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Original Article

Methylation status of TRAF2 is associated with the diagnosis and prognosis of gastric cancer

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Abstract: The purpose was to investigate whether the expression level of *TRAF2* gene was regulated by DNA methylation and explore the role of *TRAF2* methylation in the diagnosis and prognosis of gastric cancer (GC). Firstly, we detected the expression of *TRAF2* both at mRNA level and protein level. And the up-regulated of *TRAF2* expression at two different levels were both found ($P<0.001$). Then we measured the methylated status of TRAF2 by MSP and got a result of that TRAF2 was hypomethylated in GC patients compared with healthy controls ($P<0.001$). Meanwhile, the relationship between *TRAF2* methylation and clinicopathologic characteristics was estimated through chi-square. The outcome proved that *TRAF2* methylation was impacted by age ($P=0.024$), lymph node metastasis ($P=0.046$), TNM stage ($P=0.021$), distant metastasis ($P=0.002$) and depth of invasion ($P=0.002$). The AUC of 0.795 accompanying a sensitivity of 66.7% and a specificity of 94.7% were obtained from Receiver Operating Characteristic (ROC) curve which indicated the diagnostic value of *TRAF2* methylation was high. At last, we researched the prognostic value of *TRAF2* methylation. Kaplan-Meier showed that patients with *TRAF2* hypomethylation had lived much shorter than those with *TRAF2* hypermethylation (log rank test, $P<0.001$). Cox regression analysis revealed *TRAF2* hypomethylation (HR=18.827, 95% CI=3.103-114.222, P=0.001), lymph node metastasis (HR=0.154, 95% CI=0.047-0.512, P=0.002), distant metastasis (HR=3.032, 95% CI=1.116-8.237, $P=0.030$), as well as differentiation (HR=0.287, 95% CI=0.113-0.731, $P=0.009$) were all vital prognostic factors in GC. Taken together, *TRAF2* expression was increased in GC patients by DNA hypomethylation and this methylation could be an independent diagnostic and prognostic indicator in GC.

Keywords: *TRAF2*, hypomethylation, diagnosis and prognosis, gastric cancer

Introduction

Gastric cancer (GC) is the fourth most common cancer and the second leading cause of cancer-related death in the worldwide [1]. Although the overall incidence and mortality have decreased and the diagnostic technique as well as treatment methods have been improving in recent years, the rate of diagnosis during its early stages is still low and the prognosis of GC remains poor [2-4]. It is therefore of importance to identify the new diagnostic or prognostic bio-markers for GC.

In previous studies, a various of diagnostic and prognostic markers such as genes, antigens, microRNAs, lncRNAs and so on had been found in GC [5-8]. DNA methylation as a new hot spot of research has been studied more and more widely now. In GC, epigenetic changes of many genes due to DNA methylation had been observed [9, 10]. DNA methylation can not only affect the global hypermethylation patterns, hypomethylation of proto-oncogenes, and hypermethylation of tumor suppressor genes (TSGs), but can be an crucial bio-markers in GC [11]. Tumor necrosis factor receptor (TNFR)-associated factor 2 (*TRAF2*), an adaptor protein, is a critical member of the TRAF family and can directly interact with many TNF-R super family members [12, 13]. A plenty of studies have shown that *TRAF2* is a critical mediator of multiple signal pathways such as NF-κB, c-Jun N-terminal kinase (JNK) and p38 pathway activation [14-16]. However, the studies about the *TRAF2* methylation were rarely. He et al, found

the significantly hypomethylation of *TRAF2* in GC, but the other function of this methylation had never reported.

In this study, we detected the expression of *TRAF2* and validated whether it was influenced by DNA methylation. Then we estimated the diagnostic and prognostic value of *TRAF2* methylation in GC. This research may be expected to provide a new method for the early detection and the therapy for GC patients.

Materials and methods

Ethics statement

The study was conducted at Huai'an First People's Hospital and permitted by the Ethnic Committee of the hospital. All patients had signed the written informed consents in advance.

Patients and samples

129 patients with GC including females and males were collected in the current experimental. What's more, none of the patients had received any chemical treatment and physical therapy before sampling. Besides, 72 people without GC or other cancers were taken as healthy controls.

The tumor tissues, adjacent non-cancerous tissue from patients with GC and the tissues of healthy controls were obtained, respectively. Then the tissues samples were frozen by liquid nitrogen immediately and stored at -80°C for using. Clinicopathologic characteristics such as age, gender, lauren histotype, tumor size, TNM stage, lymph node metastasis, distant metastasis, differentiation and depth of invasion were recorded in databases. The follow-up was performed to analyze the prognosis of GC patients. The information was acquired by a telephone or questionnaire every three months. The days from the surgery to the death were defined as the overall survival. Patients died from unexpected events or other disease were excluded in our study.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from tumor tissues, adjacent non-cancerous tissues and healthy tissues with TRIzol Reagent (Life Technologies). PCR was carried out using UltraSYBR Mixture (CWbio. Co. Ltd) and the sequences for *TRAF2*. The primers of *TRAF2* was as follows: forward, 5'-CCCTGAGTAGCAAGGTGCA-3' (NCBI GenBank® accession No. NM_021138); reverse, 5'-TCGCCGTTCAGGTAGATACG-3' (Tib Molbiol). Reverse transcription was done with PrimeScript RT Master Mix kit (TAKARA Biotechnology Co. Ltd., Dalian, China). Then RT-PCR was performed using a SYBR Premix Ex Taq II kit (TAKARA Biotechnology Co. Ltd.) in a StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA). The β-actin was taken as internal control and its sequences of the primers were: forward, 5'-GGGACCCTGAAAGAATACGAGA-3'; reverse-5'-CGCAGCCGTCACAAGTTAAG-3'. And the RT-PCR procedures were: pre-incubation at 95°C for 10 min, then 45 cycles for 10 s at 95°C for denaturation, annealing for 10 s at 66°C and extension at 72°C for 20 s. The relative quantification of *TRAF2* at mRNA level was calculated by the comparative cycle threshold (CT) method. Each experimental were in triplicate.

Western blot analysis

Total protein was isolated from tumor tissues, adjacent non-cancerous tissues and healthy tissues, severally. After the isolation, the protein was separated with SDS-PAGE gels and transferred into nitrocellulose membrane. Then the membrane were blocked with 5% non-fat milk (Bio-Rad) and incubated with primary anti-*TRAF2* antibody at 4°C overnight. Afterwards, the membrane was washed and added into horseradish peroxidase-conjugated secondary antibody. Reaction with the enhanced chemiluminescence kit (Forevergen Biosciences, China) was performed for detecting quantification of *TRAF2* proteins.

DNA extraction and bisulfite modification

Genomic DNA was extracted from the tumor tissues, adjacent non-cancerous tissues and healthy controls with the DNA Mammalian Genomic Purification Kit (Sigma-Aldrich Co., St. Louis, MO). Then the DNA was treated with sodium bisulfite using the EZ DNA MethylationTM Kit (Zymo Research, Orange, CA) to conduct the conversion of cytosine to uracil. The modified DNA was resuspended in elution buffer and stored at -20°C.

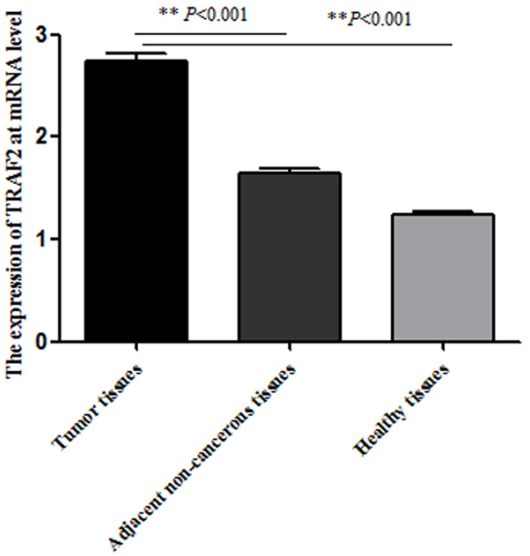

Figure 1. Expression of *TRAF2* at mRNA level. It was significantly higher of the *TRAF2* expression at mRNA level in tumor tissues than in adjacent non-cancerous tissues and healthy tissues (P<0.001).

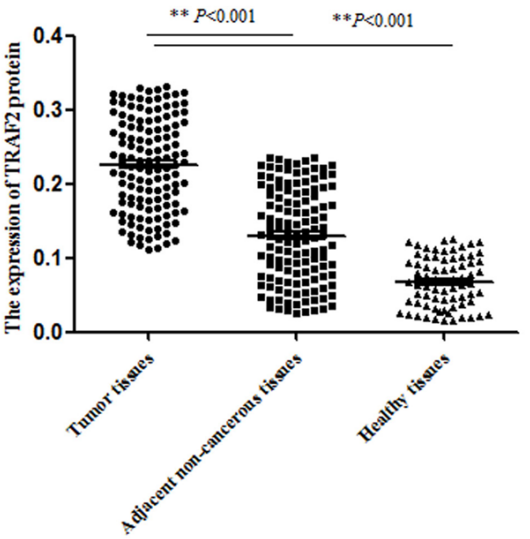

Figure 2. Expression of *TRAF2* protein. *TRAF2* protein expressed much more in tumor tissues compared with the adjacent non-cancerous tissues and healthy tissues (P<0.001).

Methylation-specific polymerase chain reaction (MSP)

The modified DNA was used to analyze the methylation status of *TRAF2* in gastric cancer using methylation-specific polymerase chain reaction on the ABI7500 PCR system (ABI Co, Norwalk, Connecticut) with the SYBR Premix

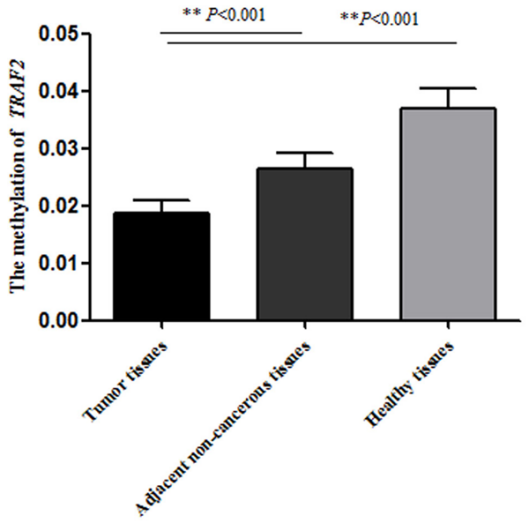

Figure 3. *TRAF2* methylation level in tumor tissues, adjacent non-cancerous tissues and healthy tissues. The *TRAF2* presented a trend of hypomethylation in GC patients (P<0.001).

Taq ExTaq Kit (TaKaRa Co Ltd, Otsu, Shiga, Japan). The PCR amplification system contained 2 μl Modified DNA, 2.5 μl 10×PCR buffer, 2.5 μl $MgCl_2$ solution, 2.5 μl dNTPs, 2.5 μl forward and reverse primer respectively, 0.1 μl 5 U/μl Taq Gold polymerase and sterile water which was supplied to total 25 μl. The procedure of MS-PCR was: initial denaturation at 95°C for 15 min, 45 cycles of denaturation for 30 s at 95°C, annealing at 55°C for 45 s, extension at 72°C for 30 s, and finally extension at 72°C for 5 min.

Statistical analysis

The statistical analysis was performed using SPSS version 13.0 software (SPSS Inc, IL, USA). The differences of *TRAF2* expression at mRNA and protein level as well as the methylation status of this gene in tumor tissues, adjacent tissues and healthy tissues were analyzed by One-way ANOVA, respectively. Receiver Operating Characteristic (ROC) curve was established to evaluate the diagnostic value of *TRAF2* methylation. The relationship between the clinicopathologic characteristics and *TRAF2* methylation was estimated with chi-square test. Kaplan-Meier and Cox regression analysis were taken to assess the association between *TRAF2* methylation and the overall survival as well as the prognosis of GC. When P<0.05, the difference was considered to be significant.

Table 1. The relationship between *TRAF2* methylation and clinicopathologic characteristics

Clinicopathologic characteristics	n	*TRAF2* methylation		P
		Hypomethylation (n)	Hypermethylation (n)	
Age				0.024
≤65	103	63	40	
>65	26	22	4	
Sex				0.387
Female	26	19	7	
Male	103	66	37	
Tumor size				0.178
≤5 cm	54	32	22	
>5 cm	75	53	22	
Lauren histotype				0.261
Intestinal	50	30	20	
Diffuse-mixed	79	55	24	
Differentiation				0.239
Low	85	53	32	
High+Moderate	44	32	12	
Lymph node metastasis				0.046
No	52	29	23	
Yes	77	66	11	
TNM stage				0.021
I+II	61	34	27	
III+IV	68	51	17	
Distant metastasis				0.002
Absent	63	33	30	
Present	66	52	14	
Depth of invasion				0.002
pT1	50	24	26	
pT2	44	32	12	
pT3-4	35	29	6	

Results

Over-expression of TRAF2 in tumor tissues compared with adjacent non-cancerous tissues of GC and healthy tissues

To detect the expression of *TRAF2* in GC patients, we measured that both at mRNA and protein level. QRT-PCR analysis demonstrated that *TRAF2* expression was significantly higher in tumor tissues than in adjacent non-cancerous tissues and healthy tissues (**Figure 1**, $P<0.001$). Meanwhile, the protein expression level of *TRAF2* was conducted by western blot analysis which showed the same trend with the expression at mRNA level. The *TRAF2* protein expression was the highest in three kinds of tissues which was more than adjacent non-cancerous tissues and healthy tissues (**Figure 2**, $P<0.001$).

Methylation status of TRAF2 in GC patients

DNA methylation was confirmed to be an important reason for the aberrant expression of many genes. We explored whether the up-regulated of *TRAF2* was related to the methylation of *TRAF2*. The result manifested that the methylation rate of *TRAF2* in tumor tissues was much lower than that in adjacent tissues and healthy tissues (**Figure 3**, $P<0.001$). Namely, *TRAF2* was hypomethylation in GC patients.

Relationship between TRAF2 methylation and the clinicopathologic characteristics

To investigate the role of *TRAF2* methylation in the development of GC, we analyzed the relationship between it and clinicopathologic characteristics. The outcome proved that the *TRAF2* methylation could be influenced by the age ($P=0.024$), lymph node metastasis ($P=0.046$), TNM stage ($P=0.021$), distant metastasis ($P=0.002$) and depth of invasion ($P=0.002$) (**Table 1**).

Diagnostic value of TRAF2 methylation in GC

In the study, we found patients with *TRAF2* methylated (completely methylated and incompletely methylated) and patients with no *TRAF2* methylated in GC patients. Based on the methylated condition, we estimated its diagnostic value of *TRAF2* methylated in GC. ROC curve indicated that the diagnostic value of *TRAF2* methylated was high with an AUC of 0.795, cor-

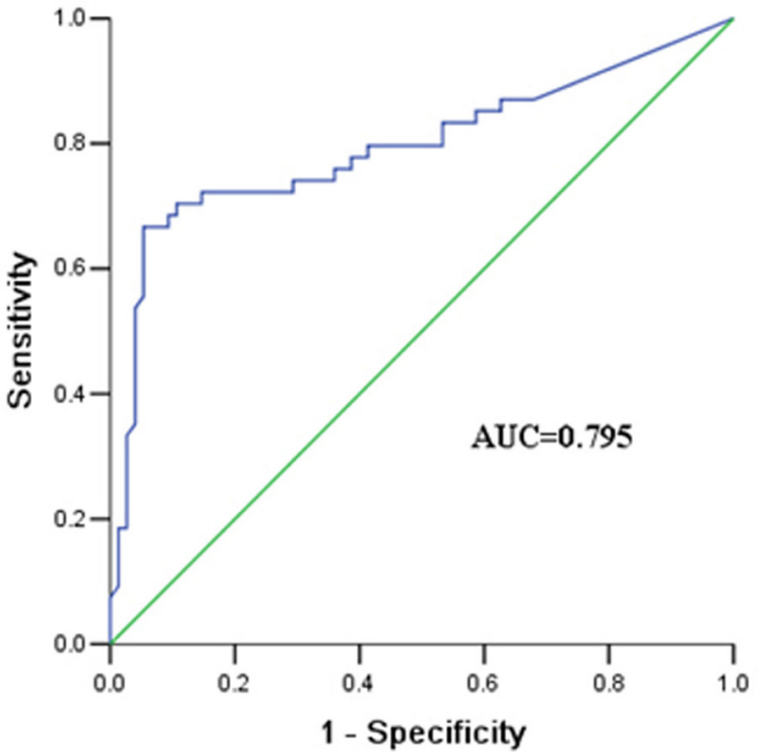

Figure 4. ROC curve for *TRAF2* methylation with an AUC of 0.795 combing a sensitivity of 66.7% and specificity of 94.7%.

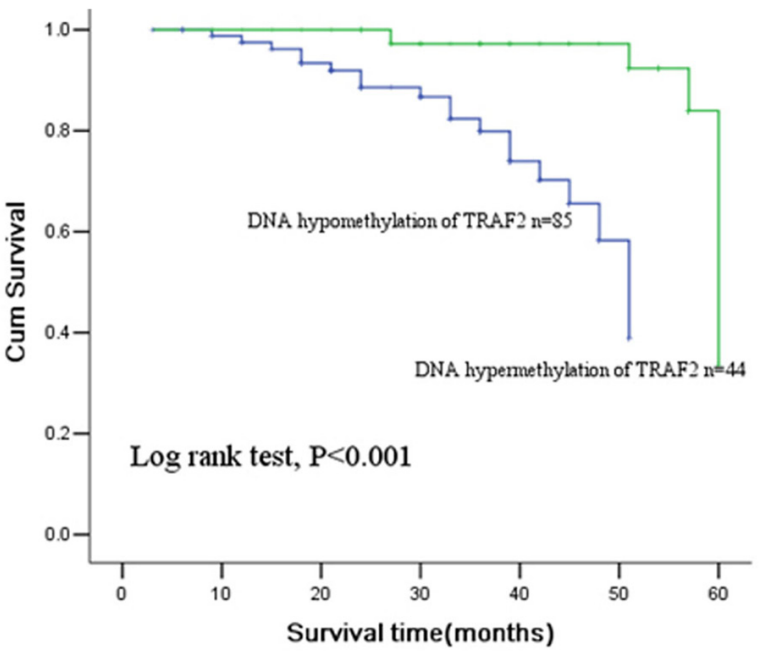

Figure 5. Overall survival of patients with GC was analyzed by Kaplan-Meier analysis (Log rank test, *P*<0.001).

responding with a sensitivity of 66.7% and specificity of 94.7% (**Figure 4**).

Association between TRAF2 methylation and overall survival of GC patients

During the follow-up, there were patients died and patients censored. Kaplan-Meier analysis revealed that patients with *TRAF2* methylated had a much lower overall survival than those without *TRAF2* methylated (**Figure 5**, log rank test, *P*<0.001). Besides, Cox regression analysis adjusted for clinicopathologic characteristics showed *TRAF2* methylated (HR=18.827, 95% CI=3.103-114.222, *P*=0.001), lymph node metastasis (HR=0.154, 95% CI=0.047-0.512, P=0.002), distant metastasis (HR=3.032, 95% CI=1.116-8.237, *P*=0.030), as well as differentiation (HR=0.287, 95% CI=0.113-0.731, *P*=0.009) were obviously linked with the prognosis of GC and they might be independent prognostic indicators (**Table 2**).

Discussion

In current study, we found the expression of *TRAF2* was distinctly enhancive in tumor tissues relative to in adjacent non-cancerous tissues and healthy tissues. These revealed that *TRAF2* was an oncogene in GC. The over-expression of *TRAF2* was also confirmed in breast cancer, multiple sclerosis, epithelial cancers, and pancreatic ductal adenocarcinoma [13, 17-19]. However, the mechanism of *TRAF2* over-express is still unclear.

DNA methylation is considered to be an important mechanism for the aberrant expression of multiple genes [20]. There were also many genes' methylation had been observed in

Table 2. Multivariate analysis for the prognostic factors of patients with GC

Parameter	Risk ratio	95% confidence interval	*P* value
Lymph node metastasis	0.154	0.047-0.512	0.002
Distant metastasis	3.032	1.116-8.237	0.030
Differentiation	0.287	0.113-0.731	0.009
TRAF2 hypomethylation	18.827	3.103-114.222	0.001
TRAF2 hypermethylation	-	-	-

GC such as APC, RASSF1A, p16, COX2, hMLH1, MGMT, CDH1, TIMP-3, DAP-Kinase, THBS1 and involved several process of GC including cell cycle, DNA repair, tissue invasion and so on [21-28]. In our study, as the increasing of *TRAF2* expression, the methylation rate became more and more low according to MSP. Therefore, we inferred that the DNA methylation was one of the main mechanisms for the abnormal expression of *TRAF2* in GC.

To further investigate the relationship between *TRAF2* methylation and the development of GC, we analyzed whether *TRAF2* methylation was related to the clinicopathologic characteristics. As it was shown that age, lymph node metastasis, TNM stage, distant metastasis and depth of invasion were all significantly influence the methylation of *TRAF2*.

As the bio-markers were vital for the diagnosis and prognosis of cancers, we then explored the diagnostic and prognostic value of *TRAF2* methylation. ROC curve manifested a high diagnostic value of *TRAF2* methylation with an AUC of 0.795 and a relative high sensitivity and specificity. According to Kaplan-Meier analysis, patients with *TRAF2* hypomethylation had a shorter overall survival compared with those with hypermethylation of *TRAF2*. Besides, we estimated the prognostic value of *TRAF2* methylation and found it also possessed a high prognostic value. These revealed *TRAF2* methylation was significantly linked with the early detection and prognosis of GC.

In conclusion, *TRAF2* gene is increased in GC and regulated by DNA methylation. And this hypomethylation of *TRAF2* can be an independent diagnostic and prognostic indicator for GC. However, because of the limitation of the sample size and the influences of other unfavorable factors, the accuracy of the study as well as further verification tests are still need.

Disclosure of conflict of interest

None.

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