Sensitivity and reproducibility of conventional qualitative and quantitative PCR assays for detection of the t(14;18)(q32;q21) chromosomal translocation in biopsy material from patients with follicular lymphoma

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Abstract. Follicular lymphoma (FL) is characterized by the t(14;18)(q32;q21) chromosomal translocation which can be detected by polymerase chain reaction (PCR) in approximately 70% of cases. The aim of our retrospective study was to evaluate the sensitivity and the reproducibility of both conventional qualitative and quantitative PCR assays for detection of the t(14;18)(q32;q21) chromosomal translocation in biopsy material. Fifty-seven formalin-fixed, paraffin-embedded tumor lymph node (LN) specimens from 50 patients with FL were included in the study. Qualitative PCR was performed with primer sets specific for the MBR, far3'-MBR and the mcr regions, respectively. Quantitative PCR was performed using the LightCycler® instrument and the LightCycler®-t(14;18) Quantification Kit (MBR). The overall detection rate of the t(14;18) in our study (52.6%) was in accordance with the literature. Of the t(14;18)-positive cases, 49.1% had breakpoints within the MBR and only 3.5% had breakpoints within the mcr. The most sensitive method was LightCycler-based PCR with a detection rate of 47.4%, followed by MBR1,2 assay (43.9%). We observed good agreement between qualitative MBR 1,2 and quantitative LightCycler-based assay with a slightly higher detection rate of the quantitative method. The sensitivities of both methods were in accordance with results from other studies. Since LightCycler-based assay detects only breakpoints within the MBR, qualitative PCR should be employed in routine diagnostic settings for detection of breakpoints within the mcr and far3'-MBR regions.

Introduction

Follicular lymphoma (FL), one of the most common non-Hodgkin’s lymphomas of B-cell type in the Western population, is characterized by the t(14;18)(q32;q21) chromosomal translocation in approximately 80% of cases (1). This chromosomal translocation juxtaposes the BCL-2 locus on chromosome 18q32.3 to the immunoglobulin heavy chain gene (IgH) locus on chromosome 14q32.33 (2,3). Breakpoints on chromosome 14 are often clustered near the 5’ end of one of the six JH gene segments (2-4). On the other hand, most of the breakpoints on chromosome 18 are clustered within two primary breakpoint regions, the major breakpoint region (MBR) and the minor cluster region (mcr) (2,4,5). Approximately 50-60% of BCL-2 rearrangements in FL occur within the MBR (5) and 10-20% of rearrangements occur within the mcr (6). Other breakpoint regions within the BCL-2 locus have also been identified (7-9). Approximately 8% of breakpoints occur within the far3'-MBR subcluster region and 3-4% of breakpoints occur within the 5'-mcr subcluster region (7-9).

These restricted breakpoint localizations have allowed the design of primers specific for 5’ regions of the MBR and mcr clusters on chromosome 18, and for conserved portions of the JH gene segments on chromosome 14, which have been widely used for standard polymerase chain reaction (PCR) assays (7,10). The combination of standard MBR and mcr primers with a JH consensus primer enables the detection of approximately 70% of BCL-2/JH rearrangements (7,10). Various methodologies in performing PCR with different primer sequences, methods of amplification and detection of amplified products have been described (7,8,10-12). Recently, quantitative real-time PCR has been extensively employed in most laboratories, particularly for detection of minimal residual disease (MRD) in follow-up of patients (13-22). The sensitivities of described methods vary greatly, mostly depending on the methodology (qualitative vs quantitative, single-round vs nested PCR) and choice of primers (10). Most previous studies were performed using DNA isolated from fresh peripheral blood or bone marrow aspirates.
(12-16,19-23) and only a few studies used DNA derived from formalin-fixed, paraffin-embedded tissue specimens (11,21,23,24).

The aim of our retrospective study on a series of archival specimens from Slovenian patients with FL was to evaluate the sensitivity and the reproducibility of both conventional qualitative and quantitative PCR assays for detection of the t(14;18) chromosome translocation. Furthermore, we compared different MBR-based PCR assays in order to determine the most useful PCR approach for detection of t(14;18) with breakpoints in the MBR on biopsy material.

Materials and methods

Study group. Fifty-seven tumor lymph node (LN) specimens from 50 patients with follicular lymphoma (FL), diagnosed and treated at the Institute of Oncology, Ljubljana, over the period 1993-2005, were included in this retrospective study. There were 16 male and 34 female patients. Median age at the time of lymphoma diagnosis was 51 years. Histological classification of FL was performed according to the World Health Organization classification. All tumor samples that had been formerly classified by the Kiel or REAL classifications were reclassified according to the WHO classification. Thirteen patients had follicular lymphoma grade I, 17 patients grade II, 15 patients grade IIIA and 5 patients grade IIIB. All tumor LN samples were fixed in phosphate-buffered formalin (PBF) and embedded in paraffin (FFPE tissue samples).

Reactive lymph nodes from 15 patients were used as a negative control group for detection of the t(14;18)(q32;q21) translocation and B-cell clonality.

DNA isolation. Five paraffin sections (10 μm) from each specimen were available for the study. DNA was isolated using the High Pure PCR Template Preparation kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer’s protocol with slight modifications in the deparaffinization step. The concentration and the purity of DNA (A260nm/A280nm) were determined spectrophoto-
deparaffinization step. The concentration and the purity manufacturer’s protocol with slight modifications in the Applied Science, Penzberg, Germany) according to the using the High Pure PCR Template Preparation kit (Roche

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Conventional qualitative PCR. Qualitative single-round PCR for t(14;18) translocation was performed with two different primer sets for detection of breakpoints in the major breakpoint region (MBR), and two primer sets for detection of breakpoints in the far3’-MBR and the minor cluster region (mcr) of the BCL-2 oncogene. MBR primer sets were MBR/JHa and, MBR1 and MBR2 in a multiplex PCR with J Ha primer. The far3’-MBR and mcr primers were also used in the two multiplex reactions with the JHa primer. The MBR primer was chosen according to Slack et al (25), MBR, and MBR2, far3’-MBR and mcr primers were proposed by P. Rombout, University Medical Centre Nijmegen (personal communication) and the JHa primer was chosen according to Segal et al (26). Expected sizes of amplified fragments are 100-270 bp in the case of breakpoints in the MBR, and 150-500 bp in the case of breakpoints in the far3’-MBR and the mcr.

All specimens were also subjected to B-cell clonality analysis using the consensus primers VH-FRIII, with a reported homology of at least 85% with published VH gene sequences, and Jμ, which anneal to variable and joining gene segments of the immunoglobulin heavy chain gene (IgH), respectively (25).

Each run included appropriate positive [DNA from cell lines Su-DHL6, SC1, OZ and K231, or patient specimens with confirmed translocation t(14;18)], negative (DNA from reactive lymph nodes) and contamination (no DNA template) controls. Furthermore, the DNA quality of each extract was tested in an internal control amplification using primers specific for the ubiquitously expressed β-globin gene (172-bp amplicon) or the exon 19 of the MLH1 gene (289-bp amplicon). All patient specimens were analyzed in duplicate by each PCR. In a case of discrepant results by particular PCR (pos/neg), analysis was repeated and the result of the third test was considered as final.

Detection sensitivity of MBR/JHa and MBR1,2 PCR assays was tested by analyzing serial dilutions of positive control DNA derived from the Su-DHL6 cell line in sterile PCR grade water (1:5, 1:10, 1:20, 1:50, 1:100, 1:10², 1:10⁴, and 1:10⁶), and by mixing Su-DHL6 DNA with the DNA derived from reactive lymph node, with previously confirmed polyclonality of B cells and the absence of t(14;18) (the same ratios as in water dilutions). Starting concentrations of both DNA preparations were adjusted to ~250 ng/µl. We did not evaluate the sensitivity of mcr and far3’-MBR PCR assays, since no positive controls other than the diluted PCR products were available.

LightCycler®-based quantitative real-time PCR (qPCR). Quantitative real-time PCR was performed using the LightCycler® - t(14;18) Quantification Kit (MBR) (Roche Applied Science), specifically adapted for detection of the t(14;18) with breakpoints in the MBR, according to the manufacturer’s instructions. The sensitivity of the method is one t(14;18)-bearing cell in 50,000-100,000 peripheral blood mononuclear cells.

Statistical analysis. Agreement between alternative MBR methods was measured using Cohen’s κ coefficient (a method of comparison study for categorical data) (27). A value of 0 for κ indicates no agreement beyond chance and a value of 1 indicates perfect agreement. κ-values <0.20 were considered as poor agreement, values between 0.21 and 0.40 as fair, values between 0.41 and 0.60 as moderate, values between 0.61 and 0.80 as good and values >0.81 as very good agreement (27). Frequencies of PCR positivity were used for statistical analysis of PCR data.

Results

Sensitivity of qualitative MBR PCR assays. The sensitivity of MBR-PCR assays varied depending on whether positive control DNA (Su-DHL6 cell line) was diluted in sterile water or with the DNA derived from reactive lymph node. The MBR1,2 PCR assay showed greater sensitivity in the water dilution experiment with the detection rate of 10⁻³ compared to the detection rate of 10⁻¹ by MBR/JHa PCR. Sensitivities of both MBR assays were lower in the dilution experiments with DNA derived from reactive lymph node, where the detection limit for both assays was 10⁻². Sensitivities of
MBR1,2 and MBR/JHa PCRs in the dilution experiments are shown in Fig. 1.

Tumor lymph node specimens. Valuable results were obtained for all 57 paraffin-embedded lymph node (LN) specimens from 50 patients.

Detection of the t(14;18)(q32;q21) chromosome translocation. The results of PCR detection of the t(14;18) were highly reproducible. Initially, all specimens were analyzed in duplicate showing the same result in parallel tests. Furthermore, at the end of the study we repeated the analysis of all specimens initially positive for MBR breakpoints and found 100% agreement of results.

We detected the t(14;18) in 30 of 57 (52.6%) specimens by using described methods. Twenty-eight specimens (49.1%) had breakpoints within the MBR and 2 (from 2 patients; 3.5%) had breakpoints within the mcr. There were no breakpoints found within the far3'-MBR. Detection rates of different MBR methods were 31.6% (18/57) for MBR/JHa PCR, 43.9% (25/57) for MBR 1,2 PCR and 47.4% (27/57) for quantitative LC-based PCR.

Comparison of different MBR methods. In order to determine the most appropriate MBR method for analysis of biopsy material, we compared the effectiveness of each assay: MBR/JHa, MBR1,2 and quantitative LightCycler-based PCR (LC) for 28 PCR-positive specimens: 64.3% (18/28), 89.3%
(25/28) and 96.4% (27/28), respectively. Eighteen of the 28 specimens (64.3%) were positive by all three MBR methods, 6/28 (21.4%) were positive by two methods and 4/28 (14.3%) were positive by only one MBR method (Table I). Very good agreement was found between the MBR 1,2 and LC method (Cohen’s $\kappa$ coefficient was 0.854). A good agreement was found between MBR/JHa and MBR1,2 PCR ($\kappa=0.737$) as well as between MBR/JHa and LC PCR ($\kappa=0.671$). Agreements expected by chance would be 92.7, 87.2 and 83.6%, respectively. Examples of qualitative MBR and mcr PCRs are shown in Figs. 2 and 3. An example of the amplification curve obtained by LightCycler-based PCR is shown in Fig. 4.

Table I. Detection of MBR breakpoints in 28 positive LN biopsy specimens.

<table>
<thead>
<tr>
<th>No. and type of method</th>
<th>No.(+)</th>
<th>%</th>
<th>Total no.(+)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Three MBR methods</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBR/JHa + MBR1,2 + LC</td>
<td>18</td>
<td>64.3</td>
<td>18</td>
<td>64.3</td>
</tr>
<tr>
<td>Two MBR methods</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBR/JHa + MBR1,2</td>
<td>0</td>
<td>0.0</td>
<td>6</td>
<td>21.4</td>
</tr>
<tr>
<td>MBR/JHa + LC</td>
<td>0</td>
<td>0.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBR1,2 + LC</td>
<td>6</td>
<td>21.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>One MBR method</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LC</td>
<td>3</td>
<td>10.7</td>
<td>4</td>
<td>14.3</td>
</tr>
<tr>
<td>MBR1,2</td>
<td>1</td>
<td>3.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBR/JHa</td>
<td>0</td>
<td>0.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>28</td>
<td>100.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

No.(+), number of cases positive by particular MBR method(s); total no.(+), total number of cases positive by three, two and one MBR method, respectively.

Clonality analysis by IgH-FRIII PCR. We determined the clonality in 54/57 (94.7%) specimens. Sixteen specimens (28.1%) were monoclonal, 33 (57.9%) were polyclonal and 5 (8.8%) were interpreted as ‘monoclonal in a polyclonal background’ (M/P). We could not determine the clonality in 3 (5.3%) specimens, since we detected neither amplified products nor smears from polyclonally rearranged IgH genes. Notably, these 3 specimens were positive for both control genes β-globin and the MLH1-exon 19 fragment, suggesting that the quality of DNA was appropriate for PCR amplification.
We compared the presence of the t(14;18)(q32;q21) chromosome translocation with the clonality status (Table II). Of the monoclonal cases, 10 were positive for the t(14;18) (9 with breakpoints within the MBR and one within the mcr), and of the polyclonal cases, 16 were positive (all with breakpoints within the MBR). Two M/P cases were also positive for the t(14;18) (one had breakpoint within the MBR and one within the mcr). Two of 3 cases for which clonality could not be determined were positive for the t(14;18), both with breakpoints within the MBR.

### Table II. Clonality status and the presence of the t(14;18) (q32;q21) in 57 LN biopsy specimens.

<table>
<thead>
<tr>
<th>Clonality</th>
<th>No. of cases</th>
<th>% of t(14;18)</th>
<th>% t+ of total</th>
<th>% t+ of M:M/P:P</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>16</td>
<td>28.1</td>
<td>10</td>
<td>17.5</td>
</tr>
<tr>
<td>M/P</td>
<td>5</td>
<td>8.8</td>
<td>2</td>
<td>3.5</td>
</tr>
<tr>
<td>P</td>
<td>33</td>
<td>57.9</td>
<td>16</td>
<td>28.1</td>
</tr>
<tr>
<td>ND</td>
<td>3</td>
<td>5.3</td>
<td>2</td>
<td>3.5</td>
</tr>
<tr>
<td>Total</td>
<td>57</td>
<td>100.0</td>
<td>30</td>
<td>52.6</td>
</tr>
</tbody>
</table>

M, monoclonal; M/P, ‘monoclonal in a polyclonal background’; P, polyclonal; ND, not determined.

We compared the presence of the t(14;18)(q32;q21) chromosome translocation with the clonality status (Table II). Of the monoclonal cases, 10 were positive for the t(14;18) (9 with breakpoints within the MBR and one within the mcr), and of the polyclonal cases, 16 were positive (all with breakpoints within the MBR). Two M/P cases were also positive for the t(14;18) (one had breakpoint within the MBR and one within the mcr). Two of 3 cases for which clonality could not be determined were positive for the t(14;18), both with breakpoints within the MBR.

**Negative control group.** All 15 reactive lymph node specimens (from 15 patients) were negative by qualitative MBR/JHa, MBR1,2, mcr and far3'-MBR PCRs and polyclonal by IgH-FRIII PCR. Notably, 4/15 (26.7%) samples were positive by quantitative LightCycler-based PCR, although in the quantification analysis normalized ratios of target to reference gene copies for those 4 samples were <0.01, suggesting the low number of t(14;18)-bearing cells.

### Discussion

Numerous studies have been undertaken to estimate the usefulness of PCR-based assays for detection of the t(14;18)(q32;q21) chromosomal translocation and relative quantification of t(14;18)-bearing cells in patients with follicular lymphoma (FL). Here we present the results of both qualitative and quantitative detection of the t(14;18) in a retrospective study on a rather large series of lymph node biopsy specimens from Slovenian patients with FL.

We evaluated the sensitivity and the reproducibility of both qualitative and quantitative approaches. The qualitative PCR assays used in our study enabled the detection of breakpoints in three important breakpoint regions: the MBR, the far3'-MBR and the mcr, while the quantitative PCR approach enabled the detection of breakpoints only in the MBR.

Before applying PCR assays on patient specimens, we estimated the sensitivity of qualitative MBR PCRs by analyzing serial dilutions of positive control DNA. The sensitivity of the MBR1,2 assay in the water dilution experiment (1 cell in 10^5) was in agreement with the results from other laboratories (11), but the sensitivity of the MBR/JHa assay in the same experiment was two logs lower (1 cell in 10^3). A higher sensitivity of MBR1,2 assay was expected, since it utilizes two MBR-primers in one reaction, in contrast to MBR/JHa assay, which is a classical single-round PCR with one forward (MBR) and one reverse (JH) primer. Johnson et al (10) showed in an international multicentre study that the cut-off in the detection by single-round PCR is 1 cell in 10^4 in most laboratories. The sensitivity of different PCR assays in this

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**Figure 4.** The amplification curve obtained by using the LightCycler® - t(14;18) Quantification Kit (MBR). Sample (~50 ng) and calibrator (DOHH2) DNA were analyzed. Lymph node samples 2, 5, 8 and 9 were positive and samples 3, 4, 6 and 7 were negative. Normalized ratios T/R (target to reference gene copies) were 0.03, 0.01, 0.06 and <0.01 for samples 2, 5, 8 and 9, respectively. The normalized ratio for the calibrator was set to value 1.0.
study (10) was tested by analyzing blood samples from healthy donors with varying numbers of t(14;18)-bearing cells added from a cell line with a breakpoint in the MBR (DOHH2). Thus, the estimation of sensitivity by analyzing serial dilutions of positive control DNA (Su-DHL6) with negative control DNA (reactive lymph node) is more accurate, although the sensitivity of both MBR assays in our hands was lower than expected [one t(14;18)-positive cell in 100 negative cells]. We did not evaluate the sensitivity of two other qualitative assays, the mcr and the far3'-MBR, because of the lack of appropriate positive controls. As positive controls for these two assays we utilized diluted PCR products obtained by amplifying DNA from cell lines with confirmed breakpoints in the mcr and the far3'-MBR. We also did not evaluate the sensitivity of the LightCycler-based PCR since we used a commercial kit with a previously defined sensitivity.

The overall detection rate of the t(14;18) in our study was 52.6% (30/57). Of the t(14;18)-positive specimens 49.1% (28/57) had breakpoints within the MBR and only 3.5% (2/57) had breakpoints within the mcr. In order to improve the overall detection rate of MBR/JH rearrangements, we employed three different PCR approaches, two qualitative (MBR/JH and MBRJ3) and one quantitative (LightCycler-based) assay. Supporting our expectations, the most sensitive method was LightCycler-based PCR with the detection rate of 47.4% (27/57), followed by MBRJ3 assay with the detection rate of 43.9% (25/57). The MBR/JH assay had the lowest detection rate in our series, 31.6% (18/57). The results of our study are in agreement with other reports. Albinger-Hegyi et al (7) reported a t(14;18) detection rate of 36% by standard MBR/JH and mcr/JH PCR assays applied on frozen tissue specimens from 59 patients with FL. In this study (7) 19 cases (32.2%) had breakpoints within the MBR and only 2 cases (3.4%) had breakpoints within the mcr. Tysarowski et al (19) detected MBR/JH rearrangements in peripheral blood of 41% (31/75) patients with FL by nested PCR. Similarly, Mahfouz et al (24) reported 45.2% (19/42) t(14;18)-positive cases by using the BCL2/JH Translocation Assay (InVivoScribe Technologies, CA, USA) on formalin-fixed, paraffin-embedded specimens. The majority of the cases in this study (24) were positive for the MBR (40.5%), whereas only 2 cases (4.8%) were positive for the mcr. Other investigators reported detection rates of 40-60% by MBR/JH PCR (26,28) and 10-20% by mcr/JH PCR (28). There are few data concerning the efficiency of qualitative real-time PCR assays. Dessars et al (24) reported a detection rate of 56% (18/32) t(14;18)-positive cases by using the BCL2/JH Translocation Assay (InVivoScribe Technologies, CA, USA) on formalin-fixed, paraffin-embedded specimens.

The results in the negative control group consisting of 15 reactive lymph node specimens (from 15 patients) are in agreement with reports from the literature. Although all specimens were t(14;18)-negative by qualitative methods, we detected 26.7% (4/15) positive specimens by the more sensitive, quantitative method. Occasional benign t(14;18)-bearing cells are present in low frequency in 30-40% of normal individuals (30,31). Usually, the low frequency of t(14;18) in benign tissues is below the level of detection using conventional qualitative PCR methods (28), but is detectable by highly sensitive quantitative tests, thus representing the potential for false-positive results (30-32).

In order to obtain additional information on our series of follicular lymphomas, we performed clonality analysis in all cases. Although we expected to find monoclonality in a larger number of cases, there were only 28.1% of monoclonal specimens. Furthermore, we could not determine the clonality in 3 specimens (5.3%), although amplification of control genes suggested that the quality of DNA was appropriate for PCR analysis. Possibly, these cases were missed by standard IgH-FRIII PCR. Several mechanisms interfere with the detection of a clonal population with IgH-FRIII PCR, including inefficient recognition of certain VH families by the consensus FRIII primer, incorrect or incomplete IgH gene rearrangements and mutations in the IgH locus (33). Furthermore, follicular lymphomas are characterized by the presence of abundant polyclonal B cells, which might mask the monoclonal population (33). To note, 2 of 3 specimens for which clonality could not be determined were positive for the t(14;18).

In conclusion, we evaluated the sensitivity and effectiveness of qualitative and quantitative PCR assays for the detection of the t(14;18)(q32;q21) in lymph node biopsy specimens. We found good agreement between qualitative MBRJ3 and quantitative LightCyclerβ-based assay with a slightly higher detection rate of the quantitative method. Sensitivities of both methods in our hands were in agreement with results from other studies. Since LightCycler-based assay detects only breakpoints within the MBR, qualitative PCR should be employed in routine diagnostic settings for detection of breakpoints within the mcr and far3'-MBR regions.

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