Epigenetic Silencing of the Putative Tumor Suppressor Gene *GLDC* (Glycine Dehydrogenase) in Gastric Carcinoma

HYAE LIM MIN[1,2], JIN KIM[1,3], WOO HO KIM[1,2,3], BO GUN JANG[3] and MIN A. KIM[3]

[1]*Cancer Research Institute, Seoul National University College of Medicine, Seoul, Republic of Korea;*
[2]*Department of Cancer Biology, Seoul National University College of Medicine, Seoul, Republic of Korea;*
[3]*Department of Pathology, Seoul National University College of Medicine, Seoul, Republic of Korea*

Abstract. *Background: The metabolic enzyme, glycine dehydrogenase (GLDC), involved in glycine metabolism, is known to be involved in non-ketotic hyperglycinemia but not in cancer. Herein, we investigated GLDC expression and its promoter methylation in gastric cancer (GC). Materials and Methods: GLDC expression and epigenetics were investigated using GC cell lines and tissues. Functional studies were also performed for identification of a correlation between methylated GLDC genes and gastric cancer progression. Results: The results of the study can be summarized as follows: (i) GLDC was silenced in GC cell lines and tissues. The down-regulation of GLDC was closely linked to promoter methylation. (ii) Knockdown of GLDC increased cell proliferation, migration, invasion, colony formation and reduced apoptosis. (iii) In GC tissues, hypermethylation of GLDC had a significant correlation with down-regulation of the GLDC protein compared to normal gastric tissues. Conclusion: GLDC is a putative tumor suppressor gene involved in gastric cancer progression and hypermethylation of the GLDC promoter regulates its transcriptional silencing.*

Gastric cancer (GC) is the second leading cause of cancer-related death worldwide (1). Multiple sequential genetic alterations occur during GC tumorigenesis and progression. Thus, in order to understand gastric carcinogenesis, it is important to study its various genetic alterations (2, 3). Although previous studies have reported multiple genes altered in human GC (4, 5), a good number of genes involved in gastric carcinogenesis and progression remain unknown.

Correspondence to: Min A. Kim, MD, Ph.D., Department of Pathology, Seoul National University College of Medicine, 101 Daehak-ro, Jongno-gu, Seoul, 03080, Korea. Tel: +82 2-20722366, Fax: +82 27435530, e-mail: everest@snu.ac.kr

Key Words: Glycine dehydrogenase, tumor suppressor gene, DNA methylation, stomach neoplasms.

Gastric carcinogenesis has multiple etiologies, including genetic and environmental factors (3, 6). In particular, DNA hypermethylation in the promoter region of tumor suppressor genes results in suppression of mRNA transcription and gene silencing and is one of the major causes of gastric carcinogenesis (7). Moreover, studies of epigenetic changes in specific tumor suppressors are clinically significant, and may be used as biomarkers for the diagnosis, prevention and treatment of GC.

Glycine dehydrogenase (GLDC) is a metabolic enzyme involved in glycine and serine metabolism. It catalyzes the reaction whereby glycine is converted to carbon dioxide, ammonia and 5,10-methylene-tetrahydrofolate (CH_2-THF) (8). In turn, CH_2-THF drives *de novo* thymidine synthesis and pyrimidine biosynthesis, thus regulating nucleotide synthesis during cell proliferation (9). A recent study reported that GLDC drives tumor-initiating cells and tumorigenesis in non-small cell lung cancer (NSCLC), suggesting that GLDC could be a therapeutic target in anticancer therapy (10). However, that study detected GLDC expression in only 26.1% of diverse cancer cell lines, including ovary, germ cell, lung, prostate, colon and brain cancers. Recently, tumor metabolism has been identified as a critical event in tumorigenesis (11, 12). For example, pyruvate kinase (PKM2) has been shown to promote tumorigenesis through a metabolic mechanism in many cancers (13). However, a metabolic role for GLDC has not been shown in carcinogenesis.

In the present study, we found that aberrant hyper-methylation of the promoter regions of *GLDC* regulated GLDC expression in GC cell lines and human gastric tissues, which suggests that GLDC has a tumor suppressive role. In the present study, we analyzed GLDC expression and methylation in gastric cancer progression, as well as its biological and clinicopathological significance in GC.

Materials and Methods

Cell lines and patient tissues. Ten gastric cancer cell lines (SNU1, 5, 16, 216, 484, 601, 620, 638, 668 and 719) and a human embryonic kidney 293 (HEK293) cell line were used. (HEK293 cell line was used as control because *GLDC* expression in HEK293 cell line is

higher than gastric cancer cell lines: http://medical-genome.kribb.re.kr/GENT/). The GC cell lines were maintained in RPMI-1640 (HyClone, Logan, UT, USA), and the HEK293 cell line was maintained in MEM (HyClone). All cell lines were obtained from the Korea Cell Line Bank and the media contained 10% fetal bovine serum (HyClone), 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma Aldrich, St. Louis, MO, USA). Cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2. Surgically resected formalin-fixed paraffin-embedded GC tissues (n=410) were collected from the archives of the Pathology Department of Seoul National University Hospital. In addition, fresh GC tissues and paired normal tissues (n=54) were obtained during surgery at the same hospital. Their clinicopathological parameters, such as World Health Organization (WHO) classification, Lauren's classification, pathologic tumor-node-metastasis (pTNM) stage, were evaluated by reviewing medical charts and pathological records. This study was approved by the Institutional Review Board of the Seoul National University Hospital.

Oligonucleotide microarray analysis. Total RNA from the ten GC cell lines was analyzed by Affymetrix U133A 2.0 GeneChip microarray (Affymetrix, Santa Clara, CA). Target preparation and microarray procedures were performed according to the Affymetrix GeneChip Expression Analysis Manual (Affymetrix). All experiments were performed in triplicate. Detailed methods for analysis were described in our previous study (14).

Illumina Infinium Human Methylation 27 BeadChip analysis. DNA was modified with bisulfite using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA, USA) and was analyzed using the Infinium Human Methylation 27 BeadChip kit (Illumina, San Diego, CA, USA). Processing and data analysis were performed using the reagent provided in the kit according to the manufacturer's instructions. All experiments were performed in triplicate. Data were analyzed using the BeadStudio v3.0 software (Illumina), and methylation values were expressed as a beta-value (β-value) ranging from 0 (completely unmethylated) to 1 (completely methylated) (15).

Reverse-transcription PCR and real-time quantitative PCR. Total RNA was prepared using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (1 μg) was reverse-transcribed to cDNA with the GoScript™ reverse-transcription system (Promega, Madison, WI, USA). Reverse-transcription polymerase chain reaction (RT-PCR) was performed as followed: 33 cycles of 95˚C for denaturation, 60˚C for annealing, and 72˚C for extension followed by a final extension at 72˚C. PCR was performed in an ABI Veriti 96-well thermal cycler (Applied Biosystems, Forster city, CA, USA). Primers for *GLDC* transcripts (forward, 5'-AACCAGGGAGCAACACATTC-3' and reverse, 5'-GCAACCA GTTCTGCAGATGA-3') and β-actin transcripts (forward, 5'-ACACTGTGCCCATCTACGAGG-3' and reverse, 5'-AGGGGC CGGACTCGTCATACT-3') were used. PCR products were electrophoresed on 2% agarose gels, stained with loading star dye (Dynebio, Seongnam, Korea), and visualized under UV light. All experiments were performed in triplicate. Real-time quantitative PCR was performed as follows: 40 cycles of 95˚C for denaturation and 60˚C for annealing. The reaction was performed in an ABI 7500 real-time PCR system (Applied Biosystems). Real-time quantitative PCR was performed using primers for *GLDC* transcripts tagged with an FAM probe and GAPDH transcripts tagged with VIC probe. (both Applied Biosystems). All experiments were performed in triplicate. After the reaction, C_T values were analyzed using the $\Delta\Delta C_T$ methods.

Western blot analysis. All cellular and tissue proteins were extracted using Pro-Prep™ for cell/tissue protein extraction solution (Intron Biotechnology, Seongnam, Korea). In order to detect apoptotic factors, such as poly-ADP ribose polymerase (PARP), cleaved-caspase 3, and cleaved-caspase 9, we extracted proteins of SNU484 shControl and shGLDC cells after treatment with 0.5 μg/ml staurosporine (STS) (Sigma Aldrich) for 4 h. The rabbit anti-GLDC (Sigma Aldrich), rabbit anti-PARP (Cell Signaling, Danvers, MA, USA), rabbit anti-cleaved-caspase 3 (Cell Signaling), rabbit anti-cleaved-caspase 9 (Cell Signaling), and mouse anti-β-actin (Sigma Aldrich) antibodies were used as primary antibodies. Following overnight incubation at 4˚C, blots were washed with TBS buffer containing 0.1% Tween-20, incubated for 1 h at room temperature with secondary antibodies, and visualized using ECL solution (Pierce). All experiments were performed in triplicate.

5-aza-2'-deoxycytidine and/or Trichostatin A treatment. GC cell lines expressing GLDC were treated with 5-aza-2'-deoxycytidine (5-aza-dc) and/or Trichostatin A (TSA) (both from Sigma Aldrich). Cells were treated with 5-aza-dc (5 μM) for 4 days or TSA (0.3 μM) for 24 h. As a control, two groups of cells were studied without the addition of drugs. For combined treatment, cells were first treated with 5-aza-dc (5 μM) for 3 days first and subsequently with TSA (0.3 μM) for 24 h.

Methylation of the cell lines and tissues. Genomic DNA was extracted from cells and tissues by proteinase K and purified by Chelex-100 (Sigma Aldrich). Isolated genomic DNA (0.5 μg) was modified using the EZ DNA Methylation-Gold™ Kit (Zymo Research). For the methylation-specific PCR, bisulfite-modified DNA was amplified using primers specific for methylated and unmethylated promoter region of GLDC. The primer sequences for the unmethylated promoter region were: forward, 5'-TGTTTTGGGTGGAGTTA TAATTTTGT-3' and reverse, 5'-CCCAACCTAAAACCCCTTTCAC-3'. The primer sequences for the methylated promoter region were: forward, 5'-GTTTTGGGTGGAGTTATAATTTTGC-3' and reverse 5'-CCGACCTAAAACCCCTTTCG-3'. PCR was performed in an ABI Veriti 96-well thermal cycler (Applied Biosystems) for 31 cycles of 65˚C annealing temperature. PCR products were loaded onto 2% agarose gels, stained with loading star dye (Dynebio), and visualized under UV light. All experiments were performed in triplicate. Bisulfite-modified DNA was used for bisulfite sequencing with specific primers (forward, 5'-TTGTTTATTTTTATTGGTTAA GGGTTTT-3' and reverse, 5'-CTCTTAACCCCTCTCCT AACCTC-3'). PCR products of 250 bp were purified using EXO-SAP (Applied Biosystems) and were directly sequenced using a BigDye terminator kit (Applied Biosystems). Sequencing reactions were run on an ABI 3130xl genetic analyzer system (Applied Biosystems), and results were analyzed using DNA sequencing analysis 3.7 software (Applied Biosystems). All experiments were performed in triplicate.

shRNA lentiviral particle transduction. All shRNAs were constructed in lentiviral particles and were obtained from Santa Cruz Biotechnology (Santa Cruz, Dallas, TX, USA). Lentiviral particles were transduction-ready and contained shRNA designed to knockdown gene expression. Cells were seeded in 60-mm

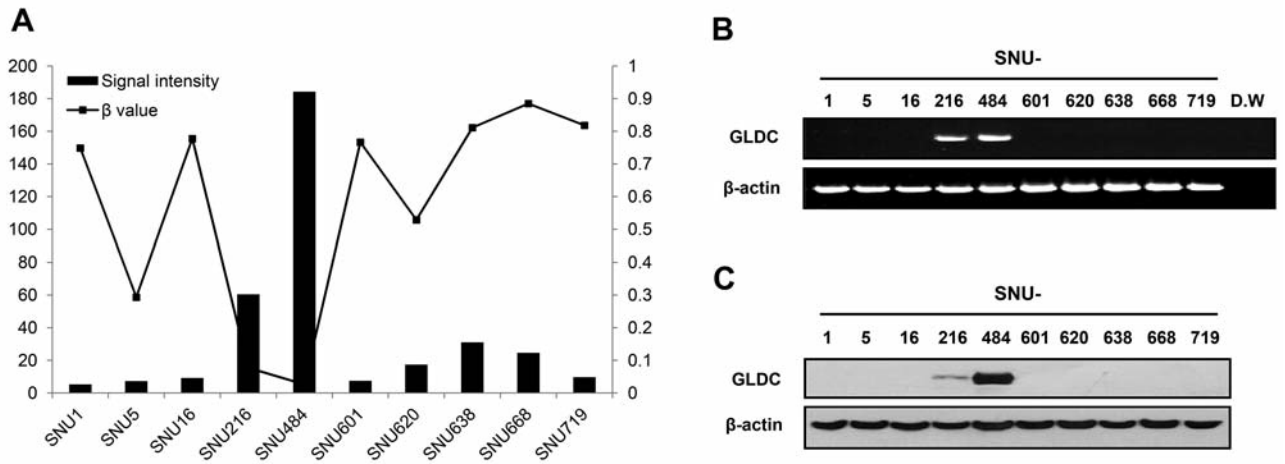

Figure 1. *Expression of GLDC in GC cell lines. (A) The oligonucleotide microarray analysis and the Infinium Human Methylation 27 BeadChip analysis were used to identify potential tumor suppressor genes in ten GC cell lines. The black bar represents GLDC mRNA expression from microarray data, and the dot and line represent methylation status. A high β-value indicates hypermethylation, while a low β-value indicates hypomethylation. (B) GLDC mRNA expression in ten GC cell lines was determined by RT-PCR. Down-regulation of GLDC was found in eight GC cell lines. Distilled water (DW) was used as a negative control, and β-actin was used as an internal control. (C) The protein expression of GLDC was performed by western blot analysis in ten GC cell lines. Protein loading was normalized using an anti-β-actin antibody.*

culture dishes and were transduced with lentiviral particles containing control and GLDC shRNA with 10 μg/ml Polybrene (Santa Cruz) according to the manufacturer's instructions. Twenty-four hours after transduction, cells were dose-dependently selected using puromycin (Santa Cruz). Silencing of GLDC was validated by RT-PCR and western blot. All experiments were performed in triplicate.

Cell biology assays. To determine the effects of shRNA lentiviral particle transduction on GC cell growth, the transduced cells were seeded in 96-well plates and incubated for 1 day at 37˚C. Cells were treated with Cell Counting Kit-8 (CCK-8) reagent (Dojindo, Tokyo, Japan) and incubated for 2 h at 37˚C, and absorbance was measured at 450 nm using a spectrophotometer (Thermo Labsystems, Beverly, MA, USA). All assays were performed in triplicate. Cell migration and invasion were compared between control and GLDC shRNA-transduced cells. For the cell migration assay, BD BioCoat Control Cell Culture Inserts in 24-well plates were used (BD Biosciences, San Jose, CA, USA) and BD BioCoat Matrigel Invasion Chamber was used for invasion assay. The assays were performed according to the manufacturer's instructions. The number of cells undergoing migration and invasion were quantified by microscopy. All experiments were performed in triplicate. Cell mobility was investigated using the wound-healing assay. The control and GLDC shRNA-transduced cells were seeded in 60-mm culture dishes, and wounds were created at three places using a sterile pipette tip. Cells were photographed under microscopy 24 h after incubation. All experiments were performed in triplicate. For the colony formation assay, the control and GLDC shRNA-transduced cells were maintained for 3 weeks. To stain surviving colonies, cells were fixed using 100% methanol for 10 min and stained using 0.5% crystal violet (Sigma Aldrich) for 20 min. After washing-off the dye, the stained colonies were counted. All experiments were performed in triplicate.

Immunohistochemistry. To quantify GLDC protein expression, immunohistochemistry (IHC) was performed using tissue microarrays (TMA) in which core tumor tissue sections (2 mm in diameter) were arranged. All IHC processing was performed using a Leica Bond-max autostainer with the Bond polymer detection kit (Leica microsystems, Wetzlar, Germany) and a GLDC primary antibody (Sigma Aldrich) diluted to 1:50. Cytoplasmic staining was scored as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong) based on intensity. Tumors with scores 0 and 1 were considered negative, and tumors with scores of 2 and 3 were considered positive. All experiments were performed in triplicate.

Statistical analysis. The Pearson's Chi-square test and Fisher's exact test (two-sided) were used to determine the significance of correlation between two factors, such as GLDC protein expression and promoter methylation or clinicopathological parameters. All analyses were performed with the SPSS PASW Statistics 18.0 software (SPSS Inc., Chicago, IL, USA), and all graphs were designed by GraphPad Prism 5.01 (GraphPad Software Inc., La Jolla, CA, USA). p-Values<0.05 were considered statistically significant.

Results

Expression of GLDC in GC cell lines. In our previous study, we performed high-throughput experiments to identify candidate tumor suppressor genes in ten GC cell lines using oligonucleotide microarray analysis and the Infinium Human Methylation 27 BeadChip. We identified candidate genes that were down-regulated by the microarray data and were hypermethylated by the methylation chip data. We excluded genes (*TWIST1* and

ADAM23) that had been previously reported to regulate gene silencing in GC by promoter methylation (4, 16). Among the novel candidate genes, we focused on *GLDC*. Comparing the mRNA expression and promoter methylation results among the ten GC cell lines, we found that two GC cell lines (SNU216 and 484) had lower β-value, indicating unmethylation, and high mRNA expression levels. The remaining eight GC cells showed high β-value, and low mRNA expression levels (Figure 1A). These data suggested a relationship between *GLDC* mRNA expression and promoter methylation status in GC cell lines. Further, RT-PCR and western blot analysis confirmed that only two GC cell lines, SNU216 and 484, expressed GLDC mRNA and protein, while the remaining eight GC cell lines did not at all (Figure 1B and C).

Epigenentic silencing of GLDC in GC cell lines. To confirm the hypermethylation status of GLDC in GC cell lines, we searched for CpG islands in the promoter region of *GLDC* (Figure 2A). Methylation-specific PCR (MSP) and bisulfite sequencing were performed, and the resulting data were similar to those obtained with the Infinium Human Methylation27 BeadChip. Two GC cell lines (SNU216 and 484) expressed GLDC mRNA, as measured by RT-PCR (Figure 1B) and showed unmethylated promoter regions, while the remaining eight GC cell lines showed silenced mRNA and methylated promoter regions (Figure 2B). Next, we confirmed the MSP results with bisulfite sequencing. Each bar in Figure 2C represents CpG sites in the promoter region. In this assay, an unmethylated cytosine is converted to uracil by bisulfite modification, while a methylated cytosine is not. The presence of TG indicated an unmethylated site in the SNU484 cell line, and the presence of CG indicated a methylated site in SNU620, 638 and 668 cell lines (Figure 2C). After treatment with 5-aza-dc and/or TSA, RT-PCR analysis demonstrated a restoration of *GLDC* mRNA expression in SNU1, 620, 638 and 719 cell lines, which had shown gene silencing (Figure 2D). These data suggested that *GLDC* silencing was associated with promoter methylation in GC cell lines.

Effect of GLDC knock-down. In order to clarify the functions of GLDC in GC, we examined the effect of GLDC knock-down on cell growth using two GC cell lines overexpressing GLDC, SNU484 and HEK293. GLDC shRNA (shGLDC) and control shRNA (shControl) lentiviral particles were transduced into the cell lines. Knockdown of *GLDC* mRNA and GLDC protein was confirmed by real-time quantitative PCR and western-blot analysis (Figure 3A). We performed a proliferation assay using CCK-8. The shGLDC cell line grew faster than the shControl cell line in both SNU484 and HEK293 cells (Figure 3B). Cell-colony formation was examined in SNU484 shControl and shGLDC cell lines. Knock-down of GLDC increased cell-colony formation in the SNU484 shGLDC cell line compared to the shControl cell line (Figure 3C). These results suggested that the inactivation of *GLDC* increased cell growth in GC and normal cell lines.

Table I. *Correlation between GLDC protein expression and clinicopathological features in GC*.

	GLDC protein expression		
	Negative (%) n=339	Positive (%) n=71	*p*-Value
Gender			0.014
Male	238 (79.9)	60 (20.1)	
Female	101 (90.2)	11 (9.8)	
WHO classification			<0.001
Papillary	1 (50.0)	1 (50.0)	
W/D	17 (63.0)	10 (37.0)	
M/D	112 (76.7)	34 (23.3)	
P/D	126 (86.3)	20 (13.7)	
Mucinous	14 (100.0)	0 (0.0)	
SRC	65 (97.0)	2 (3.0)	
Undifferentiated	3 (60.0)	2 (40.0)	
Others	1 (33.3)	2 (66.7)	
Lauren's classification			<0.001
Intestinal	128 (74.0)	45 (26.0)	
Diffuse	150 (91.5)	14 (8.5)	
Mixed	59 (84.3)	11 (15.7)	
Undetermined	2 (66.7)	1 (33.3)	
pTNM stage			N.S
I	141 (82.0)	31 (18.0)	
II	65 (79.3)	17 (20.7)	
III	70 (89.7)	8 (10.3)	
IV	63 (80.8)	15 (19.2)	
Tumor invasion			N.S
EGC	84 (80.0)	21 (20.0)	
AGC	255 (83.6)	50 (16.4)	
Lymphatic invasion			N.S
Absent	141 (86.0)	23 (14.0)	
Present	198 (80.5)	48 (19.5)	

N.S, Not significant; EGC, early gastric carcinoma; AGC, advanced gastric carcinoma; W/D, well differentiated; M/D, moderately differentiated; P/D, poorly differentiated; SRC, signet ring cell.

Table II. *Correlation of GLDC methylation status with GLDC protein expression in GC tissues and adjacent normal gastric tissues*.

Methylation-specific PCR	GLDC western blot analysis in GC tissues		
	≥Normal tissues (%)	<Normal tissues (%)	*p*-Value
Unmethylation	15 (51.7)	14 (48.3)	0.001
Methylation	2 (8.0)	23 (92.0)	
Total	17 (31.5)	37 (68.5)	

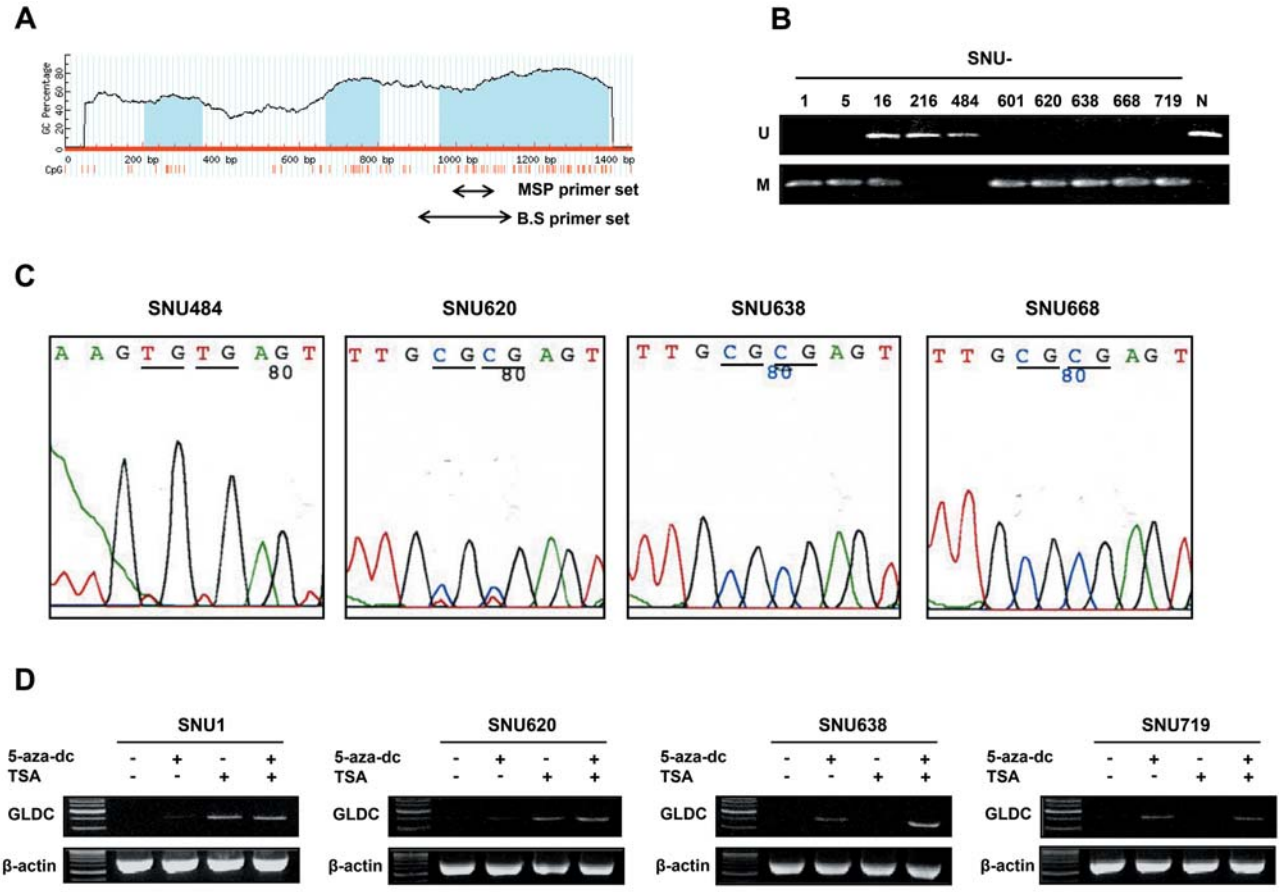

Figure 2. *Hypermethylation of the GLDC promoter region in GC cell lines. (A) Schematic of the GLDC promoter region. Sky blue areas are CpG islands in the promoter region. The two lines represent the MSP primer set and bisulfite sequencing primer set. (B) The methylation status was determined by MSP. Eight GC cell lines not expressing GLDC were methylated, and the two GC cell lines expressing GLDC were unmethylated. N represents normal gastric tissue, and normal tissue was used as an umethylated loading control. (C) GLDC promoter methylation was examined by bisulfite sequencing in GC cell lines. Each bar represents CpG sites in the promoter region. Unmethylated cytosine is converted to uracil by bisulfite modification, and methylated cytosine is not. The presence of TG indicated that these cytosines were not methylated, and the presence of CG indicated that these cytosines were methylated. (D) Treatment with 5-aza-dc and/or TSA. Drugs treatment restored GLDC mRNA expression cell lines not previously expressing GLDC. β-actin was used as an internal control.*

Next, we treated cells with *GLDC* shRNA and assessed cell migration and invasion using migration and invasion matrigel chambers. The numbers of migrating and invading cells were higher for shGLDC cells than for shControl cells in both SNU484 and HEK293 cell lines (Figure 4A). A wound-healing assay was performed to further investigate cell migration in SNU484 shControl and shGLDC cells. Twenty-four hours after scratching the cells, cell migration was increased in shGLDC cells compared to shControl cells (Figure 4B). Therefore, knockdown of *GLDC* increased cell migration and invasion in both SNU484 and HEK293 cell lines.

To detect apoptotic factors, we applied STS to SNU484 shControl and shGLDC cells. After protein extraction, we compared levels of apoptotic factors (PARP, cleaved-caspase 3 and cleaved-caspase 9) between shControl and shGLDC cells. Knockdown of *GLDC* reduced the expression of these apoptotic factors (Figure 4C).

Correlation between GLDC expression and GLDC promoter hypermethylation in GC. To determine the clinical significance of GLDC silencing in GC, IHC was performed in 410 GC tissues (Figure 5). Out of these specimens, 82.7% were negative for GLDC staining. Reduced GLDC protein was significantly correlated with WHO classification ($p<0.001$) and Lauren's classification ($p<0.001$) (Table I). However, Kaplan-Meier survival curves revealed no significant difference in overall survival between patients with GLDC-negative and GLDC-positive tumors (data not shown). We also analyzed the

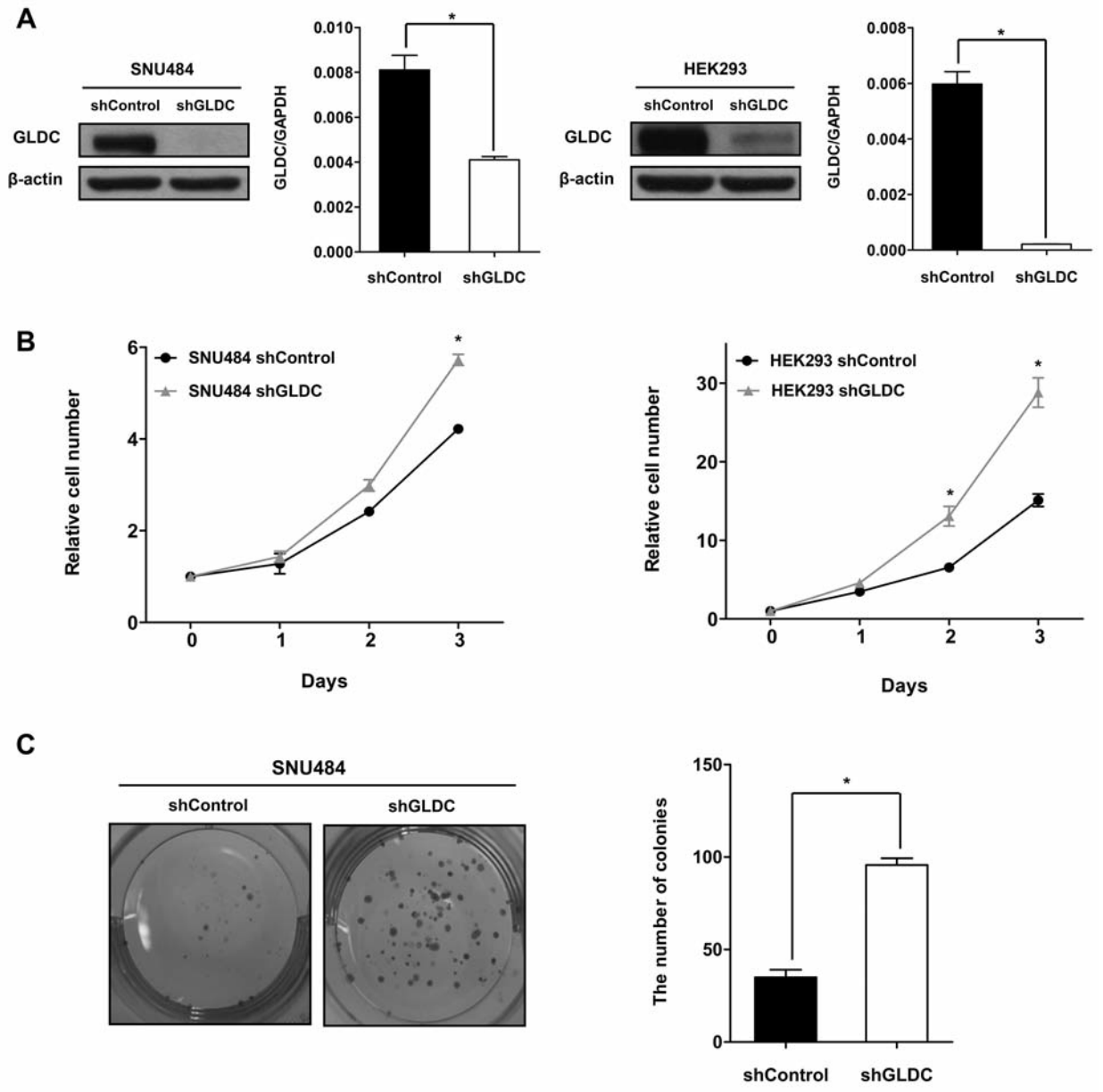

Figure 3. *Effect of GLDC expression on GC cell growth. (A) Knockdown of GLDC using shRNA lentiviral particles transduction was examined by real-time quantitative-PCR and western-blot analysis in SNU484 and HEK293 cell lines. (B) Cell proliferation assay using CCK-8. Cell proliferation was increased by GLDC knock-down. (C) Inhibition of GLDC by shRNA promoted colony formation in the SNU484 cell line. Error bars represent SD. *p<0.05.*

correlation between *GLDC* methylation and GLDC expression in GC tissues using MSP, real time-quantitative PCR, and western-blot analysis. Real-time quantitative PCR and western-blot analysis revealed that normal gastric tissues had higher *GLDC* mRNA and GLDC protein expression than paired GC tissues (Figure 6A and B). GC tissues that expressed lower GLDC protein levels than normal tissues were significantly correlated with *GLDC* promoter methylation, as assessed by MSP (Figure 6B and C, Table II).

Discussion

In the present study, we analyzed the functional significance of the metabolic enzyme GLDC, that was identified in our high-throughput screening for epigenetically silenced genes in GC. We selected potential tumor suppressor genes that had not been previously implicated in GC. We determined that *GLDC* promoter hypermethylation controlled gene silencing in GC cell lines. Using shRNA lentiviral particles, we found

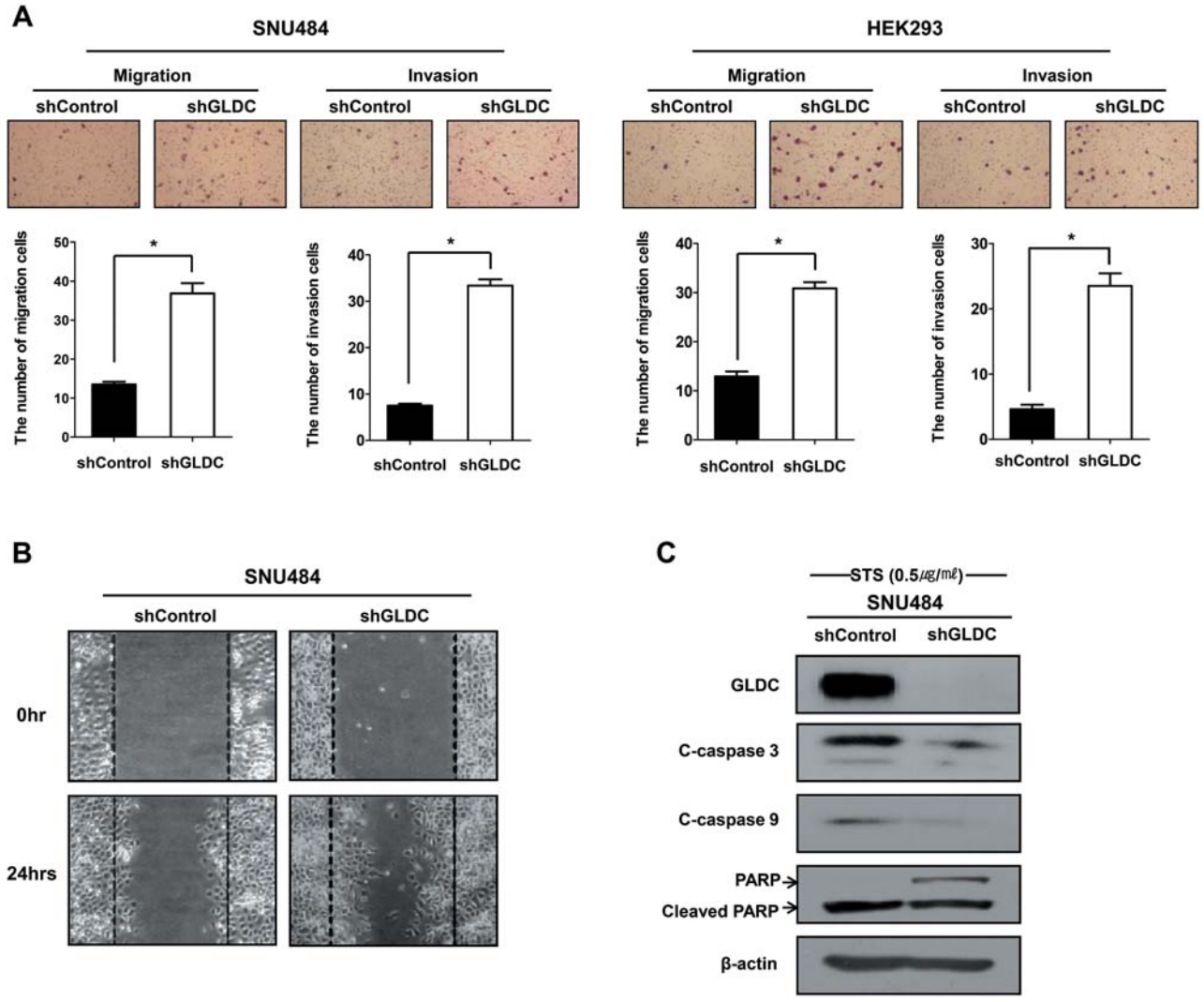

Figure 4. *GLDC inhibits cell migration, cell invasion, and induces apoptosis. (A) Cell migration and invasion were analyzed using matrigel and non-matrigel chambers. The number of migrating and invading cells was increased by GLDC shRNA in both SNU484 and HEK293 cell lines. (B) Cell migration was determined by a wound-healing assay. Twenty-four hours after scratching the cell, SNU484 GLDC knock-down cells migrated faster than control cells. (C) Western-blot analysis of apoptotic factors (PARP, cleaved-caspase 3, and cleaved-caspase 9) in SNU484 shControl and shGLDC cells 4 h after treatment with 0.5 μg/ml STS. Apoptotic factors were decreased in SNU484 shGLDC cells compared to shControl cells. Error bars represent SD. *p<0.05.*

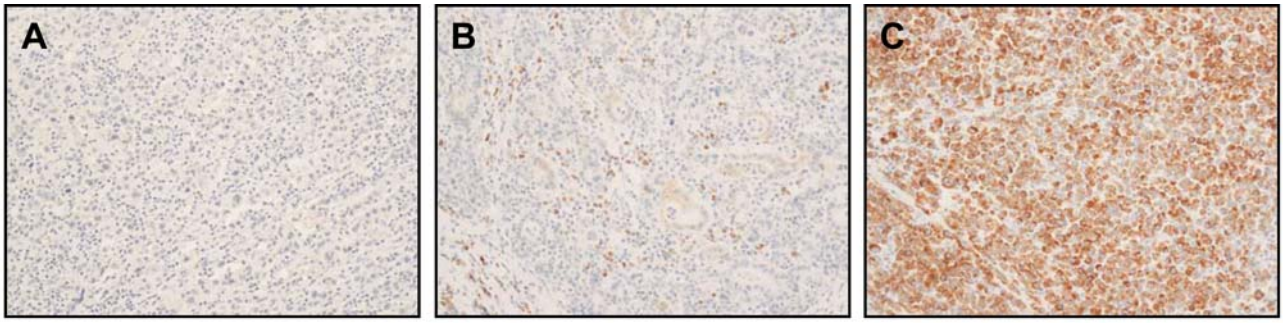

Figure 5. *GLDC protein expression in GC tissues. (A) Loss of GLDC protein in GC tissues. (B) Weakly positive staining for GLDC in GC tissues. (C) Strongly positive staining for GLDC in GC tissues. Magnification, ×100.*

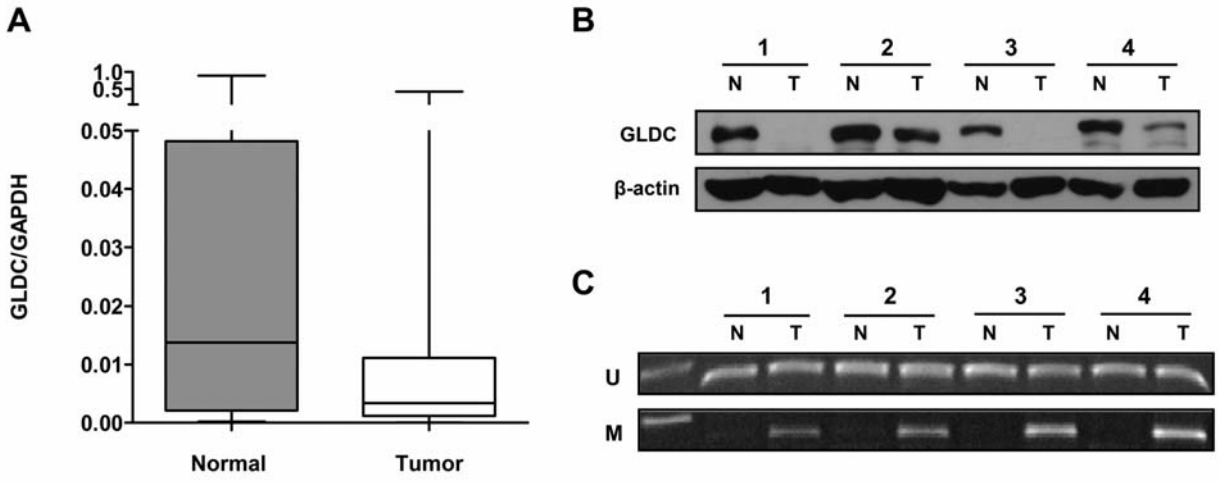

Figure 6. *Epigenetic silencing of GLDC in GC tissues and adjacent normal gastric tissues. (A) GLDC mRNA expression was evaluated by real-time quantitative PCR, and GC tissues were compared with paired normal gastric tissues. The overall values and average GLDC mRNA expression in normal gastric tissues were higher than those in GC tissues. (B) GLDC protein expression was measured by western blot analysis, and GC tissues were compared with paired normal gastric tissues. GLDC protein levels were absent or lower in GC tissues compared to paired normal gastric tissues. (C) Methylation status was assessed by MSP in GC tissues and paired normal gastric tissues. Normal gastric tissues showed promoter unmethylation, and GC tissues showed promoter methylation. Analysis of (B) and (C) used the same patient's tissues, and loss or down-regulation of GLDC was correlated with GLDC promoter hypermethylation in GC tissues.*

that GLDC suppressed GC cell growth, cell migration, cell invasion, and colony formation in GC cell lines. In contrast, GLDC induced apoptosis in GC cells.

GLDC cleaves glycine to form carbon dioxide, ammonia, and CH_2-THF, which drives cell proliferation. CH_2-THF contains a methylene group that promotes nucleotide synthesis during cell proliferation (9). Recent studies have suggested that early oncogenesis involves aberrant activation of cell proliferation, which then leads to nucleotide deficiency and replication stress (17). Up-regulation of GLDC promotes cellular transformation by overcoming this nucleotide deficiency (10).

Recently, one study reported that *GLDC* acts as a metabolic oncogene in the glycine/serine pathway in NSCLC (10). In the present study, we compared *GLDC* mRNA and GLDC protein expression in GC tissues *versus* normal gastric tissues. GLDC expression was higher in normal gastric mucosa than in GC tissues. Therefore, GLDC has different functions in different types of cancers. The previous study focused on cancer stem cell formation and early oncogenesis, while the present study focused on already established cancers. We speculate that GLDC has different effects in early carcinogenesis and cancer progression. GLDC expression was high in NSCLC formed from colonies with a cancer stem cell population. However we did not identify high GLDC expression in GC tissues.

Similar to *GLDC*, certain genes have been reported to have oncogenic and tumor-suppressive functions in different tissues. For example, angiopoietin-like 4 (ANGPTL4) is known as an oncogene in colorectal and gastric cancer (18, 19) and a tumor suppressor in lung cancer and melanoma (20, 21). Dual-specificity phosphatase 6 (DUSP6) has been characterized as an oncogene function in glioblastoma and thyroid cancer (22, 23), but as a tumor suppressor in lung cancer and esophageal squamous cell carcinoma (24, 25). Similarly, GLDC may have different functions in various cancers and stages of cancer. Therefore, future studies should investigate these functions as well as related metabolic enzymes upstream or downstream of the glycine/serine pathway in GC (26).

Gene silencing resulting from promoter hypermethylation has been reported in diverse cancers and is implicated in tumorigenesis (2, 27, 28). In the present study, we determined that promoter hypermethylation was a major cause of *GLDC* gene silencing. Loss of *GLDC* mRNA and GLDC protein expression was closely linked to promoter hypermethylation. After treatment with 5-aza-dc and/or TSA, *GLDC* mRNA expression was restored in GC cell lines. In addition, there was a significant correlation between GLDC protein expression and GLDC promoter hypermethylation in GC tissues and paired normal gastric tissues. Therefore, we concluded that promoter hypermethylation was a major cause of *GLDC* gene silencing during gastric carcinogenesis.

In summary, GLDC is a putative tumor suppressor gene in GC. GLDC expression is inhibited by promoter hypermethylation in GC cell lines, which increases cell growth, cell migration, cell invasion, and colony formation. GC tissues had lower levels of *GLDC* mRNA and GLDC protein than adjacent normal gastric tissues, which were significantly correlated with *GLDC* promoter hypermethylation. More studies are required to clarify the role of *GLDC* gene hypermethylation during gastric cancer progression.

Acknowledgements

This research was supported by grant number 03-2011-0440 from the SNUH Research Fund.

References

1 Matsuda A and Machii R: Trends in stomach cancer mortality rates in Japan, USA, UK, France and Korea based on the WHO mortality database. Jpn J Clin Oncol *42*: 154, 2012.
2 Yasui W, Yokozaki H, Fujimoto J, Naka K, Kuniyasu H and Tahara E: Genetic and epigenetic alterations in multistep carcinogenesis of the stomach. J Gastroenterol *35(Suppl 12)*: 111-115, 2000.
3 Tahara E, Kuniyasu H, Yasui W and Yokozaki H: Gene alterations in intestinal metaplasia and gastric cancer. Eur J Gastroenterol Hepatol *6(Suppl 1)*: S97-102, 1994.
4 Kang GH, Lee S, Cho NY, Gandamihardja T, Long TI, Weisenberger DJ, Campan M and Laird PW: DNA methylation profiles of gastric carcinoma characterized by quantitative DNA methylation analysis. Lab Invest *88*: 161-170, 2008.
5 Park JH, Lee BL, Yoon J, Kim J, Kim MA, Yang HK and Kim WH: Focal adhesion kinase (FAK) gene amplification and its clinical implications in gastric cancer. Hum Pathol *41*: 1664-1673, 2010.
6 Johnson IT and Belshaw NJ: Environment, diet and CpG island methylation: epigenetic signals in gastrointestinal neoplasia. Food Chem Toxicol *46*: 1346-1359, 2008.
7 Tamura G: Genetic and epigenetic alterations of tumor suppressor and tumor-related genes in gastric cancer. Histol Histopathol *17*: 323-329, 2002.
8 Kume A, Koyata H, Sakakibara T, Ishiguro Y, Kure S and Hiraga K: The glycine cleavage system. Molecular cloning of the chicken and human glycine decarboxylase cDNAs and some characteristics involved in the deduced protein structures. J Biol Chem *266*: 3323-3329, 1991.
9 Tibbetts AS and Appling DR: Compartmentalization of Mammalian folate-mediated one-carbon metabolism. Annu Rev Nutr *30*: 57-81, 2010.
10 Zhang WC, Shyh-Chang N, Yang H, Rai A, Umashankar S, Ma S, Soh BS, Sun LL, Tai BC, Nga ME, Bhakoo KK, Jayapal SR, Nichane M, Yu Q, Ahmed DA, Tan C, Sing WP, Tam J, Thirugananam A, Noghabi MS, Pang YH, Ang HS, Mitchell W, Robson P, Kaldis P, Soo RA, Swarup S, Lim EH and Lim B: Glycine decarboxylase activity drives non-small cell lung cancer tumor-initiating cells and tumorigenesis. Cell *148*: 259-272, 2012.
11 Hanahan D and Weinberg RA: Hallmarks of cancer: the next generation. Cell *144*: 646-674, 2011.
12 Teicher BA, Linehan WM and Helman LJ: Targeting cancer metabolism. Clin Cancer Res *18*: 5537-5545, 2012.
13 Yang W, Xia Y, Hawke D, Li X, Liang J, Xing D, Aldape K, Hunter T, Alfred Yung WK and Lu Z: PKM2 phosphorylates histone H3 and promotes gene transcription and tumorigenesis. Cell *150*: 685-696, 2012.
14 Jee CD, Kim MA, Jung EJ, Kim J and Kim WH: Identification of genes epigenetically silenced by CpG methylation in human gastric carcinoma. Eur J Cancer *45*: 1282-1293, 2009.
15 Van der Auwera I, Yu W, Suo L, Van Neste L, van Dam P, Van Marck EA, Pauwels P, Vermeulen PB, Dirix LY and Van Laere SJ: Array-based DNA methylation profiling for breast cancer subtype discrimination. PLoS One *5*: e12616, 2010.
16 Takada H, Imoto I, Tsuda H, Nakanishi Y, Ichikura T, Mochizuki H, Mitsufuji S, Hosoda F, Hirohashi S, Ohki M and Inazawa J: ADAM23, a possible tumor suppressor gene, is frequently silenced in gastric cancers by homozygous deletion or aberrant promoter hypermethylation. Oncogene *24*: 8051-8060, 2005.
17 Bester AC, Roniger M, Oren YS, Im MM, Sarni D, Chaoat M, Bensimon A, Zamir G, Shewach DS and Kerem B: Nucleotide deficiency promotes genomic instability in early stages of cancer development. Cell *145*: 435-446, 2011.
18 Kim SH, Park YY, Kim SW, Lee JS, Wang D and DuBois RN: ANGPTL4 induction by prostaglandin E2 under hypoxic conditions promotes colorectal cancer progression. Cancer Res *71*: 7010-7020, 2011.
19 Nakayama T, Hirakawa H, Shibata K, Abe K, Nagayasu T and Taguchi T: Expression of angiopoietin-like 4 in human gastric cancer: ANGPTL4 promotes venous invasion. Oncol Rep *24*: 599-606, 2010.
20 Galaup A, Cazes A, Le Jan S, Philippe J, Connault E, Le Coz E, Mekid H, Mir LM, Opolon P, Corvol P, Monnot C and Germain S: Angiopoietin-like 4 prevents metastasis through inhibition of vascular permeability and tumor cell motility and invasiveness. Proc Natl Acad Sci USA *103*: 18721-18726, 2006.
21 Ito Y, Oike Y, Yasunaga K, Hamada K, Miyata K, Matsumoto S, Sugano S, Tanihara H, Masuho Y and Suda T: Inhibition of angiogenesis and vascular leakiness by angiopoietin-related protein 4. Cancer Res *63*: 6651-6657, 2003.
22 Messina S, Frati L, Leonetti C, Zuchegna C, Di Zazzo E, Calogero A and Porcellini A: Dual-specificity phosphatase DUSP6 has tumor-promoting properties in human glioblastomas. Oncogene *30*: 3813-3820, 2011.
23 Degl'Innocenti D, Romeo P, Tarantino E, Sensi M, Cassinelli G, Catalano V, Lanzi C, Perrone F, Pilotti S, Seregni E, Pierotti MA, Greco A and Borrello MG: DUSP6/MKP3 is overexpressed in papillary and poorly differentiated thyroid carcinoma and contributes to neoplastic properties of thyroid cancer cells. Endocr Relat Cancer *20*: 23-37, 2013.
24 Okudela K, Yazawa T, Woo T, Sakaeda M, Ishii J, Mitsui H, Shimoyamada H, Sato H, Tajiri M, Ogawa N, Masuda M, Takahashi T, Sugimura H and Kitamura H: Down-regulation of DUSP6 expression in lung cancer: its mechanism and potential role in carcinogenesis. Am J Pathol *175*: 867-881, 2009.
25 Ma J, Yu X, Guo L and Lu SH: DUSP6, a tumor suppressor, is involved in differentiation and apoptosis in esophageal squamous cell carcinoma. Oncol Lett *6*: 1624-1630, 2013.
26 Possemato R, Marks KM, Shaul YD, Pacold ME, Kim D, Birsoy K, Sethumadhavan S, Woo HK, Jang HG, Jha AK, Chen WW, Barrett FG, Stransky N, Tsun ZY, Cowley GS, Barretina J, Kalaany NY, Hsu PP, Ottina K, Chan AM, Yuan B, Garraway LA, Root DE, Mino-Kenudson M, Brachtel EF, Driggers EM and Sabatini DM: Functional genomics reveal that the serine synthesis pathway is essential in breast cancer. Nature *476*: 346-350, 2011.
27 Das PM and Singal R: DNA methylation and cancer. J Clin Oncol *22*: 4632-4642, 2004.
28 Suva ML, Riggi N and Bernstein BE: Epigenetic reprogramming in cancer. Science *339*: 1567-1570, 2013.

Received September 30, 2015
Revised November 28, 2015
Accepted November 30, 2015