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

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Although primary mediastinal (thymic) large B-cell lymphoma has been primarily studied, its precise phenotype, molecular characteristics, and histogenesis are still a matter of debate. The International Extranodal Lymphoma Study Group collected 137 such cases for extensive pathological review. Histologically, the lymphomatous growth was predominantly diffuse with fibrosis that induced compartmentalized cell aggregation. It consisted of large cells with varying degrees of nuclear polymorphism and clear to basophilic cytoplasm. On immunohistochemistry, the following phenotype was observed: CD45⁺, CD20⁺, CD79a⁺, PAX5/

BSAP⁺, BOB.1⁺, Oct-2⁺, PU.1⁺, Bcl-2⁺, CD30⁺, HLA-DR⁺, MAL protein^{+/−}, Bcl-6^{+/−}, MUM1/IRF4^{+/−}, CD10^{−/+}, CD21[−], CD15[−], CD138[−], CD68[−], and CD3[−]. Immunoglobulins were negative both at immunohistochemistry and *in situ* hybridization. Molecular analysis, performed in 45 cases, showed novel findings. More than half of the cases displayed BCL-6 gene mutations, which usually occurred along with functioning somatic IgV_H gene mutations and Bcl-6 and/or MUM1/IRF4 expression. The present study supports the concept that a sizable fraction of cases of this lymphoma are from activated germinal center or postgerminal center cells. However, it differs from other aggressive B-cell lymphomas in that it shows defective immunoglobulin production despite the expression of OCT-2, BOB.1, and PU.1 transcription factors and the lack of IgV_H gene crippling mutations. (Am J Pathol 2003; 162:243–253)

Primary mediastinal (thymic) B-cell lymphoma (PMBL) is a distinct clinicopathological entity, originally described in 1980,¹ officially acknowledged in the REAL Classification in 1994² and more recently included in the World Health Organization book on the tumors of the hematopoietic and lymphoid tissues.³ Phenotypic and molecular studies of this entity have provided controversial results,^{4–36} thus challenging a definitive histogenetic interpretation.

In recent times, the field of B-cell lymphoma histogenesis has progressed rapidly because of the increasing availability of well-defined histogenetic markers. Genotypic markers include mutations of immunoglobulin (Ig) genes, which are somatically acquired at the time of B-cell transit throughout the germinal center (GC); muta-

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tions of BCL-6 have also been generally regarded as acquired during GC transit.^{37–40} Phenotypic markers are represented by expression of the Bcl-6 protein, which is restricted to GC B-cells, and of MUM1/IRF4 (for Interferon Regulatory Factor-4), which denotes the final step of intra-GC B-cell differentiation, as well as subsequent steps of B-cell maturation toward plasma cells.^{37–42} Finally, late stages of B-cell maturation are defined by expression of the CD138/syndecan-1 molecule.⁴¹

So far, phenotypic analysis has revealed positivity of PMBL for CD45 and CD20, but negativity for CD3 and a variety of other T-cell markers; CD79a has generally been detected, despite the usual lack of surface and cytoplasmic Ig.^{2–5,8,13,16,18,19,21,22,32} In two reported series, CD30 staining was observed in the vast majority of cases, although it was weaker and less homogeneous than in classic Hodgkin's lymphoma (cHL) and anaplastic large cell lymphoma.^{21,28} CD21 and class I and/or II histocompatibility molecules have been claimed to be absent.^{4,5,8,14} Bcl-2 protein seems to be generally expressed,³² while fragmentary data are available concerning the occurrence of some molecules, such as CD10, MUM1/IRF4, PAX5/BSAP (B-cell Specific Activating Protein), Bcl-6, BOB.1, and Oct-2.^{31,34}

In contrast to diffuse large B-cell lymphoma,^{10,31} molecular studies of small series of PMBLs have so far revealed the usual absence of *BCL-6* and *BCL-2* rearrangements/mutations,^{25,30} as well as frequent overexpression of the *MAL* gene.²⁹ The latter is located on the long arm of chromosome 2⁴³ and encodes a protein thought to play a relevant role in membrane trafficking and signaling mediated by specialized, and glycolipid- and cholesterol-enriched, membrane microdomains referred as lipid rafts,^{44–46} which might contribute to disease pathogenesis.²⁹ Mutations of the gene have not been identified and the mechanism of overexpression remains unclear yet.²⁹ Other reported oncogene abnormalities consist in *C-MYC* mutations, detection of *BCL-2* and *REL* proto-oncogene amplification and *P53* mutations.^{17,25,36} Because of the reported lack of *BCL-6* mutations, PMBL has been thought to recognize a pre-GC cell derivation.^{30–32} This assumption, however, seems to contrast with two recent reports, respectively, showing, in a small series of PMBLs, isotype-switched Ig genes with a high load of somatic hypermutations and variants in the 5'-noncoding region of *BCL-6* by single-strand conformation polymorphism analysis.^{35,36}

The aim of the present report is to focus on the morphological, phenotypic, and molecular characterization of a series of 137 PMBLs collected by the International Extranodal Lymphoma Study Group.

Materials and Methods

Case Composition and Selection

The Centers adhering to International Extranodal Lymphoma Study Group Protocol 9 on PMBL were asked to send to the Hematopathology Unit of Bologna University

the pathological material of the 426 cases enrolled in the course of a clinical trial. Histological preparations were obtained in 181 instances. The Hematopathology Units/Surgical Pathology Divisions of the following institutions contributed to the study (listed according to the number of cases submitted): Southampton University, UK; Bologna University, Italy; National Institute of Cancer, Milan, Italy; "La Sapienza" University, Rome, Italy; S. Giovanni Hospital, Bellinzona, Switzerland; Turin University, Turin, Italy; Modena University, Modena, Italy; Udine University, Udine, Italy; S. Maria Nuova Hospital, Reggio Emilia, Italy; Pisa University, Pisa, Italy; S. Giovanni Rotondo Hospital, S. Giovanni Rotondo, Italy; Bern University, Bern, Switzerland; Catanzaro University, Catanzaro, Italy; Coppito Hospital, L'Aquila, Italy; General Hospital, Dolo-Venezia, Italy; S. Maria delle Croci Hospital, Ravenna, Italy; Bianchi-Melacrino-Morelli Hospital, Reggio Calabria, Italy; Perugia University, Perugia, Italy; Bari University, Bari, Italy; S. S. Annunziata Hospital, Taranto, Italy; and General Hospital, Mestre, Italy. Hematoxylin and eosin (H&E)- and Gomori-stained sections were available in all cases, which were integrated by immunohistochemical preparations for CD20/L26 and CD3 or CD45R0/UCHL1 in 11 instances and paraffin blocks in 80 cases. The submitted material corresponded to 36 needle biopsies and 145 surgical samples. All tissue blocks had been fixed in 10% buffered formalin except 10 cases that had undergone Susa fixation. A preliminary screening of the cases was made on H&E to assess the adequacy of both the amount of evaluable material and the preservation of cytological details. Three- μ m-thick sections were cut from the paraffin blocks and stained with Giemsa and Gomori.

Immunohistochemistry

Serial sections were cut from the submitted paraffin blocks, coated on naturally charged slides, stored in a warm chamber at 56°C for at least 2 hours, and then rehydrated through repeated washes in Histoclear (National Diagnostics, Atlanta, GA) and graded alcohols. They were used for immunohistochemistry, which was performed by applying the panel of antibodies listed in Table 1; the antibodies, with the exception of the ones raised against PU.1 and MAL protein, were dispensed on a TechMate 500 immunostainer and detected by the alkaline phosphatase-anti alkaline phosphatase (APAAP) technique.^{47–49} In particular, all antigens but CD21 and MAL protein were unmasked by treating sections in 1 mmol/L ethylenediaminetetraacetic acid (pH 8.0) in a microwave oven at 900 W for 10 to 15 minutes (two to three cycles of irradiation, 5 minutes each) depending on their cytoplasmic or nuclear location.⁵⁰ The sections were then cooled at room temperature for 20 minutes. CD21 was re-exposed by incubating sections in a 0.1% solution of Protease XIV in 0.2 mol/L of Tris-buffered saline (TBS) (pH 7.5) at 37°C for 4 minutes, followed by prolonged washing in cold TBS. The anti-PU.1 antibody was revealed by the EnVision technique.⁴⁹ MAL protein expres-

Table 1. Summary of the Findings Observed at Immunohistochemistry and *in Situ* Hybridization

Marker	Cases ^{+ve} / cases ^{ev}
CD45 (mAb clone PD7/26+2B11, DAKO, Glostrup, Denmark)	80/80
CD20 (mAb clone L26, DAKO)	91/91
CD79a (mAb clone JCB117, Prof. D.Y. Mason, Oxford)	80/80
PAX5/BSAP (mAb clone 24, Transduction Lab, Lexington, KY)	80/80
CD3 (pAb) and/or CD45R0 (mAb clone UCHL1, DAKO)	0/91*
CD30 (mAb clone Ber-H2, Prof. H. Stein, Berlin, Germany)	62/72
HLA-DR (mAb clone DK22, DAKO)	61/76
Bcl-2 protein (mAb clone 124, DAKO)	55/70
CD10 (mAb clone 56C6, Novocastra, Newcastle, UK)	15/71
Bcl-6 protein (mAb clone PG-B6p, Prof. B. Falini, Perugia, Italy)	31/66
MUM1/IRF4 (mAb clone Mum-1p, Prof. B. Falini, Perugia)	46/61
CD138 (mAb clone MI15, DAKO)	0/61
Ig light chains (pAb, DAKO)	0/80
Ig light chains m-RNA (PNA probes, DAKO)	0/40
BOB.1 (pAb, Santa Cruz, Santa Cruz, CA)	73/73
Oct-2 (pAb, Santa Cruz)	69/69
PU.1 (mAb clone G148-74, PharMingen, La Jolla, CA) [†]	40/40
CD15 (mAb clone 3CD1, DAKO)	0/80
CD68 (mAb clone PG-M1, Prof. B. Falini)	0/80
CD21 (mAb clone 1F8, DAKO)	0/70
MAL protein (mAb, Dr. M. A. Alonso, Madrid, Spain)	27/44

Abbreviations: ^{+ve}, positive (all cases with a rate of neoplastic cells stained between 10% and 100% are collectively quoted as positive in the present table; deviations from the usual 70 to 100% (+) value are detailed in the result section); ^{ev}, evaluable; CD, cluster of differentiation; pAb, polyclonal antibody; mAb, monoclonal antibody.

*The total number includes 11 cases with only basic B- and T-cell markers available.

Footnote: the difference in the number of cases tested with each antibody depends on the amount of sections obtained from the submitted paraffin blocks, which varied from case to case.

[†]The determination of PU.1, performed in the light of the recently published report of Jundt and colleagues⁴⁷, was performed after the completion of all the other immunostains and was feasible in only 40 cases with left material.

sion was evaluated by using a specific monoclonal antibody,²⁹ after microwave heating in citrate buffer, pH 6.7 (one cycle of 12 minutes at 750 W followed by two cycles of 10 minutes each at 350W): the antibody was revealed by the immunoperoxidase labeled streptavidin biotin (LSAB) technique.⁴⁹ In all instances, positive and negative controls were used to assess the reliability of the results, as previously described.⁵⁰ The results were graded as follows: +, 75 to 100% of the neoplastic cells positive; +/-, 50 to 75% of the neoplastic cells positive; -/+, 25 to 50% of the neoplastic cells positive; rare, 1 to 25% of the neoplastic cells positive; -, all neoplastic cells negative. The intensity of the staining and the pattern of positivity (nuclear, cytoplasmic diffuse, dot-like, granular, membrane-bound, and so forth) were also recorded.

In Situ Hybridization

In 40 cases with abundant material available and optimally preserved tissue as judged by immunostains and morphological details, fluorescein-conjugated peptide nuclei acid (PNA) probes against κ and λ Ig light-chain mRNA (DAKO, Glostrup, Denmark) were applied according to the manufacturer's instructions. Sections from two cases of multiple myeloma with κ and λ Ig light-chain restriction, respectively, were used as positive controls.

Molecular Biology

Eight- μ m-thick sections were cut from 45 paraffin blocks with enough material available for DNA extraction and amplification. DNA purification was performed using a commercial kit (QIAamp DNA mini Kit; Qiagen, Italy) according to the manufacturer's instructions.³⁰

IgV_H gene rearrangements were amplified with two sets of six *V_H* gene family-specific primers and a *J_H* primer mix in separate reactions for each *V_H* primer, as described.^{51,52} The *V_H* primers used in this study hybridize to sequences in the framework region (FR) 1 or FR2 of the respective *V_H* families. Polymerase chain reaction (PCR) was performed for 40 cycles with an annealing temperature of 60°C. PCR products were directly sequenced using a commercially available kit (ThermoSequenase; Amersham Life Sciences) as previously reported.³⁰ Sequences were compared to the V-BASE sequence directory (MRC Centre for Protein Engineering, Cambridge, UK) using MacVector 6.0.1 software (Oxford Molecular Group PLC, Oxford, UK) for comparison of the rearranged *IgV* genes to the most homologous germline sequences.

Mutational analysis of *BCL-6* gene was performed by direct sequencing of three partially overlapping PCR products encompassing nucleotides +404 to +1142 localized in the 5'-noncoding region of the gene.³⁰ PCR fragments were directly sequenced with appropriate primers, as described above.³⁰ All mutations detected were reconfirmed on independent PCR products.

Clinical Findings and Statistical Methods

The following clinical information was available in all patients: age, sex, sites of disease, stage, presence of systemic symptoms, International Prognostic Index (IPI), lactate dehydrogenase (LDH) level, bulky disease, outcome, and therapies administered (CHOP/CHOP-like versus MACOP-B/VACOP-B/ProMACE-CytaBOM versus HDS/ABMT). Overall survival was calculated by the Kaplan-Meier method⁵³ from the date of diagnosis (starting time) until last contact or death for any cause (event). Relapse-free survival was calculated for patients who achieved a complete remission after the first-line therapy from the date of first complete remission to the date of last contact, if alive and nonrelapsed, or to relapse or death (events), whichever came first.

Univariate analysis was performed by the log-rank test⁵⁴ or Cox proportional hazard regression model,⁵⁵ as appropriate. Multivariate analysis was performed by a

Cox model using the stepwise selection method. The chi-square test was used whenever appropriate for comparison of subgroups. Two-sided *P* values were used throughout.

Results

Case Selection

Forty-four of the 181 submitted cases were excluded because of inadequate sampling or crashing artifacts, which prevented optimal cytological evaluation.

Histological Findings

At Gomori silver impregnation, features of sclerosis were appreciated in 125 of 137 cases. These varied from case to case and from field to field within the same case. In particular, they usually tended to surround groups of lymphomatous elements producing compartmentalization of the tumoral growth (Figures 1, 2, and 3). In more than half the cases, the latter finding was already evident at H&E because of the presence of thick collagen bands around clusters of neoplastic cells (Figures 1 to 3). In 14 instances, the sclerotic features were only focal. A vague nodularity was appreciated in 51 instances (Figure 3) and phenomena of necrosis, at times extensive, in 36.

On cytological grounds, in 103 cases, the tumor uniformly consisted of large cells (measuring $>20\ \mu\text{m}$ in diameter) (Figures 1 and 2), whereas in the remaining ones the size of lymphomatous elements ranged from medium to large. The nuclear contours were more often variable within the same case, resulting round, oval, indented, or multilobated (Figure 2). The nucleoli comprised within a finely dispersed chromatin were generally medium-sized, at times adjacent to the nuclear membrane (Figures 1 and 2). In all instances, the rim of cytoplasm was relatively wide: at H&E and Giemsa staining, it appeared homogeneously clear or slightly basophilic in 96 and 35 cases, respectively (Figures 1 and 2). A mixture between clear and slightly basophilic cells was appreciated in only six tumors. Mitotic figures were numerous, ranging from 4 to 9/high-power field. Within this context, elements with immunoblastic morphology were occasionally seen, while scattered multinucleated giant cells, at times with Reed-Sternberg-like features, were recorded in 33 instances.

Immunohistochemical Findings

These are summarized in Table 1 and primarily corresponded to the results obtained in other studies as far as

it concerned the expression of the most common markers.^{31,32} In particular, B-cell related antigens (CD20, CD79a, and PAX5/BSAP) as well as the leukocyte common antigen (CD45) were positive in all cases (Figure 4). However, the staining for CD79a showed some variability both in terms of intensity (which was moderate to weak in 15 cases) and number of positive cells. The latter corresponded to $<50\%$ of the neoplastic elements in 19 instances. T-cell markers were limited to reactive T-lymphocytes scattered throughout. CD30 staining was found in 62 of 72 eligible cases: it was more often moderate/weak and limited to 10 to 50% of the lymphomatous cells in 46 of 62 samples (Figure 5). HLA-DR was strongly expressed in 61 of 76 cases (Figure 6). No single case was found to express CD15, CD21, CD138, and CD68. Bcl-2 product, CD10, Bcl-6 protein, and MUM1/IRF4 were, respectively, detected in 55 of 70, 15 of 71, 31 of 66, and 46 of 61 cases with enough material left. Although some variability of the staining intensity and rate of positive elements was observed with each marker, usually most if not all neoplastic cells were marked by the corresponding antibody (Figures 7, 8, and 9). Fourteen of the 15 CD10⁺ cases displayed a reliable Bcl-6 staining. The determination of the MAL protein provided reliable results in 44 of 78 investigated cases, as judged on the basis of the existing internal controls. Twenty-seven of the interpretable cases turned out to express the molecule in question: the content of MAL-positive cells varied from case to case (from 30 to 100% of the neoplastic elements), as did the intensity of the staining (from weak to strong) (Figure 10). In eight instances, the positivity was mainly or exclusively confined to the Golgi area. The search for surface and/or cytoplasmic κ and λ Ig light-chains gave negative results in all samples except five which showed an equivocal stain for κ . The latter cases were included among those that underwent *in situ* hybridization (see below): they all turned out negative at the determination of Ig light chain mRNA (Figure 11). The transcription factors BOB.1, Oct-2, and PU.1 were detected in all of the analyzed cases (73, 69, and 40, respectively) (Figures 12, 13, and 14): the staining was usually nuclear, although simultaneous nuclear and cytoplasmic positivities were recorded in some instances. Finally, the Ki-67 marking (monoclonal antibody Mib-1; DAKO) ranged from 40 to 95% with a median value of 69.5%.

In Situ Hybridization

No positivity at the determination of κ and λ Ig light-chain mRNA was appreciated at the neoplastic level in the tested

Figure 1. Histopathology of PMBL. The tumoral population homogeneously consists of large cells with clear cytoplasm (H&E, $\times 400$).

Figure 2. Cytological details of PMBL. Neoplasm cells with clear cytoplasm show some variability both in size and nuclear contours, the latter being at times multilobated (H&E, $\times 600$).

Figure 3. Fibrotic component of PMBL. Groups of neoplastic cells are surrounded by a delicate meshwork of reticulin fibers (Gomori silver impregnation, $\times 50$).

Figure 4. Immunohistochemical features of PMBL. CD79a staining in a case characterized by almost complete positivity of neoplastic cells (monoclonal antibody JCB117; APAAP technique; Gill's hematoxylin nuclear counterstaining; $\times 600$).

Figure 5. Immunohistochemical features of PMBL. CD30 expression: the staining occurs in the majority of neoplastic cells and is weaker than in classical Hodgkin's lymphoma and anaplastic large cell lymphoma (monoclonal antibody Ber-H2; APAAP technique; Gill's hematoxylin nuclear counterstaining; $\times 400$).

Figure 6. Immunohistochemical features of PMBL. All neoplastic cells carry HLA-DR (monoclonal antibody DK22; APAAP technique; Gill's hematoxylin nuclear counterstaining; $\times 500$).

Figure 7. Immunohistochemical features of PMBL. CD10 expression by neoplastic cells (monoclonal antibody 56C6; APAAP technique; Gill's hematoxylin nuclear counterstaining; $\times 700$).

Figure 8. Immunohistochemical features of PMBL. Expression of the BCL-6 gene product by neoplastic cells (monoclonal antibody PG-B6p; APAAP technique; Gill's hematoxylin nuclear counterstaining; $\times 600$).

Figure 9. Immunohistochemical features of PMBL. The search for the MUM1/IRF4 molecule produces a staining pattern that corresponds to the one observed with the PG-B6p antibody (monoclonal antibody mum-1p; APAAP technique; Gill's hematoxylin nuclear counterstaining; $\times 600$).

Figure 10. Immunohistochemical features of PMBL. Most neoplastic cells express the MAL protein at the cytoplasmic level: some positivities confined to the Golgi area can also be seen (monoclonal antibody; LSAB technique; hematoxylin nuclear counterstaining; $\times 500$).

Figure 11. *In situ* hybridization features of PMBL. One single positive plasma cell is comprised among negative neoplastic elements (*in situ* hybridization; mRNA κ Ig light chain complementary PNA probe; development in New Fuchsin; Gill's hematoxylin nuclear counterstaining; $\times 700$).

Figure 12. Immunohistochemical features of PMBL. All lymphomatous cells react with anti-Oct-2 antibody (polyclonal antibody; APAAP technique; Gill's hematoxylin nuclear counterstaining; $\times 500$).

Figure 13. Immunohistochemical features of PMBL. The same pattern is observed at the determination of BOB.1, which is expressed both at the nuclear and cytoplasmic level (polyclonal antibody; APAAP technique; Gill's hematoxylin nuclear counterstaining; $\times 400$).

Figure 14. Immunohistochemical features of PMBL. Most neoplastic cells, intermingled with some granulocytes, do express PU.1 (monoclonal antibody; Envision technique; Gill's hematoxylin nuclear counterstaining; $\times 500$).

cases, whereas a strong staining was seen in occasional reactive plasma cells comprised within the tumor (Figure 11), as well as in the positive controls (Table 1).

Molecular Biology

Details of the results are shown in Tables 2 and 3. The *IgV_H* gene region could be amplified in 17 of 45 cases.

Presumably, the quality of DNA conservation hampered amplification of *IgV_H* genes in the majority of the remaining cases. Clonal and functional rearrangements were detected in all instances. Nine cases used genes belonging to the *V_H3* family, four cases used genes belonging to the *V_H1* family, two cases used genes belonging to the *V_H4* family, and one case each used genes belonging to the *V_H2* or *V_H6* families. Somatic mutations of *IgV_H* genes

Table 2. *IgV_H* Gene Status

Case number	<i>IgV_H</i> gene	% Homology	Frequency of <i>IgV_H</i> mutations	Technical approach utilized
560/98	VH3(DP-50 hv3)	92.50	7.53×10^{-2} bp	FR1/J _H
4111/99	VH3(DP-47)	95.70	4.3×10^{-2} bp	FR1/J _H
4365/99	VH3(DP-50 hv3)	91.90	8.1×10^{-2} bp	FR1/J _H
3898/00	VH1(DP-10 hv1)	100	0×10^{-2} bp	FR1/J _H
3906/00	VH3(VH3-8)	100	0×10^{-2} bp	FR1/J _H
28/01	VH1(DP-10 hv1)	100	0×10^{-2} bp	FR1/J _H
29/01	VH3(DP-50 hv3)	92.2	7.8×10^{-2} bp	FR1/J _H
35/01	VH3(DP-38/9-1)	100	0×10^{-2} bp	FR1/J _H
41/01	VH1(DP-8+)	91.1	8.9×10^{-2} bp	FR1/J _H
66/01	VH3(DP-58 hv3)	86.10	13.9×10^{-2} bp	FR1/J _H
72/01	VH6(DP-74)	100	0×10^{-2} bp	FR1/J _H
862/01	VH2(S12-7)	99.5	0.5×10^{-2} bp	FR1/J _H
857/01	VH1(DP-88 hv1)	100	0×10^{-2} bp	FR2/J _H
867/01	VH4(DP-63)	100	0×10^{-2} bp	FR1/J _H
2837/00	VH3(DP-51+)	81.8	18.2×10^{-2} bp	FR1/J _H
2839/00	VH4(DP-63)	90.1	9.9×10^{-2} bp	FR2/J _H
2842/00	VH3 COS3/BHG	100	0×10^{-2} bp	FR2/J _H

at a rate $\geq 2 \times 10^{-2}$ bp were found in 8 of 17 cases, whereas the remaining cases harbored unmutated *IgV_H* genes.

The analysis of *BCL-6* gene mutations could be reliably performed in 37 of 45 cases. Twenty-six of these cases (70.2%) carried gene mutations with a mutation rate ranging from 1.13×10^{-3} bp to 18.22×10^{-3} bp (mean value, 4.6×10^{-3} bp). Among the *BCL-6* mutated cases, 21 showed positivity for the Bcl-6 protein and/or MUM1/IRF4, 2 were negative for both markers, and 3 did not provide reliable immunohistochemical results. Thirteen of the *BCL-6* mutated cases expressed the MAL protein, the remaining ones being either negative ($n = 5$) or not evaluable ($n = 8$). Concerning the 11 cases without *BCL-6* mutations, 5 were positive at the determination of Bcl-6 and/or MUM1/IRF4, 2 were negative for both, and 4 were not evaluable; only 1 of them expressed the MAL protein, 4 were MAL-negative, and 6 belonged to the group of not interpretable cases.

Clinical Findings

The overall and relapse-free survival of the 137 patients calculated at 144 months corresponded to 85% and 70%, respectively. No significant differences were observed, when these data were stratified according to IPI (0 to 1 versus >2) and type of therapy administered. As to the latter, however, it should be underlined that the cases treated with MACOP-B and so forth or HDS/ABMT definitely predominated over the ones that received CHOP/CHOP-like therapy. A slightly worse outcome was observed in the patients displaying MAL protein expression or *BCL-6* gene in germ line configuration: however, the difference between these cases and those with an opposite pattern never achieved statistical significance.

Discussion

The present study provides morphological, phenotypic, and molecular data on a large series of PMBL.

At light microscopy, the vast majority of the tumors showed a diffuse growth pattern and consisted of large cells with polymorphic nuclei and a wide rim of clear cytoplasm. Fibrosis was common with frequent compartmentalization of neoplastic cells. Possible additional findings were focal nodularity, necrosis, vascular invasion, and Reed-Sternberg-like elements. These findings basically correspond to those quoted in the literature:^{1-8,12-14,16,19-21,23,26,31,32} in conjunction with immunohistochemical results, they allow a confident diagnosis in most instances, thus definitely disproving the old statement that the recognition of PMBL represents a hard task for the pathologist.^{1,6} The only problematic cases in our series were those corresponding to needle biopsies with crushing artifacts and Reed-Sternberg-like elements. Under these circumstances, the differentiation from cHL should be based on the distinctive phenotypic profile of PMBL, which is in turn CD15⁻ and positive for CD45, CD20, CD79a, MAL, BOB.1, Oct-2, and PU.1.^{2,3,56}

On immunohistochemistry, MAL protein was detected in a percentage of tumors similar to that originally reported and recently confirmed by Copie-Bergman and co-workers.^{29,57} As its detection is somewhat fixative-dependent,²⁹ it might be that the prevalence of positive cases is slightly underestimated. Interestingly, this marker, which has not been so far detected in diffuse large B-cell lymphoma, seems to be characteristic of PMBL.²⁹ Under physiological conditions it is restricted to some steps of T-cell maturation, being involved in specialized lipid raft-mediated, membrane-trafficking, and signaling pathways.^{29,45,46} Thus, in PMBL MAL protein expression might influence both the control of the neoplastic growth and the process of lymphomagenesis.²⁹

Likewise two previous studies,^{21,28} most of the PMBLs of our series showed frequent expression of the CD30 antigen.⁵⁸ In contrast, the high frequency of HLA-DR expression in our material is in conflict with previous reports that stated the lack of histocompatibility antigen expression in PMBL.^{4,5,8,32,59,60} The differences between our own and previous studies are possibly because of the

Table 3. *BCL-6* Gene Status

Case number	BCL-6 mutations	IgV _H somatic mutations ($\geq 2 \times 10^{-2}$ bp)	Bcl-6 protein	MUM1/IRF4 protein
528/95	WT	Ne	+	+
560/98	A446G, A468C, T716G, G810C	Yes	—	+
4111/99	WT	Yes	—	+
4365/99	WT	Yes	+	+
2700/00	G799A	Ne	—	—
3898/00	A581G	GL	Ne	Ne
3900/00	WT	Ne	Ne	—
3906/00	WT	GL	+	+
5463/00	WT	Ne	—	—
28/01	G590C, G629C	GL	+	—
29/01	A581C	Yes	—	+
33/01	C742T	Ne	—	+
35/01	G759C	GL	—	+
41/01	WT	Yes	—	+
47/01	A436C, G478C, G481A, T495A, G518C, G533A	Ne	+	+
64/01	G518C, C525A, T548A, A551T, C601A, T633C	Ne	—	+
65/01	G478A, G533C, G540A, A541C, A547T, T554C, G578A, etc	Ne	+	—
66/01	G790T	Yes	+	—
862/01	T482C, T554G, C584G, G758C	GL	—	—
838/01	G708A, G810A	Ne	+	+
857/01	InsG706, C763T	GL	+	+
866/01	G485A	Ne	—	+
867/01	C584T, C765T	GL	—	+
870/01	T447C, T469A, C479T, G533A, A535C, A551C	Ne	+	+
871/01	G790A, T814C, T620A	Ne	+	—
874/01	C444G, G578C, T531C, C572G	Ne	+	+
875/01	G503A, G641A, C750T, insC770, T838G	Ne	—	+
2837/00	WT	Yes	—	—
2838/00	WT	Ne	Ne	Ne
2839/00	T466C, G804C	Yes	—	+
2840/00	G766T, C769G, C777T	Ne	+	+
2841/00	WT	Ne	Ne	Ne
2842/00	C744A, T739A, G833C	GL	—	+
2843/00	G488A, A535G, G549A, A551C, T557C, A582G, G621T*	Ne	+	+
2844/00	T545G, A561C, C588T, G829A	Ne	—	Ne
3409/00	WT	Ne	—	Ne
3903/00	C704T	Ne	Ne	Ne

Abbreviations: WT, wild type; *, mutation in homozygosis; GL, germline; Yes, presence of IgV_H mutations ($\geq 2 \times 10^{-2}$ bp); +, positive result at immunohistochemistry; —, negativity at immunohistochemistry; Ne, not evaluable.

significant improvement in the antibody affinity and immunohistochemical techniques that occurred during the last few years.

In agreement with previous reports,^{22,31,32} all of the analyzed cases turned out to be negative at the determination of Ig light chains, both at the immunohistochemical and *in situ* hybridization level, despite the expression of CD79a (ie, the B-cell receptor/Ig-associated molecule).⁶¹ Herein, we provide evidence for the first time that the lack of Ig is not dependent on defective expression of either Oct-2 and BOB.1 or PU.1. Oct-2 and BOB.1 belong to a group of transcription factors, which constitute a novel class of immunohistochemical markers and whose expression correlates with cell lineage and/or the stage of cell differentiation. In particular, it has been reported that the Oct-2, Oct-1, and BOB.1/OCA-B transcription factors are required for GC formation and Ig production.^{62,63}

their down-regulation in Reed-Sternberg cells of cHL^{64,65} has been proposed as a possible explanation for the lack of Ig expression in these cells.⁶⁵ PU.1 is a tissue-restricted transcription regulator that is basic for the development of both B-lymphocytes and macrophages.^{66–68} Moreover, it binds to a large number of promoters and regulates the expression of genes required for terminal B-cell differentiation, including those encoding for Ig light and heavy chains, RAG-1, CD19, and J-chain.^{68,69} Recent studies have shown that PU.1 is not detectable in cultured and primary Reed-Sternberg cells of cHL: along with the absence of Oct-2 and BOB.1, this can contribute to defective Ig gene expression in cHL.⁵⁶ Notably, the mechanisms that can explain the lack of Ig production in most cHL cases do not pertain PMBL: in fact, neoplastic cells regularly express Oct-2, BOB.1, and PU.1. The alternative mechanism of defective *IgV_H* gene rearrange-

ments (so-called crippling mutations),⁴⁰ which may lead to the lack of Ig synthesis, cannot be applied to our cases either. Thus, the cause of Ig negativity in PMBL remains still obscure and requires further studies.

Finally, the vast majority of our cases expressed Bcl-6 and/or MUM1/IRF4. In keeping with previous observations in diffuse large B-cell lymphoma,⁴² these molecules were more often co-expressed by the same cells, an aberrant feature that does not correspond to the mutually exclusive pattern found in normal GCs.⁴² In 21% of the cases, a clear-cut CD10 staining was also seen. These findings fit with those recently reported by de Leval and colleagues³⁴ by studying 19 PMBLs for Bcl-6 and CD10 expression. Although the percentage of Bcl-6⁺ cases was lower in our series (possibly because of technical reasons), the PMBL phenotype identified in both studies reflects that of GC cells, a concept that indeed contrasts with the assumption more often reported in the literature that the tumor derives from virgin B cells.³²

Because of the histogenetic debate and the results of our phenotypic studies, we decided to focus on the status of *IgV_H* genes and *BCL-6* genes. With the exception of a very recent report³⁶ and on the basis of the results obtained in a small series of cases, it had been stated that the tumor might derive from virgin B cells.^{25,30,32} Our results unequivocally suggest a GC-cell derivation for a fraction of PMBLs. In fact, somatic mutations of functioning *IgV_H* gene are detectable in ~50% of our amplifiable cases. The latter results are in keeping with a recent publication of Laithäuser and colleagues,³⁵ who reported isotype-switched Ig genes with a high load of somatic hypermutations in 13 PMBLs. Certainly, the efficiency of the technique applied by the latter authors was higher than the one we used, because they analyzed frozen samples instead of routinely processed tissue. Nevertheless, significant analogies do occur between the two studies, because in both *IgV_H* mutations were functioning. As expected, PMBL carrying somatically mutated *IgV_H* genes also harbored *BCL-6* mutations in a fraction of cases. Curiously, though, a substantial proportion of PMBL appeared to show *BCL-6* mutations in the absence of *IgV_H* mutations. Occurrence of *BCL-6* mutations in the absence of *IgV_H* mutations has also been detected in examples of B-cell chronic lymphocytic leukemia, and has been taken to suggest that diverse pathways operate on *BCL-6* and *IgV_H* genes, at least in certain biological contexts.⁷⁰ On these basis, the sole occurrence of *BCL-6* mutations in a given tumor cannot be taken as a marker of GC-derivation in PMBL.

In summary, the present study provides indication that at least a fraction of PMBL is derived from activated GC-related B-cells. This is based on the detection of *IgV_H* gene and *BCL-6* somatic mutations, as well as the expression of peculiar molecules, such as CD10, Bcl-6, and MUM1/IRF4. Taking into consideration the results of these molecules, one might hypothesize that the neoplastic cells of GC-related PMBLs correspond to elements that have already undergone the GC selection and are nearby to exit the GC. This might explain the partial loss of CD10 and the variable expression of both Bcl-6 and MUM1/IRF4. The latter antigen in fact starts to be synthesized in the light zone of the GC by

cells that have already overcome the GC selection and are going to mature to plasma cells or memory B cells.⁴² Remarkably, these transient cells may acquire CD30 antigen expression, as often observed at the periphery of reactive GCs.⁷¹ This plausible hypothesis, however, does not completely solve the histogenetic problem because some tumors lacked both somatic mutations and GC-related protein expression. The latter results might be because of technical artifacts that cannot be completely excluded in our retrospective series, whose material was not treated according to standardized procedures and often remained stored for many years in institutional archives. Alternatively, these germ-line tumors might be derived from virgin B cells, undergoing activation and blastic transformation during the initial phases of the primary immune response.⁷² More studies are necessary to clarify this issue.

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