Evaluation of Signal Transducer and Activator of Transcription 3 (STAT-3) Protein Expression in non-Hodgkin Lymphoma Cases in Hospital Universiti Sains Malaysia

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LIST OF ABBREVIATIONS

JAK	Janus Associated Kinase
STAT	Signal Transducer and Activator of Transcription
NHL	Non-Hodgkin Lymphoma
HL	Hodgkin Lymphoma
HUSM	Hospital Universiti Sains Malaysia
FFPE	Formalin fixed paraffin embedded
NK	Natural Killer cell
IL	Interleukin
HPE	Histopathological examination
IHC	Immunohistochemistry
LDH	Lactate dehydrogenase
ESR	Erythrocyte Sedimentation Rate
OS	Overall survival
DLBCL	Diffuse Large B Cell Lymphoma
DLBCL-NGCB	Diffuse Large B Cell Lymphoma Non-Germinal centre B-cell subtype
DLBCL-GCB	Diffuse Large B Cell Lymphoma Germinal centre B-cell subtype
NOS	Not Otherwise Specified

CNS	Central Nervous System
PMBL	Primary Mediastinal large B-cell Lymphoma
ALCL	Anaplastic Large T Cell Lymphoma
ALK	Anaplastic Lymphoma Kinase
FL	Follicular Lymphoma
SLL	Small Lymphocytic Lymphoma
MZL	Marginal Zone Lymphoma
MCL	Mantle Cell Lymphoma
SPSS	Statistical Package for Social Sciences

ABSTRAK

Pengenalan: Terapi yang ditargetkan pada 'Janus Associated Kinase-Signal Transducer and Activator Transcription (JAK-STAT)' terutama yang berkaitan dengan *protein* STAT-3 dalam limfoma 'Non-Hodgkin' (NHL), menyediakan strategi perubatan terkini. Protein STAT-3 juga berkait dengan prognosa penyakit limfoma. Oleh kerana itu, kajian ini bertujuan untuk menilai ekspresi *protein* STAT-3 dalam kes NHL, yang telah didiagnos di Hospital Universiti Sains Malaysia.

Metodologi: Kajian secara retrospektif keratan rentas menggunakan 95 kes NHL daripada sampel tisu tertanam oleh lilin paraffin yang diawet formalin diperolehi. Prosedur imunostain STAT-3 dijalankan dan analisa dibuat. Perkaitan antara ciri klinikopatologi dan ekspresi *protein* STAT-3 dianalisis menggunakan ujian *Pearson chi-square* dan *Fisher's exact test.* Nilai $p < 0.05$ dianggap signifikan secara statistik.

Keputusan: Sebahagian besar kes limfoma (78.9%) menunjukkan ekspresi positif terhadap *protein* STAT-3. Enam puluh empat perpuluhan dua peratus (64.2 %) daripadanya adalah dalam kalangan agresif B sel NHL yang mana 20% daripadanya adalah subkategori *Diffuse Large B Cell Lymphoma, non-germinal centre B (DLBCL-NGCB).*Terdapat perkaitan antara *protein* STAT-3 dengan subkategori DLBCL ($p = 0.046$).

Kesimpulan: *Protein* STAT-3 mempunyai perkaitan dengan DLBCL, iaitu salah satu subkategori agresif B sel NHL. Ia berkemungkinan boleh menjadi salah satu penanda untuk prognosa dalam NHL. Walau bagaimanapun, kajian yang lebih meluas dengan gabungan *protein* JAK perlu dilakukan pada masa hadapan untuk menjelaskan mekanisme JAK-STAT dalam NHL dan perkaitan peranannya.

Kata kunci: Limfoma, limfoma non-Hodgkin, *Diffuse Large B Cell Lymphoma*, *protein* STAT-3.

ABSTRACT

Background: Evolving targeted therapy on Janus Associated Kinase-Signal Transducer and Activator of Transcription (JAK-STAT) signalling pathway especially pertaining to STAT-3 protein in non-Hodgkin lymphoma (NHL), provide new treatment strategies. STAT-3 protein also related to prognostication of NHL. Hence, we aimed to evaluate the expression of STAT-3 protein in NHL cases, diagnosed in Hospital Universiti Sains Malaysia.

Methods: A retrospective cross sectional study using formalin fixed paraffin embedded tissue blocks of 95 NHL cases were obtained. STAT-3 immunostaining was performed and evaluated. The association between clinicopathological characteristics and STAT-3 protein expression was analysed using the Pearson chi-square and Fisher's exact test. A p-value of < 0.05 is considered statistically significant.

Results: Majority of the cases (78.9 %) have positive STAT-3 protein expression. 64.2 % were among aggressive B cell NHL whilst 20 % of them are Diffuse Large B Cell Lymphoma, non-germinal centre B subtype (DLBCL-NGCB). There is significant association between STAT-3 protein expression with DLBCL subtypes ($p = 0.046$).

Conclusion: STAT-3 protein was significantly associated with DLBCL, one of the aggressive B cell lymphoma subtypes. It could be one of the possible markers to prognosis in NHL. However, a more extensive study with a combination of JAK protein needs to be done in the future to elucidate the mechanism of the JAK-STAT pathway in NHL and its associated role.

Key words: Lymphoma; Non-Hodgkin lymphoma; Diffuse Large B Cell Lymphoma; STAT-3 protein.

CHAPTER 1: INTRODUCTION

1.1 Overview of lymphoma

A heterogenous lymphoproliferative tumour known as lymphoma typically causes enlargement of lymph nodes and can also develop from extra nodal sites (1). To decide the course of treatment and forecast the prognosis, the lymphoma categorization system uses a mix of morphologic, phenotypic, genotypic, and clinical parameter (1,2). Collectively, lymphoma is mainly divided into Hodgkin lymphomas (HL) and non-Hodgkin lymphomas (NHL). The NHL categories are further subdivided based on respective cell lineages; either B cell, T cell or Natural Killer (NK) cell types (1,3).

Lymphoma was the fourth most prevalent disease in Malaysia from 2012 to 2016 according to the Malaysia National Cancer Registry, affecting 58.5% of men and 41.5% of women (4). The global burden of cancer worldwide using the GLOBOCAN 2018 estimates 2.8% and 2.6% of cancer incidence and mortality in NHL, respectively (5). In most countries worldwide, NHL ranked as the 5th to 9th most common cancer, with almost 510,000 new cases estimated in 2018 (6). The NHL, particularly follicular lymphoma, and diffuse large B cell lymphoma (DLBCL), both make up > 60% of all lymphomas, accounting the most common lymphoma types compared to HL in Malaysia (2,7).

Clinically, lymphoma patients typically have enlarged superficial lymph nodes (cervical, axillary, or inguinal lymph nodes), mediastinal nodes, or retroperitoneal

nodes (3,8). However, patients present with extra nodal mass, most commonly involving gastrointestinal (GI) tract, skin, bone marrow, sinuses, thyroid, or central nervous system (CNS) (3,9). The liver and spleen may also be enlarged because of the mass effect by the lymphoma. The other organs involvement is infrequent. “B symptoms” (fever, weight loss, night sweats) and other constitutional symptoms include weakness, fatigue, anorexia, and cachexia can be observed during presentation of the disease (1,3,8).

1.2 Overview of JAK/STAT pathway

The pathogenesis of lymphoma is relatively complexed that may involve a variety of gene mutations in multistep origin of the cancer development. Mutations that increase in tyrosine kinase activity is one of the common aberrations that drive cell growth (1). Tyrosine kinase is an enzyme that mediates intracellular signalling by phosphorylates proteins on tyrosine residues to activate Janus Associated Kinase-Signal Transducer and Activator of Transcription (JAK-STAT) signalling pathway (8). Despite the understanding of tyrosine kinase activity that become the targets of many effective new drugs in haematolymphoid malignancies, the evolution of drugs targeting JAK-STAT intracellular signalling pathway become more recognized as an adjunct to the current available treatment. JAK-STAT signalling more well-known in haematological malignancies, currently, more studies have been explored regarding its role in the pathogenesis of lymphoma and solid tumours (10).

Four recognizable Janus- associated kinase (JAK) families are JAK-1, JAK-2, JAK-3 and TYK-2 proteins, while signal transducer and activator of transcription (STAT) families consist of seven major proteins, which are STAT-1, STAT-2, STAT-3, STAT-4, STAT-5a, STAT-5b and STAT-6 (11,12).

The JAK-STAT pathway is an intracellular signalling for gene expression mediated by cytokines in the form of interferon, interleukin, and growth factors (13). The cytokine receptors receive extracellular signals once the cytokine molecules bind on them, resulting in their aggregation. The JAK protein, which associated with cytokine receptors intracellularly will phosphorylates and causes consequent activation of the tyrosine kinase domain providing docking sites for STAT protein (13). Recruited STAT monomers by JAK-mediated phosphorylation resulting in their dimerization and translocation from the cell cytoplasm across the nuclear membrane to the cell nucleus. STAT dimers enhanced transcription of specific genes in the nucleus (13). Changes in gene expression, cell proliferation, cell differentiation and prevention of apoptosis occur as a result of this intracellular complex cascade of biochemical events of JAK-STAT pathway (11–14).

The JAK-STAT pathway plays a vital role in cell proliferation, differentiation and prevention of apoptosis (11–13,15). The development of some cancers as a result of genetic mutations, amplifications, or polymorphisms causing aberrant activation of the JAK-STAT pathway and lead to continuous activation of the JAK-STAT pathway (13). Other mechanism that leads to continuous activation of JAK-STAT signalling pathway includes extracellular signals by excessive cytokines expression such as

interleukin-6 (IL-6) upregulation by tumour cells or tumour microenvironment (16–18). Therefore, the role of aberrant activation of JAK-STAT signalling contribute to carcinogenesis, either as a tumour intrinsic driver of cancer growth/metastasis, or as a modulator of immune surveillance (16).

1.3 STAT-3 and lymphoma

In numerous human malignancies, phosphorylated tyrosine kinase and STAT nuclear expression have been found, in which, both are signs of JAK-STAT pathway activation (13). JAK-1 is known to be main activator for STAT-3 activity (19), and excessive STAT-3 activation is common in lymphoma (17). In fact, aberrant STAT-3 signalling plays important role in immune evasion and become an oncogenic driver in several types of B-cell lymphoma and most of T-cell lymphomas (17). It may also present in other several solid tumours such as medulloblastoma, bladder, breast, colon and cervical cancers (16).

In healthy tissues, STAT-3 activation lasts just a short time and is tightly controlled. However, STAT-3 is aberrantly activated in cancer tissues and is triggered by certain cytokines (13). The expression of downstream proteins linked to the initiation, growth, and metastasis of malignancies is regulated by the ongoing activation of STAT-3 (13).

Previous study showed STAT-3 is expressed in B cell NHL, estimated about 25.7% of cases involving Diffuse Large B Cell Lymphoma (DLBCL) (20). It is more

frequently expressed in DLBCL non-Germinal Center B cell (DLBCL-NGCB) subtypes than that in Germinal Center B-cell (GCB) subtype, and its strong expression is correlated with poor overall survival (OS) in DLBCL (20). Other studies pertaining to DLBCL, STAT-3 protein expressions were accounted in 16-46% of cases (21,22). Among the DLBCL subtypes, 83% of DLBCL-NGCB subtype was associated with STAT-3 expression (23). In the DLBCL-NGCB subtype, STAT-3 is known to be activated by IL-6/IL-10 via JAK-1 to promote cancer cell survival (24). Previously, no significant correlation seen in between STAT-3 expression and clinicopathological parameters such as gender, age, B symptoms, lactate dehydrogenase (LDH) levels, erythrocyte sedimentation rate (ESR), stage, tumour size, subtype, and treatment (20). However, in the most recent studies, STAT-3 activity is also associated with advanced stage (stage III/IV), multiple extra-nodal sites involvement, advanced age of the patient, presence of B symptoms (presence of fever, weight loss, night seating), bone marrow involvement, bulky lymph nodes and a poor prognosis in DLBCL (21,22,24). There is lack of previous studies pertaining to the STAT-3 expression in other B cell NHL subtypes. In a small populational research done by Oghami et. al found out that follicular lymphoma (FL), small lymphocytic lymphoma (SLL), Burkitt lymphomas and Marginal Zone Lymphomas (MZL) showed STAT-3 expression in 38%, 28.5%, 20% and 20% of cases, respectively (25). In contrast, there was lacked any evidence of STAT-3 overexpression in primary mediastinal large B-cell lymphoma (PMBL) (25).

Among T/ natural killer (NK) cells NHL, activating mutations of STAT-3 had been shown to lead to constitutive activation of the JAK-STAT pathway in Anaplastic

Lymphoma Kinase Negative Anaplastic Large Cell Lymphoma (ALK-negative ALCL) (3). A few studies showed convergent activating mutations of *JAK-1* and/or *STAT-3* genes were present in approximately 20% of ALK-negative ALCL. Approximately 7-38% of ALK-negative ALCL harboured double mutations of *STAT-3* and *JAK-1* (26,27). Others T/NK cells NHL subtypes related to STAT-3 are Extranodal NK/T-cell lymphoma, Cutaneous T Cell Lymphoma including Sézary syndrome, Intestinal T-cell lymphoma and breast implant—associated ALCL (3,28).

In short, STAT-3 expression in various type of NHL may reflects the aberrant JAK-STAT signalling pathway. Targeting JAK-STAT3 signalling inhibits tumour growth and enhances antitumor immune responses (17). In addition, many researchers agreed that *STAT-3* function as an oncogene, and by inhibiting *STAT-3* activity is an effective method for treating cancer in view of aberrant *STAT-3* activation has been linked to cancer hallmarks as well as related to poor patient outcomes. Based on the outcomes of clinical trials, several STAT-3 inhibitors are well tolerated and may increase the overall survival of cancer patients including lymphoma cases (18). Therefore, aiming for JAK-STAT pathway in lymphoma have potential clinical value and promising therapeutic target in lymphoma (17,29). Thus, in line with the emerging therapeutic intervention targeting JAK-STAT intracellular signalling pathway, focusing on STAT-3 expression in this study among vast diversity of NHL types may provide local data and even additional information in evolving therapeutic intervention and prognostic information in lymphoma in the future.

CHAPTER 2: OBJECTIVES

2.1 General Objectives

To evaluate the expression of STAT-3 protein in non-Hodgkin lymphoma in HUSM and its association with the clinicopathological parameters.

2.2 Specific Objectives

1. To determine the prevalence of STAT-3 protein expression in non-Hodgkin lymphoma cases.

2. To determine the association between STAT-3 protein expression with clinicopathological parameters in non-Hodgkin lymphoma in HUSM.

CHAPTER 3: MANUSCRIPT

3.1 Title:

Evaluation of Signal Transducer and Activator of Transcription 3 (STAT-3) Protein Expression in non-Hodgkin Lymphoma Cases in Hospital USM.

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3.2 Abstract

Background: Evolving targeted therapy on Janus Associated Kinase-Signal Transducer and Activator of Transcription (JAK-STAT) signalling pathway especially pertaining to STAT-3 protein in non-Hodgkin lymphoma (NHL), provide new treatment strategies. STAT-3 protein also related to prognostication of NHL. Hence, we aimed to evaluate the expression of STAT-3 protein in NHL cases, diagnosed in Hospital Universiti Sains Malaysia. **Methods:** A retrospective cross sectional study using formalin fixed paraffin embedded tissue blocks of 95 NHL cases were obtained. The association between clinicopathological characteristics and STAT-3 protein expression was analysed using the Pearson chi-square and Fisher's exact test. A p-value of < 0.05 is considered statistically significant. **Results:** Majority of the cases (78.9 %) have positive STAT-3 protein expression. 64.2 % were among aggressive B cell NHL whilst 20.0 % of them are Diffuse Large B Cell Lymphoma, non-germinal centre B subtype (DLBCL-NGCB). There is an association between STAT-3 protein expression with DLBCL subtypes ($p = 0.046$). **Conclusion:** STAT-3 protein was significantly associated with DLBCL, one of the aggressive B cell lymphoma subtypes. It could be one of the possible markers to prognosis in NHL. However, a more extensive study with a combination of JAK protein needs to be done in the future to elucidate the mechanism of the JAK-STAT pathway in NHL and its associated role.

Key words: Lymphoma; Non-Hodgkin lymphoma; Diffuse Large B Cell Lymphoma; STAT-3 protein.

3.3 Introduction

The pathogenesis of lymphoma is relatively complexed that may involve a variety of gene mutations in multistep origin of this cancer development. Mutations that increase in tyrosine kinase activity is one of the common aberrations that drive cell growth [1]. Tyrosine kinase is an enzyme that mediates intracellular signalling by phosphorylates proteins on tyrosine residues to activate Janus Associated Kinase-Signal Transducer and Activator of Transcription (JAK-STAT) signalling pathway [2]. Despite the understanding of tyrosine kinase activity that become the targets of many effective new drugs in haematolymphoid malignancies, the evolution of drugs targeting JAK-STAT intracellular signalling pathway become more recognized as an adjunct to the current available treatment. Although JAK-STAT signalling more well-known in haematological malignancies, currently, more studies have been explored regarding its role in the pathogenesis of solid tumour including lymphoma [3].

Four recognizable Janus- associated kinase (JAK) families are JAK-1, JAK-2, JAK-3 and TYK-2 proteins, while signal transducer and activator of transcription (STAT) families consist of seven major proteins, which are STAT-1, STAT-2, STAT-3, STAT-4, STAT-5a, STAT-5b and STAT-6 [4].

The JAK-STAT pathway is an intracellular signalling for gene expression mediated by cytokines in the form of interferon, interleukin, and growth factors [5]. The cytokine receptors receive extracellular signals once the cytokine molecules bind on them, resulting in their aggregation. The JAK protein, which associated with

cytokine receptors intracellularly will phosphorylates and causes consequent activation of the tyrosine kinase domain providing docking sites for STAT protein [5]. Recruited STAT monomers by JAK-mediated phosphorylation resulting in their dimerization and translocation from the cell cytoplasm across the nuclear membrane to the cell nucleus. STAT dimers enhanced transcription of specific genes in the nucleus [5]. Changes in gene expression, cell proliferation and differentiation with prevention of apoptosis occur as a result of this intracellular complex cascade of biochemical events of JAK-STAT pathway [4–6]. The development of some cancers as a result of genetic mutations, amplifications, or polymorphisms causing aberrant activation of the JAK-STAT pathway and lead to its continuous activation [5]. Other mechanism that leads to continuous activation of JAK-STAT signalling pathway includes extracellular signals by excessive cytokines expression such as interleukin-6 upregulation by tumour cells or tumour microenvironment [7–9]. Therefore, the role of aberrant activation of JAK-STAT signalling contribute to carcinogenesis, either as a tumour intrinsic driver of cancer growth/metastasis, or as a modulator of immune surveillance [7].

In numerous human malignancies, phosphorylated tyrosine kinase and STAT nuclear expression have been found, in which, both are signs of JAK-STAT pathway activation [5]. JAK-1 is known to be main activator for STAT-3 activity, and excessive STAT-3 activation is common in lymphoma [8,10]. In fact, aberrant STAT-3 signalling plays an important role in immune evasion and become an oncogenic driver in several types of B-cell lymphoma and most of T-cell lymphomas [8]. Targeting JAK-STAT3 signalling inhibits tumour growth and enhances anti-tumour

immune responses [8]. In addition, many researchers agreed that STAT-3 function as an oncogene, and by inhibiting STAT-3 activity is an effective method for treating cancer in view of aberrant STAT-3 activation has been linked to cancer hallmarks as well as related to poor patient outcomes. Based on the outcomes of clinical trials, several STAT-3 inhibitors are well tolerated and may increase the overall survival of cancer patients including lymphoma cases [9]. Therefore, aiming for JAK-STAT pathway in lymphoma have potential clinical value and promising therapeutic target in lymphoma [8,11]. Thus, in line with the emerging therapeutic intervention targeting JAK-STAT intracellular signalling pathway, focusing on STAT-3 expression in this study among vast diversity of NHL types may provide local data and even additional information in evolving therapeutic intervention and prognostic information in lymphoma in the future. This study aims to evaluate the expressions of STAT-3 protein and to determine its prevalence as well as associated clinicopathologic characteristics in NHL cases in Hospital Universiti Sains Malaysia (HUSM).

3.4 Materials and Methods

This retrospective cross-sectional study was carried out in the Department of Pathology, Hospital USM from April 2021 until June 2023. The sampling period was between January 2015 and December 2020. The study protocol was approved by Human Research Ethics Committees Universiti Sains Malaysia (USM), USM/JEPeM/21030209.

The Power and Sample Size Calculation version 3.1.2 computer application was used to determine the sample size. The confidence interval, absolute precision error and power test were set at 95%, 0.05 and 0.8, respectively. An additional 10% for incomplete data was supplemented. The study's anticipated sample size was 155 NHL cases. The cases were retrieved from the computerized registry data (LIS and PATHORS) of the Department of Pathology, Hospital USM and via the medical record unit. However, only total of 95 NHL cases were selected in this study, that fulfil the inclusion and exclusion criteria. Inclusion criteria included formalin fixed paraffin embedded (FFPE) tissue blocks that have adequate tumoral tissue from both nodal and extra nodal site, and complete clinical information from patients' record. Referral cases from other centres were excluded from this study.

Ninety-five (95) selected FFPE-tissue blocks were sectioned at 3µm thickness. The STAT-3 primary antibody; Recombinant rabbit monoclonal [EPR787Y] from ABCAM was prediluted at 1:200 dilution. Heat mediated antigen retrieval was done using detection kit K8012 (DAKO) with high pH (pH9). Subsequently, all slides were placed in manual Stainer and incubated overnight at 4^{o}C. Then, endogenous peroxidase blocking, incubation with secondary antibody and application of substrate chromogen and haematoxylin counterstaining in sequence was applied. All samples were examined using an Olympus CX31 microscope at 400x magnification, 10 high power field (HPF). STAT-3 staining can be seen in both nuclear and cytoplasm. In this study which involved lymphoma cases, only those with nuclear staining were considered to be positive (Figure 1) [12]. The scoring method (Table 1) was utilised [12].

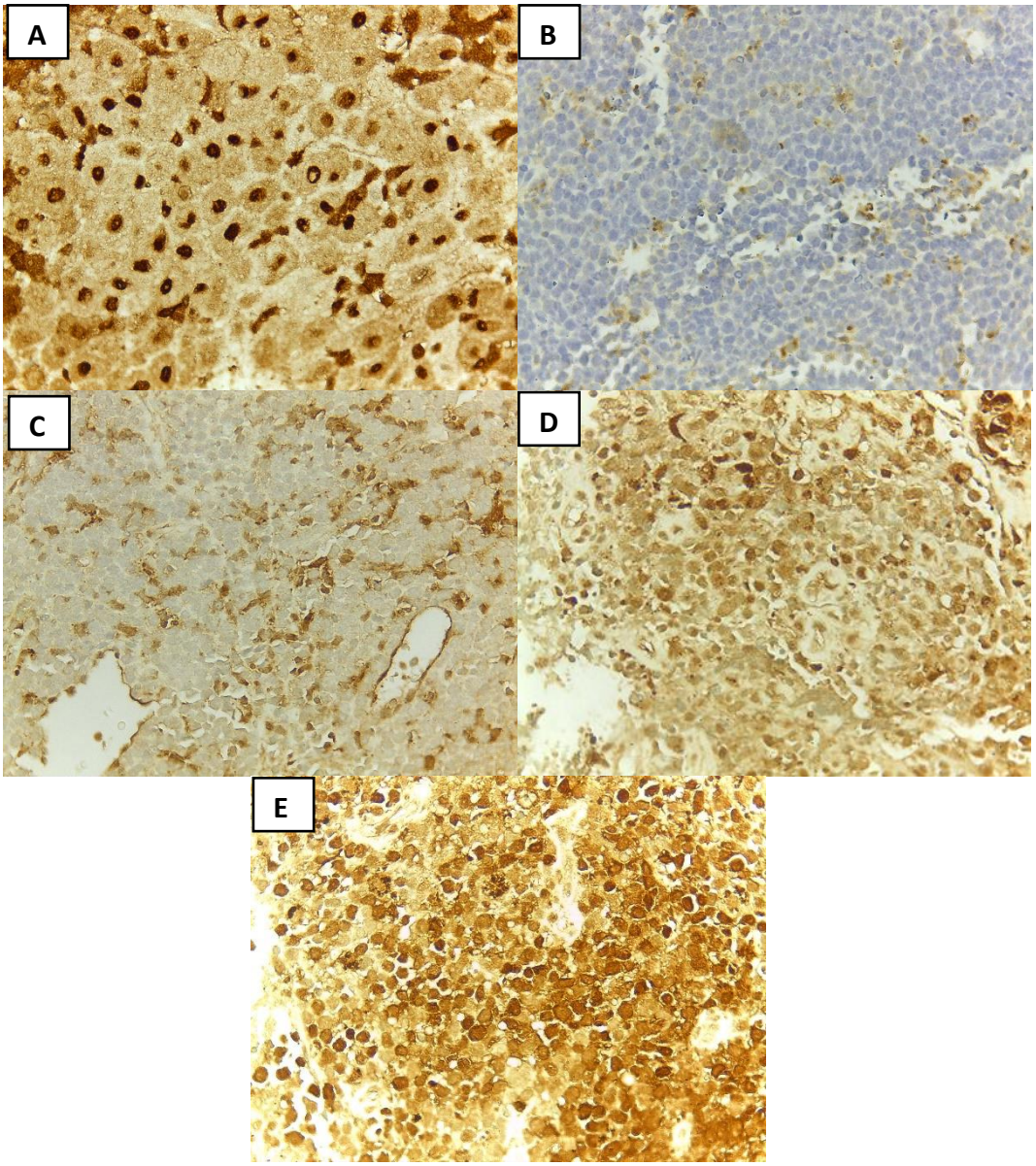

Figure 1: Expression of STAT-3 immunostaining (A) Positive control; nuclear staining in decidual placenta. (B) Negative nuclear staining. (C) Weak nuclear staining (D) Moderate nuclear staining and (E) Strong nuclear staining (400X magnification).

Table 1: Scoring system for STAT-3 stain [12].

Score	Extent of markers expression	Intensity of positive score
0	No positive cells/HPF	Nil
1	< 10% positive cells/HPF	Weak
2	10-30% positive cells/HPF	Moderate
3	> 30% positive cells/HPF	Strong

Total score = Intensity X Extent
(Values: 0 to 9, $\geq$ 3 is high expression; <3 is low expression; 0 is negative)
HPF: high power field

In view of small sample size, we adopted the above scoring system but re-classify the STAT-3 protein expression into 2 groups only: negative (for total score 0) and positive (weak positive for total score 1-2; strong positive for total score $\geq$ 3).

Age, ethnicity, gender, the localization and size of tumours, B symptoms (fever, weight loss, night sweats), lactate dehydrogenase (LDH) level, and disease stage were included as clinicopathologic features in this study. Tumour histopathological characteristics were classified based on WHO classification of Tumours of Haematopoietic and Lymphoid tissues, revised 4th edition. The NHL categories are subdivided based on respective cell lineages; either B cell, T cell or Natural Killer (NK) cell types [13]. Data was analysed using Statistical Package for Social Sciences (SPSS) version 27.0 software. Continuous data was expressed by mean and medium while descriptive and categorical data were expressed by proportion; Pearson chi-square and Fisher-exact test with appropriate application. $p < 0.05$ were considered statistically significant.

3.5 Results

A total of 95 cases with NHL of various subtypes were selected, and the clinicopathologic characteristics are shown in tables 2 and 3. The mean age of patient was 54 years old. The ethnic was mainly among Malay (90.5%) and male in gender (65.3%). Most of the patients had extra nodal sites involvement (49.5%), size of tumour less than 10 cm (63.2%), presence of B symptoms (56.8%), high LDH level (67.4%) and advanced stage (III-IV) of disease (70.5%) during first presentation. Many of them were diagnosed as aggressive B cell NHL (77.9%), largely contributed by Diffuse large B cell lymphoma (DLBCL subtypes (64.3% in total) and specifically DLBCL-NGCB subtypes (21.1%).

Table 2: Demographic and clinicopathological characteristics of patients (n = 95)

Clinicopathological parameters		Mean/median	n	(%)
Age group (years)	≤ 60	54*	51	(53.7)
	> 60		44	(46.3)
Ethnic group	Malay		86	(90.5)
	Non-Malay		**9**	**(9.5)**
	- Chinese		7	(7.4)
	- Indian		1	(1.1)
	- Others		1	(1.1)
Gender	Female		33	(34.7)
	Male		62	(65.3)
Site	Nodal		30	(31.6)
	Extra nodal		47	(49.5)
	Nodal and extra nodal		18	(18.9)
Tumour size (cm)	< 10	7#	60	(63.2)
	≥ 10		35	(36.8)
B symptoms	Absent		41	(43.2)
	Present		54	(56.8)
LDH level (U/L)	< 480 (Normal)	720#	31	(32.6)
	≥ 480 (Elevated)		64	(67.4)
Stage of disease	Stage I-II (Early)		28	(29.5)
	Stage III-IV (Advanced)		67	(70.5)

Mean*
Median#

Table 3: NHL histopathological subtypes (n = 95)

Histopathological type/subtypes		n	(%)
Type	T/Natural Killer cells	7	(7.4)
	B cells	88	(92.6)
Subtypes	**Indolent B cell**	**14**	**(14.7)**
	Follicular lymphoma	4	(4.2)
	Mantle cell lymphoma	3	(3.2)
	Extra nodal Marginal Zone Lymphoma	3	(3.2)
	Lymphoplasmacytic lymphoma	1	(1.1)
	Primary cutaneous follicular centre lymphoma	1	(1.1)
	SLL	2	(2.1)
	Aggressive B cell	**74**	**(77.9)**
	1. DLBCL group	**61**	**(64.3)**
	- DLBCL-NGCB	20	(21.1)
	- DLBCL-GCB	15	(15.8)
	- DLBCL NOS	17	(17.9)
	- DLBCL double expressions	7	(7.4)
	- DLBCL triple expressions	2	(2.1)
	2. Others aggressive B cell	**13**	**(14.0)**
	- PMBL	4	(4.2)
	- Primary DLBCL of CNS	3	(3.2)
	- EBV-positive DLBCL, NOS	1	(1.1)
	- T cell/histiocyte- rich B cell lymphoma	1	(1.1)
	- B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and classic Hodgkin lymphoma	1	(1.1)
	- Plasmablastic lymphoma	1	(1.1)
	- B-lymphoblastic leukaemia/lymphoma	1	(1.1)
	- Burkitt Lymphoma	1	(1.1)
	T cell NHL	**7**	**(7.4)**
	T-lymphoblastic leukaemia/lymphoma	3	(3.2)
	ALCL, ALK-positive	3	(3.2)
	Extra nodal Natural Killer/T cell lymphoma, nasal type	1	(1.1)

Abbreviation; SLL: small lymphocytic lymphoma; DLBCL: Diffuse large B cell lymphoma; DLBCL-NGCB: Diffuse large B cell lymphoma, Non-Germinal Centre B subtypes; DLBCL-GCB: Diffuse large B cell lymphoma, Germinal Centre B subtypes; NOS: Not Otherwise Specified; CNS: Central Nervous System; PMBL: Primary Mediastinal Large B Cell Lymphoma; EBV: Epstein–Barr virus; ALCL: Anaplastic Large B Cell Lymphoma; ALK: Anaplastic Lymphoma Kinase.

Amongst these 95 NHL cases, STAT-3 protein expressions were expressed in more than two-third (78.9%) of the cases as shown in Table 4. In overall, the largest contribution of STAT-3 protein expressions was among aggressive B cell NHL (64.2%), mainly DLBL group (54.7%) whereby 20% of them were DLBCL-NGCB, followed by DLBCL NOS (15.8%) and DLBCL-GCB (11.6%).

There were no significant association between STAT-3 protein expression with clinicopathologic characteristic; age, ethnic, gender, site and size of tumours, B symptoms, LDH level and disease staging (Table 5). Generally, there were also no significant association between STAT-3 protein expression with different NHL types based on cell lineage group and specific lymphoma subtypes. We categorized B cell NHL into indolent subtype and aggressive subtype. The later was further classified into 2 large groups: DLBCL group and others aggressive B cell lymphoma group based on WHO classification [13]. Among B cell NHL subtypes, we found there was association between DLBCL subtypes with STAT-3 protein expression ($p = 0.046$) (table 6).

Table 4: The expression of STAT-3 protein in NHL histopathological subtypes (n = 95)

Histopathological type/subtypes		STAT-3 protein expression	
		Negative n (%)	Positive n (%)
B cell NHL	**Indolent B cell**	**5 (5.3)**	**9 (9.5)**
	Follicular lymphoma	2 (2.1)	2 (2.1)
	Mantle cell lymphoma	0 (0.0)	3 (3.2)
	Extra nodal Marginal Zone Lymphoma	1 (1.1)	2 (2.1)
	Lymphoplasmacytic lymphoma	1 (1.1)	0 (0.0)
	Primary cutaneous follicular centre lymphoma	0 (0.0)	1 (1.1)
	SLL	1 (1.1)	1 (1.1)
	Aggressive B cell	**13 (13.7)**	**61 (64.2)**
	1. DLBCL group	**9 (9.5)**	**52 (54.7)**
	- DLBCL-NGCB	1 (1.1)	19 (20.0)
	- DLBCL-GCB	4 (4.2)	11 (11.6)
	- DLBCL NOS	2 (2.1)	15 (15.8)
	- DLBCL double expressions	2 (2.1)	5 (5.3)
	- DLBCL triple expressions	0 (0.0)	2 (2.1)
	2. Others aggressive B cell	**4 (4.2)**	**9 (9.5)**
	- PMBL	1 (1.1)	3 (3.2)
	- Primary DLBCL of CNS	1 (1.1)	2 (2.1)
	- EBV-positive DLBCL, NOS	0 (0.0)	1 (1.1)
	- T cell/histiocyte- rich B cell lymphoma	0 (0.0)	1 (1.1)
	- B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and classic Hodgkin lymphoma	0 (0.0)	1 (1.1)
	- Plasmablastic lymphoma	1 (1.1)	0 (0.0)
	- B-lymphoblastic leukaemia/lymphoma	1 (1.1)	0 (0.0)
	- Burkitt Lymphoma	0 (0.0)	1 (1.1)
T cell NHL	**T cell**	**2 (2.1)**	**5 (5.3)**
	T-lymphoblastic leukaemia/lymphoma	0 (0.0)	3 (3.2)
	ALCL, ALK-positive	2 (2.1)	1 (1.1)
	Extra nodal Natural Killer/T cell lymphoma, nasal type	0 (0.0)	1 (1.1)
Total		**20 (21.1)**	**75 (78.9)**

Abbreviation; SLL: small lymphocytic lymphoma; DLBCL: Diffuse large B cell lymphoma; DLBCL-NGCB: Diffuse large B cell lymphoma, Non-Germinal Centre B subtypes; DLBCL-GCB: Diffuse large B cell lymphoma, Germinal Centre B subtypes; NOS: Not Otherwise Specified; CNS: Central Nervous System; PMBL: Primary Mediastinal Large B Cell Lymphoma; EBV: Epstein–Barr virus; ALCL: Anaplastic Large B Cell Lymphoma; ALK: Anaplastic Lymphoma Kinase.

Table 5: The association between clinicopathologic characteristics with STAT-3 protein expression in NHL (n = 95).

Clinicopathological parameters		STAT-3 protein expression Negative n (%)	Positive n (%)	p value
Age group (years)	≤ 60	11 (55.0)	40 (53.3)	0.894[a]
	> 60	9 (45.0)	35 (46.7)	
Ethnic group	Malay	18 (90.0)	68 (90.7)	>0.950[b]
	Non-Malay	2 (10.0)	7 (9.3)	
Gender	Female	7 (35.0)	26 (34.7)	0.978[a]
	Male	13 (65.0)	49 (65.3)	
Site	Nodal	3 (15.0)	27 (36.0)	0.196[a]
	Extra nodal	12 (60.0)	35 (46.7)	
	Nodal and extra nodal	5 (25.0)	13 (17.3)	
Tumour size (cm)	< 10	10 (50.0)	50 (66.7)	0.170[a]
	≥ 10	10 (50.0)	25 (33.3)	
B symptoms	Absent	11 (55.0)	30 (40.0)	0.229[a]
	Present	9 (45.0)	45 (60.0)	
LDH level (U/L)	< 480 (Normal)	6 (30.0)	25 (33.3)	0.778[a]
	≥ 480 (Elevated)	14 (70.0)	50 (66.7)	
Stage of disease	Stage I-II (Early)	7 (35.0)	21 (28.0)	0.542[a]
	Stage III-IV (Advanced)	13 (65.0)	54 (72.0)	

[a]Pearson Chi Square test. [b]Fisher's exact test.
LDH: Lactate dehydrogenase

Table 6: The association between NHL type/subtypes with STAT-3 protein expression (n = 95).

NHL type/subtypes		STAT-3 protein expression Negative n (%)	Positive n (%)	p value
Type	T/Natural Killer cells	2 (10.0)	5 (6.7)	0.636[b]
	B	18 (90.0)	70 (93.3)	
Subtype	Indolent B cell	5 (25.0)	9 (12.0)	0.214[b]
	Aggressive B cell	13 (65.0)	61 (81.3)	
	T/Natural Killer cells	2 (10.0)	5 (6.7)	
B cell NHL	DLBCL group	9 (50)	52 (74.3)	**0.046[a]**
	Others B cell NHL	9 (50)	18 (25.7)	

[a]Pearson Chi Square test. [b]Fisher's exact test
DLBCL: Diffuse Large B Cell Lymphoma; NHL: Non-Hodgkin lymphoma.

3.6 Discussion

This study involved 95 cases of NHL that have been diagnosed in a 5-year periods in HUSM. The mean age of patients with NHL was 53-year-old, which was almost similar with the median age (54-year-old) among Asian population as reported in previous study [14]. In comparison, the peak incidence rate in Western region, particularly in UK is at the age of 75-year-old or older [15]. Younger age presentations across Asian populations were related to regional variation, endemic infection, and environmental exposure [15]. Lower prevalence of nodal type lymphoma was also hypothesized to be the contributing factor to presentation of lymphoma in younger population in Asian comparing to Western regions [14]. This was consistent with our study, whereby only minority (31%) of patient came with nodal site tumour involvement. Although the size of tumoral size was less than 10 cm (63.2%) in most of the cases, B symptoms (56.8%) experienced by the patients were postulated to be the reason why the patient came to seek the treatment in our centre. Pyrexia of unknown origin (PUO) warranted the physician to do extensive clinical investigation include malignancy work up.

NHL is so far, consistently more common among males, Malay ethnicity and patients tend to have advanced stage of disease during the initial presentations which were also observed in our study population as reported by Ab Manan et al [16]. Since advanced stage of disease were frequently observed among our patient, therefore baseline high LDH levels of $\geq$ 480 U/L (67.4%) reflecting the high tumour burden was expected.

In our study, majority (78.9%) of NHL cases expressed STAT-3 protein. Comparing different NHL subtypes, aggressive B cell lymphomas (64.2%) predominate the STAT-3 protein expression, particularly, DLBCL subtypes (54.7%). Most NHL

subtypes had no correlation with STAT-3 expression. However, when we specifically focus on B cell NHL subtypes, there was significant association between STAT-3 protein expression with DLBCL subtypes ($p = 0.046$).

Between B cell NHL, Ohgami et. al. found the STAT-3 expression was highest among DLBCL (36%) compared to other subtypes [17]. Furthermore, several studies focusing only on DLBCL, 41% to 84% of cases showed STAT-3 expression in which high STAT-3 expression were observed in 25.7% to 46% of cases [12,18–20]. While Wu et. al. and Kwon et. al. identified 32% and 83% of specifically DLBCL-NGCB subtype expressed STAT-3 protein, respectively, our study also demonstrated DLBCL-NGCB subtype was highest (20%) among DLBCL subtypes. Among DLBCL subtypes, higher percentage of DLBCL-NGCB subtype (83%) associated with STAT-3 expression [20]. Concerning to STAT-3 expression in DLBCL, it was related to concurrent downregulation of BCL-6 and MYC that lead to STAT-3 activation especially those with DLBCL-NGCB subtype [17,20]. The agreement of current study with the previous studies as the fact that epidemiological data of DLBCL subtypes remain the major NHL subtypes throughout the world [14]. Therefore, there were a lot of interest related to DLBCL subtypes had been explored.

Apart from DLBCL, there was little difference between other cases of lymphoma subtypes that did not express STAT-3 and those that did. Interestingly, STAT-3 expressions were present in all 3 cases of mantle cell lymphomas (MCL) and 3 out of 4 primary mediastinal large B cell lymphoma (PMBL) cases. These were contradicted with the findings of previous studies of STAT-3 expression in PMBL or MCL [17]. Our research study also found that most T/Natural Killer cells NHL expressed STAT-3 apart

from Anaplastic Large cell lymphoma, Anaplastic Lymphoma Kinase-positive (ALCL, ALK-positive). The latter barely accounted for one in three ALCL cases. However, there was lack of study using IHC technique for comparison. Nevertheless, earlier molecular studies showing that none to very small proportion of ALCL ALK-positive cases had *STAT3* gene mutation [21,22]. In contrast, wide range (6% to 72.4%) of Extra nodal Natural Killer/T cell lymphoma, nasal type cases harboured *STAT3* gene mutation [8,21,23–25]. In fact, STAT-3 IHC was reliable for surrogate marker in routine samples [12,18]. There was no previous study pertaining to the expression of STAT-3 protein in T-lymphoblastic leukaemia/lymphoma for comparison.

No association has been found between STAT-3 expression and clinicopathological parameters included in this study, which agreed with studies done by Wu et. al and Kwon et. al. [18,20]. However, in the most recent studies, STAT-3 activity was also associated with advanced stage (stage III/IV), multiple extra-nodal sites involvement, advanced age of the patient, presence of B symptoms (fever, weight loss, night sweat), bone marrow involvement, bulky lymph nodes and a poor prognosis in DLBCL [12,19,26]. The studies were comparable to current study in terms of sample size, IHC technique and interpretation of the result. The difference was our study comparing association of both non-expressed (negative IHC staining) STAT-3 and expressed (positive IHC staining) STAT-3 cases rather than comparing between among weak- and strong positive STAT-3 expressions only. Therefore, there was discrepancy among 2 groups of researchers, in view of the former category of researchers were using small sample size similar with our study while the latter category of researchers was conducting in larger scale of studies.

There are several limitations to this present study. Only small sample size (95) NHL cases were qualified in this study in single institutional centre. Furthermore, the initial propose of this study was to investigate in detail the overall JAK-STAT signalling pathway that will contribute the general understandings of lymphomagenesis. However, due to time constraint, limitation of budget and difficulty of optimising JAK-2 primary antibody, only part of the initial objectives was successfully fulfilled. In future, we hope this study can be extended in a large scale with combination of JAK protein evaluation by collaborating with other institutions and companies to establish more significant findings.

3.7 Conclusions

Our study demonstrated more frequent expression of STAT-3 protein in NHL in which DLBCL subtypes are statistically significance ($p = 0.046$). These observations may give additional local data in understanding the involvement of this protein in NHLs.