Prognostic Significance of Glutathione Peroxidase 1 (*GPX1*) Down-regulation and Correlation with Aberrant Promoter Methylation in Human Gastric Cancer

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Abstract. *Background: This study aimed at examining the association of gene silencing and promoter methylation of glutathione peroxidase 1 (GPX1) and glutathione peroxidase 3 (GPX3) in gastric cancer cells and determined the clinical significance of GPX1 and GPX3 expression loss in gastric cancer tissue. Materials and Methods: Analysis of mRNA expression was carried out by reverse transcription-polymerase chain reaction (RT-PCR). Methylation of the GPX1 promoter region was analyzed by bisulfite sequencing, and that of the GPX3 promoter region was analyzed by methylation-specific PCR (MSP). Tissue microarray-based immunohistochemistry of GPX1 and GPX3 in 1,163 resected gastric cancer specimens was performed to assess the associations with clinicopathological parameters. Results: Reduced GPX1 and GPX3 mRNA expression was associated with promoter methylation in gastric cancer cell lines. A correlation between DNA promoter methylation and loss of GPX1 expression was noted in 16 gastric cancer tissue samples (p=0.005). Loss of GPX1 and GPX3 proteins was found in 24.4% and 30.8% of gastric cancer tissues. Loss of GPX1 expression was significantly associated with advanced gastric cancer (p=0.039) and lymphatic invasion (p=0.010); loss of GPX3 expression was associated with advanced gastric cancer (p<0.001) and lymph node metastasis (p<0.001). Kaplan–Meier analysis showed that low expression of GPX1 was associated with poor cancer-specific survival (p=0.010). Conclusion: Data from this study implicate aberrant hypermethylation of promoter regions of GPX1 and GPX3 as a mechanism for down-regulation of GPX1 and GPX3 mRNA expression in gastric cancer cells. Loss of GPX1 expression was associated with aggressiveness and poor survival in patients with gastric cancer.*

Gene regulation through epigenetic modification, such as CpG methylation, appears to be an important mechanism in early gastric carcinogenesis and plays an essential role in tumor suppressor gene loss of function, affecting genes such as mutL homolog 1, colon cancer, nonpolyposis type 2 (*E. coli*) (*hMLH1*), *p14*, *p15*, *p16*, E-cadherin, runt-related transcription factor 3 (*RUNX3*), thrombospondin-1 (*THBS1*), tissue inhibitor of metalloproteinase 4 (*TIMP-3*), cyclooxygenase 2 (*COX2*), and O-6-methylguanine-DNA methyltransferase (*MGMT*) (1-4). In our previous experiment, six genes, tissue factor pathway inhibitor 2 (*TFPI2*), glutathione peroxidase 3 (*GPX3*), doublesex and mab-3-related transcription factor 1 (*DMRT1*), glutathione peroxidase 1 (*GPX1*), insulin-like growth factor binding protein 6 (*IGPFBP6*), and interferon (IFN) regulatory factor 7 (*IRF7*), were up-regulated by twofold or more by 5-aza-2'-deoxycytidine (5Aza-dC) treatment. Furthermore, these genes exhibited promoter hypermethylation in more than one gastric cancer cell line but were unmethylated in normal gastric mucosa (5).

Normal cells can handle oxidative stress through intact antioxidative systems, in which glutathione *S*-transferase (GST) and GPX play a crucial role. GPXs catalyze the reduction of hydrogen peroxide, organic hydroperoxide, and lipid peroxides by reduced glutathione, thereby protecting cells against oxidative damage (6). GPX1 is the major enzyme of glutathione (GSH)-mediated defense against

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reactive oxygen species (ROS) and reduces hydrogen peroxide at the expense of oxidizing GSH to its disulfide form, GSSG. GPX1 activity is often discussed in parallel with glutathione reductase activity, which maintains a constant supply of GSH from GSSG for enzyme activity. GPX1 is expressed in epithelial tissues of the lung and other organs (7). The enzyme is a part of the enzymatic antioxidant defense system, which prevents oxidative damage to DNA, proteins, and lipids, by detoxifying hydrogen and lipid peroxides (8). GPX3 is an oxygen radical-metabolizing enzyme and plays a critical role in the detoxification of hydrogen peroxide and other oxygen-free radicals. GPX3 is highly expressed in healthy tissues and has been suggested to exhibit tumor suppressor activity and inhibit tumor growth and metastasis. In contrast to healthy tissues, GPX3 activity is significantly reduced in the blood of patients with breast, gastric, and colorectal cancer, and GPX3 is strongly down-regulated in prostate, thyroid, and gastric cancer (9).

Chronic colonization of the human stomach by *Helicobacter pylori*, a Gram-negative bacterium, is a major cause of chronic gastritis, peptic ulcers, and gastric cancer. *H. pylori* infection of the gastric mucosa elicits an inflammatory response by the host and subsequent release of ROS by activated inflammatory cells. ROS can induce DNA damage with the accumulation of DNA mutations and contribute to the pathogenesis of gastric cancer through *H. pylori*-related inflammation. These intermediate ROS are partly responsible for increased oxidative stress in gastric epithelial cells, which may be potentiated further by the associated decrease in antioxidant levels (10). Protection of cells from ROS is accomplished through the activation of oxygen-scavenging enzymes such as superoxide dismutase (SOD), catalase (CAT), and GPX (11).

GPX1 was the first selenoprotein identified with dual-functioning UGA codons that can serve as a termination signal for protein translation, as well as a signal for the incorporation of the amino acid selenocysteine. The human genome contains 25 genes that code for selenoproteins (12). There are eight known GPXs in humans, of which five are selenoproteins. Selenium supplementation has been associated with antitumorigenesis in several animal studies (13). However, it was not shown to prevent prostate cancer in a large-scale controlled clinical trial, the Selenium and Vitamin E Cancer Prevention Trial (SELECT) (14).

GPX3 is reported to be down-regulated in several types of cancers, including that of the prostate (15), thyroid (16), and colorectum (17). Given that GPX3 is always expressed in healthy tissues of patients with these cancers, GPX3 has been suggested to exhibit tumor suppressor activity. One explanation for GPX3 down-regulation in cancer cells might be the hypermethylation of the *GPX3* gene, which has been observed in prostate cancer cells (18) and in Barrett's esophagus (19, 20).

In this study, aberrant hypermethylation of *GPX1* and *GPX3* promoter regions was explored as a regulatory mechanism of GPX expression in gastric cancer cell lines and in human gastric cancer tissue. In addition, we examined the expression of GPX1 and GPX3 and their clinicopathological significance in gastric cancer.

Materials and Methods

Cell lines and tissue samples. Ten human gastric cancer cell lines (SNU1, 5, 16, 216, 484, 601, 620, 638, 668, and 719) were obtained from the Korean Cell Line Bank (Seoul, Korea). All cell lines were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin) at 37˚C in an incubator with humidified air and 5% CO_2. Formalin-fixed paraffin-embedded specimens of 1,163 gastric cancer tissues, resected at Seoul National University Hospital from 1995 to 1996, were used in this study. Age, sex, histological type, lymphatic invasion, and pTNM stage were evaluated by reviewing medical charts, pathology reports, and glass slides. Patients' clinical outcomes were followed from the date of surgery to the date of death, or until December 1, 2000. Follow-up periods ranged from 1 to 72 months (mean=49 months). The data of patients lost to follow-up and those of patients who died of causes other than gastric cancer were excluded from the survival analysis. This study was approved by the Institutional Review Board of Seoul National University Hospital.

Reverse transcription-PCR analysis. For mRNA extraction, total RNA was isolated with the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). To generate cDNA, mRNA (5 ng) was reverse transcribed using reverse transcriptase with 2 μl of 10× reverse-transcriptase buffer, 1 μl each of 10 mM deoxynucleotides (dNTPs), 1 μl of random hexamers, 2 μl of 0.1 M 1,4-dithiothreitol, and 200 units of MMLV-reverse transcriptase (Invitrogen). The reaction was incubated at 65˚C for 10 min, 25˚C for 10 min, 50˚C for 50 min, and then 85˚C for 5 min. The PCR mixture contained 0.5 μl cDNA and 2× Premix Ex Taq (Takara, Tokyo, Japan). To test cDNA integrity, the β-actin (*ACTB*) gene was amplified for each sample.

Isolation and bisulfite modification of genomic DNA. Genomic DNA was isolated from cells and tissues (n=16) by standard phenol-chloroform extraction. To denature DNA, 2 μg of genomic DNA was incubated with 1 μg of salmon sperm DNA (Sigma Aldrich, St. Louis, MO, USA) in 0.3 M NaOH for 20 min at 37˚C in a total volume of 50 μl, diluted with 50 μl of a 3.5 M sodium bisulfite (pH 5.0)/1 mM hydroquinone solution (both Sigma Aldrich), and incubated at 55˚C for 16 h. The modified DNA was then purified using the Wizard DNA Clean Up System (Promega, Madison, WI, USA). The purified DNA was incubated with 0.3 M NaOH for 10 min at 37˚C. DNA was precipitated with ethanol, dissolved in 20 μl Tris-EDTA (pH 8.0), and stored at –20˚C. The bisulfite modification of DNA converts unmethylated cytosines to uracils, but methylated cytosines are resistant to modification.

Methylation-specific PCR (MSP). Two micrograms of genomic DNA was treated with 2.5 M sodium-bisulfite (Sigma) and 10 mM hydroquinone (pH 5.0; Sigma) and then incubated at 55˚C for 15 h. After modification, DNA was purified using the Wizard DNA

purification kit (Promega).The PCR mixture contained 1 μl bisulfite-modified DNA, 5 pmol/μl primers for *GPX1* and *GPX3*, and 2× Premix Ex Taq (Takara). PCR amplification was performed for 1 cycle at 95˚C for 5 min followed by 35 cycles at 95˚C for 30, 58-62˚C for 30, 72˚C for 1 min, and a final extension at 72˚C for 10 min in an Applied Biosystems thermal cycler (Applied Biosystems, Carlsbad, California, USA).

Bisulfite sequencing of gene promoter CpG islands. Bisulfite-modified genomic DNA was used as a template for PCR amplification using primers listed in Table I. Amplified PCR products were purified enzymatically using a pre-sequencing kit (Amersham Life Science, Cleveland, OH, USA), and then directly sequenced using the BigDye terminator sequencing kit (Applied Biosystems). Bisulfite sequencing was performed in both directions using the primers used for PCR amplification. Sequencing reactions were run on an ABI 3100 automated sequencer (Applied Biosystems), and the data collected were analyzed using DNA sequencing analysis 3.7 software (Applied Biosystems).

Tissue microarray (TMA) and immunohistochemistry. For immunohistochemical analysis, formalin-fixed paraffin-embedded tissues of 1,163 surgically resected gastric carcinoma tissues were collected at the Seoul National University Hospital from 1995 to 1996. The tumor sections were reviewed, and representative core tissue sections (2 mm in diameter) were taken from the paraffin blocks and arranged in new TMA blocks by using a trephine apparatus (Superbiochips Laboratories, Seoul, Korea). From the TMA blocks, 4-μm-thick sections were deparaffinized and rehydrated in graded alcohol. Antigen retrieval was achieved by pressure cooking in 0.01 mol/l citrate buffer for 5 min. The primary antibodies to GPX1 (sheep, polyclonal, diluted 1:600; Abcam, Cambridge, UK) and GPX3 (rabbit, polyclonal, diluted 1:400; Novus, CO, USA) were diluted and incubated at room temperature for 1 hr. The immunohistochemical reaction was visualized using the EnVision kit (Dako, Carpinteria, CA, USA) according to the manufacturer's protocol.

The intensity was semiquantitatively scored by pathologists, who were unaware of the outcome of the patients into four categories: 0, no positively stained cell; 1+, 1% to 10% positively stained cells; 2+, 11% to 50% positively stained cells; 3+, more than 50% positively stained cells. High expression levels, determined by strong, diffuse staining of the cytoplasm, was found in all normal gastric mucosa.

Table I. *Oligonucleotide primers used in reverse transcription polymerase chain reaction (RT-PCR), methylation-specific PCR (MSP), and bisulfite sequencing (BS).*

Gene			Sequence
GPX1	MSP	U-AS	5'-ATGAGGTGGGATTTTTAGGTTT -3'
		U-S	5'- ACCAAACAACACACATAACACA-3'
		M-AS	5'-GAGGCGGGATTTTTAGGTTC -3'
		M-S	5'-CTAACCGAACAACACACATAACG-3'
	BS	AS	5'-GTTGGTTTTTTGGATAATTG-3'
		S	5'-AAAAACCCAAACTCACAAACTC-3'
	RT-PCR	AS	5'-AAGGTACTACTTATCGAGAATGTG-3'
		S	5'-GTCAGGCTCGATGTCAATGGTCTG-3'
GPX3	MSP	U-AS	5'-GTGTTTGTTTTTGAAATTTTAGTTGT-3'
		U-S	5'-CTACCTAATCCCTAACCACCATC-3'
		M-AS	5'-CGTTCGTTTTTGAAATTTTAGTC-3'
		M-S	5'-CTACCTAATCCCTAACCACCGT -3'
	RT-PCR	AS	5'-CTTCCTACCCTCAAGTATGTCCG-3'
		S	5'-GAGGTGGGAGGACAGGAGTTCTT-3'

AS: Antisense, S: sense.

Results

Reduced mRNA level was associated with aberrant promoter methylation of GPX1 and GPX3 in 10 gastric cancer cell lines. We investigated mRNA expression of *GPX1* and *GPX3* by RT-PCR, and promoter DNA methylation, by MSP, in 10 gastric cancer cell lines. The presence of low mRNA levels of *GPX1* and promoter methylation was noted in SNU1 and SNU484. Low levels of *GPX3* mRNA were found in five of the cell lines, SNU1, SNU484, SNU601, SNU638, and SNU719. Methylation in the *GPX3* promoter region was detected in seven cell lines, SNU1, SNU216, SNU484, SNU601, SNU620, SNU638, and SNU719. In SNU484 and SNU620 cells, both methylated and unmethylated bands were detected; in the remaining five cell lines, only methylated bands were detected; Interestingly, SNU484 exhibited a faint band for *GPX3* mRNA expression and a strong band for methylated *GPX3*, whereas SNU620 had a strong band for *GPX3* mRNA expression and a strong band for unmethylated *GPX3* (Figure 1). These results suggest that promoter methylation was associated with silencing of *GPX1* and *GPX3* mRNA expression in gastric cancer cell lines.

Promoter hypermethylation and immunohistochemistry of GPX1 and GPX3 in 16 gastric cancer tissue samples. Methylation and immunohistochemistry of *GPX1* and *GPX3* were investigated in 16 gastric cancer tissue samples. Promoter methylation of *GPX1* was confirmed by bisulfate sequencing in five cases, four of which exhibited loss of GPX1 staining. The promoter region of *GPX1* was unmethylated in the remaining 11 cases, and loss of GPX1 expression was observed in only one of these cases (p=0.005). Promoter methylation of *GPX3* was detected by MSP in eight cases, three of which exhibited loss of GPX3 staining. Among the eight unmethylated cases, seven retained GPX3 expression. Regarding *GPX3*, no correlations between loss of immunohistochemical staining and promoter methylation were noted in gastric cancer tissue (Table II).

Correlation of loss of GPX1 and GPX3 with clinico-pathological parameters in gastric cancer tissue. Immunohistochemical analyses of *GPX1* and *GPX3* expression were performed on TMA of 1,163 gastric cancer tissue samples (Figure 3). High levels of expression of both *GPX1* and *GPX3* were found in normal gastric mucosa, as determined by strong and diffuse staining in the cytoplasm. We found that *GPX1* expression was lost in 24.4% (273 out

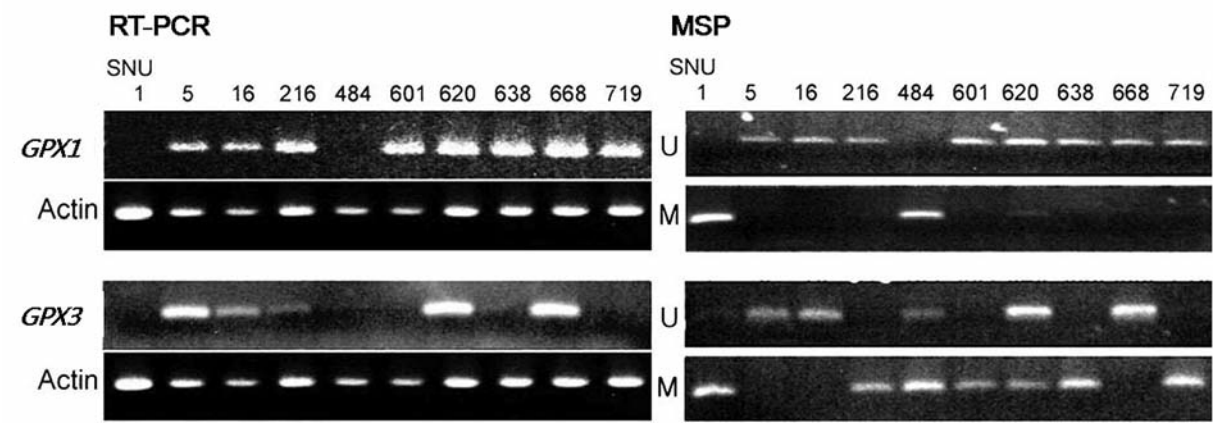

Figure 1. *Low mRNA expression and promoter methylation of glutathione peroxidase 1 (GPX1) and GPX3 in gastric cancer cell lines. SNU1 and SNU484 cell lines exhibited low levels of GPX1 mRNA expression in RT-PCR and promoter methylation of GPX1 in methylation-specific PCR (MSP). Low levels of GPX3 mRNA expression in SNU1, SNU484, SNU601, SNU638, and SNU719 cells and the presence of a methylated (M) band in SNU1, SNU216, SNU601, SNU638, and SNU719 cells were revealed in MSP. Bands were present for both unmethylated and methylated genes in SNU484 and SNU620 cells.*

Table II. *Correlation between promoter methylation and immunohistochemistry (IHC) of GPX1 and GPX3 in 16 gastric cancer tissue samples.*

	GPX1 Bisulfite sequencing					*GPX3* MSP			
GPX1 IHC	M	U	Total	*P*-Value	GPX3 IHC	M	U	Total	*P*-Value
Loss	4	1	5	0.005	Loss	3	1	4	0.248
Retained	1	10	11		Retained	5	7	12	
Total	5	11	16		Total	8	8	16	

GPX1: Glutathione peroxidase 1. GPX3: Glutathione peroxidase 3. IHC: Immunohistochemistry. MSP: Methylation-specific PCR. M: Methylation, U: Unmethylation.

of 1,119) of gastric cancer cases. Loss of *GPX1* expression was significantly associated with advanced gastric cancer (p=0.039) and lymphatic invasion (p=0.010), but not with lymph node metastasis, Lauren's classification, or vascular invasion. Loss of *GPX3* expression was noted in 30.7% (337 out of 1,095) of gastric cancer cases and was significantly associated with advanced gastric cancer (p<0.001) and lymph node metastasis (p<0.001) (Table II).

Kaplan–Meier survival plots for the 1,163 patients with gastric cancer showed significant differences in cancer-specific survival between the low- and high-expression groups of GPX1. Low GPX1 expression status was associated with poor survival (p=0.010) (Figure 4). However, low GPX3 was not associated with survival. Multivariate analysis of *GPX1* and *GPX3* expression did not reveal any correlations with cliniocopathological parameters (data not shown).

Discussion

We revealed correlations between mRNA expression and aberrant promoter methylation of *GPX1* and *GPX3*. We confirmed the down-regulation of *GPX1* and *GPX3* by immunohistochemistry on tissue microarray in 24.4% (GPX1) and 30.8% (GPX3) of gastric cancer cases and identified an association between the loss of GPX1 expression and poor cancer-specific survival.

ROS are generated as by-products of cellular metabolism, primarily in the mitochondria. When the cellular production of ROS exceeds the antioxidant capacity of a cell, cellular macromolecules such as lipids, proteins, and DNA can be damaged. Because of this, oxidative stress is thought to contribute to aging and pathogenesis of a variety of human diseases (6). Antioxidant enzymes, such as GPX, are thought to be involved in the primary cellular defense mechanism against ROS through reduction in oxidative stress, and growing data have implicated the selenium-containing cytosolic *GPXs* as determinants of cancer risk and mediators of the chemopreventive properties of selenium (21). Under certain conditions, such as nitroxidative stress and glycoxidative stress, *GPX* can be inactivated. Our results show that inactivation of *GPX1* was linked to promoter methylation in gastric cancer.

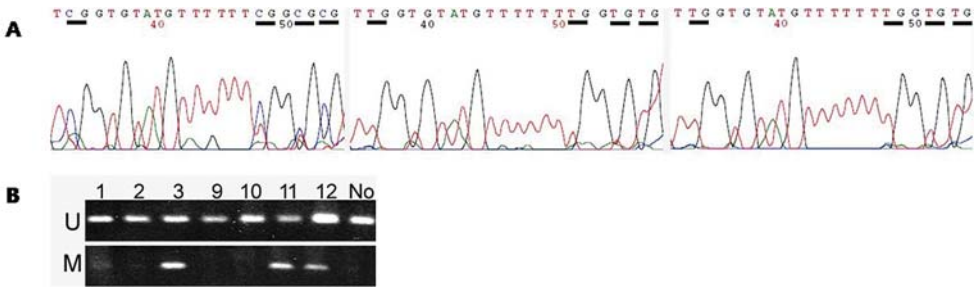

Figure 2. *Promoter methylation of glutathione peroxidase 1 (GPX1) and glutathione peroxidase 3 (GPX3) in gastric cancer tissue. A: Bisulfate sequencing of GPX1 showed methylation in 4 CpG loci in gastric cancer (left), unmethylation in gastric cancer (middle), and unmethylation in normal gastric mucosa (right). B: Methylation-specific PCR (MSP) of GPX3 in gastric cancer tissue. U: Unmethylated DNA. M: Methylated DNA.*

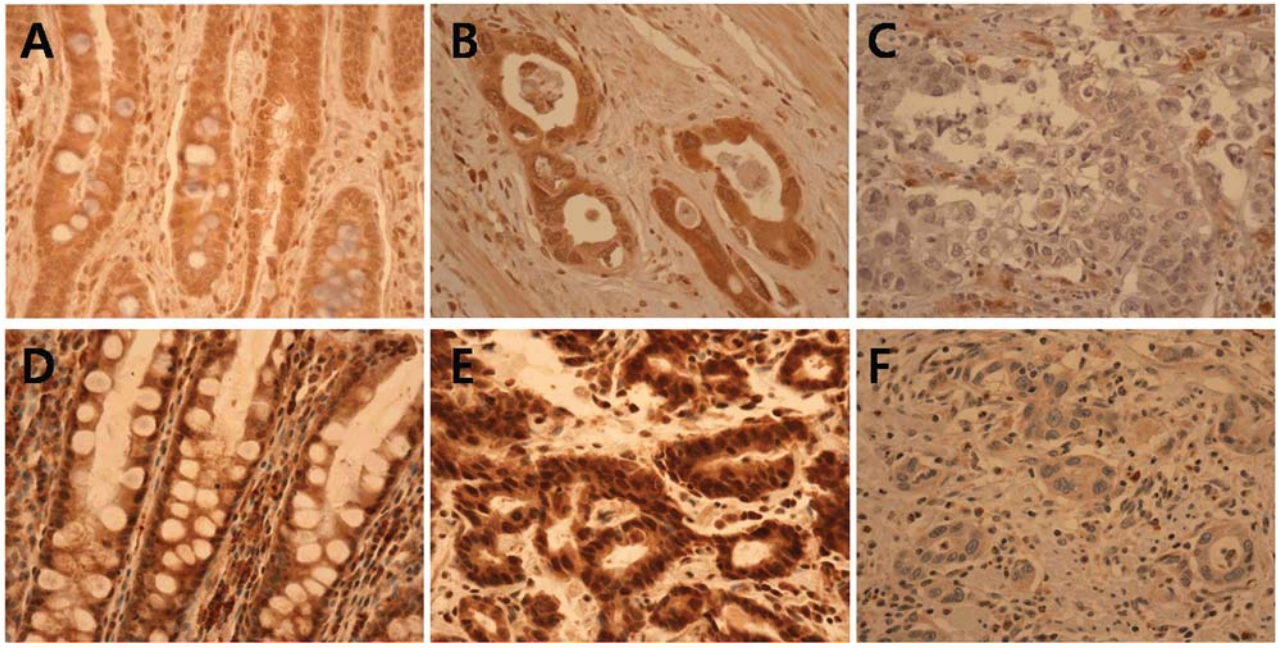

Figure 3. *Immunohistochemistry of glutathione peroxidase 1 (GPX1) and GPX3 in gastric tissue. A: Representative images of positive immunostaining of GPX1 in normal gastric mucosa. B: Gastric cancer with GPX1 expression. C: Gastric cancer negative for GPX1. D: Positive immunostaining of GPX3 in normal gastric mucosa. E: Gastric cancer with GPX3 expression. F: Gastric cancer negative for GPX3 (×400).*

There are several methylation-related genes linked to *H. pylori*-related gastritis, such as calcitonin-related polypeptide alpha (*CALCA*), cadherin 1, type 1 (*CDH1*), cellular retinoic acid binding protein 1 (*CRABP1*), cytochrome P450, family 1, subfamily B, polypeptide 1 (*CYP1B1*), death-associated protein kinase 1 (*DAPK1*), glutamate receptor, ionotropic, N-methyl D-aspartate 2B (*GRIN2B*), and twist homolog 1 (Drosophila) (*TWIST1*) (22, 23). Methylation of *GPX1* in *H. pylori*-related gastritis may play an important role during gastric carcinogenesis. Farinati *et al*. (24) found that *H. pylori* infection is apparently the single most important factor in determining the level of DNA damage in the gastric mucosa resulting from oxidative stress, as assessed from 8-hydroxydeoxyguanosine (8-OHdG) levels. 8-OHdG is considered the primary DNA modification induced by reactive oxygen metabolites and may be responsible for DNA base mutation.

GPX is a major antioxidative damage enzyme family that catalyzes the reduction of hydrogen peroxide, organic hydroperoxide, and lipid peroxides by reduction of GSH (6). GPX functions to protect cells from ROS generated during *H. pylori*-related gastritis. Based on the known functions of

Table III. *Clinicopathological correlations of GPX1 and GPX3 immunohistochemistry (IHC) in gastric cancer.*

Characteristic	GPX1 IHC						GPX3 IHC					
	Loss		No loss				Loss		No loss			
	n	%	n	%	Total	*P*-Value	n	%	n	%	Total	*P*-Value
Total	273	24.4	846	75.6	1119		337	30.8	758	69.2	1095	
AGC/EGC						0.039						<0.001
AGC	207	26.1	585	73.9	792		206	26.1	583	73.9	789	
EGC	66	20.2	261	79.8	327		131	42.8	175	57.2	306	
T Stage						0.152						<0.001
T1	66	20.3	259	79.7	325		131	43.1	173	56.9	304	
T2	139	27.0	375	73.0	514		135	26.5	374	73.5	509	
T3	66	24.6	202	75.4	268		69	25.6	201	74.4	270	
T4	2	16.7	10	83.3	12		2	16.7	10	83.3	12	
N Stage						0.101						<0.001
N0	113	27.2	302	72.8	415		160	38.6	255	61.4	415	
N1-3	217	31.9	463	68.1	680		177	26.0	503	74.0	680	
Lauren's classification						0.169						0.783
Intestinal	135	31.3	297	68.8	432		134	31.0	298	69.0	432	
Diffuse	170	28.4	429	71.6	599		181	30.2	418	69.8	599	
Mixed	25	39.1	39	60.9	64		22	34.4	42	65.6	64	
Lymphatic invasion						0.010						0.015
Absent	173	22.2	607	77.8	780		248	33.1	502	66.9	750	
Present	100	29.5	239	70.5	339		89	25.8	256	74.2	345	
Vascular invasion						0.611						1.000
Absent	263	24.6	807	75.4	1070		321	30.7	723	69.3	1044	
Present	10	20.4	39	79.6	49		16	31.4	35	68.6	51	

AGC: Advanced gastric cancer. EGC: Early gastric cancer. GPX1: Glutathione peroxidase 1. GPX3: Glutathione peroxidase 3. IHC: Immunohistochemistry.

GPXs, GPX1 and GPX3 could play an important role in neutralizing the damaging effect of ROS in *H. pylori*-related gastritis.

From our results, correlations between methylation and tissue expression or prognostic significance for GPX3 were not as strong as those observed for GPX1. Tissue-specific inactivation mechanisms other than *GPX3* promoter methylation may exist in gastric cancer. For example, frequent genomic deletions of *GPX3* are known to occur in prostate cancer (15), and such polymorphisms may also be important in gastric cancer. Recently, Wang *et al*. reported that intronic single nucleotide polymorphisms in *GPX3* can impact gene expression and influence gastric cancer risk (9).

In conclusion, expression of *GPX1* and *GPX3* is regulated by promoter methylation in gastric cancer cell lines. Loss of *GPX1* expression was significantly correlated with promoter methylation in gastric cancer tissues and was also associated with the aggressiveness of gastric cancer and poor patient survival. Our results suggest that *GPX1* is a tumor suppressor gene regulated by promoter methylation in gastric carcinogenesis.

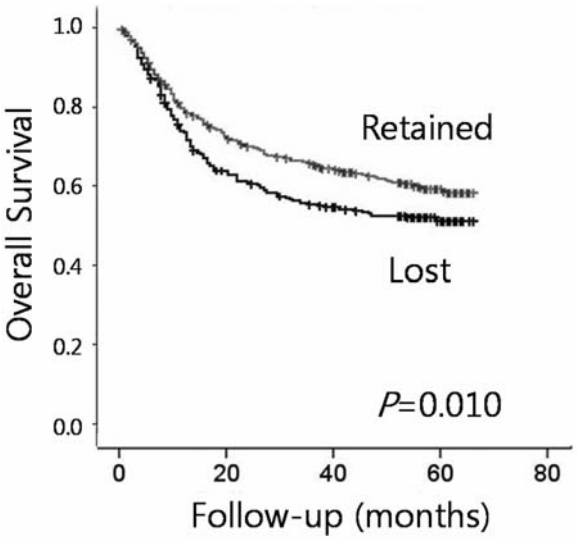

Figure 4. *Kaplan–Meier analysis of cancer-specific survival according to glutathione peroxidase 1 (GPX1) expression in gastric cancer. Loss of GPX1 expression was associated with poor survival at gastric cancer patients (p=0.010).*

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