Intratumoural and peritumoural lymphangiogenesis in canine mammary tumour linked to tumour spread and poor survival

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Canine mammary tumour (CMT) is an excellent, naturally occurring animal model for human breast cancer. Most research, so far, has focused on angiogenesis as a mode of tumour invasion and metastasis, but the role of lymphangiogenesis in mammary tumours is scarcely documented. The present study was conducted on 45 cases of CMT and the extent of lymphangiogenesis, demonstrated as intratumoural vessel density (IVD) and peritumoural vessel density (PVD) was determined by immunohistochemical staining with lymphatic markers, viz. podoplanin, LYVE-1, PROX 1 and VEGFR-3. The highest vessel density in intratumoural area was recorded with VEGFR-3 (35.94 ± 3.45), followed by podoplanin (31.95 ± 2.77), LYVE-1 (11.11 ± 2.20) and PROX 1 (7.62 ± 1.11). In peritumoural areas, the vessel density was highest with podoplanin (11.48 ± 1.32), followed by VEGFR-3 (7.69 ± 0.51), LYVE-1 (5.19 ± 0.96) and PROX 1 (3.48 ± 0.48). The lymphatics in intratumoural areas were small, thin and collapsed, whereas the peritumoural lymphatics were large and conspicuous. Overall survival was less in patients with high lymphatic density in peritumoural areas for podoplanin, and with high vessel density in intratumoural areas for VEGFR-3. The survival was also low in cases with lymphatic tumour emboli. It was concluded that both intratumoural and peritumoural lymphangiogenesis seemed to play significant role in tumour invasion and spread rather than peritumoural lymphatics. Moreover, the podoplanin and PROX 1 were found to be more specific markers of lymphangiogenesis.

Keywords: Canine mammary tumour, immunohistochemistry, Kaplan Meier survival analysis, lymphangiogenesis.

BREAST cancer is the most common malignant tumour in women, especially in the developing world[1,2]. Similarly, spontaneous mammary gland tumour is most frequent in canines, especially in the unspayed female dog[2]. Studies on canine mammary tumour have proved to be an excellent animal model for human breast cancer[2,3]. The World Health Organization has also proposed canine mammary tumour (CMT) to be one of the natural models for human breast cancer[4,5].

Nearly half of the mammary tumours that occur in bitches are malignant[6]. They affect middle-aged bitches[5,7] and often have a poor prognosis. Traditionally, the role of lymphangiogenesis in cancer metastasis has been overshadowed by greater emphasis on angiogenesis. However, the study and understanding of the lymphatic system and its role in cancer progression has been made possible in recent years by the discovery of proteins that are specifically expressed on the lymphatic endothelium and may serve as markers for it[8].

More recently, the role of lymphangiogenesis in cancer invasion and particularly metastasis has been studied in several human neoplasms, including breast cancer[9] and sparsely in feline mammary tumour[10]. However, there appears to be little information on the role of lymphatics and lymphangiogenesis in CMT. In the present study, the expression of various markers of lymphatic vessels, viz. podoplanin, LYVE-1, PROX 1 and VEGFR-3 was determined to elucidate the process of lymphangiogenesis and its role in tumour spread and prognosis in canine mammary neoplasia.

Materials and methods

Source of samples

The present study was conducted on tissue samples of 45 confirmed cases of CMT available in the Department of Veterinary Pathology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana (Punjab, India).

Histopathology

After fixation in 10% neutral buffered formalin, the tissue samples were processed and stained with routine hematoxylin and eosin (H&E) technique[11]. Multiple H&E stained

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Table 1. Panel of antibodies used for demonstration of lymphangiogenesis in CMT

Antibody	Company	Immunoglobulin class	Dilution used
Podoplanin*	GeneTex International Corporation, Taiwan	IgG	1 : 50
VEGFR-3**	GeneTex International Corporation, Taiwan	IgG	Ready to use
PROX 1*	Abgent Flanders Court, San Diego, CA	IgG	1 : 50
LYVE-1*	Novus Biologicals, Southpark Way Littleton, CO USA	IgG	1 : 50

*Polyclonal rabbit anti-human; **polyclonal rabbit anti-human/anti-mouse/anti-rat.

tissue sections from various sites in each case were screened for the presence of lymphatics and other related changes in both intratumoural and peritumoural areas.

Immunohistochemistry

Paraffin embedded tissue sections (4–5 μm) were subjected to immunohistochemistry using immPACT™ DAB peroxidase substrate kit (Vector Laboratories, Burlingame, CA, USA). The sections were dewaxed and rehydrated by dipping in EZ-AR™ common solution (BioGenex Laboratories Inc., San Ramon, California, USA). The antigen was retrieved using EZ-AR3 solution (BioGenex Laboratories Inc., San Ramon, California, USA) and later in EDTA buffer for 10 min each at 95°C. The endogenous peroxidase was quenched with a solution of 3% H_2O_2 in methanol for 15 min at room temperature in a humid chamber. The sections were then incubated with ready-to-use power block (BioGenex Laboratories Inc., USA) to block non-specific protein binding for 15 min at room temperature in a humidified chamber. Later, the sections were incubated with primary antibodies (Table 1) for 60 min at room temperature in a humidified chamber, followed by incubation in polymer HRP (BioGenex Laboratories Inc., USA) for 30 min at room temperature in a humidified chamber. After each step, three washings in PBS for 3 min were done.

The antigen-antibody-peroxidase reaction was developed with freshly prepared 3,3′-diaminobenzidine (DAB) solution. Sections were washed in distilled water for 5 min and counterstained with Gill's haematoxylin (Merck, Germany) for 30 sec and washed in running tap water for 5 min. Finally the slides were dehydrated in ascending grades of alcohol, cleared in xylene and mounted in DPX. For each antibody, a negative control was run by replacing primary antibody with phosphate buffered saline (PBS) buffer.

Assessment of immunohistochemical staining and scoring

The immunohistochemical staining score was determined semi-quantitatively for podoplanin, LYVE-1, VEGFR-3 and PROX 1 described as IRS[12]. All the sections were examined separately by two pathologists for independent subjective assessment. Expression/quantity scores from 0 to 5 were respectively assigned: 1 (1–10% positive tumour cells), 2 (11–30% positive tumour cells), 3 (31–50% positive tumour cells), 4 (51–80% positive tumour cells), and 5 (81–100% positive tumour cells) respectively. The staining intensity was rated on a scale of 0 to 3 (0 = negative, 1 = weak, 2 = moderate and 3 = strong). The raw data were then converted to Immuno Reactive Score (IRS) by multiplying the quantity and staining intensity scores. An IRS score above the median (7 or higher) was considered as high, and between 0 and 6 as low.

Assessment of vessel counts

In order to obtain comparative results from each marker, a uniform criteria as follows, was applied rather than variable criteria for different markers used in previous studies[13]. The five most vascularized areas were selected (hot spots) at 10×. In order to visualize small lymphatic vessels, besides medium and large ones, they were counted in these five intratumoural and peritumoural fields each at 40× and the final count was expressed as mean ± SE (standard error). Moreover, lymphatic vessel invasion (LVI) was determined by the presence of tumour thrombo-emboli and/or invasion of lymphatic vessel wall[14].

Statistics

The mean ± SE was calculated by one-way ANOVA post-hoc multiple comparisons (Ducnan) for the data pertaining to IRS, intratumoural vessel density (IVD) and peritumoural vessel density (PVD) for all markers. For comparison of clinicopathological parameters with intratumoural-LVD (I-LVD) and peritumoural-LVD (P-LVD) of each marker, *t*-test was done. The median value for each marker was considered and the values above and below the median were designated as high and low vessel densities. In addition, the data regarding survival in intratumoural and peritumoural areas with high and low lymphatic vessel density (using Podoplanin and VEGFR-2) was evaluated applying Kaplan Meier analysis. All the data was computed using Statistical Package for Social Sciences (SPSS) for Windows version 11.01 (SPSS INC, Chicago, Illinois).

Table 2. Comparison of IRS, I-LVD and P-LVD of different markers in subtypes of CMT

			CMT (n = 40)	Carcinoma (n = 22)	Carcinosarcoma (n = 13)	Sarcoma (n = 5)
Podoplanin	IRS	Positive cases	40	22	13	5
		Mean ± SE	10.63 ± 0.59[a]	10.64 ± 0.80[a]	11.46 ± 0.85[a]	8.40 ± 2.23[a]
	I-LVD	Positive cases	40	22	13	5
		Mean ± SE	31.95 ± 2.77[b]	31.80 ± 3.75[b]	39.82 ± 4.10[b]	12.13 ± 2.23[a]
	P-LVD	Positive cases	38	22	11	5
		Mean ± SE	11.48 ± 1.32[a]	13.56 ± 2.17[a]	8.89 ± 1.42[a]	9.00 ± 0.91[a]
LYVE-1	IRS	Positive cases	28	15	11	2
		Mean ± SE	4.60 ± 0.78[ab]	4.23 ± 0.96[ab]	6.31 ± 1.57[b]	1.80 ± 1.56[a]
	I-LVD	Positive cases	23	12	9	2
		Mean ± SE	11.11 ± 2.20[a]	10.14 ± 2.63[a]	11.87 ± 3.27[a]	13.40 ± 11.43[a]
	P-LVD	Positive cases	24	13	9	2
		Mean ± SE	5.19 ± 0.96[a]	5.82 ± 1.56[a]	5.15 ± 1.17[a]	2.53 ± 1.57[a]
PROX 1	IRS	Positive cases	25	13	10	2
		Mean ± SE	2.37 ± 0.46[ab]	2.23± 0.63[ab]	3.32 ± 0.84[b]	0.60 ± 0.40[a]
	I-LVD	Positive cases	25	13	10	2
		Mean ± SE	7.62 ± 1.11[a]	7.70 ± 1.66[a]	8.38 ± 1.40[a]	5.33 ± 3.83[a]
	P-LVD	Positive cases	24	12	10	2
		Mean ± SE	3.48 ± 0.48[a]	3.08 ± 0.64[a]	4.54 ± 0.76[a]	2.47 ± 1.58[a]
VEGFR-3	IRS	Positive cases	39	22	13	4
		Mean ± SE	9.05± 0.50[ab]	9.45 ± 0.68[b]	9.31 ± 0.73[ab]	6.60 ± 1.66[a]
	I-LVD	Positive cases	39	22	13	4
		Mean ± SE	35.94 ± 3.45[a]	40.17 ± 4.67[a]	33.87 ± 3.90[a]	22.73 ± 15.49[a]
	P-LVD	Positive cases	39	22	13	4
		Mean ± SE	7.69 ± 0.51[b]	8.12 ± 0.54[b]	8.10 ± 1.05[b]	4.73 ± 1.49[a]

Values with superscript within a row differ significantly at a significance level of 0.05.

Results

Histopathology

Only 40 cases out of 45, showed significant intratumoural and peritumoural components. These cases were selected for further immunohistochemical staining and split up into carcinoma (n = 22), carcinosarcoma (n = 13) and sarcoma (n = 5) sub-types according to latest classification of CMT[13].

Additionally, tumour infiltrating macrophages and mononuclear cell aggregates were detected in close proximity to areas showing increased intratumoural vascularization.

Immunohistochemistry

(a) Assessment of immunohistochemical staining and scoring – IRS: A comparison of different histological subtypes revealed that the carcinosarcoma had high IRS for all the markers as compared to other two subtypes (Table 2). PROX 1 (Figure 1) was expressed in the nucleus of lymphatic endothelial cells (LEC). Podoplanin expression was limited to the cytoplasm of neoplastic cells and an increased expression of podoplanin was recorded in cases showing Epithelial Mesenchymal Transition (EMT) and at the invasive front (Figure 2), whereas variable cytoplasmic (Figures 3 and 4) as well as nuclear staining was observed for VEGFR-3. In addition to the expression in tumour cells, expression for various markers was also observed in tumour emboli (Figure 5). Furthermore, LYVE-1 staining was also seen in tumour-infiltrating macrophages and mononuclear cells (Figure 6), besides tumour cells and their emboli. In the present study, most of the small intratumoural lymphatics showed a constant phase of formation by splitting or sprouting mechanism (Figure 3).

(b) Assessment of vessel counts: In general, the lymphatics in intratumoural areas were small, thin, numerous, at times collapsed and not clearly discernible, when compared with the peritumoural lymphatic vessels, which were larger, fewer and more conspicuous as determined by a specific marker (Figures 1, 3 and 4). I-LVD and P-LVD were observed highest with VEGFR-3 and podoplanin, followed by LYVE-1 and PROX 1 (Table 2).

Comparison of clinicopathological features with I-LVD and P-LVD of various markers

Various clinicopathological features (Table 3) were compared and it was found that I-LVD had a significant correlation with the increase in size (P = 0.017) of the primary tumour and the presence of EMT (P = 0.015) as revealed by podoplanin expression. In addition, grade of

the tumour ($P = 0.031$) was significantly associated with P-LVD as determined by podoplanin staining. Moreover, I-LVD was significantly correlated with lymph node (LN) metastasis ($P = 0.041$) as was elucidated by LYVE-1 immunoreaction.

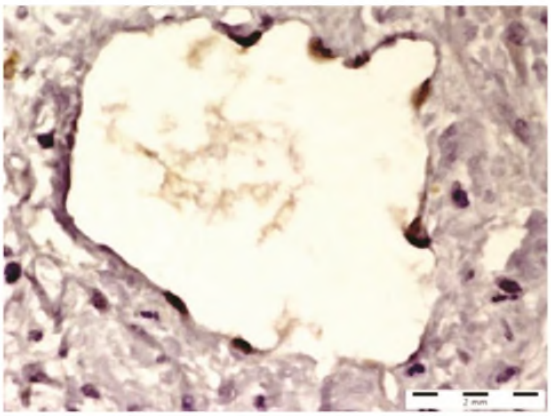

Figure 1. Nuclear staining in the lymphatic endothelial cells in peritumoural areas with PROX 1. Original magnification 1000×.

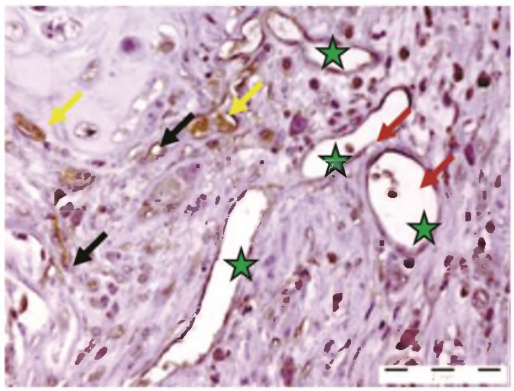

Figure 2. Podoplanin stainig showing intratumoural lymphatic vessels, small (black arrows) and medium sized (green stars) in an area showing marked EMT. Additionally, tumour cell invasion (red arrows) and thrombo-embolism (yellow arrows) is also conspicuous. Original magnification 400×.

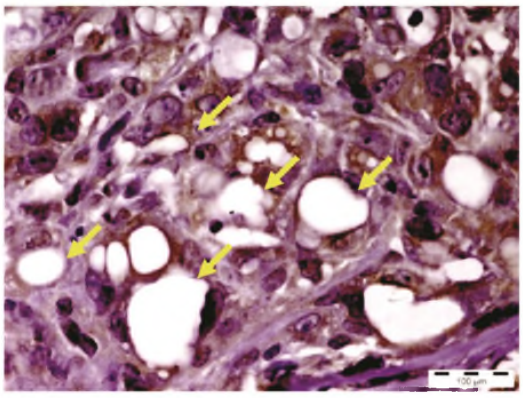

Figure 3. Moderate to marked cytoplasmic staining of tumour cells besides small intra-tumoural lymphatics (yellow arrows) with VEGFR-3. Original magnification 400×.

Survival analysis

Survival analysis was carried for podoplanin, PROX 1 and VEGFR-3. For podoplanin it was observed that the least survival period was seen in peritumoural areas having high vessel density. Similarly for VEGFR-3, survival

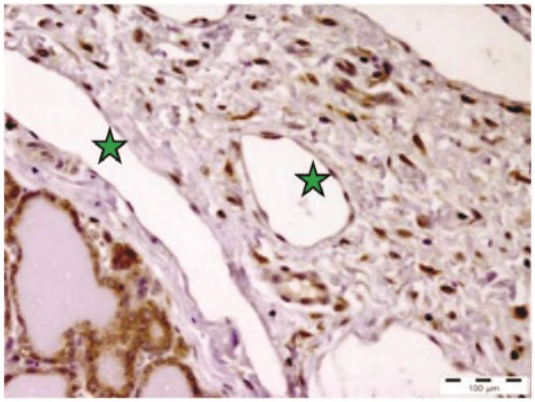

Figure 4. Prominent nuclear staining and mild cytoplasmic staining in the conspicuous peritumoural lymphatics (green stars) with VEGFR-3. In addition, neoplastic tumour cells also show positive immunostaining. Original magnification 400×.

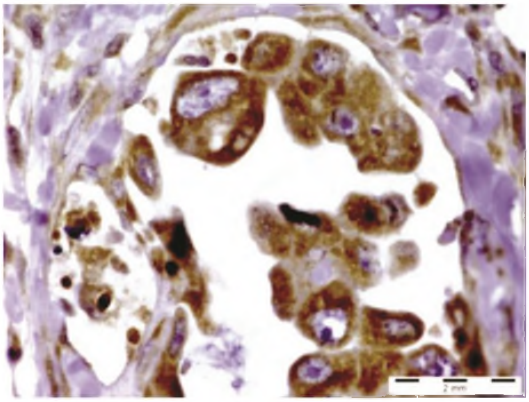

Figure 5. Tumour emboli in peritumoural lymphatic vessel besides a few fibroblasts, as stained with LYVE-1. Original magnification 1000×.

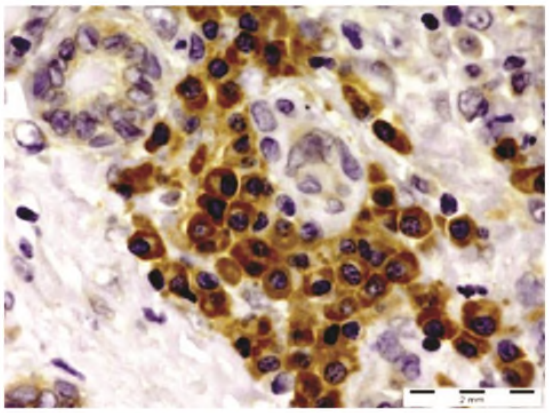

Figure 6. Tumour infiltrating macrophages and mononuclear cells, stained with LYVE-1. Original magnification 1000×.

Table 3. Comparison of various clinicopathological features with the I-LVD and P-LVD of lymphatic markers

			Podoplanin				LYVE-1				PROX 1				VEGFR-3			
		N	I-LVD	*P* value	P-LVD	*P* value	I-LVD	*P* value	P-LVD	*P* value	I-LVD	*P* value	P-LVD	*P* value	I-LVD	*P* value	P-LVD	*P* value
Age	>8 year	23	33.55 ± 4.15	0.509	13.08 ± 2.08	0.158	12.6 ± 3.11	0.829	3.88 ± 0.99	0.114	8.44 ± 0.45	0.393	3.73 ± 0.61	0.526	35.73 ± 4.76	0.947	6.98 ± 0.60	0.106
	<8 year	17	29.78 ± 3.38		9.29 ± 1.17		13.5 ± 2.90		6.96 ± 0.75		6.50 ± 0.72		3.11 ± 0.76		36.21 ± 5.10		8.64 ± 0.82	
Size	>5 cm	19	25.14 ± 3.46	0.017*	10.333 ± 0.98	0.418	12.75 ± 2.60	0.912	5.98 ± 0.56	0.440	7.19 ± 0.72	0.715	3.29 ± 0.69	0.729	30.31 ± 4.98	0.122	7.00 ± 0.60	0.198
	<5 cm	21	38.11 ± 3.84		12.508 ± 2.36		13.23 ± 3.40		4.47 ± 0.16		8.01 ± 0.44		3.63 ± 0.67		41.03 ± 4.60		8.31 ± 0.78	
Grade	I, II	28	30.13 ± 3.20	0.296	9.57 ± 0.69	0.031*	12.27 ± 2.23	0.803	5.03 ± 1.17	0.749	7.82 ± 1.38	0.588	3.55 ± 0.57	0.497	33.67 ± 3.95	0.162	7.41 ± 0.62	0.153
	III, IV	12	36.50 ± 5.33		15.69 ± 3.91		13.48 ± 5.21		5.72 ± 1.74		6.47 ± 1.93		2.83 ± 0.91		44.30 ± 6.70		9.00 ± 0.77	
Inflammation	+	16	36.43 ± 4.91	0.190	14.16 ± 3.00	0.097	16.71 ± 3.70	0.161	5.37 ± 0.24	0.878	9.18 ± 0.87	0.253	3.97 ± 0.82	0.395	42.43 ± 6.08	0.126	8.06 ± 0.62	0.557
	–	24	28.95 ± 3.20		9.68 ± 0.80		10.53 ± 2.5		5.06 ± 0.38		6.58 ± 1.34		3.13 ± 0.57		31.61 ± 3.94		7.44 ± 0.74	
LN metastasis	+	10	36.43 ± 5.57	0.357	15.00 ± 4.39	0.125	20.58 ± 5.66	0.040*	5.66 ± 1.80	0.779	11.33 ± 2.03	0.051	4.66 ± 0.87	0.151	42.80 ± 8.70	0.256	7.90 ± 1.11	0.816
	–	30	30.45 ± 3.20		10.30 ± 0.97		10.48 ± 2.01		5.03 ± 1.14		6.38 ± 1.24		3.07 ± 0.55		33.65 ± 3.57		7.62 ± 0.57	
EMT	+	21	38.26 ± 3.47	0.015*	11.74 ± 2.31	0.832	11.57 ± 2.35	0.828	5.61 ± 1.04	0.645	8.11 ± 1.30	0.650	4.14 ± 0.61	0.143	37.12 ± 4.54	0.723	7.66 ± 0.68	0.959
	–	19	24.96 ± 3.88		11.17 ± 1.16		10.59 ± 3.89		4.71 ± 1.68		7.08 ± 1.85		2.73 ± 0.71		34.63 ± 5.36		7.71 ± 0.77	
Lymphatic vessel invasion	+	22	40.39 ± 3.58	0.000*	14.45 ± 2.12	0.011*	15.93 ± 3.46	0.134	5.33 ± 1.16	0.873	9.21 ± 1.47	0.113	3.81 ± 0.58	0.433	40.16 ± 4.33	0.179	8.45 ± 0.70	0.096
	–	18	21.62 ± 2.89		7.83 ± 0.81		9.43 ± 2.01		5.01 ± 1.62		5.68 ± 1.58		3.05 ± 0.78		30.77 ± 5.43		6.75 ± 0.67	

*P value as calculated by t-test at 0.05 level of significance.

Table 4. Survival analysis of high and low vessel density in intratumoural and peritumoural area with and without censoring using Podoplanin and VEGFR-3

Area	Vessel density	Censoring	Podoplanin Mean survival time (months)	VEGFR-3 Mean survival time (months)
Intratumoral area	High vessel density	With censoring	14.56	12.04
		Without censoring	13.50	10.95
	Low vessel density	With censoring	13.92	16.57
		Without censoring	12.72	15.27
Peritumoral area	High vessel density	With censoring	10.10	13.58
		Without censoring	9.50	12.54
	Low vessel density	With censoring	18.02	14.53
		Without censoring	16.72	13.75

Table 5. Survival analysis in embolic and non-embolic subtypes

Group	Censoring	Mean survival time (months)
Embolic	With censoring	9.41
	Without censoring	9.41
Non-embolic	With censoring	15.94
	Without censoring	14.04

period was least in intratumoural areas having high lymphatic vessel density (Table 4). Further cases which showed presence of tumour emboli, survival period was low compared to non-emboli cases (Table 5).

Discussion

The role of the lymphatic vascular system in promoting cancer metastasis has received increased research effort and clinical attention in the past 15 years. The discovery of lymphatic vessel markers, VEGFR-3, LYVE-1, podoplanin and PROX 1, has enabled detailed studies on the role of the lymphatic system in human cancer.

Podoplanin, a glomerular podocyte membrane mucoprotein is expressed on LEC but not on blood vascular endothelium[15]. In the present study, the expression of podoplanin was seen mainly in cytoplasm of tumour epithelial cells and it varied from weak to marked, as also reported previously[16]. The main reason for the increased I-LVD than the P-LVD for podoplanin in our study can be that the podoplanin is expressed in smaller lymphatic vessels rather than larger ones[17], and the smaller lymphatic vessels were seen mostly in intratumoural than in peritumoural spaces. The other reasons being employing a combination of double antigen retrieval and use of podoplanin polyclonal antibody. The detection of high I-LVD by podoplanin in carcinoma and carcinosarcoma than in sarcoma, in the present study, might be due to the EMT occurring in the former two, thus invoking greater lymphangiogenesis. P-VLD was lesser than I-LVD in this study, which is in contrast to earlier findings[13]. The establishment of a microcirculation, i.e. nascent lymphatic vessels and the kinetics of tumour cells may offer many more opportunities for tumour cells to enter and metastasize[18].

LYVE-1 is a lymphatic vessel endothelial hyaluronan receptor-1 (ref. 19), that has been used extensively in clinical and experimental models as a lymphatic vessel marker, despite its low expression in mature collecting vessels[20]. LYVE-1 is present on both the luminal and abluminal sides of lymphatics[21], thus suggesting its role in shuttling across the endothelium or transcytosis. In our study, the I-LVD of LYVE-1 was more compared to P-LVD. Higher I-LVD can be attributed to the use of polyclonal antibody for LYVE-1 and higher expression of LYVE-1 in small and immature lymphatic capillaries[20], most of which were present in intratumoural regions[22]. The present study also elucidated that, in presence of inflammation, particularly in close proximity to macrophage and mononuclear cell aggregates, there was an increase in lymphatic vessel densities[23]. Lesser I-LVD and P-LVD by LYVE-1 in sarcomas may be due to the fact that sarcomas in general, are more differentiated tumours than carcinoma and carcinosarcomas, and therefore are supposed to have more mature than immature lymphatics[24].

PROX 1 is a homeobox transcription factor involved in the sprouting of lymphatic vessels from embryonic veins during development[25]. PROX 1 is being designated a 'master control gene' usually found in both nucleus and cytoplasm of LEC. But still, the location of PROX 1 is diversified, which depends on different cell type of tumours, as well as differential stages of a tumour[26]. In this study, the P-LVD as observed with PROX 1 was less compared to I-LVD. Further, PROX 1 expression was seen mostly in carcinosarcoma cases (Table 4), which are rather ill-differentiated tumours[27].

VEGFR-3 is expressed on the lymphatic vessels of the normal tissues[28]. It has also been shown to control the development and growth of the lymphatics by binding to polypeptides VEGF-C and VEGF-D[29]. In this study, expression was observed both in the cytoplasm and the nucleus of tumour cells, which is in contrast to a previous work wherein, VEGFR-3 immunoreactivity was observed in the cytoplasm of the tumour cells only[30]. The nuclear localization of VEGFR-3 may be because many receptor

tyrosine kinases function as transcription co-factors to activate gene promoters, such as those of the EGFR family[31]. This nuclear translocation of endothelial growth factor receptors has been reported to play a critical role in cancer progression.

A significant finding of the study was higher I-LVD, viz. VEGFR-3 (35.94 ± 3.45), followed by podoplanin (31.95 ± 2.77), LYVE-1 (11.11 ± 2.20) and PROX 1 (7.62 ± 1.11) than P-LVD, viz. podoplanin (11.48 ± 1.32) followed by VEGFR-3 (7.69 ± 0.51), LYVE-1 (5.19 ± 0.96) and PROX 1 (3.48 ± 0.48), in contrast to most previous studies[10,13]; however, few other reports simulated our results[23]. The detection of increased I-LVD also augments the concept that the denser is the lymphatic vasculature within or close to the tumour, the more the potential entry sites the tumour cells have to vessels that could be used as highways for metastatic spread. Moreover, intratumoural lymphatic vessels may serve as an indication of an aggressive, poorly differentiated tumour type that is more likely to metastasize[32]. In fact, tumour cells prefer to metastasize through the lymphatics more often than blood vessels because of high rate of tumour cell destruction in the blood stream due to hemodynamic shear forces and thereby the poor survival. However, because invasive tumour cells enter the lymphatics relatively easily and avoid the lethal stresses of blood circulation, the number of metastatically significant cells disseminated by the primary tumour into the lymphatics is sufficient for the establishment of a lymph node metastasis[33].

Not many studies based on the markers of lymphangiogenesis have so far been conducted for survival analysis either in humans or dogs. But in a few studies, increased P-LVD was associated with worse overall survival in human gastric cancers and squamous cell carcinomas involving the cervix and the uterus[13,34], whereas high I-LVD was associated with poor outcome in endometrial cancer in people indicating that both the intratumoural and peritumoural vessels are involved in determining the prognosis of a patient. Further in this study, cases showing lymphatic embolism had least survival period than non-embolic cases, thus validating the concept that lymphatics played a greater role in metastasis compared to blood vessels[33].

In the present study, most intratumoural lymphatics appeared to be formed by splitting or bifurfaction and sprouting mechanism as previously reported by He *et al.*[35]. In addition, in neoplasia, inflammatory cells act in concert with tumour cells, stromal cells and endothelial cells to create a microenvironment that promotes tumour spread. Cells belonging to the monocyte-macrophage lineage are important as there is growing evidence that the tumour associated macrophages promote tumour progression, and favour invasion and metastasis as also recorded in this study[36,37].

Comparative evaluation of results elucidated several salient findings with different markers and their correlates including a significant correlation between size of tumour and EMT with I-LVD by podoplanin staining, besides between lymph node metastasis and I-LVD by LYVE-1 staining, and grade of tumour and P-LVD by podoplanin Staining. Somewhat similar results with clinicopathological parameters and podoplanin expression were obtained previously[13], although these studies did not use multiple markers. It was also revealed that there was significant correlation between IRS of VEGF-D and PROX 1 of CMT in general and carcinosarcoma in particular. Furthermore it was found that IRS of PROX 1 differed significantly between carcinosarcoma and sarcoma subtypes.

The salient findings of the present study were that podoplanin was an important marker for intratumoural lymphatics but PROX 1, a 'master control gene', because of its specific nuclear staining, plays an equal and important role in mediating lymphangiogenesis. VEGFR-3 expression did not correlate with any of the clinicopathological parameters and moreover its expression in blood vessel epithelium made it rather unreliable for discrimination between lymphatics and blood vessels. An interesting observation of the study was that with increasing grade and EMT, the intratumoural lymphangiogensis showed an uprise. This seemed to be a special feature of CMT as it is known to undergo frequent desmoplastic and metaplastic transformation than human breast cancer.

Conclusion

It was concluded that a panel of markers used for detection of lymphangiogenesis in CMT should preferably include podoplanin and PROX 1, as cytoplasmic and nuclear markers respectively. Both peritumoural and intratumoural lymphatics played a major role in tumour invasion, metastasis and EMT, besides modulating the tumour microenvironment; therefore both of them appeared to supplement each other. It is also suggested that podoplanin and PROX 1 could serve as potential therapeutic targets for exploring and halting the process of lymphangiogensis in mammary neoplasia of canine and people.

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