Prediction of MicroRNAs in the Epstein–Barr Virus Reveals Potential Targets for the Viral Self-Regulation

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Abstract Studies involving miRNAs have opened discussions about their broad participation in viral infections. Regarding the *Human gammaherpesvirus 4* or Epstein–Barr virus (EBV), miRNAs are important regulators of viral and cellular gene expression during the infectious process, promoting viral persistence and, in some cases, oncogenic processes. We identified 55 miRNAs of EBV type 2 and inferred the viral mRNA target to self-regulate. This data indicate that gene self-repression is an important strategy for maintenance of the viral latent phase. In addition, a protein network was constructed to establish essential proteins in the self-regulation process. We found ten proteins that work as hubs, highlighting BTRF1 and BSRF1 as the most important proteins in the network. These results open a new way to understand the infection by EBV type 2, where viral genes can be targeted for avoiding oncogenic processes, as well as new therapies to suppress and combat the persistent viral infection.

Keywords Epstein–Barr virus · miRNA s · Self-regulation

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Introduction

The *Human gammaherpesvirus 4*, namely Epstein–Barr virus (EBV), is a species member of the *Herpesviridae* family, *Gammaherpesvirinae* subfamily and *Lymphocryptovirus* genus [1]. It infects about 90% of the human population [2], burdening the host's immune system with a life-long persistent latency. The EBV is involved in the infectious mononucleosis disease and is associated to 1.5% of registered cases of cancer worldwide [3], including nasopharyngeal carcinoma, Hodgkin's lymphoma and Burkitt's lymphoma [4]. EBVs are classified into EBV type 1 and EBV type 2, distinguished based on nuclear antigen sequences (EBNAs) [5]. Studies demonstrate that EBV type 2 frequently infects HIV-positive patients [6].

During infection, some viruses have developed strategies, such as the use of microRNAs (miRNA s), to escape the immune system of host cell [7]. The miRNAs are small molecules of non-coding RNAs that regulate gene expression through post-transcriptional repression, by pairing with the seed region of the target mRNA [8]. Viral miRNAs of EBV were identified in 2004 [9], and have been described as important regulators of viral and host genes expression, facilitating the viral latency and, in many cases, even participating of the oncogenic process [10].

Focusing on the self-regulation of the EBV type 2, we sought to predict de novo viral miRNAs and their relevant interactions with viral mRNAs, their natural targets. In addition, we aimed to construct an interaction network of the respective targeted viral proteins.

Materials and Methods

De Novo Prediction of miRNA s

Genomic and gene nucleotide sequences of the EBV type 2 (NC_009334) were extracted from GenBank database. The EBV type 2 strain AG876, has a 172,764 bp genome length, with 82 genes and a GC content of 59.5% [5]. We used the program StructRNAfinder [11] to predict the miRNAs present in the EBV type 2 genome, along with their possible RNA secondary structures. We considered the command line *cmsearch* to compare the miRNA covariance models available in the Rfam database [12], with the virus genomic sequences. It is expected from the methodology to report detections with the smallest e-values with a default cutoff of 0.01. Secondary structure identification and taxonomy of miRNA s, were predicted using the tools Infernal [13], RNAfold [14] and Krona [15], respectively through the automatic default calculations of the StructRNAfinder programs suite.

Prediction of Targeted mRNAs and Essential Compounds in the Protein Network

The command line IntaRNA 2.0 program [16] with default options was used to predict possible target sites within all coding gene sequences of the EBV type 2 genome, represented as mRNAs. Parameters such as the minimum free energy (MFE) and the accessibility of the interaction region were used for retrieving most relevant interactions. Based on a statistical test of boxplot and outliers analyses (not shown), we considered relevant the interactions with a $MFE < -26$ kcal/mol. We used the Virus Mentha database to identify the essential nodes of the protein network involved with miRNA target genes [17]. The data obtained was visualized using Cytoscape (version 3.5.1) [18] for topological analysis of the network.

Results and Discussion

In the search for the miRNAs available in the EBV type 2 genome through StructRNAfinder, we identified a total of 55 miRNA candidates with a cutoff e-value < 0.01 (Supplemental Information Table S3). The potent bioinformatic approach detected in a single run, 28 pre-miRNA candidates from the EBV BART cluster. 25 out of total BART candidates detected were positively annotated as miR-BART, the remaining three belong to the BART cluster region but the annotation process did not resolve as such. Of the total 28 pre-miRNA BART detections, 12 are unique and 16 are possible isomiRs demonstrating the

sensitivity of the detection methodology. Additionally, 6 annotated miR-BHRF candidates were detected. Also, 19 new pre-miRNAs were detected: 4 upstream of the BART region, 3 between the BHRF and the BART regions, and 12 downstream of the BHRF region.

Those pre-miRNAs had a range size between 46 and 150 nucleotides, with an average length of 98 nucleotides. This large size is due to the methodology used, in which pre-miRNA were actually searched, this means identifying all nucleotides incorporated into the relevant secondary structure of every particular miRNA [11]. Of all de novo pre-miRNAs predicted, 37% were identified as eukarya-only, 25% were identified as *Gammaherpesvirinae* sub-family exclusive, and a strikingly 38% as eukarya-virus shared. The eukarya-virus shared and the virus-only predictions adds to a 63% of de novo pre-miRNAs suspected of autoregulation. The miRNAs identified had homology in the secondary structure co-variance models, with 25 families of miRNA s, and had a wide distribution between Eukarya and viruses (Fig. 1). This ample throughput bioinformatic methodology contrasts in efficiency with comprehensive experimental strategies [19, 20] for EBV miRNA detection in the sense of having successfully detected previous experimental reported miRNAs. On the other hand, is reported that close to 50% of experimental detections are isomiRNAs [21], therefore rendering our strategy as efficient and cost-effective in terms of miRNA detection.

A miRNA detected with known function, the mir-BART5 (Rfam acc. RF00867), was found to be exclusive to EBV type 2 (Table 1). It has as target the mRNA for the pro-apoptotic protein PUMA, a protein related to the

Fig. 1 Abundance and diversity of Rfam miRNA Families detected and identified at the EBV type 2 genome. Three categories are depicted: miRNA Families regulating eukarya-only (pink bars); virus-only miRNA Families (blue bars); shared eukarya & virus miRNA Families (green bars) (color figure online)

Table 1 Most relevant EBV type 2 predicted miRs detected, matching with Rfam miRNA database

miRNA	Taxonomy	Target	Targets's function
mir-BART5	Virus (EBV)	Host's pro-apoptotic PUMA	Apoptotic resistance associated with nasopharyngeal carcinoma
mir-BART2	Eukarya & vir	Viral DNA pol BALF5	Inhibition of the replication function of BALF5
mir-BART3	Eukarya & vir	Host's tumour supressor DICE1	Repression of DICE1

apoptotic resistance in cells associated with nasopharyngeal carcinoma [22]. The mir-BART5 can be important to the maintenance of the viral latency cycle, promoting the persistence of the carcinoma process. Among the detected miRNAs common to eukarya and viruses, an interesting case is the mir-BART2 (RF00364), involved at inhibition of the viral DNA polymerase BALF5 through degradation of the viral polymerase mRNA [23], inhibiting inadvertently induced replication. The presence of this miRNA in the viral genome can be involved in the viral self-regulation during the viral infection. Another detection was the mir-BART3, also an eukarya-virus shared miRNA. It is involved in the repression of the tumor suppressor protein DICE1, which promotes the cell growth and in some cases leads to the development of tumors [24]. We suggest that the inhibition of a proper function of the host immunologic system is an active viral latency strategy, rendering as byproduct a further oncologic process.

Viral Self-Regulation

To estimate the potential self-regulation of EBV type 2 through miRNA s, we analyzed the interactions by complementarity and minimum energy analysis between the previously de novo predicted miRNAs and the mRNAs of the EBV type 2 genome. A Minimal Free Energy (MFE) value between -26.31 and -195.1 kcal/mol was accepted as a favorable interaction. In Fig. 2, we have depicted the full abundance of predicted miRNAs interactions targeting the viral EBV type 2 mRNAs diversity. All mRNAs that presented a potential to be self-regulated by the predicted miRNAs in the viral genome, as well as their respective functional annotations, are provided in Supplementary Information Table S1.

The mRNAs with the lowest energy miRNAs interactions were EBNA1, BFRF2, BILF2, BOLF1, BALF5 and BRRF2 mRNAs, with minimum free energy values of -114.6 kcal/mol, -41.99 kcal/mol, -152.1 kcal/mol, -49.16 kcal/mol, -37.33 kcal/mol and -45.31 kcal/mol, respectively (Table 2). The EBNA1 protein participates in viral replication and persistence processes of the virus. In the host cell, is involved in p53 degradation and further oncogenesis [25]. The BFRF2 protein has yet no annotated function, although, it is essential for the

production of infectious EBV particles since it is one of six viral proteins required for the transcription of late viral genes [26]. BILF2 is a glycoprotein without an annotated function [27]. It was described as “gp78/55”, being a type 1 membrane protein detected at the virion's envelope [28]. BOLF1 is a tegument protein that plays a key role during the viral outflow; in addition, it interacts with the capsid through the Large Integument Protein (LTP) and participates in its transport to the host's trans-golgi network, where secondary involvement occurs. Finally, it modulates the integumentation and capsid accumulation in the viral assembly complex [29].

The BRRF2 integument protein, located in the cytoplasm of cells infected by EBV during the lytic phase, has not annotated function, but studies have shown that suppression of BRRF2 decreases virion production. Therefore, BRRF2 is involved in the production of the infectious progenies, although it is not essential for lytic replication [30]. Due to its location in the cytoplasm, it probably participates in the maturation or transport of viral progenies [30]. The BALF5 protein, located in the cytoplasm, participates in the replication of Epstein–Barr virus (EBV), being the catalytic subunit of DNA polymerase. Therefore, it replicates the viral genomic DNA in the late phase of lytic infection. In addition, BALF5 interacts with the BMRF1 polymerase, resulting in its efficient transport to the host's nucleus [31].

The majority of the viral mRNA targeted by our de novo predicted miRNAs are part of the latent cycle, indicating that the viral strategy to negatively regulate self-expression involved in this cycle could be explained as a self-hiding strategy from the host's immune system ensuring persistence. Furthermore, miRNAs are less immunogenic, thus avoiding detection by the host immune system.

Protein Interaction Network

From the diversity of the targeted mRNAs, we constructed a protein network to better establish the biological connections during the viral self-regulation, unveiling the hallmark of the process. At Fig. 3, it is shown the protein network constructed with 331 nodes and 433 interactions (edges). This network revealed 64 nodes identified as viral proteins and 267 nodes as human proteins. We analyzed the

Fig. 2 Count of detected miR-mRNA interactions of several mRNAs of the EBV type 2 genome. The early protein EBNA-1 involved in the replication on viral latency phase is the top regulated mRNA

Table 2 Top candidates of miR-mRNA interactions for self-regulation of EBV type 2, predicted by the software IntaRNA on EBV type 2 genome

miRNA	kcal/mol of interaction	Target mRNA	Target function
mir-654	– 114.6	EBNA1	V: Replication and persistence process H: p53 degradation and oncogenesis
mir-654	– 41.99	BFRF2	V: Late Transcription
mir-BART17	– 152.1	BILF2	V: virion's Type 1 membrane protein
mir-654	– 49.16	BOLF1	Virion release; interaction with LTP; modulates integumentation and protein capsid accumulation in the viral factory
mir-BART3	– 37.33	BALF5	EBV DNA polymerase
mir-654	– 45.31	BRRF2	Its suppression decreases virion production

The viral miR-BART17 targets the BILF2 viral mRNA with a – 152.1 kcal/mol free energy

degree distribution to determine the proteins that work as hubs in the network. The results suggested that 3% of the proteins act as hubs, due to their connection abundance. Interestingly, all these hubs have a viral origin. Biological networks have a topology known as Scale-free and follow a power law distribution [29]. One of the properties of the power law is the correlation, which reveals if there is a functional relationship between a variable and the remaining variables, as well as how strong is the linkage between these variables [32]. This means that variations in one protein are linked to variations on other proteins. In the present network, we obtained a correlation of 0.915, while

the correlation value from a random network was 0.580. This result corroborates that distribution of nodes in the protein network follows a power law.

We also analyzed the betweenness centrality and closeness centrality of the network. The betweenness centrality considers the control of communication between all nodes of the network, indicating greater importance for the network structure. The higher value indicates the greater the chance of a protein connecting different clusters or biological processes. The closeness centrality is a measure of the average length of the shorter paths to access all proteins in the network. It is related to the speed that an

Fig. 3 Interaction network of proteins codified by the mRNA targets to self-regulation by EBV type 2 miRs. Human host proteins are added into this network. Human proteins are depicted as blue nodes; Target proteins as yellow nodes; and Virus proteins as red nodes (color figure online)

information takes to be shared by all nodes of the network. A higher value means that a protein will be central in the network [33].

From the 26 target proteins, the proteins BTRF1 and BSRF1 had the higher values of betweenness centrality and closeness centrality, being an important connection point in the network (Table S2 in supplementary information). Ten proteins were identified as hubs in the network: BTRF1, BSRF1, EBNA1, TK, EBNA2, LMP1, EBNA3A, BPLF1, BBLF2 and BILF1, the proteins are cited in the centrality order from the more central to less central (Table S2 in supplementary information) and their functions are described in Table 3.

The protein BTRF1 is a tegument protein not characterized, and BSRF1 do not have a known function in EBV type 2, but is similar to the protein product of the gene UL51 from HSV-1 that is involved in virus maturation and virion burst [34]. The protein EBNA1 is one of the first proteins expressed after infection. It plays an important role during the latency phase, through replication and maintenance of the episome [35]. In EBNA1 suppression, the episome can be degraded after infection in most B cells [36]. Thymidine kinase (TK) is an enzyme that participates in viral DNA replication, being present in large quantities in cancer cell proliferation [37].

Table 3 Summary of characteristics of proteins identified as hubs at the calculated Interaction Network

HUB	Temporal class	Function
BILF1	Early	Glycoprotein, g protein coupled receptor, participating in Virus-host interaction
BBLF2	Infection	One of the seven proteins required for viral DNA replication
EBNA1	Latency	A) Replication and maintenance of episome, B) KO: degradation of episome after infection In b cells. C) Development of concurrent lymphomas
EBNA2	Latency	A) Essential for the immortalization, proliferation and differentiation of b cells B) Activator of the transcription of viral and cellular genes C) Delay the progression from g2 to m phase D) Development of concurrent lymphomas
EBNA3A	Latency	A) Viral nuclear protein essential for B-lymphocytes immortalization B) Repressing the transcription of viral promoters tp1 and Cp (prom of allebna mrna). C) Development of concurrent lymphomas.
TK	Latency	A) Viral dna replication B) Found in large quantities in cancer cell proliferation
LMP1	Latency	Triggering a signal transduction cascade that can alter cell growth and survival
BPLF1	Late	Suppression of the host immune system
BSRF1	Late	Maturation and burst
BTRF1	Unknown	Unknown

EBNA-2 is essential for the immortalization, proliferation and differentiation of B cells [38], acting as activator of the transcription of viral and cellular genes. In addition, it may affect the activities of cell cycle regulators and thus delay the progression from G2 to M phase [39]. LMP1 is a protein integrated into the plasma membrane, abundant in B cells in the latent phase. It acts by triggering a signal transduction cascade that can alter cell growth and survival [27]. EBNA3A is a latency-associated viral nuclear protein essential for B-lymphocytes immortalization. It acts as a transcriptional regulator, repressing the transcription of viral promoters TP1 and Cp (promoter of all EBNA mRNAs) [40]. In addition, EBNA proteins associate with many host proteins in different signaling networks, providing a suitable platform for the life-long survival and persistence of the virus and the development of concurrent lymphomas in the infected host [41].

BPLF1 is a protein of the integument and is expressed throughout the late phase of the lytic cycle, being involved in the suppression of the host immune system [42]. BBLF2 is one of the seven proteins required for viral DNA replication in EBV [26]. BILF1 is a glycoprotein that is expressed primarily at the beginning of the lytic cycle, although it may also be expressed at low levels of latency. Finally, it acts as active G protein coupled receptor, participating in the virus-host interaction [27]. These results suggest a very complex scenario of gene self-regulation in EBV type 2, in which the virus represses the translation of

essential genes associated to the lytic cycle and, at the same time, represses essential genes associated to the host cell cycle, blocking the cell division. We suggest that this behavior is important to the stability of the latent phase, and that the participation of the EBV type 2 in the oncogenic process can be a derivate of an inadvertent deregulation of latent self-regulated genes. These results open a new way to understand the infection by EBV type 2, where the viral genes can be used as targets to new strategies to study and prevent oncogenic processes related with the persistent infection by EBV type 2, as well as, the development of new pharmacies to suppress and combat the viral infection. Evidently, although the computational methods have increased the prediction power in the last years, we indicated that the data need to experimental validation, thus, we encourage the diverse groups to test the results present in this work.

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