Expression of DNMTs and genomic DNA methylation in gastric signet ring cell carcinoma

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Abstract. The aim of the present study was to investigate the protein expression of DNA methyltransferases (DNMTs) and genomic DNA methylation status of genomes in gastric signet ring cell carcinoma (SRC). Immunohistochemistry was performed to analyze DNMT expression and methylated DNA immunoprecipitation microarray (MeDIP-chip) and MeDIP quantitative real-time PCR (MeDIP-qPCR) were performed to analyze the genomic DNA methylation status in gastric SRC tissue. An increase in DNMT1 and decrease in DNMT3A expression in SRC tissue was observed compared with matched non-cancerous tissue. However, expression of other DNMTs, DNMT2, DNMT3B and DNMT3L, was not found to differ significantly between carcinoma and control. The MeDIP-chip assay revealed that methylation of gene promoters and CpG islands in SRC was higher than those in matched control tissue. However, MeDIP-qPCR analysis demonstrated that specific tumor-related genes, including ABL2, FGF18, TRAF2, EGFL7 and RAB33A were aberrantly hypomethylated in SRC tissue. Results of the current study indicate that gastric SRC may produce complex patterns of aberrant DNA methylation and DNMT expression.

Introduction

Epigenetics is the study of inherited genetic changes that occur without altering the DNA sequence. DNA methylation is a mechanism of epigenetic change, with the most widely studied epigenetic alteration in human tumor cells being histone modification and chromatin remodeling (1). Human tumor cells exhibit aberrant DNA methylation patterns, including the hypermethylation of CpG islands in tumor suppressor genes (TSGs) and a global loss of DNA methylation in the genome (2). These changes are associated with the inactivation of TSGs and the activation of oncogenes or tumor promoter genes (TPGs), and may promote tumor progression (3). Abnormal expression of DNA methyltransferase (DNMT) may be important in the aberrant DNA methylation that occurs in tumors (4). Previous studies have identified elevated DNMT expression in tumors when compared with control tissue (5-8) and the overexpression of DNMT may contribute to tumor progression through the hypermethylation-mediated inactivation of TSGs in CpG islands (9). In the current study, expression of DNMTs (DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L) and the DNA methylation pattern of the genome in gastric signet ring cell carcinoma (SRC) was investigated. Results of this study are likely to aid future epigenetic studies investigating SRC.

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Materials and methods

Ethics statement. All experimental procedures were approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University (Chongqing, China). All patients provided written informed consent.

Clinical specimens. Twenty-eight pairs of human gastric SRC and matched non-cancerous tissue specimens (gastric mucosa tissue located >5 cm from cancerous areas) were obtained from the Department of General Surgery of the First Affiliated Hospital of Chongqing Medical University. Characteristics of these tissues are presented in Table I. Tissues were fixed in formalin and embedded in paraffin for immunohistochemistry testing, and their diagnosis was confirmed by pathological analysis. For the methylated DNA immunoprecipitation microarray (MeDIP-chip) assay, one pair of gastric tissues was selected for pathological examination to verify that the selected cancerous and matched non-cancerous tissue specimens consisted of >95% cancer and mucosal cells, respectively. In addition, five pairs of gastric tissues were selected for MeDIP quantitative real-time PCR (MeDIP-qPCR) assay to validate MeDIP-chip observations.

Immunohistochemistry. The streptavidin-peroxidase (SP) method was adopted and performed (7). Primary antibodies against DNMT1 (sc-20701), DNMT2 (sc-20702), DNMT3A

Table I. Positive DNMT expression and differential clinical characteristics of gastric signet ring cell carcinoma, n (%).

Parameter	n	DNMT1 expression (%)	DNMT2 expression (%)	DNMT3A expression (%)	DNMT3B expression (%)	DNMT3L expression (%)
Gender						
Male	12	8 (66.7)	9 (75.0)	3 (25.0)	9 (75.0)	7 (58.3)
Female	16	9 (56.3)	14 (87.5)	7 (43.8)	12 (75.0)	10 (62.5)
P-value		0.71	0.62	0.43	1.00	1.00
Age, years						
≥50	13	7 (53.8)	10 (76.9)	5 (38.5)	10 (76.9)	7 (53.8)
<50	15	10 (66.7)	13 (86.7)	5 (33.3)	11 (73.3)	10 (66.7)
P-value		0.70	0.64	1.00	1.00	0.70
Location						
Upper and middle	13	6 (46.2)	11 (84.6)	5 (38.5)	11 (84.6)	7 (53.8)
lower	15	11 (73.3)	12 (80.0)	5 (33.3)	10 (66.7)	10 (66.7)
P-value		0.25	1.00	1.00	0.40	0.70
Tumor size, cm						
≥2	16	12 (75.0)	13 (81.3)	7 (43.8)	13 (81.3)	11 (68.8)
<2	12	5 (41.7)	10 (83.3)	3 (25.0)	8 (66.7)	6 (50.0)
P-value		0.12	1.00	0.43	0.42	0.44
Depth of invasion						
Mucosa and muscular	5	1 (20.0)	4 (80.0)	2 (40.0)	2 (40.0)	2 (40.0)
Subserosa and serosa	23	16 (69.6)	19 (82.6)	8 (34.8)	19 (82.6)	15 (65.2)
P-value		0.06	1.00	1.00	0.08	0.35
Lymphnode metastasis						
Yes	17	14 (82.4)	13 (76.5)	5 (29.4)	14 (82.4)	11 (64.7)
No	11	3 (27.3)	10 (90.9)	5 (45.5)	7 (63.6)	6 (54.5)
P-value		0.01*	0.62	0.44	0.38	0.70
TNM stage						
I-II	12	4 (33.3)	9 (75.0)	4 (33.3)	8 (66.7)	5 (41.7)
III-IV	16	13 (81.3)	14 (87.5)	6 (37.5)	13 (81.3)	12 (75.0)
P-value		0.02*	0.62	1.00	0.42	0.12
H. pylori infection						
Positive	24	16 (66.7)	21 (87.5)	9 (37.5)	19 (79.2)	16 (66.7)
Negative	4	1 (25.0)	2 (50.0)	1 (25.0)	2 (50.0)	1 (25.0)
P-value		0.27	0.14	1.00	0.25	0.27

*χ^2 test and Fisher's exact test, P<0.05. DNMT, DNA methyltransferase; *H. pylori*, *Helicobacter pylori*.

(sc-20703), DNMT3B (sc-20704) and DNMT3L (sc-20705) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The SP and DAB kits were obtained from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China).

Evaluation of staining. DNMT expression was assessed by scoring the staining intensity and stained proportion of the cell nucleus. Staining intensity was recorded as negative = 0, light = 1, moderate = 2 or strong = 3. The staining proportion was recorded as 1 (≤25%), 2 (≤50%), 3 (≤75%) or 4 (>75%). The two values were multiplied for each slide to produce a terminal score. If the score was higher in cancer cells than in matched control cells, this pair of tissues was marked with a '+' (corresponding to cancer cells that expressed elevated DNMT levels). The opposite condition was marked with a '-' (corresponding to cancer cells that expressed reduced DNMT levels). If the scores were equal, the pair was marked with a '0' (corresponding to similar DNMT expression in cancer and control). Terminal scores of 0-3 were defined as negative expression; 4-12 were defined as positive expression.

MeDIP-chip assay. For the MeDIP-chip assay, the NimbleGen Human DNA Methylation 385K Promoter Plus CpG Island array was used (Roche Diagnostics GmbH, Mannheim Germany). This single array design, includes 28,226 CpG islands and all RefSeq gene promoter regions (between -800 and +200 bp of the transcription start sites), coated entirely with ~385,000 probes. Briefly, genomic DNA extraction and fragmentation, immunoprecipitation [using Biomag™

magnetic beads (Bangs Laboratories, Inc., Fisher, IN, USA) coupled to a mouse monoclonal antibody against 5-methylcytidine], whole genome amplification, DNA labeling and array hybridization, raw data scanning (with Axon GenePix 4000B microarray scanner; Axon 132 Instruments, Foster City, CA, USA), quality assessment of raw data, data normalization, data mapping to genomic features (transcripts and CpG islands) and summarizing for the selected gastric tissue, were performed by KangChen Bio-tech Inc. (Shanghai, China). Genes exhibiting differential DNA methylation in CpG islands between SRC and control tissue from the genome (based on the results supplied by KangChen Bio-tech) were separated and investigated for tumor-associated genes.

MeDIP-qPCR. Genomic DNA was extracted from 5 pairs of gastric tissue using a DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) and sonicated to random fragments of 200-1,000 bp with a Bioruptor sonicator (Diagenode, Denville, NJ, USA). Sonicated DNA fragments were divided in two, one part was used as input (normalized control) and the other was prepared for MeDIP. MeDIP was performed using Biomag magnetic beads coupled to a mouse monoclonal antibody against 5-methylcytidine. The immunoprecipitated DNA was eluted and purified by phenol chloroform extraction and ethanol precipitation for the subsequent qRT-PCR. qRT-PCR was conducted on an ABI Prism 7900 system (Applied Biosystems, Foster City, CA, USA) using PCR master mix (Qiagen) and specific primers were denatured at 95˚C for 10 min followed by 40 cycles of 95˚C for 10 sec and 60˚C for 60 sec. Following this, the comparative ΔΔCt method was performed. Each MeDIP DNA Ct value was normalized against the input DNA value for the same qRT-PCR assay (ΔCt) to account for chromatin sample preparation differences. The input percentage for each MeDIP fraction was calculated using the following formula: input percentage = $2(Ct_{input} - Ct_{MeDIP}) \times Fd \times 100$. Fd represents the input dilution factor (1/5). The input percentage values represent the DNA methylation levels of validated genes in this assay.

Statistical analysis. Standard statistical analysis was performed using the SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). The Wilcoxon signed-rank test (for immunohistochemistry analysis), χ^2 test, Fisher's exact test (for association analysis between DNMT expression and clinical parameters of SRC) and paired t-test (for MeDIP-qPCR) were used in this study. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of DNMT in gastric SRC tissue. Gastric SRC and matched mucosal tissue expressed DNMT proteins (Fig. 1). Notably, DNMT protein distribution was observed primarily in the nucleus of cancerous tissue and in the nucleus and cytoplasm of control tissue. DNMT expression in non-cancerous tissue was primarily focused on the proliferating zone of gastric mucosa. DNMTs perform DNA methylation in the nucleus; thus, nuclear staining was investigated. DNMT1 expression was elevated and DNMT3A expression was decreased in gastric SRC when compared with matched control tissue (Fig. 2). However, expression of other DNMTs did not differ significantly between cancerous and non-cancerous tissue.

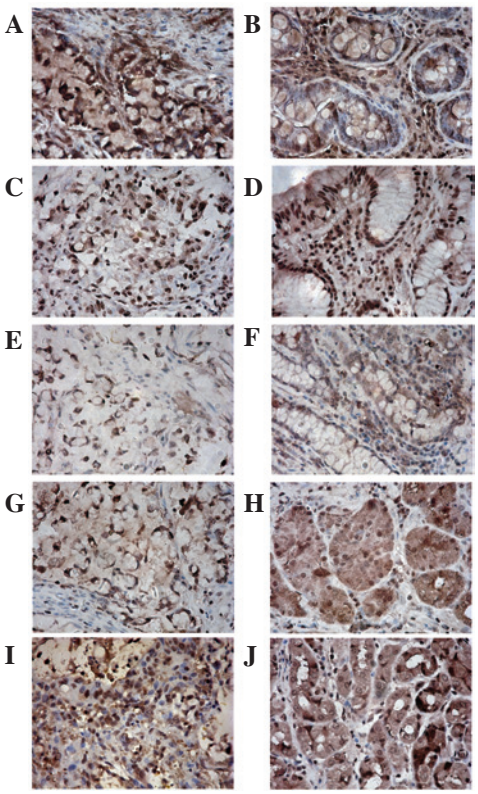

Figure 1. DNMT expression in gastric signet ring cell carcinoma and matched mucosa tissues (magnification, x200). Expression of (A) DNMT1 in carcinoma; (B) DNMT1 in mucosa tissue; (C) DNMT2 in carcinoma; (D) DNMT2 in mucosa tissue; (E) DNMT3A in carcinoma; (F) DNMT3A in mucosa tissue; (G) DNMT3B in carcinoma; (H) DNMT3B in mucosa tissue; (I) DNMT3L in carcinoma; and (J) DNMT3L in mucosa tissue. DNMT, DNA methyltransferase.

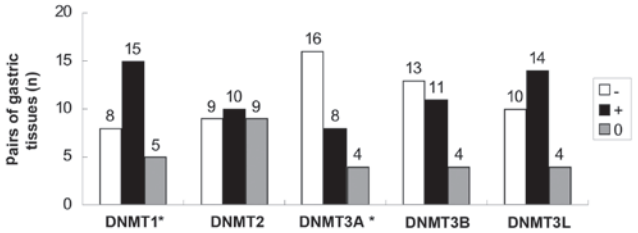

Figure 2. DNMT expression in gastric SRC and matched mucosal tissue by nuclear staining analysis. '-': lower in carcinoma than matched mucosa. '+': elevated in carcinoma than matched mucosa. '0': similar in carcinoma and in matched mucosa. DNMT1 expression was higher in gastric SRC than in matched tissues. DNMT3A expression was lower in SRC than in matched tissues (*$P<0.05$, Wilcoxon signed-rank test). Differences were not significant for other DNMTs ($P>0.05$, Wilcoxon signed-rank test). DNMT, DNA methyltransferase; SRC, signet ring cell carcinoma.

Correlation between DNMT expression and clinical characteristics of gastric SRC. According to the TNM classification of malignant tumors, expression of DNMT1 was associated with lymph node metastasis in gastric SRC (Table I). SRC samples with features of lymph node metastasis and attributes of late TNM classification were found to express DNMT1 protein at

Table II. Overview of MeDIP-chip data.

Classification	CpG islands (%)	Gene promoters (%)
Hypermethylation only in cancer	2,832 (10.03)	1,541 (8.55)
Hypermethylation only in control	2,273 (8.05)	913 (5.06)
Hypermethylation in cancer and control	1,943 (6.88)	736 (4.08)
Hypomethylation in cancer and control	21,178 (75.03)	14,838 (82.31)
Hypermethylation in cancer	4,775 (16.92)	2,277 (12.63)
Hypermethylation in control	4,216 (14.94)	1,649 (9.15)
Total	28,226	18,028

MeDIP, methylated DNA immunoprecipitation microarray.

Table III. Differential DNA methylation of tumor-related genes between SRC and control.

Classification	TSG (ref.)	Oncogene and TPG (ref.)
Hypermethylation only in cancer	BCL2L11 (10)	APCDD1 (24)
	BRMS-1 (11)	BCL11A (25)
	CARS (12)	JUN (26)
	CDKN1C (13)	LYN (27)
	CDKN2A (14)	MYB (28)
	DLC-1 (15)	MYCL1 (29)
	ING-1 (16)	REL (30)
	OVCA2 (17)	SRC (31)
	RASD1 (18)	WNT4 (32)
	RB-1 (19)	WNT9A (32)
	SYK (20)	WNT10A (32)
		WNT11 (33)
		KRAS (34)
		VEGFA (35)
		RAB6A (36)
		RAB8A (37)
		RAB27A (38)
		RAB32 (39)
Hypomethylation only in cancer	APAF1 (21)	RAB33A (36)
	CAV2 (22)	ABL2 (40)
	RASSF1 (23)	FGF18 (41)
		EGFL7 (42)
		FYN (43)
		MYCNOS (44)
		RAB3A (45)
		TRAF2 (46)
		WNT3A (47)

SRC, signet ring cell carcinoma; TSG, tumor suppressor gene; TPG, tumor promotor gene.

a higher level than control samples. There was no association noted in other DNMT expression and demographic variables of the carcinoma.

Comparison of genomic DNA methylation between gastric SRC and matched control tissue. DNMT proteins in the selected pair of gastric tissues were scored by immunohistochemistry assay as follows: DNMT1 '+'; DNMT2 '0'; DNMT3A '-'; DNMT3B '+'; and DNMT3L '-'. In carcinoma and matched mucosal tissue, characteristics of interest included local hypermethylation and global hypomethylation of genomic DNA. Specific CpG islands and gene promoters were hypermethylated only in carcinoma

Table IV. Primer sequences of validation genes.

Gene name	Primer sequences	Length (bp)
ABL2	F:5'ATTTGACAGGTGGAGGTGGGAT3'	
	R:5'CGCTGCTTGAGGTCTTTCGTC3'	162
FGF18	F:5'GGCTGGGAAACTCCACGAT3'	
	R:5'CCACATTCGCTACTCGCACT3'	135
TRAF2	F:5'GGAGAATCGCTTGAACCCG3'	
	R:5'GTGTGCTAATCTACTGGGTTGTGC3'	138
EGFL7	F:5'CTGGTTTCTGGCTGTTTTGG3'	
	R:5'ATGCTCCGTCCTGGGTAATC3'	214
RAB33A	F:5'ACCAGACAAGACTGAAGCCACC3'	
	R:5'CGACAACCGCTAGAGCTATGC3'	154

Table V. Input percentage value of validation genes in signet ring cell carcinoma and control tissues, MeDIP-qPCR.

Gene name	Carcinoma tissue	Control tissue
ABL2	0.0114±0.00408[a]	1.1698±0.22944
FGF18	0.0115±0.00125[a]	1.3419±0.15275
TRAF2	0.8152±0.20569[a]	3.5592±0.40797
EGFL7	0.3557±0.06140[a]	1.9956±0.31949
RAB33A	0.0465±0.00735[a]	2.1858±0.26880

[a]P<0.05, vs. control; paired t-test; mean ± SD, n=5; MeDIP, methylated DNA immunoprecipitation.

tissue, whereas other CpG islands and gene promoters were hypermethylated only in matched mucosa tissue (Table II), indicating that these DNA sequences were hypomethylated only in carcinoma tissue. Following a comprehensive analysis involving separation of genes exhibiting differential DNA methylation in promoters between the carcinoma and control from the genome, and searching for tumor-related genes in these separating genes, gastric SRC was observed to contain hypermethylated and hypomethylated TSGs, oncogenes and tumor-promoter genes (TPGs; Table III) (10-47).

Validation of the abnormally hypomethylated genes in gastric SRC by MeDIP-qPCR. ABL2, FGF18, TRAF2, EGFL7 and RAB33A were selected as validation genes. Their primer sequences are presented in Table IV. Using MeDIP-qPCR, the input percentage values of these genes were observed to be significantly lower in SRC than in matched mucosal tissue (Table V), indicating that the DNA was abnormally hypomethylated in gastric SRC compared with matched control tissue. This result was in agreement with the MeDIP-chip observations. The input percentage values of these genes the in negative control (non-immune serum) were <0.01.

Discussion

According to the World Health Organization, there are four predominant histological types of gastric adenocarcinoma, papillary, tubular, mucinous and SRC. SRC is characterized by the histological appearance of signet ring cells, a large vacuole full of mucin in the cytoplasm displacing the nucleus to the periphery. This adenocarcinoma originates from the undifferentiated stem cells at the gastric gland neck in gastric lamina propria and accounts for 3.14-29% of gastric cancer (48). SRC is a poorly differentiated adenocarcinoma with rapid progression and poor prognosis. To date, the etiology of SRC is unclear and therapy is mainly dependent on surgical procedures (SRC is non-responsive to chemotherapy).

Epigenetic alterations, including promoter hypermethylation, lead to chromatin remodeling and the silencing of tumor-related genes, and are crucial in tumor progression (49). DNA methylation is catalyzed mainly by DNMTs, including DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L (50-53). Previous studies have indicated that overexpression of DNMT may contribute to tumor progression through hypermethylation-mediated TSG inactivation in CpG islands. We hypothesized that overexpression of DNMT also may be detected in SRC.

In the current study, it was observed that gastric SRC and matched mucosal tissue expressed DNMT proteins. DNMT expression in non-cancerous tissue was primarily focused on the proliferating zones of gastric mucosa. An abnormal overexpression of DNMT1 was observed when nuclear staining was taken into consideration in SRC tissue compared with matched mucosal tissue. By contrast, DNMT3A expression in SRC was not significant compared with that in matched controls. For the remaining DNMTs, no expression difference between SRC and control tissue was noted. The observations indicate that overexpression of DNMT in SRC tissue was specific to DNMT1.

Following association analysis between the demographic variables and DNMT expression of SRC, positive expression of DNMT1 was associated with lymph node metastasis and late TNM stages of SRC, indicating a potential role of DNMT1 proteins in promoting SRC progression. It was hypothesized that DNMT1 may function in a similar manner to an oncogene in SRC. Considering no association was noted between DNMT3A and clinical characteristics of SRC, the hypothesis that SRC expresses a lower level of DNMT3A compared with control requires further investigation. DNMT proteins were distributed in the nucleus and cytoplasm, particularly in the proliferative zones of normal gastric mucosa. It is not yet clear if there are unknown substances in the cytoplasm that may cross-react with DNMT antibodies or if DNMT proteins function in the cytoplasm. Further investigation is required to confirm this.

The MeDIP-chip assay revealed local hypermethylation and global hypomethylation of genomic DNA in SRC and matched mucosal tissue. The number of hypermethylated CpG islands and gene promoters in SRC were increased compared with those in control mucosa (4,775 vs. 4,216 and 2,277 vs. 1,649, respectively; Table II). This observation indicated that DNA methylation of the genome increased in SRC compared with matched controls. Notably, in gastric SRC, hypermethylated and hypomethylated TSGs, oncogenes and TPGs were observed. The subsequent MeDIP-qPCR assay validated specific MeDIP-chip results. Tumor-related genes, ABL2, FGF18, TRAF2, EGFL7 and RAB33A, were abnormally hypomethylated in SRC tissue compared with matched controls. This observation is an addition to the traditional DNA methylation theory, which focuses on the hypermethylation of TSG in tumors and indicates that the aberrant DNA methylation pattern of the SRC genome is complex. Similar observations were also demonstrated in liver and pancreatic cancer (54,55). Specific tumors exhibit abnormal hypomethylation of TPGs and oncogenes (56,57) and overexpression of these genes due to hypomethylation, is potentially another epigenetic mechanism for uncontrollable cancer cell proliferation (55).

In conclusion, gastric SRCs express elevated DNMT1 protein and reduced DNMT3A protein compared with matched gastric mucosa. A difference between genomic DNA methylation between SRC and control (gastric mucosa) samples exists, however, it appears to be complex since it is not limited to hypermethylation of TSGs.

To date, there has been encouraging progression in the understanding of the role of DNA methylation in tumors. However, clinical applications based on DNA methylation theory for diagnosis and treatments of tumors remain scarce. Further investigation is required to investigate the role of DNMT inhibitors, including 5-aza-2'-deoxycytidine, in inhibiting cancer cell proliferation.

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