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ORIGINAL ARTICLE

Prognostic significance of CD 31 in breast cancer patients: A prospective study

Vivek Srivastava[1], Rohit Shyam[1], Anand Kumar[1], Mumtaz A. Ansari[1*]
[1]Department of Surgery, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India.
*Email: mumtazbhu@gmail.com

ABSTRACT
The aim of this study was to assess micro vessel density (MVD) using CD31 as vascular endothelial marker and evaluate its prognostic significance. A prospective study was done on 50 patients of Stage IIIa, IIIb and IV carcinoma breast patients. A preoperative tru-cut biopsy was done and tumor vascularity was assessed using CD 31 assay. All the patients received 2 cycles of neo-adjuvant chemotherapy (CAF) followed by mastectomy. Patients were followed for 2 years during the course of study. The histopathology, chemotherapeutic response, recurrence and metastasis data were collected and analyzed. Majority of cases were age range 30-50 years with mean age was 45.76±17.22 years. Eighteen (36%) cases showed high MVD (score ≥ 40). Fourteen cases (28%) had intermediate vascularity (score 30-40) while 18 (36%) cases had low MVD (≤ 30). The mean count was 34.24. Grade I response was seen in 26 (52%) cases, Grade II in 10 (20%), Grade III in 6 (12%) cases and complete response in 8 (16%) patients. Local recurrences were seen in 10 cases with 8 out of 10 cases having high MVD and 2 with intermediate MVD. 80% of the local recurrences occurred in the patients that showed complete or partial response following chemotherapy and they had high MVD. When comparing the local recurrence with high MVD the results were statistically significant (p=0.020). Ten cases developed metastasis, 8 (80%) cases had high MVD, while 2 cases had intermediate MVD. When comparing the metastasis with high MVD the results were statistically significant (p=0.003). Patient having high MVD had higher recurrence and poorer chemotherapeutic response in comparison to patients having low or intermediate MVD.
Keywords: *Breast cancer, micro-vessel density, immunohistochemistry, neoadjuvant chemotherapy*

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INTRODUCTION

Malignant tumors are characterized by the development of new and autonomous blood vessels by neoangiogenesis, which is recognized as a distinct sign of malignancy. Neoangiogenesisis is a significant indicator of biological activity of the tumor, hence, its assessment not only indicates primary tumor volume but also the change in vascularization can provide a precise evidence of tumor response to neoadjuvant chemotherapy [1].

There is ample literature in support of the correlation between neoplastic vascularization with tumor growth as well as the rate of metastatic dissemination of the disease [2,3]. However, the ability to exploit tumor angiogenesis as a prognostic marker is limited by the methods currently used for capillary identification and quantization [2].

Response assessment may also help to identify a subgroup of women in whom intensification of treatment might be expected to treat micro metastases more effectively [4]. This will help in assessing the extent of down staging of tumor which will identify the subset of patients in whom conservative, less mutilating surgery can be performed without increasing local recurrence [5]. Response assessment can be used as a surrogate parameter of treatment efficacy and help in early termination of inefficacious regimen [6].

Modalities of response assessment can be in vivo or in vitro. Both the methods have been variously used and shown promising results. The importance of histopathological response assessment following

neoadjuvant and adjuvant chemotherapy is one of the most important prognostic indicator for locally advanced breast cancer and responders have shown to have significantly longer survival than non-responders [5, 6].

Angiogenesis grade seems to provide an independent prognostic value when the MVD was properly assessed [7]. The evaluation of tumor angiogenesis as a prognostic indicator in terms of MVD might be introduced to the category of the criteria for determining the schedule of postoperative adjuvant therapy of breast cancer.

The microvessel density also called angiogenic index, alone is reported to be responsible for the association between tumor size and grade, the occurrence of lymph node metastasis, and ultimately early death in patients with breast cancer [8]. CD31 (PECAM1) is known to be a suitable marker for identification of angiogenic blood vessels in many tissues, including breast cancer and is used as such in the pathological analysis of breast cancer [9]. As cancer cannot grow or spread without the formation of new blood vessels, scientists are trying to find out ways to stop angiogenesis. Fox et al, reported that the ability to exploit tumor angiogenesis as a prognostic marker is limited by the methods currently used for capillary identification and quantization [10]. They evaluated all aspects of the techniques and their associated problems for assessing tumor angiogenesis in tissue sections including the area of tumor assessed, the vascular parameter measured, the method of quantization, the stratification of patients and the practical utility of computer image analysis systems.

MATERIAL AND METHODS

This is a prospective study undertaken on 50 patients of advanced breast carcinoma, who received neoadjuvant chemotherapy. Informed consent was taken from all the patients included. The study was conducted in one surgical unit of the Department of General Surgery, Institute of Medical Sciences.

Histologically proven advanced breast cancer (Stage IIIa, IIb, and IV) was included in the study. Biopsy was taken from tumor and surrounding breast tissue using a 16 G tru cut needle. Tissue specimen obtained was approximately 17 mm x 1 mm; 3-6 such pieces of tissues were collected.

All the patients received the following chemotherapy schedule: Cyclophosphamide 500mg/m^2, Adriamycin 50mg/m^2, 5-Flurouracil 500mg/m^2. Chemotherapy was given as 21 days cycle and minimum of two such cycles were given. Following chemotherapy the responders underwent simple mastectomy and axillary clearance. The histopathological details were studied in the mastectomy specimens with reference to changes in vascularity.

Assessment of tumor vascularity was done using CD31 Assay in the biopsied specimen. Immunocytochemical assessment of the tumoral vascular density was done using Monoclonal anti CD31 antibodies (PECAM-1), clone 1A10 (Novocastra Laboratories Ltd, UK). The lyophilized antibody was reconstituted using 0.1 ml sterile distilled water for the experiment. It was stored for at 4^0 Celsius. The working dilution for the primary antibody used was 1:50. The secondary antibody used was Biotinylated Goat Anti-Polyvalent anti body having specificity to Anti-Mouse IgG (H+L), Anti-Rabbit IgG (H+L). The enzyme used was biotin and the method used was as per the recommendation of the manufacturer. This technique involves the sequential incubation of the specimen with an unconjugated primary antibody, enzyme-labeled streptavidin, and substrate chromogen.

Before staining was undertaken, the procedure described was standardized to prove the reproducibility of the technique. The stained slides were first scanned for vascular “hot spots” in the low magnification and the areas showing rich vascularity were selected for the count. Endothelial cell in each high power field (400 X) were counted and the highest of all was taken as the score for tumor vascular density. The reduction in vascularity in cases following chemotherapy was then analyzed.

Single experienced pathologist who was unaware of the clinical status or the chemotherapy cycle did the MAG score. Blinding was done to eliminate intra-observer variability. Few cases were randomly selected and reanalyzed by the same pathologist. To quantify the response and compare results, the CD31 assay score was graded as: <30 – low vascularity, 30-40 – moderate vascularity and >40 – high vascularity. The treatment response was assessed by RECIST 1.1 criteria [11]: complete response, partial response, progressive disease and stable disease.

All the patients were followed up following completion of the chemotherapy regularly at 3 monthly intervals. There was no case that lost to follow up. The end point of the study was 2 years. The clinical and radiological assessment was made for recurrences every 3 monthly. The various clinicopathological parameters were correlated to vascularity assessed by CD 31.

Statistical analysis:

Statistical analysis was performed using statistical package for the social sciences (SPSS), Version 23.0. IBM Corp., NY). Simple descriptive statistics were used (mean ± standard deviation for quantitative

variables, and frequency with percentage distribution for categorized variables).The data was analyzed using Chi square test and Fisher's exact test. ANOVA test was used for comparing more than two groups of mean. P-value <0.05 is considered as statistically significant association.

RESULTS

The clinico-pathological characteristics of the patients are described in Table I.

Table I: Clinico-pathological characteristics (n=50)

Characteristics	Number
Age (years)	
30-40	13 (26.0)
41-50	24 (48.0)
51-60	9 (18.0)
>60	4 (8.0)
Symptoms at presentation	
Lump	49 (98.0)
Pain	25 (50.0)
Lump axilla	11(22.0)
Nipple discharge	9 (18.0)
Duration of symptoms (months)	
<5	33 (66.0)
6-12	14 (28.0)
>12	3 (6.0)
Menopausal status	
Premenopausal	14 (28.0)
Postmenopausal	36 (72.0)
Parity	
Nulliparous	0
One children	8 (16.0)
Two children	26 (52.0)
>Three children	16 (32.0)
Tumor size (cm)	
<5	8 (16.0)
≥5	42 (84.0)
Axillary lymph nodes	
Palpable	43 (86.0)
Not Palpable	7 (14.0)
Stage	
Stage IIIa	14(28.0)
Stage IIIb	26 (52.0)
Stage IV	10(20.0)
Histological grade	
Grade I	2 (4.0)
Grade II	32 (64.0)
Grade III	16 (32.0)

Majority of cases were in the age group of 30-50 years. The mean age of the patients was 45.76±17.22 years. Majority (72%) of the cases were postmenopausal. Pre chemotherapy histology showed ductal carcinoma in 48 (96%) cases and small cell carcinoma in 2 (4%) cases. Eight (16%) cases had tumor size less than 5 centimeters. Thirty four (68%) cases had the tumor that involved the skin in the form of fixity or ulceration. There were 4 (8%) cases of tumors fixed to chest wall with skin infiltration. Another 4 (8%) cases had inflammatory breast cancer. There were 10 (20%) cases of metastatic disease. All these cases had pulmonary metastasis with skeletal metastasis present in one. Eighteen (36%) cases showed high MVD with score more than 40. Fourteen (28%) cases had intermediate vascularity with score ranging from 30-40 while 18 (36%) cases had low MVD that was less than 30. The mean count was 34.24 (Table II).

Of the 10 cases that had metastatic disease, 8 (80%) had high MVD, while 2 cases had intermediate MVD. During follow up 10 local recurrences were seen. Of these cases, 6 (60%) cases had high MVD and 4 cases

had intermediate MVD. The response following chemotherapy was evaluated using RECIST 1.1 criteria. Complete response was seen in 23 (46%) cases following first cycle of chemotherapy. Seventeen (34%) cases showed partial response and in 8 (16%) cases had progressive disease and 2 (4%) cases showed stable disease (Table III). The mean MVD score was significantly higher in progressive as well as stable disease and vice versa with 80% of the local recurrences occurring in the patients that showed complete or partial response following chemotherapy (p<0.001).

Out of total 50 cases, 10 cases had local recurrence in the form of a palpable subcutaneous nodule at the operative site. Recurrences were noted after the patients had completed the treatment, in a follow up period of 2 years during the course of study. Recurrences were more in cases with high pre chemotherapy vascularity shown by high CD 31 score. When comparing the local recurrence with MVD assessed using CD31 assay the results were statistically significant (p=0.020) (Table IV).

Metastatic disease at presentation was seen in 10 patients. Out of these 80% showed high vascularity and none showed low vascularity. In high vascularity group with MVD of >30 metastatic disease was seen in 44.4% tumors while those with intermediate vascularity 14.3% had metastatic disease. The MVD assessed using CD31 assay showed a statistical significance for the presence of metastasis (p=0.003) (Table V).

Table II: Distribution of cases according to MVD assessed by CD31 assay pre-chemotherapy

Pre CT MVD (CD31)	Number	Percentage
<30	18	36.0
30-40	14	28.0
>40	18	36.0

Table III: Comparison of Chemotherapy response with MVD

Grade of Response	Number (%)	MVD Score (Mean)	ANOVA test (p value)
Complete clinical response	23 (46)	23.74±6.23	<0.001
Partial response	17 (34)	28.56±7.73	
Progressive disease	8 (16)	37.89±9.84	
Stable disease	2 (4)	38.96±10.21	

Table IV: Comparison of local recurrence with MVD

CD31 assay	No recurrence (n=40)	Recurrence present (n=10)
<30	18 (45.0%)	0
30-40	10 (25.0%)	4 (40.0%)
>40	12 (30.0%)	6 (60.0%)

X^2=7.812, d.f.=2, p=0.020

Table V: Comparing the presence of metastasis with MVD

CD31 assay	No metastasis (n=40)	Metastasis present (n=10)
<30	18 (45.0%)	0
30-40	12 (30.0%)	2 (20.0%)
>40	10 (25.0%)	8 (80.0%)

X^2=11.51, df=2, p=0.003

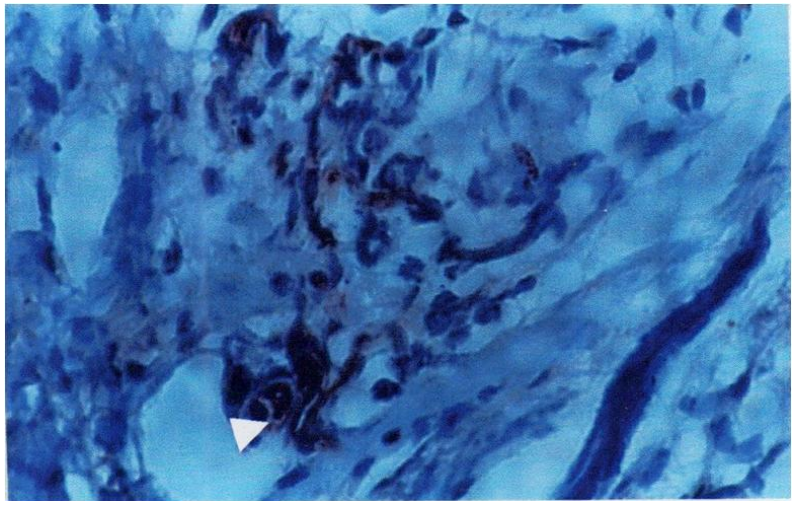

Fig 1. Photomicrograph showing high MVD (56) (400 X)(Arrowhead indicating the endothelial cells stained with CD31)

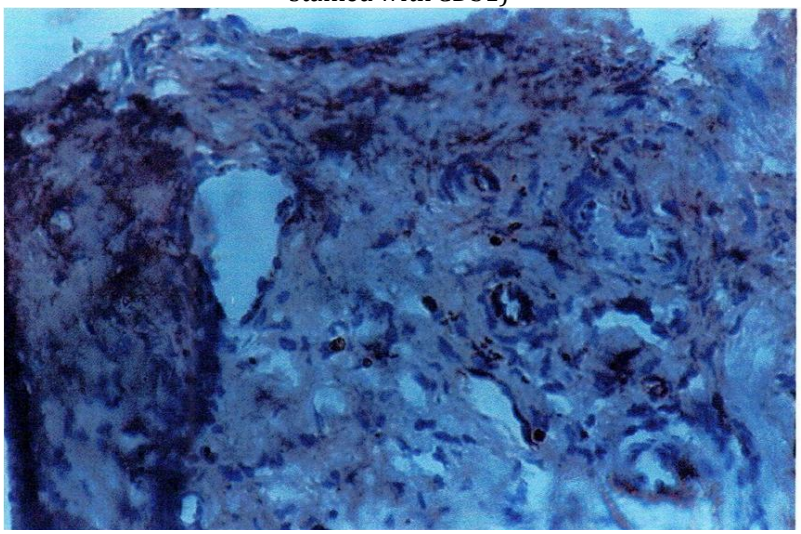

Fig 2. Photomicrograph of another patients with high MVD (52) with intensely stained endothelial cells with CD31 (200 X)

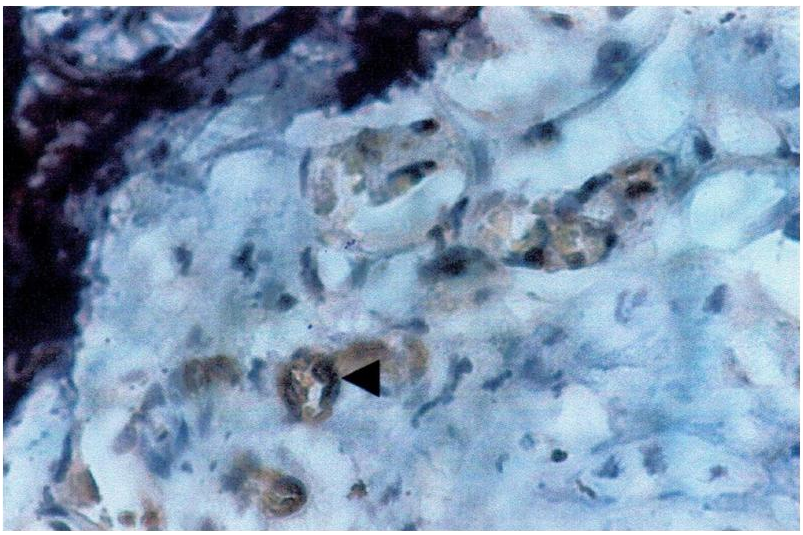

Fig 3. Photomicrograph of another patient with high MVD (48) (400X) (Arrowhead showing capillary lined with endothelial cell stained with CD31)

DISCUSSION

The real importance of blood microvessel density is still controversial. Most of the available data have some degree of discrepancy related to the significant correlation between high MVD and poor breast cancer prognosis. Several experimental and clinical studies in the past have shown that the development of blood borne metastasis is directly related to mortality [12-13]. Stromal tumor angiogenesis favors tumor growth and facilitates the entrance of the tumor cells into circulation. These metastases are dependent on the entry of tumor cells in the circulation that is directly related to the tumor neoagiogenesis [10,14-17]. Thus the role of assessing tumor vascularity cannot be better emphasized than to say that it is directly related to progression of disease, occurrence of metastasis and predicting survival [18]. Impressive promising data emerged with Folkman's findings, suggesting the MVD assessment as an independent predictor of metastatic disease either in axillary lymph nodes or at distant sites or even both [19]. Therefore the evaluation of breast cancer MVD was assumed to suspect patients with early breast cancer for aggressive therapy.

Data emerged from studies that highlighted the blood MVD as a prognostic factor to breast cancer was initially accepted as a powerful parameter to identify the more aggressive phenotypes of breast cancer [20]. However, these initial results were not confirmed and different findings obligate the revision of primary concepts. Presently the assessment of MVD by the blood and lymphatic markers is credited to be a significant unfavorable prognostic factor for long term survival in breast cancer besides being a likely therapeutic target for anti angiogenic therapy.

In principle, the angiogenic status of a tumor can be assessed by three different approaches: in vivo techniques (Immunohistochemical), indirectly by clinical chemistry based measurement of circulating biomarkers, and in vitro non-invasive imaging techniques.

The Immunocytochemical assays using anti factor VIII [21], Anti CD31/PECAM-1 [22], or Anti CD34 [23], tumor vessels can be counted in tissues sections. Angiogenesis index has been implicated in several solid tumors, the results of which show close relation of angiogenesis with the disease progression and outcome (local recurrence/metastasis). Xianghua et al [24] investigated the involvement of angiogenesis and angiogenic factors in the pathogenesis of renal cell carcinoma (RCC). The situations of tumor angiogenesis were evaluated by assessing micro vessel density (MVD) through CD31 immunostaining. The expression of vascular endothelial growth factor (VEGF), matrix metalloproteinase-2 (MMP-2) and metalloproteinase-9 (MMP-9) was detected immunohistochemically. They concluded that the degree of angiogenesis may be closely related to the tumor progression of RCC. The expression of VEGF may be responsible for angiogenesis in RCC, and both VEGF and MMP-2 expression may function as tumor associated angiogenic factors in RCC.

Cheng et al [25] studied natural and synthetic angiogenesis inhibitors, also called anti-angiogenesis agents, in the hope that these chemicals will prevent the growth of cancer by blocking the formation of new blood vessels. They concluded that there is histological, molecular and clinical evidence supporting angiogenic index as a useful 'in vivo' indicator of tumor angiogenesis, particularly for predicting lymph node metastases in cervical carcinomas. Tumor micro vessel density and tumor size were significant-independent predictors of overall survival [26]. Similarly, it has been shown that the micro vessel density of the primary tumor correlates with the pathologic stage and the presence of metastasis in patients with prostate carcinoma [27,28]. There are now data correlating micro vessel density with metastasis, recurrence, or mortality in other neoplastic disease such as colorectal carcinoma [29,30], non-small cell lung cancer [31,32], gastric carcinoma [33], squamous cell carcinoma of the head and neck [34], melanoma [35,36], testicular germ cell tumors [37], bladder cancer [38], ovarian carcinoma [39] and pediatric brain tumors [40].

In the present study high angiogenesis index in advanced breast carcinomas was associated with higher incidence of local recurrence and the presence of metastasis. Preliminary results show that there is a need for the development of wider multicentric studies to evaluate the role of angiogenesis in tumor kinetics and the development of strategies to effect its regression or elimination. There is a need to include the assessment of angiogenesis index for patients with advanced breast cancers to predict its metastatic potential and to predict outcome of treatment.

CONCLUSION

The process of tumor-associated angiogenesis is central to the growth and metastasis of malignancies. The process is complex and involves multiple steps and pathways with positive and negative signals. It is the local balance between these signals that determines whether a tumor will grow and spread or remain dormant. This process also involves interaction of tumor and endothelial cells with the surrounding tissue matrix. These processes provide a number of pathogenic steps that can be blocked or modified in an

effort to inhibit tumor-associated angiogenesis. Even if one is unable to eradicate, every tumor cell from the body, the ability to maintain tumor cells in a dormant state for years would represent a significant advance in cancer treatment. Efforts to develop more specific and more potent agents, and studies to address how to optimize use of these compound continue. Therapies affecting an end target or pathway that cannot be circumvented by alterante mechanisms may significantly enhance efficacy and broaded applicability. Newer, convenient, and reproducible methodologies for determining the biological activity of these agents is an area of future research. Although a great deal of work is required, antiangiogenic therapy may provide an additional novel cancer treatment suitable for combination with standard therapies.

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