Correlation of miR-150, hsa-let-7e, and miR-146a and gene expression of IL-6, IL-8, IP-10, and MIP-1β during dengue virus infection

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Abstract

Growing evidence suggests that microRNAs (miRNAs) play a pivotal role in viral infection. The objective of this study was to assess the association between the expression of miR-150, hsa-let-7e, and miR-146a on cytokine expression during dengue infection. Dengue virus (DENV) strain SJN-006, a serotype 2 DENV strain of the Cosmopolitan genotype, isolated in Bali, Indonesia, was used to infect peripheral blood mononuclear cells (PBMCs) isolated from healthy individuals. The relative gene expressions of miR-150, hsa-let-7e, and miR-146a as well as the gene expression of cytokines (IL-6, IL-8, IP-10, and MIP-1β) were determined using quantitative real time - polymerase chain reaction (qRT-PCR) at 6, 12 and 24 hours post infection (hpi). Correlations between the microRNAs and cytokines were analyzed by means of causality tests. Our data suggests that miR-150 and hsa-let-7e were significantly higher in infected-PBMCs after 12 hpi compared to the uninfected-PBMCs (p<0.05). The causality tests demonstrated that miR-150 and hsa-let-7e were negatively correlated with IL-8 expression, meanwhile miR-146a was the contrast. DENV infection was negatively and positively correlated with miR-150 and hsa-let-7e, respectively, after 24 hpi. In conclusion, our data demonstrates the vital role of miR-150, hsa-let-7e, and miR-146a in regulating IL-8 expression with possible different pathways.

Key words: dengue, microRNA, miR-150, hsa-let-7e, miR-146a

Introduction

Dengue fever (DF), caused by dengue virus (DENV) infection, is a global concern with 390 million cases reported each year [1]. DENV is a positive-strand RNA virus of the Flaviviridae family and there are four serotypes (DENV-1, DENV-2, DENV-3, and DENV-4) [2]. The severity of the disease varies from asymptomatic infection, dengue hemorrhagic fever (DHF) to dengue shock syndrome (DSS) with the mortality rate of DSS being potentially 50 times higher than DHF.
DHF and DSS are strongly correlated with the overexpression of an array of proinflammatory cytokines. The cascade of cytokine signaling is initiated by the recognition of pathogen-associated molecular patterns (PAMPs) by toll-like receptors (TLRs), followed by the activation of the transcription factor, nuclear factor kappa B (NFkB). The overexpression of proinflammatory cytokines leads to increased vascular permeability, plasma leakage and eventually abnormal bleeding [5]. Currently, there have been massive efforts in the scientific community to characterize the pathogenesis of cytokine-facilitated severe dengue and the involvement of microRNAs (miRNAs).

miRNAs are ubiquitously non-coding RNAs with a size of 18-25 nucleotides and play a vital role in a wide range of biological activities [6]. Recently, miRNAs have been noted to play a key role in immune regulation by inhibiting protein translation or by degrading the mRNA transcripts [7]. Several miRNAs were revealed to downregulate proinflammatory cytokines by directly binding to the 3′-untranslated region (UTR) of target mRNAs resulting in translation blockage or mRNA degradation [7]. miRNAs are also known to bind 5′-UTR of the mRNA which affects the post-transcriptional process [8]. In contrast to mRNA silencing, miRNA may induce proinflammatory cytokines by means of epigenetic modification [9]. Specific miRNAs may downregulate epigenetic regulators, such as histone deacetylases, DNA methyltransferases and polycomb-group genes [10]. Interestingly, in dengue infection, human miRNAs such as miR-21 [11] and miR-146a [12] may be hijacked by the virus to support viral replication.

Previously, we investigated the changes of expression levels of hsa-let-7e, miR-30e*, miR-150, miR-146a, and hsa-miR-4290 using peripheral blood mononuclear cells (PBMCs) infected with DENV-2 [13]. The results revealed that miR-150, hsa-let-7e and miR-146a expression was elevated to 1.74, 1.49, and 2 times at 12 hours post-infection (hpi). miR-150 has been reported to affect the expression of a major immune response inducer and modulator, interferon gamma (IFN)-γ, by regulating suppressor of cytokine signaling 1 (SOCS-1) [14]. hsa-let-7e has also been reported to play a significant role in DF pathogenesis by regulating TNFα via enhancer of zeste homolog 2 (EZH2) [15]. The host proviral miR146a may suppress IFN-β expression to support viral replication [16]. We further investigated the correlation between miR-150, hsa-let-7e and miR-146a expression and the gene expression of IL-6, IL-8, IP-10, and MIP-1β. These cytokines have been widely suggested to contribute to DF and DHF pathogenesis [17-20], in particular IL-6 and IL-8, which are responsible for increased vascular permeability [21, 22].

Materials and Methods

Infection of PBMCs with DENV-2
The PBMC samples were isolated from healthy volunteers aged 18-25 years. The virus used, SJN-006 (GenBank: KY006142.1), was a DENV-2, Cosmopolitan genotype strain isolated from Bali, Indonesia [23]. The virus was propagated in C6/36 cells, based on a previously published protocol [24] with the titer determined by plaque assay employing baby hamster kidney cells (BHK21) [25].

PBMCs were infected with DENV-2 at multiplicities of infection (MOI) of 1, and incubated for a variation of infection times from 6, 12, to 24 hpi, in parallel. To confirm that the infection had been successful, NS1 antigen produced in the culture supernatant was assayed using the Panbio Dengue Early ELISA Kit (Panbio Diagnostics). The infected PBMCs were separated from supernatant and stored at -80°C for further use.

Measurement of gene expression using qRT-PCR
The expression of miRNAs (miR-150, hsa-let-7e, and miR-146a) as well as IL-6, IL-8, IP-10, and MIP-1β were quantified using quantitative real-time RT-PCR (qRT-PCR). For miRNAs, RNA extraction was conducted using the miRCURY RNA Isolation Kit (Exiqon) and its concentration was determined using Qubit RNA BR Assay Kit (Invitrogen). The cDNA synthesis step was performed using the Universal cDNA Synthesis Kit II (Exicon). To quantify gene expression, 100ng RNA was converted into a reverse transcription template – cDNA using GoScript Reverse Transcriptase, random primer (Promega), and primer Oligo(dT). The reaction temperatures were
adjusted at 25, 42, and 70°C, respectively for 5, 60, and 15 minutes. The cDNA amplification was conducted using 2x GoTaq qPCR master mix (Promega) with 10 µM forward and reverse primers (Macrogen) (Table 1). The gene expression of miRNAs was semi-quantified based on 2−ΔΔCt of miRNA with an endogenous gene – snRNA U6 [9, 14] while the gene expressions of IL-6, IL-8, IP-10, and MIP-1β were normalized with endogenous gene - beta actin (β-actin).

Table 1. Primers used to determine the expression of miRNAs and cytokine genes using qRT-PCR

| Gene          | Primer sequence                      |
|---------------|--------------------------------------|
| snRNA U6      | Forward: 5’ CCCCACGCAAGGATGAC-3’      |
|               | Reverse: 5’-GTCGGTGCTCGAAGTGATG-3’    |
| miRNA-150e-5p | Forward: 5’-UCUCCCAACCCUUGAACUG-3’    |
|               | Reverse: 5’-GGGGUUGGGGAAGGGUUGA-3’    |
| hsa-let-7e    | Forward: 5’-GCGGTGAGGTAGGGAGTGTAT-3’  |
|               | Reverse: 5’-GCTCGTGCTGGAGTCCCGTAA-3’  |
| miRNA-146a    | Forward: 5’-UGAGAACUGAUAUUCCAUUGGUU-3’|
|               | Reverse: 5’-AGCACTGAGAICTGATTCCATT-3’|
| IL-6          | Forward: 5’-ATGAAACTCTCTCTCCACAAG-3’  |
|               | Reverse: 5’-CTACATTTCGCAAGACCTCTCAGCTGGACTG-3’|
| IL-8          | Forward: 5’-GCCCAAGGATGCTAAAG-3’      |
|               | Reverse: 5’-CTGAGAGCTTCTCCACAAG-3’    |
| IP-10         | Forward: 5’-TTCAAGGTACCTCTTCCTAG-3’   |
|               | Reverse: 5’-CTGATACATGATCTTTCC-3’     |
| MIP-1β        | Forward: 5’-CTGTCCTGATCCAGTGAATC-3’   |
| β-actin       | Forward: 5’-GCTGCGCAAGGATGAC-3’       |
|               | Reverse: 5’-CAACATGATCTGTTGACTTCTC-3’|

Statistical analysis
Distribution of the data was analyzed using Kolmogorov-Smirnov normality test. Gene expression data, with normal and non-normal distribution were comparatively analyzed using t-test and Mann-Whitney, respectively, at each infection time. The causal relationships were investigated with path analysis, correlation test, and regression tests. Direct correlation was represented by positive values, meanwhile an inverse correlation was represented by negative values.

Results

Effect of infection time against the expression of miRNAs
Expression levels of miRNAs in infected and control PBMCs are presented in Table 2. The relative expression of miR-150, hsa-let-7e and miR-146a were not significantly different within 6 and 12 hpi. The expression of miR-150 and hsa-let-7e were significantly lower in infected PBMCs compared to healthy PBMCs, 0.820 ± 0.312 vs. 1.077 ± 0.320 and 1.000 ± 0.932 vs. 1.016 ± 0.178, respectively.

Correlation between DENV-2 infection and gene expression of miRNAs and cytokines
Correlations between DENV-2 infection and the relative expression of miRNAs (miR-150, hsa-let-7e, and miR-146a) and cytokine genes (IL-6, IL-8, IP-10, and MIP-1β) are presented in Table 3. Significant, but weak, inverse correlations between miR-150 and IL-8 was found at 6 hpi (b=-0.396, p=0.011). However, this correlation was no longer found to be significant later in infection (i.e., at 12 and 24 hpi). At 12 h after infection, there was a significant correlation between miR-146a and IL-8 expression (b=0.459, p=0.002). At 24 hpi, there was a weak negative correlation between miR-146a expression and MIP-1β expression with b=−0.264 and p=0.023. There was also a moderate correlation between hsa-let-7e expression and IL-8 expression (b=0.551 and p=0.002).
Table 2. Expression of miRNAs and cytokine genes after 6, 12, and 24 hours post infection (hpi)

| MicroRNA          | 6 hours post infection | 12 hours post infection | 24 hours post infection |
|-------------------|------------------------|-------------------------|-------------------------|
|                   | Infected (±SD)         | Control (±SD)           | p-value                 | Infected (±SD)         | Control (±SD)           | p-value                 | Infected (±SD)         | Control (±SD)           | p-value                 |
| miR-150           | 0.954 ± 0.368          | 1.634 ± 1.530           | 0.323<sup>a</sup>       | 1.178 ± 0.350           | 0.991 ± 0.280          | 0.139<sup>b</sup>       | 0.820 ± 0.312           | 1.077 ± 0.320           | 0.043<sup>*,<sub>c</sub></sup> |
| hsa-let-7e        | 1.152 ± 0.453          | 1.030 ± 0.241           | 0.383<sup>b</sup>       | 1.034 ± 0.279           | 1.161 ± 0.455          | 0.629<sup>b</sup>       | 1.000 ± 0.932           | 1.016 ± 0.178           | 0.048<sup>*,<sub>c</sub></sup> |
| miR-146a          | 1.049 ± 0.392          | 1.047 ± 0.386           | 0.988<sup>b</sup>       | 1.685 ± 1.613           | 0.934 ± 0.158          | 0.646<sup>b</sup>       | 0.790 ± 0.375           | 1.029 ± 0.302           | 0.074<sup>b</sup>       |

<sup>a</sup> Analyzed using Mann-Whitney test
<sup>b</sup> Analyzed using Student t-test
<sup>*,</sup> Statistically significant at p=0.05

Table 3. Correlation of DENV-2 infection, expression of miRNAs and expression of cytokine genes after 6, 12, and 24 hours post infection (hpi)

| Correlation                  | 6 hours post infection | 12 hours post infection | 24 hours post infection |
|------------------------------|------------------------|-------------------------|-------------------------|
|                              | b coefficient<sup>a</sup> | p-value<sup>b</sup>     | b coefficient<sup>a</sup> | p-value<sup>b</sup>     | b coefficient<sup>a</sup> | p-value<sup>b</sup>     |
| DENV-2 infection → miR-150   | -0.303                 | 0.117                   | 0.287                   | 0.139                   | -0.389                    | 0.041<sup>*,</sup>     |
| DENV-2 infection → hsa-let-7e| 0.171                  | 0.383<sup>c</sup>       | -0.171                  | 0.383<sup>c</sup>       | 0.012                     | 0.048<sup>*,</sup>     |
| DENV-2 infection → miR-146a  | 0.003                  | 0.988                   | 0.322                   | 0.095                   | -0.343                    | 0.074                   |
| miR-150 expression → IL-6 expression | 0.333             | 0.067                   | 0.004                   | 0.952                   | -0.066                    | 0.623                   |
| miR-150 expression → IL-8 expression | -0.396           | 0.011<sup>a</sup>       | 0.123                   | 0.360                   | 0.200                     | 0.280                   |
| miR-150 expression → IP-10 expression | -0.217           | 0.205                   | -0.016                  | 0.911                   | -0.066                    | 0.583                   |
| miR-150 expression → MIP-1β expression | -0.029         | 0.883                   | 0.086                   | 0.090                   | 0.212                     | 0.102                   |
| hsa-let-7e expression → IL-6 expression | -0.080          | 0.612                   | -0.193                  | 0.076                   | 0.551                     | 0.002<sup>*</sup>     |
| hsa-let-7e expression → IP-10 expression | 0.017           | 0.832                   | -0.085                  | 0.666                   | -0.107                    | 0.587                   |
| hsa-let-7e expression → MIP-1β expression | 0.140           | 0.418                   | 0.007                   | 0.952                   | -0.033                    | 0.771                   |
| miR-146a expression → IL-6 expression | 0.228           | 0.233                   | -0.108                  | 0.038                   | -0.120                    | 0.361                   |
| miR-146a expression → IL-8 expression | 0.054           | 0.739                   | 0.459                   | 0.002<sup>*</sup>       | -0.211                    | 0.210                   |
| miR-146a expression → IP-10 expression | 0.042           | 0.832                   | -0.024                  | 0.903                   | -0.142                    | 0.472                   |
| miR-146a expression → MIP-1β expression | -0.045          | 0.794                   | 0.025                   | 0.837                   | -0.264                    | 0.023<sup>*</sup>      |

<sup>a</sup> Path coefficient, very weak if <0.1; weak if 0.10 ≤ b <0.50; moderate if 0.50 ≤ b <0.75; strong if 0.75 ≤ b <0.90; and very strong if b ≥ 0.90
<sup>b</sup> Otherwise stated, all analyses conducted using a linear regression
<sup>c</sup> Statistically significant using Mann-Whitney test
<sup>*,</sup> Statistically significant at p=0.05
**Discussion**

Cytokine expression has been suggested to be a good predictor of DENV disease development, since disease pathogenesis is based on the dysregulation of cytokines [17-21]. Some interesting findings have suggested the important role of miRNAs in DENV infection and are potentially to be screened to identify suitable miRNAs as DENV biomarkers [26, 27].

**miR-150**

A study has highlighted the critical role of miR-150 in the pathogenesis of DENV diseases by regulating the expression of SOCS-1 [14]. We observed the significant reduction of miR-150 in the infected PBMCs after 24 hpi. Previously, the expression of miR-150 was significantly higher in DENV2-infected peripheral blood cells of patients with DHF compared to those with DF [28]. A similar trend was also observed in another study using DENV2-infected PBMCs after 24 hpi [14].

A study based on A/H1N1 infection revealed that the upregulation of miR-150 did not occur in patients with moderate illness; only in those with severe illness [29]. Additionally, screening of miRNA from blood samples of sepsis patients revealed a reduction of miR-150 in peripheral blood leukocytes [30, 31], and was found to be correlated with pro-inflammatory cytokines [31]. Our study substantiates the potential of miR150 as a biomarker for the development and progression of DENV infection.

The suppression of miR-150 has been reported to be due to the DNA hypermethylation of its promoter, based on a study using piglet livers treated with 2-hydroxy-(4-methylthio) butanoic acid [32]. Gene expression involving miR-150 is possibly regulated via epigenetic modification [33]. Our study suggests the potential role of miR-150 as a negative regulator for IL-8 expression. Suppression of IL-8 by SOCS-3 has been reported in multiple reported cases of inflammation [34-36]. It is further corroborated by the results of the previous investigation, that revealed SOCS-1 mRNA as a hypothetical gene target of miR-150 that reduces the expression of IFN-γ gene [14].

**hsa-let-7e**

As a biomarker, the hsa-let-7 miRNA family is most notable for cancer and tumor diagnosis and prognosis [37]. Recently, hsa-let-7 is among the potent therapeutic molecules used against SARS-CoV-2 infection, targeting host angiotensin-converting enzyme 2 (ACE-2) [38]. In the case of DENV infection, hsa-let-7e has been found to be overexpressed in infected human hepatoma Huh-7 cells [39]. A previous study also found different levels of hsa-let-7e expression in DENV-infected PBMCs [9]. Nonetheless, the hsa-let-7e was upregulated only at the second and fourth day after the fever onset and downregulated at the third day [28]. The regulation pattern of hsa-let-7e is similar to our finding and our previously reported preliminary study [40]. Although after 24 hpi the expression level of hsa-let-7e was significantly lower, a further investigation is required regarding its differential expression.

It is reported that hsa-let-7e may interact with EZH2, which eventually initiates the inhibition of TNFα [15]. EZH2 is an epigenetic regulator with a critical role in histone methylation in downstream genes. NFκB was speculated to be downregulated by the EZH2-induced inhibition of NFκB activation [15]. In this regard, IL-8 can also be suppressed by hsa-let-7e since NFκB mediates the activity of IL-8 promoter [41]. Furthermore, a study reported the role of EZH2 in downregulating TNFα, IFN-β, and IL-8 during influenza A virus (IAV) strain A/WSN/33 (WSN) virus infection [42]. In the present study, our data demonstrates the possible downregulation of IL-8 by hsa-let-7e after 24 hpi.

**miR-146a**

Although, the expression of miR-146a in DENV-2-infected PBMCs was not significantly different to the control PBMCs miR-146a has been reported to be one of the only two known human pro-DENV miRNAs [16]. A previous study found that miR-146a was upregulated in THP-1 cells and primary monocyte cells, targeting tumor necrosis factor receptor-associated factor 6 (TRAF-6) and consequently suppressed IFN-β [16]. The exploitation of miR-146a is not unique to DENV infection and is observed in vesicular stomatitis virus infection by similarly targeting TRAF-6 and several other innate immune-related molecules [12].
Our data suggest the possibility of miR-146a to indirectly downregulate the expression of MIP-1β, that is induced by signal transducers and activators of transcription 1 (STAT-1) pathway [43]. This is further substantiated by the findings of a previous study [44] where IFN regulatory factor 5 and STAT-1 were shown to be the possible gene target of miR-146a.

**Conclusion**

The expression of miR-150 and hsa-let-7e were significantly lower in DENV-2 infected PBMCs compared to the uninfected PBMCs after 24 hpi. Our data suggest that the expression of proinflammatory cytokine IL-8 is associated with miR-150, has-let-7e and miR-146a. Therefore, further investigations are required to elucidate the regulation pathways.

**Declarations**

**Ethics approval**

The approval of the research protocol was granted by Ethical Review Boards of Universitas Udayana (No. 2072/UN.14.2/KEP/2017). All PBMC donors signed the informed consent prior to the enrolment.

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**Conflict of interest**

The authors declare that they have no competing interests.

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