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World J Gastroenterol 2003;9(7):1421-1426
World Journal of Gastroenterology
 ISSN 1007-9327

• GASTRIC CANCER •

Prognostic significance of expression of cyclooxygenase-2 and vascular endothelial growth factor in human gastric carcinoma

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Received: 2002-12-28 **Accepted:** 2003-02-18

Abstract

AIM: To investigate the role of cyclooxygenase-2(COX-2) and vascular endothelial growth factor (VEGF) in the development of gastric carcinoma and correlation between expression of COX-2 and VEGF and clinicopathologic features in tissues from patients with gastric carcinoma.

METHODS: 281 patients with gastric carcinoma who underwent surgical resection between 1990 and 1999 at the First Affiliated Hospital, Anhui Medical University, PRC, were followed up. Expression of COX-2 and VEGF was investigated retrospectively in 232 gastric carcinoma tissues and 60 noncancerous specimens by using immunohistochemistry.

RESULTS: The 5-year survival rates of early gastric carcinoma (EGC) and advanced gastric carcinoma (AGC) were 93.4 % and 59.0 %, respectively. Survival time was highly correlated with lymph node metastasis, vascular invasion, depth of invasion and treatment with chemotherapy. Compared with paired noncancerous tissues, expression of COX-2 and VEGF and microvessel density (MVD) value in carcinoma tissue were significantly higher. The MVD value was much higher in COX-2-positive group and VEGF-positive group than that in COX-2-negative group and VEGF-negative group. Expression of COX-2 and VEGF, as well as MVD value were highly correlated with lymph node metastasis and vascular invasion. The 5-year survival rate of patients with expression of COX-2 or VEGF was significantly lower than that of patients without COX-2 or VEGF expression. Multivariate analysis revealed that VEGF overexpression, lymph node metastasis, COX-2 overexpression, depth of invasion and vascular invasion were all independent prognostic factors of gastric carcinoma.

CONCLUSION: Overexpression of COX-2 and VEGF in patients with gastric carcinoma can enhance the possibility of invasion and metastasis, implicating a poor prognosis. They may serve as the fairly good prognostic factors to indicate biologic behaviors of gastric carcinoma.

Shi H, Xu JM, Hu NZ, Xie HJ. Prognostic significance of expression of cyclooxygenase-2 and vascular endothelial growth factor in human gastric carcinoma. *World J Gastroenterol* 2003; 9(7):1421-1426
http://www.wjgnet.com/1007-9327/9/1421.asp

INTRODUCTION

Gastric carcinoma is one of the most common malignancies worldwide. Carcinogenesis and progression of carcinoma are believed to be from multi-stage processes involving the activation of oncogenes and/or the loss of suppressor genes. Epidemiologic studies have shown that nonsteroidal anti-inflammatory drugs (NSAIDs) can reduce the incidence rate and mortality of digestive tract carcinomas, including esophageal, gastric, colon, and rectal lesions[1,2]. The prostaglandin synthetic enzyme cyclooxygenase (COX) is a target for NSAIDs therapy, and a key enzyme in the conversion of arachidonic acid to prostaglandins. Recent studies have confirmed the presence of two forms of COX, constitutively produced COX-1 and inducible COX-2[3]. COX-1 is a constitutively expressed gene in many tissues, and levels of this protein do not fluctuate in response to stimuli[4]. COX-2 is induced by pathologic stimuli, such as inflammation, various growth factors, and cytokines produced by tumor cells[5]. Human gastric mucosa, however, normally expresses barely detectable levels of COX-2 protein[6]. To date, whether COX-2 is involved in the growth of gastric carcinoma remains to be clarified, although COX-2 overexpression has recently been reported in human gastric adenocarcinoma[7].

Recently, many studies have reported on the relation between the malignant potential of neoplasms and tumor angiogenesis[8-10]. Vascular endothelial growth factor (VEGF) is one of these angiogenic factors, and is known to play a crucial role in the formation of neovasculature[11]. VEGF expression is correlated significantly with tumor vascularity and a marker for tumor angiogenesis[12,13]. In the current study, we examined COX-2 and VEGF expression in primary gastric carcinoma tissues at various stages to investigate the relations between COX-2 and VEGF expression and clinicopathologic features of these tumors. We also investigated the prognostic value of these two biologic factors in gastric carcinoma and compared them with the conventional clinicopathologic factors.

MATERIALS AND METHODS

Clinical materials

Totally 281 patients with gastric carcinoma who received gastrectomy without preoperative chemotherapy between 1990 and 1999 at our university hospital were followed up. Among the 281 patients, there were 110 patients with early gastric carcinoma (EGC) in which carcinoma invasion was confined to the mucosa or submucosa and 171 patients with advanced gastric carcinoma (AGC) that invaded beyond the submucosal layer (but not to serosa in our study) according to the criteria of the Japanese Research Society for Gastric Cancer[14]. The patients were comprised of 198 men and 83 women with an average age of 55.6 years (range, 22 to 80 years). Of these 281 patients, 66 had lymph node metastasis and 39 had vascular invasion. Patients who died of other disease were excluded from the study. 60 paired control samples (including 30 cases of chronic atrophic gastritis (CAG) and 30 cases of gastric epithelial dysplasia) were obtained from the antrum.

Immunohistochemical techniques

In brief, archival paraffin-embedded tissue specimens and controls were sectioned at a thickness of 4 μm, deparaffinized,

and rehydrated. The slides were incubated with 3 % hydrogen peroxide in methanol for 10 minutes to block endogenous peroxidase activity, and then washed in phosphate-buffered saline (PBS) and incubated in 10 % normal rabbit serum for 5 minutes to reduce nonspecific antibody binding. Rabbit polyclonal antibody specific for human COX-2 (H-62; Santa Cruz Biotechnology, Inc. Santa Cruz, CA) was applied as the primary antibody at a dilution of 1:100. Mouse monoclonal antibody against VEGF(A-20, Santa Cruz Biotechnology, Inc. Santa Cruz, CA) or Factor VIII related antigen (PC-10, Maxim Biotech, Inc.) was also applied as the primary antibody. These slides were incubated with primary antibody for 60 minutes at room temperature, followed by 3 washes with PBS. Sections then were incubated with biotinylated IgG for 20 minutes followed by 3 washes. Slides then were treated with streptoavidin-peroxidase reagent for 20 minutes and washed with PBS 3 times. Finally, slides were incubated in PBS containing diaminobenzidine and 1 % hydrogen peroxide for 5-10 minutes, counterstained with Mayer hematoxylin, and mounted. PBS was substituted for primary antibody as the negative control.

Staining analysis

COX-2 staining The expression of COX-2 was semiquantified. The degree of immunostaining for COX-2 was considered positive when unequivocal staining of the cytoplasm was observed in tumor cells[7].

VEGF staining Immunoreactivity was graded as follows[12]: Positive, unequivocal staining of the membrane or the cytoplasm was seen in more than 5 % of carcinoma cells, negative, no detectable expression or less than 5 % of tumor cells were stained.

Microvessel staining and counting Intratumoral microvessels were highlighted by immunostaining with anti-Factor VIII related antigen monoclonal antibody. Any single brownly-stained cell or cluster of endothelial cells that was clearly separated from adjacent microvessels, tumor cells, and other connective tissue elements were considered a vessel[12]. Branching structures were counted as a single vessel unless there was a discontinuity in the structure. The stained sections were screened at ×100 magnification under a light microscope to identify the 5 regions of the section with the highest vascular density. Vessels were counted in the 5 regions at ×200 magnification, and the average numbers of microvessels were recorded[12]. Two observers did the counting, and the mean value was used for the analysis.

Statistical analysis

Data were analyzed using the chi-square test for categoric variables and Student' s *t* test for continuous variables. Five-year survival was compared using the Kaplan-Meier method and analyzed by the log rank test. Factors affecting survival were analyzed by Cox proportional hazards model using the SPSS statistic package Version 10.0. Differences with *P* values <0.05 were considered statistically significant.

RESULTS

The detection rates of EGC between 1990 and 1999 fluctuated between 1.1 % and 6.6 %. The average detection rate was 4.3 % (110/2533). In patients with EGC, cardia gastric tumors more frequently occurred than corpus and antrum gastric carcinoma in 60-69 age group (50.0 % *vs* 28.9 %, 21.3 %, *P*<0.05).

Five-year survival rate

The follow-up rates of EGC and AGC were 88.2 % (97/110) and 84.8 % (145/171), respectively. The overall disease-specific 5-year survival rates for patients with EGC and AGC were 93.4 % and 59.0 %, respectively. The 5-year survival rates for patients with EGC with different tumor location were as follows: cardia, 90.9 %; corpus, 91.3 %; and antrum, 96.3 %. The 5-year survival rate for patients with EGC with different depth of invasion was 96.7 % for mucosa invasion and 90.3 % for submucosa invasion. The 5-year survival rates for patients with AGC with different tumor location were as follows: cardia, 45.0 %; corpus, 69.6 %; and antrum, 60.0 %.

Correlation between postoperative survival time and clinicopathologic factors

Table1 shows the clinicopathologic data of 106 patients who survived for ⩾5 years and 47 patients who died within 5 years. There were no differences with respect to gender, age, location of the tumor, or histology between patients with long and short survival time. But survival time was highly correlated with depth of invasion, lymph node metastasis, vascular invasion, and treatment with chemotherapy (*P*<0.05-0.01).

Table 1 Correlation between clinicopathologic factors and survival time

Variables	Alive more than 5 years *n*=106	Died within 5 years *n*=47	*P* value
Gender			
Male	76(71.7)	33(70.2)	NS
Female	30(28.3)	14(29.8)	
Age(years)			
<60	28(26.4)	18(38.3)	NS
⩾60	78(73.6)	29(61.7)	
Location of tumor			
Cardia	19(17.9)	11(23.4)	NS
Corpus	37(34.9)	17(31.9)	
Antrum	50(47.2)	21(44.7)	
Depth of invasion			
Mucosa or submucosa	57(53.8)	6(12.8)	<0.01
Muscularis propria	49(46.2)	41(87.2)	
Histology			
Differentiated	41(38.7)	19(40.4)	NS
Undifferentiated	65(61.3)	28(59.6)	
Lymph node metastasis			
Present	21(19.8)	38(80.9)	<0.01
Absent	85(80.2)	9(19.1)	
Vascular invasion			
Present	9(8.5)	26(55.3)	<0.01
Absent	97(91.5)	21(44.7)	
Chemotherapy			
Yes	69(65.1)	20(42.6)	<0.05
No	37(34.9)	27(57.4)	

Note: NS, not significant.

Immunohistochemical analysis

Correlation between expression of COX-2 and clinicopathologic factors Immunoreactivity for COX-2 protein was present in the cytoplasm of tumor cells, smooth muscle cells, and surrounding glands, but not in the surrounding stroma (Figure 1). Positive immunostaining for COX-2 was also seen in some CAG (23.3 %) and mucosal atypical hyperplasia (60.0 %) specimens (Figure 2). However, it was observed more frequently in tumor cells (Table 2), which showed that the expression of COX-2 was significantly higher in mucosal atypical hyperplasia than that in CAG (*P*<0.01).

Compared with paired noncancerous specimens, COX-2 levels in carcinoma tissue were significantly higher (P<0.05-0.01). There was no significant association between COX-2 expression and gender, age, location of the tumor, or depth of invasion. However, significant difference was noted with respect to histologic type, lymph node metastasis, and vascular invasion. The COX-2-positive rate was significantly higher in patients with lymph node metastasis or vascular invasion than that in those without such metastasis or invasion (P<0.05-0.01). Similar results were obtained in relationship between the histologic type and the COX-2-positive rate (P<0.01, Table 2).

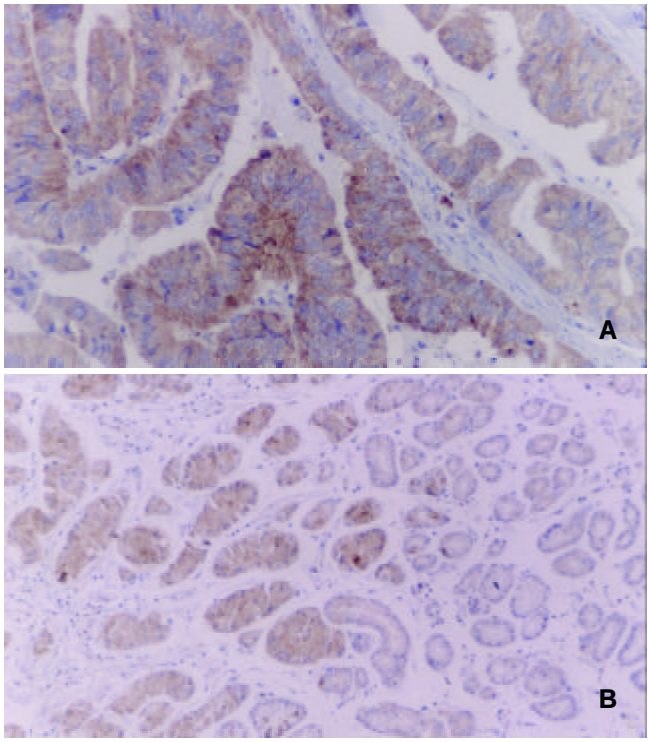

Figure 1 Immunohistochemical staining of COX-2 protein in gastric carcinoma. Immunoreactivity for COX-2 protein was present in the cytoplasm of tumor cells, smooth muscle cells (A, ×200), and surrounding glands (B, ×100).

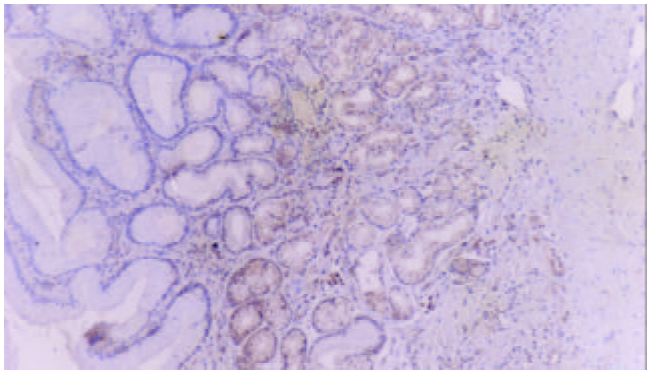

Figure 2 Positive immunostaining for COX-2 was observed in some CAG specimens (×100).

Correlations between expression of VEGF, microvessel counting and clinicopathologic factors VEGF was mainly localized in the cytoplasm or on the membrane of carcinoma cells (Figure 3). Tumor cells that were strongly immunopositive for VEGF were observed more often in the invasive front than that in the center of the tumors. Weakly positive VEGF staining was seen in some endothelial cells and noncancerous specimens. VEGF expression was detected in 122 (52.6 %) tumors and significantly higher (P<0.01) than that in noncancerous specimens (13.3 %). Correlations between VEGF expression, MVD and different clinicopathologic variables are shown in Table 3. VEGF-positive rate and MVD value were significantly correlated with depth of invasion, lymph node metastasis, and invasion of blood vessels (P<0.01). There was no significant association among VEGF expression, MVD value and histologic type. The microvessel count in COX-2-positive or VEGF-positive tumors (28.76±8.58 and 26.23±8.47, respectively) was significantly higher than that in COX-2-negative or VEGF-negative tumors (19.27±8.36 and 18.91±8.12, respectively), P<0.01.

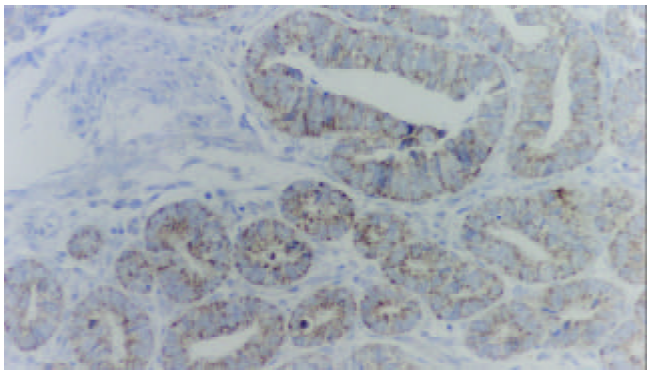

Figure 3 Immunohistochemical staining for VEGF in gastric carcinoma. VEGF was mainly localized on the membrane of the carcinoma cells or in the cytoplasm (×200).

Table 2 Correlation between COX-2 expression and clinicopathologic factors

Variables	*n*	COX-2 *n*(%)		*P* value
		Positive	Negative	
Gender				
Male	168	30(17.9)	138(82.1)	NS
Female	64	12(18.7)	52(81.3)	
Age(years)				
<60	145	25(17.2)	120(82.8)	NS
⩾60	87	17(19.5)	70(80.5)	
Location of tumor				
Cardia	61	7(11.5)	54(88.5)	NS
Corpus	77	19(24.7)	58(75.3)	
Antrum	94	16(17.1)	78(82.9)	
Histology				
Differentiated	129	7(5.4)	122(94.6)	<0.01
Undifferentiated	103	35(33.9)	68(66.1)	
Lymph node metastasis				
Present	64	2(3.1)	62(96.9)	<0.01
Absent	168	40(23.8)	128(76.2)	
Vascular invasion				
Present	39	2(5.1)	37(94.9)	<0.05
Absent	193	40(20.7)	153(79.3)	
Depth of invasion				
Mucosa or submucosa	94	19(20.2)	75(79.8)	NS
Muscularis propria	138	23(16.7)	115(83.3)	
Noncancerous tissue				
CAG	30	23(76.7)	7(23.3)	<0.01
Atypical hyperplasia	30	12(40.0)	18(60.0)	

Notes: NS, not significant; CAG, chronic atrophic gastritis.

Table 3 Correlation between VEGF, MVD and clinicopathologic factors

Variables	*n*	VEGF *n* (%)		MVD	
		Positive	*P* value	$\bar{x}\pm s$	*P* value
Gender					
Male	168	88(52.4)	NS	22.11±9.14	NS
Female	64	34(53.1)		24.21±8.85	
Tumor size (cm)					
<5 cm	163	87(53.4)	NS	23.17±9.53	NS
⩾5 cm	69	35(50.7)		23.69±8.26	
Depth of invasion					
Mucosa or submucosa	94	26(27.7)	<0.01	18.08±8.32	<0.01
Muscularis propria	138	96(69.6)		26.41±8.44	
Histology					
Differentiated	129	70(54.3)	NS	23.48±9.01	NS
Undifferentiated	103	52(50.5)		23.25±9.06	
Lymph node metastasis					
Present	64	47(73.4)	<0.01	28.52±4.39	<0.01
Absent	168	75(44.6)		19.73±8.47	
Vascular invasion					
Present	39	29(74.4)	<0.01	28.94±5.03	<0.01
Absent	193	93(48.2)		21.67±9.12	
Noncancerous tissue					
CAG	30	3(10.0)	NS	10.43±4.22	NS
Atypical hyperplasia	30	5(16.7)		11.56±6.17	

Notes: NS, not significant; CAG, chronic atrophic gastritis

Correlations between postoperative survival time and expression of COX-2 and VEGF COX-2 or VEGF positive rate was significantly higher in patients who died within 5 years (93.6 % and 78.7 %, respectively) than that in those survived ⩾5 years (69.8 % and 49.1 %, respectively, $P<0.01$). Expression of COX-2 or VEGF was highly correlated with postoperative survival time. The 5-year survival rate was 67.9 % in patients with COX-2-positive tumors and 91.4 % in patients with COX-2-negative tumors. Accordingly, the prognosis for patients with a COX-2-negative tumor was significantly better than that for patients with a COX-2-positive tumor (Figure 4, $P<0.01$). The survival curves subdivided according to VEGF expression are shown in Figure 5. The 5-year survival rate was 61.2 % in patients with VEGF-positive tumors, which was significantly lower than the rate in those patients with VEGF-negative tumors (91.5 %, $P<0.01$).

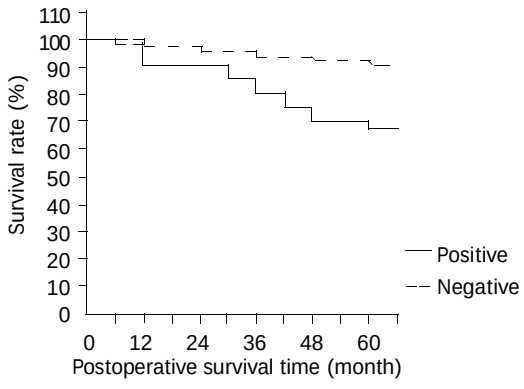

Figure 4 Kaplan-Meier survival curves of patients with gastric carcinoma with regard to COX-2 expression (positive and negative), $\chi^2=7.56$, $P<0.01$.

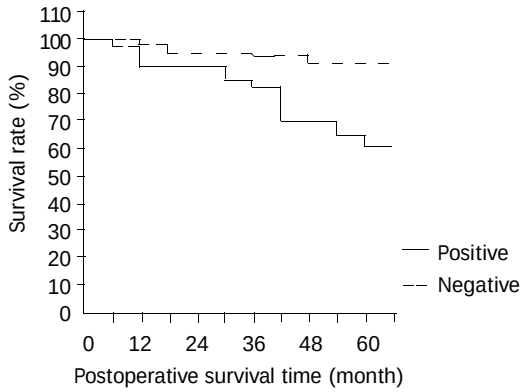

Figure 5 Kaplan-Meier survival curves of patients with gastric carcinoma with regard to VEGF expression (positive and negative), $\chi^2=16.51$, $P<0.01$.

Multivariate analysis

The effects of variables presumably associated with prognosis were studied by multivariate analysis using the Cox model. As a result, the depth of wall invasion, lymph node metastasis, vascular invasion, COX-2 expression, and VEGF expression emerged as independent prognostic factors (Table 4). Among these parameters, VEGF expression was the most important factor for predicting overall survival, followed by lymph node metastasis and COX-2 expression.

Table 4 Risk factors affecting survival determined by multivariate analysis using the Cox proportional hazards model

Variables	Regression coefficient	Standard error	Odds ratio (95% confidence interval)	*P* value
Histology (differentiated / undifferentiated)	0.564	0.337	1.758 (1.583-2.147)	NS
Depth of invasion (EGC / muscularis propria)	0.524	0.248	1.688 (1.638-1.714)	<0.05
Lymph node metastasis (present / absent)	0.796	0.1934	2.220 (1.518-3.239)	<0.01
Vascular invasion (present / absent)	0.413	0.213	2.003 (1.499-2.460)	<0.01
COX-2 expression (positive / negative)	0.776	0.194	2.173 (1.486-3.178)	<0.01
VEGF expression (positive / negative)	1.071	0.254	2.917 (1.774-4.796)	<0.01

Note: NS, not significant.

DISCUSSION

Recently, detection of gastric cancer at an early stage has been widely used in diagnostic procedures such radiography and endoscopy with targeted biopsy. In Japan, more than 50 % patients with gastric carcinoma were EGC[15]. However, in U.S., the proportion of EGC was approximately 20 %[16]. In the current study, our detection rate of EGC (4.3 %) was lower than that in above reports. This indicates the need to upgrade diagnostic efforts in the future. The incidence of adenocarcinoma of the gastric cardia has increased gradually in the West[17]. In our study, carcinoma of the gastric cardia accounted for 16.4 % of EGC and 30.4 % of AGC. The absolute number and the rates of cardia carcinoma have been increasing significantly and this increase may be derived from advances in endoscopic techniques and equipment. Our data also showed that carcinoma of the gastric cardia more frequently occurred in 60-69 age group than distal gastric cancer. Thus, we should pay attention to those patients who are older than 60 years in

the diagnosis of early carcinoma of the gastric cardia during an endoscopic examination.

The prognosis for EGC is universally excellent. Almost all Western and Japanese authors reported 5-year survival rates were over 90 % for EGC if relative survival or deaths from gastric carcinoma alone were considered. The results of the current study indicat that prognosis of patients with AGC was poorer than that with EGC and that prognosis of patients with submucosa invasion was poorer than that with mucosa invasion. The survival of patients with tumors in the upper third of the stomach was significantly worse compared with that of patients with tumors in the middle third and lower third of the stomach[16,18]. Our study disclosed that the 5-year survival rate of patients with tumors in the upper third of the stomach was lower than that of patients with tumors in the middle third and lower third of the stomach, especially in patients with AGC. However, there were no statistically significant differences among them (data not shown).

The depth of wall invasion, lymph node metastasis, and vascular invasion were reported to be the most important prognostic parameters in gastric carcinoma[19]. The current study demonstrated that long or short survival time was highly correlated not only with depth of invasion, lymph node metastasis, and vascular invasion, but also with adjuvant chemotherapy. The results were in agreement with other reports.

However, preoperative diagnosis of the extent of wall invasion or the presence of lymph node metastasis and vascular invasion is difficult in some cases. Therefore, not only conventional clinicopathologic factors but also biologic factors should be examined for the prediction of clinical outcome. Recently, some studies[20,21] found an increase in COX-2 protein levels in gastric carcinoma beyond the levels in paired normal gastric mucosa samples. The present study demonstrated that expression of COX-2 was significantly higher in mucosal atypical hyperplasia than that in CAG and that its expression was significantly higher in carcinoma tissue compared with noncancerous specimens. These indicate that COX-2 is involved in the growth of gastric carcinoma and that COX-2 promotes malignant transformation in human gastric carcinoma.

Recent studies have found that overexpression of COX-2 protein is associated significantly with lymph node metastasis[22,23] and depth of invasion[24,25] and that there is no correlation between the hisologic types of gastric carcinoma and the expression of COX-2 protein[24,26]. We found that COX-2 expression was associated with lymph node metastasis, vascular invasion, and the degree of tumor cell differentiation and did not connect to depth of invasion. The results suggest that COX-2 might enhance the metastatic potential as well as tumorigenicity and might be mainly involved in the progression of well-differentiated gastric carcinoma. The different conclusions of our study and above reports might have two explanations. First, differences in the methods employed (COX-2 mRNA level or protein immunoreactivity) and subjects may well influence the results of these studies. Second, there may have been discrepancies in the histologic type distribution among different areas.

Solid tumors need angiogenesis for growth and metastasis. Tumor angiogenesis may be regulated by angiogenic factors that are secreted by tumor cells, and VEGF is thought to be such a factor[27,28].VEGF is a selective mitogen for endothelial cells and may directly stimulate the growth of new blood vessels[27]. Numerous studies have demonstrated that the expression of VEGF is a significant predictor of an increased risk of metastatic disease and overall survival by stimulating angiogenesis in gastric carcinoma[9,28] and other carcinomas[29]. In this study, we found that VEGF expression and microvessel count were significantly associated with lymph node metastasis, depth of invasion, and vascular invasion. The finding that the microvessel count in VEGF-positive or COX-2-positive tumors was significantly higher than that in VEGF-negative or COX-2-negative tumors suggests that COX-2 as well as VEGF may faciliate tumor progression by promoting tumor angiogenesis[9,30].

With regard to prognosis, many studies have shownd that expression of VEGF is an independent prognostic indicator[12,31]. However, there have been few studies on the association of COX-2 expression and the postoperative survival rate of patients with gastric carcinoma[32]. The current study demonstrated that the 5-year survival rate in patients with COX-2-positive or VEGF-positive tumors was significantly lower than that in patients with COX-2-negative or VEGF-negative tumors. The results suggest that the presence of COX-2 or VEGF expression, as well as conventional clinicopathologic factors, are prognostic indicators in patients with gastric carcinoma. Multivariate analysis revealed five independent prognostic factors. Combination analysis of these pathologic and biologic features of gastric carcinoma will give aid to the improvement of the prognosis of some patients. If these assessments of COX-2 and VEGF expression are confirmed in long term follow-up of a larger group of patients, COX-2 and VEGF staining using endoscopically biopsied specimens prior to surgery could be used for the prediction of clinical outcome and in the preoperative selection of treatment for patients with gastric carcinoma. Accordingly, the inhibition of COX-2 activity may have an important therapeutic benefit in the control of gastric carcinoma[33].

ACKNOWLEDGMENTS

We thank Drs. Li-Xing Zhu, Ji-Fong Wu, Hong-Fu Zhang and Xi-Yu Gong for their excellent technical support.

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Edited by Xu XQ