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Genetics and Epigenetics of Human Retinoblastoma

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Keywords

retinoblastoma, RB1, genetics, epigenetics, *RB1*

Abstract

Retinoblastoma is a pediatric tumor of the developing retina from which the genetic basis for cancer development was first described. Inactivation of both copies of the *RB1* gene is the predominant initiating genetic lesion in retinoblastoma and is rate limiting for tumorigenesis. Recent whole-genome sequencing of retinoblastoma uncovered a tumor that had no coding-region mutations or focal chromosomal lesions other than in the *RB1* gene, shifting the paradigm in the field. The retinoblastoma genome can be very stable; therefore, epigenetic deregulation of tumor-promoting pathways is required for tumorigenesis. This review highlights the genetic and epigenetic changes in retinoblastoma that have been reported, with special emphasis on recent whole-genome sequencing and epigenetic analyses that have identified novel candidate genes as potential therapeutic targets.

INTRODUCTION

Retinoblastoma is a rare, pediatric cancer of the developing retina that occurs either as an inherited or as a sporadic disease (**Figure 1**). Despite its rarity, it was through the study of retinoblastoma that Knudson (1) formulated the two-hit hypothesis, proposing that retinoblastoma requires two rate-limiting events and explaining the difference in age of diagnosis of bilateral versus unilateral patients (**Figure 2**). Knudson's hypothesis was confirmed when the *RB1* gene was cloned (2) and found to undergo biallelic inactivation in virtually all retinoblastoma tumors (2, 3). Since then, hundreds of genetic lesions in human cancer have been identified. These genetic lesions can be grouped into pathways with direct or indirect mechanistic links to many of the common cellular properties, or hallmarks of cancer, including activation of growth-signaling pathways, evasion of cell death and senescence, acquisition of limitless replicative potential, sustained angiogenesis, local tissue invasion, and metastasis (4). Thus, the rate of cancer progression is related to the kinetics of acquisition of multiple genetic lesions that ultimately confer the essential cellular properties of cancer.

In the retina, *RB1* inactivation confers limitless replicative potential to retinoblasts, and these preneoplastic cells must also evade cell death and senescence, sustain angiogenesis, activate growth-signaling pathways, invade the local ocular tissues, and metastasize. If individual genetic lesions in multiple cancer pathways are required to contribute to retinoblastoma's cellular properties, then they must occur rapidly because biallelic inactivation of *RB1* is rate limiting for tumorigenesis (1).

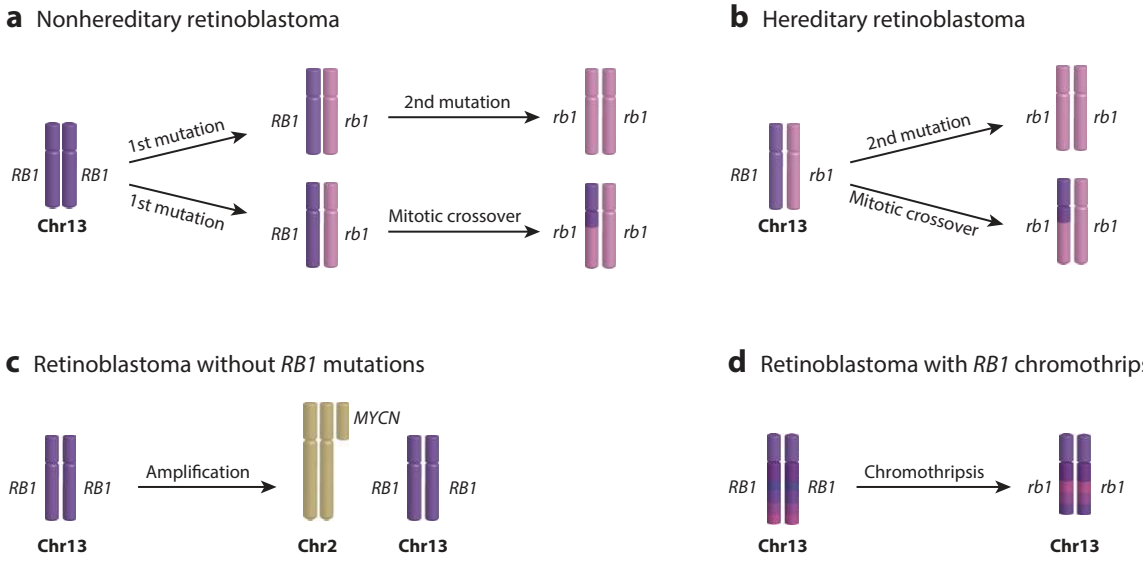

Figure 1

Hereditary versus nonhereditary retinoblastoma. (*a*) In nonhereditary retinoblastoma, noncancerous cells show no defect in either copy of *RB1*; therefore, they require two hits in a single retinal cell lineage to inactivate both copies of the *RB1* gene. These hits can be mutations, promoter hypermethylation, or loss of heterozygosity (LOH). (*b*) In hereditary retinoblastoma, all cells in the body lack one of the functional copies of *RB1*, and tumors occur where the remaining copy is lost or inactivated by a somatic mutation, mitotic crossover (LOH), or promoter hypermethylation. (*c*) A small percentage of patients have no mutations in the *RB1* gene, some of whom will develop the disease through amplification of the *MYCN* gene. (*d*) A small percentage of patients present chromothripsis, including the *RB1* gene.

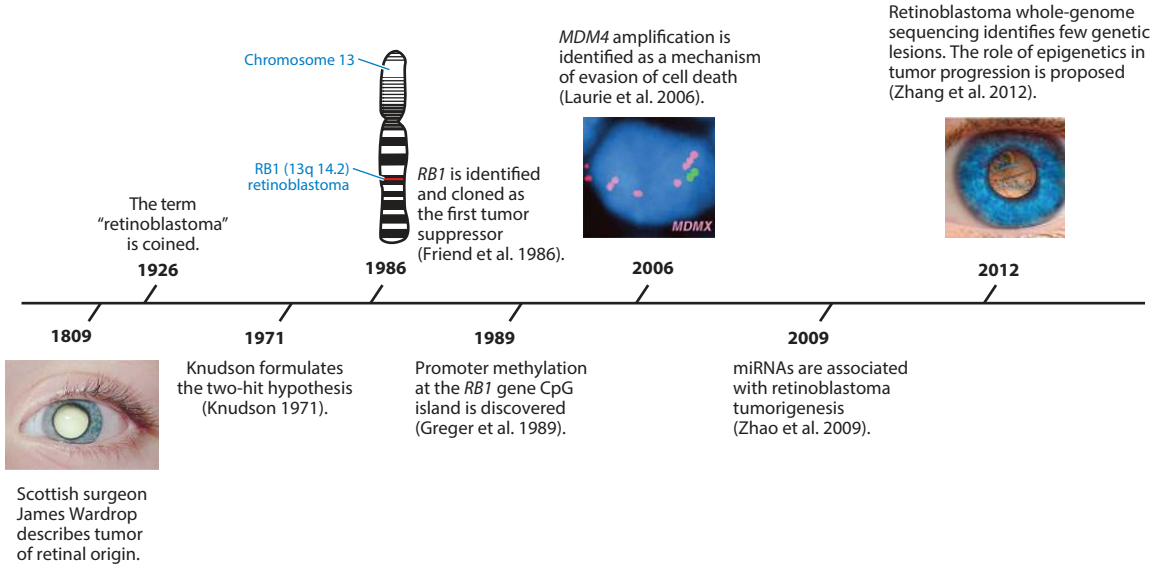

Figure 2

Genetic and epigenetic timeline of retinoblastoma.

RETINOBLASTOMA AND CHROMOSOMAL INSTABILITY

Evidence from molecular, cellular, and cytogenetic studies indicates that the RB1 protein is required for maintaining chromosomal stability (5–7). Therefore, *RB1* inactivation, which leads to chromosome instability (CIN) in cultured cells, could allow the secondary and tertiary mutations in key cancer pathways to be rapidly acquired. Thus, *RB1* inactivation could be rate limiting for retinoblastoma and still allow the cells to acquire all the hallmarks of cancer through genetic mechanisms.

Studies have suggested that loss of RB1 results in mitotic defects that can lead to aneuploidy, which in turn can contribute to CIN (5, 6, 8, 9) (**Figure 3*a***). Indeed, most cancers have suppressed RB pathways, and CIN is an important hallmark of many of the most aggressive forms of sporadic human cancers (10). This relationship between RB1 loss and aneuploidy was further strengthened by recent mechanistic data showing RB has a direct role in maintaining proper chromosome cohesion, which is important for maintaining the fidelity of chromosome segregation during cytokinesis (5). We (11) directly validated the role of RB1 in regulating proper cohesion and segregation of chromosomes in human retinoblastoma. However, three independent xenografts from different patients provided little evidence of cumulative loss of chromosomes over time, and at least some retinoblastomas had no evidence of aneuploidy (11). These findings are consistent with other studies showing that subsets of human retinoblastomas have few chromosomal lesions and that xenografts and populations of cultured retinoblastomas show little genomic variation (12–15).

Recurrent Chromosomal Abnormalities in Retinoblastoma

Isochromosome 6p, a unique chromosomal abnormality, is found in 45% of retinoblastomas (16, 17). Other common chromosomal abnormalities observed in retinoblastoma include gain of regions of chromosome 1q (44%); monosomy of chromosome 16 (18%); gain of 1p (13%) and, less

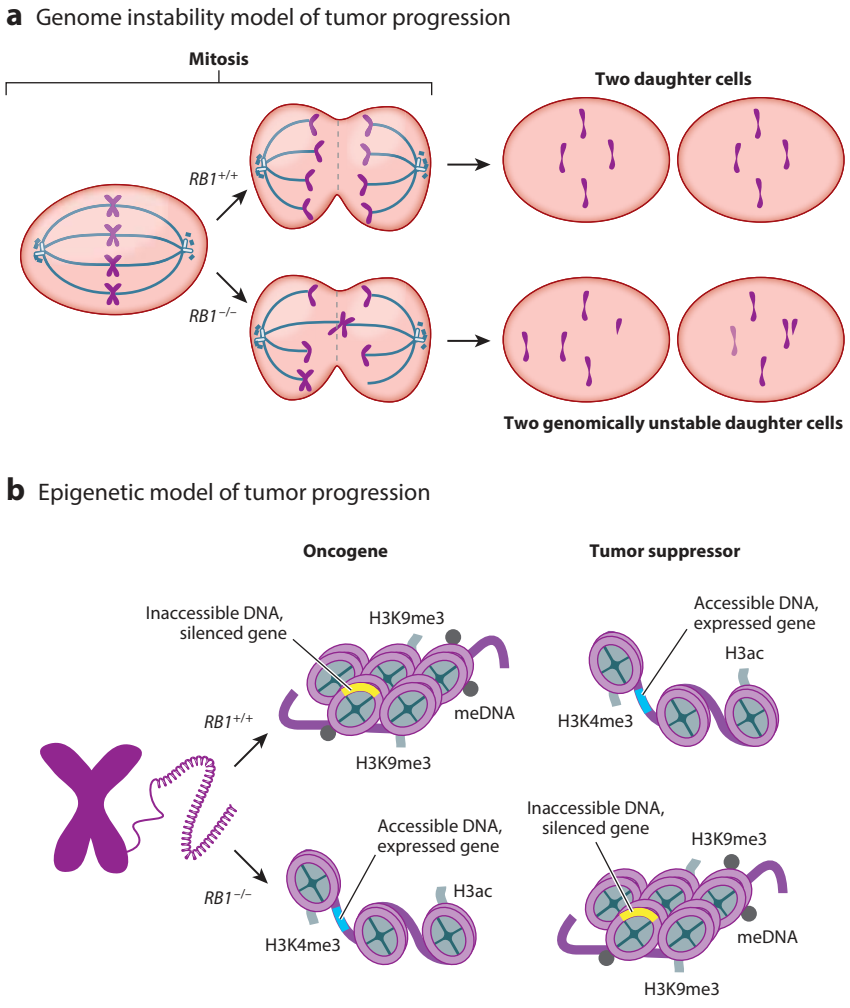

Figure 3

Models of tumor progression in retinoblastoma. (*a*) The role of the RB1 protein in maintaining proper chromosome cohesion makes it important for maintaining the fidelity of chromosome segregation during cytokinesis. Loss of RB1 could result in mitotic defects that lead to chromosome instability, allowing secondary and tertiary mutations in key cancer pathways. (*b*) RB1 has been implicated in regulating most major epigenetic processes. Loss of RB1 leads to chromatin remodeling, in which oncogenes that are normally repressed switch to active chromatin, tumor suppressors that are normally actively transcribed are repressed, or both. Abbreviation: meDNA, methylated DNA.

prevalent, loss of a sex chromosome; alterations in chromosomes 17 and 19; double minutes; and homogenously staining regions (17–20). The karyotype abnormalities found in retinoblastoma have been thoroughly reviewed elsewhere (17, 21–24). Although these reports show that the patterns of chromosomal abnormality changes present in retinoblastoma are consistent, many of these studies were performed using retinoblastoma cell lines, not primary retinoblastoma tumors, opening the possibility for clonal expansion and selection of more aggressive tumor cells that are aneuploid. Indeed, although Potluri et al. (17) identified at least one chromosomal gain or loss

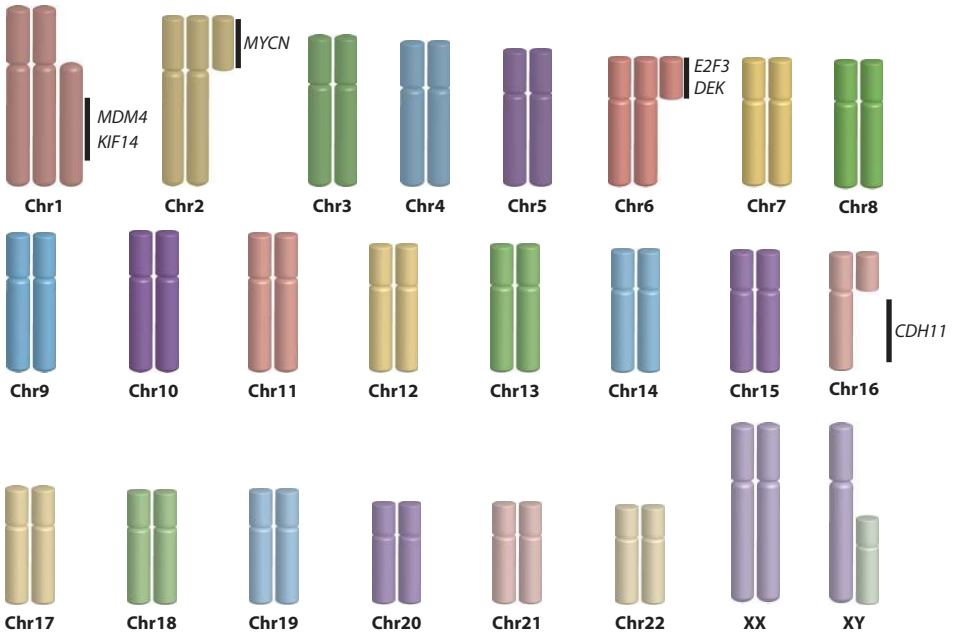

Figure 4

Recurrent genetic aberrations in retinoblastoma. Minimal chromosomal regions of gain and loss that have been validated by new technology and contain genes that are potential drivers of retinoblastoma progression.

in each of the 82 tumor karyotypes reviewed, later comparative genomic hybridization (CGH) studies on primary tumor material always found some tumors with no identifiable aneuploidy (25). The advent of new technology has allowed scientists to validate some key aberrations described in earlier years as well as identify candidate genes in those regions. A comprehensive summary of the karyotype, CGH, and microarray CGH data can be found in Corson & Gallie's review (25). The recurrent gains and losses we review here are minimal regions of gain and loss that have been validated by new technology since their initial report and contain genes that are potential drivers of retinoblastoma progression (**Figure 4**).

Isochromosome 6p: *E2F3* and *DEK*. Gains of 6p are the most common changes in retinoblastoma observed by CGH (54%) (25). The minimal region of gains on chromosome 6 was narrowed to band p22, and further gene expression analyses suggested *DEK* and *E2F3* as the potential targets of 6p gains in retinoblastoma (26, 27). *E2F3*, a family member of the E2F family of transcription factors, is regulated by the RB1 protein and is often overexpressed in cancer (28–31). *DEK* is related to chromatin reconstruction and gene transcription and plays an important role in cell apoptosis. High expression levels of *DEK* have been correlated with numerous human malignancies (32–34).

Chromosome 1q: *KIF14* and *MDM4*. Gains in chromosome arm 1q are the second most common chromosomal change in retinoblastoma (53% by CGH) (25). The minimal common region gained spans 1q31–q32, and two independent CGH studies associated gains in 1q with advanced tumors in older children (35, 36). The genes *KIF14* and *MDM4* have been validated in this region. Gene-specific analysis using fluorescence in situ hybridization confirmed that KIF14 gain was present in all retinomas studied, whereas gain of MDM4 was present in fewer cases (7).

KIF14 is a member of the kinesin superfamily of microtubule-associated motors that play important roles in intracellular transport and cell division (37). *KIF14* has been identified as a prognostic marker for gliomas and breast, lung, and ovarian cancers (38–41). Madhavan et al. (42) confirmed overexpression of *KIF14* in primary human retinoblastoma and showed that patients with an older age at diagnosis express significantly higher levels of the KIF14 protein.

MDM4 inhibits cell cycle arrest and apoptosis by binding to the transcriptional activation domain of the p53 tumor suppressor protein and inhibiting its activity. Analysis of human retinoblastoma revealed that *MDM4* was amplified in 65% of the tumors and negatively correlated with p53 levels (43). This finding provided an explanation for how retinoblastomas bypass the p53 pathway in a cancer that was previously thought to arise from an intrinsically death-resistant cell (43).

Chromosome 16: *CDH11*. CGH studies identified loss of all chromosome 16 or 16q in 32% of retinoblastoma cases and identified *CDH11* as the candidate tumor suppressor in this region (25). Chromosomal losses in this region in invasive ductal and lobular breast carcinomas have also been characterized (44). *CDH11* encodes cadherin-11, an integral membrane protein that mediates calcium-dependent cell-cell adhesion. Loss of *CDH11* in retinoblastoma cells may lead to tumor progression and invasion of the optic nerve (45).

RETINOBLASTOMA GENOMICS AND EPIGENOMICS

Whole-Genome Sequencing of Retinoblastoma

Whole-genome sequencing (WGS) of four retinoblastomas and their paired germline DNA samples showed no genetic lesions in known tumor suppressor genes or oncogenes other than *RB1* (11). More importantly, an orthotopic xenograft derived from one of the primary tumors showed no evidence of clonal variation or new coding-region mutations (11). Results from a larger tumor cohort used to validate and determine the rate of recurrence of these mutations indicated that only *BCOR* was recurrently mutated in retinoblastoma (6 of 46 samples, 13%). Five samples had *BCOR* mutations that resulted in premature truncation of the encoded protein, and one sample had a focal gene deletion. This finding suggests that retinoblastoma's genome is more stable than previously believed and that very few genetic lesions are required for retinoblastoma progression after *RB1* inactivation.

These WGS data were also used to identify somatic structural variations (SVs), including whole-chromosome gains and losses, focal deletions, insertions, inversions, intrachromosomal rearrangements, interchromosomal rearrangements, and regions of loss of heterozygosity. The average number of SVs was 10 per case, ranging from 0 to 24 events per tumor. One of the tumors exhibited a gain of a region of chromosome 2 spanning *MYCN*; in another tumor, the only chromosomal lesion was a gain of chromosome 6p, which was described above. Overall, the number of SVs was much lower than the number of tumors with genome instability (11).

Retinoblastoma-Associated Single-Nucleotide Polymorphisms

TP53 is rarely mutated in retinoblastoma (46); therefore, other mechanisms of p53 inactivation have been studied, including genomic gain and overexpression of *MDM2* and *MDM4*, two key inhibitors of p53 activity (47–49). The first study to report polymorphisms highly associated with the *MDM2* gene in retinoblastoma patients identified a T > G transversion single-nucleotide polymorphism (SNP) at nucleotide 309 in the *MDM2* promoter (rs2279744) (50). This finding

was corroborated by a later study (51); however, neither of these reports assessed whether this SNP correlated with enhanced transcription of mRNA leading to overexpression of MDM2. In fact, a study evaluating *MDM2* gene expression in 52 human retinoblastoma tumors found that *MDM2* was expressed at low levels in all tumor samples (52). Furthermore, a recent study evaluating the same SNP309 found no correlation between the presence of the SNP and *MDM2* gene and protein expression (53).

As mentioned above, cytogenetic studies of retinoblastoma have indicated that approximately 65% of retinoblastomas have genetic gain of *MDM4* (7, 43). Yet gene expression array analysis of 52 human retinoblastomas showed that *MDM4* was expressed at high levels in all tumors irrespective of the *MDM4* copy number (52). These data suggest that MDM4 expression may be elevated in retinoblastoma through mechanisms that are unrelated to the gene copy number. A genotype study of *MDM4* polymorphisms found a significantly higher frequency of SNP rs116197192G in retinoblastoma patients (51). However, no correlative study with gene and protein expression was performed. A different study examining *MDM4* SNP7 (rs1563828) and SNP34091 (rs4245739) found no significant correlation between SNP7 and *MDM4* gene and protein expression, whereas all samples with SNP34091 A/A allele had high levels of MDM4 protein expression (53). Interestingly, the distribution of MDM4 SNP34091 in this retinoblastoma cohort was not significantly different from that of the general population (53).

Epigenetics and Retinoblastoma

RB1 has been implicated in regulating most major epigenetic processes, including microRNA (miRNA) regulation, DNA methylation, histone modification, and ATP-dependent chromatin reorganization (54–59). A recent study showed for the first time that inactivation of *RB1* deregulates multiple oncogenic and tumor suppressor pathways through epigenetic mechanisms (11) (**Figure 3*b***). This epigenetic reprogramming is required for tumorigenesis, and its discovery has led to the identification of novel therapeutic targets against retinoblastoma.

Although the contribution of epigenetic deregulation of cancer pathways has been shown previously, this study in retinoblastoma provided the first example of epigenetics acting as a major driver of cancer in the absence of other genetic lesions (11). Clearly, some retinoblastomas have genetic lesions that may lead to a subtle growth advantage for a subset of tumors, but major genetic lesions that could act as driver mutations were not identified (11). This model could have important implications for cancers other than retinoblastomas and could begin to elucidate the interplay between genetic and epigenetic contributions to tumorigenesis.

MicroRNAs in retinoblastoma. miRNAs encode small noncoding RNA molecules that function in transcriptional and posttranscriptional regulation of gene expression (60). miRNAs function by base-pairing with complementary sequences within mRNA molecules, usually resulting in gene silencing via translational repression or target degradation (61). miRNA deregulation has been associated with several human diseases including retinoblastoma (62–66). Recent studies show several miRNAs as potential candidate components of oncogenic and tumor suppressor networks in retinoblastoma (**Table 1**).

Members of the let-7 family are among the most widely studied tumor suppressor miRNAs. Functionally, let-7 is involved in repressing members of the Ras family, HMGA2, and c-Myc oncogenes (67–70). As for several other cancers, reduced expression of let-7 in retinoblastoma has been reported (71, 72). Furthermore, a significant inverse correlation between let-7 downregulation and HMGA2 overexpression was documented (71). The let-7 family is highly expressed in the retina (71), suggesting that let-7 may be acting as a tumor suppressor by regulation of HMGA2.

Table 1 MicroRNAs differentially expressed in human retinoblastoma

MicroRNA	Method	Reference
let-7e, miR-513, miR-518c, miR-129, miR-198, miR-320, miR-373, miR-492, miR-494, miR-498, miR-503	Microarray analysis	62
miR-34a	qPCR, RT-PCR	65
let-7a, let-7f, miR-2, miR-7, miR-9, miR-16, miR-17a, miR-20a, miR-25, miR-26a, miR-30b, miR-30d, miR-92a, miR-93a, miR-96, miR-99b, miR-101, miR-103, miR-106b, miR-124, miR-143, miR-148b, miR-181a, miR-183, miR-216a, miR-217, miR-378, miR-1246	Microarray analysis	63
let-7c, let-7i, let-7g, miR-10a, miR-10b, miR-28-5p, miR-29a, miR-29b, miR-29c, miR-34a, miR-34b, miR-34c-5p, miR-96, miR-99a, miR-100, miR-124, miR-125b, miR-130a, miR-132, miR-135b, miR-137, miR-142-3p, miR-142-5p, miR-149, miR-181a, miR-182, miR-183, miR-193a-3p, miR-193b, miR-199a-3p, miR-214, miR-224, miR-338-3p, miR-363, miR-374a, miR-375, miR-376a, miR-505	Microarray analysis	66
let-7a, let-7b, let-7c, miR-10a, miR-10b, miR-20a, miR-21, miR-28, miR-29b, miR-30a-3p, miR-30b, miR-30c, miR-30d, miR-99a, miR-99b, miR-100, miR-103, miR-107, miR-124a, miR-125a, miR-125b, miR-133a, miR-136, miR-141, miR-145, miR-146a, miR-155, miR-181a, miR-181b, miR-182, miR-183, miR-190, miR-191, miR-206, miR-210, miR-222, miR-301, miR-302a, miR-302b, miR-320, miR-330, miR-335, miR-342, miR-368, miR-373, miR-380-5p, miR-382, miR-423, miR-433, miR-451, miR-452, miR-491	Microarray analysis	64
miR-17, miR-18a, miR-20b, miR-216a, miR-217, miR-22, miR-224, miR-25, miR-34a, miR-34c-5p, miR-93, miR-106a, miR-106b, miR-129-3p, miR-129-5p, miR-138, miR-139-3p, miR-155, miR-193a-5p, miR-196b, miR-200a, miR-200b, miR-301b, miR-330-5p, miR-342-5p, miR-370, miR-382, miR-429, miR-449a, miR-449b, miR-485-5p, miR-499-5p, miR-504, miR-518f, miR-652, miR-655, miR-758, miR-874, miR-886-5p, miR-889	qPCR	73

Another tumor suppressor miRNA, miR-34a, was identified as differentially expressed in retinoblastoma (65, 73). Studies have demonstrated that p53 transcriptionally activates the miR-34 family (74–77). Loss of miR-34a silencing functions has been identified in several human cancers, making miR-34a an attractive miRNA for therapeutic development (65). Differential expression of miR-34a and miR-34b was observed in two commonly used retinoblastoma cell lines (Y79 and Weri-Rb1) and retinoblastoma tumor samples. This study also suggests that knockdown of miR-34a by anti-miR molecules may result in increased retinoblastoma cell proliferation and chemotherapeutic resistance (65). In support of a role for miR-34a and the p53 pathway, treatment with topotecan in combination with miR-34a inhibition suppressed cell growth in retinoblastoma cells. A combined treatment using topotecan and the p53 activator nutlin-3 led to a similar inhibition of growth (43). Altogether, the evidence supports the notion that miR-34a functions as a tumor suppressor in the normal retina.

One of the best-characterized oncogenic miRNAs is the miR-17∼92 cluster (OncomiR-1). miR-17∼92 belongs to a highly conserved family of polycistronic miRNA genes, including the miR-106a∼363 and miR-106b∼25 clusters (78–80). miR-17∼92 acts to promote proliferation, inhibit differentiation, increase angiogenesis, and sustain cell survival (81). A recent report on retinoblastoma shows that members of the miR-17∼92 cluster are overexpressed in primary tumors and cell lines (63). miR-17∼92 is a target gene of E2F, and loss of *RB1* may lead to increased expression of miR-17∼92 through derepressed E2F activity (82, 83). Another study using miRNA inhibitors investigated the survival function of miR-17∼92 in human retinoblastoma cell lines (RBL15, Weri-Rb1, and Y79) (84). Their results indicated that inactivation of miR-17∼92 suppresses retinoblastoma formation in xenografts and that co-silencing of miR-17/20a and p53

cooperatively decreases the viability of human retinoblastoma cells. The authors suggested that retinoblastoma cells might be addicted to high levels of miR-17∼92a expression as a result of a synthetic lethal interaction with both p53 and RB pathways. Together, these findings identify miR-17 and miR-20a (from the miR-17∼92 cluster) as putative therapeutic targets for the selective prevention and/or treatment of retinoblastoma (84).

Jo et al. (66) investigated the differential expression of miRNAs in retinoblastoma cell lines of different growth patterns and identified other potential miRNA candidates for the treatment of retinoblastoma. In their study, the miRNA expression patterns of the SNUOT-Rb1 cell line, an adherent line with more rapid growth, were compared with those of the Y79 cell line, which displays nonadherent and slower growth (66). Their results show that more than half of the top 10 mRNA targets of miR-10b, miR-29a, miR-29b, miR-29c, and let-7c overexpressed in SNUOT-Rb1 cells, and of miR-34a, miR-34c-5p, miR-124, miR-135b, miR-142-5p, and let-7i overexpressed in Y79 cells, were related to biological processes including cell adhesion, cell cycle, cell death, and cell division, which could affect the growth patterns of cells (66). Altogether, miRNA studies on retinoblastoma have provided novel insights into the pathogenesis of this cancer and potential new therapeutic targets for decreasing oncogenic activity, increasing tumor suppressive functions, and/or promoting differentiation in cancer patients (85).

DNA methylation in retinoblastoma. A role for promoter methylation in retinoblastoma tumorigenesis was first described when methylation of a CpG island (CpG 106) overlapping the *RB1* promoter and exon 1 was discovered in these tumors (86). These initial findings were later confirmed, and hypermethylation of the *RB1* promoter was correlated with decreased gene expression (87, 88). Since then, many studies have reported *RB1* promoter methylation, leading to the first evidence of an epigenetic component in retinoblastoma tumorigenesis (11, 89–91).

Studies on retinoblastoma have also looked at the methylation status of other genes beyond *RB1*. One of the first studies to look at promoter methylation of tumor suppressor genes in retinoblastoma examined the methylation status of nine genes: *p16INK4A*, *MGMT*, *GSTP1*, *RASSF1A*, *APC*, *DAPK*, RAR_β, *CDH11*, and *CDH13* (92). Of these, *RASSF1A* (RAS-associated domain family 1A) was hypermethylated in 59% of tumors analyzed and *APC* in 6%. Other studies have confirmed the *RASSF1A* results (93); however, a functional relationship remains to be elucidated.

Epigenetic silencing of the gene O^6-methylguanine-DNA methyltransferase (*MGMT*) in retinoblastoma was also investigated (93). Promoter hypermethylation of *MGMT* was found in 15% of the retinoblastoma tumors analyzed. In addition, *MGMT* hypermethylation was associated with advanced-stage retinoblastoma (93). This finding could provide a useful prognostic tool for this disease.

A promoter methylation analysis of the tumor suppressor *p16INK4A* showed hypermethylation in most retinoblastoma tumors that also exhibited p16INK4A protein downregulation (94). Furthermore, in over half of these cases, the same alteration in *p16INK4A* expression was observed in the parents of these patients, suggesting that this alteration could be a novel inheritable susceptibility marker for retinoblastoma (94).

Until recently, promoter methylation analyses had focused on hypermethylation of tumor suppressor genes. To evaluate the general epigenetic landscape, we (11) performed a genome-wide promoter methylation analysis comparing 19 primary retinoblastoma tumor samples with 6 normal fetal retinae. In this study, we identified 118 genes that were differentially expressed (at least fivefold) and exhibited correlative DNA methylation profiles. Among these, 35 genes showed promoter hypermethylation and gene expression downregulation and 83 genes showed promoter hypomethylation and overexpression (**Table 2**). Among these 83 genes, we identified *SYK* as a potentially important oncogene in retinoblastoma (discussed below).

Table 2 Genes deregulated by DNA methylation[a]

	Gene symbol				
Upregulated	*ACOT11*	*CSTA*	*KCNS3*	*RBP4*	***SYK***
	AIF1	*CTSS*	*KCNV2*	*RBP7*	*SYNE1*
	AKR1C3	*CYP27B1*	*LY6E*	*RNASE1*	*TCIRG1*
	ANK1	***DDB2***	*LYZ*	*RNASE6*	*TFF1*
	ARHGAP15	*DEFB119*	*MATN2*	*ROBO4*	*TFPI2*
	ASTN2	*DPP4*	*MMP9*	*RS1*	*TGFBI*
	C1S	*EPHX1*	*MS4A6A*	*SERPINA3*	*TGM2*
	C7	*FGL2*	*MYL9*	*SGCD*	*TIMP3*
	CAV2	*FHL2*	*PDE6A*	*SLA*	*TMEM38A*
	CD14	*GBP1*	*PDE6C*	*SLC16A3*	*TULP1*
	CD302	*GIMAP7*	*PDE6H*	*SLC29A1*	*TYROBP*
	CD74	*GNAT2*	*PLA2G5*	*SP100*	*VMO1*
	CEL	*GPX3*	*PPL*	*SPARCL1*	*VWF*
	CFI	*GUCA1A*	*PRAME*	*SPINK4*	*WFDC1*
	CHI3L1	*GUCA1B*	*PTGDS*	*SQRDL*	*XK*
	COL5A1	*GUCA1C*	*PTGS2*	*STAB1*	
	CRISPLD2	*IFI30*	*RAC2*	*STOM*	
Downregulated	*ADAMTS18*	*DCX*	*HRC*	*PABPC5*	*SLC18A2*
	ANK3	*DKK3*	*GPR85*	*PCDH20*	***SOX2***
	BCHE	*ECEL1*	*MAB21L2*	*PLP1*	*SPON1*
	CDH10	*ELAVL4*	*MEG3*	*RAB31*	*ST6GAL2*
	COL2A1	*FOXP2*	*NR2E3*	*RBP1*	*STMN4*
	CPNE4	*GDPD2*	*NRXN3*	*SEZ6L*	*TAC1*
	CUGBP2	*GLT25D2*	*NUAK1*	*SFRP2*	*TTYH1*

[a]Cancer genes are in boldface.

Integrative epigenetic analysis of retinoblastoma. Recently, we conducted an integrative epigenetic analysis using chromatin immunoprecipitation-on-chip, DNA methylation analysis, and gene expression arrays to explore whether epigenetic deregulation of genes or pathways promotes tumorigenesis in retinoblastoma (11). We identified 60 genes that were differentially expressed and exhibited correlative histone modifications and DNA methylation profiles. Among these, 10 are known cancer genes, 3 of which are upregulated: *TFF1*, a secreted gastrointestinal mucosa protein overexpressed in some digestive tumors and breast cancers; *SYK*, a novel proto-oncogene; and *MCM5*, a protein important for DNA replication and cell cycle regulation. The downregulated cancer genes are *ASLC1*, a transcriptional regulator involved in the initiation of neuronal differentiation; *CTNND1*, a catenin involved in cell-cell adhesion; *SOX2*, a transcription factor involved in embryonic development and a cause of syndromic microphthalmia; *ADAMTS18*, a putative tumor suppressor; *GLI3*, a transcription factor and mediator of Sonic hedgehog signaling; *PCDH11X*, a member of a subfamily of cadherins; and *DKK1*, an inhibitor of the WNT signaling pathway (11).

One of the most unexpected findings from this study was the importance of SYK in retinoblastoma. This tyrosine kinase is upregulated in retinoblastoma and is required for tumor survival (11). Retinal progenitor cells and retinal neurons express very low levels of SYK, and SYK has no known function in the developing visual system. Moreover, there are no recurrent genetic lesions

in *SYK* in retinoblastoma to suggest that it is an important driver of tumorigenesis (11). Only by integrating epigenetic analyses and gene expression analysis were we able to identify SYK as an important oncogene in retinoblastoma. Treatment of retinoblastoma cell lines and animals with established orthotopic xenografts with anti-*SYK* short hairpin RNA or a small-molecule inhibitor of SYK reduced tumor growth both in vitro and in vivo (11). This example highlights the value of integrating whole-genome analyses of the genetic and epigenetic features of cancer genomes.

RARE RETINOBLASTOMA GENESIS

Retinoblastoma Without *RB1* Inactivation

Although most retinoblastoma tumors show mutations in both *RB1* alleles (or promoter methylation), a recent report estimated that 2.7% of retinoblastoma tumors present no *RB1* inactivation (95). In 52% of these $RB^{+/+}$ tumors, amplification of the *MYCN* oncogene ($RB^{+/+}MYCN^{A}$) was identified as the potential driver of tumor initiation (95) (**Figure 1*c***). Furthermore, at least one of these $RB^{+/+}$ $MYCN^{A}$ tumors displayed functional RB1 protein (95). The genetic drivers for the remaining $RB1^{+/+}$ tumors remain to be identified but show frequent gain of 19p, 17p and q, 2p, and the telomeric end of 9q (95). Retrospectively, it is not surprising that *MYCN* is implicated in $RB^{+/+}$ retinoblastomas because *MYCN*, like *RB1*, has a role in controlling the activation of the *E2F1*, *E2F3*, and *E2F3* genes (96–98). Interestingly, a $RB^{+/+}MYCN^{A}$ tumor showed no genomic aberrations other than the amplification in *MYCN*, similar to that observed in $RB^{-/-}$ tumors (11). This finding opens the possibility that, regardless of the initiating mutation, epigenetics is the driver of tumor progression in retinoblastoma and E2F transcriptional targets may mediate this process.

Chromothripsis-Driven Retinoblastoma

Chromothripsis is the phenomenon by which tens to hundreds of genomic rearrangements are acquired in a single catastrophic event in localized and confined genomic regions in one or a few chromosomes (99). We have found that, in a very small percentage of retinoblastoma tumors, the *RB1* gene has been inactivated through a chromothriptic event in chromosome 13 (**Figure 1*d***; 100). It remains to be determined whether in these rare cases epigenetics also plays a central role in tumor progression.

CONCLUSIONS

Retinoblastoma represents one of the unusual cancers in which the initiating genetic lesion (*RB1* loss of function) is known. Yet the genetic and epigenetic complexity of retinoblastoma is widely appreciated and evidenced by the intricate network of cellular and epigenetic components that modulate tumor progression. Altogether, the evidence points to a greater role for epigenetic alterations in retinoblastoma. Recent findings highlight how comprehensive genetic and epigenetic analyses of tumors can be integrated to elucidate the mechanisms underlying the progression of retinoblastoma following *RB1* inactivation. These approaches might help uncover the molecular mechanisms of tumor progression and have an impact on the diagnosis and prognosis of, drug response to, and new therapeutic approaches for retinoblastoma.

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