IDENTIFICATION OF EPIGENETIC METHYLATION SILENCING IN DIFFUSE LARGE B CELL LYMPHOMA IN EAST COAST MALAYSIA

BY

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ABSTRACT

Diffuse large B cell lymphoma (DLBCL) is the most common type of non-Hodgkin's lymphoma. It is a high grade NHL with heterogeneity observed in the morphologic appearance, immunophenotype, molecular pathogenesis and response to treatment. Epigenetic methylation has been implicated in its pathogenesis and has become a topic of considerable interest in the past few years. In Malaysia, there is very minimal data on prevalence of gene methylation in DLBCL. Hence, this study investigated the methylation status of *p16, MGMT* and *SPOCK2*. *p16* tumor suppressor gene inhibits cyclin-dependent kinase, which results in phosphorylation of retinoblastoma and blockage of cell cycle at G1 phase. DNA repair gene *MGMT* removes and transfers alkyl adduct at O^6-guanine to a cysteine residue within the protein, thus preventing lethal cross-links. *SPOCK2*, an extracellular chondroitin and heparin sulfate proteoglycans, functions mainly in extracellular matrix for cell adhesion. *SPOCK2*, which encodes for testican 2, abolishes the inhibition of membrane-type 1-matrix metalloproteinase by other testican family which might enhance the angiogenesis. Hence, the absence of *SPOCK2* methylation in the majority of cases was hypothesized to be observed. Aberrantly methylated *p16* and *MGMT* have been linked to DLBCL, but not *SPOCK2*. In this study, the extracted genomic DNA from 88 formalin-fixed paraffin embedded tissues of DLBCL and five normal lymph nodes were subjected to bisulfite conversion followed by methylation-specific PCR (MSP) analysis for *p16, MGMT* and *SPOCK2* methylation. Next, *p16* methylation was quantified in 16 samples through pyrosequencing assay. Due to unsuccessfulness in primer design, quantitative evaluation was not able to be performed for *MGMT* and *SPOCK2*. SPSS version 12.0 was utilised to perform the statistical analysis accordingly. *p16* methylation was spotted in 65/88 (74%) samples by MSP. Pyrosequencing detected *p16* methylation in all 16 samples ranging from 18% to 81%. In 6 of the 16 samples MSP failed to identify the DNA methylation. *MGMT* methylation was detected in all 88 (100%) cases. On the other hand, methylated *SPOCK2* was found in 83 (94.3%) samples. There was a significant association between *p16* methylation status with patients aged >50 years old (p= 0.04). This study indicates the contribution of *p16* and *MGMT* methylation in DLBCL. Poor expression of *p16* is believed to trigger an inappropriate cell cycle and consequently cancer cells development. According to previous report, hypothetically, unhealthy lifestyle might affect the percentage of *MGMT* methylation in this study population. On the contrary, as only five (5.6%) cases were found to be unmethylated for *SPOCK2*, then the finding seems not to be in parallel with earlier hypothesis. These preliminary discoveries may serve as a good platform in order to gain a comprehensive overview on the epigenetics contribution in the pathogenesis of DLBCL. The results also show that pyrosequencing is a robust tool in detecting and quantifying methylation.

ملخص البحث

إن لمفاويات خلايا (B) المتوسعة الانتشار (DLBCL) هي أكثر الأنواع شيوعاً من سرطان الغدد الليمفاوية غير هودجكيني. إنها عالية الدرجة في(NHL) وعديمة التجانس، تمت ملاحظتها في الظهور المورفولوجي، النمط الظاهري المناعي، التطور الجزيئي والاستجابة للعلاج. تضمنت المثيلة الجينية في آليتها التطورية وأصبحت موضوعاً لاهتمام كبير في السنوات القليلة الماضية. في ماليزيا، هناك بيانات ضئيلة جداً على انتشار مثيلة الجين في (DLBCL). بحثت هذه الدراسة في حالة المثيلة من *p16*، *MGMT* و *SPOCK2*. يثبط جين *p16* القامع للورم المفسفر المعتمد على السايكلين، مما يؤدي إلى فسفرة الشبكية وانسداد دورة الخلية في المرحلة G1. يزيل جين إصلاح الحمض النووي *MGMT* ويحول ناتج إضافة ألكيل عند O^6-guanine إلى بقايا السيستين في البروتين، وبالتالي منع الوصلات المميتة. *SPOCK2*، وهو كوندروتن وبروتيوغليكان كبريتات الهيبارين خارج الخلية، يعمل بشكل رئيسي في نسيج خارج الخلية لالتصاق الخلايا. *SPOCK2*، الذي يشفر لـtestican 2، يلغي تثبيط membrane-type 1- matrix metalloproteinase بواسطة عائلة تيستيكان التي يمكن أن تعزز الأوعية الدموية. ومن هنا، تم افتراض غياب مثيلة *SPOCK2* في معظم الحالات التي تمت ملاحظتها. تم ربط مثيلة *p16*غير طبيعي و*MGMT* إلى (DLBCL) ، ولكن ليس *SPOCK2*. في هذه الدراسة، تم إخضاع الحمض النووي الجيني المستخرج من أنسجة ٨٨ فورمالين-بارافين ثابت المضمنة من (DLBCL) وخمسة غدد ليمفاوية طبيعية لتحويل بيسلفيت تليها تحليل مثيلة PCR (MSP) محددة لمثيلة *p16* ، *MGMT* و*SPOCK2* . بعد ذلك، تم تحديد كمية مثيلة *p16* في ١٦ من خلال فحص pyrosequencing. بسبب الإخفاق في التصميم التمهيدي، لم يكن هناك قدرة على القيام بالتقييم الكمي ل *MGMT* و*SPOCK2* . وتم استخدم إصدار SPSS ١٢.٠ لأداء التحليل الإحصائي وفقاً لذلك. رُصدت مثيلة *p16* في عينات (٧٤٪) ٨٨/٦٥ بواسطة (MSP). كشف Pyrosequencing عن مثيلة *p16* في كل العينات ال ١٦وتتراوح ما بين ١٨٪ إلى ٨١٪. وفشل (MSP) في التعرف على الحمض النووي في ٦من ال ١٦عينة. أُكتشف مثيلة *MGMT* في كل ٨٨(١٠٠%) حالة. من ناحية أخرى، تم العثور على مثيلة *SPOCK2* في ٨٣ (٩٤.٣٪) عينة. كان هناك ارتباط كبير بين حالة مثيلة *p16* مع مرضى أعمارهم أكبر من ٥٠ سنة ($p= 0.04$). تشير هذه الدراسة إلى مساهمة مثيلة *p16* و *MGMT* في DLBCL. ويُعتقد أن إنتاجاً ضعيفاً من *p16* يسبب دورة خلية غير ملائمة وبالتالي تطور الخلايا السرطانية. وفقاً لتقرير سابق، نظرياً، قد تؤثر أنماط الحياة غير الصحية على نسبة مثيلة *MGMT* في هذه الدراسة السكانية. وعلى العكس من ذلك، تم العثور على خمسة حالات فقط (٥.٦٪) غير مثيلة لـ*SPOCK2* ، و من ثم لا يبدو أن يكون الاستنتاج بالتوازي مع الفرضية السابقة. هذه الاكتشافات الأولية قد تكون بمثابة منصة جيدة من أجل الحصول على لمحة شاملة عن مساهمة علم الوراثة اللاجيني في التسبب في تطورDLBCL. تظهر النتائج أيضاً أن pyrosequencing هو أداة قوية في كشف وقياس المثيلة.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Health Science.

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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LIST OF ABBREVIATIONS

ABC	Activated B-cell like
CDK	Cyclin-dependent kinase
CNS	Central nervous system
CpG	Cytosine-phosphate-Guanine
DLBCL	Diffuse large B cell lymphoma
DNA	Deoxyribonucleic acid
ECM	Extracelullar matrix
FFPE	Formalin-fixed paraffin-embedded
GCB	Germinal center B-cell like
GEP	Gene expression profiling
HL	Hodgkin lymphoma
IHC	Immunohistochemistry
IPI	International Prognostic Index
MMP	Matrix metalloproteinase
MSP	Methylation-specific PCR
NCBI	National Center for Biotechnology Information
n.d.	No date
NHL	Non Hodgkin lymphoma
PCR	Polymerase chain reaction
PPi	Pyrophosphatase
R-CHOP	Rituximab-cyclophosphamide, doxorubicin, vincristine, prednisolone
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Lymphoma is divided into Hodgkin (HL) and non-Hodgkin lymphoma (NHL). Non Hodgkin lymphoma is one of the leading cancers among adults and among children, lymphoma has been reported as one of the commonest tumours (Ye, Coulouris, Zaretskaya, Cutcutache, Rozen & Madden, 2012). According to National Cancer Registry Report 2007, there were 776 of lymphoma cases registered in the government hospitals in Malaysia. The statistic ranked lymphoma as the sixth most common cancer among males and eighth most common cancer among females. NHL is a diverse group of lymphoproliferative neoplasms arising from B or T/NK cells (Perry, Mitrovic & Chan, 2012) and is further classified using multiparameter approach by the World Health Organization (WHO) (Mozaheb, 2012). Immunodeficiency, exposure to pesticides and solvents, radiation, various infections, blood transfusion and genetic susceptibility have been recognised as the potential underlying risk factors in the development of NHL (Jiao et al., 2012; Mozaheb, 2012). Genetic factors are known to play important roles in the NHL carcinogenesis and this involves tumour suppressor genes, oncogenes and DNA repair genes (Mozaheb, 2012).

Diffuse large B cell lymphoma which accounts for approximately 40% ofNHL cases forms the largest proportion of NHL (Hiraga et al., 2006). DLBCL is an aggressive and high-grade malignancy (De et al., 2013). DLBCL can either develop as a transformation from a low grade lymphoma namely follicular lymphoma or as a first

occurrence of lymphoma termed as *de novo*. Although, it has been reported to be the most common type of acquired immunodeficiency syndrome (AIDS)-related lymphoma (Perry et al., 2012), in a large proportion of cases the etiology is still unknown. Most importantly DLBCL is a curable illness, and if left untreated or inappropriately treated is generally fatal (Flowers, Nastoupil, Bernal-Mizrachi, Rose & Sinha, 2012). About 60% of patients are responsive to the present treatment regimen consisting of rituximab combined with cyclophosphamide, doxorubicin, vincristine and prednisolone (R-CHOP) (Chambwe et al., 2014).

In the development of human malignancies, a broad configuration of aberrant genomic changes and altered patterns of gene expression have been described. Over the past few years, epigenetic modification of nucleic acid via gene methylation has been demonstrated as an alternative causal event in the tumourigenesis of various human neoplasms (Bethge et al., 2014; Bock, n.d.). In general, DNA methylation is essentially required for governing normal B cells growth and during lymphomagenesis it is recognized to be disrupted (Jiang, Hatzi & Shaknovich, 2013). The potential underlying causes of these gene methylation disturbances are largely unexplored. Despite of that, the correlation between abnormal pattern changes in gene methylation and human cancers has become increasingly clear (Chuang & Jones, 2007) through numerous published data mainly in relation to prognostic issues. Hence, many studies in the translational research areas have emphasised and focused on the assessment of the physiological and pathological states of epigenetic methylation (Claus et al., 2012).

In Malaysia, till today, epigenetic methylation specifically in DLBCL has remained unexplored. Therefore, in the present study, the methylation status of *p16*, *MGMT* and *SPOCK2* genes associated with distinguished molecular pathway are

being investigated. Though *p16* and *MGMT* share similar functions as both genes suppress the foundation of abnormal cells replication, it is demonstrated in this study that there is no overlapping involvement of those genes in any way. Gene methylation of *p16* and *MGMT* has been demonstrated to associate with the lymphomagenesis of DLBCL, but not *SPOCK2*, to the best of knowledge.

1.2 RESEARCH OBJECTIVES

The present study aimed to achieve the following objectives:

1. To identify the epigenetic methylation status of *p16*, *MGMT* and *SPOCK2* genes in diffuse large B cell lymphoma cases by using methylation-specific PCR.
2. To quantify the gene methylation (percentage) by using pyrosequencing assay.

1.3 RESEARCH HYPOTHESIS

1. The methylation status of *p16*, *MGMT* and *SPOCK2* genes in diffuse large B cell lymphoma are able to be identified using methylation-specific PCR.
2. The presence of *p16* and *MGMT* methylation are present in the majority of DLBCL cases.
3. There is absence of *SPOCK2* methylation in the majority of DLBCL cases.
4. The gene methylation is able to be quantified using the pyrosequencing assay.

1.4 LIMITATION OF STUDY

The DLBCL specimens were limited to only available and accessible archived samples of formalin-fixed paraffin embedded tissues which may contained highly degradable genomic DNA. The samples were obtained from government hospitals of Hospital Tengku Ampuan Afzan (HTAA), Pahang and Hospital Universiti Sains Malaysia (HUSM), Kelantan, located in the East Coast of Malaysia.

CHAPTER TWO

LITERATURE REVIEW

2.1 DIFFUSE LARGE B CELL LYMPHOMA

Diffuse large B-cell lymphoma (DLBCL) is an aggressive B-cell neoplasm histologically characterized by proliferation of B-lymphoid cells with a nuclear size equal to or exceeding normal histiocyte nuclei and has a diffuse growth pattern.

2.1.1 Epidemiology

The most common type of human B-cell malignancy is diffuse large B-cell lymphoma (DLBCL) which occurs in almost all parts of the world (Hunt & Reichard, 2008) with no observation of significant difference among different ethnic and racial groups (Chan & Chan, 2011). The disease may be seen in a wide range of age group including children (Javier & Ferres, 2004). The median age is 70 years, however among Asians it is lower; approximately 54 years of age (Mozaheb, 2012).

In Malaysia, a retrospective review study conducted by Praveen, Ho, Fadilah & Sagap, (2010) has demonstrated that DLBCL was the commonest type of non-Hodgkin lymphoma. Even Mancer (1990) has observed, more than two decades ago, that the highest prevalence of non-Hodgkin lymphoma in Malaysia is characterised as diffuse cells in a retrospective study regarding the spectrum of lymphoma. The study also found that the immunophenotype proportion of B cell lymphoma was four times higher than T-cell (Mancer, 1990).

2.1.2 Clinical Features and Etiology

Generally, malignant cells of lymphomas are usually detected in the lymph nodes but 24% to 84% of NHL cases are extranodal within the gastrointestinal tract followed by the head and neck (Teh & Chong, 2011). In specific to DLBCL, about 70% of patients present with nodal while the remaining (30%) have extranodal diseases (Chan & Chan, 2011). Patients may present with single or multiple rapidly enlarging, symptomatic masses in nodal or extranodal sites (Javier & Ferres, 2004). Likewise, DLBCL was noted as the most common type of extra-nodal lymphoma (Mozaheb, 2012) occurs mostly in the stomach but involvement in other sites such as bone, kidneys and testes has also been reported (Javier & Ferres, 2004).

DLBCL is characterized as aggressive and have a high degree of heterogeneity in immunophenotype, molecular pathogenesis and response to treatment (Wei et al., 2014). Patients who have the same diagnostic evaluation can express obviously different clinical behaviours and outcomes (Hiraga et al., 2006). DLBCL is a complex cancer as it can arise *de novo* from normal lymphocytes at different stages of B cell differentiation or a result of the progression of a low grade lymphoma such as follicular lymphoma (Xie, Pittaluga & Jaffe, 2015; Mozaheb, 2012).

In most patients with DLBCL, there are no known underlying risk factors (Chan & Chan, 2011). The acquired and congenital immunodeficiency conditions are recognised as the etiology of DLBCL (Mozaheb, 2012). These cases are usually showing association with Epstein-Barr virus (Chan & Chan, 2011). In addition, there appear to be increased risk of DLBCL in association with the hepatitis C virus (HCV) and Acquired Immunodeficiency Syndrome (AIDS) (Mozaheb, 2012). Chronic antigenic stimulation has been implicated in the pathogenesis of DLBCL (Shaknovich et al., 2010).

2.1.3 Morphology

Generally, DLBCL shows a diffuse architecture of mature B cell phenotype with appearance of large cell morphology (Chan, 2010). DLBCL is categorised into different variants, subgroups and subtypes by the World Health Organization (WHO) Classification of Tumours of Haematopoietic and Lymphoid tissues (Hunt & Reichard, 2008). The common morphologic variants include centroblastic (medium to large lymphoid cells with vesicular nuclei containing fine chromatin), immunoblastic (centrally located nucleolus with an appreciable amount of basophilic cytoplasm) and anaplastic variants (large round, oval or polyglonal cells with bizarre pleomorphic nuclei) (Javier & Ferres, 2004; World Health Organization, 2008). T-cell/histiocyte-rich large B-cell lymphoma, DLBCL of the CNS, primary cutaneous DLBCL, leg-type, and intravascular large B-cell lymphoma are distinctive subtypes on specific anatomic sites of presentation (Xie et al., 2015; WHO, 2008).

2.1.4 Immunophenotype and Immunohistochemical Panels

The neoplastic cells of DLBCL express various pan-B markers such as CD19, CD20 and CD22 (Javier & Ferres, 2004). In diagnosis of DLBCL cases, CD20, a mature B-cell marker has been examined for positive test, and in cases showing negative results, other markers such as CD138, CD5 and CD10 would be tested (Hunt & Reichard, 2008). Besides that, surface or cytoplasmic immunoglobulin (IgM > IgG > IgA) can be demonstrated in 50% - 75% of cases (WHO, 2008).

Immunohistochemistry (IHC) is a standard practice and relatively inexpensive method in determination of DLBCL into cell-of-origin categories (Scott et al., 2014). Hans et al. (2004) performed the immunoperoxidase staining using CD10, BCL6 and MUM1 to divide the DLBCL into prognostically important subgroups with germinal

center B-cell-like (GCB) and activated B-cell-like (ABC). CD10 and BCL6 have been demonstrated to correlate with germinal center B-cell like (GCB)-DLBCL while MUM1 and CD138 associated with activated B-cell like (ABC)-DLBCL (Hunt & Reichard, 2008; Flowers et al., 2012; Hans et al., 2004). The use of Hans algorithm could also divide patients with DLBCL into long- and short-term survivors (WHO, 2008). BCL2 positivity and lack of GCB-DLBCL immunophenotype (Hans algorithm) have been shown consistently by many large studies as relevant poor prognostic indicators (Chan & Chan, 2011).

A recent study by Eow, Kim and Peh (2006) demonstrated that low expression of CD15 and CD30 markers and common expression of Bcl-2 protein were exhibited in DLBCL among Malaysians. The study indicated that CD15 and CD30 are useful in distinguishing classical Hodgkin lymphoma and DLBCL, and besides that, Bcl-2 protein expression in DLBCL is a marker of poor prognosis (Eow et al., 2006)

2.1.5 Molecular Subgroups

Gene expression profiling (GEP), a novel molecular technique has identified the two major subgroups of DLBCL based on its cell of origin which have different clinical outcomes and responses to treatment (Lee, Kim, Abdullah & Peh, 2010; Wang et al., 2010). The GCB subtype is derived from the germinal center B cells, while ABC subtype emerges from B cells that are arrested in their differentiation toward plasma cells (Perry et al., 2012; Shaknovich et al., 2010).

The GCB-DLBCL shows frequent gains at 12q12, whereas the ABC-DLBCL subgroup displays frequent gains of 3q, 18q21-q22 and losses of 6q21-q22 as well (WHO, 2008). Additionally, those subgroups cannot reliably be recognised by morphologic appearances as the immunoblastic and centroblastic variant with

polymorphic centroblast-like cells can be seen in both groups, and even more common in the ABC-DLBCL (WHO, 2008).

2.1.6 Genomic Aberrations in DLBCL Pathogenesis

Tumorigenesis is a complex recognition consisting of genetic and/or epigenetic abnormalities (Khor et al., 2013; Bearzatto et al., 2002) which transform the normal cells into malignant derivatives (Li, Poi & Tsai, 2011). Numerous data and findings regarding genetic alterations occuring in B-cell lymphoma have been published by many research groups from a few decades ago till present. The pathogenesis of DLBCL is complex due to involvement of at least two different pathways: a transformation pathway and a *de novo* pathway (Chan & Chan, 2011).

The rearrangement of B cells antigen receptor genes which causes chromosomal breaks can increase the chance of illegitimate recombination events that may incline to the formation of lymphoma (Chan, 2010). Rearrangement of immunoglobulin heavy- and light-chain genes has been identified in DLBCL (Chan & Chan, 2011). Somatic hypermutations in multiple genes such as *PIMI*, *MYC*, *RHOH*/*TTF* and *PAX5* are implicated in more than 50% of DLBCL cases (WHO, 2008).

The important genetic modification in the pathogenesis of cancer is dysregulated tumor suppressor genes especially those involved in the cell cycle process (Chim, Liang, Tam & Kwong, 2001; Mehrzad et al., 2014). *TP53*, an important tumour suppressor gene involved mainly in the cell cycle arrest, apoptosis and cell differentiation was previously shown to be mutated in 18% to 30% of DLBCL cases (Perry et al., 2012; Chan, 2010; Hiraga et al., 2006). A recent review by Shaknovich and Melnick (2011) revealed that inactivation of tumour suppressor p300

through BCL6 or mutations appeared to play a key role in lymphomagenesis as down regulation of p300 activity would rescue DLBCL cells from synergistic killing by BCL6 inhibitors.

Mutations of *BCL2* which encodes for BCL2 anti-apoptotic protein have also been shown to be frequently associated with DLBCL development (Perry et al., 2012). The incidence of *BCL6* rearrangement occurs in nearly 30% of the cases (Chan & Chan, 2011). Other significant genomic aberrations occurring at varying frequency in DLBCL include t(14;18), amplification of 2p16 and trisomy 3 or gain/amplification of chromosome arm 3q (Perry et al., 2012). In addition, DLBCL cases have shown preferential genetic deletion involving *SPIB*, *INK4a*/*ARF* and *PTEN* (Shaknovich et al., 2010). Pasqualucci et al. (2006) has also demonstrated *BLIMP1* alterations in DLBCL. *BLIMP1* lies on chromosome 6q21-q22 which functions as a transcriptional repressor necessary for B cell differentiation and is often deleted in B cell lymphoma (Pasqualucci et al., 2006).

2.1.7 Therapy and Outcome

Systemic chemotherapy of cyclophosphamide, adriamycin, vincristine and prednisone with combination of rituximab (R-CHOP) is currently utilised as the standard anti-cancer in patients of DLBCL (Ganjoo, Moore, Orazi, Sen, Johnson & An, 2008). The combination of chemotherapy with anti-CD20 antibody, rituximab, has resulted in an increase of 2-year overall survival from 57% to 70% (Flowers et al., 2012). Nonetheless, only about 60% of patients are cured, while the remaining proportion may have primary refractory or relapsed disease with dismal outcome (Chambwe et al., 2014).

DLBCL is described as an extremely heterogeneous malignancy in which patients may have strikingly different clinical courses and treatment response. The International Prognostic Index (IPI) is one of the most broadly adopted and is considered a reliable predictor of outcome for non-Hodgkin lymphoma cases (Hiraga et al., 2006). The purpose of IPI is to figure risk stratification and clinical predictors of DLBCL patients' survival status, based on five factors: age, Ann Arbor tumor stage, serum lactate dehydrogenase, performance status and number of involved extranodal sites in order to classify patients as low risk, low intermediate risk, high-intermediate risk and high risk (Perry et al., 2012). The revised-IPI, however, provides a better prediction of outcome with the addition of rituximab to the standard chemotherapy regime (CHOP) (Perry et al., 2012; Flowers et al., 2012). In addition, lymphocyte to monocyte ratio (LMR) has been shown to be useful in predicting the prognosis of DLBCL patients who are treated with immunochemotherapy where a low LMR at diagnosis can predict a poor prognosis of non-GC DLBCL patients (Wei et al., 2014).

2.2 EPIGENETICS

Epigenetics is defined as the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequences as cited by Bird (2002). In other word, epigenetics is a study of cellular modifications which does not involve any changes in nucleotide sequences and importantly it may be reversed, unlike genetic mutations which is irreversible (CHOO, 2011; Wang et al., 2010). In lymphomagenesis, in addition to genetic alterations, epigenetic modification is another discipline which offers alternative mechanism to cancer development (CHOO, 2011). Both epigenetics and genetic mutations are events that would lead to loss or gain of biological functions (CHOO, 2011).