Am J Cancer Res 2012;2(1):116-129
www.ajcr.us /ISSN:2156-6976/ajcr0000093

Original Article

Prognostic significance of aberrant gene methylation in gastric cancer

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Received November 4, 2011; accepted November 19, 2011; Epub November 21, 2011; Published January 1, 2012

Abstract: Promoter methylation acts as an important alternative to genetic alterations for gene inactivation in gastric carcinogenesis. Although a number of gastric cancer-associated genes have been found to be methylated in gastric cancer, valuable methylation markers for early diagnosis and prognostic evaluation of this cancer remain largely unknown. In the present study, we used methylation-specific PCR (MSP) to analyze promoter methylation of 9 gastric cancer-associated genes, including *MLF1*, *MGMT*, *p16*, *RASSF2*, *hMLH1*, *HAND1*, *HRASLS*, *TM*, and *FLNc*, and their association with clinicopathological characteristics and clinical outcome in a large cohort of gastric cancers. Our data showed that all of these genes were aberrantly methylated in gastric cancer, ranging from 8% to 51%. Moreover, gene methylation was strongly associated with certain clinicopathological characteristics, such as tumor differentiation, lymph node metastasis, and cancer-related death. Of interest, methylation of *MGMT*, *p16*, *RASSF2*, *hMLH1*, *HAND1*, and *FLNc* was closely associated with poor survival in gastric cancer, particularly *MGMT*, *p16*, *RASSF2* and *FLNc*. Thus, our findings suggested these epigenetic events may contribute to the initiation and progression of gastric cancer. Importantly, methylation of some genes were closely relevant to poor prognosis in gastric cancer, providing the strong evidences that these hypermethylated genes may be served as valuable biomarkers for prognostic evaluation in this cancer.

Keywords: Gastric cancer, gene methylation, methylation-specific PCR (MSP), early diagnosis, poor prognosis

Introduction

Gastric cancer is the fourth most common cancer and the second most common cause of cancer-related death worldwide with approximately 989,600 new cases and 738,000 deaths per year, accounting for about 8% of new cancers [1]. Although the worldwide incidence of gastric cancer has declined rapidly over the recent few decades due to the recognition of certain risk factors, such as H. pylori, dietary and environmental risks, gastric cancer is still the predominant form of cancer and remains a significant cancer burden in developing world, particularly China [2,3]. Very unfortunately, most of gastric cancer patients are diagnosed at an advanced stage, and the prognosis is poor due to the limited advances in our understanding of the pathogenesis of this disease and the lack of useful diagnostic and prognostic markers [4]. Thus, there is an urgent need to identify the valuable markers for early diagnosis and prognostic evaluation of gastric cancer.

Gastric cancer is thought to arise through the accumulation of multiple genetic and epigenetic alterations, leading to gain-of-function in oncogenes and loss-of-function in tumor suppressor genes [5-7]. However, many evidences show that genetic alterations, particularly gene mutations, are relatively infrequent in gastric cancer [8]. A growing body of evidence now suggests that, in addition to genetic alterations, epigenetic alterations, such as DNA methylation, also play a critical role in the development and progression of human malignancies [9-11]. Aber-

rant promoter methylation is an important hallmark of cancer cells, and now regarded as one of the major mechanisms to inactivate tumor-related genes, contributing to gastric carcinogenesis [12-14]. However, to date, epigenetic inactivation of genes related tumor initiation and progression has not been well studied in gastric cancer as related to disease outcome.

In the present study, we used methylation-specific PCR (MSP) to evaluate promoter methylation of a panel of gastric cancer-associated genes in a large cohort of clinically well-characterized gastric cancers, including *MLF1*, *MGMT*, *p16*, *RASSF2*, *hMLH1*, *HAND1*, *HRASLS*, *TM*, and *FLNc*. These genes were potentially important in gastric carcinogenesis. Furthermore, we sought to explore the association of gene methylation with clinicopathological characteristics and clinical outcome in gastric cancer.

Material and methods

Patients and tissue samples

This study was approved by the Institutional Review Board and Human Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University School of Medicine. A total of 119 paraffin-embedded gastric cancer tissues were randomly obtained from the First Affiliated Hospital of Xi'an Jiaotong University School of Medicine. None of these patients received chemotherapy and radiotherapy before the surgery. Informed consent was obtained from each patient before the surgery. The histologic diagnosis of tumors was made and agreed upon by at least two senior pathologists at Department of Pathology of the Hospital based on World Health Organization (WHO) criteria. The routine ematoxylin-eosin (H&E) staining was used to identify residual tumor cells at the resection margins. Relevant clinicopathologic features were obtained from the patients' files or by telephone interviews with the patients or their relatives, and the details were summarized in **Table 1**.

Table 1. Clinicopathologic characteristics of gastric cancers

Characteristics	No.of patients (%)
Gender	
Male	92 (77.3)
Female	27 (22.7)
Age, years	
Mean	59.20
SD	12.726
Tumor localization	
gastric cardia	32 (26.9)
gastric body	33 (27.7)
gastric antrum	54 (45.4)
Tumor size (cm^3)	
≤3	41 (34.5)
3-5	41 (34.5)
>5	37 (31.1)
Differentiation	
well/moderate	54 (45.4)
poor/undifferentiation	65 (54.6)
Tumor invasion	
T1	12 (10.1)
T2	24 (20.2)
T3	82 (68.9)
T4	1 (0.8)
TNM stage	
I	29 (24.4)
II	19(16.0)
III	66(55.5)
IV	5(4.2)
Residual tumor	
Yes	12 (10.1)
No	107 (89.9)
Lymph node metastasis (LNM)	
Yes	73 (61.3)
No	46 (38.7)
No. of LNM	
N0	46 (38.7)
N1 (1-6)	45 (37.8)
N2 (7-15)	23 (19.3)
N3 (≥16)	5 (4.2)
Survival status	
Dead	56 (47.1)
Alive	63 (52.9)

DNA preparation

Serial sections from each tumor sample were cut at 5 μm, and stained with hematoxylin and eosin (H&E). A tumor representative tissue was marked by a senior pathologist. The other sections (8 μm) were isolated by manual microdissection under an inverted microscope using the marked H&E section for target tissue identification. DNA was extracted from tissues dissected as previously described [15]. Briefly, the tissues dissected were first treated with xylene for 12 hours at room temperature to remove the paraffin. All tissues were then subjected to digestion with 1% sodium dodecyl sulfate (SDS) and pro-

teinase K at 48°C for 48 to 72 hours with addition of several spiking aliquots of concentrated proteinase K to facilitate digestion. DNA was subsequently isolated from the digested tissues followed by standard phenol-chloroform extraction and ethanol precipitation protocol, and stored at -80°C until use.

Sodium bisulfite treatment

DNA was treated with sodium bisulfite as described previously [16]. Briefly, a final volume of 20 μl of H_2O containing 4 μg genomic DNA, 10 μg salmon sperm DNA, and 0.3M NaOH was incubated at 50 °C for 20 min to denature the DNA. The mixture was then incubated for 2-3 h at 70°C in 500 μl of a freshly prepared solution containing 3 M sodium bisulfite (Sigma, Saint Louis, MO) and 10 mM hydroquinone (Sigma, Saint Louis, MO). DNA was subsequently purified with a Wizard DNA Clean-Up System (Promega Corp., Madison, WI) following the instructions of the manufacturer, followed by ethanol precipitation, dry, and resuspension in 100 μl of deionized H_2O. After bisulfite treatment, all unmethylated cytosine residues converted to uracil, whereas the methylated cytosine residues remained unchanged. Bisulfited-treated DNA samples were stored at -80°C until use.

Methylation-specific PCR (MSP) assay

MSP assay was performed on bisulfite-treated DNA in a final reaction mixture of 20 μl containing 50 ng of bisulfite-treated DNA, 16.6 mM of ammonium sulfate, 67 mM of Tris (pH 8.8), 2 mM $MgCl_2$, 200 μM each of deoxynucleotide triphosphate mixture (dATP, dCTP, dGTP, and dTTP), 200 nM forward and reverse primers, and 0.5 U of platinum Taq DNA polymerase (Invitrogen Technologies, Inc., CA). The PCR was run in a Thermal cycler (Bio-Rad Laboratories, Inc., CA) as follows: after a 4-min denaturation at 95°C, the reaction was run 35 cycles, each comprising 45 s of denaturing at 95°C, 45 s of annealing at variable temperatures according to the primers, and 45 s of extension at 72°C, with an extension at 72°C for 5 min as the last step. Normal leukocyte DNA was methylated *in vitro* with Sss I methylase (New England Biolabs, Beverly, MA) to generate completely methylated DNA as a positive control. The primers used in the present study were presented in **Table 2**. The PCR products were electrophoresed on a 1.2 % agarose gel and visualized under UV illumination using an ethidium bromide stain.

Statistical analysis

Gene methylation associated with clinicopathological features of tumor were assessed univariately using the SPSS statistical package (version11.5; SPSS Inc., Chicago, IL, USA). Multivariate models were then developed that adjusted for the important covariates, including age, differentiation, tumor stage, lymph node metastasis and cancer-related death. Survival time was determined from the day of primary tumor surgery to the day of death or last clinical follow-up. The Kaplan–Meier method was used for survival analysis grouping with methylation status. Differences between curves were analysed using the log-rank test. Sample means were compared using unpaired student *t* test, assuming unequal variances, and the test was two-tailed. *P* values <0.05 were considered to be statistically significant. All statistical analyses were performed using the SPSS statistical package (version11.5; SPSS Inc., Chicago, IL, USA).

Results

Frequent aberrant gene methylation in gastric cancer and its association with clinicopathological features

We examined promoter methylation of *MLF1*, *MGMT*, *p16*, *RASSF2*, *hMLH1*, *HAND1*, *HRASLS*, *TM*, and *FLNc* genes using MSP approach in a large cohort of gastric cancers. As shown in **Figure 1**, all of these genes were aberrantly methylated in gastric cancer, including the *MLF1* gene in 50 of 119 tumors (42%), the *MGMT* gene in 10 of 119 tumors (8%), the *p16* gene in 53 of 119 tumors (45%), the *RASSF2* gene in 17 of 119 tumors (14%), the *hMLH1* gene in 17 of 119 tumors (14%), the *HAND1* gene in 22 of 119 tumors (18%), the *HRASLS* gene in 55 of 119 tumors (46%), the *TM* gene in 61 of 119 tumors (51%), and the *FLNc* gene in 44 of 119 tumors (37%).

The univariate analyses showed that *p16* methylation was found to be significantly associated with poor tumor differentiation (OR = 2.33, 95% CI = 1.11—4.93; *P* <0.05) (**Table 3**). Also shown in **Table 3**, *p16* methylation was signifi-

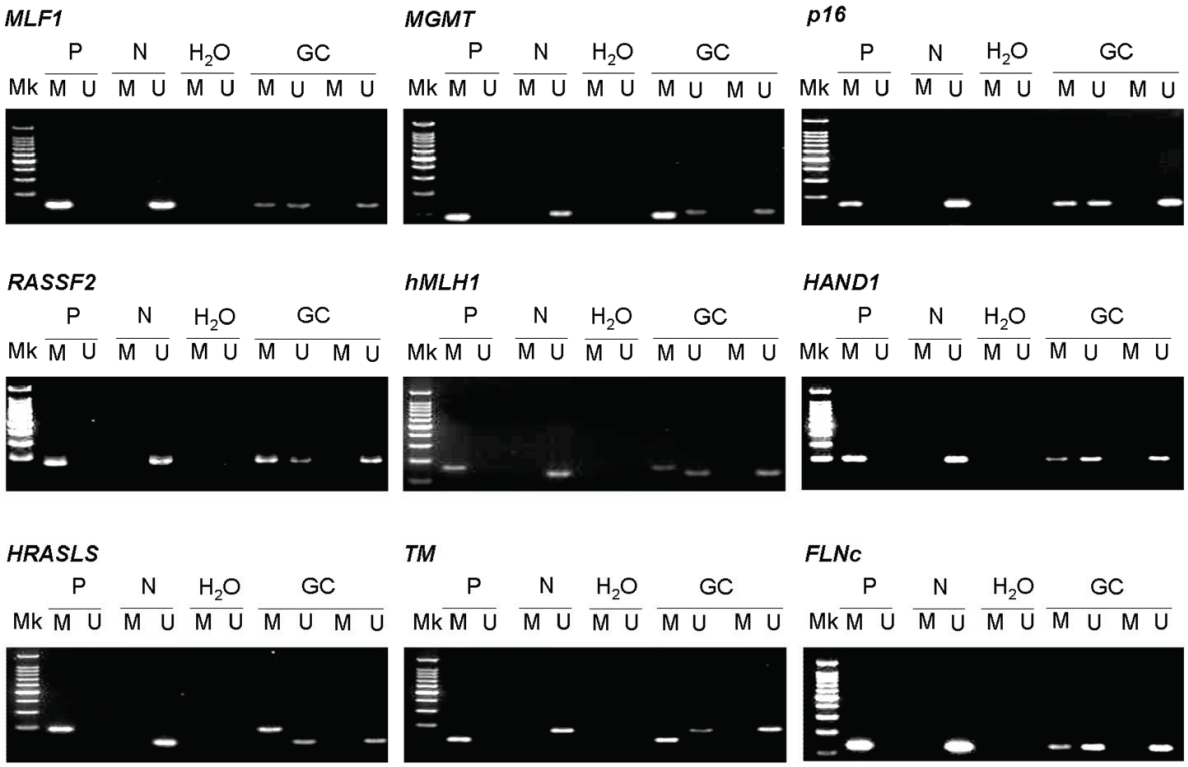


Figure 1. Representative MSP results of 9 gastric cancer-associated genes in gastric cancer. *In vitro* methylated DNA was used as positive control for methylated gene (P), bisulfite-modified normal leukocyte DNA as positive control for unmethylated gene (N), and H_2O as blank control to confirm the specificity of MSP. Details are described in the Materials and Methods. Mk, DNA marker; M, methylated gene; U, unmethylated gene. GC, gastric cancer samples.

cantly positively associated with lymph node metastasis (OR = 2.62, 95% CI = 1.20—5.71; *P* <0.05). Similar to *p16* gene, although no statistical significance was noted, there was a trend toward positive association of methylation of *MGMT* (OR = 6.33, 95% CI = 0.77—51.7) and *hMLH1* (OR = 3.40, 95% CI = 0.92—12.6) genes with lymph node metastasis. Moreover, methylation of *MLF1* (OR = 1.65, 95% CI = 1.06—2.56; *P* <0.05), *MGMT* (OR = 2.29, 95% CI = 1.11—4.75; *P* <0.05), and *p16* (OR = 1.85, 95% CI = 1.18—2.90; *P* <0.01) genes was significantly positively associated with the number of lymph node metastasis (**Table 3**). Importantly, our data showed that methylation of 5 of 9 genes, including *MGMT* (OR = 5.08, 95% CI = 1.03—25.1; *P* <0.05), *p16* (OR = 6.76, 95% CI = 3.02—15.1; *P* <0.01), *RASSF2* (OR = 6.67, 95% CI = 1.80—24.7; *P* <0.01), *hMLH1* (OR = 3.16, 95% CI = 1.04—9.64; *P* <0.05), and *FLNc* (OR = 2.52, 95% CI = 1.17—5.41; *P* <0.05), was associated with a significantly increased risk of gastric cancer-related death (**Table 3**). In order to assess the independent association of gene methylation with age, tumor differentiation, tumor stage, lymph node metastasis and cancer-related death, we conducted multiple multivariable logistic regressions (**Table 4**). Similar to univariate analysis, *p16* methylation remained significantly associated with poor tumor differentiation (OR = 2.59, 95% CI = 1.08—6.23; *P* <0.05) (**Table 4**). *MGMT* methylation remained positively associated with lymph node metastasis (OR = 8.10, 95% CI = 0.55—120) (**Table 4**). Methylation of *hMLH1* gene remained negatively associated with tumor differentiation (OR = 0.30, 95% CI = 0.09—0.97; *P* <0.05) (**Table 4**). Methylation of *MGMT*, *p16*, *RASSF2*, *hMLH1*, and *FLNc* genes also remained positively associated with cancer-related death, particularly *p16* (OR = 8.52, 95% CI = 2.99—24.2; *P* <0.01), *RASSF2* (OR = 13.6, 95% CI = 2.49—73.7; *P* <0.01), and *FLNc* (OR = 3.15, 95% CI = 1.20—8.25; *P* <0.05) (**Table 4**). However, after adjustment, *HRASLS* methylation became significantly positively associated with

Table 2. Methylation-specific PCR (MSP) primers used in the present study

Genes	Allele	Forward (5'→3')	Reverse (5'→3')	Length (bp)	Annealing temperature (°C)
MLF1	M	TCGTTTCGTTTTGGTGAC	CTAACCTCCAATACGAACGAC	127	60
	U	TTTTGTTTTGTTTTGGTGAT	AACTAACCTCCAATACAAACAAC	127	57
MGMT	M	TTTCGACGTTCGTAGGTTTTCGC	GCACTCTTCCGAAAACGAAACG	81	59
	U	TTTGTGTTTTGATGTTTGTAGGTTTTTGT	AACTCCACACTCTTCCAAAAACAAAACA	93	59
p16	M	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	150	65
	U	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	151	60
RASSF2	M	TTTTTTTTTTTTGAGTTCGC	CTAAAAAACGACGACGAACT	157	58
	U	TTTTTTTTTTTTTTTGAGTTTGT	CCTAAAAAACAACAACAAACTA	157	56
hMLH1	M	TATATCGTTCGTAGTATTCGTGT	TCCGACCCGAATAAACCCAA	153	60
	U	TTTTGATGTAGATGTTTTATTAGGGTTGT	ACCACCTCATCATAACTACCCACA	124	60
HAND11	M	AATAGTTTAGGGCGTTGGTC	AATTTTACGCTCAACCCG	184	61
	U	AATAGTTTAGGGTGTTGGTT	AATTTTACACTCAACCCA	184	56
HRASLS1	M	GGGTGTATTTATGATGGGTGTATTC	CAACGCCTACGATCAAAACG	182	60
	U	GTTGTGGGAATGTTTGAGATGT	CACAACCACCTCCACACACA	117	60
TM	M	CGTTCGTTTTTATTCGGCGTC	GCCAAACCCCATCTCATCG	118	62
	U	ATGTGTTTGTTTTTATTTGGTGTT	CATAACTAACCAAAAACCCACA	158	60
FLNc	M	GAGAGAGAGTTAGAGAGCGGTCGAGC	GACCACGAAACTCGCTACGCTACG	121	64
	U	GAGAGAGAGTTAGAGAGTGGTTGAGT	AACCACAAAACTCACTACACTACA	121	62

Table 3. Methylation of individual genes in gastric cancer — univariate associations with clinicopathological characteristics (OR† and 95%CI)

Genes	Male *vs.* Female	Age[1]	Tumor localization[2]	Tumor size[3]	Differentiation[4]
MLF1	1.14 (0.48–2.70)	0.92 (0.64–1.32)	0.77 (0.50–1.19)	1.35 (0.86–2.13)	1.27 (0.61–2.64)
MGMT	1.52 (0.37–6.32)	0.87 (0.46–1.65)	1.21 (0.54–2.70)	1.48 (0.65–3.37)	1.27 (0.34–4.76)
p16	0.82 (0.34–1.95)	1.01 (0.71–1.44)	1.12 (0.72–1.73)	1.22 (0.78–1.91)	2.33 (1.11–4.93)*
RASSF2	0.41 (0.09–1.92)	0.80 (0.48–1.34)	0.89 (0.48–1.65)	1.63 (0.85–3.15)	1.22 (0.43–3.46)
hMLH1	1.52 (0.48–4.76)	1.12 (0.68–1.86)	0.99 (0.53–1.83)	1.63 (0.85–3.15)	0.40 (0.14–1.16)
HAND1	1.00 (0.33–3.03)	1.09 (0.69–1.72)	1.00 (0.57–1.74)	1.16 (0.65–2.06)	1.25 (0.49–3.20)
HRASLS	0.50 (0.20–1.23)	1.01 (0.71–1.45)	1.15 (0.74–1.78)	0.99 (0.64–1.55)	0.76 (0.37–1.56)
TM	1.03 (0.44–2.43)	1.10 (0.77–1.56)	0.94 (0.61–1.45)	0.95 (0.61–1.49)	0.84 (0.41–1.72)
FLNc	0.81 (0.33–2.01)	0.76 (0.52–1.10)	1.30 (0.82–2.05)	1.28 (0.81–2.04)	1.15 (0.54–2.44)
(Continued)					
Genes	Tumor invasion[5]	Tumor stage[6]	Lymph node metastasis	No. of LNM[7]	Cancer-related death
MLF1	1.06 (0.62–1.82)	1.15 (0.76–1.73)	1.64 (0.76–3.50)	1.65 (1.06–2.56)*	1.86 (0.89–3.89)
MGMT	1.79 (0.53–6.12)	1.59 (0.70–3.62)	6.33 (0.77–51.7)	2.29 (1.11–4.75)*	5.08 (1.03–25.1)*
p16	1.25 (0.72–2.15)	1.42 (0.93–2.14)	2.62 (1.20–5.71)*	1.85 (1.18–2.90)**	6.76 (3.02–15.1)**
RASSF2	0.83 (0.41–1.71)	1.03 (0.58–1.82)	1.61 (0.53–4.92)	1.67 (0.94–2.98)	6.67 (1.80–24.7)**
hMLH1	1.33 (0.57–3.07)	1.86 (0.94–3.69)	3.40 (0.92–12.6)	1.67 (0.94–2.98)	3.16 (1.04–9.64)*
HAND1	1.25 (0.60–2.59)	1.10 (0.65–1.85)	1.44 (0.54–3.86)	1.28 (0.76–2.17)	1.45 (0.57–3.66)
HRASLS	0.85 (0.50–1.44)	0.97 (0.65–1.45)	1.85 (0.87–3.94)	1.38 (0.90–2.12)	1.33 (0.65–2.75)
TM	0.87 (0.51–1.48)	1.00 (0.67–1.49)	1.25 (0.60–2.62)	1.30 (0.85–1.99)	1.04 (0.51–2.14)
FLNc	0.95 (0.55–1.65)	1.13 (0.74–1.71)	1.36 (0.63–2.96)	1.54 (0.99–2.39)	2.52 (1.17–5.41)*

†OR: odds ratio with 95% confidence interval; 1 Age (per 10 years); 2 Tumor localization (gastric cardia; gastric body; gastric antrum); 3 Tumor size (≤3 cm; >3cm and ≤5 cm; >5); 4 Differentiation (well or moderate; poor or no differentiation); 5 Tumor invasion (T1; T2; T3; T4); 6 Tumor stage (I; II; III; IV); 7 No. of LNM (lymph node metastasis) (0; 1-6; 7-15; >16); *, P <0.05; **, P <0.01

Table 4. Methylation of individual genes in gastric cancer — multivariable models assessing age, differentiation, tumor stage, lymph node metastasis and cancer-related death (OR† and 95%CI)

Genes	Age[1]	Differentiation [2]	Tumor stage[3]	Lymph node metastasis	Cancer-related death
MLF1	0.91 (0.62–1.34)	1.15 (0.53–2.49)	0.85 (0.47–1.56)	1.50 (0.47–4.79)	1.74 (0.72–4.24)
MGMT	0.73 (0.35–1.53)	0.94 (0.22–4.06)	0.43 (0.14–1.34)	8.10 (0.55–120)	3.02 (0.50–18.2)
p16	1.08 (0.71–1.66)	2.59 (1.08–6.23)*	0.69 (0.35–1.35)	1.26 (0.35–4.58)	8.52 (2.99–24.2)**
RASSF2	0.79 (0.44–1.44)	1.02 (0.32–3.27)	0.53 (0.21–1.35)	1.00 (0.15–6.86)	13.6 (2.49–73.7)**
hMLH1	0.89 (0.49–1.62)	0.30 (0.09–0.97)*	1.40 (0.53–3.74)	1.72 (0.26–11.3)	2.18 (0.58–8.24)
HAND1	1.11 (0.69–1.78)	1.27 (0.48–3.38)	0.86 (0.40–1.86)	1.43 (0.32–6.35)	1.33 (0.43–4.09)
HRASLS	0.96 (0.65–1.40)	0.69 (0.32–1.51)	0.54 (0.28–1.06)	4.35 (1.19–15.9)*	1.11 (0.45–2.71)
TM	1.07 (0.74–1.55)	0.85 (0.40–1.82)	0.83 (0.46–1.50)	1.65 (0.53–5.14)	0.94 (0.39–2.25)
FLNc	0.72 (0.48–1.08)	0.92 (0.41–2.08)	0.90 (0.48–1.70)	0.90 (0.26–3.08)	3.15 (1.20–8.25)*

† OR: odds ratio with 95% confidence interval; [1] Age (per 10 years); [2] Differentiation (well or moderate; poor or no differentiation); [3] Tumor stage (I; II; III; IV); *, P <0.05; **, P <0.01

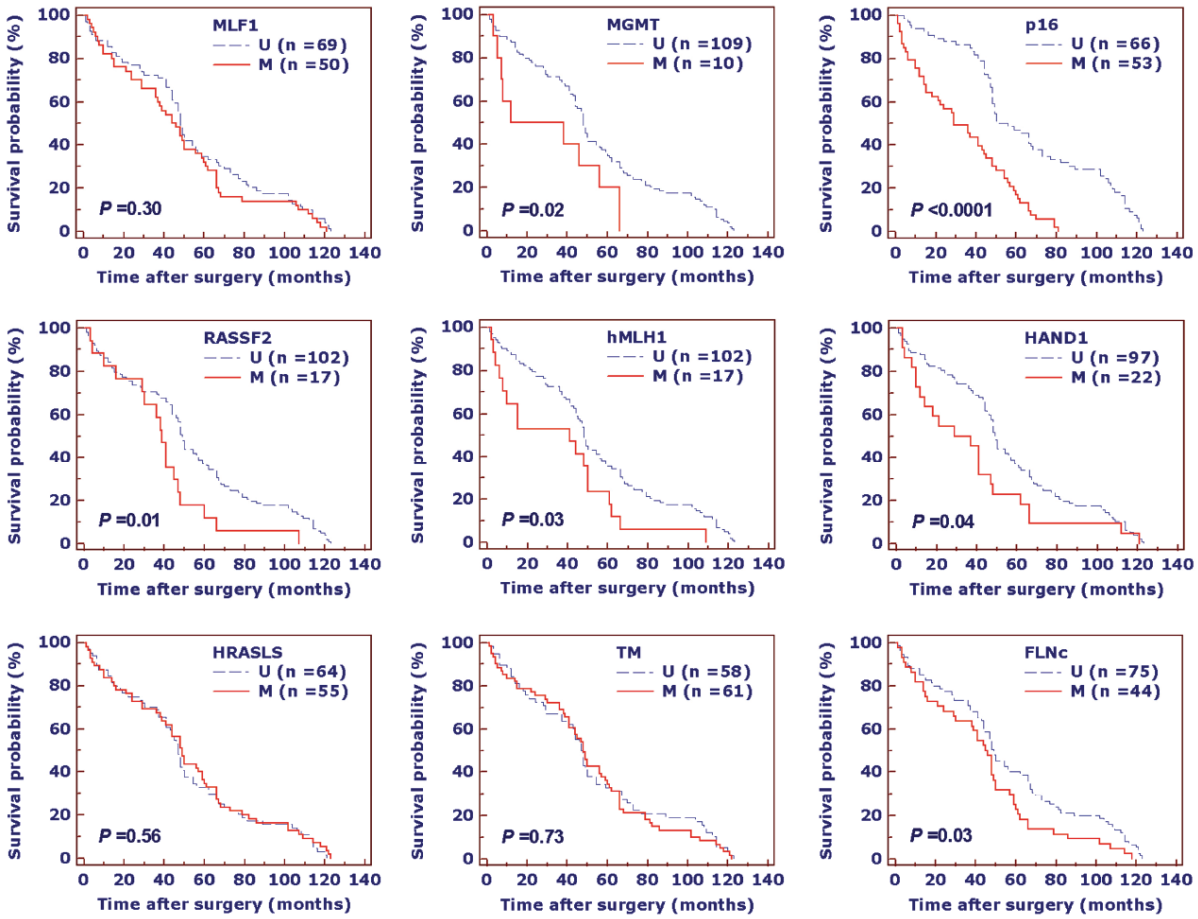


Figure 2. Effect of gene methylation on poor survival in gastric cancer. Kaplan-Meier survival curves were made according to the presence of gene methylation in a large cohort of gastric cancers. The patients with methylation of *MGMT*, *p16*, *RASSF2*, *hMLH1*, *HAND1*, and *FLNc* had significantly poorer survival times than the patients without gene methylation. Methylation of *MLF1* (*P* =0.30), *HRASLS* (*P* =0.56), and *FLNc* (*P* =0.73) was not associated with poor survival of gastric cancer patients. M, methylated genes; U, unmethylated genes.

lymph node metastasis (OR = 4.35, 95% CI = 1.19—15.9; *P* <0.05) (**Table 4**).

The effect of gene methylation on poor prognosis in gastric cancer

The Kaplan-Meier estimator of the survivorship function was used to evaluate the impact of aberrant gene methylation on the survival of gastric cancer patients. The survival of gastric cancer patients with and without gene methylation was compared using the log-rank test. As shown in **Figure 2**, methylation of *MGMT* (*P* =0.02), *p16* (*P* <0.0001), *RASSF2* (*P* =0.01), *hMLH1* (*P* =0.03), *HAND1* (*P* =0.04), and *FLNc* (*P* =0.03) was significantly associated with poor survival of gastric cancer patients. However, methylation of *MLF1* (*P* =0.30), *HRASLS* (*P* =0.56), and *TM* (*P* =0.73) did not affect the overall prognosis of gastric cancer patients.

Numerous evidences showed that residual tumor after surgery is an independent risk factor for gastric cancer patients. We thus sought to evaluate the effect of residual tumor after surgery on the survival of gastric cancer patients in the present study. As shown in **Figure 3**, the patients with residual tumor after surgery had significantly shorter survival times than the patients without residual tumor (34.4 years *vs.* 53.5 years on average; *P* =0.04). Given the impact of residual tumor after surgery on poor survival of gastric cancer patients, we excluded the patients with residual tumor to determine the association of gene methylation with poor survival in gastric cancer. Similar to the find-

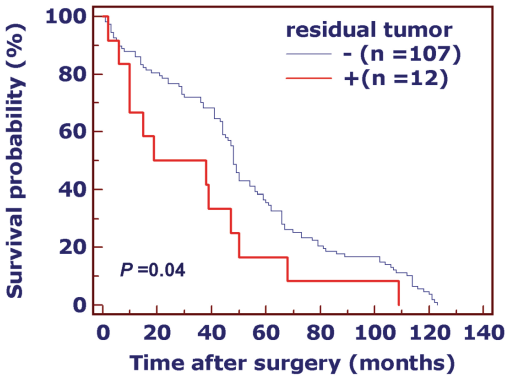
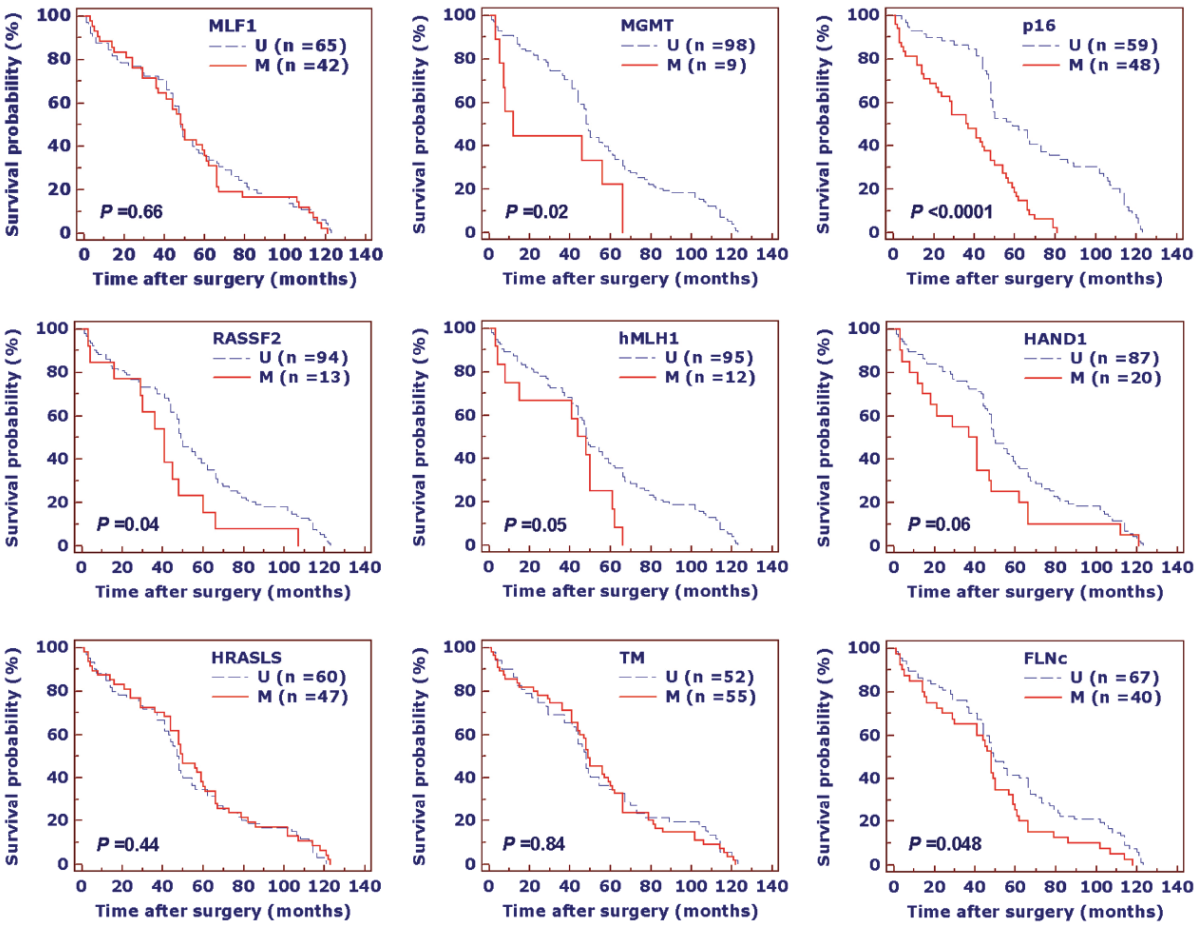


Figure 3. Effect of residual tumor after surgery on poor survival in gastric cancer. Survival was evaluated according to the presence of residual tumor after surgery in gastric cancer. Kaplan-Meier survival curves show that the patients with residual tumor after surgery had significantly shorter survival times than the patients without residual tumor (*P* =0.04). +, the patients with residual tumor after surgery; -, the patients without residual tumor after surgery.

ings in **Figure 2**, methylation of *MLF1* (*P* =0.66), *HRASLS* (*P* =0.44), and *TM* (*P* =0.84) did not have any prognostic value for gastric cancer patients. Methylation of *MGMT* (*P* =0.02), *p16* (*P* <0.0001), *RASSF2* (*P* =0.01), and *FLNc* (*P* =0.048) remained significantly associated with poor survival of gastric cancer patients (**Figure 4**). Methylation of *hMLH1* (*P* =0.05) and *HAND1*

Figure 4. Association of gene methylation with poor survival of gastric cancer patients without residual tumor. Kaplan-Meier analysis of survival was performed according to the status of gene methylation in a large cohort of gastric cancer patients without residual tumor. Methylation of *MGMT*, *p16*, *RASSF2* and *FLNc* was significantly associated with poor survival in gastric cancer. Methylation of *hMLH1* (*P* =0.05) and *HAND1* (*P* =0.06) was marginally significantly associated with poor survival of gastric cancer patients. Methylation of *MLF1* (*P* =0.66), *HRASLS* (*P* =0.44) and *TM* (*P* =0.84) did not affect poor prognosis of gastric cancer patients. M, methylated genes; U, unmethylated genes.



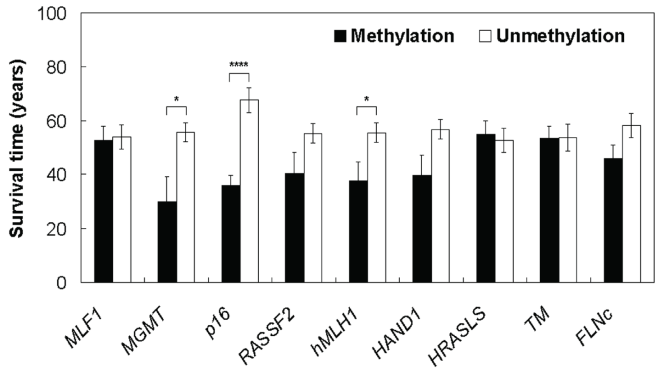


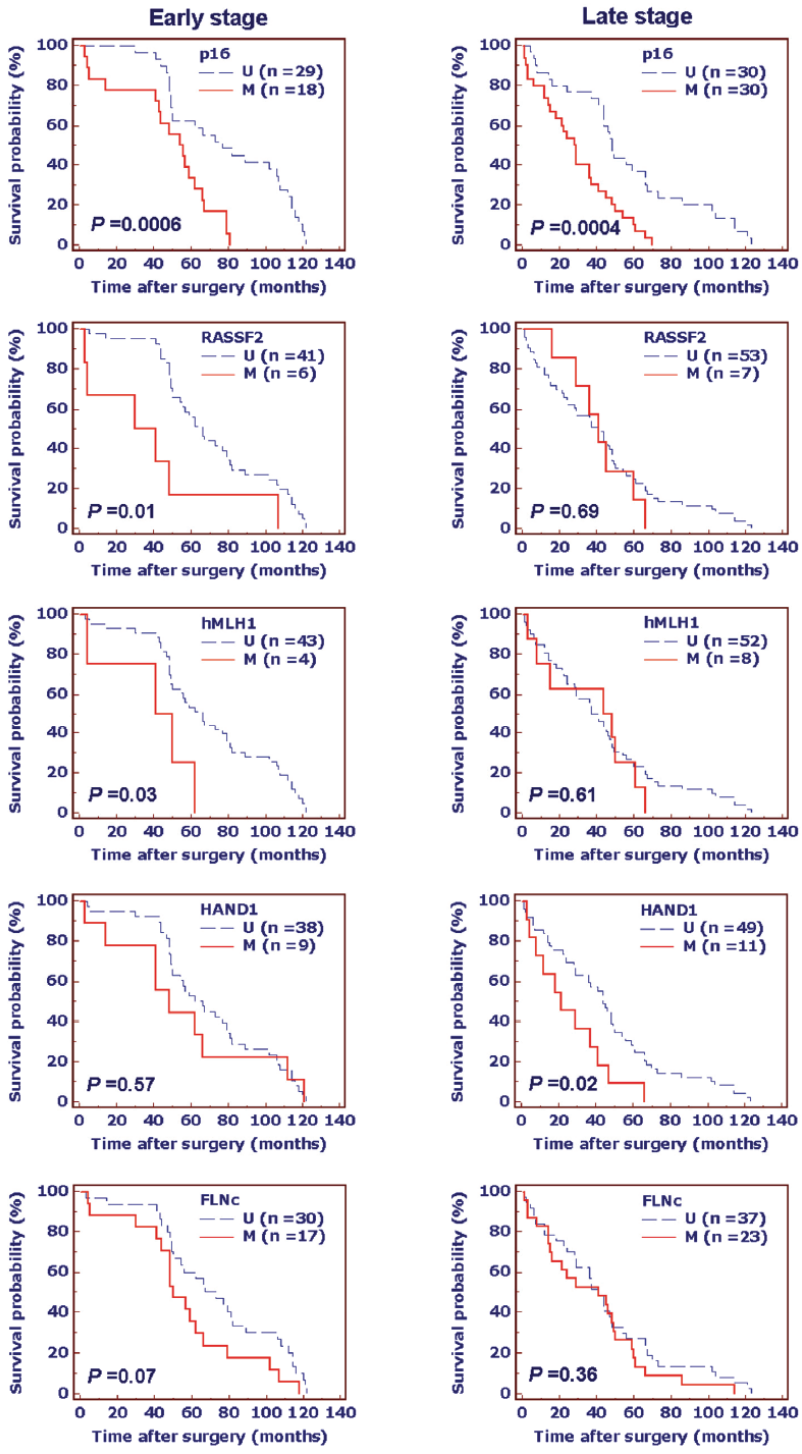
Figure 5. Effect of gene methylation on survival time of gastric cancer patients. The patients with methylation of *MGMT*, *p16*, *RASSF2*, *hMLH1*, *HAND1*, and *FLNc* had shorter survival times than the patients without methylation of these genes (*MGMT*: 29.9 years *vs.* 55.7 years on average; *p16*: 36.1 years *vs.* 67.6 years on average; *RASSF2*: 40.5 years *vs.* 55.3 years on average; *hMLH1*: 37.7 years *vs.* 55.5 years on average; *HAND1*: 39.7 years *vs.* 56.7 years on average; *FLNc*: 46 years *vs.* 58 years on average). Methylation of *MLF1*, *HRASLS* and *TM* did not affect survival time of gastric cancer patients. The data were presented as the mean ± SE. *, P <0.05; ****, P <0.0001.

(P =0.06) was still closely associated with poor survival of gastric cancer patients, although these associations did not reach statistical difference (**Figure 4**). The patients with methylation of these genes had shorter survival times than the patients without methylation of these genes, particularly *MGMT* gene (29.9 years *vs.* 55.7 years on average, P =0.03), *p16* gene (36.1 years *vs.* 67.6 years on average, P <0.0001), and *hMLH1* gene (37.7 years *vs.* 55.5 years on average, P =0.03) (**Figure 5**). The data were further stratified based on the TNM tumor stage, because it is also an independent risk factor for gastric cancer patients. As shown in **Figure 6**, *p16* methylation was significantly associated with poor survival whatever the patients who had early-stage tumors (stage I and II) or late-stage tumors (stage III and IV). Methylation of *RASSF2*, *hMLH1* and *FLNc* was more closely associated with poor prognosis in the patients who had early-stage tumors compared with the patients who had late-stage tumors, particularly *RASSF2* and *hMLH1* genes. Conversely, *HAND1* methylation was more closely associated with poor survival in the patients who had late-stage tumors compared with the patients who had early-stage tumors.

Discussion

Aberrant promoter methylation can permanently inactivate tumor-associated genes, particularly tumor suppressor genes, as mutations and chromosomal abnormalities do. Importantly, promoter methylation is more frequently present than gene mutations in gastric cancer [8]. Thus, we can use this frequent molecular event as a marker instead of gene mutations for early diagnosis and prognostic evaluation of this cancer. In the present study, we analyzed promoter methylation of *MLF1*, *MGMT*, *p16*, *RASSF2*, *hMLH1*, *HAND1*, *HRASLS*, *TM*, and *FLNc* genes using MSP method in a large cohort of gastric cancers. Although these genes were frequently methylated in gastric cancer [17-20], their association with clinicopathological characteristics and clinical outcome in gastric cancer remains largely unknown.

Similar to the previous study, our data also demonstrated that these gastric cancer-associated genes were aberrantly methylated in Chinese patients with gastric cancer, ranging from 8% to 51%. Given aberrant methylation of these genes may play a critical role in gastric tumorigenesis, we investigated their clinical significances and prognostic values in gastric cancer patients who had known survival data. Our findings showed that *p16* methylation was positively associated with poor tumor differentiation, which is consistent with a previous study [21]. Similar to the previous studies that loss of p16 protein expression was reversely associated with lymph node metastasis [22,23], *p16* methylation was positively associated with lymph node metastasis in the present study. In addition, our data showed that *MGMT* methylation was closely associated with lymph node metastasis in gastric cancer, which is consistent with the previous studies [24,25]. HRASLS is human homologue of mouse A-C1, which has been reported to inhibit growth of *ras*-transformed NIH3T3 cells [26,27]. Although the role of HRASLS in human gastric carcinogenesis still needs to be studied, our data first demonstrated that its methylation in gastric cancer was significantly positively associated with lymph node metastasis in the present study. Of note, methylation of *MGMT*, *p16*, *RASSF2*, *hMLH1*, and *FLNc*, particularly *p16*, *RASSF2*



and *FLNc*, was associated with a potentially increased risk of gastric cancer-related death. Collectively, these data suggested that aberrant methylation of these genes may contribute to oncologic outcomes of gastric cancer patients.

More importantly, like what observed in the previous studies [24,28], methylation of *p16* and *MGMT* was significantly associated with poor survival of gastric cancer patients in the present study. RASSF2, a member of the RASSF1 family, has recently been identified as a potential tumor suppressor, which is frequently hypermethylated in human cancers, including gastric cancer [18,29,30]. In the present study, we first demonstrated that *RASSF2* methylation was significantly associated with poor survival of gastric cancer patients, suggesting that this epigenetic event may be used to predict poor prognosis of this cancer. In support of this, a previous study showed that a combination of *RASSF1A* and *RASSF2* methylation was found to be significantly associated with poor disease-free survival in oral squamous cell carcinoma [31]. FLNc is a member of the filamin family, which is known to organize actin polymerization in response to various signals [32]. Methylation-associated inactivation of this gene is

Figure 6. Effect of gene methylation on poor survival of gastric cancer patients at different tumor stages. The data were stratified based on two different TNM tumor stages (early stage, stage I+II; late stage, stage III+IV). Methylation of *p16* gene significantly affected the poor survival in gastric cancer whatever the patients who had early-stage or late-stage tumors. Methylation of *RASSF2*, *hMLH1* and *FLNc* was more relevant to poor survival in the patients who had early-stage tumors compared with the patients who had late-stage tumors. Reversely, *HAND1* methylation more significantly affected poor survival in the patients who had late-stage tumors compared with the patients who had early-stage tumors. M, methylated genes; U, unmethylated genes.

frequently found in gastric cancer [17,19,33]. In the present study, we first demonstrated that *FLNc* methylation was significantly associated with poor survival and cancer-related death in gastric cancer, suggesting that abrogation of normal actin polymerization may be important in gastric carcinogenesis. In line with this finding is a recent example that simultaneous methylation of multiple genes predicted significantly worse survival in colon cancer, including *FLNc* methylation [34]. Methylation of *hMLH1* and loss of its expression has been well documented in sporadic gastric carcinomas with high-frequency microsatellite instability (MSI-H) [35,36]. A previous study showed that the overall survival was significantly shorter in the patients with *hMLH1* methylation compared to the patients without *hMLH1* methylation in colorectal cancer [37]. Similarly, our data showed that *hMLH1* methylation was closely associated with poor survival of gastric cancer patients, although no statistical significance was noted, suggesting that this epigenetic alteration may be a valuable marker for the prognosis of gastric cancer patients. *HAND1* encodes a base helix-loop-helix transcriptional factor, which is essential for placental development and cardiac morphogenesis [38]. The previous study [17] and the present study and showed that this gene was aberrantly methylated in gastric cancer, suggesting that its expression in normal gastric mucosa and silencing in gastric cancers may play a role in the maintenance of differentiated status of gastric epithelium. Moreover, *HAND1* methylation was also associated with poor survival in gastric cancer, further suggesting that this gene plays an important role in gastric carcinogenesis. Of interest, *p16* methylation affected the overall prognosis in gastric cancer whatever the patients who had early-stage or late-stage tumors, suggesting that this gene plays an important role in the multistep process of gastric carcinogenesis. Moreover, compared with the patients who had late-stage tumors, methylation of *RASSF2*, *hMLH1* and *FLNc* was more closely associated with poor survival in the patients who had early-stage tumors, particularly *RASSF2* and *hMLH1*, suggesting that these genes play a role in early stage of gastric carcinogenesis. Reversely, *HAND1* may play a role in late stage of gastric carcinogenesis.

In summary, we analyzed a panel of gastric cancer-associated genes in a large cohort of gastric cancers, and demonstrated that promoter methylation was strongly associated with certain clinicopathological characteristics, such as tumor differentiation, lymph node metastasis, and cancer-related death. More importantly, hypermethylation of some genes was closely associated with poor survival of gastric cancer patients. Thus, these aberrantly methylated genes may be served as potential biomarkers for early diagnosis and prognostic evaluation in gastric cancer.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 30901459 and 30973372) and the National Key Program for Developing Basic Research (No. 2010CB933903).

Declaration of conflicts of interest

The authors declare that they have no competing interests.

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