Cellular oncomiR orthologue in EBV oncogenesis

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A B S T R A C T

MicroRNAs are small non-coding RNAs that regulate gene expression at multiple levels. The discovery of virally encoded miRNAs attracted immense attention towards their role in viral replication and pathogenesis. Kaposi’s-sarcoma-associated herpes virus encodes miRNA that functions as an orthologue of human cellular miRNA, i.e., hsa-miR-155. Keeping the same view we extended the miRNA-homology search between the miRNAs of humans and Epstein–Barr virus. The In silico analyses show that EBV encoded miR-BART-5 has a significant ‘seed’ sequence homology to hsa-miR-18 of humans. Further, the miRNA transcripts of the human genes involved in cellular growth could potentially be targeted by both viral as well as human miRNAs. The known etiological role of hsa-miR-18 as an oncomiR suggests that miR-BART-5 may function as viral oncomiR as observed in EBV-positive gastric carcinoma patients.

1. Introduction

MicroRNAs are small non-coding functional RNAs of 21–25 nucleotides. These molecules mediate regulatory roles at both the posttranscriptional and transcriptional level. According to the degree of complementarity with their target in the 3’ untranslated region (3’UTR), they inhibit translation of mRNA to proteins or initiate their degradation and may also result in gene silencing through RNA-directed DNA methylation (Chromatin Remodeling) [1]. It is known that miRNAs play a role in the regulation of genes involved in diverse processes such as development, differentiation, apoptosis and proliferation [2]. The miRNA alterations are also reported to be involved in the initiation and progression of human cancer. The miRNAs deemed to play a crucial role in the initiation and progression of human cancer, and those with a role in cancer are designated as oncogenic miRNAs (oncomiRs). Cellular miRNA genes have been identified that might represent downstream targets of activated oncogenic pathways, or that target protein coding genes involved in cancer [3]. The participation of several oncomiRs in tumorigenesis has been proved [4] (Table 1). Deregulation of oncomiRs is associated with genetic or epigenetic alterations, including deletion, amplification, point mutation and aberrant DNA methylation [3]. The discovery of vmiRNAs, especially from a family of oncogenic herpes viruses, has raised the intriguing possibility of their role as critical modulators of viral oncogenesis. The advantages of a viral miRNA-based mechanism are multiple, as these miRNA molecules are small, non-immunogenic, and specific [5]. It is therefore not surprising that DNA viruses have been found to express several viral miRNAs [6]. Also from an evolutionary point of view, it is simpler to develop a regulatory antisense molecule rather than a regulatory protein. Lastly, the combination of a protein-mediated and miRNA-mediated posttranscriptional regulation provides a tighter evasion strategy, which is more resistant to the host immune response.

Herpes viruses can be classified into three subfamilies (α, β, or γ) based on the sequence relatedness and virus biology. Till date, the microRNA Registry [7] contains miRNA sequences from only three members of the γ (lymphotropic) subfamily – EBV and KSHV of humans and MHV68 of mice – and one from β-herpesvirus subfamily (HCMV or HHV5). EBV, causative agent of infectious mononucleosis and etiologically related to several types of malignancies like Burkitt’s lymphoma and gastric carcinoma (GC) [8], was the first virus known to encode miRNAs [9]. It expresses 39 different miRNAs [10,11] located in two clusters of the viral genome, near genes BART and BHRF, which are known to be activated during latent phase of the viral infection (Fig. 1a). As EBV encoded miRNAs reported to be differentially expressed in EBV-positive gastric carcinoma patients. Authors’s personal copy © 2011 Elsevier Ltd. All rights reserved.
as an orthologue of human cellular miR-155, a well known oncomiR [16–21]. They reported that KSHV miR-K12-11 shows significant homology to cellular miR-155, including the entire miRNA ‘seed’ region and the expression of physiological levels of miR-K12-11 or miR-155 results in the downregulation of an extensive set of common mRNA targets. This finding strengthens the idea that viral orthologue of cellular oncomiRs likely to play an important role in virus induced tumorigenesis and represents the viral evolution to exploit pre-existing gene regulatory pathway in host cells. To find whether a similar strategy might also be employed by EBV miRNAs, we have analyzed putative EBV encoded oncomiR orthologues in Humans.

2. Materials and methods

2.1. MicroRNA homology search

The sequences of 39 different miRNAs of Epstein Barr virus (EBV) from BART and BHRF miRNA clusters were extracted from miRBase...
7.0 (http://microrna.sanger.ac.uk/) [7] using accession numbers M0004988–M0004993, M0003725–M0003736 and M0001064–M0001068. The human oncomiRs (miR-17/92 cluster, BIC/miR-155, miR-21, miR-372, miR-373, miR-221 and miR-222) were obtained from miRBase 7.0 using accession numbers, M0000071 to M0000074, M0000097, M0000093, M0000681, M0000780, M0000781, M0000298 and M0000299, respectively. Both the above groups of sequences were compared with each other using EMBOSS pairwise alignment algorithms (http://www.ebi.ac.uk/emboss/align/). Since, the sequences in the query set are reasonably short and roughly of equal size (22–25 bp), we used Needleman–Wunsch algorithm [22]. The gap open penalty of 10.0 and gap extension penalty of 0.5 has been used for aligning query sequences over their entire length to find the best-matching scores and identities. Primary miRNA stemloop precursors for ebv-mir-BART5 (M0003727), hsa-miR-18a (M0000072) and hsa-miR-18b (M0001518) were similarly aligned for pairwise sequence comparison and analyzed for high-quality sequence differences. The hsa-miR-18a & 18b are found to be on mir-17–92 polycistronic miRNA cluster (Fig. 1b). To generate alignment figures, T-coffee (Combine: Regular), a multiple sequence alignment package program [23] from Swiss Institute of Bioinformatics server (http://tcoffee.vital-it.ch/cgi-bin/Tcoffee/tcoffee_cgi/index.cgi) was used. Sequence conservation between two sequences of equal length is calculated by total number of identical nucleotides at identical positions divided by total number of nucleotides compared in two sequences, multiplied by 100.

2.2. Computational prediction of human gene targets for Epstein Barr virus (EBV) encoded mir-BARTS

To identify the common targets of orthologous miRNA pair, available online resources for miRNA target predictions [24], such as TargetScan (http://genes.mit.edu/targetscan) [25]—one of the most successful programs that consider cross-species conservation and thermodynamic stability was used. As miRNAs mediate post-transcriptional regulation of gene expression by binding to the 3’UTR regions of the transcripts, a further exhaustive target finding search for ebv-mir-BART5 over the 3’UTR of all mRNA found as targets of hsa-miR-18, was performed through batch request submission to the RNA22 server (http://cbcsrv.watson.ibm.com/rna22.html) of the bioinformatics and pattern discovery group at IBM research [26]. For this, human annotated 3’UTR sequences were retrieved from BìGRe-ULB browser, using regulatory sequence analysis tools (http://rsat.ulb.ac.be/rsat/). RNA22 miRNA target detection does not rely upon cross-species conservation, and it first finds putative miRNA binding sites in the sequence of interest, then identifies the targeting miRNA. This strategy was applied to avoid the high number of false positives and the low overlapping between results set that

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<table>
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Fig. 2. Sequence comparison of mature EBV encoded viral miRNAs against cellular oncomiRs. Only those miRNAs sequence comparison have been shown that produces a score and identity above threshold value of 40% and 50%. Respectively, in EMBOSS. Scores shown generated from T-coffee alignment program.
computational methods usually yield. JAVA was installed on the system to run batch queries i.e., many miRNAs against candidate target sequences. File "batchRNA22.class" was also downloaded from RNA22 server. Three notepad files, microRNAs.txt (with the EBV encoded miRNAs listed one after another in FASTA format), targets.txt (with the potential 3’UTR target sequences in FASTA format) and parameters.txt (with four default parameters that RNA22 uses) were deposited in the same directory where batchRNA22.class was saved. To initiate batch call following command: java batchRNA22 microRNAs.txt targets.txt parameters.txt was run on a DOS prompt (WINDOWS) in the directory that contains 3 notepad files. Output HTML files were locally saved and were analyzed for results. RNA22 miRNA target detection web interface (http://cbcsrv.watson.ibm.com/ rna22_targets.html) was also used to detect target sites of ebv-mir-BART5 in complete NR3C1 gene (sequence retrieved from NCBI gene database using accession no. NM_001018077, GeneID: 2908).

2.3. Annotation of target genes

A functional analysis of the target genes was also done using the gene ontology annotation (http://www.geneontology.org/) present in GenBank records. Additional information regarding gene functions was obtained from Entrez Gene (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene), a searchable database of genes from RefSeq genomes.

Fig. 3. Sequence comparison of the primary miRNA stem-loop structures of ebv-mir-BART5, hsa-miR-18a and hsa-miR-18b (a and b, respectively). The stem-loop shown is the cellular miR-18 sequence, with the changes observed in ebv-mir-BART5 indicated. The mature miRNA sequences are shown in red. A “+” sign indicates an insertion. (A) Alignment figure generated from T-coffee alignment program. Sequence conservation is more prominent in mature microRNA sequence. (B) Alignment figure generated from T-coffee alignment program. Sequence conservation is more prominent in mature microRNA sequence. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
3. Results and discussion

3.1. EBV encoded miRNAs share sequence similarity with human cellular oncomiRs

Sequence homology search results of EBV encoded miRNAs against human oncomiRs are displayed in Fig. 2. Here, mostly the BART region encoded miRNAs show sequence similarity to the human oncomiRs tested. The small number of miRNA sequence changes observed are either at the very 5' end or present at the 3' end, which are known to contribute minimally to target mRNA recognition [27]. Of particular interest, the ebv-mir-BART3 shows significant homology against human cellular hsa-miR-18a and hsa-miR-18b, including the entire seed region that is often very critical for mRNA target recognition [28] (position 2–7 from the 5' end). The unusual one nucleotide insertion seen in ebv-mir-BART5 may imply that the miRNA targets a non-coding region, such as miRNAs 3' UTR, that can readily tolerate a 1-nt deletion or insertion.

3.2. Are the ebv-mir-BART5 and hsa-miR-18 orthologs?

In Fig. 3A and B, the primary miRNA stem-loop precursors for ebv-mir-BART5, hsa-miR-18a and hsa-miR-18b have been compared. The results shows that the ebv-mir-BART5 and hsa-miR-18a stem-loop precursors are approximately 45% conserved, the basal stems are 20%, while the terminal loops are approximately 35% conserved. Similarly, the ebv-mir-BART5 and hsa-miR-18b stem-loop precursors are approximately 46% conserved, while basal stems are 29% and the terminal loops are 13% conserved. If mature cellular miRNA, particularly seed sequence is functionally important, it is predicted that the flanking basal stem and the terminal loop should show significantly more sequence variation than the mature miRNA sequences. As it is observed there is indeed better conservation of the mature miRNA sequence when compared to the flanking basal stem and the terminal loop. More than 45% of residues in the vmiRNA precursor and human miRNA precursor are identical suggesting significant homology between them, although the non-seed regions of has-miR-18 and ebv-mir-BART5 are different, raising the possibility that they might not share all mRNA targets [29]. As these sequences have originated due to speciation event in two different organisms, it gives an understanding that these are orthologous in nature.

3.3. Are the EBV encoded miRNAs involved in virus survival and viral induced oncogenesis?

hsa-miR-18 (miR-17–92 polycistron) (Fig. 1b) is located in a region that is reported to be amplified in human B-cell lymphomas stem-loop precursors are approximately 45% conserved, the basal stems are 20%, while the terminal loops are approximately 35% conserved. Similarly, the ebv-mir-BART5 and hsa-miR-18b stem-loop precursors are approximately 46% conserved, while basal stems are 29% and the terminal loops are 13% conserved. If mature cellular miRNA, particularly seed sequence is functionally important, it is predicted that the flanking basal stem and the terminal loop should show significantly more sequence variation than the mature miRNA sequences. As it is observed there is indeed better conservation of the mature miRNA sequence when compared to the flanking basal stem and the terminal loop. More than 45% of residues in the vmiRNA precursor and human miRNA precursor are identical suggesting significant homology between them, although the non-seed regions of has-miR-18 and ebv-mir-BART5 are different, raising the possibility that they might not share all mRNA targets [29]. As these sequences have originated due to speciation event in two different organisms, it gives an understanding that these are orthologous in nature.

hsa-miR-18 (miR-17–92 polycistron) (Fig. 1b) is located in a region that is reported to be amplified in human B-cell lymphomas
and its enforced expression is known to accelerate c-myc induced lymphomagenesis [31]. The in silico search for the putative human gene targets for ebv-mir-BART5, was performed as shown in Fig. 4a. Since, ebv-mir-BART5 shows the conserved seed sequence and sequence homology with human hsa-miR-18, its functionality may be retained as evident by the above results. The analogous set of predicted targets in this study includes the genes with roles ranging from apoptosis to cell signaling. Among them three predicted targets, i.e., NR3C1 (nuclear receptor subfamily 3, group C, member 1), UBE2Z (ubiquitin-conjugating enzyme E2Z) and GAB2 (GRB2-associated binding protein) have attracted our attention.

Translational Repression of Glucocorticoid Receptor (NR3C1) by miRNA (both hsa-miR-18a and ebv-miR-BART5 binds at the same site) binding in 3’UTR is shown in Fig. 4b. This supports the view that both hsa-mir-18a and ebv-mir-BART5 share a set of common mRNA targets that are also translationally repressed by hsa-miR-18. The NR3C1 is a glucocorticoid receptor that can act both as a transcription factor as well as regulator of other transcription factors. This receptor is also reported to show a novel methylation pattern that can be further used as a cancer specific marker in colorectal carcinomas [32]. Similarly in EBVaGC also hypermethylation as discussed in epigenome modification below.

The second target predicted is UBE2Z or FLJ13855. It can be a highly potent target for ebv-mir-BART5 because it is one of the identified programmed cell death gene with proapoptotic activity [33]. Hence, by negatively regulating its expression, ebv-mir-BART5 might influence the cell death in the mammalian host cell and promotes cancer development.

The third predicted target GAB2, is reported in promoting mammary tumor metastasis [34]. Mounting evidence also indicates that overexpression of GAB2 in gastric adenocarcinomas may play a crucial role in advanced gastric cancer development as compared with early gastric carcinomas [35]. The recent findings suggest that although GAB2 overexpression may confer growth advantage to tumor cells, the functional requirement for GAB2 in mammary tumor initiation/growth can be dispensable. This may be attributed to the differential expression of ebv-mir-BART5.

![Figure 5](attachment:image.png)

Fig. 5. (a) Regions of partial sequence complementarity between NR3C1 promoter region, ebv-mir-BART5 (red) and hsa-miR-18 (blue) is shown. Sequence alignment is generated from NR3C1 and Reverse complementary ebv-mir-BART5 and hsa-miR-18 sequences in FASTA format using CLUSTALW accessible at http://align.genome.jp.
(b) Predicted targets of ebv-mir-BART5 and hsa-miR-18 in NR3C1 promoter region are shown. Images are generated from RNA22 HTML output files. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
The earlier computational studies revealed that a miRNA might bind to as many as 200 gene targets and that these targets can be diverse in their function; they include transcription factors, secreted factors, receptors and transporters [36–41]. The present study also infers that a single miRNA can target multiple mRNAs involved in physiologically significant cellular processes. It is therefore assumed that the differential expression of ebv-mir-BART5 [12,42] might regulate the expression of host genes during the early phase of EBV-induced epithelial cell transformation. The possible oncogenic potential of ebv-mir-BART5 in tumorigenesis has also been supported by recent findings in EBVaGC [42].

#### 3.4. ebv-mir-BART5 may augment tumorigenesis via epigenome modification

Epigenetic disruption of key genes occurs at the earliest stage of cancer development [43] as seen in gastric carcinoma (GC) that shows high frequency of aberrant CpG island hypermethylation. Probably EBV may also exploit the same epigenetic mechanisms [44] such as increased DNA methylation of cellular genes to downregulate their expression. Interestingly, cancer specific methylation of novel NR3C1 marker is reportedly more in EBV-positive GC than in EBV-negative GC [45]. Recent work suggests that miRNAs are able to guide heterochromatin formation at promoters that undergo overlapping transcription and possess sequence complementarity to miRNA seed region [46]. NR3C1 is known to use at least 3 different promoters, alternate splicing, and alternate translation initiation sites resulting in several transcripts encoding the same protein or different isoforms. Fig. 5 shows the result that both ebv-mir-BART5 and hsa-mir-18a has sequence complementarity (Fig. 5a) and target sites (Fig. 5b) in the NR3C1 promoter region. Thus by acting as molecular beacon for targeting chromatin-modifying complexes to specific chromosome regions, ebv-mir-BART5 and hsa-mir-18 may also play a crucial role in chromatin-dependent gene silencing.

#### 4. Summary

The viral miRNAs can function as orthologs of human cellular miRNAs, thereby downregulating the expression of numerous host (human) genes that encode particularly “troublesome” host defense factors. The present study supports this concept and further agrees with earlier findings on the critical role of seed pairing in miRNA target selection. Our in silico search analysis reveal that EBV encoded miRNA i.e., mirBART5 shows significant sequence homology to the cellular human oncomiRs i.e., hsa-miR-18 (mir-17–92 cluster). Further, the results for the putative miRNA targets (NR3C1, UBE2Z and GAB2) for both the viral and human miRNAs are found to be the same. In the case of Cancers where both the hsa-mir-18 and ebv-mir-BART5 are involved DNA hypermethylation were reported. This gives an understanding that both the miRNAs not only share the common miRNA targets but also participate in epigenetic changes for tumorigenesis. Though, the exact role of ebv-mir-BART-5 is yet to be established in vitro experimentally, with the help of this in silico results we can speculate both these miRNAs are orthogonal in nature, hence they are found to share common mRNA targets and cause tumorigenesis through epigenome modifications. Further studies are in progress for in vitro experimentation to validate the above predicted results, if the in vitro experiments verify the above hypothesis; ebv-mir-BART5 action can explain the observed gene expression changes how the EBV infection cause carcinogenesis.

### Conflict of interest statement

None declared.

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### References


