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RAPID COMMUNICATION

Relationship between vascular invasion and microvessel density and micrometastasis

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Abstract

AIM: To evaluate the relationship between vascular invasion and microvessel density (MVD) of tissue and micrometastasis in blood.

METHODS: Vascular invasion was detected by both hematoxylin and eosin staining and immunohistochemiscal staining. Blood samples were collected from 17 patients with vascular invasion and 29 patients without vascular invasion and examined for cytokeratin20 (CK20) expression by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Microvessel density of tissue samples was also determined by immunohistochemistry using antibodies to CD105.

RESULTS: CK20 was detected in 12 of the 17 patients with vascular invasion and in 9 of the 29 patients without vascular invasion. Positive RT-PCR was significantly correlated with vascular invasion (70.6% *vs* 30.0%, $P < 0.05$). The average MVD was significantly higher in patients with positive vascular invasion than in patients with negative vascular invasion (29.2 ± 3.3 *vs* 25.4 ± 4.7, $P < 0.05$). The vascular invasion detected with hematoxylin-eosin staining was less than that with immunohistochemical staining. There was a significant difference between the two staining methods (19.6% *vs* 36.9%, $P < 0.05$).

CONCLUSION: Positive CK20 RT-PCR, depth of tumor invasion, lymph node status, metastasis and MVD are significantly correlated with vascular invasion. Immunohistochemical staining is more sensitive than hematoxylin-eosin staining for detecting vascular invasion.

INTRODUCTION

Vascular invasion is one of the most important clinicopathologic characteristics of malignant tumor. Since the initial report by Brown and Warren in 1938 demonstrating an increased visceral metastasis in rectal cancer patients with vascular invasion, a number of investigators have examined the influence of vascular invasion by colorectal cancer[1].

The presence of vascular invasion which is not a consistent finding is associated with an increased incidence of lymph node and distant metastasis and a corresponding decrease in survival[1]. Since polymerase chain reaction (PCR) invented by Mullis in 1989, it has become a standard and mature laboratory technique to detect micrometastasis in patients with malignant tumor[2]. In this study, we detected cytokeratin20 (CK20) mRNA expression in portal system blood[3-5] and microvessel density (MVD) of tissue to evaluate the relationship between vascular invasion and MVD[6] of tissue and metastasis in blood.

MATERIALS AND METHODS

Blood and tissue samples

Portal system blood was obtained before operation from 27 gastric cancer patients and 19 colorectal cancer patients. A venous catheter was inserted into the right gastric omental veins of gastric cancer patients and corresponding veins of colorectal cancer patients and blood samples were collected. The initial 5 mL blood was discarded to reduce possible contamination and the following 5 mL of blood drawn using a new syringe, was used for RNA extraction[7].

Tissue samples from 27 gastric cancer patients and

Table 1 Oligonucleotide primers

cDNA	Primer	Sequence	Product length (bp)
CK20	Outer sense	5'-GAGGTTCAAC TAACGGAGCT-3'	253
	Outer antisense	5'-TCTCTCTTCCA GGGTGCTTA-3'	
	Inner sense	5'-GCCTTGAGATA GAACTCCAG-3'	
	Inner antisense	5'-ACGTCTTCTCC TTCCAGAAG-3'	
GAPDH	Sense	5'-CAGGGCTGCTT TTAACTCTG-3'	385
	Antisense	5'-CTGTTGTCGGAG TTCTAGTAG-3'	

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

19 colorectal cancer patients were formalin-fixed and parafin-embedded. The tissue samples were cut into 1 μm-thick sections, mounted onto slides coated with polylysin and examined with hematoxylin-eosin and immunohistochemical staining.

Detecting vascular invasion

Vascular invasion examined with hematoxylin-eosin staining was defined either by the presence of neoplastic cells with fibrin clots, erythrocytes, or both in endothelial cell-lined spaces without erythrocyte extravasation in the surrounding tissues or by the presence of neoplastic cells within the smooth muscle cell-lined spaces[8]. Vascular invasion examined with immunohistochemical staining was defined by the presence of at least one tumor cell cluster which was clearly visible in decorated vascular spaces where endothelial cells were stained brown[9]. According to the immunohistochemical staining, the fibrin clots or erythrocytes surrounding neoplastic cells should be considered. Vascular invasion was confirmed by at least one staning method.

Detecting CK20 mRNA in portal system blood

Isolation of mononuclear cells: Blood mononuclear cells (MNCs) were isolated by density gradient centrifugation through Ficoll-Hypaque, and washed twice with phosphate-buffered saline (PBS). Cell pellets were snap frozen in liquid nitrogen and stored at -80℃ until use.

RNA extraction: Total RNA was extracted from the MNC pellets with TRIzol reagent (Invitrogen Biotech, USA) according to the manufacturer's instructions.

Reverse transcriptase: An aliquot of 2 μg MNC RNA was pre-incubated with 0.5 μg of oligo(dT)$_{15}$ primer in 14 μL solution for 5 min at 70℃. After chilling on ice, 6 μL of 5-fold synthesis buffer, 25 U of RNase inhibitor, 1.5 μL of dNTPs (final concentration of 0.5 mmol/L) and 200 U of Moloney murine leukemia virus (M-MLV) reverse transcriptase were added. The reaction mixture was then incubated for 60 min at 42℃. The reaction was terminated by heating at 95℃ for 5 min.

Table 2 Comparison between HE and immunohistochemical staining

	Vascular invasion		χ^2	*P*
	(+)	(-)		
HE staining	9	37	19.087	< 0.05
Immunohistochemical staining	17	29		

McNemar's test for correlated proportions, $\chi^2 = 8.003$, $P < 0.05$ *vs* immunohistochemical staining.

Polymerase chain reaction (PCR): PCR was carried out as described previously[10]. The sequences of primers used are shown in Table 1. To distinguish from contaminating genomic DNA, we selected both upstream and downstream primers at different exons. Integrity of the isolated RNA was demonstrated by RT-PCR analysis of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR products were visualized after electrophoresis with ethidium bromide staining under a UV transilluminator.

Detecting microvessel density of tissue

CD105 antigen was detected by immunohistochemistry on a separate slide using a monoclonal mouse antibody following a standard protocol. Microvessel density was assessed as previously described[11].

Statistical analysis

Statistical analysis was performed using the likelihood chi-squared analysis, Fisher's exact test or Student's *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

Detection of vascular invasion

Vascular invasion was detected in 9 patients with hematoxylin-eosin staining and in 17 patients with immunohistochemical staining. There was a significant difference in vascular invasion detected by the two methods (Table 2, Figure 1A and B).

Relationship between vascular invasion, MVD and micrometastasis

CK20 was detected in 12 of the 17 patients with vascular invasion and in 9 of the 29 patients without vascular invasion. Positive RT-PCR was significantly correlated with vascular invasion. The average MVD was significantly higher in patients with positive vascular invasion (29.2± 3.31) than in those with no vascular invasion (Tables 3 and 4, Figure 2).

Comparison of clinicopathologic features

Clinicopathologic features such as depth of invasion, lymph node status and metastasis were associated with the presence of vascular invasion (Table 3).

DISCUSSION

Since vascular invasion first reported by Brown and

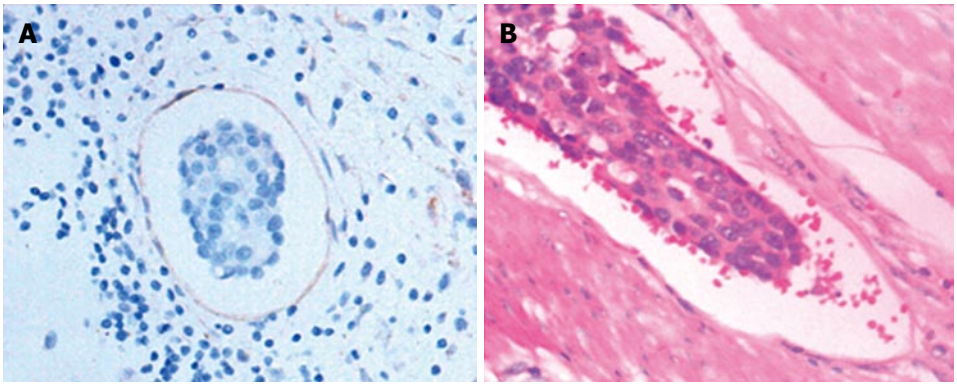

Figure 1 Immunohistochemical staining (**A**) and hematoxylin-eosin staining (**B**) of tumor cells (× 400) showing a tumor cell cluster in vascular spaces with brown-stained endothelial cells and tumor cells in blood vessel spaces with erythrocytes surrounded.

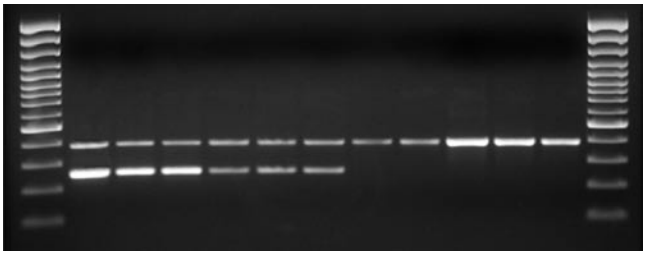

Figure 2 Expression of both CK20 mRNA and GAPDH detected in six patients and expression of only GAPDH detected in five patients.

Table 3 Comparative data on vascular invasion

	n	VI(+)	VI(-)	χ^2	*P*
CK20 mRNA					
Positive	21	12	9	6.758	< 0.05
Negative	25	5	20		
Age (yr)					
< 50	14	4	10	0.607	> 0.05
⩾ 50	32	13	19		
Size (cm)					
< 5	31	12	19	0.125	> 0.05
⩾ 5	15	5	10		
Differentiated					
Well	11	2	9	2.351	> 0.05
Moderately	20	8	12		
Poorly	15	7	8		
Serosa invasion					
Negative	14	2	12	4.440	< 0.05
Positive	32	15	17		
Lymph node metastasis					
Negative	18	3	15	5.225	< 0.05
Positive	28	14	14		
Distant metastasis					
Negative	38	9	29	16.520	< 0.05
Positive	8	8			

Statistical analysis by chi-square test. VI: Vascular invasion.

Table 4 Average number of microvessels of tissue in VI positive and negative patients

	n	MVD	*t*	*P*
VI				
Positive	17	29.2 ± 3.3	2.987	< 0.05
Negative	29	25.4 ± 4.7		

Statistical analysis of independent samples by *t* test. VI: Vascular invasion; MVD: Microvessel density.

Warren in 1938, a lot of studies have examined the influence of vascular invasion on survival[1]. Horn and colleagues found that vascular invasion is an independent prognostic factor for distant metastasis but not for survival[12]. However, Chapuis and colleagues found that vascular invasion is an independent prognostic factor for survival[13], but this was not confirmed by Wiggers *et al*[14] or Minsky *et al*[15]. In this study, we examined CK20 mRNA expression in patients with or without vascular invasion to evaluate the relationship between vascular invasion and microvessel density of tissue and micrometastasis in blood.

Vascular invasion and micrometastasis

Tumor metastasis is an orchestrated multistep process that may involve direct, hematogenous or lymphatic spread[16,17]. Tumor metastasis requires an exodus of cancer cells from the primaty site, endurance outside the hormonal and nutritional milieu of the primary site, evasion of the body' s immune surveillance, as well as adhesion, invasion, and penetration at a distant site, and organization of metastatic tissue in the secondary site with neovascularization[18]. Primary tumor invades blood and/or lymphatic vessels departing from the primary site[19]. In this study, CK20 mRNA was detected in 12 of 17 patients with positive vascular invasion, and in 9 of 29 patients with no vascular invasion, suggesting that vascular invasion is closely related to micrometastasis in blood, depth of tumor invasion, lymph node status and distant metastasis. Therefore, CK20 mRNA can be considered an indirect prognostic factor for survival. There is evidence that distant metastases are associated with the neoplastic invasion of relatively large veins at the tumor's periphery[20-22].

Vascular invasion and angiogenesis

Angiogenesis is the propelling force for tumor growth and metastasis[23-25]. To progress to a larger size, incipient neoplasms must have an angiogenic ability, which involves the sprouting of new blood vessels from preexisting capillaries, and requires the multiplication and migration of endothelial cells, remodeling of extracellular matrix, tube formation, and recruitment of surrounding structures to maintain the newly formed vessels[26]. In

this study, the average MVD was significantly higher in patients with vascular invasion than in patients with no vascular invasion, suggesting that angiogenesis is closely related with microvessel density of tissue[27] and clinical aggressiveness of tumor[28].

Detection of vascular invasion

Vascular invasion was detected with hematoxylin-eosin staining and immunohistochemical staining, respectively. The heterogeneous positive rate suggests immunohistochemical staining is more sensitive than hematoxylin and eosin staining for the detection of vascular invasion. Fibrin clots, erythrocytes, or both in endothelia-lined spaces without erythrocyte extravasation in the surrounding tissues must be concerned if detected with HE staining. However, we had to decide whether a tumor cell cluster is clearly visible in decorated vascular spaces where endothelial cells are stained brown when detected with immunohistochemical staining. Our results are consistent with the reported data[29,30].

COMMENTS

Background

Since the initial report by Brown and Warren in 1938 demonstrating an increased visceral metastasis in rectal cancer patients with vascular invasion, a number of investigators have examined the influence of vascular invasion by colorectal cancer. The presence of vascular invasion is associated with an increased incidence of lymph node and distant metastasis and a corresponding decrease in survival. However, this is not a consistent finding.

Research frontiers

Horn and colleagues found that vascular invasion is an independent prognostic factor for distant metastasis but not for survival. By multivariate analysis, Chapuis and colleagues found vascular invasion to be an independent prognostic factor for survival, but this was not confirmed by Wiggers *et al* or Minsky *et al*.

Innovations and breakthrough

Though several articles have reported the prognostic value of vascular invasion, the results are not consistent, and no study has focused on micrometastasis in patients with vascular invasion. In this study, we detected cytokeratin20 (CK20) mRNA expression in portal system blood and microvessel density of tissue to evaluate the relationship between vascular invasion and microvessel density of tissue and metastasis in blood.

Applications

We recommend vascular invasion as a method of choice for predicting prognosis of gastric and colorectal cancer patients. Patients with vascular invasion are more likely to need adjuvant therapies.

Terminology

CK20: It belongs to the epithelial subgroup of the intermediate filament family. Because of its restricted range of expression in humans, it has become an important tool for detecting and identifying metastatic cancer cells by immunohistochemistry and PCR analysis. Factor Ⅵ: Vascular invasion is usually defined by the presence of neoplastic cells with fibrin clots, erythrocytes, or both in endothelia-lined spaces without erythrocyte extravasation in the surrounding tissues or by the presence of neoplastic cells within the smooth muscle cell-lined space

Peer review

This subject is valuable for understanding the importance of vascular invasion of cancer in predicting the prognosis of such patients. It also provides a better way to increase the detection rate of vascular invasion with immunohistochemical staining.

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