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Research Article
Polymorphism of Glutamate-Cysteine Ligase Subunit Catalytic (GCLC) Gene in Pulmonary Tuberculosis Patients

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Abstract

Background and Objective: The biomarker of oxidative stress in pulmonary tuberculosis patients has not been found. Oxidative stress occurs due to the low level of antioxidants. Single nucleotide polymorphism of glutamate-cysteine ligase subunit catalytic (GCLC) gene namely -129C/T GCLC has been reported to have an association with a risk factor of oxidative stress' susceptibility. The Objective of this study was to determine the GCLC polymorphism in pulmonary tuberculosis (TB) patient. **Materials and Methods:** Blood samples of 225 pulmonary TB patients were taken from the central public health in Semarang city. The genetic test was carried out using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The isolation of DNA from 225 blood samples was performed using DNA extraction kit (Promega DNA purification kit) following the manufacturing procedure. The amplification of GCLC fragment was performed by a master mix from Thermo Scientific. Data was analyzed descriptively. Statistical analysis was performed by Chi-square test. **Results:** The results showed the existence of polymorphism-129C/T in the 5'-flanking region of GCLC genes. The frequency of C/C and C/T genotype were 63.6 and 36.4%, respectively. The C/T gene in the GCLC -129C region is a T gene promoter. There was a significant different between C/C and C/T frequencies with the value of significance of $p = 0.000$ ($p<0.05$). **Conclusion:** Therefore it was concluded that the frequency of C/T polymorphism genotype of GCLC gene in patients with pulmonary tuberculosis is 36.4%.

Key words: Gene polymorphism, GCLC gene, GCL enzyme, pulmonary TB, glutathione, -129C/T GCLC

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Glutamate cysteine ligase (GCL) catalytic subunit is a monomer of GCL gene. Glutamate cysteine ligase is an enzyme that catalyzes the formation of glutathione (GSH). Glutathione is one of the antioxidants that play a role in the regulation of the immune system and direct antimicrobial effect by improving the immunity and inhibiting the growth of *Mycobacterium tuberculosis*[1-3].

Gene polymorphism in enzymes that synthesize glutathione affects, glutathione levels[4,5] and is associated with impaired lung function[6,3,7].

Glutathione (γ-glutamylcysteine glycine) is a tripeptide composed of glutamate, cysteine and glycine. Glutathione synthesis is through two stages, each catalyzed by a different enzyme. In phase I, the formation of the dipeptide γ-glutamyl cysteine is catalyzed by the enzyme, glutamate-cysteine ligase (GCL). The II phase involves glutathione synthesis from γ-glutamyl cysteine and glycine, a reaction catalyzed by the enzyme glutathione synthetase (GSS)[8]. The enzymes that synthesize glutathione are genetically expressed by the sequence of genes that make up a protein enzyme. GCL enzyme has two components in each of its heterodimer structure, that is, the catalytic subunits (GCLC) and the modulator subunits (GCLM). GCLC and GCLM genes encode both subunits[9].

Polymorphism occurs when two or more clearly different phenotypes are present in the same population of a species. In other words, polymorphism is the presence of more than one form of morphism in the same habitat at the same time. Usually, adaptation and genetic variation are used to store various morphisms in a population living in different environments.[10] Hence, polymorphism is the result of an evolutionary process and can be genetically inherited and modified by natural selection.

Several studies suggest that genetic polymorphisms in GCLC and GCLM genes are associated with low levels of reduced glutathione (GSH) *in vitro* and lead to susceptibility to certain diseases[11-15]. Polymorphisms resulting from the expression and enzyme activity of GCL were significantly reduced and phenotype shows the severity of the disease. Research conducted by Yuniastuti and Dewi[16] found that there is GCL gene polymorphism in pulmonary TB patient at 30%. TB patients with suspected GCL gene polymorphism are more susceptible to oxidative stress, thus exacerbating the disease. So, this study was conducted to analyze the polymorphism of glutamate-cysteine ligase subunit catalytic gene (GCLC) in pulmonary tuberculosis patients.

MATERIALS AND METHODS

This study was conducted on March-November, 2013. The grading of solution used in this study was commercial grading. Materials used in this study were: Peripheral blood sample of pulmonary TB patients, $MgCl_2$, methanol+BHT 0.05%, Chloroform: Methanol+BHT+TPP, ethyl acetate, heptane, deionized water pH 3, KOH 15%, PBS (Phosphate Buffer Saline) 1×1 mL, Chelex 20% 50 µL, sterile water, Hot Star Master Mix Kit Cat Number 203443, primer GCLC forward 5'TCGTCCCAAGTCTCACAGTC-3' reverse 5'CGCCCTCCCCGCTGCTCCTC-3', *Tsp451* enzyme with cutting region at 5'⋯.-▼GTSAC⋯3' with complement side at 3'⋯CASTG▲⋯5'.

Sample collection: Blood samples were obtained from the center for central public health in semarang by consecutive non-probability sampling[17].

All patients who fulfilled the inclusion and exclusion criteria, were willing to participate in the study and signed the informed consent form were recruited as research subjects. The cohort consisted of 225 patients with pulmonary tuberculosis.

DNA extraction: The first step of DNA examination was DNA extraction process. The method of DNA extraction used was by Chelex[18].

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP): Genomic DNA was isolated from the leukocyte-rich buffy coat layer of peripheral venous blood using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA). DNA fragments of the GCLC were genotyped for the GCLC polymorphisms by PCR and amplified with the following primers, (forward) 5'TCGTCCCAAGTCTCACAGTC-3' and (reverse) 5'CGCCCTCCCCGCTGCTCCTC-3'. PCR was performed in a 25 µL reaction mixture, containing 10X PCR buffer, KAPA 2G robust hot start ready mix 12.5 µL, 10 mmol of each dNTP, ddH_2O 7.5 µL, primer forward 1.5 µL, primer reverse 1.5 µL, 3 U μL^{-1} of Taq polymerase and 2.0 µL of genomic DNA sample. The cycling conditions were pre-denaturation at 94°C for 5 min., denaturation at 94°C for 30 sec., annealing at 56.1°C for 45 sec., extension at 72°C for 2 min and a final extension at 72°C for 5 min. The cycle was repeated for 35 times. The Magnitude of the expected PCR products was 613 base pairs (bp).

The PCR fragments of the GCLC genes were analyzed by RFLP, with digestion of the fragments with *Tsp451* (Thermo Scientific, Lithuania) restriction enzyme, which was 5'...-▼

GTSAC ... 3' with the complement of the 3 '... CASTG ▲ ... 5'. Ten µL of PCR product was digested with 1 µL of FastDigest *Tsp451* restriction enzyme at 37°C for 5 min. The restriction fragments were subjected to electrophoresis on 2% agarose/ethidium bromide gel in 1× TBE buffer (0.09 M Tris-boric acid and 0.002 M EDTA). Gels were visualized under UV light and documented in Molecular Imager apparatus (BIO-RAD). The cleaved fragments were separated by electrophoresis on 2.0% agarose gel in 1× TBE buffer (89 mM tris-HCl, 2.5 mM EDTA and 89 mM boric acid pH 8.3) containing 0.05 µg mL^{-1} ethidium bromide at 75 V for 2 h.

PCR-RFLP amplification of GCLC gene: After the process of amplification, the electrophoresis was conducted using agarose gel 1.2%. It was made by dissolving 1.2 g of agarose into 100 mL 0.5X buffer TBE in Erlenmeyer flask which subsequently was heated until it became transparent. Then it was cooled until a lukewarm condition. Five µL of ethidium bromide (10 mg mL^{-1}) was added to the solution then it is molded using the tray that has been prepared before. Then the gel was put in an electrophoresis tank. 0.5x buffer TBE (108 g Tris base, 55 g Borate and 40 mL EDTA 0.5 M) was poured into the tank up to about 1 mm above the surface of the gel. After that, 3 µL PCR products were added to gel's sink. Electrophoresis tank was closed down and connected to the power supply at 100 V for 30 min. The result of PCR product's electrophoresis was observed using a gel doc[18]. The bands were visualized under ultraviolet light in a gel doc system (Thermo Scientific).

Statistical analysis: Chi-square was used to examine the difference between the wild type and the polymorphism gene of GCLC gene. Statistical analysis program used was Social Science (SPSS) software (version 20.0 for Windows, IBM Corp., NY, Armonk) with confidence interval at 95% and significance value at $p \leq 0.05$ [19].

RESULTS

Molecular approaches were conducted using the polymerase chain reaction-fragment length polymorphism (PCR-RFLP) method. The restriction site of enzyme *Tsp451* lies outside the recognition site, 5' to the G residues in each strand. PCR-RFL results of GCLC gene cut by the restriction enzyme *Tsp451* shows the shape of the genotype (Fig. 1). The differences of nucleotide were found at -129 promoter regions of genes GCLC. According to the sequencing analysis, the sequences GTGAC corresponded to the promoter region of GCLC Gene Bank. Since GTCAC turned into this position, it was recognized by the restriction enzyme *Tsp451* as a cleavage site.

The -129C/C, -129C/T and -129T/T genotype frequencies were then examined by the Chi square test. The result showed that there was a significant different between C/C and C/T frequencies with the value of significance of p = 0.000 ($p<0.05$). Hereafter, the calculation of allele frequencies was performed in order to get the proportion or percentage of the C or T allele on the loci of GCLC gene. The result of the allele percentage calculation can be used to determine the characteristic of the loci. A locus can be said as a polymorphic locus if the highest allele frequency is less or equal to 0.95. In the other hand, a locus can be said as a monomorphic locus if the highest allele frequency is more than 0.95.

Chisquare test results indicated that there were significant differences between the C/C genotype with C/T genotype of

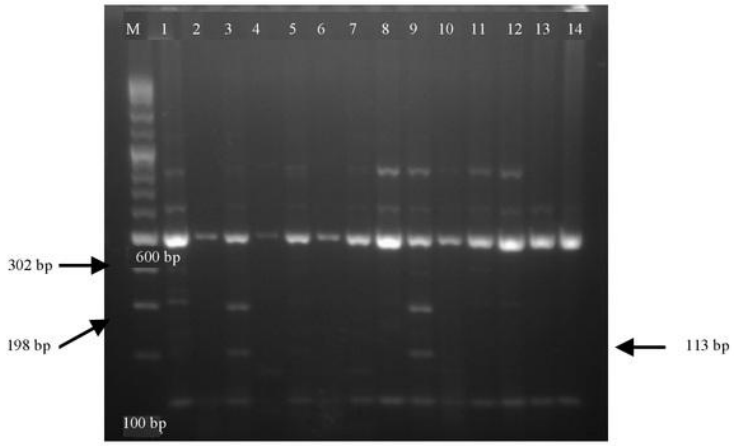

Fig. 1: Electrophoresis of PCR-RFLP products of GCLC gene

Table 1: C and T allele frequency of GCLC gene

	Genotype				
Indicator	CC	CT	TT	Total	Frequency of gene
Number of individual	143	82	0	225	
Number of C gene	286	82	0	368	368/450 = 0.82
Number of T gene	0	82	0	82	82/450 = 0.18
Number of gene total	286	164	0	450	

Table 2: Expectation and real gene frequency

		Number of individual = 225	
Genotype	Frequency of expectation genotype	Expectation	Reality
CC	$p^2 = (0.82)^2 = 0.67 \times 225$	150.75	143
CT	$2pq = 2(0.82)(0.18) = 0.295 \times 225$	66.38	82

GCLC gene with a significance p-value of 0.000 ($p<0.05$). Furthermore, in order to determine the proportion of allele C or T in GCLC gene loci, the allele frequency calculation was performed (Table 1). The allele frequency calculation results were used to determine the nature of the loci alleles located. A locus is said to be polymorphic if the frequency of an allele is similar to or less than 0.95. However, a locus is said to be monomorphic if its allele has a greater frequency than 0.95.

The results of the C allele frequency calculations in this study were 82 and 18% T allele, thus is polymorphic loci. The proportion of polymorphic loci in a population is often used as an index of genetic diversity. In other words, the C allele contributes 82% to the rest of the GCLC gene activity while the role of T allele is 18%. Furthermore, the predicted genotype frequency expectations from the real genotype frequencies are given in Table 2. Given in a sexually reproducing population, each member of the population is expected to conduct random mating. In this kind of arrangement, each genotype and phenotype have the opportunity to meet one another and be passed on to the next generation.

DISCUSSION

Polymorphism -129 C/T in GCLC gene pulmonary TB patients are likely to respond differently to oxidative stress and OAT therapy when compared with patients with no GCLC polymorphism gene. If there is a polymorphism in pulmonary TB patients, such patients are susceptible to oxidative stress which manifests on the severity of pulmonary TB diseases such as pulmonary fibrosis and lung cancer. Polymorphism -129 C/T suppress the increase in GCLC gene expression in response to oxidative stress and it is likely to weaken the activity of the enzyme to synthesize intracellular GSH in response to oxidative stress. Liu *et al.*[20] reported that polymorphisms -129/T in the promoter region of genes GCLC induce different responses to oxidants and reduce the production of glutathione. This leads to lower antioxidant capacity in lung cells and lead to increased susceptibility to oxidant injury in chronic obstructive pulmonary disease (COPD).

Glutathione is synthesized from its constituent amino acids in two sequential by GCL and GSH synthetase[9]. Glutamylcysteine, synthesized by GCL is rapidly converted to GSH by GSH synthetase. Glutamate-cysteine ligase is the rate-limiting enzyme in GSH synthesis, whereas GSH synthetase had apparently no regulatory role. When cells are challenged with sublethal oxidative stress or GSH depletion, GCLC gene expression was regulated through upward activation of oxidative stress-responsive elements in the promoter regions. This leads to GSH synthesis and provides a protective/adaptive mechanism against oxidative stress[15].

The previous study showed that the -129C/T polymorphism may suppress the increase of GCLC gene expression in response to oxidative stress[20]. The existence of -129C/T polymorphism found in this study indicated that it is also possibly weaken the intracellular production of GSH in response to oxidative stress, leading to an increase in the susceptibility to oxidant-induced susceptibility, *M. tuberculosis* and pulmonary injury, which were thought to occur as a part of TB diseases severity. The nuclear protein bound more strongly to the sequence with the C allele may be an activator of transcriptional activity. Although there is no putative enhancer element that contains the -129 position on the computer-based data research, there are several binding sites for nuclear proteins (that is, CCAAT binding protein, near the -129C/T polymorphic site). Thus, it is possible that the -129T allele might modify the binding of nuclear proteins to unidentified cis-elements around the -129 position, leading to suppression of GCLC gene expression.

In this study, the analysis of the genetic variation of GCLC genes as the indicator for the presence of oxidative stress in the pulmonary TB patients was also conducted. Moreover, the genetic variation plays a role in the severity of the pulmonary TB diseases, for example, the development of the pulmonary fibrosis as well as the pulmonary cancer. The genetic variation of GCL enzyme in pulmonary TB patients needs to be investigated to distinguish the difference of individual's genetic traits under the same conditions. The patients were infected by *M. tuberculosis* nevertheless, they gave different responses to acid-resistant bacteria changes after anti-TB drug therapy. Based on the results of this study supported with the result of study by Liu *et al.*[20], it was proven that GCLC gene polymorphism is a risk factor for susceptibility to oxidative stress and severity of pulmonary TB.

CONCLUSION

The frequency of C/T genotype in GCLC gene is 36.4%. This can be used as a basic knowledge for adding antioxidants (Vit C, Vit E, etc.) in the pulmonary TB treatment. The addition of antioxidants is useful to maintain the levels of antioxidants in the body and prevent the oxidative stress.

SIGNIFICANCE STATEMENT

This study discovers the existence of -129 C/T GCLC as the GCLC gene polymorphism in pulmonary TB patients which indicates the low level of glutathione in their body. The result of this study can be beneficial for informing the medical personnel that the provision of antioxidant is needed in pulmonary TB treatment. This study will help the researchers to uncover the critical areas of pulmonary TB treatment from the molecular analysis that many researchers were not able to explore. Thus, a new theory on others supporting treatment for pulmonary TB patients, may be arrived at the pulmonary TB treatment.

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