

Hypermethylation of the DNA Repair Gene *O*⁶-Methylguanine DNA Methyltransferase and Survival of Patients With Diffuse Large B-Cell Lymphoma

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Background: The gene encoding the DNA repair enzyme *O*⁶-methylguanine DNA methyltransferase (MGMT) is transcriptionally silenced by promoter hypermethylation in several human cancers, including diffuse large B-cell lymphoma (B-DLCL). MGMT promoter hypermethylation is a favorable prognostic marker in patients with brain tumors treated with alkylating agents. **Methods:** In a retrospective cohort study, we used methylation-specific polymerase chain reaction to analyze the MGMT promoter methylation status in tumor DNA of B-DLCL patients receiving cyclophosphamide as part of multidrug regimens. Molecular data were compared with patient response with the use of Student's *t* test. Disease-free survival and overall survival were estimated by the Kaplan–Meier method and compared with the use of the log-rank test. Multivariable survival analyses were performed with the Cox proportional hazards model. All statistical tests were two-sided. **Results:** Thirty (36%) of 84 B-DLCL patients showed MGMT promoter hypermethylation in their lymphomas. The presence of MGMT methylation was associated with a statistically significant increase in overall survival (hazard ratio for time to death for nonmethylation versus methylation = 2.8; 95% confidence interval (CI) = 1.2 to 7.5; *P* = .01) and progression-free survival (hazard ratio for time to progression for nonmethylation versus methylation = 2.6; 95% CI = 1.3 to 5.8; *P* = .02). MGMT promoter hypermethylation was both independent of and stronger than established prognostic factors, such as age, disease stage, serum lactic dehydrogenase level, and performance status. **Conclusion:** MGMT promoter hypermethylation appears to be a useful marker for predicting survival in patients with B-DLCL treated with multidrug regimens including cyclophosphamide. [J Natl Cancer Inst 2002;94:26–32]

The term diffuse large B-cell lymphoma (B-DLCL) is thought to include more than one disease entity, and patients with B-DLCL have a highly variable clinical behavior, outcome, and natural history (1,2). Although advances in treatment and the identification of clinical indicators have led to improved prognosis and have allowed some tailoring of therapy, approximately half of the patients with B-DLCL still fail the therapy and die of their disease. Because the pathogenesis of B-DLCL is a heterogeneous process involving multiple, independent molecular pathways, it has been proposed that the tumor genotype may affect the clinical behavior and the outcome of the disease (1,3). The identification of new molecular prognostic markers, therefore, may help to further stratify patients into different risk groups.

Recently, the gene encoding the DNA repair enzyme *O*⁶-methylguanine DNA methyltransferase (MGMT) has been found to be inactivated in several human cancers, including a fraction of B-DLCLs (4). The MGMT protein (E.C. 2.1.1.63), also known as *O*⁶-alkylguanine-DNA alkyltransferase (AGT), protects cells from the toxicity of alkylating agents, which frequently target the *O*⁶ position of guanine (5,6). The MGMT protein rapidly reverses the formation of adducts at the *O*⁶ position of guanine via transfer of the alkyl adduct to a cysteine residue within the protein (5,6), thereby averting the formation of lethal cross-links and other mutagenic effects. Thus, MGMT activity is a major mechanism of resistance to alkylating drugs (5,6).

In human cancer, the MGMT gene is not commonly mutated or deleted; thus, loss of MGMT function is most frequently due to epigenetic changes, specifically promoter region methylation. Hypermethylation of the MGMT CpG island as the cause of MGMT transcriptional silencing in cell lines defective in *O*⁶-methylguanine repair has been demonstrated (4,7–9). Furthermore, *in vitro* treatment of cancer cells with demethylating drugs restores MGMT expression (7,10). Aberrant MGMT methylation has been associated with loss of messenger RNA (mRNA) expression (10), lack of MGMT protein (4,11), and loss of enzymatic activity (11) in noncultured neoplasia tissue as well. The level of MGMT activity and expression varies widely in tumors; some tumors have abundant and other tumors have undetectable MGMT activity. For example, lack of MGMT activity has been detected in approximately 30% of brain tumors (12,13) and has been suggested to be associated with enhanced sensitivity to the action of alkylating agents (14–16). Recently, we (17) have reported that the presence of MGMT promoter region methylation in brain tumors was a strong predictor of response, overall survival, and time to disease progression in patients treated with the alkylating agent carmustine. B-DLCL is treated with the alkyl-

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ating agent cyclophosphamide; therefore, this study aimed at defining whether MGMT inactivation by promoter hypermethylation could provide novel prognostic information for B-DLCL patients treated with this drug.

PATIENTS AND METHODS

Patient Population and Specimen Procurement

Eighty-four patients with previously untreated B-DLCL were used for this study. The patients had been consecutively diagnosed and treated from 1986 through 1997 at three Italian institutions that had DNA available for study. Informed written consent was obtained from the patients, and tissue collection was approved by each Institutional Review Board. Clinical follow-up was obtained until August 31, 1999, or until death. The median follow-up duration from initiation of treatment for censored patients was 61 months. Patients were censored if they were alive at the date of the last follow-up visit, independent of remission status. Diagnosis was based on histopathology, immunophenotypic analysis of cell surface markers (CD10, CD19, CD20, CD22, CD79a, CD3, CD5, CD43, CD45RO, CD15, CD30, and CD45), and immunogenotypic analysis of immunoglobulin gene rearrangement, performed with the use of a J_H probe on *HindIII*, *EcoRI*, and *BamHI* digests and a J_k probe on *BamHI* digests. The histopathologic definition of B-DLCL was according to the REAL (i.e., revised European-American classification of lymphoid neoplasms) classification (1). Patients who were positive for human immunodeficiency virus were not included in the study. Disease staging included routine blood chemistry tests; blood cell counts, and differential; electrocardiogram; chest x-ray; computed tomography (CT) scan of chest, abdomen, and pelvis; and bilateral bone marrow biopsy in all patients. Disease stage was assessed according to Ann Arbor criteria (18). The International Prognostic Indicator (IPI) was calculated as described previously (19), with patients classified as low, low-intermediate, high-intermediate, and high risk.

Treatment of patients varied, depending on the stage of their disease, date of diagnosis, institution, and prognostic factors. All patients, however, were treated with cyclophosphamide and an anthracycline-containing regimen. Nine patients with localized stage of disease without adverse prognostic features were treated with a brief chemotherapy, ACOBP (i.e., a combination of doxorubicin [Adriamycin], cyclophosphamide, vincristine, prednisone, and bleomycin) or three courses of CHOP (i.e., a combination of cyclophosphamide, doxorubicin, vincristine, and prednisone), followed by locoregional radiotherapy at a dose of 36 Gy. Forty-two patients with disease at a localized stage and adverse prognostic features or advanced-stage disease were treated with CHOP (29 patients) or a third-generation chemotherapy scheme, such as MACOPB (i.e., a combination of methotrexate, doxorubicin, cyclophosphamide, vincristine, prednisone, and bleomycin) (six patients) or VACOPB (i.e., a combination of etoposide, doxorubicin, cyclophosphamide, vincristine, prednisone, and bleomycin) (seven patients). Fifteen elderly patients, older than 65 years, received PVEBEC (i.e., a combination of prednisone, vinblastine, epirubicin, bleomycin, etoposide, and cyclophosphamide). Eighteen patients with advanced-stage disease and adverse prognostic features were treated with a reduced course of standard chemotherapy (MACOPB or CHOP), followed by an intensification chemotherapy with peripheral blood stem cell harvest and high-dose BEAM che-

motherapy (i.e., a combination of carmustine, etoposide, cytarabine, and melphalan) with autologous stem cell transplantation.

Response to treatment was evaluated after the completion of the therapeutic program. Restaging tests included blood chemistries, CT scans of chest, abdomen, and pelvis in all patients, and repetition of bone marrow biopsy if abnormal at diagnosis. Complete remission (CR) was defined as the absence of any detectable disease. Patients with persistent CT abnormalities but regression greater than 75% of initial tumor volume with no signs or symptoms of active disease were considered to be in CR if the radiologic abnormalities were subsequently stable for at least 3 months. A partial remission (PR) was defined as a 50% or greater reduction in tumor volume. Failure was defined as anything less than a PR, progressive disease, or treatment-related death.

Analysis of MGMT Promoter Hypermethylation by Methylation-Specific Polymerase Chain Reaction

DNA was extracted from tumors according to standard protocols. DNA methylation patterns in the CpG island of MGMT were determined by chemical modification of unmethylated but not the methylated cytosines to uracil and subsequent polymerase chain reaction (PCR) using primers specific for either methylated or the modified unmethylated DNA as described previously (4,20). DNA (1 μ g) was denatured by NaOH and modified by sodium bisulfite. DNA samples were then purified with the use of Wizard DNA purification resin (Promega Corp., Madison, WI), again treated with NaOH, precipitated with ethanol, and resuspended in water. Primer sequences for the unmethylated reaction were 5'-TTT GTG TTT TGA TGT TTG TAG GTT TTT GT-3' (forward) and 5'-AAC TCC ACA CTC TTC CAA AAA CAA AAC A-3' (reverse); primer sequences for the methylated reaction were 5'-TTT CGA CGT TCG TAG GTT TTC GC-3' (forward) and 5'-GCA CTC TTC CGA AAA CGA AAC G-3' (reverse). The annealing temperature was 59°C. Placental DNA treated *in vitro* with *SssI* methyltransferase (New England Biolabs Inc., Beverly, MA) was used as a positive control for methylated alleles of MGMT, and DNA from normal lymphocytes was used as a negative control for methylated alleles of MGMT. Controls without DNA were performed for each set of PCR. Ten microliters of each PCR reaction was directly loaded onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

Analysis of MGMT Expression by Immunohistochemistry

The association between MGMT methylation status and MGMT protein expression was assessed in a representative panel of 26 lymphomas randomly selected on the basis of availability of sections. Sections of formalin-fixed, paraffin-embedded tissue sections were deparaffinized with xylene for 30 seconds, dehydrated by use of graded ethanols, and treated for 30 minutes in TEC [i.e., 2 mM Tris(hydroxymethyl)aminomethane, 1.3 mM EDTA, and 1.1 mM trisodium citrate dihydrate] solution (pH 7.8) in a microwave oven at 250 W. Immunohistochemistry was performed with the use of the ABC method (ABC-Elite kit; Vector Laboratories Inc., Burlingame, CA). Immunoperoxidase staining with the use of diaminobenzidine as chromogen was performed on an automated immunostainer (Ventana Medical Systems, Inc., Tucson, AZ) according to the company's protocols. Commercially available mouse anti-

MGMT monoclonal antibody (clone MT3.1; Chemicon International, Temecula, CA) at 1 : 100 was used (21). The antibody has been demonstrated previously to be useful for immunohistochemistry and to be associated with MGMT activity (4,22). Nuclear staining was determined by two authors (A. Gloghini and A. Carbone), who did not have knowledge of the molecular analysis of the samples.

Statistical Analysis

Student's *t* test was used to compare continuous variables. Contingency tables were analyzed by Fisher's exact test. Disease-free survival and overall survival were estimated by the Kaplan–Meier method and were compared with the use of the log-rank test. Multivariate survival analyses were performed with the Cox proportional hazards model, and proportional hazards assumptions were checked with the use of Schoenfeld residuals and graphical methods. Descriptive or stratified analyses always preceded parametric modeling to confirm that assumptions of the models were met. All reported *P* values are two-sided, and all confidence intervals (CIs) are quoted at the 95% level. Analyses were performed with the use of JMP 3.1 (SAS Institute, Inc., Cary, NC) and Stata 6.0 (Stata Corp., College Station, TX).

RESULTS

We examined MGMT promoter hypermethylation in tumors of 84 patients with B-DLCL. A clinical description of the patient population is given in Table 1. MGMT hypermethylation was found in 30 (36%) of the 84 samples (see examples given in Fig. 1). Similar to our previous study (4), MGMT hypermethylation was associated with absent MGMT protein expression (two-tailed Fisher's exact test, $P < .0001$), since all ($n = 17$) lymphoma samples carrying MGMT hypermethylation had failed to express the protein as tested by immunohistochemistry (not shown). Conversely, all ($n = 9$) lymphoma samples carrying unmethylated MGMT alleles and tested by immunohistochemistry expressed the MGMT protein.

The presence of MGMT methylation was not associated with any difference in clinical stage, performance status (Eastern Cooperative Oncology Group), or lactic dehydrogenase (LDH) levels (see Table 1; all *P* values .15). Among patients with MGMT

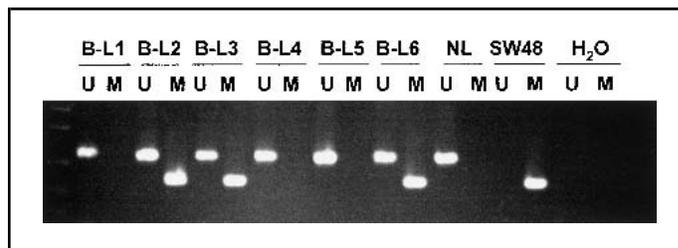


Fig. 1. Methylation-specific polymerase chain reaction (PCR) analysis of *O*⁶-methylguanine DNA methyltransferase (MGMT) promoter methylation status in tumor DNA of patients with diffuse large B-cell lymphoma. *Msp*I digest of pBR322 is shown at left as molecular weight markers. The presence of a visible PCR product in those lanes marked "U" indicates the presence of unmethylated genes; the presence of product in those lanes marked "M" indicates the presence of methylated genes. The cancer cell line SW48 was used as positive control for methylation, normal lymphocytes (NL) were used as a negative control for methylation, and water (H₂O) was used as negative PCR control. The samples B-L2, B-L3, and B-L6 show MGMT promoter hypermethylation.

methylation ($n = 30$), 23 (77%) experienced CR, four (13%) experienced PR, and three (10%) experienced no response (NR); among those not showing methylation ($n = 54$), the relative figures were 34 (63%) experiencing CR, eight (15%) experiencing PR, and 12 (22%) experiencing NR. The higher response rate in patients with tumors containing MGMT methylation was not statistically significant ($P = .3$) but is consistent with an increased sensitivity of lymphomas with MGMT methylation to alkylating agents.

However, as we had observed previously for patients with high-grade gliomas (17), the MGMT methylation status in lymphoma patients was strongly associated with overall survival and progression-free survival. Overall survival was statistically significantly increased among lymphoma patients having MGMT methylation, with a hazard ratio (HR) for nonmethylation versus methylation for the outcome of death of 2.8 (95% CI = 1.2 to 7.5; $P = .01$) (Fig. 2, A). Similarly, the HR for disease progression among those without methylation versus those with methylation was 2.6 (95% CI = 1.3 to 5.8; $P = .02$; Fig. 2, B). The traditional markers of prognosis in non-Hodgkin's lymphoma, which form the International Lymphoma Study Group classification (19)—namely, performance status, LDH levels, and disease stage—had weak or modest univariate associations with survival. However, in multivariable survival models, the MGMT methylation status was consistently the most important predictor. In addition to MGMT methylation, only disease stage was statistically significant. In a model where stage was dichotomized (stages 1 and 2 versus stages 3 and 4), the HR outcome for time to death for the higher stages was 2.4 (95% CI = 1.1 to 6.6; $P = .03$), and that for nonmethylation was virtually identical to the univariate result (HR = 2.7; 95% CI = 1.2 to 7.2; $P = .02$). Similar results were obtained for time to disease progression for stage (HR = 2.5; 95% CI = 1.2 to 5.8; $P = .01$) and nonmethylation status (HR = 2.5; 95% CI = 1.2 to 5.5; $P = .01$).

The IPI incorporates these individual factors (age, stage, bone marrow involvement, LDH levels, and the performance status) into a useful prognostic indicator (19). To determine whether MGMT methylation was still predictive of survival, we examined MGMT in relation to IPI. As demonstrated previously (19), the IPI was predictive of time to death, with an HR of 1.6 (95% CI = 1.1 to 2.3; $P = .009$) when IPI was coded as a continuous variable. MGMT remained predictive of overall survival in this

Table 1. Clinicopathologic characteristics of diffuse large B-cell lymphoma patients as a function of *O*⁶-methylguanine DNA methyltransferase promoter methylation status

	Unmethylated (n = 54)		Hypermethylated (n = 30)		<i>P</i> *
	No.	%	No.	%	
Stage					
I–II	15	28	11	37	.46
III–IV	39	72	19	63	
Performance status†					
0–1	35	65	22	73	.47
2–3	19	35	8	27	
Serum lactic dehydrogenase level					
≤450 U/L	22	41	14	47	.64
>450 U/L	26	48	13	43	
Data not available	6	11	3	10	

*All *P* values are obtained from two-tailed Fisher's exact tests.

†Eastern Cooperative Oncology Group.

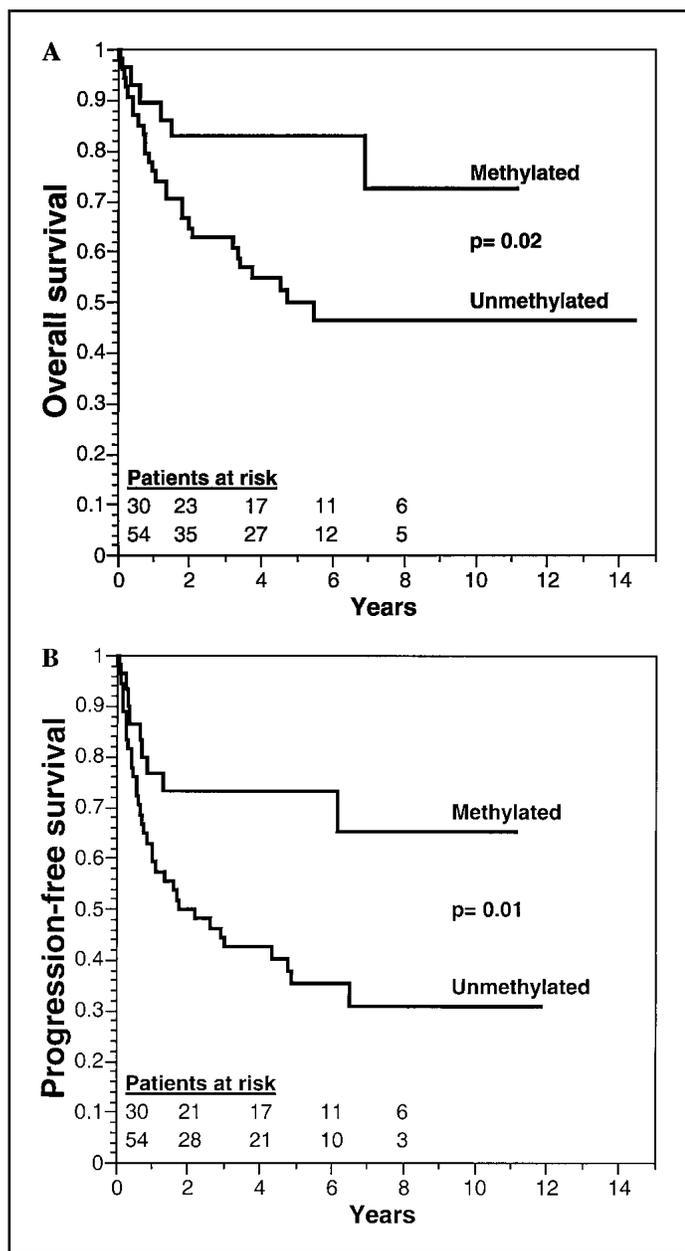


Fig. 2. Kaplan–Meier analysis of O^6 -methylguanine DNA methyltransferase (MGMT) promoter hypermethylation in patients with diffuse large B-cell lymphoma treated with cyclophosphamide and its impact on survival. Numbers of patients at risk are shown at bottom of each panel. **A)** Overall survival as a function of MGMT methylation status. At year 5, for the group with hypermethylation, overall survival is 0.83 (95% confidence interval [CI] = 0.69 to 0.97); for the group with no hypermethylation, overall survival is 0.50 (95% CI = 0.36 to 0.64). **B)** Failure-free survival as a function of MGMT methylation status. At year 5, for the group with hypermethylation, progression-free survival is 0.73 (95% CI = 0.57 to 0.89); for the group with no hypermethylation, progression-free survival is 0.35 (95% CI = 0.21 to 0.49). Log-rank test was used to determine P value.

multivariable analysis (HR = 2.3; 95% CI = 1.0 to 6.2; P = .05). For time to disease progression, the IPI as a continuous variable was also prognostically important (HR = 1.4; 95% CI = 1.0–2.0; P = .02), but MGMT methylation remained an independent predictor of time to progression (HR for nonmethylation = 2.2; 95% CI = 1.06–4.9; P = .03) in this multivariable analysis.

Fig. 3 is a graphical representation of the independent prog-

nostic role of IPI and MGMT promoter methylation. For visual clarity and because of small numbers in these eight possible categories, patients at low–intermediate, high–intermediate, and high IPI risk were combined. Fig. 3, A, shows the overall survival according to MGMT methylation status in patients with low IPI (L) and all other categories (>L) combined. It is apparent that much of the MGMT effect is seen in the low-IPI group. Likewise, much of the effect of MGMT methylation on progression-free survival was seen in patients with low IPI (Fig. 3, B).

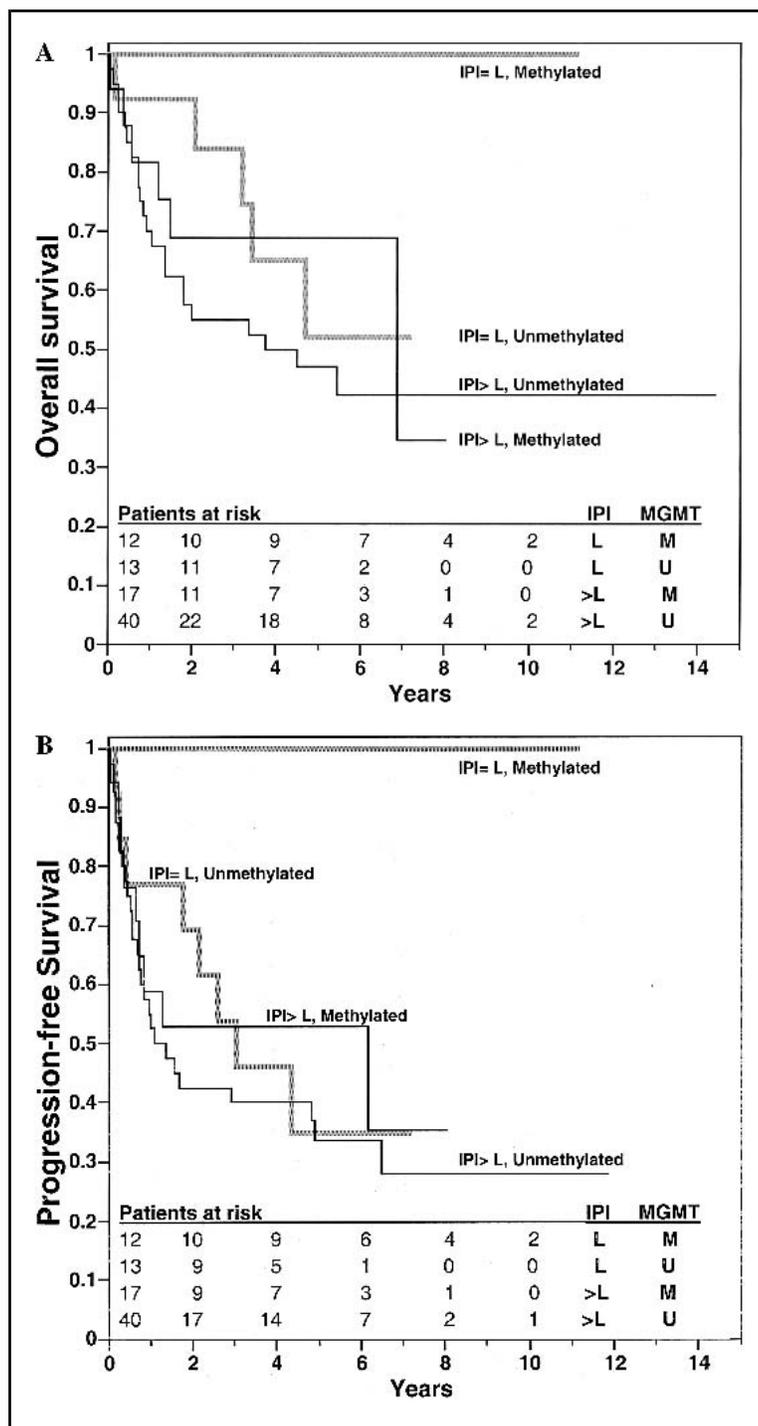
DISCUSSION

This study reports that MGMT promoter hypermethylation may provide a novel independent marker for the prognostic assessment of survival in patients with B-DLCL treated with multidrug regimens that include cyclophosphamide. In a recent report (17), we have shown that MGMT promoter hypermethylation correlates with an improved clinical response and an increase in overall survival and disease-free survival also in patients with gliomas treated with carmustine. As demonstrated for glioma patients (14–17), promoter hypermethylation, rather than enzyme activity, may be a more accurate strategy to assess MGMT status in human cancer. In fact, the presence of normal cells, including normal infiltrating lymphocytes, may make determination of MGMT activity within the tumor itself difficult. Our PCR approach eliminates the problems of infiltrating normal cells and thereby may more accurately separate tumors into those with and those without MGMT inactivation. Because hypermethylation of MGMT is associated with loss of mRNA expression and appears to be the only mechanism associated with loss of MGMT activity (4,7–10), one can study MGMT loss of function by assessing promoter hypermethylation. This approach examines the lesion itself (epigenetic inactivation of the promoter) rather than the effect of this alteration (loss of protein expression and enzyme activity).

Several hypotheses may explain the prognostic role of MGMT in predicting survival of patients with B-DLCL treated with alkylating agents. One hypothesis concerns the possibility that MGMT hypermethylation is a prognostic marker of natural history that identifies a specific pathogenetic subset of lymphomas with a more favorable outcome. While it is impossible to completely exclude this explanation, it appears to be independent of other reported prognostic markers. *A priori*, one would not expect MGMT methylation to be a positive prognostic indicator but perhaps a negative one, since MGMT hypermethylation has been associated with the formation of K-ras (also known as Kirsten ras) and p53 (also known as TP53) mutations (10,23), both of which are often negative prognostic markers. The prognostic role of MGMT hypermethylation cannot be ascribed to a clinical advantage of B-DLCL displaying a generalized methylated phenotype, since promoter hypermethylation of other genes frequently methylated in B-DLCL (24) (namely, the death-associated protein kinase gene) is not associated with outcome (our unpublished observation).

An alternative hypothesis to explain the prognostic importance of MGMT hypermethylation is that MGMT inactivation may render B-DLCL cells more prone to the genotoxic effects of alkylating agents, as it has been proposed recently in the case of glioma (17). In fact, the DNA repair protein MGMT is one of the key factors mediating resistance to these agents, and several reports suggest that MGMT does play a role in modulating the activity of cyclophosphamide at least *in vitro*, as demonstrated in

Fig. 3. Kaplan–Meier analysis of the independence of O^6 -methylguanine DNA methyltransferase (MGMT) promoter hypermethylation (U = unmethylated; M = methylated) and International Prognostic Index (IPI) in relation to survival of patients with diffuse large B-cell lymphoma. Patients were classified as being at low (L), low–intermediate, high–intermediate, or high risk according to the IPI criteria (19); for clarity of visual presentation, patients other than those in the L category were combined and categorized as >L. Numbers of patients at risk are shown **at the bottom of each panel**. **A)** Overall survival as a function of MGMT methylation status and IPI. At year 5, for the group with hypermethylation and low IPI, overall survival is 1.00 (95% confidence interval [CI] = 0.69 to 1.00); for the group with no hypermethylation and low IPI, overall survival is 0.52 (95% CI = 0.20 to 0.84). For the group with hypermethylation and IPI>L, overall survival is 0.69 (95% CI = 0.46 to 0.92); for the group with no hypermethylation and IPI>L, overall survival is 0.47 (95% CI = 0.31 to 0.63). **B)** Failure-free survival as a function of MGMT methylation status and IPI. At year 5, for the group with hypermethylation and low IPI, progression-free survival is 1.00 (95% CI = 0.69 to 1.00); for the group with no hypermethylation and low IPI, progression-free survival is 0.35 (95% CI = 0.07 to 0.63); for the group with hypermethylation and IPI>L, progression-free survival is 0.53 (95% CI = 0.29 to 0.77); for the group with no hypermethylation and IPI>L, progression-free survival is 0.34 (95% CI = 0.19 to 0.49). Log-rank test was used to determine *P* value.



lung cancer (25), medulloblastoma (26), and ovarian (CHO) cell lines (27). Thus, although MGMT has long been implicated in resistance to methylating and chloroethylating agents, it may also contribute to resistance to the cytotoxic and mutagenic effects of cyclophosphamide (28). It appears that MGMT activity is important in protecting against the toxicity of acrolein, one of the metabolites of cyclophosphamide, while the toxicity from the other metabolite, phosphoramidate mustard, is not repaired by MGMT (27). Increased sensitivity to alkylating agents conferred by MGMT inactivation may result in complete elimination of all transformed cells, which would otherwise lead to disease recurrence. The absence of statistical difference in initial response of

B-DLCL with and without MGMT hypermethylation is at variance with the behavior of glioma patients (17) and may be due to the presence of other potent and effective anticancer agents used as standard treatments for B-DLCL, such as doxorubicin, vincristine, and etoposide, that might have masked greater differences in response between methylated and unmethylated groups.

Despite these observations, the improved survival in cyclophosphamide-treated B-DLCL patients with MGMT hypermethylation cannot be attributed unequivocally to sensitivity to this alkylating agent. Such a conclusion would only be possible if cyclophosphamide were used alone and then only if a nontreat-

ment control was examined. This treatment strategy, however, is not appropriate, given the effectiveness of multidrug regimens for B-DLCL. A putative indirect approach to address the relationship between the MGMT status and B-DLCL sensitivity to cyclophosphamide may be the use of the MGMT inhibitor *O*⁶-benzylguanine (*O*⁶-BG) (26,29). *O*⁶-BG is an MGMT substrate that, by its binding to the protein in a suicide reaction, inactivates MGMT. While this inhibitor has been used primarily to enhance the response to alkyl-nitrosoureas both *in vitro* and *in vivo* (29,30), *O*⁶-BG has been shown to increase sensitivity to cyclophosphamide metabolites as well (31). The safety profile of *O*⁶-BG has allowed its use in phase I clinical trials (32). Our results prompt preclinical studies in animal models aimed at defining whether *O*⁶-BG has a role in the treatment of B-DLCL carrying unmethylated MGMT genes.

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NOTES

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Supported by Public Health Service grant P50CA58184 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services; by a grant from Ministero per l'Università e la Ricerca Scientifica e Tecnologica-Cofin 2000, Rome, Italy (to G. Gaidano); by a grant from Fondazione "Piera Pietro e Giovanni Ferrero," Alba, Italy (to G. Gaidano); and by a grant from Associazione Italiana per la Ricerca sul Cancro, Milan, Italy (to A. Carbone).

Manuscript received March 26, 2001; revised October 15, 2001; accepted November 8, 2001.