Morphologic Transformation of Follicular Lymphoma Is Associated With Somatic Mutation of the Translocated Bcl-2 Gene

By András Matolcsy, Paolo Casali, Roger A. Warnke, and Daniel M. Knowles

The t(14;18) translocation of the bcl-2 gene into the immunoglobulin heavy-chain (IgH) gene locus results in deregulated expression of the bcl-2 gene, which is involved in the regulation of programmed cell death (apoptosis). This study examines the somatic mutations of the bcl-2 gene in FL cells and their correlation with disease progression.

Follicular lymphoma (FL) is a low-grade B-cell non-Hodgkin’s lymphoma (NHL) that frequently transforms into diffuse aggressive NHL. The majority of FLs display a t(14;18) translocation that places the bcl-2 gene into juxtaposition with the IgH gene locus. Morphologically transformed malignant FL cells retain their t(14;18) translocation and may acquire additional genetic abnormalities. We analyzed serial biopsy specimens from eight patients with FL for secondary alterations of the rearranged bcl-2 gene in the breakpoint and open reading frame (ORF) regions. Two cases of FL showed no histologic alteration in the second biopsy, and six cases of FL showed morphologic transformation to diffuse large-cell lymphoma (DLL) in the second biopsy. Polymerase chain reaction (PCR) amplification, cloning, and sequencing of the junctional region of the hybrid bcl-2/IgH genes showed identical nucleotide sequences in multiple biopsy specimens of FL that did not show morphologic transformation. In patients in whom FL cells underwent morphologic transformation, DLL and autologous FL cells displayed identical bcl-2/IgH gene nucleotide sequences in five cases and different sequences in one case. In the case for which FL and DLL cells showed different bcl-2/IgH junctional sequences, DLL cells incorporated larger bcl-2 and Ig-joining (JH) gene fragments than the corresponding FL cells, suggesting that DLL clones developed by a distinct t(14;18) translocation rather than by alteration of the hybrid bcl-2/IgH gene detected in the FL cells. In all eight cases, neither FL nor DLL cells showed alterations of bcl-2 gene sequences in the breakpoint region, suggesting high conservation of the bcl-2 gene in the ORF region corresponding to the 239-amino-acid p26-bcl-2α protein. A total of 11 point mutations of the ORF were detected in DLL cells of three transformed NHLs, but no alteration of the ORF was detected in FL cells. Four of 11 mutations, at positions 29, 46, 59, and 106, yielded amino acid replacements. These findings demonstrate that DLL and FL cells may be clonally related or unrelated. They also show that transformation of FL cells may be associated with somatic point mutations of the bcl-2 proto-oncogene ORF sequence resulting in alteration of the p26-bcl-2α gene product.

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cytologic grade I. In two patients (cases no. 7 and 8), the results of the second biopsy showed no histologic alterations compared with those of the first biopsy. In six patients (cases no. 1 to 6), histology of the second biopsy was classified as DLL (Table 1).

Polymerase chain reaction and sequence analysis of the t(14;18) breakpoints. Genomic DNA preparations extracted from frozen tissue sections using a salting-out procedure were used as templates.24 Polymerase chain reaction (PCR) and sequence analysis were used to characterize the t(14;18) translocation in eight paired lymphoma samples. Major breakpoint region (mbr)− or minor cluster region (mcr)− specific bcl-2 sense primers in conjunction with a Jg antisense primer were used in the PCR, as previously described.24 PCR DNA products were cloned in pCR 1000 vector using the TA cloning system (Invitrogen Corp, San Diego, CA) following the manufacturer’s instructions. DNA sequences were analyzed using the MacVector version 4.5 (Eastman Kodak Corp, New Haven, CT) software and the GeneBank database.

PCR single-strand conformation polymorphism and sequence analysis of bcl-2 proto-oncogene ORF. PCRs were performed to amplify DNA segments of the bcl-2 ORF corresponding to p26-bcl-2α protein. Three pairs of amplification primers were designed for use in separate reactions to achieve PCR amplification of the first 717 bp corresponding to amino acids 1 to 239 of the ORF. The pairs of sense and antisense primers were as follows: (−40) AGAGGT-GCCGGTGCCCCCGTTTGG(221) GTCTGCAAGCCGGCCGGTCTC, (202) AGACCTCCGGCTGGCCAGAC(487) TGAAGGCTCTCCA-CACACATGAC, and (448) TGGATTCCGGTGGGCTAGCT(741) TGTGGGAGCCGATGAGCCG. PCR were performed with 100 ng DNA, 10 pmol of each primer, 2.5 μmol dNTP, 1 μCi [α-32P]dCTP (specific activity, 3000 Ci/mmol; New England Nuclear, Boston, MA), 10 mmol Tris (pH 8.8), 50 mmol KCl, 1.5 mmol MgCl2, and 0.5 U Ampli Taq DNA polymerase (Boehringer Mannheim Corp, Indianapolis, IN) in a final volume of 10 μL. Amplifications were performed for 30 cycles in an automated thermocycler (Perkin-Elmer Cetus, Norwalk, CT). Each cycle consisted of 95°C for 1 minute (denaturing), 58°C for 2 minutes (annealing), and 72°C for 2 minutes (extension). The reaction mixture (2 μL) was diluted 1:25 in 0.1% sodium dodecyl sulfate/10 mmol EDTA and further mixed 1:1 with a sequencing stop solution. Samples were heated at 95°C for 5 minutes, chilled on ice, and immediately loaded onto a 6% acrylamide-TBE gel containing 10% glycerol. Gels were assayed at 4 to 8 W for 14 to 16 hours at room temperature. The gels were fixed in 10% acetic acid and air-dried, and autoradiography was performed at −70°C with an intensifying screen for 6 to 24 hours. To confirm the presence of a mutation, DNAs were amplified under the same conditions as described for the SSCP, but the radioactive nucleotide was omitted. PCR products were cloned, sequenced, and analyzed as already described.

RESULTS

Molecular characterization of t(14;18) chromosomal translocations. DNAs of paired lymphoma samples from eight patients were PCR-amplified using mbr- or mcr-sequence−specific bcl-2 sense primers in conjunction with Jg−specific antisense primer. The PCR products were cloned and sequenced. A fusion was demonstrated between the bcl-2 gene and members of the Ig Jg exons in all FL and DLL samples, confirming the presence of t(14;18) translocations in these tumors. The breakpoint on bcl-2 was within the region referred to as mbr in all cases. The mbr/Jg junctional sequences of the hybrid bcl-2/IgH gene from initial FL and subsequent FL or DLL B cells were evaluated in parallel. The results are summarized in Table 1 and illustrated in Fig 1. In the initial FL and corresponding subsequent FL or DLL cells, the bcl-2 gene was juxtaposed with the Jg JgH sequences in one case, the Jh JhH gene in three cases, and the Jh JhC gene in four cases. In all but one case, Jg JgH junctional sequences of the hybrid bcl-2/IgH gene from initial FL and subsequent FL or DLL B cells were evaluated in parallel. The results are summarized in Table 1 and illustrated in Fig 1. In the initial FL and corresponding subsequent FL or DLL cells, the bcl-2 gene was juxtaposed with the Jg JgH gene in one case, the Jh JhC gene in three cases, and the Jh JhC gene in four cases. In all but one case, Jg JgH sequences showed complete identity with the published germline equivalents. In case no. 1, Jh JhC sequences of FL and DLL cells showed a single but identical bp difference when compared with the published Jh JhC sequence. The bcl-2 sequences showed no evidence of point mutations in the breakpoint regions. In seven patients (cases no. 1 and 3 to 8), FL cells of the first biopsy and the corresponding FL or DLL cells of the second biopsy showed identical bcl-2 and Jg breakpoints and intervening junctional sequences, suggesting a common clonal origin for the two tumor cell populations. In FL cells from one patient (case no. 2), the bcl-2 breakpoint was 62 bp and the Jg breakpoint was 6 bp distant from the annealing site of the sense and

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<th>Histology</th>
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<th>mcr (position)</th>
<th>Jh (position)</th>
<th>21 A/G Polymorphism</th>
<th>No. of Mutations</th>
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Abbreviations: a, first lymph node biopsy sample; b, second lymph node biopsy sample; FL-I, FL grade I, predominantly small cleaved.

* Mutation detected in only G polymorphic sequences.
antisense primers, respectively. The relatively long junctional sequence of the hybrid gene was found to contain a D21-9 gene. In DLL cells from the same patient, the bcl-2 gene breakpoint was 121 bp and the J\(_b\) gene breakpoint was 10 bp distant from the annealing site of the sense or antisense primers, respectively. The junctional sequence of the hybrid gene was 8 bp long and showed no similarity to the junctional sequence of the FL cells. The fact that the bcl-2/IgH gene hybrid of the DLL cells in case no. 2 incorporated longer bcl-2 and J\(_b\) fragments than the preexisting FL cells suggests that DLL cells developed through an additional independent t(14;18) translocation rather than by deletion of diversity (D) genes from the bcl-2/IgH gene hybrid of the FL cells.

**Frequency of ORF mutations in the bcl-2 gene.** To determine whether morphologic transformation of FL cells is associated with alterations of the ORF of the bcl-2 gene, we studied lymphoma cells in six cases of FL transformed into DLL and two cases of morphologically untransformed FL using the PCR-SSCP method. Figure 2A shows the results of PCR-SSCP analysis generated by the primers that span the region from -40 bp to 221 bp of the ORF. A/G hereditary polymorphism of the bcl-2 gene located at 21 bp into the ORF has been documented. The migration pattern of wild-type A, G, or A/G polymorphic forms of the gene are shown for comparison. Electrophoretic mobility of PCR-amplified DNA from the FL samples was identical in case no. 2 with that of the A polymorphic wild-type control, in cases no. 4 and 8 with that of the G polymorphic wild-type control, and in cases no. 1, 3, and 5 to 7 with that of the A/G wild-type controls, suggesting that somatic mutations in the first 221-bp region of the ORF are absent in FL cells. PCR products from DLL samples of cases no. 2, 4, and 6 displayed altered electrophoretic mobility compared with the respective FL cell counterparts, as shown by the results of PCR-SSCP analysis using the primers that span the region from 202 bp to 487 bp of the ORF (Fig 2B). Altered electrophoretic mobility was detected in the DLL sample of case no. 6. Figure 2C shows the results of PCR-SSCP analysis generated by the primers that span the region from 448 bp to 741 bp of the ORF. FL and DLL samples of the eight patients did not exhibit altered electrophoretic mobility compared with unmutated negative controls.

**Type and position of mutations in the bcl-2 ORF.** Bcl-2 ORFs of FL and subsequent nontransformed FL and transformed DLL cells of eight cases were reamplified in separate PCRs under the same conditions as described for the SSCP, but the radioactive nucleotide was omitted. The amplified

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**Fig 1.** Bcl-2/J\(_b\) junction sequences identified in paired serial biopsies of six cases of FL transformed to DLL (cases no. 1 to 6) and two cases of FL that did not transform (cases no. 7 to 8) by PCR-SSCP analysis. DNA sequences flanking bcl-2 and J\(_b\) breakpoints and the intervening D and N regions of initial FL (a) and corresponding subsequent DLL or FL (b) samples are clustered in each case. The distance of bcl-2 (mbr) breakpoints from the sense primers and the distance of J\(_b\) breakpoints from the antisense primers are shown in parentheses, respectively. Differences between J\(_b\) sequences and their published germline equivalents are underlined.
DNAs were cloned and sequenced. In each FL and DLL sample, sequences of 20 independent bacterial isolates were analyzed and compared with the germline sequence of the bcl-2 gene (Table 1). The sequence analysis of FL cells confirming the results of SSCP analysis showed A polymorphism in case 2, G polymorphism in cases no. 4 and 8, and A/G polymorphism in cases no. 1, 3, and 5 to 7 at position 21 of the bcl-2 ORF. None of eight initial FL biopsy specimens and second biopsy specimens of nontransformed FL samples showed nucleic acid changes compared with germ-
line bcl-2 gene sequences (not shown). A total of 11 point mutations were detected in three DLLs (cases no. 2, 4, and 6) that showed altered electrophoretic mobility by SSCP analysis, but these mutations were absent in the preexisting FLs (Fig 3). In the DLL sample of case no. 2, 6 of 20 nucleic acid sequences (clones 2, 7 to 9, 11, and 14) showed identical mutations at positions −11 and 66 (Fig 3A). In the DLL sample of case no. 4, eight sequences (clones 6, 8, 12 to 16, and 20) contained identical mutations at positions 24, 66, 85, and 137 (Fig 3A). In the DLL sample of case no. 6, nine sequences (clones 6, 12 to 16, and 18 to 20) contained identical mutations at positions 33, 175, and 176, analyzing the bcl-2 ORF region from −40 bp to 221 bp (Fig 3A). In the same DNA sample, 10 sequences (clones 1, 2, 5 to 7, 9, 10, and 16 to 18) of the bcl-2 ORF region from 202 bp to 487 bp contained identical mutations at positions 315 and 354 (Fig 3B). The frequency of identical mutant sequences was 6 to 10 of 20 independent isolates showing amplification of both mutant and wild-type clones. This observation is consistent with our recent PCR approach amplifying both the translocated and wild-type bcl-2 alleles. Furthermore, in the A/G polymorphism patient (case no. 6), the somatic mutations were consistently associated with the G polymorphic allele, suggesting that mutation is associated with only one allele. We presumed that the observed mutations are derived from the translocated bcl-2 gene, because somatic mutations of the bcl-2 gene have not been detected in a large group of normal individuals. All 11 somatic mutations were transitions (A to C or G to T), and 4 of them result in amino acid replacement at codons 29 (E to K), 46 and 59 (P to L), and 106 (R to H) of the p26-bcl-2α protein.

**DISCUSSION**

Characterization of the clonal relationship between FL and subsequent DLL and identification of molecular mechanisms associated with the morphologic transformation and clinical progression of FL are critical issues in understanding the pathogenesis, diagnosis, and management of these lymphomas. These studies demonstrate that FL and DLL cells may be clonally related or may represent different unrelated clones. They also provide the first evidence that morphologic transformation of FL may be associated with genetic alteration of the p26-bcl-2α oncoprotein.

The present report strengthens previous studies showing that FL and subsequent DLL cells may carry identical t(14;18) translocations and junctional bcl-2/IgH gene sequences suggesting a common clonal origin for the two neoplasms, and it extends them by providing evidence that FL and DLL cells in the same patient may display different t(14;18) chromosome translocations and junctional bcl-2/IgH gene sequences, thereby suggesting discrete clonal B-cell origins. In one patient (case 2), bcl-2/IgH junctional sequence analysis indicated that FL cells occurred as a mistake in V-D gene joining and autologous DLL cells developed as a mistake in J-D gene joining, suggesting that chromosomal translocations affect B cells at different stages of ontogeny. Moreover, the fact that the rearranged bcl-2 and IgH gene fragments in DLL cells are larger than those in the preexisting FL cells supports the hypothesis that these lymphoma cells arose through different t(14;18) translocations rather than by secondary alterations of the hybrid bcl-2/IgH gene of FL cells. Previous studies using highly sensitive nested PCRs have demonstrated that bcl-2/IgH translocations can occur more than once during the natural course of FL. Because t(14;18) translocation occurs in normally dividing B cells without development of a true malignant clone, it was debated whether additional t(14;18) translocations in FL are the result of development of a bicalon lymphoma or are benign t(14;18)-bearing clones detected by the sensitive nested PCR technique. The data presented here provide evidence that additional t(14;18) translocations in FL may give rise to a new neoplastic clone with different histologic features than the original FL.

Previous studies have noted small deletions or duplications of the junctional region of the translocated bcl-2 gene in FL cells. In the present study, FL and subsequent DLL cells showed no alterations of the rearranged bcl-2 gene junction sequences compared with the corresponding germ-line bcl-2 gene sequences. This finding was consistent with the previous findings of Cotter et al showing high conservation of the bcl-2 gene during t(14;18) translocation. The bcl-2/IgH junctional sequence comparison of FL and autologous DLL cells in the five patients in whom FL and DLL cells were clonally related demonstrated that junctional sequences are also preserved in the process of morphologic transformation, and additional alterations of the bcl-2/IgH junction region are not associated with morphologic transformation of FL cells.

We used PCR-SSCP and sequence analyses of the translocated bcl-2 gene in FL and subsequent DLL samples to detect structural alterations of the ORF corresponding to the p26-bcl-2α oncprotein. A total of 11 point mutations were found in three of six transformed DLL samples, but respective preexisting FL cells and FL cases that did not transform showed unmutated configuration of the gene sequence. The origin of the mutations is unknown, but their absence in the original FL cells suggests that somatic mutation of the bcl-2 gene follows the t(14;18) translocation. This conclusion is also supported by previous findings showing no somatic mutations of the ORF in normal individuals and lymphoma cell lines that lack the t(14;18) translocation. Tanaka et al suggested that somatic point mutations in translocated bcl-2 genes are attributable to the activity of the somatic hypermutation machinery characteristic of the Ig locus. The juxtaposition of the bcl-2 gene with the (D)-Jh gene sequence may mimic the events associated with physiologic rearrangement that bring Vh and D gene segments into the region of the IgH locus. In the clonal expansion of FL cells, the productively rearranged IgH chain allele also accumulates mutations. As a result of ongoing somatic mutation, the tumor cell population becomes heterogeneous, and a mutational variant having a selective growth advantage compared with parental clones gives rise to the high-grade NHL tumor cell population.

Somatic mutation of the ORF of the translocated bcl-2 gene has been demonstrated in SU-DHL-6 lymphoma cell lines carrying the t(14;18) translocation and in FL cells of different histologic subtypes, but the biologic signifi-
Fig. 3. Nucleotide sequences of bcl-2 DNA derived from PCRs. Sequences (B1) and (B2) represent two independent amplifications from the region from -40 bp to 221 bp (B1) and from 522 bp to 487 bp (B2), respectively. Sequences are grouped and show similar nucleotide sequences of over 80% homology. The top nucleic acid sequences are shown by a dot, and differences are shown by a dash.

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cance of these mutations is highly debatable. The correlation of somatic mutations with transformed DLLs suggested in our recent study may be associated with the activated proliferating phenotype of high-grade NHL cells. The enhanced biologic activity of cells carrying mutated ORF of the bcl-2 gene (amino acid 59 Pro → Ser) has been demonstrated by recent experiments by Reed and Tanaka showing a selective growth advantage for cells expressing mutant bcl-2 protein versus wild-type protein. In contrast, McDonell et al found no functional differences between B cells expressing mutant (amino acid 7, Thr → Ser; amino acid 48, Ile → Phe; and amino acid 29, Arg → Cys) or wild-type bcl-2 protein. The discordant results of these studies suggest that amino acid replacement affecting a certain region of the p26-bcl-2α protein only alters the biologic function of the cells.

Recently, cDNAs have been cloned for several novel human genes, revealing a family of bcl-2-related proteins. One of these, termed bax, encodes a 192–aminocacid protein with 21% homology with bcl-2. An additional bcl-2 homologue is bcl-x, which can generate two proteins through an alternative splicing mechanism: bcl-x-1 (longer form) and bcl-x-s (shorter form). Gene transfer studies in lymphokine-dependent hematopoietic cells indicate interactions of the bcl-2 protein with itself and other members of the bcl-2 family, including bax, bcl-x-1, and bcl-x-s. Bax is envisioned as a cell death (apoptosis) effector, with activity neutralized by binding of bcl-2. The binding of bcl-x-s to bcl-2 was hypothesized to prevent bcl-2 from interacting with bax, thus leaving bax unopposed in its apoptotic function. The use of site-specific mutagenesis and deletion mutants of bcl-2 suggests that bcl-2 homodimerization and heterodimerization with other bcl-2 family members involves interaction between distinct regions within the bcl-2 protein. Whereas BH1 (amino acid 136 to 155) and BH2 (amino acid 187 to 202) domains of bcl-2 are required for heterohybridization with bax, homodimerization of bcl-2 involves interaction of the amino acid 1 to 81 region between two bcl-2 molecules and/or interaction of amino acid 1 to 81 with the amino acid 93 to 218 region of the bcl-2 molecules. In our studies, we have shown that somatic mutation in DLL cells resulted in replacements of the amino acid at positions 29, 46, 59, and 106 of the bcl-2 protein. These structural alterations may affect interaction of bcl-2 with other bcl-2 family members and/or other oncogenes and tumor suppressor genes.

Since the precise amino acid residues of the bcl-2 protein involved in homodimerization or heterodimerization have not been determined, the mechanism(s) by which structurally altered bcl-2 protein changes the biologic behavior of FL cells is highly speculative. The demonstration of altered amino acid residues of the bcl-2 protein in morphologically transformed FL cells shown in the present studies would contribute a first step toward the investigation of the molecular bases and pathways of lymphoma transformation.

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