이학석사 학위논문

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2014 년 2 월

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지도교수 김 우 호

이 논문을 이학석사 학위논문으로 제출함.

2013 년 10 월

서울대학교 대학원

의과대학 협동과정 종양생물학 전공

민 혜 림

민혜림의 이학석사 학위논문을 인준함.

2013 년 12 월

위 원 장 _____(인)

부위원장 _____(인)

위 원 _____(인)

Epigenetic silencing of the candidate tumor suppressor gene GLDC in gastric carcinoma

By

Hyae Lim Min

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Cancer Biology at the Seoul National University College of Medicine, Seoul, Korea

December, 2013

Approved by Thesis Committee:

Professor _____Chairman

Professor _____Vice chairman

Professor_____

ABSTRACT

The metabolic enzyme, glycine dehydrogenase (GLDC) involved in glycine metabolism is known for non-ketotic hyperglycinemia but not for cancers. Recently, it has been only reported that GLDC drives tumor initiating cells (TICs) and tumorigenesis in non-small cell lung cancer. In our study, we performed the oligonucleotide microarray analysis and the Infinium Human Methylation27 BeadChip analysis to identify potential tumor suppressor genes showing down-regulated expression and promoter hypermethylation in ten gastric cancer (GC) cell lines. Among several candidate genes, the GLDC was silenced in eight out of ten GC cell lines and the down-regulation of GLDC was closely linked to the promoter methylation. Knockdown of GLDC increased the cell proliferation, migration, invasion, colony formation and reduced apoptosis. Promoter methylation of GLDC was detected in 79.4% and loss of protein expression was detected in 82.7% in GC tissues. Loss of GLDC protein expression by immunohistochemistry was found to be significantly associated with WHO classification ($p < 0.001$), Lauren's classification ($p < 0.001$) in 410 gastric cancer patients. With GC tissues and paired normal gastric tissues, we found that mRNA and protein expression was down-regulated (72.0% and 68.5%) in GC tissues compared to normal gastric tissues. In GC

tissues, hypermethylation of GLDC had significant correlation with downregulation of GLDC protein as compared with normal gastric tissues. In summary, GLDC is a candidate for tumor suppressor gene in gastric carcinogenesis and the hypermethylation of GLDC gene is the main reason for the silencing of the GLDC.

Keywods : Glycine dehydrogenase, Tumor suppressor gene, DNA methylation, Stomach neoplasms

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Introduction

Gastric cancer (GC) is the second leading cause of cancer 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 that are altered in human GC (4, 5), many genes involved in gastric carcinogenesis and progression remain unknown.

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 they 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 studied in carcinogenesis.

In the present study, we found that aberrant hypermethylation 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 this study, we analyzed GLDC expression and methylation in gastric carcinogenesis, and its biologic clinicopathological significance in GC.

Methods and materials

Cell lines and patients tissues

Ten gastric cancer cell lines (SNU1, 5, 16, 216, 484, 601, 620, 638, 668 and 719) and a kidney epithelial cell line (HEK293) were used. The GC cell lines were maintained using RPMI-1640 (HyClone, Logan, UT, USA), and the HEK293 cell line was maintained in DMEM (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). Cells were incubated at 37℃ in a humidified atmosphere containing 5% CO_2.

Surgically resected formalin-fixed paraffin-embedded GC tissues (n = 410) were collected from the archives of 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). 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) and was analyzed using the Infinium Human Methylation 27 BeadChip kit (Illumina, San Diego, CA). Processing and data analysis were performed using the reagent provided in the kit according to the manufacturer's instructions. Data were analyzed using 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 from the ten GC cell lines and HEK293 was prepared

using the Trizol reagent (Invitrogen, Carlsbad, CA). 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℃ for denaturation, 60℃ for annealing, and 72℃ for extension followed by a final extension at 72℃. PCR was performed in an ABI Veriti 96-well thermal cycler (Applied Biosystems, Forster city, CA). The PCR used primers for *GLDC* transcripts (forward, 5´-AACCAGGGAGCAACACATTC-3´ and reverse, 5´-GCAACCAGTTCTGCAGATGA-3´) and β-actin transcripts (forward, 5´-ACACTGTGCCCATCTACGAGG-3´ and reverse, 5´-AGGGGCCGGACTCGTCATACT-3´). PCR products were electrophoresed on 2% agarose gels, stained with loading star dye (Dynebio, Seongnam, Korea), and visualized under UV light.

Real-time quantitative PCR was performed as follows: 40 cycles of 95℃ for denaturation and 60℃ 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. 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 hrs. The rabbit anti-GLDC (Sigma Aldrich), rabbit anti-PARP (Cell Signaling, Danvers, MA), 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℃, 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).

5-aza-2'-deoxycytidine and/or Trichostatin A treatment

GC cell lines expressing GLDC (SNU1, 620, 638 and 719) were treated with 5-aza-2´-deoxycytidine (5-aza-dc) and/or Trichostatin A (TSA) (both 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′-TGTTTTGGGTGGAGTTATAATTTTGT-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℃ annealing temperature. PCR products were loaded onto 2% agarose gel, stained with loading star dye (Dynebio), and visualized under UV light.

Bisulfite-modified DNA was used for bisulfite sequencing with

specific primers (forward, 5´-TTGTTTATTTTTATTGGTTAAGGGTTTT-3´ and reverse, 5´-CTCTTAACCCCTCTCCTAACCTC-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 the results were analyzed using DNA sequencing analysis 3.7 software (Applied Biosystems).

shRNA lentiviral particle transduction

All shRNAs were constructed in lentiviral particles and were obtained from Santa Cruz Biotechnology (Santa Cruz, Dallas, Texas). Lentiviral particles were transduction-ready and contained shRNA designed to knockdown gene expression. Cells were seeded in 60-mm culture dishes at a density of 2.5×10^5 cells/dish 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.

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 at 5 $\times 10^3$ or 2.5 $\times 10^3$ cells/well and incubated for 1 day at 37℃. Cells were treated with 10 μl of Cell Counting Kit-8 (CCK-8) reagent (Dojindo, Tokyo, Japan) and incubated for 2 h at 37℃, and absorbance was measured at 450 nm using a spectrophotometer (Thermo Labsystems, Beverly, MA). 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) 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 dishes, and wounds were created at three places using a sterile pipette tip. Cells were photographed under microscopy 24h after incubation. All experiments were performed in triplicate.

For the colony formation assay, the control and GLDC shRNA-transduced cells were cultured, and 2.5 $\times 10^3$ or 5 $\times 10^3$ cells were seeded in 6-well plates and 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 a tissue microarray (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.

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

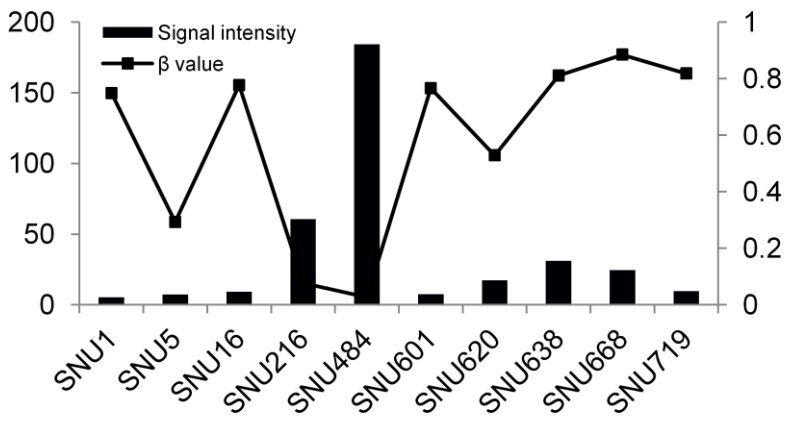
SPSS PASW Statistics 18.0 software (SPSS Inc., Chicago, IL), and all graphs were designed by GraphPad Prism 5.01 (GraphPad Software Inc., La Jolla, CA). *P* values < 0.05 were considered statistically significant.

Results

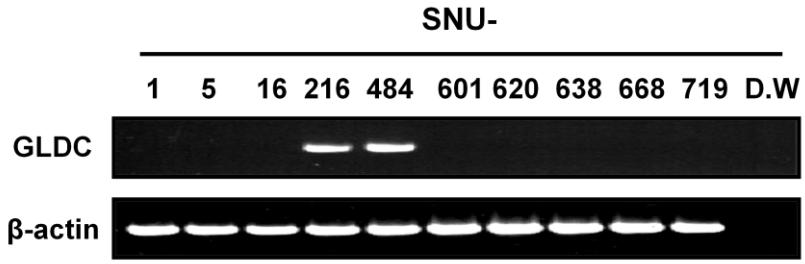
Expression of *GLDC* in GC cell lines

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 downregulated 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 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 (Fig. 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 cell lines, SNU216 and 484, expressed *GLDC* mRNA and protein, while the remaining eight cell lines did not at all (Fig. 1B and C).

A


Signal intensity
β value
200
150
100
50
0
1
0.8
0.6
0.4
0.2
0
SNU1
SNU5
SNU16
SNU216
SNU484
SNU601
SNU620
SNU638
SNU668
SNU719

B


SNU-
1 5 16 216 484 601 620 638 668 719 D.W
GLDC
β-actin

C

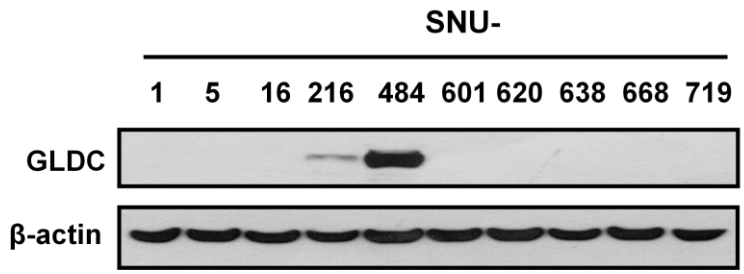

SNU-
1 5 16 216 484 601 620 638 668 719
GLDC
β-actin

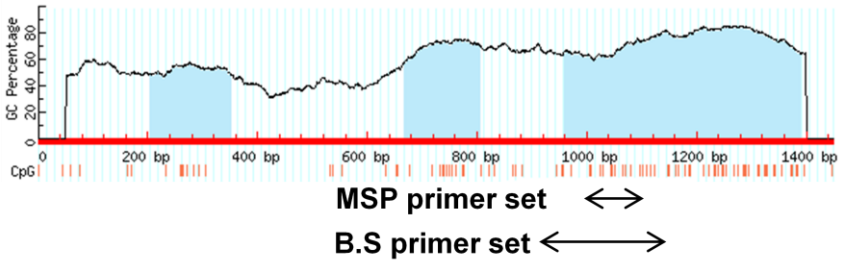
Figure 1. Expression of *GLDC* in GC cell lines

(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. Downregulation 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.

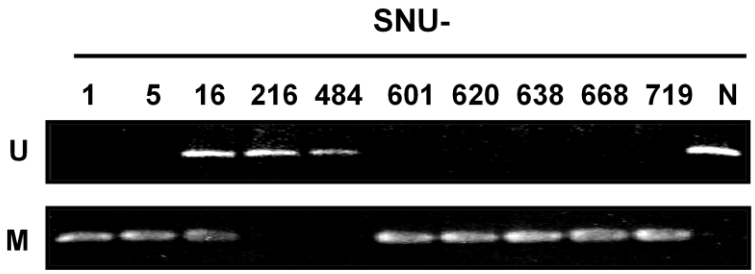
Epigenentic silencing of GLDC in GC cell lines

To confirm the hypermethylation status of *GLDC* in GC cell lines, we searched for CpG islands in the promoter region of *GLDC* (Fig. 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 gastric cancer cell lines (SNU216 and 484) expressed GLDC mRNA as measured by RT-PCR (Fig. 1B) and showed unmethylated promoter regions, while the remaining eight cell lines showed silenced mRNA and methylated promoter regions (Fig. 2B). Next, we confirmed the MSP results with bisulfite sequencing. Each bar in Fig. 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 (Fig. 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 (Fig. 2D). These data suggested that *GLDC* silencing was associated with promoter methylation in GC cell lines.

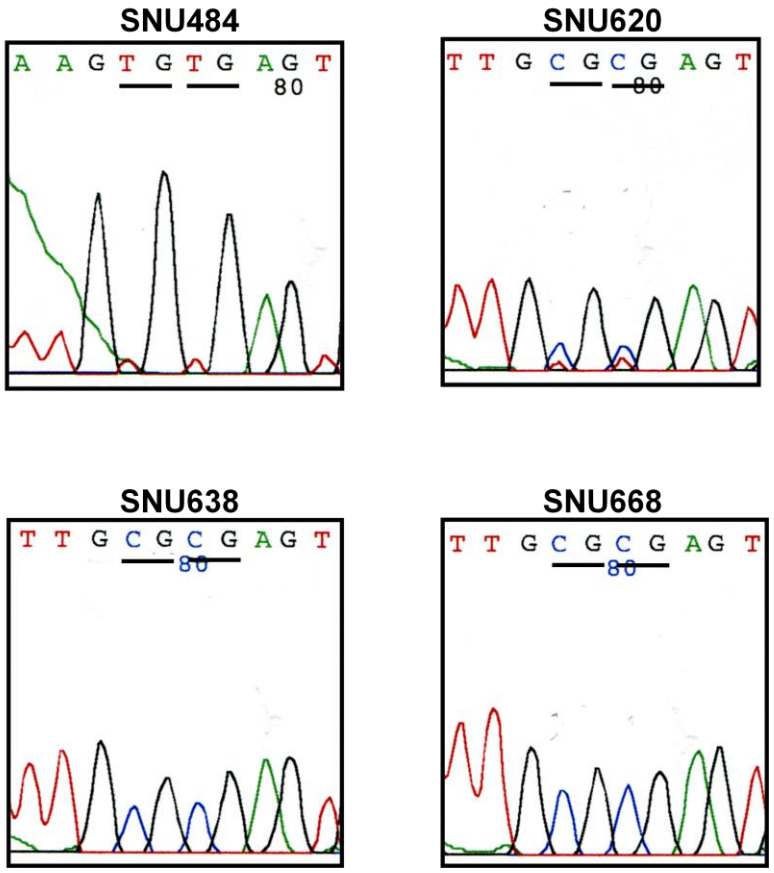
A


GC Percentage
0
20
40
60
80
0
200 bp
400 bp
600 bp
800 bp
1000 bp
1200 bp
1400 bp
CpG
MSP primer set
B.S primer set

B


SNU-
1
5
16
216
484
601
620
638
668
719
N
U
M

C


SNU484
A A G T G T G A G T
80
SNU620
T T G C G C G A G T
80
SNU638
T T G C G C G A G T
80
SNU668
T T G C G C G A G T
80

D

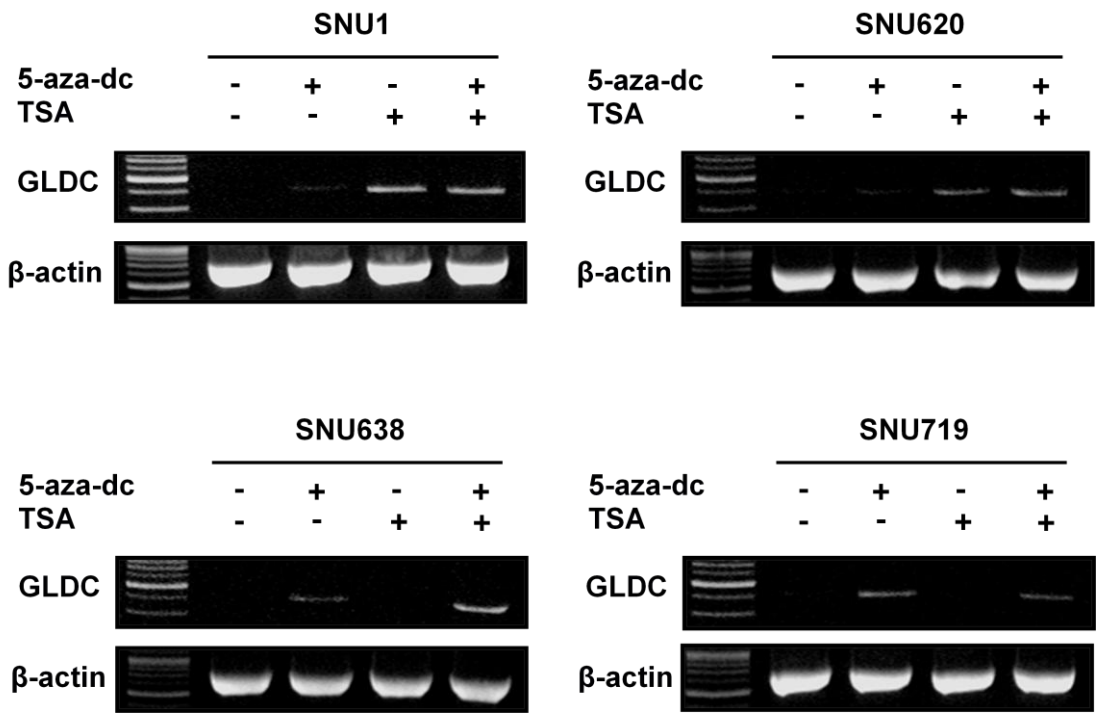

SNU1
5-aza-dc - + - +
TSA - - + +
GLDC
β-actin
SNU620
5-aza-dc - + - +
TSA - - + +
GLDC
β-actin
SNU638
5-aza-dc - + - +
TSA - - + +
GLDC
β-actin
SNU719
5-aza-dc - + - +
TSA - - + +
GLDC
β-actin

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 cell lines not expressing *GLDC* were methylated, and the two 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

Effect of *GLDC* knock-down

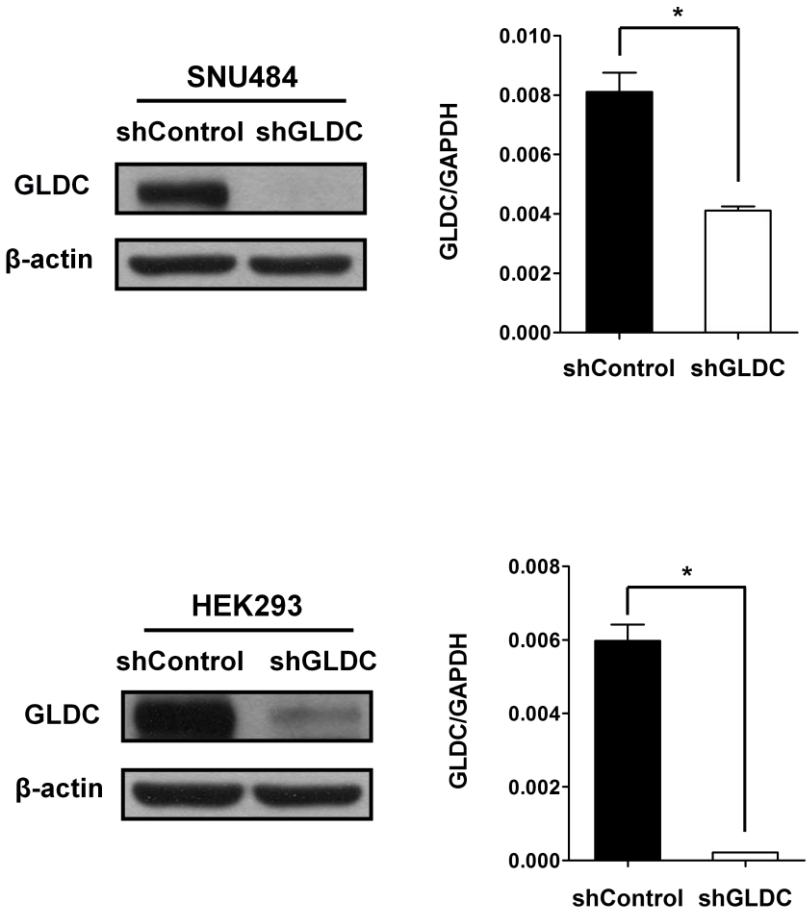
In order to clarify the functions of GLDC in GC, we examined the effect of GLDC knock-down on cell growth using two cell lines overexpressing GLDC, SNU484 and HEK293. GLDC shRNA (shGLDC) and control shRNA (shControl) lentiviral particles were transduced into the cell lines. Knockdown of *GLDC* mRNA and GLDC protein was confirmed by real-time quantitative PCR and western-blot analysis (Fig. 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 (Fig. 3B). Cell-colony formation was examined in SNU484 shControl and shGLDC cell lines. After 3 weeks, colonies were visualized by crystal-violet staining and were counted. Knock-down of GLDC increased cell-colony formation in the SNU484 shGLDC cell line as compared to the shControl cell line (Fig. 3C). These results suggested that the inactivation of *GLDC* increased cell growth in GC and normal cell lines.

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 (Fig. 4A). A wound-healing assay was performed to further investigate cell migration in SNU484 shControl and shGLDC cells.

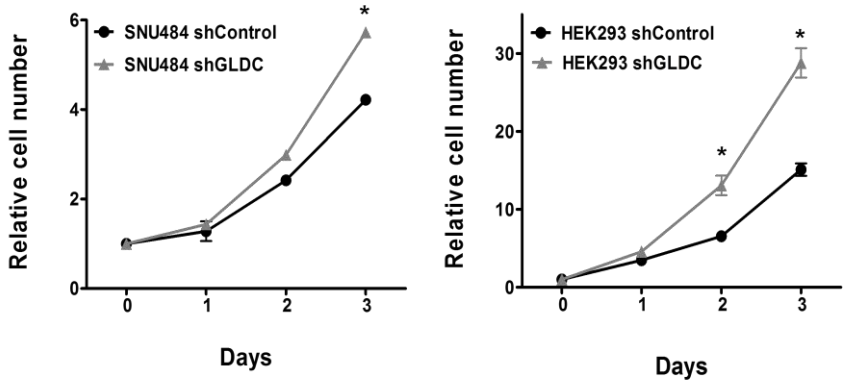
Twenty-four hours after scratching the cells, cell migration was increased in shGLDC cells as compared to shControl cells (Fig. 4B). Therefore, knockdown of *GLDC* increased cell migration and invasion in both SNU484 and HEK293 cell lines.

To detect apoptotic factors, we applied 0.5 μg/ml STS to the growth media of SNU484 shControl and shGLDC cells for 4 h. 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 (Fig. 4C).

A


SNU484
shControl
shGLDC
GLDC
β-actin
GLDC/GAPDH
0.010
0.008
0.006
0.004
0.002
0.000
*
shControl
shGLDC
HEK293
shControl
shGLDC
GLDC
β-actin
GLDC/GAPDH
0.008
0.006
0.004
0.002
0.000
*
shControl
shGLDC

B


SNU484 shControl
SNU484 shGLDC
Relative cell number
Days
HEK293 shControl
HEK293 shGLDC
Relative cell number
Days

C

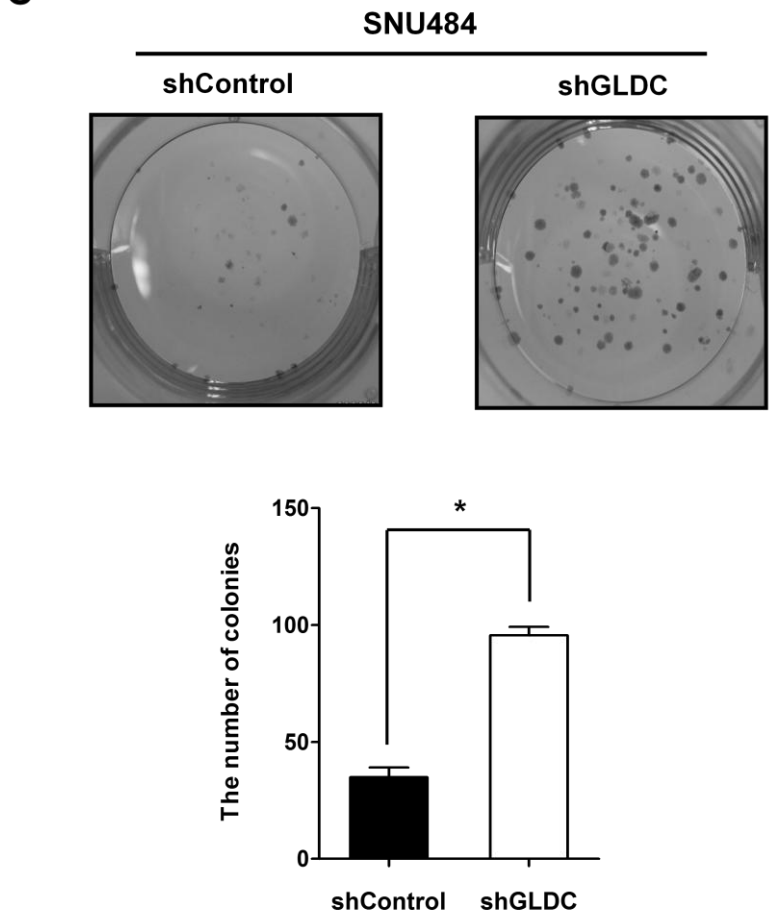
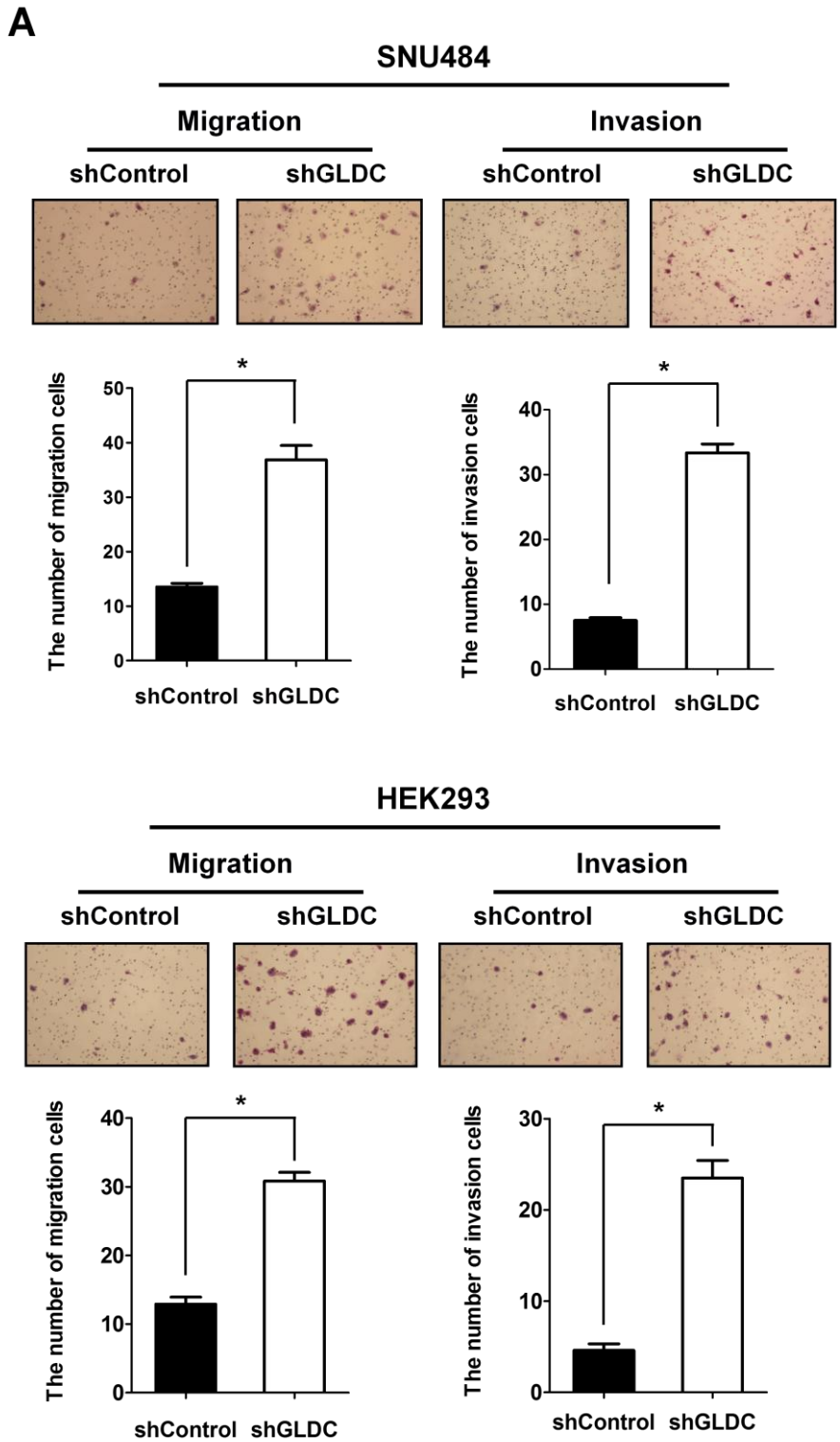

SNU484
shControl
shGLDC
The number of colonies
shControl
shGLDC

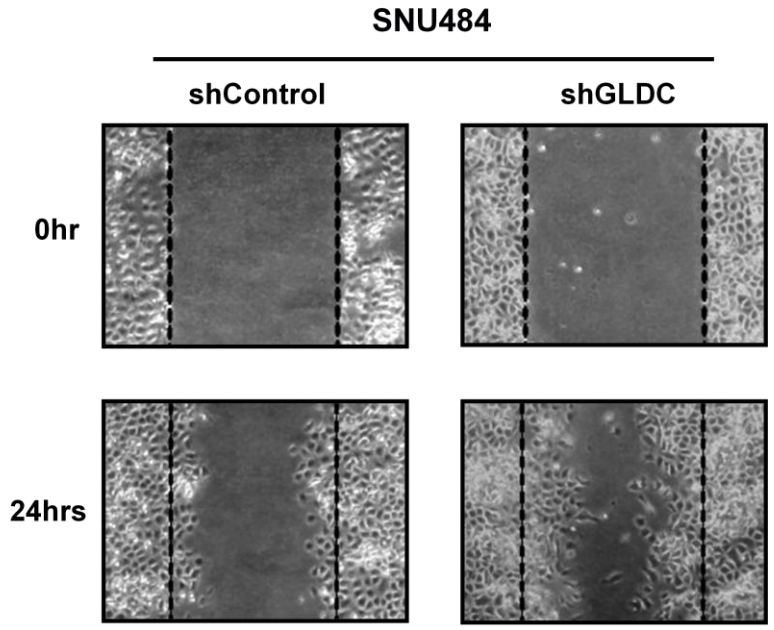
Figure 3. Effect of *GLDC* expression on GC cell growth

(A) Knockdown of *GLDC* using shRNA lentiviral 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. *$P < 0.05$.


A
SNU484
Migration
Invasion
shControl
shGLDC
shControl
shGLDC
The number of migration cells
50
40
30
20
10
0
*
shControl
shGLDC
The number of invasion cells
40
30
20
10
0
*
shControl
shGLDC
HEK293
Migration
Invasion
shControl
shGLDC
shControl
shGLDC
The number of migration cells
40
30
20
10
0
*
shControl
shGLDC
The number of invasion cells
30
20
10
0
*
shControl
shGLDC

B


SNU484
shControl
shGLDC
0hr
24hrs

C

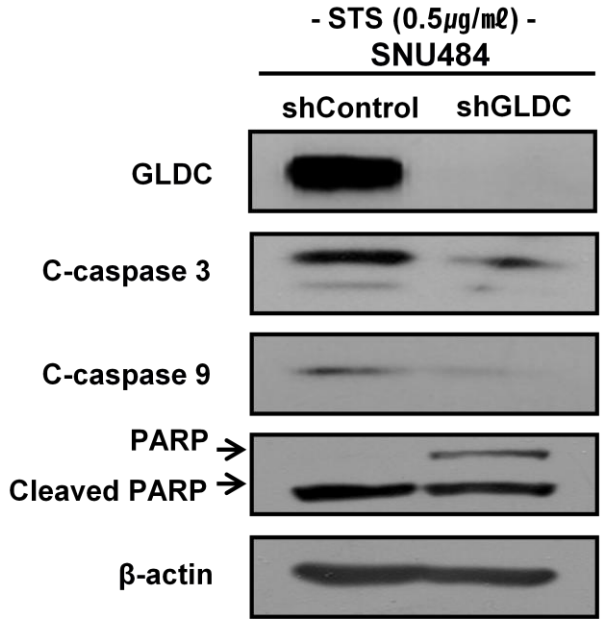

- STS (0.5μg/mℓ) -
SNU484
shControl
shGLDC
GLDC
C-caspase 3
C-caspase 9
PARP
Cleaved PARP
β-actin

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 as compared to shControl cells.

Error bars represent SD. *$P < 0.05$.

Correlation between *GLDC* expression and *GLDC* promoter hypermethylation in GC

To determine the clinical significance of *GLDC* silencing in GC, IHC for GLDC protein was performed in 410 GC tissues specimens (Fig. 5). 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 1). 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 correlation between *GLDC* methylation and GLDC expression in GC tissue 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 tissue (Fig. 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 (Fig. 6B, 6C and Table 2).

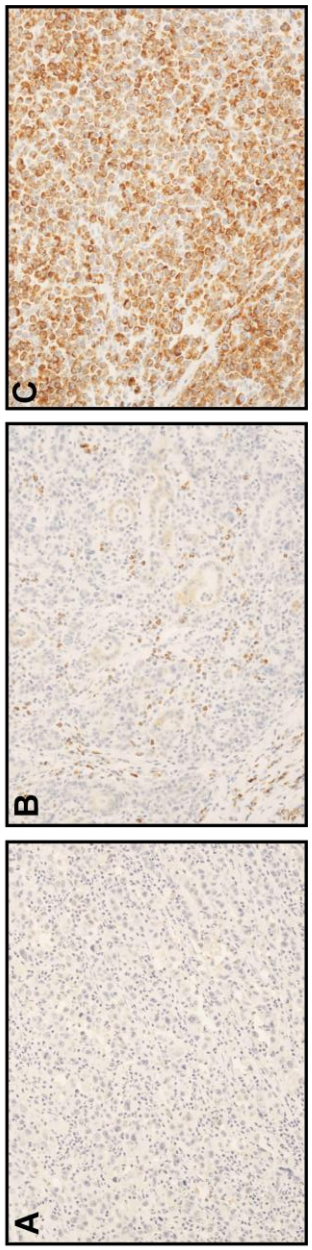

C
B
A

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×.

Table 1. Correlation between GLDC protein expression and clinicopathological features in GC

	GLDC protein expression		
	Negative (%) n = 339	Positive (%) n = 71	*P* value
Sex			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.

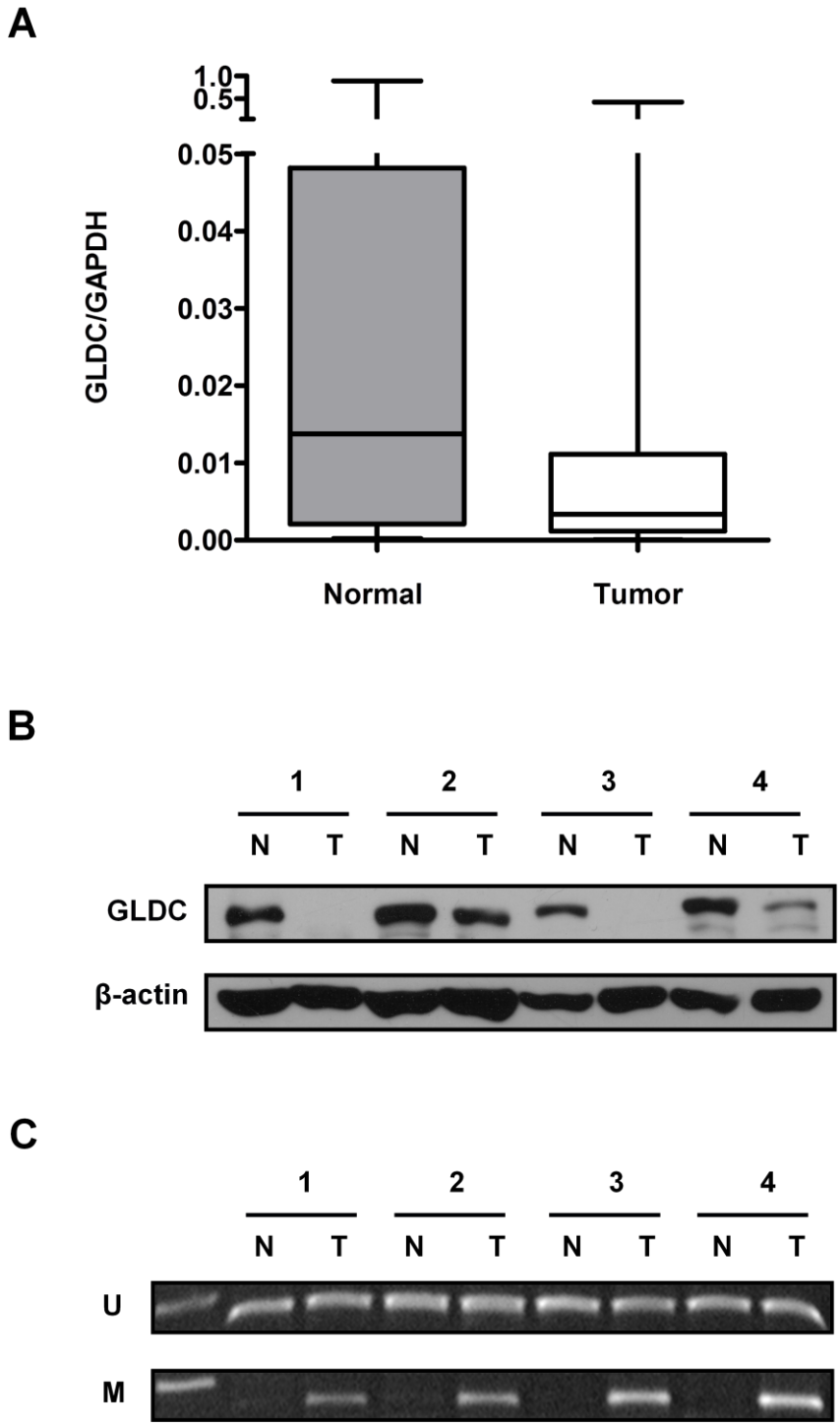

A
1.0
0.5
0.05
0.04
0.03
0.02
0.01
0.00
GLDC/GAPDH
Normal
Tumor
B
1
2
3
4
N
T
N
T
N
T
N
T
GLDC
β-actin
C
1
2
3
4
N
T
N
T
N
T
N
T
U
M

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 as 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 downregulation of GLDC was correlated with *GLDC* promoter hypermethylation in GC tissues.

Table 2. 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)	

Discussion

In this study, we analyzed the functional significance of the metabolic enzyme GLDC, which was identified in our high-throughput screen 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 that GLDC decreased cancer cell growth. Specifically, 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). Upregulation of GLDC promotes cellular transformation by overcoming this nucleotide deficiency (10).

Recently, one study reported that GLDC acted as 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 type of cancer. 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*, some 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 cancers. 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 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 tissues, which were significantly correlated with *GLDC* promoter hypermethylation. More studies are needed to understand the role of GLDC gene hypermethylation during gastric carcinogenesis.

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국문 초록

위암의 발생 원인에는 유전적, 또는 환경적인 요인 등 다양하게 존재하지만 최근에는 유전적 변화에 의한 다단계 과정 그리고 후성유전학적인 과정과 관련된 암의 발생이 주목을 받고 많은 연구가 진행 되고 있다. 본 연구에서는 위암 세포주에서 인간 메틸레이션 비드칩 분석을 실행하여 그 결과를 올리고 핵산 미세배열법 결과와 비교하였다. 그리하여 위암과 관련된 새로운 유전자들을 찾아내었다. 이 중 GLDC 는 글라이신, 세린 대사에 밀접한 연관이 있는 효소이며 이 유전자는 암의 증식과 연관이 있다고 알려져 있지만 아직 그 기능이 많이 보고 되어 있지는 않다. 10 개의 위암 세포주에서 인간 메틸레이션 비드칩 분석과 올리고 핵산 미세배열법 분석을 통해 8 개의 위암 세포주에서 GLDC 의 발현 손실이 관찰되었고 그 원인 중 하나가 GLDC 유전자의 프로모터 부분의 메틸레이션이라는 것을 밝혀냈다. GLDC 유전자가 위암에서 구체적으로 어떠한 기능을 하는지 알아보기 위해 작은 머리핀 RNA 를 GLDC 가 발현되는 위암 세포주에 형질주입 하였고 GLDC 가 위암 세포의 성장과 증식 그리고 이동과 침윤을 억제시킨다는 것을 알아냈고 세포사멸사를 유도한다는 것을 밝혀냈다. 또한 위암 조직 중 339/410 (82.7%)에서 GLDC 단백질 발현이 감소되었음을 확인하였고 이것이 여러 임상병리학적 소견과 유의한 상관관계가 있음을 확인하였다. 또한 동일 환자의 위암

조직과 그 주변 정상 조직의 메틸레이션 성상과 GLDC 단백질 발현을 비교해본 결과 정상 조직에 비해 위암 조직에서 메틸레이션 빈도가 높게 나타났으며 동시에 GLDC 단백질 발현의 소실 및 감소를 확인하였고 이 두 현상이 유의한 상관관계가 있음을 확인하였다. 이러한 결과로 유전자의 프로모터 메틸레이션에 의한 GLDC 의 발현 감소가 위암을 진단하는데 있어 유용한 표지자로 사용될 수 있음을 시사한다.

주요어 : GLDC, 종양억제유전자, DNA 메틸레이션, 위 종양

학 번 : 2012-21768