BCL-2 MOLECULAR ANALYSIS IN PARAFFIN-EMBEDDED BIOPSIES FROM DIFFUSE LARGE B-CELL LYMPHOMAS

CARLOS DE BRASI1, MARINA NARBAITZ2, ANDREA RODRIGUEZ3, IRENE LARRIPA1, IRMA SLAVUTSKY1

Abstract

Translocation t(14; 18) has been observed in 50-85% of follicular and in 30% of diffuse non-Hodgkin lymphomas. About half of follicle center lymphoma (FCL) undergo histological conversion at relapse to more aggressive diffuse large B-cell lymphoma (DLBCL). This report correlates the molecular bcl-2/IgH rearrangement by PCR and Bcl-2 immunohistochemical (IHC) expression in a series of high grade DLBCLs with and without FCL remnant. Twenty-three paraffin-embedded lymph nodes from DLBCL patients were analyzed. Eleven patients showed FCL remnant (Group A) and 12, did not (Group B). Single PCR from paraffin extracted DNA followed by Southern transfer of products, hybridisation with internal oligoprobes for the MBR/JH and MCR/JH bcl-2 rearrangements and IHC analysis of Bcl-2 expression, were performed. PCR analysis was positive in 34.8% of patients. Bcl-2/IgH gene rearrangements were observed in 8 (34%) cases and 7 (30%) showed Bcl-2 expression on large noncleaved B-cells (centroblasts). All patients from Group A showed IHC positive reaction on FCL remnant (small cleaved cells) but only 2 (18%) were positive in DLBCL areas, suggesting either the loss of the bcl-2 expression on the transformed lymphoma, or, alternatively, the development of a second disease when the first lymphoma transforms. Group B patients showed a clear correlation between PCR and IHC studies. Our results suggest a similar frequency of t(14; 18) in DLBCLs to that reported in Europe and USA series. The discordance observed between PCR and IHC, particularly in Group A, points out the necessity to perform both studies in order to detect bcl-2 gene involvement in DLBCLs.

Resumen

Análisis molecular del gen Bcl-2 en biopsias incluidas en paraffina en linfomas difusos de grandes células B. Este trabajo analiza la relación entre el rearreglo molecular bcl-2/IgH por PCR y la expresión inmunohistoquímica (IHQ) de Bcl-2 en linfomas difusos de grandes células B (LDGCB), con y sin remanente folicular. Fueron evaluadas 23 biopsias de ganglio linfático incluidas en paraffina. Once pacientes mostraron remanente follicular (Grupo A) y 12 no (Grupo B). El ADN extraído de material parafinado fue amplificado por PCR simple, transferido e hibridado con oligonucleótidos específicos para los rearreglos MBR/JH y MCR/JH del gen bcl-2. El análisis por PCR resultó positivo en el 34.8% de los pacientes. Ochos casos (34%) presentaron el rearreglo Bcl-2/IgH y 7 (30%) mostraron expresión de Bcl-2 en las células B grandes no hendidas (centroblastos). Todos los pacientes del Grupo A presentaron expresión Bcl-2 en el remanente follicular (pequeñas células B hendidas o centrocitos), en cambio sólo 2 casos (18%) fueron positivos sobre centroblastos. Esto podría deberse a que el clon neoplásico original perdería la expresión Bcl-2 en la transformación, o bien que el LDGCB se originaría a partir de células ya negativas para Bcl-2 en el linfoma primario. En el Grupo B se observó una buena correlación entre los resultados de PCR e IHQ. Estos resultados muestran una frecuencia de la t(14; 18) en esta serie de LDGCB, similar a las reportadas para Europa y Estados Unidos. La discordancia observada entre los datos de PCR e IHQ, particularmente en el Grupo A, indica la utilidad de efectuar ambos estudios para detectar la participación del gen bcl-2 en los LDGCBs.

Key words: bcl-2 gene rearrangements, Bcl-2 protein expression, diffuse large B-cell lymphomas, non-Hodgkin lymphomas

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Postal address: Dr. Carlos De Brasi, Departamento de Genética, Instituto de Investigaciones Hematológicas, Academia Nacional de Medicina, Pacheco de Melo 3081, 1425 Buenos Aires, Argentina
Fax: (54-11) 4803-9475 e-mail: lacuteci@intramed.net.ar

Cytogenetics studies in non-Hodgkin lymphomas (NHL) have shown clonal chromosome abnormalities in more than 90% of cases1. Among them, translocation t(14; 18) (q32.3; q21.3) has been observed in 50-85% of B low grade and in 30% of high grade lymphomas2-6.

At the molecular level, this translocation juxtaposes the bcl-2 proto-oncogen (18q21) with the Ig heavy chain gene (IgH) (14q32)7, resulting in an up regulation of the bcl-2 gene. Most of the breakpoints on chromosome 18 are clustered in the major breakpoint region (MBR), located in exon III of the gene in the 3'-untranslated region of the bcl-2 mRNA8. A second region termed the minor cluster region (MCR) is located approximately 30 kb downstream of the gene9. Other more sporadic breakpoints were also observed, among them a number of examples have been identified where the breakpoint...
was placed 5' to the gene\textsuperscript{10}. Bcl-2 is now known to belong to a growing family of apoptosis-regulatory gene products, which may either be death antagonist (\textit{Bcl-2, Bcl-X\textsubscript{L}, Bcl-W,} etc) or death agonists (\textit{Bax, Bak, Bcl-X\textsubscript{S},} etc)\textsuperscript{11}.

About half of follicular lymphomas undergo histological conversion at relapse to a more aggressive diffuse large B-cell lymphoma (DLBCL)\textsuperscript{12}. Moreover, among these cases, lymph node samples often show both the presence of high-grade DLBCL and areas with low-grade remnant. This histological transformation is usually associated with a rapidly progressive clinical course and a short survival\textsuperscript{12,13}.

The purpose of this study was to estimate the frequency of \textit{bcl-2} gene rearrangement in Argentine patients affected with DLBCL and to correlate immunohistochemical (IHC) expression with DNA molecular rearrangements of the \textit{bcl-2} gene in these lymphomas with remnant of follicle center lymphoma (FCL) and without it\textsuperscript{14}. Discrepancies between both groups of patients were found. It is interesting to point out that in the transformed areas \textit{bcl-2} expression was mostly negative whereas it was positive in the follicular remnant areas of the same patient.

**Material and Methods**

**Samples**

Paraffin wax blocks from lymph nodes of 23 NHL patients (9 females and 14 males) with a mean age of 55.3 years (range 26-76) were analyzed. All cases were diagnosed as high-grade DLBCL according to REAL classification\textsuperscript{14}, following current histopathological protocols. At diagnoses, 36% were in initial stages of the disease and the remaining 64% in advanced ones. Eleven patients showed remnant of FCL (Group A) and 12 did not (Group B).

**Immunohistochemistry**

The paraffin sections were dewaxed in xylene and absolute ethanol and then microwaved 3 times for 4 min in 10 mM citrate buffer, pH 6.0. After cooling, they were immunostained with an avidin-biotin peroxidase detection system according to the manufacturer's instructions (Vectastain ABC, Vector Laboratories). The following primary monoclonal antibodies were used: CD20 (Pan B, L26 DAKO) and CD3 (Pan T, clone PS1 monoclonal NOVOCASTRA) for phenotypic characterization and Bcl-2 for protein expression (DAKO).

**DNA extraction**

For the DNA analysis, paraffin-embedded tissues were used, instead of fresh ones, at risk of losing some sensitivity in order to make correlation of closes slices in the same biopsy. Five 10 µm sections were dewaxed by xylene (2x15 minutes), washed twice in 100% ethanol and air dried with few drops of acetone. The sections were resuspended in 10 mM Tris/HCl, pH 8.5 mM EDTA, 0.5% SDS; digested during 15 hr at 37 °C with 0.5 µg/µl of proteinase K; extracted with phenol-chloroform, precipitated with isopropanol; washed in 70% ethanol and resuspended in 10:1 mM TE buffer\textsuperscript{15}. The concentration and quality of extracted DNA were estimated by agarose gel minielectrophoresis.

**PCR analysis**

Reactions were carried out in 10 mM Tris/HCl, pH 8.3; 50 mM KCl; 1 mM dNTPs each; 7mM MgCl\textsubscript{2} for MBR and 8 mM MgCl\textsubscript{2} for MCR and quality control PCR (QCP), 1 µM of primers and 2 U of \textit{Taq} polymerase (Gibco BRL). One µg of DNA were subjected to 35 cycles consisted in denaturation (1 min at 95 °C, first cycle 4 min), annealing (2 min at 59 °C for MBR, 55°C for MCR and 57 °C for quality control PCR, QCP) and extension (2 min at 72 °C, last cycle 5 min). PCR products were electrophoresed in 2% agarose gels.

**TABLE 1.– Oligonucleotides used in PCR analysis**

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>Function</th>
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<tbody>
<tr>
<td>A1</td>
<td>5’-TAGAGTGCCATTCTTACCT-3’</td>
<td>JH antisense primer</td>
</tr>
<tr>
<td>A2</td>
<td>5’-TTAGAGATTTGCTTACGTG-3’</td>
<td>MBR sense primer</td>
</tr>
<tr>
<td>A3</td>
<td>5’-CCCTGCCCTTCCCTCTTCTG-3’</td>
<td>MCR sense primer</td>
</tr>
<tr>
<td>A4</td>
<td>5’-GATGGCTTGGCTGAGGATT-3’</td>
<td>QCP sense primer</td>
</tr>
<tr>
<td>A6</td>
<td>5’-GCCTGTTTCAACACAGACCC-3’</td>
<td>MCR oligoprobe</td>
</tr>
<tr>
<td>A8</td>
<td>5’-ACCACCTAACCCCTAAACCG-3’</td>
<td>QCP antisense primer</td>
</tr>
<tr>
<td>A9</td>
<td>5’-CCACTCCTCCAGAGACTGA-3’</td>
<td>MBR oligoprobe</td>
</tr>
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The sequences of primers and probes are shown in Table 1. The MBR/JH PCR gave a 200-250 bp specific signal with primers A2 for MBR and A1 for JH consensus16. The MCR/JH PCR has been designed with primers A1 and A3 to give a 300-600 bp band. The QCP for DNA samples has been designed to give specific PCR products of 200 bp with primers A4 and A8; negative cases were excluded from this study (Fig. 1). This procedure assures that all the DNA samples analyzed have enough template to avoid low and medium sensitivity false positives.

In order to improve the sensitivity and the specificity of MBR and MCR analysis, PCR products were Southern transferred and hybridized with 32P end-labeled oligoprobes: A9 for MBR and A6 for MCR. Serial dilutions of DNA extracted from t(14;18) positive control cells in normal leukocyte-extracted DNA were made in order to estimate the sensitivity of the assay. Routinely, the 10^-2 dilution gave a visible band after ethidium bromide staining and the 10^-4 dilution was only positive after hybridization (Fig. 2, lanes 1, 2 and 3).

Conditions, primers and probes (A3, A4, A6 and A8) used in QCP and MCR PCR were specially designed for this report in order to investigate bcl-2 rearrangement from paraffin-embedded samples using the MCR published sequence17.

![Figure 2](image-url)

**Fig. 2.** MBR/JH rearrangements of the t(14;18): a) Agarose gel electrophoresis from patients of Group A. Lanes 1, 2 and 3: PCR products of 10^-2 (+), 10^-3 (-) and 10^-4 (-) dilutions of the positive control respectively. Lane 4: negative control. Lane 5: case 10 showing a positive band. Lanes 6 and 7: cases 6 and 3, negatives. b) Autoradiography of gel (a) hybridized with the MBR oligoprobes showing the sensitivity 100 fold increased. Case 3 on lane 7 shows a specific band not found previously. c) Agarose gel electrophoresis from patients of Group B. Lanes 1, 2, 3 and 4 show the controls. Lanes 6 and 7: cases 18 and 21: show strong clonal bands. Lane 5: case 22 negative. d) Autoradiography of gel (c) showing specific bands on lanes 6 and 7 from cases 18 and 21 respectively.
Fig. 3.— Immunohistochemical staining patterns of Bcl-2 protein. Group B DLBCL with Bcl-2 positive centroblastic cells (200x); Insert, Bcl-2 cytoplasmic positive centroblasts (630x).

MBR/JH rearrangements were observed in 8 cases (34.8%) by PCR. None of them showed the MCR/JH rearrangement. Seven patients (30.4%) showed Bcl-2 expression in the large no cleaved B-cells (centroblasts) by IHC analysis.

All samples from Group A patients showed positive IHC reaction in the FCL remnant (small cleaved cells or centrocytes) but only 2 cases (18%) were positive on centroblasts (Cb) cells in the DLBCL areas. PCR analysis was positive in 4 patients (36%), showing a low degree of correlation between IHC and PCR studies. Case 10 was the only one positive for both reactions. Case 7 showed positive Cb cells by IHC but was negative by PCR analysis. Cases 2, 3 and 8 were positive by PCR analysis but their Cb cells did not show Bcl-2 expression.

On the other hand, patients of Group B showed Bcl-2 expression in 5 (42%) cases by IHC and 4 (33%) of them had MBR/JH rearrangements, showing clear correlation between both studies. Case 16 was the only one that showed positive Cb cells by IHC but was negative by PCR analysis. PCR studies from both groups of patients are illustrated in Fig. 2a-d showing different clonal bands.

Discussion

We report here the analysis of bcl-2 gene rearrangements and their correlation with the Bcl-2 IHC protein expression on paraffin-embedded biopsy samples from DLBCL patients. A frequency of 34.8% in the bcl-2 gene rearrangement, was observed, all of them clustering at the MBR breakpoint. Our results suggest a similar frequency of t(14; 18) in the series reported here from DLBCL patients to those observed in Europe and USA series2, 4, 18, 19.
DLBCL appears to be defined by two types of progression, some patients showing only this type of lymphoma and others with a FCL, which subsequently transforms into a more aggressive DLBCL. Moreover, this change may be with retention of the cell type or may involve a change in the predominant cell type. So far, it is not clear whether these two types of disease progression are genuinely different, or whether the former situation simply indicates that a preliminary phase of the FCL has not been observed.

We have studied cases showing a remnant of FCL (Group A) inserted in areas of the leading DLBCL and patients with only DLCBL (Group B). In Group A, our results showed a clear difference at the Bcl-2 expression between the FCL remnant and the transformed areas, showing positive centrocytes (Cs) in the former and negative Cb cells in the latter. Interestingly, all FCL remnants of Group A showed positive Bcl-2 IHC expression (on Cs) while only two (18%) of transformed areas were positive (on centroblasts). The high percentage of Bcl-2 immunopositivity observed in remnant areas could be explained either for a high correlation between Bcl-2 over-expression and transformation into a more aggressive lymphoma, or/and for random sampling in small number of cases. Thirty-six percent of these patients showed bcl-2/IgH gene rearrangement, difference probably related to the contribution of FCL remnant in the sample used for PCR analysis and/or to the suboptimal sensitivity of the PCR due to the decreased quality of the paraffin-embedded extracted DNA substrate.

The finding of negative Bcl-2 protein expression on the transformed DLBCL areas whereas it was positive in the FCL remnants lead us to suggest two alternative hypothesis: a) a Bcl-2 positive cell triggers a more aggressive clone, loosing at the same time Bcl-2 protein expression by mutational events; b) a Bcl-2 negative centroblast, which is present in the former lymphoma (FCL) since the beginning of the disease, triggers the DLBCL. Further studies using cell sorting followed by PCR or in situ analysis could be useful to clarify the biologic bases of these NHLs, relating a specific cell type (centroblasts, centrocytes, etc.) with a specific bcl-2 gene rearrangement.

On the other hand, Group B showed high correlation between IHC and PCR studies, with only one exception: a patient with positive IHC and negative PCR analysis. Discordance between PCR and IHC bcl-2 analysis have been previously reported. Cases in which protein expression occurred in absence of DNA molecular rearrangement could be related to different breakpoints of the t(14; 18) located outside MBR and MCR, or related to other mechanisms involved in the upregulation of the bcl-2 gene such as the presence of the Latent Membrane Protein-1 (LMP-1) of the Epstein-Barr virus, or the loss of repressing functions by mutations on the bcl-2 promoter.

Morphologic transformation to aggressive NHLs has been associated to different somatic mutations. Raghoeber et al. found complex alterations of both the functional and non-functional IgH allele and Matolcsy et al. showed somatic point mutations on the bcl-2 proto-oncogen open reading frame sequence, resulting in alterations of the p26-bcl-2α gene product. It could also be possible that other types of mutations affect the primers which then fail to anneal on their target sequences.

In conclusion, our results indicate that PCR and IHC analyses are complementary methodologies, both important to achieve correct diagnoses of bcl-2 involvement, particularly in DLBCL with FCL remnant, where IHC studies will be necessary to define cell types in which Bcl-2 protein expression is present.

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References

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...al dedicarme a la ciencia debía elegir entre una probable situación pecuniaria holgada y una labor científica. Elegí lo mejor, lo que vale más que el dinero, con lo que sali ganando. Pero nunca presumí que mi dedicación exclusiva a la Cátedra fuera un mérito excepcional pues, en verdad, los profesores de materias básicas que no la tienen constituyen la excepción anacrónica. Si algún mérito tuve fue el de iniciar el sistema en el país. Lo hice por respeto a mi Facultad, amor a la Ciencia y confianza en mi Patria.

Bernardo A. Houssay (1887-1971)