

RESEARCH ARTICLE

Lymphangiogenic and Angiogenic Microvessel Density in Chinese Patients with Gastric Carcinoma: Correlation with Clinicopathologic Parameters and Prognosis

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Abstract

The incidence of gastric cancer worldwide, and in particular in developing countries, has shown a marked increase. Poor prognosis of gastric cancer patients occurs due to the rapid metastasis of the disease via the lymphatic and blood vessels. The aim of this study was to investigate the expression and the clinical significance of D2-40 and CD34 in human gastric cancer. D2-40 and CD34 expression was detected in 1,072 cases of Chinese patients with gastric carcinoma using immunohistochemistry. The lymphatic vessel density (LVD) and microvessel density (MVD) were calculated and analyzed and the correlation with the clinicopathological factors and prognosis was determined. The LVD and MVD of the gastric cancer cases were significantly higher compared to those of normal tissues ($P < 0.05$). The expression of D2-40-LVD and CD34-MVD in the malignancies were positively related to the age, tumor size, invasion depth, lymphatic metastasis and pathological tumor-node-metastasis (pTNM) ($P < 0.05$); However, no statistically significant difference was identified between them with the patient gender ($P > 0.05$). Up-regulation of D2-40 and CD34 expression was significantly correlated with the poor survival rate in univariate and multivariate analyses. The LVD marked by D2-40 and the MVD marked by CD34 were positively correlated to the clinicopathological factors of the malignancies and may play important role in the development and progression of gastric cancer.

Keywords: Gastric cancer - D2-40 - LVD - immunohistochemistry - progression

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Introduction

The incidence of gastric cancer worldwide, and in particular in developing countries, has shown a marked increase (Inal et al., 2012). Poor prognosis of gastric cancer patients occurs due to rapid metastasis of the disease via the lymphatics as well as blood in advanced stages. Previous studies have shown that lymphatic and blood vessel invasion (LBVI) is closely correlated with characteristics of gastric cancer patients, including tumor stage, lymph node metastasis and histological type, which may be used as the indices to determine the metastasis and prognosis of gastric cancer patients (del Casar et al., 2008; Kim et al., 2010; Du et al., 2012). The antibodies D2-40 and CD34 are characterized as specificity labels of the endothelial cells of the lymphatic and blood vessels. However, the correlation of D2-40 and CD34 antibodies with clinicopathological characteristics and prognosis has yet to be adequately reported in the literature. Immunohistochemical staining was used to detect the malignant tissue using D2-40 and CD34 antibodies,

and the D2-40 positive lymphatic vessel density (LVD) and CD34 positive microvessel density (MVD) were measured to detect the invasion and metastasis of tumor cells in lymphatic and blood vessels. Therefore, the aim of this study was to examine the expression of D2-40 and CD34 in gastric cancer tissue and the correlation with clinical pathological parameters, thereby providing an experimental and theoretical basis for the prognosis of gastric cancer.

Materials and Methods

Patient specimens and tissue microarray construction
Specimens from a total of 1072 cases of gastric cancer were collected postoperatively and filed by the Department of Pathology of Changhai and Changzheng Hospital (Shanghai, China), and tissue chips were successively established. The patients included 757 males and 315 females; median, 59 years. Complete follow-up records were available for more than 5 years. In addition, corresponding non-tumor normal tissues were also

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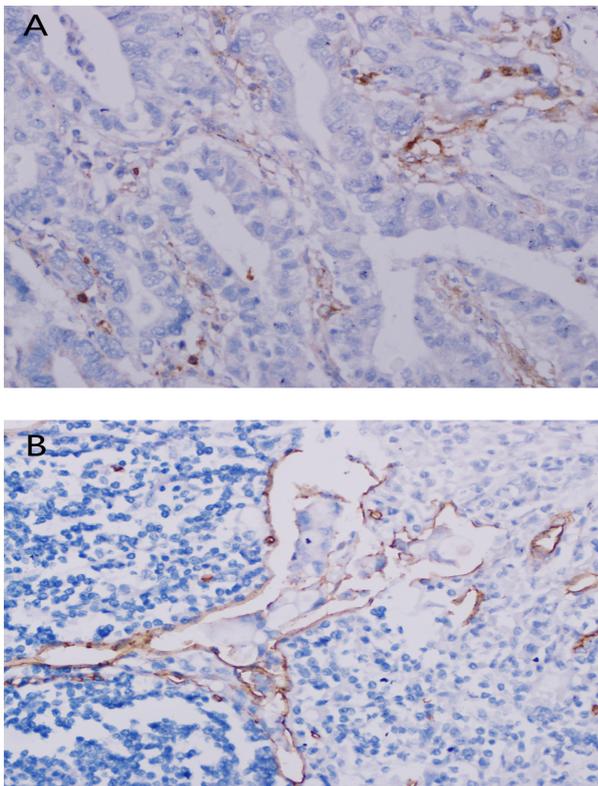


Figure 1. Analysis of Lymphatic Vessel Density (LVD) and Microvessel Density (MVD) in Human Gastric Cancer. (A) A sample of gastric cancer with high LVD, assessed by anti-D2-40 immunostaining. (B) A sample of gastric cancer with high MVD, assessed by anti-CD34 immunostaining. (Original magnification, x400)

collected from these patients as the control group. The pathological results were confirmed by two pathologists to ensure the quality of tissue blocks. Two paraffin-embedded tissue microarray blocks of normal and tumor specimens obtained from the patients were created using a manual tissue arrayer (Beecher Instruments, Sun Prairie, WI, USA). The tissue microarray consisted of 1072 primary tumor sites, which had one 1.5-mm core of non-neoplastic mucosal tissue and one 1.5-mm core of primary tumor tissue.

Main reagents

Source of antibodies: Mouse anti human D2-40 monoclonal antibody (instant type), mouse anti human CD34 monoclonal antibody (instant type) and SP immunohistochemical kit all are bought from Fuzhou Maixin Biotech Development Co.,Ltd.

Immunohistochemistry and evaluation of immunostaining

The SP immunohistochemical technology was utilized as described below. Specimens were routinely de-waxed and hydrated. Citrate buffer solution was added and heated in a microwave for antigen retrieval, then 3% H₂O₂ endogenous peroxidase inhibitor was dripped. Animal non immune serum was then incubated for 10 min, rinsed with phosphate-buffered saline (PBS) solution, and D2-40 and CD34 primary antibodies were dripped and maintained overnight at 4°C. On the second day, secondary antibodies and streptomycin avidin peroxidase solution were dripped,

stained with diaminobenzidine (DAB) and restained with hematoxylin. Sections were then dried and stored. PBS was used to replace the primary antibody and served as the negative control.

LVD and MVD were measured according to the Weidner method (Weidner, 1995). Sections were visualized under a low-power microscope (magnification, x100) and observed by two doctors blinded to the clinicopathological data. The 'hot spot' area with the highest density was identified, then three views for each case were measured under a high power microscope (magnification, x200) and the mean thereof was considered as the LVD or MVD of each case. Endothelial cells or endothelial cell clusters were measured as one blood capillary or lymphatic vessel.

Statistic analysis

Data are presented as the mean \pm standard deviation (SD). Analyses were performed using the SPSS statistical software program for Windows (SPSS Inc., Chicago, IL, USA). Measured data were tested using the χ^2 test. Comparison of the means was performed using the two-sample group design t-test. Comparison of the means of multiple samples was analyzed using one-factor analysis of variance. The Kaplan Meier method was used to estimate survival rates and the Cox proportional hazards model for the multivariate survival analysis was employed to assess predictors associated with survival. Two-sided $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of D2-40 and CD34 in the gastric cancer tissue

D2-40 was markedly expressed in gastric tissue with a positive rate of 73.8% (791/1072). The endothelial cell membrane and cytoplasm in the stained lymphatic vessel were brown yellow, mainly located in the mesenchymal tissue surrounding the tumor and had irregular shapes, such as dilated polygons and closed strips. In the control group, the positive expression rate of D2-40 was 24.3% (261/1072), which was below the expression rate ($\chi^2 = 524.250$, $P = 0.000$) in the gastric cancer group, while the positive expression rate of CD34 in the gastric cancer tissue was 79.5% (852/1072). The endothelial cell membrane and cytoplasm in the blood vessel appeared brown or brown yellow with a clearly shaped cavity of sinusoid dilatation or in the shape of closed strips. In the control group, the positive expression rate of CD34 was 29.9% (321/1072), below that of the gastric cancer group ($\chi^2 = 530.758$, $P = 0.000$; Figure 1A and B).

Correlation of D2-40 and CD34 expression with clinicopathological factors

In the 1072 cases of gastric cancer tissues, the positive rate of D2-40 was 73.8% and the LVD was 10.28 ± 5.21 micro-lymphatic vessels/field; while in the control group, the positive rate of D2-40 was 24.3% and the LVD was 4.18 ± 2.13 micro-lymphatic vessels/field ($P = 0.000$). In gastric cancer tissues, LVD was correlated with tumor size, invasion depth, lymph node metastasis and tumor-node-metastasis (TNM) stage ($P < 0.05$), but not gender

Table 1. Correlation Between LVD/MVD and Various Clinicopathological Features (mean \pm SD)

Features	n	MVD	P	LVD	P
Age(years)			<0.05		<0.05
<60	537	42.50 + 14.06		9.64 + 5.33	
>60	535	45.61 + 13.65		10.89 + 5.18	
Sex			0.144		0.097
Male	757	42.48 + 16.30		9.63 + 5.34	
Female	315	44.01 + 13.85		10.23 + 5.42	
Tumor size			<0.05		<0.05
<6cm	825	38.23 + 15.12		8.21 + 4.90	
>6cm	247	51.12 + 13.10		11.05 + 5.41	
Invasion depth			<0.05		<0.05
T1	151	24.05 + 14.28		4.96 + 3.86	
T2	161	35.26 + 12.47		8.82 + 4.72	
T3	664	43.26 + 11.37		11.52 + 5.10	
T4	96	55.52 + 9.04		10.13 + 6.55	
Lymphatic metastasis			<0.05		<0.05
N0	356	37.41 + 14.87		6.80 + 4.34	
N1-3	716	44.20 + 12.41		9.90 + 5.18	
TNM stage			<0.05		<0.05
I	217	27.44 + 14.20		4.93 + 4.09	
II	210	39.68 + 4.14		8.42 + 3.90	
III	469	43.21 + 9.42		11.50 + 4.42	
IV	176	55.85 + 11.02		14.19 + 5.12	

Table 2. Cox Proportional Hazards Model Analysis of Prognostic Factors

	Be	SE	Wald	Exp(B)	95.0% CI		Sig.
					Lower	Upper	
Age	0.257	0.077	11.103	1.294	1.112	1.505	<0.05
Sex	0.251	0.0861	8.462	1.285	1.085	1.522	0.054
Tumor size	0.336	0.054	38.438	1.399	1.258	1.556	<0.05
Invasion depth	1.048	0.072	213.092	2.853	2.478	3.284	<0.05
Lymph metastasis	0.954	0.044	476.499	2.595	2.382	2.827	<0.05
TNM	1.137	0.053	468.104	3.116	2.811	3.454	<0.05
MVD	1.232	0.094	170.802	3.426	2.849	4.121	<0.05
LVD	1.437	0.097	221.179	4.206	3.481	5.083	<0.05

SE, standard error /CI, confidence interval

and age ($P > 0.05$; Table 1).

In the 1072 cases of gastric cancer tissues, the positive rate of CD34 was 79.5% and the MVD was 43.92 ± 13.94 micro-blood vessels/field, while in the control group, the positive rate of CD34 was 29.9% and the MVD was 5.31 ± 1.45 micro-blood vessels/field, indicating a significant difference ($P = 0.000$). With the exception of gender, the differences from the comparisons among the groups with regard to parameters such as age, tumor size, invasion depth, lymph node metastasis and TNM stage ($P < 0.05$) were statistically significant ($P < 0.05$; Table 1).

Expression of D2-40 and CD34 with survival prognosis of patients with gastric cancer

The 1072 cases of patients with complete follow-up records were respectively classified into the multi-lymphatic and few-lymphatic vessel groups, as well as the multi-blood and few-blood vessel groups, with medians of LVD and MVD. Results of the Kaplan Meier survival rate analysis suggested that the median survival time of the few lymphatic and multi-lymphatic vessel groups was 64 and 32 months, respectively, whereas that of the few-blood and multi-blood vessel groups was 63 and 33 months, respectively. Differences were statistically significant ($P < 0.05$; Figure 2A and B).

Results of the Cox proportional hazards model analysis

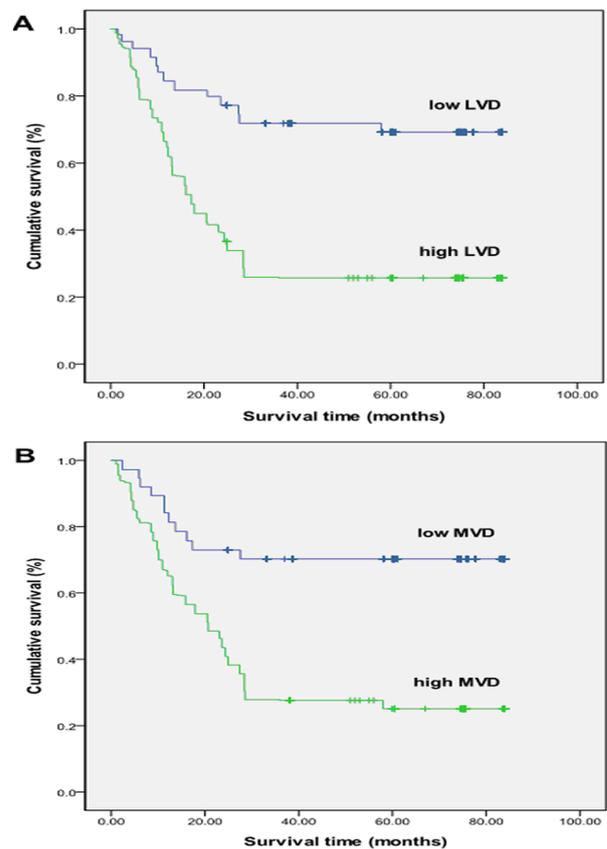


Figure 2. Kaplan-Meier Curves of Survival Durations in Patients with Gastric Cancer Treated with Curative Surgery. (A) Patients with high lymphatic vessel density (LVD) expression had shorter survival durations than did patients with low LVD expression in their gastric cancers ($P < 0.05$). (B) Patients with high microvessel density (MVD) expression had shorter survival durations than did patients with low MVD expression in their gastric cancers ($P < 0.05$)

demonstrated that tumor size, invasion depth, lymph node metastasis, TNM stage in gastric cancer, as well as the expression of MVD and LVD are all independent prognostic factors affecting the survival of patients with gastric cancer ($P < 0.05$; Table 2).

Discussion

In recent years, the incidence of gastric cancer worldwide, and in particular in developing countries like China, has shown a marked increase and has become a worldwide health burden. Statistics show that, in 2008, there were 989,600 new cases of gastric cancer, with 738,000 patients succumbing to the disease. Thus, the disease poses a major health concern (Milne et al., 2009; Jemal et al., 2011). LBVI is the main pathway for the metastasis of gastric cancer, with lymph node metastasis being the principal pathway of invasion for gastric cancer in the early stages and is closely associated with poor prognosis in patients with gastric cancer. Therefore, blocking lymphatic and blood vessel metastasis is effective in controlling gastric cancer invasion, metastasis and relapse.

D2-40 is a type of IgG antibody belonging to sialomucin that is distributed in the cell membrane and has a molecular mass of 38 kDa. It has a potential

specificity combination with one antigen fixed on the epitope in the glycoprotein M2A expressed by lymphatic vessel endothelial cells by identifying it in order to denote the lymphatic vessel endothelial cells; it also has strong specificity and sensitivity (Breiteneder-Geleff et al., 1999; Marks et al., 1999; Kaiserling, 2004). The results of this study have shown that D2-40 is expressed in gastric cancer and non-cancer normal tissue, with a positive rate of 73.8% for D2-40 in the gastric cancer tissue, and LVD in gastric cancer cases (10.28 ± 5.21) is markedly higher than that (4.18 ± 2.13) in the non-cancer normal tissue ($P = 0.000$). Additionally, the positive LVD was mainly located in the tissue area surrounding the tumor exhibiting irregular shapes such as dilated polygons, whereas the lymphatic vessels located in the central area were mainly small cavity or closed strips. Padera et al (Padera et al., 2002) observed through animal model experiments that the lymphatic vessels in the central area of the cancer, subject to the compression of the reproduced tumor cells, decrease in size, collapse and appear as closed shapes and in a functionless state. The lymphatic vessels surrounding the tumor, due to low pressure, are beneficial to the formation of micro-lymphatic vessels and thus become functional lymphatic vessels and play a role in the metastasis of tumors in the lymphatic duct. This observation is consistent with the results of this experiment. Results of the statistical analyses demonstrate that LVD in gastric cancer tissues is correlated with age, tumor size, invasion depth, lymph node metastasis and TNM stage ($P < 0.05$), but not gender ($P > 0.05$). Moreover, gastric cancer patients with high LVD have a notably poor prognosis ($P < 0.05$).

Various studies have indicated that the growth and spread of tumors are closely associated with newly formed blood vessels that provide blood and gas nutrition for the growth and reproduction of tumor tissues, as well as reliable paths for the invasion and metastasis of tumor cells. CD34 is a type of blood vessel endothelial cell label with an extremely high specificity; by combining with the corresponding endothelial cells via the antibody, CD34 may directly reflect the generation status of MVD (Teo et al., 2003; Vieira et al., 2005). Findings of the present study have demonstrated that the positive expression rate of CD34 in the gastric cancer tissue was 79.5%. Similarly, the MVD (43.92 ± 13.94) of CD34 in gastric cancer was markedly higher than that of the MVD (5.31 ± 1.45) of CD34 in the non-tumor normal tissue, indicating a significant difference ($P = 0.000$). The results of this study have shown a marked increase in newly born blood vessels in gastric cancer tissue, as well as an elevated presence in the area surrounding the cancer nest; the older the patients get, the larger the tumor focus, the deeper the invasion, the more the number of the transferring lymphatic nodes, the more the gastric cancer micro-blood vessels of the advanced TNM stage and the worse the survival prognosis ($P < 0.05$).

To sum up, lymphangiogenesis and angiogenesis are two common pathways by which tumor cells spread (Zhang et al., 2009), the positive expression of D2-40 and CD34 is associated with the marked positivity of the malignant biologic action of the gastric cancer. It has

been previously reported that LVD and MVD also result in poor prognosis in invasive ductal carcinoma (IDC) and colorectal cancer (Saad et al., 2006; El-Gohary et al., 2008), which is consistent with the results of this study. Therefore, D2-40 and CD34 are crucial to the generation and development of gastric cancer and may be used as effective indices to evaluate gastric cancer invasion, metastasis and prognosis.

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