Journal of Cellular Biochemistry 96:622–631 (2005)

Differential Expression of Antioxidant Enzymes in Various Hepatocellular Carcinoma Cell Lines

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Abstract Recent evidence suggests that reactive oxygen species (ROS) play an important role in the pathogenesis of various illnesses, and the ROS and antioxidant enzymes are highly associated with cell differentiation and diseases. In this study, we tested the hypothesis that specific antioxidant enzymes are differentially expressed in hepatocellular carcinoma (HCC) cell lines with various degrees of differentiation. We compared the expression of several antioxidant enzymes including catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GRx), and glutathione peroxidase (GPx) in five HCC cell lines with well (Hep G2 and Hep 3B) or poor (HA22T/VGH, HA55T/VGH, and SK-Hep-1) differentiation. Our results showed that both well-differentiated HCC cell lines expressed extremely higher CAT and GRx enzyme activities than all three poorly differentiated ones. Moreover, the protein and mRNA levels of CAT were much higher in two well-differentiated HCC cell lines than in all three poorly differentiated ones. Both well-differentiated HCC cell lines also showed a higher protein or mRNA expression of Cu/ZnSOD and MnSOD than three poorly differentiated ones. Our results demonstrate that specific antioxidant enzymes (especially, CAT and GRx) are differentially expressed in HCC cell lines with well or poor differentiation. These findings suggest that CAT and GRx are two potential differentiation markers for HCC. J. Cell. Biochem. 96: 622–631, 2005.

Key words: hepatocellular carcinoma; antioxidant enzymes; catalase; glutathione reductase; reactive oxygen species; superoxide dismutase

Accumulating evidence indicates that reactive oxygen species (ROS) and antioxidant enzymes are closely linked with cell differentiation and diseases [Durak et al., 1994; St. Clair et al., 1994; Di Giovanni et al., 2001; Su et al., 2001; Chen et al., 2003; Llurba et al., 2004]. Tissues of cancerous bladders exhibit lower activities of antioxidant enzymes xanthine oxidase (XO), superoxide dismutase (SOD), and catalase (CAT) than normal control bladders [Durak et al., 1994]. C3H10T1/2 cells with a manganese superoxide dismutase (MnSOD) transgene express a higher degree of differentiation characterized by the emergence of polynucleated cells and Z-bands when compared with normal C3H10T1/2 cells [St. Clair et al., 1994]. In the disease of cytochrome c oxidase (COX) deficiency, the enzyme activity of antioxidant glutathione peroxidase (GPx) increases significantly in COX-deficient muscle fibers expressing SOD [Di Giovanni et al., 2001]. In

Grant sponsor: NSC; Grant numbers: 89-2320-B-038-077, 93-3112-B-038-005, 92-2320-B-038-055; Grant sponsor: SKH-TMU-93-27.

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Received 14 March 2005; Accepted 25 April 2005
DOI 10.1002/jcb.20541

vascular smooth muscle cells (VSMCs), the expression of ROS, hydrogen peroxide in this case, corresponds positively with the appearance of VSMC differentiation markers such as α-actin and calponin [Su et al., 2001]. These lines of evidence strongly suggest that the levels of ROS or antioxidant enzymes in tissues are tightly correlated with diseases and can be used as a diagnostic index to distinguish healthy tissues from diseased ones.

Compelling evidence shows that ROS participate in the pathogenesis of liver diseases, especially hepatoma. It has been demonstrated that 7721 human hepatoma cells produce superoxide and transfection of 7721 human hepatoma cells with the *MnSOD* gene inhibits tumor cell propagation [Dong-Yun et al., 2003]. Evidence also indicates that ROS promote the invasive ability of hepatoma cells [Kozuki et al., 2000, 2001; Miura et al., 2003]. Moreover, the ob/ob mice with homozygous knockout of leptin gene develop non-alcoholic steatohepatitis (NASH) and their liver mitochondria produce more superoxide than those of normal mice [Laurent et al., 2004]. The above findings strongly suggest that ROS play an important role in the development of hepatic diseases.

Hepatocellular carcinoma (HCC) is the most frequent hepatic tumor and one of the most important causes of cancer death around the world [Kew, 2002; Pawlik et al., 2004]. Patients with well-differentiated HCC have a longer survival period than those with poorly differentiated HCC [Lerose et al., 2001]. The degree of HCC differentiation predicts its prognosis, however, no general agreement on a single staging method for HCC has made differential diagnosis of HCC and selection of the most suitable treatment difficult [Wildi et al., 2004]. Thus, the development of an easier, faster, and more reliable method to distinguish well-differentiated HCC from poorly differentiated HCC can help the physicians accurately stage the HCC and choose the most appropriate treatment for each HCC patient.

Based on the findings that ROS are closely correlated with hepatic diseases and antioxidant enzymes are also tightly associated with illnesses, we proposed the hypothesis that specific antioxidant enzymes are differentially expressed in HCC cell lines with various degrees of differentiation. In this study, we tested this hypothesis by examining the expression of antioxidant enzymes CAT, SOD, GPx, and glutathione reductase (GRx) among five HCC cell lines including two well-differentiated cell lines (Hep G2 and Hep 3B) and three poorly differentiated ones (HA22T/VGH, HA55T/VGH, and SK-Hep-1). Our results showed that the enzyme activities of CAT and GRx were dramatically higher in Hep G2 and Hep 3B cells than in HA22T/VGH, HA55T/VGH, and SK-Hep-1 cells. Moreover, Hep G2 and Hep 3B cells expressed much higher levels of CAT protein and mRNA than HA22T/VGH, HA55T/VGH, and SK-Hep-1 cells. Our findings demonstrate that CAT and GRx are differentially expressed in HCC cell lines with well or poor differentiation to a great extent and strongly suggest that CAT and GRx are two potential differentiation markers for HCC.

MATERIALS AND METHODS

Cell Culture and Protein Quantification

Five human hepatoma cell lines with well (Hep G2 and Hep 3B) or poor (HA22T/VGH, HA55T/VGH, and SK-Hep-1) differentiation were incubated with continuous aeration of 5% CO_2 and 95% air at 37°C, and maintained in complete Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), 2 mM L-glutamine, and 1 mM non-essential amino acids. The protein levels in cell lysates were quantified by measuring the absorbance of 595 nm with a spectrophotometer (Bradford method).

Activity Assays for Antioxidant Enzymes

Catalase (CAT) activity assay. The enzyme activity of CAT (U/mg protein) was quantified according to the protocol published by Beers and Sizer [1952]. In 1 ml of reaction solution containing 50 mM potassium phosphate buffer (pH 7.0), 14 mM hydrogen peroxide, and cell lysates (0.2–0.4 mg) at 37°C, the amount of 240 nm absorbance changed in 30 s reflecting the decomposition of hydrogen peroxide was used to calculate the enzyme activity of CAT by using the formula: $\log (A_0/A_1) \times [H_2O_2] \times 1$ ml/0.5 min/mg protein (Unit: μmol/min/mg protein). A_0 and A_1 stand for the readings measured at the wavelength of 240 nm with a spectrophotometer at the beginning of the test and 30 s after the start of the test, respectively.

Superoxide dismutase (SOD) activity assay. Based on the ability of SOD to suppress

auto-oxidation of pyrogallol, the SOD enzyme activity in HCC cell lines was quantified by measuring the absorbance at the wavelength of 420 nm with a spectrophotometer. In 1 ml of solution with 10 mM Tris-carcodylate (pH 8.4), 1 mM diethylene-triamine pentaacetic acid (DTPA), 0.2 mM potassium phosphate buffer (pH 7.2), 2 mM pyrogallol, and cell lysates from different HCC cell lines at 37°C, the total SOD enzyme activity (U/mg protein) in different HCC cell lines was measured by detecting the auto-oxidation rate of pyrogallol compared with the SOD standards.

Glutathione reductase (GRx) activity assay. The enzyme activity of GRx was determined based on its ability to oxidize the NADPH, which can be measured at the wavelength of 340 nm using a spectrophotometer. In 1 ml of reaction solution that contains 100 mM potassium phosphate buffer (pH 7.2), 2 mM oxidized form of glutathione (GSSG), 0.5 mM EDTA (pH 7.2), 0.16 mM β-NADPH, and cell lysates from different HCC cell lines, the enzyme activity of GRx (U/g protein) was measured at 37°C and calculated by comparing the absorbance at 340 nm with GRx standards.

Glutathione peroxidase (GPx) activity assay. According to the coupling assay, the enzyme activity of GPx was positively proportional to the degree of NADPH oxidation. The reaction solution containing 100 mM potassium phosphate buffer (pH 7.2), 5 mM EDTA, and 0.33 mM *t*-butyl-hydroperoxide was left at 37°C for 10 min, and then 1.5 mM (100 μl) β-NADPH, and cell lysates from different HCC cell lines were added to the solution. The absorbance at 340 nm was recorded for 3 min and afterward 10 mM *t*-butyl-hydroperoxide (100 μl) was applied to the medium. The 340 nm absorbance was measured for another 5 min and the enzyme activity of GPx was calculated by comparing the absorbance at 340 nm with GPx standards.

Western Blotting and Radioimmunoprecipitation

The protocol for Western blotting technique has been described in our earlier study [Shih et al., 2003]. In brief, HCC cells were washed with cold PBS and lysed with lysis buffer (0.5M NaCl, 25 mM HEPES, 5 mM EDTA, 0.1 mM sodium deoxycholate, 1.5% Triton X-100, and 0.1% SDS) [Simizu et al., 1998] including a cocktail of protease inhibitors (Roche) on ice for 20 min. The cell lysates were then subjected to centrifugation at 15,000*g* and 4°C for 15 min and the amount of proteins in the supernatant was measured by Bradford method. An appropriate volume of sampling buffer was added into each lysate and 40 μg of each HCC cell lysate was electrophoresed in each of two 12% SDS–PAGE gels. All proteins in one gel were then transferred onto a nitrocellulose paper, which was immunoblotted with sheep anti-Cu/ZnSOD antibody (CAT. No. 8474–9510, Biogenesis, Inc., Kingston, NH) or rabbit anti-human CAT antibody (CAT. No. K50805R, Biodesign International, Saco, Maine). Afterward, the nitrocellulose paper was incubated with HRP-conjugated donkey anti-sheep IgG or donkey anti-rabbit IgG and the protein of interest on the nitrocellulose paper was detected by the enhanced-chemiluminescence method (Amersham Pharmacia Biotech). The proteins on the second gel were stained with Coomassie blue and the total amount of proteins was used to normalize the expression of target protein detected by Western blotting for each cell line.

For the experiment of radioimmunoprecipitation (RIP), five HCC cell lines were cultured in methionine free RPMI medium for 1 h, and then ^{35}S-Met and ^{35}S-Cys (CAT. No. AGQ0080, Amersham Pharmacia Biotech) were added to the medium for 6 h. Subsequently, HCC cells were washed, resuspended in 0.5 ml NETT buffer at room temperature for 30 min, and centrifuged at 15,000*g* and 4°C for 5 min. After quantification of the ^{35}S radioactivity, the supernatant with the equivalent ^{35}S radioactivity from five different HCC cell lines was incubated with protein A sepharose, which has reacted with 5 μg sheep anti-MnSOD antibody (CAT. No. 8474–9550, Biogenesis, Inc., Kingston, NH) in NETT buffer for 4 h. Following 12–16-h incubation with sheep anti-MnSOD antibody conjugated protein A sepharose, the samples were centrifuged and the supernatant was discarded. Next, 20 μl of SDS–PAGE sampling buffer was added to the pellet, and the solution was boiled for 5 min and loaded for electrophoresis in a 12% SDS–PAGE gel. The gel was then incubated with the enhance solution (EN^{3}HANCE^{TM}, PerkinElmer, Inc., Wellesley, MA) in darkness for 1 h, dried and autoradiographed on a Kodak X-ray film. The amount of ^{35}S-labeled MnSOD for each cell line was quantified by measuring the intensity of exposed spots on the X-ray film with a densitometer (Gel Doc 2000 Systems, Bio-Rad Labora-

tories, Hercules, CA) and normalized with the amount of ^{35}S radioactivity in the same quantity of loading samples electrophoresed on a separate 12% SDS–PAGE gel, which was dried, exposed to an X-ray film for 1 h and quantified by a densitometer.

RNA Preparation and Northern Blotting

RNA was purified by RNeasy Mini Spin Columns (CAT. No. 74104, QIAGEN, Inc., Valencia, CA) according to the protocol provided by QIAGEN. The RNA samples (20 μg) from five HCC cell lines were diluted by RNA sampling buffer, heated at 65°C for 5 min, electrophoresed in a formaldehyde-agarose gel and then transferred onto a nylon paper according to the published protocol [Sambrook and Russell, 2001]. Hybridization was performed using appropriate nick-translated cDNA probes (MnSOD, CAT, and GPx), which were cloned by PCR based on their respective cDNA sequences [Lu et al., 2003], and human G3PDH cDNA control probe (CAT. No. 636830, BD Biosciences Clontech, Palo Alto, CA). After hybridization, the nylon membrane was washed, dried, and autoradiographed on a Kodak X-ray film at −70°C for 20 h. The respective amount of MnSOD, CAT, GPx, and G3PDH mRNA was quantified by measuring the intensity of exposure for each mRNA on the X-ray film with a densitometer. The amounts of MnSOD, CAT, and GPx mRNA for each cell line were normalized with the quantity of G3PDH mRNA and were expressed relative to those of HA22T/VGH cell line.

Statistics

The activity data of antioxidant enzymes were obtained from three independent experiments for each cell line, expressed as mean ± SEM and analyzed by one-way ANOVA followed by Bonferroni/Dunn post-hoc tests. The α level for all statistical tests performed in this study was 0.05.

RESULTS

Our data demonstrated that well-differentiated HCC cell lines (Hep 3B and Hep G2) expressed much higher enzyme activities of CAT and GRx than poorly differentiated HCC cell lines (HA22T/VGH, HA55T/VGH, and SK-Hep-1). Generally speaking, there was no difference in the enzyme activities of CAT, SOD, and GRx among poorly differentiated HA22T/VGH, HA55T/VGH, and SK-Hep-1 cell lines, except that HA22T/VGH cell line expressed a lower CAT enzyme activity than SK-Hep-1 cell line. Similarly, there was no difference in the enzyme activities of SOD and GRx between well-differentiated Hep 3B and Hep G2 cell lines.

Our results showed that there was a significant difference in the CAT enzyme activity (U/mg protein) among five HCC cell lines [$F(4, 10) = 541.1$, $P < 0.0001$, Fig. 1]. Both Hep 3B and Hep G2 cell lines exhibited a dramatically higher CAT activity than HA22T/VGH, HA55T/VGH, and SK-Hep-1 cell lines ($P < 0.0001$, Fig. 1). In addition, the CAT enzyme activity of Hep G2 cell line was significantly higher than that of Hep 3B cell line ($P < 0.005$, Fig. 1). Moreover, the SK-Hep-1 cell line showed a higher CAT enzyme activity than HA22T/VGH cell line ($P < 0.005$, Fig. 1).

A significant difference in the SOD enzyme activity (U/mg protein) existed among five HCC cell lines [$F(4, 10) = 10.909$, $P = 0.0011$, Fig. 2]. Post-hoc comparisons showed that Hep G2 exhibited a higher SOD enzyme activity than HA22T/VGH, HA55T/VGH, and SK-Hep-1 cell lines ($P < 0.001$ or 0.0005, Fig. 2). There was no

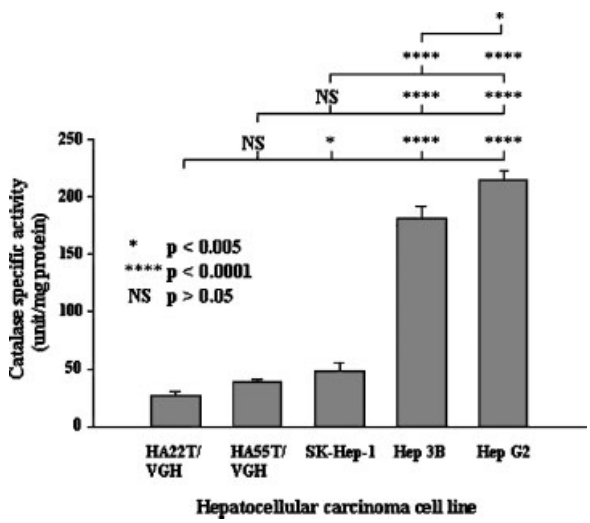

Fig. 1. The catalase (CAT) enzyme activity of two well-differentiated hepatocellular carcinoma (HCC) cell lines (Hep 3B and Hep G2) and three poorly differentiated ones (HA22T/VGH, HA55T/VGH, and SK-Hep-1). Both Hep 3B and Hep G2 cell lines had a much higher CAT enzyme activity than either HA22T/VGH, HA55T/VGH, or SK-Hep-1 cell line. In addition, the CAT enzyme activity in the Hep G2 was significantly higher than that of Hep 3B. SK-Hep-1 cell line showed a slightly higher CAT enzyme activity than HA22T/VGH cell line. Data were obtained from three independent experiments and expressed as mean ± SEM for all cell lines. *$P < 0.005$, ****$P < 0.0001$, NS $P > 0.05$.

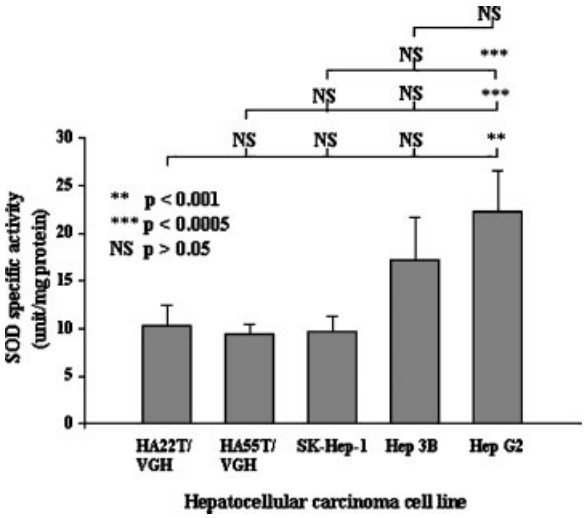

Fig. 2. The superoxide dismutase (SOD) enzyme activity of two well-differentiated HCC cell lines (Hep 3B and Hep G2) and three poorly differentiated ones (HA22T/VGH, HA55T/VGH, and SK-Hep-1). The Hep G2 cell line had a significantly higher SOD enzyme activity than the poorly differentiated ones (HA22T/VGH, HA55T/VGH, and SK-Hep-1). Data of all cell lines were derived from three independent replications and expressed as mean ± SEM. $^{**}P < 0.001$, $^{***}P < 0.0005$, NS $P > 0.05$.

difference in other comparisons among these five HCC cell lines.

One-way ANOVA indicated that there was a drastic difference in the GRx enzyme activity (U/g protein) among five HCC cell lines [$F(4, 10) = 243.452$, $P < 0.0001$, Fig. 3]. Post-hoc tests

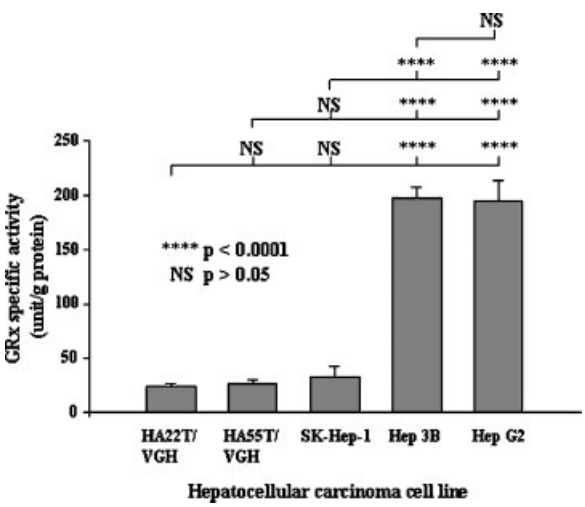

Fig. 3. The enzyme activity of glutathione reductase (GRx) in five HCC cell lines. Both Hep 3B and Hep G2 HCC cell lines with well differentiation expressed a dramatically higher GRx enzyme activity than any of the poorly differentiated HCC cell lines (HA22T/VGH, HA55T/VGH, and SK-Hep-1). Data were obtained from three independent experiments and expressed as mean ± SEM for all cell lines. $^{****}P < 0.0001$, NS $P > 0.05$.

revealed that both Hep 3B and Hep G2 cell lines had a much higher GRx enzyme activity than HA22T/VGH, HA55T/VGH, and SK-Hep-1 cell lines ($P < 0.0001$, Fig. 3). There was no difference in the GRx enzyme activity among other comparisons.

For the GPx enzyme activity, there was a significant difference among five HCC cell lines [$F(4, 10) = 75.360$, $P < 0.0001$, Fig. 4]. The GPx enzyme activity of HA55T/VGH was significantly higher than that of the other four cell lines ($P < 0.005$ or 0.0005, Fig. 4). The Hep G2 cell line expressed a more elevated GPx enzyme activity than that of HA22T/VGH, SK-Hep-1, and Hep 3B cell lines ($P < 0.0005$, Fig. 4). No other significant difference was found among the rest of comparisons.

The Western blotting and RIP data showed that well-differentiated HCC cell lines (Hep G2 and Hep 3B) expressed more CAT, Cu/ZnSOD, and MnSOD proteins than poorly differentiated HCC cell lines (HA22T/VGH, HA55T/VGH, and SK-Hep-1) (Figs. 5,6,7). The relative expression of CAT, Cu/ZnSOD, and MnSOD proteins for each cell line was normalized with the appropriate loading controls (Figs. 5A,6A,7A). The

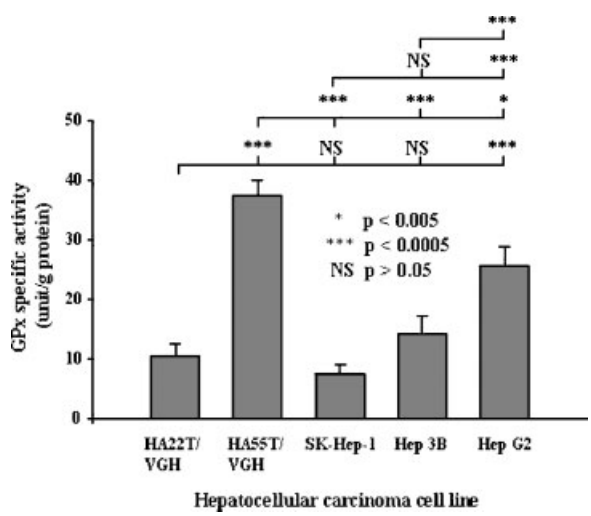

Fig. 4. The enzyme activity of glutathione peroxidase (GPx) in two well-differentiated HCC cell lines (Hep 3B and Hep G2) and three poorly differentiated HCC cell lines (HA22T/VGH, HA55T/VGH, and SK-Hep-1). Not all poorly differentiated HCC cell lines showed a lower GPx enzyme activity when compared with the well-differentiated HCC cell lines. The HA55T/VGH cell line had a significantly higher GPx enzyme activity than any other HCC cell lines. The HA22T/VGH, SK-Hep-1, and Hep 3B cell lines showed a lower GPx enzyme activity than the Hep G2 cell line. Three independent experiments were performed for each cell line and data represented mean ± SEM. $^{*}P < 0.005$, $^{***}P < 0.0005$, NS $P > 0.05$.

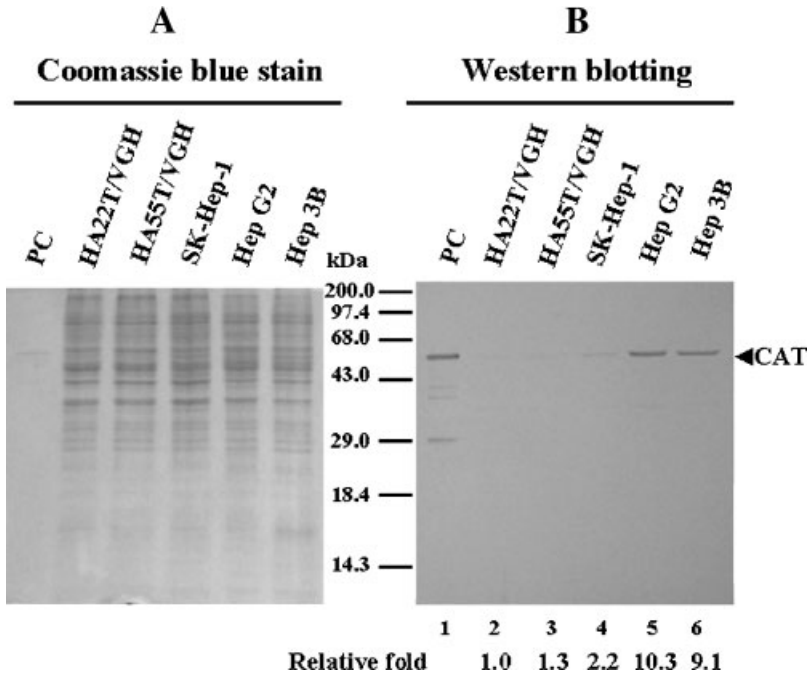

Fig. 5. **A**: An equivalent amount of proteins (40 μg) from the cell lysates of five HCC cell lines was loaded for electrophoresis using a 12% SDS–PAGE gel. The proteins on the SDS–PAGE gel were stained with Coomassie blue and the proteins on each lane were quantified by a densitometer. All five cell lines showed similar amount of proteins. PC, positive control (bovine erythrocyte CAT). **B**: The Western blotting data showed the CAT protein in five HCC cell lines. Both Hep G2 and Hep 3B HCC cell lines with well differentiation expressed a much higher amount of CAT protein than three poorly differentiated HCC cell lines (HA22T/VGH, HA55T/VGH, and SK-Hep-1). PC, positive control (bovine erythrocyte CAT).

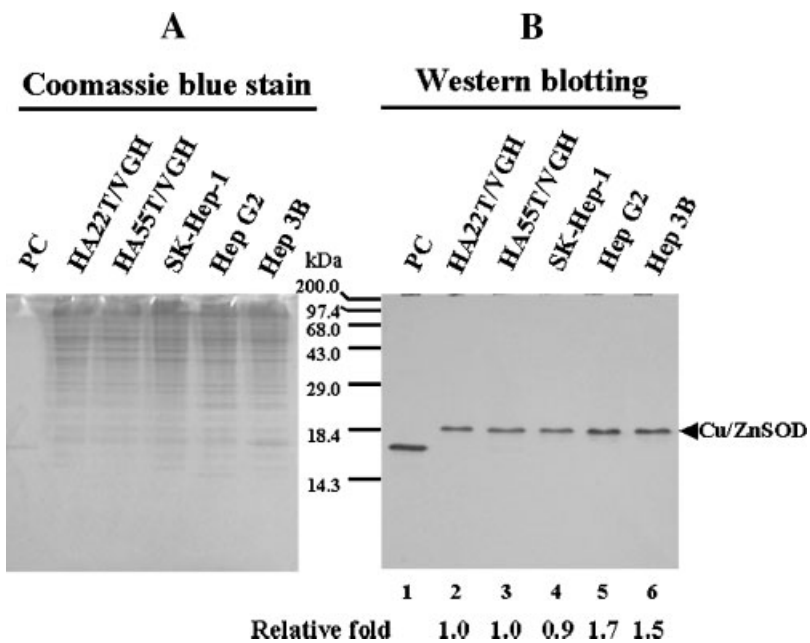

Fig. 6. **A**: Forty μg of proteins from each cell lysate of five HCC cell lines was electrophoresed in a 12% SDS–PAGE gel. Coomassie blue was used to stain the proteins on the SDS–PAGE gel and the protein level for each cell line was quantified by a densitometer. The amount of proteins was quite similar among the five HCC cell lines. PC, positive control (bovine erythrocyte Cu/ZnSOD). **B**: The Western blotting data showed the Cu/ZnSOD protein in five HCC cell lines. Both well-differentiated HCC cell lines (Hep G2 and Hep 3B) exhibited a slightly higher expression of Cu/ZnSOD protein when compared with the poorly differentiated HCC cell lines (HA22T/VGH, HA55T/VGH, and SK-Hep-1). PC, positive control (bovine erythrocyte Cu/ZnSOD).

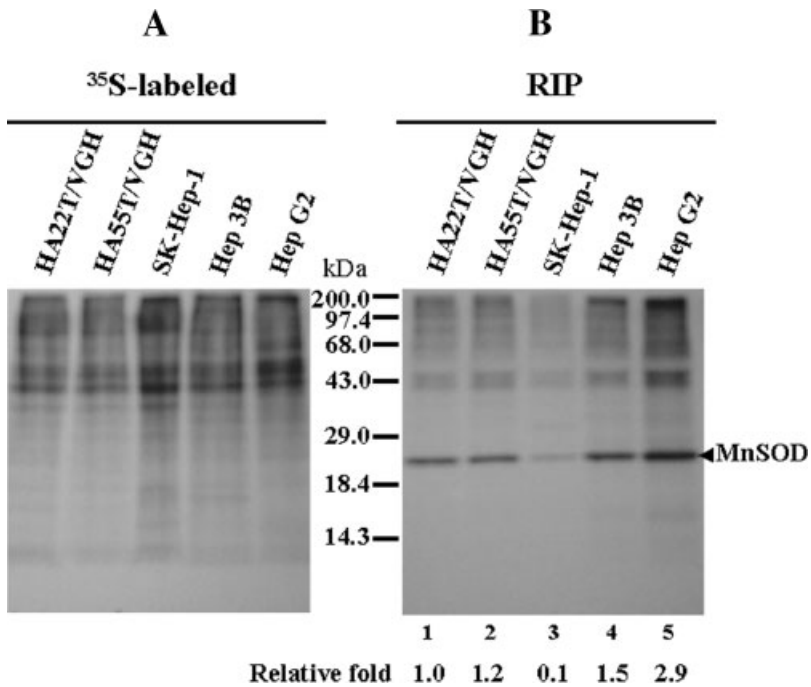

Fig. 7. **A**: The proteins with an equivalent amount of ^{35}S radioactivity from five HCC cell lines were loaded for electrophoresis in a 12% SDS–PAGE gel and the SDS–PAGE gel was then exposed to an X-ray film for 1 h. The amount of ^{35}S radioactivity for each lane was quantified by measuring the exposure density with a densitometer. **B**: The amount of MnSOD protein for each cell line was quantified by measuring the exposure density induced by ^{35}S-labeled MnSOD on the X-ray film and normalized with the amount of proteins loaded. Both Hep 3B and Hep G2 cell lines expressed more MnSOD protein than three poorly differentiated HCC cell lines.

CAT protein level in the Hep G2 and Hep 3B cell lines was 10.3 and 9.1 times the amount of HA22T/VGH cell line, respectively (Fig. 5B), whereas the CAT protein level in HA55T/VGH and SK-Hep-1 cell lines was only 1.3 and 2.2 times the quantity of HA22T/VGH cell line, respectively (Fig. 5B). The relative Cu/ZnSOD protein level for HA22T/VGH, HA55T/VGH, SK-Hep-1, Hep G2, and Hep 3B cell lines was 1.0, 1.0, 0.9, 1.7, and 1.5, respectively (Fig. 6B). The RIP data showed that the protein level of MnSOD in HA55T/VGH, SK-Hep-1, Hep 3B, and Hep G2 cell lines was 1.2, 0.1, 1.5, and 2.9 times the amount of HA22T/VGH cell line, respectively (Fig. 7B).

The Northern blotting results indicated that the expression of MnSOD and CAT mRNA was much higher in the well-differentiated HCC cell lines (Hep G2 and Hep 3B) than in the poorly differentiated ones (HA22T/VGH, HA55T/VGH, and SK-Hep-1). The relative amount of MnSOD mRNA in the HA22T/VGH, HA55T/VGH, SK-Hep-1, Hep G2, and Hep 3B cell lines was 1.0, 0.9, 0.4, 4.3, and 2.5, respectively (Fig. 8A). Similarly, the CAT mRNA in the HA55T/VGH, SK-Hep-1, Hep G2, and Hep 3B cell lines was 0.9, 1.1, 5.4, and 2.7 times the amount of HA22T/VGH cell line, respectively (Fig. 8B). In contrast, not all poorly differentiated HCC cell lines expressed a lower GPx mRNA level when compared with well-differentiated ones. The expression of GPx mRNA in the HA55T/VGH, SK-Hep-1, Hep G2, and Hep 3B cell lines was 2.2, 1.1, 2.4, and 1.7 times the quantity of HA22T/VGH cell line, respectively (Fig. 8C). The amount of G3PDH mRNA was used as an internal control to normalize the expression of MnSOD, CAT, and GPx mRNA for each cell line (Fig. 8D).

DISCUSSION

Our findings strongly support the hypothesis that HCC cell lines with various degrees of differentiation exhibit varying levels of specific antioxidant enzymes. Our results demonstrated that both well-differentiated HCC cell lines expressed extraordinarily higher CAT and GRx enzyme activities, and much more CAT protein and mRNA than three poorly differentiated ones, suggesting that both transcription and translation activities of the *CAT* gene are

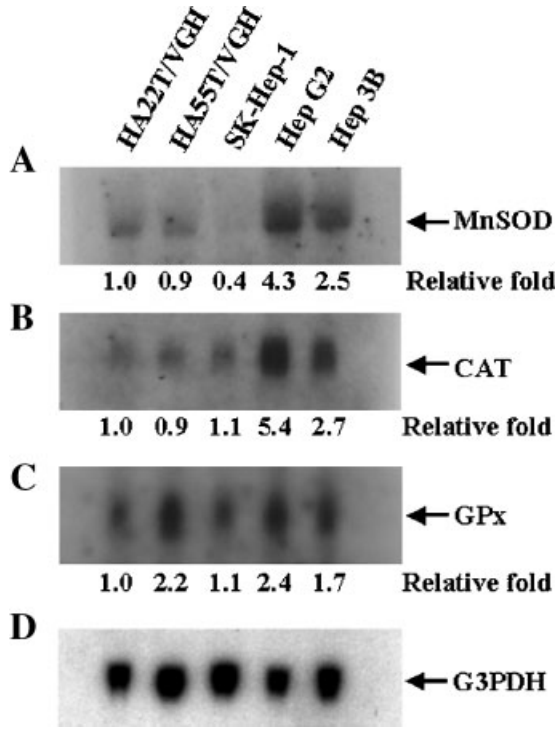

Fig. 8. Northern blotting data of (**A**) manganese superoxide dismutase (MnSOD) mRNA, (**B**) CAT mRNA, (**C**) GPx mRNA, and (**D**) G3PDH mRNA in five HCC cell lines. The amounts of MnSOD, CAT, GPx, and G3PDH mRNA for all cell lines were quantified by a densitometer. (A) Both Hep G2 and Hep 3B HCC cell lines with well differentiation showed a dramatically higher amount of MnSOD mRNA than three poorly differentiated HCC cell lines (HA22T/VGH, HA55T/VGH, and SK-Hep-1). (B) Both Hep G2 and Hep 3B HCC cell lines expressed a much higher expression of CAT mRNA than three poorly differentiated HCC cell lines. (C) Hep G2 and Hep 3B HCC cell lines expressed a higher GPx mRNA than HA22T/VGH and SK-Hep-1 cell lines. HA55T/VGH cell line exhibited GPx mRNA, which was comparable to that of Hep G2 and Hep 3B HCC cell lines.

significantly higher in well-differentiated HCC cell lines than in poorly differentiated ones. These findings also strongly suggest that the activity of CAT and GRx may provide invaluable information on the cell differentiation of HCC and even the prognosis of HCC.

The lower activities of certain antioxidant enzymes in poorly differentiated HCC cell lines may explain the higher metastasizing ability and poor prognosis of HCC with poor differentiation. As stated previously, ROS play an important role in the pathogenesis of hepatic diseases [Kozuki et al., 2000, 2001; Dong-Yun et al., 2003; Miura et al., 2003; Laurent et al., 2004]. Hepatitis C virus often leads to HCC [Di Bisceglie, 1997; Kew, 2002] and its NS5A protein has been shown to stimulate the production of ROS (the superoxide anion) [Gong et al., 2001]. Hypoxanthine (HX) and XO, which convert oxygen to the superoxide [Droge, 2002], enhance the invading activity of AH109A hepatoma cells [Kozuki et al., 2000, 2001; Miura et al., 2003]. Transfection of 7721 human hepatoma cells with the *MnSOD* gene decreases the production of intracellular ROS and inhibits the proliferation of these tumor cells, whereas knockdown of the *MnSOD* gene by the antisense technique enhances the generation of intracellular ROS and promotes the propagation of tumor cells [Dong-Yun et al., 2003]. Hepatoma cells with poor differentiation have a stronger invasive ability than those with well differentiation and poorly-differentiated HCC has a higher tendency to recur and often results in a shorter survival time [Ito et al., 1999; O'Brien et al., 2004]. In this study, we demonstrated much lower activities or expression of specific antioxidant enzymes (especially CAT and GRx) in poorly-differentiated HCC cell lines when compared with well-differentiated ones. All the above findings together strongly support the idea that poorly differentiated HCC cells are more likely to proliferate and metastasize because the much lower activities or expressions of specific antioxidant enzymes fail to effectively scavenge the ROS produced in these poorly differentiated HCC cells.

The lower expression or activities of MnSOD, CAT, and GRx in poorly differentiated HCC cell lines may result from the negative regulation or a defect of gene expression of these enzymes. The normal activities of Cu/ZnSOD (or SOD) and CAT enzymes in rat liver are around 23–80 and 60–348 U/mg protein, respectively [Casalino et al., 2002; Kalender et al., 2005; Zou et al., 2005]. The enzyme activities of SOD and CAT in well-differentiated HCC cell lines were within the normal range of hepatic SOD and CAT enzymes, whereas those in poorly differentiated ones were below the normal range in this study. It has been demonstrated that protein binding to the silencer element of the *CAT* gene is expressed more copiously in de-differentiated hepatoma cell lines than in well-differentiated ones [Takeuchi et al., 2000], suggesting that this silencer element contributes markedly to the downregulation of the *CAT* gene expression in de-differentiated hepatoma cell lines. In addition, evidence suggests that a defect exists in gene expression of MnSOD in cancerous cells [Borrello et al., 1993] and that expression of the *MnSOD* gene

promotes differentiation of murine fibrosarcoma [Zhao et al., 2001] and C3H10T1/2 cells [St. Clair et al., 1994]. The above findings together strongly suggest that the negative regulation or a defect of MnSOD, CAT, and GRx gene expression contributes notably to the lower expression or enzyme activities of MnSOD, CAT, and GRx in the poorly differentiated HCC cell lines.

CAT and GRx are two excellent differentiation markers for HCC cell lines and may be used to distinguish among HCC with various degrees of differentiation and to predict the prognosis of HCC in the future. At present, there are several complex staging systems for HCC [Wildi et al., 2004], which require a lot of time to process the samples and the certified pathologists to interpret the results. Therefore, it is indispensable and urgent to develop a fast, easy, and reliable method to classify the HCC with good or poor prognosis. As we showed in this study, two well-differentiated HCC cell lines expressed approximately four to eight-fold increase in CAT and GRx enzyme activities when compared with any of the three poorly differentiated HCC cell lines (Figs. 1,3). The more advanced HCC is expected to contain more poorly differentiated cells and consequently is anticipated to express lower enzyme activities of CAT and GRx. Thus, the CAT and GRx are two great potential markers to distinguish well-differentiated HCC from poorly differentiated one.

ACKNOWLEDGMENTS

This research was supported by Grants NSC 89-2320-B-038-077 (L.Y.Y.), NSC 93-3112-B-038-005 (National Research Program for Genomic Medicine) (L.Y.Y.), SKH-TMU-93-27 (L.Y.Y.) and NSC 92-2320-B-038-055 (C.M.S.).

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