LIBYAN JOURNAL OF MEDICINE
2021, VOL. 16, 1909902
https://doi.org/10.1080/19932820.2021.1909902

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ORIGINAL ARTICLE

Differential expression of miRNAs in a human developing neuronal cell line chronically infected with Zika virus

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ABSTRACT

Zika virus (ZIKV) is a serious public health concern that may lead to neurological disorders in affected individuals. The virus can be transmitted from an infected mother to her fetus, via mosquitoes, or sexually. ZIKV infections are associated with increased risk for Guillain-Barré syndrome (GBS) and congenital microcephaly in newborns infected prenatally. Dysregulations of intracellular microRNAs (miRNAs) in infected neurons have been linked to different neurological diseases. To determine the potential role of miRNAs in ZIKV infection we developed a chronically infected neuroblastoma cell line and carried out differential expression analyses of miRNAs with reference to an uninfected neuroblastoma cell line. A total of 3192miRNAs were evaluated and 389 were found to be upregulated < 2-fold and 1291 were downregulated < 2-fold. In particular, we determined that *hsa-mir-431-5p*, *hsa-mir-3687*, *hsa-mir-4655-5p*, *hsa-mir-6071*, *hsa-mir-762*, *hsa-mir-5787*, and *hsa-mir*-6825-3p were significantly downregulated, ranging from -5711 to -660-fold whereas, *has-mir-4315*, *hsa-mir-5681b*, *hsa-mir-6511a-3p*, *hsa-mir-1264*, *hsa-mir-4418*, *hsa-mir-4497*, *hsa-mir-4485-3p*, *hsa-mir-4715-3p*, *hsa-mir-4433-3p*, *hsa-mir-4708-3p*, *hsa-mir-1973* and *hsa-mir-564* were upregulated, ranging from 20-0.8-fold. We carried out target gene alignment of these miRNAs with the ZIKV genome to predict the function of the differentially expressed miRNAs and their potential impact on ZIKV pathogenesis. These miRNAs might prove useful as novel diagnostic or therapeutic markers and targets for further research on ZIKV infection and neuronal injury resulting from ZIKV infectivity in developing fetal brain neurons.

ARTICLE HISTORY
Received 9 December 2020
Accepted 25 March 2021

KEYWORDS
Chronic infection; dysregulation; human neuronal progenitor cell line; microRNA; Zika virus

1. Introduction

Zika virus was discovered in 1947 [1]. The first report of humans infected with ZIKV was from Nigeria in 1954, following an outbreak of jaundice in Afikpo [2]. From 1954 until 2015, ZIKV infection was considered an innocuous virus being either asymptomatic or occasionally causing mild symptoms in infected individuals [3]. However, in early 2015, a serious ZIKV outbreak was recorded in eastern regions of Brazil (i.e. Camaçari, Bahia) [4] and similar illnesses in several neighboring states prompted the Ministry of Health to issue a ZIKV alert in April [5]. By the end of the year the virus had spread to much of the northeastern part of the country. Subsequently, the virus migrated throughout most other South and Central American countries [6] and the pandemic swept into the Caribbean with autochthonous transmission in at least six island states as well as in French Polynesia [5,6]. The rapid movement of ZIKV together with troubling reports of association between ZIKV and infants born with microcephaly prompted the WHO to issue an 'International Health Emergency' on 1 February 2016. A direct link between ZIKV infection and increased incidence of neurologic disorders and microcephaly were established (reviewed in 4–6). More recently, sexual transmissions of ZIKV, particularly in men who have cleared the virus from the blood and other bodily fluids, have been documented [5,7].

Since the beginning of the ZIKV pandemic, thousands of cases presenting ZIKV symptoms have been documented [8]. The virus causes an acute febrile illness and is characterized by mild headache, fever, maculopapular rash, joint and back pain, and general malaise, sometimes accompanied by conjunctival hyperemia, anorexia, dizziness, diarrhea, and constipation [8]. The incubation period is between 3 and 12 days and symptoms may last for 2 and 7 days. Only ~20% of presenting patients infected with ZIKV exhibit symptoms and the virus has never been reported to cause hemorrhagic fever or death, often resulting in misdiagnosis as dengue [7–9]. Due to still unexplainable reasons the number of cases with ZIKV has precipitously declined in recent years in highly affected countries [10]. For example, in mid-August 2017 there was a single case of ZIKA infection as

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compared to hundreds of cases in the previous year. Similarly, ZIKV infections have plummeted in Latin America and the Caribbean, where it caused havoc in 2015–2016 [10]. It is hypothesized that this decline is due to cross immunity from Dengue infection [10,11].

Previously, we reported on genome-wide binding sites in ZIKV766 for miRNA that shared mutually homologous sequences with microcephaly (MCPH) genes, using computational analyses. We identified six miRNAs that shared mutual homology to 12 MCPH genetic motifs [12] and hypothesized that ZIKV genes regulated numerous host miRNAs, resulting in miRNA-based modifications of neuronal genetic pathways. miRNAs are small noncoding RNAs that act as gene regulators, acting directly or indirectly on many cellular functions owing to their ability to target mRNAs for degradation or translational repression [13]. Experimental evidence shows that miRNAs have a direct role in different cellular processes, such as immune function, apoptosis, and tumorigenesis. In a viral infection context, miRNAs have been linked to the interplay between host and pathogen, occupying a major role in pathogenesis. While numerous viral miRNAs from DNA viruses have been identified, characterization of functional RNA virus-encoded miRNAs, and their potential targets as therapeutic agents against numerous viral infections is still ongoing [14,15]. How cellular miRNAs are regulated, and their functions controlled during ZIKV infection, is largely unknown. miRNAs are critical regulators of gene expression that utilize sequence complementarity to bind to and modulate the stability or translation efficiency of target mRNAs. In the case of ZIKV profound modulations of miRNAs was observed in a chronically infected neuronal cell line. Our sequence-based data suggest that ZIKV-mediated miRNAs appear to modulate a wide range of gene functions in ZIKV-infected neurons, perhaps being responsible for a wide variety of neuronal functions in developing fetal brain that lead to microcephaly and an array of other neurologic illnesses observed in newborns from ZIKV infected mothers [13].

In the present study, we have conducted a full miRNA expression profiling utilizing Qiagen miR-array and identified 12 miRNAs that were significantly upregulated and 7 miRNAs that were significantly downregulated. We carried out an homology alignment with modulated miRNAs with the ZIKV genome to determine the viral gene sequences that may be responsible for the molecular pathogenesis of ZIKV-induced neurologic effects in developing fetuses. Our findings will underpin further studies of miRNAs' roles in ZIKV replication and may identify potential candidates for antiviral therapies against ZIKV as well as mechanisms of microcephaly found in ZIKV infected newborns [14,15].

2. Material and methods

2.1. Reagents and cell lines

Five neuroblastoma (NB) cell lines were purchased from ATCC (New York, NY). CRL-2267, CCL-127, CRL-2271, CRL-2266, CRL- 2149, and CRL-2142, were cultured in Eagle's Minimum Essential Media (EMEM) (ATCC, Manassas, VA) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS), and L-Glutamine-penicillin-streptomycin solution (designated complete media) (Sigma, St. Louis, MO) at 37° C, 5% CO_2. The stock cell cultures were grown in 25 mL or 75 mL flasks (Thermo-Scientific, Nunc, Rochester, NY). ZIKV strains KU501215 and MR 766 were provided by the CDC (kind gifts from Brandy Russel, Fort Collins, CO).

2.2. Propagation of ZIKV

ZIKV766 strain was propagated in Vero cells (ATCC). The cells were grown in EMEM at 70% confluency in 25 mL tissue culture flasks (Nunc Inc., USA). Following removal of the culture media, virus inoculum was added to give a Multiplicity of Infection (MOI) of 0.1 to 0.05/cell. Flasks were incubated at 37°C, 5% CO_2 with gentle agitation for 30 minutes. After incubation, 5 mL of complete media was added, and the cells were maintained for 5–9 days or until cells exhibited cytopathic effects (CPE). Cells were trypsinized and cryopreserved in 50% FBS and 10% DMSO at −80° C and used in subsequent studies [16].

2.3. Infection of neuroblastoma cell lines with ZIKV

Previously, we have demonstrated the permissiveness of NB cell lines to ZIKV [16]. All 6 undifferentiated cell lines were grown in 25 mL tissue culture flasks to ~70% confluency, media was removed and immediately exposed to an estimated 0.1–0.05 MOI or at 1.0 to 1.5 in 1.0 mL serum free media. For maximum infectivity, the flasks were intermittently gently agitated for 1 h and then 5 mL of pre-warmed media was added. Control cultures were treated identically, except no ZIKV was added to the inocula. The cells were incubated at 37°C, 5% CO_2. The cell cultures were observed every 12 h for CPE and images recorded on a digital camera. In addition, each cell line was cultured in 12-well culture plates (Cellstar, Greiner Bio-one, CAT #665-180) in triplicates and infected with ZIKV at 0.1 MOI. A parallel 12-well

plate each line was cultured without viral infection. Each week the CPE as well the cellular viability was determined by Eosin-Y viability method [17]. Each experiment was repeated at least three times for low MOI and twice for high MOI infected cells [7].

2.4. Development of chronically infected cell line

All 6-cell lines were observed for CPE and monitored for several weeks. After 6–8 weeks of culture few cells survived in five out of six cell lines. Only CRL-127 survived and grew without exhibiting in CPE (Table 1). CRL-127 cell line was passaged approximately every 5 days and observed for further growth, replication CPE. As a result, a cell line chronically infected with ZIKV was established from the CCL-127 cell line. This cell line was grown with weekly passage for at least 8 months. The control cell line was grown in parallel with the same growth conditions and passage simultaneously to decrease any inadvertent miRNA modulations by environmental factors. The supernatant fluid of both cell lines (control vs chronically infected) was tested for presence of ZIKV by PCR [17].

2.5. PCR

The ZIKV RNA from the chronically infected cell lines and from the supernatants were detected first by using one-step RT-PCR. Viral RNA was extracted from culture pellet using GenElute Mammalian Total RNA Miniprep kit (Sigma, St. Louis, MO) according to the manufacturer's protocol. Two sets of primer pairs were designed to target the 5′UTR region of the virus genome (Forward primer, ZIKV_F′- 5′-TTGGTCATGATACTGCTGATTGC-3′ and reverse primer, ZIKV_R, 5′-CCTTCCACAAAGTCCCTATTGC-3′) and (Forward primer, Zika E_F, 5′AAGTTTGCATGCTCCAAGAAAAT-3′ and reverse primer, Zika E_R,-5′CAGCATTATCCGGTACTCCAGAT-3′). The thermal cycling profile of this assay consisted of a 10 min cDNA synthesis step at 50°C, 5 min of iScript reverse transcriptase inactivation at 95°C, followed by 35 cycles of PCR at 95°C for 10 sec [16].

Table 1. Cell Viability* of the six cell lines infected with ZIKV at 0.1 MOI.

Cell line	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
CRL-2266	22	8	2	0	0	0
CRL-2267	29	11	3	<1	0	0
CRL-2271	19	9	4	1	0	0
CRL-2142	25	10	3	0.9	0.1	0
CRL-2149	16	7	2	<.4	0.1	0
CRL-127	31	13	19	37	59	66

*Each cell line was infected with an estimated 0.1 to 0.05 MOI of ZIKV.

2.6. Micro RNA profiling

The miRNA profiling was carried out by a QIAseq miRNA Library Kit library construction process; individual miRNA molecules were tagged with Unique Molecular Indices (UMI). Following sequencing, raw reads were mapped to individual miRNA molecules instead of just individual miRNAs. This allowed for true quantification of the miRNAs by eliminating any library amplification and sequencing bias [17].

Total RNA sample quality controls were performed by two sequencing steps: first, the quantity and purity were evaluated using a Nanodrop 2000 spectrometer (Thermo Scientific). UV absorption at 260 nm was used to measure RNA quantity. Absorption 260/280 ratio ≥ 2.0 and 260/230 ratio ≥ 1.8 were used as indicators of acceptable purity. Low 260/280 ratios are generally due to phenol and/or protein contaminations. Whereas, low 260/230 ratios are due to salt (e.g. guanidine isothiocyanate) and/or phenol contaminations. Contaminants may result in downstream problems by inhibiting enzymatic labeling reactions. Second, RNA sample integrity was evaluated using an Agilent Bioanalyzer 2100 (Agilent). A high-quality eukaryotic RNA sample was ensured by determining the band intensity ratio of 28S rRNA to 18S rRNA of two [13].

Since we aimed to discover differential expression of miRNAs between uninfected control vs chronically infected cell lines, all samples were prepared using the same procedure. Multiple species array chips were used in our study. Each sample passed quality controls: UV absorption 260:280 ratio ≥ 2.0, UV absorption 260:230 ratio ≥ 1.8, Bioanalyzer (Agilent) RNA Integrity Number (RIN) ≥ 8 for human samples. A complete and detailed protocol is described in QIAseq miRNA handbook [18].

3. Results and discussion

In the present study we developed a chronically ZIKV-infected cell line from a human progenitor neuronal cell line and isolated miRNAs that were either upregulated or downregulated.

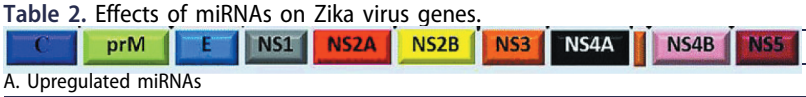
In order to develop chronically infected cell line(s) that that can maintain ZIKV infection we infected 6-NB cell lines at 0.05–0.1 MOI of ZIKV. We determined the MOI as we described previously [17]. After infection we regularly observed each of the infected cell line for CPE as well as determined the cell viability by Eosin-Y method. The results of our weekly viability assay are described in Table 1. As it is shown in Table 1, the cytopathic effects of ZIKV on each of the neuroblastoma cell line was significant around third week after the infection. And, between 4–6 weeks post infection all the neurons in 5 cell lines have died, except for CRL-127. In CRL-127 the infected cells, which initially

exhibited a significant CPE, began to develop resistant to ZIKV. This cell line developed intracellular mechanism to ZIKV-induced CPE. We continue to culture CRL-127 (designated CRL-127Z*) for 8 months. To determine the intracellular resistance to ZIKV we carried out miR analyses. In our previous report we have shown the potential role of miR in ZIKV [13].

To identify differentially expressed miRNAs in the chronically ZIKV-infected CRL-127 neuronal cell line, QIAseq miRNA NGS data analyses was carried out. Of 3192 miRNAs detected, 278 miRNAs were upregulated ranging from 2- to 451.55-fold. We selected 12 miRNAs that were upregulated, ranging from 20- to 10.8-fold (Table 2). We found 1287 miRNAs were downregulated ranging from 2- to 5611-fold. We selected the top seven downregulated miRNAs (Table 2) that were significantly downregulated, ranging from −1895 to −5711-fold. We carried out sequence alignment for each of the 19 dysregulated miRNAs and identified the locations of each of the miRNAs on the ZIKV genome using mirbase (http://www.mirbase.org/). The location of each of the miRNAs are shown in Figure 1.

ZIKV is a positive sense, single-strand ribonucleic acid (ssRNA) virus with a genome size of approximately 10.8 kilobases [7]. This viral RNA is translated into a single polyprotein of 3423 amino acids in length which encodes 3 structural proteins – capsid (C), envelope (E), and membrane (M). The M is generated from its precursor premembrane (prM). In

Table 2. Effects of miRNAs on Zika virus genes.



A. Upregulated miRNAs

Name of miRNA	Degree of upregulation (fold)	Target gene
has-miR-4315	19.75	NS4B
has-miR-568-1b	16.57	NS3
has-miR-6511a-3p	16.01	Nucleocapsid
has-miR-1264	14.37	NS1
has-miR-4418	14.17	Envelope
has-miR-4497	13.46	NS1
has-miR-4485	13.42	NS1
has-miR-4433-3p	13.25	NS4B
has-miR-4715-3p	13.17	NS5
has-miR-4708-3p	11.66	Membrane
has-miR-1973	11.04	NS5
has-miR-564	10.8	NS5

B. Downregulated mRNAs

Name of miRNA	Degree of downregulation (fold)	Target Gene
has-miR-431-5p	−5711	Nucleocapsid
has-miR-3687	−1895	Membrane
has-miR-4655-5p	−1780	NS4A
has-miR-6071	−1011	NS1
has-miR-762	−745	Envelope
has-miR-5757	−677	Nucleocapsid
has-miR-6825-3p	−660	Envelope

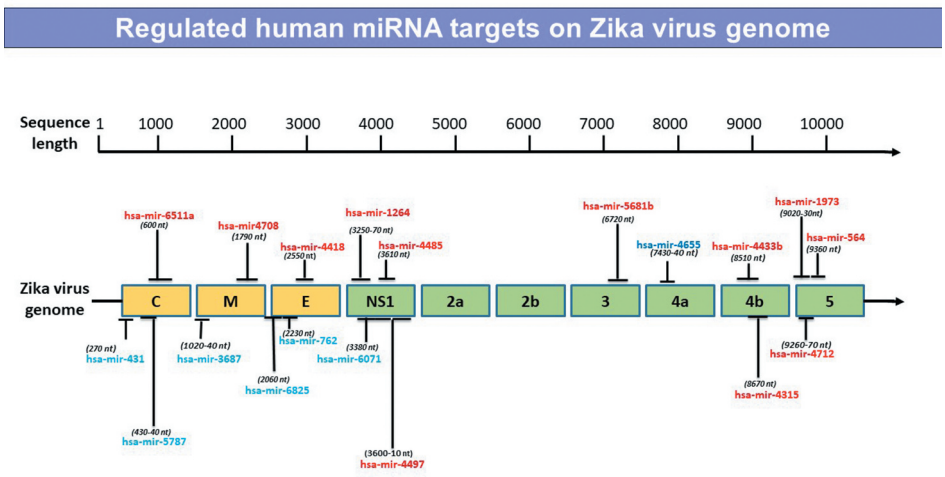


Figure 1. Locations of 19 miRNA to ZIKV766 genome. The illustration shows the 10.79KB genomic map of ZIKV766 while the exact nucleotide locations of each of the 19 miRNAs are showed on the ZIKV genome. The upregulated miRNAs are shown in red color whereas, the downregulated miRNAs are shown in blue. The genomic map of ZIKV is illustrated in two color: yellow boxes represent structural genes and green boxes represent non-structural genes. The alignment of each of the miRNA also shows the ZIKV nucleotide binding sites.

addition, the ZIKV ssRNA also encodes for seven non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. The structural proteins play a crucial role in replication and packaging of the viral genome as well as in disrupting the host pathways in favor of the virus. The generation of the 10 individual proteins from the polyprotein is regulated by viral and host proteases, as well by host miRNAs [7]. ZIKV replication occurs on complex virus-induced membrane structures incorporating host and viral factors [7,19,20] where it is believed that the replication process of ZIKV encounters host regulatory factors and the intracellular defense system of the host including miRNAs [13]

As illustrated in Figure 1, for CRL-127 neurons chronically infected with ZIKV, the intracellular immune system - miRNAs- are able to suppress viral infection by upregulating several hundred miRNAs to the point that the virus is no longer pathogenic to the neurons, even though CRL-127 represents progenitor neurons that are constantly replicating and represent neurogenetic neurons [21]. *has-mir-6511a, has-mir 4708* and *has-4418* show upregulation to apparently suppress initial viral replication steps by quelling C, M and E synthesis. However, it appears that the viral replication mechanisms may have counter strategies to block host defenses by downregulating other miRNAs including *has-mir-431, has-mir3687, has-mir-6825* and *has-mir-762*. Of note, the degree of downregulations are strikingly high as compared to the upregulated miRNAs that the host can mount (Table 2). We are not certain yet if these relatively mute miRNA upregulations are due to the counteractive downregulation. Of note, the 50-nm mature ZIKV particle is made of 180 copies of the E and M proteins embedded in the viral membrane [7,19,20]. Therefore, the precise numbers of E and M protein synthesized is an essential step in the final assembly of the viral particle [19].

As shown in Figure 1 and Table 2, the host intracellular molecular immune system, also mounted by suppressing structural protein synthesis, results in upregulation of *has-mir-1264* and *has-mir-4485* to suppress the ZIKV structural protein NS1. Two host miRNAs, *has-mir-6071* and *has-mir-4497*, are downregulated, and both bind to NS1 of the viral genome, the former down-stream of *has-mir-1264*, whereas, has-*mir-4497* at almost the identical site where *has-mir-4485* is upregulated, apparently, to counteract the suppression of NS1. In addition, several other miRNAs: *has-mir-5681b, has-mir-4433b, has-mir-1973* and *has-mir-564*, that bind to three structural proteins, NS3, NS4B and NS5, are upregulated whereas, *has-mir-4655* which binds to NS4A, is downregulated (Figure 1).

3.1. Capsid protein and miRNAs

Capsid protein is a ~ 12-kDa protein comprising the first ~105 residues of the ZIKV polyprotein. Capsid is the primary structural protein that interacts with the viral genome within virus particles and is essential for efficient packaging. Capsid dimers can bind a wide range of nucleic acid templates including the host RNAs, interfering in RNA splicing and RNA transcription. Capsid also enter the nucleolus and interacts with miRNAs and, therefore, may quell the molecular immune response against ZIKV [20,22]. Capsid protein expresses hydrophobic and hydrophilic regions that appear to play important roles in the pathogenesis of the virus. Capsid also causes significant dysregulation of host ribosomal biogenesis [20,22–25]. The hydrophobic regions of capsid proteins interact with the membrane of the endoplasmic reticulum (ER) whereas the hydrophilic region interacts with viral RNA. Binding of RNA to capsid starts particle formation by initiating aggregation of capsid. The aggregation of membrane-associated capsid into the nucleocapsid structure induces budding into the ER and the formation of immature virus particles. Capsid protein binds ssRNA, dsRNA and DNA in a sequence-independent manner via electrostatic interactions with the negatively charged phosphate backbone [20]. The packaging inside ER membrane compartments precludes capsid from packaging host RNAs.

The capsid interacts with the viral genomic RNA within virions. Interestingly, prior to encapsulation, capsid dimers are stored on lipid droplets. Thus, capsid's ability to interact with lipid droplets is crucial for efficient production of viral particles [22–25].

has-mir-6511a-3p, which shares homology to the ZIKV nucleocapsid gene is significantly upregulated, whereas two miRNAS-*has-mir-431-5p* and *has-mir -5757* which share homologies to the ZIKA nucleocapsid RNA, are significantly downregulated. We do not know the precise roles of these miRNAs in ZIKV replication, nor the pathways of ZIKV synthesis that are affected by dysregulation in these miRNAs. Recently, Zeng et al [26] have identified a capsid mutant that loses interaction with Dicer-mediated miRNA and disrupts neurogenesis in neurogenesis in neural stem cell. Further studies will be needed to decipher the precise effects of these miRNAs' *in vitro* [23,27].

3.2. Envelop protein synthesis

Protein E protrudes from the viral surface and is the primary antigen associated with the host adaptive immune system [24,25,27]. The E protein is an important aspect of host cell receptor recognition, virus entry and viral assembly [25]. Of note, E goes through immediate conformational changes due to the sudden change in pH from the extracellular to the endosomal environment upon entry [27]. The host's adaptive immune system to E protein has an important role in development of neutralizing antibodies [27]. Therefore, any interference to E protein synthesis

by the host's immune systemthat is, *has-mir-4418*-would represent an important defense mechanism. It is unclear as to whether upregulation of this miRNA breaks up the virus-host cell membrane fusion process, an essential step in viral entry [27]. In reaction to the host's defensive response, the ZIKV may neutralize and downregulate *has-mir-762* and *has-mir-6825* with sequence homologies to E mRNA (Figure 1).

3.3. Non-structural protein NS1

The NS1 protein is a glycosylated 48-kDa homodimer protein and a key in ZIKV replication, pathogenesis, and modulation of host immune response [7,28]. Within an infected cell, following translocation into the ER lumen, NS1 becomes a membrane-associated dimer, where it plays a role essential in viral genome replication. The replication complex at the ER membrane includes NS1 on the lumen side, viral transmembrane proteins (NS2a, NS2b, NS4a, NS4b), and viral enzymes (NS3 protease helicase, NS5 capping enzyme and RNA-dependent RNA polymerase) on the cytoplasmic side. NS1 is also secreted as a hexameric lipo-protein particle which can be detected in the serum of infected individuals, with serum levels correlating with the severity of the disease. Secreted NS1 (sNS1) interacts with the host's innate immune system (complement system proteins) and adaptive immune system, disrupting immune regulation [26–28].

ZIKV NS1 includes three distinct regions, an N-terminal β-roll, an epitope-rich wing region, and a C-terminal β-ladder [28]. Twelve invariant cysteines form six disulfide bonds per monomer. The fundamental unit is formed via the intertwined β-roll and end-to-end β-ladders. On the 'inner' face of the dimer, the β-roll region and an adjacent, so called 'greasy finger' loop, is hydrophobic and is the primary space for membrane interaction, and it associated with viral transmembrane proteins [29]. The dimer 'outer' face is polar and contains the glycosylation sites, polar faces point outward and hydrophobic faces point inward, where they can interact with lipid molecules in the secretory NS1 lipo-protein particle [29].

We discovered that *hsa-mir-1264, hsa-mir-4497* and *hsa-mir-4485* are upregulated 14.37, 13.46, and 13.42-fold, respectively, and share homologies to ZIKV. This is noteworthy, and it will be interesting to further explore the potential use of either of these miRNAs as therapeutic agents. *hsa-mir-6071* was significantly downregulated (Table 1 and Figure 1).

3.4. Non-structural proteins NS2B-NS3

NS3 represents an important component of the ZIKV replication complex. NS3 is anchored in the ER by strong interactions of its N-terminal with the hydrophilic region of integral membrane protein NS2B. The NS3 C-terminal domain possesses helicase and NTPase activities and is known as the helicase domain [7,24]. The N-terminal, together with its cofactor, NS2B, forms a protease involved in the cleavage of the ZIKV polyprotein. Because of its multiple roles in the virus cycle, N2B-NS3 protein may be an important target against ZIKV. One miRNA: *hsa-mir-4315*, was found to be ~20-fold upregulated, but we did not discover any RNA that was significantly downregulated.

3.5. Non-structural proteins NS4A and NS4B

The transmembrane protein NS4B in ZIKV and NS5 polymerase, are essential for viral replication and modulating the pathogenesis and host immune cell response [7,24]. NS2B-NS3 protease is responsible for all cytoplasmic cleavages at junctions between NS2A/NS2B, NS2B/NS3, NS3/NS4A and NS4B/NS5 proteins and within the capsid, NS2A and NS4A proteins. In other flaviviruses, NS2B-NS3 also plays several roles, being the basis of the formation of the virus replication complex, interacting with transmembrane protein NS4B and NS5 polymerase, and regulating the pathogenesis and host immune cell response [7,24]. We discovered two miRNAs: *hsa-mir-4315* and *hsa-mir-4433-3p*, which were upregulated. *hsa-mir-4315* at ~20-fold whereas *hsa-mir*-4433 was over 13-fold upregulated. We also discovered that *hsa-mir-4655-5p*, that shared homology to NS4A, was significantly downregulated.

3.6. NS5 protein

NS5 is the largest and most conserved protein encoded by the members of the flaviviruses [24,27–-27–30] and it is ~900 amino acids in size. This protein plays a crucial role in ZIKV replication and is an important target for drug development. Based on structural primary sequence analysis, the composition of NS5 can be divided in two major structural and functional domains: an RNA methyltransferase domain (MTase domain) in the N-terminal, and a RNA-depended RNA polymerase domain (RdRp domain) in the C-terminal. ZIKV NS5 has 70% primary sequence identity and possesses a remarkably similar tertiary structure to Japanese encephalitis virus (JEV) NS5. Of note, the role of nuclear NS5 has been investigated for several other flaviviruses [28–32]. Interestingly, the ZIKV NS5 protein was found to form discrete spherical shell-like nuclear structures, which is distinct from other flaviviral NS5 structures, which are diffuse throughout the nucleus [30–32]. We discovered three RNAs: *hsa-mir-4712-3p, hsa-mir-1973* and *hsa-mir-564* were upregulated from 13- to 11-fold. All three appear to be targeting the N-terminal

methytransferase domain. However, *hsa-mir-564* shared homology to the C-terminal domain that codes for RdRp [33–34].

3.7. Conclusion

We have described miR modulations in a chronically infected human progenitor neuronal cell line. We believe that our findings may help to discover new RNA-based therapeutics against ZIKV. There may be broader applications of these miR-based therapeutics against other flaviviruses, specially Dengue, JEV, yellow fever, and West Nile virus, which share significant sequence and function homologies to ZIKV. Even though the focus of our studies was ZIKV, many of the flaviviruses pose emerging global health threats to tropical and subtropical regions due to rapid expansion of mosquito vectors [27]. Our studies may identify pan-flaviviral targets to quell serious emerging threats from these flaviviruses.

In summary, we have identified several miRNAs that are significantly dysregulated in a chronically infected neuronal progenitor cell line. However, while the precise contribution of each of the miRNAs in pathogenesis of ZIKV-induced microcephaly, Guillain-Barre' syndrome, and disruption in corticogenesis, neurogenesis and other neurologic and non-neurologic symptoms are presently unknown, future investigations will shed further light on the roles of each of the deciphered dysregulated miRNAs.

Author's contributions

OB initially conceived the idea, designed, and planned the study, and performed some of the experiments. OB and PP contributed in performing experiments. NS contributed in development and refinement of the concepts, and in preparation of the manuscript. EM contributed in preparation and refinement of the manuscript.

Disclosure statement

All authors declare no conflict of interest.

Funding

This study was partially supported from funding from US Department of Education HBCU Masters grant is P382G170110 – 20.

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