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Original Article:

Prognostic Significance of Lymphatic Vessel Density by D2-40 Immune Marker and Mast Cell Density in Invasive Breast Cancer: A Cross Sectional Study at Tertiary Care Hospital in South India

Authors:
Dhanlakshmi B, Postgraduate Department of Pathology, Adichunchanagiri Institute of Medical Sciences,
Amita K, Professor, Department of Pathology, Adichunchanagiri Institute of Medical Sciences,
Prashantha K, ACU- Centre for Research and Innovation, Faculty of Natural Sciences,
Adichunchanagiri University, B.G.Nagara, Mandya District, Karnataka 571448, India.

Address for Correspondence
Dr. K Amita,
Professor,
Department of Pathology,
Adichunchanagiri Institute of Medical Sciences,
Adichunchanagiri University,
BG Nagara, Nagamangala Taluk, Mandya district - 571448,
Karnataka, India.
E-mail: dramitay@bgsaims.edu.in.

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Abstract: Background: Tumour induced lymphangiogenesis plays a crucial role in metastasis and tumour progression. The intratumoural and peritumoral lymphatics are supposed to have different biological effects. The aim of present study was to investigate the correlation of intratumoral lymphatic vessel density (I LVD), peritumoral lymphatic vessel density (P LVD), intratumoral mast cell density (I MCD) and peritumoral mast cell density (P MCD) with prognostic parameters in primary breast carcinoma. **Methods:** Lymphangiogenesis was detected using D2-40 monoclonal antibody and mast cell by using toludine blue stain in 50 cases of primary breast carcinoma. Positively stained lymphatic vessels were counted at 40 x in dense lymphatic vascular foci (hot-spot) within the tumour. Chi square, ANOVA test and Pearson's correlation was applied to determine the relationship amongst various variables, with statistical significance set at $p < 0.05$. **Results:** Mean P LVD was significantly higher than I LVD (6.25±21 vs 2.75±2.27, $p < 0.005$). Significant correlation was noted between I LVD and P LVD and age, tumour laterality, tumour size and overall staging. However, there was no correlation between I LVD and P LVD with other important clinicopathologic prognostic markers like grade, lymph node status and lymphovascular invasion. MCD was higher in both intratumoral and peritumoral location as compared to normal tissue. There was an association noted between P MCD with pathological staging and perineural invasion. However, there was no significant association of I MCD and P MCD with other prognostic markers like grade and lymphnode status. No significant correlation was noted between I LVD, P LVD, I MCD and P MCD. **Conclusion:** The evidence from our study supports the utility of D2-40 stain in determining the lymphatic density in IBC. The study findings also establish the existence of lymphangiogenesis in both intratumoral and peritumoral location. For now, the data presented herein do not permit us to promote the utility of LVD and MCD as predictors of prognosis in invasive breast carcinoma.

Key Words: Mast cell, Lymphangiogenesis, Breast cancer, Prognostic factors

Introduction:

Breast cancer is the most common malignancy in females and despite efforts to early diagnosis and adjuvant therapy the morbidity and mortality continue to rise.[1] Recent research focuses on the effects of tumour microenvironment, especially angiogenesis and inflammation in tumour progression in breast cancer.[2] Lymphangiogensis is formation of new lymphatics in cancer bed and is linked to lymph node metastasis. Lymph vascular density (LVD) has been shown to be associated with large tumour size, high grade, lymph node metastasis and poor prognosis in breast cancer.[2] Mast cells have been linked to angiogenesis, lymphangiogenesis and there by tumour metastasis. In breast cancer, mast cell density correlates with poor prognostic factors.[2] Similarly, peritumoral mast cells are associated with poor prognostic factors as compared to intratumoral mast cells. An increase in MCD has been noted in lymph nodes with metastatic deposits as compared to uninvolved lymph nodes. However, few studies have documented a protective role of mast cells in breast cancer. [3] Mast cells stimulate angiogenesis and lymphangiogenesis by releasing vascular endothelial growth factor and endostatin. [2] Several studies on breast carcinoma have shown a positive correlation between MCD and microvascular density with various prognostic factors. [3] However, the relationship of mast cells and lymphangio-genesis in breast carcinoma has been explored only in a few studies. [1,4] Keser SH et.al., observed a positive correlation

between MCD and lymphangiogensis. [2] Further, Lopez AA et.al., in their study reported the anti lymphangiogenesis effect of mast cells. [3] Literature survey clearly indicates that reports on the correlation of mast cells and lymphangiogenesis in breast carcinoma is very limited. Hence, present study aims to determine the role of mast cells and LVD in breast carcinoma. Further the correlation between location of mast cells with other clinicopathologic prognostic markers is also carried out.

Materials and Methods

The present study was observational-analytical study conducted over a duration of 18 months from November 2018 to May 2020 at The Department of Pathology, Adichunchanagiri Institute of Medical Sciences, BG Nagara, India. The sample size was 50 and sampling was done by convenient sampling technique. All the surgically resected mastectomy specimens diagnosed as primary invasive breast carcinoma during the study period. Recurrent breast carcinoma, patients of primary invasive breast carcinoma who had undergone neoadjuvant chemotherapy or radiotherapy and patients with concurrent malignancy at other sites were excluded during the study.

Brief clinical history, age, site of lesion (quadrant), laterality, unifocal or multifocal, local examination findings, systemic examination clinical diagnosis were recorded, and ultrasound findings were recorded wherever necessary. Formalin fixed, paraffin embedded blocks for the tumour, peritumoral area and lymph node were prepared and stained with Haematoxylin and Eosin for histopathology analysis. Histopathological typing of tumour was done by recent WHO classification (2019) and grading was done by Nottingham modification of the Scarf Bloom Richardson method. Viable areas of tumour without necrosis were taken for counting mast cells and lymph vessels. For mast cell density, sections were stained with 1% of toluidine blue for high lighting the mast cells. Mast cells were counted in intratumoral and peritumoral area in ten high power fields under 400 x magnification and the mean of this was expressed as mean ± standard deviation. Peritumoral area was defined as peripheral tissue within 2 mm of the tumour margins while intratumoral area was defined as area within the tumour and surrounded by tumour cells.

LVD was evaluated by immunohistochemical staining with D2-40 antibody using standard procedure reported elsewhere. The LVD was counted under light microscopy using Weidner's method as mentioned in the study by Hoon Tan P et al. [8] The "hot spot" was then examined under high power field (X 400) to count the lymphatic vessels. Areas of necrosis were not considered for evaluation. Immunohistochemistry (IHC) was done as per the standard technique described in the literature. [9]

Statistical test applied: Continuous variables were expressed as mean ± standard deviation, while categorical variables were expressed as frequency and percentages.

Correlations between MCD and LVD were analyzed by Pearson's (r) correlation. ANOVA tests and independent t-test were applied for correlation. Correlation between MCD and LVD with other clinicopathologic prognostic markers were analyzed by chi square test with $p < 0.05$ significance. Statistical analysis was performed with SPSS version 19.0 software.

Results

Clinical and demographic details: In the present study, the majority of the patients belonged to the age group of 51 to 60 years (36%) and minimum number of cases belonged to 31 to 40 years (12%) of age group. Mean age was 42 ± 2 SD years. Higher number of cases presented with right sided tumour (60%) as compared to left side (40%) presentation. Maximum number of cases (16/50, 32%) occupied the central quadrant, followed by upper outer and upper inner quadrant in (8/15, 16% each).

Pathology details: Out of total 50 cases, maximum number of cases had tumour size between 2-5 cm. Most frequent histological type of breast carcinoma was infiltrating ductal carcinoma not otherwise specified (IDC NOS) (47/50, 94%), in the present study. There was one case each of invasive papillary carcinoma, invasive lobular carcinoma and metaplastic carcinoma reported during the study period.

Among all the 50 cases in the present study, the maximum number of cases were 24 (48%) in grade 2, whereas the minimum number of cases were 10 (20%) in grade 3. Tumour margin was involved in 39/50 cases (78%) while ductal carcinoma in situ (DCIS) was present in 13/50 cases (74%) and perineural invasion was noted only in 3 (6%) cases. In just two cases (4%) necrosis was present while in 32 cases (64%) lymphovascular invasion was present. 19 out of 50 cases (38%) did not have lymph node metastasis, while in 12 cases (24%) each, lymph node metastasis was noted in 1-3 and 4-9 lymph nodes. 25 out of 50 cases (50%) were in the stage 2 (48%) while 24 (48%) cases were in the stage 3 and one case was classified as stage 1(2%) as per the American Joint cancer committee Tumor Node Metastasis (AJCC TNM) classification system. In the present study, tumour-infiltrating lymphocytes were present in 33 (66%) cases.

Correlation of Lymphovacular density and Mast cell density and various clinicopathologic prognostic parameters:

ANOVA test was applied to determine the correlation between I LVD, P LVD, I MCD and P MCD with age (Table 1), site (Table 2) tumor laterality (Table 3) and tumor size (Table 4). As observed in Table 1, $p<0.029$ for I LVD, indicates a significant correlation observed between I LVD and age of the patient. However, there was no significant correlation between P LVD, I MCD, P MCD and age of the patient. There was no significant correlation between. I LVD, P LVD I MCD, P MCD with a tumour site. (Table 2)

There was a significant association between the tumour laterality and I LVD and P LVD (Independent t-test, $p<0.027$ $p<0.042$ respectively) (Table 3). However, there was no correlation between tumour laterality and mast cell density.

There was a significant correlation between tumour size and I LVD and P LVD with p value <0.001 in the variables respectively (Table 4)

There was no significant correlation between margin involvement and histologic type with I LVD, P LVD, I MCD and P MCD (Independent t test, $p >0.05$) (Table 5 & 6). There was no significant association between tumour histological grade and I LVD, P LVD and I MCD and P MCD (ANOVA test) (Table 7).

There was a significant correlation between I LVD and P LVD with tumour pathological T staging. (ANOVA test, p - 0.01) (Table 8). Similarly, there was a significant association between I LVD, P LVD and overall stage ($p < 0.001$) (Table 9). Further, it was observed that, there was a significant association between perineural invasion and P MCD with p value <0.023 (Independent t-test)

Table 1: Association between age and I LVD, P LVD, I MCD and MCD

	Age in years	N	Mean	Std. Deviation	95% Confidence Interval for Mean		p value
					Lower Bound	Upper Bound	
I LVD	31-40	6	1.367	.7941	.533	2.200	0.029
	41-50	17	1.876	1.3935	1.160	2.593	
	51-60	18	3.656	3.0492	2.139	5.172	
	>60	9	3.556	1.4689	2.426	4.685	
	Total	50	2.758	2.2795	2.110	3.406	
P LVD	31-40	6	2.467	2.1713	.188	4.745	0.525
	41-50	17	5.412	5.1120	2.783	8.040	
	51-60	18	4.794	5.7331	1.943	7.645	
	>60	9	3.467	1.4071	2.385	4.548	
	Total	50	4.486	4.6651	3.160	5.812	
I MCD	31-40	6	1.733	1.7374	-.090	3.557	0.725
	41-50	17	2.271	4.9198	-.259	4.800	
	51-60	18	1.100	1.2504	.478	1.722	
	>60	9	1.289	1.8306	-.118	2.696	
	Total	50	1.608	3.0934	.729	2.487	
P MCD	31-40	6	2.283	1.2497	.972	3.595	0.486
	41-50	17	3.235	2.5916	1.903	4.568	
	51-60	18	2.350	1.7099	1.500	3.200	
	>60	9	3.289	1.9953	1.755	4.823	
	Total	50	2.812	2.0575	2.227	3.397	

ANOVA tests was applied to determine the correlation between I LVD, P LVD, I MCD and P MCD with age of the patients. Table 19 shows that there was a significant correlation between I LVD and age of the patient ($p<0.029$). However, there was no significant correlation between. P LVD, I MCD, P MCD and age of the patient.

Table 2: Correlation between tumour site and I LVD, P LVD, I MCD and MCD

	Tumour Site	N	Mean	Std. Deviation	95% Confidence Interval for Mean		p value
					Lower Bound	Upper Bound	
I LVD	Central	16	3.988	2.5113	2.649	5.326	0.126
	Upper outer	8	1.800	1.1904	.805	2.795	
	Lower outer	3	3.067	.2309	2.493	3.640	
	Upper inner	8	2.800	3.1821	.140	5.460	
	Lower inner	1	2.400				
	Multi quadrant	14	1.836	1.6463	.885	2.786	
	Total	50	2.758	2.2795	2.110	3.406	
P LVD	Central	16	6.825	6.9147	3.140	10.510	0.190
	Upper outer	8	4.663	4.3084	1.061	8.264	
	Lower outer	3	4.067	.5774	2.632	5.501	
	Upper inner	8	1.950	1.5446	.659	3.241	
	Lower inner	1	2.200				
	Multi quadrant	14	3.414	1.7724	2.391	4.438	
	Total	50	4.486	4.6651	3.160	5.812	
I MCD	Central	16	2.163	5.1506	-.582	4.907	0.795
	Upper outer	8	1.950	1.5739	.634	3.266	
	Lower outer	3	2.933	2.5403	-3.377	9.244	
	Upper inner	8	.600	.6047	.094	1.106	
	Lower inner	1	.200				
	Multi quadrant	14	1.171	1.0462	.567	1.775	
	Total	50	1.608	3.0934	.729	2.487	
P MCD	Central	16	2.619	2.7862	1.134	4.103	0.946
	Upper outer	8	2.813	1.5459	1.520	4.105	
	Lower outer	3	3.000	2.4249	-3.024	9.024	
	Upper inner	8	2.350	1.0128	1.503	3.197	
	Lower inner	1	3.800				
	Multi quadrant	14	3.186	1.9607	2.054	4.318	
	Total	50	2.812	2.0575	2.227	3.397	

ANOVA tests was applied. There was no significant correlation between. I LVD, P LVD I MCD, P MCD with tumour site.

Table 3: Correlation between tumour laterality and I LVD, P LVD, I MCD and MCD

	Specimen laterality	N	Mean	Std. Deviation	95% Confidence Interval of the Difference		p value
					Lower	Upper	
I LVD	Right	30	3.263	2.7110	-.0222	2.5488	0.027
	Left	20	2.000	1.0839			
P LVD	Right	30	5.440	5.5672	.0881	4.6819	0.042
	Left	20	3.055	2.2862			
I MCD	Right	30	1.507	3.8207	-2.0659	1.5592	0.78
	Left	20	1.760	1.5350			
P MCD	Right	30	2.743	2.3847	-1.3772	1.0339	0.756
	Left	20	2.915	1.4897			

ANOVA tests was applied. There was no significant correlation between. I LVD, P LVD I MCD, P MCD with tumour site.

Table 4: Correlation between tumour size and I LVD, P LVD, I MCD and MCD

	Tumour Size	N	Mean	Std. Deviation	95% Confidence Interval for Mean		p value
					Lower Bound	Upper Bound	
I LVD	1-2cm	2	8.500	4.9497	-35.972	52.972	0.001
	2-5cm	36	2.600	1.9584	1.937	3.263	
	>5cm	12	2.275	1.5440	1.294	3.256	
	Total	50	2.758	2.2795	2.110	3.406	
P LVD	1-2cm	2	20.000	0.0000	20.000	20.000	<0.001
	2-5cm	36	3.997	3.8093	2.708	5.286	
	>5cm	12	3.367	2.2055	1.965	4.768	
	Total	50	4.486	4.6651	3.160	5.812	
I MCD	1-2cm	2	.800	1.1314	-9.365	10.965	0.933
	2-5cm	36	1.656	3.5465	.456	2.856	
	>5cm	12	1.600	1.5374	.623	2.577	
	Total	50	1.608	3.0934	.729	2.487	
P MCD	1-2cm	2	1.700	2.1213	-17.359	20.759	0.722
	2-5cm	36	2.814	2.1780	2.077	3.551	
	>5cm	12	2.992	1.7594	1.874	4.110	
	Total	50	2.812	2.0575	2.227	3.397	

ANOVA tests was applied. There was a significant correlation between tumour size and I LVD and P LVD with p value <0.001 in the variables respectively.

Table 5: Correlation between margin involvement and I LVD, P LVD, I MCD and MCD

	Margins	N	Mean	Std. Deviation	95% Confidence Interval of the Difference		p value
					Lower	Upper	
I LVD	Involved	11	2.190	2.9122	-2.3539	.9390	0.392
	Uninvolved	39	2.897	2.1413			
P LVD	Involved	11	3.860	3.4332	-4.2242	2.5339	0.617
	Uninvolved	39	4.705	4.9982			
I MCD	Involved	11	1.620	1.5390	-2.2646	2.2328	0.989
	Uninvolved	39	1.636	3.4262			
P MCD	Involved	11	3.050	1.8769	-1.0850	1.8363	0.607
	Uninvolved	39	2.674	2.0869			

Independent t-test was applied. There was no significant correlation between margin involvement with I LVD, P LVD, I MCD and P MCD.

Table 6: Correlation between histologic type and I LVD, P LVD, I MCD and MCD

	Histological type	N	Mean	Std. Deviation	95% Confidence Interval of the Difference		p value
					Lower	Upper	
I LVD	IDC	47	2.785	2.3424	-2.3026	3.2062	0.743
	Others	3	2.333	.9018			
P LVD	IDC	47	4.598	4.7828	-3.7530	7.4820	0.508
	Others	3	2.733	1.4742			
I MCD	IDC	47	1.609	3.1521	-3.7336	3.7506	0.996
	Others	3	1.600	2.4331			
P MCD	IDC	47	2.804	2.1059	-2.6178	2.3597	0.916
	Others	3	2.933	1.3013			

Independent t-test was applied. There was no significant correlation between margin involvement with I LVD, P LVD, I MCD and P MCD.

Table 7: Correlation between histological grade and I LVD, P LVD, I MCD and MCD

	Histological Grade	N	Mean	Std. Deviation	95% Confidence Interval for Mean		p value
					Lower Bound	Upper Bound	
I LVD	Grade 1	10	2.580	1.4921	1.513	3.647	0.296
	Grade 2	24	3.258	2.9615	2.008	4.509	
	Grade 3	16	2.119	1.1542	1.504	2.734	
	Total	50	2.758	2.2795	2.110	3.406	
P LVD	Grade 1	10	3.020	1.9966	1.592	4.448	0.487
	Grade 2	24	5.150	5.3576	2.888	7.412	
	Grade 3	16	4.406	4.7481	1.876	6.936	
	Total	50	4.486	4.6651	3.160	5.812	
I MCD	Grade 1	10	2.080	1.8648	.746	3.414	0.726
	Grade 2	24	1.733	4.1926	-.037	3.504	
	Grade 3	16	1.125	1.3404	.411	1.839	
	Total	50	1.608	3.0934	.729	2.487	
P MCD	Grade 1	10	2.280	1.6033	1.133	3.427	0.634
	Grade 2	24	2.858	1.9556	2.033	3.684	
	Grade 3	16	3.075	2.4813	1.753	4.397	
	Total	50	2.812	2.0575	2.227	3.397	

ANOVA tests was applied. There was no significant association between tumour histological grade and I LVD, P LVD and I MCD and P MCD.

Table 8: Correlation between pathological staging and I LVD, P LVD, I MCD and P MCD

	Staging PT	N	Mean	Std. Deviation	95% Confidence Interval for Mean		p value
					Lower Bound	Upper Bound	
I LVD	T1	2	8.500	4.9497	-35.972	52.972	<0.01
	T	29	2.703	2.0960	1.906	3.501	
	T3	16	2.050	1.4431	1.281	2.819	
	T4	3	3.233	.7506	1.369	5.098	
P LVD	T1	2	20.000	0.0000	20.000	20.000	<0.01
	T	29	4.459	3.9275	2.965	5.953	
	T3	16	3.000	2.5841	1.623	4.377	
	T4	3	2.333	1.3614	-1.049	5.715	
I MCD	T1	2	.800	1.1314	-9.365	10.965	0.747
	T	29	2.000	3.9042	.515	3.485	
	T3	16	1.213	1.3613	.487	1.938	
	T4	3	.467	.6429	-1.130	2.064	
P MCD	T1	2	1.700	2.1213	-17.359	20.759	0.283
	T	29	2.907	2.2846	2.038	3.776	
	T3	16	3.150	1.6191	2.287	4.013	
	T4	3	.833	.7638	-1.064	2.731	
I LVD	N0	21	2.771	2.6148	1.581	3.962	0.939
	N1a	10	2.620	1.7422	1.374	3.866	
	N2a	9	2.478	1.6277	1.227	3.729	
	N3a	10	3.120	2.7312	1.166	5.074	
P LCD	N0	21	4.881	4.4400	2.860	6.902	0.901
	N1a	10	4.660	5.6222	.638	8.682	
	N2a	9	3.467	3.2465	.971	5.962	
	N3a	10	4.400	5.6796	.337	8.463	
I MCD	N0	21	2.400	4.4627	.369	4.431	0.471
	N1a	10	.680	.8066	.103	1.257	
	N2a	9	1.156	1.1949	.237	2.074	
	N3a	10	1.280	1.7819	.005	2.555	
P MCD	N0	21	3.329	2.0105	2.413	4.244	0.121
	N1a	10	3.360	2.6896	1.436	5.284	
	N2a	9	1.633	.6481	1.135	2.131	
	N3a	10	2.240	1.9363	.855	3.625	

ANOVA tests was applied. There was a significant correlation between I LVD and P LVD with tumour pathological T staging. (p - 0.01).

Table 9: Correlation between overall stage and I LVD, P LVD, I MCD and MCD

		N	Mean	Std. Deviation	95% Confidence Interval for Mean		p value
					Lower Bound	Upper Bound	
I LVD	Stage 1	1	12.000				<0.01
	Stage 2	25	2.304	1.5331	1.671	2.937	
	Stage 3	24	2.846	2.1613	1.933	3.758	
	Total	50	2.758	2.2795	2.110	3.406	
P LVD	Stage 1	1	20.000				<0.01
	Stage 2	25	4.132	4.0231	2.471	5.793	
	Stage 3	24	4.208	4.3353	2.378	6.039	
	Total	50	4.486	4.6651	3.160	5.812	
I MCD	Stage 1	1	1.600				0.507
	Stage 2	25	2.120	4.1465	.408	3.832	
	Stage 3	24	1.075	1.3652	.499	1.651	
	Total	50	1.608	3.0934	.729	2.487	
P MCD	Stage 1	1	3.200				0.339
	Stage 2	25	3.228	2.3149	2.272	4.184	
	Stage 3	24	2.363	1.7383	1.628	3.097	
	Total	50	2.812	2.0575	2.227	3.397	

ANOVA tests was applied. There was a significant association between I LVD, P LVD and overall stage ($p < 0.001$).

Table 10: Correlation between lymphovascular invasion and I LVD, P LVD, I MCD and MCD

	Peri vascular invasion	N	Mean	Std. Deviation	95% Confidence Interval for Mean		p value
					Lower Bound	Upper Bound	
I LVD	Absent	18	2.347	1.5183	1.566	3.128	0.366
	Present	32	2.970	2.5822	2.054	3.885	
	Total	50	2.758	2.2795	2.110	3.406	
P LVD	Absent	18	3.188	3.0352	1.628	4.749	0.16
	Present	32	5.155	5.2310	3.300	7.009	
	Total	50	4.486	4.6651	3.160	5.812	
I MCD	Absent	18	.976	.9431	.492	1.461	0.305
	Present	32	1.933	3.7265	.612	3.255	
	Total	50	1.608	3.0934	.729	2.487	
P MCD	Absent	18	2.247	1.7008	1.373	3.122	0.166
	Present	32	3.103	2.1861	2.328	3.878	
	Total	50	2.812	2.0575	2.227	3.397	

Independent t-test was applied. There was an association of lymphovascular invasion with P LVD and P MCD, however it was not statistically significant ($p < 0.16$). There was no significant association of lymphovascular invasion with I LVD and I. MCD.

Table 11: Correlation between mean I LVD, P LVD, I MCD and P MCD

		Intra tumoral LVD	Peritumoral LVD	Intra tumoral MCD	Peri tumoral MCD
I LVD	Pearson Correlation	-----	.434	.095	-.206
	Sig. (2-tailed)	------	.002	.512	.152
	N	50	50	50	50
P LVD	Pearson Correlation	.434	-----	.102	.224
	Sig. (2-tailed)	.002	------	.481	.119
	N	50	50	50	50
I MCD	Pearson Correlation	.095	.102	-----	-.170
	Sig. (2-tailed)	.512	.481	------	.237
	N	50	50	50	50
P MCD	Pearson Correlation	-.206	.224	-.170	-----
	Sig. (2-tailed)	.152	.119	.237	------
	N	50	50	50	50

Pearsons correlation test was applied. There was a positive correlation between I LVD and P LVD and its statistically significant ($r = .434$, $p < 0.02$). There was a no correlation between I LVD and mean P LVD and it was not statistically significant ($r = -.170$, $p < 0.0237$).

There was an association of lymphovascular invasion with P LVD and P MCD, however it was not statistically significant (Independent t test, $p < 0.16$). There was no significant association of lymphovascular invasion with I LVD and I. MCD (Table 10).

There was a positive correlation between I LVD and P LVD and it is statistically significant (Pearson's correlation test, $r = .434$, $p < 0.02$). There was a no correlation between I LVD and mean P LVD and it was not statistically significant (Pearson's correlation test, $r = -.170$, $p < 0.0237$) (Table 11).

Discussion

Literature on the pathogenesis of breast carcinoma is expanding at a rapid pace. Recent experimental studies have focused on the role of tumour microenvironment (TME) in cancer progression. [5] Tumour microenvironment includes the stroma, immune cells, non-immune cells which has both pro carcinogenic and anticarcinogenic effects on the tumour. The two important components of TME in breast carcinoma are the lymphatics and the mast cells. Lymphatic invasion is an important predictor of metastasis in breast carcinoma. [6]

Several studies have approved lymph node metastasis as the single most important prognostic factor affecting clinical management and the patient's outcome. [7] Among the various cells present in the stroma of breast carcinoma, the role of mast cell is beginning to be explored recently.[3] In the present study, we have attempted to determine the role of intratumoral lymphatic vessel density (I LVD), peritumoral lymphatic density (P LVD), intratumoral mast cell density (I MCD) and peritumoral mast cell density (P MCD) as a predictor of prognosis in invasive breast carcinoma (IBC).

Intratumoral and peritumoral lymphatic density in invasive breast carcinoma:

In the last decade, several studies have been conducted to determine the role of LVD and its relationship with various clinicopathological prognostic parameters. [8,9] However, studies focussing on the location of lymphatics in the tumour are reported to be few. The concept of existence of lymphangiogenesis within the tumour area has been ruled out by few authors. The explanation they provide is that in the intratumoural area, the pressure exerted by the proliferating tumour cells and the interstitial fluid does not favour lymphangiogenesis. [8] Several markers to reveal lymphatics have been discussed in the literature like VEGFR-3, FLT4, LYVE-1, however none are as specific and sensitive as D2-40. [8] D2-40 specifically stains lymphatic endothelium with a high sensitivity. The D2-40 antibody is a monoclonal antibody directed towards podoplanin antigen, a mucin-type transmembrane protein which has affinity to lymphatic endothelial cells. [4] This antibody stains lymphatic endothelium in several tumours including lymphatics of lymphangioma, albeit sparing the endothelium of blood vessels, angioma and angiosarcomas. Arnaout-Alkarain A, et al [10] failed to demonstrate intratumoural lymphatics by using of D2-40 antibody in IBC. However various studies have now revealed the existence of intratumoral lymphatics and that I LVD and P LVD has different prognostic connotations. In the present study mean LVD in intratumoural area was 2.75±2.27 and the LVD was significantly higher in peritumoural area as compared to that within the intratumoural area. (Fig 1 A & B) These findings were in accordance with the literature. [8]

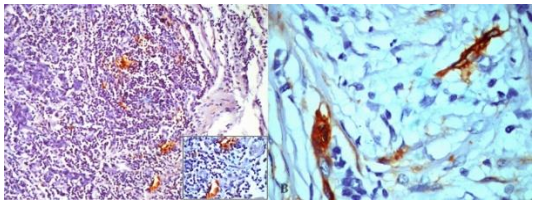

Figure 1 A: D2-40 positive staining of lymphatic vessels in peritumoral area (IHC, 100). **Inset shows high power view of 1A. Figure 1B:** D2-40 positive staining of lymphatic vessels in intratumoral area (IHC, 400)

I LVD was seen in 80% of the cases in the present study. Vleugel MM. et al [11] reported similar finding of presence of I LVD in 80% of non-inflammatory IBC and 82.5% of inflammatory IBC. However various other studies reported lower frequency of I LVD ranging from 12% to 50%. [11,12] Surprisingly in recent studies Padera TP et al, [13] Jackson DG et al,[14] and Charijs R et al[15] in cases of IDC, authors did not observe any intratumoural lymphatics and in all the above mentioned studies, LVD was demonstrated by D2-40 antibody. Similarly, Vleugel et al. [11] and Williams et al. [16] failed to demonstrate lymphatics in breast cancer by LYVE1 lymphatic marker. The contrasting results discussed herewith can be explained by the technical and interpretational variances. One of the major limitations in demonstrating LVD is the interobserver variation in counting the vessels. [16] Our study favours the existence of intratumoral lymphatics.

LVD was seen in 100% of cases in the present study. The mean value of P LVD was significantly higher than the I LVD. Similar findings were reported by the other studies. [9]

Morphology of the lymphatics differ in the peritumoral and intratumoral area that evince attention. In the present study intratumoral lymphatics were usually collapsed and found to be small. In contrast, in peritumoral area, the lymphatics were large and dilated. (Fig 2 A & B))These findings were similar to that reported by several other authors in the literature. [11,17]

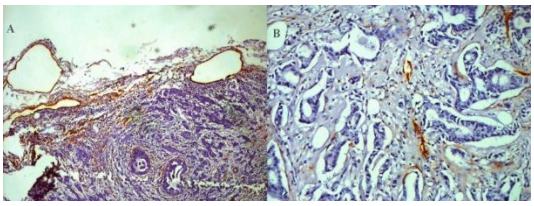

Figure 2 A: D2-40 positive lymphatics in peritumoral area. Note-The lymphatics are large and open in peritumoral area. (IHC, 100) **Figure 2 B:** D2-40 positive lymphatics in intratumoral area. Note- The lymphatics are small and collapsed in intratumoral area. (IHC, 100)

Taking into consideration the pressure exerted by the proliferating cells in the center of the tumour, the intratumoral lymphatics are small and collapsed. Correspondingly, the dilated and large lymphatics in the peritumoral area picks up the tumour cells from the invasive edges of the tumour and paves way for metastatic deposits. [18]

LVD and clinicopathological prognostic parameters:

Among the various clinical prognostic parameters analyzed in the present study, LVD showed significant correlation with the age and tumour laterality. (Table 1) The mean LVD was observed to increase with age of the patient. Similar findings were reported by Norhisham N F et al. [18] The correlation between tumour laterality and LVD has not been discussed in detail in the currently existing literature.

In the present study, both I LVD and P LVD were strongly correlated with tumour size. (Table 4) There was decrease in LVD with increase in the size of tumour. Zhao Yet al [19] reported similar findings. Increase in the intratumoural pressure or destruction of lymphatics by expanding tumour cells would have led to these observations. [29]

Apart from this, in the present study, significant correlation of I LVD and P LVD was noted with overall staging of the tumour. This finding is substantiated by many other studies reported in the literature. [2]

Several studies have established significant correlation between LVD and various well established clinicopathological parameters. In cohort of 69 IDC cases, Kandemir N O et al [9] observed a significant correlation between P LVD and lymphovascular invasion (LVI), tumour stage, tumour grade and lymph node status. However, they could not find any association between I LVD and the above discussed parameters. Similar findings were described by Norhisham N F et al [18],who reported P LVD as a significant predictor of distant metastasis in a cohort of 58 cases of IDC.

In the present study no correlation was noted between LVD and histological grade, LVI and lymph node status. Lymphangiogenesis and its role in promoting lymph node metastasis are related to the newly formed lymphatics being functional or not. The functionality could be assessed by determining the Ki67 activity in the lymphatic endothelial cells. In a study reported by Wang X et al [20], lymphatics did not show Ki67 positivity which in turn was not associated with clinicopathological prognostic parameters.

In the study involving 92 cases, Britto A V et al [21] did not demonstrate any association between LVD and other clinicopathological prognostic parameters. Few other studies have also reported similar findings. [2] The simple fact that more lymphatics at tumour periphery would provide a route for more tumour cells to metastasize is disproved by these studies. Correspondingly, authors explained that the process of metastasis is a complex phenomenon and it may not be possible to depict the intricacies of this complex process by the current static methods used. Guldur M E et al [22] did not find any association between LVD and other clinicopathological prognostic parameters in renal cell carcinoma. These findings were corroborated by Mohseni M G et al. [23]

Lymphovascular invasion

The term LVI in breast carcinoma correlates with the involvement of lymphatics or blood vessels or both by tumour cells in the peritumoral area. Introduced by Gallon Committee in 2009, several studies have independently established the value of LVI in predicting prognosis in lymph node negative breast cancer patients. [7] Despite these facts, LVI has not been incorporated in many internationally recognized staging systems. The reason being that several studies have failed to establish the clinical significance of LVI especially its use in therapeutic decision making. The discrepancies in the studies can be explained by several factors like the small sample size, discrepancies in the methods used for differentiating lymphatic and vascular invasion and failure to establish significant relationship of LVI with various clinicopathologic prognostic parameters as well as their significance in different clinical subgroups. [7] Attempts were made by several authors to use special stains including immunohistochemistry to improve sensitivity and specificity for detection of LVI. D2-40 antibody introduced in last decade has been studied by several authors to detect LVI. (Fig 3) Lymphovascular invasion has been reported in 22 -48% of cases. [24]

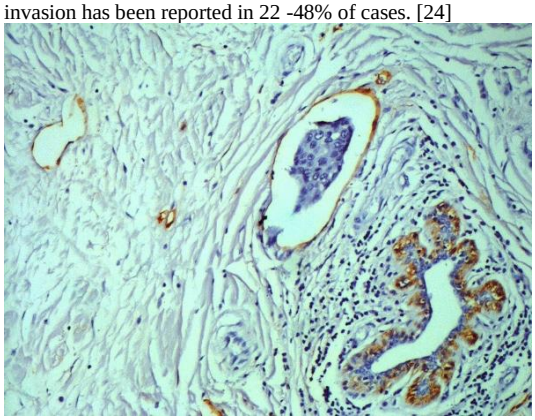

Figure 3: D2-40 positive lymphatics with tumour emboli (IHC, 100)

In the present study, lymphovascular invasion was noted in 64% of cases (32/50). There exists a large discrepancy in the lymphovascular invasion noted among various studies thereby necessitating further studies to standardize the reporting of BVI across various centres around the globe. Though use of IHC would increase the accuracy in reporting LVI, its routine use in detecting LVI is restricted in resource poor setup.

Literature on D2-40 application in identifying LVI is expanding with several studies establishing D2-40 positive lymphatic channels as independent prognostic marker for lymph node metastasis in early breast carcinoma. [6,25,26] In a cohort of 360 patients, Gujam et al [24] observed significant association of D2-40 LVI with tumour recurrence and cancer free survival, especially in node negative and triple negative breast cancer.

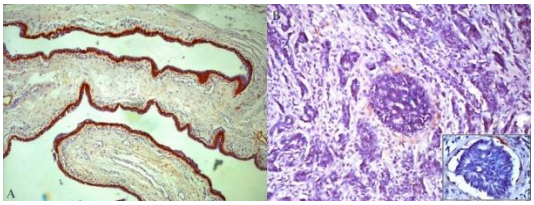

Figure 4 A: D2-40 immunostaining of myoepithelial cells in benign breast tissue. (IHC, 100). **Figure 4 B:** Section shows weak and discontinuous staining of myoepithelial cells ductal carcinoma in situ by D2-40 immunostaining (IHC, 100).Inset shows high power view of 1B.

Concern has been raised regarding the staining of myoepithelial cells by D2-40. The myoepithelial cells in normal breast (Fig 4 A), usual ductal hyperplasia (UDH), atypical ductal hyperplasia (ADH) and ductal in situ carcinoma (DCIS) (Fig 4 B) take up the D2-40 stain. [6] In solid type of DCIS, the small ducts filled with the tumour cells and surrounded by D2-40 positive myoepithelial cells may mimic LVI. Likewise, the retraction artifact surrounding the tumour cells due to tissue handling and fixation, especially when the myofibroblast in the stromal cells take D2-40 stain, makes the distinction difficult. However, awareness of the staining pattern would eliminate the false positive results. D2-40 staining of myoepithelial cells is weak and discontinuous compared to strong reactivity noted in the lymphatic endothelium (Fig 5). [18] Also, the shape and size of the individual structure helps to delineate the entity with confidence. In the present study, apart from staining the lymphatic endothelium, the D2-40 stain was seen to highlight the myoepithelial cells in normal ducts, UDH, ADH with few DCIS foci, as noted in the literature cited above.

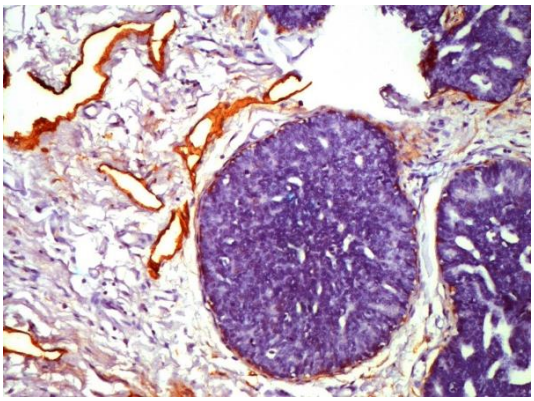

Figure 5: Comparison of D2-40 staining of ductal carcinoma in situ component and the lymphatics (IHC, 400)

Substantial evidence suggests a strong relation between LVI with tumour size, tumour grade, lymph node status and recurrence. LVI has also been shown to impact cancer free survival in early breast cancer. He et al in a study of 255 patients, reported LVI to be an independent prognostic factor in not only lymph node negative and positive patients but also in the various molecular subtypes of cancers. Ansari et al [27] though reported an association between LVI and tumour grade, tumour size, the association was not statistically significant. Similarly, they didn't find any association of LVI with tumour stage and hormonal receptor status. Some studies have shown variable association between LVI and other known prognostic characteristics. In present study there was no association between LVI and any other known clinicopathological variables. In a study by Gujam et al [24], application of univariate analysis showed significant

correlation between tumour grade, lymph node status with LVI, however the multivariate analysis on the same parameters did not reveal any such correlation. Similarly, the findings of a recent study by Bent Ejlertsen et al [28], involving longitudinal cohort of 16,178 breast cancer patients, did not support the association of LVI with prognostic parameters. They reported that the presence of LVI should not be used to categorize the patients from low risk to high-risk category. Another study involving 240 lymph nodes with invasive breast cancer cases, Gudlaugsson et al [25] observed that LVI detected by D2-40/P63 could predict prognosis only in patients more than 53 years old. The findings in the present study corroborate with the findings of studies are mentioned above. The discrepancy in the association between LVI and various other clinicopathological parameters has been mentioned in the literature. This inconsistency has been attributed to the variation in the batch of monoclonal antibodies used, method of sampling and the sample size.

No significant association was noted between the histologic type and LVD in the present study, it is in accordance with the reported literature

Lymphovascular invasion and lymphatic vessel density:

Lymphangiogenesis in the peritumoural area occurs due to stimulation of the VEGFR 3 receptor, a phenomenon explained by increased expression of VEGF C and D in the peritumoral area. These peritumoral lymphatics in tumour capture tumour cells and promote the spread. LVD correlated significantly with vascular lymphatic invasion in various studies in the literature. [8] In the present study, strong correlation existed between P LVD and LVI (p- 0.16) though it was not statistically significant. The crucial prognostic role of peritumoral lymphangiogenesis in LVI is supported by our data. Nakamura Y et al reported association of Flt-4 positive LVD with lymph node status and poor prognosis [13] Ansari et al [27] observed LVD to be comparatively higher in LVI positive patients as compared to those who were negative for LVI.

Mast cell density:

The role of chronic inflammation in carcinogenesis was postulated way back in the early part of the 19th century. [3] Inflammatory cells in the tumor stroma affect the microenvironment of the tumor either by encouraging tumor progression to more progressive stages or by engaging in a defensive effect. Paul Ehlrich in 1978, for the first time reported the presence of mast cells in tumour tissue. [8] Since then I MCD and P MCD has been reported in several tumours like thyroid, pancreas, stomach, prostate and breast carcinoma. Good number of studies has shown mast cells to be associated with poor prognosis in breast carcinoma. [2] Mast cell secrete MMP 9 and other proteases which degrade extracellular matrix in breast carcinoma favouring invasion. Albeit equal number of studies have provided evidence for its protective role in breast carcinoma. [2] Substantial amount of experimental data also favour several antitumoral properties of mast cells. [3]

Several stains and markers like toluidine blue, alcian blue, tryptase, carboxypeptidase have been used to demonstrate mast cells in cancer tissue. In the present study 1% toluidine blue was used to highlight mast cells and it was observed that, MCD was significantly higher in the breast carcinoma as compared to normal tissue (Fig 6 A &B). This finding is similar to that reported by several other studies. [2 In contrast, a study by Kabiraj A et al[29], mast cells were lower in oral squamous cell carcinoma as compared to that in normal tissue. This phenomenon though rare was attributed to the failure of mast cells to migrate owing to the modification in the tumour microenvironment during the process of tumour evolution. In the present study, the mast cells were seen in all the tumors both in the peritumoural and intratumoural location (Fig). However, there was no significant difference in the mean mast cell density in both the locations. The stem cell factors and chemokines secreted by tumour cells acts as chemoattractant for the mast cells, which in turn affects TME by stromal remodelling. [2,30]

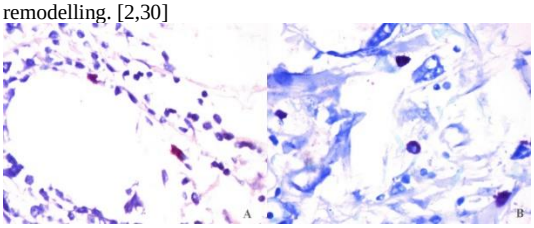

Figure 6A: Mast Cell seen in intratumoral area in infiltrative ductal carcinoma (toluidine blue,× 400)**Figure 6B:** Mast Cell seen in peritumoral area in infiltrative ductal carcinoma (Toluidine blue,× 400)

In the present study, no significant association was noted between MCD and various clinicopathological parameters like age, tumour size, grade and histological type. In studying mast cells in renal cell carcinoma, M0hseni MG et al [23] analysed the correlation of MCD with various clinicopathological parameters. But, the author failed to demonstrate any association between mast cells and various clinicopathological factors. Ren Shuyue et al [26] in their study involving 48 of breast cancer cases attempted to correlate mast cell count, area occupied by mast cells positive for tryptase and microvessel density and endothelial area. They observed a strong association between mast cell count and microvessel density. However, there was no significant association between mast cell density with main clinicopathological factors.

In the present study, there was an association noted between peritumoural MCD with pathological staging and LVI, though this was not statistically significant. Further, P MCD was also significantly associated with perineural invasion. Location of mast cells within the tumour reflects its distinct role in tumour progression. [2,4,28,30] Though Keser SH et al[2] documented an association of I MCD with poor prognostic factors in breast cancer, the association of P MCD with prognostic parameters was insignificant.

Evidence for the role of mast cells in promoting angiogenesis has been documented in several studies. [4,29,30] In the present study, no significant association was observed between mast cell density and LVD. Lack of prognostic significance of mast cells in promoting angiogenesis has been noted in several studies. [22,23] The explanation for this occurrence has been linked to the massive degranulation of the mast cells leading to diagnostic difficulty. However, these studies have elucidated the role of mast cells in angiogenesis and studies on its role on lymphangiogenesis are few. Keser SH et al[2] observed a strong association between MCD and LVD in breast carcinoma. Contrastingly, Britto et al[25] did not find any association between MCD and LVD. This conflicting data may be due to variation in the methodology of staging, counting techniques, sampling procedure and interobserver variability.

The lack of association between LVD, MCD and select clinicopathologic progrnostic parameters in the present study, as noted in few of the studies in literature [20-23], is not attributed to selection bias or reporters bias, as all the cases followed unform selection process. All cases were of IDC and interobserver variation was minimum. However, the deviation reported could be explained by the retrospective nature of the study, where in loss of anitgen expression could have occurred. Similalry, use of antibodies from different sources may have led to the discrepant results noted in the present study.

Conclusion

The evidence from our study supports the utility of D2-40 stain in determining the lymphatic density in IBC. Present findings

also establish the existence of lymphangiogenesis in both intratumoral and peritumoral locations. Significant association between lymphangiogenesis and peritumoral mast cell density with pathological staging of breast cancer makes it an important predictive factor of disease prognosis. Similarly, presence of higher mast cell density in both intratumoral and peritumoral location, as compared to normal tissue, establishes its role in carcinogenesis.

Further longitudinal prospective studies with large sample and follow up are required to determine the effect of lymphangiogenesis and mast cell influence on potential positive clinical, prognostic and therapeutic impact on breast cancer.

Limitations: The major limitation of the present study was the small sample size and the retrospective nature of the experimental design, as a result of which we could not determine the relationship of lymphangiogenesis and mast cells on the survival of the patients.

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