

Correlation between Carcinoma Percentage (CP) and Lymphatic Microvessel Density (LMVD) Based on D2-30/Podoplanin as Metastatic Prognostic Factor to Lymph Nodes in not Otherwise Specified (NOS) Colorectal Adenocarcinoma

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ABSTRACT

Background: In colorectal carcinoma (CRC), myofibroblast are the main component cells in tumour stroma which have an important role in the metastases process. The percentage between carcinoma and desmoplastic stroma known as carcinoma percentage (CP), can be used as an independent predictor metastases. D2-40/Podoplanin (PDPN) known as a spesific marker for lymphatic endothelial cell (LEC), which used to assess lymphatic microvessel density (LMVD) and lymphatic vessel invasion (LVI). This study aims to determine correlation and association between CP, LMVD and LVI with the metastases process to lymph node (LN).

Method: CP assessment conducted on 44 samples of adenocarcinoma not otherwise specified (NOS) colorectal were divided into 22 cases with CP-High and 22 cases with CP-Low and examination D2-40/Podoplanin to assess LMVD and LVI. The statistical test is performed to find the correlation between CP and LMVD, as well as the relationship between CP, LVI and lymph node metastasis.

Results: There were a strong correlation between CP and LMVD intratumoral and peritumoral area with the negative correlation. There were a significant association ($p = 0.00$) between LMVD (intratumoral and peritumoral area) with the LVI. There was a significant association between LVI and lymph node metastases ($p = 0.03$). Intratumoral area showed significant association with lymph node metastases ($p = 0.04$), whereas peritumoral area showed no significant association ($p = 0.17$).

Conclusion: CP examination in histopathology specimen can be used to predict high/low rate of tumour cells metastases to the lymph node, based on a strong correlation between CP and LMVD.

Keywords: carcinoma percentage, lymphatic microvessel density, lymphatic vessel Invasion, lymphatic endothelial cell

ABSTRAK

Latar belakang: Pada karsinoma kolorektal, miofibroblas merupakan komponen sel utama dalam stroma desmoplastik yang memiliki peran penting dalam proses metastasis. Persentase antara karsinoma dengan stroma

desmoplastik dikenal sebagai persentase karsinoma yang berperan sebagai prediktor independen metastasis. D2-40/Podoplanin (PDPN) merupakan marker spesifik sel endothelial limfatik, digunakan untuk menilai lymphatic microvessel density (LMVD) dan lymphatic vessel invasion (LVI). Tujuan penelitian untuk mengetahui korelasi dan hubungan persentase karsinoma, LMVD dan LVI dengan kejadian metastasis sel tumor ke kelenjar getah bening (KGB).

Metode: Dilakukan penilaian persentase karsinoma terhadap 44 sampel adenokarsinoma not otherwise specified (NOS) kolorektal yang terbagi menjadi 22 kasus persentase karsinoma-high dan 22 kasus persentase karsinoma-low dan pulasan D2-40/Podoplanin untuk menilai LMVD dan LVI. Uji statistik dilakukan untuk mencari korelasi antara CP dan LMVD, serta hubungan antara CP, LVI serta metastasis KGB.

Hasil: Terdapat korelasi kuat antara CP dan LMVD area intratumoral dan peritumoral dengan arah korelasi negatif. Terdapat hubungan bermakna ($p = 0,00$) antara LMVD (area intratumoral dan area peritumoral) dengan adanya LVI. Terdapat hubungan bermakna antara LVI dengan kejadian metastasis KGB ($p = 0,03$). Area intratumoral menunjukkan hubungan bermakna dengan kejadian metastasis KGB ($p = 0,04$), sedangkan area peritumoral tidak menunjukkan hubungan bermakna (nilai $p = 0,17$).

Simpulan: Pemeriksaan CP pada sediaan histopatologi dapat digunakan untuk memprediksi tinggi/rendahnya kejadian metastasis sel tumor ke KGB, didasarkan adanya korelasi kuat antara CP dan LMVD.

Kata kunci: persentase karsinoma, lymphatic microvessel density, lymphatic vessel invasion, sel endotelial limfatik

INTRODUCTION

Colorectal carcinoma (CRC) is one of malignant epithelial tumors originating from colon, more than 90% is adenocarcinoma.¹⁻³ The most important risk factor for CRC is not only age but also the location that involved the carcinogenesis.⁴⁻⁶ CRC is the second leading cause of death in America and the third in worldwide after lung cancer and breast cancer.⁴ According to Globocan data in 2012, approximately 470,000 new cases of CRC were found in the world every year and 244,000 of them died.⁷ Pathological examination of CRC resection specimen plays an important role.⁸ Currently, the staging system used is based on the American Joint Committee on Cancer (AJCC), named the classification of tumor-node-metastasis (pTNM) and histological tumor grading based on gland formation, as a determining factor in the prognosis of adenocarcinoma not otherwise specified (NOS) Colorectal. Lymph node involvement in CRC staging system is an important part. The process of spreading of tumor cells through the lymphatic system to lymph nodes is an initial step of tumor cell metastasis and being a key to the staging system and determination of subsequent therapy on CRC.^{1,9,10}

A study conducted by Li H et al explained that the tumor microenvironment (TME) plays role in regulating the growth of tumor cells, metastatic potential and affect the outcome of therapy.¹¹ Tumor microenvironment consisting of malignant cells and desmoplastic stromal components are major

factors in the development of tumor cells. The main component of the cells in the desmoplastic stroma is myofibroblast.¹² Some of the cytokines produced by myofibroblast, which are transforming growth factor $\beta 1$ (TGF- $\beta 1$), platelet derived growth factor (PDGF), interleukin 4 (IL-4) and insulin-like growth factor 2 (IGF-2) can induce myofibroblastic differentiation in tumor desmoplastic stroma. TGF- $\beta 1$ -mediated Ras/Smad signaling in contact with type I collagen in the desmoplastic stroma, has an important role in the process of epithelial to mesenchymal transition (EMT) in CRC. During EMT, stromal cells can lose the bond and polarity, resulting in tumor cells infiltrating the surrounding tissue and metastasize to distant organs.¹³ Research by Mesker et al introduced carcinoma percentage (CP) as an independent predictor of metastasis which is assessed based on the percentage between carcinoma and desmoplastic stroma, especially myofibroblast.¹³ Pro-angiogenic molecules secreted by myofibroblast named vascular endothelial growth factor-C (VEGF-C) and VEGF-D are major factors that can induce proliferation of blood vessels and lymphatic vessels.^{14,16}

Zidar et al reported a strong correlation between myofibroblast in the desmoplastic stroma and lymphatic microvessel density (LMVD) that plays role in the process of tumor cell metastasis.¹⁷ Lymphangiogenesis process which is the active path of a carcinoma metastasis can be measured through an assessment of lymphatic microvessel density (LMVD).¹⁰ Weidner

et al in their study explained that LMVD value can be determined based on the area of Hot-Spot.¹⁸ Hot-Spot area is a calculation of the number of lymphatic vessels in the intratumoral and peritumoral area of the primary tumor. LMVD important components named Podoplanin (PDPN) can be secreted by myofibroblast, lymphatic vessels and tumor cells. Podoplanin is a 43-kd glomerular podocyte membrane mucoprotein that can be used to detect lymphatic involvement in a variety of malignancies, including CRC.¹² The difficulties in studying lymphangiogenesis in previous studies are due to lack of a lymphatic endothelial cells (LEC) specific marker. Lack of LEC specific marker made it difficult to distinguish the lymphatic vessels and blood vessels by routine hematoxylin-eosin staining (HE). The current study used a monoclonal antibody D2-40/podoplanin as an LEC specific marker in studying lymphangiogenesis, especially in the calculation LMVD and lymphatic vessel invasion detection (LVI) as determinants of metastasis of tumor cells to lymph nodes.^{10,12,19} The purpose of this study is to determine the correlation between CP and LMVD and the relationship between LMVD and LVI which were assessed based on D2-40/Podoplanin staining on adenocarcinoma NOS subtype of CRC, as a prognostic determinants of the risk of tumor cell metastasis to lymph nodes.

METHOD

The study was conducted as a cross sectional analytic study in the Department of Anatomic Pathology of Cipto Mangunkusumo Hospital, Universitas Indonesia, from April to May 2016. The sample calculation used the formula of unmatched categorical analytic. The samples used were 44 cases of adenocarcinoma NOS colorectal from 2011 to 2016 which were selected based on inclusion and exclusion criteria.

Carcinoma percentage (CP) assessment was done by staining of the deepest invasion HE slides in adenocarcinoma NOS resection. Carcinoma percentage (CP) determination performed on the entire area of the primary tumor using 40x magnification, then area with the most invasive stromal involvement is selected. Tumor cells in the determination of scoring must be present on each side of the field of view (top, bottom, right and left) (Figure 1).²⁰ Then by using 100 x magnification the percentage area of CP is determined. In this study, the value percentage of CP is determined by three most invasive areas of primary tumor mass and the lowest CP percentage which is used as the CP.

cut-off values of CP is 50%, a score of $\geq 50\%$ is CP-High showing a good prognosis and a score of $< 50\%$ is CP-Low and is associated with poor prognosis. CP value obtained on the entire sample, then used as the basis for assessment of LVI and LMVD using IHK D2-40/Podoplanin staining.

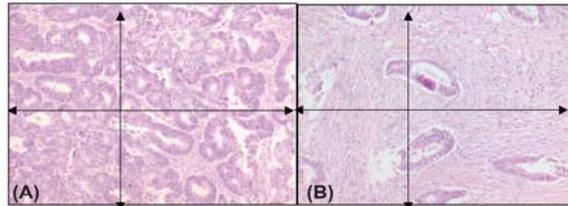


Figure 1. Assessment of carcinoma percentage (CP) on HE staining slide shows the most invasive area of the primary tumor. Tumor cells must present in every side of the field of view (top, bottom, right, left). 1.(A) CP-High (80%) and 2.(B) CP-Low (20%).²⁰

RESULTS

Resection cases with a diagnosis of adenocarcinoma NOS colorectal with lymph nodes amount of at least 12 selected as samples in this study. Samples were obtained from the archives of the Department of Anatomic Pathology, Faculty of medicine/Cipto Mangunkusumo Hospital by consecutive sampling. The calculation of sample size used unmatched categorical analytic ($p_2 = 0.25$; $p_1 = 0.65$). The total number of samples was 44 cases consisting of 22 cases of CP-High and 22 cases of CP-Low. The relationship between the clinicopathologic variables with CP can be seen in Table 1.

Table 1. Correlation between clinicopathologic variables, lymphatic vessel invasion (LVI), lymph nodes and carcinoma percentage (CP)

Variable/Category	CP-Low	CP-High	Total (%)	p
Gender				0,55
Male	11	13	24 (54,5)	
Female	11	9	20 (45,5)	
Age				0,46
≤ 40 Years	6	3	9 (20,5)	
> 40 Years	19	16	35 (79,5)	
Location				0,74
Right	7	6	13 (29,5)	
Left	16	15	31 (70,5)	
Stadium				0,61
pT2	3	1	4 (9,1)	
pT3	19	21	40 (90,9)	
Histologic Grading				1,00
Good	15	15	30 (68,2)	
Moderate-Poor	7	7	14 (31,8)	
Lymphatic Vessel Invasion				0,01**
Negative	3	11	14(31,8)	
Positive	19	11	30(68,2)	
Lymph Nodes				0,07
N0	6	12	18(40,9)	
N1	16	10	26(59,1)	

N0: Lymph nodes without tumor cells metastasis and N1: Lymph nodes with tumor cells metastasis. (***) Statistically significant

Statistical test between clinicopathological variables (gender, age, tumor location, stadium, histologic grading), lymph nodes, and CP values obtained the results of $p > 0,05$. The analysis showed that there was no significant relationship between the clinicopathological variables and lymph nodes with the high/low value of CP. While between LVI and CP, the result value of $p = 0.01$ ($p < 0.05$), which explains the existence of a significant relationship between LVI with the high/low value of CP as a prognostic factor of tumor cell metastasis to the lymph node.

Table 2. Pearson correlation between carcinoma percentage (CP) and lymphatic microvessel density (LMVD) in the intratumoral and peritumoral areas.

Variables	$\bar{x} \pm SD$	p	r
Carcinoma percentage (CP)	42,6 ± 20,9		
Intratumoral area LMVD	9,6 ± 5,9	0,00**	-0,617
Peritumoral area LMVD	10,9 ± 5,9	0,00**	-0,524

**Statistically significant, LMVD: lymphatic microvessel density

Shapiro-Wilk normality test showed normal distribution ($p > 0,05$), then the Pearson correlation test was used (Table 2). The analysis results showed that the correlation between CP and intratumoral LMVD was $p = 0,00$ ($p < 0,005$), negative correlation was $p = -0.617$, have a strong correlation. The correlation between CP and peritumoral LMVD was $p = 0,00$ ($p < 0,05$), negative correlation was $p = -0,524$, have a moderate correlation.

Table 3. Correlation between lymphatic vessel invasion (LVI) and not otherwise specified (NOS) colorectal adenocarcinoma subtype with or without tumor cell metastasis to lymph nodes.

Variables	Lymph nodes N0	Lymph nodes N1		p
	n (%)	n (%)	n (%)	
LVI				0,03**
Negative	9 (20,5)	5 (11,4)	14 (31,8)	
Positive	9 (20,5)	21 (47,4)	30 (68,2)	

N0: Lymph nodes without tumor cells metastasis and N1: Lymph nodes with tumor cells metastasis; LVI: lymphatic vessel invasion; **Statistically significant

The correlation between LVI and lymph nodes (Table 3) with Chi-square statistical test showed the p value of 0,03 ($p < 0,05$) which means that there is a correlation between LVI with the incidence of tumor cell metastasis to lymph nodes. Lymphatic vessel invasion (LVI) (Figure 2) is easier to detect with IHK D2-40/Podoplanin staining compared with HE staining.

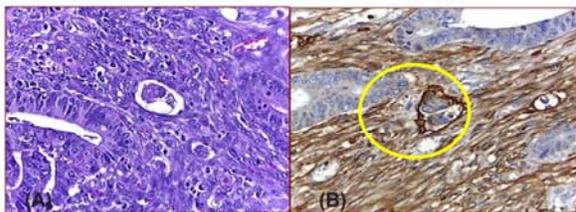


Figure 2. (A) LVI with HE staining. (B) D2-40/Podoplanin staining shows tumor mass within podoplanin positive lymphatic vessel (LVI). (Yellow circle). (400x magnification)

Table 4. Correlation between lymphatic microvessel density (LMVD) and lymphatic vessel invasion (LVI)

	Negative LVI	Positive LVI	Total	p
	Total (%)	Total (%)	%	
Mean Intratumoral				0,00**
LMVD-Low	12 (27,3)	9 (20,5)	21 (47,3)	
LMVD-High	2 (4,6)	21 (47,3)	23 (52,3)	
Mean Peritumoral				0,00**
LMVD-Low	11 (22,7)	8 (20,5)	19 (43,2)	
LMVD-High	3 (2,3)	22 (54,6)	25 (56,8)	

** Statistically significant; LVI: lymphatic vessel invasion; LMVD: lymphatic microvessel density

The correlation between LMVD and LVI (Table 4) was assessed using Chi-square statistical test and obtained the result of $p = 0,00$ ($p < 0,05$) between intratumoral area LMVD (high/low) and peritumoral area with the detection of LVI. Positive LVI was found more with LMVD-High case (Figure 3) in both tumor areas.

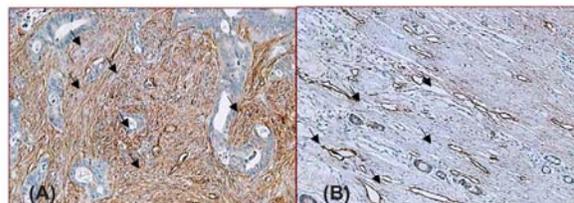


Figure 3. (A) D2-40/Podoplanin staining of intratumoral area shows LMVD-High (black arrows) with open and collapsed lumens. (B) Peritumoral area with LMVD-High (black arrows) shows wide lumens and partially overlap (200x magnification)

Chi-square statistical test (Table 5) shows a significant correlation with the $p = 0,04$ ($p < 0,05$) between intratumoral LMVD with the incidence of tumor cell metastasis to lymph nodes, whereas peritumoral LMVD and the incidence of tumor cell metastasis to lymph nodes shows no significant correlation with $p = 0,17$ ($p > 0,05$).

Table 5. Correlation between lymphatic microvessel density (LMVD) and lymph nodes

	Lymph nodes (N0)	Lymph nodes (N1)	Total	p
	n (%)	n (%)	n (%)	
Intratumoral LMVD				0,04**
LMVD-Low	12 (27,3)	9 (20,5)	21 (47,7)	
LMVD-High	6 (13,6)	17 (38,6)	23 (52,3)	
Peritumoral LMVD				0,17
LMVD-Low	10 (22,7)	9 (20,5)	19 (43,2)	
LMVD-High	8 (18,2)	17 (38,6)	25 (56,8)	

**Statistically significant; LMVD: lymphatic microvessel density; N0: Lymph nodes without tumor cells metastasis; N1: lymph nodes with tumor cells metastasis

In both tumor areas, LMVD-High is more frequently found in cases with tumor cell metastasis to lymph nodes (lymph nodes N1), while LMVD-Low

(Figure 4) is found most in cases without tumor cell metastasis to lymph nodes (lymph nodes N0).

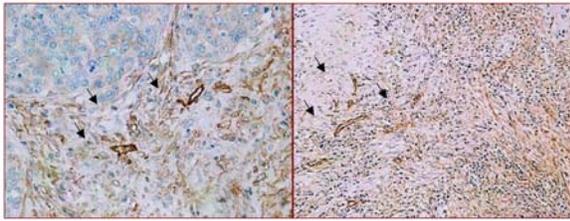


Figure 4. (A). LMVD-Low with D2-40/Podoplanin staining of intratumoral area and (B) peritumoral area. A few lymphatic vessels are easily identified (black arrows). (200x magnification)

DISCUSSION

According to literature, the number of male (24 cases/54.5%) were found more than women in the overall sample. In the men's higher mortality rate of 30-40% compared with female.²¹ CRC incidence continues to increase in accordance with increasing age, especially > 40 years, while those aged ≤ 40 years are related to hereditary factors and are often diagnosed at later stages.^{1,22} More than 50 % sample of this research was discovered at the age > 40 years (35 cases/79.5%). The most frequent location found in this study was in the left colon (31 cases/70.5%). Left side location is more easily detected at an early stage (PT1) related to the polypoid type microscopic appearance, while the right colon is more horizontal.^{2,23} (Table 1).

Table 2 shows a strong correlation between CP and LMVD intra and peritumoral area ($p = 0.00$) with the negative direction in both the tumor areas. Negative direction of correlation explains the inverse relationship between CP and LMVD, meaning the lower the value of CP (CP-Low) the higher LMVD (LMVD-High).

Mesker et al stated that CP-Low CRC with the higher amount desmoplastic stromal components than carcinoma was important in the process of invasion and metastasis of tumor cells. Myofibroblast as a major component in the stromal cells may express proangiogenic molecules named VEGF molecules. High levels of these cytokines associated with poor patient outcome results, because it can interfere with the function of the endothelial defense role in the process of tumor cell extravasation and metastasis.^{13,24}

The main component of LMVD is PDPN. Podoplanin expression (PDPN) by Cancer-Associated-Fibroblast (CAF) in the desmoplastic stroma affects tumor progression and is used as a prognostic indicator of metastasis in many malignancies. The literature describes PDPN is secreted by CAF in desmoplastic stroma associated with tumor development process,

which explains that PDPN-positive CAF can be generated by the LEC through the epithelial to mesenchymal transition (EMT).²⁰ Research by Pula et al reported an association between podoplanin expression CAF and LMVD in the process of metastasis and prognostic factors.²⁵

Lymphatic invasion or LVI is a predictor of the tumor cell metastasis to lymph nodes and markers of overall survival in CRC. Positive LVI detection in cases of N0 lymph nodes is very important, because recurrence can occur in 40% of patients.²⁶ There is a significant relationship ($p = 0.03$) between LVI with the incidence tumor cell metastasis to lymph nodes in this study, in accordance with the research of Ishii et al.^{26,27}

Lin et al and Filho et al reported that LMVD and LVI were important predictors to the process of invasion and metastasis of tumor cells to the lymph nodes and is used as an indicator of poor prognosis.^{26,28} The migration of tumor cells into the lymph nodes is mediated by lymphangiogenesis process and facilitated by lymphangiogenic factors along with the receptors.¹⁰ VEGF-C is a major lymphangiogenic cytokines that induce the growth of lymphatic vessels and contributes to the increase in the density of lymphatic vessels or LMVD-High in the intratumoral area and peritumoral area in primary tumor mass. VEGF-C along with tumor-induced lymphangiogenesis is able to promote the spread of tumors through lymphatic vessels, the general expression of VEGF-C was found to increase in tumors with tumor cell metastasis to lymph nodes (lymph nodes N1).^{29,30}

High density of lymphatic vessels or LMVD-High in both areas of the tumor is associated with the increased number of LVI. The high density of lymphatic vessels (LMVD-High) can extend the route of entry of tumor cells into the vessel by increasing the contact surface between the lymphatic vessels and the tumor cells in the process metastasis.²⁶ In this study, cases with positive LVI-High LMVD intratumoral and peritumoral areas were found more frequent. The Chi-square statistical between LVI and LMVD obtained the result of $p = 0.00$ in both areas. This explains the existence of a significant relationship between high/low value LMVD with the of LVI found. These results are consistent with the research of Saad et al and Lin et al which explained that LMVD-High especially in peritumoral area is associated with the LVI and has an important role in the process of tumor cell metastasis to lymph nodes or to distant organs.^{10,26}

Currently there is no definite consensus to clarify whether the main line of the the lymphatic spread

is via intratumoral or peritumoral area of primary tumor.³¹ The role of intratumoral lymphatic vessels remains controversial, several literature reports that intratumoral area does not contribute to the process of cell proliferation tumor.³² Some studies comparing the importance of the role intratumoral and peritumoral LMVD in the incidence of tumor cell metastasis to lymph nodes.^{26,31,33} Peritumoral area LMVD-High is associated with prognostic factors as it may facilitate the spread of primary tumor cells to regional lymph nodes when compared to intratumoral area. Kostis et al also reported that in prostate cancer, peritumoral area LMVD is closely related to the incidence of tumor cell metastasis to lymph nodes, but not intratumoral area LMVD.³³ Other literatures report different results explaining that LMVD-High in peritumoral area does not have a significant correlation with the occurrence of tumor cell metastasis to lymph nodes and the survival rate.^{10,30,32} The identified intratumoral and peritumoral lymphatic vessels by D2-40/Podoplanin based on hybridome induced tumor explains that both intratumoral and peritumoral lymphatic vessels play role in LEC adhesion process, migration, and tumor cell invasion.³²

Matsumoto et al stated that intratumoral area LMVD was associated with poor outcome in CRC.³⁴ Lee et al study in gastric cancer reported that intratumoral area LMVD was associated with the incidence of lymph nodes metastasis, whereas peritumoral area LMVD showed no significant relationship either at the early stage or the advanced stage.³⁵ While research Gao et al and Khan et al in gastric cancer report different things, explained that intratumoral and peritumoral were associated with the incidence of tumor cell metastasis to lymph nodes and poor prognosis.^{36,37}

In this study, statistical tests were done to assess the correlation between LMVD (intratumoral and peritumoral area) and lymph nodes. The analysis results showed that there are statistically significant correlation between intratumoral area LMVD with the incidence of tumor cell metastasis to lymph nodes ($p = 0,04$) while no significant correlation found in peritumoral area LMVD ($p = 0,17$). Although there was no statistically significant correlation between peritumoral area LMVD with the incidence of lymph node metastasis, but LMVD-High in peritumoral area is more frequent to be found in cases with tumor cell metastasis to lymph nodes (lymph nodes N1).

CONCLUSION

A strong correlation between carcinoma percentage (CP) and lymphatic microvessel density (LMVD) showed that CP value can be used as LMVD marker. The significant correlation between LMVD and LVI, LVI and LN, explains that LMVD and LVI can be used as prognostic factors of tumor cell metastasis to lymph nodes in NOS colorectal adenocarcinoma. This study also reports that LMVD-high in peritumoral area were found more frequent in cases with tumor cell metastasis to lymph nodes (lymph nodes N1).

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