

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 chemo-resistance.

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 ($p = 0.002$).

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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.

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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.¹⁰⁻¹³ Genetic instability demonstrated in transformed NHLs^{14,15} and the hypermutational mechanisms that take place in germinal centers play a fundamental role in the acquisition of these molecular lesions.^{12,14-20} Mutations in the tumor suppressor gene p53, structural alteration of the MUM1 gene, the p16^{INK4a} 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.²¹⁻²⁴ 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.²⁵⁻²⁹

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.¹⁰ 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.³¹⁻³³ 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 the cases in which more than one sample was studied.

On the basis of the WHO classification of NHLs originating from the germinal center,³⁴ 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 precipi-

tation with ethanol (bone marrow and peripheral blood).³⁵ 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.^{32,33} 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 (TaKaRa LA Taq polymerase, Takara Shuzo) in a final volume of 50 μ L. 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.^{32,33} 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.*²¹ PCR-SSCP analysis of the p53 gene was performed on five PCR fragments corresponding to exons 5-9 following a well established protocol.³⁶ PCR-SSCP of the *bcl-6* gene was performed using 100 ng of genomic DNA, 10 pmol of each primer, 2.5 μ mol/L dNTPs, 1 μ Ci of α -³²P dCTP (Amersham, Amersham, UK), 10 mmol/L Tris-HCl (pH 8.8), 50 mmol/L KCl, 1 mmol/L MgCl₂, 0.01% gelatin, and 0.5 U AmpliTaq polymerase (Perkin-Elmer, Norwalk, CT, USA) in a final volume of 10 μ L. 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, Qia-gen, 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 - ^{33}P] 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 χ^2 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 bcl6 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,⁴ 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/Jh 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 REAL grade	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 IAPVP-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+PBSCT RT	PR PR	Dead 8 months	MBR-10 Kb	E1.11 T-G +775 T-A +783 G-A +805 E1.12 Insert.T +876 polym	Codon 273 CGT→TGT R273C	+++	+++	+++
3	35	FL II	IVA	1	LN	CHOP+CL+FN Rituximab	CR R,CR	Alive 44 months	MBR-4Kb	Neg	Neg	+++	++	Neg
4	55	FL I	IVA	1	LN	CHOP	CR	Alive 88 months	MBR-6 Kb	E1.10 Insert.C +441	Neg	+++	++	Neg
5	35	FL-DLCL II	IVB	4	LN	CHOP	PD	Dead 10 months	MBR-4 Kb	E1.11 T-A +807 E1.12 C-A +1053 Delect G +1111	Codon 242 TGC→TAC C242Y	+++	++	++
6	48	FL II	IA	1	LN	RT	CR	Alive 20 months	MBR-4 Kb	E1.10 Insert.C +561	Neg	+++	+	Neg
7	78	FL I	IVA	1	LN	CHOP+CL	CR	Alive 148 months	MBR-5Kb	Neg	Neg	+++	+	Neg
8	30	FL-DLCL II	IVA	4	LN LN LN	CHOP IAPVP-16 DHAP+ESHAP BEAM+PBSCT	PD PD	Dead 9 months	MBR-20 Kb	E1.11 G-A +759	Codon 248 CGG→CAG R248H	+++	+++	+++
9	58	FL I	IVA	2	BM	CHOP+IFN IAPVP16	CR PR	Alive 20 months	MBR-4.4 Kb	Neg	Neg	++	Neg	Neg
10	46	FL II	IIIA	1	LN	CHOP+IFN	CR	Alive 36 months	MBR-4.4 Kb	Neg	Neg	++	+++	Neg
11	30	FL II	IVA	1	LN	CHOP+IFN Rituximab+CVP	CR PR	Alive 35 months	mcr-3 Kb	E1.11 C-G +780	Neg	+++	++	Neg
12	68	FL I	IVB	3	LN BM PB BM	CHOP Rituximab	PR PR,PD	Alive 9 months	mcr-3 Kb	E1.12 G-A +895	Codon 234 TAC→TGC Y234C	+++	+++	+
13	41	FL II	IVA	1	LN BM	CHOP	CR	Alive 9 months	mcr-16 Kb	Neg	Neg	+	+	Neg
14	70	FL I	IA	1	PB	Surgery	CR	Alive 18 months	MBR-4.4 Kb	Neg	Neg	++	+++	Neg
15	59	FL II	IVB	2	PB	CHOP Rituximab+CNOP	CR	Alive 40 months	MBR-5 Kb	E1.11 C-T +786	Neg	--	--	--
16	?	FL	?	?	PB	?	?	Alive	MBR-5 Kb	E1.11 G-C +754 G-A +791	Neg	--	--	--
17	30	FL I	IVA	3	PB	FCM+PBSCT	CR	Alive 23 months	MBR-4 Kb	E1.11 G-A +752 G-C +754 T-A +817	Neg	--	--	--
18	50	FL II	IVA	1	BM	CHOP FCM Rituximab	PR PR In course	Alive 3 to 5m	MBR-4.4 Kb	E1.10 G-C +479	Neg	--	--	--
19	46	FL I	IVB	1	BM PB BM/PB	CHOP IAPVP16+PBSCT Rituximab+CVP	CR PR CR	Dead 3 to 9 m	mr-3 Kb	Neg	Neg	--	--	--

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20	37	FL I	IVA	2	BM	CHOP IAPVP16+PBCST Rituximab+CNOP	NR PR CR	Alive with disease	mbr-4.4 Kb	Neg	Neg	--	--	--
21	?	Burkitt	IVB	4	BM	Palliative	--	Dead 4 days	mbr-4 Kb	Neg	Neg	--	--	--
22	29	FL I	IVA	1	PB	CHOP IAPVP16+PBCST FCM+PBCST	PR CR PR	Dead 36 months	mbr-4 Kb	Neg	Neg	--	--	--
23	51	FL I	IVB	1	BM	CHOP IAPVP16+PBCST FCM	PR CR ?	Alive 26 months	MBR-4.4 Kb	Neg	Neg	--	--	--
24	44	FL II	IVB	2	PB	CHOP+RT ESHAP+PBCST Rituximab	PR CR NR	Dead 40 months	MBR-4 Kb	Neg	Neg	--	--	--
25	42	FL I	IVA	2	BM	EPOCH+ minialloSCT CNOP+surgery IAPVP16	PR PR CR	Dead 44 months	mcr-9 Kb	Neg	Neg	--	--	--
26	44	FL II	IVB	2	PB	FCM+alloSCT CHOP+RT ESHAP PBCST Rituximab EPOCH MiniALOHCT	RP RP RP RC REC NR	Dead 34 months	MBR-4.4 Kb	Neg	Neg	--	--	--
27	50	DLCL	IIA	2	LN	ESHAP EPOCH	PROG PROG	Dead 2 months	mcr-2.4 Kb	Neg	Neg	+	+	Neg
28	58	FL I	IVA	1	BM	CHOP PBCST+ Rituximab	RP RC	Alive 12 months	mcr-2.5 Kb	E1.12 A-G+970	Neg	+	+	Neg
29	55	FL II	IVA	2	BM	CHOP+RT	RP	Alive 19 months	MBR 3.8 Kb	Neg	Neg	--	--	--
30	68	FL ?	IVA	2	BM	?	?	?	MBR-5 Kb	E1.1 G-C+779	Neg	--	--	--
31	55	FL I	IIA	0	LN	CHOP	RP-PROG	Alive 185 months	MBR-4.4 Kb	Neg	Neg	Neg	+	Neg
32	62	FL II	IVA	3	BM	CHOP	RP-PROG in course	Alive 16 months	MBR-4.4 Kb	Neg	Neg	--	--	--
33	54	FL I	IVA	1	LN	FCM	RP	Alive 7 months	MBR-4.4 Kb	Neg	Neg	+	+	Neg
34	46	FL I	IVA	2	BM	CHOP+IFN	RP	Alive 46 months	MBR-3.8 Kb	Neg	Neg	--	--	--
35	53	FL I	IVA	1	PB	CHOP+CL Rituximab	RP	Alive 12 months	MBR-4 Kb	Neg	Neg	--	--	--
36	59	FL-BL I	IVA	2	BM	CL CHOP ESHAP CHOP	RP-PROG RP PROG PROG	Dead 111 months	MBR-3 Kb	Neg	Neg	--	--	--
37	70	FL II	IVB	4	PB	ESHAP CHOP	PROG PROG	Dead 6 months	mcr-9.4 Kb	Neg	Neg	--	--	--
38	39	FL II	IIIA	?	LN	?	?	?	MBR-4 Kb	Neg	Neg	+	+	Neg
39	38	FL I	IIIA	1	LN	FCM	in course	Alive 7 months	MBR-4 Kb	E1.11 G-C+833 E1.12 G-A+975	Neg	+	+	Neg
40	57	FL II	IIA	1	LN	CHOP	in course	Alive 5 months	mcr-6 Kb	E1.10 T-C+559 E1.11 G-C+793 E1.12 T-A+927	Codon 232 ATC→ACC I232T	+	+	±
41	39	FL I	IA	1	BM	FCM	in course	Alive 7 months	MBR-4 Kb	Neg	Neg	+	+	Neg
42	56	FL II	IIA	2	LN	CHOP	PROG	Alive 8 months	MBR-4 Kb	Neg	Neg	+	+	±
43	55	FL I	IVB	2	PB	CHOP+IFN	CR	Alive 11 months	mcr-6 kb	E1.11 T-A+783 E1.12 T-A+1093	Codon 232 ATC→ACC I232T	--	--	--

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; PBCST: peripheral blood stem cell transplantation; IPI: international prognostic index.

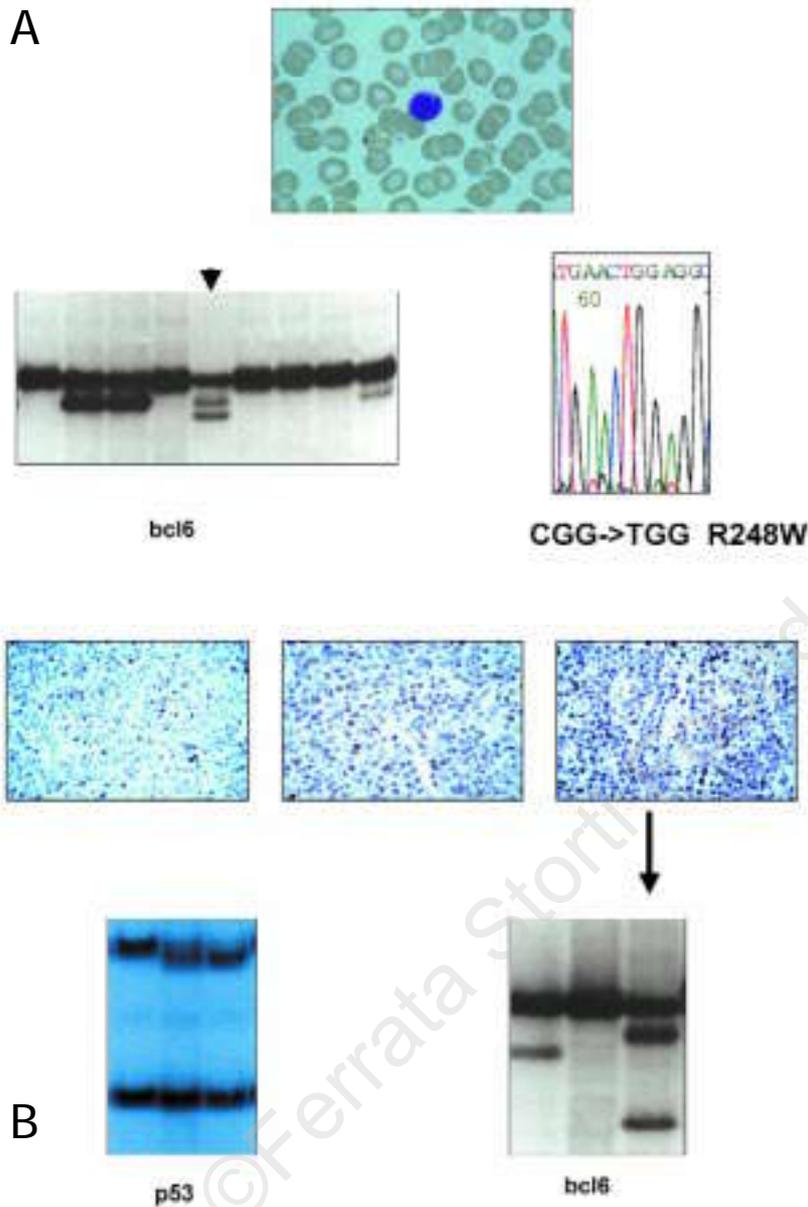


Figure 1.
A. Patient 1 (UPN 1): Multiple genetic lesions can be present in cytologically indolent FL (top). Both bcl-6 (bottom left) and p53 mutation (bottom right) were detected at diagnosis of this case. The bcl-6 mutation disappeared whereas the p53 mutation was maintained.
B. Patient 2 (UPN 8): Sequential analysis of three lymph node samples from a patient with FL showed a progressive increase in the percentage of p53⁺ cells (top). In the second sample a heterozygous p53 mutation was detected which became dominant in the last sample (bottom left). The third sample, obtained when the patient developed a DLCL transformation, also showed a bcl-6 mutation (bottom right).

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 tak-

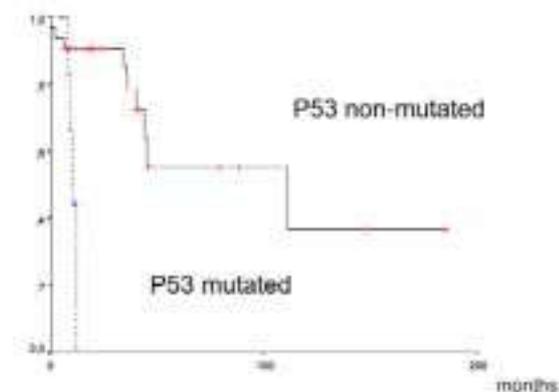


Figure 2. Probability of survival in patients with FL and bcl-2 rearrangements according to p53 mutations.

ing 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 those 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 ≥ 3 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 not 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-association of a 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, ie: allogeneic 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.*^{11,13} 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.^{10,12,14,15,17} 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.*²¹ 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.³⁷

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

for FL varied between 42–60%.^{25,38-41}

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.⁴²⁻⁴⁵ High levels of expression of Bcl-2 and Bcl-6 were demonstrated in FL with bcl-2 rearrangements⁴⁶ even in samples without bcl-6 mutations. The role of bcl-6 mutations in an intronic 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 deregulated bcl-2 gene may exert an influence on the development of genetic abnormalities of the bcl-6 gene.

The detection of mutations in other non Ig genes,⁴⁷ the absence of structural similarities between the Bcl-6 and the Ig gene sequences and the clustering in regulatory and phylogenetically conserved regions of the bcl-6 suggest an expansion 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 to patients suffering from FL with the MBR bcl-2 rearrangement, even before the transformation to a high grade lymphoma. Mutational analysis of bcl-6 and p53 should be investigated in larger series of FL in order to establish their potential value as prognostic factors.

Contributions and Acknowledgments

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.

We are very grateful to Drs. M. García, J.A. Hernández and P. Romero for identifying patients for this study and to Dr. G. Perea for providing clinical data.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Funding

This study was supported by a grant from the Fondo de Investigación Sanitaria, Spain, FIS (97/1118).

References

- Anonymous. A clinical evaluation of the International Lymphoma Study Group classification of non-Hodgkin's lymphoma. The Non-Hodgkin's Lymphoma Classification Project. *Blood* 1997; 89:3909-18.
- Tsujimoto Y, Croce CM. Analysis of the structure, transcripts, and protein products of bcl-2, the gene involved in human follicular lymphoma. *Proc Natl Acad Sci USA* 1986; 83:5214-8.
- Stamatoullas A, Buchonnet G, Lepretre S, Lenain P, Lenormand B, Duval C, et al. De novo acute B cell leukemia/lymphoma with t(14;18). *Leukemia* 2000; 14:1960-6.
- Meijerink JP, Raemaekers JM, Mensink EJ. New type of t(14;18) in a non-Hodgkin's lymphoma provides insight in molecular events in early B-cell differentiation. *Br J Haematol* 1995; 91:630-9.
- Meijerink JP. t(14;18), a journey to eternity. *Leukemia* 1997; 11:2175-87.
- Korsmeyer SJ. Regulators of cell death. *Trends Genet* 1995; 11:101-5.
- Korsmeyer SJ. Bcl-2 initiates a new category of oncogenes: regulators of cell death. *Blood* 1992; 80:879-86.
- Biagi JJ, Seymour JF. Insights into the molecular pathogenesis of follicular lymphoma arising from analysis of geographic variation. *Blood* 2002; 99:4265-75.
- Cleary ML, Galili N, Sklar J. Detection of a second t(14;18) breakpoint cluster region in human follicular lymphomas. *J Exp Med* 1986; 164:315-20.
- Gronbaek K, de Nully Brown P, Moller MB, Nedergaard T, Ralfkiaer E, Moller P, et al. Concurrent disruption of p16INK4a and the ARF-p53 pathway predicts poor prognosis in aggressive non-Hodgkin's lymphoma. *Leukemia* 2000; 14:1727-35.
- Lossos IS, Levy R. Higher-grade transformation of follicle center lymphoma is associated with somatic mutation of the 5' noncoding regulatory region of the BCL-6 gene. *Blood* 2000; 96:635-9.
- Sander CA, Yano T, Clark HM, Harris C, Longo DL, Jaffe ES, et al. P53 mutation is associated with progression in follicular lymphomas. *Blood* 1993; 82:1994-2004.
- Szereday Z, Csernus B, Nagy M, Laszlo T, Warnke RA, Matolcsy A. Somatic mutation of the 5' noncoding region of the BCL-6 gene is associated with intraclonal diversity and clonal selection in histological transformation of follicular lymphoma. *Am J Pathol* 2000; 156:1017-24.
- Gaidano G, Carbone A. MUM1: a step ahead toward the understanding of lymphoma histogenesis. *Leukemia* 2000; 14:563-6.
- Nagy M, Balázs M, Ádám Z, Petkó Z, Timár B, Szereday Z, et al. Genetic instability is associated with histological transformation of follicle center lymphomas. *Leukemia* 2000; 14:2142-8.
- Otsuki T, Yano T, Clark HM, Bastard C, Kerckaert JP, Jaffe ES, et al. Analysis of LAZ3 (BCL-6) status in B-cell non-Hodgkin's lymphomas: results of rearrangement and gene expression studies and a mutational analysis of coding region sequences. *Blood* 1995; 85:2877-84.
- Boland R, Ricciardiello L. How many mutations does it take to make a tumor? *Proc Natl Acad Sci USA* 1999; 96:14675-7.
- Pasqualucci L, Migliozza A, Fracchiolla N, William C, Neri A, Baldini L, et al. Bcl-6 mutations in normal germinal center B cells: evidence of somatic hypermutation acting outside Ig loci. *Proc Natl Acad Sci USA* 1995; 95:11816-21.
- Möller MB, Ino Y, Gerdes AM, Skjodt K, Louis DN, Pedersen NT. Aberrations of the p53 pathway components p53, MDM2 and CDKN2A appear independent in diffuse large B cell lymphoma. *Leukemia* 1999; 13:453-9.
- Herranz M, Urioste M, Santos J, Martínez-Delgado JB, Rivas C, Benitez J, et al. Allelic losses and genetic instabilities of PTEN and p73 in non-Hodgkin lymphomas. *Leukemia* 2000; 14:1325-7.
- Capello D, Vitolo U, Pasqualucci L, Quattrone S, Migliaretti G, Fassone L, et al. Distribution and pattern of BCL-6 mutations throughout the spectrum of B-cell neoplasia. *Blood* 2000; 95:651-9.
- Ohno H, Fukuhara S. Significance of rearrangement of the BCL6 gene in B-cell lymphoid neoplasms. *Leuk Lymphoma*

- 1997; 27:53-63.
23. Muramatsu M, Akasaka T, Kadowaki N, Ohno H, Yamabe H, Edamura S, et al. Rearrangement of the BCL6 gene in B-cell lymphoid neoplasms: comparison with lymphomas associated with BCL2 rearrangement. *Br J Haematol* 1996; 93:911-20.
 24. Michaud GY, Gascoyne RD, McNeil BK, Anderson ME, Horsman DE. Bcl-6 and lymphoproliferative disorders. *Leuk Lymphoma* 1997; 26:515-25.
 25. Migliazza A, Martinotti S, Chen W, Fusco C, Ye BH, Knowles DM, et al. Frequent somatic hypermutation of the 5' noncoding region of the BCL6 gene in B-cell lymphoma. *Proc Natl Acad Sci USA* 1995; 92:12520-4.
 26. Bernardin F, Collyn-d'Hooghe M, Quief S, Bastard C, Lepince D, Kerckaert JP. Small deletions occur in highly conserved regions of the LAZ3/BCL6 major translocation cluster in one case of non-Hodgkin's lymphoma without 3q27 translocation. *Oncogene* 1997; 14:849-55.
 27. Lossos IS, Levy R. Mutation analysis of the 5' noncoding regulatory region of the BCL-6 gene in non-Hodgkin lymphoma: evidence for recurrent mutations and intraclonal heterogeneity. *Blood* 2000; 95:1400-5.
 28. Peng HZ, Du MQ, Koullis A, Aiello A, Dogan A, Pan LX, et al. Nonimmunoglobulin gene hypermutation in germinal center B cells. *Blood* 1999; 93:2167-72.
 29. Noppe SM, Heirman C, Bakkus MH, Brissinck J, Schots R, Thielemans K. The genetic variability of the VH genes in follicular lymphoma: the impact of the hypermutation mechanism. *Br J Haematol* 1999; 107:625-40.
 30. Knudson CM, Tung KS, Tourtellotte WG, Brown GA, Korsmeyer SJ. Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science* 1995; 270:96-9.
 31. Buchonnet G, Lenain P, Ruminny P, Lepretre S, Stamatoullas A, Parmentier F, et al. Characterisation of BCL2-JH rearrangements in follicular lymphoma: PCR detection of 3' BCL2 breakpoints and evidence of a new cluster. *Leukemia* 2000; 14:1563-9.
 32. Akasaka T, Akasaka H, Yonetani N, Ohno H, Yamabe H, Fukuhara S, et al. Refinement of the BCL-2/immunoglobulin heavy chain fusion gene in t(14;18)(q32;q21) by polymerase chain reaction amplification for long targets. *Genes Chromosomes Cancer* 1998; 21:17-29.
 33. Akasaka T, Muramatsu M, Ohno H, Miura I, Tatsumi E, Fukuhara S, et al. Application of long-distance polymerase chain reaction to detection of junctional sequences created by chromosomal translocation in mature B-cell neoplasms. *Blood* 1996; 88:985-94.
 34. Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J, et al. World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting-Airlie House, Virginia, November 1997. *J Clin Oncol* 1999; 17:3835-49.
 35. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; 16:1215.
 36. Gaidano G, Ballerini P, Gong JZ, Inghirami G, Neri A, Newcomb EW, et al. p53 mutations in human lymphoid malignancies: association with Burkitt lymphoma and chronic lymphocytic leukemia. *Proc Natl Acad Sci USA* 1991; 88:5413-7.
 37. Prokocimer M, Unger R, Rennert HS, Rotter V, Rennert G. Pooled analysis of p53 mutations in hematological malignancies. *Hum Mutat* 1998; 12:4-18.
 38. Capello D, Carbone A, Pastore C, Gloghini A, Saglio G, Gaidano G. Point mutations of the BCL-6 gene in Burkitt's lymphoma. *Br J Haematol* 1997; 99:168-70.
 39. Gaidano G, Carbone A, Pastore C, Capello D, Migliazza A, Gloghini A, et al. Frequent mutation of the 5' noncoding region of the BCL-6 gene in acquired immunodeficiency syndrome-related non-Hodgkin's lymphomas. *Blood* 1997; 89:3755-62.
 40. Gaidano G, Capello D, Gloghini A, Fassone L, Vivenza D, Ariatti C, et al. Frequent mutation of bcl-6 proto-oncogene in high grade, but not low grade, MALT lymphomas of the gastrointestinal tract. *Haematologica* 1999; 84:582-8.
 41. Gaidano G, Capello D, Cilia AM, Gloghini A, Perin T, Quatrone S, et al. Genetic characterization of HHV-8/KSHV-positive primary effusion lymphoma reveals frequent mutations of BCL6: implications for disease pathogenesis and histogenesis. *Genes Chromosomes Cancer* 1999; 24:16-23.
 42. Capello D, Gaidano G. Molecular pathophysiology of indolent lymphoma. *Haematologica* 2000; 85:195-201.
 43. Merup M, Spasokoukotskaja T, Einhorn S, Smith CI, Gahrton G, Juliusson G. Bcl-2 rearrangements with breakpoints in both vcr and mbr in non-Hodgkin's lymphomas and chronic lymphocytic leukaemia. *Br J Haematol* 1996; 92:647-52.
 44. Volpe G, Vitolo U, Carbone A, Pastore C, Bertini M, Botto B, et al. Molecular heterogeneity of B-lineage diffuse large cell lymphoma. *Genes Chromosomes Cancer* 1996; 16:21-30.
 45. Akasaka T, Akasaka H, Ueda C, Yonetani N, Maesako Y, Shimizu A, et al. Molecular and clinical features of non-Burkitt's, diffuse large-cell lymphoma of B-cell type associated with the c-MYC/immunoglobulin heavy-chain fusion gene. *J Clin Oncol* 2000; 18:510-8.
 46. Raible MD, Hsi ED, Alkan S. Bcl-6 protein expression by follicle center lymphomas. A marker for differentiating follicle center lymphomas from other low-grade lymphoproliferative disorders. *Am J Clin Pathol* 1999; 112:101-7.
 47. Storb U, Peters A, Klotz E, Kim N, Shen HM, Hackett J, et al. Cis-acting sequences that affect somatic hypermutation of Ig genes. *Immunol Rev* 1998; 162:153-60.

PEER REVIEW OUTCOMES

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Francesco Lo Coco, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Professor Lo Coco and the Editors. Manuscript received April 4, 2002; accepted July 16, 2002.

What is already known on this topic

In spite of a common molecular lesion (Bcl-2/IgH rearrangement) follicular lymphomas are characterized by a heterogeneous clinical course.

What this study adds

The authors' data provide a better dissection, at the molecular level, of these lymphomas.

Potential implications for clinical practice

Additional lesions such as Bcl-6 and p53 mutations may identify more advanced stage disease in FL, and p53 alteration in particular might correlate with aggressive clinical behavior requiring more intensive therapy. The clinical significance of Bcl-6 mutations is less clear.

Francesco Lo Coco, Associate Editor