargardt disease,[22] Leber congenital amaurosis,[23] Usher syndrome,[24] autosomal recessive RP,[25] and adRP.[7] Different from Arrayed Primer Extension technology used in most of previous microarray studies, the HEDEP in this study was based on target exon capture technology. HEDEP was able to capture 371 hereditary eye disease genes, which cover 53 RP associated genes, 7 Stargardt associated genes, 19 Leber congenital amaurosis associated genes, 11 Usher syndrome associated genes, 33 Cone and rod dystrophy associated genes, 13 Chorioretinal atrophy associated genes, 35 Microphthalmia associated genes, 46 Congenital cataract associated genes, 4 Glaucoma associated genes, 6 Familial exudative vitreoretinopathy associated genes, and 35 RP related syndrome associated genes etc. It is applicable to a broader inherited eye disease genetic study. Sanger sequencing in this study confirmed the mutation previously detected with the HEDEP, which proved the sensitivity and specificity of the HEDEP for application in molecular diagnosis of inherited eye diseases.

We presented a successful genetic diagnosis with a specific Hereditary Eye Disease Enrichment Panel (HEDEP), and identified two novel mutations i.e. one splice site mutation (Int10 c.1074-2 A>T; p.Y359SfsX29) and one insertion (c.824_825insA; p.Y275X) of PRPF31 in two Chinese adRP families with incomplete penetrance. Both mutations resulted in PTC before the last exon, thus insufficient functioning due to haploinsufficiency was the most probably reason in these two families. Our finding broaden the spectrum of PRPF31 mutations causing adRP and the phenotypic spectrum of the disease in Chinese patient, which will be helpful for genetic consultation and genetic diagnosis in the future.

Supplementary Material

Please feter to Web version on PubMed Central for supplementary material.

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Author contributions

Conceived and designed the experiment: LP Yang; HR Zhang; GL Li and ZZ Ma.

Performed the experiments: LP Yang; XB Yin; LM Wu; NN Chen.

Analyzed the data: LP Yang; LM Wu; NN Chen.

Wrote the paper: LP Yang; ZZ Ma;

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FIGURE LEGENDS

Figure 1. Pedigrees of two Chinese adRP families with PRPF31 mutations and co-segregation in available family members. Filled symbols represent affected, unfilled unaffected, dotted asymptomatic carrier. Question marks indicate that it is not clear whether the individual is affected or not. Square signify male, circles females. Arrows mark the index patients. M refers to the mutant allele, and + means normal allele.

Figure 2. Fundus photographs of two probands with mutations in the PRPF31 gene. (A-B) Proband Ⅳ14 is from family adRP-19. (C-D) Proband Ⅲ9 is from family adRP-61. Typical retinitis pigmentosa changes can be seen.

Figure 3. Sequencing results of the PRPF31 mutations in the two families. (A) Family adRP-19 carried the mutation Int10 c.1074-2 A>T; p.Y359SfsX29. (B) Family adRP-61 carried the mutation c.824_825insA; p.Y275X.

APPENDICES

Supplementary Figure 1. The sequence comparison details between wild type and mutated is listed. RP19-2_134 from patient □3; RP19-2_146 from patient□3; RP19-2_136 from unaffected family member □15; RP19-2_145 and 147 from normal controls not related to the family. It yielded a 590bp product from unaffected family member and control samples, One 590bp fragment and another 517bp product were amplified from the affected individuals.

Figure 1A—adRP 19

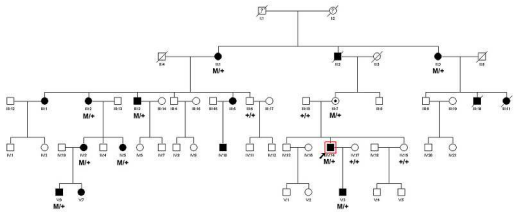
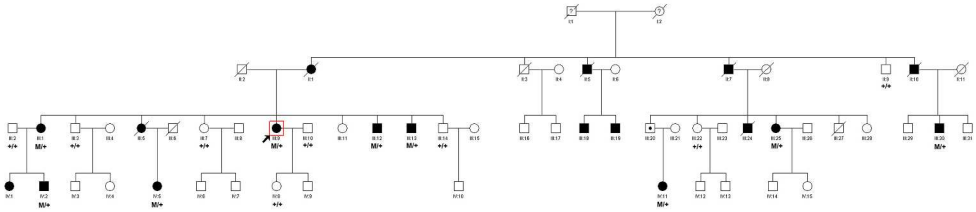


Figure 1B—adRP 61



Pedigrees of two Chinese adRP families with PRPF31 mutations and co-segregation in available family members. Filled symbols represent affected, unfilled unaffected, dotted asymptomatic carrier. Question marks indicate that it is not clear whether the individual is affected or not. Square signify male, circles females. Arrows mark the index patients. M refers to the mutant allele, and + means normal allele.
254x164mm (300 x 300 DPI)

Figure 2

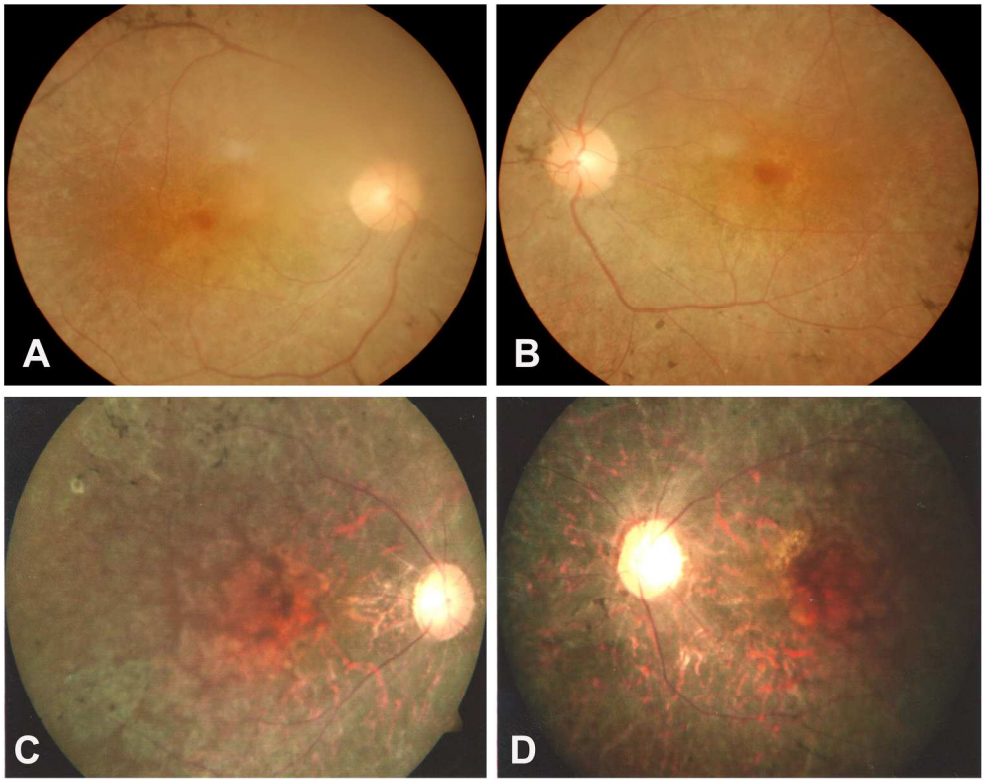
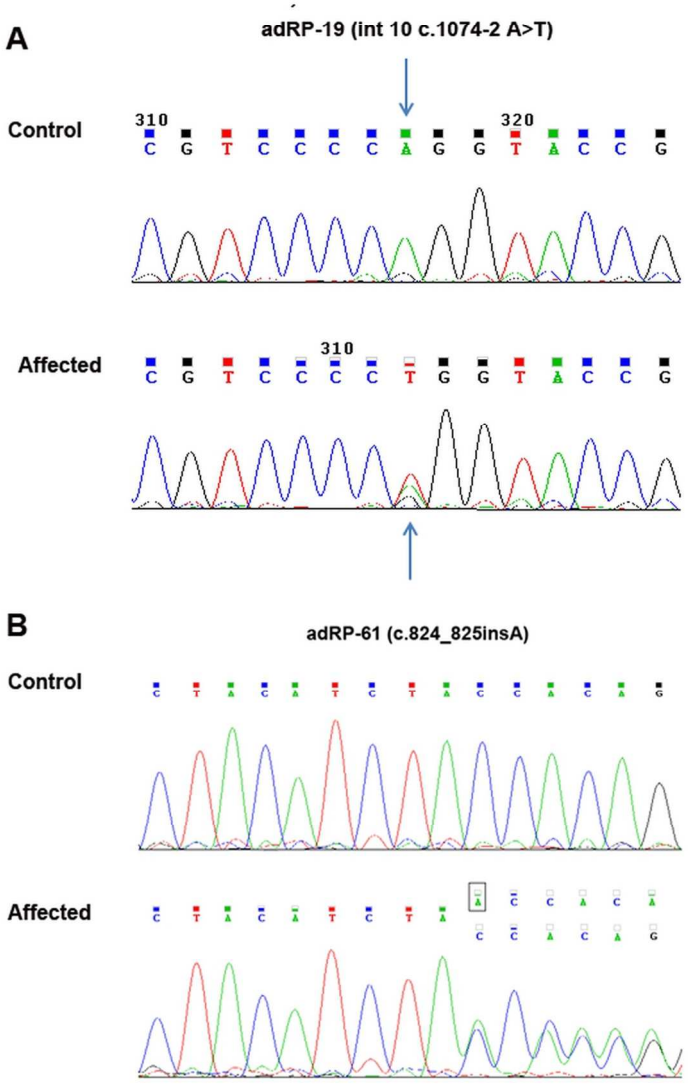


Figure 2. Fundus photographs of two probands with mutations in the PRPF31 gene. (A-B) Proband Ⅳ14 is from family adRP-19. (C-D) Proband Ⅲ9 is from family adRP-61. Typical retinitis pigmentosa changes can be seen.
220x192mm (300 x 300 DPI)

Figure 3



Sequencing results of the PRPF31 mutations in the two families. (A) Family adRP-19 carried the mutation Int10 c.1074-2 A>T; p.Y359SfsX29. (B) Family adRP-61 carried the mutation c.824_825insA; p.Y275X.
90x139mm (300 x 300 DPI)