Bcl-6 and p53 mutations in lymphomas carrying the bcl-2/Jh rearrangement

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Background and Objectives. The t(14;18)(q32;q21) chromosomal translocation is the hallmark of follicular lymphomas (FL). The translocation induces the overexpression of the Bcl-2 protein and prolongs the survival of clonogenic cells. Tumor cells may acquire additional molecular alterations that may be associated with histologic progression or with chemoresistance.

Design and Methods. We analyzed the distribution and association of bcl-6 and p53 mutations in 55 consecutive bcl-2/Jh+ lymphoma samples derived from 43 patients obtained at the time of diagnosis and, in 5 of these patients, during follow-up. A total of 29 bcl-6 point mutations were detected in seventeen patients (40%) associated with major or minor breakpoints of the bcl-2/Jh fusion gene. In seven cases a p53 mutation was detected. Three cases corresponded to FL with the minor breakpoint in the bcl-2 gene and these patients had a favorable clinical evolution, whereas the 4 patients with p53 mutations and the major breakpoint had a bad clinical outcome with morphologic transformation to high-grade lymphoma in three cases. The sequential analysis of 5 patients showed a different timing in the acquisition of mutations: one patient showed bcl-6 and p53 mutations at diagnosis, another patient showed bcl-6 mutations at diagnosis and acquired a p53 mutation later whereas the third patient had a p53 mutation before the appearance of the bcl-6 mutation.

Results. We did not find significant differences in survival between patients with FL who showed exclusively bcl-6 mutations and those without bcl-6 mutations, but those patients with a high International Prognostic Index score and p53 mutations showed the lowest overall survival (p = 0.002).

Interpretation and Conclusions. These findings suggest that bcl-2/Jh lymphomas show molecular heterogeneity and that bcl-6 and p53 mutations may be acquired during the evolution of such lymphomas. Bcl-6 mutations, by themselves, do not seem to be associated with a bad prognosis. Rearrangements at the minor bcl-2 locus may have a different molecular evolution.

Key words: follicular lymphoma, bcl-6 gene, p53 gene, mutations, bcl-2 rearrangements.

Follicular lymphoma (FL) is the most common non-Hodgkin’s lymphoma (NHL) in adults.1 The t(14;18)(q32;q21) chromosomal translocation is the hallmark of FL, being detected in 85% of cases.2 This translocation is not, however, specific to FLs, being also detected in NHLs transformed from a FL, in 20% of diffuse large B-cell lymphomas (DLBCL), in Burkitt’s lymphomas (BL) and, even in rare cases of de novo acute B-cell lymphoblastic leukemia.3 This translocation results from an illegitimate recombination during VDJ rearrangements in a pre-B-cell stage in bone marrow.4,5 Deregulation of bcl-2 induced by Ig enhancer leads to the overexpression of the Bcl-2 protein which prolongs the survival of clonogenic cells.6,8 Proliferating tumor cells are subjected to new molecular lesions that may be associated with malignant progression.10,12 Genetic instability demonstrated in transformed NHLs14,15 and the hypermutational mechanisms that take place in germinal centers play a fundamental role in the acquisition of these molecular lesions.14,16-20 Mutations in the tumor suppressor gene p53, structural alteration of the MUM1 gene, the p16INK4a and ARF genes, 6q deletions and point mutations that alter the regulatory sequences of proto-oncogenes such as c-myc and bcl-2 have all been described in transformed FL. Recently, alterations of the bcl-6 gene have also been reported in this setting.13,16,21
The bcl-6 gene is located on chromosome 3q27 and encodes a POZ/zinc finger protein which functions as a sequence-specific DNA-binding transcriptional repressor. Several lines of evidence suggest that structural alterations of the regulatory regions of the gene are involved in lymphomagenesis. Chromosomal rearrangements affecting the bcl-6 gene are associated with 40% of diffuse large cell lymphomas (DLCL) and 10% of FL. The Bcl-6 gene may be altered by somatic point mutations clustering within the 5’ non-coding regions of the gene in lymphomas which display a GC phenotype.

The bcl-2/Jh rearrangement takes place at an early stage of B-cell differentiation, but the overexpression of the antiapoptotic protein Bcl-2 is not sufficient for the development of lymphomas. We analyzed the distribution and the association of genetic lesions such as bcl-6 point mutations and p53 mutations in lymphomas carrying the bcl-2/Jh rearrangement.

Design and Methods

Tumor samples and DNA extraction

This study was based on fifty-four consecutive lymphoma samples derived from a total of 43 patients. Samples from patients with a lymphoma proceeding from the germinal center and with the t(14;18)(q21;q32) chromosomal translocation were included in the study. Diagnosis was based on morphology and immunophenotypic analysis of cell surface markers. The samples were obtained from bone marrow (n = 19), peripheral blood (n = 15) and fresh lymph node (n = 20). All the samples were selected if a positive bcl-2/Jh rearrangement was detected using molecular methods. These patients represented about 85% of all newly diagnosed cases of follicular lymphomas. Patients with other molecular lesions (variant breakpoints or bcl-2 amplification) were not included in this series. In most cases, the fraction of malignant cells was > 75% and in all cases > 20%. The specimens were collected at diagnosis with the exception of the lymphomas transformed from a follicular phase and in all cases > 20%. The specimens were collected at diagnosis with the exception of the lymphomas transformed from a follicular phase and in all cases > 20%.

On the basis of the WHO classification of NHLs originating from the germinal center, the samples were classified as FL (n = 38), BL (n = 2), one case diagnosed as de novo and the other one derived from a previous FL) and DLCL (n = 3, two cases de novo and one arising from a FL). DNA extraction was performed by digestion with proteinase K, extraction by the salting out method and precipitation with ethanol (bone marrow and peripheral blood). DNA was obtained from lymph-node samples using the standard phenol-chloroform method.

Analysis of bcl-2/Jh rearrangements by long distance-polymerase chain reaction (LD-PCR)

To analyze the presence of the bcl-2/Jh rearrangements, we used a modified PCR method (LD-PCR), which was more suited to amplifying long PCR targets. LD-PCR amplification was performed in an automated thermal cycler (DNA PCR Thermal Cycler 480; Perkin-Elmer, Norwalk, CT, USA), using 100 ng of genomic DNA, 2.5 mM of each dNTP, 20 pmol of each primer and 2.5 U Taq DNA polymerase (TaqKaRa LA Taq polymerase, Takara Shuzo) in a final volume of 50 µL. LD-PCR conditions were: one cycle of denaturation (94°C for 1 min); 30 cycles of denaturation (98°C for 20 s) and annealing (68°C for 20 min) followed by one cycle of extension (72°C for 16 min). The sequence of the primers used to detect the bcl-2/Jh fusion genes has been reported previously. The sensitivity threshold for the LD-PCR was 1/100 cells.

Analysis of bcl-6 and p53 mutations

The bcl-6 5’ region and the p53 gene were analyzed by a combination of polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and DNA direct sequencing. PCR-SSCP analysis of the bcl-6 gene was performed on three partially overlapping PCR fragments (fragments E1.10, E1.11, E1.12), spanning 739 bp, located downstream of the first non-coding exon of the gene, as reported by Capello et al. PCR-SSCP analysis of the p53 gene was performed on five PCR fragments corresponding to exons 5-9 following a well established protocol. PCR-SSCP of the bcl-6 gene was performed using 100 ng of genomic DNA, 10 pmol of each primer, 2.5 µmol/L dNTPs, 1 µCi of α-32P dCTP (Amersham, Amersham, UK), 10 mM Tris-HCl (pH 8.8), 50 mM/L KCl, 1 mM/L MgCl2, 0.01% gelatin, and 0.5 U AmpliTaq polymerase (Perkin-Elmer, Norwalk, CT, USA) in a final volume of 10 µL. Thirty cycles of denaturation (95°C for 1 min), annealing (56°C for fragments E1.10 and E1.11 and 54°C for fragment E1.12 for 1 min) and extension (72°C for 1 min) were performed in a DNA thermal cycler (Perkin-Elmer). After heating at 95°C for 5 min, the samples were chilled on ice and loaded onto a 6% acrylamide gel containing 10% glycerol. Gels were run at 8W for 15 hours at room temperature, dried and analyzed by autoradiography with an intensifying screen after exposure for 24 hours at -80°C. For all samples subjected to DNA sequencing, the DNA PCR products were purified using a
commercial kit (QIAquick PCR purification kit, Qiagen, CA, USA) and directly sequenced using the Thermosequenase Kit (Thermosequenase, Amersham, Life Sciences, UK) following the manufacturers' recommendations. Briefly, the sequencing reaction was performed using 2 µg of purified DNA, 1 pmol of the appropriate primer (forward and reverse) and [α-33P]-labeled terminator dideoxynucleotides (Amersham Life Sciences). Autoradiographs were developed after 2 days of exposure at -80°C.

Immunohistochemistry

Expression of bcl-2, Bcl-6 and p53 was detected with commercially available monoclonal antibodies purchased from Dako (Dako A/S, Glostrup, Denmark). Reactions were scored as positive using the following scale: weak positivity, when 5-10% of cells were positive; moderate positivity, when there were 10-50% positive cells; and strong positivity, when more than 50% of the cells were positive (<5% of neoplastic cells was used as a cut-off to define tumors negative for Bcl-6 expression). All samples had a high expression of Bcl-2 protein and all samples had at least 15% of neoplastic cells showing Bcl-6 protein expression.

Statistical analysis

The distribution of patients according to biological and clinical characteristics was compared by the χ2 test for categorical variables and the U Mann-Whitney test for continuous variables. Univariate overall survival curves were computed using the Kaplan-Meier technique. The log-rank test was used to compare the survival curves. Overall survival was calculated from the date of diagnosis to the date of death or the last follow-up. Multivariate analysis was done using the Cox regression technique, considering death as a dependent variable and the following as independent variables: age, stage, B symptoms, bcl-2 rearrangement, bcl-6 mutation, p53 mutation and the product between p53 mutation and bcl-6 mutation. Statistical results were considered significant if p<0.05.

Results

Characterization of patients and the tumor panel

Of the 43 patients included, we could not obtain clinical information about three patients (UPN 16, 30 and 38). The patients evaluated in the study had a median age of 55 (29-78) years, 3 were in stage I disease, 3 in stage II, 4 in stage III, 32 in stage IV and disease stage was unknown for one. One patient had an International Prognostic Index (IPI) score of 0 (2%), 18 (42%) had an IPI score of 1, 12 (30%) had an IPI score of 2, 4 (9%) had an IPI score of 3, 5 (12%) had an IPI score of 4 and in two patients the IPI score was unknown. The percentage of patients with B symptoms at diagnosis was 30% (n=12).

According to the WHO classification, histologic sections from the 54 samples (from 43 patients) were diagnosed as follows: 1) FL: 49 tumor samples derived from 38 patients (20 lymph nodes), 2) DLCL: 3 samples from 3 patients (two of them were diagnosed with de novo DLCL whereas the other was diagnosed with transformed FL), 3) BL: two samples (one de novo and the other derived from FL). The FL samples were further graded according to the WHO classification: 21 samples were judged to be grade I and 17 samples grade II. Two samples were not assigned to a specific grade.

We studied more than one tumor sample from 5 patients (UPN 1, 8, 12, 13, 19). In one patient (UPN 13), two tumor samples were obtained at diagnosis from lymph node and bone marrow whereas in 4 patients tumor samples from lymph node, bone marrow and/or peripheral blood were obtained at the time of diagnosis and during the follow-up.

Analysis of bcl-2/J H rearrangements

Analysis of the bcl-2 rearrangement revealed the presence of the bcl-2 major breakpoint (MBR) in 33/43 cases (77%) and the bcl-2 minor breakpoint (mcr) in 10/43 cases (23%).

Mutational analysis of bcl-6 and p53 genes

The samples investigated displayed a total of 29 PCR-SSCP variants (3 polymorphisms) which were unique to individual tumor samples. Cases of lymphomas arising from germinal centers were considered positive for mutations when one or more PCR-SSCP showed a variant pattern which could not be ascribed to a population polymorphism. On this basis, mutations of bcl-6 scored positive in 17/43 (40%) cases when considering all the multiple tumor samples available for each patient. Comparison of the distribution of the bcl-6 mutations and the bcl-2 rearrangements confirmed that mutations occur in lymphoma cases originating in germinal centers, both those with bcl-2 major and minor breakpoints (Table 1).

Twelve patients showed abnormal migration patterns in only one PCR fragment (E1.10, E1.11 or E1.12). Five patients (UPN 2, 5, 39, 40, 43) had abnormal patterns in more than one PCR fragment reflecting multiple mutations. The most frequently mutated fragment was E1.11 (14 positive patients); followed by fragment E1.12 (8 positive patients)
### Table 1. Characteristics, molecular lesions and immunohistochemistry of patients with bcl-6 and/or p53 mutations.

| UPN | Age | Diagnosis | Stage | IPI | Sample | Therapy | Outcome | Survival | BCL-2 rearrang. | BCL-6 mutations | p53 mutations | BCL-2 protein | BCL-6 protein | p53 protein |
|-----|-----|-----------|-------|-----|--------|---------|---------|---------|----------------|----------------|--------------|--------------|--------------|--------------|-------------|
| 1   | 62  | FL II     | IVB 4 | PB BM PB LN | ChOP+RT IPA/PI-16 | PR PD | Dead | 12 months | MBR-4 Kb | E1.12 T-A +911 | Codon 248 CGG→TGG R248W | +++ | ++ | +++ |
| 2   | 59  | DLCL IIIB | 3 LN  | ChOP BEAM→PBSTC RT | PR PR | Dead | 8 months | MBR-10 Kb | E1.11 T-G +775 T-A +783 G-A +685 E1.12 | Codon 273 GGT→TGT R273C | +++ | +++ | +++ |
| 3   | 35  | FL IVA 1 | LN  | ChOP+CL+FN Rituximab | CR CR | Alive | 44 months | MBR-4 Kb | Neg Neg | Codon 242 CGG→CAC R248H | +++ | ++ | Neg |
| 4   | 55  | FL IVA 1 | LN  | ChOP | CR | Alive | 36 months | MBR-6 Kb | E1.10 | Neg Neg | Codon 248 CGG→CAC R248H | +++ | ++ | Neg |
| 5   | 35  | FL-DLCL II | IVB 4 | LN  | ChOP IPA/PI-16 DM/P+ESAP BEAM→PBSTC | PD PD | Dead | 10 months | MBR-4 Kb | E1.11 T-A +687 E1.12 C-A +1053 | Codon 242 TCC→TAC C242Y | +++ | ++ |  |
| 6   | 48  | FL IA 1 | LN RT | ChOP+CL+FN Rituximab | CR CR | Alive | 20 months | MBR-4 Kb | E1.10 | Insert C +661 | Neg +++ | + | Neg |
| 7   | 78  | FL I | IVA 1 | LN  | ChOP | CR | Alive | 148 months | MBR-5 Kb | Neg Neg | Codon 248 CGG→CAC R248H | +++ | ++ | Neg |
| 8   | 30  | FL-DLCL II | IVA 4 | LN  | ChOP IPA/PI-16 DM/P+ESAP BEAM→PBSTC | PD PD | Dead | 9 months | MBR-20 Kb | E1.11 G-A +759 | Codon 248 CGG→CAC R248H | +++ | +++ | +++ |
| 9   | 58  | FL IVA 2 | BM  | ChOP+FN IPA/PI-16 | CR PR | Alive | 20 months | MBR-4.4 Kb | Neg Neg | Codon 234 TAC→TGC Y234C | +++ | ++ | Neg |
| 10  | 46  | FL IIIA 1 | LN  | ChOP | CR | Alive | 36 months | MBR-4.4 Kb | Neg Neg | Codon 234 TAC→TGC Y234C | +++ | ++ | Neg |
| 11  | 30  | FL IVA 1 | LN  | ChOP+FN Rituximab+CVP | CR PR | Alive | 35 months | mcr-3 Kb | E1.11 C-G +780 | Codon 234 TAC→TGC Y234C | +++ | ++ | Neg |
| 12  | 68  | FL IVB 3 | LN BM PB BM BM | ChOP | PR,PD | Alive | 9 months | mcr-3 Kb | E1.12 G-A +895 | Codon 234 TAC→TGC Y234C | +++ | ++ |  |
| 13  | 41  | FL IVA 1 | LN BM | ChOP | CR | Alive | 9 months | mcr-16 Kb | Neg Neg | Codon 234 TAC→TGC Y234C | +++ | ++ | Neg |
| 14  | 70  | FL IA 1 | PB | Surgery | CR | Alive | 18 months | MBR-4.4 Kb | Neg Neg | Codon 234 TAC→TGC Y234C | +++ | ++ | Neg |
| 15  | 59  | FL IVB 2 | PB | ChOP Rituximab+CHOP | CR | Alive | 40 months | MBR-5 Kb | E1.11 | C-T +766 | Codon 234 TAC→TGC Y234C | +++ | ++ |  |
| 17  | 30  | FL IVA 3 | PB | FCM→PBSTC | CR | Alive | 23 months | MBR-4 Kb | E1.11 G-A +752 G-C +754 T-A +817 | Codon 234 TAC→TGC Y234C | +++ | ++ |  |
| 18  | 50  | FL IVA 1 | BM  | ChOP FCM Rituximab | PR PR | Alive | 3 to 5m | MBR-4.4 Kb | E1.10 G-C +479 | Codon 234 TAC→TGC Y234C | +++ | ++ |  |
| 19  | 46  | FL V6 1 | BM | ChOP IPA/PI-16+PBSTC | CR CR | Dead | 3 to 9 m | mcr-3 Kb | Neg Neg | Codon 234 TAC→TGC Y234C | +++ | ++ |  |

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FL: follicular lymphoma; BM: bone marrow; LN: lymph node; PB: peripheral blood; MBR: major breakpoint region; mcr: minor cluster region; CR: complete remission; PR: partial remission; PD: progressive disease; R: relapse; FL: follicular lymphoma; DLCL: diffuse large cell lymphoma; PBSCT: peripheral blood stem cell transplantation; IPI: international prognostic index.
and fragment E1.10 (4 positive patients). The characteristics of the bcl-6 mutations detected in these samples are summarized in Figure 2.

The frequency of mutations in different lymphoma cases ranged from 1 to 4. All cases showed heterozygous mutations. Twenty-six cases (60%) were negative both for bcl-6 and p53 mutations. Ten cases (23%) showed only bcl-6 mutations whereas 7 cases (16%) harbored both bcl-6 and p53 mutations.

When considering cases for which multiple tumor samples were available (n = 5, UPN 1, 8, 12, 13 and 19), three patients showed bcl-6 mutations. Two of the 5 patients did not show bcl-6 mutations despite the fact that >20% cells were tumor cells in the different samples. The Bcl-6 mutations were single point mutations in E1.11 (one patient) and E1.12 (2 patients).

1. One patient (UPN 1) showed bcl-6 mutations at diagnosis but these were not detected during follow-up despite analyzing consecutively bone marrow, peripheral blood and lymph node samples.
at intervals of six months. In these samples, sequential analysis showed the acquisition of p53 mutations. This patient had a rapid clinical evolution despite the fact that we could not demonstrate evidence of histologic transformation to a high grade lymphoma (Figure 1a).

2. Another patient (UPN 8) showed bcl-6 mutations only in the last sample analyzed, in which a histologic transformation was evident, indicating an acquisition of this mutation during the evolution (Figure 1b).

3. The last patient (UPN 12) had bcl-6 mutations in the lymph node sample at diagnosis but not in peripheral blood and bone marrow samples obtained 2 weeks and 1 month and 5 months after specific therapy, despite the demonstration of the mcr breakpoint in the same samples.

All samples included in this study were investigated for mutations in p53 exons 5 through 9 by PCR-SSCP. Mutations in the p53 gene were detected in 7/43 patients (16%) (UPN 1, 2, 5, 8, 12, 40 and 43). The characteristics of the p53 mutations are shown in Table 2. A histologic transformation was evident in patients UPN 2 and UPN 8. Patients with bcl-6 mutations associated with the MBR breakpoint in the bcl-2 gene and p53 mutations (UPN 1, 2, and 8) died with one year from diagnosis. Patients with the UPN 12, 40 and 43 are still alive and, interestingly, they had bcl-6 and p53 mutations associated with the mcr breakpoint in the bcl-2 gene.

Relationship between bcl-6 mutations and p53 mutations and clinical outcome

None of the patients with bcl-6 mutations associated exclusively with bcl-2 rearrangements (n = 10) showed disease transformation to a high grade lymphoma. Lymphoma cases with bcl-6 mutations have an indolent clinical evolution whereas patients with both bcl-6 and p53 mutated and MBR bcl-2 showed a poor outcome (Figure 2).

The median follow-up was 19.5 months. Thirty-five per cent of patients achieved a complete remission (CR). Thirteen patients (32.5%) died, 10 of them from disease progression or relapse, 2 from infection and one from graft-versus-host disease. In an attempt to define the factors that could influence the achievement of complete remission, a univariate analysis of a number of biological and clinical features was performed with available data from 40 patients. Univariate analysis did not reveal a significant association between p53 mutation and failure to obtain a CR (p = 0.39). The patients’ distribution between the groups with p53 mutations and with bcl-6 mutations was homogeneous taking into account age and stage. Patients with p53 mutations had a higher frequency of B-symptoms (p = 0.017) and a higher frequency of IPI score ≥ 3 (p = 0.018) although it was not possible to demonstrate a higher death rate (p = 0.18). These differences were also not found in patients who presented exclusively bcl-6 mutations. There were no differences in overall survival (OS) between patients with major or minor bcl-2 rearrangements and bcl-6 mutations. However, there was a marked difference in OS between patients with p53 mutations (p = 0.001) and whose with a high IPI score (p<0.001).

To ascertain whether the presence of genetic lesions was associated with an independent increase in the risk of death, a Cox regression analysis was performed. Clinical and biological features including age, stage, B-symptoms, IPI score, breakpoint in the bcl-2 gene, presence of p53 and/or bcl-6 mutations and outcome were analyzed. Of these factors, only the IPI score estimated the proportion of patients with a poor evolution. IPI ≥ increased the risk of death by 10.3-fold (p<0.001, 95% CI 2.8-38.1) and there was a positive correlation between the highest values of the IPI and p53 mutations (p = 0.002).

The interaction between these mutations was analyzed to determine whether bcl-6 mutations together with the presence of p53 mutations influenced prognosis. This analysis did not show any significant survival difference between these two groups of patients. In the absence of p53 mutations, the outcome of patients with bcl-6 mutations did no differ significantly from that of patients without bcl-6 mutations.
Relationship between Bcl-6 mutations, p53 mutations, and protein expression

Expression of the Bcl-2, Bcl-6 and p53 proteins was analyzed from lymph nodes (n=21, 19 FL and 2 DLCL). Bcl-2 and Bcl-6 proteins were expressed in all the cases. The expression of p53 protein was clearly positive in 5 cases with p53 mutations and was weakly expressed in 2 samples with p53 mutations and 4 samples without p53 mutations.

Discussion

The aim of this study was to analyze and describe the distribution of bcl-6 mutations in NHLS carrying the bcl-2/Jh rearrangement, and the relationship between bcl-6 mutations and p53 mutations in these lymphomas. We found that bcl-6 point mutations affected 40% of these lymphomas and that bcl-6 mutations could be associated with both bcl-2 major (MBR) and minor (mcr) breakpoints. Furthermore, p53 mutations were detected in both groups and when associated with bcl-6 point mutations in the Bcl-2 MBR cases they were associated with a short survival rate regardless of a histologic documentation of transformation.

The study of the relationship between survival and clinical-biological factors revealed that the co-occurrence of high IPI score with p53 mutations is the worst prognostic factor for patients with FL and bcl-2 rearrangements, whereas bcl-6 mutation is not an adverse survival risk factor. This observation suggests that patients with FL and bcl-2 rearrangements with a high IPI score at diagnosis may be monitored for the presence of p53 mutations during disease evolution. If a p53 mutation appears, these patients could be candidates for more aggressive therapy, i.e.: autologous bone marrow transplantation. It was clear that all samples with p53 mutations also had multiple (57%) or single (43%) bcl-6 mutations, whereas single point bcl-6 mutations were detected in 88% of the samples in which the p53 mutation was not detected.

The timing of the acquisition of bcl-6 and p53 mutations was variable. Multiple consecutive samples were studied in 5 patients. A p53 mutation was detected prior to the acquisition of bcl-6 mutations in one patient with FL (UPN 1), whereas in another patient (UPN 8) one p53 mutation appeared after the acquisition of bcl-6 mutations during the transformation to a high-grade lymphoma. Patient UPN 12 showed bcl-6 and p53 mutations at diagnosis from the analysis of a lymph node sample, but a bone marrow sample analyzed 5 months later showed neither mutation. Although this result was interpreted as an effect of successful therapy since the percentage of CD19 positive cells was 3% and there was no light chain restriction by flow cytometry, the bcl-2 rearrangement was still detected by PCR. A dynamic acquisition and/or loss of mutations in specific target genes cannot be ruled out in lymphomas arising from germinal centers. In the last two patients (UPN 13 and 19) we did not detect any mutation in any sample at diagnosis or during evolution of the patients’ disease.

We analyzed three cases of DLCL. One patient (UPN 2) with de novo DLCL had both Bcl-6 and p53 mutations at the time of diagnosis, the second patient (UPN 27) did not present any mutation, whereas in the third case (UPN 8) a p53 mutation was acquired during the follicular phase and the bcl-6 mutations appeared during the evolution to the transformed phase of FL. The patient identified as UPN 5 showed multiple bcl-6 and p53 mutations but the transformation to a high grade lymphoma was only evident on completion of the study.

These observations generate some information regarding bcl-6 mutations: bcl-6 mutations may be acquired (or disappear) during the evolution of lymphomas arising from germinal centers, suggesting that ongoing mutations take place in the bcl-6 gene which could favor the transformation of FLs. Interestingly there are recent reports suggesting the acquisition of bcl-6 mutations during the clonal evolution of FL. Lossos & Levy and Szereday et al. have demonstrated that new mutations in the 5’ non-coding regulatory region of the bcl-6 gene developed during the clonal evolution of FL and that they accumulated during the transformation of these lymphomas. Moreover, preliminary reports have confirmed the association of genetic instability of FL, lending support to the view that multiple mutations in NHLS produce the tumor. Some bcl-6 mutations (i.e. in the E1.11 and E1.12 fragments) could predispose to the acquisition of p53 mutations. In fact, all the patients with p53 mutations also had bcl-6 mutations. The coexistence of bcl-6 and p53 mutations has already been described. Capello et al. reported 3 out of 3 cases of FL (100%) transformed to DLCL with bcl-6 and p53 mutations. In contrast, patients with de novo DLCL presented both types of mutations in 2 out of 32 cases (6%). It could be hypothesized that bcl-6 and p53 mutations may act in a synergistic manner in lymphoma evolution.

The frequency and the nature of bcl-6 mutations found in this study are comparable with those in other reports: the frequency in our study was 40%, that reported frequency in DLCL was 70%, and that
Expression of Bcl-2 and Bcl-6 protein was studied in all the samples because these two genes are the most frequently affected in mature B-cell neoplasms. High levels of expression of Bcl-2 and Bcl-6 were demonstrated in FL with bcl-2 rearrangements even in samples without bcl-6 mutations. The role of bcl-6 mutations in an intron region of this gene is, to date, unknown. However, the high level of Bcl-6 expression in FL carrying bcl-2 rearrangements suggests that the deregulation of the hypermutational mechanism.

In conclusion, our study reveals that bcl-6 mutations could be associated with both bcl-2 major and minor breakpoints. The association of p53 and bcl-6 mutations confers a very poor prognosis. M utational analysis of bcl-6 and p53 should be investigated in larger series of FL in order to establish their potential value as prognostic factors.

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MB performed the experimental work and wrote the first draft of the paper. DC supervised the PCR, AA the statistics, CE the molecular methods, and RP and RB the pathology. GG, MB, GS and JS discussed the results. JFN designed the study and wrote the final version.

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