Value of endothelial markers in accurate assessment of microvessel density (MVD) and thus angiogenesis in invasive ductal breast carcinoma

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Abstract- Background: The prognosis of breast cancer is dependent on several clinicopathological factors and molecular markers. Among these, angiogenesis is required for growth, invasion and metastasis of tumor. Microvessel density (MVD) assessment aids to quantify angiogenesis which was measured by Haematoxylin and eosin (H&E), Vascular Endothelial Growth Factor (VEGF) and CD31 in the present study, with an objective to find the best stain among the three.

   Material and Methods: The study included forty seven proven invasive ductal breast carcinoma cases. Manual Tissue Microarray (TMA) prepared slides were stained with H&E, VEGF and CD31. MVD were quantified according to Weidner's method and the results were compared with different grades of breast carcinoma and between the peritumoral (PT) and intratumoral (IT) areas.

   Results: The mean MVD for H&E, VEGF and CD31 were 4.1±2.7, 4.5±2.7 and 18.35±7.4, 95% CI [15.1, 20.1] respectively. Statistically the CD31 stained tumor had highest MVD, which also showed increasing MVD counts with increasing grades (p<0.001) with a significant difference between grade 1 & 3, and 2 & 3(p<0.001) indicating that high MVD is a predictor of poor prognosis. CD31 IT–MVD (11.3±5.7; 95% CI [9.1, 12.7]) was almost two folds higher than the PT–MVD (6.2±2.6; 95% CI [5.2, 6.9]). CD31 specifically attaches to the glycoprotein in the vascular endothelial cells that helped in the recognition of the proliferating immature vessels in the tumor centre and mature PT vessels accounting for overall highest MVD, while H&E and VEGF recognized only the mature PT vessels.

   Conclusion: We conclude that CD31 recognises both immature and mature vessels by specifically attaching to the glycoprotein in the vascular endothelial cells, thereby measures accurate MVD thus angiogenesis in invasive ductal breast carcinoma.

Index Terms- CD31, Breast carcinoma, MVD, angiogenesis, peritumoral, intratumoral

I. INTRODUCTION

Breast cancer is one of the leading causes of death in women. Clinical and pathological parameters like the stage, grade, angiogenesis and several molecular biological criteria have prognostic significance. Among these prognostic markers, the role of angiogenesis and the degree of maturity of microvessel are of great interest [1].

   Angiogenesis is the development of new blood vessels from the existing blood vessels. It is evident that tumors have a limited capacity to grow without vascular supply which is obligatory to sustain the influx of essential nutrients to the growing cancer mass [2]. It is the fundamental requirement for the growth, invasion and metastasis of solid tumors [1]. Hence research on related factors such as an accurate assessment of microvessel density (MVD), which is used to quantify angiogenesis [3], can help predict tumor behaviour [4,5] and note the effect of antiangiogenic therapy [1].

   Quantification of MVD is done by identifying the blood vessels by their morphology on Haematoxylin and Eosin (H&E) sections or by staining the blood vessels with pan-endothelial markers including CD31, CD34, CD105, factor VIII or Vascular endothelial growth factor (VEGF) [6-8]. VEGF is one of the most prominent growth factor that controls both physiological and pathological angiogenesis. CD31 is a marker that selectively detects the glycoprotein of 130kDa [9] in vascular endothelial cells and thereby contributes to the assessment of vascularisation and consequently note the tissue vessel density. Correlation between the degree of angiogenesis, the proliferative activity of endothelial cells forming vessels and the MVD count is not completely understood [10].

   The conflicting results in several studies reflect the low specificity of various markers in accurate assessment of MVD and thus angiogenesis. In the present study the role of endothelial markers – VEGF and CD31 in accurate assessment of MVD and thus angiogenesis in invasive ductal breast carcinoma has been verified with an additional emphasis on noting the peritumoral (PT) and intratumoral (IT) MVD.

II. MATERIAL AND METHODS

   The study included forty seven cases of radical mastectomy specimens that were routinely processed and proven as carcinoma breast on H&E stained sections. Breast carcinomas were graded as grade 1 to 3 based on tubule formation, mitoses and nuclear pleomorphism according to Elston and Ellis

Modified Scarff Bloom Richardson grading system [11]. Institution ethical committee approval and individual patient's informed consent were obtained.

CD31 and VEGF IHC staining

The sections from all the 47 cases were included in construction of 3 manual tissue microarray (TMA) sections that were immunohistochemically (IHC) stained with VEGF and CD31. In brief procedure of IHC was: Endogenous peroxidase of formalin fixed paraffin embedded tissue sections were inhibited by incubating with 3% hydrogen peroxide for 5 minutes. Heat induced antigen retrieval was performed and sections were incubated for 5 to10 minutes at room temperature (RT) with blocking reagent. They were incubated with the primary antibodies – CD31 BC2 monoclonal antibody (Biocare medical, Concord CA) and VEGF (Biogenex, AR483 10R) for 30 minutes. Further they were incubated for 30 minutes with a secondary conjugated polymer. Subsequently a chromogen was added and were counterstained with haematoxylin, dehydrated with graded alcohol, cleared with xylene and mounted. For positive control capillary haemangioma was included.

Microvessel density scoring

The H&E, VEGF and CD31 IHC slides were studied to quantify the MVD. The blood vessels were identified based on their morphology in H&E stained sections, while cell membrane and cytoplasmic immunoreactivity of endothelial cells were considered as positive when stained with CD31 and VEGF respectively. The MVD was determined by two independent observers, the screening was done by hot spot method in which the slides were initially screened in low power (x10 objective lens, x 10 ocular lens) to identify the areas with the highest number of micro vessels [12]. The micro vessel count was then performed in these fields under high power (x40 objective lens, x10 ocular lens). The mean value of 10 most vascularised areas at x400 field was considered as MVD for the sample.

Statistical analysis

Statistical analysis by SPSS version 23.0 was calculated. According to the Kolmogorov-Smirnov test, H&E, VEGF and CD31 MVDs had a normal distribution. Statistically MVD obtained by H&E, VEGF and CD31 were assessed for linear association by Pearson's correlation coefficient between each other and by one-way analysis of variance (ANOVA) with the different grades of breast cancer. A p-value of less than 0.05 was considered as significant.

III.   RESULTS

Out of 47 cases of breast cancer 4.2% (2) were grade 1, 36.2% (17) were grade2 and 59.6% (28) were grade 3. The MVD ranges for all three stains were shown in Table 1. The mean MVD±SD for H&E, VEGF and CD31 were 4.1±2.7, 4.5±2.7 and 18.35±7.4, 95% CI [15.1, 20.1] respectively (Table 1). Pearson's correlation statistical analysis showed correlation of 0.769 (p<0.001) between H&E MVD and VEGF MVD, correlation of 0.390 (p=0.007) between VEGF MVD and CD31 MVD and correlation of 0.449 (p=0.002) between H&E MVD and CD31 MVD (Table 2).

There was a significant correlation between the CD31 MVD and grades with a p-value of <0.001. Post Hoc test by Bonferroni showed a significant difference between grade 1 & 3, and 2 & 3 with an overall p-value of <0.001 (Table 3).

PT (6.2±2.6, 95% CI [5.2, 6.9]) and IT (11.3±5.7, 95% CI [9.1, 12.7]) CD31 MVD correlation had statistically significance with a p-value of <0.001. H&E MVD (p<0.001) and VEGF MVD (p<0.001) showed significant correlation with PT CD31 MVD. The range and mean PT and IT CD31 MVD values in all three grades of breast carcinoma and their correlation analysed was shown in Tables 1 and 3.

DISCUSSION

Angiogenesis is a complex multistep process involving extracellular matrix remodelling, endothelial cell migration, proliferation, microvessel differentiation and anastamosis [13]. Reported data suggest that angiogenesis is an independent prognostic factor in several cancers [14]. Despite several studies there is no definitive angiogenic marker identified for an accurate MVD assessment and investigators have failed to explain the difference of PT and IT MVD.

MVD of a tissue is a reflection of the angiogenesis and in the present study on correlating the MVD counts obtained by three different stains, though there was a highly significant correlation between H&E and VEGF scores (0.769), the high CD31 MVD had low significant correlation with either the H&E (0.449) or VEGF (0.39) scores (Table 2). The mean MVD by CD31 was four folds higher than the H&E and VEGF implicating that CD31 was able to identifying all tumor blood vessels thus a better stain of the three, with respect to estimation of angiogenesis in breast carcinoma.

In present study H&E stained sections, the endothelial markers VEGF and CD31 showed increasing MVD counts with increasing grades independently indicating that high MVD is a predictor of poor prognosis (Table 1). These findings are similar to that of Gunasundari and Bhaskar [15] who have reported that there is a significant difference in the mean values of MVD of the various groups defined by Bloom Richardson Grading system. On analysis only CD31 MVD (p<0.001) had statistically significant scores compared to H&E MVD (p=0.225) and VEGF MVD (p=0.769) when correlated with grades reinforcing that CD31 MVD is remarkable. The CD31 MVD in grade3 breast carcinoma (22.7±5.3) was higher than grade2 (12.5±4.7) and was approximately four folds higher than in grade1 (6.5±2.6), (Figure 2). The differences in angiogenic activity were statistically significant between grade2&3 and grade1&3 hence reaffirming that CD31 MVD is a good prognosticator as compared with the other two stains (p<0.001), (Table 3).

Further correlating the MVD counts in the PT and IT areas, there was no significant difference noted on H&E and VEGF stains. While there was a significant difference noted with the CD31 stain (p<0.001), and this is similar to the findings of Marwah N et al. [6]. The IT MVD (11.3±5.7, 95% CI [9.1, 12.7]) was almost two folds higher than the PT MVD (6.2±2.6, 95% CI [5.2, 6.9], Figure 1c). The high IT MVD suggests that there is relative hypoxia in the central areas of breast carcinoma which would stimulate the expression of HIF-1alpha and therefore up regulate a number of downstream angiogenic factors like VEGF, MMP2 etc secreted by both tumor cells and stromal cells [16]. It

was also noted that MVD counts by H&E (p<0.001) and VEGF (p<0.001) stains correlated with the PT CD31 MVD indicating that these stains have identified predominantly peritumoral blood vessels. This difference in the MVD counts in the PT and IT areas is similar to the finding of Margaritescu C et al. [3], Marwah N et al. [6], Schimming and Marmé [17], which is predominantly due to high neoangiogenesis in the tumor centre (Table 1). Though Avdalyan A et al. [1] have recorded a substantial spread of mean values; a study on MVD counts in leiomyosarcoma has shown association of PT MVD with survival, hence an independent prognostic indicator. While Eshghyar N et al. [4] had shown a significant association of IT MVD with the lymph node metastasis.

Neoangiogenesis involves sprouting of endothelial cells that initially form immature vessels that are irregular, tortuous and lack organization [18]. The unlimited tumor growth and therefore the continuous stimulation of blood vessels prevent the endothelial cells from generating a mature vasculature but expand the vascular compartment. Neoangiogenesis is thus a natural consequence in the rapidly growing tumor centre.

In H&E stained sections, blood vessels were identified by their morphology which showed a complete lumen lined by endothelial cells and the presence of RBCs within (Figure 1a). The IT immature vessels lack organization of endothelial cells and failed to be recognized with H&E stain. The lowest H&E MVD of 4.1 ± 2.7 thus recognises only the mature vessels which are predominantly seen in the PT area and fails to recognise the IT immature neovascular vessels.

VEGF was expressed by both the endothelial cells and tumor cells and thus the diffuse staining of the tissue obscures the endothelial cell and fails to recognise the immature vessels in the proliferating tumor centres (Figure1b). Therefore VEGF though a potent angiogenic marker, is not specific to the endothelial cells. This explains the lower VEGF MVD of 4.5 ± 2.7 which is close to that observed by H&E stain. Hence VEGF stain has no advantage over H&E stain with respect to MVD assessment.

In present study the mean CD31 MVD has the highest value of 18.35 ± 7.4, 95% CI [15.1, 20.1] and found to stain both the mature and immature blood vessels (Figure1d). CD31 specifically attaches to the glycoprotein in the vascular endothelial cells that helps recognition of the immature vessels also in the proliferating tumor centre and accounts for the highest IT MVD counts. High vessel density, cell immaturity and structurally abnormal vessels within the tumor tissue have also been cited by Holleman D et al. [19] and Avdalyan A et al. [1].

The variability in the MVD assessment between our study and other authors could be attributed to differences in the patient population studied, methods of MVD assessment and different endothelial markers used such as CD34, CD105 and factor VIII (Table 4), [20-28]. The main limitation of our study was inability to follow-up the patients, hence could not correlate with survival statistics.

Folkman J [29] has proposed that the growth of a tumor and its metastasis are dependent on angiogenesis and hence blocking angiogenesis could serve as a strategic way of arresting tumor growth, hence accurate assessment of MVD is of prime practical importance for today's clinical repertoire, which can be obtained easily by CD31 staining. Eshghyar N et al. has

suggested that patient with higher MVD are candidates for additional therapy with antiangiogenic drugs and follow up [4].
We conclude that CD31 which is expressed by both immature and mature vessels is more appropriate for an accurate assessment of MVD a measure of neoangiogenesis and thereby prognosis in invasive ductal breast carcinoma. A further study with image analysis is essential to eliminate intra and inter observer variability in MVD assessment and the implicit role of angiogenesis in carcinoma breast needs further substantiation.
All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional ethical committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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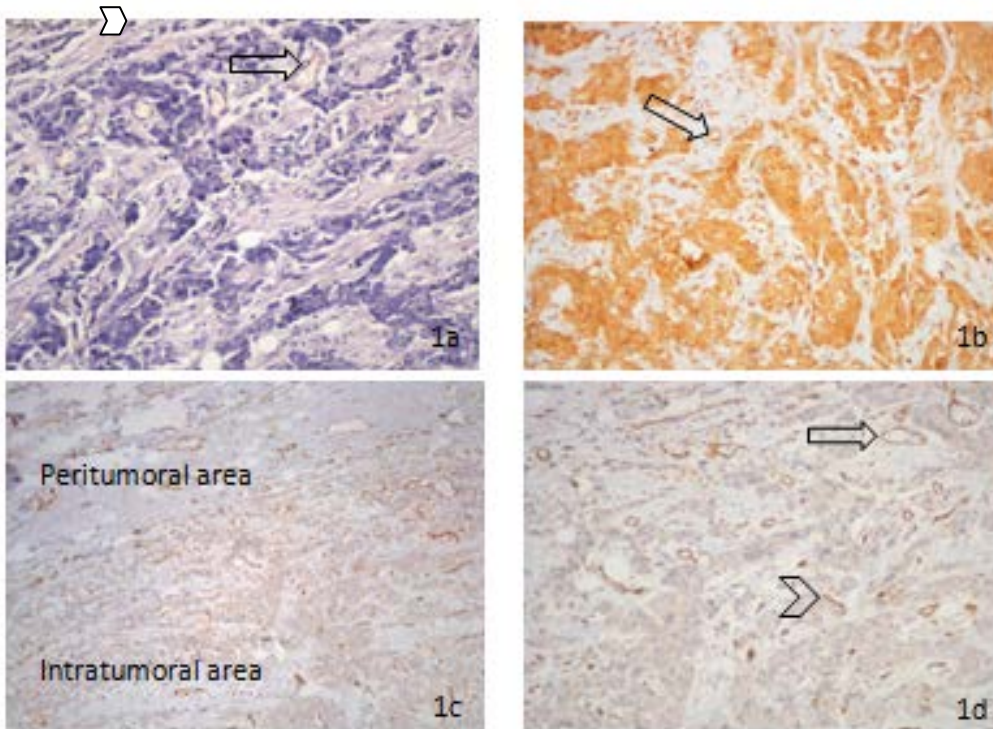
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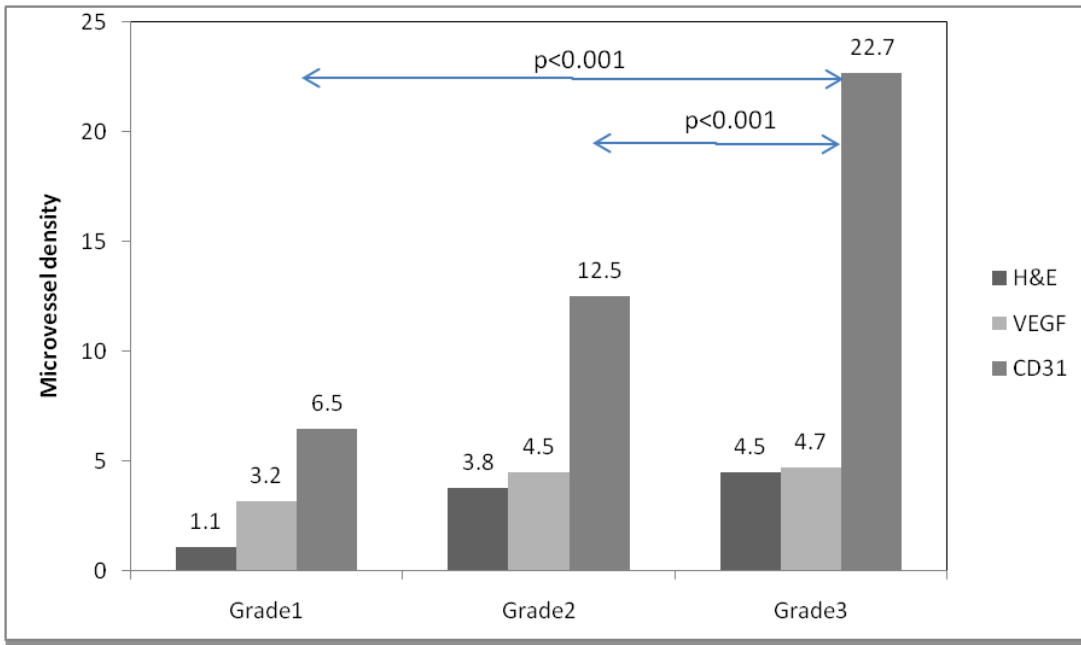Contact No - +91 9448608149

Figure 1. Identifying the blood vessels (⟹) (a) H&E 10X A complete lumen lined by endothelial cells and the presence of Red Blood Cells within, (b) VEGF 10X staining of both endothelial cells and tumor cells, (c) CD31 4X showing higher IT MVD compared to PT MVD, (d) CD31 10X stain both the mature ( ) and immature ( ) blood vessels.

H&E – Haematoxylin and Eosin; VEGF – Vascular Endothelial Growth Factor;

PT – Peritumoral; IT – Intratumoral; MVD – Microvessel density

Figure 2. The microvessel density scores of the three different grades of breast carcinoma estimated by H&E, VEGF and CD31 stains and correlation between the three grades of CD31 microvessel density

H&E – Haematoxylin and Eosin; VEGF – Vascular Endothelial Growth Factor; MVD – Microvessel

Table1. The range and mean microvessel density of three different stains, peritumoral and intratumoral areas in different grades of breast carcinoma

	Grade 1	Grade 2	Grade 3	Total
H&E MVD range	0.6-1.6	0.4-9.2	0.4-12.6	0.4-12.6
H&E mean MVD±SD	1.1±0.7	3.8±2.4	4.5±2.9	4.1±2.7
VEGF MVD range	1.6-4.8	0.8-10.2	0.4-9.8	0.4-10.2
VEGF mean MVD±SD	3.2±2.2	4.5±2.9	4.7±2.6	4.5±2.6
CD31 MVD range	4.8-8.4	4.8-18	12-33.8	4.7-33.8
CD31 mean MVD±SD	6.5±2.6	12.5±4.7	22.7±5.3	18.3±4.7
PT CD31 MVD range	1.1-3.2	1.7-10	4.4-13.2	1.1-13.2
PT CD31 mean MVD±SD	2.1±1.4	4.5±2	7.5±2	6.2±2.6
IT CD31 MVD range	3.6-5.2	2.3-13.9	7.5-25.3	2.3-25.3
IT CD31 mean MVD± SD	4.4±1.1	7.5±3.5	14.2±5.1	11.3±5.7

H&E – Haematoxylin and Eosin; SD – Standard Deviation; VEGF – Vascular Endothelial Growth Factor;

PT – Peritumoral; IT – Intratumoral; MVD–Microvessel density

Table 2. Correlations between the three different stains in breast carcinoma

	H&E MVD	VEGF MVD	CD31 MVD
H&E MVD Pearson Correlation	1	0.769	0.449
Sig. (2 tailed)		0.000	0.002
VEGF MVD Pearson Correlation	0.769	1	0.39
Sig. (2 tailed)	0.000		0.007
H&E MVD Pearson Correlation	0.449	0.39	1
Sig. (2 tailed)	0.002	0.007	

H&E – Haematoxylin and Eosin; VEGF – Vascular Endothelial Growth Factor; MVD – Microvessel density

Table 3. Correlation of H&E, VEGF, CD31, peritumoral CD31 and intratumoral CD31 microvessel density with different grades of breast carcinoma

	H&E MVD	VEGF MVD	CD31 MVD	PT CD31 MVD	IT CD31 MVD
Grade 1 & 2	0.546	1.000	0.368	0.382	1.000
Grade 2 & 3	1.000	1.000	<0.001	<0.001	<0.001

Grade 1 & 3	0.293	1.000	<0.001	0.002	0.017
Total p-value	0.225	0.769	<0.001	<0.001	<0.001

H&E – Haematoxylin and Eosin; VEGF – Vascular Endothelial Growth Factor; PT – Peritumoral;

IT – Intratumoral; MVD – Microvessel density

Table 4. Comparison of CD31 microvessel density data of our study with other authors study outcome

Authors	CD31 MVD Mean	CD31 MVD range	CD31 MVD and grade
Rajesh L [20]	NA	NA	NA
Yilmazer D [21]	NA	NA	No correlation
Arihiro K [22]	NA	NA	No correlation
Comşa S [23]	NA	NA	p=0.019
Raica M [24]	31.68	9.8-60.2	NA
de la Taille A [25]	60.1	6-184	NA
Gehani KE [26]	80.29	NA	p=0.0001
Choi WWL [27]	29.2	12.8–94.3	p=0.0107
Massi D [28]	17.37	NA	NA

| Our study | 18.3±4.7 | 4.7-33.8 | p<0.001 |

MVD – Microvessel density; NA – Not available