Direct Sequence Analysis of the 14q+ and 18q– Chromosome Junctions in Follicular Lymphoma

By Finbarr Cotter, Christopher Price, Emanuele Zucca, and Bryan D. Young

Although the t(14;18) chromosome translocation has been demonstrated to be a highly consistent feature of follicular lymphomas, the underlying mechanism generating this fusion has remained uncertain. To examine this question further, a polymerase chain reaction strategy has been devised to permit the amplification and direct sequencing of the resultant 14q+ and 18q– reciprocal junctions. Direct sequence analysis of amplified 14q+ junctions established that 7 of 11 tumors contained a bcl-2 (mbr) sequence fused to an immunoglobulin J, region (five were Jb and two were Jg). One of these junctions had an unusual configuration with the bcl-2 and Jg sequences separated by a recognizable Dg region. This finding suggests that at least some of the junctional sequences, previously thought of as N insertions, may be fragments of unrecognized Dg regions. It was also possible to amplify and sequence 18q– junctions using a primer based on the Dr recombination signal sequences. Several 18q– junctions were shown to consist of Dg/bcl-2 (either mbr or mcr) fusions. In two tumors the 14q+ and 18q– junctions were fully sequenced, and it was demonstrated that the bcl-2 sequence was conserved during mbr and mcr translocations. This contrasts with previous analyses that demonstrated either loss or duplication of several bases at the breakpoints in the bcl-2 gene.

Materials and Methods

Enzymatic amplification. DNA was extracted as previously described14 from 11 patients with histologically proven centroblastic/centrocytic follicular lymphoma. Enzymatic amplification was performed by the PCR procedure of Saiki et al15 using Thermus aquaticus (Taq) DNA polymerase and an automated Perkin-Elmer DNA Thermal Cycler (Cetus, Emeryville, CA). The final reaction volume included 1 µg of tumor DNA, oligonucleotide primers (1 mmol/L) (Boehringer Mannheim, FRG), 1.5 U of Taq DNA polymerase (Ampliqaq-Cetus), and gelatin (0.01% wt/vol) in 100 µL of Taq buffer (50 mmol/L KCl, 10 mmol/L Tris-C1 pH8.3, 1.5 mmol/L MgCl2). Amplification of the mbr-Jh junctions on chromosome 14q+ was performed with primers BC1 and JH1 and the Dg-mbr junctions on chromosome 18q– with primers DH1 and BC2 (see Table 1). The mer-Jh junctions on chromosome 14q+ were amplified with primers BC5 and JH1 and the Dg-mer junctions on chromosome 18q– with primers DH1 and BC6. An initial denaturing step of 10 minutes at 94°C was followed by 30 cycles of 15 seconds at 55°C (annealing), 1 minute at 70°C (extension), and 30 seconds at 94°C (denaturing). The final extension period was lengthened to 10 minutes. Tag polymerase (Cetus), 1.5 U was added to the reaction after the initial denaturing step. Aliquots (10 µL) from the reaction mixture were analyzed by electrophoresis in a 2% agarose gel in Tris borate electrophoresis (TBE) buffer and stained with ethidium bromide.

Direct sequence analysis. Direct nucleotide sequence analysis by the dyeoxy chain termination method16 was performed on PCR products. DNA from the reaction mixture was purified using a Sephadex G50 column followed by ethanol precipitation, freeze drying, and resuspension in distilled water. Sequencing was performed using a primer based on part of the recombinant signal sequences known to flank germ line Dg sequences. Thus, it was possible to sequence junctions produced by translocations in either the mbr or mcr regions in a series of follicular lymphomas.

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Table 1. Position, Sequence, and Use of Synthetic Oligonucleotides for Analysis of t(14;18) Junctions

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Use</th>
<th>Position (strand)</th>
<th>Orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>JH1</td>
<td>5'ACTGAGGAGACGCTGACC-3'</td>
<td>PCR</td>
<td>3' J, consensus</td>
<td></td>
</tr>
<tr>
<td>DH1</td>
<td>5'-CCTTACGGAGTTGTTACGT-3'</td>
<td>PCR</td>
<td>5' flanking region</td>
<td></td>
</tr>
<tr>
<td>BC1</td>
<td>5'-ATATATGTCACAGTTAGGT-3'</td>
<td>PCR</td>
<td>5' of mbr</td>
<td></td>
</tr>
<tr>
<td>BC2</td>
<td>5'-ACACAGACACCAAGGGCCC-3'</td>
<td>PCR</td>
<td>5' of mbr</td>
<td></td>
</tr>
<tr>
<td>BC3</td>
<td>5'-GGTCTGACATTGTCTGTTTCT-3'</td>
<td>PCR</td>
<td>5' of mbr</td>
<td></td>
</tr>
<tr>
<td>BC4</td>
<td>5'-GATGGCAGCTGAGAGGTAT-3'</td>
<td>PCR</td>
<td>5' of mbr</td>
<td></td>
</tr>
<tr>
<td>BC5 (MC12)</td>
<td>5'-TACCTTACGGAGTTGTTACGT-3'</td>
<td>PCR</td>
<td>5' of mbr</td>
<td></td>
</tr>
<tr>
<td>BC7 (MC7)</td>
<td>5'-TCAGCAGCATCCTCTGAGAGGGACC-3'</td>
<td>PCR</td>
<td>5' of mbr</td>
<td></td>
</tr>
<tr>
<td>BC8</td>
<td>5'-TCCTCTCCTCTGAGAGGGACC-3'</td>
<td>PCR</td>
<td>5' of mbr</td>
<td></td>
</tr>
</tbody>
</table>

Oligonucleotides BC5 and BC7 correspond to MC12 and MC7 used by Ngan et al. The heptamer recombination signal is underlined in oligonucleotide DH1.

RESULTS

The majority of breakpoints on chromosome 18 due to the t(14;18) translocation have been shown to occur in either of two regions within the bcl-2 gene (ie, the mbr or mcr). The corresponding breakpoints on chromosome 14 have been shown to occur at or close to members of the Jx joining regions of the Ig heavy chain locus. The high degree of clustering of both breakpoints renders the 14q+ junctions suitable for PCR amplification using either mbr+ or mcr- oligonucleotides in combination with a Jx consensus oligonucleotide. Similar amplification of the 18q- junction is more problematic, because in the few tumors analyzed7,10 a diversity region (D) has been found fused to bcl-2 sequence. However, a common feature of diversity regions is that they are flanked by heptamer-nonamer recombination signal sequences. We have sought to use this feature to design an oligonucleotide (DH1) for amplification from the chromosome 14 side of the 18q- junction. The full set of primers is shown in Table 1.

Analysis of mbr junctions in bcl-2. DNA was prepared from a series of 11 follicular lymphoma samples. Enzymatic amplification using primers BC1 and JH1 was performed on each DNA sample, and electrophoresis showed specific products from 7 of 11 samples (data not shown). Under the conditions of amplification, normal human DNA yielded no fragments. Direct sequence analysis using primer BC3 showed the structure of these products, and the results are summarized in Fig 1. In all cases a fusion was demonstrated between a bcl-2 sequence and members of the Ig heavy chain joining

Fig 1. Sequences of the chromosome 14q+ junctions in seven follicular lymphomas. B-c1 sequence is shown in upper case and joining region sequence is in upper case italics, with the coding exons in bold type. Differences between the Jx sequences and their germline equivalents are underlined. The intervening sequences between bcl-2 and Jx are indicated in lower case. The part of the intervening sequence that is identical to a previously identified D region is underlined (patient E).
region exons (JH), confirming the presence of the t(14;18) translocation in these tumors. The breakpoint on bcl-2 fell within the range of previously determined7 breaks in the mbr region. There was a clear preponderance (5 of 7) for the JH member of the JH family. Most of the JH sequences had some single base differences when compared with their germline equivalents. This could be due to either polymorphic variation or somatic mutation, which is known to occur during D-J recombination. In contrast, the bcl-2 sequence showed no evidence of mutation. In every junction there was an intervening sequence between bcl-2 and JH, although in patient D this consisted of only a single base. In patient E there was a particularly large intervening sequence which, on further analysis, was found to contain a recognizable Dn region (Fig 1). The Dn (diversity) regions recombine with JH in normal Ig gene rearrangement; thus, this junction (bcl-2-Dn-JH) could result from translocation after Dn-JH recombination. Thus, this junction consisted of bcl-2 fused to Dn, which in turn was fused to JH. None of the other intervening sequences had significant homology to each other or to any other known Dn or putative N insertion sequences.

The primer DH1, in combination with the bcl-2 primer BC2, was used for amplification of the reciprocal junctions on chromosome 18q-. Although, on electrophoresis, bands were obtained from tumor samples, direct sequencing was successful from only a proportion of tumors. Thus, these bands may represent spurious products, unrelated to the IgH/bcl-2 junction on chromosome 18. The sequence of an 18q- junction is shown in Fig 2, in which a putative Dn region is fused to the mbr region of bcl-2. In the sample from patient B, direct sequencing also showed the structure of the 18q- junction, and this has been compared with the 14q+ junction. The sequence of the reciprocal 14q+ junction is similar to those reported by others, with mcr sequence fused to a member of the JH family. Because the junction is well within the JH exon, it is not possible to identify uniquely which JH gene is involved. There is very little (at most a single base) sequence that could be called an N-insertion, and the breakpoint in mcr is precisely at a position which has been broken in two previously analyzed tumors. The sequence of the reciprocal 18q- junction is also shown in Fig 4, and it is clear, as in Fig 3, that there has been no net loss or gain of bcl-2 sequence. Furthermore, as with mbr translocations, this junction is composed of a Dn sequence fused to bcl-2.

**DISCUSSION**

The use of the Dn-based primer (DH1) offers a means to amplify and analyze rapidly a proportion of the 18q- junctions. As a means of detecting the presence of the t(14;18), it appears to be less efficient than the equivalent strategy for the 14q+ junctions. Because this approach requires the presence of the 5' recombination signals next to the junctional Dn exon, lack of amplification may be due to removal of the 5' recombination signal sequence by a preceding VJ-Dn recombination. In addition, the Dn primer includes part of the intervening sequence between the heptamer and nonamer, which will tend to be less conserved. Although these features may tend to reduce the proportion of tumors in which the 18q- junctions can be successfully amplified, it is possible that other strategies, such as the use of a VH consensus primer15, might be successful.

Previous studies have shown that the 14q+ junctions are often characterized by short stretches of unrecognizable sequences, which, because of their position adjacent to a joining region, have been thought of as N-insertions. However, there are several features of these sequences that question this interpretation. Firstly, these translocation N-regions tend to be longer than normal N-region sequences, which rarely exceed 10 bases and are usually shorter.18 Secondly, the normal N-regions are GC rich whereas translocation N-regions are less so.18 Finally, Ngan et al15 noted that two different tumors had an identical stretch of 11 bases in
their putative N-regions, and speculated that such regions could be derived instead from previously unrecognized D<sub>n</sub> sequences. The tumor from patient E (Fig 1) has been shown to contain a complete D<sub>n</sub> sequence within its putative N-region. This finding supports the idea that at least some of the putative N-regions could contain fragments of D<sub>n</sub> regions. Interestingly, this D<sub>n</sub> sequence is identical to one previously shown to be located 150 bases 5' to a D<sub>n</sub> involved in a follicular lymphoma translocation. Comparison with this sequence indicates that the germline recombination signals are missing from the 5' end of the D<sub>n</sub> sequence (Fig 1), suggesting that either the translocation has occurred as a mistake in V-D joining with a subsequent N insertion, or that V-D-J recombination had taken place before translocation. The former explanation has been invoked to explain a translocation in the Daudi Burkitt lymphoma cell line as a mistake in V-D-J joining with the D-J already rearranged. However, subsequent analysis of two tumors has shown small deletions, rather than duplications, of bcl-2 sequence at junctions. Moreover, in our study both tumors analyzed showed conservation of bcl-2 sequence, and therefore the duplication of junctional bcl-2 sequence is not a general feature of the t(14;18) translocation.

Interestingly, the breakpoint in mbr on chromosome 18 for patient B is at a location identical to that found in a tumor analyzed by Tsujimoto et al. in which a deletion of 2bp had occurred. This effectively rules out the possibility that the gain or loss of bcl-2 sequence is, in some way, dependent on the position of the breakpoint. The analysis of the reciprocal junctions for the translocation in mcr (Fig 4) showed a similar structure to that found in mbr breakpoints. In particular, the fusion of a D<sub>n</sub> to mcr sequence forming the 18q- junction has the same configuration as that found in mbr translocations. This suggests that similar mechanisms are involved in mcr translocations. This breakpoint lies at exactly the position of two previously analyzed breaks in mcr which themselves were part of a tight cluster of five breaks within 4 bases of each other. An interesting feature of this cluster is that it is bounded by two direct repeats (CTGCAAAC) 7 bases apart. Although the underlying mechanism for the t(14;18) translocation in follicular lymphoma remains unclear, similar factors appear to operate for breaks in both the mbr and mcr regions. Our sequence data suggest that, at least in some cases, it is possible that translocation has taken place after D-J rearrangement.

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