ASSOCIATION BETWEEN TUMOR-INFILTRATING LYMPHOCYTES AND PD-L1 EXPRESSION IN HUMAN INVASIVE BREAST DUCTAL CARCINOMA, NST

by

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DECLARATION

I hereby declare that this research is submitted to Universiti Sains Malaysia (USM) for the partial fulfilment of the degree of Master of Science in Medical Research. It has not been submitted to other universities.

Sincerely,

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LIST OF ABBREVIATIONS

AJCC	American Joint Committee on Cancer
APC	Antigen presenting cell
DCIS	Ductal Carcinoma in Situ
DFS	Disease-free survival
EFS	Event-free survival
ER	Estrogen receptor
FFPE	Formalin fixed paraffin embedded
H&E	Haematoxylin and eosin
HER-2	Human epidermal growth factor receptor-2
ICC	Intraclass correlation coefficient
IFN	Interferon
IHC	Immunohistochemistry
LPBC	Lymphocyte predominant breast cancer
MHC	Major histocompatibility complex
MNCRR	Malaysian National Cancer Registry Report
NK cells	Natural killer cells
NST	No special type
OS	Overall survival
pCR	Pathological complete response
PD-L1	Programmed death-ligand 1
PD-1	Programmed cell death protein 1
PR	Progesterone receptor
SHP2	Src Homolog 2 domain-containing tyrosine Phosphatase 2
SPSS	Statistical Package for the Social Sciences
TCR	T cell receptors
TGF-α	Tumor growth factor-α
TIL	Tumor-infiltrating lymphocytes
TNBC	Triple-negative breast cancer
UICC	International Union for Cancer Control

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ABSTRAK

Perkaitan di antara limfosit-infiltrasi-tumor dan ekspresi PD-L1 dalam kanser payudaya duktus invasif.

Pengenalan: Kanser payudara adalah kanser kedua terbiasa didiagnos selepaas kanser paru-paru. Ia adalah penyebab kematian paling biasa dikalangan wanita. Beberapa bukti mengaitkan peranan limfosit dan PD-L1 dengan immuniti antitumor. Kajian ini bertujuan untuk menyiasat perkaitan di antara limfosit-infiltrasi tumor, pernyataan PD-L1 dan parameter klinikopatologi dalam kanser duktus payudara yang invasif.

Metodologi: Sebanyak 80 kes kanser payudara dari tahun 2016 hingga 2018 telah digunakan untuk menyiasat kehadiran limfosit infiltrasi tumor. Maklumat klinikopatologi yang berkaitan telah diekstrak. Kaedah imunohistokimia digunakan untuk menyiasat ungkapan PD-L1 dalam 67 bahagian tisu kanser payudara. Perkaitan antara limfosit infiltrasi tumor, pernyataan PD-L1 dan parameter klinikopatologi dianalisis.

Keputusan: Lebih separuh daripada pesakit berusia lebih daripada 40 tahun. Majoriti pesakit adalah orang Melayu. Sebanyak 42.5% kes dikelaskan sebagai kanser payudara utama limfosit (skor ≥50%). Limfosit infiltrasi tumor mempunyai persamaan yang signifikan dengan gred tumor, status reseptor ER, status reseptor PR dan indeks Ki-67. Ekspresi PD-L1 di kawasan tumor ditunjukkan dalam 22 daripada 67 kes dan 28 daripada 67 kes di kawasan stromal. PD-L1 secara signifikan dikaitkan dengan gred tumor yang lebih tinggi dan paras tumor infiltrasi-limfosit yang lebih tinggi.

Kesimpulan: Limfosit infiltrasi tumor dan ekspresi PD-L1 merupakan penunjuk penting ketidak seimbangan imunologi dan dikaitkan dengan ciri-ciri kanser payudara yang tidak memuaskan.

ABSTRACT

Association between Tumor-Infiltrating Lymphocytes and PD-L1 Expression in Human Invasive Breast Ductal Carcinoma, NST

Introduction: Breast cancer is the second common cancer diagnosed after lung cancer. It is the commonest cause of cancer-related mortality among women. Several lines of evidence link the roles of tumor infiltrating lymphocytes and PD-L1 expression with host antitumor immunity. This study aims to investigate the association among tumor-infiltrating lymphocytes, PD-L1 and clinicopathological parameters in invasive breast ductal carcinoma.

Methods: A total of 80 cases of breast cancer from year 2016 to 2018 were utilized to investigate the presence of tumor-infiltrating lymphocytes. Relevant clinicopathological information was extracted. Immunohistochemistry methods were used to investigate the expression of PD-L1 in 67 breast cancer tissue section. The association between tumor infiltrating lymphocytes, PD-L1 expression and clinicopathological parameters were analysed.

Results: Over half of the patients were over 40 years of age. Majority of the patients were Malays. In addition, 42.5% of cases were classified as lymphocyte predominant breast cancer (score ≥50 %). Tumor-infiltrating lymphocytes had a significant association with tumor grades, ER receptor status, PR receptor status and Ki-67 index. The expression of PD-L1 in tumor area was shown in 22 of 67 cases and 28 of 67 cases in stromal area. PD-L1 was significantly associated with higher tumor grades and higher levels of tumor infiltrating-lymphocytes.

Conclusion: Tumor infiltrating lymphocytes and PD-L1 expression is an important indicator of immunological imbalance and they are associated with unfavourable characteristic of breast cancer.

CHAPTER I

INTRODUCTION

1.0 Introduction

Cancer burden continues on the rise worldwide, particularly in the low and middle-income countries. By 2025, it is estimated that there will be more than 20 million new cancer cases annually (Bray *et al.*, 2012). After lung cancer, breast cancer is the most common cancer diagnosed worldwide. The incidence reported in 2012 accounts for 838, 000 new cases in the less developed countries whereas 794, 000 new cases occur in developed countries. Breast cancer is the commonest cause of cancer-related mortality among women with 324, 000 death in the less developed regions (Ferlay *et al.*, 2015). Advancement in technology and treatment leads to favorable survival rate in the developed countries.

Notwithstanding increasing global incidence of breast cancer, Asian countries have the highest incidence. In Malaysia, breast cancer is the commonest cancer with 18,343 new incidences reported by Malaysian National Cancer Registry Report 2007- 2011 (MNCRR). Chinese women have the highest incidence as compared to other ethnicities (Azizah *et al.*, 2016). In Malaysia, breast cancer cases are commonly diagnosed at a late stage of the disease, particularly in the Malays ethnicity. This is due to lack of awareness, reluctance for early screening and beliefs in traditional medicine (Yip, Pathy and Teo, 2014). Malaysians also presented with higher tumor grades, more aggressive and unfavorable characteristics such as negative hormonal receptor tumors (Bhoo-Pathy *et al.*, 2012). This explains the low survival rates among Malaysians. Nonetheless, survival rates vary among different hospitals and ethnicities. The Malays

ethnicity has the poorest survival rate as compared to the Chinese and Indian (Yip, Pathy and Teo, 2014).

Various components in the microenvironment of the breast tissue dynamically interact during tumor development. The microenvironment consists of extracellular matrix and various stromal cell types including endothelial cells, fibroblasts, adipocytes and immune cells. The interactions influence the initiation, progression, and metastasis of the tumor (Place, Huh and Polyak, 2011). Various studies suggested that stromal environment affects the patient outcome. For example, inflammatory infiltrates within breast cancer and surrounding stroma indicate a better prognosis. It also increases the survival rates of the patients (Dieci *et al.*, 2018). Wang *et al.* (2017) showed that there is a correlation between the microenvironment of breast cancer and immune factors around the tumor cells that promotes the progression of the tumor. The examination of the microenvironment can therefore provide a better insight into tumor progression and the discovery of prognostic biomarkers. Among immune cells, TILs are regarded as an important indicator of immune interaction in breast cancer due to their roles in immunoediting (Romagnoli *et al.*, 2017). During the immunosurveillance process, failure to keep a balance between tumor growth and elimination leads to the progression of the tumor (Wang *et al.*, 2017). PD1/PD-L1 checkpoint pathway is one of the pathways involved in TILs' pathophysiology in cancer evasion (Xu-Monette *et al.*, 2017). Binding of PD-L1 to its receptor on T cells, PD-1, will dampen the immune activity (Kassardjian, Shintaku and Moatamed, 2018). This study is performed in such a scientific background to look into the relationship between TILs and PD-L1 expression by employing routine histological methods, i.e. H&E staining
and immunohistochemical method on the local breast cancer samples, aiming to clarify the pertinent features related to our local population.

1.1 The objective of study

1.2 General objectives:

To investigate on the roles of tumor-infiltrating lymphocyte (TILs) and expression of PD-L1 protein within breast cancer clinicopathological data

1.3 Specific objectives

The specific objectives of this study are

a) To evaluate tumor-infiltrating lymphocyte in breast cancer using H&E slide according to the International TILs Working Group 2014

b) To evaluate the association between tumor-infiltrating lymphocyte and clinicopathological parameters

c) To measure the expression of PD-L1 in breast cancer by using immunohistochemistry

d) To determine the association between PD-L1 expression and clinicopathological parameters

e) To determine the association between TILs and PD-L1 expression in breast cancer tissue.

CHAPTER II

LITERATURE REVIEW

2.0 Overview: clinicopathological features of breast cancer

Breast cancer is the second leading cancer diagnosed worldwide; it is the most common cancer among women. The number of newly diagnosed cases worldwide amounts to more than 1.67 million cases in the year 2012 as estimated by GLOBOCAN. The age-standardized incidence ranges from 31.1 per 100,000 in the less developed regions to 74.1 per 100,000 in the more developed regions. The Western Europe has the highest incidence with 96.0 per 100,000 and the Middle Africa and Eastern Asia have the lowest incidence with 27 per 100,000. The age-standardized incidence for Southeast Asia countries including Malaysia is 34.8 per 100,000. Breast cancer is ranked as the fifth cancer-related death with 522,000 mortality worldwide (Ferlay et al., 2015).

In Malaysia, breast cancer is the most common cancer diagnosis among Malaysian female residents followed by colorectal cancer and lung cancer. Based on the Malaysian National Cancer Registry Report 2007-2011(MNCRR), the age-standardized incidence for breast cancer was 31.1 per 100,000. Among the major ethnic groups, the incidence of breast cancer was more common in the Chinese (41.5 per 100,000) followed by the Indians (37.1 per 100,000) and the Malays (27.2 per 100,000). In the lifetime of Malaysian women, approximately 1 in 30 will encounter breast cancer. Different in lifetime risk among the main ethnic groups showed the lowest among the Malays (1 in 35) followed by the Indians (1 in 24) and the Chinese (1 in 22) (Azizah *et*

al., 2016). Despite having the lowest incidence of breast cancer, the Malays women tend to have with a poorer prognosis as compared to their Chinese and Indian counterparts. A multi-ethnic study conducted in the tertiary centers (University Malaya Medical Centre, Malaysia and National University Hospital Singapore) shows that the Malays had breast cancer presented with advanced stages, larger tumor sizes, and poorly differentiated tumors as compare to the Chinese and the Indians (Bhoo-Pathy *et al.*, 2012). In Malaysia, 55 years old is the peaked age of incidence in breast cancer according to the MNCRR. In addition, the incidence rate risen after the age of 25 (Azizah et al., 2016). In comparison to Western countries, Asian women commonly suffered from breast cancer at earlier ages before 50 years. The most prevalent age group reported from the Singapore and Malaysia Breast Cancer Registries ranged between 40-49 years (Pathy *et al.*, 2011). The mortality rate of breast cancer is in decreasing trend for Western countries as compared to that in the Asian countries (Toi *et al.*, 2010). The 5-year overall survival rate in advance stages in Asian patients was worse as compared to that in the United States (Pathy *et al.*, 2011). The 5-year overall survival rate for Singaporean patients was significantly higher as compared to that of Malaysian patients. However, Malaysia had a better improvement in survival rates as compared to Singapore (Saxena *et al.*, 2012). The cumulative overall survival rate by ethnicity shows that the Chinese had the highest survival rate followed by the Indians and the Malays (Bhoo-Pathy *et al.*, 2012). Heterogeneity of breast cancer is reflected in the histopathological classification of breast cancer. In addition, breast cancer is now molecularly sub-classified into hormonal receptor-positive breast cancer [HR+, HER2- (ER+/PR+, HER2-)], Her2-amplified breast cancer [HR+/-, HER2+ (ER+/-, PR+/-, Her-2+)] and triple-negative breast cancer (HR-, HER2-) (Telli *et al.*, 2011). Variations among ethnic groups and countries have been reported. Asian women had a higher

prevalence of hormone receptor-positive tumors as compared to the triple negative subtype. However, Western countries have a higher percentage of hormonal receptor-positive tumors as compared to Asian countries (Toi *et al.*, 2010). For example, the California Cancer Registry (2002-2007) recorded that 58,555 (66%) out of 89,009 cases of Asian origins in California were HR+/HER2-. Japanese women had the most favorable subtypes (a high frequency of HR+/HER2- and a low rate of HER2+ subtype). The Korean, Philippine, Vietnamese and Chinese women nonetheless had more HER2 overexpressed tumors (Telli *et al.*, 2011). HER2+ breast cancer is markedly associated with aggressive presentation, higher tumor grades, lymph node metastasis, hormone receptor negativity, and poorer survival as compared to other breast cancer subtypes (Tan *et al.*, 2009), As a comparison, Malaysian women were more likely to have a hormonal receptor-negative tumor and presented at a later stage (III & IV) as compared to the Singaporeans. Among the major ethnic groups, the Malay and Indian women were more commonly associated with a hormonal receptor-negative tumor whereas the Chinese women were likely to have favorable tumor characteristics such as ER positivity (Bhoo-Pathy *et al.*, 2012). Certain genetic mutations confer a higher lifetime risk to develop breast cancer. BRCA gene mutations increase the risk by 47% to 55%. The prevalence of BRCA1/2 mutation in Asian varies among countries. A study done by Kim and Choi (2013) revealed that 12.7% of 1,183 Asian patients carried BRCA1/2 mutations. Mutation in familial breast cancer was lower in Asian patients as compared to the Caucasian and Ashkenazi-Jews. However, the prevalence of early-onset breast cancer associated with BRCA mutation was equivalent. In Malaysia, the prevalence of BRCA 1 and BRCA 2 mutation were 2.7% and 5.4% (Toh *et al.*, 2008). The Malay ethnic group has a higher mutation rate of BRCA 2 whereas the incidence of BRCA 1 was higher in the Indians (Thirthagiri *et al.*, 2008).

Westernization of Asian women in terms of lifestyle changes (diet, exercise, alcohol, BMI), breastfeeding, parity, and hormonal exposure increases the risk of developing BRCA-associated breast cancer (Bruin *et al.*, 2012).

2.1 Molecular classification of breast cancer

Epigenetic, genetic and transcriptomic changes result in the heterogeneity of breast cancer (Eliyatkın *et al.*, 2015). Differences in biological changes of breast cancer underscore the divergent natural histories and responses of breast cancer to treatment, necessitating personalized and more targeted therapies in managing this disease (Dai *et al.*, 2015) (Blows *et al.*, 2010). Breast cancer is traditionally characterized by morphological classification. Biological aggressiveness of breast cancer is determined via histological grading using semi-quantitative method - the Nottingham Modification of the Bloom-Richardson system. Three indexes are evaluated with regard to tubule formation, nuclear polymorphism and mitotic rate (Eliyatkın *et al.*, 2015; Galea *et al.*, 1992). The extent of breast cancer is evaluated by the TNM staging system, which is maintained by the American Joint Committee on Cancer (AJCC) and the International Union for Cancer Control (UICC). This system uses the anatomical extent of cancer for staging. Three main parameters code for primary tumor (T), regional lymph nodes (N) and distant metastasis (M) respectively.

With the advancement and understanding of breast cancer biology, the Eighth Edition of the AJCC Cancer Staging Manual incorporates biologic factors that affect the outcome and response of breast cancer treatment (Amin *et al.*, 2017). Therefore, the molecular classification of breast cancer is included and will complement the traditional anatomical and histological classification.

With regard to the molecular classification of breast cancer, Perou and Sorlie first introduced this classification by showing the differences in gene expression profiling in the year 2000. They divided breast cancer into several sub-groups, which are ER-positive- luminal A (Luminal A), ER-positive- luminal B (Luminal B), HER-2 overexpressed (overexpressed ERBB2-genes), basal type (ER, PR, and HER-2 negative) and normal like tumor. HER2- overexpressed and basal-like subtypes are hormonal receptor-negative (Perou *et al.*, 2000) (Sørlie *et al.*, 2016). Subsequently, immunohistochemistry methods were attempted to classify the molecular subtypes of breast cancer as it has the advantage of being lower in cost than the gene expression profiling. Cheang *et al.* (2009) showed the immunohistochemical surrogate markers were comparable with the gene expression profiling. They incorporated Ki-67 index, a nuclear marker for cell proliferation, together with estrogen receptor, progesterone receptor, and HER-2.

The cut-off value of 14% for Ki-67 labeling index was used to distinguish between Luminal A and Luminal B sub-types. Hormone receptor-positive cases with Ki-67 index less than 14% were classified as Luminal A and hormone receptor-positive with Ki-67 more than 14% index as Luminal B (Feeley *et al.*, 2014) (Cheang *et al.*, 2009). During the St Gallen Consensus Conference 2013, the panel of experts agreed on the usage of immunohistochemical surrogate markers without molecular diagnostics to define molecular sub-types. Based on the St Gallen Consensus, the recommended intrinsic subtypes of breast cancer by the panel are provided in table 2.1 (Goldhirsch *et al.*, 2013).

Table 2.1 Breast cancer subtypes. Source: (Goldhirsch *et al.*, 2013)

Intrinsic subtypes of breast cancer	Definition
Luminal A	ER positive, PR positive, HER-2 negative, Ki-67 <14 % (low)
Luminal B	Luminal B (HER-2 negative) ER-positive, HER-2 negative, and at least Ki-67 > 14 % (high) or PR negative/low (<20%) Luminal B (HER-2 positive) ER-positive, HER-2 positive and any Ki-67 or PR positive
HER-2 overexpressed	HER-2 positive ER and PR negative
Basal-like	ER negative, PR negative, HER-2 negative

2.2 Breast cancer and immunity

In the 19th century, Rudolf Virchow had observed and speculated on the relationship between the immune cells and tumors. Several latest studies have shown that inflammatory microenvironments play a significant part in the growth of tumors (Mantovani *et al.*, 2008). Different forms of chronic inflammation coupled with the predisposition in genetic make-up will increase the risk of tumor development as well as promote tumorigenesis (Grivennikov *et al.*, 2010). In contrary to common understanding, cancer cells do not act autonomously; they interact with the stroma surrounding them. Diversity of immune cells presents in the tumor microenvironment includes both the innate and adaptive immune cells. As a whole, the tumor microenvironment is made up of a mixture of cancer cells, immune cells such as neutrophils, macrophages, dendritic cells, mast cells, T and B lymphocytes, as well as

the stromal cells such mesenchymal cells, endothelial cells, fibroblast and pericytes (Place *et al.,* 2011).

In guarding against tumor development, the immune system serves a major role. This is achieved by a process called immunosurveillance, whereby pathogens or cancerous cells are being detected and destroyed by the immune system (Carvalho *et al.*, 2014). According to this theory, the immune system recognizes and eliminates the nascent tumor. However, failure or insufficient action of the immune system to eradicate tumor formation will result in progression of the emerging tumor with ensuing micrometastasis and late-stage tumor (Hanahan and Weinberg, 2011).

In addition, the immune system demonstrates dual functionality either promoting or suppressing tumor formation. Disturbance in the maintenance of the equilibrium will favor the direction of the tumor progression (Vinay *et al.*, 2015). Transforming growth factor (TGF-ß), interferon (IFN-.) and tumour necrosis factor α (TNF-α) are some of the cytokines and chemokines produced during the inflammatory process will have an anti-tumor effect initially. However, the crosstalk of the establish cancer cells with these immune mediators in the chronic inflammatory microenvironment will stimulate the growth and metastasis of tumor (Nagarajan and Mcardle, 2018). Robert Schreiber introduced the theory of cancer immunoediting in 2002 to replace the concept of immunosurveillance that was originally proposed based solely on the protection of immune system for the host. Tumor-sculpting actions by the immune system are refined by the three stages of elimination, equilibrium, and escape (Dunn *et al.*, 2002).
The elimination process acts like the immunosurveillance process. This involves an integrated action of innate and adaptive immune responses to reject the developing

tumor. This response is initiated by the innate immune system in the presence of growing tumor and stromal remodeling. Immune mediators like IFN- γ and pro-inflammatory molecules are stimulated and produced by these events. As a result, recruitment of immune cells such as T cells, NK cells, and macrophages occurs. These immune cells will eventually recognise and destroy the tumor. Acquirement of the tumor antigens by the activated dendritic cells will stimulate the adaptive immune response. This will induce tumor-specific immunity via the development of CD4+ and CD8+ T cells. The tumor-specific CD8+ T cells will efficiently recognise the tumor antigens and facilitate the destruction of the tumor (Dunn *et al.,* 2004).

In the equilibrium phase, the lymphocytes and IFN-γ action are barely enough to control the tumor cells but unable to eliminate them totally. The survived cancer cells during the elimination phase carry mutated phenotypes. With unstable genomic integrity, tumor variants with low immunogenicity will be generated (Lengauer *et al.,* 1998). In the escape phase, the epigenetic and genetic changes occur in tumor cells would give rise to immunogenic phenotype variants of tumor cells. Various strategies are utilised by the tumor cells to evade the powerful actions of innate and adaptive immune cell. Several mechanisms are shown to be used by the tumor cells such as employing the immunosuppressive cytokines and involvement of T cell activities (Khong and Restifo, 2002).

Some of the immune system's elements are accountable for immunoediting. Tumor-infiltrating lymphocyte (TILs) are a heterogeneous group of immune cells and form a part of immunosurveillance system. The roles of T lymphocytes are still contradictory. The activation of TILs are regulated by cytokines and chemokines produced by the

tumor or immune cells in the intratumoral lesion (Wang *et al.*, 2017). Various studies have shown the association of T lymphocytes with a favorable prognosis. This has been noted in various cancer kinds including melanoma (Lee *et al.*, 2016), lung cancer (Dieu-Nosjean *et al.*, 2008) and breast cancer (Carvalho *et al.*, 2014). In contrary, some of the studies have shown the relationship of TILs with a reduced overall survival rate. The previous study by Dieci *et al.* (2018) shows that macrophages and T lymphocytes within the breast cancer and surrounding stroma indicates a better prognosis and increased the overall survival rate for patients with triple-negative breast cancer (TNBC).

A study involving 3771 patients with primary breast cancer who had received neo-adjuvant combination therapy demonstrated that higher TILs levels will increase the pathological complete response in all breast cancer subtypes (Denkert *et al.*, 2018). Subsets of TILs, CD+8 T cells which is responsible for the immune response of IFN-γ, have been shown to have antitumor activity, and they correlate with a favorable survival (Mahmoud *et al.*, 2011).

A research carried out by Kim *et al.,* (2013) has shown that a lower number of CD8+ was significantly associated with lymph nodes metastasis, higher tumor grades and immunopositivity of Ki-67. On contrary, higher infiltration of Foxp3+ Tregs T-cells was associated with lymph node metastasis, immunopositivity of p53 and higher proliferation Ki-67 index. Wang *et al.,* (2011) disclosed in their research that Th17 cells and T reg cells were present in the breast tumor microenvironment in the early phases of cancer. Th17 infiltration decreased while T reg cells increased as the disease progressed.

The paradoxical action of immune cells could be due to failure in immune cell recognition ability and lymphocytes dysfunction. These include absent of costimulatory molecules needed by cytotoxic T cells and different functional activities of T lymphocyte subsets. (Cavallo *et al.*, 2011; Sheu *et al.*, 2008). For example, CD8+ T cells, T helper 1(Th1) and NK cells involve in anti-tumor action whereas T helper 2(Th2) and T reg cells involve in tumor development and progression. The host's overall immune status regulates the equilibrium of this immune mechanism (Curigliano *et al.*, 2011).

2.3 Evaluation of tumor-infiltrating lymphocytes (TILs)

Various studies have assessed the significance of TILs in breast cancer for their prognostic and predictive (Dieci *et al.*, 2015; Jang *et al.*, 2018; Salgado *et al.*, 2015). Differences in methodological approaches in evaluating TILs in these studies have been an issue for comparison across different studies. As there was a need to standardize the evaluation methods for TILs, the international TILs working group had been formed with the aims to formulate consensus recommendations for TIL evaluation. This group has published the standardized methodology for visual assessment of TILs in the tumor and the surrounding stroma based on hematoxylin and eosin (H&E) sections. Their recommendations among other, address the issues of which area to evaluate the tumor, methods to score and clinical rationale of TILs evaluation.

Table 2.2 shows the method of evaluating tumor infiltrating lymphocyte (TILs) as recommended by the International TILs Working Group 2014.

Table 2.2 Recommendation for assessing tumor-infiltrating lymphocytes (TILs) in breast cancer (Salgado *et al.*, 2014)

No	Protocol
1)	Stromal TILs are reported as the percentage of the tumor stroma occupied by lymphocytes (the denominator used for the % is the area of the stromal tissue).
2)	Evaluate the TILs within the border of the invasive tumor, and exclude the one outside of the tumor border, DCIS and normal lobule.
3)	Avoid area with crush artifacts, necrosis, regressive hyalinization, and the biopsy site.
4)	Assess all mononuclear cell including lymphocytes and plasma cell and exclude the polymorphonuclear leukocytes.
5)	Standard 4–5 μm slide from formalin fixed and paraffin embedded tissue (FFPE) are considered optimal.
6)	One section is sufficient and microscope magnification of 200-400x (ocular x10, objective x20-x40) is recommended.
7)	A full section is preferred over cores biopsies.
8)	Avoid only assessing the hotspots.
9)	Score as a continuous variable and should round up to the nearest 5-10% and can be categorized later.
10)	There is no recommended clinically relevant TILs threshold; lymphocyte-predominant breast cancer can be used as the term for tumors that contain more lymphocytes than tumor cells.

Several studies had reported the clinical utility of TILs in breast cancer. In these studies, various methods are being used for assessment of the TILs, which include immunohistochemistry and gene expression analysis. Most of the previous studies evaluated both stromal and intratumoral TILs. However, stromal TILs were found to be more reproducible and not affected by the growth pattern of the tumor nest. Although intratumoral TILs were usually less in number, they were more heterogeneous and difficult to observe using H&E slide. Stromal TILs (sTILs) are defined as mononuclear cells within the tumor stroma, not in direct contact with tumor cells. In contrast, intratumoral TILs (iTILs) are defined as mononuclear cells within the tumor nest or in direct contact with tumor cells that had no intervening stroma (Salgado *et al.*, 2014).

2.4 Evaluation of tumor-infiltrating lymphocyte: technicalities

Interobserver agreement study between pathologists has been performed using the methodology proposed by the International TILs Working Group (Swisher *et al.*, 2016). This study confirmed that the proposed methodology is reliable to enumerate the TILs and therefore it can be used to facilitate TILs as a biomarker in research and clinical trial settings. In this study, a great interobserver agreement in enumerating the intratumoral TILs was also observed. However, the scores of intratumoral TILs were mostly parallel to the stromal TILs.

Among other technical issues, the accuracy and difficulty in assessing the TILs were addressed. This study recommended the usage of immunohistochemical staining for cytokeratin and CD45 in difficult cases to facilitate the accuracy of TILs enumeration. CD45 is used to differentiate TILs and apoptotic tumor cells whereas anti-cytokeratin

antibody cocktail to delineate tumor cells (Swisher *et al.*, 2016). Another issue is the continuous scoring of the TILs. In a routine clinical setting, it is difficult for pathologists to give the score and they usually will round the scores up to nearest 5-10%. Suggestion has been made to categorize the parameter into three categories of ≤10%, 10% - 50% and ≥50%. Hida and Ohi (2015) have also performed a similar evaluation that categorized ≤10%, as low, 10% - 50% as intermediate and ≥50% as high.

In conclusion, this methodology of evaluating the TILs depends on the subjectivity of the scoring pathologists. Variability could be due to differences in skill and experience of the pathologists. On the other hand, detailed methods of scoring will consume more time and cost if they are to be implemented on routine basis. Automated digital analysis is being explored to overcome the issue of higher variability and less accuracy in manual counting.

2.5 Clinical significance of TILs

The clinical validity of the TILs in triple negative breast cancer was confirmed in a study done by Adams *et al.* (2014). They evaluated 481 archived sample of TNBC in two adjuvant phases III randomized trials (ECOG 2197 and ECOG 1199) recruited by Eastern Cooperative Oncology Group (ECOG) were evaluated. It was observed that higher TILs scores are associated with reduced risks of death, overall mortality and distant recurrence. Using the data from the phase III trial, BIG 02-98 involving 2009 sample, it was noted that higher stromal lymphocytic infiltrations in HER2+ breast cancer subtypes are associated with better anthracycline-only treatment response. In

addition, an increase in 5-year disease-free survival and 5-year overall survival were observed in TNBC lymphocyte predominant breast cancer phenotype.

There has been no significant connection with overall survival for the luminal subtypes (Loi *et al.*, 2013).

Table 2.3 Studies that have assessed TILs according to the recommended guidelines with reported prognosis.

Reference	Study level	Regimen	Tumor tissue assay	Sample size(n)	Correlation with outcome
Denkert et al., 2018	6 randomised multicentre clinical trials: GeparDuo, GeparTrio, GeparQuattro, GeparQuinto, GeparSixto, and GeparSepto	Docetaxel, Paclitaxel, Nab-paclitaxel Neoadjuvant anti-HER2: Trastuzumab, Lapatinib, Trastuzumab+ lapatinib and Trastuzumab+ pertuzumab	TILs in H&E core biopsy	Luminal –HER2-negative 1366 HER2+ 1379 TNBC 906	Stromal TILs and LPBC associated with (pCR) in all molecular sub-sets of BC. Higher TILs associated with increased OS in TNBC.
Salgado et al., 2015	The NeoALTTO trial	Lapatinib Trastuzumab Lapatinib + Trastuzumab Followed by paclitaxel therapy & anthracycline chemotherapy (Fluoracil, epirubicin hydrochloride, cyclophosphamide)	TILs in H&E core biopsy	HER2+ 387	Higher TILs level are associated with higher pCR and (EFS) in all treatment group.
Jang et al., 2018	Institutional cohort	Sub-group analysis: treatment with anthracycline	Full section H&E	Luminal A: 424	Higher TILs are found in TNBC &

				Luminal B: 467 HER2+: 278 TNBC: 308 Sub-group analysis: 1024	HER2+ and associated with: longer DFS in all patient and longer OS with anthracycline -treated patients
Dieci et al., 2015	Multicentric phase III trials at Gustave Roussy	Adjuvant anthracycline (5-fluoracil, doxorubicin/ epirubicin & cyclophosphamide) vs no chemotherapy	Full section H&E	TNBC 199 ER+: 463 HER2+: 112	High level of TILS in TNBC and HER2+ are associated with longer OS
Luen et al., 2017	CLEOPATRA randomized phase III	Trastuzumab+ docetaxel plus either pertuzumab or placebo	Full section H&E	HER2+: 678	Higher TILs value are associated with improved OS in advance HER2+

pCR; pathological complete response, EFS; event-free survival, DFS; disease-free survival, OS; overall survival, LPBC; lymphocyte-predominant breast cancer

2.6 PD-L1/PD-1 and anti-tumor immunity

T lymphocytes are the main executor in adaptive immunity. Professional antigen presenting cells (APCs) such as dendritic cells and macrophages aid the recognition of antigens by the T cells. The processed antigens will be presented on the major histocompatibility complexes (MHCs). This will enable the T cell receptors (TCRs) to recognize the antigens and bind to them. However, binding of MHCs and TCRs solely is insufficient to activate the cytotoxic activity of the T cells. To completely activate the activity of T cells, co-stimulation by primarily B7 family proteins is required. Components of B7 family molecules needed for the co-stimulatory effect on T cell includes CD80 (B7-1) and CD86 (B7-2). Binding of these molecules with the CD28 on the T cells at the immunological synapse will prevent T cells from apoptosis and subsequently proliferate the antigen recognition signal (Greenwald *et al.,* 2005).
B7 homolog 1 (B7-H1) was found later in 1999 and it exhibits a contradict response to CD80 and CD86 (Dong *et al.*, 1999). In addition, their receptor, PD-1, on T cell was found in 2000 (Freeman *et al.*, 2000). The binding of B7-H1 or known as PD-L1 to PD-1 will dampen the TCR signal. Upon binding by PD-L1, PD-1 and phosphatase SHP2 (Src homology 2 domain–containing tyrosine phosphatase 2) will cluster with the TCR and result in dephosphorylation of TCR signaling molecules. This will block the TCR signal and reduce the activation of T cells (Yokosuka *et al.*, 2012).
Furthermore, PD-L1/PD-1 binding will increase the secretion of IL-10, which is a cytokine inhibitory factor and subsequently will suppress the T cells activation (Dong *et al.*, 1999). PD-L1 was found to be expressed in several kinds of cell types including dendritic cells, macrophages, vascular endothelial cells and immune cells (T cells, B cells, regulatory T cells) (Guan *et al.*, 2017). The upregulation of this pathway can occur through different types of cancer. Upregulation of PD-L1 expression in cancer

cells is induced by an inflammatory reaction such as INF-γ and TNF-α. Thus, this forms the basis of its association with tumors with high infiltration by inflammatory cells (Garcia-diaz *et al.*, 2017).

Another pathway involving the expression of PD-L1 is through oncogenic signaling. PTEN/P13K pathway is involved majorly in primary breast tumors (Gonzalez-angulo *et al.*, 2011). In addition, The Cancer Genome Atlas Network (2012) revealed that the majority of TNBC are associated with the silencing of PTEN and the activation of phosphoinositide 3-kinase (PI3K) pathway.

2.7 PD-L1/PD-1 expression in breast cancer

Recent studies have demonstrated that some cancers associated with PD-L1 overexpression had a poorer clinical outcome. These include gastric cancer, hepatocellular cancer, renal cell carcinoma, and ovarian cancer. In contrast, for breast cancer and Merkel cell carcinoma, PD-L1 overexpression is associated with better clinical outcome (Wang *et al.*, 2016). Significance of PD-L1 expression in breast cancers is still controversial despite various studies been reported to determine the clinical association between PD-L1/PD-1 and breast cancer.

For example, a research using 167 HER2 + breast cancer samples, for instance, discovered that greater PD-L1 expressions was correlated with greater concentrations of tumor-infiltrating lymphocytes. In HER2+ breast cancer subtype, the expression of PD-L1 found to increase disease-free survival. (Kim *et al.*, 2017). On the other hand, for TNBC subset, a study on 54 subjects undergoing neoadjuvant chemotherapy showed significant pathological complete response (pCR) with overexpression of PD-L1 (Cerbelli *et al.*, 2017).

Looking into PD-L1 expression using a microarray composed of 650 FFPE breast cancer tissue samples, the study found that there was a significant association with age, tumor size, tumor grade, lymph node metastasis, high Ki-67 and absence of ER expression. Moreover, this research also proposed the expression of PD-L1 as a negative prognostic factor (Muenst *et al.*, 2014). Another research endorsed these results with comparable connection of PD-L1 with clinicopathological parameters. Patients with younger ages, larger tumors, positive lymphovascular invasion, and advanced stages were associated with PD-L1 overexpression. Furthermore, they had significantly poorer disease-free survival and overall survival (Qin *et al.*, 2015).

A meta-analysis involving 2061 patients in five studies indicated that higher PD-L1 overexpression had negative predictive value for breast cancer. PD-L1 expression was significantly associated with positive lymph node metastasis, poorer nuclear grades, and negative ER expression (Guo *et al.*, 2016).

In conclusion, PD-L1 can act as a significant adverse prognostic biomarker in breast cancer. This also immediately implies that PD-L1 expression can facilitate future management of breast cancer as to meet the needs of immunotherapy using PD-L1 immune checkpoint inhibitors in breast cancer management.

CHAPTER IV

METHODOLOGY

3.0 Study design

This was a cross-sectional study to explore the association between tumor-infiltrating lymphocytes (TILs) and programmed death- ligand 1 (PD-L1) expression with clinicopathological data in human invasive breast ductal carcinoma. In this study, histopathology slides and pathology reports for breast cancer cases diagnosed from 2016 to 2018 at the Advanced Medical and Dental Institute (AMDI), Universiti Sains Malaysia Human were employed. Research Ethics Committee USM (HREC) had exempted this research from the need of ethical review prior to data collection with the study protocol code: USM/JEPeM/18100576. The letter of the ethical review exemption is attached in the Appendix 1.

3.1 Study sample

This study focused on samples diagnosed with invasive breast ductal carcinoma from year 2016 to 2018. The sampling method was purposive sampling whereby the samples were retrospectively selected with the diagnosis of invasive breast ductal carcinoma, NST within the stated period. Archived histopathological slides and the corresponding formalin-fixed paraffin embedded (FFPE) tumor samples from wide local excision and mastectomy specimens from Histopathology and Cytology unit, Advanced Diagnostic Laboratory, Advanced Medical and Dental Institute (AMDI), Universiti Sains Malaysia were retrieved. Pathologists from the Histopathology and Cytology unit had examined the specimens and formal pathology reports had been issued for these samples previously.

3.2 Sample size

This was a pilot study where a total of eighty cases that met the inclusion criteria were obtained from archive:

Inclusion criteria:

i. All surgical specimens from wide local excisions and mastectomy diagnosed as invasive breast ductal carcinoma from 2016 to 2018.
ii. All formal pathology reports corresponding to the specimens were retrieved.

Exclusion criteria:

i. Cases that fulfilled the inclusion criteria but the corresponding tissue blocks were not available, missing or inadequate for serial sections were excluded.
ii. Any overlapping cases were excluded.
iii. Pure in situ carcinoma cases were excluded

3.3 Clinicopathological data and pathology report

Archived formal pathology reports were retrieved and relevant information of clinicopathological parameters were acquired from each case. The data of interest included age, gender, ethnicity, size of tumor, grade of tumor, nuclear score, tubule formation, mitotic rate, number of lymph nodes retrieved and positive for metastasis, presence of lymphovascular invasion, hormonal receptor status (estrogen receptor, progesterone receptor), human epidermal growth factor receptor 2 (HER-2) expression status and Ki-67 labelling index.