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Viral miRNAs confer survival in host cells by targeting apoptosis related host genes

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ABSTRACT

miRNAs are small non-coding RNAs that regulate gene expression by RNA silencing. Like eukaryotic organisms, some viruses also produce miRNAs. While contribution of host miRNA in preventing viral pathogenesis has been studied, how viral miRNA confers survival within the host is poorly understood. Here, we hypothesize that viral miRNAs confer pathogenicity by binding to host target genes to down-regulate specific pathways that threaten cell survival. In order to identify such pathways, we performed functional enrichment analysis using targets of 168 viral miRNAs from 13 different viruses. We identified specific immune system and host defense pathways targeted by viruses via miRNA mediated gene silencing. Analysis and integration of publicly available RNA-seq data revealed that viruses target the apoptosis in the host by switching off pro-apoptotic genes through miRNA-induced mechanisms, thus ensuring cell survival. In conclusion, our findings reveal an important function of viral miRNA in downregulating host apoptosis machinery.

1. Introduction

miRNAs are ~22 nucleotide, small, non-coding RNAs that are present in the vertebrates, plants, invertebrates as well as in a wide range of viruses [1]. The primary function of miRNAs is to regulate the expression of genes post-transcriptionally, via base pair formation with the 3′-untranslated region (3′-UTR) of specific messenger RNAs (mRNA). Viral miRNAs play subtle roles in the survival and proliferation of viral particles through host immune system evasion [2], establishing micro-environment for viral replication [3], regulation of innate immune system, differentiation of adaptive immune cells [4], etc.

Like most animals and plants, some viruses (mostly DNA viruses), can also produce miRNAs that grant different selective advantages required for their survival, including host immune system evasion, regulation of host and viral genes [5], viral replication [6], influencing viral latency [4], and diminishing host antiviral responses [5]. Viral miRNAs were initially discovered in the Epstein-Barr virus (EBV) [7] and were subsequently found in other viral families like Herpesvirus, Polyomavirus, Ascovirus, Iridovirus, Baculovirus, Adenovirus, Retrovirus [8,9]. At present, more than 20 human viruses are predicted to produce miRNAs, among which around 10–12 have been validated experimentally. The human viruses produce about 135 miRNAs [10], which are thought to be beneficial for the virus survival in the human body.

The viruses produce miRNAs that can control the expression of corresponding viral genes and some human genes as well. The miRNA sequences found from these viruses have been analyzed and their respective target sites have been found at the 3′-UTR regions of many human genes [10]. It has been hypothesized that the miRNAs provide some selective advantages to the viruses by facilitating evasion of host defense machineries. Since viruses need to replicate inside host cell, they may initiate mechanisms to prevent host cell death by apoptosis or immunologic mechanisms. Therefore, we hypothesized that viral miRNAs suppress apoptosis through downregulation of apoptosis-related genes, thereby prolonging survival of the virus by sustaining the host cell.

2. Materials and methods

2.1. Identification of viral miRNAs and their target genes

In order to understand the functions of viral miRNAs in providing selective advantages to viruses, we first identified human viruses which produce miRNA. We explored miRbase [11] to extract FASTA sequences

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of the mature viral miRNAs for further analyses. We selected three different target identifying tools. TargetScan predicts the targets of miRNA sequences by searching for the presence of conserved regions within mRNAs, where genes are ranked using cumulative weighted scores of the target sites [12]. RNAhybrid is software that finds the minimum free energy hybridization of a short and a long RNA [13], and thus effectively identifies target mRNAs. PITA measures site accessibility for predicting putative miRNA targets [14]. Using TargetScan, RNAhybrid and PITA, we scanned the 3′-UTR regions of the human protein coding genes for putative miRNA targets, and from UTRdb [15] and miRTarBase [16] databases we directly obtained the putative and experimentally validated targets of viral miRNA.

We combined target genes obtained from these databases and targets identified by scanning UTRs to create a unique set of viral miRNA targets.

2.2. Functional enrichment analysis

We annotated genes in terms of the Biological Processes (GOBP), Cellular Locations (GOCL) and Molecular Functions (GOMF) from the Gene Ontology Consortium (GO) [17,18]. The pathways involved were obtained from Kyoto Encyclopedia of Genes and Genomes (KEGG) [19]. In all cases Ensembl genes (Release 79: March 2015) [20] were used.

We used Gitools [21] (Version: 1.8.4) to perform enrichment analysis and to generate the corresponding p-values and heatmaps for the targeted GOBPs, GOCLs, GOMFs and KEGGs. Gitools is a framework that helps in analyzing and visualizing genomic data. It represents data in a browsable heatmap format, where probability (such as the right *P*-value used here) for the presence of a specific gene in a biological pathway is calculated.

2.3. Gene expression microarray data analysis

Gene Expression Omnibus (GEO) [22] is a curated, public reservoir of microarray gene expression data at National Center for Biological Information (NCBI). The RNA-seq dataset GSE44769 was generated on the platform Illumina Genome Analyzer IIx (GPL10999) and contains Epstein-Barr virus (EBV) cell line in data in different conditions. The “No BART, EtOH” sample was taken as the control of this dataset and the “BART, Dox” was taken as the most appropriate sample. We then employed in-house R script to convert absolute expression into Log2 expression values. We then analyzed the differential expression of genes between experimental condition and the control.

We extracted the “Apoptosis” related gene set from the KEGG database “apoptosis pathway” term and the expression values for those genes were obtained from the RNA-seq data. We selected apoptotic genes which were down-regulated two-fold or greater for further analysis, and to identify viral miRNAs targeting them. We studied the functions of these significantly down-regulated genes to better understand the selective advantages that downregulation of these targets by miRNAs provide to viruses.

3. Result

3.1. From 13 human viruses 168 miRNAs and their targets were retrieved from various databases

We obtained miRNAs of human viruses from miRbase and various publications [4,5,7,10,23–27]. Among these viruses, we selected the 13 whose putative miRNAs were already published (**Supplementary file 1**). This resulted in a total of 168 miRNAs from 13 viruses. We then obtained the sequences of all viral miRNAs using miRBase, and identified human target genes using the RNAhybrid [13] and miRTarBase [16] databases as well as the online softwares PITA [14] and TargetScan [12]. We combined the target genes obtained from these databases and tools to generate a set of target genes in potentially comprehensive manner. This resulted in almost 22,000 unique genes targeted by the viral miRNAs. Among those, EBV targeted about 16,000 genes, HIV, HBV, HSV1, HSV2, KSHV and HCMV targeted about 15,000 genes, and BKV, JCV and MCV targeted about 7000 genes.

3.2. Functional enrichment analysis identifies biological processes and pathways targeted by viral miRNAs

We performed enrichment analysis to identify particular biological processes and pathways in the host cells targeted by viruses. The cut-off value for the significance was set to FDR corrected *P*-value 0.05 (Fig. 1). We then studied previously published experimental evidence to guide further analyses. The selected pathways were: Calcium mediated signaling, MAPK cascade, Wnt signaling pathway and Apoptosis (Fig. 2).

3.3. Enrichment analysis of apoptosis provided 84 enriched genes targeted by viral miRNAs

Enrichment analysis revealed that apoptosis was an enriched biological process. There were 84 enriched genes (corrected p-value $<$ 0.05) observed in the KEGG database term “apoptosis pathway”, which were targeted by the viral miRNAs. Enriched apoptosis-related genes were retrieved and represented in a color coded heatmap (Fig. 3).

3.4. Microarray analysis of EBV cell line provided significant downregulation of 49 genes associated with apoptosis

Analyzing the microarray data (GSE44769), we found that 75 apoptosis genes showed deviated expression level compared to the control condition. Among these 75 genes, 24 genes were upregulated and the remaining 51 genes were downregulated. As we assumed that viral miRNA would down-regulate apoptosis process, we selected the genes which were down-regulated at least two-fold. We observed 49 such genes when they were compared to the control (Fig. 4). Assigning the EBV miRNAs to their corresponding target genes revealed several miRNAs targeting an apoptosis gene (**Supplementary file 2**) (Fig. 5).

3.5. miRNA-targeted apoptosis genes provide insights into viral mechanisms of silencing host genes

The genes that were most significantly downregulated upon viral infection (the bottom 20% all expressed genes, or Log2 ratio < -2.74) included 20 apoptosis genes targeted by EBV viral miRNAs (**Supplementary file 2**). The protein database UniProt [28,29] was used to ascertain the fitness benefit of inhibition by miRNAs (Table 1).

4. Discussion

In the fields of molecular and cell biology, miRNAs have drawn a lot of research interest. Recent work has revealed the significant role of miRNAs in regulating gene expression as it relates to various cellular processes. Vertebrates, invertebrates [1], plants [1], viruses etc. Produce miRNAs to regulate the expression of their genes, thereby controlling their physiological functions. At present there are more than 9000 annotated miRNA genes, among which about 700 miRNA genes are human [30]. These miRNAs play various roles to ensure proper homeostatic conditions of the organism. One such role includes the host-pathogen relationship.

Previous experimental works have established that human miRNAs target viral genes [31,32] and function as antiviral mediators to suppress viral pathogenesis. By silencing the disease-causing genes of a virus, human miRNAs ensure the prevention of any deleterious events caused by the virus. On the other hand, growing evidences suggest that a few viral miRNAs may also effectively target and regulate host genes. To evade host defense molecules, viruses might have further evolved to produce miRNAs to silence host genes. The silencing can provide viruses

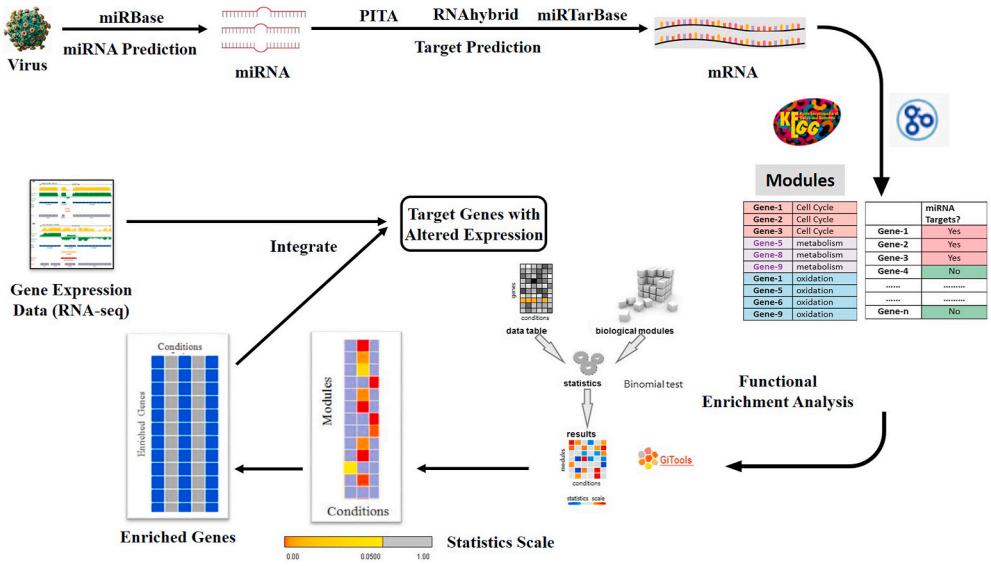

Fig. 1. Enrichment of gene ontology biological processes: Representation of (selected) Gene Ontology Biological Process (GOBP) enriched terms related to immune system obtained from Gitools. Statistical significance (False Discovery Rate, FDR corrected p-values) is represented in a color-coded scale. Color towards red indicates more significant and color towards yellow means less significant, and gray color indicates non-significance (>0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

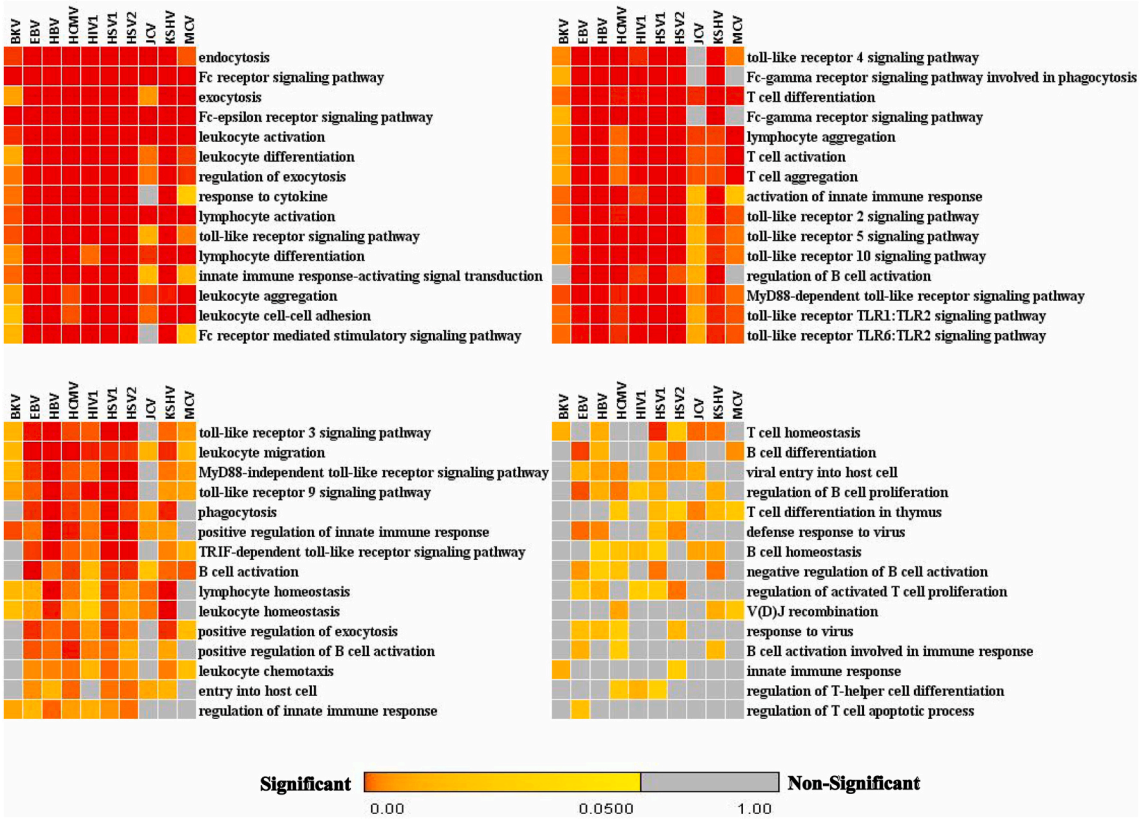

Fig. 2. Enrichment analysis of pathways: Overrepresentation of Biological processes of (A) MAPK cascade, (B) Wnt signaling pathway, (C) calcium mediated signaling, (D) Apoptosis obtained from Gitools. Color scale and legend as in Fig. 1. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

with various selective advantages including host defense evasion, viral replication [5], and diminishing antiviral responses. Whether viruses effectively target and control host genes to promote their own survival is a compelling question which several scientific works from different laboratories is sought to address.

After performing enrichment analysis, we observed that various physiological processes are enriched at a high level. We found viruses targeting immune system-related pathways, Wnt pathway, MAP kinase cascade, apoptosis etc. at a significantly high level through their miRNAs. We predicted that switching these pathways off may provide some selective advantage in their survival inside human host.

Carl et al. [10] worked with several viruses and associated miRNAs to hypothesize that viral miRNAs target host pathways by regulating the host gene expressions. They looked for the predicted miRNAs from different databases to find the target genes. They have obtained the enriched GO biological processes and predicted the pathways targeted

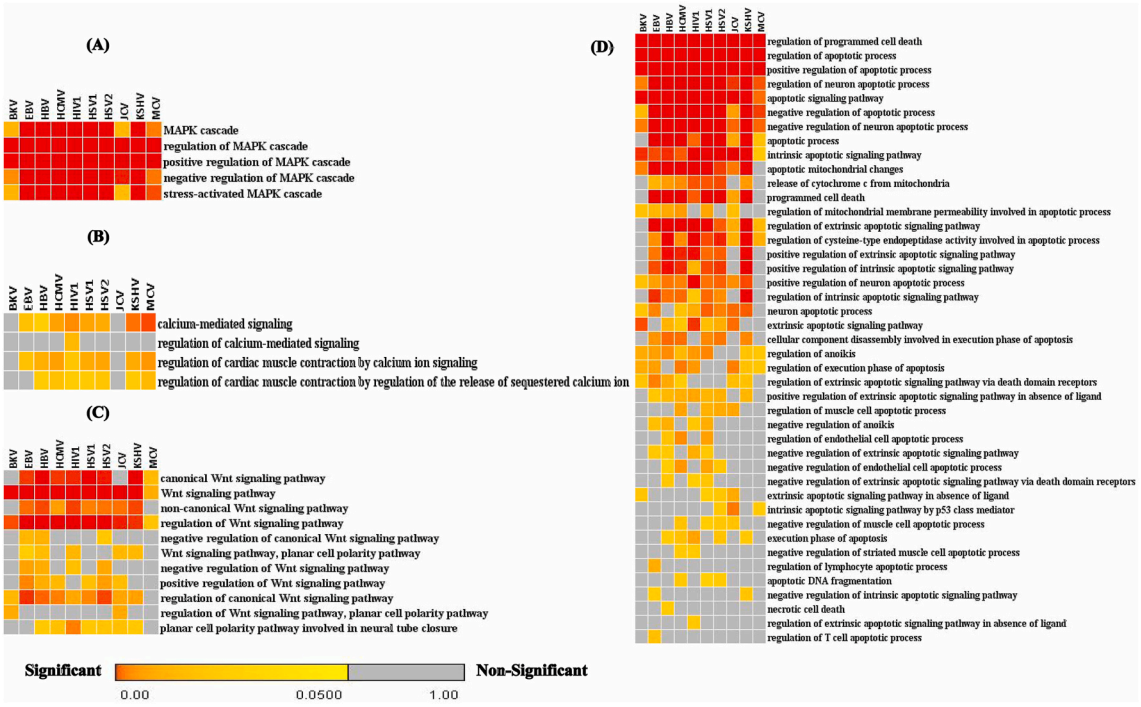

Fig. 3. Enriched apoptosis genes targeted by viral miRNAs: Enriched genes related to apoptosis process obtained from Gitools. The Blue colored hits express the genes that are targeted by viral miRNAs and the Gray colored hits are the genes that are not targeted by viral miRNAs. Vertical lines express viral miRNAs and the horizontal lines express corresponding target genes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

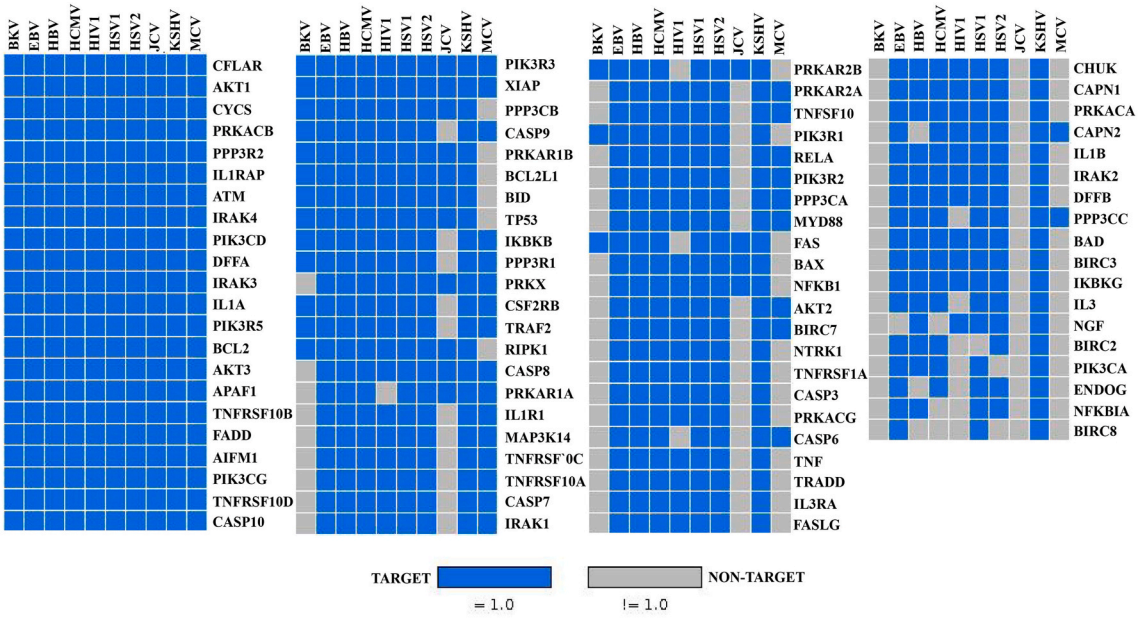

Fig. 4. Expression level of the down-regulated genes: Down-regulated genes are represented in a color-coded scale based on their expression level. The genes that are down-regulated at least two-fold (Log2 ratio < -0.5) are considered. The significance level of down-regulation is taken at -1.5 of the Log2 ratio, which is represented as White color. Genes having Log2 ratios less than -1.5 are represented as Green color and genes having values more than -1.5 are represented as Blue color. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

by viral miRNAs but lacked a link to experimental evidence. We identified the pathways and their corresponding genes targeted by viral miRNAs, and further validated their misregulation in cells infected with Epstein-Barr virus from experiments by Vereide and co-authors [33]. Our results provide evidence that viral miRNAs target specific human genes and control pathways like apoptosis associated with viral fitness.

Apoptosis is a natural physiological phenomenon where cells undergo programmed death when they reach maturity or are infected by any pathogen [34–36]. This process is very important to maintain homeostasis of an organism. Apoptosis prevents various deleterious effects like the progression of viral pathogenesis and cancer. As we have already found that viral miRNAs target host genes for their own survival, we

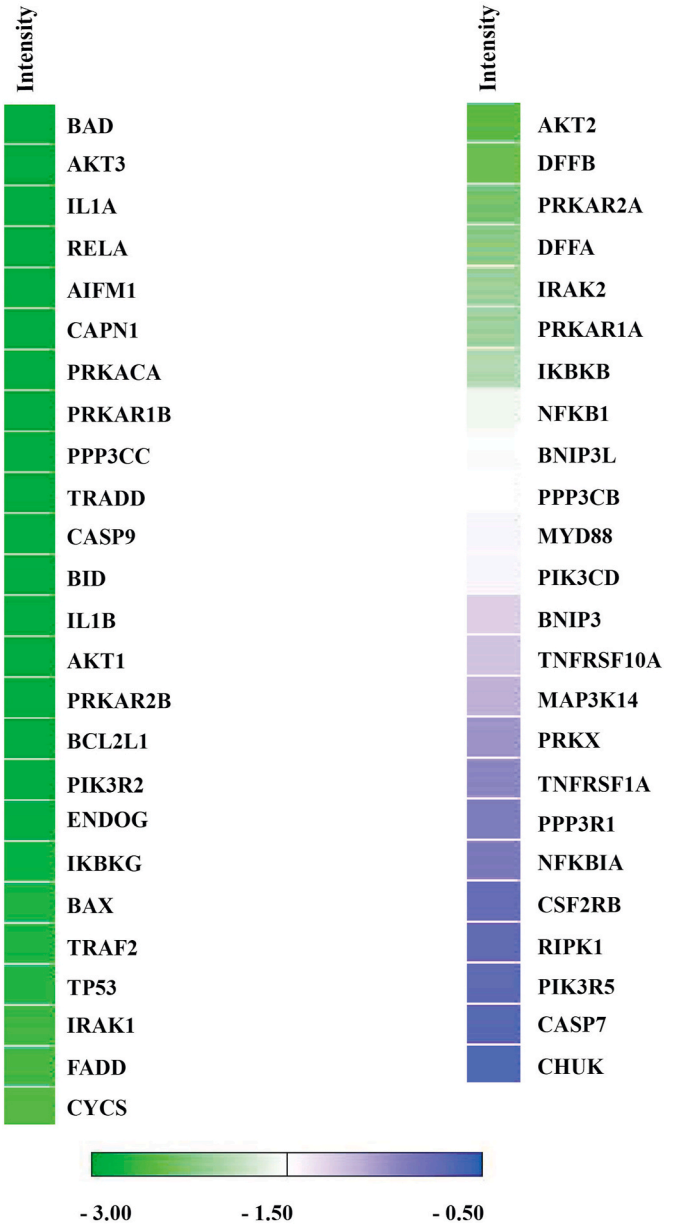

Fig. 5. Intensity of expression of the down-regulated genes: Down-regulated genes are represented by different colors based on their expression level. The genes that are down-regulated at least two-fold (Log2 ratio < -0.5) are considered. The significance level of down-regulation is taken at -1.5 of the Log2 ratio, which is represented as White color. Genes having Log2 ratios less than -1.5 are represented as Green color and genes having values more than -1.5 are represented as Blue color. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

predicted that viruses may specifically target and regulate the host apoptosis process to ensure their refuge and prolonged survival in host cells. Indeed, we found apoptosis genes among top viral miRNA targets that were most significantly downregulated in virus infected cells. These findings strengthened the hypothesis that viruses selectively target host apoptosis pathway for their own survival.

There are viruses that cause various deleterious effects in humans. EBV (also known as Human Herpesvirus-4 (HHV-4)), the causative agent of a common disease named Infectious mononucleosis, can result in several forms of cancer including Hodgkin's lymphoma, Burkitt's lymphoma, gastric cancer, nasopharyngeal carcinoma etc. There are several evidences showing that EBV is associated with autoimmune diseases as

Table-1
Functions of most significantly down regulated genes obtained from UniProt.

Serial	Gene Symbol	Encoded Protein	Function
1	*BAD*	Bcl2-associated agonist of cell death	Promotes cell death. It has cysteine-type endopeptidase activator activity, which is involved in apoptosis process.
2	*AKT3*	RAC-gamma serine/ threonine-protein kinase	Regulates metabolism, cellular proliferation, cell survival, growth and angiogenesis.
3	*IL1A*	Interleukin-1 alpha	Involved in the inflammatory response. Can stimulate the release of prostaglandin and collagenase from synovial cells.
4	*RELA*	Transcription factor p65, RELA protein	Involved in NF_KB heterodimer formation, nuclear translocation and activation.
5	*AIFM1*	Apoptosis-inducing factor 1, mitochondrial	Functions as an NADH oxidoreductase and regulates apoptosis. Acts as a proapoptotic factor in a caspase-indipendent pathway when released into cytosol and nucleus from the mitochondria.
6	*CAPN1*	Calpain-1 catalytic subunit	Catalyzes limited proteolysis of substrates involved in cytoskeletal remodeling and signal transduction.
7	*PRKACA*	cAMP-dependent protein kinase catalytic subunit alpha	Subunit of Protein Kinase A, which regulates glucose metabolism, cell division etc. PKA phosphorylates other proteins, thereby changing their activities.
8	*PRKAR1B*	cAMP-dependent protein kinase type I-beta regulatory subunit	Regulatory subunit of cAMP-dependent protein kinases that are involved in cAMP signaling in cells.
9	*PPP3CC*	Serine/threonine-protein phosphatase 2B catalytic subunit gamma isoform	Calcium dependent, calmodulin stimulated protein phosphatase, which has a role in calmodulin activation in calcineurin.
10	*TRADD*	Tumor necrosis factor receptor type 1-associated DEATH domain protein	Acts as a tumor suppressor by preventing ubiquitination. It's over expression leads to apoptosis and NF_KB activation.
11	*CASP9*	Caspase-9	Involved in apoptosis.
12	*BID*	BH3-interacting domain death agonist	Induces apoptosis. It is a major proteolytic product, which releases cytochrome *c*.
13	*IL1B*	Interleukin-1 beta	It is a proinflammatory cytokine which induces prostaglandin synthesis, neutrophil influx and activation, T-cell activation and cytokine production, B-cell activation and antibody production, and fibroblast proliferation and collagen production.
14	*AKT1*	RAC-alpha serine/ threonine-protein kinase	It regulates cell survival via the phosphorylation of MAP3K5 (apoptosis signal-related kinase). Has an important role in the regulation of NF_KB-dependent gene transcription and positively regulates the activity of CREB1 (cAMP-response element binding

(*continued on next page*)

Table-1 (*continued*)

Serial	Gene Symbol	Encoded Protein	Function
			protein). The phosphorylation of CREB1 induces the binding of accessory proteins that are necessary for the transcription of pro-survival genes such as BCL2 and MCL1.
15	*PRKAR2B*	cAMP-dependent protein kinase type II-beta regulatory subunit	Mediates membrane association by binding to anchoring proteins, like MAP2 kinase.
16	*BCL2L1*	Bcl-2-like protein 1	Prevents apoptosis by inhibiting caspases. Regulates cell death by blocking the voltage-dependent anion channel (VDAC) by binding to it and preventing the release of the caspase activator, CYC1, from the mitochondrial membrane. Also acts as a regulator of G2 checkpoint and progression to cytokinesis during mitosis.
17	*PIK3R2*	Phosphatidylinositol 3-kinase regulatory subunit beta	Phosphorylates Phosphatidylinositol 4,5-bisphosphate to generate PIP_3 (phosphatidylinositol 3,4,5-trisphosphate). PIP_3 plays a key role in activating signaling cascades involved in cell growth, survival, proliferation, motility and morphology.
18	*ENDOG*	Endonuclease G, mitochondrial	Cleaves DNA at specific sites. Also has an RNase and RNase H activity. Can generate primers during mitochondrial DNA replication.
19	*IKBKG*	NF_KB essential modulator	Essential for viral activation of IRF3. Involved in TLR3- and IFIH1-mediated antiviral innate response.
20	*BAX*	Apoptosis regulator BAX	Accelerates Apoptosis by binding and repressing BCL2, activating CASP3, and releasing cytochrome *c* from mitochondria.

well [37]. Despite causing significant adverse effects in humans, very little information is known about the pathogenesis of EBV inside the host.

Our study suggests that EBV pathogenesis involves silencing the host genes via EBV miRNAs. We found several apoptosis related genes that are significantly controlled by EBV miRNAs. Similarly, Pfeffer et al. [38] studied the miRNAs of the Herpesvirus family viruses, where they adopted computational methods to predict pre-miRNAs and then experimentally obtained the miRNAs produced by these viruses. The data from both studies accentuates the fact that miRNA mediated gene silencing promotes viral pathogenesis. We studied the functions of the apoptosis related genes that are top targets of viral miRNAs and found that most of them are pro-apoptotic genes. Reduction in expression of these genes results in continued survival of the host cells and provides prolonged refuge to the viral particles when these genes are silenced by viral miRNAs.

Genes like *BAD, CASP9,* and *BID* are associated with induction of apoptosis in human cells, all of which are highly targeted by viral miRNAs. By observing the experimentally validated expression levels of these genes after introduction of viral miRNAs, we predict that viruses selectively target genes that positively control apoptosis and reduce their expression through miRNA.

miRNAs are emerging as an important molecule in the fields of molecular biology, genetic engineering etc. It is gaining attention as a regulator of genes and gene products. The viral miRNAs and the host targets identified in our study need further experimental validation. We predict that poorly understood pathogens can be fought with increased understanding of miRNA-gene interactions. Here, we showed the role of miRNAs in the establishment of viruses inside the human host through suppression of apoptosis which ensures their prolonged refuge. This finding can be further strengthened using appropriate experimental models to learn more about the host-pathogen interactions and combat emerging pathogens.

5. Availability of data and materials

The datasets supporting the conclusions of this article are included in the article and in the Supplementary files.

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Conflict of competing interest

All authors declared that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.imu.2020.100501.

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