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The role of gene polymorphisms of glutamate-cysteine ligase catalytic (GCLC) enzyme against antioxidants and oxidative stress status of Individual who had contacted infectious tuberculosis

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ABSTRACT

Aims: Glutamate cysteine ligase (GCL) enzyme is involved in the synthesis of glutathione, which functions as an antioxidant. Polymorphisms in the sequence of amino acids making up the gene GCLC will cause differences in enzyme expression and GCLC activity. Gene expression that is influenced by oxidative stress can be used to measure markers such as F₂-isoprostanes. This study aims to examine the association between the polymorphism in the GCLC gene with glutathione plasma level and F₂-isoprostanes in contacts of person with infectious tuberculosis (TB).

Methodology and results: Samples are taken from the family members of pulmonary TB patients who seeks treatment at the Pulmonary Centre (Lung Health Center for Public = BBKPM) and Polyclinic of Dr Wahidin Sudirohusodo Hospital, Makassar. Total of approximately 4 mL of venous blood are taken from each person with pulmonary TB contacts and further analyzed using genomic PCR-RFLP method and ELISA. Our results described that contacts of person with infectious TB for approximately 6 months have polymorphism C/C genotype at 80.3%, C/T of 18.3% and T/T for 1.4% of the total 71 samples with high levels of glutathione from 0.167 to 0.548 mM/mL and F₂-isoprostanes level 72.4 - 1343.9 pg/mL.

Conclusion, significance and impact of study: There are no significant association between GCLC gene polymorphism with glutathione and F₂-isoprostanes levels of individual who had contacted infection TB. In this study the elevation of F₂-isoprostanes equal to the decrease levels of glutathione.

Keywords: pulmonary TB contacts, GCLC gene, glutathione levels, F₂-isoprostanes

INTRODUCTION

Currently, pulmonary tuberculosis (TB) is not merely described as an acute infectious disease but widespread in chronic infectious diseases, given the occurrence of several cases of anti-tuberculosis drug resistance in patients with TB, known as MDR / Multidrug resistance (resistance to several kinds of anti-tuberculosis drug). Some researchers reported that administration of the anti-tuberculosis drug in patients with suspected TB yields a wide range of Reactive Oxygen Species / ROS (free radicals). Elevated ROS level can cause oxidative stress (Chowdhury *et al.*, 2001; Wiid *et al.*, 2004; Tostmann *et al.*, 2007). TB oxidative stress is a state of the redox imbalance between oxidants and antioxidants in the lungs.

Oxidative stress are measured by assessing the biochemical marker such as malondialdehyde (MDA), oxidized low-density lipoprotein (Ox-LDL) and F₂-isoprostanes (Milne *et al.*, 2011). F₂-isoprostanes is a

product of lipid peroxidation formed by non-enzymatic and can be measured in blood, urine or tissue (Montuschi *et al.*, 2004). Lipid peroxidation is an oxidative damage to lipid biomolecule by ROS. F₂-isoprostanes is currently regarded as a reliable biomarker of oxidative stress in vivo due to its stability and high specificity, and how it is conveniently measured (Janicka *et al.*, 2010; Milne *et al.*, 2011).

The defense mechanism of host body against infection of *Mycobacterium tuberculosis* (MTB) is one of the causes of ROS. The cellular immune system serves as the self-defense mechanism against MTB infection in patients with TB. Oxidative antimicrobial response from phagocytic cells actively occurs during the phagocytosis via activation of NADPH oxidase. NADPH oxidase reduces oxygen into free radicals, a process that is known as respiratory burst (Voskuil *et al.*, 2011). Elevating ROS

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level, as well as the provision of anti-tuberculosis drug and improvement of the immune system in patients with TB will increase the use of endogenous antioxidants to neutralize ROS. When the detoxification of endogenous antioxidant capacity remains or decrease, it results to oxidative stress (Akiibinu *et al.*, 2011). Kaur *et al.* (2005) state that the decrease in antioxidant levels in TB patients happens due to an increase of ROS.

Glutathione (GSH) is one of the antioxidants that involved in the regulation of the immune system, acting as a major component in the respiratory burst (Seres *et al.*, 2000), protects lung cells from inflammation, protecting cells from the toxicity of ROS and directly acts as antimicrobials to boost the immune system and inhibit the growth of MTB. It also controls the intracellular growth of MTB in macrophages, having antimicrobial activity that acts as a carrier of NO, and as an effector molecule in cellular immunity to the body's defense against MTB infection (Venketaraman *et al.*, 2005; Dayaram *et al.*, 2006; Connell and Venketaraman, 2009).

Several studies have reported that the strain of MTB is sensitive to the antioxidant glutathione (Venketaraman *et al.*, 2005; Dayaram *et al.*, 2006). Glutathione can affect cell proliferation, prevent lipid peroxidation of unsaturated to neutralize ROS, an important process in the defense against bacterial infections MTB (Connell and Venketaraman, 2009). Glutathione synthesis regulated through two stages, each catalyzed by different enzymes. Phase I is the formation of the γ -glutamylcystein dipeptide of glutamic acid and cysteine which catalyzed by the enzyme glutamate-cysteine ligase (GCL). Phase II is the glutathione synthesis of γ -glutamylcystein and glycine which catalyzed by the enzyme glutathione synthetase (GSS). The enzymes that synthesize glutathione are genetically expressed by the sequence of the genes that make up a protein enzyme. Glutamate cysteine ligase (GCL) consists of a catalytic subunit encoded by the gene modifier GCLC and subunits encoded by GCLM genes.

Glutamate cysteine ligase catalytic (GCLC) is one of the genes that are important for enzyme catalysis, protein synthesis, allowing responses and protection against oxidative stress (Koide *et al.*, 2003). Polymorphism resulted GCL enzyme expression and activity are significantly reduced. Moreover, phenotype indicates the severity of the disease, such as myocardial infarction, cancer, diabetes and HIV / AIDS (Koide *et al.*, 2003; Wang *et al.*, 2012). The GCL gene polymorphism and the level of glutathione and F₂-isoprostanes in the TB patients and normal individuals have been described (Nwanjo and Oze, 2007; Yuniastuti *et al.*, 2013), but for the contacts of person with infectious TB have never been reported. That is why in this study we want to examine the association between the polymorphism in the GCLC gene with glutathione plasma level and F₂-isoprostanes in contacts of person with infectious TB. Thus, it is expected that transmission of pulmonary TB disease may be recognized earlier before going on a broader contagion.

MATERIALS AND METHOD

Research population

The population in this study were all contacts of person with infectious TB who have TB patient in their house who visited Pulmonary Health Center Society (BBKPM) and the Polyclinic in Dr. Wahidin Sudirohusodo Hospital, Makassar, Indonesia, for treatments.

Research samples

Samples in this study were blood specimen taken from contacts of person with infectious TB (approximately 6 months) who met the inclusion and exclusion criteria and willing to participate in this study, proven by signing the written informed consent.

Extraction of DNA from Blood

The process of Genomic DNA extraction procedure is conducted using the mini kit for blood (Geneaid) with several modifications. Total of 200 μ L vacuocyte blood sample in EDTA tubes taken and added in eppendorf tube then mixed with 20 μ L proteinase K (vortex for 15 min and incubated at 60 °C for 10 min). At the end incubation, 200 μ L of ethanol absolute was added and vortexed for 10 sec. The sample was transferred into the GD column and centrifuged at 16000 \times g for 2 min. The supernatant was discarded, 600 μ L washing buffer was added and centrifuged at 16000 \times g for 30 sec. After thorough drying, the GD column was transferred into a new eppendorf tube. Next, we added 100 μ L of elution buffer that has been heated right in the matrix and incubated for 5 min. After a centrifugation step at 16000 \times g during 30 sec, the results of DNA elution in eppendorf tube are ready for PCR.

GCLM gene amplification

Two oligonucleotide primers were used to amplify a 613 bp fragment of the GCLC promoter (forward: 5'-TCGTCCCAAGTCTCACAGTC-3') (reverse 5'-CGCCCTCCCCGCTGCTC CTC-3') (Koide *et al.*, 2003). PCR amplification was conducted in 25 μ L of a reaction mixture containing 12.5 μ L HotStar MMX, 0.5 μ L of each primer, 6.5 μ L nuclease-free water and 5 μ L of extracted DNA. The amplification conditions included an initial denaturation at 95 °C for 15 min, denaturation at 95 °C for 1 min, annealing at 67 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 15 min followed by 35 cycles.

RFLP and electrophoresis

The result of PCR product from GCLC gene with 613 bp length was cut with the addition of Tsp45I restriction enzyme by adding PCR products and then incubated at 65 °C for 1 h. After incubation, the PCR product is qualified for electrophoresis using 2% agarose gel that contains ethidium bromide. Further electrophoresis

results are observed under UV light. Positive results GCLC genes is indicated by the formation of two bands of 500 bp and 113 bp for C/C allele, 4 bands of 500 bp, 113 bp, 198 bp and 302 bp for C/T allele, 3 band along 113 bp, 198 bp and 302 bp for T/T allele (Koide *et al.*, 2003).

Biochemical examination

The Examination of glutathione levels and F₂-isoprostanes was performed using ELISA cusabio kit by comparing to the normal levels of glutathione of 1-10 mM (Hamilton *et al.*, 2003) and F₂-isoprostanes of 10-70 pg/mL (Montuschi *et al.*, 2004).

Data analysis

The results of GCLC RFLP test are served to compare the levels of glutathione (GSH) and F₂-isoprostanes levels of contacts of persons with infectious TB. The obtained data are tested using Chi-square statistical analysis and Pearson correlation.

RESULTS

RFLP-PCR of GCLC gene

Amplification of GCLC gene using specific primers shown positive results and was detected in the band with a size of 613 bp (Figure 1).

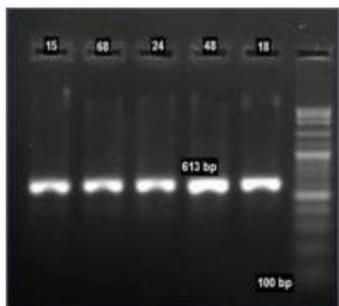


Figure 1: The results of electrophoresis of PCR products of GCLC genes in samples of contacts with infectious TB.

The results of cutting the DNA using restriction enzymes at the Tsp45I 5' GTSAC 3' site with complementary side 3' CASTG 5' using PCR-RFLP showed that: Genotype C/C cut DNA segment into two parts consist of Segment 500 bp and 113 bp. Genotype C/T cut into 4 sections of DNA segments consist of segments of 500 bp, 302 bp, 198 bp and 113 bp. Genotype T/T to cut a segment of DNA into 3 parts consisting of segments 302 bp, 198 bp and 113 bp (Figure 2).

In this study, from the total of 71 samples, the distribution of GCLC gene polymorphism shown 57 (80.3%) samples of contacts have C/C allele, 13 (18.3%) samples of contacts have C/T allele, and 1 (1.4%)

samples of contact have T/T allele (Table 1). Chi-Square test results showed that there were significant differences between genotypes CC, CT and TT with a significance value of $p = 0.000$ ($p < 0.05$).

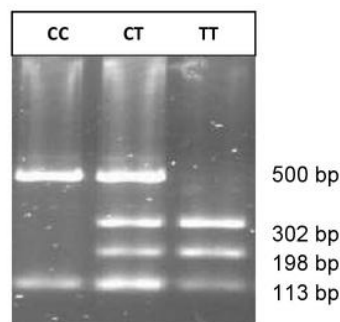


Figure 2: Results of PCR-RFLP electrophoresis with Tsp45I GCLC genes in samples of contacts with infectious TB.

Table 1: Distribution of genotype GCLC genes in samples of contacts with infectious TB.

Genotype	Frequency	Percent	Valid Percent	Cumulative Percent
C/C	57	80.3	80.3	80.3
C/T	13	18.3	18.3	98.6
T/T	1	1.4	1.4	100.0
Total	71	100.0	100.0	

Levels of glutathione (GSH)

Based on the results of glutathione level by using ELISA, 71 samples obtained minimum and maximum GSH levels of 0.167 mM/mL and 0.548 mM/mL, respectively. The normal level of GSH is 2-8 mM/mL. Chi-square analysis results between glutathione levels and GCLC gene polymorphism were obtained value $p = 0.262$ which means there is no significant correlation between gene polymorphism GCLC to decreased levels of GSH.

Levels F₂-isoprostanes

The results of F₂-isoprostanes examination using ELISA from 71 individuals shown average level of F₂-isoprostanes (616.1 pg/mL) with a minimum and maximum value of 72.4 pg/mL and 1343.9 pg/mL, respectively. The value of standard deviation is 255.5 with normal values of F₂-isoprostanes in the blood is 20-80 pg/mL.

Chi-square analysis results between levels of F₂-isoprostanes and GCLC gene polymorphism obtained $p = 0.000$ ($p < 0.05$), which shown significant differences in the distribution of F₂-isoprostanes levels with gene polymorphism GCLC from contacts of person with infectious TB. From the results above, it can be observed that the examination of F₂-isoprostanes levels are found

within normal level on 3 people. Meanwhile, we got 68 people from contacts of person with infectious TB with high level of F₂-isoprostanes.

Pearson correlation test between the level of GCLC gene polymorphisms towards the level of F₂-isoprostanes obtained the Sig. (2-tailed) = 0.630, which means there is no significant correlation between gene polymorphism GCLC to increased levels of F₂-isoprostanes.

DISCUSSION

The host defense against infection of *Mycobacterium tuberculosis* through respiratory burst in macrophage generates reactive oxygen species (ROS). Increased ROS led to an increase in the use of antioxidants such as glutathione to neutralize ROS. If an increase in oxidants are higher than a number of antioxidants in the cells, it will result in oxidative stress. These studies demonstrated GCLC gene polymorphism at C/T and T/T allele with lower percentages compared to the GCLC gene polymorphism found in patients with lung disorders (Chang *et al.*, 2008; Siedlinski *et al.*, 2008; Yuniastuti *et al.*, 2013). This discovery has shown that contacts of person with infectious TB is still susceptible to oxidative stress. Most of the samples with the history of contact with pulmonary TB patients experiencing oxidative stress that is characterized by high levels of F₂-isoprostanes. However, not all oxidative stresses are caused by GCLC gene polymorphism that reduces the production of glutathione in the body. It may also be caused by several factors such as the presence of other infections, low intake of vitamins C and E, poor lifestyle such as heavy cigarette consumption, and also the presence of chronic diseases (Nwanjo and Oze, 2007; Siedlinski *et al.*, 2008).

GCLC gene is a gene cluster in the catalytic formation of glutathione (an antioxidant cellular). Glutathione is naturally present in the human body cells, has a dominant role in the regulation of the main cells in the intracellular redox reaction, and protect the body from oxidative stress by binding with free radicals (Koide *et al.*, 2003). Various reports have reported that the levels of cellular antioxidant glutathione are significantly decreased in TB patients as well as other lung diseases (Nwanjo and Oze, 2007; Venketaraman *et al.*, 2008; Akiibinu *et al.*, 2011; Yuniastuti *et al.*, 2013).

Glutathione levels that were obtained from samples had low levels compared to normal values of 1-10 mM GSH/mL. However, these levels are still higher compared to previous studies in patients with active TB before taking the anti-tuberculosis drug in which they shown an average level of glutathione (Nwanjo and Oze, 2007; Yuniastuti *et al.*, 2013). This result indicates the different levels of glutathione between active TB and contacts of person with infectious TB due to the difference in the number of germs, levels of infection, and the imbalance between oxidants and antioxidants in the body. Glutathione in TB patients are widely used to neutralize germs, free radical and oxidative stress response. Furthermore, the presence of glutathione can be seen with the increased levels of F₂-isoprostanes

(Venketaraman *et al.*, 2005). In addition, several compounds that can induce oxidative stress, such as TGF-β₁, H₂O₂, menadione, cigarette substances, and okadaic acid has been displayed to decrease gene expression of GCL and induce the early depletion of GSH (Jardine *et al.*, 2002).

F₂-isoprostanes is one of the metabolites of arachidonic acid oxidation by free radicals plasma membrane that resembles prostaglandin. F₂-isoprostanes can be used as a marker of oxidative stress because it is also stable. Several studies have reported that the use isoprostane as an indicator of oxidative stress in human disease, especially in lung disorders (Morrow *et al.*, 1995; Repine *et al.*, 1997). Isoprostane concentrations have shown to escalate in individuals with chronic obstructive pulmonary disease (Repine *et al.*, 1997; Pratico *et al.*, 1998).

Based on the results of the examination F₂-isoprostanes of individual who had contacted infection TB shown the high levels of F₂-isoprostanes. This data is consistent with the results of Glutathione (GSH) which shown low levels of cellular antioxidant that is accompanied by high levels of cellular oxidants. However, this study found no correlation between GCLC gene polymorphism in TB contacts against the high levels of glutathione (cellular antioxidant) and low levels of F₂-isoprostanes (cellular oxidants). This result is very different from the results in several cases of TB patients (Yuniastuti *et al.*, 2013). Other studies have shown the significant differences in the high levels of oxidants in TB patients compared to the population of controls, and there is a significant difference to the decreased levels of antioxidants in TB patients compared with the population of controls (Akiibinu *et al.*, 2011). Currently, there are limited works of literature reporting GCLC gene polymorphism in pulmonary TB contacts. Therefore, further research needs to be conducted to discover other markers as an early detection of pulmonary TB contacts that is vulnerable to oxidative stress.

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