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Epstein-Barr Virus-Encoded miRNAs in Epstein-Barr Virus-Related Malignancy

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1. Introduction

In 1958, Denis Burkitt described B cell lymphomas in 2- to 14-year-old African children from malaria-endemic areas.[1] In 1964, Michael Anthony Epstein and Yvonne Barr found that immortalized B lymphocyte cell lines derived from these tumors spontaneously released a herpesvirus.[2] Thus, Epstein-Barr virus (EBV) was discovered by examining electron micrographs of cells cultured from Burkitt's lymphoma; its unusual geographic distribution indicated a viral etiology. It was Gertrud and Werner Henle who demonstrated that EBV is ubiquitous in the human population.[3] Far from having a restricted distribution, EBV, a member of the γ-herpesvirus family, was found to be widespread in all human populations and to persist in the vast majority of individuals as a lifelong, asymptomatic infection of B lymphocytes. Therefore, EBV is usually the cause of clinically inconspicuous infections, although it can cause infectious mononucleosis. The most severe, albeit rare, result of EBV infection is malignant transformation and the development of cancer in various forms, including Burkitt's lymphoma and nasopharyngeal carcinoma, the latter of which is one of the most common cancers in China.[4] The link between EBV and "endemic" Burkitt's lymphoma proved constant and became the first of an unexpectedly wide range of associations discovered between this virus and tumors.[5] As a ubiquitous human pathogen, EBV is responsible for several lymphoid malignancies, including a subset of Burkitt's lymphoma, acquired Immune deficiency syndrome (AIDS)-associated lymphoma, Hodgkin's lymphoma, post-transplant lymphoma, age-associated B cell lymphoma, and peripheral T and NK cell lymphomas.[6,7]

1.1 EBV infection

The primary site of EBV infection is the oropharyngeal cavity.[8] Children and teenagers are often infected after oral contact, hence the nickname "kissing disease". Like other herpesviruses, infection with EBV can exhibit two distinct patterns, or states, of gene expression. During acute (lytic) EBV infection, the virus sequentially expresses its entire repertoire of genes. In this lytic state, linear, double-stranded viral genomes are produced and packaged into virions that spread infection from cell to cell. Shortly after the initial

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infection, EBV enters into a latent state, whereupon only select "latent" genes are expressed, thereby evading host immune surveillance mechanisms, and establishing a lifelong, persistent infection in the host.[9] During latency, only a few viral genes are transcribed, no viral progeny are produced, and infected cells are protected from apoptotic stimuli and, in some circumstances, driven to proliferate. Based on serology, about 95% of the world's adult population is infected with EBV, and following primary infection, hosts remain lifelong carriers of the virus.[10] In developed countries, exposure to EBV occurs relatively late; only 50–70% of adolescents and young adults are EBV seropositive. About 30% of seronegative individuals will later develop infectious mononucleosis as a result of primary EBV infection. This disease is characterized by fever, pharyngitis, generalized lymphadenopathy, splenomegaly, intense asthenia, hyper-lymphocytosis (>50%) with atypical lymphocytes, and elevated transaminase levels. In developing countries, EBV antibodies are acquired early in life and the disease is mostly asymptomatic.

1.2 EBV-related cancer

EBV has been etiologically linked to a variety of human cancers, such as Burkitt's lymphoma, nasopharyngeal carcinoma (NPC), Hodgkin's disease, and more recently with sporadic cases of gastric adenocarcinoma and invasive breast carcinoma.[11,12] Nearly 100% of NPC tumors, 90% of Burkitt's lymphoma tumors of African origin, and 40–60% of Hodgkin's and non-Hodgkin's lymphomas contain EBV episomes. Clonality of the EBV genome has been confirmed in these tumors, suggesting that the tumors arose from a single EBV-infected cell, and that EBV infection is a very early, if not causal, event. EBV is also commonly associated with lymphoproliferative diseases in patients with congenital or acquired immunodeficiencies. Examples include X-linked lymphoproliferative syndrome, human immunodeficiency virus (HIV)-related non-Hodgkin's lymphoma, and perhaps most importantly post-transplantation lymphoproliferative disease.[5]

Burkitt's lymphoma is a malignant tumor associated with EBV that is endemic to central parts of Africa and New Guinea with an annual incidence of 6–7 cases per 100,000 and a peak incidence in children of 6 or 7 years of age. The epidemiological involvement of EBV in Burkitt's lymphoma was first suspected due to the presence of the EBV viral genome in tumor cells and elevated antibody titers against EBV viral capsid antigen in cancer patients. The highest prevalence of Burkitt's lymphoma occurs in the "lymphoma belt," a region that extends from West Africa to East Africa, between the 10th degree north and 10th degree south of the equator, and continues south along the eastern coast of Africa. This area is characterized by high temperature and humidity, which is likely the reason why an association between malaria and Burkitt's lymphoma was once suspected. In African countries within the lymphoma belt, such as Uganda, the association of Burkitt's lymphoma with EBV is very strong (97%), whereas it is less so elsewhere (e.g., 85% in Algeria and 10–15% in France and the USA). (World Health Organization, WHO)

NPC incidence rates are less than 1 per 100,000 in most populations, except for those in southern China, where an annual incidence of more than 20 cases per 100,000 is reported.[4] Isolated northern populations, such as Eskimos and Greenlanders, also have high incidences. Moderate incidences occur in North Africa, Israel, Kuwait, the Sudan, and parts of Kenya and Uganda. Men are twice as likely to develop NPC as women. The rate of incidence generally increases at ages 20–50 years. In the USA, Chinese-Americans comprise

the majority of NPC patients, along with workers exposed to fumes, smoke, and chemicals, implicating a role for chemical carcinogenesis. Studies assessing nutrition and diets have demonstrated an association between eating highly salted foods and NPC. Vitamin C deficiency at a young age may also be a contributing factor. Finally, a study of human leukocyte antigen (HLA) haplotypes revealed a genetically distinct subpopulation in southern China, with an increased frequency of haplotype A-2/B-Sin-2, which may account for the higher disease incidence in that area. (WHO)

1.3 Cytotoxic T lymphocyte therapy for EBV-related cancer

EBV, together with human herpesvirus (HHV)-8 (also known as Kaposi sarcoma-associated virus), belongs to the genus *Lymphocryptovirus* in the subfamily Gammaherpesvirinae of the family Herpesviridae. These are complex, enveloped, DNA viruses, which multiply in the nucleus of the host cell. EBV infects resting human B lymphocytes and epithelial cells, multiplies in the latter, and establishes latent infection in memory B lymphocytes. Thus, infected individuals may produce virions, carry virus-specific cytotoxic T lymphocytes (CTLs), produce EBV-specific antibodies, and yet harbor latently infected memory B cells. EBV-infected individuals maintain the latent EBV genome as an episome that expresses only part of its genetic information, including EBV nuclear antigens (EBNA)-1 (a latent DNA replication factor), EBNA-2 (a transcriptional activator), and EBNA-3A and -3C (involved in the establishment of latency). Also expressed are latent membrane protein (LMP)-1 and LMP-2, which play major roles in the maintenance of latency and escape from the host immune response. Latently infected cells do not express the B7 coactivator receptor and, therefore, are not targeted by CTLs. When peripheral blood from an infected individual is cultured, latently infected B cells replicate and become immortalized lymphoblasts that can be indefinitely propagated in the laboratory.[13]

In a previous study, CTL therapy was proven to be safe and effective as a treatment for patients with EBV-related cancers, and was found to enable the complete remission of patients who failed all previous standard treatments. The first clinical trials using EBV-specific CTLs tested their utility for both prophylaxis and treatment of post-transplant lymphoproliferative diseases arising in stem cell transplant or solid organ transplant recipients.[5]

Nucleoside analogs, such as acyclovir (ACV) and ganciclovir (GCV), are often used as antiviral drugs against acute EBV and other herpesvirus infections.[14] The virally encoded thymidine kinase enzyme converts these analogs into their phosphate forms, which, after conversion into their triphosphate form by host kinases, are then incorporated into newly synthesized DNA, leading to the premature termination of DNA synthesis and apoptosis of the infected cell. The EBV thymidine kinase, however, is only expressed during lytic replication of the virus. Because EBV maintains a latent state of replication in all EBV-associated malignancies, nucleoside analog drugs have very limited, or no, cytopathic effect on virus-infected cells. Novel therapeutic approaches to target EBV-infected tumor cells, which include inducing lytic replication of EBV followed by treatment with nucleoside analogs, have been proposed.

Arginine butyrate induces the expression of the viral thymidine kinase gene in EBV-positive, immunoblastic, non-Hodgkin's lymphoma cell lines and lymphoblastic cell lines (LCLs) and acts synergistically with GCV to inhibit cell proliferation and decrease cell

viability.[15] Various other agents have also been used to induce lytic replication of the EBV genome. For example, treatment of EBV-positive lymphoblastoid cells, or primary central nervous system lymphoma, with γ-irradiation promotes GCV-susceptibility of target cells.[15] Other studies successfully used 5-azacytidine, gemcitabine, doxorubicin, or a combination of anti-CD20 monoclonal antibody (Rituximab) and dexamethasone to induce lytic-phase gene expression and sensitize EBV-infected tumor cells to GCV or other nucleoside analogs.[15]

Butyric acid, a short-chain fatty acid, and its derivatives have been experimentally employed in attempts to treat leukemias and other diseases. Butyrate induces the expression of certain EBV lytic proteins, including the thymidine kinase enzyme, from latent EBV-infected cells.[16] The inhibitory effect of butyrate on histone deacetylase (HDAC) is required for this effect. In previous clinical studies, systemic administration of arginine butyrate was used to induce expression of the latent EBV thymidine kinase in the tumors of patients with EBV-positive post-transplantation lymphoproliferative disease or non-Hodgkin's lymphomas, followed by treatment with GCV.[16,17]

Bortezomib, a proteasome inhibitor, also activates EBV lytic gene expression.[18] Bortezomib leads to increased levels of CCAAT/enhancer-binding protein β (C/EBPβ) in a variety of tumor cell lines.[18] C/EBPβ activates the promoter of the EBV lytic switch gene *ZTA* (BZLF1). Bortezomib treatment leads to increased binding of C/EBPβ to sites within the *ZTA* promoter. Knockdown of C/EBPβ inhibits bortezomib activation of EBV lytic gene expression.[18] Bortezomib also induces the unfolded protein response (UPR). Thapsigargin, an inducer of the UPR that does not interfere with proteasome function, also induces EBV lytic gene expression.[18,19] The effect of thapsigargin on EBV lytic gene expression is also inhibited upon C/EBPβ knock-down.[18] Therefore, C/EBPβ mediates the activation of EBV lytic gene expression associated with bortezomib and thapsigargin.[18]

Pretreatment of naturally infected EBV tumor cell lines (from Burkitt's lymphoma and gastric carcinoma) with bortezomib activates viral gene expression.[20] Marked changes in tumor growth are also achieved in naturally infected Kaposi's sarcoma herpesvirus tumors after pretreatment with bortezomib.[20] Bortezomib-induced, enzyme-targeted radiation therapy illustrates the potential of pharmacological modulation of tumor gene expression for targeted radiotherapy.

There is increasing interest in the pharmacologic activation of lytic viral gene expression in tumors. Several therapeutic strategies requiring activation of EBV lytic genes for tumor cell lysis have been described, but concerns have been raised about the possible adverse effects of viral gene activation patients treated with pharmacologic activators.

2. EBV-encoded miRNA

2.1 miRNA

Micro (mi)RNAs are small, non-coding, single-stranded RNAs of approximately 21 to 25 nucleotides (nt) in length. They post-transcriptionally regulate mRNA expression in animals and plants and are transcribed from the non-coding regions of genes in all multi-cellular organisms and certain viruses and are often phylogenetically conserved across species.[21,22] EBV was the first human virus found to encode miRNA.[23] EBV encodes 44 viral miRNAs and a small RNA. EBV-encoded miRNAs are located within the *BHRF1* and BamHI A

rightward transcript (*BART*) loci of the EBV genome. The BHRF1 cluster of miRNAs includes BHRF1-1, BHRF1-2, BHRF1-3, and BHRF1-4.[22-24] The other EBV-encoded miRNAs are encoded by BART cluster 1 and BART cluster 2, except miR-BART2, which is expressed from a sight outside of the BART clusters.[22-25] miRNAs bind to the 3′ untranslated region (UTR) of mRNA and interfere with their translation, leading to downregulated protein expression levels. EBV-encoded miRNAs have been found in various EBV-associated carcinomas and lymphomas, such as NPC, gastric carcinoma, diffuse large B cell lymphoma, nasal NK/T cell lymphoma, and Hodgkin's lymphoma.[23,26] Viral miRNAs play vital roles in immunogenesis, host cell survival and proliferation, differentiation, lymphomagenesis, and regulation of viral infection and latency.[23,27-29]

2.2 EBV miRNA-mediated regulation of viral infection states

During lytic infection, EBV genomes are amplified into 1000 copies per cell with the help of replication proteins.[30] EBV expresses six replication proteins, the most important of which are BZLF1 and BALF5. BALF5 is a catalytic DNA polymerase encoded by the *balf5* gene during lytic infection;[30] it is not present in latent infection. This DNA polymerase is a single-stranded DNA binding protein, which functions within viral replication factories in the nucleus, likely generating replication forks on the replicating EBV genome. EBV-encoded miR-BART2 is expressed at low levels during latency, prevents aberrant expression of BALF5 mRNA, and prevents inadvertent viral replication.[31] The sequence of miR-BART2 is perfectly complementary to the 3′UTR of BALF5 mRNA. Therefore, miR-BART2 serves as an inhibitor of viral DNA replication through the degradation of BALF5 mRNA. The miRNA-guided cleavage of mRNA requires an association with Ago2,[32] which is a member of the Argonuate family of proteins and a part of the RNA-induced silencing complex (RISC). Upon its association with Ago2, miR-BART2 guides the sequence-specific cleavage of BALF5 mRNA. This miR-BART2-guided cleavage is substantially reduced after induction of the lytic cycle in EBV-infected cells.[31] The amount of miR-BART2 is reduced during lytic infection, and this causes a de-repression of BALF5 protein expression.[31] However, it is unclear whether the miR-BART2-mediated regulation of viral replication is fully controlled by BALF5 protein or not.

Another regulator of the shift from EBV latency to lytic infection is miR-BART6, which itself is regulated by RNA editing.[33] Editing of the wild-type primary (pri)-miR-BART6 sequence dramatically reduces the loading of miR-BART6-5p onto RISC, without affecting the processing of precursor (pre) or mature miRNAs.[33] Editing of pri-miRNA might affect the selection and loading of the guide strand onto RISC.[34] miR-BART6-5p silences Dicer through multiple target sites located in the 3′UTR of Dicer mRNA, but miR-BART6-3p is unable to perform this function.[33]

In EBV-infected human cells, Dicer protein levels are substantially reduced by miR-BART6-5p,[33] suggesting that miR-BART6-5p may indirectly regulate the biogenesis of all miRNAs. It may even affect the latency of EBV by modulating the expression of viral proteins, including EBNA2, LMP1, RTA, and ZTA. EBNA2 is required for the transition from the less immunologically confrontational type I or type II latency to the more immunity-stimulating type III latency, which occurs through the upregulation of all latent EBV genes and the transformation of infected B lymphocytes.[35,36] However, EBNA2 deficiency is observed in type I and type II latency.[35,36] Low-level expression of LMP1 is also observed in type II latency, but is absent in type I. LMP1 controls the NF-κB signaling pathway and the growth and apoptosis

of host cells. RTA and ZTA proteins initiate lytic infection of EBV. Thus, it is clear that miR-BART6-5p regulates EBV infection and latency by suppressing RTA and ZTA protein expression. To modulate protein expression, miR-BART6-5p downregulates viral promoters, such as Cp and Wp, which are characteristic of type III latency, and reduces transcriptional activity via its silencing effect on Dicer. Mutation and adenosine-to-inosine (A-to-I) editing are adaptive mechanisms that antagonize miR-BART6 activities and affect the latent state of viral infection.[33] Therefore, we conclude that miR-BART6-5p, and its mutant or edited versions, are critical for the establishment and maintenance of latent EBV infection.

2.3 EBV miRNA-mediated host cell survival

The miR-BART5 miRNA promotes host cell survival by regulating the p53 upregulated modulator of apoptosis (PUMA) protein.[37] PUMA is an apoptotic protein belonging to the "BH3-only" group of the Bcl-2 family and is encoded by the *BBC3* gene.[38-40] PUMA is regulated by the tumor suppressor p53 and is involved in both p53-mediated and non-mediated apoptosis via independent signaling pathways. PUMA is an important downstream regulator of p53, both of which are master regulators of host cell growth and apoptosis. PUMA function is downregulated or absent in cancer cells, but the absence of PUMA activity alone is not sufficient for the spontaneous formation of malignancies.[41-45] PUMA has four isoforms (i.e., α, β, γ, and δ), which share the same 3'UTR.[39] Only PUMA-α and PUMA-β have pro-apoptotic activity. The PUMA 3'UTR sequence is perfectly complementary to miR-BART5. Thus, binding of miR-BART5 and the PUMA 3'UTR suppresses the expression of the pro-apoptotic protein. PUMA-β protein expression is also reduced by pre-miR-BART5.[37] Abundant expression of miR-BART5 in NPC cells is correlated with significant downregulation of PUMA in 60% of NPC tissues.[37] By this mechanism, miR-BART5 induces anti-apoptotic activity in NPC cells, EBV-infected gastric carcinoma cells, and EBV-infected epithelial cells.[37] Therefore, miR-BART5 may be a good target for anti-cancer therapy in EBV-infected cancer cells.

LMP1 is a viral protein expressed during the type III latency period of EBV infection.[35,36] LMP1 promotes cell growth, resistance to serum deprivation-induced apoptosis, and phenotypic changes in epithelial cells and B cell transformation. It activates the NF-κB, JNK, JAK/STAT, p38/MAP, and RAS/MAPK pathways and regulates host gene expression.[5] NF-κB transcription factors influence proliferation, apoptosis, oncogenesis, and inflammation.[46] Low levels of LMP1 activate NF-κB, but with increasing amounts of LMP1, NF-κB activation reaches a plateau, after which small increases in LMP1 reduces NF-κB activity.[47] Thus, a threshold level of LMP1 can maintain peak NF-κB activity. LMP1 regulates the level of NF-κB activity by modulating the UPR pathway and autophagy. *BARTI* cluster miRNAs negatively regulate LMP1 expression, limiting inappropriately high levels, thereby preventing apoptosis that would otherwise result from LMP1-mediated changes in the UPR. Such *BART1* cluster miRNAs include BART16, BART17-5p, and BART1-5p, which target sites within the 3'UTR of LMP1 mRNA.[47] These miRNAs regulate LMP1 expression at the post-transcriptional level, regulating NF-κB-mediated gene expression. Therefore, the negative regulation of LMP1 expression by *BART1* cluster miRNAs may affect EBV-associated cancer development by balancing the effect of LMP1 on cellular proliferation.

BHRF1 is a latent protein expressed in growth-transformed cells that contributes to virus-associated lymphomagenesis.[48] miR-BHRF1 downregulates this protein, modulates cell transformation,[49] and promotes B cell proliferation after EBV infection. EBV-infected B cells

lacking miR-BHRF1 progress less efficiently into the cell cycle and eventually die by apoptosis.[49] miR-BHRF1 is constitutively expressed in LCLs.[49] Without miR-BHRF1, the proportion of G1/G0 cells increases while the numbers of S-phase cells decreases,[49] indicating a definite role of miR-BHRF1 in the control of proliferation of latently infected cells. miR-BHRF1 acts at a stage of the EBV life cycle when multiple EBV-encoded oncogenes become activated.

2.4 EBV-encoded miRNAs regulate immune evasion

Major histocompatibility complex (MHC) class I polypeptide-related sequence B (MICB) protein is a ligand for the NKG2D type II receptor, which is a stress-induced immune molecule.[50,51] B cells and endothelial cells, which are targets of EBV, both express this protein. Binding of MICB activates NK, $CD8^+$ αβ, and γδ T cells.[52] MICB is upregulated at the cell surface due to various insults, such as viral infection, tumor transformation, heat shock, and DNA damage. Thus, it would be beneficial for a virus to downregulate the expression of this protein ligand to avoid immune detection. Previous studies have shown that downregulated MICB expression leads to reduced lysis of infected cells by NK cells.[53] EBV-expressed miR-BART2-5p has potential binding sites in the MICB mRNA 3'UTR.[54] EBV downregulates MICB via miR-BART2-5p, resulting in decreased NK cell-mediated lysis, to avoid detection by immune cells.

miR-BHLF1-1 is expressed from the 5'UTR, and miR-BHLF1-2 and miR-BHLF1-3 are expressed from the 3'UTR, of the *bhrf1* gene in EBV-infected cells.[28] miR-BHLF1-3 is markedly elevated in EBV-infected, type III latent cell lines[28] and is also detected in EBV-positive primary effusion lymphoma and AIDS-related diffuse large B cell lymphoma.[28] BHRF1 miRNA is characteristic of EBV type III latent infections.[55] EBV miR-BHRF1-3 regulates host immunity by downregulating the interferon (IFN)-inducible T cell attracting chemokine (I-TAC; also known as CXCL-11). CXCL-11/I-TAC belongs to the CXC family of chemokines, and both IFN-β and IFN-γ strongly induce its transcription.[56] CXCL-11/I-TAC promotes cell-mediated immunity by attracting activated T cells. The 3'UTR of CXCL-11/I-TAC mRNA is 100% complementary to the sequence of miR-BART1-3 and, therefore, serves as a target of miR-BART1-3. miR-BART1-3 inversely regulates the expression of CXCL-11/I-TAC; the anti-sense sequence of miR-BART1-3 has the reverse effect.[23] miR-BART1-3 significantly reduces the expression of CXCL-11/I-TAC at both the mRNA and protein levels.[28] Thus, as cellular chemokines can be targets of viral miRNA, EBV-mediated regulation of antigen processing and presentation, and the downregulation of CTL cytokine networks, may occur through such a mechanism.

2.5 Small nucleolar RNAs encoded by the EBV genome

A small nucleolar (sno)RNA, named v-snoRNA1, has been identified within the EBV genome in EBV-infected B lymphocytes.[57] snoRNAs, 60–300 nt in length, guide nucleotide modifications of ribosomal (r)RNAs, i.e., 2'O-ribose methylation or pseudouridylation, that are located in subnuclear compartments.[58,59] snoRNAs are subdivided into the C/D box and H/ACA box classes. The majority of snoRNAs located within introns of protein-encoding genes are processed by splicing, followed by endo- and exonucleolytic cleavage.[60-62] However, some of them are orphan snoRNAs that lack rRNA or small modulatory

(sm)RNA targets. v-snoRNA is processed into 24 nt long miRNAs, which then target the 3′UTR of viral DNA polymerase mRNA.

The *v-snoRNA1* gene is located within the *BART* sense stand of the EBV genome.[57] Both v-snoRNA1 and miR-BART2 arise from the same intron. Although v-snoRNA1 is an integral part of the EBV latent transcription program, it is highly expressed during lytic infection. The 3′UTR of the BHLF5 mRNA is fully complementary to v-snoRNA1, so v-snoRNA1 binds and cleaves BALF5 mRNA, enabling its exonucleolytic degradation.[57] It is unclear whether v-snoRNA1 serves an important function during the viral life cycle.

3. Editing and mutation of EBV-encoded miRNAs

Recently widespread RNA-DNA differences in the human transcriptome were found. It also occurs to miRNAs including EBV-encoded miRNAs.[63]

3.1 RNA editing

RNA editing is carried out by enzymes that target mRNA post-transcriptionally, such as adenosine deaminases that act on RNA (ADARs, which convert adenosine to inosine, which is subsequently recognized by translation machinery as a guanosine, i.e., A-to-G mutation) and apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like (APOBEC) proteins, which convert cytidine to uridine (i.e., C-to-U mutation). Editing of pri-miR-142, the primary transcript form of miR-142 that is expressed in hematopoietic tissues, results in suppression of its processing by Drosha.[64] The mutated pri-miR-142 is degraded by Tudor-SN, a component of RISC and also a ribonuclease that is specific to inosine-containing double-stranded (ds)RNAs. Mature miR-142 is substantially upregulated in ADAR1- or ADAR2-null mice,[64] demonstrating that RNA editing helps control miRNA biogenesis. Kawahara et al. found that primary transcripts of certain miRNA genes are subject to RNA editing that converts adenosine to inosine. By way of ADAR, tissue-specific A-to-I editing of miR-376 cluster transcripts lead to the predominant expression of edited miR-376 isoform RNAs. One highly edited site is located in the middle of the 5'-proximal "seed" region of miR-376 critical for its hybridization to its targets, providing evidence that the mutated miR-376 specifically targets a set of genes that is different than those targeted by wild-type miR-376.[65] Mutated miR-376 represses phosphoribosyl pyrophosphate synthetase 1, an enzyme involved in the uric-acid synthesis pathway.

Iizasa et al. reported that the primary transcripts of four EBV miRNAs, including miR-BART6, are subject to A-to-I editing. Moreover, it was demonstrated that editing of pri-miR-BART6, as well as mutations of miR-BART6, found in latently EBV-infected cells prevented its loading onto functionally active RISC.[33] As mentioned, miR-BART6 targets Dicer and affects the latent state of EBV viral infection. Therefore, regulation of miR-BART6 expression and function through A-to-I editing may be critical for the establishment or maintenance of latent EBV infection.

3.2 Mutation of the EBV genome affects encoded miRNAs

Sequence variation in the EBV genome has been extensively studied for a long time; in particular, *BLRF1* and other genes have been reported to have sequence variation in EBV-infected cancer patients.[66] Mechanistic analysis of this sequence variability has recently been

reported by Suspène et al.[67] Human APOBEC3 cytidine deaminases target and edit single-stranded DNA, which can be of viral, mitochondrial, or nuclear origin. Retroviral genomes, such those of HIV, deficient in the *vif* gene, and hepatitis B virus, are particularly vulnerable.

The genomes of DNA viruses, such as herpesviruses, are also subject to editing. This is the case for herpes simplex virus type 1 (HSV-1), at least in tissue culture, where APOBEC3C (A3C) overexpression reduces viral titers and the particle/plaque forming unit (PFU) ratio by approximately 10-fold. A3A, A3G, and activation-induced cytidine deaminase (AICDA) can edit what is thought to be a small fraction of HSV genome in an experimental setting without seriously impacting viral titers. Hyper-editing was found to occur in HSV genomes recovered from four of eight uncultured buccal lesions, but the phenomenon was not restricted to HSV; hyper-mutated EBV genomes were readily recovered from four of five established cell lines, indicating that episomes are also vulnerable to editing [67]. These findings suggest that the widely expressed A3C cytidine deaminase can function as a restriction factor for some human herpesviruses.

Other studies reported sequence variation in BART miRNAs.[68] The significance of these mutations and their effect on miRNA processing, as well as the mechanism of mutation, whether it is mediated by A3C, members of other APOBEC families, or other mechanisms, have yet to be determined.

4. Regulation of EBV-encoded miRNA processing

4.1 Processing of miRNAs under normal versus cancerous conditions

The mechanism of miRNA biosynthesis involves sequential endonucleolytic cleavages mediated by two RNase III enzymes, Drosha and Dicer (Fig.1). Following transcription by RNA pol II, Drosha processes the primary miRNA transcript (pri-miRNA) into a 60–100 nt hairpin structure, termed the precursor miRNA (pre-miRNA), in the nucleus (Fig. 1). Following cleavage by Drosha, the pre-miRNA is transported out of the nucleus through an interaction with Exportin-5 and Ran-GTP. Then, the pre-miRNA undergoes further processing catalyzed by Dicer (Fig. 1). This cleavage event gives rise to an approximately 22 nt dsRNA product containing the mature miRNA guide strand and the miRNA* passenger strand (Fig. 1). Then, the mature miRNA guide strand is loaded onto the RISC, while the passenger strand is degraded (Fig. 1).

Although substantial progress has been made in understanding the basic mechanism of miRNA biogenesis, less is known about the mechanisms that regulate miRNA biogenesis and how these systems might be deregulated during oncogenesis. Several studies have reported that various regulatory mechanisms of miRNA biosynthesis are potentially involved in carcinogenesis.[69]

The tumor suppressor protein p53 was recently found to modulate miRNA processing through its association with p68 and Drosha.[70,71] Under conditions of DNA damage, several miRNAs, such as miR-143 and miR-16, are post-transcriptionally induced. This process requires p53, as p53-null HCT116 cells do not induce miRNAs in response to DNA damage.[72] Co-immunoprecipitation studies have indicated that p53 is present in a complex with both Drosha and p68, and the addition of p53 to *in vitro* pri-miRNA processing assays enhances the activity of Drosha. Interestingly, several p53 mutant-containing cells that are linked to oncogenesis have low post-transcriptional miRNA expression.[72]

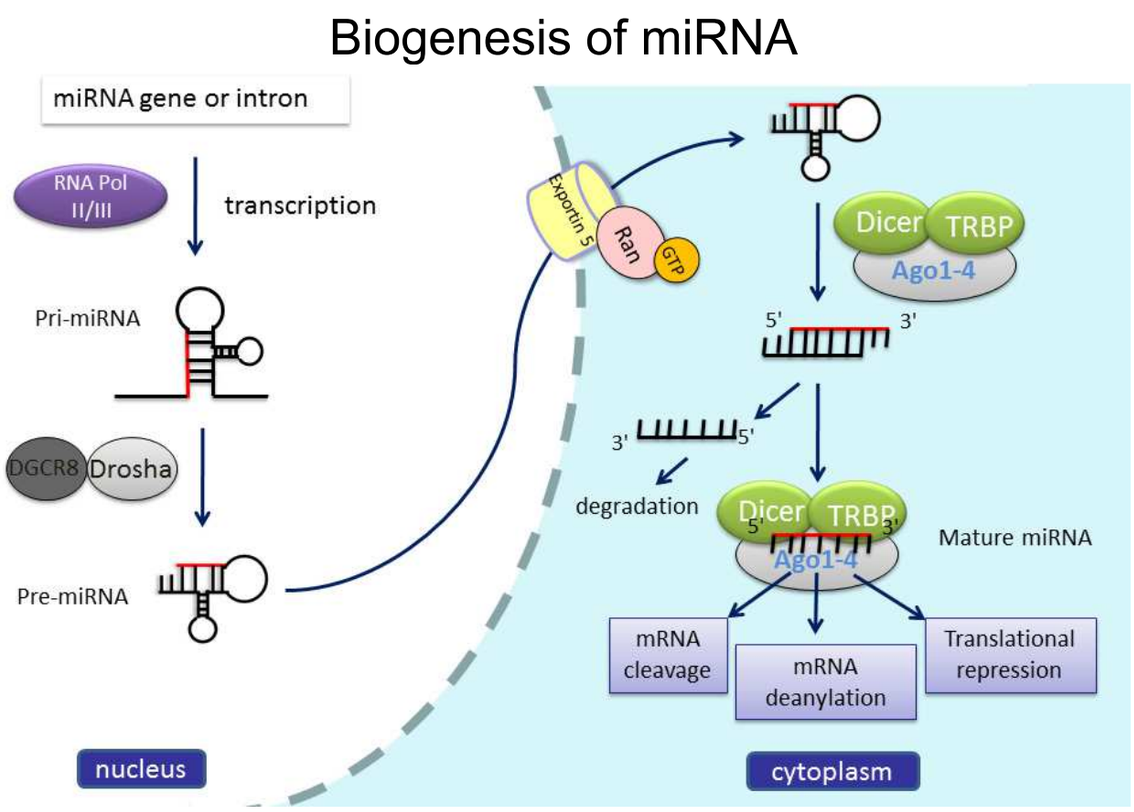


Fig. 1. Processing machinery of miRNA
miRNA genes are transcribed by RNA polymerase II or III into long primary (pri) miRNA transcripts, processed by the nuclear nuclease Drosha into ~60 bp hairpins termed precursor (pre) miRNAs, and further cleaved in the cytosol by the Dicer nuclease into mature miRNAs. Mature miRNAs are then incorporated into the multiprotein RNA-induced silencing complex (RISC), exerting post-transcriptional repression of target mRNAs, either by inducing mRNA cleavage, mRNA degradation or blocking mRNA translation.

4.2 Processing of EBV-encoded miRNAs

For EBV-encoded miRNAs, several regulatory processes have been reported[68]. Almost all of the EBV-encoded miRNAs originate from one of three sequence clusters. Two of the three clusters of miRNAs are made from the BARTs, a set of alternatively spliced transcripts that are highly abundant in NPC, but have not been shown to produce a detectable protein. Edwards et al. investigated the mechanism of BART-derived miRNA processing by comparing the processed miRNAs with the original BART transcript and residual transcripts after processing.[68] First, they showed that residual pieces of the intron sequence were detectable in the nucleus of cells that express the miRNAs. Characterization of these residual pieces indicated that the miRNAs were produced from one large initial transcript prior to splicing and that a specific spliced form of the transcript favored the production of miRNAs. Second, they found that miR-BART12 is not detected at all, even though the primary transcript is abundant. Third, pre-miR-BART5 could be detected in all cell lines and tumors tested, despite low or undetectable expression of the mature miR-BART5, indicating that the processing of pre-miR-BART5 was inhibited.

	function	target viral	Host target	
BLHF1-1	transformation	BFLF2	LILRB-5,E2F1,p53,CBFA2T2	BHRF1
BLHF1-2	transformation	BFLF2	PIK3R1	BHRF1
BLHF1-3	transformation	BFLF2	CXCL11,PRF1,TGIF,NSEP1	BHRF1
BART1-5p	Cancer development	LMP1	CXCL12	BART Cluster1
BART2-5p	viral replication	BALF5、LMP1	MIC B, Bim	
BART3		LMP1	IPO7, Bim	BART Cluster1
BART4		LMP1	Bim	BART Cluster1
BART5	Host cell survival	LMP1	PUMA, Bim	BART Cluster1
BART6	maintain viral latency	LMP1	Dicer, Bim	BART Cluster1
BART7		LMP1	Bim	BART Cluster2
BART8				BART Cluster2
BART9				BART Cluster2
BART10				BART Cluster2
BART11				BART Cluster2
BART12				BART Cluster2
BART13				BART Cluster2
BART14				BART Cluster2
BART15				BART Cluster1
BART16	Cancer development	LMP1	TOMM22	BART Cluster1
BART17	Cancer development	LMP1		BART Cluster1
BART18				BART Cluster2
BART19				BART Cluster2
BART20				BART Cluster2
BART21				BART Cluster2
BART22		LMP2		BART Cluster2

Table 1.

Amoroso et al. reported that the levels of the different BART miRNAs vary up to 50-fold within a given cell line.[73] However, this variation cannot be explained by differential miRNA turnover, as all EBV miRNAs appear to be remarkably stable, suggesting that miRNA maturation is a key step in regulating steady-state levels of EBV miRNAs. Future studies should further investigate the mechanism of miRNA transcript processing in EBV-infected cells, highlighting any differences between the three types of latent infections.

5. Secretory EBV-encoded miRNAs

5.1 Secretory miRNAs

Cellular and viral miRNAs control gene expression by repressing the translation of mRNAs into protein, a process that is tightly regulated in healthy cells, but is deregulated in cancerous and virus-infected cells. Curiously, miRNAs are not strictly intracellular, but are also secreted through the release of small vesicles called exosomes and, therefore, exist extracellularly in the peripheral blood and in cell culture media.[74] It has been suggested that exosome-associated miRNAs play a role in intercellular communication [74], although concrete evidence for this has been lacking. The dynamics of miRNA secretion via exosomes and the proposed transfer mechanisms remain poorly understood. In addition, it is unclear whether miRNAs are secreted in physiologically relevant amounts.

5.2 Existence of secretory EBV-encoded miRNAs

Pegtel et al. were the first to show that exosomes deliver viral miRNAs to non-infected cells.[75] They used EBV B95.8-immortalized LCLs and demonstrated that exosomes contained

BHRF1 miRNAs, which could target the *CXCL11/ITAC* gene in nearby uninfected cells. Furthermore, they showed that non-B cells in EBV-infected patients with elevated viral loads contained EBV miRNAs, demonstrating that exosomes apparently transfer miRNAs *in vivo* to uninfected cells. These findings were confirmed by two studies that demonstrated the release of exosomes from NPC cells. Gourzones et al. showed that EBV miR-BARTs present within exosomes can be detected in the serum of mice xenografted with human NPC cells and that the sera of NPC patients also contain BART miRNAs.[76]

6. Concluding remarks

EBV-related cancers are generally difficult to cure. Despite extensive studies based on well-known concepts and methods, the molecular basis by which EBV mediates tumorigenesis and eludes immunosurveillance remains unclear. Mouse models of EBV-mediated lymphoproliferative disease have recently revealed that EBV infection of B cells is necessary, but not sufficient, for tumorigenesis, as all peripheral mononuclear cells are needed to generate tumors in these mice.[77] Immune cells are also indispensable for EBV-mediated tumorigenesis . The relationship between these cells and EBV-infected cells with regard to tumorigenesis remains unclear. Moreover, the mechanism of drug resistance, which causes poor prognosis of EBV-related tumors, has not yet been elucidated. Therefore, it is important to study the tumor biology of EBV-related tumors from a fresh perspective, such as EBV-encoded miRNAs.

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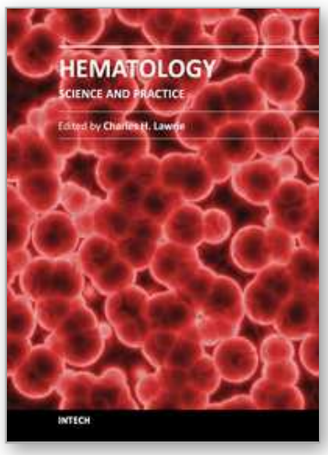
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Hematology encompasses the physiology and pathology of blood and of the blood-forming organs. In common with other areas of medicine, the pace of change in hematology has been breathtaking over recent years. There are now many treatment options available to the modern hematologist and, happily, a greatly improved outlook for the vast majority of patients with blood disorders and malignancies. Improvements in the clinic reflect, and in many respects are driven by, advances in our scientific understanding of hematological processes under both normal and disease conditions. Hematology - Science and Practice consists of a selection of essays which aim to inform both specialist and non-specialist readers about some of the latest advances in hematology, in both laboratory and clinic.

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