ORIGINAL RESEARCH PAPER

Pathology

EVALUATION OF MICROVASCULAR DENSITY AS A PROGNOSTIC INDICATOR IN BREAST CARCINOMA BY USING IMMUNOENDOTHELIAL MARKER CD-34

KEY WORDS: Angiogenesis, CD 34,Micro vascular density(MVD).

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ABSTRACT

Breast Carcinoma (BC) is the most frequent neoplasm causing death in women between 35-55 years of age .Out of the existing prognostic indicators axillary lymph node status is the most important one. But 20-30% lymphnode negative patients develop a recurrence within 10 years of initial treatment .One such potential indicator is Micro vascular density(MVD) as it has some crucial role in tumour cell survival. With this back ground the present study was undertaken to study the MVD in breast carcinoma as a potent prognostic indicator.

MATERIALS & METHODS: The present study is conducted in the department of Pathology S.C.B Medical College, Cuttack, Odisha from August 2013 to July 2015 taking all histopathologically confirmed cases of breast carcinoma. The clinical details , presence of axillary lymphadenopathy,HP grading ,staging and Nottingham Prognostic Index (NPI) were recorded .Each case was subjected to IHC for CD 34 to quantitatively measure the number of microvessels (MVS) in tissue section.

RESULTS: - This study included 53 cases in the age group of 27-80 years with a mean age of 46.6 years. Of this 49(92%) were infiltrating adenocarcima not otherwise specified,01(2%) were papillary carcinoma ,02(4%) were lobular carcinoma and 01(2%) was mucinous carcinoma .28(56%) cases show micro vascular invasion and 45 (90%) cases have more than 200 micro vassels /10 HPF. MVD correlated significantly with age, tumour size, tumour grade LVI,NPI,LN stage and TNM stage.p(<0.05).

CONCLUSION :- MVD can be regarded as a potential prognostic indicator of breast carcinoma.

INTRODUCTION :-

Carcinoma of breast (BC) is the most frequent malignancy in women worldwide. Finding out valuable means for its evaluation has always been a challenge for the medical science. Many prognostic indicators like tumour size, age, histopathological (HP) grade, axillary lymphadenopathy, TNM staging, Nottinghams Prognostic Indicator (NPI), lympho vascular invasion (LVI) are of great value for breast carcinoma.[1] Out of all these lymph node (LN) metastasis is the most important one. It is seen that 20-30% of LN negative patients show recurrence within 10 yrs of initial treatment.

Angiogenesis is the growth and formation of new blood vessels from the existing one. Tumour angiogenesis is clinically important as a prognostic and predictive marker for targeted therapy. Hence tumour angiogenesis or microvasculardensity (MVD) can be used as a potent prognostic indicator. Count of MVD using CD34 immuno-stain is the most easy and widely used method.[1,2] Studies showed that CD34 and CD31 are better endothelial marker than VWF. Out of CD31 and CD34, CD31 is more sensitive and superior on paraffin sections, but cross react with plasma cells and markedly obscure the micro vessels in sections. So in the present study CD34 was taken as endothelial immune marker. The number of micro vessels within a tumour will provide an estimate of the angiogenic potential of tumour cells and thus the probability of tumour growth, invasion and metastasis . CD34 is an 115KDa single chain trans- membrane glycoprotein present on surface of all endothelial cells. With the development of highly specific endothelial markers that can be assessed in histological archival specimens, several quantitative studies have been performed in various solid tumours.

With this back ground the present study was undertaken to study the MVD in breast carcinoma as a potent prognostic indicator & to detect node negative patient of high grade category. The present study includes MVD count and it's correlation with clinicopathological parameters to establish any relations amongest them.

MATERIALS & METHODS :-

This is a prospective study conducted in the department of Pathology S.C.B .Medical College, Cuttack, from August 2013 to July 2015. The present study was approved by the institutional Ethical Committee, S.C.B. Medical college , Cuttack . Written informed consent from the patient, the MRM specimen histopathologically confirmed as breast carcinoma, were selected for the study as per the inclusion and exclusion criteria.

Serial number of the MRM specimen, name, age, sex **,** OPD/IPD number**,** clinical presentation, clinical diagnosis**,** side of specimen**,** location of tumour**,** size of tumour **,** involvevement of axillary lymphnodes**,** histopathological diagnosis**,** BR grading**,** LVI**,** lymph node staging**,** NPI**,**TNM staging were noted in the master chart spreaded over the excel sheet.

After the data entered in master chart ,tissue processing, paraffin embedded block formation, section cutting, fixation on slide, H and E staining and DPX mounting was done. Tumour typing and grading were done under microscope. Paraffin blocks which were most representative of tumour tissue were chosen for performing immunohistochemistry. It was done for evaluation of Cd34.

Biogenex ready to use mouse monoclonal antibodies were utilized for this purpose. CD 34 is an endothelial marker that stains the endothelial cells of vessels and micro vessels. Each case was subjected to IHC for CD 34 to quantitatively measure the number of MVs in tissue section. Under scanner view the area showing maximum number of MVs was considered as tumour "Hot spot" [1]. The number of micro vessels counting was done in ten different hot spots under high power objective of field diameter 0.44mm**.** A higher magnification improves the detail of the image and allows the identification of more single endothelial cells. Counting under of 200-400X with field size 0.12 to 1.00 mm is ideal. Any dark stained endothelial cell or cell clusters separated from adjacent structures were considered as a single vessel. Vessel with thick muscular wall and vessel containing >8 RBC in lumen were excluded from the count.

In this study we consider 10 high power fields as a unit area. The number of hot spots analysed should be 10 to reduce the chance

of missing the most vascular areas. Number of micro vessels per unit area is microvasculardensity (MVD) .In the present study MVs/10HPF was MVD with 10 HPF as a single unit area. For easy correlation they were divided into three groups. Those containing <200micro vessels/10HPF is low MVD. Those containing 200-270 micro vessels/10 HPF are medium MVD. Those containing >270 micro vessels/10HPF is high MVD.

Statistical analysis

The MVDs were correlated with the prognostic indicators like age, tumour size, lymph node stage, HP-grade, sex, LVI, TNM staging, and NPI. The P-values were calculated by putting the data in software IBM SPSS-15 version. The results were considered statistically significant, if P-value is ≤ 0.05.

OBSERVATIONS:-

Fifty three patients within 27-80 years of age with a mean age of 46.6 years were taken into study .Majority (60%) of patients were in the age group of 31 – 50 years. Among them 51 (96%) were females and 2(4%) were males. In 37(70%) cases right breast was involved and in 16(30%) cases the left was involved. Maximum number of tumours were detected in upper and outer quadrant (57%) followed by upper inner quadrant (13%),lower outer quadrant(11%), subareolar(11%), lower inner quadrant(3.5%) and diffuse pattern(3.5%).Forty nine patients (92%) were infiltrating duct carcinoma NOS,01(2%) were papillary carcinoma, 02(4%) were lobular carcinoma and 01(2%) was mucinous carcinoma. This study showed that 09(17%) cases were of size less than 2cm,22(41.5%) cases were 2 to 5 cm and 22(41.5%) cases were > 5cm.Mean tumour size was 5.51 cm with a SD of 3.383 cm .Regarding lymphnode metastasis, out of 53 cases, 18 cases (34%) showed no axillary lymphadenopathy, 9 cases (17%) had 1-3 number of axillary lymphnode(LN) metastasis and 26 cases had more than 3 LN metastasis .This study revealed twenty five cases (47%) had no lymphovascularinvasion (LVI) whereas 28 cases (53%) had LVI.In the present study 8 cases (15%) had NPI <3.4 with good prognosis ,17 cases (32%) had good to poor prognosis with NPI 3.4 -6.4 and 28 cases (53%) had poor to very poor prognosis with NPI >6.4. Eight cases (15%) were grade I tumour,27(51%) were grade II tumour and 18 cases (34%) were grade III tumour .Out of 53 cases, 8 cases (15%) had less than 200 micro vessels per 10 HPF ,21 cases (40%) had 200-270 micro vessels per 10 HPF and 24 cases (45%) had more than 270 micro vessels per 10 HPF (Table –1) .

(TABLE 1) DISTRIBUTION OF CASES ACCORDING TO NUMBER OF MVD

MVD value MVs/10 HPF	Number of cases	Percentage (%)
<200(low)	8	15%
200-270(Medium)	21	40%
>270	24	45%
Total No. of cases	53	100%

Mean 258.83 ± 48.2 (SD)

While correlating with age in the age group of 21-30 years we encounter 5 cases out of which 3 cases (60%) had medium MVD and 2 cases (40%) had high MVD. In age group of 31-40 years we noted 16 cases out of which 8 cases(50%) had medium MVD and 7 cases (43.75%) had high MVD .In age group of 41-50 years we found 16 cases out of which 5 cases (31.25%) had medium MVD and 9 cases (56.25%) had high MVD .In age group 51-60 years we had 9 cases out of which 3 cases (33.3%) had medium MVD and 3 cases (33.3%) had high MVD .In age group of 61-70 years we had 5 cases out of which none had medium MVD and 3 cases (60%) had high MVD . In age group of 71-80 years we found 2 cases out of which 1 cases (50%) had medium MVD and 1 cases (50%) had high MVD .Thus it was seen that in each group the combined value of medium MVD and high MVD is > 60% and it is having a significant correlation (P value =0.017). (Table -2).

(TABLE-2) CORRELATION OF AGE WITH MVD

Age group In years	Number of cases	MVD		
		<200	200-270	>270
21-30	5	0	3(60%)	2(40%)
31-40	16	1(6.25%)	8(50%)	7(43.75%0
41-50	16	2(12.5%)	5(31.25%)	9(56.25%)
51-60	9	3(33.3%)	3(33.3%)	3(33.3%)
61-70	5	2(40%)	0%	3(60%)
71-80	2	0(0%)	1(50%)	1(50%)

P-Value – 0.017, significant correlation .

When MVD is compared with tumour size it is noticed that all 8 cases of low MVD were small tumour <2 cm,(T_1). Maximum number of medium MVD,16 out of 21 was medium size of 2-5 cm. (T_2).Maximum number of high MVD ,20 out of 24 were larger size of > 5cm,(T_3) . Thus it is found that as size of the tumour increased microvasculardensity also increased and this is significant, (P=0.025) (Table-3).

TABLE -3 CORRELATION OF MVD WITH TUMOUR SIZE

MVD	Number of cases	Tumour size		
		T (≤2cm)	T2(2-5cm)	T3(>5cm)
<200 (low)	08	08(100%)	0	0
200-270(Medium)	21	01(4.76%)	16(76.19%)	04(19.04%)
>270 (High)	24	0	04(16.66%)	20(83.34%)

P Value – 0.025, significant correlation.

When MVD is correlated with histopathological grade of tumour it is found that out of 8 cases of low MVD, seven cases (87.5%) were grade 1 tumour and 1 case (12.5%) had LVI. Out of 21 cases of medium MVD ,twenty cases (95.25%) were grade II tumour and 6 cases (57%) had LVI .Out of 24 cases of high MVD ,eighteen cases (75%) were grade III tumour and 21 cases (87.5%) had LVI .Thus it is clearly seen that as tumour grade increased ,the MVD also increased and as MVD increased LVI also increased proportionately (P=0.444) (Table -4).

Table -4 CORRELATION OF MVD WITH HISTOPATHOLOGICAL GRADE OF TUMOURS

MVD	No.of cases	Histopathological Grade			LVI
		Grade-I	Grade-II	Grade-III	
<200 (Low)	08	07(87.5%)	01(12.5%)	0	01(12.5%)
200-270 (Medium)	21	01(4.75%)	20(95.25 %)	0	06(28.57 %)
>270 (High)	24	0	06(25%)	18(75%)	21(87.5%)

P-Value — 0.044, significant correlation

It was also observed that out of 8 cases of low MVD ,7 cases (87.5%) were in stage 1,LN-0 (no lymphnode metastasis) and one case(12.5%) was in stage 2(1-3 LN metastasis) .Out of 21 cases of medium MVD, 8cases (38%) were in stage-1, LN-0 (no LN metastasis) ,7 cases (33%) were in stage 2 (1-3 LN metastasis) and 6 cases (29%) were in stage 3(>4 LN metastasis). Out of 24 cases of high MVD ,3 cases (12.5%) were in stage 1(no LN metastasis),4 cases (16.6%) were in stage 2 (1-3 LN metastasis) and 17 cases (70.83%) were in stage 3.(>4 LN metastasis). 8 cases (38%)of lymphnode negative patients showed medium MVD and 3 case (12.5%) of LN negative patients showed high MVD explained that some high stage patients showed high MVD without lymphadenopathy. These patients showed recurrence after few years of initial treatment due to in adequate treatment as per low stage patients. Thus correlation of MVD with LN staging is significant (P=0.018) (Table -5).

Table 5 CORRELATION OF MVD WITH LYMPH NODE STAGING.

MVD	No.of cases	Lymph Node Stage		
		Stage-1N0 (node- 0)	Stage-2 N1 (node 1-3)	Stage-3 N2 (node 4-9) and N3 (node >9)
<200 (Low)	8	7 (87.5%)	1 (12.5%)	0
200-270 (Medium)	21	8 (38%)	7 (33%)	6 (29%)
>270 (High)	24	3 (12.5%)	4 (16.66%)	17(70.83%)

P – Value—0.018, Significant correlation.

When MVD is correlated with NPI it is found that all 8 cases of low MVD had NPI < 3.4 with good prognosis. All 21 cases of medium MVD had NPI 3.4 to 6.4 (good to poor prognosis) . Out of 24 cases (29.17%)7 cases (29%) had NPI 3.4 to 6.4 (good to poor prognosis) and 17 cases(70.83%) had NPI >6.4 means poor to very poor prognosis This implies that as MVD increased the prognosis goes down,(P=0.001-significant)(Table 6).

Table-6 CORRELATION OF MVD WITH NPI

MVD	No.of cases	NPI		
		≤3.4	≤6.4	>6.4
<200(Low)	8	8(100%)	0	0
200-270 (Medium)	21	0	21 (100%)	0
>270 (High)	24	0	07 (29.17%)	17 (70.83%)

P – Value—0.011, Significant correlation.

When MVD is correlated with TNM staging we found that all 8 cases of MVD were in stage I. Out of 21 cases of medium MVD, 18 cases (85.71%) were in stage II and 3 cases were in stage III. Out of 24 case of high MVD, 8 cases were in stage II and 18 cases (75%) were in stage III. It showed that as the stage of the tumour increased the MVD also increased with a significant correlation. (P=0.047). Finally we found that our study has a significant correlation with age ,tumour size, tumour grade, LVI,NPI,LN stage and TNM stage having P Value<0.05.

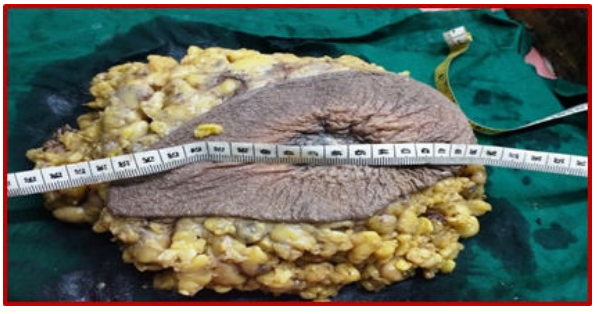
Table 7 CORRELATION OF MVD WITH TNM STAGING

MVD	No.of cases	TNM Stage		
		Stage-I (A+B)	Stage-II (A+B)	Stage-III (A+B)
<200(Low)	08	08(100%)	0	0
200-270 (Medium)	21	0	18(85.71%)	3 (14.29%)
>270High	24	0	08 (25%)	18 (75%)

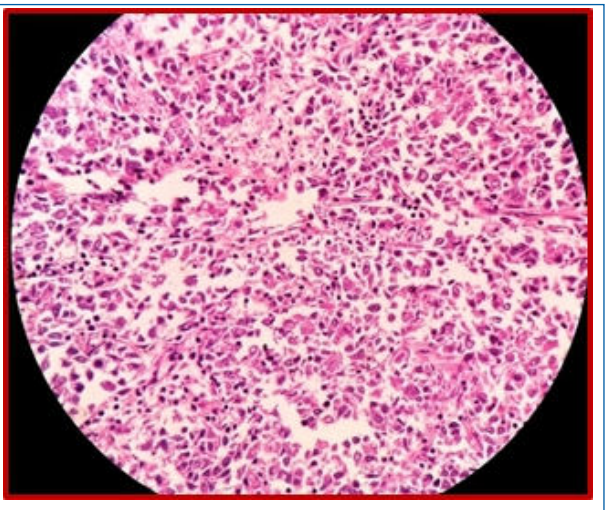
P-Value –0.047, significantly correlated

Gross, H and E Section,Tumour Hot spots and Micro vessel count of Breast Cancer specimen are shown in following diagrams (Image I-IV)

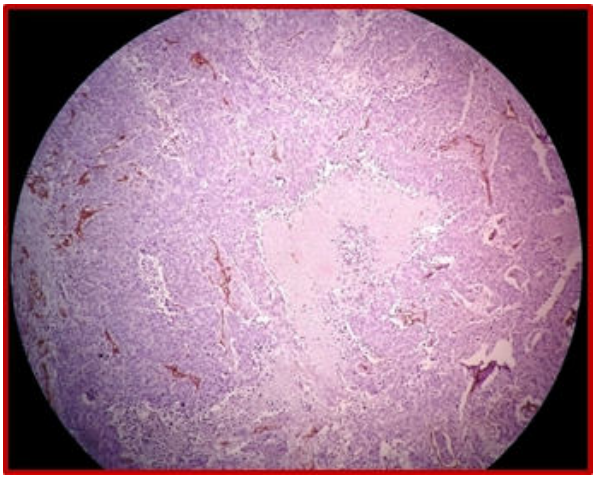
Histopathology Images I-IV



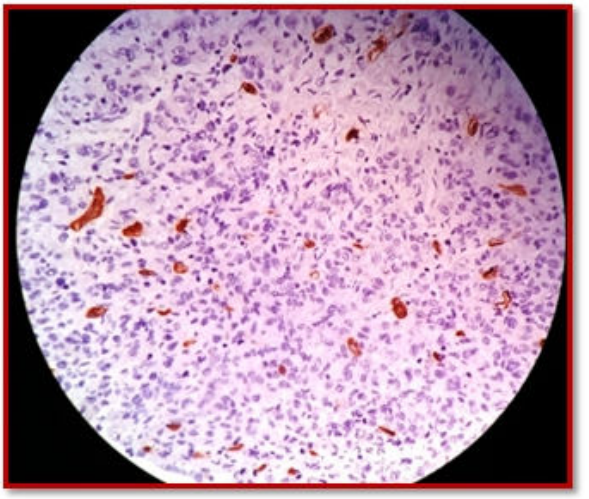
[Image I 45/F MRM specimen M 15x15x14cm with a tumour M8x5x3 cm.]



[Image II: Micro section shows IDC-NOS Grade –III BC.(H and E, X 400)]



[Image III: Micro section showing Tumour "Hot –Spots"(CD 34,X100)]



[Image IV :Micro section shows Endothelial cells positivity. Micro vessels count -32.(CD34,X400)]

DISCUSSION :-

Breast cancer is one of the leading causes of cancer death in women. Tumour progression and metastasis in the breast cancer is angiogenesis dependant. Tumour angiogenesis is generally measured by quantifying micro vascular density in sections

immune stained for vascular endothelial marker such as CD34, CD31 or Factor VIII.[1] MVD is a significant independent prognostic indicator of breast carcinoma especially in node negative patients.[1] In this study we used CD34 as immune marker as CD 31 cross reacts with plasma cells.[2] Zhang et al [3] found out that a significant correlation was found between MVD and CD34. Our study showed the values of MVD vary from 156 to 369. We have divide them in to 3 groups for easy correlation as group-1 or low MVD having less than 200 micro vessels(MV), group-2 or medium MVD having 200-270 MVS and Group-3 or high MVD having more than 270 MVS. We found 8 cases (15%) were in low MVD, 21 cases (40%) were in medium MVD and 24 cases (45%) were in high MVD (Table-1). In this study we correlated MVD with age of the patients and found a positive correlation (P=<0.017). In all age groups the combined value of medium MVD and high MVD constitute >60% cases in that age group. This is supported by Maha M Amin et al. [4] Pykurel D et al [5] also found that MVD positively correlated with age of the cases but did not find a significant value . (P=0.9). We compared MVD with tumour size and found a significant correlation between them.(P=0.025).We found as tumour sizes were increased the number of MVD were also increased .This is supported by Pykurel D et al [5] who found out that MVD positively correlated with age, tumour grade ,LVI and NPI. While correlating MVD with tumour HP grade we found a significant correlation between them. (P=0.04).Other authors like william WL choi et al [6] and Gasparin et al [7] have also found similar observation. We correlated our study with LVI; though significant correlation did not present, simple correlation was found out. (P=0.07). It clearly indicated that tumours showing high MVD had more LVI as compared to low and medium MVD. Similar findings have been observed by other authors like Tanigawa et al[8]. We correlated MVD with lymph node metastasis staging and found a significant correlation among them. (P=0.018). In table 5, it was clearly shown that 8 cases (38%) showed medium MVD without lymphnode metastasis and 3 cases (12.5%) showed high MVD without lymphnode metastasis. So these cases could be treated as high grade and high TNM stage tumours considering the MVD count and whatever the lymphnode metastasis was. Again it was also seen that as MVD increased so also the lymphnode metastasis increased in majority of cases. Similarly Meert et al [9] in 2002 also found that angiogenesis is critical to tumour growth ,metastasis and neoplastic progression .The values of MVD were correlated with the value of NPI (Table-6) and it was seen that a significant correlation exist between them.(P=0.011).It was also found out that as MVD value increased, the progression of the tumour decreased from good to poor. Similar results has been published by Pykurel D et al [5] .Lastly we compared the MVD with TNM staging and found a significant ,correlation between them(P=0.04)(Table-7).It showed that as MVD increased from low to high so also TNM stage increased in stage I to III similar to study by Beatric et al [10] .Our study had a significant correlation with age, tumour, size, tumour grade, LVI,NPI,LN stage and TNM stage(P< 0.005).So MVD can be used as a highly significant prognostic tumour .

CONCLUSION:-

By reviewing all available literatures we found that in most of studies MVD had significant correlation with clinicopathological parameters similar our study. So MVD is a potent prognostic indicator in solid tumour like breast carcinoma.

LIMITATIONS OF THE STUDY: -

- The small sample size may account for the discrepancies with other studies which have been observed during the comparison of various parameters.
- Due to economical constraints, we could not use CD31, CD105 which would have helped us for accurate counting of micro vessels by comparing CD34 stained section with CD31 & CD105 stained sections from the same paraffin embended block of BC specimen.

CONFLICT OF INTEREST: Nil

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