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Polymorphism in the 3'-untranslated region of the thymidylate synthase gene and sensitivity of stomach cancer to fluoropyrimidine-based chemotherapy

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Abstract To investigate the relationship between polymorphism in the 3'-untranslated region (3'-UTR) of the thymidylate synthase (TS) gene and sensitivity of gastric cancer to 5-fluorouracil (5-FU)-based chemotherapy, 106 cases of advanced gastric cancer were analyzed. All patients were treated with 5-FU-based chemotherapy; DNA from peripheral blood leukocytes was obtained before therapy. TS 3'-UTR genotypes were detected by PCR-RFLP. Polymorphism in the TS 3'-UTR can be classified into three groups according to the presence or absence of a 6 bp nucleotide fragment: the -6/-6 bp, -6/+6 bp and +6/+6 bp groups. The response rate of the -6/-6 bp and -6/+6 bp groups was found to be significantly higher than the +6/+6 bp group. These results show that the presence of the TS 3'-UTR 6 bp nucleotide fragment can be correlated with the sensitivity of gastric cancer to 5-FU-based chemotherapy, and that the TS 3'-UTR polymorphism profile can be used to guide the choice of 5-FU-based chemotherapy in advanced gastric cancer.

Keywords Stomach neoplasms · Thymidylate synthase · Chemotherapy · Genotypes

Introduction

Recently, global statistics have shown gastric cancer to be the second largest cause of malignant tumor, second

only to lung cancer. About 60% of cases occur in developing countries, and about 38% of cases are in China (Parkin et al. 1999). Most gastric cancer patients are diagnosed at an advanced stage, with poor prognosis and a 5-year survival rate between 5 and 15% (Berardi et al. 2004). Treatment of early stage, or locally advanced, gastric cancer is mainly surgical. However, treatment of gastric cancer at an advanced stage or with metastasis relies mainly on chemotherapy; chemotherapy is also the method of choice in the alleviative treatment of gastric cancer (Kohne et al. 2000).

5-Fluorouracil (5-FU) is one of the most widely used and most effective drugs in the treatment of advanced gastric cancer. The response rate of this single drug in the treatment of advanced gastric cancer is about 20% (Findlay and Cunningham 1993). Life span of gastric cancer patients treated exclusively with 5-FU is about 5–7 months (Kim et al. 1993; Coombes et al. 1994). Currently, most combination chemotherapy regimens for gastric cancer contain 5-FU. For instance, 5-FU with doxorubicin and mitomycin (FAM), 5-FU with doxorubicin and high-dose methotrexate (FAMTX), 5-FU with etoposide and leucovorin (ELF), 5-FU continuous infusion with epirubicin and cisplatin (ECF), 5-FU with leucovorin, cisplatin and epirubicin (PELF). The response rate of these various chemotherapies ranges from 9 to 71% (Tsai and Safran 2003). In recent years, many new chemotherapeutics have been developed. Stage I–II clinical trial results show a response rate on gastric cancer for docetaxel, cisplatin and 5-FU (DCF) of 56% (Ajani 2002). The response rate of leucovorin, 5-FU, paclitaxel and cisplatin (LFP-P) is 51% (Kollmannsberger et al. 2000), and the response rate of oxaliplatin (L-OHP), leucovorin and 5-FU (FOLFOX) is 44.6% (Louvet et al. 2002).

Upon entering the human body, 5-FU is transformed to 5-fluorine-2-deoxyuridylic acid—a reaction catalyzed by thymidine kinase. 5-Fluorine-2-deoxyuridylic acid exerts its antitumor effect via two pathways. First, it can become directly incorporated into RNA or DNA; second, it can form a kind of co-molecular compound with

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methylenetetrahydrofolic acid and thymidylate synthase (TS), blocking the synthesis of thymidylate thus interfering with the synthesis and repair of DNA. This latter pathway is believed to be the main antitumor pathway of 5-FU.

Despite the success rates cited above, chemotherapeutic programs for gastric cancer remain unsatisfactory. A regimen may be completely ineffective or achieve complete remission (CR) in the treatment of a given patient, depending on individual differences in drug sensitivity. Thus, it is important to find a reliable marker with which to forecast drug sensitivity, and to use this marker to direct individualized treatment. Such a marker would also be significant in determining the efficacy and safety of clinical medication.

Thymidylate synthase can transform deoxyuridylic acid to deoxythymidylic acid in the presence of 5,10-methylenetetrahydrofolic acid. Deoxythymidylic acid is the only resource of cell DNA biosynthesis and repair (Marsh et al. 1999; Horie et al. 1995). In cancer therapy, TS is an important target of many antimetabolites, including 5-FU. Polymorphism of the TS gene is known to occur at a 28 bp nucleotide fragment of the 5'-untranslated region (5'-UTR), or with the presence or absence of a 6 bp nucleotide fragment in the 3'-untranslated region (3'-UTR; Ulrich et al. 2000). These two kinds of polymorphism can influence TS expression by influencing the stability of the TS mRNA, and thus affect the drug sensitivity of cancer patients (Calascibetta et al. 2004; Mandola et al. 2004; Merkelbach-Bruse et al. 2004). In the present work, we studied the relationship between polymorphism of the 3'-UTR of the TS gene and drug sensitivity of gastric cancer to 5-FU-based chemotherapy.

Materials and methods

Patients

We collected 106 cases of advanced gastric cancer defined by pathology in the Department of Medical Oncology, Jiangsu Province Institute of Cancer Research from May 2001 to December 2004 (Table 1). We measured all of the tumors by computed tomography (CT). Prior to starting chemotherapy, all routine blood, liver, and renal functions were within normal ranges, no electrocardiogram was abnormal, and all functional conditions scored more than 60 points on the Karnofsky scale.

Collection of specimens

Samples of venous blood (2 ml) were taken before chemotherapy and placed in EDTA-containing anticoagulation tubes. Leukocytes were then separated and DNA extracted from white cells using a QIAamp DNA Extraction Kit (Qiagen, Hilden, Germany).

Table 1 Characteristics of the 106 patients and the therapeutic effect of chemotherapy. CFL calcium folinate (CF) + 5-FU + oxaliplatin (L-OHP), CFH CF + 5-FU + hydroxycamptothecin (HCPT), CFLH CF + 5-FU + L-OHP + HCPT, L-FP low-DDP (cisplatin) + 5-FU, CFPT CF + 5-FU + DDP + paclitaxel (TXT)

	Total (%)	Response (%)	No response (%)	<i>P</i> ^a
Gender				
Male	78(73.6)	26(33.3)	52(66.7)	0.370
Female	28(26.4)	12(42.9)	16(57.1)	
Age (years)				
< 58	52(49.1)	19(36.5)	33(63.5)	0.885
≥58	54(50.9)	19(35.2)	35(64.8)	
Adjuvant chemotherapy				
With	23(21.7)	7(30.4)	16(69.6)	0.543
Without	83(78.3)	31(37.4)	52(62.7)	
Metastasis location				
Lymph nodes	55(51.9)	21(38.2)	34(61.8)	0.588
Liver	15(14.2)	5(33.3)	10(66.7)	
Lung	4(3.8)	1(25.0)	3(75.0)	
Liver and lymph nodes	9(8.5)	5(55.6)	4(44.4)	
Others	23(21.7)	6(26.1)	17(73.9)	
Regimen				
CFL	29(27.4)	11(37.9)	18(62.1)	0.439
CFH	19(17.9)	6(31.6)	13(68.4)	
CFLH	10(9.4)	4(40.0)	6(60.0)	
L-FP	31(29.3)	8(25.8)	23(74.2)	
CFPT	17(16.0)	9(52.9)	8(47.1)	

^a*P* value of the χ^2 -test

Genotyping

We analyzed TS 3'-UTR genotypes by PCR-RFLP (Gao et al. 2004) using 5'-CAAATCTGAGGGAGCTGAGT and 5'-CAGATAAGTGGCAGTACAGA as primers. PCR products were resolved by agarose gel (3%) electrophoresis; products with two bands of 158 bp (+6 bp) and 152 bp (−6 bp) were classified as the heterozygote genotype +6/−6 bp (B). A single band was obtained for the two homozygous genotypes +6/+6 bp (A) and −6/−6 bp (C). Digestion of this single band with the restriction enzyme *DraI* yielded two bands of 88 and 70 bp in the case of the +6/+6 bp (A) homozygous genotype.

Treatment regimens

All patients were treated with subclavian vein puncture catheters or percutaneous subclavian venous catheters. All were kept on intravenous infusion of 5-FU for 24 h and intravenous infusion of other chemotherapy drugs routinely. All of the chemotherapy regimens used here are commonly used in our clinic, including CFL [calcium folinate (CF) + 5-FU + L-OHP], CFH [CF + 5-FU + hydroxycamptothecin (HCPT)], CFLH (CF + 5-FU + L-OHP + HCPT), L-FP [low-DDP (cisplatin) + 5-FU] and CFPT [CF + 5-FU + DDP + paclitaxel (TXT)]. Clinical response was assessed by CT scan 1 month after starting chemotherapy, according to World Health Organization (WHO) criteria.

Evaluation of standard of therapeutic effect and side effects

In accordance with the WHO solid tumor evaluation standard (Miller et al. 1981), clinical response can be classified as CR, partial remission (PR), no change (NC) and progress (PD). We defined CR and PR as response, NC and PD as no response. Observation and assessment of toxicity were according to unified WHO standards (Miller et al. 1981). Toxicity can be classified into four degrees, the level of degrees III–IV including:

- Hemoglobin < 80 g/l.
- White blood cells < 2.0×10^9 /l.
- Platelets < 50×10^9 /l.
- Emesis requiring therapy or cannot control.
- Diarrhea requiring therapy or bloody diarrhea.
- Dental ulcer: cannot take food, or only liquid diet.
- GOT/GPT > 5×N, paresthesia intolerant.
- Notable motor disorder or paralyzed.

Statistical analysis

We carried out statistical analyses using SAS software. Chi-square tests and Fisher's exact test were applied to analyze the relationships among genotype, therapeutic effect and toxicity.

Results

Test of balance

Of the 106 patients, 78 were male and 28 female. The response rate of chemotherapy in female patients was a little higher than in male patients but without statistical significance ($\chi^2=0.805$, $P=0.370$). The age of patients ranged from 21 to 75, with a median age of 58 years old. There was no significant deviation in the response rate of chemotherapy between patients in the older than 58-year group and patients in the younger than 58-year group ($\chi^2=0.021$, $P=0.885$). Adjuvant chemotherapy and location of tumor metastasis also did not affect response rate of chemotherapy (Table 1).

Relationship between TS genotype and chemotherapeutic effect

The relationship between TS gene 3'-UTR polymorphism and therapeutic effect can be seen in Table 2. The frequency of -6/-6 bp, -6/+6 bp and +6/+6 bp variant forms of the TS gene 3'-UTR polymorphism are 48.1, 44.3 and 7.6%, respectively, and the corresponding chemotherapy response rates were 37.3% (19/51), 40.0% (19/47) and 0% (0/8). The response rate among the three groups did not reach statistical significance ($\chi^2=4.896$,

Table 2 Relationship between thymidylate synthase (TS) gene 3'-untranslated region (3'-UTR) polymorphism and therapeutic effect of chemotherapy

TS 3'-UTR genotype	Cases (%)	Response (%)	No response (%)
-6/-6 bp	51(48.1)	19(37.3)	32(62.8)
-6/+6 bp	47(44.3)	19(40.4)	28(59.6)
+6/+6 bp	8(7.6)	0(0)	8(100.0)
Total	106(100.0)	38(35.8)	68(64.2)

$P=0.086$). However, the response rates of the -6/-6 bp and -6/+6 bp groups are significantly higher than that of the +6/+6 bp group ($P=0.045$, 0.040 Fisher's exact test).

Relationship between TS genotype and chemotherapeutic effect of different treatment regimens

Although the response rate of the CFPT regimen group was the highest (52.9%), there was no statistical difference when compared with the other four regimens: CFL group ($\chi^2=0.983$, $P=0.332$), CFH group ($\chi^2=1.685$, $P=0.194$), CFLH group ($\chi^2=0.422$, $P=0.516$) and L-FP group ($\chi^2=3.534$, $P=0.060$). To exclude the effect of different regimens on therapeutic effect, we analyzed the relationship between TS genotype and chemotherapeutic effect of different regimens (Table 3). No individuals with the +6/+6 bp genotype were found in CFLH regimen group. Individuals with the -6/+6 bp or -6/-6 bp genotypes were distributed among all regimen groups. The response rates of the -6/-6 bp and -6/+6 bp groups were higher than that of the +6/+6 bp group for all regimens, but mostly without statistically significant differences. Only in the case of the -6/-6 bp group was the response rate significantly higher than that of the +6/+6 bp group in the CFPT regimen group ($\chi^2=4.00$, $P=0.046$; Table 3).

Relationship between TS genotype and chemotherapeutic side effects

The relationship between TS gene 3'-UTR polymorphism and chemotherapeutic side effects can be seen in Table 4. Patients with the TS -6/-6 bp variant had a significantly higher incidence of toxicity above III–IV degree than patients with the -6/+6 bp or +6/+6 bp genotypes. The TS -6/+6 bp group also had more side effects than the +6/+6 bp group, but there is no statistical significance between these differences.

We also analyzed the relationship between TS gene 3'-UTR polymorphism and side effects in different regimens (Table 5). Under most regimens, although the incidence of side effects was higher in the TS -6/-6 bp

Table 3 Relationship between TS genotype and chemotherapeutic effect of different regimens. Genotypes: A -6/-6 bp, B -6/+6 bp, C +6/+6 bp

Regimen	Genotype	Cases	Therapeutic effect (%)		χ^2	df	P
			Response	No response			
CFL	A	15	5(33.3)	10(66.7)	1.119	2	0.571
	B	13	6(46.2)	7(53.8)			
	C	1	0(0.0)	1(100.0)			
CFH	A	8	1(12.5)	7(87.5)	4.665	2	0.097
	B	9	5(55.6)	4(44.4)			
	C	2	0(0.0)	2(100.0)			
CFLH	A	3	1(33.3)	2(66.7)	0.079	1	0.778
	B	7	3(42.9)	4(57.1)			
L-FP	A	16	6(37.5)	10(62.5)	2.576	2	0.276
	B	13	2(15.4)	11(84.6)			
CFPT	C	2	0(0.0)	2(100.0)	4.156	2	0.125
	A ^a	9	6(66.7)	3(33.3)			
	B	5	3(60.0)	2(40.0)			
	C ^a	3	0(0.0)	3(100.0)			

^aThe response rate of the -6/-6 bp group is significantly higher than that of the +6/+6 bp group under the CFPT regimen ($\chi^2=4.00$, $P=0.046$)

Table 4 Relationship between TS gene 3'-UTR polymorphism and side effects of chemotherapy

TS genotype	Cases	Myelosuppression (%)	Diarrhea (%)	Emesis (%)	Oral mucositis (%)	Damage of liver (%)	Peripheral neuritis (%)
-6/-6 bp	51	24(47.1)	11(21.6)	28(54.9)	13(25.5)	7(13.7)	6(11.8)
-6/+6 bp	47	20(42.6)	5(10.6)	18(38.3)	8(17.0)	4(8.5)	3(6.4)
+6/+6 bp	8	2(25.0)	1(12.5)	3(37.5)	2(25.0)	0(0)	0(0)
Total	106	46(43.4)	17(16.0)	49(46.2)	23(21.7)	11(10.4)	9(8.5)

Table 5 Relationship between TS gene 3'-UTR polymorphism and III-IV degree side effects under different regimens. Genotypes: A -6/-6 bp, B -6/+6 bp, C +6/+6 bp

Regimen	Genotype	Cases	Myelosuppression (%)	Emesis (%)	Diarrhea (%)	Oral mucositis (%)
CFL ^a	A	15	8(53.3)	8(53.3)	3(20.0)	5(33.3)
	B	13	2(15.4)	1(7.7)	1(7.7)	3(23.1)
	C	1	1(100.0)	0(0.0)	0(0.0)	0(0.0)
			$\chi^2=5.955$, $P=0.051$	$\chi^2=7.244$, $P=0.027$	$\chi^2=1.053$, $P=0.591$	$\chi^2=0.761$, $P=0.683$
CFH ^a	A	8	4(50.0)	5(62.5)	0(0.0)	0(0.0)
	B	9	7(77.8)	4(44.4)	1(11.1)	2(22.2)
	C	2	1(50.0)	2(100.0)	1(50.0)	1(50.0)
			$\chi^2=1.571$, $P=0.456$	$\chi^2=2.192$, $P=0.334$	$\chi^2=4.253$, $P=0.119$	$\chi^2=3.541$, $P=0.170$
CFLH ^b	A	3	3(100.0)	3(100.0)	1(33.3)	2(66.7)
	B	7	6(85.7)	4(57.1)	2(28.6)	2(28.6)
	C					
			$\chi^2=0.476$, $P=0.490$	$\chi^2=1.837$, $P=0.175$	$\chi^2=0.023$, $P=0.880$	$\chi^2=1.270$, $P=0.260$
L-FP ^a	A	16	5(31.3)	8(50.0)	4(25.0)	3(18.8)
	B	13	1(7.7)	7(53.8)	0(0.0)	0(0.0)
	C	2	0(0.0)	0(0.0)	0(0.0)	0(0.0)
			$\chi^2=3.063$, $P=0.216$	$\chi^2=2.047$, $P=0.359$	$\chi^2=4.306$, $P=0.116$	$\chi^2=3.114$, $P=0.211$
CFPT ^a	A	9	3(33.3)	4(44.4)	3(33.3)	3(33.3)
	B	5	4(80.0)	2(40.0)	1(20.0)	1(20.0)
	C	3	0(0.0)	1(33.3)	0(0.0)	1(33.3)
			$\chi^2=5.440$, $P=0.066$	$\chi^2=0.119$, $P=0.942$	$\chi^2=1.438$, $P=0.487$	$\chi^2=0.302$, $P=0.860$

^adf=2

^bdf=1

group than in other groups, the difference was not statistically significant. Only in the CFL regimen group was the rate of chemotherapeutic side effects in the TS -6/-6 bp group significantly higher than that in the -6/+6 bp and +6/+6 bp groups ($\chi^2=7.244$, $df=2$, $P=0.027$).

Discussion

Polymorphism exists on a tandem repetitive sequence in the 5'-promoter of the TS gene. Recent research has shown that this TS 5'-UTR 28 bp sequence repeat

polymorphism can forecast the therapeutic effect of 5-FU, with two tandem repeat sequences (2R) and three tandem repeat sequences (3R) being the most important allelotypes (Marsh et al. 1999; Horie et al. 1995). However, the frequency distribution of the TS 5'-UTR 28 bp polymorphism differs in different ethnic populations. In East Asia, the frequency of 2R/2R is 4%; in Caucasian populations, the frequency of 2R/2R is 20%. However, in China, the frequency of 2R/2R is only 2% (Marsh et al. 1999). Zhang reported the frequency of 2R/2R and +6/+6 bp as 5.6 and 15.7% (Zhang et al. 2005). Since the gene frequency of 2R/2R in China is so low, our research analyzed only the relationship between TS 3'-UTR polymorphism and sensitivity of gastric cancer to 5-FU-based chemotherapy.

Although the presence or absence of the TS 3'-UTR 6 bp nucleotide fragment does not affect the coding of any TS amino acids, as in the case of the TS 5'-UTR 28 bp sequence repeat polymorphism, the 3'-UTR polymorphism can lead to changes in TS genetic structure, by potentially affecting the stability of TS mRNA, thus affecting its expression. Mandola and co-workers found that the absence of the TS 3'-UTR 6 bp nucleotide fragment was correlated with reduced mRNA stability in vitro, and with lower expression of TS in tumors in vivo (Mandola et al. 2004). However, a study by Merkelbach-Bruse et al. (2004) found no correlation between TS 3'-UTR polymorphism and TS mRNA expression levels. Thus, the effect of the presence or absence of the TS 3'-UTR 6 bp nucleotide fragment on TS genetic function requires further study. The incidences of TS -6/-6 bp, -6/+6 bp and +6/+6 bp found in this study were 48.1, 44.3 and 7.6%, consistent with our previous report (Gao et al. 2004). We found the response rate of the -6/-6 bp and -6/+6 bp groups to be significantly higher than the +6/+6 bp group. These results show that the presence or absence of the TS 3'-UTR 6 bp nucleotide fragment can be correlated with the sensitivity of gastric cancer to 5-FU-based chemotherapy.

Many studies have also observed a relationship between gene polymorphism and chemotherapeutic side effects. Lecomte et al. (2004) found more side effects in 2R/2R genotype patients than in 3R/3R genotype patients in their study on the TS 5'-UTR 28 bp sequence. However, there was no statistical difference between the different genotypes in our study of the TS 3'-UTR 6 bp polymorphism. The incidence of chemotherapeutic side effects in TS -6/-6 bp genotype patients was higher than in patients of the other two genotypes. In our study, the side effect rate in the TS -6/-6 bp group was higher than in the other groups, although there was no statistical difference. In the CFL regimen group, the incidence of chemotherapeutic side effect in the TS -6/-6 bp group was significantly higher than in the -6/+6 bp and +6/+6 bp groups ($\chi^2=7.244$, $df=2$, $P=0.027$). Overall, our study shows a correlation between the incidence of side effects of 5-FU-based chemotherapy and the presence or absence of the TS 3'-UTR 6 bp nucleotide fragment.

Our studies indicate that detection of TS 3'-UTR polymorphism can be used to guide the choice of 5-FU-based chemotherapy on advanced gastric cancer. Detection of TS 3'-UTR polymorphism can also forecast the therapeutic effect and side effects of 5-FU-based chemotherapy.

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