Pathobiologic Roles of Epstein–Barr Virus-Encoded MicroRNAs in Human Lymphomas

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Abstract: Epstein–Barr virus (EBV) is a human γ-herpesvirus implicated in several human malignancies, including a wide range of lymphomas. Several molecules encoded by EBV in its latent state are believed to be related to EBV-induced lymphomagenesis, among which microRNAs—small RNAs with a posttranscriptional regulating role—are of great importance. The genome of EBV encodes 44 mature microRNAs belonging to two different classes, including BamHI-A rightward transcript (BART) and Bam HI fragment H rightward open reading frame 1 (BHRF1), with different expression levels in different EBV latency types. These microRNAs might contribute to the pathogenetic effects exerted by EBV through targeting self mRNAs and host mRNAs and interfering with several important cellular mechanisms such as immunosurveillance, cell proliferation, and apoptosis. In addition, EBV microRNAs can regulate the surrounding microenvironment of the infected cells through exosomal transportation. Moreover, these small molecules could be potentially used as molecular markers. In this review, we try to present an updated and extensive view of the role of EBV-encoded miRNAs in human lymphomas.

Keywords: Epstein–Barr Virus; microRNA; human lymphoma; BamHI-A rightward transcript; BART; Bam HI fragment H rightward open reading frame 1; BHRF1

1. Epstein–Barr Virus as an Oncovirus in Human Lymphomas

It is believed that viruses contribute to 15–20% of human malignancies [1]. Among the discovered oncoviruses to date, Epstein–Barr Virus (EBV), which is also named human herpesvirus 4 (HHV-4), is a key player in human malignancies [2]. It is a human γ-herpesvirus that is widely distributed in the human population, infecting more than 90–95% of its natural host for the entirety of its life [3]. As the first described human oncovirus, it was discovered in 1964 by Doctors. Anthony Epstein and Yvonne Barr (hence the name) in cultured cells isolated from endemic Burkitt lymphoma (BL) cases in sub-Saharan Africa [4,5]. Later, it became evident that an EBV infection has an oncogenic role not only in endemic BL (eBL), but also in several other cancers. Considering the scope of this paper, the described entities include eBL, sporadic BL (sBL), HIV-related BL (HIV-BL), Hodgkin lymphoma...
(HL), plasmablastic lymphoma (PBL), chronic lymphocytic leukemia (CLL), natural killer/T-cell lymphoma (NKTL), diffuse large B-cell lymphoma (DLBCL) of the elderly, HIV-related DLBCL (HIV-DLBCL), post-transplant lymphoproliferative disorder (PTLD), pyothorax-associated lymphoma (PAL), and methotrexate-associated lymphoproliferative disorder (MTX-LPD), among others [3,6–11].

**EBV Latency Programs**

Like other members of the herpesvirus family, EBV displays different patterns of gene expression, which are known as latency programs [12,13]. In fact, the latency state(s) allow the virus to remain almost inactive in the host cells and maintain its genome with the minimum number of genes expressed, helping it, for example, to hide from the host’s immune system [14]. Upon the emergence of the appropriate conditions, the virus changes its state to the lytic phase, where it uses the host cell’s machinery for the production of viral progenies, and thus, infecting other cells horizontally [15,16]. More importantly, unlike α- and β-herpesviruses, which cause diseases in their lytic form, EBV, as a γ-herpesvirus, is known to induce most of its associated human diseases in the latent forms [17].

Based on the expression patterns of the main EBV latent proteins, several latency programs have been described for the virus (Table 1). These latent proteins include EBV-encoded nuclear antigen 1 (EBNA1), EBNA2, EBNA3s (3A, 3B and 3C), latent membrane protein1 (LMP1), and LMP-2s (A and B) [18,19]. Furthermore, EBV encodes two RNA molecules named Epstein–Barr virus (EBV)-encoded small RNAs (EBERs), which are used as targets for the detection of the virus in clinical samples by means of in situ hybridization (ISH) [20]. Regarding the topic of this review, i.e., the two families of microRNAs (miRNAs) encoded by EBV (BamHI-A rightward transcript or BART; BamHI fragment H rightward open reading frame 1 or BHRF1), there are some differences in their expression patterns between different latency types (Table 1).

Along with the three main latency types of EBV, atypical instances have been reported too. For example, the BamHI W promoter (Wp)-restricted latency program was found in a subset of BLs, where a deletion of the EBNA2 gene in the viral genome led to the expression of EBNA1 and EBNA3s, granting the infected cells an elevated resistance to apoptosis [21,22].

**Table 1.** Expression patterns of EBV-encoded products in different latency programs with references to selected cancers. Some entities may represent two types of latencies.

| Latency Type | Expressed Product | Example | Reference |
|--------------|-------------------|---------|-----------|
| Type I       | EBNA1, EBERs, BART miRNAs | BL, MTX-LPD, PBL | [9,23,24] |
| Type II      | EBNA1, LMP1, LMP2s, EBERs, BART miRNAs | HL, PBL, MTX-LPD, NKTL, CLL, PTLD, DLBCL of the elderly | [6,23–28] |
| Type III     | EBNA1, EBNA2, EBNA3s, LMP1, LMP2s, EBERs, BART miRNAs (non-to-low), BHRF1 | PTLD, DLBCL of elderly, PAL, HIV-DLBCL, LCL | [3,8,28] |

*BART*: BamHI-A rightward transcript; *BHRF1*: BamHI fragment H rightward open reading frame 1; *BL*: Burkitt lymphoma; *CLL*: chronic lymphocytic leukemia; *DLBCL*: diffuse large B-cell lymphoma; *EBER*: EBV-encoded small RNA; *EBNA*: EBV-encoded nuclear antigen; HIV-DLBCL: HIV-related diffuse large B-cell lymphoma; HL: Hodgkin lymphoma; LCL: lymphoblastoid cell line; LMP: latent membrane protein; MTX-LPD: methotrexate-associated lymphoproliferative disorder; NKTL: natural killer/T-cell lymphoma; PAL: pyothorax-associated lymphoma; PBL: plasmablastic lymphoma; PTLD: post-transplant lymphoproliferative disorder.

**2. MicroRNAs**

MicroRNAs are a class of small RNAs (almost 22 nucleotides in length) which were discovered in 1994 by Lee et al. in *C. elegans* [29]. They are used by cells to “fine-tune” gene expression at the post-transcriptional level, and they play a substantial role in different physiological processes such as differentiation, immune signaling, apoptosis, and proliferation [30,31].
miRNAs are transcribed as long pri-miRNA molecules (usually by RNA polymerase II), which are then cleaved by a complex containing RNase type III Drosha. The resulting molecules, pre-miRNAs, are almost 85 nucleotides in length and show a hairpin structure. After being transferred to the cytoplasm—a process mediated by exportin-5—the activity of another RNase (named Dicer) yields a miRNA/miRNA* duplex that is almost 20–22 nucleotides long. A mature miRNA (one of the two strands of the duplex) is then loaded onto an RNA-induced silencing complex (RISC) and guides it to the target site on the related mRNA molecules. In animals, the target sites are usually located on the 3′ UTR of mRNAs and exhibit full or partial complementary sequences to regulatory miRNAs. The final result is either the degradation of the target mRNA or the blockage of its translation, both of which lead to lowered expression levels of the related protein [32,33].

Being important regulatory molecules, the altered expression of miRNAs can play an important role in different human diseases such as cancers [33,34]. In addition, based on their target gene(s) and altered expression levels, these molecules are divided into two categories: onco- and tumor-suppressor miRNAs [33,35]. To date, numerous miRNAs with such characteristics have been described; however, the discovery of the fact that miRNAs are not solely produced by metazoans and plants, but also by viruses, has opened a new window in the field. The first ever reported viral miRNAs were described by Pfeffer and colleagues in EBV-infected B cells in 2004 [36].

3. EBV-Encoded MicroRNAs in Human Lymphomas

Among the human and animal viruses with their own miRNAs, herpesviruses are the main viruses. In the latest release of miRBase, 502 viral microRNAs have been annotated, 44 of which belong to EBV [37]. EBV microRNAs are transcribed from two regions: BART, comprising two clusters plus pre-miRNA for miR-BART2, and BHRF1 cluster, encoding 40 and four mature miRNAs, respectively [38]. Figure 1 displays a schematic figure of the EBV genome encoding different latent products, including miRNAs, and the related promoters (Figure 1).

![Figure 1](image-url)

**Figure 1.** The structure of the EBV genome encoding latent products. Orange arrows indicate promoters. Blue boxes indicate miRNA coding regions. BART: BamHI-A rightward transcript; BHRF1: BamHI fragment H rightward open reading frame 1; Cp: C promoter; EBERs: EBV-encoded small RNAs; EBNA1: EBV-encoded nuclear antigen 1; EBNA2: EBV-encoded nuclear antigen 2; EBNA3: EBV-encoded nuclear antigen 3; EBNA-LP: EBV-encoded nuclear antigen leader protein; LMP1: latent membrane protein 1; LMP2: latent membrane protein 2; P1: promoter 1; P2: promoter 2; Qp: Q promoter; Wp: W promoter.
The transcription of the BamHI region produces large polyadenylated RNA molecules harboring several exons and introns, which are further alternatively spliced. The transcription is under the control of promoter 1 and 2 (P1 and P2), which are TATA-less promoters located approximately 400 nucleotides upstream of BART exon 1. Besides being subjected to reduced activity due to methylation, these promoters are regulated by several cellular transcription factors (further discussed below) [39]. Although the first four introns of the transcripts harbor the codons for the BART miRNAs, a study suggests that the processing of the pre-miRNAs occurs before the completion of the splicing reaction [40]. It is worth mentioning that for a long time, it was believed that these miRNAs were the only functional products of the transcription of the BART region [41]. Recent evidence, however, identifies functional long noncoding RNAs as other possible products [42].

On the other hand, the transcription of the BHRF1 cluster of miRNAs is under the control of C and W promoters (Cp and Wp). The products of transcription are polycistronic transcripts which, apart from BHRF1 miRNAs, encode the BHRF1 protein and some EBNA proteins as well [40,43].

3.1. EBV miRNA Expression Patterns

Several groups have applied high throughput technologies such as expression microarrays and next generation sequencing (NGS) methods in order to draw the expression patterns of EBV miRNAs in associated tumor samples, patient sera, and cell lines carrying different latency types. Some examples of the studied cancers include BL [6,9,38,44–47], HL [8,38], CLL [10], NKTL [11,48], PBL [6,38], DLBCL (of the elderly and HIV-related) [8], PTLD (systemic and central nervous system) [3,49], MTX-LPD [8], PAL [8], and lymphoblastoid cell lines (LCLs) [38,50,51].

Considering the heterogeneity observed in the reported data, creating a general consensus might seem overwhelming. The observed differences could be attributed to the usage of different technologies (e.g., microarray versus NGS), the sample type (cell line or tissue), or the sample quality (fresh versus formalin-fixed, paraffin-embedded) besides the number of the analyzed samples. Some patterns, however, are observable across these reports.

It seems that the expression of BART miRNAs is more specific to the latency types I and II, with decreased or no expression in latency type III, in contrast to the expression of the BHRF1 miRNA cluster in latency type III [3,8]. Meanwhile, BHRF1 miRNAs can be detected in Wp-restricted latency too [52]. As mentioned before, different promoters are responsible for the transcription of BART or BHRF1 miRNAs, which are regulated by several cancer- and immune system-related transcription factors. For example, c-MYC and CCAAT-enhancer-binding (C/EBP) proteins regulate P2 in a positive manner, while interferon regulatory factor (IRF) transcription factors IRF7 and IRF5 suppress P1-related expression [53]. On the other hand, NF-κB components positively regulate both promoters [54]. Hence the cellular context plays an important role in the regulation of the expression of these miRNAs which is mediated by both the viral latent proteins and/or (deregulated) cellular components, such as the NF-κB pathway, which is activated by LMP1 [54], and c-MYC, that is known to be constitutively expressed in BL [44]. It remains unclear, however, how the presence of LMP1 in latency type II and III does not lead to similar levels of BART miRNA expression. Although the reported methylation observed in EBV promoters could provide a possible hint for this phenomenon [55], more complex regulatory networks cannot be excluded.

Another notable observation in these reports is the various expression levels observed between the different members of the BART or BHRF1 families in the same cell or tumor context. This difference could be as high as 50 times in the quantity of single miRNAs, despite each of the families being transcribed as single transcripts [40,43,52]. Several possibilities have been proposed, such as the effect of thermodynamics governing the secondary structure of pre-miRNA molecules on the efficiency of miRNA maturation [52,56], and a possible temporal sequence of cleavage of original transcripts [40]. Strikingly, recent data propose how different strains and types of EBV exhibit differences in their miRNA expression patterns, at least partially due to the presence of single nucleotide polymorphisms
(SNPs) in their encoding DNA [57,58]. Thus, an interaction between different strains of the virus and the background of the infected cells most likely determines the expression level for each miRNA.

Conventional methods for the detection of EBV in patient samples mainly include immunohistochemistry (IHC), ISH, or serological methods such as anti-EBNA1 immunoglobulin G (IgG) detection [7,59]. These methods, however, have their intrinsic flaws, and in some cases they might be unsuccessful in identifying the target. For example, a very recent work published by us showed that while ISH and IHC failed to identify EBV presence in some BL cases (hence diagnosed as EBV-negative), Illumina high throughput sequencing recognized EBV genome presence and even some EBV miRNA expression in those cases [7]. More importantly, our results provided new and substantial support for the hit-and-run hypothesis in BL, which suggests that eliciting a heritable change in the gene expression pattern of the host cell may be followed by a complete loss of the genome of tumor viruses [7]. Similarly, other researchers reported detectable EBV miRNAs in CLL patients without an apparent history of an EBV infection [10]. These researchers, however, used anti-EBNA1 IgG detection as a means of revealing a previous EBV infection, which might not be the most appropriate method, since such a test would turn out negative for patients with viral chronic infection [60]. Alternatively, secondary anti-EBNA1 negativity in immunocompromised convalescent individuals such as tumor patients could provide another possible explanation for these researchers’ reported results [61].

3.2. Pathologic and Biologic Role of EBV miRNAs

In 90–100 million years of evolution, EBV has acquired the tools to survive in host cells and spread to other individuals [62]. It is thus perceivable that the miRNAs must have fundamental advantages for the virus since they have been retained in the viral genome [63]. Based on this observation, it is possible that the oncogenic roles attributed to EBV miRNAs could only be secondary effects of the mechanisms primarily developed for the long-term persistence of EBV in the host.

One notable study supporting the essentiality of EBV miRNAs in its biology was recently published, where the authors found rarely occurring mutations in the BART- and BHRF1-encoding regions in EBV-associated lymphomas [64]. The viruses replicate and evolve much faster than their hosts; thus, this information suggests that EBV miRNAs are evolutionarily conserved genes. According to the reported results, it is believed that EBV-encoded miRNAs play key roles in the pathobiology of the EBV life cycle and its associated cancers [39]. By targeting both cellular and viral miRNAs, they can modulate the latency/lytic phases of the EBV life cycle and also interfere with key cellular pathways engaged in fundamental cancer-related processes such as immunosurveillance, apoptosis, proliferation, cell cycle progression, transforming capacity, and other tumor-related mechanisms (discussed below).

3.2.1. EBV miRNAs Contribute to Evasion from Immunosurveillance

Being able to hide from the host immune system is an indispensable prerequisite for the latent virus. Some EBV proteins have immunoevasin characteristics; in other words, they have the potential to repel both the innate and the adaptive immune systems [65]. For instance, the most widely expressed viral protein, EBNA1, which is necessary for maintaining the viral genome inside the nucleolus, is protected from major histocompatibility complex (MHC) class I antigen presentation and the recognition of the infected cell by cytotoxic T cells—a property attributed to the Gly–Ala repeat (GAr) in its primary structure [66]. By targeting both the cellular components and the self-components, the miRNAs could provide the virus with further means to evade the immune system. This would potentially be more important in latency types I and II, since the latency type III is primarily observed in immunosuppressed or immunocompromised patients [3].

The reported cellular targets constitute important molecules that are engaged in both the innate and the adaptive immune system. This ensures the sustainability of the virus in all phases of the infection from its entrance into the host cell to lytic replication, latency, and lytic reactivation. Pattern recognition receptors are important components of innate immunity during the early phases of the
infection and are responsible for detecting molecular patterns expressed by the invading pathogens [67]. Retinoic acid-inducible protein 1 (RIG-I) is a member of this superfamily which functions as a detector of viral RNA and is targeted by miR-BART6-3p [68]. Similarly, miR-BART15 interacts with the mRNA of another member named NLR family pyrin domain containing 3 (NLRP3). This affects the associated inflammasome complex, which is involved in the control of viral infections and normally plays a role in stimulating an immune response through the production of interleukin-1β (IL-1β) and interleukin-18 (IL-18) [69,70].

There are other molecules related to the innate immunity that are manipulated by EBV miRNAs. The interleukin 6 (IL-6) receptor B (IL-6RB), as demonstrated by us and others, is targeted by miR-BART6-3p [45,71]. Concordantly, the importin 7 (IPO7) inhibition by miR-BART16 and miR-BART3 could reduce the production of IL-6, which stresses the importance of the IL-6-related pathway in an EBV infection [72–74]. In a similar manner, the interleukin 1 (IL-1) receptor 1 is suppressed by miR-BHRF1-2-5p [75], and CREB-binding protein (CREBBP), a key transcriptional coactivator in type I Interferon (IFN) signaling, is a direct target of miR-BART16 [76].

Interferon regulatory factors are a family of transcription factors which are important players in the innate immune response. More importantly, as mentioned above, IRF7 and IRF5, which are induced by type I IFNs, can negatively regulate the expression of BART miRNAs through the P1 promoter, with the former suppressing Q promoter (Qp) -related transcription (connected to EBNA1 expression) as well [53]. Thus, by targeting these pathways, the BART miRNA(s) impair the innate immunity and also possibly guarantee their own and (some) latent protein expression.

The expression of viral proteins, at least during latency type II (considering EBNA1 as the sole EBV protein expressed in latency type I and immunosuppressed or immunocompromised status of patients expressing latency type III) and the lytic phase should normally trigger the cellular and humoral responses of the adaptive immune system. The signaling pathways related to the responses by the two main cell types of adaptive immunity (CD4 and CD8) are targeted by several EBV miRNAs of both families. The reported targets for the T helper cell response include interleukin-12 subunit beta (IL-12B) and lysosomal proteases such as legumain (LGMN), cathepsin B (CTSB), and interferon γ inducible protein 30 (IFI30). In addition, the suppression of a peptide transporter subunit named transporter associated with antigen processing 2 (TAP2), as well as the subsequent downregulation of another subunit, TAP1, provide evidence for the manipulation of the cytotoxic T cell response [77,78].

Besides influencing the host macromolecules, microRNAs are employed by EBV to control self-gene expression. In this way, the virus can regulate the latency or latency/lytic phase transition and limit the presentation of its antigens to the host immune system. Feederle et al. observed a higher expression of EBV latent proteins in cells infected with a mutant virus lacking BHRF1 miRNAs, indicating a potential role of this cluster of microRNAs in the decrease of the antigenic load of virus-infected cells and in the regulation of viral latency [79]. Very interestingly, although it is capable of hiding from cytotoxic T cells, EBNA1 is also targeted by EBV miRNAs, probably to limit its targeting by effector T cells [77]. Furthermore, the downregulation of the viral DNA polymerase, BALF5, by miR-BART2 is suggested to regulate lytic phase activation [80]. A paper by Sesto and colleagues, however, contradicts the latter findings, as the levels of the viral protein, BZLF1, that is necessary for lytic activation remained unchanged in the cells infected with mutant viruses lacking BHRF1, BART, or both miRNA families [81]. Further investigations are necessary to clarify this matter.

Overall, this information highly supports that EBV miRNAs manipulate the immune response in different ways. In most cases, this simply leads to the impairment of antiviral responses with a nonlethal outcome, but it represents a major concern when the presence of EBV contributes to development of cancer. Above all, considering the importance of the cellular response of CD8 cells in combating cancer cells, EBV might be a major contributor to cancer progression.
3.2.2. EBV miRNAs Interfere with Several Other Cancer-Related Mechanisms

The inhibition of apoptosis is one of the main processes employed by the virus that leads to its pathogenesis because the virus needs the host cell to survive and also needs to avoid transmitting potential danger signals that are released upon apoptosis such as viral particles, which could make the virus “more visible” to the human immune system [82,83]. Caspase 3 (CASP3), the main regulator of the execution of apoptosis, is targeted by several viral miRNAs consisting of miR-BART1-3p, miR-BART1-5p, miR-BART2-5p, miR-BART3-3p, miR-BART4-5p, miR-BART7-3p, miR-BART8-5p, miR-BART13-3p, miR-BART16, and miR-BART22 [72,84]. There is most likely no other gene that has been reported to be targeted by so many viral miRNAs, which suggests the importance of this pathway for the virus. Furthermore, the translocase of outer mitochondrial membrane 22 (TOMM22) mRNA interacts with miR-BART16, resulting in the inhibition of the association between the pro-apoptotic protein BCL2 associated X (BAX) and the mitochondria, with the final outcome of BAX-induced apoptosis prevention [74]. Another example is the inhibition of PR/SET domain 1 (PRDM1) expression by miR-BHRF1-2 in LCLs [85]. In line with the latter information, EBNA1-induced hsa-miR-127 has previously been reported to downregulate PRDM1 [32].

As well as avoiding apoptosis, the virus is also able to utilize its miRNAs to improve host cell proliferation, sometimes targeting important tumor suppressors. This can potentially increase the anti-apoptotic effect. For instance, phosphatase and tensin homolog (PTEN), a well-characterized tumor-suppressor gene which acts as a master regulator of cell proliferation, is a target of miR-BART6-3p, as well as the BHRF1 cluster, while the latter can suppress the other known tumor suppressor, p27 (protein 27, also known as CDKN1B) [45,86,87]. Another example is sphingosin-1-phosphate receptor 1 (SIPR1) which is downregulated by miR-BART16. In addition, the latter improves cell mobility as well—an important prerequisite for metastasis [48].

3.2.3. Molecular Networks and Circuits Define More Complex Roles for EBV-Encoded MicroRNAs

Although the identification of single viral miRNA targets is a key step in understanding their pathologic role, it is known that one single mRNA could be targeted by several miRNAs, which suggests complex miRNA–miRNA networks, the effect of which could be additive/synergic [88,89]. Viral miRNAs are no exception, and very interestingly, this phenomenon could be extended to cellular transcripts cotargeted by viral and cellular deregulated miRNAs in the presence of the virus [90].

A paper by our group indicated that several host mRNAs which were differentially expressed between EBV-positive or EBV-negative HIV-BL could potentially be targeted by both viral and deregulated cellular miRNAs. We showed that the combined expression levels of the targets of both viral and cellular miRNAs could discriminate the two tumor categories better than each one alone, as indicated by gene expression profiling patterns [9]. In accordance with these results, a study conducted on Jijoye (Latency III) EBV-transformed B cells using high-throughput sequencing and crosslinking immunoprecipitation (HITS-CLIP) identified 90% of the targets of the 12 most copious EBV miRNAs to be also targeted by human miRNAs, though this occurred via distinct binding sites [91]. The synergism of hsa-miR-197 and miR-BART6-3p in targeting the IL-6Rβ and, similarly, hsa-miR-142 and miR-BART6-3p in the downregulation of PTEN provide us with more specific examples [71,86]. This can be extended even further, as in some cases, an EBV miRNA could interact with an mRNA at the same binding site of a cellular miRNA, e.g., targeting the NLRP3 by hsa-miR-223 and EBV miR-BART15 at a shared 3′ UTR site [70].

MicroRNAs can also cooperate in the regulation of signaling circuits and networks by targeting different molecules related to them. A report described a circuit regulated by cellular and viral miRNAs in nasal natural killer cell lymphoma, which consisted of T-box Protein Expressed in T cells (T-betPTEN, AKT, and Rapamycin-Insensitive Companion of Mammalian Target of Rapamycin (RICTOR), and was regulated by miR-BART20-5p, hsa-miR-494-3p, and hsa-miR-142-3p [92]. This is a very good example of circuitous architecture of molecular interactions [89]. More extensively, Callegari and colleagues found more than 500 molecules of small ubiquitin-like modifier (SUMO) interactome to
be potentially suppressed by EBV miRNAs [93]. This is a very interesting example, since important processes such as chromatin organization, stress, DNA damage, immune responses, and apoptosis are correlated to it. Interestingly, the extent of these networks and circuits is not limited to miRNAs and might include other noncoding RNAs as well, such as EBERs and miR-BART16, which are reported to corepress sphingosin-1-phosphate receptor 1 (S1PR1)—a process which contributes to NKTL tumor formation [48].

Furthermore, some researchers have tried to evaluate the function of EBV miRNA families from a more systematic point of view, evaluating the two miRNA families using mutant viruses that lacked either or both of them. In this regard, Vereide and colleagues found out that in the absence of any EBV-encoded product other than BART miRNAs, BL cells showed decreased apoptosis levels in comparison to non-BART-complemented cells. Furthermore, an increased proliferation mediated by BART miRNAs was observed [72]. Other researchers used a mutant EBV strain that lacked all three BHRF1 miRNAs to infect B cells in vitro. Very interestingly, they found a 20-fold decrease in the B-cell transforming capacity of the mutant virus, slower growth, and a two-fold reduction in the entrance to the S phase of the cell cycle, as compared to the wild type virus [79]. Concordantly, the BHRF1 cluster was proposed to play an important role in the promotion of cell cycle progression and the prevention of apoptosis [81]. It seems that such an effect is exerted by the BHRF1 miRNA cluster targeting PTEN mRNA [87]. It is possible that studying each of these miRNAs separately, without the presence of other ones, would have resulted in different results, reflecting the importance of a systematic study of microRNAs.

With the advent of humanized mouse models for EBV infections, the systematic study of the EBV miRNAs has entered a new era, and it is hoped that light will be shed upon the matter, as in vivo systems provide researchers with considerable advantages. Upon infecting humanized mice with wild-type or mutant virus lacking the BHRF1 miRNA cluster, Wahl et al. found that although BHRF1 miRNAs do not enhance the overall oncogenic potential of EBV in vivo, they do facilitate the development of an acute systemic EBV infection [94]. This is somewhat in contrast to the reports described in the previous paragraph. Thus, further studies are necessary to confirm this data.

Considering the increasing number of discovered noncoding RNAs, such as long noncoding and circular RNAs, as well as newly identified miRNAs, the molecular networks describing their interactions should be the upcoming trend of noncoding RNA research, achieved above all by means of tools and techniques such as mathematical models and computer simulation.

3.2.4. The Pathogenetic Effect of Viral miRNAs Could Be Observed at Global Gene Expression Profile Level

The miRNA profiling data that was obtained using NGS technologies indicates that EBV-encoded miRNAs constitute a high percentage of the total miRNAs in the cells. This percentage could be as high as 34% [8]. Given that a single miRNA could target several mRNA molecules (primary targets) and the potential downstream genes affected by these deregulated targets (secondary targets) [32], the pathogenetic effect of these molecules should be observable in a global gene expression profile of the infected cells. In order to demonstrate such an effect, we have tried to use the targets of miR-BART6-3p (as a proof of principle) in some EBV-associated cancers. These targets were identified using transfection experiments of the miRNA or its inhibitor, followed by an assessment using gene expression profiling microarrays [3,44]. In this regard, we evaluated the expression of these targets in the context of EBV-positive versus EBV-negative BL gene expression profiles, and found them to be enriched in EBV-negative BL cases, which was in line with miR-BART6-3p absence of expression in this tumor [44]. In similar experiments where we compared EBV-positive BL (latency type I) versus EBV-positive PTLD (latency type III), these targets were upregulated in EBV-positive PTLD, where the expression of this miRNA was suppressed [3]. Considering these results, the role of BART miRNAs seemed to be more substantial in BL, which was in accordance with their expression levels, since, as opposed to PTLD, the immunocompetent state of a BL patient would not allow EBV to express a full
array of its latent proteins. In PTLD, however, the viral protein might somehow surrogate the effect of the suppressed miRNAs [3].

3.2.5. Exosomal Shuttle MicroRNAs Help EBV to Manipulate Its Surrounding Microenvironment

One of the means used for communication by adjacent cells in many eukaryotes, including humans, are exosomes—small (40–100 nm) vesicles that are secreted by parental cells (especially tumor cells) and are fused to the membrane of target cells. In doing so, exosomes transport a wide range of biological molecules, including proteins and nucleic acids, which could be delivered in their functional status [95]. In this light, exosomal microRNAs (also called exosomal shuttle microRNAs), which are available in different body fluids including serum, have recently become an important trend in tumor biology, and a considerable body of published literature deals with their role in carcinogenesis [96,97].

A primary study showed the transfer of some viral and cellular miRNAs from B cells to T cells after 1.5 h in a co-cultured system, giving the possibility of direct contact between the cells [98]. Later, however, it became evident that such a transfer does not necessarily need a direct contact, since primary immature monocyte-derived dendritic cells could be labeled using exosomes released from co-cultured LCL that were not in direct contact with them [99]. It is believed that these exosomes are taken up via caveola-dependent endocytosis [100].

Theoretically, the described effects of EBV miRNAs could be extended to the neighboring cells, giving the virus the potential to manipulate the tumor microenvironment for its benefit [101]. The targeting of the NLRP3-related inflammasome by miR-BART15 was also reported to happen in uninfected cells exposed to exosomes of virus-infected cells, leading to a weakened inflammatory response in neighboring uninfected cells [70]. In addition, Yogev et al. recently described a reduction in mitochondrial respiration characterized by a 25% decrease in oxygen consumption by fibroblasts exposed to such exosomes. This is a widespread cancer-related phenomenon known as the Warburg effect, which was followed by an increase in vascular endothelial growth factor A (VEGFA) expression [101]. It is possible, however, that the observed effect was related to other biomolecules transferred by exosomes.

More importantly, recent data have suggested the possibility of a specific viral miRNA packaging process in exosomes. In viral miRNA profiling experiments performed on LCLs and their exosomes, it was found that two viral miRNAs, i.e., miR-BART3 and miR-BHRF1-1, were more abundant in exosomes [50]. Another study, by contrast, reported that some viral miRNAs were enriched in LCLs, with none more expressed in exosomes, which suggests a tendency for these molecules to remain cell-associated [102].

In any case, there seems to be several mechanisms which can selectively accumulate specific miRNAs in exosomes. These exosomes can contain, apart from miRNAs, other EBV products such as latent proteins. Hence, this mechanism can be exploited by the virus to somehow dictate its signature to the surrounding non-infected cells, which also consist of cells that normally do not host it. To date, most of the conducted research has been focused on main hosts of EBV, i.e., B cells and fibroblasts. Studying other cells constituting tumor microenvironments can possibly help to further elucidate the tumorigenesis described for the virus.

3.3. EBV microRNAs as Molecular Markers for Classification, Diagnosis, and Prognosis

Human miRNAs have been extensively used as diagnostic and prognostic molecular markers in different human cancers because of their abundance and stability [103,104]; thus, similar research has been applied to EBV microRNAs. For instance, high levels of several EBV microRNAs, including miR-BART2-5p, miR-BART7-3p, miR-BART13-3p, and miR-BART1-5p in the sera of patients with nasal NKTL turned out to be valuable for discriminating patients from healthy controls [11]. Similarly, the survival in CLL patients was found to be correlated with the expression of BHRF1-1 [10]. Concerning BL, we showed that based on EBV-encoded miRNA expression, it was possible to easily discriminate between EBV-positive and EBV-negative cases [9,44,45]; however, although EBV may
certainly have a pathogenetic role in the first group, the clinical/epidemiological classification (endemic versus sporadic versus immunodeficiency-associated) still retains a substantial value, allowing for a broader view of these tumors.

4. Conclusion Remarks

In this paper we evaluated the role of EBV-encoded miRNAs in B cell lymphomas and provided the readers with a brief description of the recent studies which indicate the importance of such a function. A summary of the discussed data is presented in Table 2.

Table 2. A summary of the discussed EBV miRNAs and their targets.

| MicroRNA | Target | Related Process/Application | Reference |
|----------|--------|----------------------------|-----------|
| **BART and BHRF1 Families** | EBNA1 | Latency Regulation | [77] |
| **BHRF1 Family** | PTEN | Cell Proliferation, Apoptosis | [87] |
| | p27 | Cell Cycle Progression | [87] |
| | Unidentified | Transformation Capacity, Cell Cycle Progression | [79] |
| | Unidentified | Acute Systemic EBV Infection | [94] |
| | Latent Proteins | Latency Regulation | [79] |
| miR-BHRF1-1 | Not Applicable | Survival Marker in CLL | [10] |
| miR-BHRF1-2 | CT5B | CD4⁺ T Cell Response | [77,78] |
| | PRDM1 | CD4⁺ T Cell Response | [77,78] |
| | IL-12B | Cell Cycle Progression, Apoptosis | [85] |
| miR-BHRF1-2-5p | IL-1 receptor | Innate Immunity | [75] |
| miR-BHRF1-3 | TAP2 | CD8⁺ T Cell Response | [77,78] |
| miR-BART1-3p | IL-12B | CD4⁺ T Cell Response | [77,78] |
| | IFI30 | CD4⁺ T Cell Response | [77,78] |
| | CASP3 | Apoptosis | [72] |
| miR-BART1-5p | IFI30 | CD4⁺ T-Cell Response | [77,78] |
| | Not Applicable | Diagnostic Marker for NKTL | [11] |
| miR-BART2-5p | CTSB | CD4⁺ T-Cell Response | [77,78] |
| | LGMN | CD4⁺ T-Cell Response | [77,78] |
| | IL-12B | CD4⁺ T-Cell Response | [77,78] |
| | CASP3 | Apoptosis | [84] |
| | BALE5 | Latency Regulation | [80] |
| | Not Applicable | Diagnostic Marker for NKTL | [11] |
| miR-BART3-3p | IPO7 | Innate Immunity | [72,74] |
| | CASP3 | Apoptosis | [84] |
| miR-BART4-5p | CASP3 | Apoptosis | [84] |
| miR-BART6-3p | PTEN | Cell Proliferation, Apoptosis | [86] |
| | IL-6RB | Innate Immunity | [71] |
| | RIG-I | Innate Immunity | [68] |
| miR-BART7-3p | CASP3 | Apoptosis | [84] |
| | Not Applicable | Diagnostic Marker for NKTL | [11] |
| miR-BART8-5p | CASP3 | Apoptosis | [84] |
| miR-BART10-3p | IL-12B | CD4⁺ T Cell Response | [77,78] |
| miR-BART13-3p | CASP3 | Apoptosis | [84] |
| | Not Applicable | Diagnostic Marker for NKTL | [11] |
| miR-BART15 | NLRP3 | Innate Immunity | [70] |
| miR-BART16 | S1PR1 | Cell Growth/Mobility | [48] |
| | CREBBP | Innate Immunity | [76] |
| | IPO7 | Innate Immunity | [72] |
| | CASP3 | Apoptosis | [72] |
| | TOMM22 | Apoptosis | [74] |
| miR-BART17 | TAP2 | CD8⁺ T-Cell Response | [77] |
| miR-BART20-5p | T-bet | Transcription Regulation of Cytotoxic NK Cells | [92] |
| miR-BART22 | CASP3 | Apoptosis | [84] |
| | IL-12B | CD4⁺ T Cell Response | [77,78] |

* Synergism with has-miR-142; ** Synergism with has-miR-197; *** EBERs and miR-BART16 corepress the target; **** Along with hsa-miR-494-3p and hsa-miR-142-3p regulates PTEN–AKT–mTOR/RICTOR circuit.
With the advent of animal models for EBV infections, we expect more accurate and cancer-applicable results to come out. Furthermore, recent data obtained from NGS experiments of EBV strains could provide us with new insights relating to their different oncologic roles [57,105]. On the other hand, mechanisms exploited by other miRNA-encoding herpesviruses such as Kaposi sarcoma-associated herpesvirus and murine γ-herpesvirus 68 could guide researchers towards clues extendible to EBV [106].

It must be noted, however, that several other tumors such gastric carcinoma, nasopharyngeal carcinoma, breast cancer, and leiomyosarcomas might be correlated with EBV [107–114], further highlighting the importance of this virus in carcinogenesis. In addition, an increasing number of publications suggest that EBV-miRNA-related pathogenesis might not be limited to cancers or other known conditions such as infectious mononucleosis, since autoimmune diseases like multiple sclerosis and primary Sjögren’s syndrome have also been connected to EBV infection [115–117]. Altogether, these data point out the importance of the development of anti-EBV vaccines (reviewed in [118]). We believe that the development of such vaccines is not a choice, but is a necessity, and thus, we encourage more research on the issue. Similarly, the evidence that EBV-encoded miRNAs retain a pathogenetic role in human lymphomas may also represent the opportunity to target them with novel therapeutic agents, much like the approach taken with miR-BART7-3p and hsa-miR-155 [119,120].

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**Abbreviations**

- **BART** BamHI-A rightward transcript
- **BAX** BCL2 Associated X
- **BHRF1** BamHI fragment H rightward open reading frame 1
- **BL** Burkitt Lymphoma
- **CASP3** Caspase 3
- **CDKN1B** Cyclin Dependent Kinase Inhibitor 1b
- **C/EBP** CCAAT-Enhancer-Binding Protein
- **CLL** Chronic Lymphocytic Leukemia
- **CREBBP** CREB-Binding Protein
- **CTSB** cathepsin B
- **Cp** C Promoter
- **DLBCL** Diffuse Large B Cell Lymphoma
- **EBERs** EBV-Encoded small RNAs
- **eBL** endemic Burkitt Lymphoma
- **EBNA** EBV-Encoded Nuclear Antigen
- **EBNA-LP** EBV-Encoded Nuclear Antigen Leader Protein
- **EBV** Epstein–Barr Virus
- **HHV-4** Human Herpesvirus 4
- **HIV-BL** HIV-related Burkitt Lymphoma
- **HIV-DLBCL** HIV-related Diffuse Large B Cell Lymphoma
- **HL** Hodgkin Lymphoma
- **IFI30** Interferon Gamma Inducible Protein 30
- **IFN** Interferon
- **IgG** Immunoglobulin G
- **IHC** Immunohistochemistry
- **IL-1** Interleukin-1
- **IL-1β** Interleukin-1β
- **IL-12B** Interleukin-12 Subunit Beta
- **IL-18** Interleukin 18
- **IL-6** Interleukin-6
- **IL-6RB** Interleukin-6 Receptor B
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