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ORIGINAL ARTICLE

High CD4+/CD8+ Intratumour Ratio is Associated with Favourable Outcome in Triple-negative Breast Cancer

THIAN QZ[1], REENA RMZ[2]*, THONG QX[3], MUAATAMARULAIN M[2], NURWAHYUNA R[2], ADAM A[4]

[1]*Department of Biology, Faculty of Science, Universiti Sains Malaysia, Pulau Pinang, Malaysia*

[2]*Department of Pathology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Yaacob Latif, Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Malaysia*

[3]*Ultimed Scientific Sdn Bhd, No.B-15-1, Jln C180/1, Dataran C180, 43200 Cheras, Selangor, Malaysia.*

[4]*Center for Artificial Intelligence Technology, Faculty of Information Science and Technology, Universiti Kebangsaan Malaysia, Bangi, Selangor, Malaysia*

ABSTRAK

Kanser payudara tripel-negatif (TNBC) ialah subjenis kanser payudara yang sangat kompleks dan menyumbang kepada 10-15% daripada semua kanser payudara yang didiagnos. Terdapat peningkatan rintangan TNBC terhadap kemoterapi konvensional dan terapi hormon kerana kekurangan reseptor estrogen (ER), reseptor progesteron (PR) dan ekspresi reseptor faktor pertumbuhan epidermis manusia -2 (HER2). Hubungan dan interaksi antara kedua-dua limfosit T CD4+ dan CD8+ adalah sangat penting untuk merangsang sistem imun adaptif semasa tindak balas imun anti-kanser. Kajian ini bertujuan untuk menentukan nisbah limfosit T CD4+ dan CD8+ dalam TNBC melalui ujian imunohistokimia dan mengkaji hubung kait antara limfosit penyusupan tumor (TIL) CD4+ dan CD8+ dengan kemandirian hidup. Kuantifikasi kedua-dua subset TIL melalui pewarnaan imunohistokimia dilakukan menggunakan mikroskop cahaya konvensional. Pelbagai jenis subset CD4+ dan CD8+ TIL ditemui dalam stroma intratumor TNBC dengan skor min populasi masing-masing antara 0.93 dan 0.53. Walau bagaimanapun, perbezaan purata populasi antara kedua-dua subset TIL adalah tidak signifikan secara keseluruhan (nilai P CD4+ = 0.484; CD8+ = 0.835) apabila dibanding secara statistik menggunakan ujian-t bebas. Nisbah CD4+/CD8+ dalam stroma intratumor adalah berkisar antara 0.50-5.88 di antara semua subspesies TNBC. Nisbah CD4+/CD8+ yang tinggi dalam stroma intratumor menunjukkan hubungan yang baik dengan

Address for correspondence and reprint requests: Prof. Dr. Reena Rahayu Md Zin. Department of Pathology, Faculty of Medicine, Universiti Kebangsaan Malaysia, Jalan Yaacob Latif, Bandar Tun Razak, 56000 Cheras, Kuala Lumpur, Malaysia. Tel: +603-91455359 Email: reenarahayu@ppukm.ukm.edu.my

hasil kemandirian hidup dalam susulan rawatan selama 2 tahun.

Kata kunci: biomarker prognostik, CD4+, CD8+, kanser payudara tiga kali ganda negatif (TNBC), limfosit penyusupan tumor (TILs), patologi digital

ABSTRACT

Triple-negative breast cancer (TNBC) is a heterogenous breast cancer subtype which accounts for 10-15% among all diagnosed breast cancers. There is increased resistance of TNBC to conventional chemotherapy and hormonal therapy due to lack of oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) expression. Mutual relationship and interactions between both $CD4^+$ and $CD8^+$ T lymphocytes are very crucial for eliciting adaptive immune system during anti-cancer immune response. This study aimed to determine the ratio of $CD4^+$ and $CD8^+$ T lymphocytes in TNBC by immunohistochemistry assay and to investigate the association of $CD4^+$ and $CD8^+$ tumor-infiltrating lymphocytes (TILs) with survival outcome. Quantification of both immunostaining TILs subset was done using conventional light microscope. A wide range of $CD4^+$ and $CD8^+$ TILs subset was found within intratumor stroma of TNBC with population mean score of 0.93 and 0.53, respectively. However, the difference of mean population between both TILs subsets was insignificant overall (P-value of $CD4^+$= 0.484; $CD8^+$= 0.835) when compared statistically using independent t-test. The ratio of $CD4^+/CD8^+$ in intratumor stroma ranged from 0.50-5.88 among all TNBC subtypes. The high $CD4^+/CD8^+$ ratio within intratumor stroma showed favorable association with survival outcome during the 2 years' follow-up.

Keywords: $CD4^+$, $CD8^+$, digital pathology, prognostic biomarkers, triple-negative breast cancer (TNBC), tumor-infiltrating lymphocytes (TILs)

INTRODUCTION

More than two-thirds of breast cancers express progesterone receptor (PR) and oestrogen receptor (ER) while approximately 20% show overexpression of human epidermal growth factor receptor 2 (HER2) gene (Howlader et al. 2014). Lacking of ER/PR expression and HER2 amplification in triple-negative breast cancer (TNBC) account for 10-15% of all diagnosed breast cancers (Dawson et al. 2009) and this underlie the main reason of their resistance to most conventional chemotherapy (Lin et al. 2012). In cases which are subjected to chemotherapy, more than 50% of TNBC patients do not have complete pathologic response (cPR) in addition to high risk of local recurrence or distant relapse (Soni et al. 2015; Wang

et al. 2017). Due to the high rates of non-responders, alternative treatment other than chemotherapy are highly sought following by the need to identify potential novel biomarkers in improving the treatment outcome.

Recently, T lymphocyte infiltration into primary tumors had been proven as favorable prognostic factor in variety of solid tumors such as colorectal carcinoma, ovarian carcinoma and non-small cell lung carcinoma (Gooden et al. 2011). The tumor-infiltrating lymphocytes (TILs) is an indication that a local immune system is activated in response to tumor progression (Canna et al. 2005). In breast cancer, most of the TILs are mainly composed of $CD4^+$ and $CD8^+$ T lymphocytes, T regulatory cells, mast cells, plasma cells and macrophages (Bense et al. 2017). However, most of the TILs were mainly $CD4^+$ and $CD8^+$ T lymphocytes which mediated pro-inflammatory type 1 immunity. Majority of them expressed effector cytotoxic ($CD8^+$) phenotype according to previous studies that involved patients with various carcinoma and melanoma (Ben-Hur et al. 2002; Leong et al. 2006). The TILs association and their prognostic significance with treatment cPR had also been highlighted in several cohorts studies, both under adjuvant and neoadjuvant settings (Adams et al. 2014; Ali et al. 2014; Loi et al. 2014; Salgado et al. 2015).

The $CD4^+$ T lymphocytes has become increasingly highlighted for their pivotal role in effective anti-tumor immunity and to activate the $CD8^+$ cytotoxic T lymphocytes (CTL) response in cancer immunotherapies. $CD4^+$ T lymphocyte could differentiate into diverse functional subtypes due to their polyfunctional properties, which in turn allowing them to play the central "coordinator" role in eliciting appropriate effector immune response (Tay et al. 2021). $CD8^+$ effector T lymphocytes play essential role in cellular-mediated immune response by killing cancer cells upon interaction with specific antigen presenting cells (APC) whereas $CD4^+$ T lymphocytes facilitate $CD8^+$ anti-tumor function within the tumor microenvironment (Dobrzanski et al. 2006). Mutual relationship and interactions between both $CD4^+$ and $CD8^+$ T lymphocytes are very crucial for eliciting adaptive immune system during anti-cancer immune response.

Interleukin-10 (IL-10) is an anti-inflammatory cytokine that suppresses the inflammation arising from immune response. IL-10 deficiency could result in development of inflammatory diseases or autoimmune diseases (Wilson et al. 2005). Originally, IL-10 is synthesised by macrophages, dendritic cell (DC), B lymphocytes, $CD4^+$ and $CD8^+$ T lymphocytes. In previous study, IL-10 was secreted to inhibit the activity of helper T-cells, natural killer (NK) cells and macrophages during infection to prevent tissue damage due to hyperinflammatory responses (Couper et al. 2008). The immunosuppressive effect of IL-10 is mediated by heterodimeric IL-10 receptor (IL-10R1, IL-10R2) which is expressed on effector immune cells through activated JAK/STAT signaling pathway upon receptor binding (Wills-

Karp et al. 2010). This in turn, decreases the release of pro-inflammatory cytokines and concomitantly decreases the immune cell activities. Thus, IL-10 is often expressed aberrantly in cancer cells as one of the mechanism to evade immunosurveillance.

The TIL subsets were previously explored in a cohort of 42 TNBC cases at the tumor-host interface within intratumoral stroma and $CD4^+/CD8^+$ ratio was found to be significantly associated with TNBC patients overall survival (OS) (Wang et al. 2017). Intratumoral TILs (iTu-Ly) refer to those lymphocytes which have direct contact with tumor cell with no intervening stroma (Khoury et al. 2018). Recently, more data has shown that TILs infiltration has been used as a favorable prognostic indicator in a wide range of solid primary tumors such as colorectal, ovarian and non-small cell lung carcinomas (Gooden et al. 2011). Presence of TILs has often been associated with better OS and has response to cancer treatments. The number of TILs could be used as a predictive biomarker for adjuvant treatment outcomes indicating strong association between TILs and better overall survival rates (Adams et al. 2014; Gooden et al. 2011; Liu et al. 2011).

The aim of this study was to investigate the potential of TILs and IL-10 as prognostic biomarkers by correlating their expression with patients' OS. The relationship between TILs subsets with the level of IL-10, how IL-10 regulated the level of intratumor TILs in TNBC and possibility of cancer progression caused by impaired IL-10 function were elucidated in this study. We used conventional immunohistochemistry assay to identify TILs and IL-10 within the TNBC intratumor stroma.

The other objective of this study was to quantify the ratio of $CD4^+/CD8^+$ lymphocytes in TNBC and their correlation with level of IL-10. Semi-quantitative scoring systems were used to convert subjective IHC-marker expression into quantitative data, which was then used for statistical analysis by computer software as adapted from previous study (Akbar et al. 2015; Moratin et al. 2021; Wang et al. 2017).

MATERIALS AND METHODS

Clinicopathologic Characteristics

Samples were identified and obtained from Universiti Kebangsaan Malaysia Medical Center (UKMMC) database via ILMSV5503_PPUKM software. A total number of 50 samples were obtained from patients' archived list dated between 2012 and 2018. All samples were scored as negative for ER, PR and HER2 status in immunohistochemical expression. The HER2 status was also considered negative if the ratio of HER2/Chr17 was less than 2.0 (non-amplified) when tested by fluorescent in-situ hybridisation (FISH) or dual-clour dual hapten in-situ hybridisation (DDISH) test, which was performed when HER2 score was equivocal (Wolff et al. 2018). All of the cases met the inclusion criteria, which included 28 mastectomy specimens, 15 lumpectomy specimens and 7 wide-local excisional specimens.

Biopsies specimen were excluded from this study because they were too scanty to detect adequate amount of TILs for investigation (Wang et al. 2017). All patients had received conventional chemotherapy treatment and radiotherapy.

Immunohistochemistry Assay to Identify IL-10, CD4$^+$ and CD8$^+$ TILs in Paraffin-embedded Tissue Sections

Primary antibidies included; (i) Rabbit monoclonal CD8$^+$ antibody, Clone SP16 Ready-to-use (Ref. No. RMPD012, Diagnostic BioSystems California USA); (ii) Rabbit monoclonal CD4 antibody, Clone EP204 Ready-to-use (Ref. No. RMPD083, Diagnostic BioSystems California USA); (iii) Mouse monoclonal IL10 antibody, Clone E-10 (Cat. No. sc-8438, Santa Cruz Biotechnology, Inc Texas USA) was used at a dilution of 1:50. Lymph node tissue was used as a positive control.

Immunohistochemical staining was performed on the tissue sections using the protocol from Mouse/Rabbit PolyVue Plus™ HRP/DAB Detection System (Cat. No. PVP250D, Diagnostic BioSystems California USA). Primary antibodies were diluted to an optimal concentration using Antibody Diluent, Dako REAL (Code No. S2022, Dako Agilent Denmark). Washing steps between each reagent were performed using EnVision FLEX Wash Buffer 20X (Code No. K8007, Dako Agilent Denmark) which was diluted to a working solution with deionised water. The DAB-containing Substrate Working Solution was prepared by diluting the 50x concentrated EnVision FLEX DAB+ Chromogen with Envision FLEX Substrate Buffer (Code No. K8023, Dako Agilent Denmark).

Tissue blocks were sectioned approximately 3 µm thickness and mounted on adhesive glass slide, Platinum Pro White (Product No: PRO-01, Matsunami Japan). The slides were left to be air-dried in room temperature overnight. The tissue slides were then incubated on hot plate at 60ºC for 1 hour. Deparaffinisation and pre-treatment step were performed in the Decloaking Chamber™ NxGen (Ref. No: DC2012-220V, Biocare Medical California USA) using the Dako Target Retrieval Solution High pH (Code No. K8023, Dako Agilent Denmark) in the conditions of 110ºC for 30 minutes followed by another 30 minutes of cooling at room temperature and then were rinsed with running tap water for 3 minutes. The slides were subsequently incubated with Tissue Primer (Cat. No. PVP250D, Diagnostic BioSystems California USA) for 10 minutes followed by washing step and then were incubated with Background Blocker (Cat. No. PVP250D, Diagnostic BioSystems California USA) for 10 minutes. No washing step was done after this incubation.

Slides were then directly incubated with primary antibody for 30 minutes at room temperature followed by washing step. Next the slides were incubated with PolyVue™ (Enhancer) (Cat. No. PVP250D, Diagnostic BioSystems California USA) for 30 minutes followed by washing step. The slides were then incubated with PolyVue™ (HRP) (Cat. No. PVP250D, Diagnostic BioSystems

California USA) for 30 minutes in room temperature and followed by washing step. Slides were then incubated with DAB-containing Substrate Working Solution for 7 minutes and then rinsed with washing buffer.

The slides were then counterstained with Hematoxylin 2 (Richard-Allan Scientific™ Signature Series™ 16 oz, Ref. No. 7231, Thermo Fisher Scientific USA) for 3-dips after the procedures had been completed followed by dehydration step with increasing alcohol solutions (80%, 90%, and 100%) (Pureview Alcohol 100% Gal. Cat. No. PV1000, Cancer Diagnostics USA) and two times with Xylene (Richard-Allan Scientific™ Xylene, Ref. No. 6615, Thermo Fisher Scientific USA). Finally, the slides were mounted using CoverSeal™-X xylene-based mounting medium (Cat. No. FX2176, Cancer Diagnostics USA).

Evaluation and Quantification of IL-10, CD4+ and CD8+ T Lymphocytes

Expression of IL-10, CD4+ and CD8+ lymphocytes were identified using immunohistochemistry according to the manufacturer's protocols. The three strongest intensity of CD4+ and CD8+ T lymphocytes at three regions were selected to access CD4+ and then IL-10. The IL-10 and both TILs subsets at these regions were compared and quantified manually under a 20 high-power-fields (HPFs) scoring method (20x magnification) using a light microscope (OLYMPUS Model BX53) and expressed as mean population, respectively. In this study, semi-quantitative scoring system (modified H-score) which included degree of lymphocytic infiltration and IL-10 immunostaining intensity were used to convert IHC expression into quantitative data (0 to 1) (Table 1). The degree of TILs infiltration was considered positive if brown color staining was presented at the region and negative if no staining presence. An image processing software ImageJ (NIH, Bethesda, MD, USA) was used to validate the surface area and regional IHC intensity covered by IL-10, CD4+ and CD8+ TILs, thus confirming the degree of immunostaining. The calculated area of IL-10 and lymphocytes were divided by the area of fields to obtain the mean value for each sample. The counting step was performed twice for each slide. The final score was obtained by summing all scores from three regions before being expressed as CD4+ and CD8+ TILs' mean population, respectively.

Statistical Analysis

Besides descriptive statistics, population means of TILs among patients were compared statistically using independent t-test to determine any significant difference between population means (high CD4+/CD8+ ratio versus low CD4+/CD8+ ratio). All data and statistical calculation were

Table 1: Scoring system used to quantify degree of lymphocytic infiltration and IL-10 immunostaining intensity

Immunostaining Intensity	Value
Strong	1
Moderate	0.5
None	0

performed using SPSS version 16.0 (IBM Corp, Armonk, NY, USA). All data were expressed in the form of mean, median and standard deviation (SD). Disease-free survival (DFS) was calculated from the date of diagnosis until the date of death or follow-up cut off point (24 months). The clinicopathological parameters for a total of 50 patients including age, tumor size, histologic grade and histologic subtypes were evaluated with Chi square test which test theirs' significance with population of IL-10, TILs subset as well as $CD4^+$/$CD8^+$ ratio. The association between IL-10 population with both TILs subset and $CD4^+$/$CD8^+$ ratio were analysed using Chi square test as well. Overall survival was analysed using the log-rank statistical test prior for plotting Kaplan-Meier survival curves. All patients who survived until the study cut off point were censored in the analysis as they did not demonstrated event of interest. P values of <0.05 was considered statistically significant in all analyses.

RESULTS

Quantification IL-10, $CD4^+$ and $CD8^+$ T Lymphocytes

Characteristics of all patients were detailed in Table 2. The TILs were found predominantly in intratumor stroma which consisted of $CD4^+$ and $CD8^+$ TILs subset (population mean score of 0.93 and 0.53 respectively). IL-10 was also found within intratumor stroma which correlated negatively to level of both $CD4^+$ and $CD8^+$ TILs (population mean score of 0.33) surface area. Regional IHC intensity covered by immunohistostaining region for IL-10, $CD4^+$ and $CD8^+$ TILs (Figure 1A, 1B & 1C) were annotate manually by the pathologist in order to confirm the degree of immunostaining under set scale (power of magnification = 20 X = 200 μm scale ; 620 pixel and 3.1 pixel /μm). Then, ImageJ software was used to calculate the region's area in pixel. The use of ImageJ software improved the data accuracy as it enhanced the quantification process of intratumoral IL-10 and TILs as the intensity of immunostaining was ambiguous (Fedchenko & Reifenrath 2014). This was done through calculating the surface area (pixel/μm) of IHC immunostaining using standardised set scale in this study.

Immunostaining region of both TILs subset in intratumor (Figure 2A & 2B) and control tissue were visualised and quantified. IHC expression of IL-10, $CD4^+$ and $CD8^+$ TILs for several TNBC samples were compared at the same area of intratumor TNBC tissues where low IL-10 expression correlated with high infiltration of both $CD4^+$ and $CD8^+$ TILs subsets (Figure 3A) or vice versa (Figure 3B). The population means of TILs among patients between those survived and deceased were compared statistically using independent t-test. The result showed that the difference of means population between both subsets were insignificant (P-value of $CD4^+ = 0.484$; $CD8^+ = 0.835$) among patients from survived and deceased groups (Table 3). This was most probably due to the small cohort of samples involved in this study.

Table 2 : Demographic information and clinical characterisation of 50 TNBC patients

Patients' Lab Number	Gender	Age	Biopsy Site	Histopathology	Histologic Grade
CASE 1	F	55	Left breast lump	Invasive carcinoma, NST	Modified Bloom-Richardson grade 2
CASE 2	F	57	Left breast	Invasive carcinoma NST	Modified Bloom-Richardson grade 3
CASE 3	F	50	Right breast lump	Invasive carcinoma NST	Modified Bloom-Richardson grade 2
CASE 4	F	62	Left mastectomy	Metaplastic carcinoma	Modified Bloom and Richardson grade 3
CASE 5	F	37	Mastectomy specimen	Invasive carcinoma NST	Modified Blooms and Richardson grade 3
CASE 6	F	43	Left breast wide local excision	Invasive carcinoma NST	Modified Bloom and Richardson grade 3
CASE 7	F	64	Right mastectomy	Invasive carcinoma (NST) with medullary like differentiation	Modified Bloom and Richardson grade 3
CASE 8	F	61	Right breast tumour	Invasive carcinoma, NST	Modified Bloom and Richardson grade 3
CASE 9	F	46	Right breast wide local excision	Invasive carcinoma of NST	Modified Bloom and Richardson grade 3
CASE 10	F	74	Left mastectomy	Invasive carcinoma of NST	Modified Bloom and Richardson grade 2
CASE 11	F	49	Left breast lump	Invasive carcinoma with medullary features	Modified Bloom and Richardson grade 3
CASE 12	F	58	Right breast	A. Invasive carcinoma of NST	Modified Bloom and Richardson grade 1
CASE 13	F	33	Right breast	Invasive carcinoma NST, Tumour size 4 cm pT2 pN1a pMx (1/19 lymph nodes show evidence of metastasis)	Modified Bloom and Richardson grade 3
CASE 14	F	46	Right breast	Invasive carcinoma of NST, with metastasis in one of the two intramammary lymph nodes	Modified Bloom and Richardson grade 3
CASE 15	F	78	Right breast	Metaplastic carcinoma with squamous cell differentiation	Modified Bloom and Richardson grade 2
CASE 16	F	68	Right mastectomy	Invasive carcinoma of breast, NST (pT4b)	Modified Bloom and Richardson grade 3
CASE 17	F	64	Right mastectomy	Invasive carcinoma, NST	Modified Bloom and Richardson grade 3
CASE 18	F	55	Right mastectomy	Metaplastic carcinoma with squamous differentiation, Small foci of invasive carcinoma of NST	Modified Bloom and Richardson grade 3
CASE 19	F	67	2 o'clock breast lump	Adenoid cystic carcinoma, pT2	Modified Bloom and Richardson grade 1

CASE 20	F	83	Left breast	Invasive carcinoma of NST	Modified Bloom and Richardson grade 3
CASE 21	F	46	Left breast lump	Metaplastic carcinoma with squamous differentiation	Modified Bloom and Richardson grade 3
CASE 22	F	74	Right breast	Invasive Carcinoma, NST	Modified Bloom and Richardson grade 3
CASE 23	F	48	Left breast	Invasive carcinoma with medullary features	Modified Bloom and Richardson grade 3
CASE 24	F	53	Right breast	Invasive carcinoma with medullary features	Modified Bloom and Richardson grade 3
CASE 25	F	65	Left breast lump	Invasive carcinoma of NST	Modified Bloom and Richardson grade 3
CASE 26	F	63	Left breast lump	Invasive carcinoma, NST	Modified Bloom and Richardson grade 2
CASE 27	F	82	Left breast tissue	Invasive carcinoma, NST	Modified Bloom and Richardson grade 2
CASE 28	F	49	Left breast lump	Metaplastic carcinoma	Modified Bloom and Richardson grade 2
CASE 29	F	41	Right breast lump	Invasive carcinoma, NST	Modified Bloom and Richardson grade 3
CASE 30	F	59	Breast	Invasive carcinoma of NST	Modified Bloom and Richardson grade 2
CASE 31	F	52	Left breast	Multicentric invasive carcinoma, NST	Modified Bloom and Richardson grade 3
CASE 32	F	48	Breast	Right breast tissue: Invasive carcinoma, NST with skin (Advanced with septicemia)	Modified Bloom and Richardson grade 3
CASE 33	F	54	A. Right mastectomy B.Left breast tissue	Invasive carcinoma of NST	Modified Bloom and Richardson grade 3
CASE 34	F	47	Right breast	Invasive carcinoma with squamous differentiation	Modified Bloom and Richardson grade 3
CASE 35	F	45	Breast lump with surrounding tissue	Infiltrating Ductal Carcinoma	Modified Bloom and Richardson grade 3
CASE 36	F	54	Left breast	Infiltrating ductal carcinoma	Modified Bloom and Richardson grade 3
CASE 37	F	43	Right breast (wide local excision specimen)	Invasive Ductal Carcinoma, NOS	Modified Bloom and Richardson grade 3
CASE 38	F	55	Right breast	Infiltrating ductal carcinoma	Modified Bloom and Richardson grade 3
CASE 39	F	55	Right breast tissue	Infiltrating ductal carcinoma	Modified Bloom and Richardson grade 2
CASE 40	F	69	Left breast	Invasive carcinoma	Modified Bloom and Richardson grade 2
CASE 41	F	60	Right breast tissue	Infiltrating ductal carcinoma	Modified Bloom and Richardson grade 3
CASE 42	F	39	Right breast	Infiltrating ductal carcinoma	Modified Bloom and Richardson grade 3

CASE 43	F	34	Left breast lump	Infiltrating ductal carcinoma	Modified Bloom and Richardson grade 3
CASE 44	F	77	Right breast lump	Infiltrating ductal carcinoma	Modified Bloom and Richardson grade 2
CASE 45	F	55	Right breast tumor	Metaplastic carcinoma	Modified Bloom and Richardson grade 1
CASE 46	F	40	Right Breast Lump	Poorly differentiated infiltrating ductal carcinoma	Modified Bloom and Richardson grade 3
CASE 47	F	47	Left Mastectomy	Infiltrating ductal carcinoma	Modified Bloom and Richardson grade 2
CASE 48	F	67	Left Breast	Infiltrating ductal carcinoma	Modified Bloom and Richardson grade 3
CASE 49	F	56	Left Breast	Infiltrating ductal carcinoma,	Modified Bloom and Richardson grade 3
CASE 50	F	59	Right breast lesion	Infiltrating ductal carcinoma	Modified Bloom and Richardson grade 3

NST= no special type; NOS= not otherwise specified

Meanwhile, the ratio of $CD4^+$/$CD8^+$ in intratumor stroma ranged between 0.50-5.88 among all TNBC subtypes (Table 4). $CD4^+$ TILs count was generally significantly higher than $CD8^+$ TILs within intratumor stroma of TNBC. Consequently, the $CD4^+$/$CD8^+$ ratio at intratumor stroma of TNBC was high (mean score of 1.95>1) particularly among invasive carcinoma of non-special type (NST) subtypes which account for 68% of cases (34/50). The median/normal point of < 2 was selected as low ratio and high ratio represented those with scores of 2. On top of that, the data spread between population mean of IL-10 (SD=0.214); $CD4^+$ TILs (SD = 0.167) and $CD8^+$ TILs (SD = 0.158) was minor. In addition, the standard deviation for

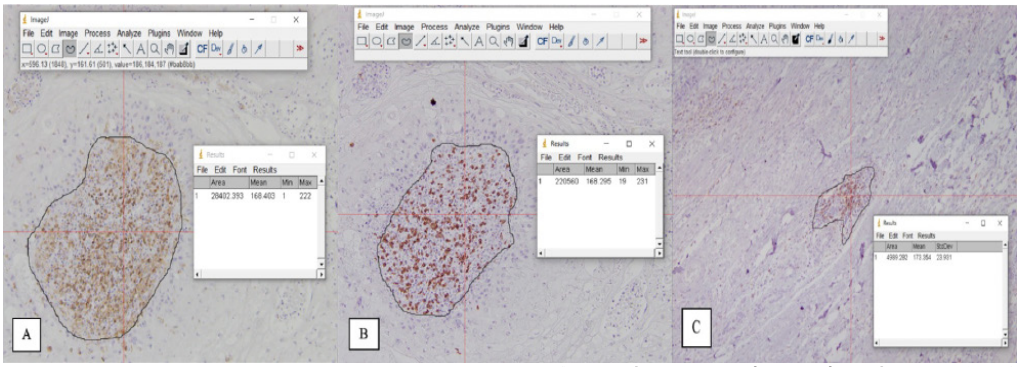

Figure 1: Surface area of immunostaining region covered by both tumor-infiltrating lymphocytes (TILs) subsets was calculated using ImageJ software (20X magnification). A) Surface area of immunostaining region covered by $CD4^+$ TILs in one of the samples; B) Surface area of immunostaining region covered by $CD8^+$ TILs in one of the samples; C) Surface area of immunostaining region covered by IL-10 in one of the samples.

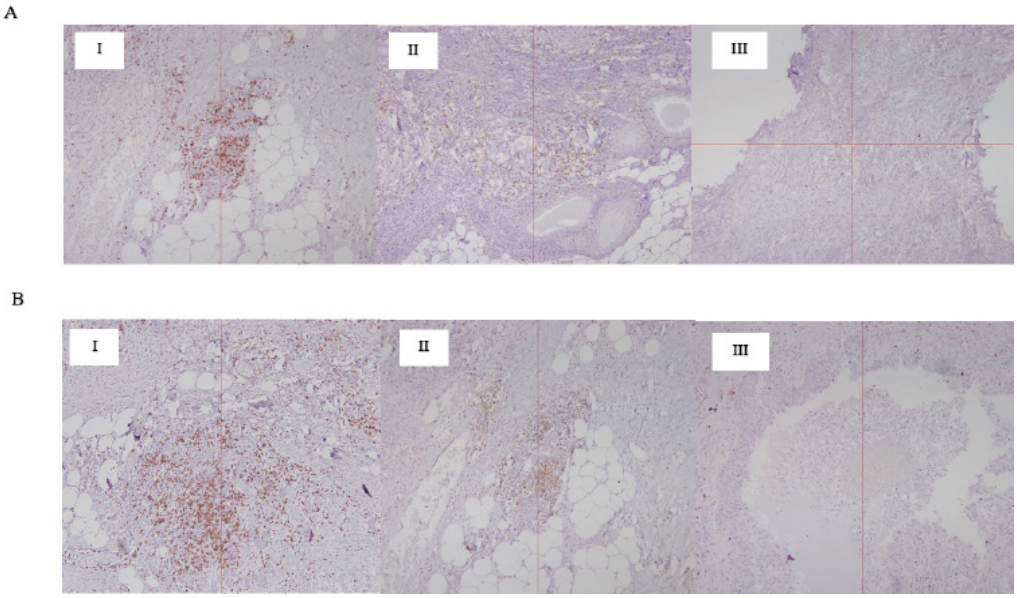

Figure 2: A) Degree of immunohistochemical staining of intratumoral $CD4^+$ TILs evaluation in triple-negative breast cancer (20x magnification) - (I) High $CD4^+$ TILs; (II) moderate $CD4^+$ TILs; (III) low $CD4^+$ TILs. B) Degree of immunohistochemical staining of intratumoral $CD8^+$ TILs evaluation in triple-negative breast cancer - (I) High $CD8^+$ TILs; (II) moderate $CD8^+$ TILs; (III) low $CD8^+$ TILs.

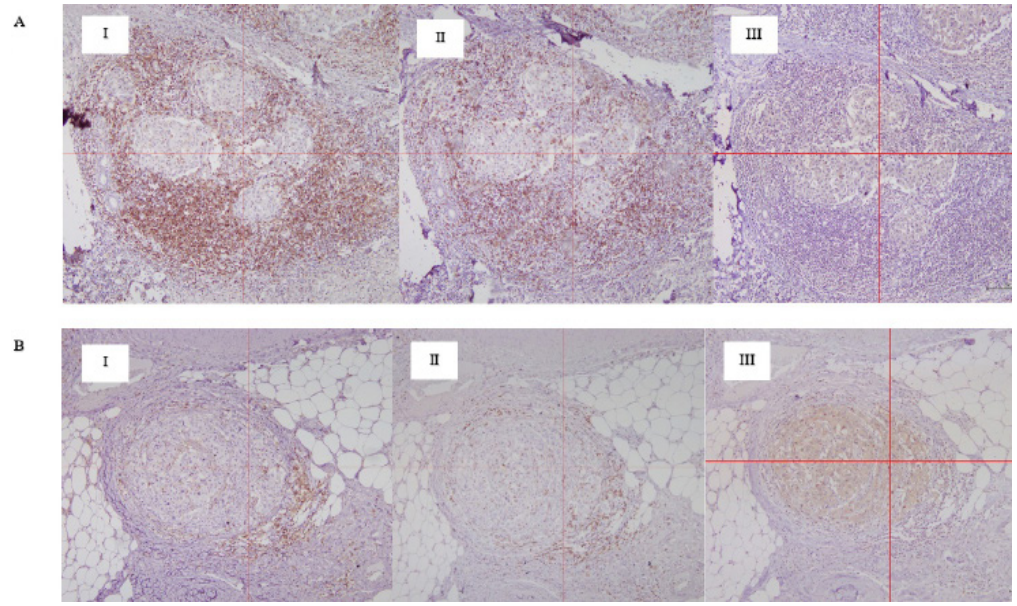

Figure 3: A) Degree of immunohistochemical staining of intratumoral IL-10, $CD4^+$ and $CD8^+$ TILs evaluation in triple-negative breast cancer (20x magnification) - (I) high $CD4^+$ TILs; (II) moderate $CD8^+$ TILs ; (III) low IL-10. B) Degree of immunohistochemical staining of intratumoral IL-10, $CD4^+$ and $CD8^+$ TILs evaluation in triple-negative breast cancer - (I) moderate $CD4^+$ TILs; (II) low $CD8^+$ TILs; (III) high IL-10.

Table 3 : Independent t-test analysis of population mean difference of both $CD4^+$ and $CD8^+$ between survival and death groups

	Outcome	N	Mean	P
Population Mean of $CD4^+$	Survived	43 (86%)	0.930	0.699
	Deceased	7 (14%)	0.881	
Population Mean of $CD8^+$	Survived	43 (86%)	0.535	0.576
	Deceased	7 (14%)	0.549	

$CD4^+/CD8^+$ ratio in intratumor stroma was 0.932 with less deviation among ratio spread as well.

Characterisation of Survival Outcomes and Statistical Analysis in relation to $CD4^+/CD8^+$ ratio

Log-rank test showed high number of $CD4^+$ TILs to low number of $CD8^+$ TILs (high $CD4^+/CD8^+$ ratio) within intratumor stroma was found to be associated with favorable survival outcomes particularly among low-grade patients (Table 5) during the 2 years follow-up (cutoff point). In addition, high $CD4^+/CD8^+$ ratio within intratumor stroma also had significant influence on TNBC patients' survival outcomes or disease-free survival (DFS) as shown in Kaplan-Meier survival curve (Figure 4). Nevertheless, the overall survival between TNBC patients with high $CD4^+/CD8^+$ ratio compared with those with low $CD4^+/CD8^+$ ratio was not considered statistically significant using log-rank test ($P = 0.055$), possibly due to small sample size. Overall, the prognosis of TNBC patients was 72% (36/50) with high $CD4^+/CD8^+$ ratio (excluding the deceased and low/inversion $CD4^+/CD8^+$ ratio) within intratumor stroma survived until the end of study. A total of 7 patients (14%) was found deceased, while remaining 7 patients (14%) were either lost to follow-up or no longer retaining their medical records at the end of study (July 2021).

There was a total of 68% of invasive carcinoma of NST subtypes in this cohort study which demonstrated a relatively high $CD4^+/CD8^+$ ratio (>2) within intratumor stroma. Survival outcomes of these patients were longer than follow-up cut off time (24 months) particularly being discharged after mastectomy surgery compared with other TNBC subtypes. Further investigation had found trend of a favorable prognosis among patients with low grade NST subtype TNBC and high $CD4^+/CD8^+$ ratio intratumorally. However, there was no prognostic significance found among patients at advanced TNBC stage even though with high $CD4^+/CD8^+$ ratio intratumorally.

In addition, IL-10 had no significant influence ($P=0.714$) on TNBC patients' survival outcomes or disease-free survival (DFS) as shown in Kaplan-Meier survival curve (Figure 5). However, patients with low IL-10 level had slightly higher survival period compare to those with high IL-10 level. Finally, no significant link was found between IL-10 with both $CD4^+$ and $CD8^+$ TILs population means with different clinicopathological features

Table 4: Statistical and ratio analysis of different tumor-infiltrating lymphocytes (TILs) and IL-10 among 50 samples

Patients' Lab Number	Population Mean of $CD4^+$	Population Mean of $CD8^+$	Ratio of $CD4^+$ to $CD8^+$	Population Mean of IL-10
CASE 1	1.00	0.50	2.00	0
CASE 2	0.50	1.00	0.50	0
CASE 3	1.00	0.50	2.00	0
CASE 4	1.00	0.50	2.00	0
CASE 5	1.00	0.50	2.00	0.17
CASE 6	1.00	0.50	2.00	0.33
CASE 7	1.00	0.50	2.00	0.33
CASE 8	1.00	0.50	2.00	0.50
CASE 9	1.00	0.50	2.00	0.50
CASE 10	1.00	0.50	2.00	0.33
CASE 11	1.00	0.50	2.00	0.33
CASE 12	1.00	0.50	2.00	0.67
CASE 13	1.00	0.50	2.00	0
CASE 14	1.00	0.50	2.00	0.50
CASE 15	1.00	0.50	2.00	0.33
CASE 16	0.83	0.67	1.24	0.17
CASE 17	1.00	0.50	2.00	0
CASE 18	1.00	0.50	2.00	0.17
CASE 19	0.33	0.33	1.00	0.33
CASE 20	0.67	0.50	1.34	0.50
CASE 21	1.00	0.50	2.00	0.50
CASE 22	0.67	0.83	0.81	0.50
CASE 23	1.00	0.50	2.00	0.17
CASE 24	1.00	0.50	2.00	0.50
CASE 25	1.00	0.50	2.00	0
CASE 26	0.67	0.83	0.81	0.33
CASE 27	1.00	0.50	2.00	0.50
CASE 28	1.00	0.50	2.00	0.33
CASE 29	1.00	0.50	2.00	0.50
CASE 30	1.00	0.50	2.00	0.50
CASE 31	0.50	1.00	0.50	0.50
CASE 32	0.67	0.67	1.00	0.33
CASE 33	1.00	0.50	2.00	0.67
CASE 34	0.83	0.67	1.24	0
CASE 35	1.00	0.50	2.00	0.33
CASE 36	1.00	0.50	2.00	0.50
CASE 37	1.00	0.17	5.88	0.50
CASE 38	1.00	0.50	2.00	0
CASE 39	1.00	0.17	5.88	0.67
CASE 40	1.00	0.50	2.00	0
CASE 41	1.00	0.50	2.00	0.33
CASE 42	1.00	0.50	2.00	0.33
CASE 43	1.00	0.50	2.00	0.17
CASE 44	1.00	0.50	2.00	0.33
CASE 45	1.00	0.50	2.00	0.83
CASE 46	1.00	0.50	2.00	0.33
CASE 47	1.00	0.50	2.00	0.5
CASE 48	1.00	0.50	2.00	0.33
CASE 49	1.00	0.50	2.00	0.33
CASE 50	0.50	1.00	0.50	0.50
Total	46.17	26.84	96.70	16.50
Mean	0.93	0.53	1.95	0.33
SD	0.17	0.16	0.93	0.21

Table 5: Survival outcome among 50 TNBCs patients using Kaplan-Meier survival analysis

Case ID	Age During Admission	$CD4^+/CD8^+$ Ratio	Time of First Diagnosed	Survival Status	Follow-up (months)	Event Type (1 = Event , 0 = Censored)
CASE 1**	55	H	2004	Alive	24	0
CASE 2	57	L	Dec-17	Alive	24	0
CASE 3***	50	H	May-18	Deceased	16	1
CASE 4	62	H	May-18	Alive	6	0
CASE 5***	37	H	Apr-16	Deceased	23	1
CASE 6	43	H	Jan-18	Alive	24	0
CASE 7	64	H	Jul-18	Alive	24	0
CASE 8	61	H	Mar-18	Alive	6	0
CASE 9	46	H	Jan-17	Alive	24	0
CASE 10	74	H	Jun-17	Alive	10	0
CASE 11	49	H	Mar-17	Alive	24	0
CASE 12	58	H	Apr-17	Alive	8	0
CASE 13	33	H	May-17	Alive	24	0
CASE 14***	46	H	Dec-16	Deceased	15	1
CASE 15	78	H	Sep-17	Alive	24	0
CASE 16	68	L	Aug-17	Alive	24	0
CASE 17	64	H	Mar-17	Alive	24	0
CASE 18	55	H	Jul-17	Alive	24	0
CASE 19	67	L	Nov-17	Alive	5	0
CASE 20*	83	L	Oct-17	Deceased	7	1
CASE 21	46	H	Nov-17	Alive	24	0
CASE 22	74	L	Feb-16	Alive	24	0
CASE 23	48	H	Mar-16	Alive	18	0
CASE 24***	53	H	Aug-15	Deceased	12	1
CASE 25	65	H	Aug-16	Alive	24	0
CASE 26	63	L	Jun-16	Alive	24	0
CASE 27	82	H	Jul-16	Alive	24	0
CASE 28	49	H	Aug-16	Alive	24	0
CASE 29	41	H	Aug-16	Alive	24	0
CASE 30	59	H	Oct-16	Alive	24	0
CASE 31	52	L	Mar-15	Alive	24	0
CASE 32*	48	L	Feb-15	Deceased	11	1
CASE 33	54	H	Dec-12	Alive	24	0
CASE 34*	47	H	24/3/2016	Deceased	5 days	1
CASE 35	45	H	Mar-14	Alive	24	0
CASE 36	54	H	Jul-15	Alive	24	0
CASE 37	43	H	May-14	Alive	5	0

CASE 38	55	H	Oct-13	Alive	10	0
CASE 39	55	H	Jun-14	Alive	24	0
CASE 40	69	H	Feb-16	Alive	23	0
CASE 41	60	H	Nov-14	Alive	24	0
CASE 42	39	H	May-13	Alive	24	0
CASE 43	34	H	Nov-14	Alive	24	0
CASE 44	77	H	May-14	Alive	24	0
CASE 45 **	55	H	Feb-12	Alive	24	0
CASE 46	40	H	Mar-14	Alive	22	0
CASE 47	47	H	Oct-12	Alive	24	0
CASE 48	67	H	Sep-12	Alive	24	0
CASE 49	56	H	Sep-12	Alive	24	0
CASE 50	59	L	Sep-12	Alive	15	0

H = high ratio ; L = low ratio
*Patients with worst prognosis outcome that corresponding to low $CD4^+/CD8^+$ ratio.
**Patients survived more than 10 years with diagnosed breast cancer.
***Patient dead despite high $CD4^+/CD8^+$ ratio.

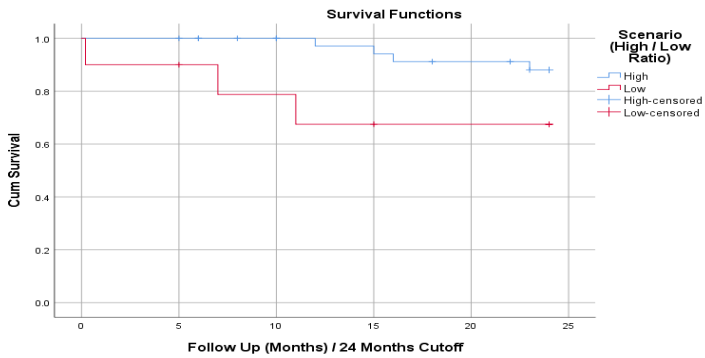

Figure 4: Kaplan-Meier survival curve showed survival outcome among TNBC patients with different $CD4^+$ to $CD8^+$ ratio using log-rank statistical test

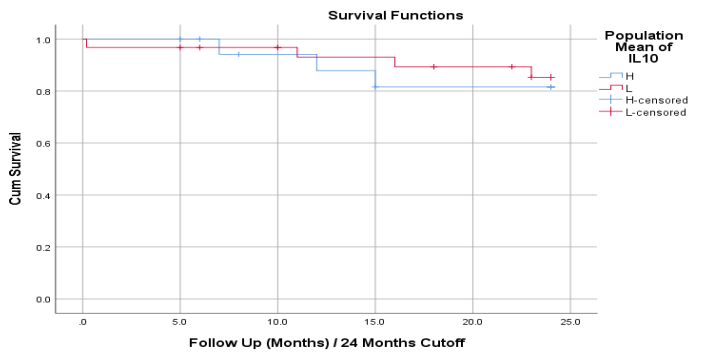

Figure 5: Kaplan-Meier survival curve showed survival outcome among TNBC patients against IL-10 population mean using log-rank statistical test

(Table 6). However, age parameter was nearly considered significant with $CD4^+/CD8^+$ ratio from Chi-square test. No significant association was observed on both TILs subset and $CD4^+/CD8^+$ ratio with IL-10 population mean for all samples (Table 7).

DISCUSSION

In this study, both $CD4^+$ and $CD8^+$ TILs subsets were found in the TNBC tissues sections as shown by IHC staining. Generally, the population of $CD4^+$ TILs was higher than the population of $CD8^+$ TILs within all intratumor stroma of TNBC samples, resulting in higher $CD4^+/CD8^+$ ratio. However, some cases showed inversion of this ratio, indicating lower $CD4^+/CD8^+$ intratumor stroma. The ratio of $CD4^+/CD8^+$ TILs was found to have some significance on TNBC patients overall survival and progression rate, which could be potentially applied to indicate the effectiveness of treatment and cPR towards treatments given.

To quantify the IHC staining, we used scoring system which involved combination of quantitative and qualitative parameters as suggested in previous study (Fedchenko & Reifenrath 2014). The scoring system included measurement of area which was covered by positively stained TILs region in percentage. In addition, we also measured the surface area of the region of interest (ROI), where immunopositive TILs cells were stained to confirm our scoring accuracy based on IHC intensity. The result showed that high $CD4^+/CD8^+$ ratio indeed led to longer OS and better prognosis outcome (Table 3). This could be attributed to better response of TNBC tumor to surgery, cancer treatments regimes, recovery and vice versa. On the contrary, majority of patients were found to have worst prognosis, progression-free survival (PFS) and OS in case of inversion ratio of $CD4^+/CD8^+$. However, there were some exceptions whereby certain patients with high ratio had short-lived whereas certain patients who had inversion ratio lived longer than expected. This phenomena was largely attributed to other factors beyond control.

From the result, the ratio of $CD4^+/CD8^+$ intratumorally did not have much significant effect on OS or prognosis of patients with advanced stage of TNBC. This was likely due to the rate of proliferation among poorly differentiated cancer cells was faster than the anti-cancer immune response. In other words, the secretion of immune cytokines, activity of phagocytosis, stimulation of antibodies production and lysis caused by CTL could not cope with the rate of tumor growth and regeneration. Consequently, this led to patients' early demise in less than 2 years follow-up cut-off period. In addition, Chi-square test revealed that there was association between age with $CD4^+/CD8^+$ ratio as immune system was getting weaker with age progression. In other words, there was tendency that high ratio of $CD4^+/CD8^+$ among younger individuals. This research also highlighted the increased of $CD8^+$ TILs population as a result of acute inflammatory condition, which led to sepsis after tumor removal surgery.

Previous study used TIL subsets in a cohort of 42 TNBC cases to compare $CD4^+/CD8^+$ ratio in tumor-host interface and within intratumoral stroma. The result showed that the $CD4^+/CD8^+$ ratio at the tumor-host interface was found significantly associated with OS, while no association was observed for the $CD4^+/CD8^+$ ratio within the intratumoral stroma (Wang et al. 2017). However, our findings supported the contention that TILs could be one of the important prognostic biomarker in TNBC progression intratumorally. Our result showed that increased $CD4^+/CD8^+$ ratio within intratumor had improved the OS of TNBC patients, as shown in the Kaplan-Meier survival curve (Figure 4) although they were not statistically significant due to small sample size. This study further proved that TILs acted as favorable prognostic biomarkers for TNBC patients as well as good pCR under adjuvant and neoadjuvant chemotherapy setting. Therefore we proposed that identification of TILs in treatment-resistant TNBC was important because they could be potential biomarkers in predicting treatment outcome for standard adjuvant or neoadjuvant therapies and immunotherapy in TNBC patients in the future.

However, there were some TNBC patients' survival rate and treatment outcomes that did not conform to the theory due to presence of outlier. These outlier data were probably attributed to artefact during immunostaining protocols, heterogenous nature of breast cancer tissue, location of tumour during lesion tissue being taken for biopsy or other uncontrollable variables, which led to unusually low or almost none of $CD8^+$ TILs infiltration into intratumoral breast tissue. For instance, uneven hematoxylin counterstaining was the common tissue processing artefact where strong staining could impeded the evaluation of both TILs subsets' immunostaining regions. On the other hand, deep-seated tumor was expected to have lower TILs due to poor perfusion and compromised immune system due to chemotoxicities. This would result in false-favorable survival outcome among TNBC patients.

Prognostic significance of $CD8^+$ TILs remained debatable. This was due to other modifiable risk factors such as diet, lifestyle, environmental and menopausal status of the TNBC patients, which were beyond control of researchers during follow-up. For instance, some patients passed on even though they had high $CD4^+/CD8^+$ ratio. On the other hand, some patients survived despite of the low $CD4^+/CD8^+$ ratio. Some patients with high ratio lived shorter than expected. This might due to breast cancer 1 (BRCA1) genetic predisposition carried by these subset of patients (Peshkin et al. 2010). Patients who carried BRCA1 predisposition might experience higher rate of genetic mutation due to altered DNA repairing mechanism via homologous recombination (Gudmundsdottir & Ashworth 2006). Consequently, the TNBC tumors progress faster than normal to advanced stage the more risk factors being carried despite carrying favourable $CD4^+/CD8^+$ ratio. On the contrary, TNBC patients who lived longer than expected despite low ratio

Table 6: Relationship between IL-10/CD4+/CD8+ population means and CD4+/CD8+ ratio with clinicopathological features

Clinicopathological Parameters	$CD4^+$ TILs			$CD8^+$ TILs			$CD4^+/CD8^+$ Ratio			IL-10		
	Low	High	Significance	Low	High	Significance	Low	High	Significance	Low	High	Significance
Age (years) (Mean 56, Median 55, Range 30 – 85												
< Mean age	3 (6%)	24 (48%)	0.155[b]	24 (48%)	3 (6%)	0.444	3 (6%)	24 (48%)	0.089[a]	15 (30.%)	12 (24%)	0.309[b]
≥ Mean age	7 (14%)	16 (32%)		18 (36%)	5 (10%)		7 (14%)	16 (32%)		16 (32%)	7 (14%)	
Tumor size (mm)												
≤ 20	1 (2%)	2 (4%)	0.496[b]	3 (6%)	0	1.000	1 (2%)	2 (4%)	0.496[b]	1 (2%)	2 (4%)	0.549[b]
> 20	9 (18%)	38 (76%)		39 (78%)	8 (16%)		9 (18%)	38 (76%)		30 (60%)	17 (34%)	
Histologic grade												
Grade I and II	2 (4%)	13 (26%)	0.702[b]	14 (28%)	1 (2%)	0.407	2 (4%)	13 (26%)	0.702[b]	9 (18%)	6 (12%)	0.849[b]
Grade III	8 (16%)	27 (54%)		28 (56%)	7 (14%)		8 (16%)	27 (54%)		22 (44%)	13 (26%)	
Histologic subtypes												
Non-special type (NST)	9 (18%)	31 (62%)	0.663[b]	32 (64%)	8 (16%)	0.184	9 (18%)	31 (62%)	0.663[b]	24 (48%)	16 (32%)	0.722[b]
Others (Metaplastic, Invasive carcinoma with medullary features and adenoid cystic carcinoma)	1 (2%)	9 (18%)		10 (20%)	0		1 (2%)	9 (18%)		7 (14%)	3 (6%)	

(n = 50)
a=Statistically significant; b=Not statistically significant

Table 7: Correlation between IL-10 mean population with both TILs subset and $CD4^+$/$CD8^+$ ratio

IL-10 Mean Population	$CD4^+$ TILs			$CD8^+$ TILs			$CD4^+/CD8^+$ Ratio		
	Low	High	Significance	Low	High	Significance	Low	High	Significance
Low	6 (12%)	25 (50%)	1.000[b]	26 (52%)	5 (10%)	1.000[b]	6 (12%)	25 (50%)	1.000[b]
High	4 (8%)	15 (30%)		16 (32%)	3 (6%)		4 (8%)	15 (30%)	

b = Not statistically significant

of $CD4^+/CD8^+$. This may be attributed to their normal gene composition that governed the genetic stability and diet with rich of antioxidants might be the factors.

In contrast to the good prognosis associated with higher level of $CD8^+$ T lymphocytes aforementioned, previous study consisted of larger cohort had shown that high $CD8^+$ T lymphocyte density infiltration intratumourally or peritumourally actually had no beneficial effect on OS or PFS among breast cancer patients (Liu et al. 2011). This phenomenon was further investigated in tissue microarray, which latter found out that $CD8^+$ T lymphocytes only demonstrated prognostic significance to breast cancers when high advanced histologic grade and lacked of ER expression (Baker et al. 2011). The information on prognostic significance of $CD4^+$ TILs had not been fully elucidated. Increased $CD4^+$ TIL was associated with better prognosis and survival outcomes, but other subsets had shown no prognostic significance (Chung et al. 2017). Conversely, two earlier studies had demonstrated that higher $CD4^+$ T lymphocyte level correlated with worse prognosis among breast cancer patients (Macchetti et al. 2006; Matkowski et al. 2009).

Undeniably, decreased $CD4^+/CD8^+$ ratio could associate with impaired cellular-mediated immunity. On the other hand, high $CD4^+/CD8^+$ ratio was associated with good prognosis in the cases of non-small cell lung cancer and cervical squamous cell carcinoma (Shah et al. 2011). Increased $CD4^+$ T lymphocytes and declined of $CD8^+$ T lymphocytes population were observed in regional lymph nodes that was invaded by breast cancer cells (Macchetti et al. 2006). Furthermore, high $CD4^+/CD8^+$ ratio was also associated with reduced cancer recurrence in hepatocellular carcinoma after liver transplant (Unitt et al. 2006). Remarkably, a study showed early pre-treatment with $CD4^+$ resulting increased $CD4^+/CD8^+$ ratio and inversion of $CD4^+/CD8^+$ ratio after treatment would lead to prolonged survival under metastatic melanoma setting (Hernberg et al. 2004). Similarly, inversion of $CD4^+/CD8^+$ ratio was also associated with better pCR and prognosis of breast cancer in one study (García-Martínez et al. 2014).

Generally, the population of IL-10 found in TNBCs tissue sections in our study correlated negatively with both $CD4^+$ and $CD8^+$ TILs population.

Negative correlation affected both subsets population together whereas influence on either one subset only as single entity was not observed in this study. The inflammatory inhibition of IL-10 appeared to have greater effect on $CD4^+$ TILs compared to $CD8^+$ TILs population. High IL-10 associated with lower $CD4^+$ TILs in some samples, which led to ratio inversion. However, the influence of IL-10 on overall population of both TILs subsets and $CD4^+/CD8^+$ ratio was not significantly associated.

IL-10 appeared to have some significant influence on TNBC patients OS. Decreased IL-10 tended to correlate with higher ratio of $CD4^+/CD8^+$ which led to favorable prognosis among TNBC patients. To further elaborate this, IL-10's function was to inhibit APC and protect tissue against damage from pro-inflammatory responses from T helper cell type 1 (Th1) and T helper cell type 2 (Th2) responses during pathogen infections (Schopf et al. 2002). This was achieved by reducing expression of MHC class II and co-stimulatory molecules such as B7-1 or B7-2 on monocytes and macrophages. Subsequently, this further reduced the downstream pro-inflammatory cytokines and chemokines production such as interleukin-6 (IL-6), interleukin-12 (IL-12), interleukin-18 (IL-18), tumour necrosis factor α (TNF-α), chemokine (C-C motif) ligand 2 (CCL2), CCL12 and Chemokine (C-X-C motif) ligand 2 (CXCL2) (Moore et al. 2001). In previous study that used mice model, it was found that neutralisation of interferon gamma (IFN-γ), signal transducer and activator of transcription 1 (STAT1) gene (one of the main transcription factor that regulated IFN-γ signaling pathway) gene expression will sensitise the mice to methylcholanthrene (MCA) induced carcinogenesis compared to their wild-type counterparts (Kaplan et al. 1998). In addition, cytolytic protein, perforin was synthesised by $CD8^+$ TILs had also been found to inhibit B cell lymphoma development (Bolitho et al. 2009). These key findings suggested that less immuno-competent or immunocompromised individual were more likely to experience high risk of tumorigenicity as well as cancer progression.

Most cancers depended on 3Es of cancer immuno-editing to refine cancer immunosurveillance i.e. elimination, equilibrium, and escape (Dunn et al. 2004). This was attributed to nature of cancer cell to sculpture their immunogenicity during development, which plays an important role during interaction with host immune cells in later stage. The more immune-selection pressure underwent by cancer cells, the lesser the immunogenicity developed by tumors, resulting in recognition escape by adaptive immune system in later stage. Immuno-editing generated tumors with lesser immunogenicity, which resulted in recognition escape by adaptive immune system. This was also the reason that some cancer cells developed resistance to certain immunotherapy. This situation exacerbated through inhibition of acute pro-inflammatory responses by IL-10 in order to protect tumour tissue against damage from Th1 and Th2

responses. As a result, the cancer cells escaped immunosurveillance as well as anti-tumor effect from TILs, leading to worst prognosis.

Mutual relationships and interactions between both $CD4^+$ and $CD8^+$ T lymphocytes were very crucial for eliciting adaptive immune system during anti-cancer response. In other words, tumor specific antigen (TSA) expressed on cancer cells were required by $CD4^+$ T lymphocyte to activate APC via CD40-CD40L interactions during priming stage prior to generate a more tumour-specific targeting CD8+ T-lymphocyte population (Feau et al. 2012). In the anti-cancer immune cycle, these T lymphocytes' crosstalk generated tumor-directed T cells in the final stage (Chen & Mellman 2013). This process was induced by macrophages that serve as APC after phagocyting TSAs, followed by presenting their antigens to $CD4^+$ T helpers cells in lymph node. Subsequently, tumor-specific $CD8^+$ T lymphocytes was activated by $CD4^+$ T helpers cells were selected and clonally expanded prior migration into tumor site through lymphatic vessels to eliminate tumor cells. However, tumor growth could only be inhibited and eliminated if the lymphocytes had undergone a fully completed immune cycle. This meant that any disruption of cross-talk event during the cancer immune cycle progression could allow cancer cells to escape immunosurveillance, resulting in failure to control tumor growth.

Meanwhile, chronic inflammation caused by dietary and environmental carcinogens and exposure, was also linked intimately with tumor-promoting microenvironment. It contributed to cancer initiation and progression by inducing cellular proliferation, metastasis through enhanced angiogenesis and invasion and mutagenesis by generating oxidative genotoxic species (Grivennikov et al. 2010). Most of the solid tumor depended on intrinsic inflammation to build "pro-tumor" microenvironment (Mantovani et al. 2008). For instance, lung tumor could promote inflammation by secreting proteoglycan versican which acted as macrophage activator through Toll-like receptor (TLR) 2 to enhance metastatic growth (Kim et al. 2009). The inflammatory microenvironment also aided cancer cell renewal and proliferation continuously through secretion of cytokines and chemokines. Furthermore, cancer-related inflammation also led to silencing of mismatch repair which associated with increased genetic instability that caused by microsatellite instability (Colotta et al. 2009). By suppressing inflammation in non-immunocompetent individuals, it will eliminate the "metastatic-niche" for tumour and impede the progression process. However, this paradox role of inflammation which in this case-dampening inflammation would causelow TILs infiltration, resulting in poorer prognosis of TNBC patients.

CONCLUSION

This study highlight novel emergent aspects of anti-tumor by $CD4^+$ and $CD8^+$ T lymphocytes. Presence of TILs and absence of IL-10 within

intratumor stroma strongly correlated with good prognosis and longer PFS outcomes among TNBC patients. Hence, TILs and IL-10 could serve as potent prognostic markers to overcome some of the limitations of current prognostic biomarker for TNBC. In attributing to this finding and translating this knowledge into clinical management, we proposed that pre-treatment with immunotherapy such as autologous T-cell therapy, chimeric antigen receptor T cells (CART) and autologous immune enhancement therapy (AIET) were crucial during pre-surgery neoadjuvent chemotherapy or after adjuvant post-surgery chemotherapy. We would also suggest that prior injection of non-reactive/ autologous-cultured T lymphocytes at local tumor area may complement the chemotherapy and radiotherapy for better prognosis and treatment outcomes. Beta-adrenergic drugs family appeares to be contraindicated with the prognosis of TNBC patient as these beta agonists induced an increased in the IL-10 production both in vitro and in vivo in opposed to beta-blockers.

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