Frequent silencing of protocadherin 8 by promoter methylation, a candidate tumor suppressor for human gastric cancer

DANJIE ZHANG, WEI ZHAO, XINHUA LIAO, TIEQIANG BI, HAIJUN LI and XIANGMING CHE

Department of General Surgery, First Affiliated Hospital of Medical College of Xi'an JiaoTong University, Xi'an 710061, P.R. China

Received May 10, 2012; Accepted July 30, 2012

DOI: 10.3892/or.2012.1997

Abstract. The cadherins are a family of cell surface glycoproteins responsible for cell adhesion which play an important role in cell morphology, contact inhibition and signal transduction during tumorigenesis. Protocadherin 8 (PCDH8), a member of the cadherin family, has been reported to act as a tumor suppressor involved in oncogenesis in breast cancer. In this study, we aimed to investigate the epigenetic inactivation of PCDH8 and its tumor suppressor function in gastric cancer. The expression of PCDH8 was markedly reduced or silenced in gastric cancer cell lines compared with normal gastric cells or tissues. Methylation of the PCDH8 gene promoter was observed in 100% (4/4) of cell lines and 55.38% (36/65) of the primary gastric cancer by methylation-specific PCR, but not in normal gastric mucosa (0/10). Methylated PCDH8 was significantly associated with lymph node metastasis in a logistic regression analysis. The demethylation reagent 5-aza-2'-deoxycytidine was able to restore or upregulate PCDH8 expression in gastric cancer cell lines. Ectopic expression of PCDH8 in silenced gastric cancer cells significantly inhibited cell migration and induced apoptosis. For the first time, our study demonstrates the epigenetic inactivation of PCDH8 by promoter methylation and its tumor suppressor function in human gastric cancer. Thus, PCDH8 could be identified as a candidate tumor suppressor in human gastric cancer.

Introduction

Gastric cancer is the second leading cause of cancer death in the world behind lung cancer (1,2). Gastric carcinogenesis is a multistep process including many genetic and epigenetic alterations, such as abnormalities in DNA mismatch repair genes, growth factors/receptors, angiogenic factors or cell cycle regulators. These abnormalities can also define biological characteristics of gastric cancer, which can play a role in therapy (3,4). Although genetic abnormalities which include gene mutation and deletion are remarkable in causing oncogene activation and tumor suppressor gene inactivation, epigenetic silence of tumor suppressor genes through aberrant promoter hypermethylation have also been confirmed to be frequent in gastric carcinoma (5,6). Gene silencing by promoter hypermethylation has been affirmed in several genes in gastric cancer, including CDH1, which is involved in cell adhesion, and hMLH1, which is associated with DNA mismatch repair and the cell cycle regulator p16. In addition, promoter hypermethylation of MAL has been shown to be an independent prognostic marker for gastric cancer (7-9). DNA high methylation of tumor suppressor genes frequently occurs in the early stage of human carcinogenesis, so investigating the methylation of these gene promoters may contribute to the diagnosis, prognosis and target therapy in gastric carcinoma.

Cadherin molecules are pivotal in producing and preserving tissue structure in tumorigenesis (10,11). Such as E-cadherin, it is a classical tumor suppressor which is mutated in gastric carcinoma and lobular breast carcinoma (12). Accumulating data have suggested that protocadherins can function as tumor suppressors. Protocadherins 10 and 20 are frequently silenced in carcinomas of the nasopharynx and lung due to promoter methylation and inhibit cell migration and proliferation (13,14). Protocadherin 17 in esophageal squamous cell carcinoma is also frequently silenced because of promoter methylation (15). The protocadherin 8 (PCDH8) gene localizes on the human chromosome 13q14.3, is an adhesion protein with six cadherin repeats that organizes the formation and polarity of developing cellular structures in embryos (16).

It has been shown that PCDH8 is epigenetically silenced caused by promoter hypermethylation in most of breast tumors and it can suppress breast epithelial migration and proliferation (17). To the best of our knowledge, the expression of PCDH8 and its promoter hypermethylation has not yet been shown in gastric cancers. Therefore, in this study, we first confirmed the expression of PCDH8 and the methylation of its gene promoter in human gastric cancer cell lines and determined the role of 5-aza-2'-deoxycytidine (5-AZA, a drug that inhibits the DNA methyltransferase (DNMT)-mediated hypermethylation of promoter region CpG islands) in regulation of PCDH8 expression in gastric cancer cells. We also detected the methylation of PCDH8 gene promoter

Correspondence to: Professor Xiangming Che, Department of General Surgery, First Affiliated Hospital of Medical College of Xi'an JiaoTong University, Yanta West Road 277, Xi'an 710061, P.R. China
E-mail: renpengyu2001@163.com

Key words: protocadherin 8, gene methylation, 5-aza-2'-deoxycytidine, gastric cancer

in tissue specimens and found the association between PCDH8 gene promoter methylation and clinicopathological characteristics of gastric cancer.

Materials and methods

Human gastric samples. A total of 65 primary gastric adenocarcinomas and their adjacent non-cancer specimens, and 10 normal gastric specimens from patients with normal endoscopy results were obtained from the Department of General Surgery, First Affiliated Hospital of the Medical College of Xi'an Jiaotong University, Xi'an, China between January 2009 and January 2010. All specimens were immediately frozen in liquid nitrogen and stored at -80˚C until use. The gastric cancer cases were clinically and pathologically verified. Standard protocols established by the Hospital's Protection of Human Subjects Committee were followed in this study.

Cell lines and culture. A total of four gastric cancer cell lines (SGC7901, MKN45, MKN28, and BCG823) and the immortalized gastric mucosal epithelial cell line (GES-1) were purchased from the Laboratory Animal Research Centre of the Fourth Military Medical University at Xi'an, China. All cell lines were cultured in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum in a humidified incubator with 5% CO_2 and 95% air at 37˚C.

These cells were passaged at a ratio of 1:3 with trypsin once they reached confluence (~10^6 cells) into 752 cm culture flasks (Sarstedt, Newton, NC). For treatment with 5-aza-2'-deoxycytidine, these cell lines were split and cultured at a low density (30% confluence) overnight and then treated with 5-aza-2'-deoxycytidine (Sigma, St. Louis, MO) at a concentration of 1 μmol/l for up to 72 h. The growth medium was refreshed every 24 h, and at the end of the treatment, DNA and RNA from these cells were isolated as described below.

Bisulphite treatment of DNA, methylation-specific polymerase chain reaction (MSP). Genomic DNA from these cell lines and tissue specimens were extracted by a DNA mini kit (Qiagen, Valencia, CA, USA). Methylation status of PCDH8 in gastric tissues and cell lines were determined by MSP. Briefly, 2 mg of genomic DNA was bisulphite-treated with Zymo DNA Modification kit (Zymo Research, Orange, CA, USA). Bisulphite-treated DNA was used as a template for MSP and quantitative PCR by ABI 2700 thermocycler (Applied Biosystems, CA, USA) and LightCycler (Roche Diagnostic), respectively. Oligo 6 software (Molecular Biology Insights, CO, USA) was used for designing primers specific for methylated of each amplification. The extracted DNA was then dissolved in Tris-EDTA (TE) buffer and stored at -20˚C. To assess the methylation levels of the PCDH8 gene promoter, genomic DNA from gastric cancer cell lines and tissue specimens were first subjected to bisulfite treatment and then methylation-specific polymerase chain reaction (MSP) as described previously (18). The MSP primers for PCDH8 were designed and synthesized according to genomic sequences skirting the presumed transcription start sites for PCDH8. The primer sequences were: PCDH8-UN 5' CCT ACG CGG GCA GCT ACC T 3', PCDH8-UN-AS 5' CGC GTT GTC GTT CTC GTC G 3', PCDH8-ME-S 5' GTG CGT TGC GTT TTT TAT GG 3' and PCDH8-ME-AS 5' CGC GTT ATC GTT CTC GTC G 3'. Each MSP reaction incorporated ~100 ng of bisulfite-treated DNA, 25 pmol of each primer, 100 pmol dNTPs, 2.5 μl 10X PCR buffer, and 1 U of JumpStart RedTaq Polymerase (Sigma) in a final reaction volume of 25 μl. The PCR amplification conditions were an initial 95˚C for 5 min and then 35 cycles of 95˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 30 sec and a final extension at 72˚C for 5 min and then stored at 4˚C. The MSP products were separated on 2% agarose gel electrophoresis and visualized under the ultraviolet (UV) light.

RNA isolation and semi-quantitative reverse transcription PCR. Total cellular RNA from the cell lines was isolated using the TRIzol reagent (Invitrogen) according to the WJG manufacturer's instructions. RNA quality and quantity were assessed using agarose gel electrophoresis (1%) and spectrophotometric analysis of 260/280 ratios. The RNA was stored at -70˚C prior to use. The first strand cDNA was synthesized with oligo-(dT) primer using a reverse transcriptase kit from Invitrogen. RNA (2 μg) was subjected to the first strand cDNA synthesis, and 1 μl cDNA from RT reaction was subjected to PCR amplification of gene expression in a total 25-μl reaction volume. The PCR amplification was carried out using primer sets derived from the published PCDH8 gene sequences: PCDH8 primers: 5'-TGGCGGTGTGGAAAGGACA-3' and 5'-CGGAGTGACCTGTATATGTG-3' (17). This primer set, designed to cross the intronic sequences, can prevent from amplification of genomic DNA for control of genomic DNA contamination during RNA isolation. A total of 32 cycles of PCR amplification were performed based on our pre-experiment for semi-quantitative measurement of PCDH8 gene expression levels. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified for 25 cycles as an internal control of equal loading and cDNA quality and quantity. The sequence of GAPDH primers: 5'-GACCACAGTCCATGCCATCAC-3' and 5'-GTCCACCACCCTGTTGCTGTA-3'. The PCR products (PCDH8, 251 bp; GAPDH, 150 bp) were then electrophoresed in 1.5% agarose gels containing ethidium bromide and reviewed under the UV light. Primers were designed according to GenBank, NCBI. For the validation, each experiment was done in triplicate.

Protein extraction and western blotting. The cells were grown and treated with or without 1 μM 5-aza-2'-deoxycytidine for 72 h and total cellular protein was then extracted from these cells in 200 μl ice-cold mild lysis buffer containing 10 μl nonidet P-40, 0.15 mol/l NaCl, 0.01 mol/l sodium phosphate (pH 7.2), 2 mmol/l EDTA, 50 mmol/l sodium fluoride, 0.2 mmol/l sodium vanadate, and 1 μg/ml aprotinin. The cell mixture was centrifuged at 20,000 r/min for 15 min and supernatants were then collected. The concentration of protein was quantified by the BCA protein assay from Pierce (Rockford, IL, USA) and an equal amount of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto PDVF membranes (Millipore, Billerica, MA, USA). Western blot analyses were then carried out using mouse monoclonal anti-PCDH8 antibody (JK-19) (sc-81817 Santa Cruz Biotechnology, Inc., USA) or an anti-β-actin antibody (Boster, Wuhan, China). The blots were developed with chemiluminescence substrate solution from Pierce and exposed to X-ray film.

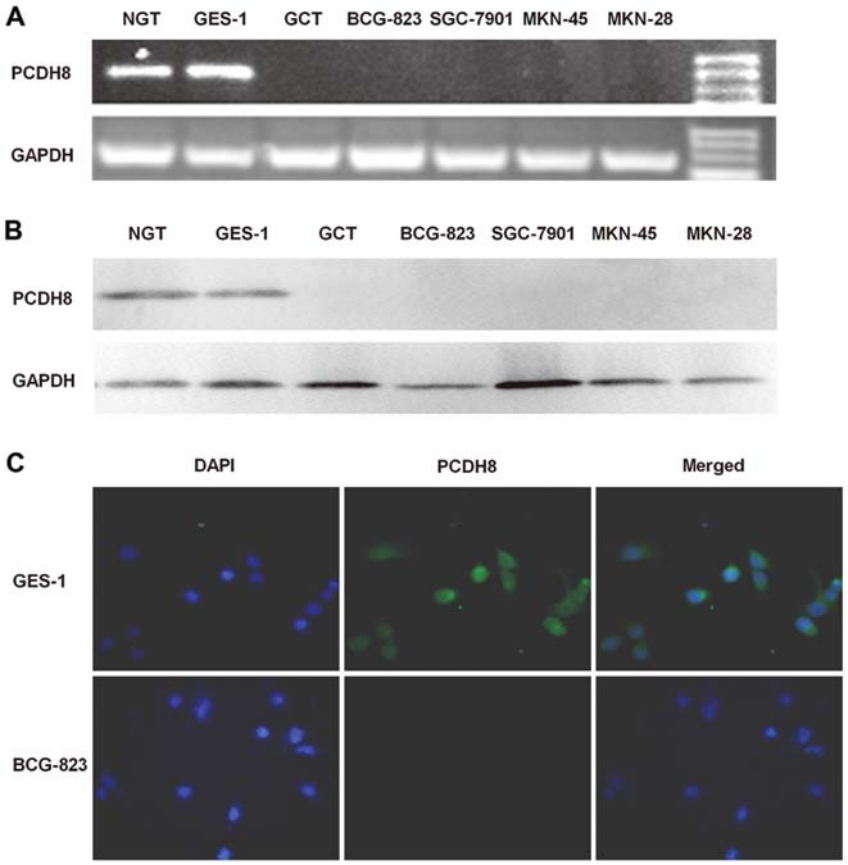

Figure 1. The expression of PCDH8 in normal gastric and gastric cancer cell lines or tissues. The expression of PCDH8 was lost in multiple gastric cancer cell lines: SGC7901, MKN45, MKN28, and BCG823 and gastric cancer tissue (GCT), but expressed in GES-1 cell line and normal gastric tissue (NGT), determined by (A) RT-PCR and (B) western blotting as described in Materials and methods, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as control. (C) Subcellular localization of PCDH8 were determined by immunofluorescence using anti-PCDH8 antibody and 4,6-diamidino-2-phenylindole dihydrochloride (DAPI). PCDH8 localizes at the cytoplasm and is concentrated in perinuclear regions in GES-1 but not expression in BCG-823 cells (x400).

Immunofluorescence. BGC-823 cells expressing PCDH8 epitope-tagged proteins were plated onto sterile cover slips in a 6-well dish. Sixteen hours after plating, cells were fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) pH 7.4 for 30 min at room temperature. Cells were washed for 20 min in PBS, permeabilized for 1 h in buffer A (5% goat serum, 0.1% Triton X-100 in PBS), and incubated with 1:1000 dilution of mouse monoclonal anti-PCDH8 antibody (JK-19) (sc-81817 Santa Cruz Biotechnology) in buffer A. Cells were washed in PBS, and incubated with 1:600 dilution of Alexafluor 568 antimouse antibody (Molecular Probes, Invitrogen Corp., Carlsbad, CA, USA) and counterstained with 40,6-diamidino-2-phenylindole dihydrochloride (DAPI; 0.15 mg/ml in water).

Detection of apoptosis. Gastric cancer cells were treated with or without 1 μM 5-aza-2'-deoxycytidine for up to 72 h. FCM was performed with PI and fluorescein isothiocyanate (FITC)-labeled annexin V (Joincare Biosciences, Zhuhai, China). After the treatment, the remaining intact cells were incubated at 37˚C for 24 h, and then the cells were washed with cool PBS at 4˚C. After a centrifugation at 1500 rpm for 5 min, 500 μl of 1X binding buffer, 5 μl of FITC-labeled Annexin V and 10 μl of PI were added to the cell suspension and gently mixed. After incubation at 25˚C for 10 min in the dark, the cells were analyzed by FCM.

Migration assays. Equal numbers of cells were plated on a 6-well plate. A single wound was introduced using a P20 pipette tip and media was replaced. Migration was assessed at indicated times.

Statistical analysis. The results were expressed as mean ± SD. The statistical analyses of the experimental data were carried out using SPSS 16.0 software for Windows (Chicago, IL). P-values for dichotomous variables were 2-tailed and based on the Pearson χ^2 test or the Pearson χ^2 test with continuity correction. Statistical differences were estimated by One-way analysis of variance (ANOVA) followed by Dunnett's test. $P<0.05$ was considered statistically significant.

Results

Silence of PCDH8 expression through methylation of PCDH8 gene promoter and 5-aza-2'-deoxycytidine induction of PCDH8 gene expression in gastric cancer cell lines.

We detected PCDH8 expression in normal gastric and gastric cancer cell lines or tissues and found that PCDH8 mRNA or protein was lost in multiple gastric cancer cell lines: SGC7901, MKN45, MKN28, and BC- G823 cell lines and gastric cancer tissue, but expressed in GES-1 cell line and normal gastric tissue. The immunofluorescent results also confirmed the above findings (Fig. 1). To find out whether the silence of PCDH8

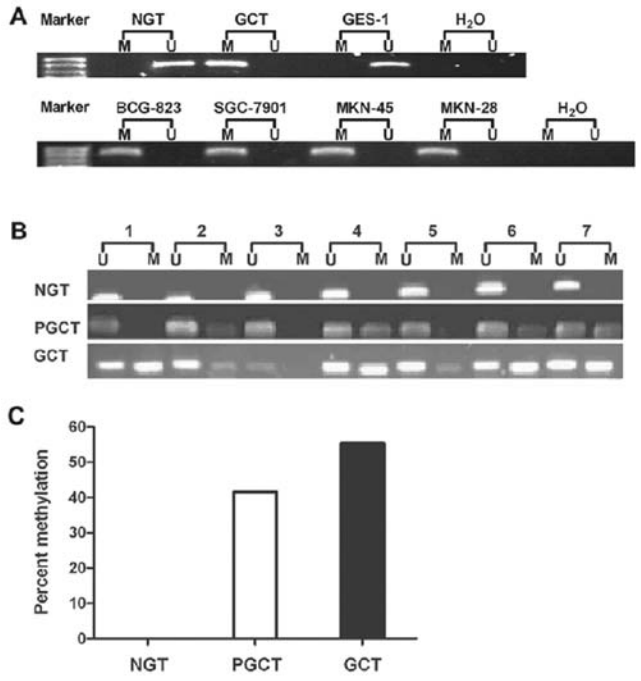

Figure 2. Analysis of the methylation status of the PCDH8 promoter by MSP in normal gastric and gastric cancer cell lines or tissues. (A) The MSP analysis showed that PCDH8 gene promoter was highly methylated in SGC7901, MKN45, MKN28, and BCG823 cell lines and gastric cancer tissue, but not methylated in GES-1 cell line and normal gastric tissue. M, methylated alleles; U, unmethylated alleles. (B) MSP analysis of PCDH8 gene promoter in 65 patients with human gastric carcinoma. MSP analysis showed that methylation of the PCDH8 gene promoter was frequently detected in gastric cancer tissue (GCT) (55.38%, 36/65) and para-carcinoma tissue of gastric (PGCT) (41.54%, 27/65), but not in normal gastric tissue (NGT). (C) Comparison of PCDH8 gene promoter methylation among NGT, PGCT and GCT. Pearson χ^2 test by SPSS 16.0 software; a, NGT vs GCT, P=0.005; b, NGT vs PGCT, P=0.002.

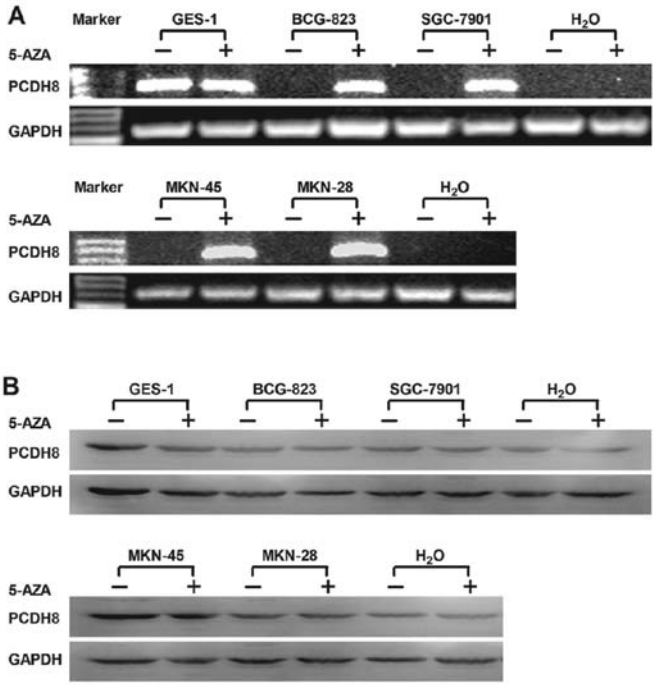

Figure 3. The expression of PCDH8 in gastric cancer cell lines after treatment with 5-aza-2'-deoxycytidine (5-AZA). PCDH8 expression was induced or upregulated in SGC7901, MKN45, MKN28, and BCG823 cell lines after treated with 5-AZA (1 μM for 72 h). (A) PCDH8 mRNA levels were measured by RT-PCR, and (B) PCDH8 protein expression was determined by western blotting, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as control.

gene expression is caused by methylation of the PCDH8 gene promoter, we investigated the methylation status of PCDH8 promoter in 4 gastric cancer cell lines. The MSP analysis showed that PCDH8 gene promoter was highly methylated in SGC7901, MKN45, MKN28, and BCG823 cell lines, but not methylated in GES-1 cell line (Fig. 2A). PCDH8 mRNA or protein expression was induced or upregulated in these cell lines after we treated them with 5-aza-2'-deoxycytidine (Fig. 3). PCDH8 expression was induced after the effect of demethylating agent, which demonstrated the silence of PCDH8 expression in gastric cancer cell lines was due to methylation of PCDH8 gene promoter.

Aberrant hypermethylation of PCDH8 gene promoter in primary gastric carcinomas. To translate this *in vitro* finding into *ex vivo* tissue specimens, MSP analysis of PCDH8 gene promoter was conducted in 65 patients with human gastric carcinoma (42 male and 23 female). MSP analysis showed that methylation of the PCDH8 gene promoter was frequently detected in gastric cancer tissue (55.38%, 36/65) and para-carcinoma tissue of gastric (41.54%, 27/65), but not in normal gastric tissue. Statistically, there was no difference in methylation of the PCDH8 gene promoter between gastric cancer and para-carcinoma tissue. However, there were statistically significant differences between gastric cancer and normal gastric tissue, and between para-carcinoma tissue and normal gastric tissue (Fig. 2B and C, P=0.002 and 0.005, respectively).

Induction of gastric cancer cell apoptosis and inhibiting migration with 5-aza-2'-deoxycytidine treatment. Previous results showed that treatment with 1 μmol/l of 5-aza-2'-deoxycytidine for up to 72 h significantly upregulated expression of PCDH8 in 4 gastric cancer cell lines. To investigate the relation between PCDH8 expression and cell development. We evaluated cell apoptosis and migration with or without PCDH8 expression. Fig. 4 shows that following the 5-aza-2'-deoxycytidine treatment (1 μM for 72 h), FCM demonstrated an obvious increase in Annexin-FITC positive apoptotic tumor cells (apoptosis rate: BCG-823, 3.73±0.30 vs 9.27±0.26%; SGC-7901, 4.82±0.32 vs 10.63±0.26%; MKN-45, 3.92±0.23 and 8.7±2.0%; MKN-28, 5.4±0.21 and 12.92±2.0%; P<0.05) (Fig. 4A). And after the treatment, the average number of migrated cells were obviously decreased in four gastric cancer cell lines [migrated cells ($x10^3$): BCG-823, 33.67±5.38 vs 81.56±6.85; SGC-7901, 38.32±4.25 vs 84.06±7.65; MKN-45, 32.51±3.14, 82.63±7.42; MKN-28, 45.36±6.57, 85.67±6.13; P<0.05] (Fig. 4B). These data suggested that PCDH8 is able to induce gastric cancer cell apoptosis and to inhibit cell migration.

Association of PCDH8 gene promoter methylation with clinicopathological data in gastric cancer patients. To determine the role of PCDH8 methylation status in gastric cancer, we examined the correlation of methylation status with the clinicopathological features. There was no significant difference in the distribution of patients with methylation or unmethylation of PCDH8 in terms of age, sex, distant metastasis, tumor size, or TNM stage. Methylation of the PCDH8 gene was detected in 80.0%

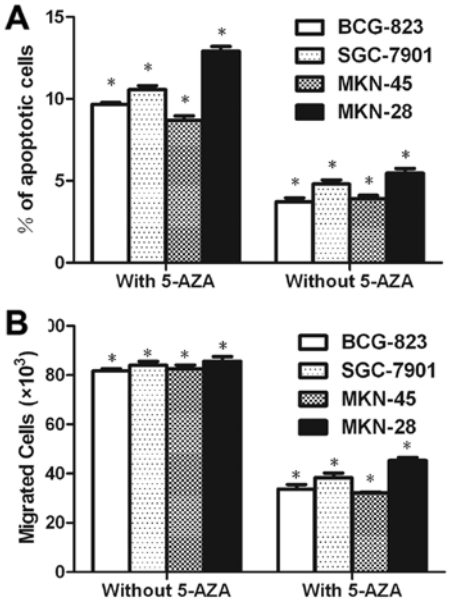

Figure 4. Induction of gastric cancer cell apoptosis and inhibiting migration with 5-aza-2'-deoxycytidine (5-AZA) treatment. Following the 5-AZA treatment (1 μM for 72 h), (A) FCM demonstrated an obvious increase in Annexin-FITC-positive apoptotic tumor cells, and (B) the average migrated cells were obviously decreased in four gastric cancer cell lines. Each value is presented as the mean ± SE of four independent experiments. *P<0.05 was considered as statistically significant with ANOVA followed by Dunnett's test.

(28 of 35) of the patients who did not have lymph node metastasis, whereas it was detected in 27.6 (8 of 29) of the patients who had lymph node metastasis. Methylation of the PCDH8 gene was also detected in 71.0% (22 of 31) of the patients who were moderate/poor, whereas it was not detected in 39.4% (13 of 33) of the patients who were well. Our statistical data showed that the methylated status of the PCDH8 gene was significantly correlated with the lymph node metastasis and tumor differentiation (Table I).

In the multivariate logistic regression analysis with backward selection, independent variables with P<0.05 in the univariate analyses were included. The variables (tumor differentiation and lymph node metastasis) were chosen for multivariate logistic regression analysis. Methylated PCDH8 was significantly associated with lymph node metastasis in a logistic regression analysis (odds ration 9.78, CI 1.12-86.84, P=0.026) (Table II). These results suggest that negative lymph node metastasis was significantly associated with methylated PCDH8.

Discussion

In this study, we determined PCDH8 gene expression and the methylation status of the PCDH8 gene promoter in gastric cancer cells. We found that the expression of PCDH8 mRNA

Table I. Association of PCDH8 gene promoter methylation with clinicopathological data in gastric cancer patients.

Variable	Patients	PCDH8 methylation	PCDH8 unmethylation	P-value
Sex				0.43
Male	42	22	20	
Female	23	14	9	
Age (years)				0.43
≤50	24	14	10	
>50	41	22	19	
Tumor size (cm)				0.24
<5	35	21	14	
≥5	29	15	14	
Tumor differentiation				0.01[a]
Moderate/poor	31	22	9	
Well	33	13	20	
TNM stage				0.68
I-II	23	12	11	
III-IV	39	22	17	
Lymph node metastasis				0.0038[a]
-	29	8	21	
+	35	28	7	
M (distal metastasis)				0.86
M0	60	31	29	
M1	5	5	0	

[a]χ^2 test. P<0.05.

Table II. Significant variables associated with the methylation of PCDH8 gene by the logistic regression analysis.

Variable	Odds ratio	(95% CI)	P-value
Tumor differentiation			0.128
Moderate/poor	1		
Well	3.832	(0.68-21.10)	
Lymph node metastasis			0.026[a]
-	1		
+	9.783	(1.12-86.84)	

[a]P<0.05; CI, confidence interval.

was lost in gastric cancer cells. MSP analysis revealed high methylation of the PCDH8 gene promoter in these tumor cells. 5-aza-2'-deoxycytidine treatment induced PCDH8 expression, but reduced viability of gastric cancer cells. Furthermore, *ex vivo* data demonstrated that the PCDH8 gene promoter is frequently methylated in gastric cancer and the para-carcinoma tissues, but not in normal gastric tissue. Therefore, the PCDH8 gene promoter methylation may be further evaluated as a biomarker for early detection of gastric cancer. Furthermore, methylated PCDH8 was significantly associated with lymph node metastasis in a logistic regression analysis. It also revealed that PCDH8 restrained tumor metastasis *in vivo*.

Inactivation of tumor suppressor genes contributes to cancer development. Such inactivation may be caused by genetic or epigenetic alterations, including gene mutation, deletion, promoter methylation, abnormal splicing, deregulation of imprinting and haploinsufficiency (4). Among these abnormalities, loss of heterozygosity (LOH) was shown to cause inactivation of most candidate tumor suppressor genes in the critical regions of chromosomes 3p, 5q, 8p and 9p (19-22). However, changes in methylation status of these genes also frequently occur. The PCDH8 gene plays an important role in organizing the formation and polarity of developing cellular structures in embryos. PCDH8 can also suppress tumor cell migration and invasion, and induces apoptosis in breast cancer cell lines (17).

PCDH8 is located on chromosome 13q14.3 and is within a cluster of protocadherins (PCDH8, PCDH9, PCDH17 and PCDH20) spanning 13q14-21 that is conserved between humans and mice. It is interesting to note that PCDH20 is methylated and homozygously deleted in lung cancer, and when reintroduced into an altered tumor cell line reduces proliferation (13). PCDH17 is methylated and homozygously deleted in esophageal squamous cell carcinoma (15).

Our present data demonstrated aberrant methylation of PCDH8 gene promoter regions and subsequent loss of PCDH8 expression in gastric cancer cell lines and tumor tissue specimens. These results are consistent with previous studies on other cancers (17). PCDH8 gene promoter is frequently methylated in gastric cancer 55.38% (36/65) and the para-carcinoma tissues 41.54% (27/65), but not in normal gastric tissue which indicated that it is a common feature of gastric cancer and may be the early stage accident in gastric carcinogenesis.

Aberrant promoter hypermethylation has been shown to be a common event in human cancer mainly due to the loss of function of tumor suppressor. This neoplasia-related event is thought to occur early in carcinogenesis, and hence, promoter hypermethylation is being widely studied as a biomarker for the diagnosis and detection of early lesions. In this context, PCDH8 was frequently methylated in gastric carcinoma adjacent tissues but not in normal gastric mucosa. It suggests that PCDH8 methylation may represent the field defect of gastric carcinoma. PCDH8 gene promoter methylation may be an early event in gastric cancer (23-25).

We found that methylation of PCDH8 gene promoter only occurred in gastric cancer but not in normal gastric tissue. 5-aza-2'-deoxycytidine induced expression of PCDH8, which is associated with reduced viability of gastric cells, indicating that PCDH8 plays an important role in suppressing gastric carcinogenesis. However, we cannot rule out whether other tumor suppressor genes are also induced and restored by 5-aza-2'-deoxycytidine, which plays a role in regulation of tumor cell viability. The latter warrants further studies because it has been shown that epigenetic modification of pro-apoptotic genes is one of the mechanisms by which the tumor cells are resistant to chemotherapy (26). Therefore, treatment with a demethylating agent such as 5-aza-2'-deoxycytidine prior to chemotherapy may help improve the therapeutic efficacy for gastric cancer.

In conclusion, silence of PCDH8 expression is achieved through the gene methylation in gastric cancer. PCDH8 can suppress gastric tumorigenesis *in vitro* and *in vivo*. Thus, PCDH8 is a candidate tumor suppressor in gastric cancer. Future studies will evaluate whether PCDH8 gene promoter methylation can be used as a biomarker for the early detection of gastric cancer.

Acknowledgements

The authors acknowledge Dr Xue Yang of the Hepatobiliary Department of First Affiliated Hospital and the Institution of Genetic Disease Research and, Xi'an Jiaotong University for technical assistance.

References

1. Herszényi L and Tulassay Z: Epidemiology of gastrointestinal and liver tumors. Eur Rev Med Pharmacol Sci 14: 249-258, 2010.
2. Brenner H, Rothenbacher D and Arndt V: Epidemiology of stomach cancer. Methods Mol Biol 472: 467-477, 2009.
3. Arkenau HT: Gastric cancer in the era of molecularly targeted agents: current drug development strategies. J Cancer Res Clin Oncol 135: 855-866, 2009.
4. Yasui W, Sentani K, Motoshita J, *et al*: Molecular pathobiology of gastric cancer. Scand J Surg 95: 225-231, 2006.
5. Herman JG and Baylin SB: Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 349: 2042-2054, 2003.
6. Wang JF and Dai DQ: Metastatic suppressor genes inactivated by aberrant methylation in gastric cancer. World J Gastroenterol 13: 5692-5698, 2007.
7. Machado JC, Oliveira C, Carvalho R, *et al*: E-cadherin gene (CDH1) promoter methylation as the second hit in sporadic diffuse gastric carcinoma. Oncology 20: 1525-1528, 2001.
8. Esteller M, Corn PG, Baylin SB, *et al*: A gene hypermethylation profile of human cancer. Cancer Res 61: 3225-3229, 2001.
9. Buffart TE, Overmeer RM, Steenbergen RD, *et al*: MAL promoter hypermethylation as a novel prognostic marker in gastric cancer. Br J Cancer 99: 1802-1807, 2008.

10. Nishikawa Y, Miyazaki T, Nakashiro K, *et al*: Human FAT1 cadherin controls cell migration and invasion of oral squamous cell carcinoma through the localization of β-catenin. Oncol Rep 26: 587-592, 2011.
11. Gumbiner BM: Regulation of cadherin-mediated adhesion in morphogenesis. Nat Rev Mol Cell Biol 6: 622-634, 2005.
12. Batlle E, Sancho E, Franci C *et al*: The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nat Cell Biol 2: 84-89, 2000.
13. Imoto I, Izumi H, Yokoi S, Hosoda H, *et al*: Frequent silencing of the candidate tumor suppressor PCDH20 by epigenetic mechanism in non-small-cell lung cancers. Cancer Res 66: 4617-4626, 2006.
14. Ying J, Li H, Seng TJ, Langford C, *et al*: Functional epigenetics identifies a protocadherin PCDH10 as a candidate tumor suppressor for nasopharyngeal, esophagealand multiple other carcinomas with frequent methylation. Oncogene 25: 1070-1080, 2006.
15. Haruki S, Imoto I, Kozaki K, *et al*: Frequent silencing of protocadherin 17, a candidate tumour suppressor for esophageal squamous-cell carcinoma. Carcinogenesis 31: 1027-1036, 2010.
16. Strehl S, Glatt K, Liu QM, *et al*: Characterization of two novel protocadherins (PCDH8 and PCDH9) localized on human chromosome 13 and mouse chromosome 14. Gemomics 53: 81-89, 1998.
17. Yu JS, Koujak S, Nagase S, *et al*: PCDH8, the human homolog of PAPC, is a candidate tumor suppressor of breast cancer. Oncogene 27: 4657-4665, 2008.
18. Herman JG, Graff JR, Myöhänen S, *et al*: Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 93: 9821-9826, 1996.
19. Gorringe KL, Ramakrishna M, Williams LH, *et al*: Are there any more ovarian tumor suppressor genes? A new perspective using ultra high-resolution copy number and loss of heterozygosity analysis. Genes Chromosomes Cancer 48: 931-942, 2009.
20. Franko J, Krasinskas AM, Nikiforova MN, *et al*: Loss of heterozygosity predicts poor survival after resection of pancreatic adenocarcinoma. J Gastrointest Surg 12: 1664-1672, 2008.
21 Chang YC, Yeh KT, Liu TC, *et al*: Molecular cytogenetic characterization of esophageal cancer detected by comparative genomic hybridization. J Clin Lab Anal 24: 167-174, 2010.
22. Ye Y, McDevitt MA, Guo M, *et al*: Progressive chromatin repression and promoter methylation of CTNNA1 associated with advanced myeloid malignancies. Cancer Res 69: 8482-8490, 2009.
23. Hukriede NA, Tsang TE, Habas R, *et al*: Conserved requirement of Lim1 function for cell movements during gastrulation. Dev Cell 4: 83-94, 2003.
24. Medina A, Swain RK, Kuerner KM and Steinbeisser H: Xenopus paraxial protocadherin has signaling functions and is involved in tissue separation. EMBO J 23: 3249-3258, 2004.
25. Unterseher F, Hefele JA, Giehl K, *et al*: Paraxial protocadherin coordinates cell polarity during convergent extension via Rho A and JNK. EMBO J 23: 3259-3269, 2004.
26. Tian K, Jurukovski V, Wang XP, *et al*: Epigenetic regulation of WTH3 in primary and cultured drugresistant breast cancer cells. Cancer Res 65: 10024-10031, 2005.