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Research Article
Evaluation of Angiogenesis by Using CD105 and CD34 in Sudanese Breast Cancer Patients

[1]Anass M. Abbas, [1]Manar G. Shalabi, [1]Shawgi A. Elsiddig, [2]Zakaria Eltahir, [3]Asaad M.A. Babker and [4]Hussain G. Ahmed

[1]Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Jouf University, Kingdom of Saudi Arabia
[2]Department of Medical Laboratory Technology Sciences, College of Applied Medical Sciences, Taibah University, Medina, Saudi Arabia
[3]Department of Medical Laboratory Sciences, College of Health Sciences, Gulf Medical University, Ajman, United Arab Emirates
[4]Department of Histopathology and Cytology, Faculty of Medical Laboratory Sciences, University of Khartoum, Sudan

Abstract

Background and Objective: Angiogenesis is a mechanism by which new blood vessels are developed in healing and tumour tissues, where it is necessary for regeneration growth, tumour cells survival and metastasis. This study aimed to assess the angiogenesis mechanism among Sudanese females with breast cancer using anti-CD34 and anti-CD105 markers. **Materials and Methods:** Three hundred female representative Formalin-Fixed Paraffin-Embedded (FFPE) breast tissue blocks were included in this study. Of the 300 representative tissue blocks, 200 were breast cancer patient's tissues (confirmed cases) and 100 were normal breast tissues (controls). Their ages mean±SD, 47.3±12.9 years. **Results:** The results showed the MVD of CD34 significantly increased in malignant lesions as compared to normal breast tissues. The mean of MVD CD34 and MVD CD105 showed statistical differences among different histologic types of breast cancer. Also, a strong positive correlation was detected between the manual and automated MVD counting methods. Also, the current study revealed no significant differences were observed in mean MVD counting for both markers and menopausal status or the age groups of the study population. **Conclusion:** The MVD is a good tool for assessing prognostic markers. The CD105 marker has a high specificity to the new evolving tumour vessels and is a useful predictor for angiogenesis and breast cancer metastasis.

Key words: Angiogenesis, MVD, breast cancer, CD105, CD34, immunohistochemistry molecular subtypes, image analysis

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Corresponding Author: Asaad M.A. Babker, Department of Medical Laboratory Sciences, College of Health Sciences, Gulf Medical University, Ajman, United Arab Emirates

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cancer is a major burden of disease worldwide. Globally, 18.1 million new cancer cases were reported and 9.6 million cancer deaths in 2018. Breast cancer has been raised as the most commonly diagnosed cancer and the leading cause of cancer death Among females[1]. In Sudan, the incidence of breast cancer has been raising to be the most common cancer[2]. This is in addition to the fact that most patients are detected at a later stage of the disease due to the lack of awareness and absence of screening programs[3]. Many methods are available for demonstrating the obtained tissue from breast lumps. Conventional histopathology is the initial method of diagnosis and is then followed by other confirmatory methods to achieve the best management. Immunohistochemistry is a novel technique to identify specific markers associated with different types of cancers. The molecular techniques; which detect the patterns of gene alterations in different types of cancer are highly valuable[4]. Angiogenesis is a process when a formation of a new blood vessel network is initiated by certain body tissues and organs in case of healing and indeed in granting cancer cells survival. Certainly in cancer tissues is to provide the sufficient nutrients supply for their rapid growth. The scale of angiogenesis determines the growth, invasion and metastasis in almost any sold tissue cancer[5,6]. Microvascular density (MVD) remains one of the most reliable methods in use for quantitation of the degree of neovascularization[7]. The intensity of immunohistochemical staining microvessel density is closely correlated with the prognosis of breast cancer and recurrence of breast cancer as well as, the response to treatment and occurrence of micro-metastases[8,9]. CD34 is a surface glycol phosphoprotein found to be expressed in new blood vessels lining endothelial cells. CD34 is reported as a sensitive, well-studied marker for angiogenesis and is useful in the determination of microvessel density in all breast cancer tissues. The immunohistochemical staining of microvessels using anti CD34 has greater sensitivity and more intensity compared to anti CD31 and anti-factor VIII-related antigen[10,11]. High expression of anti CD34 marker, is associated with higher breast tumour stage, grade and may predict poor prognosis[12]. Endoglin CD105 is a cell membrane glycoprotein predominantly overexpressed on proliferating endothelial cells that is mostly associated with neo-vascularization[13]. The intensity of expression of CD105 in breast carcinomas has been found to reflect the neo-angiogenesis and endothelial activation[14,15]. The immunohistochemical expression of CD105 is highly restricted to the breast carcinomas, specificity of about 100%, rather than the normal breast tissue[16,17]. Nevertheless, the angiogenic microvessel density can be assessed by automated Computer-Assisted Morphometric analysis (CAM), which is a software system for image analysis that quantifies the positive immunohistochemical staining within tissue sections. CAM is reproducible and a reliable technique therefore, has shown more acceptance in clinical and pathological applications in the recent few years[18].

To the best of our knowledge, there is no unique study that assessed the angiogenesis pattern among Sudanese female patients with breast cancer. Therefore, this study aimed to evaluate the pattern of angiogenesis through the determination of MVD in different histological, molecular types and grades of Sudanese patients with breast carcinomas using CD34 and CD105, employing the CAM technology.

MATERIALS AND METHODS

Study area: The study was carried out at the Surgical Pathology Lab at Khartoum Referral Oncology Hospital, Khartoum-Sudan from March, 2019 October, 2020.

Methodology: A total of 300 FFPE breast tissue blocks were used in this study. Of the 300 breast tissue blocks, 200 were patient's blocks with breast cancer (ascertained as cases) and the remaining 100 were with normal breast tissues (ascertained as control). All-female patients at any age diagnosed as having breast malignancies of any type, stage or grade were included in this study. However, any metastatic tumours in the breast, patients with breast carcinoma who receive chemotherapy or hormone therapy were excluded from the study.

Ethical approval: Ethical consented approval was obtained from the ethical research committee at the Faculty Research Board of Omdurman Islamic University and Health authority associated with ethical consented approval (No. 9845402MD).

Immunohistochemical technique: Immunohistochemistry was performed on an adjacent tissue section on which haematoxylin and eosin with molecular subtypes profiles were made. Staining for endoglin (CD105) and CD34 was performed on 4-µm-thick sections from the paraffin-embedded breast tissue blocks. Immunostaining was carried out using the EnVision polymeric System-HRP (Dako Cytomation, Denmark). The tissue sections were mounted on coated slides and dried for 1 hr at 60°C. Briefly, after deparaffinization in xylene and rehydration in graded alcohol to water, the sections were exposed to Dako retrieval solution (PT link) for 30 min under

pressure and at a boiling temperature of 100 . Then sections were cooled for 20 min at room temperature and then incubated with 0.3% hydrogen peroxide for 5 min to block the endogenous enzyme activity. After Tris-buffered Saline (TBS) washing, the blocking solution was used (Protein block Serum-free Ready to use, Dako) the slides were washed with TBS again. Primary antibodies of rabbit monoclonal anti endoglin (CD105) and anti CD34 Dako, ready to use in 1:200 dilutions were applied to the sections for 45 min (the negative control was incubated in the same manner but the primary antibody was omitted). This step was followed by washing in TBS and 40 min incubation with anti-rabbit dextran polymer conjugated with horseradish peroxidase. Visualization was done with 3-amino-9-ethylcarbazole containing H_2O_2 as substrate, for 10 min. Sections were counter-stained with Mayer's haematoxylin, dehydrated in alcohol and cleared in Xylene, then mounted and coverslipped. All the details of the procedure were according to the protocol of Dako™. Interpretations of the immunohistochemical reactions were performed first manually and independently by three expert assessors who were blinded to tumour type or stage. Appropriate positive and negative controls were performed before all immunohistochemical staining procedures were applied.

Microvessel manual quantification (MVD): The counting procedure was performed in a blinded manner without knowledge of the patient status and stains used. Furthermore, the slides were reviewed in nonconsecutive order, preventing direct comparison of individual cases. The area of most intense micro-vascularisation was selected by scanning on low magnification (×10) to identify three areas with the highest density of microvessels (hotspots). Each hotspot was then evaluated at high power magnification (×40 magnification: 0.15 mm^2 field) for the number of stained microvessels per field. Any brown-staining endothelial cell containing a visible nucleus and separate from adjacent microvessels, tumour cells and other connective-tissue elements, was considered a single, countable microvessel, without the requirement for a lumen or the presence of erythrocytes. Larger vessels with muscular walls were excluded from counting. The mean of three fields was chosen from each slide, to best reflect the overall immunostaining of the vessels. Counting was performed by two independent observers as mentioned earlier[15,19].

Computer-assisted morphometric analysis (CAM): A careful scan of the immunostained slides with a light microscope (Axiphote microscope, Zeiss, Germany) with ×100 magnification was used to identify the three most immuno-positive CD34 and CD105 vascular regions in the tumour (hotspots), disregarding any preexisting mature vessels. From each hotspot, photomicrographs of 1292×968 pixels were obtained from two to five fields depending on the hotspot size, at a magnification of X400, with Zeiss AxioCam MRc5 Digital Microscopy camera®, adjusted to these parameters. The pixel size was measured to be 0.3436 µm using a stage micrometre. The illumination was kept constant during all image capture. Digitized pictures were visualized on a high-resolution colour display. Automatic vessel identification of The CD34 and CD105 immuno-positive cells in the images were identified using a segmentation algorithm in the software program. The MVD areas were quantitatively measured using digital image processing and analysis software for professional microscopy (AxioVision LE Fujitsu, Germany). The collected measurements were presented as Mean±standard deviation. The automatic vessel count was compared to a manual count made by an experienced pathologist for quality control measures as mentioned by Safwat *et al.*[17] and Mikalsen *et al.*[20].

Statistical data analysis: Analysis of data was performed using Statistical Package for Social Sciences (SPSS) version 20. The significant differences and association between the MVD counting of CD34, CD105 and the Clinicopathological parameters (age, histological types and cancer grade) were analyzed using a Two-tailed T-test, Chi-square test and one-way analysis of variance (ANOVA). Statistical significance ($p<0.05$), with the 95% confidence level and confidence intervals were used.

RESULTS

This study investigated 300 subjects (200 were cases and 100 were controls). Their ages ranging from 24-85 years with Mean±SD, 47.3±12.9 years as shown in Table 1. The majority 141 (70.5%) of the study subjects were diagnosed as invasive ductal carcinoma followed by invasive lobular carcinoma constituting 21 (10.5%), then in situ carcinoma 16 (8%). Moreover, invasive mucinous carcinoma and invasive papillary carcinoma represented, 15 (7.5%) and 7 (3.5%), respectively as shown in Table 2. CD34 immunoexpression was weakly expressed in blood-vessel endothelial cells of control tissues with a mean MVD count of 2.091. Widespread staining for CD34 was detected in all cases with a mean MVD count of 42.6, there is a significant difference between the two means of $p = 0.001$. On contrary, CD105 was not expressed in the vascular endothelial cells of the control subjects but was

Table 1: Mean of MVD CD34 and CD105 by age groups among the studied population

Age group	N	Mean±SD MVD CD34	Mean±SD MVD CD105
<30	17	44.82±8.19	27.65±6.51
30-40	55	42.02±8.27	25.36±6.23
41-50	63	43.43±7.39	27.05±5.89
51-60	31	41.07±6.24	25.48±5.17
61+	34	42.29±8.24	25.76±6.26
Significance	p	0.745	0.875

MVD: Microvascular density

Table 2: Mean of MVD CD34 and CD105 by histological type of breast cancer among the studied population

Histological type of breast cancer	Mean±SD MVD CD34	Mean±SD MVD CD105
Invasive ductal carcinoma (n = 141)	44.82±6.83	27.68±5.32
Invasive lobular carcinoma (n = 21)	41.71±4.67	26.48±4.21
Invasive papillary carcinoma (n = 7)	36.14±2.79	22.43±1.4
Invasive mucinous carcinoma (n = 15)	41.47±5.13	26±3.3
In situ carcinoma (n = 16)	28.13±1.2	14.31±0.71
Significance	0.001	0.001

MVD: Microvascular density

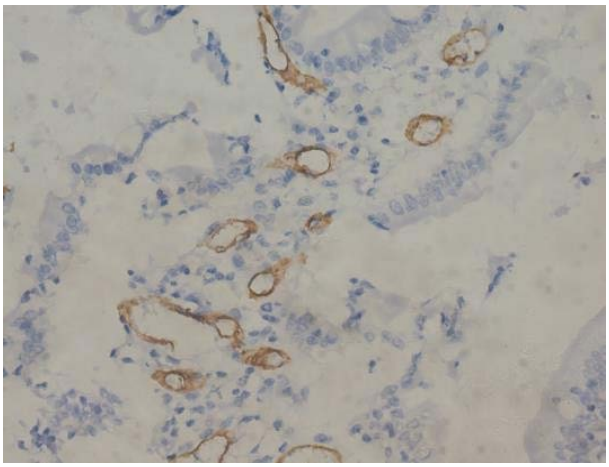

Fig. 1: Breast cancer tissue showing anti-CD34 immuno expression (×400)

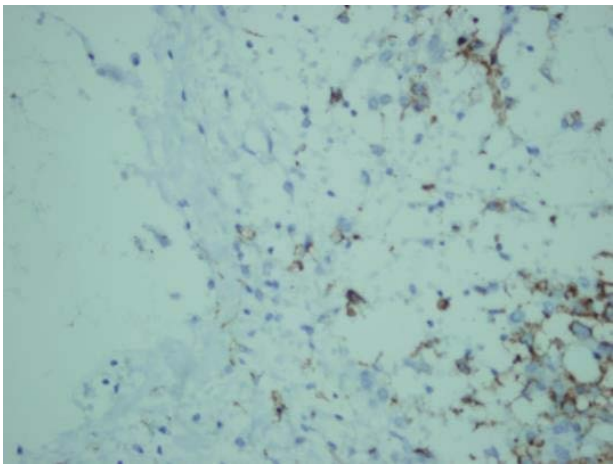

Fig. 2: Intratumoral breast cancer tissue showing CD34 microvessels staining (×400)

expressed only in the vascular endothelial cells of cases with a mean MVD of 26.18, as shown in Table 3 (Fig. 1-2). The invasive ductal carcinoma was found to be expressing the highest mean for both markers MVD CD34 (44.8) and MVD CD105 (27.68), followed by invasive lobular carcinoma (41.71) and (26.48) for CD34 and CD105, respectively (Fig. 3-4). Then, invasive mucinous carcinoma with mean MVD (41.47) for CD34 and (26.00) for CD105, followed by the invasive papillary carcinoma mean MVD CD34 (36.14) and CD105 (22.43). However, the lowest mean of MVD CD34 (28.13) and MVD CD105 (14.31) was found in the in-situ carcinoma, as shown in Table 2. This was found to be statistically significant (p = 0.001). The mean MVD of CD34 counts among the study population, the highest mean (44.82) was found in the ages below 30 years old, followed by 43.43 in the age group 41-50 years and then the mean was relatively similar in age groups 30-40 years and more than 60 years 42.02 and 42.29, respectively. The lowest mean MVD of CD34 (41.07) was found in the age group 51-60 years. Hence no evidence of a relationship between the age group and MVD CD34 count (p = 0.742) as shown in Table 1. Regarding the mean of MVD CD105 among the study population, the highest mean (27.65) was detected in the age group less than 30 years and the lowest mean (25.36) was in the age group 30-40 years. No statistically significant differences were found between the different age groups (p = 0.875), as shown in Table 1. Regarding the relationship between the mean of MVD CD34 and menopausal status, the mean was (42.85) in premenopausal and (42.03) in a postmenopausal breast cancer patient (p = 0.710). Moreover, no significant differences were detected in the MVD counting ofCD105 between premenopausal (26.31) and postmenopausal patients (25.87) as shown in Table 4. There is a strong positive correlation between manual

Table 3: Mean microvascular density count of CD34, CD105 among cases and controls in the studied population

Angiogenesis marker	Normal breast tissue (n = 100)	Breast cancer tissue (n = 200)	Significance
Mean±SD MVD CD34	2.091±0.84	42.60±7.69	p = 0.001
Mean±SD MVD CD105	Not expressed	26.18±6	-

MVD: Microvascular density

Table 4: Relationship between MVD CD34, CD105 and menopausal status among the studied population

Angiogenesis markers	Premenopausal (n = 139)	Postmenopausal (n = 61)	Significance
Mean±SD MVD CD34	42.85±7.83	42.03±7.41	0.710
Mean±SD MVD CD105	26.31±6.12	25.87±5.77	0.882

MVD: Microvascular density

Table 5: Correlation between manual MVD CD105 and automated MVD CD105 counting

Counting method	Mean±SD	Correlation
Manual counting method	34.15±0.41	$r^2 = 0.778$
Automated counting method	25.70±0.31	Sig. = 0.001

MVD: Microvascular density, r^2: Spearman's correlation factor, Sig.: Significance of p-value

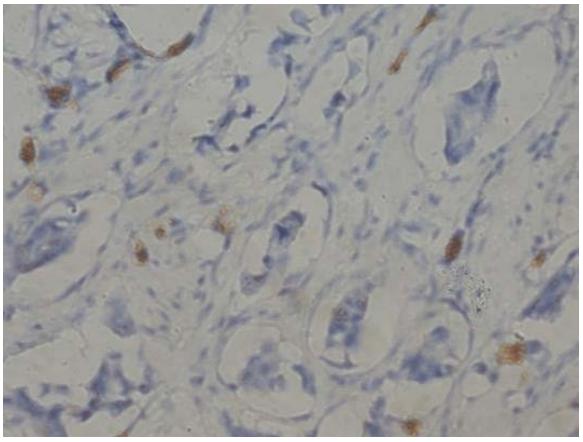

Fig. 3: CD105 (endoglin) immunoexpression showing microvessels staining intratumoral breast cancer tissue (×400)

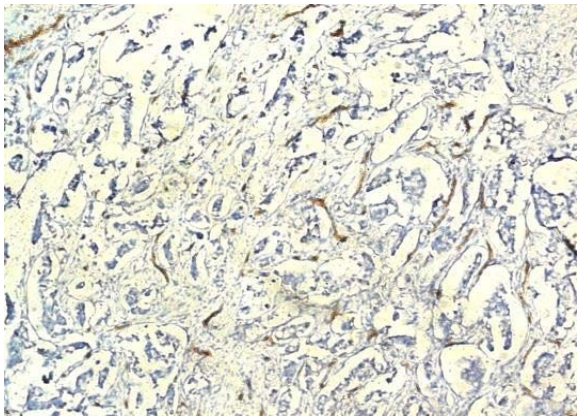

Fig. 4: CD105 (endoglin) immunoexpression showing microvessels staining hotspot intratumoral breast cancer tissue (×100)

MVD CD105 counting Method and automated MVD CD105 counting ($r^2 = 0.778$), as shown in Table 5.

DISCUSSION

In this study, CD34 and CD105 markers were found to be significantly expressed in the malignant breast cancer tissues, hence not expressed or weakly expressed in normal breast tissues. However, there is no previous study had reported this finding in Sudanese patients or the neighbouring geographic countries. However, various studies either prospective or retrospective in the developed countries have concluded significant expressions of CD34 and CD105 in breast cancer patients. Moreover, MVD assessment using anti CD34 and CD105 is a known procedure for prognostic factors assessment in invasive breast cancer patients[8,21,22]. In this study, CD34 angiogenic marker was weakly expressed in normal breast tissues and the mean of MVD was 2.09. However, Safwat *et al.*[17] and Dales *et al.*[18] found it expressed with MVD 1.07 and 2.48 respectively. Whereas, in this study, the CD105 angiogenic marker was not expressed in normal breast tissues and expressed only in the malignant breast tissues. This was following the studies of Ding *et al.*[23] and Berseford *et al.*[24]. However, there are no studies reported a positive or a weak expression of CD105 in normal breast tissue. The mean MVD count of CD34 in breast cancer cases was 42.60. However, Safwat *et al.*[17] reported the mean microvessel density CD34 was 84.12 in cases of invasive ductal carcinomas stained with an anti-CD34 marker. Dromain *et al.*[25] reported that the median value of the CD34 intra-tumoural microvessel density in breast carcinomas was 79.20. Furthermore, Kamlesh *et al.*[13] demonstrated the MVD using CD34 in invasive breast cancer cases and was in the range of 16 to 32 with a Mean of 24.19. On the other hand, the mean MVD of CD105 marker in breast cancers cases was 26.18 in this study. Safwat *et al.*[17] reported the mean of MVD CD105 in invasive ductal carcinoma cases to be 19.10. However, Białas *et al.*[6] reported that the Mean intratumoral CD105 positive vessel count was 37.84 for malignant cases. The discrepancies between these studies in mean MVD counts

may be due to the small sample size, histological type of breast cancer, grades, stages of the disease and MVD counting methods. There are significant differences between the histological types of breast cancers and the mean MVD count of CD34 and CD105. Invasive ductal carcinoma was found to be expressing the highest mean for both markers. These findings are following Hansen *et al.*[26], who reported significant correlations between high CD34 MVD counts and histological type. In contrast to Dales *et al.*[27], who found that no significant correlation between histological types of the breast carcinomas and the mean MVD count of CD105 staining microvessels. In the present study, there is no evidence of a significant relationship between the age group and MVD CD34, as well as, MVD CD105. Similarly, Frangou *et al.*[28] reported that there was no significant difference in MVD between levels of the different age groups of the patients. In contrast, Kamlesh *et al.*[13] found statistically significant differences between the mean of microvessel density in less than 45 years age group (28.0) and (21.90) in more than 45 years age group. In the present study, there is no significant difference in the mean MVD count of CD34 and CD105 between the premenopausal and postmenopausal breast cancer patients. These findings support the study by Safwat *et al.*[17], they found no significant differences were detected in MVD count between premenopausal and postmenopausal breast cancer patients. However, Kamlesh *et al.*[13] reported that the mean microvessel density in the premenopausal age group was 28.0, whereas it was 21.90 in the postmenopausal age group. This difference was statistically significant, $p = 0.001$. In the current study, the difficulty to find the complete clinicopathological parameters of the patients, lack of clinical stage and inability to follow up the participants in this study to determine the overall survival rates, are the main limitations in this study. However, there is a strong positive correlation between the manual MVD CD105 counting method and automated MVD CD105 counting. These findings support a few studies including a study by Mikalsen and colleagues, Cioca and colleagues in renal carcinoma and Zhang and colleagues in hepatocellular carcinoma[20,29,30], where Mikalsen compared automatic and manual vessel counts in intra-class correlation coefficient were $r^2 = 0.96$ and a 95% confidence interval for the percentage difference between the counts from -26.1-10.8%. The method was also found to have a sensitivity approaching 100%. This is also in agreement with Dales *et al.*[18] they mentioned that to provide more standardized data for the quantification of immunocytochemical studies, diverse computerized image analysis systems have been employed and were found to correlate well with semi-quantitative histological scoring methods and with biochemical data[31]. However, Saponaro *et al.*[32] concluded that the evaluation of tumour angiogenesis using MVD automated and manual counting methods does not provide additional prognostic information in breast cancer patients. Therefore, this study recommends that such MVD protocol is an accurate prognostic marker and useful predictor for angiogenesis and breast cancer metastasis.

CONCLUSION

MVD protocol using CD34 and CD105 angiogenic markers can reflect the status of the tumour angiogenesis cascade. This study found that angiogenesis is a very active mechanism among Sudanese female patients with breast cancer. The study found angiogenesis is increasing with invasive breast carcinomas and cancer grades. CD105 appears to be much more specific for new tumour vessels.

SIGNIFICANCE STATEMENT

This study discovers the automated use of the CD34 and CD105 as angiogenic markers can reflect the status of the tumour angiogenesis cascade. This study will help the researcher and clinician, especially oncologists in developing the new protocol to use CD105 and CD34 in the assessment of Microvascular Density and angiogenesis among patients with breast cancer.

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