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IDENTIFICATION OF METALLOTHIONEIN GENE IN HUMAN PLASMA: A MOLECULAR ANALYSIS OF CADMIUM AND LEAD POLLUTION IN GAS STATION ENVIRONMENT

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ABSTRACT

Metallothionein (MTs) is a protein that binds to metals, cysteine-rich and has low molecular weights. Metallothionein binds metals and has some essential cellular functions, such as transportation, storage, and metal detoxification. When metallic lead (Pb) and cadmium (Cd) are absorbed, it will immediately be bounded by metallothionein and transported by the blood to the liver. Metallothionein gene is encoded by MTs. This study aimed to identify the MTs gene in blood of fuel station workers which can further be used as a vulnerability biomarker of the body as a result of exposure to heavy metals like Pb and Cd in humans. This study was a cross-sectional study, conducted for 8 months and took place in Fuel Stations in the city of Semarang. Samples were fuel station workers who met the study criteria, they sought to fulfill as much as 52 samples. Blood of samples was taken approximately 5 ml and then the MT2 gene was analyzed using Polymerase Chain Reaction (PCR). The data were analyzed descriptively. The results showed that 96% of MT2 gene expression had a resemblance to normal human gene sequences by Blast GenBank. MT2 gene expression did not always reflect the accumulation of lead and cadmium in the blood of fuel station workers. The MT2 gene is likely to have a limitation when used as a biomarker for early detection of the heavy metals exposure in humans to determine the susceptibility of heavy metals exposure.

Keywords: biomarker, metallothioenian gene, lead, cadmium

INTRODUCTION

The use of a biological marker (biomarker), is needed to detect and monitor the presence of heavy metals in the human body. Additionally, the use of biological markers is able to provide data of heavy metal intensity absorbed by the sample organisms with toxicity and disruption inflicted on biological systems at certain levels. With the study of biomarkers, heavy metal pollution in humans can be controlled as a preventative action against disease severity.

Metallothionein (MT) is a material that belongs to protein compounds or polypeptide. Metallothionein (MT) is a polypeptide which has many cysteine (Cys) bonds encoded by the gene, has a low molecular weight, and functions as a metal binding peptides (Zatta, 2008). Metallothionein expression indicates exposure to certain metals (Hanson, 2008). The existence of metallothionein has at least two main functions,

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i.e cleaning free radical materials in the body and detoxifying metals to reach a state of homeostasis (Ryvolova et al., 2011; Sakulsak, 2012; Ruttkay et al., 2013). Induction of metallothionein (MT) expression is involved in metal homeostasis and detoxification (Chen et al., 2014; Wang et al., 2014).

Semarang is one of the metropolitan cities where the number of motor vehicles increases up to 6% per year. The growth of vehicles in this city has a large potential for air pollution which will impact human's health (Mifbakhuddin & Mumpuni, 2010). Increasing the number of motor vehicles will cause a high level of metal pollution which is estimated to derive from motor vehicle exhaust emissions. According to research by Sunoko et al. (2011), Semarang is a city with a high level of lead pollution which is 2.41 μg / Nm3 at one point in the city of Semarang. According to the ambient air quality standards of Central Java province, based on the Decree of the Governor of Central Java No. 8 of 2001 April 23, the limit of Pb air emissions is 2 μg / m3. Pb impact in human health can be prevented if Pb content in the air does not exceed this quality standard.

Most air pollution comes from motor vehicle exhaust gases. Gas station officers are one of the groups who are vulnerable to exposure to lead (Pb) from motor vehicle exhaust gas because gas stations are places visited by many vehicles. It may also be exposed to cadmium from the air. Cadmium in the air can be carried by different processes into the environment. The main natural sources of cadmium in the air are from mountains, evaporation, soil particles which are carried into the air, and forest fires. Other sources come from human behavior such as vehicle emission and cigarettes. Cadmium (Cd) and lead (Pb) from air entering the body through breathing (derived from cigarette smoke and vehicles).

Accumulation of lead (Pb) in the body can be detected through blood, bones, and hair. The Centers for Disease Control and Prevention in Hanna-Attisha et al. (2016) determined permissible levels of lead in children's blood by 10 μg / dL, whereas according to WHO (2005) the normal levels in adults' blood averaged 10-25 μg / dL. If the Pb content is more than 80 μg / dl, it will be harmful to health. The level of lead in the blood rises within a few hours after exposure and will remain high for several weeks afterward (Gillis et al., 2012; Meikawati & Mumpuni, 2010).

Exposure to heavy metals such as Cd and Pb causes free radicals and oxidative stress, and MRE is the metal response element (MRE)-binding transcription factor-1 (MTF-1) factor will be activated and an active MTF-1 bond occurs to the MRE region and initializes the gene transcription. Metallothionein is encoded by the MTs gene. Exposure to heavy metals, oxidative stress, and others can cause MTs gene polymorphism, and it can increase the incidence of aging (Kayaaltı et al., 2011; Ruttkay et al., 2013), damage to placental tissue (Tekin et al., 2012) and autopsy kidney tissues (Kayaaltı & Söylemezoglu, 2010). Kayaaltı et al. (2011) reported that there was a significant relationship between the -5 A / G core promoter SNP in the MT2A gene and Cd, Pb and Zn levels ($p = 0.004$, $p = 0.012$ and $p = 0.002$, respectively), but no association was found with Cu level ($p = 0.595$).

Various types of metal and heavy metal are able to compete on the metallic bond in metallothionein. These circumstances provide an advantage in the distribution mechanism of various types of metal for the body's biological system of organisms. In various studies, it is known that there is a competition among the heavy metals to bind to from metallothionein. Therefore, this study was conducted to determine whether the MT gene can be used as a biomarker for early detection of the heavy metals exposure in humans that indicate susceptibility to heavy metals exposure.

Identification, level measurement and structure characteristics of MTs are important to determine the role of metalloproteins in the body of organisms. Measurement of MT levels in body fluids can be used as a biological indicator of metal exposure in humans. Several studies have reported that examination using enzyme-linked immunoassays (ELISA) can be used to measure metallothionein-1 (MT-1) and MT-2 levels in humans and experimental animals (Chen & Song, 2009; Nakazato et al., 2014). Identification, level measurement and structure characteristics of MTs are important to determine the role of metalloproteins in the body of organisms. MT gene identification can be used as early detection of heavy metal exposure in humans or other living things.

METHODS

This study used a cross-sectional design and was conducted over 10 months in the city of Semarang. Plasma samples were examined at the Laboratory of Molecular Biology, Biology Department, Faculty of Mathematics and Science, Universitas Negeri Semarang. The study sample as many as 52 people are all workers of the sample fuel station in Semarang city who were eligible and willing to participate in this study and signed the informed consent. The sampling technique was purposive non-random sampling. Inclusion criteria are men or women aged 15-55 years old, willing to become respondent until the research is completed. Exclusion criteria are: suffering from diabetes mellitus, acute renal failure or chronic renal failure, heart failure/arrhythmias cardiac, liver disease chronic/acute lung tumors or other malignancies, degenerative diseases, discontinue therapy, GI disease, pregnancy or lactation, and hormonal contraception, HIV/AIDS.

MT-2 gene identification was performed through several steps such as DNA isolation and extraction, electrophoresis, PCR and sequencing. DNA isolation of MT-2 gene using QIA amp blood DNA mini-kit (Qiagen, Hilden, Germany) referred to the method recommended by the manufacturer. The obtained DNA stored at -20 ° C for polymerase chain reaction (PCR) analysis. The amplification of the MT-2 gene using the PCR with MT-2A: Forward 5' CGC CTG GAG CCG CAA GTG AC-3 'and Reverse: 5'-TCC TGG GCA CCA TCT GCC TA-3'.DNA amplification in PCR method using master mix kappa with a cocktail total of 50 ul reaction mixture containing 200 μMofdNTPs, 10 pmol each of Forward (F) and Reverse (R) primers, 1 U Hot Star Taq DNA polymerase (Qiagen), 1 XPCR buffer (Qiagen) and 100 ng genomic DNA. The PCR cycling conditions consisted of an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a nal extension step at 72°C for 10 min. The PCR produced was 241 bp. The PCR products were separated on a 2% agarose gel electrophoresis, visualized by ethidium bromide staining under an ultraviolet illuminator. A good PCR product followed by sequencing stage The good PCR product continued with the sequencing stage. DNA sequencing was carried out to determine the nucleotide sequence in the MT-2 gene region. Sequencing is carried out at PT. Indonesia Science Genetics, St. Duri Raya No. 5D West Jakarta, 11510, Indonesia. The sequencing method used in this study was the Sanger method.

Sequencing result data in the form of ABI files of MT-2 were edited manually using the BioEdit version 7.0.9 program. The sequence editing results were included in the NCBI BLAST (Basic Local Alignment Search Tool). Sample sequences were compared with GenBank MT-2 of Human Homo Sapiens is Lead and cadmium levels were measured using Atomic Absorption Spectrophotometry (AAS) with wavelengths of 283.3 nm and 540nm.

RESULTS AND DISCUSSION

The sampling took place at the gas station situated on West, East, and South Semarang. The total of fuel station workers that became the samples was 15 people from East Semarang, 17 people from West Semarang, and 20 people from South Semarang. Thus, the total sample was 52 people.

The average lead levels of gas station workers in Semarang was 8.8 mg/dl. Based on WHO (2005), normal blood lead levels in adults are 10-25 mg/dl. This results indicated that the blood lead level of gas station workers was low. While the average of cadmium level was 0.14 mg/L means that it was still below the normal blood cadmium level of 0.42mg/L.

Isolation and Purification of DNA

MTs gene DNA in the blood of gas station workers was isolated using Qiagen DNAeasy human Mini Kit. The results of 2% agarose gel electrophoresis showed the presence of DNA bands. It indicated that the MT2A gene had been isolated seen from the presence of two intact bands of 241bp DNA. The quantity of DNA that had been isolated was measured by spectrophotometric methods. The measurement results of the DNA quantity which was obtained from the ratio A 260/280 was about 1.86-2.01. It showed that the sample had a high purity against protein contaminants.

The results of DNA isolation and purification was continued with PCR and electrophoresis to obtain the DNA strands figure. The DNA strands figure of one of the workers is presented in Figure 1.

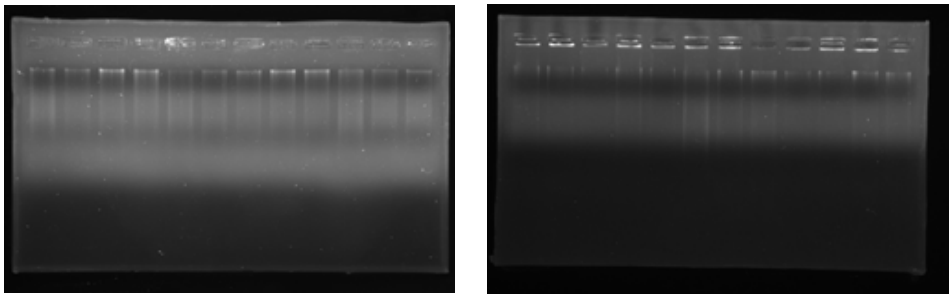


Figure 1. The Fuel Workers' Isolation and Purification of Blood DNA

The Results of MT-2A Gene Sequencing

A total of 6 samples as the representative to be sequenced was sent to the laboratory of PT. Indonesia Science Genetics, St. Duri Raya No. 5D West Jakarta, 11510, Indonesia.

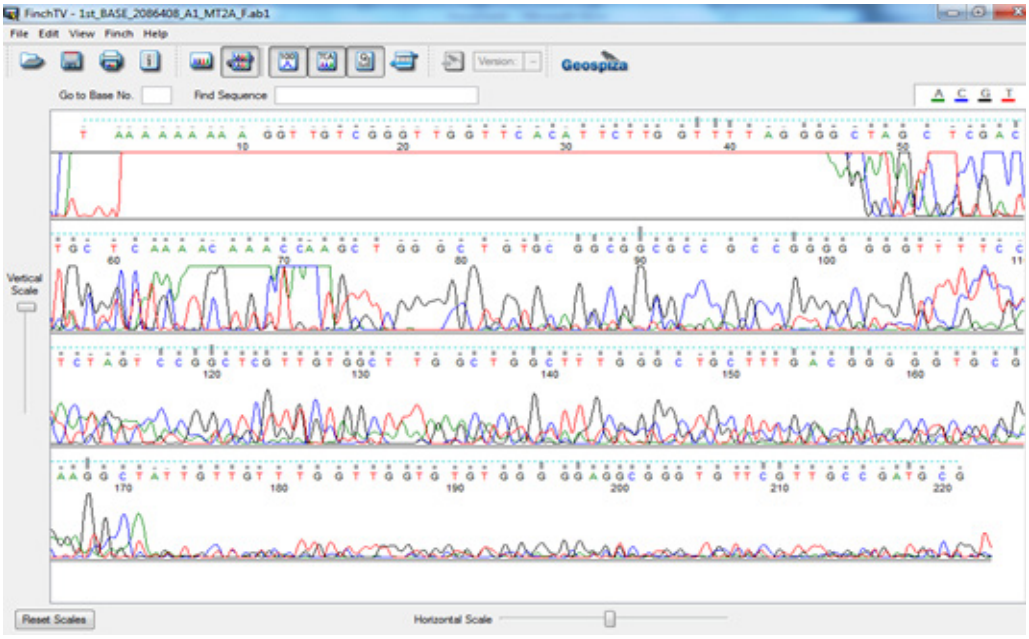
Sequencing examination was done to determine the metallothionein gene sequence on the fuel station workers. Then, it compared with genes that already confirmed from the National Center for Biotechnology Information (NCBI Bethesda, MS, USA).

DNA sequencing is the process or technique to determine the nucleotide-based sequence in a DNA molecule. DNA sequencing can be used to determine the identity and function of a gene or other DNA fragments by comparing the samples sequences with other DNA sequences which are already known. Sequencing methods are useful to identify a gene mutation and can compare homologous genes between species. In 1977, the sequencing method was developed in America, spearheaded by Maxam and Gilbert and 1974 in England by Sanger. There are two sequencing methods; Maxam-Gilbert and Sanger.

Sequencing results were obtained in the form of electropherogram. On the electropherogram, there were several curves in four different colors. The blue color indicates the cytosine (C), black for guanine (G), green for adenine (A) and red for Thymine (T). The electropherogram is read based on the colors that make up the highest top. If there is a curve, then it is assumed that in this position, there is a pair of homozygotes allele. If there are two top of curves which have relatively the same height, it is assumed that there was a pair of heterozygous alleles.

These results indicated that the MTs encoding gene from the blood of gas station officers was not successfully isolated. Figure 2 shows the sequence of MT2A encoding genes that had been isolated. The size of the gene obtained could not be ascertained exactly. Alignment analysis of DNA fragment sequence between forward and reverse using the BioEdit computer program had not been able to bring together the ends of the fragment sequence (data not shown).

FORWARD



REVERSE

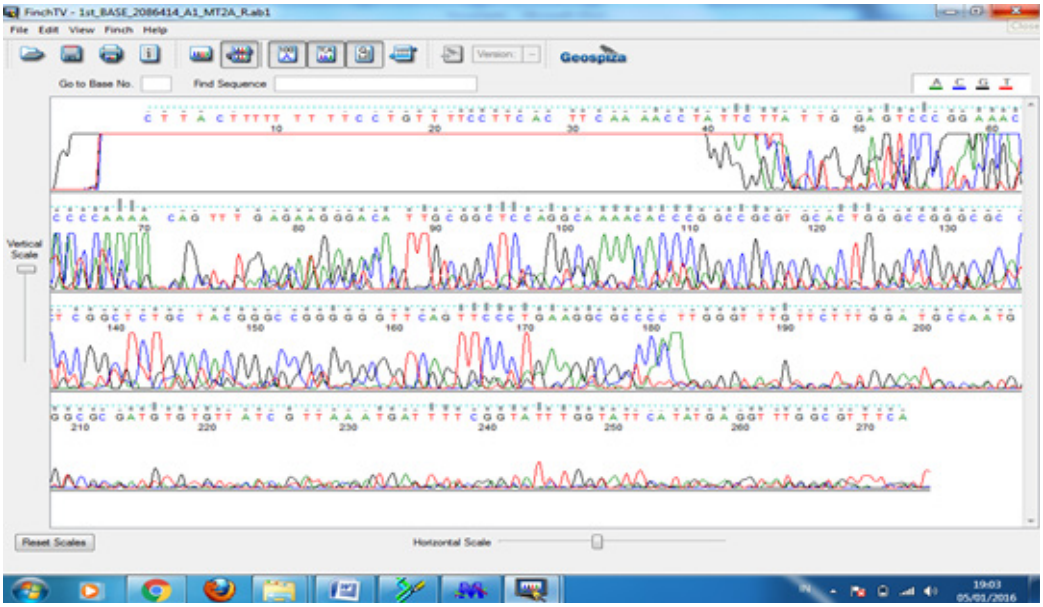


Figure 2. The MT2A Gene Elektroforegram of Fuel Workers

Based on the results of BLAST analysis, the nucleotide variant in MT-2A gene that was compared with GenBank Acc Number GQ154088.1 was not found. The nucleotide sequences contained in the 388 and 608 base sequence (Figure 2).

Semarang is one of the metropolitan cities where the average number of motorized vehicles increases by 67 vehicles, reaching growth of 5-9% per year. The growth of motorized vehicles has the potential to increase air pollution which will impact the health. (Mifbakhuddin & Mumpuni, 2010). Hadi (2007) stated that the vehicles growth in Semarang reached 6% per year and the 77% was dominated by private vehicles and motorbikes, while cars were only 19%. Thus, the transportation sector becomes greenhouse gas emissions sources and contributes up to 25%. In addition to air pollution, transportation activities produce noise, dust, vibration which can decrease the quality of health and work productivity. The more crowded of vehicles which use gasoline fuel will increase lead levels in the air. In a site of the Semarang area, the lead levels in the air up to 2.41 µg/Nm3, this indicates that the level of vehicle traffic at that site is high too (Sunoko et al., 2011).

Lead (Pb) exposure from the environment enters the human body through the respiratory and digestive tract. The danger caused by air depends on the particles size. Particles smaller than 10 mg can be swallowed to the lungs, and larger particles settle in the upper airways (Hartini, 2010). Most of the inhaled lead will enter the lungs blood vessels. Lead absorption through the airways is influenced by three processes which consist of deposition, mucociliary cleansing, and alveolar cleansing. Deposition occurs in the nasopharynx, tracheobronchial and alveolar ducts. Particles which are smaller than 10 µm can be retained in the lungs, while larger particles settle in the upper airway (Neal & Guillarte, 2012).

Low levels of lead exposure that persist for a long time will cause bad effects to the health, such as hypertension, anemia, decreasing of ability brain and could inhibit red blood cell (erythrocytes) formation (Mifbakhuddin & Mumpuni, 2010).

In this study, the obstacle was the difficulty in determining the annealing optimum temperature to make the primer sticks perfectly so that DNA could be amplified. Therefore, optimization temperature was done by trying some temperatures ranging from 51°C to 55°C to obtain the annealing optimum temperature at 53 °C. Moreover, the best PCR reagent composition to thicken the amplification DNA strand might also be an obstacle.

The size of the DNA fragment which obtained from the amplification process using a specific primer cannot be ascertained. Therefore, it was necessary to analyze DNA fragments which had been amplified by sequencing, then the sequences were further analyzed by bioinformatics. In this DNA amplification, discovered was the best DNA strand with the half of cocktail reac-

tion. The good DNA amplification results continued to the next stage, namely DNA sequencing through the help of the Genetica science service agency in Jakarta.

The sequencing results in the form of ABI file were edited using Bio edit of 7.0.9 software. Editing was needed to eliminate unnecessary bases by comparing the sequence with the chromatogram. The results were then compared in Clustal W. The edited sequence was inserted in the NCBI BLAST to found out samples sequence homology with closest species from GenBank collection.

The BLAST results showed the homology values of 96% on MT-2A gene with GenBank Acc Number GQ154088.1. Nucleotide differences between MT-2A were illustrated by the nucleotide sequence from BLAST alignment results. So, the MT-2A gene of the fuel station workers was similar to Gen Bank, which is normally MT-2A gene sequences without exposure. The resemblance was about 96% or approaching close to 100%. Thus MT-2A gene cannot be used as a biomarker for early detection to know the existence of the vulnerability on heavy metals exposure of Pb and Cd.

The results of the amino acid alignment showed similarities in the amino acid structure of MT-2A genes between samples with others in Genbank. These results explained that even at the nucleotide level, there was a mutation (transition or transversion), yet the mutations on nucleotides that did not cause any change of amino acid. Therefore, it did not change the function of the oxidase I cytochrome gene (Zein & Prawiradilaga, 2013). Zhang et al. (2010) added that other characteristics of mtDNA found in the bases content percentage.

In humans, MTs was encoded by a group of genes located on 16q13 chromosome consists of ten functional isoform MT. The encoded protein was divided into four groups: MT1, MT2, MT3 and MT4 protein (Krześlak et al., 2014; Mehus et al., 2014). Humans' MT isoforms have tissue-specific expression patterns. MTs expression increases in the response of variety inducers such as metals, interleukins, interferons, tumor necrosis factor alpha and glucocorticoid hormones (Subramanian et al., 2016; Dziegiel et al., 2016; Maret, 2011). MT could resist stress due to metal exposure by non-specific binding through metal exchange reactions in protein binding sites (Niederwanger et al., 2017). Moreover, high constitutive expression levels can also contribute to the tolerance of heat and cold shock by MTs genes (Gonçalves et al., 2016; Pedrini-Martha et al., 2016; Baurand et al., 2016; Salice et al., 2017).

The analysis results of DNA fragment sequence with the BLAST program indicated that the isolated MT2A gene was still uncertain. This was shown by the score obtained which was less than 150. The level of homogeneity was measured by scores (bits) and E value. If the score value (bits) was higher, the level of homology was also higher. Otherwise, if E value was lower, the gene was more similar with those in the Gene Bank. Similarity indicates the homology of a gene or protein. Nucleotide alignment results elucidate that the sequence of metallothionein coding genes has a similarity of 25% with encoding genes sequences. Two genes from DNA fragments are homologous if 70% of the nucleotide or 25% of the amino acids sequence are identical, with a minimum sequence length is 100 (Claverie & Notredame, 2003). Thus the MT2A gene isolation results showed that MT2A gene was not found because there were no similarities with Gene Bank.

This isolation failure might be caused by several things, such as laboratory equipment contamination, reagent and primary contamination, isolation protocols which were less obeyed, and the use of PCR cycles which have not been matched. Nevertheless, another possibility was that the low level of lead and cadmium in blood; thus, it did not induce the appearance of the metallothionein gene, because of a limited ability to induce metallothionein synthesis. Klaassen et al. (2009) stated that exposure of heavy metal cadmium will induce metallothionein when the concentration is above the threshold. Gas stations officers are likely to be exposed to lead and cadmium metals, causing various biological dysfunctions because cadmium affects some biological processes. If workers are exposed to lead and cadmium with concentrations below the threshold, symptoms of long-term cadmium accumulation may begin to appear gradually and must be monitored carefully. Therefore, there are still efforts to use the transcription level of the blood metallothionein gene (mRNA) as a metal exposure biomarker that has been applied for almost two decades (Miura, 2009). Furthermore, Ryvolova et al. (2011) said that due to the increase in metal binding capacity, it was suggested to use MT as a biomarker for environmental and biological monitoring of metal exposure. The sensitivity of the MTs gene expression regulation by heavy metals exposure makes it possible to use the MTs gene transcription quantification as a biomarker to evaluate heavy metal pollution (Wang et al., 2014).

CONCLUSION

Based on the molecular analysis, it concluded that the MTs gene cannot be used as early detection of susceptibility to heavy metals lead and cadmium exposure in the blood of gas station workers. Further research through RNA examination is required to see the expression of MTs genes.

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