รายงานวิจัยฉบับสมบูรณ์

โครงการ

ระดับไซโตไคน์ในกลุ่มการสร้างหลอดเลือดและกระบวนการอักเสบในผู้ป่วยชิคุนกุนยาระยะเฉียบพลัน (Expressions of Angiogenic Cytokines and Inflammatory Mediators in Acute Chikungunya Virus Infection)

โดย

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สัญญาเลขที่ MRG5980203

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ผู้วิจัย

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สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษาและสำนักงานกองทุนสนับสนุนการวิจัย

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ชื่อโครงการ: ระดับไซโตไคน์ในกลุ่มการสร้างหลอดเลือดและกระบวนการอักเสบในผู้ป่วยชิคุนกุนยาระยะเฉียบพลัน

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บทคัดย่อ

ไข้ปวดข้อชิคุนกุนยาเป็นโรคที่เกิดจากยุงลายเป็นพาหะ ซึ่งเป็นปัญหาสำคัญหนึ่งสำหรับประเทศไทย สาเหตุของโรคนี้เกิดจากการติดเชื้อไวรัสชิคุนกุนยา โดยมักจะทำให้เกิดอาการมีไข้และผื่นโดยไม่ทราบสาเหตุ และโดยเฉพาะปวดตามข้อซึ่งอาจจะเป็นแบบเฉียบพลันหรือเรื้อรังส่งผลต่อคุณภาพการดำรงชีวิตของผู้ป่วย กลไกที่ทำให้เกิดพยาธิสภาพของโรคยังไม่ชัดเจนนัก อีกทั้งยังไม่มีการศึกษาเกี่ยวกับไซโตไคน์ในกระบวนการสร้างหลอดเลือดในผู้ป่วยที่ติดเชื้อนี้ระยะเฉียบพลัน นอกจากนี้เมื่อ 2-3 ปีที่ผ่านมามีการระบาดของโรคนี้ขึ้น ดังนั้นผู้วิจัยจึงสนใจที่จะศึกษาการแสดงออกของไซโตไคน์ที่เกี่ยวข้องกับการสร้างหลอดเลือดและการอักเสบในผู้ติดเชื้อชิคุนกุนยาอีกทั้งศึกษาลักษณะทางพันธุกรรมของไวรัสชนิดนี้

การศึกษาไซโตไคน์ทำในผู้ป่วยชิคุนกุนยาระยะเฉียบพลันและผู้ป่วยที่ไม่ได้ติดเชื้อชิคุนกุนยาจำนวน 85 ราย โดยวัดระดับของไซโตไคน์ที่เกี่ยวข้องกับการสร้างหลอดเลือดและการอักเสบจำนวน 11 ชนิดนั่นคือ CXCL-10, IFN-α, GCSF, PECAM, HGF, VEGF, Leptin, PDGF, Angiopoietin, Follistatin, และ IL-8 ด้วยเทคนิค multiplex ELISA และทำการศึกษาลักษณะทางพันธุกรรมของเชื้อไวรัสในผู้ป่วยที่สงสัยติดเชื้อ CHIKV จำนวน 127 ราย ด้วยวิธี real time RT-PCR และ sequencing ตามด้วยการวิเคราะห์ phylogenetic

ผลการศึกษาพบว่า ระดับของ leptin ในกลุ่มผู้ติดเชื้อ CHIKV ต่ำกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ (P=0.044) และเมื่อแบ่งผู้ป่วยเป็น 3 ระยะคือระยะที่มีเชื้อไวรัสในกระแสเลือด (viremia), ระยะที่สร้างแอนติบอดีชนิด IgM, และระยะที่พบทั้งไวรัสและแอนติบอดี พบว่าระดับของ CXCL-10 มีระดับลดลงอย่างมีนัยสำคัญทางสถิติตามลำดับ ส่วนการศึกษาลักษณะทางพันธุกรรมของเชื้อ CHIKV พบว่าเชื้อที่ระบาดเมื่อ พ.ศ. 2561-2562 ที่ผ่านมาเป็นสายพันธุ์ ECSA (East/ Central/ South/ Africa) ซึ่งเป็นสายพันธุ์เดียวกับที่เคยระบาดในประเทศไทยเมื่อ 10 ปีก่อน

จากการศึกษานี้สรุปได้ว่า CXCL-10 และ leptin ซึ่งเป็นไซโตไคน์ที่เกี่ยวข้องกับกระบวนการอักเสบมีความเกี่ยวข้องกับการเกิดพยาธิสภาพของโรค อาจจะสามารถใช้ไซโตไคน์นี้เป็นตัวบ่งชี้การเกิดความรุนแรงของโรคหรือเป็นเป้าหมายในการรักษาได้ อีกทั้งเชื้อไวรัสที่มีการระบาดนี้ยังเป็นสายพันธุ์เดิม ข้อมูลนี้อาจจะช่วยเพิ่มความตระหนักหรือช่วยให้เกิดการรณรงค์เกี่ยวกับการป้องกันการแพร่ระบาดของโรคนี้ต่อไป

คำหลัก : จำนวน 3-5 คำ

ชิคุนกุนยา, ความชุก, ไซโตไคน์เกี่ยวกับการสร้างหลอดเลือด, ไซโตไคน์เกี่ยวกับการอักเสบ

Abstract

Project Code : MRG5980203

Project Title : Expressions of Angiogenic Cytokines and Inflammatory Mediators in Acute Chikungunya Virus Infection

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Project Period : 2 years

Abstract:

Chikungunya fever is a mosquito's borne disease. It is one of the most health problem in Thailand. This disease cause by chikungunya virus (CHIKV) infection. The commonly symptoms are fever, rash, and especially joint pain leading to poor quality of life. The pathophysiology of the disease is not completely clear. There are some reports on inflammatory cytokines associated with the disease, but no report has been revealed on the angiogenic mediators in the acute CHIKV patients. Furthermore, a few year ago, there is the new outbreak of CHIKV in Thailand. Therefore, the researcher attempted to study the genetic characteristic of the virus and the level of angiogenic and inflammatory cytokines in acute CHIKV patients.

Eighty-five acute CHIKV patients and patients without CHIKV were enrolled in the study. The concentrations of 11 angiogenic and inflammatory mediators were determine by multiplex ELISA. The genetic characteristic of CHIKV was performed in 127 CHIKV suspected patients. CHIKV RNA were extracted and amplified by real time RT-PCR. The positive CHIKV samples were sequenced and illustrated phylogenetic analysis.

The results found the significantly decreased leptin concentration in the acute CHIKV patients when compared with controls. Subgroup classification as viremia, IgM, and both viremia and IgM analysis, we found the CXCL-10 level was highest in viremia and gradually decreased in IgM and both. Additionally, the genetic characteristic of CHIKV during 2018-2019 outbreak was ECSA (East/ Central/ South/ Africa) linage, which was similar to previous outbreak in Thailand.

The CXCL-10 and leptin expressions, the mediators in angiogenic and inflammatory processes, were associated with the pathogenesis of CHIKV. These cytokines might be biomarkers or targets for disease diagnosis or therapy. Moreover, we revealed the old strain of CHIKV like the last 10 years, this data might be important to CHIKV surveillance consideration and start CHIKV activity to prevent the outbreak in the future.

Keywords : 3-5 words

Chikungunya infection, Prevalence, Angiogenic cytokine, Inflammatory cytokine

Introduction

Chikungunya is a mosquito-borne disease that is transmitted to human via the bites of infected *Aedes aegypti* and *Aedes albopictus* mosquitoes. The first outbreak of chikungunya was identified in Tanzania in 1952. Then the epidemic area continues to spread throughout several countries such as on island of La Reunion in the Indian Ocean, in Europe, Kenya, and Southeast Asia including in Thailand. The first Chikungunya isolation in Thailand was reported in 1958 and the currently large outbreak in Thailand was found in 2008 to 2009 (1). The chikungunya virus (CHIKV) infection has been considered as a major public health problem in many countries. There is no specific treatment for CHIKV infection disease. The CHIKV is a single-strand RNA virus belonging to family *Togaviridae*, genus *Alphavirus*. CHIKV infection is characterized by high fever, skin rash, and severe arthralgia.

Although the clinical manifestation of chikungunya has been clarified, the pathogenesis of the disease is still unclear. The host innate and adaptive immune responses are stimulated after the CHIKV infection in order to eliminate the virus with divergent pathways. Inflammatory pathway is one of the important processes that benefit the host from viral elimination. Various previous studies showed the contribution of inflammatory cytokines and pathogenesis of CHIKV infection (2, 3). Some inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and interferon-gamma (IFN-γ) were increased in the CHIKV infected patients (2, 3). There are evidences indicated that angiogenesis and inflammation are closely associated processes in many diseases like cancer, tissue injury, and infectious diseases. Angiogenesis can facilitate inflammation process and inflammation is capable of angiogenic stimulation. The site of inflammation is often hypoxic, meanwhile hypoxia is a potent pro-angiogenic signal activating angiogenesis (4). Although some reports have shown the cytokine/ chemokine profiles in chikungunya infection, there is no evidence in association between angiogenic cytokine profiles during chikungunya virus infection.

Treatment of CHIKV infection is based on anti-inflammatory drug or symptomatic therapy. Moreover, the vaccinations against CHIKV are not yet available. The intensive understanding in the cytokine hyper responses contributing to chikungunya virus infection may give a better insight into the pathology and possible therapeutic routes. Therefore, the aim of this study will to investigate the levels of inflammatory mediators and angiogenic cytokines in acute chikungunya viral infection.

Literature review

Prevalence and biology of chikungunya (1, 5-9)

Chikungunya disease is first identified in Tanzania in 1952.CHIKV subsequently spread to La Reunion, America, Europe, India, Sri lanka, Southeast Asia (Malaysia, Cambodia, Singapore, Philippines and Thailand). The first country in Southeast Asia isolated CHIKV occurs in Thailand. In Thailand, the first report has been in Bangkok in 1958. Then epidemic also spread to the Northeastern and the most recent outbreak is in the Southern of Thailand. The reemerge of chikungunya virus is unpredictable, with interval of seven to twenty years. Therefore, we could not prevent or break the sporadic of the disease.

Chikungunya virus, an alphavirus belonging to *Togaviridae* family, is transmitted to human by mosquitoes, with *Aedes aegypti* and *Aedes albopictus* being the main vector. CHIKV is a small positive single strand RNA genome of approximately 12 kb bases. The genome structure encodes two open reading frames (ORFs) that encodes for non-structural protein (nsP1 to nsP4) and structural protein (capsid protein, envelope protein: E1 and E2, accessory peptide: E3 and 6K). Clinical features of chikungunya are high fever, skin rash, headache, nausea, and severe joint pain. Normally, the disease is self-limiting and resolves itself. It is not considered as life-threatening disease, however; severe outcomes often occur in neonates, elderly people (more than 65 years old), and the patient underlying medical conditions. Acute CHIKV infection lasts for a few days to a couple weeks. Meanwhile joint pain may linger for week, months or years. Unfortunately, there is no specific therapeutic and vaccine for CHIKV infection treatment. Therefore, the pathophysiological of the disease is still needed more consideration.

Pathogenesis of chikungunya

Pathogenesis of chikungunya is poorly understood. Following transmission, CHIKV replicates in the skin and then disseminates to the liver, muscle, spleen, and joint presumably through the blood circulation (10). Chikungunya virus is able to replicate in the human adherent cells such as epithelial cell, endothelial cells, fibroblast, dendritic cells and macrophage. The infected cells are death via apoptosis pathway (7, 9, 11, 12). When chikungunya pathogenesis has occurred, the host immune response is responded to control the amount and activity of virus using both innate and adaptive immunity. Various pathways are involved in antiviral defense such as NK-cell, B cell, and T cell overresponses, apoptosis, and high productions of cytokines, and inflammatory mechanism (3).

Cytokine levels in Chikungunya

There is a little evidence on pathology of CHIKV infection and immune responses. The cytokine expression patterns have reported. Proinflammatory cytokines such as IL-6, interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1) were elevated in the CHIKV infection patients. Moreover, IL-6 and MCP-1 in plasma are reliable as biomarkers of high viral load in the patients (13). The production of inflammatory cytokine profile is different in each stage of infection. Interferon-α (IFN-α), interleukin-1 receptor (IL-1R), and IL-6 levels were increased in acute phase. On the other hand, the concentration of IL-17 became prominent expressed during chronic phase (14). The similar result was done by Chirathaworn C *et al*. They found that IL-6 and MCP-1 concentrations were stimulated in acute phase patients and were then decreased after the disease developed to the recovery stage (15). Kelvin A A and his colleague had concentrate on chikungunya disease progression. They found that IL-6, chemokine (C-X-C motif) ligand 10 (CXCL10), and MCP-1 were high concentration in acute phase patients compared to follow up samples. Additionally, IL-1ß, tumor necrosis factor-α (TNF-α), IL-12, IFN-γ levels were low during acute phase and were high at later time point (16). The expressions of cytokines studied in Thailand were shown that increased concentrations of IL-6 and MCP-1 with reduced concentration of IL-8 may be an indicator of severe chikungunya virus infection (17). All mention above imply that inflammatory cytokine production, which is one of the host immune response to viral defense, is important in pathogenic mechanism during CHIKV infection.

IFN-α is one of the members in type I interferon have been well regarded as antiviral cytokine (18). IFN-α is associated with pathology of CHIKV infection. Previous studies suggested that the concentration of IFN-α was early determined and correlated with viral load in acute CHIKV infection (3, 19). Moreover, based on both human and animal experiment, IFN-α was triggered by CHIKV infected nonhematopoietic cells especially fibroblast (20). CXCL-10 or interferon gamma inducible protein-10 (IP-10) plays roles in chemotactic, regulate cell growth and proliferation, angiogenesis in infectious disorder, and inflammation. CXCL-10 is capable of both prevent and stimulate infection. For example, CXCL-10 have a protective effect on coronavirus-induced severe acute respiratory syndrome (SARS), while it promotes the HIV infection by stimulate viral replication (21). Elevated CXCL-10 concentration was shown in acute phase CHIKV infection and gradually then decreased in follow up samples (14, 16). There is a model described the coordinate function between IFN-α and CXCL-10. Entry of virus into the body induces the viral elimination cascade starting with IFN-α stimulated the production of CXCL-10 (innate stimuli). Then innate stimuli moves to adaptive stimuli (such as IFN-γ, cytotoxic T cell) leading to viral

clearance. However, difference virus species have distinguished antiviral pathway. Therefore, the collaboration of IFN-α and CXCL-10 need to shed light on pathology of CHIKV infection (22).

According to inflammation in pathogenesis of CHIKV infection, a closely associated process is angiogenesis. Angiogenesis tends to prolong and intensity the inflammation. In inflammation, the elevating cell proliferation at affected site leading to hypoxic and hypoxia is a potential signal to promote angiogenesis (4, 23). Imhof B A *et al.* has reviewed the codependence of angiogenesis and inflammation. For instance, angiopoietin-2, a regulator of blood vessel formation, can prompt upregulation of inflammation. They explain the two processes mechanism that pathogen entering the host body induces secretion of cytokine/chemokine which stimulate recruitment of immune cells and endothelial cells. These cells move to the pathogenic site via blood circulation and lead to inflammation (24). Although there are some evidences on cytokine/chemokine concentration in CHIKV infection, no report in the expression of angiogenic cytokine profile has been studied.

Therefore, we will focus on the angiogenic cytokine and inflammatory mediator expressions in acute chikungunya virus infection in the South of Thailand. Moreover, there is over 10 years that CHIKV infection has been disappeared from Thailand and the new outbreak of CHIKV infection was found in the South of Thailand in 2018 to 2019. Hence, we also concentrate on the genetically of the chikungunya virus.

Objectives

1) To investigate the levels of angiogenesis and inflammatory cytokine in acute chikungunya patients

2) To assess the association between angiogenesis and inflammatory cytokine in acute chikungunya compared with controls

3) To perform the molecular characteristic of chikungunya virus outbreaked in the Southern Thailand

Methodology

Patients and Sample collection

Eighty five participants in Narathiwat province, located in the South of Thailand, were enrolled into the study for cytokine level assessment. All subjects lives in the closely area including in neighborhood or in family. Seventy-one suspected patients infected with CHIKV who have fever, joint pain, rash, myalgia, conjunctival redness, and headache were recruited in chikungunya group. Moreover, there were 14 control volunteers who have negative for chikungunya confirmation test (negative for CHIKV RNA and IgM). The CHIKV infection was confirmed by detection of CHIKV specific IgM antibodies using an ELISA and/or CHIKV RNA by reverse transcriptase-polymerase chain reaction (RT-PCR). Data on demographic characteristic was obtained from questionnaire. Blood samples (3–5ml of blood) were collected from the 85 subjects, serum was separated and stored in aliquots at -70°C until all tests analysis. Furthermore, when the new outbreak of CHIKV in 2018-2019 was occur, we collected another group of samples for chikungunya molecular characteristic evaluation from 127 CHIKV suspected patients went to the hospital in Satun province. Serum was separated and performed DNA extraction and CHIKV antibody detection.

Multiplex Microbead Immunoassay for Cytokine

The concentrations of nine angiogenic cytokines including granulocyte-colony stimulating factor (G-CSF), hepatocyte growth factor (HGF), platelet endothelial cell adhesion molecule-1 (PECAM-1), leptin, vascular endothelia growth factor (VEGF), platelet derived growth factor-BB (PDGF-BB), angiopoietin-2, follistatin, and interleukin-8 (IL-8) will determined in the serum samples using Bio-plex pro human angiogenesis 9-plex assay kit (Bio-rad) according to manufacturer's instruction. Results were analyzed using the Bioplex® 200 system instrument (Bio-rad), with Bioplex manager^{TM} software, based on standard curves plotted through a 5-parameter logistic curve setting.

ELISA for Inflammatory mediator detection

The CXCL-10 and IFN-α levels in all subjects were assessed by Duoset CXCL10 ELISA kit and VeriKine human interferon alpha multi-subtype serum ELISA kit, respectively followed by protocol's recommendation.

Viral RNA extraction

The viral RNA was extracted from the patient's serum using Ribospin^{TM} vRD II viral RNA purification kit (Geneall, Korea). The 100 μl of serum was added to the mini spin column and went on the process followed by the manufacturer's procedure.

Real time reverse transcriptase-polymerase chain reaction (real time RT-PCR) for CHIKV RNA detection

The CHIKV RNA was amplified using the QuantiTect Probe RT-PCR kit (Qiagen, Germany) as previously described (25). The five microliter of RNA virus was mixed with the mastermix buffer and specific primers for CHIKV. The thermal cycler was set as 50^{O}C for 30 min and 95^{O}C for 15 min, followed by 45 cycles of 95^{O}C for 15 s and 60^{O}C for 1 min.

CHIKV E1 amplification

CHIKV positive sample were converted into cDNA by ImProm II Reverse Transcription System (Promega, Madison, WI, USA). E1 gene was partially amplified using nested-PCR as previously report (26). The 485 bp of E1 amplicon was sent to sequencing by Sanger method.

Phylogenetic analysis

We analysed the CHIKV sequence to establish genetic relationship among the CHIKV strains using BLAST (http://blast.ncbi.nlm.nih.gov). Sequences were aligned using the BioEdit program v7.2.5 (http:// www.mbio.ncsu.edu/bioedit/bioedit. html). Phylogenetic analysis was created by using the maximum likelihood method implemented in MEGA5.

Statistical analysis

Statistical analysis was analyzed using the Statistical Package for the Social Sciences (SPSS) software, version 16.0 for Windows (SPSS Inc., Chicago, IL, USA). Independent samples Mann-Whitney U test used to compare the means of two independent groups (CHIKV and control groups). In addition, the independent samples Kruskal-Wallis test was conducted to determine the different of cytokine levels in 4 groups: control, viremia, IgM positive, and both viremia + IgM. Data were expressed as median ± standard error of the mean (SEM). P values<0.05 will be considered to be statistically significant for differences and correlations.

Result

Part I: Angiogenic and inflammatory cytokine evaluation

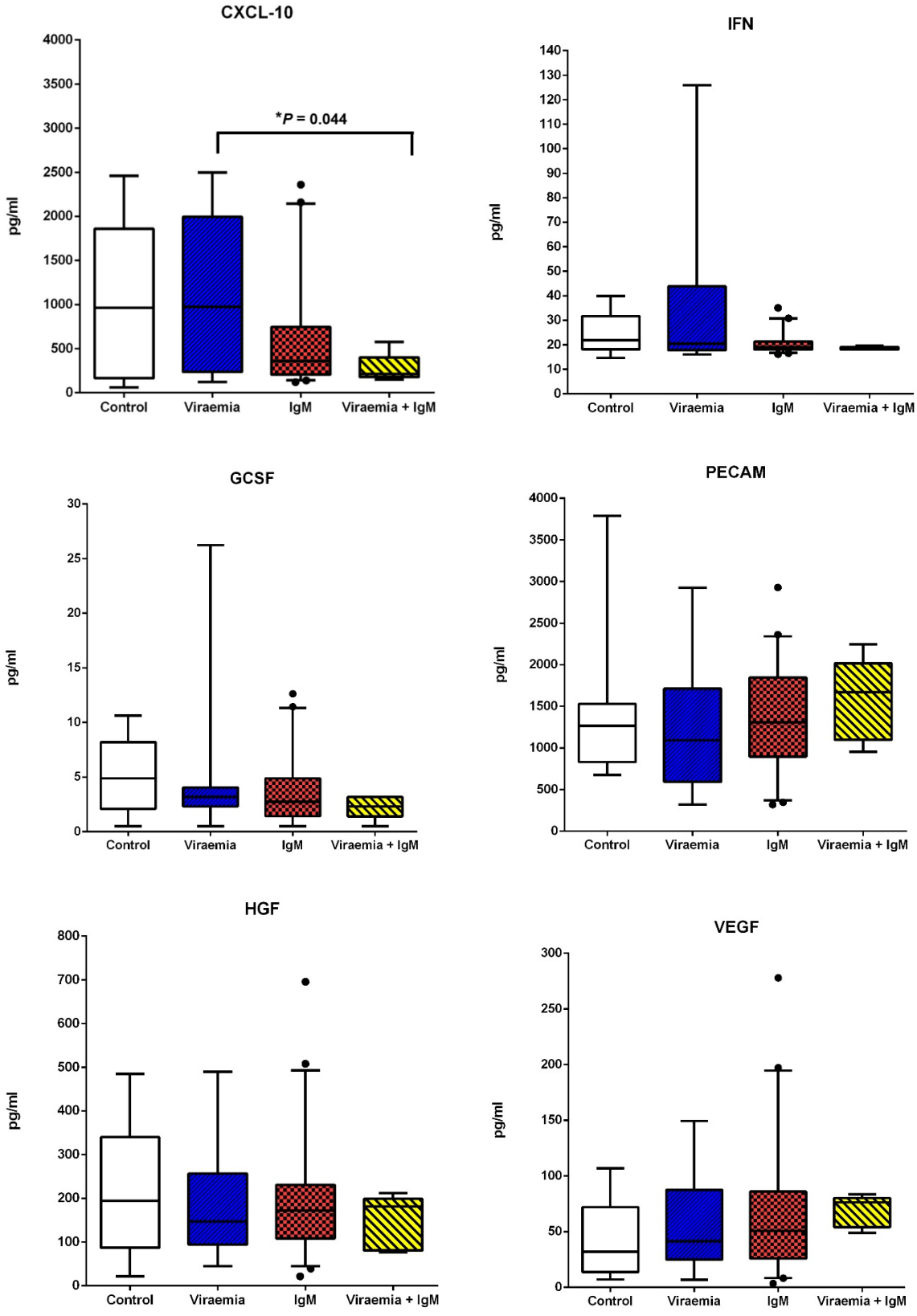
The multiplex angiogenic and inflammatory cytokines such as CXCL-10, IFN-α GCSF, PECAM, HGF, VEGF, Leptin, PDGF, Angiopoietin, Follistatin, and IL-8 levels were investigated in aged-match acute CHIKV infection patients and normal controls. The mean age of patient and control were 59.8 ± 13 years old and 52.0 ± 20 years old, respectively. We classified the patients into 3 groups including 18 viremia (CHIVK viral RNA positive), 48 CHIKV IgM antibody positive and 5 both viremia and IgM expression. The levels of almost 11 cytokines were not significant difference when compare between CHIKV patients and controls except the leptin concentration. The significantly decreased leptin level was found in CHIKV group (P = 0.44) showed in **Table1**.

Table 1 Angiogenic and inflammatory cytokine levels in CHIKV patient and control groups

Cytokines (pg/ml)	**CHIKV**		**Control**		**P-value**
	Median	**IQR (Q1 – Q3)**	**Median**	**IQR (Q1 – Q3)**	
CXCL-10	376.83	213.50 - 1010.17	965.17	166.00 - 1858.92	0.648
IFN-α	19.08	18.00 - 21.69	21.85	18.12 - 31.74	0.253
GCSF	2.98	1.43 - 4.05	4.89	2.10 - 8.20	0.110
PECAM	1242.63	855.52 - 1784.88	1263.83	830.08 - 1529.18	0.804
HGF	169.22	98.54 - 227.70	194.14	87.14 - 339.72	0.767
VEGF	51.37	26.96 - 85.80	32.11	13.85 - 71.95	0.100
Leptin	815.66	300.66 - 1698.11	2296.57	1091.26 - 4197.45	**0.044***
PDGF	923.27	474.04 - 1577.10	791.48	474.58 - 1689.06	0.767
Angiopoietin	198.23	147.09 - 291.46	218.92	155.34 - 346.10	0.362
Follistatin	28.44	21.05 - 53.75	21.39	14.12 - 31.78	0.072
IL-8	133.59	45.87 - 265.09	95.75	45.58 - 335.09	0.915

* indicated the significant different between CHIKV patients and controls

Additionally, we stratified the patients into 3 groups: viremia, CHIKV IgM positive, and both. The concentration of CXCL-10 was highest in viremia group followed by CHIKV IgM positive and both (P = 0.44). However, the other cytokine levels were not significantly different when compared between groups. The data was shown in **Figure 1**.



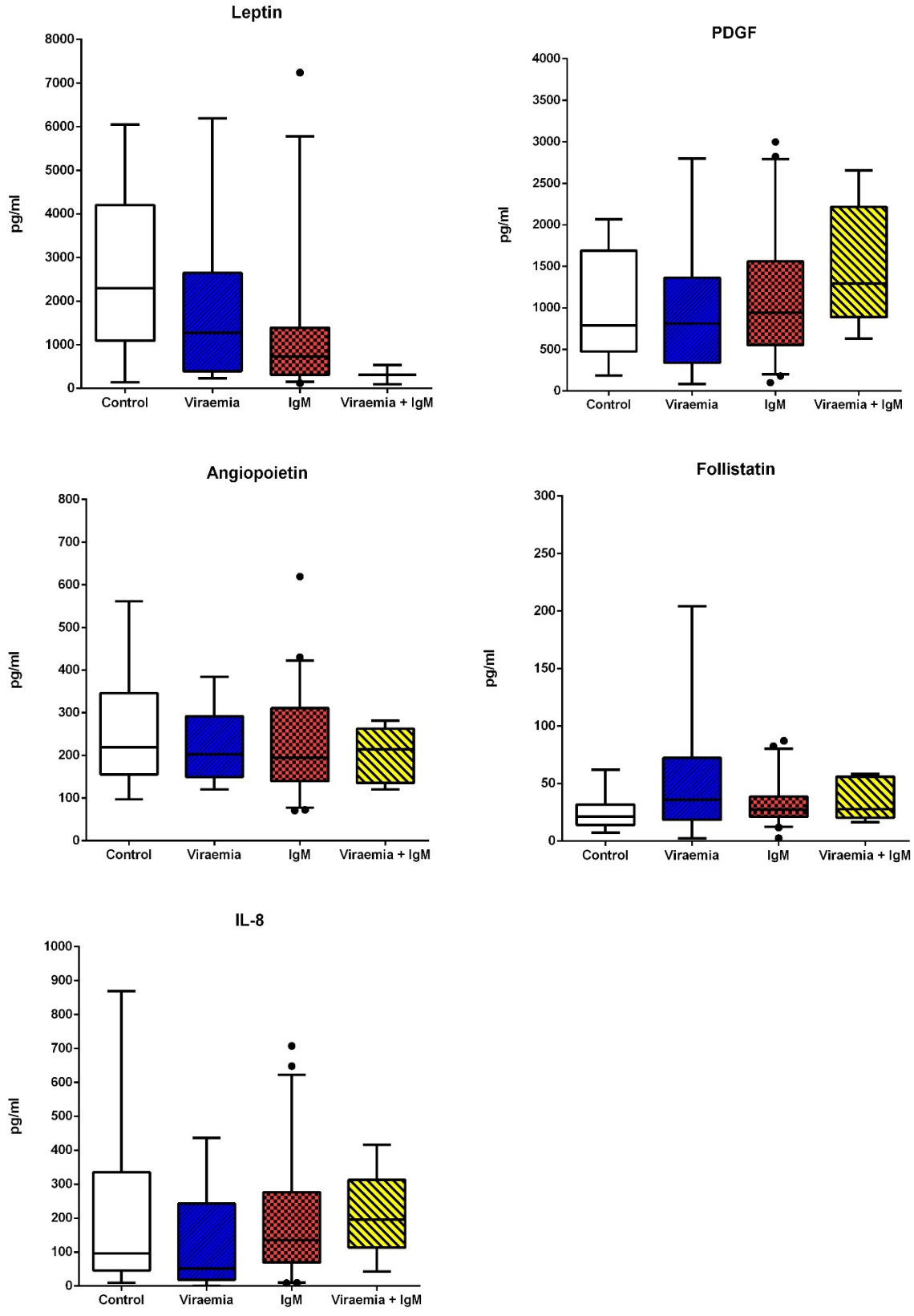


Figure 1 The levels of inflammatory and angiogenic cytokines in controls and CHIKV patients classified into viremia, IgM, and both viremia and IgM

Part 2: Genetically analysis of chikungunya virus

The 127 samples of patients who suspected chikungunya viral infection during 2018-2019 were determined the viral RNA infection. Twenty one samples were positive with real time PCR detection and nucleotide sequences from 15 patients with high viral load were submitted to the GenBank database under the accession numbers MK061363-MK061377. Moreover, phylogenetic analysis showed that the ECSA (East/ Central/ South/ Africa) lineage were identified in this study. The data was showed in **Figure 2**.

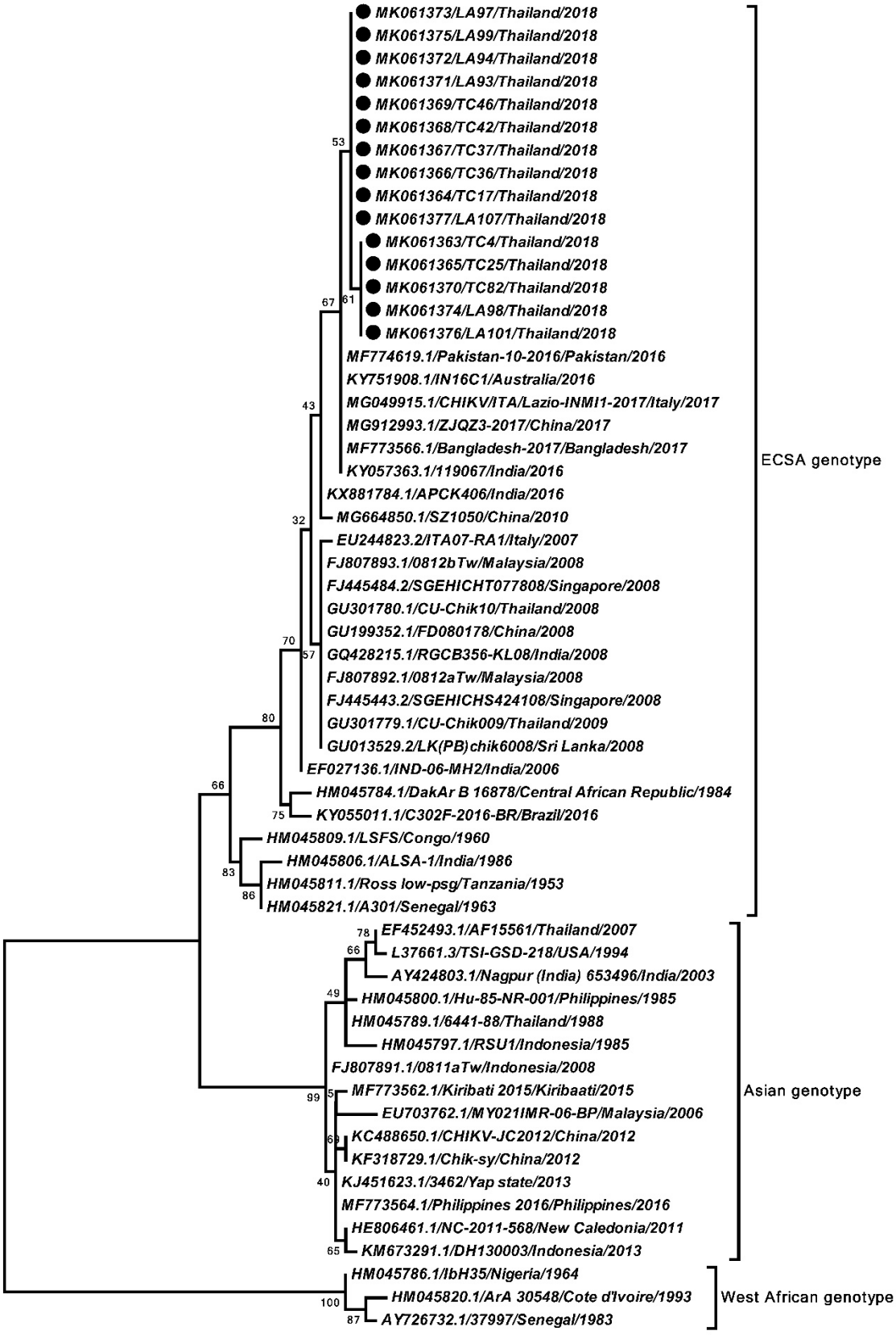


Figure 2 Phylogenetic analysis of the partial E1 gene sequences of CHIKV. Strains identified in this study were compared to the global CHIKV sequences available from GenBank. Black circles represent CHIKV strains from this study

Discussion

The prevalence of CHIKV infection in Thailand was disappear over 10 years. However, the new outbreak was established during 2018 to 2019. In this time, we found that the strain of virus was identified as ECSA lineage. It was similar to the more recent strains identified from previous study (26, 27). The prevalence data from our study revealed the existence of chikungunya virus in the country and it may be important to CHIKV surveillance consideration and start CHIKV activity to prevent the outbreak in the future.

The pathophysiology of CHIKV associated with angiogenic and inflammatory processes have been small documented. Some previous studies have been reported the immune response in CHIKV infection revealed the associations of a lot of cytokines, chemokines, and growth factors in those patients either increase or decrease expression ways (14, 28). This indicated that the inflammatory and angiogenesis are involved in pathogenesis of acute CHIKV infection. Deregulation of innate immune response including inflammation may play a role in clinical signs of CHIKV infection. Our results found the level of leptin, which is one of the inflammatory cytokines, was significant decreased in acute chikungunya patients when compared with control. Additionally, subgroup CHIKV infection classification as viremia, IgM, and both analysis demonstrated the concentration of CXCL-10 was highest in viremia group and gradually decreased in IgM and both, respectively. Otherwise, angiogenic mediators were found no significant difference between CHIKV and control groups.

Leptin, a regulator of both innate and adaptive immunity, functions as pro-inflammatory cytokine and angiogenesis. Previous study indicated that leptin deficiency leaded to increase susceptibility to intracellular infection such as HIV, influenza, and respiratory syncytial virus (29). However, there had been no report of the concentration of leptin in chikungunya infection; our data was the first documented. Reduced leptin level may contribute to decrease other inflammatory cytokine or T cell function in order to lost of immune response to CHIKV infection (29).

CXCL-10 is the chemokine associated with pro-inflammatory pathway. This chemokine was promoted by interferon γ which response to viral infection. Our result showed the highest CXCL-10 level in viremia which is the most severity stage in this study. The concentration of CXCL-10 was reduced after immune response (IgM production). The data was correlated with the report from Kelvin AA *et al*. (16) that CXCL-10 were associated with the severity of CHIKV disease manifestation. On the contrary, the CXCL-10 level was elevated in CHIKV infected patient group compared with healthy control (13). Unchangeable of various angiogenic cytokines in this study when compared between CHIKV and controls might be non-

severe tissue damage in the CHIKV patients. There was the result from Ng LF *et al.* illustrated the elevated levels of HGF and VEGF in CHIKV patient may reflect a physiological response to tissue destruction resulting from viral infection (30). Moreover, the different patient groups have reported different patterns of inflammatory mediators, indicating these mediators may differ according to the genetic variation of population leading to the contrast of our result to other previous reports. Other reason may explained the small sample size in each group and all subjects in control group had negative for CHIKV test but some have symptoms such as joint pain without CHIKV. Therefore, they might not be an ideal control group.

In conclusion, epidemic CHIKV in Thailand will be important to consideration CHIVK surveillance effort and track CHIVK activity. In field of pathology of chikungunya infection, the immune response developed at the beginning of CHIKV infection, therefore helping in the discovery of acute phase biological marker for the diagnosis and monitoring the treatment of this emergent chikungunya viral infection disease.

References

1. Weaver SC, Forrester NL. Chikungunya: Evolutionary history and recent epidemic spread. Antiviral research. 2015; 120:32-9.

2. Assuncao-Miranda I, Cruz-Oliveira C, Da Poian AT. Molecular mechanisms involved in the pathogenesis of alphavirus-induced arthritis. BioMed research international. 2013; 2013:973516.

3. Hoarau JJ, Jaffar Bandjee MC, Krejbich Trotot P, Das T, Li-Pat-Yuen G, Dassa B, et al. Persistent chronic inflammation and infection by Chikungunya arthritogenic alphavirus in spite of a robust host immune response. Journal of immunology. 2010; 184(10):5914-27.

4. Granger DN, Senchenkova E. Angiogenesis. Inflammation and the Microcirculation. 2: Morgan & Claypool Life Sciences; 2010. p. 1-87.

5. Lo Presti A, Lai A, Cella E, Zehender G, Ciccozzi M. Chikungunya virus, epidemiology, clinics and phylogenesis: A review. Asian Pacific journal of tropical medicine. 2014; 7(12):925-32.

6. Morrison TE. Reemergence of chikungunya virus. Journal of virology. 2014; 88(20):11644-7.

7. Schwartz O, Albert ML. Biology and pathogenesis of chikungunya virus. Nature reviews Microbiology. 2010; 8(7):491-500.

8. Pulmanausahakul R, Roytrakul S, Auewarakul P, Smith DR. Chikungunya in Southeast Asia: understanding the emergence and finding solutions. International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases. 2011; 15(10):e671-6.

9. Smith DR. Global protein profiling studies of chikungunya virus infection identify different proteins but common biological processes. Reviews in medical virology. 2015; 25(1):3-18.

10. Dupuis-Maguiraga L, Noret M, Brun S, Le Grand R, Gras G, Roques P. Chikungunya disease: infection-associated markers from the acute to the chronic phase of arbovirus-induced arthralgia. PLoS neglected tropical diseases. 2012; 6(3):e1446.

11. Petitdemange C, Wauquier N, Vieillard V. Control of immunopathology during chikungunya virus infection. The Journal of allergy and clinical immunology. 2015; 135(4):846-55.

12. Gasque P, Couderc T, Lecuit M, Roques P, Ng LF. Chikungunya virus pathogenesis and immunity. Vector borne and zoonotic diseases. 2015; 15(4):241-9.

13. Reddy V, Mani RS, Desai A, Ravi V. Correlation of plasma viral loads and presence of Chikungunya IgM antibodies with cytokine/chemokine levels during acute Chikungunya virus infection. Journal of medical virology. 2014; 86(8):1393-401.

14. Chow A, Her Z, Ong EK, Chen JM, Dimatatac F, Kwek DJ, et al. Persistent arthralgia induced by Chikungunya virus infection is associated with interleukin-6 and granulocyte macrophage colony-stimulating factor. The Journal of infectious diseases. 2011; 203(2):149-57.

15. Chirathaworn C, Poovorawan Y, Lertmaharit S, Wuttirattanakowit N. Cytokine levels in patients with chikungunya virus infection. Asian Pacific journal of tropical medicine. 2013; 6(8):631-4.

16. Kelvin AA, Banner D, Silvi G, Moro ML, Spataro N, Gaibani P, et al. Inflammatory cytokine expression is associated with chikungunya virus resolution and symptom severity. PLoS neglected tropical diseases. 2011; 5(8):e1279.

17. Lohachanakul J, Phuklia W, Thannagith M, Thonsakulprasert T, Ubol S. High concentrations of circulating interleukin-6 and monocyte chemotactic protein-1 with low concentrations of interleukin-8 were associated with severe chikungunya fever during the 2009-2010 outbreak in Thailand. Microbiology and immunology. 2012; 56(2):134-8.

18. Stifter SA, Feng CG. Interfering with immunity: detrimental role of type I IFNs during infection. Journal of immunology. 2015; 194(6):2455-65.

19. Wauquier N, Becquart P, Nkoghe D, Padilla C, Ndjoyi-Mbiguino A, Leroy EM. The acute phase of Chikungunya virus infection in humans is associated with strong innate immunity and T CD8 cell activation. The Journal of infectious diseases. 2011; 204(1):115-23.

20. Schilte C, Couderc T, Chretien F, Sourisseau M, Gangneux N, Guivel-Benhassine F, et al. Type I IFN controls chikungunya virus via its action on nonhematopoietic cells. The Journal of experimental medicine. 2010 207(2):429-42.

21. Liu M, Guo S, Hibbert JM, Jain V, Singh N, Wilson NO, et al. CXCL10/IP-10 in infectious diseases pathogenesis and potential therapeutic implications. Cytokine & growth factor reviews. 2011; 22(3):121-30.

22. Groom JR, Luster AD. CXCR3 ligands: redundant, collaborative and antagonistic functions. Immunology and cell biology. 2011; 89(2):207-15.

23. Jackson JR, Seed MP, Kircher CH, Willoughby DA, Winkler JD. The codependence of angiogenesis and chronic inflammation. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 1997; 11(6):457-65.

24. Imhof BA, Aurrand-Lions M. Angiogenesis and inflammation face off. Nature medicine. 2006; 12(2):171-2.

25. Lanciotti RS, Kosoy OL, Laven JJ, Panella AJ, Velez JO, Lambert AJ, et al. 222 Chikungunya virus in US travelers returning from India, 2006. Emerging Infectious Disease. 223 2007;13(5):764-7.

26. Rianthavorn P, Prianantathavorn K, Wuttirattanakowit N, Theamboonlers A, 239 Poovorawan Y. An outbreak of chikungunya in southern Thailand from 2008 to 2009 240

caused by African strains with A226V mutation. Internation Journal of Infectious Disease. 2010;14 Suppl 241 3:e161-5.

27. Pongsiri P, Auksornkitti V, Theamboonlers A, Luplertlop N, Rianthavorn P, Poovorawan Y. Entire genome characterization of Chikungunya virus from the 2008-2009 outbreaks in Thailand. Tropical Biomedicine. 2010;27(2):167-76.

28. Teng TS, Kam YW, Lee B, et al. A Systematic Meta-analysis of Immune Signatures in Patients With Acute Chikungunya Virus Infection. The Journal of Infectious Disease. 2015;211(12):1925-1935.

29. Maurya R, Bhattacharya P, Dey R, Nakhasi HL. Leptin Functions in Infectious Diseases. Frontiers in Immunology. 2018;9:2741.

30. Ng LF, Chow A, Sun YJ, et al. IL-1beta, IL-6, and RANTES as biomarkers of Chikungunya severity. PLoS One. 2009;4(1):e4261.