

## EXPRESSION AND GENOTYPE OF MANGANESE SUPEROXIDE DISMUTASE IN LUNG CANCER CELLS OF SMOKER PATIENTS

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### Abstract

Although the most common cause of lung cancer is long-term exposure to tobacco smoke, the role of genetic factor for the cell defense mechanism, such as MnSOD, should also be considered. This study aims to analyze the expression and genotype of MnSOD in lung cancer cells of smoker patients. Samples were normal and lung cancer cells of patients operated in Persahabatan Hospital from May to December 2008, as well as lung cancer cells extracted from FFPE collection. Leukocyte cells of healthy smoker subjects were used as controls. The MnSOD mRNA expression was analyzed using Real Time RT-PCR and the specific activity using xantin oxidase inhibition assay. The genotyping was performed using PCR-RFLP. The result showed that the MnSOD specific activity in lung cancer of smoker patients is higher than in leukocyte cells of smoker controls. Compared to the expression of MnSOD in the normal lung cells of patients, in the lung cancer cells the level of MnSOD mRNA was lower, whereas its specific activity was higher (1.988 times). The samples from lung cancer patients have a Val/Val genotype frequency of 100%. In this study, we could conclude that MnSOD expression is altered in lung cancer cells.

*Keywords: enzyme specific activity, genotype, gene expression, lung cancer, MnSOD, smoker*

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### Introduction

Lung cancer is the most common cause of cancer-related deaths in men (80%) and women (75%) all over the world. The incidence of lung cancer increased in line with the increase in cigarette consumption.<sup>1</sup> Cigarette/tobacco contains about 4000 organic substances potentially generating ROS which are toxic, reactive and carcinogenic.<sup>2</sup> Relative risk of an active smoker to suffer from lung cancer is 12,8-20 greater than that of non-smoker.<sup>1,2</sup> Although most of lung cancer patients are smoker or ex-smoker (~90%)<sup>2</sup>, but in fact many of them are also non-smoker.<sup>3</sup> Thus, the role of genetic factor for the cell defense mechanism against lung cancer should also be considered.

Superoxide Dismutase (SOD) is an antioxidant enzyme which could hydrolyze the reactive anion superoxyde ( $O_2^-$ ) free radical into less reactive hydrogen peroxide ( $H_2O_2$ ). Among three isoforms of human SOD enzymes, MnSOD (*Manganese Superoxide Dismutase*), the major isoform found in mitochondrial matrix, is the major endogenous antioxidant that primarily protects cell

against the anion superoxide -the most reactive oxygen species (ROS)- since mitochondria is the main source of ROS production in the cell.<sup>4</sup> Previous studies have reported that lowering MnSOD level in mitochondria would increase the ROS level which could induce oxidative stress and lead to oxidative damage of biological molecules, such as protein, lipid, carbohydrate and DNA. Oxidative stress could implicate various degenerative diseases, particularly cancer.<sup>5</sup>

Several studies showed that tumor cells have MnSOD activity lower than the normal one.<sup>6</sup> Coursin et al. has reported that the activity of MnSOD in lung cancer was almost not detected.<sup>7</sup> It has also been demonstrated that the increase in MnSOD expression could suppress the growth of various cancer cells, inhibit malignant transformation induced by radiation, and inhibit metastasis of cancer cells, thus suggesting the pivotal role of MnSOD as a tumor suppressor.<sup>8-10</sup>

However, this suggestion is still controversial since other studies showed that high MnSOD expression was

correlated with the bad prognosis.<sup>11</sup> MnSOD gene consists of 5 exons and 4 introns and is located in 6q25.<sup>9</sup> MnSOD gene polymorphism has been reported in human population, i.e. Ala16Val and Ile58Thr. In the Ala16Val polymorphism, Alanin (GCT) is substituted by Valin (GTT) at the 16<sup>th</sup> amino acid of *mitochondrial targeting sequence* (MTS) of human MnSOD. This mutation affects the MnSOD transport into mitochondrial matrix. Sutton et al. (2003) has reported that Ala-MnSOD 30-40% is more active in mitochondrial matrix than the Val-MnSOD.<sup>10</sup> Furthermore, Val homozygote alleles were also related to lung cancer among Caucasians.<sup>11</sup>

Our previous study in 196 healthy subjects in Jakarta demonstrated that Val allele of MnSOD was the dominant allele (allele frequency of 98%; *in press*), whereas the frequency of Ala allele was only 2%. The most frequent MnSOD genotype found in population in Jakarta was Val/Val (95,9%), and the heterozygote genotype (Ala/Val) was only 4,1%.<sup>12</sup> Therefore, further studies should be done in order to determine whether the dominant Val allele of MnSOD in Jakarta correlates with the risk factor of cancer or other degenerative diseases.

Until now, there is no data about the genetic polymorphism of MnSOD in lung cancer patients in Indonesia. The aim of this study is to analyze the genotype and gene expression (mRNA and enzyme specific activity) of MnSOD in lung cancer cells. The history as a smoker (more than 10 years) known to be a risk factor for lung cancer was included in the criteria for the subjects used in this study. We hope that through this study we could recommend the use of MnSOD genotyping as a diagnostic test to determine the risk factor for lung cancer in smokers, as well as the MnSOD supplementation or gene therapy for the prevention and treatment of lung cancer patients.

## Methods

This study which is designed as a cross-sectional and case-control study was carried out in the Biochemistry and Molecular Biology Laboratory, FKUI, from May 2008 to February 2009. Samples were lung cancer and normal cells of patients operated in Persahabatan Hospital Jakarta from May to December 2008, and lung cancer cells extracted from formalin fixed paraffin-embedded (FFPE) collection of Persahabatan Hospital. Leukocyte cells of healthy subjects aged more than 40 years with a history as smoker for more than 10 years were used as smoker controls.

Extraction of DNA, total RNA and protein from tissue samples was carried out using *Tripure Isolation Kit* (Roche®) and from FFPE samples using *Highpure Isolation Kit* (Roche®). Ala16Val genotyping analysis

of MnSOD was performed for the lung cancer and normal cells of patients, as well as for the leukocyte cells of smoker control, using PCR-RFLP according to Cai et al. (2004).<sup>13</sup> The PCR components were 25µL of PCR Master Mix® (Promega) which contained 50 U/mL Taq DNA polimerase in buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dTTP and 3mM MgCl<sub>2</sub>, and 40 pmol of primers: 5'-ACCAGCAGGCAGCTGGCGCCGG-3' and 5'-GCGTTGATGTGAGGTTCCAG-3'. PCR reaction started with denaturation at 95 °C for 15 minutes, followed by 35 cycles of denaturation (94 °C; 30"), annealing (60 °C; 30") and extension (72 °C; 30") and finally the last extension at 72 °C for 7 minutes. In order to differentiate the Ala and Val alleles, the 107 bp of amplicon was subsequently restricted using 1 Unit of *NgoMIV* endonuclease for 16 hours at 37 °C. The restriction products were visualized using 3% agarose gel electrophoresis and ethidium bromide staining. Analysis of mRNA expression of MnSOD was performed using *Real Time RT-PCR* and *iScript Sybr Green One Step RT-PCR kit*® (BioRad).

Reference gene used in this study was 18SrRNA gene. MnSOD and 18SrRNA primers were designed using *Primer3* program based on nucleotide sequences in the gene bank NM\_000636 and X03205. Level of mRNA expression in lung cancer cells was relatively determined using Pfaffl formula and normalized against normal lung cells of particular patients. Aquabidest was used as a negative control (NTC) to reduce the false positive result. The cDNA were 216 bp for MnSOD and 155 bp for 18SrRNA.

The MnSOD enzyme specific activity was biochemically determined using RanSOD® kit. To inhibit the Cu/ZnSOD, firstly natrium cyanide (5 mM) was added into each sample and the mixture was incubated for 5 minutes in room temperature. Xantin oxidase was then added into the mixture, followed by the measurement of light absorbance using spectrophotometer at 505 nm after 30 seconds and 3 minutes. The enzyme activity was calculated as a percentage inhibition of the samples plotted to the standard curve. The specific activity of MnSOD enzyme was calculated as enzyme activity (in Unit) per mg protein. Protein concentration was measured using spectrophotometer at 280 nm and plotted to the BSA (Bovine Serum Albumin) standard curve.

## Results and Discussion

Ethical clearance of this study has been approved by Medical Ethics Committee FKUI No: 186/PT02.FK/ETIK/2008.

Lung cancer samples collected from patients within 8 months were totally only 3 and from the formalin fixed

paraffin-embedded were 15 samples. Vein blood samples from smoker controls were 15 samples.

MnSOD expression was analyzed through a relative mRNA level and an enzyme specific activity. The relative mRNA level was determined using *Real-Time RT-PCR*. To compare the result, total RNAs used as templates for the whole RT-PCR experiments should have an equal amount (~300 ng). The melting curve of RT-PCR products showed one peak for MnSOD or 18SrRNA (reference gene), suggesting that the primers used for both genes were adequate and specific.

As shown in Figure 1, one band of DNA was observed for MnSOD (216 bp) and 18SrRNA (155 bp), but no band appeared for the blank, indicating that the bands shown in this gel electrophoresis were specific primers targeted cDNAs for both genes.

The ratio of relative mRNA level of MnSOD in the lung cancer cells was lower (0.382) than that in normal lung cells of patients (Figure 2). The suppression of mRNA synthesis of MnSOD suggests the role of this enzyme as a tumor suppressor.<sup>14</sup> Surprisingly, the mean ratio of MnSOD specific activity in the lung cancer cells was higher (1.988) compared to that in the normal lung cells (Figure 2). This contradictory result indicates that the MnSOD enzyme activation could occur during post-translation, through covalent modification or the effect of activator. The enhancement of MnSOD specific activity in lung cancer cells might be one of the cell responses against the exaggerated attack of ROS particularly in smokers.

It could also be assumed that this would be a defense mechanism of cancer cells to avoid the apoptosis and indicated a bad prognosis of cancer.<sup>11</sup>

If the MnSOD specific activity in lung cancer cells of smokers was compared to that in the leukocyte cells of control, we obviously found that this activity in lung cancer cells was significantly higher (6.6261 X), as shown in Figure 3. This result also supports the assumption that the enhancement of MnSOD activity was rather caused by its role on the defense of cancer cells to escape from apoptosis.

Figure 4 shows the result of MnSOD genotyping. In the 3% agarose gel electrophoresis, Ala/Val genotype appears as 2 bands (107 and 89 bp), whereas Val/Val genotype as 1 band (107 bp).

Similar with our previous result about MnSOD genetic polymorphism in healthy subjects in Jakarta, in this study Ala/Ala genotype could not be found in all samples. Data of the whole genotyping analysis of MnSOD were summarized in Table 1 and compared with our previous result. Obviously, we found only Val/Val genotype (100%) in lung cancer cells either from patients or from FFPE, and also in normal lung cells.

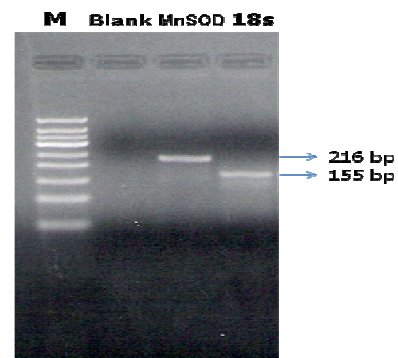


Figure 1. Electrophoresis (3% Agarose gel) of Real-Time RT-PCR Product

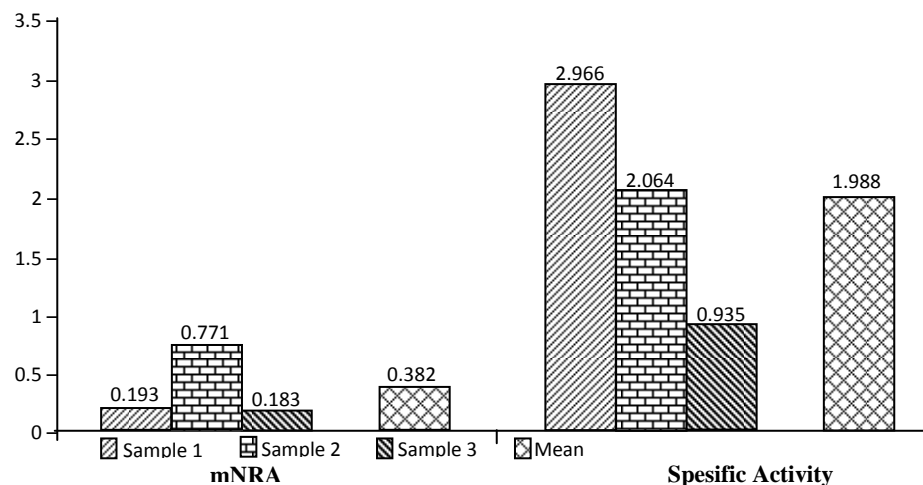


Figure 2. Ratio of MnSOD mRNA and Specific Activity In Lung Cancer Cells Compared to Normal Lung Cells of Patients

Compared to the data above, in leukocyte cells of smoker controls the frequency of Val/Val genotype was 86.3%, whereas Ala/Val was 13.3%. It should be noted that the Ala/Ala genotype could not be found in all the samples of this study and also in our previous studies analyzing the MnSOD genetic polymorphism in healthy subjects and other cancer patients, such as glioma, cervix cancer and retinoblastoma (Wanandi, et al., *in press*).<sup>12</sup>

In this study, Ala/Val and Ala/Ala genotypes could be found neither in lung cancer cells of patients nor from FFPE (Table 1). This result seems to confirm the previous study which reported that homozygote Val alleles were related to the incidence of lung cancer among Caucasians.<sup>15</sup> However, this would not be consistent with the result of MnSOD specific activity, which showed a higher activity in lung cancer samples compared to the activity in control samples. This might be the reason why previous studies about MnSOD genetic polymorphism correlate the frequency of genotype/ alleles merely with the incidence of diseases, but not with the enzyme activity. The MnSOD specific activity is not merely determined by its genotype, but the involvement of other factors on the enzyme activity

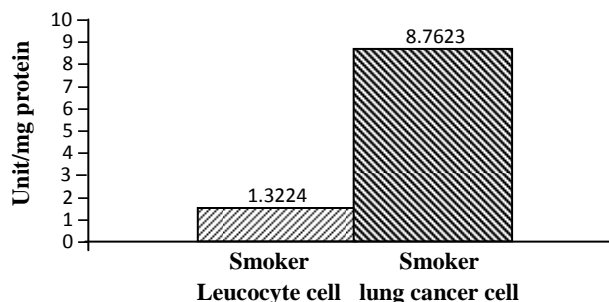


Figure 3. MnSOD Specific Activity in Lung Cancer Cells and Leukocyte Cells of Smokers

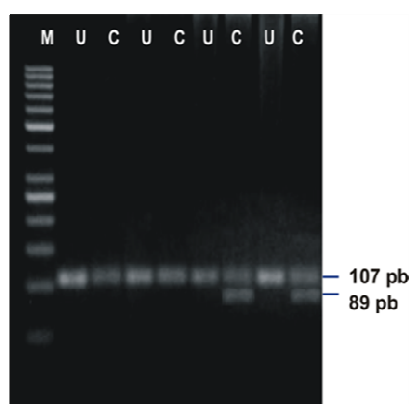


Figure 4. Genotyping Analysis of MnSOD Using PCR-RFLP. Visualized by 3% Agarose Gel Electrophoresis. M: Marker DNA; U: Uncut DNA (PCR Product); C: Cut DNA (RFLP Product)

Table 1. Genotype Frequency of Ala16Val-MnSOD

Samples	Ala/Ala	Ala/Val	Val/Val
Lung cancer cells of patients (3 samples)	0%	0%	100%
Normal lung cells of patients (3 samples)	0%	0%	100%
Lung cancer cells of FFPE (15 samples)	0%	0%	100%
Leukocyte cells of smoker controls (15 samples)	0%	13.3%	86.7%
Leukocyte cells of healthy population in Jakarta (Wanandi et al., <i>in press</i> ) (196 samples)	0%	4.1%	95.6%

should also be considered, such as nutrition supplement (in particular  $Mn^{2+}$ ), exposure to ROS from environment, enzyme activator or inhibitor and other epigenetic factors.

Nevertheless, this study was only supported by the data from lung cancer cells of 3 patients. Obviously, most of the lung cancer patients in Persahabatan Hospital were found in advanced stages, thus they could not be operated (*inoperable*). This problem would certainly limit the amount of lung cancer samples obtained in this study. It was initially thought that the use of lung cancer samples from FFPE would solve this problem, but in fact the FFPE lung cancer samples from Persahabatan Hospital did not include the normal lung cells. Therefore, we could not use these samples for the determination of mRNA level, since the normal lung cells were required as a calibrator to calculate the relative mRNA level. In this study, the gene expression of MnSOD in FFPE lung cancer samples could only be analyzed at the level of enzyme specific activity.

## Conclusion

In this study, we conclude that the mRNA expression of MnSOD in lung cancer is lower than in normal lung cells, thus supporting the role of MnSOD as tumor suppressor. However, its specific activity in lung cancer is higher than in normal lung cells, indicating the involvement of other factors (including the epigenetic factors) on the activation of enzyme post translation. Lung cancer cells observed in this study have a Val/Val-MnSOD genotype frequency of 100%, and no Ala/Val genotype in contrast to the leukocyte cells of smoker controls (13.3%). The MnSOD specific activity in lung cancer of smoker patients is higher than in leukocyte cells of smoker controls, suggesting the cell response against the exaggerated attack of ROS particularly in smokers. Further studies are required to analyze the correlation of MnSOD specific activity with oxidative stress in leukocyte cells of smoker, as well as with the

epigenetic factors which influence the MnSOD specific activity.

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