RESEARCH ARTICLE

Clinical Significance of Detecting Lymphatic and Blood Vessel Invasion in Stage II Colon Cancer Using Markers D2-40 and CD34 in Combination

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Abstract

This research was conducted to compare differences in colon cancer lymphatic vessel invasion (LVI) with D2-40 antibody labeling and regular HE staining, blood vessel invasion (BVI) with CD34 antibody labeling and HE staining and to assess the possibility of using D2-40-LVI/CD34-BVI in combination for predicting stage II colon cancer prognosis and guiding adjuvant chemotherapy.Anti-D2-40 and anti-CD34 antibodies were applied to tissue samples of 220 cases of stage II colon cancer to label lymphatic vessels and small blood vessels, respectively. LVI and BVI were assessed and multivariate COX regression analysis was performed for associations with colon cancer prognosis. Regular HE staining proved unable to differentiate lymphatic vessels from blood vessels, while D2-40 selectively labeled lymphatic endothelial cell cytosol and CD34 was widely expressed in large and small blood vessels of tumors as well as normal tissues. Compared to regular HE staining, D2-40-labeling for LVI and CD34-labeling for BVI significantly increased positive rate (22.3% vs 10.0% for LVI, and 19.1% vs 9.1% for BVI). Multivariate analysis indicated that TNM stage, pathology tissue type, post-surgery adjuvant chemotherapy, D2-40-LVI, and CD34-BVI were independent factors affecting whole group colon cancer prognosis, while HE staining-BVI, HE staining-LVI were not significantly related. When CD34-BVI/D2-40-LVI were used in combination for detection, the risk of death for patients with two or one positive results was 5.003 times that in the LVI(-)&BVI(-) group (95% CI 2.365 - 9.679). D2-40 antibody LVI labeling and CD34 antibody BVI labeling have higher specificity and accuracy than regular HE staining and can be used as molecular biological indicators for prognosis prediction and guidance of adjuvant chemotherapy for stage II colon cancer.

Keywords: Colon cancer - prognosis - blood vessel invasion - lymphatic vessel invasion

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Introduction

The Asia Pacific Cohort Studies Collaboration (APCSC) (Woodward et al., 2006) has reported on risk factors for mortality from total colorectal cancer (Asia pacific Cohort Studies Collaboration, 2007). Height, body mass index (BMI) and cigarette smoking were all found to be significantly associated with increased risk of mortality from colorectal cancer while physical activity was found to be protective (Asia Pacific Cohort Studies Collaboration, 2007).

To our knowledge, post-surgery adjuvant chemotherapy is important for some patients with stage II colon cancer. Lymphatic vessel invasion (LVI) and blood vessel invade (BVI) are known to be key bases for guiding stage II colon cancer post-surgery adjuvant chemotherapy (Merchant et al., 1995). However, the evaluation of LVI and BVI in colon cancer tissue samples by regular HE staining gives significant false positive and false negative results (Merchant et al., 1995; Kikuchi et al., 1995; Nakai et al., 2004). Therefore, exploring specific markers for LVI and BVI has become a hot topic of recent years (Pinho et al., 2007).

The focus of this study is the presentation of tumor LVI and BVI in stage II colon cancer as stained by specific markers D2-40 and CD34 antibody (compared with regular HE staining) as well as their relation with prognosis.

Materials and Methods

Tissue samples

This is a retrospective case comparison study involving primary stage II colon cancer patients who were treated during 1993-2011 in our hospital (Fujian Medical University Union Hospital).

Paraffin-embedded tissue samples were taken from the pathology department. This study has obtained approval of institutional medical ethics committee, and clinical study informed consent forms were signed by the patients.

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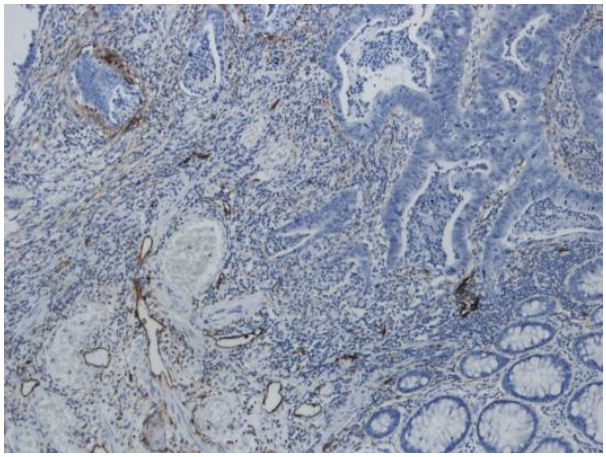

Figure 1. Anti-D2-40 Monoclonal Antibody Staining Reveals LVI in a Colon Cancer Sample (×200). The staining reveals the presence of lymphatic vessels. Many lymphatic vessels with small lumen can be seen at tumor mesenchyme, they mainly distribute in the peripheral of tumor, or the boundary between tumor and normal tissue. Compared to peripheral normal tissue, tumor has more lymphatic vessels in tumors are greater in numbers but have smaller lumen and have branches. Tumor cell invasion can be seen within part of mesenchyme

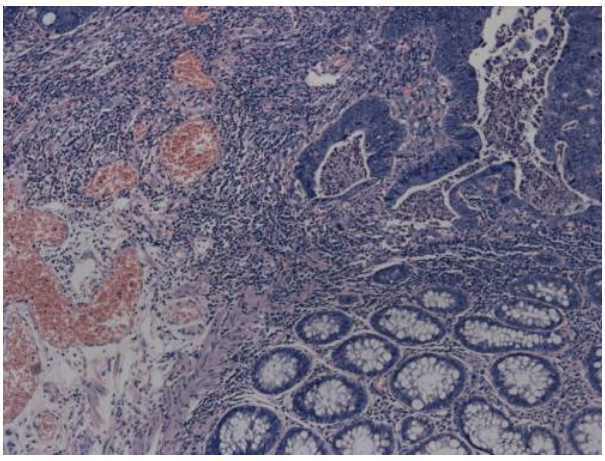

Figure 2. HE Staining Reveals LVI and BVI in a Colon Cancer Sample. HE staining could not differentiate lymphatic and blood vessels. At the site of tumor, irregular glands can be seen, and some glands are fused. Cell nucleus is large, deeply stained, irregular, and the nucleolus is visible. At the tumor, many newly generated capillaries are seen, which have small and incomplete lumen, lack part of endothelial cells, have branches, and often are distributed at the periphery part of neoplastic glands. Some blood vessels are adjacent to glands which almost projects into the lumen. Blood vessels at tumor site mainly distribute at the mucus layer of intestinal canal, and few are at the muscle layer. The blood vessels of surrounding normal mucus distribute mainly in the lower mucus layer, and have larger lumen

Study inclusion criteria

Case inclusion criteria: (1) colon cancer radical resection performed in our hospital and confirmed by pathology as stage II; (2) lymph nodes in sample taken ≥13; (3) resection confirmed by pathology as R0 (complete tumor removal, negative at far and near end as well as mesocolon rim of the resection); (4) follow-up period for at least five years with complete clinical and pathology data; (5) ECOG score 0-2; (6) have not received new adjuvant radio- or chemo-therapy. Exclusion criteria: died within 28 days of surgery; died of other causes during follow-up; association with Crohn's disease, ulcerative colitis, a second malignant tumor, colon cancer with multiple primary foci, familial multiple adenomatous polyposis or hereditary non-polyposis colon cancer. As a result, of 242 patients screened, 220 patients met the inclusion criteria and were included in the study.

Histopathology criteria: UICC colon cancer pTNM classification system, histology staging (Galon et al., 2013; Ueno et al., 2013); WHO colon cancer histopathology classification and staging (Maak et al., 2013; Merkow et al., 2013; Mrak et al., 2013) .

Clinical data

The 220 cases of colon cancer patients included 130 males (59.1%) and 90 females (40.9%), male/female ratio is 1.4 : 1; age 27-85 years (medium 62); 92 cases of right-sided colon cancer (41.8%) and 121 cases of left-sided (55.0%); 79 cases (35.9%) of papillary adenocarcinoma, 55 cases (25%) of tubular adenocarcinoma, 21 cases (9.5%) of mucinous adenocarcinoma, 25 cases (11.3%) of signet ring cell carcinoma, and 40 cases of poorly differentiated or undifferentiated cancer (18.2%). For the depth of intestinal wall invasion, there were 103 (46.8%) T3 cases and 117 (53.2%) T4 cases. The follow-up was performed by medical record department using letters, telephone interviews and hospital visits. Follow-up started from the day of diagnosis and lasted for 38~122 months (median 63 months). Of the cases, five were lost and the follow-up rate was 98.7%; for the whole group the five-year cumulative survival rate and median survival time were 70.37% and 71 months, respectively.

Table 1. Comparison of Lymphatic Vessels Invasion (LVI)/Blood Vessel Invasion (BVI) in Whole Group Colon Cancer by D2-40/CD34 or HE Staining

	Staining method	Result of Stainig		χ^2 value	*P*-value
		Negative(n)	Positive(n)		
LVI	HE staining	198	22	12.24	0.0001
	Anti-D2-40 McAb	171	49		
BVI	HE staining	200	20	9.087	0.0001
	Anti-CD34 McAb	178	42		

Immunohistochemistry

Mouse anti-human monoclonal antibody anti-D2-40 (1:1000 Santa Cruz Biotechnology) was used for detecting LVI and mouse anti-human monoclonal antibody anti-CD34 (1:1000 Santa Cruz Biotechnology) was used for detecting BVI. For each patient, 3 paraffin-embedded tissue samples from different places of center of tumor (avoiding the necrotic tissue) were examined. Detection of the primary antibody was done with PowerVision two-step histostaining reagent

Criteria for result reading

Criteria for LVI: Following D2-40 staining (located in

Table 2. Univariate Analysis Results for Risk Factors of Stage II Colon Cancer Prognosis

Risk factor	Cases (%)	5 year survival (% ±SD)	HR (95%CI)****	*P*-value
Gender			1.437 (0.716-2.048)	0.334
Female	90 (40.5)	73 ± 5.0		
Male	130 (59.5)	69 ± 4.1		
Age			2.823 (1.071-3.814)	0.012
>40 y	145 (65.9)	74 ± 2.4		
≤40 y	75 (34.1)	59 ± 2.8		
Tumor location*			1.731 (0.828-2.171)	0.091
Right colon	99 (45.0)	70 ± 3.4		
Left colon	121 (55.0)	73 ± 5.7		
Histopathology type**			4.723 (2.529-6.459)	0.001
Low malignancy potential	134 (60.9)	71 ± 3.2		
High malignancy potential	86 (39.1)	49 ± 3.6		
Intestine cavity invasion range			2.560 (1.545-3.671)	0.003
≤1/4 circle	86 (39.1)	74 ± 3.2		
>1/4 circle	134 (60.9)	60 ± 3.9		
Histology staging			4.859 (1.412-5.238)	0.001
G1- G2	149 (67.7)	76 ± 3.6		
G3- G4	71 (32.3)	49± 4.3		
Depth of intestine wall invasion			5.523(2.138-6.093)	0.001
T3	103 (46.8)	77 ± 2.9		
T4	117 (53.2)	38 ± 3.6		
Pre-surgery CEA level			2.479 (1.345-4564)	0.008
<5μg/L	73 (33.2)	74 ± 4.2		
≥5μg/L	147 (66.8)	60 ± 4.8		
D2-40				
-LVI			3.348 (1.946-5.124)	0.001
-	171 (77.7)	74 ± 4.1		
+	49 (22.3)	56 ± 3.9		
HE staining-LVI			1.369 (0.92-2.149)	0.097
-	198 (90.0)	73 ± 3.4		
+	22 (10.0)	68± 3.5		
CD34-BVI			3.669 (2.561-5.721)	0.001
-	178 (80.9)	75 ± 3.3		
+	42 (19.1)	52± 3.7		
HE staining-BVI			2.871 (1.347-4.566)	0.025
-	200 (90.9)	74± 5.1		
+	20 (9.1)	62± 3.5		
LVI+BVI combined detection				
Double negative	149 (67.7)	73 ± 4.3	4.564 (2.638-6.327)	0.001
Others***	71 (32.3)	35 ± 4.1		
Adjuvant chemotherapy				
Yes	121 (55.0)	75 ± 3.6	4.522 (2.324-5.83)	0.001
No	99 (45.0)	48 ± 3.2		

*Right-side colon (Including ileocecal colon, ascending colon, transverse colon), left side colon (including the transverse colon splenic flexure, descending colon, sigmoid colon); **High malignancy potential (including lowly or un-differentiated cancer, mucinous adenocarcinoma, and signet ring cell carcinoma,); ***refers to patients with either LVI or BVI positive or both positive results. ****HR, hazards ratio

cytosol or cell membrane and appearing as dark brown), under microscope, endothelial cell monolayer surrounded ring-shaped lumen structures can be observed, which are one D2-40 positive lymphatic vessels. LVI positive is defined as at least 1 tumor cells observed within a D2-40 positive ring-shaped lumen structure under microscope (Di Tommaso et al., 2010; Drozdz et al., 2012).

Criteria for BVI diagnosis: under light microscope, after anti-CD34 mAb staining, blood vessel ECs appear brown and at least 1 cancer cell is found in the lumne structure formed by linear arrangement of these CD34-positive ECs (Pruneri et al., 2002; Wang et al., 2010).

To understand the difference in result reading by different readers, 30 cases were first selected in random, and the sections were read, measured and scored by two researcher. The resulting LVI and BVI data agreement rate was 98.2% and 97.8%, respectively.

Statistics

The data were analyzed with SPSS 13.0. χ^2-test was used to analyze differences in LVI/BVI by different staining methods; Kaplan-Meier analysis was used to determine survival rate and Lok-rang time sequence test was performed to analyze differences in survival rate between analysis groups. The univariate and multivariate analysis (Walk recession) methods in COX regression model were used to screen independent factors affecting colon cancer prognosis.

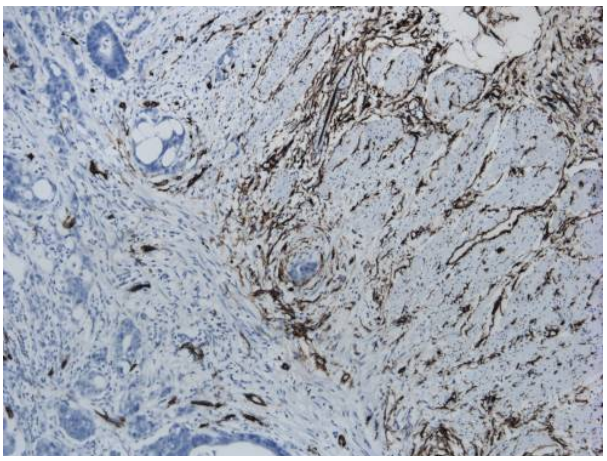

Figure 3. Staining with anti-CD34 Monoclonal Antibody Reveals Blood Vessels and BVI in a Colon Cancer Sample (×100). Vessels were marked by CD34, and blood vessel invasion can be seen in tumor tissues. The veins were formed by endothelial cells arranged linearly and were stained brown by immunohistochemistry; within their lumen cancer cells were found. In the whole group colon cancer patients, 25.4% were BVI positive. Blood vessels of normal tissues were also stained

Table 3. Results of Multivariate Analysis of Risk Factors for Stage II Colon Cancer Prognosis

Clinical pathology factor	*P*-value	HR-value	95%CI of HR value	
			Low	High
Depth of intestinal wall invasion (T)	0.001	5.582	2.848	10.147
LVI+BVI detection	0.001	5.003	2. 365	9.679
Histopathology type	0.001	4.651	3.552	7.368
Adjuvant Chemotherapy	0.008	3.746	2.324	8.037
CD34-BVI	0.001	3.582	2.331	6.6259
D2-40LVI	0.008	2.457	2.032	4.652
HE staining-BVI	0.963	1.620	0.468	2.213
HE staining-LVI	0.961	1.543	0.876	1.992

Results

Staining of lymphatic vessels in colon cancer tissue by marking D2-40

D2-40 positive staining is localized in cytosol or cell membrane. The lymphangiogenesis structure positive for D2-40 consists of flat tubes or cavities formed by EC monolayer; the lumens are irregular in size and shape and contain lymphocytes in some portion, but do not contain red blood cells, platelets and other blood vessel contents (Figure 1)

Staining of blood vessels in colon cancer tissues by marking CD34

Anti-CD34 stained blood vessels in tumor tissue are brown. CD34 is strongly expressed in small and micro-vessels as wells as large vessels of tumor tissue, and they have a clear boundary with other tissues. Cd34 is also expressed in blood vessels of normal tissues. In some blood vessels, tumor embolus could be observed (Figure 3).

Comparison of D2-40-LVI/CD34-BVI and HE staining detection of LVI /BVI

D2-40-LVI positive rate was 22.3% (49/220), evidently higher than the 10.0% (22/220) LVI rate detection by regular HE staining (Table 1).

The CD34 antibody marked BVI positive rate was 19.1% (42/220), also markedly higher than the 9.1% (20/220) BVI positive rate detected by HE staining (Table 1).

COX regression univariate analysis for factors affecting colon cancer prognosis

For univariate analysis, histopathology type, tumor site, range of intestinal wall infiltration, depth of tumor invasion, scope of enteric cavity infiltration, CEA, age, histological grading, HE staining BVI, D2-40-LVI/CD34-BVI, and adjuvant chemotherapy have significant correlation with whole group colon cancer prognosis, while gender and HE staining-LVI have no significant correlation with colon cancer survival (Table 2).

COX regression multivariate analysis for factors affecting colon cancer prognosis

The indicators in Table 2 above were input to the multivariate logistic regression model and the screening results were: depth of tumor invasion, LVI+BVI combined detection, histopathology type, adjuvant chemotherapy, CD34-BVI, and D2-40-LVI are independent high-risk factors affecting the whole group prognosis, while HE staining BVI and HE staining LVI are not significantly related to colon cancer prognosis (Table 3).

Discussion

Studies have demonstrated that LVI and BVI correlate positively with colon cancer metastasis to lymph nodes of liver and lung and to other remote places via blood circulation (Kikuchi et al., 1995; Nakai et al., 2004). The United Sates National Comprehensive Cancer Network (NCCN) stated that LVI or BVI are high risk factors for stage II colon cancer. The current study have also demonstrated that LVI and BVI correlated negatively with colon cancer early phase post-surgery survival (HR value = 3.582, 2.457), and are important independent factors for predicting prognosis. Therefore, detection of LVI/BVI has clinical significance for understanding the route of colon cancer metastasis, for guiding the development of treatment plans and deciding whether to perform adjuvant chemotherapy.

Evaluation of LVI/BVI by regular HE staining, however, have many difficulties due to tumor-induced fibrosis, fixation related artificial false appearances, cancer caused mucus lakes and destruction of vascular structures (Nielsen et al., 2011). pathology test tries to tell by staining if there is cancer embolus in the lumen cavity formed by a ring of EC monolayer, but it could not accurately differentiate the walls of lymphatic vessels from walls of capillary, and also it could not be certain of cancer embolus, resulting in both high false positive and high false negative rates (Bynum et al., 1976).

D2-40 marker can detect the antigenic determinants on salivary glycoproteins distributed on ECs of lymphatic vessels, and is a specific marker for lymphatic vessel ECs (Margaritescu et al., 2010; Skalova et al., 2012). In this

study, D2-40 was found to be specifically localized in lymphatic vessel ECs of colon tissue and it is compatible with application on formalin-treated paraffin-embedded tissue sections, which is easier to use and has a wider application range than other lymphatic vessel markers only applicable for frozen sections, and this result is consistent with that of Rajaganeshan et al. (2007). In the current study, in samples from 220 cases of primary colon cancer, the LVI positive rate by D2-40 immunohistochemistry is much higher than regular staining; in the 198 cases of LVI negative cases by regular staining, 27 cases (13.6%) were found by D2-40 staining as positive for LVI; on the other hand, in the 22 cases diagnosed as LVI positive, 2 cases (9%) were found by D2-40 staining as LVI false positive. It is thus clear that D2-40 staining could avoid false positive LVI due to sample fixation resulted tissue shrinkage and tumor cell aggregation, and missed diagnosis due to lymphatic vessel blockage by tumor embolus (Debald et al., 2010).

In this study, use of the anti-CD34 monoclonal antibody to stain blood vessel ECs improved accuracy of evaluation, it has no cross-reaction with lymphatic vessel ECs or stromal cells, and both its sensitivity and specificity were high. It improved BVI positive rate from the 9.1% of HE staining to 19.1%.

When D2-40-LVI/CD34-BVI were used in combination, the degree of death risk for double positive cases or single positive cases was 5.003 times that of LVI/BVI double negative patients, which is distinctly higher than other indicators. Therefore, it is suggested that for early phase post-surgery colon cancer samples, immunohistochemistry with anti-D2-40 and anti-CD34 monoclonal antibodies, which is simple and affordable, shall be routinely performed to detect LVI and BVI (Van den Eynden et al., 2006; Naoi et al., 2007).

In summary, anti-D2-40 and CD34 antibody marked LVI and BVI could be used as important molecular biology indicators for predicting stage II colon cancer prognosis, and for deciding whether to perform adjuvant chemotherapy. The combined detection of the two markers helps to improve accuracy of prognosis estimation, and to better guide personalized chemotherapy.

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