Short Communication

Neutrophil Extracellular Traps Contribute to the Disease Severity of Dengue Virus Infection

*Cucunawangsih Cucunawangsih[1], Ratna Sari-Wijaya[1], Nata Pratama Hardjo-Lugito[2], Ivet Suriapranata[3]

[1]Department of Microbiology, Faculty of Medicine, Pelita Harapan University, Tangerang, Indonesia
[2]Department of Internal Medicine, Faculty of Medicine, Pelita Harapan University, Tangerang, Indonesia
[3]Division of Immunology, Mochtar Riady Institute for Nanotechnology and Medical Science Group, Pelita Harapan University, Tangerang, Indonesia

*Corresponding author: Dr Cucunawangsih Cucunawangsih, E-mail: cucunawangsih.fk@uph.edu

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Abstract

Background: The spectrum of dengue infection ranges from asymptomatic or mild to severe disease. The pathogenic mechanisms are not fully understood. A viral infection can induce the neutrophil extracellular traps (NETs), and the excessive NETs lead to increased vascular permeability, coagulopathy, and platelet dysfunction, a hallmark of severe dengue.

Methods: To evaluate the association of NETs formation with disease severity using a human public transcriptomic dataset (GSE17924) and clinical samples from dengue patients with different disease severity.

Results: Based on the transcriptomic analysis, the whole blood gene expression functional in neutrophil activities and NETs formation was upregulated with dengue disease severity. The serum concentration of citrullinated histone H3 (CitH3), a NETs marker, was measured in 28 dengue patients, of whom 18 classified as dengue fever (DF) and 10 as dengue hemorrhagic fever (DHF) grade 1 and 2. A significantly higher CitH3 concentration was found in DHF compared to DF patients. The level of CitH3 was negatively correlated with platelet counts.

Conclusion: Our results suggest NETs have contributed to the disease severity of dengue infection. Future studies on the predictive value of NETs markers and the potential NETs as a targeted therapy in dengue disease should be prioritized.

Keywords: Neutrophil extracellular traps; NETs; Neutrophil; Dengue

Introduction

Dengue is the most common arboviral infection worldwide (1). Although most infected individuals are asymptomatic or show mild febrile known as dengue fever (DF), it can manifest as a severe disease, dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) (2). The pathogenesis of DF and severe dengue is not fully understood. In response to viral infection, including dengue, neutrophils can produce neutrophil extracellular traps (NETs) by secreting the decondensed chromosomal DNA composed of histones and antimicrobial proteins (3, 4). The NETs can inhibit viral dissemination by entrapping and inactivating viral particles (4). However, the excessive NETs formation has detrimental effects on endothelium and subsequently leads to increased vascular permeability, coagulopathy, and platelet dysfunction, a hallmark of severe dengue (5, 6). To better understand the relationship of NETs formation (NETosis) with disease severity, we performed transcriptome analysis and determined the serum concentration of NETs formation circulating marker in dengue patients.

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Materials and Methods

Data acquisition, processing, and analysis

The microarray data from the whole blood of dengue patients collected between 3 and 7 days after fever onset were downloaded from the publicly available database (Gene Expression Omnibus) with accession number GSE 17924 (7). The GSE17924 dataset consists of 29 samples of non-severe dengue patients (DF, DHF grade 1 and 2) and 19 samples of severe dengue patients (DSS). Gene expression levels were available as normalized and log2 transformed data. The limma R package was used to calculate the differential expressed genes (DEGs). The enrichment analysis of DEGs into biological process (BP) gene ontology (GO) terms and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were performed using the web-based Enrichr tool (https://maayanlab.cloud/Enrichr/#) (8). The statistically significant threshold for enrichment analysis was adjusted $p< 0.05$. The predefined gene set of NETosis (extracted from the KEGG database; hsa04613) was used in the gene set enrichment analysis (GSEA) of the whole blood transcriptome to get a robust biological interpretation of the role of NETosis in disease severity. An enrichment with FDR q-value< 0.25 was considered significant.

Clinical sample collection and analysis

This study used samples of dengue patients admitted to Siloam Hospital, Tangerang, Indonesia, between January and April 2021. Upon hospital admission, blood samples were collected, and sera were separated by centrifugation and kept frozen at -80 °C until further processing. The dengue infection was confirmed by RT-PCR (Bioneer, South Korea) and diagnostic tests, including NS1 test (STANDARD F dengue NS1 Ag FIA, SD Biosensor, South Korea), dengue IgG and IgM capture ELISA (Panbio, Australia). The serum concentration of citrullinated histone H3 (CitH3), a marker specific for NET remnants, was quantified using the CitH3 (clone 11D3) ELISA kit (Cayman Chemicals, USA). The haematology and clinical data were obtained from hospital medical records.

Results

To determine whether neutrophils contribute to dengue infection severity, we first analyzed the DEGs between whole blood transcriptomic data of severe and non-severe dengue patients. We observed 73 DEGs between two groups of patients, of which 60 were upregulated and 13 were downregulated in the severe compared to the non-severe dengue patients (Fig. 1A and Table 1). Most of the top ten upregulated genes in severe dengue patients were related to neutrophil granules protein (ARG1, DEFA3, DEFA4, CEACAM8, LTF, MMP8, OLFM4) (Fig. 1A). The involvement of NETosis in the disease severity was indicated by: [1] the association of the upregulated genes in the severe group with neutrophil activation and immune response (Fig. 1B), [2] The KEGG pathway analysis revealed that the upregulated genes in the severe group were significant relevant to NETosis **(**Fig. 1B), [3] In gene set enrichment analysis (GSEA) based on the 32947 protein-coding genes of DF, DHF, and DSS patients from GSE17924 dataset, the genes related to NETosis was enriched in severe disease (DSS > DHF > DF, Fig. 1C).

To gain more evidence of the role NETosis in disease severity, we measured the serum level of CitH3 of 28 hospitalized acute dengue patients. A single blood collection was obtained between days 3 and 4 after the onset of the fever. The mean age of dengue patients was 32 years, and 16 (57%) dengue patients were male (Table 2). The patients were clinically and haematological classified as having DF (n= 18; platelet count, mean [±SD]= 138.66 [20.51]x 10^3/μL) and DHF grade 1 and 2 (n=10; platelet count, mean [±SD]= 90.50 [7.59]x 10^3/μL). Based on the IgM/IgG serology profile, DF patients had 4 (14%) primary and 14 (86%) secondary infections, whereas DHF patients

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had 3 (30%) primary and 7 (70%) secondary infections.

Significantly increased levels of CitH3 were detected in DHF compared to DF patients (mean: 2.35 vs 0.91 ng/mL, p< 0.01, Fig.2A). Although a higher CitH3 concentration was observed in DHF than in DF patients both in primary and secondary dengue infection, the significant difference was only detected in a secondary infection (Fig. 2B). When we compared clinical laboratory parameters with CitH3 concentration, we detected negative correlation with platelet count (r= -0.49, p= 0.008, Fig. 2C). Age and sex were not significantly related to CitH3 levels (Supplementary Fig. 1) and dengue severity in the current study (Table 2). The level of CitH3 in serum remained significantly associated with dengue severity in multivariable logistic analysis, suggesting CitH3 level as an independent factor related to dengue severity (Table 3).

Table 1. List of differentially expressed genes (DEGs) with cut-off absolute fold change 1.2 and adjusted p< 0.05 between severe dengue versus non-severe dengue patients

Gene Symbol	LogFC	AveExpr	adj.P.Val
OLFM4	3.684792	8.269305	0.002244
VSIG4	3.233939	9.225941	5.39E-06
CEACAM6	2.93709	6.242674	0.001896
CEACAM8	2.713956	8.152327	0.000713
ARG1	2.671185	9.731862	0.001703
MMP8	2.60388	5.264401	0.001207
DEFA4	2.548078	10.67043	0.009165
DEFA3	2.524389	15.37699	0.003247
LTF	2.510175	10.63133	0.004329
COL17A1	2.466595	5.109461	0.003999
PCSK9	2.463472	3.317676	0.000729
CTSG	2.422616	9.580116	0.003999
ABCA13	2.409258	4.510567	0.001207
ELA2	2.357228	8.015215	0.001703
SPP1	2.201098	4.265368	0.000503
INHBA	2.026761	6.147368	0.000503
ID1	2.021664	7.154604	0.008697
MS4A3	1.988204	5.663081	0.009165
BPI	1.968611	8.125207	0.007603
PCOLCE2	1.947325	4.022112	0.003999
MPO	1.914549	9.768209	0.022949
SERPINB10	1.907951	4.456415	0.002043
WFDC1	1.904594	3.836671	0.027283
CRISP2	1.890101	5.494897	0.001501
OLAH	1.76522	4.469275	0.013442
LCN2	1.74598	9.35606	0.012127
CHIT1	1.702572	6.146512	0.000237
OLR1	1.690919	3.565179	0.005028
HPR	1.66247	8.804823	0.01154
IL1R2	1.651249	12.21126	0.012123
HP	1.627844	12.17435	0.01658
COL1A2	1.626149	3.809335	0.02827
ATP2C2	1.607181	4.707934	0.002244
PPARG	1.60684	5.324916	0.00776
TACSTD2	1.559584	4.90034	0.023341
PGLYRP1	1.554255	9.402735	0.01658
HTRA3	1.552225	5.745499	0.008463

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Table 1. Continued ...

CAMP	1.531532	12.2778	0.015029
GPER	1.525024	8.61035	0.003999
DAAM2	1.447164	6.274214	0.002638
TPST1	1.437648	8.709978	0.000713
MMP9	1.431306	14.36497	0.013774
PDK4	1.42939	8.093026	0.004793
ORM2	1.417116	7.842388	0.017603
DEFA1	1.411795	2.868559	0.022802
BEX1	1.400399	5.252747	0.027518
C19orf59	1.371096	11.28802	0.02817
ORM1	1.337805	9.199384	0.027283
EMP1	1.335258	7.667397	0.013774
S100A12	1.326687	13.59089	0.04103
SLCO2B1	1.323894	4.431284	0.016061
FN1	1.312212	3.847997	0.044188
MS4A4A	1.295942	9.569594	0.02029
ACOX2	1.287754	6.757037	0.016061
MSR1	1.27792	6.411289	0.022583
TCN1	1.240569	8.392428	0.023853
GPNMB	1.237105	5.575871	0.034603
CA4	1.224573	8.804537	0.029418
DPY19L1P1	1.211452	5.042407	0.01154
KCNE1	1.20187	6.198571	0.037637
MYL4	-1.21462	10.67644	0.03268
GPR44	-1.33118	6.564852	0.04788
GPR56	-1.38586	12.47741	0.001501
S1PR5	-1.39393	10.15042	0.000329
ZNF683	-1.39537	10.47343	0.004605
SLC1A7	-1.63002	8.801973	0.000329
VIT	-1.65065	5.464438	0.000503
EPB49	-1.6514	6.887229	0.011623
HBQ1	-1.69088	14.56433	0.037194
C1orf173	-1.83108	4.345844	0.017603
IDO1	-1.87036	8.75015	0.049163
EFNB3	-1.98601	13.54877	0.027769
LOC283177	-2.03835	12.07841	0.043354

Abbreviations; LogFC, log2-fold-change; AveExpr, average expression; adj.P.Val, adjusted p-value

Table 2. Characteristics of dengue patients

	Total (n=28)	**DF (n=18)**	**DHF (n=10)**	**p-value**
Age, mean (±SD), years	32 (11)	31 (13)	32 (10)	0.80
Sex, n (%)				
Female	12 (43)	7 (58)	5 (42)	0.69
Male	16 (57)	11 (69)	5 (31)	
Laboratory findings				
Platelet count (10^3/µL)	121.46 (28.92)	138.66 (20.51)	90.50 (7.59)	<0.0001
Neutrophil count (10^3/µL)	2.75 (1.62)	3.19 (1.88)	1.96 (0.63)	0.13

P-value was calculated using the Mann-Whitney U test for numerical variables and the Fisher exact test for categorical variables

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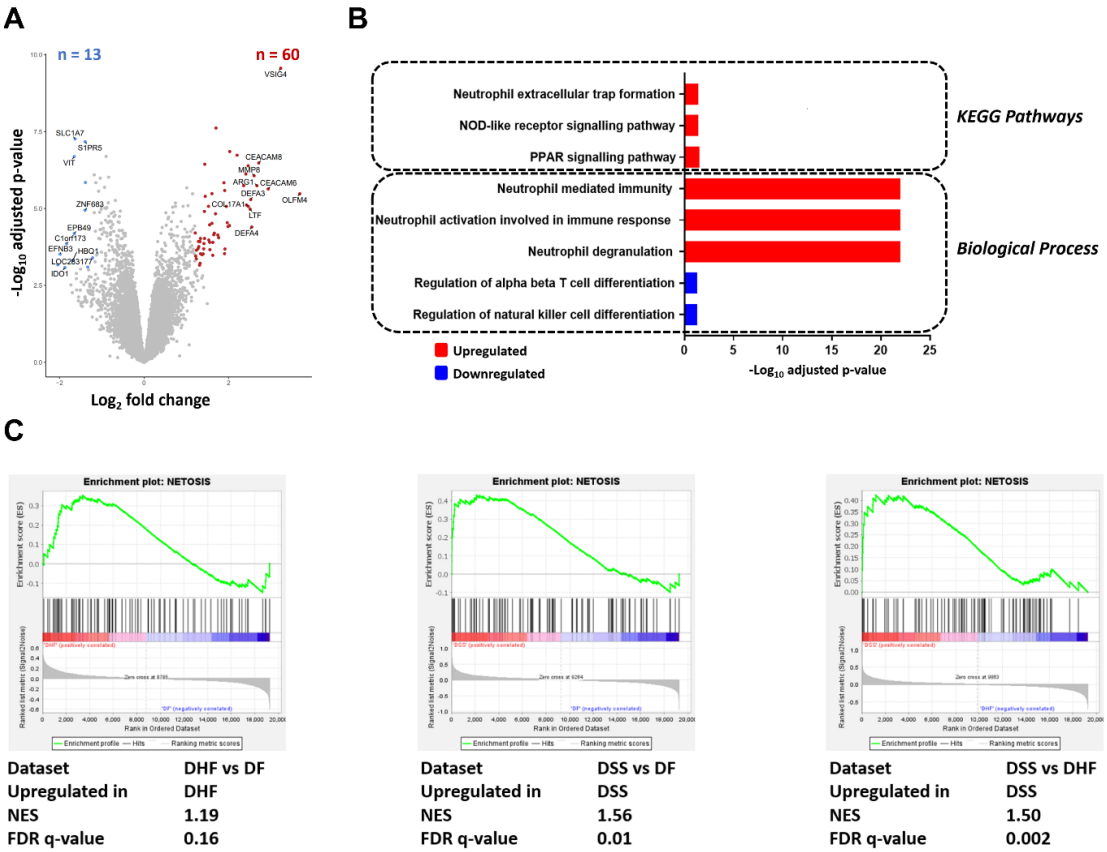
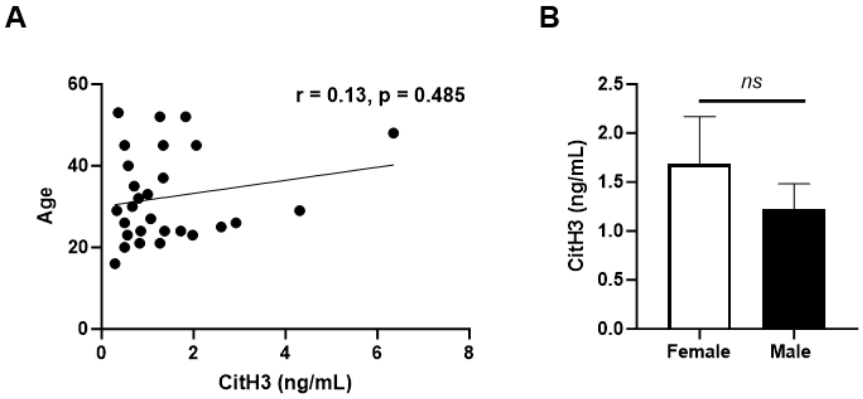


Fig. 1. Expression of genes functional in neutrophil extracellular traps (NETs) formation related to dengue disease severity. The GSE17924 dataset consists of 29 samples of non-severe dengue patients and 19 severe dengue patients. (A) The volcano plot showed that 73 genes were differentially expressed between severe and non-severe dengue patients based on a cut-off of absolute log2 fold change 1.2-fold difference and adjusted p< 0.05. The downregulated genes are labelled in blue color, and the upregulated genes are labelled in red color. The top 10 highly and lowly expressed genes were indicated in the plot. (B) The enrichment analysis of gene ontology (GO) terms in the biology process and KEGG pathway are based on genes upregulated and downregulated in severe dengue patients. The cut-off for the threshold was adjusted p< 0.05. (C) In whole transcriptome expression, the gene signatures related to NETs formation (NETosis) were positively correlated with dengue disease severity. FDR q-value < 25% after 1000 random permutations was set as the cut-off criterion. NES, normal enrichment score; FDR, false discovery rate



Supplementary Fig. 1. Association of age and sex with citrullinated histone H3 (CitH3) level in serum. (A) Spearman correlation analysis was performed to determine the association between age and CitH3 level. (B) The Mann-Whitney U test was done to evaluate the difference level of CitH3 based on sex

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Published Online: June 30, 2024

Table 3. Multivariable logistic regression analysis of citrullinated histone H3 (CitH3) in dengue severity

	Odds ratio	95% Confidence interval	p-value
CitH3 (ng/mL)[#]	4.99	1.56-31.04	0.03

[#]Adjusted for age and sex

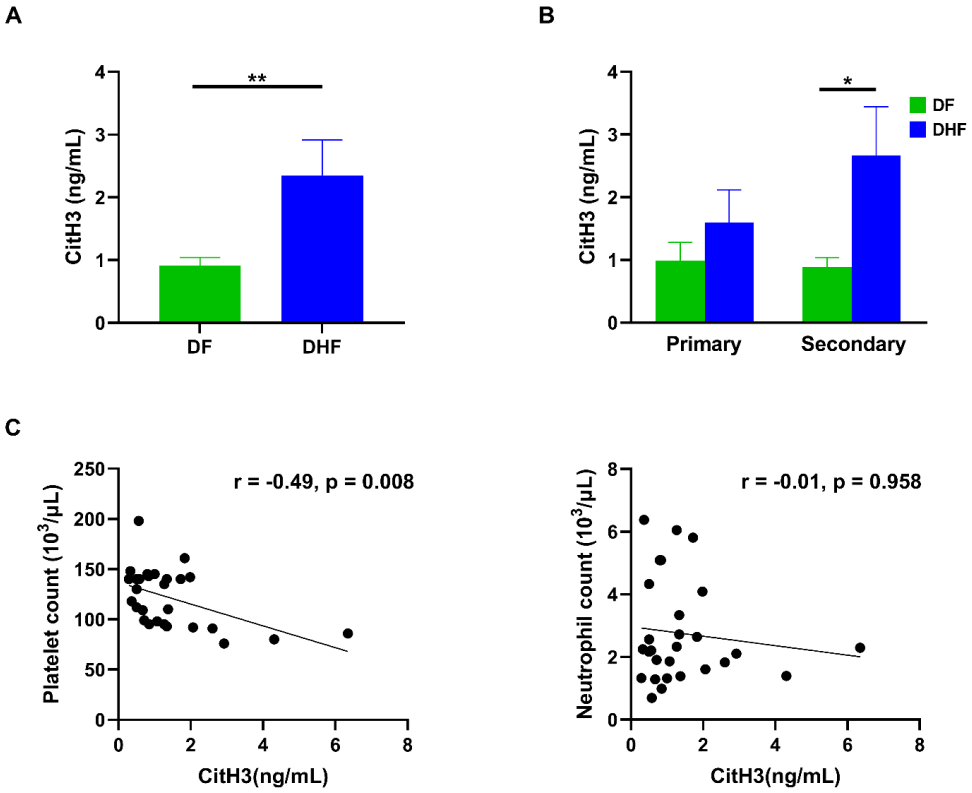

Fig. 2. Detection of neutrophil extracellular traps (NETs) formation marker, citrullinated histone H3 (CitH3), in sera of dengue patients. Sera from dengue patients (dengue fever/DF, n=18; dengue haemorrhagic fever/DHF, n=10) were assessed for CitH3, NETs marker (A). The citH3 concentration of dengue patients in primary and secondary dengue (B). The association of CitH3 with clinical laboratory results; platelet and neutrophil counts (C). The following statistical analysis was performed: Mann-Whitney (A and B) and Spearman's rank-order correlation test (C). Data are shown as mean ± SEM, *p< 0.05, ** p< 0.01

Discussion

Recent studies have shown that NETosis during dengue infection can be induced through platelet-neutrophil interactions (9, 10) or a high level of pro-inflammatory cytokines IL-8 and TNF-α induced during dengue infection (3). Significant elevation levels of CitH3 were observed in serum DHF grade 1 and 2 compared to DF patients, highlighting that NETosis plays a role in dengue severity. This finding was similar to a previous study that detected a higher level of NETosis marker, MPO-DNA complexes, in the serum of DHF than in DF patients (3).

The negative correlation between CitH3 concentration and platelet count is likely due to the increased consumption due to massive platelet adhesion, activation, and aggregation by NETs formation (11, 12). Furthermore, we found the levels of CitH3 were not only increased in DHF patients but also higher in secondary infection of DHF patients, indicating [1] the NETosis

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Published Online: June 30, 2024

may contribute to increased disease severity in secondary infection, [2] the possibility of memory in NETs immune response presence after primary dengue infection, as described in a previous study (13).

Overall, based on transcriptome and serum analysis, we found that the host immune response, NETosis, is related to the disease severity of dengue infection. Future large cohort studies that investigate the predictive power of circulating NETs markers in the early phase during the disease's evolution are required to confirm the prognostic utility of NETosis markers in clinical practice. In addition, further investigation to determine whether the dengue serotype has influenced the CitH3 level is also important since the dengue serotype has been described previously as a factor related to disease outcome (14, 15). Considering no specific treatment for dengue infection exists so far, understanding the impacts of NETs formation in the pathogenesis of dengue virus infection would be an essential step toward uncovering the potential therapeutic benefits of targeting NETosis in controlling disease progression.

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Ethical consideration

The study protocol was reviewed and approved by the Research Ethics Committee of the Mochtar Riady Institute for Nanotechnology (No: 014/MRIN-EC/ECL/XI/2019).

Conflict of interest statement

The authors declare there is no conflict of interest.

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