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• *GASTRIC CANCER* •

DNA ploidy analysis and expression of MMP-9, TIMP-2, and E-cadherin in gastric carcinoma

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Abstract

AIM: To investigate DNA ploidy and expression of MMP-9, TIMP-2, and E-cadherin in gastric carcinoma and to explore the mechanism of invasion and metastasis of gastric carcinoma.

METHODS: Immunohistochemical methods were used to detect the expressions of MMP-9, TIMP-2, and E-cadherin in 156 cases, including 99 cases of gastric carcinoma, 16 cases of adjacent noncancerous mucosa, 16 cases of distant metastases and 25 cases of metastatic lymph node (LN) from gastric carcinoma. Flow cytometry DNA ploidy and S-phase fraction (SPF) analysis were performed on 57 cases, including 47 cases of gastric cancer, 6 cases of adjacent noncancerous mucosa, and 4 cases of distant metastatic cancer.

RESULTS: The expression of MMP-9 was significantly correlated with Lauren's classification, Borrmann's classification, LN metastasis, tumor metastasis, and TNM stage, as well as depth of invasion (all $P<0.05$). The positive rate was lower in noncarcinoma than in carcinoma (31.3% *vs* 66.7%, $P<0.01$). The expression of TIMP-2 was significantly correlated with Borrmann's classification, LN metastasis, and the depth of invasion (all $P<0.05$). The expression of E-cadherin was significantly correlated with differentiation, Lauren's classification, Borrmann's classification, and LN metastasis, as well as the depth of invasion ($P<0.01$ or $P<0.05$). E-cadherin was less expressed in carcinoma than in noncarcinoma (42.4% *vs* 87.5%, $P<0.01$). There was a positive correlation between MMP-9 and TIMP-2 and a negative correlation between MMP-9 and E-cadherin, but no correlation between TIMP-2 and E-cadherin. Also there was a positive correlation between DNA aneuploid rate and differentiation and LN metastasis. SPF that was higher than 15% was positively correlated with tumor size, differentiation and LN metastasis. And there was a significant difference between carcinoma and noncarcinoma in DNA aneuploid rate and SPF.

CONCLUSION: With tumor progression and development of heterogeneity, the abnormal expressions of MMP-9, TIMP-2, and E-cadherin or DNA aneuploid rate or high SPF gradually increases, suggesting that they play a crucial role in gastric carcinoma progression.

Key words: MMP-9; TIMP-2; E-cadherin; DNA ploidy; Gastric carcinoma

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INTRODUCTION

Gastric cancer is the second most common malignancy worldwide. Although the achievement in early diagnosis and treatment of gastric cancer has improved the patients' outcome, it is still among the leading causes of mortality in countries such as China and Japan; even in the developed Western countries the 5-year survival rate for gastric cancer is only 10-19%[1]. The mechanisms of its development, progression, and metastasis are under investigation[2].

Breakdown of cell-cell adhesion and degradation of extracellular matrix (ECM) represent an essential step for tumor metastasis and invasion. The cadherin superfamily is a major class of adhesion molecules that play an important role in homotypic cell-cell interactions and hence cancer cell metastasis and invasion[3]. E-cadherin is a member of the cadherin family that is expressed in all epithelial cells. It is a calcium-dependent cell adhesion molecule and has been recognized as an important suppressor gene. A variety of human cancers exhibit altered expression of E-cadherin, which correlates with high grade and advanced stage of tumor.

Matrix metalloproteinases (MMPs) are a large family

of zinc-dependent proteolytic enzymes, which are believed to play a pivotal role in malignant behavior of cancer cells such as rapid growth, invasion, and metastasis by degrading ECM[4,5]. Increased expression of MMPs has been found in various carcinomas. The family of human MMPs comprises mainly four classes according to their structure and *in vitro* substrate specificity: collagenases, gelatinases, stromelysins, and membrane-type MMPs. MMP-9 belongs to gelatinases and most investigations supported that there was a positive correlation between MMP-9 and tumor progression[9]. Tissue inhibitors of matrix metalloproteinases (TIMPs) are the major natural inhibitors of MMPs. To date, four types of TIMPs have been recognized[6]. TIMPs are secreted proteins that are complex MMPs and are involved in the inhibition of individual MMPs and regulation of their activity. Indeed, it is thought that the balance between activated MMPs and TIMPs determines the overall MMP activity and proteolysis *in vivo*. However, recent experimental data suggest that TIMPs not only have MMP inhibitory functions, but also are multifunctional molecules, with apparent paradoxical effects on tumor progression. Recently some studies suggest a positive correlation between TIMP-2 level and poor outcome in certain types of tumors[7,8].

In this study, we detected the expression of MMP-9, TIMP-2, and E-cadherin proteins in gastric cancer, its adjacent noncancerous mucosa, metastatic lymph nodes (LNs), and metastatic cancer by using immunohistochemical staining. Moreover we detected DNA ploidy and S-phase fraction (SPF) in gastric cancer by flow cytometry to explore the molecular and cellular mechanisms of tumor progression so as to develop novel therapies and provide scientific basis for prognostic evaluation of gastric carcinoma.

MATERIALS AND METHODS

Patients and specimens

One hundred and fifty-six surgically removed specimens from the year 2000-2003 were collected from the Department of Pathology, Affiliated Hospital of Medical College, Qingdao University, including 99 cases of gastric carcinoma, 16 cases of adjacent noncancerous mucosa, 25 cases of metastatic LNs, and 16 cases of metastatic carcinoma from gastric cancer. Patients consisted of 87 male and 28 female with a mean age of 58.2 years (range 32-82 years) and were untreated before surgery. Sections from the surgical specimens were fixed in 4% formaldehyde solution for 24-48 h, and embedded in paraffin. A 4-μm section from each specimen block was stained with H&E for histological evaluation, and representative blocks were chosen for immunohistochemical study. In addition, normal gastric epithelium, colon adenocarcinoma, and breast carcinoma were used as positive controls for E-cadherin, TIMP-2, and MMP-9, respectively. For negative controls, sections were incubated with PBS (0.01 mol/L, pH 7.4) instead of the primary antibodies.

For flow cytometry, 57 specimens were selected from immunohistochemical study cases, including 47 cases of gastric carcinoma, 6 cases of adjacent noncancerous mucosa, and 4 cases of metastatic carcinoma from gastric cancer. For each case, we selected the most representative area of the specimen for flow cytometry analysis with H&E section. Five 50-μm sections from each specimen block and one 50-μm section from normal LN block, which was fixed and embedded with the same method and used for internal standard were cut for preparation of single cell suspensions.

Antibodies and other chemicals

The goat anti-human polyclonal antibody against TIMP-2, rabbit anti-human monoclonal antibodies against MMP-9 and E-cadherin were from Santa Cruz (CA, USA); Histostain^{TM}-Plus kit was from Zymed (CA, USA); Coulter DNA-Prep Reagent kit was from Beckman Coulter (Miami, USA).

Immunohistochemical staining

Immunohistochemical staining was performed with SP method, according to instruction of the kit and antibodies against MMP-9, TIMP-2, and E-cadherin were used at a concentration of 1:60, 1:30, and 1:120, respectively.

Sample preparation for flow cytometry analysis

Modified Hedley protocol was a workable method for making nuclear suspensions from paraffin blocks. Sections were deparaffinized and rehydrated through changes of xylene and graded alcohol, then immersed in water, and converted to a nuclear suspension by digestion in 0.5% pepsin (pH 1.5). The dispersive nuclear suspension was pelleted by centrifugation (1 500 r/min, 5 min) and filtered through a nylon filter, and then incubated with DNA-prep reagents kit for 30 min for DNA quantitation. The exciting spectrum was 488 nm and about 20 000 signals were collected per specimen with a coefficient of variation (CV) of 2% or less. Data collection was performed on a flow cytometer (FCM, Beckman Coulter EPICS XL).

Evaluation of immunostaining

Clear brown staining was restricted to cytoplasm, and was considered as positive for MMP-9 and TIMP-2. Slides were scored semi-quantitatively based on staining intensity and distribution. Two investigators without knowledge of the clinical data independently performed analysis of immunohistochemical staining. Staining was graded based on the method by Massi *et al*.[9], as follows: negative (-): no positive cells were found; focal (+): positive tumor cells accounted for <20% of the total; moderate (++): 21-50% of tumor cells were positive; diffuse (+++): >50% tumor cells were positive. The positive staining of E-cadherin appeared pale brown and was located in the cell membrane. Staining was scored according to the method described by Li *et al*.[10]: 0 score: no expression; 1 score: expression in cytoplasm; 2 scores: decreased expression; 3 scores: expression in cell membrane, namely normal expression. A score of 0-2 was considered as negative expression.

Evaluation of flow cytometry

FCM-derived parameters were DNA ploidy and SPF. According to DNA index (DI), aneuploid and diploid tumors were evaluated[11].

Statistical analysis

Statistical analysis was performed using the χ^2 test. A P value lesser than 0.05 was considered as statistically significant.

All statistical analyses were performed using the SPSS 11.0 statistical software.

RESULTS

Immunohistochemical staining of MMP-9

Immunoreactivity for MMP-9 was present in the cytoplasm of neoplastic and non-neoplastic mucosa cells. MMP-9 expression showed moderate or diffuse distribution in neoplastic cells (Figure 1A), but was single or focal in noncancerous mucosa cells (Figure 1B). In the invasion front, MP-9 showed strong positive staining (Figure 1C) and positive staining was also noted in vascular endothelial cells and inflammatory cells (Figure 1D). The expression of MMP-9 was significantly correlated with Lauren's classification (in intestinal and diffuse types, the positive rate was 58.2% and 90.9%, respectively, $\chi^2 = 8.025$, $P<0.05$), Borrmann's classification (in Borrmann's types I-II, III, and IV, the positive rate was 33.3%, 65.7%, and 85.0% respectively, $\chi^2 = 7.554$, $P<0.05$), LN metastasis (in negative and positive cases, the positive rate was 54.3% and 79.2% respectively, $\chi^2 = 6.986$, $P<0.05$), metastasis (in primary gastric carcinoma, metastatic LN, and metastatic carcinoma, the positive rate was 66.7%, 92.0%, and 81.7%, respectively, $\chi^2 = 7.121$, $P<0.05$) and TNM stage (in stages II, III, and IV, the positive rate was 37.5%, 50.0%, and 73.3%, respectively, $\chi^2 = 6.647$, $P<0.05$, Tables 1 and 2). A significant association between the depth of invasion and expression of MMP-9 (in submucosa, superficial muscular layer, and deep muscular layer or serosa, the positive rate was 28.6%, 59.3%, and 73.8% respectively, $\chi^2 = 6.743$, $P<0.05$) was detected. The positive rate was lower in noncarcinoma than in carcinoma (31.3% *vs* 66.7%, $\chi^2 = 7.314$, $P<0.01$).

Immunohistochemical staining of TIMP-2

Immunoreactivity for TIMP-2 was also present in the cytoplasm of neoplastic and non-neoplastic mucosa cells. TIMP-2 expression showed moderate or diffuse distribution in neoplastic cells (Figure 2A), but focal or moderate distribution in noncancerous mucosa cells (Figure 2B). The expression of TIMP-2 was significantly correlated with Borrmann's classification (in Borrmann's types I-II, III, and IV, the positive rate was 66.7%, 47.1%. and 20.0% respectively, $\chi^2 = 6.839$, $P<0.05$) and LN metastasis (in negative and positive cases, the positive rate was 56.5% and 32.1% respectively, $\chi^2 = 5.992$, $P<0.05$). A significant association between the depth of invasion and expression of TIMP-2 (in submucosa, superficial muscular layer, and deep muscular layer or serosa, the positive rate was 71.4%, 59.3%, and 33.8% respectively, $\chi^2 = 7.417$, $P<0.05$) was also revealed (Tables 1 and 2).

Immunohistochemical staining of E-cadherin

Immunoreactivity for E-cadherin was present in the membrane of neoplastic and non-neoplastic mucosa cells. In noncancerous mucosa epithelia, E-cadherin expression showed strong membranous staining, especially at the intercellular border (Figure 3A), but abnormal expression was observed in neoplastic tissues with reduction or loss of membranous expression and cytoplasmic or nuclear staining (Figures 3B-D). The expression of E-cadherin was significantly correlated with differentiation (in well-, moderately-, and poorly-differentiated, and signet ring cell carcinoma, the positive rate was 75.0%, 61.1%, 30.6%, and 27.6% respectively, $\chi^2 = 14.215$, $P<0.01$), Lauren's classification (in intestinal and diffuse type, positive rate was 53.7% and 13.6% respectively, $\chi^2 = 11.603$, $P<0.05$), Borrmann's classification (in Borrmann's

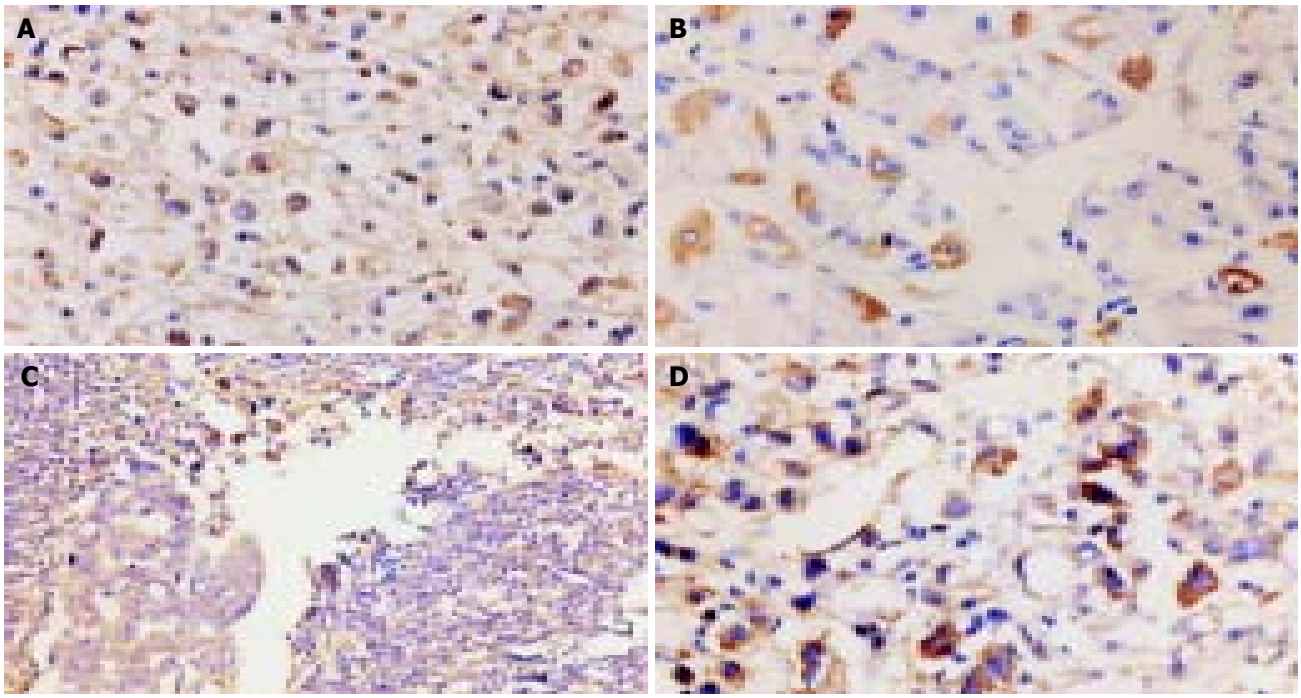

Figure 1 MMP-9 expression in gastric carcinoma. **A:** MMP-9 expression showed moderate or diffuse distribution of immunostaining in neoplastic cells (×200); **B:** single or focal distribution in noncancerous mucosa cells (×400); **C:** in the invasion front, MMP-9 showed strong positive staining (×200); **D:** positive staining was also noted in vascular endothelial cells and inflammatory cells (×400).

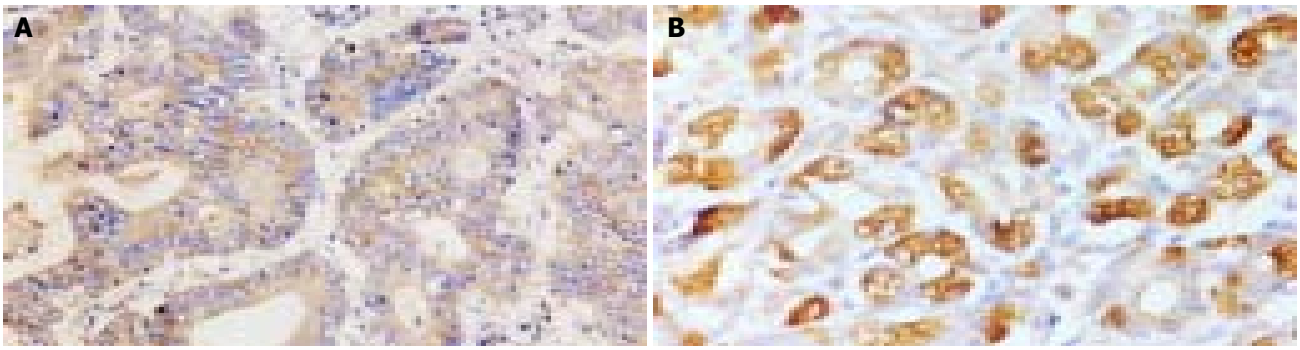

Figure 2 TIMP-2 expression in gastric carcinoma. **A:** TIMP-2 expression showed moderate or diffuse distribution in neoplastic cells (×200), **B:** focal or moderate distribution in noncancerous mucosa cells (×400).

Table 1 Relationship between expression of MMP-9, TIMP-2 or E-cadherin, and histopathological features in gastric carcinoma

		Positive			Rate (%)		
	n	MMP-9	*P*	TIMP-2	*P*	E-cadherin	*P*
Differentiation							
Well	16	50.0	>0.05	62.5	>0.05	75.0	<0.01
Moderate	18	66.7		55.6		61.1	
Poor	36	69.4		38.9		30.6	
Signet ring cell type	29	72.4		31.0		27.6	
Tumor recurrence							
Primary carcinoma	91	65.9	>0.05	44.0	>0.05	44.0	>0.05
Recurrent carcinoma	8	75.0		37.5		25.0	
Carcinoma and non-neoplastic							
Gastric carcinoma	99	66.7	<0.01	43.4	>0.05	42.4	<0.001
Non-neoplastic gastric mucosa	16	31.3		31.3		87.5	
Lauren's classification							
Intestinal type	67	58.2[a]	<0.05	46.3	>0.05	53.7[a]	<0.05
Diffuse type	22	90.9		36.4		13.6	
Mixed type	10	70.0		40.0		30.0	
Borrmann's classification							
Types I–II	9	33.3[c]	<0.05	66.7	<0.05	55.6[c]	<0.05
Type III	70	65.7		47.1		42.9	
Type IV	20	85.0		20.0		25.0	

[a]$P<0.05$ *vs* diffuse type; [c]$P<0.05$ *vs* type IV.

Table 2 Relationships between expression of MMP-9, TIMP-2 or E-cadherin, and clinical features in gastric carcinoma

		Positive			Rate (%)		
	n	MMP-9	*P*	TIMP-2	*P*	E-cadherin	*P*
Tumor size							
⩾5 cm	53	66.0	>0.05	41.5	>0.05	39.6	>0.05
<5 cm	46	67.4		45.7		45.7	
LN metastasis							
Negative	46	54.3	<0.01	56.5	<0.05	54.3	<0.05
Positive	53	79.2		32.1		32.1	
Metastasis							
Primary carcinoma	99	66.7	<0.05	43.4	>0.05	42.4	>0.05
Metastatic LN	25	92.0		36.0		20.0	
Metastatic carcinoma	16	81.7		43.8		37.5	
Depth of invasion							
Submucosa	7	28.6[c]	<0.05	71.4[c]	<0.05	85.7[c]	<0.05
Superficial muscular layer	27	59.3		59.3		48.1	
Deep muscular layer or serosa	65	73.8		33.8		35.4	
TNM stage							
Stage II	8	37.5[e]	<0.05	50.0	>0.05	62.5	>0.05
Stage III	16	50.0		43.8		50.0	
Stage IV	75	73.3		42.7		38.7	

[c]$P<0.05$ *vs* deep muscular layer or serosa; [e]$P<0.05$ *vs* stage IV.

types I-II , III, and IV, the positive rate was 66.7%, 44.3%, and 25.0% respectively, $\chi^2 = 7.097$, $P<0.05$) and LN metastasis (in negative and positive cases, the positive rate was 54.3% and 32.1% respectively, $\chi^2 = 5.012$, $P<0.05$). A significant association between the depth of invasion and expression of E-cadherin (in submucosa, superficial muscular layer, and deep muscular layer or serosa, the positive rate was 85.7%, 48.1%, and 35.4% respectively, $\chi^2 = 7.051$, $P<0.05$) was observed. E-cadherin was less expressed in carcinoma than in noncarcinoma (42.4% *vs* 87.5%, $\chi^2 = 11.223$, $P<0.001$, Tables 1 and 2).

Correlation between MMP-9 and TIMP-2

The expression of MMP-9 was significantly and positively correlated with expression of TIMP-2 (Table 3). Positive rate of TIMP-2 in cases with MMP-9 positive was higher than those with MMP-9 negative, and the coefficient of correlation was 0.22.

Table 3 Correlations between MMP-9 and TIMP-2 expression

	TIMP-2	
MMP-9	-	+
+	32	34
-	24	9

Correlation between E-cadherin and MMP-9

The expression of MMP-9 was significantly and negatively correlated with the expression of E-cadherin (Table 4). Negative rate of E-cadherin in MMP-9 positive cases was higher than in MMP-9 negative cases, and the coefficient of correlation was -0.33.

Correlation between E-cadherin and TIMP-2

The expression of E-cadherin was not correlated with the expression of TIMP-2 (Table 5). The coefficient of correlation was only 0.07 without significance by χ^2 test.

Table 4 Correlations between E-cadherin and MMP-9 expression

	E-cadherin	
MMP-9	-	+
+	46	20
-	11	22

Table 5 Correlations between TIMP-2 and E-cadherin expression

	TIMP-2	
E -cadherin	-	+
+	22	20
-	35	22

FCM results

Typical DNA histogram of non-neoplastic mucosa showed a large diploid peak and a smaller tetraploid peak, and SPF was less than 15% (Figure 4A), but the histogram from gastric carcinoma showed a definitely abnormal (aneuploid or hyperdiploid) peak (Figure 4B) or/and increased SPF (Figure 4C). Aneuploid rate was significantly correlated with differentiation (in well- or moderately- and poorly-differentiated or signet ring cell carcinomas, the aneuploid rates were 42.1% and 75.0%, respectively, $\chi^2 = 5.183$, $P<0.05$) and LN metastasis (in negative and positive cases, the aneuploid rates were 80.0% and 29.4% respectively, $\chi^2 = 11.752$,

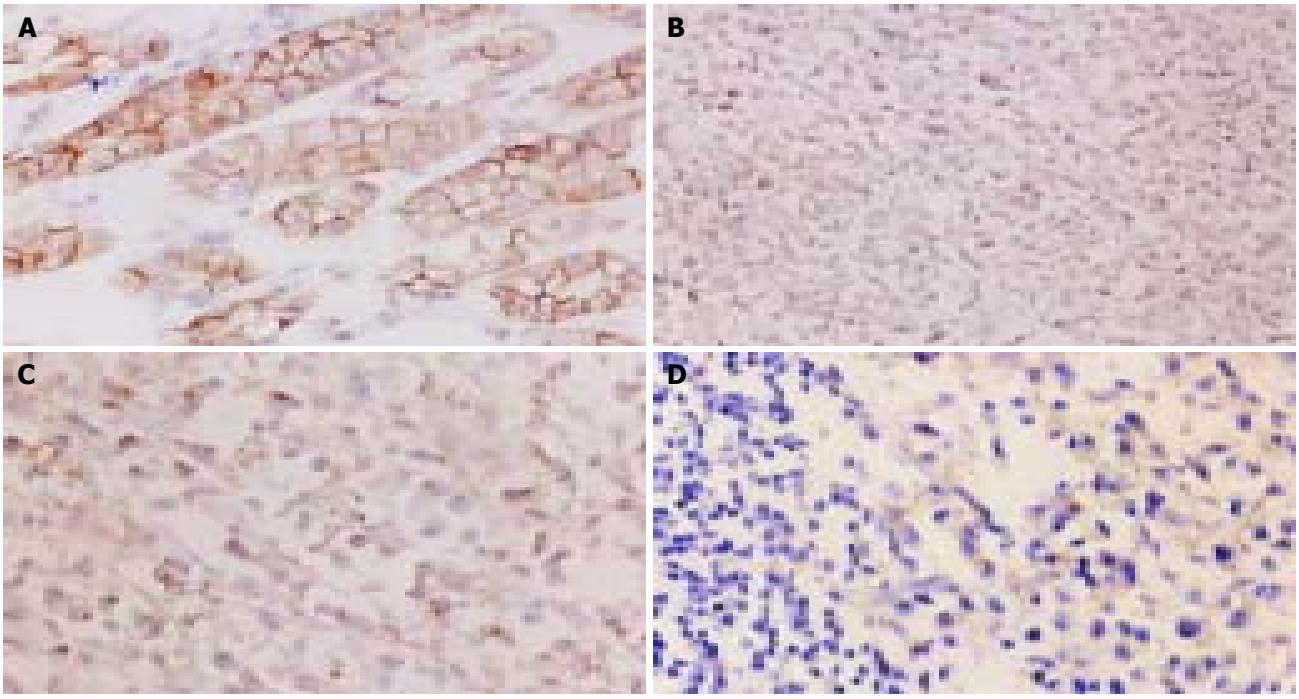

Figure 3 E-cadherin expression in gastric carcinoma. **A:** E-cadherin expression showed strong membranous staining in noncancerous mucosa cells, especially at the intercellular border (×400); **B-D:** abnormal expression was observed in neoplastic tissues which showed reduction or loss of membranous expression and showed cytoplasmic or nuclear staining (**B** and **D**, ×200; **C**, ×400).

$P<0.001$, Table 6). It was significantly different between carcinoma and noncarcinoma (aneuploid rate were 61.7% and 16.7%, respectively, $\chi^2 = 4.393$, $P<0.05$). SPF was significantly correlated with tumor size (the number of cases with SPF>15% and tumor size <5 cm was 9 and it was 23 in tumors with SPF>15% and tumor size >5 cm, $\chi^2 = 4.931$, $P<0.05$), differentiation (the number of cases with SPF>15% and well- or moderately-differentiated carcinoma was 8 and it was 21 in poorly-differentiated or signet ring carcinoma, $\chi^2 = 6.299$, $P<0.05$) and LN metastasis (the number of cases with SPF>15% and negative LN metastasis was 24 and it was 8 in cases with SPF>15% and positive LN metastasis, $\chi^2 = 5.419$, $P<0.05$). And the number of cases with SPF higher than 15% was significantly different between carcinoma and noncarcinoma (32 *vs* 1, $\chi^2 = 5.987$, $P<0.05$).

DISCUSSION

One of the typical characteristics of malignant tumor is invasion and metastasis, which is the main cause for their lethality. Tumor progression is considered to be a dynamic, complex, and a multi-step process, but the essential steps are the breakdown of cell-cell adhesion and degradation of basement membrane (BM) and ECM.

In the present study, immunohistochemical analysis revealed a progressive increase in expression of MMP-9 with increasing severity of lesions of carcinoma (Figure 1A). And in tumor invasion front or the surrounding of tumor nests, MMP-9 showed a strong staining (Figure 1C), while in non-neoplastic tissues it was weakly positive (Figure 1B). Although the antibodies do not distinguish between proenzyme and active forms of MMP-9, an increase in intensity of immunohistochemical reactions of MMPs may play a crucial role in the progression of carcinoma. MMP-9 upregulation will confer on tumor cells the ability to degrade BM and ECM and lead to tumor progression. MMP-9 immunoreactivity in vascular endothelial cells and inflammatory cells was also noted (Figure 1D), suggesting that those cells might secrete MMP-9 induced by malignant cells, the so-called host reaction[12]. As reported, MMP-9 promoted inflammatory reaction by upregulation of cytokines such as IL-1β[13], and this then stimulated host reaction, thus forming a pernicious cycle. Therefore, in view of MMP-9, it is not a good phenomenon that tumor cells can induce inflammatory reaction. MMP-9 participates in the whole process of carcinogenesis and development of gastric carcinoma and even in intramucosal gastric carcinomas MMP-9 showed strong staining and promoted LN metastasis[14,15], suggesting an early tumor progression. Our result demonstrated that MMP-9 activity increased as the depth of invasion extended,

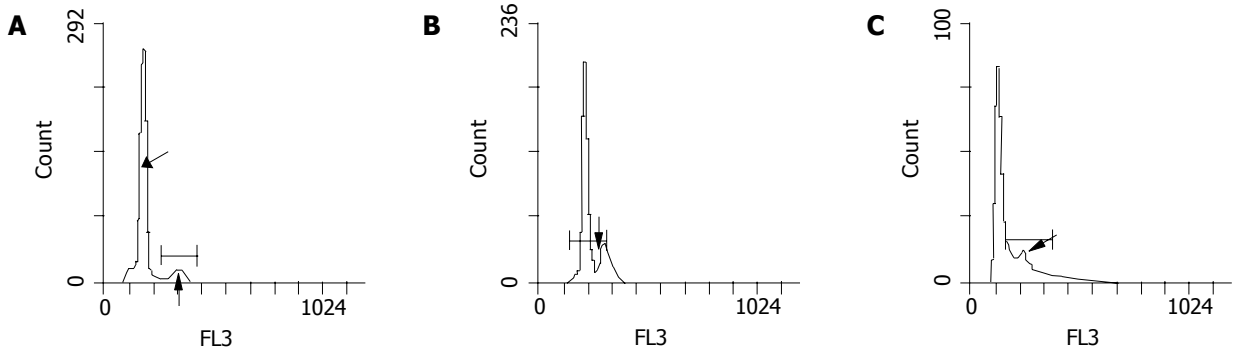

Figure 4 Histograms from FCM. **A:** A large diploid peak and a smaller tetraploid peak; **B:** an aneuploid peak (see arrow); **C:** higher SPF (see arrow).

Table 6 Correlation between DNA ploidy or SPF and histopathological or clinical features in gastric carcinoma

	n	Aneuploid rate (%)	*P*	*n* (SPF>15%)	*P*
Tumor size					
⩾5 cm	29	65.5	>0.05	23	>0.05
<5 cm	18	55.6		9	
Differentiation					
Well or moderate	19	42.1	<0.05	9	<0.05
Poor or signet ring cell type	28	75.0		23	
Depth of invasion					
Submucosa	7	28.6	>0.05	3	>0.05
Muscular layer or serosa	40	67.5		29	
LN metastasis					
Negative	30	80.0	<0.001	24	<0.05
Positive	17	29.4		8	
Metastasis					
Primary carcinoma	47	61.7	>0.05	32	>0.05
Metastatic carcinoma	4	100		3	
Carcinoma and noncarcinoma					
Gastric carcinoma	47	61.7	<0.05	32	<0.05
Noncarcinoma gastric mucosa	6	16.7		1	

indicating that MMP-9 participated in tumor invasion. It was also revealed that MMP-9 activity increased as LN metastasis progressed, indicating that MMP-9 participated in tumor metastasis. Expression rate of MMP-9 was significantly different between carcinoma and noncarcinoma (66.7% and 31.3% respectively), implying that MMP-9 may be related to carcinogenesis. Recent findings support the view that MMPs are not only responsible for the proteolytic degradation of BM as an important step in metastasis, but also essentially regulate the growth of tumor by maintaining its access to growth factors from ECM and regulation of angiogenesis[16]. Single MMP strong expression does not necessarily suggest invasion or metastasis, because its activity is influenced by its inhibitors such as RECK and TIMPs[17]. TIMPs are endogenous specific inhibitors of MMPs. They have such abilities to form tight binding, non-covalent inhibitory complexes with multiple members of the MMP family that they inhibit ECM degradation by MMPs and have anti-metastasis function[18]. TIMP-2 inhibits active and proenzyme forms of MMPs. It was reported that transfection of TIMP-2 gene into human gastric cancer cell line SGC-7901 by lipofection technology reduced their invasion potential *in vivo* and *in vitro*[19]. The expression of type IV collagenase in the untransfected group was higher than in the transfected group. The high expression of TIMP-2 may represent a mechanism by which tumor cells control the proteolysis and remodeling of ECM that occurs during invasion and progression of tumors. Another report suggested that transfection of TIMP-2 into the liver tissue could treat colorectal cancer liver metastasis[20]. However, some studies suggested that high expression level of TIMP-2 related to poor prognosis. It showed that high level of TIMP-2 mRNA correlated with the development of distant metastasis[21]. A study on gastric cancer showed that TIMP-1 and TIMP-2 were identified in 41% and 57% of tumors, respectively, whereas normal gastric mucosa was negative. No correlation was observed between the presence of TIMP-2 and tumor stage, histological type, LN status or survival[22]. However, our results demonstrated that there was correlation between the presence of TIMP-2 and depth of invasion and LN metastasis and different from MMP-9; there was no correlation between TIMP-2 and other clinical or histopathological features. Our results also suggested that as MMP-9 activity increased during tumor progression a corresponding increase in TIMP-2 level also occurred. Therefore, it appears that the expression of MMPs and TIMPs during tumor progression is coordinately regulated. Imbalance between MMP-9 and TIMP-2 could be an essential factor in tumor progression and may be more important than the concentrations of single components of MMPs and TIMPs[23,24]. The increased ratios could be interpreted as a sign of proteolytic inequilibrium in the early stage of carcinogenesis and in the advanced stage of tumor progression. We believed that the high expression of MMP-9 was the basis of tumor invasion and metastasis and that relatively high level of TIMP-2 was induced by MMP-9. Our results are contradictory to previous reports. It might be due to different study methods and antibodies used, and double effects of TIMP-2. TIMP-2 not only inhibits MMPs activities but also takes part in other activities such as cell growth and survival that cannot always be clearly reconciled with their abilities to abrogate MMP activity[25,26]. Therefore, further investigations are required to explore this controversy.

E-cadherin plays a critical role in the establishment and maintenance of intercellular adhesion, cell polarity, and tissue architecture. Abnormalities of E-cadherin adhesive system have been detected in a number of cancers. Loss of E-cadherin adhesive function has been found in premalignant conditions such as colorectal adenoma, Barrett's esophagus, and gastric dysplasia[27] and in tumors such as ductal carcinoma *in situ* of the breast, prostate carcinoma, and gastric carcinoma. In gastric carcinoma, the overall expression of E-cadherin ranges from 20% up to 90% due to different score systems and different antibodies used. The present study showed that the expression rates of E-cadherin in carcinoma and noncarcinoma were 42.4% and 87.5% respectively, suggesting that E-cadherin played a role in carcinogenesis and tumor progression. The downregulation of expression of E-cadherin may contribute to disruption of normal cell-cell adhesion in malignantly transformed cells, which may associate with tumor cells' enhanced migration and proliferation, leading to invasion and metastasis. It was also revealed that the expression of E-cadherin was significantly correlated with tumor differentiation and Lauren's classification. That is, abnormal expression rate was significantly higher in poorly differentiated (or diffuse-type) adenocarcinomas than in well-differentiated (or intestinal-type) ones, in another word, strong E-cadherin staining correlated with well-differentiated cancers and tumor cell cohesiveness, while loss of staining or changes in localization were associated with poorly differentiated, discohesive and highly invasive tumors. Multiple *in vitro* and *in vivo* studies demonstrated that a loss of E-cadherin expression correlated with the development of metastasis[28]. We proved that abnormal expression rate of E-cadherin was higher in LN metastasis positive cases than in negative ones. E-cadherin participates in the whole process from early stage to advanced stage of tumor development and in addition to its interaction with β-catenin, experimental work has also indicated that E-cadherin is involved in inducing cell-cycle arrest, at least partially, through upregulation of the cyclin-dependent kinase inhibitor, p27[29]. These interactions provide a direct link between the proliferative phenotype, presumed to be a precursor of neoplasia, and E-cadherin. It was reported[30] that the presence of paranuclear E-cadherin in cancer-associated benign epithelium suggested that alteration in E-cadherin molecule responsible for the paranuclear distribution might be an early change in gastric adenocarcinoma progression. Our result also showed the presence of paranuclear E-cadherin in cancer, especially in low-differentiated tumors (Figures 3B-D). E-cadherin has been associated with dedifferentiation, loss of adhesion and invasion in tumor cells, suggesting a role for loss of E-cadherin expression in the acquisition of potentially invasive and metastatic phenotype. Despite some contrary results, the bulk of evidence from clinical studies suggests that loss of E-cadherin is linked to prognosis and has been found to be an independent predictor in patients with various cancers[31]. Level of serum soluble E-cadherin is a potentially valid prognostic marker for gastric cancer and high concentration predicts advanced stage and indicates palliative/conservative treatment[32]. However, the reasons that E-cadherin showed strong expression in

inflammatory breast cancer are still under investigation[33].

Correlation between E-cadherin and MMP-9

It was reported that MMP-9 degraded membranous E-cadherin and caused an increase of serum soluble E-cadherin fragment which could upregulate the level of MMPs, forming a vicious cycle[34]. The present study showed that expression of E-cadherin and MMP-9 was reversely or negatively correlated, supporting previous findings. MMP-9 and E-cadherin interact and accelerate tumor progression. Llorens[35] investigated the correlation between MMP-9 and E-cadherin from another perspective, and detected that the activity of enhancer for MMP-9 was upregulated in rat skin carcinoma HaCa[Ecad(-)] cell line, accompanied with upregulation of the expression of MMP-9 mRNA and MMP-9 protein, but was downregulated in HaCa[Ecad(+)] cell line. The expression of MMP-9 protein and its activity were upregulated while signal transmission was blocked by cDNA for anti-E-cadherin monoclonal antibody. E-cadherin regulates MMP-9 at transcriptional level, which aggravates tumor invasion and metastasis. From this point of view, E-cadherin seems to be an initiating factor and more important than MMP-9. The reason may lie in Snail or SIP1, because during HCC progression, Snail/SIP1 directly represses E-cadherin gene transcription and activates cancer invasion via upregulation of MMP gene family[36].

In brief, E-cadherin and MMP-9 form a malignant cycle and detecting the levels of those two molecules simultaneously would have important significance for predicting prognosis[37]. However, some scholars reported that E-cadherin down-regulated MMPs in high-invasive bronchogenic carcinoma cell line[38], which is controversial to our report.

Correlation between E-cadherin and TIMP-2

According to our investigation, a positive correlation existed between MMP-9 and TIMP-2 and negative correlation between MMP-9 and E-cadherin, thus there is supposed to be a negative correlation between E-cadherin and TIMP-2. E-cadherin and TIMP-2 genes both belong to tumor suppressor gene (TSG)[39], however we did not find any association between them. It may be limited by our experimental method and further investigation is required.

DNA ploidy analysis

Normal human cells undergo a series of sequential changes culminating in mitotic division. These complex changes can be roughly identified using DNA measurements in which the cell population can be divided into three basic cell-cycle compartments: G_1, S, and G_2+M. Figure 4A shows typical DNA distribution of normal cells. The first peak on the left represents the G_0/G_1 population and the smaller peak to the right represents the G_2+M populations. The S phase cells are residing in between these two peaks. In contrast to normal tissues, neoplastic lesions often undergo chromosomal aberrations resulting in the appearance of nondiploid (aneuploid) clones within the tumor population and/or an increase in the proportion of S-phase cells. Numerous investigations suggested that slight changes of DNA content would lead to malignant tumors. Therefore, detecting DNA ploidy and content would be of great value for diagnosis of malignant tumors. Recent studies showed that DNA content of carcinoma cells is an independent prognostic factor[40-42]. According to our investigation result that since SPF was significantly correlated with tumor size, differentiation, LN metastasis, and depth of invasion, we conclude that percentage of cells synthesizing DNA (S phase cells) is an indirect reflection of tumor proliferation, hence aggressive behavior. However, some scholars reported that SPF was not correlated with tumor differentiation. Normal or near-normal DNA modes often correlate with a high degree of histological differentiation and abnormal DNA modes with low degree of histological differentiation. In carcinoma aneuploid rate was 61.7%, much higher than in noncarcinoma (16.7%). These results indicate that the DNA pattern is a valuable predictor of early stage of cancer and prognosis.

In summary, with tumor progression and development of heterogeneity, the abnormal expressions of MMP-9, TIMP-2 or E-cadherin or the rate of DNA aneuploidy or high SPF gradually increase correspondingly, suggesting that they play a crucial role in gastric carcinoma progression and can be objective markers for biological behaviors of gastric carcinoma. Furthermore, these factors correlate with one another, accelerating the process of tumor progression. In early stage of cancer or precancerous lesion, we may detect those factors to prevent tumor development and improve prognosis.

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