RESEARCH ARTICLE

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Tissue Microvessel Density as a Potential Predictive Marker for Vascular Invasion in Colorectal Cancer

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Abstract

Objective: This study aimed to determine whether microvessel density (MVD) in the tumor tissues could be a potential predictive marker for vascular invasion (VI). **Methods:** Surgical specimens of 73 patients with colorectal adenocarcinoma in Phramongkutklao Hospital were analyzed. Tissues of patients receiving preoperative radiation or prior anti-angiogenic therapy were excluded. Tumor MVD was determined using the average number of counted CD34-stained endothelial cells from two selected fields at 200x magnification in each slide. The presence of VI was defined by tumor involvement of endothelial cell-lined spaces. The optimal cut-off value of MVD to predict VI was examined using receiver operating characteristic analysis to assess the area under the curve and accuracy. **Result:** VI was detected in 17 of 73 specimens (23.3%). Colorectal cancer (CRC) specimens were classified according to MVD as low (61 specimens, 83.6%) and high density (12 specimens, 16.4%). Average MVD was slightly higher in specimens with VI (81.3±9.3) than those without VI (76.3±7.6), but without statistical significance (p = 0.736). The MVD's cut-off value of 60 vessels/200x field provided 88% sensitivity, 40% specificity, and 57.5% accuracy, with the area under the ROC curve of 0.5788. Patients with CRC having MVD of > 60 vessels/200x field were at significantly higher risk of VI than those with CRC having MVD of <60 vessels/200x field (P=0.009, Fisher's exact test). Univariate analysis revealed that MVD, nodal involvement and AJCC tumor stage were associated with the presence of VI (p <0.05). Further multivariate analysis of these three potential variables demonstrated MVD (OR, 11.994; 95% CI, 2.197 to 65.483; p <0.01) and nodal involvement (OR, 10.767; 95% CI, 1.973 to 58.748; p <0.05) as independent prognostic factors associated with VI. **Conclusion:** Based on our study, MVD immunostaining was an angiogenic marker that potentially be a predictive marker for VI.

Keywords: Vascular invasion- angiogenesis- VEGF- microvessel density- predictive marker.

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Introduction

Colorectal cancer (CRC) is a common cause of cancer-related death worldwide. Despite the great advances in surgical therapies and novel adjuvant treatments, long term survival of patients with CRC is still unpredictable and depends on several factors. A certain histopathologic variable associated with CRC metastasis is lymphatic invasion (LI) or vascular invasion (VI) (Huh et al., 2010), defined as the presence of tumor cells within the lymphatic or vascular channels (Al-Sukhni et al., 2017). Both LI and VI have been accepted as a parameter indicating lymph node involvement and enhancing the risk for micrometastasis among patients with CRC (Barresi et al., 2012). Based on related studies, the incidence rate of LI or VI was 8.5% among patients with CRC stage I (Kim et al., 2020). Remarkably, this rate was increased to 12.3 to 22% among patients with CRC stage II (Al-Sukhni et al., 2017; Nikberg et al., 2016; Jiang et al., 2019; Wang et al., 2021). The highest incidence rate of LI or VI that has been reported was 39.7% among patients with CRC stages I to III (Wang et al., 2021). Maehara (2000) found that the rate of lymph node involvement and metastasis in gastric cancers increased in relation to the extent of VI. The presence of VI has also been associated with an increased risk of nodal involvement and visceral metastasis among patients with rectal cancer (Minsky and Cohen, 1999). Furthermore, small vessel invasion has been proved to be an independent indicator of adverse outcome (Lim et al., 2010; Santos et al., 2013). A study found the expression of vascular endothelial growth factor (VEGF) and the microvascular density (MVD) were more prominent in tumor tissues in relation to the extent of VI (Wang et al., 2021), suggesting a close relationship between VI and intratumoral angiogenesis.

Tumor angiogenesis or neovascularization is the process of new blood vessel formation and recruitment of blood vessels from surrounding stroma that originates

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in the endothelium of the existing vasculature (Uzzan et al.,2004). In malignant tumors, the existence of abnormal angiogenesis facilitates tumor growth. An increment of metabolic needs has been observed for further tumor growth when a tumor reaches the size of 1 to 2 mm (Uzzan et al.,2004). Tumoral angiogenesis is mediated by various angiogenic factors and is associated with survival in numerous tumors (Des et al., 2006; Svagzdys et al., 2009). VEGF is an essential mediator of the new vessel formation process that is mainly produced by macrophage, vascular smooth muscle cells and tumor cells. The expression of VEGF and its receptor was demonstrated in gastrointestinal adenocarcinoma (Guang-Wu et al., 2000). Weidner (1991) developed an MVD assessment method to quantify intratumoral angiogenesis and concluded that the number of microvessel per 200x field could be an independent predictor of metastasis. Tumors with higher MVD are considered aggressive with poor prognosis due to the high angiogenic activity. Increasing MVD may consequently lead to tumor spreading via the penetration of cancer cells in the circulation (Uzzan et al.,2004). MVD has been used as a surrogate marker for tumoral angiogenic activity (Wang et al., 2008) and is associated with aggressiveness of various types of cancer (Iakovlev et al., 2012; Wang et al., 2007).

The immunohistochemistry has been widely used to measure VEGF expression and MVD in primary tumor specimens; both have been proved to be tissue biomarkers for the angiogenic activity. Several studies have examined the potential prognostic value of these two angiogenic markers. Among patients with gastric cancer, the degree of VEGF expression was proved to be a useful indicator for the prognosis and for the risk estimation of tumor recurrence (Maeda et al.,1996). In nasopharyngeal cancer, the metastatic potency of tumor tissues and the prognosis can be estimated by measuring MVD and VEGF expression in tumor tissues (Guang-Wu et al., 2000). A related study found that angiogenesis was related to prognosis and the average MVD was significantly higher among patients with VI than among those with no VI (Wang et al., 2007). The aim of this study was to determine whether MVD in the colorectal cancer tissues could be a potential predictive marker for VI.

Materials and Methods

The present study was reviewed and approved by the Institutional Review Board of the Royal Thai Army Medical Department before initiating. This study included surgical specimens of 73 patients with CRC undergoing potentially curative resection in Phramongkutklao Hospital. Tissues of patients receiving preoperative radiation or prior anti-angiogenic therapy were excluded. Medical records were reviewed to ascertain information on clinical follow-up and treatment outcomes.

Immunohistochemistry

Formalin-fixed, paraffin-embedded, 5-μm tissue sections were deparaffinized with xylene, dehydrated in ethanol and incubated with 3% hydrogen peroxidase for 5 minutes. After washing with phosphate-buffered saline (PBS), tissue sections were incubated in 10% normal horse serum, followed by an overnight incubation with either an anti-VEGF monoclonal antibody (1:200, Diagnostic Biosystem, USA) or an antiCD34 antibody (1:500, Dako, Denmark). On the following day, the slides were incubated with a drop of the superenhancer TM, washed with working PBS wash buffer, incubated with a drop of labeled dextran polymer conjugated horseradish peroxidase (HRP) for 30 minutes and washed twice with cold PBS. Finally, a drop of freshly prepared DAB (32-Diamino benzidine tetrahydrochloride a substrate chromogen) was added on the sections. Slides were then washed in running distilled water to remove excess DAB and counter-stained with hematoxylin.

Assessment of MVD

Each tissue section was initially examined at 50x magnification, and two areas with the highest MVD were identified. Individual vessel counts were then performed at 200x magnification (Fig. 1). Any single CD34-stained cell that indicated an endothelial cell was counted as a single vessel. A branching structure was counted as a single vessel unless a break in the continuity of the structure was noted. Tumor MVD was determined by an average number of counted CD34-stained endothelial cells from two selected fields at 200x magnification in each slide and categorized in two groups: low (<60) and high (>60).

Assessment of VI

Hematoxylin-eosin-stained slides were examined for the presence of VI, specifically small vessel invasion, which was defined by tumor involvement of endothelial cell-lined spaces, encompassing capillaries and postcapillary venules (Fig. 2). Tumor involvement of endothelial cell-lined spaces with an identifiable smooth muscle layer or elastic lamina, which is also known as venous (large vessel) invasion, was excluded in this study.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics, Version 26.0 (IBM Corp., Armonk, NY, USA). Patients' demographic data were analyzed using an independent t-test for normally distributed variables or a Mann-Whitney U test for non-normally distributed variables. The Chi-square test or Fisher's exact test was used for categorical data. The correlation between MVD and VI was analyzed using a Spearman's correlation method. The correlation between individual histopathologic features and the MVD was determined using either Pearson correlation or Spearman rank correlation method according to data types. A probability value of less than 0.05 was significant. A series of univariate analyses was performed. to determine the variables associated with VI. Variables associated with VI with a value of $p < 0.05$ in the univariate analysis were selected for stepwise logistic regression. Potentially associated variables were then tested using multivariate logistic regression analysis with a Wald statistic backward stepwise selection. The results of the logistic regression are reported as odds ratios (ORs) with 95% confidence intervals (CIs).

Results

The clinicopathologic features of 73 patients with CRC are shown in Table 1. VI was detected in 17 of 73 specimens (23.3%). The specimens were classified according to MVD as determined by CD34 immunostaining as low (61 specimens, 83.6%) and high density (12 specimens, 16.4%). Average MVD was shown to be slightly higher in specimens with VI (81.3±9.3) than those without VI (76.3±7.6), but without statistical significance (p = 0.736). Patients with VI had a higher recurrence rate than those with no VI (68.7 and 44.6%, respectively) as shown in Table 2.

Relationship among VI, MVD, and tumor stage

The Spearman's correlation test, as summarized in Table 3, indicated a low correlation between MVD and VI (correlation value of 0.337, p <0.01) with moderate correlations between the AJCC tumor stage and VI (correlation value of 0.226, p <0.05). The univariate analysis is summarized in Table 2. MVD, nodal involvement and AJCC tumor stage were found to be associated with the presence of VI (p <0.05). Further multivariate analysis of these potential variables, as shown in Table 2, demonstrated MVD (OR, 11.994; 95% CI, 2.197 to 65.483; p <0.01) and nodal involvement (OR, 10.767; 95% CI, 1.973 to 58.748; p <0.05) as independent prognostic factors associated with VI.

Optimal cut-off value for MVD to estimate risk of VI

A receiver operating characteristic (ROC) curve was created to determine an optimal cut-off value of MVD to predict VI. The MVD value of 60 vessels/200x field provided 88% sensitivity, 40% specificity and 57.5% accuracy, with the area under the curve of 0.578 (Fig. 3). Patients with CRC having MVD of > 60 vessels/200x field were at significantly higher risk of VI than those with CRC having MVD of < 60 vessels/200x field (P=0.009, Fisher's exact test).

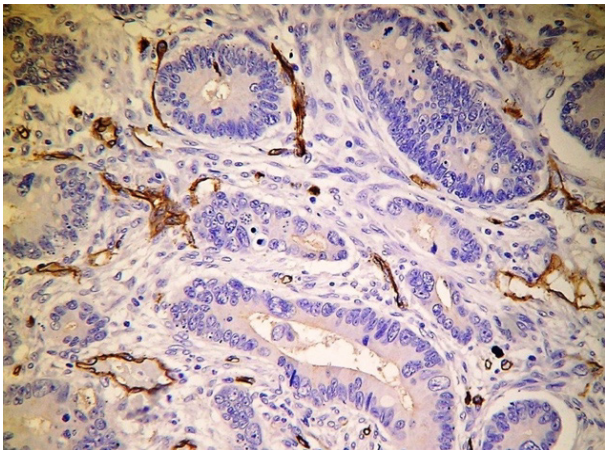

Figure 1. Microvessel Density is assessed by CD34 Immunostaining.

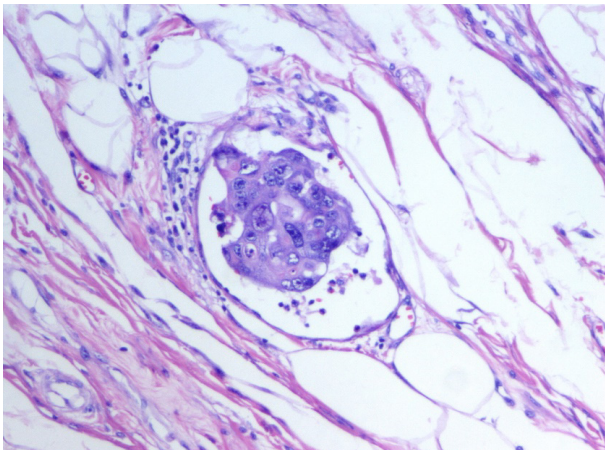

Figure 2. Vascular Invasion is Defined as Tumor Involvement of Endothelium-Lined Spaces.

Discussion

Several histopathologic features have been identified as predictors of treatment failure and disease recurrence

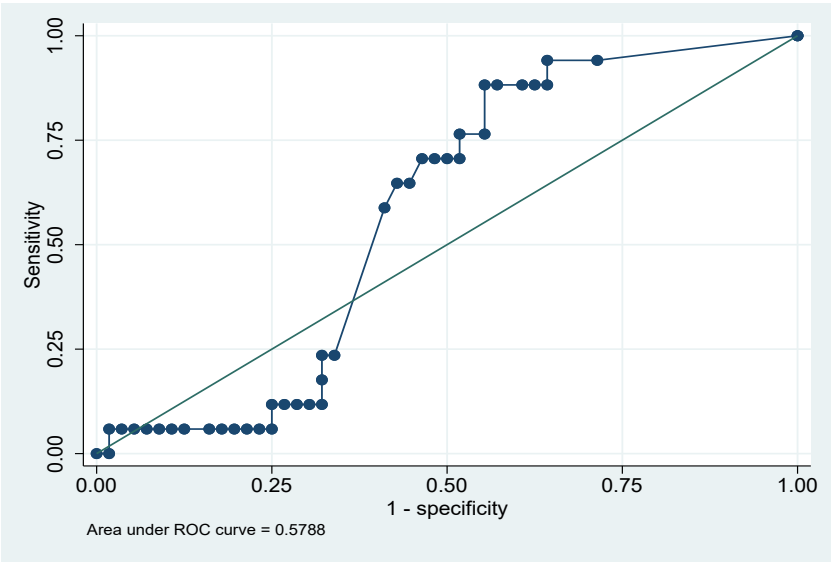

Figure 3. A Receiver Operating Characteristic (ROC) Curve. The MVD value of 60 vessels/200x field provided 88% sensitivity, 40% specificity, and 57.5% accuracy, with the area under the curve of 0.5788.

Table 1 Demographics and Clinicopathologic Characteristics of Patients

Variables	Number N (%)
Gender	
Male	46 (63%)
Female	27 (37%)
Age (Years)	
Mean ± SD	60 ± 12.8
Pre-operative CEA (ng/ml)	
Median (Min, Max)	7.69 (0.99, 117.6)
Pathological features	
Lymphatic invasion	23/73 (31.5%)
Vascular Invasion	17/73 (23.3%)
Mucinous component	13/73 (17.8%)
MVD (CD34)	
Low < 60	61 (83.6%)
High >/= 60	12 (16.4%)

including the degree of tumor differentiation, the depth of local invasion, the presence of nodal metastases and the extramural VI (Rajaganeshan et al., 2007). The first step of tumor progression is the process by which tumor cells enter the circulation through lymphatic or vascular vessels (Chung and Mahalingam, 2014). VI is defined as the presence of tumor cells within the endothelium-lined vascular channels. VI has been accepted as a histologic index of nodal involvement, visceral metastasis and advanced stage among patients with colorectal cancer (Minsky and Cohen, 1999; Huh et al., 2010; Wang et al., 2021). VI has also been an independent prognostic factor for distant metastasis (Horn et al.,1991) and for survival (Chapuis et al.,1985) in colorectal adenocarcinoma.

Angiogenesis is a fundamental process for tumor growth and metastasis (Folkman 1986), which is associated with clinical aggressiveness of tumors (Mineo et al., 2004). Angiogenesis has been measured by determining microvessel density (MVD) (Hasan et al., 2002) and has been recognized as a prognostic indictor in several types of cancers (Ushijima et al 2001; Ribatti et al 2003; Uzzan et al., 2004). MVD and VEGF expressions are two biomarkers that have been used to quantify the level of angiogenesis. Studies in various kinds of tumors tissues to determine the correlation of these two angiogenic markers are inconclusive (Decaussin et al., 1999; Chung and Mahalingam 2014). A study in colorectal

Table 2. Univariate Analysis and Multivariate Analysis for Variables Associated with Vascular Invasion

Variables	Intratumoral vascular invasion N (%)		Crude OR	95% CI	p-value	Adjusted OR	95% CI	p-value
	No	Yes						
Pathological tumor staging								
Stage I-II	19 (33.9%)	1 (5.9 %)	Ref					
Stage III-IV	37 (66.1%)	16 (94.1%)	8.216	1.012-66.737	0.049*			
Nodal involvement								
No	31 (55.4%)	3 (17.6%)	Ref			Ref		
Yes	25 (44.6%)	14 (82.4%)	5.787	1.495-22.404	0.01*	10.767	1.973-58.748	0.006*
MVD(CD34)								
Mean (SD)	81.3 (9.3)	76.3 (7.6)			0.736			
Low < 60	50 (89.3%)	10 (52.9%)	Ref			Ref		
High >/= 60	6 (10.7%)	7 (5.9%)	5.833	1.615-21.075	0.007*	11.994	2.197-65.483	0.004*
Recurrence								
No	31 (55.4%)	6 (35.3%)	Ref					
Yes	25 (44.6%)	11 (68.7%)	2.273	0.738-7.007	0.147			
Histologic grading								
Low	53(94.6%)	16(94.2%)	Ref					
High	3(5.4%)	1(5.8%)	1.104	0.107-11.36	0.663			
Mucinous component								
No	45(80.4%)	15(88.2%)	Ref					
Yes	11(19.6%)	2(11.8%)	0.545	0.108-2.745	0.719			

*Statistically significant at the 0.01 level (2 tailed)

Table 3. Correlation among VEGF Immunoreactivity, MVD, Vascular Invasion, and AJCC Staging.

Variables	MVD(CD34)	Vascular invasion	AJCC staging
MVD (CD34)	1	0.337**	-0.115
Vascular invasion	0.337**	1	0.266*
AJCC staging	-0.115	0.266*	1

*, Spearman's correlation test is significant at the 0.05 level (2 tailed); **, Spearman's correlation test is significant at the 0.01 level (2 tailed)

cancer specimens did not show a significant difference in MVD between the VEGF-positive and VEGF-negative groups (Anannamcharoen and Nimmanon, 2012). The mean MVD was not correlated with both the percentage of positive immunoreactive cells and intensity of VEGF immunoreactive staining (Anannamcharoen and Nimmanon, 2012).

In breast cancer, both average microvessel count and blood vessel invasion were independent prognostic factors for 20-year disease free recurrence (Kato et al., 2003). In colorectal cancer, MVD was found as an independent risk factor for distant metastases (Cho et al., 2017). A related study revealed that VEGF expression and MVD were more prominent in tumor tissues related to the extent of vascular VI (Wang et al., 2021). A study in gastric cancers found that tumors with VI had a greater intratumoral angiogenesis, and tumors with VEGF overexpression were associated with intratumoral angiogenesis and metastases to distant organs (Maehara et al., 2000). The findings may indicate a close relation between VI and intratumoral angiogenesis. The study of Wang (2007) in tissue samples from gastric and patients with colorectal cancer revealed that the average MVD was significantly higher among patients with VI than among those with no VI. In our study, MVD was found to be an independent indicator significantly related to VI. Therefore, it could imply that MVD as determined by CD34 immunostaining could be a potential angiogenic marker indicating the risk of VI.

In conclusion, based on our study, MVD immunostaining was an angiogenic marker that potentially be a predictive marker for VI.

Author Contribution Statement

Dr. Sahaphol reviewed the literature for this manuscript, collected data, performed statistical analysis, prepared the manuscript, provided description of the introduction, results, discussed the study findings, approved the final manuscript. Dr. Chinnakrit collected data, discussed the study findings, participated in preparing the manuscript, read and approved the final manuscript. Dr. Thirayost reviewed the literature, reviewed tissue specimens, collected data, provided a description of the results, and participated in preparing the manuscript and read, and approved the final manuscript.

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Ethics statement

This study was reviewed and approved by the Institutional Review Board of The Royal Thai Army, Medical Department before initiating.

Statement of Conflict of Interest

The authors declare they have no conflict of interest.

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