Computational Analysis Revealed miRNAs Produced by Chikungunya Virus Target Genes Associated with Cellular Proliferation and Cell Cycle Regulation

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Research Article

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Abstract

Chikungunya virus (CHIKV) that causes chikungunya fever, is an alphavirus that belongs to the Togaviridae family containing a single-stranded RNA genome. Mosquitoes of the Aedes species act as the vectors for this virus and can be found in the blood, which can be passed from an infected person to a mosquito through mosquito bites. CHIKV has drawn much attention recently because of its potential of causing an epidemic. As the detailed mechanism of its pathogenesis inside the host system is still lacking, in this in silico research we have hypothesized that CHIKV might create miRNAs, which would target the genes associated with host cellular regulatory pathways, thereby providing the virus with prolonged refuge. Using bioinformatics approaches we found several putative miRNAs produced by CHIKV. Then we predicted the genes of the host targeted by these miRNAs. Functional enrichment analysis of these targeted genes shows the involvement of several biological pathways regulating cellular proliferation and cell cycle, thereby provide themselves with prolonged refuge and facilitate their pathogenesis, which in turn may lead to disease conditions. Finally, we analyzed a publicly available microarray dataset (GSE49985) to determine the altered expression levels of the targeted genes and found four genes (FLNA, GATA6, HES6, and TP53) associated with transcription factor binding, which have significant (Adjusted p-value <0.05) altered expression level. Our finding presents novel miRNAs and their targeted genes, which upon experimental validation could facilitate in developing new therapeutics to combat CHIKV infection and minimize CHIKV mediated diseases.

Introduction

Chikungunya virus (CHIKV) is a single-stranded, positive-sense RNA virus that causes a tropical disease called chikungunya fever which, in 1952, occurred first in Tanzania. It contains an icosahedral capsid, which is covered by a lipid layer. CHIKV is an alphavirus that belongs to the Togaviridae family and mosquitoes of the Aedes species act as the vectors for this virus. CHIKV can be found in the blood and can pass from an infected person to a mosquito through mosquito bites during the first week of infection. Specific cell types that are particularly susceptible to infection include human epithelial and endothelial cells, monocyte-derived macrophages, and primary fibroblasts. Following CHIKV infection, RNA and proteins of CHIKV have been found in synovial tissue and fluids, with synovial fibroblasts and macrophages susceptible to the infection. Infected macrophages are the preferred site for viral replication of CHIKV, contributing to viral persistence and chronic symptoms. Despite several kinds of research on this virus, the pathogenesis of persistent manifestations after CHIKV infection is still unclear. Proteins of chikungunya virus have been detected in macrophages and muscle cells of patients with relapse of chronic pain, suggesting that low replicative viruses or non-replicative CHIKV debris may persist. In vitro infection of human cells has exhibited the susceptibility of microglial cells, neuroblastoma cells, and glial cells, such as astrocytes. Yet, it is still unclear if the pathogenesis of the nervous system is directly connected with the infection of the neurons and glial cells or is circuitously connected triggering the immune-mediated effects.
MicroRNAs (miRNAs) are novel ideal models in the field of the molecular regulation of gene expressions. It is turning into a magnificent research topic day by day for different researchers engaged with molecular biology. miRNAs are ~22 nucleotide, brief, non-coding RNAs that are available in the vertebrates, invertebrates, plants, and in a wide range of viruses\textsuperscript{11,12}. The essential capacity of miRNAs is to regulate the expression of genes post-transcriptionally, through the base-pair formation with the 3'-untranslated region (3'-UTR) of distinct messenger RNAs (mRNA). miRNAs assume indispensable jobs in different biological processes, including the development of an organism, regulation of the immune system, cell proliferation, oncogenesis, customized cell passing or apoptosis, and so on\textsuperscript{13-17}. Previously, human miRNAs were accounted for quelling viral pathogenesis by targeting their genes\textsuperscript{18}. Additional examinations uncovered the possibility of viral miRNAs targeting their host genes\textsuperscript{19,20}, assuming unobtrusive jobs in the endurance and proliferation of viral particles through host immune system evasion, building up microenvironment for viral replication, regulation of the innate immune system, differentiation of versatile immune cells\textsuperscript{21-24}. In this study, to explore the mechanism of action of CHIKV mediated pathogenesis, we hypothesize that CHIKV-encoded miRNAs modulate host immune system and various physiological functions that provide the viruses selective advantages for prolonged refuge and disease pathogenesis within the host.

**Methods**

**Prediction of pre-miRNAs & Mature miRNAs**

From the National Center for Biotechnology Information (NCBI)\textsuperscript{25} we obtained the complete genome sequence of CHIKV (NCBI Reference Sequence: NC_004162.2). To predict the presence and positions of the pre-miRNAs in the genome sequence we used the miRNAFold\textsuperscript{26,27} tool using in-house Perl scripts with default parameters. Stem-loop secondary structure is one crucial feature to distinguish between pri-miRNA & pre-miRNAs. We used the Triplet SVM Classifier\textsuperscript{28} tool to find the true pre-miRNAs among a set of conserved stem-loops. The minimum bases for the stem-loop was set to 22 for the Triplet SVM Classifier\textsuperscript{28} tool. In addition to this, using FOMmir\textsuperscript{29} and matureBayes\textsuperscript{30}, we predicted the mature miRNAs from the pre-miRNA sequences. We utilized the default parameters to predict these sequences.

**Prediction of miRNA Target Genes**

We utilized RNAhybrid\textsuperscript{31} to obtain the genes targeted by the predicted miRNAs. Gene symbols and Ensembl gene IDs of the targeted genes were extracted from Ensembl\textsuperscript{32,33}. The annotated functions of all the genes were identified from the Gene Ontology Consortium (GO)\textsuperscript{34,35} where the Biological Processes (GOBP), Cellular Locations (GOCL), and Molecular Functions (GOMF) of the annotated genes are provided. The pathways involved in the physiology of humans were obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG)\textsuperscript{36,37}.

**Functional Enrichment Analysis**
We employed the online functional annotation tool Database for Annotation, Visualization, and Integrated Discovery (DAVID)\textsuperscript{38,39} to obtain the enriched biological processes and pathways involving the miRNA target genes. Specific processes related to similar functions were clustered together.

**Expression Profile Analysis**

Gene Expression Omnibus (GEO)\textsuperscript{40} is a public reservoir of microarray datasets. To obtain the expression level of each of the target genes the microarray dataset GSE49985 of CHIKV infected HEK293T cells using the platform GPL15207 Affymetrix Human Gene Expression Array was used\textsuperscript{41}. Processing the data showed the Log2 fold changes in the expression level of the total human genes and the expression levels of target genes were analyzed together with their significance levels (Adjusted p-value). A schematic diagram depicting the complete methodological strategy of this study is provided in Figure 1.

**Results**

**Putative miRNAs Produced by CHIKV**

To identify whether CHIKV produces any miRNAs, we used the tool miRNAFold that yielded 200 putative pre-miRNA candidates (data not shown). Triplet SVM Classifier predicted all of them to be truly positive. Finally, using FOMmir and matureBayes we found a set of 48 putative mature miRNA sequences from the CHIKV genome (Supplementary File 1).

**Genes Targeted by Putative CHIKV miRNAs**

After obtaining the putative miRNAs we uploaded them to the online tool RNAhybrid. This online tool provided a total of 339 genes that were targeted by the CHIKV miRNAs among which, 234 genes were unique for *Homo sapiens*. We considered these 234 genes for further analyses. A table containing the putative CHIKV miRNAs and their target genes is provided in the Supplementary File 2.

**Functional Enrichment Analysis**

To understand the functions of the genes targeted by the putative miRNAs produced by CHIKV we used the online functional annotation tool DAVID that provided us with the functionally enriched biological processes and pathways. DAVID revealed a myriad of important biological processes and pathways associated with the regulation of cell cycle arrest, protein kinase activity, brain development, and cell proliferation. Interestingly, the putative miRNAs of CHIKV targeted four genes (*CDKN2A, CRLF3, GATA6,* and *TP53*) that are associated with positive regulation of cell cycle arrest, while 11 other targeted genes (*SSTR5, CTBP1, CDKN2A, CNOTB, CDKN2B, SPEG, WDR6, TP53, NPPC, PTH2, and ADORA1*) are associated with negative regulation of cell proliferation (Figure 2).

Moreover, Gene Ontology Molecular Function (MF) showed that 10 of the targeted genes (*SPDYE2B, CDKN2A, CDKN2B, GATA6, BCAR1, TP53, CALM3, SPDYE4, SPDYE5, and SPDYE6*) are associated with protein kinase binding, eight genes (*CTBP1, CDKN2A, ESRRB, GATA6, TP53, HES6, RUNX1,* and *FLNA*) are
associated with transcription factor binding and five genes (CYBA, SLC48A1, HBM, ABCB6, and MB) are associated with heme binding (Figure 3). While investigating the functional category of the genes, we found five (SPDYE2, SPDYE2B, SPDYE4, SPDYE5, and SPDYE6) targeted genes, products of which are cell cycle regulatory proteins (p-value: 0.00000984).

**Expression Profile Analysis**

To investigate the differential gene expression level of the targeted genes we used the microarray dataset GSE49985 of CHIKV infected HEK293T cells. Fold change values of gene expression were calculated comparing the uninfected control replicates to the CHIKV infected replicates of HEK293T cells. Out of 234 targeted genes, 28 genes showed significant differential expression levels (Table 1). Among these genes, 25 were significantly down-regulated, whereas, only three genes were significantly up-regulated due to CHIKV infection. Interestingly, we found four genes (FLNA, GATA6, HES6, and TP53), which are associated with transcription factor binding and positive regulation of cell cycle arrest.

**Discussion**

CHIKV has become a global health concern for its potential of causing epidemic and thus has drawn much attention recently. Though a lot of research has been done or currently ongoing, the detailed mechanism of its pathogenesis inside the host system is still wanting. In this in silico research we have mainly focused on the possibility that CHIKV might create miRNAs, which would target the genes associated with host cellular regulatory pathways, thereby providing the virus with prolonged refuge.

It is known already that miRNAs produced by humans target viral genes so that they can prevent potential viral pathogenesis. A previous study on the Zika virus (ZIKV) was also performed by us based on similar strategy. By switching the disease-causing genes of a virus off the host system ensures its disease suppression. Pathogenic viruses cause several diseases in human and human defense machinery to continuously encounter and remove these pathogenic viruses from the system. To evade these host defense molecules viruses might have further evolved to produce miRNAs to silence host genes. This silencing can provide them with various selective advantages including host defense evasion, viral replication, and diminishing antiviral responses. To accentuate this event whether CHIKV effectively targets and controls host genes we proceeded with several scientific works from different laboratories and gained insight into the role of CHIKV miRNAs in their pathogenesis.

To observe the presence of putative precursor-miRNAs by CHIKV we used the miRNAFold tool and we found 200 such pre-miRNA sequences. These pre-miRNA sequences obtained from miRNAFold were then validated by Triplet SVM Classifier. Then, we obtained 48 mature miRNAs that might be produced by CHIKV using FOMmir and matureBayes. After that, we used RNAhybrid that yielded 234 target genes unique for Homo sapiens. Some of these target genes (CDKN2A, CRLF3, GATA6, and TP53) were associated with regulating the progression of cell cycle arrest, while some other genes (SSTR5, CTBP1, CDKN2A, CNOT8, CDKN2B, SPEG, WDR6, TP53, NPPC, PTH2, and ADORA1) were found to be associated
with preventing cellular proliferation. From this observation it can be inferred that miRNAs of CHIKV target those genes that are associated with regulating the cellular proliferation and cell cycle, thus ensuring their prolonged refuge while inside the host system. Additionally, some target genes function in the transcription factor binding and protein kinase binding activities. Viral miRNAs might target these genes to facilitate their own replication, transcription, and/or translation. Surprisingly, the functional category of five of the targeted gene products (SPDYE2, SPDYE2B, SPDYE4, SPDYE5, and SPDYE6) were cell cycle regulatory proteins. Finally, analyzing the differentially expressed genes of CHIKV infected cells we found we found four genes (FLNA, GATA6, HES6, and TP53), which are associated with transcription factor binding and positive regulation of cell cycle arrest. These findings substantiate our hypothesis that CHIKV miRNAs may target the host genes associated with cell cycle regulation. Besides, the functions of the target genes associated with cell cycle regulation were identified using the UniProt protein database to better understand the mechanism of disease prognosis in humans by CHIKV infection through miRNA production (Table 2). Based on these findings we propose a mechanism of CHIKV pathogenesis through miRNA-mediated gene silencing (Figure 4).

Conclusion

In this study, we propose a mechanism, which portrays that CHIKV may progress its pathogenesis through producing miRNAs that target and downregulate essential genes involved in regulating cellular proliferation and cell cycle. We predicted several novel miRNAs, which may be produced by CHIKV, and interestingly, the genes targeted by these miRNAs are associated with regulating cell cycle as well as cellular proliferation. This study will serve as an important pathfinder for the researchers in identifying the pathogenic pathways that this virus may employ. Further experimental analysis of these miRNAs would enhance our understanding to better combat this virus in the future through novel therapeutic interventions.

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**Declarations**

**Data availability**

All data generated or analyzed during this study are available in online public repositories, within the article, and in the supplementary files.
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Author Contributions

MSI conceived the project and designed the study. MSI and MAAKK performed the analyses. MSI wrote the manuscript. All authors reviewed the manuscript.

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Competing Interests Statement

The authors declare no competing interests.

Tables

**Table 1:** List of genes having significant differential expression level
| Gene   | Log (Fold Change) | Adjusted p-value |
|--------|------------------|------------------|
| MGAT4B | -1.56927         | 0.01338          |
| UBE2S  | -1.36694         | 0.004            |
| ASNA1  | -1.20582         | 0.00959          |
| TNPO3  | -1.15124         | 0.00744          |
| FLNA   | -0.94781         | 0.00562          |
| STARD10| -0.7669          | 0.01529          |
| SLC38A7| -0.76396         | 0.00765          |
| RNF167 | -0.76223         | 0.00589          |
| KLC1   | -0.76181         | 0.01521          |
| EIF5A  | -0.71907         | 0.00659          |
| XXYLT1 | -0.71621         | 0.00952          |
| R3HCC1 | -0.66903         | 0.00602          |
| PUM1   | -0.66749         | 0.00976          |
| ATPAF1 | -0.66607         | 0.04837          |
| REPIN1 | -0.6544          | 0.01659          |
| UCKL1  | -0.58521         | 0.00901          |
| CD276  | -0.548           | 0.03846          |
| ISYNA1 | -0.51783         | 0.03705          |
| VPS53  | -0.50421         | 0.04513          |
| PLEKHJ1| -0.47574         | 0.01977          |
| NOP2   | -0.45989         | 0.037            |
| TP53   | -0.44728         | 0.02388          |
| MSRB1  | -0.42097         | 0.03218          |
| PXMP2  | -0.36449         | 0.03163          |
| ATP5D  | -0.34768         | 0.02705          |
| GATA6  | 0.400998         | 0.04212          |
| HES6   | 0.514125         | 0.04796          |
| ZNF83  | 0.942298         | 0.01181          |

**Table 2:** Functions of genes associated with cell cycle regulation targeted by CHIKV
| Gene Name | Protein Name | Protein Function |
|-----------|--------------|-----------------|
| **ADOR1** | Adenosine receptor A1 | Receptor for adenosine. The activity of this receptor is mediated by G proteins which inhibit adenylyl cyclase. |
| **CDKN2A** | Cyclin-dependent kinase inhibitor 2A | Acts as a negative regulator of the proliferation of normal cells by interacting strongly with CDK4 and CDK6. This inhibits their ability to interact with cyclins D and to phosphorylate the retinoblastoma protein. |
| **CDKN2B** | Cyclin-dependent kinase 4 inhibitor B | Interacts strongly with CDK4 and CDK6. Potent inhibitor. Potential effector of TGF-beta induced cell cycle arrest. |
| **CNOT8** | CCR4-NOT transcription complex subunit 8 | Has 3’-5’ poly(A) exoribonuclease activity for synthetic poly(A) RNA substrate. Its function seems to be partially redundant with that of CNOT7. Catalytic component of the CCR4-NOT complex which is linked to various cellular processes including bulk mRNA degradation, miRNA-mediated repression, translational repression during translational initiation, and general transcription regulation. During miRNA-mediated repression, the complex seems also to act as a translational repressor during translational initiation. Additional complex functions may be a consequence of its influence on mRNA expression. Associates with members of the BTG family such as TOB1 and BTG2 and is required for their anti-proliferative activity. |
| **CRLF3** | Cytokine receptor-like factor 3 | May play a role in the negative regulation of cell cycle progression. |
| **CTBP1** | C-terminal-binding protein 1 | Corepressor targeting diverse transcription regulators such as GLIS2 or BCL6. Has dehydrogenase activity. Involved in controlling the equilibrium between tubular and stacked structures in the Golgi complex. Functions in brown adipose tissue (BAT) differentiation. |
| **FLNA** | Filamin-A | Promotes orthogonal branching of actin filaments and links actin filaments to membrane glycoproteins. Plays a role in cell-cell contacts and adherens junctions during the development of blood vessels, heart, and brain organs. |
| **GATA6** | Transcription factor GATA-6 | Transcriptional activator. Regulates SEMA3C and PLXNA2. Involved in gene regulation specifically in the gastric epithelium. May regulate genes that protect epithelial cells from bacterial infection. Involved in bone morphogenetic protein (BMP)-mediated cardiac-specific gene expression. Binds to BMP response element (BMPRE) DNA sequences within cardiac activating regions. |
| **HES6** | Transcription cofactor HES-6 | Promotes cell differentiation. |
| **NPPC** | C-type natriuretic | Hormone which plays a role in endochondral ossification through regulation of cartilaginous growth plate chondrocytes proliferation and differentiation. May also be vasoactive and natriuretic. Specifically binds and |
| **peptide** | **stimulates the cGMP production of the NPR2 receptor. Binds the clearance receptor NPR3.** |
|-------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| **PTH2**    | **Peptidyl-tRNA hydrolase 2** The natural substrate for this enzyme may be peptidyl-tRNAs which drop off the ribosome during protein synthesis. Promotes caspase-independent apoptosis by regulating the function of two transcriptional regulators, AES and TLE1. |
| **SPEG**    | **Striated muscle preferentially expressed protein kinase** May have a role in regulating the growth and differentiation of arterial smooth muscle cells. |
| **SSTR5**   | **Somatostatin receptor type 5** Receptor for somatostatin 28 and to a lesser extent for somatostatin-14. The activity of this receptor is mediated by G proteins which inhibit adenyly cyclase. Increases cell growth inhibition activity of SSTR2 following heterodimerization. |
| **TP53**    | **Cellular tumor antigen p53** Acts as a tumor suppressor in many tumor types; induces growth arrest or apoptosis depending on the physiological circumstances and cell type. Involved in cell cycle regulation as a trans-activator that acts to negatively regulate cell division by controlling a set of genes required for this process. One of the activated genes is an inhibitor of cyclin-dependent kinases. Apoptosis induction seems to be mediated either by stimulation of BAX and FAS antigen expression or by repression of Bcl-2 expression. |
| **WDR6**    | **WD repeat-containing protein 6** Enhances the STK11/LKB1-induced cell growth suppression activity. Negative regulator of amino acid starvation-induced autophagy. |

**Figures**
Figure 1

Schematic diagram summarizing the study.
Figure 2

Enriched biological processes by DAVID. The x-axis denotes the p-value, while the y-axis represents the enriched processes.
Figure 3

Enriched molecular functions by DAVID. The x-axis denotes the p-value, while the y-axis represents the enriched functions.
Figure 4

Schematic diagram illustrating the mechanism of CHIKV pathogenesis. After entering into the host cell CHIKV releases its genomic RNA, which is translated into necessary proteins. Some of the RNAs are converted to miRNAs that target specific host mRNAs, thereby alter the expression level of the genes as well as the pathways associated with them. This results in viral pathogenesis.

Supplementary Files

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- SupplementaryFile2.docx