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Genetic and Epigenetic Discoveries in Human Retinoblastoma

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Abstract

Retinoblastoma is a rare pediatric cancer of the retina. Nearly all retinoblastomas are initiated through the biallelic inactivation of the retinoblastoma tumor susceptibility gene (*RB1*). Whole-genome sequencing has made it possible to identify secondary genetic lesions following *RB1* inactivation. One of the major discoveries from retinoblastoma sequencing studies is that some retinoblastoma tumors have stable genomes. Subsequent epigenetic studies showed that changes in the epigenome contribute to the rapid progression of retinoblastoma following *RB1* gene inactivation. In addition, gene amplification and elevated expression of p53 antagonists, MDM2 and MDM4, may also play an important role in retinoblastoma tumorigenesis. The knowledge gained from these recent molecular, cellular, genomic, and epigenomic analyses are now being integrated to identify new therapeutic approaches that can help save lives and vision in children with retinoblastoma, with fewer long-term side effects.

Keywords

SYK; retinoblastoma epigenetics; whole genome sequencing; translational research

I. INTRODUCTION

Retinoblastoma is a rare pediatric cancer of the retina. It is the most frequent neoplasm of the eye in childhood, representing 2.5% to 4% of all pediatric cancers. The average age-adjusted incidence rate of retinoblastoma in the United States and Europe is 2 to 5 per million children (approximately 1 in 14,000 to 18,000 live births).^{1,2} Retinoblastoma is diagnosed at a very early stage of a child's life: two-thirds before 2 years of age, and 95% before 5 years.¹ For these reasons, therapeutic approaches need to consider curing the disease as well as preserving vision with minimal long-term side effects.

Retinoblastoma can occur as either bilateral or unilateral disease. Most bilateral tumors are caused by the presence of germline mutations of the *RB1* gene that are either inherited from an affected survivor (25%) or the result of a new germline mutation (75%). Importantly,

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patients with a new germline mutation can be mosaic, and patients with low-level mosaicism can now be identified by whole-genome sequencing of their blood cells. In contrast to bilateral disease, unilateral retinoblastomas account for 75% of all cases, and the majority of those are thought to be the result of *RB1* somatic mutations in the developing retina. Some of these unilateral cases, however, are likely to be new germline mutations that are mosaic and below the level of detection with current clinical *RB1* sequence analysis. Whether patients with low-level germline mosaic *RB1* mutations have an increased likelihood of developing a second cancer later in life is currently not known. This is an important issue to consider as clinical genomic efforts are launched for children with cancer.

In 1971 Alfred G. Knudson hypothesized that retinoblastoma is caused by 2 mutational events to inactivate 2 copies of a tumor suppressor gene rather than a single oncogenic lesion. He proposed that in nonhereditary or sporadic retinoblastoma, mutations in both alleles of this putative tumor suppressor gene had to occur in the same developing retinal cell. Because inactivation of 2 copies of a wild-type gene was less likely to occur than inactivation of a single copy of the tumor suppressor in a patient who inherited one mutant copy, patients with unilateral disease are diagnosed later in life than those children with bilateral disease.³ We now understand that the putative tumor suppressor gene described by Knudson is the retinoblastoma susceptibility 1 gene or *RB1*.⁴ One of the important and unexpected consequences of the identification of the *RB1* gene was the discovery that this pathway is mutated in virtually every human cancer.⁵⁻⁷ Thus, by studying a rare pediatric cancer, Knudson and others identified one of the most important tumor suppressor pathways in human cancer.

II. RETINOBLASTOMA GENOMICS

Biallelic inactivation of the *RB1* gene is considered to be the initiating genetic lesion in nearly all retinoblastomas. Since its discovery, researchers have focused on identifying secondary genetic lesions that cooperate with *RB1* inactivation and contribute to tumor progression.⁸ Several cytogenetic and array comparative genome hybridization studies have identified regions of the genome that are recurrently gained or lost in retinoblastomas. It has been postulated that genes in those regions may contribute to tumorigenesis.⁸ Indeed, candidate oncogenes and tumor suppressor genes have been identified in recurrently altered genomic regions, and changes in copy number correlate with changes in gene expression. For example, one study reported that the *DEK* oncogene is located in a 0.6-Mb minimal region of 6p22 that is gained in retinoblastoma, and its expression is slightly increased (~2.5-fold) in tumors with a 6p22 gain compared with those with 2 copies of 6p22.⁹ Similar results have been reported for *NUPI53*, *E2F3*, and *TTRAP*.¹⁰

Another recurrent copy number variation (CNV) in retinoblastoma is the amplification of a region on chromosome 2p spanning the *MYCN* oncogene. This amplification is present in ~10% of retinoblastomas.¹¹ How *MYCN* amplification contributes to human retinoblastomas that harbor *RB1* mutations is not known, but genetically engineered retinoblastoma mouse models suggest a role in metastasis.¹¹ Rushlow et al.¹² proposed that approximately 1.5% of human retinoblastomas have wild-type *RB1*, and the tumors are initiated by *MYCN* amplification. However, whole-genome sequencing was not performed

on these samples, making it impossible to rule out other mechanisms or *RB1* inactivation such as chromothripsis (see section III). This novel mechanism of retinoblastoma initiation by *MYCN* in the absence of *RB1* inactivation was directly demonstrated by whole-genome sequencing, fluorescence in situ hybridization (FISH), and immunohistochemistry.¹³

In the past 5 years, next-generation sequencing approaches have made it possible to achieve nucleotide resolution for much of the retinoblastoma genome. The first retinoblastoma genomic study was carried out in 2012 using 4 primary retinoblastomas and their matched germline DNA.¹⁴ Despite the small size of the cohort, many common retinoblastoma genomic features were identified in these tumors. Both sporadic and heritable retinoblastomas were represented, and biallelic inactivation of *RB1* occurred by diverse mechanisms, including nonsense and frameshift mutations, splice-site mutations, loss of heterozygosity (LOH), and promoter hypermethylation.¹³ Those tumors also had several of the most common large chromosomal lesions, including gains on 6p, 1q, and 2q and loss of 13q. The number of chromosomal lesions was also heterogeneous, which is consistent with data from a large cohort of tumors analyzed by SNP 6.0 chips.¹⁴ One unexpected result from the first whole-genome sequencing study was the discovery that *BCOR* is recurrently mutated in approximately 13% of retinoblastomas. *BCOR* is a BCL6 corepressor that regulates gene expression by epigenetic mechanisms.^{14–17} This is the only other gene besides *RB1* that is recurrently mutated in retinoblastoma, and genetically engineered mouse models are currently being used to evaluate the contribution of *BCOR* mutations to retinoblastoma progression. Mutations in *BCOR* are not limited to retinoblastoma; other genomic studies have identified *BCOR* mutations in 2.7% of cancers from different tissues.¹⁸ This sequencing study provided important new insights into the genomic landscape of the retinoblastoma, identified novel initiating genetic lesions (*MYCN*), and highlighted the potential importance of epigenetics in retinoblastoma progression (*BCOR*).¹⁴

III. *RB1* INACTIVATION, CHROMOSOMAL INSTABILITY, AND CHROMOTHRIPSIS

To explore the mechanisms of *RB1* gene inactivation in greater detail, the *RB1* locus was analyzed in 46 primary retinoblastomas. SNP 6.0 arrays were used to identify deletions or LOH, and custom DNA capture and Illumina sequencing was performed on all 27 exons to identify single nucleotide variants.¹³ Surprisingly, 10 of the 46 retinoblastoma tumors had no detectable single nucleotide variants and did not have deletion of the *RB1* locus. These 10 tumors and their matched normal germline DNA then were analyzed by whole-genome sequencing. Most of the 10 tumors had complex structural variations (SVs) inactivating the *RB1* gene, and 3 of these tumors had focal chromothripsis spanning the *RB1* locus.¹³ Break-apart FISH and Rb1 immunohistochemistry confirmed *RB1* inactivation and loss of RB1 protein in 9 of 10 tumors.¹³ Only 1 tumor had wild-type *RB1* and nuclear RB1 protein, and that tumor also had an *MYCN* amplification. These data provide confirmation, using whole-genome sequencing, that a rare subset (~1%) of retinoblastomas has wild-type *RB1* and *MYCN* amplification. Therefore, in addition to promoter hypermethylation, nonsense mutations, splice-site mutations, and LOH, we now understand that chromothripsis is a mechanism of *RB1* inactivation in retinoblastoma¹³ (Fig. 1). Importantly, the standard

methods for analyzing *RB1* mutations do not detect chromothripsis. Retinoblastomas with chromothripsis usually have 2 copies of the *RB1* coding region and all exons intact. Only by performing whole-genome sequencing, FISH analysis, and RB1 immunohistochemistry can the retinoblastomas with wild-type *RB1* be unambiguously identified.

One of the major discoveries from the retinoblastoma sequencing studies was that not all retinoblastoma tumors are driven by genomic instability. In earlier studies researchers proposed that *RB1* loss leads to defects in chromosome segregation that result in aneuploidy and chromosomal instability (CIN).¹⁹ Indeed, an increase in kinetochore distance and sister chromatids was observed in the sequenced retinoblastomas; however, many retinoblastomas have remarkably normal karyotypes.¹⁴

This result was surprising because previous culture studies suggested that *RB1* inactivation can contribute to genomic instability or CIN.^{19–23} It is possible that retinal cells may be more sensitive to CIN mediated by *RB1* loss and, as a result, cells with instability may die; therefore, chromosomal lesions may not significantly contribute to tumorigenesis. Alternatively, differences with respect to CIN following *RB1* inactivation may occur across species (mouse versus human). This is important because some of the studies suggesting that *RB1* inactivation can contribute to CIN were carried out in genetically engineered mouse cells. A recent study showed that genetically engineered retinoblastoma mouse models exhibit aneuploidy.²⁴ In either case, genetic lesions that accumulate in retinoblastomas as a result of CIN are not required for tumor progression in humans. Genetic lesions, such as *MYCN* amplification, possibly provide a selective growth advantage to retinoblastoma cells but are not required for retinoblastoma progression.

While some human retinoblastomas have more CNVs and SVs than others, this does not necessarily mean they have unstable genomes. A common misconception is that tumors that have large numbers of CNVs and SVs are genomically unstable. Since CIN is a dynamic cumulative process, genome stability cannot be accurately measured at a single time point. Ideally, a tumor should be sampled over the course of several months or years to get a comprehensive view of the genomic landscape and the changes that occur. Sequential sampling of retinoblastoma in patients is impossible because biopsies are not routinely performed and diagnoses are made without histological assessment. However, orthotopic xenografts can be used to study genome stability in retinoblastoma.¹⁴ In one orthotopic retinoblastoma xenograft, whole-genome sequencing and SNP 6.0 analysis showed that there were no significant changes in the genome between the original patient sample and the xenograft tumor that grew continuously for 9 months in the eyes of mice. This suggests that a subset of retinoblastomas has stable genomes, which is consistent with previous retinoblastoma cytogenetic studies using cell lines and xenografts.^{27,28}

IV. THE EPIGENOME OF RETINOBLASTOMA

Epigenetic changes (alterations in DNA structure that influence gene function independent of the underlying DNA sequence) also play a role in retinoblastoma tumorigenesis. Numerous studies have identified a role for *RB1* in regulating many different epigenetic processes, including ATP-dependent chromatin organization, DNA methylation, and histone

modifications.^{29–34} Integrative analysis of DNA methylation, activating and repressive histone modifications, and gene expression data revealed epigenetic deregulation of multiple oncogenes and tumor suppressor genes in retinoblastoma. For example, the spleen tyrosine kinase (*SYK*) proto-oncogene was upregulated in retinoblastoma and was required for tumor cell survival by stabilizing the antiapoptotic protein MCL1 (myeloid cell leukemia sequence 1) (Fig. 2).¹⁴ This was a surprising discovery because SYK is not normally expressed in the developing retina.¹⁴ Since there were no recurrent mutations found in the *SYK* gene, it would have initially been overlooked as a possible driver of tumor progression and a target for retinoblastoma therapy. This is true for the other oncogenes and tumor suppressors that were identified by this integrative analysis. These new data highlight the importance of integrating genetic and epigenetic data to shed light on underlying mechanisms in retinoblastoma progression and lead to new therapeutic discoveries.

V. THE P53 PATHWAY IN RETINOBLASTOMA

The p53 pathway is believed to be inactivated in virtually all human cancers. Approximately half of human cancers have mutations in the *TP53* gene itself, and tumors with wild-type *TP53* often have mutations in other genes in the pathway. One example of p53 pathway deregulation is increased expression of p53 antagonists such as MDM2 and MDM4.^{38–42} Increased expression may be achieved through somatic gene amplification in the tumors or germline polymorphisms at the *MDM2* or *MDM4* locus, which lead to higher basal levels of expression and predispose individuals to cancer.^{39–42} There are also a variety of splice variants of *MDM4* messenger RNAs (mRNAs) that produce more stable forms of MDM4 and subsequently suppress p53.⁴³ Other proposed mechanisms of p53 silencing through MDM2 and MDM4 include changes in the transcriptional regulation of *MDM2*, changes in mRNA stability, perturbations in protein translation mediated by microRNAs, or a combination of several mechanisms. Clearly, this important tumor suppressor pathway is tightly regulated at multiple levels.

Retinoblastomas express wild-type p53,⁴⁴ and cytogenetic studies have indicated that approximately 65% of retinoblastomas have a genetic gain of *MDM4* associated with increased mRNA and protein expression.^{23,45} A more recent gene expression array analysis of 52 human retinoblastomas, however, showed that *MDM4* was expressed at high levels irrespective of the *MDM4* copy number.⁴⁶ These gene expression data are consistent with elevated MDM4 protein levels in retinoblastoma cell lines and primary human orthotopic xenografts.⁴⁷ MDM2 is expressed at lower levels than MDM4 in human retinoblastomas.^{46,47} Transcriptome analysis of primary retinoblastoma revealed that the *MDM4* transcripts are alternatively spliced to produce the MDM4-S and MDM4-A forms of the protein, which are believed to be oncogenic and more stable.⁴⁷ Together, these data further establish the importance of MDM4 in suppressing the p53 pathway in retinoblastoma, but a role for MDM2 cannot be ruled out because it is expressed at low levels in the tumors. Consistent with these data, it has been shown that retinoblastomas are sensitive to MDM2/MDM4 antagonists, and there are ongoing efforts to target these proteins using small-molecule antagonists.^{37,45}

VI. THE RETINOBLASTOMA CELL OF ORIGIN

Human and murine retinoblastoma cells express gene signatures of multiple cell types. At the single cell level they have a hybrid gene expression signature of photoreceptors, amacrine/horizontal interneurons, and retinal progenitor cells (Fig. 3).⁴⁶ The most consistent gene signature observed in both human and mouse retinoblastoma is the amacrine cell-type signature. This is remarkable because these gene signatures are usually incompatible during normal development. Also, many of the epi-genetically deregulated genes in previous integrated epigenomic analyses were these retinal differentiation genes found in the tumor cells.¹⁴ Therefore, it is possible that inactivation of *RBI* leads to a massive perturbation in the epigenome such that oncogenes are upregulated, tumor suppressor genes are down-regulated, and developmental pathways are uncoupled. It is impossible to infer a retinoblastoma cell of origin from these gene expression array studies. More direct methods of visualizing tumor initiation in vivo are required to identify the cells of origin and the molecular pathways that regulate the initiation and progression of retinoblastoma.

VII. CONCLUSION

Since the *RBI* gene was first cloned in 1986, there have been several landmark discoveries in the field.

We have come to understand that retinoblastoma is a simple tumor that requires only a single genetic lesion to initiate disease in most patients. We now understand that a small subset of retinoblastomas can initiate with *MYCN* amplification and have wild-type *RBI*. Importantly, retinoblastomas progress very quickly after the initiating genetic lesion. We also recognize that there are few secondary genetic lesions, and some tumors have no detectable oncogenic lesion beyond *RBI* loss. These tumors progress quickly after *RBI* loss as a result of epi-genetic deregulation of multiple pathways important for tumorigenesis. As our understanding of epigenetic mechanisms for retinoblastoma advances, new therapies may be identified for incorporation in clinical trials, such as histone deacetylase inhibitors.

One of the most important questions that remains unanswered in the field is, Why do children who inherit *RBI* mutations develop retinoblastoma but not other cancers as young children? It is possible that unique retinal development programs or the microenvironment contribute to retinoblastoma susceptibility. Identification of the cell of origin for retinoblastoma would help provide a deeper understanding of the factors that predispose those cells to transformation following *RBI* inactivation. By answering these questions and exploiting the new drug-gable pathways identified through epigenomic profiling, we may be able to more effectively target therapy for children with retinoblastoma.

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ABBREVIATIONS

CIN	chromosomal instability
CNV	copy number variation
FISH	fluorescence in situ hybridization
LOH	loss of heterozygosity
MCL1	myeloid cell leukemia sequence 1
mRNA	messenger RNA
RB1	retinoblastoma tumor susceptibility 1
SV	structural variation
SYK	spleen tyrosine kinase

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FIG. 1.

RB1 Inactivation by chromothripsis. An example of *RB1* inactivation by chromothripsis in a primary human retinoblastoma tumor (SJRB031). **A:** A CIRCOS plot of the SJRB031 genome showing multiple intrachromosomal translocations and loss of heterozygosity (LOH) on chromosome 13. CNV, copy number variation; INDEL, insertion/deletion; SV, structural variation. **B:** Half-oval plot of copy number demonstrating focal chromothripsis spanning the *RB1* gene. **C, D:** Two-color fluorescence in situ hybridization is used to validate chromothripsis, showing separation of the 5' and 3' region (red and green probes, respectively) spanning the breakpoints in the *RB1* gene (blue arrowheads). **D:** In this example, one of the *RB1* alleles is not intact and the other allele has a deletion of the 5' region. Reprinted with permission from Oncotarget.¹³

FIG. 2.

Targeting the SYK pathway in retinoblastoma. SYK expression promotes cell survival through phosphorylation activation of protein kinase C delta (PKCδ) and inhibition of glycogen synthase kinase-3 (GSK3), resulting in stabilization of MCL1.^{35,36} In a human retinoblastoma orthotopic xenograft, inhibiting SYK activity with small-molecule inhibitors (R406 and BAY 61-3606) resulted in caspase-mediated cell death. Targeting the SYK pathway may be a novel therapeutic approach to treat patients with retinoblastoma.

FIG. 3.

Hybrid molecular signature in single retinoblastoma cells. Each class of retinal neurons expresses a distinct gene signature and exhibits specific morphological, neuroanatomic, and neurochemical features. *RB1* inactivation in the human retina results in expression of developmental programs specific to progenitors, rod and cone photoreceptors, and amacrine and horizontal interneurons. Single-cell gene expression profiling reveals that multiple cell type-specific developmental programs are coexpressed in a single human retinoblastoma cell. This progenitor/neuronal hybrid cell type makes it difficult to infer the cell of origin.