The correlation between TAM, MVD, VEGF and MMP-9 expressions among various histological progression, histological grading and staging of breast cancer

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ABSTRACT

Breast cancer may progress from an atypical lesion. Angiogenesis has an important role in the growth, invasion and metastasis of breast cancer, which can be reflected through the microvascular density (MVD). Tumor associated macrophages (TAMs) are stromal cells that can produce pro-angiogenic factor such as vascular endothelial growth factor (VEGF), and induce matrix metalloproteinase-9 (MMP-9) for degrading extracellular matrix and basement membrane. Angiogenesis in breast cancer progression and its relationship with histological grading as well as its staging need to be defined and thus, cancer therapy and prognosis can be determined more accurately. The aim of this study was to investigate the correlation between TAM, MVD, VEGF and MMP-9 expressions among fibrocystic lesion, atypical lesion and breast cancer, and its correlation with histological grading and staging of breast cancer. Using a cross-sectional study, a total of 50 paraffin embedded tissues of fibrocystic lesion, atypical lesion and breast cancer were chosen in this study. Those specimens were stained immunohistochemically with monoclonal antibody (MoAb) anti CD68, von Willebrand factor (vWF), VEGF and MMP-9. The expressions of VEGF and MMP-9 were counted from the mean numbers of positive tumor cells. TAMs were counted from numbers of macrophages which expressed CD-68. MVDs were counted from numbers of microvessels whose endothelial cells expressed vWF, using the Average Microvessels Count (AMC) method. The correlation of both markers and different type of breast lesions were analyzed by using Pearson correlation. There were statistically significant correlations between TAM ($r = 0.760$; $p = 0.000$), MVD ($r = 0.659$; $p = 0.000$), and MMP-9 ($r = 0.518$; $p = 0.000$), among several breast lesions and histological grade of breast cancer. The highest of their expressions was found in the poor grade of cancers. There were statistically significant correlations between TAM ($r = 0.581$; $p = 0.000$), VEGF ($r = 0.443$; $p = 0.001$) and MVD ($r = 0.566$; $p = 0.000$) among fibrocystic, atypical lesion and stage II - III of breast cancer. VEGF expression was not significantly correlated with several histological grade of breast cancer and the highest of its expression was only found in atypical lesion. This study has suggested that TAM, MVD, VEGF and MMP-9 expressions might play an important role in the histological progression, histological grading and staging of breast cancer. The highest expression of VEGF in atypical breast lesion supported the fact that angiogenic switch already started in the early stage and grade of breast cancer.

ABSTRAK

Karsinoma payudara dapat berkembang dari lesi fibrokistik dengan proliferasi atipi sel epitel. Angiogenesis berperan penting dalam perkembangan, invasi dan metastasis karsinoma payudara dan proses tersebut ditunjukkan dari jumlah kepadatan mikrovaskular (MVD). *Tumor associated*

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macrophages (TAMs) adalah sel radang di dalam stroma yang dapat menghasilkan faktor pro-angiogenik seperti VEGF, dan memacu MMP-9 untuk mendegradasi matriks ekstraselular dan membran basal. Angiogenesis pada karsinoma payudara dan hubungannya dengan derajat histologis serta stadium perlu diteliti agar terapi dan prognosis karsinoma dapat ditentukan lebih tepat. Penelitian ini bertujuan mengkaji korelasi antara TAM, MVD, ekspresi VEGF dan MMP-9 antara lesi fibrokistik, lesi atipi dan karsinoma payudara, serta korelasinya dengan derajat histologis dan stadium. Dengan uji *cross-sectional*, diteliti 50 blok parafin dari lesi fibrokistik, lesi atipi dan karsinoma payudara. Spesimen dicat dengan metode imunohistokimiawi dengan MoAb anti CD68, vWF, VEGF dan MMP-9. Ekspresi VEGF dan MMP-9 dihitung dari jumlah sel karsinoma yang positif mengekspresikan marker tersebut. TAMs dihitung dari jumlah makrofag yang mengekspresikan CD68. MVD ditentukan dari jumlah mikrovaskular yang sel endotelnya meng-ekspresikan vWF, dan ditentukan dengan metode *Average Microvessels Count* (AMC). Korelasi antara marker-marker tersebut dengan berbagai tipe lesi payudara dan dengan berbagai derajat histologis serta stadium dianalisis dengan korelasi Pearson. Hasil penelitian menunjukkan adanya korelasi bermakna antara TAM (r = 0,760; p = 0,000), MVD (r = 0,659; p = 0,000), MMP-9 (r = 0,518; p = 0,000) dengan berbagai lesi payudara dan derajat histologis karsinoma. Ekspresi tertinggi marker tersebut didapatkan pada karsinoma derajat histologis buruk. Didapatkan korelasi bermakna antara TAM (r = 0,581, p = 0,000), VEGF (r = 0,443; p = 0,001) dan MVD (r = 0,566; p = 0,000) antara lesi fibrokistik, lesi atipi dan karsinoma stadium II-III. Ekspresi VEGF tidak berkorelasi dengan derajat histologis karsinoma, namun ekspresi tertinggi VEGF didapatkan pada lesi atipi. Dari penelitian ini disimpulkan bahwa TAM, MVD, ekspresi VEGF dan MMP-9 berperan penting pada perkembangan, derajat histologis dan stadium karsinoma payudara. Ekspresi tinggi VEGF pada lesi atipi menunjukkan bahwa *angiogenic switch* sudah dimulai sejak awal stadium dan derajat histologis karsinoma payudara.

Keywords : TAM - MVD - VEGF - MMP-9 - fibrocystic lesion - atypical lesion - breast cancer

INTRODUCTION

Invasive duct carcinoma is the most common type of breast cancer and it may derive from fibrocystic lesion, especially with atypical proliferation of epithelial cells. There is a significant correlation between the degree of cell proliferation and its progression into *in situ* and invasive carcinoma.[1,2] Histological grading of this cancer is classified into well, moderate and poor grade, based on the tubular formation, number of mitosis and cell pleomorphism. Many studies show that there is a correlation between histological grading and prognosis.[1] Prognosis of breast cancer is also determined by its staging that consists of tumor size, number of lymph-nodes involved and distant metastasis. In Yogyakarta Special Region, Indonesia 70% of breast cancer is found in stage III B, poorly differentiated and with 38% already having metastasis of more than 3 lymph-nodes.[3]

Breast cancer progression depends on vascularization. Cancer cannot enlarge beyond 1 to 2 mm in diameter or thickness unless they are vascularized. There are some factors that influence the angiogenesis process, such as tumor associated macrophages (TAMs).[4] TAMs are a group of leucocytes that belong to the mononuclear phagocytes. TAMs are derived from monocyte and then differentiated further into resident tissue macrophages.[5] In the setting of tumors, TAMs have pleiotrophic functions, which can influence tumor growth, both in terms of progression and regression. This differential effect of TAMs is believed to be regulated by modulation of the host immune system. Tumor growth reduction by TAMs can be mediated by non-specific antitumor cytotoxic mechanisms or induction of specific cell lytic effects. On the other hand, as the effect of tumor progression, TAMs produce growth factors that promote cancer cell proliferation and dissemination, and

enhance angiogenesis.[6,7] The most important tumor growth factor is vascular endothelial growth factor (VEGF) which can induce proliferation and maturation of endothelial cells.[8] VEGF can attract TAMs into the hypoxic tumor areas.[9] The expression of TAMs can be detected with monoclonal antibody against CD_{68}.

TAM and VEGF can induce microvascular density (MVD). MVD is highlighted by staining endothelial cells for von Willebrand factor (vWF)-related staining. MVD indicates representative angiogenesis of the tumor. The expression of MVD is higher in breast cancer than in benign lesion and the height of MVD is correlated with metastasis and worse prognosis.[10] TAM and tumor cells can produce a proteolytic enzyme, matrix metalloproteinase-9 (MMP-9), that has an important role in tumor cells invasion to the basement membrane and stroma, blood vessel penetration, and metastasis. MMP-9 has also been implicated in the process of angiogenesis both as the inhibitor and the activator.[4]

Many studies have proven that interaction of TAMs and tumor cells is important as the new alternative marker and treatment of cancer. Thyroid cancer with high TAMs expression has a better prognosis compared to the lower one.[11] In prostatic cancer, there is a reverse correlation between the number of TAMs and clinical stage, and it seems that the decrease of total number of TAMs can be used as a new prognostic marker.[12] In melanoma maligna, the high number of total TAMs is statistically correlated with worse treatment responses.[13] In a breast cancer mouse model, vaccin legumin-based DNA can induce cytotoxic T cell (CD_8) that can decrease the density of TAMs and restrain TAMs to produce pro-angiogenic factors.[14] However, the role of angiogenesis in relation with tumor progressivity, histological grading and staging of breast cancer remains unclear.

This study aimed to investigate the correlation between different tumor markers (TAM, MVD, VEGF, MMP-9) and different types of breast lesions (fibrocystic lesion, atypical lesion and breast cancer of different stages and histological grading).

MATERIALS AND METHODS

The design of this study was a quantitative non-experimental, performed by cross sectional method. Samples of this study were paraffin-embeded tissue of fibrocystic lesion, atypical lesion and breast cancer, which had been taken from Department of Anatomical Pathology, Faculty of Medicine/Dr. Sardjito General Hospital, Universitas Gadjah Mada, Yogyakarta, in the year 2008. Histological grading of cancer was classified based on the Ellis and Elston criteria into well, moderate and poor grade.[1] Staging of cancer was grouped into stage I-IV based on the TNM classification.

The specimens were stained immunohisto-chemically with monoclonal antibody (MoAb) anti CD_{68} (524H12 Novocastra, dilution 1:100), MoAb anti vWF (36B11 Novocastra, dilution 1:50), PoAb anti VEGF (clone:RB-9031 Neomaker, Lab Vision) and PoAb anti MMP-9 (Neomaker, Lab vision, dilution 1:200) using chromogen DAB and counter stained Hematoxyllin Mayer. The expressions of VEGF and MMP-9 were counted from mean number of cytoplasmic positive tumor cells/100 tumor cells in 5 microscopic fields. Numbers of TAM were counted from number of macrophages which expressed CD_{68} in the 5 hot spot areas. MVD was counted from the number of microvascular whose endothelial cells expressed vWF, using the average microvessels count (AMC) method. This method counted the average microvessel count per square millimeter and recorded the number of microvessels in the area along border between

cancer nests and the stroma.[15] Immunohisto-chemical results were analyzed by two independent observers.

The correlation of mean number of TAM, MVD, VEGF and MMP-9 with different types of breast lesions (fibrocystic lesion, atypical lesion and breast cancer of different stages and histological grading) was analyzed with Pearson Correlation test.

RESULTS

The characteristic of samples

Fifty breast cancer specimens were used in this study. These samples consisted of 10 fibrocystic lesion, 10 atypical lesion and 30 well grade breast cancer (8 samples), moderate grade (11 samples), and poor grade (11 samples). Based on the cancer stage, 13 samples belonged to stage II, 15 samples to stage III and 2 samples to stage IV. There was no sample of breast cancer for stage I.

FIGURE 1 shows the TAMs expressions among CD_{68} positive cells, cytoplasmic cancer cell's VEGF expressions, MVD showed by endothelial vWF cells expressions and cytoplasmic cancer cell's MMP-9 expressions obtained by immunohistological examination from breast cancer specimens with 400x magnification.

TABLE 1 shows statistically significant correlations between TAM, MVD and MMP-9 expressions ($p<0.05$) among fibrocystic lesion, atypical lesion and breast cancer of well, moderate and poor grade respectively. The highest of their expressions was found in the poor grade cancer. VEGF expression showed no significant correlation with different type of breast lesions, and the highest VEGF expression was found in atypical lesion.
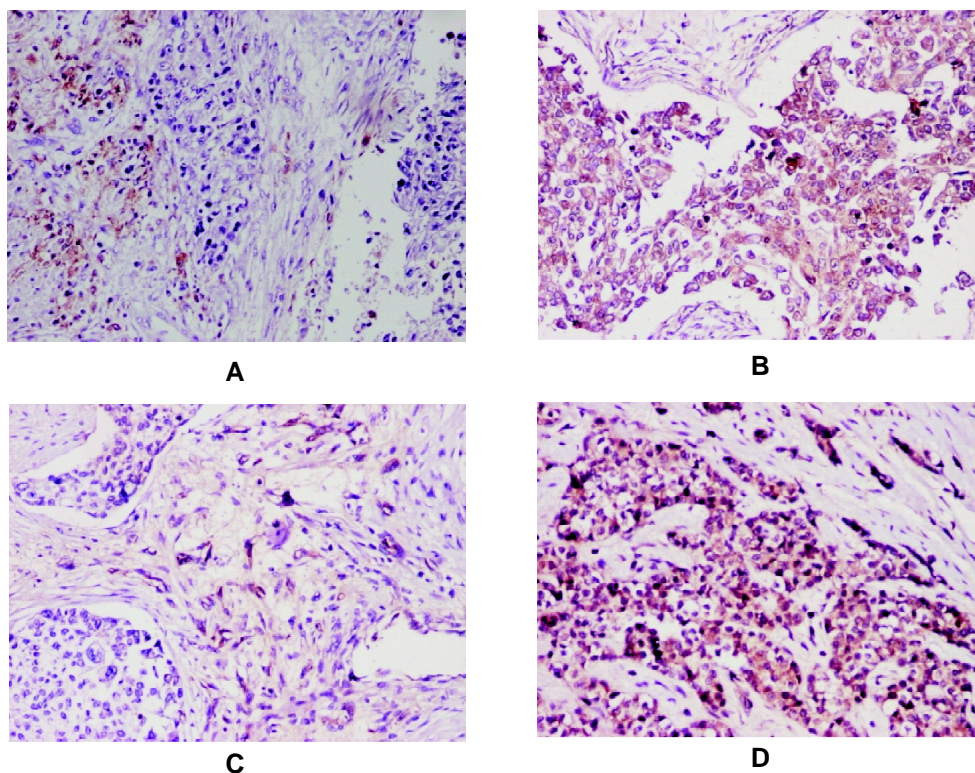

FIGURE 1.   A. TAMs Expressions among CD 68 Positive Cells (magnification 400x); B. Cytoplasmic Cancer Cell's VEGF Expressions (magnification 400x); C. MVD showed by endothelial vWF cells expressions (magnification 400x); D. Cytoplasmic Cancer Cell's MMP-9 Expressions (magnification 400x).

TABLE 1. Correlation of mean number of TAM, MVD, VEGF and MMP-9 expressions among fibrocystic lesion, atypical lesion and several histological grading of breast cancer

Characteristic	TAM	VEGF	MVD	MMP-9
Fibrocystic	7.22±2.77	85.00±18.90	3.22±0.97	28.20±20.12
Atypical lesion	15.3±7.66	88.60±24.55	7.00±2.82	61.00±25.11
Well grade cancer	21.28±10.33	35.28±29.71	10.28±2.92	32.57±25.21
Moderate grade cancer	50.00±22.91	56.25±30.57	9.5±3.03	64.25±28.04
Poor grade cancer	68.58±31.76	78.50±21.99	11.00±3.38	84.50±30.67
Statistical analysis	r = 0.760	r = -0.191	r = 0.659	r = 0.518
	p = 0.000	p = 0.185	p = 0.000	p = 0.000

TABLE 2 shows statistically significant correlation between TAM, MVD, and VEGF expressions ($p<0.05$), among several stage of breast lesions. The highest expression of TAM and MVD was found in stage III of breast cancer, while the highest expression of VEGF was found in fibrocystic and atypical lesions.

TABLE 2. Correlation of mean expression of TAM, MVD, VEGF and MMP-9 among fibrocystic and atypical lesion, stage II and III of breast cancer

Characteristic	TAM	VEGF	MVD	MMP-9
Fibrocystic and atypical lesion	11.47±7.06	86.89±21.53	5.21±27.96	45.36±27.96
Stage II of cancer	46.78±35.95	62.85±30.62	10.21±3.02	63.92±34.82
Stage III of cancer	58.53±23.18	60.93±32.91	10.86±3.07	38.50±50.51
Statistical analysis	r = 0.581	r = -0.443	r = 0.566	r = 0.255
	p = 0.000	p = 0.001	p = 0.000	p = 0.074

DISCUSSION

The most common types of breast cancer in this study were cancers with moderate and poor histological grade, as well as stage III cancer. There were no cases of cancer stage I. This means that breast cancer cases studied were mostly in the late stage and high grade. This result is similar to that of a previous study in Yogyakarta Special Region.[3]

This study showed that TAM, MVD and MMP-9 expressions were significantly higher in breast cancer than in fibrocystic and atypical lesion. It was indicated that the highest of their protein expressions, the highest of cancers grade and stage were observed (TABLE 1 and 2). This result suggests that TAM, MVD and MMP-9 expressions have an important role in the histological progression, histological grading and staging of breast cancer. The MVD and TAM were counted in the hot spot areas, which is biologically important because they provide a route through which tumor cells can metastasis. Hot spot areas may include necrosis and hypoxia. Hypoxic cells release bFGF and HIF1-α which can attract TAM to those areas. TAM can induce proliferation, migration and differentation of endothelial cells[4,9] and so as to induce angiogenesis.

TAM plays a role in the immune response via the elaboration of cytokines and growth factors. The presence of TAM has been reported to be in various tumors including breast cancer.

TAMs are recruited to tumors through the local expression of potent chemoattractants, such as colony stimulating factor-1 (CSF-1) and macrophages chemoattractant protein-1 (MCP1). Increased MCP1 was proven to correlate with increased TAM.[7] A study in breast cancer shows that high expressions of MCP1 can be used as an indicator of early relapse, while the abundance of TAMs are associated with poor prognosis.[16] It seems that TAM is recruited into tumor tissue before its conversion to malignant.[17]

Several studies support that inflammatory cells, especially TAM, secrete MMP-9 has emerged as an important modulator of angiogenesis and tumor development.[4] MMP-9 is associated with degradation of the extra-cellular matrix, including the basement membrane. Disruption of basement membrane integrity allows tumors to spread locally and distantly.[18] MMP-9 expression in this study was correlated with histological grading, but not with tumor stage. Pellikainen *et al.*[19] found that high expression of MMP-9 in carcinoma cells was associated with a small tumor, whereas positive stromal expression of MMP-9 was associated with aggresive factors. It seems that positive stromal expression of MMP-9 predicts poor survival, whereas positive tumor cells MMP-9 expression predicts favors survival. Thus, evaluation of MMP-9 expression seems to add valuable information on breast cancer prognosis. Another study found no correlation between the level of MMP-9 and histological grading as well as cancer staging.[20] Different antibody and scoring system of protein expression may highlight different results.

Many studies have proven that TAMs have an important role in angiogenesis which can be detected from MVD. A research in breast cancer found that MVD was correlated with the level of TAM, being significantly higher in ductal carcinoma with diffuse TAM infiltration than tumor without TAM in the stroma.[21] This study showed similar result with several researches that found a correlation of MVD with histological grading and staging of breast cancer,[22,23] even though others found different results.[24,25] In the early stage of breast cancer, MVD in the area containing high neovascularization can be used as an independent prognostic indicator of overall and relapse-free survival.[26] In male breast cancer, MVD is not an independent prognostic factor. As the age of diagnosis and tumor size increases, the mortality of male breast cancer increases as well.[27]

This study showed no significant correlation between VEGF expression with the histological grade and stage. The highest expression of VEGF was found in atypical lesions (TABLE 2 and 3). This result supported that angiogenic switch already started in early grade and stage of breast cancer.[28] A research conducted by Bluff *et al.*[17] found that there was a significant increase of VEGF expressions among benign, pre-invasive and invasive breast cancer. It seems that angiogenic switch already starts in the very early dysplastic breast lesion and it has an important role in the progression and invasion of cancer. Different result was found by Shankar *et al.*[29] which used MAGS score as a method for counting of VEGF expression. The method consists of vasoproliferative score, endothelial cells hyperplasia and endothelial cytology. A study done by Leek *et al.*[30] showed a significant positive correlation between high vascular grade of breast cancer with the increase of TAM index and reduced relapse-free survival. In colon cancer, there was no association between VEGF expression and the survival, but the increase of median survival had a relation with VEGF-expressing TAM in the stroma. VEGF-expressing TAM revealed an imbalanced anti-tumor activation of macropages that was

influenced by receptor flt-1 and induced by MCP1. In colon cancer, VEGF-expressing TAM was mostly found in stage II and III.[31]

Many studies propose that angiogenesis as a new alternative treatment can be used to inhibit the conversion of benign lession into malignant. An *in vivo* angiogenic therapy such as linomide, works by inhibiting the invasion of TAM into the tumor nests and the production of TNF-α, whereas anti-epidermal growth factor (EGFR) therapy can inhibit the activation of VEGF.[32]

CONCLUSION

This research shows a significant correlation between TAM, MVD and MMP-9 expressions among several histological progression, histological grading and staging of breast cancer. The highest expression of VEGF in atypical breast lesion supports that angiogenic switch already starts in early stage and grade of breast cancer.

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