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Drosha, DGCR8, and Dicer mRNAs are down-regulated in human cells infected with dengue virus 4, and play a role in viral pathogenesis

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ABSTRACT. Dengue virus (DENV) and its four serotypes (DENV1-4) belong to the *Flavivirus* genus of the Flaviviridae family. DENV infection is a life-threatening disease, which results in up to 20,000 deaths each year. Viruses have been shown to encode trans-regulatory small RNAs, or microRNAs (miRNAs), which bind to messenger RNA and negatively regulate host or viral gene expression. During DENV infections, miRNAs interact with proteins in the RNAi pathway, and are processed by ribonucleases such as Dicer and Drosha. This study aims to investigate Drosha, DGCR8, and Dicer expression levels in human A-549 cells following DENV4 infection. DENV4 infected A-549 cells were collected daily for 5 days, and RNA was extracted to quantify viral load. Gene expression of Drosha, Dicer, and DGCR8 was determined using quantitative PCR (RT-qPCR). We found that DENV4 infection exhibited the highest viral load 3 days post-infection. Dicer, Drosha, and DGCR8 showed reduced expression following

DENV4 infection as compared with negative controls. In addition, we hypothesize that reduced expression of DGCR8 may not only be related to miRNA biogenesis, but also other small RNAs. This study may change our understanding regarding the relationship between host cells and the dengue virus.

Key words: Dengue; mRNA; RNAi; Arbovirus; DGCR8

INTRODUCTION

The dengue virus (DENV) belongs to the *Flavivirus* genus in the Flaviviridae family, which consists of positive-single-stranded RNA viruses. Currently, five serotypes have been discovered in the DENV group (DENV1-5). DENV infections in humans range in severity from the undifferentiated, acute, self-limited febrile dengue fever to life-threatening conditions including dengue hemorrhagic fever and dengue shock syndrome (Guzman and Harris, 2015). More than 390 million annual DENV infections have been estimated to occur with up to 20,000 deaths (Sam et al., 2013).

Clinical features of primary dengue fever include fever, headache, myalgia, arthralgia, and petechial rash. Patients rapidly develop high viremia for up to 6 days following the onset of fever. However, rapid initiation of the hosts' innate defense systems may be a limiting factor in the development of DENV infection. In selected number of patients, complications such as plasma leakage and coagulation disorders may occur, which can lead to a fatal outcomes (Dalrymple et al., 2015).

Dengue virus serotype 4 (DENV4) was first introduced to Brazil in 1982 in the northwestern region of the Brazilian Amazon. Aside from a brief focal epidemic at the time, DENV4 infection has not been detected in the country until 2010, when it reemerged in the municipalities of Boa Vista and Cantá in the Roraima State of Brazil (Bartlett et al, 2009). The virus quickly spread to different regions in north, northeast, and southeast Brazil (Nunes et al., 2012).

Understanding how viruses regulate their gene expression is an active area of research with relevance to numerous diseases. Viruses with RNA genomes typically rely on RNA binding proteins and cis-acting RNA elements to control gene expression (Liu et al., 2009). DNA and RNA viruses encode trans-regulatory small RNAs, otherwise known as microRNAs (miRNAs), which bind to messenger RNA (mRNA) and negatively regulate host or viral gene expression. Over 200 viral miRNAs have been identified, mostly from large DNA genomes herpesvirus family and small DNA genome tumor viruses that undergo nuclear replication cycles (Sullivan, 2008).

The miRNA family consists of 21-25-nucleotide small RNAs that have been shown to negatively regulate gene expression at the post-transcriptional level (Ambros, 2003).

Similar to host-encoded miRNAs, the functions of the majority of viral miRNAs are unknown. Few viral miRNAs are homologous with host miRNAs, which implies that most viral miRNAs either regulate viral or host transcripts via unique binding sites. It is possible that viral miRNAs may also have novel and yet unknown functions (Grundhoff and Sullivan, 2011).

During DENV infections, miRNAs interact with proteins in the RNA interference (RNAi) pathway, and are processed by ribonucleases such as Dicer, Drosha, Argo1, and Argo2. Interestingly, knock-down of these proteins during DENV infections in mammalian cells resulted in increased viral replication, suggesting that the RNAi pathway may play

an important role in cellular anti-DENV responses (Kakumani et al., 2013). Furthermore, miRNAs are also induced during viral infections, thereby exerting control on host innate immune responses (Green et al., 2014).

The human Drosha enzyme is part of the microprocessor, a large multi-protein complex, which comprises of DGCR8, a double-stranded RNA-binding protein that is deleted in the DiGeorge syndrome, and several RNA-associated proteins including RNA helicases and hnRNP proteins (Han et al, 2004). *In vitro* studies have shown that the minimally active microprocessor comprises of Drosha and DGCR8 (Hassan et al., 2015). The proteins present in the larger microprocessor complex may modulate the activity of specific subsets of miRNAs either directly or via the recruitment of additional regulatory factors. Indeed, miRNA biogenesis is heavily regulated at the post-transcriptional level (Davis and Hata, 2009).

The efficiency of Drosha cleavage has been shown to be increased in the presence of the heme protein, which promote the formation of highly ordered DGCR8 structures upon binding to RNA (Zhang et al, 2013).

This study aims to determine the effect of Drosha, DGCR8, and Dicer expression level in human A-549 cells following DENV4 infection.

MATERIAL AND METHODS

Cell culture

The A-549 epithelial cells obtained from the Rio de Janeiro Cell Bank (BCRJ - UFRJ) were cultured at 5% CO_2 and 37°C in DMEM (Sigma-Aldrich) containing L-glutamine, HEPES buffer (5%), and 10% fetal bovine serum (FBS, Gibco-BRL).

Viral bank

The H778494 strain (GenBank accession No. JQ513335) of DENV4 was grown in C6/36 cells using L15 medium containing 5% heat-inactivated FBS at 28°C at room temperature for 7 days. Cells were harvested when >75% cells demonstrated the cytopathic effect. Supernatants were recovered from the cultures and stored at -80ºC.

Infection of A-549 cells

The A-549 cells were infected with DENV4 supernatants for 1 h at 37°C, using the adsorption method. Cells were then washed with phosphate-buffered saline, placed in fresh medium, and cultured for further analysis. Infected cells were collected daily for up to 5 days.

RNA extraction

RNA was extracted using the commercial Maxwell 16 Lev simplyRNA Cells kit (Promega) according to the manufacturer recommendations, and was stored at -80°C until use.

Viral load quantification

Quantification of viral load was performed as described by Johnson et al. (2005).

Absolute quantification was carried out using the standard curve method. PCR products were cloned into pGEM Easy vectors (Promega).

RT-qPCR

The primer sequences used for mRNA quantification of Dicer, Drosha, and DGCR8 are listed in Table 1 (Jafari et al., 2013). RPL38 and GAPDH are used as endogenous controls. RT-qPCR was performed using the commercial GoTaq 2-Step RT-qPCR System kit (Promega) according to manufacturer instructions. Briefly, reverse transcription (RT) was carried out using the random primers provided and the GoScript reaction mix (Promega). Quantitative PCR was performed on a ViiA 7 platform (Life technologies). The final reaction mixture (20 µL) was composed of 10 µL 2X GoTaqqPCR Master Mix, 3 µL 200 nM forward and reverse primers (Table 1), 3 µL cDNA, and 4 µL DNase/RNase-free water. The qPCR cycling conditions were as follows: 95°C for 2 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min.

Relative mRNA expression in samples were calculated and normalized to the reference genes RPL38 and GAPDH using the comparative Ct method, as previously described by Livak and Schmittgen (2001).

Table 1. Forward (F) and reverse (R) primer sequences for Drosha, DGCR8, Dicer, RPL38, and GAPDH.

ID	Sequence	Reference
Dicer F	5'-TTAACCTTTTGGTGTTTGATGAGTGT-3'	Jafari et al., 2013
Dicer R	5'-GGACATGATGGACAATTTTCACA-3'	
Drosha F	5'-CATGTCACAGAATGTCGTTCCA-3'	
Drosha R	5'-GGGTGAAGCAGCCTCAGATTT-3'	
DGCR8 F	5'-GCAAGATGCACCCACAAAGA-3'	
DGCR8 R	5'-TTGAGGACACGCTGCATGTAC-3'	
RPL38 F	5'-TCACTGACAAAGAGAAGGCAGAGA-3'	
RPL38 R	5'-TCAGTGTGTCTGGTTCATTTCAGTT-3'	
GAPDH F	5'-TCA GTG GTG GAC CTG ACC TG-3'	
GAPDH R	5'-TGC TGT AGC CAA ATT CGT TG-3'	

Statistical analysis

Data were obtained and exported using the Expression Suite v1.0 software (Applied Biosystem), and statistical computing was performed in the R Project program with qPCR. Ct pack. Comparisons between groups were performed using the Student *t*-test.

RESULTS

Viral load per day after infection

The initial viral inoculum used to infect A-549 cells that contained 3.2 $x10^{10}$ viral particles/µL. As shown in Figure 1, levels of viral load found in the samples showed peak titers three dpi, as measured by RT-qPCR.

mRNAs expression levels of Dicer, Drosha, and DGCR8

Expression level of the Dicer protein was found to be lower in infected cells as

compared with non-infected control cultures. Significant differences were observed on the second, third, and fourth days post-infection (Figure 2).

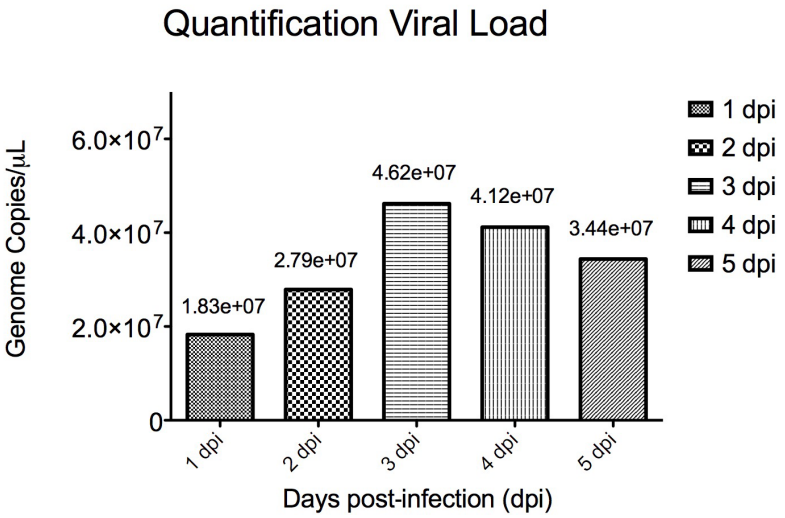

Figure 1. Quantification of viral load in different days post-infection of A-549 cells by DENV4, using RT-qPCR.

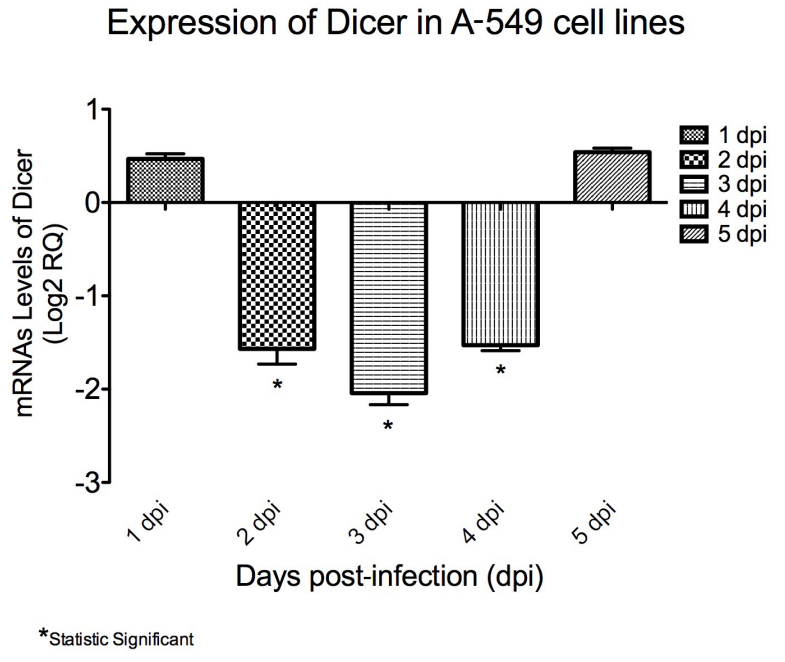

Figure 2. Dicer mRNA expression following VDEN4 infection into A-549 cell lines.

We also observed similar patterns in Drosha expression, where infected cells demonstrated significantly down-regulated Drosha mRNA as compared with control cells on the second, third, and fourth days following infection (Figure 3).

In addition, mRNA expression of DGCR8 was also altered due to viral infection, where significant reduction in DGCR8 expression was observed in the first 4 days following infection, with the third day showing the most significant reduction in mRNA expression (Figure 4).

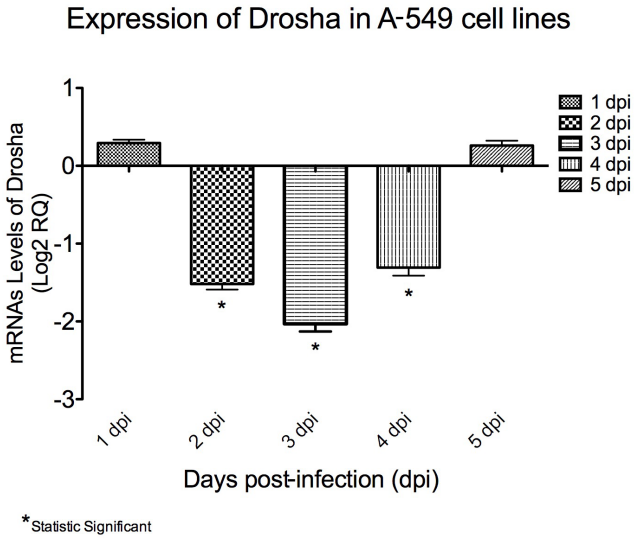

Figure 3. Drosha mRNA expression following VDEN4 infection into A-549 cell lines.

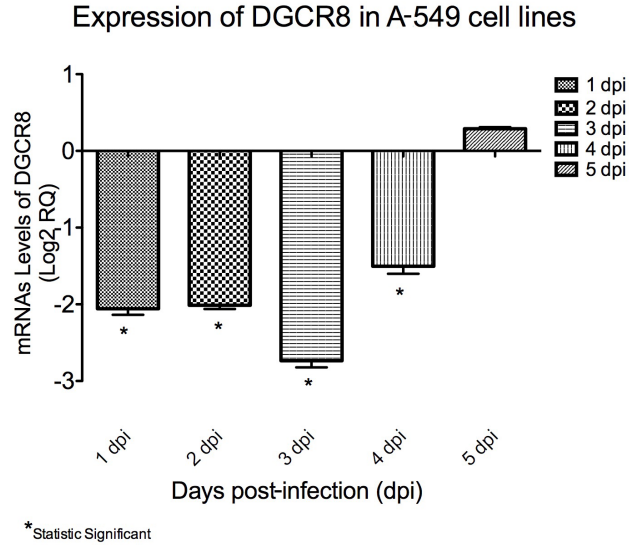

Figure 4. DGCR8 mRNA expression following VDEN4 infection into A-549 cell lines.

Statistical analysis

All analysis performed using ANOVA tests yielded statistically significant results ($P = 0.012$ and $P \leq 0.001$). In addition, all results were also subjected to the reliability test, where acceptable values were set as ≥90%.

DISCUSSION

This study aims to monitor miRNA regulation during *in vitro* DENV4 virus infection in human A-549 cells in order to determine changes in mRNA and miRNA expression during DENV4 replication.

As shown by the results, the amount of DENV4 viral particles in infected cells was the highest 3 days post-infection. Interesting, Umareddy et al. (2007) demonstrated that during DENV2 infection, this cell line presented an elevated viral load between the third and fourth day post-infection, which was correlated with high level of type-1 interferon.

Sullivan (2008) reported that in dsRNA viruses such as HBV, expressions of Dicer and Drosha mRNAs were similar, and show similar characteristics as they regulate the same groups of miRNAs.

Small RNAi have an interesting relationship with miRNAs during DENV viral infections. Using DENV2 strains, Kakumani et al. (2013) demonstrated that these small RNAs are used by the host to control cellular gene expression in response to viral infections. These authors also showed that suppression of Dicer, Drosha, Argo1, and Argo2 during DENV2 infection resulted in an increase in viral replication, suggesting that RNAi may play important roles in cellular responses against this pathogen.

In the present study, we showed that miRNAs not only regulate TLR and IL-1 signaling, but can also be induced during DENV4 infection, thereby exerting control on the innate immune system of the host.

We found that DGCR8 show similar expression pattern as Drosha. As previously described by Kim (2008), a cofactor of DGCR8 regulates Drosha expression. Therefore, low DGCR8 expression in A-548 cells may have contributed to the reduced Drosha expression during DEVN4 infection. However, Macias et al. (2012) described that DGCR8 also exhibit other functions. Indeed, aside from regulation of Drosha, DGCR8 may also be associated with other endonuclease involved in the control of other classes of RNAs.

Our results indicated that viral load in infected cells peaked on the third day of DENV4 infection. In support of this, expression of DGCR8 was the lowest 3 days post-infection. Similar to findings by Macias et al. (2012) and Kakumani et al. (2013), we have also noted that the low DGCR8 expression is correlated with increased viral replication. It is possible that DGCR8 may facilitate viral replication, and play a role in the pathogenesis of DENV4-induced diseases.

In this study, we examined the expression profile of vital components for miRNA biogenesis during an acute DENV4 infection in A-549 cells. A reduction in the expression levels of the components involved in miRNA biogenesis may be lead to a reduction in miRNAs associated with DENV4 infection. It is possible that the reduced expression of DGCR8 during DENV4 infection may also be due to other small RNAs, such as small nucleolar RNAs.

Conflicts of interest

The authors declare no conflict of interest.

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