Evidence of c-myc Gene Abnormalities in Mediastinal Large B-Cell Lymphoma of Young Adult Age

By Aldo Scarpa, Lorena Borgato, Marco Chilosi, Paola Capelli, Fabio Menestrina, Franco Bonetti, Giuseppe Zamboni, Giovanni Pizzolo, Setsuo Hirohashi, and Luciano Fiore-Donati

Six cases of mediastinal large B-cell lymphoma (MLCL) with sclerosis were analyzed for the presence and patterns of c-myc and bcl-2 loci rearrangements, and for the presence of Epstein-Barr virus DNA sequences by Southern blot hybridization. c-myc gene alterations were found in three of six cases. Two cases showed the presence of mutations or small rearrangements at the 3' end of the first exon. The c-myc gene abnormalities found in these two cases are similar to those observed in sporadic Burkitt's and in acquired immunodeficiency-associated lymphomas. None of the cases displayed bcl-2 gene rearrangements or contained viral sequences. Our data suggest a possible role for a translocation-mediated c-myc activation in the pathogenesis of MLCL. Conversely, bcl-2 gene and Epstein-Barr virus do not appear to be involved in the pathogenesis of these peculiar lymphomas. The association between c-myc structural modifications and MLCL also seems to be of relevance in light of the peculiar tendency of this tumor to involve unusual extranodal sites (eg, kidney), reminiscent of the spreading attitude of Burkitt's lymphomas.

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MEDIASTINAL large cell lymphoma (MLCL) with sclerosis has been recently recognized as a peculiar clinicopathologic entity among non-Hodgkin lymphomas (NHLs), affecting mainly young adult patients with an aggressive clinical behavior.1-4 Its B-cell nature has been suggested by immunophenotypic studies5-7 and confirmed at the genotypic level.3 The derivation from a peculiar intrathyroidic B-cell population has been proposed.8 Recent clinical studies have demonstrated that patients affected by this type of lymphoma, when properly diagnosed and conveniently treated with a combined modality chemotherapy, can experience prolonged disease-free survival.7,8 Information concerning the involvement of cellular oncogenes, whose activation is thought to be a major event in the pathogenesis of different types of lymphomas, is currently unavailable for this disease.

Chromosomal abnormalities, some of which are known to be responsible for proto-oncogene activation, characterize virtually all primary as well as progressive NHLs.5,9 A large proportion of B-NHLs exhibit one of three types of translocations involving either c-myc, or bcl-2 or bcl-1 cellular genes, that are displaced to the Ig genes loci.1,11 Of these, the two involving bcl-2 and c-myc genes exhibit strong association with histologic subtypes. The t(14;18) (q32;q21), involving the bcl-2 gene, is recognized in approximately 85% of follicular lymphomas originating from the germinal center13,16 and in a proportion (30%) of LCLs11,15,16; the latter possibly representing the evolution of previous follicular lymphomas.3 Three variants of the translocation involving the c-myc gene exist: the t(2;8) and t(8;22), in which the k and λ Ig light chain locus is dislocated to chromosome 8; and the most frequent t(8;14) (q24;q32), in which the c-myc locus is dislocated to the Ig heavy chain locus on chromosome 14. The t(8;14) is a feature of small non-cleaved cell (Burkitt's and non-Burkitt's) lymphomas and of L3-type of B-acute lymphocytic leukemia,9,11,13,19 frequent in acquired immunodeficiency syndrome (AIDS)-associated lymphomas,9,20 and is found in a proportion (11% to 28%) of diffuse LCLs.9,13,14 However, the t(2;8) and t(8;22) have only been found associated to small non-cleaved cell histology. The third type of translocation, t(11;14) (q11;q32), involving the bcl-1 gene, has been found predominantly in small cell lymphocytic lesions.9 The availability of molecular probes recognizing DNA sequences flanking or included within the translocation junction regions enables the detection of such genomic abnormalities using the Southern blot hybridization technique.

The aim of this study was to explore the presence of alterations of the two cellular genes, c-myc and bcl-2, most frequently translocated in LCLs, in six cases of MLCL. In addition, the possible role of Epstein-Barr virus (EBV)11,22 which has been involved in the pathogenesis of different types of NHL, has been investigated. We demonstrate, in three cases, the presence of c-myc gene structural alterations similar to those characteristic of translocated c-myc genes, which suggests that a translocation-mediated c-myc activation may be a major event in the pathogenesis of MLCL.

MATERIALS AND METHODS

Pathologic Samples and Immunohistochemical Analysis

Representative tumor samples were obtained at mediastinoscopy from six untreated patients suffering from a mediastinal mass (Table 1). Fresh tissue fragments were divided into three parts. One portion was fixed in 10% formol-saline and processed for conventional histology. The other portions were snap-frozen in liquid nitrogen for immunohistochemistry and DNA analysis.
Immunophenotyping on cryostat and paraffin sections was performed as previously described. Diagnosis of MLCL was established in each case by standard clinical and histopathologic criteria and by cell marker analysis. In all cases, the histologic examination showed the peculiar morphology of MLCL. Large neoplastic cells were arranged in nests and ribbons frequently compartmentalized by thin collagen bands; necrosis was generally absent. In case 3, the fibrosis was particularly marked and large areas of necrosis were found. The immunohistochemical analysis confirmed the lymphoid nature of the neoplastic cells, which expressed panleukocyte antigen (CD45) and several B-cell-related markers, including CD20 and CD22. In no case could surface Igs be detected.

**DNA Extraction and Southern Blot Analysis**

High molecular weight DNA was purified as previously described. DNAs were digested with appropriate restriction enzymes (see below), electrophoresed in 0.8% to 1.6% agarose gels, denatured, neutralized, transferred to nitrocellulose filters, and hybridized according to established procedures in 50% formamide/5X SSC at 42°C for 18 hours. Filters were washed to a final stringency of 0.2X SSC/0.5% sodium dodecyl sulphate (SDS), at 60°C to 68°C and exposed for 18 to 48 hours at ~80°C to Kodak X-AR-5 films (Eastman Kodak Co, Rochester, NY) with two lightening-plus intensifying screens (DuPont, Dreieich, Germany).

Restriction enzymes were purchased from Boehringer Mannheim (Mannheim, Germany); all the other reagents were obtained from Sigma (St Louis, MO).

**DNA Probes**

The organization of c-myc (Fig 1) was analyzed by hybridization of the human c-myc probe MC413RC, representative of the third exon of the c-myc locus, to EcoRI-, HindIII- and BamHI-digested DNAs. The point mutations or small internal rearrangements of c-myc loci, which characterize the translocated c-myc genes of the endemic form of Burkitt's lymphoma, are detectable in a high proportion of cases (60%) as polymorphisms of the Pvu II restriction site located at the 5' end of exon 1. The presence of such alterations in the six MLCLs was therefore analyzed by hybridization of Pvu II-restricted DNAs to 5'-Pv probe (Fig 1).

**Table 1. Patients and Clinical Data**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex/Age</th>
<th>Stage</th>
<th>Site</th>
<th>Therapy</th>
<th>Response</th>
<th>DSF (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F/30</td>
<td>IAb bulky</td>
<td>Mediastinum</td>
<td>MACOP-B + RTIF</td>
<td>CR</td>
<td>&gt; 21</td>
</tr>
<tr>
<td>2</td>
<td>F/25</td>
<td>IIb</td>
<td>Mediastinum, SCLN right</td>
<td>MACOP-B + RTIF</td>
<td>CR</td>
<td>&gt; 36</td>
</tr>
<tr>
<td>3</td>
<td>M/30</td>
<td>IIa</td>
<td>Mediastinum</td>
<td>MACOP-B + RTIF</td>
<td>CR</td>
<td>&gt; 58</td>
</tr>
<tr>
<td>4</td>
<td>F/25</td>
<td>IIb</td>
<td>Mediastinum, SCLN left</td>
<td>MACOP-B + RTIF</td>
<td>CR</td>
<td>&gt; 39</td>
</tr>
<tr>
<td>5</td>
<td>F/37</td>
<td>IIb</td>
<td>Mediastinum</td>
<td>MACOP-B + RTIF</td>
<td>CR</td>
<td>&gt; 18</td>
</tr>
<tr>
<td>6</td>
<td>M/23</td>
<td>IV bulky</td>
<td>Mediastinum, lung</td>
<td>MACOP-B + ABMT</td>
<td>CR</td>
<td>&gt; 46</td>
</tr>
</tbody>
</table>

Abbreviations: DSF, disease-free survival; SCLN, supraclavicular lymph nodes; RTIF, involved-field radiotherapy; ABMT, autologous bone marrow transplantation; CR, complete remission.
Table 2. Results of Molecular Hybridization Studies

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>JH</th>
<th>κ</th>
<th>Cp</th>
<th>Tβ</th>
<th>3’myc</th>
<th>5'-Pv</th>
<th>bcl-2</th>
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<tbody>
<tr>
<td>1</td>
<td>−</td>
<td>+/+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
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<td>−</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
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<td>+/+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

Patient numbers correspond to those of Table 1. Abbreviations: 3’myc, probe MC413RC representative of myc exon 3; 5'-Pv, myc probe used to detect mutations within myc exon 1; +, rearranged; −, germline; +/+, indicates the presence of rearrangement of both alleles.

Moreover, PBL were available for patients 1 and 5 and were used as internal controls for Pvu II site polymorphisms.

RESULTS

Clinical data are reported in Table 1. The results are summarized in Table 2.

Ig and TCR Gene Analysis

DNAs from all cases but one (case 1) yielded one or two bands in addition to the germline band when hybridized to JH probe. Four specimens (including case 1) showed one or two rearranged bands other than the germline at 12 kb when hybridized to the probe specific for the κ-light chain Ig gene, after BamHI and Bgl II digestions. Cases 2 and 4 showed a rearranged band when hybridized to Cμ probe. All the TCR-β genes of our cases were present in a...
germline configuration on EcoRI-, HindIII-, and BamHI-digested DNAs.

c-myc Gene Analysis

Modifications of the c-myc gene were observed in three of the six cases (Table 2). A major rearrangement of one c-myc allele was detected in case 3 by Southern blot hybridization of EcoRI-, HindIII-, and BamHI-digested DNA to the c-myc third exon probe, whereas in the other five cases the c-myc loci appeared intact (Fig 2). However, cases 2 and 5 showed the presence of mutations or other small internal rearrangements involving the 3' end of the first exon.

c-myc loci in two MLCLs contain mutations or other small internal rearrangements. The presence of mutations or other small rearrangements at the 3' end of c-myc exon 1 was investigated by hybridization of Pvu II-digested DNAs to the 5'-Pvu probe. Cases 2 and 5 each presented an additional band other than the 0.86-kb Pvu II germline fragment (Fig 3), measuring approximately 1.1 kb and 1.75 kb, respectively. To demonstrate which of the two Pvu II sites analyzed was lost, Pvu II-Xho I-digested DNAs of cases 2 and 5 were hybridized to Pvu-X and X-Pvu probes. The hybridization with Pvu-X probe showed the Pvu II-Xho I fragments to be intact in both alleles (Fig 4). Hybridization with X-Pvu probe showed one unmodified allele and an additional band of about 0.65 kb in case 2 and 1.3 kb in case 5 (not shown), demonstrating that the Pvu II site lost was consistently the one at the 3' end of the first exon. The same DNAs (cases 2 and 5) digested with Pvu II and hybridized to the 3'-Pvu probe showed a new band at 0.56 and 1.75 kb, respectively (Fig 4). Moreover, the hybridization of Pst I-digested DNAs to the 5'-Pvu probe showed a new band of about 2.6 to 2.65 kb in case 2, indicating the loss of some 50 to 100 bp in one c-myc allele, and a germline band in case 5 (Fig 5).

In conclusion, our data show the presence of c-myc gene monoallelic alterations clustered in and around the 3' end of exon 1 in these two cases. Our findings include the loss of the Pvu II site within the first exon in both cases, along with the deletion of some 50 to 100 bp and the generation of a new Pvu II site in the first intron in case 2. The occurrence of mutations or other small internal rearrangements (such as deletions, substitutions, or insertions) could account for the alterations found in the segment spanning between the Xho I site of the first exon and the Pvu II site of the first intron of case 2, and for the loss of the first exon Pvu II site in case 5. Indeed, additional cases carrying mutations in the first exon may exist but remain undetected by the Pvu II site analysis. The small quantity of available biopic material obtained by mediastinoscopy did not yield enough DNA for all the digestions and hybridizations required to demonstrate this hypothesis.

PBL of patients 1 and 5 as well as 30 DNA samples from either PBL of normal individuals (10 cases) or tumors other than B-cell neoplasia (20 cases) showed no alterations of the Pvu II fragments. This finding rules out the possibility of inherited restriction fragment length polymorphism and confirms the data obtained in 60 North American and in 11 European control cases.

Characterization of c-myc rearrangement of case 3. The c-myc gene rearrangement in case 3 was further examined by Southern blot analysis, using a combination of different restriction enzymes and probes, to explore different segments of the c-myc locus. By this approach it is possible to map approximately the recombination site corresponding to the breakpoint of a reciprocal chromosomal translocation. Sst I- and Pst I-digested DNA hybridized to Pvu II probe showed that the rearrangement was detectable only in Pst I-digested DNA (Fig 6), whereas the 13-kb Sst I fragment was intact (Fig 6). Hybridization of Pst I- and Sst I-digested DNA to MC413RC again showed intact frag-
Fig 3. Pvu II-digested DNAs from MLCL cases hybridized to 5'-Pv probe (see Fig 1). Cases 2 and 5 show an additional band other than the germline 0.86-kb band. Case numbers correspond to those of Table 1. N is a DNA from normal PBL used as a negative control. Fragment sizes are given in kilobases.

Fig 4. Characterization of c-myc abnormalities in cases 2 and 5. The enzymes and probes used are indicated below the figure. Pvu II-Xho I-digested DNAs hybridized to Pvu-X probe yielded germline fragments. Pvu II-digested DNAs showed a new band other than the 0.86-kb germline band in both cases. Case numbers correspond to those of Table 1. N is a DNA from normal PBL used as a negative control. Fragment sizes are given in kilobases.
Fig 5. Characterization of c-myc abnormalities in cases 2 and 5. Pst I-digested DNAs hybridized to 5'-Pv probe showed a new band of approximately 2.6 to 2.65 kb in case 2 and a germ-line sized band of 2.7 kb in case 5. Case numbers correspond to those of Table 1. N is a DNA from normal PBL used as a negative control. Fragment sizes are given in kilobases.

Fig 6. Characterization of the c-myc locus rearrangement in tumor 3. The enzymes and probes used are indicated below the figure. The rearranged allele is detectable in Pst I-digested DNA hybridized to 5'-Pv probe and in Pvu II digest hybridized to 3'-Pv probe. N is a DNA from normal PBL used as a negative control. Fragment sizes are given in kilobases.

Additional band at 2.0 kb (Fig 6). Together, these data suggest that the breakpoint was located in the Sst I-Pvu II region within the Sst I-Sst I fragment in the first intron of the c-myc locus. The finding of the truncation of c-myc led us to examine whether this rearrangement corresponded to the juxtaposition of c-myc with the Ig heavy-chain (IgH) locus on chromosome 14. We attempted to identify the chromosomal breakpoint by studying the linkage between c-myc and various IgH regions (JH, Cμ, and the switch μ region) in Southern blot hybridization experiments using Bam HI-, HindIII-, and EcoRI-digestions. No linkage was found between c-myc and JH loci. Hybridizations with Cμ probe yielded only germline fragments.

Lack of bcl-2 Rearrangements and Viral Sequences

Southern blot hybridization of the tumor DNAs was performed with probes from the major and minor bcl-2 breakpoint regions that identify the t(14;18) encountered in follicular lymphomas as well as in diffuse LCLs. No rearrangement was detected by any of these probes in the six MLCLs. All of the cases were also negative when tested for EBV sequences after hybridization with the EBV internal repeat probe.

DISCUSSION

In the present study we analyzed the organization of c-myc and bcl-2 genes, and explored the presence of EBV DNA sequences in six cases of MLCL of young adult age. The major finding of our study is the demonstration of c-myc gene anomalies in three cases: (1) the presence of mutations or small rearrangements involving the 3' end of
the first exon in two cases, and (2) the truncation of the \( c-myc \) gene within its first intron in an additional case. These \( c-myc \) gene modifications are similar to those that represent specific features of \( c-myc \) alleles joined to Ig gene loci by a reciprocal translocation. Namely, mutations of sequences clustered around the boundary of \( c-myc \) exon 1 and intron 1, a region that is believed essential for regulation of transcription,\(^{3,6,7}\) are constantly present in \( c-myc \) genes involved in the \( t(8;14) \) of the type found in endemic Burkitt’s lymphoma (eBL) as well as in \( t(2;8) \) and \( t(8;22) \).\(^{2,22}\) On the other hand, the truncation of the \( c-myc \) gene within its first intron, first exon, or flanking sequences is a characteristic of \( t(8;14) \) encountered in the sporadic form of Burkitt’s (sBL; undifferentiated non-Burkitt’s)\(^{3,9,19} \) and in AIDS-associated lymphomas.\(^{20}\)

The \( c-myc \) gene appears to play an important role in the control of cell proliferation and its normal regulation is remarkably complex. This regulation can be disrupted in a number of ways,\(^{22}\) such as following the translocation with one of the Ig genes, leading to the abnormal expression of the gene. A role for an abnormal expression of the \( c-myc \) gene in malignant B-cell transformation is strongly suggested at the experimental level.\(^{3,6,19}\)

Based on these considerations, our findings support the view that (1) the \( c-myc \) gene is likely to be translocated in MLCLs, (2) the translocation could possibly be with an Ig chain gene locus carrying chromosome, and (3) the activation of \( c-myc \) gene may play an important role in MLCL pathogenesis. However, a translocation involving a chromosome other than those harboring the Ig loci or a different chromosomal abnormality could be responsible for the named \( c-myc \) modifications. Cytogenetic analysis alone can aid in clarifying this topic. Unfortunately, cytogenetic data are available neither in our cases nor in reported cases of MLCLs. Available data on cytogenetic abnormalities\(^{41,42} \) in human neoplasias indicate that band q24 of chromosome 8, in which \( c-myc \) gene is localized, has been found to be uniquely involved in reciprocal translocations detected in lymphomas. The partners of chromosome 8 were most frequently chromosomes 14, 2, and 22, and in a few instances chromosomes 11\(^a\) (one case), 9\(^{41,43} \) (two cases), and 3\(^a\) (one case). All these translocations were associated with small non-cleaved cell histology. The two reported cases showing a \( t(8;9) \) were a follicular lymphoma and a diffuse lymphoma not otherwise specified containing a \( t(14;18) \) as well, respectively. Of the named translocations, only the \( t(8;14) \) has also been found in diffuse LCLs. However, the anatomical site of the LCLs containing a \( t(8;14) \) (ranging from 11% to 28% of the cytogenetically studied cases)\(^{9,13,14} \) have not been documented well enough to determine whether there is an excess number of mediastinal primary sites. Relevant information regarding these patients’ ages is unaccessable as well. Further, while the molecular abnormalities of the genes involved in the \( t(8;14) \) of small non-cleaved cell lymphomas (eBL, sBL, AIDS-associated) have been extensively studied, the molecular anatomy of the \( t(8;14) \) found in LCLs remains unexplored. It is worth noting that this study is the first time that \( c-myc \) exon 1 abnormalities have been explored and shown to exist in lymphomas other than the small non-cleaved cell ones.

The association between \( c-myc \) anomalies and MLCL seems to be of relevance in light of recent studies indicating that a number of genetic abnormalities, molecularly and/or cytogenetically characterized,\(^{3,9,18} \) are closely associated with morphologically and clinically distinct subsets of leukemia and lymphoma, and have been shown to be useful markers for diagnostic and prognostic purposes.\(^{44} \) The frequent tendency of MLCL to involve unusual extranodal sites such as the kidney and adrenal cortex (accounting for 40% of the cases examined at the University of Verona, Italy),\(^4 \) along with the absence of bone marrow involvement, is significant in its similarity to the spreading attitude of the Burkitt’s lymphoma, which shares the anomalies of \( c-myc \) genes with MLCL.

We are aware that additional genetic alterations are probably necessary to complement \( c-myc \) activation in the complex multistep process of the transformation of a normal cell into a cancer cell. We showed that these alterations are not represented by bcl-2 rearrangement or EBV infection in the case of MLCLs.

In addition to its implication for the pathogenesis of MLCL, the finding of a specific association between \( c-myc \) abnormalities and MLCL may have a practical significance in achieving a better diagnostic definition of such cases and in establishing the nature of post-therapy relapses, as well as in adding information that might prove useful for prognostic evaluation and therapeutic choices.

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REFERENCES


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