Gene Rearrangements in Follicular Lymphoma Among Indonesian

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ABSTRACT

Aim: *Gene rearrangement has an important role in the management of lymphoma. We investigated the rearrangements of B-cell leukaemia/lymphoma 2 (BCL2), BCL6 and Paired homeobox 5 (PAX5) genes in Indonesian follicular lymphoma (FL) patients.*

Methods: *We examined gene rearrangements using various kinds of polymerase chain reactions (PCRs) on 24 patients' peripheral blood DNA.*

Results: *BCL2 rearrangement was found in 58% (14 of 24 patients), 8 at mbr (major breakpoint region), 2 at mcr (minor cluster region) and 4 at icr (intermediate cluster region), respectively. No rearrangement in BCL6 and PAX5 was detected. There was a significant difference in the incidence of spleen involvement between patients with BCL-2 rearrangement and without it (50% vs. 11%, p=0.04). BCL-2 rearrangement was correlated with spleen involvement (OR=9) and anemia (OR=2.3).*

Conclusion: *BCL2 rearrangement in Indonesian FL was higher than previous reports from other Asia countries (58% vs. 48%, respectively). Our method using peripheral blood DNA might be useful for the molecular diagnosis of FL.*

Key words: *Gene rearrangement, BCL2, BCL6, PAX5, follicular lymphoma.*

INTRODUCTION

Molecular diagnosis of lymphomas is necessary for the diagnosis, accurate evaluation of prognosis, and as a marker for detection of minimal residual disease (MRD).

The molecular genetic abnormalities of malignant lymphoma are often not easily detectable by routine karyotype analysis, and molecular diagnostic tests are necessary for evaluation. Polymerase chain reaction (PCR) has replaced many of the traditional Southern blot tests. This method requires only a small amount of DNA, is relatively rapid, and can detect abnormalities at very low level.[1]

B-cell leukaemia/lymphoma 2 (BCL2) rearrangement caused by translocation of BCL2 gene at chromosome 18q21 to immunoglobulin heavy chain gene (IgH) locus at 14q32 (t(14;18)(q32;q21)) was found in 85% follicular lymphoma (FL). Consequently, the BCL2 gene is subjected to the control of the IgH Em enchancer leading to the over-expression of the anti-apoptotic protein BCL2. The resulting suppression of apoptosis probably represents the initial step of malignant transformation that facilitates the accumulation of further genomic lesions eventually leading to uncontrolled cell proliferation and progression to a FL.[2,3] The frequency of BCL2 translocation in FL varied with region, Asia (Japan and Hongkong) 48%, Europe 55% and US 79%.[4]

Other rearrangements or mutations are needed for FL development, such as translocation of myc, paired homeobox 5 (PAX5), and BCL6 or p53 inactivation. The BCL6 gene on 3q27 generates corresponding protein that specifically regulates B-cell progression and differentiation within the germinal center. As a result of BCL6 translocation, physiological BCL6 gene down-regulation following germinal center transition does not occur, preventing subsequent lymphocyte differentiation. This is one of the most common mutations in diffuse large B-cell lymphomas. In FL, a BCL6 translocation is also recognized, but at a much lesser prevalence.[4]

The PAX5 gene encodes the B-cell-specific activator protein (BSAP) transcription factor which is a key regulator of B-cell development and differentiation. PAX5 rearrangement revealed juxtaposition of the PAX5 to the IgH gene: t(9;14)(p13;q32).[5,6] This translocation has been reported in cases of lymphoplasmacytoid lymphoma. The high level of BSAP also found in large-cell lymphomas and in some FL may be a consequence of deregulated gene expression and suggest a possible involvement of PAX5 in certain B-cell malignancies.[7]

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The monoclonality of IgH gene rearrangement has been applied to distinguish malignant B cells from normal B cells. In particular, the complementarity determining regions (CDRs) in variable region of the IgH gene encodes the antigen binding site and is unique for each B cell clone. CDR III in CDRs is a useful clonal signature of an individual B cell.[8,9,10] In a previous study, we reported a new sensitive method using seminested PCR and heteroduplex analysis to detect the monoclonality of IgH rearrangement in peripheral blood leukocytes DNA from FL. This method can detect 1 malignant cell in 10,000 normal peripheral blood mononuclear cells (PBMC). Using this method, we found circulating lymphoma cell in 79% (19 of 24) patients.[11]

As far as we know, there is no report about gene rearrangement among Indonesian with FL. We report the BCL2, BCL6 and PAX5 rearrangement using the same peripheral blood DNA samples with the previous study.[11]

METHODS

Patients

Twenty-four patients with histologically verified FL at the Hematology and Medical Oncology Division of the Department of Internal Medicine of Hasan Sadikin Hospital, Bandung, between August-November 2003, were recruited in our study. The study was conducted according to the principles of the Declaration of Helsinki and approved by the Hospital Ethics Committee. Clinical characteristics of the patients are listed in table 1.

Table 1. Clinical Characteristics of 24 FL Patients

Clinical characteristic	n	%
Sex		
Male	18	75
Female	6	25
Ann Arbor clinical stage		
I	3	13
II	2	8
III	11	46
IV	8	33
B symptoms		
Yes	18	75
No	6	25
Spleen involvement		
Yes	8	33
No	16	67
Extra-nodal involvement		
Yes	7	29
No	17	71
Hematological abnormalities		
Anemia (Hb < 12 g/L)	10	42
Leukopenia (WBC < 4 x 10^9/L)	3	13
Thrombocytopenia (Platelet <150x 10^9/L)	1	4

DNA Extraction

For isolation of genomic DNA, high molecular weight genomic DNA was extracted by a modified salting out method from peripheral blood leukocyte in heparin anti-coagulated blood.[12] The DNA extraction was done at the Department of Clinical Pathology of Hasan Sadikin Hospital, Bandung.

PCR Amplifications

PCR was performed with iCycler Bio-Rad (Bio-Rad, Hercules, CA, USA) at the Department of Microbiology and Immunology of Aichi Medical University School of Medicine, Nagakute, Aichi, Japan. The oligonucleotide primers were synthesized by Rikaken Co., Nagoya, Japan. The primer sequences are summarized in table 2.

BCL2 Rearrangement

The PCR for BCL2 rearrangement were performed using 0.5 unit Taq polymerase (Ex Tag™, Takara Bio Inc, Otsu, Japan) in 20 ml reaction containing 2 ml 10x Ex Tag™ buffer, 1.6 ml dNTP mixture (2.5 mM each), 1.0 mM of each primer and 1 mg DNA template. The primers used for amplifying the BCL-2 gene were s- Major Breakpoint Region (s-MBR), s-Minor Cluster Region (s-mcr), and s-Intermediate Cluster Region (s-icr) for forward primers and s-JH for backward primer. The PCR conditions were: for MBR: initial denaturation at 98°C for 3 min; 30 cycles of 10 sec at 98°C, 30 sec at 61°C, 30 sec at 72°C; for mcr: initial denaturation at 98°C for 3 min; 30 cycles of 10 sec at 98°C, 30 sec at 58°C, 1 min at 72°C; for icr: initial denaturation at 98°C for 3 min; 30 cycles of 10 sec at 98°C, 30 sec at 58°C, 30 sec at 72°C. To control the PCR reaction, we used vExon3up and vExon3low primers (house-keeping gene).[2]

BCL6 and PAX5 Rearrangement

The long distance PCR (LD-PCR) for BCL6 and PAX5 gene rearrangement were performed using 2.5 unit LA Tag polymerase (TaKaRa LA Tag™) in 50 ml reaction containing 5 ml 10x LA PCR™ Buffer II (Mg^{2+} free), 5 ml 25 mM $MgCl_2$, 8 ml dNTP Mixture (2.5 mM each), 1.0 mM of each primer and 1 mg DNA template.

BCL-6 gene rearrangement were evaluated in 7 locations using primer combinations: BCL6/04-Cm/03, BCL6/04-Cg/01, BCL6/04-Cg/02, BCL6/04-Ca/01, BCL6/04- Ce/01, BCL6/02-Ck/01 and BCL6/02-Cl/02.[13]

PAX-5 gene rearrangement were evaluated at 5 locations using primer combinations: KIS/01-Cm/03, KIS/01-Cg/01, KIS/01-Cg/02, KIS/01-Ca/01, and KIS/01-Ce/01.[13]

The PCR conditions to detect both rearrangements were: initial denaturation at 94°C for 2 min; 5 cycles of

Table 2. Sequences of Oligonucleotide Primers for PCR

Primer	Nucleotide sequence 5'>3'	Reference
s-MBR	GAGAGTTGCTTTACGTGGCC	2
s-mcr	CGCTTGACTCCTTTACGTGC	2
s-icr	TCGTTCTCAGTAAGTGAGAGTGC	2
s-JH	ACCTGAGGAGACGGTGACC	2
vExon3up	CAACGATGCCTACTTTGCCACCC	2
vExon3low	ATCCCGTACATGACGTCACAGCG	2
BCL6/02	CAATCCCATCGGCTCCAAGGTTAGTGTGTGCATGT	13
BCL6/04	TTCATACGACCCCAGACATGGAATCACTCTTTAGA	13
KIS/01	AAGTTGTCCTGGGCTTGGGGTCTAAGTTTATCCTT	13
Cμ/01	TGCTGCTGATGTCAGAGTTGTTCTTGTATTTCCAG	13
Cγ/01	AAGATGGTGATGGTCGTCCACAGCCCGTCCAG	13
Cγ/02	AGGGCACGGTCACCACGCTGCTGAGGGAGTAGAGT	13
Cα/01	TCGTGTAGTGCTTCACGTGGCATGTCACGGACTTG	13
Cε/01	CCAGGCAGCCCAGAGTCACGGAGGTGGCATTGGAG	13
Cκ/01	GCGTCAGGGTGCTGCTGAGGCTGTAGGTGCTGTCC	13
Cλ/02	TGGCCGCGTACTTGTTGTTGCTTTGTTTGGAGGGT	13

20 sec at 98 °C, 20 min at 72 °C; 5 cycles of 20 sec at 98°C, 20 min at 70 °C; 4 cycles of 20 sec at 98 °C, 20 min at 68 °C; 16 cycles of 20 min at 98 °C, 20 min at 68 °C with 00.15 of auto-segment extension; 1 cycle of 10 min at 72 °C.[13]

Statistical Analysis

Clinical characteristics and molecular findings were tabulated and compared using Fisher's Exact test. The correlation between them was calculated using odds ratio.

RESULTS

BCL2 Translocation

BCL2 translocation was found in 58% (14/24) patients. The breakpoint locations were MBR in 33% (8 /24), mcr 8% (2 /24) and icr 17% (4 /24) patients. The representative figures of PCR results can be seen in figure 1. The clinical comparison between patient with and without BCL2 rearrangement can be seen in table 3.

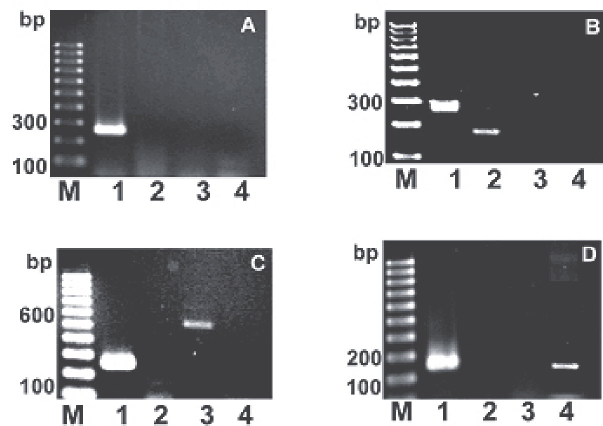

Figure 1. PCR Results of BCL2 Gene Rearrangement. No rearrangement (A), BCL2 rearrangement in Major Breakpoint Region (MBR) (B), BCL2 rearrangement in minor cluster region (mcr)(C) and BCL2 rearrangement in intermediate cluster region (icr) (D). M, 100 bp DNA size marker; line 1, house keeping gene; line 2, MBR (180-275 bp); line 3, mcr (580-630 bp); line 4, icr (73-134 bp).

BCL6 and PAX5 Gene Rearrangements

BCL6 gene rearrangement was examined in 5 regions, PAX5 gene rearrangement was examined in 7 regions. We did not find any sample with either BCL6 or PAX5 gene rearrangement.

DISCUSSION

Most patients with FL, even those in early stages, have circulating cells that carry BCL2 translocation providing a unique opportunity to identify and classify them according to their BCL2 translocation without necessarily having to directly study their tumor tissue. The agreement between the result from bone marrow (BM)/peripheral blood (PB) and lymph node was 94.2%.[14] BCL2 translocation has been describe in PB of healthy individuals. However, when only 1 ug DNA is loaded, as we have done in this study, the BCL2 translocation in PB is only detected in 6% of normal individuals.[15] When whole PB white cells are tested and not B lymphocytes, this proportion is expected to be much lower.[14] In the previous study, we detected the circulating malignant cell using monoclonality of IgH rearrangement at CDRIII in 79% (19 of 24) PB DNA of FL patients.[11] Thus, we further studied gene rearrangements using the same DNA samples.

Geographic variation in the frequency of BCL2 translocation has been reviewed in 14 studies. The rates

Table 3. Comparison of Patient Characteristic According to BCL2 Rearrangement

Characteristics	BCL2 rearrangement (+) n =14	BCL2 rearrangement (-) n = 10	P
Age: mean	48.2 ± 15.2 yrs	59 ± 13.1 yrs	> 0.05
median	54 yrs	55 yrs	> 0.05
Range	16-66 yrs	40-78 yrs	
Sex: male	79% (n=11)	70% (n=7)	> 0.05
Female	21% (n=3)	30% (n=3)	
Stadium: I	14% (n=2)	10% (n=1)	> 0.05
II	7% (n=1)	10% (n=1)	> 0.05
III	43% (n=6)	50% (n=5)	> 0.05
IV	36% (n=5)	30% (n=3)	> 0.05
Extranodal involvement	21% (n=3)	40% (n=4)	> 0.05
B symptoms	71% (n=10)	80% (n=8)	> 0.05
Spleen involvement	50% (n=7)	10% (n=1)	0.04
Hematology:			
- anemia	50% (n=7)	30% (n=3)	> 0.05
- Leukopenia	21 % (n=3)	0% (n=0)	> 0.05
- Thrombocytopenia	36 % (n=5)	10% (n=1)	> 0.05

of BCL2 translocation in Asia, Europe and United States are 48%, 55% and 79% respectively.[4] The rate of BCL2 translocation in Indonesian FL patients (58.3%) is slightly higher than in the Asia region, about the same as in Europe and lower than in the United States. This different result might be caused by adding the icr region breakpoint examination in our study, which hadn't been examined in previous studies. Therefore, we found 4 patients who have BCL2 breakpoint outside the common regions (MBR and mcr).

The result of BCL2 translocation breakpoint locations in this study using DNA from PB was about the same with previous studies using the same primer and using DNA from tissue. The comparison of BCL2 rearrangement location between previous studies and our study were: MBR (32.2% vs. 33%), mcr (3.4% vs. 8%), and icr (17% vs. 17%), respectively.[2] These results show that PB DNA samples can be used to detect BCL2 rearrangement with comparable results as tumor tissue DNA.

The comparison between previous study by Lopez Guilermo et al. and this study in examining BCL2 translocation using PB were MBR (71% vs. 33%), mcr (11% vs. 8%), total translocation 81% vs. 58%.[14] The previous study using PB did not examine icr location. The lower rate of BCL2 translocation in this study may be due to the difference in geographical location (US vs. Indonesia/Asia).

By combining data in our previous study and this study, we found 11 patients with both IgH and BCL2 rearrangement, 3 patients with only BCL2 rearrangement, 8 patients with only IgH rearrangement and 2 patients without any gene rearrangement.[11] Thus, by examining IgH and/or BCL2 we could find gene rearrangement in 92% (22 of 24) patients.

The clinical significance of BCL2 rearrangement in FL is controversial. Previous studies only found that FL patients with BCL2 rearrangement were younger than those without. No difference was found according to BCL2 rearrangement in terms of complete response rate, time to treatment failure, overall survival or other clinical characteristics.[16] In our study, patients with BCL2 rearrangement tend to be younger than those without it (48 vs. 59 years) but it is not statistically significant. We found that there is a significant difference in spleen involvement between patients with BCL2 rearrangement and without it (50% vs. 10%, p 0.04). (Table 3) BCL-2 rearrangement was correlated with spleen involvement (OR 9) and anemia (OR 2.3). It is not known yet how the BCL2 rearrangement is correlates with these abnormalities.

PAX5 gene rearrangement is detected in approximately half of lymphoplasmacytic lymphomas, also in rare cases of marginal zone lymphoma and diffuse large B cell lymphoma. BCL6 gene rearrangement found in one-third of diffuse large B cell lymphomas, also occur infrequently in FL and marginal zone lymphoma.[1] In this study, we could not find either PAX5 nor BCL6 gene rearrangement. This might be cause by different ethnicity, geographic location, or other genes rearrangements involved in Indonesian FL.

CONCLUSION

BCL2 rearrangements are commonly found among Indonesian with FL, at a slightly higher rate than in other Asia regions. BCL6 and PAX5 rearrangement were not found in our study. DNA from PB could be used as an alternative specimen to detect gene rearrangement.

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