Flow Cytometric Analysis of LEF1 for Distinguishing Chronic Lymphocytic Leukemia from other CD5-positive B-Cell Lymphoproliferative Disorders

การตรวจแอลอีเอฟวัน ด้วยหลักการโฟลไซโทเมทรี เพื่อแยกโรคมะเร็งเม็ดเลือดขาวลิมโฟไซต์ชนิดเรื้อรังออกจากกลุ่มโรคที่มีความผิดปกติในการเพิ่มจำนวนของบีเซลล์ที่ให้ผลซีดีห้าเป็นบวก

Sarinna Rahad (สรินนา ระหัด)* Apinya Sangnin (อภิญญา แสงนิล)**
Paisarn Boonsakan (ไพศาล บุญสะกันต์)*** Dr.Teerapong Siriboonpipattana (ดร.ธีระพงศ์ ศิริบูรณ์พิพัฒนา)***
Karan Paisooksantiwattana (การันต์ ไพสุขศานติวัฒนา)****

ABSTRACT

This study aimed to evaluate the utility of Lymphoid Enhancer Binding Factor 1 (LEF1) by flow cytometry for distinguishing Chronic Lymphocytic Leukemia (CLL) from other CD5-positive B-cell lymphoproliferative disorders (BCLPD). A total of 67 peripheral blood and bone marrow aspirate samples were included and comprised 25 typical CLL, 27 non-CLL and 15 inconclusive-immunophenotype cases. Compared to the non-CLL group, analysis of LEF1 in typical CLL yielded 100% sensitivity and 96.3% specificity. As such, flow cytometric analysis of LEF1 can be a potential tool in distinguishing CLL from other CD5-positive BLCPDs. Furthermore, among the inconclusive-immunophenotype cases with LEF1-positive, a distinct pattern was observed and overlapped with that found in the atypical immunophenotype CLL. LEF1 may play a role in this group but further investigation is warranted.

บทคัดย่อ

วัตถุประสงค์ของการศึกษานี้คือการประเมินผลการตรวจ Lymphoid Enhancer Binding Factor 1 (LEF1) ด้วยหลักการ flow cytometry ในการแยกโรค Chronic Lymphocytic Leukemia (CLL) ออกจากโรคในกลุ่ม CD5-positive B-cell lymphoproliferative disorders (BCLPD) ผลศึกษาตัวอย่างเลือดและไขกระดูกจำนวน 67 ราย ประกอบด้วย typical CLL 25 ราย กลุ่มที่ไม่ใช่ CLL 27 ราย และ inconclusive-immunophenotype 15 ราย เมื่อเปรียบเทียบกลุ่ม typical CLL และกับกลุ่มที่ไม่ใช่ CLL พบว่าการตรวจ LEF1 มีความไวร้อยละ 100 และมีความจำเพาะร้อยละ 96.3 ดังนั้นการตรวจ LEF1 ด้วยหลักการ flow cytometry อาจเป็นเครื่องมือที่ดีในการแยกโรค CLL ออกจากโรคในกลุ่ม CD5-positive BCLPD นอกจากนี้พบว่ากลุ่ม inconclusive-immunophenotype ที่ให้ผล LEF1 บวกจะมีรูปแบบที่จำเพาะและมีลักษณะเป็น atypical immunophenotype CLL ดังนั้น LEF1 อาจมีบทบาทมากขึ้นในกลุ่มนี้แต่ยังต้องมีการศึกษาเพิ่มเติมต่อไป

Keywords: Chronic Lymphocytic Leukemia (CLL), LEF1, CD5-Positive B-cell lymphoproliferative disorders
คำสำคัญ: โรคมะเร็งเม็ดเลือดขาวลิมโฟไซต์ชนิดเรื้อรัง แอลอีเอฟวัน กลุ่มโรคที่มีความผิดปกติในการเพิ่มจำนวนของบีเซลล์ที่ให้ผลซีดีห้าเป็นบวก

* *Student, Master of Science Program in Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University*
** *Staff, Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University*
*** *Assistant Professor, Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University*
**** *Lecturer, Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University*

Introduction

Chronic Lymphocytic Leukemia (CLL) is a clonal disorder of CD5-positive mature B-cells. CLL is classified as a non-Hodgkin lymphoma (NHL). The incidence of CLL is more prevalent in the Western countries than Asians (Swerdlow et al., 2017). In Thailand, NHL is the sixth most common cancer. Yet, CLL is rare (Information Technology Division Nation Cancer Institute, 2018; Intragumtornchai et al., 2018). Despite its small proportion, distinguishing CLL is of importance because its prognosis and patient management scheme are different from other diseases in the NHL group. Diagnosing CLL requires at least 5000 cells per microliter of monoclonal B-cell, in the peripheral blood, with a CLL immunophenotype. Typical CLL can be diagnosed by its specific morphologic characteristics in combination with immunophenotype by flow cytometry. That is, small-sized lymphocytes with round nucleus, dense chromatin, sparse nucleoli, and scant cytoplasm. Flow cytometric analysis illustrates CLL immunophenotype as followed: monoclonal B-cell with positivity for CD5 and CD23; dim positivity for CD20 and surface immunoglobulin (sIg), and negativity for CD10 (Swerdlow et al., 2017; National Comprehensive Cancer Network [NCCN], 2018a).

Nonetheless, it is found that 17% of CLL do not follow this pattern of expression, making the diagnosis of CLL difficult (Morice et al., 2008). That is, CD23 can be negative or dimly positive while CD20 and sIg can be brightly positive. The CLL cases with these unusual patterns are collectively called atypical CLL. Because of the overlapping characteristics, the use of the mentioned markers is not sufficient to differentiate CLL from other CD5-positive B-cell NHLs. To rule out such differential diagnosis of CLL as Mantle Cell Lymphoma (MCL), further investigations, namely, morphologic examination, immunohistochemistry (IHC) and Fluorescence In Situ Hybridization (FISH) are required (Swerdlow et al., 2017; Jaffe et al., 2017; NCCN, 2018a; NCCN, 2018b). However, the afore-mentioned analytical techniques suffer from such limitations as inappropriateness of the specimen type, long processing time and subjectivity. In this light, flow cytometry is a promising technique in assisting the diagnosis of CLL, of which expression is mainly found in the peripheral blood and bone marrow. By this technique, multiple cellular markers can be investigated simultaneously, rendering itself a significant role in the current diagnosis of hematologic malignancies (Stevenson, 2003; Craig et al., 2008).

Pertaining to the diagnosis of CLL, many cellular markers have been studied, including Lymphoid Enhancer Binding Factor 1 (LEF1) (Jaffe et al., 2017; El-Sewefy et al., 2014; Rahman et al., 2017; Jain et al., 2002; Menter et al., 2015; Elnenaei et al., 2001). LEF1 is a nuclear protein, functioning as one of transcription factors. Previous studies about the role of LEF1 were performed but solely by the IHC method. To date, few flow cytometric LEF1 analyses in CLL have been carried out (Amador-Ortiz et al., 2015; Menter et al., 2015; Tandon et al., 2011). Because of this, this study aims to evaluate the utility of LEF1 by flow cytometry for distinguishing CLL from other CD5-positive B-cell Lymphoproliferative disorders (BCLPD). It is expected that LEF1 will be a potential marker for CLL diagnosis.

Objectives of the study

To evaluate the utility of LEF1 by flow cytometry for distinguishing CLL from other CD5-positive B-cell lymphoproliferative disorders (BCLPD)

Methodology

Ethics

Research ethics has been reviewed and approved by the Committee on Human Rights Related to Research Involving Human Subjects, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, based on the Declaration of Helsinki (No MURA2018/112).

Sample selection

The material used in this study was leftover specimens, which were sent to the Flow Cytometry Laboratory, Division of Hematology, Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, for NHL investigation between April and November 2018. Types of specimen included peripheral blood and bone marrow aspirate, expressing the phenotype of CD5-positive BCLPD. For peripheral blood, the required clonal B cells was at least 5,000 cells per microliter. For the specimen that was bone marrow, the number of lymphocytes required was at least 30% of the nucleated cells.

Tools for CLL diagnosis in this study

Regarding diagnostic strategy employed in this study, among CD5-positive BCLPDs, the diagnosis of typical CLL, Inconclusive immunophenotype and non-CLL was made according to WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues 2017 (following algorithm; Figure1).

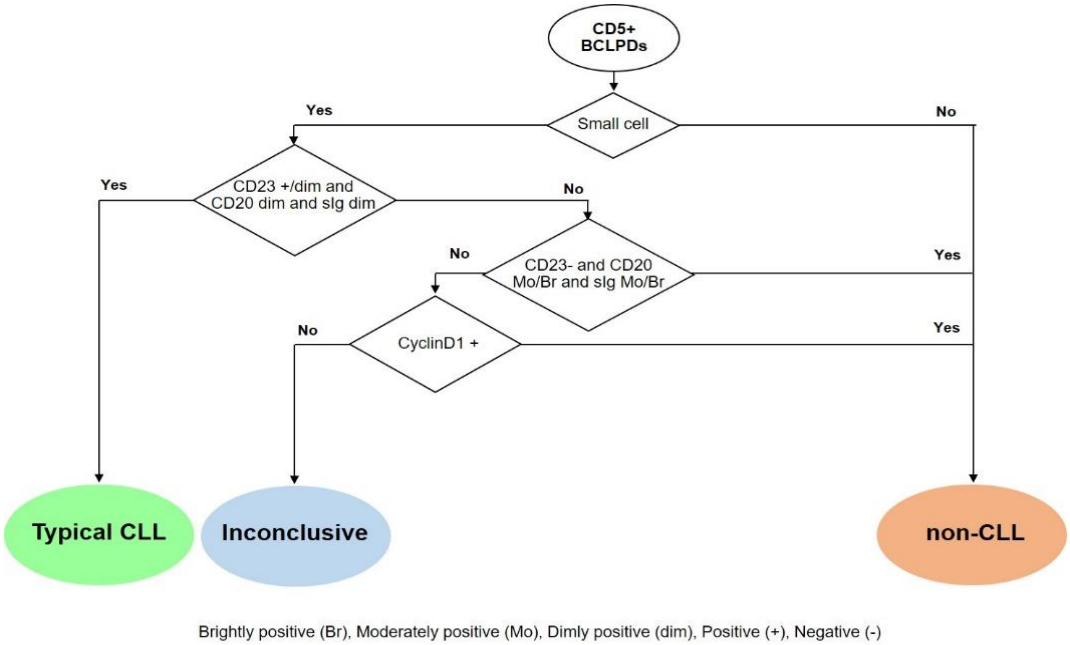

Figure 1 Diagnostic strategy

Flow cytometric analysis of LEF1

Flow cytometry analysis was performed using a BD LSR Fortessa cell analyzer (Becton Dickinson Bioscience, SanJose, CA). Samples were stained with BV605 mouse anti-human CD5 (UCHT2; Becton Dickinson Bioscience, SanJose, CA) and BV421 mouse anti-human CD19 (HIB19; Becton Dickinson Bioscience, SanJose, CA).

The test (case) was further stained with Alexa Fluor®647 rabbit anti-human LEF1 (C12A5; Cell Signaling Technology, Beverly, MA) while Alexa Fluor®647 rabbit (DA1E; Cell Signaling Technology, Beverly, MA) mAb IgG XP® Isotype Control was used for the isotype control staining.

Data of LEF1 in a CD5-positive BCLPD case was acquired by FCS Express 4 IVD edition analysis software (De Novo Software; Becton Dickinson Bioscience, SanJose, CA) with the following steps (Figure 2). The isotype control, natural killer (NK)-cell and T-cell populations were used to establish cut-off values for LEF1-positive and LEF1-negative. Further, T-cells and NK-cells were used to identify internal control populations as LEF1 is positive only in normal T-cells while absent in NK-cells.

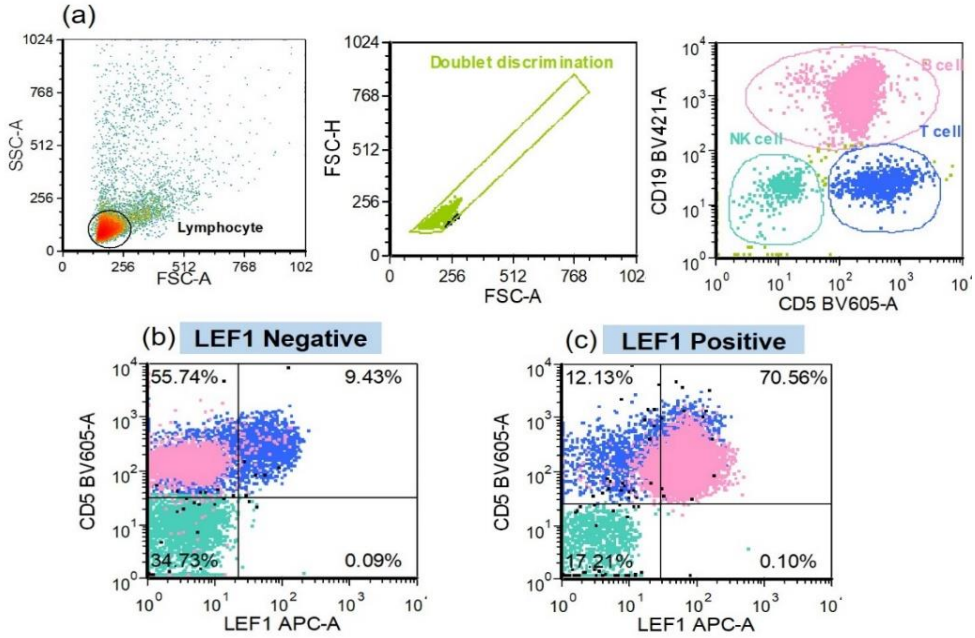

Figure 2 (a) Gating strategies used in the flow cytometric analysis of LEF1 in CD5-positive BCLPD case; (b) LEF1-negative pattern; (c) LEF1-positive pattern

Data analysis

SPSS 18.0 software program (SPSS Inc, Chicago, IL) was used for the data analysis. Normally distributed data was presented in terms of mean (standard deviation; SD) while non-normally distributed data was presented in terms of median (interquartile range; IQR). Diagnostic performance of LEF1 was analyzed by MedCalc 18.11 software program (MedCalc, Ostend, Belgium)

Results

The characteristics of the cases were shown in Table 1. The total of 67 cases comprised 25 typical CLL, 15 inconclusive immunophenotype, and 27 cases of non-CLL (Table 2). LEF1 results in typical and non-CLL cases yielded 100% for both sensitivity and negative predictive value (NPV) while specificity and positive predictive value (PPV) yielded 96.3% and 96.2%, respectively.

Table 1 Characteristics of the cases by disease groups

Characteristics		Typical CLL	Inconclusive immunophenotype	Non-CLL
Gender (*n*)	Male	14	13	20
	Female	11	2	7
Age (year)		64.8 (SD = 8.7)	61.0 (IQR =20.0)	66.5 (SD = 9.8)
Specimen types (*n*)	PB	15	8	17
	BMA	10	7	10
WBC count ($x10^3$cells/μL)	PB	31.3 (IQR = 51.1)	135.1 (SD = 133.4)	27.8 (IQR = 50.3)
	BMA	50.5 (IQR = 129.5)	81.0 (IQR = 90.0)	63.0 (IQR = 69.5)
Absolute clonal B-cell ($x10^3$cells/μL)	PB	20.8 (IQR = 38.3)	115.8 (SD = 122.7)	16.1 (IQR = 47.0)
	BMA	26.0 (IQR = 104.07)	50.4 (IQR = 38.1)	25.4 (IQR = 44.6)
sIg Kappa or Lambda restriction (*n*)	Kappa	14	10	16
	Lambda	11	5	11
LEF1 MFI		24.7 (IQR =21.5)	11.2 (IQR = 18.9)	2.5 (IQR = 0.60)

Chronic Lymphocytic Leukemia (CLL), White Blood Cells (WBC), Bone Marrow Aspirate (BMA), Peripheral Blood (PB), Surface Immunoglobulin (sIg), Lymphoid Enhancer Binding Factor 1 (LEF1), Mean (Geometric mean) Fluorescence Intensity (MFI), Standard Deviation (SD), Interquartile range (IQR)

Table 2 Distribution and expression of LEF1 in CD5-positive BCLPDs (*n* = 67)

Cases	LEF1 Expression		Total
	LEF1 Positive	LEF1 Negative	
Typical CLL (*n* = 25, 37.3%)	25	0	25
Inconclusive immunophenotype (*n* = 15, 22.4%)	8	7	15
Non-CLL (*n* = 27, 40.3%)	1	26	27

Table 3 Expression of LEF1 in inconclusive immunophenotype cases

LEF1 Expression		sIg expression	CD20 expression	CD23 expression	Cyclin D1 by IHC	Lymphocyte (small size)
LEF1 Negative (7 cases)	1	Br+	Mo/br +	Dim+	-	Yes
	2	Dim+	Mo/br +	Dim+	Inconclusive	Yes
	3	-	Dimly+	-	-	Yes
	4	Dim+	Mo/br +	-	-	Yes
	5	Mo+	Mo/br +	Dim+	-	Yes
	6	Dimly+	Mo/br +	Dim+	-	Yes
	7	Mo+	Mo/br +	Dim+	-	Yes
LEF1 Positive (8 cases)	1	Dim+	Dim+	-	-	Yes
	2	Dim+	Dim+	-	-	Yes
	3	Dim+	Dim+	-	-	Yes
	4	Dim+	Dim+	-	-	Yes
	5	Dim+	Dim+	-	-	Yes
	6	Dim+	-	-	-	Yes
	7	Dim+	Mo/br +	Dim+	-	Yes
	8	Dim+	Dim+	-	-	Yes
Total	N	15	15	15	15	15

Surface Immunoglobulin (sIg), Lymphoid Enhancer Binding Factor 1 (LEF1), Immunohistochemistry (IHC), Brightly positive (Br+), Moderately positive (Mo+), Dimly positive (dim+), Moderately to Brightly positive (Mo/br +), Negative (-)

Table 4 Comparison of previous and current studies of LEF1

Studies	Method	Sample size	Specimen type	Expression of LEF1 in cases of CLL and non-CLL		Sensitivity	Specificity
				CLL	Non-CLL		
B. Tandon et al. (2011)	IHC	290	Paraffin-embedded tissue section	CLL/SLL = 92/92 (CLL without Richter's transformation = 84/84 CLL with Richter's transformation = 8/8)	MCL (0/53) MZL (0/31) FL (6/43; FL grade3 6/12) DLBCL (27/71)	100%	83.3%
T. Menter et al. (2015)	IHC	606	Paraffin-embedded lymph node and extra-nodal lymphoid tissues	CLL = 39/56	MCL (2/17) MZL (1/102) FL (1/60) DLBCL (35/318) Transfromed DLBCL (3/14) LPL(0/4) PMBCL (1/35)	69.6%	92.2%
C. Amador-Ortiz et al. (2015)	FC	64	Peripheral blood, Bone marrow, Lymph node	CLL/SLL = 25/25	MCL (0/6) MZL (0/4) LPL (0/4) MBL (5/5) Others (0/7) No evidence of lymphoma (0/13)	100%	87.2%
T. Menter et al. (2017)	IHC	178	Bone marrow trephines , Lymph node biopsy	CLL 78/81 (CLL Richter transformation 1/1))	MCL (0/10) MZL (2/36) FL (1/38) DCBCL(1/3; DLBCL background of FL 1/1) B-ALL (2/2) T-ALL (1/1) Others (1/7)	96.3%	91.8%
This study (2018)	FC	67 (Inconclusive =15)	Peripheral blood, Bone marrow aspirate	CLL 25/25 (Typical CLL immunophenotype)	Non-CLL (1/27)	100%	96.3%

Immunohistochemistry (IHC), Flow Cytometry (FC), Chronic Lymphocytic Leukaemia (CLL), Small Lymphocytic Lymphoma (SLL) , Mantle Cell Lymphoma (MCL), Follicular Lymphoma (FL), Marginal Zone Lymphoma (MZL), Lymphoplasmacytic Lymphoma (LPL), Monoclonal B Lymphocytosis (MBL), Diffuse Large B -cell Lymphoma (DLBCL), Primary Mediastinal B Cell Lymphoma (PMBCL), Acute Lymphoblastic Leukemia (ALL)

Discussion

According to the World Health Organization classification, CLL is also known as Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma (CLL/SLL). Both are said to be the same disease entity but demonstrate different expressions. In general, CLL is expressed in the peripheral blood and bone marrow while SLL involves a lesser number of such cells in the blood and its expression is also found on other tissues, particularly the lymph node.

Presently, although CLL has no specific genetic marker, the diagnosis based on immunophenotype is of importance (Swerdlow et al., 2017).

Apparently, there has been a number of studies introducing the use of LEF1 in diagnosing CLL/SLL (Table 4), reporting that LEF1 expression, by the IHC, demonstrated approximately 69-100% sensitivity in CLL/SLL. However, by this method, only one marker can be investigated at a time. Therefore, apart from the limitation in terms of specimen for examination, interpretation of LEF1 result that was positive in multiple groups of cell was difficult. Hence, flow cytometric analysis of LEF1 can be a potential tool in the diagnosis of CLL though there are not many studies performed presently (Menter et al., 2017; Menter et al., 2015; Amador-Ortiz et al., 2015; Tandon et al., 2011).

The results demonstrated that all of typical immunophenotype CLLs were LEF1-positive and virtually all non-CLLs were LEF1-negative. This emphasized the significant role of LEF1 in assisting CLL diagnosis. From previous studies, LEF1 expression was absent in normal mature B-cells (Amador-Ortiz et al., 2015) so it can be a useful marker in Minimal Residual Disease (MRD) follow-ups after treatment in CLL patients. This study further found that immunophenotypic patterns of the inconclusive-immunophenotype cases that were LEF-positive differed from those in LEF1-negative group (Table 3). Among the fifteen inconclusive-immunophenotype cases, eight cases were LEF1-positive. Six of these cases illustrated such a repetitive immunophenotypic pattern as dimly positive sIg and CD20 and negative CD23, which overlapped with one of the characteristics of atypical immunophenotype CLL (Swerdlow et al., 2017; NCCN, 2018a). Additionally, such findings reflected that the inconclusive-immunophenotype cases shared a particular biology with the typical CLLs. Hence, LEF1 may play a role in this group, giving an opportunity for the future study to explore the gene expression profile of this group.

Importantly, for the inconclusive-immunophenotype cases that were cyclin D1-negative, FISH for translocation (11;14) detection should be performed in order to distinguish MCL from CLL according to the recommendation of the NCCN Guidelines for B-cell Lymphoma version 5.2018.

Conclusion

In conclusion, flow cytometric analysis of LEF1 yielded high sensitivity and specificity in the diagnosis of CLL. Hence, it was included in the flow cytometry panel for CLL case confirmation. In addition, this marker can be beneficial in MRD follow-ups as well as CLL treatment monitoring.

Acknowledgements

I would like to express my deep gratitude to advisor and co-advisors for their patient guidance, enthusiastic encouragement and thoughtful critiques for this research. Special thanks are given to Duangkamon Wattanatranon, M.D. and the staff of Flow Cytometry, Cytology, Histopathology and Immunohistochemistry laboratories, Faculty of Medicine, Ramathibodi Hospital for their warm support and kind assistance.

References

Amador-Ortiz C, Goolsby CL, Peterson LC, Wolniak KL, McLaughlin JL, Gao J, et al. Flow cytometric analysis of lymphoid enhancer-binding factor 1 in diagnosis of chronic lymphocytic leukemia/small lymphocytic lymphoma. Am J Clin Pathol. 2015; 143(2): 214-22.

Craig FE, Foon KA. Flow cytometric immunophenotyping for hematologic neoplasms. Blood. 2008; 111(8): 3941-67.

Elaine S. Jaffe MD DAAM, Elias Campo MD, Nancy Lee Harris MD and Leticia Quintanilla-Martinez MD. Hematopathology. 2nd ed. Philadelphia, PA: Elsevier; 2017.

Elnenaei MO, Jadayel DM, Matutes E, Morilla R, Owusu-Ankomah K, Atkinson S, et al. Cyclin D1 by flow cytometry as a useful tool in the diagnosis of B-cell malignancies. Leuk Res. 2001; 25(2): 115-23.

El-Sewefy D, Khattab D, Sallam M, Elsalakawy W. Flow cytometric evaluation of CD200 as a tool for differentiation between chronic lymphocytic leukemia and mantle cell lymphoma. The Egyptian Journal of Haematology. 2014; 39(2): 42-6.

Information Technology Division Nation Cancer Institute. Hospital-based cancer registry annual report 2016. Bankok: Pornsup Printin Co.,LTD.; 2018.

Intragumtornchai T, Bunworasate U, Wudhikarn K, Lekhakula A, Julamanee J, Chansung K, et al. Non-Hodgkin lymphoma in South East Asia: An analysis of the histopathology, clinical features, and survival from Thailand. Hematol Oncol. 2018; 36(1): 28-36.

Jain P, Giustolisi GM, Atkinson S, Elnenaei MO, Morilla R, Owusu-Ankomah K, et al. Detection of cyclin D1 in B cell lymphoproliferative disorders by flow cytometry. J Clin Pathol. 2002; 55(12): 940-5.

Menter T, Dirnhofer S, Tzankov A. LEF1: a highly specific marker for the diagnosis of chronic lymphocytic B cell leukaemia/small lymphocytic B cell lymphoma. J Clin Pathol. 2015; 68(6): 473-8.

Menter T, Trivedi P, Ahmad R, Flora R, Dirnhofer S, Tzankov A, et al. Diagnostic Utility of Lymphoid Enhancer Binding Factor 1 Immunohistochemistry in Small B-Cell Lymphomas. Am J Clin Pathol. 2017; 147(3): 292-300.

Morice WG, Kurtin PJ, Hodnefield JM, Shanafelt TD, Hoyer JD, Remstein ED, et al. Predictive value of blood and bone marrow flow cytometry in B-cell lymphoma classification: comparative analysis of flow cytometry and tissue biopsy in 252 patients. Mayo Clin Proc. 2008; 83(7): 776-85.

National Comprehensive Cancer Network. NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines®) B-Cell Lymphomas version 5.2018 [online] 2018b [cited 2018 Dec 1]. Available from: https://www.nccn.org/professionals/physician_gls/pdf/b-cell.pdf.

National Comprehensive Cancer Network. NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines®) Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma version 2.2019 [online] 2018a [cited 2018 Dec 7]. Available from: https://www.nccn.org/professionals/physician_gls/pdf/cll.pdf

Rahman K, Kumar P, Gupta R, Singh MK, Nityanand S. Role of CD200 in differential diagnosis of mature B-cell neoplasm. Int J Lab Hematol. 2017; 39(4): 384-91.

Stetler-Stevenson M. Flow cytometry in lymphoma diagnosis and prognosis: useful? Best Pract Res Clin Haematol. 2003; 16(4): 583-97.

Swerdlow SH CE, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J (Eds). WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues (Revised 4th edition). Lyon: International Agency for Research on Cancer (IARC); 2017.

Tandon B, Peterson L, Gao J, Nelson B, Ma S, Rosen S, et al. Nuclear overexpression of lymphoid-enhancer-binding factor 1 identifies chronic lymphocytic leukemia/small lymphocytic lymphoma in small B-cell lymphomas. Mod Pathol. 2011; 24(11): 1433-43.