Protein arginine methyltransferase 5 is associated with malignant phenotype and peritoneal metastasis in gastric cancer

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Abstract. Identification of novel gastric cancer (GC)-related molecules is necessary to improve management of patients with GC in both diagnostic and therapeutic aspects. The aim of the present study was to determine whether protein arginine methyltransferase 5 (*PRMT5*) acts as an oncogene in the progression of GC and whether it serves as a novel diagnostic marker and therapeutic target. We conducted global expression profiling of GC cell lines and RNA interference experiments to evaluate the effect of *PRMT5* expression on the phenotype of GC cells. We analysed tissues of 179 patients with GC to assess the association of *PRMT5* mRNA levels with clinicopathological factors. Differential expression of *PRMT5* mRNA by GC cell lines correlated positively with the levels of *GEMIN2*, *STAT3* and *TGFB3*. *PRMT5* knockdown reduced the proliferation, invasion and migration of a GC cell line. *PRMT5* mRNA levels were significantly higher in GC tissues than the corresponding adjacent normal tissues and were independent of tumour depth, differentiation and lymph node metastasis. High *PRMT5* expression was an independent risk factor of positive peritoneal lavage cytology (odds ratio 3.90, P=0.003) and decreased survival. *PRMT5* enhances the malignant phenotype of GC cell lines and its expression in gastric tissues may serve as a biomarker for patient stratification and a potential target of therapy.

Introduction

Although the incidence of gastric cancer (GC) is declining, it is the fifth most common cancer after cancers of the lung, breast, colon and prostate, and GC is the third leading cause of cancer deaths (1-3). Although palliative chemotherapy and surgery are beneficial, the outcome of GC remains dismal (4-6). The high mortality of GC is explained by our poor understanding of its mechanism of progression and the lack of suitable diagnostic markers that hinder diagnosis before the disease reaches an advanced stage (7,8). GC represents a biologically and genetically heterogeneous group of tumours that are induced by multiple factors that deregulate cell signalling pathways, which leads to the acquisition of malignant phenotypes such as increased cell proliferation, inhibition of apoptosis and enhanced invasiveness (9-11). Identification of novel molecules is therefore required to improve diagnosis and therapy.

Protein arginine methyltransferase 5 (PRMT5) catalyses the symmetrical dimethylation of arginine residues of histone and non-histone substrates (12-14). Because *PRMT5* is implicated in diverse cellular and biological processes such as transcriptional regulation, RNA metabolism, ribosome biogenesis, Golgi apparatus homeostasis and cell cycle regulation, *PRMT5* is generating increased interest in the field of cancer research (12,15,16). For example, *PRMT5* is expressed at higher levels compared with normal tissues in leukemias, lymphomas and gliomas as well as in ovarian, breast, prostate and lung cancers (13,14,17-20). In contrast, the expression levels of *PRMT5* in gastroenterological malignancies and the biological function of *PRMT5* remain to be determined.

To address these questions, we hypothesized that *PRMT5* is linked to the malignant phenotype of GC. The aim of the present study was to determine whether *PRMT5* acts as an oncogene in GC. This study provides insight into the contribution of *PRMT5* to the malignant behavior of GC, and provides a rationale for targeting this enzyme in GC.

Materials and methods

Sample collection. Ten GC cell lines (AGS, KATOIII, MKN1, MKN45, MKN74, N87, NUGC2, NUGC3, NUGC4 and SC-6-JCK) and the non-tumourigenic epithelial cell line (FHs74) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA), Tohoku University, Japan, or were established at our institute. Cell lines were

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cultured at 37˚C in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) in an atmosphere containing 5% CO_2 (21). Primary GC tissues and the corresponding non-cancerous adjacent tissues were collected from 179 patients who underwent gastric resection for GC without neoadjuvant therapy at Nagoya University Hospital between 2001 and 2010. The tissue samples were immediately frozen in liquid nitrogen and stored at -80˚C. The specimens were classified histologically according to the 7th edition of the Union for International Cancer Control (UICC) classification system. Since 2006, adjuvant chemotherapy using S-1 (an oral fluorinated pyrimidine) is used to treat all patients with UICC stage II/III GC unless contraindicated by the patient's condition (22-24). This study conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki, Ethical Principles for Medical Research Involving Human Subjects. Written informed consent for the use of clinical samples and data, as required by the institutional review board at Nagoya University, Japan, was obtained from all patients.

Analysis of PRMT5 mRNA expression. PRMT5 mRNA levels were determined using a quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) assay. Total RNAs (10 μg per sample), which were isolated from 12 cell lines and 179 primary GC tissues as well as the corresponding non-cancerous adjacent tissues, were used to generate cDNAs. The cDNAs were amplified using PCR primers specific for *PRMT5* as follows: sense 5'-TCTCATGGTTTCCCATCC TC-3' in exon 16 and antisense 5'-CCTTCTTGGAATTGCT GCAT-3' in exon 17, which amplify a 102-bp product. The RT-PCR amplification reaction was performed as follows: initial denaturation at 95˚C for 10 min, 40 cycles at 95˚C for 10 sec and at 60˚C for 30 sec. All samples were tested in triplicate, and samples without template were included in each PCR plate as negative controls. A SYBR-Green PCR Core reagents kit (Applied Biosystems, Foster City, CA, USA) was used to perform qRT-PCR, and real-time detection of the SYBR-Green fluorescence emission intensity was conducted using an ABI StepOnePlus Real-Time PCR system (Applied Biosystems). The levels of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA were quantified in each sample for standardization. The expression level of each sample was calculated as the value of *PRMT5* mRNA divided by that of *GAPDH* mRNA (25).

PCR array analysis of differential gene expression. To analyse gene expression by 10 GC cell lines and the FHs74 cell line, we used the Human Epithelial to Mesenchymal Transition (EMT) RT^2 Profiler PCR Array (Qiagen, Hilden, Germany) of 84 key genes, including those that encode the proteins as follows: transcription factors, extracellular matrix proteins as well as proteins involved in the EMT, cell differentiation, morphogenesis, growth, proliferation, migration, cytoskeleton and major signalling pathways (26).

Knockdown of PRMT5 expression using a small interfering RNA (siRNA). NUGC3 and AGS cells were cultured in a 24-well plate ($5x10^4$ cells ml^{-1}). Cells were transiently transfected the next day with either 30 nM of an siRNA specific for *PRMT5* (si*PRMT5*; 5'-CAGCCACUGAUGGACAAUCUGG AAU-3' and 5'-CCGGCUACUUUGAGACUGUGCUUUA-3') or a control siRNA (siControl) using LipoTrust EX Oligo (Hokkaido System Science, Sapporo, Japan). After transfection, cells were cultured in serum-free DMEM for 72 h and used in the western blot and functional analyses. Western blot analysis using a rabbit anti-PRMT5 polyclonal antibody (Cell Signaling Technology, Beverly, MA, USA) diluted 1:1,000 was performed as previously described (27).

Cell proliferation assay. Cell proliferation was evaluated using the Premix WST-1 Cell Proliferation assay system (Takara Bio Inc., Shiga, Japan). NUGC3 and AGS cells ($5x10^3$ cells/well) were seeded into 96-well plates in DMEM supplemented with 2% FBS for 7 days. The optical density of the solution in each well was measured on days 1, 3 and 5 for NUGC3 cell, and 1, 3, 5 and 7 for AGS cell following addition of 10 μl of WST-1. In addition, the % decrease by siPRMT5 in proliferation was calculated as [1 - (fold change of siPRMT5/fold change of untransfected)] x 100 on every measurement day.

Cell invasion assay. The ability of GC cells to invade Matrigel was determined using BioCoat Matrigel invasion chambers (BD Biosciences, Bedford, MA, USA) according to the manufacturer's protocol. NUGC3 and AGS cells ($2.5x10^4$ cells/well) were seeded into the upper well of the chamber in serum-free DMEM. After 48 h, cells on the lower surface of the membrane were fixed, stained and counted using a microscope (x200 magnification, five randomly selected fields).

Wound-healing assay. The migration of GC cells was evaluated using wound-healing assays. NUGC3 and AGS cells ($2x10^4$ cells/well) were seeded into 12-well plates in serum-free DMEM using the ibidi Culture insert method (ibidi GmbH, Martinsried, Germany) to establish wound gaps of a defined width. After 24 h, the insert was removed, and the width of the wound was measured at 200-μm intervals (10 per well, x40 magnification) according to cell-dependent time intervals.

Statistical analysis. The Chi-square and Mann-Whitney tests were used to compare the differences between the two groups. The significance of a correlation between two variables was assessed using Spearman's rank correlation coefficient. Risk factors for positive peritoneal lavage cytology were evaluated using binomial logistic analysis. Overall survival rates were calculated using the Kaplan-Meier method, and the difference in survival curves was analysed using the log-rank test. We performed multivariable regression analysis to detect prognostic factors using the Cox proportional hazards model, and variables with a P-value of <0.05 were entered into the final model. All statistical analyses were performed using JMP v.10 software (SAS Institute, Inc., Cary, NC, USA). A P-value of <0.05 was considered statistically significant.

Results

Differential gene expression by GC cell lines. Expression levels of *PRMT5* mRNA were heterogeneous among GC cell lines. The levels of *PRMT5* mRNA were >2-fold higher in AGS, KATOIII, MKN1, MKN45 and NUGC3 cells compared

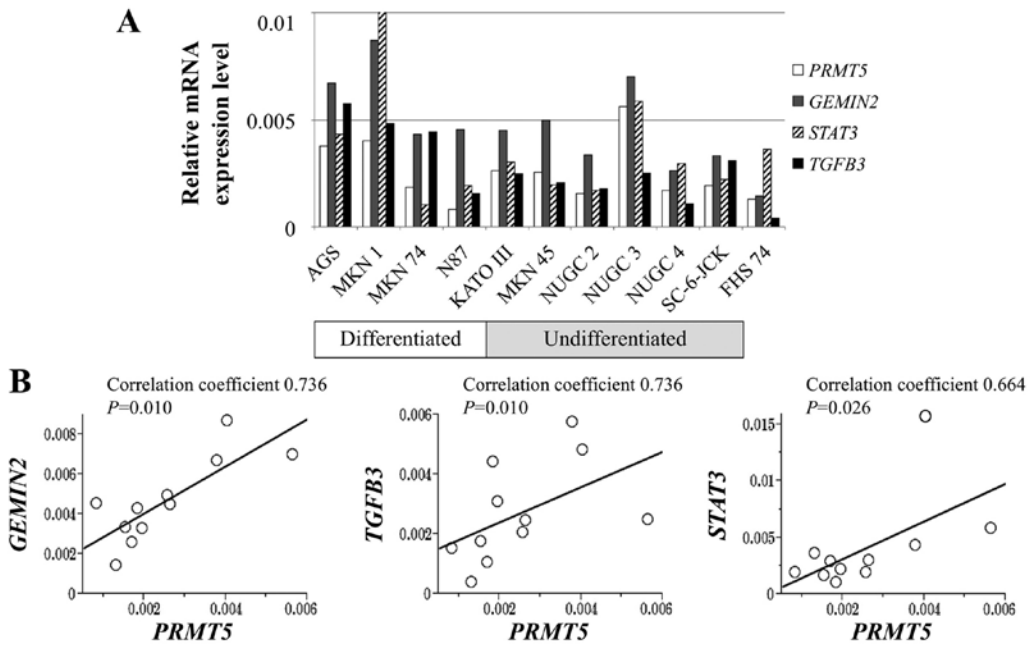

Figure 1. Expression analysis of cell lines. (A) Expression of *PRMT5* and genes expressed at similar differential levels were identified using PCR array analysis. (B) Analysis of the correlation between the mRNA expression levels of *PRMT5* and those of *GEMNIN2*, *TGFB3* and *STAT3*.

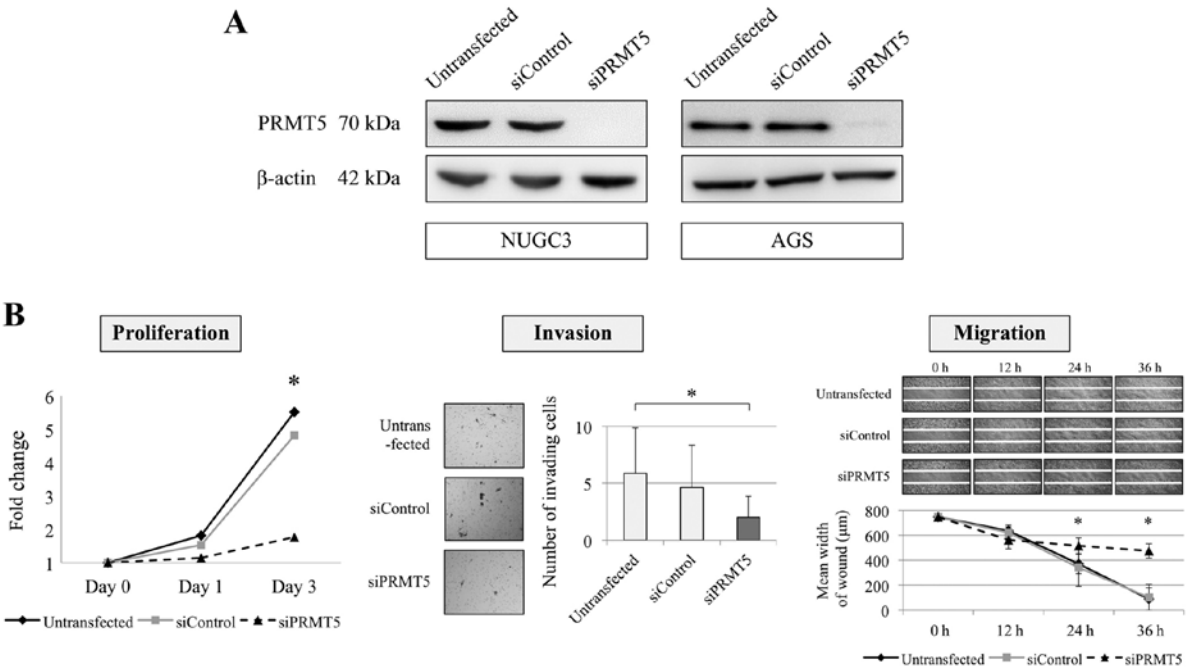

Figure 2. Effects of siRNA-mediated knockdown of *PRMT5* expression. (A) Confirmation of siRNA-mediated *PRMT5* knockdown was determined using western blot analysis. (B) Cell proliferation, invasion and wound healing assays for the NUGC3 cell. *PRMT5* siRNA inhibited significantly the proliferation, invasion and migration of NUGC3 cells. *P<0.05.

with the control FHs74 cells, whereas N87 cells showed lower *PRMT5* expression level than FHs74 cells (Fig. 1A). *PRMT5* mRNA levels did not differ according to the extent of differentiation of the GC cells. PCR array analysis revealed that mRNAs encoding gem (nuclear organelle) associated protein 2 (*GEMIN2*), signal transducer and activator of transcription 3 (*STAT3*) and transforming growth factor beta 3 (*TGFB3*) were expressed at levels that correlated significantly with those of the mRNA encoding *PRMT5* (Fig. 1B).

Effect of PRMT5 knockdown on the malignant phenotype of GC cells. Inhibition of *PRMT5* expression using a specific siRNA was conducted to evaluate the function of *PRMT5* in GC cells. NUGC3 (undifferentiated) and AGS (differentiated) cells were selected as *PRMT5*-overexpressed cells from the qRT-PCR results. The effect of *PRMT5* knockdown was confirmed using western blotting assay (Fig. 2A). We evaluated the proliferation, invasion and migration of NUGC3 and AGS cells. Knockdown of *PRMT5* expression significantly

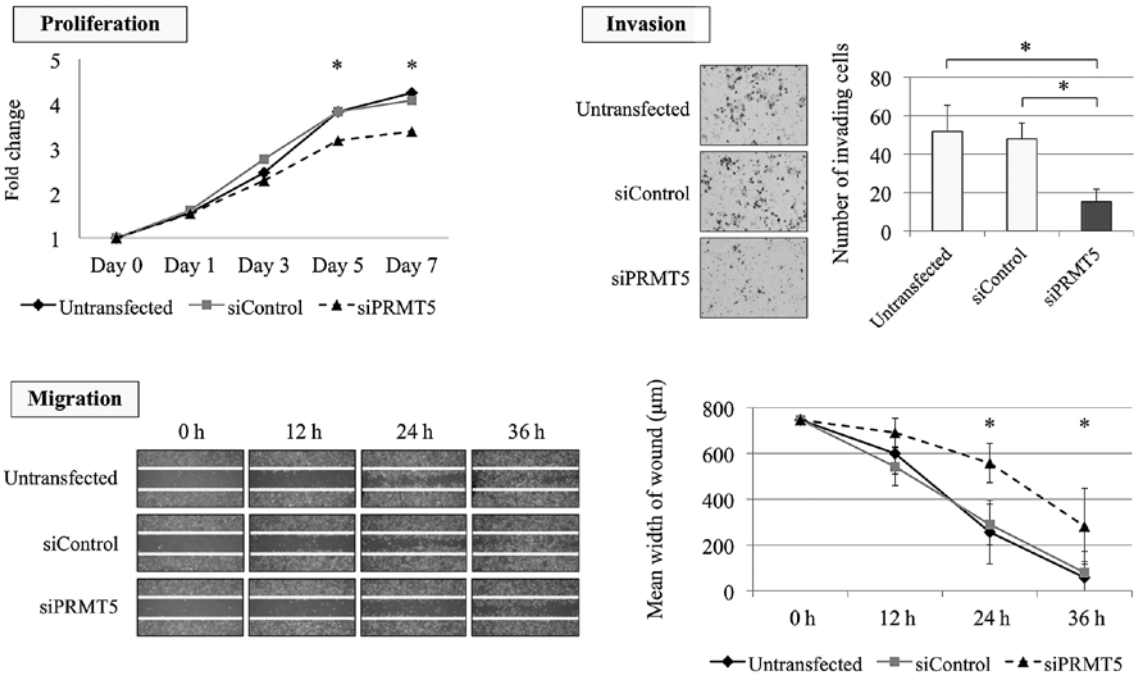

Figure 3. Cell proliferation, invasion and wound healing assays for the AGS cells. Knockdown of *PRMT5* significantly reduced the proliferation, invasion and migration abilities of AGS cell. *P<0.05.

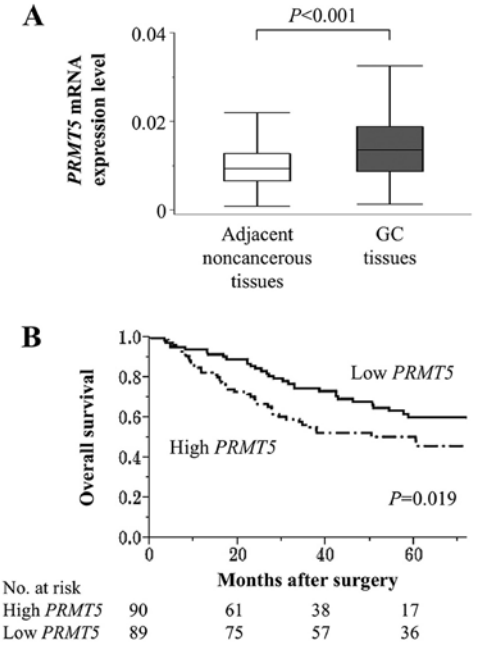

Figure 4. Expression of *PRMT5* in clinical samples. (A) The levels of *PRMT5* mRNA were elevated in GC tissues compared with the corresponding adjacent non-cancerous tissues. (B) Patients in the high *PRMT5* expression group were more likely to have poorer overall survival compared with those in the low *PRMT5* expression group. GC, gastric cancer.

decreased the proliferation ability of NUGC3 cell with 82 and 83% decrease on day 1 and 3, respectively (Fig. 2B). Invasion and migration abilities of NUGC3 cells were also reduced by inhibition of *PRMT5* compared with the untransfected and siControl-transfected cells (Fig. 2B). Similarly, the proliferation ability of AGS cells was significantly decreased by *PRMT5* knockdown with 23 and 27% decrease on day 5 and 7, respectively (Fig. 3). The invasion and migration of AGS cells were also significantly decreased after inhibition of *PRMT5* expression (Fig. 3).

Clinical implications of PRMT5 expression in tumour tissues. The mean level of *PRMT5* mRNA was significantly higher in 179 GC tissues compared with those of the corresponding adjacent normal tissues (Fig. 4A). Patients were classified into high and low *PRMT5* expression groups according to the median value of *PRMT5* mRNA levels. *PRMT5* mRNA levels were independent of tumour depth, differentiation and lymph node metastasis, whereas they were significantly and specifically associated with peritoneal lavage cytology (Table I). To investigate further the relationship between high *PRMT5* mRNA levels in GC tissues and positive peritoneal lavage cytology, multivariate binomial logistic analysis was conducted and revealed that high *PRMT5* mRNA levels were significantly associated with positive peritoneal lavage cytology (odds ratio, 3.90, 95% confidence interval 1.59-10.2, P=0.003; Table II). Patients with high levels of PRMT5 mRNA (n=90) were more likely to survive for shorter times compared with those with low levels (n=89), and their 5-year survival rates were 51 and 60%, respectively (P=0.019; Fig. 4B). In the multivariate analysis, lymph node metastasis and positive lavage cytology were identified as independent prognostic factors, but *PRMT5* expression was not (hazard ratio 1.54, 95, confidence interval 0.94-2.54, P=0.086).

Discussion

Arginine methylation is an important posttranslational modification of nuclear and cytoplasmic proteins and plays a vital role in cellular function (14,28). The human genome encodes 11 *PRMT* isoforms that covalently modify arginine

Table I. Association between *PRMT5* mRNA expression level and clinicopathological parameters in 179 patients.

Variables	High *PRMT5* mRNA in GC tissue (n)	Low *PRMT5* mRNA in GC tissue (n)	P-value
Age (years)			0.493
<65	42	37	
≥65	48	52	
Gender			0.756
Male	68	69	
Female	22	20	
Carcinoembryonic antigen (ng/ml)			0.730
≤5	72	73	
>5	18	16	
Carbohydrate antigen 19-9 (IU/ml)			0.267
≤37	70	75	
>37	20	14	
Tumour location			0.306
Entire	10	4	
Upper third	16	21	
Middle third	30	27	
Lower third	34	37	
Tumour size (mm)			0.586
<50	39	35	
≥50	51	54	
Tumour depth (UICC)			0.261
pT1-3	42	49	
pT4	48	40	
Histology			0.387
Papillary	1	1	
Well differentiated	4	6	
Moderately differentiated	26	31	
Poorly differentiated	53	48	
Signet ring cell	3	3	
Mucinous	3	0	
Differentiation			0.258
Differentiated	32	39	
Undifferentiated	58	50	
Lymphatic involvement			0.134
Absent	10	17	
Present	80	72	
Vessel invasion			0.503
Absent	40	44	
Present	50	45	
Infiltrative growth type			0.237
Invasive growth	37	29	
Expansive growth	53	60	
Lymph node metastasis			0.601
Absent	31	34	
Present	59	55	
Peritoneal lavage cytology			
Negative	60	78	<0.001[a]
Positive	30	11	
UICC stage			0.092
I	19	20	
II	14	16	
III	21	32	
IV	36	21	

[a]Statistically significant (P<0.05). GC, gastric cancer; UICC, Union for International Cancer Control.

residues in histone and nonhistone proteins that contribute to diverse cellular regulatory networks (20). Types I and II *PRMTs* catalyse monomethylation at the ω-NH2 group of arginine; however, they differ in their ability to add the second methyl group, either asymmetrically (type I) or symmetrically (type II) (13,14,29). *PRMT5* is a type II *PRMT* that catalyses the transfer of methyl groups from S-adenosyl methionine to the arginine residues of histones or non-histone proteins and is involved in numerous cellular processes (19,30). Because *PRMT5* possesses multiple cellular functions, it is an important determinant of oncological properties of various malignancies (12-14,17,18). In the present study, analyses of the expression levels of *PRMT5* and their effects on the phenotypes of GC cell lines, patient characteristics and outcomes were performed to assess the potential of *PRMT5* as a novel biomarker and therapeutic target for patients with GC.

Ibrahim *et al* (18) found that *PRMT5* is likely involved in the EMT of lung adenocarcinomas; however, the roles of *PRMT5* in the EMT are unknown. In the present study, we conducted PCR array analysis to evaluate the involvement of *PRMT5* in the oncological processes, particularly in the EMT that occurs in GC cells. *GEMIN2*, *STAT3* and *TGFB3* were identified as genes that were expressed in concert with *PRMT5*. *GEMIN2* (synonym, *SIP1*) encodes a zinc-finger transcription factor targeting the E2-box on the E-cadherin promoter and acts as a direct transcriptional repressor of E-cadherin (31,32). *GEMIN2* acts downstream in EMT-inducing signal transduction pathways activated by growth factors as well as in integrin engagement and hypoxia (33). *STAT3* was discovered as a latent transcription factor that is robustly activated by interleukin-6 and epidermal growth factor (34,35). Numerous studies indicate that *STAT3* modulates the expression of important EMT tran-

Table II. Predictive factors of peritoneal lavage cytology in 179 patients with gastric cancer.

	Univariate		Multivariate		
Variables	OR	P-value	OR	95% CI	P-value
Age (years)					
<65	1.12	0.746			
Gender					
Female	1.27	0.567			
CEA (ng/ml)					
>5	1.83	0.158			
CA19-9 (IU/ml)					
>37	2.59	0.024	2.17	0.76-6.32	0.145
Tumour location					
Lower third	0.80	0.529			
Tumour size (mm)					
≥50	3.17	0.003	1.47	0.53-4.17	0.460
Tumour depth					
pT4	15.8	<0.001	8.51	2.66-34.4	<0.001[a]
Differentiation					
Undifferentiated	2.46	0.020	1.51	0.48-4.88	0.479
Lymphatic involvement					
Present	9.29	0.003	1.38	0.06-13.7	0.803
Vessel invasion					
Present	2.28	0.025	1.45	0.55-3.91	0.452
Infiltrative growth					
Invasive	5.67	<0.001	3.43	1.26-10.0	0.015[a]
Lymph node metastasis					
Present	10.3	<0.001	3.71	0.95-19.8	0.060
PRMT5 expression					
High	3.55	<0.001	3.90	1.59-10.2	0.003[a]

[a]Statistically significant in multivariate analysis (P<0.05). CY, peritoneal lavage cytology; OR, odds ratio; CI, confidence interval; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; UICC, Union for International Cancer Control.

scription factors that integrate signals generated by multiple extracellular stimuli that influence EMT phenotypes (34-36). Mammalian TGF isoforms (TGF-β1, TGF-β2 and TGF-β3) share 97% amino acid sequence identity and signal through activation of TGF-β receptors (28). TGF-β isoforms play major roles in tumourigenesis mediated by the EMT through regulating cell growth, migration, invasion and metastasis (28,37,38). Although pathway analyses should be mandatory to further identify the involvement *PRMT5* in EMT process, our present results might suggest that *PRMT5* partially participate in EMT programs through its coordinate expression with other EMT-inducing molecules in GC cells.

There are no reports, to the best of our knowledge, that identify the function of *PRMT5* in the malignant phenotypes of GC. We show here that *PRMT5* contributed to the cell proliferation in addition to the migration and invasion abilities of GC cell lines. In NUGC3 cells, a significant decrease of proliferation ability by knockdown of si*PRMT5* was shown on day 3, whereas the differences in proliferation ability were exhibited from day 5 in the AGS cells. The possible explanations of this time lag were differences in cell-dependent efficacy, quickness and duration of action of siRNA transfection. Although analyses of apoptosis, cell cycle and interaction with the components of intracellular signalling pathways may contribute to understanding the functions of *PRMT5* in the malignant phenotypes of GC, our findings support the hypothesis that *PRMT5* acts as an oncogene when expressed at high levels and serves as a candidate target for the therapy of GC. To verify our *in vitro* results, we evaluated the expression of *PRMT5* and its clinical implications using surgically resected gastric tissues. *PRMT5* was significantly overexpressed in GC tissues compared with the corresponding adjacent normal tissues. Further, patients with high levels of *PRMT5* mRNA were more likely to survive for shorter times compared with those without.

We were intrigued by our finding here that *PRMT5* expression in patients' GC tissues was strongly associated with positive peritoneal lavage cytology. Peritoneal metastasis is a most frequent and dismal condition in patients with advanced GC and is diagnosed only when macroscopic disseminated

nodules are found during staging laparoscopy or from findings of positive peritoneal lavage cytology (6,39). Therefore, the availability of specific biomarkers that predict or enable early detection of peritoneal metastasis is anticipated to enhance management of GC. As an important step toward achieving this goal, we show here that the level of *PRMT5* mRNA in GC tissues is an independent risk factor for positive peritoneal lavage cytology. Linitis plastica, serosal invasion (T4) and lymph node metastasis have been reported to be risk factors for peritoneal metastasis of gastric cancer (40-42). In this study, high *PRMT5* expression was strongly associated with positive cytology, but independent of these well-known risk factors. This reveals the unique predictive value of *PRMT5* for peritoneal metastasis and indicates that GC patients with increased expression of *PRMT5* can be categorized into a high-risk group with peritoneal metastasis regardless of tumor types, T and N status. The value of *PRMT5* expression as a tool to screen for peritoneal metastasis will be enhanced by the development of assays to detect *PRMT5* expression in serum and peritoneal fluid.

The present study has certain limitations. First, extensive expression analyses of proteins, particularly ones related to EMT process, that potentially interact with *PRMT5* as well as apoptosis assays must be conducted to further understand the biological functions of *PRMT5* in GC. Second, this study was limited by the relatively small sample size. To determine the usefulness of *PRMT5* expression as a biomarker for GC, analysis of a larger cohort using multiple clinical samples, such as gastric tissues, ascites fluids and serum samples, will be required to deepen our knowledge on clinical significance of *PRMT5*.

Taken together, our findings indicate that *PRMT5* acts as an oncogene in GC by enhancing the malignant phenotype of a cancer cell line. *PRMT5* expression in gastric tissues may represent a promising biomarker for identification of patients at high risk, particularly for peritoneal metastasis.

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