

Correlative studies on bFGF mRNA and MMP-9 mRNA expressions with microvascular density, progression, and prognosis of gastric carcinomas

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Abstract

AIM: To investigate the mRNA expressions of bFGF and MMP-9 in gastric carcinomas so as to reveal their correlations with tumor microvascular density (MVD), invasion, metastasis, and prognosis.

METHODS: *In situ* hybridization and immunohistochemical techniques were used to detect the expressions of bFGFmRNA and MMP-9mRNA and the proteins of CD34 in 105 specimens of gastric carcinomas.

RESULTS: *In situ* hybridization study showed that positive rates of bFGF mRNA and MMP-9mRNA expressions were 60.95% and 59.19%; the mean MVD was 46.09 ± 11.52 and 43.75 ± 13.41 , respectively in piece/0.72 mm² in tumors with bFGFmRNA and MMP-9mRNA positive expressions, which were significantly higher than those with negative expression (29.41 ± 12.47 ; 33.45 ± 13.92 piece/0.72 mm², respectively). The positive expression rates of bFGFmRNA and MMP-9mRNA were correlated to the tumor invasion depth ($r_s = 0.211, P = 0.031$; $r_s = 0.335, P = 0.001$), growing pattern ($r_s = 0.324, P = 0.001$; $r_s = 0.267, P = 0.006$), vessel invasion ($r_s = 0.579, P = 0.001$; $r_s = 0.209, P = 0.032$), lymph node metastasis ($r_s = 0.405, P = 0.001$; $r_s = 0.343, P = 0.001$) and distant metastasis ($r_s = 0.474, P = 0.001$; $r_s = 0.468, P = 0.001$), but not correlated to tumor type ($r_s = 0.134, P = 0.173$; $r_s = 0.103, P = 0.145$) and differentiations ($r_s = 0.096, P = 0.332$; $r_s = 0.102, P = 0.298$). The mean MVD was much higher in the tumors with infiltrating growth at stage T₃-T₄, with vessel invasion, lymph node metastasis and distant metastasis than those with expanding growth type ($t = 10.105, P = 0.001$) at stage T₁-T₂ ($t = 5.961, P = 0.001$), with non-vessel invasion ($t = 7.394, P = 0.001$), non-lymph node metastasis ($t = 3.819, P = 0.01$) and non-distant metastasis ($t = 10.578, P = 0.001$). Positive correlation was observed between MVD and the expressions of bFGFmRNA and MMP-9mRNA ($t = 3.207, P = 0.002$; $t = 7.035, P = 0.001$, respectively). The mean survival time and 5-year survival rate were

lower in cases with MVD over 39.5 and the positive expressions of bFGFmRNA and MMP-9mRNA than those with MVD less than 39.5 and the negative expressions of bFGFmRNA and MMP-9mRNA.

CONCLUSION: bFGF and MMP-9 promote the angiogenesis of the gastric cancers. Detection of the expressions of bFGF and MMP-9 can serve as a useful index to determine the angiogenesis, invasion, metastasis, and prognosis of gastric cancers.

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Key words: Gastric neoplasm; bFGF; MMP-9; Microvascular density; Prognosis

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INTRODUCTION

The growth of solid carcinomas depends on the angiogenesis. Induction of angiogenesis is the premise of the proliferation, infiltration and metastasis of carcinomas. Among all known regulating factors, bFGF is one of the most potent inducers of the angiogenesis of carcinomas^[1-7]. Matrix metalloproteinase-9 (MMP-9) expression and distribution is also being observed in carcinoma angiogenesis^[8-15]. In some epithelial carcinomas, MMP-9 can indirectly induce angiogenesis of carcinomas and is correlated with carcinoma progression. Microvascular density (MVD) is a quantitative index for carcinoma angiogenesis and could serve as a marker for carcinoma prognosis^[16-18]. Simultaneous study of bFGF and MMP-9 is of practical value to reveal the mechanism and progression of gastric carcinoma angiogenesis and its relationship to MVD using *in situ* hybridization and immunohistochemical techniques, we observed the expressions of bFGF and MMP-9 in gastric carcinomas so as to explore the relationship of the carcinoma angiogenesis with its filtration, metastasis, and other carcinoma biological behaviors as well as the prognosis.

MATERIALS AND METHODS

Patients and tumor tissues

One hundred and five gastric carcinoma samples were collected in the hospital from October 1986 to November

1998. All the samples were fixed with formaldehyde and paraffin embedded. Complete over 5-year follow-up data are available for all these cases (follow-up ended in October 2002). Survival rate that was calculated from the day of operation to the end of the follow-up or the date of death was used due to the recurrence and metastasis. bFGF and MMP-9 mRNA *in situ* hybridization and CD34 immunohistochemistry were carried out in all samples. The average age of the cases was 57.6 years (38-78 years) and the ratio of male to female was 2:1 (70:35). According to the classification standard of WHO in 1997, 17 cases had papillary adenocarcinomas while 37, 34, 9, and 8 had the tubular adenocarcinoma, poorly-differentiated adenocarcinomas, mucinous adenocarcinomas, signet-ring cell carcinomas. Highly and intermediately differentiated carcinomas were found in 63 cases while poorly and undifferentiated carcinomas in 42 cases. Forty-eight cases had expansive growth carcinomas while 57 cases had infiltrative growth carcinomas, and 20, 24, 32, and 29 cases had T1, T2, T3, and T4 carcinomas, respectively. Carcinomas with vascular invasion were found in 76 cases while non-vascular invasion carcinomas in 29 cases. Seventy cases had lymph node metastasis while 35 cases had no lymph node metastasis. Distant metastasis carcinomas were found in 42 cases (liver metastasis 18 cases, peritoneum metastasis 24 cases) while no distant metastasis in 63 cases. Twenty control samples were collected from the same gastric mucosa 5 cm away from the carcinoma tissues.

Histological treatment

In order to avoid the RNase contamination, all the glass slides, slide covers and stain containers were treated with 0.1% DEPC for 24 h. Gloves must be used when handling tissue cutting and 10% SDS was used to clean the cutter. All the sections were spread with 0.1% DEPC treated ddH₂O. The tissues were cut into 5-7 μ mol/L in thickness and kept at 4 °C, and foil covered for HE stain, immunohistochemistry and *in situ* hybridization.

Reagents

(1) bFGF probes: Digoxin label oligonucleotides bFGF probes were from Boshide Biological Technology Limited Company, Wuhan, China, No. MK1054. The sequences were 5'-GCCGT CGGG TGGAT GCGCA GGAAG AAGCC-3'; 5'-TTGAT AGACA CAACT CCTCT CTCTT CTGCT-3'; 5'-ACCGG TAAGT ATTGT AGTTA TTAGA TTCCA-3' (2) MMP-9 probes: Digoxin label nucleotides MMP-9 probes were from Boshide Biological Technology Limited Company, Wuhan, China, No. MK1540. The sequence were: 5'-TCCCT GCCCG AGACC GGTGA GCTGG ATAGC-3'; 5'-CAACT CGGCG GGAGA CTGT GCGTC TTCCC-3'; 5'-CCAGG TGGAC CAAGT GGGCT ACGTG ACCTA-3' (3) Immunohistochemical reagents: CD34 (mouse anti-human) and Sp kit were from Zhongshan Biotech Co., Beijing, China. The working concentration of CD34 was 1:100.

In situ hybridization

All the slides, cover-slips and other containers were autoclaved and treated with 0.1% DEPC ddH₂O for 24 h. All the buffers were also treated with 0.1% DEPC. The

tissues were routinely treated before *in situ* hybridization. DEPC (0.1%) treated ddH₂O was used to spread out the sections, and the moderate temperature was used for drying the sections, gradient ethanol was used for dehydration with 3% H₂O₂ incubation for 10 min at room temperature. Digestion was enabled with pepsin at 37 °C for 20 min, 0.5 mol/L PBS wash thrice at 5 min each, 20 mL hybridization was used for each group with Probes, sealed and incubated at wet chamber for 20 h at 45 °C. Then the slides were washed with 2×SSC for 5 min×3 min, and followed by 20 mL hybridization stabilization solution (2 mL A solution, 18 mL B solution) at 45 °C for hours incubation at wet chamber. Post-hybridization washing was employed with 2×SSC-0.05×SSC for 2 h, following which the slides were blocked with normal serum at 37 °C for 30 min. After directly adding mouse-anti-digoxin antibody for 1 h at 37 °C, slides were washed with 0.5 mol/L PBS for 5 min×3 min, then added SABC at 37 °C for 20 min and biotin-peroxidase at 37 °C for 20 min. At last, the slides were washed with 0.5 mol/L PBS for 5 min×3 min, stained with DAB for 10 min and counter-stained with hematein for 8 min. No probes hybridization solution and RNase treated sample served as negative controls.

Immunohistochemistry

SP method was adopted according to the manual including briefly, paraffin sectioning, antigen recovering, 3% H₂O₂ treatment to block endogenous peroxidase, goat serum for blocking nonspecific reaction, 1st and 2nd antibodies followed by streptavidin conjugated to horseradish with DAB as the substrate. The negative is PBS used as the 1st antibody, and the positive is from the kit.

Results evaluation

The cytoplasm of the bFGF and MMP-9mRNA appeared as brown in color. Two hundred cells were chosen under microscope to evaluate the stained cell number against the total cell number in the field. Based on the positive cell number, the criteria were set as follows: the negative (-) having the positive cell number <10% or without positive staining (-); (+) having 11-50% positive cells; (++) having 51-75% positive cells; and (+++) having >75% positive cells. The MVD was calculated in the carcinoma tissues as follows: first find the clear area under microscope with endothelial cells and carcinoma cells, good background contrast; among the carcinoma cells, the brown color staining single cell or endothelial cell plexus is considered as one vessel, then in ×200 fields, choose five unrepeated areas to count the vessel number CD 34 positive vessels were used as MVD expressed in mean±SD.

Statistical analysis

Statistical evaluation was performed using χ^2 test or Fisher's exact test to differentiate the rates of different groups, *t*-test was used to analyze quantitative data, rank sum correlation was analyzed with Spearman's test. The survival rate was estimated by the Kaplan-Meier method and analyzed by means of log-rank test. *P*<0.05 was considered statistically significant. SPSS 11.0 software for windows was employed to analyze all the data.

Table 1 Correlation of bFGF and MMP-9 mRNA expression and pathological parameters in 105 gastric carcinoma cases

Groups	n	bFGFmRNA				r_s	P	MMP-9mRNA				r_s	P
		-	+	++	+++			-	+	++	+++		
Growth pattern						0.324	0.001					0.267	0.006
Expansive	48	27	8	8	5			27	12	9	0		
Infiltrative	57	14	7	20	16			17	13	21	6		
Invasive depth						0.211	0.031					0.335	0.001
T ₁ -T ₂	44	31	5	5	3			26	11	6	1		
T ₃ -T ₄	61	10	10	23	18			18	14	24	5		
Vessel invasion						0.579	0.001					0.209	0.032
Absent	29	25	2	2	0			17	7	4	1		
Present	76	16	13	26	21			27	18	26	5		
Lymph node metastasis						0.405	0.001					0.343	0.001
Absent	35	25	4	4	2			21	6	7	1		
Present	70	16	11	24	19			23	19	23	5		
Distant metastasis						0.474	0.001					0.468	0.001
Absent	63	37	14	8	4			40	9	10	4		
Present	42	4	1	20	17			4	16	20	2		

RESULTS

bFGF mRNA expression and its correlation with pathological parameters of gastric carcinoma

Two (10%) of the 20 cases of non-cancer gastric mucosa had bFGF mRNA expression, and the light positive staining was mainly located in the cytoplasm of the epithelium around the neck area of gastric crypt, while 64 (61%) of the 105 cases of gastric carcinoma had positive expression with significant difference between the two groups (Figure 1, $\chi^2 = 9.25, P = 0.025$), The carcinoma cells had brown staining in the cytoplasm and invaded the muscular layer, peritoneum and greater omentum (Figure 1). According to the clinicopathological parameters of gastric

cancer progression, bFGFmRNA positive expression had different expressions in the different stages of the gastric carcinomas (Table 1). No correlation was found between bFGFmRNA positive expression and the histological types of the carcinomas ($r_s = 0.134, P = 0.173$), differentiation ($r_s = 0.096, P = 0.332$).

MMP-9mRNA expression and its correlation with gastric carcinoma pathological parameters

Among the 105 gastric carcinoma cases, 61 cases showed MMP-9mRNA positive expression cases where, accounting for 58.1%, negative result was found in normal gastric mucosa. The early stage of gastric carcinomas had positive MMP-9mRNA expression (Figure 2), and the positive

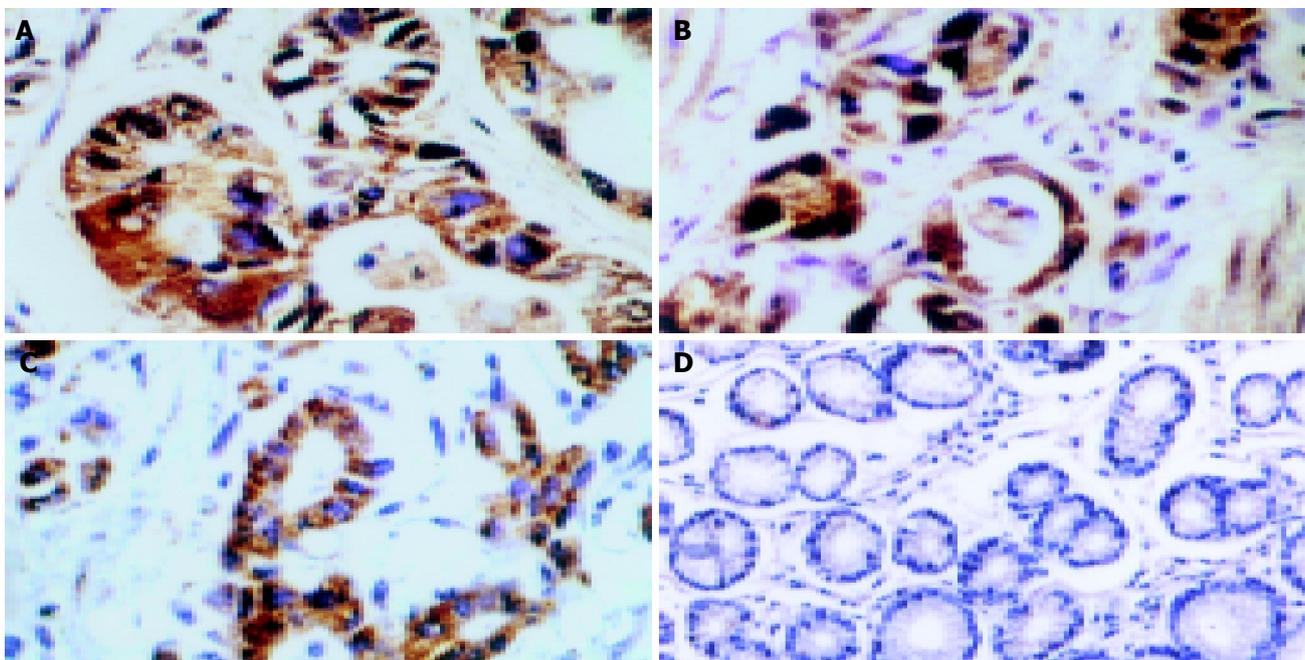


Figure 1 Expression of bFGFmRNA in gastric carcinoma tissues. **A:** Positive expression (+++) of bFGFmRNA in the plasma of gastric adenocarcinoma, ISH $\times 250$; **B:** Positive expression (++) of bFGFmRNA in the plasma of gastric adenocarcinoma, infiltrating stomach muscularis, ISH $\times 180$; **C:** Positive

expression (++) of bFGFmRNA in the plasma of gastric adenocarcinoma, infiltrating greater omentum, ISH $\times 250$; **D:** Negative expression of bFGF mRNA in normal gastric mucosa, ISH $\times 120$.

carcinoma cells were usually located near the edge of the carcinoma filtration areas, and the involved greater omentum and peritoneum were usually positive in MMP-9mRNA expression (Figure 2). The correlation between MMP-9mRNA positive expression and the pathological parameters of gastric carcinoma is shown in Table 1. No statistical correlation was revealed between MMP-9mRNA positive expression and the carcinoma types ($r_s = 0.103$, $P = 0.145$) and differentiation ($r_s = 0.102$, $P = 0.298$).

Correlation of MVD with gastric carcinoma pathological parameters (Table 2 and Figure 3)

Table 2 MVD in different gastric carcinoma pathological parameters (number/0.72 mm², mean±SD)

Groups	n	MVD	t	P
Tumor differentiation			0.965	0.337
High-moderate	63	38.33±14.83		
Low-undifferentiated	42	41.09±13.97		
Growth pattern			10.105	0.001
Expansive	48	34.70±15.23		
Infiltrative	57	43.42±12.64		
Invasive depth			5.961	0.001
T ₁ -T ₂	44	30.84±13.66		
T ₃ -T ₄	61	45.64±11.69		
Vessel invasion			7.394	0.001
Absent	29	25.69±10.11		
Present	76	44.68±12.33		
Lymph node metastasis			3.819	0.01
Absent	35	27.07±11.33		
Present	70	45.62±11.69		
Distant metastasis			10.578	0.001
Absent	63	31.30±12.97		
Present	42	52.19±6.42		

Correlation of MVD with bFGFmRNA, MMP-9mRNA expression (Table 3 and Figure 3)

Table 3 Correlation of MVD with bFGFmRNA, MMP-9mRNA expression in 105 cases of gastric cancer

Groups	n	MVD(number/0.72 mm ²)	t	P
bFGFmRNA			3.207	0.002
+----	64	46.09±11.52		
-	41	29.41±12.47		
MMP-mRNA			7.305	0.001
+----	61	43.75±13.41		
-	44	33.45±13.92		

Correlation of bFGFmRNA and MMP-9mRNA expression

Positive correlation was observed between bFGFmRNA and MMP-9mRNA expression ($r_s = 0.298$, $P = 0.045$).

Correlation of MVD, bFGF, and MMP-9mRNA expression with survival rate (Table 4 and Figure 4)

Table 4 Correlation of MVD, bFGF, and MMP-9mRNA expression with survival rate

Groups	n	Mean survival time (mo)	Five-year survival rate (%)	P
MVD				0.035
<39.5	67	70.42±5.82	63.30 (42/67)	
≥39.5	38	18.57±3.76	16.95 (6/38)	
bFGFmRNA				0.001
-	41	118.04±6.52	87.06 (38/41)	
+----	64	33.06±3.57	16.21 (10/64)	
MMP-mRNA				0.002
-	44	107.28±7.20	81.82 (36/44)	
+----	61	42.42±8.25	16.02 (9/61)	

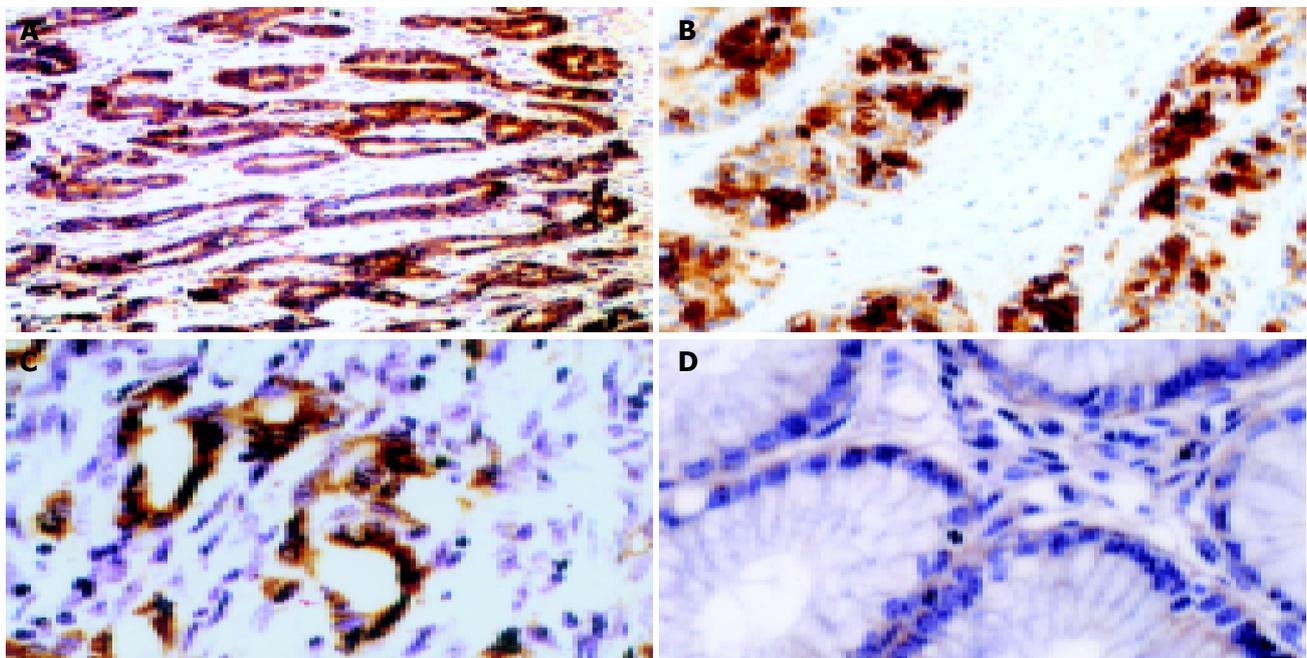


Figure 2 Expression of MMP-9mRNA in gastric carcinoma tissues. **A:** Positive expression (+++) of MMP-9mRNA in the plasma of early gastric adenocarcinoma, ISH ×100; **B:** Positive expression (++) of MMP-9mRNA in the plasma of gastric adenocarcinoma, infiltrating stomach muscularis, ISH ×120; **C:** Positive

expression (++) of MMP-9mRNA in the plasma of gastric adenocarcinoma in peritoneum cancer nodule, ISH ×180; **D:** Negative expression of MMP-9mRNA in normal gastric mucosa, ISH ×180.

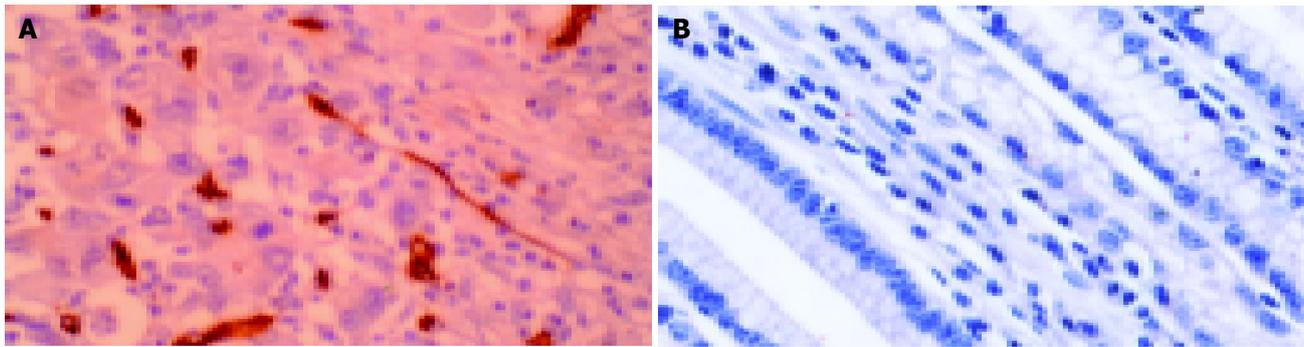


Figure 3 Expression of CD34 protein in gastric carcinoma tissues. **A:** Positive expression of CD34 in vascular endothelial cells of gastric adenocarcinoma. SP

×400; B: NO neovascularization and negative expression of CD34 protein in normal gastric mucosa. SP **×280.**

DISCUSSION

Carcinoma angiogenesis is induced by some angiogenesis factors secreted by the carcinoma cells. bFGF is a patent inducer for the mitosis of endothelial cells of vessels and increases the chemiotaxis. Experiments confirmed that once the carcinoma reached 2 mm in diameter (>1 million cells), continued growth depends on the vascularization of the carcinoma tissues to get fresh nutrients from the host, and the newly formed vessels are infiltrative in nature^[19-26]. The newly formed vessels make the basal membrane incomplete, forming broken spaces, and immature, resulting in carcinoma cell metastasis. Our present study revealed that higher expression of bFGF in carcinoma tissues than in the control gastric mucosa correlated with the pathological parameters of the tumor. Positive correlation was observed between high expression of bFGF and short survival, indicating that the angiogenesis of carcinoma is positively correlated with the malignant biological behaviors. Also, the positive correlation was found between bFGF expression and MVD, indicating that bFGF is one of the important angiogenesis factors for angiogenesis of carcinoma. Our data also showed that in the primary carcinoma tissues and stroma around the carcinomas, the average MVD was 44.68 in the 76 cases of gastric carcinoma with vascular invasion, which was much higher than that without vascular invasion (25.69), indicating that angiogenesis of the carcinoma tissues is one of the main biological behaviors of the carcinomas. We have found

that bFGF is mainly distributed in the cytoplasm of the carcinoma cells, indicating that the carcinoma cells can secrete bFGF. Further experiments demonstrate that bFGF may be transmitted through the following routes to invade the vessels and metastasize: (1) promote the proliferation of the endothelial cells with faster blood supply to the carcinoma tissues; (2) directly act on the carcinoma cells and the cancer cell secretes all the enzymes to make the metastasis easy^[27]; (3) bFGF-mediated basal membrane is immature and deficient^[28] with higher permeability, making it possible for the carcinoma cells to shed off and metastasize into the blood system.

MMP-9 is one of the MMP members and plays an important role in the degradation of extracellular matrix (ECM). However, the degradation of the basal membrane and matrix is important to immigration of the endothelial cells in the angiogenesis. We believe MMP is a key factor in the carcinoma angiogenesis^[29]. MMPs can degrade ECM, and participate in carcinoma angiogenesis. The carcinoma angiogenesis must degrade not only the basal membrane, but also the ECM. The carcinoma cells can secrete angiogenesis factors such as bFGF, inducing the formation of MMPs. Our study indicated that MVD is high when MMP-9 mRNA was positive. The infiltration and metastasis is accompanied with higher expression of MMP-9mRNA and higher angiogenesis capability. In our study, the non-cancer gastric tissue presented negative MMP-9 mRNA, but when the

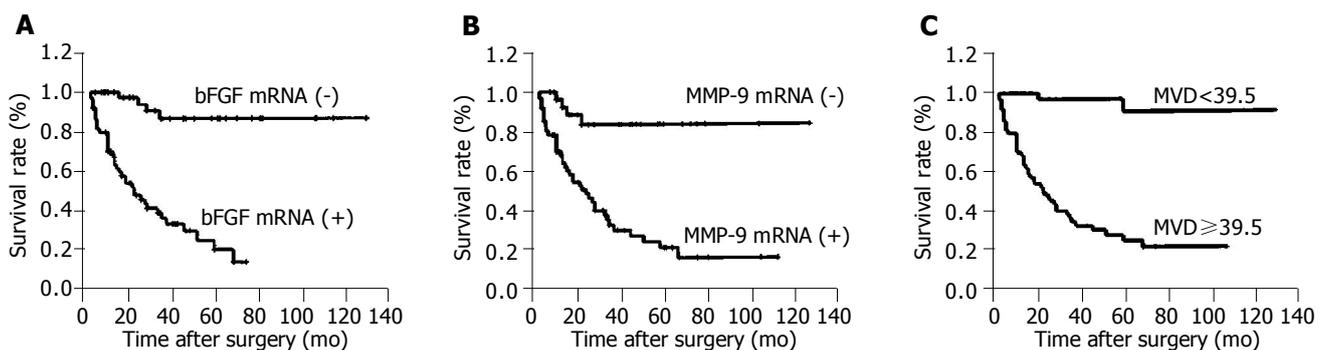


Figure 4 Correlative studies on bFGF mRNA and MMP-9 mRNA expressions with microvascular density, progression and prognosis of gastric carcinoma. **A:** Kaplan-Meier survival curves of groups with positive and negative bFGFmRNA expression in gastric adenocarcinoma ($P<0.05$); **B:** Kaplan-Meier

survival curves of groups with positive and negative MMP-9mRNA expression in gastric adenocarcinoma ($P<0.05$); **C:** Kaplan-Meier survival curves of groups with $MVD \geq 39.5$ and $MVD<39.5$ in gastric adenocarcinoma ($P<0.05$).

carcinoma metastasized into peritoneum and liver, MMP-9 mRNA became more highly expressed than those without metastasis. MVD was higher in 61 carcinoma cases with positive MMP-9 mRNA expression than in the 41 cases with negative MMP-9 mRNA, indicating that MMP-9 mRNA correlated with MVD and MMP-9 induced carcinoma angiogenesis. MMP-9 positive expression is the basis of carcinoma infiltration and metastasis. All these showed that MMP-9mRNA positive expression correlated not only with the filtration, but also with the degradation of ECM barrier to invade blood vessels and metastasis, and MMP-9 can be used as a marker of carcinoma metastasis and prognosis.

The solid carcinoma growth reached silent state when its diameter reached 1.0-2.0 mm, when the growth and death of the carcinoma cells reached a balanced state. But when the angiogenesis begins, the carcinoma cells grow unlimitedly, the blood vessels provided the nutrients that the carcinoma cells need, which resulted in the faster infiltration and metastasis. Our study showed that MVD was positively correlated with carcinoma infiltrative growth, metastasis to the muscular layers, lymph nodes, liver, and peritoneum, indicating the infiltration and metastasis are related to angiogenesis. MVD can be used as an prognosis marker which corresponded to the study of Tomanek^[30]. We also found the positive correlation between bFGFmRNA and MMP-9mRNA in gastric carcinoma tissues, both promoting carcinoma angiogenesis. That is, bFGF promotes angiogenesis while the endothelial cells secret enzymes such as MMP to promote metastasis.

The Kaplan-Meier survival curve was drawn and log-rank test indicated that bFGF and MMP-9 positive expression was different from negative expression. No report has been seen as to the relationship between bFGF, MMP-9, and MVD and gastric carcinoma prognosis. The 41 bFGFmRNA negative cases in our study showed that the mean survival rate was longer than that of 64 positive cases. Statistical difference was revealed in the five-year survival rate between the two groups. The 44 MMP-9 mRNA negative carcinoma cases had longer survival rate than the 61 MMP-9 mRNA positive cases, indicating that bFGF and MMP-9 mRNA positive expression and MVD value ≥ 39.5 will result in worse prognosis and can be used as an independent prognosis marker.

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