Frequency and Structure of t(14;18) Major Breakpoint Regions in Non-Hodgkin’s Lymphomas Typed According to the Kiel Classification: Analysis by Direct DNA Sequencing

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ABSTRACT

We have examined 165 unselected cases of non-Hodgkin’s lymphomas for rearrangements involving the t(14;18) major breakpoint region using a polymerase chain reaction (PCR) and direct sequencing of amplified major breakpoint region bcl-2/JH junctional regions. The lymphomas, diagnosed according to the updated Kiel classification, consisted of 33 centroblastic-centrocytic, 37 centroblastic, 27 immunocytic, 14 peripheral T-cell, and 4 unclassified lymphomas. In addition 18 chronic lymphocytic leukemias, 2 hairy cell leukemias, and 6 plasmacytomas were studied. In 17 cases a bcl-2/JH gene fusion sequence was amplified by PCR. A bcl-2/JH gene fusion was detected only in three lymphoma subgroups: 13 of 33 centroblastic-centrocytic (39%), 2 of 37 centroblastic (6%), and 2 of 27 immunocytic (8%) were positive. In two cases, major breakpoint region bcl-2 rearrangements verified by genomic Southern analysis were not detected by PCR. Direct sequencing of all 17 PCR-amplified, previously uncharacterized t(14;18) junctional regions provided corroborating evidence for the specificity of the assay. The procedure gave sequencing results even from limited amounts of lymphoma cells as obtained by fine needle aspiration of lymph nodes or from clinically uninvolved sites. Clone-specific sequences were identified due to the involvement of different JH segments, the variations among the exact JH and bcl-2 breakpoint positions, and the extensive incorporation of junctional region (D-) N-nucleotides. These clone-specific sequences allow accurate identification of clinically occult lymphoma cells and reduce the threat of false positive results. The finding of exceptionally long intervening sequences in some of the junctions and the partial homology with published Dm segments in three cases support the view that some of the putative N-regions harbor Dm regions.

INTRODUCTION

The t(14;18)(q32;q21) chromosomal translocation is the most frequent karyotypic abnormality, characterizing the majority of follicular and some diffuse B-cell lymphomas (1-29). Analysis of the breakpoint junctions revealed that in most cases the break on chromosome 14 occurs within the 5' portion of one of the six JH segments. On chromosome 18, the majority of breakpoints (~60%) are clustered within a 150-base pair mbr, located in the long untranslated 3' portion of the bcl-2 transcript (6, 10, 21-27). In most studies the t(14;18) chromosomal translocation has been detected by cytogenetics or by genomic Southern blot analyses of bcl-2 gene rearrangements (1-5, 7, 8, 10-20, 22, 24, 27). These techniques, however, are time consuming and require fresh, undegraded material. The focusing of the t(14;18) chromosomal breakpoints within clustered regions and the high sequence homology among the 3' portion of each of the six JH segments makes the bcl-2/JH fusion gene well suited for in vitro amplification by PCR, which allows detection of occult lymphoma cells in morphologically or cytogenetically undegraded bone marrow or peripheral blood with extraordinary sensitivity (28-34). PCR procedures, which permit direct visual detection of amplified bcl-2/JH fusion gene sequences in ethidium bromide-stained agarose gels, have been described (29, 31-34). Coupling of asymmetrical PCR for single-stranded DNA preparation with direct sequencing of the product permits rapid and precise nucleotide sequence analysis of previously uncharacterized mbr t(14;18) junctions (32). The relative ease and speed of the method allow analysis of a large number of lymphoma samples. In searching for minimal residual disease, high sensitivity as well as high specificity of the used assay are absolutely required, due to the low copy number of clone-specific gene sequences in the DNA sample. There is also a great risk of obtaining false positive results by PCR, due to contamination of the laboratory environment and reagents with previously amplified DNA (32). These complications may influence the results seriously, when PCR is used for staging or for clinical follow-up studies of patients. False positive PCR signals, derived from previously amplified mbr bcl-2/JH junctions, can easily be identified by virtue of clone-specific identification sequences, which are generated by the use of different JH segments, variations in the exact breakpoint positions on both chromosomes, and the incorporation of extra nucleotides in the junctional region N-segments (32).

The histological classification of NHL was based on the International Working Formulation (35) in almost all previous studies investigating the t(14;18) chromosomal translocation by cytogenetics, genomic Southern blot, or PCR. However, malignant lymphomas, typed according to the updated Kiel classification (36), have not been analyzed extensively. Furthermore, the frequency of mbr bcl-2 rearrangements in the different histological subsets has not yet been defined in detail (11, 20). Therefore, we screened 165 unselected cases, which were subdivided into the different groups of the updated Kiel classification of NHL, for the presence of mbr bcl-2 rearrangements, by PCR and partly by genomic Southern analysis. This study defines the Kiel-NHL subsets in which the mbr t(14;18) PCR might be useful for monitoring the extent of the disease during staging and clinical follow-up studies. For confirmation of the results each of the amplified mbr bcl-2/JH chromosome junctions was sequenced. Another purpose of this study was to analyze the molecular structure of the junctional regions at the DNA sequence level in a number of previously uncharacterized mbr bcl-2/JH fusion genes. Detection of clone-specific mbr t(14;18) sequences in DNA extracted from biopsy specimens of clinically uninvolved sites allowed reliable identification of occult lymphoma cells. In addition we demonstrate that oligonu-
cleotides, complementary to the junctional N-region target sequences, can be used as primers in a clone-specific PCR. PCR gives sequencing results within a short time even from limited amounts of material, such as fine needle lymph node aspirates.

MATERIALS AND METHODS

Lymph node biopsies, lymph nodes, bone marrow aspirates, and peripheral blood were obtained from patients at the University Hospital of Göttingen. Part of the biopsy material was submitted for diagnostic purposes to the Institute of Pathology in Berlin. Clinical details and PCR results of patients 4-5, 7-9, 10, 12, 13, and 20 have been described previously (32, 33). All patients with a histological diagnosis of NHL from which DNA was available were included in the study. No other exclusion criteria were applied.

NHL was diagnosed according to the updated Kiel classification (36). In some cases with leukemic low-grade lymphoma the diagnosis was based on bone marrow or peripheral blood smear cytology and immune cytology. Immunohistological analysis was performed with monoclonal antibodies from DAKO (Copenhagen, Denmark) against the antigens CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD19, CD22, CD23, HLA-DR, IgM, κ, and λ. Of the 165 cases 151 were B-cell and 14 were peripheral T-cell lymphomas. The B-NHL cases included 96 low-grade lymphomas (33 CB-CC, 10 CC, 18 chronic lymphocytic leukemias, 27 immunocytic lymphomas, 2 hairy cell leukemias, and 6 plasmacytomas) and 55 high-grade lymphomas (37 CB, 10 immunoblastic lymphomas, 2 lymphoblastic lymphomas, 2 Ki-1-positive anaplastic large cell, and 4 unclassified). High-molecular-weight DNA was extracted according to standard procedures (37) from frozen tissue blocks, whole peripheral blood, or bone marrow cells. Mononuclear cells were isolated by Ficoll-density-gradient centrifugation (Biochrom AG, Berlin, Germany). Lymph node aspirates yielded 10^5-10^6 mononuclear cells/sample, of which 10^5-10^6 cells were directly subjected to PCR without a DNA extraction step.

Genomic Southern Blot Analysis. Genomic DNAs were cut with appropriate restriction enzymes (EcoRI or HindIII for JH; BamHI, SstI, or PstI for bcl-2), followed by electrophoresis in 0.8% agarose gels (type I agarose; Sigma, Deisenhofen, Germany). Filters were hybridized with complementary DNA probes which were radiolabeled with [32P]dCTP (Amersham-Buchler, Braunschweig, Germany) by random priming (Multiprime kit; Amersham-Buchler). The JH probe (38) was kindly provided by Dr. P. Leder (Boston, MA), and the mbr probe (2.1-kilobase EcoRI/HindIII fragment; probe b of Ref. 10) was kindly provided by Dr. Tsujimoto and Dr. C. Croce, The Wistar Institute, Philadelphia, PA.

PCR. Forty cycles of PCR were performed on an automated thermocycler (Biomed, Theres, Germany) in 100 μl PCR buffer (10 mM Tris-HCl, pH 8.3-5.0, 1.5 mM KCl, 50 mM MgCl2, 200 μM concentration of each deoxynucleotide-0.02% gelatin) containing 1 μg of DNA or 10^5-10^6 isolated mononuclear cells, 2.5 units of recombinant Taq polymerase (AmpliTag; Perkin Elmer-Cetus, Emerville, CA), and approximately 50 pmol of each primer as described previously (32, 33). The oligonucleotide primers used in this study are listed in Table 1. The relative positions of the primers and the internal bcl-2 hybridization probe are shown in Fig. 1. Primers 1 and 2 were routinely used, followed by sequential amplification of 2% aliquots with primers 1 and 3. Cycle conditions with primers 1 and 2 (or 9) were 60 s at 92°C (denaturing) and 30-60 s at 65-70°C (annealing and chain elongation) with the denaturation step extended to 7 min at the beginning of the first cycle. Electrophoresis of amplified DNA was carried out in 2.5% agarose gels, containing 0.1% ethidium bromide, photographed under UV (254 nm) illumination, and transferred to nylon filters (Biodyne B, Pall Portsmouth, England) in 0.4 N NaOH. Filters were prehybridized for 4 h at 55°C in a solution containing 6× SSC, 1% SDS, 10× Denhardt’s, and 50 μg/ml salmon sperm DNA and hybridized for 12 h at 55°C with a 32P-labeled internal bcl-2 oligonucleotide probe ([32P]dCTP; specific activity, >1 × 10^5 cpm/μg) in 6× SSC and 1% SDS. The filters were washed at 55°C in a buffer containing 1× SSC and 1% SDS, followed by a final stringent washing at 60°C in 0.1× SSC and 0.1% SDS.

Table 1 Oligomers used for polymerase chain reaction and internal bcl-2 hybridization probe

<table>
<thead>
<tr>
<th>Oligo-primers</th>
<th>Nucleotide positions</th>
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<tr>
<td>1: 5’ GTTACGAGGGTCCCTTGCCAGCC 3’</td>
<td>2866-2898</td>
</tr>
<tr>
<td>2: 5’ GCAATTCCGATATTATCTGATTACGGATGAT 3’</td>
<td>3008-3022</td>
</tr>
<tr>
<td>3: 5’ CATTAGGATACATGC 3’</td>
<td>2888-2905</td>
</tr>
<tr>
<td>5: 5’ ACCTGAAGAGAGCCG 3’</td>
<td>2992-3007</td>
</tr>
<tr>
<td>6: 5’ TGCCTTTGGGCCCCAGC 3’</td>
<td>2930-2947</td>
</tr>
<tr>
<td>7: 5’ TTAGAGATTGCTTTA 3’</td>
<td>2997-3011</td>
</tr>
<tr>
<td>8: 5’ GAGGGAGCCAGCCAGGTTGCCC 3’</td>
<td>3020-3065</td>
</tr>
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DNA Sequencing. Direct sequencing of purified double- or single-stranded DNA was done according to the dideoxy chain termination method (39) with the T7 sequencing kit (Pharmacia, Freiburg, Germany), following the previously described protocol (32).

RESULTS

Genomic Southern Blot Analysis. Of the 165 lymphoma patients DNA samples from 59 were investigated by genomic Southern blot analysis for the presence of mbr bcl-2 rearrangements. A rearranged bcl-2 restriction fragment was found in 11 cases, with 9 of 19 (47%) CB-CC positive and 2 of 14 (14%) CB-NHL positive. All 59 cases analyzed showed rearranged JH segments, indicating that enough tumor cells and sufficient DNA were present in the samples (data not shown).

mbr bcl-2/JH PCR. Oligonucleotide primers of different lengths and positions on the bcl-2 and JH gene segments were used for amplification of mbr bcl-2/JH fusion genes. Specific PCR bands were obtained after 40 cycles of PCR with primers 1 and 2 (alternatively, primers 1 and 9) at MgCl2 concentrations between 2.5 and 10 mM (routinely 2.5 mM). Sequential amplification of a 2% aliquot of the 40-cycle PCR product for a further 25 cycles with the JH primer 1 and the nested bcl-2 primers 3, 7, or 10 yielded bcl-2/JH PCR fragments 142-157 base pairs smaller than the products obtained during reactions with primers 1 and 2. After reamplification with the nested primers 3, 7, and 10 at an annealing temperature of 50°C, unspecified bands, ranging from 230 to 600 base pairs, were observed in addition to the specific bands of approximately 142-157 base pairs.
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180–220 base pairs (see Fig. 2). Raising the annealing temperature from 50 to 55°C reduced the number of unspecific bands significantly. However, the sensitivity is reduced near the melting point at 60°C (data not shown). At 63°C the specific PCR bands vanished almost completely, and numerous unspecific bands appeared, ranging from about 200 to 1000 base pairs. Optimal results were obtained with the bcl-2 primers 3 or 7 together with JH primer 1 at an annealing temperature of 55°C.

PCR with genomic DNAs from the 165 lymphoma samples resulted in 17 cases of distinct bcl-2/JH fusion gene segments, which could be visually detected after electrophoresis in ethidium bromide-stained agarose gels. These results could be verified by Southern blot analysis, using an internal bcl-2 hybridization probe. Representative PCR results for 17 lymphoma DNA samples are shown in Fig. 3. Specific bcl-2/JH signals were obtained in 13 of these 17 lymphomas. No amplified band was seen in three negative controls without genomic DNA (Fig. 3, Lane 1) and in two lymphoma DNAs lacking bcl-2 rearrangements on genomic Southern blots (patients 2 and 3). Our PCR techniques failed to produce specific products in two cases (Fig. 3, Lanes 11 and 18) which showed clearly detectable bcl-2 rearrangements on genomic Southern blot analysis. In one case (patient 15) PCR amplification with bcl-2 primers 2 or 9 together with JH primer 1 yielded a distinct PCR fragment. However, sequential amplification with the nested primer 3 failed to amplify the 142-base pair shorter DNA fragment. Filter hybridization with the radiolabeled bcl-2 probe also failed to detect a specific band in this case. Because the PCR fragment was rather short (~200 base pairs) compared to the other bcl-2/JH PCR fragments (range, 250–350 base pairs with primers 1 and 2) it seemed likely that in this particular case the break on chromosome 18 had occurred 5' to the target sequences for primer 3. The specificity of the mbr bcl-2/JH PCR was confirmed by DNA sequencing of the amplified mbr junctions. DNA sequences from both strands of the chromosomal t(14;18) junctions were obtained by using bcl-2 sequencing primers 3 and 7 or, alternatively, the JH primers 6 or 8. The results are summarized in Fig. 4. In all cases (including patient 15) a fusion gene could be demonstrated between one of the six JH segments (38) and bcl-2 sequences (25). Because the bcl-2 breakpoint in patient 15 was in the 5' position relative to primer 3 and was not covered by our bcl-2 oligonucleotide probe, no specific signals were obtained with either method in this particular case. Without exception the bcl-2 breakpoints occurred within the known 150-base pair mbr region between nucleotide positions 3073 and 3225 of the published bcl-2 sequence (25). Six of the 17 breakpoints were focused in a 5-base pair area. In three pairs of cases (patients 14 and 19, 8 and 22, and 20 and 24) each pair of patients shared identical bcl-2 breakpoint positions, but these could be easily distinguished by virtue of their differing JH breakpoints and unique extra nucleotide junctional (D-) N-regions. The structure of the bcl-2/JH junctions is summarized in Fig. 4. The 17 intervening sequences between the breaks on bcl-2 and JH differed extensively in size (1–40 base pairs), and none of these insertions had significant homology to the others. Some of them are longer and less GC rich than normal N-regions (40, 41). Three cases (cases 8, 10, and 19) contained stretches of strong homology with known Dλ regions (Table 2). Interestingly, our patient 8 and Cotter’s patient E, whose junctional region contained a complete recognizable Dλ element (31), share an identical stretch of 11 bases in their intervening sequence (Table 2; Fig. 4). The finding of long N-regions with homology to Dλ sequences (Fig. 4) supports the hypothesis that some putative N-regions may harbor previously unrecognized Dλ sequences. At present the exact number of human D segments is still not known, and it is not clear whether these short sequences of variable length might also combine with each other (38, 42–46). Comparison of the intervening (D-) N-sequences with Genebank and EMBL data bank sequences (Genepro; Riverside Scientific Enterprises, Seattle, WA) revealed further similarities to published Dλ heavy chain diversity segments and published CDRIII sequences (data not shown).

On chromosome 14, the breakpoints were located at different positions within the 5' portions of the J5 segment. Ten of 17 cases showed an involvement of a J5Δ in the recombination event, while only two J5Δ5, four J5Δ, and one J5Δ3 fusion to bcl-2 could be found. JHΔl, J5Δ2, and the J5 pseudogenes were not involved in any of the 17 fusion genes. A point mutation (A to T transition) was found in J5Δ6 of patient 16 in comparison with the embryonic germ line J5Δ6 sequence. In three cases we observed an identical triplet exchange within J5Δ6 (GGT versus TAC in cases 8, 10, and 14) which changes a phenylalanine codon to a glycine codon. Identical exchanges within the J5Δ6 coding region have been observed in three previously published cases (24, 38, 47) and therefore likely represent a polymorphic variation within J5Δ6. All identified bcl-2 breakpoints in the mbr are shown in Fig. 5 relative to their positions to the bcl-2 PCR primers 2, 3, and 9 and the bcl-2 probe.
The considerable potential of the PCR amplification and sequencing procedure could be demonstrated in two cases where only fine-needle lymph node aspirates were available. In one patient with stage IV CB-CC lymphoma (Fig. 6, W. F.), 1.2 × 10⁸ mononuclear cells were obtained by fine-needle biopsy of an enlarged lymph node followed by Ficoll density centrifugation. Some of these cells (10⁶) were directly subjected to 100 µl PCR relation mixture with primers 1 and 9. A clearly visible PCR fragment was obtained after 40 cycles of amplification. The specific fragment was asymmetrically amplified and sequenced directly (Fig. 6). Another patient (Fig. 6, K. U.) was in complete clinical remission 6 months later. After a total of 65 months and relapsed in 1988 with an enlarged right submandibular and preauricular lymph node (33). Fine-needle aspiration cytology of the preauricular lymph node was consistent with a CB-CC NHL. DNA extraction from an aliquot of the aspirated lymph node cells yielded about 1 µg of DNA, insufficient for genomic Southern blot analysis. However, PCR amplification of the t(14;18) chromosomal breakpoint enabled direct DNA sequencing. Sequence analysis revealed a unique gene fusion between J₅ and bcl-2, linked by an extremely G-rich N-region (Fig. 6).

The frequency of the mbr bcl-2/J₅ gene fusion in the various types of lymphomas, subdivided according to the updated Kiel classification (36), is summarized in Table 3. As expected, mbr bcl-2/J₅ is most frequent in the CB-CC group, in which 13 of 33 cases (39%) were positive. Only 2 of 37 (6%) centroblastic lymphomas and 2 of 27 (8%) immunocytomas had a mbr bcl-2/J₅. In 69 other lymphomas, including 18 chronic lymphocytic leukemias and 10 immunoblastic, 10 centrocytic, and 14 T-cell lymphomas, no mbr bcl-2/J₅ gene fusion was visualized by PCR. The PCR detection of mbr bcl-2/J₅ was complemented by Southern blot analysis, using a complementary DNA mbr probe for hybridization in 59 cases (19 CB-CC, 14 CB, 9 immunocytic lymphoma, 9 chronic lymphocytic leukemia, 6 CC, 1 anaplastic large cell, and 1 immunoblastic lymphoma). A bcl-2 mbr rearrangement was found in 9 (47%) CB-CC and 2 (14%) CB lymphomas. The other NHL subtypes in this group were negative for bcl-2. In two CB-CC lymphomas, demonstrating a clear mbr bcl-2 rearrangement on Southern blot analysis, PCR failed to detect a mbr bcl-2/J₅ gene fusion (Fig. 3, 11 and 18). On the other hand, PCR detected four CB-CC cases with an mbr bcl-2/J₅ gene fusion, where Southern analysis failed to show a bcl-2 rearrangement (patients 11, 12, 14, and 20) which was due to size-degraded DNA in patient 14 or a limited number of tumor cells in the sample (bone marrow aspirate in patient 12 and lymph node aspirate in patient 20). In one case (patient 13), where intact and sufficient tumor cell DNA was available, no mbr bcl-2 rearrangement was detectable in Southern blot experiments after digestion with PstI, SstI, and BamHI. In summary, combined PCR and genomic Southern blot analysis detected a bcl-2 rearrangement in 13 of 19 CB-CC (68%) and 2 of 14 CB (14%) NHLs.

Detection of Occult Lymphoma Cells. We recently demonstrated that lymphoma cells were still circulating in the blood of a patient with stage IB CB-CC non-Hodgkin's lymphoma during complete clinical remission (33). Amplification of bcl-2/J₅ fusion gene sequences was performed with DNA, extracted from the involved lymph node at diagnosis and with DNA from peripheral blood mononuclear cells, which were collected in complete clinical remission 6 months later. After a total of 65
rounds of amplification (40 cycles with primers 1 and 2, followed by 25 cycles with primers 1 and 3) a faint band was seen in ethidium bromide-stained agarose gel after electrophoresis. It was the same size as the intense bands seen with lymph node DNA (Fig. 7). The specific PCR bands, obtained from the lymph node and peripheral blood DNA, were excised from the gel, asymmetrically amplified by PCR (32), and directly sequenced. The bcl-2 and JH breakpoints and the unique N-Region clone-specific PCR. Sequencing every PCR-derived DNA fragment is not a practical approach when analyzing serial blood or bone marrow samples from individual patients during long-term follow-up studies. Therefore we constructed clone-specific PCR primers, which were designed on the basis of the sequence data from the unique (D-) N-regions and the flanking bcl-2 and JH segments. Their sequences were complementary inverse to the coding strand of the junctional region sequences. The “N” primers were chosen to be between 17 and 29 base pairs in length and replaced the JH primer in a PCR together with the appropriate bcl-2 primer. This approach was successfully applied to five lymphomas exhibiting specific junctional region sequences. Amplification with clone-specific PCR primers was seen only when using genomic DNA of lymphomas from which the clone-specific sequences had been derived (Fig. 8). The specificity of the amplification was not influenced by the inclusion of up to 17 bases at the 5’ portion of the germ line J56 gene (patient 7) or six bases (patient 4) of bcl-2 sequences at the 3’ portion of the clone-specific primers (Fig. 7).

**DISCUSSION**

Laboratories using the extremely sensitive PCR technique as a diagnostic instrument must follow stringent precautions to avoid false positive results due to contamination with previously amplified target sequences or cross-contamination with truly positive samples (48). We describe PCR amplification in search for mbr bcl-2/JH fusion in a series of 165 unselected NHLs. In 17 cases a gene fusion could be demonstrated between bcl-2 and one of the six functional JH segments by direct sequencing of amplified DNA. The chromosome junctions of the 17 cases differed significantly from each other by virtue of their unique (D-) N-region sequences and different chromosome 14 and 18 breakpoint positions.

Since all mbr t(14;18) PCR products were characterized by DNA sequencing, the results reported here show the highest possible degree of accuracy and reliability.

Crescenci et al. (29) were the first to characterize a previously unrecognized bcl-2/JH junction by PCR amplification and direct sequencing. They observed a marked increase in specificity of bcl-2/JH by shortening their primers from 24-mers to 15-mers and by raising the annealing temperature to 61°C. In contrast, our experimental setup yielded specific bcl-2/JH bands on ethidium bromide-stained agarose gels using relatively long primers (a 25-mer for JH and a 33-mer for bcl-2) and high annealing temperatures (65–70°C) in a rapid two-temperature step PCR procedure (33). Use of the universal JH primer 1, which differs by only 1–3 base pairs from the 6 JH segments and which shares 6 identical bases on its 3’ end with each of the JH segments, amplified bcl-2 sequences fused to JH1, JH2, JH3, JH4, JH5, and JH6. Since primer 1 should also anneal to JH1 and JH2 with equal efficiency, the lack of bcl-2 sequences fused to JH1 and JH2 in our study reflects the low frequency of those fusions and cannot be explained by technical causes.

We observed a clear preponderance of the J56 segment (10 of 17 cases) in the bcl-2/JH junctions. Recently, Cotter et al. (31) sequenced mbr bcl-2/JH junctions and found the J56 segment to be involved in 5 of 7 cases. The breakpoints on bcl-2 in our study fell within the known 150-base pair mbr region, and some of the breakpoints lie exactly at the position of
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Fig. 7. PCR analysis of DNA, extracted from the diagnostic lymph node (LN) at first presentation and from peripheral blood lymphocytes (PBL) collected 6 months later during complete clinical remission in a patient with stage IB CB-CC lymphoma (Fig. 4, patient 5). A faint band is seen with PBL-derived DNA after amplification for 40 cycles with primers 1 and 2, followed by sequential amplification for a further 25 cycles with primer 1 and the nested primer 3. This faint band is of the same size as the intense band seen with lymph node DNA (LN) and is also identical, with respect to the intervening N-region and bcl-2 and JH breakpoints (bottom right), to the intense band seen with lymph node DNA (bottom left).

Our results provide further evidence that some of the putative N-insertions may indeed harbor fragments of unrecognized D H segments (31). The recently described PCR-based method for the determination of heavy chain immunoglobulin gene CDR III region sequences (49) will yield a substantial amount of new data regarding as yet unrecognized D H region sequences in the near future. It can be expected that comparison of such data with the t(14;18) junctional region sequences will also provide more detailed insights regarding the structure and underlying mechanism of the bcl-2/J H fusion.

PCR may fail to detect a mbr bcl-2/J H fusion if the break on bcl-2 lies outside the range of the bcl-2 primer used in this study (Ref. 23, case 2). With the exception of this one case, however, all of the 15 previously published mbr bcl-2/J H fusions would have been amplified by primers 1 and 2. Sequential amplification with a nested bcl-2 primer was useful as a rapid test for the specificity of the reaction.

We detected mbr bcl-2/J H fusion genes by PCR amplification in only three lymphoma subtypes of the updated Kiel classification. The highest incidence was observed in the low-grade CB-CC lymphomas (13 of 33 cases; 39%) and was rare in CB (2 of 36 cases; 6%) and immunocytic (2 of 25 cases; 8%) lymphomas. In contrast, cytogenetic and Southern blot analyses detected the t(14;18) chromosomal translocation in 30–93% of the low-grade follicular lymphomas and in 3.6–44% of the diffuse large-cell lymphomas (1–5, 8, 10–12, 14–16, 18, 19). The break on chromosome 18 occurs at positions outside of the mbr in a substantial portion of cases (4, 27, 34). Therefore, the lower detection rate of the mbr t(14;18) PCR, compared to Southern or cytogenetic analyses, is to be expected.

Recently PCR was used to detect mbr bcl-2/J H fusions in formalin-fixed paraffin-embedded tissue biopsies of 96 non-Hodgkin's lymphomas, 6 Hodgkin's diseases, and 11 reactive lymph nodes (13). In this series again, the incidence was highest in the low-grade follicular lymphomas (23 of 43 cases; 53%). Intermediate-grade (2 of 24 cases; 8%) and high-grade (1 of 24; 4%) lymphomas were rarely positive, which is in good agreement with the results of the present study. Inclusion of mcr primers with mbr primers into one reaction vessel may allow the simultaneous screening of lymphoma samples for bcl-2/J H fusions involving the mbr and mcr by PCR (31, 34).

Two studies determined the frequency of bcl-2 mbr rearrangements by Southern blot analysis in 56 (11) and 61 (20) NHLs, subdivided according to the updated Kiel classification. In one study (20), the frequency of mbr bcl-2 arrangements for CB-CC lymphomas was 37.5% (3 of 8 cases), and in CB lymphomas it was 10% (1 of 10 cases). In the other study (11), the incidence of bcl-2 rearrangements analyzed with mbr and mcr probes was 69% (18 of 26 cases) in the CB-CC subgroup and 55% (11 of 20 cases) in the CB subgroup. In 56% of the cases with bcl-2 rearrangement, the mbr region was involved. With the exception of four lymphoblastic lymphomas, two of which had a coexisting t(8;14) c-myc rearrangement, a bcl-2 rearrangement was found only in the CB-CC and CB subgroups.

Previously determined breaks (6, 23, 25, 29, 31).

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Two studies determined the frequency of bcl-2 mbr rearrangements by Southern blot analysis in 56 (11) and 61 (20) NHLs, subdivided according to the updated Kiel classification. In one study (20), the frequency of mbr bcl-2 arrangements for CB-CC lymphomas was 37.5% (3 of 8 cases), and in CB lymphomas it was 10% (1 of 10 cases). In the other study (11), the incidence of bcl-2 rearrangements analyzed with mbr and mcr probes was 69% (18 of 26 cases) in the CB-CC subgroup and 55% (11 of 20 cases) in the CB subgroup. In 56% of the cases with bcl-2 rearrangement, the mbr region was involved. With the exception of four lymphoblastic lymphomas, two of which had a coexisting t(8;14) c-myc rearrangement, a bcl-2 rearrangement was found only in the CB-CC and CB subgroups.

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Percutaneous fine-needle aspiration is a safe, rapid, and atraumatic technique for the cytological examination or Southern analysis of enlarged lymph nodes (50–53). Some lymphoma subtypes, such as CB-CC, have been considered impossible to recognize by cytological examination alone (50, 52). PCR should improve the diagnostic accuracy of fine-needle aspiration for this group of aspiration specimens, since CB-CC lymphomas have the highest incidence of PCR-amplifiable mbr bcl-2/JH junctions. The major advantage of applying PCR to fine-needle aspiration specimens is the extreme sensitivity of this technique. In addition, multiple sites can be conveniently and atraumatically sampled by DNA, facilitating more complete staging or restaging of patients during the course of their disease. It should be noted, however, that a positive (t(14;18) PCR signal does not permit precise histological subtyping of lymphomas. Most importantly, it cannot detect or rule out the presence of histological conversion into a high-grade malignancy. Twelve of the 33 analyzed CB-CC lymphomas contained areas with histological conversion from low-grade CB-CC to a high-grade CB lymphoma. Of these, five were positive for mbr bcl-2/JH. On the other hand, eight of the 21 CB-CC NHLs which lacked such a histological conversion were also mbr (t(14;18) positive. Only a coexisting (t(14;18) translocation seems to be rather specific for a high-grade lymphoblastic lymphoma emerging from a low-grade CB-CC non-Hodgkin's lymphoma (11, 54–56). It can be speculated that the high-grade malignant CB lymphomas carrying the (t(14;18) may have progressed from a clinically undetected low-grade CB-CC NHL (“secondary” CB-NHL). This view is supported by the finding of additional chromosomal aberrations during clinical progression in a substantial portion of lymphoma patients (4, 14–18) and by the fact that, like CB-CC, the secondary CB lymphomas and especially those carrying the (t(14;18) are mostly incurable by chemotherapy (4, 5).

The mbr (t(14;18) breakpoint regions are especially useful as clonal markers at the DNA sequence level for the detection of occult lymphoma cells and for the generation of diagnostic oligonucleotide probes that do not coamplify or cross-hybridize with the bcl-2/JH sequences of unrelated (t(14;18) clones. In the search for occult lymphoma cells or in other situations, where high sensitivity as well as high specificity are absolutely required, these clonal markers will reduce the risk of obtaining the false positive results inherent to PCR-based methods (32, 48).

The presence of circulating lymphoma cells in complete clinical remission or in patients with stage I or II disease has been demonstrated in 20–60% of the low-grade follicular lymphomas by immunoglobulin gene rearrangement studies or by flow cytometric k/λ analysis (57–60). The sensitivity of PCR far exceeds the detection limit of these techniques, and thus it is not surprising that PCR detects occult (t(14;18) cells in virtually all of the cases which have been studied thus far (28–30, 33). However, at present it is still unknown whether the minor populations of clinically occult (t(14;18) cells detected by PCR represent fully malignant clones causing clinical relapse in every case (29, 30). This open question may be answered only in prospective clinical trials. The ability to amplify (t(14;18) junctional regions by PCR from fixed paraffin-embedded tissue (13, 61) now allows us to analyze the largely available archival samples at the DNA sequence level. Thus, the described methods will also open up the possibility of studying patients in long-term clinical remission from CB-CC NHL.

In a very recent study an unexpectedly high frequency (32%) of mbr bcl-2/JH fusions have been detected by PCR in Hodgkin's disease (62). The DNA amplification and sequencing strategy, as described here, should be helpful in resolving this important issue of specificity in this unobserved experiment.

REFERENCES

Frequency and Structure of t(14;18) Major Breakpoint Regions in Non-Hodgkin's Lymphomas Typed According to the Kiel Classification: Analysis by Direct DNA Sequencing


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