Malignant Lymphomas • Research Paper

Primary mediastinal B-cell lymphoma: hypermutation of the *BCL6* gene targets motifs different from those in diffuse large B-cell and follicular lymphomas

[haematologica]
2004;89:1091-1099

Giorgio Malpeli
Stefano Barbi
Patrick S. Moore
Maria Scardoni
Marco Chilosi
Aldo Scarpa
Fabio Menestrina

A B S T R A C T

Background and Objectives. Somatic hypermutation of the *BCL6* gene and its expression in lymphoma represent specific markers for B-cell transit through the germinal center. Thus, analysis of *BCL6* may aid in clarifying the relationship between primary mediastinal B-cell lymphoma (PMBL) and other non-thymic diffuse large cell lymphomas (DLCL).

Design and Methods. Twenty-four PMBL were analyzed for *BCL6* status, including first intron mutations, by quantitative reverse transcription polymerase chain reaction (RT-PCR), and immunohistochemistry. We also performed a meta-analysis of reported *BCL6* mutations in PMBL (n=141), DLCL (n=233), and follicular lymphoma (n=120).

Results. Thirteen PMBL (54%) showed hypermutation of *BCL6*. All cases showed bcl6 mRNA and immunohistochemical expression. Meta-analysis demonstrated that the preferentially altered sequence motifs of *BCL6* in PMBL were TA (p=0.002) and AT (p=0.0008) dinucleotides and TAT trinucleotides (p=0.001). GC and RGYW/WRCY motifs were a target in DLCL and FL but not in PMBL. Moreover, the DNA stretch spanning nucleotides 150-270 was highly targeted only in PMBL.

Interpretation and Conclusions. The consistent expression of bcl6 protein and occurrence of hypermutation indicate that PMBL should be considered of germinal center origin. The fact that the hypermutation sites and mutational spectrum of *BCL6* in PMBL differ from those found in FL and DLCL might suggest that the maturation block of the transforming cells differs among these tumor types, and that the characteristic mutational pattern is present before neoplastic transformation. Thus, our findings strengthen the hypothesis that PMBL originate from an already defined sub-population of B-cells, which are different from those leading to either DLCL or FL.

Key words: mediastinal lymphoma, thymic lymphoma, *BCL6*, hypermutation.

From the Dipartimento di Patologia, Sezione di Anatomia Patologica, Università di Verona, Verona, Italy.

Correspondence: Aldo Scarpa, M.D. Dipartimento di Patologia, Sezione di Anatomia Patologica Università di Verona, Strada Le Grazie I-37134 Verona, Italy. E-mail: aldo.scarpa@univr.it

Primary mediastinal B-cell lymphoma (PMBL) is a clinical entity distinct from diffuse large B-cell lymphoma (DLCL).[1] Available evidence suggests that PMBL originates from thymic medullary B cells,[2,3] and recent analysis of gene expression by microarrays has suggested a connection between PMBL and Hodgkin's lymphoma rather than with DLCL.[4,5] However, the exact relationship between PMBL and DLCL remains unclear, and relatively little, from a molecular standpoint, is known about PBML with respect to other types of lymphoma.[4,6-9] The expression of *BCL6* gene has been recently identified as a marker for DLCL of germinal center origin and has been associated with a favorable clinical outcome.[10,11] This gene has also been shown to be a target of somatic hypermutation in a large proportion of DLCL.[12-14] The *BCL6* gene, like the immunoglobulin genes (*Ig*), accumulates mutations in B cells in the germinal center.[15,16] As a consequence, normal post-germinal center (post-GC) B cells and about 40% of memory B cells contain mutations in *BCL6*, while naive B and pre-GC cells contain wild-type genes.[16,17] Somatic hypermutation of *BCL6* primarily involves a 1 kb sequence containing part of exon 1 and the first intron.[15,16,18]

As the mutational frequency in *BCL6* gene of DLCL is higher than in normal GC cells,[16,17,19] it has been suggested that these mutations may play a causative role in cellular transformation.[16,18] Indeed, some mutations affecting putative control sequences in untranslated exon 1 have been shown to up-regulate *BCL6* mRNA, although these mutations are infrequent in lymphomas.[20] In a recent report, mutations in specific clusters of the first intron of *BCL6* were associated with higher mRNA expression and a more

favorable prognosis.[21] However, as pointed out by other authors,[22] many of the somatic mutations in *BCL6* may be irrelevant and probably have no functional consequences on the expression of the *BCL6* gene.

In PMBL, available immunohistochemical data has shown that bcl6 is expressed in the vast majority of cases,[9,23] while somatic mutations in the *BCL6* gene have been reported to occur in up to 60% of cases.[24,9,25] The analysis of the somatic mutations of *BCL6* may help to clarify whether or not the tumor has a germinal center origin or arises from other cells. These studies may be complemented by comparison with DLCL and follicular lymphoma (FL), further aiding in clarifying the relationship between PMBL and other non-thymic diffuse large B-cell lymphomas.

In the present study, we investigated the status of the BCL6 gene in 24 cases of PMBL by mutational analysis, immunohistochemistry and quantitative RT-PCR. We also performed a meta-analysis of the distribution of mutations in *BCL6* from PMBL, DLCL, and FL. We found that the preferentially altered sequence motifs were different in PMBL from those in DLCL and FL. The patterns of mutation at specific motifs suggest that cells giving rise to PMBL different from those in DLCL and FL.

Design and Methods

Tumor samples

Twenty-four frozen PMBL samples from untreated patients from the archives of the Department of Pathology at the Verona University Hospital were analyzed. The diagnosis of PMBL was established by standard clinical and histopathologic criteria as well as by cell marker analysis.[26-29] In all cases, immunohistochemical analysis confirmed the lymphoid B-cell nature of the neoplastic cells, which expressed panleukocyte antigen CD45, and several B-cell related markers, including CD20, CD79a, and CD22. Surface Ig were not detected in any case.

Single-strand conformation polymorphism analysis and DNA sequencing

The intronic region of *BCL6* prone to somatic mutations (bp 1 to 620 in GenBank sequence AF191831) was amplified by polymerase chain reaction (PCR) with four different pairs of overlapping primer sets. The primer pairs were: *BCL6*-Af 5'-CCGCTGCTCATGATCATTATTT and *BCL6*-Ar 5'-ACAATCTATATCCTATGGTGGG; *BCL6*-Bf 5'-ATAAATGCCGAAGATTAGTCCC and BCL6-Br 5'-CACCTC-CTTTCCAAAAACCAAA; *BCL6*-Cf 5'-AGGCTTTTGCCACC-CTCCCTTGT and *BCL6*-Cr 5'-AGAGAGATCACAAGCCG-TACGC; *BCL6*-Df 5'-TCTCGCTCTTTCTGCTGCTGCT and *BCL6*-Dr 5'-CCCTTTTTGCCTCCCGGAGTTA. PCR reactions contained 10mM Tris, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 25 μM dNTP, 0.5 mM each primer, 1 U of AmpliTaq Polymerase (Taq Gold, Perkin-Elmer, Milan, Italy), 20 ng of genomic DNA and 1 μCi α^{32}-dCTP 3000 Ci/mmol (Amersham Life Sciences, UK) in a final volume of 10 μL. The conditions used for all primer pairs were 94°C, 3 min; 30 cycles of 94°C for 30 sec, 57°C for 30 sec, 72°C for 45 sec. Samples were diluted to 40 mL with buffer containing 95% formamide, 20 mM EDTA, 10 mM NaOH, 0.05% bromophenol blue and 0.05% xylene cyanol. After denaturation at 96°C for 5 min, samples were chilled on ice and 2 μL were then loaded on a 6% acrylamide/TBE gel. Gels were run with 0.5X TBE buffer at 30W for two hours at room temperature, vacuum dried on 3M paper, and analyzed by autoradiography. Bands exhibiting aberrant migration were excised from the gel, resuspended in 25 mL of water, and incubated at 80°C for 20 min. Each sample was then reamplified with the same primers and analyzed by direct DNA sequence analysis on an ABI Prism 377 instrument.

Quantitative RT-PCR analysis of mRNA expression

Using the guanidinum-isothiocyanate method total RNA was purifed from tumor samples and three preparations of germinal center B cells isolated from tonsillar tissues of three individuals, as reported elsewhere.[30] cDNA was synthesized with the First Strand cDNA Synthesis Kit (AMV) (Roche Diagnostic, Mannheim, Germany). Real-time analysis was performed on an ABI Prism 7000 SDS (Applied Biosystems, Foster City, CA, USA) using the SYBR Green PCR Master Mix (Applied Biosystems). Oligonucleotide primers were: *BCL6*, GGACACCAGGTTTTGAGCA and CCGGAGACGATTAAG-GTTGA; glyceraldehyde-3-phosphate dehydrogenase (*GAPD*) ATCATCAGCAATGCCTCCT and GGACTGTGGT-CATGAGTCCT. The expression levels of *BCL6* were calculated by relative quantification using *GAPD* transcript levels for normalization. Data were analyzed as indicated in the User Bulletin #2 (Applied Biosystems). Real-time PCR reactions included 320 nM of each primer and 10 ng of RNA of the samples in triplicate. Five successive five-fold dilutions of cDNA (from 50 to 0.08 ng) were run in triplicate for the calibration curve of *BCL6* and *GAPD* and the control with no template.

Immunohistochemical staining and antibodies

Tissue sections (4-5 μm thick) were mounted on adhesive-treated glass-slides. After removal of paraffin, slides were re-hydrated, treated for antigen retrieval (in 0.01M citrate buffer, pH 6, boiled in a microwave oven for 5 min at 750 W three times, and 10 min at 600 W once), and then kept for 15 min at room temperature before further PBS washing.

Immunostaining was performed using a sensitive avidin-streptavidin-peroxidase technique (Biogenex San Ramon, CA, USA). All samples were processed using a semi-automated cell staining system (GenoMx i6000, BioGenex, S.Ramon, CA, USA) and standardized procedures. Consecutive sections of all cases of PMBL and controls of our series were analyzed with an antibody panel for characterizing PMBL. Antibodies included anti-cytokeratin 5 (CK5, clone XM26, Novocastra, Newcastle, UK), anti-CD3 (clone PS1, Novocastra), anti-terminal deoxynucleotidyl transferase (rabbit antibody, Dako, Glostrup, Denmark), anti-CD20 (clone L26, Dako), and two anti-bcl6 specific antibodies (Clone PG-B6p, Dako; Bcl6 (N-3), Santa Cruz Biotech, CA, USA).

Statistical analysis

The frequency of mutations in each class of dinucleotides and trinucleotides and RGYW/WRCY motifs was normalized on the basis of the base composition of the wild-type *BCL6* gene sequence (bases 1-620 in GenBank AF191831). The number of expected mutations was obtained by multiplying the frequency of occurrence of each motif in the normal sequence by the total number of observed mutations. For the analysis of dinucleotides, each mutation was counted twice as it may be part of two dinucleotide sequences formed by the mutated base and the adjacent nucleotides. Accordingly, each mutation was counted three times for the analysis of trinucleotides. Comparisons between observed and expected mutations at di- and trinucleotide motifs were performed using a Poisson distribution for rare events as the null distribution.

Literature data sources for meta-analysis of BCL6 mutations

A meta-analysis of literature data on *BCL6* first intron mutations in PMBL, FL and DLCL was performed to assess the presence of mutation biases for specific motifs. The two latter types of lymphomas are in fact those for which there was a sufficiently large number of reported mutations to permit comparison,[21,24, 31-36] while reports of only a few mutations were available for chronic lymphocytic leukemia, Burkitt's lymphoma, mantle B-cell lymphoma, Hodgkin's disease and multiple myeloma. Thus, the data included in our meta-analysis were 141 base substitutions for PMBL (64 in our series and 78 in the series studied by Pileri *et al.*,[25] 233 mutations reported in the two largest series of DLCL analyzed,[21, 24] and 120 mutations in FL (25 from our unpublished data and 95 mutations from literature data).[24,31-36] The meta-analysis was limited to the stretch spanning nucleotides 49-620, this being the longest sequence common to all the data sets.

Results

Relevant clinicopathologic data of the 24 PMBL cases and the results of the analysis of *BCL6* are summarized in Table 1, which also contains previously reported molecular data.[37] All cases showed nuclear immunostaining for bcl6 with from 20 to 80% of the cells being positive.

Mutational screening of BCL6 by PCR-SSCP and DNA sequence analysis

The 24 PMBL from untreated patients were analyzed for mutations in the *BCL6* gene in an intronic region spanning 620 bp using four overlapping fragments by PCR-SSCP. This region was chosen as it contains >90% of the somatic mutations identified to date.[18] Typical results are shown in Figure 1. Eighteen cases showed aberrantly migrating bands in at least one of the four fragments amplified. Each sample was independently analyzed twice with identical results. The abnormally migrating SSCP bands were reamplified and sequenced. Five of the 18 cases only showed sequence variants corresponding to known polymorphisms,[15,16,32] namely, 397 G-C (cases 1 and 32) and deletion 520delT (cases 10, 18 and 24). In the remaining 13 PMBL, a total of 68 sequence alterations were observed. The vast majority of mutations 64/68 (94%) were single base substitutions. The remaining 4 mutations (6%) consisted in 3 deletions and 1 insertion (cases #4, 7, 11 and 25). The individual sequence variants found are listed in Table 2. The number of mutations per case ranged from 1 to 11, with an overall mutation frequency of 2.3×10^{-3}/bp (range 0.08-9×10^{-3}/bp/case), a value that compares favorably with previous reports for *BCL6* somatic mutations in PMBL[25] and in other lymphoma types.[16,17] A total of 59 different positions were affected by substitutions in the 620 bp analyzed.

Expression of BCL6 mRNA is not related to protein expression or mutations at regulatory sites

RNA was available from 11 of the 24 PMBL and the relative expression levels of *BCL6* mRNA were determined by real-time PCR. These 11 cases included 4 with a wild type sequence (cases #5, 18, 31 and 32) and 7 with mutations (cases #6, 7, 17, 19, 21, 25 and 26). As reported in Table 3, the expression level of *BCL6* varied from 0.6 to 4 times with respect to the average expression level in germinal center B cells from tonsillar tissue. For comparison, the protein expression of bcl6 as assessed by immunohistochemistry is indicated in addition to the presence of mutations in the regions spanning residues 97 to 232 and 158 to 293. These latter are considered regulatory regions which have been suggested to contain a silencer element.[35, 38] Notably, there was no appar-

Table 1. Clinical and molecular features of the 24 primary mediastinal B-cell lymphomas.*

			Ig gene							*bcl-6*	
Patient	*Age*	*Sex*	*JH*	*K*	*EBV*	*p53*	*p16*	*c-myc*	*c-myb*	*translocation*	*#mutations°*
1	30	F	R	R	–	–	methyl	–	G	–	-
2	30	F	G	R	–	–	–	–	G	+	-
3	25	F	R	G	–	–	–	–	nd	–	-
4	25	F	R	R	–	I255F	–	exon 1	G	–	11
5	41	F	R	R	–	–	–	exon 1	nd	–	-
6	27	M	R	R	–	–	–	exon 1	G	–	1
7	52	F	R	R	–	–	–	promoter	G,R	–	10
10	30	F	R	R	–	–	–	–	G	–	-
11	67	M	R	R	–	Y220L	methyl	–	G	–	9
12	26	F	R	R	–	–	–	–	G	–	1
15	37	F	R	R	–	R273L	–	exon 1	G	–	4
17	28	M	R	G	–	–	–	–	G	–	5
18	29	F	R	G	–	–	–	promoter	G	–	-
19	26	F	R	R	+	–	–	–	G	–	3
20	27	M	R	R	–	–	–	–	nd	–	-
21	36	F	R	R	+	–	–	–	G	–	1
22	29	M	R	R	–	–	–	–	nd	–	7
23	20	M	R	R	–	–	–	–	G	–	-
24	32	F	R	G	–	–	intronic	exon 1	G	–	-
25	29	M	R	R	–	–	–	–	G	–	6
26	35	F	R	R	–	–	A148T	–	nd	–	4
28	30	F	R	G	–	–	–	exon 2-3	R	–	6
31	30	M	R	R	–	–	–	–	nd	–	-
32	33	F	R	R	–	nd	nd		exon 1	nd	- -

**R, rearranged; G, germline; +, positive; -, negative; nd, not done; methyl, methylated 5' CpG island; °Mutations include single substitutions, deletions and insertions.*

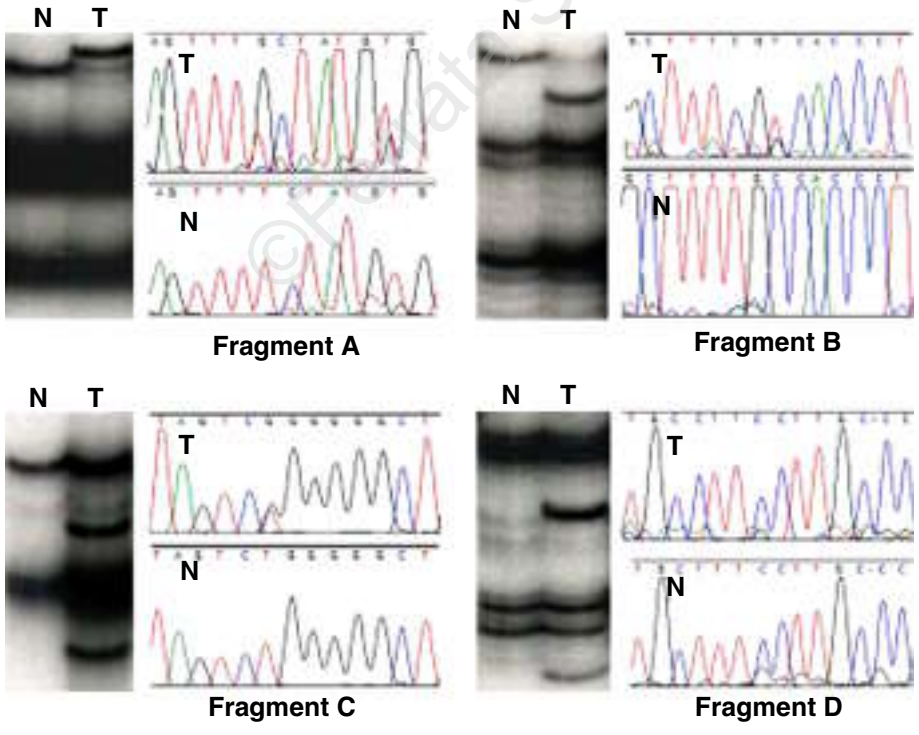

Figure 1. Representative results of PCR-SSCP analysis of the *BCL6* 5' non-coding sequence in PMBL. As an example, the patterns of migration of mutated PCR-amplified fragments BCL6 A, B, C and D are shown (M). For comparison, the migration of the corresponding wild type sequence is also shown (N).

Table 2. Mutations in the 5' noncoding region of *BCL6* in 13 PMBL.

Case	*Substitutions*	*Deletions*	*Insertions*
4	T23C, T59A, T77G, A86G, C19G, T210G, C507G, G509A, C510A, T511A		264insT
6	G518C		
7	T249C, C251T, T372G, C411A, A445T, G454A, T511C, T550C, C568A	466-470 TTCGT (520delT)	
11	A96G, A112C, A121T, G122C, G184T, A208T, T246, T267G	448-455 GCTTGTGA	
12	G369C, (G397C)		
15	A191G, G274C, G292C, C423G	(520delT)	
17	A195G, G211T, G222A, G224A, C250A		
19	C461G, G464A, T480G	(520delT)	
21	T480G	(520delT)	
22	T175C, T198C, A202G, T204G, G244T, C251T, C256G		
25	C54G, T152G, C164G, T372C, C378A		94insG
26	T189G, A205T, G230C, T248G		
28	T279C, (G397C), T418A, C425G, T426C, C435G, T541C		

The sequence variants in parentheses are known polymorphisms.

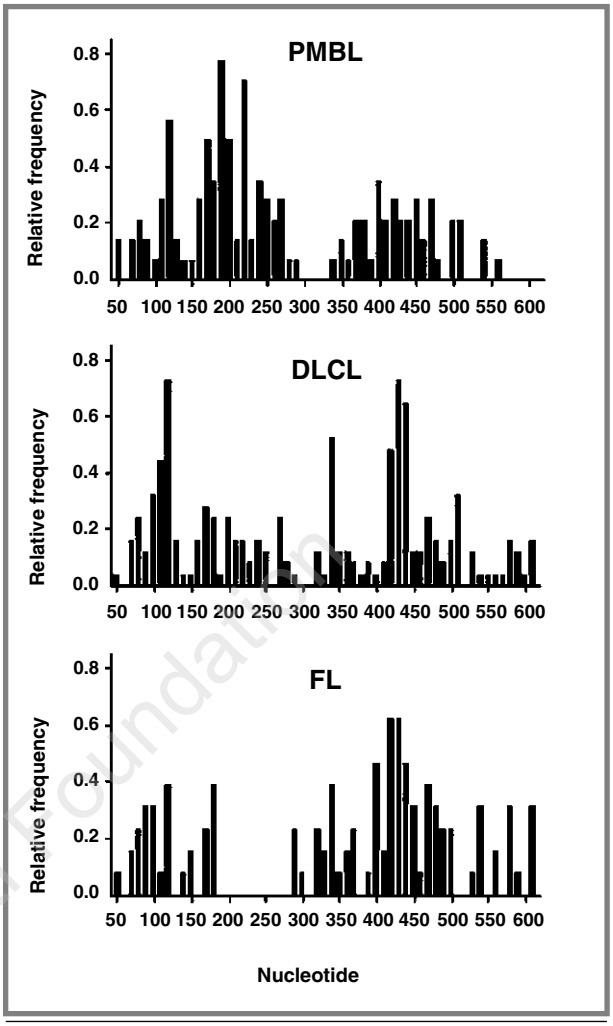

Figure 2. Spatial distribution of *BCL6* mutations within the 5' non-coding region in DLCL, FL and PMBL. Only the region common to each set (49-620) is considered. Each bar corresponds to the frequency of mutations observed in an interval of ten nucleotides divided by the total number of mutations observed for each type of lymphoma.

Table 3. Relative mRNA expression of *BCL6* in 11 PMBL assessed by real-time quantitative RT-PCR.

1	*2*	*3*	*4*	*5*	*6*	*7*
GC	nd	1	nd	nd	nd	–
5	wt	0.93	50	0	0	–
6	m	2.32	80	0	0	–
7	m	4.00	80	0	2	del520T
17	m	1.30	50	4	5	–
18	wt	3.38	70	0	0	del520T
19	m	2.86	40	0	0	del520T
21	m	0.61	50	0	0	del520T
25	m	2.36	30	2	1	–
26	m	3.69	50	3	4	–
31	wt	2.93	40	0	0	–
32	wt	1.60	40	0	0	397 C-G
Mean value		2.36				

1:case number; 2: Status; 3:mRNA expression[a]; 4:% Protein expression[b]; 5: mutations 97-232[c]; 6: mutations 158-293[d]; 7: polymorphism; a:BCL6 mRNA expression relative to germinal center B-cells (GC) normalized with GAPD; b:% of positive cells; c: according to Lossos IS et al.; d: according to Kikuchi M et al.; nd: not determined; m: mutated; wt: wild type.

ent correlation between mutations at these regulatory sites and the expression of mRNA for *BCL6*.

Distribution and spectrum of BCL6 mutations in 61 PMBL

Considering the 24 cases in the present manuscript and the 37 cases reported by Pileri *et al.*,[25] a total of 61 PMBL have been analyzed for mutations in *BCL6*, and of these 64% were found to have alterations. The vast majority (>95%) of mutations detected in the first intron of *BCL6* gene were single base substitutions. In PMBL, a total of 141 base substitutions have been found in the region of *BCL6* spanning nucleotides 49-620. These include the 63 described herein and the 78 reported by Pileri *et al.*[25] In order to determine whether the hypermutations targeting *BCL6* in PMBL exhibited either site or sequence-specific preferences, we analyzed the distribution and spectrum of these 141 base substitu-

Table 4. Frequency of mutations of dinucleotides in PMBL, DLBCL and FL.

	PMBL (n = 141)				DLBCL (n = 233)				FL (n = 120)			
	Obs	Exp	R*	P	Obs	Exp	R*	P	Obs	Exp	R*	P
AA	3	11.4	0.26		11	18.0	0.61		5	9.3	0.54	
AC	2	4.9	0.40		12	8.3	1.45		1	4.3	0.23	
AG	16	14.3	1.12		25	21.8	1.15		17	11.2	1.51	
AT	29	14.8	1.96	0.0008	27	26.3	1.03		11	13.5	0.81	
CA	0	5.9	0.00		4	10.5	0.38		6	5.4	1.11	
CC	12	19.8	0.61		26	33.1	0.79		11	17.0	0.65	
CG	14	12.8	1.09		8	21.0	0.38		9	10.8	0.83	
CT	28	29.1	0.96		65	47.4	1.37		30	24.4	1.23	
GA	15	14.8	1.01		7	23.3	0.30	0.003	8	12.0	0.67	
GC	26	18.8	1.39		67	30.8	2.17	10^{-8}	32	15.9	2.02	0.0003
GG	18	25.7	0.70		28	39.1	0.72		19	20.1	0.94	
GT	21	15.8	1.33		26	24.8	1.05		12	12.8	0.94	
TA	26	13.3	1.95	0.002	33	22.5	1.46		10	11.6	0.86	
TC	16	23.7	0.67		29	39.1	0.74		19	20.1	0.94	
TG	25	22.7	1.10		36	36.1	1.00		16	18.6	0.86	
TT	31	34.1	0.91		62	63.9	0.97		34	32.9	1.03	

**Ratio between observed and expected events. Only p values less than 0.01 are shown.*

Table 5. Frequency of mutations of specific trinucleotides in PMBL, DLCL and FL.

	PMBL (n = 141)				DLCL (n = 233)				FL (n = 120)			
	Obs	Exp	R*	P	Obs	Exp	R	P	Obs	Exp	R	P
ATT	20	10.4	1.93	0.005	18	19.2	0.94		8	9.9	0.81	
AGC	5	1.5	3.37		17	2.3	7.52	10^{-10}	8	1.2	6.87	3×10^{-5}
TTA	17	7.4	2.29	0.002	18	14.7	1.22		5	7.6	0.66	
TAT	24	11.9	2.02	0.001	29	19.2	1.51		9	9.9	0.91	
TAG	12	4.5	2.70	0.002	15	6.8	2.21	0.004	6	3.5	1.72	
TCC	2	13.4	0.15	0.006	13	21.5	0.60		8	11.1	0.72	
TGC	13	9.6	1.35		33	15.8	2.08	0.0001	11	8.2	1.35	
CTA	5	3.0	1.68		13	4.5	2.87	0.0008	2	2.3	0.86	
CTG	13	9.6	1.35		35	15.8	2.21	2x10-5	17	8.2	2.08	0.004
GTG	8	2.2	3.59	0.002	7	11.3	0.62		7	5.8	1.20	
GCA	14	14.8	0.94		4	3.4	1.18		8	1.7	4.58	0.0004
GCT	14	5.9	2.36	0.003	67	26.0	2.58	10-11	30	13.4	2.24	6×10^{-5}

**Ratio between observed and expected events. Only p values less than 0.01 are shown.*

tions (Figure 2). Mutations were clustered into two broad regions separated by a relatively unaffected stretch approximately located between nucleotides 290 and 340, which is characterized by a minor sequence complexity rich in G nucleotides. Transitions were more frequent than expected, with a ratio of transitions over transversions of 0.72, which is significantly different from the expected ratio of 0.5 (χ^2 test, p=0.02).

As somatic mutations have been shown to be associated with specific microsequences,[39,40] we analyzed the frequency of observed mutations in each of the possible combinations of dinucleotides and trinucleotides, as well as at RGYW motifs. Mutations of *BCL6* in PMBL were not randomly distributed in either dinucleotide or trinucleotide sequences. The data are detailed in Tables 4 and 5, in which all dinucleotides and the statistically significant biased trinucleotides are listed, respectively. Among dinucleotides showing a frequency of mutations higher than expected there was the AT motif (p=0.0008) and its complementary motif TA (p=0.002), with the A nucleotide within these motifs being the prevalent mutated base (69% and 67%, respectively). The dinucleotides AA (p=0.04) and CA (p=0.05) had a lower frequency of mutations than expected. Some of the 64 possible trinucleotides were mutated with a frequency that was significantly higher than expected and included TAT (p=0.001), TTA, GTG, TAG, and GCT. Moreover, the TAT trinucleotide was more frequently mutated at the A site (χ^2 test, p=0.002), in agreement with what was observed in the AT and TA dinucleotides. The

trinucleotide sequences that were under-represented included TCC and CCT. Thirty-one of the 141 (22%) single base substitutions were found at RGYW sequences and their complementary WRCY quadruplet motifs. The mutation bias for RGYW/WRCY motifs was not statistically significant as assessed by the χ^2 test.

Site and spectrum of BCL6 mutations in PMBL differ from those in DLCL and FL

To determine whether the hypermutation targeting *BCL6* exhibited site or sequence specific preferences with respect to other types of lymphoma, the distribution and spectrum of nucleotide substitutions in PMBL were compared to those in DLCL and FL. The distribution of substitutions occurring in the first intron of *BCL6* is shown in Figure 2. Three regions contained the vast majority of mutations. The nucleotide stretches from 50-130 and 350-500 appear to be mutational hotspots in common among PMBL, DLCL and FL, while the stretch spanning nucleotides 150-270 is highly targeted only in PMBL. In particular, the stretch 350-500 includes 38%, 35%, and 46% of the mutations in PMBL, DLCL and FL, respectively; while the stretch 150-270 contains 43%, 18% and 9% of mutations in PMBL, DLCL and FL, respectively.

The frequency of mutation at each dinucleotide in PMBL, DLCL and FL is reported in Table 4. In each of the three lymphoma types, the number of mutations at dinucleotides was significantly different from expected (χ^2 test; $p = 5\times10^{-5}$ in PMBL, $p=10^{-12}$ in DLCL, $p=0.01$ in FL). There was also a significant difference between PMBL and DLCL (χ^2 test, $p=0.002$) and between PMBL and FL (χ^2 test, $p=0.05$), while there was no significant difference between DLCL and FL. In particular, a major contributor to the difference was the AT dinucleotide, which was the most significantly affected dinucleotide in PMBL but not a significant mutational target in either DLCL or FL. Another difference between PMBL and DLCL or FL resides in the GC dinucleotide, which is mutated at a frequency close to the expected in PMBL, while in DLCL and FL the GC dinucleotide is mutated significantly more frequently than would be expected.

The analysis of trinucleotides showed that several motifs were significantly targeted with frequencies higher or lower than those expected in the three types of lymphoma. Table 5 lists the trinucleotides with significant biases in the three different types of lymphoma. The AGC motif and its inverted complement GCT were among the most frequently mutated motifs in DLCL and FL. Conversely, the trinucleotides ATT, TTA, TAT and GTG represented specific mutation hotspots for PMBL. Within significantly affected motifs containing A and T, mutations in A occurred more frequently than those in T. The RGYW/WRCY motifs are a mutation target in the *BCL6* sequence under examination of DLCL and FL.

In fact, 80 of the 233 (34.3%) single base substitutions in DLCL and 31 of the 120 (25.8%) single base substitutions in FL were located at RGYW sequences and their complement WRCY quadruplet motifs. The mutation bias for RGYW/WRCY motifs was statistically significant in both DLCL (χ^2 test, $p=10^{-13}$) and FL (χ^2 test, $p= 0.008$). Although these motifs did not represent a significant target in PMBL, there was no statistically significant difference in RGYW targeting between PMBL and DLCL or FL.

Discussion

In the present study, we demonstrated that (i) 54% of PMBL possessed mutations in the intronic region of *BCL6* prone to hypermutation; (ii) there was no correlation between the expression of *BCL6* mRNA or protein and the presence and site of mutations; (iii) the mutational spectrum in PMBL is different from that found in either DLCL or FL; (iv) these mutations exhibited different spatial distributions among PMBL, DLCL and FL. Taken together, these data suggest the singularity of PMBL, in terms of both mutational hotspots and nucleotide bias.

Initially, PMBL was considered anomalous among various DLCL subgroups because of the low mutational frequency of the *BCL6* gene.[24] However in subsequent studies, the frequency of *BCL6* mutations was found to be similar to that observed in DLCL.[9,25] In our series, somatic mutations in *BCL6* were present in 13 of 24 cases (54%), in agreement with the range observed in other PMBL series.[9,25] The frequency of mutation in our PMBL was $0.08\text{-}9\times10^{-3}$/bp/case, with an average of 2.3×10^{-3}/bp, and the vast majority of alterations were single base substitutions while deletions/insertions represented only 5.9% of mutations. These data are not dissimilar to those collected in other types of lymphomas.

The expression levels of *BCL6* mRNA have been previously determined in various non-Hodgkin's lymphomas,[41] in particular in FL[35,41] and DLCL,[10,21,35,41] but not in PMBL. Relative to germinal center B cells, the mean value of the expression of the *BCL6* transcript was 2.4, similar to that found in DLCL (2.5) but significantly lower than that determined in FL (9.1).[41] Although previous studies suggested that the occurrence of mutations in specific stretches of the intronic *BCL6* sequence was related to higher levels of *BCL6* mRNA and protein expression,[32,38] we found no correlation between the expression level and the presence of mutations in these purported autoregulatory control regions.

Our data showed that the dinucleotide motifs AT and TA were mutational targets in PMBL, while these motifs are mutated at the expected frequency in DLCL and FL.

Thus, alterations of AT and TA motifs are a feature of *BCL6* hypermutation in PMBL. This was also confirmed by the analysis of trinucleotides. In fact, most of the trinucleotide motifs containing AT and TA dinucleotides were mutated significantly more frequently than expected in PMBL, but not in DLCL and FL. On the other hand, the GC dinucleotide motif is a target in DLCL and FL but not in PMBL. These findings suggest the existence of differences in the hypermutation mechanism between PMBL and other types of lymphomas. The spatial distribution of *BCL6* mutations in PMBL also differed from that in either DLCL or FL. In particular, the *BCL6* stretch spanning nucleotides 150-270 was more frequently hit by mutations in PMBL than it was in the other two lymphoma types. Notably, this region is characterized by a higher density of AT dinucleotides with respect to the rest of the sequence.

The consistent expression of bcl6 protein and occurrence of hypermutation represent specific markers of B-cell transit through the germinal center and, accordingly, PMBL should be considered of germinal center origin. The fact that the hypermutation sites and mutational spectrum of BCL6 in PMBL differ from those found in FL and DLCL might suggest that the maturation block of the transforming cells differs among these tumor types. The specific pattern of somatic mutations observed in the three lymphomas could reveal a different history of clonal selection that promotes arrest at a specific step of differentiation. However, the absence of ongoing immunoglobulin gene mutations in PMBL reflects the occurrence of tranformation at a late step in B-cell differentiation, when hypermutation has been switched off. This implies that the characteristic mutational pattern observed in PMBL is present before neoplastic transformation. In other terms, our findings strengthen the hypothesis that PMBL may originate from an already defined sub-population of B-cells, which are different from those leading to either DLCL or FL. The characteristic topology of PMBL is in accordance with this hypothesis.

GM: data analysis and article drafting; SB: statistical analysis and interpretation of data, drafting of corresponding results and figures; PSM: study design and finalization of manuscript; MS: sequencing and controls, drafting of tables; MC: immunohistochemical analysis, interpretation of data and critical revision of manuscript; AS: study conception and design, finalization of manuscript; FM: study conception, interpretation of data and critial revision for intellectual content. All of the authors approved the the final version. The authors reported no potential conflicts of interest. *Supported by the Fondazione Cassa di Risparmio di Verona (Bando 2001), Verona, Italy; Associazione Italiana Ricerca Cancro (AIRC), Milan, Italy; Ministero Università, Rome, Italy.*

Manuscript received May 31, 2004. Accepted July 2, 2004.

References

1. Todeschini G, Secchi S, Morra E, Vitolo U, Orlandi E, Pasini F, et al. Primary mediastinal large B-cell lymphoma (PMLBCL): long-term results from a retrospective multicentre Italian experience in 138 patients treated with CHOP or MACOP-B/VACOP-B. Br J Cancer 2004;90:372-6.
2. Isaacson PG, Norton AJ, Addis BJ. The human thymus contains a novel population of B lymphocytes. Lancet 1987;2: 1488-91.
3. Copie-Bergman C, Plonquet A, Alonso MA, Boulland ML, Marquet J, Divine M, et al. MAL expression in lymphoid cells: further evidence for MAL as a distinct molecular marker of primary mediastinal large B-cell lymphomas. Mod Pathol 2002;15:1172-80.
4. Rosenwald A, Wright G, Leroy K, Yu X, Gaulard P, Gascoyne RD, et al. Molecular diagnosis of primary mediastinal B cell lymphoma identifies a clinically favorable subgroup of diffuse large B cell lymphoma related to Hodgkin lymphoma. J Exp Med 2003;198:851-62.
5. Savage KJ, Monti S, Kutok JL, Cattoretti G, Neuberg D, De Leval L, et al. The molecular signature of mediastinal large B-cell lymphoma differs from that of other diffuse large B-cell lymphomas and shares features with classical Hodgkin's lymphoma. Blood 2003;102:3871-9.
6. Scarpa A, Moore PS, Rigaud G, Menestrina F. Genetic alterations in primary mediastinal B-cell lymphoma: an update. Leuk Lymphoma 2001;41:47-53.
7. Barth TF, Leithauser F, Joos S, Bentz M, Moller P. Mediastinal (thymic) large B-cell lymphoma: where do we stand? Lancet Oncol 2002;3:229-34.
8. Copie-Bergman C, Gaulard P, Maouche-Chretien L, Briere J, Haioun C, Alonso MA, et al. The MAL gene is expressed in primary mediastinal large B-cell lymphoma. Blood 1999;94:3567-75.
9. Palanisamy N, Abou-Elella AA, Chaganti SR, Houldsworth J, Offit K, Louie DC, et al. Similar patterns of genomic alterations characterize primary mediastinal large-B-cell lymphoma and diffuse large-B-cell lymphoma. Genes Chromosomes Cancer 2002;33:114-22.
10. Lossos IS, Jones CD, Warnke R, Natkunam Y, Kaizer H, Zehnder JL, et al. Expression of a single gene, BCL-6, strongly predicts survival in patients with diffuse large B-cell lymphoma. Blood 2001;98:945-51.
11. Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature 2000;403:503-11.
12. Kerckaert JP, Deweindt C, Tilly H, Quief S, Lecocq G, Bastard C. LAZ3, a novel zinc-finger encoding gene, is disrupted by recurring chromosome 3q27 translocations in human lymphomas. Nat Genet 1993;5:66-70.
13. Baron BW, Nucifora G, McCabe N, Espinosa R 3rd, Le Beau MM, McKeithan TW. Identification of the gene associated with the recurring chromosomal translocations t(3;14)(q27;q32) and t(3;22)(q27;q11) in B-cell lymphomas. Proc Natl Acad Sci USA 1993;90:5262-6.
14. Ye BH, Rao PH, Chaganti RS, Dalla-Favera R. Cloning of bcl-6, the locus involved in chromosome translocations affecting band 3q27 in B-cell lymphoma. Cancer Res 1993;53:2732-5.
15. 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.
16. Pasqualucci L, Migliazza 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 1998;95:11816-21.
17. Shen HM, Peters A, Baron B, Zhu X, Storb U. Mutation of BCL-6 gene in normal B cells by the process of somatic hypermutation of Ig genes. Science 1998;280: 1750-2.
18. Peng HZ, Du MQ, Koulis A, Aiello A, Dogan A, Pan LX, et al. Nonimmunoglobulin gene hypermutation in germinal center B cells. Blood 1999;93:2167-72.
19. Gordon MS, Kanegai CM, Doerr JR, Wall R. Somatic hypermutation of the B cell receptor genes B29 (Igβ, CD79b) and mb1 (Igα, CD79a). Proc Natl Acad Sci USA 2003;100:4126-31.

20. Pasqualucci L, Migliazza A, Basso K, Houldsworth J, Chaganti RS, Dalla-Favera R. Mutations of the BCL6 proto-oncogene disrupt its negative autoregulation in diffuse large B-cell lymphoma. Blood 2003;101:2914-23.
21. Artiga MJ, Saez AI, Romero C, Sanchez-Beato M, Mateo MS, Navas C, et al. 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. Am J Pathol 2002;160:1371-80.
22. Seitz V, Hummel M, Anagnostopoulos I, Stein H. Analysis of BCL-6 mutations in classic Hodgkin disease of the B- and T-cell type. Blood 2001;97:2401-5.
23. de Leval L, Ferry JA, Falini B, Shipp M, Harris NL. Expression of bcl-6 and CD10 in primary mediastinal large B-cell lymphoma: evidence for derivation from germinal center B cells? Am J Surg Pathol 2001;25:1277-82.
24. 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.
25. Pileri SA, Gaidano G, Zinzani PL, Falini B, Gaulard P, Zucca E, et al. Primary mediastinal B-cell lymphoma: high frequency of BCL-6 mutations and consistent expression of the transcription factors OCT-2, BOB.1, and PU.1 in the absence of immunoglobulins. Am J Pathol 2003; 162:243-53.
26. Paulli M, Lazzarino M, Gianelli U, Strater E, Orlandi E, Klersy C, et al. Primary mediastinal B-cell lymphoma: update of its clinicopathologic features. Leuk Lymphoma 1997;26 Suppl 1:115-23.
27. Menestrina F, Chilosi M, Bonetti F, Lestani M, Scarpa A, Novelli P, et al. Mediastinal large-cell lymphoma of B-type, with sclerosis: histopathological and immunohistochemical study of eight cases. Histopathology 1986;10:589-600.
28. Moller P, Lammler B, Eberlein-Gonska M, Feichter GE, Hofmann WJ, Schmitteckert H, et al. Primary mediastinal clear cell lymphoma of B-cell type. Virchows Arch A Pathol Anat Histopathol 1986;409:79-92.
29. Addis BJ, Isaacson PG. Large cell lymphoma of the mediastinum: a B-cell tumour of probable thymic origin. Histopathology 1986;10:379-90.
30. Morgan JW, Kouttab N, Ford D, Maizel AL. Vitamin D-mediated gene regulation in phenotypically defined human B cell subpopulations. Endocrinology 2000; 141:3225-34.
31. 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.
32. 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.
33. 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.
34. Bellido M, Capello D, Altes A, Estivill C, Gaidano G, Pujol R, et al. Bcl-6 p53 mutations in lymphomas carrying the bcl-2/Jh rearrangement. Haematologica 2002;87:908-17.
35. Lossos IS, Warnke R, Levy R. BCL-6 mRNA expression in higher grade transformation of follicle center lymphoma: correlation with somatic mutations in the 5' regulatory region of the BCL-6 gene. Leukemia 2002;16:1857-62.
36. Jardin F, Bastard C, Contentin N, Parmentier F, Picquenot JM, Tilly H, et al. Intronic BCL-6 mutations are preferentially targeted to the translocated allele in t(3;14)(q27;q32) non-Hodgkin B-cell lymphoma. Blood 2003;102:1872-6.
37. Scarpa A, Moore PS, Rigaud G, Inghirami G, Montresor M, Menegazzi M, et al. Molecular features of primary mediastinal B-cell lymphoma: involvement of $p16^{INK4A}$, p53 and c-myc. Br J Haematol 1999;107:106-13.
38. Kikuchi M, Miki T, Kumagai T, Fukuda T, Kamiyama R, Miyasaka N, et al. Identification of negative regulatory regions within the first exon and intron of the BCL6 gene. Oncogene 2000;19:4941-5.
39. Rogozin IB, Kolchanov NA. Somatic hypermutagenesis in immunoglobulin genes. II. Influence of neighbouring base sequences on mutagenesis. Biochim Biophys Acta 1992;1171:11-8.
40. Dorner T, Foster SJ, Farner NL, Lipsky PE. Somatic hypermutation of human immunoglobulin heavy chain genes: targeting of RGYW motifs on both DNA strands. Eur J Immunol 1998;28:3384-96.
41. Jardin F, Buchonnet G, Parmentier F, Contentin N, Lepretre S, Lenain P, et al. Follicle center lymphoma is associated with significantly elevated levels of BCL-6 expression among lymphoma subtypes, independent of chromosome 3q27 rearrangements. Leukemia 2002; 16:2318-25.