Association of Polymorphisms in C-Reactive Protein (CRP) Promoter -821 A>G, -390 C>A/T, and Plasma Interferon-α (IFN-α) with Plasma CRP Level in Javanese Systemic Lupus Erythematosus (SLE) Patients

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ABSTRAK

Latar belakang: *Sebagai reaktan fase akut, CRP diperlukan untuk membersihkan sel apoptosis dan kompleks imun pada SLE. CRP yang tidak responsif ini mungkin disebabkan oleh variasi genetik dan IFN-α yang melimpah yang dapat menghambat sekresi CRP. Penelitian ini bertujuan untuk menganalisis hubungan polimorfisme nukleotida tunggal (SNP) pada promotor CRP dan IFN-α plasma dengan kadar CRP pada pasien SLE di Jawa. Kami juga menganalisis hubungan SNP ini dengan SLE.* ***Metode:*** *Empat puluh SLE dan 40 pasien spondyloarthritis (sebagai kontrol) dimasukkan. Subjek SLE menjalani pemeriksaan laboratorium rutin, kadar CRP, IFN-α serum, dan sekuensing DNA untuk mendeteksi SNP pada promotor CRP. Kelompok kontrol hanya menjalani sekuensing DNA.* ***Hasil:*** *Median usia pasien SLE adalah 31,5 tahun. Skor rata-rata SLAM adalah 8,5. Usia rata-rata kelompok kontrol adalah 39 tahun. CRP rata-rata 5,19 SB 2,69 mg / L, IFN-plasma median adalah 46,02 pg/ml. Tidak ada perbedaan signifikan SNP di CRP -821 (rs2794521) atau -390 (rs3091244) antara SLE dan kontrol. SNP baru ditemukan pada CRP -456 A>G pada 5 pasien SLE, tetapi tidak ada pada kontrol. SNP ini akan meningkatkan risiko SLE 2.143 kali lipat. Ada korelasi negatif sedang antara tingkat IFN-α dan CRP plasma. Regresi linier hanya menunjukkan tingkat IFN-α (tidak juga dengan SNP) berkorelasi dengan CRP serum.* ***Kesimpulan:*** *IFN-α plasma berhubungan dengan kadar CRP. Tidak ada hubungan SNP di CRP -821, -390, dan -456 dengan level CRP. SNP CRP -456 A>G akan meningkatkan risiko SLE dengan rasio odds 2.143.*

Kata kunci: *promotor CRP, interferon-α, kadar CRP, lupus eritematosus sistemik, manusia, penyakit.*

ABSTRACT

Background: *As an acute-phase reactant, CRP is needed to clear apoptotic cells and immune complexes in SLE. This unresponsive CRP may be caused by genetic variation and abundant IFN-α that might inhibit CRP secretion. This study aims to analyze the association of single nucleotide polymorphisms (SNP) in CRP promoter and plasma IFN-α with CRP level in Javanese SLE patients. We also analyzed the association of these SNPs with SLE.* ***Methods:*** *Forty SLE and 40 spondyloarthritis (as control) patients were included. SLE subjects underwent routine laboratory test, CRP level, serum IFN-α, and DNA sequencing to detect SNPs in CRP promoter. The control group only underwent DNA sequencing.* ***Results:*** *The median age of SLE patients was 31.5 years. The median SLAM score was 8.5. The median age of the control group was 39 years. The average CRP was 5.19 SD*

2.69 mg/L, median plasma IFN-α was 46.02 pg/ml. There was no significant difference of SNPs in CRP -821 (rs2794521) or -390 (rs3091244) between SLE and control. New SNP was found in CRP -456 A>G in 5 SLE patients, but none in controls. This SNP would increase SLE risk 2.143 times. There was a moderate negative correlation between IFN-α level and plasma CRP. Linear regression only showed IFN-α level (not either SNP) correlated with serum CRP. ***Conclusion:*** *Plasma IFN-α correlates with CRP level. There was no association of SNPs in CRP -821, -390, and -456 with CRP level. SNP CRP -456 A>G would increase the risk of SLE with an odds ratio of 2.143.*

Keywords**:** *CRP promoter, interferon-α, CRP level, systemic lupus erythematosus, human, health.*

INTRODUCTION

C-reactive protein (CRP) is an acute-phase reactant that increases during inflammation due to IL-6 stimulation. Its function is to clear apoptotic cells, increase phagocytosis, and release inflammatory cytokines. Therefore, it is used as a biomarker for infection, atherosclerosis, inflammation, and autoimmune process. Unlike other autoimmune diseases, CRP level in SLE is normal or slightly elevated despite high disease activity and abundant IL-6 unless there is a bacterial infection. Low CRP level may cause inadequate apoptotic cells clearance, and this uneliminated product will induce immune reaction by producing autoantibodies, forming immune complex deposited in tissues and cause further inflammation cascade. Indeed, protective effects of CRP in the disease process have been demonstrated in animal models of lupus.[1–3] There are three hypotheses as to why CRP is unresponsive in SLE: variation of CRP gene, the anti-CRP antibody that binds CRP, and IFN, inhibiting CRP hepatocytes' CRP secretion. There are currently no studies elucidating which factor was more dominant.[1,4]

There are several studies in various population showing CRP gene polymorphisms related to SLE and CRP level. SNPs in promoter CRP -390 (or 1440, rs3091244) and -821 (or 1009, rs2794521) are those showing correlation with SLE and CRP level in Korean, Filipino, and Afro-American population.[5–7] These SNPs have never been studied in our population (Javanese ethnic in the Indonesian population).

The anti-CRP antibody is often found in SLE, but its relation with plasma CRP level is doubted lately. It is said that anti-CRP correlates with SLE activity but not with CRP level. When produced by hepatocytes, CRP is in pentamer form (native-CRP). In a special condition like inflammation, it will dissociate into monomer CRP (mCRP) in the tissues that is more functional biologically. Serum anti-CRP is an antibody against mCRP. On the other hand, measured plasma CRP is pentameric CRP.[4,8,9] In viral infection and SLE, we find a high level of IFN-α so it is presumed that IFN-α may inhibit CRP production by hepatocytes.[2,4,10] This study aims to analyze the association of SNPs in promoter CRP and plasma IFN-α level with CRP level in Javanese SLE patients.

METHODS

The sample size was determined using the formula for linear regression study. Previous study in the Philippines showed β-coefficient = 0.45 for SNPs correlation with plasma CRP level. Therefore, we included 40 SLE patients as sample group, and 40 spondyloarthritis (SpA) patients as control. SpA patients were used as control because like other autoinflammmatory disorders, in severe SpA patients we generally would see an increase of plasma CRP level.

All SLE patients came to either the rheumatology outpatient clinic for routine control or admitted to the hospital ward due to SLE flares. We included all SpA patients from the rheumatology outpatient clinic. All of the patients were from dr. Soetomo Academic General Hospital Surabaya, Indonesia. Diagnosis of SLE was made using ACR classification criteria for SLE 2019.[11,12] Demographic data and clinical manifestation were obtained from history taking, physical examination, and medical record. SLAM (Systemic Lupus Activity Measurement) and SLEDAI (SLE Disease Activity Index) were

used to measure the SLE disease activity score.

Blood Samples

Blood samples were taken from SLE subjects to check CBC, ESR, CRP, complement C3, C4 level and other routine laboratory tests for SLE. All of the examinations were done at Clinical Pathology Laboratory at dr. Soetomo Academic General Hospital Surabaya. CRP level was measured using the immunoturbidimetry method with an upper normal limit level was 5 mg/L. Complement C3 and C4 level were measured using radial immunodiffusion technique, and the normal limit for C3 was 90-180 mg/dL and for C4 was 9-36 mg/dL. Plasma samples were stored at -800C to measure IFN-α level until ELISA was performed. ELISA kit was Human IFN alpha ELISA Kit Invitrogen BMS216/BMS216TEN. Upon completing a sample assay using the kit protocol, absorbance was determined at 450 nm on Microplate reader: iMark (BioRad). Both SLE subjects and control underwent DNA sequencing to detect SNPs in CRP promoter through DNA isolation from peripheral blood mononuclear cells (PBMC). PCR machine was Perkin Elmer PJ 2000, QIA quick gel extraction kit (Qiagen, cat.no 28704) was used for DNA purification. The forward and reverse primer is shown in **Figure 1**. Numbering system of SNPs in this study was based on *GenBank accession number* AF449713.[13]

Ethics

This study had received approval from the Ethics Committee of dr. Soetomo Academic General Hospital with Ethical Clearance Number 1014/KEPK/III/2019 dated March 8, 2019.

Statistical Analysis

SPSS.21 was used for data analysis. Firstly, data distribution was tested. If homogenous, Pearson correlation was used, and if not, the Spearman correlation test was used to analyze the association between SNPs CRP -390, -821, plasma IFN-α level and plasma CRP level. Genotypes of promoter CRP were also compared between SLE and the control group using the Mann-Whitney test. Then multivariate analysis using linear regression was done to analyze the association of some independent variables (SNPs, IFN-α) with plasma CRP level.

RESULTS

Forty Javanese SLE patients (26 from the rheumatology outpatient clinic, 14 patients from the internal medicine ward) and 40 Javanese SpA patients from the outpatient clinic were included in this study from August 2019 to February 2020. Diagnosis of SLE was made based on SLE classification criteria from ACR 2019, and diagnosis of SpA was based on ASAS classification criteria 2010 [11,12]. All patients with median age were 31,5 years old (range 18-59) for the SLE group and 39 years old for the control group (range 21-60). Clinical characteristic of SLE subjects is shown in **Table 1**. Laboratory result of SLE patients is revealed in **Table 2**.

Positive PCR result as CRP gene amplification with suitable primer on several SLE subjects can

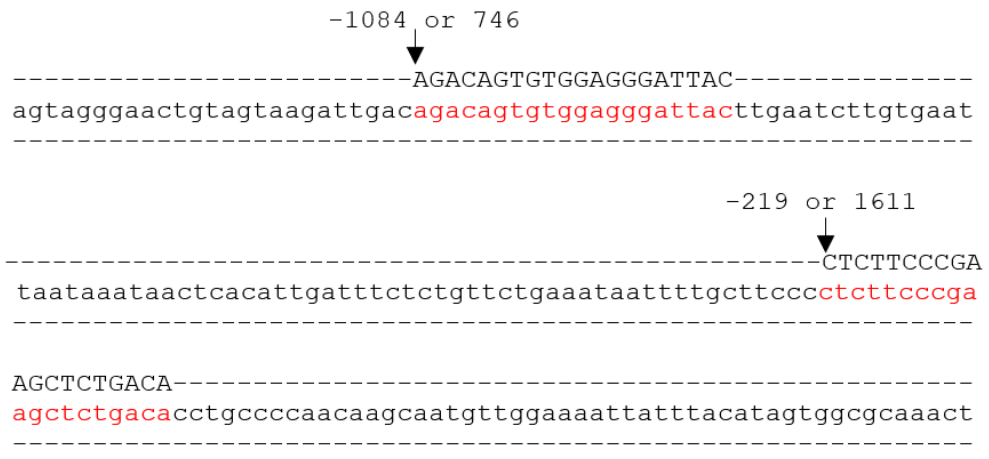

Figure 1. Forward and reverse primer.

Table 1. Clinical characteristic of subjects

Clinical manifestation	Median	Number (%)
Duration of illness (months)	23	
Body mass index	23.19	
Underweight		4 (10)
Normal		22 (55)
Overweight		14 (35)
Skin and mucosa manifestation		
Malar rash		9 (22,5)
Discoid rash		6 (15)
Oral ulcer		4 (10)
Hair loss		17 (42.5)
Photosensitivity		1 (2.5)
Musculoskeletal manifestation		
Arthritis		22(55)
Muscle pain		2 (5)
Fatigue		2 (5)
Panniculitis		2 (5)
Lung manifestation		2 (5)
Heart manifestation		
Heart failure		2 (5)
Hypertension		2 (5)
Serositis		5 (12.5)
Nephritis		13 (32.5)
Neuropsychiatric		2 (5)
SLAM score	8.5 (0-26)	
Mild (<7)		16 (40)
Moderate (7-20)		21 (52.5)
Severe (20)		3 (7.5)
SLEDAI	2 (0-28)	
Mild (1-5)		28 (70)
Moderate (6-10)		3 (7.5)
Severe (>10)		9 (22.5)

be seen in **Figure 2**. It shows DNA ribbon with 865 bp nucleotides.

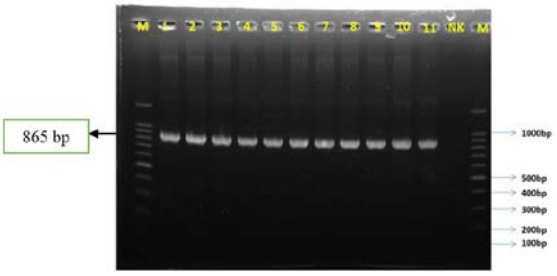

Figure 2. CRP gene electrophoresis showing DNA ribbon 865 bp.

During DNA sequencing, we found a new SNP in promoter CRP -456 A>G (nucleotide 1374 from the front). To our knowledge, this SNP has never been reported in any publication before. Five SLE patients had the SNP, but none in the control group. This SNP would increase SLE risk 2.143 times compared to wildtype. Genotype and allele distribution of CRP gene in both groups are shown in **Table 3** and **Table 4**.

Since Shapiro-Wilk test showed $p<0.05$ for IFN-α level, Spearman correlation test was used. It revealed a significant association between plasma IFN-α and CRP level ($p=0.003$ and $r= -0.455$ (moderate negative correlation). Spearman test also showed a moderate positive correlation between plasma IFN-α level and SLE disease activity (SLAM score) with $p=0.001$ and $r=0.568$ and with SLEDAI $p=0.004$ and $r=0.440$. There was no significant association between

Table 2. Laboratory characteristic of subjects.

Laboratory examination	Mean (SD)	Median	Number (%)
Positive anti ds-DNA			13 (32,5)
Hematology			
Hb (g/dl)	10.78 (2.92)		
Anemia			22 (55%)
Leukocytes (x10^3/ml)	8.92 (4.68)		
Leukopenia			5 (12.5)
Lymphocyte		1715	
Lymphopenia			17 (42.5)
Thrombocyte	252,150 (144.462)		
Thrombocytopenia			11 (27.5)
Serum complement			
C3	88.41 (44.28)		
low (<90 mg/dL)			16 (40)
C4	20.76 (12.22)		
low (<9 mg/dL)			7 (17.5)
ESR (mm/h)		24 (6-155)	
High (>20mm/h)			26 (65)
C-reactive protein (mg/L)	5.19 ± 2.69 (0.5-9.8)		
IFN-α (pg/ml)		46.02 (16.43-177.96)	

Table 3. Genotype distribution of CRP gene in SLE group and control

Location	Genotype	SLE group (%)	Control (%)	p
-821	AA (wildtype)	22 (55)	21 (52.5)	p = 0.996 *
	AG	15 (37.5)	18 (45)	
	GG	3 (7.5)	1 (2.5)	
-390	CC (wildtype)	26 (65)	29 (72.5)	p = 0.387 *
	CA	7 (17.5)	6 (15)	
	CT	5 (12.5)	5 (12.5)	
	AA	2 (5)	0	
-456	AA (wildtype)	35 (87.5)	40 (100)	p = 0.027 **
	GG	5 (12.5)	0	(OR 2.143)

* Not significant with Mann-Whitney Test (p>0.05)
** Significant difference with Fisher Exact test (p<0.05)

Table 4. Allele distribution of CRP gene in SLE group and control.

Location	Allele	SLE group (%)	Control (%)	p
-821	A	59 (73.75)	60 (75)	p=0.856*
	G	21 (26.25)	20 (25)	
-390	C	64 (80)	69 (86.3)	p=0.436**
	T	5 (6.3)	5 (6.3)	
	A	11 (13.8)	6 (7.5)	
-456	A	70 (87.5)	80 (100)	p = 0.001***
	G	10 (12.5)	0	

* Not significant with chi-square test (p>0.05)
** Not significant with Mann-Whitney test (p>0.05)
*** Significant difference with Fisher Exact test (p<0.05)

CRP level and SLE disease activity (p=0.903). Neither of the SNPs showed a significant correlation with plasma CRP level (p>0.05). All of the variables showed in **Table 5** related to plasma CRP level.

Linear regression study also revealed only IFN-α correlated with plasma CRP level multivariately, as shown in **Table 6**, with mathematic model was CRP = 6.910 – 0.025 IFNα.

Table 5. Correlation of each variable with plasma CRP level.

Variables	Plasma CRP level
SNP at promoter CRP -821	p=0.546*
SNP at promoter CRP -390	p=0.711*
SNP promoter CRP -456	p=0.161*
Plasma interferon-α	p=0.003** r= -0.455

* Not significant with Spearman test
** Significant Spearman test

Table 6. Linear regression analysis for this study.

Variables	β	b	p
IFN-α	-0.025	-0.421	0.005
SNP -456	-0.956	-0.119	0.439
Constanta	6.910		0.001

DISCUSSION

Baseline characteristics of the subjects involved in this study were slightly different than the South Korean and Phillipine cohort, but based on the sample size formula for a minimal patient number for linear regression study, 40 subjects were sufficient. Other studies also used normal subjects as control, but in this study, we used another autoinflammatory rheumatic disease as the control because there was increasing CRP in high disease activity SpA patients.[5,7] The mean CRP level in this study was 5.19 (SD 2.69) mg/L (range 0.5-9.8 mg/L), a little higher than other studies such as in Mexico with a mean CRP was 1.46 mg/L (0.5-4.77 mg/L) and the Philippines with 1.5 mg/L.[7,14] This might be because SLE subjects were from the outpatient clinic and those admitted at the hospital due to flare. All the patients from other study were from the outpatient clinic only (a stable condition with remission or low disease activity).

There was no association found in this study between SNPs at -821 nor -390 with SLE or plasma CRP level. Kim et al.[5] showed a significant difference of SNP -390 between

the SLE group and control (p=0.033), but not SNP -821. A study by Kim et al. also revealed a correlation between SNP -390 (not for SNP -821) with plasma CRP level (p=0.03). A more extensive study in the Philippines showed a moderate correlation between SNP -390 with plasma CRP level (β-coefficient = 0.45), but not for SNP promoter CRP -821.[5,7] A USA study involving the Caucasian race only showed a significant correlation between SNP -390 promoter CRP with plasma CRP level (p=0.012), but not for SNP -821. Other studies in the USA also showed a significant difference between the SLE group and control for SNP -390 with OR 1.43 (p=0.001).[6,15] Studies in China and Taiwan involving healthy subjects showed a correlation between SNP promoter CRP -821 (not CRP -390) with plasma basal CRP level.[16] New SNP on promoter CRP -456 (1374) was found in this study, and none of those studies reported this SNP before. SNP promoter CRP -456 A>G had a significant difference between the SLE group and controls with OR 2.143, but it did not correlate with plasma CRP level.

Hepatocytes synthesize CRP molecules as a response to IL-6 during inflammation. In viral infection and SLE flare, plasma CRP is normal though there is increasing in IL-6. It is caused by abundant IFN-α during SLE flare and viral infection. All subtypes of IFN-α can inhibit CRP promoter gene activity. This inhibition depends on the dose and is mediated by type-I IFN receptors. The IFN-α-dependent inhibition of CRP promoter activity was confirmed by studies of CRP secretion in primary human hepatocytes. IL-1β–induced CRP secretion was inhibited by 49.2%, and IL-6–induced secretion was inhibited by 51.5%, whereas the inhibition induced by IL-1β plus IL-6 was moderate (21.1%). After preincubation of IFN-α for 6 hours, there is suppression of promoter activity despite stimulation of IL-6 and IL-1β. CRP itself also may inhibit the production of IFN-α by pDC induced by the immune complex. As of writing, there is no study directly compares serum IFN-α and CRP level. A study by Enocsson et al.[2] showed inhibition of the transcription process in CRP promoter during CRP synthesis by hepatocytes.[2,4,8] This study directly correlated plasma IFN-α level with plasma CRP level, and it showed moderate correlation in both bivariate and multivariate analysis.

In a longitudinal study, it is reported that there is increasing of IFN-α in active SLE patient serum. Plasma IFN-α level also correlates with SLEDAI score. There is also increasing IFN-α gene expression in active SLE patients, and there is a significant difference in SLEDAI scores between patients with high and low plasma IFN-α level (p=0.0038). Direct measurement of plasma IFN-α has shown more accurate and specific than IFN-α signature.[17–19] All type I IFN will increase during SLE flare related to higher disease activity. IFN signature gene expression is also high in severe organ disturbance in lupus-like nephritis or neuropsychiatry.[20,21] Our study also showed a moderate positive correlation between IFN-α and disease activity in SLE patients.

CONCLUSION

This study showed the correlation between plasma IFN-α with CRP level in both bivariate and multivariate analysis. There was a new SNP found in this study at CRP promoter -456 A>G. There was no association of SNPs in promoter CRP -821, -390, and -456 with CRP level, but SNP promoter CRP -456 A>G would increase the risk of SLE (OR 2.143).

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CONFLICT OF INTEREST

There is no conflict of interest in this study.

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