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Down-regulation of thyroid hormone receptor β1 gene expression in gastric cancer involves promoter methylation

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ABSTRACT

Hypermethylation has been shown in the promoter region of the thyroid hormone receptor β1 (TRβ1) gene in several human tumors. However, its role in gastric cancer formation is still unclear. In the study, we analyzed mRNA expression of TRβ1 gene using real-time quantitative PCR (qPCR). A quantitative methylation-specific PCR (Q-MSP) assay was used to determine the methylation status of the TRβ1 gene promoter region in 46 pair-matched gastric neoplastic and adjacent non-neoplastic tissues. The results showed that TRβ1 mRNA expression was significantly reduced in gastric cancer specimens. The methylation of promoter of TRβ1 gene in gastric cancer tissues was significantly higher than in adjacent normal tissues. Promoter hypermethylation of the TRβ1 gene correlated with tumor infiltration, lymph node metastasis, and distant metastasis, but it was not associated with other clinicopathological characteristics. We treated gastric cancer cell lines MKN-45, MKN-28, SGC-7901, NCI-N87, and SNU-1 with 5-Aza-2-deoxycytidine (5-Aza-dC). The results showed the expression of TRβ1 mRNA was increased in MKN-45, MKN-28, SGC-7901, but not increased in NCI-N87 and SNU-1. These results suggest that the TRβ1 gene plays important roles in the development of gastric cancer partially through epigenetic mechanisms.

1. Introduction

Although the incidence of gastric cancer is declining, it still remains the fourth most common type of cancer and the second leading cause of cancer-related death worldwide [1]. In China, gastric cancer is the most commonly occurring cancer, with peak incidence at age 50 and above. Most patients are first diagnosed when they have late stage gastric cancer. Their subsequent death makes gastric cancer a lethal disease. It is now apparent that multiple genetic alterations, including *Helicobacter pylori* infection, oncogene activation, and tumor suppressor gene inactivation, are necessary steps in gastric cancer development. However, the genetic mechanisms underlying this lethal cancer have not yet been fully elucidated. Recently, there has been a growing interest in the role of epigenetic changes in carcinogenesis, especially DNA cytosine methylation in gene promoter regions. The focus on methylation is due to it being relatively easy to detect and it is a potential therapeutic target that can be reversed by drug treatment [2,3]. In fact, 41% of gastric cancers have been reported to exhibit the CpG island methylator phenotype, along with epigenetic silencing of multiple tumor suppressor genes [4].

The thyroid hormones control the development and the homeostasis of several organs in vertebrates. These cellular effects are mediated through the binding of T3 to thyroid hormone receptors (TRs), which are located within the nuclei of target cells [5]. The TRs are ligand-dependent transcription factors that bind to thyroid hormone response elements (TRE) in the promoter regions of the target genes [6–8]. The three major TR isoforms (TRβ1, TRβ2 and TRα1) have tissue specific expression. TRβ1 has the broadest tissue distribution. It was chosen for examination in the present study to increase the likelihood of identifying its presence in gastric samples. Alterations in the level of expression and integrity of TRβ1 genes has been shown to occur in different human neoplasms [9–12]. However, the association between TRβ1 and gastric cancer remains little known, although the gastrointestinal tract is a well characterized target for thyroid hormones and TRβ1. Epigenetic inactivation of the TRβ1 gene through aberrant promoter methylation has been found in breast cancer, thyroid cancer, and acute lymphoblastic leukemia [13–15] .We discovered that TRβ1 promoter hypermethylation occurred frequently in breast cancers [16]. Considering the high prevalence of the CpG island methylator phenotype in gastric cancer [17], we hypothesized that

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Table 1
Correlation between methylation status and clinicopathological features of gastric cancer patients.

Clinicopathological features	Number	NMR (M ± SD)	*p*-Value
Gender	46		
Male	32	0.44 ± 0.23	0.415
Female	14	0.50 ± 0.18	
Age			
⩽50	15	0.42 ± 0.25	0.369
>50	31	0.48 ± 0.20	
Histological type			
Intestinal	14	0.44 ± 0.26	0.931
Diffuse	19	0.47 ± 0.22	
Mixed	13	0.47 ± 0.18	
Differentiation			
Well	13	0.52 ± 0.18	0.498
Moderate	15	0.45 ± 0.25	
Poor	18	0.43 ± 0.21	
T-stage			
T1	11	0.30 ± 0.21	0.000
T2	13	0.38 ± 0.18	
T3	15	0.56 ± 0.16	
T4	7	0.66 ± 0.14	
N-stage			
N0	7	0.28 ± 0.19	0.000
N1	16	0.37 ± 0.21	
N2	17	0.56 ± 0.15	
N3	6	0.66 ± 0.14	
Distant metastasis			
M0	31	0.42 ± 0.17	0.035
M1	15	0.56 ± 0.27	

epigenetic silencing of TRβ1 was likely to be involved in gastric carcinogenesis. To investigate this hypothesis, we analyzed promoter methylation status and mRNA expression levels of the TRβ1 gene in normal and malignant gastric tissue specimens. Treatment of gastric cancer cell lines with 5-Aza-2-deoxycitidine (5-aza-dC) was performed in order to assess the dynamic association between promoter methylation and TRβ1 mRNA expression status.

2. Materials and methods

2.1. Cell lines and tissue samples

Gastric cancer cell lines used in this study were MKN-45, MKN-28, SGC-7901, NCI-N87, and SNU-1. The immortalized human gastric mucosa cell line GES-1 was used as a nontumor gastric control. These cell lines were purchased from Cell Bank of Shanghai Institute of Biochemistry & Cell Biology, Chinese Academy of Sciences. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum at 37 °C and 5% CO_2 in a humidified incubator. Tumor specimens and apparently normal adjacent tissue were obtained from 46 patients following surgical resection at Lanzhou General Hospital of Lanzhou Military Command of PLA from 2008 to 2012. Serving as controls, twenty-five noncancerous gastric mucosa tissues were obtained from patients who underwent routine endoscopy without detectable malignancy. All samples were fresh-frozen in liquid nitrogen after surgical removal and stored at −80 °C until use. No patients received any antitumor treatment before the operation. All diagnoses were histologically confirmed. All samples were obtained with informed consent and approved by the hospital institutional review board.

2.2. Real-time quantitative RT-PCR (qPCR) analysis of TRβ1 expression

Total RNA from tissues and cells in the logarithmic growth phase was extracted using Trizol solution. Reverse transcription (RT) was performed in a 20-μL reaction system according to the manufacturer's recommendation. To quantitate TRβ1 gene mRNA expression, qPCR was performed. In brief, qPCR amplification was carried out for each sample in a final reaction mixture of 12.5 μl containing: 1 μl cDNA; 10 nM of each primer; 3 nM TaqMan probe; 0.6 U platinum Taq polymerase; and 5.5 mM $MgCl_2$. After an initial denaturation step at 95 °C for 1.5 min, 40 cycles of 15 s at 95 °C and 56 s at 60 °C for annealing and extension were run on an iQ5 PCR machine (BioRad, Hercules, CA). TRβ1 mRNA levels were normalized to β-Actin. Primer sequences are presented in Table 2. All reactions were performed in duplicates. The relative expression of TRβ1 was analyzed by the comparative Ct method. Thermal dissociation plots were examined for biphasic melting curves.

2.3. Quantitative methylation-specific PCR (Q-MSP) analysis of TRβ1 gene methylation status in gastric cancer tissues

Genomic DNA was extracted using a DNeasy minicolumn kit (Qiagen, Valencia, CA). Bisulfite treatment of genomic DNA was performed using a Methylamp DNA modification kit (Sigma, Phoenix, AZ, USA), according to the manufacturer's protocol. The Q-MSP assay was performed to test methylation status of TRβ1 gene. Briefly, Q-MSP amplification was carried out for each samples in a final reaction mixture of 20 μl containing: 3 μl bisulfite-treated DNA; 600 nM of each primer; 200 nM TaqMan probe; 0.5 U platinum Taq polymerase; and 5.5 mM $MgCl_2$. After an initial denaturation step at 95 °C for 2 min, 40 cycles of 15 s at 95 °C for annealing and 60 s at 60 °C for extension were run on an iQ5 PCR machine CpGenome. Universal Methylated DNA (Chemicon International, Temecula, CA) was used as a positive control. Duplex PCR with β-actin primer and probe sequences was performed for normalization. Primer and probe sequences were presented in Table 2. As described previously [18], the normalized methylation ratio (NMR) representing the ratio of densely methylated DNA in the sample at the target sequence to the positive control DNA was calculated as follows: NMR = (Ts/Tc)/(As/Ac), where Ts and Tc represent levels of target gene methylation in the sample and control DNAs, respectively, while As and Ac correspond to amplified β-actin levels in the

Table 2
Primer and probe sequences were used in this study.

	Forward primer sequence (5′ → 3′)	Probe sequence (5′ → 3′)	Reverse primer sequence (5′ → 3′)
Real-time RT-PCR			
TRβ1-	CCAGAAGACATTGGACAAGCA	FAM-ATCATCACACCAGCAATTACCAGAGTGGTGT-AMRA	GCAGCTCACAAAACATAGGCA
β-actin	GCTCGTCGTCGACAACGGCTC	FAM-TGGCGGCACCACCATGTACC-AMRA	CAAACATGATCTGGGTCATCTTCTC
Q-MSP			
TRβ1	GGTAATTTGGTTAGAGGATCGCGT	FAM-GATCCAGAATGATTACTAACCT-AMRA	CACCCCTCCGATTCTTACGACG
β-actin	TGGTGATGGAGGAGGTTTAGTAAGT	FAM-ACCACCACCCAACACACAATAACAAACACAT-AMRA	AACCAATAAAACCTACTCCTCCCTTAA
MSP			
M-TRβ1	GGTAATTTGGTTAGAGGATCGCGC		CACCCCTCCGATTCTTACGACG
U-TRβ1	GGTAATTTGGTTAGAGGATTGTGT		CACCCCTCCAATTCTTACAACA

sample and the control DNAs, respectively. A specimen was defined as methylated when its NMR value exceeded 0.2.

2.4. Analysis of methylation-specific PCR (MSP) in gastric cell lines

Genomic DNA from cell lines was extracted using a DNeasy minicolumn kit (Qiagen, Valencia, CA). Genomic DNA was treated with sodium bisulfate (Sigma, Phoenix, AZ, USA), and then analyzed by MSP. Primer sequences were presented in Table 2. PCR amplification was performed with HotStar Taq DNA Polymerase (Qiagen) and consisted of 12 min at 95 °C, followed by an initial denaturation step of 15 min at 95 °C and then 7 cycles consisting of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. 40 cycles were performed. PCR products were separated in 3% agarose gels containing ethidium bromide and visualized by ultraviolet (UV) illumination. SssI methylase-treated DNA (M-DNA) and normal human genomic DNA were used as a positive and negative control for methylation, respectively. Amplified bisulfate PCR products were subcloned into the TA vector system (Promega) according to the manufacturer's protocol. DNA sequencing was performed on five individual clones.

2.5. DNA demethylation

A total of 2×10^5 cells were seeded per 6-well plate. Medium was supplemented with either water (negative control) or 5 μM of 5-Aza-2-deoxycytidine (5-Aza-dC). Cells were cultured for 96 h at 37 °C in 5% CO_2. 5-Aza-dC containing medium was replaced every 24 h with freshly prepared medium. The cells were harvested. The TRβ1 gene mRNA expression was assayed by qPCR.

2.6. Statistical analysis

Statistical analysis was performed using SPSS 10.0 (SPSS Inc., USA). Data are expressed as the mean ± standard deviation. The values of mRNA expression level were analyzed by independent-samples *T* test or paired-samples *T* test. The detection of methylation was analyzed with one-way analysis of variance, and between-group differences were detected with post hoc Student-Newman–Keuls test. The relationship between methylation status of TRβ1 gene and pathology parameters were analyzed by non-parametric correlations (spearman). The relationship between methylation status of TRβ1 gene and its mRNA expression level was analyzed by pearson correlation. Statistical significance was defined as p-value < 0.05.

3. Results

3.1. TRβ1 mRNA expression was decreased in a majority of gastric cancer samples and cell lines

We analyzed the mRNA expression of the TRβ1 gene in 46 pairs of gastric cancer specimens and matched normal gastric tissues by qPCR. The TRβ1 mRNA expression was reduced in 38 of the 46 (82.6%) gastric cancer tissues samples compared to the matched normal gastric tissue (Fig. 1). For the gastric cancer tissues samples, the mean mRNA expression level was 3.89 ± 3.32 compared to 11.72 ± 5.22 for the matched normal gastric tissues. The reduction of TRβ1 gene mRNA expression in gastric cancer tissue was statistically significant ($p = 0.000$). To more fully understand the mechanism of TRβ1 reduced transcription during gastric tumorigenesis, TRβ1 gene mRNA expression in a nontumor gastric cell line GES-1 and the five different gastric cancer cell lines MKN-45, MKN-28, SGC-7901, NCI-N87, and SNU-1 was analyzed by qPCR. Moderate TRβ1 expression was observed in GES-1. Transcript levels below the GES-1 cell line were observed in all five cancer cell lines.

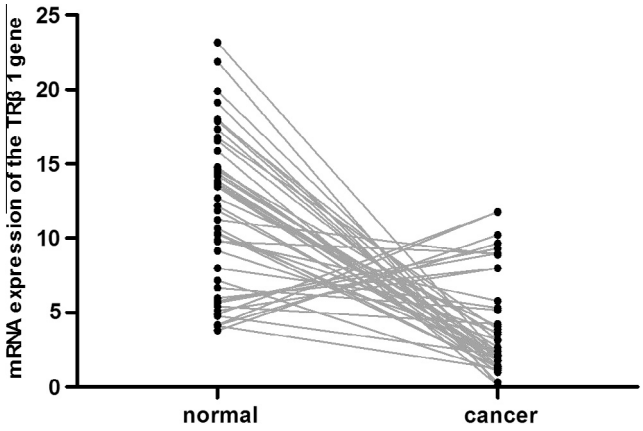

Fig. 1. Detection of TRβ1 mRNA expression in gastric cancer samples (tumor), and non-neoplastic adjacent tissues (normal) by real-time RT-PCR. Each dot represents an individual specimen. A line links each matching pair of cancerous and noncancerous specimens.

3.2. Analysis of the methylation status of the TRβ1 gene promoter in tissues

The methylation status of the TRβ1 gene promoter was determined in 46 gastric cancer tissues and adjacent non-malignant tissue samples by Q-MSP. Promoter methylation levels were represented by normalized methylation ratio (NMR). The results showed the methylation of the promoter of TRβ1 gene in gastric cancer tissues was 0.46 ± 0.22; while in adjacent non-malignant tissue samples, it was 0.12 ± 0.05. The difference between these values is statistically significant ($p = 0.000$). This result indicates that methylation of TRβ1 gene promoter frequently occurs in gastric cancer.

3.3. Correlation between methylation of TRβ1 gene promoter and clinical pathologic parameters

We analyzed the relationship between TRβ1 gene methylation and clinicopathological characteristics in the 46 tumor specimens (Table 1). Although age is a well-known factor associated with promoter hypermethylation in gastric mucosae [17], TRβ1 gene methylation status did not significantly correlate with patient age in the present study. Our data demonstrate that promoter hypermethylation of TRβ1 gene correlated with tumor infiltration, lymph node metastasis, and distant metastasis ($p < 0.05$). However, it was not associated with other clinicopathological characteristics, such as gender, stomach location, histological type, or tumor differentiation ($p > 0.05$).

3.4. Relationship between the methylation of TRβ1 gene promoter and its mRNA expression profiles in gastric cancer patients

There was a strong correlation between TRβ1 gene methylation and mRNA expression levels in the gastric cancer patients. In the 34 cases in which TRβ1 gene promoter methylation occurred, the mRNA expression level was 2.41 ± 1.90. In the 12 cases in which no methylation of the TRβ1 gene promoter was observed, the mRNA expression level was 8.10 ± 2.84. The difference between TRβ1 gene methylation status and mRNA expression is statistically significant (pearson correlation) (OR = −0.727, $p = 0.000$).

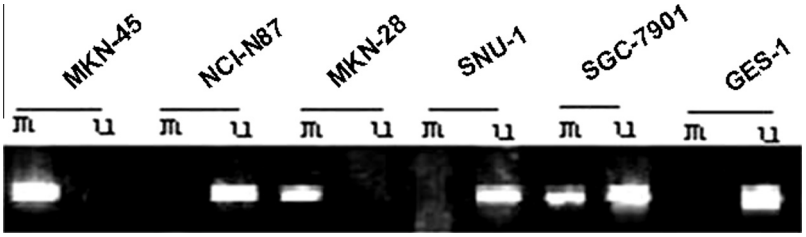

Fig. 2. Analysis of methylation status of the TRβ1 in gastric cancer cell lines. The promoter region was methylated in MKN-45, MKN-28 and SGC-7901 cell lines. In contrast, TRβ1 gene methylation was absent in the non-tumor cell line GES-1 and in the tumor cell lines NCI-N87 and SNU-1.

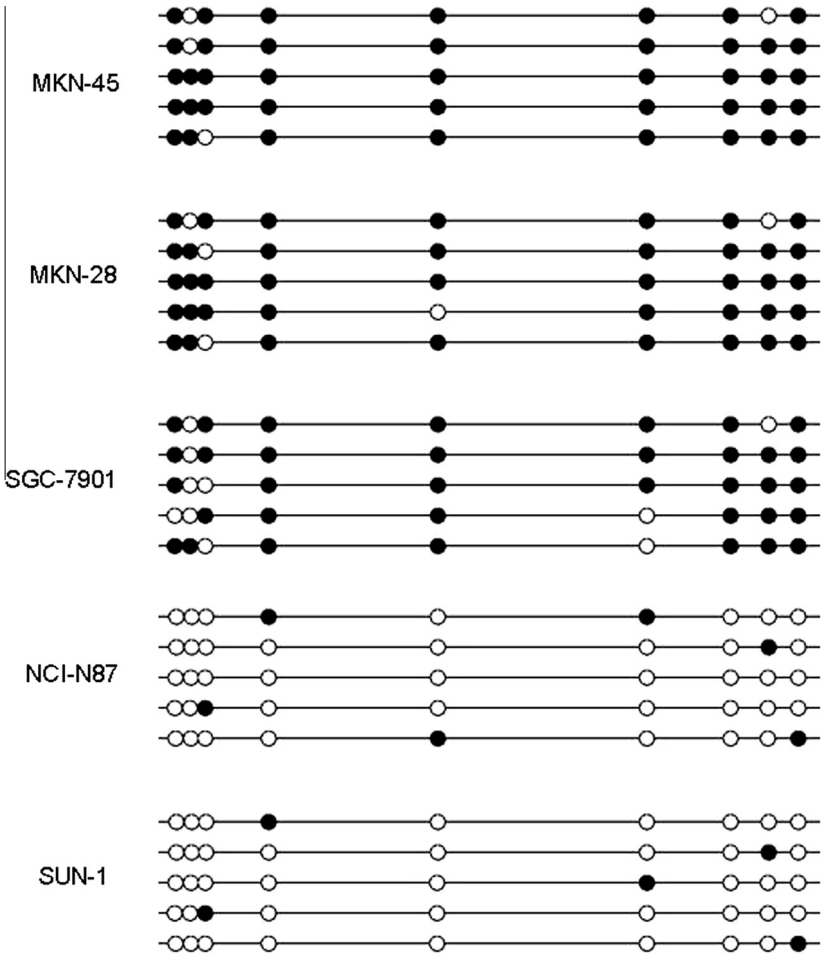

Fig. 3. Methylation status of 9 CpG sites in the promoter region of TRβ1 gene. Methylation analysis was peformed in five clones for each cell line. Each row of circles represents a single clone, and each circle represents a single CpG site. Open circle represents unmethylated cytosine; filled circle methylated cytosine.

3.5. Methylation status of the TRβ1 gene promoter in cell lines

The methylation statuses of the promoter region were examined by means of methylation-specific PCR (MSP). Promoter hypermethylation of the TRβ1 gene was found in 3 (MKN-45, MKN-28 and SGC-7901) of 5 (60%) gastric tumor cell lines. In these three cell lines, TRβ1 mRNA expression was low. In contrast, TRβ1 gene methylation was absent in the non-tumor cell line GES-1 and in the tumor cell lines NCI-N87 and SNU-1 (Fig. 2). Subsequent numbers of independent recombinant plasmids were DNA-sequenced. The result showed amplified promoter region covered 9 CpG sites within the CpG island of TRβ1 gene. There were very low methylation levels at a few sites in NCI-N87 and SNU-1 compared with MKN-45, MKN-28 and SGC-7901 gastric cell lines (Fig. 3).

3.6. Re-expression of the TRβ1 gene by 5-Aza-dC treatment

To test whether aberrant expression of TRβ1 gene in gastric cancer cells was responsible for CpG island demethylation, we treated cancer cell lines with 5-Aza-2-deoxycytidine (5-Aza-dC). After 4 days, the change in mRNA expression level was determined by qPCR. The mRNA expression of TRβ1 gene was increased in MKN-45, MKN-28, SGC-7901gastric cancer cell lines compared to the control ($p < 0.05$, Fig. 4). However, the mRNA expression was not increased in either the NCI-N87 or SNU-1 gastric cancer cell lines.

4. Discussion

Increasing evidence supports the notion that TRβ1 gene could play an important role in carcinogenesis [9–12]. It has been shown

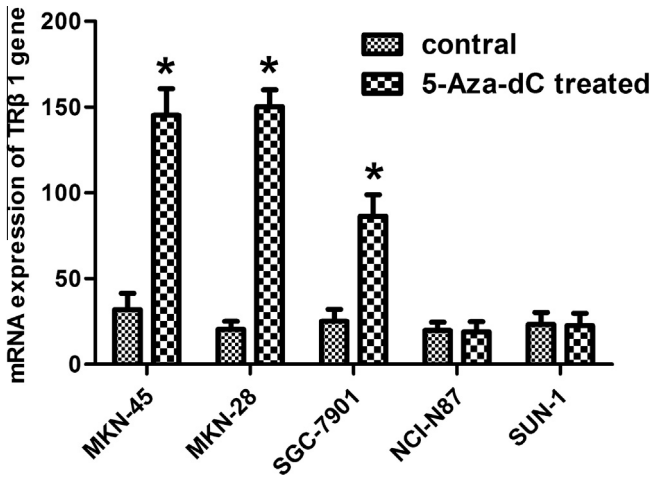

Fig. 4. Real-time quantitative RT-PCR analysis of the expression levels of the TRβ1 gene before and after treatment with 5-Aza-dC in gastric cancer cell lines. The mRNA expression of TRβ1 gene was increased in MKN-45, MKN-28, and SGC-7901 gastric cancer cell lines. The mRNA expression was not increased in NCI-N87 and SNU-1 gastric cancer cell lines $^{*}p < 0.05$. Each bar represents mean ± SD.

that Loss of Heterozygosity (LOH) of the 3p21-p25 region, where the TRβ1 gene is located, was observed in up to 100% of small cell lung cancers, 60% of uveal melanomas, and 64% of nonfamilial renal cell carcinomas [19–21]. These results have lead to the proposal that the TRβ1 gene may function as a tumor suppressor. Subsequent studies have been identified changes in TRβ1 gene expression in different cancers. Most research has indicated a positive correlation between decreased mRNA expression of TRβ1 gene and cancer [22–25]. However, TRβ1 gene has rarely been addressed for gastric cancer. Only one non-quantitative study has demonstrated a decrease in TRβ1 gene expression in gastric carcinoma specimens by reverse transcription–polymerase chain reaction [26]. In the current study, for the first time TRβ1 mRNA expression in gastric cancer has been quantified using qPCR. Statistically significant reduced mRNA expression was observed in 38 of 46 gastric cancer tissue samples compared to matched normal tissue. In addition, it was shown that TRβ1 mRNA expression is low in gastric cancer cell lines compared to a non-tumor gastric cell line. These findings suggest the possibility that TRβ1 gene is critically involved in gastric cancer development.

We investigated the mechanisms of TRβ1 mRNA expression in gastric cancer. One study detected a homozygous mutation at the TRβ1 gene locus in patients with gastric cancer; however, the authors believed TRβ1 gene to be a genetically unstable region on chromosome 3p [27]. Chia-Siu Wang et al. [28] showed that mutation alterations of TRβ1 gene could not detect in gastric cancer. These studies made us expect the possibility of other mechanisms that repress TRβ1 expression. Epigenetic changes have been discovered to be major contributing factors in carcinogenesis. Methylation-mediated gene silencing is a common epigenetic alteration in human gastric cancers [29]. The inactivation of the TRβ1 gene through aberrant promoter methylation has been found in breast cancer, thyroid cancer, and acute lymphoblastic leukemia [13–15]. For these reasons, detection of epigenetic aberrations of the TRβ1 gene in gastric cancer was undertaken. We first demonstrated aberrant promoter methylation of the TRβ1 gene in gastric cancer samples. The results showed the methylation of promoter of TRβ1 gene in gastric cancer tissues was significantly higher than in adjacent normal tissues.

We also analyzed the relation between TRβ1 methylation status and patient clinical pathological data. Silva et al. [30] reported no significant association between abnormal expression or deletion of TRβ1in breast tumors and any clinical parameters. Iwasaki et al. [31] reported the methylation status was not significantly associated with any clinicopathological parameters of non-small cell lung cancer (NSCLC) patients. Similarly, our earlier study has demonstrated the methylation status was not significantly associated with any clinicopathological parameters of breast patients [16]. In the present study, our data demonstrate that promoter hypermethylation of TRβ1 gene was not correlated with some clinicopathological characteristics, such as, age, gender, stomach location, histological type, or tumor differentiation. However, it was associated with other clinicopathological characteristics, such as tumor infiltration, lymph node metastasis, and distant metastasis. These results suggested that the progression of gastric cancer may attribute to the hypermethylation of the TRβ1 gene, which may serve as a prognostic marker in this disease. Specifically, distant metastasis is the major pattern of cancer recurrence related to the cause of death after operation. On account of its close association with distant metastasis, we believed aberrant methylation of TRβ1 gene might have a negative impact on patients' survival.

In order to determining whether methylation of the CpG island of the TRβ1 promoter region contributes to mRNA expression, we also analyzed correlation of TRβ1 gene mRNA expression with promoter methylation status. Thirty-four cases with promoter methylation showed reduced expression of mRNA compared with twelve cases in which no methylation in TRβ1 promoter was detected. This result demonstrated an inverse relationship between TRβ1 promoter methylation and mRNA expression. Notably, however, promoter methylation was not observed in 6 of the 38 patients with reduced mRNA expression of TRβ1 gene. The current study showed that promoter methylation could not explain all cases that lost expression of TRβ1 mRNA. A recent study of papillary thyroid cancer has provided evidence that the expression of the TRβ1 gene could also be repressed via micro RNAs regulatory mechanisms [32]. Although, the present results indicated that its partial inactivation through aberrant methylation may be a relevant event in the gastric cancer, other mechanisms need to be considered.

To further assess whether TRβ1 gene is functionally methylated in gastric cancer cells, we examined the effect of a demethylating agent, 5-Aza-dC, on TRβ1 low expression in five gastric cancer cell lines. We found TRβ1 gene mRNA expression was increased after treated with 5-Aza-dC in three cancer cell lines and confirmed that promoter methylation leads to loss of TRβ1 expression in these cancer cells. However, the TRβ1 expression levels remained low after 5-aza-cytidine treatment in the other two cell lines, indicating the existence of other regulation mechanisms that repress TRβ1 expression.

In summary, this is the first study to demonstrate epigenetic inactivation of TRβ1 gene through aberrant promoter methylation in gastric cancer. Since TRβ1 gene actions are complex, tissue- and time-specific, aberrant expression has different effects and is associated with different tumor types. The aim of our current study was to determine the roles of TRβ1 gene in gastric cancer. Further studies by our group and other researchers should aid in establishing the role of repressing TRβ1 expression in tumor development.

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