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Elevated Serum Level and Gene Polymorphisms of TGF-β1 in Gastric Cancer

Xue Li,[1] Zhi-Chao Yue,[1] Yun-Yan Zhang,[2] Jing Bai,[1] Xiang-ning Meng,[1] Jing-Shu Geng,[2] and Song-Bin Fu[1]*

[1]*Laboratory of Medical Genetics, Harbin Medical University, Harbin, China*
[2]*The Third Affiliated Hospital of Harbin Medical University, Harbin, China*

Transforming growth factor (TGF)-β1, as a candidate tumor marker, is currently of interest. In this study, serum TGF-β1 levels in gastric cancer (GC) patients and healthy volunteers were measured using enzyme-linked immunosorbent assay (ELISA). In addition, single nucleotide polymorphisms (SNPs) of the TGF-β1 gene at codon 10 and codon 25 were identified by means of amplification refractory mutation system–-polymerase chain reaction (ARMS-PCR) and sequence analysis. Our results indicated that serum concentrations of TGF-β1 in GC patients were significantly higher than those in the control, and positively correlated with tumor mass, invasion, metastasis, and clinical stage. The serum TGF-β1 levels of patients recovering from radical resection were markedly lower than those before surgery. Meanwhile, no deoxyribonucleic acid (DNA) sequence variation at codon 25 of the TGF-β1 gene was found and a TGF-β1 gene polymorphism at codon 10 did not show obvious correlations with either TGF-β1 expression or clinicopathological parameters of GC. Our evidence suggested that serum concentration of TGF-β1 might be a novel tumor marker for GC and the polymorphisms of TGF-β1 gene did not play a role as a determinant of serum TGF-β1 concentration or as a genetic risk factor in the gastric carcinogenesis and progression. J. Clin. Lab. Anal. 22:164–171, 2008.

Key words: transforming growth factor-beta 1; SNPs; ARMS-PCR; ELISA

INTRODUCTION

As one of the most common malignant tumors in the world (1), gastric carcinogenesis and development involves genetic alterations of multiple genes. However, the exact molecular mechanism underlying gastric cancer (GC) remains to be fully elucidated. Malignant tumors always release some proteins and polypeptides into the blood circulation, which act as helpful markers for the diagnosis, therapy, and prognosis of cancers, including human chorionic gonadotropin, carcinoembryonic antigen, and alpha-fetoprotein. Despite the lack of significant clinical tumor markers for GC, transforming growth factor (TGF)-β1 as a candidate tumor marker is now of interest (2,3).

TGF-β, as a pluripotent cytokine, was first discovered in an assay based on its ability to transform fibroblasts phenotypically in culture (4). In mammals, TGF-β has three isoforms: TGF-β1, TGF-β2, and TGF-β3. TGF-β1 is the predominant form in the human body. The TGF-β1 gene, located on chromosome 19q13, is translated to a precursor, which is bound to the latent TGF-β1 binding protein (LTBP) to form a latent complex whose activation is modulated by plasmin, cathepsin D, and so on (5–7). TGF-β1 signal transduction relies upon the type I and II TGF-β1 receptors (TβRI and TβRII) and intracellular Smads proteins, which propagate the signal and subsequently regulate specific TGF-β1-responsive gene transcriptions in the nucleus (8,9). Noticeably, TGF-β1 shows biphasic effects on contributing to the gastric carcinogenesis and progression. In the initial stage of tumorigenesis,

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Xue Li and Zhi-Chao Yue contributed equally to this publication.

*Correspondence to: Dr. Song-Bin Fu, Professor, Laboratory of Medical Genetics, Harbin Medical University, Harbin 150081, China. E-mail: fusb@ems.hrbmu.edu.cn

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TGF-β1 may play a negative or tumor suppressor role by inhibiting cellular proliferation or by promoting cellular differentiation and apoptosis. However, as cancer develops, most GC cells become resistant to the growth-inhibitory properties of TGF-β1 due to the genetic changes of TβR (10,11). And the paracrine TGF-β1 produced by the cancer cells and the stromal cells stimulates angiogenesis and cell motility, suppresses immune response, and increases the interaction of tumor cells with the extracellular matrix (ECM), finally leading to progressive invasion and metastasis of the GC (12,13).

Several reports have made a connection between the significantly elevated serum concentration of TGF-β1 and the progression and recurrence of many carcinomas such as breast cancer, prostate cancer, hepatocellular cancer, and colorectal cancer (2,14–17). However, studies concerning serum TGF-β1 levels in GC are controversial (18,19). Moreover, individual discrepancies in TGF-β1 expression may result from certain differential genetic backgrounds. Recently, the relationship between polymorphisms of functional genes and genetic susceptibility of cancer has attracted more and more researchers. For the human TGF-β1 gene, more than 10 polymorphic loci are presently known, distributed in exons, introns, and the 5′-flanking region (20), and the hotspots focus on the single nucleotide polymorphisms (SNPs) of codon 10 and codon 25. It has been reported that TGF-β1 SNPs were associated with neurodegeneration as well as susceptibilities to hepatitis C virus (HCV) infection, many kinds of cancer, and so on (21–24). In this study, we combine the detection of serum TGF-β1 levels, analysis of TGF-β1 gene polymorphisms, and clinicopathological parameters of GC patients by means of enzyme-linked immunosorbent assay (ELISA) and amplification refractory mutation system–polymerase chain reaction (ARMS-PCR) to evaluate the possible associations among them as well as the feasibility of serum TGF-β1 level as a GC biomarker. We also found that serum concentration of TGF-β1 might be a novel tumor marker, although it is still different to set the standard value. However, the polymorphisms of TGF-β1 do not show association with serum concentration, gastric carcinogenesis, and development.

MATERIALS AND METHODS

Patients

A total of 53 consecutive patients with GC (40 men and 13 women, mean age of 56.2 years, range 24–78 years) who underwent surgery in the Second and Third Hospitals of Harbin Medical University were included in the study. A total of 50 age-matched healthy volunteers (30 men and 20 women, mean age 52.3 years, range 25–73 years) also participated in the study as a control group.

Deoxyribonucleic Acid and Serum Preparation

Venous blood samples were taken from patient before surgery and healthy control, allowed to clot for 3 hr, and centrifuged at 3,000 *g* for 20 min at 4°C. The resulting serum was transferred to polypropylene microtubes and stored at –70°C until assayed. Genomic deoxyribonucleic acid (DNA) was extracted from the clot by the classical phenol-chloroform method. Serum samples of five patients who underwent radically curative surgery were also taken on postoperative day 7 and prepared in the same manner.

ELISA for TGF-β1

Serum TGF-β1 levels were detected using the human recombined TGF-β1 ELISA kit (Jingmei, Beijing, China). The sera were activated by acidification/neutralizing and tested at 1:50 dilution. The optical density (OD) was measured by a Model 550 microplate autoreader set to 450 nm (Bio-Rad, Hercules, CA). The concentration of TGF-β1 was determined from a standard curve according to the manufacturer's instructions. The detection of patients was also determined considering the mean +1 standard deviation (SD) or 2 SD of the control group as a cutoff limit.

Genotyping of TGF-β1 Codon 10 and Codon 25 by ARMS-PCR

For detection of the polymorphisms of TGF-β1 gene at codon 10 (T+29C) and codon 25 (G+74C), ARMS-PCR was applied. The information of allele-specific primers used and SNPs in detail are listed in Table 1. Each PCR reaction contained 100 ng/μL genomic DNA, 10 pmol/L of each primer, 2.5 mmol/L dNTPs, 25 mmol/L $MgCl_2$, $10 \times NH_4^+$ PCR buffer and 5 μg/μL Taq DNA polymerase (Promega, Madison, WI). PCR was performed on the PE 480 (PE, Boston, MA) under the following conditions: first at 95°C for 5 min; 30 cycles at 95°C for 30 sec, at different annealing temperature for 30 sec (66°C for codon 10 and 63°C for codon 25), at 72°C for 30 sec; then at 72°C for 10 min, and finally hold at 4°C. PCR products were detected by electrophoresis on 1.5% agarose gel containing ethidium bromide (EB). Negative and positive controls for the target allele were included and the experiments repeated three times independently to confirm the genotype of each subject.

Sequence Analysis

Selecting the heterozygous or homozygous subjects at the two SNPs sites identified above by ARMS-PCR, a

TABLE 1. Information of TGF-β1 SNPs and primers for ARMS-PCR

SNPs	dbSNP in NCBI	Product length	Substitution		Primer sequence (capital letters represent mutation sites)
			Nucleotide	Amino acid	
Codon 10	rs1982073	346−bp	cTg→cCg	leucine→proline	T: 5′-ctccgggctgcggctgctgcT-3′ CTCCGGGCTGCGGCTGCTGCT C: 5′-ctccgggctgcggctgctgcC-3′ Common: 5′-gttgtgggtttccaccattag-3′
Codon 25	rs1800471	233−bp	cGg→cCg	arginine→proline	G: 5′-gtgctgacgcctggccG-3′ C: 5′-gtgctgacgcctggccC-3′ Common: 5′-ggctccggttctgcactc-3′

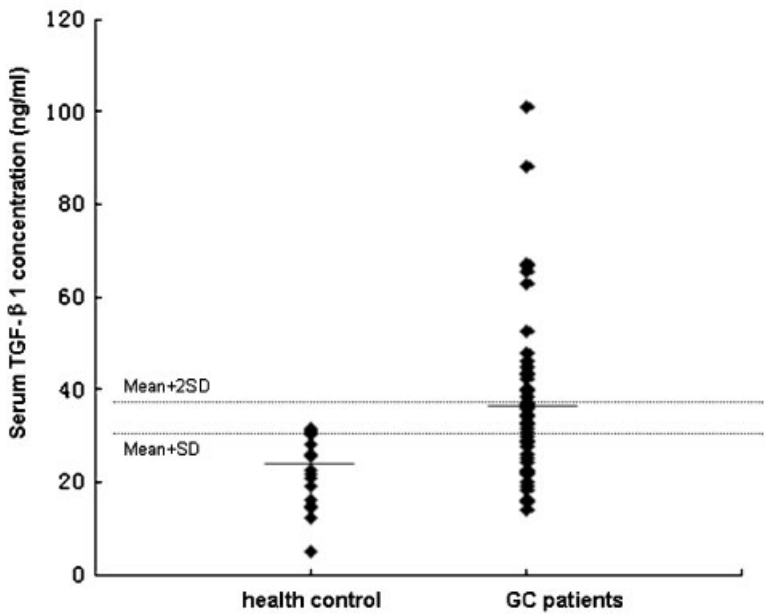

Fig. 1. TGF-β1 serum levels in GC patients and healthy donors. (Horizontal bars and dotted lines represented median values and cutoff values of TGF-β1, respectively).

590-base pair (bp) DNA region covering TGF-β1 codon 10 and 25 was amplified with the same PCR reaction and cycle condition as in ARMS-PCR for codon 10. The primers used were as follows: forward primer, 5′-atctctctccgacctgccac-3′ and reverse primer 5′-cttggcgtag-tagtcggcctc-3′. After being purified by a PCR purification kit (Promega), the PCR products were sequenced with the Big DYETM Terminator Cycle Sequencing Ready Reaction kit (PE-Applied Biosystems, Foster City, CA) using an ABI PRISMTM 377 XL DNA sequencing system (PE-Applied Biosystems) to confirm the results of ARMS-PCR.

Statistical Analysis

Data were summarized as mean ± SD and analyzed using the homoscedasticity test, *t*-test, analysis of variance, *q*-test, and the chi-squared test. $P<0.05$ was considered statistically significant.

RESULTS

Elevated Serum Concentration of TGF-β1 in GC Patients

Serum concentrations of TGF-β1 (36.3 ± 17.6 ng/mL) were significantly higher in GC patients than those in healthy volunteers (22.1 ± 7.4 ng/mL, $P<0.01$), as shown in Fig. 1. According to the pathological tumor-node-metastasis (PTNM) classification of International Union Against Cancer (IUCC), there were significant differences in serum concentrations of TGF-β1 among patients with I, II, III, or IV clinical stage ($P<0.01$). Considering 29.5 ng/mL (mean + 1 SD of controls) as a cutoff limit, the positive frequency of serum TGF-β1 levels in GC patients (64.2%) was greater than that of

TABLE 2. Relationship between serum concentrations of TGF-β1 and clinicopathological parameters in patients with GC (mean ± SD)

Variables	Number	TGF-β1 (ng/mL)	*P*-value
Gender			>0.05
Male	40	35.2 ± 16.8	
Female	13	39.7 ± 20.3	
Age			>0.05
⩽55	24	39.4 ± 21.3	
>55	29	33.7 ± 13.7	
Tumor location			>0.05
Antrum	42	38.3 ± 18.6	
Corpus and fundus	11	28.6 ± 10.8	
Histological type			>0.05
Well and moderately differentiated	37	37.6 ± 19.4	
Poorly differentiated, signet and mucinous	16	33.3 ± 12.5	
Tumor size			<0.01
<100 cm3	28	29.0 ± 11.0	
>100 cm3	25	44.5 ± 20.1	
Depth of invasion			<0.05
T1-2	11	29.9 ± 16.0	
T3	12	28.8 ± 9.7	
T4	30	41.7 ± 19.1[a]	
Lymph node metastasis			<0.05
Negative	15	28.9 ± 11.5	
Positive	38	39.2 ± 18.9	
Distant metastasis			<0.01
Negative	37	29.9 ± 10.3	
Positive	16	51.1 ± 22.1	

[a] $P<0.05$ vs. T1-2 or T3 group.

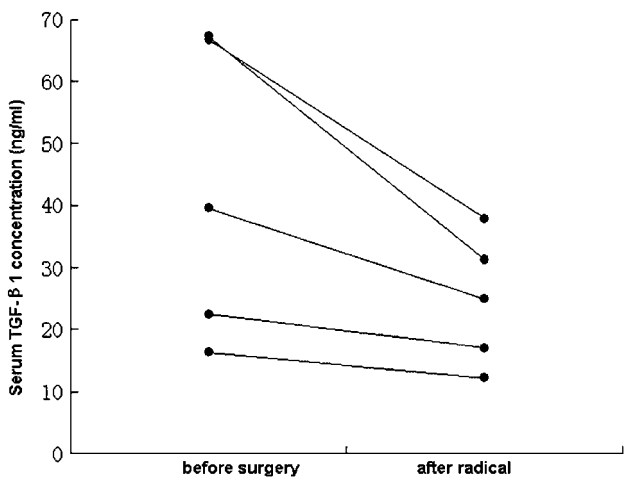

Fig. 2. Serum TGF-β1 levels in five GC patients before and after radical surgery.

healthy volunteers (22.7%, $P<0.01$). Using a cutoff limit of 37.0 ng/mL (mean + 2 SD), the positive frequency of TGF-β1 was 37.7% in GC patients and none of the controls had TGF-β1 levels higher than 37.0 ng/mL (Fig. 1). The detectability of serum TGF-β1 levels increased as GC stage progressed. As shown in Table 2, no significant differences in serum TGF-β1 levels were found in GC patients with different age, sex, tumor location, and histological type ($P>0.05$). However, Serum concentration of TGF-β1 was positively correlated with tumor mass ($P<0.01$), depth of invasion ($P<0.05$), lymph node metastasis ($P<0.05$), and distant metastasis of GC ($P<0.01$).

On postoperative day 7 the serum TGF-β1 levels of five patients recovering from radically curative resection (24.63 ± 10.4 ng/mL) were markedly lower than those before surgery (42.4 ± 24.0 ng/mL, $P<0.05$) and similar to those of controls ($P>0.05$) (Fig. 2).

TGF-β1 SNPs Analysis

The result of ARMS-PCR showed that there were three genotypes CC, CT, and TT at codon 10 of the TGF-β1 gene in the GC patients and the control patients, which were also confirmed by sequencing analysis. However, the study population was not polymorphic at codon 25 (Fig. 3 and Fig. 4). The frequency distribution of the genotype and alleles of

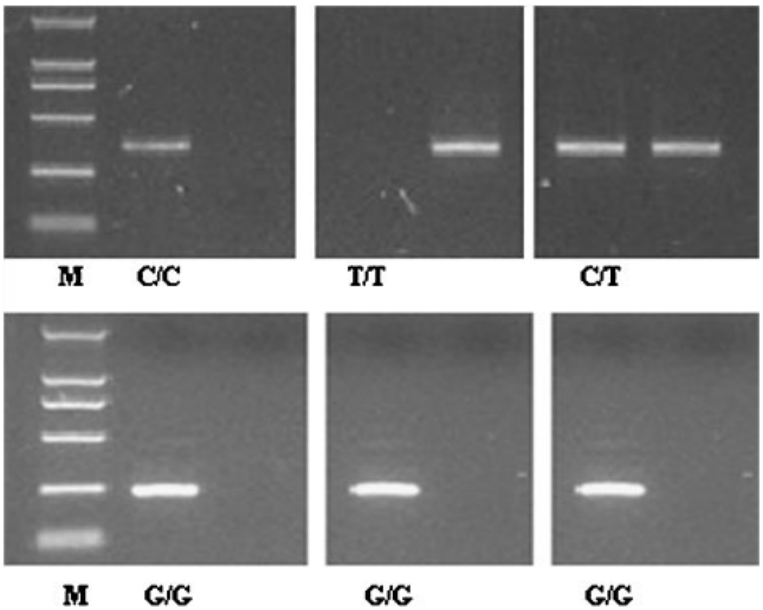

Fig. 3. Genotyping of the TGF-β1 gene polymorphisms at codon 10 and codon 25 by ARMS-PCR. Top: Gene polymorphism at codon 10 of TGF-β1 in GC samples. C and T homozygotes had only one product band in their respective lanes (left and center plate). CT heterozygote had one band in each lane (right plate). Bottom: Gene polymorphism at codon 25 of TGF-β1 in GC samples. All plates indicated the GG homozygotes. M in top and bottom panel represents a DL 2000 molecular marker.

codon 10 was not profoundly different between GC patients and control group ($P > 0.05$). Furthermore, TGF-β1 genetic polymorphism at codon 10 did not show obvious correlations with either serum concentration of TGF-β1 or clinicopathological parameters of GC patients ($P > 0.05$) (data not shown).

DISCUSSION

It has been reported that a marked increase in mRNA and protein amounts encoding TGF-β1 was detected in various tumors of epithelial origin including GC, esophageal cancer, hepatoma, prostate cancer, colorectal cancer, and ovarian cancer (25,26). This overexpression of TGF-β1 in cancer tissues may be a negative feedback response due to the TGF-β1 resistance caused by the decreased or lack of expression of TβR (26). Furthermore, immunohistochemistry analysis demonstrated that from normal mucosa, intestinal metaplasia, and dysplasia to GC, the expression of TGF-β1 increased gradually as disease stage progressed (19). In this study, we found significantly elevated serum concentrations of TGF-β1 in GC patients compared with those in healthy volunteers. In addition, this increase serum level of TGF-β1 was found to be in proportion to tumor size. Therefore, we suggested upregulation of TGF-β1 expression in GC patients' sera might result from the overexpression of TGF-β1 in GC cells. Most importantly, our result indicated that the serum TGF-β1 levels of patients after radical surgery were markedly lower than those before surgery and close to normal, further supporting that the serum concentration of TGF-β1 might partly have root in primary cancer and reflect the over expression of TGF-β1 gene in cancerous tissue.

Further correlation study revealed no significant differences in serum TGF-β1 levels of GC patients with different age, sex, and tumor location, suggesting that these parameters were not the major determinants of TGF-β1 yield. Similarly, there was no obvious correlation between the serum TGF-β1 level and histological type in GC patients, which is consistent with the conclusions obtained by previous studies on GC and colorectal cancer (2). However, Niki et al. (12) found that the serum TGF-β1 level was higher in the invasive type than in the noninvasive type in advanced GC. In addition, increased serum TGF-β1 in breast cancer was shown be related to the poorer histological grade (17). Therefore, the relationship between serum TGF-β1 concentration and histological type of tumor remains to be further investigated.

Results obtained in the current study showed that serum concentration of TGF-β1 was positively correlated with depth of invasion, lymph node metastasis and distant metastasis of GC, which is agree with Lin et al. (18) and Saito et al.'s (13) reports in GC (8). Previous studies have also reported that serum levels of TGF-β1 were higher in GC, breast cancer, and renal cancer,

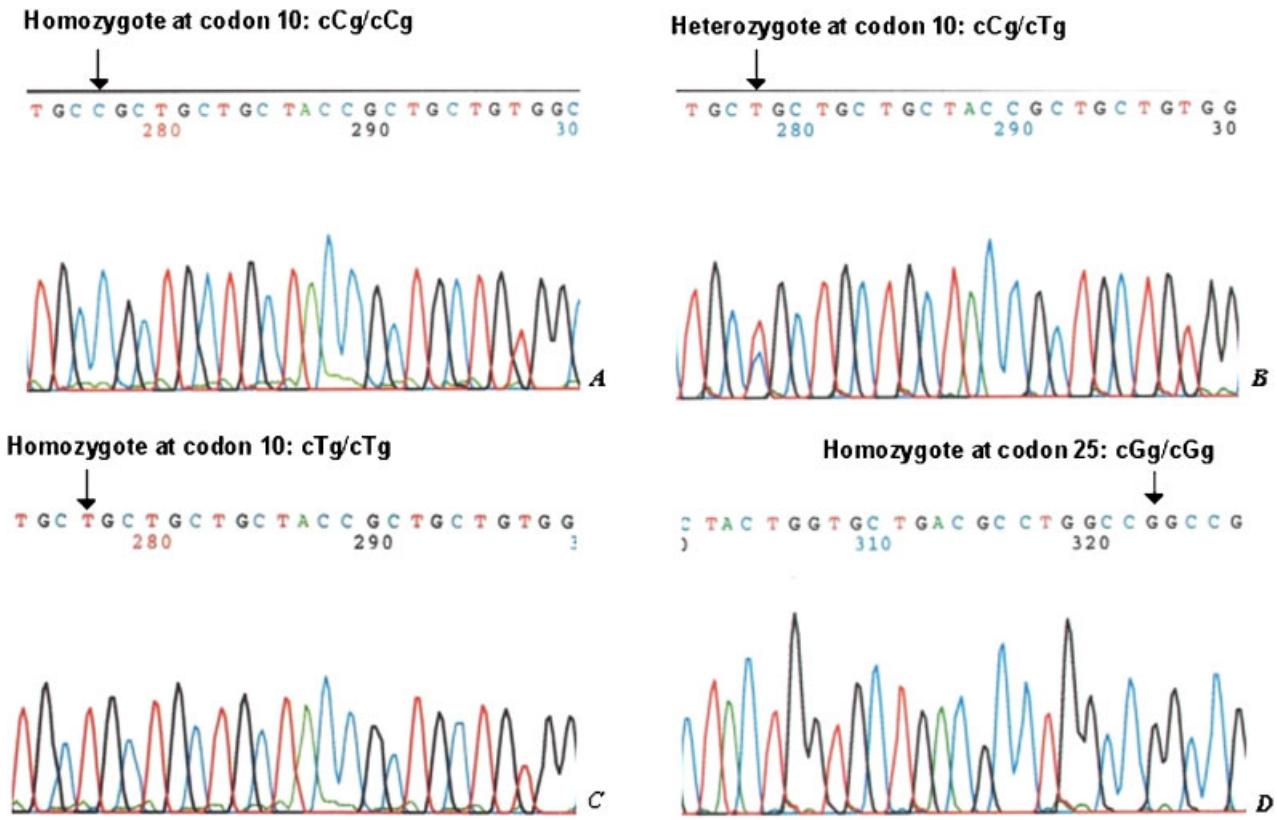

Fig. 4. Sequence analysis of SNPs at codon 10 and codon 25 in TGF-β1 gene. **A**,**B**,**C:** CC homozygote, CT heterozygote, and TT homozygote at codon 10, respectively. **D:** GG homozygote at codon 25.

with invasive type or with lymph node metastasis and peritoneal recurrence (17,25). Taken together, it suggested that over expression of TGF-β1 might be useful for predicting recurrence patterns and prognosis in patients with GC.

In this study, serum TGF-β1 levels increased gradually as GC stage progressed and this phenomenon was more evident in patients with III and IV stage whereas there were no obvious differences in TGF-β1 levels between patients with I or II stage and healthy volunteers, suggesting that there is unregulated secretion of TGF-β1 protein, which is possibly caused by insensitivity to TGF-β1, occurred in the late stage of tumorigenesis. Similar conclusions regarding the TGF β1 expression level and cancer progression have been reached in colorectal cancer, breast cancer (2,17).

When the cutoff limit increased from mean + 1 SD to mean + 2 SD of the controls, the positive frequency of serum TGF-β1 levels in healthy volunteers decreased from 22.7% to zero; however, the positive frequency of TGF-β1 in GC patients also reduced from 64.2% to 37.7%. Therefore, we need to take into consideration the possible problematic issues if serum TGF-β1 concentration is to be used as a tumor progression marker. First of all, it is difficult to achieve both detectability and specificity at the same time due to the fact that TGF-β1 is secreted from not only GC cells but numerous other cell types, and its production is influenced by various factors in vivo. Moreover, there is the possibility that individual discrepancy in amount of TGF-β1 expressed by GC tissue may lead to decreased detectability. On the other hand, the range of serum concentrations of TGF-β1 of the controls differed in various studies (15,17). Therefore, it is necessary to enlarge the samples involved and use uniform serum sample preparation and assay methods so as to establish the cutoff value of serum TGF-β1 levels for clinical applicability of GC detection. Taking one with another, no matter the origin of elevated TGF-β1 and the possible causes for this increased protein, serum TGF-β1 levels were significantly higher in patients with GC than in controls, reflected the progression of GC associated with GC invasion and metastasis, and returned to normal level after radical excision of tumor, all of which strongly pointed to the possibility of their clinical use as a GC marker.

It is because of the individual differences in amount of TGF-β1 that we adopted ARMS-PCR (27) and sequence analysis to investigate the possible genetic background of this discrepancy. Our data showed that

there were three genotypes, CC, CT, and TT, at codon 10 of the TGF-β1 gene in the GC patients and controls, while no DNA sequence variation at codon 25 of the TGF-β1 gene was defined in our study. Together with a report involving more than 1,000 Chinese breast cancer patients (24), it suggested that the Chinese population might be monomorphic at codon 25 of the TGF β1 gene. The frequency distribution of the genotype and alleles of codon 10 was not profoundly different between GC patients and the control group. Further, TGF-β1 gene polymorphisms did not show obvious correlations with either serum concentration of TGF-β1 or clinicopathological parameters of GC patients.

Numerous data obtained from the studies on other diseases have shown that serum levels of TGF-β1 were higher in subjects with the CC genotype than in those with the TT genotype at codon 10 of the TGF-β1 gene (28,29), as well as in people with the GC genotype, than in those with the GG genotype at codon 25 (30,31). The SNPs of the TGF-β1 gene at codon 10 and codon 25 lead to substitutions of amino acid residue in the signal peptide sequence, which is thought to target newly synthesized proteins in the endoplasmic reticulum (32). Transfections of HeLa cells with constructs encoding either the Pro or Leu forms of TGF-β1 indicated that the signal peptide with Pro at codon 10 caused a 2.8-fold increase in secretion compared with the Leu form, which suggested that the substitutions of amino acid residue might affect the function of the signal peptide, possibly by influencing intracellular trafficking or export efficiency of the TGF-β1 protein (33). However, it has also been observed that serum levels of TGF-β1 were lower in subjects with the proline homozygote than in those with the leucine homozygote at codon 10 of TGF-β1 gene (34). Despite the fact that polymorphisms at codon 10 and 25 of the TGF-β1 gene are involved in the upregulation of TGF-β1 secretion, there are other studies which have failed to reveal any association between serum TGF-β1 levels and SNPs at codon 10 and 25 (34), similar to what we have found in our study. This variability suggested that the discrepancy of genetic background such as race and region as well as other genetic factors might influence TGF-β1 expression in gastric carcinogenesis and progression.

Although an increasing body of literatures showed that SNPs at codon 10 and 25 of TGF-β1 gene were widely implicated in different diseases such as the 5-year disease-free survival rate in breast cancer, radiation-induced fibrosis, and osteoporosis in postmenopausal women (24), as well as the risk of suffering prostate cancer or benign prostatic hyperplasia (22) and the risk of myocardial infarction (35), our result indicated that the gene polymorphisms of the TGF-β1 gene did not function as a genetic risk factor in the carcinogenesis and clinical progression of GC. More credible results may be obtained with further studies involving a larger GC patient population and other TGF-β1 gene polymorphisms. In summary, our data supported that TGF β1 played an important role in the gastric carcinogenesis and progression and its serum level might be used as a useful clinical marker for GC patients.

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