Relationship between vascular invasion and microvessel density and micrometastasis

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

METHODS: Vascular invasion was detected by both hematoxylin and eosin staining and immunohistochemical 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.

Key words: Vascular invasion; Reverse transcriptase-polymerase chain reaction; Microvessel density; Micrometastasis

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 of tissue and metastasis in blood.
Table 1  Oligonucleotide primers

| cDNA  | Primer          | Sequence               | Product length (bp) |
|-------|-----------------|------------------------|---------------------|
| CK20  | Outer sense     | 5'-GAGGGTACA          | 253                 |
|       | TAACGGAGCT-3'   |                        |                     |
|       | Outer antisense | 5'-TCTCTCTCTCA        |                      |
|       | GGGTCTCT-3'     |                        |                     |
|       | Inner sense     | 5'-GCCTGAGATA         |                      |
|       | GAACTCCAG-3'    |                        |                     |
|       | Inner antisense | 5'-ACGTCTTCTC         |                      |
|       | TCTCCAGAAG-3'   |                        |                     |
| GAPDH | Sense           | 5'-CAGGGCTGCTT        | 385                 |
|       | TTAACCTCG-3'    |                        |                     |
|       | Antisense       | 5'-CTGTTTGCGGAG       |                      |
|       | TTCTTAGAG-3'    |                        |                     |

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

19 colorectal cancer patients were formalin-fixed and paraffin-embedded. The tissue samples were cut into 1 μm-thick sections, mounted onto slides coated with polylysine 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. 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. According to the immunohistochemical staining, the fibrin clots or erythrocytes surrounding neoplastic cells should be considered. Vascular invasion was confirmed by at least one staining 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°C 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)18 primer in 14 μL solution for 5 min at 70°C. 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°C. The reaction was terminated by heating at 95°C for 5 min.

Polymerase chain reaction (PCR): PCR was carried out as described previously. 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.

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
Warren in 1938, a lot of studies have examined the influence of vascular invasion on survival. Horn and colleagues found that vascular invasion is an independent prognostic factor for distant metastasis but not for survival. However, Chapuis and colleagues found that vascular invasion is an independent prognostic factor for survival, but this was not confirmed by Wiggers et al. or Minsky et al. 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. Tumor metastasis requires an exodus of cancer cells from the primary 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. Primary tumor invades blood and/or lymphatic vessels departing from the primary site. 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.

### Vascular invasion and angiogenesis

Angiogenesis is the propelling force for tumor growth and metastasis. 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.

### Table 3: Comparative data on vascular invasion

|             | $n$ | $VI(\pm)$ | $VI(-)$ | $\chi^2$ | $P$  |
|-------------|-----|-----------|---------|---------|------|
| CK20 mRNA   |     |           |         |         |      |
| Positive    | 21  | 12        | 9       | 6.758   | < 0.05|
| Negative    | 25  | 5         | 20      |         |      |
| Age (yr)    |     |           |         |         |      |
| < 50        | 14  | 4         | 10      | 0.607   | > 0.05|
| $\geq$ 50   | 32  | 13        | 19      |         |      |
| Size (cm)   |     |           |         |         |      |
| < 5         | 31  | 12        | 19      | 0.125   | > 0.05|
| $\geq$ 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

| $VI$   | $n$ | MVD  | $t$  | $P$   |
|--------|-----|------|------|-------|
| 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.

Figure 1: Immunohistochemical staining (A) and hematoxylin-eosin staining (B) of tumor cells ($\times$ 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 4: Average number of microvessels of tissue in $VI$ positive and negative patients

| $VI$   | $n$ | MVD  | $t$  | $P$   |
|--------|-----|------|------|-------|
| 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.

Wang YD et al. Prognosis in vascular invasion positive patients
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[28,29].

**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[22,24].

**REFERENCES**

1. Minsky BD, Cohen AM. Blood vessel invasion in colorectal cancer—an alternative to TNM staging? *Ann Surg Oncol* 1999; 6: 129-130
2. Mullis KB. Target amplification for DNA analysis by the polymerase chain reaction. *Ann Biol Clin (Paris)* 1990; 48: 579-582
3. Majima T, Ichikura T, Takayama E, Chochi K, Mochizuki H. Detecting circulating cancer cells using reverse transcriptase-polymerase chain reaction for cytokeratin mRNA in peripheral blood from patients with gastric cancer. *Ipn J Clin Oncol* 2000; 30: 499-503
4. Wild S, Kleeff J, Maruyama H, Maurer CA, Friess H, Büchler MW, Lander AD, Korc M. Characterization of cytokeratin 20 expression in pancreatic and colorectal cancer. *Clin Cancer Res* 1999; 5: 2840-2847
5. McDonnell CO, Hill AD, McNamara DA, Walsh TN, Boucher-Hayes DJ. Tumour micrometastases: the influence of angiogenesis. *Eur J Surg Oncol* 2000; 26: 105-115
6. Duff SE, Li C, Garland JM, Kumar S. CD105 is important for angiogenesis: evidence and potential applications. *FASEB J* 2003; 17: 984-992
7. Vulms FA, Diepstra JH, Cornelissen RM, Ruers TJ, Ligtenberg MJ, Punt CJ, van Kriekien JH, Wobbes T, van Muijen GN. Limitations of cytokeratin 20 RT-PCR to detect disseminated tumour cells in blood and bone marrow of patients with colorectal cancer: expression in controls and downregulation in tumour tissue. *Mol Pathol* 2002; 55: 156-163
8. Hyung WJ, Lee JH, Choi SH, Min JS, Noh SH. Prognostic impact of lymphatic and/or blood vessel invasion in patients with node-negative advanced gastric cancer. *Ann Surg Oncol* 2002; 9: 562-567
9. Birner P, Obermair A, Schindl M, Kowalski H, Breitenecker G, Oberhuber G. Selective immunohistochemical staining of blood and lymphatic vessels reveals independent prognostic influence of blood and lymphatic vessel invasion in early-stage cervical cancer. *Clin Cancer Res* 2001; 7: 93-97
10. Sooth E, Röder C, Juhl H, Krüger U, Kremmer B, Kalthoff H. The detection of disseminated tumor cells in bone marrow from colorectal-cancer patients by a cytokeratin-20-specific nested reverse-transcriptase-polymerase-chain reaction is related to the stage of disease. *Int J Cancer* 1996; 69: 278-282
11. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis—correlation in invasive breast carcinoma. *N Engl J Med* 1991; 324: 1-8
12. Horn A, Dahl O, Morild I. Venous and neural invasion as predictors of recurrence in rectal adenocarcinoma. *Dis Colon Rectum* 1991; 34: 798-804
13. Chapuis PH, Dent OF, Fisher R, Newland RC, Pheils MT, Smyth E, Colquhoun K. A multivariate analysis of clinical and pathological variables in prognosis after resection of large bowel cancer. *Br J Surg* 1985; 72: 698-702
14. Wiggers T, Arends JW, Schutte B, Volovics L, Bosman FT. A multivariate analysis of pathologic prognostic indicators in large bowel cancer. *Cancer* 1988; 61: 386-395
15. Minsky B, Mies C. The clinical significance of vascular invasion in colorectal cancer. *Dis Colon Rectum* 1989; 32: 794-803
16. Fidler IJ. Critical factors in the biology of human cancer metastasis: twenty-eighth G.H.A. Clowes memorial award lecture. *Cancer Res* 1990; 50: 6130-6138
17. Weiss L, Bronk J, Pickren JW, Lane WW. Metastatic patterns and target organ arterial blood flow. *Invasion Metastasis* 1981; 1: 126-135
18. Liotta LA, Kleinerman J, Saidel GM. Quantitative relationships of intravascular tumor cells, tumor vessels, and pulmonary metastases following tumor implantation. *Cancer Res* 1974; 34: 997-1004
19. Van Trappen PO, Pepper MS. Lymphatic dissemination of tumour cells and the formation of micrometastases. *Lancet Oncol* 2002; 3: 44-52
20. DeVita VT, Hellman S, Rosenberg SA. Cancer: principles and
practice of oncology. Philadelphia: Lippincott-Raven, 1997: 211

Roder JD, Böttcher K, Siewert JR, Busch R, Hermanek P, Meyer HJ. Prognostic factors in gastric carcinoma. Results of the German Gastric Carcinoma Study 1992. Cancer 1993; 72: 2089-2097

Ichikura T, Tomimatsu S, Ohkura E, Mochizuki H. Prognostic significance of the expression of vascular endothelial growth factor (VEGF) and VEGF-C in gastric carcinoma. J Surg Oncol 2001; 78: 132-137

Weidner N. Intratumor microvessel density as a prognostic factor in cancer. Am J Pathol 1995; 147: 9-19

Takahashi Y, Kitadai Y, Bucana CD, Cleary KR, Ellis LM. Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. Cancer Res 1995; 55: 3964-3968

Weidner N. Tumoural vascularity as a prognostic factor in cancer patients: the evidence continues to grow. J Pathol 1998; 184: 119-122

Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000; 100: 57-70

Hasan J, Byers R, Jayson GC. Intra-tumoural microvessel density in human solid tumours. Br J Cancer 2002; 86: 1566-1577

Mineo TC, Ambrogi V, Baldi A, Rabitti C, Bollero P, Vincenzi B, Tonini G. Prognostic impact of VEGF, CD31, CD34, and CD105 expression and tumour vessel invasion after radical surgery for IB-IIA non-small cell lung cancer. J Clin Pathol 2004; 57: 591-597

Sakuragi N, Takeda N, Hareyama H, Fujimoto T, Todo Y, Okamoto K, Takeda M, Wada S, Yamamoto R, Fujimoto S. A multivariate analysis of blood vessel and lymph vessel invasion as predictors of ovarian and lymph node metastases in patients with cervical carcinoma. Cancer 2000; 88: 2578-2583

Tanaka F, Otake Y, Yanagihara K, Kawano Y, Miyahara R, Li M, Yamada T, Hanaoka N, Inui K, Wada H. Evaluation of angiogenesis in non-small cell lung cancer: comparison between anti-CD34 antibody and anti-CD105 antibody. Clin Cancer Res 2001; 7: 3410-3415