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Incidence of BCL-2 gene rearrangements in Argentinean non-Hodgkin lymphoma patients: increased frequency of breakpoints outside of MBR and MCR

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Abstract

The t(14;18)(q32;q21) is the most frequent cytogenetic abnormality observed in follicular lymphoma (FL), but also occurs in diffuse large B-cell lymphoma (DLBCL). We have evaluated the frequency of this translocation in 106 Argentinean non-Hodgkin lymphoma (NHL) patients: 83 with diagnosis of FL and 23 with DLBCL. Nested (N) and long-distance PCR (LD-PCR) approaches were used. By N-PCR, a total of 42 (51%) FL cases showed BCL-2 rearrangement: 28 (34%) for major breakpoint region (MBR) and 14 (17%) for minor cluster region (mcr). By LD-PCR, additional 23 (28%) new positive cases were found: 10 (12%) for MBR and 13 (16%) for mcr. These data make a total of 65 (78%) positive cases for BCL-2 gene rearrangements. In DLBCL cases, N-PCR detected two (9%) cases with the MBR breakpoint and one (4%) with mcr. Seven (30%) new positive cases by LD-PCR were found: four (17%) for MBR and three (13%) for mcr, showing a total of 10 (43%) positive cases. Thus, both FL and DLBCL had high frequencies of breakpoints located between MBR and mcr clusters. Our N-PCR results in FL (51%; 95% CI, 40–62%) showed statistical differences with respect to the pooled data from USA ($P < 0.0001$) and overlapped with the frequencies from Asia and Europe. In DLBCL, no significant differences with respect to the literature were found. This data support the idea that FL may be a heterogeneous malignancy with distinct molecular pathogenesis and suggest that the geographic differences may be related with the distribution of breakpoints that are widely spread throughout the sequence stretch between MBR and mcr.

Keywords: Non-Hodgkin lymphomas; Follicular lymphomas; DLBCL; BCL-2 rearrangements

Introduction

The most frequent nonrandom chromosome rearrangement in non-Hodgkin lymphoma (NHL) of B-cell type is t(14;18)(q32;q21), which is found in approximately 50–85% of follicular lymphomas (FL), 15–30% of diffuse large B-cell lymphomas (DLBCL), and occasionally in other histological subtypes of NHL [1]. Molecular studies of this translocation have disclosed a juxtaposition of *BCL-2* gene in 18q21.3 with the immunoglobulin heavy-chain (IgH) gene *locus* at 14q32.33 [2,3]. Consequently, the *BCL-2* gene is subjected to the control of the IgH Eμ-enhancer leading to the overexpression of the anti-apoptotic protein BCL-2 [4,5]. On chromosome 18, the breakpoints occur at two *loci*, the major breakpoint region (MBR) in the 3′ -untranslated region of the third exon of the *BCL-2* gene (approximately 70% of cases) [2], and the minor cluster region (mcr) in a DNA stretch approximately 30 kb downstream of the *BCL-2* locus (approximately 15% of cases) [6]. On chromosome 14, breakpoints are predominantly found within the joining region (JH) of the IgH gene [2,7]. A recent study [8] suggests that t(14;18) is a more complex process than previously thought, which involves two distinct mechanisms: V(D)J recombination, mediating the breaks on chromosome 14 and an additional unidentified mechanism creating the initial breaks on chromosome 18.

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The tight clustering of the breakpoints at t(14;18) translocation makes this a particularly favorable target for polymerase chain reaction (PCR) amplification on genomic DNA using either MBR or mcr oligonucleotides in combination with JH consensus oligonucleotide primer [9–11]. However, this approach is limited to those cases in which the breakpoints fall within the well-defined regions flanked by the primer target sequences. The breakpoints on chromosome 18 are not always clustered within the MBR or mcr loci, but are often scattered between these two cluster regions [12–14]. Long-distance PCR (LD-PCR) provides a powerful advantage in the analysis of chromosomal translocations in which breakpoints are distributed over a large region [12].

In addition, it has been observed that there are marked differences in the incidences of *BCL-2* translocations in FL across geographic regions. Thus, the incidence of t(14;18) translocation appears to be significantly lower in patients with FL from Asia than in patients from Western countries [15–19]. Moreover, it has been recently suggested that different etiologic or genetic factors might influence the development of FL across separate regions [20].

In the present study, we focused our interest in the estimation of the frequency of the t(14:18) translocation in Argentinean patients with FL, and compared it with data reported from different parts of the world. Significant differences with respect to the frequencies observed in USA series were found. Moreover, the incidence of *BCL-2* rearrangements in DLBCL was also evaluated.

Materials and methods

Patients

For this study, we have analyzed a total of 106 NHL patients: 83 with diagnosis of FL (34 females and 49 males; mean age, 51 years; range, 29–82 years) and 23 with de novo DLBCL (14 females and 9 males; mean age, 58 years; range, 26–76 years), admitted to our Institute between years 1993 and 2003. None of DLBCL patients had a prior history of FL, and their histopathological studies did not show follicular structures. Histological classification was performed according to the World Health Organization classification [21].

DNA extraction

Genomic DNA was isolated from fresh tissues and peripheral blood lymphocytes by conventional phenol-chloroform extraction and ethanol precipitation [22]. DNA concentration and purity were estimated by agarose gel electrophoresis and UV spectrophotometry. The quality of all DNA samples was further checked by PCR amplification of a target DNA within a β-globin gene fragment (536 bp).

Nested-PCR

PCR detection for the MBR-JH and mcr-JH rearrangements of the t(14;18) translocation were performed in 50 μl of final volume using 1–1.5 μg DNA, 1 μM of each oligonucleotide primer (Table 1), 200 μM of all dNTPs, 2.5 mM $MgCl_2$, and 2.5 U *Taq* DNA polymerase (Promega). PCR reactions were performed as described by Gribben et al. [19] with the following modifications: the first PCR reaction consisted of 25 cycles that included 1 min at 94°C, 1 min at 55°C for MBR or 58°C for mcr, and 1 min at 72°C, followed by 10 min at 72°C for tailing. The second round of PCR was performed on 5 μl of the first product and consisted of 30 cycles that included 1 min at 94°C, 1 min at 53°C for MBR or 59°C for mcr, and 1 min at 72°C, followed by 10 min at 72°C for tailing (MJResearch minicycler PTC). PCR products were analyzed by 2% agarose gel electrophoresis. Specific PCR products give bands at 150–300 bp for MBR-JH and at 400–700 bp for mcr-JH. Each experiment included the clinical samples, a positive control consisting in DNA tenfold serial dilution from SU-DHL6 cells in normal cells, and a negative control DNA from a healthy individual. These control reactions detect PCR product contamination, avoid false negativity caused by suboptimal PCR efficiency, and standardize the variation in PCR efficiency.

Table 1
Sequences of oligonucleotide primers for nested and long-distance PCR

Primers (reference)	Nucleotide sequences 5′→3′
Nested-PCR [19]	
MBR external	CAG CCTTGAAACAATGATGG
MBR internal	TATGGTGGTTTGACCTTTAG
mcr external	CGTGCTGGTACCACTCCTG
mcr internal	GGACCTTCCTTGGTGTGTTG
JH external	TATGGTGGTTTGACCTTTAG
JH internal	ACCAGGGTCCCTTGGCCCCA
LD-PCR	
MBR/01 [6]	CACAAGTGAAGTCAACATGCCTGCCCCAAACAAAT
mcr/01 [12]	GGTAGAGGTGAATACCCCAGGGCTGAGCAGGAAGG
Eμ/01 [23]	CTAGGCCAGTCCTGCTGACGCCGCATCGGTGATTC

Table 2
Frequencies of *BCL-2* gene rearrangements in Argentinean FL and DLBCL patients

Diagnostic	Number of patients	*BCL-2* rearrangements (%)					
		MBR	mcr	LD-MBR	LD-mcr	Total	NR (%)
FL	83	28 (34)	14 (17)	10 (12)	13 (16)	65 (78)	18 (22)
DLBCL	23	2 (9)	1 (4)	4 (17)	3 (13)	10 (43)	13 (57)

NR: no *BCL-2* rearrangement.

Long-distance-PCR

Genomic DNA samples were subjected to LD-PCR using primers MBR/01 or mcr/01, and Eμ/01 as described by Akasaka et al. [12] (Table 1). The LD-PCR reactions were performed with the following modifications: 36–44 ng/μl of genomic DNA, 2.25 mM $MgCl_2$, 0.1 μM of each primers, 500 μM of all dNTPs, and 1.3 U *Taq/Pwo* DNA polymerase mixture (Expand Long Template DNA Polymerase, Roche) in a total volume of 25 μl. Thermal cycling was preceded by 94°C for 2 min, followed by 3 cycles of 94°C for 20 s and 72°C for 13 min, 3 cycles of 94°C for 20 s and 70°C for 13 min, and 30 cycles of 94°C for 20 s and 68°C for 13 min with addition of 15 s per cycle at 68°C (DNA PCR thermal cycler Techne). Long amplimers were analyzed by 0.8–1% agarose gel electrophoresis. LD-PCR products ranged from 1.9 to 10.7 kb for MBR-IgH fusion and from 1.9 to 9.4 kb for mcr-IgH. *Hin*dIII digested λ phage DNA was used as molecular size marker.

Bioinformatics

Third exon DNA sequences of the human BCL-2 gene and its downstream region spanning all breakpoint clusters, and the DNA sequences from the IgH gene (JH and Eμ regions), were obtained from GenBank (accession numbers AF325194 and BX97051.1, respectively). The annotations of target sequences for primers, breakpoint clusters, exons and exon–intron boundaries, and the determination of physical distances were performed using EditSeq and MapDraw software (LaserGene, DNA Star). The distribution of BCL-2 clusters was based in an accurate analysis of the molecular breakpoints described by Albinger-Hegyi et al. [14].

Statistical analysis

The 95% confidence intervals (CI) frequencies were calculated using the following standard formula:

$$a \pm 1.96[a(1-a)/n]^{1/2}$$

where a is the frequency of positive cases in the studied population and n is the total number of cases under analysis. The statistical comparisons among populations were evaluated by Chi-square test.

Results

Evaluation of t(14;18) in FL

The translocation t(14;18) was studied at the MBR and mcr breakpoints in 83 patients with diagnosis of FL by N- and LD-PCR. By N-PCR, 28 (34%) cases showed the MBR rearrangement and 14 (17%) cases showed the mcr, producing an overall frequency of 51% (42/83) in Argentinean FL patients (Table 2) (Figs. 1a, b). Negative patients for the

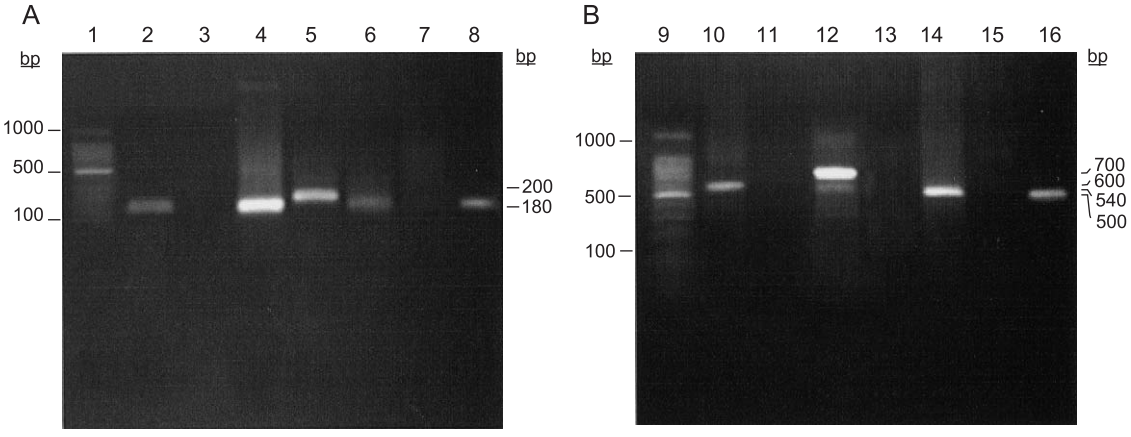

Fig. 1. EtBr-stained 2% agarose gel electrophoresis showing the MBR-JH (A) and mcr-JH (B) *BCL-2* gene rearrangements using nested-PCR. Lanes 1 and 9: ladder of 100 bp. Lanes 2 and 10: positive controls. Lanes 3 and 11: negative controls. Lanes 4–6 and 8: positive cases for MBR. Lane 7: a negative case for MBR. Lanes 12, 14, and 16: positive cases for mcr. Lanes 13 and 15: negative cases for mcr.

former PCR protocol were further evaluated by LD-PCR. Using this assay, additional 23 (28%) new positive cases were found: 10 (12%) for MBR rearrangement and 13 (16%) for mcr breakpoint (Figs. 2a, b). Thus, our series of follicular NHL showed BCL-2 gene rearrangements in 65 (78%) cases. Due to each of the LD-PCR positive samples showed a specific size, false-positive results related to PCR-product contamination or unspecific amplification can be ruled out. Furthermore, several FL samples, which were positive for the nested approach, were also detected by LD-PCR. In all tested cases, the length of LD-PCR products were consistent with the molecular size predicted from the N-PCR results.

Comparison with the literature

The frequency of *BCL-2* gene rearrangements from our follicular NHL patients was compared with data from different geographic regions. For this evaluation, only the N-PCR frequencies were used. In order to optimize the analysis of data, we have limited the comparison of PCR results to those reports in which MBR and mcr loci were examined. This particular issue was previously evaluated by Biagi and Seymour [20]. Recent reports were included in the present study [14,24].

The pooled frequencies and 95% CI from each geographical region and the data of the present study are shown in Table 3. Our results (51%; 95% CI, 40–62%) showed statistical differences with respect to the pooled data from United States ($P < 0.0001$) and overlapped with the frequencies from Asia and Europe. When the BCL-2 breakpoint clusters were analyzed, our data for MBR showed statistical differences with respect to those observed from the Europe and US series ($P < 0.02$ and $P < 0.0001$, respectively), while mcr exhibited differences compared to European and Asian reports ($P < 0.0001$ and $P < 0.05$, respectively).

Evaluation of t(14;18) in DLBCL and comparison with the literature

BCL-2 rearrangements were also evaluated in 23 patients with diagnosis of de novo DLBCL by N- and LD-PCR (Table 2). By nested approach, two (9%) cases showed rearrangements at the MBR and only one case (4%) at the mcr. Negative patients for the standard PCR protocol were evaluated by LD-PCR. Seven (30%) new positive cases were found: four (17%) for MBR rearrangement and three (13%) for mcr breakpoint. Thus, our series of DLBCL showed *BCL-2* gene rearrangements in 10 (43%) cases. We have also compared our N-PCR data (3; 13%) with the frequencies reported in the literature in which MBR and mcr breakpoints were evaluated (Table 4). Pooled data from European and US series revealed frequencies of 17% and 15%, respectively. No significant differences with respect to our data were observed.

Distribution of BCL-2 breakpoints

The specific results from each PCR approach and their molecular sizes obtained from the sequential analysis of

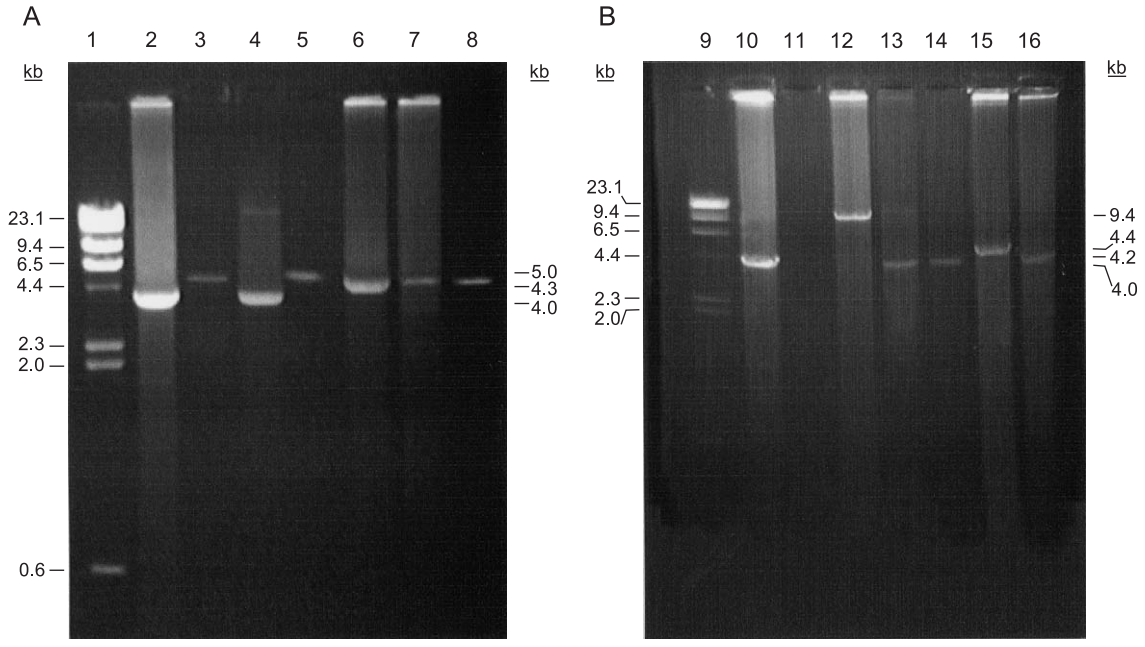

Fig. 2. Representative EtBr-stained 1% agarose gel electrophoresis showing MBR-IgH (A) and mcr-IgH (B) DNA fusions using long-distance PCR. Lanes 1 and 9: *Hin*dIII digested λ phage DNA as a molecular size marker. Lanes 2–8: positive cases for MBR-IgH rearrangements. Lane 11: a negative case for mcr. Lanes 10 and 12–16: positive cases for mcr-IgH.

Table 3
BCL-2 gene rearrangements in FL from different geographical regions

Reference	Number of patients	*BCL-2* rearrangements (%; CI)		
		MBR	mcr	Total
United States				
Zelenetz et al. [25]	40	22 (55)	6 (15)	28 (70)
Liu et al. [26]	48	24 (50)	5 (10)	29 (60)
Gribben et al. [19]	88	56 (64)	18 (20)	74 (84)
Guilley et al. [27]	8	4 (50)	1 (12)	5 (62)
Lopez-Guillermo et al. [28]	139	105 (70)	13 (9)	118 (79)
Pooled data (%; CI 95%)	323	211 (65; 60–70)	43 (13; 9–17)	254 (79; 75–83)
Europe				
Montoto et al. [24] (Spain)	60	39 (65)	1 (2)	40 (62)
Séité et al. [29] (France)	64	30 (47)	8 (13)	38 (59)
Pezzella et al. [17] (UK, Denmark)	51	18 (35)	3 (6)	21 (41)
Lee et al. [30] (UK)	20	7 (35)	1 (5)	8 (40)
Lambrechts et al. [31] (Holland)	21	12 (57)	1 (5)	13 (62)
Poetsch et al. [32] (Germany)	28	18 (64)	0 (0)	18 (64)
Johnson et al [18] (Sweden)	50	30 (60)	0 (0)	30 (60)
Albinger-Hegyi et al. [14] (Switzerland)	59	19 (32)	2 (3)	21 (36)
Pooled data (%; CI 95%)	353	173 (49; 44–54)	16 (5; 3–7)	189 (54; 49–59)
Asia				
Mitani et al. [15] (Japan)	30	12 (40)	1 (3)	13 (43)
Loke et al. [16] (Hong Kong Chinese)	16	8 (50)	1 (6)	9 (56)
Chen et al. [33] (Taiwan Chinese)	17	8 (47)	1 (6)	9 (53)
Pooled data (%; CI 95%)	63	28 (44; 32–56)	3 (5; 0–10)	31 (49; 37–61)
South America				
Present study (Argentina) (% CI 95%)	83	28 (34; 24–44)**	14 (17; 9–25)***	42 (51; 40–62)*

* Statistical differences with respect to pooled data from US: $P < 0.0001$.
** Statistical differences with respect to pooled data from US and Europe: $P < 0.0001$ and $P < 0.02$, respectively.
*** Statistical differences with respect to pooled data from Europe and Asia: $P < 0.0001$ and $P < 0.05$, respectively.

MBR-N-PCR, mcr-N-PCR, MBR-LD-PCR, and mcr-LD-PCR allowed us to easily recognize the *BCL-2* breakpoint clusters and thus draw a simple classification. Therefore, our results compared to those reported by Albinger-Hegyi et al. [14] allowed us to establish the following distribution of the *BCL-2* breakpoints (Table 5): (a) MBR nested (150–300 bp); (b) mcr-nested (400–700 bp); (c) LD-MBR-proximal that corresponds to *BCL-2* breakpoints vicinal to the 150-pb-long MBR cluster but cannot be detected by nested-PCR

Table 4
BCL-2 gene rearrangements in DLBCL from different geographical regions

Reference	Number of patients	*BCL-2* rearrangements (%; CI)		
		MBR	mcr	Total
United States				
Weiss et al. [34]	30	3 (10)	1 (3)	4 (13)
Jacobson et al. [35]	45	7 (16)	2 (4)	9 (20)
Gascoyne et al. [36]	102	11 (11)	3 (3)	14 (14)
Pooled data (%; CI 95%)	177	21 (12; 7–17)	6 (3; 0.5–5.5)	27 (15; 10–20)
Europe				
Lee et al. [30]	10	1 (10)	0 (0)	1 (10)
Volpe et al. [37]	70	12 (17)	1 (1)	13 (19)
Pooled data (%; CI 95%)	80	13 (16; 8–24)	1 (1; 0–3)	14 (17; 9–25)
South America				
Present study (%; CI 95%)	23	2 (9; 0–21)	1 (4; 0–12)	3 (13; 0–27)

Table 5
Distribution of *BCL-2* breakpoints

	FL		DLBCL	
	Number of patients (%)	Size band in kb (number of patients)	Number of patients (%)	Size band in kb (number patients)
MBR nested	28 (34)	0.15–0.3 (28)	2 (9)	0.18 (1), 0.23 (1)
MBR-LD-proximal (<8 kb)	9 (11)	1.9 (1), 2.3 (1), 4.0 (3), 5.0 (2), 5.5 (1), 6.6 (1)	3 (13)	3.9 (1), 4.0 (1), 4.3 (1)
MBR-LD-distal (>8 kb)	1 (1)	10.7 (1)	1 (4)	10.7 (1)
mcr nested	14 (17)	0.4–0.7 (14)	1 (4)	0.6 (1)
mcr-LD	13 (16)*	1.9 (2), 2.3 (1), 2.5 (1), 3.9 (1), 4.0 (2), 4.1 (1), 4.3 (2), 4.4 (1), 5.0 (1), 9.4 (1)	3 (13)**	4.1 (1), 4.3 (1), 4.4 (1)
Total *BCL-2* positives	65 (78)		10 (43)	

* 12/13 may correspond to icr region.
** 3/3 may correspond to icr region.

(band size <8 kb); (d) LD-MBR distal that exclusively corresponds to large LD-PCR signals (band size ≥8 kb); and (e) LD-mcr breakpoints that are only detected by the LD approach and include both mcr breakpoints and the intermediate cluster region (icr) defined by Albinger-Hegyi et al. [14]. Our results in both FL and DLBCL patients showed that most of LD-MBR breakpoints corresponded to the proximal region (<8 kb) (9/10, 90% and 3/4, 75%, respectively). Simultaneously, when LD-mcr breakpoints were analyzed, most of patients had breakpoints that may correspond to the icr region (12/13, 92% for FL and 3/3 for DLBCL).

Discussion

Different studies have demonstrated a marked geographic variation in the incidence rates of FL between Asian and Western countries [38–40]. Simultaneously, data from previous studies have also suggested geographic variation in the frequencies of *BCL-2* rearrangements, with relatively low frequencies among Asian and European populations and high rates for US series [20,41]. To the best of our knowledge, no data from South American populations have been previously published.

In this report, we have studied the frequency of t(14;18) translocation in a series of Argentinean FL patients using N- and LD-PCR. By N-PCR, the overall frequency of t(14;18) from our patients was similar to those found in series from Europe and Asia but significantly lower than those observed in reports from US patients. Thus, our results confirm the presence of a significant gradient in the frequency of *BCL-2* gene rearrangement from a relatively low rate from 49% to 54% among Asian, Argentinean, and European studies to 79% among the US series.

As for the breakpoints, our frequency of MBR was significantly lower than those reported in US and European series, and no differences were found compared to Asian reports. Besides, although the sample sizes were not too large, the incidence of mcr breakpoints in Argentinean patients was significantly lower than those reported from US series but higher than those observed among Asian reports. No difference with the European frequency was found.

Moreover, patients without *BCL-2* gene rearrangement by nested-PCR were evaluated by LD-PCR, and 23 new t(14;18) positive patients were found, showing the high frequency of breakpoints located between MBR and mcr (28%) breakpoints. Thus, an overall frequency of 78%, similar to those observed in USA series, was found [20]. These results are quite similar to those previously described by Albinger-Hegyi et al. [14] in European patients, who reported 36% of positive cases by N-PCR and an overall frequency of 71% when LD approach was applied.

These data support the idea that FL may be a heterogeneous malignancy, morphologically similar but with distinct molecular pathogenesis [20], and suggest that the geographic differences may be related with the distribution of breakpoints that are widely spread throughout the sequence stretch between MBR and mcr. Particularly, our cases showed a high proportion of LD-MBR proximal breakpoints as well as points that may correspond to icr, region in which has been recently observed three times more rearrangements than in the mcr [14]. An analogous situation can be drawn in Burkitt's lymphoma, in which endemic and sporadic forms differ at a molecular level on both chromosomes 8 and 14 [42]. In addition, molecular epidemiological studies showed a geographic variance in the pattern of chromosomal breakpoint locations, suggesting the existence of pathogenetically distinct subtypes in different world regions [43].

We have also evaluated BCL-2 gene rearrangements in de novo DLBCL. Our study by N-PCR showed similar frequencies than those reported in the literature, without differences among geographic regions. In addition, negative patients were analyzed by LD-PCR and seven (30%) new positive cases were observed, increasing to 43% the frequency of this genomic alteration. This finding also shows a high frequency of breakpoints located between MBR and mcr.

DLBCL represent a heterogeneous group of lymphoid malignancies in which there are no reliable indicators, morphological, clinical, immunohistochemical or genetic, that can be used to define subgroups. Gene expression patterns can delineate two distinct subgroups of de novo DLBCL: one with the germinal center B-cell gene expression profile and the other with the activated B-cell-like gene expression signature [44]. In addition, Huang et al. [45] have found that t(14;18) translocation defines a subset of DLBCL with germinal center B-cell expression profile, suggesting this alteration as an important event in the pathogenesis of this subgroup of DLBCL, just as it does in follicular lymphomas.

Thus, our findings confirm the importance of an accurate detection of t(14;18) translocation in both FL and DLBCL by using LD-PCR, a useful tool to determine the appropriate initial treatment and the subsequent management of these neoplasms. The comparison of our rates of *BCL-2* gene rearrangements with data of the literature suggests that the geographical variance in different world regions may be related with the distribution of *BCL-2* breakpoints located between MBR and mcr.

Finally, a molecular subdivision of t(14;18) rearrangements based on the *BCL-2* breakpoint clusters is proposed. The *BCL-2* rearrangement site was shown to be an important prognostic factor in indolent FL, useful to identify patients who may require different treatment [46]. Consequently, more studies using this new set of molecular categories to evaluate possible correlations with clinical data may provide a better understanding of the biology of these neoplasias.

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