POLYMORPISM ANALYSIS OF IL-17RA GENE TO THE IL-17RA CONCENTRATION AND CHRONICITY DIFFERENCES IN NEFRITIS LUPUS (NL) PATIENTS

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ABSTRACT

Nefritis lupus (NL) is autoimmune disease and one of serious complication from Systemic Eritematosus Lupus (LES). Clinical manifestation of NL was variated, there are hematuria microscopic asymptomatic until renal failure. In addition of clinical manifestation, the NL disorder degree also measured from histopathological grade. Although, the mechanism that mixed up with pathogenesis of NL was known, however the cytokine also play a role to the disease process. The cytokine that assumpted have a role to the inflammation is IL-17RA, the increase production of IL-17RA influenced to the inflammation and NL chronicity degree. The aim of this study to analyzed the relationship between polymorphism of IL-17RA gene, blood IL-17RA concentration with the NL disorder degree. These studies were used cross sectional with control case design. The sample were used 40 patients consist NL patients and Lupus patient without nephritis as a control. The polymorphisms of IL-17RA gene were investigated by using PCR method and gene sequence analysis. The alterations of allele frequency of IL-17RA gene were analyzed by bioinformatics method. The bloods IL-17RA concentration were investigated by ELISA method. The NL chronicity degrees were investigated by NL histopathological grade. The hypothesis were proofed by data normality test and homogeneity test, Chi-square and Odds ratio, Spearman correlation by using SPSS 17.0 for windows. The result of showed that there are significantly different between IL-17RA gene mutant genotype frequency of NL patient (NL) with the control patient with value OR 8.48. There are significantly different between G allele of IL-17RA gene of NL patient (case) with the control patient with the value OR 4.17. There are no significantly different between IL-17RA concentrations of NL patient (case) with the different chronicity value. There are positive correlation with the OR value 4.17 between IL-17RA concentrations with the chronicity value index.

***Key word**: polymorphism, IL-17RA gene, Nefritis Lupus (NL)*

INTRODUCTION

Nephritis lupus (NL) is kidney disruption that caused by systemic eritematosus lupus complication, with the criteria there is glomerulus damage and the progressive decrease of kidney function. This manifestation occur in 40-50% of LES patient that become extinct with End Stage Renal Disease (ESRD) for 5 years although was given aggressive immunosuppressant therapy. Based on Kusworini research, the kidney biopsy of 31 LES patient showed that there is 58% of NL that approximately become extinct with renal failure (Kusworini, 2010). The Asia and black skin population more over to get NL than another *Ras* (Fauci, 2009). The acute NL patient has a 5 years survival of < 70-80% (Avihingsanon, 2010).

Based on the ACR (American College of Rheumatology) criteria, NL is a clinical condition and laboratory that appropriate with the proteinuria persistent 0.5 gm per day or greater than 3 by dipstick, and/or cellular casts including red blood cells [RBCs], hemoglobin, granular, tubular, or mixed. The gold standard for NL diagnostic is kidney biopsy (Bawazier 2009; Dooley 2007; Hahn, 2009). The NL pathophysiology related to macrophage complicity. A research showed that macrophage is NL mediator, because intra renal macrophage will increase with the compliance of Kidney complicity degree at the experimental animal with NL tissue (Cheng, 2005). The production of MCP-1 is a principal factor that influence the macrophage accumulation at Kidney tissue (Tesch, 2008). IL-17 is a potential pro-inflamatory cytokine, this cytokine will increase the chemo attractant to the monocyte and neutrofil on the target organ, with the increase of local chemokine production, including MCP-1 (Saxena, 2008). The mechanism that serve as a basis of this condition not yet fully understood, the IL-17RA was estimated have a big role at the collagen and fibrosis matrix deposition (Zuccardi, 2007; Vuong, 2009).

Based on Alunno (2012) research, at the LES patient group showed that there are increase of serum or plasma IL-17RA concentration, also the increase of IL-17 production by T cell at peripheral blood, and Th17 infiltration at the target organ such as Kidney. The IL-17 concentration correlated with the disease activity and Kidney disruption. Gene polymorphism can cause the protein structure change of cytokine, and that structure change will influence at the interaction between cytokine protein and receptor. Based on that case, was made an assumption that genetic variation will influence the expression or activity of IL-17. The recent study related to IL-17RA gene polymorphism and IL-17RA receptor related to the risk of Rheumatoid arthritis, alopecia areata and colitis ulserativa (Lew, 2012). The aim of this study to know the relation of IL-17RA gene polymorphism to the IL-17RA concentration and the chronicity differences in Nephritis Lupus (NL) patient.

MATERIALS AND METHOD

Analytic observational studies were conducted with cross sectional study. The research and data collection we-

re done in RSSA (Saiful Anwar Hospital), Malang: 1) Department of Rheumathology and Nephrology section for sampling the sample, 2) Department of Pathology and Anatomy for observation of renal failure histology degree, 3) Genetics Laboratory of Islamic State University (UIN) Malang for ELISA, PCR and sequencing of DNA. The LES diagnose were established based on ARA criteria in 1997. The patient with congenital kidney disorder, renal trauma history, and co-morbid disease such as Diabetes Mellitus were excluded from this study. These studies were conducted after obtaining an approval from Ethics Committee of Medicine Faculty, Brawijaya University or RSSA Malang. The entire patient that included in this study was asked to sign an informed consent.

Polymorphism Analysis of IL-17RA gene receptor

The polymorphisms of IL-17RA gene analysis were done through DNA extraction. Measurement of purity and concentration of DNA, IL-17RA gene primer design, gene amplification by PCR method and DNA sequencing.

DNA isolations were done by using DNA isolation Kit (Wizard Genomic DNA Purification Kit from Promega). 0.3 ml of blood were put in 2 ml tube that containing 1.5 ml of Buffer Cell Lysis Solution. The sample were incubated at room temperature for 10 minutes, and then were centrifuged at 2000 g for 10 minutes. The supernatant were discarded, and then the pellet were added 1 ml of Nuclei Lysis and were homogenated by using vortex. The samples were added 0.3 ml of Protein Precipitation solution, and then were centrifuged at 2000 g for 10 minutes. The supernatant were taken and put at a new tube that containing 1 ml of isopropanol, then the sample were homogenized by using vortex and centrifuged at 20-00 g for 1 minute. The supernatant were discarded, and the pellets were added 1 ml of ethanol 70%, and then were centrifuged at 2000 g for 1 minute. The supernatant were discarded and the pellets were dried. Then the pellets were dissolved by adding 75 µl of DNA Rehydration solution. The sample were incubated at 65 ^{0}C for 1 hour, and then were stored at -20^0 until use.

The primer were designed based on IL-17RA gene with specific locus of IL-17RA gene (rs2275913) that contained in the NCBI *database* (*National Center for Biotechnology Information*). The primer were designed by using Oligo Analyzer 1.0.2. Version, Oligo Explorer 1.1.0. Version and BLAST (*Basic Local Alignment Search Tool*) software which can be accessed online at the NCBI. The design primer were tested by Oligo Analyzer 1.0.2 Version software, include percentage of GC content, *Tm* (*time melting*), dimmer test and multiplex test between forward and reverse primer. The primer design of IL-17RA draws of Lew, dkk. (2012) research; *forward* primer (5'- CCTCAGTTG- GGTTTCTCAGC-3') and *reverse* primer (5'-AGGTCCTGGATGCGGAAGTA-3').

Amplification of IL-17RA gene polymorphism genotyping were done by Polymerase Chain Reaction (PCR) technique using thermal cycler machine. The composition of PCR with 20 µl/*tube* total volume consist of 6 µl dd-H_2O, 10 µl *PCR* kit GoTaq® Green Master Mix (10 x buffer taq polymerase, dNTP, $MgCl_2$, primer, Taq DNA polymerase, ddH_2O), forward primer 1 µl, reverse primer 1 µl and 2 µl of genomic DNA samples. The determination of amplification temperature refers to Correa (2012), in sequence amplification process were conditioned on pre-denaturation at 95 ^{0}C for 5 minutes 1 cycle, and 30 cycle on denaturation at 95 ^{0}C for 30 second, 52 ^{0}C for 30 second, and 72 ^{0}C for 30 second, then followed by 1 cycle of extention at 4 ^{0}C for 5 minutes.

Sequencing were used purified of DNA samples from PCR product. The sequencing composition consists of 2 µl of purified gene amplicon sample. ddH_2O 10 µl, Big Dye terminator 8 µl (ABI PRISM® BigDye® terminator v3.1cycle sequencing kits), 5 µl buffer solution (buffer with EDTA (Applied Biosystem), and 2 µl of primer 20 pmol, so that the total volume is 25 µl. Furthermore, the PCR amplicon were added buffer solution (Hi-Di™ Formamide (Genetic Analysis Grade-Applied Biosystem)) and sequenced by using ABI PRISM® 310 Genetic Analyzer.

Polymorphism analysis with bioinformatics method such as collecting data bases of IL-17RA, the data selection with alignment, and polymorphism analyzed on base sequence of nucleotides. The data base of IL-17RA gene were obtained through *national Centre of biotechnology Information* (NCBI) (http://www.ncbi.nlm.nih.gov/) with the key word data accordance with nucleotide base SNP data that used in research. The obtained data were stored in FASTA file for data selection use. The alignment of data selection by using Bio-edit program. The mutated polymorphisms were analyzed by homozygote and heterozygote interpretation by looking at the bases sequence and base curve of sequencing result.

Blood (serum) IL-17RA Analysis

Blood IL-17RA were analyzed by sandwich enzyme-linked immunosorbent assay (ELISA) method using specific antibodies for Human IL-17RA. Serum samples were taken from venous blood then placed in sterile container and stored in -80 °C until analyzed. In micro titer wells that had been coated with anti-human IL-17RA antibody (peroxide conjugated polyclonal anti-human IL-17RA antibody) were added to the samples that have been diluted. The samples were washed and added biotinylated antibody HRP-conjugated streptavidin. Then the samples were washed and added TMB substrate solution to give color

to the IL-17RA bound. The reaction were stopped by adding of stop solution, and then read with a spectrophotometer at 450 nm. The results were plotted on a standard curve to get the IL-17RA concentration.

Renal biopsy analysis and histopathological grade examination

A renal biopsy were performed by USG (ultrasonography)-guided. Tissue samplings were done twice. The tissue were cut with a thickness 2-3 mm. The tissue were fixed in 10% formalin and embedded in paraffin by Tissue-Tek Processor automatic machines (Tissue-Tek®, Torrance, CA, USA). The samples were cutted by using microtome (Leica Microsystems (SEA) Pte Ltd, Ayer Rajah Industrial Estate, Singapore) with a thickness 3-5 µm. The tissues that have been cut were stained with Haematoxilyn-Eosin (HE). Histopathological grade examinations were conducted by Pathology specialist doctor by using light microscope. NL histopathological grade classifycations were determined based on criteria of the ISN / RPS on 2004. NL grade 1 showed by normal glomerolus. NL grade II showed by pure mesangial hypercellular with various degrees or mesangial matrix expansion with mesangial immune deposits. NL grade II showed by endo glomerulonephritis or active and inactive focal capillary extra, segmental or global that involving < 50% of the glomerulus. NL grade IV showed by endo glomerulonephritis or antive or inactive diffuse calipary extra, segmental or global that involving < 50% of the glomerulus. NL grade V showed by global sub epithelial deposits immune or segmental or their morphologic sequelae, with or without mesangial changes. The NL degree were grouped into serious NL grade (grade III-V) and small NL grade (grade 1-II).

Statistical Analysis

The research hypotheses were evidenced by parametric statistical test analysis. The data were analyzed by prerequisite parametric test, that is normality test and data homogeneity test (Kolmogorov-Smirnov test). Statistical analyses were performed by using SPSS for Windows software 17.0 Version. The difference in genotype and IL-17RA gene allele frequencies in case and control were analyzed with Chi-square test and Odds ratio. The differences of IL-17RA ratio in NL subject with different chronicity index score were analyzed by student t-test, when normality is not eligible then to be tested by Mann Whitney test. The relationships between polymorphism of IL-17RA gene with serum IL-17RA level were analyzed by using Spearman correlation test. P < 0.05 indicates statistically significant differences.

RESULT

The total sample is 40 subjects, consist 24 NL patient (62.5%) and 16 control (37.5%) that obtained from Rheumatology Polyclinic and treated in the Rheumatology and Nephrology Section of RSSA Malang.

Based on table 1 showed that OR value was obtained 8,48 (p=0,09). This means, the NL patient with mutan genotype (AG, GG) are at risk for experiencing chronicity 8.48 times heavier than the Wild genotype (TC) patients.

Table 1. Analysis of relationship between polymorphism of IL-17RA genotype gene and Chonicity

Genotype	Case N(%)	Control N(%)	Total N(%)	Direction
Mutan	22 (55)	7 (17,5)	29 (72,5)	P value = 0,009
Wild	3 (7,5)	8 (20)	11 (27,5)	OR = 8,48
Total	25 (62,5)	15 (37,5)	40 (100)	

Direction: *p-value* > 0.05 there is no significantly difference, *p-value* < 0.05 there is significantly difference

Table 2. Analysis of Relationship between Polymorphisme of IL-17RA gene G Allele and Chronicity Incidence

Allele	Case N(%)	Control N(%)	Total N(%)	Direction
G	32 (40)	9 (11,25)	29 (72,5)	p-value = 0,005
T	18 (22,5)	21 (26,25)	41 (27,5)	OR = 4,17
Total	50 (62,5)	30 (37,5)	80 (100)	

Direction: *p-value* > 0.05 there is no significantly difference, *p-value* < 0.05 there is significantly difference

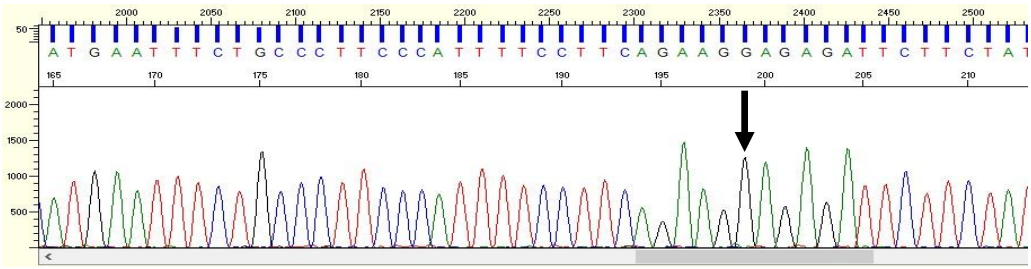

Figure 1. IL-17RA mutan area on Nephritis Lupus patient (arrow)

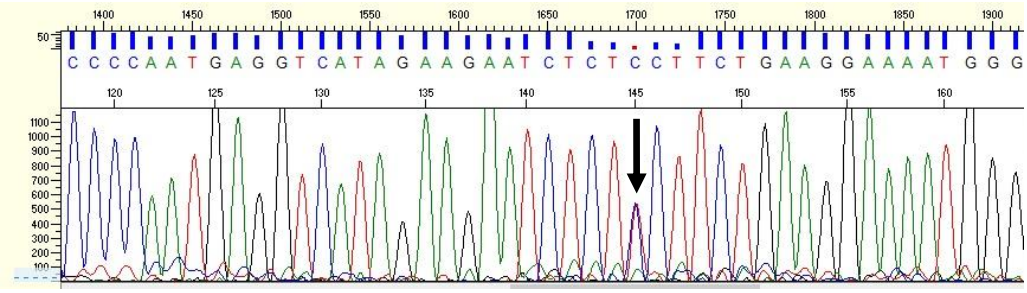

Figure 2. Wild genotype sequence area on Nephritis Lupus patient (arrow)

Based on the table 2 showed that OR value that obtained is 4.17 (p=0.09). this indicate that NL patient with G allele at risk for experiencing chronicity 4.17 times heavier than subject with T allele.

The relationship of chronicity incidence and serum IL-17Ra level, in the current study was compared IL-17RA level on NL patient with chronicity difference that is high chronicity index (score > 4) and low chronicity index (score <4) (Figure 3).

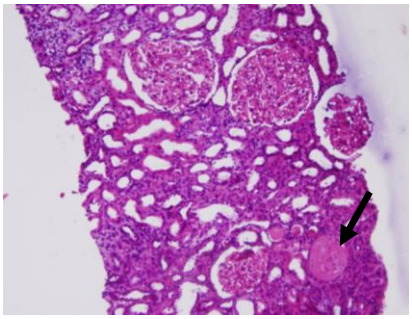

Figure 3. Histology of renal biopsy of research subject with high chronicity index (score 5/12). Showed there is total sclerosis on glomerulus, interstitial fibrosis and tubule atrophy.

DISCUSSION

Based on the result showed that the average of patient age 18 years for NL patient and 25 years for control. This is consistent with the research that stated that majority of LES patient are childbearing age woman (15 to 40 years). It also consistent with a theory that woman have prevalence more often to get LES, because it influenced by sex hormones factor so that will influence on the susceptibility to the development of LES (Dooley, 2007).

Of the 25 samples that was examined chronicity index, show that 80% of that patient on Low chronicity index (1-4) and 20% of the patient on high chronicity index (>4), as shown in table 1. It could be because of most of research subject have not shown advance phase of the disease. Renal failure is dominated by widespread renal fibrosis on kidney tissue. The chronicity index is one of component that used for rating of renal fibrosis. The increase of renal fibrosis generally have increased chronicity index score. Renal biopsy is difficult to determine the chronicity index in patients with renal failure (Dooley, 2007).

Relationship of IL-17RA gene polymorphism and chronicity incidence

The comparison of G allele frequency distribution of IL-17RA gene in case and control is 40% and 11.25%, respectively. The comparison of T allele frequency distribution of IL-17RA gene in case and control is 22.5% and 26.25%, respectively. The G allele frequency is found on case group and increasethe risk for chronicity. This is accordance with Wu *et al.,* (2010) research, stated that genetic co-existence variation that is homozygous CG genotype and G allele, its possible as a significantly risk factor for the occurence of COPD in Chinese Han population. However, the distribution of IL-17RA gene polymorphism appear to differ among different ethnic group that need further investigation in accordance with their respective ethnic (Wu *et al.,* 2010).

The distribution of G allele of IL-17RA gene on case group and control was found only ne sample (CG). The frequency of T allele is found in the case group and control. According to Wang *et al* (2007), IL-17RA gene has many polymorphism, including C-988A, G-800A, and C-509T in the promoter region, 72 position (C insertion) in the non-translated region, and C263T (Thr/Ile), T869C (Leu/Pro), G915C (Arg/Pro) in the coding region. Each population has a particular polymorphism. The Japan population, especially has T869C polymorphism, but lacks of IL-17RA gene polymorphism. The result of this study also showed that little of subject study which have IL-17RA gene polymorphism.

Relationship of IL-17RA gene polymorphism and Serum IL-17RA levels.

The IL-17RA comparison test in mutant patient group and wild patient group showed that there is no significant different although there are increase of serum IL-17RA levels on mutant group. This result are not in accordance with Saxena (2008) research which reported that IL-17RA levels that measured from kidney tissue strongly correlate with the chronic renal failure, especially with tubulointerstitial disease and glomerulosclerosis. The Rus (2009) research support the previous research, which stated that IL-17RA strongly correlate in chronic renal failure compared with acute renal failure. Similary, Shao

(2011) reported that serum IL-17RA plays an important role in the clinical figure in severe Nephritis Lupus.

Relationship of Chronicity and Serum IL-17RA levels.

The histology of Nephritis Lupus renal fibrosis will appear on histopathological examination of kidney tissue biopsy. The nephritis histopathological examination include grade and activity index, and the chronicity as one of standard for diagnosis, prognosis and appropriate therapy for Nephritis Lupus (Mok, 2010).

The result of this study showed that there is no significant different of IL-17RA levels between low chronicity index of research subject and high chronicity index of research subject, although the mean of IL-17RA levels was higher in subject with a high chronicity index. The result of IL-17RA mRNA in urine sediment of LES patient significantly increase in the disease active condition and associated with activation status and histopathology activity degree, and also parallel decrease with the reduce of disease activity in patients. But there are also study which stated that serum IL-17RA did not correlate with activity status and histophatology activity degree of NL (Chan, 2006; Colucci, 2006). The patient in this study did not have significant increased of IL-17RA levels, its possible because of the sampel were measured on blood, not in urine. The urine sample more directly describe the occurrence of renal insufficiency than serum samples.

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