The role of miRNAs and EBV BARTs in NPC

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Abstract

The BamHI A Rightward Transcripts are a set of alternatively splicing transcripts produced by Epstein-Barr Virus that are highly expressed in nasopharyngeal carcinoma. These transcripts contain several open reading frames as well as precursors for twenty two miRNAs. Although the putative proteins corresponding to these open reading frames have not been detected, several studies have identified properties that are interesting and potentially significant with respect to cellular transformation. The miRNAs, however, are very abundant in all nasopharyngeal carcinomas and several potentially significant functions have been identified for some of the miRNAs. This article will focus on the nature of this complicated set of transcripts and the evidence that they contribute to the development of nasopharyngeal carcinoma.

Keywords

EBV; BARTs; miRNAs; NPC

1. Introduction

The Epstein-Barr Virus (EBV) is a ubiquitous member of the herpesvirus family that is considered an etiologic factor in the development of several human malignancies. A major malignancy is nasopharyngeal carcinoma (NPC) which occurs globally at a rate of 1 in 100,000 adults with highly elevated incidence of 20-50 per 100,000 in areas of southern China. The first evidence of an association of EBV infection with NPC was the identification of IgA antibodies to the EBV viral capsid antigen in NPC patient serum [1]. Subsequently EBV DNA was identified within the malignant epithelial cells of undifferentiated NPC regardless of geographic location while in Southern China, EBV is detected in all examples of NPC regardless of the differentiation subtype [2,3]. Furthermore, a clonal EBV infection was found in early, preinvasive lesions suggesting a role in initiation of NPC tumors [4]. The detection of the viral genome and viral proteins and RNAs in all cells within a tumor strongly suggest a causative role for EBV infection in NPC.

In NPC, EBV expression is restricted to a subset of the latent transcripts that are expressed in EBV-transformed B-lymphocytes and in transplant-associated lymphoproliferative disorders. Of the six EBV latent nuclear antigens, only EBNA1 expression is detected in NPC tumor biopsies [5]. However, the latent membrane proteins LMP1 and LMP2A [5,6], as well as two highly abundant, small noncoding RNAs (EBERs) [7], are consistently expressed in NPC. In addition to these latent genes, BARF1, an early lytic protein in infected lymphocytes, has been detected in NPC [8,9]. Importantly, the BamHI A rightward transcripts (BARTs) are consistently and highly expressed in NPC [10]. The BARTs are an overlapping group of differentially spliced RNAs that are 3' coterminal and possibly initiate from two transcriptional start sites [11]. The complex differential splicing produces multiple open reading frames (ORFs) for proteins and precursors for microRNAs (miRNAs). The potential contribution of the BARTs in the pathogenesis of NPC is likely to be continuously illuminating, however, this review will summarize our current understanding of the nature and function of the BARTs with particular emphasis on relationships to NPC.

2. Identification and Structure of the BARTs

2.1. Initial Characterization and Expression Patterns of the BARTs

The BARTs were first identified as abundant viral transcripts in cDNA libraries prepared from the C15 NPC tumor that is serially passaged in nude mice [12,13,14] Northern blotting revealed a predominant band of 4.8kb that was expressed in this tumor, although several other sized bands are also apparent [13,15]. Subsequent studies suggested that these transcripts were present in other NPC xenografts as well as in NPC biopsies [10]. These transcripts were striking in that they were highly abundant in NPC but had not been identified in EBV transformed lymphocytes. However they were antisense to the several known open reading frames encoding lytic proteins, including BALF5, the viral DNA polymerase [14]. It was suggested that the transcripts may contribute to the inhibition of replicative genes and were referred to as complementary strand transcripts (CSTs) [15].

Interestingly, this region was shown to be abundantly transcribed in early studies of viral transcription in NPC and Burkitt's lymphomas (BL) biopsies [16,17]. Although BART transcription can be detected using RT-PCR in all latently infected cell types, their relative abundance is highest in the more restricted Type 1 and Type 2 patterns of latency found in the tumors associated with EBV and low to undetectable in EBV transformed lymphocytes expressing the Type 3 full pattern of latent genes [18,19]. Interestingly, most of the DNA encoding the BART transcripts is deleted in the prototype B95-8 strain of EBV [20]. The B95-8 cell line was selected for high virus production and the virus produced from it is efficient in lymphocyte transformation. Additionally, a recombinant virus that is deleted for the entire region of the genome encoding the BARTs is still capable of infecting and transforming B lymphocytes *in vitro* [21]. These findings indicate that the BART transcripts are not required for B-lymphocyte transformation which is in agreement with the negligible levels of expression in Type 3 latency. However, the abundant expression of the BART transcripts in EBV associated tumors that do not express most of the potent EBNA proteins suggests that they are major contributors to tumor development or growth. It is also possible that the BARTs contribute to the maintenance of latency through antisense effects on the replicative genes on the opposite strand of the genome for the BARTs. Importantly, the highest expression of the BARTs is detected in infected epithelial cells, including NPC and gastric cancer, which may indicate that the BARTs have specific properties in epithelial cells.

2.2. Structure and splicing patterns of the BARTs

The BARTs are a series of transcripts that share a common 3' terminus and are produced by a complicated series of alternative splicing. A variety of experimental techniques including sequencing DNA clones, RT-PCR, and RNase protection assays have been used to map the various forms of the transcripts that exist in NPC, as well as other EBV-associated tumors [11,22,23,24]. The general structure is one of seven exons (named I through VII), although significant variety exists at multiple locations (Figure 1). Exons III, V, and VII contain additional splicing sites that can result in excision of parts of those exons. Exons II and VI are not always included in the final transcript and there are an additional two exons (Ia and Ib) that are sometimes included between exons I and II.

There is no large ORF that spans the length of any of the identified BARTs, however, there are several smaller ORFs that may encode for proteins. All of the transcripts contain exon VII, which has a small ORF indicated by blue in Figure 1, that could potentially encode a 174 amino acid protein termed BamHI A rightward frame 0 (BARF0) [10]. This ORF is particularly unusual in that the stop codon is embedded within a repeated polyadenylation signal such that only a subset of the transcripts would encompass the stop codon. A subset of these transcripts that contain alternative splicing in exon V and are directly spliced into the BARF0 ORF in exon VII, which would produce a larger version of this protein (279 amino acids) that has been referred to as RK-BARF0 [11]. An ORF, indicated in green, that spans exons IV and V would encode for a 103 aa protein, which has been called RPMS1 or RK103 [24]. Perhaps most interestingly, a consistent splice pattern that spans exons V, VI, and a distinct spliced form of exon VII results in an ORF indicated in yellow that is named A73 or RB2, which would produce a 126 aa protein [23]. Evidence for and against the production of the proteins from these ORFs will be reviewed in section 3.

2.3. The BART promoter and regulation of expression

Through sequencing of cDNA clones and 5'RACE the major transcriptional initiation site (P1) for the BARTs has been mapped to nucleotide 138357 of the wild type EBV sequence [11,24,25]. An alternative start site (P2) upstream from this has also been identified at position 138067 [25]. These two transcriptional start sites appear to be under differential regulation. The P1 site can be stimulated by binding of Jun family members to the consensus AP-1 site directly upstream and can be suppressed by binding of IRF family members to an IRF site [25]. The P2 site can be stimulated by c-myc and C/EBP family members and there are potential myc and C/EBP binding sites directly upstream of P2 [25]. C/EBP family members may be of significant interest since they are highly expressed in cells of epithelial origin, thus, it has been hypothesized that upregulation of the transcription from the P2 site may be responsible for the high levels of BARTs seen in NPC and other epithelial cells [25].

Many EBV latent promoters are regulated by methylation of CpG dinucleotides, with decreased levels of methylation corresponding with increased levels of transcription [26]. In NPC tumors and cell lines, the promoter region of the BARTs has been shown to be hypomethylated compared to the rest of the genome, consistent with high levels of expression [27,28]. Also, a recent report demonstrated that treatment of EBV-infected B lymphocytes with a DNA methyltransferase inhibitor led to an induction of the BARTs, consistent with a role of DNA methylation in regulating the BART promoter [29].

3. Potential BART-encoded proteins

Several open reading frames exist within the BARTs that have the potential to produce proteins with interesting functions with respect to the development of NPC. However, whether these proteins are actually translated *in vivo* remains a point of controversy. Evidence for and against the relevance of these ORFs is presented below.

3.1. BARF0 and RK-BARF0

All BARTs share the same 3' end, and, therefore, share an ORF named BARF0, which is located in the last exon of each transcript [10]. However, differential splicing can produce an N-terminal extension of this ORF named RK-BARF0 [11]. In both of these ORFs, there is a canonical poly-A addition sequence that occurs just upstream of the stop codon and 3'RACE of these transcripts revealed that a majority terminate before the stop codon [11]. This calls into question whether this ORF is actually translated to produce a protein in infected cells.

Currently, the experiments designed to determine whether the BARF0 or RK-BARF0 gene products are actually made have been inconclusive. *In vitro* translated BARF0 could be immunoprecipitated using serum from NPC patients suggesting these patients produced antibodies directed against the protein [10]. In addition, cytotoxic T lymphocytes from EBV-seropositive patients, but not –seronegative, exhibited a response when stimulated with a BARF0 peptide [30]. However, a BL cell line expressing recombinant BARF0 did not elicit a similar response [30]. A rabbit antibody generated to a peptide in BARF0 that bound the exogenous BARF0 and RK-BARF0 on Western blots revealed evidence of 30 and 35 kD proteins in many EBV-infected cell types [31]; however, these bands were also seen in some EBV negative cell lines, suggesting a lack of specificity for this antibody [32] and subsequent analysis revealed that this antibody also bound to HLA-DR β chain [33]. Affinity purification of this antibody removed this cross reactivity, but the affinity-purified antibody could not consistently detect BARF0 protein in EBV-infected cells by Western blotting [34].

Despite a lack of an ability to clearly demonstrate production of protein directly from the BARTs, exogenous BARF0 and RK-BARF0 do have interesting biological properties. Through both immunofluorescence of FLAG-tagged constructs and direct imaging of GFP-tagged constructs, BARF0 and RK-BARF0 localized to the nucleus of transfected HeLa or BL cell lines [32]. In addition, RK-BARF0 has been shown to interact with Notch family members by yeast two-hybrid as well as immunoprecipitation [35]. Expression of RK-BARF0 sequesters Notch in the nucleus, as well as promotes its proteasome-mediated degradation, preventing its normal maturation and expression as a cell surface receptor and, thus, also preventing its interaction with RBP-Jκ/CBF1 [34,35]. This is a potentially interesting interaction given that the EBV latent protein EBNA2 requires binding RBP-Jκ to activate transcription of genes such as the EBV oncoprotein LMP1 [36,37,38], and in fact, expression of RK-BARF0 leads to an increased level of LMP1 [35].

3.2. RPMS1

The most proximal potential ORF in the BARTs spans the junction between exons IV and V and would code for an 103 aa protein that has been termed RPMS1 (also known as RK103) [11,24]. Given that the most abundant BARTs contain both RPMS1 and BARF0 on the same transcript [23], a reasonable prediction would be translation of RPMS1 would be favored as the proximal transcript on the message. However, detection of native RPMS1 has also not been possible in EBV cell lines and tumors, despite the production of two monoclonal antibodies that recognize the recombinant protein by immunofluorescence and Western blotting [27].

However, similar to BARF0, RPMS1 also has interesting biological properties when produced exogenously in cell lines. Overexpression of RPMS1 in HEK293 cells resulted in increased growth in soft agar and cells that can form tumors in nude mice [39]. Flag-tagged RPMS1 localized to the nucleus in both 293T and HeLa cells [23,40]. RPMS1 has been shown through a variety of assays to bind to RBP-Jκ/CBF1 as well as with members of a repressive histone deacetylase complex that interacts with RBP-Jκ [23,41]. This interaction

has been shown to be able to inhibit RBP-Jκ-dependent Notch or EBNA2 gene activation [23,41]. It is interesting that two potential proteins produced by the BARTs have the ability to downregulate Notch signaling, suggesting that this pathway might be of particular interest in EBV biology. Depending on context, Notch signaling can have both an oncogenic or tumor suppressive phenotype [42], thus, it is not clear at this point how affecting this pathway might contribute to NPC development.

3.3. A73

Alternative splicing within exon VII can produce an additional ORF that spans exons V, VI, and VII that would code for a 126 aa protein that has been named A73, also known as RB2 [11,23]. As with the other potential BART proteins, monoclonal antibodies have been unable to identify the native protein for this ORF in either EBV-infected cell lines or tumors [27]. Unlike BARF0 and RPMS1, exogenously expressed A73 is a cytoplasmic protein. A yeast two-hybrid screen identified interactions with both RACK1 (receptor for activated C kinase 1) and β5-integrin [23]. RACK1 has been shown to regulate release of calcium ions from intracellular stores and expression of A73 in 293 cells resulted in an increase of UTP stimulated calcium release [27]. At this time it is unclear if this or another function of A73 might play a role in the development of NPC.

4. BART-encoded miRNAs

Perhaps the biggest finding with respect to the function of the BARTs was made when the first viral miRNAs were cloned from an EBV-infected B cell line. Five miRNAs were identified as being derived from the EBV genome, including two from the introns of the BARTs [43]. Three of the miRNAs were located near the BHRF1 ORF that encodes a bcl2 homologue. These miRNAs have subsequently been shown to only be present in cells in Type 3 latency that express the primary EBNA transcript that initiates from the Wp or Cp promoters [44]. miRNAs are approximately 22 nucleotide RNAs processed from hairpin structures of larger RNA that function in protein repression through a mechanism that is similar to siRNAs [45]. As the putative protein products for the BART ORFs have not been identified, the finding that the BARTs could serve as a template for miRNAs was a major discovery. This initial study used cells infected with the B95-8 laboratory isolate, an unusual strain of EBV that has a 12 kb deletion that spans many of the 5' noncoding exons of the BARTs [20]. Subsequent studies using cell lines infected with undeleted EBV as well as the use of high throughput sequencing methods, have demonstrated that the BARTs encode for 22 miRNA precursors and because each miRNA hairpin can in principle code for two miRNAs (one from each arm), as many as 44 mature BART miRNAs can be found in EBV-infected cells[44,46,47,48].

4.1. BART miRNA nomenclature and expression patterns

The BART miRNAs have been named ebv-mir-BART1-22 with the numbers corresponding to the order in which they were original discovered. In typical miRNA biogenesis, one strand of the miRNA hairpin is selected to be the mature miRNA, while the other strand is quickly degraded [45]. However, this is not always the case and in some instances, including rather frequently for the BART miRNAs, both strands of the original hairpin persist and can, therefore, be functionally relevant. To distinguish between the two potential miRNAs that can be produced by each hairpin the suffixes -5p and -3p have been used to designate the 5' end of the hairpin and 3' end of the hairpin for cases in which there appears to be significant mature miRNAs produced from each hairpin. In cases where one side of the hairpin is dramatically favored over the other in terms of abundance, the less abundant miRNA is designated with a *, consistent with the nomenclature used for all miRNAs, when referring to the opposite, less abundant strand miRNA [45]. A complete listing of the EBV miRNAs

with both mature and precursor sequences, as well as accepted nomenclature can be found at www.mirbase.org.

A significant amount of effort has been made to determine the expression patterns of the BART miRNAs. This is of interest primarily because the effectiveness of any particular miRNA is necessarily tied to its abundance, as miRNA function is a process dependent upon stoichiometric relationship of miRNA to target mRNA. While it is clear that in general the BART miRNAs are expressed higher in cell lines and tumors in which the BARTs themselves are expressed higher [44], it is also true that the concentrations of each of the individual miRNAs vary by several orders of magnitude from each other.

Two methods have been used to determine the abundance of BART miRNAs in NPC tumors and cell lines. The first is a step loop PCR method used to perform RT PCR from very small RNA templates such as miRNAs [49]. This method has been used to profile the expression levels of the BART miRNAs from both the NPC derived cell line, C666-1, as well as directly from NPC biopsy samples [50,51,52,53]. In addition, relative abundance can be determined by the relative representation in directly sequenced small RNA libraries; and several groups have used both traditional and high throughput methods to sequence miRNAs from NPC cell lines, xenografts, and biopsies [47,48,54]. Estimations can be made from both of these methods with regards to copies of miRNA/cell; either from miRNA abundance in a standard amount of total RNA in the case of PCR, or from the percentage abundance of a particular miRNA with respect to the total pool in the case of sequencing. Both approaches have determined that the individual BART miRNAs vary greatly in abundance in NPC, with the most abundant being present at concentrations in the thousands of copies per cell and the least abundant being expressed at a few copies per cell or less (Figure 2). Comparison of these studies indicates that while some miRNAs appear to be consistently abundant in NPC and others are hardly expressed at all, there is a significant percentage of the BART miRNAs that vary greatly across studies. This variation appears to be due to more than just differences in the samples used, as the profile of the relative abundance of miRNAs present in the C666-1 cell line varies considerably. For example, the relative abundance of miR-BART15 as determined by PCR differs considerably among three studies and is different if determined by sequencing [50,51,54]. Strikingly one of the highest expressing miRNAs in C666 cells by sequencing, miR-BART10 [54], only showed modest expression in the same cells by RT-PCR [51]; whereas miR-BART19-3p, which appeared to be highly abundant in PCR studies of cells or tumors [51,53], only appeared at low levels or was not detected at all in sequencing studies [47,48,54]. In some instances, different studies yield opposite results with respect to which strand of a miRNA is the dominant form. For example, in the two PCR studies that have examined both miR-BART2-5p and -3p, one found the -5p version to be several order of magnitude higher in abundance while the other study found the opposite [51,53]; despite this neither appears in very high abundance in the sequencing studies [47,48,54]. It is true that each method of estimating miRNA abundance comes with its own caveats. The step loop PCR method relies on precise knowledge to the 3' end of the miRNA, which in the case of the BARTs has been shown to be variable throughout the population [48]. Additionally, sequence-specific biases are also introduced by the RNA ligation step used to generate small RNA libraries for sequencing [55].

The reason for the apparent differences in abundance of the individual BART miRNA within a single infected cell is not known. Theoretically, such differences in steady state levels could be due to differences in stability, differences in precursor transcription, or differences in the processing to produce the mature miRNA. Relative stability of the various BART miRNAs has not been evaluated to this date, so the extent to which that plays a role in steady state abundance is not clear. It would seem unlikely that differential rates of transcription could account for the different relative abundances as the miRNAs are tightly

clustered in the BART introns, and should in theory all be transcribed together. The differential splicing that occurs in the transcripts could possibly account for some of the differences if specific splicing events favor or inhibit the excision of the miRNA hairpins based on their location. In support of this possibility cell lines that accumulate higher levels of the BART miRNAs also favor a version of the BART mRNA in which exon I is spliced directly to exon III [22]. This is true despite the fact that the miRNAs appear to be processed out of the initial transcript prior to completion of the splicing reaction, as stable RNAs resulting from miRNA excision span the exon/intron boundaries [22]. It is presently unknown whether the final spliced mRNAs are a product of splicing that occurs after the excision of the miRNAs or they are made from a pool of transcripts that are distinct from those that are the template for miRNA production. Further experimentation into the role splicing plays in the production of the miRNAs will be necessary to clarify this issue. Another possibility to explain the different levels of the BART miRNAs would be if each miRNA is recognized with a different affinity for the enzyme responsible for the excision of the hairpin, the Drosha/DGCR8 microprocessor complex [56]. This is another hypothesis that has not been fully explored, but it is worth noting that the stable BART RNA fragments that can be isolated from nuclei of EBV infected cell lines often have particular miRNAs excised, while others have not been processed out, suggesting there is some order or bias to the processing by the microprocessor complex [22].

4.2. Viral targets for BART miRNAs

In order to fully understand the potential role for the BART miRNAs in the development of NPC, it is necessary to determine the targets of the miRNA-mediated downregulation. In principle the targets could be other viral transcripts or transcripts from the host cell. Targeting viral transcripts may be an important way to maintain latency by targeting of lytic transcripts or to avoid immune detection through maintaining low levels of other latent gene products. Perhaps the most straightforward function for a BART miRNA is in the case of miR-BART2. miR-BART2 is located apart from the two clusters of miRNAs further upstream and is directly antisense to the 3'UTR of BALF5, the viral DNA polymerase [43]. Given the obvious perfect complementarity of miR-BART2 to the BALF5 transcript, it is not surprising that expression of the miRNA was shown to downregulate BALF5 protein expression, through a mechanism that involves cleavage of the BALF5 mRNA at the site of miR-BART2 binding [57]. There is also evidence that other BART miRNAs can regulate the latent transcripts of EBV that are expressed in NPC. Several BART miRNAs from cluster I have been shown to be able to target the 3'UTR of LMP1 and result in downregulation of the protein [58]. In addition, a cluster II miRNA, miR-BART22, has been shown to downregulate LMP2 at the protein level [54]. In all of these cases, the consequences of miRNA action for the viral life cycle or for cancer progression have not been fully explored. It will be interesting to determine whether any of these potential functions for BART miRNAs contribute to EBV oncogenesis *in vivo*.

4.3. Cellular targets for BART miRNAs

For a majority of the BART miRNAs, a clear viral transcript target is not apparent. The most likely function of these miRNAs would be downregulating transcripts from the host cell. In the past few years, some progress has been made in identifying such targets. Excitingly, many of the targets identified at this point have roles as tumor suppressors, further highlighting the potential important role the BART miRNAs may play in the development of NPC.

Several of the cellular targets identified to this date have roles in promoting apoptosis and, thus, the miRNAs that target them have anti-apoptotic affects. miR-BART5 was predicted to target the BH3-only protein PUMA, which helps mediate cytochrome c release from the

mitochondria in response to apoptotic stimuli, through complementary with the 3'UTR of the PUMA mRNA [59]. Downregulation of PUMA by miR-BART5 was confirmed using 3'UTR reporter assays as well as Western blots . Another BH3-only protein, Bim, was identified by microarray analysis looking for transcripts that decreased in abundance in cells expressing the BART miRNAs [60]. Bim was also been shown to be targeted by multiple BART miRNAs from cluster I through reporter assays and Western blots. Interestingly, Bim is a tumor suppressor that is targeted by a number of oncogenic cellular miRNAs in other forms of cancer [61,62,63]. In addition, in a study looking for EBV miRNA targets by immunoprecipitating the RNA-induced silencing complex (RISC), TOMM22 was identified as a potential target for miR-BART16 [64]. TOMM22 is a part of a mitochondrial pore complex that serves as a receptor for the proapoptotic protein Bax; knockdown of TOMM22 by siRNA has been shown to be anti-apoptotic [65]. Targeting of TOMM22 was confirmed by the 3'UTR reporter assay, although direct regulation of protein levels was not assessed [64].

In addition to genes involved in apoptosis, other tumor suppressor genes have also been implicated as targets for the BART miRNAs. Multiple tumor suppressors that target the Wnt signaling pathway were shown to be downregulated by Western blots in cells expressing the appropriate miRNAs, including WIF1 (miR-BART19-3p) and APC (miR-BART7, 19-3p, and 17-5p) [53]. Other targets for BART miRNAs have also been identified in which the role in NPC would be less clear including IPO7 for miR-BART3 [64], Dicer for miR-BART6-5p [66], and MICB for miR-BART2-5p [67]. As each individual miRNA could target multiple genes and pathways at the same time, it is likely that many more cellular targets will be affected by the BART miRNAs.

4.4. BART miRNAs in the serum

In addition to being functionally relevant in the tumor cells they are produced in, tumor-associated miRNAs can also be secreted from cells and are present in high copies in the serum [68,69,70]. This is also true of the BART miRNAs, which are present in high concentrations in the serum of NPC patients [53,71]. It is possible that the detection of BART miRNAs in the serum of NPC patients might be used as a biomarker as tumor specific cellular RNAs have proven to be useful in other types of cancer [72]. Given that miRNAs in the serum are in a cell-free compartment that is highly stable and resistant to RNAse [70], and the fact that miRNAs have been shown to be secreted from cells in small vesicles known as exosomes [73], the current hypothesis is that most if not all of serum miRNAs are contained within exosomes. The BART miRNAs have been shown to be packaged into exosomes from both NPC xenografts and cell lines[71,74]. Interestingly, the BART miRNAs can be transferred to recipient uninfected cells, and the transferred miRNAs remain functional in downregulating protein expression [74,75]. This opens up the possibility that the BART miRNAs may affect not only the EBV-infected cells of a tumor, but the surrounding uninfected cells as well.

5. Conclusions and Future Directions

The EBV BARTs are a highly expressed and complicated set of transcripts that encode for a large number of miRNAs, as well as potentially interesting proteins in NPC. Considering the high levels of BART miRNAs that are detected in clinical samples and the lack of evidence of BART protein production, it seems likely that the major role for these transcripts is the production of miRNAs. It is not clear, however, if there is a situation in which the ORFs are also translated. It is presently unknown whether one transcript can be processed for miRNA production and also translated. A study of the BHRF1 miRNA production and effects on BHRF1 mRNA and protein synthesis indicated that Drosha inhibition did not affect the abundance or stability of the unspliced BHRF1 mRNA and suggested that the mRNAs might

be produced from a distinct template [76]. It would be interesting to know if inhibiting miRNA production by knocking down Drosha for example would enable translation of the downstream ORFs. There appears to be a correlation between different splicing patterns and the amount of miRNAs produced, which leads to the interesting hypothesis that production of the miRNAs may influence the structure of the transcripts themselves and perhaps then even the translation of potential ORFs. It could also be that forcing different splicing patterns would result in a different balance of miRNAs/proteins produced as end products. A better understanding of the mechanisms regulating the maturation of the BARTs might identify factors that favor protein production over miRNA generation.

Many of the potential cellular gene products targeted by the BART miRNAs have important functions and several possibly modulate apoptosis. The determination of whether the BART miRNAs are necessary or sufficient for the growth properties characteristic of NPC will be difficult. In addition to the difficulty in transforming epithelial cells by EBV infection, the large number of BART miRNAs suggests the possibility that they may work in concert. Thus, standard antisense inhibition experiments to determine if they are required for growth or maintenance of tumors will be daunting. The identification of the expression patterns and the functional capabilities of each individual miRNA will help in selecting candidate miRNAs that possibly function as oncomirs. Despite the considerable effort in profiling BART miRNA abundance, there are still large disparities in the various data sets. While some of these disparities may be due to various biases in the techniques used, it is probably also true that there are more complexities that have yet to be considered. For example, the stability of each miRNA is completely unknown at this point, as is what factors might affect that stability.

Considering the highly restricted patterns of viral protein expression in most of the tumors associated with EBV, the abundance of the BART miRNAs suggests that they are likely to be a major factor in the contribution of EBV to oncogenesis and in particular to NPC. Although the overall biologic effects of these miRNAs are presently unknown, it is likely that BART miRNA directed therapies will be developed as their molecular properties are experimentally defined and their potential contribution to the pathogenesis of NPC is clarified.

Acknowledgments

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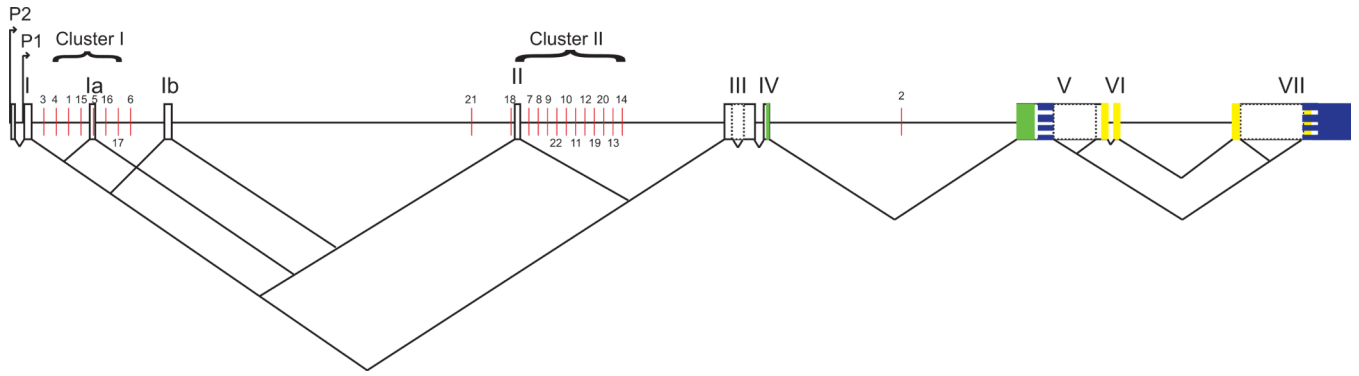
Figure 1. Diagram of the BART transcripts

A diagram of the 22 kD BART locus drawn to scale. The exons are indicated as boxes and are labeled with Roman numerals. The introns are indicated as lines between the boxes. The positions of the potential open reading frames are indicated by color boxes. BARF0 is shaded in blue, with the RK-BARF0 extension indicated by stripes. RPMS1 is indicated by the green box and A73 with yellow. The position of the BART miRNAs are indicated by red lines, and each are numbered according to the accepted nomenclature. The diagonal lines below the transcript indicate the various splicing events that have been observed.

Figure 2. Expression of the BART miRNAs from RT-PCR and small RNA sequencing studies in NPC

A table comparing the available expression data for the BART miRNAs in NPC tumors and the NPC cell line, C666. For each study, the expression levels of each miRNA were broken into four categories, high (green), moderate (yellow), low (blue), or very low (purple). The white boxes on the PCR half of the table indicate a miRNA that wasn't evaluated in that study. The median values of each category differ by an order of magnitude. For the PCR studies the green miRNAs indicate those expressed at approximately 10,000 copies/10μg RNA, yellow ~1,000 copies, blue ~100 copies, and purple ~10 copies. For the sequencing

studies, green indicates miRNAs that appear at a frequency of approximately 10% of the total pool of miRNAs, yellow ~1%, blue ~0.1%, and purple ~0.01% to undetected.