

## RESEARCH ARTICLE

# Hypermethylation and Clinicopathological Significance of *RASAL1* Gene in Gastric Cancer

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### Abstract

**Background:** Recent studies have suggested that expression of the RAS protein activator like-1 gene (*RASAL1*) is decreased in gastric carcinoma tissues and cell lines, indicated a role in tumorigenesis and development of gastric cancer. Reduced expression of *RASAL1* could result in aberrant increase of activity of RAS signaling pathways in cancer cells. However, the exact mechanism which induces down-regulation of the *RASAL1* gene remains unclear. This study aimed to determine the methylation status and regulation of *RASAL1* in gastric cancer. **Materials and Methods:** Using the methylation-specific polymerase chain reaction (MSP), the methylation status of CpG islands in the *RASAL1* promoter in gastric cancers and paired adjacent non-cancerous tissues from 40 patients was assessed and its clinicopathological significance was analyzed. The methylation status of *RASAL1* in gastric cancer lines MKN-28, SGC-7901, BGC-823, as well as in normal gastric epithelial cell line GES-1 was also determined after treatment with a DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (5-Aza-CdR). RAS activity (RAS-GTP) was assessed through a pull-down method, while protein levels of ERK1/2, a downstream molecule of RAS signaling pathways, were determined by Western blotting. **Results:** The frequencies of *RASAL1* promoter methylation in gastric cancer and paired adjacent non-cancerous tissues were 70% (28/40) and 30% (12/40) respectively ( $P < 0.05$ ). There were significantly correlations between *RASAL1* promoter methylation with tumor differentiation, tumor size, invasive depth and lymph node metastasis in patients with gastric cancer (all  $P < 0.05$ ), but no correlation was found for age or gender. Promoter hypermethylation of the *RASAL1* gene was detected in MKN-28, SGC-7901 and BGC-823 cancer cells, but not in the normal gastric epithelial cell line GES-1. Elevated expression of the *RASAL1* protein, a decreased RAS-GTP and p-ERK1/2 protein were detected in three gastric cancer cell lines after treatment with 5-Aza-CdR. **Conclusions:** Aberrant hypermethylation of the *RASAL1* gene promoter frequently occurs in gastric cancer tissues and cells. In addition, the demethylating agent 5-Aza-CdR can reverse the hypermethylation of *RASAL1* gene and up-regulate the expression of *RASAL1* significantly in gastric cancer cells *in vivo*. Our study suggests that *RASAL1* promoter methylation may have a certain relationship with the reduced *RASAL1* expression in gastric cancer.

**Keywords:** Gastric cancer - *RASAL1* - methylation

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### Introduction

The carcinogenesis and development of gastric cancer is a complex, multistep process which involving in a lot of oncogenes, tumor suppressor genes, DNA repair genes, cell cycle regulators and signaling molecules undergoing genetic and epigenetic alterations. Studies (Pino et al, 2010) have shown that the RAS p21 protein is one of the hub molecular which controlling many signaling cascades such as "RAS-RAF-MEK-ERK" pathway (related to cell proliferation), "RAS-APKKKs-MKK4/7-JNK" pathway

(related to growth, differentiation, survival, apoptosis), "RAS-TIAM1-Rac-Rho" pathway (related to cytoskeleton organization, cell motility), "RAS-PI3K-PDK1-AKT/PKB" pathway (related to apoptosis, cell survival)", "RAS-Ral GDS-Ral-PLD" pathway (related to cell transformation, vesicle) and "RAS-Ral GDS-TBK1-NF  $\kappa$ B" pathway (related to cell survival, inflammation). Obviously, RAS p21 plays an important role in the process of carcinogenesis and development of malignant tumor. The RAS p21 have two distinct states: the inactive GDP-bound state and the active GTP-bound state, of which

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transition are controlled by RAS GTPase-activating proteins (GAPs) and RAS guanine nucleotide exchange factors (GEFs). RAS protein activator like-1 (*RASAL1*) is a newly found gene. The protein encoded by *RASAL1* is a member of the GAP family of GTPase-activating proteins. The *RASAL1* protein could stimulate the intrinsic GTPase activity of RAS p21 resulting in the inactive GDP-bound form of RAS, thereby allowing control of processes of differentiation, proliferation, apoptosis, cell adhesion and cell migration.

On the study of colon cancer, it was found that the expression of *RASAL1* was down-regulated which leading to the aberrant activating RAS p21 protein and RAS p21 regulating signaling pathways. The decreased expression of *RASAL1* gene was also verified in hepatic cancer, bladder cancer and so on (Jin et al., 2007; Calvisi et al., 2011). So the *RASAL1* gene was considered as a candidate tumor suppressor gene. Our early study had demonstrated that the expression of *RASAL1* is also markedly decreased in gastric carcinoma tissues and cell lines, which is associated with gastric carcinoma differentiation degree and progression, suggesting *RASAL1* may contribute to gastric carcinogenesis (Jin et al., 2007; Chen, et al., 2012). This study is aimed to investigate the mechanism which inducing the decreased expression of *RASAL1*, in order to explore whether this were relevant to the methylation of *RASAL1* gene promoter.

## Materials and Methods

### *Patients and Tissue Samples*

Gastric cancer and paired adjacent non-cancerous tissue specimens were obtained from 40 patients who underwent surgically partial or total gastrectomy between March 2011 and December 2011 in the Affiliated Zhongda Hospital of Southeast University (Nanjing, China), with clinical and pathological data (such as age, gender, tumor size, differentiation, location, depth of tumor invasion and lymph node metastasis) available. All the patients did not receive any treatment before operation. The adjacent non-cancerous tissue were derived from the tissues which leaving the tumor edge 5.0 cm or more. All procedures were permitted by the ethics committee of Zhongda Hospital, Southeast University. Written informed consent was obtained from all patients.

### *Cell Lines*

The well differentiated gastric adenocarcinoma cell MKN-28, the moderately differentiated gastric adenocarcinoma cell SGC-7901 and the poorly differentiated gastric adenocarcinoma cell BGC-823 were all obtained from the Shanghai Institute of Biochemistry and Cell Biology, China. The immortalized normal gastric epithelial cell line GES-1 was obtained from the Shanghai Institute of Digestive Disease, China. The cell lines were cultured and maintained in RPMI-1640 media supplemented with 10% fetal bovine serum, 100U/ml penicillin and 100 µg/ml streptomycin in a humidified cell incubator with an atmosphere of 5% CO<sub>2</sub> at 37°C. Each cell line was divided into three groups: (1) Control group: the cells were cultured in medium without DNA

methyltransferase inhibitor 5-Aza-CdR for 48h. (2) Group treated by low concentration 5-Aza-CdR: the cells were cultured in medium containing 10 µmol/L 5-Aza-CdR for 48h. (3) Group treated by high concentration 5-Aza-CdR: the cells were cultured in medium containing 20 µmol/L 5-Aza-CdR for 48h.

### *Methylation-specific PCR (MSP) Assay*

Genomic DNA was extracted from fresh frozen tissues or cultured cells by Genomic DNA isolation kit (Biyuntian, China) according to the manufacturer's protocols. Bisulfite modification of DNA was carried out by an EpiTect Bisulfite kit (Qiagen, China). MSP assay was applied to detect the methylation status of the *RASAL1* promoter in clinical samples and cells. The specific *RASAL1* primers used were as reported by Seto et al. (2011). The unmethylated primer were 5'-aatttattaggagtgtaggttat (forward) and 5'-cacaacaaactcttaccacaaaca (reverse); the methylated primer were 5'-gtgtattttgtttctgctgcttc (forward), and 5'-caacgaactcttaccgaaacg (reverse). When MSP products appeared unmethylation band only, the result was recorded as methylation negative. Meanwhile, as long as MSP products appeared methylation band, the result was recorded as methylation positive regardless of unmethylation band appeared or not.

### *Western blotting*

Western blotting analysis was performed to detect *RASAL1*, total ERK1/2 and p-ERK1/2 protein expression in gastric cancer cell lines. The protein concentration was standardized by the BCA assay (Pierce, USA). Protein samples were subjected to SDS-PAGE (8%) gel, and electrophoresis and membrane transfer was performed following the manufacturer's protocol (Bio-Rad, Hercules, CA). Anti-*RASAL1* (1:200), anti total ERK1/2 (1:1,000), anti p-ERK1/2 (1:500) and rabbit anti-β-actin antibodies (1:8000, Sigma, USA) were used as primary antibodies, and goat anti-rabbit Ig (H+L)-HRP secondary antibodies (KeyGen Biotech Co, Nanjing, China) were used. ECL reagents were used to show the positive bands on the membrane. An enhanced chemiluminescence detection system (Millipore, USA) was used for detection. The results were showed as the ratio of target protein/loading control.

### *Pull-down assay*

RAS-GTP, the active form of RAS protein, was checked by RAS-GTP pull-down assay (Cell Biolabs, USA). Briefly, cells growing on 100 mm culture plate were washed three times with cold PBS and were lysed in lysis buffer (25 mM Hepes, pH 7.5, 0.5% NP-40, 150 mM NaCl, 10 mM MgCl<sub>2</sub>, 10% glycerol, and 1 mM EDTA) containing 60 mg/300 mL PBD-GST. Lysates were then cleared by centrifugation 14000 × g at 4°C for 10 min, and the supernatants were incubated with 20 µg of RAS RBD fusion conjugated with glutathione beads at 4°C for 1 h. The beads were washed 3 times with lysis buffer and subjected to SDS-PAGE. Western blotting was then used to analyze the amount of RAS-GTP. The experiments were repeated three times and the mean value was used for subsequent analysis.

**Table 1. The *RASAL1* Promoter Methylation and Clinical Features in Gastric Cancer Tissues**

	Number	Frequency of <i>RASAL1</i> promoter methylation	X <sup>2</sup>	P
Gender				
Male	24	75.0% (18/24)	0.243	0.622
Female	16	62.5% (10/16)		
Age (yr)				
<60	12	58.3% (7/12)	1.905	0.168
≥60	28	57.1% (16/28)		
Tumor size (cm)				
<4	20	45.0% (9/20)	3.75	0.043
≥4	20	75.0% (15/20)		
Differentiation				
Well, moderately	28	35.7% (10/28)	7.619	0.006
poorly	12	83.3% (10/12)		
Lymph node metastasis				
Negative	18	55.5% (10/18)	6.599	0.01
Positive	22	90.9% (20/22)		
TNM stages				
1,2	26	46.1% (12/26)	5.934	0.015
3,4	14	85.7% (12/14)		

### Statistics

The results were presented as the mean ± standard deviation (SD). Statistical analysis was performed by SPSS 18.0 software. T-test is adopted between two group's comparison, and more than two groups were compared with one-way ANOVA. Values of  $P < 0.05$  were deemed to indicate statistical significance.

## Results

### The methylation of *RASAL1* promoter in gastric cancer tissues and the paired adjacent non-cancerous tissues

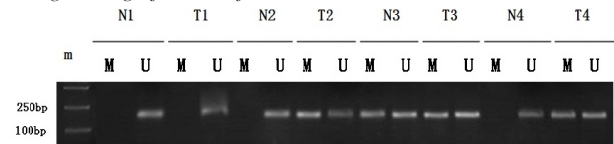
MSP was applied to detect methylation of the *RASAL1* promoter in 40 cases of gastric cancer tissue and the paired adjacent non-cancerous tissue. The results indicated that the frequency of *RASAL1* promoter methylation was 70% (28/40) in gastric cancer tissues and 30% (12/40) in the paired adjacent non-cancerous tissues. The result indicated the frequency of *RASAL1* promoter methylation in gastric cancer tissues was higher than in non-cancerous tissues ( $P < 0.001$ ) (Figure 1).

### Association of the *RASAL1* promoter methylation with clinicopathologic features

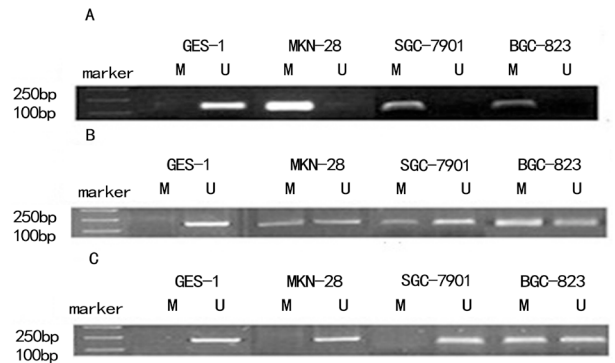
The relationship between methylation status of *RASAL1* promoter and known clinicopathologic factors in 40 tumor tissues were examined. The analyses were summarized in Table 1. The results of statistical analysis indicated that no correlation was found about *RASAL1* promoter methylation in age or gender, but there was significant correlation between *RASAL1* promoter methylation and tumor differentiation, tumor size, invasive depth and lymph node metastasis in patients with gastric cancer (all  $P < 0.05$ ).

### The methylation status of *RASAL1* promoter before and after treatment with 5-Aza-CdR

MSP assay showed that before and after treated by 5-Aza-CdR, the methylation of *RASAL1* promoter were



**Figure 1. The Methylation Status of the *RASAL1* Promoter in Gastric Cancer Tissues and Paired Adjacent Non-cancerous Tissues Was Analyzed by Methylation-specific PCR (MSP).** It was shown the unmethylation was detected in N1, T1, N2, N4 and the methylation was detected in T2, N3, T3 and T4. (m: marker. M: methylated PCR products; U: unmethylated PCR products. N1-N4: the paired adjacent non-cancerous tissues; T1-T4: gastric cancer tissues)

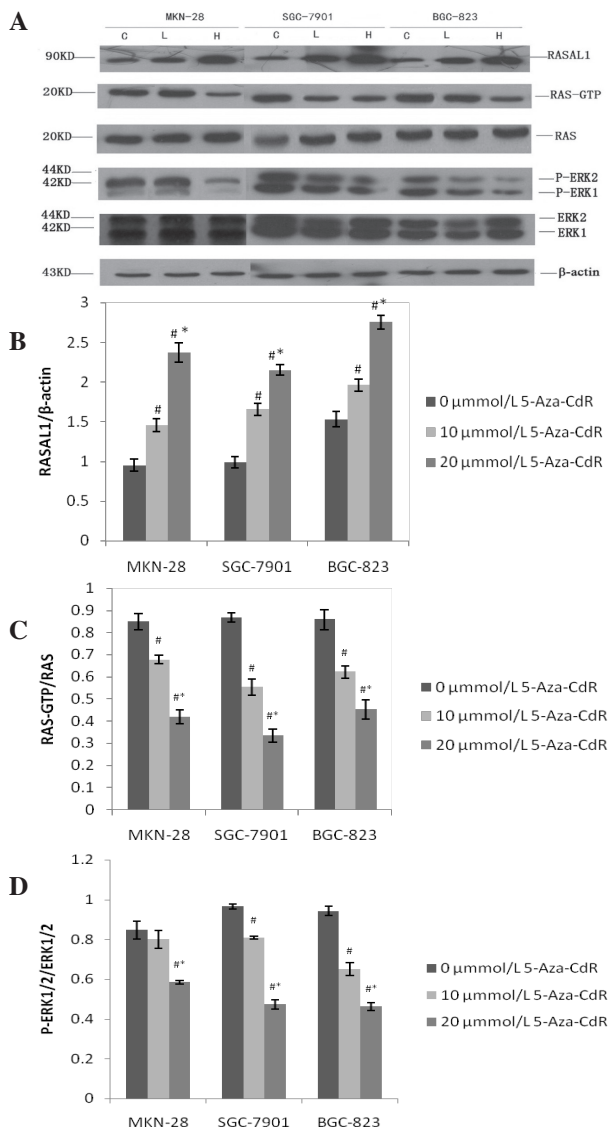


**Figure 2. The Methylation Status of the *RASAL1* Promoter in Gastric Cancer Cells and Normal Gastric Epithelial Cell Before and after Treatment with 5-Aza-CdR.** (M: Methylation, U: Unmethylation. A: Control group; B: Group treated by low concentration 5-Aza-CdR; C: Group treated by high concentration 5-Aza-CdR)

all undetectable in normal gastric epithelial cell. However, in three kinds of gastric cancer cells, MKN-28, SGC-7901 and BGC-823, the methylation of *RASAL1* promoter were all detectable. It was also found that the methylation of *RASAL1* in three gastric cancer cells could be reversed by 5-Aza-CdR (Figure 2).

### Expression of *RASAL1* protein, RAS-GTP and ERK1/2 protein in gastric cancer cells before and after treatment with 5-Aza-CdR

Figure 3A showed the expression of *RASAL1* protein, RAS-GTP and ERK1/2 protein in three gastric cancer cells after treated by 5-Aza-CdR (0, 10, 20 μmol/L, respectively). It was found that the expression of *RASAL1* protein increased after treated by various concentrations 5-Aza-CdR. The higher concentration of 5-Aza-CdR treated, the more *RASAL1* protein expression got. Meanwhile, It was found that the expression of RAS-GTP protein, the active form of RAS, was decreased after treatment with various concentrations of 5-Aza-CdR. Furthermore, the total ERK1/2 and p-ERK1/2 protein, the downstream signal molecules of "RAS-RAF-MEK-ERK" signaling pathways, were detected. The results showed that the expression of p-ERK1/2 protein decreased after treatment with various concentrations of 5-Aza-CdR. The ratios of *RASAL1*/β-actin (Figure 3B), RAS-GTP/RAS (Figure 3C), p-ERK1/2 and ERK1/2 (Figure 3D) were showed respectively to indicated the difference (mean±SD).



**Figure 3. Expression of RASAL1 Protein, RAS-GTP and P-ERK1/2 Protein in Gastric Cancer Cells Before and after Treatment with 5-Aza-CdR Were Detected.** C: Control group, without treatment with 5-Aza-CdR; L: Group treated by low concentration 5-Aza-CdR (10 μmol/L); H: Group treated by high concentration 5-Aza-CdR (20 μmol/L). Figure 3B showed the ratio of RASAL1/β-actin in three gastric cancer cell lines (#*P*<0.01, compared to control; \**P*<0.05, compared to group treated with 10 μmol/L 5-Aza-CdR). Figure 3C showed the ratio of RAS-GTP/RAS in three gastric cancer cell lines (#*P*<0.01, compared to control; \**P*<0.05, compared to group treated with 10 μmol/L 5-Aza-CdR). Figure 3D showed the ratio of P-ERK/ERK in three gastric cancer cell lines (#*P*<0.01, compared to control; \**P*<0.05, compared to group treated with 10 μmol/L 5-Aza-CdR)

**Discussion**

Studies had found that there is a close relationship between the oncogenesis and development of human malignant tumors with the abnormal of “RAS-RAF-MEK-ERK” signal transduction cascade, and the role of the RAS gene in the related process has been getting extensive studies. There are many research works indicated that RAS gene mutation is a very important reason which resulting in the abnormal RAS hyperactivity and together with

other gene changes ultimately inducing the malignancy progression. The frequencies of RAS mutations reported range from 20 to 100% that occurring in the tumor progression model, of which pancreatic cancer has the highest incidence of RAS mutations. However, the frequency of RAS gene mutation was not common in some other malignant tumors included gastric cancer. So it is supposed that there must be other mechanisms that resulting in the anomalous RAS hyperactivities in gastric cancer.

RAS proteins are a subfamily of the super family of regulatory GTP hydrolases (G proteins), which consists of the Ras, Rho, Rab, Arf, Sar1, and Ran families. The RAS protein switches between two conformations, the inactive structure (GDP bounding) and the active structure (GTP bounding). The process of switching is controlled by two classes of molecular, the RAS GTPase-activating proteins (RAS GAPs) and the RAS guanine nucleotide exchange factors (RAS GEFs). The RAS GEFs catalyze the release of GDP, allowing GTP to bind and causing RAS activation; whereas RAS GAPs enhance the intrinsic RAS-GTPase activity, leading to RAS inactivation through the conversion of GTP into GDP (Jin et al., 2007). Though the switching of GDP bounding or GTP bounding, the RAS gene family mediates pleiotropic effects including cell proliferation, survival and migration

The RASAL1 protein is a member of the GTPase activating proteins (GAPs) family. It enhances the intrinsic GTPase activity of Ras proteins, resulting in the inactive GDP-bounding form of Ras, thereby inhibiting cell proliferation and differentiation. The RASAL1-related cancer suppressing mechanism has been reported in some cancers, including prostate cancer, lung cancer and liver cancer (Liu et al., 2005; Bernards et al., 2009; Györfy et al., 2010). Seto et al. (2011) has reported that RASAL1 expression was reduced in 6 of the 10 cell lines and in 23 of 48 (48%) gastric cancers, but in none of the gastric adenomas.

In our previous study (Chen et al., 2012; Qiao et al., 2012), we had reported that RASAL1 expression of in mRNA and protein level were all reduced in human gastric cancer cell lines BGC-823, SGC-7901 and MCG-803 compared with the normal gastric epithelial cell GES-1. We also investigated the RASAL1 protein level by western blotting and the RASAL1 mRNA expression level by qPCR in 50 pairs of tumor and matched non-tumor tissue specimens, the results shown the frequency of RASAL1 protein down-regulation was 64% (32/50), and the RASAL1 mRNA down-regulation was 58.8% (20/34) which is consistent with the protein expression level. These data indicated that decreased RASAL1 expression may play an important role in gastric tumorigenesis.

It is unknown the exact mechanism of RASAL1 decreased expression in cancer. In the study of colorectal cancer, it was found that the possible mechanisms including CpG methylation of RASAL1 DNA promoter, intracellular calcium ion dysregulation, the upstream gene PITX1 (pituitary homeobox 1) silence and so on (Kolfshoten et al., 2005; Ohta et al., 2009). In this

research, MSP was supplied to detect *RASAL1* promoter methylation in gastric cancer tissues and cell lines. The three gastric cancer cell lines showed a characteristic DNA methylation status in the promoter region of *RASAL1* gene. However, there was no DNA methylation found in the promoter region of *RASAL1* gene in normal gastric epithelial cell line. The frequency of *RASAL1* promoter methylation in gastric cancer tissues and paired adjacent non-cancerous tissues were 70% (28/40) and 30% (12/40) respectively ( $P < 0.05$ ). Besides, we found that the methylation of *RASAL1* in three gastric cancer cell lines was reversed and raised expressions of *RASAL1* protein was detected after treatment with 5-Aza-CdR. Our findings demonstrated that reduced *RASAL1* expression, owing in part to *RASAL1* promoter methylation, may contribute to gastric carcinogenesis. Furthermore, the activity of RAS (RAS-GTP) and RAS signaling pathway downstream signaling molecules ERK1/2 and p-ERK1/2 were determined in three gastric cancer cell lines after treatment with 5-Aza-CdR. It was found the RAS-GTP and protein of p-ERK1/2 decreased. Based on the above findings, the expression of *RASAL1* could get up-regulating due to the demethylation effect of 5-Aza-CdR which further activate the activity of RAS GTPase-activating proteins (GAPs) and reduce the RAS activity. At last the “RAS-RAF-MEK-ERK” signal path transduction is blocked which is illustrated by the decreased p-ERK1/2, a RAS signaling pathway downstream signaling molecules.

In conclusion, our findings suggest that *RASAL1* promoter methylation have a certain relationship with the reduced *RASAL1* expression in gastric cancer. Our results also reemphasize the importance of RAS signaling in gastric cancer development. These findings may provide a new clue to understand the molecular mechanism on the carcinogenesis and development of gastric cancer, which provide new opportunities to explore the therapeutic strategies for gastric cancer.

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