DISTRIBUTION PATTERNS OF ANGIOGENESIS IN COLORECTAL CANCER: STUDY PROTEIN EXPRESSION OF ENDOGLIN (CD105)

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ABSTRACT

Introduction*: Angiogenesis plays an important role in the growth and metastasis of colorectal carcinoma, which is currently one of the targets of cancer therapy. It has been reported that the Endoglin (CD105) involved in angiogenesis and is a powerful marker for angiogenesis in colorectal carcinoma. Level Quantitative angiogenesis in peritumor and an intratumor area is important to know because it is closely related to the micro-environmental factors that influence the occurrence of cancer angiogenesis. The goal of this study to analyze the distribution pattern of angiogenesis in colorectal cancer by comparing the distribution of angiogenesis in peritumor and intratumor areas between well, moderate and poorly differentiated colorectal carcinoma, and between metastasis and non-metastatic colorectal cancer.* ***Methods****: This study analyzed fifty samples of resected adenocarcinoma colorectal. Angiogenesis was assessed by the immunohistochemical method using a primary monoclonal antibody Endoglin (CD105). Positive expression of CD105 was assessed through the CD105 protein expression in neovascular endothelial cells, while the distribution pattern of angiogenesis assessed by counting the positive expression of CD105 protein in hot spots by using the MVD (microvessel density) in the peritumor and intratumor areas and then performed statistical analysis.* ***Results****: There is a significant difference between the quantitative level of angiogenesis in peritumor and intratumor areas of well (P<0.01), moderate (P<0.01) and poorly (<0.05) differentiated adenocarcinoma. The significant difference between the quantitative levels of angiogenesis in peritumor and intratumor areas of non-metastatic colorectal cancer (P<0.01) and lymph node metastases (<0.05) was found, but not in colorectal cancer with liver metastasis.* ***Conclusion:*** *Angiogenesis pattern is more concentrated in peritumor compared to intratumor areas. This showed the role of stromal cells in-angiogenesis. There is significant expression between angiogenesis in peritumor and intratumor areas.*

Keywords: Angiogenesis, Colorectal cancer, Endoglin (CD105)

INTRODUCTION

Colorectal carcinoma is a malignancy derived from the large intestine that caused by uncontrolled cell growth.[1] Colorectal cancer is the third most common cancer in men (746.000 cases, 10.0% of the total) and second in women (614.000 cases, 9.2% of total) worldwide. Almost 55% of the cases occur in more developed regions. Mortality is 694.000 death (8.5% of the total) with more deaths (52%) in developed regions of the world.[1,2]

In the 1980s researchers proposed a four-step progression of gene alterations in colonic epithelium. This phenomenon starts from transformation of normal epithelium to an adenoma, proceeding to in situ carcinoma, and ultimately to an invasive and metastatic

tumor. In 1990, Fearon and Vogelstein elucidated specific pathways essential to the development of CRC, consisting of accumulated mutations in multiple genes that regulate cell growth and differentiation.[2] Both genetic and epigenetic alterations, the latter leading to aberrant methylation of tumor suppressor genes, result in inactivation of these genes and subsequent promotion of neoplasia.[3]

The main causes of death in colorectal carcinoma is due to metastasis. Metastasis is the process by which tumors spread from the primary organ and appear in other organs. There are four mechanisms of metastasis in colorectal carcinoma is through direct invasion, transperitonial, hematogen, and lymphonia. Many factors can occur initiated a process of metastasis, among others; damage to cells adhesion molecule, release of angiogenic growth factors, release lymphangiogenic of growth factors, mechanical factors, release of proteolytic enzymes and motility of cancer cells.[4,5]

It was reported, tumor growth and metastasis depend on tumor angiogenesis, where tumor would not grow over 1-2 mm in diameter without angiogenesis. Angiogenesis is a formation of a new blood vessel in peritumor and intratumor from established blood vessel through sprouting mechanism. Neovascularization due to angiogenesis allow tumor growth and metastasis by providing nutrition and oxygen for tumor cell, and also a medium for budding from the main tumor through blood vessel to metastasize to another organ.[6,7]

Due to induction of angiogenesis in solid tumors is related to tumor progression, angiogenesis becomes an important target for the detection, diagnosis, and treatment of cancer. Currently, angiogenesis emerged as targets of cancer therapy that has been used in the treatment of cancer, including colorectal cancer and until now still being investigated.[8-10]

CD105 (Endoglin) was reported as a marker of endothelial cell in neovascularization. CD105 (Endoglin), a cell adhesion molecules, was expressed in cell surface located in 9q34, an 180 kDa homodimeric transmembrane glycoprotein, a component of TGF-beta (Transforming Growth Factor-Beta) receptor complex, a pleiotropic cytokine which modulated angiogenesis through cell regulation including proliferation, differentiation, and migration. CD105 bind TGF-beta 1 and 3 then modulate TGF-beta signal through interaction with TGF receptor 1 and/or II.[11]

CD105 exclusively expressed in colorectal carcinoma angiogenesis and few other organ carcinomas through its positive expression in endothelia cell in de novo blood vessels, either in the intratumor or peritumor area. In contrast, CD105 almost not expressed in endothelia cell in normal tissue capillary around the tumor. Furthermore, CD105 expression in endothelia cell is very selective without any significant cross-reactivity with cell inflammatory, stroma cell in neoplasm tissue, even in a lymph vessel endothelial cell.[13] Many reports, neovascular count through MVD (microvessel density) method with CD105 marker is better estimator for prognosis and tumor survival compared to MVD method using panendothelial marker, such as CD31.[12,13]

It has been reported that CD105 is involved in the formation of angiogenesis and seem to be a powerful marker for neovascularization in colorectal carcinoma. At present, CD105 emerged as a major vascular target for anti-angiogenic cancer therapy.[12,14] However, how it was expressed and level of angiogenesis in peritumor and the intratumor area is important due it is closely related to known environmental factors that affect the occurrence of tumor angiogenesis. To our knowledge, has not been reported the distribution patterns of angiogenesis in intratumor and peritumor areas of colorectal carcinoma and its relation to histopathologic finding, The purposes of this research is to evaluate the distribution pattern of angiogenesis in peritumor and intratumor of colorectal carcinoma and its relation to the histopathologic finding.

METHODS

Tumor sample

Fifty (50) surgical resection of colorectal carcinoma derived from Hospital in Makassar during 2007-2008. All specimens/samples were fixed in formalin and paraffin blocks then stained with HE (hematoxylin-eosin) to diagnose colorectal carcinoma. All samples are adenocarcinoma subtype based on histopathology sample stratified by Japanise classification for colorectal cancer, comprising; 13 well-differentiated, 37 moderate differentiated, 10 poorly differentiated.

Surgical resection of colorectal carcinoma tissue obtained from Pathology Anatomy Department. The tissues were cut in the central region of cancerous tissue and 1-2 cm surrounding non-cancerous tissue in the oral and anal margin. Tissue have been cut then made to paraffin blocks and cooled in the refrigerator and cut with a microtome with a thickness of 3 microns. Object glass smeared with albumin to glued the tissue. Then, cut tissue put in a water bath (<60°C). Tissue in a water bath then taken with an albumin smeared glass object, slide then drained then placed on the slide warmer (<60°C) for 15 minutes. The slide then ready for staining. Slide contain paraffin block tissue soaked in xylol for 5 minutes, performed 2 times in 2 different containers, then soaked in 95% alcohol for 2 minutes, also done 2 times in 2 different containers. After that, soaked in 70% alcohol for 2 minutes, rinsed with running water for 5 minutes. Then, soaked for 15 minutes with Hematoxylin Mayer, rinsed with running water until turned blue. After that, soaked slide with eosin 1% for 5 minutes, 70% alcohol for 2-5 minutes, 95% alcohol for 2-5 minutes, performed 2 times in 2 different containers, soaked with Carbol Xylol for 5 minutes, soaked with xylol for 2-5 minutes. After that, the slide dried, then cover with glass deck. The slide is ready to be viewed under a microscope.

Immunohistochemisry staining is done with an indirect immuno-enzyme technique using labeled streptavidin complex. Before staining process begins slide are differentiated with xylene for 15 minutes dan rehydrated with 100% alcohol and alcohol concentration is diluted to 90%, 80%, 70% and 60% for 10 minutes each. After that, the slides are washed with dH20 2 times for 5 minutes and incubated with PBS solution for 5 minutes. Then, slides are put into glass box containing EDTH (pH8) and heated in the microwave for 6 minutes for optimized its antigenicity. The slide is cooled at room temperature for 1 hour, and after briefly dried, the slide is marked using a pap pen. Slides washed with dH20 and PBS solution for 5 minutes each before incubation with 0.3% hydrogen peroxidase for 15 minutes. After endogenous peroxidase blocked, the slides were incubated with blocking solution for 30 minutes to block avidin in the tissue. Then, the slides were incubated overnight at -40C temperature with primary CD105 antibody (Santa Cruz) diluted to 1:50. Slides are washed again 3 times with dH20 before incubated with secondary antibody (goat anti-mouse) and streptavidin during each 30 minutes at room temperature. For staining, 3.3 diaminobenzidine tetrahydrochloride used for approximately 10 minutes to obtain the staining reaction that can be detected by microscopic examination. After that stained again with hematoxylin to clarify the cell nucleus for 30 seconds and washed with running water for 5 minutes, the slide is dehydrated by using the alcohol concentration increased gradually from 70%, 80%, 90% to 100% for 2 minutes each. After that, slides soaked into xylene for 5 minutes. Last, slides were given malinol before it covered with the glass cover.

The CD105 level of immunoexpression was assessed by using the method of intratumoral and peritumoral MVD (microvessel density). With low magnification (×40), each peritumor and intratumor area selected five most populated microvessel/ neovascular areas (hotspots) expressing the positive CD105 marker. In each area, with high magnification (×100), microvessel count is conducted in microvessel/neovascular CD105 positive expression. Each microvessel where one or more of endothelial cells stained positive (brown) by immunohistochemical CD105 marker, is counted as one microvessel/ neovascular. Blood vessels with lumen-looking, defined as non-neovascular, it was not calculated. The intensity of CD105 expression grouped as: 0 (negative): none or <20% of endothelial cells with an expression of weak positive CD105; 1+ (weak):> 20% of endothelial cells with expression of weak positive CD105; 2+ (strong):> 20% of endothelial cells with expression of strong positive CD105. Characterization of angiogenesis distribution pattern in peritumor and the intratumor area is designated after the conclusion of microvessel counts. Immuno-histochemical evaluation will be carried out individually by the researcher (RM and UM) to get accurate results

Data were statistically analyzed using Spearman 'p' correlation and multivariate analysis. The quantity difference in microvessel count is compared using paired 't' test. All statistical tests were performed using SPSS 17.0, windows. The statistical is significant if $p < 0.05$.

RESULTS

Fifty (50) surgical resection of colorectal carcinoma derived from Hospital in Makassar during 2007-2008. All specimens/samples were fixed in formalin and paraffin blocks then stained with HE (hematoxylin-eosin) to diagnose colorectal carcinoma. All samples are adenocarcinoma subtype based on histopathology sample stratified by Japanise classification for colorectal cancer, comprising; 13 well-differentiated, 37 moderate differentiated, 10 poorly differentiated. Thus, the clinical pathology data samples are in the table 1.

Based on tumor metastasis, patients with tumors without lymph node metastasis are 13 subjects (8 men and 5 women), with an average age of 58±2. Metastasis to lymphonodus was found in 29 subjects (24 men and 5 women) with a mean age of 63±6, and metastasis to the liver was found in 8 subjects (6 men and 2 women) with an average age of 63±8. Well, differentiation Colorectal cancer was found in 7 subjects, metastasis to lymph nodes was found in 1 subject, and metastasis to the liver was found in 2 subjects. Colorectal cancer with moderate differentiation without metastasis are found in subjects, metastasis to lymph node are found 27 subjects and metastasis to the liver are found in 4 subjects. No poorly differentiated colorectal cancer without metastasis was found, metastasis to lymph nodes in 1 subject and metastasis to the liver 2 subjects (Tabel.2).

Table 1. Clinical pathology and histopathology of samples

	No.
Number of patients	50
Age (yrs)	
Range (median)	39-78 (62)
Gender	
Male	38
Female	12
Adenocarcinoma	50
Histopathology diagram	
Well differentiated	10
Moderately differentiated	37
Poorly differentiated	3
Metastasis to lymph nodes	28
Metastasis to other organ	8

Expression of CD105 (Endoglin)

Fifty samples were used, all of the samples (100%) showed positive expression of CD105 on neovascular endothelial cells in peritumor; 46 (92%) strong expression and only 4 (8%) weak expression. There is 31 (62%) of the total sample show positive expression of CD105 on neovascular endothelial cells in the intratumor area; 17 (34%) strong expression, and 14 (28%) weak expression. (Table. 2)

There is a significant difference of CD105 expression on neovascular endothelial cell in peritumor and intratumor areas based on the grading histopathology of adenocarcinoma

Table 2. Distribution of samples based on metastasis

	Patient		
	LN meta(+) n=29	**LN meta(-) n=13**	**Hepar meta(+) n=8**
Gender	24 M / 5 F	8 M / 5 F	6 M / 2 F
Age			
Range (years)	40-71	39-68	56-69
Mean (years)	63±6	58±2	63±8
Grade			
Well differentiated (n=10)	1	7	2
Moderatelly differentiated (n=37)	27	6	4
Poorly differentiated (n=3)	1	0	2

LN meta; Limfonodus metastasis, M; male, F; female, WD; well differentiated, MD;moderatelly differentiated, PD; poorly differentiated

Table 3. Distribution of CD105 positive expression on neovascularization in peritumor and intratumor area of colorectal carcinoma

Location	n	Positive expression of CD105 (%)
Peritumor	50	50 (100)
Strong		46 (92)
Weak		4 (8)
Intratumor	50	31 (62)
Strong		17 (34)
Weak		14 (28)

colorectal, wherein well-differentiated adenocarcinoma there is 674 positive expression of CD105 on neovascular endothelial cells in peritumor and 297 in the intratumor area with $p<0.01$. In moderate differentiated, 1028 expressed positive CD105 in peritumor and 589 on intratumor with $p<.001$. In poorly differentiated adenocarcinoma, there are 128 expressed positive CD105 in peritumor and 84 in intratumor with $p<.005$. (Table.4)

In non-metastatic adenocarcinoma, there is 689 positive expression of CD105 on neovascular endothelial cells in peritumor and 104 in intratumor. In lymph nodes, metastatic colorectal cancer shows 1423 positive expression of CD105 on neovascular endothelial cells in peritumor and 583 in intratumor. We found 798 positive expressions of CD105 on neovascular endothelial cells in peritumor and 207 in the intratumor area of liver metastatic colorectal cancer.

We found a significant difference between level expression of CD105 on neo-vascular endothelial cell in peritumor and intratumor areas, either in non metastasis and lymphoid metastatic adenocarcinoma colorectal with a p-value of $p<0.01$ and $p> 0.05$, respectively, but no significant differences of CD105 expression between peritumor and intratumor area in liver metastatic adenocarcinoma was found ($p=0.07$). (Table.4)

DISCUSSION

By using CD105 as an exclusive marker to angiogenesis, this study proves that there are significant differences in protein expression of CD105 on peritumor and intratumor area nor in well, moderately and poorly differentiated tumor with p-value $p<001$ for well and moderately differentiated, and $p<005$ for poorly differentiated. It was suspected due to the difference in environmental factor surrounding the tumor effect the number nor rate of angiogenesis in peritumor and intratumor are, both in well, moderately, and poorly differentiated tumor. This study also found increased number of neovascularization expressed positive CD105 in well differentiated to moderately differentiated, both in peritumor and intratumor with p-value <0.01, respectively.

Angigenesis is controlled by the balance between proangiogenetic and inhibitor factor. Disturbance of the balance caused the disturbance on angiogenesis process. The involvement of a cascade complex begins with

Tabel 4. The levels of protein expression of CD105 on peritumor and intratumor area based on histopathology grading dan correlation between the level of expression of CD105 protein with histopathological grading in colorectal carcinoma

	Protein CD105 Expression		
	Peritumor	Intratumor	p
Grade			
Well differentiated (n=10)	674	297	<0.01
Moderatelly differentiated (n=37)	1028	589	<0.01
Poorly differentiated (n=3)	128	84	<0.05
LN non-meta (n=8)	689	104	<0.01
LN meta (n=29)	1423	583	<0.05
Liver meta (n=13)	798	207	0.07

LN meta; Lymph node metastasis, M; male, F; female, WD; well differentiated, MD;moderately differentiated, PD; poorly differentiated

activation of vascular endothelial with proangiogenetic factor nor cytokine, followed by proliferation and invasion of angiogenesis with the involvement of extracellular remodeling matrix. Next step is maturation and stabilization.[15]

This study also proved the significant difference between angiogenesis level in peritumor and intratumor areas, both on non-metastases and lymph node metastatic adenocarcinoma colorectal, $p<0.01$ dan $p<0.05$, respectively. In liver metastatic adenocarcinoma colorectal, there is no significant difference of CD105 expression on neovascular endothelial cell in peritumor and intratumor ($p=0.07$), it was suspected due to a low number of samples (n=3). This study also found an increased number of neovascularization which marked with positive expression of CD105 in non-metastatic and lymph nodes metastatic adenocarcinoma colorectal in peritumor and intratumor areas, $p<0.01$ dan $p<0.05$, respectively (Table.4)

Angiogenesis was reported as an important process for tumor growth, invasion, and metastasis. Previously, angiogenesis has been reported occur on peritumor and intratumor areas in the certain type of tumor, including colorectal carcinoma.[16]

CONCLUSION

Based on this study, we conclude that Angiogenesis pattern is more concentrated in peritumor compared to intratumor areas. This showed the role of stromal cells in angiogenesis. There is significant expression between angiogenesis in peritumor and intratumor areas. The results of this study provide a fundamental data for a strategy of antiangiogenic cancer therapy.

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