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STATUS OF TRIPLE NEGATIVE BREAST CANCER PATIENTS AND ITS CORRELATION WITH TUMOR VASCULARITY IN INDIAN SCENARIO

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ABSTRACT

Background: Triple negative breast cancer (TNBC) patients have poor prognosis in terms of disease-free and overall survival. In an attempt to predict the fate of patients, various prognostic factors have been identified among which tumoral vascularity is an important parameter.

Methods: The primary aim of this study was to *evaluate triple negative receptor status in breast cancer patients being treated in our University hospital between January 2011 to December 2012 and comparison between TNBC and non TNBC patients in terms of* the color Doppler parameters (RI, PI and Vmax) and micro vessel density (MVD) by immunohistochemistry using anti human CD31 antibody. The secondary aim was to compare these two groups with presently established clinicopathological prognostic parameters in breast cancer like age, duration, tumor size, lymph node status, stage and grade of disease.

Results: The total number of patients enrolled was 40 with mean±SD age of 44.40±10.37 years. The incidence of TNBC was 17.5%. The mean±SD of RI, PI, Vmax and MVD were 0.89±0.12, 2.42±0.12, 20.80±8.27 and 8.37±2.59 respectively in TNBC group compared to 0.65±0.18, 1.46±0.12, 16.44±6.28 and 5.28±1.89 in non TNBC group (p value<0.05). Younger age of presentation, tumor size, number of positive lymph nodes, stage and grade of tumor was significantly higher in TNBC group.

Conclusion: The present study revealed TNBC breast cancer is not uncommon. Triple negative cases are aggressive and have higher vascularity. Clinico-pathologically they have poor prognosis as compared to non triple negative cases.

KEYWORDS: Triple Negative, Breast Cancer (TNBC), Prognostic and Indian Scenario

INTRODUCTION

Breast cancer is a heterogeneous disease with varied morphological appearance, molecular features, behavior and response to therapy (Rakha E.A et al, 2011).The disease encompasses heterogeneous entities that behave differently in terms of biological aggressiveness. Traditionally, breast cancers are classified into different subtypes based on the expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (HER- 2 neu). Tumors without ER, PR and HER2 neu expression are referred to as triple negative breast cancer (TNBC). They have a poor prognosis in terms of disease free and overall survival [Haffty BG et al 2006, Rakha EA et al 2007 and Dent R et al 2007]. TNBC comprises around 15% of all breast cancer and is characterized by its aggressive clinical behavior and insensitivity toward available targeted treatment strategies with endocrine and anti-HER2 neu therapies (Bauer et al, 2007; Rhee et al, 2008). Development of novel treatment strategies is, therefore, needed and the study of other putative targets in TNBC, like angiogenesis is a topic of interest, which can serve as therapeutic targets in this subset of breast cancer (Cleator et al, 2007; Pal et al, 2009).

TNBC is associated with higher angiogenesis. Mohammed et al in his study found micro vessel density was significantly higher in the TNBC group than in the non TNBC group (Mohammed RAA et al, 2011). Apart from being a prognostic factor this assessment of tumor angiogenesis can have therapeutic applications also in the form of development of new agents with antiangiogenic properties (Rayson D et al 1999), vascular targeting drugs, assessment of response to primary chemotherapy and enhancement of anti-tumor activity of cytotoxic drugs by combining with anti-angiogenic therapy or antiangiogenic scheduling (Browder T et al, 2000).

When non-invasive methods are taken into account for the assessment of breast carcinoma, mammography provide no additional useful information over clinical examination in majority of cases and CT, MRI though are sensitive imaging modalities are costlier. In this back ground Color doppler ultra sonography is useful for functional assessment of tumor angiogenesis and neo vascularization. Osanai T et al (2003) and Kumar A et al (2007, 2010) in their study confirmed that preoperative color doppler ultrasonography is useful for the assessment of intra tumoural blood flow analysis, correlates well with histological grade and degree of malignancy of the breast cancer. Carcinoma breast have been found to be associated with higher values of doppler parameters like Resistivity Index (RI), Pulsatility Index (PI) and Maximum Flow Velocity (Vmax) (Chao TC et al, 1999).

Aim of Study

The objective of the present study is to define the incidence of Triple Negative status among breast cancer patients in this part of country. Differences in the demographic profile between TNBC & non TNBC patients are described with regards to clinic pathological parameters and angiogenesis. An attempt has been made to quantify and correlate the vascularity assessed by color doppler ultrasonography and angiogenesis measured by using anti human CD-31 antibody in breast carcinoma and to compare them between triple negative & non- triple negative breast cancer patients. The other common prognostic parameters like stage of the disease, histological grade of the tumor, receptor status and lymph node involvement were also taken into consideration in the study.

METHODS

The present study is a prospective study undertaken on 40 established breast cancer patients treated in one surgical unit in Department of General Surgery in collaboration with Department of Radio-Diagnosis, Radiotherapy and Pathology,

IMS, BHU between July 2011 to May 2013. A detailed history and clinical evaluation was done in all patients as per enclosed proforma. Breast lump was measured along two perpendicular diameters using Vernier calipers and the mean diameter was calculated. The work up included complete blood count, blood urea, serum creatinine, blood sugar, liver function tests, ECG, X-ray chest, USG abdomen, FNAC/Tru-cut biopsy from breast lump. Other investigations were done according to the suspicious site of metastasis.

Color Doppler examination of the breast lump and axilla was done by a single experienced radiologist. It was done using 7.5 MHz Doppler probe (Xario Toshiba).The color Doppler was done prior to surgery. Following parameters were recorded in tumor mass and lymph nodes: Maximum flow velocity (Vmax), Resistivity index (RI) = Peak systolic velocity- End diastolic velocity/ Peak systolic velocity, Pulsatility index (PI) = Peak systolic velocity - End diastolic velocity / Average velocity (mean velocity). A detailed histological evaluation, receptor status estimation and assessment of angiogenesis using the specific endothelial marker CD31 was done by in the paraffin embedded histological sections. In histological examination, size of tumor, type of malignancy, differentiation (grade), and lymph node status were studied. After his to pathological examination the blocks were sent for ER, PR & HER2Neu receptor status examination. Immuno his to chemical assessment of tumor micro vessel density was done using mouse anti human CD-31 antibody. The secondary antibody was biotinylated goat anti-mouse IgG. Antibody and detection kit was obtained from "BIOGENEX, Netherlands".

Weidner criteria (1991) were used for determining microvessels density. A vessel was defined as the vessels, which have endothelium with immunopositivity and a vascular lumen. The sections were initially scanned at low magnification (40x), thereby finding area with the highest number of microvessel in all the fields of each slide at the periphery of the tumour (hot spot). MVD was then determined by counting all CD-31immunostained vessels at 400x magnification. Counting was done in three 'hot spots' selected at low power magnification. Determination of staining was strictly confined to hot spots. Microvessel counts were done by two independent observers, naive to the patient's pathologic and clinical status. The mean value of microvessel densities observed by both investigators in each patient was entered into further calculations. In the case of inter observer differences >30% in microvessel count, the respective slides were reinvestigated by both observers using a discussion microscope. Statistical analysis was done using SPSS 16.0 version. The various parameters studied during observation period were compared using Chi- square test for non-continuous variables. The critical value of 'p' indicating the probability of significant difference was taken as <0.05 for comparison.

RESULTS

Fifteen (37.5%) patients of age below forty years of age. Seventeen (42.5%) patients had duration of symptoms less than 6 months. Twenty six (65%) patients were pre menopausal. Seven (17.5%) patients were using oral contraceptive pills for more than 1 year. Thirty patients (75%) had parity three or more, while only one patient was nulliparous. Two (5%) patients had positive family history of breast malignancy. Lump in breast was the most common complaint and was present in all patients at the time of presentation. Lump axilla was associated with breast lump in nine (22.5%) patients and associated nipple discharge was present in four (10%) patients. Pain was associated in seven (17.5%) patients. Twenty four (60%) patients had clinical tumor size of 6 cm or more. Thirty (75%) patients had clinically palpable ipsilateral axillary lymph nodes at presentation. Maximum (60%) patients were of advanced stage, out of them only 1 patient had stage IV disease, sixteen patients (40%) had IIa/IIb lesion. There was no case of stage I breast cancer.

All patients on his to pathological back ground were divided into two groups, differentiated and undifferentiated. Differentiated group contained mild and moderately differentiated tumors and were considered as low grade. Undifferentiated tumors were considered as high grade. Fourteen out of forty (35%) had high grade tumor. Low grade tumor was observed in 65 % cases. Mean number of his tologically positive lymph nodes was six. Above or equal to six was considered high and below that was considered low. Lymph nodes were negative for metastatic deposits in 17.5% (n=7) of cases. Twenty seven cases (67.5%) had more than six positive lymph nodes. Receptor status assessment was done in all forty cases. Eleven patients were ER/PR positive (27.5%). HER2-neu over expression was found in thirty one out of forty patients (77.5%). Nineteen patients (47.5%) were found to be negative for both ER & PR.

Out of forty patients ten (25%) patients were triple receptor status positive and seven (17.5%) patients were triple negative. The Doppler variables and Microvessel density were categorized with reference to their mean values. Subsequently the mean value was considered as standard for stratification as low or high for further correlation. Resistivity Index was found to be high in twenty three patients (57.5%). Pulsatility Index was found to be high in thirteen patients (32.5%) while it was low in twenty seven patients (67.5%). Maximum flow velocity was found to be high in eighteen patients (45%) while it was low in twenty two patients (55%). Microvessel density was found to be high in fifteen out of forty patients (37.5%).

Out of forty diagnosed breast cancer patients' seven patients were triple negative (17.5%).Significant difference were noticed when age distribution, duration of presentation, tumor size, oral contraceptive pill use, histological grade, RI & microvessel density Score were compared between TNBC & non-TNBC patients table 1.

In this study no significant difference was found clinical axillary lymphnode status, his to pathological lymphnode status, PI and Vmax.

Table 1: Comparison of all Prognostic Parameters between TNBC and Non TNBC Cases

Prognostic Parameters	TNBC (N=7)	Non TNBC (N=33)	P Value
Age distribution <40 years ≥40 years	 5 2	 10 23	**0.041**
Duration of complain <6 months ≥6 months	 6 1	 11 22	**0.011**
Distribution according to oral contraceptive pill use (>1yr) Yes No	 4 3	 3 30	**0.002**
Distribution according to tumor size ≥6 cm <6 cm	 7 0	 17 16	**0.029**
Clinically palpable axillary lymph node Positive Negative	 6 1	 24 9	0.656
Clinical stage distribution Early invasive(I+II) Advanced(III+IV)	 2 5	 13 20	0.591
RI distribution High Low	 7 0	 16 17	**0.014**

Table 1: Contd.,

PI distribution High Low	 3 4	 10 23	0.519
Vmax distribution High Low	 2 5	 16 17	0.336
Distribution of histologically positive lymphnodes Negative High Low	 1 5 1	 6 22 5	0.964
Distribution of histological grade High Low	 5 2	 9 24	**0.026**
Distribution of microvessel density Score High Low	 6 1	 9 24	**0.007**

DISCUSSIONS

TNBC represent a consistent subgroup of breast cancer with heterogeneous clinical presentation, clinical behavior, histology and response to therapy. Little is known about the etiological profile of TNBC which is associated with high mortality and inadequate therapeutic options.

TNBC accounts for 10 to 20% of all breast cancer [Rakha EA et al, 2007; Dent R, et al, 2007; Carey LA et al, 2006], depending on the thresholds for ER, PR and the HER2 assessment method, as well as the age groups, racial groups and tumour grade/type/stage in different study cohorts. Among Asian women more than 30% of breast cancer patients are of triple negative subtype (Kim MJ et al, 2006).Only few studies have been done showing the incidence of TNBC from India. Notably among them is a study done in Grant Medical College and Sir JJ Group of Hospitals, Mumbai by Patil VW et al,2011 where in a study group of 683 patients 59.9% were ER-positive, 40.1% were PR-positive, 25.6% were HER-2/neu-positive (18% were HER-2/ neu-undetermined) and19.9% had triple negative status. Similar study done at Tata Memorial Hospital, Mumbai by Ghosh J et al in 2011 showed among 2001 breast cancer patients registered in the year 2008, triple negative status was found in 29.8% cases. In our study thirteen patients (32.5%) were ER-positive, nineteen patients (47.5%) were PR-positive, thirty-one patients (77.5%) were HER-2/neu-positive and seven out of forty patients were found to be triple negative i.e.17.5%.

Several population-based studies have shown that TNBC often presents at younger age and more frequently in African American women and black ethnicities (Carey LA et al, 2006). Similar result was seen in a large study of The California Cancer Registry, which revealed that women with TNBC are significantly more likely to be aged, <40 years. This clearly shows that triple negative subgroup of breast cancer patients are younger as compared to their receptor positive counterparts. Tan GH et al, 2009 in their study in Asian women found that the TNBC patients were more likely to be under age 40 years old. Among Indian patients study done by Patil et al showed significant number of patients were even younger i.e.<35yrs. Similar results were reproduced from study done by Tata Memorial group where they have shown the median age of presentation of patients with TNBC in India is approximately a decade younger than western literature and as a corollary to this the chances that younger patients to have a TNBC phenotype is fairly high as compared to older patients. In the present study also among the triple negative group 71.4% patients were less than 40 yrs of age as compared

to only 30.3% among non triple-negative group (p=0.041). Among triple negative patients mean age of presentation was 39.85 years.

Breast lump was most common complaint and was present in all TNBC as well as non TNBC patients at the time of presentation. Dent R et al, 2007 in his study found TNBC is more likely to be detected through clinical examination than through serial imaging. This may reflect a more rapid growth rate or may be due to intrinsic differences in detectability. Collett K et al in 2005 found that TNBC were more likely to present in the interval between regular mammograms than other breast cancers. This may relate to differences in the density of the breast tissue in women with the triple negative phenotype, rendering them more difficult to identify on traditional mammography.

The other explanation could be that triple-negative tumors may grow rapidly in relation to the screening interval. Since mammographic detection rate in this part of the country is very low we have found that both the TNBC and non TNBC patients were diagnosed with a clinically detectable lump. Although both the groups were detected with palpable lump yet, in our study most of the TNBC patients (85.7%) presented with shorter duration (<6months) as compared to non-TNBC patients (p=0.011) showing the aggressive nature of tumor.

Millikan RC et al (2008) showed a beneficial effect of longer duration of breastfeeding, and an increasing number of children breastfed. However, Phipps A et al, 2008 did not find an association between parity and breast cancers being more likely to be TNBC but showed that women who had breastfed for ≥ 6 months had reduced risks of triple-negative disease relative to parous women who had never breastfed. Yang X et al (2007) also didn't find any relation between parity and TNBC. In our study also we failed to find any correlation of parity and breast feeding with triple negative and non triple negative status.

The relationship between oral contraceptive use and breast cancer risk has been the subject of extensive research (White E et al 1994, Brinton LA et al 1995, Harris NV et al 1982, Malone KE et al 1993, Marchbanks PA et al 2002). Unlike well-established risk factors such as family history, early menarche, nulliparity, and lack of breastfeeding, the relationship between oral contraceptive use and breast cancer risk has remained less clear. A large pooled analysis by Calle EE et al, 1996 and recent meta-analysis by Kahlenborn C et al, 2006 found an increased risk for breast cancer in relation to oral contraceptive use among premenopausal women.

Previous studies have also shown risk in relation to oral contraceptive use to be concentrated among younger premenopausal women (Rookus MA et al 1994, Rosenberg L et al 1996). In a study done by Dolle JM et al (2009) showed that the risk of TNBC among longer (>1 year) oral contraceptive users was more as compared to recent users and there was a 2.7-fold increased risk for TNBC. In our study four (57.1%) out of seven TNBC patients were using oral contraceptive pill for more than 1yr, as compared to three (9.1%) out of thirty three non TNBC patients. Although the difference is significant the low number of study population limits us to draw a conclusion

Tumor size is an important prognostic factor. It stands second only to axillary lymph node status as an independent prognostic factor. The influence of primary tumor size on prognosis can be appreciated in both node-negative and node-positive cases. This relationship probably reflects that larger tumor size increases vascular and lymphatic dissemination. Size is directly related to an increasing probability of regional metastasis (Donegan WL, 1992). TNBC patients present with larger tumor size as compared to non -TNBC (Rakha EA et al, 2007). Dent R et al, 2011 in their study found 7.77% of TNBC patients had T3 lesion, as compared to 1.12% of non TNBC patients. In the present

study all the TNBC patients presented with larger tumor size (>6cm) as compared to 51.5% of non TNBC patients (p=0.029).

Axillary lymph node status is one of the most powerful prognostic factors signifying that the malignancy has gained access for systemic spread and eventually distant metastasis (Fisher et al, 2001; Wenger et al, 1993). The relationship of triple negative status and lymph node spread is not well defined in literature. Rakha EA et al (2008) found no relation, while Dent R et al (2007) showed the rate of node positivity was slightly higher in the TNBC group compared with non TNBC (54.6% versus 45.6%, respectively; P = 0.02). In some studies a negative association was found between TNBC and lymph node status (Kusinska et al, 2005, Crabb et al, 2008; Tan et al, 2008). In our study six out of seven (85.7%) TNBC patients had positive axillary lymph nodes as compared to twenty four out of thirty three (72.7%) non TNBC patients, but the correlation was not significant (p=0.656). The lack of difference in our study could be explained by the advanced stage of presentation in our patients.

Different studies have shown that vast majority of the TNBC patients (about 90%) are poorly differentiated with high proliferative activity and large primary tumor size (Carey LA et al, 2006, Thike AA et al, 2010, Rakha EA et al, 2007). Dent R et al (2007) showed that patients in the TNBC group were more likely to have grade III tumors as compared to non TNBC tumors (66% versus 28%; P < 0.0001). Similarly Patil VW et al 2011 found that among Indian patients the TNBC correlated with higher his to pathological and nuclear grade compared to non TNBC (p =0.001 and p= 0.001, respectively). In this study five (71.4%) out of seven TNBC patients had high histological grade, as compared to nine (27.3%) out of thirty-three non TNBC patients. The correlation between these two were statistically significant (p=0.026).

Use of the color doppler ultra sonography in patients with breast cancer has been reported to be useful for distinguishing whether a tumor is benign or malignant (Choi HY et al, 2000). However, there has been controversy regarding the usefulness of this method for determining the aggressiveness of the tumor by measuring the degree of neo vascularization (Weidner N et al, 1991). Since the color doppler test result represents the degree of blood circulation in the inner part of the tumor, this value can be an indirect reflection of the degree of neo vascularization (Zhu Q et al, 2011). Using color doppler ultra sonography, studies have shown higher values of RI, PI and Vmax in more vascular lesions like malignancy (Chao TC et al, 1999). Malignant breast lesions typically show increased signals on color doppler due to tumor neo vascularization (Choi HY et al, 2000; Raza S et al, 1997; Kook SH et al, 1999). In breast tumors with high vascularity, differentiation between benign and malignant could be done on the basis of flow patterns. The malignant tumors have much higher RI and PI values (Peter-Engl C et al, 1995; McNicholas MMJ et al, 1993). Increase in resistance may be due to occlusion and stenosis in the tumor vessel network structure produced by vascular encasement due to tumor growth. Wang Y et al (2010) showed correlation between power color doppler index with the levels of VEGF protein (marker of angiogenesis) and hence, the pre-operative color doppler examination could be used to evaluate tumor angiogenesis indirectly and to provide a useful reference regarding treatment selection and prognosis. Shin et al (2011) found marked vascularity shown on colour doppler is associated with a high grade and negative hormone receptors. Although another study by Kojima et al (2011) showed majority of TNBC presented with few color spots (36.3%) or vessel formations (41.2%), but rarely showed marked vascularisation (12.5%). Similarly Lacroix MB et al (2012) did not find any relationship between color doppler parameters and TNBC. In the present study resistivity index, pulsatility index and maximum flow velocity was obtained in all patients. When comparison was done between TNBC and non TNBC

patients, RI was found to be high (>0.92) in TNBC cases (p=0.014) but no correlation could be made out when PI and Vmax compared.

Angiogenesis and lymphangiogenesis are essential for tumor growth, invasion and metastasis (Nathanson SD et al, 2003; Folkman J et al, 1971). Recent studies have shown that intra tumoral microvessel density is an important prognostic marker of survival in breast cancer and for prediction of the likelihood of systemic metastases (Choi WW et al, 2005). Hence, the measurement of neo vascularisation can be exploited as useful tool in prognostication of breast cancer patients preoperatively. Mohammed et al (2011) in their study found out of ninety-nine TNBC cases fifty seven patients had high microvessel density as compared to hundred eight out of three hundred thirty-four non-TNBC cases (p<0.001). In the present study histological slides were examined for angiogenesis assessment done by CD-31 antibody. Mean micro vessel density was 13.17±4.13. Six out of seven (85.7%) TNBC patients had high score as compared to nine out of thirty-three (27.3%) non TNBC patients (p=0.007).

Thus with the present data we can conclude that TNBC patients have poorer clinic opathological prognostic markers, higher vascularity and angiogenesis as compared to non TNBC patients.

CONCLUSIONS

Thus with the present data we can conclude that TNBC is not uncommon. Triple negative cases are aggressive and have higher vascularity. Clinicopathologically they have poor prognosis as compared to non triple negative cases, evidenced by younger age of presentation, larger tumor size, higher histological grade, higher doppler resistivity index and higher microvessel density score. This study has also highlighted the importance of color doppler study in breast cancer which can be an important modalitity of preoperative investigation and prognostic marker.

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