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# Whole exome sequencing identifies a novel *NRL* mutation in a Chinese family with autosomal dominant retinitis pigmentosa

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**Purpose:** To investigate the genetic basis and its relationship to the clinical manifestations in a four generation Chinese family with autosomal dominant retinitis pigmentosa.
**Methods:** Ophthalmologic examinations including fundus photography, fundus autofluorescence imaging, fundus fluorescein angiography, optical coherence tomography, and a best-corrected visual acuity test were performed to define the clinical features of the patients. We extracted the genomic DNA from peripheral blood samples. The proband's genomic DNA was submitted to the whole exome sequencing.
**Results:** Whole exome sequencing and the subsequent data analysis detected six candidate mutations in the proband of this pedigree. The novel c.146 C>T mutation in *NRL* was found to be the only mutation that co-segregated with the disease in this pedigree. This mutation resulted in a substitution of proline by a leucine at position 49 of NRL protein (p.P49L). Most importantly, the proline residue at position 49 of NRL is highly conserved from zebrafish to humans. The c.146 C>T mutation was not observed in 200 control individuals. What's more, we performed the luciferase activity assay to prove that this mutation we detected alters the NRL protein function.
**Conclusions:** The c.146 C>T mutation in *NRL* gene causes autosomal dominant retinitis pigmentosa for this family. Our finding not only expands the mutation spectrum of NRL, but also demonstrates that whole-exome sequencing is a powerful strategy to detect causative genes and mutations in RP patients. This technique may provide a precise diagnosis for rare heterogeneous monogenic disorders such as RP.

Retinitis pigmentosa (RP; OMIM 268000) is a major cause of adult blindness, and it is one of the most common forms of inherited retinal degeneration. The prevalence of RP is about 1 in 4000 people worldwide [1]. Three common modes of inheritance including autosomal dominant (adRP), autosomal recessive (arRP) and X-linked (XLRP) have been reported. Additionally, RP can also be inherited in the rare mitochondrial or digenic form [2,3]. With all of these possible modes of disease transmission, it is has becoming increasingly important to better define the etiology of this disease for genetic counseling.

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Previous genetic studies show that RP is a heterogeneous set of inherited retinopathies with many disease causing genes, many known mutations and highly varied clinical consequences [4]. Most patients, they develop night blindness and are unable to adapt to darkness in the early stage. Then, it progresses from peripheral to central vision loss, and finally tunnel vision or blindness with attenuation of the retinal vessels [5]. RP patients may show some considerable symptoms overlap with the other types of inherited disease. And the variations among the individuals sharing the same mutations have been discovered in the RP patients.

RP can occur alone or as a part of a more complex syndrome. Even for the nonsyndromic RP, the genetic and clinic features are strikingly complicated [6]. We take the autosomal dominant RP we studied here as an example to illustrate it. The nonsyndromic RP encompasses 65% of

all RP cases, and adRP accounts for about 30% of the total number of nonsyndromic RP cases. According to the RetNet, 26 genes including NRL, Rho, BEST1, Crx and SEMA4 have been identified as the causative genes of adRP. Although many RP causative genes and mutations have been identified over the past decades, the pathogenic mutations in about half of the RP patients still remain unclear, indicating that there are still many RP linked genes waiting to be found.

Next generation sequencing has becoming a powerful strategy to apply to RP. Although the human genome has been completely sequenced, but only less than 10% of it has been well defined. Consequently with whole exome sequencing, one can focus on the coding regions or exons of known genes, which contain 85% of disease-causing mutations. It also includes many disease-predisposing SNPs throughout the genome [7]. These features make it a practicable tool for us to conduct genetic analyses effectively, and besides that it has a lower cost.

In this study, we investigated a four-generation Chinese pedigree with autosomal dominant RP. We performed the whole exome sequencing and identified a novel c.146C>T mutation in *NRL*. This mutation co-segregated with the disease in the pedigree and was not detected in the 200 normal controls. Our work may provide valuable information to make a genetic diagnosis in patients and be able to study the pathogenesis of RP caused by *NRL* mutations.

## METHODS

*Subjects and DNA preparation:* A four-generation Chinese pedigree in Hubei province was investigated in this study. The proband and other members of this pedigree received ophthalmologic examinations including visual acuity testing, Goldmann visual field test, fundus photography, full-field electroretinogram (FERG), optical coherence tomography (OCT) and fundus autofluorescence (FA) at the Union Hospital affiliated to Tongji Medical College, Huazhong University of Science and Technology. After written informed consent was obtained from the participants of the family members, peripheral blood samples from all of the participants were taken, and the genomic DNA was extracted following the manufacturer's description.

*Whole-exome sequencing:* To construct the library, the samples were randomly fragmented into 150–200 bp, end repaired, and then ligated with specific adaptors at both ends. The extracted DNA was amplified by ligation-mediated polymerase chain reaction (LM-PCR), purified and hybridized to the Agilent SureSelect Human All Exon Kit 51 (v4; Agilent Technologies, Santa Clara, CA) for enrichment, and the non-hybridized fragments were washed out. The captured LM-PCR products were subjected to quantitative PCR to estimate the magnitude of enrichment. The captured library was loaded on the Illumina Hiseq 2000 platform (Illumina Inc., San Diego, CA) and subjected to next generation sequencing (NGS). The read length of the sequencing was 90bp and the read depth was 50×.

*Whole exome data analysis:* The sequences were aligned to the human reference genome (hg19) with BWA [8]. GATK was used to conduct the base quality score recalibration, insertion/deletion (indel) realignment, duplicate removal. Additionally, standard hard filtering parameters were used to perform SNP and INDEL discovery and genotyping [9]. ANNOVAR was used to annotate the variation [10]. Nonsynonymous variants (NSVs) including single nucleotide variants (SNVs) splicing sites and indel variants were selected and filtered by the dbSNP137 database. The genes listed on the RetNet have been proved to be associated with the retinal diseases. Furthermore, we chose the variants which could be mapped at genes that listed on the RetNet to perform segregation analysis first, and then the rest of the variants.

*Segregation analysis:* Genomic sequences related to the candidate mutations were downloaded from the UCSC genome browser. The Primer3 online tool was used to design the specific primers used for PCR amplification and direct sequencing of the surrounding region of each selected variant. PCR products were examined by agarose gel electrophoresis and subjected to Sanger sequencing. All the participants of the family were included in the segregation test.

*Dual luciferase reporter assays:* Site-direct mutagenesis was performed to create the NRL (P49L) mutant. The WT and P49L mutant NRL cDNA were cloned into the p3X-FLAG-CMV-7.1 vector. The Rho promoter (pGL3-Rho) containing the NRL-responsive element (NRE) was amplified using the primers containing two restriction sites (Mlu I and Hind III) in the end of both sides. Then we sub-cloned the PCR products into pGL3-basic (Promega) vectors and verified it by direct DNA sequencing. WT and p.P49L mutant NRL plasmids were transiently co-transfected with the pGL3-Rho promoter plasmid into the human embryonic kidney 293 (HEK-293) cells.. The pRL-TK vector containing renilla luciferase (Promega) was also co-transfected into the cells as internal reference. Cells were cultured for 36 h after transfection and then harvested for the luciferase activity assay. The relative luciferase activity was normalized with the activity of pRL-TK renilla.

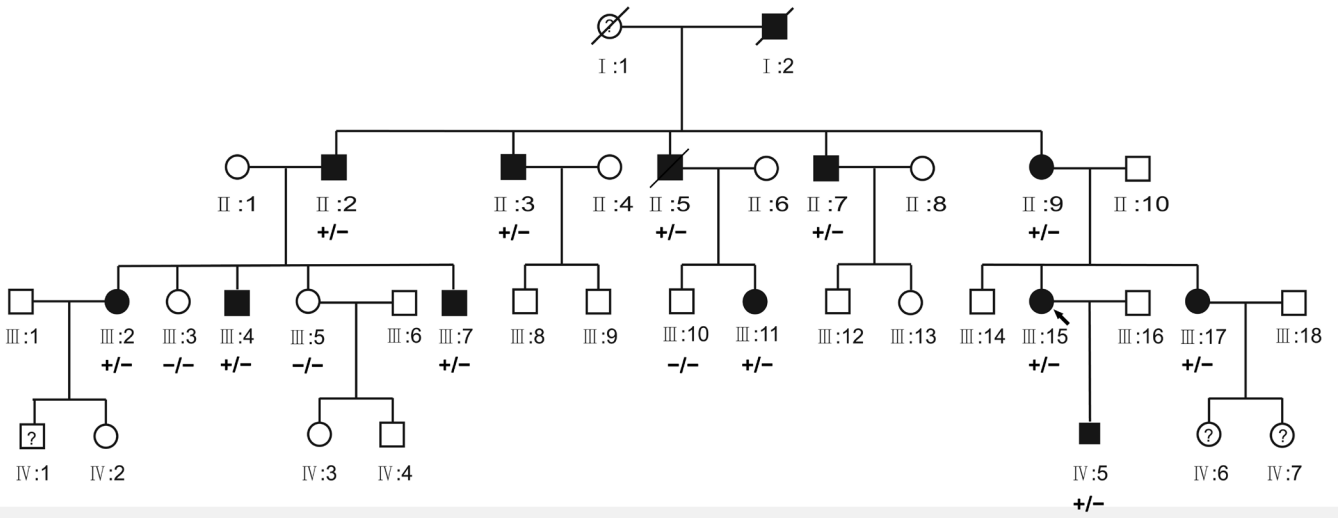



Figure 1. Pedigree structure of the Chinese family with autosomal dominant retinitis pigmentosa (adRP). The squares and cycles represent males and females respectively. Filled symbols represent affected patients while the empty symbols represent unaffected individuals. Wild-type (−/−), heterozygous (+/−) carrier of the mutation. The proband is indicated by arrow. The symbols with a question mark represent the individuals with unknown phenotype.

## RESULTS

*Clinical examinations and pedigree analysis:* In this study, we characterized a four-generation non-consanguineous Chinese pedigree (Figure 1). According to the family members description, I:2 became completely blind in his 30s. There were 11 members in this pedigree had visual problems, and 10 of them participated in our study. Additionally, our study also enrolled 4 normal controls from this pedigree. All of them donated their peripheral blood samples. Six affected members and 4 normal members had ophthalmologic examinations; the detailed clinical features were summarized in Table 1.

All the affected members of this pedigree showed typical RP symptoms. Although RP is clinical heterogeneous disease, the clinical features between different patients in this pedigree were similar to each other. We took the patient II:3 as an example to display some symptoms clinically findings of this pedigree. The fundus photography revealed that the patients had typical RP symptoms including attenuated retinal arterioles, peripheral intra-retinal pigment deposits in a bone-spicule configuration, waxy optic disc, macular degeneration and diffuse mottling of retinal pigmented epithelium (Figure 2B). The OCT images showed that the patients got marked thinning and disruption of the photoreceptor layer and the retinitis pigment epithelium (RPE; Figure 2D). Based on the family history and the clinical examinations, we concluded that the patients inherited retinitis pigmentosa from their parents in an autosomal dominant pattern.

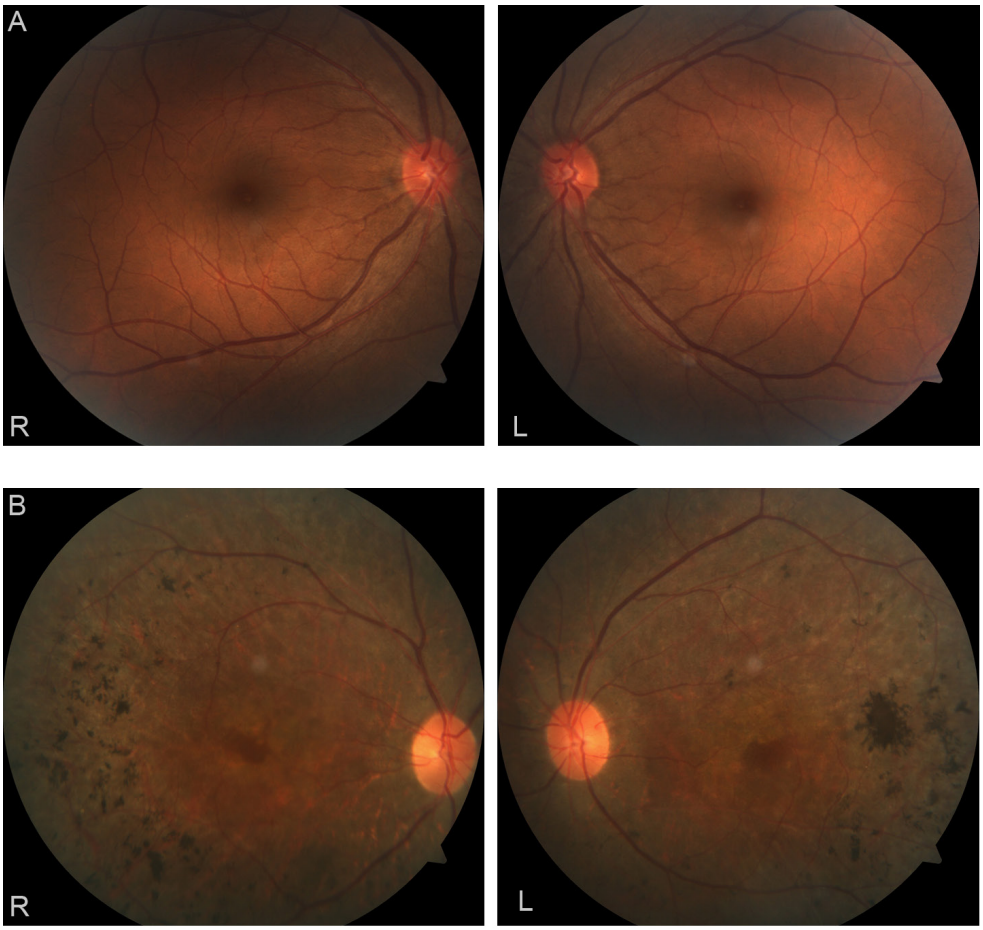
*Whole exome sequencing:* Whole exome sequencing was selectively performed on the proband. We obtained 4.35 billion bases of sequence as paired-end 90-bp reads from the whole exome sequencing. After mapping to the human reference genome (hg19), we achieved 2.3G base mapped to the targeted exome region with a mean depth of 44.41×. On average, 85.89% of the exome was covered at least 10×. After conducting the base quality score recalibration, indel realignment, duplicate removal, SNP and INDEL discovery and genotyping, we detected 86,078 variants. Based on the annotation from the ANNOVAR, we only focused on the 460 candidate nonsynonymous variants (NSVs) including the SNVs, splicing sites and indel variants, which were more likely to be pathogenic. For the next step, we filtered out the variants that already existed in dbSNP137. The genes listed on the RetNet (url:https://sph.uth.edu/retnet/) were the target genes for our research. Finally, we selected 480 candidate variations which could be mapped at genes that listed on the RetNet. Among them, 6 variations were probably the pathogenic gene in this family (Table 2).

*Identification of a novel mutation in NRL in this family:* Six candidate genes including *NRL*, *CERKL*, *TRIM32*, *STL2*, *TREX1* and *BEST1* were detected in this pedigree by our exome sequencing analysis. Among them, the *BEST1* variant (R563C) affects an arginine that is evolutionarily not conserved. The other three genes were associated with complicated syndromes. Mutations in *TREX1* could cause dominant retinal vasculopathy with cerebral leukodystrophy,

**Table 1. Clinical features of the participants in this study.**

| ID | Age (yrs) | Sex | Disease status | Age at onset (yrs) | Visual acuity | Fundus appearance | Color vision | ERG | OCT | Visual field |
|---|---|---|---|---|---|---|---|---|---|---|
| II:2 | 68 | M | Aff | 30s | <0.1/0.2 | PD, AA, MD, POD | BB | Diminished | Thinning PL and RPE; Disrupted choroid | visual field loss |
| II:3 | 63 | M | Aff | 30s | <0.1/0.1 | PD, AA, MD, POD | BB | Diminished | Thinning PL and RPE; Disrupted choroid | visual field loss |
| III:2 | 48 | F | Aff | 20s | <0.1/0.1 | PD, AA, MD, POD | BB | Not examined | Thinning PL and RPE; Disrupted choroid | Not examined |
| III:3 | 43 | F | Un | - | Not examined | No obvious RP symptoms | Normal | Normal | Not examined | Normal |
| III:4 | 40 | M | Aff | 20s | <0.1/0.1 | PD, AA, MD, POD | BB | Not examined | Thinning PL and RPE; Disrupted choroid | visual field loss |
| III:5 | 39 | F | Un | - | Not examined | No obvious RP symptoms | Normal | Normal | Normal | Not examined |
| III:7 | 34 | M | Aff | 10s | LP | PD, AA, MD, POD | BB | Diminished | Thinning PL and RPE; Disrupted choroid | visual field loss |
| III:10 | 26 | M | Un | - | Not examined | No obvious RP symptoms | Normal | Normal | Normal | Normal |
| III:11 | 21 | F | Aff | 20s | Not examined | No obvious RP symptoms | Normal | Diminished rod response, normal cone response | Normal | Normal |
| III:15 | 31 | F | Aff | 20s | <0.2/0.2 | PD, AA, MD, POD | BB | Not examined | Thinning PL and RPE; Disrupted choroid | Not examined |

Abbreviations: M: male; F: female; Aff: affected; Un: unaffected; ERG: electroretinography; LP: light permission; OCT: optical coherence tomography; PD: pigment deposition; AA: attenuated retinal arterioles; MD: macular degeneration; POD: pale optic discs; BB: blue color blindness; PL: photoreceptor layer; RPE: retinal pigment epithelium



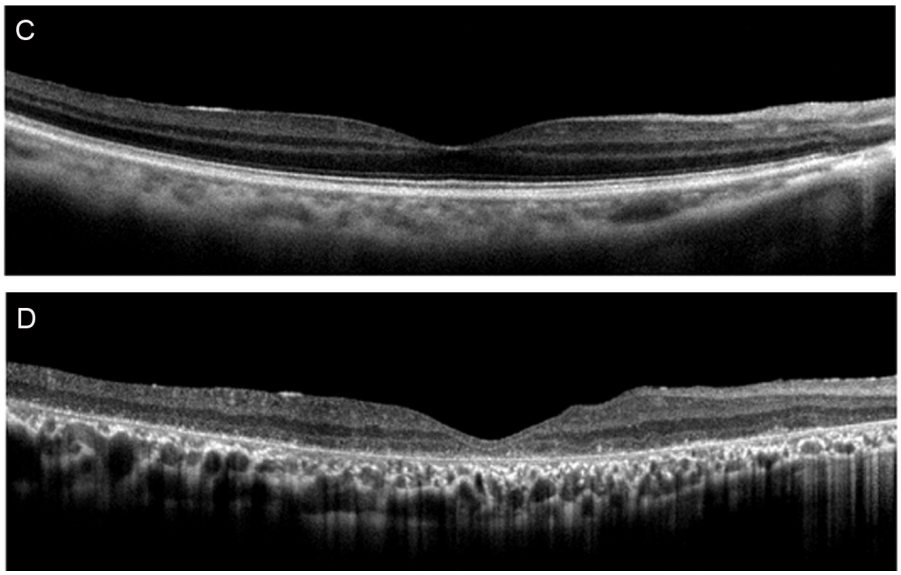




Figure 2. Fundus photography and OCT test of normal and affected member of this pedigree. Fundus photograph of the normal individual III:10 (**A**) and the patient II:3 (**B**) revealed that the patients had typical RP symptoms including attenuated retinal arterioles, peripheral intraretinal pigment deposits in a bone-spicule configuration, degenerated macula and diffused mottling of retinal pigmented epithelium. Optical coherence tomography (OCT) images of the normal individual III:10 (**C**) and the patient II:3 (**D**) showed that the patient had marked thinning and disruption of the photoreceptor layer and the retinitis pigment epithelium.

Table 2. Six candidate mutations detected from the whole exome sequencing.

| Gene | Gene Bank ID | Exon | Nucleotide change | Amino acid change |
|---|---|---|---|---|
| NRL | NM_006177 | 2 | c.146C>T | p.P49L |
| TRIM32 | NM_012210 | 2 | c.284T>C | p.V95A |
| CERKL | NM_ 201548 | 2 | c.346A>G | p.T116A |
| STL2 | NM_001854 | 33 | c.2645G>A | p.R882H |
| TREX1 | NM_016381 | 1 | c.161C>A | p.P54Q |
| BEST1 | NM_001139443 | 9 | c.1687C>T | p.R563C |

dominant Aicardi-Goutiere Syndrome 1 or dominant chilblain lupus. *TRIM32* was a causative gene of recessive Bardet-Biedl Syndrome and recessive limb-girdle muscular dystrophy. Also, mutations in *STL2* could cause dominant Stickler Syndrome type II or dominant Marshall Syndrome. Yet, the clinical expression in this family is non-syndromic RP, suggesting that variations in *NRL* and *CERKL* would probably be pathogenic mutations.

To see if these variations co-segregated with the disease, primers were designed to amplify the sequences that contained the mutation sites (Table 3). DNA samples from participants of the pedigree were subjected to PCR amplification and subsequent Sanger Sequencing. After studying all of the candidate mutations, we identified the novel mutation (c.146C>T: p.P49L) in *NRL*. It was the only mutation that co-segregated with the disease in this family (Figure 3). Furthermore, this mutation was absent in 200 normal controls.

The novel mutation identified in this study results in a substitution of a proline by a leucine at protein position 49 (p.P49L). We performed sequence alignment and identified that the proline residue at position 49 (P49) is highly conserved (Figure 4). The PROVEAN project was used to predict the effect of the amino acid substitution. The predicted PROVEAN score equal to or below the default threshold (−2.5) was considered to be damaging. This mutation was predicted to be deleterious according to the PROVEAN score (−4.427). These results suggested that P49 may be very important for the NRL protein, and the P49L mutation is a candidate pathogenic mutation.

*The p.P49L mutation in NRL increased its ability to transactivate Rho expression:* To investigate whether the mutation we identified affected the NRL protein function, we performed the luciferase activity assay to examine the ability of the p.P49L mutant NRL to transactivate the Rho expression. By compared to the WT NRL, p.P49L mutant showed a statistically significant increase in transactivating the rhodopsin expression (Figure 5). Thus, our results indicated that the novel mutation we identified here affects the protein function and supported the conclusion that the p.P49L mutant in NRL is the pathogenic mutation in this adRP pedigree.

## DISCUSSION

Whole exome sequencing of the proband detected six candidate mutations. All of these genes were subjected to the segregation test. The novel c.146C>T mutation in *NRL* was identified to be the only mutation that co-segregated with the disease in this family. The *NRL* gene which mapped to chromosome 14q11.1–11.2 encodes a highly conserved basic motif leucine zipper transcription factor of the Maf subfamily [11,12].

*NRL* is the third gene that has been linked to adRP and the S50T mutant was the first disease causing mutation

Table 3. Primers used for the identification of the pathogenic mutation in this family.

| Primers | Forward | Reverse | Product (bp) |
|---|---|---|---|
| NRL | CAGGGCACTTGGGCTTTGAG | CCTCTGGGCTCCCTGGGTAGTA | 477 |
| TRIM32 | CCGCATAACCAGCTTGACCC | AGGGAGTGTACAGTGGCCAGG | 208 |
| CERKL | CCCTTTGCTCACTTTCTACCC | GGAACAGAAGGAAACTATCTCAA C | 459 |
| STL2 | GCTGTGTGAAAATCACTCTGTTC | TGCCAGACAAATCCAAAGAC | 584 |
| TREX1 | CGCGGGAGAGTGTGCAGC | GGTGGTGGAGGAACTGTGGGAG | 580 |
| BEST1 | CCTAGAACCATCAGCGCC | TCAAAGTAAGTTGGGGCCAG | 652 |

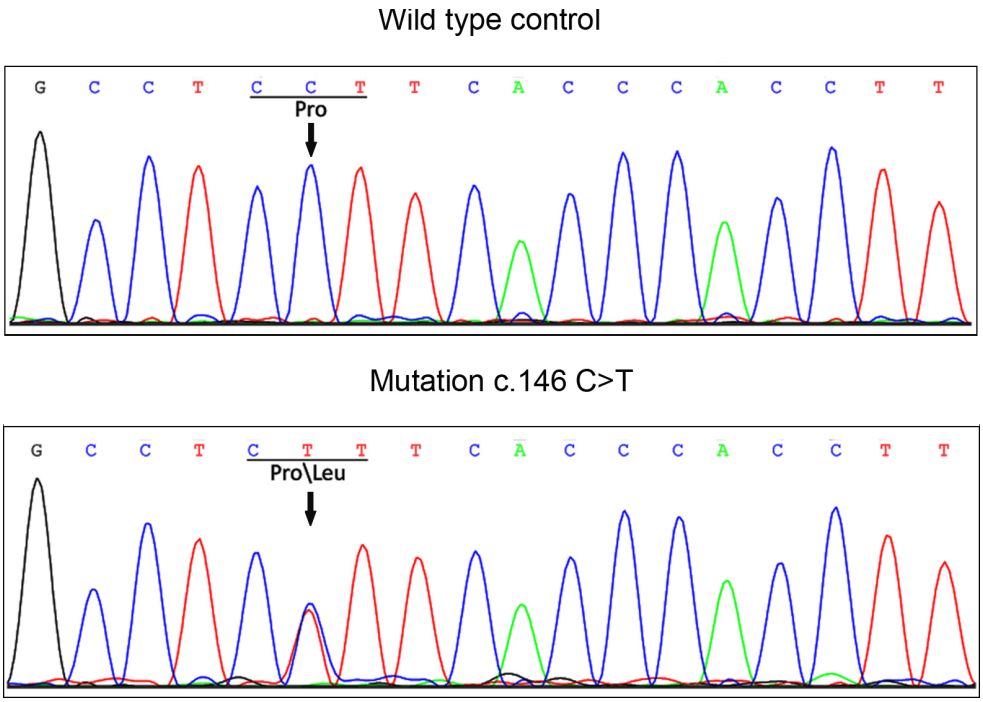



Figure 3. Identification of a novel *NRL* mutation in this pedigree. Conventional sequencing profile around the position c.146 from the normal individual (III:5) and the affected individual (II:7).

identified in *NRL* [13]. There are six pathogenic mutations identified in the highly conserved S50 and P51 site. The compound heterozygous mutations L75fs and L160P were detected in two arRP patients. Other missense mutations including E63K, A76V, S225N, H125Q, L235F, G122E lead to changes in amino acid residues that are not conserved among the members of the Maf protein family. Consequently, they were classified as nonpathogenic variants [14]. The mutation M96T was also detected in an evolutionarily not conserved site. Clinical and genetic studies suggest that this mutation may show an incomplete penetrance or is not a causative mutation of RP [15]. These genetic studies revealed that almost all the identified pathogenic mutations were mapped in the S50 and P51 site. Thus, the S50 and P51 site seemed to be a hot spot in *NRL*.

The novel mutation (c.146 C>T; p.P49L) in *NRL* gene we identified in this study caused a substitution of a proline by a leucine at protein position 49 (p.P49L). The mutation site P49 was adjacent to the mutation hot spot S50 and P51. Besides that, this mutation mapped to a minimal transactivation domain (MTD, 40–74), which is essential for the protein function [16]. These findings suggested that the novel mutation we detected in this study was most likely to be pathogenic. And there may be a potential pathogenic mutation hot spot (P49, S50, P51) in the MTD region of *NRL* gene.


Figure 4. The amino acid sequences alignment of NRL in different species. The box indicates the P49 residue is highly conserved during the evolution. The symbol * is used to represent that this position is conserved, while the symbol: is used to represent the less conserved position.

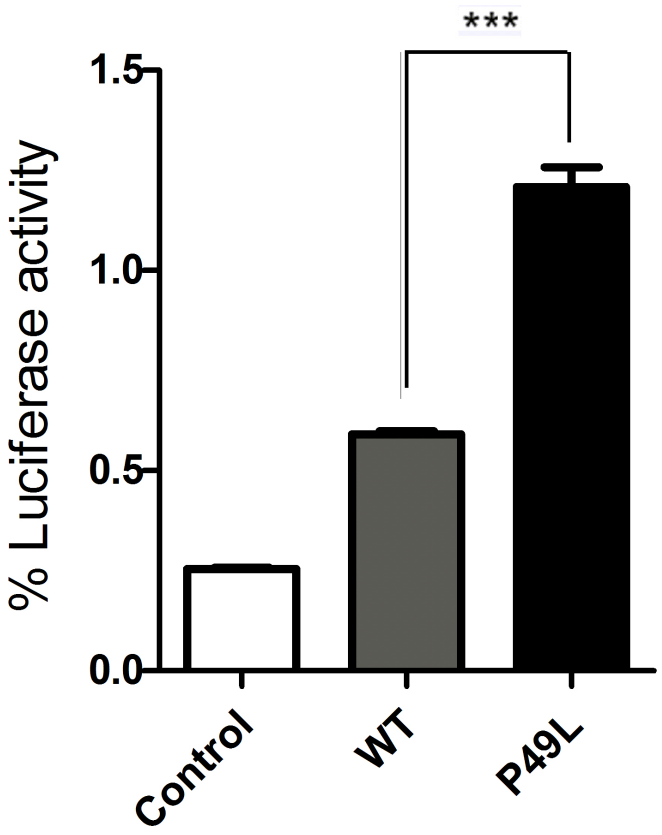



Figure 5. The p.P49L mutation in NRL increased its ability to transactivate Rho expression. WT or p.P49L mutant NRL expression constructs were co-transfected into HEK293 cells with pGL3-Rho-promoter and pRL-TK plasmids. The empty p3X-FLAG-CMV-7.1 expression vector was used as a negative control. The luciferase activities of whole cell lysates were measured. Data are the average of three replicate experiments and error bars show +/−1.00 SD. The asterisk indicates a significant difference.

Dysfunction and loss of rod photoreceptors followed by the cones has been thought to be the main cause of RP. The *NRL* gene we investigated in this study is specifically localized to the rod photoreceptor and acts as a vital regulator in the rod photoreceptor development [17]. In the developing retina, *NRL* is transactivated by Crx. Then, coordinately with Crx, NRL can positively regulate many rod photoreceptor specific genes' expression including *RHO*, *Nr2e3p* [18,19]. *NRL* knockout mouse models resulted in complete transformation of rods to functional cones. Furthermore, expression of *NRL* in the post mitotic photoreceptors leads to a functional retina with only rod photoreceptors [20]. Interestingly, in the developing retina NRL, Crx and Nr2e3 can synergistically function as a transcription factor network to regulate the development and maintenance of photoreceptors [21]. These studies might define the molecular mechanisms as to how the mutations in *NRL* contribute to the development of retinitis pigmentosa.

RP is such a complicated disease, which encompasses many different diseases with many distinct causes, and diverse biologic pathways with overlapping symptoms have similar clinical consequences [6]. Subsequently, it is challenging for the RP patients to get a precise diagnosis. For instance, patient III:11 is a 21 year old female and she developed retinoblastoma and had a surgical intervention to her left eye. Yet, she was determined not to be affected. Fundus photography of her right eye seems to be normal to date. However, the genetic diagnostics showed that she inherit the pathogenic c.146C>T mutation. Based on this result, she was referred for further clinical exams. The ERG test revealed that the rods responses were gone while the cone responses were not affected. These findings indicate that she should be diagnosed with RP based on the genetic diagnosis. The clinical features showed that she was at an early stage of the disease and referred urgently for medical intervention.

In conclusion, we have identified a novel mutation in *NRL* gene by using the whole exome sequencing. The novel c.146C>T mutation in *NRL* was identified to be the only mutation that is co-segregated with the disease in this family. What's more, we also proved the novel c.146C>T mutation in *NRL* increased protein's ability to transcactivate the expression of the Rho. These results suggested the c.146C>T mutation in *NRL* gene was the pathogenic mutation in this pedigree.

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