

Essentials in Ophthalmology

Series Editor: Arun D. Singh

Jasmine H. Francis

David H. Abramson *Editors*

Recent Advances in Retinoblastoma Treatment

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Series Editor
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Preface

The chapters in this book represent most of the lectures that were delivered at the celebration honoring 100 years of retinoblastoma in New York (1914–2014). During those 100 years, there was a complete reversal in the field. In 1914, more than 95 % of children with retinoblastoma died of metastatic disease and the majority of the eyes removed for retinoblastoma did not contain cancer. By 2014, more than 99 % of children in New York survived and for more than 40 years no eye in New York has been removed for retinoblastoma incorrectly. In 1914, retinoblastoma management consisted of enucleation, exenteration, and low energy external beam irradiation and few children retained an involved eye and no retained eye had useful vision. By 2014, the therapeutic options included enucleation, high energy external beam irradiation, brachytherapy, laser photocoagulation, cryotherapy, intravenous, periocular, intravitreal, and intra-arterial chemotherapy and largely as a result of intra-arterial chemotherapy and intravitreal chemotherapy more than 95 % of eyes could be saved and more than 90 % of children retain 20/20 vision. In 1914, retinoblastoma was classified as a “glioma” of the retina and by 2014 elegant studies revealed that the cell of origin was a cone precursor (the name retinoblastoma was introduced 90 years ago).

Retinoblastoma survival is now the highest in all of pediatric oncology and issues of long-term consequences (physical and psychological) are influencing present therapeutic decisions. The story of second malignancies in this disease has served as paradigm for oncology and strategies for preventing and detecting (and treating) these (usually) lethal cancers were presented at the meeting.

While metastatic retinoblastoma is now rare in the USA, it remains a major issue worldwide where it is estimated that 50 % of children still die. Metastatic retinoblastoma is now largely curable in countries with advanced medicine but second cancers, pineal malignancies, and secondary sarcomas are now the major cause of death of retinoblastoma survivors in the USA.

More than 175 health care workers from almost 20 countries participated in this meeting/celebration (with others electronically listening in from Africa).

In these 100 years, many physicians have worked, studied, contributed and help make the New York program the leading center in the world. There have been three directors: Algernon B. Reese, M.D., Robert M. Ellsworth, M.D., and me. I had the opportunity to learn from both of these giants and my work is simply built on their pioneering efforts. I hope that after reading this book you can say the same.

New York, NY, USA

David H. Abramson, MD

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Scott D. Walter and J. William Harbour

1.1 The First Tumor Suppressor

The existence of hereditary cancer syndromes, such as familial retinoblastoma, attracted the attention of early investigators seeking to understand the genetic basis of cancer. Early notions regarding the hereditary basis of familial cancer assumed that such genes would function in a dominant manner, and the concept of a recessive or “tumor suppressor” gene was initially met with skepticism (Neel and Falls 1951). The retinoblastoma susceptibility gene, *RBI*, was the first bona fide tumor suppressor gene to be identified.

The pivotal breakthrough occurred in 1971, when Alfred Knudson proposed his “two hit” hypothesis: that retinoblastoma results from the mutational inactivation of both alleles of a putative recessive oncogene (Knudson 1971). The retino-

blastoma locus was mapped to chromosome 13q through its linkage to esterase D, an enzyme that had previously been mapped to this region and which could be assayed for its enzymatic activity (Franke 1976). Esterase D activity was found to be about half the normal level in retinoblastoma patients with karyotypically visible deletions on chromosome 13q (Sparkes et al. 1980). In another study, esterase D activity was 50 % of normal in healthy tissues of retinoblastoma patients but was undetectable in tumor tissue (Benedict et al. 1983), providing further support for Knudson’s hypothesis. The recessive nature of the retinoblastoma locus was conclusively demonstrated by loss of heterozygosity in tumor tissue (Cavenee et al. 1983), a technique that subsequently has become a standard for studying tumor suppressor genes. The identification and subsequent characterization of this prototypical tumor suppressor gene spurred a revolution in our understanding of cell biology and tumor genetics, as illustrated in the historical timeline below (Fig. 1.1).

The significance of the Rb tumor suppressor pathway is twofold. We now recognize that inactivation of the Rb pathway is a threshold event initiating neoplastic transformation not only in retinoblastoma, but also in many other cancers. Also, the retinoblastoma pathway paved the way for the discovery of countless other tumor suppressor genes, including p53, PTEN, p16^{INK4a}, WT1, ARF, and others. Tumor suppressor genes have since been implicated in virtually all known hereditary cancer syndromes.

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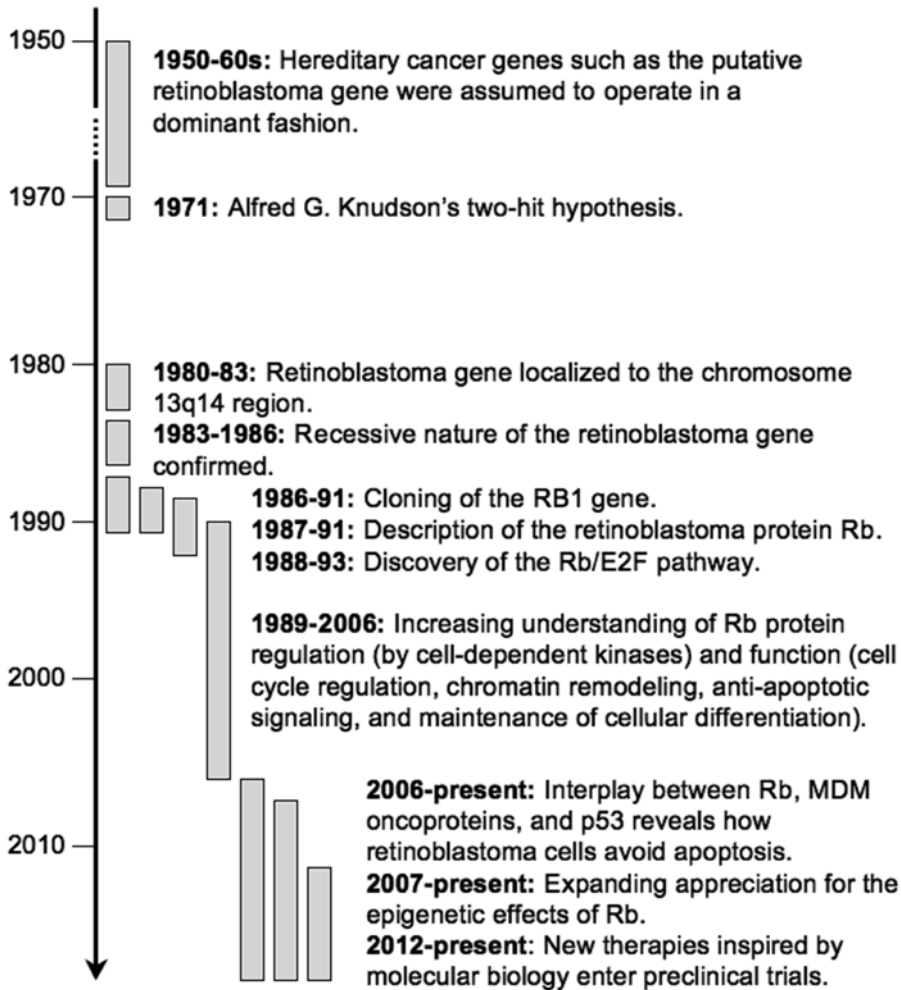


Fig. 1.1 Timeline illustrating key discoveries relating to the Rb pathway

1.2 Structural Biology of the Rb Pathway

1.2.1 The *RB1* Gene

The retinoblastoma gene (*RB1*) spans over 200 kilo bases of DNA and contains 27 exons (Blanquet et al. 1991; Wiggs et al. 1988). Germline mutations tend to cluster at CpG areas, but are also spread throughout the gene with no mutational hot-spot (Harbour 1998; Mancini et al. 1997). Germline mutations can include deletions, frameshift and nonsense mutations, splicing mutations, and mutations in introns

resulting in a truncated protein product (Harbour 1998). The second “hit” is usually a nondisjunction or recombination event during mitosis, resulting in reduction to hemizyosity of the mutant allele (Zhu et al. 1992).

1.2.2 The Rb Phosphoprotein

The retinoblastoma protein (Rb) is composed of 928 amino acids and has three functional domains: the N-terminus, the central pocket domain, and the C-terminus. The pocket, which is formed by the highly conserved A and B boxes, is required for binding to E2F, viral oncoproteins,

and chromatin remodeling proteins, and it is required for tumor suppression (Chow and Dean 1996; Qin et al. 1992). Most pathogenic *RB1* mutations affect the pocket (Mancini et al. 1997). The C-terminus is also required for efficient binding to E2Fs, and it has binding sites for the oncoproteins MDM2 and c-abl (Hiebert 1993; Hsieh et al. 1999; Welch and Wang 1993; Xiao et al. 1995). The function of the N-terminus is less clear, and it does not appear to be required for tumor suppression (Xu et al. 1994).

Rb protein activity is modulated during the cell cycle by phosphorylation, which can occur on 16 cyclin-dependent kinase phospho-acceptor sites. Rb phosphorylation varies throughout the cell cycle, with hypophosphorylated Rb predominating in G_0 and G_1 , and hyperphosphorylated Rb in S-phase and G_2/M (DeCaprio et al. 1989, 1992; Lee et al. 1987). Rb usually becomes hypophosphorylated during differentiation and senescence, whereas it becomes hyperphosphorylated in cells re-entering the cell cycle (Chen et al. 1989; Ikeda et al. 1996; Stein et al. 1990).

1.3 Functions of the Rb Pathway

Regulation of cell cycle progression is considered a fundamental function of all tumor suppressor genes, with deregulation predisposing to neoplasia through uncontrolled cell division. In the most simplistic terms, Rb can be thought of as a “gatekeeper” regulating passage through the G_1 -to-S phase transition of the cell cycle (Harbour and Dean 2000a). However, in order to fully understand the myriad functions of the *RB1* pathway, one must individually consider several distinct molecular functions of the Rb protein.

1.3.1 Regulation of E2F-Responsive Gene Transcription

A major binding partner of Rb is the E2F family of transcription factors (E2Fs) (Chellappan et al. 1991). Rb does not have a DNA binding domain, but is brought to specific promoters through its interaction with E2Fs (Fig. 1.2). E2F-responsive

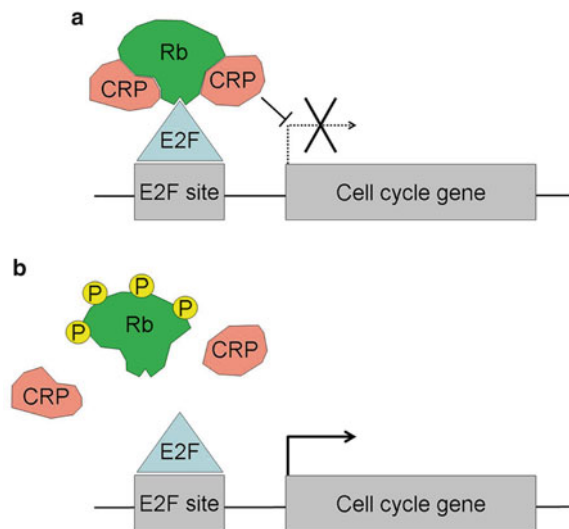


Fig. 1.2 The molecular mechanism of action of Rb. (a) When Rb is hypophosphorylated and active, it binds to E2F transcription factors that bind to E2F sites in the promoter regions of genes involved in cell cycle progression and apoptosis. Rb directly inhibits E2Fs by binding and masking the transactivation domain, and it also recruits

chromatin remodeling proteins (CRPs) that alter local chromatin structure into a conformation that is not permissive for transcription. (b) When Rb is hyperphosphorylated, it does not bind efficiently to chromatin remodeling proteins or E2Fs, which allows E2Fs to transactivate cell cycle genes

genes contain one or more E2F binding sites in their promoters, and these genes play an important role in cell cycle regulation, DNA repair, apoptosis, and other key regulatory functions (DeGregori and Johnson 2006). In addition to a DNA binding domain, E2Fs 1-5 also have a transactivation domain by which they promote the transcription of specific genes necessary for DNA and chromosome replication (Fig. 1.3). Phosphorylated Rb (pRb) binds and inhibits E2Fs by masking the E2F transactivation domain (Flemington et al. 1993; Helin et al. 1993). A more complicated picture of Rb-E2F interactions is emerging in which E2Fs 1-3 assume paradoxical activating and repressing roles in dividing versus differentiating cells, and in normal versus cancer-like cell cycles (Chong et al. 2009).

1.3.2 Epigenetic Effects, Including Active Repression of Other Genes Through Chromatin Remodeling

pRb actively represses promoter and enhancer elements independently of its effect on the E2F transactivation domain (Harbour and Dean 2000b; Weintraub et al. 1995). This so-called “active repression” is thought to result from the recruitment by Rb of chromatin remodeling proteins that reconfigure local chromatin into a non-permissive state for transcription (Harbour and Dean 2000c). These proteins include histone deacetylases, DNA methyltransferases, and polycomb complexes (Brehm et al. 1998; Dahiya et al. 2001; Luo et al. 1998; Magnaghi et al. 1998; Nielsen et al. 2001; Robertson et al. 2000). Active repression by Rb is required for the full tumor suppressor activity of Rb, including its inhibition of the G₁-to-S phase transition (Zhang et al. 1999).

Rb appears to play a critical role in regulating the epigenetic program of cancer genomes. A family of Rb binding proteins, including RBP2, functions as histone demethylases. Histone methylation regulates chromatin structure and transcription, defining the epigenetic program of the cell. RBP2 specifically erases trimethylation of histone H3 at lysine 4, a key site for

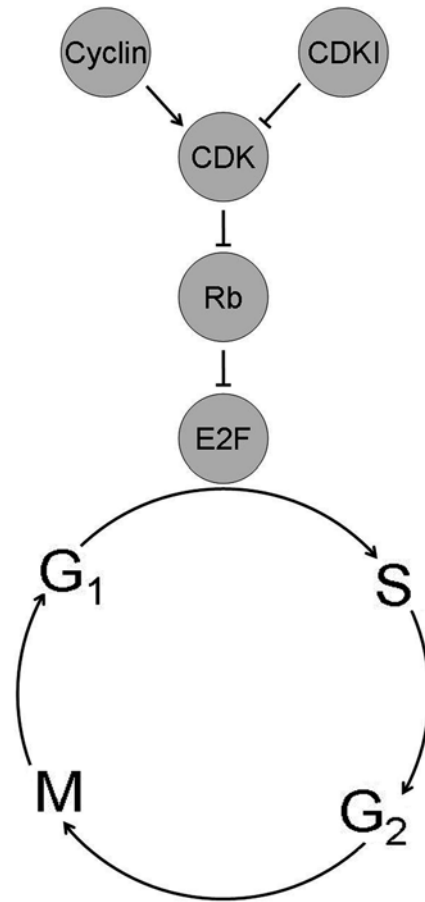


Fig. 1.3 The retinoblastoma tumor suppressor pathway. E2F transcription factors promote cell cycle progression in part by transactivating genes involved in DNA synthesis (S) phase of the cell cycle. Hypophosphorylated, active Rb inhibits cell cycle progression from G₁ phase into S phase by directly inhibiting E2Fs and by actively repressing transcription of cell cycle genes. Rb can be inhibited by hyperphosphorylation catalyzed by cyclin-dependent kinases (CDK). CDKs, in turn, require activation by cyclins, and they can be inhibited by CDK inhibitors (CDKIs) such as p16. Cancers tend to up-regulate cyclins and CDKs and down-regulate CDKIs through genetic mutation or functional perturbation

transcriptional regulation of genes critical to cellular differentiation and development (Christensen et al. 2007; Klose et al. 2007). Analysis of chromatin immunoprecipitation, DNA methylation, and gene expression data from retinoblastoma tumors reveals a strong signal of epigenetic deregulation in 15 oncogenes (Zhang et al. 2012).

1.3.3 Cell Cycle Inhibition

The most well-characterized function of Rb is to inhibit the G₁-to-S phase transition of the cell cycle (Harbour and Dean 2000a). Several key experiments implicated Rb as the regulatory “gatekeeper” of this particular restriction point in cell division. First, microinjection of unphosphorylated Rb into cells was shown to result in G₁ arrest when injected early in G₁ phase (Goodrich et al. 1991). This observation was later confirmed by overexpression of Rb via exogenous *RB1* cDNA (Hinds et al. 1992). Thus, Rb expression restrains cell cycle progression at the G₁ phase transition and effectively maintains cells in a terminally differentiated state.

The mechanism has to do with the interaction between hypophosphorylated Rb and E2F, which functionally represses transcription of genes required for S phase entry and DNA synthesis (Zhang et al. 1999). Cyclin-dependent kinases phosphorylate Rb during late G₁ phase (Sherr 1996). pRB predominates throughout S, G₂, and M phase (Chen et al. 1989), weakening Rb’s interaction with E2F (Harbour and Dean 2000b). Thus, once a cell passes through the G₁-to-S phase checkpoint, active repression is released and the cell is committed to DNA synthesis and cell division.

1.3.4 Maintenance of Cellular Differentiation

Rb promotes maintenance of cellular differentiation by promoting G₀ arrest and preventing cell cycle re-entry. Rb potentiates the function of C/EBP proteins; a family of transcription factors is implicated in the control of terminal differentiation in a variety of tissues, including adipocytes (Chen et al. 1996a) and leukocytes (Chen et al. 1996b). Rb also augments the function of MyoD, a key regulator of muscle differentiation (Gu et al. 1993). Perhaps most importantly for understanding retinoblastoma, Rb is known to effect neuronal differentiation through a complex interaction with Id2. The Id family of proteins inhibits differentiation by sequestering basic helix-loop-helix

transcription factors. Rb in turn sequesters Id2, relieving the inhibition of these transcription factors, thereby promoting differentiation (Lasorella et al. 2000). Thus, loss of Rb function is predicted to result in loss of differentiation, another critical aspect of carcinogenesis. Indeed, Rb-knockout mice demonstrate abnormal neuronal differentiation, incomplete axonogenesis, and decreased expression of neuronal differentiation markers (Lee et al. 1994). This molecular mechanism may partially explain why retinoblastoma exhibits histologic features of primitive undifferentiated neuroectoderm.

Novel roles are being defined for Rb as an extrinsic regulator of cell differentiation and migration. In the hematopoietic system, Rb inactivation has been shown to alter the bone marrow microenvironment in a manner that promotes mobilization of hematopoietic stem cells to extramedullary sites (Walkley et al. 2007). The potential role of Rb in regulating the tumor microenvironment in retinoblastoma and other cancers remains an area for further study.

1.3.5 Anti-apoptotic Function

In addition to its role as a cell cycle inhibitor, Rb also inhibits apoptosis (Harbour and Dean 2000c). Rb is phosphorylated in several hierarchical steps, and the phosphorylation events regulating the cell cycle can be differentiated from those regulating apoptosis (Harbour et al. 1999; Ma et al. 2003). It seems likely that Rb is only partially phosphorylated and inhibited during normal cell division, and that this incomplete inactivation is sufficient to block its cell cycle inhibitory function and to allow cell cycle progression without triggering apoptosis (Delston and Harbour 2006). This residual anti-apoptotic activity may persist unless Rb is more completely phosphorylated (or unless the *RB1* gene is mutated), suggesting that Rb may serve as a buffer against apoptosis during normal cell division and a checkpoint for triggering apoptosis under abnormal stress conditions such as excessive mitogenic stimulation, DNA damage, and hypoxia in which apoptosis may be favored for survival of the organism.

1.3.6 Maintenance of Chromosomal Stability

Multiple genetic alterations underlie the process of tumorigenesis in retinoblastoma and other cancers. In addition to its role in cell cycle regulation, Rb plays a crucial role in the control of mitotic chromosomes during M-phase, including organization of centromere function, chromosome condensation, and segregation (Coschi et al. 2010; Manning et al. 2010). Cells lacking Rb are more susceptible to chromosome breakage (van Harn et al. 2010). Activation of the Rb pathway induces replication stress through its combined effects on dysregulation of the G₁-to-S phase transition and M-phase chromosome dynamics, leading to a state of cellular nucleotide deficiency (Bester et al. 2011). Nucleotide deficiency promotes replication-induced DNA damage and cancer genome instability. Thus, Rb inactivation may predispose to multiple genetic alterations that ultimately underlie tumorigenesis.

1.4 Disruption of the Rb Pathway

Although inactivating *RB1* gene mutations are common in only a few types of cancer, such as retinoblastoma and small cell lung cancer, the Rb pathway is functionally disrupted in virtually all human cancers (Harbour et al. 1988; Sherr 1996). A variety of mechanisms have been described, including modulation of Rb phosphorylation, viral oncoproteins, loss of maternal imprinting, and tumor micro-RNAs.

1.4.1 Modulation of Rb Phosphorylation State

Rb is phosphorylated by cyclin-dependent kinases (CDKs), which are activated by interaction with cyclins (Ewen et al. 1993; Sherr 1996). Therefore, overactivity of cyclins or CDKs can lead to functional inactivation of Rb. For example, cyclin D1 is a bona fide oncogene that is overexpressed and functionally inhibits Rb in many cancer types (Arnold 1995; Brantley and Harbour 2000). A constitutively active CDK4

mutant that maintains Rb in a phosphorylated state has also been identified in some cancers (Wolfel et al. 1995). On the other hand, CDKs are inhibited by proteins such as p16^{INK4a}, which is a tumor suppressor that is mutationally inactivated in some cancers (Okamoto et al. 1994).

Interestingly, the tumors associated with complete Rb inactivation by genetic deletion (e.g., retinoblastoma and small cell lung cancer) have a different phenotype than those associated with partial Rb inhibition by loss of p16^{INK4a} or overexpression of cyclin D (e.g., melanoma, most solid tumors). The former tumors tend to exhibit rapid proliferation, high apoptotic rates, and sensitivity to chemotherapy, whereas the latter tumors tend to have slow rates of proliferation and apoptosis and tend to be resistant to chemotherapy (Shackney and Shankey 1999). This may reflect fundamental differences in the biologic activity of Rb at different gene dosages. Alternatively, selective pressures may favor a degree of Rb activation in the context of different tumor genomes. In colorectal cancer for instance, Rb activity is needed to repress E2F1, a potent inhibitor of beta-catenin (Morris et al. 2008).

1.4.2 Viral Oncoproteins

Some viruses have evolved proteins that transform their host cells into immortal cell lines. For example, the adenoviral E1a and E1b proteins bind to Rb and p53, respectively, resulting in stable transformation and immortalization of the host cell. Viral oncoproteins that inhibit Rb have been implicated in human cervical carcinoma (viz. human papilloma virus E7 protein) and various solid tumors in nonhuman primates (viz. SV40 large T antigen). Various mechanisms of Rb interaction exist, including direct binding to the Rb pocket domain (preventing interaction with E2F) or hyperphosphorylation of Rb (promoting inappropriate cell cycle progression) (DeCaprio et al. 1988; Dyson et al. 1989; Hume et al. 2008; Whyte et al. 1988). In addition to Rb inactivation, the adenoviral E1a protein has an independent epigenetic effect through global relocalization of Rb binding, which serves to promote S-phase entry and repress cellular differentiation (Ferrari et al. 2008).

1.4.3 Epigenetics and Imprinting

Loss of function mutations in *RB1* may represent only the tip of the iceberg, as a more complete picture of the *RB1* locus as an epigenetic “hotspot” emerges. Recent data have revealed that the *RB1* locus is imprinted and primarily expressed from the maternal allele. A CpG island located in intron 2 is unmethylated on the paternal allele and functions as a promoter of an alternative *RB1* transcript (Kanber et al. 2009). As a result, there is normally transcriptional dominance of the maternal *RB1* allele via its regular promoter. Loss of maternal imprinting represents a novel epigenetic mechanism for *RB1* pathway inactivation and has been implicated in human hepatocellular carcinoma (Anwar et al. 2014). Hypermethylation of the *RB1* promoter has been implicated in the development of some unilateral, sporadic ocular tumors (Greger et al. 1989). Tumor micro-RNAs that interact with different components of the Rb pathway also represent a means by which epigenetic modifiers might disrupt Rb function (Reis et al. 2012).

1.5 Clinical Importance of Retinoblastoma Molecular Genetics

The molecular biology of retinoblastoma has informed our clinical perspectives on the disease in myriad ways.

1.5.1 Heritability

About 93 % of new cases of retinoblastoma are sporadic with no family history. Among sporadic cases, about 60 % have the non-heritable form and 40 % have the heritable form of retinoblastoma. In patients with non-heritable disease, the eye cancer is typically unilateral and usually diagnosed between 1 and 2 years of age. There is no increased risk of other cancers, and if the primary cancer is treated effectively, these patients have a normal life expectancy (Wong et al. 1997). Patients with the heritable disease are usually diagnosed at an earlier age, typically within the

first year of life. About 85 % of these patients will have multiple, bilateral eye tumors, and upwards of 5 % will develop a midline intracranial tumor similar in embryonic origin and histologic appearance to retinoblastoma (Blach et al. 1994; de Jong et al. 2014; Marcus et al. 1998). This condition is commonly referred to as “trilateral retinoblastoma.” Patients with heritable retinoblastoma are also at risk of many other types of second primary cancers throughout life. Therefore, it is critically important to identify and monitor patients with the heritable form of the disease.

Although age of onset, bilaterality, and other clinical features may help to distinguish the heritable and non-heritable forms of the disease, genetic testing for germline *RB1* mutations is now commonly employed. No single testing strategy is sufficient to detect all germline *RB1* mutations, because of the variety of types and locations of mutations that occur (Harbour 1998). By combining multiple complementary tests, molecular evaluation of peripheral blood can identify germline *RB1* mutations in greater than 95 % of individuals with a hereditary predisposition to retinoblastoma. If a proband with unilateral retinoblastoma tests negative for a germline *RB1* mutation, the risk of developing a tumor in the other eye is quite low. In this clinical scenario, serial examinations under anesthesia may be deferred in favor of less invasive surveillance methods, such as routine clinical examination and ultrasound.

In families with a history of hereditary retinoblastoma, mutation status is helpful in identifying asymptomatic siblings at risk, for whom close screening is required during the first years of life. Negative genetic testing may reduce the need for costly screening procedures (e.g., examinations under anesthesia) in family members who do not carry the disease-causing mutation (Noorani et al. 1996).

When individuals with a known disease-causing *RB1* mutation reach reproductive age, prenatal diagnosis may in some cases help inform the family’s decisions about whether to continue the pregnancy. Alternatively, in vitro fertilization can be combined with preimplantation genetic diagnosis to select non mutation-carrying embryos for implantation (see Chap. 2 for further details).

1.5.2 Second Primary Tumors

Patients with heritable retinoblastoma have a systemic predisposition to cancer resulting from the germline *RBI* gene mutation, which is present in most or all cells in the body. Multiple other types of second primary cancers can be seen, including osteosarcomas, soft tissue sarcomas, hematologic malignancies, melanomas, and carcinomas (Moll et al. 1997). The finding of bi-allelic *RBI* gene mutations in osteosarcomas lends support to the “two-hit” hypothesis of tumorigenesis for these tumors (Friend et al. 1986). The incidence of second primary tumors reaches roughly 25 % at 40 years follow-up (Eng et al. 1993), with a median age at diagnosis of 13 years (Woo and Harbour 2010). With improvements in diagnosis and treatment for retinoblastoma, second primary tumors are now the leading cause of death in patients with heritable retinoblastoma (Eng et al. 1993).

If a patient receives radiation therapy, the risk of second malignancies increases significantly (Roarty et al. 1988). Cells lacking Rb are more susceptible to chromosome breakage (van Harn et al. 2010), perhaps increasing susceptibility to radiation-induced DNA damage. Sarcomas, in particular, are more common inside the radiation field after external beam radiation therapy (Woo and Harbour 2010). If possible, any radiation (including X-ray, CT scan, and external beam radiation) should be avoided in individuals with heritable Rb to minimize the lifetime risk of developing secondary malignancies (see Chap. 10 for further details).

1.5.3 Age of Onset

Retinoblastoma usually manifests before 5 years of age, and fewer than 25 cases have been reported in adults 20 years or older (Singh et al. 2011). The molecular genetics of the Rb pathway helps explain why retinoblastoma arises almost exclusively in pediatric patients. In most human tissues, total loss of Rb would lead to activation of p53-mediated apoptosis. However, during development human cone precursors express high levels of MDM2 under the control of RXR γ ,

a cone-specific transcription factor (Xu et al. 2009). Because MDM2 antagonizes p53-mediated apoptosis, cone precursors are protected against the pro-apoptotic effects of *RBI* deletion. There is strong selective pressure for amplification of MDM2 and MDMX gene copy number during retinoblastoma progression, leading to increased expression of proteins that abrogate the p53-mediated apoptosis response in retinoblastoma (Laurie et al. 2006).

1.5.4 Retinocytoma and Low Penetrance Retinoblastoma

The benign variant of retinoblastoma is referred to as retinoma or retinocytoma (Gallie et al. 1982; Margo et al. 1983). These benign retinal tumors can be distinguished clinically from retinoblastoma by features such as small size, surrounding chorioretinal atrophy, and lack of seeding or retinal detachment. Histopathologically, retinocytomas exhibit relatively well-differentiated retinal-like cells that form fleurettes and rosettes. Retinocytomas have been documented in the parents of children with sporadic heritable retinoblastoma and in patients with low-penetrance forms of retinoblastoma.

Most *RBI* mutations result in >90 % penetrance, but some mutations have been associated with low penetrance retinoblastoma or retinocytoma (Abouzeid et al. 2009). These low penetrance mutations can be divided into those that cause a reduction in the amount of normal Rb protein (usually promoter mutations) and those that result in a partially functional Rb protein (truncating or missense mutations) (Abouzeid et al. 2009; Harbour 2001a, b, c). Interestingly, large or complete deletions of the *RBI* gene are also associated with low or no tumor risk, as there appears to be an essential survival gene (*MED4*) located in the region of *RBI* (Dehainault et al. 2014). Recent work examining patterns of gene deletion in *RBI* and other tumor suppressor genes showed that homozygous deletions with one large and one small deletion far outnumber other possible patterns of homo- or hemizygous deletion (Bignell et al. 2010). This suggests that cancer genomes are subject to selective pressures

against large homozygous deletions of tumor suppressor genes. According to the “survival gene” hypothesis, coevolution of the *MED4/RBI* locus may reflect a genomic adaptation to mitigate the risk of tumorigenesis from large deletions of the *RBI* locus.

1.5.5 Vitreous Seeding

Advanced intraocular disease with vitreous seeding is considered one of the classic indications for enucleation in retinoblastoma. Despite advances in intra-arterial and periocular chemotherapy (Abramson et al. 2010; Harbour et al. 1996; Munier et al. 2013), vitreous seeding remains a threshold event in the progression of the disease, associated with poorer rates of local control and overall survival. Vitreous seeding occurs when retinoblastoma cells acquire the ability to detach from the primary retinal tumor and proliferate as small cell clusters within the vitreous cavity. In other cancers, loss of cell-to-cell contact is often associated with mutations in cadherin genes that encode cell adhesion molecules. In retinoblastoma, loss of heterozygosity in the 16q24 region is highly associated with diffuse vitreous seeding (Gratias et al. 2007; Gustmann et al. 2011). A cadherin gene, *CDH13*, is located in this region and its expression is significantly lower in retinoblastoma cell lines and primary retinoblastomas compared to normal human retina (Gustmann et al. 2011). However, further investigation is needed to determine whether the association between 16q24 deletion and vitreous seeding is in fact cadherin-mediated.

1.5.6 Future Treatments

Retinoblastoma is one of the great clinical success stories in pediatric oncology. A century ago, retinoblastoma was uniformly fatal, but today the survival rates are greater than 95 % in developed countries. This dramatic improvement in patient outcomes is the result of advances in diagnostic and treatment modalities (Harbour 2001a, b).

Despite enormous progress in understanding the molecular biology of retinoblastoma, there are currently no treatments commercially available which specifically target the Rb pathway. Drugs designed to mimic Rb function would likely generate untoward off-target effects, given Rb’s diverse regulatory functions and ubiquitous expression in human tissues. *RBI* gene replacement therapy would also be challenging, considering the unpredictable effects of gene dosage with current gene delivery strategies. Therefore, the next generation of rational pharmacotherapies for retinoblastoma will likely target secondary mutations or other pathways downstream of Rb.

Recent discoveries have suggested new, more promising molecular targets for retinoblastoma pharmacotherapy. Loss of Rb function results in genome-wide alterations in histone methylation, chromatin structure, and gene expression. The epigenetically modified spleen tyrosine kinase (*SYK*) proto-oncogene is upregulated in retinoblastoma and required for tumor survival (Zhang et al. 2012). Small molecule Syk inhibitors have been developed by the pharmaceutical industry and are currently being tested in early phase clinical trials for a variety of chronic inflammatory conditions (Geahlen 2014). The fact that retinoblastoma cells are sensitive to Syk inhibitors has engendered efforts to develop an inhibitor suitable for ocular delivery (Pritchard et al. 2014).

Regulators of p53, such as the MDM family of oncoproteins, represent another potentially promising target for retinoblastoma therapy. Unlike adult cancers, where loss-of-function mutations in p53 are common, p53 mutations are observed only rarely in retinoblastoma (Kato et al. 1996). Nevertheless, retinoblastoma and other pediatric cancers often employ other mechanisms of p53 inactivation (van Maerken et al. 2014). MDM2 and MDMX, which are amplified in 75 % of retinoblastoma tumors (Laurie et al. 2007), are critical negative regulators of p53. Amplification of MDM2 and MDMX gene copy number during retinoblastoma progression abrogates the p53-mediated apoptosis response in retinoblastoma (Laurie et al. 2006). The nutlins are a

class of small molecule inhibitors of p53-MDM interaction, which have shown promise in restoring wild-type p53 function in pre-clinical models of retinoblastoma (Elison et al. 2006; Laurie et al. 2006) and other pediatric cancers (van Maerken et al. 2014).

1.6 Conclusion

In the four decades following Knudson's landmark "two hit hypothesis," scientists have elucidated an incredibly complex picture of Rb's molecular biology. The Rb pathway is an important nexus of cellular control over cell cycle progression, cellular differentiation, and apoptosis. The Rb protein exerts genome-wide effects on gene expression via its interactions with transcription factors, histone deacetylases, DNA methyltransferases, and polycomb complexes. The influences of secondary mutations, epigenomic factors, and the tumor microenvironment continue to complicate the picture, but may offer new insights into tumor biology, behavior, and treatment. New therapeutic approaches inspired by molecular biology, such as Syk inhibitors and nutlins, are now in development.

Retinoblastoma continues to provide important new insights into cancer biology and treatment. New discoveries will continue to emerge from the study of retinoblastoma and the Rb pathway, and these will likely play an important role in the continued search for better cancer therapies.

Compliance with Ethical Requirements Conflict of Interest

Scott D. Walter, M.D., M.Sc. and J. William Harbour, M.D. declare that they have no conflict of interest.

No human or animal studies were carried out by the authors for this article.

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Challenges of Genetic Susceptibility Testing for Retinoblastoma

2

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2.1 Introduction

The traditional presentations of retinoblastoma include both heritable and non-heritable forms. Approximately 90 % of germline *RB1* mutations are thought to be *de novo* or caused by a new mutation in the first affected child (the proband) and were not inherited from the parents (Lohmann and Gallie 2004). Retinoblastoma (RB) is inherited in an autosomal dominant manner with a 50 % risk to inherit an *RB1* mutation. Up to 90 % of individuals with an *RB1* mutation will develop retinoblastoma. However, 10 % will not develop tumors and will be unaffected carriers of the *RB1* mutation. The children of these unaffected carriers will be at risk to develop RB, even if their parent did not. Table 2.1 shows heritable and non-heritable forms of retinoblastoma. Retinoblastoma was the prototype for Knudson's two-hit hypothesis that is well known (Knudson 1971). Mutations in both *RB1* alleles are necessary for the development of disease. The first mutation may be either a germline or somatic mutation, while the second mutation is always somatic. The second mutation or "hit" occurs in the retinal cell that progresses to form the retinoblastoma tumor (Knudson 1976).

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Given the complexities involved with *RB1* penetrance, genetic counseling for these families requires reliance on the interpretation of clinical data and genetic testing that is accurate. The primary aim of genetic counseling and *RB1* testing is to improve the disease management and provide options for family planning to both the immediate and extended family (Richter et al. 2003). Application of Knudson's hypothesis to individual families with retinoblastoma has the potential to significantly reduce the morbidity and mortality of children in these families.

2.2 Definition of Genetic Counseling

Genetic counselors work in varied settings and specialize in areas such as prenatal, pediatric, oncology, or cardiac genetics. Genetic counseling has been defined by the National Society of Genetic Counselors (NSGC) as the "process of helping people understand and adapt to the medical, psychological, and familial implications of genetic contributions to disease. This process integrates: (1) the interpretation of family and medical histories to assess the chance of disease occurrence or recurrence; (2) education about the inheritance, testing, management, prevention, resources, and research; and (3) counseling to promote informed choices and adaptation to the risk or condition" (National Society of Genetic Counselors

Table 2.1 Heritable and non-heritable forms of retinoblastoma

Feature	Non-heritable	Heritable
Retinoblastoma tumor	Unilateral	Usually bilateral or multifocal
Family history	None	~20 % of cases
Average age at diagnosis	~2 years	<1 year
Increased risk of second primary tumors	No	Osteosarcoma, soft tissue sarcomas, melanoma, others
<i>RB1</i> mutations found	In tumors only (somatic)	In germline and in tumors

Abramson and Frank 1998, American Society of Clinical Oncology 2004, Kleinerman et al. 2012

2005). “With an emphasis on education and patient empowerment, every genetic counseling encounter and diagnosis of a hereditary syndrome raises a distinct set of patient and family issues. The master’s level education and training of genetic counselors is multidisciplinary in origin combining medical and behavioral sciences and provides these professionals with the necessary skills to interact and connect with both the lay public/patients and other healthcare providers in a unique way” (Salo-Mullen and Guillem 2012).

2.3 Referrals for Genetic Counseling in Retinoblastoma Clinics

In most cancer genetics settings, the first step in the process for genetic counseling is the identification of patients who warrant an evaluation. For retinoblastoma, our center (MSKCC) offers genetic testing to every patient with bilateral, unilateral multifocal, or unilateral unifocal disease. Genetic counselors are considered an essential part of the multidisciplinary team in the thrice-weekly ophthalmic oncology clinics. The ophthalmic oncologists, medical oncologist, nurses, and administrative staff help to identify new patients and introduce genetic counseling as an option and important consideration for these families. The families are provided with the contact information to make an appointment for genetic counseling and the genetic counselors are provided with the patient’s name, medical record number, and a brief summary of the patient’s history at the time of the referral.

During the ophthalmic oncology clinics, genetic counselors approach and offer to provide

on-site counseling for the parents of patients with retinoblastoma soon after the patient’s diagnosis. The goal is to provide ease of access and facilitate this process during the patient’s treatment period. In this clinical setting, the counselors also provide follow-up to patients who have undergone testing in the past, those who have tested at an outside institution, and to patients who have previously been referred for genetic counseling but who are now approaching young adulthood.

2.4 Elements of Genetic Counseling

The purpose of genetic counseling for retinoblastoma is to educate patients and their families about the heritability, risks for secondary tumors in mutation carriers, screening protocols, recurrence risk for offspring, and reproductive options (Canadian Retinoblastoma Society 2009). Traditionally, genetic counseling is provided prior to undergoing genetic testing and after having undergone genetic testing (i.e., pre-test and post-test genetic counseling, respectively) (Riley et al. 2012). The components of pre-test and post-test genetic counseling will be described with a focus on the aspects introduced during the pre-test session.

2.5 Components of Pre-test Genetic Counseling

During the initial consultation with the genetic counselor, an overview of the genetics of retinoblastoma is provided. The genetic counselor obtains a detailed family history and draws the

pedigree, identifying at-risk relatives. In addition to heritable retinoblastoma, the counselor provides an evaluation for other cancer predisposition syndromes. When individuals at risk for a different cancer predisposition are identified, individualized genetic counseling and consideration of testing is recommended (Robson et al. 2010). Pre-test genetic counseling has been referred to being equivalent to the informed consent process (Offit and Thom 2010). Specifically, the risks, benefits, and limitations including sensitivity and ability to interpret the results of genetic testing accurately are discussed. The potential medical, psychosocial, and reproductive implications of *RB1* test results are also explained.

Since many of the parents of a proband with retinoblastoma are of reproductive age themselves, this testing has particular relevance to them so that *their* options, when applicable, as well as their child’s future options are reviewed. The types of test results and the risks, benefits, and limitations of *RB1* testing will be reviewed in this chapter. For a more comprehensive review of the medical, psychosocial, and ethical aspects of cancer predisposition genetic counseling and the risk assessment process, the

National Cancer Institute PDQ® is available (National Cancer Institute 2014).

2.6 Genetic Testing and Types of *RB1* Results

Genetic testing for *RB1* germline mutations is central to predicting disease and reducing risks (Temming et al. 2013). Testing is typically performed on a tumor tissue sample when available and blood sample from the affected proband or a blood sample alone. The genetic counselor describes the types of test results to adult patients with retinoblastoma or to the parents of patients who are minors with retinoblastoma during the pre-test counseling session. A positive result can be defined as having a causative, or deleterious, mutation identified on one allele in the *RB1* gene in a patient’s germline. This result type is expected to be identified in patients affected with bilateral or multifocal disease and in approximately 15 % of patients with unilateral disease (Dimaras et al. 2012; Richter et al. 2003; Rushlow et al. 2009). Table 2.2 gives a summary of the interpretation of *RB1* test results.

Table 2.2 Summary of the interpretation of *RB1* test results

Type of <i>RB1</i> test result	Possible interpretation	Recommendations/Options
Positive ^a	Pathogenic, deleterious mutation identified=thought to be causative of disease	Surveillance and risk-reduction strategies
		Predictive testing for family members
		Family planning options
Variant of uncertain significance ^a	Pathogenic; polymorphism; <i>or</i> no significance	Segregation and functional studies may be performed on a new blood sample
		Medical management based on history
		No testing available for family members (exception of segregation studies on a research basis)
Negative (no variant or mutation identified)	For patients with bilateral or multifocal RB = Uninformative (i.e., may be an occult mutation or a different gene involved)	Medical management based on personal history
	For patients with unifocal unilateral RB = Reduces likelihood of a mutation (i.e., may be sporadic)	No testing available for family members
	For unaffected relatives of a proband with an <i>RB1</i> mutation = Considered “true” negative	

^aMosaicism is explained to probands and/or their parents as being a possibility for both positive and variation of uncertain significance results in the proband (germline mosaicism) or one parent (gonadal mosaicism)

Cytogenetic testing is often performed when a child with retinoblastoma has developmental delay or birth defects. A chromosome deletion involving band 13q14 is reported to be found in approximately 7.5 % of probands with multifocal RB and up to 5 % of probands with unifocal retinoblastoma (Castéra et al. 2013; Mitter et al. 2011).

2.7 Potential Risks of RB1 Testing

The risks of any genetic testing, including RB1 testing, can include a physical risk from a blood draw. Genetic testing may also have a psychological effect such as emotional distress on the proband or family members who are undergoing testing. Some members of the family may decline to have their own genetic counseling or choose not to pursue their own genetic testing even after having individualized genetic counseling. There is a possibility of genetic discrimination. The federal government and some states have legislation that forbids the use of genetic test results against individuals (Genetic Information Nondiscrimination Act 2008). Some other potential risks of testing include human error in labeling or handling of a patient’s sample, laboratory error in performing analyses, and laboratory error with the interpretation or reporting of test results.

2.8 Explanation of the Potential Benefits and Uses of RB1 Genetic Testing

During the pre-test genetic counseling session and often during the referral from the ophthalmic oncology physicians and team, the potential benefits and uses of RB1 testing are outlined for patients and/or their parents. RB1 testing may impact not only the probands, but also have implications for their parents, unaffected siblings/future siblings, extended family members, and their children/future children. Table 2.3 lists the potential benefits and uses of positive RB1 germline test results.

Table 2.3 Potential benefits and uses of positive RB1 germline test results

<i>Patients with bilateral or unilateral multifocal RB who test positive for an RB1 mutation:</i>
• Confirms the explanation for the diagnosis in the patient • Pending the type of mutation (e.g., mosaic, low penetrant), confirms or assists with the recommendation for long-term follow-up and screening for secondary tumors • Allows the identification of the mutation in parents, siblings, children, extended relatives and allows true negative result for the proband’s siblings and children • Provides reproductive options now or in the future for the proband and also any of their relatives whom are identified to harbor the same RB1 mutation
<i>Patients with unilateral unifocal RB whom test positive for an RB1 mutation:</i>
• Provides an explanation for the diagnosis in the proband • Supports current medical management for the risk to the contralateral eye (regular EUA’s until at least 44 months of age) • Guides future medical management for secondary tumors and long-term follow-up • Allows the identification of the mutation in parents, siblings, children, extended relatives and allows true negative result for the proband’s siblings and children • Provides reproductive options now or in the future for the proband and also any of their relatives who are identified to harbor the same RB1 mutation
<i>Unaffected relatives who test positive for an RB1 mutation:</i>
• Guides future medical management for secondary tumors and long-term follow-up • Allows the identification of the mutation in other relatives and allows true negative result for their own children • Provides reproductive options now or in the future and also any of their other relatives who are identified to harbor the same RB1 mutation

2.9 Referral to Prenatal Genetic Counselor and Options for Prenatal Testing

Prenatal testing is possible if an RB1 mutation has been identified in the affected proband in a family. Reproductive genetic counseling is offered to young adult probands or the parents of

young probands to discuss the potential risks to offspring and the options for testing during or prior to pregnancy. Prenatal diagnosis (PD) for retinoblastoma may involve testing a fetus before birth through procedures called chorionic villus sampling (CVS) or amniocentesis. Preimplantation genetic diagnosis (PGD) is one means of assisted reproductive technology (ART) wherein embryos that do not inherit a familial mutation are selected for implantation and gestation (Offit et al. 2006a, b; Xu et al. 2004).

2.10 Explanation of the Limitations of *RB1* Genetic Testing

As an element of the pre-test genetic counseling session, the limitations of testing are reviewed in detail with the patient and/or family. In order to provide accurate consent for *RB1* gene testing, it is essential for individuals to understand the limitations including the potential intricacies of this testing. Although the sensitivity of *RB1* germline testing on blood samples alone (no tumor tissue available) has greatly improved in the past decade as updated technologies have become available, the methods utilized are laboratory-dependent. None of the current methods utilized for *RB1* germline analysis, even using a multi-method approach, have a complete detection rate including sensitivity for picking up very low level mosaicism in the blood (Rushlow et al. 2009; Price et al. 2014). Mosaicism can be defined as a situation when the genetic status of cells in the body differs from other cells of the body. Rarely, this can involve the gametes (egg or sperm cells) and is referred to as gonadal mosaicism (Carlson and Desnick 1979; Sippel et al. 1998). The complexities of *RB1* testing are introduced in pre-test setting and reviewed in detail during the post-test counseling session pending the outcome of a given patient's result.

In probands with unilateral retinoblastoma, the best way to begin testing for *RB1* gene mutations is by analyzing a sample of the tumor tissue, when available. Identification of two mutations or two copies of the same *RB1* mutation in tumor tissue

allows for targeted testing of the blood sample. Although a small chance, it is possible that very low level mosaicism in the blood sample will not be detected and the proband may wish to consider cytogenetic analysis (constitutional chromosomes) in the context of reproductive genetic counseling in the future (Lohmann et al. 1997). When no tumor tissue is available for unilateral patients (as is frequent given the success of currently available treatment modalities), testing can be performed first on the DNA from a sample of the proband's blood. The sensitivity of this testing is not as high as when tumor tissue is available.

For patients with bilateral or unilateral multifocal retinoblastoma, the test sensitivity on blood samples alone is not 100 %. When an *RB1* mutation is not identified in the DNA from the blood sample, a new blood sample for RNA analysis may be requested for additional studies by an outside laboratory. It is possible that there is an *RB1* mutation that cannot be detected by the testing methods utilized and no single method will find all possible gene mutations. In individuals that meet the diagnostic criteria for hereditary retinoblastoma, an occult *RB1* mutation or other mechanism is suspected (Thériault et al. 2014).

Another potential limitation of genetic testing, including *RB1* gene testing, is the possibility of a variation of uncertain clinical significance (VUS). VUS can also be called a variant. Some genomic variation among individuals worldwide is common (Weitzel et al. 2011). Variants in different genes are often detected in populations that have not been tested as often as other groups. When a variation of uncertain significance is identified in the *RB1* gene, a new blood sample for cDNA analysis and functional studies is often requested by the outside laboratory in order to obtain more information for the patient and family. With information from additional studies and time, the variant may be reclassified from uncertain to polymorphic, or rarely, deleterious (Culver et al. 2013).

Most *RB1* null alleles (mutant copy of the gene) show nearly complete penetrance (>99 %) and most of these families have frameshift or nonsense mutations present (Lohmann et al. 1996). It has been estimated that less than 10 % of families with hereditary retinoblastoma show

incomplete penetrance and reduced expressivity. This is called a “low penetrance” phenotype and is associated with *RB1* mutations which are in-frame or missense, one of a few specific splice-sites, or within the promoter region (Bremner et al. 1997; Otterson et al. 1997). Lastly, some families appear with lower penetrance but not

lower expressivity (Klutz et al. 2002). Alternative penetrance and expressivity in retinoblastoma and mechanisms of sporadic retinoblastoma are reviewed in case scenarios (Figs. 2.1, 2.2, 2.3, and 2.4 summarize four clinical case scenarios). The noncritical clinical features in these cases have been changed to preserve confidentiality.

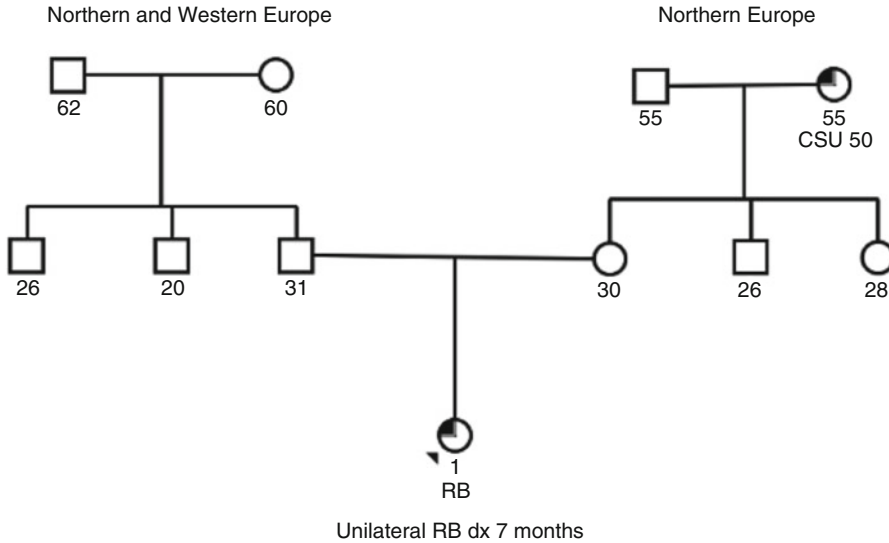


Fig. 2.1 Large germline *RB1* deletion in a patient with unilateral retinoblastoma

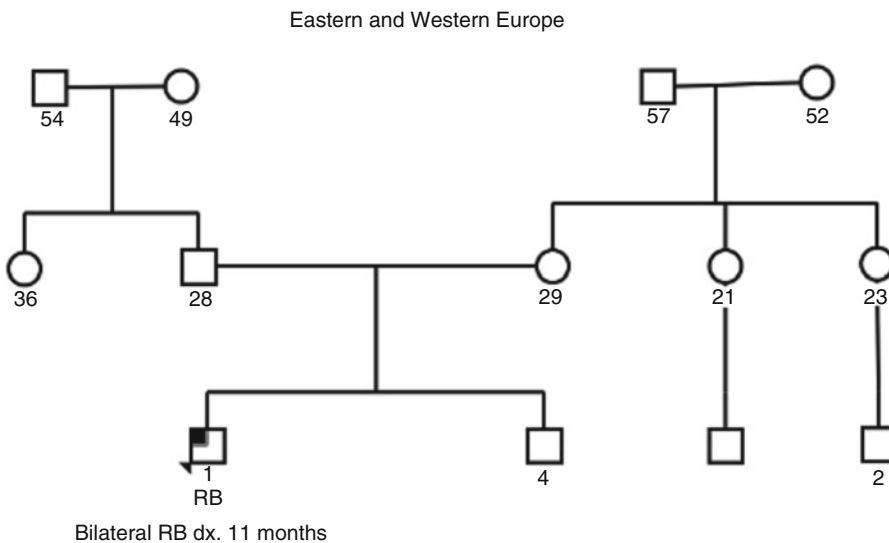


Fig. 2.2 Inheritance of the paternal allele in a patient with bilateral retinoblastoma

Fig. 2.3 Recurrence risk for parents of children with bilateral retinoblastoma

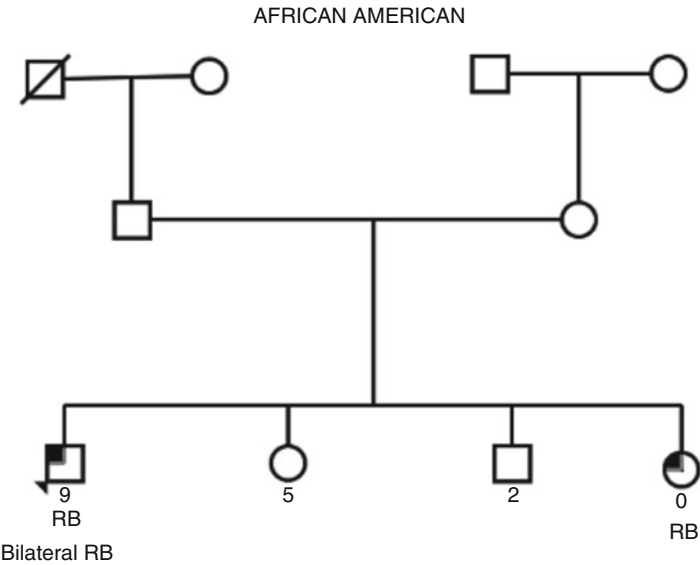
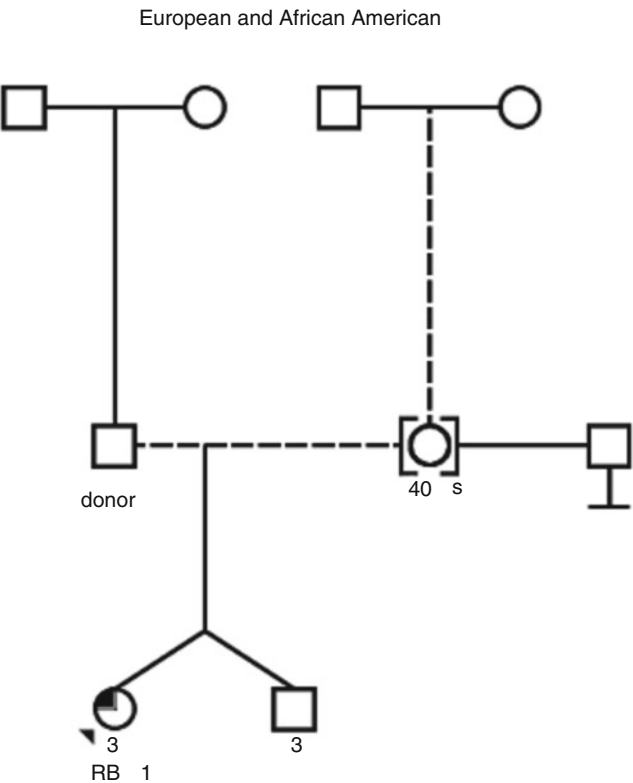


Fig. 2.4 Promoter hypermethylation in a patient with unilateral retinoblastoma



2.11 Case Scenarios

Patient Case 1: Large Germline *RB1* Deletion in a Patient with Unilateral Retinoblastoma (See also Fig. 2.1)

A 7-month-old female with unilateral retinoblastoma of the left eye, diagnosed at 7 months of age, was seen in clinic. There was no family history of retinoblastoma, her maternal grandmother had a malignancy (unknown primary) diagnosed at 60 years of age. *RB1* germline testing on a blood sample alone in the proband revealed a deletion of the entire *RB1* gene (from the promoter through exon 25) on one allele. Tumor tissue was not available.

A new blood sample from the proband was subsequently sent for high-resolution SNP-array and karyotype analysis. However, there were a lack of analyzable metaphases and it was considered a sample failure. There was a sufficient sample for FISH analysis. The FISH analysis performed on this sample of the proband's blood confirmed a deletion of the region containing *RB1*. Neither of her parents were found to harbor the deletion in their blood samples.

It has been reported that large and complete germline *RB1* deletions can be associated with low risk, or in some cases, no tumor risk (Mitter et al. 2011). The mechanisms to explain this phenomenon are currently unknown, although a recent study (Dehainault et al. 2014) defined a genomic region associated with low penetrance. This specific region includes a few genes including the *MED4* gene. *MED4* was described in this report as a “survival gene” in retinoblastoma since *RB1*–/– cells cannot survive in the absence of *MED4*.

Therefore, individuals with *RB1* deletions, such as this patient, may not have the risks typically associated with hereditary

retinoblastoma. Additional research is ongoing in this area. The patient's parents were encouraged to remain in contact with the genetics professionals for updates and to pursue submitting a new blood sample for a repeat of the high-resolution SNP-array and karyotype analysis.

Patient Case 2: Inheritance of the Paternal Allele in a Patient with Bilateral Retinoblastoma (See also Fig. 2.2)

At 14 months of age, a male patient previously diagnosed with bilateral retinoblastoma at 11 months of age presented in clinic. He had no known family history of cancer. *RB1* germline testing identified a splice-site mutation called IVS6+1G>T in his blood sample. Single amplicon testing of his parents was performed and his unaffected father was found to carry the *RB1* splice-site mutation.

This specific mutation has been previously reported to have an unusual characteristic: inheritance from the maternal allele leads to reduced penetrance, while inheritance from the paternal allele leads to almost complete penetrance. Segregation to his paternal grandparents was recommended, but testing has not been performed to date. Testing of the proband's sibling was negative for this *RB1* mutation.

The mechanism for this inheritance may be due in part to post-transcriptional events rather than transcriptional silencing. However, it is not completely understood. Specifically, post-transcriptional mechanisms may account for the low relative abundance of nonsense mRNA that is transcribed from paternally inherited mutant alleles (Klutzt et al. 2002).

Patient Case 3: Recurrence Risk for Parents of Children with Bilateral Retinoblastoma (See also Fig. 2.3)

A now 9-year-old male patient was diagnosed with bilateral retinoblastoma in infancy. *RB1* testing was performed at that time and identified a frameshift mutation. His parents were both tested and neither was identified to harbor the mutation in their blood samples. The couple went on to have two unaffected children, both of whom tested negative for the proband's *RB1* mutation during their first few weeks of life.

The couple's fourth child was diagnosed with bilateral retinoblastoma prior to 1 month of age. She was tested and identified to carry the proband's *RB1* mutation. There are a few possible explanations for this second child's diagnosis, including non-paternity, but that is unlikely in this family. This couple was educated during the post-test counseling sessions about the residual risk for future pregnancies based on gonadal mosaicism in one of the parents, and they were encouraged to meet with a reproductive genetic counselor to discuss their options in detail prior to each pregnancy.

Although gonadal mosaicism has not been proven in this family, it is suspected to be the explanation for the retinoblastoma diagnoses in this sib ship given that both children share the same *RB1* mutation (Carlson and Desnick 1979; Sippel et al. 1998).

Patient Case 4: Promoter Hypermethylation in a Patient with Unilateral Retinoblastoma (See also Fig. 2.4)

At 26 months of age, the female proband presented in clinic. She had been diagnosed with unilateral retinoblastoma of the right eye at 23 months of age. She was status post enucleation of her right eye and the tumor

tissue was available. Her mother underwent in-vitro fertilization (IVF) with donor sperm sample due to her partner's infertility issues. The proband had a dizygotic twin brother who was healthy. The tumor tissue was obtained in order to send both tumor and blood samples for *RB1* testing for this patient with unilateral retinoblastoma.

Promoter hypermethylation was identified in both alleles of the *RB1* gene in her tumor tissue and not in her blood sample. This is a well-described mechanism (Zeschnigk et al. 2004) and confirmed her sporadic disease. The family was pleased with this information and that they did not need to pursue any additional *RB1* testing themselves, for their unaffected son or for their affected daughter.

Of note, the histology of the patient's retinoblastoma tumor in patient case 4 (Fig. 2.4) was reviewed prior to sending the sample to the laboratory for genetic testing. The tumor showed the characteristic rosettes and no RB protein expression. However, if a different patient's tumor histology had included multiple nucleoli and blast cells, *MYCN* amplification may be suspected to have played a role (in the presence of non-mutated *RB1* genes in this other patient's tumor sample). Approximately 2 % of unilateral RB tumors do not have evidence of *RB1* mutations, promoter hypermethylation, or loss of heterozygosity. These tumors have been described to be molecularly, histologically, and clinically distinct from RB-/- tumors and instead have amplification of the *MYCN* oncogene. The age at diagnosis of children with *MYCN* amplification has been observed to be earlier (on average 4.5 months vs. 24 months). The possible therapeutic options such as targeting *MYCN* remain to be defined (Rushlow et al. 2013; Thériault et al. 2014). Tumor tissue from a proband with unilateral retinoblastoma is required in order to identify *MYCN* involvement and tumor samples from a larger cohort of patients are required for further study.

2.12 Other Considerations of *RB1* Testing

The genetic counselor addresses the privacy and confidentiality of *RB1* test results during the pre-test counseling session. Genetic test results are kept within a patient's medical record and only are released with written permission by the adult patient or young patient's parents. An institution's privacy practices are provided to patients at the time of registration for services including genetic counseling.

Prior to undergoing testing in the United States, the proband or the parents of the proband are provided with the cost of *RB1* testing and current procedural terminology (CPT) codes if they choose to contact their insurance carrier about coverage for *RB1* genetic testing. In the United States, many insurers have covered the cost of genetic counseling and *RB1* gene testing. The cost of *RB1* testing varies between laboratories in the US and other countries and is based on the proband's diagnosis (unilateral vs. bilateral), sample type (blood and tumor vs. blood sample only), whether a familial mutation is known, whether this is a prenatal test or not, and whether it is institutional billing or insurance billing (BioReference Laboratories 2014).

2.13 Components of Post-test Genetic Counseling

During the post-test genetic counseling session, the genetic counselor discloses the genetic test results and provides an interpretation of the *RB1* results for the proband and family members. The medical implications for the patient and family members based on the test results are reviewed in detail and recommendations for testing of parents, siblings, or more extended relatives are made. Pending the type of result and the family dynamics, there may also be a discussion about how to approach communicating the genetic test results with at-risk relatives and the potential psychosocial consequences of sharing these results with other family members.

Commercial DNA banking (the storage of DNA usually from leukocytes) is available to patients and their families for possible use of the sample in the future. Given that the testing methodologies and our understanding of genes, mutations, and retinoblastoma will likely improve in the future, this type of storage may be recommended for affected individuals and particularly when an occult *RB1* mutation is thought to be the explanation for the proband's personal history of retinoblastoma (Lohmann et al. 2000).

2.14 Conclusion

Reviewing the risks, benefits, and limitations of *RB1* testing is at the crux of the pre-test genetic counseling and informed consent process. Some of the challenges posed in the clinical cancer genetics setting, with a focus on retinoblastoma, have been reviewed. The genetics professional plays a role in providing information to patients and families during both the pre-test and post-test consultations. As the proband and/or family members reach the age of reproductive decision-making, they are encouraged to reach out to their original genetic counselor for a local referral to reproductive genetics. Also as the proband matures, a follow-up consultation may be warranted to review the proband's previous *RB1* test results and the implications of this in further detail. The genetic counselor remains a resource to the proband and family and continues to be available to them for questions.

At our institution, genetic counselors participate in multidisciplinary retinoblastoma clinics and are available to patients and families to introduce the option for testing, to provide education, and to follow-up on their understanding of the information. It is anticipated that this role and availability will continue now and into the future as patients begin their own families.

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Compliance with Ethical Requirements

Megan Harlan Fleischut declares that she has no conflict of interest. No animal or human studies were carried out by the author for this article.

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Technique of Ophthalmic Artery Chemosurgery for Retinoblastoma

3

Y. Pierre Gobin

3.1 Introduction

Following the report in 2008 of the treatment of ten children with retinoblastoma by selective ophthalmic artery chemotherapy (OAC) (Abramson et al. 2008), IAC has become an essential technique in the treatment of advanced intra-ocular retinoblastoma. The modern IAC technique comes from a long thought process: In 1955, Reese et al. (1955) was the first to describe intra-arterial chemotherapy for retinoblastoma by “instillation under direct observation into the internal carotid artery on the side of the involved eye”. Krementz et al. later reported using the same technique in a few cases (Krementz et al. 1966). The idea was forgotten for almost 30 years, until in 1993 Mohri et al., from the Tokyo University and the Japan National Cancer Center, started routinely using IAC. Their technique consisted of the infusion of melphalan into the cervical internal carotid artery while a balloon was (temporarily) inflated to occlude the intra-

cranial internal carotid distal to the take-off of the ophthalmic artery (Mohri 1993; Yamane et al. 2004; Suzuki and Kaneko 2004). In May 2006, our group performed the first direct intra-ophthalmic artery catheterization using modern microcatheters for selectively infusing chemotherapy. Our initial goal was to offer an alternative to enucleation in eyes that had failed prior treatments. However, we soon realized that IAC was effective, had acceptable ocular complications and minimal systemic toxicity (Abramson et al. 2008, 2010), and that many treated eyes could retain or even improve retinal function (Brodie et al. 2009). Therefore, we expanded our indications for IAC as a first line treatment as an alternative to (instead of failure of) intravenous chemotherapy and radiotherapy (Gobin et al. 2011). Since our first report multiple groups worldwide have adopted and then published on IAC (Muen et al. 2012; Shields et al. 2014; Peterson et al. 2011; Thampi et al. 2013; Grigorovski et al. 2014; Hadjistilianou et al. 2014; Jabbour et al. 2012).

In this chapter, we describe our experience with 1000 intra-arterial infusions for treating 229 children with retinoblastoma, the difficulties we have encountered, and the precautions that we are taking to optimize the efficacy and minimize the complications from IAC.

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3.2 Material and Methods

3.2.1 Patient Population

From May 30, 2006 to April 24, 2014, we treated 229 children with unilateral or bilateral intraocular retinoblastoma with ophthalmic artery chemotherapy. All procedures were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Patients were enrolled into a Phase 1/2 protocol until July 2009 and have since been enrolled in a prospective registry. Our local institutional review board has approved these protocols, and informed consent was obtained from the parents.

3.2.2 Protocol

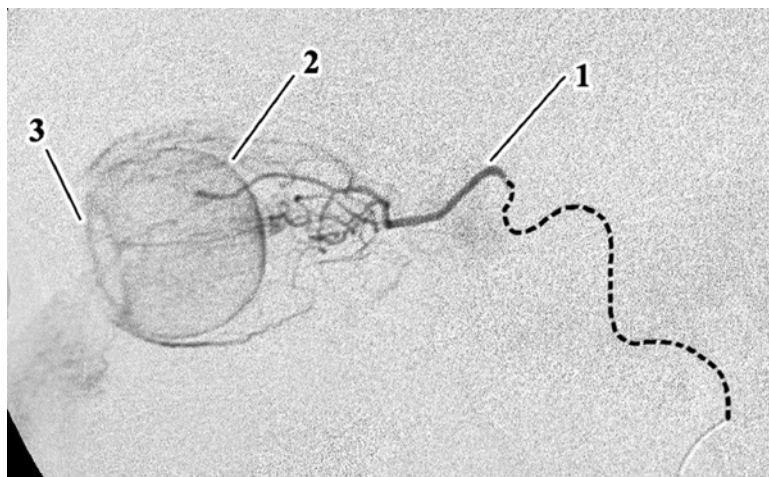
In our center, the decision to use IAC is decided during an ophthalmologic examination under anesthesia (EUA), usually performed the previous day (Gobin et al. 2011). EUA includes indirect ophthalmoscopy, fundus photography, ophthalmic ultrasound, and electroretinography. Cryotherapy (for peripheral tumor) or laser (for posterior tumor) is performed as a curative treatment of small tumors; in large tumors the goal is to increase the permeability of the blood-retina barrier prior to chemosurgery if no retinal detachment is present. At the end of the EUA, each case is discussed to decide which chemotherapy drugs will be given.

3.2.3 Intra-arterial Chemotherapy Procedure

The procedure is performed under general anesthesia with endotracheal intubation. As soon as the child is asleep, we use two topical vasoconstrictors to reduce the extra-ocular territory of the ophthalmic artery. A nasal vasoconstrictor (oxymetazoline hydrochloride 0.05 %) is sprayed into the nostril on the side of IAC (bilateral if both eyes are treated). The vasoconstrictor decreases

the blood flow from the ophthalmic artery (through the anterior and posterior ethmoidal arteries) to the nasal mucosa during the procedure. This has two benefits: first, by preventing the chemotherapy drug from being delivered to the nasal mucosa, it indirectly increases the amount delivered to the orbit; second, it prevents epistaxis due to the vascular toxicity of the melphalan when it contacts the capillaries of the nasal mucosa. In addition, when melphalan is given, another vasoconstrictor (two drops of AK-Dilate 10 %) is rubbed on the skin of the mesial forehead and upper eyelid, i.e., the usual cutaneous territory of the ophthalmic artery. This is done to decrease the vascularization of the skin during the infusion and limit the redmark that melphalan can induce (Marr et al. 2010). Following this, one femoral artery (alternatively right/left) is punctured with a 4 F micropuncture set (Cook, Bloomington, IN), using palpation of the femoral artery or in difficult cases ultrasound guidance. The 4 F micropuncture set is attached to a Tuohy Borst adapter connected to a pressurized bag of isotonic saline (with heparin 1000 IU/L). A standard dose of heparin of 80 International Units/Kg is administered intravenously. An activated clotting time (ACT) is measured after 7 min; the desired ACT value is twice the baseline and the heparin is adjusted to achieve this. The internal carotid is catheterized with a microcatheter (Magic 1.5, Balt, Montmorency France or Marathon, EV3, Irvine, CA) and a micro guide-wire (Mirage, EV3) shaped with a mild curve; a reference cerebral angiogram is then performed. In most cases, the ophthalmic artery lies in a straight line from the last curve of the carotid siphon and the microcatheter (with no curve) is easily placed at the ostium of the ophthalmic artery (Fig. 3.1). The most typical catheterization technique is to advance the microcatheter with the microguide-wire distal to the ophthalmic artery, withdraw the microguide-wire, then pull the microcatheter while simultaneously injecting contrast with a 1 cm³ syringe to flip the microcatheter tip into the ophthalmic artery ostium. It is important to keep the microcatheter at the ostium of the ophthalmic artery and not further, because distal catheterization of

Fig. 3.1 Direct ophthalmic artery catheterization. The lateral view of the selective ophthalmic artery angiogram shows the ophthalmic artery (1), the choroid blush (2) and the blush from the Ciliary body (3). The dotted line highlights the microcatheter



the ophthalmic artery risks vasospasm or worse, dissection or embolus, and should be avoided. Once the microcatheter is in place, an angiogram of the ophthalmic artery is performed to visualize the angioanatomy, paying special attention to the choroidal blush, blush from the Ciliary body, the lacrimal gland, as well as flow to the extra-ocular branches. Then, test injections are performed to determine the rate of injection and bolus volume necessary to obtain the injection of all ocular structures with no reflux into the internal carotid artery. Brisk anterograde flow is desired, while slow flow or worse, wedge flow, must be avoided as it increases the time of contact of the drug with the tissue and may lead to severe toxicity.

When the ophthalmic artery is diminutive, has a high origin, and makes a sharp angle with the carotid, direct catheterization of the ophthalmic artery may not be possible; therefore, other techniques must be used. We do not like the technique of catheterization through the posterior communicating artery as it has the inconvenience of passing through the posterior circulation where the consequences of the smallest arterial occlusion could be severe (Pham et al. 2012). Using the route through the deep temporal artery anastomoses to the orbit is possible but, in our opinion, is generally too indirect to be usually effective (Amans et al. 2014). In our center we use the two following alternatives to ophthalmic artery catheterization: the middle meningeal artery technique (Fig. 3.2) and the balloon tech-

nique (Klufas et al. 2012) (Fig. 3.3). The orbital branch of the middle meningeal artery connects the middle meningeal artery with the lacrimal branch of the ophthalmic artery; in young children, this artery is often large enough to be catheterized. The 1.5 F microcatheter must be advanced as close as possible to the lacrimal artery to reach a wedge flow state, so that the contrast (and later the chemotherapy drugs) injected refluxes into the proximal ophthalmic artery. The second alternative, the balloon-assisted technique, is similar to the Mohri technique (Mohri 1993), where a balloon microcatheter (Hyperform 4×7, EV3) occludes the ICA above the ophthalmic artery, while a guide catheter (DAC 4.2 F, Concentric Medical, Mountain View CA) is advanced up to the petrous segment of the internal carotid artery.

All precautions are taken to reduce radiation doses (Vijayakrishnan et al. 2010; Gobin et al. 2012a). We use a specific pediatric low dose protocol developed on our General Electric angiography machine. Fluoroscopy is performed with the minimal magnification compatible with adequate visualization of the microcatheter, using further collimation to reduce the field of view to the necessary minimum; additionally, the image detector is placed as close to the patient as possible. Fluoroscopy is performed in anteroposterior view for catheterization of the common carotid artery from the aortic arch, then in lateral view only (with the x-ray tube on the side of the treated eye)

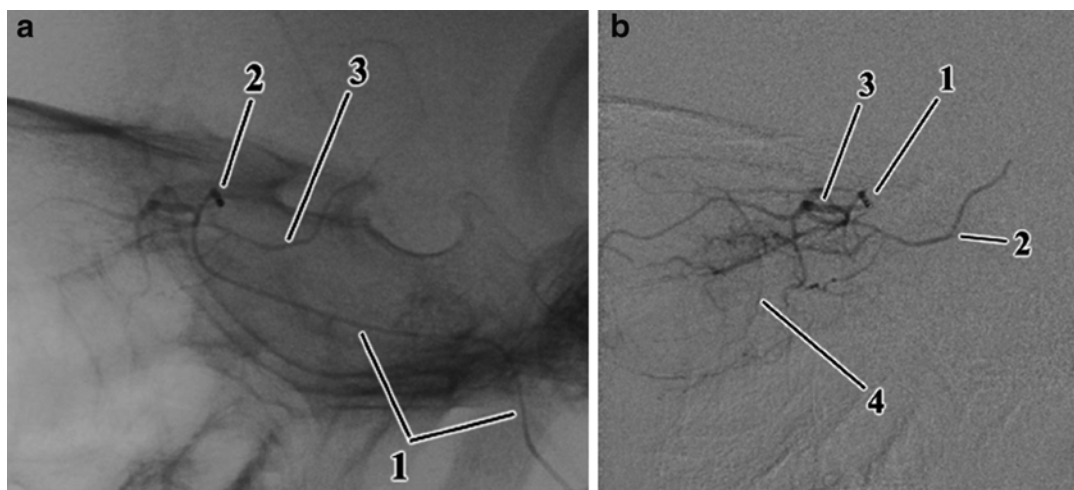


Fig. 3.2 Middle meningeal artery technique. Lateral view of the catheterization of the orbital branch of the middle meningeal artery, unsubtracted (**a**) and subtracted (**b**) angiograms. (**a**) Shows the microcatheter (1), the microcatheter tip wedged into the orbital branch of the middle meningeal

artery (2) and the retrograde opacification of the ophthalmic artery (3). (**b**) Shows the tip of the microcatheter (1), reflux into the ophthalmic artery (2), the anastomosis between the orbital branch of the middle meningeal artery and the ophthalmic artery (3) and the choroid blush (4)

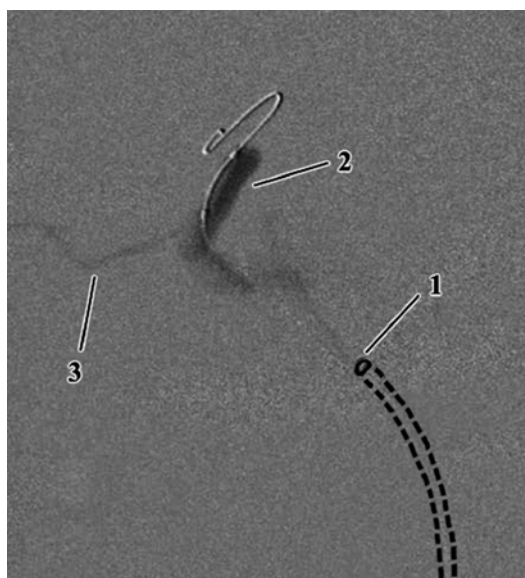


Fig. 3.3 Balloon-assisted technique. Lateral view angiogram of the internal carotid artery with the micro-balloon (2) inflated in the supra-clinoid segment of the internal carotid artery. Contrast injected from the guide catheter at the catheter tip (1) opacifies the internal carotid artery and the ophthalmic artery (3)

for catheterization of the internal carotid artery followed by the ophthalmic artery. All ocular angiograms are performed by subtracted fluoroscopy rather than digital subtracted angiography.

Lateral fluoroscopy is collimated to avoid directly irradiating the eye. Biplane simultaneous fluoroscopy is performed only when catheterizing the middle meningeal artery or using the balloon technique. Using all these steps, the resulting irradiation doses are extremely low, much lower than the doses received when performing a regular cerebral angiogram or neuro-interventional procedure.

The chemotherapy drugs received from the hospital pharmacy are diluted with saline to obtain a 10 cm³ solution that is injected manually by repeated small bolus—pulsatile injection—at a rate of 1 cm³/min. If several drugs are used, they are injected sequentially. Melphalan is more delicate to use than carboplatin or topotecan because it easily crystallizes when in solution, and these crystals should not be injected into the retinal circulation. Melphalan needs to be injected within 1 h of its preparation, which requires tight coordination between the angiography suite and the pharmacy; it also has to be filtered just before injection, which we do in the angiography suite, immediately before usage, with a 0.22 μm high pressure filter (Interlink System Extension Set, Baxter, IL). After drug delivery, another angiogram of the ophthalmic artery is performed. The microcatheter is then pulled and a final cerebral

angiogram is performed. When using balloon assistance, each drug is diluted to a total volume of 5 cm³. The balloon is inflated and the first drug is injected in the guide catheter within 2 min; the balloon is then deflated for 3 min of reperfusion; this sequence is repeated for each drug. At the end of the procedure, all catheters are removed and hemostasis of the femoral artery is obtained with manual compression for 15 min. The child is awoken and kept in the post-anesthesia recovery unit for 5 h before being discharged home. Corticosteroids are given during and after the procedure when carboplatin and melphalan are given simultaneously: dexamethazone 0.5 mg/kg IV during the procedure, oral prednisone 1 mg/kg/day for 3 days followed by half dose for 2 days. A complete blood count with platelets is performed 7–10 days after the procedure.

Drug dosage: The drug(s) are dosed according to age (which provides an estimate of the ocular size) (Fledelius and Christensen 1996) and the angioanatomy of the ophthalmic artery and its branches. Three drugs are used: melphalan, topotecan, and carboplatin. The drug doses are not based on body weight. Body weight is taken into account only for limiting the total systemic dose in the youngest children with bilateral treatment, particularly with melphalan (Abramson et al. 2010). In our experience, a dose of melphalan of more than 0.4 mg/kg was often complicated with neutropenia (Dunkel et al. 2014). At the beginning of our experience, we were using mostly melphalan, sometimes in association with topotecan. We now reserve single drug chemotherapy for the simplest cases (Abramson et al. 2012a; Francis et al. 2012a) and most often perform triple drug chemotherapy (Marr et al. 2012a). In bilateral cases, we cannot use melphalan on both sides because of systemic toxicity; so in general the worst side receives triple drug IAC with melphalan, while the contralateral side receives double drug IAC with carboplatin and topotecan only. For each drug, we have established a standard dose (Table 3.1) for a “standard angioanatomy” of the ophthalmic artery (Marr et al. 2012b). The dosage does not increase further after 3 years of age, as at this age the eye has almost reached adult size (Fledelius and Christensen 1996). The dose

Table 3.1 Drug dosage

Age/Drug	3–6 month	6–12 month	1–3 year	>3 year
Melphalan (mg)	2.5–3	3	4	5
Topotecan (mg)	1	1	2	2
Carboplatin (mg)	30	30	40	50
Increase dose:	• OA has large extra ocular branches • Insufficient result from previous cycle • Balloon and MMA technique			
Decrease dose:	• Recent IV chemo/EBR • Wedge flow • ERG decrease after previous cycle • Inflammatory reaction previous cycle • Total dose of Melphalan would be >0.4 mg/kg			

must be adapted to the angioanatomy and increased by up to 50 % if large extra-ocular branches (e.g., middle meningeal artery) are seen on ophthalmic artery angiogram. In case of “wedge flow” (stagnation of the contrast) in the ophthalmic artery beyond the microcatheter, the dose is reduced by 30–50 %. The dose is also reduced by 20–30 % when the eye had received multiple therapies, especially recent radiation therapy. Finally, the dose should be adapted on a case-by-case basis according to the response from the previous chemosurgery. Efficacy and toxicity are evaluated during the EUA performed 4 weeks after treatment. Retinal toxicity is best estimated on the electroretinogram, as it is very difficult to measure clinically the visual acuity in preverbal children (Brodie et al. 2009; Gobin et al. 2011). In addition, toxicity must be suspected if an inflammatory reaction (palpebral swelling and conjunctival hyperemia) is observed in the days following the chemosurgery, and the drug dosage should be decreased for subsequent procedures.

Anesthesia considerations: IAC is associated with a high incidence of serious respiratory reactions, which is specific to IAC and has not been described previously during neurological catheterization and interventions in young children. We had some difficulties at the beginning of our experience to differentiate this reaction from allergic

reactions to contrast. However, we later characterized this reaction as an acute decrease in lung compliance (Kato et al. 2015). The respiratory reaction appears before the injection of any contrast medium, when the microcatheter reaches the cavernous segment of the internal carotid artery or the ophthalmic artery. In very sensitive patients, it can even appear when the catheter reaches the low cervical internal carotid artery. This decrease in lung compliance has a bimodal distribution, with one group exhibiting only mild changes and a second group exhibiting severe changes. Severe decrease in compliance occurs in approximately 30 % of all procedures and is more likely to happen during the second and subsequent procedures. This syndrome is easily recognized by an increase in the inspiratory pressure and/or decrease in the tidal volume occurring as soon as the microcatheter has reached the arterial trigger point. If not treated adequately, it can be followed by hypoxemia, hypotension, and bradycardia (Phillips et al. 2013). It is managed very efficiently with the administration of IV epinephrine (0.25 mcg/kg at the first sign of compliance changes; if more severe changes are observed then 0.5–1 mcg/kg is administered) and stopping the catheterization until the lung compliance has normalized, at which point catheterization can resume. Other drugs, such as atropine, albuterol, diphenhydramine, or dexamethasone, are either marginally or not effective and should not be used for this indication. Curiously, in case of bilateral IAC, when the lung compliance decrease has occurred at the catheterization of one side, it will not recur for the catheterization of the contralateral side despite the short half-life of epinephrine. In our series, no child suffered morbidity or longer recovery room time from this respiratory reaction.

3.3 Results

From June 30, 2006 to April 24, 2014, we performed 1000 intra-arterial chemotherapy infusions during 856 procedures (144 were bilateral) for the treatment of 290 eyes with retinoblastoma in 229 patients. There were 98 males and 131

females, aged from 1 month to 21 years (median=15 months). Most eyes had advanced tumors: according to the Reese-Ellsworth classification, 50 (17 %) eyes were Group I–IV and 240 (83 %) eyes Group V; according to the International classification 50 (17 %) eyes were Group A–C, 204 (70 %) eyes were Group D, and 36 (13 %) eyes were Group E. Many patients had prior treatment before being referred to our center, including: 22 contralateral enucleations (10 % of 229 patients), 88 intravenous chemotherapy (38 % of 229 patients), 20 IAC performed in another center (7 % of 290 eyes), 14 external beam radiation (5 % of 290 eyes). Because we reserve IAC to children older than 3 months and who weight more than 7 Kg, 21 children (9 % of 229 patients) who presented in the neonatal period were first treated with intravenous carboplatin until they reached the required age and weight. This bridging intravenous-intra-arterial protocol has been previously reported (Gobin et al. 2012b).

The average number of IACs per eye was 3.4 (range 1–9). Catheterization was successful in 988 cases and failed in 12 (1.2 % failure). Failures were mostly at the beginning of our experience when we were not using alternative techniques to direct ophthalmic artery catheterization. IAC was achieved by direct ophthalmic artery catheterization in 772 cases (77 %), catheterization of the MMA in 78 (8 %), and using a balloon in 138 (14 %). In 11 cases (all of which were included into successful catheterization), not all drugs could be injected for various reasons: unstable catheter in the ophthalmic artery (4), unstable balloon (1), vasospasm of the internal carotid artery while using the balloon (1), thrombosis of the middle meningeal artery (1), reverse flow into the ophthalmic artery (3), and finally severe allergy to carboplatin (1).

One patient died from a trilateral retinoblastoma, two patients who developed metastasis were treated with intensive chemotherapy and are doing well.

Eye survival: The Kaplan–Meyer estimate of ocular survival was 85 % at 2 years, 77 % at 5 years, and 71 % at 7 years.

Complications were various and are detailed below. Note that there were few serious ocular and extra-ocular complications.

Neurological complications: One patient developed a mild hemiparesis after balloon-assisted infusion. The 24-h MRI was slightly abnormal with a doubtful DWI hypersignal in the ipsilateral basal ganglia. The patient made a full recovery in a few weeks. We have no definite explanation regarding the mechanism of this complication involving the arterial territory of the anterior choroïdal artery: embolic, flow reduction secondary to balloon inflation, or toxicity of the chemotherapy are possible explanations.

Ocular complications: An inflammatory reaction following the procedure was frequent, usually appearing the second or third post-operative day and generally lasting 1–3 weeks (Marr et al. 2010). Reactions included simple eye swelling in 26 (9 %), or more severe swelling with ptosis in 12 cases (4 %) (Fig. 3.4), VI nerve paresis in four cases from which one did not fully recover, transient ON swelling in four cases. Inflammatory reactions are minimized by steroids and by reducing the dosage of drug for the subsequent procedures.

A red mark on the mesial part of the forehead, above the eye, is frequent after infusion of melphalan (approximately 20 % of cases) (Fig. 3.4). It appears approximately 1 week after the procedure and may persist for 3–4 weeks (Marr et al. 2010). It is almost always transient, but was complicated in three cases in our series: One ulceration, which healed leaving a small scar, one skin fold due to subcutaneous atrophy, and one persistent pigmentation. Recently, we have been able to reduce its incidence and intensity by rubbing the skin of the forehead with two drops of phenylephrine (AK-Dilate 10 %) immediately before the infusion of melphalan. Madarosis or loss of eyelashes, especially on the medial third of the upper eyelid, is also frequent; however, it has been transient in our experience.

Four eyes became blind after developing an occlusive arteriopathy of the retina; on examination the findings were similar to what can be

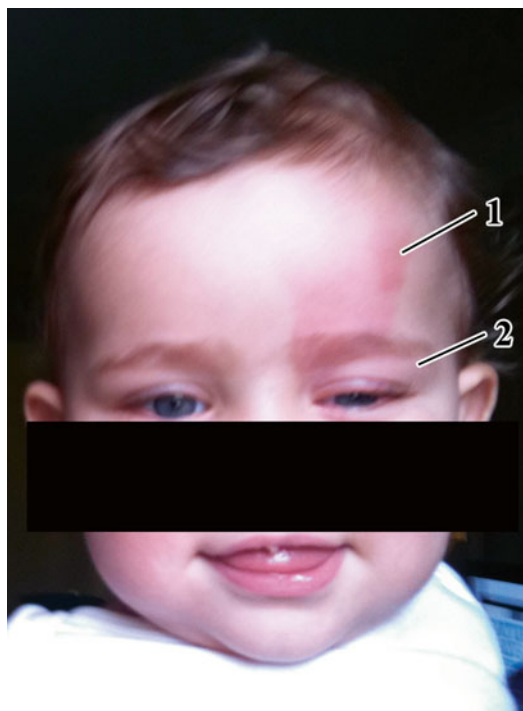


Fig. 3.4 Complication of intra-arterial chemotherapy. This photo taken 10 days after intra-arterial chemotherapy with melphalan shows erythema (1) and ptosis (2). This complication is almost always transient

observed after external beam radiation. All four cases occurred early in our experience, in eye that had been extensively previously treated and received multiple sessions of high dose (7.5 mg) melphalan. In three of the four cases, severe inflammatory reactions had developed after previous IAC. Following these complications, we decided to not (or exceptionally) give more than 5 mg melphalan per IAC and to consider the inflammatory reaction as a warning for potential retinal toxicity.

Three children experienced vascular retinal complications: the first patient developed bilateral central retinal artery occlusion immediately following bilateral IAC. Fortunately, one eye regained vision. Coagulation workup demonstrated an elevated plasminogen activator inhibitor (Francis et al. 2012b). A second child with sickle cell trait developed a large sub-retinal hemorrhage; the eye was subsequently enucleated (Abramson et al. 2012b). In a third case, a

hemi-central retinal vein occlusion developed 2 days after high dose IAC in a child who was refusing post-procedure steroids.

Arterial complications: We observed one occlusion and two stenosis of the ophthalmic artery, all developing after repeated catheterization and infusion of multiple drugs, including melphalan. Similarly, after IAC was performed through infusion into the orbital branch of the middle meningeal artery, two of the arteries stenosed and one thrombosed. None of these complications were symptomatic; this is likely due to the fact that the arterial lesions progressively appeared, giving time for collaterals to develop. We also observed one dissection (visible flap) of the supraclinoid segment of the internal carotid artery while using the balloon technique. The procedure was aborted and the artery normalized on a follow-up angiogram performed 15 min later. This last child was placed on aspirin for 3 weeks and was never symptomatic.

Access complications: There were two occlusions of the femoral artery. One was diagnosed the day following the procedure due to pallor of the foot. Daily aspirin was given and a Doppler performed 1 week later showed that the femoral artery had reopened. Another femoral occlusion was found incidentally on the clinical examination during the following IAC procedure. There were very few groin hematomas, only one was significant enough to warrant overnight admission for observation. Finally, petechia in the foot were observed in three cases.

Hematologic complications: At least one episode of Grade 3 or 4-toxicity (neutropenia <1000 was the most frequent) was observed in 30 % children. Twenty hematologic episodes required intervention: infusion of filgrastim in eight cases, transfusion in five cases, admission for filgrastim, and antibiotherapy in seven cases.

Allergy: Two patients progressively developed a severe allergy to iodine; one patient needed retreatment and the procedure was performed without using contrast. One patient developed a severe

allergy to carboplatin and further IACs were performed with melphalan and topotecan only.

There were three episodes of epistaxis after IAC with melphalan, occurring 7–10 days after the procedure; one, combined with thrombopenia, required platelet transfusion; all episodes of epistaxis were benign and did not necessitate more than anterior packing. All were at the beginning of our experience, before we started giving nasal vasoconstrictors; no episodes of epistaxis have occurred since.

3.4 Conclusion

As our experience has been building over more than 8 years, we have learned the following key points:

1. We can minimize access (groin) complications by decreasing the size of the introducer sheath to 3 F. This requires to forgo using an intermediary 4 or 5 F catheter and to perform the entire catheterization using the microcatheter only.
2. We can minimize irradiation by doing the entire procedure with digital subtracted fluoroscopy instead of digital subtracted angiography.
3. The drugs and doses of chemotherapy are determined according to age (as an approximation for eye size) then adapted to each patient and each procedure, according to the angioanatomy, position of the microcatheter, and results of the former IAC.
4. The respiratory reaction to catheterization consists of a decrease in lung compliance; it should be differentiated from an allergic reaction (contrast media or carboplatin); it is very effectively treatable with epinephrine.
5. The microcatheter should be positioned at the ostium of the ophthalmic artery and not advanced further.
6. Failure of catheterization is rare when one uses the three techniques of catheterization (ophthalmic artery, middle meningeal artery, and balloon-assisted infusion).

Compliance with Ethical Requirements Y. Pierre Gobin declares that he has no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

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Vitreous Disease and the Technique, Safety and Toxicity of Intravitreal Chemotherapy for Retinoblastoma

4

Jasmine H. Francis

Historically, there has been trepidation surrounding the placement of needles into the eyes of patients diagnosed with retinoblastoma. Many of these concerns derive from earlier literature that reported the grave consequences of diagnostic needle biopsies of retinoblastoma eyes (Chévez-Barrios et al. 2005; Henkes and Manschot 1963; Karcioğlu 2002). However, injections for therapeutic purposes permit smaller gauge needles and do not aspirate the intraocular contents. With the adoption of safety-enhanced techniques and good efficacy data, intravitreal injections of chemotherapy for retinoblastoma are becoming increasingly common. This chapter describes the nature of vitreous disease, including the classification of vitreous seeds. It then discusses the methods, rationale, safety and toxicity for the current technique of intravitreal injections of chemotherapy in retinoblastoma eyes.

4.1 Vitreous Disease

Describing vitreous disease with the umbrella statement “seeds,” does not capture the heterogeneity that is observed clinically. Hence, our group believes differentiating vitreous seeds into three

categories is a strong clinical tool and a classification has been proposed (Munier 2014; Francis et al. 2015). The classification system is based on morphological characteristics of seeds and divides them into three groups: dust (class 1), spheres, (class 2), and clouds (class 3). Further investigations have discovered that each class of seeds responds to intravitreal melphalan in a distinct manner, and these findings are summarized below (see Fig. 4.1) (Francis et al. 2015).

4.1.1 Dust

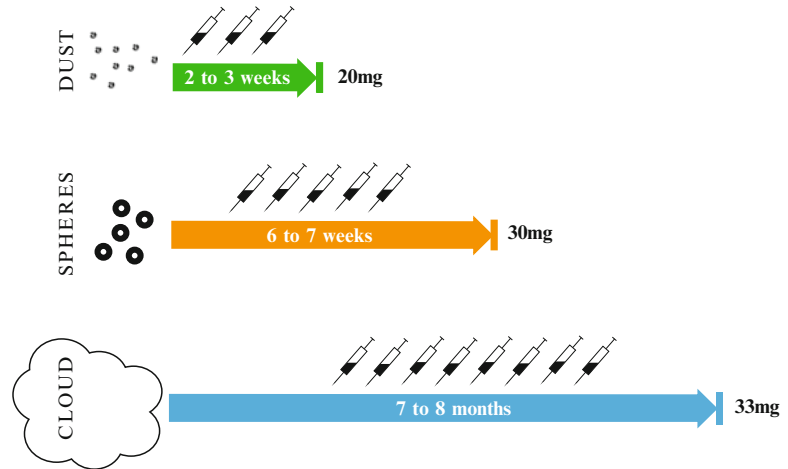
Dust appears as small granules of vitreous opacities, often seen as a vitreous haze overlying tumor. It results from a displacement of tumor cells into the vitreous (Munier 2014). It responds in the least amount of time to intravitreal melphalan, at approximately 2 weeks following the first weekly injection. Eyes with dust receive the fewest number of injections, least amount of cumulative melphalan and lowest mean melphalan dose before regression is noted (Francis et al. 2015). Dust typically regresses into a type 0 pattern, meaning it is not detectable via ophthalmoscopy.

4.1.2 Spheres

Spheres are the result of translocation of tumor cells into the vitreous, which undergo clonal expansion into spheres (Munier 2014; Kivelä et al. 2011;

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Fig. 4.1 Illustration of vitreous seed classifications and response to intravitreal melphalan



Francis et al. 2015). They are spherically shaped opacities, sometimes intermixed with dust. They can appear as homogeneously opaque, or can have a translucent outer shell with relatively transparent or whitish center (or vice versa). They respond to intravitreal melphalan in a medium amount of time, specifically at 6–8 weeks. Similarly, their regression is apparent after significantly fewer injections and less melphalan than a cloud, but more than dust (Francis et al. 2015). Initially, spheres disperse (which has been described as pseudo-growth) and eventually disappear by ophthalmoscopy, or became calcific (regression type I) or amorphous in shape (regression type II) or a combination of the latter (regression type III).

4.1.3 Clouds

Clouds are seen as a dense collection of punctuate vitreous opacities and form from massive transference of tumor cells into the vitreous (Munier 2014). They can appear as a sheet or globule of seed granules sometimes with wispy edges, and dust and spheres can be present. They respond to intravitreal melphalan in a prolonged manner, calculated at approximately 7–8 months (Francis et al. 2015). In part due to this long duration, they are treated with more injections, the highest amount of cumulative melphalan and highest mean melphalan dose. They typically regress to calcific or amorphous

granules and, with time, can become undetectable to ophthalmoscopy.

4.2 Technique

4.2.1 History

Recently, there has been increase in the use of intravitreal injections for retinoblastoma. Over a 50-year period prior to 2011, the literature reported 77 injections into retinoblastoma eyes. This compares to the past three years since 2011, when the number of reported injections has surpassed 1300. Intravitreal injections for retinoblastoma began with Ericson et al. in 1961 and the use of thio-TEPA, an alkylating agent derived from aziridine and thiophosphoryl chloride (Chávez-Barrios et al. 2005; Ericson and Rosengren 1961). The same agent was used again, three and then four decades later by two separate groups (Munier et al. 2012a; Seregard et al. 1995; Lee et al. 2003). The ensuing years brought about experimentation with a number of other drugs and agents including thymidine kinase delivered via virus particles (Chávez-Barrios et al. 2005; Francis et al. 2014a), bevacizumab (Seregard et al. 1995; Lee et al. 2003), methotrexate (Kivelä et al. 2011; Smith et al. 2012), carboplatin (Smith et al. 2012) and even topotecan (Kivelä et al. 2011; Ghassemi et al. 2014).

However, the vast majority of the reported cases in the world's literature involve the intravitreal use of melphalan. Specifically, 85 % (1209 of 1420) of the reported injections into retinoblastoma eyes are comprised of melphalan. This effort is spearheaded by the extensive experience of Kaneko and his colleagues (Munier et al. 2013; Kaneko and Suzuki 2003). They previously demonstrated melphalan to be a powerful agent against retinoblastoma in vitro (Munier et al. 2013; Inomata and Kaneko 1987), and it became the drug of choice for both their selective ophthalmic arterial injection therapy and intravitreal techniques. Subsequently initiated by the work of Munier (Chávez-Barrios et al. 2005; Munier et al. 2012b, 2013), other groups have now published their results using intravitreal melphalan.

4.2.2 Safety Enhancement

Many of the concerns regarding extraocular retinoblastoma dissemination with therapeutic intravitreal injections have been tempered by the adoption of safety-enhanced techniques. The literature shows a low risk of extraocular dissemination. In a review of 1304 reported injections, there are 12 cases of extraocular extension in eyes receiving intravitreal injections, but only two of these had propagation along the needle tract and were attributable to the injection

(Francis et al. 2014b; Smith and Smith 2013). Smith et al. calculated the proportion of eyes with extraocular extension from intravitreal injections to be 0.007 (Francis et al. 2014b; Smith and Smith 2013). This low risk has been explained by the implementation of various techniques to improve safety, which are outlined below.

4.2.3 Decreasing Needle Size and Volume of Drug

Boyle's law suggests that introducing volume into the closed space of the globe, should increase pressure and thereby heighten the risk of effluxing intraocular contents outwards. Therefore, over the years, there has been a drive to use increasingly smaller volumes of intravitreal drug (Table 4.1). In the 1960s and 1990s, volumes of 0.3–0.5 mL of fluid were injected into retinoblastoma eyes (Ericson and Rosengren 1961; Seregard et al. 1995). This has since been reduced to volumes on the order of 0.1 mL and even as low as 0.05 mL (Kivelä et al. 2011; Francis et al. 2014a; Munier et al. 2012b). In addition to a decreased volume, needles with a smaller diameter are used where the assumption is that a small needle reduces the corresponding needle puncture site, and provides less space for tumor cells to escape the confines of the globe. The needles that were initially used in the 1960s measured

Table 4.1 Previous published literature on intravitreal melphalan and injection characteristics

Author	Year	<i>n</i> inj	Drug	Dose	Needle	Volume
Ericson et al.	1961	38	Thio-TEPA	NR	0.3 mm	0.3 cm ³
Seregard et al.	1995	17	Thio-TEPA	2 mg	NR	0.5 cm ³
Lee et al.	2003	1	Thio-TEPA	NR	NR	NR
Chevez-Barrios et al.	2005	21	Thymidine kinase	10 ¹¹ vp	30 g	NR
Wells and Hubbard	2011	1	Bevacizumab	NR	NR	NR
Kivelä et al.	2011	131	MTX	400 µg	30 g	0.1 cm ³
Kaneko et al.	2011	896	Melphalan	8–40 µg	30/32 g	0.05 cm ³
Smith et al.	2012	2	Carboplatin	3–6 µg	32 g	0.05–0.1 cm ³
Munier et al.	2012	135	Melphalan	8–30 µg	32 g	0.1 cm ³
Ghassemi et al.	2012	33	Melphalan	8–50 µg	30 g	0.05 cm ³
Sun et al.	2013	6	Melphalan	20 mg [SIC]	30/32 g	0.05 cm ³
Francis, Schaiquevich et al.	2014	107	Melphalan	30 µg	33 g	0.07 cm ³
Ghassemi et al.	2014	33	Melphalan and topotecan	40 µg	30 g	0.08 cm ³

n inj number of injections, *NR* not recorded, *vp* virus particles, *g* gauge

0.3 mm in diameter (Ericson and Rosengren 1961). Since 2011, groups report using either 30- or 32-gauge needles, the latter of which has an outer diameter of 0.24 mm. Our group currently uses a 33-gauge needle, which has an outer diameter of 0.21 mm (Francis et al. 2014a).

4.2.4 Lowering Intraocular Pressure

With the same considerations for Boyle's Law, there has been a move towards lowering intraocular pressure prior to retinoblastoma eyes receiving an intravitreal injection. In the first reports on intravitreal injections into retinoblastoma eyes, Ericson et al. wrote, "an increase in pressure arises, disappearing after 15–20 min" (Ericson and Rosengren 1961). In 2005, Chevez-Barrios et al. was the first to introduce an anterior chamber tap as a means of lowering intraocular pressure in these retinoblastoma eyes (Chávez-Barrios et al. 2005), and this was used again in 2011 by Kivelä et al. (2011). Munier and colleagues revisited this concept and asserted the benefit of a paracentesis to create a transient hypotony and prevent efflux (Munier et al. 2012a). Other groups have adopted a similar technique. Our group prefers to use digital massage to lower the intraocular pressure to lower than 10 mmHg (Fig. 4.2) (Francis et al. 2014a).



Fig. 4.2 Digital massage is performed to the eye to lower the intraocular pressure below 10 mmHg prior to the intravitreal injection

This can be achieved in less than a minute, immediately prior to the injection and confirmed by TonoPen. It has the advantage of achieving an adequate result without the introduction of an additional needle into a tumor-laden eye.

4.2.5 Injection Site Precautions

Some groups have paid specific attention to the needle injection site, believing this to aid in risk reduction. Chevez-Barrios et al. elected to use "a transcorneal approach" as opposed to the pars plana location used by the majority of other groups, "to minimize the risk of tumor spread during the injection" (Chávez-Barrios et al. 2005). This approach required them to insert the needle through the iris and zonules while avoiding the lens (Chávez-Barrios et al. 2005). In addition, supplementary techniques have been adopted to sterilize the injection site area. For instance, Kivela et al. describe applying a swab to the injection site "to wipe away any vitreous from the needle when retracting it" (Kivelä et al. 2011). Others, such as first reported by Chevez-Barrios et al. (2005), have chosen to perform cryotherapy at the needle site. The Swiss group does this in a triple-freeze-thaw fashion over the injection site, immediately after the needle is withdrawn from the eye and without indenting the eye (Munier et al. 2012a). Our group uses a single-freeze-thaw cycle by placing the cryoprobe adjacent to the needle and allowing the ice formation to encompass (and thereby seal and sterilize) the injection site as the needle is removed (Fig. 4.3) (Francis et al. 2014a).

A number of groups have elected to deposit chemotherapy in the subconjunctival or subtenon space as a means of further decontamination. This was first reported by Seregard et al. following intravitreal thio-TEPA, "and a small amount was left subconjunctivally at the injection site" (Seregard et al. 1995). Smith et al. report two cases where they deposited "subtenon" carboplatin prior to chemotherapy to "mitigate the risk of orbital tumor seeding" (Smith et al. 2012). They found no evidence of viable tumor cells on histopathologic examination and no signs of orbital recurrence at 37.5–45.4 months of follow-up

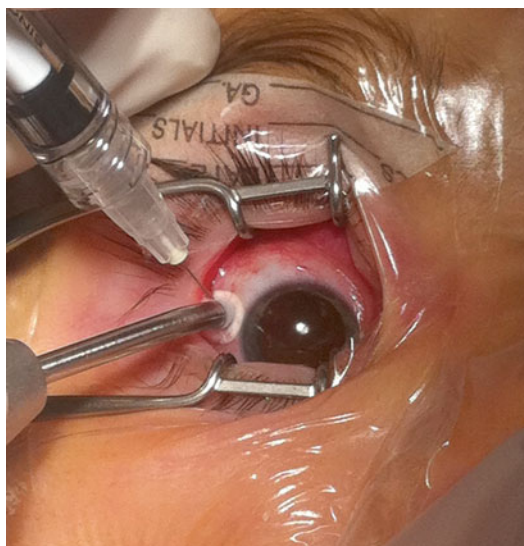


Fig. 4.3 Cryotherapy is performed to the injection site prior to needle removal, such that the ice encompasses the puncture site in order to seal and sterilize it

(Smith et al. 2012). Similarly, the Japanese group with the most extensive experience described expelling a small amount of melphalan in the subconjunctival space as the needle is withdrawn, for the purpose of sterilizing this space.

4.2.6 Exclusion Criteria

Careful selection of appropriate eyes and injection sites has also been recognized as an important safety strategy. Kivela and colleagues would purposefully choose an injection site “where the conjunctiva adhered to the sclera” in an area with some distance from a tumor focus (Kivelä et al. 2011). Munier et al. have generated a number of injection site exclusion criteria as determined by clinical exam and with the aid of an ultrasound biomicroscopy (UBM) (Munier et al. 2013). Specifically they note certain characteristics should serve as exclusion criteria for an appropriate injection site, including the presence of vitreous seeds, tumor, retinal detachment, an anterior hyaloid detachment or invasion of the anterior or posterior chamber (Munier et al. 2013). The rationale is that injecting into a location with one of these characteristics would conceivably

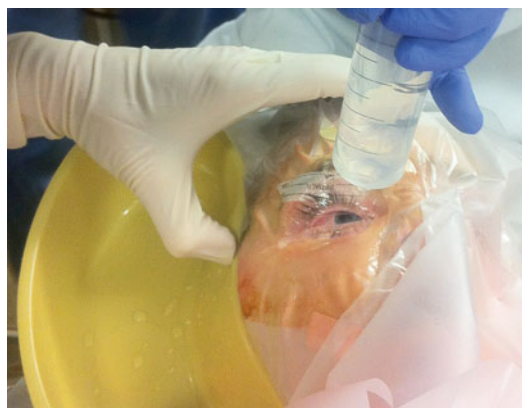


Fig. 4.4 The eye is submerged in irrigating sterile water for 3 min and the effluent is collected and sent to cytology

increase the risk of extraocular extension or risk to the eye.

4.2.7 Irrigation/Water Submersion

Besides cryotherapy, other post-injection measures include irrigating the needle tract area, as was reportedly done copiously by Chevez-Barrios and colleagues (2005). Our group subjects the eye to a similar procedure, however, we have data to support the importance of selecting an optimal irrigating solution. Submerging in vitro retinoblastoma cells in deionized, distilled water results in their nonviability: 95 % are nonviable by 2 min and greater than 99 % are nonviable by 3 min (Francis et al. 2014b). In contrast, placing these same cells in saline solution does not have the same effect on their viability (Francis et al. 2014b). We now apply this principle clinically. Following the intravitreal injection, we submerge the eye in sterile water for three minutes and collect the effluent in a kidney basin that has been placed along the adjacent lateral orbital wall and cheek (Fig. 4.4) (Henkes and Manschot 1963; Karciloglu 2002; Francis et al. 2014b). These efforts are thought to kill any tumor cells that may have escaped from the eye at the time of the injection. It also allows us to collect the ocular surface washings for further evaluation.

4.2.8 Ocular and Needle Washings

No malignant cells were recovered from 200 ocular surface effluent samples from retinoblastoma eyes submerged in sterile water following an intravitreal injection of melphalan. This predicts with 95 % certainty that no extraocular malignant cells would be found with this method. However, there are a number of caveats with this technique: such as the possibility of not capturing effluxed cells that trap in the subconjunctival space or incomplete capture of fluids draining from the eye. Despite this, it is certainly reassuring to find an absence of cells outside the eye with this method, and is in keeping with calculated data that suggests a low risk of extraocular extension following intravitreal injections.

In addition, there were no malignant cells observed in any sample from 156 needle washing specimens. Following the injection, the needle was immediately washed and agitated in Cytolyt. This same Cytolyt fluid was aspirated into the syringe and expelled out through the needle to recover any cells that may have become trapped in the needle bore. This suggests that, at least with this examining technique, tumor cells rarely if ever collect on the needle in a manner that externalizes them outside the eye.

4.2.9 Tethered Seeds

While we have evidence to suggest that tumor cells rarely get outside the eye following an intravitreal injection, we have a couple of cases that demonstrate the intraocular cells and the adjacent vitreous can come very close to the puncture site (Munier 2014; Francis et al. 2014c). Specifically, we have two cases where the vitreous seed has become tethered to the site of the injection (Francis et al. 2014c). One case resolved with continued intravitreal injections of melphalan and the second case required both intravitreal melphalan and cryotherapy for complete resolution (Francis et al. 2014c). The mechanism for the seed tethering is unknown although one could speculate that the negative pressure generated by needle withdrawal allows the intraocular contents to move towards

the injection site (Chévez-Barrios et al. 2005; Francis et al. 2014c). Clearly it is important to perform a 360° exam of the peripheral exam to check for seed tethering.

4.3 Ocular Toxicity

Before intravitreal melphalan, the treatment option for eyes with vitreous seeding was limited, and in many cases was restricted to enucleation. However, intravitreal melphalan has drastically changed the salvage rates for eyes with vitreous disease: eyes that were once lost are now being saved. Along with this advancement comes the question about vision, and specifically, what is the impact of intravitreal melphalan on visual function?

Our understanding of the retinal toxicity of intravitreal melphalan comes from both clinical and preclinical data. A 50 µg dose of intravitreal melphalan in a human eye results in appreciable retinal toxicity including gliosis, vascular occlusion, retinal necrosis, neovascularization, and hemorrhage (Lee et al. 2003; Ghassemi and Amoli 2014). In animal models, electroretinogram (ERG) monitoring in rabbits eyes demonstrated normal responses at a human equivalent of 23 µg of melphalan injected directly into the eye (Kivelä et al. 2011; Ueda et al. 1995); and abnormal responses using a human equivalent dose of 35 µg melphalan in a vitrectomy infusion (Smith et al. 2012; Shimoda et al. 2008). This suggests a human eye's threshold for melphalan toxicity lies between 23 and 35 µg.

4.3.1 Electroretinogram: Clinical

The most recent data on our cohort looked at electroretinogram recordings in 25 retinoblastoma eyes receiving 30 µg injections of melphalan for vitreous disease. 161 weekly injections were given to 25 eyes, with each eye receiving a median of seven injections and a median follow-up of 8.4 months. ERGs were performed as described in Chap. 5.

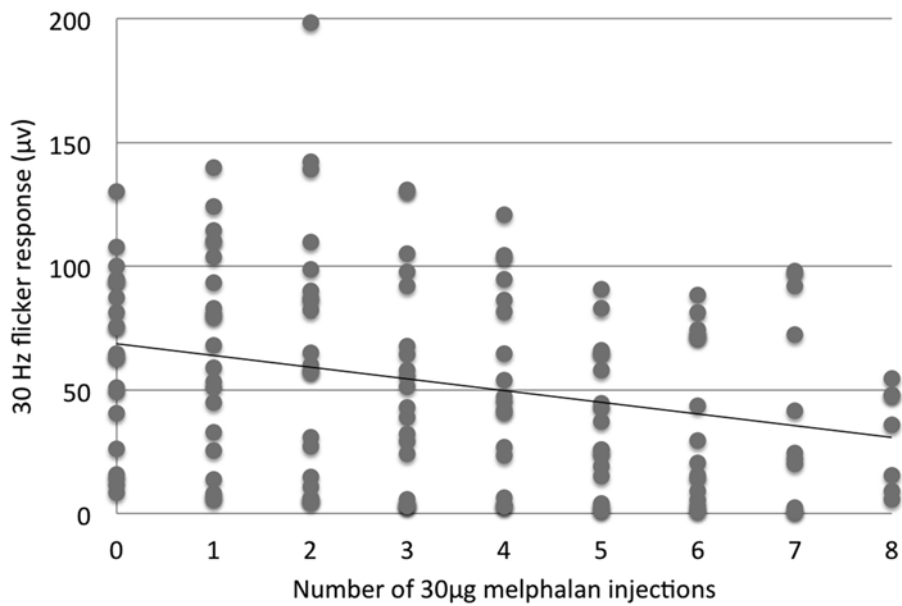


Fig. 4.5 Scatterplot demonstrating change in electroretinogram response to weekly injections of 30 µg intravitreal melphalan

By linear regression analysis, it was determined that for every 30 µg melphalan injection, the ERG response decreased by about 5 µV (Fig. 4.5). And over the injection course, the mean change in ERG response resulted in a degradation of 34 µV. After the injection course was completed, the ERG response remained constant: it did not degrade over time nor did it improve. Table 4.2 shows the other factors that were evaluated for associations with ERG changes. Neither age at first injection, weight at first injection, or iris pigmentation were significantly associated with changes in ERG response. Furthermore, it has previously been demonstrated that concomitant ophthalmic artery chemosurgery is not associated with ERG degradation (Francis et al. 2014a). However, this cohort confirms previous findings that the extent of retinal pigment epithelial changes (akin to salt-n-pepper retinopathy) is significantly associated with a change in ERG response (Fig. 4.6). One explanation is the proximity of the drug with the retina results in a higher likelihood of salt-and-pepper retinopathy and more degradation on ERG.

Table 4.2 Factors related to the toxicity of intravitreal melphalan as evaluated by linear regression analysis

Variable	P-value
Age at first injection (mos)	0.6
Weight at first injection (kg)	0.2
Iris pigmentation (blue, light brown, dark brown)	0.4
Concomitant ophthalmic artery chemotherapy	0.4
Extent of retinal pigment epithelial changes	<0.5

4.3.2 Electroretinogram: Preclinical

In a collaborative effort with the group from Argentina, we confirmed these clinical findings using a rabbit model (Francis et al. 2014a). Twelve rabbits were given 15 µg/0.1 cm³ melphalan, which is equivalent to a human dose of 30 µg. All eyes that were treated with 3 weekly injections of intravitreal melphalan had degradations in their ERG responses, measured at 1 week after the Second injection and then 2 and 4 weeks following the Third injection. By contrast, the vehicle treated eyes and untreated left eyes maintained



Fig. 4.6 Salt-n-pepper retinopathy after intravitreal melphalan. Note retinal pigment epithelial changes in the periphery extending across the 12 o'clock meridian

responses equivalent to baseline throughout the treatment course (Francis et al. 2014a; Inomata and Kaneko 1987). Histopathological evaluation of the eyes treated with three injections of melphalan demonstrated a severely atrophic retina with loss of photoreceptors and outer nuclear layers (Francis et al. 2014a; Munier et al. 2012b, 2013). Furthermore, there was toxicity observed in the optic nerve with histocytic-like cells containing granular material and disorganized nerve fibers and glial cells with scant blood vessels. Clinical examination of these rabbit eyes confirmed the presence of retinal toxicity: both funduscopy and fluorescein angiography depicted retinal whitening and sclerotic vessels in the melphalan treated eyes (Francis et al. 2014a; Smith and Smith 2013). Clearly, in rabbits and humans, weekly injections of 30 μ g melphalan may result in an abrupt ERG degradation that appears to be nonprogressive once the injection course is complete. Further studies will determine how the injection technique (e.g. placement of needle in the eye) will influence these findings of retinal toxicity.

4.3.3 Clinical Signs of Toxicity

Clinical reports on intravitreal melphalan describe clinical features of toxicity. The most commonly reported abnormalities include retinal pigment epi-

thelial changes akin to salt-and-pepper retinopathy and varying degrees of hemorrhage, whether vitreous, subretinal, or subconjunctival (Kivelä et al. 2011; Francis et al. 2014a; Munier et al. 2012b; Smith and Smith 2013; Ghassemi and Shields 2012; Suzuki et al. 2011). Lens and vitreous opacities have been described including fluctuations in intraocular pressure from hypotony to elevation (Chávez-Barrios et al. 2005; Ericson and Rosengren 1961; Seregard et al. 1995; Ghassemi and Shields 2012). At the higher doses of drugs, there have even been accounts of phthisis (Kivelä et al. 2011; Francis et al. 2014a; Munier et al. 2012b; Ghassemi and Shields 2012).

On the subject of toxicity, Smith reviewed the cases described in ten previously published clinical reports (Ericson and Rosengren 1961; Smith et al. 2014). The study included 295 eyes and was differentiated between 261 eyes that received standard melphalan dose (defined as 8–30 μ g), and 34 eyes that had high-dose melphalan (doses greater than 30 μ g) (Francis et al. 2014a; Smith et al. 2014). They divided side effects into “significant” (visual loss, RD, sight-threatening vitreous hemorrhage, cataract, CR or iris atrophy or phthisis) and minor (transient increase in IOP, conjunctivitis, keratitis, vitreous opacity, vasculitis, RPE changes, or hemorrhage) (Ericson and Rosengren 1961; Smith et al. 2014). In the standard melphalan group, 3 % of patients suffered significant side effects, compared to 26 % of the eyes receiving high-dose melphalan (Chávez-Barrios et al. 2005; Smith et al. 2014). Overall 7.1 % of all eyes experienced side effects defined as minor (Kivelä et al. 2011; Smith et al. 2014). This demonstrates that intravitreal melphalan is not without clinical signs of toxicity and the severity of these changes appears to be dose dependent.

4.4 Systemic Toxicity

Knowledge regarding the systemic toxicity of intravitreal melphalan is limited to findings from a single paper in the literature. Specifically, 46 blood specimens were collected and evaluated for complete blood counts (Francis et al. 2014a). The data was divided into two groups to

determine whether there was any impact from concomitant OAC. Therefore, there were instances ($n=16$) where patients received an injection and CBC analysis within 21 days of prior OAC, and those where the injection and analysis were beyond that window ($n=30$). There were no cases of grade 3 or 4 neutropenia in either group (Francis et al. 2014a). In addition, further analysis demonstrated that increasing injections were not significantly associated with changes in absolute neutrophil count levels. This confirmed our low expectation for systemic toxicity with a 30 μg injection into the vitreous, since this dose is well below the 0.4 mg/kg threshold for neutropenia that we have measured in patients receiving ophthalmic artery chemosurgery (Dunkel et al. 2014).

4.5 Conclusion

In conclusion, despite historical trepidation regarding intravitreal injections into retinoblastoma eyes, the adoption of safety-enhanced techniques has reduced their associated risks. In fact, they appear to have a satisfactory safety profile both by calculations in the literature and as demonstrated by our data: neither our ocular surface nor needle washings revealed evidence of extra-ocular malignant cells. Despite local toxicity, we had no cases of Grade 3 or 4 neutropenia in our cohort, suggesting these injections do not result in severe systemic toxicity. However, 30 μg intravitreal melphalan injections are locally toxic as measured by ERG. For every injection, the ERG amplitude decreases by 5 μV , and this is unrelated to age, weight, iris pigmentation, or concomitant OAC. Instead, the salt-n-pepper RPE changes are significantly associated with degradation in ERG responses. Further studies will determine what correlation these degraded ERG responses have to visual function.

Compliance with Ethical Requirements Jasmine H. Francis declares that she has no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (Memorial Sloan Kettering Cancer Center) and with the Helsinki Declaration of 1975, as

revised in 2000. Informed consent was obtained from all patients for being included in the study. All institutional and national guidelines for the care and use of laboratory animals were followed.

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Scott E. Brodie

5.1 Introduction

The historic goals of treatment for retinoblastoma have been: first, prevention of mortality from retinoblastoma; second, retention of eyes with retinoblastoma; and only third, preservation and optimization of vision in retained eyes with retinoblastoma. The goal of prevention of mortality has largely been met over the last century, at least to the extent that prompt recognition and diagnosis of retinoblastoma followed by enucleation is curative in nearly all cases. Indeed, the encapsulation of retinoblastoma within the eye, until late in the course of the ocular disease (in nearly all cases), allows for a cure rate by means of local resection which is nearly unique in the spectrum of human cancer.

Nevertheless, enucleation entails loss of all visual potential in the operated eye, and carries a high cosmetic burden, which is all but unacceptable in many cultures. Accordingly, there has been a long history of efforts to extirpate retinoblastoma in situ, including external beam radiation, brachytherapy, which limits the exposure of adjacent tissues to the radiation, photocoagulation (with xenon arc or laser light sources), cryotherapy,

systemic chemotherapy (generally more successful as chemoreduction than as the primary therapeutic modality), more recently, localized chemotherapy modalities, including periocular injection, selective and super-selective intra-arterial chemotherapy infusion, and intravitreal injection. Historically, the therapeutic goal of most of this local therapy has been primarily retention of the globe, as preservation of vision was a rare and sporadic outcome. Indeed, visual outcomes have rarely been measured in retinoblastoma clinics, and reports of visual outcomes are sparse.

Historically, the likelihood of retention of vision in eyes with advanced stages of retinoblastoma has been discounted to the extent that enucleation has been the preferred treatment until recent decades. Writing in the 1970s, Reese recommended enucleation “in the usual advanced unilateral case,” and enucleation of the “more advanced eye” in “most bilateral cases,” with therapy to be attempted only in the fellow eye. [It is to be noted that these “advanced eyes” constitute the majority of cases in most series. For example, at Reese’s clinic in the early 1960s, eyes in the favorable Groups I–III constituted only 76 of 192 cases.] [1].

Intra-arterial chemotherapy infusion and intravitreal chemotherapy injection have substantially changed these patterns of therapy for retinoblastoma in the last decade. With increasing likelihood of the salvage of eyes with more

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advanced disease [2], only the very most advanced eyes are enucleated *per primum*. Retention of most eyes with retinoblastoma raises the prospect of preservation of vision in these eyes, an important factor in the balance between the risks and benefits, as well as the considerable expense, of these treatment options.

Measurement of vision in patients with retinoblastoma, mostly non-verbal infants, is frequently challenging. Snellen acuity testing is of course impossible. Formal “preferential looking” methods are time-consuming and difficult, and have not been practical in the hectic environment of our clinic, where the exigencies of preparing children for examination under anesthesia take priority. Traditional qualitative assessments of fixation are regularly performed in our clinic, but are often unreliable, and reflect primarily foveal vision, which may or may not reflect the extent of retinal disruption by tumor or therapy.

Since the introduction of intra-arterial chemotherapy (and later, intravitreal injection of chemotherapy) in our center in 2006, we have elected to monitor retinal function (and, thus monitor for retinal toxicity) by means of electroretinography (ERG) [3, 4]. The full-field ERG provides a summed, global estimate of retinal function, and is frequently used to detect retinal injury from drug toxicity, as well as structural disruption or genetic defects. ERG recording does not require verbal participation by the patient, and is easily performed during examination under anesthesia. Of course, the ERG can serve only as an imperfect proxy for measurements of visual function. For example, subjective acuity may remain excellent in eyes with widespread disease of the retina, but with macular sparing, where the ERG will be greatly attenuated, or may be greatly impaired following central macular injury, when the ERG will be essentially normal. The ERG, as a measure of retinal function, is insensitive to isolated injury to the optic nerve or more central visual pathways. Nevertheless, we have found that these selective insults to the visual system are atypical in our retinoblastoma patients, in whom the effects of tumor burden, local therapies, and the toxicity of chemotherapy are quite generally reflected in reductions in ERG ampli-

tudes (which are grossly proportional to the degree of retinal disruption or toxicity). We have thus been able to follow ERG amplitudes as an important source of feedback in the planning of individualized treatment regimens and the adjustment of chemotherapy dosage [2].

Visual Evoked Potential (VEP) recording is not helpful in this situation. The VEP is generated by the cortical neurons, which are most sensitive to the suppression of neuronal activity by general inhalation anesthesia. There is thus no reliably recordable VEP signal during examination under anesthesia, regardless of the functional status of the retina and optic nerve.

This Chapter will summarize our experience with ERG monitoring for treatment of retinoblastoma in the era of intra-arterial and intravitreal chemotherapy.

5.2 Ophthalmic Artery Chemotherapy Infusion

Systemic chemotherapy is traditionally administered by intravenous infusion, distributing toxic medication throughout the body by means of the general circulation. Therapeutic selectivity depends on differential sensitivity of the target tumor tissue to the cytotoxic effects of the drug, compared with its action on the other tissues in the body, as essentially all the tissues are exposed to the same concentration of drug. Typically, dosing is limited by the toxicity of the agent to rapidly dividing normal tissues, such as bone-marrow and intestinal epithelium, not to mention hair follicles. By selectively delivering the drug as precisely as possible into the arterial blood supply of the target tumor tissue, it becomes possible to obtain much greater specificity of action, as the drug is exposed to the tumor tissue at high concentration during the “first-pass” circulation, but subsequently diluted into the full vascular volume (if not the entire aqueous compartment).

In the case of ophthalmic artery infusion, the anatomy is particularly favorable, with a single artery providing the main blood supply to only a small block of tissue (the orbit) containing the target tumor, which contains few rapidly dividing

cell populations which would be susceptible to the action of the drug. While we have not attempted to measure drug levels in the orbital tissues of our patients, we can estimate a dilution effect of at least the ratio of the volume of the orbit to the volume of the vascular compartment. For a 6.0 kg infant, this yields an estimated dilution of

$$13-15\text{ cm}^3 / 480\text{ cm}^3 \approx 0.03.$$

If the drug is distributed in the entire aqueous compartment (perhaps 2000 cm^3 in a 6 kg infant), the dilution would be approximately a factor of 0.007. If, as seems likely, the drug were confined to a smaller portion of the orbital volume during the initial pass, the dilution factor would be even greater.

In practice, the dosing of our mainstay chemotherapy agent, melphalan, has been limited by the threshold of bone-marrow toxicity [5]. At the beginning of our experience with ophthalmic artery chemosurgery, it was not at all clear whether the corresponding concentration of drug (presumably at least 30-fold higher than the maximal tolerated systemic concentration) would be tolerated by the retina and other ocular and orbital tissues. Our initial series of ten patients were monitored by ERG in an attempt to answer this question [3].

5.3 ERG Protocol

The widely-adapted standards for ERG recording promulgated by the International Society for Clinical Electrophysiology of Vision (ISCEV) [6] do not formally apply to examination under anesthesia. We have attempted to adapt the ISCEV standard protocol to our clinical environment with as few changes as necessary, compatible with efficient and safe practice. We use the standard ISCEV photopic and scotopic stimuli, but have reduced the recommended intervals for light- and dark-adaptation in order to avoid unwarranted prolongation of the duration of general anesthesia [3, 4] (see Fig. 5.1 and Box 5.1):

We initially performed most ERG testing after the initial examination of patients during examination under anesthesia by indirect oph-

thalmoscopy with scleral depression. This results in a high degree of light-adaptation from the illumination of the fundus by the indirect ophthalmoscope. As noted below, we subsequently observed that scleral depression and other ocular manipulations reduce the ERG [7]. We have since modified our routine so as to obtain the ERG recordings immediately following anesthesia induction, before ophthalmoscopy. We noticed no difference in photopic ERG amplitudes without the initial light exposure from the ophthalmoscope.

Dark adaptation is potentially more problematic. The ISCEV standard of 20 min in the dark is utterly impractical in our clinic. [We examine as many as 15 or more patients, and perform up to 10 or 12 ERGs, per day—full 20-min adaptation on each patient would extend the schedule by up to 4 h!] Careful review of the literature confirms that, in the absence of extensive bleaching of the retina



Fig. 5.1 ERG recording during examination under anesthesia using Espion ColorBurst stimulator. Note use of mask for inhalation anesthesia; Eyelids are opened with lid speculum. ERG-jet contact lens electrode is in place on the right eye. ERG waveform appears on screen of laptop computer (at rear)

Box 5.1 ERG Protocol

- Modified from ISCEV standard ERG protocol
- ERG-jet contact lens electrodes
- Diagnosis Espion system with ColorBurst hand-held ganzfeld stimulator
- Standard flash stimuli:
 - Light-adapted 3.0 flash
 - Light-adapted 30-Hz flicker
 - Hand-held ganzfeld, background luminance 30 cd/m²
 - Dark-adapted 0.01 flash (rod-isolating dim flash)
 - Dark-adapted 3.0 flash
 - (Dark-adapted 0.01 red flash (non-standard stimulus))
- Light-adapted recordings are obtained after dilation, with adaptation to ordinary room lighting (ISCEV standard is 10 min)
- Dark-adapted recordings are obtained after 5 min of dark adaptation (ISCEV standard is 20 min)

by prior light exposure, scotopic ERG responses reach between 80 and 90 % of their final amplitudes after the initial 5 min of dark adaptation [8, 9]. This has been felt adequate for our clinical purposes. Indeed, after several years of experience, we observed that in this setting of patients presenting with retinoblastoma, or following treatment for retinoblastoma, selective injury to rod or cone systems of the retina does not occur, so that there is essentially no additional information obtained in this context from scotopic ERG testing beyond what is learned from photopic recordings (see below); we thus now seldom obtain scotopic ERGs in retinoblastoma patients.

5.4 ERG Testing Schedule

We attempt to obtain ERG recordings from all our patients with retinoblastoma according to the schedule shown in the accompanying Box:

ERGs are occasionally unobtainable due to failure of the recording instrument, absence of an electrophysiologist, or other exigencies.

Box 5.2 ERG Testing Schedule

- At initial presentation to MSKCC
- At next EUA after each ophthalmic artery chemotherapy infusion (OAC) treatment
- 3 months after most recent OAC treatment
- Annually after most recent OAC treatment
- Prior to each intravitreal injection
- At next EUA after most recent intravitreal injection
- 3 months after most recent intravitreal injection
- Annually after most recent intravitreal injection

In general we try to obtain recordings before and after each chemotherapy session, so as to be able to note the effect of the treatment on retinal function. In most cases, with patients seen and treated with ophthalmic artery chemosurgery on a monthly basis, ERGs are obtained monthly until tumor control is achieved. A follow-up is obtained three months later to observe possible late effects of treatment, or possible recovery, after the acute effects of chemotherapy have dissipated. ERGs are obtained annually thereafter. For patients undergoing intravitreal chemotherapy injections, treatment and evaluation typically occurs on a weekly basis. ERGs are obtained immediately after anesthesia induction at each visit, prior to injection. After tumor control is achieved, follow-up ERGs are recorded at three months and annually thereafter.

5.5 ERG Recording During Examination Under Anesthesia: Technical Considerations

5.5.1 Baseline Noise

Recording ERGs in the procedure room from patients under Sevoflurane general anesthesia raises technical issues not encountered during

ERG recording from awake patients. A few of these merit specific comments.

The electrical baseline obtained under Sevoflurane anesthesia is rarely comparable to the flat line with superimposed high-frequency noise of a few microvolts in amplitude seen in routine clinical settings with awake patients. Instead, one sees slowly varying baselines with excursions as large as 100 μV , and a power spectrum with a roughly $1/f$ frequency dependence, and only a very small component of “line noise” at 60 Hz (Fig. 5.2).

Extensive investigation confirmed that this baseline fluctuation was not an artifact of poor electrode connections or leakage from external sources. In order to investigate the possibility that it originated as a physiologic signal from the central nervous system (apparently disinhibited by the effect of general anesthesia), we compared the RMS amplitude of this baseline fluctuation with an estimate of brain activity as measured by the “BIS” bispectral index monitor. This instrument uses a proprietary algorithm to provide a number (from 1 to 100) summarizing the level of activity of the EEG signal as detected by adhesive electrodes placed over the forehead. A correlation of 0.731 ($p < 10^{-8}$) was observed between the RMS amplitude of the ERG baseline and the BIS score (Fig. 5.3).

This observation suggests that this $1/f$ baseline noise is intrinsic to ERG recordings under these conditions, presumably leaking from deep brain centers into the ERG circuit. It can be suppressed by adequate signal averaging—we typically work with averages of at least ten successive individual ERG response tracings, and use grand averages of ten of these averaged responses (i.e., averages of 100 original response tracings) in cases where small signals (as amplitudes of 5 μV or less) are difficult to distinguish from noise. In the most difficult such cases, we prefer to work from the responses to 30 Hz flicker, rather than the responses to single flashes, as the periodic nature of the stimulus assists in the distinction between true responses (which must show an identical periodicity) and underlying noise.

5.5.2 Effect of Ocular Manipulation on the ERG

After observing both increases and decrements in ERG signal strength following intra-arterial chemotherapy for retinoblastoma, we began to inquire, for comparison, as to the effects of local therapies, such as laser and cryotherapy, on the ERG responses. It quickly became apparent that cryotherapy caused an acute reduction in ERG amplitudes. On further investigation, control experiments demonstrated that even routine scleral depression, without associated cryoablation, also causes acute reductions in the ERG. In fact, to our astonishment, manipulation (either scleral depression or cryotherapy) of one eye, was found to cause consistent reductions in the ERG of the untouched fellow eye (Fig. 5.4) [7].

Thus, when recording during EUA, we make every attempt to record the ERG as the first procedure immediately following induction of general anesthesia (“First-thing ERGs”), and generally avoid recordings following manipulations such as cryotherapy or scleral depression.

We do not know if this effect is unique to ERGs obtained under general anesthesia—it is possible that it has not been previously noted due to the absence of indications for scleral depression prior to ERG testing in most electrophysiology clinics, or perhaps the lack of occasions to record ERGs from the same patient both before and after scleral depression.

5.5.3 Alternating Flicker Responses

On occasion, we have observed alternation in the amplitude of the individual response peaks in response to 30-Hz flicker stimulation during our ERGs under anesthesia (Fig. 5.5).

This is sometimes seen as an acute response to cryotherapy or TTT laser treatment, but is also noted in First-thing ERGs. The effect has not been reported in routine clinical ERGs in awake patients. The mechanism is unknown, though theoretical considerations suggest a possible role for reduced effectiveness of intraretinal feedback circuits.

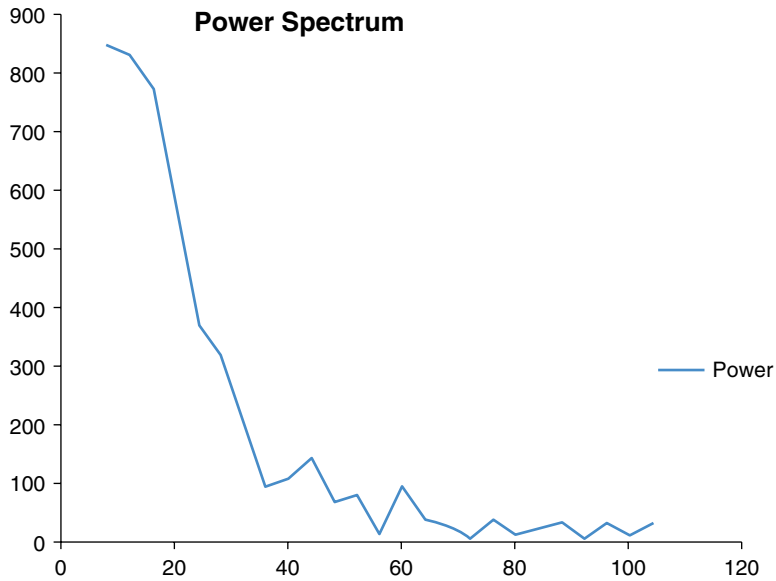
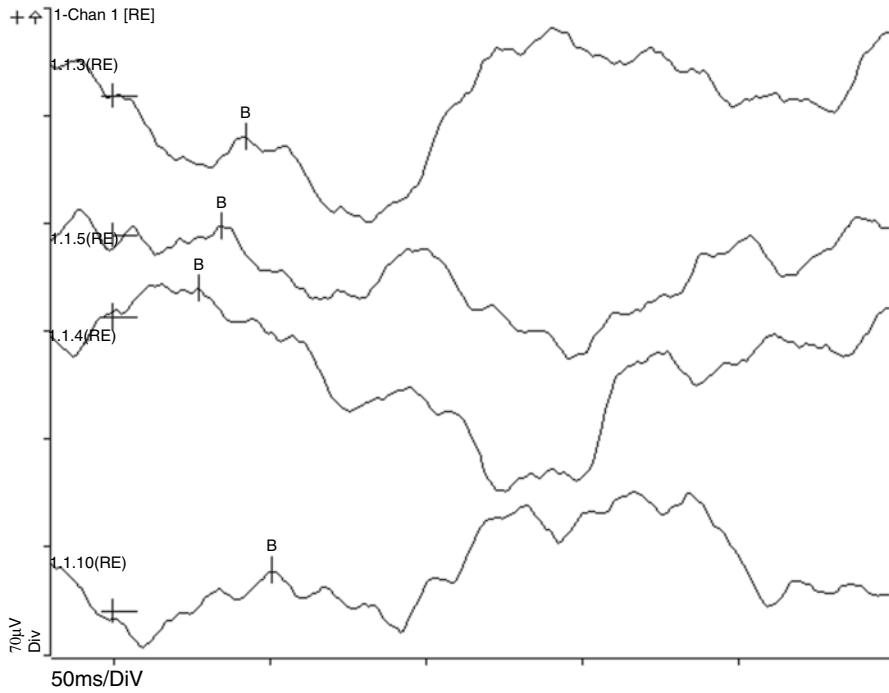


Fig. 5.2 Baseline ERG noise under Sevoflurane anesthesia. Typical baseline recordings (without photic stimulation) are shown at *top*. Typical power spectrum of the baseline noise is shown at *bottom*. Note approximately $1/f$

frequency dependence, and very small peak at 60 Hz due to slight contamination with interference from ambient electrical devices (“line noise” or “mains hum.”)

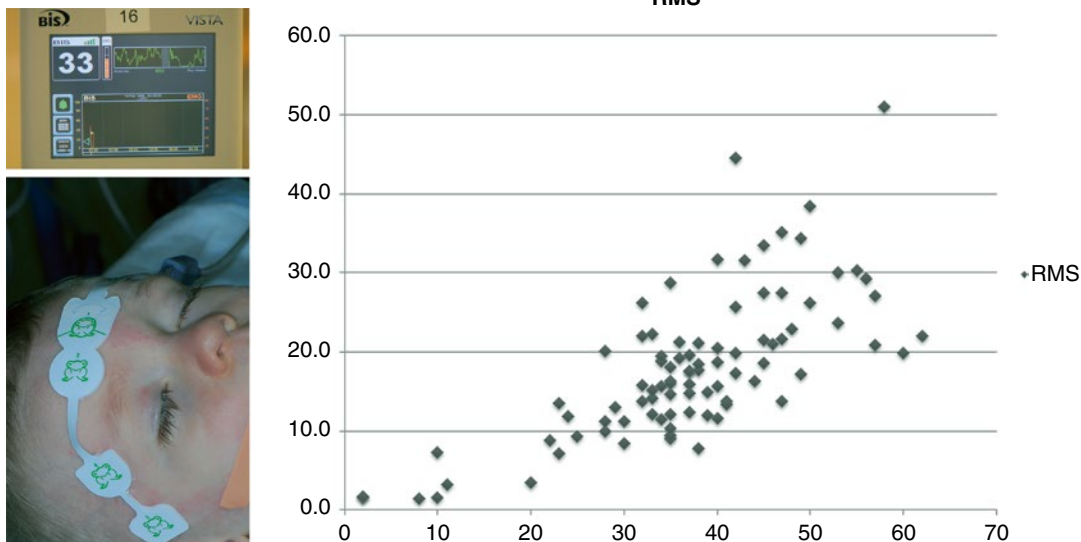


Fig. 5.3 BIS bispectral analyzer instrument, and placement of BIS adhesive electrode strip on forehead are shown at *left*. Scatter plot comparing BIS activity estimate (horizontal axis) with RMS noise amplitudes (vertical axis) is shown at *right*

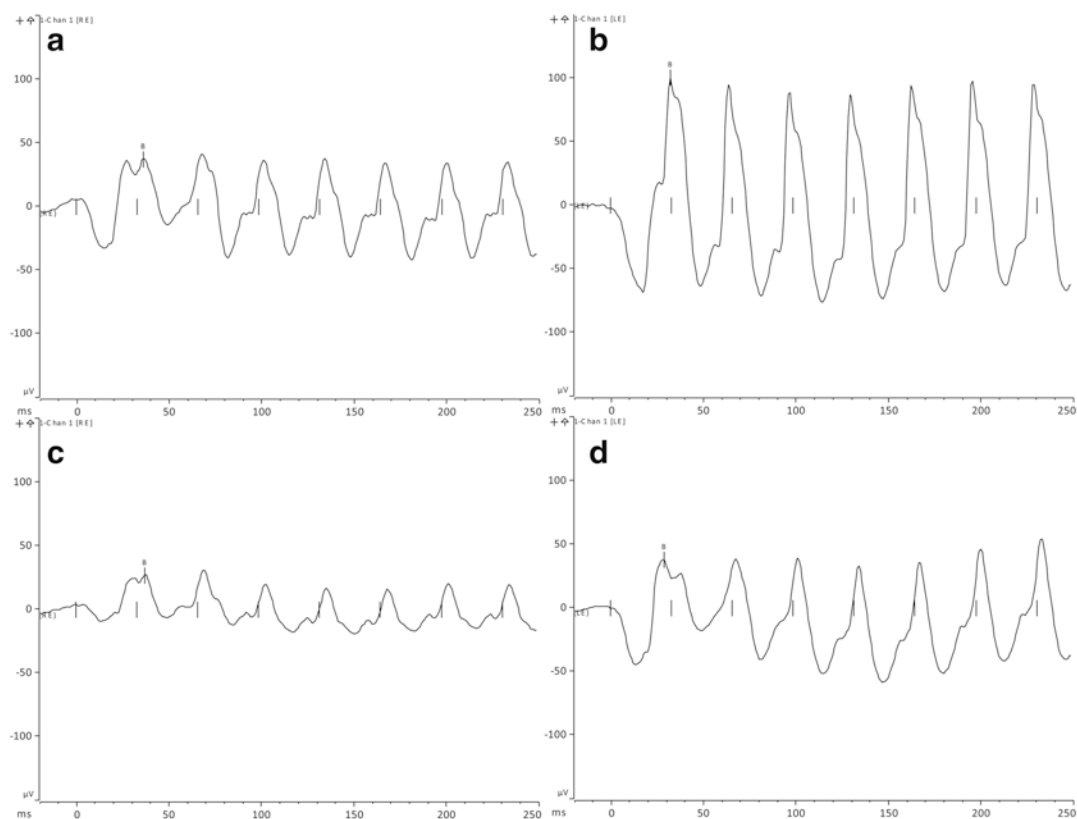


Fig. 5.4 Effects of scleral depression on 30 Hz flicker ERG amplitudes of both the eye undergoing scleral depression (*left hand column*) and untouched fellow eye (*right hand column*). Baseline response tracings are at top (**a, b**); response tracings after scleral depression are below (**c, d**)

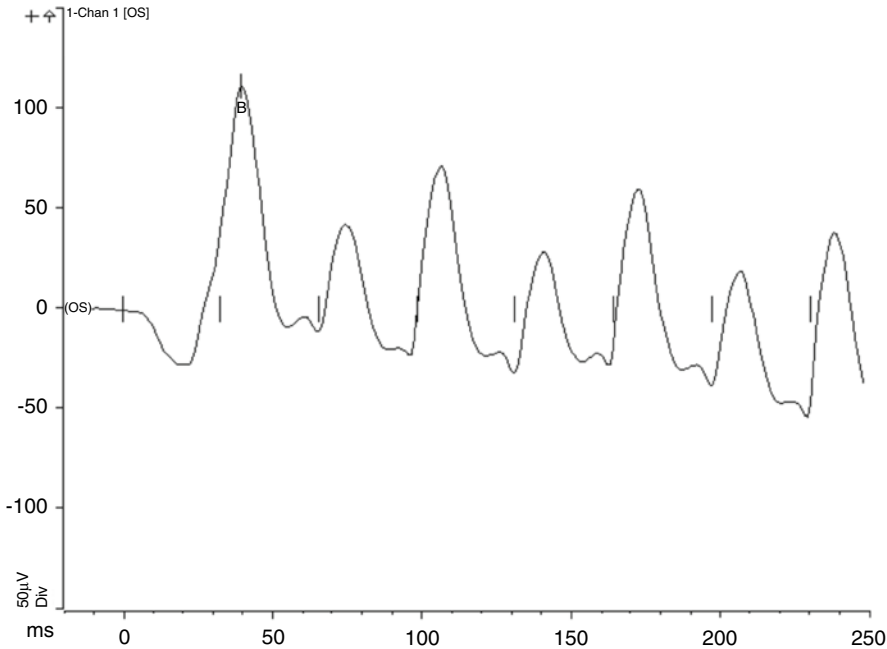


Fig. 5.5 Alternating peaks in responses to 30-Hz flicker under Sevoflurane anesthesia

In cases of alternation of flicker response peaks, we take the average peak amplitude as the measure of the response.

5.5.4 Increased Responses from Normal or Minimally Involved Eyes

We routinely record ERGs from both eyes of our patients, even in cases of unilateral disease, to verify normal function of the apparatus, as well as to provide an individualized basis for comparison. Under these conditions, the photopic response amplitudes from normal, healthy eyes (and, occasionally, eyes with minimal tumor involvement), are often far greater in amplitude than those routinely recorded from awake children or adults, at times exceeding 200 μV , or, rarely, even 300 μV . These large responses are not always consistently seen in successive recordings from the same patients. It is not clear why the responses are occasionally so large, but it seems likely that they represent an effect of the anesthetic agent.

We have continued to use traditional normative values for photopic ERG responses, and thus treat any response greater than 100 μV as normal, and have not differentiated between these typical normal responses, and the occasional supra-normal responses.

5.5.5 Response Variability

ERG response norms are notoriously wide, with great variations in response amplitudes, even among age-matched subjects with healthy eyes. Standardization of ERG recording protocols, especially widespread adoption of the ISCEV standard ERG protocol, has improved comparison of results obtained at different laboratories, and can produce very repeatable results with careful attention to testing conditions, but under routine conditions, test–retest variability remains problematic.

We have had similar experiences with ERG testing under general anesthesia in our retinoblastoma patients. Using responses from normal, fellow eyes, our initial estimate of the test–retest

standard deviation of the photopic response amplitudes was 24.7 μV . We have recently repeated this analysis, using data only from recordings obtained from normal, fellow eyes immediately following induction of anesthesia (“First-thing ERGs”). Elimination of the effects of ocular manipulation on the ERG reduces the test–retest standard deviation to 19.4 μV . We have thus adopted a simple classification of ERG responses into broad amplitude categories (Extinguished; between 0 and 25 μV (“Poor”); 26–50 μV (“Fair”); 51–75 μV (“Good”); 76–100 μV (“Very Good”), and greater than 100 μV (“Normal”), and taken 25 μV as the threshold for clinically significant changes in ERG amplitudes.

While it is tempting to suggest that variability in the depth of the general anesthesia may explain at least some of the variations in ERG amplitudes, we have not been able to confirm this. It is generally conceded that it is not possible to estimate the depth of anesthesia in the clinical setting, as the clinical indicators monitored by anesthesiologists (respiration rate, blood pressure, blood oxygenation, etc.) are known to correlate poorly with estimates of neurological activity. We do not observe consistent variation in ERG amplitudes during repeated recordings during the termination of anesthesia, up to the point of regaining of consciousness.

5.5.6 Delay of ERG Responses

ERG response peak implicit times are almost always prolonged in recordings obtained under general anesthesia. Accordingly we have not ascribed any clinical importance to our implicit time measurements.

5.5.7 Correlation of Photopic and Scotopic Responses

Comparison of photopic ERG responses, derived from retinal cones, with scotopic responses

derived mostly from retinal rods, is a mainstay of clinical ERG interpretation, particularly for patients with certain genetic retinal disorders, which tend to preferentially and selectively affect rods (as in retinitis pigmentosa) or cones (as in achromatopsia). We have not found this to be the case with our retinoblastoma patients: the destruction or replacement of retinal tissue by the tumors at presentation, retinal detachments, and possible paraneoplastic effects of the cancer on retinal responses appear to affect rod and cone systems with equal severity (3). Similarly, the effects of our treatment modalities (ablation by TTT laser therapy or cryotherapy), systemic chemotherapy, intra-arterial chemotherapy, and intravitreal chemotherapy, likewise appear to cause only nonselective retinal damage.

In order to verify this observation, we compared photopic and scotopic responses from over 100 patients with retinoblastoma, before, during, and after completion of treatment. The resulting correlation matrix showed excellent correlations between photopic and scotopic responses, as shown in the following matrix of correlation plots [10] (Fig. 5.6).

These findings confirm our impression that there is little additional information to be obtained in this cohort of patients from scotopic recordings beyond that obtained (with greater efficiency) from the photopic recordings alone. We have accordingly discontinued scotopic recordings in most of our retinoblastoma patients, and routinely report only the results of the ERGs obtained under photopic conditions.

5.5.8 Other Factors Affecting ERG Recordings

ERG amplitudes during EUA also reflect variations in other aspects of the recording conditions, as in routine clinical testing. These may include degree of pupillary dilation, rotation of the eye behind the eyelids due to effects of general anesthesia (even with a lid speculum in place), diurnal variation, patient age, and variations in core temperature.

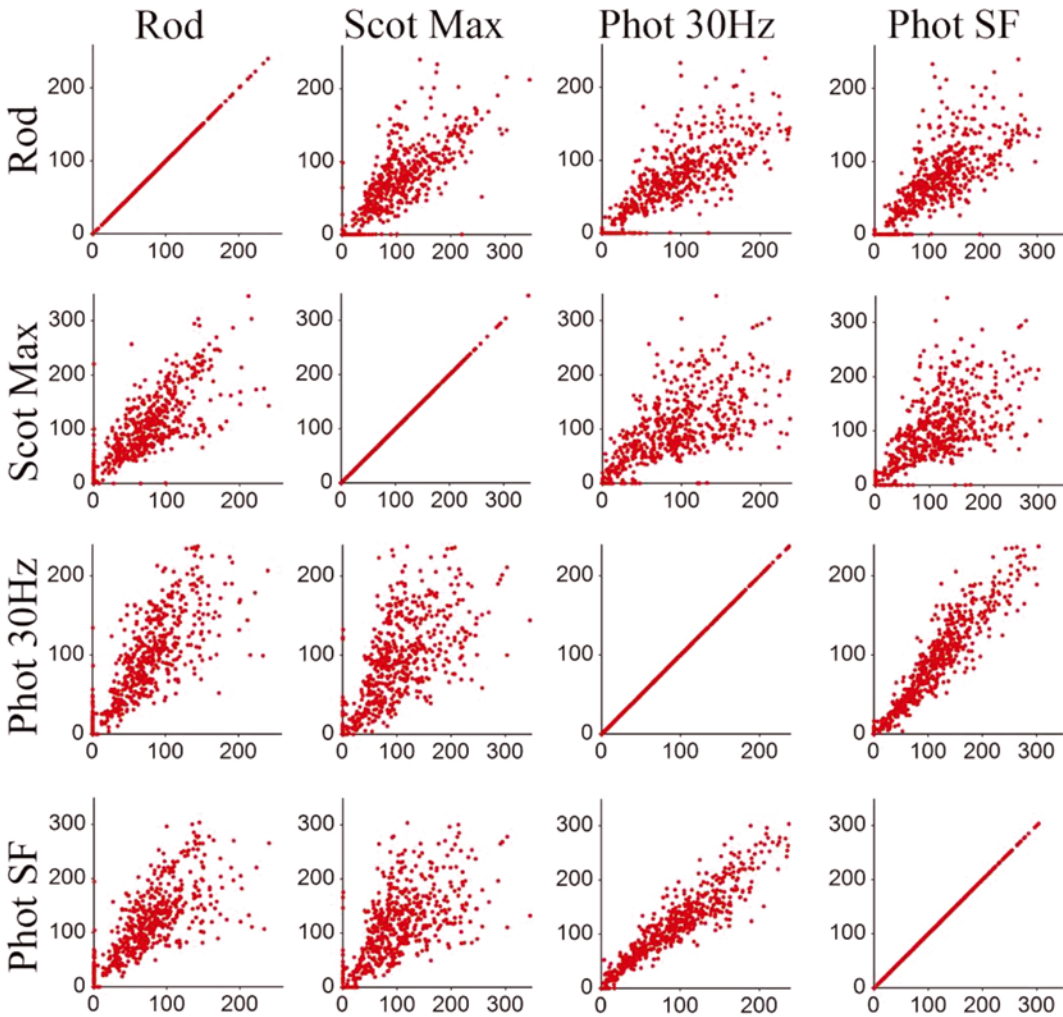


Fig. 5.6 Matrix of scatter plots comparing ERG response amplitudes to the four major ERG stimulus conditions: Dark-adapted 0.01 (“Rod”), Dark-adapted 3.0 (“Scot

Max”), Light-adapted 3.0 (“Phot SF”), and Light-adapted 30-Hz flicker (“Phot 30 Hz”)

5.5.9 Intra-arterial Chemotherapy: Initial Experience

Our first ten cases treated with ophthalmic artery chemosurgery were published in 2009 [3, 4]. Initial ERGs were available for nine patients: five were extinguished; two were poor or fair, and two were normal. Of the ten eyes in this initial cohort, all of which were destined for enucleation but for the option of ophthalmic artery chemosurgery, seven were salvaged, and three were enucleated. Of the three eyes that were lost, two had extin-

guished initial ERGs—no ERG was available for the third.

Of the seven eyes which were salvaged with intra-arterial chemotherapy, two, with persistent retinal detachments, had extinguished initial ERGs, which never recovered during treatment; one eye with initially extinguished ERG improved to fair; of three eyes with initial fair ERGs, one became extinguished after developing a persistent retinal detachment, and two improved to at least good; one eye with normal initial ERG retained its excellent retinal function throughout treatment.

Overall, of the seven salvaged eyes, ERGs were worse in one, same in three, and improved in three. We interpreted this initial experience as suggesting that, in favorable cases without retinal detachment, intra-arterial chemotherapy is compatible with preservation, and perhaps even improvement of retinal function.

5.5.10 Subsequent Experience with Intra-Arterial Chemotherapy

Our results after 3 years of experience with primary intra-arterial treatment (28 eyes of 23 patients) was reviewed in 2010 [11]. ERG data were available for 23 patients: at completion of treatment, ERGs were available for 26 eyes. The final 30-Hz flicker ERG response was extinguished in six patients, poor (0.2–25 μ V) in five, fair (25.1–50 μ V) in 3, good (50.1–75 μ V) in two, and excellent (>75 μ V) in ten. In eight cases, the ERG improved after treatment. One eye with an excellent ERG at baseline developed a poor ERG upon completion of treatment.

Results in eyes with mild disease (Reese-Ellsworth Groups I, II, III; International Classification Groups B, C) were summarized after 5 years of experienced [12]. Compared with initial results, 30 Hz ERG responses were unchanged in seven patients, improved by at least 25 μ V in seven patients, and decreased by at least 25 μ V in five patients. Of those ERGs that deteriorated, four remained in our “Good” category (>75 μ V), which is considered a normal or near-normal 30 Hz ERG response amplitude under routine conditions in awake patients. Results in eyes with extensive (>50 %) retinal detachments proved surprisingly favorable, in marked contrast to prior practice, when such eyes were routinely enucleated [13]. Reattachment rates reached 76 %. ERGs upon completion of treatment were stable in 70 % of cases, and improved in 19 % of cases, deteriorating in only 11 % of cases.

5.5.11 Toxicity Thresholds

We have carefully monitored our ERG outcomes in comparison with the dosages of intra-arterial

chemotherapy. Significant trends have been difficult to confirm, as we have generally reduced the dosage (consistent with adequate tumor control) as soon as ERG outcomes appeared to deteriorate, preferring to make every effort to preserve visual function rather than put additional eyes at risk just to achieve statistical significance. Eventually, we were able to accumulate evidence for a threshold for ERG toxicity, at least for cumulative dose of melphalan: while many eyes tolerated even higher doses, decrements of ERG amplitude of 50 μ V or more were not seen at cumulative doses below 14 mg, or below 20 mg in eyes free from alterations in retinal architecture (such as retinal detachments) (Fig. 5.7) [14].

We have recently reviewed our ERG outcomes with intra-arterial chemotherapy by comparing the ERG amplitudes at baseline to those obtained 3 months after completion of treatment, a measure of the initial impact of intra-arterial treatment on retinal function, with the subsequent changes in ERG amplitudes at the time points 1, 2, 3, 4, and 5 years after completion of treatment. At the 3 months time point, of 215 eyes, ERGs were worse in 25.6 %, same in 58.1 %, and improved in 16.3 %. At the 1 year time point, the comparison with the 3-month data (available for 131 eyes) was worse: 10.7 %; same: 71.0 %; better: 18.3 %. At the 2-year time point of 69 eyes, ERGs were worse: 17.4 %; same: 59.4 %; better 23.2 %. After 3 years, ERGs from 33 eyes were worse: 21.2 %; same: 57.6 %; better: 21.2 %. At 4 years (14 eyes), the comparison was worse: 21.4 %; same: 50 %; better: 28.6 %. At 5 years, of 3 eyes 2 remained stable; 1 improved.

Overall, over 5 years of follow-up, ERGs are worse in about 20 % of cases, unchanged in 60 %, and better in 20 %—that is, the same or better in perhaps 80 % of cases.

5.5.12 ERGs After Intravitreal Chemotherapy Injection

Intravitreal chemotherapy injections for refractory cases of retinoblastoma (most with vitreous seeds) have been employed at MSKCC since 2012, after the pioneering examples of Munier and Kaneko [15, 16]. Our initial patient’s ERGs remained stable

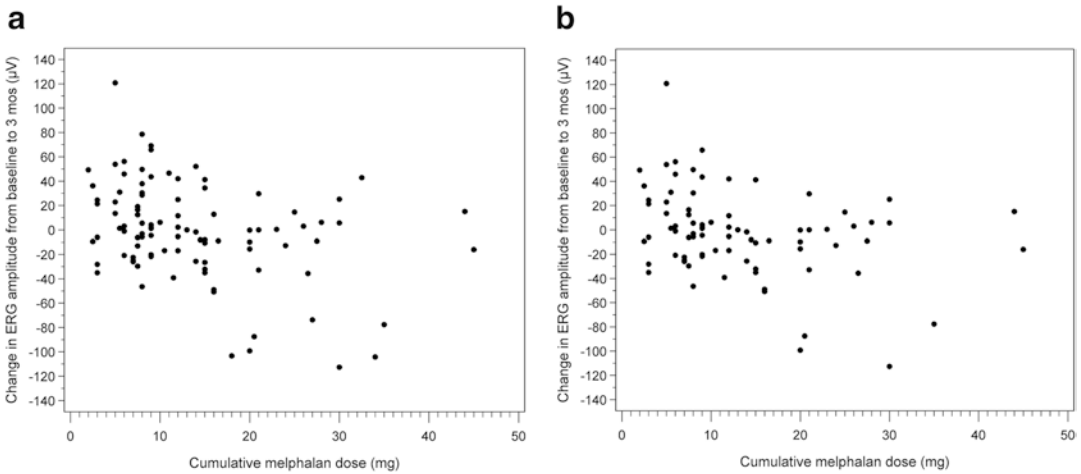


Fig. 5.7 Thresholds of retinal toxicity for cumulative dose of intra-arterial melphalan. Large decrements in ERG amplitude were seen only in patients with cumula-

tive melphalan doses exceeding 14 mg, or 20 mg among patients without gross alterations in retinal architecture. Long-term stability of ERG outcomes

after four intravitreal injections with melphalan [17]. After a subsequent recurrence, this patient received treatment with a ruthenium plaque, and an additional course of four intravitreal melphalan injections. ERGs were slightly decreased.

Our subsequent experience has revealed a general tendency for reductions in ERG amplitudes with each successive intravitreal melphalan injection. Our linear regression analysis estimates a decrement of 5 μV of photopic ERG amplitude with each intravitreal injection of 30 μg of the drug [18]. The range of effective dosing of intravitreal melphalan consistent with retention of retinal function appears to be quite narrow. See Chap. 4 for further details.

5.5.13 ERGs After Systemic Chemotherapy for Retinoblastoma in Small Infants (Bridge Therapy)

At our center, we do not perform intra-arterial chemotherapy on infants younger than 3 months of age, or smaller than 6.0 kg in weight, to avoid risks associated with arterial punctures in smaller, younger infants. In these smallest patients, tumor control is maintained with systemic chemotherapy

(“bridge therapy”) until age and weight thresholds for intra-arterial treatment are reached. ERG monitoring of retinal function in this cohort has shown preservation or improvement in ERG amplitudes in nearly all cases, perhaps in part due to maturation of the retina during the first few months of life [19].

5.6 Conclusions

In the 8 years of ERG monitoring of patients undergoing treatment for retinoblastoma, we have performed over 5300 hundred ERG studies. We have been able to document persistence of retinal function in many cases, and improvement in ERG amplitudes in 20–30 % of cases, especially with resolution of retinal detachment. ERG monitoring has proven to be an effective guide to the adjustment of chemotherapy dosage.

Compliance with Ethical Requirements Scott E. Brodie declares that he has no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

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Pharmacokinetics of Chemotherapy Delivered by Ophthalmic Artery Chemosurgery

6

Paula Schaiquevich and Guillermo L. Chantada

6.1 Introduction

Since the introduction of super-selective infusion of chemotherapy into the ophthalmic artery (OAC, ophthalmic artery chemosurgery) in retinoblastoma patients, enucleation has been avoided in an increasing number of eyes (Abramson et al. 2008, 2012; Abramson 2014; Gobin et al. 2012). Systemic infusion of chemotherapy and other local routes for chemotherapy delivery (periocular, subconjunctival) have shown clinical limitations due to the relatively poor penetration of drugs from the blood into the target ocular structures, thought to be due to the blood-retinal barrier and rapid orbital clearance (Abramson 2005; Carcaboso et al. 2007; Chantada et al. 2009; Gaudana et al. 2009). An alternative to reach therapeutic levels may be increasing the dose; however, this approach will raise the incidence of systemic toxicity, especially myelosuppres-

sion, and subsequent infections. Hence, innovative routes for chemotherapy delivery are being studied and OAC has been a major breakthrough in such approaches: allowing chemotherapy to be delivered locally to the ocular structures affected by the tumor while minimizing systemic drug exposure.

Pharmacokinetics is the study of the temporal changes in the amount of a drug or the metabolite with pharmacological activity in tissues, cells, organs, or the whole body. In a broad sense, the term refers to how the body affects the drug and includes the kinetics of drug absorption, distribution, and elimination (metabolism and excretion) (Ritschel and Kearns 2004).

After intravenous administration, the whole dose of the drug is delivered to the bloodstream and subsequently, distribution and elimination from the body starts (Fig. 6.1a). However, the oral or any extravascular route of administration starts with the passage of the drug across membranes or the absorption process across membranes usually by passive diffusion, and thereafter, the drug is subject to distribution to the rest of the body and elimination (Fig. 6.1b). In the case of intravenous infusion, there is an initial rise in the concentration vs. time profile during the infusion and when it is interrupted, the concentration starts to decline as the drug is eliminated from the body (Fig. 6.1c) (Ritschel and Kearns 2004). The pharmacokinetics of drugs injected directly into the vitreous may be

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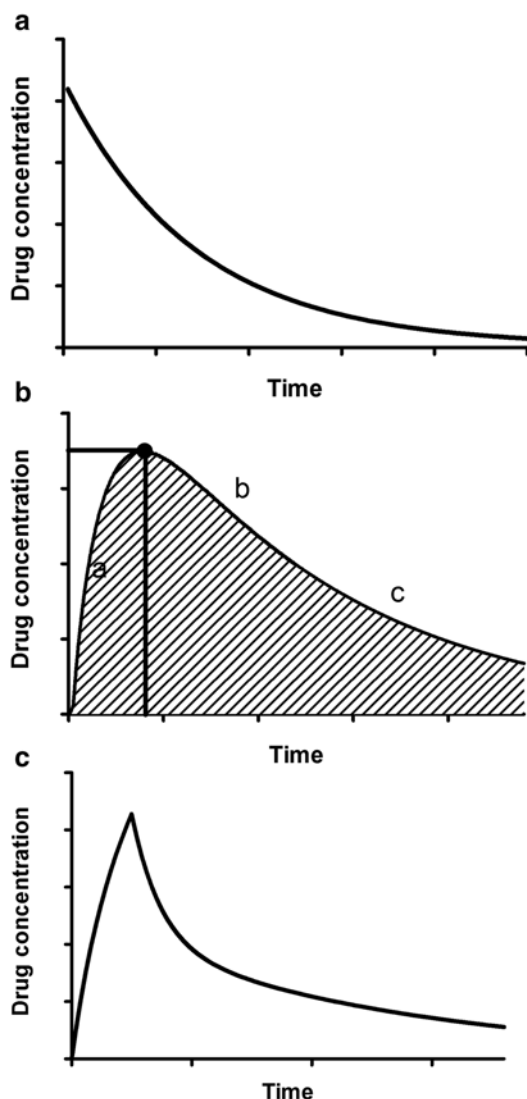


Fig. 6.1 Representative concentration vs. time profile for a drug after an intravenous bolus (a), oral (b) or intravenous infusion (c). Three different phases are represented after the oral administration, (a) absorption or a rise in the concentrations until the maximum concentration $C_{\max}$ at the time $T_{\max}$; (b) distribution and (c) elimination phase

modeled as an intravenous bolus, while in the case of the OAC of a drug, the concentration vs. time curve may follow the same pattern as after an extravascular administration due to the passage across different ocular tissues until reaching the vitreous (Schaiquevich et al. 2012a).

6.1.1 Pharmacokinetic Terminology

After intravenous injection, the drug becomes available in the systemic circulation, and hypothetically at the target site. Nevertheless, the administration of drugs by other routes, introduces the passage of the drug across membranes or the absorption process from the site of injection and the distribution to the rest of the body. In this sense, the bioavailability consists of two components: the rate and the amount of the dose of the drug that becomes available at the target site. In general, the systemic bioavailability of a drug is equal to one after intravenous administration as 100 % of the dose reaches the systemic circulation. Nevertheless, this concept is true when the target site is at the bloodstream or a tissue with rapid equilibrium with the systemic compartment. In the case of the eye, and specifically the vitreous compartment, the bioavailability fraction would be equal to one if the drug is injected directly into the vitreous while after intravenous administration it would be less than one due to the limited passage through the blood-ocular barriers (Cunha-Vaz 2004). Thus, in ocular pharmacology it is of interest to compare the bioavailability of the drugs administered to the eye by different routes, to maximize the ocular and minimize the systemic exposure. In addition, the bioavailability of a drug may vary because of patient-related factors including demographics, genetic or pathological conditions and this variability is referred to as inter-individual variability (Ritschel and Kearns 2004). The pharmacokinetic parameter expressing bioavailability is the area under the drug concentration-time curve or AUC as it measures the total amount of the drug that reaches the studied compartment. Another measure of drug exposure is the maximum concentration attained in the site of interest ($C_{\max}$). In this chapter, we will use the term “exposure” as a synonym of the former (AUC).

It is important to assess the amount or concentrations of drugs at the target site or in the bloodstream (if the target site is in rapid equilibrium with the systemic circulation) for correlation with the pharmacodynamic effect (efficacy or

safety). Once this relationship has been established, it is possible to assess the amount of a chemotherapeutic agent after a certain time of administration and determine if it is pharmacologically active. In addition, by knowing the pharmacokinetics of a drug given in a certain schedule and dose, it is possible to simulate and predict what may happen under different conditions including a change of dose or frequency of drug administration. Thus, the pharmacokinetics of a drug may be closely related to safety and efficacy and, once this relationship has been established, it becomes an important tool for optimizing the pharmacotherapy of the patient.

Each drug is characterized by its own pharmacokinetics, depending on many factors that include the route of administration, pharmaceutical characteristics and physiological, pathological, treatment, genetic, and environmental aspects related to the patient receiving the drug (Ritschel and Kearns 2004). Therefore, the pharmacokinetics of a drug should be studied if a new route for drug delivery is considered.

Another important pharmacokinetic parameter is clearance, usually defined as the volume of blood or plasma cleared of the drug in a certain interval of time. Clearance is inversely related to systemic exposure, and thus gives an estimate of the systemic exposure when also taking into account the administered dose. If a drug is administered concomitantly with a second one, we can study whether the clearance is affected, since it will impact systemic exposure. Potential pharmacokinetic drug–drug interactions among chemotherapeutic agents used for retinoblastoma after local delivery have not been extensively studied, but may have considerable impact on the efficacy and safety of the treatment.

The concentration that inhibits 50 % of the cell growth (IC₅₀) obtained from *in vitro* studies of cytotoxicity in retinoblastoma cell lines may be used as a surrogate for the drug concentration needed to reach the target ocular site, and the time above that threshold would be the interval of time during which the drug would be expected to exert antitumor activity. The IC₅₀s for topotecan, melphalan, and carboplatin in

different retinoblastoma cell lines have been extensively assessed and are available in the literature.

6.1.2 Animal Models Used for Pharmacokinetic Characterization

Based on the clinical results in patients that received OAC of chemotherapy, we may hypothesize that the excellent results achieved in terms of tumor control were due to higher bioavailability of the drug delivered by this route, compared to other local or systemic routes of administration (Schaiquevich et al. 2012a). A larger amount of the drug may have made its way to the tumor in the eye or was not cleared so easily from the ocular structures, resulting in a greater response. Nevertheless, further studies should be performed to support this hypothesis. In addition, while eyes with retinal detachment and subretinal seeds respond particularly well to OAC chemotherapy, eyes with vitreous seeding remain difficult to cure (Abramson et al. 2012; Kaneko and Suzuki 2003; Palioura et al. 2012). Therefore, these clinical observations may be related to differences in disposition between the retina/choroid and the vitreous of the infused eye. A better understanding of chemotherapy pharmacokinetics in the different ocular tissues may be useful to guide management tailored to the specific condition of the patient.

One of the obstacles in the treatment of intraocular retinoblastoma is the control of vitreous seeds. Therefore, in order to manage tumor growth, bioavailability of the chemotherapy should be increased in the vitreous and the challenge is to find active drugs with good penetration (Cunha-Vaz 2004). It is possible to design new drug delivery routes and study *in vitro* release, but *in vivo* the amount that reaches the vitreous would remain unknown. Procurement of vitreous tumor samples from patients for pharmacokinetic studies is not possible and thus the only alternative is to study ocular pharmacokinetic behavior in animal models and to extrapolate the findings to humans. Therefore, in order to

study the ocular pharmacokinetics of chemotherapy after OAC, a suitable animal model is required.

The first requirement for this animal model is adequate body size and diameter of the ophthalmic artery making catheterization technically feasible. Thus, small animals, such as rabbits, mice, and rats, are not suitable, but monkeys and pigs are good models for OAC pharmacokinetics assessment (Tse et al. 2013). Although anatomical and physiological differences between these two models and humans exist, the pig model has the advantage of previous use in eye research and of having a well-characterized vascular anatomy (Dondelinger et al. 1998; Moren et al. 2009). Both pig and the nonhuman primate models have been used for ocular pharmacology studies after OAC of chemotherapy, however, up to now only data on pigs have been reported (Schaiquevich et al. 2012a, b). Since the best anatomical correlate is large animals, it excludes the possibility of studying pharmacokinetics in tumor-bearing models, such as mice. Although the use of animal models is a useful approach for the study of alternative and innovative treatments of retinoblastoma, interspecies differences in the anatomy and physiology of the eye and the absence of blood-retinal barrier disruption in non-tumor-bearing animal models may limit the translation of these findings to humans (Dyer et al. 2005).

6.1.3 Microdialysis

Over the last decade, microdialysis has been increasingly used to study the pharmacokinetics of drugs in the eye. Briefly, the principle of the technique is based on the diffusion of the drug from the study tissue through a dialysis membrane. The membrane has a diameter similar to a blood capillary and is inserted into a probe connected to inlet and outlet tubing for continuous exposure with a perfusion fluid. This perfusion fluid intentionally resembles the ionic composition of the surrounding tissue of interest (e.g., phosphate buffer saline, pH 7.4) (Hocht et al. 2004). Subsequently, the fluids are

serially collected for quantification of the drug using an appropriate analytical technique. The microdialysis technique allows serial vitreous sampling from each eye and hence, a complete pharmacokinetic study can be carried out with a reduced number of animals compared to the number needed if a single sample were taken by aspiration of vitreous humor. This is a versatile technique since a variety of probes are available with different microdialysis membrane cut-offs, that determine the size of the compounds that can cross by passive diffusion. It should be emphasized, however, that depending on the study site, the technique is invasive and thus only experimental data in animals can be obtained.

The current treatment of retinoblastoma using the OAC consists of the concomitant or single administration of three agents—melphalan, topotecan, and carboplatin—to one eye or both eyes in tandem, alternating the drug association between cycles of treatment according to tumor response and other factors (see Chap. 3 for further details) (Abramson 2014; Marr et al. 2012). Although all three agents are commonly used, the pharmacokinetics of topotecan and melphalan has been well characterized, however, no data are available for carboplatin after OAC.

Here we review the current knowledge of the pharmacokinetics of chemotherapeutic agents after OAC in animal models and patients with retinoblastoma, with emphasis on treatment safety and efficacy based on *in vitro* data.

6.2 Topotecan Pharmacokinetics

The topoisomerase I inhibitor, topotecan, has *in vivo* and *in vitro* activity against retinoblastoma (Chantada et al. 2004; Laurie et al. 2005; Qaddoumi et al. 2012). Topotecan is subject to spontaneous chemical conversion dependent on the pH of the biological medium, from the lactone structure to the open-ring carboxylate devoid of antitumor activity (Abramson et al. 2010; Herben et al. 1996). Lactone or total—lactone plus carboxylate—concentrations in plasma are routinely

measured to quantify exposure in the patient after the intravenous drug infusion and to guide the next dose based on the proven relationship between exposure and the incidence of severe myelosuppression (Santana et al. 2003).

The currently available data on ocular and systemic topotecan pharmacokinetics after OAC were obtained in the pig. After the infusion of 1 mg of OAC topotecan over 30 min into domestic Landrace pigs, the attained vitreous concentrations were above the IC₅₀ reported in retinoblastoma cell lines of 20–30 nM (equivalent to 9 ng/mL–14 ng/mL, respectively) (Laurie et al. 2005), and remained well above this pharmacodynamic threshold for at least 4 h as shown in Fig. 6.2a. In addition, the vitreous-to-plasma exposure ratio was 29 indicating the favorable penetration into the vitreous or the difficulties for topotecan to leave the posterior chamber after OAC in the animal. In this study, the authors also compared these findings with the pharmacokinetics of topotecan after periocular injection of the same 1 mg-dose to the fellow eye of the animals after a washout period to ensure the lack of carry-over effect. As it was hypothesized from the clinical observation of the lack of tumor control after

periocular injection in the clinics, topotecan vitreous exposure was only 16 % of that attained after the same dose was infused into the ophthalmic artery of the animal (Schaiquevich et al. 2012a, 2014). Interestingly, the ratio between topotecan vitreous exposure after OAC to the value obtained after periocular injection of the same dose was six, demonstrating the higher selectivity of the OAC technique to deliver topotecan to the vitreous in the animal model. In addition, a comparable or even lower systemic exposure was attained after OAC compared to the periocular route. It is clear that the higher vitreous-to-plasma $C_{\max}$ and AUC for topotecan after OAC are mainly based on limiting the anatomical barriers of the trans-scleral routes and avoiding the orbital clearance involved in the periocular route. Altogether, these findings favor the use of OAC topotecan over the periocular route (Schaiquevich et al. 2012a, 2013, 2014).

Based on clinical judgment, topotecan was seldom used as a single treatment agent but often combined with other agents in the same session of OAC. This empirical practice may be supported, at least in part, by the synergistic toxicity found in retinoblastoma cell lines and

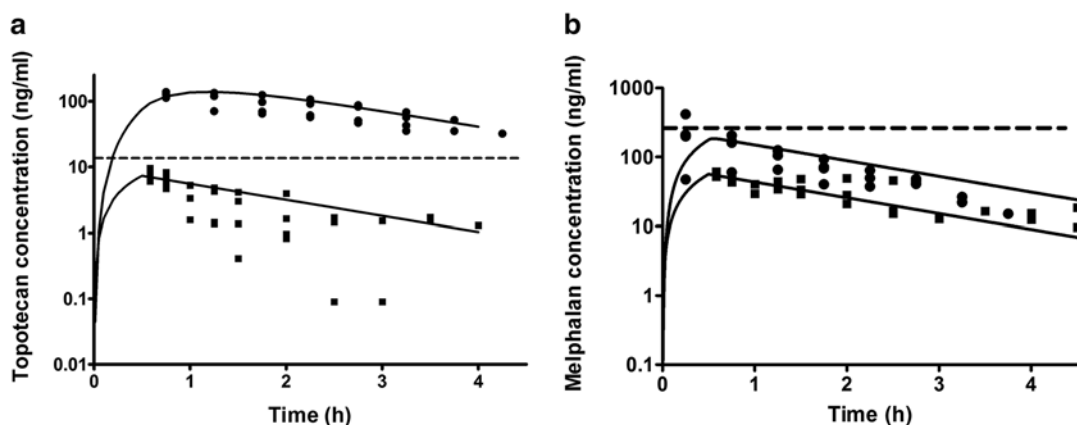


Fig. 6.2 Vitreous and plasma disposition after super-selective ophthalmic artery infusion of topotecan (a) and melphalan (b) in the pig. Circles and squares represent individual data points for vitreous and plasma total topotecan concentrations, respectively. Lines represent the best-predicted concentration for vitreous and plasma total

topotecan or melphalan for a representative animal. Dashed line represents topotecan and melphalan IC₅₀ obtained in retinoblastoma cell lines. Note how close the IC₅₀ is to the maximum vitreous concentration of melphalan while for topotecan this pharmacodynamic value was highly exceeded in the vitreous after OAC

animal models when concomitant treatment with topotecan and melphalan or carboplatin was assessed (Athale et al. 2002; Francis et al. 2013; Nemeth et al. 2011). Indeed, the only data available on the systemic disposition of topotecan after OAC in retinoblastoma patients was obtained in pharmacokinetic studies where topotecan was infused before or after concomitant melphalan (Taich et al. 2014). Nonetheless, it is interesting to comment on the agreement between topotecan pharmacokinetic parameters after OAC and those previously reported in pediatrics. Assuming a mean body weight of 12 kg, based on the reported clearance of 0.67 L/h/kg, the mean clearance of total topotecan in children with retinoblastoma after OAC is about 8 L/h. This value is closely related to a clearance of 9–10 L/h previously calculated in patients after a 30-min intravenous infusion for the treatment of different pediatric malignancies (Tubergen et al. 1996).

As we previously discussed, the importance of pharmacokinetic characterization is to obtain a surrogate marker for pharmacodynamic efficacy or toxicity and this threshold for the increased incidence of severe neutropenia is attained at a systemic lactone topotecan exposure of $180 \text{ ng} \times \text{h/mL}$ (Santana et al. 2003). Thus, taking into account that the median systemic exposure for total topotecan after OAC was $95 \text{ ng} \times \text{h/mL}$ and around one-third of the exposure could be in the lactone form, topotecan delivery into the ophthalmic artery at routine clinical doses hardly results in exposures expected to be myelosuppressive.

Although a favorable vitreous disposition with high penetration or slow clearance from this compartment has been found, clinical evidence for the efficacy of topotecan as a single-agent therapy using OAC is still scarce and different schedules should be further evaluated (Panetta et al. 2008).

6.3 Melphalan

Melphalan is the prototype chemotherapeutic agent for OAC in retinoblastoma. The drug belongs to the nitrogen mustards. It alkylated different molecular structures in the cell including

nucleic acids and finally leading to disruption of DNA function, protein synthesis, and cell death. Although it is used in several adult malignancies, the indication in pediatrics has been limited to hematologic malignancies mainly due to the substantial toxicity after systemic administration. Nonetheless, back in 1987, Inomata et al. showed a significant cytotoxic effect in retinoblastoma cells, and subsequently introduced this drug into clinical practice for the treatment of retinoblastoma (Inomata and Kaneko 1987). In order to limit the severe adverse events of this alkylating agent, it had to be administered through a local route for ocular delivery and this was attained by the selective ophthalmic artery infusion or intravitreal injections (Francis et al. 2014; Kaneko and Suzuki 2003; Munier et al. 2012). Thereafter, melphalan was introduced to the general community of retinoblastoma clinicians using the super-selective ophthalmic artery infusion technique. In only a few years, super-selective ophthalmic artery infusion of melphalan became an established therapy for advanced retinoblastoma.

Despite being widely used in the clinical practice for the treatment of retinoblastoma, there was limited information on the pharmacokinetics of melphalan after OAC. The first published data were obtained from the pig model (Schaiquevich et al. 2012b). These data were of particular importance to assess the penetration of the chemotherapeutic agents into the ocular structures, specifically the vitreous humor, and the extent of systemic exposure after OAC in spite of being a local route for drug delivery.

Based on *in vitro* sensitivity studies in commercial retinoblastoma cell lines, the melphalan IC₅₀ was found to be between 1.1 and 1.6 μM equivalent to 376 ng/mL and 547 ng/mL, respectively (Inomata and Kaneko 1987; Schaiquevich et al. 2012b). The studies reported by Schaiquevich et al. (2012b) showed that after 7 mg of melphalan OAC the median (range) maximum vitreous concentration ($C_{\text{max, vit}}$) was 170 ng/mL (47–416) (Schaiquevich et al. 2012b). These studies were performed using microdialysis technique. Hence, this local route for drug delivery barely reaches pharmacological levels in the vitreous that reach the IC₅₀ (Fig. 6.2b).

This pharmacokinetic–pharmacodynamic observation is in line with findings in clinical practice in which response to melphalan was shown to be good in eyes with retinal detachment caused by the tumor and subretinal seeds, but less impressive results were achieved in those with vitreous seeding (Abramson et al. 2012; Palioura et al. 2012).

Interestingly, median (range) vitreous and systemic exposure (AUC) was 391 ng×h/mL (163–472) and 134 ng×h/mL (102–284), respectively. Thus, vitreous exposure after OAC was threefold greater than systemic exposure favoring local delivery of melphalan (Schaiquevich et al. 2012b). However, this ratio is much smaller than that previously discussed for topotecan, even though it was obtained in the same animal model. It is important to emphasize that the pharmacokinetics of each drug after OAC has to be studied separately and that the findings of one study cannot be extrapolated to different drugs. The penetration and residence in the vitreous compartment after OAC depend on several factors related to the infusion technique, but also on the physical and chemical properties of the infused drugs: such as the affinity of the drugs to the vitreous components and membrane transporters that influence the passage through the blood-retinal barrier.

Of additional interest was the presence of melphalan in the retinal pigment epithelium (RPE)-choroid of the animal model. Three out of four animals showed detectable levels at the RPE-choroid, but only one treated eye had detectable melphalan in the retinal tissue (Klaase et al. 1994). This may be the result of the preferential distribution of melphalan, an L-phenylalanine mustard, in the pigmented tissues such as the RPE (since phenylalanine is a metabolite of melanin). The accumulation of melphalan in the RPE-choroid may explain the choroidal toxicity observed in patients. Conversely, the low or even undetectable disposition in the retina of the animals could be the result of low affinity to the LAT-1 transporter impairing the passage from the choroid to the retina (Hosoya et al. 2008).

Currently, there are two pharmacokinetic studies in the literature that investigate melphalan OAC in children with retinoblastoma. These studies provide data on the systemic exposure to

melphalan after unilateral and tandem OAC in children. They suggest that melphalan shows linear pharmacokinetics in doses ranging from 3 to 7 mg expressed as total doses. This observation is important in the sense that under linear assumption, the dose is proportional to the systemic exposure and thus, increasing the dose for treating more advanced tumor or tandem patients may also lead to an increase in the systemic exposure and to toxic levels (Schaiquevich et al. 2012b; Taich et al. 2014).

It was previously shown that melphalan attains a low systemic exposure after OAC (median, range, 165 ng×h/mL (84–398)) suggesting a low risk for associated sterility and secondary leukemia (Hartmann et al. 1984; Nath et al. 2007). Even though little is known about the threshold for gonadal toxicity in young children receiving alkylating agents, in stem-cell transplant patients receiving melphalan, dosages of 160 mg/m² (equivalent to 5.3 mg/kg for a mean body weight of 30 kg) may cause sterility (Hobbie et al. 2005). Nonetheless, toxicity-related exposure may be reached only after ten cycles of OAC, especially at the higher melphalan doses (Pritchard et al. 2005). Additionally, leukemia-related events have been observed in children with retinoblastoma receiving systemic etoposide infusion and secondary leukemia in adults with multiple myeloma receiving melphalan (Gombos et al. 2007; Hobbie et al. 2005; Wahlin et al. 1982). In all cases intravenous infusion of alkylating agents led to higher systemic exposure than melphalan OAC (Nath et al. 2007); however, the clinician should bear in mind that cumulative toxicity cannot be ruled out as a risk factor.

Inter-patient variability in melphalan pharmacokinetics may explain the wide variability in systemic exposure at a fixed dose of drug. Adjusting dose to body weight may correct this inter-patient variability and thus, should be taken into account at the time of individualizing the dose. As was previously reported by Gobin P. et al., the selection for subsequent dosing can be influenced by clinical observations (Gobin et al. 2012). Additionally, dose-related myelotoxicity is the most frequent side effect of intravenous melphalan; however, only about one-third of the

patients experienced grade III/IV neutropenia after melphalan OAC (Abramson et al. 2008). Altogether, considering the relationship between systemic exposure and dosage (linear pharmacokinetics) after melphalan OAC and the significant importance of body weight in the inter-patient variability of melphalan pharmacokinetic parameters, a threshold dose for melphalan corrected by body weight should be determined in order to reduce the possibility of post-OAC myelosuppression. Of note, in this clinical pharmacokinetic study it was also shown that children receiving dosages higher than 0.48 mg/kg had a 50 % chance of presenting (Common Terminology Criteria for Adverse Events (CTCAE)) grade 3–4 neutropenia compared with a 0.04 % likelihood in children receiving lower dosages (Taich et al. 2014). Currently, in clinical practice dose-corrected-by-weight is a useful tool to tailor melphalan OAC treatment to each individual patient.

Interestingly, melphalan pharmacokinetic parameters obtained after almost a 20-fold higher intravenous dose (100 mg IV vs. 5 mg OAC) are comparable to those obtained in retinoblastoma patients after OAC, confirming that melphalan follows linear pharmacokinetics in this wide range of doses (Table 6.1).

At least three aspects of the previously cited studies are important to highlight. First, in agreement with those previous reports on intravenous melphalan, in which body weight was the most important clinical factor related to melphalan pharmacokinetic parameters, we observed a similar dose-weight association after melphalan OAC

(Nath et al. 2007). Second, melphalan is metabolized mainly by spontaneous chemical conversion to hydroxy metabolites and eliminated by the kidney. As expected, these pharmacokinetic studies of endovenous melphalan reported a wide inter-patient variability in melphalan pharmacokinetics explained by glomerular filtration rate as a surrogate for kidney function (Nath et al. 2007). Finally, an important observation was the significant decrease in melphalan clearance in patients previously treated with carboplatin (Nath et al. 2007). These studies strongly suggest that carboplatin impairs melphalan clearance by inducing renal damage; however, further studies should be conducted to determine the mechanism of the interaction. It would be interesting to assess whether melphalan pharmacokinetics after OAC is affected by kidney function mainly in heavily treated patients receiving a combination of melphalan, topotecan, and carboplatin, tandem patients, or in cases of bridge therapy with particular emphasis on young children receiving carboplatin for initial tumor control (Gobin et al. 2012; Marr et al. 2012).

Another important aspect to discuss is the dose assignment in tandem patients. When prescribing total dosages (the sum of the doses for both eyes) that are greater than 0.5 mg/kg melphalan, clinicians should be aware of the risk for potentially severe neutropenia. It has been reported that three out of the four patients that developed severe myelosuppression were tandem administrations with melphalan dosages greater than the threshold of 0.48 mg/kg (Schaiquevich

Table 6.1 Pharmacokinetic parameters of melphalan and hematological toxicity incidence after local and systemic routes for drug delivery

Pharmacokinetic parameter	Super-selective ophthalmic artery infusion (Schaiquevich et al. 2012b; Taich et al. 2014)		Intravenous infusion ^a
	Single-agent	Concomitant with topotecan	
CL (L/h/kg)	0.51 (0.03)	0.44 (0.02)	0.47 (0.36–0.57)
AUC/D ((ng × h/mL)/mg)	165.5 (83.8–397.6)	150.8 (68.6–321.4)	^b
% Incidence of severe neutropenia ^c	16.3	12.1	^c

^aPatients received a single high dose (140–180 mg/m²) or divided dose regimens (e.g., three consecutive days of 70 mg/m²)

^bA target AUC of 9000 ng × h/mL after a dosage of 140 mg/m² to patients with a median BSA of 0.77 m²

^cSevere myelosuppression is expected in all patients (Nath et al. 2007)

et al. 2012b). An option in this case may be to lower the dose, although this may be less effective. Therefore, the development of new treatment strategies would be helpful using new drug combinations for tandem patients or those requiring intensification of chemotherapy due to poor response. Ideally, these strategies would focus on melphalan dose reduction without losing pharmacologically active exposure in the vitreous of each single agent, while also avoiding higher systemic toxicity. Indeed, the combination therapy approach is the basis for all antineoplastic treatments in pediatric solid tumors aimed at circumventing tumor resistance and attaining greater efficacy based on synergistic effects and may want to be applied for the local treatment of retinoblastoma (Nemeth et al. 2011; Qaddoumi et al. 2012; Schaiquevich et al. 2012b).

It is interesting to note that an *in vitro* synergistic effect of topotecan and melphalan was observed in retinoblastoma cell lines based on the combination index less than one according to the Chou and Talalay approximation (Chou 2010). This synergistic effect was not surprising, considering the previous experience of combining an alkylating agent and a topoisomerase inhibitor in other pediatric neuroectodermal malignancies (Saylor et al. 2001). Based on this *in vitro* synergistic effect and the previously discussed favorable ocular pharmacokinetic disposition of topotecan in an animal model, concomitant infusion of melphalan and topotecan would theoretically make a good treatment option. It is no surprise that the regimen of melphalan OAC sequentially infused with other agents (including topotecan and carboplatin) has been recommended in different clinical settings with satisfactory results (Marr et al. 2012). An interesting point was to assess potential pharmacokinetic drug–drug interactions between the two agents after sequential OAC at the level of the kidney, which may affect their disposition compared with the single-agent pharmacokinetics. This interaction may result in increased systemic exposure of melphalan causing more hematologic toxicity than expected. Moreover, administering two myelosuppressive drugs may lead to pronounced hematologic toxicity. Finally,

not only is the drug interaction influential, but the sequence of drug administration may also affect the pharmacokinetics of the infused drugs since vascular effects exerted by the first infused drug may alter the disposition of the following one (Schrijvers et al. 2000). All these factors require a better understanding of the pharmacokinetics and toxicity of OAC chemotherapeutic combinations in clinical practice. Taich et al. (2014) analyzed all these factors in a cohort of 27 eyes from 26 patients treated with concomitant melphalan and topotecan, and compared them to a historical cohort of patients treated with single-agent melphalan OAC (Taich et al. 2014). All patients were administered less than the maximum dosage of 0.5 mg/kg of melphalan concomitant to 0.5–1 mg topotecan, depending on age. The study statistically showed that melphalan pharmacokinetics in terms of systemic drug exposure and clearance (inversely related to the exposure) was not affected by the association with topotecan or the sequence of drug infusion. In other words, systemic melphalan exposure after a certain dose delivered through OAC is the same whether infusing the drug alone or concomitantly with topotecan in the same session, and no expected effect on melphalan hematologic toxicity.

Combining two agents in the same procedure obliges the interventional neuroradiologist to reduce the length of infusion of each drug by half so that the time of angiographic exposure is not increased. Interestingly, melphalan plasma pharmacokinetics after a 15-min infusion was similar to that found in a historical cohort of patients infused for 30 min.

The question as to whether topotecan results in hematologic toxicity by itself at the working dosages is important, since this may also have an impact on the pharmacodynamics of melphalan OAC. The study showed that the combination therapy of melphalan and topotecan was well tolerated with an incidence of severe neutropenia of 12 % (Table 6.1). Compared to the historical control of patients treated with melphalan as a single agent, the incidence of severe hematologic toxicity was 16 %. If working with melphalan OAC dosages less than 0.5 mg/kg, this study demonstrated

Table 6.2 Comparison of pharmacokinetic parameters between melphalan and topotecan after OAC in pigs

Drug	Dose (mg)	AUC _{vit} /AUC _{pl}	C _{max, vit} /C _{max, pl}	IC50 (μM) ^a
Melphalan	7	3.1 (0.6–4.2)	3.4 (0.4–7.9)	1.0–1.6
Topotecan	1	29 (25.8–36.5)	16 (14.5–16.3)	0.02–0.03

^aThe reported IC50 was obtained in Y79 and WERI-RB1 cell lines (Laurie et al. 2005; Schaiquevich et al. 2012b)

AUC area under the concentration vs. time profile for vitreous (vit) and plasma (pl) samples, C_{max} maximum concentration obtained after OAC in vitreous (vit) or plasma (pl), IC50 concentration of topotecan or melphalan that inhibits 50 % of the growth of retinoblastoma cell line

that the addition of concomitant topotecan had no statistically significant influence on systemic toxicity. Concomitant melphalan and topotecan OAC resulted in the same pattern and incidence of hematologic toxicity as those reported for single-agent melphalan. Thus, considering the favorable ocular pharmacokinetics of topotecan and the low incidence of severe hematologic toxicity after combination topotecan-melphalan OAC, this regimen was selected for the use in clinical practice (Table 6.2). These encouraging findings have added incentives to combine different active chemotherapy drugs that may work synergistically against this ocular tumor.

6.4 Final Discussion

Previously reported data support the pharmacokinetic advantages of super-selective ophthalmic artery infusion of chemotherapy over intravenous infusion or other local routes, such as periocular injections. A higher vitreous bioavailability was attained after topotecan OAC than after periocular injection of the same dose while the selectivity of the route in terms of vitreous-to-systemic exposure was significantly lower after periocular administration. For melphalan, this ratio was not so favorable and vitreous levels barely attaining the IC50 as a surrogate for antitumor activity. We emphasize that all available ocular pharmacokinetic data were obtained from animal models. Ocular exposure studies are only feasible in an animal model mainly due to ethical constraints and fears of extraocular tumor dissemination.

Although OAC is a local route for drug delivery, quantifiable systemic exposure of topotecan

and melphalan was assessed in patients. Hence, while limited systemic toxicity including mild myelosuppression has been reported, the potential cumulative systemic exposure after multiple OAC sessions with unknown long-term effects should not be disregarded. A statistically significant association between systemic melphalan exposure and the incidence of severe neutropenia was reported at a threshold dosage of 0.5 mg/kg melphalan. Using this threshold and considering the synergistic cytotoxic effect of the combination of melphalan and topotecan, the favorable disposition of topotecan in the eye, as well as the lack of pharmacokinetic and pharmacodynamic interaction between the two drugs, combination therapy of melphalan and topotecan OAC is an interesting option for the treatment of retinoblastoma in clinical practice (Table 6.2). New drugs and combinations delivered through OAC should be further studied and assessed for pharmacokinetic characteristics for additional optimization of retinoblastoma treatment.

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The present study followed the tenets of the Declaration of Helsinki and institutional review board approval was obtained. Written informed consent was obtained from the parent/guardian for being included in the study.

All experiments adhered the tenets of Hospital Garrahan Institutional Committee for Animal Care and the ARVO Statement for the use of animals in ophthalmic and vision research. Also, all institutional and national guidelines for the care and use of laboratory animals were followed.

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7.1 Introduction

Retinoblastoma (Rb) is a pediatric intraocular malignancy, either hereditary or sporadic, caused by a mutated *RBI* gene, and affects approx. 5,000 children around the world each year (Shields and Shields 2004; Grossniklaus

2014). The current treatment options for Rb include delivery of chemotherapy via intra-arterial, periocular, intravitreal, or even intravenous routes. Although advances in genomic research and improved methods of localized drug delivery have enabled up to a 95 % survival rate in developed countries, the efficacy and toxicity profiles could be improved for these current therapies (Nishimura et al. 2001; Klein et al. 2003; Shields and Shields 2004; Rizzuti et al. 2008; Abramson 2014). As of July 2014, *ClinicalTrials.gov* reports 56 Rb-related interventional studies, a majority of which were either suspended or produced inconclusive outcomes. The question arises as to why be it so critical and challenging to discover novel drugs for Rb? Primarily, the treatment has to be administered to children less than 5 years of age, who are still in their early stages of growth and development, and standard chemotherapy can have significant side effects at this young age. In addition, the current chemotherapeutic agents require to be delivered at high systemic or consistent localized (ocular) concentrations to produce an effect, especially to treat vitreous seeds, which in turn presents the issue of considerably high toxicity (Francis et al. 2014). There is an added difficulty to manage those Rb

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patients who have developed multidrug resistance. Since Rb is a pediatric cancer, there is also a lack of sufficient subjects for clinical trials, and therefore, a heavy dependence on pre-clinical animal studies in mice and rabbits for safety and efficacy assessments, respectively (Houston et al. 2013).

It is quite ironic that, although *RBI* was the first tumor suppressor gene to be discovered (1971) and cloned (1984), we still do not have a targeted cure for Rb (Grossniklaus 2014). This could perhaps be explained by the accumulation of a variety of unknown genomic aberrations and differential gene expression patterns in the cells of origin, rendering a growth advantage to these tumors and making them resistant to target-based therapies. To this end, multiple groups around the world have undertaken dedicated research to identify alternate defective targets in Rb. For example, over-expression of oncogene SYK and p53 regulators MDM2 and MDM4 have been implicated in disease development and progression and explored as likely targets for therapeutic intervention (Zhang et al. 2012; Theriault et al. 2014). Similarly, modulations in the Wnt/ β -catenin, NF- κ B, p53, and Akt/PKB pathways have also been reported (Laurie et al. 2006;

Houston et al. 2013; Zheng et al. 2014). Nutlin-3 (inhibitor of mdm2-p53 interaction) and calcitriol (inhibitor for cell cycle arrest) have been successfully investigated for their antiproliferative properties against Rb in Y79 cells (Wagner et al. 2003; Laurie et al. 2006). Despite such advances in research, our realm of knowledge with regard to the Rb interactome remains limited and specific treatments are yet to ensue. At the same time, conventional drug discovery methods for development of novel cancer therapeutics have become increasingly associated with high cost, poor safety, limited efficacy and bioavailability, and a lengthy design and testing process. Typically, the entire process of Research & Development would require approx. 1.8 billion dollars of investment and 13 years of time commitment (Paul et al. 2010), a luxury we cannot afford given the urgency to improve current treatment options. Therefore, although various methods could be adopted to discover drugs for Rb (Table 7.1), this chapter details only three such approaches of drug discovery that are productive, fast, and innovative—(1) Drug repurposing using classical pharmacology, (2) Companion drug therapy, and (3) Drug discovery in three-dimensional disease models.

Table 7.1 Summary of drug discovery approaches for retinoblastoma treatment

Drug discovery approaches		Benefits	Challenges
Targeted screens		• Rational drug discovery approach	• Limited by druggable genome
		• Avenue for target-driven therapy	• Limited by chemical diversity
			• Unknown poly-pharmacology of “target-specific” drugs • Multiple unknown genomic alterations in <i>cells of origin</i> • Long and expensive journey from bench to clinic

(continued)

Table 7.1 (continued)

Drug discovery approaches		Benefits	Challenges
Phenotypic screens	Re-purpose old drugs	• Accelerated drug discovery approach	• Conflicting IP
		• Rapid translation from bench to clinic	
		• Drug efficacy and toxicity information handy	
		• Clinical trial statistics available	
	Companion drugs	• Nutraceuticals for combination therapy	• Might antagonize with anti-cancer drugs
		• Improve patient's immune system, metabolism and health	• Non-respondent patient population
		• Negligible toxicity relative to anticancer drugs	
	3D culture systems	• Mimics tumor's microenvironment	• Currently low-throughput
		• Potential to study hard to treat vitreous seeds	• Currently immature technology
		• Realistic drug response assessment	
	Natural products screening	• High chemical diversity	• Difficult to purify active ingredient
		• Low cytotoxicity	
Orthotropic animal models		• Represent realistic disease state	• Very expensive
			• Very time intensive

7.2 Discovery and Repurposing Through Automated High-Throughput Screening

High-throughput screening (HTS) methodologies, together with state-of-the-art robotics, advanced instrumentation, and innovative technologies, has opened doors to rapidly test hundreds and thousands of compounds for therapeutic activity in a matter of hours. Alongside, the classical drug discovery approaches gradually gave way to targeted approaches, also known as “reverse pharmacology,” which aimed to measure the effects of compounds on a purified target protein or gene of interest (Takenaka 2001). However, this preferred method of identifying novel inhibitors require characterized targets in context of a disease state, presenting a major caveat towards its applicability in drug

discovery for Rb; one, Rb is caused by loss of target gene *RB1*, and second, understanding of the alternate targets potentially driving Rb is incomprehensive (Houston et al. 2013). A more beneficial approach for Rb would be to rather revisit classical pharmacology and conduct unbiased phenotypic screens in disease-relevant cell models. This would allow researchers to assess differential physiological responses with minimal emphasis on molecular targets or signaling pathways.

Implementing fruitful HTS, a grand endeavor with a colossal financial investment, in itself faces a huge roadblock. Although, collections of up to 400,000 chemicals are commercially available for screening, the number of new chemical moieties entering clinic has declined over the years (Djaballah 2013). The issue lies in the limited scaffold diversity within and among distinct

libraries synthesized using man-made combinatorial chemistry and represent only a miniscule of the total chemical space. This missing link could be found in Mother Nature, as more natural products (NPs) get added to the screening libraries and have shown some promising outcomes (Djaballah 2013; Bhinder and Djaballah 2014). As an example, Piperlongumine, obtained from Southeast Asian pepper plant, was recently identified through a chemical screen to exhibit anti-proliferative properties in breast cancer (Bharadwaj et al. 2015). Although NPs bear great potential, isolation and purification of their active ingredient have proven to be a complex and time-consuming process. In the case of cancers like Rb, where time is of essence, an efficient approach to find improved therapy is critical.

It is believed that different diseases might share commonality at the level of pathways and molecular targets. This ideology encouraged the notion of drug repurposing, best conceptualized as discovery of *unknown benefits of known drugs*. The roots of repurposing lie in the accidental discovery of an erectile dysfunction indication for Sildenafil (Viagra®) originally developed for the treatment of angina. Since then many drugs have been repurposed for various diseases, and few such examples pertaining to cancer include Ibuprofen, Metformin, and Rapamycin (Gupta et al. 2013). Research efforts also focus on exploring anti-tumorigenic properties of non-cancer drugs, such as recently identified antiproliferative effects of antidepressant Imipramine in Small Cell Lung Cancer and antihelmintic drug Mebendazole in metastatic colon cancer (Jahchan et al. 2013; Nygren and Larsson 2014). Use of off-label prescription drugs in cancer has also garnered a lot of attention. The NIH established a dedicated division called National Center for Advancing Translational Medicine (NCATS), to foster drug-repurposing projects. Since data on drug toxicity, pharmacodynamics, and pharmacokinetics is already available through pre-clinical and Phase 1 trials, repurposing can indeed be recognized as the fastest route available to translate drugs to patient population; an excellent avenue to develop therapy for Rb.

With this motivation, we executed the first dedicated HTS to rediscover novel applications of existing FDA-approved drugs and bioactives for treatment of Rb (Antczak et al. 2009). Briefly, we utilized two well-established Rb cell lines Y79 and RB355 to test a focused library of 2640 compounds in an alamar blue-based cellular viability assay, measuring fluorescence as the end point readout. The screen was run in duplicate at a screening concentration of 10 μ M and the assay was deemed robust and reproducible before proceeding. The output was quantified into percent inhibition relative to the controls, and the threshold was set at a minimal of 95 % inhibition in both of the cell lines being tested, so as to enable identification of scaffolds with a generic activity against Rb; a total of 11 potent compounds, defined as hits, were identified through this criterion. Nine out of these 11 hits were also found to be pan-active across a panel of cell lines that included Rb cell lines Y79, RB355, and WERI-Rb-1 and uveal melanoma cell lines C918 and Mum2b. The nine hits belonged to either of the two distinct pharmacological classes; (1) five ion pump effectors—most potent, and (2) four antimicrobial agents.

Surprisingly, the five ion pump effectors (peruvoside, ouabain, neriifolin, digoxin, and digoxigenin) identified in the screen shared a common chemical scaffold to that of a cardenolide core. This observation led us to explore the structure-activity relationship of cardenolides as a novel antiproliferative class of compounds for treatment of Rb. For the purpose, we collected 35 cardenolides and derivatives to perform drug titration studies in four ocular cancer cell lines (Y79, RB355, WERI-Rb-1, and C918). Interestingly, 23 cardenolides produced an IC_{50} of <10 μ M in a minimal of two cell lines, indicative of their strong antiproliferative effect, and 21 out of these 23 potent cardenolides also had a distinct structural similarity—a glycoside substituent at their 3-hydroxy group. Among these hits were two FDA-approved cardiac glycosides, ouabain and digoxin, that exhibited generic potency across all four ocular cell lines tested and were therefore selected as suitable

candidates for further follow-up to assess their repurposing potential.

Repurposing ouabain: Ouabain, a representative cardenolide from the screen, is a cardiac glycoside originally indicated for treatment of congestive heart conditions. First, the *in vitro* potency of ouabain was tested in comparison to that of the six known agents against Rb (vincristine, etoposide, carboplatin, cisplatin, nutlin-3, and calcitriol). Relative to others, ouabain exhibited a significantly higher potency with an IC_{50} 's of $0.65\ \mu\text{M}$ in Y79 cells and $0.40\ \mu\text{M}$ in RB355 cells. Furthermore, an apoptosis assay measuring immunofluorescence of cleaved caspase-3 was performed in Y79 cells so as to assess whether the antiproliferative effects of ouabain were mediated via apoptosis. Relative to the baseline levels, vincristine and etoposide (also tested here) induced significant apoptosis at $100\ \mu\text{M}$ and $10\ \mu\text{M}$, respectively, while ouabain-induced significant apoptosis at a much lower concentration ($0.5\ \mu\text{M}$), and at higher concentrations eradicated Y79 cells. Ouabain was further investigated for its *in vivo* efficacy in Rb xenograft mouse models with Y79 tumors. Results of bioluminescent imaging and quantification of the tumor size revealed significant tumor shrinkage ($18\ \text{mm}^3$ average size) and near eradication by day 14, without affects on the average body weight of mice treated with continuous infusion of $15\ \text{mg/kg}$ ouabain; these observations represented high potency and low toxicity of this drug. On the contrary, the vehicle-treated control mice population showed continuous tumor proliferation, reaching $1000\ \text{mm}^3$ in size by day 14 (Antczak et al. 2009).

Repurposing digoxin: Digoxin, originally approved for treatment of cardiac arrhythmia, was selected for an off-label assessment directly in clinic and repurposed to a non-respondent 4-year-old patient with Reese-Ellsworth Classification Vb. The tolerated oral dose of serum digoxin levels ($0.5\text{--}1.5\ \text{ng/mL}$) results in $<0.0025\ \mu\text{g/mL}$ of the drug in the vitreous, a concentration much below the IC_{50} of digoxin ($1.56\ \mu\text{g/mL}$). Clearly, oral treatment was unlikely to produce sufficient intraocular concen-

tration of digoxin without considerable systemic toxicity. Therefore, an alternate route of intra-arterial administration of digoxin was sought. With a hope to achieve the desired ocular concentration of $5.5\ \mu\text{g}$, incrementing doses of digoxin ($25, 125, 250\ \mu\text{g}$) were delivered to the ostium of the ophthalmic artery over three infusions. These high doses were well tolerated by the patient and a moderate reduction in tumor size was observed. Previously, we had found that continuous infusion of the drug had led to a dramatic tumor regression in mice. Therefore, it was concluded that this modest response, *in lieu* of a complete remission, was perhaps due to the intermittent nature of drug administration, which may have limited a sustained intraocular bioavailability of digoxin (Patel et al. 2011).

Overall, these observations made for ouabain and digoxin do establish cardenolides as an exciting new class of drugs against Rb and the findings support the feasibility of achieving a high dose tolerance via intra-arterial infusion. Recently, a nonsteroidal anti-inflammatory drug (NSAID) indomethacin, clinically used for the treatment of rheumatoid arthritis, was also shown to have an antiproliferative activity against Y79 cells (Zheng et al. 2014). Taken together, translational and retrospective studies indicate that an HTS-based strategy to identify and repurpose approved drugs is a promising, rapid, and robust approach to develop novel treatments for Rb.

7.3 In Search of a Companion Drug: Case Study for Resveratrol

Anticancer drugs are often used in combinations to enhance their therapeutic efficacy and to salvage single drug resistance by means of target diversification (Kummar et al. 2010; Knight et al. 2010). However, there are challenges in combining these multiple cytotoxic drugs in therapy, primarily due to their combined toxic effects on normal tissue, unknown polypharmacological properties, and lack of target specificity. In addition, rational designs to test multiple approved drugs in combinations of two prove to be very

time-consuming and expensive with uncertain outcomes. A compelling alternative would be to incorporate nutraceuticals, such as dietary supplements, and herbal products, as companion drugs to accompany standard chemotherapy. As the name suggests, nutraceuticals nourish the body with key nutrients, strengthening the individual's immune system and metabolism, with an added benefit of negligible systemic toxicity. Therefore, it is of no surprise that cancer patients in therapy or remission often turn to nutraceuticals with hopes to attain better prognosis and improved health (Tripathi et al. 2005; Gupta et al. 2010). One such widely popular nutraceutical, resveratrol, has been reported to possess antitumorogenic properties against Rb cells while being non-toxic in normal cells, making it an excellent candidate for companion therapy (van Ginkel et al. 2008; Sareen et al. 2006; Fulda 2010; Mahida et al. 2013). However, the feasibility of using resveratrol directly in clinic is restricted due to its poor bioavailability and low potency; fortunately, these issues could be readily addressed by seeking drugs that would enhance its clinical efficacy. For the purpose, we developed an HTS approach to enable a rapid in vitro identification of chemical agents that either enhanced (synergizers) or rescued (antagonists) the retinal tumor cells from the antiproliferative effects of resveratrol (Mahida et al. 2013).

The chemical screens were conducted to test a collection of 6912 FDA-approved drugs and bioactives at 10 μ M against the absence or presence of resveratrol at 80 μ M, in Y79 and RB355 cells, and the residual cellular viability was assessed in an alamar blue assay (Mahida et al. 2013). After evaluation of its aqueous solubility limit, 80 μ M of resveratrol was deemed optimal for screening. The assay was quite robust with high signal to noise ratios and the screens run in duplicate exhibited good reproducibility, with the exception of the screen using RB355 cells in the presence of resveratrol. The observed fluorescence values for cellular viability were quantified as percent inhibition relative to the controls. The compounds that produced ≥ 50 % inhibition in the presence and ≤ 50 % inhibition in absence of resveratrol were scored as synergizers, while

those with an opposite effect were scored as antagonists. The focus was to identify positives with broad-spectrum potency, and therefore, 11 unique synergizers and 14 unique antagonists with activities across both Y79 and RB355 cells were selected. Interestingly, the antagonists showed an enrichment of positives belonging to either the microtubule inhibitor or the ion channel inhibitor classes.

The positives that were readily available for resupply were followed-up for drug titration studies in combination with resveratrol at 0, 30, and 80 μ M. Among the synergizers, an antibiotic berberine chloride, also found in some eye drop formulations, emerged as most potent with about a tenfold decrease in IC_{50} 's in the presence of resveratrol compared to its treatment as a single agent. Among the antagonists, the most prominent positive was a microtubule inhibitor, nocodazole, which increased the IC_{50} values in the presence of resveratrol by a drastic 100-fold. Upon retesting the antagonists in a battery of 13 distinct cells of various tumor origins, all three microtubule inhibitor positives (nocodazole, chelidonine, and albendazole) from the screen were confirmed, and so did two additional microtubule inhibitors, vincristine, and colchicine. These results were particularly interesting as they complement the previous reports that emphasized the role of resveratrol in blocking the cytotoxic effects of microtubule inhibitors vincristine, vinblastine, and taxol, routinely used chemotherapy drugs (Ahmad et al. 2004; Mao et al. 2010).

In summary, the approach described above represents a novel and unbiased phenotypic screening platform by virtue of evaluating the combinatorial effects of cancer drugs with nutraceuticals. On one hand, the identified synergizers would be beneficial as companion effectors of chemotherapy, fostering development of innovative lines of therapy with minimal associated toxicity. On the other hand, the finding with regard to antagonists, if confirmed in vivo, would partially explain why some patient populations might be non-responsive to ongoing treatments, bringing into bear the far-fetched clinical implications of the blindfolded use of nutraceuticals.

7.4 Overcoming 3D Cell Formation as a Novel Approach to Treat Rb

Approximately 95 % of the promising drug candidates' fail in clinical trials, putting to waste years of dedicated effort, billions of dollars, and hopes for a novel treatment (Hartung 2013). The problem perhaps lays in the heavy reliance on two-dimensional (2D) cell culture models during the early stages of drug discovery. 2D models represent a homogenous monolayer of cells, and therefore, are not a realistic representation of complex tissue physiology; the extracellular architecture and microenvironments that surround native tissues play a key role in driving their phenotypic functions and response to drug treatment (Xu et al. 2014). On the contrary, three-dimensional (3D) models closely mimic *in vivo* cellular organization including development of extracellular matrix, concentration gradients of nutrients and oxygen, and consequently, differential cellular proliferation (Benien and Swami 2014; Xu et al. 2014). 3D models have begun to gather great interest in cancer research; several techniques to form 3D cultures have already been established, some of which either use scaffolds such as inert matrices, or are scaffold-free such as hanging drop, and magnetic levitation (Yamada and Cukierman 2007; Ong et al. 2010; Haycock 2011; Daquinag et al. 2013; Haisler et al. 2013; Lamhamedi-Cherradi et al. 2014). 3D culture systems have been successfully developed to form multi-cellular tumor spheroids (MCTS) for various types of cancer such as prostate, ovarian, breast, colorectal, and cervical cancer (Kelm et al. 2003; Grun et al. 2009; Matsuda et al. 2010; Cheung and Ewald 2014; Rajcevic et al. 2014; Zhao et al. 2014).

In context of Rb, 3D cell culture-based drug discovery remains widely unexplored. In fact, it wasn't until mid-2012 that Mitra and coworkers became the first and the only group to report on a 3D culture of Y79 cells, created using biodegradable polymeric microparticle scaffolds (Mitra et al. 2012). The group tested the efficacy of three routinely used anticancer drugs—doxorubicin, carboplatin, or etoposide, in 2D versus 3D cell cultures. Not only did they observe a significant drop in the IC₅₀'s of the drugs being tested, but

also found a differential gene expression pattern among the 2D and the 3D models. These observations strongly suggest that the use of a 2D model towards drug discovery is an inherently flawed approach. Instead, 3D cultures by virtue of their close resemblance to the *in vivo* cellular organization play a far more realistic and important role in understanding the clinical uptake, distribution, and efficacy of the administered drugs. Therefore, these constitute perhaps the best model systems for drug discovery and development for Rb, as they would closely mimic the 3D vitreous seeds.

The work published by Mitra and co-workers was indeed a promising first step towards culturing Rb cells in 3D. However, the polymeric microparticles used by them appeared to produce non-uniform cell clusters with variable surface area. To control for any bias in the experimental results emerging from such variabilities in size, we attempted to develop a 3D Rb model comprised of uniform MCTS. As an essential preliminary step of the process, three different technologies were sought to assess their feasibility for constructing the most uniform 3D cell cultures for Rb cells; (1) Nanoimprint technology (Scivax Life Science, Inc.)—nanoculture plates imprinted with either Micro-Honeycomb (MH) or Micro-squares (MS)-shaped ultra fine mesh, (2) Elplasia™ technology (Kuraray Co., Ltd)—plates with each well containing square-type compartments enclosing micro-spaces, and (3) Magnetic Nano Shuttle™-PL (NS) particle technology (Nano3D Biosciences, Inc.)—nanoparticles assembled through electrostatic interactions of poly-L-lysine, gold, and magnetic iron oxide. Black flat-bottom corning plates form cell monolayers and were, therefore, used as a 2D culture control. In Cell Analyzer 2000 (GE Healthcare Life Sciences) was used for whole-well bright-field imaging at a ×4 magnification, and image acquisition was performed up to day 14 post cell seeding. The study was conducted using a suspension Rb cell line, Y79.

First, the nanoimprint technology was evaluated for 3D cluster formation on the imprinted wells of both MS and MH plates in a 384-well format. The Y79 cells failed to form 3D MCTS and maintained a grape-like arrangement, which

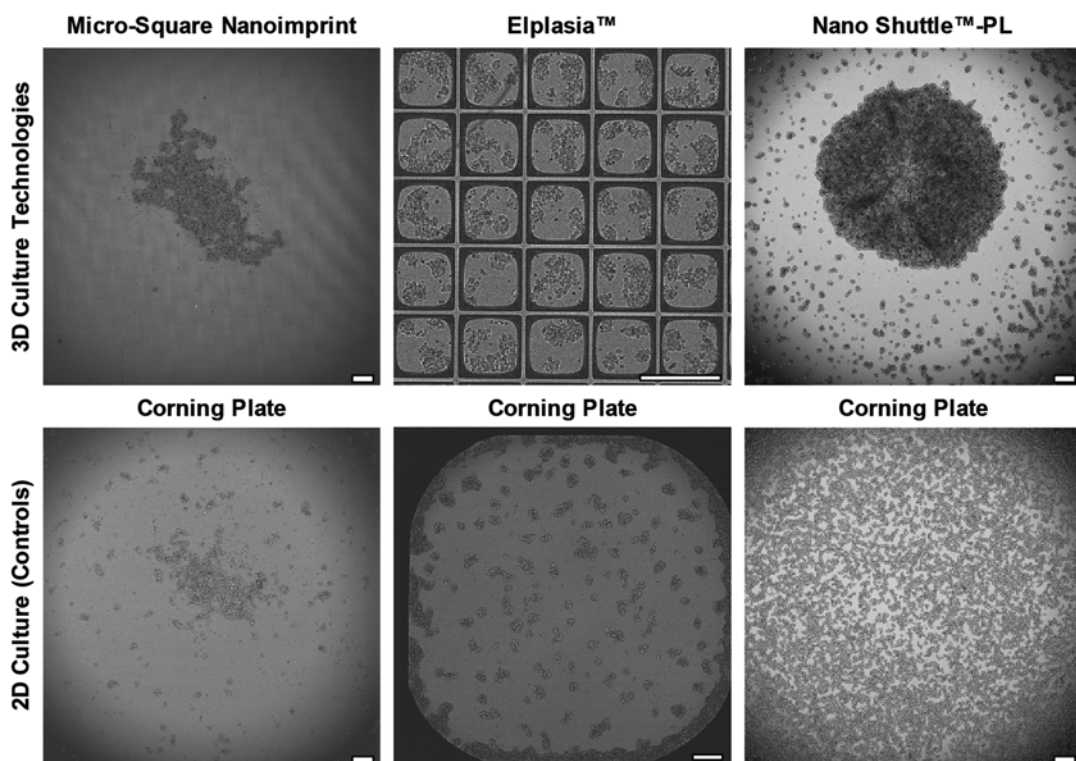


Fig. 7.1 Assessment of three leading technologies for 3D culture formation. The images show representative wells for Y79 cells grown in 3D cultures (*top panel*) vs. in control 2D suspensions (*bottom panel*). Magnetic Nano Shuttle™-PL particle technology from Nano3D

Bioscience (*top right*) formed most efficient 3D cultures relative to high surface binding micro-square nanoimprint plates from Scivax Life Sciences (*top left*) and Elplasia™ from Kuraray Co. (*top middle*). Scale bar, 250 μ m

is a characteristic of suspension cells. To determine whether this lack of 3D culture formation was associated with the anchorage properties of the cell line used, the nanoimprint technology was further evaluated in RB355 (Rb) and HeLa (adenocarcinoma) cell lines, which exhibit partial or completely adherent properties, respectively. Surprisingly, both RB355 and HeLa cells clustered by migration into multi-cellular spheroids; the spheroids formed on the MS plates were more compact and circular than those on the MH plates. Taken together, it was concluded that other technologies were needed to be explored for their suitability to form 3D clusters of suspension cells, such as Y79. At the time, Scivax Life Science Inc. introduced an improved version of the nanoimprint plates that were believed to exhibit higher surface-binding properties. We took this as an opportunity to re-test 3D cluster formation in Y79 cells using this newer version

of MS plates, made available in a 96-well format. The cells did start to aggregate and form 3D clusters, but the clusters appeared to be more erratic, mostly single and irregular in shape, with a poor surface attachment (Fig. 7.1). With a hope to address these limitations observed with the nanoimprint technology, cluster formation was tested using the second technology, that of Elplasia™. Although the micro-spaces in the fabricated 384-well plates did promote cellular assembly, the clusters lacked uniformity and high cell seeding densities led to saturation (Fig. 7.1). Intuitively, high content analysis on such a plate structure might also be very challenging.

As an attempt to improve upon the drawbacks identified in the nanoimprint and Elplasia™ technologies, the analysis was proceeded to test the NS particle technology, which are based on the principle of magnetic levitation (Bio-Assembler™). For the purpose, cells were incu-

bated with an optimal amount of magnetic nanoparticles, which conferred magnetic properties to the cells. This was followed by magnetic bioprinting, which included placing the plate containing magnetized cells on a magnetic blot drive that would allow the cells to cluster under the effect of an external magnetic field. It was observed that high cell seeding densities (approx. eightfold higher than those used in the nanoimprint technology) significantly promoted MCTS formation (Fig. 7.1). Interestingly, the 3D cultures began to form within approx. 2–3 days of treatment, making it a considerably faster approach. Furthermore, magnetized 3D models are also believed to be easier to handle and image. Studies are ongoing to further investigate the validity and efficacy of this system.

7.5 Future Perspectives

Recent advances in Rb treatment and management have proven successful in saving young patients' lives and saving more eyes, but at the expense of losing or harming vision in many cases. There is room for improving our current treatment methods for retinoblastoma and the missing pieces of the puzzle could lie in translational research, which is gradually ushering the drug discovery world into new possibilities of accelerated clinical discovery. FDA-approved non-cancer drugs, tested through classical pharmacology-driven HTS, can be rapidly repurposed by virtue of either a common target engagement or the polypharmacology of the drug and independent of any a priori knowledge with regard to a disease relevant target. With no time wasted in pre-clinical and Phase 1 trials, the drug is ready to make an expedited entry into the clinic, an approach best suited for Rb research as was exemplified by digoxin, and to be pursued dedicatedly in near future (Fig. 7.2).

Besides testing FDA-approved drugs for newer indications, it will also be critical to pay special attention to NP research, mainly due to their limited systemic toxicity at appropriate doses. NPs have been tested in the past for antiproliferative effect in Rb cells; matrine, a phytochemical found in the dry roots of the *Sophora*

flavescens, targeting the caspase-dependent signaling pathway, and combination of okadaic acid and parthenolide, targeting the PTEN/Akt/Mdm2/p53 pathway, have been identified as effective antitumor agents in Rb (Di Fiore et al. 2013; Shao et al. 2014). Such promising findings need to be investigated further with a hope of developing improved and non-toxic therapies. Furthermore, nutraceuticals in the form of dietary supplements are not prescribed with ongoing treatments, yet ailing patients tend to consume them to feel better and complement their treatment. The observations reported in the previous sections regarding resveratrol highlight that nutraceuticals might actually hamper the efficacy of some routinely used cancer drugs. Therefore, similar studies need to be undertaken in future to identify options most suitable for patient's well-being; nutraceuticals that could assist in increasing the potency of the ongoing chemotherapy could be prescribed often as companion effectors, as well as, also being wary of those which render the patients resistant to ongoing treatments, and therefore excluded from dietary intake.

A major shift in the drug discovery for Rb requires adoption of a more realistic approach by using 3D cell culture models to test novel drugs. In 1997, Bissell and co-workers provided experimental evidence to show that breast cancer cells tend to become non-cancerous when grown in 2D monolayers versus 3D; a seminal finding that for the first time substantiated the unappreciated potential of 3D culture models in drug discovery (Weaver et al. 1997). As the best methods for 3D cluster formation are being explored in depth for a suspension Rb cell line Y79, perhaps it would be beneficial to assess feasibility of different 3D culturing techniques for adherent Rb cell lines, such as the recently described adherent Rb cell lines SNUOT-RB1 and SNUOT-RB4 (Kim et al. 2008). Subsequently, better methodologies and specialized automated equipment are required to handle 3D clusters so as to protect them from breakage and to maintain their integrity. This would promote the feasibility of testing 3D models using HTS platforms instead of the current low-throughput strategies. Consequentially, FDA-approved drugs and NPs could be rapidly tested against these models, which would also serve as



Fig. 7.2 Drug discovery for retinoblastoma—a broad perspective. The illustration shows key steps in the drug discovery pipeline for retinoblastoma. The process is initiated by fishing for active compounds through HTS. The confirmed hits are re-tested in mouse and rabbit disease

model systems for toxicity and efficacy, respectively. Promising candidates would then enter clinical trials, with a potential to develop novel therapy and to treat young patients afflicted with retinoblastoma

in vivo representatives of the detached vitreous seeds. As a unique advantage, inhibitors targeting hypoxia, angiogenesis, and cell adhesion can also be tested for an anti-tumor effect in such systems. Since the 3D-fuelled research would best identify with the tumor's local microenvironment, the probability of picking *true hitters* in a screen would be much higher translating into perhaps a much lower risk of their failure in clinical trials, thus saving valuable time and money.

For Rb, the challenge for long had been to deliver drugs at a well-tolerated systemic dose that would also result in an adequate concentration in the eye. Advances in localized delivery modalities have fortunately allowed administration of high doses of chemotherapy while circumventing the general blood circulation; intra-arterial fusion of digoxin led to modest tumor regression in a patient, while subconjunctival delivery of nutlin-3a produced encouraging outcomes in orthotopic xenografts (Brennan et al. 2011; Patel et al. 2011). There is room for improvement of these methods so as to maintain a sustained and localized dose of the chemotherapeutic agents in the eye, such as by periocular implants or eye drops. Such developments in drug delivery would also play an instrumental role in repurposing opportunities for shelved drugs, previously neglected due to their high systemic toxicities.

In conclusion, the future of discovery and development of drugs for Rb relies on multidisciplinary, multi-faceted and innovative approaches. Perhaps, along the way Rb research might also stumble upon serendipitous breakthroughs, which have led to striking discoveries in the world of medicine for centuries.

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In developed countries, retinoblastoma is diagnosed during its intraocular state and most clinical management decisions are centered on the likelihood of eye preservation (Abramson 2014). In those disease states, distant dissemination is very unlikely and conservative therapies may be undertaken without significantly increasing their risk for extraocular relapse. The tumor may be confined to the eye for a variable period of time, but if not treated or if progressing or relapsing after conservative therapy, extraretinal extension to the outer layers of the eye occurs increasing the risk of dissemination. It is accepted that the tumor should first extend from the retina to other ocular tissues for the tumor to disseminate outside the eye; however, metastatic disease from eyes without extraretinal invasion is theoretically possible and it has been clinically recognized (Rubin et al. 1985).

Retinoblastoma may invade the optic nerve, and through it, it may reach the CNS. It may also invade the uvea, which by its vascular nature

would open its way to the systemic circulation increasing the chance of systemic metastasis. After traversing the whole width of the choroid, it can invade the sclera and reach the orbit. In very advanced cases, it may penetrate the conjunctiva causing overt loco-regional dissemination to the orbit and lymph nodes. In the clinic, advanced disease states can be easily recognized by physical examination and imaging studies. Bone marrow examination usually shows typical clumps of tumor cells in microscopical examination, but it usually occurs late in the disease course, usually concomitantly to overt orbital dissemination. Invasion to the optic nerve causes changes in its appearance on MRI: initially seen as enhanced contrast uptake or heterogeneous features, but ultimately leads to enlargement, which is more easily identified (Radhakrishnan et al. 2013). Scleral involvement eventually would lead to an orbital soft tissue mass, which can also be identified by imaging with high degree of certainty.

Patients with massive extraocular spread are relatively easy to identify by imaging studies and by tissue sampling of metastatic sites. However, the major challenge is to identify those patients who still have presumed intraocular disease clinically and by imaging, where the tumor clinically still looks localized but harbors a higher risk of dissemination. Currently available imaging studies have less than optimal sensitivity and specificity for this (de Graaf et al. 2012). Therefore, patients with the usual pathology risk factors for

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extraocular relapse, such as microscopical invasion to the postlaminar optic nerve or massive extension to the choroid or sclera, are difficult to identify preoperatively. Without histopathology, it is often difficult to clinically predict which cases are at higher risk for minimal dissemination.

At diagnosis, some presenting clinical features such as glaucoma, buphthalmia, anterior chamber invasion, older age at diagnosis have been correlated [in series from developing countries where the prevalence of high risk features is higher (Kashyap et al. 2012b)], with the occurrence of higher risk factors on pathological examination (Kashyap et al. 2012a; Chantada et al. 2009a). The association of these clinical features with pathology risk factors was not so evident in smaller series from developed countries, probably because of their lower statistical power and a significantly lower prevalence of pathology risk factors (Wilson et al. 2011). In an extensive evaluation of 519 primarily enucleated eyes from a major referral center, it was found that the degree of disease extension in the eye correlated with the chance of harboring pathology risk factors after enucleation (Kaliki et al. 2013). In that series, pathology risk factors were found in 17 % of group D eyes and 24 % of group E eyes with presenting factors. However, systemic metastases occurred in 10 % of group E eyes with high risk pathology features and in 0 % of group D eyes (Kaliki et al. 2013). It is necessary to note that the definition of group D and E eyes varies slightly, but potentially meaningfully between the two versions of current classification systems. In the original classification reported by Linn Murphree, eyes of group E included those with one or more of the following features: irreversible neovascular glaucoma, massive intraocular hemorrhage, aseptic orbital cellulitis, tumor anterior to anterior vitreous face, tumor touching the lens, diffuse infiltrating retinoblastoma, phthisis, or prephthisis (Linn Murphree 2005). On the contrary, those with more than 50 % of the retina compromised by tumor were added to this group in the Philadelphia system (which was used in that report), so some patients considered as group E in that series may not have been considered group E for other groups

(Shields et al. 2006). A study of minimally disseminated disease in retinoblastoma showed that children with a history of glaucoma were at higher risk of harboring minimally disseminated disease (Laurent et al. 2013). With the advent of intra-arterial chemotherapy and its improved results in children with advanced eyes, this distinction of eyes with higher risk of occult systemic dissemination became critical (Levin et al. 2011). Some authors expressed their concerns that treatment of advanced eyes potentially harboring pathology risk factors may be put in danger of extraocular relapse if not enucleated and adjuvant therapy given according to the pathology (Levin et al. 2011).

Hence, despite advances in imaging and in the evaluation of minimally disseminated disease, current medical practice is based on factors obtained by pathological examination of the enucleated eye. It is generally accepted that eyes with higher risk of harboring microscopical invasion to extraretinal tissues should be promptly enucleated with the aim of removing the tumor mass and thereby reduce its risk of metastatic spread (Dimaras et al. 2012). However, even in these higher risk children, only a relatively small proportion would ultimately develop metastatic spread. Theoretically, in children whose extent of disease evaluation fails to show disease dissemination by standard techniques but later develop metastatic dissemination should have undetected minimally disseminated disease at some point of their disease state. Minimal dissemination to the systemic circulation would theoretically give rise to bone marrow and bone metastasis and invasion through the optic nerve would, at some point, seed the cerebrospinal fluid (CSF) causing disease dissemination in that compartment. As reported for other pediatric malignancies, it is also theoretically possible that the CSF may also be seeded from systemic dissemination (Biojone et al. 2012). Detection of minimally disseminated disease would therefore be a useful tool to determine the risk of extraocular relapse in these patients. Molecular techniques capable of detecting rare metastatic cells with a high sensitivity and specificity have been developed for other pediatric solid tumors (Beiske et al. 2009) and to

a more limited extent for retinoblastoma. A widely available and reliable molecular marker for studying minimal dissemination is yet to be described. Reported markers for minimal disease include the Rb1 mutation which can be used in children with non-germ line mutations (Dimaras et al. 2010), GD2 synthase mRNA which was used for the evaluation of minimally disseminated disease in the CSF (Laurent et al. 2013), and more recently, CRX, mRNA, a cone-derived growth factor proved to be a sensible and specific marker in the CSF and the bone marrow (Torbidoni et al. 2015). The molecular mechanisms of extraocular disease relapse, though inferred from the clinics, have not been completely elucidated for retinoblastoma. The accurate detection of minimally disseminated disease faces many limitations. As seen in other tumors, minimal dissemination may occur intermittently making it difficult to detect these minimally disseminated cells in low concentrations in highly cellular tissues like the bone marrow. On the other hand, detecting tumor cells in metastatic sites, such as the bone marrow, does not always indicate the occurrence of a subsequent inevitable metastatic relapse: for instance, these dormant metastatic malignant cells have been documented in other tumors (Edry Botzer et al. 2011). Locoregional dissemination as a starting point to metastatic seeding would be even more difficult to detect with the current technologies since minimal seeding of the orbit is not always possible to detect by current imaging or laboratory studies.

Even after accepting that pathology examination of the enucleated eye is the most useful predictor of risk of extraocular relapse, there are also many limitations to this practice. Until recently, a consensus on the processing of enucleated eyes with retinoblastoma was not available and many centers still report different degrees of invasion to critical ocular layers. In the last decade, an international consensus provided uniform guidelines for these factors (Sastre et al. 2009). Prospective randomized studies were never performed and only a few prospective studies were published, usually including a relatively low number of patients with high risk features (Aerts et al. 2013;

Chantada et al. 2004a, 2010; Sullivan et al. 2014). It is widely accepted that invasion to the choroid (when massive), the postlaminar optic nerve, and the sclera all constitute high risk factors for extraocular relapse (Eagle 2009; Sastre et al. 2009; Kashyap et al. 2012b). However, their relative weight and the potential implications of associations of these factors as predictors of extraocular relapse are controversial. It is common that eyes with high tumor burden present with more than one concomitant risk factor, so it is difficult to weigh the impact of each isolated factor as predictor of relapse. Despite that, these three risk factors stand alone as significant predictors for extraocular relapse. However, some groups consider the association of other pathology factors that alone would not qualify as a higher risk factor: for example, focal choroidal invasion and prelaminar optic nerve involvement posing an additive risk that warrants adjuvant therapy (Kaliki et al. 2013). Other putative risk factors such as the invasion to the anterior segment have been considered by some groups and not by others (Kaliki et al. 2011).

There is agreement that patients in whom initial enucleation fails to obtain a complete resection of the tumor are at higher risk for metastatic disease. Enucleation of an affected eye with retinoblastoma needs to be undertaken by an experienced ophthalmologist after a thorough clinical and imaging evaluation. Special care should be undertaken by the ophthalmologist to obtain a long optic nerve stump in order to be able to remove all tumors that extend into the optic nerve. However, in certain situations, tumor residue may be left behind in the resection margin. With current imaging studies, significant optic nerve invasion is usually detectable preoperatively and neoadjuvant chemotherapy is commonly used to treat these children. Occasionally, a long optic nerve stump is not possible to be obtained because of technical limitations to the surgeon or because of severe buphthalmia that makes it difficult to access the posterior orbit. In these swollen eyes, the risk of eye perforation during enucleation increases and leads to a high risk of seeding the orbit with tumor. Less frequently, the tumor extends through the sclera to

the orbit, so a complete resection is not achievable despite complete resection of tumor in the optic nerve. In all these instances, adjuvant therapy is mandatory to treat these residual tumor cells.

8.1 Pathology Risk Factors for Extraocular Relapse in Enucleated Eyes with Retinoblastoma

8.1.1 Choroidal Invasion

Choroidal invasion has long been recognized as a risk factor for extraocular relapse (Shields et al. 1993). For estimating the risk of extraocular relapse, it is necessary to discriminate between different degrees of invasion to the choroid (Fig. 8.1). A current consensus definition indicates that massive choroidal invasion is present when it is greater than 3 mm or when it invades its whole width of the choroid (Sastre et al. 2009). Focal choroidal invasion includes all the remaining categories. For an accurate estimation of the degree of choroidal invasion, routine histopathological examination of the enucleated eye should follow a thorough study of at least four sections, including not only the pupil-optic nerve section, but also the calottes (remainder of ocular tissue after obtaining the pupil-optic nerve section) (Sastre et al. 2009). If only a few sections of the enucleated eye are evaluated, significant degrees

of choroidal invasion may be missed. Since some groups did not use adjuvant therapy for the treatment of eyes with choroidal invasion, its natural history is known with relatively high accuracy. In a recent review of 169 children with isolated choroidal invasion (35 with massive invasion) treated with no adjuvant therapy in three subsequent prospective studies, the probability of 5-year event-free survival (pEFS) was 98.1 % and the probability of overall survival (pOS) was 98.7 % (Bosaleh et al. 2012). Children with massive invasion had a significantly lower pEFS (94.2 %) compared to those with focal invasion (99.2 %) in that series, all events included extraocular relapse; however, since a sizable proportion of children with extraocular relapse were salvaged with intensive therapy, there was no significant difference in survival (98.7 % vs. 99.2 %, respectively, $p=0.29$) (Bosaleh et al. 2012). Other groups, however, routinely recommend adjuvant therapy for children with these features. So it is likely that the estimated 6 % risk of extraocular relapse of children presenting with massive choroidal invasion and no postlaminar optic nerve or scleral invasion would be further reduced by adjuvant chemotherapy (Kaliki et al. 2011; Sullivan et al. 2014). Conversely, the remaining 94 % would receive unnecessary chemotherapy since they would have been cured with enucleation alone. In addition, any difference in survival would be difficult to prove since it is already in the range of 98–99 % when these patients are

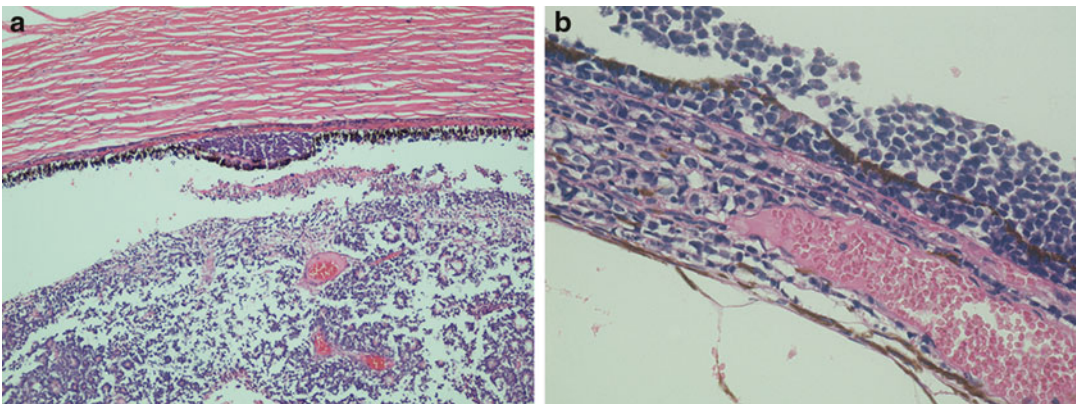


Fig. 8.1 Choroidal invasion. (a) Focal choroidal invasion (hematoxylin-eosin, original magnification $\times 2$). (b) Massive choroidal invasion (hematoxylin-eosin, original magnification $\times 4$)

treated with enucleation only and aggressive therapy of extraocular relapse. So, many groups do not recommend adjuvant therapy for isolated choroidal invasion or when it is combined with prelaminar optic nerve involvement, but others treat them with adjuvant therapy with moderately intense chemotherapy (Kaliki et al. 2011). Therefore, the identification of a subgroup of children with isolated choroidal invasion who would unequivocally benefit from adjuvant therapy is difficult. Therefore, this decision should include other factors including the family preference, the availability of effective salvage therapy, adequate follow-up, and reliability of the pathological examination as well as supportive care during the course of chemotherapy. Thus, refraining from adjuvant therapy for all children with isolated choroidal invasion and treating those few who relapse would be a reasonable alternative (Chantada et al. 2004b). However, the treatment of extraocular relapse is very intense leading to severe long-term sequelae in those who survive it. High dose chemotherapy, including autologous stem cell rescue and radiotherapy, is needed for retrieving these patients. The strategy of not using adjuvant therapy for isolated choroidal invasion is based on the assumption that an expert ocular pathologist reviewed the case to minimize the possibility of not recognizing other higher risk features such as scleral or retrolaminar invasion. In cases where such an expert pathological

examination is not available, the risk of extraocular relapse may be relatively higher and routine use of adjuvant chemotherapy may be safer (Chantada et al. 2013a). In any case, the decision to use adjuvant chemotherapy aiming to reduce a 6 % risk of extraocular relapse should be balanced with the local toxic mortality rate of chemotherapy regimens, which even for moderate risk regimens is not zero, especially in many developing countries with limited resources.

8.1.2 Scleral Invasion

Massive invasion of the whole width of the choroid is a prerequisite for scleral invasion. When not present, artifactual scleral invasion usually manifested as “floating” tumor cells on the sclera due to inadequate handling of the specimen should be suspected. Occasionally the tumor gains access to the sclera via the episcleral veins or through sites of puncture, for example when intravitreal chemotherapy is used. Two grades of scleral invasion can be recognized: intra and transscleral invasion (Fig. 8.2), and both is a major risk factor for extraocular relapse. Transscleral invasion is considered similarly to incompletely resected disease, since tumor cells already extend to the orbit. The mechanisms by which scleral invasion is related to increased risk for extraocular relapse are poorly understood.

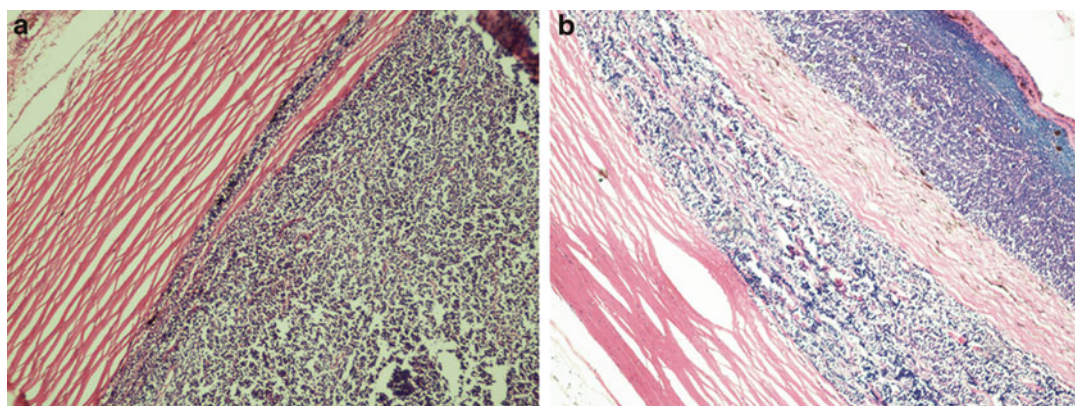


Fig. 8.2 Scleral invasion. (a) Intrasccleral invasion (hematoxylin-eosin, original magnification $\times 2$). (b) Transscleral invasion (hematoxylin-eosin, original magnification $\times 2$)

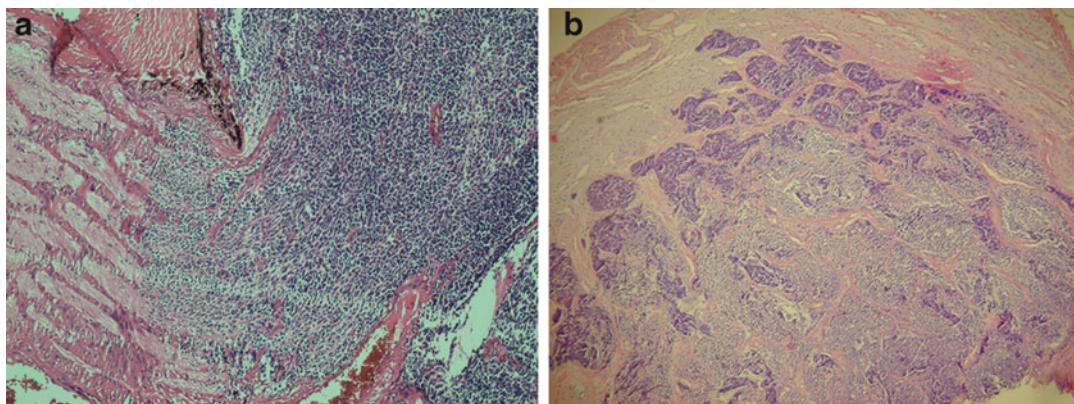


Fig. 8.3 Optic nerve invasion. (a) Post-laminar optic nerve invasion (margin negative) (hematoxylin-eosin, original magnification $\times 2$). (b) Involvement of optic nerve

in the resection margin (hematoxylin-eosin, original magnification $\times 2$)

As opposed to the choroid or the optic nerve, which are either highly vascular or are in direct proximity to the CNS, intrinsically, the fibrous structure of the sclera lacks those factors. When tumor cells invade the sclera, by proximity, they would be more prone to seed the orbit leading to a higher risk of orbital relapse. However, most reported cases of extraocular relapse in children with scleral invasion included the CSF and not the orbit (Cuenca et al. 2009). However, all these cases occurred after treatment with adjuvant chemotherapy, which may affect the natural history of the tumor by possibly preventing orbital relapse but at the same time creating a sanctuary site in the CSF. On the other hand, it is conceivable that part of the increased risk of scleral invasion is explained by the concomitant massive choroidal invasion that inevitably occurs.

There is very limited data about the treatment of patients with microscopic scleral invasion in the literature. The use of intensive chemotherapy regimens adding alkylating agents to the regular platinum-based schemas may be more effective (Antoneli et al. 2007). With the rationale of avoiding a potential orbital relapse, some authors recommend adjuvant orbital radiotherapy (Kaliki et al. 2013). However, its application is controversial since with the use of intensive regimens, the risk of orbital relapse is relatively low and most cases with extraocular relapse in such patients involve the CNS.

8.1.3 Optic Nerve Invasion

In order to assess optic nerve invasion, it is important to recognize the invasion of tumor cells through the lamina cribrosa, which is considered an anatomical boundary of the eye with the orbit. Invasion to the prelaminar optic nerve is not related significantly with a higher risk for extraocular relapse (Chantada et al. 2013b; Kaliki et al. 2013). Conversely, when the tumor extends beyond the lamina cribrosa (technically outside the eye into the orbit), the risk of extraocular relapse is higher (Fig. 8.3) (Shields et al. 1994). Since most of these patients receive adjuvant chemotherapy, their actual risk for metastatic relapse is not known. In addition, it is not uncommon that eyes presenting with postlaminar optic nerve invasion show concomitant invasion to the choroid or other pathology risk factors, making it difficult to estimate the weight of its isolated involvement to predict extraocular relapse (Shields et al. 1994). The mechanism by which invasion to the retrolaminar optic nerve would cause an extraocular relapse remains unknown since theoretically all the tumor manifestations are removed during enucleation. Since concomitant choroidal invasion is common, malignant cells may also escape the eye through the choroidal vasculature. However, isolated CNS relapse, usually involving the CSF, may also occur in these patients, so other mechanisms should be

possible (Chantada et al. 2007a). It is likely that at some point, the CSF is seeded by tumor cells from the optic nerve. As shown in a limited series, by increasing the intraocular pressure and possibly reversing the CSF flow (Killer et al. 2008), glaucoma may favor tumor seeding of the CSF from the perineural sub-arachnoid space (Laurent et al. 2013). Interestingly, historical series from the pre-CT scan era, when these patients did not receive adjuvant chemotherapy, show a 42 % survival rate (Magrann et al. 1989). So, at least a 42 % of the patients would be cured with enucleation alone. A more recent report of children with postlaminar optic nerve invasion and no concomitant massive choroidal invasion treated only with enucleation showed an increased relapse rate when the invasion through the optic nerve was less than 1 mm from the lamina cribrosa (Chantada et al. 2007a). Interestingly, relapsing patients in that series had a systemic relapse not involving the CSF (Chantada et al. 2007a). Adjuvant therapy may also influence the kinetics of relapse in these patients by creating a sanctuary site in the CSF, modifying the natural history of the tumor.

Recent reports using tailored adjuvant therapy show that more than 90 % of these children can be cured (Chantada et al. 2007a; Kaliki et al. 2011; Aerts et al. 2013). Both prospective and retrospective studies show that adjuvant chemotherapy effectively prevents relapse in more than 90 % of these patients; however, the numbers are still too small to identify patients at a lower risk who could be spared from the toxicity of chemotherapy or those with a higher risk that may benefit from a more intensive approach.

Some groups suggest that the risk of extraocular relapse may be higher when concomitant invasion to the choroid or sclera is present (Chantada et al. 2004b). In a report on 61 patients with postlaminar optic nerve disease treated on three consecutive treatment protocols, a 94 % 5-year overall survival rate with tailored therapy was achieved (Chantada et al. 2007a). That study revealed three groups of patients that were at higher risk of relapse and in whom adjuvant chemotherapy was recommended: (1) patients who had postlaminar optic nerve involvement together

with full choroidal invasion and/or scleral invasion, (2). those with greater than 1 mm of invasion through the optic nerve, or (3) greater than 30 % of extension through the optic nerve. In that series, children without other high-risk features (30 % of the study population) were not given adjuvant therapy and their relapse rate was less than 10 % (Chantada et al. 2007a).

8.1.4 Incompletely Resected Tumor at the Optic Nerve

The occurrence of a tumor residue in the optic nerve stump may be the consequence of several factors. It is usually related to massive involvement of the optic nerve, but on occasions it occurs because the ophthalmologist fails to obtain an adequate optic nerve stump. Hence, enucleation should be done by an experienced ophthalmologist to obtain a long optic nerve stump (of at least 10 mm) in order to avoid leaving tumor residue. In other instances, enucleation may be difficult in children with massive buphthalmia and high intraocular pressure (Bellaton et al. 2003). In these cases, access to the posterior orbit, necessary to obtain a long optic nerve stump, may be difficult, and in some rare occasions, eye perforation may occur. Massive optic nerve invasion is detectable by MRI but more subtle changes may go unnoticed (de Graaf et al. 2012). In cases with severe buphthalmia, especially in those in whom retrolaminar optic nerve invasion is evident in the MRI, neoadjuvant chemotherapy, followed by secondary enucleation, may be used (Bellaton et al. 2003). This approach would result in reduction of the eye volume after 2 or 3 cycles, making it easier to perform enucleation with adequate access to the posterior orbit. Some authors believe this will reduce the risk of an incomplete resection (Bellaton et al. 2003; Chantada et al. 2014).

However, in these cases, chemotherapy causes distortion of the pathological features of the enucleated eye and risk estimation based on the presence of pathology risk factors could be altered. It is not uncommon to see massive tumor response after neoadjuvant chemotherapy. Hence, most authors continue adjuvant chemotherapy after

enucleation regardless of the pathological examination.

Unfortunately, on occasions, significant optic nerve is not detected preoperatively and the patient undergoes initial enucleation and tumor is detected in the optic nerve resection margin on the pathological examination of the enucleated eye. A careful pathological examination of the optic nerve stump is mandatory for an accurate estimation of tumor invasion. A transversal section marking with China ink at the resection margin should be obtained as the first step, before opening the eyeball (Fig. 8.3). It is also important to assess tumor extension to the subarchnoidal space which, if detected, should be considered as incomplete resection because of its potential for dissemination to the CSF. Evaluation of the optic nerve margin in eyes that underwent neoadjuvant chemotherapy is complicated by the presence of tumor necrosis and gliosis, making it difficult to estimate the tumor viability in these specimens.

Despite up to 70 % of children with tumor at the resection margin of the optic nerve being cured (Chantada et al. 2009b; Stannard et al. 1979), they need multimodal, intensive therapy including orbital radiotherapy and chemotherapy. Data regarding treatment of these patients is scarce and no randomized trial has ever been reported. The use of orbital radiotherapy is controversial and some current intensive studies do not include it for the treatment of these patients. In these cases, the radiation field should include the orbit and the chiasm. The rationale for using orbital radiotherapy in these cases is aimed at optimizing local tumor control in the optic nerve, with the ultimate goal of reducing the occurrence of tumor seeding to the CSF, which is the most common event in these patients. Alternatives to treating the optic nerve and the chiasm include the use of localized radiotherapy technique using Iodine 125 seeds, which, by sparing the orbit, may be associated to less late effects (Stannard et al. 2002). Others have used intrathecal chemotherapy including methotrexate, cytarabine, and dexamethasone, which have relatively low activity against retinoblastoma (Chantada et al. 2009b; Antoneli et al. 2003). More recently, topotecan, an active drug for retinoblastoma, became an option for intrathecal use but there is limited

information about its efficacy in retinoblastoma (Potter et al. 2012). Minimally disseminated disease studies account for a higher risk of CSF dissemination in children with massive optic nerve involvement, including those with incomplete resection (Laurent et al. 2013). Hence, despite most current regimens omitting intrathecal chemotherapy and relying on intensive intravenous for the treatment of minimally disseminated disease in the CSF, intrathecal or intraventricular therapy with active agents may give some benefit.

These children are highly susceptible to severe long-term sequelae. Severe orbital hypoplasia and endocrinological dysfunction leading to growth hormone deficiency or hypopituitarism occurs in more than 80 % of surviving patients (Chantada et al. 2009b; Friedman et al. 2013).

8.2 High Risk Pathology Factors After Secondary Enucleation for Failure of Conservative Therapy

Eyes undergoing enucleation after failure of conservative therapy may have invasion to the choroid, postlaminar optic nerve, or the sclera. Their significance as predictors for extraocular relapse, compared to primarily enucleated eyes, is not known. However, most authors would use the same guidelines for their treatment. Scleral invasion was found as a predictor for extraocular relapse in this population in a large multicentric study (Chantada et al. 2007b).

8.3 Chemotherapy Regimens for Adjuvant Therapy

Carboplatin-based regimens are the preferred chemotherapy schemas used for adjuvant therapy for the treatment of pathology risk factors (Kaliki et al. 2013). Some groups add alkylating agents to these regimens, and less frequently, anthracyclines are used by some groups (Aerts et al. 2013; Chantada et al. 2010; Sullivan et al. 2014). Most groups use moderate dose chemotherapy regimens (Table 8.1) for a number of 4–6

Table 8.1 Commonly used chemotherapy regimens for adjuvant therapy

Author	Chemotherapy regimen and number of cycles	Indications	Comments
Aerts et al. 2013	Etoposide 100 mg/m ² (3.3 mg/kg ^a) days 1–5 on cycles 1 and 3.	Massive choroidal involvement and/or intra- and retrolaminar involvement and/or involvement of the anterior segment	Cycles repeated every 21 days
	Carboplatin 160 mg/m ² (15.3 mg/kg ^a) days 1–5 on cycles 1 and 3.		
	Vincristine 1.5 mg/m ² (0.05 mg/kg ^a) days 1 and 5 on cycles 2 and 4		
	Cyclophosphamide 300 mg/m ² (10 mg/kg ^a) days 1–5 on cycles 2 and 4		
Aerts et al. 2013	Etoposide 100 mg/m ² days 1–5, cycles 1, 3 and 5	Invasion of the surgical margin of the optic nerve and/or microscopic extrascleral involvement	Support with colony-stimulating growth factors needed.
	Carboplatin 160 mg/m ² days 1–5, cycles 1, 3, and 5		
	Thiotepa (intrathecal) 15 mg, day 1, cycles 1, 3, and 5		
	Vincristine 1.5 mg/m ² , day 1 on cycles 2, 4, and 6		
	Cyclophosphamide 1,000 mg/m ² days 1–3 on cycles 2, 4, and 6		
	Etoposide 350 mg/m ² days 1–5		
	Consolidation regimen		
	Carboplatin 350 mg/m ² days 1–5		
	Cyclophosphamide 1,600 mg/m ² days 2–5		
Kaliki et al. 2011	Carboplatin 18.6 mg/kg day 0, Etoposide phosphate 5 mg/kg/days 0 and 1, Vincristine 0.05 mg/kg/day 0 Median of 6 cycles	Anterior segment, posterior uveal invasion equal or greater than 3 mm, postlaminar optic nerve invasion or any posterior uveal invasion plus any optic nerve invasion	One patient with extrascleral invasion received orbital radiotherapy
Chantada et al. 2010	Etoposide 100 mg/m ² (3.3 mg/kg ^b) days 1–3 on cycles 1, 3,5 and 7.	Scleral invasion, post laminar optic nerve invasion alone if greater than 1 mm or in combination with massive choroidal invasion. Tumor at the resection margin of the optic nerve	Support with colony-stimulating growth factors needed. Patients with tumor at the resection margin of the optic nerve received orbital radiotherapy
	Carboplatin 500 mg/m ² (18 mg/kg ^b) days 1 and 2 on cycles 1, 3, 5 and 7.		
	Vincristine 1.5 mg/m ² (0.05 mg/kg ^b) day 1 on cycles 2, 4, 6 and 8		
	Cyclophosphamide 65 mg/kg, day 1 on cycles 2,4,6 and 8		
	Idarubicin 10 mg/m ² day 1 on cycles 2,4,6 and 8		
Sullivan et al. 2014	Vincristine 1.5 mg/m ² (0.05 mg/kg ^c) day 1, Cyclophosphamide 1200 mg (40 mg/kg ^c), day 1 and Doxorubicin 45 mg/m ² (1.5 mg/kg ^c) day 1. Total of 4 cycles each every 21 days	Anterior segment, posterior uveal invasion equal or greater than 3 mm, postlaminar optic nerve invasion	Patients were given GCSF support
	The same regimen alternating with Carboplatin dose targeted to an AUC of 6.5 mg/mL/min on day 1, Etoposide 100 mg/m ² (3.3 mg/kg ^c) days 1–3	Tumor at the resection margin of the optic nerve and/or scleral invasion	At the discretion of the treating group, orbital radiotherapy (45 Gy) could be given to these patients

^aDose for children younger than 1 year or weighing less than 10 kg^bDose for children weighing less than 10 kg^cDose for children younger than 12 months

cycles (Aerts et al. 2013; Kaliki et al. 2011). Other groups, after reporting cases of extraocular relapse in prospective studies using moderate dose regimens, use higher dose schemas for higher risk cases (Chantada et al. 2010). There is no study comparing these regimens. For deciding the regimen to use, it is important to consider their toxicity, especially in those subgroups with lower probability of extraocular relapse (such as those with isolated choroidal invasion), since toxic mortality may overweigh the benefit of a more intensive regimen. As in other pediatric malignancies, adjuvant chemotherapy may not prevent all instances of relapse. In most cases, moderate dose adjuvant regimens may prevent extraocular relapse in more than 90 % of the patients; however, the reported studies include a relatively low proportion of higher risk patients. Patients with a higher tumor burden may benefit from a higher intensity regimen. The adequate identification of such higher risk patients is a challenge for a most effective tailoring of adjuvant therapy.

Compliance with Ethical Requirements Guillermo L. Chantada and Maria T.G. de Davila declare that they have no conflict of interest.

No human or animal studies were carried out by the authors for this article.

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9.1 Introduction

Retinoblastoma is the most common primary pediatric ocular neoplasm with long-term survival mainly dependent upon the stage of disease involvement. In the United States and other high-income nations, the vast majority of patients present with intraocular disease and thereby have survival rates that exceed 95 %. In these countries, the risk of metastatic spread is markedly low with extraocular disease occurring in less than 5 % of patients. In contrast, extraocular retinoblastoma remains a common manifestation of disease in lower-income countries, frequently associated with poor prognoses. This chapter will focus on the diagnosis and management of extraocular retinoblastoma, including trilateral disease.

9.2 Mechanisms of Spread

In the absence of prompt diagnosis and appropriate treatment, intraocular retinoblastoma may metastasize outside of the globe via several different mechanisms. Direct invasion through the optic nerve and to the brain remains the commonest form of exten-

sion. From the optic nerve, malignant cells migrate directly towards the optic chiasm or invade through the pia mater and into the subarachnoid space with potential for leptomeningeal dissemination. In addition, the intraocular tumor may also infiltrate the adjacent choroid and sclera, growing into the orbital bone and soft tissues. Hematogenous dissemination can occur with distant metastases to the bone, bone marrow, and liver. While rare, lymphatic spread may produce regional nodal disease; however, within the eye, the only limited lymphatic drainage remains through channels in the conjunctiva. Figure 9.1 demonstrates hematogenous metastasis of malignant retinoblastoma cells.

9.3 Clinical Presentation

As stated previously, extraocular retinoblastoma remains a frequent initial clinical presentation of disease in lower-income countries, most often present at diagnosis. In contrast, patients in higher-income countries generally present with extraocular disease at the time of relapse. While intraocular retinoblastoma often presents with leukocoria of one or both eyes (white pupillary reflex instead of a normal red reflex), the diagnosis of extraocular disease often occurs after buphthalmos (enlarged eye) or gross proptotic disease invasion becomes clinically evident.

Furthermore, the manifestations of extraocular pathology are dependent upon the sites of disease

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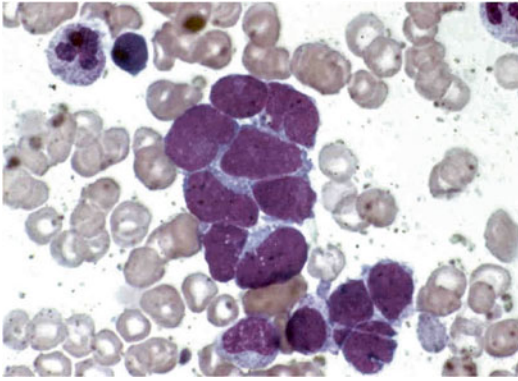


Fig. 9.1 Photomicrograph demonstrating hematogenous metastasis of malignant retinoblastoma cells shown on a bone marrow aspirate

involvement. In cases where extraocular retinoblastoma develops following enucleation of an affected eye, this often becomes apparent within several months following enucleation and usually occurs upon parental observation that a child's prosthesis no longer fits properly. Central nervous system (CNS) involvement may present with headache, lethargy, emesis, ataxia, or focal neurologic deficits. Bony involvement can present with localized pain or a visible mass, whereas bone marrow disease can manifest as pancytopenia, though may also be asymptomatic. Liver metastases are often discovered incidentally upon extent of disease workup.

9.4 Extent of Disease Evaluation

In order to properly ascertain the extent of disease involvement when extraocular pathology is suspected at diagnosis, a comprehensive evaluation should be performed to determine the anatomic sites involved. An appropriate workup includes a brain and orbit MRI (with and without contrast) and a lumbar puncture to evaluate cerebrospinal fluid composition and cytology. If CNS disease is present—a spine MRI with and without contrast should also be performed. Bony disease can be evaluated with a bone scan while liver metastases can be evaluated with an abdominal CT scan or MRI. Finally, the presence of bone marrow involvement should be ruled out with bone marrow aspirates and biopsies when distant metastases are suspected.

9.5 Staging

In 2006, a new international retinoblastoma staging system was proposed as there had previously been no widely accepted classification scheme for those patients with significant intra-ocular disease warranting enucleation and those with extraocular pathology (Chantada et al. 2006). This consensus document remains vital to standardize diagnostic procedures and enable oncologists to determine the efficacy of their therapies. With a uniformly recognized staging system, one becomes able to identify those histopathological features that put patients at risk for extraocular recurrence and allows for the identification of those patients who require adjuvant chemotherapy. In addition, this international staging system is used in the currently open Children's Oncology Group (COG) protocol, ARET0321, *A Trial of Intensive Multi-Modality Therapy for Extra-Ocular Retinoblastoma*. Table 9.1 shows the International Staging System for Extraocular Retinoblastoma.

9.6 Treatment

9.6.1 Regional Extra-ocular Retinoblastoma: Stage II or III Disease

Historically, patients who presented with disease isolated to the orbit (i.e., positive optic nerve margins) had poor outcomes when treated with surgery with or without radiation therapy. However, their prognosis improved markedly when conventional chemotherapy was added to their treatment regiment with 1-year event-free survival rates of 40 % (Doz et al. 1994). As noted above, stage II patients are those individuals with microscopic residual disease noted after enucleation of an affected eye. In contrast, stage III patients have imaging studies consistent with orbital extension (by invasion through the sclera or optic nerve enlargement) or preauricular/cervical lymphadenopathy. Given the location of their disease, stage III patients are usually considered surgically unresectable upfront and require neoadjuvant chemotherapy. Often, their

Table 9.1 International staging system for extraocular retinoblastoma

Stage 0:	Patients treated conservatively
Stage I:	Eye enucleated, completely resected histologically
Stage II:	Eye enucleated, microscopic residual tumor (i.e., optic nerve involvement to the transection line, trans-scleral invasion)
Stage III:	Regional extension
	A. Overt orbital disease
	B. Preauricular or cervical lymph node extension
Stage IV:	Metastatic disease
	A. Disseminated hematogenous metastasis (without CNS involvement)
	1. Single lesion
	2. Multiple lesions
	B. CNS extension (with or without any other site of regional or metastatic disease)
	1. Prechiasmatic lesion
	2. CNS mass
	3. Leptomeningeal and CSF disease

orbital disease responds well to chemotherapy and shrinks to a point that makes limited surgery an option after 2–4 cycles; however, additional surgery is generally unnecessary. Additionally, in our experience, an aggressive surgical approach via orbital exenteration is never warranted.

The Garrahan group in Argentina treated 15 patients with orbital or pre-auricular lymph node disease on two consecutive protocols (Chantada et al. 2003). Chemotherapeutic agents on local protocol 87 included vincristine (0.05 mg/kg), doxorubicin (0.67 mg/kg/day $\times$ 3 days), and cyclophosphamide (20–40 mg/kg). Thereafter, protocol 94 involved cycles of vincristine (0.05 mg/kg), idarubicin (10 mg/m²), and cyclophosphamide (65 mg/kg) and subsequent cycles of carboplatin (18.7 mg/kg/day or 560 mg/m²/day $\times$ 2 days) and etoposide (3.3 mg/kg/day or 100 mg/m²/day $\times$ 3 days). Patients were then treated with external-beam radiation therapy using 4500 cGy given up to the optic chiasm in patients with orbital disease and directly to involved pre-auricular nodes to those with lymphadenopathy. The group was able to achieve 5-year event-free survival rates of 84 %. A later combined report from the Argentinian and New York groups published the results of 12 patients with positive optic nerve margins who were treated with the regimens noted previously in addition to

orbital external-beam radiation therapy (4000–4500 cGy). All patients in this cohort remained event-free survivors (Chantada et al. 2004).

Investigators in Brazil published similar results to the Argentinian and New York groups described above. They evaluated patients treated on two consecutive protocols and treated from 1987 to 2000. Their regimens included cycles of vincristine (0.05 mg/kg), doxorubicin (2 mg/kg), and cyclophosphamide (30 mg/kg) alternating with cycles of cisplatin (90 mg/m²) and teniposide (100 mg/m²) in patients treated from 1987 to 1991. From 1992 to 2000, they used cycles of ifosfamide (1800 mg/m²/day $\times$ 5 days) and etoposide (100 mg/m²/day $\times$ 5 days) alternating with cycles of cisplatin (90 mg/m²) and teniposide (100 mg/m²). They then administered 4000–5000 cGy to the orbit via external-beam radiation therapy and also utilized triple intrathecal chemotherapy. This multi-modal treatment regimen produced long-term event-free survival in 20 out of 32 patients (63 %) with orbital disease and in 22 out of 29 patients (76 %) with positive optic nerve margins (Antoneli et al. 2003).

Thus, recent publications from various international groups have validated that patients with stage II or III disease can be cured with conventional, systemic chemotherapy combined with external-beam radiation therapy.

9.6.2 Stage IVa Disease

In contrast to patients with stage II and III disease who may be cured using conventional dose chemotherapy in combination with external-beam radiation therapy to affected sites, those individuals with stage IVa or IVb metastatic retinoblastoma suffer dismal outcomes when treated with the same treatment approach (Schvartzman et al. 1996). The Argentinian group described above who used vincristine, doxorubicin, cyclophosphamide, idarubicin, carboplatin, and etoposide in standard doses found that all patients with distant metastases died ($n=26$) (Chantada et al. 2003). In a similar fashion, the Brazilian investigators concluded that their treatment regimens described above resulted in survival of only 1 of 14 patients (7 %) with distant metastatic disease (Antoneli et al. 2003).

Despite these poor outcomes, recent evidence demonstrates that patients with metastatic retinoblastoma may be curable when their treatment regimen is intensified to involve high-dose chemotherapy followed by autologous stem cell rescue (ASCR). French investigators published the results of 25 patients with high-risk retinoblastoma treated with high-dose carboplatin, etoposide, and cyclophosphamide followed by ASCR. They reported that five out of eight patients with metastatic disease not involving the CNS were event-free survivors 11–70 months after high-dose chemotherapy. The remaining three patients suffered CNS relapses and died of disease 10–20 months following high-dose chemotherapy. Three separate patients had progressive disease during conventional induction chemotherapy and thus never received a high-dose regimen. Thus, their results show that 5 out of 11 patients (45 %) with initially stage IVa disease were event-free survivors (Namouni et al. 1997).

In 2005, Japanese investigators published a cohort of five patients with metastatic retinoblastoma treated with high-dose chemotherapy consisting of a melphalan backbone in combination with other agents such as cisplatin, cyclophosphamide, carboplatin, or thiotepa followed by ASCR. Three out of five patients were retinoblastoma-free survivors at 113, 107, and 38

months, respectively, from the time of ASCR. None of these three survivors had CNS involvement. The two patients who died of their disease had CNS involvement (Matsubara et al. 2005). In addition, other investigators in Germany (Kremens et al. 2003), St. Jude (Rodriguez-Galindo et al. 2003), and Children's Hospital Los Angeles (Jubran et al. 2004) published similar conclusions regarding the efficacy of high-dose chemotherapy followed by ASCR.

In 2010, the New York group reported results on a cohort of 15 patients with Stage IVa retinoblastoma (distant metastatic disease not involving the CNS) treated from 1993 to 2006 (Dunkel et al. 2010c). This cohort constitutes the largest published experience of intensive multimodality therapy for this subset of patients with Stage IVa, high-risk disease. In this group of patients, anatomic sites of disease included bone marrow ($n=14$), bone ($n=10$), orbit ($n=9$), and/or liver ($n=4$). Their induction chemotherapy regimens generally consisted of vincristine, cyclophosphamide, cisplatin, and etoposide. Subsequently, their high-dose regimen included carboplatin and thiotepa ($n=1$) or with etoposide ($n=5$) or topotecan ($n=7$). They noted that bone marrow disease cleared at the first post-induction chemotherapy examination in all patients. Stem cells were then harvested after a median of 3.5 cycles of chemotherapy (with a range of 3–6 cycles). Two of 15 patients suffered disease progression prior to starting high-dose chemotherapy and died. The remaining 13 patients all received high-dose chemotherapy at a median of 6 months after diagnosis of metastatic disease (with a range of 4–8 months). In follow-up analysis, ten patients remain disease free in their first remission at a median follow-up time of 103 months (with a range of 34–202 months), while three had recurrent disease (two in the CNS, one in the mandible) in 14–20 months post-diagnosis of metastatic disease. These investigators found that 67 % of patients had 5-year retinoblastoma-free survival (95 % CI: 38–85 %). Six out of the ten survivors received radiation therapy and three patients developed secondary osteosarcoma in a range of 4–14 years after their diagnosis of metastatic disease. Thus, the investigators concluded

that intensive multimodality therapies including high-dose chemotherapy followed by ASCR was associated with improved survival for patients with Stage IVa metastatic disease; however, the contributing role of external-beam radiation therapy in these remains unclear and in need of further investigation.

9.6.3 Stage IVb and Trilateral Disease

There is less data available in the published literature regarding the outcomes of patients with retinoblastoma involving the CNS (Stage IVb or trilateral disease) who are treated with high-dose chemotherapy followed by ASCR. Trilateral retinoblastoma refers to a disease entity whereby a primary intracranial midline primitive neuroectodermal tumor develops in a patient with intraocular retinoblastoma (Dunkel et al. 2010b). Approximately 5 % of children with germline *RB1* mutations are at risk of developing this high-risk disease subtype. Historically, patients with trilateral disease suffered dismal outcomes despite multi-modality treatment consisting of conventional chemotherapy, external-beam radiation therapy, and surgery.

A multi-center retrospective review described a series of 13 patients with trilateral disease who were treated with intensive therapy (high-dose chemotherapy with ASCR) (Dunkel et al. 2010b). Of the 13 patients, trilateral disease sites included pineal ($n=11$) and suprasellar ($n=2$) locations. Seven of these patients had localized (M-0) disease, whereas six of the patients had leptomeningeal dissemination (M-1+). Of this cohort, five patients had trilateral disease at the time of original diagnosis of intraocular retinoblastoma, while eight patients later developed a trilateral disease focus at a median of 35 months (range 3–60 months) post-diagnosis of intraocular retinoblastoma. Induction chemotherapy for this group usually included vincristine, cisplatin or carboplatin, cyclophosphamide, and etoposide. Stem cells were then generally harvested from peripheral blood after one or two cycles of induction chemotherapy. Subsequently, high-dose chemo-

therapy included thiotepea-based regimens ($n=7$) or melphalan and cyclophosphamide ($n=3$)-based treatments. Out of 13 patients, one died during induction from septicemia and multi-organ system dysfunction. Three patients had progressive disease prior to starting high-dose chemotherapy regimens. The remaining nine patients received high-dose chemotherapy at a median of 5 months (range 4–9 months) after diagnosis of trilateral disease. Five of these patients had event-free survival at a median of 77 months (range of 36–104 months) and never underwent external-beam radiation therapy. Of note, four out of seven patients with M-0 survived event-free in contrast to only one of six patients with M-1+ disease. Thus, the investigators concluded that high-dose chemotherapy with ASCR remains a potential curative treatment for patients with trilateral disease with improved survival associated with those patients who did not have leptomeningeal dissemination.

A recent meta-analysis conducted by investigators in the Netherlands and Finland sought to provide a systematic review of published data for patients with trilateral retinoblastoma in order to evaluate changing survival patterns (de Jong et al. 2014). Out of 90 studies, which included 174 patients with trilateral retinoblastoma, the authors found that 5-year survival in patients with pineal trilateral disease increased from 6 % (95 % CI: 2–15 %) in patients diagnosed prior to 1995 to 44 % (95 % CI: 26–61 %, $p<0.0001$) in those patients diagnosed after 1995. After 1995, patients with non-pineal trilateral retinoblastoma had 5-year survival rates of 57 % (95 % CI: 30–77 %, $p=0.035$). In their analysis, hazard ratios adjusted for presence of leptomeningeal metastases and location of trilateral disease, suggesting that both conventional and high-dose chemotherapy regimens with ASCR contributed strongly to this improvement in outcome. The absence of leptomeningeal disease was associated with improved survival (HR 2–13, 95 % CI: 0.98–4.60 %, $p=0.055$) corroborating the findings in previous retrospective reviews. They also found that while non-pineal trilateral retinoblastomas were larger than pineal tumors, both had similar outcomes since 1995.

In a similar fashion to trilateral disease, patients with metastatic retinoblastoma with CNS involvement (Stage IVb disease) also suffered poor outcomes. In 2009, a multi-center retrospective case series of eight patients with Stage IVb disease was published (Dunkel et al. 2010a). Seven of these patients had leptomeningeal disease with one patient having optic nerve infiltration with direct extension into the CNS. Three of eight patients had Stage IVb disease at the time of initial diagnosis, while the remaining five patients were diagnosed with CNS involvement at a median of 12 months (range of 3–69 months) following initial intraocular retinoblastoma diagnosis. Induction chemotherapy regimens consisted of cyclophosphamide and/or carboplatin with a topoisomerase inhibitor. Subsequently, their high-dose chemotherapy regimens utilized carboplatin and thiopeta with or without etoposide ($n=3$) or carboplatin, etoposide, and cyclophosphamide ($n=2$). One of these patients died from septicemia and multi-organ system dysfunction during induction, while two others had disease progression prior to starting high-dose chemotherapy. A total of five patients were eventually treated with high-dose chemotherapy at a median of 6 months (range of 4–6 months) after initial diagnosis of Stage IVb disease. Two of these patients had event-free survival at 40 and 101 months. One of the survivors received external-beam radiation therapy after recovering from high-dose chemotherapy. The authors thus concluded that intensive multi-modality regimens hold some potential for improving outcomes in this subset of high-risk patients. Figure 9.2 demonstrates a trilateral disease focus.

9.7 Current COG Trial

The current COG clinical treatment protocol (A Trial of Intensive Multi-Modality Therapy for Extra-Ocular Retinoblastoma—ARET0321) opened in 2008 and is accruing patients from COG institutions as well as collaborating elite centers in Argentina, Brazil, Chile, and Egypt. This prospective study attempts to confirm the results discussed above for patients with

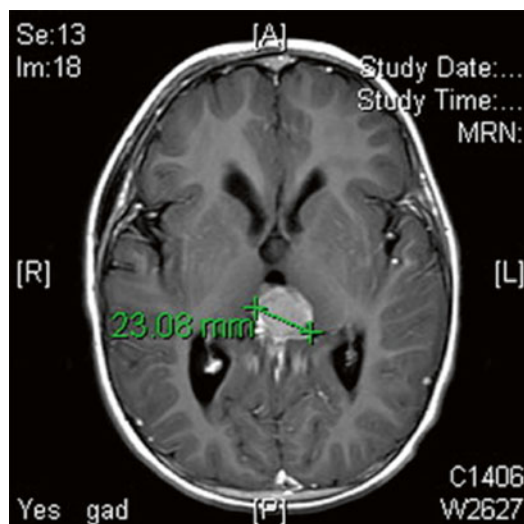


Fig. 9.2 Brain MRI demonstrating a trilateral disease focus

extraocular disease. Furthermore, it will seek to affirm the position that conventional chemotherapy in combination with targeted external-beam radiation therapy is appropriate and sufficient for patients with regional extra-ocular disease. In contrast, those patients with metastatic and trilateral disease will likely achieve improved survival outcomes when their therapy is intensified to include conventional induction chemotherapy followed by high-dose therapy and ASCR along with external-beam radiation therapy (Children's Oncology Group 2007).

Compliance with Ethical Requirements Ratna Behal, M.D. declares that she has no conflict of interest. Ira Dunkel declares no conflict of interest regarding the content of this chapter, but has had some consulting agreements with pharmaceutical companies.

No human or animal studies were carried out by the authors for this article.

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10.1 Introduction

Retinoblastoma is a rare childhood malignancy that is caused by mutations in the *RB1* gene, a prototypical tumor suppressor gene. Approximately 300 cases are diagnosed each year in the United States, and the incidence rates have remained stable over the past three decades (Howlader et al. 2011). Approximately 40–50 % of children diagnosed with retinoblastoma have a germline mutation in the *RB1* gene, whereas the other 50–60 % have acquired somatic mutations in *RB1*.

In the United States, the current 5-year survival is 96.5 % for retinoblastoma patients (Howlader et al. 2011). Improvements in treatment account for the excellent survival; however, retinoblastoma survivors face the risk of second cancers due to a combination of risk factors. The incidence of second cancers has been rising in retinoblastoma survivors, and they are now the leading cause of

death in this population, exceeding deaths due to retinoblastoma (Yu et al. 2009).

In this review, we describe key risk factors for second cancers after retinoblastoma. The study of second cancer risk factors requires long-term follow-up of large populations of survivors in order to reliably estimate risks. Three large cohort studies of long-term survivors of retinoblastoma in the United States, United Kingdom, and the Netherlands have provided very informative and consistent estimates of the risk of second cancers by hereditary status compared to the general population of each country (Eng et al. 1993; Kleinerman et al. 2005, 2007, 2012b; Wong et al. 1997, 2014; Yu et al. 2009; Marees et al. 2008, 2009, 2010; Reulen et al. 2011; MacCarthy et al. 2013). We define hereditary patients as those with bilateral retinoblastoma or unilateral with a family history of retinoblastoma; all of these patients presumably have a germline *RB1* mutation. All other patients with unilateral retinoblastoma are considered non-hereditary and presumably do not have a germline *RB1* mutation. In describing his two-hit model to explain the sequence of *RB1* mutations that cause retinoblastoma, Knudson observed that germline mutations account for most of the retinoblastoma diagnoses at a very early age (often under 12 months), whereas somatic mutations result in slightly older ages at retinoblastoma diagnosis (Knudson 1971).

In each cohort, hereditary survivors experienced much higher risks of second cancers

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Table 10.1 Risk of second cancers in cohort studies of long-term survivors of retinoblastoma by hereditary status

Cohort	Hereditary		Non-hereditary	
	No. second cancers	SIR ^a	No. second cancers	SIR
United States (n=1601) ^b Kleinerman et al. 2005	260	19*	17	1.2
United Kingdom (N=1062) Reulen et al. 2011	113	13*	19	1.7*
Netherlands (n=668) Marees et al. 2008	62	20*	12	1.9

^aSIR standardized incidence ratio. This is the number of observed cancers compared to the number of cancers expected in the general population

^bNumber in parentheses is the number of subjects in the cohort

*p<0.05

compared to the general population (Table 10.1). Kleinerman et al. (2005) reported a standardized incidence ratio (SIR) of 19 (95 % CI 16–21) for hereditary survivors and SIR=1.2 (95 % CI 0.7–2.0) for non-hereditary survivors. Marees et al. (2008) observed similar risks estimates of SIR=20 (95 % CI 16–26) for hereditary survivors and SIR=1.9 (95 % CI 0.96–3.2) for non-hereditary survivors in the Dutch cohort. Reulen et al. (2011) found a SIR=13.4 (95 % CI 11–16) for hereditary and SIR=1.7 (95 % CI 1.1–2.7) for non-hereditary survivors in the United Kingdom cohort.

The United States cohort, which will be the focus of this review, is comprised of 1850 1-year retinoblastoma survivors, diagnosed and treated at hospital centers in New York (1914–2006) and Boston (1937–1984). Information on second cancers was obtained from medical records, interviews with survivors or their families, and the National Death Index. The median follow-up in the United States cohort is 29 years, ranging from 1 to 70 years, and 74 % of the cohort is still alive. Most of the patients were treated with various combinations of radiotherapy, chemotherapy, and enucleation. Treatment varied by hereditary status, with 87 % of hereditary patients receiving radiotherapy compared with only 18 % of non-hereditary

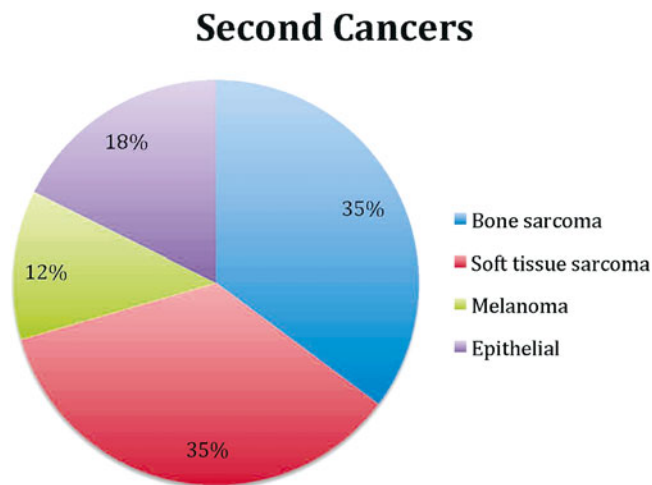
patients. Half of the patients receiving radiotherapy also received chemotherapy.

The most common second cancers noted in hereditary survivors were soft tissue sarcomas (35 %), bone sarcomas (35 %), and melanoma (12 %) (Fig. 10.1). The remaining were various types of epithelial cancers (18 %). Very high risks of sarcomas and melanoma were noted for hereditary survivors, and it was striking that none of these malignancies were diagnosed in non-hereditary survivors in this cohort. With the exception of breast cancer, risks were not increased for any of the few other second cancers diagnosed in non-hereditary survivors. The cumulative incidence of any second cancer 50 years after treatment for hereditary retinoblastoma was 36 % (95 % CI 31–41 %) compared to 5.7 % (95 % CI 2.4–11 %) in non-hereditary survivors (Kleinerman et al. 2005). The cumulative mortality from second cancers 50 years after retinoblastoma diagnosis was 25.5 % (95 % CI 21–30 %) in hereditary compared to 1.0 % (95 % CI 0.2–1.8 %) in non-hereditary survivors (Yu et al. 2009).

10.2 Causes of Second Cancers

The *RB1* gene is a high-risk cancer susceptibility gene and many studies have reported that hereditary patients with a *RB1* germline mutation have a high risk for second cancers compared to the general population (Kleinerman et al. 2005; McCarthy et al. 2009; Marees et al. 2008). In addition, somatic mutations in this gene have been linked to the diagnoses of sporadic sarcomas and several epithelial cancers, including lung, bladder, breast, ovary, and prostate cancers (Burkhart and Sage 2008). Both past chemotherapy and external beam radiotherapy have been associated with increased risks of second cancers in retinoblastoma survivors (Marees et al. 2008; Wong et al. 2014) as well as other pediatric cancer populations (Henderson et al. 2012; Le Vu et al. 1998). Host, medical, lifestyle, and other environmental factors such as tobacco have been related to the risk of second cancers; however, these factors play a much less important role in the development of second cancers in pediatric

Fig. 10.1 Distribution of second cancers among irradiated, 5-year survivors of hereditary retinoblastoma



cancer survivors compared to adult cancer survivors (Morton et al. 2014; Bhatia and Sklar 2002).

Radiotherapy increased the risk of second cancers in hereditary patients by threefold (95 % CI 2.0–5.3) (Wong et al. 1997). The cumulative risk of a second cancer in irradiated hereditary patients at 50 years rose to 38 % (95 % CI 33–44 %) compared to a cumulative risk of 21 % (95 % CI 9.4–36 %) in non-irradiated hereditary patients for the same time period (Kleinerman et al. 2005). Yu et al. (2009) noted similar patterns for cumulative mortality risks at 50 years from a second cancer (26.8 % for irradiated hereditary patients compared to 17.2 % for non-irradiated hereditary patients).

Wong et al. (1997) demonstrated a significant radiation dose–response relationship for the risks of both bone and soft tissue sarcomas in hereditary survivors. Most of the bone and soft tissue sarcomas occurred in the head, which received high doses of radiotherapy (mean dose=41.7 Gy to the skull and 45.2 Gy to soft tissues of the orbit). The significant radiation dose–response relationship for bone sarcomas observed in this cohort was consistent with previous reports of increased risk of bone sarcomas with increasing radiation dose in retinoblastoma survivors (Hawkins et al. 1996; Tucker et al. 1987). A radiation dose–response for soft tissue sarcomas has also been observed in other pediatric cancer populations (Berrington de Gonzalez et al. 2012).

Chemotherapy was often used in combination with radiotherapy, and more recent research has focused on the joint effect of radiotherapy with chemotherapy on the risk of second cancers, showing increased risks for bone sarcomas and soft tissue sarcomas, particularly leiomyosarcoma (Wong et al. 2014).

10.3 Risks for Specific Second Tumors

10.3.1 Soft Tissue Sarcomas

Retinoblastoma survivors have 184-fold increased risk of developing soft tissue sarcomas compared with the general population, and these sarcomas were diagnosed both inside and outside the radiation field (Kleinerman et al. 2007). The occurrence of soft tissue sarcomas outside the radiation field points to a genetic predisposition to soft tissue sarcomas independent of radiation, supported by the observation that *RBI* somatic mutations have been observed in sporadic soft tissue sarcomas (Friend et al. 1987). Leiomyosarcoma was the most common subtype, representing 30 % of the soft tissue sarcomas. It was notable that 78 % of leiomyosarcomas were diagnosed 30 or more years after retinoblastoma. The location of these leiomyosarcomas varied with many in the head and neck, but the majority

of leiomyosarcoma in females were diagnosed in the uterus (Francis et al. 2012). The cumulative risk for any soft tissue sarcoma 50 years after radiotherapy for retinoblastoma was 13.1 % (95 % CI 9.7–17 %). In addition to the radiation-related risks, Wong et al. (2014) recently found that the cumulative incidence of leiomyosarcoma at 40 years was significantly higher in survivors who had received both radiotherapy and alkylating agent chemotherapy compared to those who received radiotherapy alone (5.8 % vs. 1.6 %, $p=0.01$).

10.3.2 Bone Sarcomas

Bone sarcomas are one of the most common second cancers reported in studies of pediatric cancer survivors (Bhatia and Sklar 2002). It is well known that high-dose radiotherapy is associated with bone sarcomas in other pediatric and adult cancer patients (Berrington de Gonzalez et al. 2013; Henderson et al. 2012; Schwartz et al. 2014). Retinoblastoma survivors have 360-fold increased risk of developing bone sarcomas compared with the general population, and these sarcomas were diagnosed primarily in the head, but interestingly almost 25 % of the bone sarcomas were also diagnosed in the lower legs that received no scatter radiation (Kleinerman et al. 2005). Similar to the finding for soft tissue sarcomas outside the radiation field, genetic predisposition likely accounts for the occurrence of bone sarcomas in the lower legs, and *RBI* somatic mutations have been identified in sporadic bone sarcomas (Friend et al. 1986). Analyses in bilateral survivors indicated that both radiotherapy and chemotherapy increased the risk of bone sarcomas, but sex, age at retinoblastoma diagnosis, and family history of retinoblastoma were not significantly associated with risk of bone sarcomas in this cohort (Kleinerman et al. 2012a). Similar to soft tissue sarcomas, the cumulative incidence of bone sarcomas at 40 years in hereditary survivors was higher in patients treated with both alkylating agents and radiotherapy compared to radiotherapy alone (12.7 % vs. 9.0 %, $p=0.07$) (Wong et al. 2014).

10.3.3 Melanoma

Cutaneous melanoma is the third most common malignant neoplasm diagnosed in retinoblastoma survivors (Kleinerman et al. 2005; MacCarthy et al. 2013; Marees et al. 2008). Increased risks of similar magnitude have been observed in both irradiated and non-irradiated survivors, which indicates the involvement of genetic factors independent of radiation exposure. In addition, survivors of hereditary retinoblastoma are prone to dysplastic nevi (Traboulsi et al. 1988), a risk factor for melanoma (Goldstein and Tucker 2013). Further analyses comparing melanoma risk in 5-year survivors following radiotherapy alone ($SIR=32.3$, 95 % CI 20–50) vs. radiotherapy with alkylating agents ($SIR=23.8$, 95 % CI 12–42) indicated no significant differences ($p=0.42$) (Wong et al. 2014). However, risks for melanoma appeared to be three times higher in bilateral patients reporting a family history of retinoblastoma compared to bilateral patients without a family history ($RR=3.08$, 95 % CI 1.2–7.2, $p=0.02$) (Kleinerman et al. 2012b). Melanoma risk was also significantly related to older attained age of retinoblastoma survivors compared to younger survivors (25 years and older compared to less than 25 years, $RR=6.28$, 95 % CI 2.6–18, $p<0.001$), which is consistent with the increase in melanoma incidence beginning at age 20 in the general population.

10.4 Epithelial Tumors

Alterations of the *RBI* gene have been documented in several common adult cancers including lung, prostate, breast, bladder, leukemia, brain, esophageal, and liver cancer (Burkhart and Sage 2008). Similarly increased risks for adult onset epithelial tumors combined were noted following radiotherapy alone ($SIR=6.2$, 95 % CI 4.0–9.2) and radiotherapy plus alkylating agents ($SIR=6.8$, 95 % CI 4.2–10.3) in 5-year hereditary survivors (Wong et al. 2014). Cancers of the lung and breast were the two most common epithelial tumors noted in the United States cohort; the other epithelial tumors included cancers of

the bladder, colorectal, kidney, nasal cavity, prostate, retroperitoneum, thyroid, tongue, and uterus (Wong et al. 2014).

In an earlier study of lung cancer mortality following retinoblastoma, elevated risks of lung cancer were noted in both irradiated (SIR=8.1, 95 % CI 1.0–29) and non-irradiated hereditary survivors (SIR=35, 95 % CI 7–102), but not in non-hereditary survivors (Kleinerman et al. 2000). Although the study was based on only five lung cancer deaths, it was striking that all deaths occurred in females, 3 of 5 were under age 50 years, 4 out of 5 smoked, and 3 of 5 were small cell lung cancer. Somatic mutations and deletions in the *RB1* gene have been detected in small cell lung cancer (Harbour et al. 1988). Following up on this observation, survivors were queried about their tobacco use, and hereditary and non-hereditary survivors reported similar patterns of tobacco use (Foster et al. 2006), leading the investigators to conclude that survivors with a germline *RB1* mutation may be highly susceptible to smoking-related lung cancer.

An increased risk of breast cancer was observed in both hereditary and non-hereditary retinoblastoma survivors. Radiation is a known risk factor for breast cancer, but lower increased risks were noted for hereditary survivors who received radiotherapy (SIR=3.89, 95 % CI 2.3–6.1) compared to those without radiotherapy (SIR=8.72, 95 % CI 3.5–18), whereas the opposite pattern was noted in non-hereditary survivors, with a higher risk of breast cancer (SIR=6.19, 95 % CI 2.0–14) in irradiated compared to non-irradiated survivors (SIR=2.09, 95 % CI=1.00–3.84) (Little et al. 2014). Based on these observations, Little et al. (2014) conducted a case–control study of breast cancer after retinoblastoma combining data from the United States and the United Kingdom cohort study. They reported an increasing dose of radiotherapy significantly related to increasing breast cancer risk in non-hereditary RB survivors, but not in hereditary survivors. Little et al. concluded that the *RB1* germline mutation may have contributed to an underlying susceptibility in hereditary survivors, which is consistent with known associations of *RB1* mutations with sporadic primary breast cancer (Bosco and Knudsen 2007).

10.4.1 Benign Tumors

Li et al. (1997) noted an increased incidence of lipomas, benign tumors of adipose tissue, among hereditary survivors, and observed that lipomas may serve as an early marker of soft tissue sarcomas based on an analysis of second cancers among those with a lipoma. Twenty (3.6 %) of 556 hereditary patients and two (0.6 %) of 342 non-hereditary patients reported lipomas (Odds ratio=6.3, 95 % CI 1.5–56, $p=0.003$) in the United States cohort. Preferential locations of lipomas included the face, neck, shoulders, and upper chest. Six of the 20 (30 %) hereditary patients with a lipoma also developed a second primary neoplasm (half of which were soft tissue sarcomas) compared with 62/536 (12 %) of hereditary patients without a lipoma, suggesting that germline *RB1* mutations may increase the risk of both lipomas and second cancers. Following up on this observation, Rieder et al. (1998) reported cytogenetic and molecular evidence of a link between germline *RB1* and lipomas by somatic loss of an *RB1* allele in a hereditary survivor.

10.4.2 Summary

Based on numerous studies of the risk of second cancers in retinoblastoma patients, it has been consistently observed that hereditary survivors have a greater risk of second cancers compared to non-hereditary survivors. The most common second cancers diagnosed in hereditary survivors were bone sarcomas, soft tissue sarcomas, and melanoma. These three cancers accounted for 82 % of second cancers diagnosed in hereditary retinoblastoma survivors. Lung and breast cancers were the most frequently reported epithelial cancers. Increasing doses of radiation significantly increased the risk of bone and soft tissue sarcomas, but not melanoma. Chemotherapy in combination with radiotherapy increased the risk of bone sarcomas and soft tissue sarcomas, particularly leiomyosarcoma. Risks of bone sarcoma, soft tissue sarcoma, and melanoma persist into adulthood, decades following initial retinoblastoma diagnosis.

Genetic studies have been conducted or are underway to identify specific germline *RB1* germline mutations that may be related to the risk of specific second cancers in hereditary survivors (Dommering et al. 2012). Identification of specific *RB1* germline mutations would identify those survivors who may be at high risk of second cancers. Future work would also include identification of molecular changes that characterize specific second cancers in relation to radiotherapy and chemotherapy.

10.5 Conclusion

Improvements in treatment over the past century have greatly increased survival for retinoblastoma patients. However, as survival has improved, the risk of second cancers has increased. Second cancers are now a major cause of morbidity and mortality in retinoblastoma survivors. Past treatments including radiotherapy and alkylating agent chemotherapy have contributed to this high risk of second cancers, in addition to genetic predisposition to second cancers related to germline mutations in the *RB1* gene. The studies described in this review have helped to identify and understand second cancer risks, particularly those associated with radiotherapy. With the recognition of risk due to past radiotherapy and transition to newer treatments such as inter-arterial chemotherapy (Gobin et al. 2011), the risk for second cancers in the head region could decrease. However, hereditary patients will likely remain at risk for second cancers due to their germline *RB1* mutation, and future studies should evaluate the risk of second cancers with newer treatments. Hereditary survivors who received radiotherapy in the past should be informed of their possible risk of second cancers, and they should be encouraged to follow cancer prevention strategies.

Pooling of epidemiologic and genetic data from large follow-up studies of long-term survivors of retinoblastoma would also allow further exploration of factors that may affect or modify the risk of second cancers in retinoblastoma survivors.

Summary

- Retinoblastoma survivors with a germline *RB1* mutation have an increased risk for second cancers
- Second cancers are a major cause of morbidity and mortality
- Radiotherapy and chemotherapy have contributed substantially to risk of second cancers
- Genetic predisposition enhances treatment-related risk
- Second cancer risks persist for decades

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Danielle Novetsky Friedman

11.1 Introduction

Curative therapy for retinoblastoma has resulted in a growing cohort of young adult and adult retinoblastoma survivors who are at risk for a variety of long-term health problems. While survivors' ocular and visual problems, as well as the increased lifelong risk for subsequent malignancies in those with the genetic form of the disease (Wong et al. 1997; Kleinerman et al. 2005, 2007, 2012a; Francis et al. 2012), are well-documented, data on the long-term health and health status of retinoblastoma survivors are limited. The current chapter provides background on childhood cancer survivorship and summarizes the available data on retinoblastoma survivors' long-term health. It also highlights the gaps in the literature where further investigation is warranted.

11.2 Background: Childhood Cancer Survivorship

Due to advances in the diagnosis and treatment of childhood cancer, 5-year survival rates now exceed 80 % (DeSantis et al. 2014). Improved

survival, however, has led to increased recognition of survivors' excess risk of premature morbidity and mortality due to treatment-related complications, which are collectively known as "late effects" (Oeffinger et al. 2006; Geenen et al. 2007; Mertens et al. 2008; Diller et al. 2009; Hudson et al. 2009, 2013). While many treatment-related chronic conditions impact survivors shortly after therapy, others rarely become apparent until much later in life. The identification of such conditions has been made possible by the continued long-term follow-up of large childhood cancer survivor cohorts, such as the Childhood Cancer Survivor Study (CCSS).

The CCSS is a North American multi-institutional, retrospective cohort study that tracks the longitudinal health of over 14,000 survivors of pediatric and adolescent cancer diagnosed between 1970 and 1986 (Robison et al. 2002). Recruitment of an expansion cohort of patients treated between 1987 and 1999 is in its final stages as well. Enrolled CCSS participants periodically complete detailed questionnaires about their medical conditions, health behaviors, and health status; conditions are then graded for severity on a scale of 1 [mild] through 5 [fatal] using the National Cancer Institute's Common Terminology Criteria for Adverse Events (National Cancer Institute 2010).

A 2006 landmark study from the CCSS demonstrated that nearly three-fourths of childhood cancer survivors will have a chronic health condition

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within 30 years after the primary cancer diagnosis; 42.4 % will experience a serious, life-threatening, disabling, or fatal condition during that time (Oeffinger et al. 2006). When compared to a group of matched unaffected siblings, survivors were found to be three times more likely to have a chronic health condition; eight times more likely to have a severe, disabling, or life-threatening condition; and nearly five times more likely to have two or more chronic conditions.

More recently, the St. Jude Lifetime Cohort Study, which prospectively performs exposure-based medical assessments on over 1700 adult survivors of pediatric malignancies (median age: 32 years), found an even higher estimated cumulative prevalence of chronic conditions among childhood cancer survivors. The study demonstrated that by the age of 45, as many as 95.2 % of childhood cancer survivors will have at least one chronic condition, while 80 % will have a severe, debilitating, or life-threatening condition (Hudson et al. 2013).

Data from these studies have allowed for the formulation of clinical guidelines related to childhood cancer survivors and their potential therapy-related late effects, which are publicly available at: www.survivorshipguidelines.com (COG 2014). These guidelines may be used by healthcare teams to ensure the provision of appropriate lifelong risk-based care for childhood cancer survivors. Patient education materials called “Health Links” are publicly available at this website as well.

11.3 Retinoblastoma Survivorship: General Medical Outcomes

While data from the CCSS and other cohort studies have been instrumental in elucidating the long-term health risks faced by survivors of childhood cancer, it is important to note that survivors of retinoblastoma were not included in the CCSS cohort. Small numbers of retinoblastoma survivors have, however, participated in other survivorship studies. The St. Jude Lifetime Cohort ($n=1713$) includes 66 adult retinoblastoma survivors (3.9 % of the overall cohort);

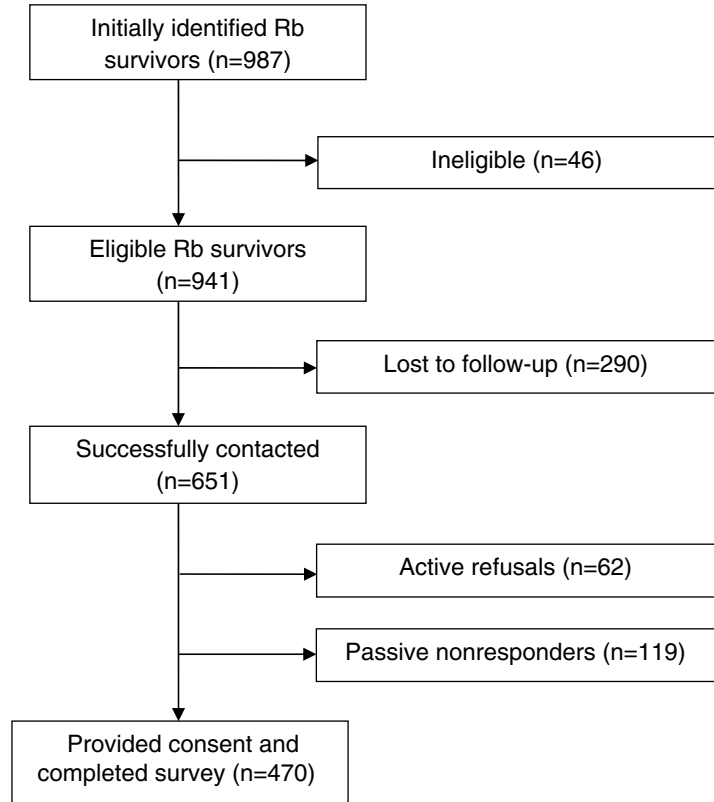
specific retinoblastoma-related data and/or recommendations, however, have not yet been presented. One other study on long-term complications in a cohort of 21 retinoblastoma survivors treated between 1976 and 1994 found the most frequent late effects in this population to be orbital deformation and neurocognitive disabilities (Nahum et al. 2001).

The vast majority of other reports on retinoblastoma survivors’ chronic medical conditions have focused exclusively on long-term oculo-visual outcomes (Kaste et al. 1997; Peylan-Ramu et al. 2001; Desjardins et al. 2002; Ek et al. 2002; Abramson et al. 2004) and the elevated risk of subsequent malignancies in those with the genetic form of the disease (Eng et al. 1993; Moll et al. 1997, 2001; Wong et al. 1997; Abramson and Frank 1998; Abramson et al. 2001; Kleinerman et al. 2005, 2007, 2012a; Marees et al. 2008; MacCarthy et al. 2009, 2013; Francis et al. 2012; Rodjan et al. 2013). Data are otherwise lacking on adult retinoblastoma survivors’ general health and overall risk of chronic medical conditions.

In an effort to fill this gap, our group designed the Retinoblastoma Survivor Study (RBSS), which is the first large retrospective cohort study of adult retinoblastoma survivors. Modeled after the CCSS, eligible participants were asked to complete a detailed questionnaire about their medical conditions, health behaviors, psychosocial well-being, and general health as well as validated vision questions from the National Eye Institute’s 25-Item Visual Function Questionnaire (Mangione et al. 2001). Siblings of CCSS participants ($n=2271$), who completed identical questionnaires except without added vision questions, served as a comparison group.

Four hundred seventy survivors diagnosed with retinoblastoma between 1932 and 1994 ultimately provided informed consent and completed the questionnaire (see Fig. 11.1); 53.6 % of participants had a history of bilateral disease and 52.1 % were female. The mean age at study enrollment was 43 years and the median length of follow-up was 42 years (range: 15–75). More than half of the cohort was treated with radiation therapy (56.5 % of all participants; 91.7 % of those with bilateral disease; 15.7 % of those with

Fig. 11.1 Participation and contact in the Retinoblastoma Survivor Study



unilateral disease) and a little over one-quarter received systemic chemotherapy (26.0 % of all participants; 37.3 % of those with bilateral disease; 11.5 % of those with unilateral disease). Data collection has been completed and the analysis is currently underway.

11.4 Retinoblastoma Survivorship: Health-Related Quality of Life and Psychosocial Outcomes

11.4.1 Health-Related Quality of Life

While data are limited on retinoblastoma survivors' medical outcomes, a number of previous studies have been conducted on their health-related quality of life. Overall, survivors' health-related quality of life appears to be similar to normative populations. In a cohort of 87 adult retinoblastoma survivors who completed a

generic quality of life questionnaire, survivors were found to experience an overall good, but slightly decreased, quality of life, when compared to a healthy Dutch reference group; the documented decrease in quality of life perception was primarily related to increased mental health concerns such as anxiety, feelings of depression, and loss of control (van Dijk et al. 2007b).

Similarly, in a group of 65 children and adolescent retinoblastoma survivors (ages 8–18 years) and their parents, survivors were found to have very good health-related quality of life, when compared to a Dutch reference group (van Dijk et al. 2007a). In fact, survivors self-reported better health-related quality of life than did the reference group on questions regarding “moods and emotions” and “autonomy.” Interestingly, survivors' perceptions and parents' perceptions correlated poorly on all health-related quality of life domains with parents judging the health-related quality of life of their children to be worse than the children themselves.

In a recent large study of health-related quality of life among 1593 Swiss childhood cancer survivors ($n=37$ with retinoblastoma), and 695 sibling comparators, retinoblastoma survivors reported lower scores in the Physical Component Summary when compared to other childhood cancer survivors (Rueegg et al. 2013). In general, childhood cancer survivors with health problems were found to have lower health-related quality of life, regardless of primary diagnosis.

11.4.2 Activities of Daily Living

In a cohort study of 156 Dutch retinoblastoma survivors, 55 % of the young survivors (ages 8–17 years) and 54 % of adult survivors (ages 18–35 years) considered themselves to be restricted to some extent in school or professional career, mobility, self-care, or relationships (van Dijk et al. 2010). Survivors were found to have lower educational attainment, but were no different from the general population in terms of employability or marital status.

A more recent study from Israel of 46 retinoblastoma survivors (ages 2–18 years), and their parents, found that the overall health-related quality of life of retinoblastoma survivors was comparable to that of a normative sample of healthy children (Weintraub et al. 2011). However, the subgroup of patients with a history of bilateral disease participated less in daily activities and had lower emotional quality of life when compared to those with a history of unilateral disease. Again, parent-report and survivor self-report correlated poorly, with parents reporting their children's general health and emotional quality of life as worse than survivors themselves via self-report (although only the latter was statistically significant).

11.4.3 Behavioral Outcomes

In a study of behavioral outcomes in 148 Dutch survivors of retinoblastoma (8–35 years of age), 30 % of survivors were reported by their parents to have behavioral problems. Again, a much

lower percentage of survivors self-reported such problems [9 % of adolescents; 12 % of adults] (van Dijk et al. 2009). Despite the disagreement in prevalence rates of behavioral problems between survivors and their parents, both groups agreed on the nature of the reported behavioral issues, which included internalizing behaviors (anxious/depressed, withdrawn, somatic complaints) and somatic complaints.

11.4.4 Limitations

While multiple studies have been published on the health-related quality of life and psychosocial outcomes of Dutch retinoblastoma survivors, and more recently Israeli survivors, data on survivors from other parts of the world, including North America, are lacking. The RBSS, described above, will provide data on self-reported psychosocial outcomes and health status in 470 adult retinoblastoma survivors treated in the New York area.

11.5 Developmental and Cognitive Functioning in Survivors of Retinoblastoma

Previous small studies in the 1960s and 1970s reported superior cognitive abilities in patients with retinoblastoma (Eldridge et al. 1972). A more recent study of 22 retinoblastoma survivors diagnosed between January 1992 and December 1994 in Sweden found that survivors had neither cognitive nor motor delay at 4 or 6 years of age. Interestingly, those with a history of bilateral disease were found to have better cognitive abilities, when compared to patients with a history of unilateral disease (Ek et al. 2002). All children in the study were in mainstream education.

In contrast to these positive results, however, a recent prospective study of 94 retinoblastoma patients who underwent serial assessments of adaptive and cognitive functioning at ages 6 months and 1, 2, 3, and 5 years demonstrated that at age five, survivors' developmental functioning

was in the low-average range, which was significantly less than what would be expected on the basis of population means (Willard et al. 2014). The trajectory of both developmental functioning and adaptive functioning declined among all survivors over time, but patients treated with enucleation alone were found to have the greatest decline in cognitive functioning across all subscales and over time (after excluding those with 13q deletion). It is unclear how to explain these findings and results will need to be replicated in future follow-up studies of retinoblastoma survivors.

11.6 Late Effects in Survivors of Extra-Ocular Retinoblastoma

While patients with intra-ocular retinoblastoma have excellent survival rates, those with extra-ocular disease have historically had very poor outcomes. Over the past few decades, however, the use of intensive multimodality therapy has resulted in improved prognosis with a resultant increase in the number of survivors. In an effort to clarify the late effects for which extra-ocular retinoblastoma survivors are at risk, our group performed a single-institutional retrospective analysis of long-term medical outcomes in 19 survivors of extra-ocular retinoblastoma treated between 1992 and 2009 (Friedman et al. 2013).

In this study, all 19 patients were treated with intensive multimodality therapy, which included conventional chemotherapy ($n=19$), external beam radiotherapy ($n=15$), and/or high-dose chemotherapy with autologous stem cell transplant ($n=17$). After a median follow-up of 7.8 years from diagnosis of extra-ocular disease, survivors were found to have a number of late effects, but the majority were mild-moderate in severity. Survivors' severe, life-threatening, or disabling conditions were primarily related to severe ototoxicity ($n=8$; $n=4$ require hearing aids) and subsequent malignancies ($n=6$). Five of the six patients who developed subsequent malignancies carried a known RB1 mutation. Long-term cardiac, pulmonary, hepatobiliary, or renal conditions were not identified in any survivors.

11.7 Screening for Subsequent Malignancies in Survivors of Hereditary Retinoblastoma

In those with the genetic form of retinoblastoma, the cumulative incidence of a subsequent malignancy has been reported to be 28 % within 40 years of the primary diagnosis, and 36 % within 50 years of the primary diagnosis (Kleinerman et al. 2005; Marees et al. 2008). Treatment factors and family history may further amplify this risk (Wong et al. 1997, 2014; Kleinerman et al. 2012b). Nonetheless, screening regimens that facilitate the early detection of subsequent malignancies have not yet been implemented in this high-risk population. It has been theorized that whole-body magnetic resonance imaging (MRI), an ionizing radiation-free imaging modality, might serve as an ideal screening tool for new primary malignancies in survivors of the genetic form of retinoblastoma.

In an effort to assess the utility of whole-body MRI screening in this population, our group implemented an institutional pilot study in which 41 whole-body MRIs were performed in 25 adolescent and young adult survivors of the genetic form of retinoblastoma (Friedman et al. 2014). Median age at first MRI was 16 years. Among all whole-body MRIs performed, new osseous lesions suspicious for malignancy were detected in five patients (see Fig. 11.2 for details). After further evaluation, which included dedicated imaging and biopsy when indicated, two patients were found to have localized high-grade osteosarcoma of the extremity and three were diagnosed with benign osseous abnormalities. Additionally, one patient was diagnosed with a high-grade osteosarcoma three months after a normal whole-body MRI scan. Given these findings, the sensitivity of detecting subsequent malignancies using whole body MRI in this cohort was 66.7 % and the specificity was 92.1 %.

Based on these limited data, it is unclear whether whole-body MRI screening programs have the potential to decrease second cancer-related mortality in retinoblastoma survivors. Further study in larger survivor cohorts is required

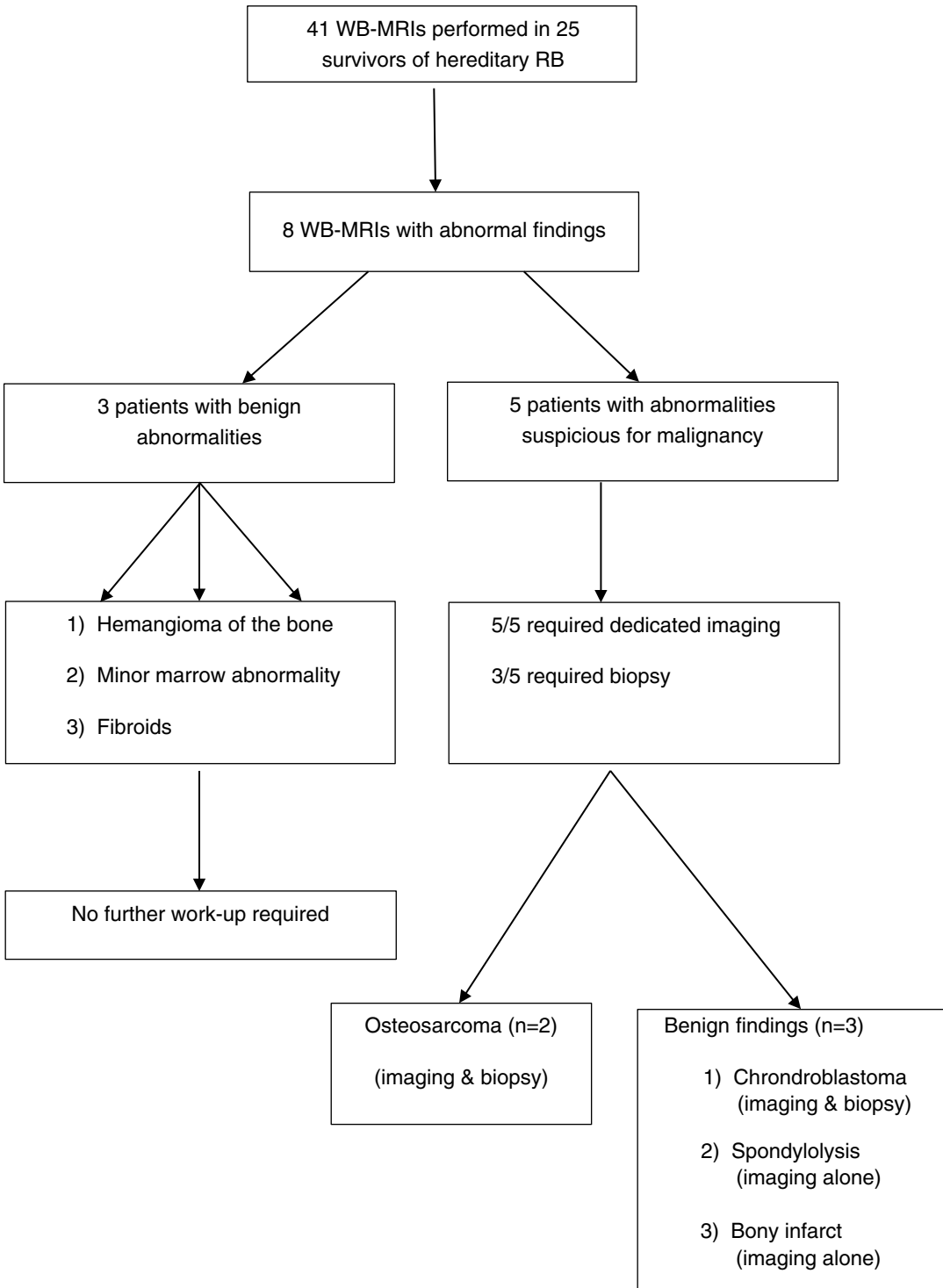


Fig. 11.2 Abnormal whole-body MRI (WB-MRI) screening results in 25 retinoblastoma survivors with the genetic form of the disease. Adapted from Friedman DN, *Pediatric Blood & Cancer*, 2014

to determine the utility of this modality in those with hereditary cancer predisposition syndromes, such as the genetic form of retinoblastoma.

11.8 Future Directions

In an effort to maximize overall survival, ocular survival, and vision preservation, paradigms for the treatment of retinoblastoma have rapidly evolved over the past few decades. As such, survivors' potential late effects will continue to evolve as well. While the RBSS, described above, will provide the first report of adult retinoblastoma survivors' general health and health status, further research will be required to elucidate the potential late effects related to contemporary treatments such as intra-arterial chemotherapy. Institutional and multi-institutional cohort studies of contemporarily treated patients will be necessary to determine how best to provide long-term care to survivors treated with newer therapeutic modalities.

11.9 Conclusions

Given the dramatic advances in treatment for retinoblastoma over the past century, there is a growing cohort of long-term retinoblastoma survivors who are at risk for a variety of treatment-related medical and psychosocial late effects. The current chapter summarizes the limited published data on non-ophthalmic, non-second tumor-related late effects in survivors of retinoblastoma. Analyses are currently underway to delineate these late effects in a large cohort of adult retinoblastoma survivors treated in the New York area, but continued future investigation is warranted in survivors treated with newer therapies.

Until these analyses are complete, however, retinoblastoma survivors should receive risk-based screening in accord with the Children's Oncology Group's published survivorship guidelines (COG 2014) as well as continued ophthalmic care. Careful screening for the development of subsequent malignancies is indicated in those with the genetic form of the disease; further

research is indicated in order to establish evidence-based, consensus guidelines on optimal surveillance protocols in high-risk survivors. Continued, systematic investigation of late effects in this population is warranted and will inform the optimal long-term care of retinoblastoma survivors as they age into adulthood.

Compliance with Ethical Requirements Danielle Novetsky Friedman declares that she has no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being in the study.

No animal studies were carried out for this article.

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