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Prognostic Correlation of Color Doppler Ultrasonography and Lymphangiogenesis in Breast Carcinoma

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Abstract: **Tumor vascularity and lymphangiogenesis are two established prognostic parameters however detecting a proxy marker could help in prognosticating patients in an early stage. We tried to investigate color doppler and Lymphatic vessel immunohistochemical staining in breast cancer patients and correlate it with established prognostic markers in breast cancer. 30 patients were included in the study with the mean age of 44.40±10.37 years (range 30-65 years). In color doppler, high RI value significantly correlated with T3 tumor size, number of lymph nodes (>10), grade (grade III), hormonal status, LMVD (high) and LVI D2-40 positivity with a p-value of 0.0004, 0.001, 0.007, 0.006, 0.008 and 0.008 respectively. On correlation with PI, number of lymph nodes (>10), grade (grade III), hormonal status and LVI D2-40 positivity were significantly correlated with high PI value (p=0.044, p=0.012, p=0.013 and p=0.046 respectively). In Lymphatic vessel immunohistochemical staining high LMVD value significantly correlated with tumor size, number of lymph nodes, tumor grade and hormonal status (p=0.002, p=0.001, p=0.0006 and p=0.015) while LVI significantly correlated with tumor size, number of lymph nodes and hormonal status prognostic factors (p=0.008, p=0.013 and p=0.046 respectively).**

Index Terms: **Breast cancer, Color Doppler, Immunohistochemical staining, Lymphangiogenesis, Tumor vascularity.**

I. INTRODUCTION

Breast cancer (BC) is the third most common tumor in world and represents 9% of global cancer burden (Lopez-Knowles et al, 2004). In India it is the second commonest cancer amongst women next to cancer cervix with a rising incidence in young premenopausal women (Raina et al, 2005). Over the past few decades, BC management has undergone significant changes, characterized by less aggressive and rationalized approaches in diagnosis and treatment. Current management of primary BC involves the need for assessment of various predictors and prognostic markers. The prognostic markers are clinical or biological parameters associated with disease free or overall survival. In an attempt to predict the fate of patient, various prognostic factors have been identified.

The functional assessment of tumor angiogenesis and neovascularization has now become possible with the use of color Doppler. Osanai et al (2003) and Kumar et al (2007, 2010) in their study confirmed that preoperative color Doppler ultrasonography is useful for the assessment of intratumoural blood flow analysis, correlates well with histological grade and degree of malignancy of the BC. BC 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 et al, 1999).

Estimation of lymphovascular invasion and tumor lymphangiogenesis assessment are current emerging prognostic indicators (Hasebe et al, 2004 & Schoppmann et al, 2004). Lymph node (LN) status is the most important independent clinical prognostic factor for patients with BC (Fischer et al, 1995). The presence and the extent of axillary LN metastases reflect the probability that the cancerous process has spread through the body and both are strongly correlated with the development of distant metastases and with shortened disease-

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free and overall survival. However, the process of lymphatic invasion and metastasis to regional lymph nodes, and whether tumors promote lymphangiogenesis (i.e., new lymphatic vessel growth) in a manner similar to angiogenesis, remains poorly understood. There are speculations that lymphangiogenesis plays a similar role as that of angiogenesis.

Studies with regard to quantification and correlation of angiogenesis and lymphangiogenesis in breast carcinoma could be a research area in order to establish the role of these two markers of prognosis. The present study explores the correlation of angiogenesis and lyphangiogenesis in breast cancer and how these correlate with other established prognostic markers in BC. The primary aim of this study was to correlate the color Doppler ultrasonography findings with lymphangiogenesis by estimating lymphatic microvessel density (LMVD) and lymphovascular invasion (LVI) by immunohistochemistry using D2-40 antibody. The secondary aim was to correlate these two parameters with presently established clinicopathological prognostic parameters in BC.

II. METHODS

A prospective study was conducted in a single surgical unit of a University hospital over a period of 2 year. All patients of BC admitted in the unit during study period for surgery as primary modality of treatment were included in study. The patients who received any prior treatment like, lumpectomy, neoadjuvant chemotherapy or radiotherapy were excluded. Patients who had any kind of breast surgery in past or who did not gave consent were also excluded. The diagnosis was made by FNAC from breast lump after detailed clinical evaluation. Color doppler examination of the breast lump and axilla was done by a single experienced radiologist.

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. Standardized machine settings were used for Color Doppler imaging to optimize sensitivity to low velocity and low volume blood flow (wall filter: low frequency; dynamic range: 60 dB). The Color Doppler ultrasonographic data acquisition was limited to a region of interest containing the tumor and the Doppler parameters RI, PI and Vmax were recorded. Patients were categorized as having 'high' or 'low' RI, PI and Vmax depending on whether their individual RI, PI or Vmax is higher or lower than the mean RI, PI or Vmax value.

All patients underwent modified radical mastectomy. A detailed histological evaluation, receptor status estimation and assessment of lymphangiogenesis by determining the lymphatic micro-vessel density (LMVD) and Lympho-vascular invasion (LVI) was done by using the specific lymphatic endothelial marker D2-40 antibody obtained from Dako (Agilent pathology solutions), USA in the paraffin embedded histological sections. The following histological characteristics were studied: size of tumor, type of malignancy, differentiation (grade), lymph node status, lymphovascular invasion and receptor status. The secondary antibody was biotinylated goat anti-mouse antibody. A block of archival tonsillar tissue was taken as positive control for D2-40 IHC assays. For negative control, a slide was prepared from same tissue block and a preimmune serum was used instead of primary antibody.

Mastectomy specimen were cut into slices at approximately 0.5 cm and the specimen were kept in 10% buffered formalin for 18-24 hours for proper fixation, then the specimen were grossed by trained pathologist to obtain representative tissue sections which were processed routinely in the conventional way for embedding in paraffin wax. 4μm section were cut and placed on glass slide, one slide of each tissue was stained with Hematoxylin and Eosin (H & E). After H & E staining these sections were evaluated under light microscopy for histopathological details.

Blocks of the viable tumor representative area were selected for immuno-histochemistry (IHC) by staining with D 2-40. The 3 μm sections were taken on 1% Poly L-lysine coated slides. De-waxing was done by dipping the slides in Xylene-1 and then in Xylene-2 for five minutes and then rehydrated in absolute alcohol (95%) for 1-2 minutes. Antigen retrieval was done in microwave using citrate buffer (pH 6.0) at 95^0C and second at 97^0C for 10min each. Slides washed in TRIS buffer (pH 7.6) and then endogenous peroxidase blocking by 3% H_2O_2. Sections were finally incubated in primary antibody solution and washed. Diamino benzidine dihydrochloride (DAB) Chromogen applied and then treated with secondary antibody. After final blotting and drying, slides observed under microscope.

Weidner criteria (1991) were used for determining microvessels density. A lymphatic vessel was defined as the vessel, 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). Lymphatic microvessel density (LMVD) was then determined by counting all D2-40 immunostained vessels at 400 x magnification. Counting was done in three 'hot spots' selected at low power magnification. Microvessel counts were done by two independent observers and their mean value was entered into further calculations. In the case of interobserver difference of >30% in microvessel count, the respective slides were reinvestigated by both observers using a discussion microscope. Mean of all values were taken and patients having higher LMVD than mean value were considered as LMVD positive, those with lower value LMVD negative.

Lymphatic vessel Invasion (LVI) was recognized as tumor cell nests floating within empty spaces, which were surrounded by thin, spindle shaped endothelial cells. A lymph vessel that showed positive staining of the endothelium for D2-40 and surrounded the

tumor cells was diagnosed as positive for lymphatic invasion. The cases were categorized as LVI (+) positive or LVI (-) negative. The D2-40-stained slides were also assessed for lymph vessel invasion.

The Color Doppler parameters (RI, PI, Vmax), LMVD and LVI were correlated with other well-known prognostic parameters like Tumor size, Lymph node status, Histological type, Grade, Stage of disease and Hormonal receptor status. LMVD and LVI were correlated with color Doppler parameters and also with each other.

The data were analyzed using SPSS 16.0 software (SPSS, Inc., Chicago, IL). The mean difference between two groups was calculated by student t test. Categorical variables were evaluated by the two-tailed Chi-square test or two-tailed Fisher's exact test. $P<0.05$ was considered statistically significant. The level significance was considered at 5% cut-off point. The significance level more than 5% written as $p>0.05$ was taken as statistically insignificant.

III. Results

A total of 30 patients were included in the study with the mean age of 44.40±10.37 years (range 30-65 years). Majority of patients (53.3%) were pre-menopausal and rest were postmenopausal (46.7%). Lump was present in all patients at the time of presentation. Seven patients (23.3%) had associated lump in the axilla; 1 patient had associated nipple discharge while only 4 (13.3%) patients complained of pain in the lump. The clinical and pathological characteristics of patients are presented in Table 1.

Receptor status assessment was done in 26 cases only and HER2/neu status in 22 cases. Three patients were ER/PR positive (11.5%). HER2-neu over expression was found in 16 out of 22 patients (72.7%). Sixteen patients (61.6%) were found to be negative for both ER & PR. Among 22 patients, 3 patients were triple negative (18.2%). Receptor status of patients was further categorized into four groups with reference to prognostic significance.

The Doppler parameters i.e. RI, PI and Vmax were categorized (low / high) on the basis of their mean value (Fig. 1). The mean RI of 30 patients was 0.89. The RI value >0.89 (high) was present in 19 (63.33%) patients while 9 (30%) patients had PI value >2.43 (high) have higher PI value than mean (2.43) and 10 patients had Vmax value >20.81 (high) have higher PI value than mean (20.81). The correlation of color Doppler indices with various prognostic factors, LMVD and LVI D2-40 are shown in Table 2. The high RI value is significantly correlated with T3 tumor size, number of lymph nodes (>10), grade (grade III), hormonal status, LMVD (high) and LVI D2-40 positivity with a p-value of 0.0004, 0.001, 0.007, 0.006, 0.008 and 0.008 respectively. On correlated with PI, number of lymph nodes (>10), grade (grade III), hormonal status and LVI D2-40 positivity were significantly correlated with high PI value (p=0.044, p=0.012, p=0.013 and p=0.046 respectively). No correlation was observed between Vmax value and all the prognostic factors. The LMVD was categorized (low / high) on

Table 1: Patient Characteristics

Characteristics	
Age (years), mean±SD	44.40±10.37
Menstrual status	
Premenopausal	16 (53.3)
Postmenopausal	14 (46.7)
Tumor size (cm)	
T 2	8 (26.7)
T 3	22 (73.3)
N-status	
N0	10 (33.3)
N1	16 (53.4)
N2a	4 (13.3)
Stage	
Early invasive (IIa+IIb)	14 (46.7)
Locally advanced (IIIa)	16 (53.3)
Grade	
Low (I + II)	11 (36.7)
High (III)	19 (63.3)
No of Lymph Nodes	
Negative	8 (26.7)
1-3	3 (10.0)
4-9	3 (10.0)
>10	16 (53.3)
ER	
Positive	3(11.5)
Negative	23 (88.5)
PR	
Positive	10 (38.5)
Negative	16 (61.5)
HER2	
Positive	16 (72.7)
Negative	6 (27.3)

Table 2: Correlation of color Doppler indices with various prognostic factors

	RI			PI			Vmax		
	Low (< 0.89)	**High (>0.89)**	**p-value**	**Low (< 2.43)**	**High (>2.43)**	**p-value**	**Low (< 20.81)**	**High (>20.81)**	**p-value**
Tumor size (cm)									
T 2	9 (81.8)	2 (10.5)	0.0004	10 (47.6)	1 (11.1)	0.057	7 (35.0)	4 (40.0)	0.788
T 3	2 (18.2)	17 (89.5)		11 (52.4)	8 (88.9)		13 (65.0)	6 (60.0)	
No of Lymph Nodes									
Negative									
1-3	8 (72.7)	0	0.001	8 (38.1)	0	0.044	4 (20.0)	4 (40.0)	0.57
4-9	2 (18.2)	1 (5.2)		3 (14.3)	0		2 (10.0)	1 (10.0)	
>10	0	3 (15.8)		1 (4.8)	2 (22.2)		3 (15.0)	0	
	1 (9.1)	15 (79.0)		9 (42.8)	7 (77.8)		11 (55.0)	5 (50.0)	
Grade									
Low (I + II)	9 (81.8)	2 (18.2)	0.007	11 (52.4)	0	0.012	6 (30.0)	5 (50.0)	0.285
High (III)	2 (10.5)	17 (89.5)		10 (47.6)	9 (100)		14 (70.0)	5 (50.0)	
Stage									
Early invasive (IIa+IIb)	6 (54.5)	8 (42.1)	0.510	8 (38.1)	6 (66.7)	0.150	9 (45.0)	5 (50.0)	0.795
Locally advanced (IIIa)	5 (45.5)	11 (57.9)		13 (61.9)	3 (33.3)		11 (55.0)	5 (50.0)	
Hormonal status									
ER+ PR+HER2/neu-	3 (42.8)	0	0.006	3 (23.0)	0	0.013	2 (14.3)	1 (12.5)	0.542
ER± PR± HER2/neu±	3 (42.8)	2 (13.3)		5(38.5)	0		3 (21.4)	2 (25.0)	
ER- PR- HER2/neu+	1 (4.4)	10 (66.7)		5(38.5)	6(66.7)		6 (42.9)	5 (62.5)	
ER- PR- HER/2neu-	0	3 (20.0)		0	3(33.3)		3 (21.4)	0	
LMVD									
Low	10 (90.9)	8 (42.1)	0.008	15 (71.4)	3 (33.3)	0.050	13 (65.0)	5 (50.0)	0.429
High	1 (9.1)	11 (57.9)		6 (28.6)	6 (66.7)		7 (35.0)	5 (50.0)	
LVI D2-40									
Positive	2 (18.2)	13 (68.4)	0.008	8 (38.1)	7 (77.8)	0.046	8 (40.0)	7 (70.0)	0.121
Negative	9 (81.8)	6 (31.6)		13 (61.9)	2 (22.2)		12 (60.0)	3 (30.0)	

the basis of mean value 8.37 into low (n=18) and high (n=12). The LVI D2-40 was positive in 15 (50%) cases and rest 15 cases were negative. The LVI D2-40 was positive in 15 (50%) cases compared to just 9 cases which showed LVI on H&E (30%). So there was 20% increase of in detection of LVI on D2-40 compared to H&E. There were 7 cases who were positive for LVI on D2-40 and negative on H&E compared to one case vice versa. 8 cases were positive on both D2-40 and H&E (p=0.005). The correlation of LMVD and LVI assessed by D2-40 with various prognostic factors are shown in Table 3. The high LMVD value was significantly correlated with tumor size, number of lymph nodes, tumor grade and hormonal status (p=0.002, p=0.001, p= 0.0006 and p=0.015). The LVI D2-40 positivity was found to be significantly correlated with these three i.e. tumor size, number of lymph nodes and hormonal status prognostic factors (p=0.008, p=0.013 and p=0.046 respectively). When comparison was done between LMVD and LVI D2-40, twelve out of fifteen (80%) cases with low LMVD had negative LVI and 12 out of 15 cases with high LMVD had positive LVI. The correlation was statistically

significant (p=0.001).

IV. Discussion

Breast cancer is the most common cancer among women, with a lifetime risk of up to 12% and a risk of death of up to 5% (Parkin et al, 2002).

Its incidence has been increasing but after a period of continuous rise in many industrialized countries breast cancer mortality has been stable or has even decreased in the last 10–15 years (Botha et al, 2003). This has been the result of mass mammographic screening programs resulting in earlier detection of small tumors in combination with therapeutic improvements. Although earlier diagnosis and better treatment are now available, many of the mechanisms underlying the ability of cancer cells to metastasise are poorly understood. Prognostic factors can be used to predict the natural history of breast cancer. In the last 30 years, intensive efforts have been made to identify tools to improve prognostication. The well-known prognostic

Table 3: Correlation of LMVD and LVI assessed by D2-40 with various prognostic factors

	LMVD			**LVI D2-40**		
	Low < 8.37)	**High (>8.37)**	**p-value**	**Positive**	**Negative**	**p-value**
Tumor size (cm)						
T 2	11 (61.1)	0	0.002	2 (13.3)	9 (60.0)	0.008
T 3	7 (38.9)	12 (100)		13 (86.7)	6 (40.0)	
No of Lymph Nodes						
Negative	8 (44.4)	0	0.001	1 (6.7)	7 (46.7)	0.013
1-3	3 (16.7)	0		1 (6.7)	2 (13.3)	
4-9	3 (16.7)	0		1 (6.7)	2 (13.3)	
>10	4 (22.2)	12 (100)		12 (80.0)	4 (26.7)	
Grade						
Low (I + II)	11 (61.0)	0	0.0006	3 (20.0)	8 (53.3)	0.058
High (III)	7 (38.9)	12 (100)		12 (80.0)	7 (46.7)	
Stage						
Early invasive (IIa+IIb)	10 (55.6)	4 (33.3)	0.232	7 (46.7)	7 (46.7)	1.00
Locally advanced (IIIa)	8 (44.4)	8 (66.7)		8 (53.3)	8 (53.3)	
Hormonal status						
ER+ PR+HER2/neu-	3 (25.0)	0	0.015	0	3 (33.3)	0.046
ER± PR± HER2/neu±	5 (41.6)	0		2 (15.4)	3 (33.3)	
ER- PR- HER2/neu+	3 (25.0)	8 (80.0)		8 (61.5)	3 (33.3)	
ER- PR- HER/2neu-	1 (8.4)	2 (20.0)		3 (23.1)	0	

parameters are age, menstrual status, parity, stage, tumor size, nodal status, histological type, grade, lymphovascular invasion and ER/PR/HER2-neu status.

Angiogenesis and lymphangiogenesis are essential for tumor growth, invasion and metastasis (Nathason, 2003). Previous studies have shown that intratumoral microvessel density is an important prognostic marker of survival in breast cancer and for prediction of the likelihood of systemic metastases (Choi et al, 2005). Hence, the measurement of neovascularisation can be exploited as useful tool in prognostication of breast cancer patients preoperatively. Apart from prognostic importance, this might be useful for making therapeutic decision.

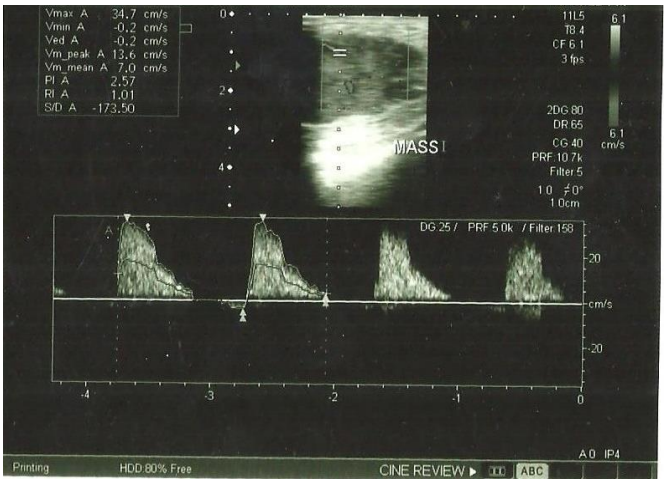
Tumor angiogenesis is complex and highly heterogenous that can be indirectly evaluated by color Doppler ultrasonography as a cost effective measure because of the superficial location of tumor. The multiplicity of vessels, their disordered pattern, and the arteriovenous shunts of these tumor give rise to flow that can be detected as high-velocity signals with a distinctive rasping sound on color Doppler ultrasonography (Yang et al, 2000). The measurement of color Doppler indices (RI, PI, and Vmax) can have a prognostic value in determining the nature of breast cancer.

The presence of metastatic tumor in regional lymph nodes is an independent single most important prognostic factor in breast carcinoma. Tumor lymphangiogenesis can be a tool to prognosticate in such node negative patients in whom an important prognostic parameter in form of lymph node involvement is not available. It can be studied by various parameters like estimation of expression of VEGF by immunohistochemistry or Quantitative RT-PCR for VEGF-Family gene products, lymphatic vessel density assessment by immunohistochemistry by using antibodies against proteins specifically expressed on lymphatic endothelium. Several lymphatic endothelial markers have been established recently like podoplanin, desmoplakin, Prox 1, the receptor for VEGF-C, VEGF-D (VEGFR-3), and the LYVE-1 antigen (Cursiefen et al, 2002 & Erovic et al, 2003). Specific lymphatic endothelial markers are available, making possible analysis of lymphatics in cancer. D2-40, an IgG2a monoclonal antibody generated against an oncofetal membrane antigen M2A, has been identified in ovarian carcinoma cell lines and germ cell neoplasia and has been reported to be a specific marker for lymphatic endothelium in normal and neoplastic tissue (Marks et al, 1999). It has been shown to stain endothelium of lymphatic vessels and lymphangiomas but not that of blood vessels or hemangiomas.This study was done on 30 patients of breast carcinoma treated in single surgical unit to quantify angiogenesis by color Doppler ultrasonography and lymphangiogenesis by assessing microvascular density using the specific lymphatic endothelial marker D2-40, and correlate these measurements with other known clinicopathological parameters and with each other.

The mean age of presentation in our subset of patients were 44.40±10.37 years with majority of patients belonging to the 3rd and 4th decade (46.7%). In an Indian study by Raina et al (2005), the mean age of presentation of the breast cancer in India is less than 50 years, which is lower than that in the developed countries. The incidence of breast cancer in our study was more in premenopausal (53.3%) than in postmenopausal (46.7%) women. Datta et al (2012) reported that now majority of new cases in India diagnosed in premenopausal women. The most common complaint of patient was breast lump. Breast lump was most common complaint and was present in 94.3% of patients at time of presentation, as reported in other Indian studies (Nagpal et al, 1980).

The use of the color Doppler ultrasonography in patients with breast cancer has been reported to be useful for distinguishing whether a tumor is malignant or benign (Choi 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 neovascularization (Weidner 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 neovascularization. Malignant breast lesions typically show increased signals on color Doppler due to tumor neovascularization (Choi et al, 2000) correlate angiogenic and lymphangiogenic microvessel density in breast carcinoma with clinicopathological prognostic parameters and found a strong correlation between microvessel density and tumor size, grade, stage, and lymph node metastasis. Similar findings were observed by other studies also (Tsutsuiet al, 2003 & Uzzan et al). Qingli et al (2011) study on breast cancer showed color doppler flow imaging being capable of evaluating breast tumor angiogenesis. Wang et al (2010) in his study showed that in breast cancer patients, the increased blood flow shown by color power Doppler index was found to correlate with the levels of VEGF protein (marker of angiogenesis) and hence, the pre-operative color doppler

Fig. 1: Color Doppler ultrasonography of breast mass showing high RI, PI and Vmax



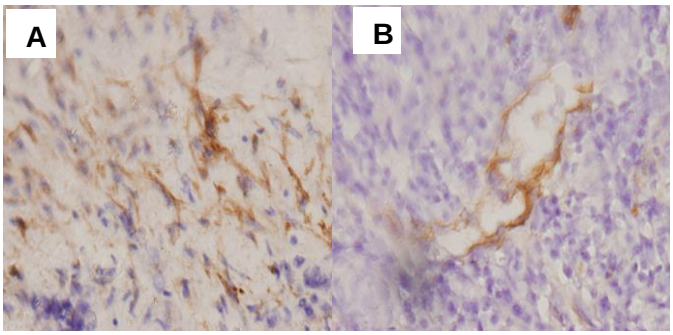
examination could be used to evaluate tumor angiogenesis indirectly and provide a useful reference regarding selection and prognosis.

Using color Doppler ultrasonography, studies show higher values of RI, PI and Vmax in carcinoma and indicates high vascularity (Chao 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 (Jose et al, 2005). Increase in resistance may be due to occlusion and stenosis in the tumor vessel network structure produced by vascular encasement due to tumor growth. Tumor vascularity as revealed by Doppler correlated strongly with detection of lymph node involvement & lymphatic vascular invasion (Mehta & Raza, 1999). Jose et al (2005) found color Doppler as a useful tool to predict the prognosis of patients with breast cancer.

Tumor size is one of the important predictor of breast cancer for local recurrence, regional and systemic spread and therefore overall survival (Jagsi et al, 2005). In the present study RI

correlated with tumor size (p=0.0004). Although PI increases with the increase in tumor size but correlation was not significant and peak systolic velocity (Vmax) did not show any correlation with size (p=0.057; p=0.788). Similar results were seen in a study done by Chao et al [30], where they found significant correlation between RI and tumor size while non-significant between PI and size. Mehta et al (1999) in their study found that the tumors with vascularity detectable by color Doppler US were larger than those without. Color power Doppler sonographic vascularity showed a significant positive correlation with tumor size (Yang & Chang, 2000). The observations suggest that the progressive tumor growth has proportionate increase in neovascularisation.

Fig. 2: Same patient in Fig. 1 with positive immunostaining of lymph vessels for D2-40 antibody showing (A) high LMVD (200X) and (B) lymphatic vessel invasion(400X).



The presence of lymph node metastasis represents a major criterion for evaluating the potential prognosis of breast cancer patients and predicts the choice of additional adjuvant therapy after surgery (Schoppmann et al, 2004). In a study done by Watermann et al [31], the strongest predictors of overall survival were number of tumor arteries on color Doppler and number of positive axillary lymph nodes. A positive correlation was found between nodal metastases and measured tumor flow velocity (Vmax) in T1 (< 2 cm) breast tumors (Lee et al, 1995). However no significant correlation was observed for tumors more than 2 cm signifying the importance of lymph node metastasis in malignancy irrespective of tumor size. In our study, high RI was associated with higher chances of lymph node metastasis (P=0.001). Similarly, PI also correlated with lymph node metastasis (p=0.044). In the present study, the peak systolic velocity could be correlated in 60% patients with higher Vmax &were histological lymph node positive as compared to 80% patients with low Vmax and node positive status (p=0.57). Thus RI and PI significantly correlated with Lymph node metastasis but Vmax did not correlated. Chao et al (2001) showed a trend for axillary nodal metastases in vascular tumors. Other studies are also in concordance with similar observations (Kubek et al, 1996 & Mehta et al, 1999). By a study on invasive breast cancer, Yasser et al (2008) concluded that microvascular density (MVD) measured by CD-31 for evaluation of angiogenesis, showed significant correlation with lymph node status, tumor size, nuclear grade, and clinical stage.

The three strongest prognostic determinants in operable breast cancer used in routine clinical practice are lymph node status, primary tumor size, tumor histologic grade. The most widely used histologic grading system of breast cancer is the Nottingham combined histologic grade. Several studies have shown an independent prognostic significance of tumor grade in breast cancer. In our study, significant correlation was found between tumor grade and RI & PI (p=0.007; p=0.032). Peak systolic velocity (Vmax) did not show any correlation with grade (p=0.285).Though the increase in clinical stage showed higher RI values but correlation was not significant (p=0.510). PI and Vmax did not showed any correlation with stage (p=0.150; p=0.795). This observation further contributes to the observations made earlier that increasing size does not have much bearing with reference to Doppler indices. Vascularization displayed with color Doppler sonography was found to be greater in grade III breast cancers than in the grade I/II (Holcombe et al, 1995). Yang et al (2002) found a positive correlation between power Doppler measurement of tumor vessel number and histologic grade. But Jose et al (2005) was unable to found any significant differences in the RI and PI of tumors with different grades. Yasser et al (2008) found significant correlation of CD-31 detected microvessel density with lymph node metastasis, nuclear grade, histologic grade, stage, and vascular invasion. Intratumoral blood flow analysis assessed by color Doppler ultrasonography correlates well with histological grade and Nottingham prognostic index (Takayuki et al, 2003).

The presence of hormone receptors in the cancer cell is important in guiding prognosis and treatment. Hormone-positive tumors have a more indolent course and are responsive to hormone therapy. Her2/neu positive patients have more aggressive disease. In our study, Receptor status were classified based on worsening prognosis. On comparison, high RI and PI were correlated with poorer receptor status and low RI and PI with better receptor status (p=0.006; p=0.013). All patients with triple negative status (n=3) had high RI and PI values. But the correlation of Vmax with receptor status was non-significant (p=0.542).The limitation to this statement has been less number of patients in the study group. Probably the observations made can be substantiated with more number of triple negative patients who carry poor prognosis. Since no such correlation is available in the literature, it will be difficult to draw a definite conclusion. However, in a study done by Yang et al (2002), color power Doppler sonographic vascularity showed a significant positive correlation with PR negativity. Choi et al (2005) showed positive correlation with ER, PR and Her2/neu status but Chao et al (2001) did not found significant correlation between Color Doppler indices (RI, PI, and Vmax) and ER/PR status.

A. LMVD:

Using immunohistochemical and biochemical methods, several studies have shown a worse prognosis for tumors with high lymphangiogenic activity. A correlation of lymphatic vessel density (LVD) detected by immunohistochemistry with an unfavorable prognosis has been observed.

In 30 patients studied, LMVD was calculated using D2-40 antibody. LMVD ranged from 5/hpf to 15/hpf with mean LMVD of 8.37±2.59 and median LMVD of 7.5. Previously reported studies have analyzed LMVD either as continuous data (Gasparini & Harris, 1994) or as dichotomous data based on the median score as cut off. In our study the mean LMVD score was 8.37±2.59 and the patients were classified into low and high LMVD based on LMVD value <8.37 or >8.37 respectively. Further other clinico-pathological parameters were compared between these two groups.

Tumour size is considered as a prognostic marker in breast carcinoma (Jagsi et al, 2005). The observations made by Tsutsui et al (2003), in a study over 252 patients showed a significant correlation between tumour size and LMVD (p= 0.0487). Bono et al [38] had also found positive correlation between tumour size and LMVD. The results of above studies corroborated with our observation in the study and 61.1% patients with T2 lesion had low LMVD and 100% patients with T3 lesion had high LMVD. It showed that larger tumor size was associated with high LMVD (p=0.002).

In our study LMVD score was significantly correlated with higher chances of lymph node involvement (p=0.001).The results obtained in the present study were concordant with study done by Choi et al (2005) the mean lymphatic microvessel density correlate strongly with lymph node metastasis (P=0.0558). This significant association between LMVD and lymph node involvement could be explained through a lymphangiogenesis-induced increase of the lymphatic vessel. Tumor-associated lymphatic vessels are considered as the main route of tumor cells to axillary lymph nodes and tumor cells exposed to more microvessels are more likely to spread to distant sites and to lymph nodes.

Schopmann et al (2004) showed that high LMVD was associated with a higher differentiation grade tumor. It led to speculation that fast growing tumors produced more growth factors and offer a bigger clonal variety of tumor cells capable of involving lymphatic vessels compared with well differentiated slow growing tumors. Similar results were obtained in this study and high LMVD correlated with higher grade of tumor (p=0.0006).

Choi et al (2005) in a study on breast cancer patients found ER/PR to be strongly correlated with LMVD (p=0.0435**;** p=0.0650 respectively).In our study also all patients with high LMVD belonged to poor prognostic hormonal receptor status combination (ER-/PR-/HER2Neu- or ER-/PR-/HER2Neu +), whereas only 33.4% patients with low LMVD belonged to these groups (p=0.015).

B. Lymphovascular Invasion (LVI):

Invasion of lymphatics by tumor cells are important for the lymph node metastasis. Thus, the recognition of peritumoral lymphatic vessel invasion (LVI) on histological sections is very important. In the International Consensus Panel during the St-Gallen Conference, 2005 (Goldhirsch et al, 2005) peritumoral vascular invasion, especially lymphovascular invasion (LVI), was included as a novel adverse prognostic factor and was used as guideline for postoperative adjuvant systemic therapies in early breast cancer.

Kahn and Marks (2002) in their study on 50 breast cancer cases reported an increase of 18% in LVI detection on D2-40 when compared to LVI detection on H&E staining. The increase in sensitivity of detection of lymph vessel invasion is attributed to the demarcation of lymphatic endothelium that stains positively for D2-40 around the tumor emboli. Van Eynden's study using D2-40 also showed that lymphovascular invasion was missed on H&E in 20% (peritumorally) and in 65% (intratumorally) of breast cancer cases. Marinho et al showed similar detection rates with 28.5% and 13.8% of LVI on D2-40 and H&E staining respectively. We also found similar results and LVI was identified by D2-40 immunostaining in 50% of the study population, while only 30% cases were detected on by routine H&E staining. Thus there was a significant increase of 20% Lymphovascular invasion detection rate. D2-40 identified tumor emboli that completely obliterated the lumen of the lymphatics. These tumor emboli could not be differentiated from nests of tumor cells on H&E sections. The study highlights the importance of D2-40 in the study of LVI. The significant increment in LVI detection using D2-40 as compared with H&E staining can be explained by the better visualization of lymphatic endothelium.

Tsutsui et al (2003) in their study on 252 breast cancer patients showed a significant correlation between tumor size and lymphatic vascular invasion (p=0.031). In our study the similar results obtained and the larger tumors were associated with more chances of lymphatic invasion and the correlation was statistically significant (p=0.008).

Van den Eynden et al (2006) showed that peritumoural LVI, but not peritumoral blood vessel invasion correlated with the presence of lymph node metastasis, which is still the only most important prognostic factor in breast cancer. There was a significant correlation between the LVI positivity and metastatic lymph node status (p=0.011). Further the LVI positivity increased with the number of positive lymph nodes on histopathology. This supports the use of LVI on predicting metastatic axillary lymph node status. Kahn et al (2002) demonstrated lymphatic invasion in 44% of LN negative and 86% of LN positive breast cancer

patients. In our study 63.3% cases with positive lymph nodes showed LVI compared to only 12.5% cases with negative lymph node. Similarly 80% cases having more than 10 positive lymph nodes showed LVI (P=0.013).

Another marker of aggressive behavior of the tumour is the histopathological grade, which independently affects disease-free and overall survival (Kuru et al, 2003). Gurleyik et al (2007) reported that of LVI increases with the grade of tumor. In our study, higher grade tumor was present in 80% of cases with LVI positive compared to 46.7% of patients with LVI negative. So there was more chance of lymphatic invasion in higher grade tumors but the correlation was statistically insignificant (p=0.058).

Gurleyik et al (2007) reported significant correlation between ER/PR status and lymphatic vascular invasion. In our study we found 84.6% LVI positive patients belonged to poorer prognostic hormonal receptor status combination (ER- PR- HER2/neu- or ER- PR- HER2/neu+), 15.4% belonged to intermediate prognostic group (ER± PR± HER2/neu±) and no patients in good prognostic group (ER+ PR+ HER2/neu) (p=0.046).

C. LMVD versus LVI

Schopmann et al (2004) showed association between LVI and mean LMVD in 374 cases. They observed positive correlation between LMVD and lymphatic vascular invasion (p=0.001). This significant association between LMVD and LVI could be explained through a lymphangiogenesis-induced increase of the "lymphatic window" providing tumor cells with more opportunities to enter into lymphatic vessels. The risk of developing lymph node metastasis increases significantly with the presence of lymphovascular invasion, so it can be regarded as the precursor of nodal involvement. Study done by Yasser et al (2008) also showed significant correlation between LMVD and LVI. Our result were similar to their results and 80% cases with low LMVD had negative LVI and 80% cases with high LMVD had positive LVI (p=0.001). Here we showed that breast cancers with high peritumoural lymphangiogenesis, measured with peritumoural LMVD, significantly more often invade these lymphatic vessels.

D. Color Doppler ultrasonography Vs. Lymphangiogenesis:

Yasser et al (2008) found significant correlation between microvessel density measured by CD31 with lymphatic microvessel density measured by D2-40 in breast cancer patients. Yang et al (2002) evaluated the correlation between color power Doppler sonographic measurement of breast tumor vasculature and immuno-histochemical analysis of microvessel density for the quantification of angiogenesis and a significant correlation was obtained ($P < 0.05$). But, to the best of our knowledge, to date very few studies in the literature reporting a correlation between color Doppler parameters (RI, PI, Vmax; indirectly assessing degree of angiogenesis) and lymphangiogenesis (LMVD & LVI). In our study, 90.9% patients with low RI had low LMVD and 57.9% with high RI had high LMVD (P=0.008). RI was also significantly correlated with Lymphatic vessel invasion (p=0.008). This is because of neovascularisation leads to increase in resistivity index and lymphatic microvascular density. Also, the chances of lymphatic vascular invasion by tumor cells increases with increasing RI value. Significant correlation between pulsatility index (PI) and lymphatic microvascular density (p=0.050) and lymphatic microvascular invasion (p=0.046) was found. When peak systolic velocity was correlated with LMVD and LVI, 65% of low Vmax tumors had low LMVD but tumors with high Vmax had equal numbers of high and low LMVD. It means there is linear correlation between Vmax and LMVD for low but not for high peak velocity tumors (p=0.429). Significant correlation was not found between Vmax and lymphatic vessel invasion (p=0.121).

CONCLUSION

In conclusion, in the present study, the degree of blood flow determined by color Doppler ultrasonography in terms of RI and PI was found to correlate with the well-known prognostic parameters while peak systolic velocity (Vmax) showed least correlation. The system of angiogenesis and lymphangiogenesis represents a potential new target for development of anti-cancer strategies. The data presented herein support the importance of LVI and LMVD assessment using D2–40 breast cancer for prognostic purpose. The higher positivity of LMVD and LVI correlated with known poor prognostic markers and color doppler. This highlights the use of these two in identification of patients who will have a poor prognosis even if they have early cancer without nodal involvement.

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