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

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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 1/16,000 to 18,000 worldwide (1). Retinoblastoma results from biallelic mutation of the *RB1* gene (OMIM:180,200), 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

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radiation (9,10). These include soft tissue sarcomas, osteosarcoma, 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 8 × 60K 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) CAACGATAGTGGTGG GAATGAA, 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) GGTGGGAATGAAGG AACAATAAC, 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-examination 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