Detection of Chromosomal Translocation t(14;18) Within the Minor Cluster Region of bcl-2 by Polymerase Chain Reaction and Direct Genomic Sequencing of the Enzymatically Amplified DNA in Follicular Lymphomas

By Bo-Yee Ngan, Jamison Nourse, and Michael L. Cleary

A majority of t(14;18) translocations have been shown to cluster at one of two sites on chromosome 18, called the major breakpoint region (mbr) or the minor cluster region (mcr), which map within or flanking the bcl-2 proto-oncogene, respectively. We have determined the nucleotide sequence for a portion of the mcr, and constructed oligonucleotides that were used to perform the polymerase chain reaction (PCR) in conjunction with universal immunoglobulin primers to specifically amplify t(14;18) breakpoints in DNA obtained from follicular lymphomas. Eight of 10 breakpoints that were detectable on Southern blots using DNA probes for the mcr could be detected due to specific amplification by the PCR technique using an mcr-specific primer. Direct nucleotide sequencing of the enzymatically amplified DNAs showed that the breakpoints clustered within a 500 nucleotide region, and five occurred within three nucleotides of each other. These data show a remarkable clustering of some t(14;18) breakpoints at a site on chromosome 18, at least a 30-kb distance from the bcl-2 gene. Our findings also indicate that mcr-specific primers may be used in conjunction with previously described mbr-specific primers in a highly sensitive DNA amplification technique to detect a large fraction of t(14;18) breakpoints.

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Nucleotide sequence analyses. The DNA sequence for the germline configuration of the t(14;18) mcr was determined using a modified T7 DNA polymerase (Sequenase) with the dideoxy chain termination method on DNA fragments subcloned into M13 single-stranded phage vectors. The subcloned and sequenced chromosome 18 DNA fragments (Fig 1) consisted of portions of a 4.5 kb EcoRI fragment containing the previously described pFL2 fragment.4 For direct sequencing of amplified t(14;18) breakpoint DNA, products of the above described PCR reactions were purified by Centricon filtration; then one fifth was used as template for an asymmetrical amplification using identical conditions as above, except that only one oligonucleotide (usually MC4) was added to the reaction. Following Centricon purification of the product, one fifth was directly sequenced using either MC7 or MC11 as sequencing primers with the modified T7 DNA polymerase, a three-minute labeling reaction at 8°C, and a three-minute termination reaction at 56 to 58°C.

RESULTS AND DISCUSSION

Several previous studies have shown that approximately 60% of t(14;18) breakpoints occur in the bcl-2 gene at the mbr site. Of the remaining breakpoints, most have been shown to occur at various sites flanking the bcl-2 gene at either its 5’ or 3’ ends.2,4-6,15 We have reported that many breakpoints not located in the mbr are detectable by routine Southern blot analysis using a single chromosome 18 DNA probe (pFL-2).4 The region of chromosome 18 defined by this probe could not be shown to have any structural relationship to the bcl-2 gene since it mapped at least 20 kb away, thus defining a distant second site (mcr) for clustering of a minority of t(14;18) breakpoints.

To further investigate this region of chromosome 18 and the translocation breakpoints that were detectable with the pFL-2 probe, selected nucleotide sequencing was carried out on germline chromosome 18 DNA containing and flanking the pFL-2 DNA fragment. The resulting sequence (Fig 1) was used to construct oligonucleotides for use in PCR reactions in combination with a consensus Ig JH primer. These primers were used to study DNA specimens from a set of lymphomas that were selected solely because they had detectable chromosome-18 DNA rearrangements on Southern blots using the mcr-specific probe pFL-2. When a chromosome 18 primer (MC8) was used with the Ig JH primer on these DNA specimens, a majority was found to contain amplified DNA bands (Fig 2) as detected with a radiolabeled oligonucleotide homologous to the amplified segment of chromosome 18 (MC12). Most of the specimens showed a major hybridizing band of approximately 550 to 600 bps, but two had larger bands of about 1,000 bps in size. The hybridizing DNA bands corresponded to t(14;18) breakpoints.

Fig 1. Sequence of the t(14;18) minor breakpoint cluster region showing locations of PCR oligonucleotides and translocation breakpoints. The nucleotide sequence of the t(14;18) minor cluster region is shown oriented in a 5′ to 3′ direction and represents the same DNA strand as the sense DNA strand of the bcl-2 gene. This region of chromosome 18 maps approximately 30 kb downstream of the last bcl-2 exon. Underlined sequences indicate specific oligonucleotides used for PCR and sequencing reactions as described in Materials and Methods. Arrows denote locations of t(14;18) breakpoints as determined by direct nucleotide sequencing of amplified DNA (Table 1).
evident based on sequence composition as to what is the basis for
amer signal sequences that flank Ig coding segments. We noted that the chromosome-18 sequence in the region of the breakpoints is relatively A-T rich; however, it is not clear if the sequences flanking sites of the breakpoints did not reveal any specific markers. Examination of the chromosome-18 DNA showed that all eight amplifiable breakpoints occurred within a 500 bp region of chromosome-18 DNA. In addition, five of the breakpoints occurred within three nucleotides of each other, and three breakpoints (C.B., L.K., E.K.) appeared to be in joining segment J4 (L.K., B.E., C.D.), and the others appeared to be in J6. Between the clearly identifiable chromosome-18 and -14 portions of each sequence, insertions of variable lengths were observed, ranging in size from 11 to 24 nucleotides. As suggested earlier, these may represent N-insertions similar to those found at the V-D and D-J joints of Ig and TCR genes. However, we cannot rule out that at least some of the inserted nucleotides actually represent D segments, particularly since patients L.K. and C.D. share a string of 11 nucleotides of identical sequence in their inserted regions. Although the latter does not match any of the published human D-region sequences, confirmation that all of the mcr breakpoints shown here actually occurred as errors in the D-J, or possibly the V-D, joining reactions must await analyses of their reciprocal translocation products. If the insertions observed at these breakpoints do correspond to N-insertions without associated D segments, they are notable for their length, which is larger than that normally observed at coding or signal joints of IgH genes. The sequence composition of the putative N insertions showed only a slight preponderance of G-C over A-T nucleotides. The structural features of the t(14;18) breakpoints studied here are consistent with previous suggestions that they were mediated at least in part by Ig recombinases. Although the majority of mcr breakpoints were detected by our methods, at least two in the current series were not amplifiable, even though they could be shown by Southern blots to have breakpoints within ~15 kb of the pFL.2 probe (MW and MAW in Table 2). Additional studies have shown that these breakpoints likely map more proximal to the bcl-2 gene by at least 5 to 10 kb (Ngan et al, unpublished observations, November 1988). Thus, although most of the breakpoints in this general region of chromosome 18 tend to cluster within about 500 nucleotides of each other, it appears that at least a few are distributed more diffusely and remain

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**Table 1. Sequence of Translocation Breakpoints That Occur in the t(14;18) MCR**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Chromosome 18 . . . N segment . . . Chromosome 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.B.</td>
<td>CATGCTGCAAAAAAGGGGGGGGCTACTACTACTACGGTATGG</td>
</tr>
<tr>
<td></td>
<td>920</td>
</tr>
<tr>
<td>L.K.</td>
<td>CATGCTGCAAAAAACGTTGGGACCCCACCCTCTACTTTGACTATTG</td>
</tr>
<tr>
<td></td>
<td>920</td>
</tr>
<tr>
<td>E.Z.</td>
<td>CATGCTGCAAAAAACTTGGGACCCCACCCTCTACTTTG</td>
</tr>
<tr>
<td></td>
<td>920</td>
</tr>
<tr>
<td>B.E.</td>
<td>ATGCTGCAACACTTATTATTGAGTTGGTGGGACCCCCTGTCACCC</td>
</tr>
<tr>
<td></td>
<td>922</td>
</tr>
<tr>
<td>C.D.</td>
<td>TGCTGAACAGGGGACCCCACCCTCTCAAGAACGGACGGGAGGAGCTACTACT</td>
</tr>
<tr>
<td></td>
<td>1023</td>
</tr>
<tr>
<td>P.C.</td>
<td>GGTTACACCGGCGGCTATTAGCCCTCTCTGAGAAATACGGATGG</td>
</tr>
<tr>
<td></td>
<td>1417</td>
</tr>
<tr>
<td>M.V.</td>
<td>AGCCTTTCCGGCCTTCATGCGCTACTACTACGGTATGGA</td>
</tr>
<tr>
<td></td>
<td>1422</td>
</tr>
<tr>
<td>B.B.</td>
<td>TTGGTGCTGCTGATTATTCTCAACATACTACTACGGG</td>
</tr>
</tbody>
</table>

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**Fig 2. Southern blot analysis of amplified t(14;18) breakpoint DNAs in follicular lymphomas.** DNAs obtained from follicular lymphoma biopsies were subjected to polymerase chain reactions using mcr primer MCB and Ig JH primer MCA as described in Materials and Methods. Reaction products were electrophoresed through 0.8% agarose gels, transferred to nylon membranes and hybridized with kinased mcr-specific oligonucleotide MC12. Size markers consisted in Hindlll digested phage lambda DNA.

breakpoint DNAs, which were specifically amplified due to the configurations of the primers flanking any potential junctions of the Ig JH region of chromosome 14 and the mcr region of chromosome 18, as shown in Fig 1. DNA from sources not carrying a t(14;18) failed to result in detectable hybridizing bands. Likewise, DNA from 20 lymphomas carrying t(14;18) breakpoints that localized to the mbr of bcl-2 also failed to show amplification of their breakpoints under identical conditions (data not shown).

Amplification products from each PCR were directly sequenced to determine the precise locations of each breakpoint on chromosome-18 DNA. Nucleotide sequences determined for the eight amplifiable breakpoints are shown in Table 1, and the locations of breakpoint sites within germline chromosome-18 DNA are shown as arrows in Fig 1. The data show that all eight amplifiable breakpoints occurred within a 500 bp region of chromosome-18 DNA. In addition, five of the breakpoints occurred within three nucleotides of each other, and three breakpoints (C.B., L.K., E.K.) appeared to have occurred at the same nucleotide on chromosome-18 DNA. Although identical in their chromosome 18 portions, these latter three breakpoints could be distinguished from each other since their chromosome-14 portions and their N-insertions (underlined in Table 1 and discussed below) were unique. Examination of the chromosome-18 DNA sequences flanking sites of the breakpoints did not reveal any significant similarities to the conserved heptamer and nonamer signal sequences that flank Ig coding segments. We have noted that the chromosome-18 sequence in the region of the breakpoints is relatively A-T rich; however, it is not evident based on sequence composition as to what is the basis for clustering of t(14;18) breakpoints in this small segment of DNA, which is at least 20 kb distant from the bcl-2 gene.

All eight breakpoints occurred at or within an Ig JH segment, as might be expected by their ability to be amplified using a consensus JH primer that could hybridize to the 3' portion of each JH segment during the amplification procedure. The chromosome-14 crossover sites for three of the breakpoints appeared to be in joining segment J4 (L.K., B.E., C.D.), and the others appeared to be in J6. Between the clearly identifiable chromosome-18 and -14 portions of each sequence, insertions of variable lengths were observed, ranging in size from 11 to 24 nucleotides. As suggested earlier, these may represent N-insertions similar to those found at the V-D and D-J joints of Ig and TCR genes. However, we cannot rule out that at least some of the inserted nucleotides actually represent D segments, particularly since patients L.K. and C.D. share a string of 11 nucleotides of identical sequence in their inserted regions. Although the latter does not match any of the published human D-region sequences, confirmation that all of the mcr breakpoints shown here actually occurred as errors in the D-J, or possibly the V-D, joining reactions must await analyses of their reciprocal translocation products. If the insertions observed at these breakpoints do correspond to N-insertions without associated D segments, they are notable for their length, which is larger than that normally observed at coding or signal joints of IgH genes. The sequence composition of the putative N insertions showed only a slight preponderance of G-C over A-T nucleotides. The structural features of the t(14;18) breakpoints studied here are consistent with previous suggestions that they were mediated at least in part by Ig recombinases. Although the majority of mcr breakpoints were detected by our methods, at least two in the current series were not amplifiable, even though they could be shown by Southern blots to have breakpoints within ~15 kb of the pFL.2 probe (MW and MAW in Table 2). Additional studies have shown that these breakpoints likely map more proximal to the bcl-2 gene by at least 5 to 10 kb (Ngan et al, unpublished observations, November 1988). Thus, although most of the breakpoints in this general region of chromosome 18 tend to cluster within about 500 nucleotides of each other, it appears that at least a few are distributed more diffusely and remain.
undetectable with the primers used here. Further studies are needed to demonstrate whether these nonamplifiable breakpoints define an additional mini-cluster site amenable to PCR detection with another chromosome-18 primer.

Our results also confirm the findings of others\textsuperscript{10-12} that the PCR method applied to t(14;18) breakpoints is a more sensitive method for detecting occult disease in specimens that otherwise would be judged free of involvement by B-cell lymphoma. Patients BE and HB in Table 2 are a direct demonstration of this since specimens from both were found not to contain mcr rearrangements by Southern analysis, yet did contain detectable PCR products. In BE, a previous lymph node had Southern-blot-detectable disease, but a blood sample taken later in the disease course contained a low number of circulating t(14;18)-carrying cells, detectable only by PCR. The mcr-specific oligonucleotide MC8 described in this report can be used in the same reaction vessel with an mbr-specific primer and a consensus J\textsubscript{H} primer to provide a general PCR test that detects the vast majority of reported t(14;18) breakpoints. These findings should facilitate PCR detection of minimal residual disease in non-Hodgkin’s lymphoma patients and further efforts to elucidate the biology of these disorders.

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