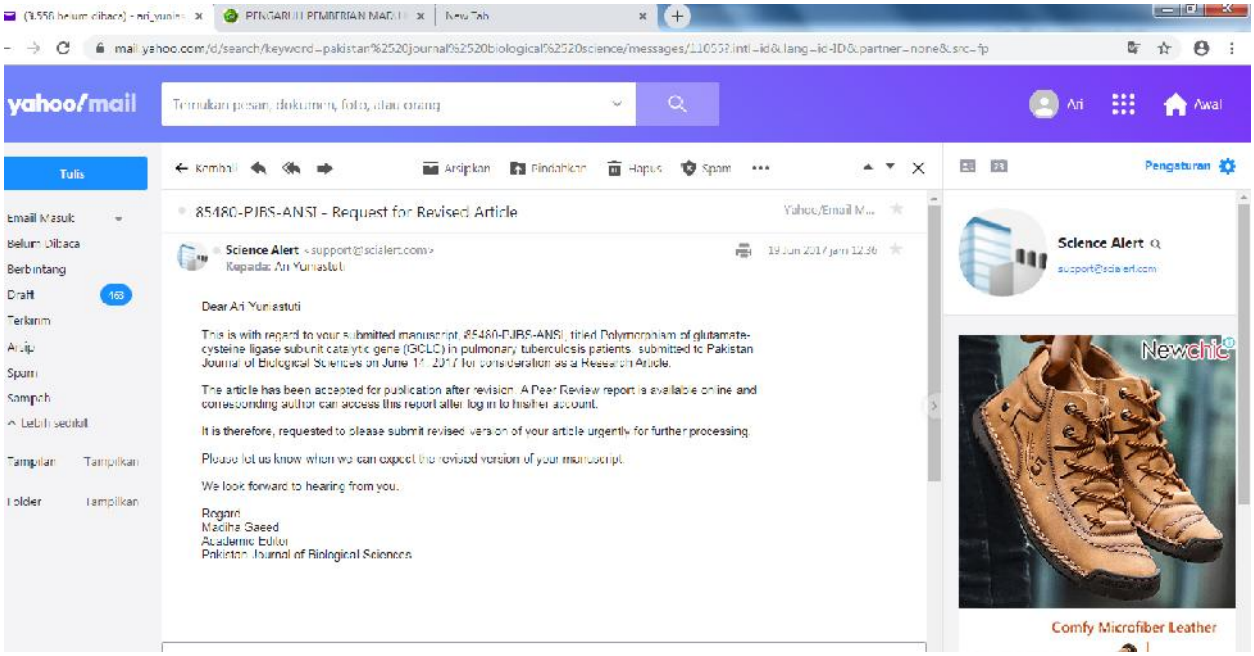
PERMOHONAN REVISI DAN HASIL REVISI PADA PAKISTAN JOURNAL OF BIOLOGYCAL SCIENCE

19 JUNI 2017



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The authors are obliged by the publishing agreement to carry out all the changes advised by reviewers within a deadline set by the editor. Refusal or not providing the amended document on time will result in rejecting your paper for publication.

Serial No.	Part of the Manuscript	Reviewer's Comments	Formatted Table
1	Scope of journal	Submitted manuscript does not fall in the scope of the journal. It is therefore requested to please submit your article in a relevant journal. This article should be submitted in **JMS (journal of medical science)** as it is specifically related to this area of interest	I think this article can be included in the scope of Pakistan journal Biological science because it is included in the study of biotechnology and molecular biology
2	Write-up	Author is advised to re-write the full text carefully with the help of English Language Expert and correct the spelling, grammar, punctuation and vocabulary usage errors. Provide English Language Editing certificate	I have improved some part of this article with better sentences
3	Authors	Write the complete name of each author without abbreviations.	All authors' name is already with no abbreviations. R. Susanti

			is the real complete name of the author
4	Corresponding Author	• Do provide the Contact details(Mobile Number) of Corresponding Author for faster communication and updation. • From January 1, 2017, its compulsory for all corresponding authors submitting papers to any Science Alert Journal to provide LiveDNA iDs (livedna.net) before final publication of their articles. With this standard identifier, you can create a profile of your research activities to distinguish yourself from other researchers with similar names, and make it easier for your colleagues to find your publications. To get LiveDNA, please go to the link: http://livedna.net/form.php	I have added the phone number in the manuscript I am in process of making LiveDNA ID
5	Running title	Provide the running title of the article as it is necessary according to the format of the journal.	I have added the running title in the manuscript
6	Significance statement	A statement about the significance of this research work should be included in the manuscript. The significance statement should provide the novelty aspect and significance of this research work with respect to the existing literature and more generally to the society. It should be a short summary which describe what this paper adds to and what was already known. **Start this statement with the following words:** This study discover the ---------- that can be beneficial for …… **And the last sentence of this statement could be such as:** This study will help the researcher to uncover the critical areas of ---------- that many researchers were not able to explore. Thus a new theory on ---------- may be arrived at. **A Model Significance Statement:** This study discovers the possible synergistic effect of vitamin E, calcium, and vitamin D combination that can be beneficial for osteoporosis-induced ovariectomized rats. This study will help the researcher to uncover the critical area of postmenopausal bone loss that many researchers were not able to explore. Thus, a new theory on these micronutrients combination, and possibly other combinations, may be arrived at.	I have added the statement in the manuscript

7	Abstract	Precisely present background of study	I already present the background of the study
8	Key words	• Provide at least five key words. Key words should be unique and a concise summary of your paper's content and should capture the most important aspects of your paper	I have added more keywords in the manuscript
9	Introduction	• Briefly mention the context that concerned the same studied aspect related to study the polymorphism in TB that became rationale behind the work. • Split long statements in sentences of considerable length • References must be cited in the text in superscript digits at the end of sentence or paragraph before punctuation or full stop[1]. In case of two or more references, separate the superscript digits by comma[1,2,6]. Moreover, If there are more references but in continuous numbers then use dash between superscript digits[2-6].	I have made some improvements according to the suggestions
10	Materials and Methods	• When the study was carried out? • Do not start the sentence by using numeric values. • If author did not do any modification in original procedure then there is no need of complete description of methodology only the reference is enough • Mention grading of solutions and chemicals. Whether these are of analytical or commercial grade? • Provide the description about pH of buffer • Please indicate both the manufacturer's name and location (including city, state, and country) for all specialized software used in the experiment. E:g Social Sciences (SPSS) software (version 13.0 for Windows; SPSS Inc., Chicago, IL)	I have made some improvements according to the suggestions
11	Results	• No need to repeat the Table values in the text. Delete the data from the text that have already been presented in the table • Don't mix-up the results with methodology and discussion	I have made some improvements according to the suggestions

	Discussion	• Discussion section should be comprised as given the example: In the current study, periodontal disease and dental tartar were the most common affections in donkeys. These affections mainly involved cheek teeth. Similar findings were reported in previous studies[4,15] • What are the implications, applications, recommendations and limitations of the study? • Sentence is too long, split it into considerable length with meaningful approach.	I have made some improvements according to the suggestions
	Conclusion	Rewrite your conclusions clearly and concisely, explain why your study is important to the reader. After reading this section, a reader should have a good idea of what you have investigated and discovered. Mention the effect of this polymorphism on TB	I have made some improvements according to the suggestions
	Acknowledgement	Is there any source of funding that supported this research work. Funding source with relevant grant number should be mentioned in the Acknowledgement.	I have mentioned the source of funding
	Authors contribution	There is a series of questions that will enable you to state the contributions of each author. Each author listed on the manuscript should have a real and concrete contribution to the submission. Every single person who contributed to the manuscript should be listed. More information about authorship can be collected from *Editorial Policies*	I have made some improvements according to the suggestions
	References	Some of the references are incomplete or not formatted properly. Such type of references are not acceptable. Go to Http://scialert.net and consult "references" from "guide to author" for having idea of reference categories and formatting	I have made some improvements according to the suggestions
	Figures and Tables	• Define "lanes" of figure 1 carefully • It's better to remove tables having small data and present the results of those tables in text.	

Don't forget to make the changes

One of the biggest mistakes made by authors is to respond to all the comments, but forget to actually update the paper.

Don't be that person: one way to make sure you remember is to always include line numbers in your changes. That way, you actually have to make the change first before including the line numbers in your response.

How to prepare the revised paper?

1. Before you start editing the document, make sure Tracking changes is enabled! In MS Word go to Review menu and enable 'Track changes'.
2. You have to address every comment from both reviewers.
3. Mark your every change by adding a comment, excluding typos or similar minor editorial errors.

The comments should include a justification of the change (or lack of change!). In other words, answers to two questions:

- Why I made this change?
- What is the result?

If you decide to explain or justify why you believe your change (or lack of it) adds to the paper, always make sure to clearly indicate parts of paper you refer to (e.g. I mentioned this fact already in the Introduction, p. 1 paragraph 2. I believe bringing up this information there clarifies the issue from the very beginning).

Don't add comments with questions. Bear in mind that:

- Our editors won't answer them
- There will be only two stages of corrections – after major and minor review. There is no space for discussions with the reviewers.

If you remove something, ~~don't mark it like this~~. Instead, mark the whole paragraph by adding a comment with explanations of what you did and how it helps to improve the paper.

Summary: Track changes + put a comment with justification on every change you make

b. Cover letter

It is a good academic practice to attach a cover letter to submissions of new and revised papers to journals. Cover letter sent with the paper after major review usually includes:

- Courteous thanks to the editor, etc.
- General confirmation on carrying out all minor editorial corrections (typos etc.)
- Confirmation of implementing the requested changes. Explanations on general character of changes. You may mention 1-3 fundamental changes you made and explain how you dealt with it.
- A list of tasks given by the reviewer with precise indication where in the document you included the changes.
- Other comments those are important to the editor.

Polymorphism of glutamate-cysteine ligase subunit catalytic gene (GCLC)- in pulmonary tuberculosis patients

[1]Ari Yuniastuti, -[1]R. Susanti and [2]Dewi Mustikaningtyas

Comment [ZA1]: Write the complete name of each author without abbreviations.

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Running title: GCLC gene polymorphism

Significance statement: This study discover the existence of GCLC gene polymorphism namely -129C/T GCLC in pulmonary TB patients that can be beneficial for informing the medical personnel that antioxidant is needed to add in the treatment of pulmonary TB patients. This study will help the researcher 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 treatment suggestions may be arrived at.

ABSTRACT

Background and Objective: Glutamate cysteine ligase subunit catalytic (GCLC) gene is a candidate gene for glutamate cysteine ligase, which an enzyme that catalyzes the formation of glutathione (GSH). Single nucleotide polymorphism of GCLC gene namely -129C/T GCLC has been reported to be an association with a risk factor for susceptibility to oxidative stress and severe pulmonary tuberculosis (TB). The Objective of this study was to determine the GCLC polymorphism in pulmonary tuberculosis patient. **Methodology**: Blood samples of 225 pulmonary TB patients ~~with pulmonary TB~~ 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

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Comment [U5]: A statement about the significance of this research work should be included in the manuscript. The significance statement should provide the novelty aspect and significance of this research work with respect to the existing literature and more generally to the society. It should be a short summary which describe what this paper adds to and what was already known. Start this statement with the following words: This study discover the ---------- that can be beneficial for …… And the last sentence of this statement could be such as: This study will help the researcher to uncover the critical areas of ---------- that many researchers were not able to explore. Thus a new theory on ---------- may be arrived at.
A Model Significance Statement: This study discovers the possible synergistic effect of vitamin E, calcium, and vitamin D combination that can be beneficial for osteoporosis-induced ovariectomized rats. This study will help the researcher to uncover the critical area of postmenopausal bone loss that many researchers were not able to explore. Thus, a new theory on these micronutrients combination, and possibly other combinations, may be arrived at.

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Comment [MAI6]: Precisely present background of study

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purification kit) following the manufacturing procedure. The amplification of GCLC fragment was ~~done~~ performed by a master mix from Thermo Scientific. Data was analyzed descriptively. Statistical analysis was ~~done~~ ~~using~~performed by Chi-square test. **Results**: ~~The results showed that the samples from pulmonary TB patients showed~~The frequency of C/C and C/T ~~polymorphism~~ genotype ~~C/C of~~were 63.6% and ~~a C/T of~~ 36.4% respectively. The ~~polymorphism genotype,~~ C/T gene in the ~~region, namely~~ GCLC -129C region is a T gene promoter. **Conclusion:** the frequency of C/T polymorphism genotype ~~C/T~~ of GCLC gene in patients with pulmonary tuberculosis is 36.4%.

Key words: Polymorphism, GCLC gene, GCL enzyme, P~~p~~ulmonary ~~tuberculosis~~TB, glutathione.~~.~~

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[6,7].

Comment [Mi8]: For a better sentence

Comment [Mi9]: For a better sentence

Comment [Mi10]: For a better sentence

Comment [U11]: Provide at least five key words. Key words should be unique and a concise summary of your paper's content and should capture the most important aspects of your paper

Comment [U12]: This key word is not suitable for indexing

Comment [U13]: Briefly mention the context that concerned the same studied aspect related to study the polymorphism in TB that became rationale behind the work.

Comment [MAI14]: This statement is too long. Split into sentences of considerable length

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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 second phase involves glutathione synthesis from γ-glutamyl cysteine and glycine, a reaction catalyzed by the enzyme glutathione synthetase (GSS).[8,4] 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.[9,5]

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.[6,10] 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.[7,11,11-15] 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] ~~(2012)~~ 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 that, this study was conducted to analyze the polymorphism of glutamate-cysteine ligase subunit catalytic gene (GCLC) in pulmonary tuberculosis patients.

MATERIALS AND METHODS

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 (Phosphat Buffer Saline) 1x 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 on March-November 2012 by consecutive non-probability sampling.[17]

All patients who ~~The subjects of the study met the~~fulfilled the inclusion and exclusion criteria~~. They willfully~~, were willing to participate~~d~~ in the study and

Comment [MAI15]: References must be cited in the text in superscript digits at the end of sentence or paragraph before punctuation or full stop[1]. In case of two or more references, separate the superscript digits by comma[1,2,6]. Moreover, If there are more references but in continuous numbers then use dash between superscript digits[2-6].

Comment [ZA16]: Introduction section should logically end at the purpose of the study. By the end of the introduction the reader should know exactly what you are trying to achieve with the article.

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signed the informed consent form were recruited as research subjects. ~~Blood samples~~The cohort consisted of 225 patient~~subject~~s ~~were obtained from the Center for Central Public~~f Health in Semarang by consecutive non probability ~~sampling.~~[13] with pulmonary tuberculosis.

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DNA extraction

Three~~3~~ ml of blood sample was taken from each subject. Genomic DNA was extracted from the whole blood according to the method described by Chelex ~~with minor modification~~. Briefly, 800 µL of PBS was added to 200 µL of blood samples and mixed for 30 s, then incubated for 10 min at 4°C. After incubation, they were centrifuged at 10.000 rpm for 10 min at 37°C. The supernatant was discarded, then 500 µL of PBS was added to the pellet and mixed for 15 s then centrifuged at 4000 rpm for 5 min; this process was repeated three times. After that, the supernatant was discarded and 50 µL of Chelex 20% and 75 µL of sterile Aquadest were added to the pellet and mixed until homogeneous. It was then incubated at 100°C for 10 min and centrifuged at 10.000 rpm for 10 min at 37°C. Then, 50 µL of supernatant was transferred to Eppendorf tube ready to be used or saved at -20°C.[18~~14~~]

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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 25µl reaction mixture, containing 10x PCR buffer, KAPA 2G Robust Hot Start Ready Mix 12.5 µl, 10mmol of each dNTP, ddH_2O 7.5 µl, Primer Forward 1.5 µl, Primer Reverse 1.5 µl, 3 U/µl of Taq polymerase, and 2.0 µl of genomic DNA sample. The cycling conditions were pre-denaturation at 94^0C for 5 min., denaturation at 94^0C for 30 sec., *annealing* at $56,1^0$C for 45 sec., extension at 72 ^{0}C for 2 min., and a final extension at 72^0C 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 microliters 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 1x 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 mLG ethidium bromide at 75 V for 2 hours.

PCR-RFLP amplification of *GCLC* gene

After the process of amplification, 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 microliters of ethidium bromide (10 mg/ml) 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, 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 volts for 30 min. The result of PCR product's electrophoresis was observed using a gel doc. [18~~14~~] The bands were visualized under ultraviolet light in a gel-doc system (Thermo Scientific).

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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]

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RESULTS

Molecular approaches were conducted using the polymerase chain reaction-fragment length polymorphism (PCR-RFLP) method. The restriction site of enzyme Tsp45I lies outside the recognition site, 5' to the G residues in each strand. PCR-RFL results of GCLC gene cut by the restriction enzyme Tsp45I shows the shape of the genotype (Figure 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 Tsp 45I as a cleavage site. The data of -129C/C, -129C/T and -129T/T genotype frequencies are shown in Table 1. ~~The -129C/C genotype was found in 143 patients with pulmonary TB (63,6%), whereas -129C/T genotype was found in 82 patients (36,4%). However, there was no -129T/T genotype found in all of the 225 patients. Data are shown in Table 1.~~

Comment [MAI26]: No need to repeat the Table values in the text. Delete the data from the text that have already been presented in the table

Comment [Mi27]: According to the suggestion

Chisquare test results indicated that there were significant differences between the C/C genotype with C/T genotype of 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 2). 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 is18%. Furthermore, the predicted genotype frequency expectations from the real genotype frequencies are given in Table 3. 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. However, these conditions cannot always be found. The calculation of the expectation and the real gene frequencies is used to find out that calculation could be compared with the expected proportion of gene frequencies.

Genotype frequency expectations are in accordance with the real genotype frequencies. Some of the PCR products samples of pulmonary tuberculosis have been examined by DNA sequencing to confirm the results that have been obtained from the PCR-RFLP.

Results of PCR-RFLP are in accordance with the results obtained through the examination of a representative sample sequencing several samples of pulmonary tuberculosis. Based on research data, the calculation of allele frequencies and description of gene sequencing showed that it is polymorphic enough.

Comment [MAI28]: Don't mix-up the results with methodology and discussion

DISCUSSON

Comment [ZA29]: What are the implications, applications, recommendations and limitations of the study?

Comment [U30]: Discussion section should be comprised as given the example: In the current study, periodontal disease and dental tartar were the most common affections in donkeys. These affections mainly involved cheek teeth. Similar findings were reported in previous studies[4,15]

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] ~~(2007)~~ 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).[16]

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Glutathione is synthesized from its constituent amino acids in two sequential by GCL and GSH synthetase.[95] 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.[15~~11~~]This leads to GSH synthesis and provides a protective/adaptive mechanism against oxidative stress.[15~~11~~]

The present study ~~demonstrated~~ showed that the -129C/T polymorphism may suppress the increase of~~in~~ GCLC gene expression in response to oxidative stress~~,~~ ~~and it~~ It ~~may~~ 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 ~~is~~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 conducted. Moreover, the genetic variation plays a role in the severity of the pulmonary TB diseases, for example, the development of the pulmonary fibrosis

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as well as the pulmonary cancer was investigated. 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 and some previous research studies, it was explained that GCLC gene polymorphism is a risk factor for susceptibility to oxidative stress and severity of pulmonary TB.

CONCLUSION

The frequency of ~~polymorphism genotype~~ C/T genotype in GCLC gene ~~GCLC amounted to~~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

Comment [U33]: Rewrite your conclusions clearly and concisely, explain why your study is important to the reader. After reading this section, a reader should have a good idea of what you have investigated and discovered. Mention the effect of this polymorphism on TB

CONFLICT OF INTERESTS

The authors did not declare any conflict of interest.

ACKNOWLEDGMENTS

A very special gratitude goes out to the Ministry of Research Technology and Higher Education for providing the funding for this study through the Fundamental Research via the Head of Institute for Research and Community Service Universitas Negeri Semarang.

Authors' contribution:

Comment [U34]: Is there any source of funding that supported this research work. Funding source with relevant grant number should be mentioned in the Acknowledgement.

Comment [ZA35]: There is a series of questions that will enable you to state the contributions of each author. Each author listed on the manuscript should have a real and concrete contribution to the submission. Every single person who contributed to the manuscript should be listed. More information about authorship can be collected from Editorial Policies

Ari Yuniastuti: Conception and design, analysis and interpretation of the data and statistical expertise.
R. Susanti: Drafting of the article, analysis and Interpretation of the data.
Dewi Mustikaningtyas: Collection and assembly of data and dministrative, technical or logistic support.

REFERENCES

Comment [U36]: Some of the references are incomplete or not formatted properly. Such type of references are not acceptable. Go to Http://scialert.net and consult "references" from "guide to author" for having idea of reference categories and formatting

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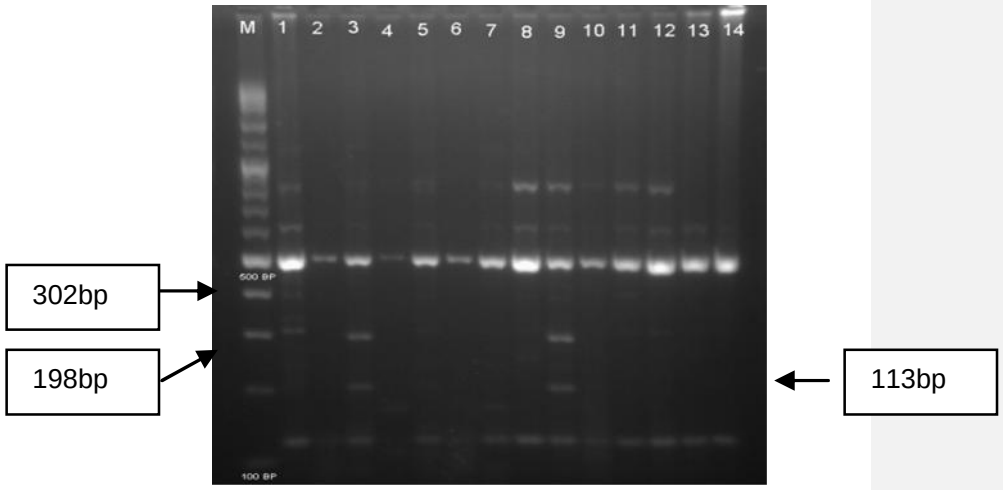

Figure 1. Electrophoresis of PCR-RFLP products of GCLC gene.

Comment [U37]: Define "lanes" of figure 1 carefully

Table 1. Genotype frequency of GCLC gene.

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Genotype frequency	GCLC gene		P-value (Chi square test)
	Frequency	(%)	
C/C Homozygote	143	63.6	0.000
C/T Heterozygote	82	36.4	
Total	225	100	

$p < 0.05$.

Table 2. C and T allele frequency of GCLC gene.

Indicator	Genotype				Frequency of gene
	CC	CT	TT	Total	
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 3. Expectation and real gene frequency.

Comment [U39]: It's better to remove tables having small data and present the results of those tables in text.

Genotype	Frequency of expectation genotype	Number of individual 225	
		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

JOURNAL REVISION COVER LETTER

June 22, 2017

Madiha Saeed
Academic Editor
Pakistan Journal of Biological Sciences

Dear Madiha,

It is with excitement that I resubmit to you a revised version of manuscript of85480-PJBS-ANSI, titled Polymorphism of glutamate-cysteine ligase subunit catalytic gene (GCLC) in pulmonary tuberculosis patients.

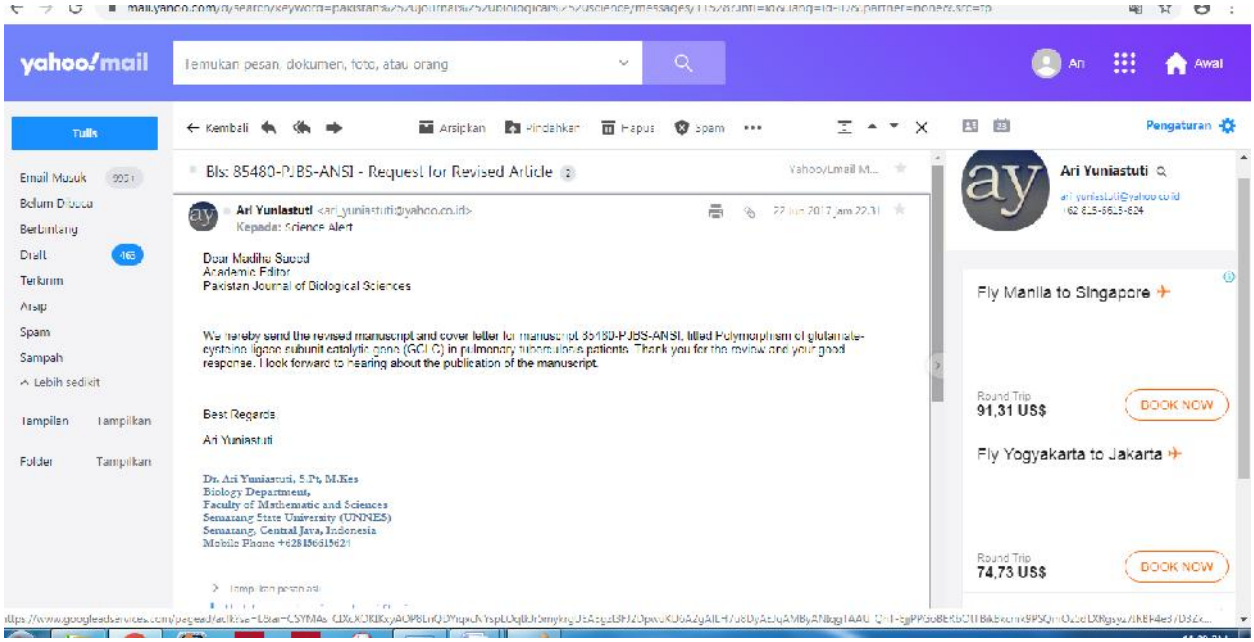
Thank you for giving me the opportunity to revise and resubmit this manuscript. I am resubmitting this revision before the agreed upon deadline. I appreciate the time and detail provided by each reviewer and by you and have incorporated the suggested changes into the manuscript to the best of my ability. The manuscript has certainly benefited from these insightful revision suggestions. I look forward to working with you and the reviewers to move this manuscript closer to publication in the Pakistan Journal of Biological Sciences.

I have responded specifically to each suggestion in the **Reviewer's Comments Sheet**, along with the revised manuscript. Thank you.

Best Regards,

Ari Yuniastuti

22 JUNI 2017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Bls: 85480-PJBS-ANSI - Request for Revised Article
Ari Yuniastuti
Kepada: Science Alert
Dear Madiha Saeed
Academic Editor
Pakistan Journal of Biological Sciences
We hereby send the revised manuscript and cover letter for manuscript 85480-PJBS-ANSI, titled Polymorphism of glutamate-cysteine ligase subunit catalytic gene (GCLC) in pulmonary tuberculosis patients. Thank you for the review and your good response. I look forward to hearing about the publication of the manuscript.
Best Regards
Ari Yuniastuti
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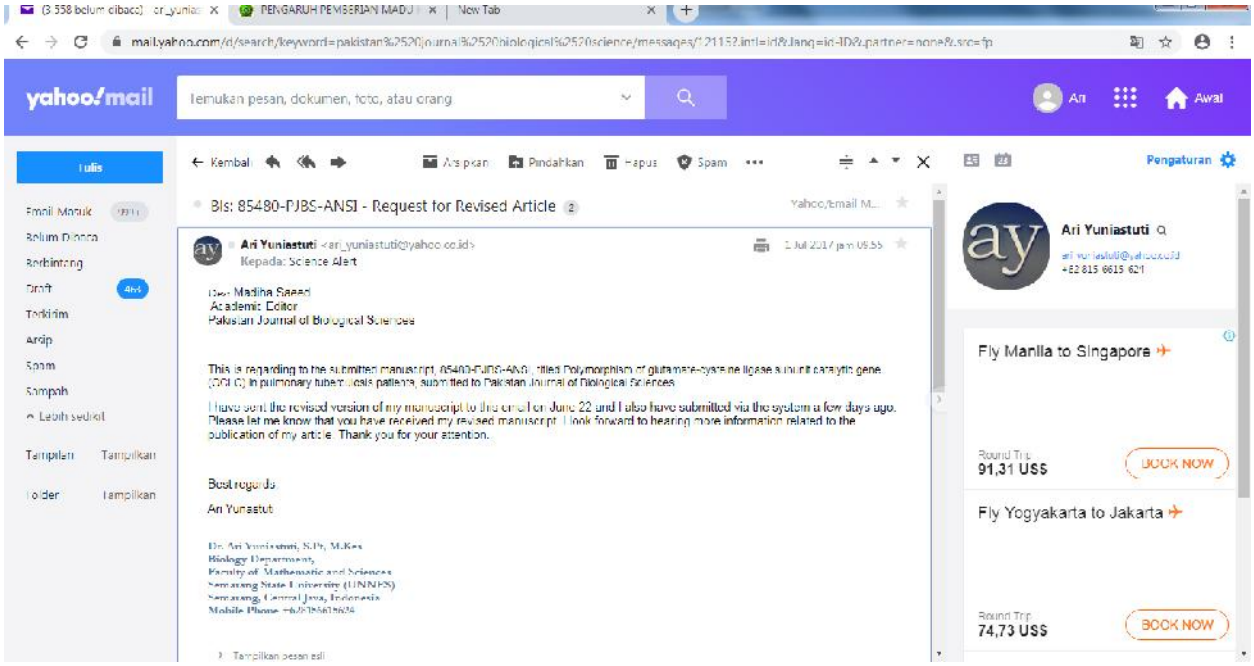
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Ari Yuniastuti <ari_yuniastuti@yahoo.co.id>
Kepada: Science Alert
Dear Madiha Saeed
Academic Editor
Pakistan Journal of Biological Sciences
This is regarding to the submitted manuscript, 85480-PJBS-ANSI, titles Polymorphism of glutamate-cysteine ligase catalytic gene (GCLC) in pulmonary tuberculosis patients, submitted to Pakistan Journal of Biological Sciences.
I have sent the revised version of my manuscript to this email on June 22 and I also have submitted via the system a few days ago. Please let me know that you have received my revised manuscript. I look forward to hearing more information related to the publication of my article. Thank you for your attention.
Best regards,
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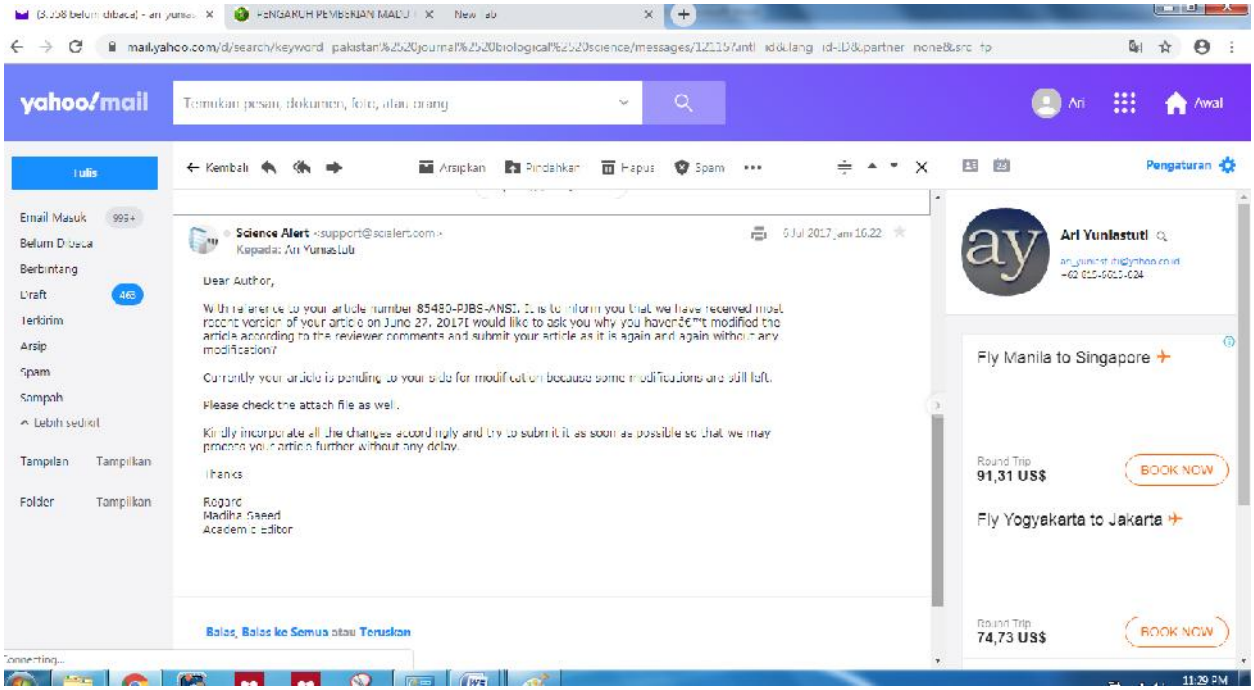
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Polymorphism of glutamate-cysteine ligase subunit catalytic (GCLC) gene in pulmonary tuberculosis patients

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Running title: GCLC gene polymorphism in pulmonary TB patients

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 researcher 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.

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 antioxidant. Single nucleotide polymorphism of 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 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

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[6,7].

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 second 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 (Phosphat Buffer Saline) 1x 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 25µl reaction mixture, containing 10x PCR buffer, KAPA 2G Robust Hot Start Ready Mix 12.5 µl, 10mmol of each dNTP, ddH_2O 7.5 µl, Primer Forward 1.5 µl, Primer Reverse 1.5 µl, 3 U/µl of Taq polymerase, and 2.0 µl of genomic DNA sample. The cycling conditions were pre-denaturation at 94^0C for 5 min., denaturation at 94^0C for 30 sec., *annealing* at $56,1^0$C for 45 sec., extension at 72 ^{0}C for 2 min., and a final extension at 72^0C 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 microliters 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 1x 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 mLG ethidium bromide at 75 V for 2 hours.

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 microliters of ethidium bromide (10 mg/ml) 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, 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 volts 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 Tsp45I lies outside the recognition site, 5' to the G residues in each strand. PCR-RFL results of GCLC gene cut by the restriction enzyme Tsp45I shows the shape of the genotype (Figure 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 Tsp 45I 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 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 is18%. 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.

DISCUSSON

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.[15]This leads to GSH synthesis and provides a protective/adaptive mechanism against oxidative stress.[15]

The ~~present~~ previous study showed that the -129C/T polymorphism may suppress the increase of GCLC gene expression in response to oxidative stress. The existence of -129C/T polymorphism found in this study indicated that ~~I~~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.

~~This study found the existence of -129C/T polymorphism in pulmonary TB patients. The results of PCR-RFLP are in accordance with the results obtained through the examination of a representative sample sequencing several samples of pulmonary tuberculosis. Based on research data, the calculation of allele frequencies and description of gene sequencing showed that it is polymorphic enough.~~

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 ~~was investigated~~. 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 ~~and~~ supported with the result of study by Liu et.al[20] ~~some previous research studies~~, it was proven ~~explained~~ that GCLC gene polymorphism is a risk factor for susceptibility to oxidative stress and severity of pulmonary TB.

Comment [u1]: Which studies?

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Co relate to previous latest studies

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

CONFLICT OF INTERESTS

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENT

A very special gratitude goes out to the Ministry of Research Technology and Higher Education for providing the funding for this study through the Fundamental Research via the Head of Institute for Research and Community Service Universitas Negeri Semarang. The grant number of funding is DIPA-023.04.2189822/2013, December 5, 2012.

Authors' contribution:
Ari Yuniastuti: Conception and design, analysis and interpretation of the data and statistical expertise.
R. Susanti: Drafting of the article, analysis and Interpretation of the data.
Dewi Mustikaningtyas: Collection and assembly of data and dministrative, technical or logistic support.

REFERENCES

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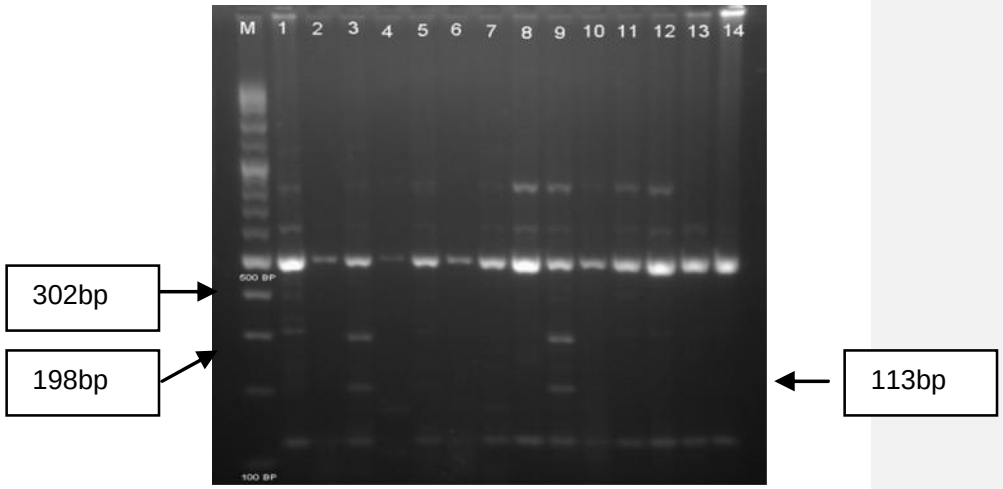

Figure 1. Electrophoresis of PCR-RFLP products of GCLC gene

Table 1. C and T allele frequency of GCLC gene

Indicator	Genotype				Frequency of gene
	CC	CT	TT	Total	
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

Genotype	Frequency of expectation genotype	Number of individual 225	
		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

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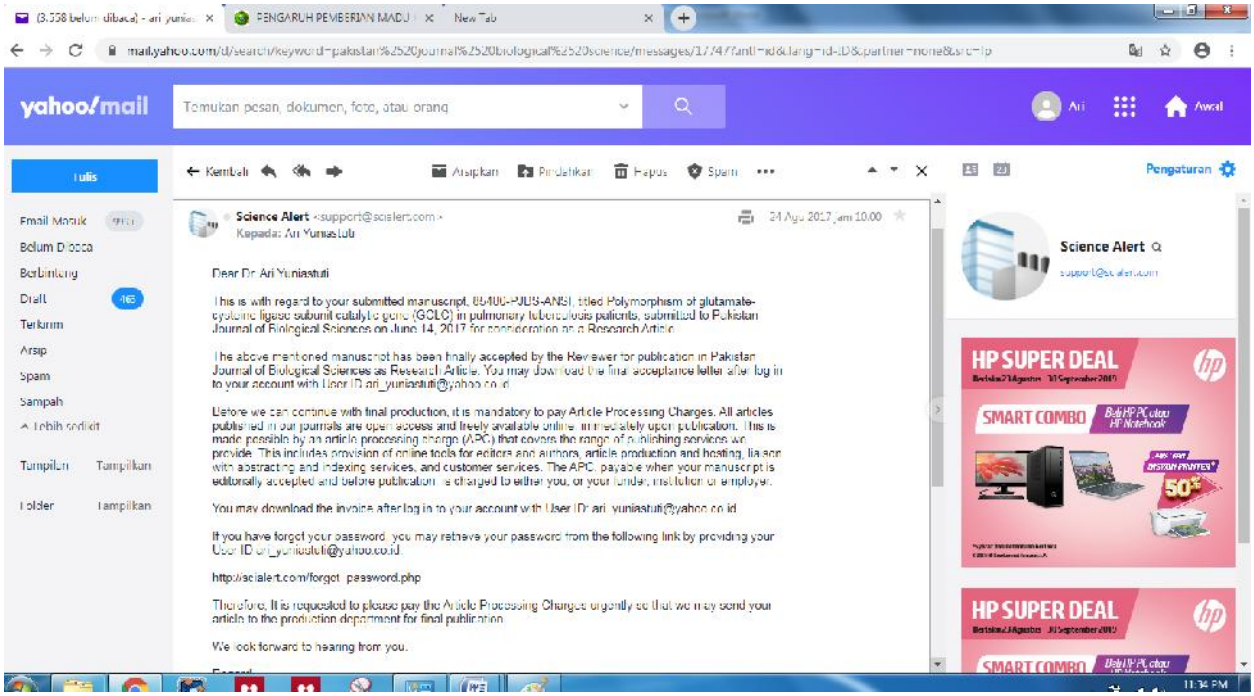

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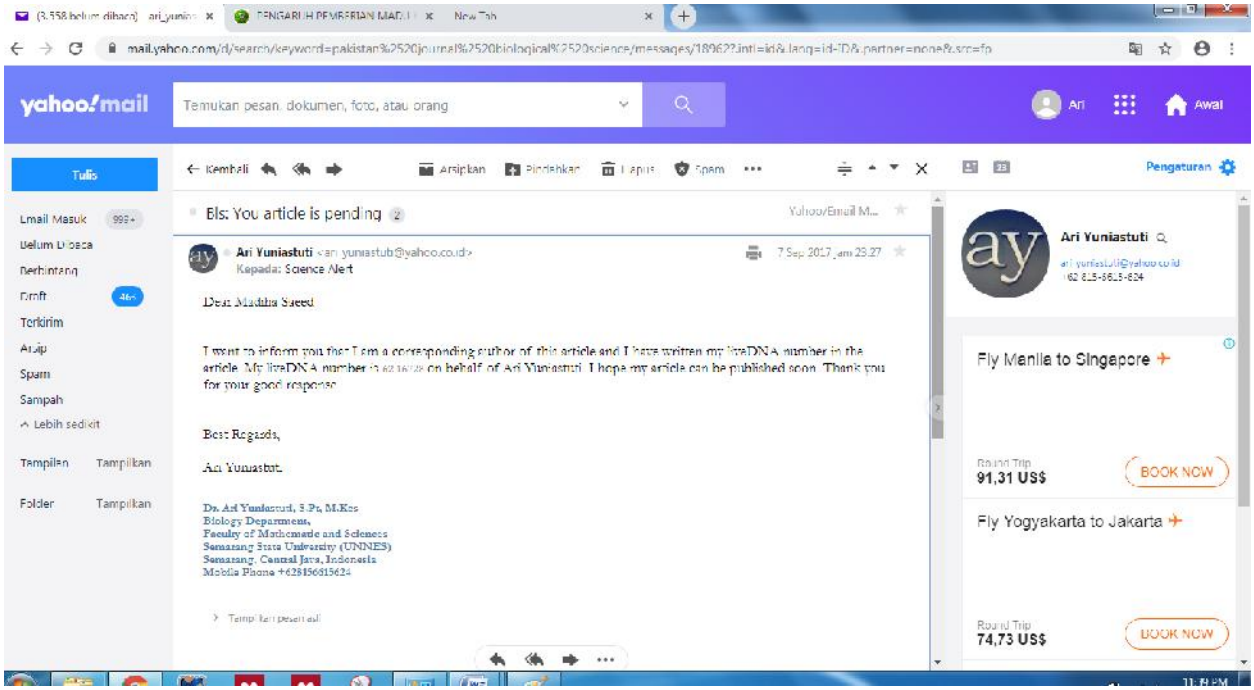

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Research Article
Polymorphism of Glutamate-Cysteine Ligase Subunit Catalytic (GCLC) Gene in Pulmonary Tuberculosis Patients

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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

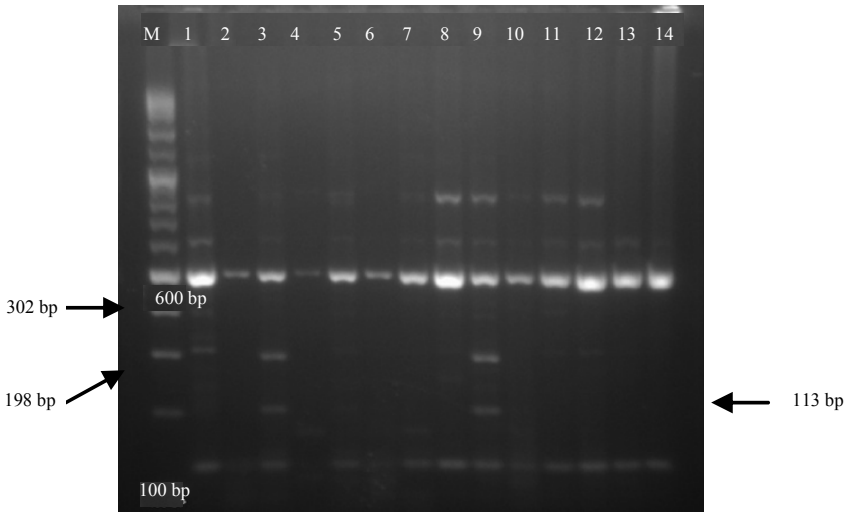

Fig. 1: Electrophoresis of PCR-RFLP products of GCLC gene

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	Genotype				
Indicator	CC	CT	TT	Total	Frequency of gene
Number of individual	143	82	0	225	
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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.

ACKNOWLEDGMENT

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