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The Role of Vascular Endothelial Growth Factor in Vascular Leakage in Children with Dengue Infection

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ความเป็นมา การรั่วของพลาสมาออกนอกหลอดเลือดในผู้ป่วยโรคไข้เลือดออกเป็นสาเหตุสำคัญที่นำไปสู่ภาวะระบบไหลเวียนเลือดล้มเหลว ซึ่งขณะนี้กลไกการเกิดนั้นยังไม่เป็นที่ทราบแน่ชัด การศึกษานี้จึงทำขึ้นเพื่อหาความสัมพันธ์ของการเกิดการรั่วของพลาสมากับสารวีอีจีเอฟ

วิธีศึกษา ผู้ป่วย 41 คนที่ได้รับการตรวจยืนยันทางห้องปฏิบัติการว่าเป็นไข้เลือดออก จะถูกจำแนกเป็น 3 กลุ่ม คือ ไข้เดงกี, ไข้เลือดออกเดงกี และไข้เลือดออกเดงกีที่ช็อค ผู้เข้าร่วมวิจัยจะได้รับการเจาะเลือดเพื่อตรวจนับเม็ดเลือด, การทำงานของเอนไซม์ในตับ และปริมาณสารวีอีจีเอฟในวันแรกที่เข้าร่วมวิจัย, วันที่ไข้ลง, ก่อนกลับบ้าน และวันที่นัดติดตามอาการ ทุกคนจะได้รับการอัลตราซาวด์เพื่อประเมินค่าชี้วัดของน้ำในช่องเยื่อหุ้มปอด และการทำงานของกล้ามเนื้อหัวใจ

ผลการศึกษา พบว่าค่าวีอีจีเอฟในเลือดต่ำในผู้ป่วยที่มีระดับความรุนแรงของโรคไข้เลือดออกที่มากกว่า และค่าวีอีจีเอฟในวันที่นัดติดตามอาการสูงขึ้นในผู้ป่วยทุกคน ยกเว้นในผู้ป่วย 1 รายที่เป็นไข้เดงกี จากการศึกษาไม่พบความสัมพันธ์ระหว่างค่าวีอีจีเอฟกับค่าชี้วัดน้ำในช่องเยื่อหุ้มปอด, การทำงานของหัวใจ หรือเอนไซม์ในตับ

สรุปผลการศึกษา การที่วีอีจีเอฟสูงขึ้นอย่างมีนัยสำคัญทางสถิติ ในช่วงที่มีการฟื้นตัวของโรคน่าจะสัมพันธ์กับกระบวนการซ่อมแซมเซลล์บุผนังหลอดเลือดในโรคไข้เลือดออก ดังเช่นที่พบการเปลี่ยนแปลงดังกล่าวในโรคอื่นๆที่เคยได้มีการศึกษาไว้

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สาขาวิชา กุมารเวชศาสตร์ ลายมือชื่ออาจารย์ที่ปรึกษา.....
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KEY WORD: dengue fever / dengue hemorrhagic fever / dengue shock syndrome / vascular endothelial growth factor / vascular leakage / capillary permeability

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Background: In dengue hemorrhagic fever (DHF), the major cause of hypovolemic shock and circulatory failure is increase in vascular permeability. Although many cytokines were reported to play a role, the pathogenesis of vascular leakage remains unclear. In this study, we investigated the role of VEGF in the pathogenesis of DHF and dengue shock syndrome (DSS).

Methods: 41 serologically confirmed dengue infected children were assigned to be dengue fever (DF), DHF without shock and DSS. Serum samples for vascular endothelial growth factor (VEGF), complete blood count and liver enzymes (ALT and AST) were collected at first day of fever, defervescence, discharge and follow-up time. All patients underwent ultrasonographic studies for pleural effusion index and cardiac index at each stage of disease.

Results: During the illness, serum VEGF levels were lower in patients of more severe illness. At follow-up period, significant increment of serum VEGF was found in all patients, except 1 with DF. There was no relationship between serum VEGF level and cardiac index, pleural effusion index or liver enzymes.

Conclusion: We hypothesized that VEGF could have a role in repairing process of endothelial damage in recovery phase of dengue infection, similar to many other diseases that had been previously studied.

Department of Pediatrics Student's signature.....

Field of study Pediatrics Advisor's signature.....

Academic year 2004 Co-advisor's signature.....

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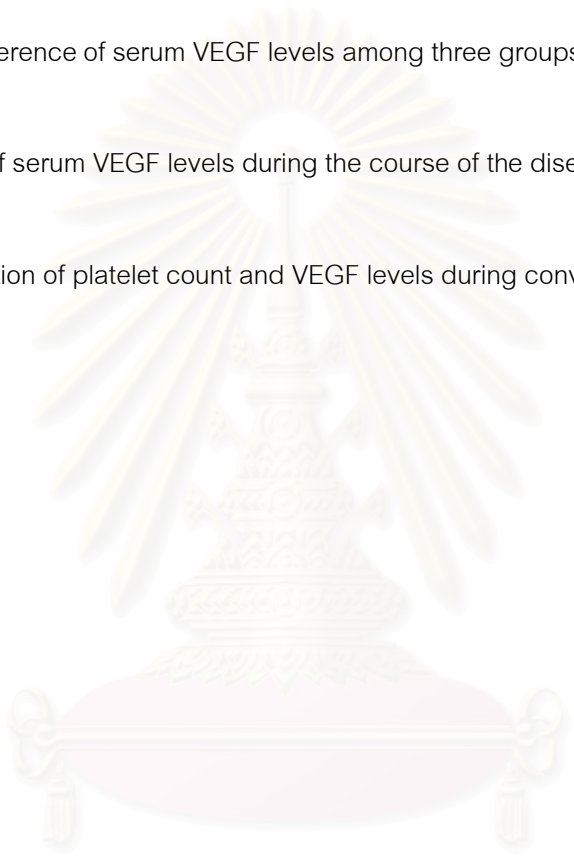


List of Abbreviations and Symbols

ALT	for	alanine aminotransferase
AST	for	aspartate aminotransferase
Ca^{2+}	for	calcium ions
CI	for	cardiac index
DV	for	dengue virus
DF	for	dengue fever
DHF	for	dengue hemorrhagic fever
DSS	for	dengue shock syndrome
D+ HUS	for	D-positive hemolytic uremic syndrome
IFN	for	interferon
IL	for	interleukin
LFT	for	liver function test
PEI	for	pleural effusion index
Plt.	For	platelet
TNF	for	tumor necrosis factor
TNFR	for	tumor necrosis factor receptor
VEGF	for	vascular endothelial growth factor
WHO	for	World Health Organization

CHAPTER I

INTRODUCTION

Background and Rationale

Dengue hemorrhagic fever is one of the most important diseases in Thailand and other tropical regions of the world. The disease is caused by four serotypes of dengue viruses (DV), arthropod-borne flaviviruses. Diseases caused by dengue virus infection were reported throughout the nineteenth and early twentieth centuries. The incidence and affected areas have steadily increased over the past 40 years. Annually, it is estimated that there are 20 million cases of dengue infection, resulting in approximately 24,000 deaths around the world *(1)*.

Most infected patients are asymptomatic. In those who have symptoms, their presentations are classified into 3 groups; undifferentiated fever (UF), dengue fever (DF), and dengue hemorrhagic fever (DHF). Dengue shock syndrome (DSS) is a severe complication of dengue hemorrhagic fever, characterized by massive increase in vascular permeability leading to hypovolemic shock and circulatory failure. The cause contributing to progression of dengue virus infection into DHF remains unclear. Major factors believed to play a role in progression into DHF are hyperendemicity with multiple serotypes or sequential infection and host factors. Good nutritional status may lead to enhanced immunological responses resulting in secretion of multiple cytokines and chemokines *(1-3)*.

In dengue hemorrhagic fever / dengue shock syndrome, intravascular volume decreases due to increase in vascular permeability. Evidences of vascular leakage are hemoconcentration, ascites, pleural effusion, hypoalbuminemia and low central venous pressure (CVP). However, the pathogenesis of the vascular leakage induced by DV infection is still not clearly understood. Releases of cytokines and chemokines from monocytes and macrophages are believed to target the endothelium and play a key role

in the pathogenesis of vascular leakage. These mediators include TNF-alpha, IL-1 beta, IL-2, IL-6, interferonγ and sTNFR p 75 *(4-8).*

Vascular endothelial growth factor (VEGF), originally named vascular permeability factor, is a homodimeric heparin-binding glycoprotein with potent angiogenic, endothelial-cell-specific mitogenic and vascular permeability-enhancing activities. There are four different molecular species of VEGF designated as VEGF121, 165, 189 and 206, whose molecular weight ranges from 34 to 42 kDa. VEGF expression has been found in activated macrophages, keratinocytes, renal glomerular visceral epithelium and mesangial cells, hepatocytes, smooth muscle cells, Leydig cells, embryogenic fibroblasts and bronchial and choroid plexus epithelium. The mechanisms of VEGF in increasing capillary permeability are mediated by phospholipase C activation and rapid increases of free cytosolic Ca^{2+}. It acts directly on the endothelium to promote extravasation of plasma fibrinogen, a substrate for endothelial and tumor cell growth. Modified extracellular matrix caused by fibrin deposition subsequently promotes migration of macrophages, fibroblasts and endothelial cells. VEGF has been shown to play roles in normal angiogenesis in embryonic development *(9-11).* Plasma extravasation in pathological angiogenesis associated with many tumors, intraocular neovascular disorders, rheumatoid arthritis and psoriasis *(12,13).* Several studies showed that it played a pathogenic role in the capillary hyperpermeability that characterized ovarian hyperstimulation syndrome*(14-16)* , preeclampsia*(17)*, and the plasma leakage in cirrhotic patient with spontaneous bacterial peritonitis (SBP)*(18).* One study demonstrated that plasma VEGF level was a predictor for the severity of postoperative capillary leak syndrome, the syndrome characterized by extravascular fluid accumulation, secondary intravascular volume depletion and significant organ dysfunction in children undergoing cardiopulmonary bypass *(19).* The pathogenesis may be comparable to those of DHF/DSS. These findings encourage us to determine the possible role of VEGF in the pathogenesis of DHF/DSS.

Research question

Does VEGF have a role in the pathogenesis of vascular leakage in dengue hemorrhagic fever?

Objectives

1. To compare serum level of VEGF in dengue fever (DF), dengue hemorrhagic fever without shock (DHF) and dengue shock syndrome (DSS).
2. To correlate serum VEGF levels with severity of dengue hemorrhagic fever measured by:
    - Amount of pleural effusion at 36-48 hours after treatment of DHF
    - Cardiac index (measured by echocardiography)
    - Severity of liver involvement measured by liver enzyme and portal blood flow study.

Limitation

Because up to now, there is no previous study of VEGF in dengue infection, sample size cannot be calculated exactly. We decided to include 15 patients from each group by expert opinion. The small sample size may not empower the results as it should be.

Operational Definition

Dengue fever

- Probable : an acute febrile illness with two or more of the following manifestations :
  - Headache
  - Retro-orbital pain
  - Myalgia
  - Arthralgia
  - Rash
  - Haemorrhagic manifestations
  - Leukopenia
- Confirmed : a case confirmed by laboratory criteria

Dengue hemorrhagic fever

The following must all be present:

- Fever, or history of acute fever, lasting 2-7 days
- Haemorrhagic tendencies, evidenced by at least one of the following:
  - a positive tourniquet test
  - petechiae, ecchymoses or purpura
  - bleeding from the mucosa, gastrointestinal tract, injection sites or other locations
  - hematemesis or melena.
- Thrombocytopenia (100,000 cells per mm^3 or less)
- Evidence of plama leakage manifested by at least one of the following
  - A rise in haematocrit equal to or greater than 20% above average for age, sex and race

- A drop in the haematocrit following volume-replacement treatment equal to or greater than 20% of baseline
- Signs of plasma leakage such as pleural effusion, ascites and hypoproteinemia

Grading for severity of DHF

- Grade 1: No shock, positive tourniquet test only
- Grade 2: No shock, with evidence of bleeding such as epistaxis, gastrointestinal bleeding
- Grade 3: The patient is in shock
- Grade 4: Profound or prolonged shock, unmeasurable blood pressure, pulse not palpable

Dengue shock syndrome

All of the above four criteria for DHF must be present, plus evidence of circulatory failure manifested by

- Rapid and weak pulse, and
- Narrow pulse pressure (<20 mmHg) or
- Hypotension for age, and
- Cold, clammy skin and restlessness.

Tourniquet test

Tourniquet test was performed by inflating a blood pressure cuff on the upper arm to a point midway between the systolic and diastolic pressures for 5 minutes. A test was considered positive when 20 or more petechiae per 2.5cm (1inch) square were observed.

Laboratory criteria for confirmation of dengue fever in this study:

The demonstration of a rise in dengue-specific IgM and IgG by ELISA according to the previously published report was considered to have dengue virus infection *(20)*.

Defervescence

The day of defervescence was defined as the day that the temperature dropped below 38°C without a subsequent elevation.

CHAPTER II

LITERATURE REVIEWS

Chemical mediators related to vascular leakage in dengue virus infection

Several cytokines and chemokines have been studied in patients with dengue hemorrhagic fever. Kurane I., et al. reported elevation of IL-2 and interferon (IFN)-γ in children with dengue infections compared with healthy controls *(21)*. Other studies have found the elevation of serum levels of tumor necrosis factor (TNF)-α, IL-1β, IL-6, and sTNF receptor p 75 in patients with severe dengue illness *(4-8)*. Sharon Green, et al. reported that the plasma levels of soluble tumor necrosis factor receptor (sTNFRs) and IFN-γ were higher in children with DHF as compared to those with DF or other febrile illnesses and were correlated with the degree of subsequent plasma leakage *(22)*. At present, there is no study of the correlation between VEGF and plasma leakage in DHF.

Pathogenic role of VEGF in comparable conditions

1) In 1995, Philip N. Baker reported higher serum VEGF levels in the patients with preeclampsia compared with normal pregnant women. This finding suggested that VEGF might be implicated in the pathogenesis of preeclampsia, the disease characterized by changes in glomerular capillary endothelial morphology, increased capillary permeability, and hypercoagulation. *(17)*

2) In 1995, Abramov Y, et al. reported high levels of VEGF in plasma of patients with severe ovarian hyperstimulation syndrome (OHSS). It's level dropped along with clinical improvement. Ascitic fluid from these patients also contained high VEGF levels. This study suggests the role of VEGF in the pathogenesis of capillary leakage in OHSS. *(15)*

3) In 1997, Rizk B, et al. reported high VEGF concentration in serum, peritoneal fluid and follicular fluid of patients with OHSS, the syndrome characterized by massive cystic enlargement and third space fluid shift resulting in ascites and pleural effusion, believed to be influenced by VEGF and interleukins. *(14)*

4) In 2000, D. Abrahamov, et al. found that preoperative VEGF levels correlated along with capillary leak syndrome in patients undergone cardiopulmonary bypass. They reported the VEGF level as a predictor of the severity of postoperative capillary leak syndrome, a serious complication in children undergoing cardiopulmonary bypass. This syndrome characterized by extravascular fluid accumulation and significant organ dysfunction. This finding elucidates the relationship between permeability properties of VEGF and systemic inflammation. VEGF was thought to be the cause of the disease. *(19)*

5) In 2000, Zhang ZG, et al. reported enhancing angiogenic activity of VEGF in ischemic brain of rats leading to increased blood brain barrier leakage and improvement of neurological outcome. *(23)*

6) In 2001, Cejudo-Martin P, et al. reported higher production rate of VEGF by peritoneal macrophages of patients with spontaneous bacterial peritonitis (SBP) compared with those of non-infected cirrhotic patients. The results suggested that locally released VEGF might cause increased vascular permeability and plasma leakage in the peritoneal vessels of cirrhotic patients with SBP. *(18)*

7) In 2002, Grove CS and Lee YC reviewed that there were high quantities of VEGF in pleural effusion and concluded that VEGF was a potent inducer of vascular permeability. *(24)*

8) In 2002, David R. Thickett, et al. measured VEGF levels in epithelial lining fluid (ELF) in 40 patients with acute respiratory distress syndrome (ARDS) and 28 patients at risk of ARDS. They reported lower VEGF in ELF from ARDS patients and increased VEGF level in ELF at day 4 associated with recovery. *(25)*

9) In 2003, Teralm M, et al. reported a role of VEGF in vascular leakage in Kawasaki disease. They found that after starting intravenous gamma globulin (IVGG), the IVGG-resistant group had higher VEGF and lower albumin levels compared with the IVGG-responsive group. This corresponded with the existing knowledge of extensive plasma leakage in fatal Kawasaki disease. *(26)*

10) In 2004, D.Maraeska te Loo, Nienka Bosma, et al. evaluated VEGF levels in the serum of 40 D^+ hemolytic uremic syndrome (HUS) patients in acute phase and during the course of the disease. They reported elevation of serum and plasma VEGF levels in children with D^+HUS compared with controls. The VEGF levels increased during the 2^{nd} and 3^{rd} week after HUS onset and there was significant relationship between VEGF levels and the severity of the disease. *(27)*

CHAPTER III

METHODOLOGY

Subjects

Sample size cannot be predicted in this study because there was no previous comparable data. The estimated patient to be enrolled was 45 cases. (15 in each group)

- *Inclusion criteria*

All patients aged 5 to 15 years who were admitted with fever more than 38 ^{o}C for 3 days or longer duration and no definite source of infection were enrolled in this study. All patients were assigned to be DF, DHF without shock (DHF grade 1 and 2) and DSS (DHF grade 3 and 4) according to the WHO criteria.

- *Exclusion criteria*
  - Patients with underlying diseases that might altered the serum VEGF level such as neovascularized tumor, rheumatoid arthritis, psoriasis and liver cirrhosis
  - Patient whose serologic studies were not done
  - Patient whose parent did not give inform consent to the study

Procedure

1. History taking, physical examination and blood sample collection

After informed consent was obtained, the history of illness were taken from the patient and parents including demographic data; day of illness; presenting symptoms such as fever, headache, nausea, vomiting, abdominal pain and bleeding; drugs taken and underlying diseases. The patient's weight, height, blood pressure, pulse rate, chest width and circumference, presence of edema, liver size and tourniquet test result were recorded. The history taking and physical exmination were done by the study physicians. The blood samples were drawn for complete blood count, serum albumin, ALT and AST on the first date of enrollment, at defervescence and at follow-up time. Serum samples for IgM and IgG of dengue virus were collected on the first date of enrollment and discharge date (or at follow-up time). Blood samples for VEGF level were collected at all of the following stages (if possible)

- First day of enrollment (as febrile stage of the disease)
- The day of defervescence (as toxic stage of the disease)
- Before discharge (as convalescent stage of the disease)
- follow-up period (as recovery stage of the disease)

2. Imaging studies

Quantitation of pleural effusion was done by ultrasonographic studies of right pleural cavity on the first day of enrollment and daily until discharge date. A pleural effusion index (PEI) was designed in order to evaluate the severity of plasma leakage. The pleural effusion index was calculated as the width of pleural effusion divided by the width of chest wall. The width of pleural effusion was measured at the level of xyphoid process, with the probe placed at mid-axillary line and directed posteriorly as patient was in supine position. The width of chest wall was measured at the level of xyphoid. Echocardiography was done to measure cardiac index by

standard M-mode technique. Cardiac index was calculated as cardiac output divided by body surface area. Clinical information was not provided to the physicians reading echocardiogram to prevent bias from the investigators.

3. Measurement of VEGF

Three to five ml of blood sample was collected, using a standard tube for clot blood sampling, and centrifuged at approximately 1000 G for 10 minutes after clot occurred. Then the serum was removed and stored at $\leq$ -70°C for subsequent serum analysis.

The VEGF level was measured by solid-phase ELISA (Quantikine R&D Systems, Minneapolis). This immunoassay was designed to measure VEGF165 levels in cell culture supernate, serum and plasma. The VEGF kit contained a polyclonal antibody against VEGF and a monoclonal antibody specific to VEGF. The assay employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for VEGF had been pre-coated onto a microplate. Standards and samples were pipetted into the wells and any VEGF present would be bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for VEGF was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color develops in proportion to the amount of VEGF bound in the initial step. The color development was stopped and the intensity of the color was measured. Comparing the optical density of the samples to the standard curve, prepared by plotting optical density versus concentration of VEGF in the standard wells, the concentration of VEGF in samples was determined.

4. Serologic study for dengue virus infection

The serologic study for dengue virus infection was done at The Arm Force Research Institute of Medical Sciences (AFRIMS) by ELISA technique. For single

specimens, 40 U of IgM to dengue (with dengue IgM greater than JEV IgM) was considered evidence of a dengue infection (30 U if from paired sera with <15 U of antibody in the acute specimen). With serial specimens, a 2-fold increase in IgG to dengue with an absolute value of ≥100 U indicated a secondary infection in the absence of IgM to dengue of ≥ 40 U.

5. *Clinical course*

The patients were treated by attending staffs and residents of the Department of Pediatrics, King Chulalongkorn Memorial Hospital according to the Guidelines of the Royal college of Pediatrician and the Ministry of Public Health of Thailand.

The history taking and physical examination were done daily by the study physicians to obtain the same data as the first day of enrollment. The first-24-hour and total amount of intravenous fluid given was recorded. Type of fluid used was labeled as crystalloid, colloid or blood components.

Data Collection and Analysis

All data were collected and recorded by the study physicians. The data were analyzed using SPSS for Windows version 11.5. Continuous variables were expressed as mean ± standard deviation and were compared using one way analysis of variance (ANOVA). Categorical variables were compared by chi-square test or Fisher exact test as appropriate. Comparisons of VEGF within the 3 groups and during the course of disease were done by one way analysis of variance (ANOVA). Associations between serum VEGF levels and pleural effusion indices, cardiac indices and liver enzymes were assessed by Pearson's correlation coefficient and linear regression analysis. $P < .05$ was considered significant.

Ethical Committee Approval

The study was approved by the ethical committee of the Faculty of Medicine, Chulalongkorn University. Written informed consent was obtained from all subjects, either parents or the patients themselves if they were more than 13 year old.

CHAPTER IV

RESULTS

Clinical Data at Study Entry

Forty-one patients were enrolled (16 famales and 25 male). Mean age was 11.13±3.1 years. There were 15 children with DF, 14 with DHF without shock and 12 with DSS. Among the DHF group, 11 were grade 1, 3 were grade 2, 9 were grade 3 and 3 were grade 4. There was no significant difference in age, sex, body weight, height and duration of fever among the 3 groups (Table1). Children with more severe diseases had significantly higher value of maximum hematocrit and pleural effusion index at discharge and lower platelet count and cardiac index at toxic stage. Demographic and clinical data of all patients were shown in table1.

Measurement of Serum VEGF

We used VEGF kit to measure VEGF concentration by solid-phase ELISA (Quantikine R&D Systems, Minneapolis). Using comparation of the optical density of the samples to the standard curve in figure 1, the serum VEGF concentration was determined.

Difference of Serum VEGF Levels Among the Three Groups. (figure2)

During febrile, toxic and convalescent stages of the disease, serum VEGF levels were lower in the more severe group. The serum VEGF levels during each stage were shown in table 2. The difference of VEGF levels among groups was statistically significant during the toxic stage (156.93±70.8, 108.66±47.6 and 81.76±38.8 pg/ml in DF, DHF without shock and DSS respectively, p=0.038). During the follow-up period, there was no difference in serum VEGF levels among the three groups. The difference among the three groups was shown in figure 2.

Changes of Serum VEGF Levels During the Course of Disease

Among 25 subjects who had serum VEGF samples at follow-up time, everyone had increasing of serum VEGF levels when compared with that of the toxic and convalescent stage, except 1 patient with DF. There was statistically significant difference in the follow-up level of VEGF compared to toxic stage (p=0.007, n=13) and follow-up level versus convalescent level (p=0.012, n=14). Trend of serum VEGF levels during the course of disease was demonstrated in figure 3.

Correlation of Serum VEGF with Other Factors

Using different parameters as indicator for the severity of DHF, the followings were found (Table3): no correlation between VEGF levels and 1) cardiac indices during any stages of the disease, 2) pleural effusion indices during convalescent stage and 3) serum level of AST, ALT and albumin during toxic stage. To quantify the importance of platelet number and serum VEGF level, the correlation between platelet counts and serum VEGF levels were also analyzed. There was no significant relationship between VEGF levels and platelet counts during toxic and follow-up date (p=0.154 and 0.063 respectively), but significant correlation was found during convalescent stage (p=0.002, n=5) as shown in figure 4.

Table1. Demographic and clinical data of all patients

	DF	DHF	DSS	p-value
Sex (M:F)	8:6	9:5	8:4	.9
Age (yr)	10.54±3.3	12.08±1.9	10.71±3.9	.366
Body weight (Kg)	35.57±13.0	43.65±16.1	38.11±18.2	.383
Height (cm)	138.75±20.0	151.35±14.2	142.12±25.6	.251
Duration of fever (days)	5.07±1.2	5.14±0.9	5.18±1.2	.967
Max.Hct (%)	40.05±3.9	44.70±4.4	45.02±6.0	.014
Lowest Plt. ($x10^3/mm^3$)	96.2±41.7	54.5±33.2	34.6±19.9	.006
Cardiac index (L/min/m2)	2.75±0.6	2.28±0.6	1.93±0.6	.025
PEI at D/C	0	0.65±0.5	1.12±1.1	.002

NOTE: DF = dengue fever

DHF = dengue hemorrhagic fever without shock

DSS = dengue shock syndrome

Hct = hematocrit

Plt = platelet number

PEI = pleural effusion index

Table2. The serum VEGF levels (pg/ml) during each stage.

stage	group	N	Mean	Std. Deviation	Minimum	Maximum
febrile	DF	4	177.09	70.51	98.46	254.15
(admission)	DHF	4	139.65	85.17	27.20	226.34
	DSS	0	.	.	.	.
	Total	8	158.37	75.10	27.20	254.15
Toxic	DF	7	156.93	70.80	107.86	298.56
(defervescence)	DHF	9	108.66	47.68	58.42	207.81
	DSS	8	81.76	38.89	36.36	127.76
	Total	24	113.77	59.09	36.36	298.56
Convalescence	DF	9	299.74	286.18	70.27	791.11
(discharge)	DHF	5	195.95	137.90	102.92	434.17
	DSS	6	73.36	42.27	35.75	144.07
	Total	20	205.88	220.68	35.75	791.11
Recovery	DF	8	474.51	203.71	245.00	783.98
(follow-up)	DHF	10	388.21	351.58	92.06	1319.02
	DSS	7	638.57	575.59	107.03	1772.08
	Total	25	485.93	390.00	92.06	1772.08

Table3. Correlation of serum VEGF levels with other factors

FACTORS	N	r	R^2	p-value
PEI at convalescence	17	.300	.090	.241
Cardiac index at				
• Enrollment date	4	.839	.704	.161
• Defervescence	17	.284	.080	.270
• Discharge	15	.029	.001	.917
• Follow-up	22	.260	.068	.242
Platelet number at				
• Defervescence	15	.387	.150	.154
• Discharge	5	.986	.963	.002
• Follow-up	3	.995	.990	.063
Serum AST, ALT, albumin at defervescence	21	.284	.081	.688

Figure1. Standard curve: performed by comparing the known VEGF samples and optical density of the samples

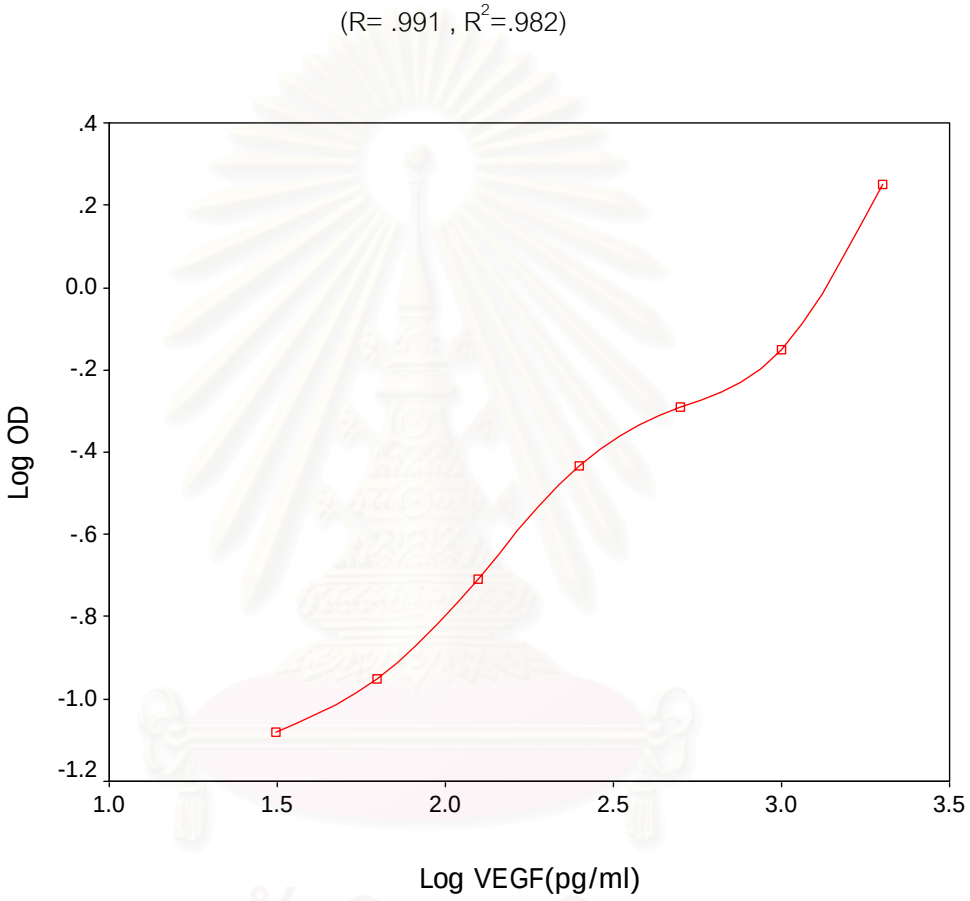


Figure2. The difference of serum VEGF levels among three groups during each stage.

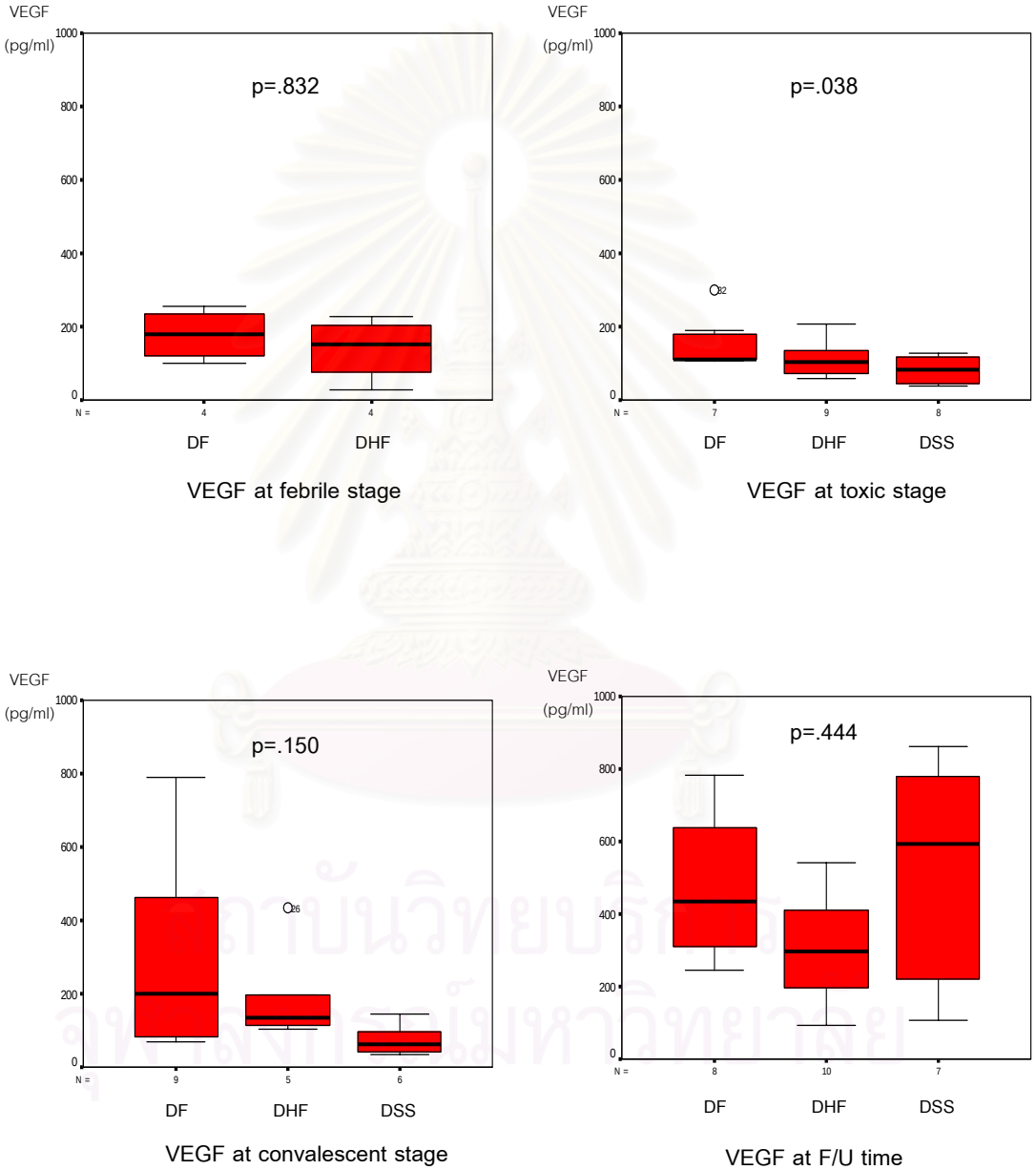


Figure3. Trend of serum VEGF levels (pg/ml) during the course of the disease.

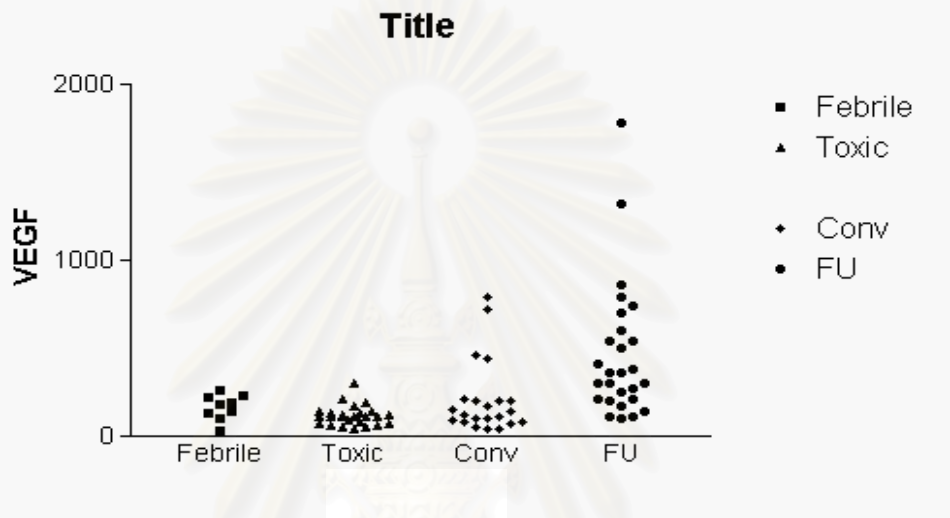
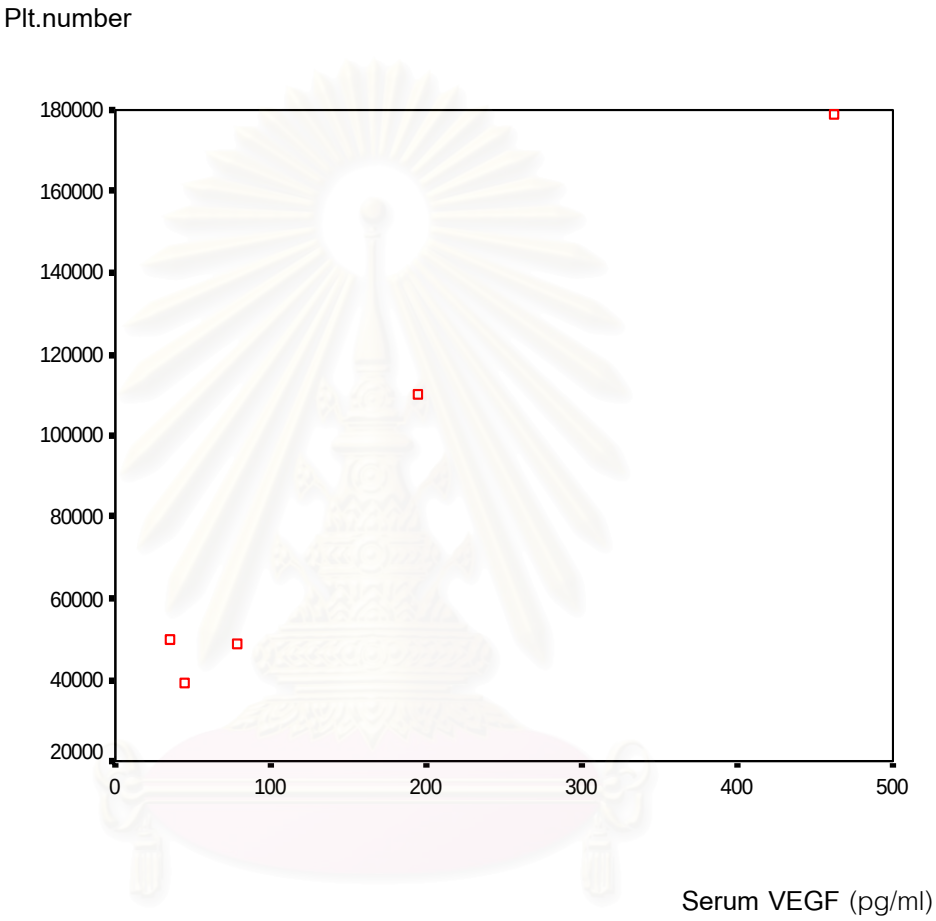


Figure4. Correlation of platelet count and VEGF levels during convalescent stage.



CHAPTER V

DISCUSSION

Major cause of hypovolemic shock and circulatory failure in DHF/DSS is believed to be increase in vascular permeability. The pathogenesis of vascular leakage in DHF/DSS remains unclear. Many cytokines and chemokines have been reported to be involved. Vascular endothelial growth factor (VEGF) has been reported to play a role in many diseases that have comparable pathogenesis to DHF. Up to now, there is not much data about the role of VEGF in the pathogenesis of vascular leakage in DHF. At first, we hypothesized that VEGF may have a role in vascular leakage in DHF/DSS as one of its properties was to enhance the vascular permeability. The original research idea assumed that the patients at follow-up could be used as normal controls.

Serial determination of serum VEGF levels demonstrated higher level at follow-up compared to toxic stage which was contradictory to the original hypothesis. The level at follow-up appeared to be higher than normal value reported in other studies. There were several studies reported normal serum VEGF values in children. In 2004, D.Maroeska te Loo, Nienke Bosma, et al. reported mean values of serum VEGF as 290±130 pg/ml in 39 normal control children (compared to patients with hemolytic uremic syndrome) *(27)* and in 2005, Ootaki Y, Yamaguchi M, et al. reported mean values as 203±221.6 pg/ml in 61 normal control children compared to those with congenital cyanotic heart disease *(28)*.

In the present study, we demonstrate that serum VEGF levels tended to be lower in more severe groups (with statistical significance shown only during toxic stage). All patients, except 1 in DF group, had elevated serum VEGF level during the follow-up time when compared with toxic and convalescent stage.

There appeared to be correlation between the severity of disease and serum VEGF levels, but not in the way we expected. In contrast to previous knowledge of strong permeability-enhancing activities of VEGF, we found that less VEGF levels were detected in more severe groups. Vascular leakage in DHF may not be explained by VEGF from the present study. One study in ARDS patients reported decrease production of VEGF in lung tissue and correspond with the apoptotic endothelial cell counts, this support that VEGF promotes endothelial survival by inhibiting apoptosis.*(29)* One hypothesis in the present study was that severely damaged endothelial cells were incapable of producing VEGF at that time.

During the follow-up period, the serum VEGF levels appeared to be increased. This implys that VEGF may play a key role in the recovery phase of dengue infection. The VEGF exerts its properties as both endothelial growth factor and permeability factor. Apart from studies reporting the pathologic role of VEGF in vascular leakage, there were also several studies reporting that VEGF enhances endothelilal cell proliferation and neovascularization. The study in D+HUS showed significant elevation of both serum and plasma VEGF level during recovery phase of the disease *(27)*. VEGF elevation was also found in epithelial lining fluid in patients with ARDS with resolution of lung injury *(25)*. The increased level of serum VEGF in the recovery phase of dengue infection in this study may represent the role of VEGF in the repair process of endothelial cells in this disease.

Limitation of the study

1) A few recent studies reported possible relation of serum VEGF with platelet count *(30,31)*. In this study, we found no correlation between serum VEGF level and platelet count in most stages of the disease except in convalescent stage (with sample size of only 5). Similarly, no correlation between platelet count and VEGF were found in patient with D^+ HUS *(27)*. It remains to be established if the changes in plasma VEGF

levels were similar to the serum levels due to possible effect of low platelets in dengue infection.

2) Injury to liver parenchyma leads to production of VEGF by hepatocytes and Kupffer cells was reported in 1999 by Ishikawa K, Mochida S, et al *(32)*. In this study, we quantify the relationship of hepatic involvement and serum VEGF levels and found no correlation found between serum VEGF levels and AST, ALT or albumin during toxic stage of dengue infection.

3) More subjects may need to be enrolled to empower the correlation between serum VEGF and severity of the disease.

CHAPTER VI

CONCLUSION

From the present study, we found that during the course of dengue virus infection, serum VEGF was lower in patients of more severe groups, although the difference was only statistically significant during the toxic stage. During the follow-up period, there was statistically significant increment of serum VEGF levels in most patients when compared to that of the toxic and convalescent stages. We hypothesized that VEGF may have a role in repairing process of endothelial damage in recovery phase of dengue virus infection. Further study is needed to validate the results.

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APPENDICES

ข้อมูลสำหรับผู้ปกครอง

โครงการวิจัย เรื่อง " การศึกษาการทำงานของตับ ขนาดของหลอดเลือดในตับ และระดับสารที่ก่อให้เกิดการรั่วของน้ำออกนอกหลอดเลือด ในผู้ป่วยโรคไข้เลือดออก "

ที่มาและวัตถุประสงค์

โรคไข้เลือดออกเป็นโรคที่พบมีการระบาดในประเทศไทย ลักษณะของโรคที่สำคัญคือ มีไข้ร่วมกับอาการเลือดออก มีตับโต และมักจะมีภาวะช็อค ซึ่งอาจทำให้ถึงตายได้ การรักษาประกอบด้วยการให้สารน้ำเข้าไปทดแทนในปริมาณที่เพียงพอ โดยไม่เกิดภาวะน้ำเกินหรือน้ำขาด ได้มีการศึกษาเกี่ยวกับกลไกการเกิดโรคและพยาธิสภาพที่เกิดกับผู้ป่วยในแง่มุมต่าง ๆ พบว่าผู้ป่วยบางคนมีการทำงานของตับผิดปกติทำให้เกิดภาวะการแข็งตัวของเลือดผิดปกติ หรือมีตับวายในรายที่รุนแรงได้ และมีข้อสันนิษฐานเกี่ยวกับสารบางตัวที่ก่อให้เกิดการรั่วของน้ำออกนอกหลอดเลือดทำให้เกิดมีภาวะน้ำในช่องเยื่อหุ้มปอดหรือช่องท้อง และเกิดภาวะช็อกได้ การวิจัยนี้จึงทำขึ้นเพื่อศึกษากลไกการเกิดโรคดังกล่าว

สิ่งที่จะได้รับเมื่อเข้าร่วมโครงการฯ

หลังจากผู้ปกครองลงนามในใบยินยอมเข้าร่วมโครงการฯ แล้ว แพทย์ในโครงการฯ จะ

- ทำการตรวจร่างกาย, ตรวจอัลตราซาวด์หัวใจ เยื่อหุ้มปอด และตับในขณะแรกรับ และหลังจากนั้น 24 ชั่วโมง และวันที่ผู้ป่วยจำหน่ายออกจากโรงพยาบาล
- เก็บตัวอย่างเลือดขณะแรกรับ และ หลังจากนั้น24 ชั่วโมงและวันที่ผู้ป่วยจำหน่ายออกจากโรงพยาบาล พร้อมกับการเจาะเลือดตามปกติของการรักษา ประมาณครั้งละ 5 ซีซี (1 ช้อนชา) เพื่อพิสูจน์ว่าเป็นไข้เลือดออกจริง, ตรวจการทำงานของตับ และ หาปัจจัยเสี่ยงของการเกิดภาวะน้ำรั่วออกนอกหลอดเลือด
- ตรวจอัลตราซาวด์โพรงเยื่อหุ้มปอด เพื่อตรวจหาภาวะน้ำในช่องเยื่อหุ้มปอดก่อนที่ผู้ป่วยจำหน่ายออกจากโรงพยาบาล (ประมาณ 2 วัน หลังการรักษา)
- ทำการตรวจร่างกาย ตรวจอัลตราซาวด์หัวใจ เยื่อหุ้มปอด และตับ และ เก็บตัวอย่างเลือดในขณะนัดติดตามอาการเป็นเวลา 1 เดือน ที่หอผู้ป่วยนอก ประมาณ 5 ซีซี (1 ช้อนชา) เพื่อพิสูจน์ว่าเป็นไข้เลือดออก

แพทย์ในโครงการฯ จะทำการดูแลเด็กของท่านเป็นอย่างดี ตลอดระยะเวลาที่อยู่โรงพยาบาล ประมาณ 2-4 วัน

มีความเสี่ยงหรือความไม่สบายเกิดขึ้นจากการเข้าร่วมโครงการฯ หรือไม่

การตรวจหัวใจด้วยอัลตราซาวด์ไม่ทำให้มีความเจ็บปวดหรือมีอันตรายใดๆ การเจาะเลือดอาจทำให้บุตรหลานของท่านเกิดความกลัว หรือ เจ็บบ้าง แต่ผู้ทำการวิจัยจะเก็บตัวอย่างเลือดไปพร้อมๆกับการเก็บตัวอย่างเลือดซึ่งทำเป็นปกติเพื่อการรักษา ยกเว้นในวันที่ท่านกลับมาพบแพทย์ภายหลังจากหายป่วยแล้ว ซึ่งในบางรายอาจต้องได้รับการเจาะเลือดเพิ่มเติมอีก 1 ครั้ง

ประโยชน์ที่จะได้รับจากการเข้าร่วมโครงการฯ

ผู้เข้าร่วมโครงการอาจได้รับประโยชน์จากการตรวจหัวใจในกรณีที่อาการช็อกเกิดจากหัวใจทำงานผิดปกติอย่างมากและแพทย์ไม่สามารถวินิจฉัยได้จากการตรวจตามปกติ

ความรู้ที่คณะแพทย์ในโครงการฯ ได้รับจากการศึกษานี้ จะช่วยในการปรับปรุงแนวทางการบำบัดรักษาโรคไข้เลือดออกให้มีประสิทธิภาพมากยิ่งขึ้น

ท่านต้องเสียค่าใช้จ่ายใด ๆ หรือไม่

ท่านไม่ต้องเสียค่าใช้จ่ายใด ๆ สำหรับการตรวจเลือดเพิ่มเติมจากการรักษาปกติ และการตรวจด้วยเครื่องมือพิเศษของแพทย์ในโครงการฯ

คำชี้แจงเกี่ยวกับสิทธิของผู้ป่วย

โครงการฯ นี้ ได้ผ่านการพิจารณาจากคณะกรรมการจริยธรรมการวิจัย คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัยแล้ว ท่านสามารถตัดสินใจยินยอมให้เด็กของท่านเข้าร่วมในโครงการฯ ด้วยตัวท่านเอง และสามารถนำเด็กของท่านออกจากโครงการฯ ได้ตลอดเวลา โดยไม่จำเป็นต้องมีเหตุผลใด ๆ ด้วยการแจ้งให้แพทย์ในโครงการฯ ทราบ เด็กของท่านจะยังคงได้รับการบำบัดรักษาเช่นเดิม ข้อมูลส่วนตัวของบุตรหลานของท่านในการวิจัยจะไม่ถูกเปิดเผย และตัวอย่างเลือดจะไม่ถูกใช้ในวัตถุประสงค์อื่น ยกเว้นจะได้รับอนุญาตจากผู้ป่วยหรือผู้ปกครองเป็นลายลักษณ์อักษรก่อน

กรณีมีปัญหาต้องการซักถามเกี่ยวกับงานวิจัย กรุณาติดต่อโดยตรงที่ นพ. อภิชัย คงพัฒนะโยธิน โทร (02) 256-4966 ในเวลาราชการ และ (02) 258-6053 นอกเวลาราชการ

กรณีที่มีปัญหาซักถามเกี่ยวกับอาการของบุตรหลานของท่าน กรุณาติดต่อ _____ ซึ่งเป็นแพทย์ประจำหอผู้ป่วย หรือติดต่อ แพทย์เวร นอกเวลาราชการ หรือติดต่อแพทย์ที่ร่วมในโครงการซึ่งให้คำแนะนำในการรักษาผู้ป่วยคือ_____โทร _____

คำยินยอมจากผู้ปกครอง

ข้าพเจ้าได้รับคำอธิบายเกี่ยวกับโครงการฯ ได้อ่านข้อความข้างต้น ได้รับโอกาสให้ซักถามปัญหาต่าง ๆ จนเป็นที่พอใจแล้ว ข้าพเจ้ายินยอมที่จะให้บุตร ของข้าพเจ้าเข้าร่วมในโครงการ โดยได้รับการเข้ารับการซักประวัติ ตรวจร่างกาย จะเลือด และเข้ารับการตรวจหัวใจดังกล่าว ด้วยความสมัครใจ โดยไม่มีการบังคับหรือให้อามิสสินจ้างใดๆ

ผู้ปกครอง..... วันที่ .....

(.....ตัวบรรจง)

ชื่อผู้ป่วย..... (เฉพาะผู้ป่วยที่อายุมากกว่า 13 ปี)

(.....ตัวบรรจง)

ชื่อพยาน .....

(.....ตัวบรรจง)

ผู้ได้รับอนุญาต .....แพทย์ผู้รับผิดชอบโครงการ หรือ แพทย์ผู้ทำการวิจัย

(.....ตัวบรรจง)

CLINICAL REPORT FORM

I. Patient's Data

Name		Patient NO. ___ ___ ___
Age ___ ___ yr	Sex (M/F) _____	HN _____/___
Birth Date ___/___/___	Admission Date___/___/___	Onset Date ___/___/___
Address *available in informed consent*		Tel.

II. Data on Admission

Day of Fever ____ ____	BTmax (if avalable) ____ ____ . ____ ^{o}C
Weight ___ ___ . ___ kg	Height ____ ____ ____ cm
Chest circumference ___ ___ cm	BSA ____ . ____ ____ m^2
Underlying Diseases (Y/N, detail of diseases)	Previous History of Dengue Infection (Y/N, when)

III. Data on Discharge

Clinical Classification of Dengue Infection (WHO): DF, DHF, DSS	
Grading of Dengue Infection: 1, 2, 3, 4	

IV. Appointment

Appointment date _____/_____/_____

V. Data on Laboratory Confirmation

Date	DEN-IgM	DEN-IgG	JE-IgM	JE-IgG
1st Serum (__/__/__)				
2nd Serum (__/__/__)				
3rd Serum (__/__/__)				
Interpretation	( ) Not dengue	( ) Dengue	( )Primary	( )Secondary

INSTRUCTIONS FOR RECORDING DATA

I,II,VI record on hospitalization
III,IV record on discharge
V record after laboratory confirmation result (Chitsanu)
VII record once daily during hospitalization

NOTE: *Please complete this form and send it to: Dr Chitsanu Pancharoen:*

VI. Clinical findings on Admission	YES	NO	Comments
1. Positive tourniquet test			
2. Petechii, ecchymosis, purpura			
3. Mucosal bleeding (gum,epistaxis)			
4. Bleeding at injection site			
5. GI bleeding/melena			
6. Maculopapular rash			
7. Injected pharynx			
8. Headache			
9. Cough			
10. Rhinitis			
11. Generalized lymphadenopathy			
12. Hepatomegaly (…. cm below RCM, span …. cm)			
13. Splenomegaly			
14. Vomiting			
15. Abdominal pain			
16. Constipation			
17. Diarrhea			
18. Myalgia/arthralgia			
19. Seizure			
20. Abnormal reflex			
21. Restlessness			
22. Coma			
23. Shock			

Signature	**Date _____/_____/_____**

VII. Daily Record Form

Day of Illness	1	2	3	4	5	6	7	8
Date								
Stage of Disease (1,2,3)								
BTmax (^{o}C)								
BP (mmHg)								
PR (/min)								
RR (/min)								
BW (kg)								
Abd. circumference (cm)								
Chest circumference (cm)								
Lymph node								
Heart/Lung								
Skin rash								
Hepatomegaly (….cm below RCM)								
Petechii, ecchymosis, purpura								
Mucosal bleeding: gum, epistaxis								
Bleeding at injection site								
GI bleeding								
Shock								
Neurological abn.								
Pleural effusion								
Ascites								
Hb/Hct (highest value)								
WBC (cells/mm^3)								
N (%)								
L/AL (%)								
Mo (%)								
Eo (%)								
B (%)								
Platelet (/mm^3)								
PT/aPTT (sec)								

III. Echocardiogram (Apichai)

Day of Illness		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Date									
Echo. (time)									
Alb (g/dL)									
ALT/AST (IU/L)									
Pleural effusion (U/S)									
Ascites (U/S)									
Liver span (U/S, cm)									
BP (during echo., mmHg)									
Intake (cc)	Oral Fluid								
	IV Fluid								
	Plasma								
	PRC/ FWB								
Output (cc)									

Note: Intake and output filled by physicians.

Appointment Date	**Date ___/___/___**	Time _ _._ _
Signature	**Date ___/___/___**	

VITA

NAME: Miss Patcharapa Sathupan

BIRTHDATE: 11th April, 1978

PLACE OF BIRTH: Bangkok.

EDUCATION: Graduated with a Bachelor's degree of Medicine (first class honor) from Faculty of Medicine, Siriraj Hospital, Mahidol University, in 2000.

Continue study for the Master of Science program in Pediatrics, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University in 2004.

EXPERIENCE IN WORKING: 3 years of internist at Thammasat hospital, Patum thani in 2000-2003.

Now training in residency program in Pediatrics, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University.