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Pathology Section

Utility of D2-40 Immunohistochemistry in Detecting Lymphovascular Invasion in Colorectal Carcinoma: A Cross-sectional Study

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ABSTRACT

Introduction: The detection of Lymphovascular Invasion (LVI) has been recognised as an important prognostic factor in Colorectal Carcinoma (CRC), particularly in determining the adjuvant chemotherapy and further treatment plan. LVI on routine Haematoxylin and Eosin (H&E) section are often underdiagnosed, hence immunohistochemistry in detecting lymphatic invasion has a greater role. The majority of endothelial markers stain lymphatics and blood vessels without discrimination. D2-40 is a recent marker that is specific for lymphatic endothelium.

Aim: To assess the utility of D2-40 immunohistochemical marker in identifying LVI in Colorectal Carcinoma (CRC).

Materials and Methods: This hospital-based observational cross-sectional study was conducted at Department of Pathology, SCB Medical College, Cuttack, Odisha, India between the period July 2022 to June 2024 which included 42 resected CRC cases. Immunohistochemical staining using D2-40 was performed. Statistical analysis was carried out using International Business Machine (IBM) Statistical Packages of Social Sciences (SPSS) version 25.0; Chi-square test and Independent t-test were applied to evaluate the association of LVI, intratumoural and peritumoural Lymphatic Vessel Density (LVD) detected by D2-40 Immunohistochemistry (IHC) with lymph node status and clinicopathological parameters.

Results: D2-40 demonstrated a higher detection rate of LVI compared to H&E staining, identifying 6 (14.28%) additional cases with statistical significance (p=0.031). A strong association was observed between LVI and lymph node involvement, with 88% of node positive cases showing LVI on D2-40 (p<0.001). Peritumoural LVD showed significant association with nodal metastasis (p<0.001).

Conclusion: D2-40 enhances the identification of LVI and provides valuable prognostic information in CRC. LVI detected by D2-40 showed strong association with lymph node metastasis, suggesting its role in predicting disease progression. Peritumoural LVD was significantly associated with nodal status, indicating its potential role in tumour dissemination. Incorporating D2-40 into routine diagnostic practice may aid in identifying high-risk patients and guiding adjuvant therapy decisions.

Keywords: Lymphangiogenesis, Lymph node involvement, Microvessels, Podoplanin, Prognostic markers, Tumour metastasis

INTRODUCTION

The CRC poses a serious health problem as the world's third most frequently diagnosed malignant neoplasm and the second most fatal cancer globally [1]. The annual burden of colorectal cancer is expected to rise to 3.2 million new cases per year (an increase of 63%) and 1.6 million deaths per year (an increase of 73%) by 2040 [2]. It mostly affects elderly persons, with the majority of cases occurring in people aged 50 and above. The male-to-female ratio is 1.7:1 and males are more predisposed than females. A rising trend of incidence in the younger age group has been noted in recent times [3]. The aetiopathogenesis of CRC is an intricate interplay of environmental and genetic factors. Multiple studies have demonstrated that risk factors for CRC include diet and lifestyle, family history and chronic inflammation [4,5].

Pathological staging is presently the most decisive predictor of prognosis in CRC. The frequently used staging system for CRC, including Dukes and Tumour Node Metastasis (TNM) rely on the degree of depth of invasion and number of lymph nodes involved in metastasis and assist as a benchmark for anticipating clinical outcome [6,7]. Although TNM staging has been proven to be a reliable prognostic tool [8], there are situations in which it appears to only represent the anatomical extent of the tumour with no correlation with patient's survival, as disease progression with locoregional or distant metastases followed by adverse outcome has also been observed in a percentage of stage I CRC [9]. Therefore, the detection of novel histopathologically relevant prognostic markers in addition to the currently used clinical-pathological system would be beneficial to identify those patients with early stage of CRC at high risk of progression in order to submit them to adjuvant therapies.

The lymphatic system is proved to be one of the most significant pathways for tumour cell dissemination. In fact, it is predicted that tumour cells can enter lymphatic microvessels more easily than blood microvessels because the former shows a discontinuous or completely absent basement membrane and are devoid of pericytes [10]. Lymphatic vessel invasion in CRC is reported to be a significant risk factor that is associated with dismal prognosis [11]. It is also known that lymphatic infiltration by the tumour poses a serious danger for lymph node metastases as well as survival in CRC [12].

The blood vessels can be identified on routine H&E staining methods with the endothelial lining and presence of red blood cells within the lumen. Controversies over LVI detection arise mainly from the difficulty in visualising lymphatic vessel walls [13]. There can be interobserver variations in H&E stained sections because of artifactual spaces mimicking such lymphatic vessels. Recently, lymphatic endothelial specific markers have become available, which facilitate the analysis of lymphangiogenesis in cancer [14]. D2-40 is a monoclonal antibody against a 40-kDa O-linked transmembrane sialoglycoprotein podoplanin, a specific marker for lymphatic endothelial cells. D2-40 clearly demarcates tumour emboli in lymphatic vessels in paraffin sections of primary tumours and hence has the potential for increasing the accuracy of detection of lymphatic invasion in CRC. Despite the established prognostic significance of LVI in CRC [15,16],

it remains frequently underdiagnosed on routine H&E staining due to tissue retraction artifacts and interobserver variability. While several studies have explored the utility of D2-40 in detecting LVI, data on simultaneous assessment of both intratumoural and peritumoural LVD across all TNM stages of CRC remains limited [14,17]. The present study addresses this gap by evaluating D2-40 immunohistochemistry not only for LVI detection but also for quantifying LVD in both compartments across all stages of CRC, thereby providing a more comprehensive assessment of lymphatic involvement and its association with nodal metastasis. The objective of the present study was to assess the lymphatic invasion using D2-40 immunostaining in CRC, compare the detection of LVI by H&E staining with D2-40 immunostaining and to evaluate the association of LVI and LVD (peritumoural and intratumoural) with lymph node status.

MATERIALS AND METHODS

The present study was designed as a hospital-based observational cross-sectional study carried out in the Department of Pathology of SCB Medical College, Cuttack, Odisha, India on cases collected between July 2022 to June 2024 to evaluate the role of D2-40 immunohistochemistry in detecting lymphatic invasion in CRC. This design was considered appropriate as it allows assessment of histopathological and immunohistochemical parameters in resected specimens at a single point in time without intervention. Given the relatively limited availability of suitable cases within the study duration, a consecutive sampling approach was adopted to minimise selection bias and ensure inclusion of all eligible cases. Similar study designs have been widely employed in previous histopathological and immunohistochemical studies evaluating prognostic markers in CRC. Ethical clearance was obtained from Institutional Ethics Committee (IEC No:-1292).

Sample Size: The sample size of 42 was based on all consecutive cases of CRC received during the study period that fulfilled the inclusion criteria.

Inclusion and Exclusion criteria: The present study included cases of resected specimens with CRC from all age groups, both men and women irrespective of distant metastasis. Poorly preserved or-fixed tissues and neoadjuvant treated cases were excluded from this study.

Study Procedure

Clinical data such as patient's age, gender and other relevant details like tumour site, tumour extent, histological type and grade were noted. Specimens received were thoroughly examined and fixed overnight in 10% neutral buffered formalin within 30 minutes of collection and not more than 48 hours. Representative bits were taken from main tumour areas and peritumoural areas to include the infiltrative border with adjacent pericolic pad of fat as the chance of finding LVI is higher in areas just outside the tumour. Formalin-fixed paraffin-embedded blocks were prepared after routine processing in automated tissue processor. H&E stained sections of 4 µm thickness were analysed from each block. Tumour typing was done according to the World Health Organisation (WHO) classification of tumours [15]. Grading and Staging of the tumour was done according to American Joint Committee on Cancer (AJCC) 8th edition. (Pathologic Stage Classification i.e., pTNM) [16]. Full thickness sections were subjected to IHC using D2-40 (company-Quartett, Monoclonal mouse antibody). to identify the lymphatic vessels. Tonsil tissue was taken as positive control. Omission of primary antibody in a test case in each batch of staining was taken as negative control.

Method of reporting by Immunohistochemistry (IHC): Staining pattern of D2-40 is cytoplasmic and membranous and it stains only lymphatic endothelial cells. LVD was assessed as described by Weidner N et al., with slight modification. For LVD assessment, microvessel was considered to be a single endothelial cell or a cluster of endothelial cells positive for D2-40 and was located around a visible lumen, which was easily separated from adjacent microvessels and from other connective tissue components [17]. Intratumoural lymphatic vessels were defined as vessels within the main tumour mass, surrounded by tumour cells, with no Red Blood Cells (RBCs) in lumina. Vessels more than one High Power Field (HPF) away from the tumour front was considered as peritumoural lymphatic vessels. First, the sections were examined at low magnification 40x three areas with greatest number of distinct lymphatic foci (hot spots) were selected by 2 blinded observers and then independently evaluated for microvessel count using 400x magnification. In the absence of apparent hotspots, three or more randomly selected areas were counted. The highest number of vessels in both intratumoural and peritumoural areas were recorded for data analysis. The average of intratumoural or peritumoural vessel count was regarded as intratumoural or peritumoural LVD, respectively. LVD was classified as Low (<10 vessels/HPF), Moderate (10-15 vessels/HPF) and high (>15 vessels/HPF). LVI was considered present when at least one tumour cell cluster was clearly visible inside a D2-40 positive vessel.

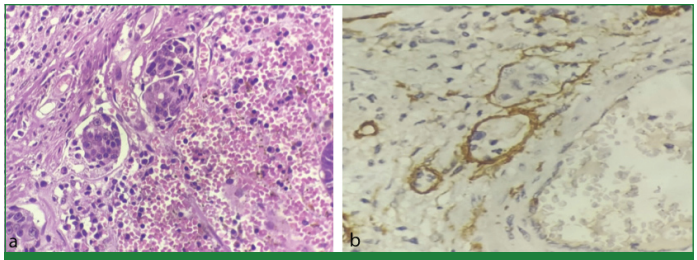
STATISTICAL ANALYSIS

All data were analysed using statistical software IBM SPSS version 25.0. All continuous variables were expressed in mean±SD, whereas categorical variables were analysed by frequency percentage. Fisher's-exact test was used to assess the association between LVI detected by D2-40 and lymph node status, given the small sample size. McNemar's exact test was applied to compare LVI detection rates between H&E and D2-40 immunostaining, as both methods were performed on the same cases (paired data). Independent samples t-test was used to compare intratumoural and peritumoural lymphatic vessel counts between lymph node positive and negative groups. The p-value ≤0.05 was set for the level of statistical significance.

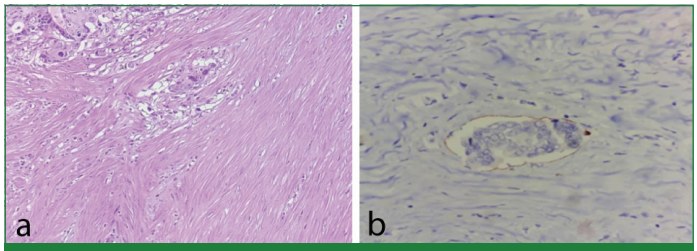
RESULTS

A total of 42 cases were included in the study, age distribution ranging from 26-89 years with a mean age of 59.2±12.5 years. 28 (66.7%) cases were males and 14 (33.3%) were females. Out of 42 cases, 16 tumours (38.1%) were located in the ascending colon, 8 (19%) in rectosigmoid region, 4 (9.5%) in sigmoid colon, 4 (9.5%) in the rectum, 3 (7.1%) in transverse colon, 3 (7.1%) in descending colon, 2 (4.8%) in ileocecal region and 2 (4.8%) in the caecum. Total 8 cases (19%) were in stage pT1, 21 (50%) in pT2, 8 (19%) in pT3 and 5 (11.9%) in pT4. Of the 42 cases, 31 cases (73.8%) were moderately differentiated, followed by 7 (16.7%) cases were well-differentiated and 4 (9.5%) cases were poorly differentiated.

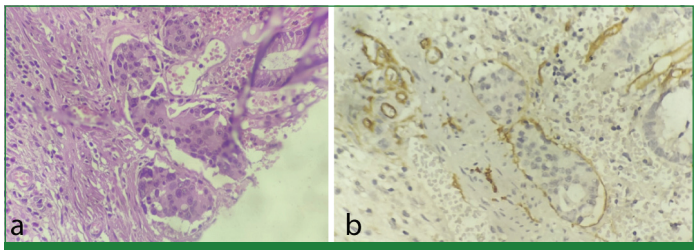
Around 25/42 (59.5%) of the cases show lymph node metastasis. A total of 22 (52.4%) out of 42 cases were positive for LVI by H&E [Table/Fig-1a]. A 28 (66.7%) showed LVI with D2-40 [Table/Fig-1b]. D2-40 detected LVI in six additional cases (14.28% of the total 42 cases) that were negative on H&E staining. (p-value=0.031) [Table/Fig-2-4]. Out of 25 cases with lymph node involvement, 22 of them showed lymphatic invasion which was identified through D2-40 showing significant association (p-value=0.001) between LVI and frequency of tumour cell metastasis to lymph node [Table/Fig-5]. Peritumoural lymphatics were found to be present in all cases and intratumoural lymphatics were identified in only 40(95%) cases. The two cases in which intratumoural lymphatics were absent were assigned a LV count of zero and categorised as low LVD for the purpose of analysis.Intratumoural lymphatics were small, irregular and flattened [Table/Fig-6] while peritumoural lymphatics were enlarged and dilated [Table/Fig-7]. The mean number of vessel identified by D2-40 in intratumoural region is 4.07±2.72. On the whole, the mean LV in peritumoural region was significantly higher than in intratumoural region (7.36±3.16 vs 4.07±2.72). Lymphatic vessel counts in peritumoural region showed significant association (p-value <0.001) with nodal metastasis while intratumoural LV count showed no significant association with nodal metastasis (p-value=0.157) [Table/Fig-8]. Peritumoural LVD showed significant association (p-value=0.001) with nodal metastasis while intratumoural LVD showed no significant association (p-value= 0.405) with nodal status [Table/Fig-9].



[Table/Fig-1]: a) Photomicrograph from colon carcinoma showing LVI with (H&E), (400x). b) Corresponding photomicrograph showing LVI with D2-40 (400x).



[Table/Fig-2]: a) Photomicrograph from colon carcinoma showing LVI not apparent on H&E surrounded by desmoplastic stroma (100x). b) Corresponding photomicrograph showing LVI identified with D2-40 (400x).



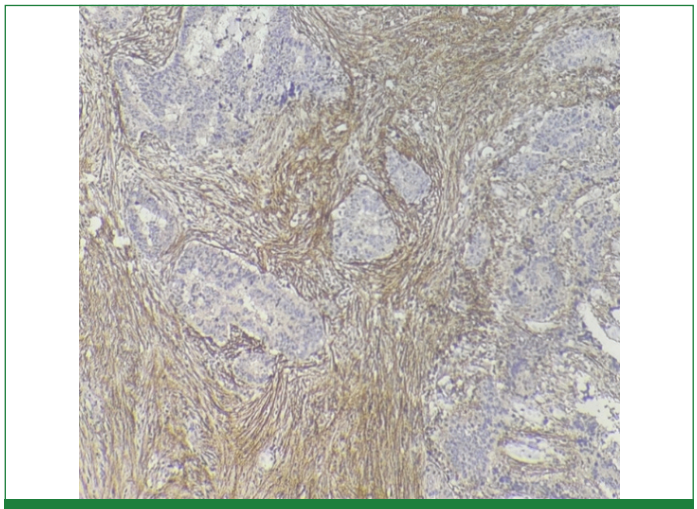
[Table/Fig-3]: a) Photomicrograph from colon carcinoma showing LVI with (H&E) (400x). b) Corresponding photomicrograph showing LVI with D2-40 (400x).

Lymphatic invasion (H&E)	Positive D2-40 IHC	Negative D2-40 IHC	McNemar exact test (p-value)*
Identified	22	0	0.031
Not Identified	6	14	

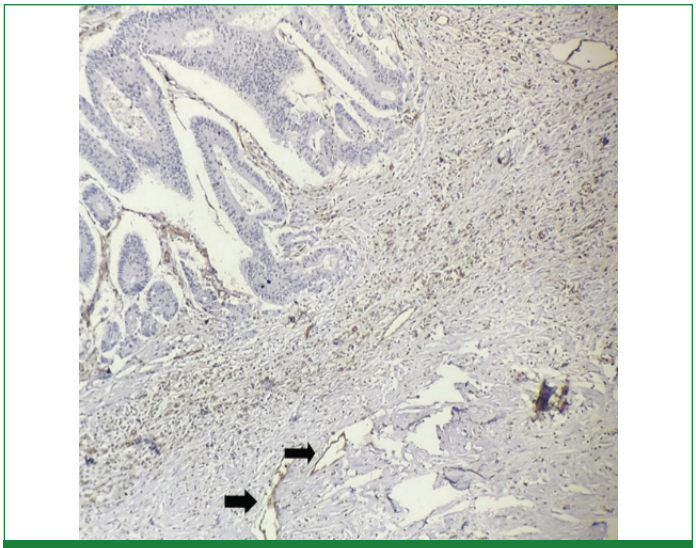
[Table/Fig-4]: Comparison between Lymphovascular Invasion (LVI) detected on H&E and D2-40 immunohistochemistry.
McNemar exact test applied as data represents paired observations on the same cases assessed by two methods. p ≤0.05 considered statistically significant

Lymph node status	Lymphatic invasion (D2-40)		
	Positive	Negative	Fisher's-exact test (p-value)*
Positive	22 (88.0%)	3 (12.0%)	0.001 Odds ratio=13.44
Negative	6 (35.3%)	11 (64.7%)	

[Table/Fig-5]: Association between LVI detected by D2-40 immunohistochemistry and lymph node status.
*Fisher's-exact test was applied in place of chi-square given the small sample size (n=42). A two-sided p-value ≤0.05 was considered statistically significant.



[Table/Fig-6]: Photomicrograph showing intratumoural lymphatics, D2-40 (100x).



[Table/Fig-7]: Photomicrograph showing peritumoural lymphatics (arrows) away from focus of tumour, D2-40 (100x).

Lymph node status	LV count			
	Intratumoural	p-value*	Peritumoural	p-value*
Positive	4.68±2.63	0.082	8.96±2.88	<0.001
Negative	3.18±2.77		5.35±2.47	

[Table/Fig-8]: Association of lymph node status with intratumoural and peritumoural lymphatic vessel count.
*Independent samples t-test; p ≤0.05 considered statistically significant

Lymph node status	LVD intratumoural			LVD peritumoural		
	Low	Mod	Fisher's-exact (p-value)	Low	Mod	Fisher's-exact (p-value)
Positive	25 (100%)	0	0.405	14 (56.0%)	11 (44.0%)	0.001
Negative	16 (94.1%)	1 (5.9%)		17 (100%)	0 (0%)	

[Table/Fig-9]: Association of lymph node status with intratumoural and peritumoural Lymphatic Vessel Density (LVD).

DISCUSSION

Colorectal cancer is one of the commonest cause of cancer related death worldwide and in India it still remains the important cause of mortality and morbidity despite advanced treatment options. The lymphatics make up an important pathway of metastasis for majority of cancers and extent of lymph node involvement is an important prognostic factor [18].

The LI is a significant finding in evaluating primary tumours and it helps in predicting survival. LI identification carries even more significance when the lymph nodes show no tumour deposits and the presence of LI can help the oncologist to decide upon further treatment. Studies have shown the prognostic value of LVD [14]. The study of tumour-associated lymphangiogenesis and its possible role in tumour progression has been made easier by the discovery of novel markers for distinguishing blood and lymphatic vessels. D2-40 is a specific marker for lymphatics to identify LVI and assess LVD [19,20].

In present study, maximum number of cases was in age group of 41-60 years (50%) and mean age of 59.2±12.5 years. Kawaura K et al., (2007) and Amritha PS et al., (2022) also found similar age distribution [21,22]. In present study, 28 (66.7%) cases were males and 14 (33.3%) were females with male to female ratio of 2:1. Study conducted by Bajaj S et al., (2017) also had same gender distribution [23]. Among 42 cases, 59.5% cases had lymph node involvement in the present study. Similar to the present study, Bajaj S et al., (2017) and El-Aziz A et al., (2019) also found more cases with lymph node involvement [23,24]. In contrast to present study, Amritha PS et al., (2022) and Ishii M et al., found relatively less cases with lymph node involvement [22,25].

In the present study, D2-40 immunostaining detected LVI in 6 additional cases compared with H&E, showing a 14.2% increase in detection. Similar improvement in LVI detection by D2-40 has been reported by Saad et al., (2006), Kawaura K et al., (2007), Barresi V et al., (2012), Bajaj S et al., (2017) and El-Aziz A et al., (2019) [Table/Fig-10] [14,21,23,24,26]. The results of the examinations by H&E staining and immunohistochemical staining with D2-40 monoclonal antibody can be explained by a number of factors. Tumour emboli in microlymphatic vessels are sometimes difficult to detect by H&E staining because they are in invasive areas with a strong host reaction, such as fibrocollagenous tissue proliferation. Spaces around nests of tumour cells that are caused by inevitable retraction of tissue shrinkage artifacts after tissue fixation may be confused with true tumour emboli floating in lymphovascular spaces. D2-40 monoclonal antibody proved to be quite helpful in rectifying the error in recognising the actual tumour in lymphatic vessels, as it only reacts with the endothelium of lymphatic vessels.

Author and year	No. of cases	LVI (H& E)	LVI (D2-40)	No of more cases identified by D2-40 than H&E
Saad RS et al., (2006) [14]	90	34.4%	53.3%	D2-40 identified 17 more cases than routine H&E (19%).
Kawaura K et al., (2007) [21]	122	14.8%	34.4%	D2-40 detected 24 more cases with LI than H&E (19.6%).
Barresi V et al., (2012) [26]	82	22%	28%	D2-40 detected 5 more cases with LI than H&E. (6.09%)
Bajaj S et al., (2017) [23]	40	40%	67.5%	D2-40 detected 11 more cases with lymphatic invasion than H&E (27.5%).
El-Aziz A et al., (2019) [24]	60	33.3%	50%	D2-40 detected 10 more cases with lymphatic invasion than H&E (16.7%)
Present study, 2026	42	52.4%	66.7%	D2-40 detected 6 more cases with lymphatic invasion than H&E (14.2%).

[Table/Fig-10]: Comparison of LVI detection by H&E and D2-40 immunohistochemistry in the present study and previous studies [14,21,23,24,26].

Lymphatic invasion was correlated with lymph node status in this study. Out of 25 node positive cases, 88% had LVI. And out of 17 node negative cases, 35.3 % had LVI. There is significant association between LVI (IHC) with lymph node status (p-value=0.001) in present study. This study is similar to Kawaura K et al., (p-value=0.0415) and Barresi V et al., (p=value= <0.0001) [21,26]. Similar findings were also noted by Ishii M et al., i.e., in LVI-positive (D2-40) patients, lymph node metastasis was observed more frequently than in LVI-negative (D2-40) patients (13/45 vs 5/91, p<0.001) [25].

It has been well established that lymphangiogenesis can occur both within the tumour mass and/or in the tumour periphery, leading to formation of intratumoural and peritumoural lymphatic vessels respectively. However, the functional significance of intratumoural and peritumoural lymphatics involved in the pathology of tumours remains controversial. Peritumoural lymphatics were more numerous than intratumoural lymphatics in the present study. Peritumoural lymphatics were enlarged and dilated and intratumoural lymphatics were small, irregular and flattened similar to other studies [14]. Lymphatic vessel counts in peritumoural region showed a significant association (p-value <0.001) with lymph node metastasis and Peritumoural LVD had a significant association (p-value=0.001) with nodal metastasis. On the contrary, no statistically significant association was seen between intratumoural LVD and nodal status (p-value=0.405). Similar to present study, Barresi V et al., and Zhang Y et al., also reported that high peritumoural LVD had a significant association with lymph node metastasis [26,27]. But in contrast to the present study, Bajaj S et al., concluded that intratumoural lymphatics showed significant association with lymph node status (p-value=0.024) while Lin M et al., reported no significant association between intratumoural and peritumoural LVD with lymph node metastasis [23,28]. Such divergent results could be caused by the various antibodies and techniques used to highlight and assess lymphatics and LVD quantification. Most studies have mainly focused on a particular subset of CRC patients with either early or late TNM stage [29]. Based on present results from 42 cases, spanning all TNM stages, we found that peritumoural LVD, rather than intratumoural LVD, was substantially associated with lymph node metastasis. This indicates that peritumoural LVD plays a more significant role in lymph node metastasis than intratumoural LVD.

Limitation(s)

There were few limitations in the present study. This was a single Institutional study and convenience sampling of cases was done. Hence, this single study is not sufficient to give generalised result to comment on whole population. All histological sub-types of CRC could not be represented owing to the limited number of cases. In the present study, survival analysis of patients was not possible as follow up period was short.

CONCLUSION(S)

The LVI in CRC is a strong stage-independent prognostic factor and is closely associated with lymph node metastasis and poorer survival. D2-40 immunostaining enhances the detection of LVI compared with routine H&E staining. In the present study, LVI showed a strong correlation with nodal status, supporting its role in tumour dissemination and risk stratification. Peritumoural LVD was also significantly associated with nodal metastasis, suggesting that increased peritumoural lymphatics may facilitate lymphatic spread. Thus, D2-40 may serve as a useful adjunct in routine histopathology to identify high-risk patients and guide clinical decision-making. Larger studies are required to confirm its prognostic value and clarify the mechanisms of tumour-associated lymphangiogenesis in CRC.

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