Published in final edited form as:

Biochem Biophys Res Commun. 2008 March 7; 367(2): 420–426. doi:10.1016/j.bbrc.2007.12.156.

Identification and functional characterization of a novel splicing mutation in RP gene *PRPF31*

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Abstract

A six-generation Chinese family with autosomal dominant retinitis pigmentosa (adRP) was identified and characterized. Genome-wide linkage analysis linked the family to markers *D19S601* to *D19S605*, which span the *PRPF31* gene on chromosome 19q13.33–13.43 (RP11) (LOD = 5.03). Direct DNA sequence analysis identified a novel splicing mutation (IVS1 +1 G > T) in affected family members, but not in unaffected family members and 200 normal controls. The splicing mutation occurs at the splicing donor of intron 1. Real time PCR with lymphoblastoid RNA samples from family members showed that in comparison to normal family members, the splicing mutation reduced the expression level of the *PRPF31* mRNA by 57% in symptomatic patients and by 28% in clinically asymptomatic carriers. Our studies identify a novel splicing mutation in *PRPF31* associated with adRP and suggest that the penetrance of RP11 mutations may be correlated with the expression level of the *PRPF31* mRNA.

Keywords

Autosomal dominant retinitis pigmentosa; *PRPF31*; Splicing mutation; Genetics; Linkage

Retinitis pigmentosa (RP) is a genetically heterogeneous disorder characterized by progressive degeneration of the peripheral retina, night blindness, constriction of visual field, and eventual loss of visual acuity. It is one of the leading causes of blindness in adults. Other typical features of RP include bone spicule-like retinal pigmentary deposits and reduced amplitudes or absent electroretinographic responses [1]. RP is the most frequent form of inherited retinopathies and has an incidence of 1 in 3,500 individuals worldwide [2,3]. The incidence is about 1 in 1,000

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elderly Chinese people in Northern China [4]. RP can be transmitted as a dominant, recessive, X-linked, or non-Mendelian trait [5]. One of the major genes for autosomal dominant RP (adRP) is the RP11 gene, *PRPF31*, which is located on chromosome 19q13.4 (RP11) and comprised of 14 exons spanning approximately 16 kb of genomic DNA. The RP11 gene encodes a ubiquitously expressed 61 kDa splicing protein, PRPF31 [3,6].

The splicing of pre-mRNA in the nucleus is catalyzed by a large ribonucleoprotein complex (spliceosome). The spliceosome is composed of the pre-mRNA substrate, small nuclear ribonucleoproteins (snRNPs) U1, U2, U4, U5 and U6, and other splicing factors. The PRPF31 is an integral component of the U4/U6+U5 tri-snRNP. It is thought to be responsible for tethering the tri-snRNP to the spliceosome rather than forming or maintaining individual snRNP complexes [6–8]. Over 20 different mutations in the *PRPF31* gene have been reported to be associated with adRP. The mutations identified to date include missense mutations, deletions, insertions and splicing mutations [2,3,9–12]. In this study, we studied a large Chinese adRP family with over 77 living members and identified a novel splicing in the *PRPF31* gene. Functional studies showed that the mutation affected the splicing of PRPF31 and the differences of the expression level of *PRPF31* could be a cause for the interesting finding of the “all or none” type of penetrance associated with RP11.

Materials and methods

Study subjects and isolation of genomic DNA

The study participants were identified and enrolled at Maternal and Child Health Clinic of Tanghe City. Informed consent was obtained from the participants in accordance with the study protocols approved by the Ethics Committee of Huazhong University of Science and Technology. The participants were clinically examined at Eye Hospital of Nanyang. Ocular examinations were performed by slit lamp biomicroscopy, and direct or indirect ophthalmoscope. Fundus photographs were obtained by a Fundus camera (RC-XV3, Kowa Company. Ltd. Japan).

DNA was extracted from peripheral whole blood samples using Wizard Genomic DNA Purification Kit (Promega, Madison, WI).

Linkage analysis

A genome wide linkage scan was carried out with the ABI PRISM Linkage-Mapping set version 2.5 (Applied Biosystems, Foster City, CA). A total of 382 fluorescent microsatellite markers from the ABI Mapping Panel MD-10 were genotyped in the adRP family and they are distributed over the entire human autosomes with a resolution of ~10 cM. The genotyping of markers was carried out as described [13].

Markers for fine mapping were selected from the Marshfield Medical Genetics database (<http://research.marshfieldclinic.org/genetics/>) and genotyped as described above.

Pairwise logarithm of the odds (LOD) scores were calculated with the Linkage Package 5.2 program assuming an autosomal dominant model, a gene frequency of 0.0001, a penetrance rate of 78% in the family, and an allele frequency of 1/n, where n equals the number of alleles observed. Haplotype was constructed using the Cyrillic program and manual prediction.

Mutational analysis

All exons and exon-intron boundaries of *PRPF31* (GenBank accession no. [AC012314](#)) were amplified by PCR and mutational analysis was carried out using direct DNA sequence analysis. The proband DNA sample was used for initial mutational analysis. PCR products were run on

a 2.0% agarose gel, purified using the QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA), and sequenced with both forward and reverse primers. DNA sequencing analysis was carried out using the BigDye Terminator Cycle Sequencing v3.1 kit (ABI, Foster City, CA).

Restriction fragment length polymorphism (RFLP) analysis was used to determine whether the mutation co-segregated with the disease in the family and whether the mutation was absent in 200 normal controls. The wild type *PRPF31* allele contains an *Hph* I restriction site, whereas mutation IVS1+1G>T disrupts the site. The first exon/intron boundary of the *PRPF31* gene was amplified using PCR primers 5' aacgctagaacagtgtgc 3' (forward primer) and 5' aggttgcaatgagccgagatc 3' (reverse primer). The 222 bp PCR product was digested with 1 unit of *Hph* I (NEB, USA) at 37°C overnight, and the resulting products were separated on a 2.0% agarose gel.

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

Total RNA was extracted from peripheral whole blood samples from individuals III-13, III-15, IV-5, IV-13, IV-15, V-2, V-3, V-6, V-12, V-16 and V-24 (see Fig. 1) using the Trizol reagent (Invitrogen, USA). The RNA samples were treated with DNase I (Promega), and used for RT-PCR analysis with the Titanium One-Step RT-PCR kit (BD Clontech, USA). Primers for RT-PCR were designed to amplify exons 1 to 4 of *PRPF31*. The forward primer is 5'-AGGCCTTGCTTTCTTGTCTAAC-3', and the reverse primer is 5'-TTGGCTTGCTTGCTGATATAC-3'. The RT-PCR products were separated on 2% agarose gels, purified, and sequenced as described above.

Real-time PCR analysis

Real-time PCR was performed by the SYBR Green I assay with a Lightcycler 2.0 Instrument (Roche). The housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as internal control. Primers for *PRPF31* were 5'-AGCGGGGACCAATCAGAGAG-3' and 5'-CCGCGCACCAGTCTTCTAG-3'. For the GAPDH gene, the primers were 5'-CATGAGAAGTATGACAACAGCCT-3' and 5'-AGTCCTTCCACGATACCAAAGT-3'. All samples were run in duplicate. All experiments were repeated at least three times. The level of gene expression was calculated using the $\Delta\Delta C(t)$ method [14].

Results

Identification of a novel splicing mutation IVS1+1G>T in *PRPF31* in a Chinese family linked to RP11

We identified and characterized a large, six-generation, non-consanguineous Chinese family with 77 living members, among whom 21 individuals were affected with RP (Fig. 1A). The inheritance pattern in the family is autosomal dominant. Incomplete penetrance exists in the family as individuals III-2, III-10, III-13, III-15, IV-6, and IV-16 are obligate carriers, but do not exhibit any RP phenotype. The proband (Fig. 1A, V-6) was a 32-year-old female with night blindness since the age of 3 years. Her visual acuity declined gradually in >20 years, with present right and left visual acuity of 15/250. The visual field loss was first noticed at the age of 5 years. Fundus examinations (Fig. 1B, C) revealed advanced RP with heavy bone spicule pigmentation and severe atrophy, wax yellow optic disc and attenuation of retinal blood vessels.

A genome-wide linkage scan was carried out to identify the chromosomal location of the disease-causing gene in the family. Significant linkage was identified with multiple markers on chromosome 19. The results of haplotype analysis are shown in Fig. 1D. Linkage analysis showed that the causative gene in the family was linked to markers *D19S601-D19S605* with a LOD score of 5.03 (data not shown). The *PRPF31* is located between marker *D19S924* and *D19S418* (Fig. 1D), and became the strong candidate gene for RP in the family.

Direct DNA sequence analysis revealed a single nucleotide change (G>T) at the position +1 in intron 1 (IVS1+1G>T) of *PRPF31* in the proband. DNA sequence analysis also identified the mutation in the proband's father, family member IV-2 and other obligate carriers III-13, III-15, V-12 and V-35 (Fig. 2A). The mutation occurs at the highly conserved G residue at the 5' donor site of intron 1, and represents a novel splicing mutation.

RFLP analysis showed that all patients in the family carried both the wild type allele and the mutant allele, whereas normal individuals showed only the wild type allele (Fig. 2B). Similar RFLP analysis showed that the IVS1+1G>T mutation was not present in 200 normal control individuals (data not shown). Together, these results provide strong genetic evidence that the IVS1+1G>T mutation of *PRPF31* gene causes adRP in the family.

Functional characterization of the IVS1+1G>T mutation using RT-PCR analysis

To determine whether the IVS1+1G>T splicing mutation has any effect on mRNA splicing, we performed RT-PCR analysis for *PRPF31* using total RNA samples isolated from peripheral blood samples from the proband (V-6), six other patients (IV-5, IV-13, IV-15, V-2, V-16, V-24), three carriers (III-13, III-15, V-12), one unaffected family member (V-3), and seven normal controls not related to the family (Fig. 1).

As shown in Fig. 2C, RT-PCR yielded a 649-bp product from a normal control sample as expected with normal splicing. One 606-bp fragment and another short 360-bp product were amplified from the sample from affected individuals. DNA sequence analysis showed that the 606-bp and 360-bp fragments from the patients resulted from alternative splicing that led to a fusion of truncated exon 1 to exon 2 (isoform 1) or a fusion of truncated exon 1 to exon 4 (isoform 2), respectively (Fig. 2D). Interestingly, in normal samples we found another novel splicing isoform that fuses the truncated exon 1 to the middle of exon 4 (deletion of 42 bp fragment at the 5'-terminus) (isoform 3, 318 bp) (Fig. 2D). The various alternative splicing types are shown in Fig. 2E.

We estimated the stability of various mRNA structures resulted from alternative splicing in patients and normal individuals by RNA secondary structure prediction (www.genebee.insu.su/services/rnaz-reduced.html) (Fig. 3A). The wild type mRNA (Fig. 3Aa) from normal individuals and patients, splicing isoforms 1 and 2 (Fig. 3Ab, 3Ad) from patients are predicted to be stable with negative free energy (−550.7 kkal/mol, −540.7 kkal/mol and −482.8 kkal/mol, respectively), but isoform 3 (Fig. 3Ac) from normal individuals may be unstable with positive free energy (0.6 kkal/mol).

We analyzed the expression level of the *PRPF31* gene in the patients, asymptomatic mutation carriers, and a normal member in the adRP family using real-time PCR. Real-time PCR was performed for the wild type form of splicing products only. The reverse primer is located within the 43 bp region deleted in the other forms of splicing products, isoforms 1, 2, and 3, which makes the product of real-time PCR specific to the wild type splicing form. As shown in Fig. 3B and Table 1, the expression level of *PRPF31* mRNA was reduced by 57% in patients compared with normal individuals ($p=0.0005$) and by 28% in asymptomatic carriers ($p=0.014$).

Discussion

RP11 has been reported as the second most common locus for adRP [15]. *PRPF31* mutations were identified in Spanish, Chinese, Japanese, and British families with adRP as summarized in Fig. 3C [2,3,9,10]. In this study, we studied a large Chinese family with adRP. A genome-wide scan linked the family to RP11 on chromosome 19q13.4 with a significant LOD score of 5.03. Mutational analysis identified a novel splicing mutation (IVS1+1G>T) in *PRPF31* in the family. The mutation co-segregated with 13 RP patients in this family, but not with 18

unaffected family members and 200 controls. These results supports the conclusion that IVS1+1G>T may be the cause of adRP in this family. One of the most prominent features of RP11 is that it shows “all or none” form of incomplete penetrance [9]. Mutation carriers usually present with either fully symptomatic phenotype or completely asymptomatic phenotype. In the Chinese RP family, we found that 4 mutation carriers did not exhibit any phenotype of RP. This finding is also consistent with our linkage results that the family is linked to RP11.

The IVS1+1G>T mutation is located in intron 1 and close to exon 1, which encodes the 5'-UTR of *PRPF31*. RT-PCR results showed that the mutation was associated with reduction of *PRPF31* mRNA expression by 57% in patients and by 28% in asymptomatic carriers (Fig. 4B). These results suggest that the peculiar, “all or none” phenotypic manifestation of *PRPF31* mutations may be linked to the expression level of *PRPF31*. Rivolta et al. studied the effect of five mutations on the expression levels of the *PRPF31* gene, and found that asymptomatic carriers had *PRPF31* mRNA levels similar to normal control individuals [10]. A similar finding was also made in lymphoblasts from a large RP11 family with an 11-bp deletion in exon 11 [16]. Together, these results may provide an important clue to understanding of the molecular mechanism explaining the phenotypic variability among RP11 patients.

In summary, we have studied a large Chinese family with adRP and identified a novel splicing mutation responsible for RP in the family. Further analysis suggests that the expression level of the *PRPF31* gene is a potential determining factor for the peculiar “all or none” form of incomplete penetrance associated with RP11.

Acknowledgments

We are grateful to the family members for their participation in this study. This work was supported by the Chinese Ministry of Science and Technology National High Technology “863” Program of China No. 2006AA02Z476 (to Q.K.W.), the Chinese National Natural Science Foundation grants No. 30670736 (to J.Y.L.) and No.30671158 (to S.).

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Fig. 1. (A) Pedigree structure of a large Chinese family with adRP. (B) Fundus photographs from the proband left eye (left) and right eye (right). Typical RP appearance of the fundus can be seen. (C) Fundus photograph and fluorescein angiograms from individual V-12 in the family. No sign of RP was observed. (D) Haplotype analysis of the large Chinese family with markers at the RP11 locus. Subjects were genotyped for 8 markers within and surrounding the *PRPF31* gene.

Fig. 2. Identification of a novel splicing mutation in the *PRPF31* gene. (A) A single nucleotide change (G>T) at the position +1 of intron 1 was identified in the Chinese family. Left, mutation; right, normal. (B) Mutation IVS1+1G>T co-segregates with patients in the family. M, molecular weight marker; N, normal individual; P, affected individual (patient). C, asymptomatic mutation carrier. The IVS1+1G>T mutation generates an *Hph* I restriction site. All patients and carriers are heterozygous for the wild type allele, identified by a 177-bp fragment, and for the mutated allele characterized by two fragments of 222-bp and 45-bp (not detectable in this gel). (C) Representative results from RT-PCR are shown for the proband (P) and one normal family member. (D) DNA sequence analysis of wild type and the truncated transcripts from the proband and normal family member: isoform 1 (606 bp); isoform 2 (360 bp); isoform 3 (318 bp). (E) Schematic representation of wild type and three alternative splicing isoforms.

Fig. 3. (A) The predicted structure and estimated free energy for different isoforms of *PRPF31* mRNA: a, wild type; b, isoform 1; c, isoform 2; d, isoform 3. (B) Real-time PCR analysis for expression of the wild type splicing isoform of *PRPF31* mRNA: P, patient with the *PRPF31* mutation; C, carrier with the *PRPF31* mutation; N, normal family member without the *PRPF31* mutation. (C) The schematic diagram showing the location of the *PRPF31* mutation in relation to other RP11 mutations: black histograms and numbers are exons of the *PRPF31* gene; gray histograms, UTR regions; asterisk, the novel splicing mutation identified in this study.

Table 1
The expression level of *PRPF31* mRNA as measured with real-time PCR*

P (n=7)	C (n=3)	N (n=8)	Ratio P/N	Ratio P/C	Ratio C/N	p-value Pvs.N ^a	p-value Pvs.C ^a	p-value Cvs.N ^a
43±3	72±2	100±6	0.43	0.60	0.72	0.0005	0.014	0.034

* Values were normalized so that the average expression level for each gene in control individuals is 100. The results are shown as mean ± standard error of the mean.
^a p-values were computed with the *t*-test.
P, symptomatic patients; C, asymptomatic carriers; N, normal individuals.