The genomic landscape of retinoblastoma: a review

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Abstract

Retinoblastoma is a paediatric ocular tumour that continues to reveal much about the genetic basis of cancer development. Study of genomic aberrations in retinoblastoma tumours has exposed important mechanisms of cancer development, and identified oncogenes and tumour suppressors that offer potential points of therapeutic intervention. The recent development of next-generation genomic technologies has allowed further refinement of the genomic landscape of retinoblastoma at high resolution. In a relatively short period of time, a wealth of genetic and epigenetic data has emerged on a small number of tumour samples. These data highlight the inherent molecular complexity of this cancer, despite the fact that most retinoblastomas are initiated by the inactivation of a single tumour suppressor gene. Here, we review the current understanding of the genomic, genetic and epigenetic changes in retinoblastoma, highlighting recent genome-wide analyses that have identified exciting candidate genes worthy of further validation as potential prognostic and therapeutic targets.

Keywords
retinoblastoma; cytogenetics; epigenetics; microRNAs; single nucleotide polymorphisms
INTRODUCTION

Retinoblastoma – a genetic disease

Retinoblastoma is a paediatric eye tumour arising in the retina, representing the most common childhood intraocular malignancy.\(^1\) Retinoblastoma was the first disease demonstrating a genetic basis for cancer development,\(^2\) initiated by biallelic inactivation of the \(RB1\) gene.\(^3\) More recently, as discussed later in this review, a second genetic form of retinoblastoma has been discovered: that initiated by amplification of the \(MYCN\) gene.

Retinoblastoma is either heritable or non-heritable. The heritable form can result in tumours affecting either one (unilateral, 60% of all cases) or both (bilateral) eyes, while the non-heritable form leads only to unilateral tumours. All bilateral retinoblastoma is heritable and tends to present at an earlier age, whereas unilateral retinoblastoma is heritable in only a small percentage (15%) of cases.\(^3\)–\(^5\) All heritable retinoblastoma results from biallelic \(RB1\) inactivation; the first \(RB1\) mutation (M1) is constitutional, while the second mutation (M2) occurs somatically in one or more retinal cells.\(^3\) In a small proportion of cases, M1 occurs in one cell of the multicell embryo, resulting in mosaicism in the proband.\(^5\) Most non-heritable retinoblastomas are caused by biallelic \(RB1\) loss where both \(RB1\) mutational events (M1 and M2) arise in a single somatic retinal cell. A small fraction of non-heritable retinoblastoma result from \(MYCN\) amplification with normal \(RB1\), also arising somatically in a single retinal cell (see below). A predisposing constitutional \(RB1\) mutation leads to earlier age of presentation (15 months for bilateral vs. 27 months for unilateral in developed countries).\(^3\)

With an incidence of 1 in 15,000 to 20,000 live births, translating to approximately 9,000 new cases every year worldwide,\(^3,6\) the impact of retinoblastoma on health care systems continues after initial diagnosis and treatment. Constitutional mutation of the \(RB1\) gene predisposes individuals to second cancers later in life, such as lung, bladder, bone, skin and brain cancers.\(^7\) The heritable nature and second cancer susceptibility associated with retinoblastoma translates into a need for life-long follow-up, such as genetic testing and counseling for families and offspring to determine heritable risk, and to monitor for and treat second cancers.

Discovery of a tumour suppressor and initial genomic profiling

Over 40 years ago, Knudson proposed that retinoblastoma was initiated by inactivation of a putative tumour suppressor gene.\(^1\) His mathematical study of the discrepancy in the time to diagnosis between unilateral and bilateral patients led to the hypothesis that two mutational events are rate limiting for the development of retinoblastoma. This postulate was further refined by Comings in 1973 to suggest that mutation of two alleles of a single gene was the cause.\(^8\) These studies informed the discovery of the first tumour suppressor gene, \(RB1\) on chromosome 13q14.\(^9\)–\(^11\) We later confirmed that both alleles of the \(RB1\) gene are indeed mutated in retinoblastoma.\(^12\) Study of the benign, non-proliferative precursor lesion retinoma led us to discover that loss of function of the \(RB1\) gene can initiate retinoma, but is insufficient for the development of retinoblastoma.\(^13\)

We postulated that additional genetic changes, termed M3-Mn in keeping with Knudson’s nomenclature, are required for the progression of benign retinoma to malignant retinoblastoma,\(^13\)\(^,\)\(^14\) Early genomic profiling through karyotype analyses and comparative genomic hybridization (CGH) studies indeed revealed that retinoblastomas also contained many genomic changes, including recurrent gains of chromosome 1q, 2p and 6p, and losses of chromosome 13q and 16q.\(^14\) We and others went on to map specific regions of gains/losses to develop a genomic signature of putative M3-Mn events, subsequently identifying
oncogenes and tumour suppressors in these regions that could facilitate tumour progression.\textsuperscript{15,16}

**New genomic technologies, new horizons**

These initial efforts in the genomic profiling of retinoblastomas led to an explosion in the study of the molecular pathogenesis of this cancer, but the importance of these findings translates beyond retinoblastoma, as many similar genomic changes have been identified in other cancers.\textsuperscript{17–20} Recent advances in genomic (single nucleotide polymorphism [SNP] analysis and next-generation sequencing) and epigenetic (methylation and miRNA) analysis methodologies now allow us a “high-resolution” view of specific aberrations. These techniques open the door to enhanced understanding of retinoblastoma development and progression, moving towards potentially curative therapeutic interventions. However, a higher-resolution view has also uncovered an even more complex genomic landscape in individual retinoblastomas that requires careful validation.

This review summarizes our current understanding of the retinoblastoma genome. We highlight the candidates that have emerged as the most tangible therapeutic targets. We also examine in detail the emerging genome-wide expression, sequencing and epigenetic data that will contribute to a greater understanding of initiation and progression of retinoblastoma, and possibly offer even better targets for prevention and cure in the future (Figure 1).

**RECURRENT GAINS AND LOSSES REVEAL CANDIDATE ONCOGENES AND TUMOUR SUPPRESSORS IN RETINOBLASTOMA**

We have previously reviewed in detail\textsuperscript{14} the first karyotypic, CGH and array CGH (aCGH) studies leading to the identification of minimal regions of gain (MRGs) and minimal regions of loss (MRL) frequent in retinoblastomas, including chromosome gains at 1q32, 2p24, 6p22, and losses at 13q and 16q22–24. New technology has subsequently validated these initial discoveries. Candidate oncogenes in the retinoblastoma genome include \textit{MDM4} (also known as \textit{MDMX}),\textsuperscript{16,21} \textit{KIF14},\textsuperscript{18} \textit{MYCN},\textsuperscript{15} and \textit{DEK} and \textit{E2F3},\textsuperscript{22} plus a candidate tumour suppressor, \textit{CDH11} (Table 1).\textsuperscript{23} There is mounting evidence implicating these genes as drivers in retinoblastoma progression.

**\textit{MDM4} – mouse double minute 4, human homolog (1q32.1)**

\textit{MDM4} is a nuclear protein that binds through its transcriptional activation domain to inhibit p53 activity. It also binds and inhibits the activity of the E3 ubiquitin ligase \textit{MDM2}, which mediates the degradation of p53.\textsuperscript{24} Genomic amplification and overexpression of \textit{MDM4} have been noted in multiple cancers, including glioblastoma, cutaneous melanoma, osteosarcoma, breast and colorectal carcinomas; these changes are more frequent in tumours with wild-type \textit{TP53}.\textsuperscript{25} Amplification and overexpression of \textit{MDM4} has been observed in 65\% of retinoblastomas in comparison to fetal retina,\textsuperscript{21} and could explain inactivation of the p53 pathway without any genetic alteration of the \textit{TP53} gene in retinoblastoma.\textsuperscript{14}

Functional analyses of \textit{MDM4} have shown that in mice lacking \textit{Rbl1} and \textit{Rbl2} (p107), \textit{MDM4} could promote tumorigenesis, and that treatment of retinoblastoma cell line xenografts with the small molecule nutlin-3, which targets the p53-\textit{MDM2/4} interaction, can reduce tumour growth.\textsuperscript{21} Subconjunctival delivery of nutlin-3 in preclinical models of retinoblastoma demonstrated some efficacy in mediating p53-dependent cell death in retinoblastoma.\textsuperscript{26} However recent evidence has shown that only a small number of retinoblastomas overexpress \textit{MDM4} mRNA and protein vs. normal retinal tissues,\textsuperscript{27} suggesting that other mechanisms of p53 pathway inactivation, such as loss of the \textit{p14ARF}
tumour suppressor protein expression could be responsible for progression of some retinoblastomas.28

**KIF14 – Kinesin Family Member 14 (1q32.1)**

KIF14 is a mitotic kinesin and molecular motor essential for the last stages of cytokinesis.29,30 KIF14 is overexpressed in over 50% of primary retinoblastomas. In retinoma lesions, gain of 1q32 is the most prevalent karyotypic abnormality following loss of the RB1 gene. These findings point to KIF14 gain as a possible M3 event.13,18,19 Gene-specific analysis of retinomas via fluorescence in-situ hybridization (FISH) confirmed that KIF14 gain was present in all retinomas studied, while gain of MDM4 was present in a smaller proportion of cases. This highlights the significance of the KIF14 oncogene as a potential driving event in the progression of retinoblastoma.13 Genomic gain of 1q is seen in many other cancer types, including breast, lung, liver, papillary renal cell, esophageal, glioblastoma, ovarian cancers and meningiomas.31 The Kif14 locus was also gained in the SV40 large T antigen-induced model of retinoblastoma (Tag-RB),32 and KIF14 showed low-level amplification in a human retinoblastoma.15

KIF14 mRNA is overexpressed in retinoblastomas,18,33 in TAg-RB, and in many other cancers,17,19,20,32,34,35 Expression correlates with poor prognosis in breast,17 lung,19 and ovarian cancers.20 Stable or transient knockdown of KIF14 significantly reduces proliferation, migration and colony formation in established cancer cell lines in vitro,19,20,36 pointing to a crucial role for KIF14 in tumour formation and progression, perhaps separate from its role in cytokinesis. KIF14 was recently shown to interact in a specific manner with Radil, a crucial mediator of Rap1a–mediated integrin inside-out signalling.36 KIF14 controls the amount of Radil-Rap1a activity at the cell membrane to promote cell adhesion and migration, favouring metastatic progression in breast cancer cells. These studies demonstrate KIF14 as an important oncogene promoting tumorigenesis in multiple cancers, offering opportunities for therapeutic disruption of specific cancer-causing protein interactions.37

There is still debate as to which 1q gene is important in retinoblastoma development. MDM4 and KIF14 are located within 4 Mbp of each other at chromosome 1q32.1. Via high-resolution quantitative multiplex PCR of five sequence-tagged sites spanning 1q25.3–1q41, we identified in retinoblastoma and breast tumours a 3.06 Mbp MRG spanning 1q31.3–1q32.1.18 This region contained KIF14, but excluded MDM4. Via CGH, others have identified MRGs in retinoblastomas encompassing both candidate genes.16,38,39 Two studies concluded that MDM4 was the candidate gene within the MRG; although KIF14 was located within the defined MRG, its genomic expression was not tested.16,40 In any case, the fact that we and other groups have shown gene-specific gain of both MDM4 and KIF14 in retinomas and retinoblastomas13,18,21,41 underscores the importance of both genes in this 1q region of gain in the pathogenesis of retinoblastoma.

**MYCN - v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (2p24.3)**

MYCN encodes N-Myc, a basic helix-loop-helix protein (bHLH) that binds with other bHLH proteins, acting as a transcription factor to control the expression of cell cycle genes that promote proliferation.42 It is frequently amplified in tumours of neuroectodermal origin, including neuroblastoma, retinoblastoma, glioblastoma, medulloblastoma, rhabdomyosarcoma and small cell lung carcinoma,42–49 and is associated with poor prognosis in neuroblastoma.50 MYCN is also amplified in the archetypal retinoblastoma cell line Y79,51 and has been reported amplified in approximately 3% of primary retinoblastomas.14,15
Through an international collaboration with four other clinical RB1 testing centres, we have recently discovered a subset of retinoblastomas that possess a wild type RB1 gene and a full-length, functional pRb protein (RB1+/+, as evidenced by presence of hypo- and hyperphosphorylated forms of pRb that bind to E2F1), but demonstrate high-level amplification of the MYCN gene (28 to 121 copies; MYCN^A). These RB1+/+, MYCN^A tumours, which represent approximately 1% of all retinoblastomas, have a relatively stable genome by aCGH, apart from MYCN amplification. The frequency of copy number alterations was significantly reduced in RB1+/+, MYCN^A tumours vs. RB1−/− or RB1+/− tumours. The minimal amplicon was found in two primary tumours to contain only the MYCN gene, in contrast to previous studies in RB1−/− retinoblastoma and neuroblastoma tumours where MYCN co-amplified with genes NAG and DDX1.

Of 15 RB1+/+, MYCN^A tumours evaluated, three showed unusual changes at chromosome 17q (17q21.3-qter or 17q24.3-qter gain), while two tumours showed 11q loss. Both regions are commonly altered in neuroblastoma, but rare in RB1−/− retinoblastoma. Other changes included gains at 14q and 18q, and losses at 11p. These unilateral, MYCN^A tumours possess histologic features similar to neuroblastoma (large prominent nucleoli) and present with large, invasive tumours at a young age (4 to 5 months) compared to RB1-inactivated, unilateral, non-familial retinoblastoma (27 months). This new subset of retinoblastoma challenges the dogma that this cancer is always initiated by the loss of both copies of the RB1 gene. These findings have immediate clinical impact, as patients with RB1+/+, MYCN^A tumours have MYCN amplification only in the tumour cells. These children have no special risk for retinoblastoma or second cancers later in life, and may benefit from future MYCN-directed therapies to save vision.

### DEK - oncogene, DNA binding (6p22.3), and E2F3 – E2F transcription factor 3 (6p22.3)

Genes for DEK, a chromatin remodelling factor and histone chaperone protein, and E2F3, a pRb-regulated transcription factor crucial in cell cycle control, are located within a small region on chromosome 6p22.3. Genes on 6p are frequently gained (54% by CGH) and overexpressed in retinoblastomas, manifest as an isochromosome 6p (i(6p)). However, spectral karyotyping and multicolour banding analyses identified novel 6p rearrangements and recurrent translocations in many retinoblastoma cell lines, pointing to additional mechanisms of gain for the short arm of chromosome 6p and activation of the DEK and E2F3 oncogenes.

Although frequently gained and overexpressed together, there are instances where gain of only one gene is present. In some retinomas, gene-specific gain of DEK is present while E2F3 remains 2-copy, while primary retinoblastomas show a higher proportion of tumours with E2F3 gain (70%) than gain of DEK (40%); this ratio is similar in the TAg-RB mouse model. Furthermore some evidence points to transcriptional regulation of DEK by E2F3, adding a level of complexity to the regulation of expression of these oncogenes in cancer. Both DEK and E2F3 have important oncogenic roles in multiple cancers. DEK was first discovered in acute myeloid leukemia (AML) as a fusion gene with nucleoporin (NUP214) in 1% of the leukemic cells, and is overexpressed in multiple cancers including melanoma, hepatocellular carcinomas, brain tumours, and breast cancers. Furthermore, DEK overexpression may confer stem cell-like properties on cancer cells that facilitate tumour progression and chemoresistance, demonstrating its importance as a tumour-initiating oncogene. E2F3 is gained and overexpressed in bladder, prostate, lung and breast cancers, and siRNA-mediated knockdown of E2F3 in bladder, prostate and breast cancer cells significantly reduced their proliferative capacity. These results point to inactivation of E2F3 as an attractive therapeutic target in multiple cancers.
**CDH11 – Cadherin 11 (16q21)**

CDH11 (also called osteoblast cadherin) encodes a type II classical cadherin, an integral membrane protein that mediates calcium-dependent cell-cell adhesion that is involved in bone development and maintenance. Loss of CDH11 is common in osteosarcoma, and correlates with tumour invasion and metastasis.\(^6^4,^6^5\) CDH11 has also been identified as a candidate tumour suppressor gene in invasive ductal and lobular breast carcinomas\(^6^6\) and is involved in invasive gliomas.\(^6^7\) The CDH11 promoter is methylated and effectively silenced in many different cancer cell lines, demonstrating its role in tumorigenesis.\(^6^8\) Furthermore, hypermethylation of the CDH11 promoter was identified in metastatic cell lines derived from melanomas and head and neck cancers in comparison to their respective primary tumours, strengthening the postulate that loss of CDH11 expression is important in metastatic progression.\(^6^9\)

We identified a hotspot loss of chromosome 16q, and narrowed the MRL to the CDH11 gene, lost in 58% of retinoblastomas tested. We also studied expression of Cdh11 in the TAg-RB murine model, and demonstrated that many TAg-RB tumours exhibited loss of Cdh11.\(^2^3,^3^2\)

Subsequently, Laurie et al. documented that loss of Cdh11 expression correlated with optic nerve invasion in a murine transgenic model of retinoblastoma with functional, retinal progenitor-specific inactivation of p107, pRb and p53 proteins.\(^7^0\) By crossing the TAg-RB mouse with a Cdh11-null mouse, we showed that tumour formation was significantly reduced, and in the tumours that did form, cell proliferation was increased while apoptotic marker expression greatly decreased.\(^7^1\) These results clearly indicate a tumour suppressive role for Cdh11 in retinoblastoma development and progression, at least in mice. However, in addition to loss of 16q22, Gratias et al. Identified, by conventional and matrix CGH, loss of heterozygoty at 16q24, an MRL encompassing the potential tumour suppressor CDH13.\(^7^2\) Loss of this region also associated with intraocular seeding, implicating CDH13, perhaps in addition to CDH11, as an important tumour suppressor in retinoblastoma.\(^7^2\)

**EMERGING TARGETS FROM GENOMIC AND EXPRESSION ARRAY ANALYSES**

**Genomic analyses**

Since our previous review,\(^1^4\) high-resolution aCGH and SNP array analyses of retinoblastomas have revealed novel regions of genomic imbalance pointing to new target genes (Table 2; Figure 1). One study profiled tumour from 10 bilateral and 8 unilateral patients, 2 who had retinoma. In addition to the characteristic genomic changes such as gain at 1q, 2p, and 6p, and losses at 13q and 16q, there were gains in two small regions of chromosome 9 (9q22.2 and 9q33.1) and loss at 11q24.3 in retinoblastomas, along with gains at 6p, 5p and 5q in retinomas.\(^4^1\) The MRGs defined in this study encompassed larger chromosomal regions than were previously characterized (1q12 – 25.3, 6p25.3 – 11.1 and 16q12.1 – 21), but also excluded previously characterized regions, such as 1q32.1. Thus additional candidate genes were identified on 1q such as MUC1, a membrane-bound protein overexpressed in many epithelial cancers that confers resistance to apoptosis, MCL1, a member of the Bcl-2 family of anti-apoptotic proteins (previously shown to be overexpressed in retinoblastoma\(^4^0\)), and SHC1, a signalling adapter molecule that mediates the transforming activity of oncogenic tyrosine kinases, and also identified as a candidate target gene from a previous study.\(^1^6\)

On chromosome 11, the novel candidate ETS1, a well-known transcription factor involved in proliferation, senescence and tumorigenesis was also identified. On chromosome 13q, a recently characterized tumour suppressor gene, ARLTS1, was identified as being within the MRL.\(^7^3\) It encodes a Ras family pro-apoptotic protein, and its loss of function (through
deletion, SNPs and methylation) has been demonstrated in multiple cancers including breast, lung and ovarian cancers.41

In addition to loss of CDH11 on chromosome 16q, the MRL encompassed the tumour suppressor gene RBL2 encoding the RB family member p130. RBL2 was previously identified as an important tumour suppressor gene involved in the progression of human13,74 and mouse retinoblastomas.75 One study found high frequency (close to 60%) loss of 16q22 specifically encompassing the RBL2 gene in 19 primary retinoblastomas, further implicating this gene as an important player in the progression of retinoblastoma.76 Sampieri and colleagues evaluated chromosomal gains common between retinoma and retinoblastoma that included the oncogenes DEK and E2F3, in agreement with previous findings.13 Novel gains of 5p and 5q were also found in both retinoma and retinoblastoma tissues; potential candidate genes identified within these regions included the oncogene SKP2 (p45; 5p13) known to be overexpressed in many cancers and involved in the ubiquitin-mediated degradation of p27, and BIRC1 (on 5q13.2), an inhibitor of apoptosis that suppresses caspase activity.41

Using a whole genome sampling array (WGSA), Ganguly et al. also identified novel regions of gain/loss in 25 unilateral retinoblastomas compared to their matched normal tissues.38 Novel regions of gain included chromosomes 1q44, 3p25, 11q14, 11q25, 14q23, 15q21, 16p13, 17p11, 19q13 and 20q13, while regions of loss included 6p22, 7p21 and 21q2. On chromosome 1q, MDM4 and GAC1, genes involved in the regulation of p53 activity were identified. SMYD3 was identified as a novel target, located on 1q44. It is a histone methyltransferase that regulates the transcriptional activity of the RNA polymerase complex. Another novel gain on chromosome 14q23 identified the target gene CEP170, a protein involved in centriole architecture that gives rise to chromosomal abnormalities during mitosis. SIX1 and SIX4, homeobox proteins, were also identified as novel target genes within this region. Along with CEP170, these genes are all associated with bilateral anophthalmia, further pointing to the potential importance of developmental genes in the formation and progression of retinoblastoma.38 To our knowledge, further expression and functional analyses of these genes in retinoblastoma have not yet been reported.

A recent study by Livide et al. identified a number of novel targets in retinoblastomas.77 Using a methylation specific multiplex ligation probe assay (MS-MLPA), they analysed a total of 39 genes in 12 tumours with corresponding normal retinal tissues, and found alterations in 25 genes.77 These included gains in TNXB (6p21), an anti-adhesion extracellular matrix glycoprotein involved in tumour progression, and deletions in TP53, CDH11, GATA5, CHFR, TP73, IGSF4 and BRCA2, as well as changes in the methylation status of a number of additional genes (see below).

**Microarray expression studies**

Surprisingly few studies have taken a comprehensive look at differential gene expression patterns in retinoblastomas (Table 2). Chakraborty et al. conducted a comparative microarray analysis between 10 retinoblastomas and 3 adult retina samples. They identified deregulated genes in functional classes including the insulin and JAK/STAT signalling pathways, axon guidance, extracellular matrix–receptor interactions, proteasome, sugar metabolism, ribosomes, cell adhesion molecules, and tight junction complexes.78 Confirmatory semi-quantitative RT-PCR analysis validated insulin signalling pathway genes (PIK3CA, AKT1, FRAP1 and RPS6KB1) as significantly upregulated in tumours vs. normal tissues, suggesting that the PI3K/AKT/mTOR/S6K1 signalling pathway is dysregulated in retinoblastoma. Other upregulated genes included CDC25A, a cell cycle progression gene, and ERBB3, involved in cancer development and progression.
LATS2, a serine-threonine kinase and tumour suppressor, and CHFR, a mitotic checkpoint pathway gene, were found downregulated in retinoblastomas. Regional biases of gene expression were also found, where gene expression changes mapped to particular chromosomal regions, including clusters of upregulated genes mapping to chromosomes 16 and 17, and clusters of downregulated genes mapping to chromosome 1. Interestingly, none of the previously validated retinoblastoma candidate genes on chromosomes 1 and 16 (KIF14, MDM4, CDH11) were identified as differentially expressed in this study, perhaps due to the small sample size or use of adult retina as comparator. Nonetheless, these data encourage future testing of known pathway inhibitors for retinoblastoma treatment.

A recent study undertook a microarray expression comparison between matched normal retina and retinoblastoma tissues of 6 patients. Increased expression was seen for 1116 genes, and 837 genes showed decreased expression in tumours vs. normal retina. These genes fell into functional groups including cell cycle regulation, cell death, DNA replication, recombination and repair, cellular growth and proliferation, and cellular assembly and organization. Among these groups, the DNA damage response pathway genes were most differentially expressed and included previously identified players such as breast cancer associated genes BRCA1 and 2, AHR and ATM signalling genes, and G2/M DNA damage checkpoint regulation genes, polo-like kinase (PLK1), E2Fs, and checkpoint kinase 1 (CHK1). The identified kinases are of particular interest, as there are drugs targeting these kinases that could be tested for therapeutic relevance. Interestingly, the authors also found overexpression of cone-cell-specific markers in retinoblastomas, supporting previous reports of a cone progenitor cell of origin for retinoblastoma, or that cone differentiation is a “default” pathway in failed retinal differentiation.

SINGLE NUCLEOTIDE POLYMORPHISMS ASSOCIATED WITH DEVELOPMENT OF RETINOBLASTOMAS

Since TP53 is rarely mutated in retinoblastoma, other mechanisms of p53 inactivation in these tumours have been discovered, including the genomic gain and overexpression of key inhibitors of p53 activity, MDM2 and MDM4 (see above). MDM2 was the first modifier gene identified in retinoblastoma (Table 3), when Castera et al. identified a T>G transversion SNP at nucleotide 309 in the MDM2 promoter (rs2279744) to be highly associated with the incidence of bilateral and unilateral retinoblastoma in RB1 mutation carrier families. This allele confers enhanced transcription of mRNA leading to overexpression and accumulation of the MDM2 protein, effectively abrogating the function of the p53 protein.

The p.Arg72Pro substitution in p53 protein (c.215G>C,) decreases the ability of p53 to induce apoptosis, in essence causing functional inactivation. In development of retinoblastoma, a significant association of the Pro/Pro variant of p.Arg72Pro has been documented, while only a weak negative association was seen with MDM2–309.

MDM4, another key regulator of p53 activity found to be gained and overexpressed in retinoblastomas (see above) is also a genetic modifier in retinoblastoma. Genotype studies of 104 retinoblastoma patients found that both the MDM2 rs2279744G (vs T) and MDM4 rs4252668C (vs T) SNPs were present at a higher frequency in control patients, while MDM2 rs2279744TG and GG genotypes, and the MDM4 rs116197192G allele were present at high frequency in retinoblastoma patients and associated with poor survival.

To elucidate the relationship between MDM2/4 SNPs and gene/protein expression, McEvoy and colleagues performed sequencing and expression analysis for MDM2/4 in 44 retinoblastomas and 3 orthotopic xenografts derived from primary tumours, in comparison
to normal blood and retina tissues. The MDM2-309 and MDM4-7 (rs1563828C>T) SNPs did not correlate with elevated gene expression in retinoblastomas. However, the orthotopic xenografts demonstrated high levels of MDM4 protein associated with the MDM4 SNP 34091C>A. This variant was first documented in ovarian cancer to cause high overexpression of MDM4 due to loss of regulation by miR-191. The orthotopic xenografts had the 34091AA genotype, and this genotype was associated with high levels of expression of the MDM4 protein while maintaining mRNA levels similar to those in fetal retina. These results suggest that SNPs may enforce functional changes in tumour suppressive pathways to promote tumorigenesis in retinoblastoma.

The first whole genome sequencing (WGS) study of retinoblastomas was recently presented (Table 3). Zhang et al. characterized the genetic and epigenetic alterations of 4 primary retinoblastomas along with matched normal tissues. They validated 668 somatic sequence mutations and 40 structural variations, with on average 167 mutations per case, but only 11 genes were found to harbour mutations leading to amino acid changes. Their calculated mutation rate was 15-fold lower than in other tumour types, except for AML. The only structural variations that were identified were loss RB1 and gain of MYCN, suggesting very few genomic changes are present after loss of the RB1 gene. These results diverge with many other reports demonstrating genomic instability and presence of characterized genomic alterations following loss of RB1 (see above). The 11 genes containing somatic mutations were further sequenced in 46 retinoblastomas. BCOR, a transcriptional corepressor involved in BCL6 repression, was the only gene that showed recurrent mutation in 13% (6 out of 46) of cases. As BCOR mutations are recurrent in AML patients, and it is expressed in the developing retina, this gene may be an important player in some retinoblastomas.

**EPIGENETIC CHARACTERIZATION OF RETINOBLASTOMAS**

**Methylation analyses of retinoblastomas**

Methylation of the RB1 promoter was first demonstrated in 1989 by Greger et al., who identified CpG 106, an island overlapping the promoter and exon 1, to be methylated in some retinoblastomas, thus silencing gene expression. Since then, multiple CpG islands within the RB1 promoter and gene have been identified and characterized in retinoblastomas, demonstrating an epigenetic component to RB1 inactivation and subsequent development of retinoblastoma. Methylation of the RB1 promoter is the causative M1 in 8% of unilateral non-germline tumours.

Aberrant methylation of additional genes has also been shown in retinoblastomas (Table 4). RASSF1A, a tumour suppressor involved in microtubule stability, is inactivated by promoter hypermethylation in anywhere from 59 to 80% of retinoblastomas in comparison to normal retinal tissues. It is inactivated by methylation in multiple cancers. MGMT, encoding an O6-alkylguanine-DNA alkyltransferase, was also found hypermethylated, but in a smaller proportion of retinoblastomas (58% and 35% in two studies). p16INK4A (CDKN2) has long been implicated as a tumour suppressor in retinoblastoma development. Recently, Indovina and colleagues studied p16INK4A expression and promoter methylation in a cohort of retinoblastomas along with peripheral blood from both patients and their parents. Fifty-five percent of retinoblastoma patients showed a downregulation of p16INK4A expression in blood. In over half of these, one of the parents possessed the same downregulation of p16INK4A in their blood cells. Interestingly, methylation analysis of the CDKN2 promoter in this cohort revealed that patients and parents harbouring the same alteration showed promoter hypermethylation, suggesting that this alteration could be heritable, and therefore could become a novel susceptibility marker for these patients.
Additionally, these results begin to provide a basis for the investigation of demethylating agents for therapeutic interventions.\textsuperscript{2,96}

Livide et al. recently identified a novel set of hypermethylated genes in multiple retinoblastomas, in addition to confirmation of hypermethylation in previously identified genes \textit{MGMT}, \textit{RB1} and \textit{CDKN2}.\textsuperscript{77} Hypermethylation was found in 7 novel genes, including \textit{MSH6} (50%), a post-replication DNA repair mismatch gene commonly mutated in cancer; \textit{CD44} (43%), a cell surface glycoprotein involved in cell-cell and cell-matrix interactions; \textit{PAX5} (42%), a member of the paired box family of transcription factors involved in developmental processes that is deregulated in lymphomas; \textit{GATA5} (25%), a transcription factor involved in cardiac smooth muscle cell diversity; \textit{TP53} (8%); \textit{VHL} (8%), a tumour suppressor involved in the predisposition to Von Hippel-Lindau syndrome via the ubiquitin-mediated degradation of HIF1; and \textit{GSTP1} (8%), a glutathione \textit{S}-transferase enzyme playing a role in susceptibility to many diseases including cancer.\textsuperscript{77} Although these studies were done on microdissected tumour and matched normal retina, it remains possible that observed methylation “changes” reflect the methylation status of the undefined retinoblastoma cell of origin.

Zhang et al. conducted a chromatin immunoprecipitation-on-chip and methylation analysis of 4 primary retinoblastomas and one orthotopic xenograft.\textsuperscript{88} They identified a total of 104 genes that were differentially expressed and which also exhibited correlative histone modifications in retinoblastomas when compared to normal tissues. Only 15 of these genes have been identified as known cancer genes.\textsuperscript{88} Upregulated genes with activating histone modifications included \textit{TFF1}, a secreted gastrointestinal mucosa protein overexpressed in some digestive tumours and breast cancers; \textit{SYK}, a novel proto-oncogene involved in breast cancer; and \textit{MCM5}, important in DNA replication and cell cycle regulation. Downregulated genes with associated inactivating histone modifications included \textit{CTNN\textsubscript{D1}}, a catenin involved in cell-cell adhesion; \textit{SOX2}, involved in embryonic development and a cause of syndromic microphthalmia; and \textit{ADAMTS18}, thought to act as a tumour suppressor. The authors validated the proto-oncogene \textit{SYK}, a druggable kinase.\textsuperscript{88} This kinase has no documented role in the developing retina, but has importance in several haematological malignancies.\textsuperscript{88,91} The \textit{SYK} promoter showed high activating histone and RNA polymerase binding activity, and real-time RT-PCR and immunohistochemical analysis of primary tumours and xenografts demonstrated high SYK expression. Treatment of retinoblastoma cell lines and animals with established xenografts with anti-\textit{SYK} shRNA or a small-molecule SYK inhibitor reduced tumour growth both \textit{in vitro} and \textit{in vivo}, suggesting a potential new therapeutic target for retinoblastoma.

Differential microRNA expression in retinoblastomas

MicroRNAs (miRNAs) are a large class of small non-coding RNAs that regulate gene expression by targeting mRNAs to either inhibit transcription or destabilize the transcript, effectively downregulating protein expression. miRNAs have been implicated in many crucial cellular pathways in normal and cancer cells, and their role in retinoblastomas has only recently been examined (Table 5 and Table 6).

We were the first to profile miRNA expression in retinoblastoma by microarray,\textsuperscript{97} and showed substantial downregulation of let-7b in three tumours versus normal retina, as well as decreased let-7c, miR-24, miR-125b, miR-191, miR-181a and miR-423. No miRNA was uniformly overexpressed in these samples. Downregulation of let-7b was confirmed in a larger cohort by qPCR, and putative let-7b target genes \textit{CDC25A} and \textit{BCL7A} were upregulated in tumours. Importantly, this overexpression was abrogated by exogenous let-7b. Mu et al. recently confirmed that the let-7 family was highly expressed in retinal tissues, with significantly decreased expression in 39% of primary tumours tested.\textsuperscript{98}
Furthermore, a significant inverse association between let-7 expression and expression of high mobility group proteins HMGA1 and HMGA2 was found, indicating that let-7 may be acting as a tumour suppressor in retinoblastoma. let-7 has been documented to regulate the expression of HMGA2.91

Subsequent studies identified additional miRNAs involved in the progression of retinoblastoma (Table 5). One microarray analysis of human retinoblastoma tissues identified 13 highly expressed miRNAs compared to normal retina, including miR-373, previously identified as a putative “oncomiR” in testicular germ cell tumours.91,99 Another tumour suppressor miRNA, miR-34a, was identified as differentially expressed in retinoblastomas and cell lines.100 Loss of miR-34a has been shown in various cancers, including brain, breast, colorectal, lung, pancreatic, and prostate.91 miR-34a demonstrated variable expression in two primary retinoblastomas and two cell lines, and treatment of Y79 and WERI-Rb1 cells with miR-34a decreased cell growth and increased apoptosis; this effect was improved with topotecan co-treatment, suggesting that miR-34a acts as a tumour suppressor in retinoblastoma cells, mediating proliferation and chemotherapeutic resistance.100

Conkrite and colleagues demonstrated that the miR-17~92 cluster, one of the first “oncomiR” clusters to be characterized, is a potential therapeutic target in retinoblastoma.101 By studying a murine model of retinoblastoma initiated by mutations in Rb1 and Rbl1, they identified through aCGH a focal amplification in 14qE (syntenic to human 13q32) which contains the miR-17~92 cluster. One mouse tumour exhibited a 14 Mbp gain at 5qG2–3, containing the miR-17~92 paralog, miR106b~25.101 miR-17~92 has been implicated in tumorigenesis, as it promotes proliferation, inhibits differentiation, and increases angiogenesis in lymphomas.91 However, out of 32 human retinoblastomas, only one demonstrated low-level gain of the human miR-17~92 locus, but 4 tumours exhibited low-level gain of miR106b~25, thus suggesting relevance of this cluster to both murine and human diseases. Interestingly, microarray analyses and deep sequencing (RNA-Seq) of both murine and human retinoblastomas revealed high expression of miR-17~92. Overexpression of miR-17~92 in mice was by itself insufficient for tumour formation, however deletion of both Rb1 and Rbl1 and overexpression of miR-17~92 accelerated retinoblastoma development, with presence of frequent brain metastases.101 miR-17~92 increased the proliferative capacity of pRb/p107-deficient cells by suppressing p21Cip1 expression, thereby promoting proliferation and development of retinoblastoma.

Nittner et al. extended these findings to human cell lines, investigating whether miR-17~92 could promote survival in human cells with inactivated RB1. In RBL15, WERI-Rb1 and Y79 retinoblastoma cell lines, inhibition of miR-17~92 suppressed tumour formation in xenografts. Co-silencing of miR-17/20a and p53 was cooperative in decreasing the viability of human retinoblastoma cells.102 Together, these studies identify miR-17~92 as an interesting therapeutic target worthy of further study.

Retinoblastoma cell lines have also been probed to assess the biological implications of some differentially expressed miRNAs. Jo et al. conducted an expression analysis of two retinoblastoma cell lines with different growth properties.103 The authors found that the miRNA expression pattern of SNU/OT-Rb1 cells (overexpressing miR-10b, miR-29a, and let-7c), which are adherent and demonstrate rapid growth, is completely different from the miRNA expression pattern of Y79 cells (overexpressing miR-34a, miR-124, and miR-135b), which are non-adherent and slowly growing. Bioinformatics analysis of these differentially expressed miRNAs showed direct relation to gene expression control of biological processes such as cell adhesion, proliferation, death and division,103 suggesting targets that could be validated in clinical samples.
For example, a target for miR-10b is the tumour suppressor HOXD10. Its transcriptional inhibition leads to activation of Rho kinase activation and tumour cell invasion. Using a novel functional linkage method of analysing publicly available STRING protein-protein interaction data, Li et al. identified a set of 53 differentially expressed miRNAs in retinoblastomas in comparison to normal retinal tissue. This study confirmed previously identified miRNAs, such as let-7a, let-7c, miR-20a, miR-124, and miR-125, but also identified novel miRNAs such as miR-21, −155 and −301.

Expression analysis of 12 retinoblastomas identified several other novel miRNAs that may play a role in tumorigenesis. Through microarray analysis, Martin et al. identified 41 differentially miRNAs as compared to normal retinal tissues. They went on to validate five of these (miRs-129-3p, −129-5p, −382, −504, and −22) as highly downregulated in 12 primary tumours, two cell lines and two mouse retinoblastomas. Many of these identified miRNAs have demonstrated roles in other cancers. For instance, the most downregulated miRNA, miR-129, is involved in cell cycle regulation by inhibiting the cell cycle regulatory genes CDK4 and CDK6, and is silenced in gastric, esophageal and colorectal cancers.

Another interesting target, miR-382, is involved in repressing MYC transcription. In osteosarcoma, loss of miR-382 leads to upregulation of c-Myc, in turn activating the miR-17−92 cluster involved in human and mouse retinoblastomas (see above). However, when miRNA expression levels were associated with clinical variables, no significant correlations could be found with optic nerve invasion or intraocular neovascularization, warranting the analysis of a greater patient cohort.

Finally, one recent study performed an in silico analysis of the miRNA expression profile of 14 late-stage retinoblastoma patient serum samples with publicly available miRNA expression data on retinoblastomas, to identify miRNA and gene targets that could be used as potential serum biomarkers. A total of 33 miRNAs, including 25 upregulated and eight downregulated miRNAs in both serum and retinoblastoma tumours were identified. Real-time PCR of an additional 20 retinoblastoma serum samples validated a total of five miRNAs, three upregulated (miR-17, miR-18a and miR-20a) and two downregulated (miR-19b, and miR-92a-1). Although the first study of its kind, these results postulate exciting candidate miRNAs for development into serum biomarkers, and will pave the way for other studies to help define predictive biomarkers for retinoblastoma patients.

IMPACT OF THE RETINOBLASTOMA GENOMIC LANDSCAPE ON CLINICAL PRACTICE

As evidenced by the number of studies presented, the genomic landscape of retinoblastoma is becoming increasingly complex. However, most of the studies reviewed here are based on a small number of clinical samples, and therefore the data needs to be functionally validated in cell-based and animal models, as well as clinically validated in larger patient cohorts before being included in standard of care recommendations. That said, ophthalmologists should be aware of other genomic changes beyond RB1 mutation with the distinct potential to affect therapeutic decisions and long-term surveillance and care:

1. MYCN status

Retinoblastoma tumours can now be subdivided into two molecular classes: RB1−/−, and RB1+/+, MYCN+. This latter subset of retinoblastoma tumours has a wild-type, functional RB1 gene, but high level amplification of the MYCN gene present only in the tumour cells. This means that these children have no special risk for retinoblastoma or second cancers later in life, and may not need life-long surveillance. These tumours are highly aggressive, are histologically different, and arise earlier with a very young age of presentation compared...
to RB1−/− retinoblastomas. Because these tumours are highly aggressive, enucleation is likely the optimal therapy. If a MYCN retinoblastoma is confirmed molecularly, any disseminated disease might be treatable with future therapies that target MYCN. Currently, this changes genetic counseling for survivors with this type of tumour, as they carry no additional risk for tumours in the other eye, second cancers, and no risk of affecting the next generation.

2. Candidate genes as surveillance markers and therapeutic targets

Gene-specific copy number gains or losses, and/or changes in gene expression, such as KIF14, MDM4, MYCN, DEK, E2F3, CDH11, miR-17–92, and SYK have demonstrated importance in retinoblastomas via multiple lines of evidence (Table 1). These genes have demonstrated functional importance in cell lines, animal models and patient tumours, and may be associated with progression and/or poor outcomes. These genes could be developed into markers that would facilitate surveillance of tumour recurrence or metastasis (as shown for RB1109, KIF14 and E2F315), as well as being the target of new therapies to treat recurring retinoblastomas.

CONCLUSIONS

The study of retinoblastoma has revealed guiding principles of the molecular initiation and progression of cancer. Similarly, ongoing studies via higher resolution genomic technologies will continue to facilitate our exploration into the molecular intricacies of this model cancer, with the hopes of refining causative molecular pathogenic pathways and offering opportunities for therapy. Next-generation sequencing (NGS) in particular holds the promise of read depth that could possibly identify mechanisms of convergent evolution of gene mutations and gene copy number changes. This type of technology has the potential to reveal the identity of initiating vs. progression changes, facilitating diagnosis and therapeutic management. While NGS is being used as a tool for mutation discovery, its current sensitivity and accuracy for identifying RB1 mutations is still not published, let alone clinically validated. Although NGS technologies promise to be more affordable for clinical mutation testing for retinoblastoma, detection of all kinds of mutation has yet to be demonstrated.

Although RB1 loss is the causative genetic alteration underlying most retinoblastoma development, it is becoming increasingly evident that other genetic, genomic, epigenetic and gene expression events are also necessary for tumour development (Figure 1). Complementing and complicating these studies, the discovery of MYCN+ RB1+/+ retinoblastoma opens new avenues for comparing the molecular progression of this subtype with “classic” retinoblastoma.

Integrating the results from different approaches will contribute to a greater understanding of this cancer. Especially, pan-“omics” approaches incorporating whole-genome sequencing, epigenetics, expression, proteomics and even metabolomics on large numbers of tumours will help define crucial molecular events in retinoblastoma (Figure 2). However, some of these technologies have already begun to open the door to a wealth of genetic, expression, and epigenetic data, revealing an inherent molecular complexity for this disease. Careful scrutiny, compilation of multiple data sources, functional validation in animal and cell culture models, and most important, clinical studies, promise to define clinically relevant associations that can impact care. Importantly, it is noteworthy that most of the studies reviewed here focus on small numbers of tumours with very little associated clinical information. The study of larger cohorts of patient tumours and their clinical data is crucial. Given the wide distribution of retinoblastoma cases worldwide, multi-centre, international collaboration is a necessity to enable robust, powerful studies.
The concept of precision medicine has been applied to clinical retinoblastoma management for more than 20 years. The discovery of the genetic nature of retinoblastoma decades ago led to the development of genetic testing of patients and families at risk, ushering in the concept of individualized medicine to guide patient management, treatments and outcomes, now often across generations of a family. In many respects, the framework is already in place to evolve such individualized testing to incorporate new genetic and genomic technologies into the standard of care, providing refined information on disease severity, molecular pathophysiology, and prognosis for responses to treatments. Only then will we gain a clear insight into the important and targetable tumorigenic pathways of retinoblastoma, many of which will be applicable to other malignancies, as has already been repeatedly demonstrated. Most importantly, individualized testing and analysis will also provide prospective validation of the emerging candidates enabling therapeutic strategies with improved outcomes for retinoblastoma patients.

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Figure 1.
Towards a broadened genomic landscape of retinoblastoma. miRNAs, microRNAs; SNPs, single nucleotide polymorphisms. All patients consented to have their clinical images used for education and research.
Figure 2.
The future of retinoblastoma management in a post-genomic era.
Table 1

Candidate oncogenes and tumour suppressor genes in retinoblastoma. Included genes have multiple lines of evidence (4 or more) supporting involvement

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Symbol</th>
<th>Gene type</th>
<th>Cytoband of minimal region</th>
<th>Change</th>
<th>Frequency of change (%)</th>
<th>Evidence</th>
<th>Reference</th>
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<tr>
<td>Kinesin family member 14</td>
<td>KIF14</td>
<td>oncogene</td>
<td>1q32.1</td>
<td>genomic gain</td>
<td>50</td>
<td>G, Ex, C, A</td>
<td>13, 15, 16, 18–21, 32, 33, 36, 38, 39, 41</td>
</tr>
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<td>Mouse double minute 4, human homolog</td>
<td>MDM4</td>
<td>oncogene</td>
<td>1q32.1</td>
<td>genomic gain</td>
<td>65</td>
<td>G, M, Ex, C,</td>
<td>13, 16, 18, 21, 25, 40, 41, 85–87</td>
</tr>
<tr>
<td>v-myc myelocytomatosis viral related oncogene, neuroblastoma derived</td>
<td>MYCN</td>
<td>oncogene</td>
<td>2p24.3</td>
<td>genomic gain or amplification</td>
<td>13 – 34 (gain) 3 – 30 (amplification)</td>
<td>G, Ex, C, A</td>
<td>14, 15, 32, 51, 52</td>
</tr>
<tr>
<td>DEK oncogene, DNA binding</td>
<td>DEK</td>
<td>oncogene</td>
<td>6p22.3</td>
<td>genomic gain</td>
<td>40 – 54</td>
<td>G, Ex, C, A</td>
<td>13–15, 56, 57</td>
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<td>E2F transcription factor 3</td>
<td>E2F3</td>
<td>oncogene</td>
<td>6p22.3</td>
<td>genomic gain</td>
<td>70</td>
<td>G, Ex, C, A</td>
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<td>Cadherin 11</td>
<td>CDH11</td>
<td>Tumour suppressor</td>
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<td>58</td>
<td>G, Ex, C, A</td>
<td>23, 32, 70, 71</td>
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<td>miR-17–92 cluster</td>
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<td>13q32</td>
<td>genomic gain</td>
<td>15</td>
<td>G, Ex, Ep, C, A</td>
<td>101,102</td>
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<td>spleen tyrosine kinase</td>
<td>SYK</td>
<td>oncogene</td>
<td>9q22</td>
<td>Overexpression</td>
<td>NA</td>
<td>Ex, Ep, C, A</td>
<td>88</td>
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</table>

NA, not available; G, genomic studies (aCGH, MS-MPLA, NGS); M, mutational studies (SNP); Ex, expression studies (RT-PCR, real-time QPCR, microarray, protein in tumours and/or cell lines); Ep, epigenetic studies (methylation, miRNA); C, cell-based assays; A, animal studies (transgenic, xenograft)
Table 2
Emerging targets from recent genomic and expression analyses of retinoblastoma

<table>
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<tr>
<th>Study Type</th>
<th>Gene name</th>
<th>Symbol</th>
<th>Minimal chromosomal region</th>
<th>Study type</th>
<th>Change</th>
<th>Primary tumours studied (#)</th>
<th>Frequency of change (%)</th>
<th>Involved in cancer</th>
<th>Reference</th>
</tr>
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<td>Genomic</td>
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<td>GAC1</td>
<td>1q32</td>
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<td>1q44</td>
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<td>Y</td>
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<td>SIX1</td>
<td>14q23</td>
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<td>N</td>
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<td>MUC1</td>
<td>1q12-q25.3</td>
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<td>22</td>
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<td>41</td>
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<td>MCL1</td>
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<td>18</td>
<td>22</td>
<td>Y</td>
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<td>SHC (Src homology 2 domain containing) transforming protein 1</td>
<td>SHC1</td>
<td>1q12-q25.3</td>
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<td>18</td>
<td>22</td>
<td>Y</td>
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<td>S-phase kinase-associated protein 2, E3 ubiquitin protein ligase</td>
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<td>5p15.33</td>
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<td>nucleotide-binding oligomerization domain, leucine rich repeat and BIR domain containing 1</td>
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<td>5q13.2</td>
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<td>16q12.1-q21</td>
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<td>12q24.33</td>
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<td>16</td>
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<td>phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha</td>
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<td>Primary tumours studied (#)</td>
<td>Frequency of change (%)</td>
<td>Involved in cancer</td>
<td>Reference</td>
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<td>NA</td>
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<td>microarray</td>
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NA, not available; WGS, whole genome sampling array; aCGH, array comparative genomic hybridization; MS-MLPA, methylation-specific-multiplex ligation probe assay
### Table 3

SNPs and gene mutations associated with retinoblastoma

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Symbol</th>
<th>SNP/mutation</th>
<th>RefSeq gene</th>
<th>Position</th>
<th>Nucleotide change</th>
<th>Amino acid change(s)</th>
<th>Effect of mutation</th>
<th>Risk</th>
<th>Odds Ratio</th>
<th>P value</th>
<th>Number of patients studied</th>
<th>Reference</th>
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<tbody>
<tr>
<td>mouse double minute 2</td>
<td>M2</td>
<td>2279</td>
<td>NG_0</td>
<td>56</td>
<td>T&gt;G</td>
<td>intronic</td>
<td>enhancement of mRNA expression associated with incidence of retinoblastoma</td>
<td>4</td>
<td>0.0</td>
<td>0.01</td>
<td>336</td>
<td>82</td>
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<tr>
<td>mouse double minute 2</td>
<td>M2</td>
<td>2279</td>
<td>NG_0</td>
<td>56</td>
<td>T&gt;G</td>
<td>intronic</td>
<td>enhancement of mRNA expression associated with incidence of retinoblastoma</td>
<td>0.6</td>
<td>0.0</td>
<td>4</td>
<td>104</td>
<td>85</td>
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<td>mouse double minute 4</td>
<td>M4</td>
<td>1161</td>
<td>9719</td>
<td>26</td>
<td>T&gt;G</td>
<td>intronic</td>
<td>missense mutation in predicted casein kinase II ligation site associated with incidence of retinoblastoma</td>
<td>5.41</td>
<td>0.0</td>
<td>0.01</td>
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<td>mouse double minute 4</td>
<td>M4</td>
<td>4245</td>
<td>739</td>
<td>38</td>
<td>G&gt;A</td>
<td>D153G</td>
<td>protein stabilization, insensitive to miR-191 mRNA inhibition associated with retinoblastoma xenografts</td>
<td>NA</td>
<td>N</td>
<td>A</td>
<td>44 (plus 3 orthotopic xenografts)</td>
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<td>tumor protein 53</td>
<td>TP</td>
<td>1042</td>
<td>NM 00054</td>
<td>41</td>
<td>G&gt;C</td>
<td>R72P</td>
<td>decreased induction of apoptosis P/P variant associated with incidence of retinoblastoma</td>
<td>3.58</td>
<td>0.0</td>
<td>0.02</td>
<td>111</td>
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<tr>
<td>BCL6 corepressor</td>
<td>BC-OR</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>premature truncation of protein mutations, including 1 deletion, present in 6/46 tumours (13%)</td>
<td>NA</td>
<td>N</td>
<td>N</td>
<td>46</td>
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</table>

NA, not available
Table 4

Differentially methylated genes in retinoblastoma

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Symbol</th>
<th>Methylation status in tumours</th>
<th>Assay</th>
<th>Frequency (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ras association (RalGDS/AF-6) domain family member 1</td>
<td>RASSF1A</td>
<td>hypermethylation</td>
<td>MSP</td>
<td>82</td>
<td>92</td>
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<tr>
<td></td>
<td>RASSF1A</td>
<td>hypermethylation</td>
<td>MSP</td>
<td>59</td>
<td>93</td>
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<tr>
<td></td>
<td>RASSF1A</td>
<td>hypermethylation</td>
<td>MSP</td>
<td>60</td>
<td>94</td>
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<tr>
<td></td>
<td>MGMT</td>
<td>hypermethylation</td>
<td>MSP</td>
<td>58</td>
<td>77</td>
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<tr>
<td>O-6-methylguanine-DNA methyltransferase</td>
<td>MGMT</td>
<td>hypermethylation</td>
<td>MSP</td>
<td>35</td>
<td>92</td>
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<td></td>
<td>MGMT</td>
<td>hypermethylation</td>
<td>MSP</td>
<td>35</td>
<td>95</td>
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<tr>
<td>cyclin-dependent kinase inhibitor 2A</td>
<td>p16INK4A</td>
<td>hypermethylation</td>
<td>MSP</td>
<td>91</td>
<td>96</td>
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<td>mutS homolog 6</td>
<td>MSH6</td>
<td>hypermethylation</td>
<td>MS-MLPA</td>
<td>50</td>
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<td>CD44 antigen</td>
<td>CD44</td>
<td>hypermethylation</td>
<td>MS-MLPA</td>
<td>43</td>
<td>77</td>
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<tr>
<td>paired box 5</td>
<td>PAX5</td>
<td>hypermethylation</td>
<td>MS-MLPA</td>
<td>42</td>
<td>77</td>
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<td>GATA binding protein 5</td>
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<td>MS-MLPA</td>
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<td>tumor protein 53</td>
<td>TP53</td>
<td>hypermethylation</td>
<td>MS-MLPA</td>
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<tr>
<td>von Hippel-Lindau tumor suppressor</td>
<td>VHL</td>
<td>hypermethylation</td>
<td>MS-MLPA</td>
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<td>77</td>
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<tr>
<td>glutathione S-transferase pi 1</td>
<td>GSTP1</td>
<td>hypermethylation</td>
<td>MS-MLPA</td>
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<tr>
<td>trefoil factor 1</td>
<td>TFF1</td>
<td>hypomethylation</td>
<td>Human methylation BeadChip</td>
<td>NA</td>
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<td>Spleen tyrosine kinase</td>
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<td>Human methylation BeadChip</td>
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<tr>
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<td>Human methylation BeadChip</td>
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<td>catenin (cadherin-associated protein), delta 1</td>
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<td>hypermethylation</td>
<td>Human methylation BeadChip</td>
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<td>sex determining region Y</td>
<td>SRY</td>
<td>hypermethylation</td>
<td>Human methylation BeadChip</td>
<td>NA</td>
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<td>SRY (sex determining region Y)-box 2</td>
<td>SOX2</td>
<td>hypermethylation</td>
<td>Human methylation BeadChip</td>
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<td>ADAM metallopeptidase with thrombospondin type 1 motif, 18</td>
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<td>hypermethylation</td>
<td>Human methylation BeadChip</td>
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MSP, methylation-specific PCR; MS-MLPA, methylation-specific-multiplex ligation probe assay; NA, not available
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<th>miRNAs</th>
<th>Expression in tumours</th>
<th>Method</th>
<th>Primary tumours studied (#)</th>
<th>Frequency of change (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>let-7b, let-7c, miR-24, miR-125b, miR-191, miR-181a, miR-423</td>
<td>downregulated</td>
<td>microarray and qPCR</td>
<td>3</td>
<td>100</td>
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<td>let-7 family</td>
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<td>RT-PCR</td>
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<td>miR-494, let-7c, miR-513-1, miR-513-2, miR-518c, miR-129-1, miR-129-2, miR-198, miR-492, miR-498, miR-320, miR-503, miR-373</td>
<td>upregulated</td>
<td>microarray</td>
<td>9</td>
<td>100</td>
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<tr>
<td>miR-34a</td>
<td>variable</td>
<td>qPCR</td>
<td>2 (plus 2 cell lines)</td>
<td>NA</td>
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<tr>
<td>miR-17-92 and miR-106b-25 (paralog), let-7a, let-7f, miR-2, miR-7, miR-9, miR-16, miR-17a, miR-20a, miR-25, miR-26a, miR-30b, miR-304, 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</td>
<td>differentially expressed</td>
<td>computational</td>
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<td>miR-129-3p, miR-382, miR-304, miR-22, miR-874, miR-139-3p, miR-758, miR-655, miR-129-5p, miR-200a, miR-370, miR-485-5p, miR-193a-5p, miR-330-5p, miR-429, miR-889, miR-499-5p, miR-342-5p, miR-448, miR-200b, miR-196b, miR-518, miR-34c-5p</td>
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<td>microarray</td>
<td>12</td>
<td>100</td>
<td>106</td>
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<td>miR-1305, miR-424, miR-532-3p, miR-663b, miR-633, miR-194, miR-299-3p, miR-142-5p, miR-144, miR-93, miR-545, miR-374a, miR-374b, miR-665, miR-146b-5p, miR-194, miR-892b, miR-32, miR-501-5p, miR-513c, miR-513b</td>
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<td>microarray</td>
<td>12</td>
<td>100</td>
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<td>miR-138, miR-135, miR-106b, miR-216a, miR-217, miR-20b, miR-17, miR-106a, miR-25, miR-652, miR-301b, miR-886-5p, miR-93, miR-34a, miR-18a, miR-49a, miR-449, miR-224</td>
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<td>microarray</td>
<td>12</td>
<td>100</td>
<td>106</td>
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<td>miR-1305, miR-424, miR-532-3p, miR-663b, miR-633, miR-194, miR-299-3p, miR-142-5p, miR-144, miR-93, miR-545, miR-374a, miR-374b, miR-665, miR-146b-5p, miR-194, miR-892b, miR-32, miR-501-5p, miR-513c, miR-513b</td>
<td>upregulated</td>
<td>microarray</td>
<td>14 serum samples</td>
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<td>miRNAs</td>
<td>Expression in tumours</td>
<td>Method</td>
<td>Primary tumours studied (#)</td>
<td>Frequency of change (%)</td>
<td>Reference</td>
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<td>-------------------------</td>
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<td>let-7a, let-7d, let-7c, let-7i, miR-98, miR-let-7b, miR-1254, let-7e, miR-122, miR-221, miR-1299, miR-198-2, miR-486-3p, miR-375, miR-1260, miR-1287, miR-720, miR-124, miR-133a, miR-379, miR-129, miR-328, miR-335, miR-1228</td>
<td>downregulated</td>
<td>microarray</td>
<td>14 serum samples</td>
<td>100</td>
<td>108</td>
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<tr>
<td>miR-199a-3p, miR-99a, miR-125b, miR-214, miR-10b, miR-29b, miR-100, miR-224, miR-505, miR-29a, miR-363, miR-10a, miR-137, let-7c, miR-193a-3p, miR-374a, miR-130a, miR-29c, miR-335, miR-18la, miR-28-5p, miR-376a</td>
<td>upregulated</td>
<td>microarray</td>
<td>SNUOT-Rb1 cell line</td>
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<td>miR-124, miR-142-3p, miR-34a, miR-135b, miR-96, miR-142-5p, miR-183, miR-338-3p, miR-193b, let-7i, miR-182, miR-149, miR-let-7g, miR-34c-5p, miR-132, miR-34b</td>
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<td>microarray</td>
<td>Y79 cell line</td>
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NA, not available
### Table 6

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<th>miRNA</th>
<th>Expression in tumours</th>
<th>Primary tumours studied (#)</th>
<th>Frequency of change (%)</th>
<th>Target genes</th>
<th>Target gene expression</th>
<th>Correlation with outcome/cellular function</th>
<th>Reference</th>
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<tbody>
<tr>
<td>let-7b</td>
<td>downregulated</td>
<td>3</td>
<td>100</td>
<td>CDC25A, BCL7A</td>
<td>upregulated</td>
<td>NA</td>
<td>97</td>
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<tr>
<td>let-7 family</td>
<td>downregulated</td>
<td>44</td>
<td>39</td>
<td>HGMA1, HGMA2, CCND1, CCNE2, CDK4, E2F3, EMP1, MDMX, SIRT1, MLLT3, SLC30A3, CTNN2, PLCG1, ACTR1A, CDC25A, EFNB1, KCN2</td>
<td>upregulated</td>
<td>poor tumour differentiation, invasion and proliferation</td>
<td>98</td>
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<tr>
<td>miR-34a</td>
<td>variable</td>
<td>2 (plus 2 cell lines)</td>
<td>NA</td>
<td>CDC45, CDC24, CCND1, CCNE2, CDK4, E2F3, EMP1, MDMX, SIRT1, MLLT3, SLC30A3, CTNN2, PLCG1, ACTR1A, CDC25A, EFNB1, KCN2</td>
<td>downregulated in response to miR-34a induction</td>
<td>NA</td>
<td>100</td>
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<tr>
<td>miR-17–92 and miR-106b–25 (paralog)</td>
<td>upregulated</td>
<td>32</td>
<td>15</td>
<td>CDKN1A (p21Cip1), CDK4 and CDK6 (miR-129), MYC (miR-382), TP53 (miR-504), HDAC4 and MYCP (miR-22)</td>
<td>downregulated in response to miR-17–92 inhibition</td>
<td>inhibition of miR-17–92 decreased cell line proliferation and tumour formation in mice</td>
<td>101</td>
</tr>
<tr>
<td>miR-129-3p, miR-129-5p, miR-382, miR-504, miR-22</td>
<td>downregulated</td>
<td>12 (plus 2 cell lines and mouse tumours)</td>
<td>100</td>
<td>CDKN1A (p21Cip1), CDK4 and CDK6 (miR-129), MYC (miR-382), TP53 (miR-504), HDAC4 and MYCP (miR-22)</td>
<td>downregulated in response to miR-17–92 inhibition</td>
<td>inhibition of miR-17–92 decreased cell line proliferation and tumour formation in mice</td>
<td>101</td>
</tr>
<tr>
<td>miR-17, miR-18a, miR-20a</td>
<td>upregulated</td>
<td>via consensus regulatory network analysis: PCNA, CDKN2A, CDC25A, CDC25A, KIF15, ERBB BAX, RAF1, MAP3K5</td>
<td>NA</td>
<td>signature of advanced stage tumours</td>
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<td>miR-19b, miR-92a</td>
<td>downregulated</td>
<td>20 serum samples</td>
<td>NA</td>
<td>signature of advanced stage tumours</td>
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<td>108</td>
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NA, not available