

Regulation of viral oncogenesis by microRNAs

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Abbreviations: CHB, chronic hepatitis B; EBV, Epstein–Barr virus; HBV, hepatitis B virus; HBx, hepatitis B virus x protein; HCC, hepatocellular carcinoma (HCC); HCV, hepatitis C virus; HD, Hodgkin's disease; HPV, human papilloma virus; miRNA, microRNA; NPC, nasopharyngeal carcinoma; UTR, untranslated region

Viral infection may play a causative role in human cancers, for example hepatitis B virus (HBV) or hepatitis C virus (HCV) in liver cancer, human papilloma virus (HPV) in cervical cancer, and Epstein–Barr virus (EBV) in nasopharyngeal carcinoma. Virally infected cells express viral-encoded genes that are critical for oncogenesis. Some viruses also encode microRNA (miRNA) species. miRNAs are small noncoding RNA molecules that play an important role in cancer development and progression. Recent studies indicate an important interplay among viral oncoproteins, virus-encoded miRNAs, cellular miRNAs, and cellular genes. This review focuses on modulation of HBV-, HCV-, HPV-, and EBV-associated cancers by cellular and/or viral miRNA. An understanding of the mechanisms underlying the regulation of viral carcinogenesis by miRNAs may provide new targets for the development of specific viral therapies.

Introduction

It has been estimated that 20–25% of human cancers are caused by viral infections.^{1,2} The best-known examples are liver cancer caused by persistent infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) and cervical cancer caused by human papilloma virus (HPV). In 2008, Dr. Harald zur Hausen of Germany was awarded the Nobel Prize in Medicine and Physiology for his discovery in 1983 that HPV causes cervical cancer. Other types of cancer can also be induced by viruses. For example, the Epstein–Barr virus (EBV), one of the most common viruses in humans, is linked to Burkitt's lymphoma, nasopharyngeal carcinoma (NPC), Hodgkin's disease (HD), and gastric carcinoma. HBV, HPV, and EBV are DNA viruses that contain DNA as their genetic material and replicate using a DNA-dependent DNA polymerase. In contrast, HCV is an enveloped, positive-sense single-stranded RNA virus that replicates using RNA-dependent RNA polymerase. The genomic RNA of HCV can be immediately translated by host cells in a similar manner to cellular mRNA. In this review, we will focus on these important viruses.

MicroRNAs (miRNAs) are small (approximately 22 nucleotides) noncoding RNA molecules that interact preferentially with the 3'-untranslated regions (3'-UTRs) of target mRNAs and regulate gene expression at the post-transcriptional level by either degradation or translational repression of the target mRNA.^{3,4} miRNAs are able to recognize their target mRNAs through as few as 6–8 nucleotides (the seed region) at the 5' end of the miRNA. miRNAs have been found in plants, animals, and some viruses. Many miRNA-encoding genes are intergenic or oriented antisense to neighboring genes and are thus considered to be transcribed as independent units. However, up to 40% of miRNA genes lie within the introns and exons of non-protein coding genes or in the introns of protein-coding genes. These are usually, though not exclusively, found in a sense orientation, and are typically modulated together with their host genes. Other miRNA genes reside in clusters of polycistronic units containing the information for several microRNAs. miRNA genes are usually transcribed by RNA polymerase II (Pol II), generating a transcript that is capped at the 5' end, polyadenylated to give a (poly)A tail, and spliced to form a primary transcript (pri-miRNA). Pri-miRNAs, which can be several hundreds to thousands of nucleotides (nt) in length, contain an RNA hairpin within which one of the two strands includes the mature miRNA. Pri-miRNAs are cleaved in the nucleus by the double strand-specific ribonuclease Droscha to yield hairpin precursor miRNAs (pre-miRNAs) of approximately 70 nt. The resulting hairpin pre-miRNAs are transported to the cytoplasm by an exportin 5/RAN-GTP complex. In the cytoplasm, the pre-miRNA is further cleaved by another double-stranded endonuclease called Dicer to generate a short double-stranded RNA in which one strand is the mature miRNA. The mature miRNA is incorporated into a RNA-induced silencing complex (RISC) with Argonaute and other cellular proteins, and then guides the RISC to target sites to regulate gene expression by degradation of target mRNA through direct cleavage or by inhibiting protein synthesis. Although less common, RNA polymerase III (Pol III), rather than Pol II, can transcribe some miRNAs, especially those with upstream Alu sequences, tRNAs (tRNAs), and mammalian wide interspersed repeat (MWIR) promoter units.

Cellular miRNAs have been reported to play a variety of roles in many biologic processes, including control of embryonic development, cell proliferation, differentiation, and apoptosis. Cellular miRNAs are aberrantly expressed in many types of human cancers,⁵ including liver cancer, cervical cancer, and nasopharyngeal

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Table 1. Cellular miRNAs with known targets in HBV- or HCV-associated cancers

miRNA	Target gene	Up/downregulated	Function	Ref
miR-1	, <i>E2F5</i>	regulated in HBV infection	Increases HBV replication s cell cycle, inhibits cell proliferation	12
miR-18a	<i>ERα</i>	Upregulated in HCC	Increases HCC cell proliferation	13
miR-21	<i>PTEN</i>	Upregulated in HCC	Promotes proliferation, metastasis	14
miR-101	<i>DNMT3A</i>	ownregulated in HBV-related HCC	Aberrant DNA methylation Repressed by HBx	44
miR-122	<i>Cyclin G1</i> <i>HCV RNA</i>	regulated in HBV infection Unknown	Loss of control of HBV replication Repressed by HBx Enhances HCV replication	15 16
miR-125a	Unknown	Unknown	Suppresses HBsAg expression, inhibits cell proliferation	17
miR-132	Unknown	regulated in HBV-related HCC	Suppresses proliferation and colony formation of HCC cells	45
miR-152	<i>DNMT-1</i>	in HCC	Induces aberrant DNA methylation	18
miR-210	Unknown	Unknown	Suppress HBV replication by binding to pre-S1 region Accelerates progression of cholangiocarcinoma	19
miR-221	, <i>Bmf</i>	Upregulated in HCC	s cell growth and apoptosis	20
miR-223	<i>STMN1</i>	Downregulated in HCC	Inhibits HCC cell viability	21
miR-224	<i>PAK4, MMP9</i>	Elevated Upregulated in HCC	s cell proliferation and metastasis	22
miR-602	<i>RASSF1A</i>	Elevated Upregulated in HCC	s cell proliferation and apoptosis	23
Let-7	<i>STAT3</i>	in HCC	s cell proliferation and metastasis Repressed by HBx	24
miR-29a	<i>PTEN</i>	in HCC	Upregulated by HBx Enhances cell migration	25
miR-143	<i>FNDC3B</i>	in HCC	Upregulated by HBx Promotes cell invasion and metastasis	47
miR-148a	<i>HPIP</i>	Downregulated in HBV-positive HCC	Downregulated by HBx Inhibits cell proliferation, migration, and EMT	26
miR-338	<i>Cyclin D1</i>	in HBV-positive HCC	Inhibits cell proliferation	30
miR-196b	HCV RNA	Upregulated in HCC	Inhibits HCV replication	27
miR-199a	HCV RNA	Upregulated in HCC	Inhibits HCV replication Antitumor activity	28
miR-29	HCV RNA	in HCV-infected cells	Represses tumorigenesis	29

carcinoma. These miRNAs can function as either oncogenes or tumor suppressors, and some represent potentially promising biomarkers for cancer patients. The first virus-encoded miRNA was reported in 2004 in human EBV⁶ and since then hundreds of viral miRNAs have been discovered. It seems that almost all DNA tumor viruses encode viral microRNAs⁷ whereas investigations have so far failed to identify any viral miRNAs in a wide range of RNA viruses, including HCV. The criteria for authentic viral miRNAs include miRNAs derived from viral genomes with characteristics of cellular miRNAs.⁸ The viral miRNA expression pattern may vary with the nature of the infected cell and the type of viral latency. Unlike cellular miRNA sequences, viral miRNA sequences may have a high rate of mutation. In general, viral miRNAs and cellular miRNAs do not share seed homology; however, some viral miRNAs may have seed homology in common with cellular miRNAs. Although many viral miRNAs have been identified, the function and clinical significance of

most viral miRNAs remains to be elucidated. Cellular miRNAs can modulate the expression of various viral genes and play an important role in the virus–host interaction network. Viral miRNAs can protect viruses against the cellular antiviral response. Moreover, viruses may exploit the cellular miRNA pathway to their own advantage. The present review discusses current issues related to the modulation of HBV-, HCV-, HPV-, and EBV-associated cancers by cellular and/or viral miRNA.

miRNAs in HBV-Associated Cancer

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide.⁹ There are many risk factors for HCC, including alcohol abuse, chemical contamination by aflatoxin B1, obesity, gender, and viral infection. Of these, chronic infection with HBV and/or HCV is responsible for the majority

of HCC risk.¹⁰ The mechanism by which HBV induces transformation of normal hepatocytes to HCC remains poorly understood. Recently, numerous studies have highlighted miRNAs as new regulators in HBV-related HCC.

Virus-encoded miRNAs in HBV-associated liver cancer

Although almost all DNA viruses encode their own miRNA species, HBV-encoded miRNAs have not been confirmed experimentally. One research group analyzed potential candidates for HBV-encoded miRNAs using computational approaches and found that HBV putatively encoded only one candidate pre-miRNA. They matched the deduced mature miRNA sequence from this pre-miRNA against a database of 3' UTRs from the human genome and surprisingly found no cellular transcripts that were potential targets of the viral miRNA sequence. A search of targets among viral mRNAs revealed one viral mRNA that was targeted by a viral miRNA.¹¹ However, whether this miRNA exists *in vivo* is questionable. First, it was screened by computation analysis without any experimental confirmation. Second, the biologic function of this miRNA in the process of HBV infection requires further investigation. Last, but not least, whether the function of this miRNA is associated with progression of HBV infection to HCC remains unclear. Nonetheless, this work raises the possibility that HBV might use viral miRNAs as a means to regulate its own gene expression for its benefit. A deeper understanding of HBV and more effective technology to discover and verify miRNAs might lead to the identification of HBV-encoded miRNAs in the future.

Cellular miRNAs in HBV-associated liver cancer

Although viral miRNAs encoded by HBV have not been verified, the products of HBV were shown to alter cellular miRNA expression profiles. Numerous studies focusing on miRNA profiling in HBV-related HCC have identified a number of deregulated miRNAs that are critical for the multistep process of HCC development (Table 1). However, the miRNA profiles in serum or liver tissues of HBV-related HCC reported by various studies are complicated.³¹⁻³³

A number of plasma and serum miRNAs were found to have potential as diagnostic and prognostic markers of HCC. Tomimaru et al. measured the plasma miR-21 levels of various subjects including patients with HCC or chronic hepatitis³⁴ and showed that plasma miR-21 was significantly reduced after curative resection for HCC, and that its level in patients with HCC was significantly higher than that in patients with chronic hepatitis and healthy controls. Thus, miR-21 could differentiate HCC from healthy controls with high sensitivity and specificity. In this regard, miR-21 might be superior to α -fetoprotein (AFP), a well-known biomarker of HCC, in the diagnosis of HCC. Li et al. used Solexa sequencing and qRT-PCR to screen and validate miRNAs in serum samples.³³ They found that 13 miRNAs could accurately distinguish HBV cases from healthy individuals, and also HBV-positive patients with HCC from those with HBV chronic infection. Additionally, in a comparison of miRNA expression in the serum of HCC subjects and healthy controls, 6 miRNAs were found to be significantly upregulated in samples from patients with HCC compared with cases with HBV infection only. Three miRNAs (miR-25, miR-375, and let-7f) could

be used to distinguish HCC cases from healthy controls. In the prediction of HCC, miR-375 had an area under the receiver operating characteristic (ROC) curve of 0.96 (specificity: 96%; sensitivity: 100%). These data suggest that plasma and serum miRNAs are promising candidates for detecting HCC or HBV-positive HCC.

Certain miRNAs have been shown to be dysregulated in liver tissues in a similar manner to that in serum. miR-145 and miR-199b are frequently downregulated in liver tissues with malignant dysplasia with little sign of allelic loss, suggesting that miRNA dysregulation may occur earlier than genetic alterations during the early phase of transformation. Downregulation of miRNAs such as miR-145 and miR-199b might be indicative of early pre-malignant transformation, whereas the progressive upregulation of miR-224 could imply further malignant transformation to HCC.³⁵ Downregulation of miR-145 is involved in the immortalization of nontumorigenic cells and its overexpression inhibits proliferation and cell migration. Thus, miR-145 may be a candidate tumor suppressive miRNA in the early steps of HBV-related hepatocarcinogenesis.

A number of reports have indicated that the expression of miRNAs could predict the prognosis of HCC. Li et al. showed that high expression of miR-125b is associated with good survival: forced expression of miR-125b in HCC cell lines repressed cell growth and phosphorylation of Akt. miR-29a-5p was shown to be upregulated in HCC patients with early tumor recurrence compared with those without early tumor recurrence.³⁶ Budhu et al. generated a unique 20-miRNA metastasis signature that could significantly differentiate HCC tissues with venous metastases from metastasis-free solitary tumors. Moreover, they could not identify significant miRNAs in the corresponding noncancerous liver tissues. These new findings, if further validated, may have great clinical value in the diagnosis and treatment of HBV-related HCC.³⁷

Cellular miRNA regulates HBV replication

Cellular miRNAs can affect HBV replication directly or indirectly, thus affecting the progression of chronic hepatitis B (CHB) to HCC. For example, Li et al. showed that highly redundant HBV transcripts are involved in HBV-mediated miR-122 suppression.³⁸ They further identified pituitary tumor-transforming gene 1 binding factor (PBF) as a target of miR-122 and demonstrated that HBV replication causes an obvious increase in PBF levels. Furthermore, miR-122 levels were decreased and PBF was upregulated in CHB and HCC. In addition, miR-122 could inhibit HBV replication by binding to the viral target sequence. Overexpression and knockdown studies revealed that PBF enhances proliferation and invasion of HCC cells, and silencing of PBF results in a dramatic reduction of HCC tumor growth *in vivo*. These studies therefore identified a novel HBV mRNA-miR-122-PBF regulatory pathway that facilitates malignant hepatocyte growth and invasion in CHB and HCC. This work underscores the reciprocal interplay of host miRNA and viral mRNAs, which may contribute to cancer related to chronic infection. There are other examples of host miRNAs that alter HBV replication leading to HCC, such as miR-141 and miR-152. Recently, many HCC-related miRNAs, including miR-15a/

miR-16–1,³⁹ the miR-17–92 cluster,⁴⁰ and miR-224, were shown to target HBV mRNAs directly and inhibit HBV replication.⁴¹

HBx regulates HBV-related HCC via miRNAs

Despite its small size, the HBV x protein (HBx) plays a critical role in the malignant transformation of CHB-infected cells. Some studies have found that Hbx plays a role in HBV transcription and replication by enhancing the activities of viral promoters and enhancers. HBx is involved in many important cancer-related cellular processes, such as apoptosis, cell division, stress response, protein degradation, inflammation, and the immune response.^{42,43} HBx can modulate HBV-associated HCC via miRNAs. miR-29a is overexpressed in HBx-transfected hepatoma cells and in transgenic mouse models, compared with control cells and wild type mice, respectively. HBx might therefore upregulate the expression of miR-29a, which positively correlates with metastasis potential. miR-29a overexpression results in increased migration ability of HCC cells by directly targeting the tumor suppressor phosphatase and tensin homolog (PTEN). Downregulation of PTEN increases phosphorylation of Akt at Ser473, activating a pathway that leads to increased cell migration.²⁵ The HBx protein may also exert an oncogenic effect via its indirect role in epigenetic modification through miRNAs. HBx-expressing HepG2.2.15 liver cancer cells in which the HBV genome is integrated into several sites of HepG2 cellular DNA exhibit significantly lower miR-101 expression than control HepG2 cells that do not express the HBx protein. miR-101 directly binds to and targets the 3' UTR of DNA methyltransferase 3A (DNMT3A), which catalyzes addition of a methyl group to the 5'-CpG dinucleotide of the cytosine ring leading to epigenetic gene silencing. The inverse relationship between DNMT3A and miR-101 is further validated by decreased mRNA expression levels and increased methylation in the promoter regions of 6 tumor suppressive genes when miR-101 is inhibited. HBx may therefore enhance tumorigenesis by decreasing miR-101 expression levels, ultimately leading to epigenetic silencing of tumor suppressive genes.⁴⁴ miR-132 is downregulated in HepG2.2.15 cells and HBV-related HCC tissue samples compared with HepG2 cells and adjacent noncancerous liver tissues respectively. HBx induces DNA methylation of the promoter region of miR-132, leading to repression of its expression. miR-132 decreases cell proliferation, possibly through inactivation of the Akt signaling pathway. Serum levels of miR-132 were found to correlate with expression in the tumor tissues, implicating miR-132 as a noninvasive candidate diagnostic biomarker of HBV-related HCC.⁴⁵ These findings demonstrate the multiple roles of miRNAs in the epigenetic modulation of HBV-related HCC.

let-7 miRNAs, which are often downregulated in HCC, are involved in cellular differentiation and proliferation. Ectopic expression of let-7a significantly decreases proliferation in liver cancer cells. Let-7a targets STAT3, a protein mediating the JAK/STAT pathway involved in cell proliferation.²⁴ HBx negatively regulates let-7a. We recently showed that the p53 tumor suppressor increases miR-148a expression and that HBx decreases the expression of miR-148a through its interaction with p53. We further identified human PBX1 interacting protein (HPIP)

as a direct target of miR-148a and showed that the miR-148a/HPIP axis regulates expression of mTOR, a protein kinase that induced cell proliferation, migration, and invasion, through the AKT/ERK/FOXO4/ATF5 pathway. miR-148a overexpression suppresses proliferation, migration, and epithelial-mesenchymal transition (EMT) of HCC cells through regulation of the HPIP-mediated mTOR pathway. HBx enhances liver cell growth and migration through inhibition of miR-148a. Moreover, miR-148a expression is downregulated in patients with HBV-related liver cancer and negatively correlates with HPIP, which is upregulated in patients with liver cancer.²⁶

HBx also regulates miRNA-related machinery by decreasing the expression of Drosha, an RNase III enzyme that catalyzes the biogenesis of miRNAs. HBx inhibits Drosha promoter activity although the exact mechanism of this inhibition has yet to be elucidated. However, HBx may repress Drosha by phosphorylating and inactivating the transcription factor SP1, thus ultimately downregulating Drosha expression.⁴⁶

Taken together, the HBx protein, which is essential for the multistep progression of CHB to HCC, has been found to be associated with alterations in the host miRNA profiles through different mechanisms. miRNAs that are upregulated by HBx include miRNA-29a²⁵ and miR-143⁴⁷ whereas miRNAs that are downregulated by HBx include miR-101,⁴⁴ miR-122,¹⁵ miR-132,⁴⁵ miR-148a,²⁶ miR-152,¹⁸ let-7,²⁴ and the miR-16³⁹ family.

miRNA Regulates HCV-Related HCC

HCV, a small enveloped positive-strand RNA virus of the *Flaviviridae* family, is the most common etiologic agent underlying chronic hepatitis. Approximately 3% of the world population is infected with HCV. Chronic HCV infection leads to cirrhosis of the liver and ultimately to the development of HCC. Moreover, anti-HCV treatments show varied efficiency among different viral genotypes and depending on the ethnic origin of the infected patients.⁴⁸

Recent advances in our understanding of virus–host interactions have uncovered multiple host factors used by HCV to infect cells. As a major factor controlling different cellular processes, miRNAs represent an interesting line of investigation with respect to HCV infection and replication. The first publication in this field described the positive role of miR-122 in HCV replication.⁴⁹ This liver-specific miRNA, representing 50% to 70% of all miRNAs expressed in the liver, has been a focus of numerous research projects investigating the interaction between the liver and HCV. miR-122 has 4 binding sites in the HCV genome and is implicated in the regulation of different metabolic pathways in liver cells (e.g., cholesterol metabolism). An anti-HCV treatment based on miR-122 inhibition by antisense oligonucleotides has been tested in chimpanzees and yielded very promising results. The same anti-miR-122 molecule is also the first miRNA-targeting treatment to enter into human clinical trials for the treatment of patients infected with HCV (miravirsin, Santaris Pharma A/S).⁵⁰ Miravirsin is a locked nucleic acid-based antisense oligonucleotide that is delivered to the liver and effectively represses

Table 2. Representative miRNAs with known targets in EBV-associated cancers

miRNA or c-miRNA	Target gene	Up/downregulated	Function	Ref
miR-BART1-5p/ miR-BART16/ miR-BART17-5p	<i>LMP1</i>	Upregulated in NPC	Suppresses LMP1, induces NF-κB signaling	55
miR-BART5-5p/ miR-BART19-5p	<i>LMP1</i>	Unknown	EBV latency	56
miR-BART22-3p	<i>LMP2A</i>	Upregulated in NPC	Immune evasion	71
miR-BART10-3p	<i>BHRF1</i>	Unknown	EBV latency	56
miR-BART6-5p	<i>Dicer</i>	Unknown	Viral latency	72
miR-BART3-5p	<i>DICE1</i>	Downregulated in NPC	Increases cell proliferation	70
miR-BART5-5p	<i>PUMA</i>	Upregulated in NPC	Anti-apoptosis	69
miR-BART15-3p	<i>BRUCE</i>	Unknown	Inhibition of cell proliferation Induction of apoptosis	73
Let-7	<i>c-MYC</i>	Downregulated in NPC	Inhibition of cell proliferation	73
miR-155	, <i>c-SKI, JMJD1A, BACH1</i>	Upregulated in NPC	Unknown	74, 75
miR-424	, <i>c-MYB</i>	Unknown	Upregulation of β-catenin signaling	74
miR-150	<i>c-MYB</i>	Unknown	Induction of Burkitt lymphoma differentiation	76
miR-203	, <i>/CCNG1</i>	Downregulated in NPC	Inhibition of cell cycle entry and transformation	77
miR200b /miR429	, <i>/ZEB2</i>	Downregulated in NPC	Induction of lytic replication	78

miR-122 following intravenous injection without inducing liver toxicity. The inhibitor is currently in phase II clinical trials with preliminary data presented at an academic meeting on November 2011 demonstrating promising results from a group of patients with chronic HCV genotype 1 infection. Patients were randomly allocated to placebo groups or one of three multiple ascending dose groups (3 mg/kg, 5 mg/kg, 7 mg/kg miravirsen). Patients were subjected to 5 weekly doses of miravirsen via subcutaneous injection for 4 wk, and 4 out of 9 patients receiving the highest dose showed a significant reduction in HCV RNA. Further trials investigating the safety and pharmacokinetics of miravirsen will be performed in other countries such as Germany and the Netherlands. In addition to miR-122, other miRNAs may have promising potential as therapeutic targets, such as miR-196b,⁵¹ miR-199a-3p,³² and miR-29,⁵² which inhibit HCV replication in several models. However, these miRNAs have not been as intensively studied as miR-122.

miRNAs in EBV-Associated Cancer

3.1. EBV miRNAs regulate the expression of EBV-encoded oncogenes

EBV is associated with Burkitt's lymphoma, NPC, HD, and gastric cancer.⁵³ EBV expresses many non-coding RNAs and many proteins, such as latent membrane protein-1 (LMP1),

LMP2A, Epstein-Barr nuclear antigen-1 (EBNA1), Epstein-Barr RNA (EBER), BamHI fragment H rightward open reading frame 1 (BHRF1), BamHI-A reading frame-1 (BARF1), and the BamHI-A rightward transcripts (BARTs). LMP1 is a viral oncogene that can transform rodent fibroblasts and induces a wide range of phenotypic changes in epithelial cells and B cells. Like LMP1, BARF1 has malignant transforming activity in rodent fibroblasts. The BHRF1 protein reveals strong functional homology to the human bcl-2 proto-oncogene product. BHRF1 protects human B lymphocytes from apoptosis and is implicated in EBV-mediated B-cell transformation. BARTs are highly expressed in NPC. Although BARTs contain several open reading frames, whether these transcripts are actually translated into proteins in vivo remains controversial. EBV miRNAs are grouped into two clusters located around the *BHRF1* gene and within the *BART* transcripts. EBV miRNAs are generally more strongly upregulated than cellular microRNAs in NPC biopsies.⁵⁴ BART miRNAs (e.g., miR-BART16, 17-5p, 1-5p, 5-5p, or 19-5p) target the LMP1 3' UTR and inhibit the expression of LMP1 protein,⁵⁵ possibly activating downstream NF-κB signaling, a key contributor to LMP1-induced cell transformation. In addition, miR-BART10-3p can directly inhibit expression of the BHRF1 protein.⁵⁶ Although members of the EBV BHRF1 microRNA cluster cooperate to transform B lymphocytes, whether the BHRF1 microRNAs target EBV oncogenes remains unknown.

EBV-encoded oncogenes regulate the expression of cellular miRNAs

LMP1 and BARF1 are two important EBV-encoded oncogenes. LMP1 can be localized in the cytoplasm, membrane, and/or nucleus depending on the cell type. Through microarray analysis, Cameron et al. identified several cellular miRNAs that are modulated by LMP1. The most highly regulated of these is miR-146a.⁵⁷ LMP1 stimulates miR-146a expression predominantly through two NF- κ B binding sites in the miR-146a promoter, and miR-146a represses interferon-responsive gene expression. These results suggest that LMP1-mediated induction of miR-146a may result in suppression of the interferon-mediated antiviral response, thus protecting the EBV virus from host immunity. miR-155 is an oncogenic miRNA that is important for B-cell maturation and immunoglobulin production in response to antigen. Through activation of the NF- κ B pathway LMP1 can stimulate miR-155 expression, which is much higher in EBV-immortalized B cells than in EBV-negative B cells.⁵⁸ miR-155 expression is upregulated in EBV-positive NPC tissue samples.⁵⁹ miR-155 overexpression promotes NPC cell proliferation, migration, and invasion. Overexpression of miR-155 decreased the activities of a luciferase reporter fused to the 3' UTR of JMJD1A or BACH1 as well as the expression of JMJD1A and BACH1 in NPC cells, identifying JMJD1A and BACH1 as targets of miR-155.⁶⁰ Reduced JMJD1A expression is associated with poor prognosis of NPC patients. However, the roles of miR-146a and miR-155 in LMP1-mediated oncogenesis remain to be elucidated.

LMP1 has been shown to be a positive regulator of the metastasis of NPC cells. High levels of miR-10b expression were observed in EBV-positive LMP1-expressing NPC cells.⁶¹ LMP1 induces Twist expression, which in turn increases the transcription of miR-10b in NPC cells. miR-10b overexpression enhances the metastasis of NPC and accelerates the death of tumor-bearing nude mice, suggesting that miR-10b plays an important role in LMP1-mediated metastasis of NPC cells.

In addition to LMP1-mediated induction of cellular miRNAs, LMP1 can also inhibit the expression of cellular miRNAs. EBV infection decreases the expression of miR-203 in epithelial cells and is associated with the downregulation of miR-203 in NPC tissues.⁶² Overexpression of miR-203 induces G1/S cell cycle arrest in EBV-infected cells and represses the growth of tumors induced by EBV in vivo. Whether LMP1 promotes tumor growth through inhibition of miR-203 remains to be determined. miR-204 is downregulated in NPC tissues and reduced expression of miR-204 is associated with a more aggressive and poor prognostic phenotype of NPC.⁶³ LMP1 indirectly inhibits miR-204 expression by activating the transcription factor Stat-3. miR-204 may inhibit invasion and metastasis of EBV-positive cells through direct targeting of Cdc42.

BARF1 is an intracellular and secreted protein that functions as a viral oncogene and immune modulator in EBV-driven carcinogenesis. BARF1 regulates the expression of genes involved

in cell proliferation, mitosis, and cell cycle regulation, such as *CCND1* and *bcl-2*. The regulatory relationship between BARF1 and miRNAs is unclear.

EBV miRNAs regulate the expression of host genes

By processing the *BHRF1* and *BART* transcripts, EBV generates several tens of viral miRNAs called BHRF1 miRNAs and BART miRNAs. The targets of most BHRF1 miRNAs and BART miRNAs are unclear. The viral miRNAs of the *BHRF1* locus have been shown to inhibit apoptosis and facilitate cell cycle progression and proliferation during the early phase of infected human primary B cells.⁶⁴ miR-BHRF1-1 is involved in EBV late lytic infection and directly inhibits the tumor suppressor p53 in NPC cells through its 3' UTR.⁶⁵ miR-BHRF1-1, miR-BHRF1-2, and miR-BHRF1-3 contribute to B-cell transformation.^{66,67} The underlying mechanisms of these activities remain to be investigated.

The majority of BART miRNAs have been shown to enhance cell proliferation in gastric cancer cells, although 5 BART miRNAs (miR-BART15-3p, -5-5p, -16-5p, -17-3p, and -20-3p) inhibit cell proliferation.⁶⁸ Among these 5 miRNAs, miR-BART15-3p represses cell proliferation most strongly. Moreover, miR-BART15-3p inhibitor enhances cell proliferation and suppresses apoptosis in EBV-infected cells. miR-BART15-3p reduces the expression of baculovirus inhibitor of apoptosis repeat-containing ubiquitin-conjugating enzyme (BRUCE) by directly targeting the *BRUCE* 3' UTR. Similar to miR-BART15-3p, knockdown of *BRUCE* induces cell death in gastric cancer cells. p53 upregulated modulator of apoptosis (PUMA) has been shown to be a direct target of miR-BART5, which is abundantly expressed in NPC and EBV-GC cells.⁶⁹ PUMA, a proapoptotic protein belonging to the "BH3-only" group of the Bcl-2 family, is significantly downregulated in approximately 60% of human NPC tissues. miR-BART5 inhibition of PUMA in NPC cells confers resistance to apoptosis. Although miR-BART15-3p and miR-BART5 can directly regulate the expression of apoptosis-related genes, whether the miR-BART15-3p/BRUCE or miR-BART5/PUMA axis plays an important role in EBV-associated carcinogenesis remains unknown.

In addition to apoptosis-related genes, the tumor suppressor DICE1 can be directly repressed by miR-BART3-5p.⁷⁰ Overexpression of miR-BART3-5p stimulates NPC cell proliferation. DICE1 is downregulated in NPC tumor tissues and there appears to be an inverse correlation between miR-BART3-5p and DICE1 expression.

Cellular miRNAs in EBV-associated cancer

Cellular miRNAs play a critical role in the development and progression of EBV-associated cancer (Table 2). As described above, cellular miRNAs can be regulated by viral oncogenes; however, direct modulation of cellular miRNAs by viral miRNAs is uncommon. EBV infection has a profound impact on cellular miRNA expression.⁷⁴ miR-424, -223, -199a-3p, -199a-5p, -27b, -378, -26b, -23a, and -23b are upregulated and miR-155, -20b, -221, -151-3p, -222, -29b/c, and -106a are downregulated after EBV-infection of diffuse large B-cell lymphoma. Many host miRNAs, such as members of the let-7 and miR-200 families, are also downregulated by EBV infection of gastric cancer cells.^{79,80}

Table 3. Representative cellular miRNAs with known targets in HPV-associated cancers

c-miRNA	Target gene	Up/downregulated	Function	Ref
miR-23b	<i>uPA</i>	in cervical cancer	es cell cycle arrest and apoptosis	83
miR-375	<i>SP1</i>	in cervical cancer	Inhibits cell proliferation, migration, and invasion Induces cell cycle arrest	84
miR-129-5p	<i>SP1</i>	intraepithelial lesions	Inhibits cell proliferation Promotes apoptosis Blocks cell cycle progression	85
miR-218	<i>LAMB3</i>	in cervical cancer	Inhibits cell proliferation, migration, and invasion	86
miR-497	<i>IGF-1R</i>	in cervical cancer	Inhibits cell proliferation, migration, and invasion	87
miR-34a	<i>p18Ink4c</i>	in cervical cancer	Inhibits cell growth Promotes apoptosis.	88
miR-92	<i>PTEN</i>	in cervical cancer	Promotes cell growth and invasion	89

The dysregulation of cellular miRNAs (e.g., miR-155 or miR-424) results in alteration of the expression of both oncogenes and tumor suppressor genes (e.g., c-MYB or SIAH) in host cells.⁷⁴ However, the targets and functions of the majority of these cellular miRNAs in EBV-associated carcinogenesis remain to be determined. Additionally, we cannot exclude the possibility that cellular miRNAs whose expression is not changed by EBV infection also play roles in EBV-associated oncogenesis.

Cellular miRNAs in HPV-Associated Cervical Cancer

More than 100 different HPV types have been identified. Among high-risk HPVs, HPV16 and HPV18 are the principal causes of cervical cancer as well as several other tumor types.⁸¹ HPV encodes two important oncoproteins, E6 and E7, which disrupt natural tumor suppressor pathways, including the p53 and Rb pathways. E6 and E7 oncoproteins regulate several cellular processes, including cell proliferation, apoptosis, migration, invasion, and immune evasion. It is generally accepted that, unlike other DNA viruses, HPVs do not encode any miRNAs. However, a recent study demonstrated that HPVs do encode their own microRNA species:⁸² for example, 2 are encoded by HPV16, 1 by HPV38, and 1 by HPV68. The function of these HPV-encoded miRNAs remains to be investigated. Recent studies indicate an important level of interaction among the E6/E7 oncoproteins and cellular miRNAs. Cellular miRNAs play important roles in HPV-related cervical oncogenesis (Table 3).

HPV-encoded oncogenes regulate the expression of cellular miRNAs

Using miRNA array analysis and small RNA sequencing (miRNA-Seq), Wang et al. performed a comprehensive examination of miRNA profiles in raft cultures derived from human foreskin keratinocytes (HFKs) or human vaginal keratinocytes (HVK) with or without productive HPV16 or HPV18 infection, and identified a group of cellular miRNAs that are regulated by the HPV infection.⁸⁸ The viral oncoprotein E6/E7 is responsible for an increase in miR-16, miR-25, miR-92a, and miR-378, and a decrease in miR-22, miR-27a, miR-29a, and miR-100. Examination of expression of these 8 miRNAs in 158 cervical specimens, including 38 normal, 52 cervical intraepithelial

neoplasia, and 68 cervical cancer tissues, demonstrated a remarkable increase in miR-25, miR-92a, and miR-378 with lesion progression but no obvious change in miR-22, miR-29a, and miR-100 among the HPV-infected tissues. The targets and roles of miR-25, miR-92a, and miR-378 in HPV-associated cervical cancer development and progression remain to be investigated.

Wang et al. showed that cervical cancer tissues and cervical cancer-derived cell lines containing oncogenic HPVs exhibit decreased expression of tumor-suppressive miR-34a.⁹⁰ Expression of HPV E6 inhibits miR-34a expression by destabilizing p53. Expression of miR-34a in HPV18-positive cervical cancer cells leads to growth retardation, cell cycle arrest at G2/M, and apoptosis. miR-23b has been shown to be downregulated in HPV-associated cervical cancer.⁸³ The E6 oncoprotein decreases the expression of miR-23b and increases the expression of urinary plasminogen activator (uPA), a direct target of miR-23b that plays an important role in tumor cell migration, invasion, and metastasis. E6 thus promotes migration of cervical carcinoma cells through miR-23b and uPA. p53 is involved in the transcriptional activation of miR-23b; E6 inactivates p53, thus repressing miR-23b. The E2F transcription factor is an important positive regulator of the cell cycle, and is used by HPV to regulate proliferation of infected cells. The E7 protein of HPV interacts with Rb, a negative regulator of E2F, and blocks its binding to E2F. The expression of miR-15b is strongly associated with the expression of several E2F-regulated genes in anal carcinoma biopsies.⁹¹ Additionally, E7 inhibits miR-15b expression in cervical cancer cells. Ectopic expression of miR-15b increases the expression of cyclin E, a E2F-regulated gene.

Cellular miRNAs regulate the expression of HPV-encoded oncogenes

Examination of the expression of miR-375 in 170 cervical cancer tissues and 68 normal cervical tissues revealed downregulation of miR-375 in cervical cancer tissues. miR-375 expression negatively correlates with lymph node metastasis, FIGO stage, deep stromal invasion, lymphovascular space involvement, and vaginal wall extension.⁸⁴ Overexpression of miR-375 inhibits cell proliferation, induces G1/S cell-cycle arrest, and reduces cell migration and invasion in human cervical cancer cells. miR-375 negatively regulates SP1 gene expression by directly targeting its 3' UTR. Consistent with the effects of



Figure 1. miRNAs may regulate the hallmarks of virus-related cancer.

miR-375 overexpression, knockdown of SP1 reduces proliferation, migration, and invasion in cervical carcinoma cells. Moreover, SP1 expression is inversely correlated with miR-375 expression in cervical cancer tissues. No difference in miR-375 expression was found between 32 normal cervical tissues with high-risk HPV infection and 36 normal cervical tissues without high-risk HPV infection. Moreover, HPV16 E6/E7 does not alter miR-375 expression. Interestingly, miR-375 targets HPV type 16 and 18 transcripts and represses E6 and E7 expression.⁹² HPV-positive cervical cancer contains wild type p53 and/or Rb, and inactivation of these tumor suppressors is driven by E6 and E7 oncoproteins produced from high-risk HPVs. Cellular E6AP forms a complex with E6, repressing p53 by ubiquitin-dependent degradation. E7 degrades Rb, thus activating cell proliferation genes. miR-375 increases the expression of p53 and Rb in HPV16- and 18-positive cervical cancer cells. These data suggest that rescue of tumor suppressors by miR-375-mediated repression of E6 and E7 oncoproteins may result in reduced proliferation of HPV-positive cervical cancer cells.

Conclusion and Perspective

Great progress has been made by research into the role of miRNAs in human carcinogenesis since the initial discovery of miRNAs in the laboratory model organism nematode *Caenorhabditis elegans* in 1993.⁹³ DNA viruses encode their own miRNA species, whereas RNA viruses seem to rarely encode miRNAs. It is conceivable that temporal and spatial interplay exists among virus-encoded oncoproteins, virus-encoded miRNAs, cellular miRNAs, and cellular genes. Indeed, EBV miRNAs can regulate the expression of EBV-encoded oncogenes and cellular (host) genes, EBV-encoded oncogenes can modulate the expression of cellular miRNAs, and cellular miRNAs also play a role in EBV-associated cancer. Viral oncoproteins, viral miRNAs, cellular miRNAs, and cellular genes may cooperate to control normal cell transformation and cancer cell proliferation, differentiation, apoptosis, migration, invasion, and metastasis, although the detailed mechanisms underlying miRNA modulation of viral oncogenesis remain to be elucidated. Additional functional studies on viral oncogenesis require

relevant models of viral infection. The hallmarks of cancer include sustained proliferative signaling, evasion of growth suppressors, resistance to cell death, enabling of replicative immortality, induction of angiogenesis, activation of invasion and metastasis, deregulation of cellular energetics, avoiding immune destruction, genome instability and mutation, and tumor-promoting inflammation (Fig. 1).⁹⁴ Whether viral oncogenesis is caused by alteration of these hallmarks through the action of miRNAs remains to be further investigated.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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