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The Association of ICAM-1 Detected by Immunohistochemical Staining with Triple Negative and Non-Triple Negative Breast Carcinoma

(Perkaitan ICAM-1 Dikesan oleh Pewarnaan Imunohistokimia dengan Karsinoma Payudara Ganda Tiga Negatif dan Bukan-Ganda Tiga Negatif)

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ABSTRACT

*Metastasis of tumour cell greatly contributes to the cause of mortality. Tumour-associated macrophage (TAM) and the intercellular adhesion molecule 1 (ICAM-1) were associated with metastases of breast carcinoma. However, the relationship between lymphatic vessel densities and invasion with TAM and ICAM-1 remained unclear. The aim of this study was to investigate the relationship of lymphovascular densities and invasions with patient's clinicopathological data. The roles of TAM and ICAM-1 influencing lymphovascular invasions were also investigated. Haematoxylin and eosin (H&E) and immunohistochemical (IHC) staining on a consecutive section of 37 formalin fixed-paraffin embedded (FFPE) breast invasive carcinoma samples were carried out. The D2-40, CD34, CD163, and ICAM-1 antibodies were used to stain lymphatic vessel, blood vessel, TAM, and ICAM-1 receptor, respectively. The total lymphatic vessel density (LVD) was significantly reduced on increased tumor size (*p*=0.045). The increase of intra-tumoral LVD and lymphatic vessel invasion (LVI) was significantly associated with human epidermal growth factor receptor 2 (HER2) negative status (*p*=0.022 and p=0.05, respectively). The percentage of LVI was higher than blood vessel invasion (BVI) in 18.5%. Lymphovascular invasions detected in H&E were missed in 49.76% compared with those detected in IHC-stained tissues (206/410). ICAM-1 scores were significantly associated with non-triple negative breast cancer (non-TNBC) (p=0.008). ICAM-1 is significantly overexpressed on non-TNBC sample. Therefore, ICAM-1 might be clinically useful as a targeted molecule for non-TNBC patients. In histological reporting, in addition to H&E staining, IHC staining using D2-40 and CD34 should be considered to increase the accuracy of diagnosis.*

Keywords: Breast carcinoma; CD34; D2-40; ICAM-1; lymphovascular invasion

ABSTRAK

*Metastasis sel tumor menyumbang kepada mortaliti. Makrofaj berkaitan tumor (TAM) dan molekul lekatan antara sel 1 (ICAM-1) dikaitkan dengan metastasis karsinoma payudara. Walau bagaimanapun, hubungan antara ketumpatan dan pencerobohan saluran limfa oleh TAM dan ICAM-1 masih tidak jelas. Tujuan kajian ini adalah untuk mengkaji hubungan antara ketumpatan dan pencerobohan limfovaskular dengan data klinikopatologi pesakit. Peranan TAM dan ICAM-1 yang mempengaruhi pencerobohan limfovaskular juga dikaji. Pewarnaan hematoksilin dan eosin (H&E) dan imunohistokimia (IHC) pada hirisan berterusan 37 sampel awetan formalin dan benaman parafin (FFPE) karsinoma invasif payudara telah dijalankan. Antibodi D2-40, CD34, CD163, dan ICAM-1 masing-masing digunakan untuk mewarna saluran limfa, saluran darah, TAM, dan reseptor ICAM-1. Ketumpatan saluran limfatik (LVD) berkurangan dengan ketara pada saiz tumor yang meningkat (*p*=0.045). Peningkatan LVD dan pencerobohan saluran limfatik (LVI) intra-tumoral berkait dengan reseptor faktor pertumbuhan epidermis manusia 2 (HER2) negatif (*p*=0.022 dan p=0.05). Peratusan LVI adalah lebih tinggi daripada pencerobohan saluran darah (BVI) pada 18.5%. Pencerobohan limfovaskular yang dikesan dalam H&E adalah 49.76% berbanding yang dikesan dalam tisu yang diwarnakan dengan IHC (206/410). Skor ICAM-1 adalah berkaitan secara signifikan dengan kanser payudara bukan tiga negatif (bukan-TNBC) (p=0.008). ICAM-1 secara signifikan meningkat pada sampel bukan-TNBC. Oleh itu, ICAM-1 mungkin berguna secara klinikal sebagai molekul sasaran untuk merawat pesakit bukan-TNBC. Dalam laporan histologi, sebagai tambahan kepada pewarnaan H&E, pewarnaan IHC menggunakan D2-40 dan CD34 perlu dipertimbangkan untuk meningkatkan ketepatan diagnosis.*

Kata kunci: CD34; D2-40; ICAM-1; karsinoma payudara; pencerobohan limfovaskular

INTRODUCTION

Breast carcinoma refer to the malignant growth of breast tissue that affects the milk-producing ducts or glands of the breast (Abduelkarem et al. 2015). In general, woman's breast consists of glands and lobules that produce milk, ducts that carry milk to the nipple, adipose tissue, and blood and lymphatic vessels. The major cause of mortality from breast carcinoma is due to dissemination and metastases of the primary tumor to the other part of the body (Schoppmann et al. 2004). Tumor-induced

lymphangiogenesis, lymphatic vessel invasion (LVI), regional spread, and distant metastases in breast carcinoma had been associated with the involvement of axillary lymph node (Ran et al. 2010; Viale et al. 2005). Compared with blood vessel invasion (BVI), the role and mechanism of LVI in breast carcinoma metastasis is not well established. Tumour-associated macrophage (TAM) is described as an important inflammatory cell playing a significant role in tumour progression (Obeid et al. 2013). The involvement of TAM in local proteolysis was shown to promote invasion and metastatic of breast carcinoma cells (Volodko et al. 1998). High macrophage count is associated with neovascularisation and primary tumour growth (Storr et al. 2012). Further study proved that cytokines released by TAM might regulate tumour cell invasion to the lymphatic vessel (Safuan et al. 2012). However, the mechanism driving this regulation is still unclear.

Cell adhesion molecule (CAM) is the protein located on the surface of the cell, which is involved in cell to cell and cell to extracellular matrix (ECM) binding or adhesion to maintain the integrity of the cell structure, tissue repair, and wound healing (Lachapelle & Foulkes 2011). Currently, there are 50 types of CAMs that have been discovered, including integrin, cadherin, selectins, and immunoglobulin (Ig) family (Drivalos et al. 2011; Farahani et al. 2014; Okegawa et al. 2004). Adhesion molecules play an important role in the activation of cancer-related biological processes such as survival, migration, extravasations, homing, and metastasis of malignant cell (Hein et al. 2011; Schroder et al. 2011). The ICAM-1, ICAM-4, and ICAM-5 genes located on chromosome 19p13.2 might influence the risk of breast and prostate cancer (Kammerer et al. 2004). ICAM-1 molecules have the highest expression in both normal and tumor breast tissue compared to ICAM-4 and ICAM-5 (Kammerer et al. 2004). The expression of ICAM-1 also increases in the inflammatory environment (Hua 2013). The inflammatory cytokines such as tumor necrosis factorα (TNF-α) and interleukins upregulate the expression of ICAM-1 in various cell types including breast carcinoma (Hua 2013; Muro & Muzykantov 2005). Higher ICAM-1 expression levels were significantly associated with the active event of breast carcinoma cell invasion *in vitro* (Rosette et al. 2005)2005. However, the role of ICAM-1 in influencing lymphatic vessel invasion (LVI) in breast carcinoma was not established. The aim of this study was to investigate the roles of TAM influencing lymphovascular invasion via adhesion molecule, ICAM-1.

Materials and Methods

Patients and Specimens

This study was conducted on 37 consecutive FFPE archival specimens of breast carcinoma obtained from Universiti Sains Malaysia Hospital (HUSM). The specimens within the year 2009 to 2014 were included in this study. Ethical approval for this study was granted by the Human Research Ethics Committee (HREC), Universiti Sains Malaysia (FWA Reg. No.: 00007718; IRB Reg. No.: 00004494). Clinical data of the patients were summarized in Table 1.

TABLE 1. Clinicopathological data of patients

N=37	Clinicopathological data
Age (Years)	
Mean ± Standard deviation	53.57 ± 10.18
Upper-lower boundaries	50.18 – 56.96
Range	36 – 76
Tumor grade	
I	4 (10.8%)
II	19 (51.4%)
III	14 (37.8%)
Tumor size in diameter (mm)	
1 – 30	11 (29.7%)
31 – 50	13 (35.1%)
>51	13 (35.1%)
ER status	
Positive	15 (40.5%)
Negative	21 (56.8%)
Not determine	1 (2.7%)
PR status	
Positive	19 (51.4%)
Negative	18 (48.6%)
HER2 status	
Positive	18 (48.6%)
Negative	19 (51.4%)
Number of positive node	
0	13 (35.1%)
1 – 4	13 (35.1%)
≥5	11 (29.7%)

ER, oestrogen receptor; HER2, human epidermal receptor 2; mm, millimetre; PR, progesterone receptor

Haematoxylin and Eosin (H&E Staining)

Slides with 4 μm thicknesses of FFPE tissue were placed on a 60ºC hotplate (Electrothermal, UK) for 30 min. The clearing steps were carried out by dipping the slides in two changes of xylene for 5 min each, continued with rehydration in a series of descending ethanol concentrations (100%, 90%, 80%, 70%, and 50% in water for 1 min at each concentration). Next, the slides were dipped in two changes of distilled water for 5 min each. Slides were stained with filtered Harris haematoxylin (Sigma, UK) for 5 min. Next, the slides were washed in running tap water for 5 min, continued with differentiation in 0.3% acid alcohol for 3 s. Slides were washed under running tap water for 5 min. Bluing was carried out in 0.5% ammonia water. Slides were rinsed in running tap water for 5 min, continued with eosin (Sigma, UK) counterstain for 2 min. Dehydration step was carried out in a series of ascending ethanol concentrations (50%, 70%, 80%, 90% and 100%) in water for 1 min each.

Next, the slides were fixed in two changes of xylene and mounted with DPX (Merk, Germany). Slides were left to dry overnight before viewing under the microscope.

IMMUNOHISTOCHEMICAL (IHC) STAINING

Four consecutive FFPE breast carcinoma sections from each block were stained with CD34 (Dako, Germany), D2-40 (Abcam, UK), CD163 (GeneTex, USA), and ICAM-1 (GeneTex, USA) monoclonal antibodies to stain blood vessels, lymphatic vessels, macrophages, and ICAM-1, respectively.

Staining optimisation was conducted previously on tonsil tissue and breast carcinoma composite whole sections before using them in the main breast carcinoma cohort. Antigen retrieval (pH6.0) (DAKO, Germany) was carried out using a pressure cooker (WMF, Germany) for 3 min at full power (121ºC). Next, 3% hydrogen peroxide in methanol was used to block endogenous hydrogen peroxidase activity for 5 min. The slides were incubated with primary antibodies (1:100 of CD34, 1:500 of D2-40, 1:500 of CD163, and 1:300 of ICAM-1) diluted in antibody diluent (Dako, Germany) for 1 h at room temperature. Phosphate buffer saline (PBS), pH7.2 to pH7.6 was used to wash the unbound primary antibodies, followed by the addition of DAKO Real Envision Detection System (DAKO, Germany) for another hour at room temperature. The slides were then washed with PBS and immunohistochemical reactions were developed by using 1:20 of Diaminobenzidine (DAB) Substrate Chromogen System (DAKO, Germany) in DAB buffer for 5 min. Counterstained was carried out by using filtered Harris Haematoxylin for 30 s. Excess counterstained was removed under running tap water for 5 min. Sections were then dehydrated in a series of ascending ethanol concentrations (50%, 70%, 80%, 90% and 100% in water for 1 min at each concentration). Next, the slides were fixed in two changes of xylene and mounted with DPX. Slides were left to dry overnight before viewing under the microscope. Tonsil sections were used as both positive and negative controls each time staining was conducted. Positive controls were treated by using the same procedure as above. For the negative controls, the primary antibody was excluded.

ASSESSMENT OF LYMPHATIC AND BLOOD VESSELS

Evaluation of lymphatic and blood vessel densities across the whole sections stained with D2-40 and CD34 were done within the tumor (intra-tumoral) and around the tumor (peri-tumoral). Each tumor section was examined by using the low power microscopic field (40× magnification) and divided into intra-tumoral and peri-tumoral areas. Three hotspots which consist of areas with the highest count stained vessels at 100× magnification of a light microscope were evaluated for each slide. The practice of hotspots enables the collection of information, especially vessel density and macrophage count across the whole tissue section (Mohammed et al. 2007). The hotspot technique applied on the assessment of vessel density and macrophage count have been practiced by most previous studies on FFPE samples (Goddard et al. 2001). The mean value of the three hotspots for both lymphatic and blood vessels density in each sample was used for analysis. Total vessel density for each sample equal to the sum of the intra-tumoral vessel and peri-tumoral vessel densities.

The detection of lymphatic and blood vessel invasion was carried out in CD34, D240, and the H&E stained slides. In IHC staining, LVIs were counted across the whole section and divided into intra-tumoral and peri-tumoral areas. As CD34 can also stain the lymphatic vessels subset, LVI was identified as the presence of tumor cells within both positive D2-40 and CD34-stained vessels. In contrast, BVI was identified as the tumor cells within the vessels that were positively stained with CD34 but negative with D2-40. H&E staining was compared with IHC staining to evaluate the frequency of the lymphovascular invasion and to determine the false positive or false negative staining. The false positive lymphovascular invasion was reported when H&E-stained tissues were scored as a positive invasion but negative in the IHC slides. The false negative lymphovascular invasion was reported when H&E-stained tissues were scored as a negative invasion but scored positive in CD34 and D240 slides.

ASSESSMENT OF MACROPHAGE COUNT

The macrophage count was evaluated by using sections stained with CD163. Three hotspots which consist of areas with the highest count stained macrophage at 100× magnification of a light microscope was evaluated for each slide.

ASSESSMENT OF ICAM-1 SCORES

Positively stained sections with ICAM-1 were viewed under 100× magnification of light microscope and graded by using the histochemical score (H-score). The H-score is a method comprises of the percentage of staining intensity multiplied by the ordinal scores of the staining; scores 0 to 3 corresponding to negative, weak, moderate, and strong intensity of the staining. Total scores were obtained by the formula of (0 × negatively stained %) + (1 × weakly stained %) + (2 × moderately stained %) and (3 × strongly stained %), given a range of 0 (no staining in the section) to 300 (strongly stained cells in the section).

STATISTICAL ANALYSIS

Lymphovascular densities, macrophage count, and ICAM-1 intensity were plotted in distribution histograms and classified into two groups according to the median value (high>median, low<median). Statistical analysis was performed using grouped clinicopathological parameters. The relationship between lymphovascular densities and invasions, with clinicopathological data, were assessed using Pearson Chi-Square test of association (X^2) (or Fisher's Exact test if a cell count was <5). Statistical

analysis was carried out using Statistical Package for the Social Sciences (SPSS) (IBM SPSS Statistics version 22.0).

Results and Discussion

The aim of the current study was to investigate the relationship between LVI and BVI in breast carcinoma cells dissemination using IHC staining and the association of these characters with clinicopathological criteria of breast carcinoma. Furthermore, conventional assessments of lymphovascular invasion by using H&E staining was compared with those assessed in IHC staining with specific endothelial antibodies. The role of TAM in influencing lymphovascular density as well as lymphovascular invasion in breast carcinoma was investigated by studying the associations between macrophage counts and characteristics of the vessels. In addition, the study aimed to distinguish the relationship between TAM, LVI, and ICAM-1 in breast carcinoma.

Relationship between lymphovascular density and lymphovascular invasion

Based on the scoring, the mean value of intra-tumoral LVD and peri-tumoural LVD were 8.24 and 9.11 (data not shown). The total LVD ranged from 5 to 60. The BVD is majority intra-tumoral area, ranged between 18 and 150 per hotspot. The peri-tumoral BVD ranged from 7 to 120. The total BVD which indicates the sum of intra- and peri-tumoral BVD ranged from 47 to 245. The BVD is significantly higher than LVD, $p<0.001$ (131.3 and 17.62, respectively). LVI was mainly peri-tumoural, with the mean of 2.08. The mean of intra-tumoral LVI was 1.84. The total LVI which indicates the total of intra- and peri-tumoral LVI shows the mean of 3.92. BVI was located mainly in the peri-tumoural area with the mean of 4.19. The intra-tumoral BVI mean is 2.97. The total BVI which indicates the total of intra- and peri-tumoral BVI shows the mean of 7.16. In general, the total BVD from 37 breast carcinoma samples is higher than LVD (4858/642). However, the invasions are higher in lymphatic vessel (145/642, 24.0%) compared to blood vessels (265/4858, 5.5%).

Lymphatic vessels were involved in the spread of metastatic tumor cells but not in the proliferation and expansion of the tumor size (Nishida et al. 2006). Tumor mass could be the reason why LVD less observed intra-tumorally. However, without blood circulation, tumor cells can only grow up to 2 mm in diameter, angiogenesis makes it possible to grow more than 2 mm (Nishida et al. 2006). This could be the reason why BVD was higher than LVD. Current study showed that LVD is reduced with increased of tumour size. A study by Vleugel et al. (2004) showed that increases tumour mass increase BVD but reduce intra-tumor LVD in invasive breast carcinoma sample.

There was a statistically significant difference of mean score between LVI and BVI (7.16 and 3.92, respectively). From the 37 breast carcinoma samples, a total of 652 lymphatic vessels and 4858 blood vessels were counted. The results showed that BVD was much higher than LVD. Even though the mean score of BVI was significantly higher than the LVI, the percentage of LVI was higher than BVI among vessel densities, 22.24% (145/652) and 5.45% (265/4858), respectively. High LVD was significantly associated with distant metastases, tumor stage, and poorer Nottingham prognostic index (Mohammed et al. 2011). The current study showed that increase of LVD is significantly increased LVI. This statement is supported by the study of Bono et al. (2004), showing that increases of peri-tumoural LVD significantly associated with poor prognosis of ductal breast carcinoma.

Relationship between lymphatic vessel density and invasion with clinicopathological variables

Table 2 shows the association of LVD with clinicopathological data. The intra-tumoral LVD was significantly associated with HER2 negative status (Fisher's Exact test, $p=0.022$). Peri-tumoral LVD was not associated with any clinicopathological characteristics. Total LVD was significantly reduced on larger tumor size (Mann-Whitney U test, $X^2=6.193$, df=2, $p=0.045$). Intra- and peri-tumoral, as well as total blood vessel densities, were not associated with any clinicopathological characteristics.

The HER2 gene is located on the long arm of chromosome 17 and encodes a 185-kDa transmembrane protein found in breast cells. Overexpression of HER2 is often related to aggressive tumor phenotype, reduced survival rate, and poor prognosis of breast carcinoma patients (Hudis 2007; Meric-Bernstam & Hung 2006). Breast carcinoma patients with HER2 positive tumour often receive Trastuzumab, the humanized monoclonal antibody targeting the HER2 receptor as therapy (Mitri et al. 2012). The current study showed that increased intra-tumoral LVD and LVI were significantly associated with HER2 negative status. Basal-like breast carcinoma patients have been found to develop visceral metastasis and shorter relapse-free survival times (Minn et al. 2005; Perou et al. 2000; Rodríguez-Pinilla et al. 2006).

Table 3 shows the association of LVI with clinicopathological data. The increased of intra-tumoral LVI was significantly associated with HER2 negative status (Fisher's Exact test, $p=0.05$). Peri-tumoral and total LVI were not associated with any clinicopathological characteristics. Intra-tumoral and peri-tumoral, as well as total BVI, were not associated with any clinicopathological characteristics (data not shown).

Association between lymphovascular invasions detected by IHC and H&E staining (data not shown)

The intra-tumoral lymphovascular invasion detected in IHC-stained tissues was significantly associated with those detected in H&E-stained tissues (Fisher's Exact test, $p<0.001$), with the median of 2.0 and 1.0, respectively. Peri-tumoral invasion detected on IHC-stained tissues was significantly higher than those detected on H&E stained tissues (Fisher's Exact test, $p<0.001$), with the median

TABLE 2. Association between LVD with clinicopathological characteristics

Categories		Lymphatic vessel density n(%)								
		Intra-tumoral			Peri-tumoral			Total		
		Low	High	*p* value	Low	High	*p* value	Low	High	*p* value
Age	36 – 50	8(33.3)	7(47.4)	0.85	7(41.2)	8(40.0)	0.95	6(46.2)	9(37.5)	0.80
	51 – 60	6(50.0)	8(26.3)		6(35.3)	8(40.0)		4(30.8)	10(41.7)	
	>60	4(16.7)	4(26.3)		4(23.5)	4(20.0)		3(23.1)	5(20.8)	
Tumor Size (mm)	0 – 30	4(22.2)	7(36.8)	0.18	4(23.5)	7(35.0)	0.11	2(15.4)	9(37.5)	0.045
	31 – 50	5(27.8)	8(42.1)		4(23.5)	9(45.0)		3(23.1)	10(41.7)	
	>50	9(50.0)	4(21.1)		9(52.9)	4(20.0)		8(61.5)	5(20.8)	
Tumor Grade	I	0(0.0)	4(21.1)	0.11	3(17.6)	1(5.0)	0.47	0(0.0)	4(16.7)	0.28
	II	11(61.1)	8(42.1)		8(47.1)	11(55.0)		7(58.3)	12(50.0)	
	III	7(38.9)	7(36.8)		6(35.3)	8(40.0)		6(46.2)	8(33.3)	
Number of Positive	0	4(22.2)	9(47.4)	0.12	5(29.4)	8(40.0)	0.73	3(23.1)	10(41.7)	0.50
Node	1 – 4	6 (33.3)	7(36.8)		7(41.2)	6(30.0)		5(38.5)	8(33.3)	
	≥5	8(44.4)	3(15.8)		5(29.4)	6(30.0)		5(38.5)	6(25.0)	
ER Status	Negative	9(50.0)	7(36.8)	0.52	6(35.3)	10(50.0)	0.5	6(46.2)	10(41.7)	1.00
	Positive	9(50.0)	12(63.2)		11(64.7)	10(50.0)		7(53.8)	14(58.3)	
PR Status	Negative	10(55.6)	9(47.4)	0.75	6(35.3)	13(65.0)	1.0	7(53.8)	12(50.0)	1.00
	Positive	8(44.4)	10(52.6)		11(64.7)	7(35.0)		6(46.2)	12(50.0)	
HER2 Status	Negative	5(27.8)	13(68.4)	0.022	9(52.9)	9(45.0)	0.75	5(38.5)	13(54.2)	0.50
	Positive	13(72.2)	6(31.6)		8(47.1)	11(55.0)		8(61.5)	11(41.8)	
Triple Negative	No	17(94.4)	14(73.7)	0.18	15(88.2)	16(80.0)	0.7	12(92.3)	19(79.2)	0.39
	Yes	1(5.6)	5(26.3)		2(11.8)	4(20.0)		1(7.7)	5(20.8)	

ER, oestrogen receptor; HER2, human epidermal receptor 2; mm, millimetre; PR, progesterone receptor

TABLE 3. Association between LVI with clinicopathological characteristics

Categories		Lymphatic vessel invasion n(%)								
		Intra-tumoral			Peri-tumoral			Total		
		Low	High	*p* value	Low	High	*p* value	Low	High	*p* value
Age	36 – 50	7(36.8)	8(44.4)	0.43	7(33.3)	8(50.0)	0.43	7(38.9)	8(42.1)	0.98
	51 – 60	9(47.4)	5(27.8)		8(38.1)	6(37.50)		7(38.9)	7(36.8)	
	>60	3(15.8)	5(27.8)		6(28.6)	2(12.5)		4(22.2)	4(21.1)	
Tumor Size (mm)	0 – 30	4(21.1)	7(38.9)	0.46	6(28.6)	5(31.3)	0.91	3(16.7)	8(42.1)	0.22
	31 – 50	8(42.1)	5(27.8)		7(33.3)	6(37.5)		8(44.4)	5(26.3)	
	>50	7(36.8)	6(33.3)		8(38.1)	5(31.3)		7(38.9)	6(31.6)	
Tumor Grade	I	3(15.8)	1(5.6)	0.60	4(19.0)	0(0.0)	0.16	3(16.7)	1(5.3)	0.13
	II	9(47.4)	10(55.6)		9(42.9)	10(62.5		11(61.1)	8(42.1)	
	III	7(36.8)	7(38.9)		8(38.1)	6(37.5)		4(22.2)	10(52.6)	
Number of Positive	0	6(31.6)	7(38.9)	0.62	9(42.9)	4(25.0)	0.49	7(38.9)	6(31.6)	0.90
Node	1 – 4	6 (31.6)	7(38.9)		7(33.3)	6(37.5)		6(33.3)	7(36.8)	
	≥5	7(36.8)	4(22.2)		5(23.8)	6(37.5)		5(27.8)	6(31.6)	
ER Status	Negative	8(42.1)	8(44.4)	1.00	9(42.9)	7(43.8)	1.00	7(38.9)	9(47.4)	0.74
	Positive	11(59.9)	10(55.6)		12(57.1)	9(56.3)		11(61.1)	10(52.6)	
PR Status	Negative	9(52.6)	10(55.6)	0.75	10(47.6)	9(56.3)	0.74	7(38.9)	12(63.2)	0.19
	Positive	10(44.4)	8(44.4)		11(52.4)	7(43.8)		11(61.1)	7(36.8)	
HER2 Status	Negative	6(31.6)	12(66.7)	0.05	12(57.1)	6(37.5)	0.33	7(38.9)	11(57.9)	0.33
	Positive	13(68.4)	6(33.3)		9(42.9)	10(62.5)		11(61.1)	8(42.1)	
Triple Negative	No	17(94.4)	14(73.7)	0.41	16(76.2)	15(93.8)	0.21	15(92.3)	16(79.2)	1.0
	Yes	2(5.6)	4(26.3)		5(23.8)	1(6.3)		3(7.7)	3(20.8)	

ER, oestrogen receptor; HER2, human epidermal receptor 2; mm, millimetre; PR, progesterone receptor

of 4.0 and 1.0, respectively. The total lymphovascular invasion detected in IHC staining was significantly higher than the lymphovascular invasion detected in H&E staining (Fisher's Exact test, $p<0.001$), with the median of 8.0 and 3.0, respectively. Intra-tumorally, vessel invasions detected in H&E was missed 41.57% (104/178) compared with vessel invasions detected in IHC. Peri-tumorally, vessel invasions detected in H&E was missed in 56.03% (102/232) compared with vessel invasions detected in IHC. Generally, total lymphovascular invasion detected in H&E was missed by 49.76% (206/410). Figure 1 shows the representative of true positive lymphatic vessel invasion which was scored as positive invasion in the H&E and D2-40 but scored negative in the CD34-stained tissue.

The current study shows that the specific endothelial antibodies such as CD34 and D2-40 for detection of blood and lymphatic vessel endothelial cells, respectively, was more sensitive than H&E staining which have been reported by other studies (Braun et al. 2008; Storr et al. 2012). Detection of lymphovascular invasion in IHC was increased from 8 to 30% compared to H&E staining only (Storr et al. 2012). Routine H&E was shown to have 64% false positive rate (Storr et al. 2012). The current study showed that observation of total lymphovascular invasion was missed in 49.76% in H&E-stained tissue (206 vessel invasions) compared to those stained with IHC (410 vessel invasions). The IHC staining improves the accuracy of diagnosis up to 33.12%. Therefore, IHC with CD34 and D2-40 should be considered in histological reporting for accurate individual interpretation. At the same time, it could help to overcome missed observation of scanty positive invasive vessels.

RELATIONSHIP BETWEEN MACROPHAGE COUNT AND ICAM-1 SCORES WITH CLINICOPATHOLOGICAL CHARACTERISTICS

Figure 2 shows the representative of ICAM-1-stained tissue viewed under 400× magnification of a light microscope. Table 4 shows the association of macrophage count and ICAM-1 scores with clinicopathological characteristics. Macrophage infiltration was not associated with any clinicopathological characteristics. However, ICAM-1 scores were significantly increased in non-triple negative breast carcinoma (Fisher's Exact test, $p=0.008$).

Triple-negative breast cancer (TNBC) consists of a group of heterogeneous tumours structured by various histological characteristics and genetics, consequently produced the features of low expression of hormonal receptors specifically ER, PR, and HER2 (Lachapelle & Foulkes 2011). Through observation of IHC-stained tissue from the current study, non-TNBC showed overexpression of ICAM-1. Furthermore, in the current study of IHC staining, ICAM-1 and lymphovascular invasion showed no significant association with individual tumor grades. These results were unexpected as we hypothesised that ICAM-1 will have a significant increase in TNBC and that macrophage is the main influence. Study of Guo et al. (2014) showed that ICAM-1 level is high in human TNBC cell line. They also found that ICAM-1 is overexpressed in 26 TNBC tissue microarrays. ICAM-1 was also increased in breast carcinoma patients serum compared to benign breast disease (Maksoud et al. 2017). However, this study also supports the current findings, showing that there is no association between ICAM-1 level with patients clinicopathological data including tumour stage, tumour size, lymph node involvement, and hormonal receptor status (Maksoud et al. 2017). The variation of findings among studies might be due to the differences

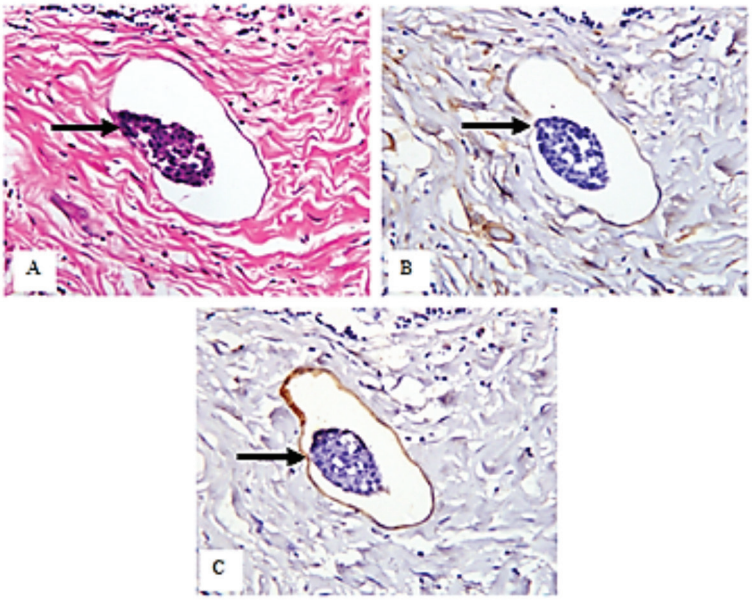

FIGURE 1. True positive LVI (black arrows) determined in H&E and D2-40-stained tissues. H&E-stained tissues scored as positive lymphovascular invasion (A). In CD34-stained tissues (B), BVI was scored as negative. At the same field of microscopic field view (400× magnification), LVI was clearly observed in D2-40-stained tissues (C)

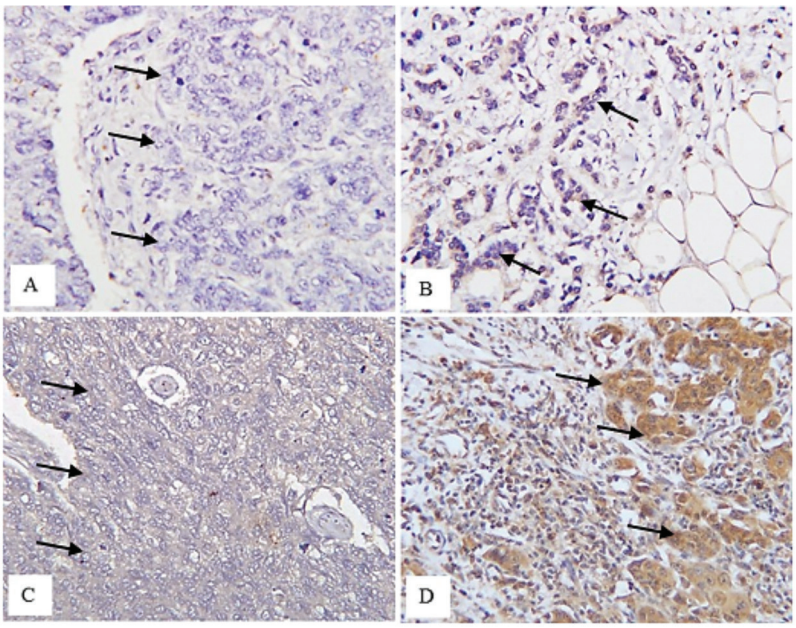

FIGURE 2. ICAM-1 immunoreactivity patterns in various scores (black arrows). ICAM-1 intensities were evaluated by using the H-score method. A: ICAM-1 intensity scored as 0 (negative). B, C, and D were scored as 1+ (scanty), 2+ (moderate), and 3+ (strong), respectively. All images were taken at 400× magnification of a light microscope

TABLE 4. Association between macrophage count and ICAM-1 scores with clinicopathological characteristics

Categories		Macrophages count n(%)			ICAM-1 Scores n(%)		
		Low	High	*p* value	Low	High	*p* value
Age	36 – 50	7(41.2)	8(40.0)	0.48	8(44.4)	7(36.8)	0.76
	51 – 60	5(35.3)	9(40.0)		7(38.9)	7(36.8)	
	>60	5(23.5)	9(20.0)		3(16.3)	5(26.3)	
Tumor size (mm)	0 – 30	4(23.5)	7(35.0)	0.11	4(22.2)	7(36.8)	0.46
	31 – 50	9(52.9)	4(20.0)		8(44.4)	5(26.3)	
	>50	4(23.5)	9(45.0)		6(33.3)	7(36.8)	
Tumor grade	I	1(5.9)	3(15.0)	0.58	1(5.6)	3(15.8)	0.60
	II	10(58.8)	9(45.0)		10(55.6)	9(47.4)	
	III	6(35.3)	8(40.0)		7(38.9)	7(36.8)	
Number of positive node	0	9(52.9)	4(20.0)	0.11	7(33.3)	6(36.8)	0.66
	1 – 4	4(23.5)	9(45.0)		5(27.8)	8(42.1)	
	≥5	4(23.5)	7(35.0)		6(38.9)	5(21.4)	
ER status	Negative	7(41.2)	9(45.0)	1.00	9(50.0)	7(36.8)	0.52
	Positive	10(58.8)	11(55.0)		9(50.0)	12(63.2)	
PR status	Negative	8(47.1)	11(55.0)	0.75	9(50.0)	10(52.6)	1.00
	Positive	9(52.9)	9(45.0)		9(50.0)	9(47.4)	
HER2 status	Negative	7(41.2)	11(55.0)	0.52	10(55.6)	8(42.1)	0.52
	Positive	10(58.8)	9(45.0)		8(44.4)	11(57.9)	
Triple negative	No	14(82.4)	17(85.0)	1.0	12(66.7)	19(100.0)	0.008
	Yes	3(17.6)	3(15.0)		6(33.3)	0(0.0)	

ER, oestrogen receptor; HER2, human epidermal receptor 2; mm, millimetre; PR, progesterone receptor

in specimens' pathological characteristics such as the lymph node involvement and the hormone receptors status. Sample preparation and techniques used in each study might influence the final results obtained. Immediate tissue fixation after removal is crucial.

Several studies proved that protein extraction from archived FFPE sample are non-degraded and could be applied to suitable methods such as western blot and protein microarray, and two-dimension (2D) gel electrophoresis (Addis et al. 2009; Espina et al. 2003; Wolff et al. 2011).

However, delayed tissue fixation might destroy the quality of the sample (Wolff et al. 2011). The major challenge in IHC study includes critical timing of pre-treatment of target antigen retrieval and elimination of unspecific binding. Consequently, these situations might affect the quality of microscopic examination. Therefore, a special research on proper sample preparation, collection, and handling for IHC study is suggested in the future. The biomarkers used in vessels detection and differences of reagents and solutions product used might also affect the final IHC results.

In term of the tumour microenvironment, macrophages play an important role in promoting metastasis by secreting numerous cytokines including interleukin 1 beta (IL-1β) and tumour necrosis alpha (TNF-α) (Martinez et al. 2008). These cytokines might induce the secretion of ICAM-1 on malignant cells and vascular endothelial cell during inflammation (Ley et al. 2011). The current study showed no significant increase of macrophage infiltration on TNBC nor non-TNBC samples. Therefore, in this case, overexpression of ICAM-1 on non-TNBC might be related to inflamed vascular endothelial cells but not macrophage itself.

RELATIONSHIP BETWEEN LYMPHOVASCULAR DENSITY AND INVASION WITH MACROPHAGE COUNT AND ICAM-1 SCORES

The lack of association between TAM and total LVI in this study was not expected. Hypothetically, it was postulated that TAM increases LVI via ICAM-1 receptor (p=0.25 and p=0.87, respectively). The other cytokines such as interleukin 10 (IL-10) which was proven to suppress the other cytokine including interleukin 6 (IL-6) in in tumour microenvironment (Kozłowski et al. 2002). In this situation, IL-10 might have the potential to suppress the other cytokines based on individual tumour development. Another study proved that breast carcinoma patients with ER-negative showed overexpression of IL-10. Further studies are required to investigate the connection of ICAM-1 with other cytokines, such as IL-1β, IL-10 and IL-6.

CONCLUSION

ICAM-1 targeted molecule could be the possible alternative therapeutic target for non-TNBC treatment. Specific lymphatic vessel endothelial antibody (D2-40), as well as blood vessel endothelial antibody (CD34), should be considered in histological reporting. The current study results could strengthen our understanding of lymphovascular invasion and the role of ICAM-1 in breast carcinoma. Inhibition of the ICAM-1 molecule could reduce the tumor metastasis event.

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