Expressions of MMP-2, MMP-9 and VEGF are Closely Linked to Growth, Invasion, Metastasis and Angiogenesis of Gastric Carcinoma

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Abstract. *Background: Gastric carcinoma is still a major leading cause of cancer death in East Asia. Since angiogenesis is a necessary condition for invasion and metastasis, its regulation is of essential significance. Materials and Methods: Expressions of MMP-2, MMP-9 and VEGF were examined with microarray of gastric carcinoma tissue samples (n=249) by immunostaining. In addition, microvessel density (MVD) was assessed after labelling with the anti-CD34 antibody. Data were cross-compared with clinicopathological parameters of tumors, including PTEN expression. Results: Expressions of MMP-2, MMP-9 and VEGF were positively correlated with tumour size, depth of invasion, lymphatic and venous invasion, lymph node metastasis, UICC staging and MVD of gastric carcinomas ($p<0.05$). VEGF expression was positively linked with levels of MMP-2 and MMP-9 ($p<0.05$), but negatively with PTEN ($p<0.05$). The latter was also inversely associated with the MVD in gastric carcinomas ($p<0.05$). Conclusion: MMP-2, MMP-9 and VEGF largely contribute to the angiogenesis and progression of gastric carcinomas. PTEN might inhibit the processes by down-regulating VEGF expression. These parameters should be regarded as good markers to indicate pathobiological behaviours of gastric carcinomas.*

Like most solid tumors, gastric carcinomas require neovascularisation, if they are to grow beyond a few millimeters in diameter. The new vessels not only help to meet the growing metabolic demands of the tumor by supplying additional nutrients, but also provide potential routes for tumor dissemination and metastasis. As the gastric carcinoma continues to develop, so does angiogenesis, since tumor-stroma interaction enhances neovascluarisation through angiogenic factors, such as matrix metalloproteinases (MMPs) and vascular epithelial growth factor (VEGF). When chronic exposure to these angiogenic factors either supports proteolysis of the basement membrane or antagonizes endothelial-pericyte functions, the network of vessels will become highly permeable, facilitating extravasation and ultimately metastasis of the tumour cells (1, 2).

MMPs are a family of enzymes that proteolytically degrade various components of the extracellular matrix (ECM). High expression levels of certain MMPs, either by the tumour cells themselves, by stromal fibroblasts, or by infiltrating inflammatory cells, are closely correlated with tumour invasive and metastatic potential. Furthermore, they participate in the degradation of the vascular basement membrane and remodelling of the ECM during angiogenesis. The 72 kDa MMP-2 and 92 kDa MMP-9 have been shown to play critical roles in the "angiogenic switch" and tumor cells could synthesise and secret large amounts of MMP-2 and MMP-9 in a paracrine and/or autocrine manner to stimulate angiogenesis and increase VEGF release (1). VEGF itself is a 45 kDa homodimeric glycoprotein, which acts as a potent and selective endothelial mitogen, inducing rapid and complete angiogenic response *via* binding to its receptor (2). Recently, evidence was presented that PTEN (phosphatase and tensin homology deleted from human chromosome 10) inhibits endothelial tube formation *in vitro* and vascular sprouting in an *ex vivo* model of angiogenesis, due to its down-regulating effect on VEGF (3).

Although the morbidity and mortality due to gastric cancer have shown dramatic downward trends in most countries world-wide, it continues to be the second leading cause of death from malignant neoplasms. Invasion and metastasis determine the survival time and life quality of patients with the tumour (4). Since angiogenesis is of prime importance in this regard, the expressions of MMP-2, MMP-9, VEGF and PTEN were examined here and the microvessel density

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(MVD) was labelled with the anti-CD34 antibody, to access the roles of these proteins in the angiogenesis and progression of gastric carcinoma.

Materials and Methods

Patients. Gastric carcinomas (n=229: 166 male and 63 female, 38-88 years, mean=66.5 years) were collected from our affiliated hospitals between 1993 and 2002. Of the total, 100 cases were accompanied with lymph node metastasis. None of the patients had undergone chemotherapy or radiotherapy before surgery. All provided consent for use of tumor tissue for clinical research and our University Ethical Committee approved the research protocol.

Pathology. All tissues were fixed in 4% neutralised formaldehyde, paraffin-embedded, cut into 4-μm sections and stained by hematoxylin-and-eosin (H&E) to confirm their histological diagnosis and other microscopic characteristics. The staging for each gastric carcinoma was evaluated according to the UICC system for extent of tumour spread (5). Furthermore, tumour size, depth of invasion, lymphatic and venous invasion and lymph node metastasis of tumours were determined.

Tissue microarray (TMA). Representative areas of solid tumour were identified in H&E stained sections of the selected tumour cases and a 2-mm diameter tissue core per donor block was punched out and transferred to a recipient block with a maximum of 48 cores using a Tissue Microarrayer (AZUMAYA KIN-1, Japan). Four-μm-thick sections were serially cut from the recipient block and transferred to poly-lysine-coated glass slides. H&E staining was performed on TMA for confirmation of the tumour tissue (Figure 1a).

Immunostaining. Consecutive sections were deparaffinised with xylene, dehydrated with alcohol and subjected to immunostaining by microwave intermittent radiation, as described previously (6). Mouse anti-MMP-2 (Daiichi Fine Chemical. Co. Lt, Japan; 1:50), mouse anti-MMP-9 (Daiichi Fine Chemical. Co. Lt; 1:150), rabbit anti-VEGF (LAB VISION, USA; ready to use), mouse anti-PTEN (NovoCastra, UK, 1:150) and mouse anti-CD34 (DAKO, USA, 1:100) antibodies were employed for the detection of the respective proteins, with anti-mouse or anti-rabbit Envison-PO (DAKO) secondary antibodies. Binding was visualised with 3,3'-diaminobenzidine (DAB) and slides were examined after counterstaining with Mayer's hematoxylin. Omission of the primary antibody was used as a negative control and appropriate positive controls were utilised as recommended by the manufacturers.

Evaluation of immunostaining. The immunoreactivity for MMP-2, MMP-9 and VEGF was localised in the cytoplasm and in the nucleus for PTEN (Figure 1b-e). One hundred cells were randomly selected and counted from five representative fields of each section blindly by two independent observers. The positive percentage of counted cells was graded semi-quantitatively using the four-tier scoring system: negative (–), 0~5%; weakly positive (+), 6~25%; moderately positive (++), 26~50%; and strongly positive (+++), 51~100%.

Microvessel density counting. CD34 expression in the cytoplasm and membrane of vascular epithelial cells (Figure 1f) was selected as a marker for the microvessel density counting, although it was occasionally localised in tumour cells and fibroblasts. A modified Weidner's method was employed to calculate the microvessel density of gastric carcinoma by anti-CD34 immunostaining (7). In brief, observers selected five areas and counted individual microvessels on a x400 magnification (0.1885 mm^2 per field) after the area of highest neovascularisation was identified. Any brown-stained endothelial cell or endothelial cell cluster that was clearly separated from the adjacent microvessel, tumor cells and other connective tissue elements was considered as a single, countable microvessel. The counts were performed independently by two investigators.

Statistical analysis. Statistical evaluation was performed using Spearman correlation test to analyse the rank data and Mann-Whitney *U*-test to differentiate non-parametric means of different groups. $P<0.05$ was considered as statistically significant. SPSS 10.0 software was employed to analyse all data.

Results

As summarized in Tables I-V, the expressions of MMP-2, MMP-9 and VEGF were positively correlated with tumour size, depth of invasion, lymphatic and venous invasion, lymph node metastasis, UICC staging and MVD of gastric carcinomas ($p<0.05$). VEGF expression showed positive association with MMP-2 and MMP-9 expressions ($p<0.05$), but was negatively correlated with PTEN expression ($p<0.05$). PTEN expression itself was inversely linked to the MVD in gastric carcinomas ($p<0.05$).

Discussion

Angiogenesis, the process of new capillary formation from pre-existing vessels, plays an essential role in invasion and metastasis of malignancies, because it provides oxygen and nutrients to tumour and a channel for its hematogenous metastasis (8). In our present study, expressions of the angiogenic factors MMP-2, MMP-9 and VEGF were found to be positively linked to the tumour size, invasive depth, lymphatic and venous invasion, lymph node metastasis and UICC staging, in line with their contribution to development of a tumour vasculature allowing solid tumour growth and metastatic spread (8).

Current opinion holds that MMPs promote tumour growth and metastasis by a variety of mechanisms that include ECM degradation and possibly regulation of tumour cell growth itself. It was documented that MMP-2 and MMP-9 contributed to extracellular release of tumour necrosis factor (TNF)-alpha and soluble Fas ligand, which prevents the cancer cells from undergoing lymphocyte-induced apoptosis (9-11). Thus, MMP-2 and MMP-9 were involved in not only progression, but also in the growth of gastric carcinoma through proteolysis of ECM and inhibition of apoptosis. VEGF is a potent multifunctional cytokine that exerts several potentially independent actions on the vascular endothelium, including endothelial mitogenesis, permeability, vascular tone, production of vasoactive molecules and anti-apoptosis of endothelial cells in newly formed vessels (12).

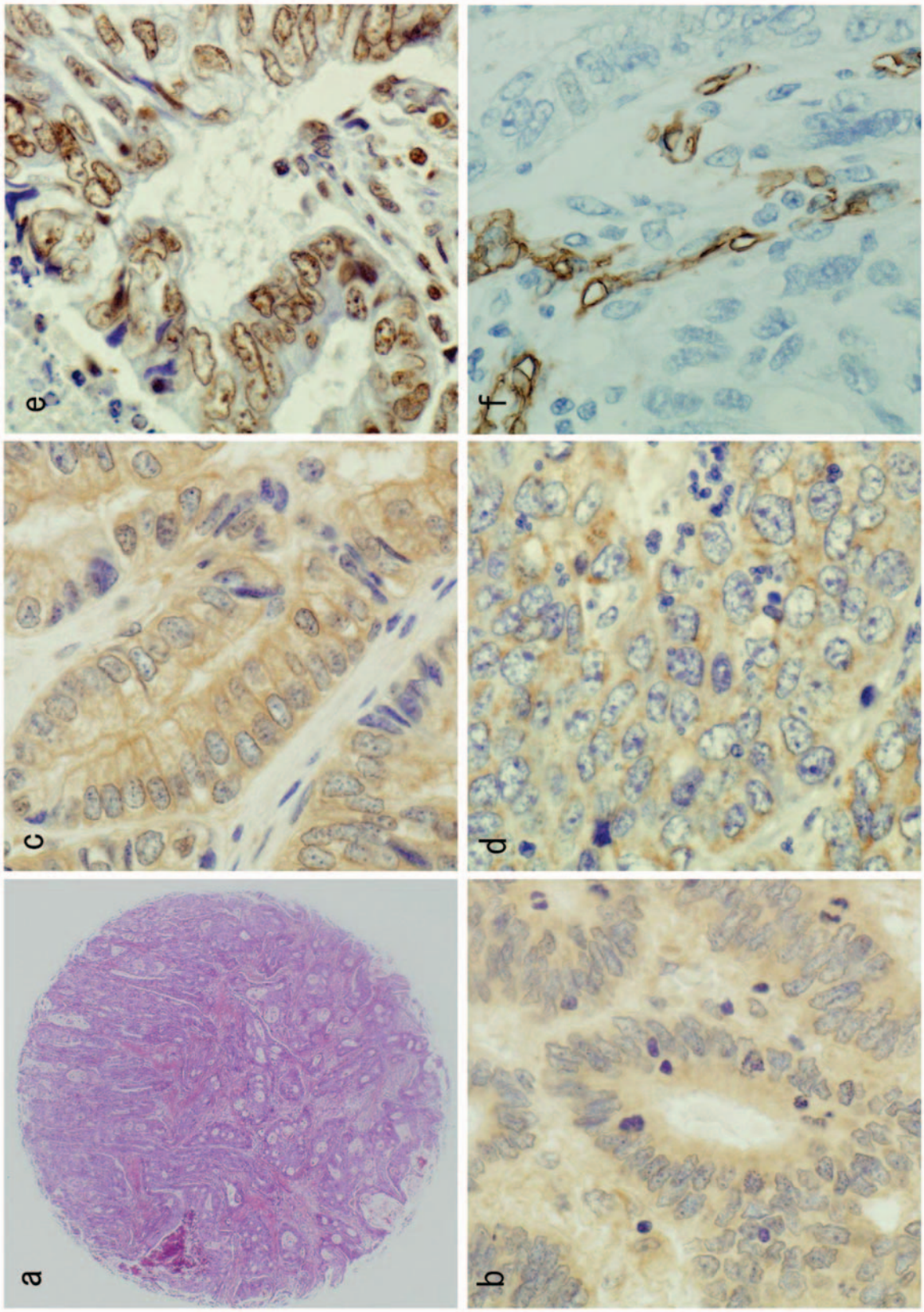

Figure 1. *HE staining and immunostaining of gastric carcinoma. HE staining of TMA of gastric carcinoma (a) and immunohistochemical demonstration of MMP-2 (b), MMP-9 (c), VEGF (d) (in the cytoplasm) and PTEN (e)(in the nucleus) of gastric carcinoma cells and CD34 (f) (in the cytoplasm and membrane) of vascular epithelial cells.*

Table I. *Relationship between MMP-2 expression and clinicopathological features of gastric carcinomas.*

Clinicopathological features	n	MMP-2 expression						
		–	+	++	+++	PR(%)	rs	*P*-value
Tumour size							0.293	<0.001
<4 cm	103	16	23	35	29	84.5		
≥4 cm	116	5	13	37	61	95.7		
Depth of invasion							0.309	<0.001
T_{is}-T_1	108	14	28	36	30	82.7		
T_2-T_4	111	7	8	36	60	95.5		
Lymphatic invasion							0.224	<0.005
–	134	18	30	38	48	86.6		
+	85	3	6	34	42	96.5		
Venous invasion							0.269	<0.001
–	193	21	35	67	70	88.6		
+	26	0	1	5	20	100.0		
Lymph node metastasis							0.204	<0.005
–	126	15	29	38	44	88.1		
+	93	6	7	34	46	93.5		
UICC staging							0.256	<0.001
O-I	136	18	30	42	46	86.8		
II- IV	83	3	6	30	44	96.4		

PR: positive rate.
T_{is}: carcinoma *in situ*; T_1: lamina propria and submucosa; T_2: muscularis propria and subserosa; T_3: exposure to serosa; T_4, invasion into serosa.

Table II. *Relationship between MMP-9 expression and clinicopathological features of gastric carcinomas.*

Clinicopathological features	n	MMP-9 expression						
		–	+	++	+++	PR(%)	rs	*P*-value
Tumour size							0.136	<0.05
<4 cm	106	34	16	27	29	67.9		
≥4 cm	122	31	15	24	52	74.6		
Depth of invasion							0.168	<0.05
T_{is}-T_1	112	35	19	30	28	68.8		
T_2-T_4	116	30	12	21	53	74.1		
Lymphatic invasion							0.157	<0.05
–	139	44	23	31	41	68.3		
+	89	21	8	20	40	76.4		
Venous invasion							0.166	<0.05
–	201	61	28	47	65	69.7		
+	27	4	3	4	16	85.2		
Lymph node metastasis							0.198	<0.005
–	129	43	21	30	35	66.7		
+	99	22	10	21	46	77.8		
UICC staging							0.187	<0.01
O-I	140	46	22	32	40	67.1		
II- IV	88	19	9	19	41	78.4		

PR: positive rate.
T_{is}: carcinoma *in situ*; T_1: lamina propria and submucosa; T_2: muscularis propria and subserosa; T_3: exposure to serosa; T_4, invasion into serosa.

We compared these angiogenic factors with MVD and found positive associations with all three, MMP-2, MMP-9 and VEGF. Additionally, a strong correlation of VEGF expression with MMP-2 and MMP-9 levels was found. It is well-known that angiogenesis features three main steps: proliferation of endothelial cells, breakdown of the ECM and endothelial cell migration (13). Angiogenic factors control these three aspects through various mechanisms. For example, MMP-2 and MMP-9 can remodel the ECM and promote the mobility of vascular epithelial cells (14). VEGF might enhance the proliferation of vascular epithelial cells and inhibit their apoptosis (15).

To investigate regulatory mechanisms, we compared PTEN expression with VEGF expression and MVD and found that gastric carcinomas without PTEN expression displayed higher VEGF expression and MVD, in line with the finding that PTEN inhibits angiogenesis through the down-regulation of VEGF expression (16). The detailed mechanisms may be as follows: (i) lowering VEGF expression by decreasing expression of hypoxia-inducible factor-1a, required for VEGF transactivation *via* binding to VEGF promoter; (ii) restraining activation of phosphatidylinositol 3 kinase, which is also targeted by the VEGF pathway (17).

In conclusion, MMP-2, MMP-9 and VEGF appear to be intimately involved in the growth, angiogenesis and progression of gastric carcinomas. In contrast, PTEN could inhibit angiogenesis, conceivably by down-regulating VEGF

Table III. *Relationship between VEGF expression and clinicopathological features of gastric carcinomas.*

Clinicopathological features	n	VEGF expression						
		–	+	++	+++	PR(%)	rs	*P*-value
Tumour size							0.262	<0.001
<4 cm	107	26	28	22	31	75.7		
≥4 cm	122	21	14	15	72	82.8		
Depth of invasion							0.287	<0.001
T_{is}-T_1	113	31	28	19	35	72.6		
T_2-T_4	116	16	14	18	68	86.2		
Lymphatic invasion							0.268	<0.001
–	139	36	33	21	49	74.1		
+	90	11	9	16	54	87.8		
Venous invasion							0.267	<0.001
–	202	46	41	34	81	77.2		
+	27	1	1	3	22	96.3		
Lymph node metastasis							0.223	<0.005
–	129	31	31	22	45	76.0		
+	100	16	11	15	58	84.0		
UICC staging							0.242	<0.001
O-I	140	35	32	23	50	75.0		
II- IV	89	12	10	14	53	86.5		

PR: positive rate.
T_{is}: carcinoma *in situ*; T_1: lamina propria and submucosa; T_2: muscularis propria and subserosa; T_3: exposure to serosa; T_4, invasion into serosa.

Table IV. *Relationship between expressions of VEGF, MMP-2, MMP-9 and PTEN.*

Groups	n	VEGF expression						
		–	+	++	+++	PR(%)	rs	*P*-value
MMP-2 expression							0.499	<0.001
–	21	8	8	5	0	61.9		
+	36	15	7	8	6	58.3		
++	72	15	15	12	30	79.2		
+++	89	5	11	10	63	94.4		
MMP-9 expression							0.341	<0.001
–	65	19	14	6	26	70.8		
+	31	10	9	7	5	67.7		
++	51	13	12	12	14	74.5		
+++	80	4	7	11	58	95.0		
PTEN expression							0.226	<0.005
–	65	10	5	8	42	84.4		
+	48	7	9	11	21	85.4		
++	44	12	11	8	13	72.7		
+++	71	18	17	9	27	74.6		

PR: positive rate.

Table V. *Relationship between expressions of VEGF, MMP-2, MMP-9 and PTEN and MVD in gastric carcinoma.*

Groups	n	MVD(mean±SD)	*P*-value
VEGF expression			<0.01
–	47	26.7±15.3	
+ ~ +++	181	33.7±18.0	
MMP-2 expression			<0.001
–	21	19.7±11.5	
+ ~ +++	197	33.7±18.0	
MMP-9 expression			<0.05
–	64	28.4±17.1	
+ ~ +++	163	33.9±17.7	
PTEN expression			<0.001
–	63	40.5±21.0	
+ ~ +++	164	30.3±19.0	

SD: standard deviation.

expression. These parameters should, thus, be regarded as good markers for the pathobiological behaviours of gastric carcinomas.

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