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Genetic instability and CpG methylation in the 5'-flanking region of the PAI-1 gene in Chinese patients with gastric cancer

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ABSTRACT. We explored a possible correlation of genetic instability and CpG methylation in the 5'-flanking region of the PAI-1 gene with clinicopathologic features of gastric cancer in Chinese patients and looked for molecular markers for diagnosing gastric tumor development. Microsatellite instability and loss of heterozygosity of the PAI-1 gene locus *D7S515*, *D7S471* and *pai-1* in 50 specimens of gastric cancer and relevant pericancerous tissues were detected by PCR-single strand conformation polymorphism (PCR-SSCP) with sliver staining. Methylation-specific PCR was used to detect CpG methylation in the 5'-flanking region of the PAI-1 gene. Microsatellite instability was significantly more common in the negative than in the positive serosa infiltration group of gastric cancer (42.86 *vs* 2.33%). The frequency of microsatellite instability was significantly lower in the cases with lymph node metastasis than in those without metastasis (18.18 *vs* 2.56%); however, it was significantly higher in the low differentiation group than that in the middle or high differentiation groups (21.05 *vs* 0.00%). CpG methylation in the 5'-flanking region of the PAI-1 gene did not differ significantly. Microsatellite instability and loss of heterozygosity

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of the PAI-1 gene apparently regulates the development of gastric cancer through different pathways. Microsatellite instability could be used as a molecular marker for the development of gastric cancer. CpG methylation in the 5'-flanking region of the PAI-1 gene appears not to be involved in the development of gastric cancer.

Key words: Gastric cancer; MSI; LOH; PAI-1 gene; DNA methylation

INTRODUCTION

Gastric cancer remains one of the commonly seen tumors showing the highest mortality in clinical practice in the Chinese population. Statistical data provided by the World Health Organization (WHO) in 2002 showed that the death rate from gastric cancer accounts for 5% of the total annual mortality for all diseases in China. Most patients with gastric cancer are diagnosed at an advanced stage with a 5-year survival of less than 20% (Jemal et al., 2008). Therefore, great attention should be paid to finding new early markers and effective treatment methods for this disease.

Carcinogenesis is a multistep process involving the activation of oncogenes and the loss or inactivation of tumor suppressor genes. Genetic instability of oncogenes, such as microsatellite instability (MSI) and loss of heterozygosity (LOH), is probably associated with mutations of genes responsible for carcinogenesis, which play an important role in cancer pathology (Chakrabarti et al., 2006). Studies on MSI and LOH of digestive system cancer have focused on the genetic instability of P53 (Juvan et al., 2007), P16 and FHIT (Xiao et al., 2006), and nm23H1 (Yang et al., 2008). However, there are only a few studies evaluating the association between the PAI-1 gene and gastric cancer. In addition, the epigenetic regulation of gene expression plays a major role in cancer. Aberrant transcriptional silencing of tumor suppressor genes by epigenetic deregulation is a common occurrence in human cancers (Baylin and Herman, 2000; Esteller, 2003). In the present study, we explored the correlation of genetic instability and CpG methylation of the PAI-1 gene with clinicopathologic features of gastric cancer in Chinese patients.

The plasminogen activator inhibitor-1 (PAI-1) gene is located on chromosome 7 and regulates the activities of tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) (Wind et al., 2002). tPA and uPA catalyze the proteolytic conversion of the zymogen plasminogen into the active protease plasmin, which can degrade fibrin and other extracellular matrix proteins. Plasmin-catalyzed degradation of proteins of the basement membrane is believed to enable cancer cells to invade normal tissues (Andreasen et al., 2000). The causal and important role of uPA-catalyzed plasmin generation in cancer cell invasion has been suggested in many studies. High levels of uPA have been found to correlate with poor prognosis in some tumors (Andreasen et al., 2000). However, the amounts of PAI-1 were found to be much higher in tumors than the corresponding normal tissues. Therefore, a high level of PAI-1 is even a better marker of poor prognosis in tumors (Durand et al., 2004).

In this study, we investigated the MSI and LOH of the PAI-1 gene at three loci (*pai-1*, *D7S471* and *D7S515*) in Chinese patients with gastric cancer, and the methylation status of CpG in the PAI-1 gene 5'-flanking region in gastric cancer.

MATERIAL AND METHODS

Tissue samples

Fifty tissue specimens of gastric cancer and 50 paired normal gastric tissue specimens were obtained after informed consent from patients who underwent curative resection for gastric cancer in the Zhejiang Provincial People's Hospital between 2004 and 2006. The specimens were snap-frozen in liquid nitrogen and stored immediately after surgery until further use. No patient received radiotherapy or chemotherapy prior to operation. All of the samples were carefully reviewed at the Department of Pathology of the hospital to confirm the diagnosis and to determine the cellular composition of the tumor.

LOH and MSI analysis

PCR amplification

DNA was extracted with a Qiagen kit according to manufacturer instructions. Primers were designed as previously described (Sourla et al., 1996) and synthesized by Shanghai Invitrogen Company (Table 1). The PCR mixture contained 200 ng template-DNA, while the PCR buffer contained 50 mM KCl, 10 mM Tris-HCl, pH 8.4, 1.5 mM $MgCl_2$, 0.5 μM each of two fragment-specific primers, 100 μM dATP, dGTP, dTTP, and dCTP, and 2 U Taq DNA polymerase (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd.) for a reaction volume of 50 μL. The conditions for PCR amplification were as follows: a pre-denaturation at 94°C for 10 min, then 35 cycles at 94°C for 30 s, at 53°-57°C for 30 s, and at 72°C for 45 s, and a final extension at 72°C for 10 min. The amplified fragments were run on a 2% agarose gel.

Table 1. Primer sequences for single-strand conformation polymorphism (SSCP) and methylation specific polymerase chain reaction (MS-PCR).

Primer	Primer sequence	Annealing temperature (°C)
SSCP		
pai-1 locus		
Forward	5'-GATAGCAGCAAGAGGCTG-3'	57.0
Reverse	5'-GGGGACAGAGCAAGAATCT-3'	
D7S471 locus		
Forward	5'-AGCAGCTATTATGGAATTGC-3'	57.0
Reverse	5'-CAACATATGCAAGGTGCCTA-3'	
D7S515 locus		
Forward	5'-GGGAGTTACTACCCTCAQCTTAATG-3'	53.0
Reverse	5'-GGACTGGGCAGCAAAG-3'	
MS-PCR		
M-Forward	5'-TGTTTGGTTGTAGGGTTAAGAGC-3'	57.0
M-Reverse	5'-AAATACCTTACGATTAACGATTCGT-3'	
U-Forward	5'-GTTTGGTTGTAGGGTTAAGAGTGT-3'	55.0
U-Reverse	5'-AATACCTTACAATTAACAATTCATC-3'	

Single-strand conformation polymorphism (SSCP) analysis

SSCP analysis of fragments was performed on a mini electrophoresis unit (Bio-Rad Company, USA). Four microliters of the PCR product were diluted with 4 μL sample buffer containing 90% formamide, 0.05% bromophenol blue dye and 0.05% xylene cyanol. The

samples were heated at 100°C for 8 min, transferred to an ice-cold water bath for 3 min, and analyzed by 10% polyacrylamide gel electrophoresis (PAGE) in 45 mM Tris-borate, pH 8.0, and 1 mM EDTA (TBE) buffer under 13 V/cm at 10°C.

DNA silver staining

Gels were fixed in 100 mL/L alcohol for 10 min, and then oxidized in 100 mL/L nitric acid for 3 min. After washing for 1 min with double-distilled water, they were stained in 2 g/L silver nitric acid for 5 min and washed for 1 min with double-distilled water. Gels showed an appropriate color in 15 g/L anhydrous sodium carbonate and 4 mL/L formalin. The reaction was terminated by 7.5 mL/L glacial acetic acid, and finally, the gels were washed with double-distilled water.

Methylation analysis

Methylation-specific PCR (*MS-PCR*)

Genomic DNA was treated with sodium bisulfite using the CpGenome Fast DNA Modification kit (S7824). The primer sequences used for MS-PCR analysis of the PAI-1 5'-flanking region were designed as previously described (Gao et al., 2005) and synthesized by Shanghai Invitrogen Company (Table 1). PCR mixtures (25 μL) contained 10 μM of each primer, covering +10 to +129, 0.2 mM of each dNTP, 1X PCR buffer and 1 U HotStarTaq DNA polymerase (TaKaRa), 0.2 mM cresol red, 12% (w/v) sucrose, and 50-100 ng bisulfite-treated DNA. Reactions were started with an initial denaturation at 95°C for 9 min, followed by 40 cycles at 94°C for 30 s, the appropriate annealing temperature for 30 s and 72°C for 30 s. The annealing temperatures for the unmethylated and methylated reactions were 55° and 50°C, respectively. The PCR products were resolved on 2% agarose gels.

DNA silver staining

DNA silver staining was performed with the same method as described above in LOH and MSI analysis.

Statistical analysis

Statistical analysis was performed using analysis of variance (one-way ANOVA) and the Student *t*-test by SPSS16.0. $P < 0.05$ was considered statistically significant.

RESULTS

The amplified fragments were run on a 2% agarose gel to detect the results of PCR (Figure 1). Three loci (*pai-1*, *D7S471* and *D7S515*) of 50 tumors and 50 paired normal gastric tissue samples were amplified by the designed primers as previously described (Sourla et al., 1996). MSI was positive as tumor samples added an allele band as compared to normal tissue (Figure 2); LOH was positive as tumor samples lacked an allele band as compared to normal tissue (Figure 3).

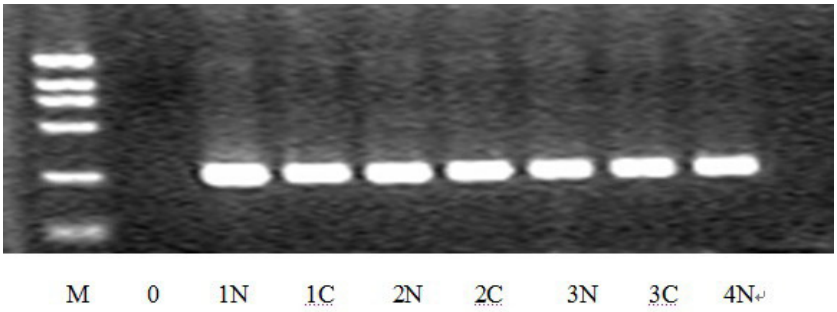

Figure 1. Polymerase chain reaction product of locus pai-1 on 2% agarose gel electrophoresis. *Lane M* = DNA marker; *lane 0* = control; *lane N* = normal gastric tissue; *lane C* = tumor tissue.

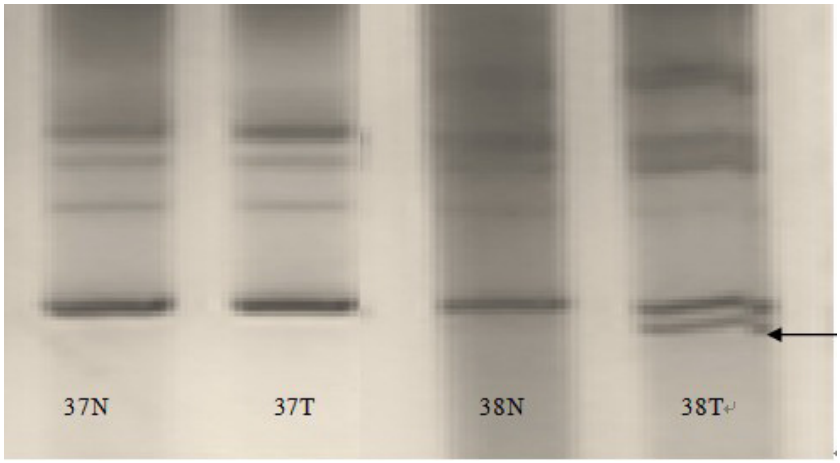

Figure 2. Microsatellite instability was positive as tumor samples added an allele band as compared to normal tissue. *Lane N* = Normal gastric tissue; *lane T* = tumor tissue; the arrow show that 38T added an allele band when compared with 38N.

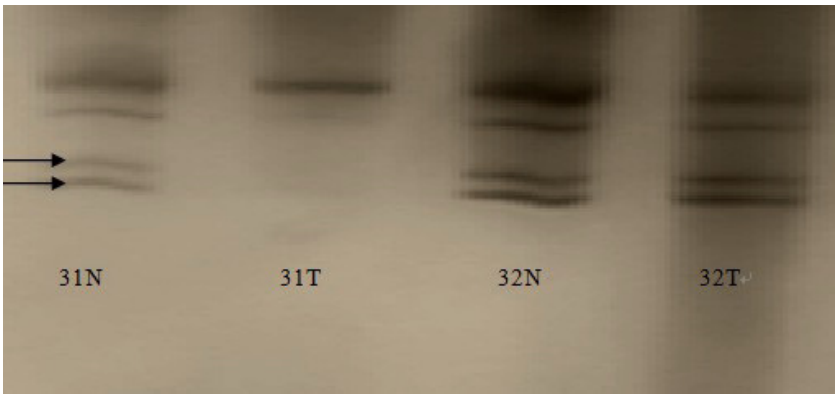

Figure 3. Loss of heterozygosity was positive as tumor samples lacked an allele band as compared to normal tissue. *Lane N* = Normal gastric tissue; *lane T* = tumor tissue; the arrows show that 31T lacked two allele bands when compared to 31N.

LOH and MSI

pai-1 locus

The MSI and LOH were independent of the differentiation degree, serosal infiltration and lymph node metastasis. MSI was related to serosal infiltration. The frequency of MSI was higher in the negative than in the positive serosal infiltration group (42.86 *vs* 2.33%, P = 0.031). LOH did not show a significant difference with regard to serosal infiltration (14.29 *vs* 6.98%, P > 0.05). In addition, MSI tended to decrease with lymph node metastasis (2.56 *vs* 18.18%, P = 0.007), while LOH did not (0.00 *vs* 10.26%, P > 0.05) (Table 2).

Table 2. Relationship between clinicopathologic parameters and pai-1 locus genetic instability in gastric cancer.

Clinicopathologic factors	Cases	Frequency of MSI cases (%)	P	Frequency of LOH cases (%)	P
Differentiation degree					
High differentiation	8	1 (12.50%)	P > 0.050	0 (0.00%)	P > 0.050
Moderate differentiation	23	3 (13.04%)		1 (4.35%)	
Low differentiation	19	0 (0.00%)		3 (15.79%)	
Serosa infiltration					
Positive	43	1 (2.33%)	P = 0.031	3 (6.98%)	P > 0.050
Negative	7	3 (42.86%)*		1 (14.29%)	
Lymph node metastasis					
Positive	39	2 (2.56%)	P = 0.007	4 (10.26%)	P > 0.050
Negative	11	2 (18.18%)#		0 (0.00%)	

Microsatellite instability (MSI); loss of heterozygosity (LOH). #P < 0.01, *P < 0.05.

D7S471 locus and D7S515 locus

The frequency of MSI and LOH in *D7S471* showed no statistical difference with regard to serosal infiltration or lymph node metastasis in gastric cancer. However, the frequency of MSI in the low differentiation group was higher than that in the moderate or high differentiation group (21.05 *vs* 0.00%, P = 0.027), while the frequency of LOH did not show such results (5.26 *vs* 8.70 and 0.00%, P > 0.05) (Table 3).

Table 3. Relationship between clinicopathologic parameters and D7S471 locus genetic instability in gastric cancer.

Clinicopathologic factors	Cases	Frequency of MSI cases (%)	P	Frequency of LOH cases (%)	P
Differentiation degree					
High differentiation	8	0 (0.00%)	P = 0.027	0 (0.00%)	P > 0.050
Moderate differentiation	23	0 (0.00%)		2 (8.70%)	
Low differentiation	19	4 (21.05%)*		1 (5.26%)	
Serosa infiltration					
Positive	43	3 (6.98%)	P > 0.050	2 (4.65%)	P > 0.050
Negative	7	1 (14.29%)		1 (14.29%)	
Lymph node metastasis					
Positive	39	4 (10.26%)	P > 0.050	3 (7.69%)	P > 0.050
Negative	11	0 (0.00%)		0 (0.00%)	

Microsatellite instability (MSI); loss of heterozygosity (LOH). *P < 0.05.

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The frequency of MSI and LOH in *D7S515* showed no statistically significant difference with regard to differentiation degree, serosal infiltration or lymph node metastasis in gastric cancer (Table 4).

Table 4. Relationship between clinicopathologic parameters and D7S515 locus genetic instability in gastric cancer.

Clinicopathologic factors	Cases	Frequency of MSI cases (%)	P	Frequency of LOH cases (%)	P
Differentiation degree					
High differentiation	8	0 (0.00%)	P > 0.050	0 (0.00%)	P > 0.050
Moderate differentiation	23	1 (4.35%)		3 (13.04%)	
Low differentiation	19	4 (21.05%)		1 (5.26%)	
Serosal infiltration					
Positive	43	4 (9.30%)	P > 0.050	3 (6.98%)	P > 0.050
Negative	7	1 (14.29%)			
Lymph node metastasis					
Positive	39	4 (10.26%)	P > 0.050	4 (10.26%)	P > 0.050
Negative	11	1 (9.09%)		0 (0.00%)	

Microsatellite instability (MSI); loss of heterozygosity (LOH).

CpG methylation in 5'-flanking region

DNA of tumor tissues and paired normal gastric tissues was amplified by the designed primers as previously described (Gao et al., 2005) (Table 1). CpG methylation was found in the 5'-flanking region of the PAI-1 gene by MS-PCR analysis with primers covering +10 to +129. All samples were detected with the methylated sequence-specific primers and the unmethylated sequence-specific primers (Figure 4). The percentage of methylated samples observed in gastric cancer and in paired normal gastric tissues was 55.3 and 57.4%, respectively. There was no statistically significant difference between methylation rates in the two groups of tissues. The frequency of methylation rate showed no correlation with lymph node metastasis in gastric cancer (50.0 *vs* 80.0%, P > 0.05) (Table 5).

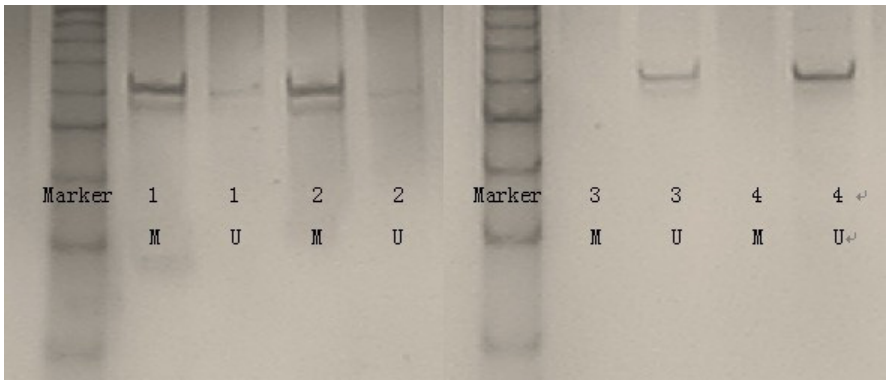

Figure 4. MS-PCR of PAI-1. *Lane marker* = DNA marker; *lanes 1*, *2*, *3* and *4* = number of tumor tissues. *Lane M* = MS-PCR for methylated sequence-specific primer; *lane U* = MS-PCR for unmethylated sequence-specific primer. All samples were detected with the methylated sequence-specific primers and the unmethylated sequence-specific primers. The samples 1, 2 are methylated and 3, 4 are unmethylated.

Table 5. Relationship between clinicopathologic parameters and CpG methylation in 5'-flanking region of PAI-1 in gastric cancer.

Clinicopathologic factors	Cases	Methylation frequency cases (%)	P
Differentiation degree			
High differentiation	7	5 (71.43%)	P > 0.05
Moderate differentiation	23	13 (56.52%)	
Low differentiation	17	8 (47.06%)	
Serosal infiltration			
Positive	41	24 (58.54%)	P > 0.05
Negative	6	2 (33.33%)	
Lymph node metastasis			
Positive	37	18 (48.65%)	P > 0.05
Negative	10	8 (80.00%)	
TNM stage			
? + ?	37	20 (54.05%)	P > 0.05
? + ?	10	6 (60.00%)	

DISCUSSION

Microsatellites are short tandem repeat sequences of unknown function scattered throughout the human genome. LOH and MSI are the phenotypes of genetic instability caused by abnormalities of tumor suppressor and DNA mismatch repair genes, respectively. MSI is associated with slippage of DNA polymerase during DNA synthesis resulting in changing units of repetitive sequences, while LOH is allelic loss in a certain region of chromosome. Previous studies have reported that genetic instability of oncogenes is probably associated with mutations of genes responsible for tumorigenesis, and plays an important role in tumor pathology (Storchova and Pellman, 2004).

However, it is still unclear how cancer cells acquire genetic instability. Several studies using endogenous loci have demonstrated increased mutation frequencies in cells grown in tumors. In this study, we detected three loci (*D7S515*, *D7S471* and *pai-1*) associated with gastric cancer in a Chinese population. We further analyzed genetic instability using the *pai-1* marker and *D7S471*, a marker telomeric to the PAI-1 gene. We also used the *D7S515*, a marker centromeric to PAI-1.

Ozisik et al. (1993) investigated the cytogenetic patterns of uterine leiomyomas and found frequent abnormalities of chromosome 7, particularly del (7)(q22). Gopalan et al. (2010) reported that increasing copy number of the GAEC1 gene located at 7q22 is related to the clinicopathologic parameters of digestive system cancers. Because the locus of the PAI-1 gene is on chromosome 7q22, we therefore analyzed the genetic instability of the PAI-1 gene using the *pai-1* marker, *D7S471* and *D7S515*.

Many studies have proven that PAI-1 is closely associated with high metastatic potential and poor prognosis of human cancer. Sakakibara et al. (2006) found that TNM staging and lymphatic metastasis are significantly associated with PAI-1 in gastric cancer. In our experiment, the MSI frequency was significantly correlated with serosal infiltration, lymph node metastasis and differentiation degree of gastric cancers at the *pai-1* and *D7S471* loci. The lower the MSI frequency was, the poorer the prognosis. Our data indicated that the occurrence of MSI at the *pai-1* and *D7S471* loci can be considered a molecular marker of early stage gastric cancer. However, LOH frequency did not show similar results, indicating that MSI and LOH can control carcinogenesis and metastasis through different pathways.

Epigenetic alterations are heritable changes in gene expression without an accompanying change in primary DNA sequence. Histone modification and DNA methylation, two major epigenetic processes, can control the expression of genes at the transcriptional level (Wu and Morris, 2001). There is increasing evidence that aberrant DNA methylation and histone acetylation are useful independent molecular prognostic markers. For example, hypermethylation of CpG islands in promoter regions of the gene occur earlier than malignant proliferation. Thus, cancer could be diagnosed at an early stage of development (Nuovo et al., 1999). Since epigenetic changes are reversible, inhibitors of DNA methylation could raise the possibility of exploiting new therapeutic targets (Sato et al., 2003). Although DNA methylation in CpG islands is believed to play an important role in the regulation of gene expression, it has been suggested that methylation of CpG islands localized at exonic sites with a great distance from the 5'-flanking region cannot affect transcription (Gonzalez-Zulueta et al., 1995; Strathdee et al., 2004). Gao et al. (2005) proved that the PAI-1 gene transcription may be regulated by methylation of less dense CpGs in the 5'-flanking region rather than methylation of upstream CpG islands. We analyzed the correlation between CpG methylation in the 5'-flanking region of the PAI-1 gene and gastric cancer development.

In our study, CpG methylation in the 5'-flanking region of the PAI-1 gene showed no correlation with serosal infiltration, lymph node metastasis or differentiation degree of gastric cancer, indicating that the 5'-flanking region methylation does not control gastric cancer development.

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