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A Leaky Deep Intronic Splice Variant in *CLRN1* Is Associated with Non-Syndromic Retinitis Pigmentosa

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INTRODUCTION & AIM

Inherited retinal diseases (IRDs) encompass a large group of retinal phenotypes char -acterized by extensive clinical variability and large genetic heterogeneity [1,2]. Retinitis pigmentosa (RP) is the most prevalent and heterogeneous IRD and can be inherited in autosomal dominant (AD), autosomal recessive (AR), digenic, mitochondrial and X-linked (XL) modes.

Although it is accepted that most IRD-associated genes have been identified, next generation sequencing (NGS) used as gene panels, whole exome sequencing (WES) or whole genome sequencing (WGS) fail to identify the causative genes in about 30% of IRD cases. Although genes currently not associated with IRDs, and therefore not well covered in such analyses, might cause a fraction of these cases, elusive variants might play an important role in such cases. One example might be deep intronic variants that affect splicing, usually by introducing pseudo-exons into the transcripts. A second possibility might be novel variants in genes that are associated with a similar, but not identical, phenotype.

CLRN1canproduce11transcripts through various mechanisms, and therefore, exon numbering might vary between studies. The gene, originally named USH3A, was found to have five exons and two splice variants: the main form included exons 1, 2, 3 (3a), and 4 (3b) with a 120-aa open reading frame (ORF), while the second variant included exon 1b, resulting in a 30-aa ORF. Subsequent studies refined it to CLRN1, revealing that the initial four-exon form was a rare splice variant. The main splice form consists of exons 0, 2, and 3 (3a extending into the intron between 3a and 3b), with a 232-aa ORF. All known mutations causing USH3 are located within this three-exon variant. In the current study, we report such an elusive, deep intronic variant in the CLRN1 gene, variants which are known to cause mainly USH type 3. This variant was found in compound heterozygosity with a well-established CLRN1 pathogenic variant that causes USH3. The intronic variant introduces a pseudo-exon in a leaky fashion, allowing normal hearing in a 51-year-old (YO) patient presented with non-syndromic retinitis pigmentosa.

METHODS

2.1**. Patient Recruitment:** Blood samples were collected from the index cases and unaffected family members. The sample collection process adhered to ethical standards, receiving approval from the Hadassah Hospital Institutional Review Board. Informed consent, obtained in writing, ensured compliance with all relevant ethical regulations and safeguarded the rights and privacy of participants. Genomic DNA extraction was carried out from blood samples using the Promega kit and Promega Maxwell (Madison, WI, USA) DNA extraction system.

2.2. Sanger and Next-Generation Sequencing (NGS): WES was performed by Pronto Diagnostic (Tel-Aviv, Israel), and an IRD gene panel including 113 genes that cause RP and Leber congenital amaurosis was performed using the smMIPsplatform. Fastq files were uploaded to the Genoox pipeline and analyzed using Franklin as previously reported [6]. For segregation analysis, primers were designed using primer3 web For: TCTAATGGTCTGTCTTCTCCCA; Rev: AGCCTTTAATGAC CTTTCTCGG.Bothvariants

2.3. In Silico Splicing Analysis:were verified by Sanger sequencing. 2.3. The effect of the deep intronic c.254-643G>T CLRN1 (NM_174878.2) variant on its pre-mRNA splicing was predicted using Alternative Splice Site Predictor (ASSP), Berkeley Drosophila Genome Project and the SpliceAI prediction tool.

2.4. Cloning: Aminigene exontra pplasmid (pET01)was purchased from MoBiTec(Eupen, Belgium). A792bpCLRN1intron 1 sequence flanking the c.254-643G>T variant was PCR amplified from the genomic DNA of the patient and a control individual using PCR Taq Mix Red (PB045625-071-0, London, UK). In total, 1 µg of each PCR product and 1 µg of the pET01 plasmid were digested with BamHI (1 µL) and XhoI (1 µL) restriction enzymes for 2 h at 37 ◦C. For ligation, the insert and the plasmid were mixed in a 3:1 ratio and ligated using T4 DNAligase (NEB, Cat. no: M0202S) for 2 h at room temperature. In total, 50 µL of DH5 α-competent cells were added to each ligation mixture and incubated for 30 min on ice. After the incubation, the bacteria were heat shocked at 42c for 90 s. After heat shock, 1 ml of LB media was added to bacteria and grown for 45 min at 37c (NEB, Cat. no: C2987H).

2.5. Transfection, RNA Isolation and cDNA Synthesis: For splicing analysis, HeLa cells were seeded into a 24-well plate and cultured until they reached 70% confluence. Cells were transfected separately with empty pET01 plasmid, wild-type and mutant CLRN1 plasmids using (TransfeX™ Transfection Reagent, ACS 4005™, ATCC, Manassas, VA, USA) transfection reagent. Cells were harvested 48 h post transfection, and the total RNA was isolated using (Quick-RNA MiniPrep, R1055, ZYMO RESEARCH, Tustin, CA, USA) by following the manufacturer's protocol. cDNA synthesis was carried out using a qScript cDNA Synthesis Kit (Cat. No: 733-1178).

2.6. Automated Electrophoresis: TapeStation Quality control of the RT-PCR products was assessed using the Agilent Technolo gies D1000 ScreenTape system kit and Agilent Technologies 4200 TapeStation instrument (Agilent Technologies, Waldron, Germany). After automated electrophoresis, the results were analyzed on the TapeStation Controller Software version 5.1 to detect product size and quantity with a sensitivity of 0.1 ng/µL and sizing accuracy of ±10%. The primer sequence used for PCR amplification and Sanger sequencing of CLRN1 cDNA analysis was F-GGATTCTTCTACACACCC;R-AGGTGGGTCGAGGTCAAC.

RESULTS & DISCUSSION

1. Clinical and molecular analysis

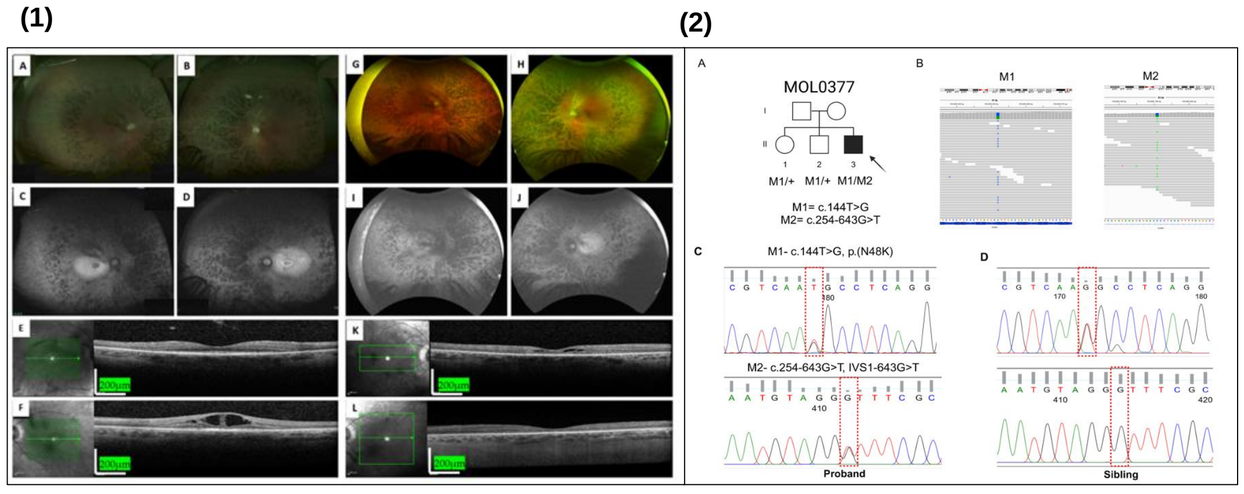

Figure 1: Retinal imaging of MOL377-1 at the age of 40 (A–F) and 50 (G–L). A-B and G-H repre sent ultra-wide-field pseudocolor and autofluorescence (FAF) fundus photos, respectively, taken using the Optos Panoramic 200 Optomap Fundus Camera. Characteristic peripheral dense BSPs mixed with retinal atrophy encroaching the temporal vascular arcades can be seen**. Figure 2**: Variant detection and familial segregation analysis. (A) Two-generation family pedigree; (B) BAM files showing the two CLRN1 variants: c.144T>G and c.254-643G>T; (C,D) Familial segregation analysis in proband (C) and one of his unaffected siblings (D). Dotted red lines shows the identified variants.

2. Functional analysis of *CLRN1* deep intronic variant

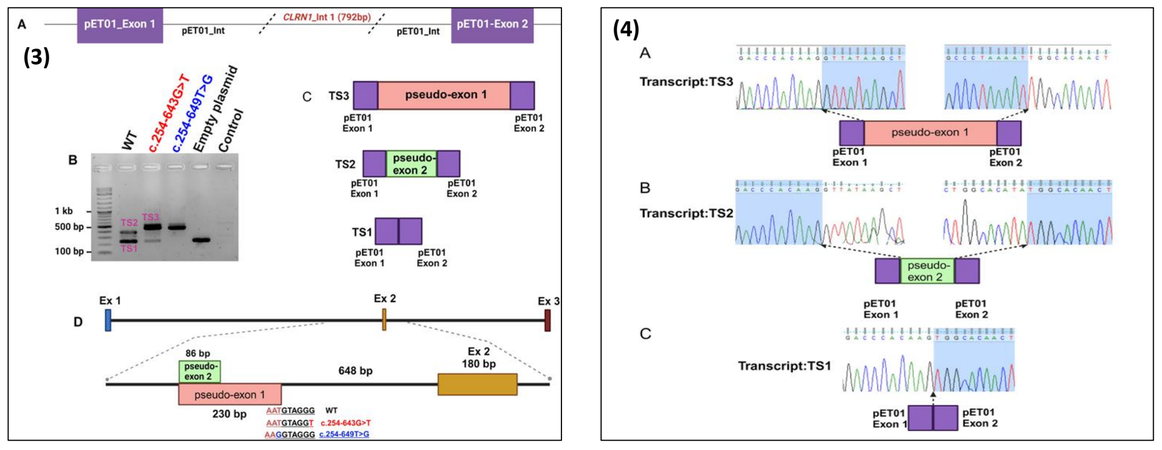

Figure3: Analyzing the effect of two CLRN1 variants (c.254-643G>T and c.254-649T>G) on its pre mRNA splicing. **Figure 4:** Sanger sequencing of CLRN1 mutant transcripts. **Figure 5:** Detailed analysis of CLRN1 transcripts

CONCLUSION

We report on a novel deep intronic variant in CLRN1 causing non-syndromic RP. The non-syndromic phenotype observed in this index case may be attributed to the leaky nature of this variant, which is causing some normal transcripts to be produced.

FUTURE WORK / REFERENCES

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