Downregulation of catalase by reactive oxygen species via hypermethylation of CpG island II on the catalase promoter

Ji Young Min, Seung-Oe Lim, Guhung Jung*

The Department of Biological Sciences, College of Natural Sciences, Seoul National University, Seoul 151-742, South Korea

ABSTRACT

Catalase, which decomposes reactive oxygen species (ROS), is reduced in hepatocellular carcinoma (HCC); however, the reasons are poorly defined. In this study, it is demonstrated that prolonged exposure to ROS induced methylation of CpG island II on the catalase promoter and downregulated catalase expression at the transcriptional level in HCC cell lines. In addition, hypermethylation of CpG island II was also observed in tumor tissues, together with a decrease in catalase mRNA and protein expression levels when compared to non-tumor tissues. From these data, we suggest that ROS may downregulate catalase through the methylation of promoter during the development of HCC.

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1. Introduction

Catalase is a 240-kDa homotetrameric enzyme that catalyzes the decomposition of extracellular and intracellular hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) and plays a key role in protecting cells against reactive oxygen species (ROS) [1]. The expression of catalase is decreased in hepatocellular carcinoma (HCC), as well as in other types of cancers [2,3]. A loss in catalase activity during cancer development is associated with tumor formation and metastasis [4-6].

ROS, such as superoxide radicals, hydroxyl radicals, and H\textsubscript{2}O\textsubscript{2}, contribute to tumor progression by promoting DNA damage and/or altering cell signaling pathways [7]. ROS are involved in tumor metastasis, which is a complex process involving epithelial-to-mesenchymal transition, migration, invasion, and angiogenesis within the tumor microenvironment [8].

Epigenetic gene silencing can be caused by an aberrant methylation of gene promoters [9-11], and it plays an essential role in the regulation of chromatin structure formation and gene expression in general. DNA methylation, which can result in the loss of gene function, also promotes cancer development by repressing tumor suppressor genes [12].

HCC is a common fatal malignancy that results in approximately one million deaths per year, worldwide [13]. Oxidative stress is the most important causative factor of HCC [14].

The present study shows that catalase is epigenetically silenced in HCC cell lines following prolonged exposure to ROS. Furthermore, catalase was found to be downregulated in tumor tissues when compared with non-tumor tissues. This difference in expression level may be attributed to differences in the methylation status of the catalase promoter.

2. Materials and methods (see Supplementary data)

2.1. Cell culture

The human hepatoma cell lines Hep3B and Huh7 were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA). For ROS treatment, the medium was changed daily for fresh medium with 10% FBS and the cells were incubated for 4 days with 300 \textmu M H\textsubscript{2}O\textsubscript{2} and 2 \textmu M phenazine methosulfate (PMS). For some experiments, the cells were pretreated with 5 \textmu M N-acetylcycteine (Sigma–Aldrich, St. Louis, MO, USA) and 2 \textmu M 5-aza-2’-deoxycytidine (5-aza-dC) (Sigma–Aldrich) for 30 min before the addition of H\textsubscript{2}O\textsubscript{2} and PMS.

Abbreviations: 5-aza-dC, 5-aza-2’-deoxycytidine; DNMT, DNA methyltransferase; HCC, hepatocellular carcinoma; MSP, methylation-specific polymerase chain reaction; ROS, reactive oxygen species; PMS, phenazine methosulfate; BS, sodium bisulfite genomic sequencing

* Corresponding author. Address: Department of Biological Sciences and Seoul National University, 504-408 Daehak-dong, Gwanak-gu, Seoul 151-742, South Korea. Fax: +82 2 872 1993.
E-mail address: djung@snu.ac.kr (G. Jung).
2.2. Methylation-specific polymerase chain reaction (MSP)

One microgram of genomic DNA was treated with sodium bisulfite using the EpiTect Bisulfite kit (Qiagen, Valencia, CA, USA). To analyze the methylation status of the catalase promoter, primers for MSP were designed using the Methyl Primer Express software (Applied Biosystems, Foster City, CA, USA). The sequences of the oligonucleotide primers are provided in Supplementary Table 1.

2.3. Immunoblot analysis

The detailed methods for immunoblot analysis have been described previously [15]. Antibodies used in this study included

Fig. 1. Reactive oxygen species downregulate catalase expression in hepatocellular carcinoma (HCC) cell lines. (A) Distribution of CpG islands on the catalase promoter and the location of PCR primer binding sites. (B) Hep3B and Huh7 cells were treated with 300 μM H₂O₂ and 2 μM phenazine methosulfate (PMS) for 2, 4 and 6 days, after which methylation-specific PCR (MSP) was performed. SM, Size marker; U, unmethylated DNA; M, methylated DNA; PMS, phenazine methosulfate. (C) Bisulfite-modified DNA was amplified with sodium bisulfite genomic sequencing (BS) primers. h, Unmethylated CpG site; j, methylated CpG site. (D) Catalase expression was assessed by real-time RT-PCR. (E) Catalase expression was assessed by immunoblot analysis of cell lysates prepared as described in (B). (F) Catalase activity was measured using cell lysates prepared as described in Section 2. (G) The catalase promoter deletion constructs are schematically represented (left). Huh7 cells were cotransfected with each of the different methylated or non-methylated pGL3-serially-deleted catalase promoters, together with a Renilla luciferase expression vector (pRL-SV40) as a control for transfection efficiency. Cells were lysed 48 h after transfection, and the luciferase activity in the cell lysates was measured. Error bars represent the mean standard deviation (S.D.) of at least three experiments performed in duplicates.
anti-catalase (Abcam, Cambridge, MA, USA) and anti-β-actin (Sigma–Aldrich).

3. Results

3.1. ROS induce methylation of the catalase promoter

To determine the effects of long exposure to ROS, HCC cell lines were treated with H2O2 and PMS as ROS sources [15] for 6 days. The effect of these ROS on the methylation of the catalase promoter was analyzed using MSP analysis (Fig. 1A and B). We specifically selected the CpG island II region located in the catalase promoter and excluded the CpG island I because it was already methylated in HCC cells (see Supplementary Fig. 1). The methylation of CpG island II was observed 4 days after ROS treatment, as determined by MSP analysis and sodium bisulfite genomic sequencing (BS). The mean methylation status of control cells was approximately 1.11%, whereas that of H2O2-treated cells was 28.52% (Fig. 1C). Real-time RT-PCR, immunoblot analysis and catalase activity assay, performed with the same cells, demonstrated that catalase messenger RNA (mRNA) (Fig. 1D), protein (Fig. 1E) and catalase activity (Fig. 1F), respectively, were also reduced.

Thus, these results showed that prolonged exposure to ROS induced methylation of CpG island II on the catalase promoter and simultaneously downregulated catalase expression and activity in HCC cell lines.

To assess whether methylation of the catalase promoter causes any changes in its transcriptional activity, serial deletion analysis of catalase promoter constructs was performed using a dual luciferase assay in combination with an in vitro methylation assay. Each promoter-luciferase expression plasmid was mock-methylated or methylated with the CpG methyltransferase, M. SssI (see Supplementary Fig. 2), and used to transfet Huh7 cells. Methylation of the human catalase promoter suppressed the expression of the reporter gene although different promoter constructs had different levels of promoter activities (Fig. 1G). However, the activity of the M. SssI-methylated pGL3–basic plasmid was almost the same when unmethylated, implying that the M. SssI-mediated methylation reduced the catalase promoter-driven transcription specifically without affecting the vector. In addition, the insert DNA was digested, gel-purified, and then methylated or mock-methylated in vitro before religation with the KpnI/BgIII-digested pGL3 vector (see Supplementary Fig. 3); the recombinant plasmid was then transfected into Huh7 cells. Since, M. SssI-methylated not only the catalase promoter fragment but also the open reading frame (ORF) of the luciferase gene, we performed additional experiment.

These data show that the methylation of CpG sites on the catalase promoter caused a reduction in the promoter activity.

3.2. N-Acetylcysteine and 5-aza-dC restore catalase expression

Methylation of the catalase promoter was inhibited following the treatment of cells with N-acetylcysteine, an antioxidant or with

![Fig. 2. N-Acetylcysteine and 5-aza-2'-deoxycytidine restore catalase expression in ROS-treated HCC cell lines. (A) Hep3B and Huh7 cells were treated with 300 μM H2O2, 5 mM N-acetylcysteine and 2 μM 5-aza-dC for 4 days, after which MSP was performed using genomic DNA isolated from these cells. (B) Catalase expression was assessed by real-time RT-PCR. (C) Catalase expression was assessed by immunoblot analysis. (D) Catalase activity was measured using cell lysates of the cells treated as described in (A).](image-url)
5-aza-dC, a DNA methyltransferase 1 (DNMT1) inhibitor (Fig. 2A). Concomitantly, catalase mRNA (Fig. 2B), protein (Fig. 2C), and catalase activity (Fig. 2D) were restored in these cells. These results suggest a correlation between prolonged exposure to ROS stress, downregulation of catalase, and methylation of the catalase promoter. To determine whether catalase downregulation by ROS was reversible, cells were treated with H₂O₂ for 4 days and changed the fresh medium lacking H₂O₂, and then incubated for an additional 4 days. MSP analysis was performed to examine the methylation status of CpG island II on the catalase promoter. After removal of H₂O₂ from the medium, CpG island II was found to be demethylated (see Supplementary Fig. 4A). Catalase mRNA, protein expression and activity (see Supplementary Fig. 4B–D) were decreased upon ROS treatment, but 4 days after the removal of ROS treatment, these levels had again increased.

From these data, it was observed that ROS-mediated methylation of the catalase promoter and downregulation of catalase expression is reversible in HCC cell lines.

3.3. Methylation of CpG island II on the catalase promoter and downregulation of catalase expression in HCC tissues

To determine the methylation status of the catalase promoter, the promoter region in 30 samples of grade III HCC tissues were analyzed. CpG island I on the catalase promoter was found to be completely methylated in both tumor and non-tumor tissues. However, CpG island II was methylated at a greater frequency in tumor tissues than the non-tumor tissues (Fig. 3A). Twenty-one (70%) of the 30 tissues showed a decrease in catalase expression, while MSP analysis showed methylation of the catalase promoter in 73.33% (22/30) of the grade III tissues revealed (Fig. 3A). In the 21 cases in which catalase was downregulated, nineteen (63.33%) were accompanied by promoter methylation (Fig. 3A, Table 1). BS of the HCC tissues revealed that CpG island II was methylated to a higher extent in tumor tissues than in non-tumor tissues (Fig. 3B and see Supplementary Fig. 5). Because mRNA from these tissues was not available, we did not perform real-time RT-PCR on these tissues to determine the methylation status.

Table 1 Correlation between the decrease in catalase expression and methylation of CpG island II on the catalase promoter in HCC tissues.

| Grade | Characteristics | N = 30 |
|-------|-----------------|-------|
| HCC G III | Methylation of CpG island II on catalase promoter | 22/30 (73.33%) |
| HCC G III | Decrease in catalase expression | 21/30 (70%) |
| HCC G III | Decrease in catalase expression accompanied by promoter methylation | 19/30 (63.33%) |

G III, Edmondson-Steiner grade III.

Fig. 3. Methylation status of the catalase promoter and the expression of catalase in HCC tissues. (A) The methylation status of CpG islands I and II on the catalase promoter was determined by MSP of genomic DNA from grade III HCC tissues. SM, Size marker; N, non-tumor tissue; T, tumor tissue; U, unmethylated DNA; M, methylated DNA. (B) Methylation analysis of CpG island II present on the catalase promoter in tumor tissue and non-tumor tissue pairs as evaluated by BS (Supplementary data and Section 2). Ten clones from tumor tissue and 10 clones from non-tumor tissues were analyzed for CpG dinucleotide methylation. Each arrow represents a primer binding site. ☐, Unmethylated CpG site; ■, methylated CpG site. (C) Catalase expression was assessed by real-time RT-PCR. N, Number of cases. *P < 0.05. (D) Immunoblot analysis using catalase and β-actin-specific antibodies was performed with tumor and non-tumor tissues. An anti-β-actin antibody was used as a control for protein loading.
tissue samples was unavailable, we used other grade III tissues, in which CpG island II was methylated. Catalase expression was lower in grade III HCC tissues than in non-tumor tissues (Fig. 3C). This decrease in catalase expression in the tumor tissues was also confirmed by immunoblot analysis (Fig. 3D).

Altogether, these results indicate that a significant correlation exists between prolonged exposure to ROS stress, downregulation of the catalase expression, and methylation of the catalase promoter in HCC tissues.

4. Discussion

Here, we showed that ROS may downregulate the expression of catalase at the transcriptional level in HCC cells. We observed that DNA methylation abolished the transcriptional activity of the catalase promoter. Furthermore, we found downregulated catalase mRNA levels in human HCC tissues. These findings suggest that catalase downregulation by ROS occurred in both our ROS-treated cells and in patient tumor tissues. From the data of HCC cells and human HCC tissues we thus confirmed that exposure to ROS stress is significantly associated with catalase downregulation and methylation of the catalase promoter during the development of HCC.

In our previous study, we showed that changes in ROS stress markers, decreased the expression of antioxidant enzymes such as Cu/Zn superoxide dismutase (SOD) or catalase, and increased DNA damage and that these changes positively correlated with the differentiation grade of HCC, which, in turn, correlated with vascular invasion. An overexpression of catalase can inhibit the invasiveness of HCC cells as well as decrease the intracellular ROS levels [15]. These data, together with the data for changes in the degree of differentiation, imply that the greater the increase in ROS stress, the more extensive is the methylation of the catalase promoter. Thus, catalase plays an important role in inhibiting vascular invasion during tumor progression.

Many studies have shown that catalase expression may be regulated by aberrant methylation during the process of mouse skin tumors and that catalase activity is attenuated during malignant progression of mouse keratinocytes [16,17]. These studies, however, did not address the epigenetic relationship between ROS and the methylation status of the catalase promoter in tumor tissues. In the present study, we confirmed this relationship in human HCC cell lines, but not in mouse cell lines. Moreover, this is the first study to validate that the catalase promoter is methylated in cases of human HCC.

Ding et al. reported that 3.3% of the catalase promoter sequence is hypermethylated in HCC. In our study, however, 73.33% of the promoter sequence was methylated in HCC cells. This discrepancy in the methylation status is probably because different sites were analyzed in the two studies. Unlike the previous study, which focused on the region downstream (+118 to +372) of the transcription initiation site, our study focused on the region upstream (−204 to −14) of the transcription initiation site, on CpG island II [18].

The methylation frequency of the catalase promoter is substantially lower than that of promoters of other tumor suppressor genes that undergo methylation-based regulation, such as p21 and E-cadherin [15,19]. Previous studies also verified that methylation interferes with the binding of transcriptional activators to the promoter, thereby reducing the level of transcription. Several transcriptional activators bind to the catalase promoter [20,21]. Catalase downregulation could be regulated by an analogous mechanism in which the binding of the activator to the promoter is hindered by methylation.

Short-term exposure of HCC cells to ROS increases the expression of catalase (see Supplementary Fig. 6). However, prolonged exposure to ROS decreases catalase expression because the methylation of the catalase promoter. This finding implies that the cells probably lose their defense mechanism against ROS, which facilitates the progression of cancer. Because of the reduced capacity to eliminate ROS, ROS levels are increased. This process is accompanied by significant intracellular changes, such as reduction in E-cadherin levels, as observed by changes in the E-cadherin repressor Snail [15].

In this study, we did not investigate whether ROS directly cause methylation of the catalase promoter in tumor tissues. Further studies should focus on identifying the putative ROS-mediated pathway that affects DNA methylation. Nevertheless, our results strongly suggest that ROS affect the methylation status of the catalase promoter during carcinogenesis.

Thus, we propose the presence of a functional pathway involving ROS-induced epigenetic changes in which persistently elevated ROS induce methylation of CpG island II of the catalase promoter in HCC cells. We have validated our hypothesis with data from human tissues. Our findings may suggest new therapeutic approaches for curing HCC by targeting the catalase promoter.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.04.048.

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