

Original Article

# Immunoglobulin Gene Rearrangement of Lymphoid Malignancies : Application of the PCR Technique to Formalin-Fixed, Paraffin-Embedded Tissues

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Every B-lymphocyte carries a unique sequence of the immunoglobulin heavy chain (IgH) gene, which serves as a marker for tumor cells derived from clonal B-lymphocyte expansion. Southern blot analysis for IgH gene rearrangement is not suitable for small biopsies and formalin-fixed, paraffin-embedded tissues. We aimed to assess the application of PCR techniques to demonstrate clonal IgH gene rearrangement in B-cell non-Hodgkin's lymphoma (B-NHL). A series including 119 B-NHL, 10 T-cell NHL, 6 reactive lymphoid hyperplasia, and 15 non-lymphoid malignancies were examined. DNA was extracted from paraffin-embedded tissues by proteinase K digestion. A semi-nested PCR amplification targeting two framework regions (Fr2A and Fr3A) and the joining region ( $J_H$ ) of the variable gene segment of the IgH gene was performed. Clonal IgH gene rearrangement was detected in 30% and 63% of B-NHL with Fr2A and Fr3A assays, respectively, and 74.8% when both assays were combined. Clonal IgH gene rearrangement was not demonstrated in any of the reactive lymphoid hyperplasia and non-lymphoid malignancies. However, 20.0% of the T-NHL showed clonal IgH gene rearrangement. Differences were noted among different subtypes of B-NHL. Clonal IgH gene rearrangement was detected in all of the low-grade B-NHL, except follicular lymphoma. High-grade B-NHL generated heterogeneous results.

**Key words** immunoglobulin gene rearrangement, PCR, B-NHL, paraffin-embedded tissue

## INTRODUCTION

B-lymphocytes generate diverse antibodies through the mechanism of immunoglobulin (Ig) gene rearrangement and somatic hypermutation<sup>1,2</sup>. The immunoglobulin heavy chain (IgH) gene consists of three highly variable complementarity determining regions (CDR) and three rather conserved framework regions (FR), interspersed among each other. The antigen-binding site, known as CDRIII, is the most variable region of the rearranged IgH gene. It is generated by recombining an assortment of one

of the 200 to 300 variable ( $V_H$ ) segments to one of the 20 to 30 diversity ( $D_H$ ) segment, and one of the 1 to 6 joining ( $J_H$ ) segments<sup>3</sup>. Further variability is introduced by random removal and addition of nucleotides at the junction between the segments. This rearrangement is specific to an individual B cell and its clonal progeny, and thus may serve as a marker for clonally derived tumor cells.

Southern blot analysis for IgH gene rearrangement has been used previously as a *gold standard* in laboratory tests. Besides being cumbersome, this technique requires at least 30 to 50 (g of 30 to 50 kilobase-sized DNA fragments extracted from fresh tissue<sup>4</sup>. It is not suitable for small biopsies and formalin-fixed, paraffin-embedded samples because the DNA from the latter is often degraded. The sensitivity and reproducibility of the polymerase chain reaction (PCR) tolerates small quantities of poor quality DNA, allowing amplification of DNA extracted from fixed and processed materials<sup>5-8</sup>. PCR is as

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sensitive as Southern blotting for detection of clonality, and both techniques are equivalent in being able to detect approximately 2% to 5% of a clonal population admixed in a larger polyclonal population<sup>9,10</sup>. PCR has been optimized to detect a clonal population of lymphocytes in as little as 0.1% of the total cells in a tissue sample<sup>11</sup>.

However, the PCR technique is unable to detect clonal IgH gene rearrangement in all B-cell lymphoproliferative diseases. The Southern blot technique is superior to PCR in detecting some unusual IgH gene configurations. These configurations may arise from partial rearrangement, chromosome translocation into the J<sub>H</sub> locus and other abnormal rearrangements, which do not correctly align a V<sub>H</sub> segment to the D<sub>H</sub>-J<sub>H</sub> sequence<sup>12</sup>. Deletion or point mutation of primer target sequences would prevent primer binding. Diffuse large B-cell lymphoma and follicular lymphoma, for instance, are known to undergo somatic hypermutation in the V<sub>H</sub> gene<sup>13</sup>. Even though the consensus primers are homologous to most of the known IgH sequence, about 20% to 30% of the rearrangement could still be missed due to differential annealing or mismatch of the primers<sup>5,9,10,12,14</sup>.

The aim of this study was to evaluate the application of a simple PCR method to demonstrate clonal IgH gene rearrangement in lymphoid malignancies using DNA extracted from formalin-fixed, paraffin-embedded tissues. A series of B-cell non-Hodgkin's lymphoma (B-NHL) was analyzed using two pairs of consensus primers. T-cell non-Hodgkin's lymphoma (T-NHL) and non-lymphoid malignancies were included to examine for the specificity of this method.

## MATERIALS AND METHODS

### *Patients' materials*

A total of 119 cases of B-NHL, of various subtypes, were retrieved from the files in the Department of Pathology, University of Malaya Medical Centre (UMMC). These cases were reviewed by a pathologist (Peh SC) for confirmation. Six cases of reactive lymphoid hyperplasia, 10 T-NHL, 15 non-lymphoid malignancies (9 carcinomas, 4 sarcomas, and 2 malignant melanomas) were included to assess the

specificity of the method.

### *Immunophenotype*

Immunohistochemical staining was performed to confirm the phenotype of the NHL using a panel of antibodies for B- and T-cell markers. The antibodies included CD20 (L26, DAKO, Denmark), CD3 (polyclonal, DAKO, Denmark), CD2 (NCL-CD2-271, Novocastra, United Kingdom), CD8 (CD8/144B, DAKO, Denmark), CD43 (MT1, a gift from S. Poppema), and CD79 $\alpha$  (a gift from D. Mason). Antigens were retrieved by a microwave heat-inducing method for all of the antibodies. Three-step immunoenzymatic staining using an ABCComplex (DAKO, Denmark) was used to localize the antigen. A liquid DAB+ substrate-chromogen system (DAKO, Denmark) was used to develop color. The tissues were lightly counterstained with hematoxylin.

### *DNA extraction*

DNA was extracted by proteinase K digestion based on published protocols for fixed, paraffin-embedded tissues with minor modifications<sup>15,16</sup>. The supernatant containing crude DNA was used directly for PCR amplification.

### *Primers and PCR conditions*

Published primers flanking the framework II (Fr2A) and framework III (Fr3A) of the V<sub>H</sub> and J<sub>H</sub> (LJH) regions were adapted for this study<sup>8,12,17</sup>. Semi-nested PCR was performed using an inner primer at the J<sub>H</sub> region (VLJH).

Aliquots of 1 to 2  $\mu$ l of the extracted DNA were used as template. Each 20  $\mu$ l PCR reaction mixture contained 500 nmol/l of each primer, 200 (mol/l of dNTPs mix (BIOTOOLS, Spain), 1 unit of *HotStarTaq* DNA polymerase (QIAGEN, Germany) in 1X PCR buffer. The MgCl<sub>2</sub> concentration for Fr2A and Fr3A reaction was 2.0 and 3.0 mM, respectively. The cycling conditions for Fr2A/LJH were 15 min at 95°C to activate *HotStarTaq* DNA polymerase, followed by 30 cycles of 1 min at 95°C, 2 min at 63°C and 2 min at 72°C, and a final extension for 4 min at 72°C in a PTC-200 Peltier Thermal Cycler (MJ Research, USA). The same cycling conditions were applied to Fr3A, except at annealing temperature of 55°C. Products from Fr2A/LJH and Fr3A/LJH ampli-

fications were diluted in sterile distilled water (1 : 100) as a template for subsequent PCR. The same reaction mixture was applied to amplification of Fr2A/VLJH and Fr3A/VLJH, and the annealing temperatures were 65°C and 60°C, respectively. PCR products were analyzed on 2% agarose gel (HISPANLAB, Spain) containing 5 µg/ml ethidium bromide (GIBCO BRL, USA), electrophoresed and were then visualized on an ultra-violet light illuminator. Clonal rearrangement was determined when one or two distinct bands were detected after electrophoresis, depending on whether one or both alleles were rearranged.

**Amplification of housekeeping genes**

To reduce the false negative rate, amplification of housekeeping genes, *i. e.*  $\beta$ -globin (GH20 and PC04)<sup>18</sup> and exon 5 of *p53* genes<sup>19</sup>, were performed on cases that were not amplified by both Fr2A and Fr3A assays. Cases that are not amplified by these housekeeping genes indicate that the DNA extracted is not suitable for PCR analysis and will be excluded from the study.

**Direct sequencing of PCR product**

One B-lymphoblastic lymphoma and one Burkitt's lymphoma that showed clonal amplification for Fr2A and Fr3A, respectively, were sequenced to determine the specificity of the amplified fragments. The PCR products were separated on 1% low-melting point agarose gel (GIBCO BRL, USA) and the band of interest was

excised from the gel. It was purified using an Agarose Gel DNA Extraction Kit (Roche Molecular Biochemicals, Germany) according to the manufacturer's instructions. Sequencing was carried out in the Advanced Materials Characterization Allied Laboratories (AMCAL), University of Malaya, using an ABI 377 DNA Sequencer (Perkin-Elmer, USA). The sequences were compared to the deposited sequences using the BLAST program in the PubMed Center.

**RESULTS**

**Patients' materials and immunophenotype**

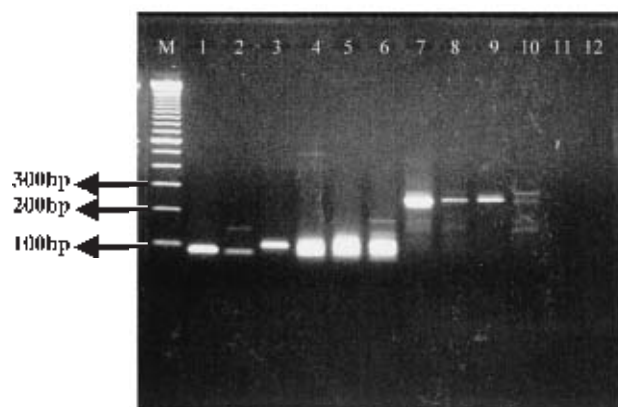
All of the B-NHL expressed CD20 and/or CD79 $\alpha$ , but did not express CD3. These B-NHL were subtyped as 35 diffuse large B-cell lymphomas (26 unspecified, 7 T-cell rich B-cell, 2 mediastinal/thymic), 28 follicular lymphomas, 22 Burkitt's lymphomas, 21 extranodal marginal zone MALT-type lymphomas, 4 chronic/small lymphocytic lymphomas, 3 mantle cell lymphomas, and 2 each of B-lymphoblastic lymphoma, lymphoplasmacytic lymphoma, and plasmacytoma. The subtypes of B-NHL in this series are presented in Table 1. In all the T-NHL, the tumor cells expressed CD3 and other T-cell markers, but none expressed CD20 and/or CD79 $\alpha$ .

**IgH gene rearrangement**

Clonal IgH gene rearrangement was demon-

**Table 1.** Clonal IgH gene rearrangement according to the subtypes of B-cell non-Hodgkin's lymphoma from paraffin-embedded tissues

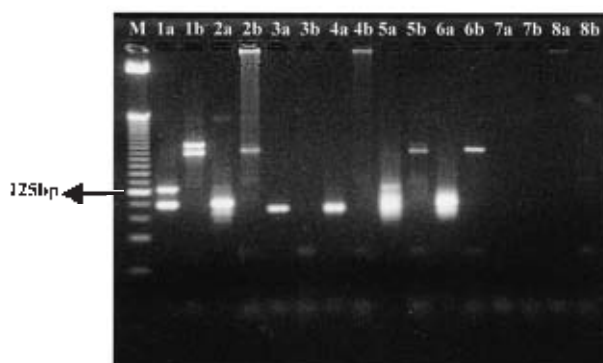
B-NHL subtypes	Fr3A/JH (%)	Fr2A/JH (%)	Combined clonality (%)
B-lymphoblastic lymphoma	2/2 (100.0)	1/2 (50.0)	2/2 (100.0)
Chronic/small lymphocytic lymphoma	4/4 (100.0)	2/4 (50.0)	4/4 (100.0)
Lymphoplasmacytic lymphoma	1/2 (50)	2/2 (100.0)	2/2 (100.0)
Mantle cell lymphoma	1/3 (33.3)	1/3 (33.3)	1/3 (33.3)
Extranodal, marginal zone lymphoma (MALT-type)	12/21 (57.1)	11/21 (52.4)	18/21 (85.7)
Burkitt's lymphoma	21/22 (95.5)	3/22 (13.6)	21/22 (95.5)
Follicular lymphoma	14/28 (50.0)	6/28 (21.4)	17/28 (60.7)
Diffuse, large B-cell lymphoma	19/35 (54.3)	10/35 (28.6)	23/35 (65.7)
Plasmacytoma	1/2 (50.0)	0/2 (0.0)	1/2 (50.0)
Total	75/119(63.0)	36/119(30.3)	89/119(74.8)



**Fig. 1.** Results for amplification of Fr3A/LJH/VLJH and Fr2A/LJH/VLJH on Malaysian B-cell lymphomas. Lanes 1-6 represent amplifications from Fr3A (80-120bp) and Lanes 7-12 are Fr2A amplifications (240-280 bp). Mono-clonality is indicated by the presence of one or two distinct band(s), and polyclonality is considered when there is a smear or no detectable product. M: 100 bp DNA ladder, Lane 1: chronic lymphocytic lymphoma; Lane 2: Burkitt's lymphoma; Lane 3: extranodal marginal zone lymphoma (MALT-type); Lane 4: T-immunoblastic lymphoma; Lanes 5-6: reactive hyperplasia; Lane 7: B-lymphoblastic lymphoma; Lane 8: T cell-rich B cell lymphoma; Lane 9: small lymphocytic lymphoma; Lane 10: T-immunoblastic lymphoma; Lanes 11-12: reactive lymphoid hyperplasia.

strated in 30.3% (36/119) and 63.0% (75/119) of the B-NHL with Fr2A and Fr3A assay respectively, with a combined results of 74.8% (89/119). The Fr3A assay demonstrated clonal IgH gene rearrangement in more cases when compared to Fr2A assay. Examples of the pattern of IgH gene rearrangement are included in Figures 1 and 2. Clonal IgH gene rearrangement was demonstrated in all cases of B-lymphoblastic lymphoma (2/2), chronic/small lymphocytic lymphoma (4/4) and lymphoplasmacytic lymphoma (2/2).

Burkitt's lymphoma and extranodal marginal zone MALT-type lymphoma also generated satisfactory results. Clonal IgH gene rearrangement was demonstrated in 3/22 (13.6%) and 21/22 (95.5%) of Burkitt's lymphoma with Fr2A and Fr3A assay respectively, generating a combined percentage of 95.5% (21/22). Demonstration of clonal IgH gene rearrangement in MALT-type lymphoma was significantly improved by combining the Fr2A and Fr3A assays. The Fr2A assay detected clonal IgH gene rearrangement in 11/21 (52.4%) of MALT-type lymphomas, and Fr3A assay in 12/21 (57.1%) of this tumor, gener-



**Fig. 2.** Pattern of clonality shown using the combination of Fr3A and Fr2A primers. Lanes (a) and (b) represent amplifications from Fr3A and Fr2A, respectively. Lanes 1 & 2 were 2 cases showing mono-clonality with both Fr3A and Fr2A; Lanes 3 & 4 represent 2 cases showing mono-clonality with Fr3A, but not Fr2A; Lanes 5 & 6 were 2 cases with mono-clonality by Fr2A, but not Fr3A; Lanes 7 & 8 represent 2 cases that were negative for both Fr3A and Fr2A. M: 25bp DNA ladder, Lanes 1a & 1b: Burkitt's lymphoma; Lanes 2a & 2b: extranodal marginal zone lymphoma (MALT-type); Lanes 3a & 3b: diffuse large cell lymphoma; Lanes 4a & 4b: T cell-rich-B cell lymphoma; Lanes 5a & 5b: lymphoplasmacytoid lymphoma; Lanes 6a & 6b: extranodal marginal zone lymphoma (MALT-type); Lanes 7a & 7b: mantle cell lymphoma; Lanes 8a & 8b: follicular lymphoma.

ating a combined result of 85.7% (18/21).

Clonal IgH gene rearrangement was demonstrated in 65.7% (23/35), 60.7% (17/28), and 50.0% (1/2) of diffuse large B-cell lymphoma, follicular lymphoma and plasmacytoma, respectively. Heterogeneity was observed in diffuse large B-cell lymphomas. Clonal rearrangement was demonstrated in all cases of some specific subtypes, *i. e.* T-cell rich B-cells (7/7) and mediastinal/thymic (2/2) lymphomas. Clonality was only demonstrated in 1 (33.3%) of the 3 cases of mantle cell lymphoma.

None of the 6 reactive lymphoid hyperplasias showed clonal amplification. These cases showed either a broad smear (sizes within the range of 240 to 280 bp for Fr2A assay, or 70 to 140 bp for Fr3A assay), multiple bands or no detectable product. Two (20.0%) of the 10 T-NHL showed a clonal band by the Fr3A assay, but none was clonal with the Fr2A assay (Table 2).

A faint band was detected on agarose gel in 3 of the 15 non-lymphoid malignancies with the Fr3A assay. Two of these cases were nasopharyngeal carcinoma and another was hepatocel-

**Table 2.** Results for clonal IgH gene rearrangement analysis of reactive lymphoid hyperplasia, T-NHL and non-lymphoid malignancies

Types of malignancies	Fr3A/JH (%)	Fr2A/JH (%)	Combined clonality (%)
Reactive lymphoid hyperplasia	0/6 (0)	0/6 (0)	0/6 (0)
T-NHL	2/10(20)	0/10(10)	2/10(20)
Sarcomas	0/4 (0)	0/4 (0)	0/4 (0)
<i>Leiomyosarcoma</i>	0/1 (0)	0/1 (0)	0/1 (0)
<i>Ewing's sarcoma</i>	0/1 (0)	0/1 (0)	0/1 (0)
<i>Myxoid liposarcoma</i>	0/1 (0)	0/1 (0)	0/1 (0)
<i>Rhabdomyosarcoma</i>	0/1 (0)	0/1 (0)	0/1 (0)
Carcinomas	0/9 (0)	0/9 (0)	0/9 (0)
<i>Infiltrating ductal carcinoma</i>	0/1 (0)	0/1 (0)	0/1 (0)
<i>Adenocarcinoma</i>	0/1 (0)	0/1 (0)	0/1 (0)
<i>Prostate Carcinoma</i>	0/1 (0)	0/1 (0)	0/1 (0)
<i>Nasopharyngeal carcinoma</i>	0/2 (0)	0/2 (0)	0/2 (0)
<i>Hepatocellular carcinoma</i>	0/1 (0)	0/1 (0)	0/1 (0)
<i>Endometrial carcinoma</i>	0/1 (0)	0/1 (0)	0/1 (0)
<i>Gastric carcinoma</i>	0/1 (0)	0/1 (0)	0/1 (0)
Malignant Melanomas	0/2 (0)	0/2 (0)	0/2 (0)

lular carcinoma. A case of malignant melanoma showed a clonal band on agarose gel with the Fr2A assay.

#### ***Amplification of housekeeping genes***

Thirty cases (30/119, 25.2%) showed no clonal IgH gene rearrangement by either the Fr2A and Fr3A assays. Of these cases, some showed a broad smear, instead of clonal band(s) (Figure 2, Lanes 5a and 6a), and some did not show any detectable product (Figure 2, Lanes 7 and 8). All of these cases were amplified with  $\beta$ -globin and exon 5 of p53 genes. Amplification with the housekeeping genes showed that these cases contain suitable DNA for PCR analysis (Figure 3). These cases represent true negatives, *i. e.* without clonal IgH gene rearrangement.

#### ***Direct sequencing of PCR products***

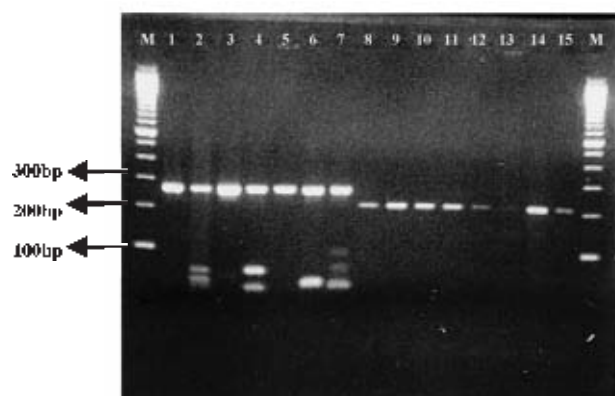
Representative cases for amplification by Fr2A (B-lymphoblastic lymphoma) and Fr3A (Burkitt's lymphoma) assays were subjected to direct sequencing. The results from the sequencing, when compared to the sequences deposited in

the PubMed Center, produced significant alignment with immunoglobulin heavy chain gene sequences. The sequence and alignment for the cases of B-lymphoblastic lymphoma and Burkitt's lymphoma can be retrieved by request ID 1024919174-02834-158806 and 1024919131-02462-7493, respectively, in the BLAST program.

Cases of nasopharyngeal carcinoma, hepatocellular carcinoma and malignant melanoma were also subjected to direct sequencing. However, the sequencing analysis failed to yield a readable sequence. A repeated investigation on these four cases confirmed the absence of a clonal B-cell population.

### **DISCUSSION**

This study showed that the Fr3A assay is superior to Fr2A the assay in demonstrating IgH gene rearrangement. Even though the detection rate with the Fr2A assay was rather low, it helped to increase the overall clonality rate to 74.8%, an increase of 15.7%. A lower rate for the Fr2A assay is in accord with other reports (5, 8, 17). Diss *et al.*<sup>5</sup> reported a significantly lower clonality rate with the Fr2A assay in paraffin-



**Fig. 3.** Amplification of housekeeping genes for cases that were not amplified by both Fr2A and Fr3A assays. M denotes 100 bp DNA ladder; Lanes 1-7 represent amplification for the  $\beta$ -globin gene (268 bp) and Lanes 8-15 represent amplification for exon 5 of the  $p53$  gene (209 bp). Lane 1, mantle cell lymphoma; Lane 2, MALT-type lymphoma; Lanes 3 & 4, diffuse large B-cell lymphoma; Lanes 5-7, follicular lymphoma; Lane 8, MALT-type lymphoma; Lane 9, Burkitt's lymphoma; Lane 10, plasmacytoma; Lanes 11-13, diffuse large B-cell lymphoma; Lanes 14 & 15, follicular lymphoma.

embedded tissues (67%) and haematoxylin-stained materials (36%), compared to fresh/frozen tissue (86%). The fixation process has been shown to inhibit PCR reactions, and amplification of longer fragments is more difficult for degraded and impure DNA<sup>20</sup>.

Differences in clonal IgH gene rearrangement rates are noted among the various subtypes of B-NHL. Clonal IgH gene rearrangement was demonstrated in all cases of B-lymphoblastic, chronic/small lymphocytic, and lymphoplasmacytic lymphomas. Other studies also reported that these subtypes are easier to amplify using the VH/JH assay<sup>12,13</sup>. Segal *et al.*<sup>13</sup> demonstrated IgH gene rearrangement in the majority of low grade lymphomas, *i. e.* 100% of chronic lymphocytic lymphoma, 96% of small lymphocytic lymphoma, and 100% of mantle cell lymphoma, using the Fr3A/JH combination. Our results agree with their findings, except for mantle cell lymphoma, where the detection rate was only 33.3% in our cases. However, this figure may not be a true reflection, due to the small number of cases studied. Nonetheless, the absence of clonal amplification may be attributed to translocation involving the  $bcl-1$  gene on chromosome 11q13 and the IgH gene, which is present in approximately 35% to 66% of mantle

cell lymphomas<sup>21</sup>. Bertoni *et al.* reported 44% (7/16) of the mantle cell lymphomas studied were clonal for  $bcl-1$ /IgH translocation and another 44% (7/16) were clonal for CDR3/JH<sup>22</sup>. Therefore, the application of primers for both the translocation and IgH gene rearrangement may help to improve the detection of clonality in mantle cell lymphoma.

Follicular lymphoma is one of the more difficult low grade subtypes for evaluation with the standard VH/JH assay. It is known to give a false negative rate of about 44%<sup>12</sup>. Clonal IgH gene rearrangement was demonstrated in 50.0% and 21.4% of the cases using Fr3A and Fr2A assays, respectively, in this study, and a combined result of 60.7%. Early studies also documented overall poor results, of 52% to 54%, demonstrating clonal rearrangement<sup>12,23</sup>. This may be due to chromosomal translocation involving the  $bcl-2$  gene on chromosome 18 and the IgH gene locus, which is present in 85% to 90% of follicular lymphomas<sup>24,25</sup>. Segal *et al.* was able to improve the detection rate to 82% when the result from the Fr3A assay (60.0%) was combined with that of the  $bcl-2$ /JH assay<sup>23</sup>.

Studies on diffuse, large B-cell lymphomas reported variable results, ranging from 17% to 70% with the Fr3A/VH assay<sup>12,14,17</sup>. The 54.3% result found in this study falls within the reported range. The poor results may be attributed to chromosomal translocations involving the  $bcl-2$  or  $bcl-6$  gene and the IgH gene. The detection rate can be significantly improved by employing family-specific primers<sup>12,17</sup>. However, this method involves multiplex PCR, and is not suitable for diagnostic purposes.

Failure to detect clonal IgH gene rearrangement in a proportion of follicular lymphomas and diffuse, large B-cell lymphomas is due to the presence of somatic hypermutation in the  $V_H$  gene segments. Somatic hypermutation occurs in the germinal center during the differentiation of B cells, targeting the FR and CDR of the  $V_H$  gene segment<sup>1,2</sup>. The distribution of the mutation with marked accumulation of replacement (R) mutations in the CDR and more silent (S) mutations in the FR is necessary to produce antibodies of high affinity and to conserve the necessary structural component of the antibodies<sup>26</sup>. Somatic hypermutation is known to occur in germinal center cell- and post-germinal center cell-derived lymphomas, such as follicular lymphoma<sup>27,28</sup> and

in diffuse large B-cell lymphomas<sup>29</sup>, but not in pre-germinal center cell-derived mantle cell lymphoma<sup>30</sup>.

Analyses of V<sub>H</sub> gene segments show that MALT-type lymphoma also harbor somatic hypermutations, indicating that it might be derived from the post-germinal center cells<sup>26,31,32</sup>. Addition of the Fr2A assay significantly increased the detection rate of MALT-type lymphoma to 85.7% from 57.1% with Fr3A alone. Similar to our findings, Diss *et al.*<sup>12</sup> also reported that the Fr2A assay (85%) yielded a higher clonal rate than the Fr3A assay (77%) in their series. Studies on somatic hypermutation in high grade MALT-type lymphoma reported a higher mutation frequency in FR3, and lower frequency in FR1 and FR2 with no difference between these two regions<sup>31</sup>. The efficiency of the Fr3A assay will, therefore, be greatly reduced by the failure of primers to anneal to the mutated FR3 in this lymphoma. The specificity of PCR for confirmation of neoplastic rearrangement in MALT-type lymphoma was estimated to be only 45.7%<sup>33</sup>, because clonal IgH gene rearrangement was also found in normal gastric MALT, in the edge and base of gastric ulcer and in chronic *Helicobacter pylori* gastritis<sup>33-35</sup>. The extremely high sensitivity of the PCR technique has, unfortunately, resulted in low specificity in distinguishing benign reactive conditions from malignant lesions.

Demonstration of clonal IgH gene rearrangement in Burkitt's lymphoma was reported to be low using Fr3A but improved when using Fr2A<sup>7</sup>. Failure to demonstrate clonality in Burkitt's lymphoma has been attributed to the chromosomal translocation involving the *c-myc* gene on chromosome 8 and the IgH gene<sup>36</sup>. This study however, was able to show clonal IgH gene rearrangement in most of the cases (95.5%) by the Fr3A assay, but in only 13.6% by Fr2A assay. It is speculated that this may be due to geographical difference in the breakpoint region of chromosomal translocation t(8; 14)<sup>37</sup>.

The extremely high sensitivity of PCR method detected minor (less than 1% of the cells) clonal non-neoplastic B-cell populations in the infiltrates<sup>33,38</sup>, as reported by Ling *et al.* on normal infiltrates in a case of colonic adenocarcinoma<sup>10</sup>. Because Southern blot analysis is less sensitive, it reduces the incidence of false positives. Clonal IgH gene rearrangement had also

been reported in other non-neoplastic diseases, such as atypical lymphoid hyperplasia<sup>39</sup>, a reactive lymph node from a patient with toxoplasmosis<sup>4</sup>, EBV-associated lymphoproliferative diseases<sup>40,41</sup>, and a minor fraction of Castleman's disease<sup>42</sup>. Even though a clonal band was detected on agarose gel in 3 carcinomas and one malignant melanoma, direct sequencing of the PCR products did not confirm clonal IgH gene rearrangement. The PCR products may actually contain heterogeneous bands, which could not be separated by agarose gel. Hence, a sequencing reaction will not yield readable results due to heterogeneity of the PCR products.

Clonal IgH gene rearrangement was demonstrated in 20.0% of T-NHL. Dual rearrangement involving the IgH gene and the T-cell receptor (TCR) gene on B-cell lymphomas had been reported previously<sup>43-46</sup>. The frequency of illegitimate Ig gene rearrangement in mature T-cell neoplasms of the lymph node is estimated to be around 10% for most morphological subtypes<sup>47</sup>. It is postulated that the dual rearrangement may be caused by aberrant activation of the recombinase machinery<sup>47-49</sup>. This hypothesis is supported by the existence of similar structural configurations and a common recombinase enzyme for both IgH and TCR gene rearrangements<sup>47,49,50</sup>.

## CONCLUSION

Application of the PCR technique to demonstrate clonality in lymphoid malignancies has generated satisfactory results. This study showed that the clonality rate with the PCR technique differs among subtypes of B-NHL. A combination of Fr2A and Fr3A assays have been successful in demonstrating clonal IgH gene rearrangement in a large proportion of B-lymphoblastic, small/chronic lymphocytic, lymphoplasmacytic, T-cell rich B-cell, mediastinal/thymic, Burkitt's and MALT-type lymphomas.

Mantle cell, follicular, and diffuse large B-cell lymphomas yielded poorer results. Supplement primers targeting the breakpoints of the putative translocations, or family-specific primers should be applied to improve the detection rate in these lymphomas. Demonstration of clonal IgH gene rearrangement in lymphoid malignancies by PCR should, however, be used with caution, as the PCR technique contains some

inherent flaws. Extremely high sensitivity of the PCR technique can even detect small clonal populations in the non-neoplastic infiltrates. The result may be difficult to interpret in cases of benign proliferation. Therefore, for diagnostic purposes, clonality, as demonstrated by clonal IgH gene rearrangement, should only be a complement to the clinical and immunohistopathological parameters to ensure diagnostic accuracy.

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