

Expression profile of the *PIWI* mRNAs protein family in human cells experimentally infected with Dengue Virus 4

Perfil da expressão dos mRNAs da família de proteínas *PIWI* em células humanas infectadas experimentalmente pelo Vírus Dengue 4

Perfil de expresión de mRNAs de la familia de proteína *PIWI* en células humanas infectadas experimentalmente por el Vírus Dengue 4

Received: 02/26/2021 | Reviewed: 03/06/2021 | Accept: 03/09/2021 | Published: 03/17/2021

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Abstract

Objective: Evaluate the messenger RNAs (mRNA) PIWI proteins expression during infection with VDEN 4 in human hepatocyte cells. **Materials and Methods:** VDEN4 strain H778494 (JQ513335) was used, which was stored in *Aedes albopictus* cell culture (Clone C6 / 36) at the Arbovirology and Hemorrhagic Fevers section of the Evandro Chagas Institute. The techniques of cell cultures, stock (C6/36), inoculation, extraction, viral load quantification, RTqPCR and statistical analyzes were performed at the Viral Biogenesis Laboratory. **Results:** According to the results obtained, two cells (HepG2 and Huh7.5) demonstrated a higher level of viral replication at 72 hours post infection (hpi). The PIWI 2 target alter its mRNA expression during VDEN4 infection. The PIWI 4 target expression, was observed an altered expression in the infected cells. Thus, it was found that they are in different poles, while the viral load in the first three days showed high expression. The expression of mRNA was low in relation to the normal rate given by the uninfected cells. **Conclusion:** In our findings, it was observed that PIWI2 and 4 proteins have an inverse relationship to the viral infection process by VDEN4, when there is an increase in viral replication, these two proteins end up having a significant reduction in their expression. Probably, this reduction of expression is involved with the biogenesis process of apoptosis-regulating microRNAs.

Keywords: PIWI; mRNA; Dengue Vírus; microRNA.

Resumo

Objetivo: Avaliar a expressão dos RNAs mensageiros (mRNA) das proteínas PIWI durante a infecção por VDEN 4 em células de hepatócitos humano. **Materiais e Métodos:** Foi utilizada a cepa H778494 (JQ513335) do VDEN4 que se encontrava estocada em cultura de células *Aedes albopictus* (Clone C6/36) no acervo da seção de Arbovirologia e Febres Hemorrágicas do Instituto Evandro Chagas. As técnicas de cultivos celulares (C6/36, HepG2 e Huh7.5), estoque (C6/36), inoculação, extração, quantificação de carga viral, RTqPCR e análises estatísticas foram todas realizadas no Laboratório de Biogênese Viral da mesma seção. **Resultados:** De acordo com os resultados obtidos observou-se que duas células HepG2 e Huh7.5) demonstraram um maior nível de replicação viral a 72 horas pós

infecção (hpi). O alvo PIWI 2 demonstrou que altera sua expressão de mRNA durante a infecção por VDEN4. Na expressão do alvo PIWI 4 foi observado uma expressão alterada nas células infectadas. Assim, verificou-se que os mesmos se encontram em polos diferentes, enquanto o título viral nos três primeiros dias apresentaram alta expressão, a expressão de mRNA foi baixa em relação a taxa normal dada pela amostra não infectada. **Conclusão:** Em nossos achados observou-se que as proteínas PIWI2 e 4 possuem uma relação inversa ao processo de infecção viral por VDEN4, isto é, quando ocorre um aumento da replicação viral essas duas proteínas acabam por ter uma redução significativa de sua expressão. Estes dados acabam por nos levar a considerar que esta redução da expressão está envolvida com o processo de biogênese de microRNAs reguladores de apoptose.

Palavras-chave: PIWI; mRNA; Dengue Vírus; microRNA.

Resumen

Objetivo: Evaluar la expresión de los ARN mensajeros (ARNm) de las proteínas PIWI durante la infección por VDEN 4 en células de hepatocitos humanos. **Materiales y Métodos:** Se utilizó la cepa VDEN4 H778494 (JQ513335), la cual se almacenó en cultivo celular de *Aedes albopictus* (Clon C6 / 36) en la colección de la sección de Arbovirología y Fiebres Hemorrágicas del Instituto Evandro Chagas. Las técnicas de cultivo celular, stock (C6/36), inoculación, extracción, cuantificación de carga viral, RTqPCR y análisis estadísticos se realizaron en el Laboratorio de Biogénesis Viral de la misma sección. **Resultados:** De acuerdo con los resultados obtenidos, se observó que dos células (HepG2 and Huh7.5), demostraron un mayor nivel de replicación viral a las 72 horas post infección (hpi). Se ha demostrado que la diana PIWI 2 altera su expresión de RNAm durante la infección por VDEN4. En la expresión de la diana PIWI 4, se observó una expresión alterada en las células infectadas. Así, se encontró que se encuentran en polos diferentes, mientras que el título viral en los primeros tres días mostró alta expresión, la expresión de RNAm fue baja en relación a la tasa normal dada por la muestra no infectada. **Conclusión:** en nuestros hallazgos se observó que las proteínas PIWI2 y 4 tienen una relación inversa con el proceso de infección viral por VDEN4, es decir, cuando hay un aumento en la replicación viral, estas proteínas terminan teniendo una reducción significativa en su expresión. . Estos datos terminan por llevarnos a considerar que esta reducción de expresión está involucrada en el proceso de biogénesis de los microRNA reguladores de la apoptosis.

Palabras clave: PIWI; mRNA; Dengue Vírus; microRNA.

1. Introduction

Viruses characterized by their association with arthropods, by their means of transmission, are described as arboviruses and stand out due their enzootic amplification and their impact at the epidemiological level, responsible for causing outbreaks in tropical centers, as if observed in Dengue, constituting a disease with a broad seasonal spectrum, associated with multifactorial dependence on determinants that influence its corresponding virus, such as temperature, humidity and climate (Amorim et al, 2020; Weaver, Reisen, 2010).

The dengue virus (VDEN) is described as one of the most relevant arboviruses today, responsible for affecting human beings worldwide. According to the World Health Organization, approximately one million cases are reported annually in more than 100 different countries, responsible for more than 250,000 hemorrhagic cases and 25,000 deaths (Whitehorn; Farrar, 2010). Dengue is an acute febrile disease, caused by the dengue virus (VDEN) belonging to the family *Flaviviridae*, genus *Flavivirus* and is widely distributed on all continents, except Europe. It is transmitted to humans through the bite of an infected female *Aedes aegypti* (Franz et al., 2006).

VDEN is divided into four serotypes VDEN1, VDEN2, VDEN3 and VDEN4, morphologically they are spherical viruses, measuring about 40 - 70 nm in diameter. It contains an lipoproteic envelope. Its genome is composed of a single-stranded RNA, positive polarity, 10,158 to 10,173 base pairs (bp), including two untranslated regions (100 and 450 base pairs) that flank a coding region of 3,386 to 3,391 amino acids (aa). The viral organization takes two forms, an immature intracellular form, which is characterized by the presence of the prM membrane protein, and the mature extracellular form which is characterized by the presence of the M protein (Umarredy et al., 2007).

The positive polarity viral genome serves as messenger RNA (mRNA) for the translation of viral proteins. A single reading frame encodes a large polyprotein, which is co- and post-translationally cleaved into 10 proteins. From the N-terminal region, a quarter of the polyprotein encodes structural proteins (C-prM-E), followed by non-structural proteins, NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 (Umarredy et al., 2007). As for the immune response against infections caused by VDEN, the

innate system uses MDA5, toll-like receptors and the RIG-I complex as the first mechanisms for recognizing viral RNA. After adsorption of the virus and shortly after acidification of the endosome that encompasses it, type I interferon is produced through TLR3 that recognizes viral dsRNA, where β interferon is related to MDA5 and RIG-I during host defense. In some cases in which cells have virus receptors, low TLR expression occurs, causing suppression of interferon and the MDA5 and RIG-I signaling cascade (Guzman & Harris, 2014).

In addition to the mechanisms mentioned, VDEN can use alternative strategies, such as the inhibition of antivirals through interference RNA (RNAi), producing subgenomic flavivirus RNA, originating from the 3' untranslated region that prevents the dsRNA cleavage by the Dicer enzyme, it can also suppress RNAi through the expression of the NS4B protein (Guzman & Harris, 2014). Then, the adaptive response produces specific antibodies for the cell membrane epitopes, which is one of the problems in subsequent infections by different serotypes, that leads to a severe secondary plasma response (Guzman & Harris, 2014).

Evidence has shown that the expression of ncRNAs from intronic regions sense or antisense transcriptional orientation in relation to mRNA from the same gene locus and from active intergenic regions is a very general phenomenon, observed in multiple biological models and measured using different techniques (Karapetyan et al., 2013). The functionality of these transcripts is a topic under discussion by the scientific community. There is a hypothesis that ncRNAs may represent merely a transcriptional noise resulting from the illegitimate recognition of promoters by RNA polymerase II (RNAP II). On the other hand, it has been proposed that ncRNAs may perform regulatory functions (Van Bakel et al., 2010).

In fact, in recent years, several ncRNAs have been characterized, with functions associated with the regulation of a wide variety of cellular processes such as alternative splicing, editing of RNA (conversion of adenosines to inosines), gene silencing, dose compensation, genomic imprinting, DNA methylation and heterochromatin formation (Hacisuleyman; Cabili; Rinn, 2012).

The ncRNAs are classified according to their size: long ncRNAs (lncRNAs) when greater than 200 nucleotides and short ncRNAs, less than 200 nucleotides (Van Bakel et al., 2010). Short ncRNAs (<200 nt) include microRNAs (miRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), piwi-interacting RNAs (piRNAs), small RNAs associated with promoter regions, among others (TAFT et al, 2009). Long ncRNAs include RNAs with 200 to a thousand nucleotides, with or without splicing. Some lncRNAs serve as precursors for short ncRNAs such as miRNAs and snoRNAs (Hacisuleyman; Cabili; Rinn, 2012).

PiRNAs (Piwi-interacting RNAs), found associated with Argonaute proteins of the PIWI subfamily, are small regulatory RNAs (from 26 to 30 nt) that are extremely abundant in the testicles of rats and mice, involved in the spermatogenesis process in mammals, where they act directing the silencing of retrotransposon expression (Baillat; Shiekhattar, 2009). Regarding the immune system, it has been shown that the pathway using PIWI is important for the defense against attack by pathogens, with these mechanisms being better described in arthropod vectors. Where a point of relevance on this pathway is its presence in the interaction with exogenous material in the cytoplasm of the host cell (Baillat; Shiekhattar, 2009).

2. Methodology

2.1 Viral Strain

The strain H778494 (JQ513335) of VDEN4 was used, which was stored in *Aedes albopictus* cell culture (Clone C6 / 36) at the collection of the Arbovirology and Hemorrhagic Fevers section of Evandro Chagas Institute.

2.2 Viral Stock in cell culture of *Aedes Albopictus*, Clone c6 / 36.

The viral stock for was carried out on C6 / 36 cells. The propagation and maintenance of the cells, the L-15 medium was used. Where the cells were kept at around 28°C, with weekly passes of the confluent monolayers in 25 cm² plastic bottles with 10 mL of growth medium. On the tenth day after inoculation, aliquots of infected cell suspension were collected for the indirect immunofluorescence test (IFI). Subsequently the stock was kept at -70 oC until the moment of use.

2.3 Inoculation in Hepg2 and Huh7.5 cells

HepG2 and Huh7.5 cells were infected with the VDEN4 strain (5 bottles) diluted in DMEM medium (Gibco, USA) using the adsorption method for one hour at 37 ° C. After virus adsorption, the cells were washed using 1x buffered saline (PBS) 1x, placed in fresh DMEM culture medium (Gibco, USA) and cultured for further analysis. One bottle was collected every 24 hours after cell infection, totaling five collections. For a control of this experiment, two bottles of cells from the Huh7.5 and HepG2 strains were kept concomitantly, thus guaranteeing the quality of the experiment and these bottles were also used later as negative controls for the subsequent molecular tests.

2.4 RNA Extraction

The samples used were extracted using the commercial kit Maxwell 16 Lev simplyRNA Cells (Promega, USA) according to the manufacturer's specifications. The samples were quantified using the Qubit equipment (Invitrogen, USA), subsequently stored at -80°C until the moment of use.

2.5 Viral Load quantification qRT-PCR

For the viral load quantification, the method described by Johnson *et al.* (2005) was used. The GoTaq Probe 1-Step RT-qPCR System kit (Promega, USA) was used in conjunction with the absolute curve quantification method using a cloned plasmid into the pGEM Easy vector (Promega, USA) of the VDEN4 genome.

2.6 mRNA Quantification

The GoTaq 2-Step RT-qPCR System (Promega, USA) was used, having as first step the step of reverse transcription (RT) using Random primers and GoScript (Promega, USA) as described by manufacturer.

Then, a quantitative PCR was performed using the ViiA 7 platform (Life technologies, USA), with qPCR System (Promega, USA). For this step, primers for targets PIWI1, PIWI2, PIWI3 and PIWI4 were used.

The relative amounts of target mRNA in the sample were calculated and normalized to the corresponding level of the transcribed mRNA RPL38 and 18S (endogenous controls). The Ct comparative method was used to evaluate expression as described in the literature (Livak; Schmittgen, 2001).

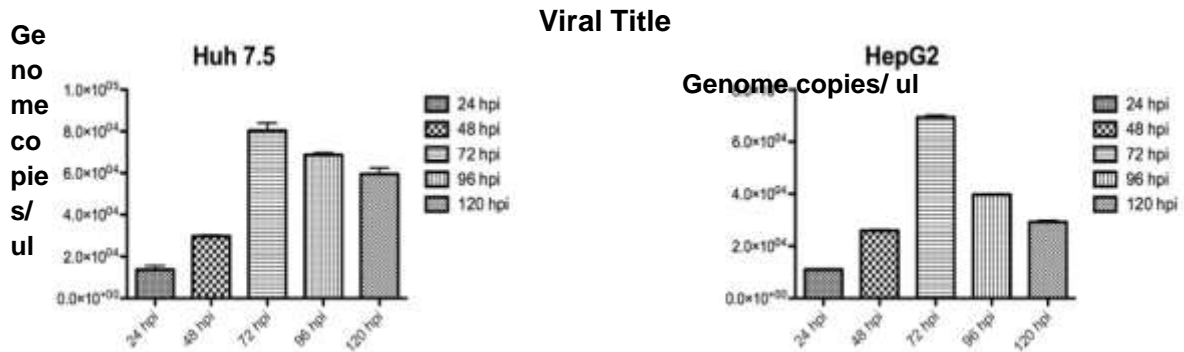
2.7 Statistical Analysis

The statistical analysis was performed using the Expression Suite v1.0 programs (Applied Biosystem, USA). The samples were compared with uninfected cells using Anova method.

3. Results and Discussion

The present study performed the quantification (RT-qPCR) of the cell supernatant of the two infected cells lines by VDEN4, demonstrating a higher level of viral replication at 72 hours post infection (hpi).

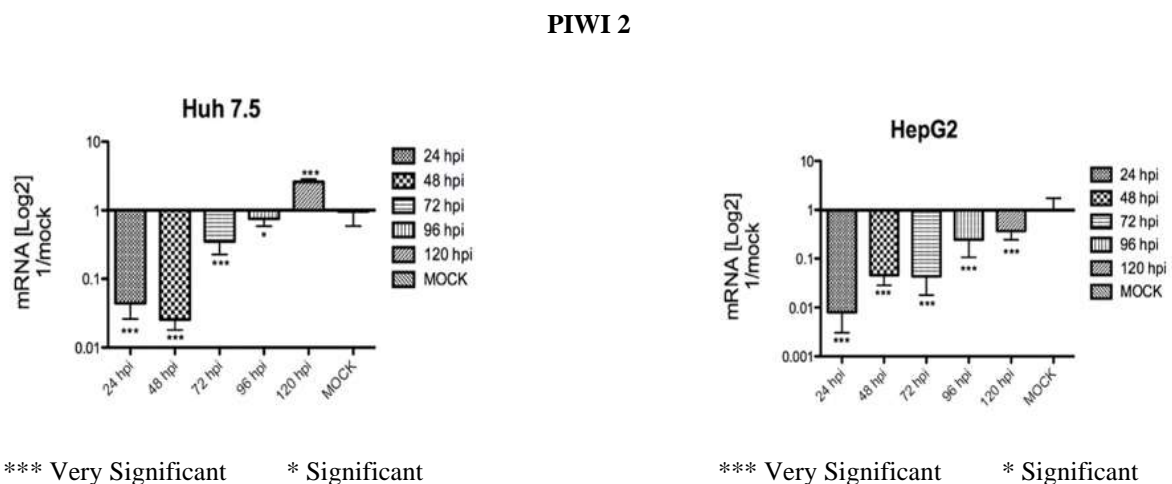
Figure 1 - Viral load quantification by RT-qPCR



Source: Authors.

After quantifying the viral load, the infected cells were extracted as well as the uninfected controls. Then, RNA quantification was performed and PIWI target expression quantification (1,2,3 and 4) was performed, in addition to exogenous controls. It was observed that the PIWI targets 1 and 3 did not have significant changes between the cells infected by VDEN4 and the negative controls for both the HepG2 and Huh7.5 cell lines, however the quantification of the PIWI targets 2 and 4 was possible to verify that they have differences in expression of their mRNAs in relation to uninfected control. The PIWI 2 mRNA target demonstrated that it occurs an expression changes during VDEN4 infection, being possible to relate a drop in its expression levels (Figure 2), and in the Huh 7.5 cell line a low expression of high significance was observed in to control between 24 to 72 hpi. The HepG2 strain showed a high significance reduction between 24 to 120 hpi.

Figure 2 - PIWI 2 mRNA expression quantification by RT-qPCR.

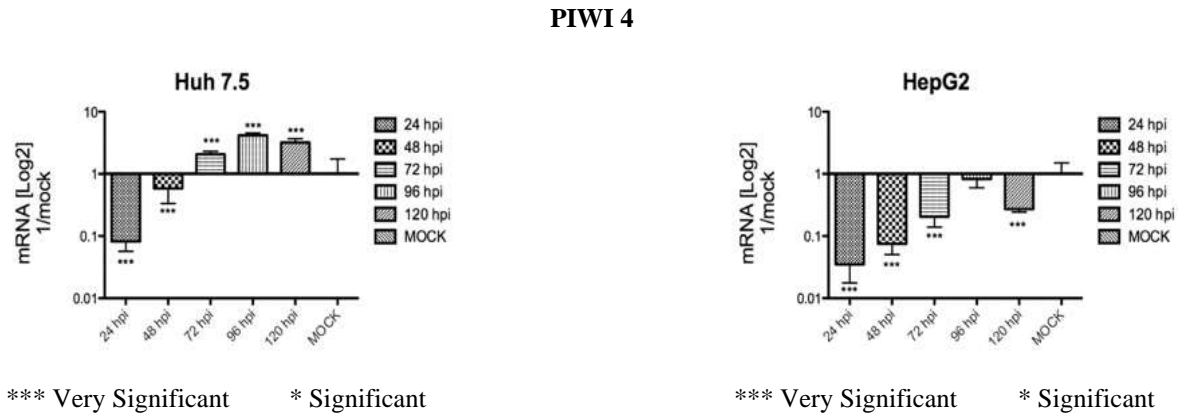


Source: Authors.

In the expression of the PIWI 4 target, an altered expression was observed in the infected cells in relation to the control (Figure 3). The Huh 7.5 cell line demonstrated a reduction in mRNA expression of high significance in relation to the control

between 24 to 48 hpi, after this period a significant increase between 72 and 120 hpi was observed. The HepG2 strain showed a high significance reduction between 24 to 120 hpi.

Figure 3 - PIWI 4 mRNA expression quantification by RT-qPCR.



Source: Authors.

PIWI proteins have been the subject of several studies, demonstrating its important role in the regulation of arthropods, mammals and plants cell biogenesis. As previously reported, only PIWI2 and PIWI4 demonstrated statistically relevant. Studies like the one by Al-Janabi, et al (2010) demonstrate that PIWI2 and 4 have a great relevance during cell regulation and mainly during cell death, contributing to our findings. Because Umareddy, et al (2007) demonstrated in their studies that during VDEN infection there is an apoptosis increase during viral infection.

Thus, the low expression of these mRNAs can justify the apoptosis caused by VDEN4, and studies related to the regulation of apoptosis with PIWI2 (Tan; Lee) have been demonstrating that low expression of this protein activates the STAT3 / BCL-XL system. During the first three days, the viral load of VDEN4 showed a high replication with a third day peak of infection, so when comparing the cell lines, the Huh7.5 showed a higher viral load than the HepG2, this occurs due to the fact that the strains are different, Huh7.5 is immunodepressed and HepG2 is immunocompetent.

However, the strains had similar viral load measurements in the same periods. Analyzing the mRNA expression of PIWI proteins, it was found that they are in opposites, while the viral titer in the first three days showed high expression, mRNA expression was low in relation to the normal rate given by the uninfected sample.

4. Conclusion

These findings show that the PIWI2 and 4 mRNA proteins have an inverse relationship to the VDEN4 viral infection process, When there is an increase in viral replication, these two proteins end up having a significant reduction in their expression. These data eventually lead us to consider that this reduction is involved with the biogenesis process of apoptosis-regulating microRNAs, as well as may also be involved with inflammatory and apoptosis regulation pathways such as STAT3. However, these data are still preliminary and new studies should take place to better describe the action of PIWI2 and 4 proteins.

References

Amorim, M. T., Ferreira, J. F. L., Luna, F. C. F. De, Franco Neto, W. F., Melo, K. F. L., Araújo, A. P. S., Borges, G. A. L., Casseb, S. M. M., Cruz, A. C. R & Holanda, G. M (2020). Estudo teórico-experimental comparativo aplicado à análise da expressão gênica de formas de DNA em células de artrópodes e de mamíferos infectadas experimentalmente pelo Vírus Dengue. *Research, Society and Development, [S. l.]*, 9 (11), 94191110687.

- Baillat, D., & Shiekhatar, R (2009). Functional dissection of the human TNRC6 (GW182-related) family of proteins. *Molecular and cellular biology*, 29 (15), 4144–55.
- Franz, A. W. E., Vargas, I. S., Adelman, Z. N., Blair, C. D., Beaty, B. J., James, A. A & Olson, K. E (2006). Engineering RNA interference-based resistance to dengue virus type 2 in genetically modified *Aedes aegypti*. *Proc. Nat. Acad. Sci. USA*, 14 (103), 4198-203.
- Guzman, M. G., Halstead, S. B., & Artsob, H. (2010). Dengue: a continuing global threat. *Nat Rev Microbiol.* 8 (12): S7-16.
- Hacisuleyman, E., Cabili, M. N., & Rinn, J. L (2012). A Keystone for ncRNA. *Genome Biology*, 13 (5) 315-321.
- Johnson, B. W., Russell, B. J., & Lanciotti, R. S (2005). Serotype-Specific Detection of Dengue Viruses in a Fourplex Real-Time Reverse Transcriptase PCR Assay. *Journal of Clinic Microbiology*, 43 (10) 4977–4983.
- Karapetyan, A. R., Buiting, C., Kuiper, R. A., & Coolen, M. W (2013). Regulatory Roles for Long ncRNA and mRNA. *Cancers*, 5 (2) 462–490.
- Livak, K. J., & Schmittgen, T. D (2001). Analysis of relative gene expression data using real-time quantitative PCR and. *Methods*, 25, 402–408.
- Umareddy, I. Pluquet, O., Wang, Q. I & Vasudevan, S. G (2007). Dengue virus serotype infection specifies the activation of the unfolded protein response. *Virology journal*, 4 (91), 167-176.
- Van Bakel, H., Nislow, C., Blencowe, B. J., & Hughues, T. R (2010). Most “dark matter” transcripts are associated with known genes. *PLoS Biology*, 8 (5).
- Weaver, S. C., & Reisen, W. K (2010). present and future arboviral threats, *Antivir., res.*, 85: 328-345.
- Whitehorn, J., & Farrar, J (2010). Dengue. *British Medical Bulletin*, 95 (1) 161–173.