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Immunohistochemical Expression of CD34, Smooth Muscle Actin and Type IV Collagen in Breast Carcinoma. A clinicopathological Study

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Abstract

Background Tumoral angiogenesis is essential for the growth and spread of breast cancer cells.

Objective To evaluate angiogenesis by measuring microvessel density (MVD) with CD34 and its maturity with smooth muscle actin (SMA) immunohistochemistry and to study invasion of basement membrane by tumor cells using type IV collagen.

Methods In the present study microvascular quantification was undertaken on 52 cases of breast carcinoma and 5 cases of benign breast lesions after immunohistochemical staining of tumor vessel, using CD34 antibody and SMA antibody. Microvessel quantification was performed at x400 magnification in the three most vascular areas of the tumors (hot spots).

Results The difference in MVD between benign and malignant cases is significant (P= 0.001). MVD is significantly correlated with L.N. involvement (P=0.004) and lymphovascular permeation (P=0.001), no statistical significant correlation between MVD and age of patient (P=0.656), tumor size (P=0.052), tumor grade (P=0.324).

Conclusion Measurements of angiogenesis may have clinical utility in the evaluation of breast cancer, particularly for estimation of metastatic risk. A high MVD may be a poor prognostic marker of breast carcinoma and a target for antiangiogenic therapy.

Key words Angiogenesis, CD34, SMA, Collagen IV, breast carcinoma.

Introduction

Breast cancer is the most common cancer affecting women in the world today. It is the leading cause of cancer related death for women aged between 35 and 55 years worldwide [(1)]. In Iraq, breast cancer is the commonest type of female malignancy, accounting for approximately one-third of the registered female cancers [(2)].

Tumor angiogenesis is the development of new blood vessels from an existing vascular network; it is a prerequisite for tumor growth beyond 2 mm in diameter and plays an important role in metastasis and prognosis of the tumors including breast cancer [(3)]. Angiogenesis can be quantitated by staining histological sections with antibodies that specifically identify endothelial cells. CD34 is a sensitive marker for vascular endothelium. The maturity of blood vessels can be assessed by using SMA which is a marker that stains smooth muscles covering mature vessels. The number of microvessels can be estimated by many methods including hot spot method [(4)]. Type IV collagen is a marker used to study the invasion of basement membrane by tumor cells. In the present study we concentrated on

the immature blood vessels which are the target of antiangiogenic (anticancer) therapy.

Methods

Fifty seven cases were included in this retrospective study, 5 benign and 52 malignant (female not receive any therapy). All formalin fixed paraffin embedded tissue blocks representing mastectomy specimens of breast carcinoma were retrieved from archived files of department of pathology of Al-Kadhimiya Teaching Hospital (for the period between Jan. 2010-Nov. 2010) and Teaching Laboratories of Medical City Hospital (for the period between Jan. 2009-Oct. 2009).

Clinical information regarding age, tumor size, grade, histological subtype, lymph node involvement, and lymphovascular permeation were studied. For each case, 4 sections of 5 μm thickness were taken, one for H&E and others were processed for immunohistochemical analysis to determine the MVD by CD34, vascular maturity by SMA and basement membrane invasion by Collagen type IV (all from Dako) using LSAB (labeled strept-avidin-biotin peroxidase) technique on paraffin-embedded sections. After deparaffinization with xylene, the slides were put in antigen retrieval then in buffer (3006) then incubated with peroxidase blocking reagent, then with primary antibody for 24 hours then with biotinylated link antibody then incubated with streptavidin\perixidase then with DAB chromogen.

Vascular count: We chose special areas of tumor in 40× magnification that did not have necrosis, ulceration or inflammation, as vascular hot-spot. we counted the number of vessels(stained by CD34) in three hot spots with 400× magnification field (area of 0.79 mm2) after counting the number of mature blood vessels in the same spot stained by SMA, we substract them from total count by CD34 then obtained average value of MVC of the three spots so that only immature vessels are included in the vessel count.

Isolated endothelial cells or cords of non-perfused endothelial cells , without lumen , for which the reaction for CD34 is positive and negative for SMA represent the immature vessels (Figure 1).

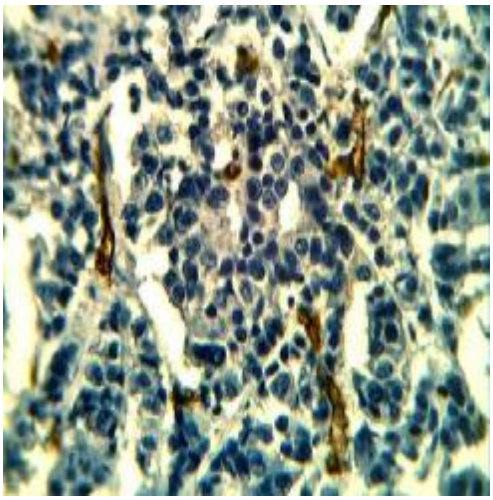

Figure 1. Invasive ductal carcinoma of breast (NOS) grade II immunostained with CD34 showing immature sprouting blood vessels (vascular hot spot)(X40).

They do not have pericytes or smooth muscle cells in their walls. The mature vessels were characterized through the co-expression of CD34 and SMA, the presence of lumen and sometimes containing RBCs (Figure 2).

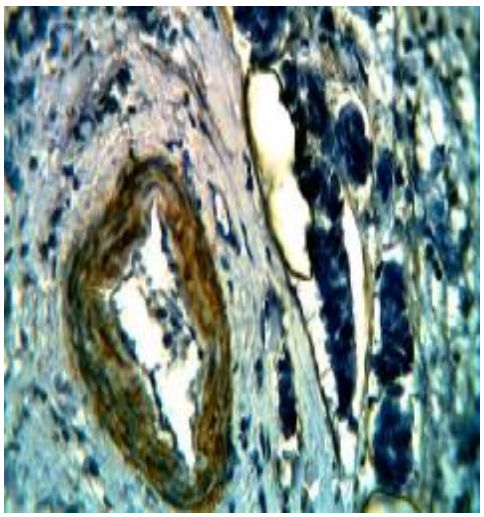

Figure 2. Invasive ductal carcinoma (NOS) grade II immunostained with SMA showing mature blood vessel with no immature blood vessels (X40).

The microvessel counts per field were converted to microvessel per square millimeters for subsequent statistical analysis.
Any endothelial cells or endothelial cell cluster positive for CD34 and separate from an adjacent cluster was considered a single countable microvessel [4].

MVD=mean MVC\ high power field area (0.79) mm^2

Collagen IV for assessment of BM
In normal section of breast tissue there is strong and well defined staining of immunoreactive type IV collagen. In carcinoma insitu, the continuity of type IV collagen staining at the interface between cancer cells and stroma was noted (Figure 3).

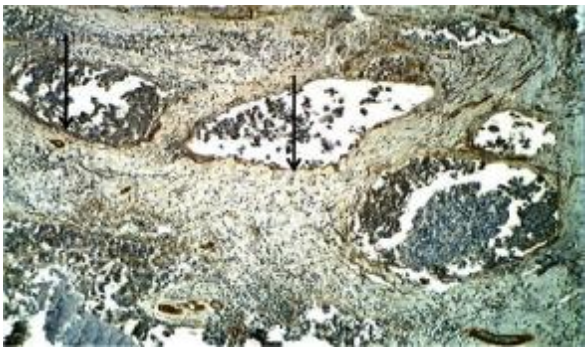

Figure 3. Invasive ductal carcinoma(NOS) with comedo carcinoma *insitu* immunostained with collagen IV showing continuity of the basement membrane at the interface between malignant cells and stroma (arrows)(X10).

Focal interruptions indicate areas of invasion. In invasive carcinoma, there is limited, very weak or absent type IV collagen staining (Figure 4).

The data were statistically analyzed using SPSS V.17 (statistical package for social sciences). The student t-test was used to assess the correlation between two parameters. Pearson correlation coefficient (r) was used to study correlation be. P-value of less than 0.05 was considered statistically significan.

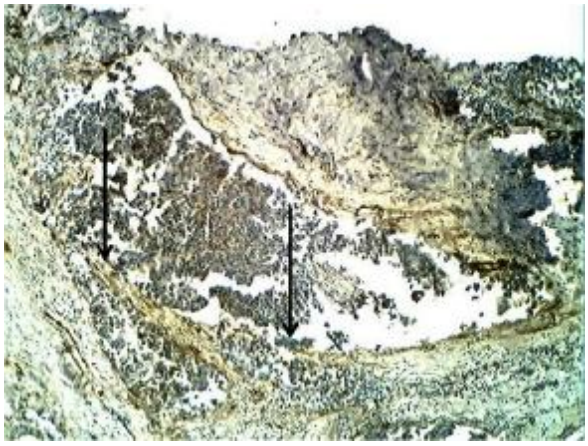

Figure 4. Invasive ductal carcinoma(NOS) immunostained with collagen IVshowing distuption of the basement membrane(arrows)(X10).

Results

The correlation between MVD and the clinicopathologic parameters are summarized in table 1. There was a significant correlation between MVD and lymph node involvement and lymphovascular permeation, MVD not significantly correlated with age, tumor size and grade. ANOVA test cannot applied to study correlation between histological subtypes and mean MVD because to 2 of the types includes 1 case only, so P-value is not calculated for this parameter.

Discussion

The ability of tumors to induce a vascular stroma is a critical requirement for tumor progression at all stages of breast cancer development [5]. Although angiogenic activity is associated with tumor proliferation and metastasis, vessel maturation also plays an essential role in the later stages of tumor development [6]. Recruited pericytes and smooth muscle cells form a protective barrier that stabilizes blood vessels and reduces apoptotic events [7,8]. Thus, there is heterogeneity in blood vessels present in tumors [9].
In the present study, more neovascularization in malignant than in benign lesions was noticed. The results were in agreement with Hammoudi, 2005 [10]. MVD values in *insitu*

carcinoma are lower than in invasive carcinoma which means more blood vessels are required by the tumor for invasion. A dense microvascular rim adjacent to the basement membranes of DCIS associated with invasive ductal carcinoma was seen in the present work. This finding is consistent with previous publications which suggested that periductal vascularization may be caused by the direct release of angiogenic factors by neoplastic cells and could be important in determining the transformation from in situ to invasive disease [(11,12)].

Higher MVD in higher grade tumors indicates more aggressive tumor although the relation was statistically not significant. Thus, tumor de-differentiation would seem to be associated with an increased pro-angiogenic activity. The results were in agreement with El-Moneim et al, 2008 [(13)].

MVD in T2 is higher than in T1 and T3, this is in agreement with El-Moneim et al [(13)], 2008 and disagree with Hammoudi, 2005 [(10)]. This could be due to the interstitial pressure in tumor would lead probably to compression closure of capillaries, and then to ischemia and transport problems that ultimately result in necrosis. Moreover, active angiogenesis occurs mainly in the tumor periphery, while maintenance of the inner vascularization is a result of continuous remodeling. Therefore, it is not surprising that the importance of angiogenesis is diminished as the tumor grows [(14)]. Also related to that the determination of MVD in the areas of highest vascular density (hotspots), in which systemic dissemination of cancer cells is more likely in the areas of highest vessel density. Since identification of hotspots is of great importance for the method, the chance of identifying the "hottest-spots" would most likely be influenced by the potential variation of vascular density between different parts of a tumor, so tumor heterogenicity play a role in variation of vascular counting [(15)].

There was a statistically significant correlation between the MVD and lymph node status, this is in agreement with El-Moneim et al, 2008 [(13)] and disagree with Rishil 2009 [(16)]. The increase in microvessel density (MVD) in and around tumors is thought to increase the chance that invasive tumor cells enter the lymphatic vasculature. In turn, it is suggested that this promotes the formation of lymph node metastases, as increased numbers of disseminating tumor cells are transported to regional lymph nodes [(17)].

The correlation between MVD and lympho-vascular permeation was statistically significant, this disagree with Johan 2002 [(15)]. The explanation of this is related to the architecture of microvessels that makes them more amenable to the entry of invasive tumor cells, they have loose overlapping cell–cell junctions, and end capillaries have no or only an incomplete basement membrane. Invasive tumors can permeate into the lymphatic vasculature locally as strings of cells, but generally traffic as emboli [(18)].

Measurements of angiogenesis may have clinical utility in the evaluation of breast cancer, particularly for estimation of metastatic risk.MVD might also have predictive value with regard to benefit from adjuvant chemotherapy, or by specific antiangiogenic drugs.

In conclusion ,MVD is significantly correlated with L.N. involvement and lymphovascular permeation. No statistical significant correlation between MVD and age of patient, tumor size, and tumor grade. Assessment of tumor vascularity by CD34, SMA immunohistochemistry is valuable in quantifying angiogenesis and its maturity in breast carcinoma. Measurements of angiogenesis may have clinical utility in the evaluation of breast cancer, particularly for estimation of metastatic risk. A high MVD may be a poor prognostic marker of breast carcinoma and a target for antiangiogenic therapy.

Table 1. Summary of clinicopathologic parameters and correlation of them with MVD of studied cases

Parameters		No. of cases	Mean MVD	P* value
Behavior	Benign Malignant	5 52	45.60±7.54 91.55±3.93	0.001
Age (27-80) year Mean = 46.52+1.46 year Median=40 year		52 (malignant)	91.55±3.93	0.656
Histological type	DCIS DCIS+LCIS IDC(NOS) IDC+comedo IDC+DCIS Mucinous	1 1 35 8 6 1	59 66 93.74 96.25 92.8 91	
Grading	Grade II Grade III	36 16	88.94±4.35 97.43±8.27	0.324
Size	<2 cm (T1) 2-5 cm (T2) >5 cm (T3)	10 22 20	78.40±3.43 102.09±6.57 86.55±6.37	0.052
LN involvement	Positive Negative	35 17	97.60±5.46 79.11±2.46	0.004
Lymphovascular permeation	Positive Negative	43 9	95.20±4.52 74.11±3.55	0.001

DCIS: ductal carcinoma *in situ*; LCIS: lobular carcinoma *in situ*; IDC (NOS): invasive ductal carcinoma not otherwise specified. P* value <0.05 is significant.

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