A Novel Nonsense Mutation in *ARR4* Leads to X-linked High Myopia: A Genetic Paradox

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Abstract

ARR4 has been associated with X-linked, female-limited, high myopia. However, using exome sequencing (ES) in a Southern Chinese family, we identified the first hemizygous high myopia case in a male patient. A novel truncated mutation (*ARR4*: c.569C>G, p.S190*), which co-segregated with the disease phenotype in affected members, was identified in this family. Because the proband's father was a hemizygote for the *ARR4* variant, the present study demonstrated a case where high myopia caused by *ARR4* is not X-linked, female-limited. This implied that a complicated X-linked inheritance pattern may exist for *ARR4*. Thus, the results of this study expanded the variant spectrum in *ARR4* and provided additional information for genetic counseling, prenatal testing and diagnosis. Moreover, we characterized the pathogenic role of *ARR4* c.569C>G (p.S190*) and demonstrated that the mutant protein accumulated under ER stress and was degraded by the proteasome.

Introduction

Myopia occurs when the axial length of the eyes is too long, typically, causing distant objects to be focused in front of the retina(Young, 2009). High myopia (HM) is a common human genetic sensory defect characterized by reduced vision, retinal degeneration and choroidal atrophy. Its global prevalence exceeds 2.9%(Holden et al., 2016; Saw, 2003). HM is defined by diopters (D) greater than -6.0 or an axial length greater than 26 mm(Young, Metlapally, & Shay, 2007).

Both environmental and genetic factors can influence the severity of myopia(Morgan, Ohno-Matsui, & Saw, 2012; Saw, Katz, Schein, Chew, & Chan, 1996), with some family studies showing that genetic factors play a crucial role in the development of HM(Cai et al., 2019; Young et al., 2007). *ARR4* (RefSeq NM_004312.3, see Materials and Methods, "Nomenclature") gene is associated with X-linked, female-limited HM because in the three families examined all affected patients were female, it has recently been reported that the *ARR4* gene is associated with X-linked, female-limited HM(Xiao, Li, Jia, Guo, & Zhang, 2016) . Even though several male family members were hemizygous, they did not possess the phenotypic features of HM thereby proving to be the second disease, after those associated with *PCDH19* , with this unusual inheritance pattern(Depienne et al., 2009).

Accumulation of misfolded and mutant proteins disrupts cellular homeostasis, which will lead to endoplasmic reticulum (ER) stress(Hiramatsu, Chiang, Kurt, Sigurdson, & Lin, 2015). ER stress causes the activation

of the unfolded protein response (UPR), which restores the homeostasis of the ER and protects cells from further damage(Walter & Ron, 2011). However, the activation of UPR hinders protein translation, and chronic stress response will cause cell apoptosis(Mendes, van der Spuy, Chapple, & Cheetham, 2005). ER stress and UPR are found to be associated with the development of various ocular diseases(Kroeger, Chiang, Felden, Nguyen, & Lin, 2019), including diabetic retinopathy(Fu et al., 2012; Li, Wang, Yu, Wang, & Zhang, 2009; Oshitari, Yoshida-Hata, & Yamamoto, 2011; Tang et al., 2011; Yan et al., 2012), glaucoma(Zode et al., 2011; Zode et al., 2014), retinal pigmentary degeneration(Lobo, Au, Kiser, & Hagstrom, 2016), and retinal dystrophy(W. C. Chiang et al., 2017; Kohl et al., 2015; Skorczyk-Werner et al., 2017) Ultimately, while protein destabilization will produce misfolded proteins and protein accumulation, proteasome and lysosomal degradation pathways play an important role in the elimination of misfolded proteins(Balchin, Hayer-Hartl, & Hartl, 2016). Previous studies have found that the UPR and cell stress caused by the pathogenic variation of Arrestin-1 are the causes of photoreceptor death (Vishnivetskiy et al., 2018) ◦

In this study, a member of a Chinese family was referred for HM. While funduscopy results were suggestive of a case of HM, exome sequencing (ES) revealed that an *ARR4* variant which submitted to the LOVD was responsible for the phenotype. This finding is in stark contrast to previous research that has suggested that *ARR4* variants associated with HM are present only in females(Xiao et al., 2016). In the family included in this study, one hemizygote male was affected, implying that *ARR4* has more complicated hereditary patterns than initially thought. These results showed that the nonsense mutation of*ARR4* gene led to ER stress and UPR, and then induced cell stress which may be the cause of photoreceptor apoptosis.

Materials and Methods

Nomenclature

The nomenclature of arrestin protein and gene is very confusing. In fact, the nomenclature of arrestin should be as follows: arrestin-1 (historic names S-antigen, 48 kDa protein, visual or rod arrestin), arrestin-2 (β-arrestin or β-arrestin1), arrestin-3 (β-arrestin2 or hTHY-ARRX), and arrestin-4 (cone or X-arrestin; for unclear reasons its gene is called "arrestin 3" in the HUGO database)(Zhan, Perez, Gimenez, Vishnivetskiy, & Gurevich, 2014). We would like to use*ARR4* (OMIM: 301770) to avoid the confusion of nomenclature, because this can be referred back to numerous previous research results, so as to have continuity in research outputs of this field.

Human subjects

This study included a family comprising 14 individuals. Of the eight members that were affected, one patient was male. All patients had HM with refractive errors greater than -6.0 D. Participants were recruited at the Division of Medical Genetics at Liuzhou Municipal Maternity and Child Healthcare Hospital, Liuzhou, Guangxi, China. The study was approved by the Ethical Review Board of the Medical Faculty of the Liuzhou Municipal Maternity and Child Healthcare Hospital.

Exome sequencing, variant calling and Sanger sequencing

Exomes sequencing (ES) was performed on the female proband and her parents in order to identify the causative mutation. The ES protocol achieved a mean coverage of 200× over 98% of the targeted regions. A minimum coverage of 20× was the standard (AmCare Genomics Laboratory, Guangdong, China) in the regions that contained 5,177 disease candidate genes. The Speedseq Toolkit was used for the data analysis by: 1) performing the human genome alignment with BWA-MEM, 2) marking duplicates using SAMBLASTER, 3) sorting and performing indexing of BAM files with Sambamba, and 4) executing variant calling using FreeBayes. Speedseq is an ultra-fast personal genome/exome analysis toolkit for next generation sequencing(C. Chiang et al., 2015). It takes roughly 2 hours to complete the analysis of one ES sample by using 7 core 14 threads of the CPU. To quickly and accurately detect pathogenic variants, we used EXOMISER to help narrow down the potential candidate variants. Variants were prioritized according to pathogenicity, quality, inheritance pattern, model organism phenotype data and phenotypes known to be associated with disease genes in humans(Smedley et al., 2015). Sanger sequencing was used to determine the

segregation across all family members. The *ARR4* c.569C>G (p.S190*) primers for PCR amplification were: 5'-CCATAGGAAAACAGGCATCAGG-3' (forward) and 5'-CCTTGTTGGTGCAGTTGTTG-3' (reverse).

Cell culture and transfection

The APRE19 human retinal pigment epithelial cell line was used in the study. DAPI staining was performed to detect whether cells were contaminated with mycoplasma. Cell culture was performed using DMEM containing 10% fetal calf serum at 37°C with 5% CO_2.

Real-time quantitative PCR, proteasome degradation pathway and ER-stress analysis

For RT-PCR experiments, total RNA was extracted from APRE19 cells using an RNA kit (Thermo GeneJET RNA Purification Kit, Thermo scientific), and then reverse-transcribed into cDNAs (RevertAid^{TM} First Strand cDNA Synthesis Kit, Thermo Scientific). The real-time primer pairs upstream of the mutation site were 5'-ACAAGCTAGGGGACAATGCC-3 'and 5'-AACCAGCCGCACATAGTCTC-3', while the downstream mutation site primers were 5'-ACAAAGAGCTGCTGGGGATC-3 'and 5'-CTAGCGGCCTCATGAGATGG-3'.

After transfection of the wild-type and mutant recombinant vectors for 48 hours, a total protein extraction was performed and the differences in the expression of ER-stress markers detected. Similarly, 48 hours after transfection of wild-type and mutant recombinant vectors, c.569C>G (p.S190*) transfected cells were pretreated with 0 μM, 25 μM, and 50 μM of the MG132 proteasome inhibitor (Sigma-Aldrich) for 24 hours. Normal APRE19 cells served as the control, total proteins were extracted and the change in ARR4 content measured. Target proteins were detected according to the following procedures. First, the harvested cells were washed with PBS and lysed with a 63 mM Tris HCl, 10% glycerol, and 2% SDS lysis buffer. The extracted proteins were subjected to 10% SDS-PAGE electrophoresis and then transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA). Second, the membrane was washed with Tris buffer, blocked using 5% skim milk powder in 0.1% Tween-20, and then incubated with the primary and secondary antibodies according to the manufacturer's instruction. Finally, an enhanced chemiluminescence blotting detection kit (GE Healthcare, Buckinghamshire, UK) was used to detect protein bands.

Statistical analysis

Three independent experiments were conducted, and the data were presented as mean ± standard deviation (SD). A Tukey's or Dunnett's test was used for one-way analysis of variance. GraphPad Prism 5 (Graphpad, Inc, San Diego, CA, USA) was used for statistical analysis. $P<0.05$ was considered to be statistically significant.

Results

Clinical features

All patients, notably the only affected male family member, had a history of early-onset HM before the age of 7 years, without other ocular diseases or systemic comorbidities. The clinical description of family members is shown on Table 1. Affected members demonstrated peripapillary atrophy, tigroid appearance and depigmentation changes after fundus photographs examination (Figure 1). The only male patient in the family (II: 1) stated that he had trouble seeing since childhood, and began to wear glasses for myopia until he was 20 years old. His spherical equivalent in the right eye (OD) is -12.00 D and the left (OS) is -11.00 D.

Genetic findings

Three family members (II-1; father, II-2; mother, and III-1; proband) were selected for ES to be performed in order to identify potential variants responsible for HM in this family. ES analysis revealed a novel heterozygous nonsense mutation in*ARR4* (c.569C>G, p.S190*) in the proband (III-1), which was predicted to produce a shorter, unfinished protein product. This mutation was verified by Sanger sequencing and was found to co-segregate with all affected individuals (Figure 2AD, Table 1). According to ACMG guidelines, and because it belongs to PVS1 (a nonsense variant in*ARR4* where a loss of function is one mechanism

of*ARR4*- linked HM), PM2 (absent from controls), and PP1 (co-segregation with HM), the novel *ARR4* mutation was classified as pathogenic.

Stability of the mutant ARR4 protein

The human ARR4 protein is composed of 388 amino acids and contains two arrestin-like domains in the N- and C-terminals (Figure 2B). There is only one peer-reviewed report describing three pathogenic *ARR4* variants. However, it is still unclear how the variants affect protein function or which pathways play a role in regulating the stability of the protein. In order to observe the effect of the identified variant on protein expression, the mutant and wild-type proteins were labeled with tag flags, transfected into APRE19 cells, and cultured for 48 hours. The mRNA expression of the mutant gene was significantly lower than that of the wild type (Figure 3A). The result showed that ARR4 mutants had significantly lower protein expression than their wild-type counterparts (Figure 3B).

The mutant ARR4 protein mediated ER-stress.

To verify whether the accumulation of mutant protein mediated ER-stress occurred, the expression of the relevant protein markers were examined. Notably, in the cells transfected with c.569C>G (p.S190*) mutant plasmids, the expression levels of ER stress markers such as CHOP, XBP1, ATF6, and GRP78 were significantly increased (Figure 4). This suggested that mutant ARR4 is associated with mediating ER-stress.

The mutant ARR4 protein is degraded via the proteasome pathway.

In eukaryotic cells, protein abnormalities which lead to incorrect folding or protein aggregation may be eliminated via the proteasome or lysosome degradation pathways. In order to determine whether abnormal ARR4 proteins could be degraded through those pathways transfected cells were pretreated with either a proteasomal or lysosomal inhibitor. Compared to wild-type ARR4 proteins, the MG-132 proteasome inhibitor could significantly restore mutant ARR4 protein levels (Figure 5A). In contrast, chloroquine, a lysosomal inhibitor, was incapable of restoring mutant protein concentrations (Figure 5B).

Discussion

In the present study, we reported an X-linked myopia case and identified the variant associated with HM in a Southern Chinese family. A heterozygous/hemizygous novel nonsense *ARR4* mutation, c.569C>G (p.S190*), was found to be associated with HM and co-segregated within affected family members. Furthermore, it was found that the unstable mutant ARR4 protein induced an ER stress response and was rapidly degraded via the proteasome degradation pathway. Combining with previous studies, we found that *ARR4* may mediate a disease with pattern of inheritance which is contrary to the conventional genetics.

ES has been shown to be remarkable at detecting variants associated with monogenic disorders. Notably, compared to 21-25% diagnostic rate in proband-only ES, a diagnostic rate of 30-37% was observed among affected individuals when ES was applied to testing trios(Farwell et al., 2015; Valencia et al., 2015). Consequently, trio-based ES analysis was used in this study involving a HM family. Following bioinformatic analysis, the *ARR4* c.569C>G (p.S190*) mutation was identified as the explanation of the proband's phenotype as evidenced by not being present in public databases and a high amino acid conservation. The *ARR4* gene, located on chromosome Xq13.1, has been linked to abundant expression in retinal tissue. To the best of our knowledge, because only three HM families have been identified harboring mutations in *ARR4* worldwide(Xiao et al., 2016), it is easy to omit pathogenic *ARR4* variants due to the lack of clinical associations to this gene. EXOMISER, an exome analysis toolkit, provided an effective way for prioritizing patients' candidate genes based on modified phenotypic databases associated with zebrafish models(Smedley et al., 2015).

As shown in Figure 2B, of the three previously described pathogenic*ARR4* mutations, two were missense mutations, while the remaining variant was a nonsense mutation. When including c.569C>G (p.S190*), domain predictions indicated that all four mutations may affect the arrestin C-/N-terminal domains (Figure 2B). Previous studies have demonstrated that both the C- and N-terminal domains play a critical role in

binding to the receptor molecule(Gurevich & Benovic, 1993; Hirsch, Schubert, Gurevich, & Sigler, 1999). Alignment of multiple ARR4 amino acid sequences have shown that the serine at codon 190 is highly conserved (Figure 2C).

Arrestin is an important family of proteins that can desensitize G-protein coupled receptors (GPCRs). Rhodopsin belongs to a class of GPCRs that can sense external light signals and transmit these to cells that produce vision. In mammals, there are two types of visual arrestins: arrestin-1 (*ARR1* , also called S-antigen[*SAG*] or 48K protein)(Kuhn, Hall, & Wilden, 1984; Pfister et al., 1985; Vishnivetskiy et al., 2018; Wacker, Donoso, Kalsow, Yankeelov, & Organisciak, 1977), and arrestin-4 (*ARR4* , also called cone arrestin [*CAR*], or X-arrestin [*ARRX*])(Craft, Whitmore, & Wiechmann, 1994; Murakami, Yajima, Sakuma, McLaren, & Inana, 1993). After being activated by light, opsins are phosphorylated by rhodopsin, and arrestin-1 and arrestin-4 can bind to phosphorylated opsins to inhibit the transduction of downstream signals(Deming et al., 2015). In murine models, these two vision-inhibiting proteins are required for normal inactivation of rhodopsin in cone cells(Nikonov et al., 2008). Misfolded proteins can be recognized on the endoplasmic reticulum and transported to the proteasome for degradation(Griciuc, Aron, & Ueffing, 2011). With elevated expression of ER-stress-related markers that can be observed, the mutant ARR4 protein was found to mediate an ER stress. Consistent with this finding, it was shown that the mutant proteins were degraded via the proteasome pathway due to the proteasome inhibition effects observed on mutant APRE19 cells.

The pathogenicity of the *ARR4* gene was predicted and its association with HM in female patients were described for the first time in 2016. Consequently, an X-linked, female-limited pattern of inheritance was considered for *ARR4* -associated HM cases; a statement strengthened by the observation that none of the hemizygous male family members were diagnosed with HM(Xiao et al., 2016). However, in our study it was demonstrated that the proband's father was affected with HM and possessed pathogenic *ARR4* mutation. This suggested that the hereditary pattern of the *ARR4* gene may not be X-linked, female-limited. HM is a complex, heterogeneous disease which is influenced by environmental and genetic factors. Occurring in females, an X chromosome is compacted during X-chromosome inactivation, which results in the random silencing of one of the X chromosomes(Galupa & Heard, 2018). X-chromosome inactivation could however not explain the phenotypes observed in this study since a nonsense mutation was identified in an affected male hemizygote, while unaffected male hemizygotes carried of a missense mutation in other families. So we speculate that the mechanism of loss of function caused by null variants may be more detrimental for the *ARR4* gene. Both *Arr1* and*Arr4* genes are expressed in mouse cone cells. If only one of the two genes is knocked out, the recovery time will slow down after being stimulated by saturated strong light, but knockout of both genes will greatly increase the delay of recovery time, which indicates that there may be functional compensation between Arr1 and Arr4 in mice(Nikonov et al., 2008). So it becomes reasonable to question that if there is also a complementary mechanism in human retina, which leads to the occurrence and absence of diseases in different hemizygous individuals? Further proof is needed in the future. In Craniofrontonasal syndrome, the female heterozygote of *EFNB1* gene located on X chromosome is usually patient, but the non-mosaic hemizygous male is usually not affected or has only mild symptoms(Niethamer et al., 2020). Some studies believe that this is a kind of "cellular interaction" caused by the mosaic state of EPHRIN-B1 protein, and the mechanism is not clear(Twigg et al., 2004; Wieacker & Wieland, 2005; Wieland et al., 2004). Polydactyly was found in female heterozygous mice (*Efnb1* +/-), but not in hemizygous males (*Efnb1* -) and homozygous females (*Efnb1* -/-). Meanwhile, six sporadic male patients with the mosaic state were found to be severely affected. Interestingly, different proportion of mosaic mutations of *EFNB1* can be detected in various tissues of them including peripheral blood(Twigg et al., 2013). However, we reviewed the exome sequencing results of high-myopia male patients in this study, and found that the sequencing depth of c.569C>G (p.S190*) site was 183X, and all of them were pathogenic variant G bases, which seemed to be inconsistent with the conclusion in the above study. However, the phenotype of high myopia caused by *ARR4* mutation is limited at the present. So, it's unknown that if *ARR4* mutation is also affected by "cellular interference" like *EFNB1* , resulting in both normal and affected hemizygous males? This is the goal of our research in the future.

Conclusions

In conclusion, a novel *ARR4* nonsense mutation, c.569C>G (p.S190*), was identified in a Southern Chinese family, with the X-linked female-limited *ARR4* hereditary pattern not observed in this study. Combined with previous studies, the high myopia caused by the pathogenic mutation of ARR4 cannot be easily considered as X-linked dominant or X-linked female-limited disease. While it was also found that abnormal ARR4 mediated ER stress and was degraded by the proteasome pathway, our results ultimately expand the *ARR4* variant spectrum and provides additional evidence for reliable genetic counseling and prenatal diagnosis of HM.

Conflict of Interest Statement

The authors declare no competing interests.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Table 1: Description of the clinical characteristics and the *ARR4* genotypes of the family

Sample ID	Sex	Age	Affected status	Age at onset	Genotype	Refractive error(D)	Refractive error(D)
						OD	**OS**
I:2	F	83	A	NA	c.569C>G (p.S190*) / WT	NA	NA
II:1	M	63	A	<7	c.569C>G (p.S190*) (HEMI)	-12.00	-11.00
III:1(P)	F	29	A	<5	c.569C>G (p.S190*) / WT	-10.00	-11.00
III:2	F	27	A	3	c.569C>G (p.S190*) / WT	NA	NA
III:4	F	25	A	3	c.569C>G (p.S190*) / WT	-12.00	-13.00
III:6	F	31	A	<5	c.569C>G (p.S190*) / WT	-17.00	-17.00
III:7	M	22	U		WT	NA	NA
IV:1	F	8	A	3	c.569C>G (p.S190*) / WT	-8.00	-7.50
IV:2	F	6	U		WT	+0.50	+1.00
IV:3	F	5	A	2	c.569C>G (p.S190*) / WT	-7.00	-8.00
IV:4	F	2	U		WT	NA	NA

A, affected; D, diopter; F, female; HEMI, hemizygote; M, male; NA, unavailable; OD, right eye; OS, left eye; P, proband; U, unaffected; WT, wild-type.

Table 2: Description of the clinical characteristics and the *ARR4* genotypes of the family

Algorithm	Score	Prediction
CADD	37	Damaging
DANN	0.997	Damaging
FATHMM_MKL	0.974	Damaging
GERP++	4.69	Conserved
phyloP	5.433	Conserved
phastCons	1.000	Conserved
SiPhy	16.026	Conserved

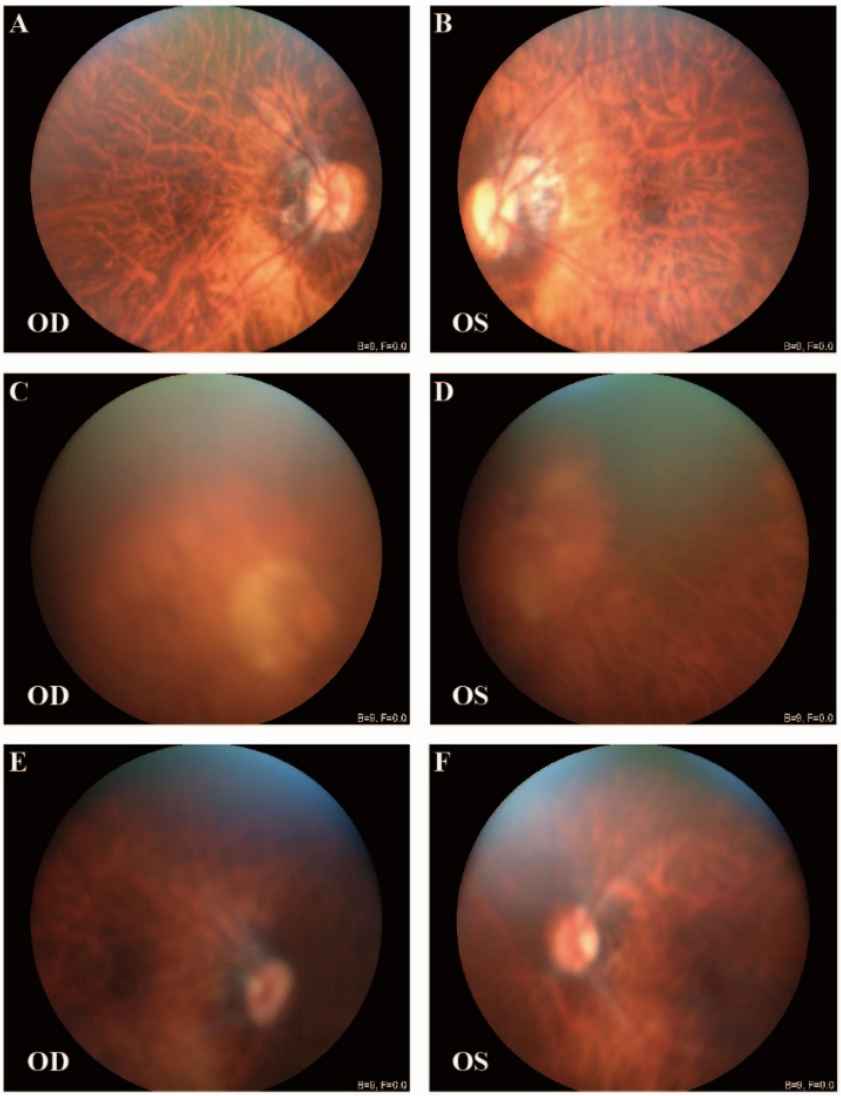

Figure 1: Fundus images of patients from the large family. (III-4: **A, B**): Peripapillary atrophy, tigroid appearance and depigmentation were observed in both eyes. (II-1: **C, D** ; III-1:**E, F**): Tigroid appearance and depigmentation were observed.

OD, right eye; OS, left eye.

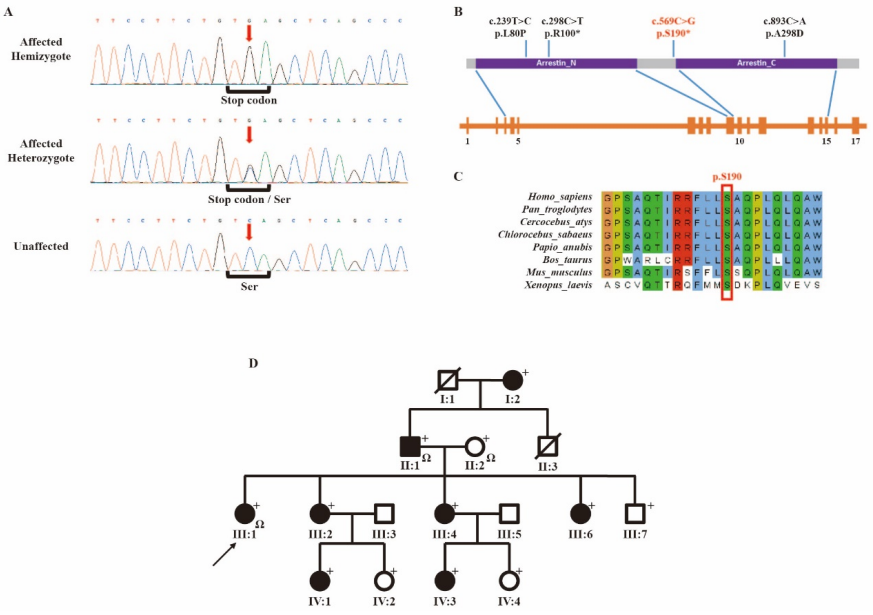

Figure 2: The *ARR4* mutation identified in myopia patients. (A) Sanger sequencing analysis for the mutation identified in the family with high myopia. (B) Location of the pathogenic *ARR4* protein. The protein contains two important functional domains: an arrestin_N domain and an arrestin_C domain. (C) The nonsense*ARR4* c.569C>G (p.S190*) mutation is highly conserved among different species. (D) The pedigree for the family with the c.569C>G (p.S190*) mutation. Square = male, circle = female, dark symbol = affected, arrow = proband, slash = deceased member, 'Ω' = samples used for exome sequencing, '+' = DNA available for this study.

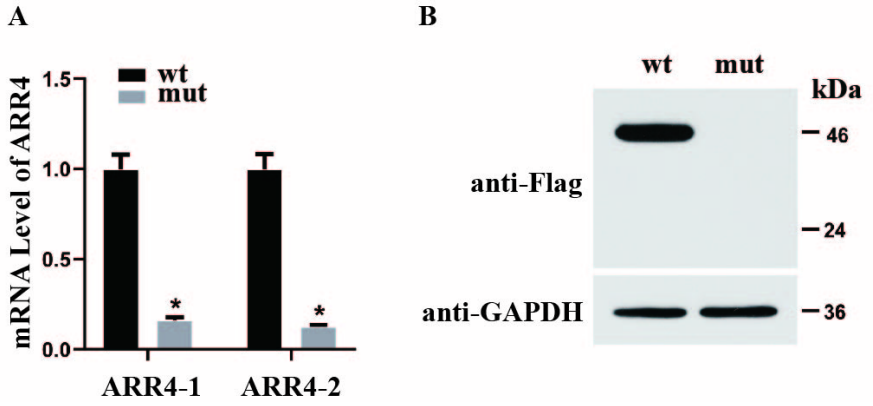

Figure 3: (A) Quantitative RT-PCR using GAPDH as the housekeeping gene demonstrated that the mRNA expression level was significantly decreased in mutant cases compared with wild-type controls. $^{*}p < 0.05$ versus WT. (B) Western blot analysis of WT and S190* mutant ARR4 in whole cell lysate.

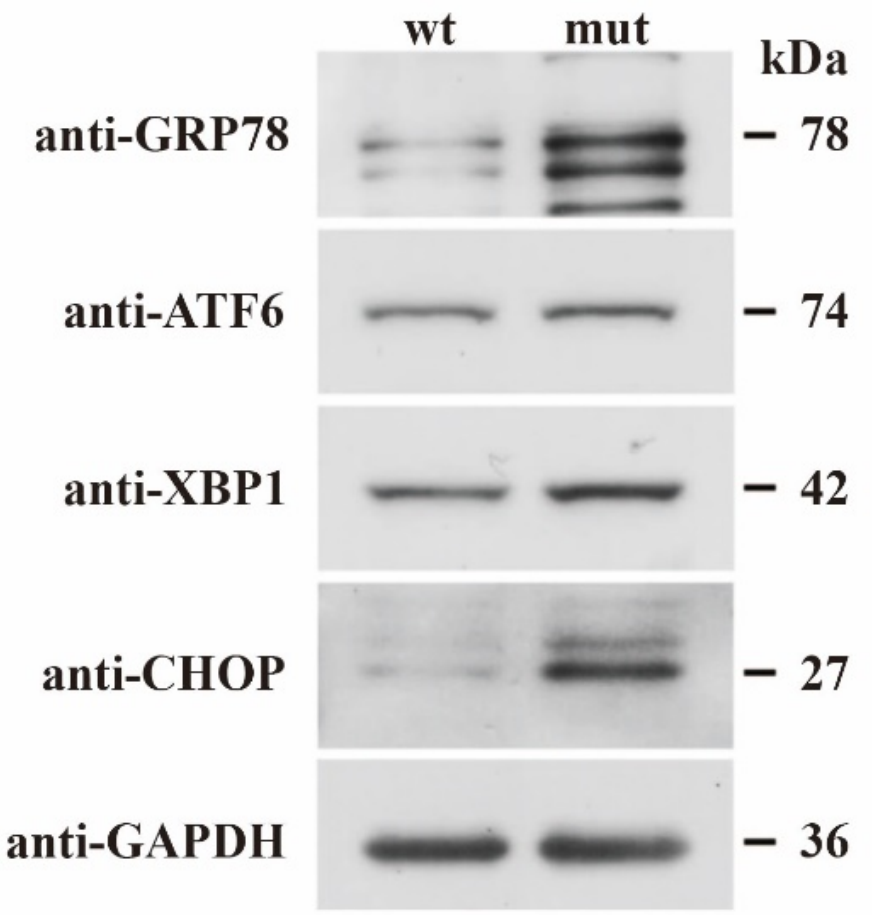

Figure 4: A typical western blot of ARR4 extracts for GRP78, ATF6, XBP1, and CHOP proteins.

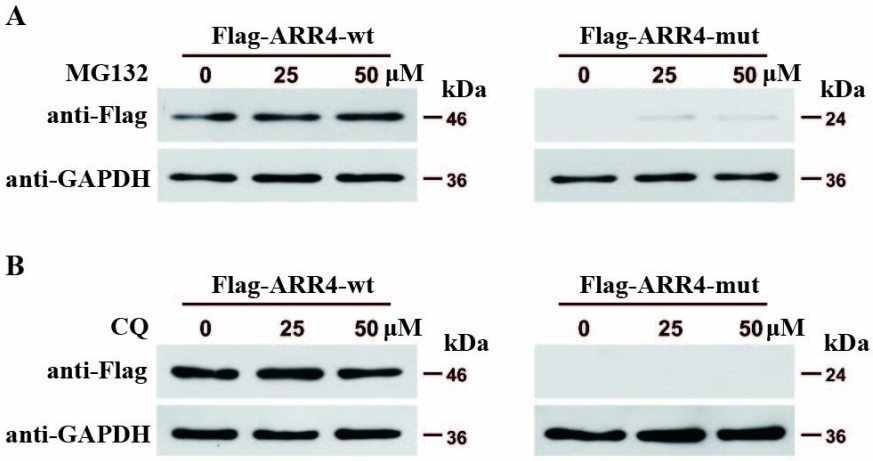

Figure 5: The *ARR4* c.569C>G (p.S190*) mutation destabilized ARR4 via the proteasome system. (A) APRE19 cells transfected with wild-type and mutated *ARR4* with flag tags were treated with 0 μM, 25 μM, and 50 μM of MG132 for 24h and the *ARR4*protein levels detected. The APRE19 cells were simultaneously treated with CQ(chloroquine). Actin protein levels served as the as endogenous control.