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ในผู้ป่วยไทยที่เป็นโรคมะเร็งต่อมน้ำเหลือง

นางสาวอัจราพร โชติพืช

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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FREQUENCY OF BCL-2 REARRANGEMENT, T(14;18), IN THAI PATIENTS WITH FOLLICULAR LYMPHOMA

Miss Atjaraporn Chotipuech

สถาบันวิทยบริการ
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โรคมะเร็งต่อมน้ำเหลืองชนิดฟอลลิคูลา (Follicular lymphoma) นั้นสามารถวินิจฉัยได้โดยใช้การเปลี่ยนที่ของยีนระหว่าง ยีนบนโครโมโซมที่ 14 คือ ยีนอิมมูโนกลอบบูลิน (*IgH* gene) กับยีนบนโครโมโซมที่ 18 คือ ยีนบีซีแอล ทู (*BCL-2* gene)ซึ่งเป็นยีนที่ต่อต้านการตายของเซลล์ ซึ่งการเปลี่ยนที่ของยีนนี้มีผลทำให้เกิดการเพิ่มการแสดงออกของยีนบีซีแอล ทู มากกว่าปกติ ทำให้ไม่มีการทำลายเซลล์ที่ผิดปกติจนก่อให้เกิดการพัฒนาไปเป็นโรคมะเร็งได้ ซึ่งปรากฏการณ์ของการเกิดโรคมะเร็งต่อมน้ำเหลืองชนิดฟอลลิคูลาในประชากรแถบเอเชียนั้นพบได้น้อยกว่ากลุ่มประชากรทางยุโรปและอเมริกา ในการศึกษาครั้งนี้ได้ทำการตรวจสอบการเปลี่ยนที่ของยีนบีซีแอล ทู จากชิ้นเนื้อของผู้ป่วยไทยที่เป็นโรคมะเร็งต่อมน้ำเหลืองชนิดฟอลลิคูลาจำนวน 40 ราย โดยใช้วิธีพีซีอาร์ (PCR) และวิธีฟิช (FISH) ในการตรวจโดยวิธีพีซีอาร์นั้นได้ใช้ไพรเมอร์ 3 คู่ เพื่อตรวจหาตำแหน่งที่เกิดการเปลี่ยนที่ของยีน พบว่า มีการเปลี่ยนที่ของยีนบีซีแอล ทู ทั้งหมด 6 ราย คิดเป็นร้อยละ 15 โดยเกิดการเปลี่ยนที่ของยีนในตำแหน่งหลัก 5 ราย คิดเป็นร้อยละ 12.5 และเกิดการเปลี่ยนที่ของยีนในตำแหน่งกลาง 1 ราย คิดเป็นร้อยละ 2.5 ในขณะที่ไม่พบการเปลี่ยนที่ของยีนในตำแหน่งรองเลย ส่วนการใช้วิธีฟิชนั้น ได้ทำในชิ้นเนื้อของคนไข้ทั้งหมด 8 รายพบว่าสามารถตรวจสอบได้ 3 ราย พบมีการเปลี่ยนที่ของยีนบีซีแอล ทู 2 รายโดยที่มี 1 รายที่ไม่สามารถตรวจสอบได้โดยใช้วิธีพีซีอาร์ จากผลการศึกษาบ่งบอกได้ว่า ความถี่ของการเปลี่ยนที่ของยีนบีซีแอล ทู ในผู้ป่วยไทยที่เป็นโรคมะเร็งต่อมน้ำเหลืองชนิดฟอลลิคูลานั้นพบได้น้อยกว่าในกลุ่มผู้ป่วยทางประเทศตะวันตก ซึ่งอาจเป็นเพราะว่ากระบวนการเกิดโรคในผู้ป่วยไทยบางรายไม่ได้ขึ้นอยู่กับการเปลี่ยนที่ของยีนบีซีแอล ทู และ/หรืออาจเป็นเพราะการเปลี่ยนที่ของยีนบีซีแอล ทู นั้นมีตำแหน่งที่แตกต่างไปทำให้ไม่สามารถตรวจสอบได้โดยวิธีพีซีอาร์

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ATJARAPORN CHOTIPUECH : FREQUENCY OF BCL-2 REARRANGEMENT, T(14;18), IN THAI PATIENTS WITH FOLLICULAR LYMPHOMA.THESIS ADVISOR : ASSOC. PROF. PONLAPAT ROJNUCKARIN. THESIS COADVISOR : PROF. TANIN INTRAGAMTORNCHAI, 49 pp. ISBN 974-53-1376-9.

Follicular lymphoma is characterized by the specific chromosomal translocation t(14;18). This translocation involves the immunoglobulin heavy chain (*IgH*) gene on chromosome 14 and the B-cell lymphoma-2 *(BCL-2)* gene on chromosome 18, resulting in dysregulated expression of the anti-apoptotic *BCL-2* gene in lymphoma cells. The incidence of follicular lymphoma in Asian population is much lower than that of Western countries suggesting the differences in pathogenesis. In this study, we analyzed t(14;18) translocation in 40 paraffin-embedded tissues from Thai patients with follicular lymphoma using polymerase chain reaction (PCR) and fluorescence in situ hybridization (FISH) assays. Three primer pairs for major breakpoint region (MBR), minor cluster region (mcr) and intermediate cluster region (ICR) were used. The t(14;18) translocation was found in 6 of 40 cases (15%) by PCR; 5 (12.5%) had translocation at the MBR breakpoint and 1 (2.5%) at the ICR breakpoint, whereas translocation in mcr breakpoint was not found. Eight cases were analyzed by FISH. Three cases were successfully probed, and two of them showed positive translocation signal. One of the these two was negative for t(14;18) translocation by PCR. The results indicate that frequency of t(14;18) translocation in Thai patients with follicular lymphoma is much lower than in Western patients. Two possible explanations are 1.) *BCL-2* translocation is not absolutely required for lymphomagenesis in Thais and/or 2.) the translocation is present but at different breakpoints undetectable by PCR.

Field of study Medical Science Student's signature ....……..

Academic year 2004 Advisor's signature ....……..

Co-advisor's signature...................................……..

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LIST OF ABBREVIATIONS

BCL-2	B-cell lymphoma-2
bp	base pair
CG	cytogenetic analysis
°C	degree Celsius
dNTPs	dATP, dTTP, dGTP and dCTP
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
FISH	fluorescence in situ hybridization
FL	follicular lymphoma
icr	intermediate cluster region
IGH	immunoglobulin heavy chain
IPTG	isopropy1-β-D-thiogalactopyranoside
kb	kilobase
LB	Luria-Bertani media
LD-PCR	long-distance PCR
mcr	minor cluster region
mg	milligram
ml	milliliter
mM	millimolar
M	molar
MBR	major breakpoint region
ng	nanogram
N	normal
NHL	non-Hodgkin's lymphoma
pmol	picomole

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PCR	polymerase chain reaction
Tris-HCl	tris-(hydroxymethyl)-aminoethane
SA	southern analysis
SSC	saline-sodium citrate
μg	microgram
μl	microliter
μm	micrometer

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CHAPTER I

INTRODUCTION

1. Background and Rationale

The development of B cells in bone marrow is initiated by the assembly of genes for the variable regions of the heavy and light chains of antibodies in B-cell progenitors by a process called V(D)J recombination (Figure 1) *(1, 2)*. Antibodies consist of two identical heavy chains and two identical light chains. In this process, the DNA located between the rearranging gene elements is deleted from the chromosome. There are many different variable (V), diversity (D), and joining (J) segments in the germ line. Therefore, each B cell combines a particular set of 3 segments (V, D and J) for its heavy chain variable region and another set of 2 segments (V and J) for its light chain variable region to encode a distinct antibody different from those of other B-cells *(3)*. These distinct gene rearrangements also equip each B-cell with an individual, molecular clonal marker (an essential feature for the analysis of B-cell lymphomas). The expression of antibody as an antigen receptor on the surface is critically important for the survival and development of B-cells. During development, the cells go through an ordered program of V(D)J rearrangements, in which the only surviving cells are those that have acquired heavy and light chain variable region genes that can be translated into proteins because the correct reading frames are preserved (in frame rearrangements). Cells with out-of-frame V(D)J rearrangements, thus, without functional antigen receptor expression, die by programmed cell death (apoptosis) *(4, 5)*. In mature B-cells, the expression of an antigen receptor is also essential for cellular survival, because induced deletion of the receptor in vivo leads to rapid cell death. In bone marrow, newly formed B-cells expressing autoreactive receptors either are removed by apoptosis or edit their receptors by means of secondary V(D)J rearrangements. Once this process of receptor editing leads to the expression of an innocent B-cell receptor, B-cells leave bone marrow to become mature, naïve B-cells

(6). The modification of the specificity of the B-cell antigen receptor by genetic means is resumed in a later phase of B-cell differentiation in germinal centers in the course of T-cell-dependent immune responses.

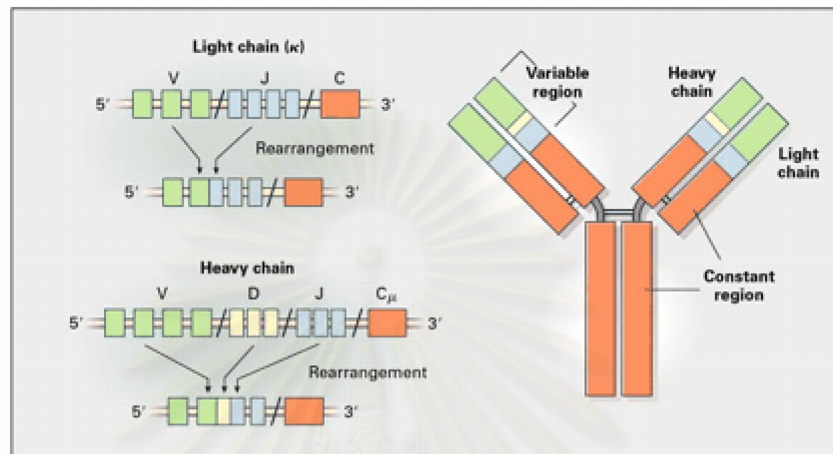

Figure 1. V(D)J recombination in B-cell development.

Naïve B-cells that recognize antigens with their membrane-bound antibody accumulate in the germinal centers of secondary lymphoid organs such as lymph nodes and spleen. The genomic DNA of the B-cells, after antigen exposure, may then be subjected to three types of modification: somatic hypermutation, immunoglobulin class switching, and receptor editing (Figure 2) *(7 - 9)*.

Somatic hypermutation is a process by which mutations (mainly single-nucleotide exchanges, but also deletions and duplications) are introduced at a high rate into variable-region genes. Some B-cell mutants in germinal centers produce antibodies with increased affinity for the immunizing antigen and are positively selected. Cells expressing favorable antibody mutants are released into the periphery as plasma cells or long-lived memory B-cells. However, cells either lose the ability to bind antigen or fail to produce a functional antibody and undergo apoptosis. Some B-cells in germinal centers switch from expression of IgM and IgD to expressing heavy chains of other classes of immunoglobulin: IgG, IgA or IgE. This results in a change in

the effector functions of the antibody but leaves the V(D)J region unaltered. Class switching is mediated by a recombination event that deletes the DNA between repeated sequences (called switch regions) located upstream of the constant region of immunoglobulin heavy chain genes. Germinal-center B-cells that acquire affinity-increasing mutations are positively selected and differentiate into antibody-producing plasma cells or memory B-cells (Figure 3).

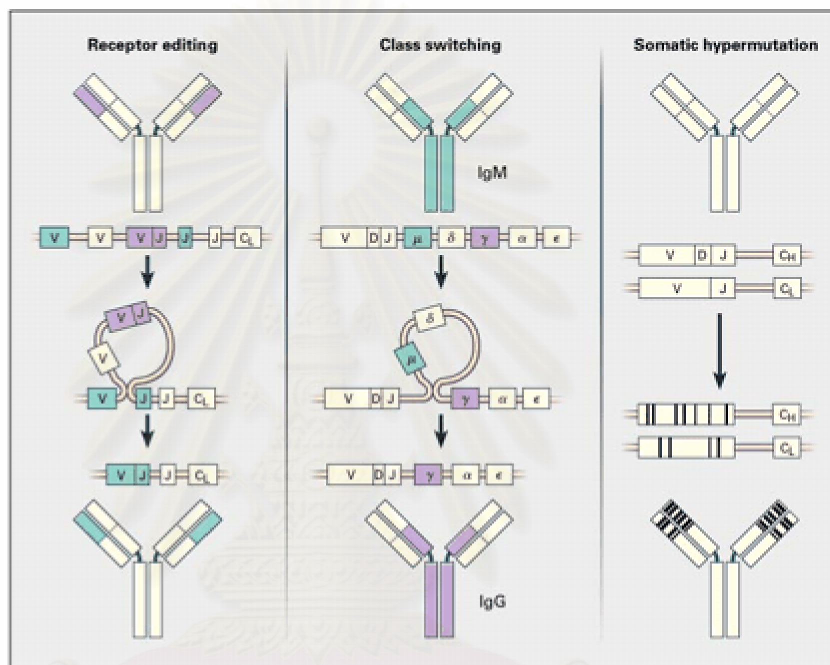

Figure 2. Molecular processes modifying the genes encoding antibody molecules.

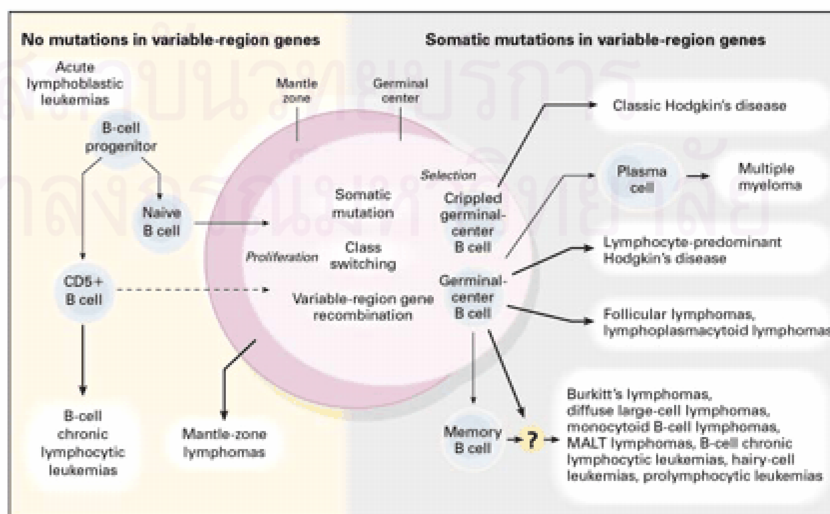

Figure3. Assignment of human B-cell lymphoma to their normal B-cell counterparts.

Lymphoma is a broad term encompassing a variety of neoplasms of the lymphatic system. The lymphatic system defends the body from foreign invasions by microbial agents such as viruses, bacteria, or fungi. The lymphatic system consists macroscopically of bone marrow, spleen, thymus gland, lymph nodes, tonsils, appendix, and a few other organs *(10).* In lymphoma, one clone of the cells in the lymphatic system multiplies uncontrollably. Lymphoma is categorized by histopathology of the tumors and the origins of cells that abnormally proliferate. (Figure 4)

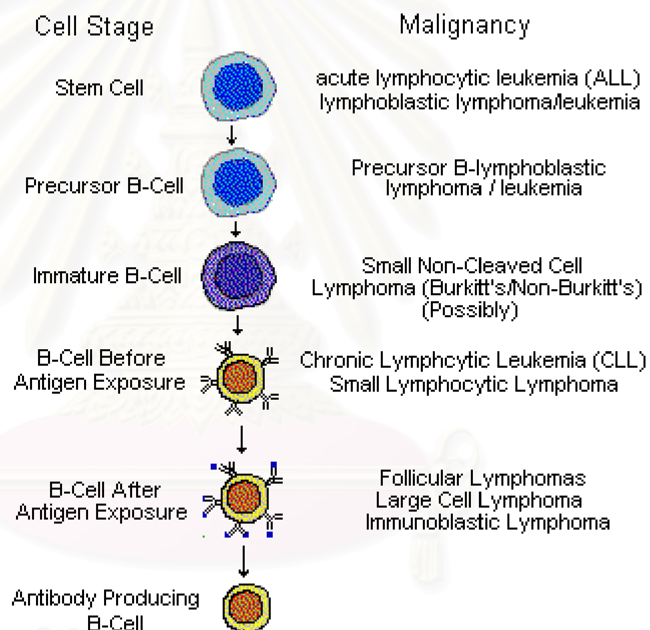

Figure 4. B cell cancers by cell development.

The two main groups of lymphoma in humans are Hodgkin's lymphomas which contain B-cell-derived Reed Sternberg cells (Figure 5) in histopathology and non-Hodgkin's lymphomas (NHL) that is derived from either B-cell or T-cell lineage but no Reed Sternberg cells. The pathogenesis of NHL is a highly complex process involving multiple genetic alterations in the tumor clone itself as well as biological

alterations in the host. Four main mechanisms of lymphomagenesis are recognized. These include accumulation of gene alterations in the tumor genome, infection of the tumor clone by an oncogenic virus, stimulation and selection of tumor cells by an antigen and immunodeficiency of the host *(11)*. The non-Hodgkin's lymphoma (NHL) comprise more than 30 types of tumors of the lymphoreticular system with distinct histomorphological and immunohistochemical appearances. However, they can be divided by their clinical courses into three major categories: low-grade, intermediate grade, and high grade lymphomas. The histological subtypes of low grade lymphomas are small lymphocytic lymphoma, follicular small cleaved cell lymphoma, and follicular mixed small cleaved and large-cell lymphoma *(12)*.

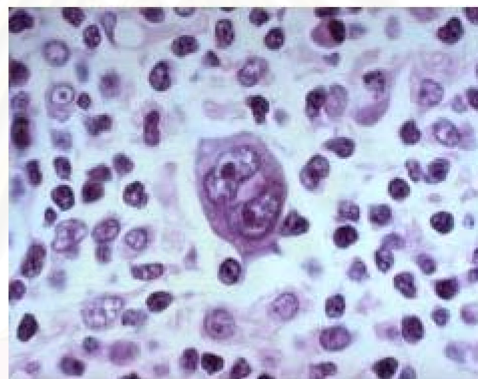

Figure 5. Reed Sternberg cells.

Chromosomal translocation represents the main mechanism of proto-oncogene activation in several type of NHL. Translocations in NHL juxtapose the proto-oncogene to the heterologous regulatory sequences derived from the partner chromosome. The heterologous regulatory regions implicated in NHL translocations are often derived from antigen receptor loci that drive high and sustained expression of these genes specifically in normal lymphoid cells *(11, 12)*. When proto-oncogenes are translocated and, thus, under control of these regulatory regions, they will be overexpressed causing lymphomas (Table 1).

Table 1. Chromosomal translocations in Non-Hodgkin lymphomas.

NHL Histologic Type	Translocation	Proto-oncogene Involved	Mechanism of Proto-oncogene Activation	Proto-oncogene Function
Lymphoplasmacytoid lymphoma	t(9;14)(p13;q32)	*PAX-5*	Transcriptional deregulation	Transcription factor regulating B-cell proliferation and differentiation
Follicular lymphoma	t(14;18)(q32;q21)	*BCL-2*	Transcriptional deregulation	Negative regulator of apoptosis
Mantle cell lymphoma	t(11;14)(q13;q32)	*BCL-1* /cyclin D1	Transcriptional deregulation	Regulator of the early phases of cell cycle
B-lineage diffuse large cell lymphoma	t(3;various)(q27;various)	*BCL-6*	Transcriptional deregulation	Transcriptional repressor implicated in formation and function of germinal centers
Burkitt lymphoma	t(8;14)(q24;q32) t(2;8)(p11;q24) t(8;22)(q24;q11)	*c-MYC*	Transcriptional deregulation	Transcription factor regulating cell proliferation, differentiation and apoptosis
T-cell anaplastic large cell lymphoma	t(2;5)(p23;q35)	*NPM/ALK*	Fusion protein	ALK is a tyrosine kinase

Translocation between chromosome 14 and 18 (Figure 6), t(14;18)(q32;q21), is found in more than 90% of follicular lymphoma, suggesting its essential role in pathogenesis. This translocation results in the juxtaposition of the immunoglobulin heavy (*IgH*) chain joining segment (J_H) on chromosome 14 to a locus designated *BCL-2* (B-cell lymphoma-2) on chromosome 18. The breakpoints on chromosome 18 commonly cluster into two groups: the major breakpoint region (MBR) are located in the middle of untranslated part of the third exon of *BCL-2* gene. It accounts for approximately 65% of the breakpoints. The minor cluster region (mcr) is located in 20 kb downstream from the gene. It accounts for 25% of the breakpoints *(13).* The effect

of this translocations is thought to be deregulation of the *BCL-2* gene by the enhancer region of the immunoglobulin heavy chain gene. This results in increased production of *BCL-2*, a mitochondrial membrane protein that inhibits programmed cell death, or apoptosis. Overexpression of *BCL-2*, in turn, causes abnormal prolongation of cell survival, leading to excessive accumulation of follicular B-cells and creating circumstances, in which further genetic events may occur *(14 - 16)*. This hypothesis is supported by the fact that t(14;18) can be found in B-cells of normal person without lymphoma, suggesting that this translocation alone is not sufficient to cause neoplasm.

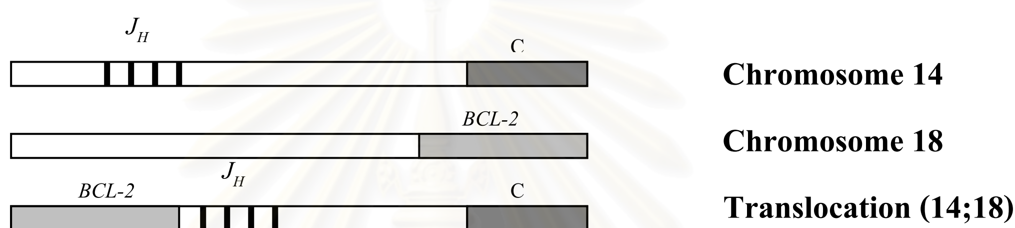

Figure 6. Translocation between chromosome 14 and 18.

The incidence of follicular lymphoma in Asia is much lower than that of Western countries. Notably, some studies found that the incidence of this translocation was significantly lower in patients with follicular lymphoma or normal populations from Asia than in patients or normal populations from Western countries *(17)*. This suggests that the pathogenesis of this lymphoma may be different in different geographic region. It is interesting to test in Thai follicular lymphoma patients for the frequency of the t(14;18) translocation at the MBR and mcr using PCR amplification for these breakpoints. This may give us deeper insights in pathogenesis of this type of lymphoma. In addition, the information of *BCL-2* breakpoints in Thai patients will be helpful in designing primers and interpreting the results of the molecular diagnosis and minimal residual disease detection in Thais.

3. Objective

To study frequency of t(14;18) chromosomal translocation in Thai patients with lymphoma

4. Hypothesis

The frequency of breakpoints of t(14;18) translocation in Thai follicular lymphoma is lower than those from Western countries.

5. Key Words

BCL-2

Follicular lymphoma

t(14;18) translocation

6. Obstacles and strategies to solve the problem

DNA fragments from paraffin block sometimes fail to be amplified. One possible reason for this is the present of inhibitors that interfere with the polymerase activity of the reaction. Therefore, DNA from paraffin blocks must be carefully extracted, are removed inhibitors by proteinase K digestion before purification using phenol chloroform and ethanol precipitation. Subsequently, samples will be appropriately diluted to lower concentrations of inhibitors, but remain amplifiable by PCR. Suitability of the sample for PCR amplification is ascertained by control amplification of the beta-globin gene. In addition, FISH study does not work in old paraffin blocks. Therefore, only specimen less than 2 years old will be used for FISH.

CHAPTER II

LITERATURE REVIEWS

The t(14;18) is commonly detected by standard cytogenetic analysis (CG) or at the molecular level by Southern analysis (SA) or the polymerase chain reaction (PCR). The use of the PCR to detect the chromosomal translocations associated with lymphoma is of particular interest to clinical laboratories because of the ability to generate rapid results with high sensitivity, less laborious and less expensive than CG and SA *(18)*. The PCR method is very sensitive technique making it possible to detect the translocation in a very small number of lymphoid/lymphoma cells in peripheral blood, bone marrow and paraffin blocks of tumors that are missed by other diagnostic methods. In this way, one can perform a quick and early diagnosis, examine the result of treatments as well as detect the remissions and the possible relapses right at the beginning *(19)*.

Yasukawa M. *et al. (20)* studied the incidence of follicular lymphoma and found a significant difference between white and Japanese individuals. The *BCL-2/*J_H translocation was detectable in peripheral blood lymphocytes by PCR from more than 50% of healthy white individuals. To clarify the reason for the difference in incidence of follicular lymphoma between whites and Japanese, the frequency of *BCL-2/*J_H translocation in peripheral blood lymphocytes of healthy Japanese individuals was compared with that of German individuals. The prevalence of *BCL-2/*J_H translocation in Japanese adults appeared to be significantly lower than that in German adults. The present data suggest that the low frequency of *BCL-2/*J_H translocation in the Japanese general population may be one of the major reasons for the difference in incidence of follicular lymphoma between whites and Japanese.

From the report in 1990, Shibata D. *et al. (21)* investigated the incidence of t(14;18)(q32;q21) chromosomal translocations detectable by a polymerase chain reaction assay on 113 American formalin-fixed, paraffin-embedded tissue biopsies

(non-Hodgkin' lymphoma, 96 cases; Hodgkin's lymphoma, 6 cases; reactive hyperplasia, 11 cases). Of the 96 non-Hodgkin' lymphoma cases, 56 had a follicular pattern and 40 had a diffuse pattern. Polymerase chain reaction amplifiable t(14;18) chromosomal translocations were detected in 23 of 43 (53%) follicular low grade lymphoma, one of eight follicular intermediate grade lymphomas, one of five follicular high-grade lymphomas, and one of ten diffuse large-cell lymphomas.

In 1992, Ladanyi M. and Wang S. *(22)* studied 30 American follicular lymphomas with the t(14;18). They found that 25 cases (83%) had a junctional fragment of *BCL-2* MBR rearrangement, demonstrable by PCR. They suggested that negative PCR results in this setting were attributable to small variations in *BCL-2* MBR breakpoint location could not be interpreted without the corresponding conventional southern blotting data. With this caveat in mind, PCR analysis for the t(14;18) remains an extremely useful technique, especially in the follow-up and monitoring for minimal residual disease in previously characterized cases of follicular lymphoma.

In 1999, Essop MF. *et al. (23)* used PCR to detect the t(14;18) translocation in 64 South African cases with follicular center cell lymphoma. DNA was purified from paraffin-embedded tissue collected from different ethnic groups namely white, black and "mixed" race patients, and primers used to detect both MBR and mcr. The overall incidence of the translocations was 45%, which is similar to that of Caucasian and Chinese patients. The ratio of rearrangements occurring at the MBR and mcr was 7:1. This ratio was three times higher for the "mixed" race group compared to whites, suggesting that there is an ethnic variation in the sites breakpoints in South African patients.

In the recent study, Montoto S. *et al. (24)* from Spain analyzed the *BCL-2/IgH* rearrangement by means of PCR technique, and correlated molecular findings with clinical characteristics and outcome. A total of 77 DNA samples were analyzed, 54 were obtained from lymph node biopsy and 23 from peripheral blood or bone marrow.

BCL-2/IgH rearrangement was assessed for both the major breakpoint region (MBR) and the minor cluster region (mcr) breakpoints by PCR technique. Thirty-nine out of sixty patients (65%) with assessable samples were found to have a *BCL-2/IgH* rearrangement in the MBR breakpoint, whereas *BCL-2/IgH* rearrangement in mcr was observed in one patient (2%) and no rearrangement at MBR or mcr in the remaining 20 patients (33%). No differences were found according to *BCL-2/IgH* rearrangement in term of complete response rate, time to treatment failure and overall survival.

Limpens J. *et al. (25)* screened blood cells from healthy blood donors of Netherland by seminested polymerase chain reaction. Each fraction of mononuclear cells, granulocytes, flow-sorted B cells and T cells was separately tested by 5-7 independently performed PCR. Six of nine individuals harbored t(14;18) breakpoints. They speculate that the translocation t(14;18) is regularly generated in bone marrow precursor B cells of healthy individuals and that t(14;18)-carrying daughter cells enter the blood stream and home to lymphoid tissues. Deregulation of *BCL-2* in these cells may enhance cell survival, thereby providing a pool of long lived, circulating, t(14;18) -carrying B cells detected in their PCR experiments. These cells may selectively accumulate additional genetic damages, because high expression of *BCL-2* can prevent apoptosis normally induced by various toxic agents. Accumulation of genetic damages in one of the multiple t(14;18)-carrying clones, probably in combination with antigen-driven proliferation within the germinal centers, may ultimately leads to the malignant phenotype of follicular lymphoma.

Further report from Liu J. *et al., (26)* investigated the ability of the PCR to detect the t(14; 18) chromosomal translocation in fixed-tissue samples. They studied 48 cases of American follicular lymphoma using DNA extracted from paired samples of fresh-frozen tissue and formalin-fixed, paraffin-embedded tissue. PCR of fresh tissue DNA revealed amplifiable products in 29 of 48 follicular lymphomas (60%). Twenty-four (83%) of these rearrangements fused the *IgH* gene with the *BCL-2* major breakpoint region (MBR), and 5 (14%) involved the minor cluster region (mcr).

However, the fixed-tissue DNA studies were positive in only 24 (83%) of 29 follicular lymphomas that were positive by PCR of fresh tissue DNA. Specifically, 20 of 25 MBR-positive samples, and 4 of the 5 mcr positive cases revealed amplifiable *BCL-2* gene rearrangement products in formalin-fixed tissue. Furthermore, they found that the extraction and precipitation steps could sometimes be avoided by attempting PCR with a diluted sample of the proteinase K extract. This dilution step probably reduces inhibitors of the PCR reaction to an inconsequential level, whereas the amount of DNA remains sufficient for amplification.

Segal MJ. *et al. (27)* tested tissue specimens from 36 Israeli follicular lymphoma patients by PCR amplification of the MBR breakpoint. Twenty-two of 36 patients (61%) tested positive. Furthermore, they searched of the literature yielding 35 papers that reported the frequency of the t(14;18) translocation in follicular lymphoma detectable by polymerase chain reaction (Table2).

Table2. Frequency of t(14:18) in follicular lymphoma by geographic region: pooled data.

Breakpoint	Far East	Europe	USA
MBR	29%	42%	55%
mcr	2%	4%	16%
total	31%	46%	71%

More recently data, using different long-range amplification methods detected some *BCL2-*J_H rearrangements 3’ from the third *BCL-2* exon between the MBR and mcr regions called the intermediate cluster region (icr) (Figure 7). These breakpoints escape the detection by conventional PCR techniques leading to a large number of false negative results. Buchonnet G. *et al. (28)* selected a group of 83 French patients with t(14;18) on cytogenetic analysis using usual probes and primers. On PCR 63% (54/83) showed MBR rearrangements, 8.4% (7/83) were mcr positive and 26.5%

(22/83) remained negative. Subsequently, they used new PCR assay in 22 negative cases to detect breakpoints located 1 to 3 kb downstream from the third *BCL-2* exon.

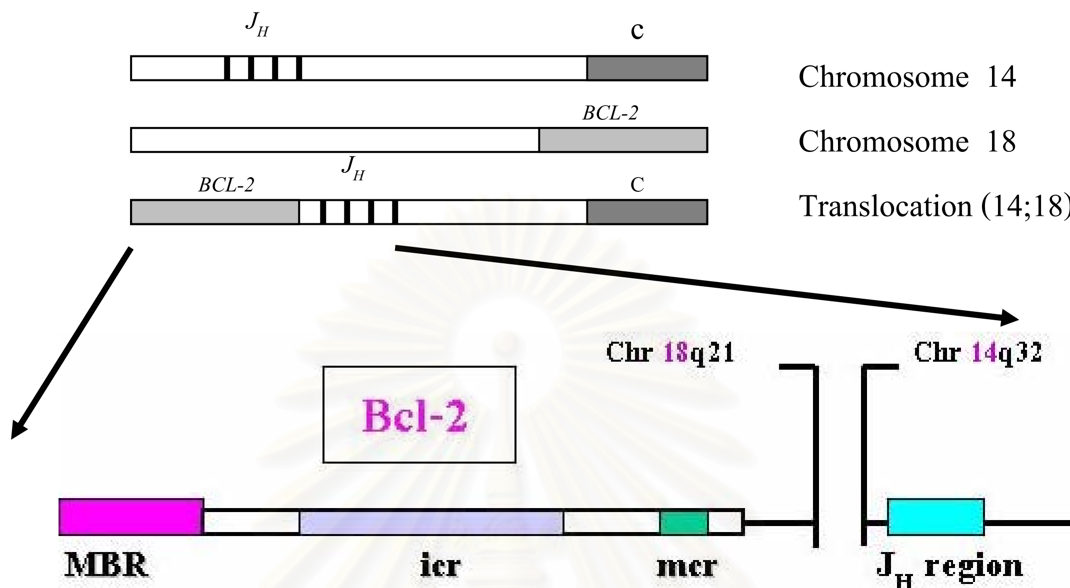

Figure 7. Translocation between chromosome 14 and 18 and breakpoints of these translocation

They found that 15.6% (13/83) are positive for icr. Suggesting that this new PCR assay is useful and efficient in detecting new breakpoints and to increase the PCR detection rate of *BCL2-*J_H rearrangements in patients with t(14;18) translocation. Albinger-Hegyi et al. *(29)* improved long-distance PCR (LD-PCR) protocols to identify breakpoints outside of MBR and mcr clusters. They designed 2 sets of new primer pairs for shorter t(14;18) target amplifications and compared with standard PCR approaches. They analyzed frozen tissues from 59 patients with follicular lymphoma for presence of a t(14;18) translocation by standard PCR with MBR/J_H and mcr/J_H primer pairs. They demonstrated a translocation in 36% of the samples (21/159) including 19 samples (32.2%) with breakpoints within the MBR cluster and 2 samples (3.4%) with involvement of the mcr locus. Because the standard PCR detect breakpoints only within the 2 cluster regions, they have performed a second screening round using a novel LD-PCR approach. They established 2 sets of LD-PCR assays that

covered the entire region between the third exon of the *BCL-2* gene and mcr allowing the detection of breakpoints outside the MBR and mcr cluster. They found that 71% (42/59) carried translocations detectable with these 2 primer sets. Furthermore, they have designed a new primer (s-icr) to supplement the widely used standard PCR with s-MBR and s-mcr primers to improved detection of t(14;18) translocation in formalin-fixed tissues. They could significantly increase the overall t(14;18) detection rate from 36 to 46%.

Many studies utilized the fluorescence in situ hybridization (FISH) method for the detection of chromosomal aberration including translocation, deletions, aneuploidy and gene amplification *(30-34)*. FISH involves the preparation of sequences of single stranded DNA, called probes, that are complementary to the target DNA sequences (Figure 8). These probes hybridize the complementary DNA because they are labeled with fluorescent tags, allow visualization of those specific sequences of DNA. There are many different types of FISH probes *(35)*. The most commonly used probes in cytogenetic analysis of hematological malignancy are: 1.) repetitive sequence centromeric probes that target tandemly repeated alpha (or beta) satellite sequences present in the heterochromatin of the chromosome centromeres. This type is useful in many types of leukemia where the chromosome morphology is poor and banding is too indistinct to tell whether the chromosome is structurally abnormal. 2.) Whole chromosome painting probes are complex mixtures of sequences from the entire length of a specific chromosome. Chromosome paints are most useful for identifying the components of highly complex rearranged chromosomes. This cannot detect small interstitial deletion, duplications or inversion. 3.) Locus-specific probes are use to detect specific sequences present in only one copy. Structural rearrangements detectable using this type of probe include translocations, inversions and specific deletions. FISH is a powerful tool for quantitative analysis of chromosomes and genes and can be applied in a variety of specimens *(36-43)* including cell cultures, isolated nuclei from fresh and fixed tissues, and histological tissue sections from paraffin

block. One of the greatest advances in cytogenetic analysis facilitated by FISH is the ability to use non-dividing cells as DNA targets, referred to as interphase FISH *(44-46)*.

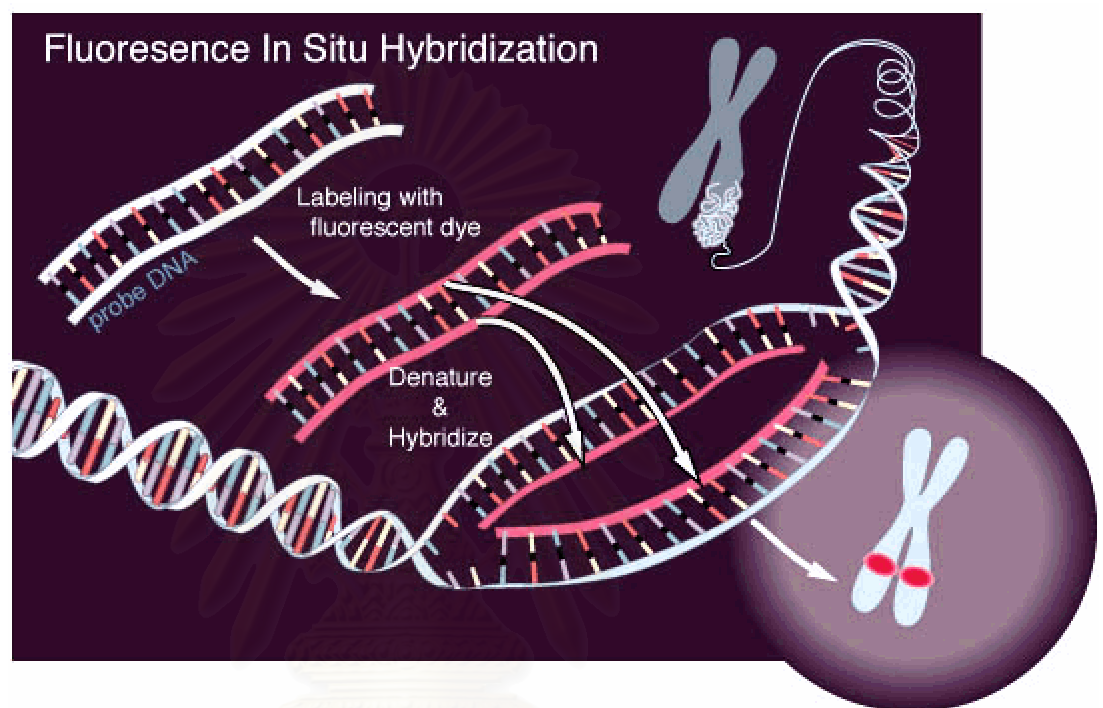

Figure 8. Fluorescence in situ hybridization (FISH)

Two different approaches have been used in the utilization of FISH on paraffin tissue; FISH on paraffin-embedded tissue section (< 6 μm) and FISH on isolated nuclei from the paraffin tissue block *(31, 47)*. FISH analysis on isolated nuclei from paraffin tissue samples was found to be limited by loss of histological architecture, contamination by benign cells and requirement for thick tissue section for digestion (>50 μm). Application of FISH on histological tissue section (< 6 μm) maintains the tissue morphology, requires a small amount of tissue material, enables analysis of tumors even though they are suspended in benign architecture, and can be very useful in cases with limited tissue block material.

Many studies are able to detect chromosomal translocations by FISH on paraffin embedded routine biopsy samples. Matsumoto *et al. (30)* studied the incidence of t(14;18)(q32;q21) in 54 Japanese patients with follicular lymphoma by dual-color FISH on paraffin-embedded tissue sections using probes for *BCL-2* and immunoglobulin heavy chain *(IGH)* genes, as well as BCL-2 protein expression. They were able to assess 50 patients (92.5%) through tissue FISH analysis. The t(14;18)(q32;q21) was detected in 56% (28/50) follicular patients, 67% (12/18) grade I follicular patients, 50% (10/20) grade II and 50% (6/12) grade III. There was no statistical differences among histologic grades and t(14;18) positivity. On the other hand, overexpression of BCL-2 protein was detected in 83% (45/54) patients. No t(14;18) was found in 17 patients showing normal BCL-2 protein expression.

Sharon L. *et al. (33)* analyzed 28 paired frozen and fixed tissues of follicular lymphoma and 20 reactive controls. The t(14;18) was detected in 82% (23/28) cases using PCR on frozen material, breakpoints were detected at the MBR 70% (16/23) and mcr in 30% (7/23). Eight of twenty (40%) cases with amplifiable DNA were positive using PCR on paraffin material. Using FISH, 92% (24/26) cases were positive on the frozen touch preparations, and 93% (26/28) were positive on the paraffin nuclei. In five cases, the efficiency of hybridization was low. In fresh tissue, 5 cases were negative for the translocation using PCR, 3 of these cases were positive using FISH. No case was positive by PCR but negative by FISH. All 20 reactive nodes were negative for the t(14;18) by PCR and 1 of 20 had occasional cells with the translocation FISH pattern.

Shaminie J. *et al. (31)* studied t(14;18) translocation in 50 Malaysian follicular lymphoma patients on paraffin-embedded tissue using PCR and FISH method. Thirty cases (60%) had t(14;18) translocation detectable by PCR amplifications, 50% (25/50) had breakpoint within MBR and 10% (5/50) involved mcr. Twenty cases without detectable t(14;18) translocation by PCR were analyzed by FISH. Eleven (55%) cases were successfully probed, and 36% (4/11) showed positive translocation signals. They

suggested that it was basically due to the ability of FISH probe to detect other different breakpoint regions on chromosome 18 and chromosome 14. The combination of PCR and FISH analysis on paraffin tissue sections for the detection of t(14;18) translocation increases the sensitivity of detection from 60 to 68%.

CHAPTER III

MATERIALS AND METHODS

1. Tissue samples

Routinely processed paraffin-embedded follicular lymphoma samples from Central laboratory King Chulalongkorn memorial hospital were used in this study. All cases were confirmed as follicular lymphoma by histomorphology and immunohistochemical staining for *BCL-2* oncoprotein.

2. DNA extraction

DNA extraction was based on published protocols for paraffin-embedded tissue *(26, 31).* Tissue of 15-20 μm thick (3-5 cuts/block, depending on size of tissue) were cut using a microtome and placed in sterile 1.5 ml microcentrifuge tube. The blade was changed after each block to avoid cross contamination. Tissues were deparaffinised with xylene and washed in alcohol. The samples were then digested overnight at 56°C in digestion buffer (50 mM KCl, 10 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.1 mg/ml gelatine, 0.45% Igepal CA 630 and 0.45% Tween 20) containing 20 mg/ml proteinase K. When completely digested, the samples were boiled for 10 minutes at 95°C to inactivate proteinase K. The supernatants were extracted by phenol and chloroform to remove protein. DNA was then precipitated with 2 volume of 100% cold ethanol and 1/20 volume of 3M sodium acetate. The pellet was allowed to be air-dried completely, dissolved in TE buffer and stored at -20°C until use. The DNA concentration was measured by UV absorbance at 260nm.

3. Amplification DNA for human-beta globin gene (Positive control)

Amplifying ability of DNA extracted from paraffin embedded tissue was assured by performing PCR of human beta-globin gene *(47)*. The inhibitor of PCR amplification was removed by serial dilution of DNA with dH_2O before amplification.

The samples that failed to detect amplifiable to beta-globin gene were considered unsuitable for further PCR analysis.

The cycling conditions included an initial denaturation for 10 minutes at 95°C followed by 40 cycles of 30 seconds for denaturation at 95°C, 45 seconds for annealing at 55°C and 45 seconds for primer extension at 72°C; followed by 10 minutes for final extension at 72°C. Each reaction mixture (20μl) contained 100-500 ng of the DNA in PCR buffer (10 mM Tris pH8.3, 50 mM KCl) with 3.75 mM $MgCl_2$, 2.5 mM dNTPs , 10 ρmol of primers (GH20 and PCO4 primer, Proligo, Singapore) (Table 3) and 1.25 U Amplitaq gold (Roche). PCR amplification was performed in Tgradients thermal cycles (Biometra®).

4. PCR detection of t(14;18) translocation

PCR amplification of the major breakpoint region (MBR), minor cluster region (mcr) and intermediated cluster region (icr) based on published primer sequences (Table 3) *(29, 48-51)*. DNA extracted from a paraffin block, which contains the t(14;18) translocation, was used for the positive control. A negative control for each run consisted of all of the reagents without the addition of any DNA.

4.1 PCR amplification of MBR

PCR was performed in a final volume 50 μl containing 100-500 ng of the DNA, 10 ρmol of primers (MBR1 and J_H primer, Invitrogen, USA) 200 μM of dNTPs, 1.77 mM $MgCl_2$, 1.5 U of Amplitaq® DNA polymerase (Roche), 1.5 U fast start taq DNA polymerase (Roche) and PCR buffer (10 mM Tris pH8.3, 50 mM KCl). PCR amplification was performed with 50 cycles consisting of 15 seconds of denaturation at 95°C and 1 minutes of annealing/extension at 60°C followed by a 10 minutes final extension at 72°C. PCR amplification was performed in Tgradients thermal cycles (Biometra®).

4.2 PCR amplification for mcr

4.2.1 PCR amplification for MC7

PCR was performed in a final volume 50 μl containing 100-500 ng of the DNA, 10 ρmol of primers (MC7 and J_H primer, Invitrogen, USA), 200 μM of dNTPs, 1.15 mM $MgCl_2$, 1.75 U of Amplitaq Gold (Roche) and PCR buffer (10 mM Tris pH8.3, 50 mM KCl). PCR amplification was performed for 50 cycles consisting of 15 seconds of denaturation at 95°C and 2 minutes of annealing/extension at 60°C followed by a 10 minutes of final extension at 72°C. PCR amplification was performed in Tgradients thermal cycles (Biometra®).

4.2.2 PCR amplification for MC8

PCR was performed in a final volume 50 μl containing 100-500 ng of the DNA, 10 ρmol of primers (MC8 and J_H primer, Invitrogen, USA), 200 μM of dNTPs, 1 mM $MgCl_2$, 1.5 U of Amplitaq® DNA polymerase (Roche), 1.5 U fast start taq DNA polymerase (Roche) and PCR buffer (10 mM Tris pH8.3, 50 mM KCl). PCR amplification was performed for 45 cycles consisting of 15 seconds of denaturation at 95°C, 1 minutes of annealing at 60°C and 2 minutes of extension at 72°C followed by a 10 minutes final extension at 72°C. PCR amplification was performed in Tgradients thermal cycles (Biometra®).

4.3 PCR amplification for icr

PCR was performed in a final volume 50 μl containing 100-500 ng of the DNA, 10 ρmol of primers (icr and J_H primer, Proligo, Singapore), 200 μM of dNTPs, 2.5 mM $MgCl_2$, 2.5 U of Amplitaq Gold (Roche) and PCR buffer (10 mM Tris pH8.3, 50mM KCl). The cycling conditions included an initial denaturation for 3 minutes at 96°C followed by 35 cycles of 94°C denaturating for 2 minutes, 58°C annealing for 1 minutes and 72°C primer extension for 2 minutes followed by a final extension at

72°C for 5 minutes. PCR amplification was performed in Tgradients thermal cycles (Biometra®).

Table3. Primer sequences for PCR amplification.

Primer	Nucleotide sequences
PCO4	5’ CAA CTT CAT CCA CGT TCA CC 3’
GH20	5’ GAA GAG CCA AGG ACA GGT AC 3’
MBR1	5’-CGG GAA TTC TTT GAC CTT TAG AGA GTT GCT T-3’
mc7	5’-CGG GAA TTC TCA GTC TCT GGG GAG GAG TGG-3’
mc8	5’-CGG GAA TTC GAC TCC TTT ACG TGC TGG TAC C-3’
icr	5’ TCG TTC TCA GTA AGT GAG AGT GC 3’
J_H	5’-CTC AAG CTT ACC TGA GGA GAC GGT GAC C-3’

4.4 Gel electrophoresis

After completion of the amplification, the products (10 μl) were analyzed by 1.5% gel electrophoresis and visualized under UV illumination by gel document (Syncgene) after ethidium bromide staining.

4.5 Interpretation of PCR amplification

A result was considered positive for t(14;18) if a band was present in the patient lane on an ethidium bromide stained gel. The amplified products using the MBR1 primer set should be in the 150 to 250 bp range. The products for MC8 primer set should be approximately 600 to 1000 or greater base pairs. The products for the MC7 primer set should be approximately 500 bp and smaller than MC8 product. A specimen that was positive for MC8 breakpoint would usually be positive for the MC7 breakpoint as well. Usually, a DNA will not amplify for MC7 breakpoint if it did not

amplify for the MC8 breakpoint as well. The products for icr primer set should be in approximately 200 bp.

5. Isolation and sequencing of the PCR products

For sequencing, we cloned the PCR products using the pGEM®-T Vector Systems (Promega). PCR reactions were analyzed on an agarose gel and then gel-purified before ligated into the pGEM®-T vector. After the ligation, pGEM®-T vector with PCR insert was used for transformation. Successfully cloned in the pGEM®-T vector, the recombinant clones could usually be identified by color screening on indicator plates. Recombinant clones that contained PCR products produced white colonies. However blue colonies could result from PCR fragments that were cloned in-frame resulting in the functional *lac-Z* gene.

5.1 Gel purification

We used the Wizard® SV Gel and PCR Clean-up System (Promega) to remove excess nucleotides and primers from the PCR products. After electrophoresis to separate the DNA fragments, the band of interested was excised and dissolved in the membrane binding solution (4.5 M guanidine isothiocyanate, 0.5 M potassium acetate pH 5.0) at 50-65 °C for 10 minutes or until the gel slice was completely dissolved. DNA could be isolated from the gel slice using microcentrifugation to force the dissolved gel slice through the membrane while simultaneously binding the DNA on the surface of silica (Wizard® SV minicolumns). After washing the isolated DNA fragment using membrane wash solution (10 mM potassium acetate pH 5.0, 80% ethanol and 16.7 μM EDTA pH 8.0), the DNA was eluted by nuclease-free water.

5.2 Cloning of the PCR products

5.2.1 Ligation of the PCR products into pGEM®-T Vector

After gel-purification, the PCR products were cloned into pGEM®-T vector. The 10μl ligation reactions contained 50 ng pGEM®-T vector, 3U of T4 DNA ligase and rapid ligation buffer (60 mM Tris-HCl pH 7.8, 20 mM $MgCl_2$, 20mM DTT and 2 mM ATP). The ligation reactions were incubated at 4°C for 16-18 hours.

5.2.2 Transformation in to *E.coli*, competent cells JM 109

The ligation reactions were transformed into competent cells at 42°C for 40-50 seconds, then, incubated in SOC medium under shaking at 37°C for 1.5 hours. The transformed cells were plated on LB agar plate with 100 μg/ml ampicilin supplemented with 100 mM IPTG and 50 μg/ml of X-gal for blue/white screening. The plate was incubated at 37°C for 16-24 hours.

5.2.3 Preparation of the plasmid DNA

The single white colony was incubated with shaking overnight in LB medium with 100 μg/ml ampicillin at 37°C. After the culture was centrifuged, the cell pellet was resuspended in STE buffer (10 mM Tris-HCl pH 8.0, 0.1 M NaCl, 1mM EDTA pH 8.0) and lysed with alkaline lysis solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0 and 10 mM EDTA pH 8.0). Subsequently alkaline lysis solution II (0.2 N NaOH, 1% w/v SDS) was added to bacterial suspension. It was, then, mixed gently and stored on ice for 10-30 minutes. After that, alkaline lysis solution III (5 M potassium acetate, glacial acetic acid and H_2O) was added. It was, then, mixed gently and stored on ice for 3 – 5 minutes. After centrifugation at 20,000 g for 10 minutes, DNA was extracted using phenol-chloroform and precipitated with ethanol. After centrifugation at 20,000 g for 10 minutes, the pellet was allowed to be completely dried, dissolved with TE buffer pH 8.0 (10 mM Tris-HCl pH 8.0 and 1 mM EDTA pH 8.0) and stored at -20°C.

5.2.4 Restriction endonuclease and electrophoresis

The presence insert in the pGEM®-T vector was verified by digestion with *Pst1*

restriction enzyme. The digestion condition contained 1 U of *Pst1* in an appropriate buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$ and 1 mM DTT pH 7.5), and incubated overnight at 37°C. After digestion, the products were analyzed by agarose gel electrophoresis for selection of the clones containing inserts for sequencing.

5.2.5 DNA sequencing

The PCR sequencing was performed using BigDye™ Terminator Cycle Sequencing Ready Reaction Kit. The reaction containing 1 μg DNA insert in final volume of 10 μl consisting of 1 ρmol of sequencing primer (T7 or SP6) in a reaction mix (Amplitaq DNA polymerase and FS with thermostable pyrophosphatase). The primer extension was performed with denaturation for 30 seconds at 95°C followed by 25 cycles of 10 seconds of denaturation at 95°C, 5 seconds of annealing at 50°C and 4 minutes of extension at 60°C. The products were precipitate with ethanol and 3 M sodium acetate pH 8.0 and the pellets were heated at 95°C for 2 minutes. Finally, the DNA pellet was resuspended in template suppression reagent (Perkin-Elmer) and loaded to the ABI PRISM sequencer.

5.2.6 Alignment and computational searching sequences analysis

The nucleotide sequences obtained from the clones of interest were compared the nucleotide sequences in online GENBANK database by using BLAST N (Basic Local Alignment Search Tool) program. Alignment of sequences were made using CLUSTAL W multiple sequence alignment program.

6. Fluorescene in situ hybridization (FISH) analysis

FISH for the t(14;18) was performed on paraffin- embedded tissue sections of lymph node or tumor tissue obtained from patients with follicular lymphoma. A commercially available LSI IGH/BCL-2 dual-color fusion translocation probe (Vysis, USA) was used. It composed of a mixture of the LSI IGH probe directly labeled with

SpectrumGreen fluorophore and the LSI BCL-2 probe labeled with SpectrumOrange fluorophore. The LSI IGH probe spanned approximately 1.5 Mb and contained sequences homologous to essentially the entire IGH locus, as well as sequences extending about 300 kb beyond the 3' end of the IGH locus (Figure 8). The LSI BCL-2 probe covered an approximately 750 kb region, including the entire BCL-2 gene with additional sequences extending approximately 250 kb both distal and proximal to the gene (Figure 8).

Cases that showed positive translocation by PCR were also performed to demonstrate the translocation by FISH study.

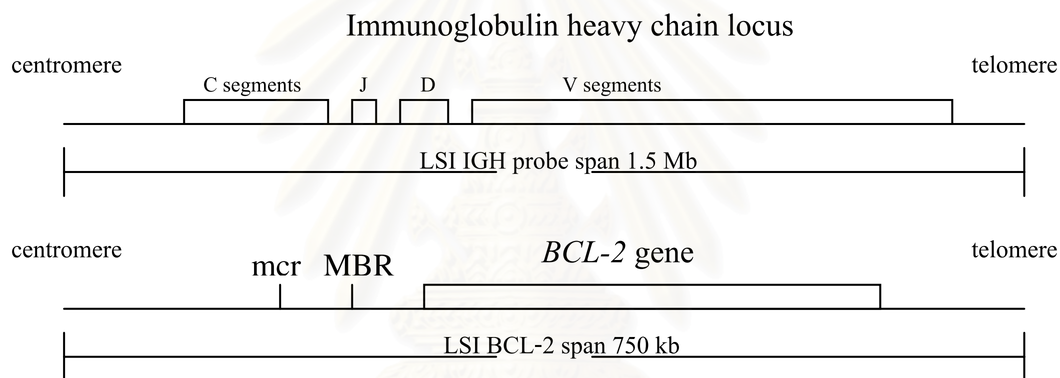

Figure 9. Approximate regions of hybridization for LSI IGH and LSI BCL-2.

6.1 sample preparation

Section 3-4 µm thick were cut and placed on slides that had been treated with 3-aminoprophyltriethoxysilane. The slides were heated overnight at 56°C. The sections must be deparaffinized by xylene to remove embedding medium, dehydrated with ethanol and washed with two changes of distilled water. Incomplete removal of paraffin was strictly avoied.

6.2 Pretreatment/Denaturation

The sections were treated with Pre-Treatment solution (MES buffer; 2-[N-morpholono]ethanesulphonic acid) at 95°C for 10 minutes. The entire jar was removed

and the slides were allowed to cool in the Pre-Treatment solution for 15 minutes at room temperature. Then, the sections were washed with wash buffer (Tris/HCl buffer) for 3 minutes at room temperature.

6.3 Digestion and Hybridization

Excess buffer was tapped off and carefully wiped around the specimen using tissue paper to remove any remaining liquid and to keep reagents within the defined area. The sections were digested by pepsin (pH 2.0) for 3 minutes at room temperature and then washed using the wash buffer. Tissue sections were dehydrated through a series of ethanol: 70%, 80% and 95% ethanol. Tissue sections were allow to be air-dried completely. 10 μl of LSI IGH/BCL-2 probe mix (1 μl of probe, 7 μl of LSI hybridization buffer and 1 μl of purified H_2O) was then applied to the center of the tissue section. Coverslip was immediately placed over the probe allowing it to spread evenly under the coverslip, avoiding air bubbles. Coverslip was sealed using coverslip sealant around the periphery. The coverslip sealant was allowed to overlap the coverslip and the slide. The slides were placed in the incubator for heating at 73°C for 5 minutes before placing in preheated humidified hybridization chamber and incubated overnight at 37°C.

6.4 Post- hybridization

After hybridization, the sections were washed in Stringent wash buffer (SSC buffer; saline-sodium citrate) at room temperature for removal of the coverslip. The sections were then incubated in Stringent wash buffer (SSC buffer; saline-sodium citrate) at 65°C for 10 minutes and washed by wash buffer. Dehydrated tissue sections through a series of ethanol: 70%, 80% and 95% ethanol. Tissue sections were air-dried completely.

6.5 Mounting

10 μl of fluorescence mounting medium containing DAPI II was applied to target area of slide and cvered with a coverslip. Because fading occurs if slides are exposed to light or high temperatures, slides were stored in the dark at -20°C until read.

6.6 Interpretation of FISH

The samples were analyzed using Olympus fluorescence microscope, equipped with a triple pass filters (Green, Orange and DAPI) under a 100x oil objective.

Cases were defined as normal if there were two green (IGH) and two red (BCL-2) signals. A t(14;18) translocation was defined when there were fusion signals of the BCL-2 and IGH probes (yellow). Signals from overlapping nuclei were ignored. The entire preparation was analyzed at least 100 nuclei were formally counted in each cases. In general, a cutoff point of 5% was used to define a t(14;18) positive case.

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CHAPTER IV

RESULTS

1. PCR detection of t(14;18) translocation

40 paraffin-embedded tissue samples from Thai patients with histologically confirmed follicular lymphoma (FL) were diagnosed from 1991 – 2004. There were 18 male and 19 female patients. The age ranged from 31 to 82 years with the average of 60 years. Four were FL stage I, 3 were stage II, 4 were stage III and 8 were stage IV. All samples were assayed for the t(14;18) translocation using PCR amplification with three primer sets. The MBR/J_H, mcr/ J_H and icr/J_H primer sets were used to detect 3 breakpoints on *BCL-2* gene that were MBR, mcr and icr breakpoint, respectively.

The amplified products using the MBR primer set were within the 150-250 basepair range (Figure 9), mcr products showed bands between 500-1000 basepair and icr amplified products showed bands ranging between 100-200 basepair. (Figure 10)

Six cases (15%) had t(14;18) translocation detectable by these PCR amplifications, 5 cases (12.5%) showed breakpoints within the MBR region and 1 case (2.5%) involved the icr region. No case was found with a breakpoint in the mcr region. All cases were positive for beta globin gene PCR (268 bp) suggesting that DNA was amplifiable.

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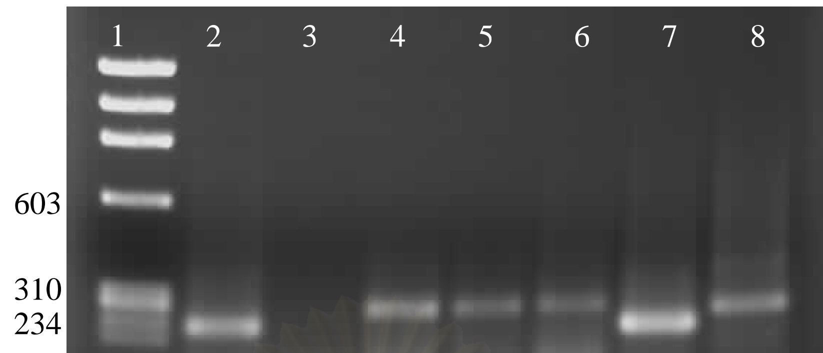

Figure 10. Ethidium bromide-stained gel for MBR/J_H amplification products from paraffin embedded tissues. Lane 1, phi x 174DNA/*Hae* III markers; Lane 2, positive control; Lane 3, negative control; Lanes 4-8, positive t(14;18) FL cases.

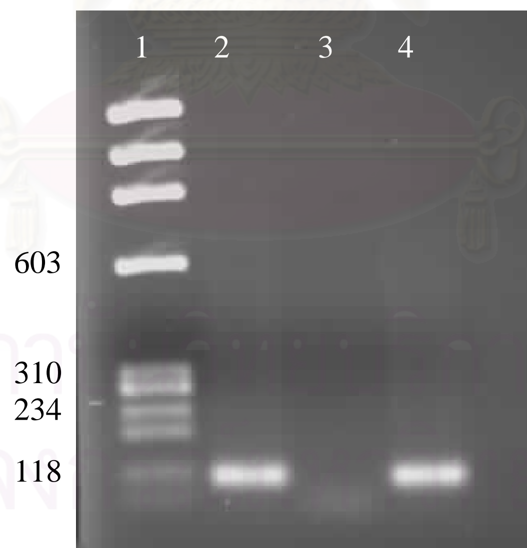

Figure 11. Ethidium bromide-stained gel for ICR/J_H amplification products from paraffin embedded tissues. Lane 1, phi x 174DNA/*Hae* III markers; Lane 2, positive control; Lane 3, negative control; Lanes 4, positive t(14;18) FL cases.

2. Isolation and sequencing of the PCR products

2.1 Sequencing of the MBR product

The products of MBR clones were sequenced and analyzed compared with GENBANK database using the BLAST N and CLUSTAL W program. The sequences of MBR product showed homologous to *BCL-2* and Immunoglobulin heavy chain joining region gene from GENBANK database. This confirmed that the PCR products were correct. Nucleotide insertions between the *BCL-2* and *IgH* were found.(Figure 12, 13).

BC1

```
BCL-2        ACGTGGCCTGTTTCAACACAAACCCACCCAGAGCCCTCCTGCC
             CTCCTTCCGCGGGGGCTTTCTCATGGCTGTCCTTCAGGGTCTT
             CCTGAAATGCAGTGGTGCTTACGCTCCACCAAGAAAGCAGGAA
             ACCTGTGGTATGAAGCCAGACCTCC

Insertion    TCCTACC

IgH          AATTACTACTACTACTACGGTATGGACGTCTGGGGG


BCL-2        ACGTGGCCTGTTTCAACACAGACCCACCCAGAGCCCTCCTGCC
BC1          ACGTGGCCTGTTTCAACACAAACCCACCCAGAGCCCTCCTGCC
             ******************** **********************

BCL-2        CTCCTTCCGCGGGGGCTTTCTCATGGCTGTCCTTCAGGGTCTT
BC1          CTCCTTCCGCGGGGGCTTTCTCATGGCTGTCCTTCAGGGTCTT
             *******************************************

BCL-2/IgH    CCTGAAATGCAGTGGTCGTTACGCTCCACCAAGAAAGCAGGAA
BC1          CCTGAAATGCAGTGGTGCTTACGCTCCACCAAGAAAGCAGGAA
             ****************  *************************

BCL-2/IgH    ACCTGTGGTATGAAGCCAGACCTCCTCCTACCAATTACTACTA
BC1          ACCTGTGGTATGAAGCCAGACCTCC-------AATTACTACTA
             *************************       ***********

IgH          CTACTACGGTATGGACGTCTGGGGG
BC1          CTACTACGGTATGGACGTCTGGGGG
             *************************
```

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Figure 12. The sequences of MBR product (BC1) showed homologous to *BCL-2* and immunoglobulin heavy chain joining region gene from GENBANK

database. The nucleotides inserted between the breakpoint were shown in italics.

BC25

```
BCL-2       TTTGACCTTTAGAGAGTTGCTTTACGTGGCCTGTTTCAACACAGACCCA
            CCCAGAGCCCTCCTGCCCTCCTTCCGCGGGGGCTTTCTCATGGCTGTCC
            TTCAGGGTCTTCCTGAAATGCAGTGGTGCTTACGCTCCACCAAG

Insertion   CTTGGATCGTGTTTCTTTCGACAATGTCGGTTTTAAGTACCGCGAAT

IgH         TTACTACTACTACTACGCTATGGACGTCTGGGGCCAAGGGACCACGGTC
            ACCGGTCTCCTCAGGTAAG


BCL-2       TTTGACCTTTAGAGAGTTGCTTTACGTGGCCTGTTTCAACACAGACCCA
BC25        TTTGACCTTTAGAGAGTTGCTTTACGTGGCCTGTTTCAACACAGACCCA
            *************************************************

BCL-2       CCCAGAGCCCTCCTGCCCTCCTTCCGCGGGGGCTTTCTCATGGCTGTCC
BC25        CCCAGAGCCCTCCTGCCCTCCTTCCGCGGGGGCTTTCTCATGGCTGTCC
            *************************************************

BCL-2       TTCAGGGTCTTCCTGAAATGCAGTGGTGCTTACGCTCCACCAAG-----
BC25        TTCAGGGTCTTCCTGAAATGCAGTGGTGCTTACGCTCCACCAAGCTTGG
            ********************************************

IgH         ------------------------------------------TTACTAC
BC25        ATCGTGTTTCTTTCGACAATGTCGGTTTTAAGTACCGCGAATTTACTAC
                                                      *******

IgH         TACTACTACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCG-TC
BC25        TACTACTACGCTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGGTC
            ********** *********************************** **
```

Figure 13. The sequences of MBR product (BC25) showed homologous to *BCL-2* and immunoglobulin heavy chain joining region gene from GENBANK database. Nucleotides inserted between the breakpoint were shown in italics.

2.2 Sequencing of the icr product

The products of icr clones were sequenced and analyzed compared with GENBANK database using the BLAST N and CLUSTAL W program. The sequences of icr product showed homologous to *BCL-2* and Immunoglobulin heavy chain joining region gene from GENBANK database. This confirmed that the PCR products were correct. Nucleotide insertions between the *BCL-2* and *IgH* were also found (Figure 14).

```
ICR

BCL-2        ATTCGTTCTCAGTAAGTGAGAGTGCAGAATCTGA

Insertion    TTCTTTTAATGTAACCTTCACGGA

IgH          GACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCCTCAGGT


ICR          ATTCGTTCTCAGTAAGTGAGAGTGCAGAATCTGATTCTTTTAAT
Bcl-2        ATTCGTTCTCAGTAAGTGAGAGTGCAGAATCTGA----------
             **********************************

ICR          GTAACCTTCACGGAGACTACTGGGGCCAGGGAACCCTGGTCACC
IgH          --------------GACTACTGGGGCCAGGGAACCCTGGTCACC
                           ******************************

ICR          GTCTCCTCAGGT
IgH          GTCTCCTCAGGT
             ************
```

Figure14. The sequences of icr product showed homologous to *BCL-2* and immunoglobulin heavy chain joining region gene from GENBANK database. The nucleotides inserted between the breakpoint were shown in italics.

3. FISH detection of t(14;18) translocation

The detection of t(14;18) translocation on paraffin-embedded tissues by FISH was successfully performed in 3 of 8 cases. One of these 3 cases that showed t(14;18) translocation detectable by PCR was confirmed to have positive translocation by FISH

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analysis, 2 of 3 cases had no detectable t(14;18) translocation by PCR. The cutoff point for positive t(14;18)translocation was 5% of cells containing fusion of BCL-2 and IgH probes. The scoring procedure eliminated cells that were uninterpretable because of artefactual phenomena, such as partial loss of the nucleus during sectioning and excessive non-specific signals.

67% (2/3) showed positive translocation signal (yellow signal)(Figure 15). One of 2 cases the t(14;18) was demonstrated using PCR but another one without detectable t(14;18) translocation by PCR. The percentage of the cells that showed translocation signals in these 2 cases were 31% and 61%. One of these 3 cases showed negative translocation by both techniques (Figure 16).

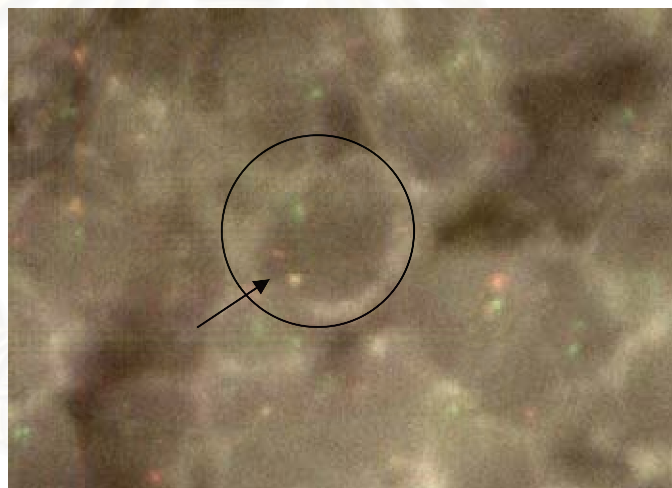

Figure 15. Probe hybridization pattern in positive cell containing the t(14;18) translocation showing the fusion yellow signals.

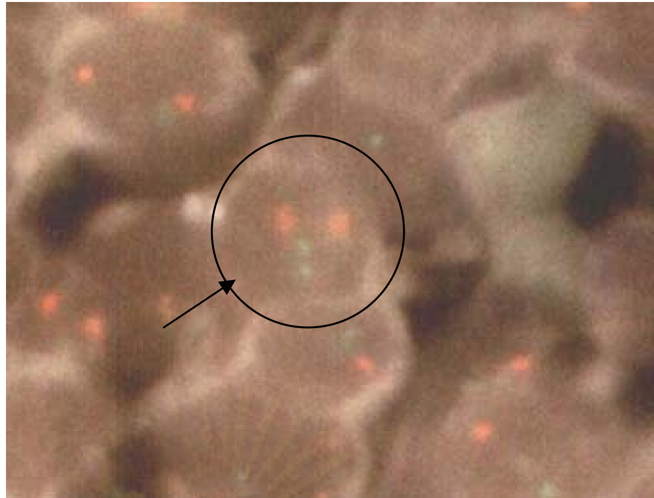

Figure 16. Probe hybridization pattern in normal cell showing the two red (*BCL-2*) and two green (*IgH*) FISH signals.

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CHAPTER V

DISCUSSION AND CONCLUSION

In our study, we performed PCR amplification of the breakpoint region using DNA that was extracted from paraffin-embedded tissues. There are many factors that affect the overall efficiency of the amplification that should be considered when studying paraffin embedded tissue *(26).* For example, optimization of the proteinase K digestion volume, the amount of prepared sample and the age of the tissues. The condition in our study was optimized to detect beta globin gene (positive control) in all samples. We found that the frequency of the t(14;18) translocation in Thai patients with follicular lymphoma (15%) is much lower than those in Western patients (40-60%), suggesting that *BCL-2* translocation is not essential for lymphomagenesis in Thais or the translocation present at different breakpoints undetectable by PCR. There was a possibility that a low frequency of the t(14;18) translocation in Thai patients with follicular lymphoma may be caused by the low products of these PCR amplification generating false-negative. This possibility should be evaluated by hybridizing all PCR products with internal J_H and BCL-2 probes. This will increase the sensitivity of detection rate of t(14;18) translocation *(18, 25).*

Unlike the MBR and icr products, the mcr products are much larger spanning approximately 500-1000 bp. The cases with breakpoints at the mcr regions might be underestimated because of the length of the PCR products involved. DNA extracted from paraffin-embedded tissues is often fragmented and is not optimal for PCR amplification for a product larger than 500 bp. In the study from Liu J., *(26)* primers designed to amplify three segments of the human beta- globin gene (175, 324 and 676 bp) were used to analyze the DNA extracted from the fixed tissue samples. They found that the percentage of cases with amplifiable DNA decreased as size of beta- globin product increased. Many studies have been published on the isolation of fixed-tissue

DNA for PCR analysis *(52-54)* that differed in the extent of digestion and purification before amplification. They used a simple boiling water extraction method and reported low PCR amplification rates *(52)*. The combination of a dilution step, phenol-chloroform extraction and precipitation should ethanol *(26, 52, 54)* that used in this study will result in the successful amplification for both of the beta- globin and *BCL-2* gene.

In addition to the PCR amplification, in this study, we used FISH analysis to detect the t(14;18) translocation on paraffin- embedded tissue sections. We used the Vysis IGH/BCL-2 probe set consist of 1.5 Mb locus specific IGH probe, (labeled with green fluorescence) spanning the entire IGH locus and a 750 kb BCL-2 probe, (labeled with orange fluorescence), spanning the entire *BCL-2* gene, and extending 250 kb both distal and proximal to the gene. The major advantages of FISH on paraffin tissue sections are that the cells carrying the translocation can be analyzed in a histological context and less patients material. We successfully used FISH analysis to assay for this translocation on 3 paraffin tissue sections that were from recent year (2004). The relatively low rate of success maybe related to a combination of factors *(55, 56)* including the small size of the targets, their degradation and/or masking during the fixation and further processing of the samples, insufficient probe penetration, partial probe hybridization and abundant auto-fluorescence *(57)*. One of 3 had t(14;18) translocation detectable by PCR and reconfirmed by FISH. In 2 of 3 cases without t(14;18) translocation by PCR, 1 was positive using FISH. This may be explained by that PCR requires absolute sequence complementary, but it is not as crucial for FISH *(33)*. In follicular lymphoma, on-going somatic hypermutation is a key feature and this may result in base changes at the primer-binding sites preventing PCR amplification. Moreover, PCR will not detect alternative breakpoints that are outside the regions covered by PCR strategy. The case that was positive by FISH but negative by PCR demonstrated the advantage of FISH over PCR for the detection of *BCL-2* gene rearrangements. However, FISH assay was helpful only in specimens processed within

1-2 years. This combination of two approaches will increase the sensitivity of detection rate of t(14;18) translocation. Because FISH analysis is more laborious, it may be performed only in cases with negative PCR.

Some follicular lymphoma cases showed BCL-2 protein overexpression but without detectable t(14;18) translocation using both FISH and PCR analysis. This suggesting that the causes of lymphomagenesis of these cases regulated by other mechanisms, such as mutation or *BCL-2* gene amplification *(58, 59)*, unknown gene that regulated *BCL-2* gene expression or an abnormality of chromosome 18 *(60).* We did not find *BCL-2* gene amplification using FISH in this study. Interestingly, Wong et al. *(61)* reported a case of follicular lymphoma with trisomy 18 and it was shown that this case overexpressed BCL-2 protein in the absence of t(14;18) translocation. More FISH assay will be performed in Thai patients with follicular lymphoma.

Detection of t(14;18) may be of value in the clinical investigation of malignant lymphoma for confirmation of diagnosis *(62)*, for assessment of disease stage and minimal disease *(63)*, to determine prognosis of follicular lymphoma *(64)*, and evaluation of investigation therapies *(65, 66).*

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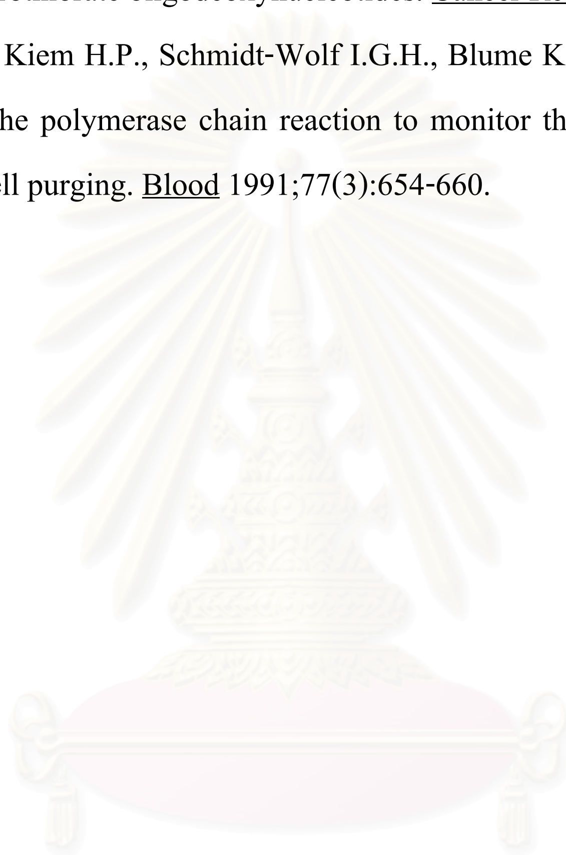

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APPENDIX

1. Equipments

Fluorescent microscope (Olympus), Andrology Unit, Department of Obstetrics and Gynecology, Faculty of Medicine, Chulalongkorn University.

Gel document (Syncgene)

Tgradient thermo cycles (Biometra®)

2. Bacteria media

2.1. LB Medium (per liter)

10 g	Bacto®- tryptone
5 g	Bacto®-yeast extract
5 g	NaCl

Adjust pH to 7.0 with NaOH

2.2. LB plates with amplification

Add 15 g agar to 1 liter of LB medium. Autoclave. Allow to cool to 50°C before adding amplicilin to a final concentration of 100 μg/ml. Pour 30-35 ml of medium into 85 mm petridishes. Let the agar harden. Store at 4°C for up to 1 mount or 1 week at room temperature.

2.3. LB plates with ampicillin/IPTG/X-Gal

Make the LB plates with ampicillin as above; then supplement with 0.5 mM IPTG and 80 μg/ml X-Gal and pour the plates. Alternatively, 100 μl of 100 mM IPTG and 20 μl of 50 mg/ml X-Gal may be spread over the surface of the amplicilin plate and allowed to absorb for 30 minutes at 37°C prior to use.

2.4. SOC medium (100ml)

2.0 g	Bacto®- tryptone
0.5 g	Bacto®-yeast extract

1 ml	1M NaCl
0.25 ml	1 M KCl
1 ml	2M Mg^{2+} stock, filtersterilized
1 ml	2M glucose, filtersterilized

Add Bacto® - tryptone, Bacto®-yeast extract, NaCl and KCl to 97 ml distilled water. Stir to dissolve. Autoclave and cool at room temperature. Add 2M Mg^{2+} stock and 2 M glucose, each to a final concentration of 20 mM. Bring to 100 ml with sterile distilled water. The final pH should be 7.0.

3. buffer

3.1. Alkaline lysis solution I

50 mM	Glucose
25 mM	Tris-Chloride, pH 8.0
10 mM	EDTA, pH 8.0

3.2. Alkaline lysis solution II

0.2 N	NaOH
1 % (w/v)	SDS

3.3. Alkaline lysis solution III

60 ml	5 M Potassium Acetate
11.5 ml	Glacial Acetic Acid
28.5 ml	dH_2O

3.4. Digestion buffer

50 mM	KCl
10 mM	Tris-HCl (pH 8.3)
2.5 mM	$MgCl_2$
0.1 mg/ml	Gelatine
0.45%	Igepal CA 630
0.45%	Tween 20

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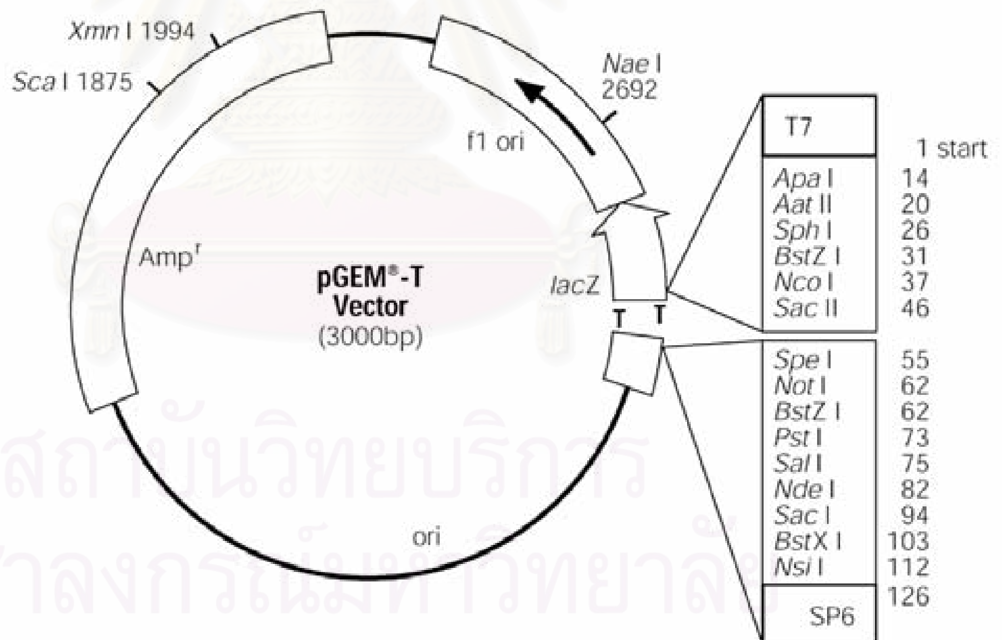
3.5. STE buffer

10 mM	Tris-Cl pH 8.0
0.1 M	NaCl
1 mM	EDTA pH 8.0

3.6. TE buffer

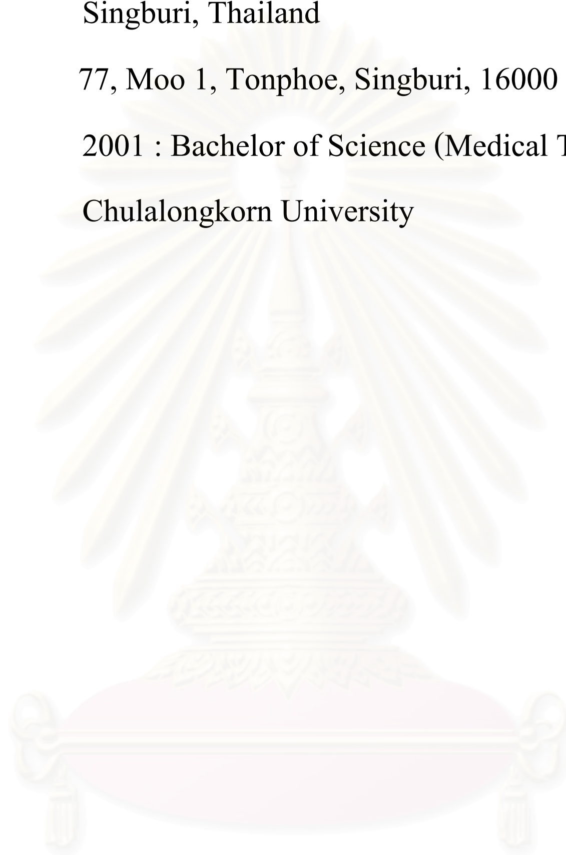
1 M	Tris-HCl (pH 8.0)
0.5 M	EDTA (pH 8.0)

4. pGEM®-T Vector Circle Map



BIOGRAPHY

Name	Miss Atjaraporn Chotipuech	**Sex**	Female
Birth date	January 27, 1980	**Age**	25
Nationality	Thai		
Place of birth	Singburi, Thailand		
Home address	77, Moo 1, Tonphoe, Singburi, 16000 Thailand		
Education	2001 : Bachelor of Science (Medical Technology)		
	Chulalongkorn University		



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