

# PGP9.5 Methylation in Diffuse-Type Gastric Cancer

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## Abstract

**Diffuse-type gastric cancer (DGC) is the most deadly form of gastric cancer and is frequently accompanied by peritoneal dissemination and metastasis. The specific molecular events involved in DGC pathogenesis remain elusive. Accumulating evidence of epigenetic inactivation in tumor suppressor genes led us to conduct a comprehensive screen to identify novel methylated genes in human cancers using pharmacologic unmasking and subsequent microarray analysis. We compared differential RNA expression profiles of DGC and intestinal-type gastric cancer (IGC) cell lines treated with 5-aza-2'-deoxycytidine using microarrays containing 22,284 genes. We identified 16 methylated genes, including many novel genes, in DGC cell lines and studied *PGP9.5* with particular interest. In primary gastric cancers, *PGP9.5* was found to be more frequently methylated in DGCs (78%) than in IGCs (36%; DGC versus IGC,  $P < 0.05$ ). Furthermore, real-time methylation-specific PCR analysis of *PGP9.5* showed relatively higher methylation levels in DGC than in IGC. Our data thus implicate a molecular event common in the DGC phenotype compared with IGC.** (Cancer Res 2006; 66(7): 3921-7)

## Introduction

Uncontrollable tumor invasion and dissemination of cancer cells around the primary organ is the neoplastic process responsible for most deaths from cancer due to inadequacy of surgical removal (1). Invasive and metastatic cancer cells have undergone numerous genetic and epigenetic changes, manifested by cytoskeletal changes, loss of adhesion, and expression of proteolytic enzymes that degrade the basement membrane (2). Among gastric cancers, diffuse-type gastric cancers (DGC) exhibit a higher frequency of invasion with dissemination to the peritoneum and lymph node metastasis compared with intestinal gastric carcinomas (IGC; refs. 3, 4). In addition, signet ring cells, which are found in DGC, show marked morphologic and phenotypic alterations from non-signet ring gastric cancer cells, such as anchorage-independent growth, resistance to cellular adhesion, and resistance to apoptosis

(5–8). Much remains to be learned about the transition from normal gastric epithelial cells to cells capable of invading surrounding tissues and metastasis. Moreover, further progress in the treatment or prevention of DGC is contingent upon identifying novel genes and pathways that are consistently and specifically altered in disseminated cells.

Differences in the clinicopathologic features between IGC and DGC have led investigators to believe that two distinct pathways are involved in their pathogenesis. Using Lauren's approach to histologic classification (DGC versus IGC), the average age of onset for DGC was reported to be 56 years, 10 years earlier than for IGC (4, 9, 10), and there was a significantly higher percentage of DGC without associated (atrophic) gastritis, a presumed causal lesion of IGC (9). The rate of IGC is more than double in men than in women; however, there is no difference in the rate of DGC between the sexes (4, 10, 11). Genetic predisposition to gastric cancer is also more commonly associated with DGC than IGC (4, 12).

*E-cadherin* is somatically mutated in ~50% of sporadic DGC cases but not in IGCs (13), and germ line mutations have been found in familial DGCs (14). Interestingly, the second hit in *E-cadherin* germ line mutation carriers is generally due to methylation (15, 16). Furthermore, *E-cadherin* was recently found to be methylated more frequently in sporadic DGCs; 80% of DGC cases harbored methylation, whereas IGCs displayed methylation in only 30% of cases (17). However, *E-cadherin* is still inactivated in a substantial number of IGC tumors and other cancers with completely different morphologies (17, 18); thus, inactivation of *E-cadherin* is unlikely to be the exclusive determinant of the DGC-specific phenotype.

Recently, hypermethylation of gene promoters has been explored as both a mechanism and marker of carcinoma progression (19–22). We have had great success in identifying novel cancer-specific methylated genes by pharmacologic unmasking [5-aza-2'-deoxycytidine (5Aza-dC) treatment] and subsequent microarray (PUM) analysis for esophageal and head and neck squamous cell carcinomas (HNSCC; refs. 23, 24). In this study, we applied this novel approach, using differential PUM between DGC and IGC, to identify novel methylated genes more specifically involved in the formation of DGC.

## Materials and Methods

**Cell lines and tissue samples.** The following gastric cancer cell lines were used: NUGC3 (undifferentiated DGC), NUGC4 (signet ring cell DGC), KATOIII (signet ring cell DGC), AZ521 (highly differentiated IGC), and MKN7 (highly differentiated IGC). These cell lines were obtained from the Cell Response Center for Biomedical Research Institute in the Department of Aging and Cancer, Tohoku University. Cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum for isolation of DNA and RNA.

Thirty-one pairs of primary gastric cancers and their corresponding adjacent normal tissue specimens were obtained from patients who had

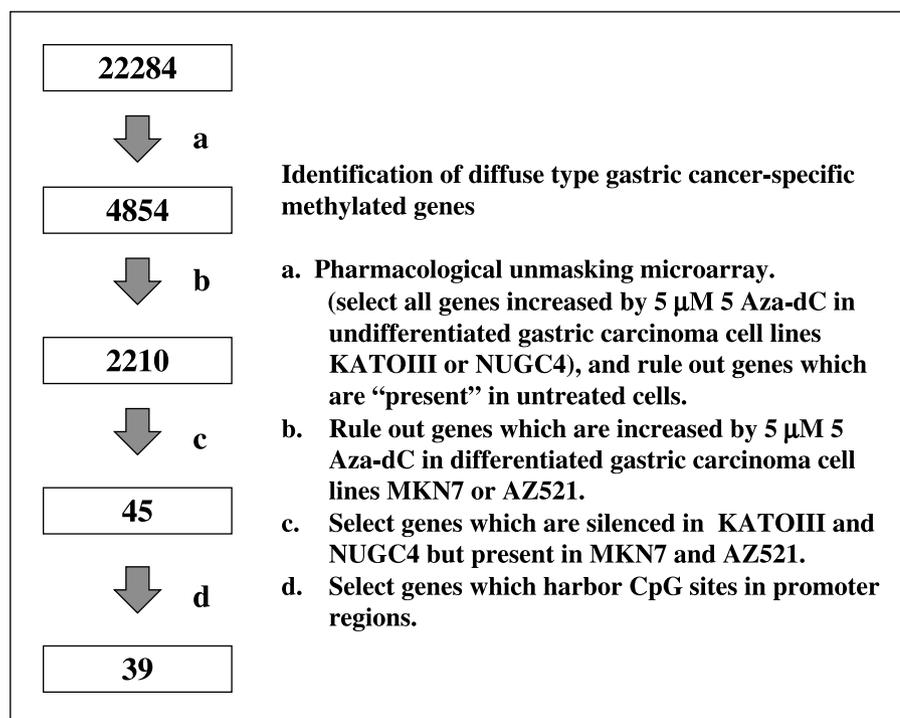
**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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**Figure 1.** Flowchart for selection of candidate tumor suppressor genes. To screen for candidate markers specific to DGC, we treated four gastric cancer cell lines with 5 μmol/L 5Aza-dC and did microarray analysis using microarrays containing 22,284 genes. We obtained 4,854 candidates that showed absence of expression in any untreated DGC cell line and an increase in expression after treatment. We diminished the number of candidates by ruling out genes also increased in IGC cell lines, and 2,210 genes remained. We further reduced the number of candidate genes by only choosing genes with restricted expression profiles (see text), and 45 genes remained. We finally selected 39 genes that contained CpG islands to examine for methylation analysis.

undergone surgery in the Department of Surgical Oncology at the Medical Institute of Bioregulation, Kyushu University and the Department of Surgery at Oita Prefectural Hospital. The 40 cases were composed of 18 DGCs and 22 IGCs. Specimens were obtained from tumors, avoiding necrotic centers, immediately after resection. Corresponding normal mucosa specimens, which were at least 5 cm away from the tumor edge, were also obtained by sharply dissecting the mucosa off the muscularis propria. All specimens were quick frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until processing.

**Pharmacologic unmasking (5AzadC treatment) of gastric cancer cells.** Cells were split to low density ( $1 \times 10^6$  per T-75 flask) 12 to 24 hours before treatment. Cells were then treated for 4 days with 5 μmol/L 5Aza-dC (Sigma, St. Louis, MO) dissolved in 50% acetic acid/PBS or were mock treated with the same amount of acetic acid/PBS as done previously (23).

**Microarray and reverse transcription-PCR analysis.** We did oligonucleotide microarray analysis on the GeneChip Human Genome U133A Array (Affymetrix, Santa Clara, CA) containing 22,284 genes as per the

**Table 1.** Promoter methylation in DGC cell lines

Gene no.	Gene name	CpG*	Chromosomal	NUGC3	KATOIII	NUGC4	AZ521	MKN7	Methods
1	NM_004181 <i>PGP9.5</i>	(+)	4p14	M	M	M	U	U	SQ
2	AL136939 <i>Homologue of yeast LCEAE</i>	(+)	6p21	U	M	M	M	M/U	SQ
3	NM_024576 <i>FLJ21079 (Opioid receptor-like)</i>	(+?)	6q13	M	M	U	U	U	SQ
4	M60485 <i>Fibroblast growth factor receptor (FGFR)</i>	(+)	8p11	U	M	M/U	M/U	U	MSP
5	NM_023929 <i>Zinc finger protein RINZF</i>	(+)	8q13-21	U	M	U	U	U	SQ
6	U38945 <i>p16</i>	(+)	9p22	M	M	D	M	U	SQ
7	NM_000170 <i>Glycine dehydrogenase</i>	(+)	9p22	D	M	M	U	U	SQ
8	NM_014368 <i>LIM-6</i>	(+)	9q34	M/U	M	U	U	U	MSP
9	AL157398 <i>Nebulette</i>	(+)	10p12	M/U	M	U	U	U	MSP
10	NM_002906 <i>Radixin</i>	(+?)	11q23	U	M	U	U	U	SQ
11	NM_014333 <i>TLSC1</i>	(+)	11q23	U	M	M	U	M/U	SQ
12	NM_006931 <i>GLUT3 (SLC14)</i>	(+)	12p13	M	M	M	M	M	SQ
13	NM_025083 <i>FLJ21128</i>	(+)	15q23-24	U	M	U	U	U	SQ
14	AI358867 <i>Apolipoprotein E</i>	(+)	19q13	D	M	M	D	U	SQ
15	NM_003098 <i>Syntrophin α 1</i>	(+)	20q11	D	M	D	U	U	SQ
16	NM_014258 <i>Synaptonemal complex protein 2</i>	(+?)	20q13	M	M	M	M	M	SQ

Abbreviations: M, methylated; U, unmethylated; D, homozygous deletion or difficult to amplify DNA; SQ, sequencing after bisulfite treatment; MSP, methylation specific PCR.

\*(+), dense CpG close to transcription start site; (+?), dense CpG island far from transcription start site (>2 kb).

manufacturer's instruction and identified genes up-regulated by pharmacologic treatment according to the manufacturer's algorithm. We isolated total RNA using Qiazol (Invitrogen, Carlsbad, CA), reverse-transcribed total RNA (8 µg) with Moloney murine leukemia virus (Invitrogen), and used one hundredth of the cDNA as a template for PCR. Reverse transcription-PCR (RT-PCR) was done for 24 to 30 cycles of 95°C for 1 minute, 54°C or 56°C for 1 minute, and 72°C for 1 minute or by touch-down PCR, depending on the gene. Primer sequences are available on request.

**Bisulfite treatment of DNA.** We extracted genomic DNA from Qiazol-treated samples and did bisulfite modification of genomic DNA as described (23). For DNA denaturing, 2 µg of genomic DNA were incubated with 5 µg salmon sperm DNA (Sigma) in 0.3 mol/L NaOH for 20 minutes at 50°C. The DNA sample was then diluted with 500 µL of a 2.5 mol/L sodium metabisulfite/125 mmol/L hydroquinone/0.4 mol/L sodium hydroxide solution and placed at 70°C for 1 hour. The sample was then applied to a column (Wizard DNA Clean Up System, Promega, Inc., Madison, WI), incubated with 0.3 mol/L NaOH for 10 minutes, and treated with 3 mol/L ammonium acetate for 5 minutes; 2.5-fold volume of 100% ethanol was added, and DNA was allowed to precipitate for 1 hour at room temperature. DNA was resuspended in 100 µL LoTE composed of 10 µmol/L Tris-HCl, pH 8 (Quality Biological, Inc., Gaithersburg, MD) and 2.5 µmol/L EDTA, pH 8 (Invitrogen) and stored at -80°C.

**PCR amplification of bisulfite-treated DNA for sequencing.** The primers were designed to recognize DNA alterations caused by the bisulfite treatment. Oligonucleotide primer pairs were purchased from Invitrogen. PCR amplifications were done as follows: a 5-minute 95°C incubation step followed by 45 cycles of 1 minute at 95°C, 1 minute at 54°C, and 2 minutes at 72°C. A 7-minute elongation step at 72°C completed the PCR amplification program. Primer sequences are listed in Supplementary Table S1.

**Methylation-specific PCR.** Bisulfite-treated DNA was amplified with either a methylation-specific or unmethylated-specific primer set for LIM homeobox protein 6 (*LIM-6*), *FGFR*, and *Nebulette*. The primers for methylated *LIM-6* were 5'-ACGACGAAACCGACGCTCG-3' and 5'-GGCGGGTCTGTTTTTCGTCG-3'. The primers specific for unmethylated *LIM-6* were 5'-CCAAAACAACAAACCAACACCTCA-3' and 5'-TTGGTGG-TGGGGTTGTTTTTGGTTG-3'. The primers specific for methylated *FGFR* were 5'-GACGCATAACGCTCGAAACG-3' and 5'-TAGCGGCGCG-TTCGCGTTCG-3'. The primers specific for unmethylated *FGFR* were 5'-CCACCAACACATAACTCAAAACA-3' and 5'-TTAAGTAGTGGTGT-GTTGTGGTTG-3'. The primers specific for methylated *Nebulette* were 5'-CGCGAACGAAACGCCAACG-3' and 5'-GGAGGGGGCGGGTTCGTCG-3'. The primers specific for unmethylated *Nebulette* were 5'-AAACACA-CAAACAAAACACCAACA-3' and 5'-GGAGGGGGTGTGTTTGTGTTG-3'. PCR reactions were done for 35 cycles of 95°C for 30 seconds, 59°C to 61°C for 30 seconds, and 72°C for 30 seconds.

**Real-time quantitative methylation-specific PCR of bisulfite-treated DNA.** For Taqman methylation-specific PCR (MSP), fluorescent probe and primer sets were designed to hybridize to the amplified region of DNA (25). The *β-actin* primer sequences were used as an internal control and previously described (24). For all reactions, 3 µL of bisulfite-treated DNA were added to a final volume of 20 µL. Serial dilutions of *in vitro* methylated human leukocyte DNA were used to construct a calibration curve, and all reactions were done in duplicate. The methylation ratio was defined as quantity of fluorescence intensity derived from *PGP9.5* promoter amplification divided by fluorescence intensity from *β-actin* amplification, multiplied by 100 (we designated this value as the Taqman methylation value: TaqMeth V).

## Results

### Differential PUM analysis between DGC and IGC cell lines.

We did a comprehensive survey for DGC-specific tumor suppressor gene candidates by comparing mock-treated and 5Aza-dC-treated gastric cancer cells. Our analysis included four cell lines, two derived from DGCs (NUGC4 and KATOIII, both signet ring cell

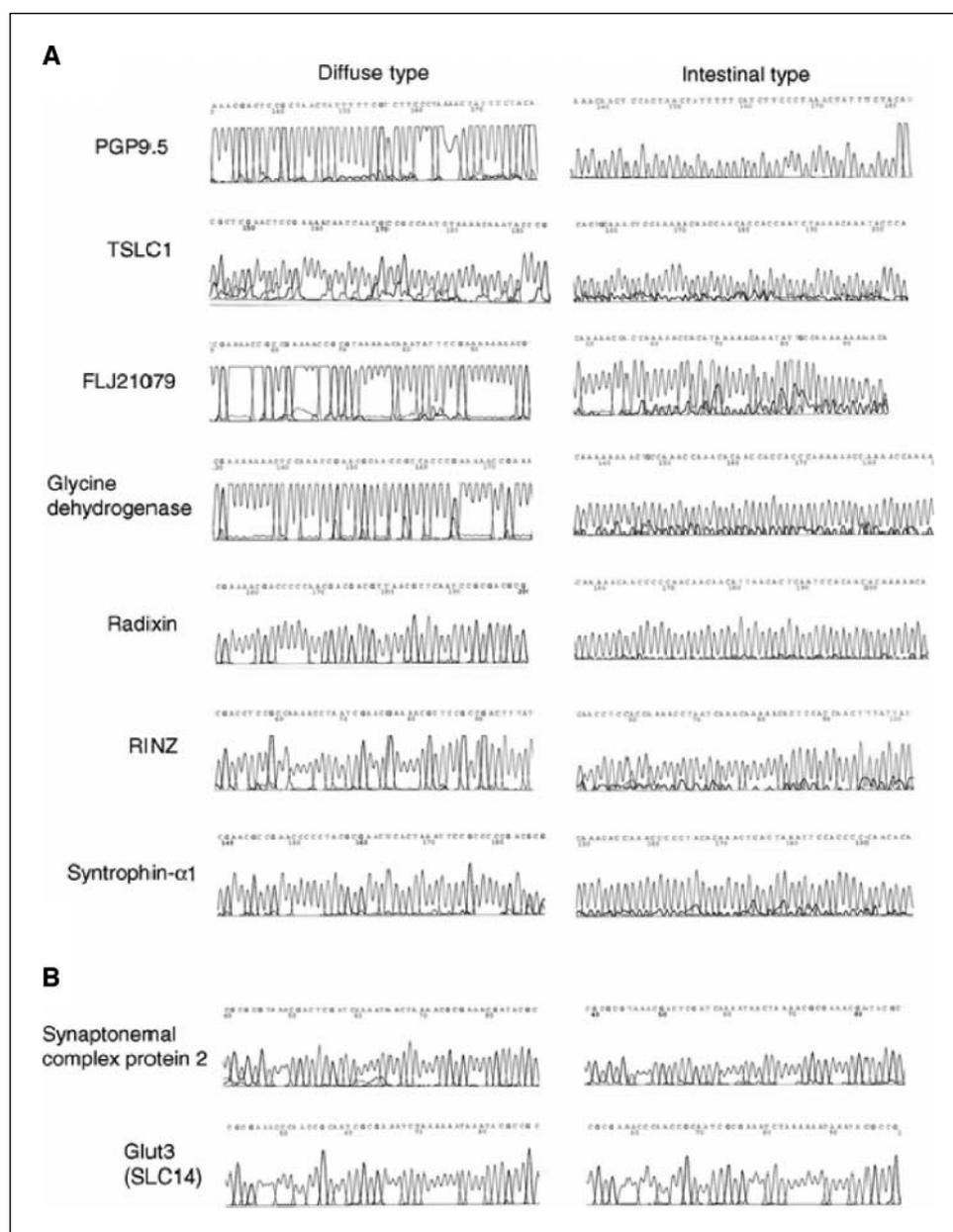
carcinomas) and two derived from IGCs (AZ521 and MKN7, both highly differentiated adenocarcinomas). We previously found that 5 µmol/L 5Aza-dC treatment resulted in reexpression of >85% of silenced transcripts also identified by more complex treatments, including either a lower dose of 5Aza-dC alone or in combination with trichostatin A (23). Thus, after treatment with 5 µmol/L 5Aza-dC for 4 days, isolated cell line RNA was hybridized to Affymetrix microarrays containing 22,284 transcripts. This procedure was nearly identical to that used in our previous studies, but the arrays used in our present study include more genes than previously contained in our esophageal squamous cell carcinoma (ESCC; ref. 23) and HNSCC (24) studies.

Complete silencing of expression is characteristic of methylated genes (23, 24); we, therefore, removed genes showing "present" expression in all four cell lines before pharmacologic treatment. After ruling out such genes, 4,854 unique genes remained, which were significantly up-regulated in at least one of the two DGC cell lines treated with 5 µmol/L 5Aza-dC compared with mock-treated cells (Fig. 1). To produce a more DGC-specific gene list, we further ruled out genes increased after pharmacologic unmasking of IGC cell lines, and 2,210 genes remained. We finally selected 45 genes that exhibited a profile of complete absence in the DGC cell lines and expression in the IGC cell lines, because putative DGC-specific tumor suppressor genes are likely to be more relevant if they are completely silenced in DGCs. Among the 45 remaining genes, 38 genes (84.4%) harbored CpG islands in their promoters by visual examination.

**Identification of genes more frequently methylated in DGC cell lines.** From the 38 genes examined for promoter DNA methylation, we identified 16 methylated genes in gastric cancer cell lines by using bisulfite DNA sequencing or MSP (Table 1). For methylation analysis, we added one more DGC cell line (NUGC3), which is not a signet ring cell carcinoma but an undifferentiated adenocarcinoma. Representative methylation is shown in Fig. 2A and B. Among the 16 genes, the methylation status of 14 genes was generally consistent with their gene expression profile in microarray (Table 1). The remaining two genes were *GLUT3 (SLC14)* and *Synaptonemal complex protein 2*, both of which were methylated in all five gastric cancer cell lines tested (Fig. 2B).

*PGP9.5* was the only gene specifically methylated in all three DGC cell lines but not in the two IGC cell lines (Table 1). Nine genes were also found to be methylated specifically in DGC cell lines, including *FLJ21079 (Opioid receptor-like)*, *Zinc finger protein RINZF*, *Glycine dehydrogenase*, *LIM-6*, *Nebulette*, *Radixin*, *FLJ21128*, *Apolipoprotein E*, and *Syntrophin α1* (Fig. 2A and C; Table 1). Thus, these 10 genes showed DGC-specific methylation in our cell line analysis. The remaining four genes (*Homologue of yeast LCEAE*, *FGFR*, *p16*, and *TSLC1*) were not specifically methylated in DGC cell lines but may still be involved in DGC pathogenesis. By RT-PCR, these latter genes were silenced in expression in DGC, reactivated by pharmacologic unmasking, and present in IGC before treatment (Fig. 3).

**PGP9.5 is more frequently methylated in DGC cell lines and primary DGC tumors.** Among these genes, *PGP9.5* and *TSLC1* were of particular interest because *PGP9.5* was the only gene methylated specifically in all the DGC cell lines tested, and *TSLC1* was the only gene that was completely silenced in signet ring cell carcinoma cell lines (NUGC4 and KATOIII) yet expressed in both IGC cell lines (Fig. 3). Previous analyses have revealed that methylation patterns of many genes in primary cancer tissues are not necessarily consistent with those in cancer cell lines; thus, we examined the methylation



**Figure 2.** Promoter methylation analysis of several candidate markers specific to DGC. *A*, representative examples of direct sequencing analysis of bisulfite-treated DNA from DGC and IGC cell lines. *PGP9.5* (NUGC3 and AZ521), *TSLC1* (KATOIII and MKN7), *FLJ21079* (NUGC3 and MKN7), *Glycine dehydrogenase* (KATOIII and MKN7), *Radixin* (KATOIII and MKN7), *RINZ* (KATOIII and MKN7), and *Syntrophin alpha 1* (KATOIII and MKN7) in DGC (*left*) and IGC (*right*). All guanines (*black peaks*) present after sequencing are derived from methyl cytosines on the complementary strand. *B*, methylation of two genes was not specific to DGC cell lines.

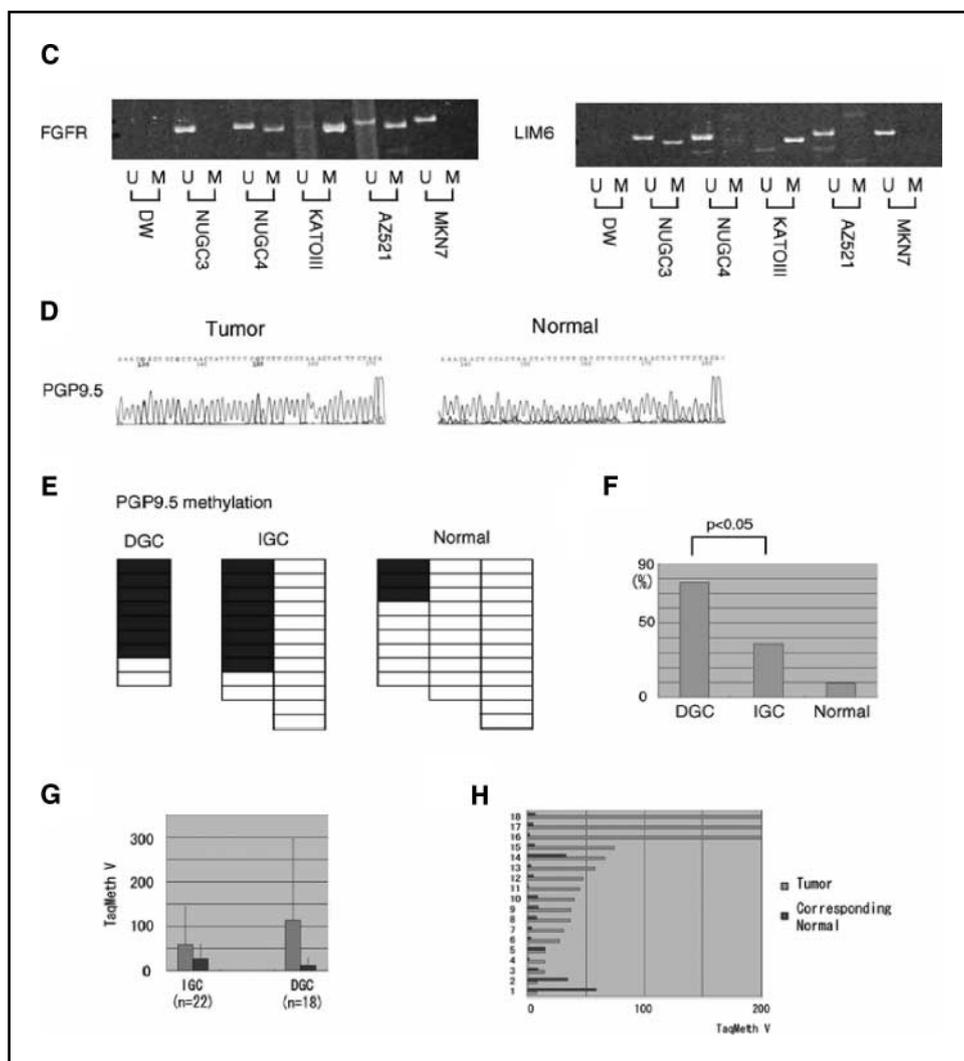
status of *PGP9.5* and *TSLC1* by direct sequencing after bisulfite treatment in primary gastric cancer tissue specimens comprised of DGCs ( $n = 9$ ) and IGCs ( $n = 22$ ), as well as their corresponding normal gastric epithelium ( $n = 31$ ). For *PGP9.5*, there was little methylation in the adjacent normal epithelium from these cases (3 of 31 cases, 9.7%), some methylation in IGC specimens (8 of 22 cases, 36.4%), and significantly more frequent methylation in DGC (7 of 9 cases, 77.8%; IGC versus DGC,  $P < 0.05$ ; Fig. 2*E* and *F*). Representative results of *PGP9.5* bisulfite sequencing for primary cancer and normal adjacent tissues are shown in Fig. 2*D*. By quantitative analysis using real-time MSP, *PGP9.5* methylation levels were higher in DGCs ( $n = 18$ ) than in IGCs ( $n = 22$ ; Fig. 2*G*). On the other hand, *TSLC1* methylation did not show any specificity in primary DGC tissue specimens (23.8% versus 20% in DGCs versus IGCs, respectively), consistent with our cell line data and the previously published data in gastric cancers (26).

## Discussion

At the time of diagnosis, DGCs generally exhibit features of a more advanced stage, including more frequent peritoneal dissemination, lymph node metastasis, and nerve permeation, than IGCs (3, 4). Clinical studies have shown that DGCs have significantly worse prognosis than IGCs when considering the same pT (pT<sub>3</sub>), sex (male), and age group. Moreover, a worse prognosis for DGC is influenced more by the incidence of regional lymph node involvement than by any other factors (27, 28). Although no difference in incidences of proximal gastric adenocarcinoma and distal adenocarcinoma has been seen, residual tumor was more frequently associated with DGCs ( $P < 0.01$ ; ref. 29). These data suggest the existence of different pathogenic processes for these two histologic subtypes of gastric cancers.

No differences in classic tumor markers, such as carcinoembryonic antigen and  $\alpha$ -fetoprotein, were found between DGC and

**Figure 2.** *Continued.* **C**, for genes that could not be analyzed by direct sequencing, we did conventional MSP to determine methylation status. MSP results for *FGFR* and *LIM-6* in five gastric cancer cell lines. *DW*, distilled water. **D**, *PGP9.5* methylation in primary DGC and corresponding normal mucosa tissue specimens. This region is the same as that examined in the cell lines shown in (A). **E**, methylation analysis for *PGP9.5* and *TSLC1* in primary gastric tumors and corresponding adjacent normal tissue. Green boxes indicate methylation of the promoter region. Nine DGCs, 22 IGCs, and 31 corresponding adjacent normal tissue specimens were examined. **F**, *PGP9.5* methylation is more commonly found in DGC (78%) than in IGC tissues (38%,  $P < 0.05$ ). **G**, quantitative evaluation of *PGP9.5* methylation levels showed that *PGP9.5* methylation (TaqMeth V) was higher in DGCs ( $n = 18$ ) than in IGCs ( $n = 22$ ). Methylation levels of the corresponding adjacent normal tissues (black bars). **H**, DGC tissues showed higher hypermethylation levels of the *PGP9.5* promoter region compared to the corresponding normal tissues by quantitative real-time MSP analysis.



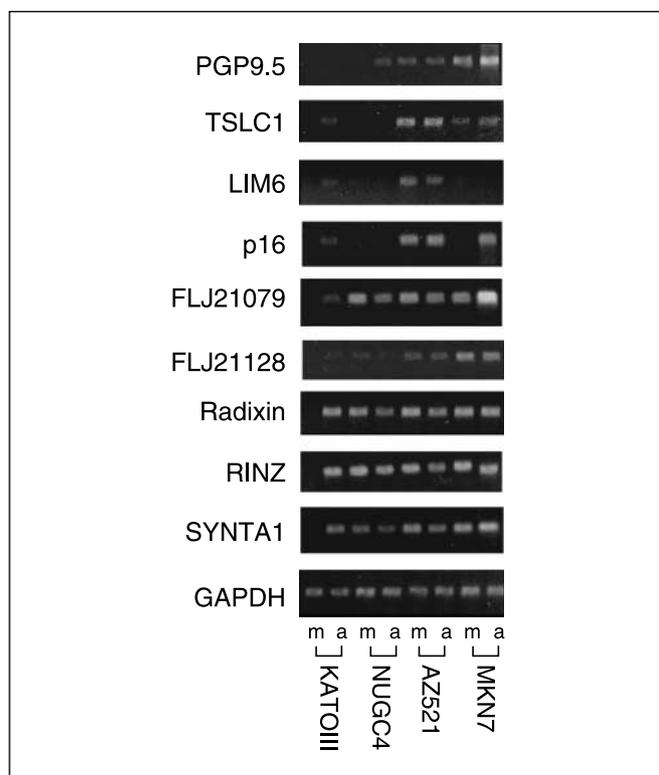
IGC (30). On the other hand, there have been reports on a number of genes that exhibit differential expression in DGCs and IGCs, such as M1, a mucin antigen (31); HLA-DR antigen (32); p53 protein accumulation (33, 34); and insulin-like growth factor-2 (35). Epigenetic events are one of the most upstream alterations, which could result in changes in expression of multiple genes, and there have been few reports of epigenetic differences between DGC and IGC, such as *E-cadherin* (17). In the current study, we found that *PGP9.5* exhibits more frequent methylation in DGCs than in IGCs and thus may be involved in DGC pathogenesis.

Less frequent methylation of *PGP9.5* was also found in the corresponding normal tissue (9.7%) as well as in IGC tumors (36.4%). Detection of *PGP9.5* methylation in matched adjacent normal-appearing tissue likely reflects surrounding field effects in patients with cancer. Normal tissue from patients without cancer remains to be tested in gastric cancer but is likely to be free of *PGP9.5* methylation (24). The level of specificity of *PGP9.5* methylation in primary DGC is similar to that of *E-cadherin*, which is also more frequently methylated in DGC, but not strictly specific (80% and 30%; ref. 17). Methylation of *E-cadherin* is also sometimes present in normal-appearing mucosa (18), whereas *E-cadherin* mutation is only seen in cancer (50% in DGC and absent in both IGC and normal tissues; ref. 13).

*PGP9.5* has recently been reported as an onco-related molecule and potential tumor suppressor gene. *PGP9.5* was identified as a cancer-specific methylated gene in pancreatic cancer (36) and HNSCC by using PUM (24). In pancreatic cancer, *PGP9.5* was found to be methylated in almost all the cases (100%). Recently, we also profiled the methylation status of *PGP9.5* in ESCCs by real-time MSP and determined that its methylation was clearly associated with worse prognosis in ESCC ( $P = 0.01$ ; ref. 25). Thus, *PGP9.5* may serve as a biomarker of more aggressive disease. We recently obtained functional evidence that *PGP9.5* inhibits log phase cell growth in culture and anchorage-independent growth and promotes apoptosis.<sup>3</sup> Hence, there is increasing evidence that *PGP9.5* is both a tumor suppressor gene and a useful biomarker for certain cancers.

Promoter methylation for six genes (*FGFR*, *TSLC1*, *GLUT3*, *Synaptonemal complex protein 2*, *Homologue of yeast LCF4E*, and *p16*) was not specifically methylated in DGC cell lines. Some of these genes could represent artifacts of the screening process in which differences in expression are exaggerated due to technical issues. Two genes [*GLUT3* (*SLC14*) and *Synaptonemal complex*

<sup>3</sup> In preparation.



**Figure 3.** Pharmacologically reactivated genes identified by microarray and selected by the algorithm outlined in Fig. 1. These genes are candidates for specific silencing in DGC. *GAPDH* expression was used to confirm equal loading in each lane. *m*, mock, acetic acid/PBS; *a*, 5Aza-dC in the same volume of acetic acid/PBS.

*protein 2*] were methylated in all five gastric cancer cell lines tested (Fig. 2B), suggesting that the promoter regions we selected for investigating methylation are not critical for regulating gene expression. *AP-2*  $\alpha$  and *trypsinogen* are such examples in which methylation of precise regions of promoter DNA correlates with gene expression status despite ubiquitous methylation of adjacent promoter regions (37, 38). In these cases, further analysis through the whole promoter region is needed to identify the exact region involved in regulating gene expression.

Surprisingly, *TSLC1* was not robustly reactivated in both DGC cell lines (slight reactivation in KATO III). This suggests differential sensitivity of the *TSLC1* promoter to demethylating agents or that other events (not necessarily epigenetic events) may also be involved in silencing *TSLC1* expression. In the DGC cell line KATO III, E-cadherin was previously found to be abrogated by gene mutation (39); it is possible that *TSLC1* expression may be influenced by the E-cadherin pathway. On the other hand, for the

other two genes (*p16* and *Homologue of yeast LCF1E*), we observed complete methylation of promoter CpG islands but abundant expression in AZ521 cells. This lack of correlation indicates that methylation is not always sufficient to cause gene silencing in cancer. For example, histone acetylation is an alternate mechanism that regulates gene expression, and synergistic suppression is well known to be required for complete silencing of several tumor suppressor genes (40). Our observation that *p16* is up-regulated by 5Aza-dC but is unmethylated in MKN7 cells suggests that *p16* may also, in some cases, be a downstream target of a methylated gene that is reactivated with treatment.

Further experiments will be required to definitively determine the molecular mechanisms through which *PGP9.5* affects the phenotype of DGC, but one of the most important ramifications of our current observation concerns its diagnostic potential. MSP after candidate gene identification allows timely and robust analysis of primary tumors and constitutes a promising molecular detection approach (18, 41–46). *PGP9.5* methylation is not strictly specific to DGCs; we observed that IGCs and corresponding normal tissues also harbored methylation but at a less frequent rate than in DGCs. In addition, background methylation in the corresponding normal tissues was lower in DGCs than in IGCs, which resulted in a more remarkable contrast between DGC cancer tissues and their corresponding normal tissues compared with IGCs (Fig. 2G). This difference in background methylation may be due to the difference in age distribution between patients with DGC and IGC. With its high sensitivity and specificity in primary DGC, *PGP9.5* methylation has potential to be developed as a biomarker to detect DGC in bodily fluids.

Finally, we showed in this study that differential PUM is a powerful tool to identify specific disease-related methylation events. This strategy enabled us to identify *PGP9.5* as a gene that is more frequently methylated in DGC than in IGC. If methylation events are involved in the process of developing resistance against chemotherapy and radiotherapy in cancer cells, this approach may be applied to identify novel biomarkers of resistance against such cancer therapies and could aid in determining the best treatment approach for individual patients. In addition, discovery of novel methylated genes, like *PGP9.5*, will further contribute to our understanding of multiple methylation events and their roles in the biological progression of human cancers.

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