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A LINE-1 mediated deletion resulting in germline retinoblastoma predisposition

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Retinoblastoma is an ocular cancer associated with genomic variation in the *RB1* gene. In individuals with bilateral retinoblastoma, a germline variant in *RB1* is identified in virtually all cases. We describe herein an individual with bilateral retinoblastoma for whom multiple clinical lab assays performed by outside commercial laboratories failed to identify a germline *RB1* variant. Paired tumor/normal exome sequencing, long-read whole genome sequencing, and long-read isoform sequencing was performed on a translational research basis ultimately identified a germline likely de novo Long Interspersed Nuclear Element (LINE)-1 mediated deletion resulting in a premature stop of translation of RB1 as the underlying genetic cause of retinoblastoma in this individual. Based on these research findings, the LINE-1 mediated deletion was confirmed via Sanger sequencing in our clinical laboratory, and results were reported in the patient's medical record to allow for appropriate genetic counseling.

Case Description

A male proband presented to an outside institution at 22 months of age with vision concerns. He was subsequently diagnosed with bilateral retinoblastoma. In an attempt to salvage both eyes, he received systemic chemotherapy with a regimen that included cyclosporin, etoposide, carboplatin, and vincristine. At 24 months of age, progressive disease was noted, and he received bilateral external beam radiation therapy of approximately 4500cGy to both eyes. Following the radiation therapy, he subsequently underwent cryotherapy to eradicate the residual tumor. Regardless of these efforts, his disease progressed, and he received 4 months of treatment (32–36 months of age) on a salvage chemotherapy regimen with topotecan and cytoxan. Despite this treatment, he developed progressive disease, primarily in the left eye, and re-treatment with the original chemotherapy regimen was attempted at 37 months of age, including 3 additional cycles of cyclosporin, etoposide, carboplatin, and vincristine. Unfortunately, he again developed progressive disease. At this point, the family made the decision to enucleate the left eye (39 months of age), followed shortly by enucleation of the right eye (45 months of age). There was no evidence of widespread metastatic disease. He has continued to receive routine follow-up care, including regular brain/orbit only MRIs which have shown no evidence of tumor recurrence. He is now in remission and 16 years off therapy.

Pathology review of the enucleated right eye revealed corneal scarring, a clouded lens, and an atrophic ciliary body and iris. A diffuse, multifocal tumor was present in the retina, with a 2 × 1 mm mass superior to the macula, and a 1.5 × 2 mm mass in the inferior retina growing into the vitreous. There was an additional tumor present in the nasal retina near the equator, measuring 2 × 2 mm. Microscopically, the tumor was composed of small, round blue cells with high nuclear-to-cytoplasmic ratios and hyperchromatic nuclei. Pyknotic cells and mitotic figures were also noted in the tumor, and in some areas, the tumor had regressed and displayed low nuclear-to-cytoplasmic ratios, eosinophilic cytoplasm, and bland nuclei. The tumor invaded into the subhyaloid space, but not into the optic nerve head. The choroid was focally fibrotic and showed no evidence of tumor invasion. Retinal scarring was also noted. These findings were consistent with diffuse retinoblastoma postradiation therapy and chemoreduction, with associated vitreous seeding and type 3 regression.

At 14 years old, the proband was referred for genetic counseling to assess for inherited cancer predisposition and discuss reproductive risks. There was no family history of retinoblastoma or other tumors associated with *RB1*-driven disease (Figure 1), but as germline variants are present in virtually all cases of bilateral disease, a germline *RB1* variant was highly suspected. The patient's initial treatment occurred

at an outside institution and the tumor sample was not immediately available for testing. Germline *RB1* sequencing and microarray-based deletion/duplication testing were performed at an outside commercial laboratory and found to be negative. At age 16, the germline testing was repeated at a different commercial laboratory whereby *RB1* gene sequencing, allele-specific PCR, methylation-specific PCR, and MLPA-based deletion/duplication were performed. This testing was also negative. Due to the absence of a germline *RB1* alteration in the setting of bilateral retinoblastoma, the proband has consented to a translational research protocol for comprehensive molecular profiling. Under this protocol, he received paired tumor/normal exome sequencing, utilizing saliva as the germline comparator sample and formalin-fixed paraffin-embedded tissue from the right eye enucleation as the disease-involved specimen. By an internal pathology review, the disease-involved specimen was estimated at 30% tumor content and 60% necrosis. Exome sequencing was performed using NEBNext Ultra II FS DNA kit with paired-end Illumina sequencing to achieve >200x depth (see Supplementary Material for additional details). RNA-sequencing was also performed on the disease-involved specimen.

Paired tumor/normal exome sequencing revealed an *RB1* nonsense variant (NM_000321.3: c.1735C >T, p.Arg579Ter) at 11% variant allele fraction in the disease-involved tumor sample. This variant is predicted to result in loss of function either through nonsense-mediated decay of the *RB1* transcript or premature truncation of the encoded protein. This somatic variant has been previously reported in the setting of hereditary retinoblastoma, is classified as pathogenic by numerous clinical laboratories (ClinVar ID: 126785), and is consistent with the diagnosis of retinoblastoma.[1–5] Copy number analysis of the disease-involved sample also revealed gains of 1q and 6p (Supplementary Figure 1), which are recurrently observed in retinoblastoma.[6,7]

In the germline saliva sample, a 63bp in-frame insertion near the splice junction of exon 23 of *RB1* (c.2483_2484insGAAATAAAAGAGGACACAAACAAATGGAAGAACATTCCATGCTCATGGGTAGGAAGAATCA), p.(Arg828_Ser829insLysTerLysArgThrGlnThrAsnGlyArgThrPheHisAlaHisGlyTerGluGluSerIle) was called by bioinformatic analysis; however, upon manual review of the sequencing reads surrounding this region, this finding likely revealed a larger event rather than a 63bp insertion. Notably, a sharp drop in coverage near the end of exon 23 and a group of soft-clipped reads that did not align to the reference sequence were observed in ~50% of reads in both the germline and tumor samples (Figure 2A), suggesting a possible structural alteration at this site and highlighting the limitations of short-read exome sequencing. Using a Blast-Like Alignment Tool, these soft-clipped reads mapped with ~100% identity to over 200 regions of the genome (Supplementary Figure 2A). Using the RepeatMasker track in the University of California Santa Cruz genome browser, this region mapped within a Long Interspersed Nuclear Element-1 (or LINE-1) sequence element (Supplementary Figure 2B). To further characterize this event, Pacific Biosciences (PacBio) HiFi long-read genome sequencing was performed on peripheral blood from the proband and saliva from both parents (see Supplementary Material for additional details). In the proband, a 4539bp deletion beginning in exon 23 and expanding into intron 23 of *RB1* (Figure 2B) was observed. The region of the deleted sequence overlaps a LINE-1 sequence and thus is supportive of a LINE-1 mediated insertion–deletion event (Supplementary Figure 2C). This event was observed in 56% (9/16) of reads, consistent with a heterozygous alteration in the germline of the proband. Although the parental samples had low depth of coverage across *RB1,* with approximately five reads in the father and approximately three reads in the mother within the deleted region, there was no evidence for this event in either parent, suggesting that the event is likely de novo in the proband. Further supporting the likely de novo nature of this event is the lack of contributory family history in either parent or siblings (Figure 1). This event is predicted to result in a premature stop of translation in *RB1*. To further characterize the transcribed product, short-read RNA-sequencing was performed on the disease-involved tumor tissue and PacBio Iso-Seq (isoform sequencing aimed at the detection of full-length transcripts) was performed on peripheral blood

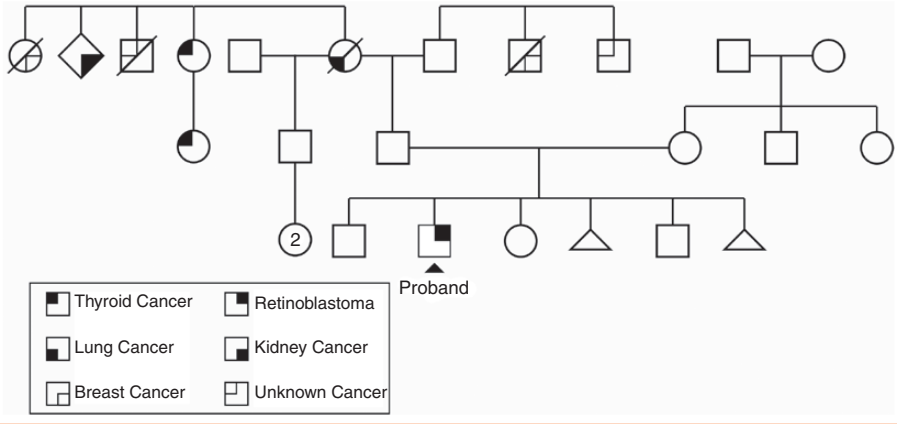

Figure 1. Three-generation pedigree. No family history of retinoblastoma was reported. Arrow indicates proband.

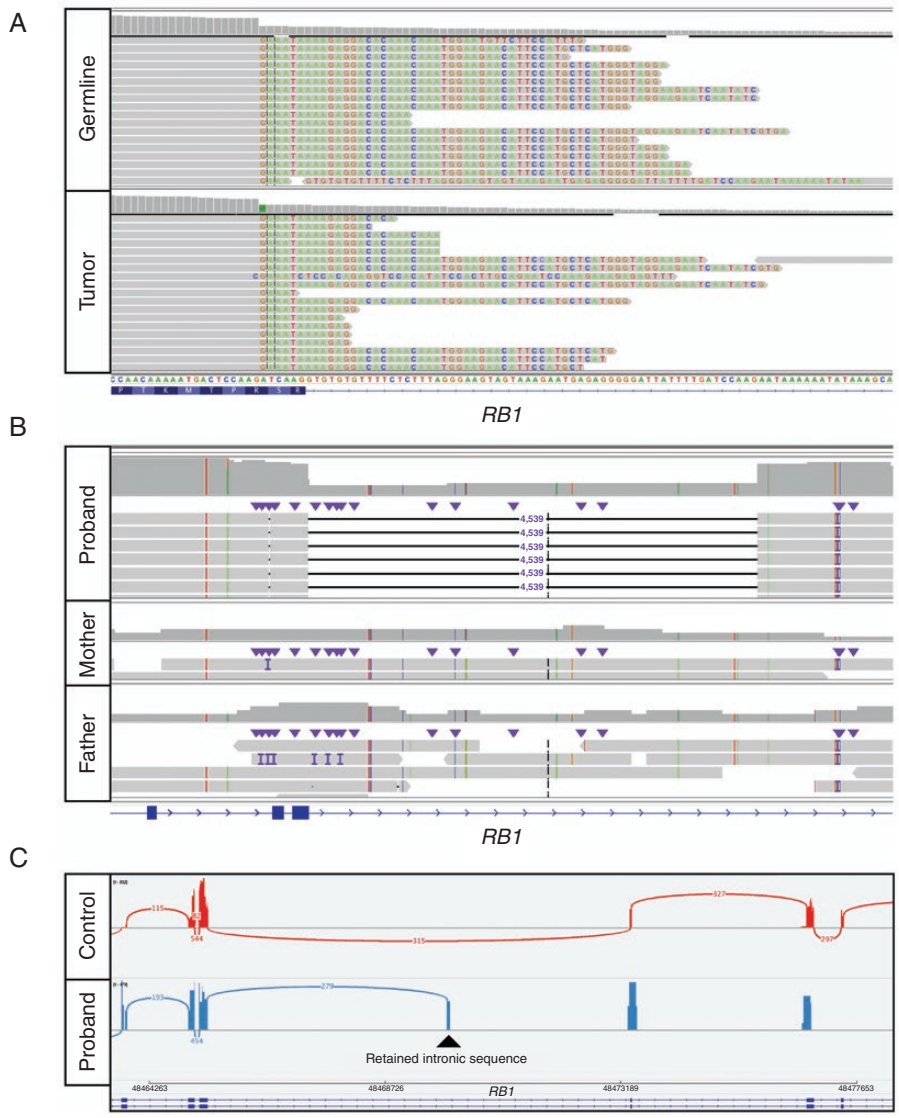

Figure 2. (A) Sequencing reads from germline (top) and tumor sample (bottom) display a sharp drop in coverage near the end of exon 23 of *RB1* and multicolored soft-clipped reads which do not align to the *RB1* reference sequence, noted at the bottom. (B) PacBio HiFi long-read genome sequencing indicates a 4539bp deletion beginning in exon 23 and expanding into intron 23 of *RB1* in the proband (top) that is absent from both parents (bottom 2 panels), suggesting this event likely occurred de novo in the proband. (C) RNA-sequencing performed on tumor sample from the proband indicates intron retention of material from intron 23 of *RB1*.

(see Supplementary Material for additional details). RNA-sequencing and Iso-Seq demonstrated disruption of the canonical splice site at the *RB1* exon 23 boundary and retention of sequence from intron 23 (Figure 2C). This event was observed in only a small number of reads by RNA-seq and in a single transcript supported by 6 reads from Iso-Seq long-read sequencing. However, premature truncation of the *RB1* transcript resulting in nonsense-mediated decay could result in these events being underrepresented at the RNA level. Additionally, diminished tumor content could also explain the low level of supporting reads in the RNA. Taken together, these data suggest that a germline likely de novo LINE-1 mediated deletion resulting in a premature stop of translation of *RB1* is the underlying genetic cause for the proband's bilateral RB.

Translational research results were returned to the clinical care team for consideration and subsequent discussion with the proband and his family. Following the disclosure of these research results, there was a desire to clinically confirm these findings and report them in the medical record. As we did not have clinically validated long-read sequencing available, and in consideration of a targeted approach, we performed clinical RT-PCR with Sanger sequencing (see Supplementary Material for additional details). We designed a primer set based on the abnormally spliced transcript identified by Iso-Seq due to the longer read sequence relative to the intron retention observed by short-read RNA-seq. A forward primer in exon 23 of *RB1* and reverse primer in intron 23 were designed to detect this germline event (Figure 3A). Confirmatory

Sanger sequencing results demonstrated a product which includes retention of intron 23 of *RB1* as demonstrated by RNA-sequencing (Figure 3B and C). Based on HGVS guidelines for sequence nomenclature,[8] when the size of the inserted sequence is not known, variants should be written using estimated size. In this case, the clinical assay used to confirm the LINE-1 mediated event could not fully deduce the variant size, despite our research-based assays fully characterizing the event. As such, this was clinically reported as (c.2484_2485ins(4686)). In accordance with ACMG/AMP guidelines,[9] this variant was classified as pathogenic. Loss of function impacting the RB1 protein is a known mechanism of disease in association with retinoblastoma (ACMG/AMP: PVS1), and the patient's presentation is highly specific for a disease with a single genetic etiology (ACMG/AMP: PP4).[10,11] Additionally, this variant is absent from gnomAD, a large-scale control population database (ACMG/AMP: PM2). A clinical report was issued into the medical record enabling informed management, surveillance, and genetic counseling regarding risk for secondary tumors and recurrence risk. Secondary cancers are known to occur in hereditary retinoblastoma survivors, with osteosarcoma and soft tissue sarcoma being 2 of the most common cancer types.[12,13] As a result, the proband continues to be followed in our survivorship clinic. He currently remains in remission. The proband was also interested in understanding his germline status for reproductive reasons, and these data were able to inform the recurrence risk for future offspring estimated at 50%. Knowledge of the specific disease-associated *RB1* variant in this proband enabled education and potential for consideration of reproductive technologies or prenatal or early childhood genetic testing for future offspring.

Discussion

Retinoblastoma is an ocular cancer associated with genomic variation in the *RB1* gene (OMIM: 180200). Retinoblastoma may present as unilateral (unifocal) or bilateral (multifocal), and typically occurs in children under the age of 5.[13] The mean age of diagnosis in individuals with unilateral retinoblastoma is 24 months, whereas bilateral retinoblastoma has a mean age of diagnosis of 15 months.[13] Nearly 100% of individuals with bilateral presentation, regardless of family history, have hereditary retinoblastoma.[13] Hereditary retinoblastoma is autosomal dominant in nature, caused by a germline heterozygous genomic alteration on 1 allele, with acquisition of somatic

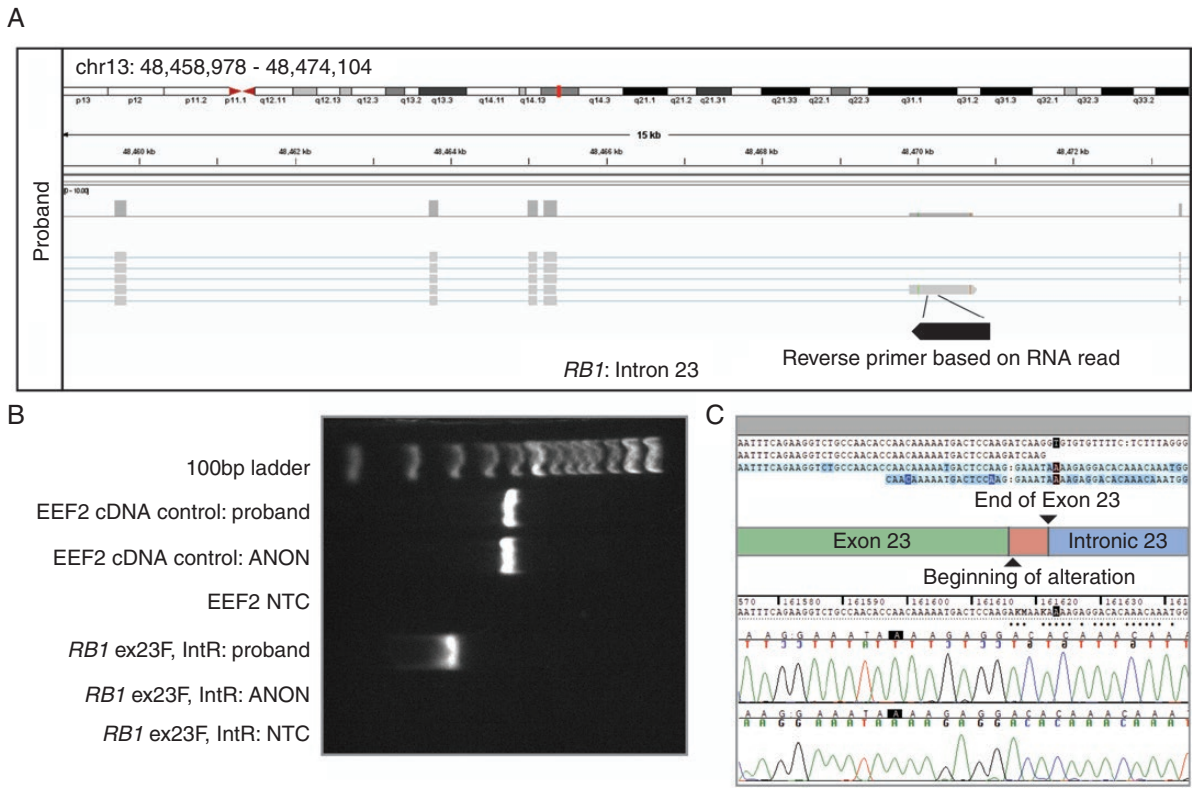

Figure 3. (A) Primers for confirmation of this event were designed based on the abnormally spliced transcript identified by Iso-Seq, with a forward primer in exon 23 of *RB1* and reverse primer in intron 23 designed from the Iso-Seq read. (B) An amplified product was obtained from the proband using these primers, but not an anonymous (ANON) control as expected. No product was observed in the no template control (NTC). Primers in *EEF2* were used as a control for RT-PCR. (C) Sanger traces (forward and reverse) from the proband confirmed the presence of LINE-1 associated material near the end of exon 23 of *RB1*.

variation on the other allele. The vast majority of pathogenic variants are loss of function and show complete penetrance.[14–16] Individuals with retinoblastoma are at increased risk of developing subsequent extraocular cancers, including bone cancers, soft tissue sarcomas, and melanoma, among others.[17–19] Herein, we present an individual with a heterozygous, likely de novo LINE-1 mediated deletion in *RB1* who presented prior to age 2 with bilateral retinoblastoma.

Transposable elements make up about 46% of the human genome.[20] They can be broadly classified into 2 major classes, DNA and RNA transposons, defined by the type of molecule used as an intermediate in their mobilization. Retrotransposons make use of an RNA-mediated transposition process by which they produce RNA transcripts, and use reverse transcriptase enzymes to convert the RNA sequences back into DNA, which is then inserted into the target site. Retrotransposons are subdivided into 2 major groups: those containing long-terminal repeats (LTR), and those that do not, termed non-LTR retrotransposons. Non-LTR retrotransposons are comprised of long interspersed nuclear elements (LINEs) and short interspersed nuclear elements and include autonomous and non-autonomous members.

LINE-1, or L1, elements are autonomous mobile elements with clear evidence of retrotranspositional activity in the human genome.[20–22] Mobile LINE-1 elements typically contain a 5′ untranslated region (UTR) with a strong internal RNA polymerase II promoter, 2 open reading frames (ORFs), and a 3′ UTR with a poly(A) tail. The first ORF encodes ORF1p, which is involved in RNA binding and is required for LINE-1 retrotransposition. The second ORF encodes ORF2p, which has endonuclease and reverse transcriptase activities essential for retrotransposition. Over time, greater than 99% of LINE-1 elements have been rendered inactive, primarily due to 5′ truncations disrupting the RNA polymerase II promoter site, or deleterious alterations within the ORFs.[21,23]

LINE-1 mediated retrotransposition events can result in various types of human structural variation.[20,21] L1 endonuclease, which nicks the DNA at target integration sites, may cause double-strand breaks, leading to genomic instability, or unequal homologous recombination between LINE-1 elements.[21,24,25] Retrotransposition events may directly disrupt exons or insert into introns to induce aberrant splicing or exon skipping.[21] Insertions and deletions are also known to occur at LINE-1 target sites and the areas surrounding where they excise.[21,24,25] While rare, these different mechanisms have been observed in the setting of germline cancer predisposition, including retinoblastoma[26] and neurofibromatosis type 1.[27,28] Additionally, LINE-1 transposition has been shown to drive tumorigenesis through somatic integration.[29–32] While the precise mechanism resulting in the insertion–deletion is not known in this proband, the presence of material corresponding to a LINE-1 transposable element provides strong evidence that LINE-1 retrotransposition or a LINE-1 mediated deletion was the driving event that predisposed this individual to retinoblastoma.

This case presents an uncommon mechanism for *RB1* loss of function which predisposes an individual to retinoblastoma and other cancers. The utility of translational research-based assays, such as RNA-seq and long-read sequencing, allowed for the characterization of this event. Confirmatory Sanger sequencing was necessary to report this finding in the proband's medical record, which ultimately required data from RNA-based assays to determine transcript structure and facilitate primer design. This individual underwent germline testing previously, including *RB1* sequencing and deletion/duplication analysis at multiple commercial laboratories, highlighting the need to understand the limitations of each assay, particularly when the clinical presentation is highly suspicious for a specific genetic etiology. These findings also highlight the utility of a manual review process of data, particularly when the common mechanism of disease is not fully explained by the findings. Through a manual review of the sequencing reads, we were able to identify that the variant initially called by our bioinformatics pipeline (63bp insertion) was in fact representative of a larger structural alteration resulting from a LINE-1-mediated insertion–deletion event. Finally, this case highlights LINE-1 activity as a unique mechanism of gene inactivation that while rare, is important to consider in prolonged diagnostic odysseys.

Supplementary material

Supplementary material is available online at *Neuro-Oncology* (https://academic.oup.com/neuro-oncology).

Keywords

cancer predisposition | long-read sequencing | LINE-1 | retinoblastoma

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Conflict of interest statement

None declared.

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Authorship statement

Conceptualization: E.L.M., C.E.C., K.M.S. Investigation: E.L.M., A.R.M., E.S., C.E.C., K.M.S. Clinical care: R.O., K.Z. Formal analysis: E.L.M., A.R.M., E.S., T.A.B., C.E.C., K.M.S. Writing (first draft): E.L.M., K.M.S. Writing (review and edit): E.L.M., A.R.M., E.S., R.O., K.Z., T.A.B., E.R.M., Y.M.N.A., C.E.C., K.M.S.

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