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Predictive value of the serum *RASSF10* promoter methylation status in gastric cancer

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Abstract

Background: This study aimed to investigate whether the detection of methylation in the promoter of the Ras association domain family 10 gene (*RASSF10*) in the serum of patients with gastric cancer (GC) by methylation-specific PCR (MSP) can be used as a diagnostic and prognostic indicator of GC.

Methods: We used MSP to examine *RASSF10* methylation levels in the serum and/or tumor samples from 100 GC patients, 50 patients with chronic atrophic gastritis (CAG), and 45 healthy controls (HC). We also analyzed clinicopathological and follow-up data.

Results: Our results showed that the rate of serum *RASFF10* promoter methylation among patients with GC (49/100) was higher than in those with CAG (1/50) or HC (0/45). Moreover, the *RASSF10* methylation status was consistent between serum and tumor tissues. GC patients with serum *RASSF10* promoter methylation had significantly shorter overall survival and disease-free survival times than GC patients without serum *RASSF10* promoter methylation. Multivariable Cox regression analysis showed that serum *RASSF10* promoter methylation and lymph node metastasis both correlated with reduced survival in GC patients.

Conclusions: Detection of the serum *RASSF10* methylation status by MSP is feasible as a diagnostic and prognostic indicator of GC.

Keywords

Gastric cancer, RASSF10, methylation, methylation-specific PCR, prognosis, diagnosis

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Introduction

Gastric cancer (GC) is one of the most common malignancies in the world. Although the overall worldwide incidence has been declining in recent years, the absolute incidence remains very high; indeed, GC has the fourth and second highest morbidity and mortality rates, respectively, among all malignancies.[1] In China, GC has incidence and mortality rates that are among the highest for any type of tumor. The number of new patients diagnosed worldwide with GC each year can reach 470,000, of which nearly 90% are in a progressive stage.[2] One reason that so many cases of GC are at an advanced stage at the time of diagnosis is related to patients' fears of invasive gastroscopy examinations. Therefore, it is of great importance to find a less invasive diagnostic method to improve the early diagnosis and prognosis of GC.

Tumor biomarkers are bioactive substances synthesized and secreted by tumor cells or by the body's response to tumor cells during the development of malignant tumors.[3] Traditional tumor markers mainly include glycoproteins, embryonic antigens, and secretory proteins in the serum, which can be detected in the peripheral blood of healthy individuals but show increased levels in patients with tumors.[4] At present, the sensitivity of commonly used markers such as carcinoembryonic antigen (CEA), cancer antigen (CA)125, and CA199 is rather low, with a detection rate of less than 30% in GC patients, thus limiting their application in GC diagnosis.

With increasing research into tumor markers, free circulating nucleic acids have attracted attention as a new type of marker. Nucleic acids are released into the blood early on during tumor occurrence and development,[5–7] and carry abnormal changes related to tumor genetics and epigenetics.[8] Free circulating nucleic acids in the blood include cell-free DNA (cf-DNA), mRNA, and micro RNA. The study of methylation markers in cf-DNA in peripheral blood has become a hot topic in tumor marker research.[9] Qualitative bisulfite sequencing (BSP) PCR and quantitative methylation-specific (MSP) PCR are among the methods used to detect DNA methylation, with MSP one of the most common and economical techniques to evaluate methylation in promoter regions.

Located on human chromosome 11p15.2, the Ras association domain family 10 gene (*RASSF10*) is the most recently identified member of the RASSF of genes. It is a tumor suppressor gene that was recently found to be inactivated by promoter methylation. *RASSF10* contains a 2,254 bp CpG island with 209 CpG sites, making it the most frequently N-terminally methylated gene in the RASSF family.[10] The RASSF10 protein is composed of 507 amino acids with a molecular weight of 57 kD.[11] In a variety of human tumor tissues, RASSF10 expression is reduced or absent because of a high level of promoter methylation. This promotes tumor cell growth,[12,13] inhibits cell apoptosis,[14] promotes cell migration and invasion, and reduces the sensitivity of tumor cells to chemotherapeutic drugs,[13,15] thus promoting the occurrence and development of the tumor. The effectiveness of MSP to qualitatively detect the methylation status of *RASSF10* in cf-DNA for the diagnosis and prognosis of GC has not been reported.

Therefore, in the present study, we used MSP to detect the methylation status of the *RASFF10* promoter in serum from patients with GC, mild chronic atrophic gastritis (CAGI), moderate chronic atrophic gastritis (CAGII), severe chronic atrophic gastritis (CAGIII), and healthy controls (HC). We combined these results with patient follow-up data to determine whether the serum *RASFF10* promoter methylation status can be used as a marker for GC diagnosis and prognosis. Our study is likely to

provide a new and accessible biomarker for the diagnosis and prognosis of clinical GC.

Materials and methods

Specimen information

The study inclusion criteria included all GC patients who underwent standard D2 lymphadenectomy from March to September 2010 and who had complete clinical pathology and follow-up data at the General Surgery Department of the Affiliated Hospital of Nantong University (Jiangsu, China). Patients with clear GC pathology did not receive neoadjuvant chemotherapy, radiation therapy, or immunotherapy before surgery. Patients who presented with other malignant tumors or distant metastasis preoperatively were excluded from this study. Paired serum and tumor tissue samples were collected from GC patients (n=100). Serum samples from 50 sex- and age-matched CAG patients (15 with CAGI, 15 with CAGII, and 20 with CAGIII [gastritis severity defined by histopathological sections]) and 45 HCs with no abnormalities detected by gastroscopy were collected during the same period. Follow-up was completed by September 2015. We obtained clinical data including age, sex, tumor diameter, tumor differentiation, CEA levels, tumor T stage, lymph node metastasis, and cell proliferation antigen (Ki67) levels from patient medical records. All study participants (including CAG patients and HCs) gave informed written consent for the collection of specimens and information. The study was approved by the Ethics Committee of the Affiliated Hospital of Nantong University.

Serum and tissue specimen treatment

A total of 5 mL peripheral venous blood was extracted from fasting participants in the morning using vacuum blood-collection tubes. As soon as possible after collection, tubes were centrifuged for 5 minutes at $1{,}000 \times g$. After centrifugation, the upper serum was transferred to a 2-mL EP tube and the remainder was kept for other experimental procedures. EP tubes were marked for serum DNA extraction and stored at –80°C. Fresh tumor tissue samples were obtained within 30 minutes after surgical resection, frozen in liquid nitrogen, and stored at –80°C.

DNA extraction and sulfite treatment

Genomic DNA was extracted from blood samples using the QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia, CA, USA) and from tumor samples using the QIAamp DNAMini Kit (Qiagen Inc.). Extracted genomic DNA samples (1 μg in a 20-μL volume) were processed by modifying with sulfite using the EZ DNA Methylation-Gold Kit (Zymo Research Corp., Irvine, CA). All extractions and processing were performed according to the manufacturers' instructions.

Methylation-specific PCR and electrophoretic analysis

Methylation-specific (M) and unmethylation-specific (U) primer sequences for PCR amplification of the *RASSF10* promoter were MF: 5′-GGGTATTTTGGGT AGAGTTAGAGC-3′ and MR: 5′-AAA CAAACTAAAAAA CGACTACGAC-3′; and UF: 5′-GGGTATTTTGGGTAGAG TTAGAGTG-3′ and UR: 5′-AAACAA ACTAAAAAACAACTACAAC-3′, respectively.[16] The reaction system contained 2.0 μL sulfite-modified DNA template, 1.0 μL 20-mM upstream and downstream primers, and 10 μL TaqMix, adjusted with double-distilled water (ddH_2O) to a final volume of 20 μL. PCR conditions were as follows: pre-denaturation at 95°C for 10 minutes followed by 40 cycles of 95°C

for 30 s, 60°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 10 minutes. After the reaction, 5 μL of the PCR product was subjected to 2.0% agarose gel electrophoresis and the results were visualized using the Bio-Rad Imaging System (Bio-Rad, Hercules, CA, USA). The GC cell line AGS and normal gastric mucosa cell line GES-1 (Genechem, Shanghai, China) were used as methylated and unmethylated controls, respectively. ddH_2O was also used as a negative control.[17,18] The production of methylation-specific primer amplification products indicated methylation, while the lack of methylation-specific primer amplification indicated unmethylation.

Cell culture

AGS and GES-1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin (Hyclone, South Logan, UT, USA) in a humidified incubator at 37°C with 5% CO_2.

Follow-up

All patients were discharged in good health and were followed up every 3 months for the first 3 years after discharge and then every 6 months for a further 3 years. Follow-up included a physical examination, laboratory analysis, computed tomography, endoscopy, and B-scan ultrasonography. The diagnosis of recurrence and metastasis was based on imaging and histological examinations. The location and time of recurrence and metastasis were recorded. Overall survival (OS) refers to the time between surgery for GC and either death or the end of follow-up. Disease-free survival (DFS) refers to the time between surgery for GC and either tumor recurrence or the end of follow-up.

Statistical analysis

All analyses were performed using IBM SPSS Statistics for Windows, Version 20.0 statistical software (IBM Corp., Armonk, NY, USA). Fisher's exact test was used to compare rates of serum *RASSF10* promoter methylation among patients with GC or CAG and HCs. The Kappa statistic was used to analyze methylation levels between serum and tumor tissue samples, and the χ^2 test was used to determine the relationship between *RASSF10* promoter methylation and clinicopathological features. Factors that were determined to be potentially important in univariate analysis ($P<0.05$) were included in a multivariable, unconditional logistic regression model. Univariate and multivariate analyses were subsequently performed using a Cox regression model to identify independent risk factors. Survival curves were determined using the Kaplan–Meier method, and log-rank tests were performed to determine significance. All tests were bilateral, with $P<0.05$ chosen as the threshold for statistical significance.

Results

Serum RASSF10 methylation status in patients with GC or CAG and HCs

MSP findings revealed serum *RASSF10* promoter methylation in 49.0% (49/100) of patients with GC and in 5% (1/20) of those with CAGIII. No serum *RASSF10* promoter methylation was detected in patients with CAGI or CAGII, or in the 45 HCs (Figure 1). The rate of serum *RASSF10* promoter methylation in patients with GC was significantly higher than in those with CAGI, CAGII, and CAGIII, and among HCs ($P<0.001$). There was no difference in the rate of serum *RASSF10* promoter methylation between patients with CAGI, CAGII, and CAGIII, and HCs.

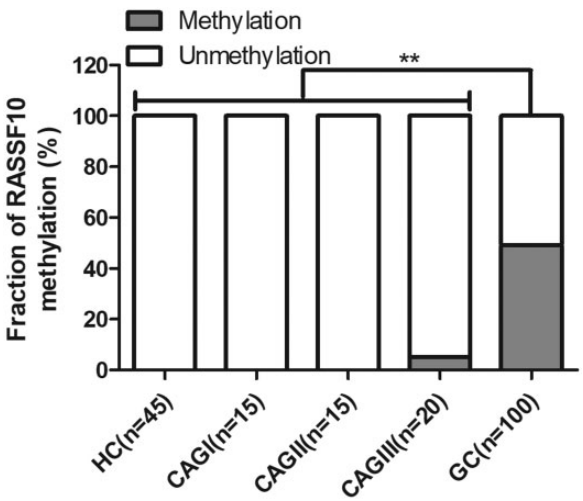

Figure 1. The number of individuals with serum *RASSF10* methylation among patients with GC, CAGI, CAGII, CAGIII, and HCs. GC: gastric cancer serum; CAGI: mild chronic atrophic gastritis serum; CAGII: moderate chronic atrophic gastritis serum; CAGIII: severe chronic atrophic gastritis serum; HC: healthy control serum. $^{**}P<0.01$, vs. HC, CAGI, CAGII, and CAGIII groups

Consistency analysis of RASSF10 methylation status in tumor tissues and serum of patients with GC

MSP revealed *RASSF10* methylation in tumor tissues from 55% (55/100) of patients with GC; 47 of GC patients had *RASSF10* promoter methylation in both serum and tissues (Figure 2), while 43 of GC patients had no *RASSF10* promoter methylation in both serum and tissues. There was a positive correlation between the methylation status of the serum and that of the GC tissue (kappa=0.8, $P<0.001$).

Correlation between the serum RASSF10 methylation status and the pathologic features of patients with GC

Univariate analysis of the serum *RASSF10* promoter methylation status and the clinicopathological characteristics of patients with GC showed that the methylation status was not correlated with patient age, sex, CEA, Ki67, or differentiation level (Table 1). The rate of serum *RASSF10* promoter methylation among patients with T stage I–II tumors was significantly lower than among those with T stage III–IV tumors ($P=0.019$). Serum *RASSF10* promoter methylation was significantly more common among patients with lymph node metastases than in those without lymph node metastasis ($P=0.001$). Multiple logistic regression analysis showed that serum *RASSF10* promoter methylation was significantly correlated with lymph node metastasis (odds ratio=3.064, 95% confidence interval [CI]=1.222–7.681, $P=0.017$).

Effect of the serum RASSF10 methylation status on postoperative survival in patients with GC

The 5-year OS and DFS rates among patients with GC with *RASSF10* promoter methylation were 24.5% and 6.1%, respectively, versus 70.6% and 64.7%, respectively, among patients without *RASSF10* promoter methylation. Clinical follow-up data and Kaplan–Meier survival curves of patients with GC showed that OS and DFS

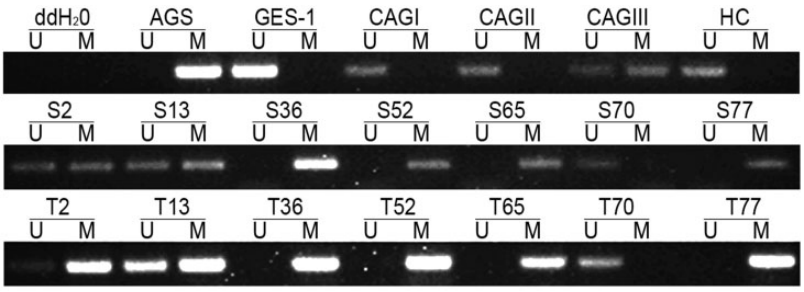

Figure 2. Typical methylation analysis of *RASSF10* promoter by MSP. T: GC tissues; S: GC serum; CAGI: mild chronic atrophic gastritis serum; CAGII: moderate chronic atrophic gastritis serum; CAGIII: severe chronic atrophic gastritis serum; HC: healthy control serum; M: methylation; U: unmethylation; AGS: gastric cancer cell line; GES-1: normal gastric mucosa cell line; ddH_2O: negative control; MSP: methylation-specific PCR; RASSF10: Ras association domain family 10. Numbers indicate the serial numbers of GC patients

Table 1. Relationship between RASSF10 methylation and clinicopathological characteristics in patients with GC

Clinicopathological characteristics	n	Unmethylation	Methylation	χ^2	*P* value
Total	100	51	49		
Sex				1.940	0.164
Male	38	16	22		
Female	62	35	27		
Age (years)				0.662	0.416
≤65	53	25	28		
>65	47	26	21		
Grade of differentiation				4.227	0.121
Low	48	21	27		
Middle	22	10	12		
High	30	20	10		
Tumor diameter (cm)				2.058	0.151
≤4	44	26	18		
>4	56	25	31		
T stage				5.525	**0.019**
I or II	36	24	12		
III or IV	64	27	37		
N metastasis				10.274	**0.001**
No	49	33	16		
Yes	51	18	33		
Ki67 level				1.986	0.159
≤15%	48	28	20		
>15%	52	23	29		
CEA level				0.008	0.93
≤5	84	28	41		
>5	16	8	8		

GC: gastric cancer.

were both significantly shorter in patients with serum *RASSF10* promoter methylation than in those without ($P<0.01$; Figure 3). Patients with lymphatic metastasis had significantly shorter OS and DFS times after D2 lymphadenectomy than those without lymphatic metastasis ($P<0.01$).

Multivariate Cox regression analysis showed that serum *RASSF10* promoter methylation was a significant independent risk factors both for shorter OS (hazard ratio [HR]=2.820, 95% CI=1.471–5.407, $P=0.002$) and DFS (HR=4.150, 95% CI=2.244–7.672, $P<0.001$; Table 2).

Discussion

Studies have shown that patients with tumors have higher serum cf-DNA levels than individuals without neoplasms.[4] Additionally, circulating DNA shares certain characteristics with tumor DNA[19] such as genetic mutations, microsatellite variation, and epigenetic changes. Furthermore, changes in cf-DNA levels are associated with tumor burden.[20] Therefore, cf-DNA may indirectly reflect the occurrence and development of tumors.

It is common practice to test tissue specimens after tumor resection; however, the acquisition of such specimens causes trauma for the patient, thus increasing the risks of complications such as bleeding, pain, and infection, and is not conducive to the early detection of tumors. Conversely, blood sampling is convenient, minimally invasive, and cheap, making it suitable for crowd screening.[21] Tumors can also be noninvasively and dynamically monitored in real-time by extracting circulating nucleic acids from peripheral blood. Blood sampling can thus provide important information for the early diagnosis, evaluation of treatment efficacy, relapse monitoring, and prognosis of GC.

Abnormal DNA methylation, including hypomethylation of the whole genome and hypermethylation of CpG islands in the promoters of multiple tumor-associated genes, is an epigenetic modification that is closely related to the occurrence of malignant tumors. Current widespread methods to detect DNA methylation include MSP,[22] hydrosulfite treatment and sequencing,[23] combined bisulfite restriction analysis,[24] methylation-sensitive high-resolution melting-curve analysis,[25] pyrosequencing,[26] and chip-based methylation analysis.[24] MSP is the most commonly used method to detect methylation in promoter regions.[22] It works on the principle that hydrosulfite treatment converts unmethylated cytosine to uracil while leaving methylated

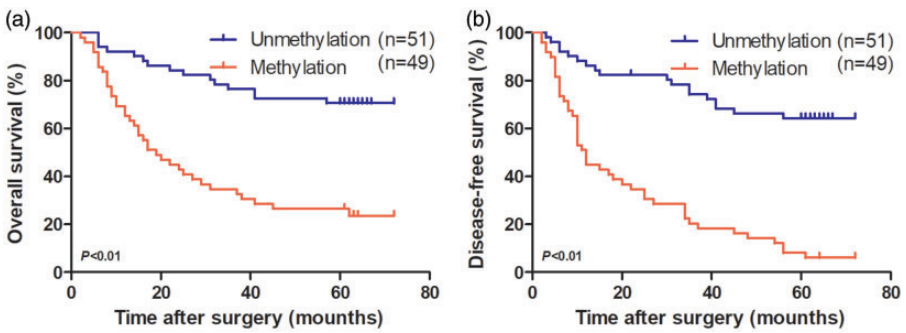

Figure 3. Survival analysis of GC patients by the Kaplan–Meier method. (a) GC patients with serum *RASSF10* methylation had shorter OS times than those with unmethylated *RASSF10*. (b) GC patients with serum *RASSF10* methylation had shorter disease-free survival than those with unmethylated *RASSF10*. GC: gastric cancer, OS: overall survival, DFS: disease-free survival

Table 2. Univariate and multivariable analysis of overall survival and disease-free survival in patients with GC

	OS			DFS		
	Univariate analysis	Multivariable analysis		Univariate analysis	Multivariable analysis	
Variable	*P*>|z|	*P*>|z|	HR (95%CI)	*P*>|z|	*P*>|z|	HR (95%CI)
RASSF10 methylation M (n=49) vs. U (n=51)	<0.001	<0.001	2.820 (1.471) U (n=51)	<0.001	<0.001	4.150 (2.244) U (n=51)
Sex Male (n=38) vs. female (n=62)	0.414			0.117		
Age (years) ≤65 (n=53) vs. >65 (n=47)	0.83			0.813		
Grade of differentiation Low (n=48) vs. middle (n=22) vs. high (n=30)	0.005			0.007		
Tumor diameter (cm) ≤4 (n=44) vs. >4 (n=56)	0.004			0.001		
T stage I or II (n=36) vs. III or IV (n=64)	0.002			<0.001		
N metastasis No (n=35) vs. Yes (n=43)	<0.001			<0.001		
Ki67 level ≤15% (n=49) vs. >15% (n=51)	0.983			0.989		
CEA level (ng/ml) ≤5 (n=84) vs. >5 (n=16)	0.365			0.425		

OS: Overall survival; DFS: Disease-free survival; GC: gastric cancer; M, methylation; U, unmethylation

cytosine unchanged.[27] PCR primers specific for methylated and unmethylated alleles can be designed accordingly and used to determine the methylation status of CpG islands in specific genes. MSP requires little DNA, no specific restriction sites, and results in no isotope contamination, making it an efficient, specific, and rapid method to detect methylation.[22] Compared with other methods, MSP is highly sensitive, simple, and affordable, so is suitable for large sample testing.[28]

The high and stable incidence of hypermethylation in the promoters of tumor suppressor genes during tumor formation and the high prevalence of promoter hypermethylation in tumor tissues mean that promoter hypermethylation is a potential tumor marker.[29] Methylation of the *RASSF10* promoter has been detected in a variety of malignant tumors such as childhood leukemia,[30] thyroid cancer,[31] cutaneous melanoma,[32] prostate cancer,[33] liver cancer,[34] lung cancer,[35] esophageal cancer,[36] and breast cancer.[15,37] Li et al.[16] used MSP to qualitatively examine the *RASSF10* methylation status of tumor specimens and adjacent healthy tissues from 86 patients with GC, and found *RASSF10* methylation in 61.6% (53/86) of tumor specimens and 38.4% (33/86) of corresponding adjacent specimens. In another study that used BSP as a quantitative method, *RASSF10* promoter methylation was detected in 62.7% of tissue samples from patients with GC compared with only 30.60% of corresponding adjacent tissues.[23] The rate of serum *RASSF10* promoter methylation was 47.84% in GC patients, compared with 11.89% and 11.35% among patients with CAG and

HCs, respectively. This study identified a correlation between serum *RASSF10* promoter methylation in serum and tumor specimens, which matched our own findings.

We used MSP to detect the serum *RASSF10* promoter methylation status. The rate of serum *RASSF10* promoter methylation was 49% (49/100) among patients with GC and 5% (1/20) among patients with CAGIII. We did not detect any serum *RASSF10* promoter methylation in patients with CAGI or CAGII or in HCs. Consistency analysis showed that the serum *RASSF10* promoter methylation status in patients with GC was consistent with that in tumor tissues from the same patients (kappa=0.8, $P<0.001$). These results therefore suggest that detection of the serum *RASSF10* promoter methylation status by MSP can be used to help diagnose GC. We also found that serum *RASSF10* promoter methylation was correlated with lymph node metastasis, and that the survival rate of GC patients with *RASSF10* promoter methylation was significantly lower than that of GC patients without *RASSF10* promoter methylation after radical D2 resection. Multivariate Cox regression analysis showed that *RASSF10* promoter methylation was an independent risk factor for poor prognosis in patients with GC. Our results suggest that the serum *RASSF10* promoter methylation status can be used as a biomarker for GC with adverse biological characteristics and poor prognosis.

Although MSP is a highly sensitive detection method, the overall rate of single-gene methylation in the serum of GC patients is not high.[38] We detected a serum *RASSF10* promoter methylation rate of 49% (49/100) in GC patients, which was higher than the rate of CEA positivity (16%, 16/100) but lower than the methylation rate of some other tumor-related genes in the serum of GC patients.[39] Therefore, the combined detection of the methylation of multiple tumor-related genes may be of greater clinical value and useful as an auxiliary means for the clinical diagnosis of GC.

One shortcoming of our research is its small sample size. Because the number of GC patients with complete clinical pathology data and prognosis follow-up data is still limited, we plan to continue increasing data collection and sample size in the future.

In conclusion, we demonstrated that it is feasible to use MSP to detect the methylation status of the *RASSF10* promoter region in patients with GC. Although plasma testing remains less sensitive than the direct testing of tumor tissues and some plasma test findings may not be consistent with those of direct detection in cancer tissues, MSP detection of plasma *RASSF10* promoter methylation has the potential to be used as an index to help determine GC diagnosis and prognosis. Our results also provide a theoretical basis for targeting CpG island methylation in the *RASSF10* promoter as a means to treat GC.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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