TNRC6 AND ARGONAUTE PROTEINS ARE OVER EXPRESSED DURING DENGUE 4 INFECTION IN HUMAN CELLS

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ABSTRACT

Dengue virus (DENV) is one of the most medical important arboviruses. About 100 million cases are reported annually in more than 100 countries worldwide, with up to 22,000 deaths. Previous works shown that miRNAs play an important role in maintaining the viral replication cycle in human cells, regulating the viral genome or cellular mRNAs. TNRC6 and Argonaute family proteins are related to gene silencing process through miRNAs. We evaluated the expression of mRNAs of Argonaute 2 protein and TNRC6 in human hepatocytes lineages, infected with DENV4. Using molecular techniques, it was detected a peak of viral infection in 72 hours post infection in both cell lineages. The mRNA levels of proteins TNRC6 expression it was statistically significant at 96 hours post-infection. While Argonaute 2 protein showed significant expression at 96 and 120 hours post-infection, a significant relationship between the Argonaute 2 and family TNRC6 proteins. Similar studies have demonstrated the TNRC6 relationship with possible arbovirus infections in insects, however this is a pioneering work to relate DENV infection with this family proteins in human cells.

KEYWORDS: Dengue, miRNA, TNRC6, Argonaute.

1. INTRODUCTION

Dengue virus (DENV) group is constituted into four serotypes, which belongs to the *Flaviviridae* family, and use *Aedes aegypti* mosquites and humans as the primary vector and host, respectively[1]. DENV has single-stranded RNA genome with positive polarity, being translated into a single polyprotein, which originates 3 structural proteins and 7 non-structural proteins[2].

The viral structural proteins are: capsid (C), membrane or pre-membrane (M / MRP) and envelope (E) while non-structural proteins are NS1, NS2a, NS2B, NS3, NS4A, NS4B[3]. About 40% of the population inhabits areas with high risk of dengue transmission. DENV is endemic in at least 100 countries in Asia, the Pacific, the Americas, Africa and Oceania. Occurs approximately 50 to 100 million apparent infections per year, 500,000 cases of dengue hemorrhagic fever and 22,000 deaths[4].

The VDEN can use RNAi mechanisms, such as microRNAs (miRNAs), developing mechanisms to inhibit interferons pathways in viral infections as a mechanism of regulating the antiviral response[5].

The miRNAs are defined as single stranded RNA molecules with approximately 23 nucleotides (nt), not encoding proteins, that act regulating post-transcriptional gene expression[6]. The cell machinery silencing components of miRNAs are prone to failure, and some studies have shown its involvement with various infections diseases including viral infections[7].

The main interference mechanism is the cleavage of mRNAs. The Argonaute protein, composed in the complex RISC (RNA induced silencing complex), catalyzes the cleavage process, influencing in the transcriptional pathways and post-transcriptional silencing[8,9].

The TNRC6 family proteins are attached to Argonaute proteins in mammalian cells. Previous studies suggest that TNRC6 family proteins are important components when associated with miRISCs, for locating cytoplasmic P bodies and mRNAs knockdown[10].

There is evidence that viruses may synthesize miRNAs and use them to escape the immune system. Another advantage would be that viral miRNAs inhibit expression of genes with antiviral properties. These can direct the viral and cell transcripts, involved synergistically, with viral proteins to reprogram or promote a favorable environment for viral replication in the cell[11].

Thus, the development of an effective diagnostic and therapeutic measure is important to make a better understanding of viral replication mechanisms. This study aimed to investigate the interaction of virus/host

through monitoring the viral infection and expression of DENV4 Argonaute 2 and TNRC6 proteins, simultaneous.

2. MATERIAL AND METHODS

Vital Stock

A DENV4 strain (BeH778494) isolated of the PAHO/WHO collaborating center for Arbovirus reference and research at the Evandro Chagas Institute was propagated in *Aedes albopictus* C6/36 clone cell lineage, in L-15 medium at 28ºC. The cell suspension was collected on the tenth day post-infection, aliquoted and stored at -80ºC.

Inoculation in Human Hepatocytes

After cultivation the HepG2 and Huh7.5 cells were infected with DENV4 using the adsorption method for one hour at 37ºC. Subsequent to adsorption, the cells were washed using phosphate buffered saline (PBS) 1x and DMEM culture medium (Gibco, USA) was used; one bottle was collected every 24 hours after cell infection. A uninfected control bottle was maintained concomitantly of each cell lineage, in order to guarantee the quality of the experiment, using them as negative controls for molecular procedures.

RNA extraction

The samples were extracted using the Maxwell simply RNA Cells Lev 16 (Promega, USA) commercial kit, according to the manufacturer's specifications. The RNAs samples were quantified using the Qubit equipment (Invitrogen, USA) with the commercial kit, and subsequently aliquoted and stored at -80°C until the time of use.

Quantification of Viral Load by RT-qPCR

For quantification of viral load, it was based on the method described by Johnson [(1)], using the commercial kit GoTaqProbe 1-Step RT-qPCR System (Promega, USA) together with the quantification method of absolute curve by a cloned plasmid vector pGEMEasy (Promega, USA) of the DENV4 genome.

mRNAs RT-qPCR

To analyze the protein level expression by RT-qPCR it was used the GoTaq 2-Step RT-qPCR system kit (Sigma, USA); for this purpose, a reverse transcription step (RT) using Random primers and M-MLV (Invitrogen, USA), as described by the manufacturer. At the qPCR stage we used primers for the TNRC6 and Argonaute 2 target mRNAs in ViiA 7 platform (Life Technologies, USA). The relative amounts of target mRNA in the sample were calculated and normalized to the corresponding level of RPL38 transcript (endogenous control). The Ct comparative method was used to evaluate the expression as described by Livak[12].

Statistical analysis

Statistical analysis was performed using the Expression Suite v1.0 software (Applied Biosystem, USA) and R Project software package.

The results of DENV4 infections samples were compared with uninfected cells and analyzed using the ANOVA statistical test, with significant value of $p < 0.05$ were considered statistically significant.

3. RESULTS

Viral Load at HepG2 and Huh7.5 cell lineages

For this experiment the initial viral stock had about 3.2 E10 copy genome/µL. The extraction of viral RNA was performed every 24 hour, until 120 hours. The two cell lineages showed increased viral load between 72 and 96 hours post infection (hpi) (Figure 1).

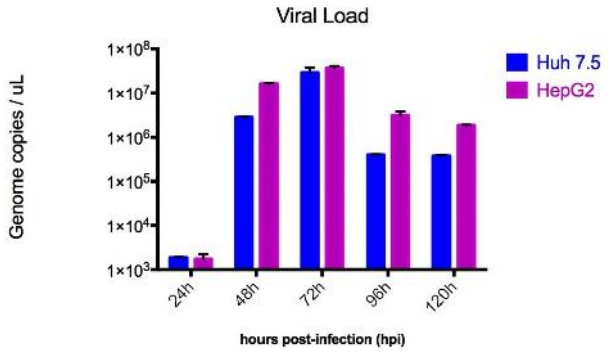

Figure 1. RT-qPCR DENV 4 viral load quantification in HepG2 and Huh 7.5 infected cells.

Expression of the TNRC6 and Argonaute 2 mRNA proteins in HepG2 and Huh 7.5 cells.

The expression levels of TNRC6 A mRNA protein in both infected cell lineages were higher than in the uninfected controls (MOCK), validating our results, and important, they were statistically significant during the 48 and 96 hpi (Figure 2).

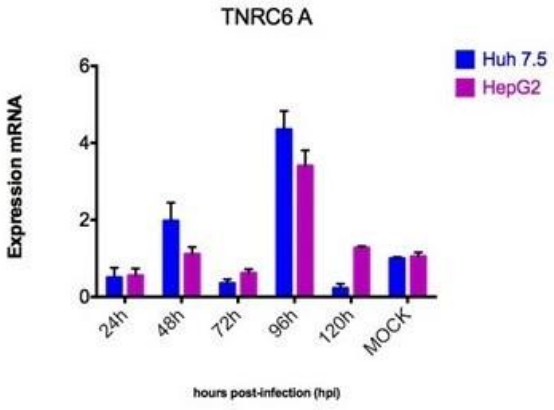

Figure 2. RT-qPCR mRNA expression quantification of TNRC6A proteins during DENV4 infection in Huh 7.5 and HepG2 cells.

It was demonstrated similar expression pattern with the TNRC6 B mRNA protein in infected cells during the same period. With significant expression at 96 hpi when also compared to MOCK (Figure 3).

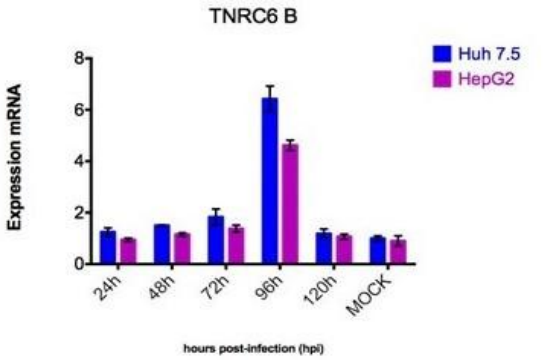

Figure 3. RT-qPCR mRNA expression quantification of TNRC6B proteins during DENV4 infection in Huh 7.5 and HepG2 cells.

It was observed that the pattern of TNRC6 C mRNA protein expression was similar, and also showed the highest level of expression at 96 hpi (Figure 4).

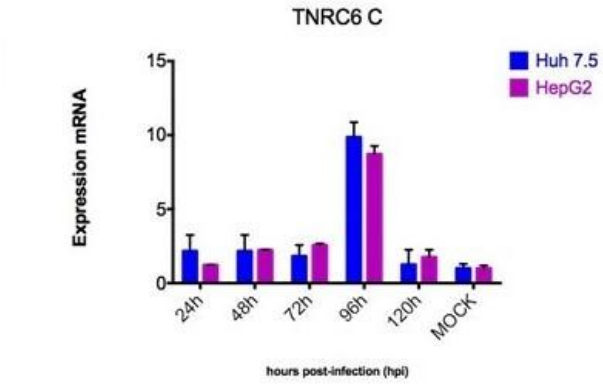

Figure 4. RT-qPCR mRNA expression quantification of TNRC6C proteins during DENV4 infection in Huh 7.5 and HepG2 cells.

The expression of mRNAs related to Argonaute 2 protein was higher than the uninfected cells, with a significantly high expression during the 96 and 120 hours post infection (Figure 5), following the peak of viral replication.

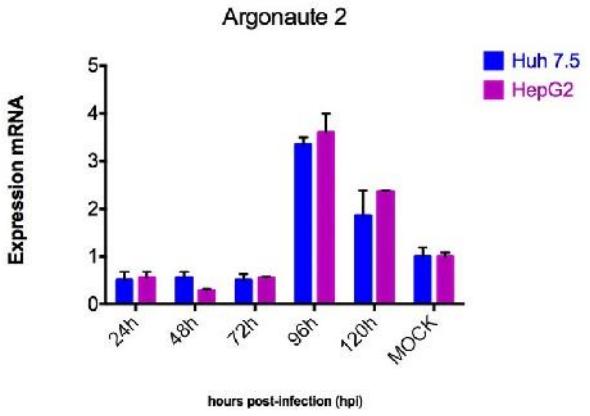

Figure 5. RT-qPCR mRNA expression and quantification of Argonaute 2 protein during DENV infection in Huh 7.5 and HepG2 cells.

Note that all values shown were P=0.014 and P=<0.05, thus becoming significant. Besides the ANOVA test, it was performed a Dundee posttest, which was compared the statistical value against the experiment control (MOCK). All results were subjected to reliability test, which the acceptable range was greater or equal to 95%.

4. DISCUSSION

It is proposed that RNAi mechanism is activated by miRNAs to direct gene silencing, and established that viruses may use their own or cell miRNAs in order to facilitate its replication[11]. In Sonkoly[13] describes that there is a direct or indirect relationship between miRNAs and viruses. By the other side, it is known that the Argonaut proteins are central effector in gene knockdown after of the incorporation into the RISC complex, where, the PIWI domain cleaves the target after miRNA and mRNA pairing[10]. Our study showed that the Argonaute 2 protein had a similar expression pattern in both cell lineages and similar to the viral infection peak, with higher expression at 96hpi.

The Argonaut proteins associated with the RISC complex, have catalytic activity of RNA silencing by miRNAs. In humans the only protein with this particular function is the Argonaute 2, because it has the ability to cleave a phosphodiester bond of a target mRNA[14].

The TNRC6 family proteins expression have showed the same pattern of Argonaute 2 and viral load, suggesting a direct relationship[14] and showed that in cases of partial complementarity with the mRNA, miRNAs activity are dependent on an Argonaute and a TNRC6 proteins.

The TNRC6 proteins are essential for the biogenesis of miRNAs, and have a direct relationship with the Argonaute proteins. These proteins have two terminals, the N-terminal, wherein interacts with Argonaute proteins and C-terminal, with the function of gene silencing. There is another correlation involving in the formation of P bodies. It is known that these bodies store degraded mRNAs and might be associated as location labels of the RISC complex and TNRC6 proteins[15].

TNRC6 Protein A has three different forms, GW220, GW195, GW182, which differ at their N-terminus. These proteins appear to be associated with the cytoplasmic adenylase, thus the recruitment of these proteins by Argonaute protein activates mRNA deadenylation and destabilization, induced by miRNAs. The GW220 protein has higher expression than the others, being able to recruit P bodies and TNRC6 B protein. This was also confirmed by the analysis and the results, which demonstrated proportional expression value between the two proteins[15].

Although few studies of TNRC6 B and TNRC6 C, Baillat & Shickhattar[10] described that these proteins are also linked to P bodies. Also demonstrated that TNRC6

B has two areas, where the I domain interacts with the PIWI domains of the Argonautes, and the III domain, with P bodies signaling, once again validating our results. Based on information previously described by other authors and the results obtained by mRNA analysis of proteins, we can suggest the miRNAs gene silencing activation in the course of Dengue viral infections.

However, when relating the quantification of viral load with the proteins expression, and observing the relationship between the data obtained in each analysis, mainly at 48hpi and 72hpi as higher expression and viral load, we suggest that DENV4 may use viral or cell miRNAs to facilitate and/or ensure their intracellular replication. Since it was used different cell lineages, we suggest that DENV4 can synthesize their own miRNAs and/or miRNA bound to cellular non-immune pathways.

5. CONCLUSION

This study presents a great originality, describing the relation between the expression profile of TNRC6 protein family during DENV4 infection, which has never been demonstrated. Thus, it is necessary further complementary studies to obtain a more detaited greater understanding of these miRNAs interference gene silencing pathways by Dengue and other viruses.

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