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O-6-Methylguanine-DNA Methyltransferase, C-MYC, and EBER Status in Diffuse Large B-Cell Lymphoma of Central Nervous System

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Article type: **ABSTRACT**

Original Article

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Diffuse Large B-cell Lymphoma (DLBCL), the most common type of primary central nervous system lymphoma (PCNSL), is a rare aggressive subtype of DLBCL with a poorly understood biology. This study aimed to investigate the prevalence of O-6-Methylguanine-DNA Methyltransferase (MGMT), *C-MYC* and Epstein-Barr virus Encoded RNA (EBER) positivity in CNS-DLBCLs. Using tissue microarray method, formalin-fixed paraffin-embedded blocks of 76 cases of confirmed PCNS-DLBCL and 2 cases of immunodeficiency-related CNS DLBCL were examined for EBER and *C-MYC* by chromogenic in situ hybridization (CISH), and for MGMT, CD10, BCL2, BCL6, MUM1 and Ki67 by Immunohistochemistry (IHC). The results were analyzed in association with histopathologic and demographic characteristics. The majority of the tumors were of non-germinal center B-cell (non-GCB) type. Loss of MGMT expression on IHC, as a surrogate marker of MGMT methylation, was detected in about 68.9% of PCNSLs. Preserved MGMT expression was found to occur more frequently in males and in MUM1-negative and GCB-type tumors. EBER positivity was exclusively seen in immunodeficient cases. Low *C-MYC* amplification was detected in 18% of cases and showed association with BCL2 and Ki67 expression. We concluded that loss of MGMT expression is a common phenomenon in PCNSLs. Epstein-Barr virus (EBV) may not be commonly detected in PCNS-DLBCL as frequently as in systemic DLBCL, but its expression is inevitable in CNS-DLBCLs of immunocompromised ones. Maintained MGMT expression is associated with less aggressive histopathologic features. Further studies are warranted to confirm the prognostic significance of loss of MGMT expression in PCNSLs and its potential use for predicting therapeutic response to alkylating agents in PCNSLs.

Keywords: Primary central nervous system lymphoma, diffuse large B-cell lymphoma, MYC, EBER, MGMT, O-6-methylguanine-DNA methyltransferase

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Introduction

Primary central nervous system lymphoma (PCNSL) is an extra-nodal non-Hodgkin lymphoma limited to brain, eyes, spinal cord and leptomeninges upon initial presentation. This tumor accounts for about 2.4-3% of intracranial neoplasms (1). Diffuse Large B-cell Lymphoma (DLBCL) constitutes up to 95% of PCNSL cases (2). A steady increase has been observed in the incidence of PCNSL, especially in immunocompetent individuals, over the past decades due to the population aging (3, 4). Little is known about the molecular biology and prognostic factors of PCNSLs. PCNS DLBCLs differ from systemic DLBCLs in their behavior, management, and prognosis, as they show a worse prognosis and a poorer response to treatment (1). The molecular pathogenesis of CNS DLBCLs is also different in immunocompetent individuals from that of immunocompromised ones (5, 6). Several studies have tried to investigate molecular pathogenesis of PCNSLs to explain the reason for the inferior prognosis of these tumors. To date, the only known prognostic factors of CNS lymphoma are clinical factors such as age, tumor multifocality, and immune status (7). No reliable molecular prognostic or therapeutic biomarker exists for CNSL in routine practice.

Emerging studies suggest implication of several markers like *C-MYC*, BCL2, O-6-methylguanine-DNA methyltransferase (MGMT) and p53 expression for predicting clinical outcome and individualizing treatment regimens (8). Owing to the substantial variability in the efficacy of novel targeted therapies in different subtypes of DLBCL, phenotypic subclassifying of DLBCLs has become of increasing clinical importance (9). Beside the therapeutic value, biologic subtyping has also prognostic implications. The well-known Hans algorithm is widely used as the standard immunohistochemical (IHC) method for molecular subtyping of DLBCL in routine diagnostic practice. Assessing IHC expression of BCL6, CD10 and MUM1, DLBCLs are assigned into germinal center B-cell (GCB) or activated B-cell (ABC) subtypes in this method (10).

MGMT gene, normally present in all body tissues, encodes a DNA repair enzyme, which protects DNA from DNA mismatch damages induced by alkylating agents. Transcriptional silencing of this gene via promoter methylation plays a role in tumorigenesis of different tumors. MGMT methylation status has been widely studied in glial brain tumors. It is known to be a predictor of prognosis and patient response to alkylating agents like temozolomide in theses tumors (11, 12). Recently, there has been an increasing interest in the role of *MGMT* methylation in lymphomas. Emerging use of temozolomide as an alternative therapeutics' modality for PCNSL treatment, makes it necessary to study the methylation status of *MGMT* gene or MGMT protein expression and to assess its prognostic value in PCNSLs.

Epstein-Barr virus (EBV) is a herpesvirus associated with multiple malignancies in human. EBV infects the majority of the adult population across the world, leaving them as lifelong asymptomatic carriers (13). The role of EBV in development of different types of lymphoma remains unclear and controversial. Studies reveal conflicting results about the role of EBV in the pathogenesis of CNS DLBCL in immunodeficient and immunocompetent individuals (5, 14, 15). It is now agreed that EBV infection plays a pivotal role in pathogenesis of CNSL in immunocompromised patients, but further studies are warranted to judge on its etiologic role in PCNSLs of immunocompetent ones.

C-MYC is a proto-oncogene, coding a transcriptional factor involved in various cellular processes, including growth, proliferation, and apoptosis. *C-MYC* overexpression is essential in the tumorigenesis of

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multiple malignancies, particularly Burkitt lymphoma and some other aggressive subtypes of B-cell lymphomas like diffuse large B-cell lymphoma. Only a few studies have investigated *C-MYC* amplification status in PCNSLs (6, 15).

The management of PCNSL still remains challenging in clinical settings. Recent rise in incidence of PCNSL along with unknown pathogenesis and absence of a molecular prognostic marker has highlighted the necessity of molecular studies in this field. To our knowledge, only few recent studies with limited sample sizes have analyzed MGMT gene methylation or protein expression status in PCNSLs (16-20). We aimed to investigate the prevalence of loss of MGMT protein expression, a surrogate marker of *MGMT* methylation, in a large number of CNS DLBCLs. Moreover, we assessed the association of MGMT expression with molecular subtype and other potential prognostic biomarkers such as BCL2, MUM1, BCL6, Ki67, *C-MYC*, and EBER. Results of this study will aid in recognizing possible molecular alterations contributing to the pathogenesis of PCNSLs. The expression status of these markers may become of major clinical application for prognostic stratification of the patients and predicting tumor sensitivity to novel therapeutics in the near future.

Materials and methods

We designed a retrospective cross-sectional study to evaluate MGMT expression in CNSLs and assess its association with the status of *C-MYC* and EBER. Further, we evaluated the relationship of MGMT expression status with age, sex, and other histopathologic characteristics of the tumor, including Ki67 proliferating index, CD10, BCL6, BCL2 and MUM1expression, and phenotypic subtype.

Tissue paraffin blocks and patients

Of the 109 histologically confirmed CNS diffuse large B-cell lymphoma cases, consecutively recruited from the archives of pathology departments of multiple centers in Tehran, Iran, in a 10-year period, 78 cases of PCNS-DLBCL and two immunodeficiency-associated CNS-DLBCL cases (an AIDS-related CNSL and a post-transplant CNSL occurring in the context of long-term immunosuppression) were enrolled in this study. Cases of DLBCL with the involvement of the dura, intravascular large B-cell lymphomas, secondary lymphomas with the evidence of systemic involvement at the time of diagnosis or with unknown history of prior systemic lymphoma were excluded. Formalin-fixed paraffin-embedded tissue blocks from all patients were retrieved and hematoxylin and eosin (H&E)-stained sections were reviewed to define and mark representative tumor areas for construction of tissue microarray (TMA) blocks. From each donor block, 1-3 tissue cylinders with a diameter of 1 mm were punched from different selected regions of the tumor and arrayed into recipient TMA blocks. Proper mapping for cores was done.

Immunohistochemical (IHC) staining and scoring

MGMT, CD10, MUM1, BCL2 and BCL6 expression status as well as Ki-67 proliferating index were determined based on IHC staining using monoclonal antibodies specific for MGMT (clone MT 3.1, ThermoFisher), CD10 (clone 56C6, Dako), MUM1 (clone MUM1p, Dako), BCL2 (clone 124; Dako), BCL6 (clone LN22, Dako) and Ki-67 antigen (clone MIB-1,Dako). Paraffin-embedded TMA blocks were sectioned at 4μm by suitable microtome. Prepared slides were deparaffinized routinely, and pretreated with heat for epitope retrieval and stained by appropriate dilutions of primary monoclonal antibodies MGMT, CD10,

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MUM1, BCL2, BCL6 and Ki67 according to the manufacture's protocol. The chromogen 3, 3′-diaminobenzidine was used to detect the immunoreactivity. Positive and negative controls were stained in parallel to ensure the reliability of staining results. The negative control included all steps of immunostaining with the exclusion of primary antibody. Normal tonsillar tissue was used as the positive control for MGMT protein staining. Two pathologists scored immunostained slides of all samples blindly without knowing the clinical characteristics. MGMT and Ki-67 were interpreted as the percentage of stained tumor cells nuclei. MGMT protein expression was considered as positive when 10% or more of tumor cells nuclei stained in IHC, as previously described by Sahara *et al* (21). A cut-off value of 30% was used for interpreting BCL6 and MUM1 protein expression on IHC as defined by Hans et al. (10). We interpreted a BCL2 immunostaining of >50% as positive according to the 2016 revision of the World Health Organization (WHO) classification of lymphoid neoplasms (1).

Determining the phenotypic subtype of the tumors

The cases were categorized into GCB or ABC (also known as non-GCB) phenotypes according to CD10, BCL6, and MUM1 immune expression as proposed by Hans et al. (10). The tumors were classified as non-GCB subtype when they were negative for CD10 and positive for MUM1, regardless of the expression status of BCL6. The tumors were assigned to GCB (ABC) phenotype if they stained positive for CD10 or displayed a pattern of immunostaining that was negative for CD10 and MUM-1, and positive for BCL-6.

Chromogenic InSitu Hybridization (CISH) for C-MYC and EBER

Detection of EBV genome was performed by chromogenic in situ hybridization (CISH) for EBV encoded mRNA including both EBER-1 and EBER-2 encoding regions, using a one-colored EBER probe (Zyto*Fast* ® EBV Probe, Prod. No. T-1114-400, Zytovision Company, Germany). Results were interpreted positive if more than 20% of tumor cells stained after counting at least 500 cells by light microscopy in comparison with the results of positive and negative controls.

MYC gene amplification also evaluated by CISH method using the one colored digoxigenin-labeled probe (Zyto*Dot* ® SPEC *MYC* Probe, Prod. No. C-3013-400, Zytovision Company, Germany) specific for the *MYC* gene region at 8q24.21. Normal diploid cells were expected to exhibit two dot-shaped signals with smooth round borders in each nucleus. It was designated as low gene amplifications or gain of chromosomes if the nuclei exhibited multiple dots or small clusters. Furthermore, nuclei demonstrating abundant dots or large clusters comprising an area exceeding five dots, would be designated as high gene amplifications. All CISH slides were investigated blindly by two expert pathologists.

Statistical analysis

Statistical analyses were performed using SPSS software version 24.0. Quantitative data were shown as mean values± standard deviation and qualitative variables were expressed as number (percent). The association of biomarkers' positivity with molecular subgroups and other histopathologic and demographic parameters were assessed using chi-square and Fisher's exact tests. Moreover, to compare the mean percentage of MGMT, Ki67, MUM1 and BCL6 expression and age between GCB and non-GCB molecular subgroups and other categorical parameters, either Mann Whitney U or t-test was used, depending on the variable data distribution pattern. The Kolmogorov–Smirnov test was used to test for the normality of data distribution for quantitative variables. The threshold for statistical significance was set at $P < 0.05$.

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Results

Participants' demographic, clinical and biomarkers characteristics

Participants included 78 patients with a mean age of 53.8 ±17.3 years, ranging from 7 to 84 years. CNSL was seen to occur mainly in the 5th-8th decade of life (78.2% of the patients) with a predilection to male sex (M/F ratio of 2:1). The most common pre-operative clinical diagnoses were astrocytoma/glioma (42.9%), lymphoma (24.5%), metastasis (24.5%) and glioblastoma (8.2%). The most prevalent anatomic location of the tumor was the supratentorial area (80.8%). Multifocality was seen in 12.5% of the patients. The most common presenting signs and symptoms included neurological motor deficit (26.9%), loss of consciousness (23.1%), and ataxia/vertigo (15.4%). Table 1 describes demographic and clinical characteristics of the study participants.

Table 1. Demographic, clinical and histologic characteristics of study population.

Parameter	Mean±SD	No. (%)	IHC/CISH findings	Mean±SD	No. (%)
Age	53.8±17.3		Ki-67 (%)	79.6±20.0	
Gender			≥90		40 (51.9)
Male		51 (65.4)	<90		37 (48.1)
Female		27 (34.6)	BCL6		
Tumor Site			Positive		35 (46.1)
Supratentorial		63 (80.8)	Negative		41 (53.9)
Infratentorial		7 (9.0)	MUM1		
Periventricular		5 (6.4)	Positive		53 (72.6)
Spinal cord		3 (3.8)	Negative		20 (27.4)
Preoperative Clinical diagnosis			MGMT		
Astrocytoma/glioma		17 (21.8)	Positive		23(31.1)
Lymphoma		12 (15.4)	Negative		51 (68.9)
Metastasis		11 (14.1)	BCL2		
Glioblastoma		4 (5.1)	Positive		69 (90.8)
Meningioma		2 (2.6)	Negative		7 (9.2)
Ependymoma		1 (1.3)	CD10		
Choroid plexus papilloma		1 (1.3)	Positive		13 (17.1)
Not specified		30 (38.5)	Negative		63 (82.9)
Presenting symptoms			C-*MYC*		
Hemiparesis/hemiplegia		7 (9.0)	Positive		14 (18.4)
Loss of consciousness		6 (7.7)	Negative		62 (81.6)
Ataxia/vertigo		4 (5.1)	EBER		
Visual impairment		3 (3.8)	Positive		2 (2.6)
Sphincter dysfunction		2 (2.6)	Negative		75 (97.4)
N/V/headache/Seizure		3 (3.8)	Phenotypic Subtype		
Paresthesia/numbness		1 (1.3)	GCB		17 (22.4)
Not provided		52 (66.7)	Non-GCB (ABC)		59 (77.6)

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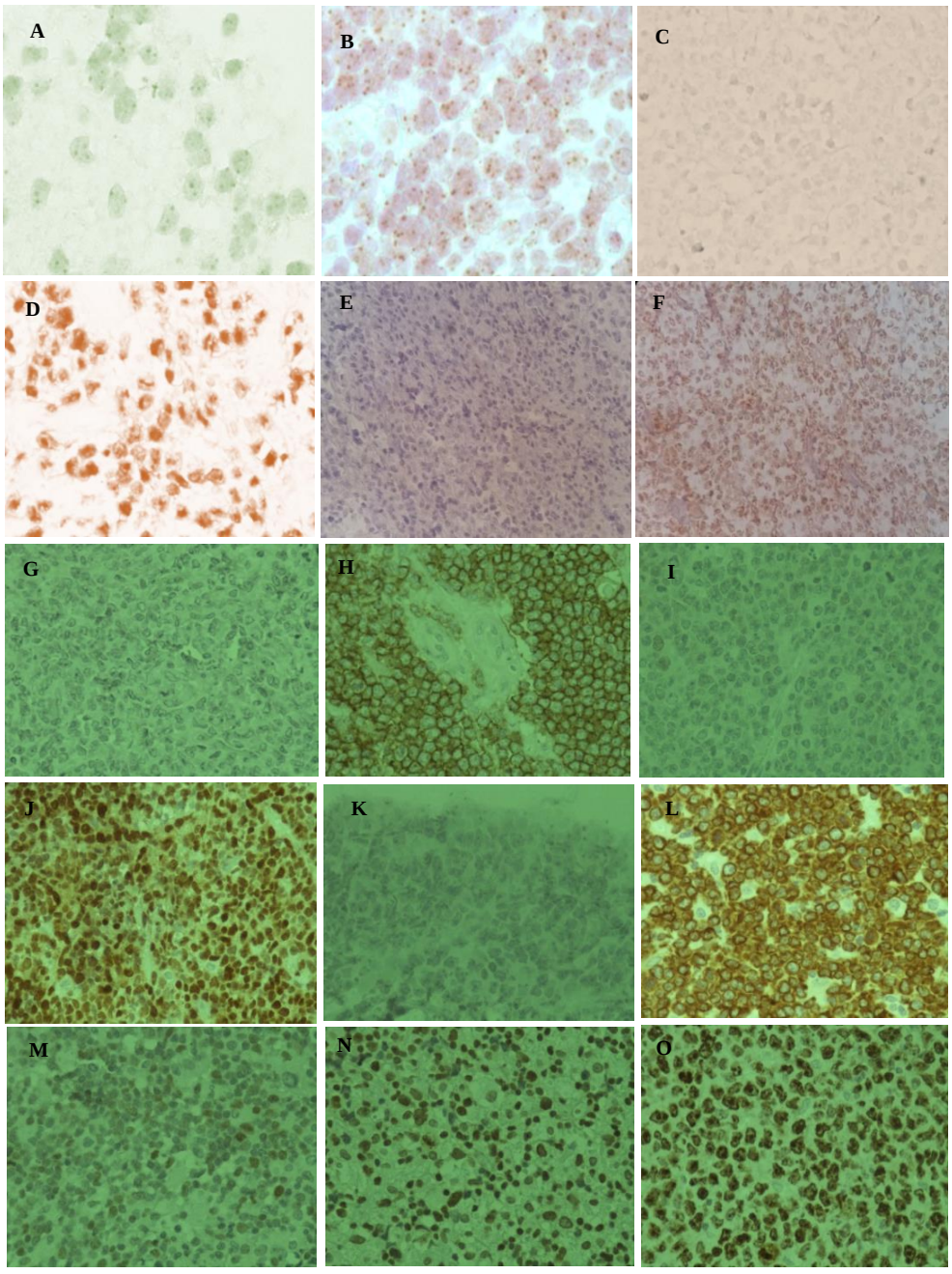

Fig. 1. Cases of CNS DLBCL that show negative *C-MYC* amplification (×1000) (A), Low *C-MYC* amplification (×1000) (B); Negative for EBER (×1000) (C), Positive for EBER (×1000) (D); Loss of MGMT protein expression (×400) (E); Preserved MGMT expression (×400) (F); Negative CD10 expression (×400) (G); Positive CD10 expression (×400) (H); Negative MUM1 expression (×400) (I); Positive MUM1 expression (×400) (J); Negative BCL2 expression (×400) (K); Positive BCL2 expression (×400) (L); Negative BCL6 expression (×400) (M); Positive BCL6 expression (×400) (N); Positive for Ki-67 with a high expression of >95% (×400) (O);

Most of the tumors (77.6%), including both immunodeficiency-related CNSLs, were of non-GCB type. The majority of the CNS DLBCLs showed loss of MGMT expression and only about one third of the tumors,

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including the two immunodeficiency-related CNSL cases, showed preserved MGMT expression on IHC. Low C-MYC amplification was seen in 18.4% of cases. No cases with high C-MYC amplification were identified. EBER was positive in the two (2.5%) patients, both of which were immunodeficient. None of the immunocompetent PCNSL cases showed trace of EBV on CISH study. MUM1, BCL6, BCL2 and CD10 were expressed in 72.6%, 46.1%, 90.8% and 17.1% of patients (Table 1 and Figure 1).

Association of MGMT expression with clinicopathologic variables

MGMT expression was associated with sex, CD10 and MUM1expression status, as well as phenotypic subtype (GCB or Non-GCB). Loss of MGMT expression was observed more in females and in MUM1-posittive, CD10-negative, and non-GCB type tumors (Table 2). Patients with loss of MGMT expression were older than those with preserved MGMT expression (54.9 ±17.2 vs. 50.8±19.1 years), though not statistically significant (P=0.42). Table 2 demonstrates MGMT expression percentage and positivity according to clinicopathologic variables**.**

Table 2. Clinicopathologic characteristics of patients in association with MGMT expression.

Variables	MGMT Reactivity No. (%) Positive	Negative	P-value	MGMT Expression (%) Mean ± S.D	P-value
Sex			0.04		0.14
Male	19 (38.8)	30 (61.2)		24.9±34.3	
Female	4 (16.0)	21 (84.0)		10.2±23.4	
MUM1 expression			<0.01		0.01
Positive	11 (21.2)	41 (78.8)		13.3±26.9	
Negative	11 (57.9)	8 (42.1)		39.7±38.1	
EBER			0.53		0.98
Positive	1(50.0)	1 (50.0)		12.5±17.7	
Negative	21 (29.6)	50 (70.4)		19.2±31.4	
C-*MYC*			0.82		0.87
Amplified	4 (28.6)	10 (71.4)		18.6±30.0	
Not amplified	19 (31.7)	41 (68.3)		20.2±32.3	
BCL2			0.67		0.59
Positive	20 (29.9)	47 (70.1)		18.7±31.0	
Negative	3 (42.9)	4 (57.1)		30.7±38.6	
BCL6			0.47		0.60
Positive	12 (35.3)	22 (64.7)		18.7±32.0	
Negative	11 (27.5)	29 (72.5)		21.2±31.7	
CD10			0.05		0.02
Positive	7 (53.8)	6 (46.2)		41.6±40.7	
Negative	16 (26.2)	45 (73.8)		15.2±27.7	
Ki67			0.92		0.45
≥ 90%	12 (31.6)	26 (68.4)		19.7±30.5	
< 90%	11 (30.6)	25 (69.4)		20.1±33.4	
Phenotypic Subtype			0.03		0.04
GCB	9 (52.9)	8 (47.1)		35.9±38.2	
Non-GCB	14 (24.6)	43 (75.4)		15.1±28.1	

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Association of C-MYC amplification and EBER status with clinicopathologic variables

C-MYC amplification showed a negative association with BCL2 expression, as 57.1 % (4/7) of BCL2-negative cases showed C-MYC amplification, while only 14.5% (10/69) of BCL2-positive cases simultaneously showed C-MYC amplification (P=0.019).

EBER positivity showed the association with immune status, as it was positive in both (100%) immunocompromised cases, but in none of the immunocompetent ones (P<0.001). Other histopathologic and demographic factors did not differ significantly between EBER-positive and EBER-negative or between C-MYC amplified and not amplified cases (P>0.05).

Association of molecular subtype with biomarkers status and clinicopathologic variables

MGMT expression was significantly higher in GCB group (P=0.04). Ki67 and BCL6 expression was not statistically different between GCB and non-GCB cases (P=0.27 and P=0.94 respectively). MUM1 was expressed more in non-GCB cases, as expected (P<0.001) (Figure 2). No difference was seen in the mean age of the patients between GCB and ABC phenotypes (P=0.17). C-MYC, EBER and BCL2 positivity, sex, and immune status showed no association with tumor subtype.

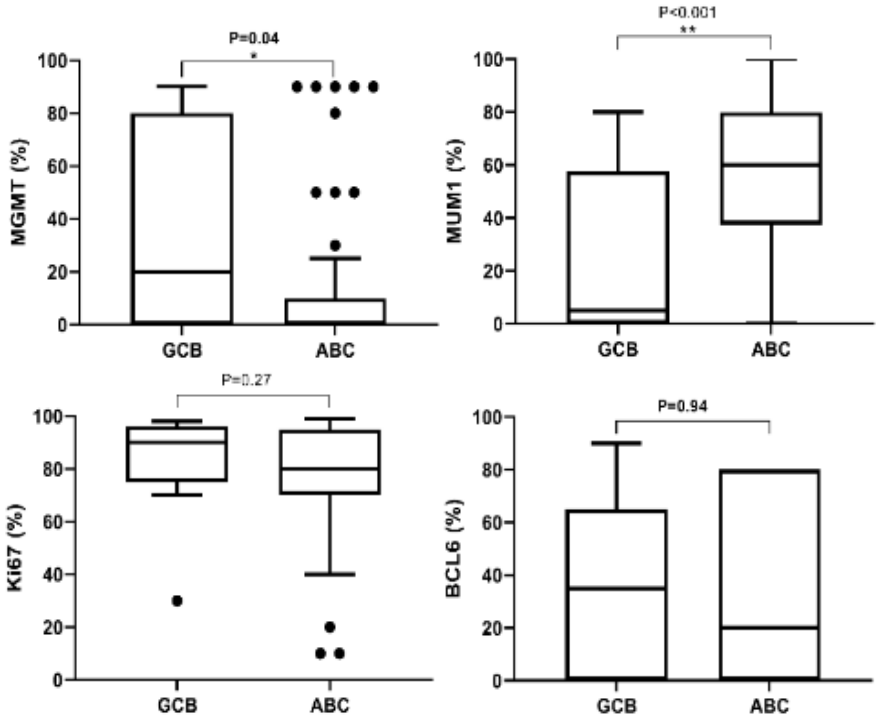

Fig. 2. Boxplots (Tukey method) comparing MGMT, MUM1, BCL6, and Ki67 % expression between GCB and ABC molecular subtypes.

Discussion

PCNSL is a rare and aggressive form of non-Hodgkin lymphoma that arises in the CNS without prior systemic involvement. Molecular alterations of PCNSL are not yet understood. The MGMT protein, a DNA damage repair enzyme, counteracts with cytotoxic effect of alkylating agents. *MGMT* methylation is claimed

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to have a prognostic significance and be responsible for sensitivity to alkylating agents such as temozolomide in different neoplasms. We found that about two-third of PCNS-DLBCLs show loss of MGMT expression on IHC. The results of the study suggest that a majority of PCNSLs are assumed to be sensitive to treatment with temozolomide, an FDA-approved drug used for glioblastoma with methylated *MGMT* (11, 12). Due to suboptimal and dose-dependent penetration of cytotoxic medications across the blood-brain barrier (BBB), PCNSLs exhibit an inferior response to chemotherapy in comparison to systemic DLBCLs. Despite the emergence of novel therapeutic options, the optimal therapeutic approach for PCNSL remains debated. Recently, Temozolomide has been effectively utilized in systemic non-Hodgkin's lymphomas. Temozolomide has been introduced as a favorable alternative for salvage therapy in patients with refractory or relapsed PCNSL or those who are unable to tolerate conventional chemotherapy regimen. *MGMT* gene promoter methylation and MGMT protein expression status may be used as promising predictors of response to alkylating agents in PCNSLs (16, 17). Evaluating MGMT expression status in CNS DLBCLs helps to screen a high proportion of PCNSL patients that would potentially benefit from alkylating agents. Future therapeutic approaches may stratify patients according to novel molecular biomarkers, including MGMT expression.

Emerging studies have recently examined MGMT expression and MGMT promoter methylation in PCNSLs (16-20). In a study by Shi *et al*., 32.9% (25/76) of PCNS-DLBCL cases showed loss of MGMT expression on IHC (18). Zheng *et al*. analyzed fifty-four cases of PCNS-DLBCL for *MGMT* methylation by pyrosequencing and reported an *MGMT* methylation frequency of 37% (20). According to Tofollati *et al*. *MGMT* gene methylation and loss of MGMT protein expression are frequent events in PCNSLs. They observed MGMT loss of expression in 54% (13/24) of cases with more occurrences in females and elderly (19). Similarly, our findings indicated that MGMT protein loss of expression in PCNSLs was more prevalent in females than males, but no association with age was found. Several other studies have also shown that *MGMT* methylation or loss of expression has a predilection for female sex in PCNSLs (18, 19).

We demonstrated that loss of MGMT expression was associated with histopathological indicators of aggressiveness in PCNSLs, i.e., MUM1 positivity, CD10 negativity and ABC molecular subtype, suggesting it as a prognostic factor in PCNSL. Prognostic impact of *MGMT* methylation is controversial. Shi *et al*. showed that in PCNS- DLBL patients aging over 60 years, the prognosis of those with MGMT-positive tumor on IHC was significantly better than MGMT-negative ones (18). However, some authors have shown no significant difference in median overall survival between methylated and unmethylated MGMT PCNS-DLBCL cases (20, 22). In contrast, studying 10 PCNSL cases who received high dose chemotherapy, Toffolatti et al. claimed that MGMT promoter methylation was associated with a prolonged overall survival. Further clinical trials are needed to confirm the prognostic significance of MGMT expression in PCNSL.

It is supposed that detection of loss of MGMT expression by IHC staining as a more feasible and accessible surrogate marker for *MGMT* gene methylation status, can be alternatively used for targeted therapy to select patients who will potentially benefit from alkylating agents. Several studies have shown that absent or a low level of MGMT protein expression on IHC, as a more applicable method, is associated with MGMT promoter methylation. Sahara et al. suggest implication of IHC with moderate diagnostic accuracy for detecting MGMT methylation status in gliomas (21). Tofollati *et al*. showed a high correlation between

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MGMT methylation with loss of protein expression in PCNSLs (19). Additional investigation is required to make a definite judgment regarding the implementation of IHC in routine clinical practice for the purpose of assessing MGMT status in PCNSLs and selecting appropriate patients for treatment with alkylating agents.

The classification of diffuse large B-cell lymphoma into GCB or ABC subtype is becoming increasingly important due to the development of innovative therapeutic strategies that specifically target the biological pathways in either subtype. Using Hans's model, 77.6% of CNS DLBCL cases were classified as non-GCB subtype in our study. GCB phenotype is uncommon in PCNSLs. In patients who present with DLBCL of GCB type in the CNS as the primary presentation, it is needed to exclude concurrent systemic lymphoma. In a study by Yin *et al*. 81.8% of PCNS-DLBCL cases were non-GCB type (15). Gill *et al*. evaluated 59 cases, out of which 41 (69%) had a non GCB cell-of-origin phenotype (23). Gandhi et al. classified 47 tissue EBV- and 44 EBV+ CNS DLBCL cases by a different method of assignment of cases into cell-of-origin groups (NanoString Lymph2Cx assay) and found that the majority (69%) of EBV- PCNSLs had ABC origin, while EBV+ cases had significantly a lower frequency of ABC phenotype (as low as 18%). Similarly, most EBV-positive systemic DLBCLs have an activated B-cell immunophenotype (24). These findings suggest a distinct origin of tumorigenesis in EBV-related DLBCLs compared to EBV-negative cases. Nevertheless, we observed an ABC phenotype in both EBER-positive CNS DLBCL cases tested in our study. We studied a large number of immunocompetent cases with PCNS DLBCL, all of whom were negative for EBER in their tumor cells. We propose that EBV might not assume a pivotal role in the pathogenesis of PCNSL in individuals with intact immunity, in contrast to systemic DLBCLs that harbor EBV in the tumor cells of a considerable number of cases without a history of immunodeficiency (25). Our study suggests the oncogenic role of EBV in the pathogenesis of CNSLs in immunocompromised individuals. However, due to the limited number of EBV-positive cases in our study, this result does not provide convincing evidence to deny a different tumorigenesis pathway for EBV-positive CNS DLBCLs compared to EBV-negative ones. Further studies with larger sample sizes are warranted to claim a definite relationship between EBER positivity in CNSL and immune status. Only a limited number of studies have investigated EBV in PCNSLs of immunocompetent patients. Contradictory results have been observed in these studies regarding the role of the Epstein-Barr virus (EBV) in the development of PCNSLs in immunocompetent individuals. While some authors argue that there is no evidence of EBV infection unless an underlying immunodeficiency is present (26, 27), others have reported a positive rate of EBER (Epstein-Barr virus-encoded small RNA) in approximately 6-30% of immunocompetent individuals with PCNS-DLBCL (15), most of which had multiple lesions. These contradictory findings can be attributed to differences in the methods (gene expression, protein expression, serology), type of sample examines (tumor tissue, cerebrospinal fluid, serum) and timing of EBV assessment. In patients with recurrent brain tumors in whom initial detection of EBV is negative but subsequent lesions show presence of EBV, the reactivation of EBV may be influenced by the administration of chemotherapy or steroids.

One noteworthy molecular alteration identified in systemic DLBCLs is the rearrangement of the *C-MYC* oncogene, a phenomenon that is correlated to an unfavorable prognosis (28). There are a few studies on *C-MYC* alterations in PCNS DLBCL. We observed that *C-MYC* amplification is not a common molecular alteration in PCNS-DLBCL. Low levels of *C-MYC* amplification was detected in 18% of our cases, similar

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to that has been reported for systemic DLBCLs (5-15%) (1). However, previous investigations have demonstrated that MYC protein expression is much more frequent in both systemic and PCNS DLBCLs (1). Yin *et al*. detected C-MYC protein expression in 12 out of 33 cases (36.4%) (15). Several authors have reported a higher incidence of C-MYC expression in PCNSLs using IHC method and have asserted that its expression is associated with a poor outcome (23, 29). We did not discover a correlation between *C-MYC* amplification and histopathologic indicators of aggressiveness, but found that *C-MYC* amplification was inversely associated with BCL2 expression. Although BCL2 overexpression has been proposed as a predictor of inferior outcome in systemic and PCNS DLBCLs by some authors (30, 31), its prognostic significance remains controversial. Taking into consideration various factors such as the methodology employed in determining marker positivity (whether gene alterations or protein expression were evaluated), the type of measurement (quantitative or qualitative), and the specified cut-offs for interpreting test results is crucial in formulating a more precise statement regarding the prognostic significance of C-MYC and BCL2 in DLBCL of CNS. The cut-off points mentioned for BCL2 expression show considerable variation across different studies ranging from 30% to 85%, thereby giving rise to divergent interpretations. It is now universally agreed that co-expression of MYC and BCL2 proteins serves as an independent predictor of outcome in DLBCLs. Limited investigations have examined the prognostic significance of double hit lymphomas in PCNS DLBCLs. Unfortunately, in our study, we were unable to assess the frequency of double-hit lymphomas due to the fact that BCL2 was evaluated at the protein expression level, while the examination of C-MYC focused on the alteration in gene copy number. Due to the limited number of *C-MYC* amplified cases in our study, the findings may not be convincing enough to make conclusive judgment about the role of *C-MYC* and BCL2 alterations in the molecular pathogenesis of PCNS DLBCL.

This study encountered several limitations that should be taken into account in future studies. Specifically, we utilized immunohistochemistry to assess the protein expression of MGMT. However, the methylation status of MGMT promoter and its association with protein expression in PCNS DLBCLs remains undisclosed. Moreover, the limited number of immunodeficiency-related CNS DLBCL cases in our study has adversely impacted the research findings regarding the pathogenesis of immunodeficiency-related CNS DLBCL. On the contrary, the strength of this study lies in the comparatively large sample size as compared to previous investigations.

Assessing MGMT, EBER and C-*MYC* status in a considerable number of CNS DLBCL cases, we have put forth a proposal regarding the prognostic significance of the loss of MGMT protein expression, a commonly observed phenomenon in PCNS DLBCLs. The contribution of EBV in the pathogenesis of PCNS DLBCL in individuals with intact immune system is questionable. Further investigation is necessary to acquire a comprehensive understanding of the molecular mechanisms underlying PCNSL in immunocompetent and immunocomprised individuals and to explore the potential role of *C-MYC* amplification and MGMT protein expression as a prognostic marker and therapeutic target in this aggressive form of lymphoma.

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