Hypermethylation and downregulation of glutathione peroxidase 3 are related to pathogenesis of melanoma

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Abstract. As a crucial antioxidant enzyme, glutathione peroxidase 3 (GPX3) has been found to be frequently repressed in many cancers due to promoter hypermethylation and is known as a possible tumor suppressor gene. In the present study, we investigated whether promoter hypermethylation of GPX3 and its repression are present in melanoma and, if so, whether GPX3 downregulation is implicated in the pathogenesis of melanoma. Our results revealed methylation of GPX3 and downregulation of its expression in both melanoma cell lines and surgical melanoma tissue samples. In melanoma cell lines, GPX3 expression was restored by treatment with 5-aza-2’-deoxycytidine both in mRNA and protein levels. Depletion of GPX3 was found to increase the proliferative ability, motility, and invasiveness of melanoma cells. Moreover, negative expression of GPX3 was related to poor prognosis in melanoma patients. These results suggest that methylation-mediated GPX3 repression may have critical implications for melanoma pathogenesis.

Introduction

As well known potential carcinogens, reactive oxygen species (ROS) are involved in both carcinogenesis and tumor progression via ROS-induced DNA damage (1-4). During normal metabolism, cells avoid oxidative damage by means of antioxidant defense mechanisms that control the balance between the generation and removal of oxygen radicals. The antioxidant system includes various enzymes, such as superoxide dismutase and catalase, as well as glutathione peroxidase (GPX) (5,6).

GPX3, a member of the GPX family, is an extracellular glycosylated enzyme that reduces both phospholipid hydroperoxides and fatty acid hydroperoxides using various electron donors, such as glutathione, thioredoxin, and glutaredoxin (7-10). It is generally known that hydroperoxides are involved in various biological behavior of cancer cells such as proliferation, motility, and invasion (11). Therefore, some investigators expected that GPX3 might play a role in cancer carcinogenesis by controlling the hydroperoxide levels inside cells (11).

The human GPX3 gene, located on chromosome 5q32, is the only known extracellular antioxidant isoform containing selenocysteine at the catalytic site (12-15). GPX3 activity is regulated by the availability of selenium, a well known cancer chemopreventive agent (16). Selenium uptake is related to decreased cancer incidence, and although there is no direct experimental evidence, this linkage may be related to the anticaner effect of selenium (17).

GPX3 is mainly synthesized by the proximal tubules of the kidney (18). GPX3 is readily detectable in extracellular body fluids such as the aqueous humor in the anterior and posterior chambers of the eye, blood plasma, and thyroid colloid (19,20). Moreover, transcripts of GPX3 are also detected in epithelial cells of the oviduct (21). The mechanisms that regulate the transcription of GPX3 remain largely unknown. Recent studies have shown that oxidative stress can induce transcriptional upregulation of GPX3 in inflammatory bowel disease and experimental colitis, in patients with asthma, and in the diabetic mouse heart (22-25). However, GPX3 expression is usually downregulated in cancer tissues compared to its expression in normal tissues (26). In contrast, overexpression of GPX3 in prostate cancer cells has been found to attenuate tumorigenic potential and spontaneous metastasis in vivo (27). GPX3 may serve as a potential tumor suppressor gene in cancer progression.

Genetic and epigenetic mechanisms lead to inactivation of tumor suppressor genes in cancers. As one of the major modifications involved in epigenetic inactivation of genes, abnormal methylation in CpG-rich promoter regions can induce transcriptional silencing of tumor suppressor genes. Promoter hypermethylation of GPX3 is frequently detected in...
various cancer tissues, such as gastric, prostate, and Barrett's esophageal cancer, and this may be a common and crucial cause of GPX3 downregulation in cancers.

Malignant melanoma is a common leading cause of cancer-related death among patients with skin cancer. The incidence of cutaneous melanoma has rapidly increased over the last 50 years (28). However, the molecular basis of melanoma is largely unknown. In the current study, for the first time, we determined whether GPX3 is also downregulated in melanoma and, if so, whether promotor hypermethylation is associated with its repression. We further investigated the influence of GPX3 on the biological behavior of melanoma cells and its clinicopathological significance in melanoma patients.

Materials and methods

Patients. Two tissue microarray (TMA) sections of melanoma samples were purchased from Pantomics, Inc. (Richmond, CA, USA) and Super BioChips (Seoul, Korea), respectively, and used for immunohistochemistry. The specimens included 70 primary melanoma and 25 metastatic melanoma tissue samples. Three normal skin tissues and 10 primary melanoma tissue samples were obtained from Yanbian University Hospital and used for quantitative real-time PCR (qRT-PCR) and methylation-specific PCR (MSP) analysis. The clinicopathological characteristics of all patients are shown in Table I. This study was approved by the Institutional Review Board of Yanbian University Hospital.

Cell culture and establishment of GPX3 knockdown SK-MEL-24 cells. The human melanoma cell lines (SK-MEL-2 and SK-MEL-24), and adult human epidermal melanocytes (HEMs) were purchased from ATCC (Rockville, MD, USA). Melanoma cell lines were cultured in microvascular endothelial cell medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml ampicillin, 100 µg/ml streptomycin, and 2 mM glutamine (Gibco BRL, Gaithersburg, MD, USA). HEMs were cultured in MPM-4 melanocyte cell basal medium (Lonza Group AG, Basel, Switzerland) supplemented with MBM-4 plus SingleQuots™. Cells were grown in a humidified atmosphere of 5% CO₂ at 37˚C. SK-MEL-24 cells were positive for GPX3 on both the mRNA and protein levels. GPX3-depleted stable SK-MEL-24 cell lines were created using GPX3 shRNA-encoding plasmid (Origin Pharmaceutical Services Ltd., Abingdon, Oxfordshire, UK). Retroviruses were generated in 293T cells and the transduced cells were selected using GPX3 shRNA-encoding plasmid (Origin Pharmaceutical Services Ltd., Abingdon, Oxfordshire, UK). Silencing of GPX3 in SK-MEL-24 cell lines was confirmed by reverse transcription polymerase chain reaction (RT-PCR) analysis (Fig. 1A).

Immunocytochemistry in TMA sections. Melanoma tissue sections were deparaffinized in xylene and rehydrated in graded alcohol. Antigen retrieval was performed by a 2 min autoclave treatment using antigen retrieval buffer (Dako North America, Inc., Carpinteria, CA, USA). Sections were treated with primary antibody against GPX3 (working dilution: 1:100; Abcam plc, Cambridge, UK) at room temperature (RT) for 1 h, and the Donkey Anti-Mouse IgG H&L (Alexa Fluor® 488) (Abcam) was used as the secondary antibody. Counterstaining was performed using 4,6-diamidino-2-phenylindole (DAPI).

In this study, sections of cell blocks from HeLa cells were used as a positive control for staining of GPX3. Rabbit IgG (R&D Systems, Wiesbaden-Nordenstadt, Germany) was used as a negative control in this study.

Immunohistochemistry in human melanoma cell lines and adult HEMs. GPX3 expression was determined in both HEMs and melanoma cell lines by immunocytochemistry. Cell lines were plated on glass coverslips containing 6-well plates on the day before staining. After fixation with 95% ethanol, the cells were blocked with 5% bovine serum antigen (BSA) at RT for 1 h. Endogenous peroxidase activity was inactivated with a mixture of H₂O₂ and methanol, in a ratio of 1:40. The cells were then treated with primary antibody against GPX3 (working dilution: 1:200; Abcam) at RT for 1 h, and the Real™EnVision™HRP Rabbit/Mouse detection system (Dako North America, Inc.) was used as the secondary antibody. After visualization with 3,3’-diaminobenzidine, counterstaining was performed using hematoxylin. HeLa cells were used as a positive control for GPX3.

Total RNA extraction and analysis for GPX3 mRNA expression. Total RNA was isolated with TRIzol reagent (for cell line samples; Invitrogen, Carlsbad, CA, USA) and the RNeasy FFPE kit (from paraffin tissue samples; Qiagen GmbH, Hilden, Germany), and cDNA was synthesized using total RNA and AccuPower® RT PreMix (Bioneer, Seoul, Korea) according to the manufacturer’s protocol. qRT-PCR was performed as previously described using 1X SYBR-Green Master Mix (Applied Biosystems, Foster City, CA, USA) and synthesized cDNA in an ABI7500 Real-time PCR system (Applied Biosystems) under the following conditions: initial denaturation for 10 min at 95°C, followed by 40 cycles of 95°C for 20 sec, 50°C for 30 sec, and 72°C for 45 sec. Conventional RT-PCR analysis was performed using AccuPower PCRPreMix (Bioneer) and the synthesized cDNA with an annealing temperature of 58°C. β-actin was used as the housekeeping gene. Oligonucleotide primers used for the PCR were: 5’-CAACCAATTTGGAACACAGG-3’ and 5’-GTG

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Table I. Characteristics of the patients with malignant melanoma.
GGAGGACAGGAGTTCTT-3' for GPX3; and 5'-ATAGC ACAGCCTGGATAGCAACGTAC-3' and 5'-CACCTTC TACAATGAGCTGCGTGTG-3' for β-actin (29).

Treatments with 5-aza-2'-deoxycytidine (5-Aza). Two melanoma cell lines (SK-MEL-24 and SK-MEL-2) were treated with 5-Aza (Sigma-Aldrich, Inc., St. Louis, MO, USA), 1 or 10 µM, for 72 h, with drug replacement every 24 h. Cells were then harvested and mRNA expression of GPX3 was detected in each group of the cells.

DNA extraction and MSP. Genomic DNA extraction was performed in HEMs and melanoma cell lines, three normal skin samples, and 10 melanoma tissue samples using the QIAamp DNA mini kit (Qiagen GmbH). Modification of DNA was performed with the EZ DNA Methylation kit (Zymo Research Corp., Irvine, CA, USA), and the methylation-specific or unmethylation-specific amplification was performed with an annealing temperature of 59°C using the following primers: 5'-TATGTTATTGTCGTTTGCAGGG-3' and 5'-GTCCGTCTAAAATATCCGACG-3' for methylation-specific amplification; and 5'-TTTATGTTATTGTTTGAGGATG-3' and 5'-ATCCATCTAAAATATCCAACAC TCC-3' for unmethylation-specific amplification. The amplified DNA products were loaded on 2% agarose gels for electrophoresis and stained with ethidium bromide.

Cell proliferation assay. To determine the influence of GPX3 on the proliferative ability of the melanoma cells, the trypan blue exclusion assay was performed in GPX3-depleted SK-MEL-24 (GPX3∆) cells and cells transfected with the control vector (Mock). Cells were seeded in 6-well plates and

Figure 1. Depletion of GPX3 using retroviral vector-based shRNA. The mRNA (A) expression was analyzed in SK-MEL-24 Mock and GPX3∆ cells. The mRNA (B) and protein (C) expression of GPX3 were detected in HEMs (i), SK-MEL-24 (ii), and SK-MEL-2 (iii) cells. GPX3 expression was decreased in melanoma cell lines compared to HEMs both in mRNA and protein levels. Promoter methylation of GPX3 was found in melanoma cell lines (E) and both protein (D) and mRNA (F) expression were restored by 5-Aza treatment.
the number of cells in each group was counted at 36 and 72 h using a hemocytometer.

Cell migration assay. To evaluate the influence of GPX3 on the migration ability of the melanoma cells, a wound healing assay was performed in GPX3∆- and Mock-SK-MEL-24 cells. The cells were seeded in 24-well culture plates in triplicate. After 24 h of culturing, linear scrape injuries were made with a yellow pipette tip on the cells that displayed growth to a confluent monolayer. Wound closing in each group was photographed at the indicated time points and analyzed with the Image J analysis software package (National Institutes of Health, Bethesda, MD, USA).

Cell invasion assay. To evaluate the influence of GPX3 on the invasiveness of the melanoma cells, an invasion assay was performed using Transwell filters (pore size, 8 µm; BD Biosciences, Bedford, MA, USA) in GPX3∆- and Mock-SK-MEL-24 cells. Both the lower and top sides of Transwell filters were coated with 8 µg/µl Matrigel (BD Biosciences, San Jose, CA, USA) and the filters were placed on 24-well culture plates with a culture medium with 15% BSA. The cells were suspended in a medium containing 3% BSA and then seeded in the coated Transwell chambers. After 36 h of culturing, the bottom side of the membrane with the invaded cells was fixed with 4% paraformaldehyde and then stained with crystal violet. The number of invaded cells was counted with a microscope and comparatively investigated between GPX3∆- and Mock-SK-MEL-24 cells.

Statistical analysis. In this study, we used commercially available software (SPSS version 15.0 for Windows; SPSS, Inc., Chicago, IL, USA) for statistical analysis. The Mann-Whitney U test was used to analyze differences between groups in the in vitro studies. The χ² test and Fisher's exact test were used to investigate differences between groups of clinical samples. Survival analysis for melanoma patients was performed using Kaplan-Meier analysis, and the differences were compared using the log-rank test. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

GPX3 is downregulated in melanoma cell lines by promoter hypermethylation. In the current study, both mRNA and protein expression of GPX3 were comparatively investigated in HEMs and melanoma cell lines. Our results showed that GPX3 expression was downregulated in melanoma cell lines to a greater extent than in HEMs on both the mRNA and protein levels (Fig. 1B and C). The methylation status of GPX3 was evaluated in both HEMs and melanoma cell lines using MSP primers against the GPX3 promoter. Full methylation (presence of only methylated CpGs) was found in the SK-MEL-2 cell line, which was negative for GPX3 on both the RNA and protein levels. Moreover, HEMs and SK-MEL-24 cells, with expression of GPX3 on both the RNA and protein levels, showed unmethylation (presence of only unmethylated CpGs) and partial methylation (presence of both methylated and unmethylated CpGs) for GPX3, respectively (Fig. 1E). To investigate whether methylation status was related to repression of GPX3 expression, melanoma cell lines were treated with the DNA demethylation agent 5-Aza, and our results showed that demethylation of the GPX3 gene by 5-Aza in both SK-MEL-2 and SK-MEL-24 cell lines restored GPX3 expression at mRNA and protein levels (Fig. 1D and F). No change was found in GPX3 expression of HEMs according to the presence or absence of 5-Aza treatment (Fig. 1D).

GPX3 promoter is methylated in primary melanoma tissues. The methylation status of GPX3 was also determined in 3 normal skin and 10 primary melanoma tissue samples. Unmethylation for GPX3 was found in the 3 normal skin samples. By contrast, full methylation and partial methylation were detected in three and seven melanoma tissue samples, respectively (Fig. 2A). Moreover, RT-PCR analysis showed that GPX3 mRNA expression was significantly decreased in primary melanoma tissues compared to normal skin tissues (Fig. 2B).

The influence of GPX3 downregulation on the biological behavior of SK-MEL-24. SK-MEL-2 showed increased
invasion ability than SK-MEL-24 cells, which was found to be negative for GPX3 on both RNA and protein levels (data not shown). However, SK-MEL-2 and SK-MEL-24 cells may have a different molecular basis. Therefore, the differences in invasive ability between the cells cannot exactly represent the role of GPX3. We also found that invasive activities were suppressed to a greater extent in SK-MEL-2 and SK-MEL-24 cells with 5-Aza treatment than without 5-Aza treatment (data not shown). However, no change was found in HEMs according to the presence or absence of 5-Aza treatment. Other tumor suppressor genes in addition to GPX3 have been found to be methylated in melanoma (30). Consequently, 5-Aza treatment can restore the RNA and protein expression of various genes that are methylated in melanoma. Therefore, we constructed GPX3-depleted cell lines to investigate the influence of GPX3 on the biological behavior of melanoma cells.

The proliferative, migration, and invasive ability of cells were comparatively investigated in the GPX3∆- and Mock-SK-MEL-24 cells. Compared to Mock-SK-MEL-24 cells, GPX3∆-SK-MEL-24 cells demonstrated 1.43- and 2.07-fold higher proliferative ability in each time point, respectively (P<0.001, and P<0.001, respectively). Moreover, GPX3∆-SK-MEL-24 cells showed 3.06-fold (P<0.001), and 6.47-fold (P<0.001) higher migration and invasive ability, respectively (Fig. 3A-D).

Clinicopathological significance of GPX3 expression in melanoma patients. GPX3 expression was detected in the cytoplasm of cancer cells in melanoma tissue samples (Fig. 4A). The expression frequency of GPX3 was significantly increased in primary sites (33.8%) compared to metastatic sites (10.8%) of melanoma tissues (P=0.036) (Fig. 4B). Kaplan-Meier analysis was performed in 46 primary malignant melanoma patients. We found that patients with GPX3 expression showed increased survival rates (median survival duration 71 months) compared to patients without GPX3 expression (median survival dura-

![Figure 3. The influence of GPX3 downregulation on the biological behavior of SK-MEL-24. The proliferative (A), invasive (B), and migration (C and D) ability were significantly increased in GPX3 depleted SK-MEL-24 cells compared to Mock-SK-MEL-24 cells. The results are shown as mean values ± SD and were analyzed by the Mann-Whitney U test (*P<0.001).](image-url)
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No significant association was found between GPX3 expression and age or gender of melanoma patients.

Discussion

The antioxidant system is the first line of defense against ROS-induced damage. GPX3, a major antioxidative enzyme, is known as a critical scavenger of ROS. Silencing of GPX3 promotes ROS production in muscle stem cells and colon cancer cell lines (31,32). GPX3 is selectively expressed in several healthy tissues, such as the esophagus, stomach, and colon (26). In the current study, GPX3 expression was detected in the cytoplasm of HEMs, but decreased or negative expression was found in melanoma cell lines. Moreover, unmethylation was found in HEMs, while full or partial methylation was observed in melanoma cell lines. Methylation of GPX3 was also frequently detected in the human melanoma tissue samples, and mRNA expression was concurrently repressed in melanoma tissue samples, in the present study. Supportively, 5-Aza treatment can restore the expression of GPX3 in melanoma cell lines. 5-Aza inhibits DNA-methyltransferase activity and thereby restores the expression of silenced genes (33). Downregulation and silencing of GPX3 in melanoma may result from promoter hypermethylation.

Many studies have shown that GPX3 is frequently methylated and downregulated in cancers compared to related normal tissues, and it has been hypothesized that GPX3 may act as a tumor suppressor in cancers. However, the effect of GPX3 on the biologic behavior of cancer cells has not been fully investigated (11). Some investigators showed that GPX3 overexpression can promote cancer cell motility but not the growth of cancer cells in gastric cancer (34). In prostate cancer cell lines, GPX3 overexpression inhibits the invasiveness of cells in vitro, and can reduce tumor volume and prevent metastasis in vivo (27). In the current study, we found that GPX3 downregulation can promote the proliferation, motility, and invasion of melanoma cells in vitro. In support of these observations, negative expression of GPX3 was more frequently detected in metastatic site than primary site of melanoma and was also found to be a negative prognostic indicator for melanoma patients in the present study. The clinical implications

Figure 4. Clinicopathological significance of GPX3 expression in melanoma patients: Negative (i) and positive (ii) patterns of GPX3 expression in melanoma tissues (A). GPX3 expression was found in cytoplasm of cancer cells in melanoma tissues (i), and was detected more often in primary site than metastatic site (B). The results were analyzed by χ²-test (P=0.036). According to the Kaplan-Meier analysis, negative GPX3 expression significantly related to poor prognosis of melanoma patients (C).
of GPX3 have also been mentioned by other investigators. In ovarian clear cell adenocarcinoma, GPX3 expression is related to cisplatin sensitivity in patients. Moreover, downregulation and promoter methylation of GPX3 were detected more often in gastric cancer patients with lymph node metastasis. Downregulation of GPX3 was also related to poor prognosis in patients with various cancers such as multiple myeloma, gastric cancer, and gallbladder cancer (35–37). Loss of expression of Nkx3.1, a well known tumor suppressor gene in prostate cancer, can induce increased oxidative damage in prostate carcinogenesis. In Nkx3.1 knockout mice, GPX3 expression strongly increased during conversion from normal prostate epithelium to prostatic intraepithelial neoplasia (PIN), and this expression is mainly found in tumor cells of PIN lesions. However, the loss of PTEN that is induced by increased oxidative damage can decrease GPX3 expression during the transformation from PIN to cancer in Nkx3.1 knockout mice (38). As in other cancers, loss of GPX3 expression may also have tumor suppressor functions in melanoma carcinogenesis and progression via failures in the cellular antioxidant system.

The epigenetic inactivation of GPX3 may be a crucial mechanism in the pathogenesis of melanoma, and GPX3 may serve as a possible predictive or prognostic biomarker and therapeutic target for melanoma patients.

Acknowledgements

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