·BASIC RESEARCH·

Correlations of Polymorphisms of TGFB1 and TGFBR2 Genes to Genetic Susceptibility to Gastric Cancer

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[ABSTRACT] **BACKGROUND & OBJECTIVE**: Transforming growth factor β (TGFβ) signaling pathway plays an important role in the genesis and progression of tumors through regulating cell proliferation and differentiation. The concentration of TGFβ1 in plasma and the expression of TGFβ receptor Ⅱ (TGFβR Ⅱ) are correlated to the development of certain tumors, including gastric cancer. This study was to explore the correlations of functional genetic variants in TGFB1 and TGFBR2 genes to the genetic susceptibility to gastric cancer. **METHODS**: A case-control study was conducted in Yixing City, a high incidence area of gastric cancer. Polymorphisms of TGFB1 C-509T, TGFB1 Leu10Pro, and TGFBR2 G-875A in 256 gastric cancer patients and 303 cancer-free controls, frequency-matched by age and sex, were determined by primer-introduced restriction analysis-polymerase chain reaction (PIRA-PCR). Crude and adjusted odds ratios (ORs) and 95% confidence intervals (95% CIs) were measured by multivariate Logistic regression analysis to evaluate the correlations of the polymorphisms to the susceptibility to gastric cancer. **RESULTS**: The TGFB1 C-509T and TGFB1 Leu10Pro were in high linkage disequilibrium (D'=0.86). Compared with wild-type homogenous genetypes -509CC and 10 Leu/Leu, variant genetypes -509CT/TT, 10 Leu/Pro, and 10 Pro/Pro decreased the risk of gastric cancer by 49% and 34% (adjusted OR=0.51, 95% CI=0.36-0.74 for -509CT/TT; adjusted OR=0.66, 95% CI=0.45-0.98 for 10Leu/Pro or 10Pro/Pro). The risk of gastric cancer was decreased along with the number of variant sites in the TGFB1 C-509T and TGFBR2 G-875A (χ^2=15.70, P < 0.001). Stratified analysis showed that the protective effects of the genotypes were obvious in the subjects of no more than 60-year old (OR=0.42, 95% CI=0.23-0.79) and in non-drinkers (OR=0.45, 95% CI=0.27-0.74). **CONCLUSION**: Genetic variants of TGFB1 and TGFBR2 genes may contribute to the risk of developing gastric cancer in an eastern Chinese population in Yixing city.

KEYWORDS: Gastric neoplasm; TGFB1; TGFBR2; Polymorphisms; Genetic susceptibility

1. Introduction

Gastric cancer is one of the most common malignant tumors in China. In 2005, there were 400,000 new gastric cancer cases, and 300,000 people died. The incidence and mortality of gastric cancer rank the third among all the malignant tumors [1]. Gastric cancer is the joint effect of risky environmental factors and individual genetic factors. It has been confirmed in epidemiological study that, H. pylori (H.P.) infection, intake of salted food, drinking and smoking, and so on. are the major risky factors for the development of gastric cancer [2]. Many people contact with the same carcinogenic substance, but only a small portion has cancer, which demonstrates that there is a susceptibility to gastric cancer in individuals. The latest studies demonstrate that, the signal pathway of transforming growth factor β (TGFβ) has important effects in the genesis and progression of various tumors, like gastric cancer. The signal pathway has become the hot issue in the tumor study [3].

TGFβ signal pathway exerts dual effects in the genesis and progression of tumors. In normal tissues and tumors at early stages [3], TGFβ inhibits the tumorigenesis by regulating the cell cycle, inhibiting cellular proliferation and inducing apoptosis. For the progressing tumors, TGFβ can promote the invasion and metastasis by immune suppression and/or immune escape, or by increasing the angiogenesis and reinforcing the interaction between tumor cells and the extracellular matrix. TGFβ 1 and TGFβ R2 are the critical genes of this pathway, and the genetic mutations of the two are demonstrated to be closely related with the intensity of TGFβ signals or whether the signals can be normally descended [3, 4]. Polymorphisms of TGFβ 1 gene may be related to the genetic susceptibility of tumors [4-8]. In this study, we investigated the correlations of polymorphisms of TGFβ 1 and TGFβ R2 genes to genetic susceptibility to gastric cancer by a case control study.

2. Materials and Methods

2.1 Study subjects

In total 256 primary gastric cancer patients who were all Han nationality in Department of General Surgery and Oncology of Yixing Peoples Hospital, Yixing, Jiangsu Province, were enrolled. All the patients were finally histopathologically diagnosed, and no radiotherapy or chemotherapy was applied before operation. There were 303 randomly selected normal controls from Han nationality who underwent physical examination with no tumor history or abnormal sign. The study pairs were made based on sex, age (± 5 years old) and case frequency. All subjects signed the informed content. The subjects were sampled 5 ml venous blood, and EDTA was used as anticoagulation. When blood samples were collected, epidemiological questionnaire was used to ask and record the demographic data, life styles and living habits of every study subject (including smoking and drinking conditions, and so on).

2.2 General characteristics

The test group and the control group had balanced sex and age conditions (Table 1). The ratio of smokers in gastric cancer patients was obviously higher than that in the control group (52.0% vs. 40.6%, P=0.007). The gastric patients with the drinking habit accounted for 38.7% of the total patients, which was superior to 31.4% of the control group (P=0.070).

Table 1 Distribution of selected demographic variables and risk factors in gastric cancer patients and controls [cases (%)]

Variable	Gastric cancer patients (n=256)	Controls (n=303)	P value[a]
Age (years)			0.200
≤60	112 (43.8)	149 (49.2)	
> 60	144 (56.3)	154 (50.8)	
Sex			0.439
Male	180 (70.3)	222 (73.3)	
Female	76 (29.7)	81 (26.7)	
Smoking status			0.007
Never	123 (48.1)	180 (59.4)	
Ever	133 (52.0)	123 (40.6)	
Drinking status			0.070
Never	157 (61.3)	208 (68.7)	
Ever	99 (38.7)	95 (31.4)	

[a]Two-sided χ^2 test

2.3 Genotyping of TGFβ 1 and TGFβ R2 polymorphisms by PIRA-PCR

Primer introduced restriction analysis-PCR (PIRA-PCR) [9] was used to analyze the polymorphisms of C-509T (rs1800469) in the promoter region, Leu10Pro (rs1982073), the first exon of TGFβ 1 gene, and G-875A (rs3067465) in the promoter region of TGFβ R2. When the DNA segments

containing the C-509T polymorphic site were amplified, we introduced a T to replace the original G two bases downstream the polymorphic site, thus to produce an AflⅡ enzyme recognition site. The primers were TGFβ 1-509, 5-GTC GCA GGG TGT TGA GTG ACAG-3 and TGFβ 1-509R, 5-AGG GGG CAA CAG GAC ACC TTA-3, which were synthesized by Invitrogen company. *Taq* enzyme was bought from Shanghai Shenneng Bocai Biotechnology Co., Ltd.. The 123 bp segment was amplified using MJ PTC-200 PCR amplification system. The reaction system was 20μl, containing about 50ng genomic DNA, 12.5 pmol primers, 0.1 mmol/L mononucleotide, 1× PCR buffer (50 mmol/L KCl, 10 mmol/L Tris HCl and 0.1% Triton X-100), 1.8 mmol/L $MgCl_2$ and 1.0 U *Taq* enzyme. Annealing temperature of the reaction was 64℃. After amplification, 5μl product was taken, mixed with 3U *Afl* Ⅱ (New England Biolabs, USA) and 1μl attached buffer. Double distilled deionized water was added to 10μl, and the mixture was digested at 37℃ for 6h. Electrophoresis was performed in a 3% agarose gel at 80 V for 100 min to determine the digestion result. No digestion site existed in the wild-type gene CC. Heterozygote CT produced three segments, 123 bp, 101 bp and 22 bp, while mutated TT produced two segments after digestion, 101 bp and 22 bp (Figure 1A). Similarly, when DNA segments containing polymorphism sites such as TGFβ 1 Leu10Pro and TGFβ R2 G-875A were amplified, the wrongly matched A and G were introduced to replace the original T and A two bases downstream and two bases upstream the polymorphism sites, thus to produce the recognition sites of *Pvu* Ⅱ and *Rsa* Ⅰ. The used primers were TGFβ 1 10F, 5-CTC CGG GCT GCG GCT GCA GC-3, TGFβ 1 10R, 5-GGC CTC GAT GCG CTT CCG CTT CA-3; TGFβ R2-875F, 5-GCA AGA AAG GAA ATT TGA AAG TTT GT-3, and TGFβ R2-875R, 5-TCA CCT GAA TGC TTG TGC TTT T-3. The reaction system was similar as the one described above. The annealing temperatures were 60℃ and 56℃. The products were 136 bp and 124 bp respectively. Products were digested by *Pvu* Ⅱ and *Rsa* Ⅰ (New England Biolabs, USA) for 6 h. The wildtype gene, Leu/Leu, of TGFβ 1 Leu10Pro, produced two segments, 119 bp and 17 bp; heterozygote Leu/Pro produced three segments, 136 bp, 119 bp and 17 bp, while the mutated type Pro/Pro had no digestion sites (Figure 1B). Wild-type gene GG of TGFβ R2 G-875A had no digestion site and it produced two segments after digestion; heterozygote GA produced three segments, 124 bp, 99 bp and 25 bp; mutated type AA produced two segments after digestion, 99 bp and 25 bp (Figure 1C). Three different bands were randomly selected for genotyping using the direct sequencing method (Figure 2). In addition, about 10% of the samples were randomly repeated, and the consistency of the results was 100%.

2.4 Statistical analysis

Chi-square criterion was used to compare the demographic characters, conditions of smoking and drinking and the distribution difference of all genomes in the two groups. Odds ratio (OR) and its 95% confidence interval (CI) were calculated by Logistic regression of single factor or multiple factors to indicate relative risk. All the statistical tests were double-side probability tests. Those who smoked at least once daily continuously for more than one year were defined as smokers. Those who drank at least three times per week continuouly for more than six months were defined as drinkers. The statistical software applied was SAS9.1.3 version.

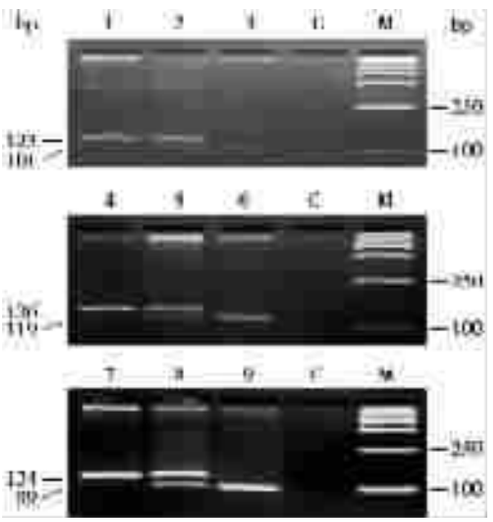

Figure 1 TGFB1 and TGFBR2 genotypes detected by primer-introduced restriction analysis-polymerase chain reaction (PIRA-PCR)

Lane C:control (water); lane M:DNA marker; lane 1:TGFB1-509CC (123 bp); lane 2: TGFB1 -509CT (123 bp and 101 bp); lane 3: TGFB1-509TT (101 bp); lane 4:TGFB1 10Pro/Pro (136 bp); lane 5: TGFB1 10Leu/Pro (136 bp and 119 bp); lane 6: TGFB1 10Leu/Leu (119 bp); lane 7:TGFBR2-875GG (124 bp); lane 8:TGFBR2-875GA (124 bp and 99 bp); lane 9: TGFBR2 -875AA (99 bp).

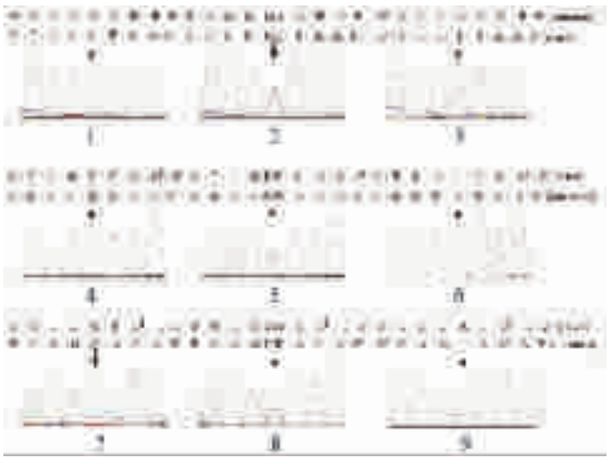

Figure 2 Genotype sequencing of the TGFB1 C-509T, TGFB1 Leu10Pro and TGFBR2 G-875A polymorphisms

Chart 1:TGFB1-509CC; chart 2:TGFB1-509CT; chart 3:TGFB1-509TT; chart 4:TGFB1 10Leu/Leu(29TT); chart 5:TGFB1 10Leu/Pro(29TC); chart 6: TGFB1 10Pro/Pro(29CC); chart 7: TGFBR2-875GG; chart 8:TGFBR2-875GA; chart 9:TGFBR2-875AA.

†A mismatched base was introduced into the primer to create an enzyme restriction site.

3. Results

3.1 Distribution of genotypes and alleles, and their correlation to the risk of gastric cancer

In 303 normal controls, the distribution frequency of genotype polymorphisms of TGFβ 1 C-509T, TGFβ 1 Leu10Pro and TGFβ R2-G875A was not significantly different calculated by Handy-Weinberg formula (C-509TL: χ^2=0.08, P =0.775; Leu10Pro: χ^2=020, P=0.651; G-875A: χ^2=031, P =0.579). The genotype distribution of TGFβ 1 and TGFβ R2 and their relations to the risk of gastric cancer are concluded in Table 2. The distribution of TGFβ 1 C-509T, TGFβ 1 Leu10Pro and TGFβ R2-G-875A genotypes as well as alleles had statistical significance in the study group and the control group. After adjusting age, sex, smoking and drinking, the gastric cancer risk was obviously decreased by 49% in individuals carrying mutant alleles (-509T) compared to those carrying homozygotic wild genotype (OR=0.57, 95% CI=0.36-0.74); while the risk of gastric cancer of individuals with at least one 10Pro was obviously decreased by 34% (95% CI =0. 45-0.98). For the TGFβ R2 G-875A site, the risk of gastric cancer of individuals carrying at least one -875A was decreased by 30% compared to those carrying -875GG genotypes (OR =0.70, 95% CI=0.48-1.01); however, the difference was not significant.

3.2 Joint analysis and stratified analysis

Linkage disequilibrium was performed for the two polymorphism sites of TGFβ 1, and high linkage disequilibrium was observed (D=0.86).

Table 2 Logistic regression analysis of correlations of TGFB1 and TGFBR2 polymorphisms to risk of gastric cancer [cases(%)]

Genotype	Gastric cancer patients(n=256)	Controls(n=303)	Crude OR(95% CI)	Adjusted OR[a](95% CI)
TGFB1 C-509T				
CC	103(40.2)	78(25.7)	1.00	1.00
CT	90(35.2)	149(49.2)	0.46(0.31–0.68)	0.46(0.31–0.69)
TT	63(24.6)	76(25.1)	0.63(0.40–0.98)	0.61(0.38–0.95)
CT/TT	153(59.8)	225(74.3)	0.52(0.36–0.74)	0.51(0.36–0.74)
T allele	216(42.2)	301(49.7)		
TGFB1 Leu10Pro				
Leu/Leu	77(30.1)	66(21.8)	1.00	1.00
Leu/Pro	123(48.1)	155(51.2)	0.68(0.45–1.02)	0.71(0.47–1.07)
Pro/Pro	56(21.9)	82(27.1)	0.59(0.37–0.94)	0.57(0.35–0.92)
Leu/Pro or Pro/Pro	179(69.9)	237(78.2)	0.65(0.44–0.95)	0.66(0.45–0.98)
Pro allele	235(45.9)	319(52.6)		
TGFBR2 G-875A				
GG	190(74.2)	202(66.7)	1.00	1.00
AG	62(24.2)	89(29.4)	0.74(0.51–1.08)	0.74(0.51–1.10)
AA	4 (1.6)	12 (4.0)	0.35(0.11–1.12)	0.34(0.11–1.09)
AG/AA	66(25.8)	101(33.3)	0.70(0.48–1.00)	0.70(0.48–1.01)
A allele	70(13.7)	113(18.6)		
Combined TGFB1 C-509T and TGFBR2 G-875A variant loci				
0	75(29.3)	52(17.2)	1.00	1.00
1	143(55.9)	176(58.1)	0.56(0.37–0.86)	0.58(0.38–0.89)
2	38(14.8)	75(24.8)	0.35(0.21–0.61)	0.35(0.21–0.60)

OR, odds ratio; CI, confidence interval. [a]Adjusted for age, sex, smoking status and drinking status.

TGFβ 1 C-509T and TGFβ R2 G-875A were chosen to analyze the influence of the joint effects of the two sites (Table 2). Compared to individuals with two sites of wild-type homozygote, the risk of gastric cancer was decreased by 42% in individuals with only one variant site (heterozygote or mutant homozygote) (adjusted *OR*=0.58, 95% *CI*: 0.38-0.89); when there was a mutant allele in the two sites, the risk of gastric cancer was decreased by 65% in the individuals (adjusted *OR*=0.35, 95% *CI*: 0.21-0.60). The protective effect was stronger when the mutant allele sites were increased (χ^2=15.70, *P*=0.001). Stratified analysis was performed with miscellaneous factors. When one or more genotypes were mutated, the protective effects of the genotypes were especially obvious in subjects younger than 60 years old (adjusted *OR*=0.42, 95% *CI*=0.23-0.79) and in non-drinkers (adjusted *OR*=0.45, 95% *CI*=0.27-0.74) (Table 3).

Table 3 Stratified analysis of the correlation of the combined genotypes of TGFB1 C-509T and TGFBR2 G-875A polymorphisms to risk of gastric cancer
[cases (%)]

Item	Cases (n=256)		Controls (n=303)		Adjusted OR[a] (95% CI)	
	Wild[b]	Variant[b]	Wild	Variant	Wild	Variant
Age (years)						
≤60	32 (28.6)	80 (71.4)	22 (14.8)	127 (85.2)	1.00	0.42 (0.23–0.79)
> 60	43 (29.9)	101 (70.1)	30 (19.5)	124 (80.5)	1.00	0.58 (0.34–1.00)
Sex						
Male	55 (30.6)	125 (69.4)	41 (18.5)	181 (81.5)	1.00	0.55 (0.34–0.89)
Female	20 (26.3)	56 (73.7)	11 (13.6)	70 (86.4)	1.00	0.44 (0.19–1.01)
Smoking status						
Never	31 (25.2)	92 (74.8)	26 (14.4)	154 (85.6)	1.00	0.50 (0.28–0.91)
Ever	44 (33.1)	89 (66.9)	26 (21.1)	97 (78.9)	1.00	0.50 (0.28–0.90)
Drinking status						
Never	48 (30.6)	109 (69.4)	34 (16.4)	174 (83.7)	1.00	0.45 (0.27–0.74)
Ever	27 (27.3)	72 (72.7)	18 (19.0)	77 (81.1)	1.00	0.69 (0.34–1.39)

[a]Adjusted for age, sex, smoking status and drinking status.
[b]"Wild" represents the subjects with both wild homozygotes (TGFB1-509CC and TGFBR2-875GG); "variant" represents the subjects with at least one variant allele of TGFB1 C-509T and TGFBR2 G-875A.

4. Discussion

In our study, the mutant alleles (-509T, 10Pro and -875A) of TGFβ 1 C-509T, TGFβ 1 Leu10Pro and TGFβ R2 G-875A could decrease the risk of gastric cancer, and -509T and 10Pro had more obvious effects. Moreover, TGFβ 1 C-509T and TGFβ R2 G-875A had dose-effect relationship with the decreased risk of gastric cancer. For example, when the number of the mutant site increased, the risk of gastric cancer obviously decreased. In addition, the joint protective effects of two combined genotypes were more significant in patients younger than 60 years old and non-drinkers.

It was indicated in various studies that, TGFβ signaling pathway had important effects in the genesis and progression of tumors. As the core genes of this pathway, the mutations of TGFβ 1 and TGFβ R2 would influence the normal descending of this pathway. Reinforced TGFβ signal could obviously lower the tumor incidence, while increased TGFβ 1 level could reinforce TGFβ signaling. Compared with the alleles of TGFβ 1-509C and 10Leu, -509T and 10Pro could increase the secretion of TGFβ 1 [4, 10]. It was indicated in the previous studies that, the polymorphisms of TGFβ 1-509C and 10Leu were related with the genesis risks of various tumors. Li *et al.* [8] indicated in a case-control study performed in Japanese hospitals (353 patients and 303 control cases) that, the risk of prostate carcinoma in people with 10 Leu/Leu gene was obviously increased compared to that of people with 10 Pro/Pro gene (*OR*=1.67, 95% *CI*=1.08-2.57), which was similar to our results.

Previous studies indicated that, the promoter area of TGFβ R2 was easy to mutate, and TGFβ R Ⅱ was lowly expressed, which would influence the normal descending of TGFβ signals [11]. G-875A site was the only polymorphism site with frequency of more than 5% in the promoter region, while -875A allele could reinforce the transcription of TGFβ R2 in epithelial cells [12]. This might be the biological mechanism for -875A allele to exert the protective effect in gastric cancer. In a case-control study carried out by Jin *et al.* [5], it was indicated that polymorphism of TGFβ R2 G-875A might not be related to breast cancer, which was inconsistent with our study. This may be caused by various factors, such as genetic background, the sample size and the mechanisms of different tumors.

Our study also demonstrated that, the distribution frequency of TGFβ 1-509T and TGFβ 1 29Pro alleles in normal Chinese Han population were 0.497 and 0.526 respectively, which were consistent with the results reported before [6]. The frequency was about 0.50 in Japanese and Korean population [7, 8], and less than 0.40 among Caucasian [4, 5]. The distribution frequency of TGFβ R2-875A allele of Han population was similar to that of Caucasian (Finland, 0.170; Poland: 0.190) [5].

The study suggests that, polymorphisms of

TGFβ 1 C-509T, Leu10Pro and TGFβ R2 G-875A may be related with the genetic susceptibility of gastric cancer in Chinese Han population. Further functional studies *in vivo* and *in vitro* and the prospective study of a large sample size are needed to confirm the conclusion.

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