

Original article

Association of microRNAs as biomarkers for severe liver dysfunction in severe dengue infection

Napat Pas-arj^{a, b, c} , Umaporn Limothai^{b, c, d} , Janejira Dinhuzen^{b, c, d} ,
Sasipha Tachaboon^{b, c, d} , Nattachai Srisawat^{a, b, c, d, f, g, *} 

^aMaster of Science Program in Medical Sciences, Faculty of Medicine, Chulalongkorn University

^bExcellence Center for Critical Care Nephrology, King Chulalongkorn Memorial Hospital, Bangkok, Thailand

^cCenter of Excellence in Critical Care Nephrology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

^dTropical Medicine Cluster, Chulalongkorn University, Bangkok, Thailand

^eDivision of Nephrology, Department of Medicine, Faculty of Medicine, Chulalongkorn University

^fCenter for Critical Care Nephrology, The CRISMA Center, Department of Critical Care Medicine, University of Pittsburgh, School of Medicine, Pittsburgh, PA, USA

^gAcademy of Science, Royal Society of Thailand, Bangkok, Thailand

Abstract

Background: The identification of early biomarkers to predict liver dysfunction in patients with severe dengue is essential. Currently, there are no available data on circulating microRNAs to predict this important complication.

Objectives: This study aimed to explore the role of circulating microRNAs in predicting severe liver dysfunction in dengue infection by identifying and validating predictive microRNAs via NanoString and quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

Methods: On the initial day of admission, serum samples were gathered from patients with dengue infection. These patients were monitored for 14 days post-admission to ascertain their final diagnosis. Following the WHO 2009 criteria, participants were categorized into severe and non-severe liver dysfunction groups. To perform transcriptomic analysis of the circulating microRNAs, we used the NanoString nCounter 1 Human v3 microRNA Expression Assays. Subsequently, we validated the levels of the selected microRNAs using qRT-PCR.

Results: In the discovery phase, 5 non-severe cases and 4 severe cases were tested, and elevated liver enzymes were found in the severe cases. In the validation phase, 11 severe cases demonstrated substantially longer durations of fever before admission; higher white blood cell counts, total bilirubin, direct bilirubin, and liver enzyme levels; and lower platelet counts and abdominal pain, compared with those of the 92 non-severe cases. Analysis revealed that only 2 of 798 microRNAs, microRNA-424-5p and microRNA-30d-5p, were upregulated in the severe liver dysfunction group compared with the non-severe liver dysfunction group. MicroRNA-424-5p had an area under the receiver operating characteristic curve of 0.67 (95% confidence interval: 0.52–0.86, $P = 0.04$) and exhibited sensitivity, specificity, positive predictive value, and negative predictive value of 63.6%, 76.1%, 16.3%, and 83.7%, respectively. Functional enrichment and gene-targeting analyses indicated that microRNA-424-5p may influence important signaling pathways, including mitogen-activated protein kinase, PI3K-Akt, and mTOR, which are involved in cellular development and metabolic regulation.

Conclusion: Notably, microRNA-424-5p exhibited modest diagnostic performance in distinguishing severe from non-severe liver dysfunction. Our research highlighted the potential of circulating microRNAs, particularly microRNA-424-5p, as biomarkers for predicting severe liver dysfunction in patients with dengue.

Keywords: microRNA, non-severe liver dysfunction, severe dengue infection, severe liver dysfunction, Thailand.

***Correspondence to: Nattachai Srisawat**, Department of Medicine, Faculty of Medicine, Chulalongkorn University, and King Chulalongkorn Memorial Hospital, Thai Red Cross, Bangkok 10330, Thailand.

E-mail: drnattachai@yahoo.com

Received: August 10, 2025

Revised: March 2, 2026

Accepted: April 8, 2026

Dengue infection is a flu-like disease that can affect individuals of all ages and may result in a diverse range of clinical outcomes. The spectrum of disease ranges from the subclinical stage, with defervescence occurring on days 3–7, to the severe stage, which causes plasma leakage and severe bleeding.⁽¹⁾

The liver is affected in patients with dengue infection, thus making it one of the most commonly involved organs. Hepatic clinical manifestations can be observed in 60.0–90.0% of patients infected with dengue, including elevated aspartate aminotransferase or alanine aminotransferase levels, hepatomegaly, and jaundice.⁽²⁾ The incidence of severe liver dysfunction in severe dengue infection is approximately 5.5%.⁽³⁾ The presence of severe hepatitis is indicative of an unfavorable prognosis among patients with dengue, even though the majority of individuals recover from their illness without requiring treatment.⁽⁴⁾

MicroRNAs (miRNAs) have been identified as potential predictive biomarkers for the diagnosis of infectious diseases, including tuberculosis and malaria. In tuberculosis, shifts in miRNA expression have been explored across the different phases of infection, highlighting their potential as diagnostic biomarkers.⁽⁵⁾ Meanwhile, in malaria, diagnosing severe malaria remains challenging due to its broad and nonspecific symptoms. In addition, current detection methods are insufficient for identifying the extent of organ damage, as complications often progress unnoticed until the window for effective treatment has closed.⁽⁶⁾

miRNAs are small non-coding endogenous RNAs with approximately 21 nucleotides. Furthermore, miRNAs are involved in many biological processes, including the control of infection and the regulation of messenger RNAs by deadenylating and decapping them for degradation.⁽⁷⁾

A previous study highlighted the potential of blood-derived miRNAs as biomarkers for dengue diagnosis and severity assessment. Differential miRNA expression patterns were observed between mild and severe dengue cases, indicating their role in disease progression. Specific miRNAs were associated with immune response modulation, vascular permeability, and liver dysfunction in patients with severe dengue; meanwhile, the expression levels of miR-24-1-5p, miR-512-5p, and miR-4640-3p exhibited substantial variation between dengue fever (DF) and patients with DF with liver dysfunction (DFL), suggesting their potential role in distinguishing disease severity. These findings suggest that miRNAs could serve as non-invasive molecular indicators for early detection and risk stratification.⁽⁸⁾

The late onset and short duration of warning signs have led to the identification of additional potential early biomarkers to predict severe dengue infection, particularly severe liver dysfunction. Moreover, the ability to predict this specific complication will allow clinicians to make early interventions for patients. Therefore, this study aimed to identify reliable predictive miRNAs and underlying molecular pathways in patients with severe liver dysfunction due to dengue infection.

Materials and methods

Ethics statement

In this study, all patients aged ≥ 18 years and parents of patients aged < 18 years provided informed consent. The research was reviewed and approved by the Institutional Review Board, Faculty of Medicine, Chulalongkorn University (IRB no. 0827/66), as well as the Ethics Committee of Banpong Hospital (REC no. 009/2562) and Photharam Hospital (REC no. 32/2562). The research adhered strictly to the principles of the Declaration of Helsinki and Good Clinical Practice guidelines.

Criteria

The inclusion criteria for this research were: (i) Admitted to the participant hospital, (ii) consented to participate in the research; and (iii) a positive test result for dengue virus non-structural protein 1 antigen and antibodies (immunoglobulin G/immunoglobulin M) against dengue virus, performed using the severe dengue (SD) BIOLINE Dengue Duo kit. Exclusion criteria were patients without dengue infection.

Sample collection

Blood samples for miRNA analysis were drawn from patients with suspected dengue on day 1, day 3 of admission, defervescence day, and day of discharge. Collection and storage of clinical data and serum samples from patients were performed at the Excellence Center of Critical Care Nephrology, King Chulalongkorn Memorial Hospital, Bangkok, Thailand. The volume of blood collected was 9 mL for patients aged < 15 years and 18 mL for patients aged ≥ 15 years.

To collect serum, a 10 mL red-top serum collection tube was used to store the whole blood, and the tube was left upright at room temperature for approximately 15–30 min to allow clotting. Thereafter, the tube was centrifuged at 3,000 rpm for 10 min. Then the

remaining liquid, either solution or serum, was transferred into a polypropylene tube. To collect plasma, a 10 mL heparinized green-top tube was used to store whole blood, and the tube was gently inverted 6–8 times. After centrifugation at 3,000 rpm for 10 min, the remaining liquid, or plasma, was transferred into a polypropylene tube. Serum and plasma components were portioned into smaller aliquots and stored at -80°C until further analysis.

Patient enrolment and study design

This research was a multicenter retrospective observational study of patients from 3 hospitals, including 2 hospitals in Ratchaburi province (Ban Pong Hospital and Photharam Hospital) and King Chulalongkorn Memorial Hospital in Thailand, between September 1, 20 and December 31, 2020.

In the discovery phase, samples were randomly selected from the non-severe and severe liver dysfunction groups to perform miRNA profiling using the NanoString technique.

Then, 9 patients were randomly selected, including 5 from the non-severe liver dysfunction group and 4 from the severe liver dysfunction group.

In the validation phase, the receiver operating characteristic (ROC) ^(9, 10) in MedCalc (MedCalc Software Ltd., Ostend, Belgium) ⁽¹¹⁾ was used to calculate the sample size. The sample size was calculated based on the following values: a type I error of 0.05; a type II error of 0.20 (power of 80.0%); an area under the curve (AUC) of 0.8; a null hypothesis of 0.5; and a ratio of sample sizes in negative to positive cases of 33:3 (non-severe liver dysfunction: severe liver dysfunction).⁽³⁾ The minimum required sample size was determined to be 96 cases, comprising 88 non-severe liver dysfunction cases and 8 severe liver dysfunction cases. Therefore, we included 103 patients in this study.

All confirmed cases of dengue were classified into three categories according to the 2009 WHO criteria: non-severe cases without warning signs (referred to as dengue infection or DI), non-severe dengue with a warning sign (DWS), and SD.⁽¹²⁾ SD was defined by the presence of at least one of the following: 1) severe plasma leakage resulting in a high or progressively rising hematocrit with an elevation of at least 20% above baseline, a pulse pressure of 20 mm Hg or less, the presence of pleural effusions or ascites, circulatory compromise or shock as characterized by symptoms such as tachycardia, cold

and clammy extremities, capillary refill time exceeding 3 s, a weak or undetectable pulse, narrow pulse pressure or, in late shock, unrecordable blood pressure; 2) severe bleeding as determined by physicians; and 3) severe organ impairment leading to alteration of consciousness, severe gastrointestinal involvement is marked by persistent vomiting, increasing or intense abdominal pain and jaundice, acute renal failure, encephalopathy, and cardiomyopathy. Severe liver dysfunction was indicated by aspartate aminotransferase or alanine aminotransferase levels ≥ 1000 U/L. Serum samples from healthy donors, without an apparent or prior history of infection, were obtained from the National Blood Center of the Thai Red Cross Society in Bangkok, Thailand. These donors were qualified by physical examinations and negative serology tests for dengue non-structural protein 1, immunoglobulin M, hepatitis B virus, hepatitis C virus, and human immunodeficiency virus.

RNA isolation and microtranscriptomic analysis

Viral RNA was extracted from 200 μL of serum sample using the microRNeasy Serum/Plasma Kit (Qiagen, Gaithersburg, MD, USA) according to the manufacturer's instructions. The NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) was used to measure RNA concentration and purity.

The nCounter 1 Human v3 miRNA Expression Assay (NanoString Technologies, Seattle, USA) was used to analyze the human miRNA expression. Nine patients were divided into two groups: 4 with severe liver dysfunction and 5 with non-severe liver dysfunction. A total of 100 ng of RNA underwent processing as per the manufacturer's instructions. The nCounter Digital Analyzer was used to record image capture across 280 fields of view, and the nSolver Analysis (version 4.0) software was used to analyze the miRNA data. Significantly different miRNAs were selected based on $P < 0.05$. After NanoString analysis, the expression patterns of 798 human miRNAs were examined, and significant miRNAs were selected based on the following criteria: $P < 0.05$, Log_2 fold-change ≥ 1.5 or ≤ -1.5 , and relevant underlying mechanisms from other studies.

Validation by quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The QIAamp Viral RNA Mini Kit (QIAGEN, Germany) was used to isolate viral RNA from serum samples and to detect dengue virus and its serotypes.

To analyze the dengue virus, a 511 bp DNA band was detected. Subsequently, nested PCR with specific primer sets was used to determine the serotype. Detection of a 482 bp, 119 bp, 290 bp, or 392 bp DNA band indicated the presence of dengue virus-1, dengue virus-2, dengue virus-3, or dengue virus-4, respectively. Validation of miRNAs was performed using TaqMan qRT-PCR. To normalize human circulating miRNA data, identifying reference genes was necessary. Numerous studies have indicated that miR-16-5p is highly stable in serum samples.^(13, 14) All procedures were performed according to the manufacturer's protocols. To measure fold changes in miRNA expression levels, the $2^{-\Delta\Delta C_t}$ method was used.

Statistical analysis

All patients' clinical data were collected in an Excel file. We applied nSolver Analysis Software (Version 4.0) for NanoString analysis. Continuous variables with a normal distribution were expressed as the mean $\pm$ standard deviation (SD), while median and interquartile range (IQR) were used for variables with a non-normal distribution. For parametric variables, Student's *t*-test or one-way analysis of variance was employed, while non-parametric variables were analyzed using the Mann-Whitney U test or Kruskal-Wallis test. A chi-square test was performed on categorical variables, and results were expressed as percentages. Factors associated with disease severity were assessed using univariate analysis, and candidate miRNA expression levels were examined for correlations with other variables using either Pearson or Spearman correlation tests. To evaluate the predictive potential of miRNAs, the ROC curve analysis was employed. Statistical significance was determined as a $P < 0.05$. SPSS version 23 was used for statistical analyses, and GraphPad Prism 9 was used to create the figures.

Results

Clinical characteristics of patients with dengue

In the discovery phase, a total of 9 patients, including 5 with non-severe liver dysfunction and 4 with severe liver dysfunction, were identified by miRNA transcriptomic analysis (**Table 1**). The results revealed that individuals with SD exhibited significantly higher levels of aspartate aminotransferase and alanine

aminotransferase in the severe liver dysfunction group than in the non-severe liver dysfunction group. Next, 103 patients in the validation phase, comprising 92 with non-severe liver dysfunction and 11 with severe liver dysfunction, were analyzed by qRT-PCR. The duration of fever before admission, jaundice, white blood cell counts, the total bilirubin, direct bilirubin, aspartate aminotransferase, and alanine aminotransferase levels were significantly higher, and the levels of abdominal pain and platelets were significantly lower in the severe liver dysfunction group than in the non-severe liver dysfunction group. The remaining clinical characteristics are described in **Table 1**. It was noted that 7 patients in the severe group experienced severe illness on day 1, 2 patients on day 2, and one patient on the day of defervescence, as shown in **Figure 1**.

Differential expression analysis and validation of miRNAs in patients with dengue

Microtranscriptomic analysis identified 798 miRNAs potentially relevant to the study. From that pool, 30 miRNAs were identified as significantly differentially expressed biomarkers in dengue infection (**Figure 2**). To identify significant differential miRNAs, miRNAs were excluded based on the following criteria: a $P < 0.05$, $\log_2$ fold-change ≥ 1.5 or ≤ -1.5 , or relevant underlying mechanisms from other studies. In the validation phase, miRNAs, including miR-424-5p and miR-30d-5p, were potential upregulation biomarkers in NanoString analysis. In qRT-PCR analysis, miR-30d-5p and miR-424-5p were upregulated in the severe liver dysfunction group compared with the non-severe liver dysfunction group (**Figure 3**).

miRNAs as potential biomarkers for severe liver dysfunction

To compare the accuracy of diagnostic tests, two miRNAs exhibited discriminatory ability between the severe and non-severe liver dysfunction groups: miR-424-5p had an area under the receiver operating characteristic curve of 0.67 (95% confidence interval (CI): 0.52–0.86, $P = 0.04$) (**Figure 4**).

The optimal threshold of miR-424-5p for identifying severe liver dysfunction was 6.9, with a sensitivity, specificity, positive predictive value, and negative predictive value of 63.6%, 76.1%, 16.3%, and 83.7%, respectively (**Table 2**).

Table 1. Clinical characteristics, including demographic data, physical examination findings, clinical signs, and laboratory parameters, with statistical comparisons, categorized into non-severe and severe cases across both the discovery and validation phases.

Clinical characteristics			Discovery phase		Validation phase		P-value
	Non-severe (n = 5)	Severe (n = 4)	P-value	Non-severe (n = 92)	Severe (n = 11)		
Demographic data							
Age; years (median, IQR)	19.0(12.0, 43.0)	19.0(17.0, 21.8)	1.000	19.0(15.0, 28.8)	24.0(21.0, 30.0)	0.115	
Gender; male (N, %)	1 (20.0)	2 (50.0)	0.343	39 (42.4)	6 (54.5)	0.442	
BMI; kg/m ² (median, IQR)	22.8(19.1, 25.9)	26.5(16.8, NA)	0.480	21.0(18.4, 23.3)	25.7(19.1, 34.8)	0.105	
Duration of fever before admission; days (median, IQR)	2.0 (1.0, 3.5)	4.0(1.5, 5)	0.258	2.0(1.0, 3.0)	5.0(3.0, 5.0)	0.010*	
Physical examination							
Body temperature; °C (median, IQR)	37.9(36.8, 39.3)	37.9(37, 39.7)	0.806	37.7(36.9, 38.7)	37.4(36.8, 38.3)	0.589	
Systolic blood pressure; mmHg (median, IQR)	120.0(103.5, 135.5)	121.0(1.5, 135.5)	0.901	110.0(100.0, 120.0)	120.0(100.0, 140.0)	0.364	
Diastolic blood pressure; mmHg (median, IQR)	70.0(61.0, 82.5)	65.0(60.5, 84.5)	0.620	70.0(60.0, 70.0)	62.0(60.0, 75.0)	0.662	
Pulse rate; bpm (mean, SD)	90.8 (10.0)	104.0 (23.5)	0.355	92.2 (16.0)	100.0 (22.9)	0.319	
Respiratory rate; bpm (median, IQR)	20.0(20.0, 23.0)	20.0(20.0, 23.0)	0.770	20.0(20.0, 20.0)	20.0(20.0, 23.0)	0.956	
Clinical sign							
Abdominal pain (n, %)	0 (0.0)	1 (25.0)	0.236	11 (12.0)	4 (36.4)	0.030*	
Persistent vomiting > 2 times (n, %)	0 (0.0)	2 (50.0)	0.073	12 (13.0)	5 (45.5)	0.060	
Jaundice (n, %)	0 (0.0)	0 (0.0)	NA	0 (0.0)	1 (0.1)	0.040*	
Hepatomegaly > 2 cm (n, %)	0 (0.0)	0 (0.0)	NA	1 (0.0)	0 (0.0)	0.728	
Laboratory							
Serotype by qRT-PCR (median, IQR)	0.0(0, NA)	1.5(0.25, 2.0)	0.711	2.0(1.0, 2.0)	2.0(0.8, 2.3)	0.699	
DENV NS1 positive (n, %)	5 (100.0)	3 (75.0)	0.236	57 (62.0)	7 (63.6)	0.914	
DENV qRT-PCR positive (n, %)	3 (60.0)	1 (25.0)	0.157	67 (72.8)	5 (45.5)	0.137	
Anti-DENV IgM positive (n, %)	2 (40.0)	0 (0.0)	0.151	51 (55.4)	4 (36.4)	0.231	
Anti-DENV IgG positive (n, %)	2 (40.0)	1 (25.0)	0.270	56 (60.9)	6 (54.5)	0.686	
White blood cell counts; cells/μL (median, IQR)	2,000.0 (1,450.0, 4,585.0)	3,390.0 (2,785.0, 15,845.0)	0.327	2,950.0 (2,200.0, 4,142.5)	5,600.0 (3,300.0, 11,330.0)	0.012*	
Neutrophil; % (mean, SD)	45.8 (9.9)	53.5 (9.7)	0.279	53.4 (17.4)	60.2 (10.3)	0.212	
Lymphocyte; % (mean, SD)	41.9 (11.8)	33.8 (5.9)	0.271	34.1 (15.0)	29.1 (9.9)	0.284	
Platelets; × 10 ³ ; cells/μL (median, IQR)	97.0(82.5, 200.5)	66.5(85.5, 113.3)	0.110	103.5(80.8, 153)	60.0(45.0, 98.0)	0.030*	
Total bilirubin; g/dL (median, IQR)	0.3(0.2, NA)	1.1(0.6, 2.2)	0.077	0.5(0.4, 0.6)	2.5 (0.9, 15.3)	< 0.001*	
Direct bilirubin; g/dL (median, IQR)	0.3(0.3, NA)	0.4(0.3, 1.0)	0.100	0.3(0.2, 0.3)	1.1(0.3, 9.9)	0.004*	
AST; U/L (median, IQR)	29.5(19.8, 44.5)	1,056.5 (1,006.4, 1,658.2)	0.021*	45.0(31.4, 67.3)	1183.8(891.4, 2,876.7)	< 0.001*	
ALT; U/L (median, IQR)	14.5(8.5, 28.8)	483.5 (286.0, 545.0)	0.020*	21.1 (14.3, 35.8)	545.0(422.0, 1,889.0)	< 0.001*	

* $P < 0.05$. ALT, alanine aminotransferase; AST, aspartate transaminase; BMI, body mass index; bpm, beats per minute; DENV, dengue virus; IQR, interquartile range; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; SD, standard deviation.

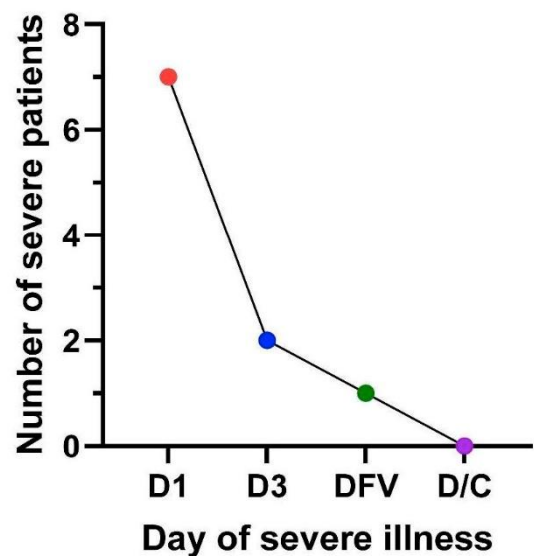


Figure 1. Timeline of disease progression in severe liver dysfunction patients.

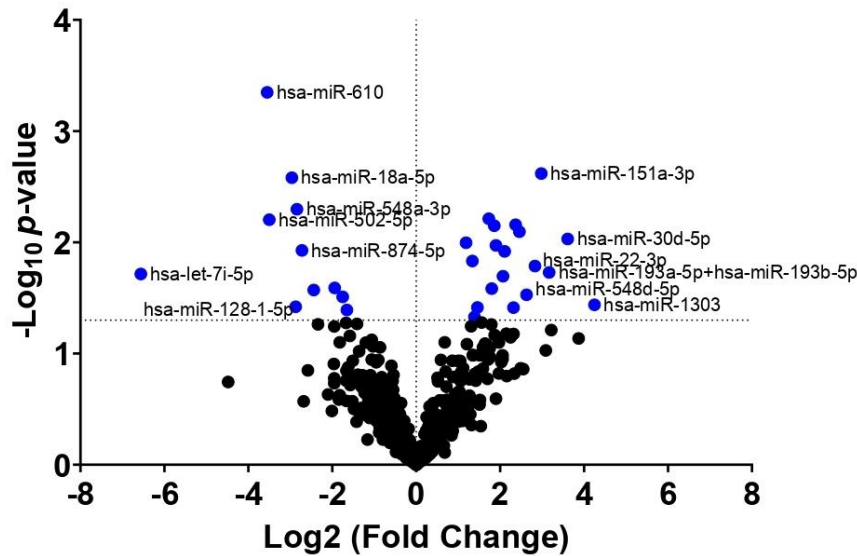


Figure 2. Profiling of circulating microRNAs in patients with severe and non-severe liver dysfunction.

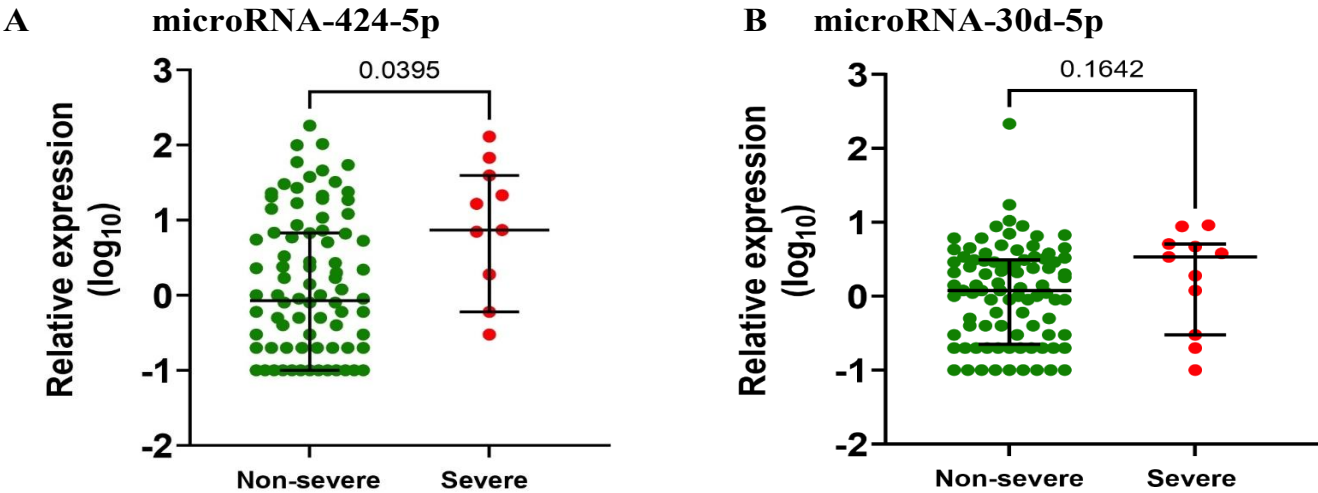


Figure 3. Serum levels of microRNA-424-5p (A) and microRNA-30d-5p (B) in severe and non-severe liver dysfunction patients.

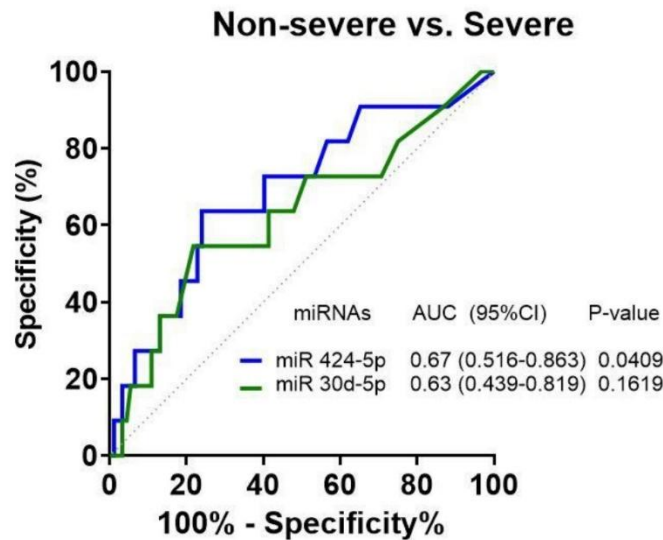


Figure 4. Predicting severe liver dysfunction with serum microRNAs: ROC curve analysis. (The optimal cut-off for microRNA-424-5p was determined by 6.92 using ROC curve).

Table 2. microRNA-424-5p performance in predicting severe liver dysfunction.

microRNA	Cut-off	Sensitivity	Specificity	PPV	NPV
microRNA-424-5p	6.9	63.6	76.1	16.3	83.7

NPV, negative predictive value; PPV, positive predictive value.

Correlation of circulating miRNAs with clinical parameters in the disease

We observed significant positive and negative correlations between three miRNAs and several clinical parameters. Specifically, miR-424-5p and miR-30d-5p exhibited a negative correlation with Dengue virus qRT-PCR positivity (**Table 3**). Whereas miR-424-5p revealed a negative correlation with gender (**Table 3**).

Analysis of serum miRNAs and many factors that could potentially affect the prognosis of severe liver dysfunction were performed using a univariate approach. The result identified prognostic indicators of severe liver dysfunction, including body mass index, duration of fever before admission, abdominal pain, persistent vomiting more than twice, white blood cell counts, and platelet counts (**Table 4**).

Gene targeting and functional profiling

To investigate the biological relationships, we performed Gene Ontology enrichment analysis. For miR-424-5p, the top four molecular functions

were protein binding, catalytic activity, which acted on a protein, and protein kinase activity. For phosphotransferase activity with an alcohol group as the acceptor (**Figure 5A**), the top four biological processes were multicellular organism development, developmental process, anatomical structure development, and regulation of primary metabolic processes (**Figure 5B**). The top four cellular components were cytosol, cytoplasm, nucleoplasm, and cell junction (**Figure 5C**).

The initial step of gene targeting by mirDIP with a high score class yielded a catalog of 1,039 target genes regulated by miRNA-424-5p. According to the enrichment analysis, miR-424-5p was associated with 27 significant pathways, including mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase-protein kinase B (Akt) signaling, and the mammalian (or mechanistic) target of rapamycin signaling pathway (**Figure 5D**). In addition, for miRNA424-5p, a significant gene identified was calcium voltage-gated channel subunit alpha1E (CACNA1E), which is part of the mitogen-activated protein kinase pathway.

Table 3. Correlation between serum microRNA Levels and clinical parameters.

Parameters	microRNA-424-5p		microRNA-30d-5p	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Age	0.100	0.310	0.180	0.076
Gender	-0.216	0.028*	-0.060	0.561
BMI	0.080	0.464	0.120	0.274
Duration of fever	0.110	0.286	0.130	0.189
Body temperature	-0.020	0.848	-0.140	0.159
Systolic blood pressure	0.020	0.834	-0.020	0.874
Diastolic blood pressure	-0.190	0.058	-0.170	0.079
Pulse rate	0.070	0.508	0.050	0.629
Respiratory rate	0.010	0.958	0	0.991
Abdominal pain	0.180	0.077	0.110	0.252
Persistent vomiting > 2 times	0	0.986	0.130	0.181
jaundice	0.080	0.403	0.090	0.366
Hepatomegaly	0.050	0.593	0.120	0.241
DENV NS1 positive	0.140	0.156	0	0.952
DENV qRT-PCR positive	-0.201	0.041*	-0.224	0.023*
Anti-DENV IgM positive	-0.020	0.808	0.100	0.317
Anti-DENV IgG positive	-0.080	0.414	0.070	0.461
WBC	-0.010	0.899	0.060	0.533
Neutrophil	0.080	0.413	0.090	0.384
Lymphocyte	-0.100	0.341	-0.100	0.342
Platelets	-0.030	0.763	0.080	0.444
AST	0.180	0.078	0.110	0.281
ALT	0.270	0.006*	0.228	0.021*

* $P < 0.05$. ALT, alanine aminotransferase; AST, aspartate transaminase; BMI, body mass index; DENV, dengue virus; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; WBC, white blood cell.

Table 4. Factors associated with severe liver dysfunction. It includes odds ratios (OR), 95% confidence intervals (CI), and *P*-values.

Variables	Univariate analysis		
	OR	95%CI	<i>P</i> -value
Age	1.01	0.96–1.06	0.652
Gender	1.63	0.46–5.73	0.446
BMI	1.14	1.00–1.30	0.046*
Duration of fever before admission	1.60	1.12–2.28	0.010*
Body temperature	0.81	0.48–1.35	0.415
Abdominal pain	4.21	1.06–16.73	0.041*
Persistent vomiting > 2 times	5.56	1.47–21.07	0.012*
Serotype by qRT-PCR	0.83	0.43–1.63	0.592
DENV NS1 positive	1.08	0.29–3.94	0.914
DENV qRT-PCR positive	0.32	0.07–1.52	0.153
Anti-DENV IgM positive	0.46	0.13–1.68	0.239
Anti-DENV IgG positive	0.77	0.22–2.72	0.686
White blood cell counts	1.00	1.00–1.00	0.005*
Neutrophil	1.03	0.99–1.07	0.212
Lymphocyte	0.98	0.93–1.02	0.282
Platelets $\times 10^3$	1.00	1.00–1.00	0.010*
microRNA-424-5p (Log10)	1.832	0.98–3.43	0.059
microRNA-30d-5p (Log10)	1.639	0.65–4.15	0.297

* $P < 0.05$. BMI, body mass index; DENV, dengue virus; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

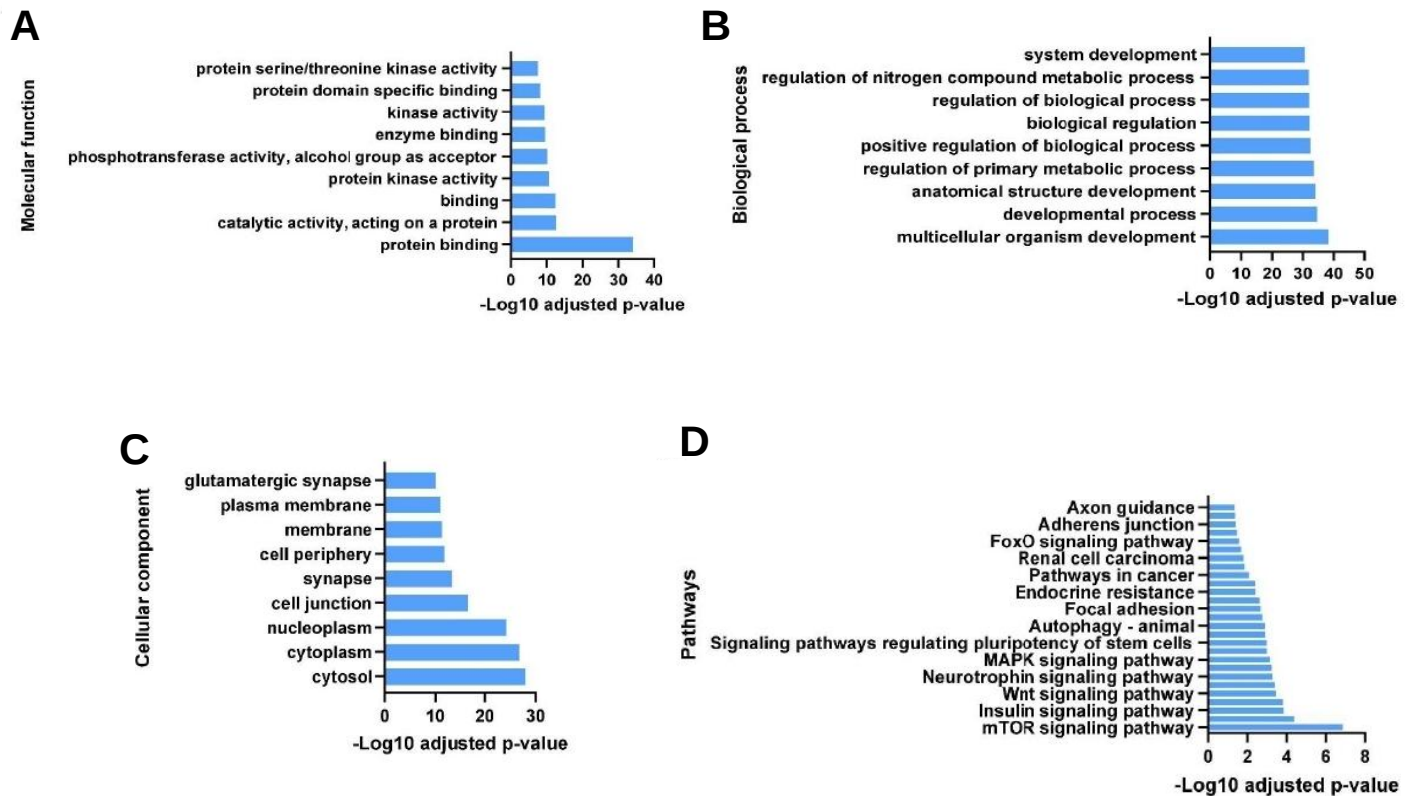


Figure 5. Significant pathway enrichment based on microRNA-424-5p, highlighting the biological processes and molecular pathways influenced by its expression. (A) Molecular function, (B) biological process, (C) cellular component, and (D) pathways.

Discussion

This study aimed to identify potential miRNAs and their associated molecular pathways in patients with severe liver dysfunction due to dengue infection. Initially, we found 798 miRNAs that could potentially predict the clinical outcome. During the discovery phase, we selected three miRNAs: miR-16-5p (as a reference gene), miR-424-5p, and miR-30d-5p. These miRNAs were then further validated by qRT-PCR analysis in 103 patients, including 92 in the severe group and 11 in the non-severe group. miRNA-30d-5p and miRNA-424-5p were notably elevated in patients diagnosed with severe liver dysfunction compared to those in the non-severe group. Demographic data revealed that the duration of fever before admission, jaundice, white blood cell counts, and levels of total bilirubin, direct bilirubin, aspartate aminotransferase, and alanine aminotransferase were significantly higher in the severe liver dysfunction group than in the non-

severe liver dysfunction group. In contrast, abdominal pain and platelets were significantly lower in the severe liver dysfunction group than in the non-severe liver dysfunction group. The ROC curve analysis demonstrated that miRNA-424-5p could distinguish cases of severe from non-severe liver dysfunction. According to ROC curves, miR-424-5p exhibited effective diagnostic performance, with an AUC of 0.67 and sensitivity, specificity, positive predictive value, and negative predictive value of 63.6%, 76.1%, 16.3%, and 83.7%, respectively.

miRNA levels showed positive correlations with aspartate aminotransferase and alanine aminotransferase and negative correlations with dengue virus qRT-PCR positivity and gender.

Furthermore, miRNA-424-5p was upregulated in this study. We evaluated the impact of suppressor of cytokine signaling 2 on liver injury caused by acetaminophen, which was a target gene of this miRNA.⁽¹⁵⁾ When a toxic dose (600 mg/kg) of

acetaminophen was administered to wild-type mice, they showed notable liver necrosis. In contrast, suppressor of cytokine signaling 2^{-/-} mice displayed significantly higher levels of necrosis, neutrophil recruitment, and chemokine (C-X-C motif) ligand 1, an active chemokine for neutrophils. Moreover, proinflammatory cytokines such as tumor necrosis factor- α and interleukin-6 were upregulated. In contrast, anti-inflammatory cytokines such as interleukin-10 and tumor growth factor- β were reduced in suppressor of cytokine signaling 2^{-/-} mice. In vitro experiments revealed that suppressor of cytokine signaling 2^{-/-} hepatocytes exhibited elevated phosphorylation of nuclear factor kappa-light-chain-enhancer of activated B cells and increased reactive oxygen species production compared with wild-type hepatocytes upon exposure to acetaminophen. These findings indicate that suppressor of cytokine signaling 2 plays a protective role in the liver by regulating oxidative stress and inflammation triggered by acetaminophen.⁽¹⁶⁾

The KEGG pathways enriched by gene targeting revealed 27 pathways related to cell regulation, including miRNA-424-5p. Furthermore, compared to a previous study, one gene, CACNA1E, was significantly associated with miRNA-424-5p. This gene was associated with neutrophil activation and maturation and may also be a target of miRNA-320a.⁽¹⁷⁾ Other significant genes, such as P53, FASLG, and MAPK, have been associated with the pathogenesis of liver involvement in patients with dengue.⁽¹⁸⁾

This study revealed many strengths. Observing aspartate aminotransferase or alanine aminotransferase levels, along with miRNA levels, over time provides a more comprehensive understanding of how these levels change throughout disease progression.

However, this study also had several limitations. It only included Thai patients with dengue. In addition, miRNA levels were assessed on the first day of enrolment, without monitoring changes during admission. Examining miRNA levels at multiple time points could provide a more comprehensive understanding of how these miRNAs change throughout the course of dengue illness. Moreover, some blood samples were not collected at the same time across the disease stages, and our study had a limited number of cases of severe liver dysfunction among patients with SD. These miRNAs should be further investigated in a larger and more diverse population.

Conclusion

MiR-424-5p may be a potential diagnostic and prognostic biomarker with modest performance for severe liver dysfunction in SD before the onset of severe liver dysfunction. However, further validation of this microRNA analysis is required in larger prospective cohorts, and multi-marker validation is recommended.

Author contributors

NP, UL, and NS contributed to the conceptualization and design of the study. UL conducted microRNA experiments and supervised data collection. JD and ST performed microRNA extraction. NP and UL were responsible for analyzing and interpreting the data and drafting the manuscript. NS provided overall research guidance and edited the manuscript critically for important intellectual content. All authors approved the final version submitted for publication and accept responsibility for statements made in the published article.

Acknowledgments

This work was supported by Ratchadapiseksompotch Fund, Graduate Affairs, Faculty of Medicine, Chulalongkorn University, Grant number GA67/036. We are grateful to the Excellence Center for Critical Care Nephrology at King Chulalongkorn Memorial Hospital for their valuable support in coordinating data collection and facilitating the clinical trial. We are thankful to Chulalongkorn University for providing research scholarships. Our appreciation also extends to the clinicians and nurses at Banpong Hospital, Photaram Hospital, and King Chulalongkorn Memorial Hospital for their support with data collection and patient follow-up. Furthermore, we sincerely thank all the patients and their families for their participation in this study.

Conflicts of interest statement

The authors have no conflicts of interest to declare.

Data sharing statement

The microRNA profiling data obtained through NanoString technology were submitted to the NCBI Gene Expression Omnibus under accession ID *GSE190749*. Access to all other data can be requested from the corresponding author.

ORCID

Napat Pas-arj  <https://orcid.org/0009-0002-2508-6624>

Umaporn Limothai  <https://orcid.org/0000-0003-1521-5592>

Janejira Dinhuzen  <https://orcid.org/0000-0001-8104-0984>
Sasipha Tachaboon  <https://orcid.org/0000-0002-7137-6764>
Nattachai Srisawat  <https://orcid.org/0000-0002-8544-8132>

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