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ASSOCIATION OF -129C/T PROMOTER GCLC POLYMORPHISM WITH GLUTATHIONE PLASMA LEVEL IN PULMONARY TUBERCULOSIS PATIENTS

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ABSTRACT

Genetic polymorphism of glutamate-cysteine ligase (GCL) could lead the changes in GCL enzyme activities. These changes could disrupt glutathione synthesis allowing the reduction of glutathione level. Furthermore, the decrease of glutathione level would change the phenotype and lead to the lack of defense against some diseases. The aim of this study was to determine the relationship between genetic polymorphism of the -129C/T promoter region in glutamate-cysteine ligase sub unit catalytic (GCLC) genes with glutathione plasma level in pulmonary tuberculosis patients. A prospective cohort study was conducted to study this association. The samples were obtained from the center of health lung community (BBKPM) and Labuang Baji Hospital, Makassar, Indonesia in accordance with the inclusion and exclusion criteria. The consecutive sampling technique was accomplished based on the order of arrival of the patient. Analysis of genetic polymorphism and glutathione level in pulmonary tuberculosis patients were performed. The results of study indicated that genetic polymorphism of C/C was increased by 11% in genotype C/C whereas in genotype C/T was decreased by 32%. According to the results, the polymorphisms of GCLC gene were associated with glutathione level in pulmonary tuberculosis patients.

Keywords: pulmonary tuberculosis, genetic polymorphism, glutamate-cysteine ligase, glutathione.

INTRODUCTION

Polymorphism in biology occurs when two or more clearly different phenotypes exist in the same population of a species. In other words, the occurrence of more than one form or morph, likewise, morphs must occupy the same habitat at the same time and belong to a panmictic population (one with random mating). Pulmonary tuberculosis (pulmonary TB) is an infectious disease that still becomes a high public health problem and causes the highest death toll in the developing countries including Indonesia. The main cause of this disease is by infection of *Mycobacterium tuberculosis*, an acid-fast (acid resistance), Gram-positive, and rod bacteria. The prevalence of TB in Indonesia in 2009 was about 520.000 individuals [1]. In 2020, TB is predicted to attack 1 billion persons with more than 70 million casualties, in case that the disease could not be controlled [2].

Some researchers have reported the administration of anti-tuberculosis drug to the pulmonary TB patients. The results suggested that the drug consequence in Reactive Oxygen Species (ROS) which might generate oxidative stress [3].

Mycobacterium tuberculosis infection might also lead the establishment of ROS by the immunity mechanism. In the pulmonary TB patients, the cellular immune system plays the role as the defense mechanisms against *Mycobacterium tuberculosis* infection [4]. This process involves macrophages as the active phagocytic cells to kill *Mycobacterium tuberculosis* bacteria.

The antimicrobial oxidative response by the active phagocytic cells occurs during the phagocytosis activity through the activation of NADPH oxidase and inducible nitric oxide synthase (iNOS) enzymes. NADPH oxidase would reduce the oxygen turns into free radicals. This process is called a respiratory burst. This process yields reactive oxygen and nitrogen species (ROS and RNS) [5-6]. Although this process is the essential part of the immune system against *Mycobacterium tuberculosis*, the excess amount of ROS generated can trigger the oxidative stress [7]. The oxidative stress in the pulmonary TB is the redox imbalance condition between oxidants and antioxidants in the lungs.

Glutathion plays a role as the main an antioxidant in protecting the lung cells from inflammation, as well as protecting the cells from the toxic effect of ROS and RNI. Glutathione has the direct antimicrobial effect by improving the immunity and inhibiting the growth of *Mycobacterium tuberculosis* [8-12]. The glutathione deficiency on pulmonary TB patients was suggested to interrupt the regulation of the immune cell function, and it might cause a failure to scavenge the ROS [9,13].

Glutamate-cysteine ligase (GCL) enzyme synthesizes the glutathione. GCL enzyme comprises of heterodimers consisting of the catalytic subunits (GCLC) and the modulator subunits (GCLM) [14]. The genetic variation (gene polymorphism) of GCL changes the function and activity of GCL enzyme. This changes cause the interference of the glutathione synthesis, allowing the lessening of glutathione level. By the reduction of glutathione level, the phenotype would show the weakness against some diseases such as hemolytic anaemia, cancers, myocardial infarction, diabetes mellitus, and HIV/AIDS

[15-19]. Also, GCL gene polymorphisms were associated with the drop of lung function [20-21]. The pulmonary tuberculosis patients with GCL gene polymorphisms were easier to get oxidative stresses [22]. This study aimed to reveal the association between the GCL gene polymorphisms with the low level of glutathione in the pulmonary tuberculosis (pulmonary TB) patients [23].

MATERIALS AND METHODS

Study population

Using an analytical observational study and prospective cross-sectional design, the pulmonary TB patients with acid-fast positive (+) were examined by the Institute for Health Lung Society (BKPM) and Labuang Baji Hospital during the study period. The sample numbers were determined by the cohort design of study formula [24]. The group consisted of 103 TB patients without accompanying diseases such as diabetes, coronary heart disease, hypertension and cancer. The examination was carried out from February 2013 to October 2013. The sampling method was followed to the "first come, first served" policy. The patients who visited BKPM and Labuang Baji Hospital during the study period which were considered to meet the criteria were selected (consecutive sampling from admission).

Acid-fast analysis of sputum

The sputum samples of 103 pulmonary tuberculosis patients were collected. The Ziehl-Nielsen (acid-fast) staining technique was then used to check the sputum. This method was used to examine the existence of acid-resistant bacteria in sputum before and after anti-tuberculosis medications.

Analysis of glutathione level

Three ml amount of perifer blood samples from pulmonary tuberculosis patients were analyzed for glutathione level using enzyme-like immune assay (ELISA) reader, Glutathione Kit (Cubios Lab, USA).

Analysis of GCL gene polymorphism

The polymorphism was investigated through several steps as follows: the DNA isolation and purification (Chelex method), qualitative and quantitative measurement of DNA, the GCL gene amplification using polymerase chain reaction (PCR) followed by DNA cutting. The amplification of GCL gene was performed using GCLC primer pair (F: 5'-TCGTCCCAAGTCTCACAGTC-3'; R: 5'-CGCCCTCCCCGCTGCTCCTC-3'). The restriction enzyme used was Tsp451. The PCR-RFLP results were further investigated by 2% agarose gel electrophoresis. In order to find the genetic variation differences between C/C genotype and C/T genotype of GCL gene among the samples, a Chi-Square (χ^2) statistical analysis was assisted by SPSS 12 for Windows application program [25]. The significance value was $p < 0.05$, with the confidential level of 95%.

RESULTS

A number of the female subjects (60 individuals, 58.3%) were slightly equal to the male subjects (43 individuals, 41.7%).The age of patients in the case group were ranging from 15 to 60 years old with an average of 59.16 ± 11.62 years old, whereas the range of the patient age in the control group was 22-60 years old with an average of 50.76 ± 14.56 years old.

The analysis of sputum before and after anti-tuberculosis medications is presented in Table-1. Data of sputum analysis shows that in C/C genotype group, 66 patients were positive to have acid-resistant bacteria before treatment whereas 61 patients were converted to be negative after treatment of anti-tuberculosis medications. However, 5 patients were still remaining positive after treatment. Moreover, in C/T genotype 37 patients were positive before medications, whereas 8 patients were converted to be negative after treatments and 29 patients were still remaining positive even after medications.

A series of research activities was carried out to understand the genetic variation of glutamate-cysteine ligase (GCL) enzyme, which catalyzes the synthesis of glutathione.

The results of glutathione level analysis showed that in the beginning, the average level of glutathione in pulmonary tuberculosis patients at GCLC gene of C/C genotype was 0.0589 ± 0.0402 mM higher than C/T genotype which was 0.0326± 0.0192 mM. At the end, the average level of glutathione GCLC gene of C/C genotype 0.0654±0.0389 mM, also higher than the average glutathione level of G/T genotype which was 0.0260± 0.0221 mM. The data of average glutathione level are shown in Table-2.

The statistical analysis using *Mann Whitney* test showed that there was a significant difference between the initial glutathione levels as well as the final glutathione levels of C/C genotype with C/T genotype (p value < 0.05). Statistically, C/C and C/T genotypes contributed differentially in the initial along with final glutathione level. The polymorphisms of GCLC gene in C/T genotype indicated the lessening of glutathione level to 32%. Whereas in C/C genotype, there was no polymorphism found at the GCL gene, the glutathione level increased to 11%.

Table-1. Results of sputum analysis before and after anti-tuberculosis medications.

Variable	Genotype	Before medications			After medications		
		(+++)	(++)	(+)	(++)	(+)	(-)
	C/C	6	10	50	-	5	61
GCLC gene		9%	15%	76%		7.6%	92.4%
	C/T	7	10	20	7	22	8
		19%	27%	54%	19%	59%	22%

Table-2. The average glutathione level of pulmonary tuberculosis patients.

Variable	Genotype	Glutathione level (mM)*					
		Initial glutathione level			Final glutathione level		
		Mean	SD	P	Mean	SD	P
	C/C	0.0589	0.0402	0.000	0.0654	0.00389	0.000
Gen GCLC	C/T	0.0326	0.0192		0.0260	0.0221	

*The values indicate the significance at or above 95% confidence level

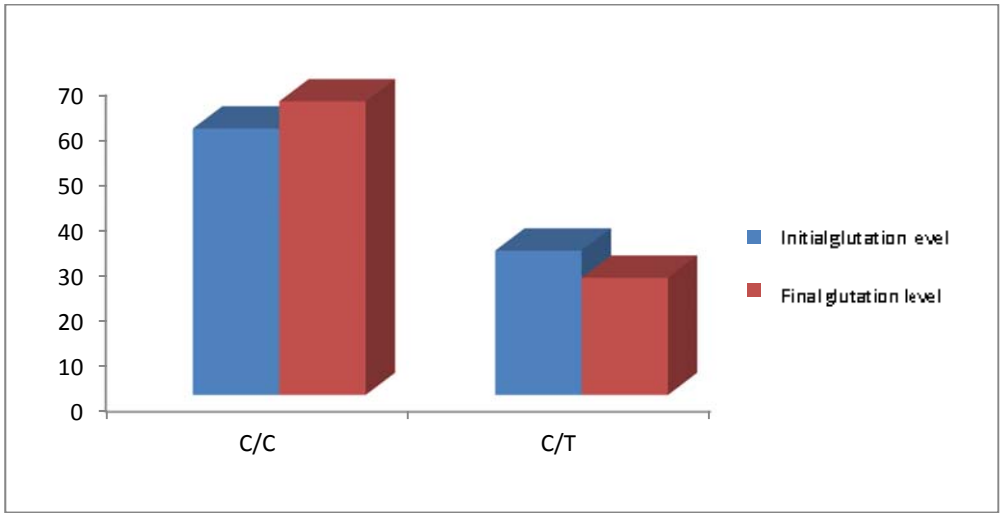

Figure-1. The initial and final glutathione levels of GCL gene polymorphisms in C/C and C/T genotypes.

DISCUSSIONS

Glutathione is synthesized from two amino acids, i.e. cysteine and glutamate in which the process is catalyzed by glutamate-cysteine Ligase (GCL) enzyme to form a glutamyl-cysteine complex [14]. Furthermore, the glutamyl-cysteine complex is converted to glutathione (GSH) in a process catalyzed by GSH synthetase enzyme. Glutamate-cysteine ligase is an enzyme synthesizing GSH in the initial reaction, whereas GSH synthetase does not play a role in the regulation of GSH synthesis [26].

When a cell is exposed to oxidant, the oxidative stress happens subsequently. This circumstance leads the GSH level to decrease, and the GCL gene expression will be regulated by the response element activation. The

regulation aimed to against the oxidative stress on the promoter area. This could initiate the synthesis of GSH, and then act as the defender or adaptation mechanism against the oxidative stress [26]. Therefore, the presence of the GCLC gene polymorphisms would possibly cause the reduction in the response against the oxidative stress. This would cause the decrease in the intracellular GSH production, which might reduce the response against the oxidative stress. As a result, the susceptibility to the induction of the oxidants will increase and lead to damage the tissues. These steps are included in the pathogenesis parts of pulmonary tuberculosis.

In some research on the GCL gene and its role in various related diseases, many single-nucleotide polymorphisms have been identified in the human GCL gene promoter. This gene has also been correlated with the increase of the susceptibility against various diseases due to oxidative stress. Sieldinski *et al.* (2008) have studied the GCL genotype and concluded that there were differences in the GCL allele distribution based on the ethnicity and the people background. The people with relatively high proportion of GCL allele would have a greater possibility to increase the predisposition on the incident of many chronic metabolism diseases, degenerative diseases, inflammation and autoimmune diseases [20].

Another research related to GCLC gene according to Koide *et al.* (2003) who found that -129T polymorphism on GCLC gene could suppress the induction of GCLC gene against the oxidant response [16]. The polymorphism could imply on the endothelial dysfunction of coronary vasomotor and myocardial infection. Other studies reported that the polymorphism of GCLC gene was related to the severity of lung fibrosis [21]. Additionally, there was the relationship among the polymorphism of GCLC gene with the smokers and the low level of the vitamin C intake which contribute on the oxidative stress [20]. Finally, Wang *et al.* [17] stated that there was an association between the expressions of mRNA from GCL gene with the hypersensitivity of the sulfamethoxazole induction on HIV patients by GCL.

CONCLUSIONS

The genetic variation of glutamate-cysteine ligase (GCL) enzyme could be used as the biomarker at the molecular level to detect the presence of oxidative stress on pulmonary TB patients effectively. Moreover, polymorphisms of GCL gene in GCLC subunits were associated with the glutathione level of pulmonary tuberculosis patients. The polymorphisms of GCL gene indicated the lessening of glutathione level.

To date, the bibliographical study has not found any study reporting the polymorphism of GCLC genes on pulmonary TB in Indonesia. The current study has a limitation in the focus on the polymorphism of GCLC genes. It was suggested to conduct further research to find the exact loci of the GCLC genes using a sequencing process. This study would be beneficial to see whether the different location of the GCL gene at a particular position plays a role as the risk factor of pulmonary TB in Indonesia. A further advanced study with a larger sample and the more diverse ethnical people is needed to confirm this issue.

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