Biomarkers in retinoblastoma

Jie Sun, Hui-Yu Xi, Qing Shao, Qing-Huai Liu

INTRODUCTION

Retinoblastoma (RB) is the most common primary intraocular malignancy tumor in pediatric patients worldwide, accounting for 6% of cancers in children[1]. It affects children under the age of 5y with an incident in one of 15 000-20 000 live births[2]. RB has adverse effects on the quality of life of infants and it is estimated that almost 9000 newly diagnosed pediatric cases are reported every year. With the development of improved surveillance and knowledge and clinical techniques and treatments, the 5-year survival rate has greatly improved. Among the plenty of clinical treatment options for RB tumors, chemotherapy has become an important therapy to restore vision, including chemoreduction and intra-arterial chemotherapy, which can reduce the rates of enucleation in RB cases[3-5]. To date, the clinical risk assessment of RB tumors has been largely determined by a combination of clinical and histopathological features[6]. But there are still a small percentage of RB cases that cannot be evaluated using these methods, and the impact of chemical drug resistance and side effects remains to be resolved. To improve therapeutic outcome, avoid enucleation, and prevent metastasis, there is an urgent need to further study the biology and molecular mechanisms underlying RB and identify the specific biomarkers that cause tumor progression. Therefore, the study of RB biomarkers would be helpful in determining the severity of this disease, early diagnosis and accurate prognosis assessment.

The occurrence of RB can be hereditary and non-hereditary, related to germline and somatic mutations in the tumor suppressor Rb1 gene, respectively[7]. As a pediatric cancer that occurs either as a sporadic or an inherited disease, it is known that RB results from the biallelic inactivation of the Rb1 gene, which is located at the 13q14 region of chromosome 13[8-9]. Evidence from the study of mechanism suggests that it confers unlimited proliferation potential to RB because the functional retinoblastoma protein (pRB) is essential for chromosomal stability[10-11]. An analysis of embryonic retinal cells identified differentiating cones as the cell of origin for RB. Studies have shown that in dissociated retinal cultures, Rb1 knockdown induced the development, proliferation and malignancy of cone precursors and formed tumors in orthotopic xenografts with histologic features and protein expression profiles typical of differentiated human RB[12-13]. In a very small percentage of RB tumors, the Rb1 gene has been inactivated by chromothripsis in chromosome 13[14]. Although most RB tumors show alteration in both Rb1 alleles, it has been shown that a subset of early-onset, unilateral, malignant RB tumors do not have the mutations in the second Rb1 allele. This RB subset is usually diagnosed in infants younger than 6mo, and is caused by the amplification of a known gene MYCN[15-16]. Although the critical role in the dysfunction of Rb1 and MYCN...
has been identified, current RB clinical treatments do not target these mutations specifically (Figure 1). Additionally, there are other identified oncogenes and suppressors. Some of them have become targets of action for the development of novel, efficient therapeutics. These genes that drive RB progression include: chromatic remodeling factors, MDM4, KZF14, DFR; transcription factor E2F3; and the tumor suppressor CDH11[17]. Although many studies have investigated the pathogenesis of RB, a thorough understanding of these mechanisms involving the cellular and molecular targets is lacking. Hence, research on the identification of RB biomarkers would be helpful to deepen our understanding of RB pathogenesis. Also, the new biomarkers could serve as potential indicators, leading to new therapeutics and help determine specific strategies for treatment.

**Epigenetic Biomarkers in Retinoblastoma** Aside from genetic mechanisms, epigenetic mechanisms play an important role in the progression of RB. It has been demonstrated that a variety of epigenetic alterations could act as potential biomarkers for RB pathogenesis. Studies have shown that Rb1 is involved in the regulation of most major epigenetic alterations, including site-specific DNA methylation, histone modification, modification of microRNA (miRNA) and long non-coding RNA (lncRNA), and ATP-dependent chromatin remodeling[18-19]. It has been shown that inactivation of Rb1 can lead to dysregulation of the tumor suppressor and oncogenic pathways through epigenetic mechanisms[20]. Moreover, the reprogramming of epigenomics is essential for tumorigenesis and provides a relatively new avenue for therapeutic targets against RB, as epigenetic modifications can be reversible[21-22]. Thus, epigenetic regulators should be integrated into approaches identifying new RB therapeutics.

**DNA methylation biomarkers of retinoblastoma** DNA methylation is one of the hallmark epigenetic events most studied in cancers[23]. DNA methylation involves the addition of a methyl group to the 5' carbon of a cytosine ring located 5' to a guanosine base in a CpG dinucleotide and is catalyzed by DNA methyltransferases (DNMTs). These CpGs are often clustered together and called CpG islands, the majority of which are found in the promoter region of genes. Hypermethylation of CpG islands in the promoter region leads to gene silencing through the inhibition of transcription or via recruitment of chromatin remodeling co-repressor complexes[24]. During RB tumorigenesis, the role of promoter methylation was first described when methylation of CpG islands (CpG 106) overlaps with the Rb1 promoter and exon1[22]. It is now known that low expression of Rb1 is associated with hypermethylation of the Rb1 promoter[25]. In RB, aberrant DNA methylation has been found to be involved in many genes beyond Rb1, including RASSF1A (tumor suppressor related to microtubule stability), MGMT and p16INK4A (tumor suppressor) etc[26].

RASSF1A, the human Ras association domain family 1A gene, is located at 3p21.3 and encodes a predicted peptide of MW 39 000 and contains a Ras association domain. Loss of RASSF1A expression by promoter hypermethylation is a common epigenetic event in RB. A high frequency (82%, 56/68) of hypermethylation at the CpG sites of the RASSF1A promoter have been detected in RB carcinoma samples. The RB cell lines WERI-Rb-1 and Y79 carried a completely methylated RASSF1A promoter and did not express RASSF1A. Such epigenetic silencing was not detected among normal or nonmalignant retinal tissues, which indicates that aberrant promoter methylation of RASSF1A, may contribute to the pathogenesis of RB[27].

MGMT, the DNA-repair enzyme O6-methylguanine-DNA methyltransferase, is transcriptionally silenced by promoter hypermethylation in several human cancers[28]. Studies have...
indicated that MGMT hypermethylation is related to poor differentiation. Hypermethylation of the MGMT promoter was found to be prominent among RB with poor tissue differentiation and was more frequently detected among patients with bilateral disease\cite{59}. It has been determined that methylation of the MGMT promoter increases the sensitivity of glioma to alkylating agents\cite{30}. In RB, alkylating agents such as carmustin or cisplatin have been commonly used for treatment. The investigation of the effects of MGMT hypermethylation on the response to chemotherapy in RB is required. Therefore, silencing of MGMT is a poor prognostic factor in RB and may be a good predictive marker for chemotherapy when alkylating agents are used.

*p16INK4A*, which encodes a CDK inhibitor that prevents cell cycle progression, has been shown to play a crucial role in RB progression\cite{1}. In fact, a high expression of p16INK4A in Rb−/− retina cells was proposed to prevent RB development by blocking transformation at the stage of the non-proliferative RB precursor lesion. In a recent study, evidence of an inherited p16INK4A downregulation has been shown in RB families, suggesting that this alteration might be a possible novel heritable susceptibility marker to RB. This study revealed a downregulation of p16INK4A in about half of the tumor and blood samples from RB patients. Furthermore, in most cases with p16INK4A downregulation at least one of the parents showed the same alteration in blood cells. More importantly, it was observed that p16INK4A downregulation seems to be due to the hypermethylation. The finding that the methylation of the p16INK4A promoter seems to be a germline and heritable alteration opens up the new possibility of monitoring high-risk families through simple blood sample analysis\cite{32}.

**TFFs**, which are required to maintain and reconstitute epithelial integrity, have been indicated in the involvement of processes including oncogenic modification, tumor growth and metastasis. Previous studies have demonstrated that *TFF1* was the only TFF peptide expressed at detectable levels RB cells. *TFF3* is the only TFF family member expressed in healthy human retina. In contrast, *TFF2* is the only TFF peptide expressed in the murine retina. After determining the TFFs promoter methylation status in RB cell lines, the correlation of endogenous TFF expression with promoter methylation was revealed. Up-regulation of *TFF1* displayed low-methylation or high demethylation of certain CpG islands in the promoter region\cite{33-34}. Many studies have observed that the *TFF1* knockdown generates lower viability of RB cells in many RB cell lines, which could lead to decreased proliferation rates and the induction of apoptosis by activating p53\cite{35}. Similarly, all RB cell lines with low-expression of endogenous *TFF3* showed high density methylation of all CpG islands. *TFF3* executes a significant pro-apoptotic, anti-proliferative, and tumor suppressive effect in RB cells, suggesting it could be a starting point for future additive chemotherapeutic approaches to reduce RB tumor size\cite{58}.

**Histone modification biomarkers of retinoblastoma** DNA exists in the nucleus in the form of chromatin, which is usually composed by DNA, histones, no-histones and a small amount of DNA. The histones H2A, H2B, H3, H4, are the basic structural proteins of chromatin, and constitute a nucleosome by wrapping about 200 base pairs of DNA\cite{37}. Post-translational modifications can be made at many sites on the N-terminal tail of histones, including methylation, acetylation, ubiquitination, and phosphorylation. All of these modifications can cause a change in the conformation of the DNA-histone complex, affecting the binding of various transcription factors to DNA and gene transcription. Among these, histone acetylation is essential for maintaining histone function and DNA transcription, which is accomplished by coordinated catalysis of histone acetyltransferase (HAT). Thus, the disequilibrium of histone acetylation will cause changes in the corresponding chromosomal structure and gene transcription levels, which affect cell cycle, differentiation and apoptosis, and ultimately lead to tumorigenesis. Methylation is also an important modification of histones, and occurs mostly on the lysine and arginine residues of H2, H3. Histone methylation is catalyzed by specific histone methyltransferases (HMTs) and this process can be modified in a regulatable dynamic way. Thus, even without the introduction of DNA mutations, the modification of histones can alter the expression of oncogenes or tumor suppressors in cancer\cite{38}. Among these changes, aberrant expression of an HMT could be a feature of RB.

Enhancer of Zeste homolog 2 (EZH2), an HMT enzyme which catalyzes trimethylation at lysine 27 on histone (H3H3K27me3), is the first epigenetic enzyme to be dysregulated in human RB. It is selectively expressed in cultured human RB cell lines and RB tumor samples, but is not present in non-proliferating cells of the normal fetal and postnatal retina. The specific presence of EZH2 in RB tissue suggested the protein might be a novel therapeutic target. Moreover, the EZH2 protein could be used to aid in the detection of invasive RB cells. EZH2 could also be used as a marker to identify single RB cell invasion into the optic nerve, a site of invasion whose involvement is an indication for systemic chemotherapy following enucleation. Indeed, inhibitors of EZH2 have been shown to kill human RB cells by impairing intracellular ATP production but did not affect primary human fetal retinal pigment epithelium (RPE). Thus, the dysregulation of EZH2 is a feature of human RB and an EZH2 inhibitor is a starting point for the development of epigenetic therapies in RB. Ubiquitin-like with PHD and ring finger domains 1 (UHRF1) is not only a critical epigenetic regulator for DNA methylation
and histone modification, but also acts as an important regulator of tumor cell proliferation and survival. UHRF1 is highly expressed in human RB tumors and human RB cell lines but there is no detectable expression in normal retina[39]. A recent study revealed that high UHRF1 expression in RB promotes tumor cell survival against genotoxic insults by enhancing DNA repair and consequently reducing apoptotic cell death. Stable knockdown of UHRF1 sensitized RB cells to chemotherapeutic drugs such as etoposide and camptothecin. X-ray repair cross complementing 4 (XRCC4) is a key mediator for the drug sensitivity upon UHRF1 depletion in RB cells[40]. Therefore, UHRF1 can serve as a potential biomarker and an efficient therapeutic target to improve the efficiency of current chemotherapy for RB treatment. 

TFF1, which has been described above, has been regarded, not only as a differentially methylated gene in RB, but also as one of the up-regulated genes with an activating histone modification in primary RB[38].

**Biomarkers from non-coding RNA regulation in retinoblastoma** Regulation of RNA-levels has emerged as one of the key epigenetic regulators for transcriptional regulation and modification of chromatin structure via formation of an RNA scaffold for RNA-protein interactions. Non-coding RNA (ncRNA) mainly consists of long non-coding RNA (lncRNA) and microRNA (miRNA). It can also be divided into two categories based on their location, nuclear and cytoplasmic RNA, which to a large extent, determines their mode of action[41]. 

miRNAs are short non-coding RNAs of about 21-22 nucleotides. It is known that miRNA is processed from a hairpin precursor and binds to the 3’ UTR of target mRNAs in the cytoplasm. It can repress gene expression by further degradation of mRNA and inhibits of translation[42]. It has been demonstrated that miRNAs can be detected as diagnostic biomarkers for many types of cancer and the expression of miRNAs can be changed by various cellular pathogenesis signals; this alteration plays an important role in various cancers including RB[43-44]. The impact of miRNAs in RB can be either as potential components of oncogenes or tumor suppressors. Hence, the use of miRNAs can make a difference as an accessible and non-invasive biomarker for diagnoses and new therapies[45]. Many studies characterizing miRNA expression profiles have been performed to identify differential expression of miRNAs in RB tissues and cells. We can figure out the expression of a subset of miRNAs is in a higher level in RB compared to the healthy retina, and there are still a lot of miRNAs are down-regulated in RB (Table 1)[46-72]. Among these highly expressed miRNAs in RB, many have been proved to play a vital role in regulating tumorigenesis and progression. miR-17 to 92, an important cluster, which was selected as oncomir-1, was shown to be related to tumor development. miR-17 to 92 can induce RB cell proliferation in the case of Rb1 inactivation and many studies have revealed the regulation mechanisms of miR-17 to 92. Indeed, RB in the miR-17 to 92 overexpression mouse model was highly invasive, with tumor cells observed in the optic nerve[73]. Epithelial cell adhesion molecule (EpCAM), is a type I transmembrane glycoprotein, which plays a role in RB tumorigenesis. It has been shown that the inhibition of EpCAM efficiently reduced miR-17 to 92 inhibition[49]. Another notable factor is single transducer and activator of transcription 3 (STAT3), which is activated in many cancers, and has been shown to be activated in RB tissues. It has been shown that activation of STAT3 triggers various cellular events by regulating the expression of genes involved in cell cycle, tumor migration and angiogenesis. Thus, STAT3 can be regarded as a signaling hub and leads to the upregulation of many oncogenic miRNAs including miR-17 to 92 clusters in RB through a positive feedback loop of miR-17 to 92/STAT3. Also, it was shown that the inhibition of STAT3 in RB reduces proliferation and migration[74]. Furthermore, the inactivation of miR-17 to 92 can restrain RB formation and co-silencing of p53 and miR-17/20a reduced the survival of RB cells. Thus, the lethal interaction between p53 and the Rb1 mutation results in the high expression of miR-17 to 92. Overall, these findings identify miR-17 to 92, especially miR17 and miR-20a as important biomarkers for therapeutic treatment of RB[75].

As we have identified above, TFF1 can affect p53 expression by regulation of miRNAs. miR-18a has been reported to regulate the p53 pathway by targeting interferon regulatory factor 2 (IRF2)[76]. miR-18a, which is encoded by the miR-17 to 92 cluster and is upregulated in RB, can be downregulated by the forced overexpression of TFF1 mentioned above[10]. Interestingly, the overexpression of miR-130 and miR-181 is involved in the upregulation of EpCAM, inhibition of miR-181c and miR-130 can change RB cell properties by inducing apoptosis and reducing viability and proliferation. The significant role of EpCAM-regulated miR-130 and miR-181 can be detected in RB progression and these target genes and miRNAs can serve as new possibilities to reduce carcinogenesis[49]. Another highly overexpressed miRNA in RB tissue is miR-21, which targets PDCD4, a tumor suppressor gene, to regulate RB progression and metastasis. Therefore, miR-21 could also serve as a therapeutic target for RB[48,77].

Studies performed in RB have also identified clusters of miRNAs which are down-regulated compared to normal retina. Several miRNAs have shown great influence on RB pathogenesis. The Let-7 family, the most studied down-regulated miRNA in RB, has been shown to repress the
functionality of oncogenes of HMGA, C-Myc, and Ras family functionally. high-mobility group A (HMGA), which modulates transcription by affecting chromatin structure, is expressed in many tumors. HMGA1 and HMGA2 are the most meaningful types of HMGA[56]. In many RB samples, the high level of HMGA1 and 2 is accompanied by the down-regulation of Let-7. Thus, it can be suggested that there is a significant inverse correlation between HMGA1 and 2 and Let-7. The function of Let-7 as tumor suppressor can be regulated by HMGA 1 and 2[78]. There are other miRNAs that are down-regulated in RB tissues and cell lines. miR-183 targeting the low-density lipoprotein receptor related protein 6 (LRP6) can suppress RB cell viability, invasion and proliferation but was down-regulated compared to healthy retina[59]. As we mentioned above, EZH2 is highly expressed in RB, but miR-101, which is down-regulated, can inhibit the expression of EZH2. miR-101 can reduce cell growth and induce cell cycle arrest and apoptosis and functions as an important tumor suppressor[57].

In addition, the up-regulation of miR-204 in RB cells and tissues is propitious to the inhibition of RB cell proliferation and metastasis, both in vitro and in vivo. Thus, miR-204 can suppress the progression of RB; its critical regulative role for RB was identified by targeting the matrix metalloproteinases-9 (MMP9) and cyclin D2 genes[60].

In addition to these upregulated and downregulated miRNAs in RB, there are a number of miRNAs which have variable expression in RB cell lines and primary RB samples. Among these, the miR-34 family is important in RB tumorigenesis and progression because they act as tumor suppressors by inducing cell cycle arrest and the inhibition of apoptosis[79]. The miR-34 family, including miR-34a and miR-34b, is related to an important tumor suppression pathway, the p53 pathway,

<table>
<thead>
<tr>
<th>Expression in RB</th>
<th>MicroRNA(s)</th>
<th>Target gene(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Upregulation</strong></td>
<td><strong>Tissue</strong></td>
<td>miR-17-92[46] EpCAM/STAT3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>miR-21[47-48] PTEN/PI3K/AKT/PDCD4</td>
</tr>
<tr>
<td></td>
<td>miR-130b, miR-181c[49] Caspase3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>miR-494, miR-513, miR-518c, miR-129, miR-198, miR-492, miR-498, miR-503[50]</td>
<td></td>
</tr>
<tr>
<td><strong>Cell lines</strong></td>
<td>miR-21[47]</td>
<td>PDCD4</td>
</tr>
<tr>
<td></td>
<td>miR-24[51]</td>
<td>p14ARF</td>
</tr>
<tr>
<td></td>
<td>miR-181b, miR-125a-3p, miR-30c-2[52] HIF, VEGF</td>
<td></td>
</tr>
<tr>
<td></td>
<td>miR-10b, miR-29, miR-34a, miR-124, miR-135b, miR-142-5p, let-7c, let-7i, miR-31, miR-200a, miR-19b, miR-195, miR-222, miR-181c[40-45,54-55]</td>
<td></td>
</tr>
<tr>
<td><strong>Serum</strong></td>
<td>miR-17, miR-18a, miR-20a, miR-103, miR-182, miR-183, miR-30a, miR-29, miR-101, miR-26a, miR-378, miR-494, miR-16[53]</td>
<td></td>
</tr>
<tr>
<td><strong>Downregulation</strong></td>
<td><strong>Tissue</strong></td>
<td>miR-let-7c[56] HMGAI/HMGAI2</td>
</tr>
<tr>
<td></td>
<td>miR-101[57] EZH2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>miR-433[48] Notch1, PAX6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>miR-183[59] LRP6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>miR-204[60] CyclinD2/MMP-9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>miR-125b[61] CDK6, CDC25A, LIN28A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>miR-25[62] BCL2L1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>miR-124[63] STAT3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>miR-145[64] ADAM19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>miR-613[65] E2F5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>miR-129-3p, miR-382, miR-22, miR-129-5p, miR-532-5p, miR-486-3p, miR-21, miR-200c, miR-143, miR-497[54,66-68]</td>
<td></td>
</tr>
<tr>
<td><strong>Cell lines</strong></td>
<td>miR-491-3p[55] HIF, VEGF</td>
<td></td>
</tr>
<tr>
<td></td>
<td>miR-26a[66] Beclin 1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>miR-125a-5p[70] TAZ-EGFR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>miR-204[66] CyclinD2, MMP-9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>miR-183[71] LRP6</td>
<td></td>
</tr>
<tr>
<td><strong>Serum</strong></td>
<td>miR-19, miR-92a, miR-10b, let-7a, let-7c, let-7e, miR-129, miR-21, miR-25, miR-198, miR-503, miR-217, miR-9, miR-320, miR-373, miR-498, miR-92a[53]</td>
<td></td>
</tr>
<tr>
<td><strong>Plasma</strong></td>
<td>miR-320, miR-21, let-7c[72]</td>
<td></td>
</tr>
</tbody>
</table>

RB: Retinoblastoma.
which transcriptionally activates the miR-34 family. miR-34a has been shown to be a tumor suppressor by targeting HMGB1 mRNA, which suppresses the translation of HMGB1. As we mentioned above, HMGB1 promotes tumor formation, invasion and migration and it can also inhibit apoptosis and induce drug resistance by regulating autophagy. Thus, miR-34a acts as autophagy inhibitor and promotes the cytotoxicity of chemotherapy drugs. miR-34b is another member of the miR-34 family, which is upregulated in RB cell lines and is regulated by posttranscriptional regulation of gene expression. In addition to this effect on the p53 pathway, miR-34 can also mediate the downregulation of p14ARF to abrogate p53-mediated surveillance. Furthermore, other studies have emphasized the potential role of miRNAs as important biomarkers of RB. It was shown that miR-302, Let-7e and miR-21 were downregulated in the plasma of RB patients compared to normal people. Similarly, various serum miRNAs are changed in serum samples. Thus, miRNAs levels can be used as new biomarkers for diagnosis and prognosis.

LncRNA is a class of non-coding RNA longer than 200 nucleotides. Unlike miRNA, LncRNA can be folded into secondary structures to form more versatile and flexible interactions between RNA and protein. The vital roles of LncRNA in tumor initiation and progression has been previously shown. In recent years, many studies have demonstrated that LncRNA are dysregulated in RB and have characterized the roles of LncRNA in RB. Also, LncRNA can compete with miRNAs by binding to the same regions of miRNAs, resulting in the downregulation of target miRNAs. The important roles of LncRNA in RB indicate that they can be critical potential diagnostic and prognostic biomarkers. In RB cell lines, it has been shown that the LncRNA colon cancer-associated transcript 1 (CCAT1) is upregulated and has an effect on RB cell migration and proliferation by negative regulation of miR-218-5p. The LncRNA actin filament-associated protein 1 antisense RNA1 (ATAPI-AS1) is elevated in RB cell lines and tissues and is related to tumor size and invasion. Moreover, the high expression of AFAP1-AS1 is an independent adverse prognostic factor for RB patients. These studies suggest that both CCAT1 and AFAP1-AS1 could serve as potential biomarkers and therapeutic targets for RB.

Another oncogenic LncRNA is brain-derived neurotropic factor antisense (BDNF-AS), which is downregulated in RB tumors and cells. When upregulated, BDNF-AS can suppress the cell-cycle transition and results in the inhibition of RB progression. Therefore, the low expression of BDNF-AS can be used as a prognostic biomarker. Maternally expressed gene 3 (MEG3), a LncRNA, is downregulated in RB tissues and its level is negatively related to nodal and distant metastasis. Moreover, the tumor suppressive role of MEG3 is regulated by negatively affecting the activity of the Wnt/β-catenin pathway. Thus, it can also be a biomarker for prognosis and a target for molecular therapeutics. LncRNA are also closely correlated with miRNAs to regulate RB biological characteristics. For instance, the 3′-UTR of the LncRNA differentiation antagonizing non-protein coding RNA (DANCR) and the mRNA of MMP9 can be targeted by miR-34c and miR-613; DANCR is upregulated in RB tissues and cells. Conversely, the LncRNA H19 is downregulated in RB and it inhibits RB cell proliferation and induces cell apoptosis and cell cycle arrest. These inhibitions of RB are due to the counteraction of the function of the miR-17 to 92 cluster by binding to each other. Through this binding, H19 represses the activation of STAT3 induced by miR-17-92 and induces p21 expression which, conversely, suppressed by the miR-17 to 92 cluster. In summary, LncRNA are prospective biomarkers for RB progression and are also therapeutic targets.

**Biomarkers from Proteomic Analysis in Retinoblastoma**

Although a number of potential biomarkers have been reported, the development in the field of proteomics enables the profiling of proteins for the identification of reliable biomarkers or specific therapeutic targets in RB. Proteomics is a systematic study for probing the expression of total proteins in cells and tissues and the analysis of proteomics is an accurate, high-throughput strategy for the identification of proteins. At first, the methods for detecting the RB proteome were gel-based approaches, such as two-dimensional (2-DE) electrophoresis and mass spectrometry (MS), but these gel-based approaches can only detect a small portion of the total proteins. Moreover, the high-throughput, gel-free proteomics technologies such as iTRAQ, ESI-MS/MS, and LC-MS/MS, combined with bioinformatics analysis can identify thousands of proteins for proteomic profiling in RB. iTRAQ (isobaric tags for relative and absolute quantitation) is a method of labeling amines in peptides; the peptides are then subjected to fractionation by hRPLC. The use of ESI-MS/MS and LC-MS/MS can then be used to identify and quantitate protein. Lastly, the identified proteins are submitted to bioinformatics analysis and annotations. Overall, these strategies can detect the comprehensive proteomic signature as well as potential biomarkers in RB.

**Biomarkers from proteomic profiling in retinoblastoma tissues**

Mallikarjuna et al. reported the proteome of fresh RB tissues using a 2-DE-MS/MS. This study revealed that twenty-seven proteins were differentially expressed in RB compared to normal retina, including 11 downregulated and 16 upregulated proteins. The detection of mRNA levels of
some of the identified proteins and immunohistochemistry were consistent with the proteomic data. Among these, proteins including CRABP2, peroxiredoxin 6, apolipoprotein A1, and recoverin were significantly more expressed in RB with invasion. Recently, with the advent of proteomics technology, more studies have been performed to investigate the proteomic profiling in RB tissue. The study performed by Danta et al\textsuperscript{[94] using high-resolution MS between RB and normal retina, quantified 3587 proteins overall and identified 899 differentially expressed proteins; 402 were upregulated and 497 were downregulated. This study corroborated the previously reported overexpressed proteins in RB, including spleen tyrosine kinase (SYK), vimentin (VIM) and stathmin1 (STMN1). Beyond that, novel proteins were identified in RB; this study validated RACGAP1, CHGA and AHSG IGF2BP1 by IHC, which were not previously reported. Among these, the functional validation of insulin growth factor 2 mRNA binding protein 1 (IGF2BP1) has been demonstrated suggesting that it plays a vital role in RB cell proliferation and migration.

In addition to these studies in general RB tissues, the comparative proteomes in both human papilloma virus (HPV) positive and negative RB patients were conducted because the important role of HPV in abolishing pRB protein function was identified\textsuperscript{[95]. The significant association between high-risk HPV subtypes and sporadic RB was revealed over the past decade\textsuperscript{[96]. Thus, this study aimed to detect potential RB-specific prospective targets and to clarify the possible role of HPV, which could help us understand the mechanism of RB progression. The analysis of this study revealed that in HPV positive RB, β-catenin (CTNNB1) was the most important regulated signaling pathway compared to HPV negative RB and suggested that glial fibrillary acidic protein (GFAP), retinol-binding protein 3 precursor (RBP3), and apolipoprotein A1 (APOA1) could act as potential targets and biomarkers for research on the therapy and support of disease mechanisms. Cellular retinoic acid binding proteins (CRABPs), the members of the fatty acid binding family of proteins, have aberrant expression in RB compared to normal retina\textsuperscript{[97]. Through the studies mentioned above, it was observed that CRABP1 was significantly downregulated but CRABP2 was overexpressed in RB. CRABP1 slowed down the proliferation of RB cells through retinoic acid receptor (RAR)-mediated transcription programs, but CRABP2 was involved in RB cell proliferation \textit{via} the activation of the PPARβ/δ and FABP5 proteins\textsuperscript{[98-99].

Alpha-crystallin-A (CRYAA), a major lens structural protein, acts as an anti-apoptotic factor by restricting the product of cytochrome C from the mitochondria, which could inhibit apoptosis in tumor cells. The increased expression of CRYAA was detected in RB tissues compared to healthy retina and this overexpression was correlated with the apoptotic index\textsuperscript{[99]. Thus, CRYAA may have a positive role in preventing apoptosis of RB cells and the understanding of that mechanism could help us target CRYAA-expressing RB cells with specific anticancer drugs\textsuperscript{[100].

APOA1, which transports cholesterol from peripheral tissues to the liver for excretion, was overexpressed in an RB cohort compared to healthy retina. APOA1 is reported to directly promote tumor survival through kinase activation in recurrent head and neck squamous cell carcinoma, but the elucidation of its precise role in RB requires more studies to elucidate\textsuperscript{[101].

VIM is a cytoskeletal intermediate filament protein located in mesenchymal cells and is expressed in astrocytes and Müller cells in healthy retina to preserve cell integrity and resist stress. VIM was overexpressed in RB tissues, especially in HPV positive tissues compared to negative RB tissues. The overexpression of VIM was accompanied by an elevation of sex-determining region Y box 2 (SOX-2) and vascular endothelial growth factor (VEGF) in high-risk factor (HRF) RB, which partially elucidated the aggressiveness of HRF RB\textsuperscript{[101]. The important role of VIM as a marker in the epithelial mesenchymal transition (EMT) could provide evidence of a connection between VIM overexpression and tumor progression, including RB cell invasiveness and poor prognosis\textsuperscript{[20].

GFAP is another cytoskeletal intermediate filament protein which was overexpressed in RB. Similar to VIM, GFAP can act as biomarker for activated Müller and astrocyte cells, but GFAP is only expressed in Müller cells after retinal injury\textsuperscript{[102]. Thus, although GFAP has been identified as a serum biomarker for glioblastoma multiforme and was recently overexpressed in RB, it is still questionable to consider GFAP as a biomarker in RB as elevated GFAP expression could be detected as a specific response to retinal injury. However, GFAP is considered to be a marker in RB glia cells, which enhance RB cell proliferation and induce RB cell apoptosis. Hence, the precise function of GFAP requires further studies.

RBP3, a glycoprotein synthesized in photoreceptors, was significantly downregulated in RB. It was shown that RBP3 is in charge of transferring retinoids between the RPE and the outer segment (OS) of photoreceptors. Further, the reduction of RBP3 resulted in impaired RAR activity and photoreceptor preservation, a condition which may lead to the proliferation of RB cells and impairment or loss of photoreceptors.

Besides these proteins discussed above, the analysis of the network and pathways of proteins demonstrates that most proteins have interactions with signaling molecules like Akt, myc, and hsp90. Both Akt, a serine threonine protein, and the transcriptional regulator myc are related to the regulation of cell development, survival, and death, and both of them are deregulated oncoproteins in many cancers. Akt is involved...
Biomarkers in retinoblastoma

In tumor suppression by MDM2-mediated proteasomal degradation. Also, the elevated expression of Akt1 and other vital factors of the PI3K/Akt/mTOR pathway have been detected in unilateral RB tumors, which suggest the possibility of these regulators as potential biomarkers and therapeutic targets for RB\textsuperscript{[103]}. The proteomic analysis of the vitreous humor in RB tumors has also been conducted. Because the vitreous humor is associated with the size of RB tumors in the eye, the vitreous humor is an auspicious source to study specific protein targets in RB. The alterations in the retina will lead to changes of both proteomic and biochemical properties in RB. Most importantly, the vitreous humor has a greater advantage for studying biomarkers in RB by acting as a medium\textsuperscript{[104-105]} for many aspects, such as lower risk of tumor seeding when collecting samples and direct delivery of chemotherapeutic drugs. Thus, this study conducted proteomic analysis of vitreous fluid to explore novel therapeutic targets and biomarkers in RB by using iTRAQ labelling coupled with ESI-MS/MS and bioinformatics analysis\textsuperscript{[106]}. This analysis showed 431 differentially expressed proteins, including 362 upregulated and 69 downregulated proteins that were detected. Several proteins like MMP2, TNC, and CRABP1 were highlighted for use as potential therapeutic targets in RB. In addition, p38MAPK and Akt signaling were revealed as important regulated pathways in RB through network and pathway analysis.

MMP2 protein is a zinc-dependent enzyme which was significantly overexpressed in RB than controls; the elevated MMP2 levels were closely associated with RB cell differentiation and optic nerve invasion in RB. Tenascin (TNC), another extracellular matrix glycoprotein, was upregulated in RB. Though the precise role of TNC in RB is still unknown, it has been reported that TNC-C could act as modulator for tumor cell growth and migration. Also, the correlation between high levels of TNC and poor prognosis has been reported for many other cancers\textsuperscript{[107]}. CTNNB1 was identified as a hub protein, which could connect proteins. In the dataset of this study, all detected proteins had an interaction with upregulated CTNNB1, suggesting the vital role of CTNNB1 in the associated pathways. SOX2, a member of high-mobility group transcription factors family, has a key role in embryotic development and cell differentiation\textsuperscript{[108]}. Interestingly, recent studies demonstrate that SOX2 is overexpressed in lung, liver, stomach and breast cancers, and is associated with tumor development, invasion and metastasis\textsuperscript{[109]}. It has been reported that the expression of SOX2 is increased in RB tissues and is correlated with the degree of RB differentiation and optic nerve invasion. SOX2 gene is highly expressed in the peripheral blood of children with RB. The level of SOX2 expression was significantly higher in the peripheral blood of the poorly differentiated group compared with the well-differentiated group\textsuperscript{[110]}. However, further research is needed to clarify the specific molecular mechanism of SOX2. To summarize, SOX2 has an important role in the incidence and development of RB, and SOX2 gene expression may be a novel target biomarker for the early diagnosis and treatment of RB.

**Membrane proteomic biomarkers of retinoblastoma**

Recently, the role of membrane proteins involved in ion transport and signal transduction has been demonstrated. Since these membrane proteins can act as secretory molecules, this makes them alternative biomarkers for tumor diagnosis and prognosis\textsuperscript{[106]}. Of the total proteome, 30% are membrane proteins including peripheral and integral proteins. Integral membrane proteins contain transmembrane domains which go through the lipid bilayer but peripheral proteins interact with the integral membrane without traversing the lipid bilayer. Peripheral proteins are much easier to extract due to the hydrophobic nature of integral membrane proteins\textsuperscript{[111-113]}. In this study, the researchers used iTRAQ but used high-resolution MS to extract and quantitate the membrane proteins in RB primary tumors and normal retina\textsuperscript{[114]}. Based on the membrane proteome analysis, 3122 proteins were detected, among which 663 proteins were dysregulated, including 282 upregulated and 381 downregulated in RB tissues compared to healthy retina. The bioinformatics analysis illustrated that most proteins were related to RB cell growth, communication and transport. Furthermore, the comparison between the membrane proteomic data and the proteomic profiling detected on whole RB proteome and vitreous humor from RB patients showed that 86 proteins were accordant in these three studies, with 314 proteins that were specifically dysregulated in the membrane protein data, suggesting that these proteins could be novel in RB. The common proteins which was present both in the membrane proteome and the vitreous humor proteome suggested that these proteins could be used as biomarkers for the diagnosis and prognosis of invasive RB. When forced to identify molecular functions, the laminB1 (LMNB1) and transferrin receptor (TFR) showed significant positive expression in RB tissues by immunohistochemistry compared to normal retina, which indicated that both could be novel biomarkers and therapeutic targets for RB.

LMNB1, an important member of the lamin protein family, is usually located in the membrane and matrix of the nuclear membrane. It plays a vital role in cellular functions, like gene expression and nuclear stability\textsuperscript{[115]}. LMNB1’s role in cancer and especially in RB is largely unexplored and its role in cell progression will be elucidated by future studies. TFR is a glycosylated protein, which can bind to diferric transferrin (TF) and forms a complex at acidic pH, releasing...
the ferric ion. It has been reported that the overexpression of TFRC could lead to the disruption of iron homeostasis, which can induce cancer progression. The overexpression of TFRC and TF have been detected in RB, indicating the disruption of iron homeostasis. However, further studies will need to be conducted to reveal the potential mechanism of TFRC in RB.

**Proteins in aqueous humor and serum of retinoblastoma**

Early diagnosis of RB is essential for reducing mortality. Current research focuses on safe and non-invasive assessment modalities that can diagnose disease accurately. Ideally, such molecular assays would be applicable to non-invasively obtained body fluids such as aqueous humor and blood. Lactate dehydrogenase (LDH) is a conventional RB marker. Studies have shown that the presence of RB is significantly associated with the aqueous humor LDH level. The LDH activity in the aqueous humor and serum did not show any correlation with each other. There was no correlation between aqueous humor LDH levels and the following clinical features such as sex, family history, bilaterality, prior treatment, presentation age, enucleation age, and metastasis.

In conclusion, detecting LDH levels in aqueous humor can be a useful adjunct to the clinical diagnosis of RB.

Survivin is a bifunctional inhibitor of apoptosis protein that has been implicated in protection from apoptosis and regulation of mitosis. It is overexpressed in most human neoplasms and has the potential to be used as a tumor biochemical marker. Recently, it was reported that coexpression of survivin and heat shock protein 90 probably plays an important role in cellular proliferation in RB. The concentration of survivin in serum and aqueous humor was significantly high in RB. Additionally, there was a positive and significant correlation between aqueous humor survivin and RB staging and optic nerve affection. The specificity of survivin in both serum and aqueous humor was >90%. However, the sensitivity of survivin photoreceptormness gene expression was related to drug resistance and poor differentiation of RB.

**Radiogenomics Biomarkers in Retinoblastoma**

Radiogenomics, also called imaging genomics, is a rapidly evolving term that can be used to identify the association between genetic variation and imaging features. The radiogenomics term was created by Andreassen et al. in 2002. In the past 20y, genomic data such as DNA microarrays, RNA and DNA-seq has gradually emerged. It allows radiogenomics to not only be used as vital images for diagnosing diseases, but also to establish new correlations with cellular genomics. Based on these studies, many researchers have used this to identify imaging biomarkers for diagnosis, especially for noninvasive genotyping. Given the promising results, these imaging features could be a substitution for normal genomics. Thus, radiogenomic biomarkers could have potential roles both in prognosis prediction and precise treatments of patients.

Since RB cannot be diagnosed by histopathologic and biopsy-based genotyping, it is necessary to use noninvasive genotyping of RB. Magnetic resonance (MR) imaging is an essential and standard method for diagnosis and examination of the extent of RB. Radiogenomics provides the imaging-genomic associations in both diagnosis and further clinical prognosis and decision in RB. In the study of Jeasen et al., they explored two different ways to identify MR imaging features as biomarkers for utilitarian gene pathways and RB progress. One of the two ways was the assessment of the predefined photoreceptormness-related gene expression signature, called photoreceptormness. As we have described above, pRB protein depletion induced RB cells to differentiate from cone precursors. RB without photoreceptormness became poorly differentiated morphologic characteristics in advanced stages of RB and loss of this photoreceptormness gene expression was related to drug sensitivity in vivo. From this study, MR features suggested that this photoreceptormness could act as an indicator for advanced stages of RB. RB with photoreceptormness loss showed larger eye size, the equal enhancement of the choroid beneath the tumor, multisite lesions and a tumor filling the entire eye. Thus, these radiogenomic biomarkers could be predictors of prognosis and assist in RB treatment. The second way to identify MR imaging features as biomarkers was the analysis of the association between MR images and gene expression profiles. The image-genomic correlation could be detected using 10 MR features and 1336 deregulated genes. It revealed that subretinal seeding in MR images showed an association with RNA processing and multifocal lesions, and was associated with low expression of cilia development genes, which could restrain RB cell growth. Thus, these results indicate that radiogenomic biomarkers could be helpful for predicting tumor process and treatment effects. Moreover, among these imaging-genomic correlations, two individual genes were relevant to RB. RB with smaller size.
was correlated with PAX2 overexpression, which could affect development of the optic stalk and the RPE. The other gene, MYCN, whose amplification could drive RB as we introduced above, showed an association with subretinal seeding. From the results of MR images, a defined radiophenotype of RB was detected, including a combination of plaque shaped, diffuse growth and multifocal lesions. This newly distinct imaging phenotype was related to the downregulation of KAL1 and the upregulation of SERTAD3. The dysregulations of both KAL1 and SERTAD3 were significantly related to shape, diffuse growth and multifocality. The stimulation of SERTAD3 to E2F may cause this more aggressive radiophenotype in RB. However, the correlation between RB and KAL1 is still unknown and requires more research.

Overall, the advantages of radiogenomics are considerable for genotyping RB because it is a noninvasive test and can be used to monitor all disease stages. However, there are limitations of RB radiogenomics, such as the limited generalizability and the difference in spatial resolution.

The Other Genetic Biomarkers in Retinoblastoma Because overall survival and vision mitigation depend on the severity of disease, early diagnosis and accurate prognostic evaluation are important. Recently, investigations have identified other genetic biomarkers such as gene polymorphism, which can affect disease progression. These genetic biomarkers can not only help us to effectively understand RB pathogenesis, but also induce the development of new therapeutic methods.

Biomarkers from gene single-nucleotide polymorphism of retinoblastoma The TP53 gene is well known to be the most frequently mutated gene in human cancer. In addition to mutations, there are >20 different coding region single nucleotide polymorphisms (SNPs) in the TP53 gene. Several of these SNPs are known to alter p53 pathway function. It has been suggested that the p53 pathway targets pRB for degradation and in physiological conditions controls the cell cycle and apoptosis in retinal cone precursor cells, from which the RB cell lineage originates. The Arg to Pro change in codon 72 (also known as p.Arg72Pro, rs1042522 G>C) is the most frequently studied functional SNP in p53. It was observed that the p53 pathway targets pRB for degradation and in physiological conditions controls the cell cycle and apoptosis in retinal cone precursor cells, from which the RB cell lineage originates.

The MDM2 gene is an important negative regulator of the p53 pathway. It was observed that MDM4 is a downstream regulator of the p53 pathway. The MDM4 gene (also known as MDMX), located on chromosome 11q22-q23, has high structural similarity which has been shown to promote the proteasome-mediated degradation of p53 and negatively regulate the p53 pathway. It was observed that MDM4 is overexpressed in RB compared with fetal retina. Recent studies have reported that both the AA genotype and the A allele at MDM4 rs11801299 were related to an increased risk of developing RB as well as tumor invasion and poor pathological differentiation. Further, the G allele at rs1380576 reduced the risk of developing RB, and was associated with low tumor aggressiveness. In addition, the G allele at rs11801299 reflected poor prognosis of RB patients. Another meaningful SNP is rs4938723 T>C, which is located in the promoter region in the mir-34b/c gene and alters mir-34b/c expression. The mir-34b/c inhibits p53 antagonists and pro-apoptotic proteins and it is a part of the p53 pathway. A recent study has implied that a germline Rho1 mutation and the SNP rs4938723CC of the mir-34b/c gene can speed RB genesis and patients with these mutations could have earlier diagnosis. Thus, the mir-34b/c SNP rs4938723 T>C may act as a potential biomarker for hereditary RB. Though the interaction of SNPs can provide novel markers for RB prognosis, diagnosis, and treatment, further studies need to be done.

Genetic biomarkers in high-risk retinoblastoma Besides biomarkers for RB diagnosis, prognosis, and therapy, genetic biomarkers to define high-risk RB are also essential to reduce mortality. Nowadays, the calculation of RB clinical
risk is mainly determined by histopathologic features combined with clinical traits. These features, called high-risk histopathologic features (HRFF), are detected in most RB cases\(^\text{[142]}\). However, there is a small portion of RB patients that do not have these typical features even after progressing to metastasis and death\(^\text{[143]}\). Thus, it is important to discover additional biomarkers to predict high risk RB and the effect of chemotherapy. Cellular anaplasia, which is different from cellular differentiation, indicated high-risk RB and predicted the RB metastases even without HRFFs\(^\text{[6]}\). Furthermore, as we all know, gene profiles could assist researchers in identifying mechanisms devoted to anaplastic severity and define genetic markers of anaplastic grades. Hudson \textit{et al}\(^\text{[144]}\) conducted gene profiles to characterize anaplastic grades in RB, and showed that different anaplastic grades have various gene expressions. Especially in severe anaplasia, photoreceptor and nucleoporin expression were highly dysregulated, including downregulation of photoreceptor genes and markedly increased nucleoporin expression. A limited gene set consisting of EXOC8, CHTOP, NUCKS1, and ADSS accurately separated severe anaplasia from all other samples, which means this gene set could predict severe anaplasia. Overall, these results contribute to identification of biomarkers for high-risk RB.

**DISCUSSION**

RB, one of the most common primary pediatric malignancies, usually develops from the immature cells of the retina. In recent years, the morbidity and mortality of RB have greatly decreased, due to accurate early diagnosis and prognosis. A better understanding of RB disease progress facilitates the identification of biomarkers for diagnosis and treatment. RB biomarkers are rapidly being discovered through investigations of processes involved in RB carcinogenesis. Previous studies have identified many promising RB biomarkers, such as epigenetic alterations. Generally, epigenetic alterations result from genetic deregulation and interact with each other in RB pathogenesis. Though these epigenetic and genetic biomarkers have emerged as important diagnostic approaches, more studies are needed for validation, better diagnoses and therapies in RB. Apart from epigenetics, the emergence of other research techniques enables us to detect other biomarkers in RB. For example, both proteomic and radiogenomics approaches were used to identify biomarkers, which implies that these approaches could play an important role in diagnosis and prognosis. Because tumor biopsy is not typically used to diagnose RB, the utilization of radiogenomics and the vitreous proteome provides a significant opportunity to reveal the biology of the RB tumor. However, it should be mentioned that the translation of proteomic and radiogenomics to therapeutic regimens has not been implanted, primarily because targeted RB therapies need further development. Furthermore, appropriate disease and treatment stratification is essential to improve the survival rate of RB patients. Hence, the identification of RB genetic markers from different anaplasia grades could be helpful in distinguishing high-risk RB.

As with all diseases, the identification of biomarkers could greatly facilitate the detection and diagnosis of RB. There are two general ways in which a detection of RB can be made: by using either an invasive or non-invasive procedure. Histopathological analysis of excised tissue is the primary invasive procedure. Non-invasive assessment modalities of RB may be broadly divided into aqueous humor, blood biomarker assessments and imaging-based technologies (Figure 2). Non-invasive biomarkers can not only diagnose at-risk patients, but also asymptomatic screening, monitoring disease recurrence and response to treatment. However, prior to the development of clinically applicable blood-based assays, confirmatory studies are needed to further modify and validate the proposed biomarkers.
In summary, this review introduces a multitude of RB biomarkers identified from different areas. So far, the data indicate that these biomarkers warrant further investigation and could not only play critical roles in RB biological processes, but also will help to correlate and confirm the diagnosis, identify therapeutic targets or serve as a prognostic indicator of treatment (Figure 3).

ACKNOWLEDGEMENTS

Foundations: Supported by the National Natural Science Foundation of China (No.81672673); the National Key Research and Development Program of China (No.2017YFA0104101).

Conflicts of Interest: Sun J, None; Xi HY, None; Shao Q, None; Liu QH, None.

REFERENCES


41 Dorn GW 2nd, Matkovich SJ. Menage a Trois: intimate relationship among a microRNA, long noncoding RNA, and mRNA. Circ Res 2014;114(9):1362-1365.


Biomarkers in retinoblastoma


Gao Y, Lu X. Decreased expression of MEG3 contributes to retinoblastoma.


Biomarkers in retinoblastoma


125 Andreasen CN, Alsn J, Overgaard J. Does variability in normal tissue reactions after radiotherapy have a genetic basis—where and how to look for it? Radiother Oncol 2002;64(2):131-140.


141 Yu F, Jiang Z, Song A. Association of rs11801299 and rs1380576 polymorphisms at MDM4 with risk, clinicopathological features and prognosis in patients with retinoblastoma. *Cancer Epidemiol* 2019;58:153-159.

