

Gene expression of manganese superoxide dismutase in human glioma cells

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Abstrak

Tujuan Menganalisis ekspresi MnSOD pada sel glioma manusia yang dibandingkan dengan sel leukosit sebagai kontrol sel normal, sehingga dapat mengetahui peran MnSOD sebagai antioksidan endogen yang diduga sebagai supresor tumor.

Metode Ekspresi MnSOD dianalisis dengan mengukur mRNA MnSOD secara kuantitatif dan aktivitas spesifik enzim MnSOD. Ekspresi MnSOD dideteksi pada 20 pasien glioma dengan menggunakan Real Time RT-PCR untuk mRNA MnSOD dan pemeriksaan biokimia untuk mengukur aktivitas spesifik enzim MnSOD (kit RanSOD). MnSOD pada leukosit digunakan sebagai kontrol. Analisis statistik yang digunakan yaitu uji Kruskal Wallis.

Hasil Kadar relatif mRNA MnSOD sel glioma 0,015–0,627 kali lebih rendah dibandingkan dengan sel leukosit sebagai kontrol pada 70 % distribusi sampel, 10 % distribusi sampel menunjukkan nilai 1,002–1,059 serta 20 % distribusi sampel menunjukkan kadar relatif mRNA MnSOD 1,409–6,915 kali lebih tinggi dibandingkan dengan kontrol. Begitu pula dengan kadar relatif aktivitas spesifik enzim MnSOD pada sel glioma 0,064–0,506 kali lebih rendah dibandingkan kontrol pada 80 % distribusi sampel serta 20 % distribusi sampel menunjukkan nilai 1,249–2,718 kali lebih tinggi.

Kesimpulan Ekspresi gen MnSOD baik mRNA maupun aktivitas spesifik enzim MnSOD pada sebagian besar sampel sel glioma manusia lebih rendah secara signifikan dibandingkan dengan sel leukosit. (*Med J Indones 2010; 19:21-5*)

Abstract

Aim This study analyze the MnSOD gene expression as endogenous antioxidant in human glioma cells compared with leucocyte cells as control.

Methods MnSOD gene expression of 20 glioma patients was analyzed by measuring the relative expression of mRNA and enzyme activity of MnSOD in brain and leucocyte cells. The relative expression of mRNA MnSOD was determined by using quantitative Real Time RT-PCR and the enzyme activity of MnSOD using biochemical kit assay (xantine oxidase inhibition). Statistic analysis for mRNA and enzyme activity of MnSOD was performed using Kruskal Wallis test.

Results mRNA of MnSOD in glioma cells of 70 % sample was 0.015–0.627 lower, 10 % was 1.002–1.059 and 20 % was 1.409–6.915 higher than in leucocyte cells. Also the specific activity of MnSOD enzyme in glioma cells of 80 % sample showed 0,064–0,506 lower and 20 % sample was 1.249–2.718 higher than in leucocyte cells.

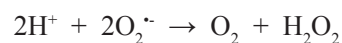
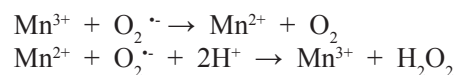
Conclusion MnSOD gene expression in human glioma cells are significantly lower than its expression in leucocytes cells. (*Med J Indones 2010; 19:21-5*)

Key words : MnSOD, glioma, gene expression

Glioma is the primary brain tumor frequently found in Indonesia. High grade gliomas such as glioblastoma multiforme often recur within 6 months after surgery, and despite radiation therapy and chemotherapy, 80 % of patients die within one year. Although ionizing radiation is the most effective treatment modality for malignant gliomas, many of these tumours are resistant to radiation therapy. Therefore, additional therapy, such as gene therapy is needed to improve therapy response.¹

Manganese Superoxide Dismutase (MnSOD) is a mitochondrial enzyme that catalyze the conversion

of O₂⁻ to oxygen (O₂) and hydrogen peroxide (H₂O₂). MnSOD is the major antioxidant enzyme that scavange superoxide radical, because electron transport chain in mitochondria is the major reactive oxygen species (ROS) production. MnSOD neutralize anion superoxide by two step dismutase reaction. At this process, Mn³⁺ is reduced and then oxidized.²



The decrease in the MnSOD level could elevate the ROS level in mitochondria, leading to oxidative stress including oxidative damage in biomacromolecules, such as protein, lipid and DNA.² For the past 20 years, MnSOD activity has been postulated to be low in malignant tumours. Tumour cells generate superoxide and other ROS, and this generation, if it also occurs *in vivo*, might generate heighten tumour cell proliferation and invasion. It has been hypothesised that the production of ROS combined to a decreased antioxidant enzyme level may be characteristic of tumour cells.³

There is a great amount of experimental data to suggest that the MnSOD expression in cancer cells were low and the malignant phenotype of a cancer cell can be suppressed by raising the MnSOD level. Therefore, it has been hypothesised that the MnSOD gene is a tumour suppressor.⁴ Overexpression of MnSOD by gene transfection has been shown to suppress a variety of disorders, including radiation-induced neoplastic transformation of mouse embryonic fibroblasts, the metastatic capacity of mouse fibrosarcoma cells and human breast cancer cells, and the growth rate of human melanoma cells, human transformed fibroblasts, rat glioma cells, hamster cheek pouch carcinoma cells, and human prostate carcinoma cells.⁵⁻⁸

Nevertheless, the role of MnSOD as a tumour suppressor is still controversial, because explain another study showed that MnSOD expression is high in some of human cancers, include in glioma.⁹ The level of MnSOD directly correlates with grade of some tumours and high MnSOD is associated with poor prognosis in some tumours.⁹ In addition, if MnSOD is really anti-apoptotic, as it has been observed, MnSOD overexpression in tumours could offer a survival advantage to a tumour cell and lead to treatment resistance.¹⁰

Regarding the difference of MnSOD gene expression in human cancer cells, we want to clarify confirm the expression of MnSOD in human glioma cells compared with leucocyte cells as control, so we can explore the role of MnSOD. MnSOD expression was analyzed with measuring mRNA of MnSOD and enzyme activity of MnSOD.

METHODS

This study was cross sectional study carried out at Biochemistry and Molecular Biology Laboratory, Faculty of Medicine University of Indonesia within 12

months (April 2007 – March 2008) using 20 of patients as subjects. The inclusion criteria were a glioma patient with informed consent, who was diagnosed glioma by radiology examination and underwent open surgery craniotomy. The exclusion criteria was a subject with the result of histological examination did not show glioma. Glioma cells samples were obtained by open surgery craniotomy, samples were stored at -70°C or homogenized directly. Leucocyte cells were obtained from blood venous of patients before radiotherapy treatment. Whole blood were collected in EDTA tube to prevent the blood from clotting. Samples were stored at 2–8 °C for not more than 24 hours prior to RNA isolation procedures. Procedures followed were approved by Research and Development Committee, Faculty of Medicine University of Indonesia (No. 235/PT.02.FK/ETIK/2006).

RNA Isolation from glioma tissues and from whole blood using Aquapure RNA Isolation Kit (BioRad®). The step of RNA isolation followed the instruction that kit were cell lysis, protein-DNA precipitation, RNA Precipitation and RNA hydration. RNA hydration samples were stored at -70°C until use.

Amplification cDNA using Reverse Transcriptase Real Time PCR

RNA samples (both from glioma tissues and whole blood) were amplified using *iScript One Step RT-PCR Kit with SYBR Green* (BioRad). cDNA synthesis and PCR amplification were carried out in the same tube. Reaction protocols were cDNA synthesis for 10 minutes on 50°C, inactivation of *iScript Reverse transcriptase* for 5 minutes on 95°C, PCR cycles (40 cycles) for 10 seconds on 95°C; 30 seconds on 59°C (after optimalization); 30 seconds on 72°C, *Melt curve analysis* for 1 minutes on 95°C; 1 minutes on 55°C; 10 seconds on 55°C (80 cycles, increase 0,5°C every cycles). Primer for MnSOD gene were gcaactagcagcatgttgagc (Forward) and actctcctcggtgacgttc (Reverse) with amplicon size were 216 bp. 18S rRNA was used as external standard, amplification procedures same as MnSOD gene. Primer for 18S rRNA were aaacggctaccacatccaag (Forward) and cctccaatggatcctcgta (Reverse) with amplicon size 155 bp. Primers for this reaction were design by Primer3 programme and Primer Analysis software. Sequence for MnSOD (NM_000636) and for 18S rRNA (X03205) were obtained from NCBI Gene bank. Aquabidest were used as a negative control (NTC) to reduce the false positive result. Level of mRNA expression in glioma cells was relatively determined using Pfaffl formula and normalized against leucocyte cells as normal control cells of particular patients.

The MnSOD enzyme specific activity

Tissue samples (10-20 mg) were homogenized in 600 μ l *Nuclei Lysis Solution*, Promega[®] on ice. The mixture was incubated on 65 °C for 10 minutes. The homogenate tissue kept at -20°C before use. Whole blood (200 μ l) was added into 600 μ l of red blood cell lysis solution (Promega). The mixture was incubated for 10 minutes in room temperature and centrifuged at 16000 rpm for 20 seconds. The white pellet containing leucocyte cells was collected and lysis with 200 μ l of nuclei lysis solution (Promega). Lysates were diluted 5 times with PBS and kept at -20°C before use.

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 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. The results of MnSOD mRNA are a ratio or relative mRNA expression, so MnSOD specific activity of human glioma cells will be compared with leucocyte cells to get the ratio value.

RESULTS

Figure 1 shows melting curve analysis for MnSOD gene (A) and 18S rRNA (B). Electrophoresis results showed the product of amplification, only one band for MnSOD gene (216 bp) and for 18S rRNA (155 bp).

The result of mRNA MnSOD was defined by calculating threshold cycle (Ct) number (figure 2). The results showed that mRNA MnSOD in 70 % sample distribution of glioma cells was 0,015 – 0,627 lower than control, 10 % sample distribution was 1,002-1,059, and 20 % sample distribution was 1,409-6,915 higher than control. Also, the specific activity of MnSOD enzyme in 80 % sample distribution of glioma cells showed 0,064-0,506 lower than control and 20 % sample distribution was 1,249-2,718 higher than control (figure 3). The value of MnSOD mRNA & specific activity every glioma samples are compared with leucocyte as control, therefore the values of glioma samples are relative expression (ratio) with value “1” for control (figure 4). Analysis of statistic for mRNA and enzyme activity of MnSOD using Kruskal Wallis test was significant between groups of distribution sample ($p < 0.01$). Spearman's correlation analysis showed that there were linier relationship between mRNA and specific activity of MnSOD enzyme with moderately relationship and statistically significant ($R 0.699, p < .01$).

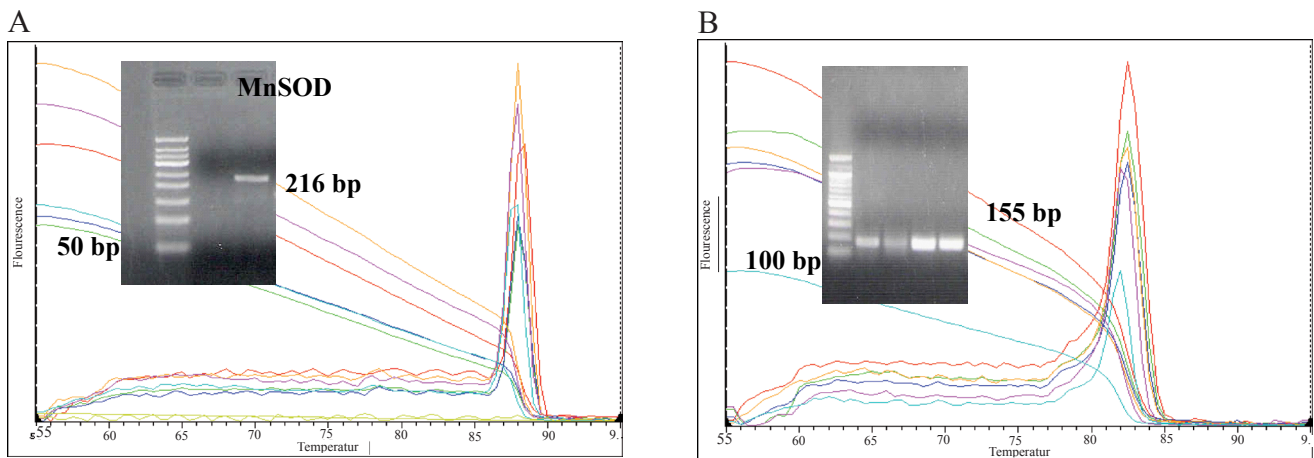


Figure 1. Melting Curve Analysis. Insert: Electrophoresis on 2 % agarose gel for (A) MnSOD gene (216 bp); (B) 18S rRNA gene (155 bp)

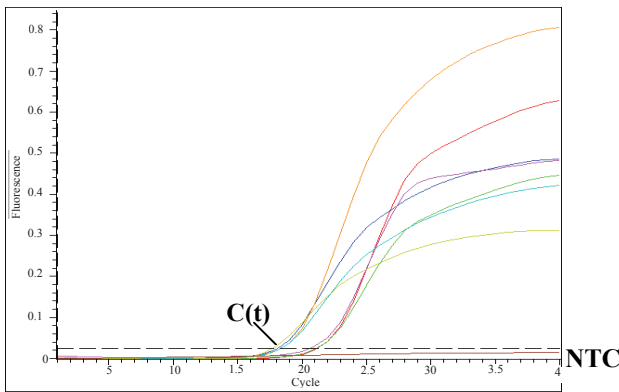


Figure 2. Fluorescent curve in RT-PCR. Ct: Cycle threshold, NTC: Non template Control

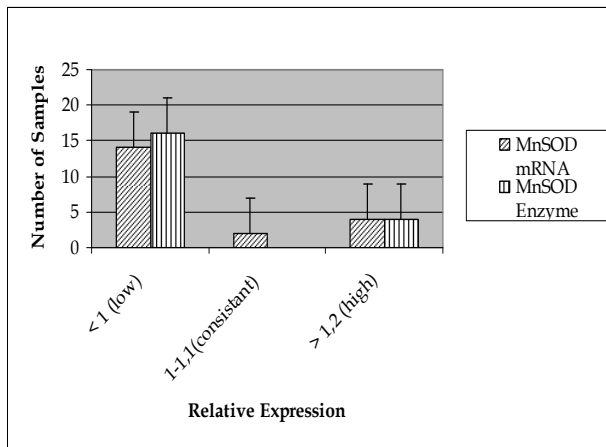


Figure 3. Samples distribution of MnSOD mRNA and MnSOD enzyme specific activity which is significantly different between groups ($p < .01$)

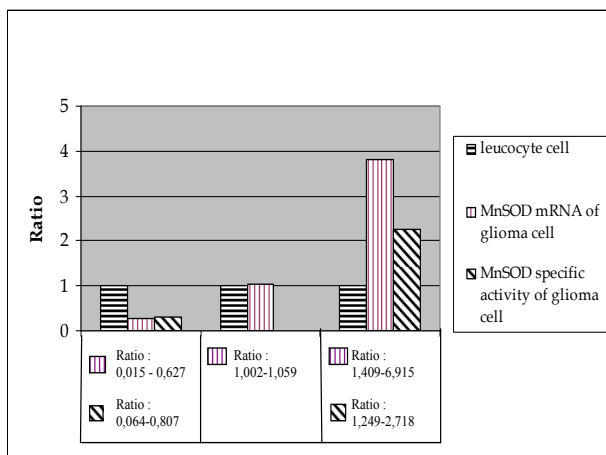


Figure 4. Relative Expression (ratio) of MnSOD mRNA and MnSOD enzyme specific activity in glioma cells which is compared with leucocytes cells as a control

DISCUSSION

The figure 1 showed one peak in melting curve analysis and one band in electrophoresis, which were meant that the primer was specific. The concept of the threshold cycle (Ct) is at the heart of accurate and reproducible quantification using fluorescence-based RT-PCR. Fluorescent values are recorded during every cycle and represent the amount of product amplified to that point in the amplification reaction. The more template present at the beginning of the reaction, the fewer number of cycles it takes to reach a point in which the fluorescent signal is first recorded as statistically significant above background. This point is defined as the Ct and will always occur during the exponential phase of amplification.

The results showed that mRNA MnSOD in 70% sample distribution of glioma cells was 0,015–0,627 lower than control, 10% sample distribution was 1,002-1,059 and 20% sample distribution was 1,409-6,915 higher than control. Also the specific activity of MnSOD enzyme in 80% sample distribution of glioma cells showed 0,064-0,506 lower than control and 20% sample distribution was 1,249-2,718 higher than control (figure 3). The value of MnSOD mRNA & specific activity in every glioma samples is compared with leucocyte as control, therefore the values of glioma samples are relative expression (ratio) with value “1” for control. (figure 4). Analysis of statistic for mRNA and enzyme activity of MnSOD using Kruskal Wallis test was significant between groups of distribution sample ($p < .01$). Correlation analysis using Spearmen’s correlation showed that there were linier relationship between mRNA and specific activity of MnSOD enzyme with moderately relationship and statistically significant ($R 0.699, p < .01$).

The results indicated that MnSOD gene expressions in most of human glioma cell are lower than leucocyte cells as a control. This study has the same result as the previous study which was explored the role of MnSOD as suppressor tumour which was found consistently low in cancer cell.⁴

The lowered of MnSOD mRNA expression has not been understood until now. Perhaps it was conducted with mutation. Mutation on MnSOD promoter can cause the MnSOD mRNA alteration. MnSOD mutation that was reported was mutation on promoter of MnSOD in breast cancer.⁴ However, the mutation on glioma MnSOD promoter has not been found until now.

The MnSOD specific activity in this research was equally low. The lowered of MnSOD specific activity may related with polymorphism and redox state. be what other study specify clearly suggest that genetic polymorphism in the human MnSOD gene may be associated with a risk of developing cancer.¹¹ Two polymorphic variants at codon 16 that lead to incorporation of either alanine or valine in the mitochondrial targeting sequence are frequently detected in humans. The valine at this position was predicted to change the mitochondrial targeting sequence conformation from the preferred amphiphilic helix to a combination of an amphiphilic helix and a β -pleated sheet, possibly interfering with the transport efficiency of the mature MnSOD protein, potentially decreasing its functional effectiveness.¹² Moreover, the lowered of MnSOD specific activity in this study maybe was related with redox state. The lowered antioxidant capacity and the oxidant-antioxidant imbalance have been considered to play a role in multistage carcinogenesis.³ Tumour cell produce high reactive oxygen species (ROS) and MnSOD as antioxidant has a function to reduce ROS. As a results, so the lowered activity of MnSOD in this study was caused by consumption this enzyme. Tumour cells may overproduce ROS because the NADPH-oxidase is regulated by the GTPase RAcI, which is a downstream of the proto-oncogene Ras.¹³

In this study, there was 20 % of sample distribution which has higher MnSOD gene expression (both mRNA and activity MnSOD). This condition may be conducted with grade of tumour malignancy and the responses of therapy treatments. Other studies also found the following results: (1) the higher MnSOD expression in many human cancers, (2) the level of MnSOD directly correlates with the grade of some tumours, and (3) high MnSOD is associated with poor prognosis in some tumours.⁹ The high levels of MnSOD expression by tumor cells could impair the patient's tumour immunosurveillance capability. Those studies would predict that malignant gliomas constitutively expressing a high level of MnSOD in vivo would be resistant to endogenous TNF α -mediated tumor killing. If this were found to be true in vivo, tumours producing MnSOD may evade the host tumor immunosurveillance mechanisms.⁹ Therefore, the further studies are required to analyze the correlation this MnSOD expression with grade of tumour malignancy and therapy response.

In conclusion, MnSOD gene expression in human glioma cells are significantly lower than its expression in leucocytes cells.

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