Molecular analysis distinguishes metastatic disease from second cancers in patients with retinoblastoma

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Abstract

The pediatric ocular tumor retinoblastoma readily metastasizes, but these lesions can masquerade as histologically similar pediatric small round blue cell tumors. Since 98% of retinoblastomas have *RB1* mutations and a characteristic genomic copy number "signature", genetic analysis is an appealing adjunct to histopathology to distinguish retinoblastoma metastasis from second primary cancer in retinoblastoma patients. Here, we describe such an approach in two retinoblastoma cases. In patient one, allele-specific (AS)-PCR for a somatic nonsense mutation confirmed that a temple mass was metastatic retinoblastoma. In a second patient, a rib mass shared somatic copy number gains and losses with the primary tumor. For definitive diagnosis, however, an *RB1* mutation was needed, but heterozygous promoter→exon 11 deletion was the only *RB1* mutation detected in the primary tumor. We used a novel application of inverse PCR to identify the deletion breakpoint. Subsequently, AS-PCR designed for the breakpoint confirmed that the rib mass was metastatic retinoblastoma. These cases demonstrate that personalized molecular testing can confirm retinoblastoma metastases and rule out a second primary cancer, thereby helping to direct the clinical management.

Keywords

retinoblastoma; metastasis; mutation detection; inverse PCR; differential diagnosis; second primary tumor

Introduction

Retinoblastoma is the most common pediatric eye cancer with an incidence of 1/16000 to 18000 worldwide [1]. Retinoblastoma results from biallelic mutation of the *RB1* gene (OMIM:180200), with a rare exception [2]. One *RB1* mutation is germline and heritable in 50% of patients [3]. Thousands of somatic and germline mutations have been identified in *RB1* in retinoblastoma tumors and patients, ranging from single nucleotide alterations to large chromosomal deletions (http://rb1-lsdb.d-lohmann.de).

When retinoblastoma is diagnosed early, >95% of cases are effectively treated [4]. However, some patients (2%) develop metastases [5, 6]. Retinoblastoma can invade optic nerve, sclera, uvea, extend extraocularly into orbit and brain, and/or metastasize through blood, especially to bone marrow [7, 8]. Survival from metastatic retinoblastoma is poor.

In addition to risk for metastasis, patients with heritable retinoblastoma also have increased risk of developing second primary cancers, particularly if treated with external beam radiation [9, 10]. These include soft tissue sarcomas, osteosarcoma, glioblastoma, melanoma, and brain tumors [11].

Distinguishing between metastatic disease and secondary cancer can be difficult in young retinoblastoma patients [12]. Metastatic retinoblastoma may have cytomorphologic features that overlap with other small round blue cell tumors, such as rhabdomyosarcoma, lymphoma, or nephroblastoma [13]. Making this distinction is important as the clinical management for metastatic retinoblastoma differs from the management of other cancers. Here, we demonstrate the utility of molecular testing for diagnosis of retinoblastoma metastases.

Materials and methods

RB1 Mutation Detection

RB1 mutations in eye tumors were identified by sequencing, AS-PCR for recurrent mutations (as seen in Patient A), and/or quantitative multiplex PCR (QM-PCR) for *RB1* and copy number of genes characteristic of retinoblastoma. These techniques were performed as previously described [14-16].

aCGH

Tumor DNA of Patient B was extracted from ten 25 μm rib tumor tissue sections, using the QIAamp DNA FFPE Tissue kit (Qiagen, Valencia, CA, USA). Array comparative genomic hybridization (aCGH) was performed on this DNA hybridized with same-sex normal reference DNA (Kreatech, Amsterdam, Netherlands), using the CytoSure ISCA 8x60K v2.0 array platform (Oxford Gene Technology, Tarrytown, NY, USA), followed by data analysis with CytoSure Interpret software v4.7.13. All nucleotide coordinates are based on the GRCH37/hg19 assemblies.

Inverse PCR

By examination of the QM-PCR and aCGH results, Patient B's breakpoint was determined to lie between the exon 11 QM-PCR primers and the right flanking, 2-copy aCGH probe, at g.48942813 and g.48945286, respectively. This corresponds to positions g.69931 and g.72404 of *RB1* (GenBank accession number NG_009009.1). *Eco* RI was chosen for restriction digestion as it does not cut within this normal sequence and 2 kbp upstream. Thus, fragments <5 kbp would not be found in normal DNA.

Tumor or normal DNA (1 μg) was digested with *Eco* RI, 3 h, 37°C, then 450 ng was self-ligated in a 450 μL reaction volume with T4 DNA ligase, 16°C overnight. After clean up, 100 ng of ligated DNA or unligated control DNA were used in a 50 μL PCR reaction containing KOD buffer, 0.5 μL KOD polymerase, 200 μM dNTPs, 2 mM $MgSO_4$, 1.25 M Betaine, and 1 μM each primer. Inverse PCR primers were chosen in the normal sequence just downstream of the putative deletion region: F (72763-72784) CAACGATAGTGGTGGGAATGAA, R (72645-72665) CTCAGTGGAATGGGACACAAA. The PCR protocol was 95°C 2 min, then 35 cycles of 95°C 20 s, 58°C 10 s, 70°C 2 min, then 10 min at 70°C. Samples were analyzed by agarose gel electrophoresis and excised bands cycle sequenced using the same PCR primers (GenScript, Piscataway, NJ, USA).

To confirm specificity, nested PCR was performed using similar conditions, with 1 μL of the first round PCR reaction as template and primers F (72773) GGTGGGAATGAAGGAACAATAAC, R (72565) GGTTAAGAACCACTGAGACAGAC.

Patient-specific AS-PCR

AS-PCR primers unique to Patient B's deletion were designed and optimized using methods previously described [17]. Specific conditions included 33 cycles, an annealing temperature of 55°C, and primers F CATCAAGACGCCAAATCTCTG, R TAATCGAACCTAAGAGGTGTC.

Results

Patient A: Temple Tumor

A 19 month old female presented with unilateral retinoblastoma (Group D, diffuse seeding of tumor below retina or into vitreous, International Intraocular Retinoblastoma Classification

[IIRC] [18]). The eye was enucleated and histopathology was interpreted to be pT2b (tumor superficially invades optic nerve head but does not extend past lamina cribrosa and exhibits focal choroidal invasion [19]), with no high risk features such as "massive" choroidal invasion (which would be pT3) (Figure 1A). Genetic analysis revealed a germline c.62delC (p.Pro21ArgfsTer43) *RB1* mutation, and a somatic c.763C>T (p.Arg255Ter) mutation. A temple mass appeared four months later and was biopsied. Multiple CNS and bone marrow masses were then discovered on imaging (Figure 1B). Although location and histology of the temple mass was suggestive of metastatic retinoblastoma (Figure 1C), molecular analysis was employed for confirmation. AS-PCR enabled confirmation of the somatic mutation in the mass (Figure 1D). Re-review of the pathology and serial sections of the whole eye revealed a focus of tumor within a scleral blood vessel (Figure 1A), which still would not be designated "high risk" according to the 2010 AJCC Cancer Staging Manual [19], where sclera is not mentioned. However, tumor invasion into the sclera has been suggested to indicate high risk [20]. With retinoblastoma metastasis confirmed, high dose systemic chemotherapy followed by autologous bone marrow transplant (BMT) was performed but with poor response. The child was started on palliation and died 25 months after initial diagnosis.

Patient B: Chest Wall Tumor

A 24 month old male presented with unilateral retinoblastoma (IIRC, group D [18]). The eye was enucleated, and histopathology revealed no high risk features (pT2a, focal choroidal invasion [19]) (Figure 2A, B). Our standard *RB1* mutation detection workflow [14] identified a deletion, promoter→exon 11, in the primary tumor. No second, tumor-specific *RB1* mutation was found, nor any constitutive *RB1* mutation. The child was followed in clinic every three months. A year later the child experienced night pains and fever, initially misdiagnosed as Kawasaki's disease

until a paravertebral mass (Figure 2C) was detected on MRI; fine needle aspiration cytology revealed a small round cell tumor (Figure 2D). The differential diagnosis included a second primary such as Ewing's sarcoma, or metastatic retinoblastoma, which was considered unlikely due to the absence of histopathological features indicating risk for metastases. Serial sections of the whole eye again confirmed pT2a with focal choroidal invasion, not considered to indicate high risk for metastasis.

Given the histopathologic uncertainty, we again employed molecular analysis to characterize this mass. We analyzed DNA from the rib mass and the primary tumor for the "hotspot" copy number change profile characteristic of retinoblastoma [16]. Both tumors shared the same pattern of common copy number changes of retinoblastoma (Figure 2E). Moreover, aCGH of rib mass DNA confirmed a pattern of genome-wide copy number changes consistent with those seen commonly in retinoblastoma (Figure 2F) [21]. This shared genomic "fingerprint" suggested that the rib mass and the primary tumor shared the same origin.

Inverse PCR Identifies a Deletion Breakpoint

To monitor this tumor, the identity of the unique deletion breakpoint was needed to enable AS-PCR. aCGH confirmed a deletion of ≥238 kbp spanning the 5′ end of *RB1* in the primary tumor (Figure 2G). Due to wide spacing of aCGH oligonucleotide probes around the deletion, a higher-resolution approach was required to identify the precise deletion breakpoint. We turned to inverse PCR for this task. Based on the known, flanking two-copy QM-PCR primer and aCGH probe locations, we designed primers for inverse PCR (Figure 2H). These primers yielded a 2.6 kbp band specific to tumor DNA that had undergone ligation (Figure 2I).

The 3′ ends of both the 2.6 kbp inverse PCR product and a confirmatory 2.5 kbp nested PCR product (data not shown) contained sequence that mapped to the *RB1* gene, as far upstream as g.71606. However, the 5′ ends of these PCR products mapped to sequence upstream of the *HNRNPA1L2* gene, confirming the breakpoint location. This gene lies ~4 Mbp telomeric of *RB1*, suggesting an unbalanced inversion. Using the breakpoint sequence, we designed primers that were specific for tumor DNA. This primer set could detect one part tumor DNA in 1000 parts normal DNA (Figure 2J), indicating a reasonably sensitive assay for minimal residual disease detection. The patient's rib mass and pre-treatment bone marrow were both strongly positive, while post-treatment bone marrow was negative (Figure 2J). With metastatic retinoblastoma diagnosis confirmed, the child received systemic chemotherapy followed by high dose chemotherapy with autologous BMT. The child remained in remission for 12 months, then brain and meningeal recurrences reappeared. The child died 18 months after presentation with metastasis, 30 months after initial retinoblastoma diagnosis.

Discussion

We describe two patients originally diagnosed with retinoblastoma who subsequently developed additional tumors. After inconclusive histology, to ascertain if these were metastases, we employed molecular genetic strategies, including a novel use of inverse PCR to develop an AS-PCR assay for the breakpoints of a large deletion.

In both cases, the *RB1* mutation originally found in the eye tumor was also present in the subsequent extraocular tumor, confirming that the disease was metastatic. In both cases, anatomic pathology failed to indicate risk of metastasis; both tumors behaved in an unusually aggressive manner that warrants further research. This report illustrates the value of innovative,

personalized molecular techniques in the differential diagnosis and management of metastatic retinoblastoma patients.

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Conflict of Interest

HR, DM, DR and BP are employees of Impact Genetics. BLG is an unpaid consultant to Impact Genetics. The other authors declare no conflict of interest.

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Figure legends

Figure 1 Molecular confirmation of retinoblastoma metastatic to temple and humerus. (A) No features scored for high risk on pT2b eye pathology (H&E stained section of eye; blue box: retinal pigment epithelium upper right corner, with artifactual implantation of loose tumor between choroid and sclera; green box: tumor in a blood vessel in sclera; red box: tumor invasion of optic disc anterior to lamina cribrosa, yellow line). (B) Clinically apparent temporal mass (arrowhead) involving orbit and extradural space (arrow). (C) Histology of the temple mass invading muscle (H&E stained biopsy) is suggestive of retinoblastoma. (D) Agarose gel of AS-PCR product confirms the presence of the somatic *RB1* mutation in the temple mass, but not in the cerebrospinal fluid (CSF).

Figure 2 Clinical features and molecular characterization of retinoblastoma metastatic to the ribs. (A) No features scored for high risk on pT2a eye pathology: green box shows small round blue cells; blue box shows intact retinal pigment epithelium and no invasion of sclera; (B) separate section of whole eye shows optic nerve dragged into the eye with no optic nerve invasion past cribriform plate. (C) MRI reveals a paravertebral mass (arrow). (D) Histology of paravertebral mass is inconclusive. (E) Quantitative multiplex PCR indicates gene gains and losses, common in retinoblastoma, shared between primary tumor and rib mass: three copies of *KIF14* (1q32) and *E2F3* (6p22), four copies of *DEK* (6p22), and one copy of *CDH11* (16q22), although *MYCN* (2p24; commonly gained) was two-copy. (F) Whole genome aCGH profile of the rib mass DNA confirms a retinoblastoma-like pattern of genomic gains and losses: large gains at chromosomes 1q, 6p, 9q, 13q and 17q, and large losses at chromosomes 1p, 13p and 16q. (G) aCGH defines a partial deletion of the *RB1* gene: arr[hg19] 13q14.2(48703647-48941658)x1. (H) Inverse PCR strategy for sequencing the breakpoint. (I) Successful

252 amplification of an inverse PCR product. T, tumor DNA; N, normal blood DNA. (J) Agarose gel
253 of AS-PCR product confirms the presence of this deletion in the rib mass and in bone marrow
254 (BM) DNA prior to therapy, and absence on indicated days after therapy.