Detection of micro RNA hsa-let-7e in peripheral blood mononuclear cells infected with dengue virus serotype-2: preliminary study

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Abstract. Pathogenesis of dengue infection is still obscure. Recently, the role of microRNA has been associated with the cytokine storm which leads to plasma leakage in endothelial cells. The objective of our study was to determine whether particular microRNA is overexpressed in PBMCs infected with DENV and to assess its correlation to the expression of suppressor of cytokine signaling 3 (SOCS3) proteins to increase the production of pro-inflammatory cytokines. We report the result of a preliminary study on the expression of microRNA hsa-let-7e. The peripheral blood mononuclear cells (PBMCs) from the healthy volunteer were infected with the clinical isolate of DENV-2. RNA was extracted with miRCURY®LNA™Exiqon. Quantitative Real-Time PCR was used to measure the relative expression of hsa-let-7e micro RNA and the mRNA of SOCS3 proteins. MicroRNA hsa-let-7e expression was increased in PBMCs upon DENV-2 infection. The relative expression of hsa-let-7e is detected at 1.46 folds relative to uninfected PBMCs in 4 hours post-infection and decreased in 19 hours post infection. In contrast, the expression of mRNA of SOCS3 was inversely expressed with hsa-let-7e expression. MicroRNA was overexpressed in PBMCs upon infection with DENV-2. This microRNA may bind the SOCS3 and contribute to the pathogenesis of dengue infection.

1. Introduction
Dengue virus (DENV) infection, which varies in clinical manifestations, is the fastest emerging arboviral infection in the world.[1] The disease affects almost 2.5 billion people in the tropical and subtropical countries in the world [2], caused 390 million new cases arising each year and almost 96 million dengue hemorrhagic fever (DHF) cases leading to hospitalization each year.[3] Countries in tropical and subtropical regions reported DENV infection as a hyperendemic whereas the four DENV serotypes found to circulate in these countries.[4-9] The DENV has four related, but distinct serotypes, known as DENV-1 thoroughly DENV-4.[10] Infection by one serotype confers life-long immunity but only partially or non-neutralizing antibody to the other serotypes.[2] Within each serotype there exist a considerable variation in the form of phylogenetically distinct subtypes or genotypes, which are DENV-1 has five genotypes [11], DENV-2 has six [12], DENV-3 has four [13], and DENV-4 has four genotypes.[14,15]
The clinical spectrum of DENV infection ranging from asymptomatic, mild dengue fever (DF), and dengue hemorrhagic fever (DHF) include the life-threatening dengue shock syndrome.[16] The hallmark of DHF is plasma leakage as manifest as increasing hematocrit as high as 20% from baseline and or fluid accumulation in the pleural cavity, peritoneal space.[16] The pathogenesis of DENV infection is not fully elucidated yet.[17,18] Antibody-dependent enhancement in the sequential DENV infection contributed to getting severe DENV infection.[19] The role of the virus strain may also provide a particular outcome, which is DENV-2 contributed to the development of SSD.[20] The cytokines storm in DENV infection associated with leakage process in the endothelial cell.[21-23] Analysis of cytokines expression in A549 cell line infected with various DENV found 7 of 26 cytokines expression were significantly higher than uninfected cell line. The induced cytokines were eotaxin, granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), IFN-α2, IL-6, IL-8, IL-15 and IP-10.[24] An in-vivo study found the level of TNF-α, IL-1β, IL-6, IL-8, IL-10, IFN-γ, c-c motif chemokine ligand (CCL)-2, CCL-3, CXCL-8, CXCL-10 were increased in DENV patients serum.[25] Negative regulatory factors of cytokines production are needed to alleviate the series of cytokine cascade during the innate immune response to DENV infection. The family of protein SOCSs consists of 8 members of cytokines induced SRC homology2 (SH2)-domain-containing protein (CIS and SOCS1-SOCS3). SOCS proteins are inhibitors of cytokine of signaling pathway.[26-28]

MicroRNA (miRNA), a short, non-coding RNA, function mainly through binding to the 3'UTR untranslated region, of target mRNA, resulted in inhibition of translation. It has diverse biological processes such as tumorgenesis, development, inflammation and immune regulation in infection.[29] MiRNA affects and regulate cytokines production, cytokine signaling and provides the balance of Th1/Th2 polarization.[30,31] Recent evidence revealed that particular miRNA such as miR-146a, miR-30e, miR150, miR-548g-3p and some others are inducible by DENV[32-34] in which augmented miR-150 expression may be depressed SOCS1 expression.[32] However, whether others miRNAs regulate SOCS1 and also SOCS3 expression during DENV infection-induced pro-inflammatory cytokines in the development of more severe DENV infection is not known. Here we reported a preliminary report of higher expression of miR-hsa-let-7e and lower expression of SOCS3 in PBMCs infected versus non-infected with local clinical isolate (Semarang DENV-2 isolate).

2. Material and methods
Institutional Review Board of the Faculty of Medicine, Udayana University, Bali has reviewed and approved this study (Approval No: 2072/UN.14.2/KEP/2017). Blood samples were drawn from a healthy donor after provided informed consent. PBMCs were isolated using Ficoll-Hypaque density gradient centrifugation. All PBMCs were washed in RPMI-1640 medium and then suspended in the same medium with 10% inactivated fetal calf serum. Finally, PBMCs were cultured and incubated at 37°C in 5% CO₂ for 6-12 hours. Local isolate (Semarang isolate) of DENV-2 were propagated in C6/36 mosquito cells, and the titer of the virus was determined using plaque assay as described in the previous report.[24] After rinsing of the PBMCs with RPMI-1640 medium, PBMCs was then infected with DENV-2 in the same medium with the multiplicity of infection (MOI) of 4 PFU/10⁶ cells. Cells were incubated at 37°C and sampled at 4 and 19 hours post-infection. Total RNA was extracted using miRCURY LNA™Exiqon (Sweden) RNA isolation kit in one step as described by the kit manufacturer’s instructions. Qubit 3 fluorophotometer was used to assess the RNA quality and quantity. Quantitative real-time polymerase chain reaction (qRT-PCR, Biorad) was used to analyze the relative expression of miRNA. The internal reference genes used were the non-coding small nuclear RNA (snRNA U6) for miRNA and β-actin for SOCS expression.

3. Results
The excessive release of pro-inflammatory cytokine during severe DENV infection is hypothesized as the pathogenesis of plasma leakages that may responsibility of low expression of SOCS protein as the negative inhibitor of cytokine regulator and production. The low expression of SOCS expression
though to be related to failure to SOCS gene translation caused by particular miRNA binding. As the preliminary study, supernatants were collected at 0, 4 and 19 hours post DENV-2 infection. The qRT-PCR result was normalized using U6 and β-actin to reduce sample variation. Expression of hsa-let-7e revealed the upregulation 1.4 folds at observation time 4h in infected samples, compared with the uninfected sample. The down expression of hsa-let-7e was observed on 19h observation. In contrast, the SOCS3 expression that was elevated at 0h observation, was down-regulated at the 4h observation. The SOCS3 expression was upregulated again in 19h (Figure 1). To ensure that the DENV-2 infection induced immunological responses of the PBMC, we also performed qRT-PCR expression profiling of genes encoding cytokines and chemokines namely IL-6, IL-8, IP-10 and MIP-1β (data not shown).

![miRNA Expression](image.png)

**Figure 1.** Expression of hsa-let-7e and SOCS3 in PBMC infected with DENV-2 assessed on 0, 4, and 19 hours post infection (p.i).

4. **Discussion**

The role of pro-inflammatory cytokines during the cytokines storm as the response to DENV infection has been widely accepted.[35] The plasma leakage of DENV infection is the consequence of the effect of cytokine storm to the infected endothelium. Loss of negative inhibition pathway includes down-regulation of SOCS protein that bind by particular miRNA may respond to the uncontrolled release of pro-inflammatory cytokines. This preliminary study revealed that association. Up-regulation of miRNA hsa-let-7e with the inverse downregulation of SOCS3 expression may be explained by the contribution of miRNA in the pathogenesis of DENV infection. In our study, the expression on hsa-let-7e was not as high as with another study.[36] This may becaused by the different time points of microRNA sampling conducted in our study compared to the previous study. Further study will be performed to identify the miRNAs expression at various time points e.g. 6, 12, and 24-hour post-infection to get more information on the pattern of expression of particular miRNAs. Others studies described the high expression of miR-146a, miR-30c*, miR-150, miR548g-3p in DENV infection which suggests the role important of miRNA in the DENV pathogenesis.[33,34,36]

5. **Conclusion**

In conclusion, our study provides novel data in the mechanism of hsa-let-7e through down regulation of SOCS3 protein expression in inducing pro-inflammatory cytokines in DENV infection that was not evaluated in the previous study.
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