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PCDH17 gene promoter demethylation and cell cycle arrest by genistein in gastric cancer

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Summary. Protocadherin 17 *(PCDH17)* is a member of the cadherin superfamily, but little is known about its functions. We focused on it as a candidate tumor suppressor gene (TSG) and hypothesized that *PCDH17* may be susceptible to promoter methylation and gene silencing. Genistein has been reported to upregulate mRNA expression in many TSGs. We further tried to determine whether genistein could increase transcriptional *PCDH17* by promoter demethylation. Gastric cancer cell line AGS and normal gastric cell line Ges-1 were treated with genistein and 5-aza-2'-deoxycytidine (5Aza-C). Fluorescence-activated cell sorting was performed to analyze cell cycle and apoptosis, and cell proliferation was examined by cell viability assay. *PCDH17* mRNA expression in pairs of gastric cancer and normal tissue samples and cell lines were determined by quantitative real-time polymerase chain reaction. Bisulfite-modified polymerase chain reaction, cloning and sequencing were used to examine promoter methylation. We found genistein has an anti-proliferative effect on cancer cell growth through induction of cell cycle arrest. *PCDH17* mRNA expression was down-regulated in cancer tissues, and *PCDH17* promoter in cancer tissues was hyper-methylated in comparison with normal ones. Genistein and 5Aza-C induced *PCDH17* mRNA expression in AGS, but not in Ges-1. Furthermore, genistein and 5Aza-C treatment significantly decreased promoter methylation in putative methylation target regions in AGS, reactivating *PCDH17* expression. These results suggest that silencing of *PCDH17* expression through promoter hypermethylation leads to loss of its tumor-suppressive activity. Genistein showed similar effects to that of 5Aza-C. Our results indicate that genistein is a novel, advantageous therapeutic agent for treating gastric cancer.

Key words: Gastric cancer, Geinstein, *PCDH17*, DNA methylation

Introduction

Gastric cancer is the second most common malignant neoplasm and the second leading cause of cancer death throughout the world, although, the mechanisms that underlie the carcinogenesis of gastric cancer are still poorly understood (Nardone, 2003). With delayed symptoms, most gastric cancer patients are diagnosed at an advanced stage, and 5-year survival remains less than 20% (Jemal et al., 2008). Since chemotherapy limits its therapeutic results for the high incidence rate of severe side effects, there has been growing interest in developing more effective chemotherapeutic agents for gastric cancer.

Carcinogenesis is a multistep process that results from the accumulation and interplay of genetic and epigenetic changes. Increased DNA methylation of CpG islands in the promoter region of genes is well established as a common epigenetic mechanism for the silencing of tumor suppressor genes (TSGs) in cancer cells (Jones and Laird, 1999; Verma and Srivastava, 2002; Baylin and Ohm, 2006). CpG islands are 0.5 to 2 kb regions rich in cytosine-guanine dinucleotides and are present in the 5' promoter region of approximately 40-50% of human genes (Momparler and Bovenzi, 2000). Epigenetic silencing of tumor-related genes due to CpG island methylation has been recently reported in gastric cancer. Loss of *CACNA2D3* expression through aberrant promoter hypermethylation is thought to contribute to

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Abbreviations: 5Aza-C, 5-aza-2'-deoxycytidine; mRNA, messenger RNA; PCR, polymerase chain reaction; TSG, tumor suppressor gene

gastric carcinogenesis (Wanajo et al., 2008). The hypermethylation of the *MAL* gene was shown to be significantly associated with gastric cancer (71-80%), concluding that detection of promoter hypermethylation may be useful as a prognostic marker (Buffart et al., 2008). Besides *CACNA2D3* and *MAL*, promoter hypermethylation of many other genes, such as *P16, Runx3, MGMT, and DAPK* were also shown to play an important role in the pathogenesis of gastric adenocarcinoma (Zou et al., 2009). There are probably many additional TSGs important in gastric carcinogenesis remaining to be identified.

Epigenetic silencing of a gene can be reversed by drugs such as 5-aza-2'-deoxycytidine (5Aza-C), which forms a covalent complex with the active sites of methyltransferase resulting in generalized demethylation. Unfortunately, the applicability of 5Aza-C is hampered by its high toxicity and instability in physiological solutions (Bender et al., 1998). Recently, genistein-a soy derived isoflavone has attracted much attention of the medical scientific community. The main advantage of genistein as a potential drug is its multidirectional action in the live cell and its very low toxicity (Polkowski and Mazurek, 2000). Many studies have demonstrated the effect of genistein on specific target molecules and signaling pathways, including but not limited to, cell proliferation and differentiation, cell cycle regulation, apoptosis, angiogenesis, cell adhesion and migration, metastasis, and activity of different enzymes (Valachovicova et al., 2004). One major effect of genistein that has recently received considerable attention is its up-regulation of TSGs' mRNA expression by epigenetic mechanisms, such as DNA methylation and/or histone acetylation (Kikuno et al., 2008; Wang and Chen, 2010).

Protocadherins are a subfamily of the cadherin superfamily, but little is known about their functions (Morishita and Yagi, 2007). Several *PCDHs*, including *PCDH8*, *PCDH10* and *PCDH20*, are frequently silenced in carcinomas of the breast, nasopharynx, and lung, respectively (Imoto et al., 2006; Ying et al., 2006; Yu et al., 2008, 2010; Narayan et al., 2009), suggesting their function as TSGs. Since *PCDH17* is in the same subgroup as *PCDH8* and *PCDH10*, we focused on it as a candidate TSG. The *PCDH17* promoter is a TATA-less promoter containing CG-rich sequences that may be susceptible to DNA methylation and gene silencing (Verma and Srivastava, 2002).

In the present study, we examined the promoter methylation status of *PCDH17* in gastric cancer tissues and cancer cell line and compared it with normal tissues and a non-malignant-immortalized cell line. We further evaluated the effects of genistein on promoter hypermethylation and compared the results with that of 5Aza-C. In addition, we investigated the effect of genistein on cell cycle, apoptosis and cell proliferation in gastric cancer cell line and non-malignant-immortalized gastric cells.

Materials and methods

Tissue samples and cell culture

Fresh tumor and paired normal gastric tissue specimens were obtained after informed consent from patients who underwent gastric resection for gastric cancer in Zhejiang Provincial People's Hospital between 2009 and 2010. The specimens were snap-frozen and stored in liquid nitrogen straight after operations untill processing. No patient received radioactive therapy or chemotherapy before the operation. All of the samples were carefully reviewed at the Department of Pathology of the hospital to confirm the diagnosis and to determine the cellular composition of the tumor.

Human gastric cancer cell line AGS and the non-malignant immortalized gastric cell line Ges-1 were purchased from the Cell Bank of Chinese Academy of Sciences. The cell lines were cultured as monolayers in RPMI Medium 1640 with 10% fetal bovine serum (Hyclone), 50 μg/ml penicillin, 50 μg/ml streptomycin (Invitrogen, Carlsbad, CA) and maintained in an incubator with a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Both cells were treated with varying concentrations of genistein (0, 10, 25 and 50 μMol/l) (Sigma, St Louis, MO) dissolved in dimethyl sulfoxide and 5-Aza-C (5 μMol/l). Cells treated with vehicle only served as control. Fresh genistein or 5Aza-C was administered every day, along with a change of media, and the cells were grown for 3 days.

DNA cell cycle and cell viability assay

The cells were harvested, washed with cold phosphate-buffered saline and processed for cell cycle analysis. Briefly, $1x10^6$ cells were resuspended by treatment with trypsin and fixed with ice-cold 75% (v/v) ethanol at -20°C for 2 h. After centrifugation, the pellet was washed with 2 ml cold phosphate-buffered saline, resuspended in 1 ml phosphate-buffered saline containing 30 μg/ml propidium iodide, 0.3 mg/ml RNase A and incubated at 25°C for 1 h in the dark. The cell cycle distribution of the cells of each sample was then determined using a fluorescence-activated cell sorting caliber instrument (BD Biosciences FACSCalibur) equipped with Cell Quest software. ModFit LT for Mac V3.0 was used to determine the percentage of cells in the different cell cycle phases.

For growth assay, gastric cells were plated in 96-well plates at a density of $2x10^3$ cells per well in 100 μl medium. Before treatment, cells were allowed to adhere to the plate for 24 h. Afterward, the medium was replaced with the same volume of medium containing genistein or 5Aza-C, including the untreated control. After 24, 48 and 72 h, 10 μl of the CCK-8 solution (Cell Counting Kit-8, Dojindo Laboratories) was added to each well of the plate and incubated at 37°C for 3 h. The absorbance at 450 nm was measured using a microplate

spectrophotometer (BIO-RAD xMark).

RNA extraction from clinical samples and cell lines

Total RNA was extracted by using TRIzol reagent (Invitrogen). Fresh gastric tissues were homogenized in 1 ml TRIzol reagent. After the addition of 0.2 ml chloroform, samples were centrifuged for 15 min at 14,000 rpm. The aqueous phase was moved to a new centrifuge tube and resuspended with one half volume of 100% ethanol. RNA was then quantified spectrophotometrically and checked by gel electrophoresis for integrity.

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

After DNase treatment, cDNA was synthesized from 2 μg total RNA using High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Target primers were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA) and were as follows: RT-*PCDH17*-sense 5'-CAACGCGCCCTCCTTCT-3' and RT-*PCDH17* -antisense 5'-GCGTTCTCCGAGATGTCCAT-3'. RT-PCR quantification was then performed using glyceraldehyde-3 phosphate dehydrogenase (GAPDH) as an internal control. The comparative Ct method was used to calculate the relative changes in gene expression in the 7900 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The relative changes of gene expression were calculated using the following formula: fold change in gene expression, $2^{-\Delta\Delta Ct}=2^{-[\Delta Ct(genistein-/5Aza-C-treated\ samples)-\varphi Ct(untreated\ control)]}$, where $^{\Delta}Ct$=Ct (detected genes)-Ct (GAPDH) and Ct represents threshold cycle number.

Sodium bisulfite modification and sequencing

Genomic DNA extraction from clinical samples and cell lines was conducted by using a combination of TIANamp Genomic DNA KIT (TIANGEN biotech, Beijing) and RNase A (TIANGEN biotech, Beijing). Genomic DNA was treated with sodium bisulfite to convert unmethylated cytosines to uracil, which then converts to thymine, leaving 5-methylcytosines unchanged using EZ DNA Methylation-Gold Kit (ZYMO Research, Orange, CA, USA) following the manufacturer's instructions. We elected to analyze CpG sites that were upstream of the longest known 5' UTR of *PCDH17* transcripts. Relative to the cDNA clone accession # ENST00000448267, the thirty two CpG sites were at -884, -870, -836, -824,-819, -814, -796, -788, -756, -749, -744, -731, -692, -678, -654, -651, -649, -645,-614, -575, -498, -458, -331, -272, -240, -207, -148, -146, -129, -111, -84 and -74 on the genomic DNA (Fig. 3). The primer sequences for CpG sites -884 to -575: BSF_F1 5'-AGATTGAGTTAGAGAAAGYGTTAGG-3'; BSF_R1 5' CTAAATAAAAACAACACATCAAAA TTTAAC 3'. For CpG sites -654 to -458: BSF_F2 5' GGGTAGGAGTTTTTGGTTGG 3'; BSF_R2 5' TACCTCTTTTCTTTCTATTCAACATC 3'; For CpG sites between -331 and -74: BSF_F3 forward 5'-CTATAAATACAAAAACTATTTCCCTTCTAA-3'; BSF_R3 5' –ATTAGTATT TGTTGTGTGAGTTT ATTG-3' (Fig 3). PCR conditions for the primers were 95°C for 10 min, followed by 94°C for 30 sec, annealing at 55°C for 30 sec, 72°C for 30sec, 35 cycles. The PCR products from bisulfite treated genomic DNA were cloned into the vector pSC-A (Stratagene, Cedar Creek, TX, USA). Ten colonies from each PCR cloning were inoculated into SeqPrepTM 96 plates (Edge BioSystems, Gaithersbureg, MD, USA). The plasmid DNA was prepared by using SeqPrepTM 96 Plasmid Prep Kit (Edge BioSystems) and sequenced according to the manufacturer's instructions for double-stranded plasmid DNA using the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) with M13 forward or reverse primers.

Statistical analysis

Statistical analysis was performed using StatView version 5.0 for Windows as needed. $P<0.05$ was considered statistically significant and is represented by "*" in the figures.

Results

Induction of cell cycle arrest by Genistein

Fluorescence-activated cell sorting analysis was done to test the effect of genistein or 5Aza-C on cell cycle distribution. As summarized in Fig. 1A, only genistein resulted in a significantly higher number of AGS cells in the G2–M phase (38%) compared with vehicle treated control (17%). In addition, 5Aza-C induced apoptosis (12%) in AGS, whereas this effect was not observed in the genistein-treated cells. No obvious cell cycle and apoptotic changes were observed in non-malignant-immortalized Ges-1 cell under the effect of either agent.

In order to examine the effect of genistein and 5Aza-C on gastric cell proliferation, we performed cell viability assay. As shown in Fig. 1B, genistein (25 μM for 72 h) and 5Aza-C (5 μM for 72 h) significantly decreased cell viability in a time-dependent manner, confirming that both agents have anti-proliferative effects on AGS. Moreover, the anti-proliferative effects of both agents were observed earlier and more obviously in AGS than in Ges-1, indicating that both agents targeted tumor cells rather than normal cells.

PCDH17 expression profile

In order to determine relative expression levels of *PCDH17* in gastric cancer cell, we performed TaqMan

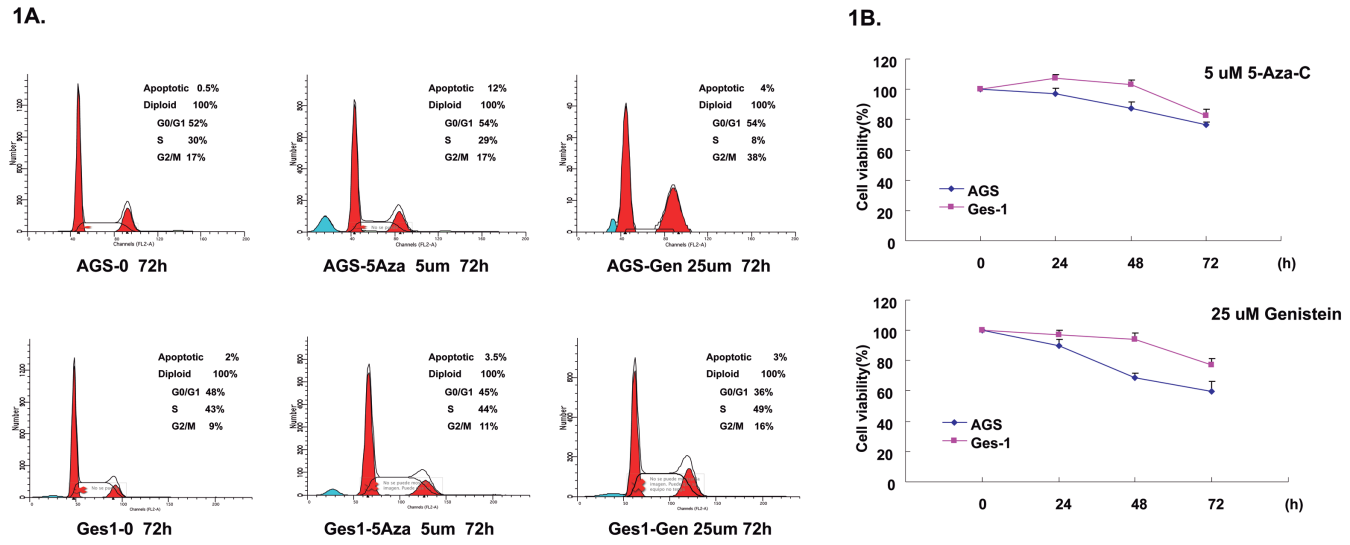


Fig. 1. Effect of genistein and 5Aza-C on cell cycle progression. DNA content and cell cycle progression were analyzed by flow cytometry. **A.** The two peaks in the fluorescence-activated cell sorting diagrams indicate G0–G1 and G2–M cells with S phase cells between peaks. Sub G1 fractions represent cells with fragmented DNA or apoptotic cells. The main panel shows a single representative result, whereas the numerical values are the mean ± SD. **B.** The 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide cell proliferation assay results. The data expressed in the graph is the mean ± SE of three independent experiments. Declines in cell viability were statistically significant ($P<0.05$).

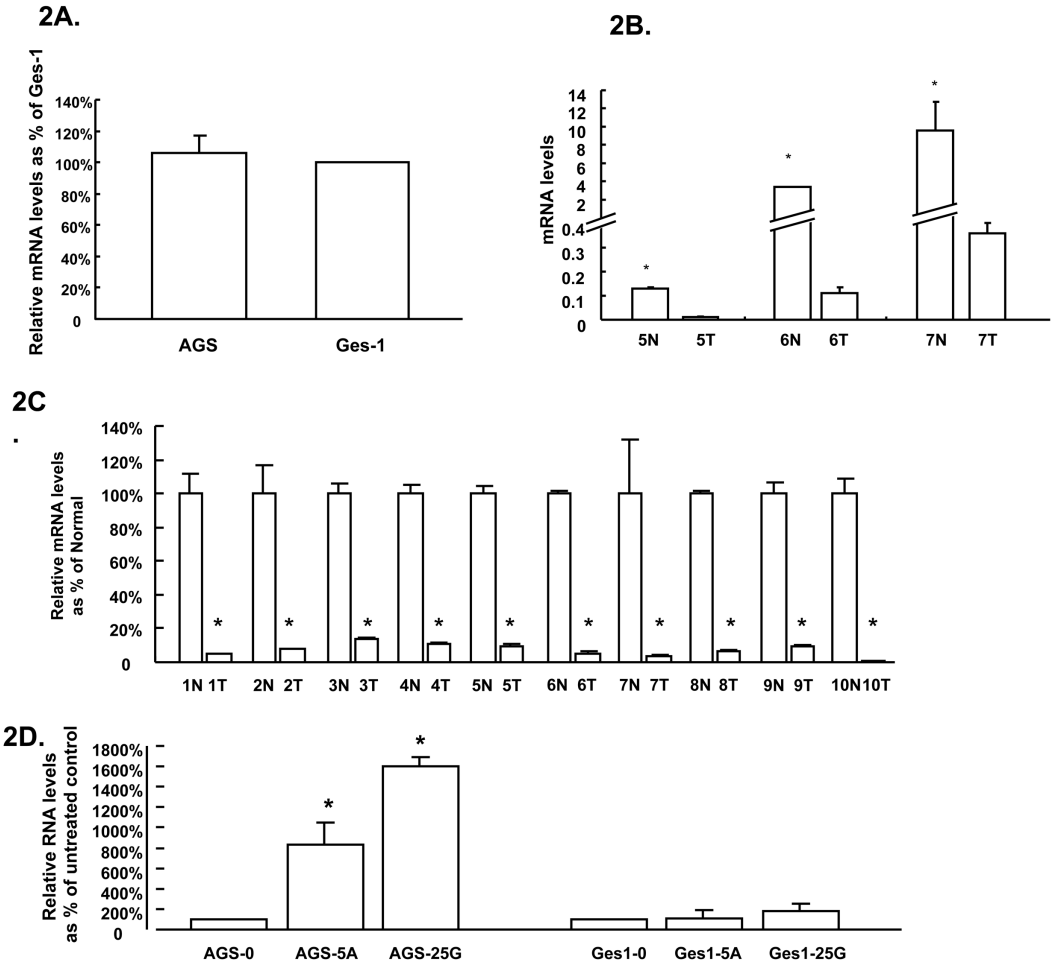


Fig. 2. *PCDH17* expression profile. **A.** Relative expression profile of *PCDH17* in gastric cancer cell AGS and non-malignant-immortalized gastric cell Ges-1. **B.** Expression profile of *PCDH17* in gastric carcinoma(T) and normal(N) clinical samples. **C.** Relative expression profile of *PCDH17* in gastric carcinoma (T) and normal (N) clinical samples. **D.** Relative expression profile of *PCDH17* gene following treatment with 0 and 25 uMgenistein (25G) and 5 uM 5-Aza-2'-deoxycytidine (5A). Relative quantification was performed by quantitative real-time PCR using the following formula: Fold change in gene expression, $2^{-\Delta\Delta Ct}=2^{-[\Delta Ct(\text{genistein-/5Aza-C-treated samples})-\Delta Ct\ (\text{untreated control})]}$, where $\Delta Ct=Ct$ (detected genes)-Ct (GAPDH) and Ct represents threshold cycle number. For untreated controls data were normalized to 1. All data are expressed as the mean ± SE(bars). Statistically significant at $P<0.05$

quantitative real-time PCR analysis for AGS and compared it with the non-malignant-immortalized Ges-1. However, no obvious difference was observed in gastric cancer cell line AGS and non-malignant-immortalized Ges-1 (Fig. 2A). We also compared the mRNA expression levels of tumor samples and normal tissue samples, and the results showed that the *PCDH17* transcription levels were significantly different in different individuals (Fig. 2B). The relative mRNA expression was significantly lower in tumor samples when compared with normal tissue samples (Fig. 2C). These results showed that the *PCDH17* gene transcription level varied widely due to individual differences, while it is transcriptionally downregulated in gastric cancer.

Effect of genistein and 5Aza-C treatment on the expression of PCDH17

Genistein significantly upregulated the relative expression level of *PCDH17* in a dose-dependent manner over vehicle control (Fig. 2D, data not shown for lower doses) in gastric cancer cells AGS. 25 μM genistein treatment increased the *PCDH17* mRNA expression level by 15 to 16-fold in AGS compared with vehicle control, while 5Aza-C treatment increased by 8 to 9-fold. The results indicate that the *PCDH17* gene can be significantly induced by genistein and 5Aza-C treatment. However, no obvious upregulated *PCDH17* mRNA level was observed in non-malignant-immortalized Ges-1 cell under the effect of either agent.

Methylation status of PCDH17 promoter

To check whether transcriptional silencing of the *PCDH17* gene is due to promoter hypermethylation, we analyzed the methylation status of the *PCDH17* promoter in pairs of tumor and normal tissue samples and cell lines by bisulfite-modified PCR followed by direct sequencing of the modified DNA samples. Selected amplicons were subsequently subcloned, and the recombinants were identified and subjected to automated DNA sequencing. The resulting sequences were compared with the parent promoter sequence from which the clones were made and the methylation status of the CpG dinucleotides within this amplicon was determined by characteristic chemical changes associated with cytosines existing in either a methylated or an unmethylated state. DNA sequencing results revealed that the promoter of the *PCDH17* gene in tumor samples was hypermethylated in some target CpG sites in comparison with normal tissue samples, such as CpG site -870, -731 and -458 in the pair of No. 1 sample. Besides, the distribution of its target sites in different individuals was also different (Fig. 4B). Under 5Aza-C and genistein treatment, methylation of several CpG sites in *PCDH17* promoter was changed in AGS cells. 5 μM 5Aza-C significantly demethylated the *PCDH17* promoter at site -84 and -74, and 25 μM Genistein significantly demethylated the CpG site of -678, -614, -458, -207 and -129. However, no significant demethylation was observed in Ges-1 cells (Fig. 4A). These results indicate that transcriptional silencing of the *PCDH17* gene is due to promoter hypermethylation that can be reversed by genistein and 5Aza-C treatment.

Discussion

Our study clearly demonstrates that *PCDH17* is transcriptionally downregulated in gastric cancer due to aberrant promoter CpG island methylation. We showed that the methylation-silenced *PCDH17* gene can be

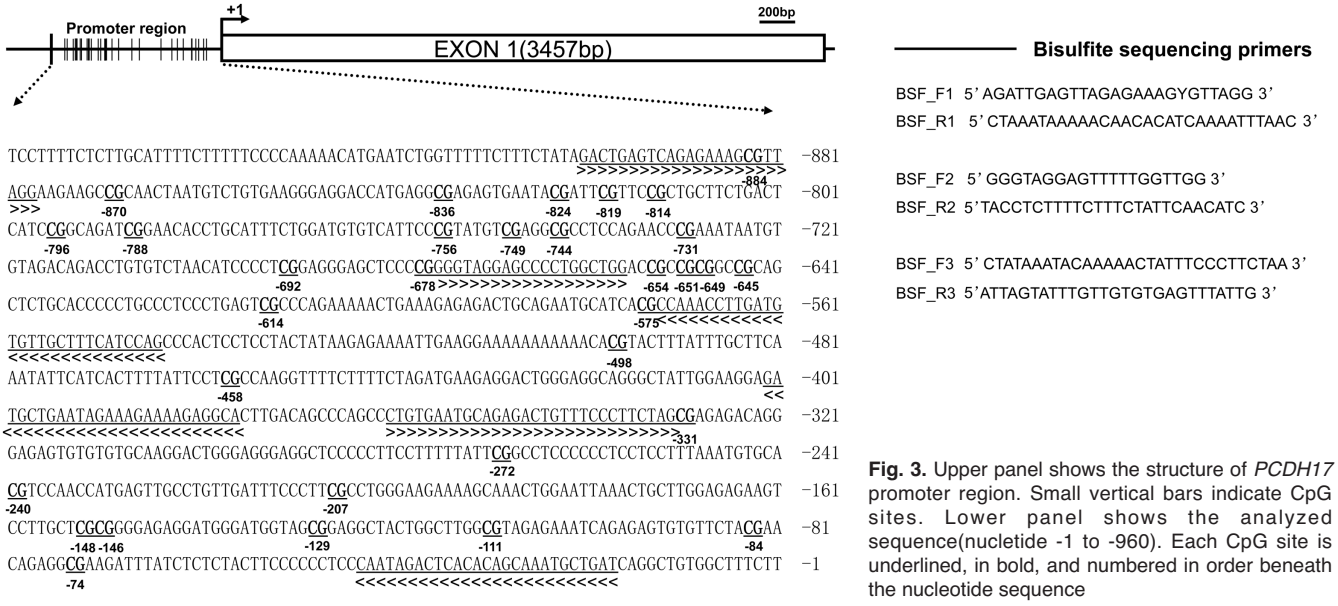
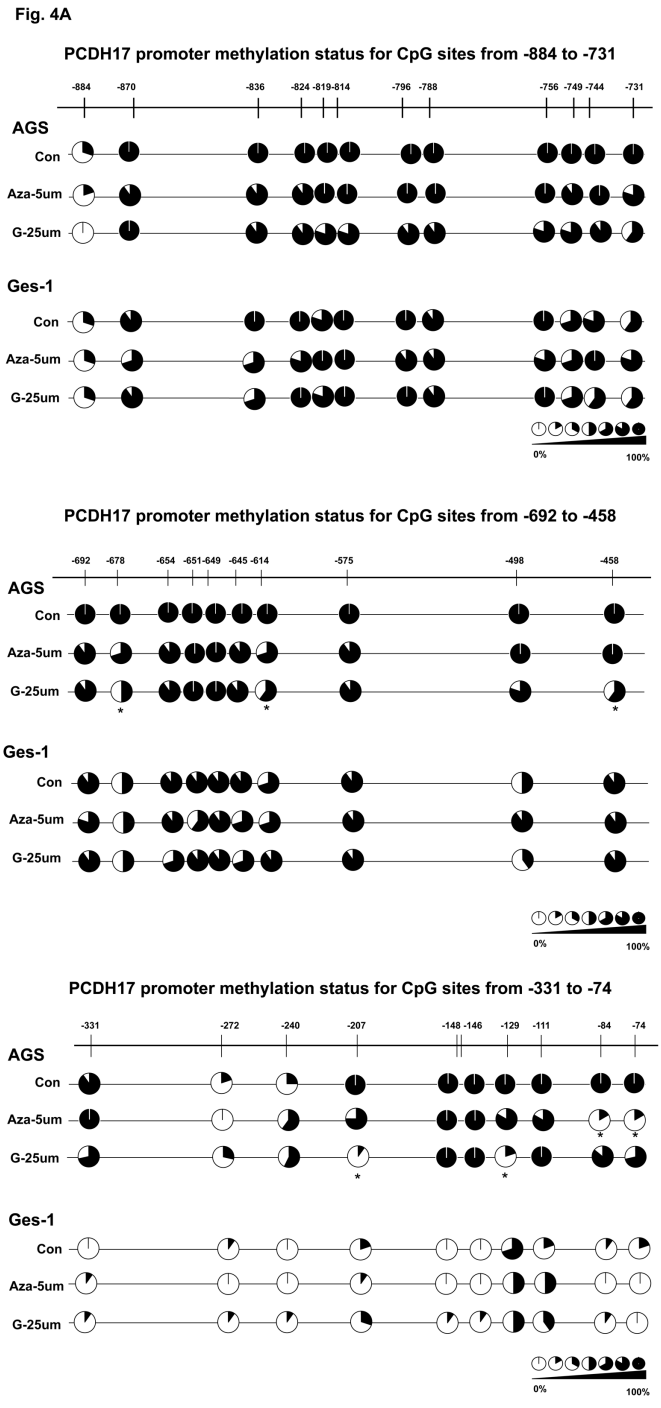


Fig. 3. Upper panel shows the structure of *PCDH17* promoter region. Small vertical bars indicate CpG sites. Lower panel shows the analyzed sequence(nucletide -1 to -960). Each CpG site is underlined, in bold, and numbered in order beneath the nucleotide sequence

reactivated by genistein, which causes CpG demethylation in gastric cancer cells. Genistein's anti-proliferative effect on gastric cells may be due to induction of cell cycle arrest rather than apoptosis. To our knowledge, this is the first report showing that the *PCDH17* gene is epigenetically silenced in gastric cancer and that it can be reactivated by genistein-induced promoter demethylation.

Control of cell cycle progression in cancer cells is considered to be a potentially effective strategy for the



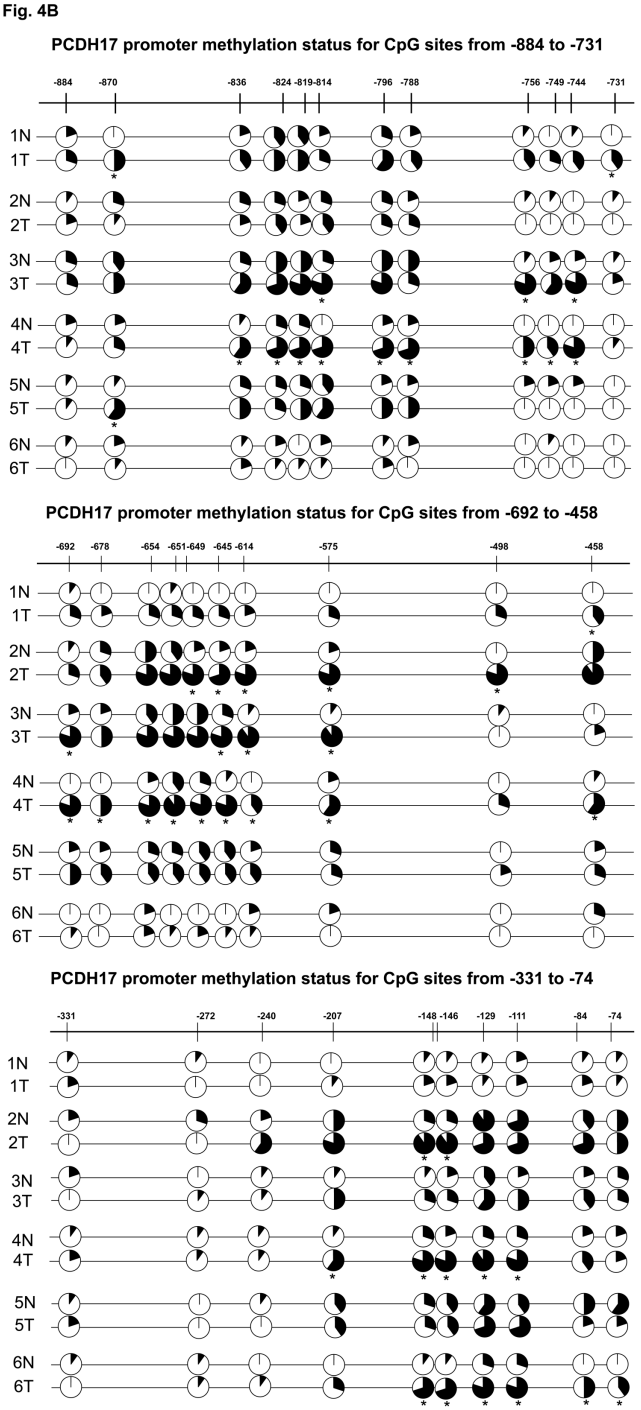


Fig. 4. A. *PCDH17* methylation status showing demethylation of CpG sites by 5Aza-C (Aza) and genistein (G) treatments in AGS cell and Ges-1 Cell. **P*<0.05, compared to Con. **B.** *PCDH17* methylation status in 6 pairs of normal and tumor samples. Each pair was obtained from the same patient and microdissected by a certified pathologist into normal and tumor. **P*<0.05, compared to normal in pair.

control of tumor growth, since molecular analysis of human cancers has revealed that cell cycle regulators are frequently mutated in many common malignancies (Molinari, 2000). Our in vitro data indicated that treatment of gastric cancer cells with genistein resulted in significant G2-M phase arrest of cell cycle progression. Genistein has been found to induce G2-M cell cycle arrest in breast (Cappelletti et al., 2000), liver (Chang et al., 2004), human ovarian cancer cells (Ouyang et al., 2009), PC3 prostate cancer (Raffoul et al., 2006) and lung cancer cells (Lian et al., 1998). Cell viability assay showed that genistein has anti-proliferative effects on gastric cancer cells and this may be due to induction of cell cycle arrest since we did not observe any obvious induction of apoptosis by genistein.

Upadhyay found that compared to normal breast epithelial cells, genistein causes a greater degree of G2-M arrest and induced apoptosis in malignant cell lines (Upadhyay et al., 2001). Chang found that genistein treatment inhibited the growth of prostate cancer cells in a dose-dependent manner, whereas no effect on normal prostate epithelial cells was observed (Chang et al., 2009). In our experiment, no obvious cell cycle changes were observed in non-malignant-immortalized Ges-1 cells under the effect of either agent. Besides, cell viability assay showed that the anti-proliferative effects of both agents was observed earlier and more obviously in AGS than in Ges-1, indicating that both agents targeted tumor cells rather than normal cells.

Both genistein and 5Aza-C inhibit the proliferation of cancer cells, but in different ways. We found only genistein resulted in an obvious cell cycle arrest, while only 5Aza-C induced apoptosis, indicating that 5Aza-C has high toxicity, whereas genistein does not. The precise molecular mechanisms responsible for such a divergence, however, are not clearly known. One potential mechanism is that they are involved in different regulation of gene activity, such as modulating epigenetic events, directly or through a receptor dependent process, which needs to be further investigated.

Carcinogenesis is a multistep process and one mechanism in the multistep model is the promoter methylation of specific TSGs. If methylation occurs within the promoter region of a suppressor gene, epigenetic silencing of this gene may lead to functional inactivation, which is a mechanism reported for various tumor entities (Jones and Baylin, 2002; Marsit et al., 2005; Tomii et al., 2007; Jung et al., 2008). In recent years, the list of TSGs that are inactivated by epigenetic events rather than classical mutation/deletion events has been growing (Dammann et al., 2003; Dote et al., 2005). Our results show that the *PCDH17* gene is hypermethylated in many CpG islands in gastric cancer samples compared with normal tissues. Promoter hypermethylation subsequently leads to the transcriptional silencing of the *PCDH17* gene (Fig. 4B).

Unlike mutational inactivation, methylation is reversible and demethylating agents are being used in clinical trials. If the methylation of a TSG is relevant for gene silencing, then reversal of methylation by demethylating agents may lead to reactivation of the gene. 5-Aza-C is a nucleoside analog and an active drug for the therapy of acute leukemia. Incorporation of 5-Aza-C into DNA blocks DNA methylation and can result in the activation of specific genes, although, it is also a teratogenic agent and a mutagen which has high toxicity and instability in physiological solutions. In this study, we used genistein, a natural, nontoxic dietary isoflavone and compared its effect with that of 5Aza-C. Our results revealed that genistein significantly induced *PCDH17* mRNA expression and its effects were similar to that of 5Aza-C in gastric cancer cell line AGS (Fig. 2D). This is consistent with other reports which have shown that genistein upregulated mRNA expression of the BRAC1, RARb, MGMT (Majid et al., 2008, 2009). Further, we tried to determine whether transcriptional repression of *PCDH17* is a consequence of promoter methylation. We found that a CpG island at the promoter of the *PCDH17* gene was hypermethylated in putative methylation target regions in tumor samples (Fig. 4B) compared with normal tissues. Treatment with 25 μM genistein caused demethylation of these regions, inducing *PCDH17* mRNA expression, though at different regions than that of 5Aza-C (Fig. 4A).

In conclusion, our study showed that *PCDH17* is epigenetically silenced in gastric cancer cells and can be reactivated by genistein-induced promoter de-methylation. In addition, genistein showed anti-proliferative effects on cancer cells through cell cycle arrest. Genistein showed similar effects to that of 5Aza-C, which is a potent demethylating agent but has high toxicity and instability in physiological solutions. Since genistein is a natural, non-toxic, dietary isoflavone and is effective in gastric cancer growth retardation, it may be a promising candidate for therapy in gastric cancer.

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Conflict of interest. The authors declare no conflict of interest related to this work.

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