A Short Mutational Hot Spot in the First Intron of BCL-6 Is Associated with Increased BCL-6 Expression and with Longer Overall Survival in Large B-Cell Lymphomas

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BCL-6 somatic mutations have been described in normal and tumoral B lymphocytes, associated with germinal center transit. We analyzed mutations in the major mutation cluster of BCL-6 in a series of 45 large B-cell lymphomas (LBCLs) and 15 Burkitt's lymphomas, and their relation to the level of BCL-6 expression and clinical outcome. Mutations in LBCL cases revealed the existence of two distinct, short mutational hot spots, spanning positions 106 to 127 and 423 to 443, in which the mutation frequency was higher than expected ($P < 0.001$). Mutations in the 423 to 443 subcluster were associated with an increased level of expression, although this was not the case with the 106 to 127 cluster. Additionally, LBCL cases characterized by the presence of mutations in the 423 to 443 cluster showed an increased overall survival ($P < 0.05$) when compared with the nonmutated LBCL cases in these positions. Burkitt's lymphoma cases showed a slightly lower frequency of mutations with a nonclustered distribution and lacked any relationship with the level of expression or any clinical characteristic. Findings from LBCLs suggest that the 423 to 443 cluster includes a regulatory region that is of importance for BCL-6 expression. Deregulation of BCL-6 expression caused by these mutations could play an important role in lymphoma genesis or progression. (*Am J Pathol* 2002, 160:1371-1380)

The BCL-6 gene was identified as a result of its involvement in chromosomal translocations in a subset of large B-cell lymphomas (LBCLs) and follicular lymphomas.¹ These translocations deregulate the expression of the BCL-6 gene after substituting the BCL-6 promoter with a variety of other gene promoters.^{2,3} This gene contains 10

exons and encodes for a 3.8-kb mRNA that is translated into a 706-amino acid protein.⁴ BCL-6 protein has been identified as being a transcriptional repressor with two important functional domains: six zinc-fingers at the carboxyl terminus that mediate its interaction with DNA, and a BTB/POZ domain, a hydrophobic region, that mediates its interactions with other proteins.⁵ The BCL-6 gene has been shown to be a multifunctional gene, regulating important genes involved in B-cell differentiation (*blimp-1*, *IP-10*, and others) and cell-cycle control (such as *c-myc*, *p27^{KIP1}*, and *cyclin D2*).⁶

In addition to translocations, the BCL-6 gene experiences somatic mutations in the first intron, 100 bp downstream from the first noncoding exon, analogous to what occurs with IgVH genes, although at a lower frequency.^{7,8} BCL-6 somatic mutations have been described in normal and tumoral B lymphocytes, being more frequently observed in LBCL than in other tumoral types.^{8,9} BCL-6 mutations are considered to be markers of germinal center transit because they are absent in benign and malignant (mantle cell lymphoma) pregerminal center B cells and characteristically present in germinal center lymphocytes and derived tumors.

It has been suggested on several occasions that BCL-6 expression is probably deregulated as a consequence of somatic mutation.⁸ However, a relationship between the frequency or localization of these mutations and the expression of the protein has not been conclusively demonstrated.

Furthermore, consequences of the accumulation of BCL-6 mutations during the genesis and progression of lymphomas have not been clarified sufficiently. The only exception is the higher grade transformation of follicular lymphoma, in which the accumulation of new mutations has been described.¹⁰ However, this accumulation has not been shown to have any pathogenic association with the mechanism of the progression.

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The mutation frequency of BCL-6 seems to be high in LBCL. An expression profile that includes the expression of BCL-6 and other germinal center markers has been found to be a reliable predictor of increased overall survival (OS) in LBCL.¹¹ Moreover, it has been recently described that the expression of BCL-6 alone can be a reliable predictor of better survival in LBCL.¹² For these reasons, we decided to determine whether BCL-6 mutations are associated with differences in the expression of the BCL-6 protein and the outcome of the patients in a group of LBCL cases. To this end, we analyzed mutations in the major mutation cluster (MMC) of BCL-6 in a series of 45 LBCL cases, relating them to the level of BCL-6 expression and clinical outcome. A group of 15 Burkitt's lymphoma (BL) cases was also included to allow comparison of the effects of mutations in these different lymphoma types.

Materials and Methods

Tissue Samples, Patients, and Clinical Variables

We studied 45 LBCL and 15 BL patients who had been diagnosed according to the REAL classification.¹³ For most tests, each histological group was studied separately, making it possible to analyze molecular differences between them. Diagnostic pretreatment samples were analyzed in all cases. Tumoral and reactive specimens were selected from the Pathology Laboratory, Hospital Virgen de la Salud (Toledo, Spain), on the basis of the availability of paraffin-embedded and frozen tissue for molecular studies and the existence of an adequate clinical follow-up (except for four patients, whose clinical history was not available). All patients were treated with chemotherapy regimes including anthracyclines. A clinical follow-up was performed in all cases. Clinical follow-up of patients alive at the time of the study included periods ranging from 11 to 122 months (mean, 75.38 months). Age, clinical stage, performance status, levels of lactate dehydrogenase (LDH) in blood and the number of extranodal sites of the disease were used as outcome criteria, as set out by the International Prognostic Index (IPI).¹⁴ Patients with 0 to 2 unfavorable variables were considered as being low risk, and those with 3 to 5 variables as high risk. Complete remission was defined as the resolution of clinical and radiological evidence of disease for a minimum of 4 weeks.

Biopsy specimens of these 60 patients were divided into two parts: one was formalin-fixed and paraffin-embedded for morphological and immunohistochemical studies, while the other was embedded in Optimal Cutting Temperature Tissue-Tek (OCT; Sakura, Zoeterwoude, Netherlands) and preserved at -80°C for molecular studies.

DNA extraction from frozen sections was performed following the standard phenol-chloroform protocol. RNA was extracted from frozen sections. Before each DNA or RNA extraction, frozen sections of the specimens were analyzed to ensure that the specimen was representative

of the tumor. The percentage of tumoral cells was greater than 80% in all cases.

To quantify BCL-6 mRNA expression, we extracted RNA from Raji cells. This cell line was obtained from the American Tissue Type Culture Collection (Rockville, MD), and maintained in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum, 2 mmol/L glutamine, and penicillin-streptomycin (Life Technologies, Inc., Grand Island, NY).

Immunostaining Techniques

BCL-6 protein was detected with monoclonal antibody for P6-B6p, a recombinant protein corresponding to amino acids 3 to 484 from DAKO, Glostrup, Denmark (1/10).¹⁵ The proliferation index was evaluated using nuclear antigen Ki67 expression, detected with the MIB1 antibody from Immunotech (Marseille, France).

Immunostaining techniques were performed in paraffin-embedded tissue sections. For antigen retrieval, before incubation with the antibodies (Ab), the slides were heated in a pressure cooker for 3 minutes in 0.01 mol/L of sodium citrate solution. Additionally, the slides were digested with proteinase K for 10 minutes at room temperature.

After incubation with the primary Ab, immunodetection was performed with biotinylated anti-mouse immunoglobulins, followed by peroxidase-labeled streptavidin (LSAB-DAKO, Denmark) and diaminobenzidine chromogen as substrate. All immunostaining was performed using the Techmate 500 (DAKO) automatic immunostaining device.

Incubations omitting the specific Ab, or with unrelated Abs, were performed to provide controls of the technique. The quality of the staining was checked on every slide, using BCL-6-positive reactive lymphocytes as an internal control.

Quantitative Studies

All scoring and interpretations of immunohistochemical results were performed independently by two of the authors (AIS and MJA) without knowledge of the clinical variables or the results of the molecular analysis.

High-magnification fields were chosen for the evaluation of BCL-6 and MIB1 expression, focusing on tumoral areas and counting up to 300 cells. All immunoreactive cells were considered to be positive. A manual cell-counting procedure was used so that all nontumoral subpopulations could be excluded on the basis of their cell morphology.

The intensity of BCL-6 immunostaining was classified into low, intermediate, or high groups when the signal was lower, similar, or higher, respectively, than reactive T lymphocytes in the tumor.

For survival analysis of BCL-6 expression, a cutoff of 50% of positive cells was used because this threshold divided the series into groups of similar size (median of percentage of BCL-6-positive cells: 50) and had the advantage of being easily reproducible.

Real-Time Polymerase Chain Reaction (PCR)

Total RNA extraction was performed using a protocol based on Trizol (Life Technologies, Inc.). cDNA was synthesized with avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI), according to manufacturer's instructions.

Real-time PCR was developed using the TaqMan technology, in an ABI Prism 7700 Sequence Detector System (PE Applied Biosystems, Norwalk, CT). As a control of the quality and quantity of the RNA, GAPDH gene was amplified in parallel with that of BCL-6. The primers and probes used in this study have been described previously.¹² Both probes were labeled at the 5' end with 6-carboxy-fluorescein phosphoramidite (FAM), and at the 3' end with 6-carboxy-tetramethyl-rhodamine (TAMRA) as quencher. The reactions were not multiplexed.

Each sample was measured from two different RNA extractions, each at two different dilutions, and each dilution in triplicate. To compare different experiments, in every PCR a standard curve (composed of different dilutions of a cDNA from Raji cells) and a calibrator (cDNA from a reactive tonsil) were derived, as suggested in the ABI 7700 User Bulletin 2 (PE Applied Biosystems). The conditions of the reactions were those recommended in the ABI 7700 User Bulletin 2 (PE Applied Biosystems).

Sequencing of BCL-6

A unique PCR product, 791 bp long, was amplified using 5'-CCGCTGCTCATGATCATTATTT-3' and 5'-TAGACACGATACTTCATCTCAT-3' primers. This fragment is located downstream of the first noncoding exon of BCL-6 and includes the entire MMC region.

The PCR reaction was performed in a 50- μ l total volume containing 50 pmol of each primer, 0.1 mmol/L dNTP, 1.5 mmol/L MgCl₂, and 2 U Taq Platinum (Life Technologies, Inc.). Conditions for amplification were as follows: 94°C, 5 minutes denaturation; 35 cycles of 30 seconds at 94°C, 30 seconds at 58°C, and 1 minute at 72°C; and a final extension step at 72°C for 10 minutes. PCR was performed in a Perkin Elmer 9700 GeneAmp PCR System (Norwalk, CT).

PCR products were purified by using the Microcon PCR kit (Millipore, Bedford, MA). Both strands were then directly sequenced, using the same primers as for the amplification and two additional internal oligonucleotides, in an ABI 370 (Perkin Elmer Applied Biosystems, Warrington, UK), following the manufacturer's procedure. Mutations were identified by comparison with the BCL-6 germline sequence (GenBank accession number AF191831). To control for potential Taq errors, all PCR and sequencing procedures were performed twice.

Statistical Analysis

Statistical study of the correlation between distributions was performed using either Fisher's exact test for categorical variables, the Kruskal-Wallis test for single-ranked data, and the Pearson correlation for double-ranked data.

The clinical variables analyzed in the survival studies were those included in the IPI¹⁴ (measured as 0 to 2, 3 to 5), these being: age (≤ 60 versus > 60 years), gender (female versus male), clinical stage (I + II versus III + IV), and LDH (normal versus $> \text{normal}$). Survival curves were calculated by the Kaplan-Meier method and compared by the log-rank test.^{16,17} Actuarial survival curves [OS and disease-free survival (DFS)] were calculated using the Kaplan-Meier method. Statistical significance was calculated using the log-rank test. Cox's proportional hazard univariate analysis¹⁸ was also performed, providing estimates of the confidence interval and the relative risk (RR) in terms of survival.

To identify the factors that might be of independent significance in influencing survival (OS and DFS), a Cox backward proportional hazard model was fitted.¹⁹ Variables included in the maximal models were IPI (0 to 2, 3 to 5) and presence of mutations inside the 423 to 443 cluster. The low-risk IPI and presence of mutations in the 423 to 443 region, found to be associated with higher survival probability, were taken as reference levels. All *P* values were two-sided, and values of 0.05 or less were considered to indicate statistical significance. SPSS 10.0 for Windows was used for all statistical analyses (SPSS Inc., Chicago, IL).

Results

BCL-6 Expression

An initial screening of BCL-6 protein expression was performed in reactive lymphoid tissue. The pattern of expression was similar to that described before: germinal center cells were variable although they generally stained strongly for BCL-6. Mantle and interfollicular cells were mostly negative, although some scattered cells were present in these areas (Figure 1A).

BCL-6 protein expression was then analyzed by immunohistochemistry in 52 cases of aggressive NHL, including 40 LBCLs and 12 BLs (Figure 1A). In the other eight cases, the results could not be evaluated because of the lack of adequate internal controls, which are usually provided by BCL-6-positive reactive lymphocytes. Variation in signal intensity and number of BCL-6-positive cells was observed and there was a strong positive correlation between the two variables ($r = 0.437$, $P = 0.001$, Spearman correlation). Therefore, we only considered the number of positive cells in the quantification of BCL-6 expression.

BCL-6 reactivity represented by fewer than 20% of positive cells was found in 5 cases (all LBCL samples), whereas 13 cases (8 LBCL and 5 BL) had more than 80% positive cells. Most cases (35 of 52) showed an intermediate level of BCL-6 expression. Table 1 and Figure 1 summarize the immunostaining results. There were only slight, nonsignificant differences in the distribution of expression levels between LBCL and BL cases.

BCL-6 protein expression was then contrasted with BCL-6 mRNA expression as measured by real-time PCR. We analyzed a group of 17 tumor samples from our series with variable protein expression detected by immunohis-

Figure 1. Expression pattern for BCL-6 protein in reactive tissue and lymphomas. **A:** Protein expression of BCL-6 in different lymphoma cases and reactive tissue. All photographs were taken at an original magnification of $\times 40$, except for those of reactive tonsil, which were taken at an original magnification of $\times 20$. **1:** Pattern of expression of a DLBCL scored as having 2% positive cells. **2:** Pattern of expression of a DLBCL scored as having 95% positive cells. **3:** Expression pattern in human reactive tonsil. BCL-6 is expressed only in germinal center cells and in scattered cells in the marginal area. **4:** BCL-6 expression in a mantle cell lymphoma case, showing that only cells originated in the germinal centers invaded by the tumor were positive. **B:** Real-time PCR results for BCL-6 mRNA expression of the cases whose protein expression is illustrated in **A**. The standard curve is represented in orange-yellow. Ct value was fixed at 0.02. The quantity of cDNA tested in this experiment was controlled and GAPDH expression was constant in the four cases (data not shown).

Table 1. Summary of BCL-6 IHC Results

	LBCL	BL	Total
Cases	40	12	52
Mean	53.6 (± 27.69)	64.0 (± 28.51)	55.9 (± 27.95)
Range	2–100	23–100	2–100

Values of mean and range are expressed as percentages of the number of BCL-6-positive cells in the total number of cells scored. Standard deviation is indicated in brackets.

LBCL, large B-cell lymphoma; BL, Burkitt's lymphoma.

tochemistry, and 4 reactive tonsil samples and 2 mantle cell lymphoma samples as controls (Figure 1B). LBCL samples showed a good correlation among protein and mRNA expression levels with the exception of a discrep-

ancy in five cases. The only striking finding was the detection in MCL cases of mild, but distinct BCL-6 mRNA expression levels, thus confirming the previous observations by Lossos and co-workers.¹²

BCL-6 Mutations

Sequencing analysis was performed in 45 LBCL and 14 BL cases identifying a wide variety of mutations that were present in the majority of cases (80% LBCL and 71% BL were mutated). Table 2 shows a summary of the mutational analysis. The frequency of mutations in our series was 3.7×10^{-3} mutations/bp (3.8×10^{-3} mutations/bp in LBCL cases and 3.7×10^{-3} mutations/bp in BL cases).

Table 2. Description of the BCL-6 MMC Mutations Found in this Series

Diagn.	Mutations	Diagn.	Mutations
LBCL	G-C (397)	LBCL	WT
LBCL	G-A (243), G-A (244), G-C (397) , G-A (448), G-C (518)	LBCL	A-G (96), G-C (125), G-A (448), A-G (455), C-T (519), C-A (586), T-C (753)
LBCL	C-G (88), T-C (89), A-C (108), G-C (122), C-G (169), C-T (172), C-A (173), G-A (211), T-C (218), A-G (226), G-A (240), G-A (241), T-C (272), G-C (397) , C-G (423), C-T (425), G-A (434), G-A (441), C-G (446), G-A (448), G-A (588), G-C (618)	LBCL	G-C (397)
LBCL	T-C (120), G-T (122), A-C (195), T-G (349), T-G (350), C-A (487), C-G (495), C-T (561), A-G (616), C-G (728), T-A (744)	LBCL	G-C (397) , C-G (438), A-G (696)
LBCL	INSERC C (36)	LBCL	WT
LBCL	G-C (397) , DEL T (520)	LBCL	G-C (397) , DEL T (520)
LBCL	A-G (106), A-T (116), G-C (122), A-G (208), C-T (348), A-G (445), G-C (448)	LBCL	G-C (122), DEL T (520)
LBCL	T-C (750)	LBCL	C-G (423)
LBCL	T-A (79)	LBCL	T-A (110), A-G (112), DEL C (164) , C-G (166), C-T (216), DUPL (383–413) , G-C (414), G-A (431), C-G (435), C-T (548)
LBCL	C-G (88), T-C (111), G-C (335), G-C (369), G-A (373), G-A (385), T-G (737)	LBCL	G-C (122)
LBCL	C-T (602)	LBCL	A-T (176), G-T (177), C-G (423), DEL T (520)
LBCL	WT	LBCL	WT
LBCL	WT	LBCL	DEL T (520)
LBCL	T-A (360), C-G (429), C-G (514), C-T (515), DEL T (520) , T-G (573), T-C (632)	LBCL	G-C (397) , DEL T (520)
LBCL	G-A (122), C-G (423), T-G (439), G-A (518), G-A (588)	LBCL	DEL T (520)
LBCL	WT	BL	T-C (43), T-G (110), G-C (122), C-T (123), T-G (133), T-G (182), T-C (194), T-C (266), G-C (398), C-A (432), G-A (464), G-A (502), DEL T (520)
LBCL	WT	BL	DEL T (520)
LBCL	WT	BL	T-C (87), G-A (398), A-C (401), C-T (495), T-C (517), G-A (736), INSERC T (755)
LBCL	G-A (443)	BL	DEL T (485) , C-A (514), DEL T (520)
LBCL	T-G (450), T-C (451)	BL	G-C (397)
LBCL	G-A (122), G-A (125), C-T (127), G-A (298), C-T (507)	BL	WT
LBCL	A-G (112), G-C (122), G-A (400)	BL	DEL T (520)
LBCL	A-G (615)	BL	WT
LBCL	DEL T (520) , T-C (582), G-A (643), A-G (727)	BL	A-G (90), A-G (112), A-T (191), A-G (196), G-C (397) , C-T (574)
LBCL	WT	BL	WT
LBCL	G-C (397) , C-G (493), C-A (432), G-A (598)	BL	DEL T (520)
LBCL	DEL T (520)	BL	WT
LBCL	C-G (54), T-C (201), C-A (251), G-C (397) , A-C (504), T-C (512), G-A (626), A-T (657)	BL	DEL T (520)
LBCL	DEL T (520)	BL	T-G (349), T-G (350), C-G (366), T-A (367), C-G (459)

Nucleotide I was arbitrarily assigned at the start of the MMC (position +356 of the gene), according to the previously published sequence (GenBank accession number AF191831). Polymorphisms are indicated in grey.

Figure 2. Distribution of MMC BCL-6 mutations per case. The number of mutations per case varied from 0 to 22.

Our series included 9 LBCL and 4 BL nonmutated cases (20% and 28%, respectively), 15 LBCL and 5 BL cases (33% and 36%, respectively) with one mutation, and 21 LBCL and 5 BL cases (47% and 36%, respectively) with more than two mutations (up to 22 sequence changes) (Figure 2). The mutations present in the series were mostly punctual substitutions. Two cases showed punctual deletions, two cases had a punctual insertion, and one case featured a long tandem duplication (31 bp) (See Table 2 for details). In agreement with previous studies,^{7,20} there were two polymorphisms in the MMC at positions 397 (C to G) and 520 (del T). Changes in these nucleotides were observed in 42% of LBCL cases and 57% of BL cases. Additionally, seven recurrent mutations were identified in more than one patient (at positions 88, 112, 349, 350, 423, 432, 588). Moreover, mutations to different nucleotides appeared at eight positions (110, 122, 125, 398, 448, 495, 514, 518) (recurrently mutated positions). These positions can thus be considered to be mutational hot spots. Hypothetical polymorphisms in these positions were excluded through the study of non-tumoral DNA for the changes in positions 88, 112, 423, 432, 448, 518, 588. Other possible polymorphisms had already been excluded by previous studies.^{7,8,10,20-23}

To compare the frequency of BCL-6 mutations in different types of aggressive B lymphomas, we analyzed the frequency of mutated cases and the number of mutations per case in each diagnostic group. There was a slight difference between LBCL and BL, because the frequency of mutated cases was higher in LBCL (80%) than in BL (71.4%). Nevertheless, when considering mutated cases alone, the mean number of mutations per case was very similar in both groups (3.78 mutations/case in LBCL, versus 3.90 mutations/case in BL) (Table 3).

Absence of Relation between Number of BCL-6 Mutations, Expression Level, and Clinical Outcome

Because of its location in the first intron of the BCL-6 gene, MMC could behave like a regulatory region, controlling the expression of BCL-6. This prompted us to search for a relation between number of mutations and protein expression. However, in our series of LBCL and

Table 3. Summary of Sequencing Results

	WT cases*	Mutated cases	Mean of number of mutations/case in mutated cases†	N
LBCL	9 (20.0%)	36 (80.0%)	3.78	36
BL	4 (28.6%)	10 (71.4%)	3.90	10
Total	13 (22.0%)	46 (78.0%)	3.80	46

*Number and percentage (in parentheses) of nonmutated and mutated cases in the different diagnostic groups.

†Mean of number of mutations/case considering only mutated cases.

BL no such relation between these two variables was found ($r = 0.127$, $P = 0.37$, Pearson correlation coefficient) (Figure 3).

We then examined whether either number of mutations per case or protein expression had any relationship with any clinical variable. There was no correlation between BCL-6 protein expression level and growth fraction, measured as MIB1 expression ($r = -0.005$, $P = 0.97$ for LBCL cases; $r = 0.32$, $P = 0.34$ for BL cases; Pearson correlation), or OS probability ($P = 0.72$ for LBCL cases and $P = 0.5$ for BL cases, log-rank test). Similar results were obtained comparing the number of mutations with MIB1 expression ($r = -0.12$, $P = 0.44$ for LBCL cases and $r = 0.10$, $P = 0.76$ for BL cases, Pearson correlation) or OS ($P = 0.06$ for LBCL cases and $P = 0.18$ for BL cases, log-rank test). This result was independent of the presence of polymorphisms.

Distribution of Mutations along the Sequence of MMC: A Clustering Effect

Sequences of regulatory regions are not absolutely functional in every base pair of their length. Usually, only small transcription factor-binding regions are important for their regulatory physiological effect. So we examined whether there were parts of the entire MMC where mutations were clustered or had an effect on BCL-6 expression. Distribution of mutations throughout the whole series is shown in Figure 4. In trying to identify small regions of potential functional relevance, we focused on the existence of

Figure 3. Absence of correlation between BCL-6 protein expression and number of mutations per case ($r = 0.127$, $P = 0.34$).

Figure 4. Distribution of mutations along the MMC sequence in the entire series. The **abscissa** represents the position along the MMC, the **ordinate** corresponds to the number of mutated cases at each position. **Black bars** indicate the number of cases mutated within the two clusters, defined by bases 106 to 127 and 423 to 443. Polymorphisms (located at positions 397 and 520) are indicated.

mutational clusters associated with changes in BCL-6 protein expression intensity. Thus, on the basis of the frequency of mutations and/or variations in expression level, we defined two clusters, spanning positions 106 to 127 and 423 to 443, that had a high mutation frequency (1.7×10^{-2} mutations/bp for the 106 to 127 cluster, and 1.3×10^{-2} mutations/bp for the 423 to 443 cluster, versus 3.7×10^{-3} mutations/bp for the entire MMC). The clustering distribution of BCL-6 mutations within MMC was statistically significant (chi-square = 58.69, d.f. = 1 for cluster 106 to 127 and chi-square = 25.10, d.f. = 1 for cluster 423 to 443, $P < 0.001$ for both clusters; Fisher's exact test). Three LBCL and one BL showed mutations in both clusters, six LBCL showed mutations in 423 to 443 cluster, and eight LBCL and one BL showed mutations in 106 to 127 cluster.

Tumors with mutations within the 106 to 127 cluster had variable BCL-6 expression (mean, 57.54%), whereas most of tumors with mutations in the 423 to 443 cluster showed a high expression level (mean, 75.38%). Thus, cases with mutations within the 423 to 443 cluster had a significantly higher probability of displaying high protein expression than cases with mutation outside of the cluster (chi-square = 6.23, d.f. = 1, $P = 0.013$, Kruskal-Wallis test). These findings prompted us to consider this cluster spanning positions 423 to 443 in further studies because the observed increase in the expression of the protein suggested a possible functional role for this region.

Because LBCLs and BLs are characterized by large differences in the molecular mechanisms and clinical outcome, we analyzed the two groups separately. The LBCL group yielded similar results to those obtained from the entire series. However, BLs showed no clustering in the BCL-6 mutation distribution, although the total number of BL cases considered was relatively small (Figure 5). Additionally, no significant relation was observed between the location of mutation and level of expression in BL.

Figure 5. Distribution of BCL-6 mutations in different diagnostic groups of the series. **Black bars** indicate the number of cases mutated within the two clusters, defined by the bases 106 to 127 and 423 to 443. Polymorphisms (located at positions 397 and 520) are indicated.

Relation between Mutational Clusters and Clinical Outcome

In the LBCL group, the Kaplan-Meier statistic obtained indicated that patients with mutations within the 423 to 443 cluster showed both an improved OS and DFS. Thus eight of nine LBCL patients with mutations within the 423 to 443 cluster were still alive (Figure 6A for OS) and in complete remission (Figure 6B for DFS) after a mean follow-up time of 110 months. This contrasts with the OS and DFS observed in the rest of the series ($P = 0.011$ for OS and $P = 0.022$ for DFS, log-rank test).

Additionally, a univariate Cox analysis confirmed that mutations in the 423 to 443 cluster were a very good predictor of greater OS in the LBCL group ($P = 0.038$, RR = 8.40). Univariate analysis also showed IPI to be a significant predictor of OS ($P = 0.035$, RR = 2.46) (Table 4).

In a multivariate analysis that included IPI and mutations within the 423 to 443 cluster, the presence of mutations in the 423 to 443 cluster (Table 4) was found to be significantly associated with a longer OS ($P < 0.05$). This effect was slightly greater than that of IPI. These relationships were absent in the BL group. Mutations within the 106 to 127 cluster had no significant effect on survival probability.

Figure 6. OS (A) and DFS (B) of LBCL patients by presence or absence of mutations in 423 to 443 cluster.

Discussion

The results of this study show that in LBCL, a significant proportion of mutations within the MMC are clustered into discrete regions spanning positions 106 to 127 and 423 to 443. LBCL cases with mutations within the 423 to 443 cluster are characterized by a higher OS probability. At the same time, mutations within this small mutational hot

spot are associated with an overexpression of the BCL-6 protein.

Besides the distribution of mutations in clusters, we found some mutations appearing in more than one case (recurrent mutations), and positions where mutations gave rise to different base changes in several cases (recurrently mutated positions), as described previously.²³ These positions are probably mutational hot spots, the possibility of them being polymorphisms having been excluded through the study of nontumoral DNA and/or in previous studies.^{7,8,10,20-23} Except for two cases, none of them coincided with the consensus sequence for mutations in IgH.

The functional relevance of mutations within an intronic region depends on its putative role as a regulatory region. This has been already demonstrated for the MMC in the BCL-6 gene, because it includes at least one region with a negative regulatory capacity.²⁴ Mutations and other structural abnormalities within these regulatory regions may play a role in the deregulation of the expression of the BCL-6 gene in lymphomas.³ This deregulatory effect of BCL-6 mutations would be critically dependent on the precise location of the mutations, those involving small regions bound by transcription factors being of potential importance. This could explain why, in this and in previous studies, the overall frequency of BCL-6 mutations does not predict the clinical behavior or BCL-6 expression level. These variables may depend more on the precise location of these mutations.⁹ This consideration prompted us to analyze the relevance of specific redundant mutations or mutations involving discrete mutational hotspots, termed subclusters, within the MMC. Thus, we have focused on regions with an increased frequency of mutations and/or associated with marked alterations in the protein expression. We identified two regions, one defined by nucleotides 106 to 127 and another by nucleotides 423 to 443. Both regions contained a significantly higher proportion of mutations than in the MMC overall (five times greater than expected under a null hypothesis of random distribution; $P < 0.001$). These clusters have not been described in previous LBCL studies, but the analysis of the data provided by those authors seems to show similar clustering phenomena.^{9,23}

Although mutations within the first subcluster were not associated with variations in the level of BCL-6 protein expression, cases with mutations inside the second sub-

Table 4. Cox Analysis of Overall Survival

	RL	RR	95% CI	<i>p</i>
A. Univariate Cox Analysis				
423-443 CLUSTER	Mutations within the cluster	8.4	1.13-62.58	0.038
IPI	Low risk	2.5	1.07-5.69	0.035
B. Multivariate Cox Analysis				
423-443 CLUSTER	Mutations within the cluster	7.5	1.00-56.49	0.049
IPI	Low risk	2.2	0.96-5.11	0.064

A, OS by univariate Cox's regression model in LBCL cases. Upper row shows the results for mutations in the 423 to 443 cluster. Lower row shows the results for IPI. B, Multivariate analysis including only the factors that influence OS in the LBCL group (i.e., 423 to 443 cluster and IPI). RL: reference level; RR: relative risk; CI: confidence interval.

cluster had a higher level of BCL-6 protein expression than did the entire series ($P = 0.005$) that strongly suggests that they involve a region of importance in the regulation of BCL-6 protein expression. Additional arguments in favor of the relevance of this region in the BCL-6 regulation were provided by a logistic regression analysis showing that mutations in nucleotides 423 and 443, considered separately, tend to be associated with a high level of BCL-6 protein expression (data not shown). This clustering effect was observed in the LBCL group, whereas BL cases displayed no clear tendency toward clustering despite the presence of mutations in the MMC.

The regions identified here as being potentially involved in BCL-6 regulation do not coincide with those recently described by Kikuchi and co-workers,²⁴ which could indicate the existence of different multiple regulatory regions within the BCL-6 MMC, identifiable through the use of alternative experimental approaches.

The importance of mutations within this cluster is further emphasized by the findings concerning clinical outcome in these patients. Thus, LBCL patients with mutations within this 423 to 443 subcluster had an increased OS probability compared with the remainder who did not have such mutations. Although this conclusion should be regarded with caution because of the small size of the series, the effect was strikingly absent in BL. Cases with mutations within the 106 to 127 subcluster showed no significant variations either in the level of expression of the protein or the clinical outcome.

These findings enable us to formulate a model in which mutations and deregulation of the level of expression of BCL-6 play a role in lymphoma genesis and/or progression. The relevance of the role of the BCL-6 gene is supported by recent data concerning BCL-6 down-regulated genes. Thus, the BCL-6 gene, described as a multifunctional regulator of lymphocyte differentiation and immune responses, has also been demonstrated to repress key genes in cell-cycle control, such as $p27^{KIP1}$, *blimp-1* (a repressor of *c-myc*), or cyclin D2,⁶ which could easily explain the clinical significance of BCL-6 mutations or alterations in the expression.

Alizadeh and co-workers,¹¹ have shown that LBCL patients with germinal center profile expression (and hence BCL-6 positivity) had greater survival probability compared with LBCL patients with activated B-cell profile. Recently, the role of the expression of BCL-6 in survival has been clarified by Lossos and colleagues,¹² showing that the high level of expression of this marker is associated with better clinical outcome. Our findings indicate a more subtle relationship between BCL-6 expression and clinical course, whereby only a high level of expression associated with mutations within the 423 to 443 cluster seems to be significantly related to the clinical course.

The physiological basis of this relationship between improved survival, increased BCL-6 expression, and clustering of BCL-6 mutations needs further investigation. Nevertheless, some recently published results show a relation between BCL-6 overexpression and increased sensitivity to apoptosis. Albagli and co-workers²⁵ transfecting BCL-6 protein into SAOS2 cells observed cell-

cycle arrest and an increased apoptotic rate. Moreover, a recent article, revealed that BCL-6, probably through the repression of *blimp-1*, and subsequent activation of *c-myc*, could also trigger apoptosis, a known effect of *c-myc* activation.⁶

Strikingly, in the BL cases analyzed here, the percentage of mutated cases was lower than in LBCL cases, although the mutation frequency was similar in both lymphoma types. Additionally, in this group, mutations within the MMC were not clearly clustered within the 107 to 126 and 423 to 443 subclusters, and neither were mutations within these regions associated with variations in the clinical course or the level of protein expression. Thus, it seems that the role of BCL-6 mutations in lymphoma is specifically dependent on histological type, probably reflecting the distinct molecular pathogenesis of each main lymphoma entity.

These results highlight the need for a more comprehensive analysis of the role of BCL-6 regulation in lymphoma genesis and progression, through the identification of putative transcription factors binding in the 423 to 443 region, and the analysis of the consequences of their binding for cell-cycle control, differentiation, and apoptosis.

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