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A Novel Intronic Deletion in *PDE6B* Causes Autosomal Recessive Retinitis Pigmentosa by Interfering with RNA Splicing

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Keywords
PDE6B · Intronic variant · Aberrant splicing · Retinitis pigmentosa · Consanguineous families

Abstract

Introduction: Retinitis pigmentosa (RP) is a rare degenerative retinal disease caused by mutations in approximately seventy genes. Currently, despite the availability of large-scale DNA sequencing technologies, ~30–40% of patients still cannot be diagnosed at the molecular level. In this study, we investigated a novel intronic deletion of *PDE6B*, encoding the beta subunit of phosphodiesterase 6 in association with recessive RP. ***Methods:*** Three unrelated consanguineous families were recruited from the northwestern part of Pakistan. Whole exome sequencing was performed for the proband of each family, and the data were analyzed according to an in-house computer pipeline. Relevant DNA variants in all available members of these families were assessed through Sanger sequencing. A minigene-based splicing assay was also performed. ***Results:*** The clinical phenotype for all patients was compatible with rod cone degeneration, with the onset during childhood. Whole exome sequencing revealed a homozygous 18 bp intronic deletion (NM_000283.3:c.1921-20_1921-3del) in *PDE6B*, which co-segregated with disease in 10 affected individuals. In vitro splicing tests showed that this deletion causes aberrant RNA splicing of the gene, leading to the in-frame deletion of 6 codons and, likely, to disease. ***Conclusion:*** Our findings further expand the mutational spectrum of the *PDE6B* gene.

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Introduction

Phosphodiesterases (PDEs) are part of the family of phosphohydrolase enzymes. They catalyze the hydrolysis of cyclic adenosine or guanine monophosphate (cGMP) into AMP and GMP, respectively. There are eleven different sub-types of phosphodiesterases (PDE1 to PDE11), each of which is, in principle, expressed in specific tissues and performs specific functions [1].

In rod photoreceptors, phosphodiesterase 6 (PDE6) is composed of three different subunits (α, β, and γ) encoded by the genes *PDE6A* (MIM#180071), *PDE6B* (MIM#180072), and *PDE6G* (MIM#180073), respectively. This enzyme participates in the phototransduction cascade by selectively degrading cGMP, which in turn leads to the closing of the cGMP-gated channels and to the hyperpolarization of the plasma membrane [2]. *PDE6B* was among the first genes to be identified in association with autosomal recessive retinal dystrophies and autosomal dominant stationary night blindness in mice, dogs, and humans [3–6]. Approximately 100 unique pathogenic/likely pathogenic mutations in *PDE6B* are currently reported in ClinVar [7], including loss-of-function mutations (n = 83) and missense variants (n = 21).

Recessive retinitis pigmentosa (recessive RP) is a rare inherited retinal disease (IRD), with a worldwide prevalence of ~1 in 6,000 individuals [8]. Typically, RP starts with nyctalopia (night blindness), followed over the years by visual field constriction. The condition then gradually worsens with time, to result in legal or complete blindness at the end stage [9], due to the degeneration of retinal photoreceptor cells [10, 11]. RP is clinically and genetically heterogeneous, with mutations detected in as many as ~70 different genes [12]. At present, molecular diagnostic yields in RP do not exceed 70% [13, 14]. The identification of novel pathogenic variants in known genes not only helps refine molecular diagnoses but also allows patients to access gene-based clinical trials or therapies.

In this study, we associate a novel homozygous rare intronic deletion in *PDE6B* to autosomal recessive RP in three Pakistani families. This variant abolishes the canonical acceptor splice site of intron 15, leading to aberrant splicing and ultimately to disease.

Materials and Methods

Patients and Their Families

Three consanguineous families with a total of 10 individuals with RP were enrolled at Hazara University, Mansehra, Pakistan, and characterized at the molecular level at the Institute of Molecular and Clinical Ophthalmology Basel (IOB), Switzerland. Specifically, subjects were recruited from three different districts of northwestern Pakistan: Peshawar (family F1), Waziristan (family F2), and Lakki Marwat (family F3). All affected individuals of these families had been experiencing night blindness and progressively deteriorating vision since childhood. With the help of a detailed questionnaire, we collected descriptive phenotypes as well as a complete medical history of each affected individual, while family F1 also underwent an ophthalmological examination at the Khyber Teaching Hospital, Peshawar, Pakistan. Saliva samples were collected from healthy and affected individuals of all three families using the Oragene DNA saliva kit (OG-500, DNA Genotek Inc., Ottawa, Canada).

Whole Exome Sequencing

Genomic DNA was extracted from saliva samples using the prepI-L2P protocol, according to the manufacturer's instructions (PT-L2P, DNA Genotek). Whole exome sequencing (WES) was performed for the probands of the three families (MAPK1960, MAPK1243, and PK106-5) using the Twist Human Core Exome Capture Kit (Twist Bioscience, San Francisco, CA, USA) and an Illumina NovaSeq 6000 PE150 instrument (Illumina, San Diego, CA, USA).

Reads were aligned to the human reference genome (hg19/GRCH37) using the NovoAlign software (V3.08.00; Novocraft Technologies). Duplicate reads were identified with MarkDuplicates (Picard) [15]. We used the genome analysis toolkit GATK (v3.8) [16] for single base quality score and recalibration, ExomeDepth (v2.1) [17] to detect copy number variations, and HaplotypeCaller [18] to generate VCF files containing all the variants. Furthermore, AutoMap [19] was used to identify runs of homozygosity (ROHs) from WES data, and an in-house developed in silico bioinformatics pipeline was applied for variant annotation and filtration [20].

Minigene Splicing Assay

In order to determine the effect of the *PDE6B* variant (c.1921-20_1921-3del) on pre-mRNA splicing, we used the DNA of one healthy control and of one proband of family F2 (MAPK1243) as templates to amplify part of the *PDE6B* gene (exon 15 – intron 15 – exon 16). We designed PCR primers to include the attB-1 and attB-2 sites at their 5′ ends; the forward primer (5′-ggggacaagtttgtacaaaaaagcaggctgtcccagaaccccttggc-3′) binds to the start of exon 15 and the reverse primer (5′-ggggaccactttgtacaagaaagctgggtttgaagtcagggccaggtcc-3′) anneals to the end of exon 16. We then confirmed the PCR-amplified regions by Sanger sequencing and cloned them first into the pDonr-221 vector (Thermo Fisher Scientific, Zurich, Switzerland) and finally shuttled them into pDest26, allowing their expression in mammalian cells via the use of a cytomegalovirus (CMV) promoter.

HEK-293 cells were revived in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum. The cells were subcultured in a 6-well plate until they reached a confluency of ~70%, at which point they were transfected with 500 ng of either wild-type or mutant *PDE6B*-containing plasmids, using the Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific). Sixteen hours post-transfection, the cells were washed with 1X PBS (phosphate-buffered saline) and cultured in fresh media.

Cells were harvested 48 h post-transfection and total RNA was extracted using the Illustra RNAspin Mini Kit (GE Healthcare, Opfikon, Switzerland). One microgram of total RNA was reverse transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems by Thermo Fisher Scientific). The resulting cDNA (15 ng) was used for PCR amplification, using primers annealing to *PDE6B* exon 15 (forward: 5′-gtcccagaa cccttggc-3′) and exon 16 (reverse: 5′-ttgaagtacagggccaggtccg-3′). The PCR-amplified products were resolved on 2% agarose gel, and their sequence was verified by Sanger sequencing (Microsynth AG, Balgach, Switzerland). The same experiments were repeated by using a different destination vector, pCI-NEO-RHO exon3,5/DEST, a kind gift by Dr. Frans P. M. Cremers (Radboud University Medical Center, Nijmegen, The Netherlands).

Results

Clinical Features

All affected individuals from these three families showed similar clinical phenotypes, with the disease onset, starting with night blindness, having taken place in early childhood. In addition, all of them presented with progressive loss of vision.

Only the three affected individuals of family F1 (MAPK1960, MAPK1961, and MAPK1962, evaluated at ages 39, 36, and 32 years, respectively) received a detailed ophthalmic examination and were diagnosed with RP. In these patients, visual acuity corresponded to the detection of hand motion. Fundus examination showed floating cell debris in the vitreous humor and degenerative peripheral retina with bone spicules. The optic discs were pale, and retinal vessels were attenuated in both eyes (online suppl. Fig. 1a; for all online suppl. material, see https://doi.org/10.1159/000530800). Optical coherence tomography B-scan at the level of fovea showed extreme thinning of the fovea and hyper-reflective foci at the level of the outer retinal layers, the Bruch's membrane, and the choroid, indicative of an advanced status of the disease (online suppl. Fig. 1b). Additionally, the patients presented with mild posterior subcapsular cataract, bilaterally.

Since no clinical examinations were performed on patients from families F2 and F3, to assess their respective phenotypes, we had to rely solely on the available medical history and the information included in the questionnaires. At the time of ascertainment, the 4 patients from family F2 were aged: 13 years (MAPK1243), 15 years (MAPK1244), 20 years (MAPK1248), and 24 years (MAPK1242), whereas PK106-5 from family F3 was 22 years old (ages of other patients from F3 were unknown). Based on their answers on the questionnaires, it was observed that in all cases, the disease started with night blindness during late childhood, followed by progressive vision loss during daytime as well, in agreement with a possible diagnosis of RP.

Genetic Analyses

In order to identify potential disease-causing variants, we performed WES in probands MAPK1960, MAPK1243, and PK106-5 of families F1, F2, and F3, respectively. The high levels of global genomic homozygosity detected by AutoMap (256.45 Mb in MAPK1960, 334.34 Mb in MAPK1243, and 400.77 Mb in PK106-5) confirmed that these patients were all the offspring of consanguineous unions (Fig. 1b). At first, we focused on rare coding variants (gnomAD frequency <0.01) but could not find any potential homozygous or compound heterozygous candidate mutations.

We then thoroughly analyzed all intronic variants in known IRD-associated genes and identified a homozygous 18 bp intronic deletion in the *PDE6B* gene (NM_000283.3:c.1921-20_1921-3del), which was also absent from all public genotype databases (e.g., gnomAD and the Greater Middle Eastern genetic variation database) [21, 22] as well as in 377 Pakistani individuals sequenced in-house. This intronic deletion was located inside the only ROH that was common to the three probands of the families (Fig. 1b). We then genotyped all available individuals of families F1, F2, and F3 by Sanger sequencing. This variant was indeed present homozygously in all affected subjects, whereas in their parents it was present at a heterozygous state. In other words, the deletion co-segregated with disease in all pedigrees according to a pattern that is typical of recessive mutations (Fig. 1a), as suggested initially by family analysis alone. Moreover, the assessment of variants surrounding this allele revealed that it was located within a common haplotype of at least 4.19 Mb in size. Since these families were from three different geographical areas, our data suggest that the deletion identified could represent a founder event within the whole Pakistani population.

Functional Validation of the Variant Identified

We hypothesized that NM_000283.3:c.1921-20_1921-3del could interfere with pre-mRNA splicing and, subsequently, cause aberrant mRNA production. The Human Splicing Finder software [23] did not identify any alteration of the splicing events in this region. However, SpliceAI [24] predicted the loss of the canonical acceptor site of intron 15, with an acceptor loss score of 0.82 and the creation of a cryptic site 18 bp downstream of it (between chr4:657576 and chr4:657577, hg19), with an acceptor gain score of 0.78. MaxEntScan [25] also predicted the disruption of this acceptor site (score in absence of the deletion: 5.15, in the presence of the deletion: 1.79).

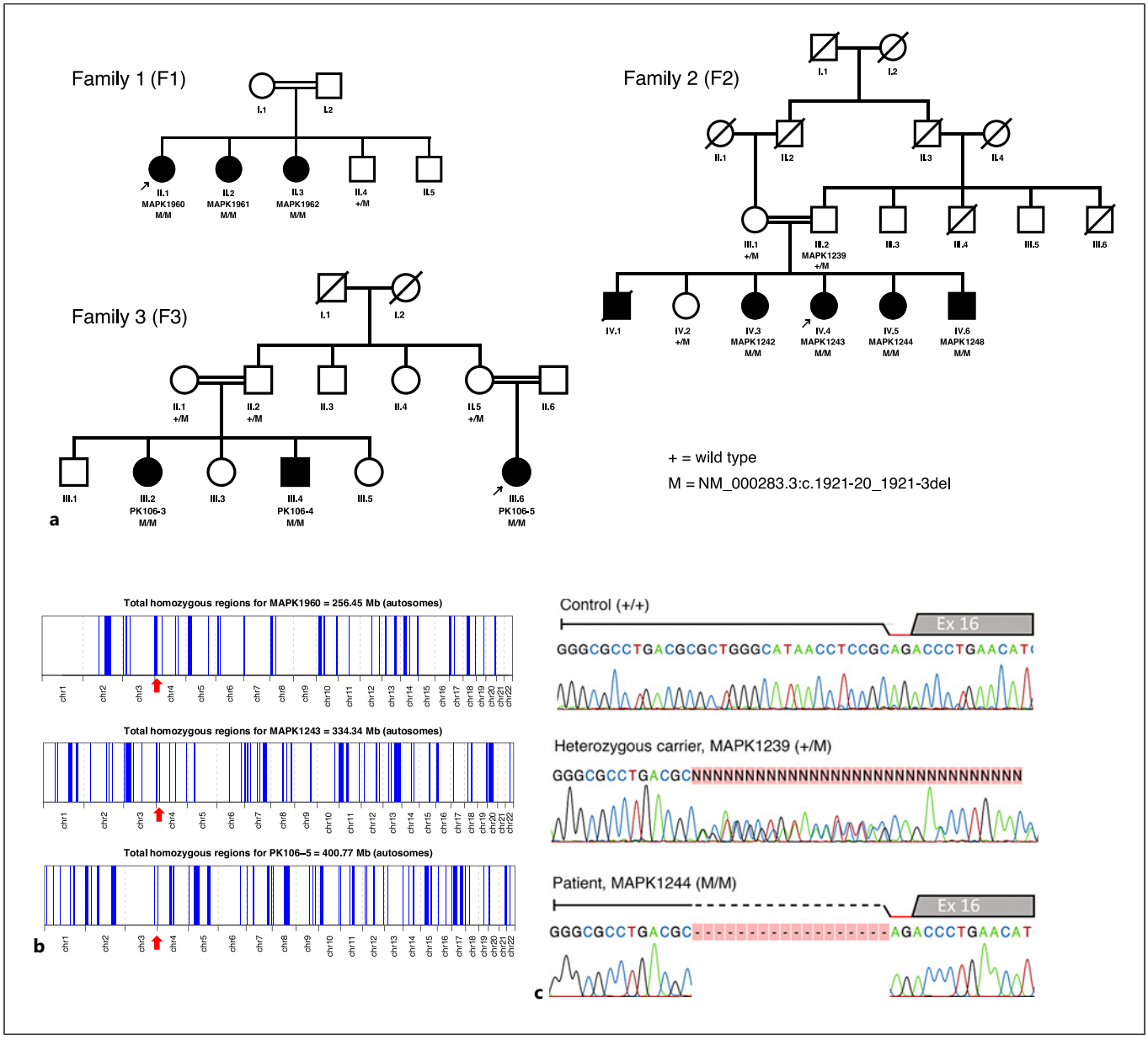

Fig. 1. Genetic features of the families investigated. **a** Pedigrees. Squares indicate males; circles indicate females. Filled shapes designate affected individuals. Double horizontal lines indicate consanguinity, whereas arrows point to the index patient of each family. **b** Homozygosity maps of all index patients. Vertical blue lines represent ROHs. Arrows indicate the homozygous region on chromosome 4 that is common to all patients and contains the mutation detected. **c** Chromatograms of a short stretch of genomic DNA encompassing the variant identified from a control individual (top), a heterozygous carrier (MAPK1239, middle), and a homozygous patient (MAPK1244, bottom). The invariant nucleotides at the acceptor site of intron 15 are indicated by horizontal red lines.

We then transfected HEK-293 cells with a CMV promoter-driven *PDE6B* minigene containing this variant, as well as with a control minigene (Fig. 2a). RT-PCRs at 48 h post-transfection yielded a slightly shorter product in HEK-293 cells transfected with plasmids carrying the intronic deletion, compared to those transfected with wild-type plasmids (Fig. 2b). Sanger sequencing of these products showed that the intronic deletion abolished the use of the canonical acceptor splice site and induced the use of an alternative site,

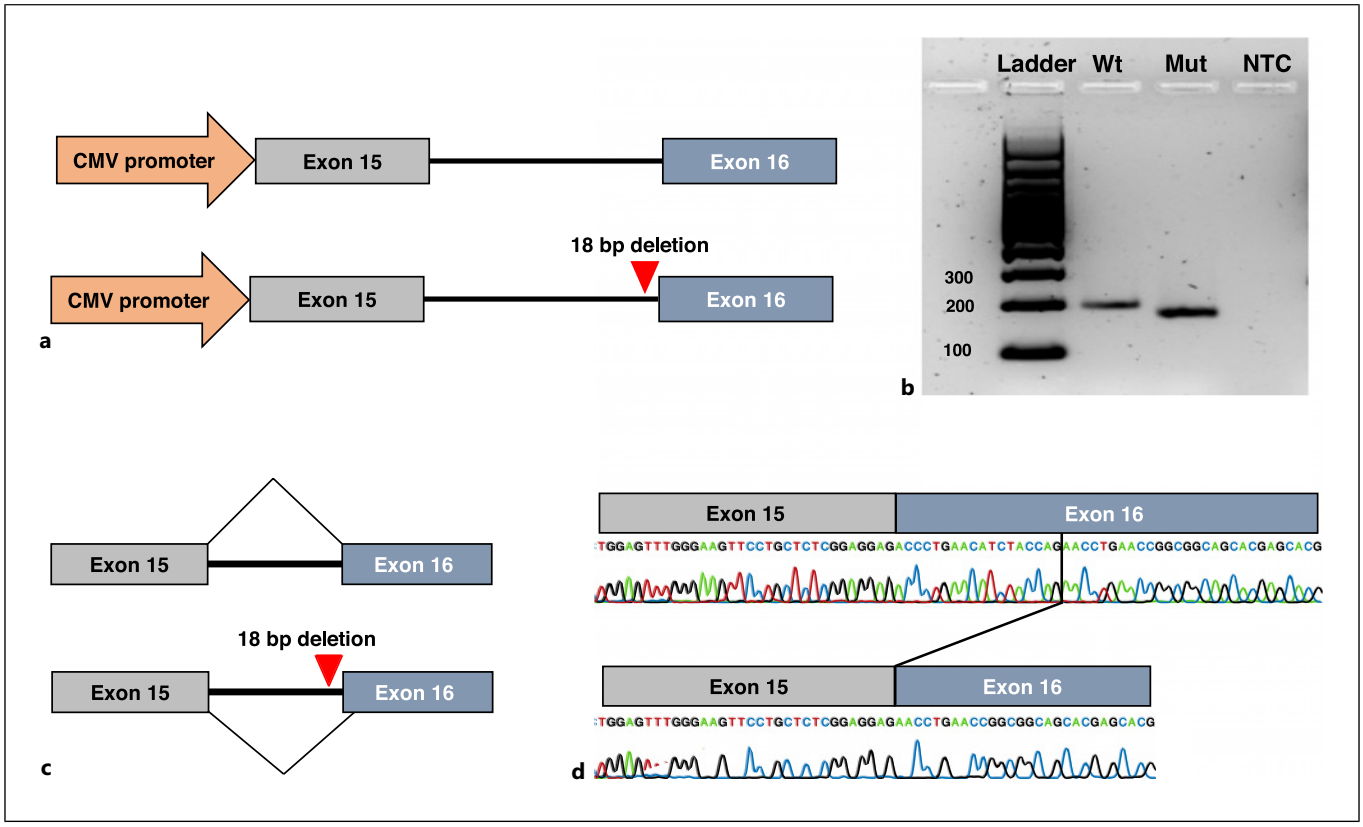

Fig. 2. Minigene assays. **a** Graphical representation of the wild-type and mutant minigene constructs used in splicing assays. **b** RT-PCRs on mRNA from HEK-293 cells, following transfection with relevant minigenes. Wt: wild-type; mut: mutant; NTC: no-template control. **c** Schematic representation of the splicing events driven by the minigenes depicted in **a**. **d** Sanger sequencing of the RT-PCR products shown in **b**.

ultimately resulting in a non-frameshift deletion of the first 18 bases [NP_000274.3:p.(Thr641_Gln646del)] of exon 16 (Fig. 2c, d). These missing triplets encode evolutionarily conserved amino acid residues located within the C-terminal catalytic domain of the PDE6B protein, which is indispensable for its enzymatic activity [26].

To confirm that the splicing pattern detected was not an artifact resulting from the specific backbone of the minigene used, we repeated these experiments by cloning the same sequences mentioned above into a different destination vector, pCI-NEO-RHO exon3,5/DEST [27]. No differences with respect to the patterns reported in Figure 2 were observed.

Discussion and Conclusions

This study associates a novel intronic variant in *PDE6B* to autosomal recessive RP in three Pakistani families. This mutation is present homozygously in 10 affected individuals, co-segregates with disease in all pedigrees according to a recessive pattern of inheritance, and is absent from all public genotype databases of controls. In addition, this deletion is part of a common haplotype, indicative of a possible founder event and therefore of particular importance for future genetic testing in the Pakistani population. Functional analysis, performed by minigene splicing, showed that this intronic deletion leads to aberrant *PDE6B* transcripts lacking 18 nucleotides, which encodes 6 amino acid residues that located within the PDE6B catalytic domain [26]. According to the American College of Medical Genetics and Genomics (ACMG) guidelines, this intronic deletion can be classified as "likely pathogenic," with the criteria PM2, PM4, PS3, and PP1. Overall, these results support the hypothesis that, in vivo, the detected intronic variant causes aberrant splicing, which, in turn, leads to the production of defective PDE6B protein and consequently to disease.

Interestingly, a previous report identified a prevalent mutation in Caucasus Jews (*PDE6B*; c.1921-9C>G)

occurring in the same stretch of DNA that is deleted in our patients [28]. More precisely, this intronic variant involved the −9 nucleotide of intron 15, creating a cryptic acceptor splice site. This event caused the retention of eight additional base pairs during pre-mRNA splicing and in the formation of a premature stop codon. Similarly, an in-frame deletion of residues Tyr645-Gln646, just 12 bp downstream of the exon 15-exon 16 junction, was described to be associated with recessive RP in a cohort of patients from the USA [29]. Of note, these two amino acid residues are included in the portion of the PDE6B protein that is predicted to be lost in our patients as well (Thr641 to Gln646). Together with our data, these findings suggest that the 3′ side of intron 15 and the 5′ side of exon 16 are particularly relevant for the molecular pathology of *PDE6B*-associated RP.

In the past years, noncoding and intronic parts of the human genome have been largely ignored in medical genetics research. Recently, however, many studies have shown that these regions may play an essential role in pathogenesis, for instance, by affecting gene regulation or pre-mRNA processing. The analysis of noncoding variants in undiagnosed IRD patients represents, indeed, a new and very effective strategy for identifying causative mutations and reducing missing heritability in these disorders [30–33]. The mutation discovered here falls into this category; despite occurring in an intronic region of a gene, it appears not to be less deleterious than more canonical truncating variants, such as nonsense or frameshift mutations.

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Statement of Ethics

The study was approved by the Ethical Committee of Hazara University, Mansehra, Pakistan (approval number: F.NO: 185/HU/Zool/2018/283), and by the Ethikkommission Nordwest-und Zentralschweiz (approval number: 2019-01660). Written informed consent was obtained from all participants prior to the study, which was conducted in agreement with the tenets of the Declaration of Helsinki and the ARVO statement on the use of human subjects in biomedical research.

Conflicts of Interest Statement

The authors declare no conflicts of interest.

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Author Contributions

M.U., A.U., and F.U. identified and recruited the families. A.R. and B.K. performed the clinical examination. M.U., A.U.R., M.Q., and M.A. analyzed the WES data. M.U. and M.F. designed and performed minigene assay. M.U. wrote the first draft, and M.Q. and C.R. revised and corrected the manuscript. M.A. and C.R. supervised the study.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material. Further inquiries can be directed to the corresponding author.

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