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Up-regulation of circulating miRNA146a correlates with viral load via IRAK1 and TRAF6 in hepatitis C virus-infected patients

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ABSTRACT

Background: Hepatitis C virus (HCV) is a life threatening human pathogen. It has been found that miRNA146a regulates innate immunity, inflammatory response and antiviral pathway.

We evaluated miRNA146a expression by real-time PCR and IL-1 receptor associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6) levels by ELISA in serum of 36 HCV viremia patients and 42 age and gender matched healthy controls.

Results: miRNA146a expression was significantly higher in HCV patients with a best cut off value 1.63 to discriminate between HCV patients and healthy controls. Meanwhile, it was negatively correlated to IRAK1 and TRAF6 levels and positively correlated to viral load in HCV patients.

Conclusions: miRNA146a has a potential role in HCV infection and viral replication through IRAK1 and TRAF6. It can also serve as a new screening method for HCV.

1. Introduction

Hepatitis C virus (HCV) is a life threatening human pathogen. The impact of HCV infection on the liver varies from minimal changes to extensive fibrosis, cirrhosis and hepatocellular carcinoma (HCC) (Wilkins et al., 2010; Rosen, 2011; Stambouli, 2014). The number of chronically infected persons worldwide is estimated to be over 170 million (Bunchorntavakul et al., 2015). Egypt has the highest HCV incidence ranging from 0.8 to 6.8/1000 persons annually and the highest prevalence (14.7%) nationwide (Mahmoud et al., 2013).

The first line of defense against invading organisms is innate immunity. Toll-like receptors (TLRs) are the key molecules of the innate immune systems and represent the primary sensors that recognizes viral components and induces antiviral responses. All TLR signaling pathways culminate in activation of Nuclear Factor Kappa B (NF-κB) (Kawai and Akira, 2007). NF-κB is a protein complex that controls transcription of DNA, cytokine production and cell survival. Upon activation by viruses, NF-κB induces the expression of specific genes leading to the given immune or a cell survival response (Nelson et al., 2004). Several miRNAs have been implicated in antiviral immunity (Shah et al., 2013; Praven and Chittkara, 2014). Recent evidence demonstrated that miRNA146a regulates innate immunity, inflammatory response and antiviral pathway (Li et al., 2015). Taganov et al. (2006) analyzed the promoter of the gene encoding for miRNA146a and detected several binding sites for NF-κB. When researchers evaluated miRNA146a levels in response to various stimuli, they elicited an increase in its expression in cell lines stimulated with lipopolysaccharide and cytokines including interleukin-1b and TNF-alpha in a NF-κB dependent manner (Taganov et al., 2006; Nahid et al., 2009).

Regarding IL-1 receptor associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6), they are two key adaptor molecules downstream of Toll-like receptor signaling pathway (Ha et al., 2009). Studies done on breast cancer cell lines demonstrated that miRNA146a can inhibit the expression of IRAK1 and TRAF6 with subsequent impairment of NF-κB activity (Bhaumik et al., 2008) and suppression of the expression of NF-kB target genes (Li et al., 2015). The previous findings were also found by Hou and Wang (2009) who concluded that miRNA146a is involved in a feedback mechanism that suppresses Toll-like receptor-triggered NF-κB activation and they suggested a role for miRNA146a in TLR signal termination.

We postulated a role for miRNA146a in HCV infection through IRAK1 and TRAF6. Therefore, we conducted this study to evaluate the expression of miRNA146a in Egyptian patients with HCV viremia in relation to the levels of IRAK1 and TRAF6. Furthermore, the relation of investigated biomarkers to the different laboratory parameters of the participants was assessed aiming at shedding light on new regulators of

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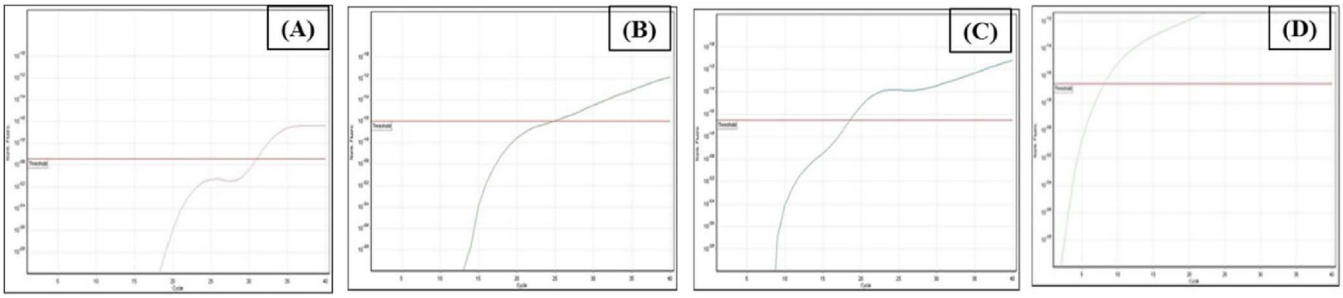

Fig. 1. Representative Amplification plot curve of miRNA146a Real time-PCR in: (A) Healthy controls, (B) Low viremia, (C) Intermediate viremia and (D) High viremia.

hepatitis c viral replication.

2. Material and methods

The study included 36 Egyptian patients with HCV viremia recruited from Internal Medicine and Hepatology Department of Ain Shams University Hospitals. Their ages ranged from 18 to 65 years old (mean ± SD was 47.44 ± 10.10 years). They were 19 males (52.8%) ranging from 29 to 56 years old and 17 females (47.2%) ranging from 20 to 59 years old with no statistical significant difference between gender groups regarding age ($p > 0.05$). They were positive for HCV diagnosed by real time quantitative polymerase chain reaction (qRT-PCR) for HCV RNA genotype 4. All patients do not receive any treatment for HCV and free from another coexisting either hepatic or extrahepatic diseases especially other viral infections like hepatitis B virus (HBV), vesicular stomatitis virus (VSV) and Epstein-Barr virus (EBV). Our patients' group was further subdivided into three subgroups according to the viral load into patients with low (< 10,000 IU/ml), intermediate (10,000–1,000,000s'IU/ml) and high viremia (> 1,000.000s'IU/ml) (Hasan et al., 2012). Forty two healthy age and gender matched subjects were recruited as controls. Their ages were ranging from17 to 65 years old (mean ± SD was 49.00 ± 18.91 years). They were 30 males (71.4%) ranging from17 to 65 years old and 12 females (28.6%) ranging from 26 to 55 years old. No statistical significant difference was found between HCV patients and healthy controls regarding age ($p = 0.774$) or between HCV patients' subgroups according to viral load (52.10 ± 58.50, 42.92 ± 12.42 and 47.33 ± 9.75 for low, intermediate and high viremia patients respectively) and healthy controls ($p = 0.14$). This study has complied with the principles laid down in the Declaration of Helsinki, adopted by the 18th World Medical Assembly, Helsinki, Finland, June 1964, and recently amended at the 59th World Medical Assembly, Seoul, Korea, October 2008. The entire protocol was approved by institutional ethical committee with a registration number: FMASU 1707/2013. All participants provided signed informed consent for participation in the study as required.

2.1. Sample collection and processing

Five milliliters of venous blood was collected from each participant under complete aseptic conditions. Samples were centrifuged at 3000 rpm for 10 min for separation of serum which was aliquoted and stored immediately at −80 °C until used in determination of the relative expression profiling for miRNA146a using qRT-PCR and in quantitative measurement of IRAK1and TRAF6 using ELISA kits designed by Cloud-Clone Crop, assembled by Uscn Life Science Inc, Houston, USA.

2.2. qRT-PCR of miRNA-146a

Total RNA was isolated using miRNeasy Serum/Plasma Kit (Qiagen, Hiden, Germany). The RNA purity and concentration were determined by spectrophotometric measurement of absorbance at 260 and 280 nm. The samples were considered with good RNA quality if RNA: Protein ratio (260:280) was more than 1.5. The miRNAs were reversely transcribed into cDNAs using miScript II RT Kit (Qiagen, Hiden, Germany). Each reaction mixture of qRT-PCR contained 10 µl of RT product (normalized to 500 ng), 12.5 µl of miScript SYBR GREEN PCR Master Mix, 0.5 µM of each primer (Forward: miRNA-specific miScript Primer Assay, Reverse: miScript Universal Primer of the miScript SYBR Green PCR Kit) and deionized water to a total volume of 25 µl. Reactions were run with the following thermal cycling parameters: 95 °C for 15 min followed by 40 cycles of 94 °C, 55 °C and 70 °C for 15 s, 30 s and 30 s respectively. Each sample was normalized based on its endogenous SNORD68-11(Qiagen, Hilden, Germany) RNA content. Relative expression of miRNA-146a was presented as fold expression in relation to the control sample; the actual values were calculated using the $2^{-\Delta\Delta Ct}$ equation, where ΔΔCt = [Ct miRNA-146a − Ct SNORD68-11RNA] (HCV patient sample)-[Ct miRNA-146a − Ct SNORD68-11 RNA] (control sample), Fig. 1.

3. Statistical analysis

The analysis was done using the Statistical Package for the Social Sciences (SPSS software version 19, SPSS Inc., Chicago, IL). Results were expressed as mean ± standard deviation (SD). Differences between continous variables were analyzed using Student's *t*-test or ANOVA test. Correlation between different variables was performed by Pearson or Sperman. Statistical significance was set at a value of $ps < 0.05$. Receiver operating characteristic (ROC) curve was used to discriminate positive from negative results. It determined the threshold value for optimal sensitivity and specificity, which was constructed by calculating the true positive fraction (sensitivity percent) and false positive fraction (100-specificity) of markers at several cut off points. Sample size was calculated using the CaTS-Power Calculator (www.sph.umich.edu/csg/abecasis/CaTS). The power of study was 80 per cent and relative risk for power calculation was set at 2.

4. Results

The laboratory parameters of the study participants are shown in Table 1. We found a significant increase of N-fold miRNA146a expression in HCV patients (14.79 ± 6.90) compared to healthy controls (1.00 ± 0.05, $p < 0.001$) with an up regulation of miRNA146a in HCV patients by 2 folds. Meanwhile, our results revealed high statistical significant difference between HCV patients subdivided according to viral load and healthy controls regarding N-fold expression of miRNA146a (7.50 ± 1.93, 13.34 ± 2.11 and 23.54 ± 3.70 for low, intermediate and high viremia patients respectively and 1.00 ± 0.05 for healthy controls, $p < 0.001$). Furthermore, we observed that the serum levels of IRAK1 and TRAF6 were significantly lower in HCV patients compared to healthy controls, Table 1.

The medians, IQR of viral load in low viremia patients (6250, 2025–9030), intermediate viremia patients (1,69,750, 1,34,100–4,17,500) and high viremia patients (2,540,000, 1,745,000–3,410,000) were significantly different ($p < 0.001$).