Detection of single and associated lesions of the Bcl-1, Bcl-2, Bcl-6, c-myc, p53 and p16 genes in B-cell non-Hodgkin’s lymphomas: value of molecular analysis for a better assignment of the histologic subtype

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ABSTRACT

Background and Objective. Molecular genetic abnormalities have been frequently described in non-Hodgkin’s lymphomas (NHL). These lesions have been associated with specific entities, allowing a better categorization of NHL. However, these abnormalities are not as specific as initially described and their association is still unknown.

Design and Methods. By Southern blot and polymerase chain reaction, we have simultaneously analyzed the proto-oncogenes Bcl-1, Bcl-2, Bcl-6, c-myc and MLL and the tumor suppressor genes p53 and p16, in 100 unselected B-cell NHL patients at diagnosis, to establish its incidence throughout the different NHL subtypes, defined both by Working Formulation and REAL classifications, and to assess the frequency of co-existence of two or more genetic lesions within each individual patient.

Results. Fifty two cases displayed some genetic abnormality. Bcl-1, altered in 12 cases, was highly specific to mantle cell lymphomas (57% of them), but 6 cases had a different histologic subtype. Bcl-2 was rearranged in 26 cases: 70% in follicular lymphomas (FL) and 20% in diffuse large cell lymphomas; these abnormalities were also present in other subtypes, i.e. marginal lymphomas (30%). Bcl-6 abnormalities were mostly found in diffuse large cell lymphomas (29%) but also found in other subgroups, like FL (14%). C-myc rearrangements were specific to Burkitt’s lymphoma. MLL gene was always germline. Deletions and/or rearrangements of p53 and p16 genes were rare (4% and 8% of all cases, respectively). Finally, association of genetic lesions was a relatively common finding (13% of cases), especially in cases with adverse prognostic morphologies according to the REAL.

Interpretation and Conclusions. Molecular abnormalities are frequent in NHL at diagnosis, not only as unique lesions but also associated. A relative high specificity of some alterations was seen, thereby contributing to a better assessment of the histological subtype.

E XPERIMENTAL data support the view that carcinogenesis is a multistep process that involves activation of proto-oncogenes as well as inactivation of tumor-suppressor genes.1 In past years, molecular characterization of non-random cytogenetic abnormalities has led to the identification of several genes whose alterations represent a very important mutational mechanism in non-Hodgkin’s lymphomas (NHL).2 That information has resulted in recognition of new entities and refinement of previously recognized disease entities.3 For example, the follicular lymphomas (FL) are characterized by the t(14;18) translocation, present in 40-85%, moving the long arm of chromosome 18 to the long arm of chromosome 14.4,5 Similarly, in about of 60% of mantle cell lymphomas (MCL), the t(11,14) translocation involves a rearrangement of the CCND1/Bcl-1 gene.4,6 In addition, almost all Burkitt’s lymphomas have a t(8;14), t(8;22) or t(2;8) in which the c-myc gene translocates from chromosome 8 to chromosomes that harbor the immunoglobulin heavy or light chain genes.9-11 Bcl-6 gene rearrangements have been recently ascribed to diffuse large cell lymphomas (DLCL).12,13 All this data has contributed to a better categorization and management of NHL, but some of these abnormalities are not as specific as initially described. For example, the t(14;18) translocation has also been found in one third of DLCL14,15 and the t(11;14) is present in some NHL different from the MCL.16,17 Thus, in order to define the real incidence and specificity of these molecular markers, comprehensive studies are needed in which several genes are simultaneously analyzed in unselected series of NHL. Moreover, this type of studies will help to reveal associations of genetic lesions which may contribute to the understanding of the pathogenesis of the disease.

In addition, there are some proto-oncogenes such as mixed lineage leukemia (MLL) and tumor sup-
pressor genes like p53 and p16 that have been shown to be altered in closely related disorders, such as lymphoid leukemias.19-22 Their real incidence NHL and their possible relationship with specific histological subtypes are still unknown.

In the present study, we have simultaneously analyzed the presence of abnormalities of the proto-oncogenes Bcl-1, Bcl-2, Bcl-6, c-myc and MLL and the tumor suppressor genes p53 and p16 in a series of 100 untreated NHL. We then correlated them with histologic subtypes defined according to two classifications (Working Formulation and REAL classifications).

Materials and Methods

Patients

High molecular weight DNA was obtained from tumor specimens of 100 adult untreated patients consecutively admitted to the University Hospital of Salamanca between July 1989 and July 1996 with the histopathological and immunohistochemical diagnosis of B-NHL. The distribution of patients into category groups was made according to the Working Formulation criteria.23 When the new REAL classification became available,24 all cases were reviewed and redistributed accordingly to these new criteria.

Tissue samples

Fresh samples from lymph nodes, bone marrow or peripheral blood were selected for the analysis based on the number of tumor cells, rejecting those with less than 25% neoplastic cells. The number of tumor cells was counted by flow cytometric analysis based on their k/λ and B cell antigen expression.24 The presence of clonal B cells was also assessed by detection of IgH clonal rearrangements with Southern blot analysis (JH6 probe)25 in order to confirm not only the B-cell clonality of the disease but also the presence of sufficient clonal cells in the sample.

DNA preparation and Southern blot (SB) analysis

High molecular weight DNA was isolated by standard proteinase K digestion, phenol-chloroform extraction and ethanol precipitation.26 For Southern blot analysis, 10 µg of DNA were digested to completion with the Eco RI, Bam HI, Hind III, Bgl II and Xba I restriction enzymes; size fractionated in 0.8% agarose gel, denatured, neutralized, and transferred to nylon membranes (Hybond-N+, Amersham, Little Chalfont, UK). The blots were hybridized with the appropriate ready to go32P-labeled probes according to the manufacturer’s specifications (Ready to go, Pharmacia Biotech, Upsala, Sweden), washed twice

<table>
<thead>
<tr>
<th>Genes</th>
<th>DNA probe</th>
<th>Size of insert in Kb (restriction enzymes)</th>
<th>Restriction enzymes used for digestion of genomic DNA</th>
<th>Size of germine restriction fragments in Kb</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgH</td>
<td>IgH6</td>
<td>1.02 (Eco RI/Hind III)</td>
<td>Bgl II, Bam HI/Hind III</td>
<td>3.8, 6.0</td>
<td>25</td>
</tr>
<tr>
<td>Bcl-1</td>
<td>MTC</td>
<td>2.3 (Sac I-Sac I)</td>
<td>Eco RI, Hind III, Bam HI</td>
<td>12.5, 11.0 and 2.8, 21.0</td>
<td>4</td>
</tr>
<tr>
<td>Bcl-2 (MBR)</td>
<td>PFL1</td>
<td>1.5 (Eco RI/Hind III)</td>
<td>Bam HI, Eco RI, Hind III</td>
<td>20.0, 3.0, 4.0</td>
<td>5</td>
</tr>
<tr>
<td>Bcl-2 (mcr)</td>
<td>PFL2</td>
<td>4.0 (Eco RI/Eco RI)</td>
<td>Hind III, Eco RI</td>
<td>13.0, 3.9</td>
<td>27</td>
</tr>
<tr>
<td>Bcl-2 (vcr)</td>
<td>pB16</td>
<td>1.6 (Eco RI/Eco RI)</td>
<td>Hind III, Bam HI</td>
<td>8.0, 4.0, 1.5</td>
<td>28</td>
</tr>
<tr>
<td>Bcl-6/LAZ3</td>
<td>Bcl-6</td>
<td>4.0 (Sac I-Sac I)</td>
<td>Bam HI, Xba I</td>
<td>11.0, 14.0</td>
<td>12</td>
</tr>
<tr>
<td>MLL</td>
<td>11q23</td>
<td>0.8 (Bam HI-Bam HI)</td>
<td>Bam HI</td>
<td>8.3</td>
<td>27</td>
</tr>
<tr>
<td>c-myc</td>
<td>cD1A</td>
<td>2.0 (EcoR1-EcoR1)</td>
<td>Hind III, Bgl II</td>
<td>10.0, 6.0, 5.8</td>
<td>38</td>
</tr>
<tr>
<td>MTS1/p16</td>
<td>p16T (cDNA)</td>
<td>0.8 (Eco RI-Xho)</td>
<td>EcoR1, Hind III</td>
<td>4.2, 6.0</td>
<td>39</td>
</tr>
<tr>
<td>p53</td>
<td>cDNA</td>
<td>1.7 (Bam HI-Bam HI)</td>
<td>Eco RI</td>
<td>15.0 &amp; 3.7</td>
<td>40</td>
</tr>
</tbody>
</table>
in 0.5% SSC, 1% sodium dodecyl sulfate (SDS) for 30 minutes at 65°C, and autoradiographed between 48 and 96 hours at –80°C. Bcl-1, Bcl-2 (MBR, mcr, vcr), Bcl-6, c-myc, MLL, p53 and p16 genes were studied using this methodology. Probes used to detect rearrangements and deletions on these genes, together with the restriction enzymes used for digestion of genomic DNA and size of germline restriction fragments detected with them are shown in Table 1.A5,12,19,25,27,31 In order to exclude the restriction fragment length polymorphisms, digestion with two or more restriction enzymes was carried out in every gene.

**Polymerase chain reaction (PCR) to detect the t(11;14) and t(14;18) translocations**

The amplification of the breakpoint regions of both translocations was performed by PCR with 1.5 μg of genomic DNA and the oligonucleotides as shown in Table 2.A6-8 using the specifications of Molot al.7 for the t(11;14) translocation and Gribben et al.6 for the t(14;18) translocation, slightly modified. PCRs for t(11;14) translocation were performed in a 50 µL final volume containing: 100 nmol/L of oligonucleotide primers, 200 µmol each of deoxynucleotide triphosphate, 1.5 mmol/L MgCl2 and 1.5 U of Taq polymerase (Promega, Madison, WI, USA) in PCR buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl, 1% Triton). The same conditions were used to detect the t(14;18) translocation, except for oligonucleotide primers (20 nmol/L) and MgCl2 (2.25 mmol/L). For t(11;14) translocation, each amplification included a first PCR product was carried out for 30 cycles using oligonucleotide primers internal to the original primers (JHc-int and Bcl-2 MBR-int or Bcl-2 mcr-int) with 1 minute of denaturation (94°C), 1 minute of annealing at 58°C and 1 minute of extension at 72°C. The initial denaturation was performed for 5 minutes at 94°C followed by 30 cycles, each cycle consisting of 1 minute at 94°C, 1 minute at 55°C for the MBR or 58°C for the mcr amplification, and 1 minute at 72°C. The final extension was developed for 10 minutes at 72°C. In each case, a re-amplification of a 5 µL of the first PCR product was carried out for 30 cycles using oligonucleotide primers internal to the original primers (JHc-int and Bcl-2 MBR-int or Bcl-2 mcr-int) with 1 minute of denaturation (94°C), 1 minute of annealing at 58°C and 1 minute of extension at 72°C. The initial denaturation and final extension were again for 5 minutes at 94°C and 10 minutes at 72°C, respectively.

PCR products from the final reactions were analyzed by electrophoresis in 1.5% agarose gel (Nu Sieve, FMC, Rockland, ME, USA) in tris-borate electrophoresis buffer and visualized by staining with the ethidium bromide under UV light. In all experiments, two negative controls (sterile distilled water and normal DNA) and a positive control (genomic DNA from a positive patient diluted 10–3, 10–4 and 10–5 in normal DNA) were used. The final sensitivity was 10–4/10–5 in all cases.

**Results**

Overall, 52% of the patients displayed some genetic abnormality. Tables 3 and 4 show these genetic lesions and their distribution along the WF and REAL classifications.

**Bcl-1**

Abnormalities of the Bcl-1 proto-oncogene were identified in a total of 12 cases, 8 of them by SB and 10 by PCR. Thus, two cases were positive by SB only, and four cases were positive by PCR only, while the remaining 6 cases were positive by both techniques. Concerning the histological subtype, four cases were diffuse small cleaved cell lymphomas (SCCL), three diffuse large cell lymphomas (DLCL) and five small lymphocytic lymphomas (SLL). According to the REAL classification, 6 of them corresponded to mantle cell lymphomas (MCL), 3 were DLCL, 2 marginal lymphomas (ML) and one small lymphocytic lymphoma (SLL). Thus, 6 out of 11 MCL (57%), 3/25 DLCL (12%) and 2/9 ML (22%) had abnormalities of the Bcl-1 proto-oncogene. Interestingly, the six MCL have both SB and PCR positive results while in the other six patients, alterations of Bcl-1 were detected either by SB or by PCR but not with both, indicating that these cases either had a different involved region

**Table 2. Oligonucleotides used for the detection of the t(11;14) and t(14;18) translocation by PCR-Nested.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Translocation</th>
<th>Localization</th>
<th>Sequence</th>
<th>Refs.</th>
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<tbody>
<tr>
<td>Bcl-1 MTC-ext</td>
<td>t(11;14)</td>
<td>MTC</td>
<td>5'cagccttgataagcctg3'</td>
<td>7</td>
</tr>
<tr>
<td>Bcl-1 MTC-int</td>
<td>t(11;14)</td>
<td>MTC</td>
<td>5'gagccttgggatgctc3'</td>
<td>8</td>
</tr>
<tr>
<td>Bcl-2 MBR-ext</td>
<td>t(14;18)</td>
<td>MBR</td>
<td>5'cagccttgataacattagc3'</td>
<td>6</td>
</tr>
<tr>
<td>Bcl-2 MBR-int</td>
<td>t(14;18)</td>
<td>MBR</td>
<td>5'tatggtgtaacctttag3'</td>
<td>6</td>
</tr>
<tr>
<td>Bcl-2 mcr-ext</td>
<td>t(14;18)</td>
<td>mcr</td>
<td>5'cgctctgtaaccatctg3'</td>
<td>6</td>
</tr>
<tr>
<td>Bcl-2 mcr-int</td>
<td>t(14;18)</td>
<td>mcr</td>
<td>5'ggacgctcttggtgctg3'</td>
<td>6</td>
</tr>
<tr>
<td>JHc-ext</td>
<td>t(11;14)</td>
<td>JH</td>
<td>5'acgagggtgatgctggtc3'</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>t(14;18)</td>
<td>JH</td>
<td>5'acgagggtgatgctggtc3'</td>
<td>6</td>
</tr>
<tr>
<td>JHc-int</td>
<td>t(11;14)</td>
<td>JH</td>
<td>5'acgagggtgatgctggtc3'</td>
<td>6</td>
</tr>
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</table>
or that of the abnormality only occurred in a subset of tumor cells, respectively. All 11 mantle cell lymphoma cases were CD19+CD5+CD23–CD10–SIg+, except one which had a CD23+ subpopulation, and presented the t(11;14) translocation.

**Bcl-2**

Alterations of the Bcl-2 proto-oncogene were noted in 26 cases. The MBR region was involved in 20 cases, the mcr in 5 cases and the vcr in 5 cases. In four patients more than one region was involved: MBR/vcr in two, mcr/vcr in one and MBR/mcr in one, the last case lacking co-migration between the rearranged bands of mcr and JH. Of these 26 cases, 15 were follicular lymphomas (FL) (groups B, C & D of WF) and 3 were DLCL (group G). Of the remaining eight cases, two corresponded to small non-cleaved cell lymphomas (SNCCL), one to a lymphoblastic lymphoma (LBL) and one to SCCL, while the other 4 cases were initially classified as SLL (group A). Globally, the incidence of Bcl-2 abnormalities was 70% in FL and 20% in DLCL. When the REAL classification was employed, the results were similar. However, a new category included in the REAL classification, the marginal NHL, also showed a high incidence of Bcl-2 rearrangements (3/9), the mbr region being affected in all of them. Interestingly, none of the five vcr rearrangements corresponded to SLL, but they were observed in FL (n=3), Burkitt’s NHL (n=1) and MCL (n=1).

**Bcl-6**

The Bcl-6/LAZ3 gene was rearranged in 12 cases: DLCL (n=4), FL (n=3), SNCCL (n=2), SLL (n=2) and IL (n=1). Rearrangements were confirmed in Bam HI, Xba I and Hind III digestions. Thus, the incidence of Bcl-6 rearrangements in DLCL was 29% but it was lower in the corresponding group of REAL classification (20%).

**c-myc**

Seven rearrangements of the c-myc proto-oncogene were observed. Six were present in cases of Burkitt’s NHL and the other in an MCL, according to REAL classification. Inversely, of 7 SNCCL (group J of WF)
there was one case without \textit{c-myc} rearrangement, but it was considered as a Burkitt-like NHL in the REAL classification. All six Burkitt’s lymphoma cases were CD19+CD22+CD5-CD23-CD10-Sig+.

**11q23/MLL**

No abnormalities of 11q23/MLL gene were found in any of the 100 NHL patients included in this series.

**p53**

Four cases showed alterations in the p53 gene using the SB methodology. There was one homozygous deletion and one hemizygous deletion, together with two rearrangements. These two rearrangements were found in high grade lymphomas (one Burkitt’s and one unclassified NHL), while the two deletions were observed in two cases of SLL, representing an incidence of 6% in the WF and 9% in the REAL classification.

**Genetic lesion associations**

Thirteen cases displayed more than one gene abnormality (Table 5). The histologic category that most frequently showed coexistence of two or more abnormalities was the SNCCCL since 4 of these cases showed another genetic lesion associated to the \textit{c-myc} rearrangement. The remaining cases did not show a particular histologic distribution. Nevertheless, upon using the REAL classification, cases with multiple genetic lesions were more frequently observed within adverse histologic categories, since only four cases corresponded to the indolent forms (2FL, 2 ML) while 9 cases corresponded to aggressive subtypes (4 Burkitt’s NHL, 3 MCL and 2 DLCL) (8% of SLL+FL+ML+HCL+LPL, versus 19% in PCL+DLCL+MCL+BL+LPL+unclassified). Moreover, all 3 cases that simultaneously displayed three genetic lesions also fell into the adverse categories: two MCL and one Burkitt’s lymphoma.

**Discussion**

Molecular characterization of non-random chromosomal translocations and other genetic abnormalities\(^2\) has largely contributed to a better definition of NHL due to the association between some clinico-

<table>
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<tr>
<th>Case WF(^2)</th>
<th>REAL(^3)</th>
<th>Molecular analysis</th>
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<tbody>
<tr>
<td>1</td>
<td>B</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td>+</td>
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<td>3</td>
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<tr>
<td>9</td>
<td>G</td>
<td>+</td>
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G: germline; R: rearranged; d: Deleted. *p53/PCR-SSCP analysis not performed.
The presence of t(14;18) has been reported in 40-85% of patients with FL, and in 20-30% of DLCL. In most of these cases, the breakpoint usually involves the untranslocated region of the exon 2 (MBR); in the remaining cases, the breakpoint is located 20 Kb downstream (mer). In our series, 20 cases had the abnormality in the MBR region and 5 in the mer region. Most of these cases corresponded to FL (groups B, C & D) or DLCL (group G), with an incidence of 71% and 20%, respectively. In addition, it should be noted that some cases of SLL and SCCL may show this translocation. The variant forms [t(2;18); t(18;22)], that involve the mer region of the Bcl-2 gene, have been classically ascribed to CLL/SLL. However, in our experience, this genetic abnormality may also be present in other histologic subtypes, such as FL, which is concordant with the observation of Tsujimoto et al. and Merup et al. This indicates that FL can deregulate the Bcl-2 gene through different mechanisms that can coexist within the same case, as we were able to observe in four patients with more than one of these regions affected.

Contradictory data has been reported concerning rearrangements of Bcl-6 gene. They are present in around one third of DLCL and, although they were initially described as specific of this NHL subtype, they can also occasionally be observed in cases of low grade NHL. In the present study, we have found Bcl-6 rearrangements in 12 out of 100 NHL; the majority of cases belonged to the groups G & H of the REAL. This data confirms the high specificity of the REAL classification. Since these two cases would not have been detected by PCR-SSCP analysis, the methodology employed most frequently in the vast majority of reports, the importance of p53 alterations should be reconsidered in low grade lymphomas.

It has been suggested that the other tumor suppressor gene analyzed here, p16, is as important as p53, and several reports have shown it to have a critical role in both in T-lineage and in B-lineage acute lymphoblastic leukemias. In B-NHLs the reported incidence of p16 alterations was very low (6%) and there is only one study that has associated the abnormalities with a particular histologic subtype (DLCL), but with an incidence of only 15%. In the present series, the overall frequency of p16 alterations was 8%, while within DLCL it was 13%. This suggests that p16 lesions, though perhaps are more frequent in DLCL, are not greatly different from other histologic subtypes. Interestingly, three of our cases with p16 abnormalities were low grade lymphomas according to the REAL classification was used. Thus, according to these results, it appears that p16 lesions are probably associated with adverse prognostic morphologic subtypes.

Co-existence of multiple genetic events in individual NHL patients has been observed during disease progression, supporting the multistep hypothesis suggested for the pathogenesis of cancer. Some reports have also found more than one genetic abnormality at diagnosis, especially in those cases with high grade histology and/or bad prognosis, and in some specific entities like acquired immunodeficiency syndrome-related NHL, although the real incidence of this finding still needs to be established. The present work shows that the incidence of more than one genetic alteration in 100 unselected NHL is relatively high at diagnosis (13%), supporting the idea that the multistep process is present not only in the latter course of the disease, but it is already present during the initial development of the tumor. In addition, the relatively higher frequency found in cases with aggressive morphologies according to the REAL, could help to support the hypothesis that the co-existence of two or more genetic lesions may contribute to the emergence of different NHL forms, probably reflecting a higher grade of genetic instability.

In summary, the present work shows that genetic abnormalities are a frequent event in NHL, some of them being associated with specific histologic types. In addition, the presence of several associated genet-
oncogene is located.

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...The order tries to take into account the time work and scientific contribution of all authors.

Contributions and Acknowledgments

RG-S was the main investigator and designed the study, managed the data and performed the literature revision; he wrote the article with MG, and both were responsible for the data interpretation, direct supervision, funding and day-to-day contact with participants. MV carried out the DNA digests, transfections and final preparations of Southern blot filters, together with PCRs of all samples. MCC and AB performed all DNA extractions, probe growing and purifications, and final hybridizations. TF was the pathologist who reviewed all the morphological pictures. MDC and JH were responsible for the clinical management and clinical data acquisition. JFSM was the main coordinator of the group and reviewed the article to obtain the final form in which it was sent for submission. The order tries to take into account the time work and scientific contribution of all authors.

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Disclosures

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