Epstein-Barr Virus microRNAs in the pathogenesis of human cancers

Short title: EBV miRNAs and human cancers

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Keywords: EBV mir-BARTs, EBV mir-BHRF1, EBERs, nasopharyngeal carcinomas, lymphomas, gastric carcinoma

Abstract

The Epstein-Barr Virus (EBV) is a gamma-herpesvirus involved with a variety of human cancers, notably the endemic Burkitt lymphoma and nasopharyngeal carcinoma. In 2004, EBV was described as the first known human oncovirus to encode viral microRNAs (miRNAs), and these molecules were found to interact with viral and host targets. EBV miRNAs modulate biological processes that are critical for carcinogenesis, contributing to cell transformation and tumor progression of EBV-associated cancers. Herein we review and discuss EBV miRNAs as modulators of viral biology and carcinogenesis, as well as their usefulness as putative markers to monitor the onset, progression, and recurrence of cancers associated with the EBV infection.

1 Epstein-Barr virus: an overview

Cancers accounted for more than 18.1 million new cases and 9.6 million deaths in 2018 and are a leading cause of morbidity and mortality worldwide [1]. The incidence and prevalence rates for specific cancers varies around the world among different geographical regions and socioeconomic contexts. The risk of developing cancers is influenced by constitutive factors (genetic background, for instance), lifestyle, and environmental factors, such as exposition to some physical, chemical, or infectious agents, most of them preventable.

In 2018, 2.2 million new cancer cases (about 13% of all cancers diagnosed) were estimated to be associated with infectious agents, mostly in developing countries [2]. For several relevant reasons, the Epstein-Barr-Virus (EBV) deserves special attention compared to other infectious agents associated with human cancers. Notably, EBV is a ubiquitous virus that causes lifelong latent infection in over 90% of the human adult population worldwide. Also, EBV infection is associated with a variety of human cancers, especially the endemic form of Burkitt lymphoma (BL) and nasopharyngeal carcinoma (NPC), but also in a variable number of cases of other malignancies, such as classical Hodgkin lymphomas (cHL), non-Hodgkin lymphomas (NHL), and gastric carcinomas (GC) [3].

EBV is a gammaherpesvirus formally designated Human Herpesvirus 4 (HHV-4). As for other herpesviruses, the EBV life cycle is essentially divided into lytic and latent infection phases. Either *in vivo* or *in vitro*, most of the EBV-infected cells are in viral latency, a phase in which only a subset of viral genes is expressed. Conversely, most of the viral genes are expressed for the production of new particles during the lytic cycle, allowing persistent infection and viral dissemination to other hosts. The lytic reactivation occurs sporadically *in vivo,* usually triggered by changes in the status of the host's immune responses. It also occurs spontaneously in a small fraction of latently infected cells *in vitro*, but higher levels of lytic reactivation can be obtained by treating infected cells with phorbol esters, such as tetradecanoylphorbol-13-acetate (TPA), or sodium butyrate [4]. The EBV proteins Zta and Rta (encoded by BZLF1 and BRLF1 genes, respectively) are key activators of viral lytic infection.

In cells latently infected by EBV three profiles of viral gene expression are typically observed, described as latency types I, II, and III (see Table 1). Some EBV products are expressed in all latency profiles, such as the EBV-encoded small RNAs (EBERs), BamHI-A rightward transcripts (BARTs), and the EBV nuclear antigen 1 (EBNA1). This minimum set of viral products expressed during the latent phase of the viral life cycle characterizes the EBV latency type I, commonly found in BL cells *in vivo*. Latency type II also encompasses the expression of EBV latent membrane proteins (LMPs 1, 2A, and 2B) and it is typically observed in NPC neoplastic cells, for instance. In latency type III, all the latency viral products are expressed: six EBNAs (1, 2, 3A, 3B, 3C, and LP) and three EBV LMPs (1, 2A, and 2B) proteins, along with EBERs, miRNAs encoded in the BART and the BamHI fragment H rightward open reading frame 1 (BHRF1) segments of the viral genome. Latency type III is usually observed in EBV-associated lymphoproliferative diseases and lymphoblastoid cell lines constitutively infected by EBV [5].

In the first years of the XXI century, the discovery that EBV encodes their ncRNAs in its genome provided valuable insights for a better understanding of the viral carcinogenesis mechanisms [6]. In this regard, recent evidence shows that the contribution of EBV in the pathogenesis of human cancers is a result not only of the activity of viral oncoproteins but also viral non-coding RNAs (ncRNAs). Thus, herein we will review the current state of the knowledge on the role of viral miRNAs in the development and progression of human cancers associated with EBV infection.

2 EBV encodes viral ncRNAs

Both EBV and the Kaposi Sarcoma Herpesvirus (KSHV) – the two known herpesviruses recognized as oncogenic to humans so far – encode and express their own ncRNAs [6], which are critical for the viral life cycle and the infection pathogenesis. Overall, viruses rely on a subset of their viral products to regulate and take advantage of cellular gene expression. Nonetheless, viral proteins may trigger immune defenses, which impose costly limitations to use them as a leading strategy for modulation of cellular gene expression. This is especially important in the context of viral carcinogenesis, because viral proteins may elicit immune responses against cells transformed by oncogenic viruses. The activity of viral ncRNAs

may enhance viral control over cellular gene expression without significantly eliciting host defenses against the infected cells [7].

The EBV-encoded RNAs (EBERs) 1 and 2 are two non-polyadenylated non-coding RNAs encoded in the viral genome that are known to contribute to EBV-induced malignant transformation for a few decades now [8]. Both EBERs are generated by the RNA Polymerase III (Pol III), processed to either short 5'-derived RNAs or 3' fragments, and expressed in all EBV latency types. Some authors refer to EBERs as EBV "small non-coding RNAs" (sncRNAs). However, based on their distinctive biogenesis compared to typical sncRNAs, others designated EBERs as "long non-coding RNAs" – even though they do not meet the usual definition criterion for lncRNAs (longer than 200 nucleotides), as they have 167 and 172 nucleotides in length, respectively. Even though there is no agreement in the literature regarding the proper classification of EBERs, in this review we will discuss EBERs in addition to EBV microRNAs (miRNAs) due to its importance in EBV pathogenesis. EBV also encodes a stable intronic sequence RNA (ebv-sisRNA), mostly derived from the W repeat region of the viral genome [9]. It is suspected that ebv-sisRNA plays a role in EBV-related diseases, although their virological and pathogenetic activities require further elucidation [10].

2.1 Biogenesis of EBERs and EBV-encoded miRNAs

Similar to that found in EBERs, EBV miRNAs are abundantly expressed in cells latently infected by the virus (see **Table 1**). There are two groups described of EBV miRNAs: ebv-miR-BHRFs and ebv-miR-BARTs, encoded within the BHRF1 and BART regions of the viral genome, respectively. To date, 25 EBV precursor miRNAs (pre-miRNAs) were identified, giving rise to 48 mature miRNAs [11]. EBV miRNAs are generated from either protein-coding or non-coding regions of the viral genome by the RNA polymerase II (Pol II), and subsequently processed by Drosha and Dicer for miRNA maturation [12].

The miRNAs encoded by EBV hijack intracellular pathways implicated in the synthesis and maturation of miRNAs of either viral or host origin. For instance, ebv-miR-BART6-5p targets four sites present within the 3'-UTR of the human Dicer mRNA, affecting the miRNAs maturation in general,

111 causing global suppression of these molecules. This is a key regulatory phenomenon in the viral life cycle,
112 contributing to the maintenance of the latent infection [13]. Dicer downregulation and the global
113 suppression of miRNAs were previously described in malignant cells, often associated with cancer
114 progression [14]. EBV products other than viral miRNAs also contribute to Dicer downregulation, such
115 as the EBNA1 oncoprotein [15].

116 The biogenesis and effects of EBV miRNAs are also affected by adenosine to inosine (A-to-I) RNA
117 editing. Briefly, adenosine in RNA molecules (double-stranded, both coding and non-coding) can be
118 converted to inosine due to the activity of adenosine deaminases acting on RNA (ADARs) enzymes, which
119 results in negative regulation of certain mature RNAs, including ncRNAs [16]. These A-to-I conversions
120 affect the stability of miRNAs or may even create alternative 3'UTR targets, impacting the properties
121 and activities of well-known mature miRNAs [17,18]. In cells latently infected by EBV, A-to-I edit of ebv-
122 miR-BART6 precursors elicits the activation of viral genes encoding EBNA2, LMP1, ZTA, and RTA, leading
123 to viral cycle transition either to type III latency or lytic reactivation [13]. In NPC cell lines, the ebv-miR-
124 BART3 is also subjected to A-to-I editing, disrupting its maturation [19]. Altogether, this data shows that
125 the EBV life cycle takes advantage of both EBV miRNAs and viral proteins to regulate the biogenesis,
126 maturation, and activity of the ncRNAs repertoire in infected cells, with important consequences for the
127 pathogenesis of EBV-associated cancers, as discussed later in this review.

128 *2.2 EBV miRNAs and EBERs are key for the regulation of the viral life cycle*

129 The life cycle of herpesviruses evolved alongside their hosts, allowing these viruses to develop
130 ways for efficient regulation of the expression of both viral and cellular genes in the infected cells. The
131 EBV lytic reactivation allows the expression of highly immunogenic viral proteins repressed during
132 latency. Nonetheless, while most viral proteins are suppressed during viral latency, the EBV ncRNAs are
133 abundantly expressed, as they play important roles for inhibition of lytic reactivation and maintenance
134 of persistent infection in the host [20]. A recent target prediction analysis *in-silico* using the seeding
135 sequences of EBV miR-BARTs showed that the EBV genes encoding for EBNA1, BFRF2, BILF2, BALF5, and

BRRF2 are putative candidates for viral autoregulatory inhibition. Noteworthy, the respective EBV proteins are crucial for the lytic cycle, being involved in the duplication of the viral genome and the production of new virions [21].

Experimentally, it was already demonstrated that the ebv-miR-BART2 inhibits the replication of the EBV genome by targeting the viral DNA polymerase BALF5, while ebv-miR-BART6 suppresses EBNA2 and inhibits ZTA and RTA [13,22], two essential EBV lytic transactivators directly targeted also by ebv-miR-BART20-5p [23]. In EBV-positive gastric carcinoma cells, the ebv-miR-BART20-5p was described to exert an important role in latency and viral persistence via inhibition of the Bcl-2-Associated Death promoter (*BAD*), essential to trigger the expression of EBV immediate early genes during lytic reactivation [24].

Recently, it was described that the latent-to-lytic switch is regulated in EBV-infected cells by ebv-miR-BHRF1-2-5p and ebv-miR-BART2-5p, which target key molecules involved in the B-cell Receptor (BCR)-mediated signaling, including Growth Factor Receptor Bound Protein 2 (*GRB2)*, MALT1 Paracaspase (*MALT1)*, Rac family Small GTPase 1 (*RAC1)*, and Rac Guanine Nucleotide Exchange Factor 1 (*SOS1)*. The BCR signaling pathway plays a key role in B-cell activation, differentiation, cellular proliferation, and EBV lytic reactivation as well. The inhibition of ebv-miR-BHRF1-2-5p and ebv-miR-BART2-5p cause viral lytic reactivation triggered by BCR engagement, highlighting the importance of these EBV miRNAs in antagonizing extracellular stimuli that elicit EBV reactivation [25]. The regulation of the EBV lytic cycle by viral miRNAs remains to be properly elucidated; the available evidence discloses important and yet overlooked mechanisms by which EBV explores a repertoire of viral miRNAs for self-regulation of its life cycle.

2.3 EBV miR-BARTs target signaling pathways involved in cell proliferation and apoptosis

The EBV miRNAs are also instrumental for the maintenance of persistent infection by reducing cytopathic effects associated with the microenvironment, modulating cell survival, and apoptosis. In this regard, EBV-encoded miRNAs were found to regulate multiple molecules involved in the Wnt signaling,

one of the key intracellular pathways that regulate cell fate, development, and stemness. For instance, in AGS-EBV and SNU-719 EBV-positive gastric carcinoma cells the EBV-miR-BART10-3p was found to downregulate the expression of the Dickkopf WNT Signaling Pathway Inhibitor 1 (*DKK1*) gene, which encodes a soluble secreted protein involved in the suppression and control of the Wnt signaling [26]. Another study reported that EBV miR-BART1-3p suppresses apoptosis and promotes cell migration via the downregulation of the Disabled Homolog 2 (*DAB2*), a putative tumor suppressor that contributes to attenuation of the Wnt/β-Catenin signaling [27]. More recently, the expression of EBV-miR-BART19-3p was reported to induce cell growth and to suppress apoptosis in EBV-negative Jurkat and CNE2 cell lines. Similar effects were also found in cells transfected with EBV-miR-BART19-3p mimics and were attributed to the downregulation of the Adenomatous Polyposis Coli (*APC*) gene, another key regulator of the Wnt signaling [28].

The contribution of EBV-encoded miRNAs for cell survival and proliferation may be also related to post-transcriptional regulation of the Tumor Protein (*TP53*), a tumor suppressor gene. This was shown for EBV-miR-BART5-3p, which directly target the *TP53* transcript, increasing cell proliferation *in vitro* and also *in vivo*, in xenograft models using gastric cancer cell lines SGC7901 (EBV-positive) and AGS (EBV-negative). EBV-miR-BART5-3p was found highly expressed in human specimens of EBV-positive gastric cancer [29]. Furthermore, an inverse correlation between the expression of EBV-miR-BHRF1-1 and TP53 was reported in patients with chronic B-cell leukemia, as well as in B-cell leukemia MEC1 and JVM3 cells *in vitro*. Inhibition of EBV-miR-BHRF1-1 upregulated TP53, while expressing the viral miRNA decreased cell cycle arrest and apoptosis, and caused an increase in cell proliferation. These effects were reported to be due to EBV-miR-BHRF1-1 binding to the 3'-UTR region of the *TP53* gene transcript [30].

The modulation of cell survival pathways by EBV miRNAs is an important matter for cancer treatment, as it may be explored to understand and management of radio- and chemoresistance phenotypes in EBV-associated malignancies. In this regard, the expression of EBV-miR-BART8-3p in 5-8F and HONE1 EBV-positive NPC cells was associated with apoptosis resistance after radiotherapy, an effect associated with modulation of the Ataxia-Telangiectasia Mutated (*ATM*) / Ataxia Telangiectasia Mutated

and Rad3-related (*ATR*) signaling pathway. A tumor xenograft model using 5-8F cells confirmed that EBV-miR-BART8-3p significantly promotes radioresistance and tumor growth in nude mice. EBV was also shown to increase phosphorylated forms of ATM and ATR proteins in irradiated cells, favoring DNA repair and apoptosis resistance [31]. Also in NPC cells, EBV-miR-BART22 has been shown to induce cisplatin chemoresistance via direct downregulation of the Mitogen-Activated Protein Kinase 4 (*MAP2K4)* transcript, inducing Myosin Heavy Chain 9 (*MYH9*) expression and GSK3β ubiquitination, promoting tumor stemness, metastasis, and chemotherapy resistance [32]. Therefore, the foregoing evidence highlights pivotal roles for EBV miRNAs in cell survival and proliferation pathways, contributing to viral persistence and, ultimately, with expected consequences for the development and progression of EBV-associated cancers.

2.4 EBV ncRNAs contribute to subversion of host immune responses

The successful persistence of viral infection depends on checks and balances with the host immune system. In the case of EBV, this can be addressed with stringent regulation of highly immunogenic viral proteins such as LMP2a, which interferes with the B Cell Receptor (BCR) signaling and is crucial for viral persistence in B-cells. The robust and specific cytotoxic responses mediated by T CD8+ cells expression elicited by EBV LMP2 can be mitigated by ebv-miR-BART22 [33]. Similar results were reported recently in a study using NOD-SCID γ_c null (NSG) and HLA-A2 transgenic NSG mice with infection by EBV variants lacking viral miRNAs. B-cells from mice infected with miRNA-deficient virus exhibited a drastic reduction in tumorigenic potential, reduced cell proliferation, and lower viral load when compared to wild type EBV. Furthermore, depletion of T $CD8^+$ cells resulted in lymphoma formation and increased viral load in the majority of mice infected with a miRNA-deficient virus. These results strengthen the idea that EBV-encoded miRNAs support tumorigenesis via immune evasion *in vivo* [34].

Other mechanisms for immune regulation by EBV-encoded miRNAs are also described. For instance, ebv-miR-BART6-3p mediates downregulation of the Interleukin-6 Receptor (*IL-6R*) and the RIG-I-Like Receptor (*RIG-I*), a Pattern Recognition Receptor (PRR) important for virus recognition by the

innate immune system [35,36]. Recently it was reported that ebv-miR-BART7 targets the MHC-I Polypeptide-Related Sequence A (*MICA*) transcript, causing impairment activity of Natural Killer (NK) cells [37]. EBV miRNAs may also regulate immune checkpoints: for instance, in EBV-negative gastric carcinoma cells, the expression of EBV-miR-BART5-5p inhibited the Protein Inhibitor of Activated Stat3 (*PIAS3*) transcript, increasing PD-L1 upregulation and ultimately causing cell resistance to apoptosis, increased cell proliferation, cellular invasion, migration, and immune escape [38]. In a similar study, ebv-miR-BHRF1-2-5p was shown to exert a context-dependent counterregulatory role by targeting LMP1-driven PD-L1 and PD-L2 expression, also supporting immunoevasion and viral persistence [39].

There are scarce data regarding the immunomodulatory effects of EBERs, essentially investigated in few pioneer works. In EBV-positive BL cell lines, the expression of EBERs induces IL-10 production mediated by the Retinoic Acid-Inducible Gene I (*RIG-I*)/*IRF-3* pathway, decreasing local inflammation and other immune responses [40]. EBER1 also inactivates the interferon-induced RNA-dependent Protein Kinase (*PKR*), a key component in the host cell mechanism against viral infection [41,42].

3 EBV ncRNAs in infection-associated cancers

Compelling evidence indicates that viral ncRNAs may influence many biological phenomena in a variety of cancers associated with EBV infection. Because a significant number of the studies relevant to this topic relies on cell lines cultivated *in vitro*, their results should be carefully evaluated based on known biological features and limitations of these models (see **Table 2**).

EBV-associated cancers may express a variable set of the viral ncRNAs, including EBERs, Small Nucleolar RNAs (snoRNAs), Stable Intronic-Sequence RNA (ebv-sisRNA), and viral miRNAs encoded in the BHRF1 and BART regions of the EBV genome [43]. Besides EBERs, EBV miR-BARTs are the most studied ncRNAs so far, but relevant data have been also published for EBV miRNAs encoded in the BHRF1 region of the viral genome lately.

As discussed in the previous section, EBV-encoded miRNAs exert a preeminent role in the regulation of viral biology, targeting both viral and human transcripts to support viral persistence in the infected cells. While these are mechanisms to regulate the EBV life cycle, they eventually contribute to cancer development and progression. In the following sections, we will delineate the current state of knowledge regarding the role of EBV ncRNAs, notably microRNAs, in the pathogenesis of human cancers associated with viral infection. For better understanding, the selected studies will be discussed considering major contributions of their results in three complementary, non-exclusive scenarios in cancer biology: 1) cell survival, proliferation, and tumorigenesis; 2) cancer microenvironment and immunobiology; and 3) cancer progression and aggressiveness.

3.1 Cell survival, proliferation, and tumorigenesis

Several EBV ncRNAs are being reported as putative regulators of cell proliferation and survival, ultimately contributing to the development of EBV-associated cancers. For instance, the expression of ebv-miR-BARTs was found to provide a growth advantage for xenografted tumors derived from the EBV-positive gastric carcinoma cells SNU-719 [44,45]. Furthermore, in the EBV-negative SGC7901 and AGS gastric carcinoma cells, the ectopic expression of ebv-miR-BART3-3p supported cell growth and inhibited

cell senescence both *in vitro* and in *in vivo* [46]. In another scenario, it was demonstrated that the expression of both ebv-miR-BHRF1-2 and ebv-miR-BHRF1-3 were positively correlated with an increase in the rate of cell growth for EBV-positive lymphoblastoid cell lines (LCLs) spontaneously established from peripheral blood cells of transplanted patients under therapeutic immunosuppression [47]. Although the specific mechanisms remain to be fully clarified, this data supports the concept that the expression of EBV ncRNAs have an impact on the regulation of cell proliferation and may support either tumorigenesis or lymphomagenesis associated with EBV infection.

Tumor suppressor genes encoding for regulators of cell proliferation and cell death pathways are important targets for EBV ncRNAs. In this regard, in the epithelial cells HNE1* and the gastric carcinoma cells SGC7901, the transcriptional and protein levels of the *TP53* tumor suppressor gene was adversely affected by the ectopic expression of ebv-miR-BART5-3p. In these models, cells expressing the viral miRNA showed accelerated *TP53* degradation and reduced levels of *TP53*-responsive genes, such as the cell-cycle regulator Cyclin-Dependent Kinase Inhibitor 1A (*CDKN1A)* and the apoptosis-related genes Fas Cell Surface Death Receptor (*FAS)*, BCL2 Associated X, apoptosis regulator (*BAX)*, and BCL2 Binding Component 3 (*BBC3),* also known as *PUMA*. Ultimately, cells expressing the ebv-miR-BART5-3p were more resistant to ionizing radiation and etoposide-induced apoptosis, showing increased growth both *in vitro* and *in vivo* [48]. In another study, the expression of ebv-miR-BARTs 1, 2, 7, 8, and 22 in HEK293 cells caused downregulation of the caspase-3 pro-apoptotic protein [49], which was also affected by ebv-miR-BARTs 1-3p and 16 in the 293 cell line and Oku-BL (EBV-positive BL cells) [50]. In the AGS-EBV cell line, the expression of ebv-miR-BART20-5p was also implicated in apoptosis inhibition by targeting the 3'-UTR of the Bcl2-associated Agonist of Cell Death (*BAD*) [24]. The tumor suppressor gene *TP53* is also targeted directly by ebv-miR-BART3-3p*,* as demonstrated by Wang and colleagues (2019) in clinical tumors of EBV-positive gastric carcinoma patients, the authors reported that the expression of ebv-miR-BART3-3p was negatively correlated with *TP53* expression. Furthermore, the expression of ebv-miR-BART3-3p in SGC7901 and AGS gastric carcinoma cell lines caused an increase in cell growth *in vitro* and tumorigenesis *in vivo* [51].

Together, these results demonstrate that EBV miRNAs can downregulate tumor suppressor genes, including those encoding regulators of cell cycle and the apoptosis pathways, though this data has emerged more recently and its clinical significance requires further investigation.

3.2 Cancer microenvironment and immunobiology

The expression of EBV ncRNAs is also relevant in terms of tumor metabolism and tumoral microenvironment. For instance, ebv-miR-BART1-5p stably expressed in CNE1* and HONE1* cells increased the expression of genes involved in the Warburg effect (aerobic glycolysis) and neoangiogenesis *in vitro* (chorioallantoic membrane model) and in *in vivo* (xenograft mouse tumor). These effects were associated with the downregulation of the α1 catalytic subunit of AMP-Activated Protein Kinase (*AMPK*), which causes an increase in mammalian-Target of Rapamycin (mTOR) signaling and Hypoxia-Inducible Factor 1-alpha (HIF-1α) expression [52]. Recently, it was reported that primary endothelial cells (HUVEC) either transfected with EBV EBERs or exposed to exosomes derived from EBV-positive C666-1 NPC cells showed an increase in angiogenic response linked to EBERs-mediated activation of Toll-Like Receptor 3 (TLR3) and *RIG-I*, causing vascular remodeling mediated by the Vascular Cell Adhesion Protein 1 (VCAM1) [53].

EBV ncRNAs can be also instrumental in setting up an inflammatory microenvironment favorable to EBV-positive malignant cells. The expression of the LMP1 oncoprotein and EBERs in EBV-negative HNE2* cells cooperatively induce the expression of the pro-inflammatory cytokines TNFα, IL-6, and IL-1α, with high levels of EBERs correlated to higher levels of TNF-α and poorer survival rates in NPC patients [54]. Additionally, ebv-miR-BARTs induce features of tumor-associated macrophages (TAMs) when transferred into regular macrophages via exosomes derived from umbilical cord blood cell lines transformed with the EBV strain B95.8 or Akata. This effect was attributed to inhibition of the Myocyte Enhancer Factor 2C (*MEF2C)* translation, causing an increase in pro-tumoral molecules, such as IL-10, TNF-α, and Arginase 1 (*AGR1*) [55]. Furthermore, EBV EBER2 was reported to increase the levels of Toll-Like Receptor 7 (TLR7), known to be involved in inflammation and carcinogenesis [56].

As mentioned earlier in this review, EBV-infected cells may express immunogenic proteins that are tightly regulated by viral ncRNAs. Beyond favoring the EBV persistence, this regulation can provide adaptative advantages for the infected malignant cells. For instance, it was previously shown that LMP1 expression upregulates the immune checkpoint ligand PD-L1 via STAT3, AP-1, and NF-κB, supporting the immunoevasion of infected cells [57]. Both the PD-L1 and PD-L2 transcripts can be directly targeted by ebv-miR-BHRF1-2-5p, disclosing a context-dependent counterregulatory mechanism to fine-tune the LMP1-driven upregulation of these immune checkpoints molecules [39]. This typifies the complex regulatory roles that EBV ncRNAs may exert in the immunobiology of EBV-associated cancers, providing relevant information to take into account when considering therapies based on immune checkpoints to manage these diseases.

As shown herein, by increasing the expression of cytokines, immune mediators, and other pro-tumoral molecules, EBV miRNAs and EBERs contribute to set up an inflammatory microenvironment that promotes vascular remodeling and immunoevasion. Although relatively scarce, the data already available provides valuable insights on the mechanisms underlying the modulation of tumoral microenvironment and immunobiology by ncRNAs encoded by EBV.

3.3 Cancer progression and aggressiveness

The traditional paradigm that implicates some viruses in the pathogenesis of cancers only recently evolved to consider the participation of oncoviruses not only in malignant cell transformation but also as modulators of the aggressiveness and progression of their associated cancers [58]. On top of that, compelling evidence published in the past decade unraveled important roles of EBV ncRNAs interfering in key pathways associated with cancer invasion, epithelial-to-mesenchymal (EMT) transition, and metastasis.

In that regard, increased rates of cell migration and invasion *in vitro* were attributed to the expression of ebv-miR-BART8-3p in CNE-1*, SUNE1, and C666-1 cells, associated with a higher metastatic potential to lungs of nude mice. The transcript for the gene encoding for RING Fingerrotein

38 *(RNF38)* was implicated as a direct target of ebv-miR-BART8-3p in these NPC cells; moreover, downregulation of *RNF38* was associated with activation of the NF-κB and Erk1/2 signaling pathways, increasing the phosphorylation of TAK1, p65, IKKα, IKKβ, and IKKα/β, while reducing the expression of both non-phosphorylated and phosphorylated forms of IκB proteins α and β [59]. The activation of NF-κB was also reported to be a result of ebv-miR-BART13-mediated downregulation of NF-κB inhibitor interacting Ras-like 2 (*NKIRAS2*) [60]. Additionally, miR-BART8-3p was recently implicated in radiotherapy resistance in NPC, as this viral miRNA promotes activation of the ATM/ATR signaling, leading to DNA repair after irradiation *in vivo* in EBV negative cell lines (HONE1 and 5-8F) and increased xenograft tumor size in nude mice [61].

The activity of EBV miRNAs eventually reflects changes in the aggressiveness of EBV-infected malignant cells *in vivo* due to its impact on various cancer progression phenomena. In patients with preclinical NPC, high levels of ebv-miR-BART2-5p were correlated with disease progression, impacting adversely the prognosis. The ectopic expression of ebv-miR-BART2-5p in EBV-negative NPC cell lines CNE2 and HONE-1 significantly increased the cell migration rate *in vitro*; otherwise, downregulation of this viral miRNA in EBV-positive NPC cells decreased the migration and invasion of these cells. Mechanistically, the ebv-miR-BART2-5p regulates negatively the transcript for Rho Family GTPase 3 (*RND3*), leading to an increase in cell motility due to the activation of the Rho signaling pathway[62]. In another recent study, the metastatic potential and stemness for NPC cell lines 5-8F, HONE1, and SUNE1, as well as CNE1* cells, were correlated to the activity of the endogenous oncomir miR-7421 induced by ebv-miR-BART22 via the PI3K/AKT/c-JUN/Sp1 signaling, ultimately inhibiting the expression of GSK3β and causing the activation of the Wnt/β-catenin pathway [63].

Cancer progression phenomena may be modulated by EBV ncRNAs also as a result of their activity over the Epithelial-to-Mesenchymal (EMT) program in epithelial cells. For instance, ebv-miR-BART10-3p downregulates the transcript for the gene encoding the Beta-Transducing Repeat Containing E3 Ubiquitin Protein Ligase (*BTCR*), involved in the WNT signaling pathway, cell cycle checkpoints, as well as EMT regulation [64]. The WNT/β-catenin axis is also affected by ebv-miR-BART9, which targets the E-

cadherin transcripts [65]. Alternatively, EMT activation can be a result of inhibition of the Phosphatase and Tensin Homolog (PTEN) by ebv-miR-BART1, upregulated in NPC at advanced stages (e.g., TNM N2-3 and clinical stages III-IV), as well as linked to an increase in the metastatic potential in nude mice tumor model [66].

In primary EBV-positive human gastric carcinoma samples, the ebv-miR-BART10-3p and ebv-miR-BART22 were found highly expressed and associated with lymph node metastasis and worse overall survival. The expression of these viral miRNAs was also implicated in the activation of the Wnt pathway by targeting *APC* and *DKK1* genes, causing increasing cell migration and invasion of EBV-positive gastric carcinoma cells [67]. In EBV-positive gastric carcinoma cell lines SNU-719 and AGS-EBV cells, the ebv-miR-BART10-3p was implicated in increased cell proliferation and migration by direct targeting the *DKK1* transcript, a negative regulator of the Wnt/β-catenin signaling [68]. Similarly, the expression of ebv-miR-BART1-3p in gastric carcinoma cells promoted cell migration due to the downregulation of the Disabled Homolog 2 (*DAB2*), an adaptor phosphoprotein implicated in the regulation of clathrin-mediated endocytosis and a variety of signaling pathways, including MAPK, WNT, and TGF-β [69]. More recently, the overexpression of ebv-miR-BART11 was shown to also increase the EMT phenotype in human gastric cell lines by targeting the transcript for Forkhead Box P1 (*FOXP1*), a transcriptional repressor involved in the regulation and expression of embryonic genes and cell differentiation [70]. These results strengthen the idea that signaling pathways that participate in the EMT program, such as WNT, are targeted by multiple EBV miRNAs, with expected consequences in the modulation of the aggressiveness of carcinomas associated with EBV infection, such as EBV-positive gastric cancers and NPC.

In a study published in 2020, Min and colleagues reported that ebv-miR-BART1-5p downregulates the O-glycan Synthase Glucosamine (N-acetyl) Transferase 3 (GCNT3) enzyme, which catalyzes the formation of O-linked glycosylation in proteins, a putative marker of poor prognosis in non-small cell lung carcinoma (NSCLC). Intriguingly, EBV-associated gastric cancer showed lower levels of the GCNT3 protein compared to EBV-negative cases. This was linked to a suppressive activity of ebv-miR-BART1-5p, ultimately causing inhibition of cell proliferation and migration *in vitro*[71]. Even though the mechanism

of *GCNT3* regulation by ebv-miR-BART1-5p remains to be better described, this data may suggest that differences in the activity of viral ncRNAs could be context-dependent, stressing the importance to discriminate pathogenetic properties of EBV ncRNAs in NPC and EBV-associated gastric carcinomas, for instance.

Besides WNT, the TGF-beta signaling is also a key pathway for EMT and the stemness phenotype. The TGF-β signaling pathway may be activated by ebv-miR-BART7-3p, which is abundantly expressed in NPC clinical samples and inhibits the expression of the Mothers Against Decapentaplegic Homolog 7 (*SMAD7*) gene by binding the 3'-UTR portion of its transcript. The inhibition of *SMAD7* by ebv-miR-BART7-3p was inversely correlated with the potential of CNE2* and 5-8F cells to form tumorspheres *in vitro*, an experimental proxy to estimate the cell stemness phenotype. Moreover, the ectopic and constitutive expression of ebv-miR-BART7-3p in CNE2* cells increased their tumorigenic potential in xenografted nude mice [72]. Furthermore, patients with advanced-stage NPC compared to patients with early-stage tumors express higher levels of ebv-BART2-5p in serum, and this is associated with worse progression-free survival. In this study, the ectopic expression of ebv-BART2-5p caused an increase in the metastatic potential of SUNE1 and HK1 cells in nude mice, an effect that was mechanistically linked to direct suppression of the transcript for the Rho Family GTPase 3 (*RND3)* gene – a negative regulator of the Rho-associated protein kinases (ROCK) signaling pathway [73]. Therefore, the expression of EBV miR-BARTs plays a role in the progression and metastatic phenotype of EBV-associated carcinomas due to modulation of the EMT program, the stemness of EBV-infected carcinoma cells, or even disrupting key signaling networks involved in cell motility, such as the Rho Kinases (ROCKs) pathway.

Another important mechanism for the activity of EBV ncRNAs in cancer development and progression could be either by mimicking cellular ncRNAs or interfering with their activity. The sequencing of NPC samples has shown that EBV miRNAs can share seed sequences with some human miRNAs while interacting with others [74]. For instance, by mimicking the human miR-513b, ebv-miR-BART8-3p expressed in NPC cells supports disease progression due to its activity on the IFN pathway [59]. Also, ebv-miR-BART6-3p binds and downregulates the *LOC553103* lncRNA, and either the

405 overexpression of this viral miRNA or knocking down *LOC553103* via siRNA caused a decrease in the
406 migration and invasion rates *in vitro* for HNE2* and other cell lines derived from NPC (5-8F and C666-1)
407 or gastric carcinoma (AGS), while both strategies inhibited metastasis of 5-8F-derived tumors in nude
408 mice *in vivo* [75]. In EBV-positive NPC cells C666-1 and the EBV-negative AGS gastric carcinoma cell line,
409 the expression of ebv-miR-BART6-3p was associated with cell proliferation inhibition, while C666-1 cells
410 transfected with ebv-miR-BART6-3p inhibitors showed an increase in cell proliferation and cell cycle
411 progression. The effects were attributed to the downregulation of the transcripts for Stathmin 1
412 (*STMN1*) and the lncRNA *LOC553103* by ebv-miR-BART6-3p, ultimately impacting the expression of cell-
413 cycle associated proteins[76].

414 In summary, the available data indicates that EBV ncRNAs may have either a positive or negative
415 effect on cancer development, interfering in key oncogenic pathways related to cell cycle regulation,
416 cellular migration and invasion, activation of the EMT program, stemness, and regulation of immune
417 responses. Considering EBV miRNAs, the available data in the literature on the activity of miR-BARTs are
418 more frequent and robust, while the effects and importance of viral miRNAs derived from the BHRF1
419 still are poorly understood.

420

4 Concluding remarks and future perspectives

The data presented in this review discloses the activities of EBV ncRNAs in the virus-induced carcinogenesis by a variety of mechanisms, with effects spanning the malignant transformation itself throughout cancer progression – at the cellular, microenvironmental, or even systemic levels. A summary of known activities of viral ncRNAs in phenomena occurring during EBV-induced carcinogenesis is presented in **Figure 1.**

Several EBV ncRNAs are emerging in the spotlight due to their potential application for diagnosis and prognosis for some virus-induced cancers, at least. In this regard, we may recall that the detection of EBV EBERs by *in situ* hybridization is the gold standard method for the diagnosis of EBV-associated malignancies for a few decades now [77]. Moreover, new data in the literature also provide evidence for the use of EBV-encoded miRNAs as biomarkers for cancer detection/progression using either blood or tumor samples. As shown in **Table 3**, several EBV miR-BARTs were suggested as putative markers to monitor the onset, progression, and recurrence of EBV-associated cancers.

The ncRNAs encoded by EBV are crucial regulators of gene expression in cells infected by this virus. Their activities are not limited to the regulation of viral life cycle, latency, and persistence of infection, as these ncRNAs also impact cellular immortalization, malignant transformation, and cancer progression. Overall, EBV-encoded ncRNAs are emerging as putative biomarkers for early detection, disease monitoring, and assessment of treatment response or disease recurrence for cancers associated with the viral infection, such as NPCs. Remarkably, the EBV ncRNAs may act within the cell, in a paracrine fashion, or even systemically, being transported via exosomes. For instance, both EBERs 1 and 2 were detected in exosomal fractions of EBV-infected cells [78,79]; nonetheless, the effects of EBERs in the phenotype infected cells and the tumor microenvironment remains to be elucidated. Also, interactions of EBERs and EBV miRNAs in the pathogenesis of EBV-associated cancers are essentially unknown.

Therapeutic approaches targeting either human or EBV ncRNAs are still a long shot because they impose important challenges, such as methodological limitations and admissibly unpredictable effects

in the clinical setting. Nonetheless, the technological advances and the increasing knowledge about cancer pathways affected by EBV ncRNAs are expected to shed more light on important and perhaps overlooked molecules that could be targeted on new therapies.

Finally, a note of caution is warranted when interpreting the new or previously published data, because the effects of the expression of specific EBV ncRNAs may be substantially different considering models using the sole expression of these molecules compared to the simultaneous activity of multiple viral ncRNAs in the natural scenarios of EBV-driven carcinogenesis *in vivo*. Furthermore, it is essential to consider the features and limitations of the experimental models used for these analyses, notably when experimental data in vitro is used to conclude about the role of the viral ncRNAs in the pathogenesis and evolution of specific cancers associated with EBV infection.

Conflict of Interest: The authors have no conflicts of interest to disclosure

459 **Table 1** - Most relevant viral products expressed by EBV during viral latency.

	EBV Latency			
	Type I	**Type II**	**Type III**	**Type "zero"**
Main viral proteins	EBNA1	EBNAs 1 & LP 1; LMPs 1, 2A, 2B	EBNAs 1, 2, 3A, 3B, 3C, LP; LMPs 1, 2A, 2B	none
Viral miRNAs	miR-BARTs	miR-BARTs	miR-BARTs and mirBHRF1	miR-BARTs
Other ncRNAs	EBERs	EBERs	EBERs	EBERs
Associated diseases	BL, PBL, PEL	cHL, DLBCL, GC, NPC, PBL, LG, PTDL	DLBCL, IM, LG, PTDL	BL

460 Abbreviations: BL - Burkitt Lymphoma; cHL - classic Hodgkin Lymphoma; DLBCL - Diffuse Large B-cell Lymphoma;
461 GC - Gastric Carcinoma; IM - Infectious Mononucleosis; KS - Kaposi Sarcoma; LG - Lymphomatous Granulomatosis;
462 MCD - Multicentric Castleman disease; NPC - Nasopharyngeal Carcinoma; PBL - Plasmablastic Lymphoma; PEL -
463 Primary Effusion Lymphoma; PTDL - Post-Transplant Lymphoproliferative Disease.

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Table 2 - Cell line models used in cited studies investigating the role of EBV ncRNAs in human cancers.

Designation	Origin and biological remarks	ID code(s)1
5-8F	Human, nasopharyngeal carcinoma, EBV-negative	CVCL_C5
AGS	Human, gastric adenocarcinoma, EBV-negative	CVCL_0139
C666-1	Human, undifferentiated nasopharyngeal carcinoma, EBV-positive. Presence of murine leukemia virus (MuLV) [80]	CVCL_7949
CNE-1*	Originally described as well-differentiated human nasopharyngeal carcinoma, EBV-positive, but evidence of HeLa genome [81,80]	CVCL_6888; ICLAC-00473.
CNE-2*	Originally described as derived from a poorly differentiated human nasopharyngeal carcinoma, EBV-positive, but evidence of HeLa genome [81,80]	CVCL_6889 ICLAC-00474
HEK293 (T)	Human, embryonic kidney cells (neuronal), with incorporated adenovirus 5 genome. HEK293T cells are transformed with the SV40 large T antigen	CVCL_0045; CVCL_0063
HeLa	Human, uterus adenocarcinoma, HPV-18-associated.	CVCL_0030
HNE1*	Originally described as derived from a human nasopharyngeal carcinoma, EBV-negative, but evidence of HeLa genome [81]	CVCL_0308
HNE2*	Originally described as derived from an EBV-negative human nasopharyngeal carcinoma, but evidence of the HeLa genome [81].	CVCL_FA07
HONE1*	Originally described as derived from a poorly differentiated human nasopharyngeal carcinoma, but evidence of HeLa genome [81,80]	CVCL_8706
MUTU1	Human, Burkitt lymphoma, EBV-positive.	CVCL_7202
NIH-3T3	Mouse, embryonic fibroblasts.	CVCL_0594
NPC/HK1	Human, differentiated nasopharyngeal squamous cell carcinoma	CVCL_7084
NP69 (a.k.a. NP69^{SV40T})	Human, nasopharyngeal epithelial cells, immortalized by large-T antigen expression for SV40 virus, EBV-negative.	CVCL_F755
Oku-BL	Human, Burkitt lymphoma, EBV-positive.	CVCL_7206
SGC7901	Human, gastric adenocarcinoma, EBV-negative	CVCL_0520
SNU-719	Human, gastric adenocarcinoma, EBV-positive	CVCL_5086
SUNE-1	Human, nasopharyngeal carcinoma, EBV-negative.	CVCL_6946

[1] Cell lines indicated with (*) are flagged as problematic in the Register of Misidentified Cell Lines [82], curated by the International Cell Line Authentication Committee (ICLAC), or elsewhere in the literature. [2] CVCL - Accession ID in Cellosaurus database [83], maintained by

the Swiss Institute of Bioinformatics (SIB) (refer to https://web.expasy.org/cellosaurus/). When available, ICLAC Registration ID is also indicated (refer to https://iclac.org/databases/cross-contaminations/).

473 **Table 3** – Summary of selected studies showing the usefulness of EBV ncRNAs to monitor the onset, progression,
474 and recurrence of EBV-associated cancers in the last 5 years.

Study	Ref#.	Key study features	Summary of conclusions
Xu *et al*., 2020	[30]	RT-qPCR[1] from peripheral blood mononuclear cells from patients with chronic lymphocytic leukemia (CLL)	The expression of ebv-miR-BHRF1-1 was correlated with aberrant p53 and prognostic marker for overall survival in patients with CLL; inhibition of ebv-miR-BHRF1-1- upregulated p53 protein expression, induced cell cycle arrest, and apoptosis.
Gao *et al*., 2019.	[84]	RT-qPCR[1] from plasma samples of NPC patients and non-cancer donors	Ebv-miR-BART19-5p levels using in blood samples were useful for the detection of primary NPC; ebv-miR-BARTs 8-3p and 10-3p were correlated with disease recurrence; ebv-BART13-5p in serum was associated with NPC progression and regional lymph node metastasis.
Jiang *et al*., 2018.	[85]	RT-qPCR[1] from serum samples of NPC patients and non-cancer donors	The levels of ebv-miR-BART2-5p to rise before the NPC diagnosis; particularly valuable for the screening of high-risk patients
Ramayanti *et al*., 2019.	[86]	Small RNA sequencing from serum, nasopharyngeal brushing, and frozen tumor biopsy samples	Expression of ebv-miR-BART13-3p had high specificity (97%) to distinguish sera from NPC patients and healthy controls. Superior diagnostic performance compared to both anti-EBNA1 IgA serology and EBV-DNA load.
Lung *et al*., 2018.	[87]	Small RNA sequencing obtained from patient-derived xenografts (PDX) and the NPC cell line C666-1	Ebv-miR-BARTs 5-5p, 7-3p, 9-3p, and 14-3p synergistically target the ATM gene. ATM downregulation increased the radiosensitivity of transfected NP69 and Hela cells and contribute to EBV latency.
Zheng *et al*., 2018.	[48]	RT-qPCR from qPCR from formalin-fixed paraffin-embedded (FFPE) tumor samples of EBV-associated gastric cancer.	Expression ebv-miR-BART5-3p was found in 10 out of 20 EBV-associated gastric carcinoma tumors, but none of the 4 EBV-negative tumors evaluated

Study	Ref#.	Key study features	Summary of conclusions
Kang *et al*., 2017.	[88]	RT-qPCR from formalin-fixed paraffin-embedded (FFPE) tumor samples of EBV-associated gastric cancer.	High intratumoral levels of ebv-miR-BART20-5p were correlated with poorer recurrence-free survival rates in EBV-associated gastric cancers.
Hirai *et al*., 2016.	[89]	RT-qPCR from serum samples NPC patients and non-cancer donors	Circulating ebv-miR-BART17-5p after NPC treatment was associated with poor prognosis and treatment outcomes
Zhang *et al*., 2015.	[90]	RT-qPCR from blood samples of newly diagnosed NPC patients	Ebv-miR-BARTs 7 and 12 diminished after radiotherapy treatment in NPC patients, highlighting their usefulness for prediction of treatment efficacy.
Zheng *et al*., 2015.	[91]	RT-qPCR from tissue samples of NPC, chronic nasopharyngitis, lymphoma, and non-NPC head, and neck tumors	Detection of ebv-miR-BART1-5p in nasopharyngeal brush samples was effective for diagnosis of early-stage NPC and disease progression, even in false-negative cases based on antibody titers, EBV DNA load, and histopathological analyses.

[1] Reverse-Transcription Quantitative Polymerase Reaction (RT-qPCR).

Figure 1 – Cancer-associated biological phenomena associated with the expression of EBV ncRNAs. The ebv-miR-BHRF1 cluster has a crucial role in the immortalization of B-cells and its expression alongside EBERs contributes to malignant transformation. The expression of some ebv-miR-BARTs was also shown to increase genomic instability, favoring tumor progression. EBV miR-BHRF1 and EBERs increase cell proliferation, contributing to tumorigenesis, while the expression of EBERs, ebv-miR-BHRF1, and ebv-miR-BARTs is associated with increased resistance to apoptosis and cell survival. Furthermore, EBERs and ebv-miR-BARTs were shown to promote immune evasion, alter cell metabolism, and increase angiogenesis, facilitating tumor growth and metastasis. Several ebv-miR-BARTs have been associated with increased cell migration and invasion, fostering EMT and cancer metastasis. The current evidence discloses the important roles of EBV ncRNAs on the initiation and progression of infection-associated cancers.

Acknowledgments

ViriCan's studies at the State University of Sao Paulo (UNESP), SP, Brazil, are sponsored by the Brazilian public agencies CAPES, CNPq, and FAPESP, which awarded scholarships to and BFRC (CNPQ DR 142294/2017-9; FAPESP DR 2017/20352-0, FAPESP BEPE DR 2019/03804-0), BGMC (FAPESP DR 2017/22312-5; CAPES DR) BASJ (FAPESP MS 2018/12164-1, FAPESP BEPE MS 2019/05061-4), as well as research funds to DEO (FAPESP Grant 2017/233393-9). The authors sincerely apologize for studies relevant to the topic but not mentioned in this review due to space and citations limits.

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Transcriptional regulation

- EBV EBERs

Cellular Immortalization

- EBV cluster BHRF1

Immune evasion

- EBV EBERs
- EBV-miR-BHF1-2-5p

Metabolic disturbances

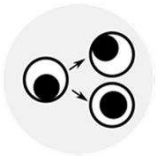
- ebv-miR-BART1-5p

Genomic instability

- ebv-miR-BART5-5p
- ebv-miR-BART7
- ebv-miR-BART9-3p
- ebv-miR-BART14-3p

EMT and stemness

- ebv-miR-BART1
- ebv-miR-BART9
- ebv-miR-BART10-3p
- ebv-miR-BART11



Cell Proliferation

- EBV cluster BHRF1
- EBV EBERs
- ebv-miR-BART10-3p

Malignant Transformation

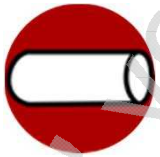
- EBV cluster BHRF1
- EBV EBERs

Cell Migration

- ebv-miR-BART1-3p
- ebv-miR-BART2-5p
- ebv-miR-BART8-3p
- ebv-miR-BART10-3p

Cell Survival

- EBV BARTs
- EBV cluster BHRF1



Angiogenesis

- EBV EBERs
- ebv-miR-BART1-5p

Cell Invasion

- ebv-miR-BART8-3p

Apoptosis

- EBV cluster BHRF1
- EBV EBERs
- EBV BARTs

Inflammation

- EBV EBERs

Cancer Metastasis

- ebv-miR-BART2-5p
- ebv-miR-BART8-3p
- ebv-miR-BART10-3p
- ebv-miR-BART22