REVIEW

Retinoblastoma, the visible CNS tumor: A review

Helen Dimaras[1,2,3,4,5] | Timothy W. Corson[6,7,8,9]

[1]Department of Ophthalmology and Vision Sciences, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada

[2]Division of Clinical Public Health, Dalla Lana School of Public Health, University of Toronto, Toronto, Ontario, Canada

[3]Department of Ophthalmology and Vision Sciences, The Hospital for Sick Children, Toronto, Ontario, Canada

[4]Child Health Evaluative Sciences Program, SickKids Research Institute, Toronto, Ontario, Canada

[5]Department of Human Pathology, College of Health Sciences, University of Nairobi, Nairobi, Kenya

[6]Eugene and Marilyn Glick Eye Institute, Department of Ophthalmology, Indiana University School of Medicine, Indianapolis, Indiana

[7]Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana

[8]Department of Pharmacology & Toxicology, Indiana University School of Medicine, Indianapolis, Indiana

[9]Indiana University Melvin and Bren Simon Cancer Center, Indianapolis, Indiana

Correspondence
Timothy W. Corson, Eugene and Marilyn Glick Eye Institute, Department of Ophthalmology, Indiana University School of Medicine, 1160 West Michigan Street, Indianapolis, IN, 46202.
E-mail: tcorson@iu.edu

Abstract

The pediatric ocular cancer retinoblastoma is the only central nervous system (CNS) tumor readily observed without specialized equipment: it can be seen by, and in, the naked eye. This accessibility enables unique imaging modalities. Here, we review this cancer for a neuroscience audience, highlighting these clinical and research imaging options, including fundus imaging, optical coherence tomography, ultrasound, and magnetic resonance imaging. We also discuss the subtype of retinoblastoma driven by the *MYCN* oncogene more commonly associated with neuroblastoma, and consider trilateral retinoblastoma, in which an intracranial tumor arises along with ocular tumors in patients with germline *RB1* gene mutations. Retinoblastoma research and clinical care can offer insights applicable to CNS malignancies, and also benefit from approaches developed elsewhere in the CNS.

KEYWORDS

cancer genetics, *MYCN*, neuroimaging, optical coherence tomography, pediatric cancer, pineoblastoma

1 | INTRODUCTION

The pediatric retinal tumor retinoblastoma is the most common eye cancer in children. It is unique among central nervous system (CNS) tumors because it is visible through the eye without requiring invasive imaging. This provides an unparalleled opportunity to image tumors as they develop or respond to treatment. Moreover, retinoblastoma provides intriguing insights into nervous system disease in its trilateral and *MYCN*-driven forms.

In this review, we introduce retinoblastoma with a focus on these features. In the first part of the review, we describe the clinical, molecular, and histological features of this tumor in the context of its treatment and prognosis. In the second section of the review, we focus on

Significance

Retinoblastoma is the most common eye cancer in children. It arises in the developing retina, part of the central nervous system (CNS). Thus, retinoblastoma is a CNS cancer viewable from outside. Retinoblastoma is usually caused by mutations in the *RB1* gene, but it can also be caused by amplification of the neuroblastoma oncogene *MYCN*. Rarely, *RB1* gene mutations cause a tumor within the brain as well as eye tumors. Retinoblastoma can be imaged by multiple means, including visually through the eye, by ultrasound, magnetic resonance, and optical coherence tomography. Together, these features make retinoblastoma a unique case study amongst CNS tumors.

innovations in the imaging of retinoblastoma to highlight what this pediatric ocular cancer tells us about CNS malignancy. We follow this with a discussion of the aforementioned trilateral and *MYCN*-driven forms of retinoblastoma, with the goal of highlighting what retinoblastoma research can gain from studies of other CNS malignancies, and vice versa.

2 | RETINOBLASTOMA OVERVIEW

2.1 | Clinical vignette

A mother in urban Canada is taking photographs of her child, when she notices a strange white reflex in the child's eye (Figure 1a). Unsure what it is, later that night she types "white eye in baby" into an Internet search engine. A list of results pops up, alerting her that a white pupil could be a sign of cancer. She immediately seeks attention at the nearest hospital, where the child is diagnosed with retinoblastoma.

Similarly, one evening a mother in rural India notices a white reflex in the eye of her child. Her baby does not seem bothered by it, so she does not worry about it too much. Later that month, a community health worker arrives to check on the family. The mother asks about the odd white reflection in her baby's eye. The health worker has just been trained on a new app that helps in the diagnosis of eye conditions. Using her smartphone, she takes a flash photo of the child's eye, and uses the app's database of eye conditions to compare her photograph. She realizes the photo she took looks similar to the database photo of retinoblastoma, so she refers the family to hospital.

2.2 | Clinical features

These hypothetical cases illustrate typical presentations of retinoblastoma. The incidence of retinoblastoma is constant worldwide at 1 in every 16,000 live births (Dimaras et al., 2012, 2015). As such, countries with higher birth rates and populations observe the highest numbers of cases. The most common first signs of retinoblastoma are leukocoria, a white reflex visible through the pupil (Figure 1a); and strabismus, misaligned eyes. Awareness of these early signs of eye cancer is crucial to good outcomes for affected children. However, where awareness of these signs is low, and access to health care is challenging, retinoblastoma is often diagnosed late. In this case, the most common sign at presentation may be proptosis, where tumor causes the eye to bulge from the orbit. Retinoblastoma can affect one or both eyes, and sometimes also the pineal, parasellar, or suprasellar regions (trilateral retinoblastoma, discussed below).

Detection of retinoblastoma by laypeople is possible because retinoblastoma is a visible tumor with distinct features. Several awareness efforts aim to improve public knowledge of the early visible signs of retinoblastoma. These include low-tech innovations, such as including images of leukocoria in Maternal Child Health booklets (Ministry of Health Kenya, 2010) provided to mothers shortly after childbirth, to
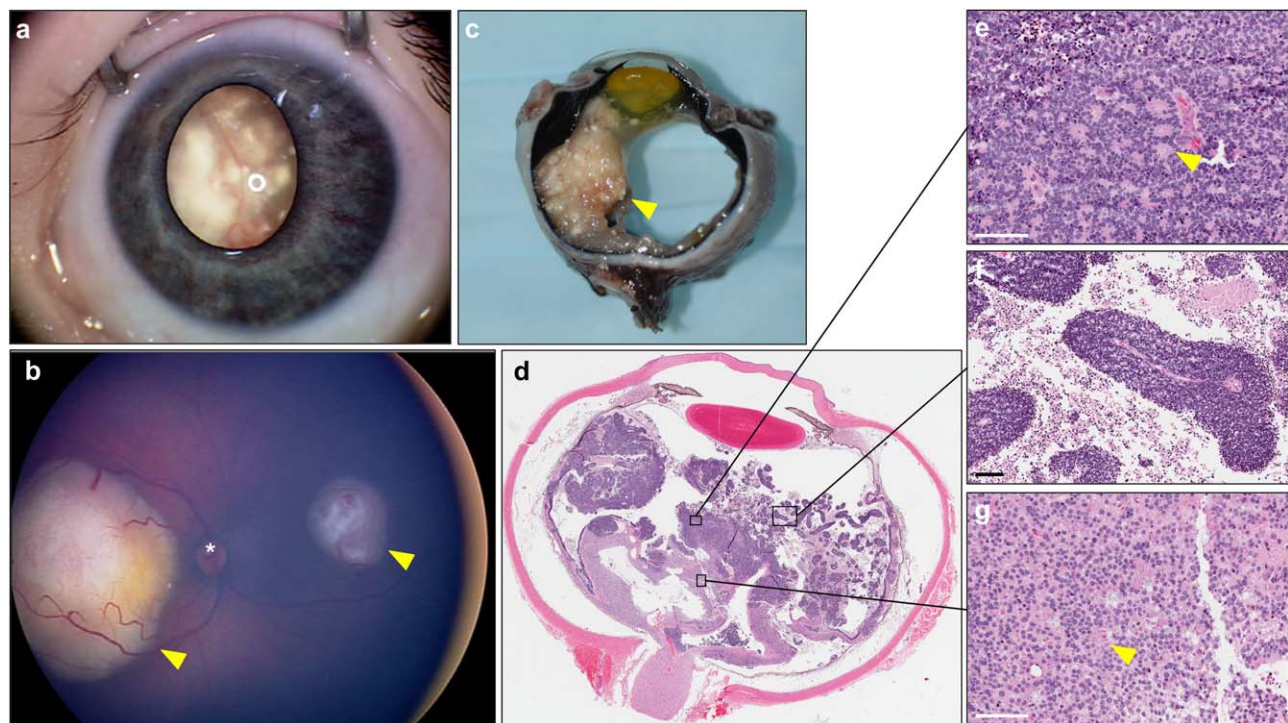

FIGURE 1 Clinical features of retinoblastoma. (a) External Retcam image of retinoblastoma; calcified tumor is visible through the pupil as leukocoria. (b) Retcam fundus image revealing two tumors (arrowheads). *Optic nerve head. (c) Gross pathology of an enucleated eye. A large, calcified tumor is apparent on the left side of the globe (arrowhead). (d) Pupillary–optic nerve section of an eye enucleated for retinoblastoma as primary treatment. (e) Homer Wright rosettes (arrowhead) and packed cells; (f) viable collars of cells around blood vessels, and abundant necroses between them; (g) an area of retinoma, with fleurettes (arrowhead). Scale bars = 100 μm

alert them of the signs of retinoblastoma. High-tech innovations include concepts like the Portable Eye Examination Kit (Peek), a smartphone application that allows users to recognize and detect eye conditions, including retinoblastoma (Bastawrous, 2016; Bastawrous et al., 2015; Lodhia, Karanja, Lees, & Bastawrous, 2016; Morjaria, Bastawrous, Murthy, Evans, & Gilbert, 2017).

Medical diagnosis of retinoblastoma is based on the clinical features of the tumors visible in the eye upon dilation of the pupil (Dimaras et al., 2012) (Figure 1b). This differs from diagnosis of other cancers, where histological confirmation via biopsy is usually required. Biopsy of retinoblastoma is not recommended because it can induce seeding and extraocular spread along the needle tract (Karcioglu, 2002).

Retinoblastoma is often highly calcified (Figure 1c) (Bullock, Campbell, & Waller, 1977). Calcification is visible to the naked eye and can also be detected by ultrasound or magnetic resonance imaging (MRI) (Rodjan et al., 2015) (see below). Calcification in retinoblastoma is "dystrophic" (Eagle, 2013)—that is, it occurs in response to tissue damage or necrosis. It is more often observed in tumors of older children and advanced retinoblastoma (Levy, Frenkel, Baras, Neufeld, & Pe'er, 2011).

Retinoma, the benign precursor of retinoblastoma, also displays calcification, along with several distinct clinical features; it is described clinically as a gray, translucent mass, often with chorioretinal atrophy and variation in the retinal pigment epithelium (Dimaras et al., 2008, 2009; Gallie, Phillips, Ellsworth, & Abramson, 1982). Retinoma is rare, but sometimes it can be observed in parents of children with retinoblastoma (Dimaras et al., 2008; Gallie, Ellsworth, Abramson, & Phillips, 1982). Its rare occurrence suggests that most retinomas undergo malignant transformation to retinoblastoma.

Growing retinoblastoma tumors produce seeds that detach and adhere below the retina (subretinal seeds) or float into the vitreous (vitreous seeds). The appearance of seeds has been described as fine dust, spheres, or clouds, each with a progressively worse prognosis, respectively (Francis et al., 2015; Francis, Marr, & Abramson, 2016; Munier, 2014). Large tumors can lift and detach the retina and cause significant inflammation and necrosis, sometimes leading to phthisis bulbi (shrinkage of the eye) (Gallie, Phillips, et al., 1982; Kashyap et al., 2011).

Tumor can spread beyond the eye by surpassing retinal boundaries, such as Bruch's membrane, to invade the choroid and reach the blood supply. Another common route for tumor dissemination is via the optic nerve to reach the brain and cerebrospinal fluid (CSF) (Mallipatna et al., 2017).

2.3 | Staging

The features of retinoblastoma at presentation provide data to accurately classify the disease in each eye, and consequently direct treatment. During the era when retinoblastoma was primarily treated by radiation, the Reese-Ellsworth Classification (Reese & Ellsworth, 1963) system was used, separating tumor classes by their predicted response to radiation treatment. As systemic chemotherapy began to replace radiation as the most common form of treatment, the International Intraocular Retinoblastoma Classification (IIRC) (Murphree, 2005)

system was developed. This system is largely based on natural progression of disease from small tumors away from the macula (easily treatable with laser therapy) and larger, central tumors with variable degrees of seeding and retinal detachment (requiring chemotherapy in addition to focal therapy). Modifications to the original IIRC were later introduced by another group (Shields et al., 2006), making it difficult to compare across studies and centers (Novetsky, Abramson, Kim, & Dunkel, 2009). The critical differences between the classification systems have previously been reviewed (Dimaras et al., 2015). Other classification schemes include staging of extraocular retinoblastoma (Chantada et al., 2006) and staging of vitreous seeding (Francis et al., 2015, 2016; Munier, 2014).

As with other cancers, retinoblastoma is also staged using the American Joint Commission on Cancer's "Tumor Node Metastasis" (TNM) classification system. The most recent (8th) edition of the TNM (Mallipatna et al., 2017) aims to provide a way forward for retinoblastoma classification, given the above eye-based classification confusion. The Tumor stage is first applied per eye based on clinical features, and the patient's score is based on the most advanced eye (Mallipatna et al., 2017). Radiology plays an important role in determining the Metastasis stage, particularly in delineating any involvement of the CNS. The 8th edition of the TNM also includes a new feature, the additional "H" or heritable trait, which denotes risk of multifocal tumors that can affect both eyes and brain (trilateral retinoblastoma). As yet, trilateral retinoblastoma is not staged by the TNM system. In fact, no other tumors of the CNS are staged by TNM, as tumor size is not a prognostic indicator, the brain and spinal cord do not have a lymphatic system, and metastases are rarely distant (Laws Jr. et al., 2017). Retinoblastoma is a unique CNS tumor in this respect because tumor size is prognostic; it can affect preauricular, submandibular, or cervical nodes; and distant metastases occur (Mallipatna et al., 2017).

2.4 | Pathology

Retinoblastoma is a small round blue-cell tumor (Figure 1d,e), and thus bears histologic similarity to other childhood nervous system tumors including neuroblastoma and medulloblastoma. Cells are primarily composed of large basophilic nuclei and little cytoplasm. The tumor is highly proliferative, displaying abundant mitoses and necrosis. Cells may take on a polygonal shape and mimic cobblestones when packed together (Tso, 1980). Often, collars of viable cells can be observed around a blood vessel, surrounded by necrosing cells (Figure 1f). Tumors display varying degrees of differentiation. Cellular differentiation is primarily in the form of Flexner–Wintersteiner rosettes and Homer Wright rosettes (Tso, 1980) (Figure 1e). Flexner–Wintersteiner rosettes are composed of an "empty" lumen surrounded by columnar cells (Wippold & Perry, 2006). Homer Wright rosettes consist of cells surrounding a central lumen made up of their processes (Wippold & Perry, 2006). While each type is individually common in other neural tumors, the dual presence of Homer Wright and Flexner–Wintersteiner rosettes is pathognomonic for retinoblastoma.

Retinoma histopathology is distinct from retinoblastoma (Figure 1g). This benign precursor is composed of sparsely spaced round cells

and does not contain rosettes. Instead, it is characterized by the presence of fleurettes, composed of clusters of cells with long, bulbous cytoplasmic processes, mimicking the fleur-de-lys (Dimaras et al., 2008, 2009). Retinoma is observed next to retinoblastoma in 16% to 20% of eyes enucleated for retinoblastoma (Dimaras et al., 2008; Eagle, 2009).

Cellular differentiation has not been conclusively linked to prognosis, and thus does not form part of the histological evaluation of tumors. However, the degree of anaplasia evident in retinoblastoma has been shown to correlate with prognosis (Mendoza et al., 2015), but this has not yet become part of routine histological evaluation. The pathology TNM (pTNM) classification determines extent of tumor involvement of the optic nerve, choroid, sclera, and anterior segment (Finger, 2009; Gallie et al., 2016; Mallipatna et al., 2017). High risk of metastasis (pT3) is conveyed by $>3\,\mathrm{mm}$ of tumor in the choroid (the vascular layer of the eye, under the retina), tumor extending beyond the lamina cribrosa of the optic nerve (the perforated or "mesh-like" structure in the sclera through which the fibers of the optic nerve exit the eye), or significant invasion of the sclera. Extraocular disease (pT4) is marked by tumor at the cut end of the nerve; involvement of the episclera; or invasion of adjacent bone, eyelid, conjunctiva, or muscle (Mallipatna et al., 2017).

2.5 | Genetics

Retinoblastoma is perhaps best known to cancer biologists as the inspiration for the "two-hit hypothesis" for oncogenesis, which states that loss of both alleles of a tumor suppressor gene is necessary to initiate cancer. Working in the pre-genomic era, Alfred Knudson noted an earlier age of onset for bilateral retinoblastoma than for unilateral (Knudson, 1971). His mathematical analysis implied that two rate-limiting steps were needed for unilateral retinoblastoma to develop, while only a single rate-limiting step was required for bilateral cases. Fifteen years later, the "retinoblastoma gene," RB1, was cloned, confirming Knudson's prediction (Friend et al., 1986; Fung et al., 1987; Lee et al., 1987). Unilateral patients (usually) have lost both wild-type alleles of RB1 in a susceptible retinal cell (two hits), leading to a single, unifocal tumor (Figure 2). Conversely, all bilateral patients have a germline mutation in RB1, and thus only require a second hit to initiate cancer, which (usually) happens in multiple cells, in both eyes. The situation is complicated somewhat by low-penetrance alleles, which can be mutated in the germline without promoting bilateral disease (Lohmann, Brandt, Hopping, Passarge, & Horsthemke, 1994). Because of this, approximately 15% of unilateral cases have a heritable mutation.

The irony is not lost on us that while being a visible tumor, retinoblastoma can rob vision from the children it affects. If patients grow up to have children of their own, they should learn whether they have a heritable mutation that could affect their offspring. For all families with heritable retinoblastoma, the "visible feature" of retinoblastoma that allows early detection is knowledge of the RB1 mutation. Prenatal genetic detection coupled with early-term delivery (37–38 weeks' gestation) of the affected infant allows clinical teams to monitor the newborn's retina for the earliest sign of tumor and treat it immediately (Soliman et al., 2016).
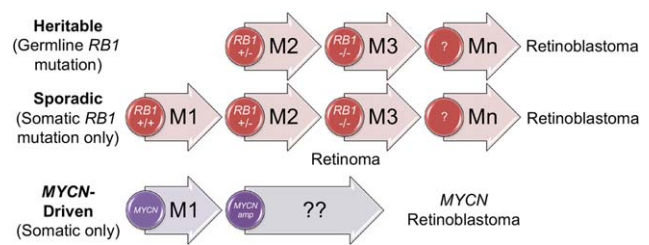

FIGURE 2 Retinoblastoma genetics. The cancer can initiate with a germline RB1 mutation followed by a single somatic hit, or with two somatic mutations (M1 and M2). Loss of RB1 leads to the benign retinoma; further mutations (M3 to Mn) are required for malignancy. Retinoblastoma can also initiate by amplification of MYCN, with other genomic changes as yet unknown

The RB1 gene encodes pRB, a key negative regulatory transcription factor governing the G_1 to S phase cell cycle transition by blocking the interactions of DNA-binding E2F and DP transcription factors with DNA (Dick & Rubin, 2013). In response to mitogen-stimulated cyclin-dependent kinase signaling, normal pRB is hyperphosphorylated, releasing it from E2F interactions, allowing these factors to activate transcription. pRB also has a growing spectrum of roles in differentiation, chromatin remodeling, genome stability, and apoptosis (Dimaras & Corson, 2016; Velez-Cruz & Johnson, 2017).

Over 1,000 mutations in RB1 have been documented in retinoblastoma tumors, including missense and nonsense mutations, splicing mutations, frameshifts, micro- and macrodeletions, and promoter methylation (Lohmann & Gallie, 2004). Despite this, a small fraction of unilateral cases lack identified RB1 mutations, so it was long postulated that an alternative mechanism of retinoblastoma genesis might be possible. As part of an international team, in 2013 we identified that 1.4% of unilateral retinoblastoma, lacking RB1 mutations, instead showed amplification of MYCN (Rushlow et al., 2013) (Figure 2), a transcription factor–encoding gene more frequently associated with the pediatric peripheral nervous system tumor, neuroblastoma (Ruiz-Perez, Henley, & Arsenian-Henriksson, 2017). This finding raises interesting questions about the parallels between retinoblastoma and other neuronal tumors (see below).

Even in retinoblastoma tumors with a defined RB1 mutation, further mutational events are needed for malignancy (Corson & Gallie, 2007). Retinal cells homozygous for RB1 loss can form the benign tumor, retinoma, but develop other genomic changes in the progression to malignancy (Dimaras et al., 2008). The spectrum of changes is characterized by relatively few mutated genes, especially compared with common adult epithelial cancers. The transcriptional corepressor BCOR (BCL6 corepressor) is the most commonly mutated gene in retinoblastoma after RB1 (Zhang et al., 2012). However, retinoblastoma displays a distinct pattern of genomic gains and losses, notably 1q, 2p, and 6p gain, and 16q loss (Kooi et al., 2016b). Candidate progression genes with functional evidence in these regions include MDM4 and KIF14 on 1q, encoding the p53 regulator mouse double minute 4 and the oncogenic motor protein kinesin family member 14, respectively. MYCN is the most commonly amplified gene on 2p, while on 6p, the best-studied genes are the DNA-binding proto-oncogene DEK and the

transcription factor *E2F3*. Genes commonly lost on 16q include *CDH11*, encoding the adhesion protein cadherin-11, and *RBL2*, encoding the retinoblastoma family member p130 (Kooi et al., 2016b; Theriault, Dimaras, Gallie, & Corson, 2014). In addition, aberrant methylation is seen in the retinoblastoma genome (Benavente & Dyer, 2015), including the oncogenic kinase *SYK* (spleen tyrosine kinase). As in other cancers, a variety of microRNAs are also dysregulated in retinoblastoma (Singh, Malik, Goswami, Shukla, & Kaur, 2016), including the miR-17~92 cluster and miR-106b~25 (Conkrite et al., 2011).

2.6 | Treatment and prognosis

Treatment of retinoblastoma is complex and varied. Eyes containing tumors that display clinical features threatening to extend beyond the globe may be surgically removed (enucleation) and histologically assessed to determine risk of metastasis. Eye salvage may be attempted on less severely affected eyes (Dimaras et al., 2015; National Retinoblastoma Strategy, 2009), with focal therapy (laser, cryotherapy) (Hamel, Heon, Gallie, & Budning, 2000), brachytherapy (Hernandez, Brady, Shields, Shields, & DePotter, 1993), and/or chemotherapy. Chemotherapy may be systemic, usually with a cocktail of vincristine, etoposide, and carboplatin (Chan et al., 1996; Gallie et al., 1996); intra-arterial (Yousef et al., 2016); and/or intravitreal (Munier, Gaillard, et al., 2012; Munier, Soliman, et al., 2012). Melphalan is the preferred drug for these latter modalities. External beam radiation is another effective treatment; however, this is not preferred because of its added second cancer risks (Temming et al., 2016). Trilateral retinoblastoma treatment and outcomes are discussed in a later section.

Left untreated, retinoblastoma will grow and extend beyond the eye. Commonly, it invades the regional lymph nodes, bone, bone marrow, and CNS. Extension into the CNS can be detected with radiologic imaging. Prognosis for CNS disease is much worse than for bone marrow because treatment with systemic chemotherapy is impeded by the blood-brain barrier (Mallipatna et al., 2017). Cytologic examination can detect tumor dissemination in CSF and bone marrow. Monitoring of CSF disease can be also done molecularly by looking for tumor-specific (nongermline) *RB1* mutations or post–*RB1* loss genomic gains and losses (Bowles et al., 2007; Dimaras et al., 2010; Racher et al., 2016), or tumor-derived *GD2* or *CRX* mRNA expression (Laurent et al., 2010, 2013; Torbidoni et al., 2015).

Individuals with a constitutional *RB1* mutation have an increased risk of developing second primary tumors, like osteosarcoma, melanoma, or lung or bladder cancer later in life (MacCarthy et al., 2013). Surveillance for second primary malignancies is not yet supported by strong evidence or broad consensus, though some suggest there may be a role for annual screening by whole-body MRI (Friedman et al., 2014; Kamihara et al., 2017). More commonly, retinoblastoma survivors are educated on second primary tumor risks and instructed to be vigilant about potential signs of new malignancies (Kamihara et al., 2017).

Guidelines for treatment in high-income (National Retinoblastoma Strategy, 2009) and low- and middle-income countries (Kenyan National Retinoblastoma Strategy Group, 2014) are similar, although

they account for differences in availability of resources and stage of disease at presentation (i.e., low- and middle-income countries see more cases of extraocular and metastatic disease). Patient outcomes are notably worse in low- and middle-income countries than in high-income ones (Dimaras et al., 2015), and further research is needed to address social determinants affecting access to quality care (Dimaras, Dimba, & Gallie, 2010).

3 | IMAGING RETINOBLASTOMA

3.1 | Clinical vignette

An adult survivor of bilateral retinoblastoma is pregnant with her first child. She undergoes prenatal genetic testing. Her fetus is found to carry her familial *RB1* mutation and is therefore at risk of developing retinoblastoma. Shortly after birth, the infant's eyes are visually examined, and the retina appears normal in each eye. Monthly examinations are scheduled, during which the ophthalmologist inspects each eye with the indirect ophthalmoscope, and an imaging specialist documents the fundus with digital images. At the second examination after birth, the ophthalmologist still does not see any tumors in the eyes. A clear fundus is documented in each eye using a high-resolution fundus camera. Next, the imaging specialist uses optical coherence tomography (OCT) to image the eyes. Surprisingly, the OCT reveals a tiny lump within the inner nuclear layer of the retina in the left eye. Looking back at the fundus images, the ophthalmologist notes a slight haze in the exact spot picked up by OCT: it is a tiny tumor, originally missed on examination of the photographic images. The tumor is treated with focal therapy, preventing its growth and sparing vision. (Based on similar cases previously reported; Rootman et al., 2013; Soliman et al., 2016.)

3.2 | Fundus imaging

Images of the fundus (interior surface of the eye), which become part of the medical record, facilitate consultation and collaboration and precise tracking of tumor response or resistance to treatment. This kind of longitudinal, visual documentation is unique among CNS tumors. Before photographic imaging equipment was a standard part of ophthalmic care, retinal drawings (Wilson, 1975) were used to map retinal topography, lesions, relevant landmarks, and treatments applied, as observed through the indirect ophthalmoscope (Dvorak & Russell, 2011). Retinal imaging has changed practice by providing a way to digitally document the eye, but retinal drawings still remain an important part of learning to understand the disease trajectory.

Digital retinal imaging via portable fundus camera has revolutionized clinical documentation of retinoblastoma (Figure 1b, 3a,d), allowing comparison of pre- and posttreatment images (Romanowska Dixon & Morawski, 2017) and facilitating consultation between centers (National Retinoblastoma Strategy, 2009). The RetCam (Clarity Medical) captures high-resolution, wide-angle photographs, and with scleral depression, allows imaging of the retina up to the ora serrata (the anterior edge of the retina) (National Retinoblastoma Strategy, 2009).
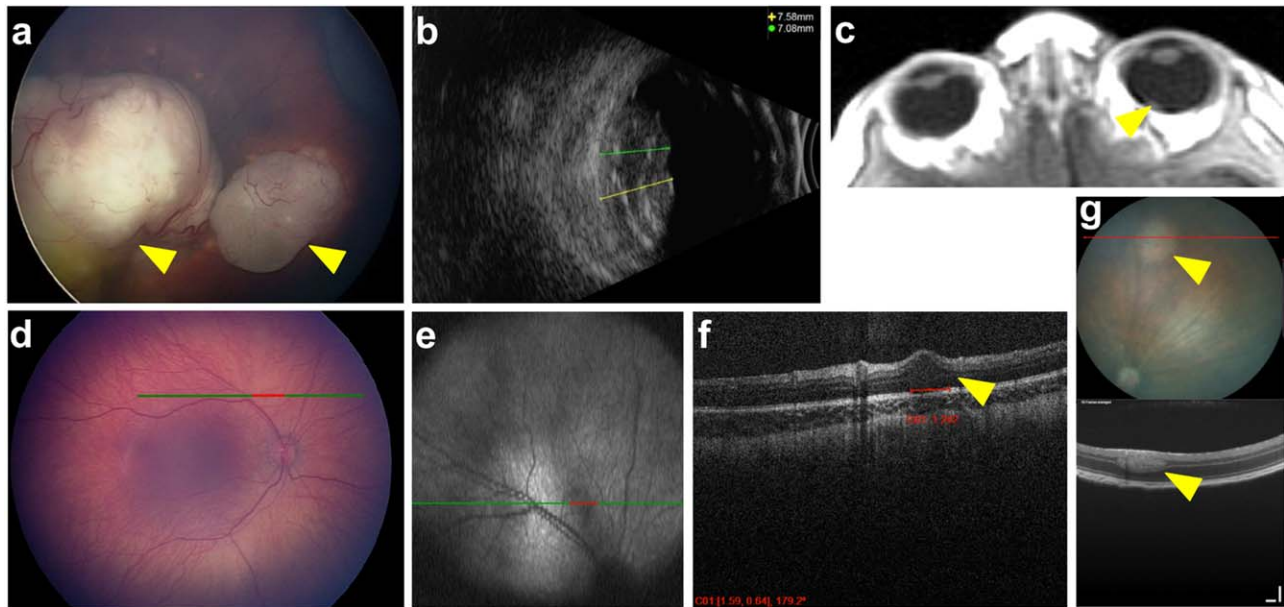
FIGURE 3 Retinoblastoma imaging. (a,b) Retcam (a) image of an eye reveals two large tumors, and ultrasound image (b) allows measurement of tumor height (yellow and green lines). (c) Axial T1 MRI post gadolinium reveals tumor at back of eye (arrowhead). (d–f) Imaging of the eye of an infant with heritable retinoblastoma by Retcam (d) shows no visible tumor. OCT scan line shown in green/red. OCT imaging (e, en face; f, transverse section) reveals a small tumor appearing to arise out of the inner nuclear layer of the retina (arrowhead). (g) Fundus photo (top) and OCT image (bottom) show a retinoblastoma-like tumor (arrowheads) arising in the TAg-RB mouse model. OCT scan line shown in red. Scale bars = 100 µm

Similarly, the ICON camera (Phoenix Technology Group) produces wide-field, high-contrast, high-resolution fundus images.

Unlike fundus drawings, which depict the entire retina in one image, fundus images are partial views of the entire retina and must be stitched together to get a whole-retina view. Fluorescein angiography with portable fundus camera imaging is also important to document vascularization, especially for follow-up of advanced tumors (Kim et al., 2014).

3.3 | Ultrasound

The B-scan (10 MHz) 2D ultrasound is commonly used for ocular imaging and has been a part of the diagnostic tool kit in ophthalmology for decades (Giglio & Sherman, 1979; Kendall et al., 2015; Mrochuk, 1990). It is able to distinguish between ocular structures such as the lens, choroid, retina, and sclera and to image and measure aberrations in the eye, such as tumors or retinal detachment. Importantly for retinoblastoma, B-scan ultrasound allows visualization of calcification, a key feature of these tumors, and thus can help confirm diagnosis (National Retinoblastoma Strategy, 2009; Novotny & Krasny, 1990) (Figure 3b).

High-frequency B-scan ultrasonography (20–50 MHz), also known as ultrasound biomicroscopy (UBM), produces images with higher resolution than traditional B-scan (Pavlin, Sherar, & Foster, 1990). It first showed promise in the imaging of anterior segment tumors more consistently than B-scan (Pavlin, McWhae, McGowan, & Foster, 1992; Reminick, Finger, Ritch, Weiss, & Ishikawa, 1998). For retinoblastoma, UBM has been shown to be essential for determining the proximity of

the tumor to the anterior portion of eye (Vasquez et al., 2011). These are important diagnostic details that assist with accurate classification at presentation.

Furthermore, like other imaging modalities, ocular ultrasonography also allows monitoring of tumor regression as treatment progresses, primarily by comparing the size of the tumor at each examination versus measurements taken at baseline. Unlike the use of intraoperative ultrasound for detection of residual disease in brain tumors (Bohringer et al., 2009), ocular ultrasound cannot currently distinguish between active and inactive retinoblastoma tumor.

3.4 | Magnetic resonance imaging

As with other CNS tumors, MRI can provide important diagnostic and prognostic information in retinoblastoma (Figure 3c) and is vital for screening for trilateral retinoblastoma (see below). MRI can also aid in characterizing the tumor in cases where it is not visible—for instance, if it is behind a cataract-containing lens.

Where available, MRI is often used as part of the workup of all patients with retinoblastoma because it can provide vital information about high-risk pathological features prior to enucleation (de Graaf et al., 2012). These include optic nerve invasion evidenced by thickening, massive choroidal invasion evidenced by thickening or irregularities, scleral invasion evidenced by signal-enhancing tissue beyond the choroid, and intracranial invasion into the suprasellar region (Razek & Elkhamary, 2011). However, sensitivity for detecting these high-risk features varies considerably (de Jong, de Graaf, et al., 2014). High-field-strength MRI can also confirm retinoblastoma diagnosis by

corroborating the calcifications observed by ultrasound or fundus imaging (Galluzzi et al., 2009) and can detect vitreous seeds or anterior segment involvement (Razek & Elkhamary, 2011). Again, these uses are particularly valuable when fundus imaging is not possible. Note that computerized tomography (CT) imaging is generally avoided in retinoblastoma because of the radiation risks, especially in patients with a germline *RB1* mutation, who are already at increased risk of second primary tumors.

In intracranial tumors like gliomas, intraoperative MRI has assumed a pivotal role, to detect removal of diseased tissue during surgery (Hlavac, Wirtz, & Halatsch, 2017). Intraoperative MRI is not necessary in retinoblastoma, as enucleation usually removes the entirety of the tumor, unless extraocular spread has been previously detected, or high-risk features are subsequently discovered on pathology. However, one retinoblastoma case report describes the use of intraoperative MRI to track the delivery and tumor penetration of intra-arterial melphalan delivered in a cocktail with gadolinium (Materin et al., 2012).

3.5 | Optical coherence tomography

OCT, a non-invasive imaging technique based on differential near-infrared light reflection, offers a powerful tool for imaging retinoblastoma, with resolution exceeding that of ultrasound or MRI. Conceptually similar to ultrasound, spectral domain OCT can generate high-resolution, cross-sectional images of tissues where light can penetrate (Fujimoto & Swanson, 2016), and therefore has found utility in dermatology, urology, and in particular, ophthalmology, where it is routinely used in adult clinics. It has also been used in the CNS for ex vivo analysis of gliomas (Valdes, Roberts, Lu, & Golby, 2016) and medulloblastoma (Vuong et al., 2015), and it is being trialed for intraoperative imaging of gliomas to detect residual tumor, as is also done via ultrasound and MRI (Bohringer et al., 2009).

Recently, with the advent of handheld intraoperative OCT systems, OCT has become popular for pediatric ophthalmology as well, including retinoblastoma assessment (Do, Do, & Berry, 2017). Initial publications were case reports indicating the value of OCT for detecting cavitary retinoblastoma (Mashayekhi, Shields, Eagle, & Shields, 2005) and reasons for visual loss (such as retinal detachment) post therapy (Shields, Materin, & Shields, 2005). Subsequently, OCT showed utility for documenting response to therapy, spotting edge recurrence, and, notably, detecting "invisible" tiny retinoblastoma (Figure 3d–f) (Rootman et al., 2013), as well as assessing foveal anatomy, which impacts the likelihood of visual function post therapy (Cao, Markovitz, Ferenczy, & Shields, 2014; Samara, Pointdujour-Lim, Say, & Shields, 2015). One case report even documented optic nerve head disease, usually detected by MRI (see above) (Yousef, Shroff, Halliday, Gallie, & Heon, 2012). OCT's ability to look "behind" small features such as vitreous seeds to the underlying tumor or normal retina can be valuable for assessing retinoblastoma comprehensively.

This growing body of work also contributed to an ongoing debate on the cell of origin of retinoblastoma, as some tiny tumors detected by OCT seem to originate in the inner nuclear layer (Rootman et al., 2013) (Figure 3f), while others are more localized to the outer nuclear layer, suggestive of a cone precursor origin for at least some retinoblastoma (Berry, Cobrinik, & Kim, 2016), which is consistent with experimental data (Xu et al., 2014).

Although not yet in use for retinoblastoma, recently developed OCT angiography holds considerable promise for documenting vascularization and blood flow in and around tumors (Gao et al., 2016). However, OCT does have limitations: it images the periphery of the retina poorly (although technology for widefield OCT is improving), and it relies on transparent media; large tumors and/or cataracts limit its usefulness. Despite this, OCT for retinoblastoma is usually successful and can guide treatment decisions (Soliman, VandenHoven, et al., 2017). With time, OCT will doubtless assume a greater role in the staging and management of retinoblastoma, taking true advantage of the accessibility of the eye to manage this cancer.

3.6 | Imaging animal models

The wealth of imaging tools available for the eye has made longitudinal studies of retinoblastoma animal models possible (Figure 3g), complementing more traditional histological analyses. The advent of small-animal intraocular imagers enabled documentation of intraocular tumors in both transgenic and orthotopic xenograft models of the cancer. In particular, coupling fluorescence and brightfield imaging of GFP-expressing xenografts allowed qualitative assessment of tumor growth and (importantly) dissemination within the eye (Corson et al., 2014). Similarly, a number of preclinical therapeutic studies have included qualitative fundus and fluorescein angiography images (Brennan et al., 2011; McEvoy et al., 2011; Zhang et al., 2012). In the future, quantification of fluorescence should allow volumetric analysis of tumor growth, as is currently done using bioluminescence of luciferase-expressing xenografts (Corson et al., 2014; Ji et al., 2009; Laurie et al., 2005).

MRI has also been employed in preclinical treatment trials, and in one report was used for volumetric estimation of tumor (McEvoy et al., 2011). Ultrasound has also been used for animal models in limited cases (Brennan et al., 2011); one study showed fundus, ultrasound, and MRI images of transgenic animals in a treatment trial but did not use these images for assessing efficacy (Nemeth et al., 2011).

OCT has become a favored method for detecting lesions, especially in genetic models. The first work in this area was done with custom-built OCT apparatus, enabling detection of intraretinal tumors in the SV40 T-antigen–driven mouse model of retinoblastoma (TAg-RB) (Ruggeri et al., 2007) and assessing their response to therapy (Cebulla et al., 2008). Even retinal tumors in utero could be detected through the uterine horn (Larina et al., 2012). TAg-RB tumors have also been documented by ultrasound (Foster & Brown, 2012).

More recently, using a commercially available small-animal OCT system, we showed that this technique can detect the earliest signs of tumors in the TAg-RB model, corresponding to their first appearance by histology (Figure 3g) (Wenzel, O'Hare, Shadmand, & Corson, 2015), and we used this finding to show that overexpression of the oncogene *Kif14* accelerates not just tumor growth but also tumor initiation in this model (O'Hare et al., 2016).

OCT has proven useful for xenografts: intravitreal xenografts could be monitored (Corson et al., 2014; Tschulakow, Schraermeyer, Rodemann, & Julien-Schraermeyer, 2016) and subretinal xenografts could be rapidly assessed by a combination of OCT and funduscopy (Lemaitre et al., 2017). Of course, OCT assessment of morphology of xenografts is less clinically relevant than the morphology of tumors arising intraretinally as in genetic retinoblastoma models. However, the prospects for rapidly assessing genetic modifiers and/or therapeutic efficacy by OCT in both types of model are appealing, especially with the advent of methods to volumetrically assess lesions on OCT (Ruggeri et al., 2009; Sulaiman et al., 2015).

4 | *MYCN*-AMPLIFIED RETINOBLASTOMA

4.1 | Clinical vignette

A 1-month-old infant with no family history of retinoblastoma has a large retinoblastoma identified in one eye. On enucleation, no rosettes are noted, but the high-risk pathological feature of optic nerve invasion is present. Because early-onset, aggressive tumors are often indicative of a germline *RB1* mutation, heritable disease is suspected, with the concomitant risk of tumors in the fellow eye and a need for lifelong surveillance and risk to offspring. But genetic analysis of the enucleated tumor fails to find any mutated *RB1* alleles, despite a >96% detection rate in specialized labs (Soliman, Racher, Zhang, MacDonald, & Gallie, 2017). However, further analysis of common copy number changes in retinoblastoma reveals that the oncogene *MYCN* is amplified to 53 copies in this tumor, and on subsequent re-review, the pathologist notices some neuroblastoma-like histological features. These findings strongly suggest *MYCN*-driven disease, which is not heritable, leaving this child at only population risk for future malignancies.

4.2 | Discovery and characteristics

MYCN retinoblastoma was identified on the basis of careful analysis of over 1,000 tumor genotypes (Rushlow et al., 2013). Although *MYCN* amplification had been documented in occasional retinoblastoma since the *MYCN* gene was first identified (Gilbert et al., 1981), this large-scale study was needed to reveal that *MYCN* amplification is much more common among unilateral retinoblastoma without a detectable *RB1* mutation. This finding has since been replicated in other cohorts (Ewens et al., 2017; Kooi et al., 2016a; McEvoy et al., 2014), but it is important to note that *MYCN* retinoblastoma is currently only identifiable after enucleation. Retrospective analysis of *MYCN* retinoblastoma revealed several further differentiating features (Figure 4). Clinically, these tumors are among the very-earliest-onset unilateral tumors and often have high-risk pathological features, although there is not yet sufficient evidence to conclude that *MYCN* amplification has a dramatic effect on aggressiveness, as it does in neuroblastoma or medulloblastoma (Ruiz-Perez et al., 2017). Histopathologically, these tumors also differ from $RB1^{-/-}$ retinoblastoma. Comparatively, they show more rounded, undifferentiated cells, with prominent nucleoli (Figure 4c), and lack the nuclear molding, differentiated rosettes, and extensive
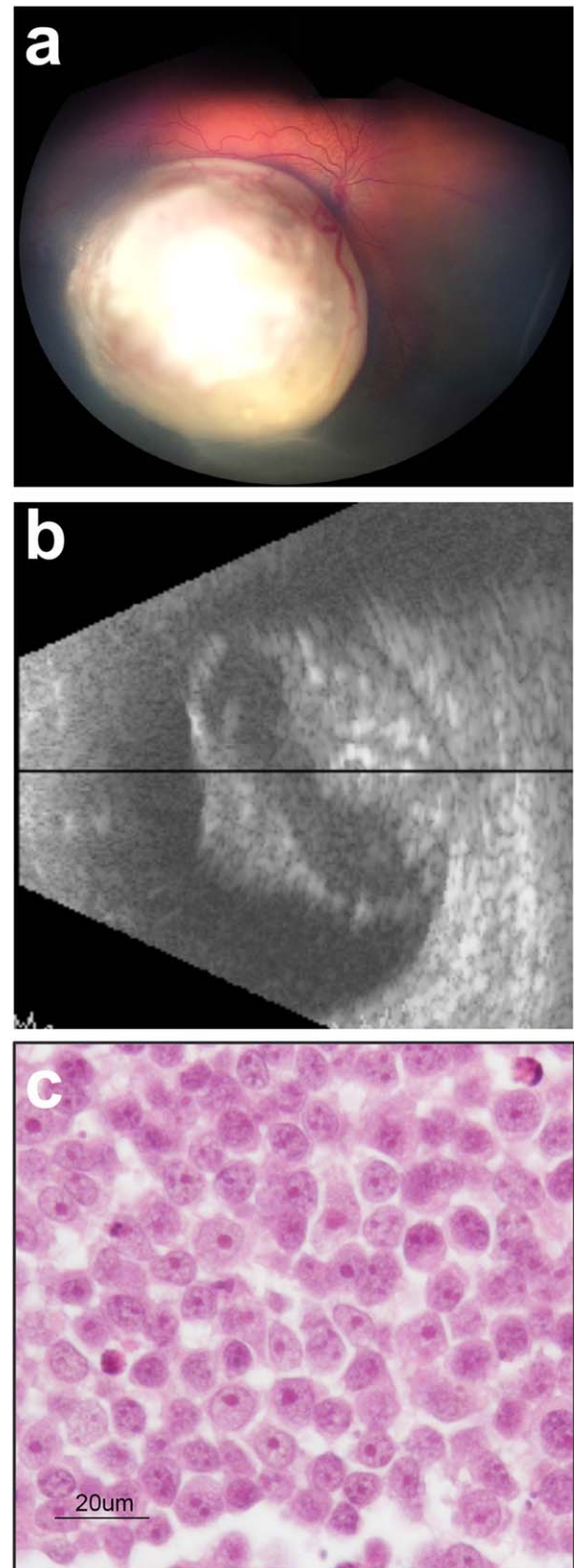

FIGURE 4 *MYCN* retinoblastoma. (a) Tumor appears retinoblastoma-like on fundus image. (b) Ultrasound reveals characteristic calcification. (c) Histopathology of tumor in enucleated eye reveals round nuclei with prominent large multiple nucleoli; contrast with $RB1^{-/-}$ retinoblastoma in Figure 1e. Adapted with permission from Rushlow et al. (2013)

calcification commonly seen in retinoblastoma. Again, the histology of MYCN retinoblastoma is more similar to neuroblastoma than it is to $RB1^{-/-}$ retinoblastoma.

4.3 | Molecular features

Beyond the lack of RB1 mutations, MYCN retinoblastoma had fewer copy number alterations than $RB1^{-/-}$ tumors in one study (Rushlow et al., 2013), although this was not seen in a subsequent report (Ewens et al., 2017). MYCN retinoblastoma was also less likely to display the characteristic chromosomal gains and losses of retinoblastoma (1q and 6p gain, 16q loss), instead showing some variations in common with neuroblastoma (17q gain, 11q loss) (Rushlow et al., 2013).

MYCN retinoblastoma has functional, hyperphosphorylated pRB (Ewens et al., 2017) and high levels of N-Myc protein (Rushlow et al., 2013). The fact that pRB is expressed, yet inactivated by post-translational modification, strongly implies that genetic changes at the RB1 locus are not driving this disease. The SKP2-p27 axis leading to pRB phosphorylation was not activated in three of four MYCN retinoblastoma (Ewens et al., 2017), suggesting that this MYCN-activated pathway is not a key driver in MYCN retinoblastoma, unlike in neuroblastoma (Evans et al., 2015).

Interestingly, high retinal Mycn levels alone (driven by the Pax6α enhancer) could not initiate retinoblastoma-like tumors in mice, but could cooperate with Rb1 loss to form tumors (which normally require loss of another retinoblastoma family member, such as p107) (Wu et al., 2017). This discovery also offered a caution toward MYCN-targeted therapy: when Mycn levels were reduced in this mouse model, tumors reoccurred with Mycn independence (Wu et al., 2017). Further insight into how MYCN amplification leads to pRB inactivation by hyperphosphorylation and drives the human tumors is needed. Perhaps the Pax6α enhancer does not drive expression in the retinal cell type susceptible to Mycn-driven retinoblastoma; Mycn overexpression alone can promote cancer in other tissues, as observed with the tyrosine hydroxylase–driven Mycn neuroblastoma model (Weiss, Aldape, Mohapatra, Feuerstein, & Bishop, 1997). In the absence of perfect mouse models, cell lines and patient-derived xenografts will be best suited for these experiments, but given the rarity of MYCN retinoblastoma, few are currently available.

4.4 | Are MYCN-amplified ocular tumors retinoblastoma?

Despite the histopathological and genetic similarities with neuroblastoma, there is no reason to believe that "MYCN retinoblastoma" is not retinoblastoma: it apparently arises in a retinal neuronal precursor and (based on a small number of tumors) has a gene expression profile not statistically distinct from that of a major group of retinoblastoma, characterized by low "photoreceptorness," but high expression of RNA biogenesis and M phase genes (Kooi et al., 2015). Thus, to date, it is unclear whether MYCN retinoblastoma derives from a different retinal cell of origin than $RB1^{-/-}$ retinoblastoma, or whether the cell of origin

is the same, but the initiating MYCN amplification event pushes these cells into a distinct phenotype.

5 | TRILATERAL RETINOBLASTOMA

5.1 | Clinical vignette

A 6-month-old presents with a 2-week history of leukocoria in one eye. Upon ophthalmic examination, the child is diagnosed with unilateral retinoblastoma. As part of routine workup, the child undergoes CT of the head and both orbits. The CT reveals an intracranial midline tumor. Cytologic examination of the CSF shows presence of malignant cells. The child undergoes treatment for trilateral retinoblastoma, which includes high-dose chemotherapy followed by autologous stem cell transplant. The child is alive and well 10 years post treatment. (Based on a previously reported case; Dimaras et al., 2011, 2015.)

A 4-month-old presents to the emergency room with atypical eye movements and overgrowth syndrome. A CT scan reveals a suprasellar tumor (Figure 5). The eyes are not examined at this time. The tumor is biopsied and shows a small blue cell tumor with abundant Flexner–Wintersteiner rosettes. The eyes are then examined, and reveal multifocal tumors in both eyes. Systemic and intrathecal chemotherapy for trilateral retinoblastoma is prescribed. During the placement of the intrathecal catheter, the treating physicians observe tumor seeding along the needle tract of the initial biopsy. The child's tumor reoccurs, and she dies. (Based on a previously reported case; Dai et al., 2008; Dimaras et al., 2011.)

5.2 | Origins and pathology

Trilateral retinoblastoma, first described in 1977 (Jakobiec, Tso, Zimmerman, & Danis, 1977) and coined in 1980 (Bader et al., 1980), is the heritable form of retinoblastoma associated with an intracranial tumor. The intracranial tumors most commonly affect the pineal gland, as well as the suprasellar or intrasellar region of the brain (Figure 5). Pineal tumors are generally smaller than non-pineal tumors in trilateral retinoblastoma (de Jong, Kors, et al., 2014). Benign pineal cysts have also been observed with retinoblastoma (Beck Popovic, Balmer, Maeder, Braganca, & Munier, 2006; Gupta et al., 2016; Karatza, Shields, Flanders, Gonzalez, & Shields, 2006). In rare cases, pineal cysts can transform into malignant tumors, suggesting that they should be monitored over time (de Jong et al., 2016). Cystic normal pineal glands, however, are also regularly observed in children; their size is often comparable to that of solid and cystic pineoblastoma (Sirin et al., 2016). This makes it challenging to know which cystic lesions require follow-up and which do not. A classification for pineal glands has been proposed to differentiate glands based on absence or presence of cysts, number of cysts, and enlargement (type 0–4) (Sirin et al., 2016). The European Retinoblastoma Imaging Collaboration proposed guidelines for follow-up of cystic pineal lesions based on this classification: they recommend monitoring type 3 and 4 lesions (multicystic with enlargement) by routine MRI for changes in size or morphology of the solid component of the lesion (Sirin et al., 2016). It is sometimes equally
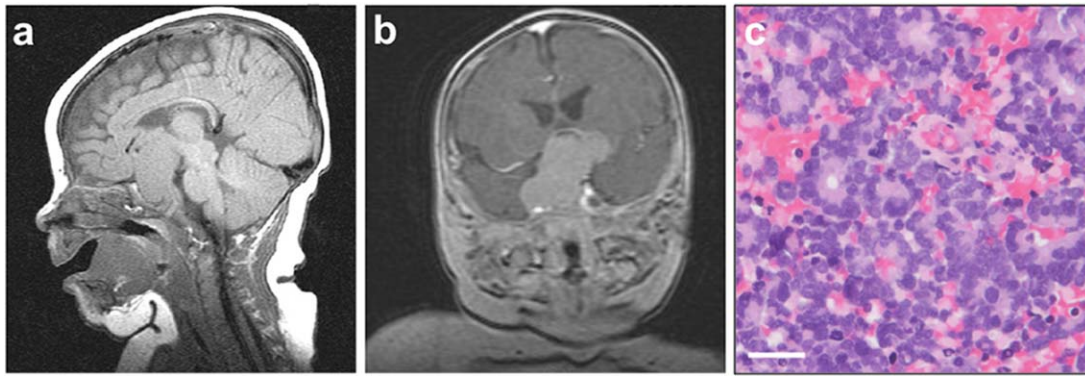
FIGURE 5 Trilateral retinoblastoma. (a) Sagittal T1-weighted and (b) coronal T2-weighted fluid-attenuated inversion recovery (FLAIR) contrast MRI reveals suprasellar mass in a patient who also has bilateral retinoblastoma. (c) Histology of this mass reveals abundant Flexner–Wintersteiner and Homer Wright rosettes. Scale bar = 25 μm

challenging to differentiate large solid pineal glands from pineoblastoma (Galluzzi et al., 2016).

Trilateral retinoblastoma often presents with leptomeningeal metastasis or CSF involvement, which is difficult to cure. The histological features of the intracranial tumor are identical to those of retinoblastoma (Figure 5c). Because of its rarity, little work on the molecular genetics of this tumor has been reported (Li, Bouffet, Hawkins, Squire, & Huang, 2005). Mouse models may yet offer some insights: 15% to 27% of TAg-RB mice develop primitive neuroectodermal tumors, analogous to the parasellar and suprasellar tumors arising in patients with trilateral retinoblastoma (Marcus et al., 1991; O'Brien et al., 1990). The trilateral tumors in mice have histological features consistent with their human counterparts (Marcus et al., 1991). However, this murine retinoblastoma model does not develop pineoblastoma (Marcus et al., 1991), although other T-antigen–driven tumor models do (Marcus et al., 1996). Also, $Rb1^{+/-}$ mice with loss of p53 develop pineoblastoma, but these mice do not develop retinal tumors (Williams et al., 1994). Despite the existence of these models, no preclinical studies on trilateral retinoblastoma have yet been reported.

In addition to predisposition by *RB1* mutation, pineoblastoma alone is associated with germline mutations in *DICER1* (de Kock et al., 2014). In murine retinoblastoma models, *Dicer1* is a haploinsufficient tumor suppressor (Lambertz et al., 2010). Further investigation is warranted into the development of pineoblastoma within and without the context of retinoblastoma.

5.3 | Epidemiology

The incidence of trilateral retinoblastoma among patients with heritable retinoblastoma is 3.5% (de Jong et al., 2015). Prior systemic chemotherapy to treat retinoblastoma is associated with decreased risk of developing an intracranial tumor (Shields, Meadows, Shields, Carvalho, & Smith, 2001). Suprasellar or intrasellar tumors are diagnosed earlier than pineal, and often concurrently with the retinoblastoma (Kivela, 1999). In contrast, 60% of pineal tumors are diagnosed within 1 year of initial retinoblastoma diagnosis, suggesting that routine screening by MRI could contribute to earlier diagnosis (de Jong, Kors, et al., 2014).

Some centers screen by MRI every 6 months until 5 years of age (Kamihara et al., 2017); however, it is unclear whether this approach is warranted or effective (De Ioris et al., 2014).

5.4 | Becoming treatable

Treatment for trilateral retinoblastoma generally involves chemotherapy delivered systemically and intrathecally via an Ommaya reservoir, followed by high-dose chemotherapy and autologous stem cell rescue (Dimaras et al., 2011). Surgical treatment has been attempted in some cases; however, complete resection is difficult because of the locations of the intracranial tumors (Dunkel et al., 2010). Craniospinal radiation, although effective, is not preferred because of its long-term developmental, neurocognitive, and endocrine damage (Duffner, Cohen, Thomas, & Lansky, 1985; Mulhern et al., 1999). Indeed, the use of radiation in the treatment of trilateral retinoblastoma has declined in recent years (de Jong, Kors, et al., 2014).

Survival from trilateral retinoblastoma has improved over time, most notably with the introduction of high-dose chemotherapy and autologous stem cell transplant (de Jong, Kors, et al., 2014). Looking at outcomes before and after 1995, when chemotherapy changed treatment for retinoblastoma, the 5-year survival for pineal trilateral retinoblastoma has increased from 6% to 44%, and nonpineal from 0% to 57% (de Jong, Kors, et al., 2014; Kivela, 1999). The presence of leptomeningeal metastases considerably reduces the probability of survival (de Jong, Kors, et al., 2014).

6 | CONCLUSIONS AND OUTLOOK

Although rarer than many other CNS cancers, retinoblastoma has offered fundamental cancer knowledge, and clinicians and researchers have taken advantage of its accessibility to carefully document and treat it using multiple modalities. We envisage several areas for future developments to improve our understanding and clinical management of this cancer.

6.1 | Trilateral retinoblastoma

Our improving knowledge of the clinical behavior of intracranial tumors in retinoblastoma has not been matched by our understanding of their molecular features. It will be valuable to determine whether there are molecular characteristics of certain heritable retinoblastoma types that might predispose to formation of trilateral tumors. These might include *RB1* genotype, *DICER1* genotype, protein marker expression, features on high-resolution imaging, or others. Trilateral retinoblastoma tumors (like their anatomical counterparts not associated with retinoblastoma) might benefit from targeted therapy, and each could benefit from discoveries in the other in this domain. The availability of mice that develop trilateral retinoblastoma–like tumors makes preclinical studies technically feasible, but the low frequency of intracranial tumor formation in these mice plus the need for histological diagnosis makes such studies challenging.

6.2 | MYCN retinoblastoma

The study of *MYCN* retinoblastoma can gain much from the wealth of knowledge available for *MYCN* neuroblastoma (Huang & Weiss, 2013). Despite caveats (Wu et al., 2017), the potential for targeted agents borrowed from neuroblastoma research to treat *MYCN* retinoblastoma is exciting. Currently, these would only be useful for metastatic disease as the *MYCN* genotype is only determined post enucleation (recall that intraocular retinoblastoma is not biopsied because of the risk of tumor dissemination). But a promising new "liquid biopsy" technique could change this because it allows for capture and molecular analysis of tumor DNA from the aqueous humor of the eye, which can be safely removed with techniques that minimize the risk of tumor spread (Berry et al., 2017). This approach could potentially be used to identify *MYCN* tumors in situ. Moreover, if distinguishing characteristics of *MYCN* tumors could be discovered in OCT, fundus, ultrasound, or MRI, or in circulating tumor cells or DNA, targeting the primary tumor would also be possible. Large, prospective studies will be needed to find these characteristics. Similarly, comparison of the molecular features (genomic, proteomic, and signaling changes) of *MYCN* retinoblastoma with *MYCN* neuroblastoma may reveal shared vulnerabilities or unique characteristics.

Deciphering the cell of origin of *MYCN* retinoblastoma is likewise important. One way to explore this would be to parallel the work that identified cone precursors as a likely origin of at least some $RB1^{-/-}$ retinoblastoma by knocking down *RB1* in dissociated human fetal retinal cells and observing which cell types can proliferate (Xu et al., 2014). A similar experiment, overexpressing *MYCN* instead of removing *RB1*, could perhaps provide insight into retinal cells susceptible to proliferation after *MYCN* gain.

6.3 | Imaging

Tumor imaging has revolutionized care of retinoblastoma, and could perhaps be further applied to research on other CNS tumors. The powerful combination of fundus imaging and OCT makes the prospect of xenografting other tumor types into the eye an appealing one, especially for neural tumors where intravitreal or subretinal injection would be more facile than intracranial xenografting. Although the intraocular microenvironment differs from the brain, it offers similarities (neuronal and glial neighboring cells, separation from the blood by a semipermeable barrier) that subcutaneous xenografts lack. Such paratopic xenografts could be easily followed using the imaging modalities above, and will be an interesting area for further exploration.

Retinoblastoma may also learn from imaging in CNS tumors. Molecular imaging via positron emission tomography or single-photon emission computed tomography is showing promise for brain tumors, especially using radiolabeled amino acid tracers (Brindle, Izquierdo-Garcia, Lewis, Mair, & Wright, 2017). But the rare research on these imaging modalities in retinoblastoma has shown little to no promise, offering no additional insight on tumor features or response to treatment (Moll et al., 2004). Further research including novel tracer development may be indicated. However, the need to keep radiation exposure low for heritable retinoblastoma cases must also be considered.

Large imaging studies might also reveal other imaging biomarkers that could help stratify retinoblastoma types for improved prognostication and treatment decisions. Such work could affect identification of both trilateral-predisposed and *MYCN* retinoblastoma, as noted above. Increased ability to look out for distinctive features in the eyes of retinoblastoma patients will continue to improve the outlook for this visible CNS tumor.

CONFLICT OF INTEREST

The authors have no conflicts of interest.

AUTHOR CONTRIBUTION

Conceptualization, H.D., T.W.C.; *Writing – Original Draft*, H.D., T.W.C.; *Writing – Review & Editing*, H.D., T.W.C.; *Visualization*, H.D., T.W.C.

ORCID

Timothy W. Corson http://orcid.org/0000-0002-1402-7875

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