• GASTRIC CANCER •

Expression of heparanase gene, CD44v6, MMP-7 and *nm23* protein and their relationship with the invasion and metastasis of gastric carcinomas

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Abstract

AIM: To explore the expression of heparanase gene, CD44v6, MMP-7 and *nm23* proteins in human gastric carcinoma as well as their relationship with the clinicopathological factors.

METHODS: Reverse transcription polymerase chain reaction (RT-PCR) was used to measure the expressions of heparanase mRNA in 43 human gastric carcinomas and 10 adjacent normal gastric tissues. Streptavidin-peroxidase (S-P) two step method was used to measure the immunohistochemical expression of CD44v6, MMP-7 and *nm23* protein in 43 human gastric carcinomas.

RESULTS: The expression of heparanase gene was positive in 29 cases of gastric cancer with a positive rate of 67.4%, which was significantly higher than those in adjacent normal gastric tissues ($P<0.05$). The heparanase mRNA expression was significantly related to advanced stage of disease, serosal infiltration, lymph node metastasis and size of tumors ($P<0.05$), but not related to tumor location, gross and histological types of the cancer, peritoneal dissemination and liver metastasis ($P>0.05$). The positive rate of CD44v6, *nm23* and MMP-7 proteins was 76.7%, 37.2% and 60.5%, respectively. The CD44v6 protein expression was significantly related to serosal infiltration, lymph node metastasis and TNM stage of disease ($P<0.05$). The *nm23* protein expression was significantly related to the tumor gross appearance, lymph node and peritoneal metastasis ($P<0.05$). The MMP-7 protein expression was significantly related to serosal infiltration and TNM stage of disease ($P<0.05$). In an attempt to measure the association between these agents, we found that the expression of heparanase mRNA had significantly negative correlation with the expression of CD44v6 and MMP-7 protein ($P<0.05$), the expression of MMP-7 protein had significantly positive correlation with the expression of CD44v6 protein ($r=0.568$, $P<0.05$), the expression of MMP-7 protein had no correlation with the expression of *nm23* protein ($P>0.05$), and the expression of *nm23* protein had no correlation with the expression of CD44v6 protein ($P>0.05$).

CONCLUSION: Despite their different mechanisms of action, heparanase, CD44v6 and *nm23* may play important roles in the invasive infiltration and lymph node metastasis
in gastric carcinomas. Instead of metastatic spreading, MMP-7 may be just related to the invasion of gastric carcinomas. However, co-detection of these factors may be important to predict metastatic spreading in such patients.

INTRODUCTION

Invasion and metastasis of gastric carcinomas involve many processes including adherence, degradation, motility, and angiogenesis and escaping from immune surveillance. However, the penetration through the barrier formed by the basement membrane (BM) and extracellular matrix (ECM) is critical for invasion and metastasis of cancerous cells. This barrier is mainly composed of structural proteins and glycosaminoglycans. The former mainly includes collagen, laminin and fibronectin, whereas the latter chiefly consists of heparan sulfate proglycans (HSPGs) which is formed by a core protein and several covalent-binding heparan sulfate (HS) side chains. In the past decade, the damage of structural proteins has been considered to be a critical step for tumors' invasion and metastasis[1-3]. Previous researches mostly focused on property of some enzymes such as matrix metalloproteinases (MMPs) which use structural proteins as substrates. However, we should never neglect the effect of hydrolytic enzymes which use HSPGs as substrates, as well as their roles in degrading the BM and ECM. Heparanase is an endoglycosidase cloned out by Vlodavsky *et al* in 1999, which degrades the HS chain of HSPGs[1,2]. Recently, protein or messenger RNA (mRNA) expression of heparanase has been identified in various cancer cells, and the overexpression of heparanase protein or mRNA in tumour cells has been reported to correlate with the metastatic potential of tumour cells *in vitro* and *in vivo* as well as with poor prognosis[4-7]. However its role in gastric carcinomas is still not clearly clarified. In the present study, the expression of heparanase mRNA in gastric carcinomas and its correlation with clinicopathological features were investigated.

nm23, a metastasis suppressor gene, was reported to be associated with the tumor invasion and metastasis[8,9]. CD44v6, a highly glycosylated cell surface protein, was reported to be involved in cell-cell and cell-matrix interactions and thought to take part in cell motility, tumor growth, and invasion[10]. MMP-7, a member of matrix metalloproteinases family, was reported to play an important role in the degradation of connective tissue which is associated with the development of tumor metastases[11]. However, their roles in gastric carcinomas have not been clearly illustrated. In current study, CD44v6, *nm23* and MMP-7 protein expression in gastric carcinomas and their relationship with each other were also investigated.

MATERIALS AND METHODS

Patients

Between Oct 2002 and May 2003, 43 patients with gastric carcinoma underwent radical gastrectomy (D2 or D3), as subtotal or total gastrectomy in the Department of Gastrointestinal Surgery at the First Affiliated Hospital of Sun Yat-sen University. The age and sex of the patients, as well as the location, macroscopic type, histological grade, stage and depth of the invasion of the tumor, histological lymph node metastasis, and type of surgical procedures were retrieved from the patients' records. Pathological diagnosis and classifications were done according to the UICC standard published in 1997. Histological grouping based on UICC TNM classification was confirmed by histological examination.

mRNA extraction

The gastric tissues were obtained from those duly informed patients whose consents were obtained for the use of their subsequent resected tissues. The present study conformed to regulations of the Ethic Committee of the First Affiliated Hospital of Sun Yat-sen University. Tissue samples of approximately 1 g were collected immediately after each gastrectomy. Non-cancerous gastric tissues were obtained from regions distant from the tumours. Half of the both cancerous and non-cancerous tissues were fixed in 40g/L buffered formaldehyde and embedded in paraffin. Sections (4 mm thick) were prepared with haematoxylin-eosin staining for histopathological diagnosis and with immunohistochemical staining. The other half of the tissues was stored in RNAlater (Sigma, USA) at 4 ℃ overnight, then transferred to a clean freezing tube and stored at -80 ℃ for mRNA extraction. Before starting the study, histopathological examination had confirmed that no cancerous cells had contaminated the non-cancerous gastric tissues. Total RNA from tissues was isolated using RNeasy Mini Kits (Qiagen, USA) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from 2 μg of total RNA in a 25 μL reaction mixture. The 25 μL reaction mixture contained 2 μg RNA solution, 1 μL oligo d(T)18 (0.5 μg/μL), 1 μL M-MLV reverse transcriptase (Promega,USA), 1 μL RNasin ribonuclease inhibitor (Promega, USA) and 2 μL dNTP. The integrity of RNA was checked electrophoretically and quantified spectrophotometrically.

Real-time reverse transcription[6,12,13]

The following primers such as forward primer 5' -TTC GAT CCC AAG AAG GAA TCA AC-3' and reverse primer 5' -GTA GTG ATG CCA TGT AAC TGA ATC-3' were used for heparanase. The PCR reaction was run for 40 cycles under the following conditions: denaturation at 94 ℃ for 3 min, denaturation at 94 ℃ for another 30 s, annealing at 50 ℃ for 30 s and extension at 72 ℃ for 1 min with an extra 10-min extension for the last cycle. We determined the nucleotide sequence of this PCR product and confirmed that it was identical to the expected fragment of cDNA of heparanase. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts were monitored as a control to quantify the transcripts of the genes in each sample. GAPDH-specific sequences were amplified using the forward primer 5' -CAG GGG GGA GCC AAA AGG-3' , reverse primer 5' - GGC AGT GGG GAC ACG GAA- 3' , which yielded a 378-bp product. After completion of these amplification cycles, 10 μL of each PCR product was electrophoresed at 50-80V for 30-40 minutes on a 10g/L agarose gel in Tris boric acid EDTA buffer together with 100 bp DNA ladder marker (TaKaRa Biotechnology(Dalian) Co.,Ltd) and was stained with ethidium bromide.

Immunohistochemistry

Streptavidin-peroxidase (S-P) two step method was used for the immunohistochemical detection of CD44v6, *nm23* and MMP-7 proteins. Immunohistochemical detection of CD44v6, *nm23* and MMP-7 was performed with monoclonal antibodies (Maixin Biological, Fuzhou, China) against CD44v6, MMP-7 and *nm23*/NDP kinase.

The sections were first washed for 3 min with phosphate buffered saline (PBS) 3 times and then blocked with a solution of 30mL/L hydrogen peroxide in ethanol for 10 min at room temperature. After that they were immersed in 30mL/L normal horse serum for 10 min also at room temperature. Primary antibodies to CD44v6, *nm23* and MMP-7 were incubated for 1 h at room temperature. The ultrasensitive S-P kit (Maixin Biological, Fuzhou, China) was used to detect the resulting immune complex. The procedures of blocking, linkage, and labeling of binding reaction were carried out according to manufacturer's instructions. The peroxidase activity was visualized by DAB kit (Maixin Biological, Fuzhou, China), the sections were finally counterstained with hematoxylin and the negative control was processed by incubation with non-immune rabbit IgG in substitution for the primary antibody.

To evaluate the expression of CD44v6, only cell membrane staining equivalent to normal control was considered positive, while for *nm23* and MMP-7, only cytoplasmic staining equivalent to normal control was considered positive. Slides were examined and scored by two pathologists ignorant of the clinical details. Those slides exhibiting diffuse immunostaining or presenting more than 50% of tumor cells were classified as (++), more than 10% but less than 50% as (+) and those with immunoreactivity less than 10% were classified as (–). (+) and (++) were combined together as positive cases for statistical analysis.

Statistics

Statistical analyses were done with the SPSS 10.0 for Windows program. Frequency tables were analyzed with the Pearson Chi-square test or Fisher's exact test. The relationships between heparanase and *nm23* and CD44v6 and MMP-7 in gastric tumor tissues were evaluated statistically using the Kendall rank correlation test. Statistical differences with *P* values less than 0.05 by two-tailed tests were considered significant.

RESULTS

Clinical findings

The subjects included 26 men and 17 women. Their average age at the time of surgery was 58.5±13.4 years (ranging from 29 to 86 years). Ten cases of gastric cancer located at the upper third of the stomach, 7 at the middle third and 26 at the lower third. Of these 43 patients, 3 had peritoneal metastasis, 2 had liver metastasis, 35 had serosal invasion, and 27 had lymph node metastasis. Histopathological diagnoses of the patients were made according to the guidelines for classification of the primary gastric cancer. Thirty patients were classified as adenocarcinoma, 5 as mucinous carcinoma and 8 as signet-ring cell carcinoma. For histological differentiation, 4 were well differentiated, 10 were moderate differentiated and 29 were poorly differentiated. For TNM staging, 6 patients belonged to stage I, 8 belonged to stage II, 13 belonged to stage III, and 16 belonged to stage IV. None of the patients had received preoperative chemotherapy.

Relationship between heparanase gene expression and clinicopathological features in gastric carcinomas

The expression of heparanase mRNA was positive in 29 cases of gastric cancer with a positive rate of 67.4%. While its expression rate in the 10 cases of non-cancerous gastric tissues

was 10.0% (1 case). The difference was statistically significant ($P<0.05$) (Figure 1).
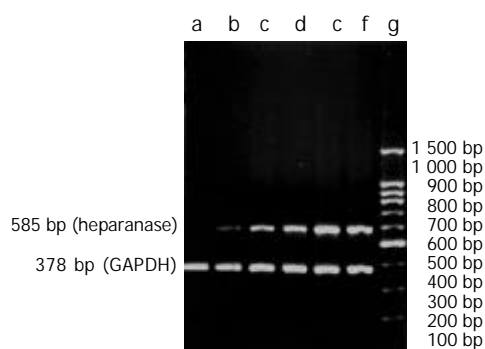

Figure 1 Expression of heparanase mRNA in gastric carcinomas. a: non-cancerous tissue, b-f: cancerous tissue, g: marker (100 bp DNA ladder marker).

From Table 1, we could find that the mRNA expression of heparanase was not correlated with tumor location, gross appearance, histological characteristics, peritoneal dissemination and liver metastasis ($P>0.05$). It was correlated with tumor size, serosal infiltration, lymph node metastasis, distant metastasis and TNM staging of gastric carcinomas ($P<0.05$). In 14 cases of negative expression of heparanase mRNA, only 5 cases had lymph node metastasis. However, in 29 cases of positive expression of heparanase mRNA, twenty-two had lymph node metastasis, among which N_1 was in 11 cases, N_2 in 3 cases and N_3 in 8 cases. The difference between these two groups was statistically significant ($P<0.05$).

Relationship between CD44v6, nm23 and mmp-7 protein expression and clinicopathological features in gastric carcinomas

The expression of CD44v6 protein was positive in 33 cases of gastric cancer with a positive rate of 76.7% and the intensity of its immunoreactivity in cancer tissue was scored as (+) in 27

Table 1 Relationship between clinicopathological features and heparanase mRNA expression and CD44v6, *nm23* and MMP-7 protein expression in primary gastric carcinomas

| Clinicopathological features	Number of patients											
	Heparanase			CD44v6			*nm23*			MMP-7		
	(+)	(-)	*P* value	(+)	(-)	*P* value	(+)	(-)	*P* value	(+)	(-)	*P* value
Gender			0.307			0.481			0.280			0.415
Female	13	4		12	5		8	9		9	8	
Male	16	10		21	5		8	18		17	9	
Tumor location			0.216			0.346			0.903			0.804
Upper third	9	1		9	1		4	6		6	4	
Middle third	4	3		6	1		3	4		5	2	
Lower third	16	10		18	8		9	17		15	11	
Gross type			0.242			0.325			0.025			0.121
Borrmann I	1	0		1	0		0	1		1	0	
Borrmann II	4	5		5	4		7	2		5	4	
Borrmann III	21	9		25	5		9	21		20	10	
Borrmann IV	3	0		2	1		0	3		0	3	
Histologic type			0.671			0.297			0.444			0.325
Adenocarcinoma	19	11		23	7		13	17		20	10	
Mucinous carcinoma	4	1		5	0		1	4		3	2	
Signet-ring cell carcinoma	6	2		5	3		2	6		3	5	
Peritoneal metastasis			0.539			1.000			0.045			0.266
Absent	26	14		30	10		13	27		23	17	
Present	3	0		3	0		3	0		3	0	
Liver metastasis			1.000			1.000			0.133			0.511
Absent	27	14		31	10		14	27		24	17	
Present	2	0		2	0		2	0		2	0	
Histologic differentiation			1.000			0.704			0.888			0.101
Well/moderate	9	5		10	4		5	9		6	8	
Poorly	20	9		23	6		11	18		20	9	
Serosal invasion			0.009			0.010			0.101			0.042
Absent	2	6		3	5		5	3		2	6	
Present	27	8		30	5		11	24		24	11	
Lymph node metastasis			0.018			0.024			0.008			0.084
Absent	7	9		9	7		10	6		7	9	
Present	22	5		24	3		6	21		19	8	
Distant metastasis			0.039			0.656			0.125			1.000
Absent	21	14		26	9		11	24		21	14	
Present	8	0		7	1		5	3		5	3	
TNM stage			0.001			0.001			0.228			0.021
I-II	4	10		6	8		7	7		5	9	
III-IV	25	4		27	2		9	20		21	8	
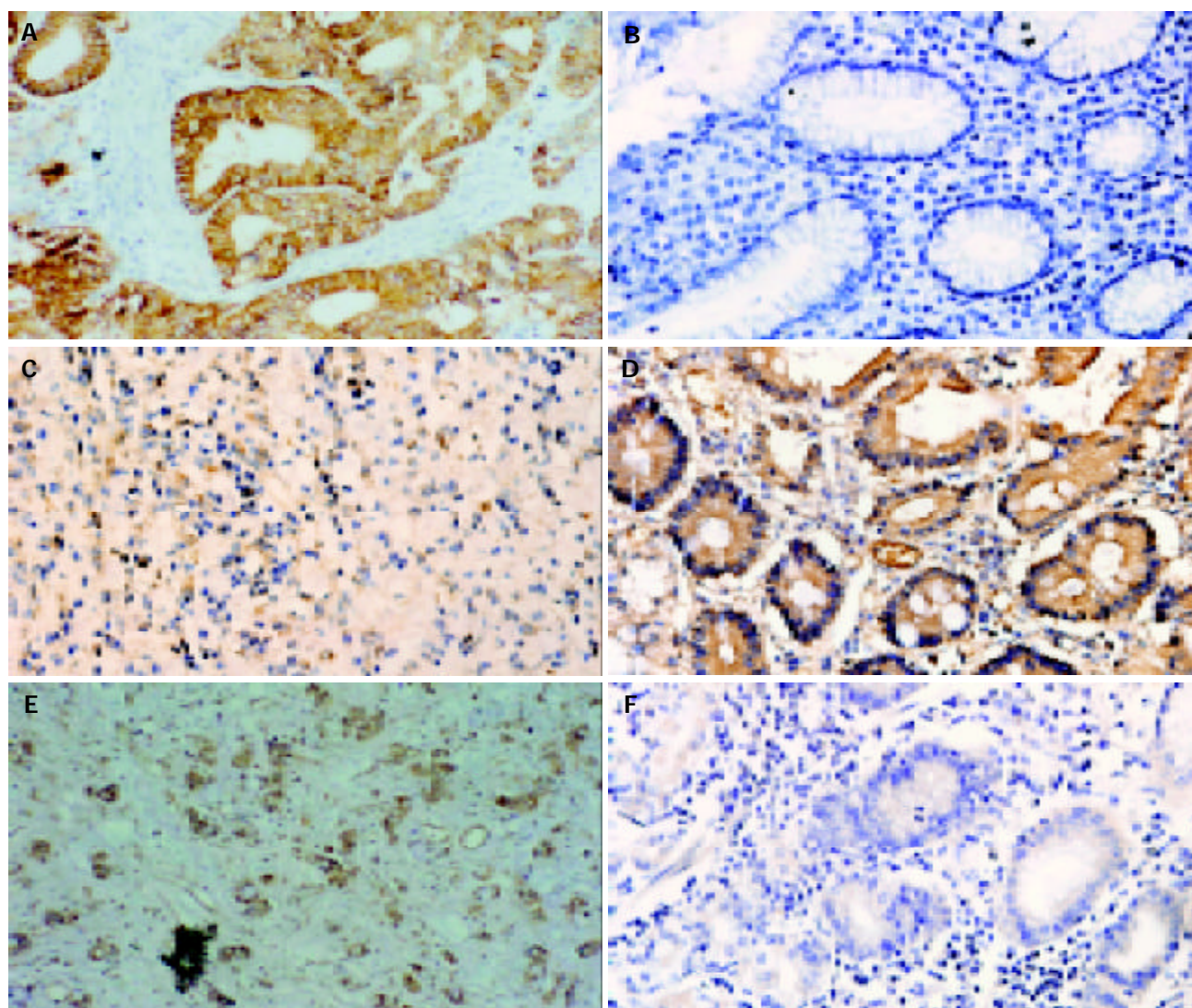
Figure 2 Expression of CD44v6, *nm23* and MMP-7 protein in gastric carcinomas and non-cancerous gastric tissues. A: gastric carcinoma stained for CD44v6(×400), B: non-cancerous gastric tissue stained for CD44v6(×400), C: gastric carcinoma stained for *nm23* (×400), D: non-cancerous gastric tissue stained for *nm23* (×400), E: gastric carcinoma stained for MMP-7 (×400), F: non-cancerous gastric tissue stained for MMP-7 (×400).

patients and (++) in 6 patients. The expression of *nm23* protein was positive in 16 cases of gastric cancer with a positive rate of 37.2% and the intensity of its immunoreactivity in cancer tissue was scored as (+) in 10 patients and (++) in 6 patients. The expression of MMP-7 protein was positive in 26 cases of gastric cancer with a positive rate of 60.5% and the intensity of its immunoreactivity in cancer tissue was scored as (+) in 20 patients and (++) in 6 patients (Figure 2).

The expression of CD44v6 protein was significantly correlated with lymph node metastasis, TNM staging and serosal infiltration ($P<0.05$). The expression of *nm23* protein was significantly correlated with the tumor gross appearance, peritoneal metastasis and lymph node metastasis ($P<0.05$). The expression of MMP-7 protein was significantly correlated with serosal invasion and TNM staging (Table 1).

Correlation between heparanase mRNA expression and CD44v6, nm23 and MMP-7 protein expression

The expression of heparanase mRNA had significant negative correlation with the expression of CD44v6 and MMP-7 protein ($P<0.05$) (Table 2). The expression of MMP-7 protein had significant positive correlation with the expression of CD44v6 protein ($\gamma=0.568$, $P<0.01$). The expression of MMP-7 protein had no correlation with the expression of *nm23* protein ($P>0.05$).

The expression of *nm23* protein had no correlation with the expression of CD44v6 protein ($P>0.05$).

Table 2 Correlation between the expression of heparanase mRNA and the expression of CD44v6, *nm23* and MMP-7 protein

Items	Number of cases	Heparanase (+)	Correlation coefficient	*P* value
CD44v6			-0.440	0.004
(-)	10	3		
(+)	33	26		
Nm23			-0.021	0.889
(-)	27	18		
(+)	16	11		
MMP-7			-0.352	0.023
(-)	17	8		
(+)	26	21		

DISCUSSION

In normal human tissues, heparanase mRNA is chiefly distributed in placenta and lymphatic tissues. Contrarily in the connective tissues and most of the epithelial cell, its expression is rare or even absent[1-3]. Vlodavsky *et al.*[1] confirmed that

heparanase could promote the metastasis of tumor by gene transfection experiments. They transfected non-metastatic Eb cells with a full-length human heparanase cDNA using a pcDNA3 expression plasmid. As a result, the transfected cells expressed high levels of heparanase mRNA and high heparanase activity. Ninety percent of the DBA/2 mice injected with heparanase-transfected Eb cells died by d 34, whereas 60% of the mice inoculated with mock-transfected cells were alive at that time. The livers of mice inoculated with hepranase-transfected cells were infiltrated with numerous Eb lymphoma cells. In contrast, metastatic lesions could not be detected by gross examination in the liver of the mice with mock-transfected and control Eb cells. Few or no lymphoma cells infiltrated the liver tissue. Furthermore, transient transfection of the heparanase gene into B16-F1 mouse melanoma cells with low metastatic potential followed by intravenous inoculation resulted in a 400-500% increase in lung metastases. All these implied that heparanase might play an important role in tumor invasion and metastasis.

In the present study, the positive rate of heparanase mRNA expression in gastric carcinomas (67.4%) was significantly higher than that of non-cancerous gastric tissues (10%) (*P*<0.05). Heparanase mRNA tended to express more in those patients with serosal invasion, lymph node metastasis, advanced stage of diseases, distant metastasis and larger size of tumors (*P*<0.05). The results indicated that heparanase mRNA was closely related with the clinicopathological features that reflected the invasion and metastatic potential and prognosis. Up to now, invasion depth, lymph node metastasis or distant metastasis and TNM stage were considered to be the prognostic factors for gastric carcinomas[14,15]. That heparanase mRNA expression statistically related with these factors implied that the invasion and metastasis induced by heparanase might indicate a poor prognosis of the gastric carcinomas. This needs to be confirmed by further survival analysis.

According to the researches on other kinds of carcinomas, the mechanisms by which heparanase accelerates invasion and metastasis can be summarized as the following two points. First, heparanase is responsible for degrading HSPGs side chain HS which is the chief component of the BM and ECM but not degraded by MMPs. Second, the degradation of HSPGs by heparanase leads to the release of various growth factors and cytokines trapped in HSPGs which regulate the angiogenesis, tissue repair, inflammation and lipid metabolism[12,16,17]. In our study, we observed that heparanase mRNA expression often occurred in patients with serosal infiltration and advanced stage of disease, which might imply the important role of heparanase in damaging and penetrating the BM in gastric carcinomas.

The metastasis in gastric carcinomas occurs not only through lymphatic and hematogenous ways but also by direct infiltration. It is still unknown of the definite roles of heparanase in the metastasis of gastric carcinomas. We found none of the patients with liver metastasis expressed heparanase mRNA, while those with lymph node metastasis were significantly related with the expression of heparanase mRNA (*P*<0.05). We supposed that heparanase was involved in lymphatic metastasis of gastric carcinomas instead of hematogenous metastasis. To draw a further conclusion, larger sample investigations on gastric carcinoma are needed, since the cases with liver metastasis in our study were few. However, Endo *et al*[12] found there was no correlation between heparanase expression and lymph node metastasis in gastric carcinomas. In their study, 65%(20/31) patients with positive expression of heparanase mRNA had venous metastasis, compared with 47%(15/32) in negative mRNA expression. They thought heparanase was involved in hematogenous metastasis of gastric carcinomas instead of lymphatic metastasis.

The *nm23* was the first identified metastasis suppressor gene. In 1988, Steeg *et al*.[18] discovered murine *nm23* cDNA by using differential colony hybridization between murine K-1735 melanoma cell lines that varied in metastatic potential *in vivo*. They found that the *nm23* mRNA levels of two low metastatic potential cell lines were quantitatively higher than that of five related but highly metastatic cell lines. Later, the examination of protein levels exhibited a similar pattern[19]. This discovery aroused much interest in the role of *nm23* in progression of carcinomas. Up to now, eight members of the human *nm23* family have been reported and are found in multiple subcellular compartments. Homologs in other species are known as nucleoside diphosphate kinases (NDP kinase or ndpk) or, in Drosophila, as abnormal wing discs (awd)[20]. Two highly homologous genes have been described so far, both located at the long arm of chromosome 17, coding for the 18.5 and 17 kD proteins *nm23*-H1 and *nm23*-H2, respectively. The *nm23*-H1 and *nm23*-H2 gene products have been shown to be identical to human nucleoside diphosphate (NDP) kinases A and B[19]. *nm23* has been considered as an antimetastatic gene for most malignancies, but the role of *nm23* gene in gastric carcinomas is still not completely known.

In our study, we found that *nm23* had a significant association with lymph node metastasis and peritoneal dissemination. In 27 patients with lymph node metastasis 21 were *nm23* negative, while in 16 patients without lymph node metastasis only 6 were *nm23* negative. The difference was statistically significant (*P*<0.01). In 40 patients without peritoneal metastasis, 27 were *nm23* negative, but in 3 patients with peritoneal metastasis all were *nm23* positive. The difference was also statistically significant (*P*<0.05). It indicates that *nm23* suppresses lymphatic metastasis and peritoneal metastasis in gastric carcinomas. Our study also demonstrated that decreased expression of *nm23* was related to tumor's gross type. Other researches showed similar results and proposed *nm23* was related to 5 year survival of the patients[21]. However Yoo *et al*.[22] found there was no significant difference in 5-year survival rates between patients with *nm23*-positive and *nm23*-negative tumors (*P*>0.05) and denied *nm23* to be a predictor of outcome of patients with gastric carcinomas.

CD44 is a highly glycosylated cell surface protein. In normal tissues, CD44 is expressed in a variety of epithelial and mesenchymal cells, as well as in blood cells and glial cells of the central nervous system[10,23]. Tremendous interest in CD44 was generated when Gunthert and colleagues conferred metastatic potential on a non-metastatic cell line by transfecting a variant of CD44[23, 24]. CD44 has two isoforms, one has been termed CD44 standard (CD44s) and the other CD44 variant (CD44v). The mRNA of CD44s contains no variant exons, while CD44v may contain one or more variant regions, such as CD44v6 or CD44v3-v7. CD44v6 now is considered to be involved in cell-cell and cell-matrix interactions and take part in cell motility, tumour growth, invasion and metastasis[10,23,25].

Our results demonstrated that CD44v6 was significantly related to lymph node metastasis, serosal invasion and TNM stage. Twenty-four out of 27 patients with lymph node metastases were CD44v6 positive, while 9 out of 16 patients without lymph node metastasis were CD44v6 positive. The difference was statistically significant (*P*<0.05). Twenty-seven out of 29 stage III and IV patients were CD44v6 positive but only 6 out of 14 stage I and II patients were CD44v6 positive. Their difference was also statistically significant (*P*<0.05). These indicated that CD44v6 might be a useful predictor of lymph node metastasis in gastric carcinomas. Joo *et al*.[26,27] obtained almost the same results as ours. But Yamaguchi *et al*.[28] tended to consider CD44v6 protein to have an important role in hematogenous metastasis of gastric carcinomas.

MMPs are a family of zinc-dependent endoproteinases whose enzymatic activity is directed against components of

the ECM. Up to now, 24 different vertebrate MMPs have been identified, of which 23 are found in humans. On the basis of substrate specificity, sequence similarity, and domain organization, vertebrate MMPs can be divided into six groups: collagenases; gelatinases; stromelysins; membrane-type MMPs; matrilysins and other MMPs. MMP-7 is also called matrilysin[29,30]. The mechanism of MMP-7 in gastric carcinomas is still unclear. Our study demonstrated that only serosal invasion and advanced stage of disease were closely related to MMP-7 protein expression and it had no correlation with other clinicopathological features. In 26 MMP-7 positive cases, 24 cases had serosal invasion while for 17 MMP-7 negative cases, only 11 cases had serosal invasion ($P<0.05$). Yonemura *et al*.[31] found MMP-7 had not only significant positive correlation with serosal involvement but also with lymph node metastasis, poor differentiation and peritoneal dissemination. Patients with MMP-7-positive tumor had significantly poorer survival and more frequently died of peritoneal recurrence than did those with MMP-7-negative tumors. So MMP-7 was considered to be a good indicator of peritoneal dissemination. This was confirmed later by the study using MMP-7-specific antisense oligonucleotide which could inhibit peritoneal dissemination in human gastric cancer[32]. However, our study did not show any correlation between MMP-7 expression and peritoneal metastasis.

According to the studies on other kinds of carcinomas, MMPs facilitate tumor cell invasion and metastasis by at least three distinct mechanisms. First, proteinase removes physical barriers to invasion through degradation of ECM macromolecules such as collagens, laminins, and proteoglycans. Second, MMPs have the ability to modulate cell adhesion. Finally, MMPs may act on ECM components or other proteins to unkown hidden biologic activities[11]. Liu *et al*.[33] investigated immunohistochemically MMP-7 expression in 214 gastric carcinomas and found MMP-7-positive tumor cells were preferentially found in deeply invading nests, especially at the invasive front. The mean MMP-7 labeling index (LI) at the invasive front was significantly higher in tumors invading or penetrating the muscularis propria and in stages II - IV than within the submucosal layer and in stage I, respectively ($P<0.001$). Our study also showed serosal invasion and advanced stage of disease were closely related to MMP-7 protein expression. Liu *et al*[34] also found Ki-67 antigen, an indicator of cell proliferation, was absent in MMP-7 positive tumor cells and vice versa and concluded that MMP-7 expression was related inversely with proliferative activity of tumor cells. So it could be deduced that MMP-7 might participate in the penetration of BM and ECM in gastric carcinomas instead of promoting cell proliferation. Recently, two studies demonstrated that MMP-7 upregulation was closely related with the *H pylori* cag pathogenicity island and this implied a certain role of MMP-7 in carcinogenesis of gastric carcinomas[35,36].

Invasion and metastasis of gastric carcinomas are a multistep process. As far as we know, the relationship between heperanase, CD44v6, *nm23* and MMP-7 has not been reported. In our study, we found the expression of heparanase mRNA had significant negative correlation with the expression of CD44v6 and MMP-7 protein. Although heparanase and MMPs both acted on BM and ECM, their substrates were different. Heparanase mainly used HS as substrate while MMPs mainly used structural proteins as substrates. This might account for the negative correlation between the expression of heparanase mRNA and MMP-7 protein. But what exactly caused this is worthy of further studying. That the expression of MMP-7 protein had significant positive correlation with the expression of CD44v6 protein further confirmed that MMPs have the ability to modulate cell adhesion. Why there was no correlation

between the expression of MMP-7 and *nm*23 protein? Might it be caused by their different mechanisms? Further studies will be needed to elucidate their relationship in tumors.

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