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The Role of Vascular Endothelial Growth Factor (VEGF) and p53 Status for Angiogenesis in Gastric Cancer

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Background : *Angiogenesis is of crucial importance for tumor growth and development of metastases. Vascular endothelial growth factor (VEGF) has a potent angiogenic activity and mutations of the p53 gene has been thought to upregulate VEGF. The purpose of our study was to evaluate the prognostic significance of these tumor biomarkers for angiogenesis relative to the information derived from established clinicopathological parameters in gastric cancer.*

Methods : *In this study, we conducted an immunohistochemical investigation of VEGF and p53 expression in 145 tissue samples obtained from gastric cancer patients undergoing curative surgical treatment. To evaluate angiogenesis, microvessel density (MVD) was counted by staining endothelial cells immunohistochemically using anti-CD34 monoclonal antibody.*

Results : *High MVD was significantly associated with depth of tumor invasion and distant metastasis (p=0.004, 0.021, respectively). Moreover, overall survival for patients with high MVD were significantly lower than that of low MVD (p=0.048). Positive expression of VEGF correlated significantly with lymph node and distant metastasis (p=0.040, 0.048, respectively). However, no significant correlation was found between p53 expression and various clinicopathological parameters. VEGF positive tumors showed a higher MVD than VEGF negative tumors (p=0.028). The expression of p53 did not correlate with VEGF expression. Also, the relationship between the status of p53 expression and MVD had not statistically significant differences. In the multivariate analysis, status of VEGF, p53 expression and MVD were not an independent prognostic factor.*

Conclusion : *VEGF seems to be an important, clinically relevant inducer of angiogenesis and angiogenesis assessed by the MVD may be a useful marker for predicting metastasis in gastric cancer. However, further studies are warranted to clarify the impact of p53 on the angiogenesis and the prognostic significance of angiogenesis in gastric cancer.*

Key Words : *Angiogenesis; Genes, p53; Stomach Neoplasms; Immunohistochemistry*

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INTRODUCTION

Angiogenesis has been shown to be a critical aspect of tumor growth and metastasis[1-3]. The induction of angiogenesis by a tumor is controlled process, influenced by angiogenic and angiostatic factors which involves a complex interaction between tumor and endothelial cells[3-5]. Among the many reported angiogenic factors, vascular endothelial growth factor (VEGF) is the most powerful endothelial-cell-specific mitogen that plays a key role in the complicated process of angiogenesis. It has been shown to be significantly upregulated in various human malignant tumors and to be associated with tumor angiogenesis and disease outcome[6-9].

Tumor growth and metastasis are characterized by uncontrolled cellular proliferation. This is usually the result of multiple genetic and epigenetic insults to the cell, particularly involving proto-oncogenes and tumor suppressor genes. The genetic and epigenetic alterations that are responsible for tumor growth and metastasis may underlie the ability of tumors to switch to an angiogenic phenotype[3-5].

p53 which encodes the tumor suppressor gene is mutated or deleted in about 50% of spontaneously arising tumors[10]. Several studies have indicated that angiogenesis may be regulated, in part, by the function of the p53 tumor suppressor gene. Functional p53 suppresses angiogenesis by downregulating angiogenic factor expression, whereas dysfunctional p53 stimulates angiogenesis by both upregulating VEGF and downregulating thrombospondin-1, an angiogenesis inhibitor[11-14].

The degree of intratumoral microvessel density (MVD) is thought to reflect the angiogenic activity generated by the neoplastic cells and the supporting stroma. Moreover, tumor angiogenesis, as quantitated by measurement of intratumoral MVD, has shown to be a significant negative prognostic factor in various human tumors, including breast carcinoma, lung carcinoma, prostate carcinoma, endometrial carcinoma, colon carcinoma and gastric carcinoma[15-20].

The purpose of our study was to evaluate the prognostic significance of these tumor biomarkers for angiogenesis relative to the information derived from established clinicopathological parameters in gastric cancer.

MATERIALS AND METHODS

Patients and tumor specimens

The study included 145 patients who underwent curative surgery for gastric cancer at Chonnam National University Hospital between January 1992 and December 1993. Formalin-fixed and paraffin-embedded tissue blocks were selected by viewing original pathologic slides and choosing blocks that show the junction between carcinoma and benign tissue. This allowed for direct comparison of carcinoma and benign tissue side by side after immunohistochemistry. Patient characteristics, including sex, age, histologic grade, stage and survival data, were obtained by medical records and pathologist and physician contact when necessary. No patient had received anticancer therapy prior to the operation. The histologic grade was classified according to the criteria of Lauren and the World Health Organization[21, 22]. The tumors were staged at the time of surgery by the standard criteria for TNM staging using the American Joint Committee on Cancer[23]. This study group comprised 99 males and 46 females. The mean age was 59.2±10.3 (mean±standard deviation) with a range from 28 to 79 years. The mean size of the tumor was 5.1±2.8 cm (mean±standard deviation) with a range from 0.5 to 15.0 cm.

Immunohistochemistry

All procedures for immunohistochemical staining were done by the Micro-Probe staining system (Fisher Scientific, Pittsburgh, PA) based on capillary action[24]. Paraffin sections, of 4 μm in thickness with mounted probe on slides, were immunostained with anti-mouse monoclonal antibodies by the avidin-biotin peroxidase complex method[24]. Sections were deparaffinized and rehydrated. They were immersed in 0.6% hydrogen peroxide for 5 minutes to block the endogenous peroxidase activity. A polyclonal antibody against VEGF (A-20; diluted 1:50; Santa Cruz Biotechnology, Santa Cruz, Calf, USA), a monoclonal antibody against CD34 (QB-END/10; diluted 1:25; Novocstra Lab., Newcastle, UK) and a monoclonal mouse antihuman p53 antibody (DO-7, diluted 1:100; Dakopatts, Glostrup, Denmark) were used as primary antibodies. The primary antibodies, in the aforementioned concentrations, were diluted in phosphate- buffered saline supplemented with 5% normal horse serum and 1% bovine serum albumin and then incubated with tissues for 15 minutes at 45℃. Anti-mouse immunoglobulin G (Sigma, St. Louis, MO) labeled with biotin was added as a secondary antibody for the detection of primary antibodies and the samples were incubated for 7 minutes at 45℃. After multiple rinses with universal buffer, streptavidin-alkaline phosphatase detection system (Biomeda, Foster, CA) was applied for 7 minutes. As the final step, the slides were developed for 7 minutes with the enzyme substrate 3 amino-9-ethyl

carbazole (AEC, Sigma, St. Louis, MO). The slides were counterstained with hematoxylin solution for 1 minute (Research Genetics, Huntsville, AL). After dehydration, the tissue was sealed with a universal mount (Research Genetics, Huntsville, AL). For negative controls, the primary antibody was omitted and replaced with phosphate-buffered saline.

Evaluation of VEGF and p53 expression

Staining intensity was classified from zero (no staining) to 3 (strong staining) and the percentage of staining area was classified as 0 for no positive staining of tumor cells, 1 for positive staining in <10% of the tumor cells, 2 for positive staining in 10% to 50% of the tumor cells, or 3 for positive staining in >50% of the tumor cells. A staining index was calculated as the product of staining intensity and staining area[18]. Assessment of the staining was evaluated by two independent observers without knowledge of the clinical outcomes, such as tumor stage, grade and survival. Consensus scores were assigned for each case by reviewing the slides with discrepancies in scoring. All sections on which the two observers disagreed were re-evaluated and, after discussion, there was total agreement on the classification. The tumors were categorized as positive expression (staining index>4) or negative expression (staining index$\leq$4).

Microvessel staining and density

Microvessels were highlighted with a monoclonal antibody against CD34 (QB-END/10; diluted 1:25; Novocstra Lab., Newcastle, UK) using the Micro-Probe staining system (Fisher Scientific, Pittsburgh, PA) based on capillary action. Microvessel quantification was performed according to an international consensus[25]. Any single brown-stained cell or cluster of endothelial cells that was clearly separate from adjacent microvessels was regarded as a microvessel (Figure 1). Neither vessel lumens nor the presence of red blood cells were used to define a micovessel. Large vessels with thick muscular walls were excluded from the counts. The stained sections were screened at ×40 magnification to identify the areas of the highest vascular density within the tumor. Vessels were counted in the 5 areas of highest vascular density at ×200 magnification. MVD was expressed as the mean number of vessels in these areas.

Statistical analysis

The x^2-test and Fisher's exact test, where appropriate, were used to compare expression of the VEGF and p53 with various clinicopathological parameters. The relationship between VEGF or p53 expression and MVD was evaluated by the Mann-Whitney U test. Actuarial survival rates of patients were evaluated according to the Kaplan-Meier method and the differences were tested with a log-rank test. The Cox regression model was used to determine the prognostic significance of each parameter by a multivariate analysis. The statistical software program used was Statistical Package for the Social Sciences (SPSS/PC+ 10.0, Chicago, IL). A p value of less than 0.05 was accepted as statistically significant.

RESULTS

Expression of VEGF and p53 in gastric cancer tissues

Normal gastric mucosa was not immunoreactive with

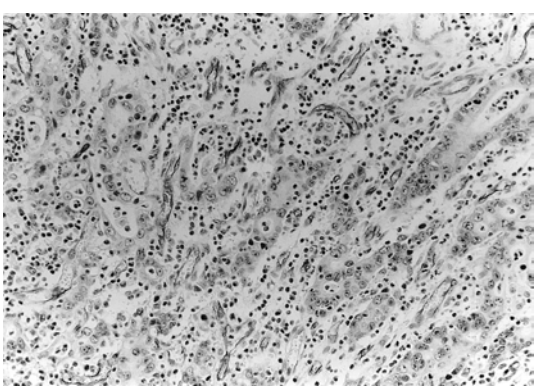

Figure 1. Immunohistochemical staining of endothelial cells with an antibody against CD-34. Individual microvessels can easily be identified (×200).

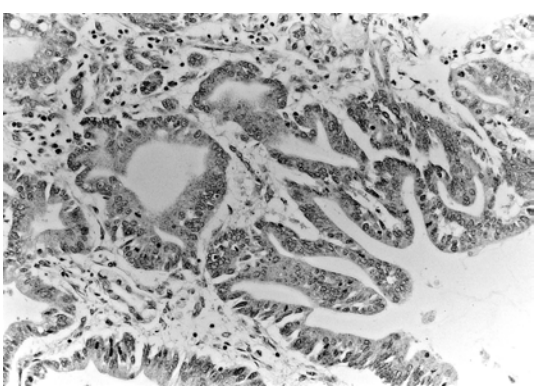

Figure 2. Typical immunohistochemical staining of VEGF in gastric cancer tissue. VEGF immunoreactivity is strongly expressed in the cytoplasm of the tumor cells (×200).

Table 1. Correlation between VEGF and p53 expression in gastric cancer

VEGF expression	p53 expression		*p*-value
	Positive (n=52)	Negative (n=93)	
Positive (n=45)	16	29	
Negative (n=100)	36	64	0.959

VEGF, vascular endothelial growth factor

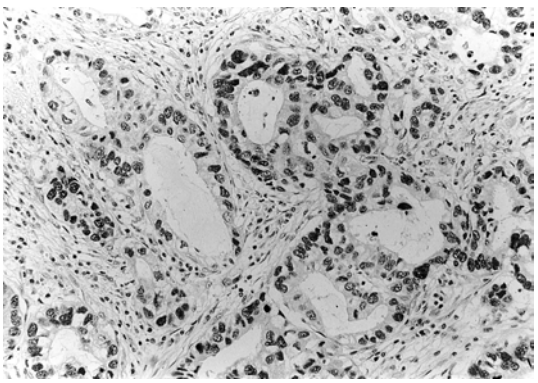

Figure 3. Typical immunohistochemical staining of p53 in gastric cancer tissue. Intense nuclear localization of p53 protein is detected in tumor cells (×200).

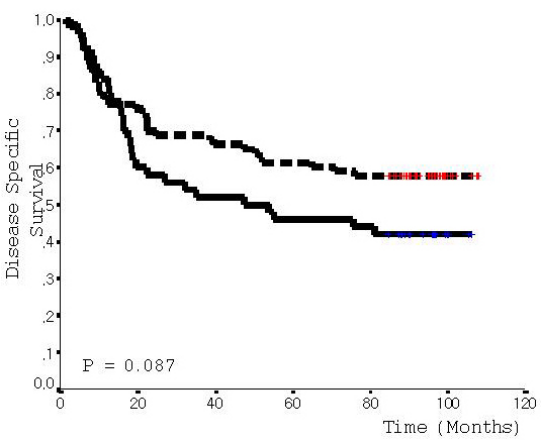

Figure 4. Kaplan-Meier survival curve correlating disease specific survival with positive (solid line) or negative (dotted line) expression of VEGF.

an anti-VEGF antibody. VEGF was mainly localized to the cytoplasm or the membrane of the tumor cells (Figure 2). Tumor cells that stained strongly for VEGF were observed more often in the invasive front than in the tumor center. In cancerous tissues, positive expression of VEGF was

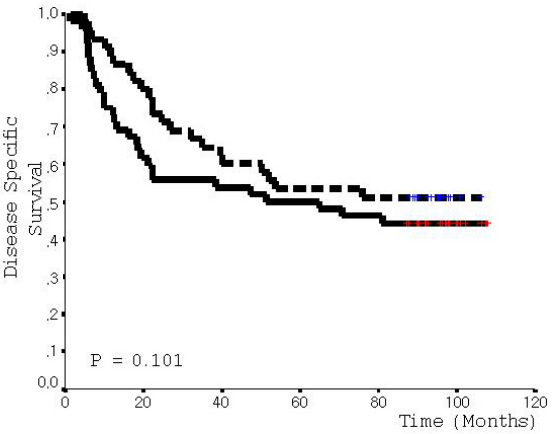

Figure 5. Kaplan-Meier survival curve correlating disease specific survival with positive (solid line) or negative (dotted line) expression of p53.

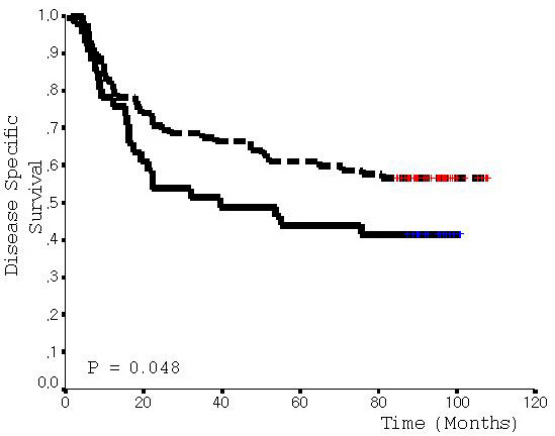

Figure 6. Kaplan-Meier survival curve correlating disease specific survival with high MVD (solid line) or low MVD (dotted line).

31.0% (45/145) (Table 1). Abnormal accumulation of the p53 protein was evident in the nuclei of tumor cells (Figure 3), and heterogenously distributed. Based on our criteria, the positive expression of p53 in cancerous tissues was 35.9% (52/145) (Table 1).

Correlation between VEGF and p53 expression and clinicopathological features

The expression of p53 did not correlate with VEGF expression (p=0.959, Table 1). The correlation between VEGF or p53 expression and clinicopathological parameters is summarized in Table 2, 3. Positive expression of

Table 2. Correlation between VEGF expression and clinicopathological parameters of gastric cancer

Clinicopathological parameters	Total (n=145)	VEGF expression Positive (n=45)	Negative (n=100)	*p*-value
Age (years)				
< 60	67	19	48	0.519
≥ 60	78	26	52	
Sex				
Male	99	32	67	0.623
Female	46	13	33	
Tumor size (cm)				
< 5.0	66	18	48	0.403
≥ 5.0	79	27	52	
Lauren Classification				
Intestinal	70	17	53	0.223
Diffuse	42	15	27	
Mixed	33	13	20	
Differentiation grade*				
WD	24	3	21	0.096
MD	45	15	30	
PD	76	27	49	
TNM stage				
I	52	10	42	0.064
II	21	8	13	
III	47	15	32	
IV	25	12	13	
Depth of tumor invasion (T)				
T1	24	7	17	0.163
T2	32	5	27	
T3	75	28	47	
T4	14	5	9	
Lymph node metastasis (N)				
N0	70	16	54	0.040
N1-3	75	29	46	
Distant metastasis (M)				
M0	125	35	90	0.048
M1	20	10	10	

VEGF, vascular endothelial growth factor;
*WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated adenocarcinoma

Table 3. Correlation between p53 expression and clinicopathological parameters of gastric cancer

Clinicopathological parameters	Total (n=145)	p53 expression Positive (n=52)	Negative (n=93)	*p*-value
Age (years)				
< 60	67	25	42	0.736
≥ 60	78	27	51	
Sex				
Male	99	37	62	0.578
Female	46	15	31	
Tumor size (cm)				
< 5.0	66	25	41	0.594
≥ 5.0	79	27	52	
Lauren Classification*				
Intestinal	70	27	43	0.280
Diffuse	42	11	31	
Mixed	33	14	19	
Differentiation grade*				
WD	24	9	15	0.721
MD	45	18	27	
PD	76	25	51	
TNM stage				
I	52	17	35	0.280
II	21	6	15	
III	47	22	25	
IV	25	7	18	
Depth of tumor invasion (T)				
T1	24	3	21	0.147
T2	32	18	14	
T3	75	25	50	
T4	14	6	8	
Lymph node metastasis (N)				
N0	70	23	47	0.456
N1-3	75	29	46	
Distant metastasis (M)				
M0	125	46	79	0.556
M1	20	6	14	

*WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated adenocarcinoma

VEGF correlated significantly with lymph node and distant metastasis (p=0.040, 0.048, respectively, Table 2). There was a trend towards an association between the positive expression of VEGF and poor survival (p=0.087, Figure 4). However, no significant correlation was found between p53 expression and various clinicopathological parameters, including survival (Table 3, Figure 5).

Correlation between MVD and clinicopathological features

The MVD for 145 tumors ranged from 23.0 to 182.0 with a mean MVD of 74.0±31.1. When a mean MVD value of 74.0 was chosen as the cut-off point for discrimination of the 145 patients into two subgroups, 74 patients were categorized as high MVD and 71 as

Table 4. Correlation between microvessel density group and clinicopathological parameters of gastric cancer

Clinicopathological parameters	Total (n=145)	Microvessel density (MVD) Group		*p*-value
		High MVD (n=74)	Low MVD (n=71)	
Age (years)				
< 60	67	33	34	0.627
≥ 60	78	41	37	
Sex				
Male	99	49	50	0.599
Female	46	25	21	
Tumor size (cm)				
< 5.0	66	30	36	0.501
≥ 5.0	79	44	35	
Lauren Classification				
Intestinal	70	32	38	0.382
Diffuse	42	25	17	
Mixed	33	17	16	
Differentiation grade*				
WD	24	7	17	0.613
MD	45	27	18	
PD	76	40	36	
TNM stage				
I	52	21	31	0.068
II	21	11	10	
III	47	23	24	
IV	25	19	6	
Depth of tumor invasion (T)				
T1	24	5	19	0.004
T2	32	16	16	
T3	75	40	35	
T4	14	13	1	
Lymph node metastasis (N)				
N0	70	35	35	0.756
N1-3	75	39	36	
Distant metastasis (M)				
M0	125	58	67	0.021
M1	20	16	4	

*WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated adenocarcinoma

low MVD. High MVD was significantly associated with the depth of tumor invasion (p=0.004) and also did correlate with distant metastasis (p=0.021) (Table 4). Moreover, the overall survival for patients with high MVD wes significantly lower than that of low MVD (p=0.048) (Figure 6).

Table 5. The relationship between microvessel density and the expression of VEGF and p53 in gastric cancer

VEGF/p53 status	Total (n=145)	Microvessel density (MVD) No.		*p*-value
		Mean±SD	Range	
VEGF				
Positive	45	78.5±1.3	30.3~182.0	0.028
Negative	100	71.2±26.3	23.0~164.5	
p53				
Positive	52	72.7±29.8	23.0~182.0	0.525
Negative	93	76.9±34.1	27.5~173.6	
VEGF/p53				
Positive/Positive*	16	75.6±30.3	45.1~182.0	0.147*
Positive/Negative	29	74.5±28.5	30.3~173.6	
Negative/Positive	36	73.7±35.5	23.0~164.5	
Negative/Negative*	64	72.3±32.0	27.5~157.3	

VEGF, vascular endothelial growth factor; No, number; SD, standard deviation

Correlation between VEGF or p53 expression and MVD

The mean MVD value of VEGF positive tumors was 78.5±31.3 and was a significantly higher MVD than that of VEGF negative tumors (p=0.028). However, the relationship between the status of p53 expression and MVD was not statistically significant (p=0.525). The mean MVD value of VEGF and p53 positive tumors was 75.6±30.3 and was higher than that of VEGF and p53 negative tumors, but the mean MVD value of both groups did not show statistically significant difference (p=0.147) (Table 5).

Prognostic value of VEGF status, p53 status, MVD and conventional clinicopathological parameters

When the status of VEGF and p53, MVD and conventional clinicopathological parameters were analyzed by the Cox regression model, the stage and status of metastasis were found to be significant, independent, prognostic factors, while the status of VEGF, p53 expression and MVD were not significant (data not shown).

DISCUSSION

Angiogenesis is crucial for normal growth and development and in protective responses, such as wound healing and inflammation. However, aberrant angio-

genesis can occur in a variety of pathological settings including growth and dissemination of tumors[1-3].

Recognition of the importance of angiogenesis for the growth and dissemination of tumors has raised fundamental questions regarding the molecular mechanisms of the angiogenic switch during tumor progression. The angiogenic switch is regulated by changes in the relative balance between inducers and inhibitors of endothelial cell proliferation and migration[5]. The switch can be activated by increasing the levels of inducers, such as VEGF and/or by reducing the concentration of inhibitors, such as thrombospondin[15]. Among the many reported angiogenic inducers, VEGF is thought to be the most powerful angiogenic inducer. In various human cancers, VEGF expression was correlated with tumor angiogenesis and prognosis[6-9]. Also, in our study, VEGF positive tumors showed a higher MVD than VEGF negative tumors. Positive expression of VEGF correlated significantly with lymph node and distant metastasis. There was a trend towards an association between the positive expression of VEGF and poor survival. These results suggest that VEGF may be an important, clinical relevant inducer of angiogenesis and a predictor of metastatic potential in gastric cancer.

The genetic alterations involved in the tumorigenesis are also responsible for the phenotypic characteristics of cancer cells. The p53 tumor suppressor gene is one of the most frequently mutated genes in human cancers[10]. Previous reports indicate that loss of p53 function, via somatic mutations or expression of viral oncoproteins, contributes to activation of the angiogenic switch during tumorigenesis[11-14]. The p53-mediated inhibition of VEGF expression, with the ability of p53 to upregulate thrombospondin-1, indicates that p53 provides dual functions that regulate angiogenesis. Thus, loss of p53 function during tumorigenesis deregulates both arms of the balance, providing a potent stimulus for angiogenesis and tumor progression[11-14]. Recent studies have shown that p53 expression correlates with tumor angiogenesis through VEGF upregulation in human gastric cancers[26-29]. However, in our study, the expression of p53 did not correlate with VEGF expression. Also, p53 expression was not related with high MVD. Our results suggest that tumor angiogenesis, through the regulation of VEGF in gastric cancer, may be not dependent on p53 status. However, these contradictory findings might be due to differences in the antibody used, staining methods or the criteria used. Also, immunohistochemistry has been shown to have a discordancy rate of 30~35% when compared with techniques that determine p53 gene status, including single strand conformation polymorphism polymerase chain reaction analysis and direct DNA sequencing[30, 31]. Thus, it should be noted that the expression of p53 as detected by immunohistochemistry does not provide adequate information about the dysfunction of the protein and gene mutation. Further studies are needed to evaluate the effect of p53 status on angiogenesis in an in vivo tumorigenesis context.

Tumor angiogenesis, as quantitated by measurement of intratumoral MVD, has recently shown to be a parameter of potential prognostic significance for various human tumors[15-20]. In our study, high MVD was significantly associated with the depth of tumor invasion, distant metastasis and poor survival. These results suggest that tumor angiogenesis may be a useful marker for predicting metastasis in gastric cancer, but MVD or VEGF expression were not found to be significant, independent, prognostic factors in a multivariate analysis by the Cox regression model. Furthermore, several reports have shown that MVD is not a reliable predictor of metastasis-free survival or overall survival in colon and pancreatic cancers[32-34].

A discrepancy still exists in the impact of tumor angiogenesis as a prognostic predictor of cancer patients, according to our and other reports. There are several possible explanations for this discrepancy. First, in various human cancers, the quantitation of tumor angiogenesis by MVD, as reported in different studies, is difficult to compare due to different score systems and different antibodies used. Also, accurately measuring MVD requires superb immunohistochemistry, representative tumor tissue, relatively standard field size and considerable experience at tumor pathology[35]. Second, this may be due in part to inadequate sample size, inappropriate multiple significance testing and arbitrary definition of patients' group[36]. Third, biological processes, such as tumor growth and metastasis, are regulated by a complex interplay of multiple factors, including angiogenic factor, growth factor, motility factor and cell adhesion molecules. Thus, the capacity of tumor cells to induce angiogenesis does not always correlate with malignant potential and it is unclear whether the growth, metastasis and clinical outcome of a tumor is angiogenesis -dependent[37].

In conclusion, VEGF seems to be an important, clinically relevant inducer of angiogenesis, and tumor angiogenesis assessed by the MVD may be a useful marker for predicting metastasis in gastric cancer.

However, further studies are warranted to clarify the impact of p53 on the angiogenesis and prognostic significance of angiogenesis in gastric cancer.

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