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研究課題名（和文）重症デング熱におけるmiRNAのバイオマーカー探索及び機能解析

研究課題名（英文）Determination of miRNA biomarkers in the pathogenesis of severe dengue

研究代表者
モイ　メンリン（Moi, Meng Ling）

東京大学・大学院医学系研究科（医学部）・教授

研究者番号：４０５９７４９９

交付決定額（研究期間全体）：（直接経費）　　3,300,000円

研究成果の概要（和文）：デングウイルス病態発現機構の解明や新規抗ウイルス薬開発に資するため、デング熱患者血中におけるmiRNAおよびサイトカインのレベルを解析し、ホスト側の免疫応答を詳細に解析した。急性期および重症患者においてはCCL5, SCF, PDGF-BB、IL-10、TNF-αの優位な誘導が確認できた。miRNA解析においてmiRNA hsa-let-7c-5pが急性期患者に優位に上昇していることが明らかとなった。また、ヒト細胞においてmiRNA hsa-let-7c-5pはウイルス抑制作用を有することを確認し、ウイルス抑制作用は本miRNAによる免疫応答の活性化によるものが示唆された。

研究成果の学術的意義や社会的意義
miRNA hsa-let-7c-5p inhibited DENV replication and is potentially associated with host response generated during viral infection. These circulating factors may represent leading signatures in acute DENV infections, that are associated with clinical severity.

研究成果の概要（英文）：In dengue, humoral and cellular immunity may play a dual role in disease pathogenesis and protection. We first analyzed 45 cytokines and other factors in serum samples from the acute phase of DENV infection from 167 patients.ignificant correlations were identified between disease severity and CCL5, SCF, PDGF-BB, IL-10, and TNF-α levels; between NS1 Ag and SCF, CCL5, IFN-α, IL-1α, and IL-22 levels; between thrombocytopenia and IL-2, TNF-α, VEGF-D, and IL-6 levels; and between primary or secondary infection and IL-2, IL-6, IL-31, IL-12p70, and MIP-1β levels. A total of 3 candidates identified from the mRNA profiles of dengue patients was synthesized. One candidate, miRNA hsa-let-7c-5p was selected for the following assays due to higher potency during infection. Virus growth was examined by using real-time PCR and plaque assay. We found that miRNA hsa-let-7c-5p inhibited dengue virus replication and is potentially associated with host response generated during DENV infection.

研究分野：Virology

キーワード：dengue　miRNA

１．研究開始当初の背景

Approximately half of the world population is estimated to be at risk of mosquito-borne infections, particularly in South-east Asia and the Americas. Global Dengue epidemics has been reported at the tropics and sub-tropical regions. Severe Dengue (Fig. 1) are one of the leading causes of morbidity and mortality, particularly among children. Because of the serious consequences that has been associated with DENV infection, it is important to understand factors that are associated with pathogenesis and protection, for the development of effective disease control measures.

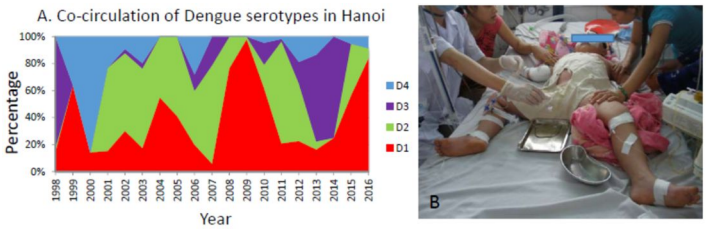


Fig. 1. Dengue circulation patterns in Hanoi, Vietnam 1998-2016. All four serotypes are co-circulating in the country, and this phenomenon is common among hyperendemic region such as Vietnam (A). DENV-associated morbidity are reported mainly in children. Ascites and pleural effusions are common in severe Dengue cases (B, No. 1 Children Hospital, Hanoi, Vietnam). When left untreated, severe Dengue has a mortality rate as high as 50%.

Ample evidence now exist to support that the macrophage/monocyte lineage and dendritic cells are the target cells of DENV *in vivo*. Whereas recent studies have demonstrated that in respond during acute virus infection, robust IFN type I response are induced. The longitudinal studies of PBMCs suggest that important chemokines, including those involved in leukocyte recruitment are comparatively lower during early time points. These results suggest that measuring time-point, and specific transcriptional-associated factors would be important to elucidate how DENV infection alters the magnitude, specificity and functionality target cells. Additionally, cytokine- and chemokine-mediated response also reflects that the hemodynamic changes of plasma leakage and clinical course of disease.

MicroRNAs (miRNAs) are small nucleic acids which disrupt translation of target messenger RNAs and alter mRNA expression patterns. It is well understood that cross-talk between DENV target cells (macrophages/monocytes lineage cells) is essential in Dengue disease outcome. In terms of the pathogenic role of miRNA in disease regulation, miRNA has been associated with virus replication *in vitro*. In clinical studies, high viremia levels are associated with disease severity (Moi et al., *J Infect Dis*., 2011; Endy et al., *J Infect Dis*., 2003). Thus, understanding the detailed patterns of miRNA during DENV infection offers a unique opportunity for elucidating the pathogenesis of severe Dengue and contribute to the development of novel biomarkers.

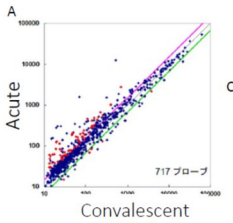
２．研究の目的

Global dengue epidemics are rapidly increasing but the disease pathogenesis is not well-understood. In Dengue, humoral and cellular immunity may play a dual role in disease pathogenesis and protection. As pre-existing immunity may lead to differential disease outcomes depending on the magnitude and quality of response, we aim to determine the global miRNA dynamics during an early and a late time point after dengue virus (DENV) infection by using clinical samples. To achieve these aims, we will use virological and immunological approaches including RNAseq to analyze and compare the miRNA transcriptional patterns from various groups of DENV patients (secondary vs primary, acute vs convalescent, blood vs ascites). Comparison of the longitudinal miRNA signatures in various stages of disease represents a novel approach and is expected to provide to a better understanding into host response to DENV infection. Thus, the proposal offers a unique opportunity to elucidate the human immune response to natural DENV infection and contribute to the identification of novel biomarkers and development of effective dengue vaccines.

３．研究の方法

To understand the immunologic cross-talk between circulating miRNA during DENV infection, we have previously characterized the miRNA and global networks involved in transition from acute (fever phase) to convalescent (recovery phase) by using blood samples from DENV patients (Fig. 2). By using a novel approach of characterizing cell free miRNA analyses and viremia titration, we have characterized the development and resolution of an *in vivo* human miRNA expression profiles in response to primary DENV infection. Our analyses of human miRNA expression profiles demonstrated that during acute DENV infection, miRNA(s) associated with immune response regulation and virus replication are altered in response to acute DENV infection.

Based on the preliminary data described above, measuring time-point and disease-type specific miRNA profiles in the blood and body fluids of individuals with and without severe Dengue can elucidate the role of miRNA during infection. Determination of miRNA profiles during different stages of infection (early vs convalescent, severe vs non-severe) would lead to a better understanding of flavivirus disease pathogenesis. In particular, our novel approach of identifying the *in vivo* human miRNA profiles that connect innate immunity and regulate the balance between protection and pathogenesis, would lead to revelation of bio-signatures that aid in disease prognosis and the development of safe and effective DENV and flavivirus vaccines.



B

Group	Levels	P<0.05 (>1.5times)	P<0.01 (>1, <1.5times)	P<0.05 (>4 times)	Total number of miRNA
Acute	Increased	65	0	4	69
Convalescent	Decreased	5	0	0	5

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Rank	miRNA ID	Symbol	発現比 (Fold change)	Target gene 数
1	hsa-let-7c-5p	let-7a-5p (and other miRNAs w/seed GAGGUAG)	6.336	132
2	hsa-miR-145-5p	miR-145-5p (and other miRNAs w/seed UCCAGUU)	3.557	44
3	hsa-miR-221-3p	miR-221-3p (and other miRNAs w/seed GCUACAU)	2.814	21
4	hsa-miR-765	miR-3584-5p (and other miRNAs w/seed GGAGGAG)	2.812	1
5	hsa-miR-23b-3p	miR-23a-3p (and other miRNAs w/seed UCACAUU)	2.172	20
6	hsa-miR-483-5p	miR-483-5p (miRNAs w/seed AGACGGG)	1.995	2
7	hsa-miR-671-5p	miR-671-5p (miRNAs w/seed GGAAGCC)	1.976	1

Fig. 2. Differential levels of cell-free miRNAs in the early phase of patients with DENV infection. (A) Using 8 samples from viremic phase and 6 samples from convalescent phase, the levels of >2000 miRNAs was screened and compared by using miRNA array. (B) Among the 7 miRNAs that significantly increased during the acute phase of infection (total differentially expressed miRNAs=69, experimentally observed), (C) let-7c miRNA, which is associated with innate immune response regulation and inhibition of DENV replication in vitro, significantly increased from disease day 1, suggesting that the immune response is regulated early during the viremic phase.

The proposed research strategy involves the following three components (clinical, virologic and immunologic): (1) Clinical characterization of samples collected from Vietnam, this will include analyses of comprehensive clinical data from patient and blood biochemistry results, (2) Virologic analyses will include virus isolation, viremia titration and gene sequence with phylogeny analyses and (3) miRNA analyses will include determination of miRNA response signatures and biological functions *in vitro*.

(1) Analyses of clinical data.

Inflammatory cytokine levels will be determined by using ELISA and Luminex. Because disease outcomes are also affected by sequence of infection, these data will be used to group patients according to disease severity, course of disease and type of infection and analyzed with levels of expressed miRNA.

(2) Determination of regulation of miRNA in dengue patients with differential viremia and clinical outcomes, and validation of miRNA expression by qRT-PCR

miRNA and cytokine profiles (upregulated and downregulated) in using longitudinal samples was determined by microarray analyses. Comparisons made by clustering each of the (1) classical dengue patient group vs severe dengue patient groups, (2) early (acute) vs late (convalescent) was determined, and we identified 3 unique miRNAs that are associated with acute infection and severity.

(3) DENV induced gene regulation in different host cell lines

A total of 3 candidates identified from the mRNA profiles of dengue patients was synthesized. One candidate, miRNA hsa-let-7c-5p was selected for the following assays due to higher potency during infection. Virus growth was examined by using real-time PCR and plaque assay.

(4) Statistical analyses

Two-group comparisons were analyzed using Student's t-test. Multiple group comparisons were analyzed by running both parametric (ANOVA) and non-parametric (Kruskal Wallis) statistical tests with Dunn's and Tukey's post hoc tests. Continuous variables are presented as the median and interquartile range (IQR) or mean ± standard deviation. Skewness was tested to assess the normal distribution of the variables. As the data did not show normal distribution, we used non-parametric tests. Data from more than two groups were compared using MANCOVA, and between two groups were compared using the Mann–Whitney U test based on distribution of data. The Spearman's test was used to identify correlations and the results are presented as the coefficients (ρ −1 to +1). p–values of < 0.05 were considered as statistically significant. PCA was conducted in R (Version 4.1.2) with plotting tools from the factoextra package.

4．研究成果

(1) Host cytokine expression

We first analyzed 45 cytokines and other factors in serum samples from the acute phase of DENV infection (within 3-5 days of symptom onset) from 167 patients. All of the patients tested positive for serum DENV nonstructural protein 1 antigen (NS1 Ag); 78.4% and 62.9% were positive for immunoglobulin M (IgM) and G (IgG), respectively; and 18.0%, 19.8%, and 11.9% tested positive for serotypes 1, 3, and 4, respectively. Although the DENV-4 viral load was significantly higher than those of DENV-1 or DENV-3, disease severity was not associated with viral load or serotype. Significant correlations were identified between disease severity and CCL5, SCF, PDGF-BB, IL-10, and TNF-α levels; between NS1 Ag and SCF, CCL5, IFN-α, IL-1α, and IL-22 levels; between thrombocytopenia and IL-2, TNF-α, VEGF-D, and IL-6 levels; and between primary or secondary infection and IL-2, IL-6, IL-31, IL-12p70, and MIP-1ϐ levels (Fig3).
Samples were grouped according to severity for further analyses.

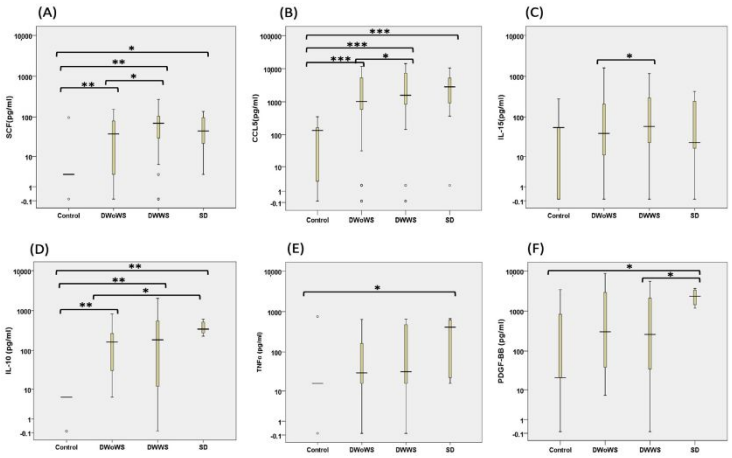

Fig. 3. Serum cytokine levels in dengue patients during the acute phase of infection. (A–F) Cytokine levels in serum samples from healthy individuals (Control; n = 10) or patients with DWoWS (n = 64), DWWS (n = 64), and SD (n = 39). (A) SCF, (B) CCL5, (C) IL-15, (D) IL-10, (E) TNF-α, and (F) PDGF-BB. Circles represent samples outliers to the median value. Box plots show the median and interquartile range. * p < 0.05, ** p < 0.01, *** p < 0.001 using MANCOVA (after controlling for Age, gender, days of fever and IgM) the Mann–Whitney U test.

Next, from the cytokine data, a total of 3 principal components were extracted, accounting for 60.8% of variation in the data. In terms of percentage contribution, there was no clear dominance by any cytokine in the first component despite the largest explained variance at 40.3%. The second and third component exhibited some overlap with considerable contributions from similar cytokines such as PIGF1 and CCL5 (Figure 4a–c). Based on this, the comparisons between components 2 and 3 were omitted in subsequent analyses. Additional subgroup analyses separating cases with positive and negative for DENV RNA were also performed (Figure 4d–i). The extracted principal components were first analyzed for the capacity of distinguish between primary and secondary infection cases and by disease severity (Figure 4d–f). While there was limited PCA distinction between the primary and secondary infections based on the selected components (Figure 4d–f), the data suggest that the first two principal components were able to distinguish cases by disease severity (Figure 4g–i), particularly among those that were negative for DENV RNA within different group means by severity (Figure 4i).

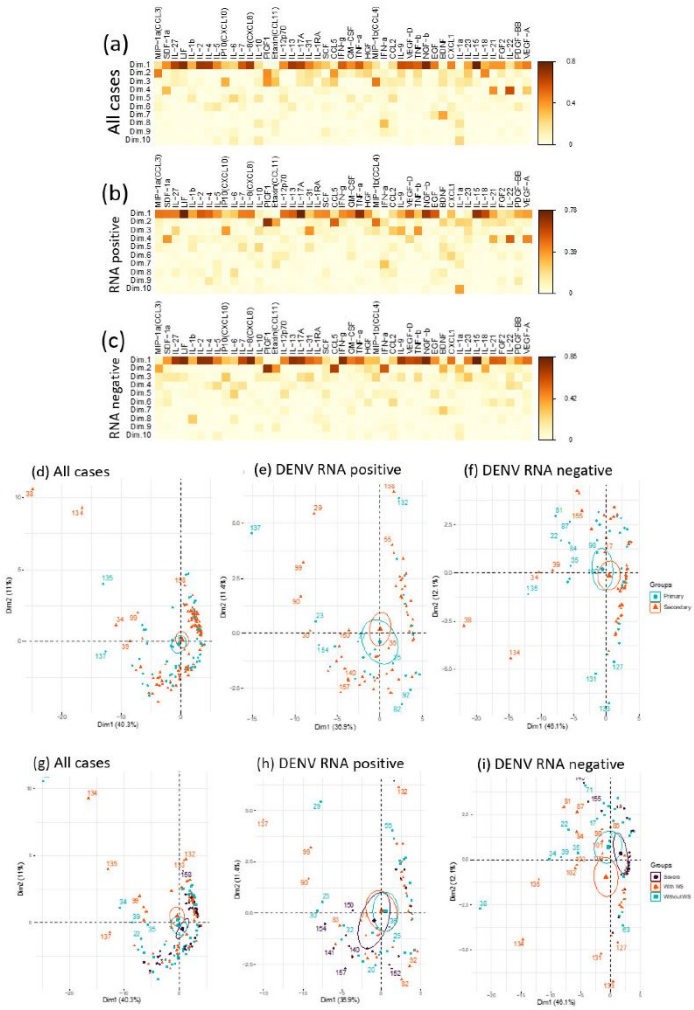

Figure 4. Principal component analysis (PCA) of different cytokine levels from dengue patients with severe and non-severe dengue. The contributions of cytokines to the top principal components (Dim.1 to Dim10) in all cases (a), dengue cases that demonstrated virus RNA (b) and in the absence of virus RNA (c). Clustering of individuals by primary and secondary infection according to principal component 1 (Dim.1) and component 2 (Dim.2) (d–f). Clustering of individuals by the severity of infection—severe, with and without warning sign (WS) according to principal component 1 (Dim.1) and 2 (Dim.2) for all cases (g), cases with positive RT-PCR (n = 83, (h)), and cases with negative RT-PCR (n = 84, (i)). Circles represent the group means. All cases (n = 167) were used in the analyses (a–i).

(2) Effects of miRNA hsa-let-7c-5p in Vero 9013 and K562 cells.

To determine the effects of miRNA has-let-7c-5p in DENV human cells, K562, HEK293T and T98G cell lines were used in this experiment. In plaque assay, miRNA has-let-7c significantly reduced DENV replication at 0.1nM and 1 nM concentrations (Figure 5), but the effect was not significant at 0.01 nM (data not shown).

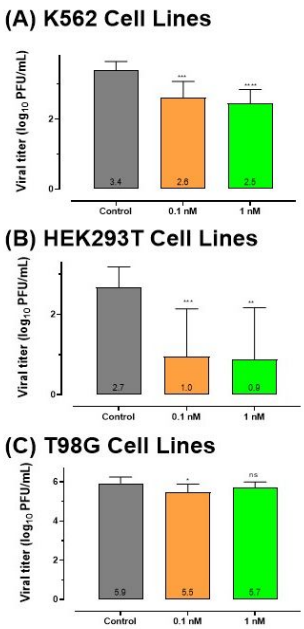

Figure 5. Efficacy of miRNA hsa-let-7c-5p at 0.1nM and 1nM concentration against DENV-2 at an MOI of 0.01, as quantified by plaque assay at post-treatment studies. Log10 transformed viral titer values of miRNA-treated viral-infected cell culture supernatants were compared with that of the untreated viral-infected control. (A) K562, (B) HEK293T (3) T98G cells. Cells were infected with virus for 1 h, viral inoculum was aspirated, cells were washed with EMEM twice and replaced with EMEM with 10% FBS. The error bars represent the standard deviation of the mean of at least 6 replicates; * $p < 0.05$; *** $p < 0.001$; ns=non-significant.

Conclusion: miRNA hsa-let-7c-5p inhibited dengue virus replication and is potentially associated with host response generated during DENV infection. Further in-vitro and in-vivo study are needed to demonstrate the efficacy of miRNA hsa-let-7c-5p against dengue viral infection and pathogenesis.

5．主な発表論文等

〔雑誌論文〕　計1件（うち査読付論文　1件／うち国際共著　1件／うちオープンアクセス　1件）

1．著者名 Nwe, K.M.; Ngwe Tun, M.M.; Myat, T.W.; Sheng Ng, C.F.; Htun, M.M.; Lin, H.; Hom, N.S.; Soe, A.M.; Elong Ngono, A.; Hamano, S.; Morita, K.; Thant, K.Z.; Shresta, S.; Thu, H.M.; Moi, M.L.	4．巻 11
2．論文標題 Acute-phase Serum Cytokine Levels and Correlation with Clinical Outcomes in Children and Adults with Primary and Secondary Dengue Virus Infection in Myanmar between 2017 and 2019.	5．発行年 2022年
3．雑誌名 Pathogens	6．最初と最後の頁 1-15
掲載論文のDOI（デジタルオブジェクト識別子） 10.3390/pathogens11050558	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

〔学会発表〕　計0件

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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6．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考
研究協力者	ヒヤンミャット　ツー (Hlaing Myat Thu)		
研究協力者	スレスター　スジャン (Shresta Sujan)		
研究協力者	チークインマイ　ル (Thi Quynh Mai Le)		
研究協力者	ニオ　フイミン (Neoh Hui Min)		

7．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関			
マレーシア	Universiti Kebangsaan Malaysia			
ミャンマー	Department of Medical Research			
ベトナム	NIHE			
米国	La Jolla Institute of Immunology			