

SINGLE-NUCLEOTIDE POLYMORPHISM OF *TNFSF4* (RS2205960) OF SYSTEMIC LUPUS ERYTHEMATOSUS PATIENTS IN BANDUNG, INDONESIA

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ABSTRACT

Objective: This study aimed to determine the genotype distribution of the *TNFSF4* (Tumor Necrosis Factor Superfamily 4) gene rs2205960 in Systemic Lupus Erythematosus (SLE) patients in Bandung, West Java, Indonesia.

Methods: This was a cross-sectional study; 84 genomic DNA samples were amplified, electrophoresed, then analyzed by DNA sequencing.

Results: The genotype distribution of the *TNFSF4* gene rs2205960 in SLE patients showed that from 84 DNA samples, 55 patients are GG (65.48%), 25 patients are GT (29.76%), and 4 patients are TT (4.76%).

Conclusion: Results indicate that SLE patients in Bandung have a genotype distribution of the *TNFSF4* rs2205960 gene that fulfills the Hardy-Weinberg equilibrium.

Keywords: Bandung, Single-nucleotide polymorphism, RS2205960, Systemic lupus erythematosus, *TNFSF4*

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INTRODUCTION

Systemic Lupus Erythematosus (SLE) is a chronic inflammatory autoimmune disease with various clinical manifestations affecting multiple organ systems [1]. SLE patients experience a loss of self-tolerance due to an abnormal immunological function, which leads to the formation of immune complexes that can damage tissues and characterized by the production of autoantibodies, activation of the complement system, and the involvement of genetic and environmental components [1, 2].

The cause of SLE is very multifactorial, between various genetic and environmental factors that contribute to disease susceptibility [3]. In recent years, many studies have shown the relationship between polymorphisms of several genes with SLE susceptibility, one of which is the Tumor Necrosis Factor Superfamily 4 (*TNFSF4*) gene. This gene is located in chromosome 1 at position 25 (1q25). The *TNFSF4* gene encodes OX40L, a ligand for the OX40 receptor, which is a member of the tumor necrosis factor (TNF) superfamily that is expressed on antigen-presenting cells, such as B cells, dendritic cells, macrophages, mast cells, endothelial vascular cells, and natural killer cells [4-8].

Single-nucleotide polymorphism (SNP) of the *TNFSF4* gene rs2205960 has the most significant association in the *TNFSF4* region in SLE patients, according to Genome-Wide Association Studies (GWAS) conducted by Chang *et al.* (2019). This intronic SNP is located 38.6 kb upstream of *TNFSF4* and implicated as a cis-acting factor for transcription [8-10]. SNP rs2205960 (G>T) causes an increase in OX40L expression, which influences the development of SLE disease. Overexpression of OX40L may increase the stimulation of CD4⁺T helper effector cells and increase the response of follicular T helper cells whose function is to help B cells proliferate and form antibodies causing inflammation and autoimmunity in specific organs [11-13]. The OX40L signaling suppresses the generation and function of interleukin-10 (IL10)-producing CD4⁺type 1 regulatory T

cells, which play an important role in maintaining peripheral resistance [14].

According to the meta-analysis conducted by Wang *et al.* (2019), the TT genotype of the *TNFSF4* rs2205960 increases the risk of SLE with a significant degree of association [11]. Another study stated that the T allele of *TNFSF4* rs2205960 had a significant association in SLE patients from Asian, Caucasian, Hispanic, and Mexican races [10, 12-19]. These results showed the influence of race and ethnicity on SNP. However, research on *TNFSF4* gene polymorphisms, especially rs2205960 in SLE patients in Indonesia, has not been reported. Based on this background, this study identified the gene polymorphism of *TNFSF4* rs2205960 and analyze the allele and genotype frequencies in SLE patients in Bandung using the direct sequencing method.

MATERIALS AND METHODS

Subjects

Blood samples from 84 patients were collected from the Hasan Sadikin Hospital in Bandung, Indonesia. Patients who were included in this study were female patient's aged 18-65 y diagnosed with SLE. Patients were willing to participate in the research and agreed to sign the informed consent. Patients excluded from this study were SLE patients with incomplete treatment data or medical records that could not be traced and patients who were not willing to participate in the study. This study protocol was approved by the Health Research Ethics Committee Universitas Padjadjaran (reference no. 128/UN6. KEP/EC/2020).

DNA isolation

DNA isolation was done using Geneaid™ DNA Isolation Kit (Geneaid™, New Taipei City, Taiwan), which is based on the precipitation method. Extracted DNA samples were then stored at 20 °C until further use.

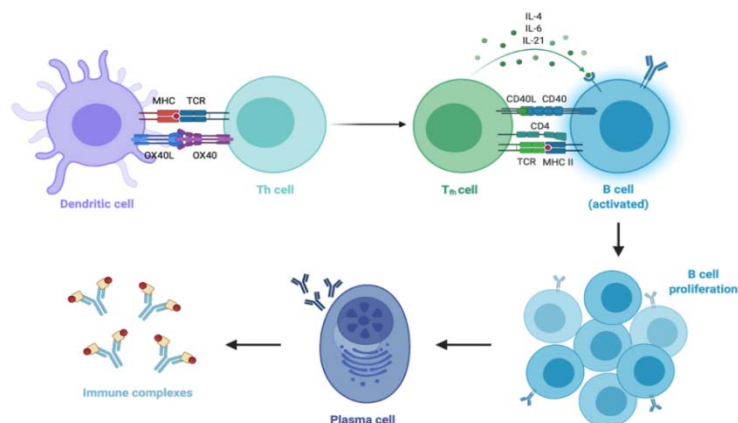


Fig. 1: Higher expression of OX40L and its interaction with OX40 correlates with the formation of immune complexes in SLE (created with BioRender.com)

Single-nucleotide polymorphism analysis

DNA samples were amplified using Promega GoTaq® Green PCR Master Mix (Promega, Fitchburg, Wisconsin). A total volume of 50 μ l of each PCR reaction mixtures was prepared, which contained 2 μ l DNA template, primer forward and reverse each 1 μ l, Promega GoTaq® Green PCR Master Mix 25 μ l, and nuclease-free water 21 μ l. Primers used in this study are based on research by Lu *et al.* in 2016 [20]. The two primer sets were as follows: forward 5'-AACCTTGGTCTCCTATAATGGGTACTCT-3' and reverse 5'-GACTTTTCCCTTTGTCATTTCAG-3 to detect *TNFSF4* polymorphic gene. The expected amplicon size was 162 bp. Amplification process was carried out in a Bio-Rad T100™ Thermal Cycler. PCR reaction was initiated with an early denaturation at 95 °C for 3 min, and 35 cycles of denaturation at 95 °C for 30 s, followed by annealing at 58,8 °C for 30 s, and extension at 72 °C for 1 min. The final step is a cycle of 72 °C final extension for 5 min. Furthermore, the polymorphism of *TNFSF4* rs2205960 was determined using Sanger sequencing method (1st BASE, Selangor, Malaysia).

The results of the DNA sequencing were then aligned with the reference sequence of the *TNFSF4* gene originating from Homo sapiens chromosome 1, GRCh38. p13 Primary Assembly (<https://www.ncbi.nlm.nih.gov/snp/rs2205960>). Alignment between the reference sequence and the resulting sequence was performed using the BioEdit 7.2 software.

Table 2: Distribution and comparison of allele frequency

Genotypes	n	G allele	T allele	Observed frequency (n/%)	Expected frequency (n/%)
GG	55	110	0	55 (65,48)	54,2 (64,52)
GT	25	25	25	25 (29,76)	26,5 (31,55)
TT	4	0	8	4 (4,76)	3,2 (3,81)
Total	84	135	33	84 (100)	84 (100)

A Chi-squared test was conducted to see if the sample populations fulfill the Hardy-Weinberg equilibrium. The rejection of the null hypothesis (H_0) indicates that there is a change in allele frequency from generation to generation which means that it does not fulfill the Hardy-Weinberg equilibrium, and the acceptance of H_0 states that the distribution in the population is consistent with the Hardy-

Statistical analysis

The genotypic distribution data were then analyzed using the Hardy-Weinberg equilibrium (HWE) equation ($HWE, df = 1$). The Chi-squared (χ^2) statistic test was used to analyze if the sample populations fulfill the Hardy-Weinberg equilibrium.

RESULTS

The results of the sequence alignment can be seen in fig. 2, which shows the similarity between the sample sequences and the reference sequences.

Table 1: Number and percentage of genotype distribution

Genotype	Total patients (n)	Percentage
GG	55	65,48%
GT	25	29,76%
TT	4	4,76%

The genotypic distribution data were then analyzed using the Hardy-Weinberg equilibrium test.

The results of the observed and expected frequency can be seen in table 2.

Weinberg equilibrium [21]. From the calculation results in table 3, the Chi-square value (χ^2) obtained is 0.2752, and the p-value is 0.599 ($P > 0.05, \alpha = 5\%$). These results indicate that the null hypothesis is accepted since it has a smaller χ^2 value than the table χ^2 and p-value greater than the significance level, which means that this study is consistent with the Hardy-Weinberg equilibrium.

Table 3: Results of chi-square test on the hardy-weinberg equilibrium

Genotypes	Observed frequency (n/%)	Expected frequency (n/%)	Chi-squared (χ^2) value	Chi-squared test p-value
Homozygote reference (GG)	55 (65,48)	54,2 (64,52)	0.2752	0.599
Heterozygote (GT)	25 (29,76)	26,5 (31,55)		
Homozygote variant (TT)	4 (4,76)	3,2 (3,81)		

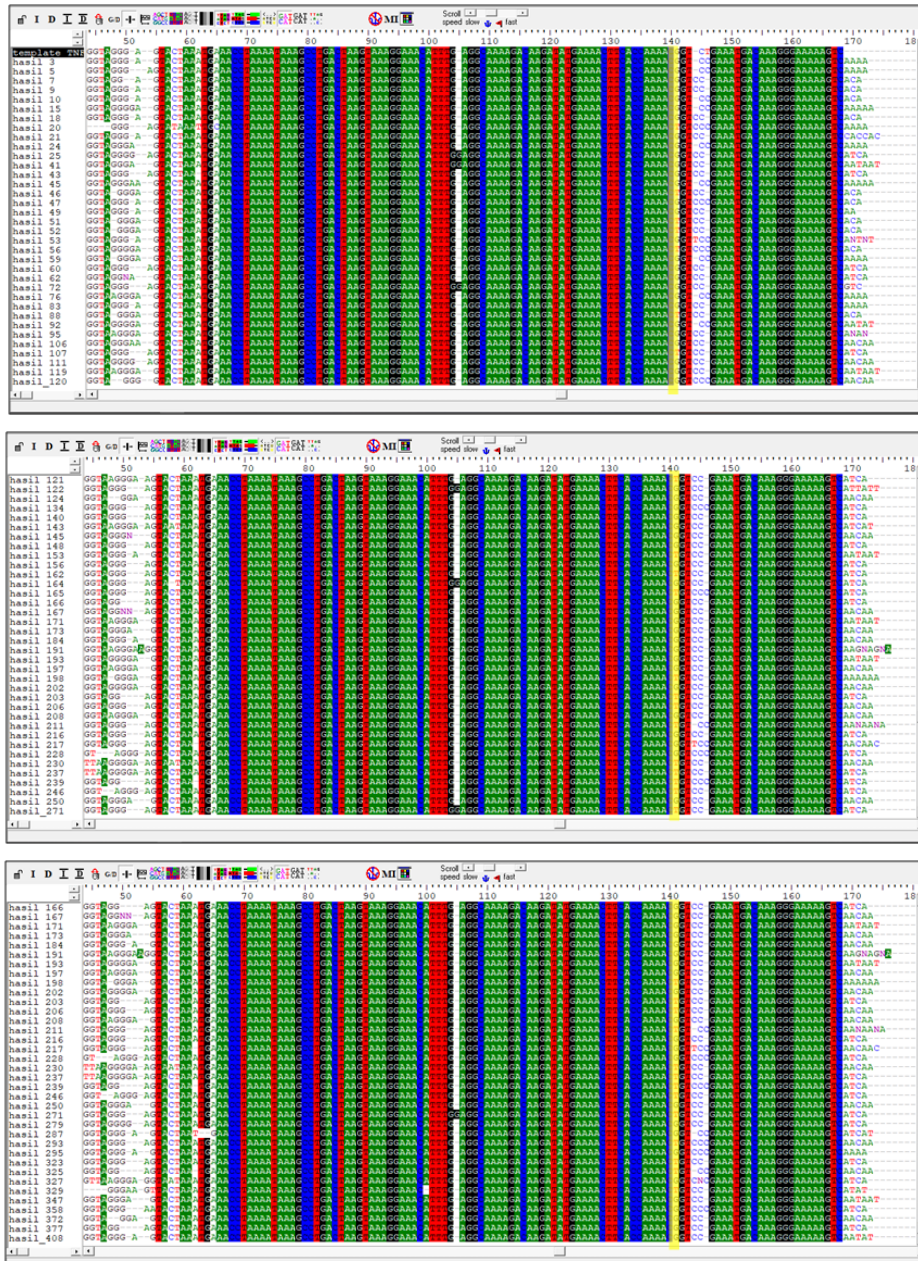


Fig. 2: Alignment results of DNA sequencing (highlighted with yellow is the mutation point/rs2205960)

The information about how to determine results that show the variations obtained can be seen in fig. 3-5.

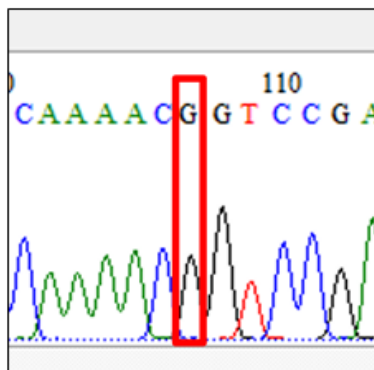


Fig. 3: DNA sequencing results that showed GG genotype

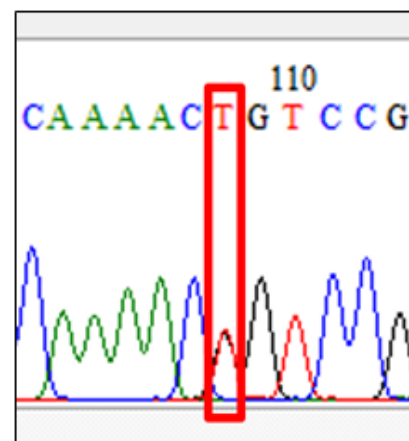


Fig. 4: DNA sequencing results that showed GT genotype

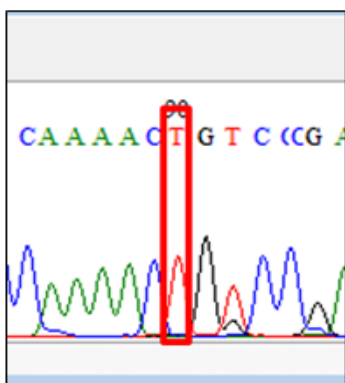


Fig. 5: DNA sequencing results that showed TT genotype

The results of DNA sequencing from 84 samples that were identified, 55 patients (65.48%) have wild-type GG genotype, 25 patients (29.76%) have GT genotype, and 4 patients (4.76%) have TT genotype (table 1).

DISCUSSION

Research on Chinese ethnicity SLE patients conducted by Chua *et al.* (2016) showed the distribution of the GG genotype was 50.9%, the GT genotype was 39.9%, and the TT genotype was 9.8%. The same study was also conducted on Malaysian patients with SLE, which showed GG genotype distribution of 65.14%, GT genotype 31.9%, and TT genotype 3.67% [12]. Asian SLE patients themselves are dominated by the wild-type GG homozygous genotype and the GT heterozygous genotype, while the TT mutant homozygous genotype frequency is still not widely found. The same thing was also found in a study conducted by Gupta *et al.* in 2018. The GG genotype was more common in SLE patients in India, which were 54.1%, while the GT mutant heterozygous genotype was 36.5%, and the TT mutant homozygous was only 9.4% [22]. However, different results were shown in the Latin American population. Research conducted by Moreno-Eutimio *et al.* (2020) showed the GT genotype was more prominent than other genotypes, which was 47.1% compared to the GG (30.4%) and TT (22.5%) [16]. The comparison between the results of this study and the genotypic distribution data in SLE patients obtained from other countries can be seen in table 4.

Table 4: Comparison of genotype and allele frequency on SLE patients based on race or ethnicity

TNFSF4 SNP	Genotypes and Allele	Chinese [10]	Malaysian [10]	North Indian [20]	Latin American [15]	Indonesian (Bandung)*
rs2205960	GG	50.9%	65.14%	54.1%	30.4%	65.48%
	GT	39.9%	31.19%	36.5%	47.1%	29.76%
	TT	9.8%	3.67%	9.4%	22.5%	4.76%
	T allele	29.5%	19.27%	27.7%	46.1%	19.64%
	G allele	70.5%	80.73%	72.3%	53.9%	80.36%

*In this research

Meanwhile, studies evinced the normal populations are dominated by GG genotype in Chinese (56.6%), Malaysian (66.67%), and Indian populations (60.5%) [12, 22]. Compared with the population of SLE patients, the normal population showed a greater number of GG genotypes, as can be seen in table 5. In the Chinese and Malaysian populations, a greater percentage of GT genotypes were present in

SLE patients compared to the normal population, and only the Chinese population showed a greater percentage of TT genotypes in SLE patients than in the normal population (9.8% and 6.1%, respectively) [12]. Another difference was found in the normal population in Latin America, where the GT genotype (46.3%) had a greater percentage than the GG genotype (37.6%) [16].

Table 5: Comparison of genotype and allele frequency on normal populations based on race or ethnicity

TNFSF4 SNP	Genotypes and Allele	Chinese [10]	Malaysian [10]	North Indian [20]	Latin American [15]
rs2205960	GG	56.6%	66.67%	60.5%	37.6%
	GT	37.3%	28.95%	34%	46.3%
	TT	6.1%	4.39%	5.5%	16.1%
	T allele	24.8%	18.86%	22.5%	39.2%
	G allele	75.2%	81.14%	77.5%	60.8%

The meta-analysis conducted study showed the T allele was associated with SLE in European and Asian ethnicities. This study also showed that the T allele is rare in populations originating from Africa, while European and Asian populations show a higher frequency of the T allele than the African populations. These differences reflect the genetic heterogeneity of SLE among the world populations [10]. Another meta-analysis was carried out by Wang *et al.* (2019) and Lee *et al.* (2012) stated similar results, which found a significant association between the T allele of rs2205960 and SLE in all European and Asian subjects. A significant association was also found in the TT genotype with SLE in the same study [12, 21]. The association of the T allele of the TNFSF4 gene rs2205960 with SLE in Hispanic and East Asian races was also found in a study by Manku *et al.* in 2013 [15].

Other research stated that the presence of T allele in both GT and TT genotypes correlates with higher expression of OX40L protein in the cell surface [8, 10]. The OX40-OX40L interaction regulates the expansion, differentiation, and activity of T regulatory (T_{reg}) cells in positive and negative ways depending on the condition or presence

of autoimmune disorders [24]. The presence of OX40-OX40L interaction causes inhibition of the formation of T_{reg} cells. Therefore, increased cell surface OX40L levels may lead to increased autoantibody and immune complex formation due to B cell proliferation and differentiation, as well as loss of immune tolerance with suppression of T_{reg} function and activation of T helper cells [23–25]. This mechanism contributes to formation of autoantibody and inflammation in SLE patients [26, 27]. It is also known that the T allele of TNFSF4 rs2205960 has a higher binding affinity for the NF-κB protein p65 compared to the G allele, indicating that the T allele has a regulatory effect on gene expression [15].

When associated with other SLE comorbidities, a case-control study showed a significantly higher increase in OX40L expression in patients with lupus nephritis compared to healthy controls and SLE patients without lupus nephritis manifestations [28]. This study also found a rise in serum creatinine levels, where high serum creatinine levels are often associated with kidney disorders [29]. A study conducted on SLE patients in China stated that a change in G>T at rs2205960 correlates with renal impairment and low C3 levels,

while low C3 levels are among the characteristics of glomerulonephritis due to immune deposition [30, 31]. This indicates that the *TNFSF4* gene has the potential as a marker of lupus nephritis and shows that certain genetic variants are associated with the development of different phenotypes in SLE [32, 33], though the detailed mechanism for its role in the pathogenesis of lupus nephritis is not known with certainty.

Gender differences are known to play a role in the development of SLE, with a higher number of cases being found in women than men. It has been observed that the disease has affected about 80-90% of women of all reported cases. These data suggest that hormones play an important role in the etiology of SLE [34, 35]. Hormones such as estrogen have anti-inflammatory and pro-inflammatory properties depending on the factors that influence them [36]. Estrogen is known to have a higher immune system reactivity in women and contributes to triggering autoimmune diseases, including SLE [34]. Consequently, only female subjects were used in this study.

This study indicates that SLE patients in Bandung have genotype variations (GG, GT, and TT), which showed the possibility that the *TNFSF4* gene rs2205960 influences susceptibility of SLE. However, these results need to be investigated further with a larger sample size and involving a healthy control group to ascertain the impact of the *TNFSF4* gene polymorphism rs2205960 as a risk factor for SLE.

LIMITATIONS

The limitation of this study is that there is a possibility of bias due to the *TNFSF4* gene polymorphism at rs2205960 also has associations with other autoimmune diseases, such as Vogt-Koyanagi Harada syndrome, systemic sclerosis, Sjögren syndrome, and primary biliary cirrhosis [19, 37, 38]. The involvement of patients with other autoimmune diseases is unknown due to limited clinical data and no records on comorbidities.

CONCLUSION

Based on this study, it can be concluded that the genotype distribution of the *TNFSF4* gene rs2205960 in SLE patients in Bandung showed three types of genotypes, namely GG, GT, and TT. From 84 DNA samples, 55 patients (65.48%) had a GG genotype, 25 patients (29.76%) had a GT genotype, and 4 patients (4.76%) had a TT genotype. Thus, the results of this study indicate a genotype distribution of the *TNFSF4* rs2205960 gene that fulfills the Hardy-Weinberg principle.

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AUTHORS CONTRIBUTIONS

AAK was responsible for data interpretation, statistical analysis, and article writing. GBK were responsible for sample collecting, sample selection, and performing genotyping analysis. EW, MSP, and DRF undertook data processing. RA supervised the writing process. LH and MIB supervised the project and revised the article.

CONFLICTS OF INTERESTS

The author(s) declared no potential conflicts of interest in this study.

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