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Case Report Open Access

Novel *RP2* Gene Deletion in a Patient with Bilateral Retinitis Pigmentosa: Insights of Technical Challenges of Next Generation Sequencing

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Abstract

Massively parallel sequencing or next generation sequencing (NGS) panel-based testing has paved the way for rapid clinical molecular diagnosis of diseases by sequencing all known involved genes. One such condition is retinitis pigmentosa (RP), which is a genetically heterogeneous disorder that can be caused by autosomal recessive, autosomal dominant, digenic, or X-linked pathogenic variants, making a genetic diagnosis challenging. Large deletions and duplications in the RP genes have been known to contribute to a significant proportion of cases, and therefore, deletion/duplication analysis by various methods are often to be added to the RP NGS panel testing. In this report, we describe the process of identifying a large novel deletion in the X-linked *RP2* gene that presented novel challenges for the NGS-based molecular testing.

Keywords: *RP2* deletion; Retinitis pigmentosa; Molecular diagnosis; Next generation sequencing; NGS; Sanger confirmation; aCGH

Introduction

Retinitis pigmentosa (RP) (OMIM # 268000) is the most commonly inherited form of retinal degeneration, with disease incidence ranges from 1 in 2500 to 1 in 7000, and prevalence varying widely depending on population [1-3]. RP is associated with progressive retinopathy and degeneration initially presenting as loss of night vision, followed by tunnel vision development and slowly progressive decreased central vision. Bone spicule pigmentation occurring mostly in the periphery, attenuation of retinal vesicles and a waxy pallor of the optic nerve head typically occur in the fundus are the hallmark signs of RP. However, the clinical presentations can be diverse, at least partially due to the genetic diversity and phenotypic overlap between RP and other inherited retinal dystrophies such as Leber congenital amaurosis (OMIM # 204000) and cone-rod dystrophy (OMIM # 120970).

The landscape of genes involved in development of RP is vast, with as many as 84 genes associated with non-syndromic RP [4]. This genetic diversity can lead to difficulty in determining molecular diagnosis. Pathogenic variants in genes associated with RP can be transmitted in various inheritance patterns i.e., autosomal recessive, autosomal dominant, digenic, or X-linked pathogenic variants; pathogenic variants in the same gene may also be inherited in dominant or recessive manner. Additionally, with phenotypic overlap, discovering the underlying genetic cause of RP can be challenging. NGS panel testing has offered a relatively inexpensive and quick method of examining several genes simultaneously. According to Genetic Testing Registry (https://www.ncbi.nlm.nih.gov/gtr/, last accessed on September 07, 2018), there are over 150 different tests offered by CLIA-certified laboratories for diagnosis of RP ranging from panels targeting four genes to panels targeting over 1000 genes with approximately half offering deletion/duplication analysis and turnaround times of up to 98 days with an average of around 30 days.

The ARL3 GTPase activating protein, also called *RP2*, is expressed in the plasma membrane of photoreceptors, retinal pigment epithelium (RPE), and other cells [5,6]. The N-terminus has high homology to tubulin cofactor C (TBCC), which is a GTPase activating protein [7]. This region interacts with ARL3, a cytoplasmic GTPase protein, which regulates key chaperone proteins important in photoreceptor function and is involved in cell signalling and protein trafficking [8].

This report describes the process of identifying a novel deletion in the intron 1/exon 2 boundary of the *RP2* gene that uncovered additional challenges for NGS-based molecular testing. The deletion was 387 nucleotides, but was located at the intron/exon boundary, and escaped comparative genomics hybridization (CGH) detection. In addition, due to the intronic location and relatively small proportion of exon 2 that was deleted with acceptable coverage, this deletion variant escaped NGS low-coverage detection when an averaged value was used for the region but can be detected when coverage was calculated for the loci, rather than averaged.

Case Report

The patient is an 18-year-old adopted male with slowly progressive bilateral retinitis pigmentosa. He has myopia, has worn glasses since kindergarten, and has decreased best visual acuity since first diagnosis. He has moderate "beaten-metal" retinal pigment epithelium (RPE), retinal sheen centrally with diffuse RPE atrophy in the peripheral macula and attenuated vessel caliber throughout. On the retinal periphery, the patient has 2-3 generalized bone spicule pigmentary clumping with intervening RPE atrophy but no hemorrhages.

Methodology

Genomic DNA extracted from patient's peripheral blood sample using the PureGene extraction kit (Qiagen Inc., Valencia, CA) was

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used for simultaneous deletion/duplication analysis and the RP NGS panel testing. Deletion-duplication analysis was performed by using a custom-designed exonic array comparative genomic hybridization or aCGH with OGT CytoSure DNA labeling technique (Oxford Gene Technology, Tarrytown, NY) and scanned on the NimbleGen MS200 using a 2-micron laser (Roche, Basel, Switzerland). Data was analyzed using Feature Extraction and Nexus software (Agilent, Santa Clara, CA, US) to determine relative concentrations of the patient sample to the control DNA, revealing copy number imbalances in the patient's DNA. The 4-plex array consisted of 53 gene panel and was designed with targeted gene regions using the Agilent e-Array software (https://earray.chem.agilent.com). For each gene, all transcripts are covered on the array flanking 2 kb on either end of the most outside of any of the transcripts. For *RP2* there is only one RefSeq transcript, NM_006915. Probes were concentrated in exons, with 3-5 probes on an average in every exon. Sixty-mer probes were placed on average every 200 bp along the gene region. The gene region is defined as extending 2.5 kb from the beginning and end of the transcripts.

DNA is enriched for regions of interest by a solution capture method (SureSelect, Agilent, Santa Clara, CA) for NGS assay and then sequenced on the Illumina NextSeq NGS platform (Illumina, San Diego, CA). Sequencing reads in FASTQ format were aligned to the human reference genome hg19 using BWA (v0.6.1) [9]. The in-house developed bioinformatics pipeline workflow consists of aligning paired-end reads to human reference build 37 with BWA, PCR duplicates identified with Picard, and GATK was used for base quality scores recalibration, realignment of reads around indels, and candidate variant discovery. Gene variants are identified using two variant-calling software tools: FreeBayes for SNVs and small insertions/deletions and Pindel for larger insertions/deletions [10-12]. Detected variants are subjected to a rigorous manual curation process including querying variant databases (dbSNP, the NHLBI Exome Sequencing Project, the Exome Aggregation Consortium, HGMD, etc.), literature review, and classified based on ACMG guidelines [13]. Sanger confirmation is performed for variants classified as pathogenic, likely pathogenic or variant of uncertain significance.

This comprehensive NGS-based panel was designed to target 53 genes involved in the development of RP and/or Leber congenital

Set	Forward	Reverse
1	TGTAAAACGACGGCCAGTGGCAGCCAATAGTCCTTTAGTTGA	CAGGAAACAGCTATGACCCCAGTTGAGTTCTCCTGACACA
2	TGTAAAACGACGGCCAGTTACTCAAGGTCTGTGTTTTGTTCC	CAGGAAACAGCTATGACCCTGCATCTTTGAACTGGAAAGCTA
3	TGTAAAACGACGGCCAGTCTGGTCTCATCTGATCCTCCCA	CAGGAAACAGCTATGACCTGGTAACTGTAGCAGAGTGATCA

Table 1: Primer sequences.

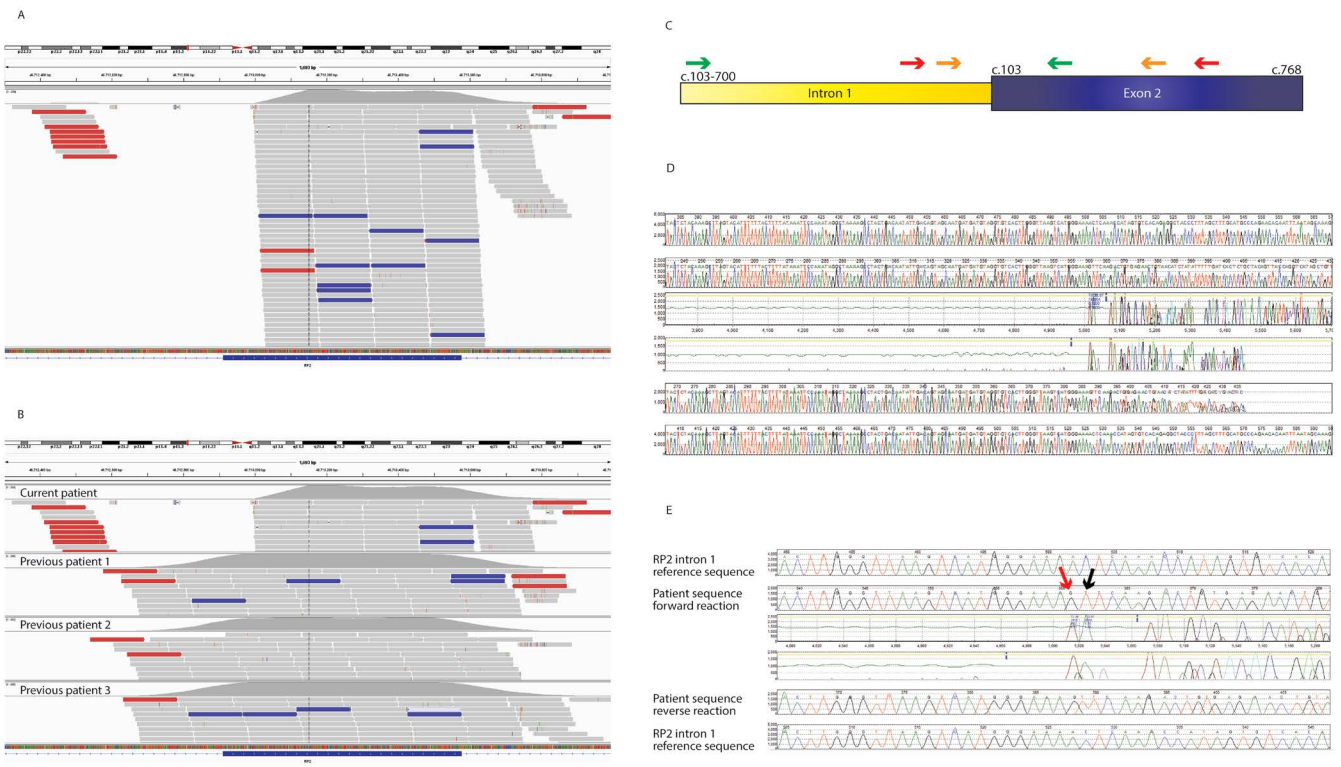

Figure 1: (A) Sequencing reads from ChrX:46,712,301-46,714,000 (by the Integrative Genomics Viewer). Manual inspection of the *RP2* exon 2 region yielded what appeared to be a large deletion extending from ChrX:46,712,612-46,712,998, encompassing at least a portion of *RP2* exon 2. (B) Comparing sequencing reads from several samples for ChrX:46,712,301-46,714,000. Manual inspection of the *RP2* exon 2 region comparing recent samples shows this is not a common deletion. (C) Primer design iterations. The first primer design is pictured in red (1), the second in yellow (2), and the third successful primer design in green (3). See Table 1 for primer sequences. (D) Sanger sequencing of *RP2* intron 1 region confirms the deletion. (E) *RP2* exon 2 Sanger sequencing data (by Mutation Surveyor V5.0.1). Red arrow indicates inserted G nucleotide and black arrow indicates c.191 with subsequent exon 2 sequence.

amaurosis. Targeted genes include: *ABCA, AIPL1, BEST1, CA4, CDHR1, CEP290, CERKL, CNGA1, CNGB1, CRB1, CRX, DHDDS, EYS, FSCN2, GUCA1B, GUCY2D, IDH3B, IMPDH1, KLHL7, LCA5, LRAT, MERTK, NR2E3, NRL, PCARE (formerly known as C2orf71), PDE6A, PDE6B, PRCD, PROM1, PRPF3, PRPF31, PRPF8, PRPH2, RD3, RDH12, RGR, RHO, RLBP1, ROM1, RP1, RP2, RP9, RPE65, RPGR, RPGRIP1, SAG, SEMA4A, SNRNP200, SPATA7, TOPORS, TTC8, TULP1,* and *USH2A*. Deep intronic and regulatory variants were not analyzed. Amino acids 737-998 of *RPGR* were not analysed.

Locations of the primers for Sanger confirmation of the detected deletion are depicted in Figure 1 and sequences are shown in Table 1. This case report did not meet the definition of human subject research by the University of Utah Institutional Review Board, thus was not subjected to the review process.

Results

The RP panel by NGS and exonic aCGH did not yield any pathogenic candidates initially by routine analysis in this patient with clinical suspicion of RP. However, upon further manual inspection of the raw sequences using Integrative Genomics Viewer (IGV, version 2.3.93 (151) Broad Institute, it was noted that the parallel sequencing did not adequately cover the boundary of *RP2* exon 2 with intron 1 (Figures 1A and 1B) [14,15]. The average coverage over all exons was 257.5X and the coverage for *RP2* exon 2 was 258.1X for other patients tested by the RP NGS panel. The exon is located from ChrX:46,712,911-46,713,576, however, the low-coverage region in this patient appeared to be located within intron 1 into exon 2 from ChrX:46,712,612-46,712,998.

In order to confirm the presence of a possible deletion discovered by manual sequence review, primers were designed to cover the region for a targeted Sanger sequencing, which detected a large deletion extending from intron 1 into exon 2 and the insertion of a single G nucleotide, c.103-299_190delinsG hemizygous in this male patient (Figures 1C, 1D and 1E) (Table 1).

Discussion

Deletions involving the *RP2* gene are rarely reported in the literature. There are only a handful of patients identified with gross deletions of varying size including a single patient with a complete gene deletion and another patient with 25 bp deletion [16,17]. All of these deletions were classified as pathogenic. The 387 bp deletion, c.103-299_190delinsG, located at the intron 1/exon 2- boundary of *RP2* gene, has not previously described in the literature or in gene-specific databases. However, this region is known to be a critical functional domain and as a mutational hot spot in humans. Deletion of this region in a mouse model results in progressive retinopathy [18,19]. The deletion observed in the current patient removes a portion of *RP2* intron 1 and exon 2, which may lead to an in-frame transcript lacking critical functional domains or may lead to an out-of-frame product due to a cryptic splice acceptor. Considering the information available and referencing ACMG variant classification guidelines, this variant is classified as likely pathogenic [13].

Bioinformatics pipelines for NGS data designed to detect small sequence alternations including small indels may still be challenged to detect large deletion/duplication variants. In general, bioinformatics pipelines for large indels >21 bp in length, structural variants (inversions and translocations), gene fusion variants and translocations, gene expression variations, epigenetic variants, copy number alterations, and other variants not defined as single nucleotide variants (SNVs) or small indels are not used widely for routine clinical diagnosis [20]. In this present study, our clinical NGS bioinformatics pipeline is not designed to detect deletions larger than 100 bp, and rarely deletions larger than 40 bp.

Deletions such as the one observed in this patient fall in the copy number variation (CNV) category or large indels, and it would not be discovered by the low-coverage indicator if the low coverage calculated using average of the exon. To our knowledge, Alu deletions have not been described in *RP2*, but at least one LINE1 insertion has been described in intron 1 [6]. Repeat Masker predicts this region contains a LINE2 low complexity region between c.103-96 and c.103-39. The aCGH platform is designed to detect whole and partial gene deletions in the targeted exonic regions. Due to the biological limitations, some exons may have limited number of probes or no probes. For these exons, the copy number variant encompasses intronic probes in order for the copy number variation to be called. The resolution of this aCGH is limited to 2.1 kb, indicating that deletions smaller than 2.1 kb might not be detected. However, if lowering the settings of "Minimum number of probes to = 2 and "Significance Threshold" to = -7, does result in calling the deletion in this *RP2* gene, but results in significantly increased numbers of uncertain calls of indels as well.

Manual review of the sequence data by IGV played a critical role in discovering the suspicious low-coverage region in this patient in comparison of the sequence data of the same region from several patients. In addition, several rounds of design for primers were performed in order to confirm the deletion due to a disconnect between identification of the deletion and primer design. Primers were initially designed to the deleted regions and Sanger failed. This highlights challenges in Sanger confirmation of variants identified by next generation sequencing and highlights the need for critical analysis of the sequence of large deletions prior to primer design for confirmation will ensure an efficient Sanger confirmation process.

Conclusion

This report describes the process of detecting a novel large deletion of *RP2* gene with modification/deviation of routine clinical NGS workflow. Both the size and the location of the deletion, together with the detection limit of both NGS and aCGH for indels, presented challenges for not only the current RP case but also can occur for any other NGS-based comprehensive testing. This case has also highlighted the importance of continuing improvement of bioinformatics pipeline for NGS data, careful manual review, and robust Sanger confirmation. Without all of these, this patient may have not obtained his molecular diagnosis as the X-linked RP due to pathogenic deletion in the *RP2* gene.

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Conflict of Interest

The authors have no conflict of interest to declare.

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