BCL-6 Expression Predicts Improved Survival in Patients with Primary Central Nervous System Lymphoma¹

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ABSTRACT

Purpose: The purpose of this study was to investigate the histogenetic origin of primary central nervous system lymphoma (PCNSL) with respect to stage of B-cell differentiation and to identify prognostic markers in a cohort of patients with PCNSL treated with i.v. high-dose methotrexate therapy.

Experimental Design: This study included 33 patients with PCNSL treated with high-dose i.v. methotrexate at the Massachusetts General Hospital for whom archival tumor tissue was available. All 33 patients tested negative for HIV. The lymphomas were morphologically subclassified according to the Kiel system, as modified in the WHO classification. Immunohistochemistry for the following antigens was performed: BCL-6; BCL-2; MUM1; CD10; vs38c; CD138; CD44; p16; and p53. Fluorescence in situ hybridization and multiplex PCR for CDKN2A/p16 were also performed.

Results: There were 17 women and 16 men enrolled, with a median age of 60 years. All tumors were diffuse large B-cell lymphomas. Of the 23 cases that could be subclassified, 22 were centroblastic, and 1 was immunoblastic. Twenty-six of 33 tumors were BCL-6+, 6 of 32 tumors were CD10+, 27 of 29 tumors were BCL-2+, 31 of 32 tumors were MUM1+, 11 of 31 tumors were CD44+, 4 of 33 tumors were vs38c+, and 0 of 32 tumors were CD138+. There were 18 of 32 (56%) complete responses and 8 of 32 (25%) partial responses to methotrexate, whereas 6 of 33 (18%) progressed during treatment. Ten patients died of disease. Expression of BCL-6 was significantly associated with longer overall survival (P = 0.002; median survival, 101 versus 14.7 months, with approximately 95% lower confidence limits of 41.7 and 8.8 months, respectively).

Conclusions: In this group of 33 patients with PCNSL, expression of BCL-6 was significantly associated with longer overall survival. BCL-6 warrants further investigation as a potentially important prognostic marker in this disease.

INTRODUCTION

PCNSLs³ comprise 1% of all lymphomas and 4% of all primary brain tumors (1). The majority are sporadic, whereas a minority are associated with congenital or acquired immunodeficiency. The incidence of PCNSL is increasing in both immunocompetent and HIV+ patient populations and at a faster rate than systemic NHL (2, 3). The site of origin of PCNSL is uncertain, although some data suggest that the tumor has a GC immunophenotype and shows features normally associated with the GC environment (4, 5). The prognosis is poor, with a median survival of <12 months when treated with WBRT alone (6) and 30–40 months when treated with methotrexate-based chemotherapy ± WBRT (2). Stratification of patients into “good risk” and “poor risk” categories based on prognostic indicators may be beneficial in targeting therapy. Currently, clinical parameters, such as age, immune status, and initial performance status, are the only known factors of prognostic importance (2, 3, 6). There have been few immunophenotypic and molecular genetic studies of PCNSL, and markers associated with prognosis have only recently become the subject of investigation.

PATIENTS AND METHODS

Patients. Thirty-three patients with PCNSL treated uniformly at the Massachusetts General Hospital from 1988–2001 for whom tumor tissue was available were included in this study. PCNSL was defined as lymphoma confined to the nervous system with or without involvement of the eye at the time of diagnosis, as shown by radiographic imaging, CSF analysis, and ocular examination. All patients were treated with i.v. methotrexate (3.5 or 8 g/m²) in induction (every 2 weeks until response or progression), consolidation (every 2 weeks for two doses), and maintenance (every month for ≥1 year) phases. This treatment regimen has been the standard recommendation for patients with newly diagnosed PCNSL treated at our institution for more than a decade. The diagnostic slides were reviewed by three pathologists without knowledge of clinical outcome and subclassified, where possible, according to the Kiel lymphoma

¹ The abbreviations used are: PCNSL, primary central nervous system lymphoma; FISH, fluorescence in situ hybridization; DLBCL, diffuse large B-cell lymphoma; CB, centroblastic; IB, immunoblastic; NHL, non-Hodgkin’s lymphoma; GC, germinal center; WBRT, whole brain radiation therapy; CSF, cerebrospinal fluid; HIC, immunohistochemistry; CNS, central nervous system.

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classification system, as modified in the WHO classification (7, 8). Clinical variables, including age at diagnosis, site and number of tumors, CSF status, ocular status, initial response to chemotherapy, overall survival, and time and site of relapse, were obtained from an institutional brain tumor patient database and from the Department of Pathology computer system.

IHC. Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue using the avidin-biotin-peroxidase technique. Sections were deparaffinized and rehydrated, and endogenous peroxidase activity was blocked with hydrogen peroxide/methanol. Antigen retrieval was carried out in EDTA buffer (1 mM, pH 8.0) by microwaving for 20 min at 95–99°C or by pressure cooking for 5 min. After antigen retrieval, tissue sections were blocked with 2% normal horse serum/1% BSA in PBS and incubated at 4°C overnight with the working dilution of primary antibody. Thereafter, tissue sections were sequentially incubated with biotinylated horse antimouse antiseraum and streptavidin peroxidase and developed with H2O2 and 3-amino-9-ethylcarbazole. Sections were subsequently counterstained with hematoxylin and mounted in Dako glycer-gel. The following mouse monoclonal antibodies were used: BCL-6 (clone PG-B6; Dako; dilution, 1:25); BCL-2 (clone 124; Dako; dilution, 1:30), vs38c (mouse monoclonal antibody; courtesy of Kevin Gatter, Oxford, United Kingdom; dilution, 1:100); MUM-1 (ICSAT m-17; Santa Cruz Biotechnology; dilution, 1:200); CD10 (clone B-B4; Serotec; dilution, 1:25); BCL-2 (clone 124; Dako; dilution, 1:30), vs38c (mouse monoclonal antibody; courtesy of Kevin Gatter, Oxford, United Kingdom; dilution, 1:100); MUM-1 (ICSAT m-17; Santa Cruz Biotechnology; dilution, 1:200); CD10 (clone B-B4; Serotec; dilution, 1:25); CD138 (clone B-B4; Serotec; dilution, 1:25). Sections were dewaxed through xylene (2 × 10 min) and ethanol (2 × 5 min) and treated in 10 mM sodium citrate for 30 min at 80°C, followed by pepsin digestion (4 mg/ml in saline; pH adjusted to 1.5 with HCl) for 15 min at 37°C. Dual-color FISH was performed for CDKN2A/p16 analysis. A plasmid artificial chromosome in-}

**RESULTS**

**Patient Characteristics and Clinical Outcome.** The 33 patients included 17 women and 16 men, with a median age of 60 years (range, 31–80 years). Eighteen of 33 patients had solitary brain lesions. Diagnosis was achieved by biopsy in 31 of 33 patients and by resection in 2 of 33 patients. There were malignant or atypical lymphoid cells in 3 of 11 patients who underwent lumbar puncture, and 7 of 30 patients had ocular involvement. All patients tested negative for HIV by enzyme immunoassay screening of blood. All patients received high-dose i.v. methotrexate as initial treatment. The median follow-up time was 33.5 months (95% confidence interval, 27.8–44.5 months) and exceeded 3.4 months for all subjects. There were 18 of 32 (56%) complete responses and 8 of 32 (25%)
partial responses to methotrexate, whereas 6 of 33 (18%) progressed during treatment. One patient could not be assessed for response (no residual tumor after surgery). Nine of 33 patients experienced relapse. Five relapses occurred within the CNS at a median time interval of 1.5 years [range, 0.5–8 year(s)]. Three patients had a systemic relapse only, one each in the adrenal gland, liver, and testis. The median interval to relapse in these three patients was 2 years [range, 1–3 year(s)]. One patient experienced relapse in the skin at 32 months, lymph nodes at 35 months, and CNS at 37 months. There were 10 deaths overall. The overall median survival for the entire group was 101 months (lower 95% confidence limit of 36.5 months).

**Histopathology.** All tumors were DLBCLs. Twenty-three tumors were subclassified according to the Kiel lymphoma classification system, whereas 10 could not be subclassified because of scant tissue and/or tissue artifact. Twenty-two tumors were CB (see Fig. 1), of which three were CB polymorphous. Six CB cases contained numerous multilobated cells. Only one case fulfilled the criteria of IB subtype (H11022/90% immunoblasts).

**Results of IHC.** Twenty-six of 33 (79%) tumors expressed BCL-6 (see Table 1 and Fig. 2). In the majority of cases (18 of 26 cases), staining was scored as 3+. Six tumors were 2+, and two cases were 1+. Twenty-seven of 29 (93%) evaluable tumors expressed BCL-2, denoted by strong membranous staining. Only four (12%) tumors showed cytoplasmic staining with vs38c. The remainder showed no staining for vs38c. All but 1 tumor (31 of 32 tumors) showed nuclear staining for MUM1, which was nearly always (28 of 31 cases) strong and diffuse, with >75% of nuclei positive. Only 6 of 32 (19%) evaluable cases showed membrane staining for CD10 (see Fig. 3); all of these were BCL-6+. No case displayed membrane staining for CD138; however, five had faint granular cytoplasmic staining. Eleven of 31 (36%) evaluable tumors had membrane staining for CD44. Six of 33 (18%) cases strongly expressed the nuclear antigen p53. Twenty-five of 31 (81%) tumors were p16−. Five tumors were slightly positive for p16, and 1 tumor was strongly positive for p16.

**Results of FISH and Multiplex PCR for CDKN2A/p16.** Molecular analysis of the CDKN2A/p16 gene was performed on 31 cases. Of these, 13 tumors had a homozygous deletion, 6 had a hemizygous deletion, and 12 tumors had no deletion. All 13 cases with homozygous deletion of the CDKN2A/p16 gene showed no expression of p16 by IHC (see Table 2). The cases with hemizygous deletion had slight (three cases) or no (three cases) expression of p16. Of the 12 tumors without CDKN2A/p16 deletion, 9 were negative for p16 expression, 2 had slight p16 expression, and 1 showed strong p16 expression.

**Statistical Analysis.** Older age at diagnosis was associated with decreased survival (P = 0.072; hazard ratio = 1.052). However, age at diagnosis did not significantly modify the probability of response to methotrexate (P = 0.555).

The morphological subgroups of CB versus CB polymorphous plus IB were compared. Cases that could not be subclassified were compared with subclassified cases. None of these comparisons demonstrated significant differences in response to methotrexate or survival.

A number of immunophenotypic groups were analyzed for association with end points, including BCL-6+/CD10+, CD10+/ CD44−, CD10+/CD44+, BCL-6+/CD138−, CD44+/vs38c−, CD44+/CD138−, and BCL-6+/vs38c−, as well as the status of each individual marker. In addition, the degree of BCL-6 staining was analyzed for significance.

After a Bonferroni correction for the multiple comparisons of interest, only BCL-6 expression demonstrated a statistically significant association with overall survival. Expression of

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**Table 1 Immunochemistry for GC-related antigens**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>No. (%)</th>
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<tbody>
<tr>
<td>BCL-6</td>
<td>26/33 (79%)</td>
</tr>
<tr>
<td>CD10</td>
<td>6/32 (19%)</td>
</tr>
<tr>
<td>MUM1</td>
<td>31/32 (97%)</td>
</tr>
<tr>
<td>CD44</td>
<td>11/31 (35%)</td>
</tr>
<tr>
<td>vs38c</td>
<td>4/33 (12%)</td>
</tr>
<tr>
<td>CD138</td>
<td>0/32 (0%)</td>
</tr>
<tr>
<td>BCL-2</td>
<td>27/29 (93%)</td>
</tr>
</tbody>
</table>

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**Fig. 1** DLBCL of the CNS, CB subtype (H&E staining).
BCL-6 was associated with longer overall survival ($P = 0.002$ using a permutation log-rank test; see Fig. 4). The median survival for patients with tumors that expressed BCL-6 was 101 months. For patients with tumors that were BCL-6−, median survival was 14.7 months. This association remained significant in a proportional hazards model controlling for age.

In analyzing the association of various factors with response to chemotherapy, only a complete response was defined as a response; a nonresponse was defined as either a partial response or progression. There was no association between any of the evaluated markers and response at the Bonferroni-adjusted significance level of 0.004, due to 13 comparisons that were examined. The BCL-6+/vs38− phenotype showed a possible association with response (odds ratio = 5.00; $P = 0.062$). No significant association of response with morphology or genetics was detected. BCL-6 expression was not associated with age, gender, location of the brain lesion(s), number of brain lesions, or the presence or absence of ocular or CSF involvement.

**DISCUSSION**

The cell of origin of lymphomas arising in the CNS, a site devoid of normal resident lymphoid tissue, has not been determined. Morphological (4), immunophenotypic (4), and molecular genetic (5) studies of PCNSL suggest that the cell of origin...
is related to the GC. Expression of BCL-6 is one feature that suggests a relationship to the GC. The BCL-6 protein is a zinc-finger transcriptional repressor encoded by the BCL-6 gene (12). It is required for the formation of the GC (13). In normal lymphoid tissue, there is nuclear expression of the BCL-6 protein almost exclusively by GC B cells. Lymphomas thought to be derived from GC cells, such as follicular lymphoma, also express BCL-6, whereas lymphomas derived from naïve (chronic lymphocytic leukemia and mantle cell lymphoma) or post-GC (marginal zone lymphomas, myeloma) B cells do not (14, 15).

Additional immunophenotypic markers that can aid in the characterization of lymphoid cells with respect to stage of B-cell differentiation were investigated in this study, both for their histogenetic relevance and as potential prognostic indicators. These included CD10, a marker that consistently reflects GC origin in both reactive lymphoid tissue and lymphomas (15); MUM1, a late GC/early post-GC marker (16); vs38c (17) and CD138 (16), which denote plasmacytic and/or post-GC differentiation; and CD44, expressed most strongly by post-GC mantle zone cells (18).

The majority (79%) of PCNSL cases in this study expressed BCL-6. This is consistent with other reports of PCNSL (4, 19, 20). Whereas expression of this marker, which is highly associated with the GC, supports the theory that the cell of origin in PCNSL is related to the GC stage of B-cell differentiation, only 6 of 32 (19%) tumors displayed a definite germinal-center like phenotype, as denoted by coexpression of two GC antigens (BCL-6 and CD10), combined with an absence of the post-GC antigens, vs38c and CD138. However, 10 additional BCL-6+ cases were CD10− but lacked CD44 and vs38c as well, possibly suggestive of late GC differentiation. Combining these two categories means that 16 of 32 (50%) of our PCNSL cases had either a definite or possible GC-like phenotype. All but one tumor expressed MUM-1, suggestive of late GC or post-GC origin. Seven tumors were BCL-6−/CD10−, consistent with a non-GC immunophenotype. True plasmacytic differentiation, as evidenced by membrane staining for CD138, was not seen, and cytoplasmic staining for vs38c was distinctly rare (four cases). Taken together, the immunophenotypic findings are suggestive of a GC or possibly late GC stage of B-cell differentiation for most cases of PCNSL. Because the CNS in humans is devoid of a GC structure, the implication of this finding is that PCNSL may represent a tumor that arises in an extraneural, GC environment. Subsequent localization to the CNS might involve the development of a “neurotropic” cellular phenotype or incomplete eradication of malignant lymphocytes in the CNS by the host immune system.

The only known prognostic factors for patients with PCNSL are clinical parameters, such as age, immune status, initial performance status, and CSF protein level (21, 22). This cohort of nonimmunosuppressed patients was not significantly different from other large series of PCNSL patients in terms of age, gender, or percentage of patients with complete response to chemotherapy (2). Aside from age, none of these parameters were found to be of prognostic significance in this series. One clinical variable not controlled for in this study was performance status at the time of diagnosis. This was not recorded for most patients in our series. In order for this variable to confound our results, it must be associated with both the genetic status of patients with PCNSL and survival. Whereas Karnofsky performance status has been associated with prognosis in PCNSL, there is no a priori evidence that functional status is associated with either the genetic or immunophenotypic status of patients with PCNSL, although such associations cannot be excluded. Furthermore, if performance status is indeed prognostic, exclusion of it from the analysis would likely dilute the effect of BCL-6 (23).

Morphological subclassification of DLBCLs according to the Kiel lymphoma classification has been found by some authors to be of prognostic significance in systemic NHL, with IB and CB polymorphous subtypes having a worse overall survival than the CB subtype (24, 25). However, this association has not been demonstrated in PCNSL (26). In this study, morphological subtype was not found to be of prognostic importance.

BCL-2 protein expression prevents cellular apoptosis and is down-regulated by normal GC cells. However, it may be up-regulated due to BCL-2 gene rearrangement, such as in follicular lymphoma. Overexpression of BCL-2 has been shown to be an independent poor prognostic indicator for systemic NHL (27). Its significance in PCNSL has not been defined. Expression of BCL-2 was not associated with response to methotrexate or survival in the present study.

Genetic alterations in tumor suppressor genes have been associated with prognosis in patients with systemic DLBCL. The CDKN2A/p16 gene is involved in control of the cell cycle and is frequently altered in neoplasia (28). Alteration of the CDKN2A/p16 gene through deletion or hypermethylation has been demonstrated in approximately 60% of PCNSL cases (29, 30). Genetic alterations of CDKN2A/p16 have not been studied as a prognostic factor in PCNSL; however, CDKN2A/p16 inactivation may be an independent prognostic indicator of poor clinical outcome in systemic DLBCL (31). Expression of p16 protein in human cancers has not been well studied. In normal tissues, numerous types of epithelium express p16; however expression of p16 by normal lymphoid tissue is limited (32). Loss of p16 protein expression has been associated with progression of indolent NHL (33). A majority (66.7%) of the PCNSL tumor specimens in this study had either hemizygous or...
homezygous deletions of CDKN2A/p16. The frequency of deletions found in this study is in concordance with other studies of PCNSL. (29, 30). This is in contrast to systemic NHL, in which deletions and mutations of CDKN2A/p16 are relatively infrequent (34, 35). Although this study did not find a correlation between CDKN2A/p16 deletion and survival, the high frequency of CDKN2A/p16 deletions lends additional support to the possibility that deregulation of the p16 cell cycle-regulatory protein may play a role in the pathogenesis of lymphomas in the CNS.

The tumor suppressor p53 regulates a number of key cellular functions, including the cell cycle, apoptosis, and DNA repair, and is frequently altered in human cancers. Like CDKN2A/p16, alterations of p53 have been implicated in the progression of indolent systemic lymphoma to high-grade lymphoma (36). Inactivating p53 gene mutations have been documented in a minority of PCNSLs (37). The significance of p53 protein expression in PCNSL is not known. The results from our study are consistent with prior data because only a minority of PCNSL cases demonstrated p53 expression (6 of 33 cases). Expression of p53 was not associated with response to methotrexate or survival in our series of PCNSL patients.

In addition to its possible relevance toward understanding the histogenesis of PCNSL, BCL-6 expression is a potential prognostic indicator. Prior studies of BCL-6 expression in systemic DLBCL have suggested that this marker may be associated with improved overall survival (38, 39). The prognostic significance of BCL-6 in PCNSL has not been defined. In this study of 33 patients with PCNSL treated with high-dose i.v. methotrexate, BCL-6 expression was associated with a significantly improved overall survival (101 versus 14.7 months). Our results are in concordance with the results of a recent study of systemic DLBCL by Lossos et al. (38). They found that BCL-6 gene expression, as assessed by the presence of BCL-6 mRNA, was associated with a statistically significant prolongation of survival in DLBCL (171 versus 24 months). BCL-6 protein expression also predicted longer overall survival in that study. Similarly, Takeshita et al. (39) found BCL-6 expression to be a good prognostic factor in patients with gastric DLBCL. In contrast, BCL-6 expression was not associated with overall survival in a study of PCNSL in 17 nonimmunosuppressed patients (19).

BCL-6 expression was not associated with response to methotrexate in this series, although the sample size may have been too small to detect such an association. However, because many patients who fail to achieve a complete response to methotrexate subsequently achieve a complete response when treated with alternative chemotherapy or WBRT, it may be that BCL-6 expression correlates with overall treatment response and not simply methotrexate response. Because our data were incomplete with respect to response to after methotrexate treatment, we could not assess this hypothesis.

In summary, this study investigated a number of immunophenotypic and molecular markers in PCNSL. The immunophenotypic profile was heterogeneous, but expression of antigens associated with the GC or early post-GC stage of B-cell differentiation was common.

In this study, BCL-6 expression was associated with a statistically significant survival advantage compared with tumors that did not express the protein. Although similar findings have been reported in patients with systemic DLBCL, this study represents the first demonstration that BCL-6 expression is associated with improved survival in PCNSL, and this observation has potentially important clinical implications. It may be possible to analyze BCL-6 as a biological marker that could separate “good risk” from “poor risk” patients with PCNSL. This may allow application of risk-adjusted therapies in this disease. For example, in a newly diagnosed PCNSL patient with a good prognosis (BCL-6+), it may be desirable to defer WBRT to avoid the radiation-induced neurotoxicity that becomes more prevalent over time. In contrast, patients who have a “poor risk” phenotype and good performance status may be candidates for more aggressive initial therapy with chemotherapy and radiation. In clinical trials comparing different treatments for PCNSL, BCL-6 expression, as a significant prognostic factor, may become an important stratification variable. This would guarantee a balance of this marker in different treatment groups and would reduce some of the heterogeneity among patients, thereby increasing the power to detect treatment effects. Before the adoption of such treatment algorithms, the preliminary observations from this study should be confirmed in larger studies with more power to analyze this potential association. If these findings are replicated, this could represent a powerful prognostic marker in this disease. Because the biological assays for this test are simple and widely available, this may represent a practical clinical advance in the management of patients with this disease.

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REFERENCES