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Identification of New Putative MicroRNAs in Epstein-barr Virus Genome for Medical and Biotechnological Applications

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Abstract

Introduction: MicroRNAs play important roles in regulating gene expression in animals and plants. Many studies used these genetic elements to up- or down-regulate human gene expression. It has been shown that viral miRNAs can target and regulate not only viral genes but also host genes. To date, 48 miRNAs have been identified in Epstein-Barr virus (human gamma-herpesvirus 4). This virus can invade and reside in many human tissues such as epithelial or immune system cells. In this study, we evaluated a novel method of finding miRNAs in the Epstein-Barr virus (EBV).
Materials and Methods: The entire genome content of human gamma-herpesvirus 4 (EBV) was searched using ab initio prediction algorithm by miRNAFold. Selected sequences screened by homology algorithm of miRBase web server.
Results: Among the identified 12 sequences, two putative miRNAs were selected and their properties were further investigated. The main human gene target of the putative miRNA-1 was BCL11A gene that is related to the B-cell lymphoma, and that of putative miRNA-2 was ZBTB18 gene which is mainly related to the mental retardation.
Previous studies used known EBV miRNAs incorporated in delivering vectors such as lentiviral vectors to target various human genes. The introduced two miRNAs can target genes in some malignancies and other types of disorders.
Conclusion: Because of the high stability of the proposed putative miRNAs, they can be used experimentally to regulate human genes.

Keywords: Epstein - barr virus, Hairpins, miRNA, RNA folding

1. Introduction

Genes must be expressed in order to perform their functions. The first step in gene expression is the transcription of DNA to RNA [1]. Some of the RNAs synthesized during the transcription process are not recognized by ribosomes and therefore not translated into polypeptides. These types of RNAs are called non-coding RNAs [2]. MicroRNAs are non-coding RNAs that regulate the gene expression in many organisms. They have 17-24 nucleotides and are initially transcripted to a pre-miRNA [3]. Previous studies have shown that these molecules bind to the 3'-UTR of their target genes. In some cases, miRNAs also bind to the 5'-UTR of the target gene [2]. MicroRNAs have three mechanisms of gene regulation. The first one is microRNA-mediated gene silencing via minimal miRNA-induced silencing complex (miRISC). The target specificity of miRISC is determined by the interaction of complementary sequences with the target mRNA [4]. The second mechanism is microRNA-mediated translational activation, in which the translation process is up-regulated by induction of the miRNA. The final action mechanism of miRNA is the microRNA-mediated post-transcriptional gene regulation within the cell nucleus [5]. The Epstein-Barr virus (EBV) that is formally called human gamma-herpesvirus 4, is one of the nine known human herpesvirus in its family [6]. It is a ubiquitous human

oncogene that is a member of the *Herpesviridae*, subfamily *Gammaherpesvirinae*. It may come as a surprise to know that almost 95% of the human population are affected by EBV virus [7]. EBV causes many malignancies such as Burkitt's lymphoma, Hodgkin's lymphoma and hemophagocytic lymphohistiocytosis [8]. In addition, many childhood diseases are secondary to the infection with this virus, including Alice in wonderland syndrome, autoimmune disorders, and acute cerebellar ataxia. It is estimated that around 200,000 cases of the cancer are due to EBV [9].

Because of the EBV involvement in various diseases and its burden on the human health system, it is important to find and examine novel treatments and growth restricting approaches for this virus [10]. One of the beneficial tools to achieve this goal is the application of miRNAs. Most of the characterized miRNA sequences are intergenic. Few examples of the miRNA in which their coding genes are located within exon regions, such as miR-20a, have also been identified [11]. It is now clear that pri-miRNA forms a hairpin structure that is common to all families of the identified miRNAs [12]. This structure is very important as it can help us to evaluate the potency of a genomic region to develop a miRNA. A polymerase III enzyme called Dorsha, processes this structure into a mature miRNA [13].

Unlike mRNA, identification of miRNA coding gene is more complicated due to the less preserved regions in its order and also in its adjacent sequences. The miRNA does not have easily recognizable signal sequences [14]. In this article, an in silico approach has been employed to detect potential miRNA sequences in Epstein-Barr virus to use for further biomedical applications. A two step miRNA finding and filtering method was used to identify the miRNA with the most stable structure and homology to the previously described sequences.

2. Materials and Methods

Total genome screening for possible hairpin sequences

The entire genome content of human gamma-herpesvirus 4 (EBV) (Accession ID: NC_009334) was retrieved from the NCBI genome bank. Search for possible miRNA sequences was performed by an ab initio prediction algorithm using miRNAFold [15]. The initial search with the default settings found 3,756 possible hairpin sequences. To apply the filter and find more stable miRNA, the thermodynamic parameter was set to -100 kcal/mol and the minimum length for hairpin sequences was adjusted to 75. The new search with these parameters yielded 12 sequences.

Screening of the hairpins for miRNAs

The obtained hairpins were screened with a homology algorithm using the miRBase web server [16]. Homology similarities were used as a second criterion to find miRNAs. The maximum error value was set at 2.0, and BLAST search was performed on all mature miRNAs sequences which are deposited in the miRBase. Often, the miRNAs are noncoding sequences; based on this fact, coding sequences were identified by performing the BLASTx of the NCBI web server, and excluded from putative sequences.

Pre-miRNA characterization

To identify and characterize the pre-miRNA sequence, hairpin sequences were found by miRNAFold. The RNAfold webserver, Vienna RNA [17], was used to determine the secondary structure of the pre-miRNA.

Determination of miRNA gene targets

Our newly found sequences were uploaded in miRDB and the gene targets for each miRNA were determined [18]. The human gene database (GeneCards) was used to evaluate the role of each gene in humans and to characterize the potential application of miRNAs [19].

3. Results

Identification of miRNAs

Screening of the entire human gamma-herpesvirus genome using the above-mentioned parameters yielded 12 very stable hairpins in the genome. All sequences were subjected to BLASTx to exclude the protein sequences, showing that all 12 sequences were non-coding. The second step was to filter these sequences against the existing miRNA database. Two sequences were identified showing similarity to miRNAs from *Hydra magnipapillata* and *Callithrix jacchus*. *Hydra magnipapillata* or *Hydra vulgaris* is a small organism living in fresh waters [20]. *Callithrix jacchus* is an animal and a member of the family *Callitrichidae* [21]. The number of bases for miRNA 1 and 2 were 24 and 19, respectively (Table 1). The result of homology alignment is shown in Figure 1.

Table 1. Identified putative miRNAs and their BLAST details

Putative miRNA No.	Putative miRNA sequence	Homologous Sequence	Similarity	Coverage	Organisms
1	AGCAUAUGCUAUCCUA AUCUAUAU	hma-miR-3006	79	92	*Hydra magnipapillata*
2	GGGCAUCCUCUGAGACC CU	cja-miR-4512	84	91	*Callithrix jacchus*

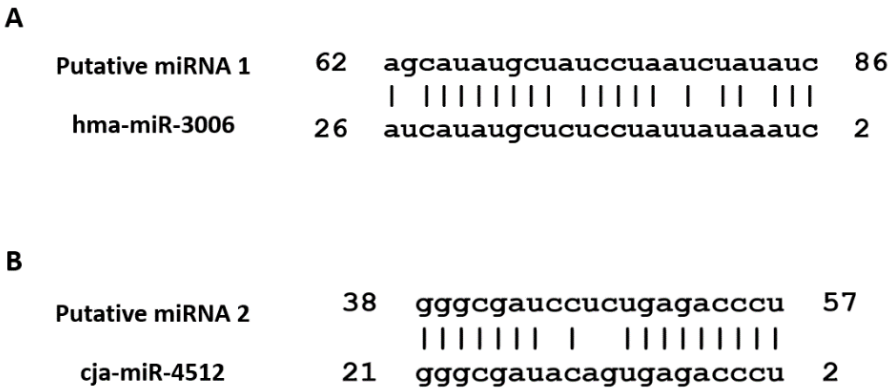


Figure 1. Alignment of two putative miRNA of EBV with their homologous sequences. A: the putative miRNA1 had highest homology with hma-miR-3006 from *Hydra magnipapillata.* B: the putative miRNA1 had highest homology with hma-miR-3006 from *Callithrix jacchus*.

Secondary structure of pre-miRNAs

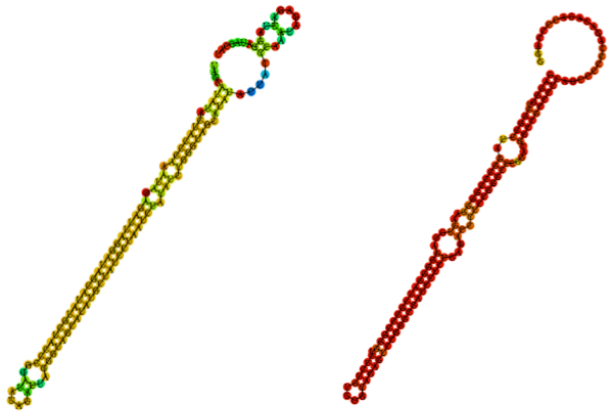
Another essential property for the sequence to be considered as an miRNA was to locate at the left arm of the putative pre-miRNA loop structure. Considering this property of miRNAs, 7 putative sequences were excluded from further evaluation. The free energy of the thermodynamic ensemble for putative miRNA no.1 sequence was determined to be -114 kcal/mol and the frequency of MFE structure was calculated to be 45.63%. The ensemble diversity was 1.36 and the centroid secondary structure in dot-bracket is seen in the Figure. For the putative miRNA No.2, the free energy of the thermodynamic ensemble was -117.26 kcal/mol; the frequency of the MFE structure was calculated to be 47.11%, and the ensemble diversity was 2.63 (Figure 2).

Functional annotation

The gene targets for both putative sequences were determined. For miRNA-1, the highest target score belonged to the *BCL11A* gene, a famous proto-oncogene. B-cell lymphoma/leukemia 11A is a protein encoded by *BCL11A* [22]. This protein is a C2H2 type zinc-finger regulatory domain specifically binding to DNA, and in association with other SWI/SNF complex proteins regulates the gene expression through chromatin remodeling [23]. The *BCL11A* gene is the common site of retroviral integration in myeloid leukemia and is therefore considered to be a leukemia disease gene [24]. Other gene targets are listed in Table 2.

By evaluating the genes that can be targeted by miRNA-1, it was shown that they fall into five categories including cancer, neurological, infectious, metabolic and autoimmune diseases (Figure 3).

For putative miRNA-2, the gene target score was significantly lower than that of putative miRNA-1. The major target gene was *ZBTB18*. This gene encodes a transcriptional repressor that plays a role in many developmental processes such as brain development and myogenesis. The second gene target is *CNDP1*, which encodes a hydrolysis enzyme referred to as beta-Ala-His dipeptidase. The protein is found in serum of healthy adults and is absent in the serum of homocarnosinosis patients, indicating the correlation between beta-Ala-His dipeptidase and this disease. Other target genes are listed Table 3. The proportion of each disease type associated with putative miRNA-2 target genes is shown in Figure 4.



Putative miRNA No.1 Putative miRNA No.2

Figure 2. Pre-miRNA structures of putative miRNAs. The stem loop numbers and positions differ in the two sequences.

Table 2. Gene targets of putative miRNA-1 and their brief description.

Target Rank	Target score	Gene Symbol	Gene description	Reference
1	99	*BCL11A*	*BCL11A*, *BAF* complex component	[24]
2	99	*AGBL3*	ATP/GTP binding protein like 3	[25]
3	99	*KCTD9*	Potassium channel tetramerization domain containing 9	[26]
4	98	*DENND1B*	DENN domain containing 1B	[27]
5	98	*DACT1*	Disheveled binding antagonist of beta catenin 1	[28]
6	97	*LETM2*	Leucin zipper and EF-hand containing transmembrane protein 2	[29]
7	96	*SETD5*	SET domain containing 5	[30]
8	96	*CPEB2*	cytoplasmic polyadenylation element binding protein 2	[31]
9	95	*SLC35G3*	solute carrier family 35 member G3	[32]
10	95	*SPESP1*	sperm equatorial segment protein 1	[33]
11	94	*IRS2*	insulin receptor substrate 2	[34]
12	94	*SH3KBP1*	SH3 domain containing kinase binding protein 1	[35]
13	94	*MEF2C*	myocyte enhancer factor 2C	[36]
14	94	*CXADR*	CXADR, Ig-like cell adhesion molecule	[37]
15	94	*KCNQ4*	potassium voltage-gated channel subfamily Q member 4	[38]
16	94	*PRKAR2B*	protein kinase cAMP-dependent type II regulatory subunit beta	[39]
17	93	*BCL2*	*BCL2*, apoptosis regulator	[40]
18	93	*BICC1*	BicC family RNA binding protein 1	[41]
19	93	*VASH2*	Vasohibin 2	[42]
20	93	*SMAP1*	Small *ArfGAP 1*	[43]

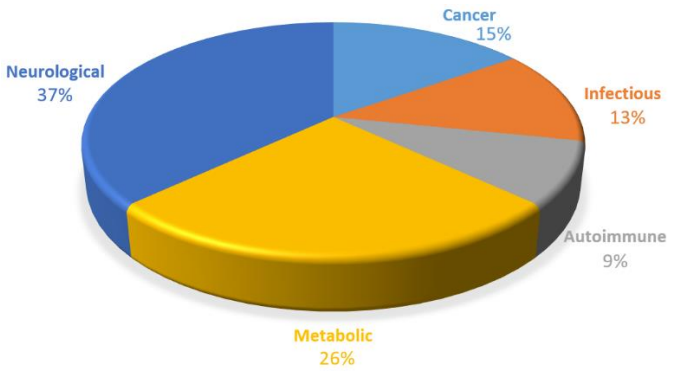


Figure 3. Share of each disease type among the putative miRNA-1 gene targets. Neurological and metabolic related genes are dominant in the target gene spectrum.

Table 3. Gene targets of putative miRNA-2 and their brief description.

Target Rank	Target score	Gene Symbol	Gene description	Reference
1	85	*ZBTB18*	zinc finger and BTB domain containing 18	[44]
2	81	*CNDP1*	Carnosine dipeptidase 1	[45]
3	81	*TOR2A*	Torsin family 2 member A	[46]
4	80	*NCDN*	Neurochondrin	[47]
5	78	*MUC17*	Mucin 17, cell surface associated	[48]
6	76	*CD44*	CD44 molecule (Indian blood group)	[49]
7	63	*PLCG2*	Phospholipase C gamma 2	[50]
8	62	*FXR1*	FMR1 autosomal homolog 1	[51]
9	61	*POLDIP3*	DNA polymerase delta interacting protein 3	[52]
10	61	*SON*	SON DNA binding protein	[53]
11	61	*YWHAG*	Tyrosin 3-monooxygenase/ activation protein gamma	[54]
12	60	*FOXK1*	Forkhead box k1	[55]
13	59	*VWA5A*	Von Willebrand factor A domain containing 5A	[56]
14	59	*ZC3H4*	Zinc finger CCCH-type containing 4	[57]
15	55	*CLSTN3*	Calsyntenin 3	[58]
16	55	*PPARGC1B*	PPARG coactivator 1 beta	[59]
17	55	*TTLL7*	Tubulin tyrosine ligase like 7	[60]
18	54	*MPZ*	Myelin protein zero	[61]
19	53	*KLHL6*	Kelch like family member 6	[62]
20	52	*GPR17*	G protein-coupled receptor 17	[63]

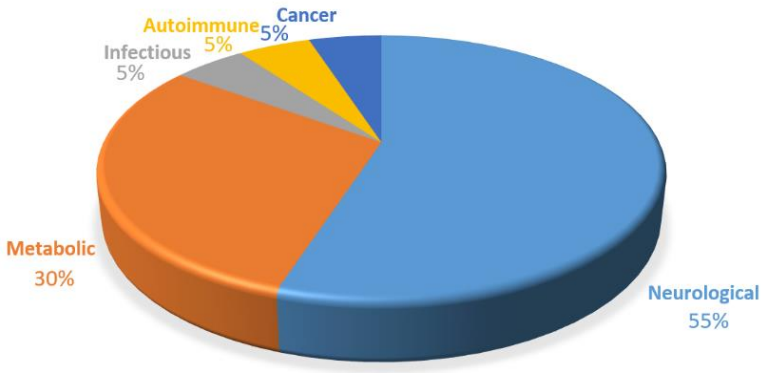


Figure 4. Share of each disease type among the putative miRNA-2 gene targets. Same as putative miRNA-1, neurological and metabolic related genes are dominant in the target gene spectrum

4. Discussion

The miRNAs perform various functions in the nature and have an important position in current medical, industrial and even agricultural biotechnology. So far, 1917 miRNA sequences have been identified in the human genome consisting of both putative and experimentally approved sequences [64]. It is estimated that approximately 30% of the human genes are regulated by miRNAs. Many of the miRNA targets are important genes whose function profoundly influences the human life [65]. Human gamma-herpesvirus 4 (EBV) can infect both B cells and epithelial cells. There are many pieces of evidence showing that EBV can invade and infect various body systems and organs such as the immune and nervous systems. This feature can be used in medical applications [6]. As we know, unlike most bacteria, viral infections can induce several changes in the function of the host cells. Our results in accordance with previous studies showed that viral miRNA can up- and down-regulate the human genes. Our two introduced putative miRNA demonstrated their intent to target genes mainly involved in nervous system functions and interactions. Cancer related genes are also one of the targets that these two putative miRNAs regulate. It is demonstrated in several studies that EBV miRNAs are related to the development of various types of lymphoma (AIDS-related DLBCL), post-transplant lymph proliferative disorder (PTLD), Hodgkin's lymphoma (HL), nasal NK/T-cell lymphoma (NNL) and Burkitt's lymphoma (BL) [10]. In EBV-associated primary central nervous system PTLD (pCNS PTLD) patients, miR-BHRF-1-1 was detected in all samples [66]. The miRNAs of the EBV are also involved in the development of carcinomas. BART miRNAs have shown to play a role in the development of nasopharyngeal cancer [67]. The first putative miRNA-1 target was predicted to be the *BCL11A* gene which is related to the B-Cell lymphoma. As a therapeutic approach, the *BCL11A* gene can be suppressed for treating beta-hemoglobinopathies. In 2014, Brendel and colleagues used lentiviral vectors to deliver miRNA scaffolds and thereby showed 100-1000 fold less Hbb-y induction using shRNA [68]. Another study on *BCL11A* gene regulation was published in 2021 by Esrick et al. They used the BCH-BB694 lentiviral vector that encodes a shRNA targeting *BCL11A* and is embedded in a microRNA to treat sickle cell disease. They observed reduction of nearly 20 percent in the manifestation of sickle red blood cells [69].

5. Conclusion

In our study, we have proposed an approach to finding the miRNA sequences. Using this method, two putative miRNA sequences are introduced which can target many gene related diseases such as cancer and neurological disorders. These miRNAs can be embedded in viral vectors, especially in their origin virus EBV, to treat various diseases.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Author's contributions

All authors equally contributed to preparing this article.

Conflict of interest

The Author declares that there is no conflict of interest.

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