·Basic Research·

Correlation of hypermethylation of TSP1 gene with TGF-β1 level and T cell immunity in gastric cardia adenocarcinoma

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[**Abstract**] **Background and Objective:** Thrombospondin-1 (TSP1) is an inhibitor of angiogenesis and its promoter hypermethylation has been found resulting in gene silencing in some primary human carcinomas. This study was to investigate the promoter methylation of TSP1 and its correlation with TGF-β1 level and T cell immunity in gastric cardia adenocarcinoma (GCA). **Methods:** Methylation specific polymerase chain reaction (MSP) approach and immunohistochemistry method were used to examine the methylation status of the 5′ CpG island and expression of TSP1 protein, respectively. The level of TGF-β1 was measured by ELISA and T cell immunity of GCA by flow cytometry analysis. **Results:** TSP1 methylation frequency was significantly higher in tumor specimens than in the corresponding normal tissues (35.4% vs. 3.1%, $P<0.001$) and significanty higher in Stages Ⅲ and Ⅳ tumor tissues than in Stages Ⅰ and Ⅱ tumor tissues ($P<0.05$). TSP1 protein expression was significantly lower in the tumor tissues than in the corresponding normal tissues ($P<0.05$) and statistically correlated with its methylation status ($P<0.01$). The total level of TGF-β1 was significanty higher in the GCA patients than in the healthy controls ($P<0.05$) and significanty higher in Stages Ⅲ and Ⅳ GCA patients than in Stages Ⅰ and Ⅱ GCA patients ($P<0.05$). The level of active TGF-β1 was significanty higher in the GCA patients with hypermethylation of TSP1 than in the GCA patients without methylation of TSP1 ($P<0.05$), but there was no statistical difference ($P>0.05$). The function of T cell immunity was significantly different between the GCA patients with hypermethylation of TSP1 and those without methylation of TSP1 ($P<0.05$). **Conclusions:** Promoter hypermethylation of TSP1 may play an important role in the development of GCA and reflect the biological behaviours of GCA.

Key words: gastric cardia adenocarcinoma, DNA methylation, TSP1 gene, TGF-β1 gene, T cell immunity

Gastric cardia carcinoma used to be classified as esophageal carcinoma or gastric carcinoma in tumor registry. Thanks to the progress in endoscopy screening and pathologic diagnosis, it has been identified as a distinct entity in recent years. Generally, the occurrence of tumor is a result of the co-action of environmental and genetic factors.[1] Besides some genetic changes, phenogenetic changes, such hypermethylation of tumor suppressor genes (TSGs), are now also considered to be involved in tumor occurrence and development and may be early events of this process.[2] Thrombospondin-1 (TSP1) is an endogenous tumor vascularization regulator. Recent studies suggested that TPS1, as a candidate TSG, could inhibit vascularization in tumors.[3] In many tumors, hypermethylation may silence this gene. However, no report has described its methylation status in gastric cardia adenocarcinoma (GCA). In the meantime, TPS1 is an important endogenous activator for TGF-β1. Absence of TSP1 expression can lead to the loss of its activation on TGF-β1, whereas TGF-β1, as an important cytokine in the body, is involved in many aspects of tumor immunity, such as inhibiting T cell proliferation and promoting vascularization.[4] In this study, we examined the methylation status of TSP1 gene and explored its correlation with serum TGF-β1 level and T-cell immunity in GCA, so as to have more understanding on the methylation status of TSP1 gene promoter in GCA, and to see whether TSP1 gene methylation and deactivation are related with TGF-β1 level, to investigate the role of TSP1 gene methylation in the occurrence and development of GCA.

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Materials and Methods

Materials

Main apparatuses and reagents Reagents and apparatuses

included protease K (Merck Company), quinhydrone (Sigma Company), sodium bisulfite (Sigma Company), Wizard DNA purification kit (Promega Company), mouse anti-human TSP1 polyclonal antibody as primary antibody (A6.1, Abcam Company), human TGF-β1 ELISA reagent kit (Jingmei Biotech Co., Ltd), PE-anti-CD3, FITC-anti-CD4, PE-anti-CD8 and FITC-anti-CD25 monoclonal antibodies (Coulter Company), FITC-anti-Foxp3 monoclonal antibody (eBioscience Company) and Epics-XL Ⅱ flow cytometer. Primers were synthesized by Beijing SBS genetech Co., Ltd.

Study subjects and samples Tumor samples were from 96 GCA patients who underwent surgical resection in the Fourth Affiliated Hospital of Hebei Medical University during 2004–2007. Of these patients, 73 were men and 23 women, with a median age of 56.5 years (range, 38–78 years). All patients did not receive preoperative chemotherapy and radiotherapy. Before resection, two blood samples were taken from each patient via peripheral vein after fasting; one was anti-coagulated and used for extraction of monocytes for the analysis on T-cell immunity, and the other was not anti-coagulated and was separated for serum, which was preserved at −80℃ for subsequent experiments. Tissue specimens were obtained from primary GCA lesion and normal tumor-adjacent mucosa in each patient, and were fixed in 10% neutral formaldehyde, paraffined and preserved as routine, then used for DNA extraction and immunohistochemical staining. Both tumor tissue and tumor-adjacent tissue were pathologically confirmed. Of the 96 cases of GCA, according to the TNM staging system by UICC, 7 (7.3%) were at stage Ⅰ, 35 (36.5%) at stage Ⅱ, 39 (40.6%) at stage Ⅲ, and 15 (15.6%) at stage Ⅳ; 43 (44.8%) were well differentiated, 35 (36.4%) were moderately differentiated, and 18 (18.8%) were poorly differentiated. During the same period, anti-coagulated and non anti-coagulated peripheral blood samples were obtained from 30 healthy individuals as controls, of which 16 were men and 14 women, with a median age of 51 years (range, 29–70 years).

Methods

Methylation-specific PCR (MSP) for measurement of TSP1 methylation Each specimen was prepared into 10–20 sections (10 μm), then digested by protease K as routine. Genomic DNA was extracted from cancer tissue and normal adjacent tissue using phenol-chloroform method. An amount of 5 μg DNA was obtained from each specimen and was denatured with 2 mol/L NaOH,[5] then incubated in 10 mmol/L quinhydrone and 3 mol/L sodium bisulfite at 50℃ for 16 h. Wizard DNA purification kit was used to purify the DNA sample that had been treated with sodium bisulfite. Primers used for the detection of methylated TSP1 were 5'-TGCGAGCGTTTTTTTAAATGC-3' (upstream primer) and 5'-TAAACTCGCAAACCAACTCG-3' (downstream primer) (74 bp); primers for detection of unmethylated TSP1 were 5'-GTTTGGTTGTTGTTTATTGGTTG-3' (upstream primer) and 5'-CCTAAACTCACAAACCAACTCA-3' (downstream primer) (115 bp). The reaction was catalyzed under 95℃ for 10 min for pre-denaturation, and then 35 cycles of 30 s at 94℃ for denaturation, 30 s at 62℃ for annealing and 30 s at 72℃ for elongation, and finally 72℃ for 10 min for further elongation. The amplified product underwent electrophoresis in 2% agarose gel. Image analyses were conducted by UV gel electrophoresis imaging and analyzing system. As positive control for MSP, genomic DNA was treated by methylase (*Sss* I; New England BioLabs, Inc., Beverly, MA) and was then used in PCR; as negative control, sterile double-distilled water was used in replacement of DNA template in PCR. As quality control for the MSP measurement, 10% of the samples were randomly selected for repeated experiments.

SP immunohistochemical detection for TSP1 protein expression Paraffin section was de-paraffinated and hydrated as routine. Endogenous peroxydase was blocked with 3% methanol and H_2O_2. The section was then recovered by microwave for 15 min. Primary antibody, corresponding biotinylated secondary antibody and horseradish peroxidase-labeled third antibody were consecutively added. The section was then treated with DAB, and cell nuclei were counterstained with hematoxylin. Routine dehydration, vitrification, and sealing with neutral gum were performed. PBS was used in replacement of primary antibody as blank control. Normal gastric mucosal tissue was used as positive control.

ELISA detection for TGF-β1 concentration Serum concentrations of total TGF-β1 and active TGF-β1 were measured respectively as described in the protocol of TGF-β1 immunohistochemical detection reagent kit. The serum samples treated by 1 mol/L hydrochloric acid were used for total TGF-β1 detection, while untreated serum samples were used for active TGF-β1 detection.

Flow cytometry for $CD3^+$, $CD4^+$, $CD8^+$ and $CD4^+$-$CD25^+$-$Foxp3^+$-Treg cells The percentages of $CD3^+$, $CD4^+$, $CD8^+$ and $CD4^+$-$CD25^+$- $Foxp3^+$-Treg cells in peripheral blood monocytes (PBMC) were measured by flow cytometry.

Statistical analyses Statistical analyses were performed by SPSS11.5 software. Intergroup differences were analyzed with chi-square test, analysis of variance and *t* test; correlation analysis was conducted with Spearman method. With two-sided test, $P<0.05$ indicated statistical significance.

Results

TSP1 gene methylation in GCA

MSP analysis was performed for 96 GCA specimens and corresponding adjacent tissues (Fig. 1). TSP1 gene methylation rate was significantly higher in GCA tissues than in normal adjacent tissues (35.4% vs. 3.1%, $P<0.001$), and significantly higher in stage Ⅲ–Ⅳ GCA tissues than in stage Ⅰ–Ⅱ GCA tissues ($\chi^2=4.40$, $P=0.04$); the differences between well, moderately and poorly differentiated tumors were not significant ($\chi^2=1.19$, $P=0.55$) (Table 1).

TSP1 protein expression in GCA

Immunohistochemically stained TSP1 protein was seen in cytoplasm and/or matrix (Fig. 2). The positive rate of TSP1 protein was significantly lower in GCA specimens than in corresponding adjacent tissues (71.9% vs. 100%, $P<0.001$), and

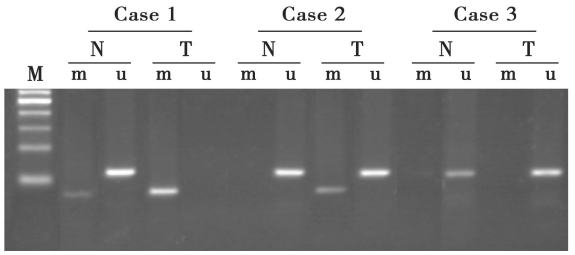

Figure 1 Methylation status of TSP1 in tumor tissue and corresponding normal tissue

T, tumor tissue; N, normal tissue; u, unmethylated gene; m, methylated gene; M, DNA marker. Case 1, the tumor is fully methylated, whereas the corresponding normal tissue has a very faint band demonstrating methylation; Case 2, tumor-specific methylation; Case 3, both of tumor and corresponding normal tissues are unmethylated.

Table 1 Methylation status of TSP1 gene and expression of TSP1 protein in patients with gastric cardia adenocarcinoma (GCA)

Group	Methylation status of TSP1		*P*	Protein expression of TSP1		*P*
	M	U		–	+	
TNM stage			0.036[a]			0.028[a]
Ⅰ	1	6		0	7	
Ⅱ	9	26		7	28	
Ⅲ	16	23		14	25	
Ⅳ	8	7		6	9	
Pathological differentiation			0.551[b]			0.463[b]
Well	13	30		10	33	
Moderate	13	22		10	25	
Poor	8	10		7	11	

[a]*P* value of Stages Ⅲ and Ⅳ patients vs. Stages Ⅰ and Ⅱ patients; [b]*P* value among three differentiation groups. M, methylated; u, unmethylated.

significantly lower in stage Ⅲ–Ⅳ GCA tissues than in stage Ⅰ–Ⅱ GCA tissues (χ^2=4.85, *P*=0.03), whereas the differences between well, moderately and poorly differentiated cancers were not significant. All the tumor tissues without TSP1 hypermethylation showed TSP1 protein expression, indicating significant correlation between methylation of TSP1 gene promoter and absent expression of TSP1 protein (*P*<0.01).

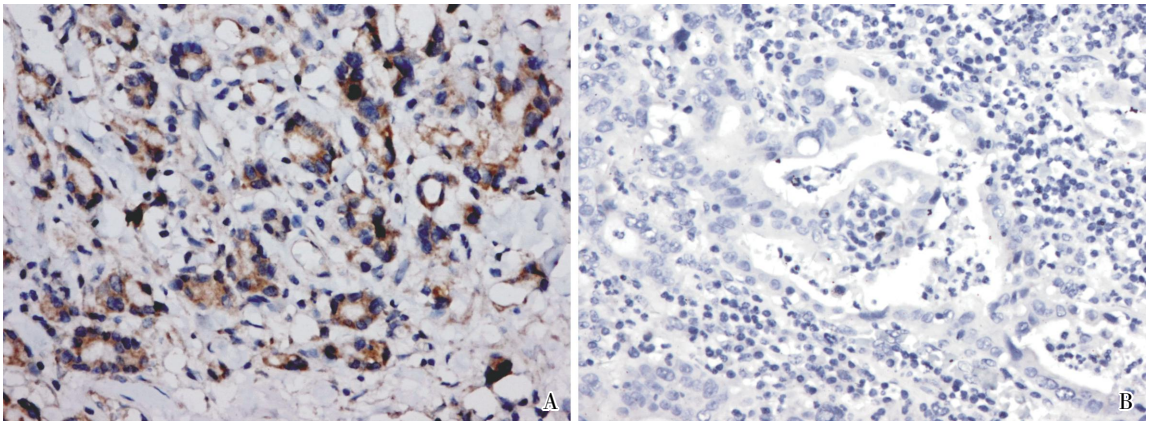

Figure 2 Expression of TSP1 protein in gastric cardia adenocarcinom tissues (SP×200)

A, positive staining; B, negative staining.

TGF-β1 level in GCA

Serum concentration of total TGF-β1 was significantly higher in GCA patients than in normal controls [(17.2±6.0) μg/L vs. (11.9±3.1) μg/L, *P*<0.05]; however, the difference in concentration of active TGF-β1 was not significant [(8.2±5.0) μg/L vs. (8.8±4.1) μg/L, *P*>0.05]. Serum concentration of total TGF-β1 was significantly higher in stage Ⅲ–Ⅳ GCA tissues than in stage Ⅰ–Ⅱ GCA tissues (*P*<0.05). The differences in serum concentration of total and active TGF-β1 between the GCA tissues with and without TSP1 methylation were not significant (*P*>0.05) (Table 2).

Correlation between cell immunity and TSP1 methylation in GCA patients

GCA patients had less $CD3^+$ and $CD4^+$ cells and lower $CD4^+/CD8^+$ ratio than normal controls (*P*<0.05), and more $CD8^+$ and $CD4^+$-$CD25^+$Foxp3$^+$-Treg cells than normal controls (*P*<0.05). The patients with stage Ⅲ–Ⅳ GCA had less $CD3^+$ and $CD4^+$ cells, lower $CD4^+/CD8^+$ ratio, and more $CD8^+$ and $CD4^+$-$CD25^+$-Foxp3$^+$-Treg cells than those with stage Ⅰ–Ⅱ GCA (all *P*<0.05). The percentage of $CD4^+$ cells and $CD4^+/CD8^+$ ratio were significantly lower in GCA patients with TSP1 methylation than in those without methylation (*P*<0.05), while the percentages of $CD8^+$ and $CD4^+$-$CD25^+$-Foxp3$^+$-Treg cells significantly higher in GCA patients with TSP1 methylation than in those without methylation (*P*<0.05); no significant difference was seen in $CD3^+$ cells (Table 3).

Table 2 The levels of TGF-β1 in patients with GCA (μg/L)

Group	Level of total TGF-β1 ($\bar{x}\pm s$)	*P*	Level of active TGF-β1 ($\bar{x}\pm s$)	*P*
Control	11.9±3.1		8.8±4.1	
GCA patients	17.2±6.0	0.002[a]	8.2±5.0	0.253[a]
Stages Ⅰ and Ⅱ GCA patients	13.0±4.8		8.0±3.2	
Stages Ⅲ and Ⅳ GCA patients	20.8±5.3	0.001[b]	8.3±4.0	0.356[b]
GCA patients with TSP1 methylation	17.0±4.1		7.9±3.1	
GCA patients without TSP1 methylation	17.3±5.2	0.423[c]	8.6±4.2	0.106[c]

[a]GCA patients vs. control; [b]Stages Ⅲ and Ⅳ patients vs. Stages Ⅰ and Ⅱ patients; [c]GCA patients without TSP methylation vs. GCA patients with TSP1 methylation.

Table 3 The status of T cell immunity in patients with GCA ($\bar{x}\pm s$)

Group	$CD3^+$(%)	$CD4^+$(%)	$CD8^+$(%)	$CD4^+/CD8^+$	Treg(%)
Control	67.7±7.5	45.8±6.9	27.6±8.1	1.6±0.3	5.2±0.7
GCA patients	55.6±8.5	33.9±7.6	38.1±6.9	0.9±0.2	7.1±1.7
Stages Ⅰ and Ⅱ GCA patients	69.2±7.7	41.4±6.9	30.7±7.3	1.2±0.3	5.9±1.2
Stages Ⅲ and Ⅳ GCA patients	40.3±6.8	27.1±7.8	42.2±7.9	0.6±0.2	8.2±2.0
GCA patients with methylation of TSP1	54.8±6.9	28.2±8.1	40.8±5.9	0.8±0.2	7.9±1.5
GCA patients without methylation of TSP1	55.9±7.6	39.1±6.9	31.9±6.8	1.1±0.3	6.2±1.1

Discussion

TSP1 is a member of thrombospondin family, which includes five members. TSP1 and TSP2 are trimers, while TSP3, TSP4 and TSP5 are pentamers. These proteins are highly homologous, but they are expressed at different levels in different tissues due to varied promoters. In 1971, TSP1 was identified as a glycoprotein with high molecular weight (450 000). Initial studies demonstrated that TSP1 was produced and secreted by platelets, but subsequent studies found that it could also be produced and secreted by fibroblasts, monocytes, macrophages, smooth muscle cells and some kinds of tumor cells.[6-8] In physiological circumstance, serum concentration of TSP1 ranges from 60 to 300 ng/mL.[9] TSP1 is involved in numerous physiological processes, including cell adhesion, migration and proliferation, intercellular interaction, vascularization, tumor cell metastasis, inflammation and hemagglutination.[10,11] Studies suggested that TSP1 produced by breast cancer cell line could inhibit tumor progresssion, and that TSP1 expression was negatively related to the malignant progression of melanoma, lung cancer and breast cancer.[12,13]

Studies have proven that TSP1 was an endogenous vascularization inhibitor, which could induce apoptosis in endothelial cells in vitro, and inhibit neovascularization in vivo and invasive growth.[12] TSP1 has complicated action mechanisms. It can bind to CD36 receptors on the surface of capillary vessels and thus exerts its effect in counteracting vascularization; it can also bind to the precursor of TGF-β and thereby releases active TGF-β in a direct or indirect manner, whereas TGF-β promotes tumor growth and invasion by stabilizing the vacular system in the tumors.[4] Some studies demonstrated that hypermethylation of TSP1 gene led to absent expression of this protein in many tumors, and was thereby involved in the occurrence and development of tumors.[14,15] But studies regarding TSP1 methylation status in GCA are rare.

Our study found highly frequent methylation of TSP1 gene (35.4%) in GCA tissues, together with absent expression of TSP1 protein, indicating that gene silence, which was resulted from methylation of TSP1 gene promoter, might be one important reason for the deactivation of this gene in GCA. TSP1 protein expression in GCA tissues was significantly lower than that in normal adjacent tissues, but protein expression could be positive even in tumor tissues with TSP1 methylation. Confounding normal tissues in tumor tissues might be one reason, and heterogeneous gene methylation and allele methylation might be another important reason. In our study, we also found positive protein expression in GCA tissues with methylation, indicating incomplete methylaiton. In addition, DNA methylation was currently considered to inhibit gene expression on transcription level. Some studies suggested that density of CpG island methylation was correlated the inhibition level on transcription;[16] a weak promoter might be completely inhibited by lower density of methylation, but when the promoter was enhanced by an enhancer, transcription activity could be partially restored. In some tumor tissues, the methylation level of TSP1 gene promoter might not be enough to inhibit transcription, leading to a scenario where gene is methylated but protein expression is still positive.

TGF-β family exerts important biological effects via TGF-β /Smad signal transduction pathway; they regulate cell proliferation, differentiation, apoptosis, migration and adhesion and the production of extracellular matrix, and also have important roles in embryonic development, repairing mature tissue and immunity regulation.[4] TGF-β is a strong inhibitor on cell growth. It inhibits cell growth by changing the expression or functions of cell cycle regulators. During the occurrence of tumors, TGF-β has biphasic effects. At early stages when cells are still responsive to the anti-proliferation effect of TGF-β, it

exerts inhibition on the tumors; while in malignant progression stages when cells develop resistance to the growth inhibition of TGF-β, it promotes tumorigenesis.[10] Our study found that serum TGF-β1 level was significantly elevated in patients with GCA, and also significantly increased with tumor progression, suggesting close correlation between serum TGF-β1 concentration and the occurrence, development, infiltration and metastasis of GCA. As an important endogenous activator of TGF-β1, TSP1 contacts and reacts with TGF-β1 latency associated peptide (LAP) and leads to the dissociation of the non-covalent bond between TGF-β1 and LAP; moreover, the binding of TSP1 and TGF-β1 prevents TGF-β1 from binding to its receptor and thus makes it inert. To examine whether TSP1 influenced the activation of TGF-β1 in GCA patients, we analyzed serum concentration of active TGF-β1 in GCA patients. The results showed that, although active TGF-β1 concentration in patients with TSP1 methylation was lower than those without TSP1 methylation, the difference was not significant. However, in the study on colon cancer cell line, Rojas *et al.*[15] found that TSP1 demethylation resulted in increased concentration of active TGF-β1 in the supernatant of cell culture. The reason for such inconsistence might be, first of all, the different types of tumor used in the studies: our study detected the serum active TGF-β1 concentration in the peripheral blood from GCA patients, while Rojas *et al.* detected active TGF-β1 concentration in the supernatant of colon cancer cell culture. In addition, influential factors for in vitro cell culture were simple, while with the complicated signal tranduction pathways in vivo, the activation and secretion of TGF-β1 might be regulated by multiple genes. The influence of TSP1 methylation alone on the activation of TGF-β1 was not obvious enough to be seen in peripheral blood. This also demonstrated that the occurrence and development of GCA was a result of the multi-stage co-action involving numerous genes.

Host anti-tumor immunity is mainly seen as cell immunity. T cell subgroups are important in maintaining immune surveillance over tumors, and are also important effector cells in the anti-tumor immunity of the body. In a healthy individual, the immune system is in stable balance, containing a lot of effector T lymphocytes and also inhibitory T lymphocytes, namely Treg cells. Using serial CD monoclonal antibody (mAb), we can recognize the antigenic determinant on the surface of T lymphocytes. Among these CD markers, CD3 is a surface marker for mature T cells, while CD4 and CD8 are surface markers of T helper cells (Th) and cytotoxic T cells (Tc), respectively. Decreased CD3 cells and inverted CD4/CD8 ratio generally reflect depressed cell immunity in the body. Treg cells, which express high level of CD25, show inhibition on immunity. Foxp3 is currently recognized as an optimal marker for $CD4^+$ Treg cells. TGF-β is in extremely close correlation with Treg cells by that it transforms $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ T cells, which is one of the important sources of Treg cells in vivo. Meanwhile, Treg cells per se also secrete TGF-β, and thus inhibit cell immunity.[17] Our study demonstrated that GCA patients had less $CD3^+$ and $CD4^+$ cells and more $CD8^+$ cells than healthy individuals, with an inverted CD4/CD8 ratio; $CD4^+$-$CD25^+$-$Foxp3^+$-Treg cells were increased, and cell immunity was further impaired as the tumor progressed; in addition, cell immunity was significantly correlated to serum TGF-β1 concentration. Further analysis revealed that cell immunity in GCA patients with TSP1 methylation was remarkably lower than in those without TSP1 methylation, suggesting that methylation deactivated TSP1 and thus affected its inhibition on vascularization. As a result, the normal balance of vascularization was interrupted, and cytokines that inhibited immunity and promoted vascularization, such as TGF-β1, were increased. In turn, TGF-β1 inhibited cell immunity in tumor patients by inhibiting the proliferation of Th cells and inducing the production of Treg cells, and thus helped tumor cells escape from the immune surveillance in the body and promoted occurrence, development, infiltration and metastasis of the tumors.

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