THE EVALUATION OF PROGRAM DEATH 1 AND PD-1 LIGAND EXPRESSIONS IN HISTOLOGICAL SUBTYPES OF PRIMARY EXTRANODAL NON HODGKIN LYMPHOMA.

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LIST OF ABBREVIATIONS

AITL	: Angioimmunoblastic lymphoma
ALCL	: Anaplastic large cell lymphoma
CLL	: Chronic lymphocytic leukaemia
CNS	: Central Nervous System
DLBCL	: Diffuse large B cell lymphoma
EBV	: Epstein-Barr virus
ENKTL	: Extranodal NK/T cells lymphoma
FDA	: United States of Food and Drug Administration
FL	: Follicular Lymphoma
GCB	: Germinal centre type DLBCL
GIT	: Gastrointestinal tract
HL	: Hodgkin lymphoma
HPF	: High power field
HREC	: Human Research Ethics Committee
IHC	: Immunohistochemistry
IPI	: International Prognostic Index
FFPE	: Formalin-fixed, paraffin embedded
LIS	: Lab information system
MALT	: Mucosa-associated lymphoid tissue
MF	: Mycosis Fungoides
MREC	: Medical Research and Ethics Committee
MZL	: Marginal zone lymphoma.

NGCB	: Non-germinal centre type DLBCL.
NHL	: Non-Hodgkin lymphoma
PD-1	: Program death- 1
PD-L1	: Program death ligand -1
peNHL	: Primary extranodal non-Hodgkin lymphoma
SPSS	: Statistical Package for Social Sciences
USM	: Universiti Sains Malaysia

ABSTRAK

Latar belakang: Kemunculan Program Death-1 (PD-1) dan ligannya, PD-L1 immunoterapi telah memberikan gambaran baru mengenai kaedah rawatan kes-kes tumor. Keberkesanan terhadap immunoterapi adalah bersangkutan dengan ekspresi PD-1 dan PD-L1. Kajian in bertujuan menganalisa ekspresi PD-1 di persekitaran mikro tumor dan PD-L1 dalam sel-sel tumor di dalam kes-kes limfoma Non-Hodgkin esktranodal primer (peNHL).

Metodologi: Kajian *cross-sectional* retrospektif menggunakan 87 blok tisu paraffin bagi pesakit yang didiagnos peNHL. Kesemua sample tersebut telah diwarnai dengan PD-1 dan PD-L1 oleh kaedah immunohistokimia dan ekspresi diberikan pernilaian. Kaitan antara ekspresi PD-1 di persekitaran mikro tumor dan ekspresi PD-L1 di dalam sel-sel tumor dengan jenis kes-kes peNHL dianalisa secara statistik.

Keputusan: Sebilangan besar kes menunjukkan ekspresi negatif PD-L1 pada sel-sel tumor (46, 52.9%), tetapi majoriti positif untuk ekspresi PD-1 di persekitaran mikro tumor (57, 65.5%). Kaitan antara ekspresi PD-1 dan PD-L1 dengan jenis-jenis kes peNHL adalah signifikan secara statistik ($p<0.05$) dengan ekspresi tertinggi di dalam kes kes DLBCL.

Kesimpulan: Kajian kami, yang pertama di Malaysia yang meneroka ekspresi PD-1 dan PD-L1 dalam peNHL, menunjukkan perkaitan yang signifikan antara ekspresi PD-1 dan PD-L1 dengan jenis jenis peNHL yang boleh memberi kesan kepada rawatan pesakit ini pada masa akan datang.

ABSTRACT

***Introduction*:** Emergence of Programmed death 1 (PD-1) and its ligands, PD-L1 immunotherapy provide new insight in the treatment modality of malignancies. The responsiveness of the patients are associated with the expressions of the PD-L1 and PD-1 protein. We aimed to evaluate the expression of PD-1 in the tumour microenvironment and PD-L1 in the tumour cells with histological subtypes of primary extranodal non-Hodgkin lymphoma (peNHL).

***Methodology*:** A retrospective cross-sectional study using 87 archived formalin fixed paraffin-embedded tissue blocks of patients diagnosed with peNHL. Samples were stained for PD-1 and PD-L1 by immunohistochemistry method and the proteins expressions were evaluated microscopically. The association between expression of the PD-1 and PD-L1 with subtypes of peNHL were statistically analysed.

***Results*:** Majority of the cases are negative expression of PD-L1 in the tumour cells (46, 52.9%), however majority are positive for PD-1 expression in the tumour microenvironment (57, 65.5%). Significant associations were found between PD-1 and PD-L1 expression with subtypes of peNHL ($p<0.05$) and higher expression was found in DLBCL.

Conclusion: Our study, the first in Malaysia to explore expression of PD-1 and PD-L1 in peNHL, demonstrates significant association of PD-1 and PD-L1 expressions with subtypes of peNHL which may impact treatment of these patients in the future.

Keywords: Primary extranodal non-Hodgkin lymphoma, PD-1, PD-L1

CHAPTER 1

INTRODUCTION

1.1 Overview of Primary Extranodal Non-Hodgkin Lymphoma (peNHL)

Lymphomas are solid tumours of the immune system. Majority of them are Non-Hodgkin lymphoma(NHL) (90%) and only 10% are Hodgkin lymphoma(HL) (Zucca, 2008). Non-Hodgkin lymphomas are heterogeneous groups of disorders originating from B-lymphocytes, T-lymphocytes or natural killer cells. At least one-fourth of NHL arise from tissues other than lymph nodes (Zucca, 2008; Vannata & Zucca, 2015) .

The definition of primary extranodal non-Hodgkin lymphoma (peNHL) is controversial, particularly in the presence of both nodal and extranodal disease. Dawson *et al,* 1961 first proposed a strict criteria, defined primary gastric lymphoma as presentation with main disease manifestation in stomach, with or without involvement of regional lymph nodes (Dawson *et al*., 1961). Later these criteria were extended to allow for contiguous involvement of other organs (e.g. liver, spleen) and for distant nodal disease providing that the extranodal lesion was the presenting site and, after routine staging procedures, constituted the predominant disease bulk, to which primary treatment must be directed (Krol *et al.*, 2003). However, Krol *et al*., (2003) also reported superior disease-free survival for patients with peNHL defined by strict criteria. Furthermore, the inclusion among peNHL of cases presenting with stage III and IV is also questionable and several authors consider only stage I and II presentation as peNHL (Zucca, 2008).

Lymphomas arising primarily in extranodal sites can be diagnostically challenging due to their morphological diversities and lack of uniformity in the histopathological classification system. There is also a compelling evidence that peNHL at some sites have rather specific

aetiologies that are site specific, such as *Helicobacter pylori* in gastric lymphomas and autoimmune thyroiditis in thyroid lymphomas (Swerdlow *et al.*, 2017). Numerous papers dealing with peNHL originating in almost every organ in the body have been published, however the literatures on peNHL as a group is limited.

The incidence of peNHL has increased substantially in the last 40 years (Zucca, 2008). This may in part be due to improved diagnostic procedures (particularly in the brain and gastrointestinal lymphomas) (Thomas *et al.*, 2011). The frequency of peNHL varies in different parts of the world. In countries where total lymphoma incidence is high the incidence of lymphomas at each extranodal site also tends to be high (Thomas *et al.*, 2011). A comparative study done in 2008 demonstrated there was a higher prevalence of peNHL cases in Arabs than in Asians (AlShemmari, Ameen & Sajnani, 2008). In Malaysia, a study done in University of Malaya from 1980 to 2012, reported 42 cases of peNHL involving the oral maxillary fascial area (Ramanathan *et al.*, 2014). However no cumulative study in Malaysia was published for peNHL.

Primary extranodal non-Hodgkin lymphoma (peNHL) have been reported to originate from almost every anatomic site of the body such as gastrointestinal tract (GIT) , head and neck, skin, central nervous system (CNS), bone, testis, thyroid, breast, orbit, and rarely adrenal, pancreas, and the genitourinary tract (Wu *et al.*, 2009; Thomas *et al.*, 2011; Padhi *et al.*, 2012; Mertsoylu *et al.*, 2014; Shen *et al.*, 2018). A Dutch study showed that the GIT was the most common site of peNHL (45.7%) (Krol *et al.*, 2003). In contrast, the CNS constituted the most common extranodal site in an Indian study (29.7%) (Padhi *et al.*, 2012). According to a study done by Wu *et al*. (2009) in China on the incidence of peNHL B-cell types accounted for the majority of peNHL cases among all races and sex group (Wu *et al.*, 2009). Among the B cells types, Diffuse large B-cell lymphoma

(DLBCL), marginal zone lymphoma (MZL) and follicular lymphoma (FL) accounted for over 75% of B-cell lymphoma subtypes (Wu *et al.*, 2009; Shen *et al.*, 2018). The incidence of T cells peNHL is rare compared to B cells, its accounted only 3% out of all type of peNHL (Summyia *et al.*, 2014). Majority of the cases are confined to the skin, such as mycosis fungoides (Padhi *et al.*, 2012).

1.2 Overview of Program Death-1 (PD-1) and Programmed Death Ligand -1 (PD-L1)

Program death-1, PD-1 (CD279) is a cell surface protein belonging to the CD28 family, encoded by *PDCD1* gene located in chromosome 2q37. It was discovered in 1992 by Tasuku Honjo and colleagues as an apoptosis-associated molecule after they cloned the *PD-1* gene from immune cell lines undergoing apoptosis (Ishida *et al.*, 1992). The main physiological role of PD-1 is in limiting autoimmunity in an inflammatory context (e.g. in response to infection) by restricting the activity of T cells in peripheral tissues (Ishida *et al.*, 1992; Keir *et al.*, 2008). PD-1 is expressed on activated T cells, with particularly high expression by tumour-infiltrating T lymphocytes. PD-1 is also expressed on activated non-T cells, including B cells, natural killer cells, and monocytes, implying that PD-1 may also modulate immunity in a T-cell–independent manner. In normal human reactive lymphoid tissue, PD-1 is expressed on germinal centre associated T cells (Keir *et al.*, 2008) .As a receptor, PD-1 has two ligands, PD-L1 and PD-L2 which are differ in their expression patterns (Yu *et al.*, 2020).

Programmed cell death ligand 1, PD-L1 (CD274 and B7-H1) is an immunomodulatory transmembrane glycoprotein of ~43kDA (290 amino acids). PD-L1 is a member of the B7 family of co-stimulatory molecules and encoded by *PDCD1LG1* in chromosome 9. The PD-L1 protein is expressed in a tissue-and cell type–specific manner and is found in heart, placenta, lung and

skeletal muscle as well as in the hematopoietic compartment, in antigen-presenting cells, and including in activated T cells (Panjwani *et al.*, 2018). Programmed cell death ligand 2, PD-L2 is another ligand of PD-1(Akinleye & Rasool, 2019). PD-L2 expression is much more restricted than PD-L1 expression. PD-L2 is inducibly expressed on dendritic cells, macrophages, and bone marrow–derived mast cells (Keir *et al.*, 2008).

Dysfunctional of PD-1/PD-L1 leads to autoimmunity like colitis, endocrinopathy and immune/inflammatory dermatoses are common complications (Anagnostou & Ansell, 2017). Given its pivotal role in preventing autoimmunity and maintenance of peripheral tolerance in normal tissues, tutor cells exploited this mechanism to evade anti-tumour immune responses and ultimately progress, disseminate, and metastasize(Anagnostou & Ansell, 2017). PD-1 is highly expressed in tumour microenvironment predominantly on tumour-infiltrating lymphocytes in a large proportion among many types of cancers(Akinleye & Rasool, 2019). While PD-L1, are constitutively expressed on different types of tumour cells including melanoma and ovarian, lung, and renal carcinomas(Panjwani *et al.*, 2018; Akinleye & Rasool, 2019).

1.3 Expression of PD-1 and PD-L1 in Non-Hodgkin Lymphoma

In lymphomas, most data on PD-1 and PD-L1s expressions and their clinical relevance have been described mainly in HL and DLBCL. PD-L1 is expressed by both DLBCL tumour cells and by non-malignant cells from their immune microenvironment, such as macrophages (Song, Park & Uhm, 2019). In contrast to PD-L1, PD-1 expression has almost exclusively been detected in the microenvironment cells of DLBCL (Gravelle *et al.*, 2017)**.** Using a large series of 1200 DLBCL samples, Kiyasu *et al*.(2015) demonstrated that patients with PD-L1 positive DLBCL had inferior

overall survival rates than PD-L1 negative DLBCL patients. Moreover, patients with PD-L1 positive tumour cells but low PD-1 positive in tumour microenvironment had poorer prognosis than patients with PD-L1 negative DLBCL and high PD-1 positive in the tumour microenvironment (Kiyasu *et al.*, 2015). In contrast to DLBCL, most follicular lymphoma (FL) tumour cells do not express PD-L1 , however PD-1 positive cells are abundant in their microenvironment(Gravelle *et al.*, 2017).

While for T cell lymphoma, PD-1 is a well-known established sensitive marker of angioimmunoblastic lymphoma (AITL) (Swerdlow *et al*., 2017). However, expression of PD-1 in the tumour microenvironment of T cells and PD-L1 expression on the tumour cells are not well explored. Study by Jo *et al* in 2017 involving 79 cases of extranodal NK/T cells lymphoma (ENKTL) demonstrated PD-L1 was expressed variably by tumour cells or infiltrating immune cells in ENKTL, while the expression rates of PD-1 in infiltrating immune cells were very low (Jo *et al.*, 2017). Furthermore, this study found out that PD-L1-positive patients had a trend toward better overall survival and the lower IPI score (Jo *et al.*, 2017).

There are four mechanisms of intrinsic signals reported in lymphoid malignancies that are associated with increased expression of PD1 and PD-L1. First is copy number alterations (CNA) of 9p24.1, including chromosomal amplification, gain, polysomy, or translocation. This is one of the hallmarks of classical Hodgkin lymphoma (HL) (Green *et al.*, 2012). Second mechanism that causes overexpression of PD-L1 and PD-1 is Epstein-Barr virus (EBV) infection. EBV-encoded latent membrane protein (LMP)-1 activates AP-1 (*via* cJUN/JUN-B components) which further activate the PD-L1 enhancer and promoter (Green *et al.*, 2012). A study done by Kiyasu *et al* in 2015, shows there is significant association between PD-L1 positive DLBCL with EBV infection. A study done in France involving 82 cases of plasmablastic lymphoma, found that EBV positive

cases exhibited a higher expression of PD-1 and PD-L1 in both malignant cells and microenvironment as compared to EBV negative cases (Laurent *et al.*, 2016).

Kataoka *et al.*, (2016) discovered *PD-L1* 3′-untranslated region (UTR) disruption in subset of DLBCL and adult T cell leukaemia/lymphoma patients. The 3′-UTR disruption produces truncated PD-L1 protein (Kataoka *et al.*, 2016). Lastly, constitutive activation of the JAK/STAT pathway also can induced expression of PD-L1 in lymphoma cells. In anaplastic large cell lymphoma (ALCL) with *NPM-ALK* rearrangement, the fusion transcript can induce PD-L1 expression mediated by activated STAT3. JAK/STAT pathway is also enhanced in DLBCL activated B cell-like (ABC) phenotype, which more commonly expresses PD-L1 compared to germinal centre (GCB) DLBCL (Ok & Young, 2017).

1.4 Immune checkpoint inhibitors as cancer therapeutics

PD-1 and PD-L1 inhibitors have shown clinical efficacies against many different solid and hematologic malignancies(Akinleye & Rasool, 2019). These inhibitors disrupt PD-1 axis reverses T cell suppression and enhances endogenous antitumor immunity to unleash long-term antitumor responses for patients with a wide range of cancers(Muhamad *et al.*, 2020). To date, five antibody-based inhibitors have been approved by the United States Food and Drug Administration (FDA), which are two anti-PD-1 antibodies (nivolumab and pembrolizumab) and three anti-PD-L1 antibodies (avelumab, atezolizumab, and durvalumab). Nivolumab has been approved for non-small-cell lung cancer, renal cell carcinoma, bladder cancer, colorectal cancer with microsatellite instability or mismatch repair deficiency, hepatocellular carcinoma, classic Hodgkin lymphoma, melanoma, and head and neck squamous cell carcinoma (Ansell *et al.*, 2015; Akinleye & Rasool, 2019).

1.5 Immunohistochemical stains for PD-1 and PD-L1

The establishment of PD-L1 and PD-1 expressions via immunohistochemical (IHC) staining has been proposed as one potential biomarker for patient response to immunotherapy as well as prognostic. IHC staining has various advantages: (1) broad availability of formalin fixed, paraffin-embedded tissue; (2) the ability to visualize the expression in various cell populations (tumour versus immune/ stromal cells) to some extent based on morphology; (3) relative test rapidity; and (4) the test's acceptable low cost and widespread use in pathology laboratories, particularly in comparison to the molecular pathology-based methods (Panjwani *et al.*, 2018) .

However, IHC-based detection of PD-L1 expression is limited by preanalytical and analytical variability including heterogeneity in antibody clones, scoring methodology and intrinsic biological variation in PD-L1 expression due to the type of specimen analysed as well as prior treatment status (Kythreotou *et al.*, 2018). PD-L1 expression on both cancer cells and/or infiltrating immune cells was found to correlate with responses to PD-L1/PD-1 inhibitors and has therefore been investigated as a potential predictive biomarker of responsiveness to these checkpoint inhibitors (Ansell *et al.*, 2015). However, the data available in literature, related to the expression of PD-L1 and PD-1 expressions in different tumour types, are not uniform and are often conflicting. This problem might be associated with the use of different antibodies clones, with variable specificity, and mainly with a score not uniquely defined.

Considering the potential availability of PD-1 and PD-L1 inhibitor, clarification of the PD-1 and PD-L1 expressions on tumour behaviour is crucial. Furthermore, most of the research was conducted in the Western countries, which may not apply to our local settings, as well as there is no data on PD-1 and PD-L1 expression in peNHL has been collected and reported in Malaysia.

Hence, it is important to embark on a local study regarding the expression of PD-1 and PD-L1 in peNHL and its correlation with the subtypes which may provide local data and even aid in building new “Malaysia specific” prediction models later.

CHAPTER 2

STUDY PROTOCOL

2.1 Study protocol

Title: The evaluation of Program Death-1 and PD-1 Ligand Expression in Histological Subtypes of Primary Extranodal Non-Hodgkin Lymphoma (peNHL).

Introduction

Lymphomas are solid tumours of the immune system. Majority of them are Non Hodgkin lymphoma (90%) and only 10% are Hodgkin lymphoma (Zucca, 2008). Non-Hodgkin lymphomas are heterogeneous groups of disorders originating from B-lymphocytes, T-lymphocytes or natural killer cells. At least one-fourth of non-Hodgkin's lymphomas (NHL) arise from tissues other than lymph nodes (Zucca, 2008; Vannata & Zucca, 2015) . The definition of primary extranodal non-Hodgkin lymphoma (peNHL) is a crucial issue, especially in patients where both nodal and extranodal sites are involved. Strict criteria were proposed by Dawson in 1961, reporting on a series of primary intestinal lymphomas. In his study, patients were only included if no palpable superficial lymphadenopathy was found at first examination, with chest X-ray showed no obvious enlargement of mediastinal lymph nodes and the white blood cell counts were within normal limits. Patients were excluded from analysis when distant abdominal lymph nodes, spleen or liver were involved (Dawson, Cornes & Morson, 1961). More recent study by Krol *et al*. used a liberal definition of peNHL where non-Hodgkin lymphoma that apparently originated at an extranodal site, even in the presence of disseminated disease, as long as the extranodal component was clinically dominant (Krol *et al*., 2003).

The incidence of peNHL has increased substantially in the last 40 years (Zucca, 2008). This may in part be due to improved diagnostic procedures (particularly in brain and gastrointestinal lymphomas). The frequency of peNHL varies in different parts of the world. In countries where total lymphoma incidence is high the incidence of lymphomas at each extranodal site also tends to be high (Thomas *et al.*, 2011). A comparative study done in 2008 demonstrated there was a higher prevalence of peNHL cases in Arabs than in Asians(AlShemmari, Ameen & Sajnani, 2008). In Malaysia, a study done in University of Malaya from 1980 to 2012, reported 42 cases of peNHL involving the oral maxillary facial area (Ramanathan *et al.*, 2014). However no cumulative study in Malaysia was published for peNHL. Primary extranodal non-Hodgkin lymphoma have been reported to originate from almost every anatomic site of the body such as gastrointestinal tract , head and neck (Waldeyer's ring, nose/paranasal sinses/nasopharynx, salivary glands, etc.), skin, central nervous system (CNS), bone, testis, thyroid, breast, orbit, and rarely adrenal, pancreas, and the genitourinary tract (Wu *et al.*, 2009; Thomas *et al.*, 2011; Padhi *et al.*, 2012; Mertsoylu *et al.*, 2014; Shen *et al.*, 2018).

Like other solid tumours, mechanisms that cause immune evasion for the tumour cells to survive are also found in lymphoma (Xu-Monette, Zhou & Young, 2018). The interaction of the programmed death-1 receptor (PD-1, CD279) at the surface of T lymphocytes, together with its programmed death-ligand-1 (PD-L1) or PD-L2, which are found at the surface of some tumour cells and macrophages is the most common dysregulated checkpoint (Gravelle *et al.*, 2017). Identifications of PD-1 and PDL-1 expression as predictive and prognostic marker in tumour cells have been one of the major achievements. With the identifications of these markers, targeted treatments have been used to improve the survivability in the affected patients. Thus, further assessment and validation of PD-L1 expression via immunohistochemical staining within this

subgroup of solid tumour has shown as one potential biomarker to predict improved outcome(Gravelle *et al.*, 2017).

Considering the potential availability of PD-1 and PD-L1 inhibitors, investigation for PD-1 and PDL-1 as a potential target for therapy and clarification of the impact of PD-1 and PDL-1 expression on tumour behaviour is crucial. Furthermore, most of the researches were conducted in the Western countries, which may not apply to our local settings, as well as there is no data on PD-1 and PD-L1 expression in primary extranodal non-Hodgkin lymphoma has been collected and reported in Malaysia. Hence, it is important to embark on local study regarding the expression of PD-1 and PD-L1 in primary extranodal non Hodgkin lymphoma and its correlation to the clinicopathological parameters which may provide local data and even aid in building new "Malaysia specific" prediction models later.

Research questions

a) What is the expressions of PD-1 and PD-L1 expression in peNHL?

b) What are the association of between the expressions of PD-1 and PD-L1 with subtypes of peNHL?

Objectives and Research Hypothesis

General

To evaluate the level of PD-1 and PD-L1 expressions and its association with the subtypes of peNHL.

Specific objectives:

1. To determine the PD-1 and PD-L1 expressions in peNHL.
2. To determine the association between PD-1 and PD-L1 expressions with demographic factors and subtypes of peNHL.

Research hypothesis:

Null: There is no association between PD-L1 and PD-1 expressions with subtypes of peNHL and demographic factors.

Alternative: There is an association between PD-L1 and PD-1 expressions with demographic factors and subtypes of peNHL.

Methodology

Study design

Retrospective Cross-sectional studies design

Study location

The study will be conducted in Hospital Sultanah Bahiyah and Hospital Universiti Sains Malaysia.

Study population and study period

Patients diagnosed with primary extranodal non Hodgkin lymphoma in Hospital Sultanah Bahiyah from

January 2015 to December 2019.

Subject criteria

Inclusion criteria

All primary extranodal non Hodgkin lymphoma diagnosed between January 2015 to December 2019

Exclusion criterias

- Patient with major nodal involvement of non Hodgkin lymphoma
- Referral case from other centres.
- Tissue blocks that are not available or insufficient specimen
- Cases with insufficient clinical data
- Overlapping cases

Sample size determinant

Sample size for this study was calculated to fulfill the following objectives:

Sample size calculation:

Objective 1: To determine the PD-1 and PD-L1 expressions in peNHL.

Sample size was calculated using a single proportion formula:

$n= (z / r)^2 p (1-p)$ (n=sample size, r=precision+5%)

z= 1.96

r=5% (0.05)

P= 11% (Kiyasu *et al.*, 2015)

$n=(1.96/0.05)^2 0.11(1-0.11)$

n= 150

Adding 20% drop out , n = 170

Objective 2 : To determine the association between PD-1 and PD-L1 expressions with demographic factors and subtypes of peNHL.

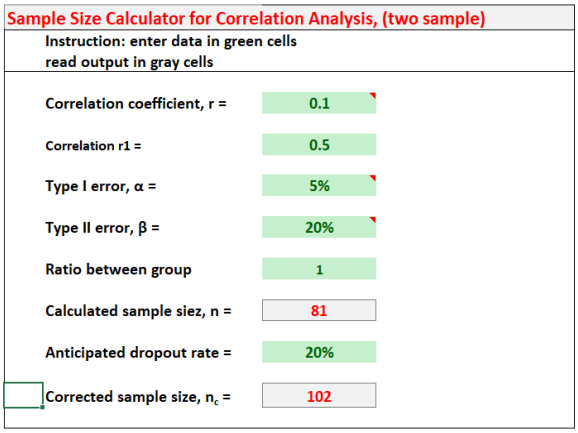
Sample size calculated using Two sample correlation by Najib MY (2015), Sample Size Calculator for Correlation Analysis, Unit of Biostatistics & Research Methodology, School of Medical Sciences, Universiti Sains Malaysia Kubang Kerian Kelantan.

Taking into consideration the following:

$\alpha = 0.05$ (Type 1 error)

$\beta = 20\%$

Correlation coefficient, r = 0.1 (According to Cohen (1992) for Large effect size)



Sample Size Calculator for Correlation Analysis, (two sample)	
Instruction: enter data in green cells read output in gray cells	
Correlation coefficient, r =	0.1
Correlation r1 =	0.5
Type I error, α =	5%
Type II error, β =	20%
Ratio between group	1
Calculated sample siez, n =	81
Anticipated dropout rate =	20%
Corrected sample size, n_c =	102

Total sample size required base on objective 1 and objective 2 is 170

Sampling method

Convenient sampling. Cases that fulfilled the inclusion criterias will be retrieved from the computerized registry database via Laboratory Information System (LIS) from Department of Pathology, HSB. Patients' demographics data of interest will be traced using electronic hospital information centre (E-HIS). Corresponding tissue blocks will be retrieved from the tissue archive and cases will be excluded from the study according to the exclusion criteria. For each case, a single representative block from the available blocks, which contain the tumour, will be utilized in the study.

Immunohistochemical analysis: Staining

Selected formalin-fixed, paraffin-embedded (FFPE) tissue blocks will be cut at 4 µm and send for staining for the PD-1 and PD-L1 IHC staining in pathology laboratory in Hospital USM. Sections will be deparaffinised, followed by epitope retrieval using EnVision Flex Target retrieval solution, high pH. All the slides subsequently placed in Squenza immunostainer incubated overnight at 4°C

with Anti-PD-L1 antibody and recombinant Anti-PD1 antibody. Both will be prediluted to 1: 500. Localization of the antigen-antibody achieves using labelled polymer, EnV FLEX/HRP. The complex will be visualised with Substrate Chromogen (Envision FLEX Substrate Working Solution). Tonsils and lymph nodes with reactive hyperplasia serve as external positive controls for both PD-1 and PD-L1.

Immunohistochemical analysis: Assessment of staining

PD-L1 staining is defined as complete circumferential or partial linear cellular membrane staining at any intensity. Cytoplasmic staining, if present, is not considered positive for scoring purposes. Non-malignant cells and immune cells (e.g., such as infiltrating lymphocytes or macrophages) may also stain with PD-L1; however, these are not included in the scoring for the determination of PD-L1 positivity. For PD-L1, based on previous studies (Qian *et al.*, 2020) , the samples with <5% stained tumours cells will be defined as negative. For >5% positive PD-L1 stained samples are defined as positive regardless of the intensity.

PD-1 was detected in the tumour microenvironment with membranous staining. The assessment and scoring was done as the previous study(Ko *et al.*, 2011). PD-1 expressions will be assessed at 3 different high power fields (HPF), counted the number of PD-1- positive cells in the tumour microenvironment, and recorded the average value. The samples are consider positive if >20/HPF and negative for ≤20/HPF.

Statistical analysis will be performed with the SPSS version 26.0. The association of clinicopathological characteristic with PD-1 and PD-L1 expression will be analysed using the chi square test or Fisher's exact test. Level of significance in this study was set as p value <0.05.

Study Protocol

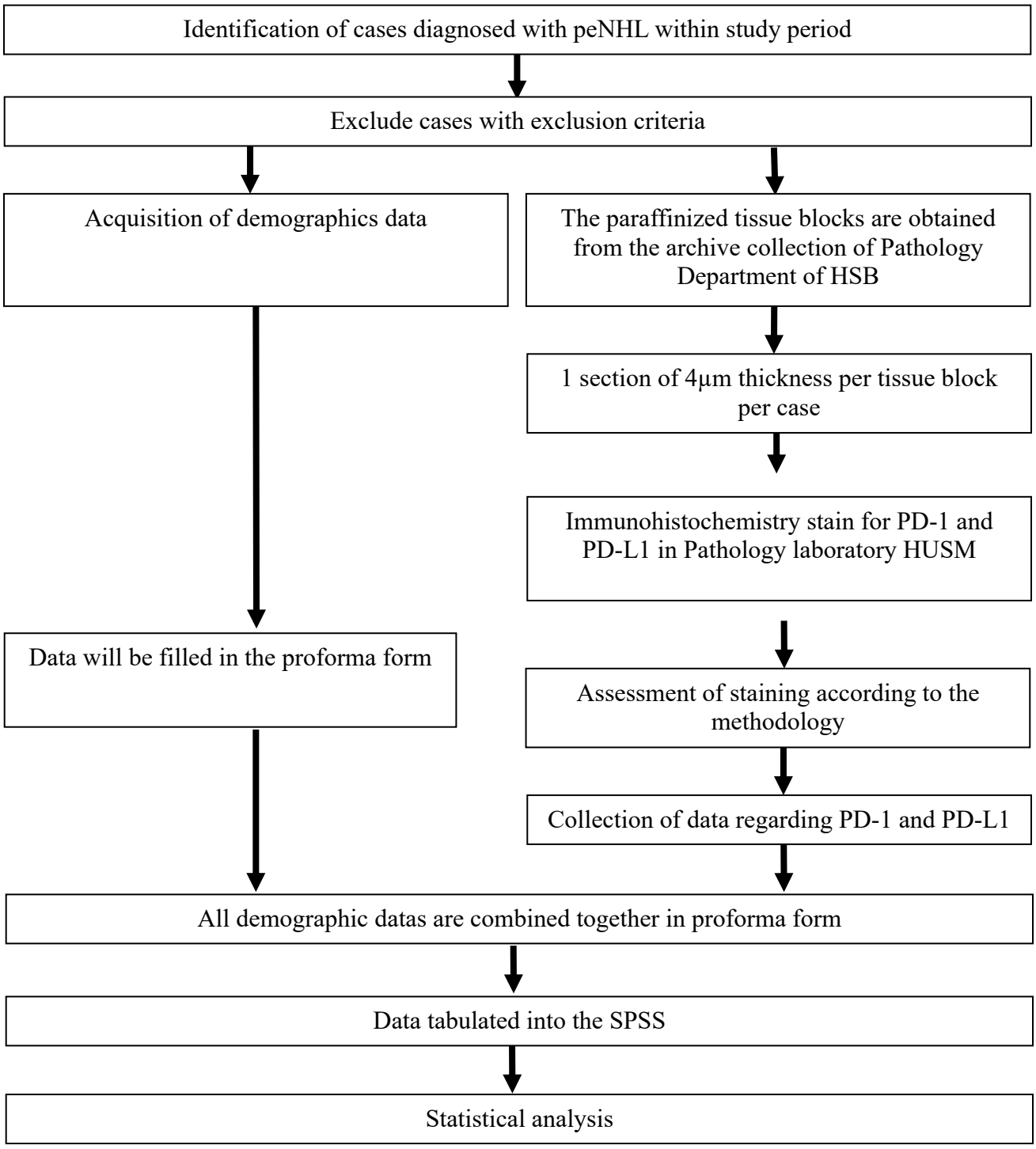


Figure 1: Study Protocol.

Research tool

Antibody:

1. Anti-PD-L1 antibody [28-8] (ab205921; Abcam)
2. Recombinant Anti-PD1 antibody [EPR4877(2)] (ab137132; Abcam).

Demographic data from HSB computerized registry database (LIS and eHIS)

Ethical Consideration

Ethical approval for this study will be applied from Medical Research and Ethics Committee (MREC) and Human Research Ethics Committee (HREC), Universiti Sains Malaysia

Expected limitation

- Biopsy specimen might not be adequate/exhausted
- Optimizing antigen retrieval might be difficult
- Interpretation of the staining of the slides may be biased
- Inadequate amount of stains for all the study sample needed.

Privacy and confidentiality

To protect the confidentiality of the patient, all tissue blocks and demographic data will be assigned with Pathology Number. Only researchers can access the data. Data will be presented as a grouped data and will identify the responders individually.

Conflict of interest: There is no conflict of interest.

Expected result

Table 1: Demographic characteristics and types of peNHL

Variables	Median	n(%)
Age group		
< 60 years old		
≥ 60 years old		
Sex		
Male		
Female		
Ethnic		
Malay		
Chinese		
Indian		
Others		
General subtype		
B cell lymphoma		
T cell lymphoma		
Locations		
GIT		
Head and neck		
CNS		
Genitourinary		
Skin and musculoskeletal		
Mediastinum		
Breast		
Retroperitoneum		
CVS		

Table 2 : The expression of PD-1 and PD-L1 in peNHL and it's association

		PDL-1 expression in the tumour cells		TOTAL	*p* value
		Negative, n(%)	Positive, n(%)		
PD-1 expression in tumour microenvironment	Negative				
	Positive				
TOTAL					

Table 3: The association between PD-L1 expression with demographic factors, general subtypes and locations of peNHL.

Variables	N	PDL-1 expression of the tumour cells			
		Positive n (%)	Negative n(%)	χ^2 (df)	*p* value
Age					
< 60					
≥ 60					
Sex					
Male					
Female					
Ethnicity					
Malay					
Chinese					
Indian					
Others					
General subtype					
B cell lymphoma					
T cell lymphoma					
Locations					
GIT					
Head&neck					
CNS					
Genitourinary					
Skin and musculoskeletal.					
Mediastinum					
Breast					
Retroperitoneum					
CVS					
Lung					

Table 4: The association between PD -1 expression with demographic factors, general subtypes and locations of peNHL.

Variables	N	PD-1 expression in the tumour microenvironment			
		Positive n (%)	Negative n (%)	χ^2 (df)	*p* value
Age					
< 60					
> 60					
Sex					
Male					
Female					
Ethnicity					
Malay					
Chinese					
Indian					
Others					
General subtype					
B cell lymphoma					
T cell lymphoma					
Locations					
GIT					
Head&neck					
CNS					
Genitourinary					
Skin&musculoskeletal					
Mediastinum					
Breast					
Retroperitoneum					
CVS					
Lung					

Table 5: The association between expression of PD-1 and PD-L1 with subtypes peNHL

Lymphoma Subtypes	n	PD-L1 expression of the tumour cells			PD-1 expression in the tumour microenvironment		
		Positive n (%)	Negative n(%)	*p* value	Positive n (%)	Negative n (%)	*p* value
Follicular							
DLBCL							
MALT							
Burkitt							
SLL							
Plasmablastic							
Large B cells							
T cells							

Milestone chart

March 2019	Completion of data collection from HSB
Sep 2019	Completion of FFPE tissue slide sectioning and send to USM for IHC staining
May 2020	Completion of immunohistochemical staining and analysis.
July 2020	Completion of report
Nov 2020	Thesis submission