

• GASTRIC CANCER •

Relationship between the expression of iNOS, VEGF, tumor angiogenesis and gastric cancer

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Abstract

AIM: To investigate the relationship between the expression of inducible nitric oxide synthase (iNOS), vascular endothelial growth factor (VEGF), the microvascular density (MVD) and the pathological features and clinical staging of gastric cancer.

METHODS: Immunohistochemical staining was used for detecting the expression of iNOS and VEGF in 46 resected specimens of gastric carcinoma; the monoclonal antibody against CD34 was used for displaying vascular endothelial cells, and MVD was detected by counting of CD34-positive vascular endothelial cells.

RESULTS: Of 46 resected specimens of gastric carcinoma, the rates of expressions of iNOS and VEGF were 58.70% and 76.09%, respectively, and MVD averaged 55.59 ± 19.39 . Judged by the standard TNM criteria, the rate of expression of iNOS in stage IV (84.46%) was higher than those in stage I, II, III (Fish exact probabilities test, $P=0.019, 0.023$ and 0.033 , respectively); the rates of expression of VEGF in stage III, IV (76.0%, 92.31%, respectively) were higher than those in stage I, II (Fish exact probabilities test, $P=0.031, 0.017, 0.022$ and 0.019). MVDs in stage III, IV ($64.72 \pm 14.96, 67.09 \pm 18.29$, respectively) were higher than those in stage I, II ($t=2.378, 4.015, 2.503$ and $2.450, P<0.05, P<0.001, P<0.001, P<0.05$, respectively). In 37 gastric carcinoma specimens with lymph node metastasis, MVD (68.69 ± 18.07) and the rates of expression of iNOS and VEGF (70.27%, 83.78%, respectively) were higher than those in the specimens with absence of metastasis ($t=2.205, c^2=6.3587, c^2=6.2584, P<0.01, P<0.05, P<0.05$, respectively). MVD and the expressions of iNOS and VEGF were not correlated to the location, size or grade of tumor, nor with the depth of invasion of tumor; MVDs in the positive iNOS and VEGF specimens ($59.88 \pm 18.02, 58.39 \pm 17.73$, respectively) were higher than those in the negative iNOS and VEGF specimens ($c^2=6.3587$ and $6.1574, P<0.05, P<0.05$, respectively); thus the expressions of iNOS and VEGF was correlated to MVD, but the expression of iNOS was not correlated to that of VEGF. In addition, of the 46 surviving patients, the 5-year survival rate of patients with positive iNOS or VEGF tumors was significantly less than that of patients with negative iNOS- or VEGF tumors ($c^2=4.3842$ and $5.4073, P<0.05, P<0.05$, respectively).

CONCLUSION: The expressions of iNOS and VEGF are closely related to tumor angiogenesis, and are involved in the advancement and the lymph node metastasis; thus MVD and the expressions of iNOS and VEGF may serve indexes for evaluating staging of gastric carcinoma and forecasting its risk of metastasis, which will help establish a comprehensive therapeutical measure of post-operative patients and provide a new approach to tumor therapy.

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INTRODUCTION

Gastric carcinoma, as one of the most common human malignant tumor, ranks worldwide as the first leading cause of gastrointestinal cancer-related mortality. In China, it now ranks the second. It had been shown that tumor angiogenesis played an important role in its growth, invasion, metastasis and recurrence^[1-9]. We studied the relationship between the expression of iNOS, VEGF, MVD and the pathological features, lymph node metastasis and clinical staging of gastric carcinoma, and evaluated the relationship between tumor angiogenesis and the expression of iNOS, VEGF as well as the relationship between tumor angiogenesis and the advancement and the metastasis of gastric cancer using immunohistochemical staining method in order to reveal the biological features of iNOS and VEGF, which will contribute to further understanding of oncogenesis and provide a new approach to tumor therapy.

MATERIALS AND METHODS

Materials

The resected specimens from 46 cases of gastric cancer confirmed pathologically were obtained from our hospital from January 1999 to October 2000. Of these, 35 patients were male, and 11 female, with a mean age of 56.96 ± 11.26 (32 to 78). All of them had not received any radiotherapy or chemotherapy. Among these specimens, 8 were situated in the upper third of the stomach, 13 in the middle third, and 25 in the lower third. Histologically, they were classified by the WHO criteria, 5 were highly differentiated adenocarcinoma, 10 moderately-differentiated, 28 poorly-differentiated, 2 undifferentiated, and 1 was gastric mucous adenocarcinoma. As regards to the size of cancer, 2 were <3 cm, 18.3-5 cm, 26 >5 cm. 34 tumors invaded to the serosa and 12 tumors did not. By TNM staging of UICC, 2 cases were in stage I, 6 in stage II, 25 in stage III, and 13 in stage IV. Only 37 cases had local lymph node metastasis.

Reagents and methods

Antibody against iNOS was purchased from Wu Han Boster Co. Ltd; antibodies against VEGF and CD34 and ready-to-use

SP immunohistochemical reagent box were purchased from FuJian Maxin Co. Ltd. Formalin-fixed, paraffin-embedded surgical specimens from 46 cases of gastric carcinoma were available and sliced sequentially with a thickness of 4 μ m. The slices carrying the detected antigen were dyed with SP immunohistochemical staining method, and those in the control group were dyed according to the above method, with the first antibody substituted by PBS.

Statistical methods

The data were presented as $x \pm s$; numerical variable by the chi-square test; enumeration data by t test; the differences of these groups were compared by analysis of variance.

RESULTS

The cytoplasm of the gastric cancer cells staining brown granules were identified to be positive iNOS or VEGF, and the slices were graded respectively according to the density and the percentage of positively stained gastric carcinoma cells into score 0,1,2 or 3. If the sum of two scores was 0-2, the slice would be considered as the negative iNOS or VEGF; whereas 3-6, it would be considered as positive iNOS or VEGF. When the cytoplasm of theirs stained brown or brownish yellow, vascular endothelial cells were CD34- positive; the microvessels were counted according to the number of single endothelial cell or endothelial cell cluster showing brownish yellow granules in the cytoplasm. The slices were observed first microscopically under the low power ($\times 40$), then selected the most dense area of microvessel under the high power ($\times 200$, the surface area of every vision field being 0.785 mm²), and the number of microvessel in 3 vision fields were counted and took the average as MVD of this specimen^[10]

The relationship between the expressions of iNOS and VEGF, MVD and pathological features of gastric carcinoma

The positive iNOS and VEGF stained were located at brownish yellow stained granules in the cytoplasm. The positive expression rate of iNOS was 58.7% (27/46) and that of VEGF was 76.09% (35/46). In addition, the positive expression of CD34 was mainly presented at brownish yellow or brownish granules in the cytoplasm of vascular endothelial cell. In all cases, MVD was 20.7 to 81.7 per vision field of high power with an average of 55.59 ± 19.39 .

As shown in Table 1, MVD and the rate of expression of iNOS in gastric carcinoma tissue had no significant differences among the site, the size, the degree of differentiation and the depth of invasion of gastric cancer. MVD and the rate of expression of iNOS in cases having lymph node metastasis was significantly higher than those having no lymph node metastasis ($t=2.205$, $\chi^2=6.3587$, $P<0.05$, $P<0.05$, respectively). The rate of expression of VEGF in the gastric carcinoma tissue also had no significant differences among the site, the size, the degree of differentiation of gastric cancer. The rate of expression of VEGF in the gastric carcinomas invading serosa was higher than that failed to invade serosa ($\chi^2=6.2584$, $P<0.05$). Likewise, the rate of expression of VEGF in cases having lymph node metastasis was significantly higher than cases having no lymph node metastasis ($\chi^2=6.1574$, $P<0.05$).

The relationship between the expression of iNOS, VEGF, MVD and TNM staging of gastric carcinoma

As shown in Table 2, the rates of expression of iNOS, VEGF, MVD, the tumor angiogenesis were all related to the clinical staging of gastric carcinoma, and increased with the progression of disease.

Table 1 The relationship between the expressions of iNOS and VEGF, MVD and pathological features of gastric carcinoma

Pathological characteristics	MVD ($\bar{x} \pm s$)	positive iNOS (%)	positive VEGF (%)	total
Site of gastric cancer lesion				
Upper one third	61.50 \pm 14.50	5(62.50)	4(50.0)	8
Middle one third	59.82 \pm 16.54	8(61.54)	10(76.92)	13
Lower one third	54.52 \pm 20.01	14(56.00)	21(84.0)	25
Size of tumor				
< 3 cm	61.50 \pm 20.51	1(50.0)	2(100.0)	2
3~5cm	59.78 \pm 16.58	11(61.11)	14(77.78)	18
> 5cm	54.86 \pm 17.99	15(57.69)	19(73.08)	26
Depth of invasion				
Invading serosa	62.35 \pm 32.97	22(64.71)	29(85.29) ^c	34
Not invading serosa	55.15 \pm 18.28	5(41.67)	6(50.0)	12
Metastasis of lymph nodes				
Positive	68.69 \pm 18.07 ^a	26(70.27) ^b	31(83.78) ^d	37
Negative	54.40 \pm 14.23	3(33.33)	4(44.44)	9
Degree of differentiation*				
Well differentiated	49.49 \pm 20.10	10(66.67)	11(73.33)	15
Poorly differentiated	59.24 \pm 16.80	17(54.84)	24(77.42)	31

Note: Well differentiated cancer cells include highly and moderately differentiated ones; poorly differentiated cancer cells include poorly differentiated and undifferentiated ones and mucous adenocarcinoma. ^a $P<0.01$ ($t=2.205$), vs MVD in cases having no lymph node metastasis; ^b $P<0.05$ ($\chi^2=6.3587$), vs the rate of expression of iNOS in cases having no lymph node metastasis; ^c $P<0.05$ ($\chi^2=6.2584$), vs the rate of expression of VEGF in gastric carcinomas not invading to serosa; ^d $P<0.05$ ($\chi^2=6.1574$), vs the rate of expression of VEGF in cases having no lymph node metastasis.

Table 2 The relationship between the expression of iNOS, VEGF, MVD and TNM staging of gastric carcinoma

Clinical staging	n	Expression of iNOS (%)	Expression of VEGF (%)	MVD ($\bar{x} \pm s$)
Stage I	2	1(50.0)	1(50.0)	51.00 \pm 7.0 ^d
Stage II	6	2(33.33)	3(50.0)	47.66 \pm 8.71 ^e
Stage III	25	13(52.0)	19(76.0) ^b	64.72 \pm 14.96 ^f
Stage IV	13	11(84.62) ^a	12(92.31) ^c	76.09 \pm 18.29 ^g

^a $P<0.05$, (Fish exact probabilities test, $P=0.019$, 0.023 and 0.033), vs the rate of expression of iNOS in stage I, II and III, respectively; ^b $P<0.05$ (Fish exact probabilities test, $P=0.031$ and 0.017), vs the rates of expression of VEGF in stage I and II, respectively; ^c $P<0.05$ (Fish exact probabilities test, $P=0.022$ and 0.019), vs the rates of expression of VEGF in stage I and II, respectively; ^d $P<0.05$ ($t=2.378$, vs MVD in stage III; $P<0.001$ ($t=4.015$), vs MVD in stage IV; $P<0.001$ ($t=2.503$), vs MVD in stage I); ^e $P<0.05$ ($t=2.450$), vs MVD in stage I.

The relationship between MVD and the rates of expression of iNOS and VEGF

The above results show that MVD (59.88 ± 18.02) in the iNOS-

positive gastric tissue was higher than that (49.64 ± 12.06) in the iNOS-negative one ($t=3.980, P<0.05$); and MVD (58.39 ± 17.73) in the VEGF-positive gastric tissue was higher than that (45.43 ± 18.21) in the VEGF-negative one ($t=4.098, P<0.05$), suggesting that the expressions of iNOS and VEGF were related to MVD and tumor angiogenesis.

The relationship between the expressions of iNOS and VEGF and the prognosis of gastric carcinoma

As shown in Table 3, among 46 patients, the 5-year survival rate of the patients with iNOS- or VEGF-positive tumors was significantly less than that of the patients with iNOS- or VEGF-negative tumors ($\chi^2=4.3842$ and $5.4073, P<0.05, P<0.05$, respectively).

Table 3 The relationship between the expressions of iNOS and VEGF and the prognosis of gastric carcinoma

	n	survival period		rate of five-year survival (%)
		< 5 years	> 5 years	
iNOS expression				
Positive	27	22	5	18.52 ^a
Negative	19	10	9	47.37
VEGF expression				
Positive	35	30	5	14.29 ^b
Negative	11	5	6	54.55

^a $P<0.05, (\chi^2=4.3842)$, vs the iNOS-negative gastric cancer patients;

^b $P<0.05, (\chi^2=5.4073)$, vs the VEGF-negative gastric cancer patients.

DISCUSSION

MVD is related to the increase of the risk of metastasis and/or the decrease of survival period of gastric carcinoma patients^[11], and being a reliable index of tumor angiogenesis^[12]. In the present study, we labeled the vascular endothelial cells with monoclonal antibody against CD34 and detected MVD in all specimens by immunohistochemical staining method, and finally found MVD averaging 55.59 ± 19.39 per vision field of high power, indicating active tumor angiogenesis. In addition, MVD in the specimens having lymph node metastasis was significantly higher than that having no metastasis, suggesting that increase of MVD and tumor angiogenesis in gastric carcinomas might result in cancer cells entering into the blood circulation, and the lymph node metastasis could be promoted when the gastric cancer cells invade lymphatic vessels. In 46 resected gastric cancer specimens, MVDs in stage III and IV were significantly higher than those in stage I and II ($t=2.378, 4.015, 2.503$ and $2.450, P<0.05, P<0.001, P<0.001, P<0.05$, respectively), indicating MVD was closely related to clinical staging of gastric carcinoma, and MVD and tumor angiogenesis increased with the invasion of gastric cancer. This result reveals MVD may reflect the advancement of gastric carcinoma and the extent of tumor angiogenesis and metastasis^[13]; thus it can serve an important index forecasting the prognosis of gastric cancer^[5, 14].

It is shown that the expression of iNOS in most tumor tissue is higher than that in the normal one^[15]; Nitric oxide produced through iNOS induction may increase the vascular permeability and accelerate the nutrient supply of tumor tissue and finally promote the tumor growth^[16, 17]. In this study, we found that the rate of expression of iNOS of gastric carcinoma in stage IV

was higher than those in stage I, II and III (Fisher exact probabilities test, $P=0.019, 0.023$ and 0.033 , respectively), revealing the expression of iNOS of gastric cancer increased with staging of the cancer, and was higher in late stage^[18], the higher the expression of iNOS of gastric cancer, the more the advancement and the worse the prognosis^[19, 20]. We also found that the rate of expression of iNOS of gastric carcinoma in those having lymph node metastasis was higher than that having no metastasis ($\chi^2=6.3587, P<0.05$), suggesting the significant increase of its expression in the gastric cancer tissue can promote its lymph node metastasis.

VEGF plays an important role in each stage of tumor angiogenesis^[21, 22], and its over expression is closely related to clinical staging, lymph node metastasis and recurrence of gastric carcinoma^[23, 24]. In the present study, we found that the rate of expression of VEGF was related to the depth of invasion, it was higher in gastric cancers with the invasion of serosa than in gastric cancers without that ($\chi^2=6.2584, P<0.05$), indicating VEGF may contribute to the invasive growth of gastric carcinoma, and is relevant to the lymph node metastasis^[28]. These are corroborated in stage III or IV lesions. These results show the over expression of VEGF in the gastric cancer tissue has prognostic significance^[25-47].

VEGF produced by tumor cell can bind with the surface acceptor of vascular endothelial cell, and promote the production of nitric oxide that can transmit messages between the cells and induce tumor angiogenesis^[48, 49]. This study showed that the rates of expressions of iNOS and VEGF of gastric cancer were related to MVD ($t=3.980$ and $4.098, P<0.05, P<0.05$, respectively), indicating iNOS and VEGF were closely related to tumor angiogenesis^[50, 51], and might be important factors involved in gastric carcinoma angiogenesis. Moreover, iNOS and VEGF and their effects on angiogenesis can promote the lymph node metastasis and the prognosis, thus iNOS, VEGF and MVD may all serve important indexes reflecting the biological behaviors, advancement and prognosis of gastric carcinoma.

In conclusion, active angiogenesis exists in the gastric cancer tissue, and MVD is closely relevant to lymph node metastasis, clinical staging and advancement of gastric carcinoma and may act as a valuable index of gastric cancer prognosis; the rates of expressions of iNOS and VEGF in gastric carcinoma are higher than those in the normal gastric tissue, and are related to lymph node metastasis and clinical staging, suggesting they are involved in the advancement and metastasis of gastric cancer which are relevant to its prognosis^[52]; the rates of expressions of iNOS and VEGF in the gastric cancer tissue are closely related to MVD, and they may be important factors involved in gastric carcinoma angiogenesis, thus iNOS, VEGF and MVD can act as important indexes reflecting the biological behaviors, advancement and prognosis of gastric cancer. In addition, further study on the mechanism of their regulation will probably offer a new approach to anticancer treatment.

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