Original Article
Middle East Journal of Cancer; April 2018; 9(2): 85-90

A Comparison of Endothelial Cell-Selective Adhesion Molecule and von Willebrand Factor Expression in Breast Cancer Growth and Metastasis

M Husni Cangara*, Mochammad Hatta♦, Upik A Miskad*, Syarifuddin Wahid***

*Department of Anatomical Pathology, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia
**Molecular Biology and Immunology Laboratory, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia

Abstract

Background: Angiogenesis is the process of new blood vessels formation that contribute to tumor growth and metastasis. Endothelial cell-selective adhesion molecule is one of the proteins that expresses in vascular endothelial cells. *In vitro* and animal studies have shown involvement of this protein in physiological and pathological angiogenesis. von Willebrand factor is a protein expressed by endothelial cells and megakaryocytes that has a role in blood clotting processes. In the current study, we investigate the expression of endothelial cell-selective adhesion molecule and von Willebrand factor in carcinoma mammae specimens and explore their correlation with tumor growth and metastasis.

Methods: We obtained 79 specimens from paraffin blocks of patients diagnosed with invasive breast carcinoma of no special type. The slides from these specimens were then stained with endothelial cell-selective adhesion molecule, von Willebrand factor, and Ki-67 antibodies to assess vascular numbers and cell proliferation.

Results: We found a total of 31 (39%) low vascularity and 48 (61%) high vascularity samples from endothelial cell-selective adhesion molecule staining. There were 34 (43%) low vascularity and 45 (54%) high vascularity samples by von Willebrand factor staining. There was a significant correlation of blood vessel numbers in the endothelial cell-selective adhesion molecule-stained samples with tumor volume, metastasis to lymph nodes, and proliferation cells. The von Willebrand factor-expressed samples only had a significant correlation of vascular number with tumor volume.

Conclusion: Endothelial cell-selective adhesion molecule and von Willebrand factor as the endothelial cell expressed proteins play a role in the angiogenesis process of breast cancer. However, endothelial cell-selective adhesion molecule expression is more consistent than von Willebrand factor in predicting the presence or absence of metastatic breast cancer.

Keywords: Endothelial cell-selective adhesion molecule, von Willebrand factor, Breast cancer

♦Corresponding Author:
Mochammad Hatta, MD, PhD
Molecular Biology and Immunology Laboratory, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia
Tel/Fax: 62-411-587464/62-411-586971
Email: hattaram@indosat.net.id

Received: December 31 2016; Accepted: July 4, 2017

Introduction

The formation of new blood vessels from pre-existing vasculature (angiogenesis) is one of the important processes in tumor growth.[1] Endothelial cell-selective adhesion molecule (ESAM) is a member of the immunoglobulin superfamily of proteins found on endothelial cells.[2] Its function is to mediate the homophilic interaction of endothelial cells.[3] A previous study has reported a role for ESAM in tumor growth with knock-out genes responsible for the ESAM expression in animal experiments.[4] ESAM is also known to play a role in mediating tumor metastasis.[5] On the other hand, ESAM controls endothelial cell permeability and leukocyte migration.[6,7]

von Willebrand factor (vWF) is a plasma glycoprotein produced by endothelial cells and megakaryocytes.[8,9] It is required in the interaction of platelets with sub- endothelium during the process of hemostasis, acts as a carrier of factor VIII blood clotting, and is involved in endothelial cell adhesion to the vascular basement membrane.[10-12] However, vWF expression varies greatly between endothelial cells in some types of blood vessels.

Previous studies have reported the expression of ESAM in metastatic lung cancer in an animal model.[5] However, no studies have been performed on the expression of this protein in human breast cancer. Therefore, our objectives in this study were to examine the immunohistochemical expression of ESAM in breast cancer tissues and compare them with vWF expression as a well-known endothelial marker, and to explore their correlation with tumor growth and metastasis.

Materials and Methods

Tissue samples

We obtained 79 formalin-fixed and paraffin-embedded specimens from patients diagnosed with invasive breast carcinoma of no special type from the histopathology files of the Department of Anatomical Pathology, Hasanuddin University, Indonesia. Exclusion criteria were specimens from patients that previously received chemotherapy or radiotherapy, and those with microscopic necrosis.

Antibodies and immunohistochemistry

The specimens were cut into 4 µm sections and immunostained using primary antibodies against ESAM (R&D Systems), vWF (Dako) and Ki-67 (Dako) with 1: 100 dilutions. Immunohistochemical procedures were performed by streptavidinbiotin-peroxidase method according to the manufacturing company's standards and guidelines. Diaminobenzidine chromogen was used to visualize ESAM, vWF, and Ki-67.

Statistical analysis

The results were analyzed statistically by one-way analysis of variance (ANOVA) with the unpaired t-test. *P*-values <0.05 were considered statistically significant.

Results

Table 1 lists the 79 breast carcinoma patients' clinicopathological parameters. Immunohisto-

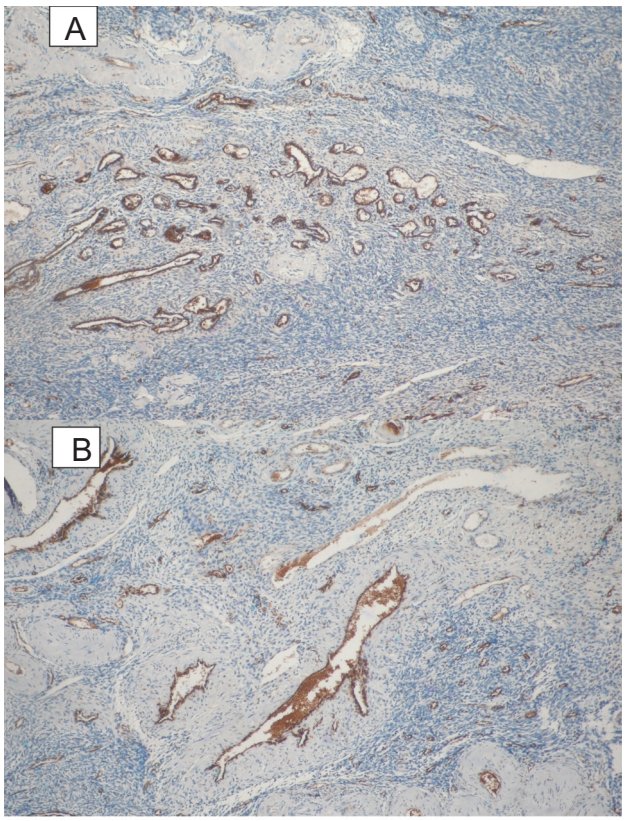

Figure 1. Vascular image in mammary carcinoma specimen with (A) endothelial cell-selective adhesion molecule (ESAM) and (B) von Willebrand factor (vWF) immunohistochemistry staining (magnification: 40×).

chemical staining using antibody ESAM, vWF, and Ki-67 were performed on 79 samples. Stained-specimens were grouped according to measured tumor volume, the degree of differentiation, and the presence or absence of lymph node metastasis.

Endothelial cell-selective adhesion molecule (ESAM) expression in tumor tissue and its significance to sample characteristics

Immunohistochemical staining of ESAM showed the morphology and degree of microvascularity inside the breast tumor specimens (Figure 1A).

The average number of blood vessels within the tumor based on ESAM expression was 66 (range: 30-120). This number served as the median value used to classify the tumor into low and high vascularization tumor groups. In this study, 31 tumor samples had less vascularization and 48 samples had high vascularization.

The number of vascularizations in the tumor were linked to assessed parameters (Table 2). From the results shown in table 2, we observed a significant correlation between the number of blood vessels with the tumor size and volume, and the presence or absence of metastases ($P<0.05$). No significant correlation existed between the total number of blood vessels (vascularity) and the degree of differentiation. However, there was a tendency that the tumor with high vascularization had moderate and severe differentiation.

Von Willebrand factor (vWF) expression in tumor tissue and its significance to sample characteristics

In this study, we stained sample specimens with vWF as another angiogenesis marker (Figure 1B). The average number of blood vessels within the tumor based on vWF expression was 58 (range: 14-262). This median value was used to differentiate between low and high vascular tumor. In this study, there were 34 tumors with low vascularization and 45 tumors with high vascularization. We correlated the numbers of vascularization as represented by vWF expression with the parameters assessed (Table 3).

Table 3 shows a significant correlation between the number of blood vessels (vWF expression) with the tumor size and volume ($P<0.05$). No significant correlation existed between the number of blood vessels (vascularity) with the degree of differentiation and the presence or absence of metastasis to the lymph nodes. However, as shown in the previous result from ESAM staining, there was a tendency that the tumors with high vascularization from vWF staining had a degree of moderate and severe differentiation.

Table 1. Clinicopathological characteristics of samples.

Characteristics	N=79	Percent (%)
Sex		
Male	3	4
Female	76	96
Age (years)		
<31	5	7
31-40	20	25
41-50	20	25
51-60	23	29
>60	11	14
Tumor volume (cm^3)		
<51	33	41
51-400	38	48
>400	8	11
Differentiation		
Well	11	15
Moderate	47	59
Poor	21	26
Metastasis to lymph nodes		
Present	12	16
Absent	67	84

Ki-67 expression in tumor tissue and its significance to sample characteristics

We examined Ki-67 expression in tumor cells as a reflection of cell proliferation (Figure 2). We used the staining results to calculate the average number of cells with Ki-67 expression from 10 different visual fields per slide and compare the results with the assessed parameters (Table 4).

This study showed a significant difference between large and small tumors in the numbers of cancer cells that expressed Ki-67. The data indicated that specimens from large tumors had numerous cells that expressed Ki-67 compared to specimens from small tumors. There was no significant difference in the number of cells that

Table 2. Correlation of vascular number by endothelial cell-selective adhesion molecule (ESAM) expression with characteristics of the samples.

Characteristics	Vascular number (ESAM) Low (N=31)	High (N=48)
Tumor volume (cm^3)		
<51	21 (64%)*	12 (36%)
51-400	8 (21%)	30 (79%)*
>400	2 (25%)	6 (75%)*
Differentiation		
Well	4 (36%)	7 (64%)
Moderate	20 (42%)	27 (58%)
Poor	7 (33%)	14 (67%)
Lymph node metastases		
Present	0 (0%)	12 (100%)*
Absent	31 (46%)	36 (54%)

*P<0.05, low vs. high. The average vascular number = 62.

Table 3. Correlation of vascular number by von Willebrand factor (vWF) expression with characteristics of the samples.

Characteristics	Vascular number (vWF) Low (N=34)	High (N=45)
Tumor volume (cm^3)		
<51	23 (69%)*	10 (31%)
51-400	9 (29%)	27 (71%)*
>400	2 (25%)	8 (75%)*
Differentiation		
Well	6 (54%)	5 (46%)
Moderate	20 (42%)	27 (58%)
Poor	8 (38%)	13 (62%)
Lymph node metastases		
Present	7 (58%)	5 (42%)
Absent	27 (40%)	40 (60%)

*P<0.05, low vs. high. Average vascular number = 58.

expressed Ki-67 when viewed from the degree of differentiation and metastasis to lymph nodes.

The significance of vascular number by endothelial cell-selective adhesion molecule (ESAM) expression with Ki-67 expressed tumor cell numbers

We analyzed the correlation of the vascular number in the tumor with the expression of Ki-67. Tumor specimens with high vascular number (ESAM expression) had a large number of cells that expressed Ki-67 compared to the tumor specimens with low vascular numbers (Table 5). From this study, we observed a trend that with a higher degree of vascularity inside the tumor, there were more cells that expressed Ki-67 (Figure 3).

Discussion

Angiogenesis is a process by which new blood vessels are formed from the branching of the pre-existing capillary blood vessels.[13,14] The process of angiogenesis includes endothelial cell activation and degradation of extracellular matrix and basement membrane, and subsequent differentiation into functional blood vessels.[15] In normal conditions, angiogenesis occurs during the process of wound healing, the reproductive cycle in women, embryo growth, organ formation and regeneration, and tissue remodeling.[16] On the other hand, abnormal angiogenesis has been found in several pathological conditions such as tumors, diabetic retinopathy, and inflammatory conditions.[17]

The ability of a tumor to form new blood vessels (angiogenesis) is an important factor for the growth of tumors at any stage of breast cancer development. Angiogenesis is not only an important stage in the growth of tumors, but also a major factor that affects the invasion and metastasis of malignant tumor cells.[18,19] This factor may be the standard for evaluating the development of breast cancer, especially whether there is a risk of metastasis.

In this study, specimens of breast cancer were immunostained with ESAM and vWF antibodies to facilitate the calculation of vascular number and Ki-67 antibody as a marker of cell proliferation. Compared to normal tissue, the mammary

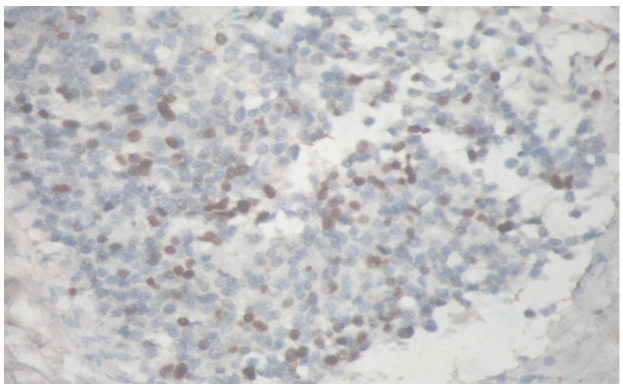

Figure 2. The expression of Ki-67 by immunohistochemistry staining in a mammary carcinoma specimen (magnification: 40×).

Table 4. Correlation of Ki-67 expression with characteristics of samples.

Characteristics	Average number of Ki-67 expressed cells
Tumor volume (cm^3)	
<51	24.91*,#
51–400	159@
>400	424
Differentiation	
Well	188.1
Moderate	127.6
Poor	186.7
Lymph node metastases	
Present	182
Absent	136

P≤0.05 @more than 400 vs. 51-400; *vs. <51; #51-400 vs. <51.

carcinoma tissues have more blood vessels. This proves that the increase in the number of blood vessels associated with neovascularization and the angiogenesis is an essential factor for tumor growth.

From this study, it appeared that a significant correlation existed between tumor volume and vascular number. There was a tendency toward higher vascularization in tumors with large size and volume. This might be explained by the unlimited ability of tumor cells to grow and multiply (proliferation). To ensure its potency, the tumor cells require a continuous supply of nutrients and oxygen. In order to warrant both supplies, the tumor cells stimulate the formation of new blood vessels through a process of angiogenesis. Finally, the new vessels will impact tumor growth as characterized by rapidly increasing tumor size and volume.

Another result found in this study was the relationship between the amount of vascularization in the tumor with the presence of cancer metastases to lymph nodes.

The new blood or lymph vessels formed by angiogenesis serves not only to supply oxygen and other essential nutrients for tumor cells. These new vessels will become a means for tumor cells to escape from the initial (primary) location and move to another location (metastases) either through the lymph vessels to some lymph nodes or through the blood vessels to distant organs. In this study, it has appeared that the tumors with high vascularity had a tendency to metastasize.

Table 5. Correlation of vascular number by endothelial cell-selective adhesion molecule (ESAM) expression with Ki-67 expression.

Characteristics	Average number of Ki-67 expressed cells
Vascular numbers	
Low	89.3*
High	194.04

*P<0.05, low vs. high

Endothelial cell-selective adhesion molecule is a protein that belongs to the immunoglobulin-like superfamily. This protein has two types of domains, V and C2, in the extracellular part. Northern blot analysis of various cell types and tissues showed expression of ESAM on endothelial cells and on tissues that known to have a high amount of vascularization such as the lungs, heart and kidneys.[2] With the use of an electron microscope, ESAM have been localized in tight junctions and function to mediate homophilic interactions between endothelial cells.[2,3]

The results of this study showed differences in the expression of ESAM and vWF as a marker of blood vessels in relation to the presence or absence of lymph node metastases. It was found that the difference in the vascularity related tumor groups between positive to negative metastatic lymph nodes were more significant in the specimens stained with ESAM. In contrast, the results from specimens stained with vWF showed no significant difference. This could be explained by the inconsistency of vWF expression in

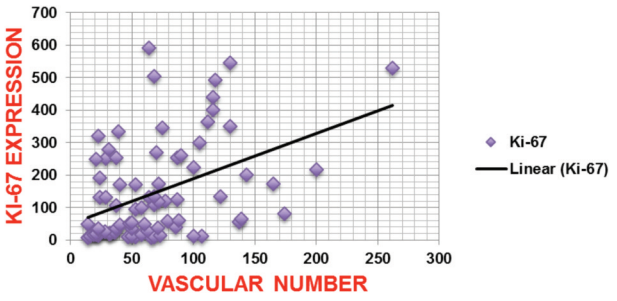

Figure 3. Correlation of tumor vascular number by endothelial cell-selective adhesion molecule (ESAM) expression with Ki-67 expressed cells in invasive breast carcinoma of no special type.

endothelial cells of various different types of blood vessels. Hence, the use of vWF for immunostaining as a marker of vascular endothelial cells should be reviewed again.

In conclusion, angiogenesis plays an important role in tumor growth through an increasing number of vascular tissues and tumor cell proliferation activity. ESAM and vWF, as endothelial cell expressed proteins, play a role in the angiogenesis process of breast cancer. Angiogenesis also plays a role in the metastatic process, which ESAM expression is more consistent than vWF in predicting the presence or absence of metastatic breast cancer.

Further research is needed to determine the role of ESAM in angiogenesis from multiple cancer samples other than breast carcinoma. Similarly, in-depth research is needed to understand the role of ESAM in pathological processes other than the growth and metastasis of cancer.

Acknoledgements

This study was supported by Hasanuddin University grants in accordance with the Implementation Agreement No. Research. 08/UN4-LK.26/2012.

Conflict of interest

No conflict of interest is declared.

References

1. Folkman J. Endothelial cells and angiogenic growth factors in cancer growth and metastasis. Introduction. *Cancer Metastasis Rev.* 1990;9(3):171-4.
2. Hirata K, Ishida T, Penta K, Rezaee M, Yang E, Wohlgemuth J, et al. Cloning of an immunoglobulin family adhesion molecule selectively expressed by endothelial cells. *J Biol Chem.* 2001;276(19):16223-31.
3. Nasdala I, Wolburg-Buchholz K, Wolburg H, Kuhn A, Ebnet K, Brachtendorf G, et al. A transmembrane tight junction protein selectively expressed on endothelial cells and platelets. *J Biol Chem.* 2002;277(18):16294-303.
4. Ishida T, Kundu RK, Yang E, Hirata K, Ho YD, Quertermous T. Targeted distruption of endothelial cell-selective adhesion molecule inhibits angiogenic processes *in vitro* and *in vivo*. *J Biol Chem*. 2003;278(36):34598-604.
5. Cangara HM, Ishida T, Hara T, Sun L, Toh R, Rikitake Y, et al. Role of endothelial cell-selective adhesion molecule in hematogeneous metastasis. *Microvasc Res.* 2010;80(1):133-41.
6. Wegmann F, Petri B, Khandoga AG, Moser C, Khandoga A, Volkery S, et al. ESAM supports neutrofil extravasation, activation of Rho, and VEGF-induced vascular permeability. *J Exp Med.* 2006;203(7):1671-7.
7. Hara T, Ishida T, Cangara HM, Hirata K. Endothelial cell-selective adhesion molecule regulates albuminuria in diabetic nephropaty. *Microvasc Res.* 2009;77(3):348-55.
8. Jaffe EA, Hoyer LW, Nachman RL. Synthesis of antihemophilic factor antigen by culture human endothelial cells. *J Clin Invest.* 1973;52(11):2757-64.
9. Nachman R, Levine R, Jaffe EA. Synthesis of factor VIII antigen by culture guinea pig megakaryocytes. *J Clin Invest.* 1977;60:914-20.
10. Sakariassen SK, Bolhuis PA, Sixma JJ. Human blood platelet adhesion to artery subendothelium is mediated by factor VIII-von willebrand factor bound to subendothelium. *Nature.* 1979;279:636-8.
11. Dejana E, Lampugnani MG, Giorgi M, Federici AB, Ruggeri ZM, Marchisio PC. Von willebrand factor promotes endothelial cell adhesion via an Arg,-gly-Asp dependent mechanism. *J Cell Biol.* 1989;109(1):367-75.
12. Lollar P. The association of factor VIII with von Willenbrand Factor. *Mayo Clin Proc.* 1991;66(5):516-23.
13. Risau W. Mechanisms of angiogenesis. *Nature*. 1997;386(6626):671-4.
14. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature*. 2000;407(6801):249-57.
15. Bendeck MP. Macrophage matrix metalloproteinase-9 regulates angiogenesis in ischemic muscle. *Circ Res.* 2004;94(2):138-9.
16. Ferrara N, Kerbel RS. Angiogenesis as a therapeutic target. *Nature*. 2005;438(7070):967-74.
17. Coultas L, Chawengsaksophak K, Rossant J. Endothelial cells and VEGF in vascular development. *Nature*. 2005;438(7070):937-45.
18. Folkman J. Angiogenesis in cancer, vascular rheumatoid and other disease. *Nat Med.* 1995;1(1):27-31.
19. Sökmen S, Sarioglu S, Füzün M, Terzi C, Küpelioglu A, Aslan B. Prognostic significance of angiogenesis in rectal cancer: a morphometric investigation. *Anticancer Res.* 2001;21(6B):4341-8.