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Lymphatic expression of CLEVER-1 in breast cancer and its relationship with lymph node metastasis

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Abstract. *Background*: Mechanisms regulating breast cancer lymph node metastasis are unclear. Staining of CLEVER-1 (common lymphatic endothelial and vascular endothelial receptor-1) in human breast tumors was used, along with *in vitro* techniques, to assess involvement in the metastatic process.

Methods: 148 sections of primary invasive breast cancers, with 10 yr follow-up, were stained with anti-CLEVER-1. Leukocyte infiltration was assessed, along with involvement of specific subpopulations by staining with CD83 (mature dendritic cells, mDC), CD209 (immature DC, iDC) and CD68 (macrophage, Mφ). *In vitro* expression of CLEVER-1 on lymphatic (LEC) and blood endothelial cells (BEC) was examined by flow cytometry.

Results: *In vitro* results showed that although both endothelial cell types express CLEVER-1, surface expression was only evident on LEC. In tumour sections CLEVER-1 was expressed in blood vessels (BV, 61.4% of samples), lymphatic vessels (LV, 18.2% of samples) and in Mφ/DCs (82.4% of samples). However, only CLEVER-1 expression in LV was associated with LN metastasis ($p = 0.027$) and with Mφ indices ($p = 0.021$). Although LV CLEVER-1 was associated with LN positivity there was no significant correlation with recurrence or overall survival, BV CLEVER-1 expression was, however, associated with increased risk of recurrence ($p = 0.049$). The density of inflammatory infiltrate correlated with CLEVER-1 expression in BV ($p < 0.001$) and LV ($p = 0.004$).

Conclusions: The associations between CLEVER-1 expression on endothelial vessels and macrophage/leukocyte infiltration is suggestive of its regulation by inflammatory conditions in breast cancer, most likely by macrophage-associated cytokines. Its upregulation on LV, related surface expression, and association with LN metastasis suggest that it may be an important mediator of tumor cell metastasis to LN.

Keywords: Breast cancer, lymph node metastasis, CLEVER-1, lymphatic, tumor cell adhesion

List of abbreviations

BV	blood vessel
CLEVER-1	common lymphatic endothelial and vascular endothelial receptor-1
DFI	disease free interval
iDC	immature dendritic cells
FFPE	formalin-fixed paraffin-embedded
HEV	high endothelial venules

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HUVEC	human umbilical vein endothelial cells
hTERT	human telomerase reverse transcriptase
IHC	immunohistochemical
LEC	lymphatic endothelial cell
LN	lymph node
LV	lymphatic vessel
LVI	lymphovascular invasion
Mφ	macrophage
mDC	mature dendritic cell
OS	overall survival

1. Introduction

Tumor growth and metastasis are associated with the formation of new blood vessels (BV) (haemangiogenesis) and lymphatic vessels (LV) (lymphangiogenesis). Blood vasculature provides the interface for the transfer of nutrients, fluid and immune cells into tumor tissues. LV in tumors, in comparison, function as a 'drainage system' to release build-up of interstitial pressure, and play an important role in the trafficking of antigen presenting cells and their 'homing' to lymph nodes (LN). Tumor cells also disseminate to LN via LV however the cellular and molecular mechanisms that regulate such cellular intra- and extravasation into and out of LV is still, in comparison to BV, largely unknown.

With the advent of lymphatic markers such as VEGFR-3 [17], prox-1 [43], LYVE-1 [4], and podoplanin (also known as D2-40 [35]), it is now possible to distinguish LV from BV. Podoplanin/D2-40 has been used as reliable lymphatic marker in immunohistochemical (IHC) studies [18, 38], with results showing a strong correlation between lymph vessel density (LVD) and tumor metastasis to LN in melanoma and breast cancer [7, 25, 29]. Such LN metastasis is associated with a worse prognosis and so it is important to understand the molecular mechanisms regulating cell transfer across LV. Identification of molecules involved in this regulatory process may identify patients at high risk of developing metastases.

CLEVER-1 (common lymphatic endothelial and vascular endothelial receptor-1, also known as Stabilin-1, MS-1, FEEL-1) is expressed in alternatively activated (type-2) macrophages and in sinusoidal (non-continuous) endothelial cell (EC) in various tissues such as liver, spleen and lymph nodes [13, 15, 24, 34]. Double immunostaining of vessels with blood and lymphatic endothelial cell markers (PAL-E and LYVE-1 respectively), in fresh frozen tissue material, have suggested that CLEVER-1 is mainly expressed in LV and is up regulated in high endothelial venules (HEV), or HEV-like vessels in LN, in inflamed conditions (skin lesions and cancerous tissues) [14, 15, 34]. CLEVER-1 has been shown to function as a scavenger receptor for ac-LDL and its derivatives [20], play a role in angiogenesis [1] and, most recently, has been hypothesized to function as an adhesion molecule for leukocyte and tumor cell trafficking in the lymphatic system [14, 15]. All information relating to CLEVER-1 tissue distribution has thus far come from fresh frozen material and therefore there has been little correlation with clinicopathological information or with regards the prognostic significance of its expression [14, 34]. The role of CLEVER-1 as an adhesion molecule for mononuclear cell or tumor cell adhesion to vessels has been assessed, *in vitro*, using human umbilical vein endothelial cells (HUVEC, [34]) and fresh whole lymph node sections [14], however its role in tumor cell adhesion to lymphatic endothelial cells *in vitro* is unknown. Although the regulation of CLEVER-1 expression is not fully understood inflammation, as discussed above, has been reported as a possible stimulatory signal [14].

This study presents, for the first time, a retrospective analysis of CLEVER-1 expression in formalin-fixed paraffin-embedded (FFPE) archival human breast cancer specimens with 120 months clinical follow-up. The involvement of different leukocyte subpopulations within the tumor inflammatory infiltrate was also examined with a view to assessing their role in regulating CLEVER-1 expression. In addition, *in vitro* assessment of CLEVER-1 expression in endothelial cell models was also investigated.

2. Materials and methods

2.1. Patients and tissue samples

148 FFPE archival specimens of primary invasive breast cancer were retrieved from the Department of Histopathology, Nottingham City Hospital. The median age of patients at the time of diagnosis was 57 years (range 32–70 years). Forty one patients (28%) were younger than 50 years. 27% of tumors were grade I ($n = 40$), 30% were grade II ($n = 44$) and 42.5%

grade III ($n = 63$). Most tumors, 67%, were stage I ($n = 99$), 24% were stage II ($n = 36$) and 8% stage III ($n = 12$) disease. Fifty (34%) of the specimens were $\leq$1.5 cm. Complete clinical follow-up information was available for all patients, with ethical approval obtained for analysis obtained from Nottingham Local Research Ethics Committee (REC C2020313). Nottingham Local Research Ethics Committee waived the need for written informed consent. Thirty four patients (23%) developed a regional recurrence by the time of their last follow-up. At the time of primary diagnosis, 45 (30%) patients had positive LN, and 49 (33%) had lymphovascular invasion (LVI). 21 (14%) patients were both LN and LVI positive. IHC determination of LVI and the expression of vascular endothelial cell growth factors VEGF-A/-C/-D have been assessed in this cohort of patients and results described previously [26, 27]. The median follow-up period was 96 months (min 3, max 120 months). Tonsil, LN and nine normal breast tissues were also included in the study. Sections of tonsil and LN were used as positive controls for CLEVER-1 staining [15, 24].

2.2. *Immunohistochemistry*

Staining with CLEVER-1 [34] (1 : 50), immature dendritic cell (iDC) marker CD209 (R&D Systems) (1 : 100), mature dendritic cell (mDC) marker CD83 (Serotech) (1 : 50) and macrophage (Mφ) marker CD68 (Abcam) (1 : 50) was carried out on sections from FFPE blocks of tonsil and breast cancer tissues. CLEVER-1 staining was, in addition, conducted using LN sections.

Paraffin Sections (4-μm thick) were de-waxed in xylene and re-hydrated in a series of descending ethanol concentrations (100–30%). Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide (Sigma). Slides were washed in tap water before antigen retrieval using proteinase K (DAKO) (15 min at room temperature). Proteinase K was used for CLEVER-1, CD209 and CD83 staining. Antigen retrieval for CD68 staining required boiling slides in sodium acetate buffer (pH 6) for 20 min using a 800 W microwave. Slides were then rinsed with TBS (pH 7.6) and non-specific binding blocked by normal swine serum (DAKO). Following incubation for 1 hr with the primary antibodies, slides were washed and incubated with biotinylated secondary antibody (StreptABComplex/HRP Duet, Mouse/Rabbit kit, DAKOCytomation) according to the manufacturer's instructions. After washing, avidin coupled to biotinylated horseradish peroxidase was added (1 h), washed and immunoreactions visualized using diaminobenzidine (DAB, DAKO). Specimens were counterstained using Haematoxylin (Gill's formula, Vector Laboratories Inc.), dehydrated, re-fixed in xylene and mounted with DPX (Fluka). Negative controls were stained using the same procedure but excluding primary antibody.

2.3. *Microscopic analysis*

CLEVER-1 expression in tumor vasculature was examined and compared with CD34, CD31 and podoplanin staining [27, 28], in parallel sections, to determine the nature of the vessel [26]. CLEVER-1 expression was observed in BV, LV and Mφ/DC and categorized into two groups according to the presence or absence of staining. All specimens contained inflammatory infiltrate (assessed by the morphology of cells, i.e. round cells in the tumor stroma and infiltrating tumor sheets). Inflammation was semiquantitatively graded, depending on the level of infiltrate, into mild or dense.

The number of Mφ and DC present was assessed using Chalkley point methodology [9]. Briefly, three hotspots of positively stained cells were identified and counted using a graticule microscope eyepiece. The mean of the three counts represented the count for each slide (sample index) and was used in further analysis. Once the Mφ/DC indices were obtained, the distributions of indices were plotted and the median used as a cut-off point for categorization: high: $\geq$median, low: <median.

Samples were examined independently by two assessors, blinded to clinicopathological data, with reanalysis of any discrepancies.

2.4. *In vitro systems*

Human telomerase reverse transcriptase (hTERT)-transfected lymphatic endothelial cells (hTERT-LEC) [31] were used, between passages 27–34, as a model of LEC and cultured in EGM-2MV medium (Clonetics). HUVEC were isolated as previously described [16], and pooled populations, from a minimum of three donors, used between passages 1–4. HUVEC were cultured in MM199 media (Sigma)

with 20% iron fortified calf serum (PAA Laboratories), L-glutamine (Sigma), HEPES buffer (Sigma), Penicillin/Streptomycin (Sigma), EGF (Sigma) and bFGF (Peprotech). Cells were incubated in a humidified atmosphere at 37°C and 5% CO_2.

2.5. *In vitro CLEVER-1 expression*

For assessment of surface CLEVER-1 expression, by FACS analysis, endothelial cells were trypsinized, washed then fixed in 4% formaldehyde solution in phosphate buffered saline (PBS) and aliquoted for further staining. For intracellular (total) expression, trypsinized cells were fixed in 4% formaldehyde solution in phosphate buffered saline (PBS) and permeabilized with saponin (40 mg/100 ml) and glucose (0.9 g/100 ml) then aliquoted for further analysis.

2×10^5 cells were either incubated with blocking buffer (PBS+0.1% bovine serum albumin (Sigma)), mouse IgG1 isotype control (R&D systems) or anti-CLEVER-1 (3–372) at 20 μg/ml. Antibody treated cells were further incubated with goat anti-mouse FITC labeled antibodies (DAKOcytomation). Cells were then analyzed using a FACSscan flow cytometer (Becton Dickinson, Sunnyvale, CA, USA) and WinMDI 2.8 software.

2.6. *Statistical analysis*

Statistical analysis of IHC results was performed by correlating CLEVER-1 expression (presence/absence) with clinicopathological parameters including estrogen and progesterone receptor (ER and PR) status, lymph node and distant metastasis, levels of VEGF-A, C and D expression, LVI, as well as density of inflammatory infiltrate. Chi-squared tests were used to assess the relationship between categorized data. Overall survival (OS) and disease free interval (DFI) analyses were performed using the Kaplan-Meier method and the statistical significance of the differences between groups assessed by the long-rank test. DFI was defined as the period from the end of primary treatment until any recurrence occurred: local (defined as tumour arising in the treated breast or chest wall); regional (defined as tumour arising in the axillary or internal mammary LNs); or distant (any remote site other than local and regional). Any of these recurrences was scored as an event, with censoring of other patients at the time of last follow-up or death. Overall survival (OS) was defined as the period from primary surgery until the death of the patient.

For *in vitro* FACS analysis the average median fluorescence intensities (MFI) of CLEVER-1 expression (with the MFI of the IgG1 isotype control subtracted) were presented ± SEM for the indicated number of experiments. A p value of ≤ 0.05 defined a significant relationship. All statistical analysis was performed using SPSS, version 15.0.

3. Results

3.1. *CLEVER-1 is preferentially expressed, in vitro, on the surface of lymphendothelial cells*

FACS intracellular staining of total CLEVER-1 expression in proliferating hTERT-LEC and HUVEC shows that both cell types express CLEVER-1 with higher expression present in LEC than in HUVEC (Fig. 1). Such an effect was not due to the different culture media as HUVEC cultured in LEC media still showed similar expression levels (data not shown). Interestingly, assessment of surface expression suggests that CLEVER-1 levels are significantly higher on LEC than HUVEC. MFI for intracellular and surface staining were 127 ± 8.8 and 17.3 ± 1.9 ($n = 11$), respectively, in hTERT-LEC and 59.5 ± 13 ($n = 4$) and 4.7 ± 1.8 ($n = 8$), respectively, in HUVEC.

3.2. *CLEVER-1 is expressed in blood vessels, lymph vessels and Mφ/DC in tonsils, lymph nodes, and breast cancer tissues but only occasionally in normal breast, and then only in Mφ/DC*

Tonsil and LN, used as positive controls for CLEVER-1 staining [15, 24], are rich in lymphatic and blood vessels, including HEV, and leukocytes with different maturation or activation status. In tonsils (Fig. 2A), CLEVER-1-positive cells were detected amongst epithelial cells of the mucosal layers, most probably Langerhan's cells, and macrophages in the submucosa. EC of lymphatic vessels or HEV were also CLEVER-1 positive (Fig. 2A). Similarly, in LN, CLEVER-1 stained ECs of the subcapsular sinusoids in lymph vessels, or HEV, and was also positive in the macrophages lining the nodal sinusoids (Fig. 2A). Nine normal breast tissues were also stained for CLEVER-1

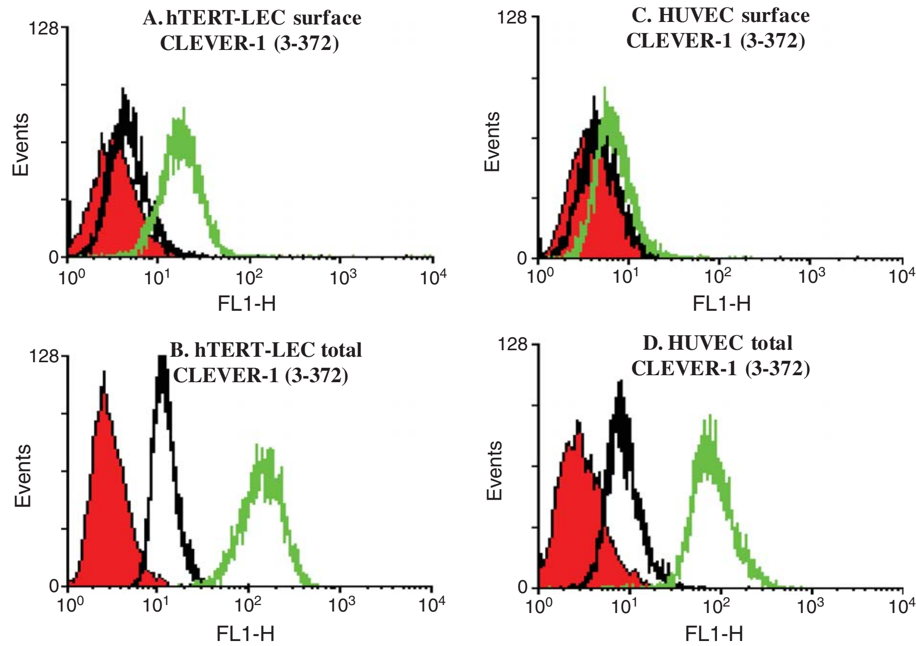

Fig. 1. Representative cytoflorograms of surface (A and C) and intracellular (B and D) staining of CLEVER-1 expression in hTERT-LEC (A and B) and HUVEC (C and D). Both EC express CLEVER-1 however surface expression is higher on hTERT-LEC. Red (solid) areas represent untreated cells, black line is the IgG1 isocontrol and green (grey) represents CLEVER-1 expression as detected via 3-372 antibody staining.

which was occasionally present in Mφ/DC found in the intra- and inter-lobular stroma, however no staining was observed in vessels (Fig. 2A).

In breast cancer sections, staining was conducted on 148 sections 5 of which did not contain any tumoral lymphatics. The nature of vessels was assessed by parallel staining with CD34, podoplanin and CD31 (Fig. 2B). There was some variation in CLEVER-1 expression within the same tumor section in that not all vessels within the section stained positively for CLEVER-1. Positive staining was more frequent and more intense on BV ($CD34^+/CD31^+/podoplanin^-$) ($n = 91$, 61% of samples) than LV ($CD34^{\pm}/CD31^+/podoplanin^+$) ($n = 27$, 18% of samples), located in both peri/intratumoral areas, and usually associated with detection of inflammatory infiltrate close by. Interestingly, 82% of samples showed positive CLEVER-1 staining of Mφ and DC with staining being particularly strong in areas of dense inflammation. Adipocytes in the periphery of the tumor sections were also positive for CLEVER-1 (Fig. 2B) – such positive staining may be related to adipocytes or to the macrophage residing in fat tissue.

3.3. *Correlations between CLEVER-1 expression in BV/LV and clinicopathological criteria*

The correlation between CLEVER-1 expression in BV or LV with different clinicopathological criteria was analyzed and results shown in Table 1. There were no significant relationships between BV-CLEVER-1 and patient clinicopathological criteria. However, the presence of CLEVER-1 in LV was significantly correlated with ER status ($p = 0.005$), PR status ($p = 0.006$) and, interestingly, LN metastasis ($p = 0.027$).

To examine whether lymph/angiogenic growth factors were involved in CLEVER-1 regulation in breast cancer, expression was correlated with VEGF-A/-C and -D expression that had previously been assessed in parallel sections [27]. No significant correlations were observed. The relationship between density of inflammation and CLEVER-1 expression was also examined (Table 1). Dense inflammatory infiltrate was significantly associated with CLEVER-1 expression in BV ($p = 0.041$) and in LV ($p < 0.001$). Moreover, a strong association resulted when CLEVER-1 expression in BV was correlated with its expression in Mφ

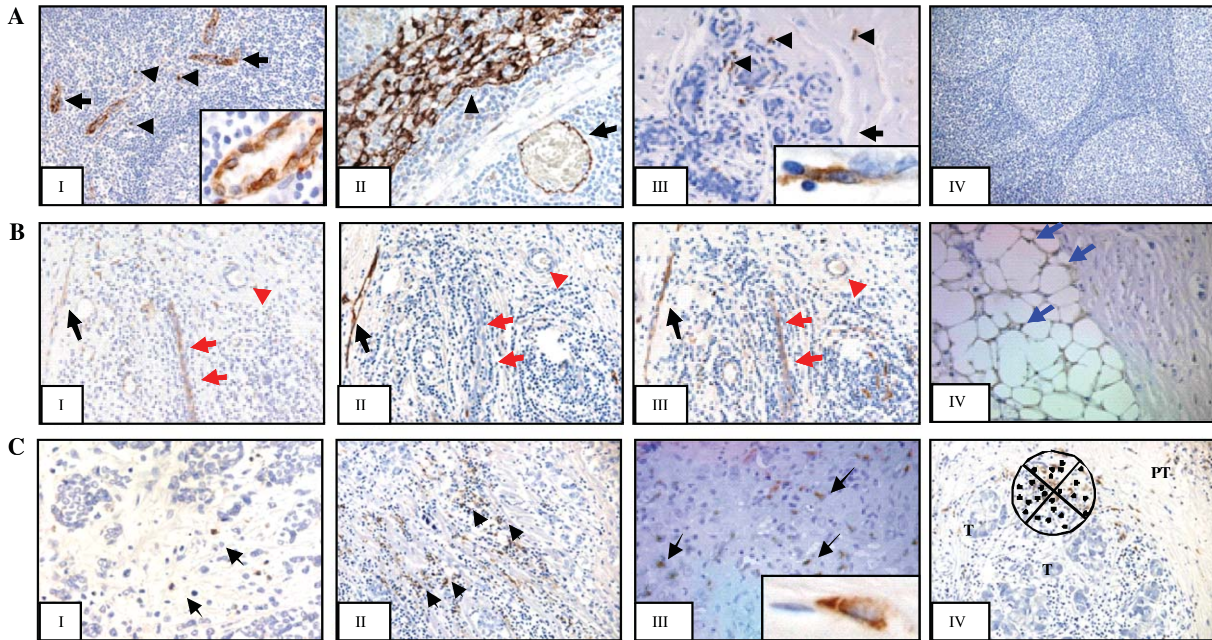

Fig. 2. (A) CLEVER-1 expression in FFPE specimens of tonsil (I), LN (II), and normal breast tissue (III). Strong CLEVER-1 staining is detected in tonsils and in LN endothelial cells of lymphatics/HEV (black arrows). Strong expression is also found in Mφ/DC (arrow heads). In normal breast tissues, occasional Mφ/DC staining with CLEVER-1 is observed but no staining in vessels is found (×400). An example of negative control staining in a tonsil section is shown in (IV) (×200). (B) CLEVER-1 expression in breast cancer. Parallel sections were stained with CLEVER-1 (I), podoplanin (II) and CD34 (III). CLEVER-1 was detected in both blood vessels ($CD34^{+}$/$podoplanin^{-}$, red/grey arrows) and lymph vessels ($CD34^{\pm}$/$podoplanin^{+}$, black arrows). CLEVER-1 expression in vessels was variable; an example is shown where positive (red/grey arrows) and negative (arrow heads) blood vessels are present in the same section. Panel IV shows CLEVER-1 positive staining of adipocytes in the tumor periphery (blue/white arrows) × 200. (C) Leukocyte immunohistochemistry in breast cancer. Panels I, II and III show mDC (CD83+ve), iDC (CD209+ve) and Mφ (CD68+ve) leukocytes, respectively. iDC and Mφ were more frequent in the tumor periphery but were also found infiltrating the tumor sheets, as mentioned in the narrative very few mDC were observed. Panel IV shows a representation of a hotspot assessment, used for determination of the iDC index. I–III ×400, IV ×200.

($p < 0.001$), with similar results being obtained with LV ($p = 0.004$) (Table 1). Such correlations are suggestive of a common signal(s) between Mφ and BV/LV that up regulates CLEVER-1 expression in the tumor environment. It was interesting, therefore, to investigate the role that specific leukocyte subpopulations might play in regulating CLEVER-1 expression. 128 breast cancer sections (of the 148 specimens) were stained for the presence of iDC and mDC and 79 (pair matched) samples stained for Mφ (Fig. 2C). Very few mDC were observed in sections (between 0 and 5 across in the whole section) and, when present, were mainly located in the stroma of peritumoral areas. All sections contained significantly more iDC and Mφ and were more frequently observed in peritumoral than intratumoral areas. The median number of iDC and Mφ, assessed per hotspot, were 3.7 and 2, respectively. Interestingly, when iDC/Mφ indices were correlated with CLEVER-1 expression in vessels, only Mφ counts were associated with LV expression of CLEVER-1 ($p = 0.02$).

In terms of OS, high Mφ indices were associated with poor survival ($p = 0.031$, data not shown), which is in agreement with previous studies in breast cancer [23, 41]. In the case of DFI, CLEVER-1 expression in BV was associated with a higher rate of recurrences ($p = 0.049$) (Fig. 3). Although iDC indices did not influence DFI of patients over 120 months follow-up the majority of recurrences occurred within 60 months (for example, a total of 28 patients had recurrences over 120 months, 20 of which occurred within 60 months) and at a 60 month cutoff high iDC indices were significantly associated with higher rates of recurrence ($p = 0.038$) (Fig. 2). Although significant for OS high

Table 1
Correlation of CLEVER-1 expression on blood and lymph vessels with clinicopathological criteria and with iDC and Mφ indices

Clinical-pathological criteria		CLEVER-1 in blood vessels			CLEVER-1 in lymph vessels		
		Absent *n* (%)	Present *n* (%)	*p*-value	Absent *n* (%)	Present *n* (%)	*p*-value
Age	≤50	14 (34.1)	27 (65.9)	0.499	31 (75.6)	10 (24.4)	0.231
	>50	43 (40.2)	64 (59.8)		90 (84.1)	17 (15.9)	
Size (cm)	≤1.5	24 (48)	26 (52)	0.09	43 (86)	7 (14)	0.378
	>1.5	33 (33.7)	65 (66.3)		78 (79.6)	20 (20.4)	
Grade	I	19 (47.5)	21 (52.5)	0.355	37 (92.5)	3 (7.5)	0.13
	II	15 (34.1)	29 (65.9)		35 (79.5)	9 (20.5)	
	III	22 (34.9)	41 (65.1)		48 (76.2)	15 (23.8)	
Stage	1	40 (40.4)	59 (59.6)	0.277	85 (85.1)	14 (14.9)	0.053
	2	14 (38.9)	22 (61.1)		28 (77.1)	8 (22.9)	
	3	2 (16.7)	10 (83.3)		7 (58.3)	5 (41.7)	
NPI	Good	28 (45.9)	33 (54.1)	0.255	55 (90.2)	6 (9.8)	0.082
	Moderate	25 (34.7)	47 (65.3)		55 (76.4)	17 (23.6)	
	Poor	4 (26.7)	11 (73.3)		11 (73.3)	4 (26.7)	
ER	≤20%	18 (36.7)	31 (63.3)	0.857	34 (69.4)	15 (30.6)	**0.005**
	>20%	37 (39.4))	57 (60.6)		83 (88.3)	11 (11.7)	
PR	≤20%	21 (33.3)	42 (66.7)	0.382	45 (71.4)	18 (26.7)	**0.006**
	>20	32 (41.6)	45 (58.4)		69 (89.6)	8 (10.4)	
DM	Absent	51 (39.5)	78 (60.5)	0.512	106 (82.2)	23 (17.8)	0.5
	Present	5 (31.3)	11 (68.8)		12 (75)	4 (25)	
LVI	Absent	40 (40.8)	58 (59.2)	0.337	82 (83.7)	16 (16.3)	0.366
	Present	16 (32.7)	33 (67.3)		38 (77.6)	11 (22.4)	
LN status	Negative	43 (41.7)	60 (58.3)	0.221	89 (86.4)	14 (13.6)	**0.027**
	Positive	14 (31.1)	31 (68.9)		32 (71.1)	13 (28.9)	
VEGF-A	Low	39 (43.3)	51 (56.7)	0.133	77 (85.6)	13 (14.4)	0.136
	High	18 (31)	40 (69)		44 (75.9)	14 (24.1)	
VEGF-C	Low	40 (43)	53 (57)	0.107	80 (86)	13 (14)	0.071
	High	16 (29.6)	38 (70.4)		40 (74.1)	14 (25.9)	
VEGF-D	Low	32 (57.1)	46 (50.5)	0.497	63 (80.8)	15 (19.2)	0.833
	High	24 (42.9)	45 (49.5)		57 (82.6)	12 (17.4)	
MVD or LVD	Low	32 (41)	46 (59)	0.437	86 (84.3)	16 (15.7)	0.206
	High	24 (34.8)	45 (65.2)		34 (75.6)	11 (24.4)	
Inflammatory density	Mild	49 (43)	65 (57)	**0.041**	101 (88.6)	13 (11.4)	**<0.001**
	Dense	8 (23.5)	26 (76.5)		20 (58.8)	14 (41.2)	
Mφ CLEVER-1	Absent	25 (96.2)	1 (3.8)	**<0.001**	26 (100)	0 (0)	**0.004**
	Present	32 (26.2)	90 (73.8)		95 (77.9)	27 (22.1)	
iDC index	Low <3.7	25 (53.2)	40 (50.6)	0.781	53 (52.5)	12 (48)	0.689
	High ≥3.7	22 (46.8)	39 (49.4)		48 (47.5)	13 (52)	
Mφ index	Low <2	16 (54.7)	22 (44.9)	0.466	35 (54.7)	3 (20)	**0.021**
	High ≥2	14 (45.3)	27 (55.1)		29 (45.3)	12 (80)	

NPI (Nottingham prognostic index); DM (distant metastasis).

Mφ indices did not correlate with DFI over 120 months, or when using 60 months as a cut-off. This may, however, be related to sample size as only 17 cases of the macrophage assessed population (from a total of 23 patients with recurrences) relapsed within this time – all 17 cases were in the high Mφ category. It is also interesting to note that although LV CLEVER-1 expression was significantly associated with LN positivity there was no association with either DFI or OS.

4. Discussion

Previous studies have shown that CLEVER-1 is a novel adhesion molecule potentially involved in tumor cell dissemination via lymphatics. Although CLEVER-1 has been studied in fresh frozen tissue materials, no retrospective analysis has been conducted to investigate its correlation with clinicopathological criteria and disease outcome. The current study

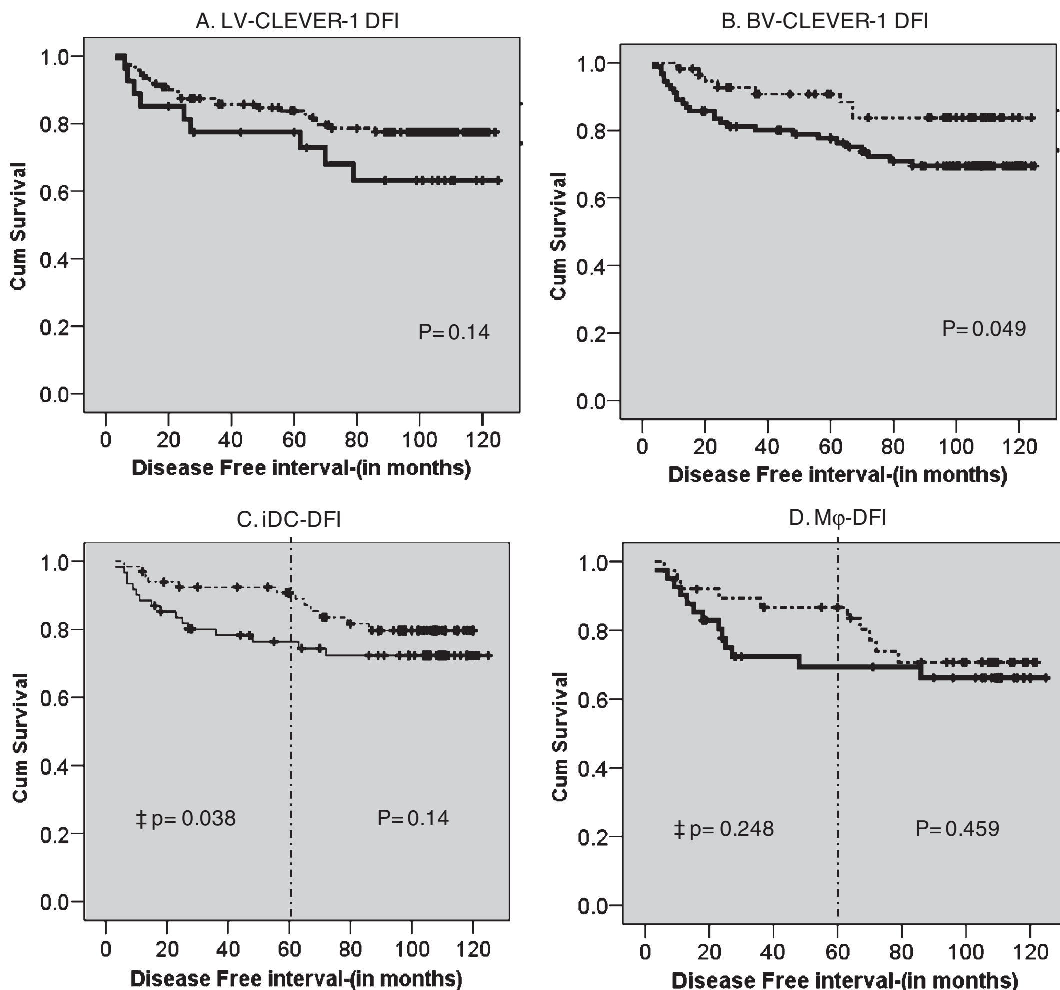

Fig. 3. Kaplan-Meier survival curves of DFI versus CLEVER-1 expression in BV and LV, and versus iDC and Mφ indices. There was no statistical significance of CLEVER-1 expression in LV and recurrence rates over 120 months ($p = 0.14$) however BV-CLEVER-1 expression may predict regional recurrence in breast cancer patients ($p = 0.049$). Dashed line represents a 60 month follow-up cut-off period. iDC index is predictive of decreased DFI at 60 months follow-up ($p = 0.038$) but not at later times. ‡ Represents the p value of iDC and Mφ at 60 months. $p < 0.05$ represents statistical significance.

presents, for the first time, the results of CLEVER-1 staining in archival FFPE breast cancer material, with long follow up (120 months).

CLEVER-1, identified by Irjala and co-workers, is present in LN on both efferent and afferent LV (characterized by VEGF-R3 and mannose receptor staining) and on HEV (expressing peripheral LN addressin) [15]. Previous studies have shown that CLEVER-1/MS-1/Stabilin-1 is upregulated in vessels in inflamed disease conditions of skin such as wound healing, melanoma and psoriasis [11, 34, 37]. In cancerous human tissues, the increase in CLEVER-1 expression has been mainly associated with tumor-associated lymphatics and not with blood vasculature [14].

In agreement with the expression pattern of CLEVER-1 in normal skin [34] IHC results from the current study show that CLEVER-1 is not expressed in vessels of normal breast. Endothelial expression *in vitro* may be related to the proliferative status of cells in culture or to the culture conditions, being exposed to the variety of growth factors and high serum concentrations, required to maintain cell viability. It would be of interest to examine whether CLEVER-1 expression correlated with EC proliferation, either in patient samples or *in vitro*. Inflammation has been suggested to up

regulate expression in blood vessels. Lymphoid organs such as tonsils and LN are important antigen presenting sites; as such they contain different types of leukocytes and at varying states of inflammation. This may explain why HEV, along with LV, in such organs express CLEVER-1 [15]. Tonsil and LN staining also confirmed that some subtypes of Mφ/DC (assessed by their localization and morphology) express CLEVER-1 [10, 12, 13, 24].

The current study of breast cancer, and concurrent staining of specimens with blood and lymphatic vessel endothelial markers (CD34, CD31 and podoplanin, respectively), allowed the identification of the vessel type. Although controversy exists regarding the topography of lymph vessels in breast cancer [16, 17] the current study supports the existence of tumoural lymphatics and that cellular interaction with lymph vessels is not a passive process [3]. It is tempting to compare current CLEVER-1 results with previous studies using fresh frozen material but it should be borne in mind, when attempting such comparisons, that previous staining of breast cancer tissues with anti-CLEVER-1, by Irjala and co-workers, did not use a similar approach to distinguish the two types of vessels [14]. PAL-E (for BV) and VEGFR-3 (for LV) were used [14] rather than CD34/CD31/Podoplanin. It has been shown that although VEGFR-3 is a robust marker to identify lymphatic vessels present in normal tissues when used in tumors it could give false positive LV results as certain BV in the tumor environment may express it [22]. Podoplanin, in comparison, is generally accepted as a robust marker for detection of LV [30, 32, 38, 42]. With such caveats in mind Irjala and colleagues showed that in breast cancer CLEVER-1 was mainly expressed in LV (100% of peritumoral and 74% of intratumoral lymphatics, respectively) [14]. In contrast, the current report identified only weak staining in LV with the majority of CLEVER-1 being present in BV. This discrepancy may, as mentioned above, be due to the use of different markers to identify vessels. CLEVER-1 staining in the current study was variable in intensity and, interestingly, was not positive in all vessels within the same tumor section. The heterogeneity in the tumor environment may be responsible for such variations.

Although CLEVER-1 staining has been reported in head and neck and breast cancer [14] and in melanoma [11] limited information is available with respect to expression and correlation with clinicopathological criteria (i.e. only with tumor grade and LN status [14]). Retrospective analysis using archival materials, as in the current study, allows relationships between CLEVER-1 expression and a variety of clinicopathological information, including survival parameters, to be analyzed. Although CLEVER-1 was more frequently expressed in BV than LV, only LV CLEVER-1 showed significant correlation with LN metastasis. Such a statistical correlation was not obtained with the previous work of Irjala et al. [14], possibly due to the sample size and, as discussed above, the staining methods and markers chosen. Similarly, previous reports of CLEVER-1 staining in breast cancer [14] did not show any expression in tumoral leukocytes. We report here that 82% of specimens have CLEVER-1^{+} macrophages/DCs infiltrating the tumor. Stabilin-1/ CLEVER-1 has been found to be inducible in alternatively-activated macrophages/monocytes (type-2 or Mφ-2) *in vivo* (animal models of wound healing, melanoma and in pancreatic cancer) and *in vitro* [37]. CLEVER-1 expression in Mφ-2 has been associated with a scavenging function to clear out foreign, and some self, antigens from the ECM [20]. In the current study, analysis of CLEVER-1 expression resulted in a significant correlation between its expression in Mφ and BV or LV. This suggests that cytokines and other inflammatory mediators available in the tumor microenvironment may play a role in CLEVER-1 upregulation in both vessels and Mφ-2. These cytokines may be secreted by tumor associated macrophage (TAM) however it is still unclear what key signals initiate CLEVER-1 expression. Mφ-2 are generated *in vitro* by treating macrophages/monocytes with IL-4 with or without glucocorticoids. Inducible Mφ-CLEVER-1 however is inhibited by IFN-γ [10]. Other inflammatory cytokines such as TNF-α are unable to produce similar regulatory effects in macrophage/monocytes [10]. Expression of CLEVER-1 in fat cells (or possibly in macrophages infiltrating adipose tissue) has not been previously reported and, like BV, LV and Mφ, its expression and role in this cell type requires verification and further investigation. CLEVER-1 expression in adipocytes could indicate a function as a scavenger receptor for an acetylated LDL derivative and storage in adipose tissue.

The reverse correlation between LV-CLEVER-1 and ER, and PR, status may be explained by the high cytokine content in such tumors i.e. IL-6, IL–8, IL-10, G-CSF, IFN-γ, MCP-1, MIP-1β and TNF-α have been shown to be expressed at high levels in comparison

to normal breast tissues and were inversely correlated with ER and PR expression [6]. The cytokine profile that correlates with CLEVER-1 expression in vessels in different diseases is unknown and needs further investigation particularly, for reasons given above, the role of IL-4/glucocorticoids and IFN-γ. Increased IL-4 and decreased IFN-γ is considered as a marker of a shift toward a Th2 CD4 and Tc2 CD8 phenotype and an immature immune response [8]. In addition, such an environment promotes the formation of alternatively activated Mφ which are associated with inducing endothelial cell proliferation (angiogenesis) and inhibition of CD4+ T cell proliferation [19, 36]. In the current findings, the scarcity of mDC and the presence of CLEVER-1 in Mφ suggests the presence of high levels of IL-4/glucocorticoids and low levels of IFN-γ in these tumors and, therefore, of a suppressed immune response in breast cancer patients. The inhibitory effect of IFN-γ on CLEVER-1 expression could be indirect as IFN-γ did not change CLEVER-1 expression *in vitro* (data not shown). Members of the VEGF family (A/C/D) seem not to play a direct role on CLEVER-1 expression. However, they could be involved indirectly via increasing MVD (inducing angiogenesis) [39] and recruiting the inflammatory infiltrate, in particular Mφ which further interact with the tumor environment to produce the cytokines involved in CLEVER-1 expression. Overall, the regulation of CLEVER-1 expression by different cytokines and the molecular mechanisms involved are still unknown and need further examination.

The current study reports the first association between CLEVER-1 expression and patient survival. Results indicate that CLEVER-1 may be involved in tumor recurrence or in spread to lymph nodes. Results are intriguing and suggest that a larger study is warranted. Spread to LN via lymphatics may be a result of the differential surface expression of CLEVER-1 on LEC as opposed to BEC, as shown from *in vitro* studies. The current study also suggests, like others, that Mφ indices may be an important prognostic marker to predict tumor outcome. Although iDC indices had no prognostic significance in terms of patient overall survival they may have a prognostic significance for early relapse (up to 5 yrs of the initial cancer). It would be interesting, as with CLEVER-1 expression, to conduct a larger study to confirm this.

It is interesting to speculate upon the role CLEVER-1 may be playing in regulating metastatic spread. No cellular ligand for CLEVER-1 has thus far been identified but the protein plays a role in scavenging, including degradation of acLDL and the matricellular protein SPARC (Secreted Protein Acidic and Rich in Cysteine) [21]. This latter function may be important as SPARC has various, differing, effects on EC, tumor cells and on the extracellular matrix – all may increase tumor metastasis. Focal adhesion is dissociated upon addition of SPARC to EC cultures (reviewed in [5]). It may well be that this is mediated via SPARC binding to CLEVER-1 and subsequent increase in EC permeability and/or tumor cell migration across EC. In melanoma, overexpression of SPARC by tumor cells was associated with loss of E-/P-cadherin which correlates with a more invasive type of tumor [33]. Moreover, SPARC can modulate the structure of ECM by playing a role as an alternative substrate of ECM proteins (e.g. fibronectin) for crosslinking enzymes (e.g. transglutaminase) making it "malleable and permissive" of cell migration, proliferation and differentiation [5].

The current report suggests, in agreement with previous studies in breast cancer [23, 41], that tumors with a high Mφ index correlate with a worse prognosis. In terms of DFI, iDC indices seem to significantly distinguish patients who are more likely to have recurrences within 60 months but not at longer follow-up (i.e. 10 yr). The lack of significance at 10 years follow-up is in accordance with previous findings in breast cancer [2]. On the other hand, iDC indices may be a valuable predictive tool for recurrence within 60 months follow-up. A shorter follow-up time was also used by Treilleix and co-workers (80 months), in breast cancer, and significant results found [40].

5. Conclusions

Limited *in vitro* information is available comparing tumor cell adhesion to blood and lymph endothelium and the potential role that CLEVER-1 may play in this process. IHC data show that CLEVER-1 expression is up-regulated on endothelial vessels by inflammatory conditions in breast cancer, most likely by macrophage-associated cytokines. With its up regulation on LV, and *in vitro* results showing that expression on such vessels is, unlike vascular endothelium, cell surface related, it is potentially playing an important role in mediating tumor cell metastasis to LN. *In vitro* and *in vivo* functional studies of the role of CLEVER-1 in tumor metastasis will be important

to verify whether it represents a therapeutic target to inhibit tumor cell metastatic dissemination.

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The author(s) declare that they have no competing interests.

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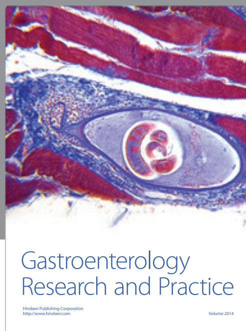

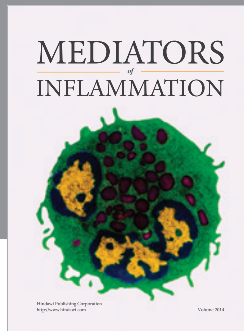

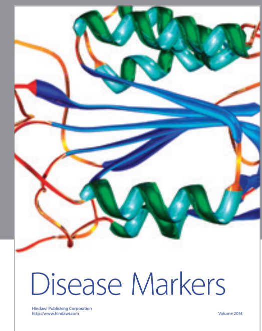

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