Novel *C8orf37* mutations cause retinitis pigmentosa in consanguineous families of Pakistani origin

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Purpose: To investigate the molecular basis of retinitis pigmentosa in two consanguineous families of Pakistani origin with multiple affected members.

Methods: Homozygosity mapping and Sanger sequencing of candidate genes were performed in one family while the other was analyzed with whole exome next-generation sequencing. A minigene splicing assay was used to confirm the splicing defects.

Results: In family MA48, a novel homozygous nucleotide substitution in *C8orf37*, c.244–2A>C, that disrupted the consensus splice acceptor site of exon 3 was found. The minigene splicing assay revealed that this mutation activated a cryptic splice site within exon 3, causing a 22 bp deletion in the transcript that is predicted to lead to a frameshift followed by premature protein truncation. In family MA13, a novel homozygous null mutation in *C8orf37*, c.555G>A, p.W185*, was identified. Both mutations segregated with the disease phenotype as expected in a recessive manner and were absent in 8,244 unrelated individuals of South Asian origin.

Conclusions: In this report, we describe *C8orf37* mutations that cause retinal dystrophy in two families of Pakistani origin, contributing further data on the phenotype and the spectrum of mutations in this form of retinitis pigmentosa.

Retinal dystrophies are a heterogeneous group of inherited eye disorders that lead to severe visual impairment and can cause blindness in a proportion of cases. Some forms are characterized by blindness at birth, while in others, symptoms appear during childhood or adulthood. The underlying pathology leading to loss of color, night, central, or peripheral vision can be accounted for by mutations in specific genes (see RetNet) known to disrupt crucial processes such as phototransduction, phagocytosis, vitamin A metabolism, cell-to-cell signaling, or gene regulation. These conditions can have dominant, recessive, X-linked, or mitochondrial modes of inheritance, and there is increased prevalence of retinal disease in communities with high levels of consanguinity [1-3]. Identifying the pathogenic mutation in the affected cases facilitates genetic counseling for prognosis and recurrence risk, as well as presymptomatic and carrier testing. It also serves to stratify and prioritize patients for an increasing number of clinical trials for gene and other forms of therapeutic intervention [4,5].

The aim of this study was to investigate the genetic basis of retinitis pigmentosa in two families of Pakistani origin, one from a rural village near Multan in the Punjab province in Pakistan and the other living in West Yorkshire in the United Kingdom (UK). Here we report previously undescribed mutations in the recently implicated gene *C8orf37* (OMIM 614477) as the cause of retinitis pigmentosa in these patients.

METHODS

Patient recruitment: Patients and their relatives were recruited after they gave informed, written consent using a process approved either by the Institutional Review Board of Quaid-i-Azam University (Project number IRB00003532)

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or by the Leeds East Research Ethics committee (Project number 03/362) that adhered to the tenets of the Declaration of Helsinki and the ARVO statement on human subjects. In total, 5 affected (4 males and 1 female) and 8 unaffected (3 males and 5 females) subjects were recruited either through a field visit to a remote village near Multan, Pakistan or through the eye clinic at St. James's University Hospital, Leeds, England, followed by a home visit. Pedigree structures are depicted in Figure 1. The patients, aged between 12 to 27 years old at the time of initial examination, were diagnosed with retinitis pigmentosa after ophthalmic assessment by an experienced ophthalmologist. Apart from problems with their vision they had no other obvious abnormalities. Peripheral blood (2–6 ml) was collected from affected patients, their parents, and unaffected relatives where they were available by venipuncture and drawn in BD Vacutainer EDTA blood collection tubes (BD Biosciences, Oxford, England). Genomic DNA was extracted from peripheral blood leukocytes according to standard procedures.

Autozygosity mapping: Whole-genome homozygosity mapping was performed using Affymetrix Gene Chip Human Mapping 250 K-*NspI* arrays. The data were analyzed using Homozygosity Mapper software applying default settings.

Whole exome sequencing: Whole exome capture was performed on 3 µg of genomic DNA using the SureSelect Target Enrichment reagent version 4 (Agilent Technologies Limited, Wokingham, England) followed by deep sequencing using paired end reads on an Illumina HiSeq 2500 (Illumina, Little Chesterford, England) according to the manufacturer's protocols. The sequencing output files were prepared and checked with FASTQ tools using the online data analysis platform Galaxy [6]. The sequencing reads were aligned to the human genome reference hg19 using Bowtie2 software [7] and processed in the SAM/BAM format [8] using Picard and

the Genome Analysis Toolkit [9]. Single-nucleotide variants and indels were called using the UnifiedGenotyper [10]. Only variants with a prediction score of >20 were included in the analysis. Annovar software was used to annotate the variants. Any variants with a minimum read depth of 10, outside the exon and its flanking splice site regions, synonymous, with a minor allele frequency >1% in the exome variant server or the 1000 Genomes database were filtered out. The resulting list of homozygous gene variants was compared to the retinal dystrophy genes found in RetNet [June 2014].

Sanger sequencing: Primers for amplifying all exons and exon–intron boundaries of *C8orf37* were designed with Primer3plus software and are listed in Appendix 1. PCR was performed on human genomic DNA with 10 pmol of forward and reverse primers, and 0.5 U of Taq DNA polymerase according to standard protocols. An initial denaturation step of 94 °C for 4 min was followed by 35 cycles consisting of 94 °C for 15 s, 55-62 °C for 15 s and 72 °C for 30 s with a final extension step of 72 °C for 5 min. The products were treated with ExoSAP-IT (GE Healthcare, Freiburg, Germany) and sequenced using the BigDye terminator v.1.1 cycle sequencing kit (Applied Biosystems, Darmstadt, Germany) on an ABI 3130 Genetic Analyzer (Applied Biosystems). Results were analyzed using Lasergene SeqMan Pro software (DNASTAR, Madison, WA). Purified plasmid DNA was sequenced directly using the BigDye Terminator v.1.1 kit according to the manufacturer's instructions.

Minigene splicing assay: To construct a minigene plasmid containing human *C8orf37* exons 2 to 4 and the intersecting introns, a ~5 kb DNA fragment was amplified from genomic DNA using a Long PCR Enzyme kit (Fischer Scientific, Schwerte, Germany), with oligonucleotide primers dATA TAA GCG GCC GCG CTT TGC CTG CAG AAG GTA G and dATA TAA GGA TCC AAA TGC CAA TGG AAT
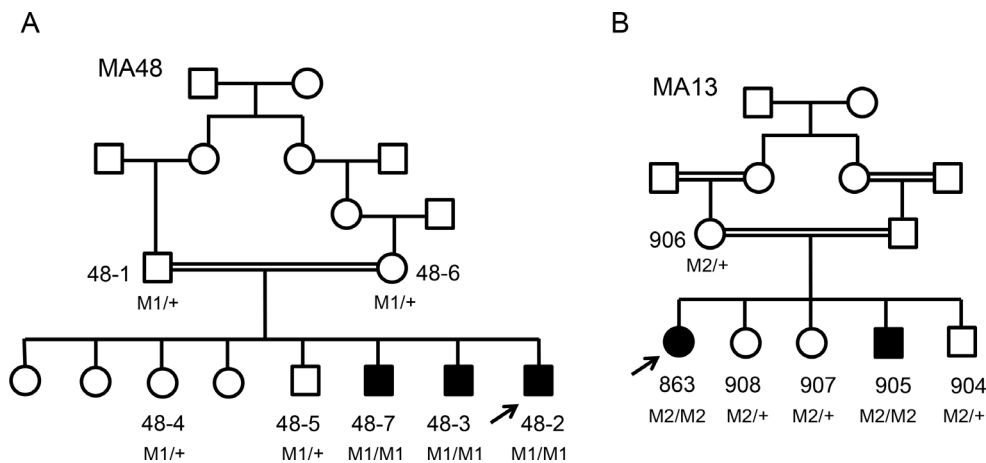

Figure 1. Pedigrees of the two investigated families with retinitis pigmentosa. **A**: Family MA48. **B**: Family MA13. The arrow indicates the proband. Genotypes of the cases from whom DNA was available for analysis is indicated. M1=c.244–2A>C and M2=c.555G>A. M1/M1= homozygous mutant genotype; M1/+=heterozygous genotype.

GTG CT that contained restriction enzyme recognition sites (underlined). This PCR fragment was digested with *BamHI* and *NotI* and then ligated into a double-digested pSPL3 plasmid vector (Life Technologies, Darmstadt, Germany) using *T4 DNA Ligase* (New England Biolabs, Frankfurt am Main, Germany) according to the manufacturer's instructions. Plasmid DNA of the wild-type minigene construct was purified from the transformed *Escherichia coli* host in the presence of ampicillin (Life Technologies), using the peqGOLD Plasmid Miniprep KitI (PEQLab Biotechnologie, Erlangen, Germany), and the integrity sequence was verified with restriction digests and sequencing as described.

To introduce a single nucleotide substitution into the minigene construct, site-directed mutagenesis was performed with oligonucleotide primers (dGGC CTT TTA TTG TTT TGC TTT TTT AAC AGA AAT TAA AAT CTA AAT CTT CAG GTA ACA C and dGTG TTA CCT GAA GAT TTA GAT TTT AAT TTC TGT TAA AAA AGC AAA ACA ATA AAA GGC C; the nucleotide mutation is underlined) using the QuikChange In Vitro Mutagenesis kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's protocol. The mutated construct was partially sequenced to confirm that the desired change was introduced.

Minigene constructs containing either wild-type or mutant sequence were transformed into confluent HEK293 cells using Lipofectamine 2000 (Life Technologies). After 24 h, the cells were harvested. Then the total RNA was extracted (PEQLAB Biotechnologie), and reverse transcribed (Roche Diagnostics GmbH, Mannheim, Germany) before PCR amplification with vector-specific primers dTCT GAG TCA CCT GGA CAA CCT CAA and dGCT CAC AAA TAC CAC TGA GAT was performed. The products were analyzed with agarose gel electrophoresis and direct sequencing.

RESULTS

Clinical phenotype of patients: Two families, one from a rural village near Multan in Pakistan (MA48) and the other living in West Yorkshire in England (MA13), both of Pakistani origin, were investigated in this study. All affected cases had early onset retinitis pigmentosa with moderate to severe loss of visual acuity between the second and third decades. The fundus examination findings were consistent with a clinical diagnosis of retinitis pigmentosa (Table 1 and Figure 2). Two of three cases (48–3 and 863) that were examined had axial myopia with refractive errors between −5.5 and −9.5 diopters. Full-field electroretinography was unrecordable in one patient (863), and another had sluggish pupillary reactions (48–2) consistent with a severe form of retinitis pigmentosa and minimally functioning retina. No other extraocular manifestations were observed in the patients who were examined. The family history and evidence of consanguinity suggested autosomal recessive inheritance (Figure 1).

Genetic analysis of families: To identify the causative mutation in family MA48, genomic DNA from the three affected cases, 48–2, 48–3, and 48–7, was analyzed with homozygosity mapping. Several homozygous regions on chromosomes 4, 5, 7, 8, 10, and 15 were shared by all affected individuals (Appendix 2). Of these, an approximate14.4 Mb interval on chromosome 8 contained the candidate gene *C8orf37*, which was recently implicated in retinal dystrophy with early macular involvement [11]. Sanger sequencing of all coding regions and flanking intron segments revealed a homozygous nucleotide substitution, c.244–2A>C in *C8orf37* [NM_177965.3], that disrupted the consensus acceptor splice junction of exon 3 (Figure 3A). This mutation segregated with the disease phenotype as expected in a recessive manner and was not present in 8,244 unrelated individuals of South Asian origin who had been whole-exome sequenced as part of various disease-specific and population genetic studies (accessed via. the Exome Aggregation Consortium [ExAC] browser, version 0.2). To analyze the precise effect of this mutation on transcript splicing, a minigene construct was generated containing exons 2 to 4 and the intervening intronic sequences of *C8orf37*. Following site-directed mutagenesis to introduce the mutation, the wild-type and mutant constructs were transfected into HEK293 cells, total RNA was extracted, and reverse-transcription PCR was performed before Sanger sequencing. This showed that the splice-site mutation activates a cryptic splice site within exon 3, causing a 22 bp deletion in the transcript that predicts a frameshift and premature termination of protein translation (Figures 3C,D).

In family MA13, genomic DNA from the proband 863 was analyzed with whole exome sequencing. The output files were processed to generate 47,391 variants with a minimum read depth of 10. These variants were filtered to include those within exons as well as the conserved two base pair flanking splice-site junctions and that have a minor allele frequency of less than 1% in the public DNA databases. After synonymous variants were excluded, 73 homozygous variants remained in the filtered list (see Appendix 3). Of these, only recessive mutations in *C8orf37* had previously been implicated in retinal dystrophy [11]. It therefore seemed likely that the homozygous null variant in *C8orf37*, c.555G>A, p.W185*, was the most suitable candidate for causing the condition (Figure 3B), and was later confirmed to segregate with disease in this family and was absent from 8,244 unrelated subjects of South Asian origin who had been accessed using the ExAC browser.

TABLE 1. CLINICAL DETAILS OF RETINAL DYSTROPHY PATIENTS EXAMINED IN THIS STUDY.

Family	Patient ID	Gender	Age at examination (years old)	Best corrected visual acuity (OD and OS)	Fundus examination	Diagnosis	Additional Findings
MA48	48–2	M	27	LP and LP	Pigmentary retinopathy, arteriolar attenuation and optic disc pallor	Retinitis pigmentosa	Alternate exotropia, sluggish pupil reaction.
MA48	48–3	M	15	CF and CF	Pigmentary retinopathy, arteriolar attenuation and optic disc pallor	Retinitis pigmentosa	Auto refraction: OD −8.0/-0.75X180; OS −5.50/-2.50X165
			25	6/36 and 6/36	Pigmentary retinopathy		High myope (−9.5D SEM correction)
MA13	863	F	46	6/36 and 6/36	Pigmentary retinopathy	Retinitis pigmentosa	Non-recordable full-field ERG
			64	LP and LP	Pigmentary retinopathy		Cataract surgery OU. Pre-operative axial lengths recorded as 25.7 and 25.4 mm.

The gender, age at examination, corrected visual acuity, ophthalmoscopy and electroretinography results, diagnosis and any additional findings are summarized. M=male, F=female, LP=light perception, CF=count fingers, OD=right eye, OS=left eye, OU=both eyes, SE=spherical equivalent.
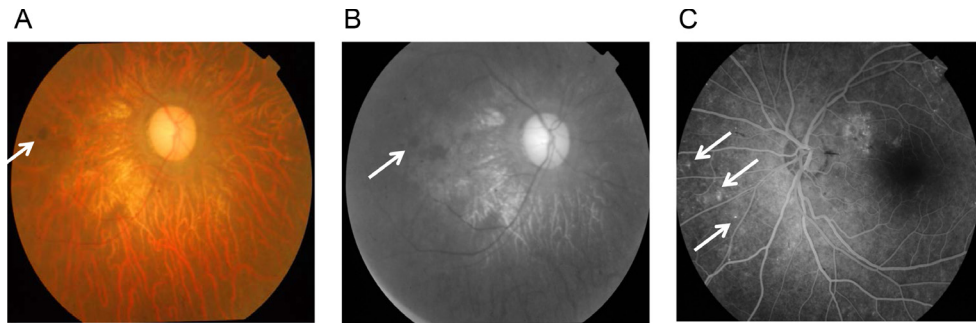
Figure 2. Fundus photographs of patient MA48–2 at 33 years old. Color (**A**) and black-and-white (**B**) photos of the right eye show diffuse nummular hyper-pigmentation at the macula (arrows), waxy pallor of the optic disc, arteriolar attenuation, and chorioretinal atrophy. **C**: Fluorescein angiography of the left eye shows bone spicule-like deposits as fluorescent areas nasal to the optic disc (arrows).

DISCUSSION

C8orf37 encodes a 207 amino acid protein of unknown function with a predicted molecular mass of approximately 23 kDa. Immunolocalization studies in retinal cross sections have shown that *C8orf37* colocalizes with γ-tubulin, a basal body marker at the connecting cilium, between the outer and inner segments in the photoreceptor layer [11]. The basal body acts as the organizing center for the cilium and permits trafficking of proteins and lipids through intraflagellar transport from the inner to the outer segment, that is necessary for the formation of the outer segment discs [12,13].

Here we describe novel mutations in *C8orf37* that caused retinitis pigmentosa in two consanguineous families of Pakistani ethnicity. Mutations in *C8orf37* are a rare cause of retinal dystrophy. To date, they account for only 0.4% of all unrelated retinal dystrophy cases (three out of 700) that have been solved in our laboratories. Previous papers on the clinical features seen in patients with mutations in this gene described six patients with a diagnosis of cone-rod dystrophy (CRD) and seven with retinitis pigmentosa (RP) with early macular involvement [11,14-17]. Distinguishing between RP, where patients experience night blindness followed by progressive visual field constriction, and CRD, where the symptoms are photophobia, loss of visual acuity, and central vision, depends on recognizing the early symptoms. However, in the most severe cases there is considerable phenotypic overlap at presentation making it difficult to diagnose these progressive conditions. Sometimes when features of both
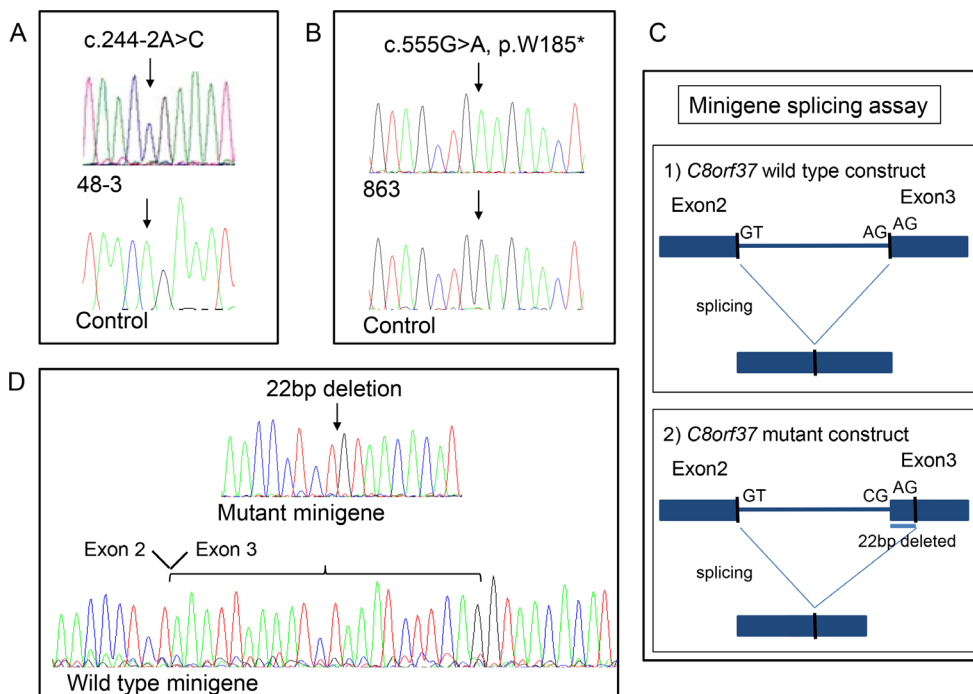

Figure 3. Genetic analysis of *C8orf37*. Sanger sequencing chromatograms depicting mutations c.244–2A>C in an affected individual (48–3) from family MA48 (**A**) and c.555G>A; p.W185* in an affected individual (863) from family MA13 (**B**). Chromatograms from a normal control subject are also shown for comparison. Diagrammatic representation of the minigene splicing assay (**C**) and sequencing chromatograms (**D**) showing that the consequence of the c.244–2A>C splice site mutation was activation of a cryptic splice site within exon 3 and deletion of 22 base pairs of coding sequence in the resulting transcript. The wild-type minigene assay spliced exons 2 and 3 correctly.

conditions are present at the early stages it may be difficult to assign a disease category. Of the 73 genes that have mutations that lead to RP and the 30 genes that have defects that lead to CRD (RetNet, October 2014), only mutations in seven genes, including *C8orf37*, account for patients with either diagnosis. These include dominant mutations in *SEMA4A* (OMIM 607292) [18] and *PRPH2* (OMIM 179605) [19,20], recessive mutations in *ABCA4* (OMIM 601691) [21-23] and *CERKL* (OMIM 608381) [24,25], X-linked mutations in *RPGR* (OMIM 312610) [26,27], and mutations in *PROM1* (OMIM 604365) that show a genotype-phenotype correlation; a dominant missense mutation, p.Arg373Cys (dbSNP: rs137853006), causes CRD [28] whereas recessive mutations lead to RP [29,30].

A summary of all *C8orf37* mutations found in patients with retinal dystrophy to date, together with the available clinical information in each case, is shown in Figure 4. Two of the patients with CRD, both harboring a homozygous splice-site acceptor change, showed unilateral postaxial polydactyly as an additional feature, which suggests the condition may be a ciliopathy [11]. The patients described in this report, including the one described by Jinda et al. who had a different homozygous splice-site mutation [15], did not present with extraocular features and polydactyly was absent. The clinical features of night blindness, visual field constriction, and progressive loss of vision appeared to be consistent in all patients with *C8orf37* mutations, whether the patients had a missense mutation or a null allele, suggesting that there does not appear to be a correlation between genotype and phenotype.

This is the first time that mutations in this gene have been described in the Pakistani population. To date, of the 132 known genes that cause non-syndromic retinal dystrophies, only 35 have been found to be mutated in cases of Pakistani

origin, and about 90% of the mutations in these 35 genes have not been found in other populations [31]. Finding the mutation in these families should facilitate counseling for recurrence risk and carrier testing. However, although approximately 60% of marriages in Pakistan are consanguineous, one of the highest rates in the world [32], which is likely to contribute to the high burden of genetic disease [33], practicing physicians are required to perform counseling duties since specialized genetic counseling services are not part of the healthcare system in Pakistan [34] whereas the family living in England will have access to appropriate infrastructure such as ophthalmologists, clinical geneticists, and social services through the National Health Service.

To summarize, we have identified novel *C8orf37* mutations in two consanguineous families who originated in Pakistan. Our findings contribute further data on the phenotype and the spectrum of mutations in this form of retinitis pigmentosa.

APPENDIX 1. PRIMERS FOR SANGER SEQUENCING THE EXON AND INTRON BOUNDARIES OF C8ORF37.

The primer names, DNA sequences, PCR product size and melting temperatures of each primer are shown. To access the data, click or select the words "Appendix 1."

APPENDIX 2. HOMOZYGOSITY MAPPING IN FAMILY MA48.

Analysis of the SNP genotyping data of affected cases 48-2, 48-3 and 48-7 identified autozygous segments on chromosomes 4 (rs10212780 – rs3755967, ~7.1Mb), 5 (rs4357016 – rs1443096, ~2.0Mb), 7 (rs6583338 – rs4724950, ~7.2Mb; rs4720204 – rs11771663, ~5.4Mb and rs6943120 – rs12706290,
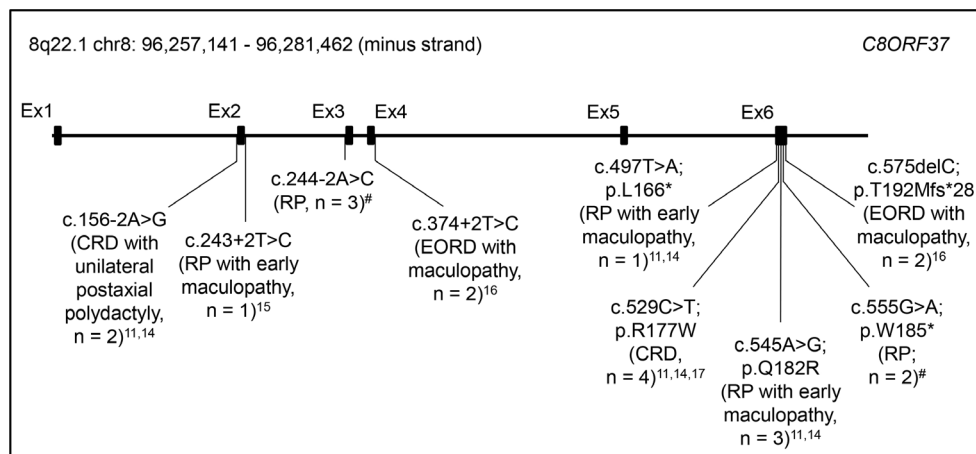

Figure 4. Diagram showing *C8orf37* mutations that have been identified to date and diagnosis of the patient(s) in whom they were found. The n value denotes the number of patients with that mutation. Thus far, 18 patients with *C8orf37* mutations have been described. The references that report the mutations are in superscript with # representing mutations identified in the current study.

~2.6Mb), 8 (rs4445224 – rs7842637, ~79.0Mb), 10 (rs6537586 – rs1973972, ~61.5Mb) & 15 (rs16940472 – rs8042228, ~2.4Mb) that are highlighted in red. The location of C8orf37 which is at ~14.4Mb on chromosome 8 is depicted. To access the data, click or select the words "Appendix 2."

APPENDIX 3. LIST OF VARIANTS IDENTIFIED AFTER FILTERING THE EXOME SEQUENCING DATA FROM PATIENT 863.

The chromosome, position, gene, coding effect, transcript accession number, exon, cDNA and protein changes as well as the minor allele frequencies in the exome variant server and the 1000 Genomes database are shown for the 73 homozygous variants. The C8orf37 mutation is highlighted in bold. To access the data, click or select the words "Appendix 3."

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