Analysis of the Expression of the Hybrid Gene bcl-2/IgH in Follicular Lymphomas

By Pierre Soubeyran, Fernando Cabanillas, and Ming Sheng Lee

To investigate the clinical and biologic significance of the circulating t(14;18)-carrying cells in follicular lymphoma (FL) patients, we analyzed the mbr/JH junction of the hybrid bcl-2/IgH gene simultaneously at the DNA and RNA levels by polymerase chain reaction (PCR) in 37 peripheral blood samples from 37 patients in different remission status: 4 before treatment, 8 during treatment, and 25 in complete remission (CR). Of these 37 patients, 22 were positive either at the DNA or RNA level (8 with active disease and 14 in CR). Among these positive patients, RNA was more often negative for patients in CR (9 of 14 [64%]) than for patients with active disease (2 of 8 [25%]; Fisher’s exact test, P = .09). Among the 14 patients in CR with residual disease, 2 of 5 with RNA positivity relapsed, whereas 1 of 9 with RNA negativity and DNA positivity relapsed with a median follow-up after sample collection of 8 months (range, 4 to 18 months). Simultaneous analysis of the bcl-2/IgH gene at the DNA and RNA level showed heterogeneous patterns of PCR positivity in regards to the evaluation of the biologic activity of the t(14;18)-carrying cells. A larger study and long-term follow-up will help in determining whether the expression patterns in turn reflect the functional status of disease activity in FL patients.

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FOLLCULAR lymphomas (FL) represent a large part of the non-Hodgkin’s lymphomas (NHL). They are characterized by the t(14;18) chromosomal translocation that juxtaposes the bcl-2 gene with the Ig heavy chain gene (IgH), which, in turn, results in the overexpression of a chimeric bcl-2/IgH message. Because the chromosomal breakpoint falls outside the translated portion of the bcl-2 gene, the protein product is identical with the normal bcl-2 protein.

Recently, Hockenbery et al showed that the bcl-2 protein is able to block programmed cell death (apoptosis). Consequently, the rearrangement of bcl-2 with IgH leads to longer survival of the concerned cells that allows secondary transforming events to occur.

Although clinical complete remission (CR) is easily achieved in low-grade FL, the relapse rate is high, raising the problem of minimal residual disease resistant to systemic treatment. Detection of minimal residual disease in FL patients in CR has been very actively pursued over the last years. Different methods have been used: detection of Ig gene rearrangement by Southern blotting,7 clonal excess methods,8 and polymerase chain reaction (PCR).10,11 Whereas Southern blotting and clonal excess methods allow the detection of 1 to 10 abnormal cells among 100 normal cells, PCR is able to recognize one abnormal cell among 100,000 normal cells.10,11 However, recent data tend to show that this minimal residual disease is not closely related to an increased risk of relapse in FL.12,13 All these methods provide information on the presence or absence of putative tumor markers characteristic of the disease, but they fail to examine the functional status of the residual disease. The circulating cells carrying the marker could either be benign or quiescent, so that there would be no imminent threat of disease recurrence. However, if they do represent a residual malignant clone with high proliferative activity, the disease would be expected to relapse early. Such speculations have led us to pursue whether there are any means to examine the biologic activities of the minimal residual t(14;18)-carrying cells, such as the expression of the chimeric bcl-2/IgH messenger RNA (mRNA). In this report, we demonstrated the feasibility of applying PCR to the detection of extremely small amounts of chimeric bcl-2/IgH mRNA produced from circulating t(14;18)-carrying cells.

Consequently, we also studied the clinical values of this RNA PCR assay in FL. Peripheral blood samples were collected and analyzed before treatment, during therapy, and in remission. Peripheral blood appears to be a good choice for residual disease detection because it is easily accessible and it has been previously shown that at least 80% of B-cell lymphomas have monoclonal circulating cells at initial presentation.14

MATERIALS AND METHODS

DNA and RNA extraction. High molecular weight DNA and total cellular RNA were simultaneously extracted from blood samples using a method previously described15 using guanidine thiocyanate, cesium chloride, and ultracentrifugation.

DNase treatment of RNA. The total RNA samples prepared as described above were further treated by DNase I (RNase free) in a total reaction volume of 35 μL (50 mmol/L Tris, pH 7.8; 1 mmol/L EDTA, pH 8.0; 10 mmol/L MgCl2, 1 mmol/L dithiothreitol, 40 U RNAse [Promega, Madison, WI]; 60 U DNase I RNase-free [Boehringer Mannheim Biochemica, Indianapolis, IN]). The reaction was incubated at 37°C for 1 hour and then heated at 95°C for 5 minutes and immediately cooled on ice.

Reverse transcription of RNA. One to 3 μg of these DNase-treated RNA was submitted to reverse transcription. The reaction was performed in a total reaction volume of 40 μL in the following conditions: 50 mmol/L Tris-HCl, pH 8.3; 75 mmol/L KCl; 3 mmol/L MgCl2; 1 mmol of each dNTP; 400 mmol/L random primers (Boehringer Mannheim Biochemica); 5 mmol/L dithiothreitol; 40 U RNase (Promega); and 400 U MMLV reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD) at 37°C overnight.

PCR methods. The sequences of the different oligonucleotide primers used are outlined in Table 1. The primer mbr3+ is derived from the sequence 5' to the major breakpoint clustering region (mbr) of the bcl-2 gene.16 JH1 primer is derived from the consensus sequence that has been described at the 3' end of the six different JH...
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RESULTS

Removal of the DNA contamination from the RNA samples. To ensure that our RNA samples were free of DNA contamination, we used DNase I treatment that was able to completely remove DNA contamination of intentionally contaminated RNA. Two RNA samples were analyzed, one contaminated by 1 μg of DNA per 1 μg of RNA and the other by 10 ng of DNA per 1 μg of RNA. After DNase I treatment, reverse transcription PCR (RT-PCR) of c-raf-1 gene was performed. As shown in Fig 1, it is efficient in removing the DNA contamination of the RNA while maintaining RNA quality. This allowed us to further study patients samples.

Verification of the intactness of RNA samples and effectiveness of reverse transcription. All RNA samples were reverse transcribed using random hexamers as primers and then tested by the c-raf-1 amplification. In all cases, amplification of the c-raf-1 cDNA showed an adequate amount of product, confirming that the RNA quality was good enough for PCR amplification and that the reverse transcription worked correctly.

Confirmation of adequate PCR efficiencies in patients' samples analyses. Sensitivity of our PCR assay was tested on serially diluted DNA samples; from 1 μg to 1 pg of positive DNA was diluted in 1 μg of negative DNA. We could easily detect the hybrid bcl-2/IgH junction in samples containing 10 pg or more of positive DNA (data not shown). When patients samples were amplified, two positive control samples (10 ng and 100 pg) were also amplified in parallel to ensure that PCR was performed at its optimal efficiency.

PCR amplification of the chimeric bcl-2/IgH DNA and RNA in FL patients' samples. Of the 37 patients (4 pre-treatment, 8 during treatment, and 25 in CR), 16 were positive at the DNA level (43%) and 11 at the RNA level (30%). Considering DNA and RNA results together, 22 patients were positive (60%); 11 with DNA positivity/RNA negativity, 5 with DNA positivity/RNA positivity, and 6 with DNA negativity/RNA positivity (Table 3 and Fig 2).

Table 1. Oligonucleotide Primers and Probes' Sequences

<table>
<thead>
<tr>
<th>Mbr3+</th>
<th>JH-</th>
<th>18q21 probe</th>
<th>Raf 8+</th>
<th>Raf 9+</th>
<th>Raf 9 probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' TTT GAC CTT TAG AGA GTT GCT TTA CG 3'</td>
<td>5' ACC TGA GGA GAG GGT GAC C 3'</td>
<td>5' CAG AGA CCC ACC CAG AGC CC 3'</td>
<td>5' GAT GCA ATT CGA AGT CAG AGC G 3'</td>
<td>5' TTT TCT CCT GGG TCC CAG A7A 3'</td>
<td>5' GTC CAG TAG CCC CAA CAA CAA TCT G 3'</td>
</tr>
</tbody>
</table>

Table 2. Main Characteristics of the Patients

<table>
<thead>
<tr>
<th>Presence of Disease</th>
<th>CR</th>
</tr>
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<tbody>
<tr>
<td>(12 patients)</td>
<td>(25 patients)</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>Mean 48</td>
</tr>
<tr>
<td></td>
<td>Range 26-72</td>
</tr>
<tr>
<td>Sex</td>
<td>Male 6</td>
</tr>
<tr>
<td></td>
<td>Female 6</td>
</tr>
<tr>
<td>Stage</td>
<td>I 1</td>
</tr>
<tr>
<td></td>
<td>II 1</td>
</tr>
<tr>
<td></td>
<td>III 3</td>
</tr>
<tr>
<td></td>
<td>IV 7</td>
</tr>
<tr>
<td>Pathology</td>
<td>FSC 11</td>
</tr>
<tr>
<td></td>
<td>FM 1</td>
</tr>
<tr>
<td></td>
<td>FLC 0</td>
</tr>
</tbody>
</table>

Abbreviations: FSC, follicular small cleaved; FM, follicular mixed; FLC, follicular large cell.

segments of the IgH gene.17 These two primers were able to amplify the crossover site sequence of the t(14;18) within the mbr region in approximately 60% to 70% of FL patients. The length of the fragments amplified ranged from approximately 150 to 400 bp. The same primers were used for DNA and cDNA amplification. Because this set of primers does not span any intron, the length of the PCR product is the same in the same patient for DNA and cDNA. Therefore, it is extremely important to rule out the problem of DNA contamination in the RNA sample. Removal of the DNA contamination was performed by DNase I treatment as described above. The effectiveness of removing DNA contamination was confirmed by PCR of c-raf-1 gene. Primer derived from exon VIII of c-raf-1 gene (raf8+) and primer derived from exon IX of c-raf-1 gene (raf9+) span an intron 110 bp in length.18 The PCR product will be 148 bp in length on cDNA amplifies and 258 bp in length in case of DNA contamination. cDNA amplification of the c-raf-1 gene also allows us to directly evaluate the effectiveness of reverse transcription. Because the expression of c-raf-1 is ubiquitous,19 the PCR product represents the whole pool of RNA.

For each patient sample, three different PCR reactions were simultaneously performed in three different tubes with the mbr3+ and JH- primers: 1 μg of DNA, cDNA generated from 0.6 to 1 μg of RNA using random hexamers, 1 μg of RNA without reverse transcription (as negative control) to rule out DNA contamination.

PCR amplification was performed as a modification of the method previously described19 for 45 cycles using a thermal cycler (Perkin Elmer Cetus, Norwalk, CT). Patients were considered positive when they were positive either at the DNA or RNA level.

We followed the recommendations of Kwok and Higushi20 concerning control of contamination. Furthermore, because the breakpoint of each patient is different, the length of the PCR product is different. In this way, PCR contamination is easier to identify than in other models. Finally, we always ran two negative controls (negative DNA and no template) to confirm that our PCR reaction was free of false-positivity secondary to contamination.

Southern blot analysis of the PCR products. Fifteen percent of PCR products were size fractionated in a 2% Nu Sieve gel (FMC Bioproducts, Rockland, ME) and then transferred to a Nytran membrane (Schleicher and Schuell, Keene, NH), as described by Sothern.21 Membranes were then hybridized with a 5′ end radiolabeled oligonucleotide probe 18q21 (Table 1) at 42°C overnight. Washing was performed in 2 SSPE/0.1% SDS at room temperature for 1 hour. Autoradiography was performed against a single intensifying screen at -70°C for 72 hours.

Patients. Thirty-seven blood samples obtained from 37 patients were analyzed, including 12 with active or persistent disease (4 before and 8 during treatment) and 23 during CR. The main characteristics of these two groups of patients are outlined in Table 2. All patients except one were treated with chemotherapy using either CHOP Bleo22 or other regimens with or without radiotherapy. The exceptional patient received exclusive radiotherapy.

The duration of CR has been computed from the date of the first negative work-up (including all initially positive exams) to the date of the sample collection.

Table 3. Oligonucleotide Primers and Probes' Sequences

<table>
<thead>
<tr>
<th>Mbr3+</th>
<th>JH-</th>
<th>18q21 probe</th>
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<td>5' GTC CAG TAG CCC CAA CAA CAA TCT G 3'</td>
</tr>
</tbody>
</table>

Abbreviations: FSC, follicular small cleaved; FM, follicular mixed; FLC, follicular large cell.
Results of samples from 12 patients collected either before or during treatment. Eight of these 12 patients were positive (66%) either by DNA or RNA testing (Table 3). DNA positivity was detected in 6, whereas RNA PCR allowed two more positive diagnoses.

Of these 12 patients, 4 were pretreatment samples. All 4 were positive either by DNA or RNA. One patient showed a strong signal for DNA without any expression of the hybrid message of bcl-2 at the RNA level.

Eight samples were collected during treatment (after 2 to 7 months of treatment by chemotherapy or α-interferon [α-IFN]). Four patients were positive either at the DNA or RNA level. Two of these presented a strong expression of the hybrid message of bcl-2 with either a weaker band (patient B, Fig 2) or not any detectable band at the DNA level (patient E, Fig 2). The remaining 2 patients presented a strong signal at the DNA level with either a weak expression in one case (patient C, Fig 2) or no expression at all at the RNA level. These 4 patients were treated with combination chemotherapy and α-IFN.

Results of 25 samples collected during CR. Fourteen of these were positive either at the DNA or RNA level, with 10 detectable at the DNA level and 5 at the RNA level (Table 3). Of the 10 DNA-positive patients in CR, only one presented a detectable level of expression of hybrid bcl-2/IgH message by PCR. Four patients in CR showed only RNA positivity. The results were further correlated with the duration of remission (Table 4). Eight of 13 patients (61.5%) in CR less than 3 years showed PCR positivity, including 5 with RNA negativity. Six of 10 patients (60%) in CR for 3 to 5 years had PCR positivity, including 4 with RNA negativity.

Our results were also correlated with the pretreatment clinical Ann Arbor stage. Of the eight patients in stages I and II, four were positive, including two at the DNA level only, one at both DNA and RNA level, and one exclusively at the RNA level. None of these localized stages relapsed.

Among 17 patients in stages III and IV, 10 were positive, including 7 exclusively at the DNA level and 3 exclusively at the RNA level.

Three of these 25 patients relapsed 4 to 10 months after the sample collection. All three were of follicular small cleaved cell histology. All of them presented residual disease at the time of PCR analysis, including 2 with hybrid bcl-2/IgH RNA expression (Table 5). All the other patients are still in CR, with a median follow-up after sample collection of 12.5 months (range, 0 to 32.5 months).

Median follow-up computed from date of diagnosis to date last known alive was 49.5 months (range, 12.5 to 91 months) for the 25 CR patients and 15 months (range, 4 to 42.5 months) for the 12 with active disease.

### Table 3. Detection of the Chimeric bcl-2/IgH Junction at the DNA and RNA Level

<table>
<thead>
<tr>
<th></th>
<th>Before Therapy</th>
<th>During Therapy</th>
<th>CR</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA+/RNA−</td>
<td>4 (100)</td>
<td>4 (50)</td>
<td>14 (56)</td>
</tr>
<tr>
<td>DNA+/RNA+</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>DNA−/RNA+</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>DNA−/RNA−</td>
<td>0</td>
<td>4 (50)</td>
<td>11 (44)</td>
</tr>
</tbody>
</table>

Percentages are in parentheses.

![Fig 1. Set-up of the DNase I treatment of RNA. Three percent NuSieve gel stained with ethidium bromide. Lane M corresponds to marker 174-Hae III digested. Lanes 1 and 3 represent, respectively, RNA samples contaminated with 1 μg and 10 ng of DNA per 1 μg of RNA. Lanes 2 and 4 represent the same samples after treatment by DNase I for 1 hour at 37°C. In all cases, 1 μg of RNA was used as template for reverse transcription. Lane 5 is the positive DNA control (1 μg of DNA) and lane 6 the negative control (no template). The DNA band (258 bp) is visible in lanes 1 and 3, whereas it is not visible in lanes 2 and 4, demonstrating the efficiency of the treatment. RNA bands are visible in lanes 1 to 4, showing the integrity of RNA as well as the efficiency of reverse transcription.](www.bloodjournal.org)
DISCUSSION

Analysis of the pattern of expression of the hybrid message of \( bcl-2/lgh \) within circulating residual cells of patients with \( t(14;18) \) to our knowledge has not been reported. PCR is well suited to this kind of study because it provides high sensitivity and can be performed in low amounts of even partially degraded RNA. Because the sequence at the juncture of mbr/IgH region is the same at the DNA and the mRNA levels, the most pertinent technical problem in the mRNA PCR assay for the \( t(14;18) \) is DNA contamination. We have undertaken multiple precautions to control this problem of DNA contamination in RNA, which is commonly observed in the extraction of RNA. First, RNA was cleaned from DNA through a DNase I treatment. Secondly, the efficiency of DNA removal was assessed in two ways: \( c-raf-1 \) amplification and negative RNA control (without any reverse transcription). The \( c-raf-1 \) amplification allowed us to assess both the quality of the RNA and the efficiency of our reverse transcription reaction. This information ensured the reliability of our mbr3-JH RNA PCR because the same pool of cDNA was used for both PCRs.

We also took special precautions to control the risk of contamination from previously amplified positive samples. Indeed, the search for residual disease is highly exposed to this kind of risk.23 The \( bcl-2/lgh \) model is easier than the others in this aspect because it provides an additional control by the size of the PCR product, which is variable from patient to patient. We used multiple negative controls in each reaction and used highly diluted positive controls to limit the production of positive PCR product. Finally, we did not try to increase the PCR efficiency by either increasing the amount of \( T\alpha q \) polymerase or using boosted PCR.12 We preferred the use of hybridization with an internal probe to increase the specificity of our PCR assay. Around 60% to 70% of FL patients with the \( t(14;18) \) have the breakpoints falling within the mbr region.24 Our PCR assay is confined to examining this subpopulation of patients. We observed 66% of positive results among the 12 treatment samples and 56% among the 25 CR samples. Among 27 patients with follicular small cleaved cell histology, 17 were positive (63%); among five follicular mixed cell samples, three were positive (60%); and among five follicular large cell samples, two were positive.

Simultaneously analyzing the mbr/JH junction at the DNA and RNA level, we have observed four patterns of results: (1) negative at both DNA and RNA level; (2) positive DNA and negative RNA; (3) negative DNA and positive RNA; (4) positive DNA and RNA. No firm conclusion can be drawn from the double-negative samples because we do not know whether this represents a tumor without...
a breakpoint at the mbr region of bcl-2 or the absence of residual disease in patients with bcl-2 rearrangement. Furthermore, PCR negativity by no means indicates complete absence of t(14;18)-bearing cells, which could be too few to be detected by PCR. Our current knowledge leads us to expect a strong expression of the hybrid message bcl-2/IgH.2 At the PCR level, we indeed frequently observe a stronger signal for RNA than for DNA. That is what we observed 3 out of 4 times in pretreatment samples (75%), 2 out of 4 times in samples collected during treatment (50%), and 5 out of 14 times in the positive samples among cases in CR (36%).

One of our pretreatment samples exhibited bcl-2 rearrangement at the DNA level without any RNA expression of the same kind. This raises the question as to whether the hybrid bcl-2/IgH message could be downregulated by either extrinsic or probably intrinsic factors to the tumor. One case of absence of expression of the chimeric message of bcl-2/IgH has been described.23 However, it represents a unique t(8;14;18) that juxtaposed the c-myc gene to bcl-2 and IgH. Finally, previous analyses3,4 clearly show that the absence of expression of bcl-2/IgH in cases of t(14;18) is expected to occur infrequently.

Among the four positive samples drawn during treatment, two exhibited an absence or a weak expression of the bcl-2/IgH message. One patient was being treated with CHOP-Bleo combination and the other was being treated with α-IFN. It raises the possibility that at least one of the therapeutic agents may downregulate the RNA expression. This is possible with most of the drugs used in the CHOP Bleo combination and also with α-IFN because it is already known to turn off the c-myc message in some tumors.26 This possibility is also supported by the results of the samples obtained during CR that showed RNA expression only in 5 of 14 positive samples.

Detection of residual bcl-2 rearranged cells in FL has not shown any predictive value on the risk of relapse. Even long-term remission patients with a low risk of relapse are frequently positive.12,13 Two hypotheses can be drawn: either the detected cells are t(14;18)-bearing cells that are not fully malignant [bearing only the t(14;18), but not the secondary genetic events leading to malignant transformation28-29] or they represent tumor cells that are quiescent (temporarily or not). RNA PCR could be informative in this regard because it examines the expression of the chimeric gene that plays an important role in the pathogenesis of the cells carrying the t(14;18). Yet, a large number of patients and longer follow-up are obviously necessary to assess the predictive value of this test in regards to risk of relapse.

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