GKN1-miR-185-DNMT1 axis suppresses gastric carcinogenesis through regulation of epigenetic alteration and cell cycle

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Supplemental Table

Table S1. Primer sequences for PCR

Primer name	Sequences
GKN1	F: 5’- CTTCGGGGTACCATGCTTGCCTACTCCTCTGTCCAC-3’ R: 5’- GTTGCCCTCGAGTTAGTTCTCCACCGTGTCTCCACA-3’
shGKN1	5’-GCCCAAACAAAGTCGATGAC-3’
DNMT1	F: 5’-CTACCAGGGAGAAGGACAGG-3’ R: 5’-CTCACAGACGCCACATCG-3’
shDNMT1	5’-CAGCACCTCATTTGCCGAATA-3’
EZH2	F: 5’-CCCTGACCTCTGTCTTACTTGTGGA-3’ R: 5’-ACGTCAGATGGTGCCAGCAATA-3’
shEZH2	5’-TATTGCCTTCTCACCAGCTGC-3’
shHDAC1	5’-GCTCCATCCGTCCAGATAACA-3’
CDKN2A	F: 5’-CATAGATGCCGCGGAAGGT-3’ R: 5’-GATGATCTAAGTTTCCCGAGGTTTC-3’
GAPDH	F: 5’-AAATCAAGTGGGGCGATGCTG-3’ R: 5’-GCAGAGATGATGACCCTTTTG-3’
miR-185_RT	5’-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGAT ACG ACTCAGGAA-3’
miR-185	F: 5’-CAATGGAGAGAAAGGCAGTTCC-3’ R: 5’-AATCCATGAGAGATCCCTACCG-3’
Pri-miR-185	F: 5’-AGACCTGCTGGCTAGAGCTG-3’ R: 5’-CAAGGGAAGGCCATAAACAG-3’
Pre-miR-185	F: 5’-CAATGGAGAGAAAGGCAGTTCC-3’ R: 5’-TGGGAGGGAAGGACCAGAGG-3’
U6	F: 5’-ATTGGAACGATACAGAGAAGATT-3’ R: 5’-GGAACGCTTCACGAATTT-3’
E-cadherin BGS	F: 5’-TTTAGTAATTTTAGGTTAGAGGGTTAT-3’ R: 5’-AAACTCACAAATACTTTACAATTCC -3’
CDKN2A BGS	F: 5’- AGATGTTTTGTGGTTGTTGTGA-3’ R: 5’-CAAAAATCTTCCATTCTTCAAAC-3’
CDKN2A_M1	F: 5’- TTATTAGAGGGTGGGGCGGATCGC-3’ R: 5’-GACCCCGAACCGCGACCGTAA-3’
CDKN2A_UM1	F: 5’- TTATTAGAGGGTGGGGTGGATTGT-3’ R: 5’-CAACCCCAAACCACAACCATAA-3’
CDKN2A_M2	F: 5’- TTATTAGAGGGTGGGGCGGATCGC-3’ R: 5’-CCACCTAAATCGACCTCCGACCG-3’
CDKN2A_UM2	F: 5’- TTATTAGAGGGTGGGGTGGATTGT-3’ R: 5’-CCACCTAAATCAACCTCCAACCA-3’

Table S2. Correlation of expression of GKN1, DNMT1 and EZH2 with histologic type in 80 gastric cancers.

Histologic type	GKN1			DNMT1			EZH2		
	+	-	*P* value	+	-	*P* value	+	-	*P* value
Lauren			0.0371			0.0047			0.1520
Intestinal	10	36		32	14		39	7	
Diffuse	1	33		33	1		33	1	
Different			0.0001			0.0004			0.0571
Well	4	2		4	2		5	1	
Moderate	6	26		20	12		26	6	
Poor	1	41		41	1		41	1	
total	11	69		65	15		72	8	

Supplementary Figure Legends

Figure S1. Effects of GKN1 on cell growth and proliferation in MKN1 and MKN28 cells. A & B. *GKN1*-transfected MKN1 and MKN28 cells showed time-dependent inhibition of cell growth and proliferation in MTT (A) and BrdU incorporation assay (B).

Figure S2. Epigenetic regulation of *CDKN2A* in AGS gastric cancer cell lines. A. Methylation status at *CDKN2A* was examined in AGS cells by methylation specific PCR. Primer sequences were described in Supplementary Table S1. The effect of 5-aza-dC treatment on AGS cells was also investigated. U, unmethylated *CDKN2A*; M, methylated *CDKN2A*. **B.** CDKN2A expression was estimated in 5-aza-dC treated AGS cells.

Figure S3. Effects of GKN1 on expression of DNMT1 and EZH2 in MKN1 and MKN28 cells. A & B. Ectopic *GKN1* expression down-regulated expression of DNMT1 and EZH2 protein (A) and mRNA (B) in MKN1 and MKN28 cells.

Figure S4. Effects of *DNMT1, ESZH2* and *HDAC1* silencing in AGS cells. A. In MTT assay, *shDNMT1, shEZH2* and *shHDAC1* transfected AGS cells showed time-dependent inhibition of cell growth, respectively. *$P < 0.05$ compared to control shRNA by student t-test. **B & C**. Silence of *DNMT1* significantly up-regulated *CDKN2A* mRNA expression (B) and cell cycle arrest at G0/G1 and G2/M phases (C).

Figure S5. Effects of GKN1 on miR-185 expression. A. *GKN1*-transfected MKN1 and

MKN28 cells induced increased miR-185 expression. **B.** *GKN1*-transfected AGS cells induced increased primary (pri)- and precursor (pre) miR-185 expression. However, silencing of GKN1 reduced pri- and pre-miR-185 expression in HFE-145 cells. **C.** *c-Myc*-transfected AGS cells induced decreased pri-, pre- and mature miR-185 expression, but overexpression of c-Myc had no effect on pri-, pre- and mature miR-185 expression in HFE-145 cells. **D.** *GKN1*-transfected AGS cells bound to and reduced c-Myc expression. Immunoprecipitated proteins were resolved by SDS-PAGE, transferred to PVDF membranes, and processed for immunoblotting with the indicated antibodies. In addition, *GKN1*-transfected AGS cells induced decreased c-Myc expression. **E**. Expression of miR-185 was strong in HFE-145 cells, but weak in AGS cells. miR-185 expression levels were increased in miR-185 mimic transfected AGS cells and down-regulated in anti-miR-185 transfected HFE-145 cells.

Figure S6. Effects of miR-185 in MKN1 and MKN28 cells. A. In MTT assay, miR-185 transfected MKN1 and MKN28 cells showed time-dependent inhibition of cell growth. **B & C.** miR-185 down-regulated expression of DNMT1 and EZH2 protein (B) and mRNA (C) in both cells.

Figure S7. Effects of miR-185 on GKN1 induced inhibited cell growth. *GKN1*-transfected MKN1 and MKN28 cells showed time-dependent inhibition of cell growth, but co-transfection of *GKN1* with anti-miR-185 resulted in recovery of cell growth.

Figure S8. Sequencing results of *E-cadherin* and *CDKN2A* promoter regions. A. Bisulfite genomic sequencing showing methylation status of the bis-*E-cadherin* region

of the *E-cadherin* 5` CpG island (underlined) in mock, *GKN1* and *GKN1* with anti-miR-185-transfected AGS cells. **B.** Bisulfite genomic sequencing showing methylation status of the bis-*CDKN2A* region of the *CDKN2A* 5` CpG island (underlined) in mock, *GKN1* and *GKN1* with anti-miR-185-transfected AGS cells.

Supplemental Experimental Procedures

Cell culture, treatment of 5-aza-dC, and transfection of *shDNMT1*, *shEZH2, shHDAC1* and *c-Myc*

AGS gastric cancer cell lines were obtained from the Korea Cell Line Bank (KCLB, Seoul, Korea). These cell lines were cultured at 37^{o}C in 5% CO_2 in RPMI-1640 medium (Lonza, Basel, Switzerland) with 10% heat-inactivated fetal bovine serum. To inhibit DNA methylation, cells were treated with 2 μM of 5-aza-deoxycytidine. To induce an autophagic response, 10 μM of ceramide (N-acetyl-d-erythrosphingosine; Calbiochem) dissolved in DMSO was added.

The *shDNMT1, shEZH2* and *shHDAC1* were cloned into the expression vector pSilencer 3.1 H1-neo (Invitrogen, Carlsbad, CA, USA), and *c-Myc* was cloned into the expression vector pcDNA3.1-His ((Invitrogen, Carlsbad, CA, USA). AGS cells were transfected in 60 mm-diameter dishes with expression plasmids (5 μg total DNA), using Lipofectamine Plus transfection reagent (Invitrogen) according to the manufacturer's recommendations.

Measurement of cell viability

For cell viability analysis, MTT assay was performed at 24, 48 and 72 hrs after transient transfection of *shDNMT1, shEZH2 and shHDAC1*. Absorbance was measured using a spectrophotometer at 540 nm and cell viability was expressed relative to mock (empty vector + Lipofectamine).

Flow-cytometry analysis of cell cycle

For cell cycle analysis, AGS cells from each experimental group were collected and stained with propidium iodide (PI) for 45 min in the dark before analysis. The percentages of cells in different phases of the cell cycle were determined using a FACSCalibur Flow Cytometer with CellQuest 3.0 software (BD Biosciences, Heidelberg, Germany). Experiments were performed in triplicate.

Measurement of *CDKN2A* and miR-185 expression

Real-time RT-PCR was performed using SYBR Green Q-PCR Master Mix (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Pri-, pre- and mature miR-185 expression was quantified by SYBR Green Q–PCR and normalized by human U6 snRNA. *CDKN2A* mRNAs were quantified by Q–PCR and normalized to mRNA of the housekeeping gene *GAPDH*. Data are reported as relative quantity according to an internal calibrator using the $2^{-\Delta\Delta CT}$ method (1). The sequences of the primers are described in Supplementary Table S1. All PCRs were done in triplicates.

Methylation specific PCR (MSP)

Methylation analysis was carried out in AGS gastric cancer cell lines. Methylation status of the promoter region of the *CDKN2A* gene was determined using sodium bisulfite treatment of the DNA followed by methylation specific PCR (MSP), as described in the literature with minor modifications (2). The bisulfite-modified DNA (5 µl) was subjected to MSP using two sets of primers for methylated and unmethylated *CDKN2A*. The primer sequences were described in supplementary Table S1. PCR was performed in a total volume of 30 µl, containing 5 µl of the template DNA, 0.5 µM of

each primer, 0.2 μM of each dNTP, 1.5 mM MgCI2, 0.4 unit of Ampli Taq gold polymerase (Perkin-Elmer) and 3 μl of 10X buffer. The reaction solution was initially denatured for 1 min at 95°C. Amplification was carried out for 40 cycles of 30 s at 95°C, 30 s at 58°C and 30 s at 72°C, followed by a final 5 min extension at 72°C. Each PCR product was loaded directly onto 2% agarose gels, stained with ethidium bromide and visualized under UV illumination.

Western blot analysis

Whole-cell extracts were prepared with radio-immunoprecipitation (RIPA) lysis buffer containing protease inhibitors. Protein concentrations were then determined using a BCA protein assay kit. RIPA lysates containing 20 μg of protein were separated by SDS-PAGE and transferred on to polyvinylidene difluoride membranes. Lysates prepared from AGS cells were analyzed by Western blotting using the CDKN2A (Cell Signaling Technology) antibodies. The ECL plus Western blotting detection system (Amersham Biosciences) was used to detect bound antibodies. The intensities of Western blot bands were quantified using an LAS 3000 densitometer (Fuji Photo Film Co.). Each blot was repeated at least twice.

Co-immunoprecipitation (Co-IP)

GKN1-transfected AGS cells were washed with PBS and lysed at 4°C with PBS, pH 7.2 containing 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10mM NaF, 1.0mM NaVO4 and 1.0% protease inhibitor cocktail (Sigma) as described (3). Equal protein aliquots (1.0mg) were immunoprecipitated with 2.0μg of antibodies to GKN1 (Sigma) plus protein A/G-agarose (Santacruz, CA, USA) as described by the

manufacturer. Immunoprecipitated proteins were resolved on 12% SDS-polyacrylamide gels and transferred to PVDF membranes (BioRad, Richmond, CA, USA). The membranes were blocked for 1 h with PBS containing 0.1% Tween 20 (PBS-T) and 5% non-fat dry milk (Sigma) and reacted with antibodies against c-Myc or GKN1 each diluted 1:1,000. The membranes were washed with PBS-T, incubated for 1h at room temperature with horseradish peroxidase-conjugated anti-mouse IgG antibody (Sigma) diluted 1:5,000 and developed with ECL plus Western blotting detection system (Amersham Biosciences). To confirm equivalent protein loading and transfer, the blots were stripped with 62.5 mM Tris–HCl, pH 6.7, 100 mM 2-mercaptoethanol and 2.0% SDS, blocked with PBS-T, 5% milk, and probed with the same antibody used for immunoprecipitation. Immunoreactive bands were identified by co-migration of prestained protein size markers (Fermentas, Glen Burnie, MD, USA). In some experiments, AGS whole cell lysates were directly probed with antibodies against c-Myc or GKN1 followed by secondary antibodies and chemiluminescence substrate. To control for protein loading and transfer, the blots were stripped and reprobed for GAPDH (Santa Cruz Biotechnology).

Supplemental References

1. Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. *29*, e45.

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3. Guang W., Ding H., Czinn S. J., Kim K. C., Blanchard T. G., Lillehoj E. P. (2010) MUC1 cell surface mucin attenuates epithelial inflammation in response to a common mucosal pathogen. J. Biol. Chem. 285, 20547–20557.