Comparison of Methods to Identify Lymphatic and Blood Vessel Invasion and their Prognostic Value in Patients with Primary Operable Colorectal Cancer

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Abstract. *Background/Aim: Lymphatic and blood vessel invasion are important independent prognostic factors in colorectal cancer, but identification of the separate components remains difficult. The aim of the present study was to compare routine hematoxylin and eosin (H&E) and elastica staining with immunohistochemistry using D2-40 and CD31. Materials and Methods: A total of 75 surgical specimens of colorectal cancer were examined for blood and lymphatic vessel invasion, by comparing stains. Results: The minimum clinical follow-up of survivors was 5 years. During that time, 45 patients died, 34 from their cancer. Lymphatic invasion by H&E was found in 19% compared to 40% detected with D2-40 ($p<0.001$). Lymphatic invasion was not associated with T-stage (H&E, $p=0.923$; D2-40, $p=0.724$) but was significantly associated with N-stage, (H&E, $p=0.001$; D2-40, $p<0.001$). No significant association between lymphatic invasion (H&E or D2-40) and cancer-specific survival was found on univariate analysis. Blood vessel invasion by elastic detection was detected in 53% compared to 32% detected with CD31 ($p=0.090$). Blood vessel invasion was associated with T-stage, (elastica, $p=0.028$; CD31, $p=0.839$) but was not associated with N-stage (elastica, $p=0.377$; CD31, $p=0.519$). On univariate analysis of blood vessel invasion was associated with cancer-specific survival (elastica, $p=0.009$) when detected by elastica, but not when detected by CD31, ($p=0.611$). Lymphatic invasion (D2-40) was associated with blood vessel invasion (elastic) ($p=0.019$). On multivariate analysis, blood vessel invasion with elastica had independent prognostic value (hazard ratio=2.55, 95% confidence interval=1.23-5.28; $p=0.012$). Conclusion: The results of the present study indicate that immunohistochemistry using D2-40 improves the identification of lymphatic invasion compared to use of H&E staining only; however, its prognostic value was limited. Elastica staining improves the detection rate of blood vessel invasion (compared to CD31) and venous invasion detected with elastica had independent prognostic value in patients undergoing curative resection for colorectal cancer.*

Colorectal cancer represents a leading cause of cancer-related death in the Western world and is by far the most common malignancy of the gastrointestinal tract (1). Despite potentially curative surgery for patients with colorectal cancer up to 50% of patients ultimately die of recurrent disease (2). Therefore, improved identification of patients at high risk of recurrence would be beneficial in the allocation of adjuvant treatment.

The principle cause of death in colorectal cancer is metastatic disease and invasion of tumor cells into lymphatic and blood vessels (intravasation) are critical steps for the establishment of metastasis (3). In colorectal cancer, invasive tumor cells either enter the lymphatic system to be transported to regional lymph nodes, or enter into the blood vasculature, where they are transported in vessels of the circulatory system to other organs, particularly the liver (4). At present, prediction of prognosis predominantly relies on TNM staging, that is used to allocate adjuvant treatment.

Lymphatic infiltration by the tumour is recognized as a significant risk factor for lymph node metastasis, as well as survival in colorectal cancer (5-7). Nevertheless detection remains difficult on routine haematoxylin and eosin (H&E) staining, while immunohistochemical staining appears to

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improve detection (7, 8). Venous invasion of the tumor is recognised as a feature of colorectal cancer progression and elastica staining can be used to improve the detection as well as prediction of survival (9, 10). A recent review of the literature concluded that further work was required to establish the best staining approaches to identify lymphatic and blood vessel invasion in clinical pathological practice (7).

The aim of the present study was to directly compare the detection of lymphatic invasion using H&E with that using D2-40, a specific marker of lymphatic endothelium, and to compare the detection of blood vessel invasion using elastica with that using CD31, a marker of vascular endothelium, in a series of resected colorectal carcinomas. The value of these different staining methods in assessing the role of lymphatic and blood vessel invasion in predicting cancer survival was then examined.

Materials and Methods

A total of 75 patients were selected for the study from a prospective database of patients with histologically proven colorectal cancer, who on the basis of laparotomy findings and preoperative abdominal computed tomography, were considered to have undergone potentially curative resection between 1997 and 2007 at a single surgical unit at the Glasgow Royal Infirmary. Patients who received neoadjuvant chemoradiotherapy, had metastatic disease at diagnosis, and those who died within 30 days of surgery were excluded. The tumours were staged using conventional Dukes' and TNM classifications (11). The Research Ethics Committee of North Glasgow University Hospitals approved the use of patient data and tissue for this study.

Routine stains: H&E. For evaluation of lymphatic invasion with H&E, routine slides were evaluated and findings recorded. Lymphatic invasion was recorded when tumour was seen within an endothelial-lined space devoid of mural smooth muscle, elastic fibres, and luminal red blood cells (Figure 1A).

Routine stains: Elastica. For evaluation of blood vessel invasion with Miller's elastica, routine slides were evaluated and findings recorded. Venous invasion was recorded if tumour was seen in a vessel with elastic fibres in its adventitia. Extramural and intramural blood vessel invasion were grouped together for analysis as "blood vessel" invasion.

Immunohistochemistry. For evaluation of lymphatic and blood vessel invasion, two parallel 4-μm-thick sections from each specimen corresponding to the same routine slides of H&E and elastica (deepest portion of tumour) were stained by D2-40 (monoclonal, SIG-3730, 1:100 dilution; Covance, USA) and CD31 (monoclonal, 0823, 1:100 dilution; Dako, Glostrup, Denmark) for confirmation of the vascular nature of the structure. Briefly, sections were de-waxed in xylene and rehydrated in a sequence of descending concentrations of ethanol. For antigen retrieval of CD31, sections were microwaved for 14 min in sodium citrate buffer (pH 6). Endogenous hydrogen peroxidase reactivity was blocked with 3% H_2O_2 for 15 min. Non-specific reactions were blocked by incubation with 10% horse serum for 30 minutes. Sections were subsequently incubated with the respective primary antibody: 60 minutes at room temperature for D2-40 and 30 minutes at 25˚C for CD31. Sites of binding were detected using the Envision technique (K5007; Dako) with 3-30 diaminobenzidine (SK 4001; Vector, Burlingame, CA, USA), a chromogenic substrate, according to the manufacturer's instruction. Slides were counterstained with haematoxylin, dehydrated and mounted with distyrene plasticizer xylene.

Table I. *The clinicopathological features of primary operable colorectal tumors (n=75).*

Characteristic	N (%)
Age (<65/65-75/>75 years)	26 (35%)/20 (27%)/ 29 (39%)
Gender (male/female)	37 (49%)/38 (51%)
Site (colon/rectum)	56 (75%)/19 (25%)
T-Stage (1/2/3/4)	1 (1%)/1 (1%)/53 (71%)/ 20 (27%)
N-Stage (0/1/2)	33 (44%)/36 (48%)/6 (8%)
TNM (I/II/III)	1 (1%)/32 (43%)/42 (56%)
Differentiation (well/poor)	70 (93%)/5 (7%)
Tumour perforation (absent/present)	74 (99%)/1 (1%)
Serosal involvement (absent/present)	57 (76%)/18 (24%)
Surgical margins (clear/involved)	72 (96%)/3 (4%)
Lymph nodes (<12 nodes/ >12 nodes)	41 (55%)/34 (45%)
H&E Lymphatic invasion (absent/present)	61 (81%)/14 (19%)
D2-40 Lymphatic invasion (absent/present)	45 (60%)/30 (40%)
Elastica venous invasion (absent/present)	35 (47%)/40 (53%)
CD31 Venous invasion (absent/present)	51 (68%)/24 (32%)
Alive/cancer death/non cancer death	30 (40%)/34 (45%)/ 11 (15%)

Evaluation of lymphatic and blood vessel invasion. D2-40 and CD31 immunostained sections were screened for the presence of lymphatic invasion. Assessment of lymphatic invasion and blood vessel invasion were conducted by the differential expression of these markers. Lymphatic invasion was discerned by the presence of tumour cells within D2-40-positive, CD31-positive vessels (11) (Figure 1B). Although CD31 was also found to be positive in lymphatic vessels, it was recorded as blood vessel invasion when tumour was seen in D2-40-negative, CD31-positive vessel. A total of 75 immunostained sections for lymphatic and blood vessel invasion were independently scored by two observers (HVW and AF) blinded to patient outcome and the other observer's score. Differences between reporters were resolved, following review at a multiheaded microscope whereby agreement was reached.

Statistical analysis. Consistency between the observers was analysed using intraclass correlation coefficient (ICCC), with values of 0.6 or higher considered acceptable and greater than 0.7 considered good. Chi-square test was used to examine associations between clinicopathological data and the presence of lymphatic invasion and blood vessel invasion. Survival rates were calculated using the Kaplan–Meier method. The influence of a given parameter on survival was assessed with the log-rank test, and the Cox proportional hazard regression model was used in multivariate analysis. Deaths up to the end of 2011 were included in the analysis.

Table II. *The relationship between clinicopathological features and lymphatic invasion assessed by H&E and D2-40*

	H&E			D2-40		
Characteristic	Absent, 61 (81%)	Present, 14 (19%)	*p*-Value	Absent 45 (60%)	Present 30 (40%)	*p*-Value
Age (<65/65-75/>75 years)	22/16/23	4/4/6	0.620	17/14/14	9/6/15	0.189
Gender (male/female)	28/33	9/5	0.173	21/24	16/14	0.371
Site (colon/rectum)	48/13	8/6	0.095	36/9	20/10	0.152
T-Stage (1/2/3/4)	1/1/42/17	0/0/11/3	0.923	0/1/32/12	1/0/21/8	0.724
N-Stage (0/1/2)	32/27/2	1/9/4	<0.001	28/17/0	5/19/6	<0.001
TNM (I/II/III)	1/31/29	0/1/13	0.003	1/27/17	0/5/25	<0.001
Serosal involvement (absent/present)	46 /15	11 /3	0.554	35/10	22/8	0.431
Surgical margins (clear /involved)	58/3	14/0	0.533	42/3	29/1	0.210
Tumor perforation (absent/present)	60/1	14/0	0.813	44/1	30/0	0.600
Alive/cancer death/non cancer death	25/27/9	5/7/2	0.817	20/18/7	10/16/4	0.590
Cancer-specific survival (months)[a]	114 (96-133)	92 (50-133)	0.358	124 (103-144)	90 (62-118)	0.073

[a]Mean (95% confidence interval).

Table III. *The relationship between clinicopathological features and blood vessel invasion assessed by elastica and CD31 staining.*

	Elastica			CD31		
Characteristic	Absent 35 (47%)	Present 40 (53%)	*p*-Value	Absent, 51 (68%)	Present, 24 (32%)	*p*-Value
Age (<65/65-75/>75 years)	16/11/8	10/9/21	0.012	16/14/21	10/6/8	0.395
Sex (male/female)	17/18	20/20	0.543	29/22	8/16	0.059
Site (colon/rectum)						
28/7	28/12	0.234	40/11	16/8	0.278	
T-Stage (1/2/3/4)	0 /0/22/13	1/1/31/7	0.028	0/1/37/13	1/0/16/7	0.839
N-Stage (0/1/2)	18/14/3	15/22/3	0.377	25/21/5	8/15/1	0.519
TNM (I/II/ III)	0/18/17	1/14/25	0.349	1/24/6	0/8/16	0.176
Serosal involvement (absent/present)	24/11	33/7	0.128	40/11	17/7	0.475
Surgical margins (clear /involved)	32/3	40/0	0.097	49/2	23/1	0.692
Tumor perforation (absent/present)	34/1	40/0	0.467	51/0	23/1	0.145
Alive/cancer death/non cancer death	19/10/6	11/24/5	0.020	24/22/5	6/12/6	0.031
Cancer-specific survival (months)[a]	136 (114-159)	89 (66-112)	0.007	113 (92-134)	103 (74-131)	0.610

[a]Mean (95% confidence interval).

p-Values of 0.05 or less were considered as statistically significant. Statistical analysis was performed using the SPSS software version 21 (SPSS Inc., Chicago, IL, USA).

Results

The pathological characteristics, presence of lymphatic invasion and blood vessel invasion of colorectal carcinomas (n=75) are shown Table I.

The patients consisted of 38 men and 37 women with a median age of 68 years. Cancer affected the colon in 56 (75%) and rectum in 19 (25%) of patients. In terms of T-stage, tumor invaded to the submucosa in one (1%) patient, to the muscularis propria in one (1%), subserosa in 53 (71%), and peritoneum in 20 (27%) patients. No positive lymph nodes were found in 33 (44%) patients, 1-3 lymph nodes contained tumor in 36 (48%), and three or more lymph nodes were positive in six (8%) patients. One patient (1%) had stage I disease, 32 (43%) stage II and 42 (56%) stage III after surgical resection.

Histology showed 70 (93%) of the tumors were well-differentiated and 5 (7%) of tumors were poorly differentiated. Tumor perforation was present 1 (1%) of tumours. Serosal involvement was recorded in 18 (24%) of tumours and surgical margins were involved in three (4%) resection specimens. In 34 (45%) specimens, more than 12 lymph nodes were retrieved.

The relationship between clinicopathological features and lymphatic invasion assessed by D2-40 and by H&E are shown in Table II. H&E detected lymphatic invasion in 19%

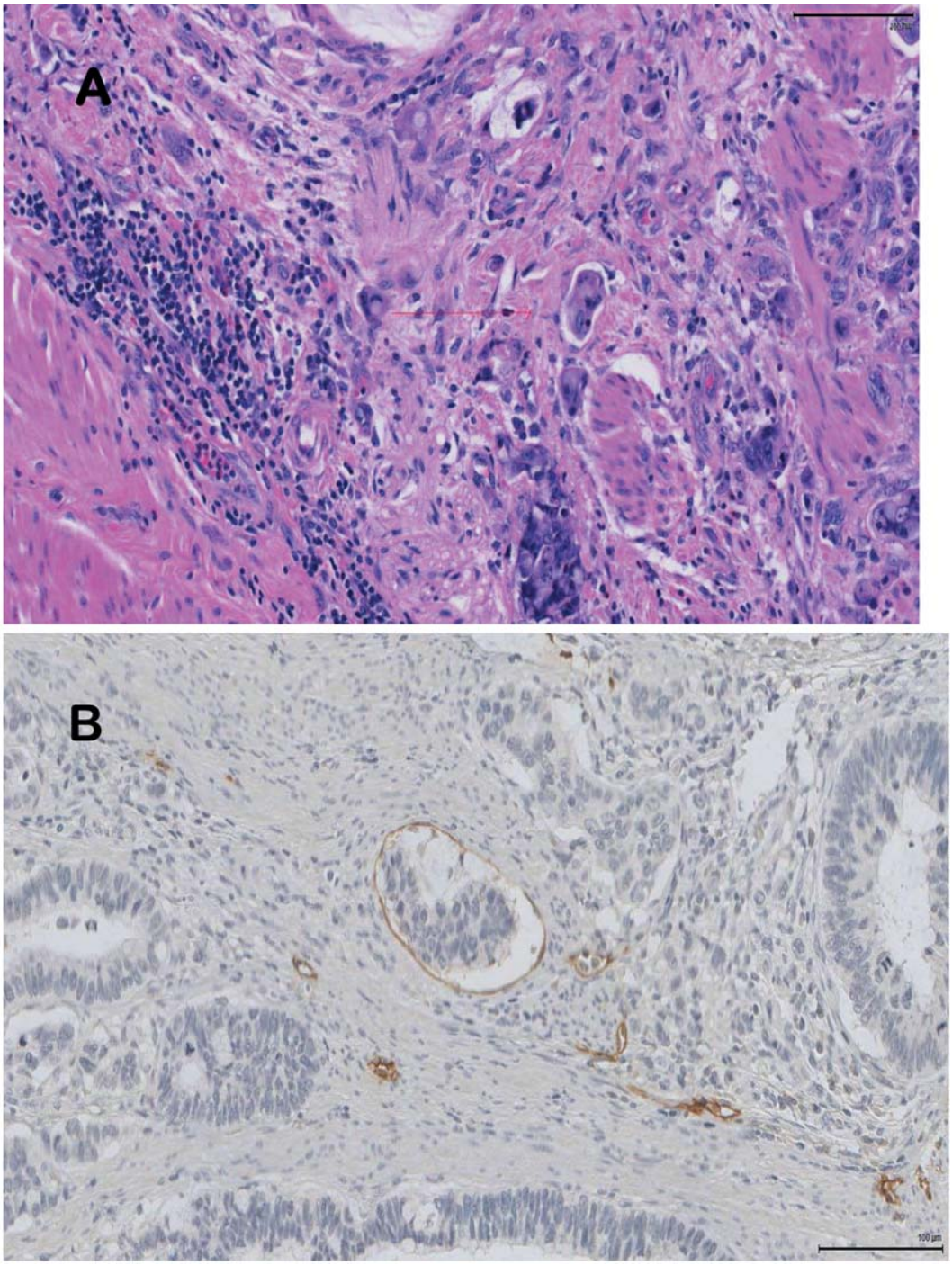

Figure 1. *Example of lymphatic invasion with H&E (A) and D2-40 (B) in colorectal cancer. Scale bar 100 μm.*

(14/75), while immunohistochemistry (D2-40) detected lymphatic invasion in 40% (30/75) of cases (p=0.001). Of the lymphatic invasions detected by immunohistochemistry, 5% (1/75) of tumors had lymphatic invasion on both D2-40 and CD31 slides. The presence of lymphatic invasion determined by H&E and immunohistochemistry (D2-40) was directly compared. Lymphatic invasion was not associated with T-stage (H&E, p=0.923; D2-40, p=0.724) but was significantly associated with N-stage, (H&E, p=0.001; D2-40, p<0.001).

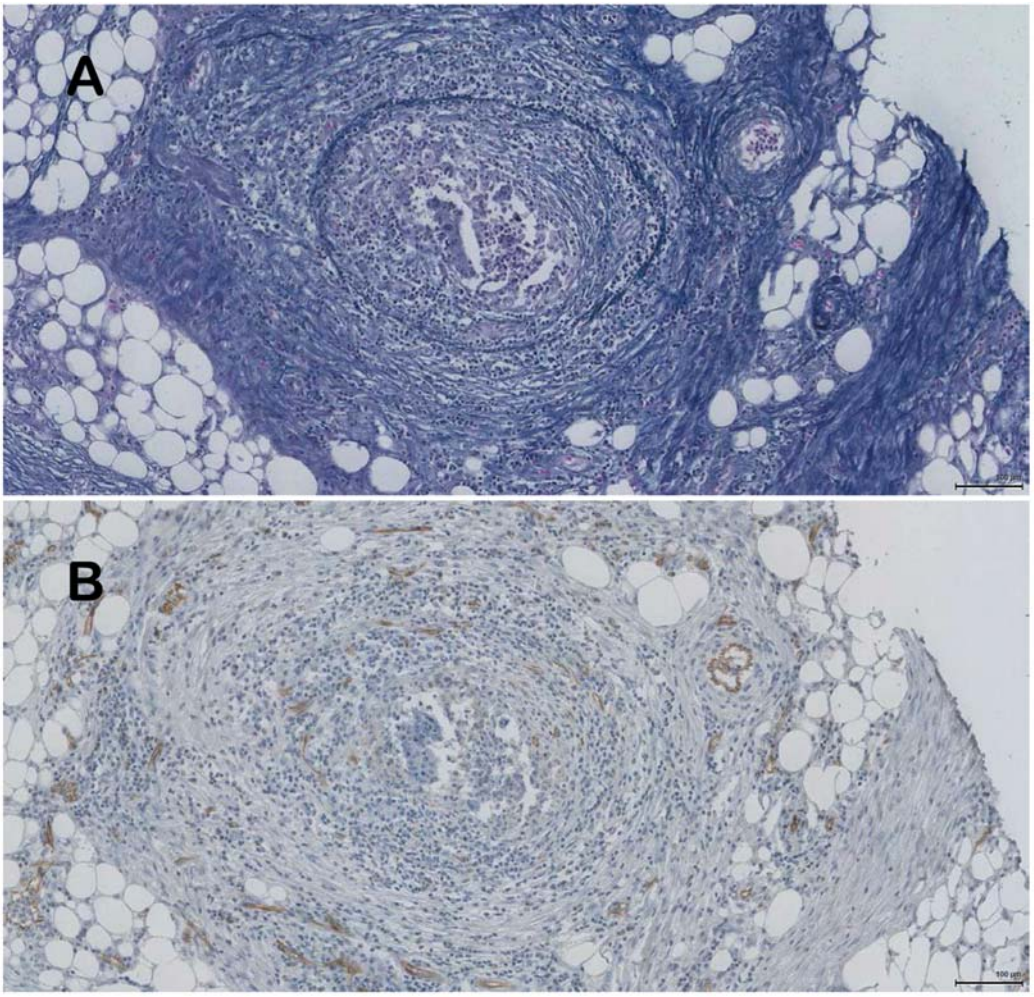

Figure 2. *Blood vessel invasion as shown by staining of elastica (A) and CD31 (B) in colorectal cancer. Tumour in blood vessel and endothelial lining destroyed are clearly visible. Scale bar 100 μm.*

The relationship between clinicopathological features and blood vessel invasion assessed by CD31 and by elastica are shown in Table III. Blood vessel invasion was detected with Miller's elastica in 53% of tumors compared to 32% detected with CD31 (p=0.090). Blood vessel invasion detected by elastica was associated with T-stage (p=0.028) but not when detected by CD31 (p=0.839). Venous invasion detected by either method was not associated with N-stage (elastica, p=0.377; CD31, p=0.519).

Elastica staining of elastic fibres in the wall of large blood vessels thus enhanced detection (Figure 2A). CD31 staining of involved blood vessels was common and seen both in intratumoural as well as peritumoural areas of the tumour. Although CD31 was used to identify vascular endothelium, it appeared more helpful in identifying lymphatic and small blood vessel invasion than venous invasion, as the endothelium of some larger veins invaded by tumour was destroyed (Figure 2B).

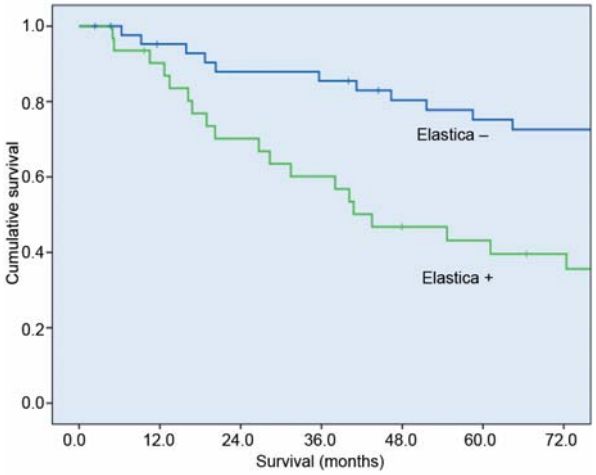

Figure 3. *Relationship between blood vessel invasion as shown by using elastica staining and cancer-specific survival of patients undergoing resection for colorectal cancer (p=0.007).*

Table IV. *The comparison between lymphatic and blood vessel invasion with routine and specific stains and cancer-specific survival in patients with primary operable colorectal cancer.*

	Cancer-specific survival Univariate survival analysis	
Characteristic	HR (95% CI)	*p*-Value
H&E Lymphatic invasion (absent/present)	1.48 (0.64-3.39)	0.361
D2-40 Lymphatic invasion (absent/present)	1.84 (0.94-3.61)	0.078
Elastica blood vessel invasion (absent/present)	2.68 (1.28-5.61)	0.009
CD31 Blood vessel invasion (absent/present)	1.20 (0.59-2.44)	0.611

HR:Hazard ratio; CI: confidence interval.

The minimum clinical follow-up of survivors was 5 years. During that time, 45 patients died, 34 from their cancer. The comparison of lymphatic and blood vessel invasion between routine and special stains and cancer-specific survival in primary operable colorectal cancer is shown in Table IV.

On univariate analysis, lymphatic invasion (H&E, $p=0.361$; D2-40, $p=0.078$) was not significantly associated with cancer-specific survival. On univariate analysis, blood vessel invasion detected by elastica was associated with poorer cancer-specific survival (elastica, $p=0.009$; CD31, $p=0.611$) as well as N-stage ($p=0.05$). Lymphatic invasion detected with D2-40 was associated with blood vessel invasion detected with elastica ($p=0.019$). On multivariate analysis, elastic blood vessel invasion remained independently associated with poorer cancer specific survival (hazard ratio=2.55, 95% confidence interval=1.23-5.28, $p=0.007$) (Figure 3).

Discussion

The results of the present study show that compared to H&E, the use of an immunohistochemical stain D2-40 improved the detection of lymphatic invasion but this feature was not significantly associated with survival. In contrast, compared to the immunohistochemical stain (CD31), elastica was more sensitive in the detection of blood vessel invasion and revealed venous invasion (but not CD31 detected venous invasion) was significantly associated with survival. Lymphatic invasion with D2-40 staining was associated with blood vessel invasion with elastica staining. Taken together these results indicate that specific stains (immunohistochemical stain D2-40 and elastica) play an important role in the detection of lymphatic and blood vessel invasion.

In the present study, the incidence rate of lymphatic invasion with H&E and D2-40 was 19% and 40%, respectively, and was within the range of previous reports. (6, 7, 12). It also confirms the difficulty in detecting lymphatic invasion with the routine H&E staining procedure and confirms the increased sensitivity of D2-40. Lymphatic invasion with D2-40 was mainly seen at the invasive margin of the tumor and not within the tumour. Despite Kenney and Jain reporting that lymphatic vessels were present within the *lamina propria* of colonic tissue in pathological states (13), controversy still exists about the importance of the exact localisation of tumour lymphatic vessels in colorectal cancer (14, 15) and further investigation of methods of detection of lymphatic vessels is warranted.

In the present study, lymphatic invasion (by both H&E and D2-40) was positively associated with nodal metastasis. This suggests that tumor cells reach lymph nodes mainly *via* the lymphatic system (12, 16, 22). Therefore, lymphatic invasion is likely to play an important role in predicting lymph node metastases in early cancer, for example in endoscopically-resected cancerous polyps (17). However, in the present small study, the association of both H&E and D2-40 with survival was relatively weak. Larger studies have reported that lymphatic invasion as shown by D2-40 was significantly associated with nodal metastases and disease progression (6, 18, 19).

In terms of blood vessel invasion, the incidence rate of blood vessel invasion with routine elastic (53%) and CD31 (32%) were within the range of previous reports (7). Blood vessel invasion, as revealed by staining of CD31, was seen in peritumoural and intratumoural areas and was more useful in demonstrating small vessel invasion as the endothelial lining of some bigger vessels was destroyed by tumour. In contrast to CD31, blood vessel invasion revealed with elastica was positively associated with tumor stage. Blood vessel invasion with elastica was also significantly associated with shorter cancer-specific survival duration in the present study. This work is consistent with and correlates with previous reports (10, 20, 21).

When the relationship between lymphatic invasion (D2-40) and blood vessel invasion (elastica) was examined, they were found to be directly associated. However, only blood vessel invasion was independently associated with cancer survival. These results would suggest lymphatic and blood vessel invasion may reflect the same process promoting

tumour dissemination. If this were proven to be the case, it would have important implications for the staging and treatment of colorectal cancer.

A limitation of the present study was that although lymphatic and blood vessel invasion are included in guidelines for current pathological reporting in colorectal cancer, lymphatic invasion on H&E-stained slides did not have any prognostic value. This may suggest that the sample size was rather small for such survival analysis. Nevertheless, there was a direct comparison with other staining approaches and blood vessel invasion did have a prognostic value. This would suggest that the present results are of value and make the case for increased appreciation of the relative prognostic value of blood vessel invasion (using elastica staining) in such patients.

In conclusion, immunostaining with D2-40 has potential as a marker of lymph node metastasis in early-stage colorectal cancer. The detection of blood vessel invasion appears to be optimised by utilising elastica stain, resulting in improved detection rates and improved prediction of survival. The present results also point to the relative role of special stains in tumour progression and dissemination in patients with colorectal cancer.

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