DNA methyltransferase DNMT3b protein overexpression as a prognostic factor in patients with diffuse large B-cell lymphomas

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Diffuse large B-cell lymphomas (DLBCL) are the most common type of aggressive lymphomas, with considerable heterogeneity in clinical presentation, molecular characteristics, and outcome. Previous studies have showed significant correlations between DNA methyltransferase (DNMT) overexpression and unfavorable prognosis in human cancers. Therefore, we investigated in this study the biological and prognostic significance of DNMT1, DNMT3a, and DNMT3b protein expression in DLBCL. DNA methyltransferase (DNMT) expression was analyzed by immunohistochemistry in 81 DLBCL cases and correlated with clinicopathological parameters. Kaplan–Meier curves were used to estimate survival rates, and the Cox proportional hazard regression model was used to evaluate the prognostic impact of DNMT expression. Our results showed that overexpression of DNMT1, DNMT3a, and DNMT3b were detected in 48%, 13%, and 45% of investigated cases, respectively. DNA methyltransferase 1 (DNMT1) and DNMT3b overexpression was significantly correlated with advanced clinical stages ($P = 0.028$ and $P = 0.016$, respectively). Moreover, concomitant expression of DNMT1 and DNMT3b was significantly correlated with resistance to treatment ($P = 0.015$). With regard to survival rates, although data was available only for 40 patients, DNMT3b overexpression was significantly correlated with shorter overall survival ($P = 0.006$) and progression-free survival ($P = 0.016$). Interestingly, multivariate analysis demonstrated that DNMT3b overexpression was an independent prognostic factor for predicting shortened overall survival ($P = 0.004$) and progression-free survival ($P = 0.024$). In conclusion, DNMT3b overexpression was identified as an independent prognostic factor for predicting shortened survival of patients with DLBCL and could be, therefore, useful in identifying patients who would benefit from aggressive therapy. (*Cancer Sci* 2010; 101: 1722–1730)

Diffuse large B-cell lymphomas (DLBCL) are the most common form of aggressive lymphoma.[1,2] Although curable in the majority of cases with anthracycline-based combination chemotherapy and the monoclonal antibody rituximab, approximately 40% of patients with DLBCL will relapse after standard first-line therapy.[1,2] Several biological markers have been studied in attempting to identify high-risk patients, but none has proved to be completely effective,[2,3] and the international prognostic index (IPI) has remained the gold standard for predicting prognosis.[4] Recently, microarray gene expression studies have identified multiple genes of potential prognostic significance in DLBCL, and have led to the subdivision of DLBCL into two major biological categories based on presumed cell of origin: germinal centre B-cell like (GCB), and non-GCB/activated B-cell like.[5] However, the prognostic value of genetic markers in DLBCL remains controversial. The identification of new molecular prognostic markers would therefore improve the prognostic predictability and understanding of the clinical behavior of those lymphomas.[2,3]

DNA methylation is a common epigenetic modification with an important role in the control of gene expression in mammalian cells.[6] Abnormal DNA methylation is thought to be the most important early event in tumor development characterized by widespread genome hypomethylation leading to chromosome instability and localized DNA hypermethylation.[7,8] DNA methylation is the result of the activity of a family of DNA methyltransferases (DNMTs) which catalyze the transfer of a methyl group from the ubiquitous methyl donor S-adenosyl methionine to the 5-position of cytosines residing in the dinucleotide sequence cytosine–guanine.[7,9–11] At least three independently DNMTs are known including DNMT1, DNMT3a, and DNMT3b which are involved in gene hypermethylation.[7,9,11]

DNA methyltransferase 1 (DNMT1) has a preference for hemi-methylated DNA and is therefore thought to be involved in maintenance methylation or copying methyl-CpG patterns after DNA replication.[7,12,13] DNA methyltransferase 3a (DNMT3a) and 3b are thought to function as de novo DNMTs and the murine enzymes are required for the methylation after embryonic implantation and the de novo methylation of newly integrated retroviral sequences.[7–9,14] The deregulation of DNMT expression (mainly DNMT1 and DNMT3b) clearly contributes to tumorigenesis and tumor suppressor gene (TSG) hypermethylation.[15] Moreover, induced DNMT overexpression in cultured cell lines gradually induces CpG hypermethylation and cell transformation.[13,16–26] Most interestingly, DNMT1 and DNMT3b overexpression has been correlated with unfavorable prognostic in several human cancers including breast and hepatocellular carcinomas, and lung cancers.[13,16,17,19,23,27–30]

To our knowledge, the expression and the prognostic significance of DNMT expression in DLBCL has never, previously, been documented. The aim of this study was therefore to analyze DNMT1, DNMT3a, and DNMT3b expression by immunohistochemistry in a series of 81 DLBCL cases and sought relationships between the overexpression of these DNMTs and clinicopathological parameters including patients' outcomes. We also sought relationships between the expression of DNMTs and well-known genes for which DNA promoter hypermethylation has been shown in DLBCL.[31,32]

Materials and Methods

Patients and tissue samples. This study included 81 newly presented DLBCL cases selected from the archives of the Department of Pathology at the Farhat-Hached hospital of Sousse (Tunisia). Diagnosis was based, for all cases, on morphology and immunohistochemical analysis according to the World Health Organization (WHO) classification.[1] All cases included

in this study were clinical cases routinely examined and diagnosed between 1996 and 2006. Criteria for selection of these cases were the availability of sufficient paraffin-embedded tumor biopsy specimens, before any treatment, for further analyses. All cases were previously classified by immunohistochemistry as GCB or non-GCB-like DLBCL using monoclonal antibodies against CD10, BCL6, and MUM1.[33] Besides, all of these cases were also characterized for the chromosomal translocation t(14;18) presence by PCR assays.[33] Clinical and follow-up data concerning performance status, number of extranodal sites, serum lactate dehydrogenase (LDH) level, international prognostic index, B symptoms, bulky tumor, response to treatment, and survival was available only for 40 patients. Those patients were uniformly treated at the same institution with standard regimes, according to the IPI scores and patients' age, and completed their planned treatment. Ten patients were treated with cyclophosphamide, hydroxydaunorubicin, vincristine, and prednisone (CHOP), eight with cyclophosphamide, oncovin, and prednisone (COP), seven with adriamycine, cyclophosphamide, vindesine, bleomycine, and prednisone (ACVBP), six with cyclophosphamide, vincristine, and prednisone (CVP), and nine with mini-cyclophosphamide, epirubicin, oncovin, and prednisone (CEOP). Radiotherapy was administered after chemotherapy to five patients who presented with a bulky mass or who had a detectable residual mass after chemotherapy. All of these 40 patients completed their planned treatment. However, none of these patients received rituximab or alemtuzumab. Patients' outcomes were evaluated according to standard international criteria.[34]

Immunohistochemical analysis. DNA methyltransferase protein expression was analyzed by immunohistochemistry on paraffin-embedded tissue sections using a rabbit polyclonal anti-DNMT1 antibody (clone H-300, dilution 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), a mouse monoclonal anti-DNMT3a antibody (clone 64B1446, dilution 1:200; Imgenex, San Diego, CA, USA), and a mouse monoclonal anti-DNMT3b antibody (clone 52A1018, dilution 1:200; Imgenex). Five-micrometer-thick tissue sections were cut, dried overnight at 56°C, deparaffinized in toluene, rehydrated through a series of alcohol, and washed in Tris–buffered saline (TBS) (0.05 mM Tris–HCl; 1.15 mM NaCl, pH 7.6). For antigen retrieval, sections were boiled in a water bath with EDTA buffer (10 mM, pH 8.0) for 20 min until the temperature reached 98°C. Sections were then allowed to cool at room temperature for 20 min, rinsed thoroughly with water, and placed in TBS. Endogenous peroxidase activity was blocked with hydrogen peroxide/methanol for 5 min. Subsequently, sections were rinsed gently with TBS and incubated at 4°C overnight with the appropriate primary antibody. Immunostaining was performed using the high-sensitive polymer-based EnVision system (DakoCytomation, Glostrup, Denmark) according to the manufacturer's instructions. Immunoreactivity was visualized with the 3,3′-diaminobenzidine etrahydrochloride. Sections were counterstained with Mayer's hematoxylin, permanently mounted, and viewed with a standard light microscope. Lymphocytes on the same slide were used as an internal positive control for DNMT immunoreactivity.[25] For negative control preparations, primary antibodies were omitted and replaced by TBS. Furthermore, sections from each case that were treated in a similar fashion using normal serum instead of primary antibodies served as additional negative controls and nonspecific binding controls for primary antibody reaction. In all cases, immunostaining results were evaluated independently by two pathologists using the scoring system proposed by Choi et al.,[35] and taken into consideration were the staining intensity obtained (0, negative; 1, mild; 2, moderate; 3, high) and the proportion of positive cells observed (0, negative; 1, positive in ≤10%; 2, positive in >10% and ≤33%; 3, positive in >33% and ≤66%; 4, positive in >66% of cells). The two scores were then combined for each slide, and the immunoreactivity was graded as negative (0), weakly positive (1 or 2), moderately positive (3–5), or strongly positive (6 or 7). In this study, for DNMT1, DNMT3a, and DNMT3b expression level, the stains were considered as ''overexpression'' if the grade was ≥6.

Immunohistochemical analysis of P53, BCL2, and Ki-67 expression was carried out as described above using mouse monoclonal antibodies anti-P53 (clone DO-7, dilution 1:50; DakoCytomation), anti-BCL2 (clone 124, dilution 1:50; DakoCytomation), and anti-Ki67 (clone MIB-1, dilution 1:100; DakoCytomation). P53 expression was considered positive if nuclear staining was observed in 10% or more of the tumor cells and BCL2 expression was scored as positive if 50% or more of the tumor cells showed cytoplasmic staining.[33] Nuclear proliferation of antigen Ki-67 was quantified by determining the number of positive cells expressing nuclear Ki-67 among the total cells. The intensity was graded as mild, when positive in less than 30% of tumor cells; moderate, when positive between 30% and 50% of tumor cells; and intense, when positive in more than 50% of tumor cells. However, immunoreactivity was considered positive when nuclear staining in more than 30% of tumor cells was observed.[36] All slides were blindly graded by two co-authors (M.T. and S.Z.), with good correlation scores between them.

Methylation-specific polymerase chain reaction. Promoter methylation status of the *GSTP1*, *SHP1*, *DAPK*, *RB1*, *P14*, *P15*, *P16*, *CDH1*, *VHL*, *RASSFA1*, and *TIMP3* genes was analyzed by methylation-specific PCR (MSP). Primer sequences and PCR conditions were described previously.[31,32] Negative and positive controls were always included in each experiment. CpG universal methylated DNA (Qbiogene, Carlsbad, CA, USA) was used as the positive control for methylated alleles, and DNA from normal lymphocytes was used as the negative control for unmethylated alleles. Negative controls without DNA were always included in each experiment. Amplified products were visualized under ultraviolet illumination after electrophoresis in 2% agarose gels containing ethidium bromide using the Gel Doc 2000 System (Bio-Rad, Marnes-la-Coquette, France). For each case, MSP results were scored when a clearly visible band on the electrophoresis gel with the methylated and/or the unmethylated primers was observed as described previously.[31]

Statistical analysis. Pairwise correlations between categorical variables were investigated by χ^2-test or Fisher's exact test where appropriate. Overall survival (OS) and progression-free survival (PFS) were estimated using the Kaplan–Meier method. The log-rank test was used to compare patients' survival time between or among groups. The Cox proportional hazards model (Cox regression) was used with backward elimination to find the most important independent prognostic factors. Overall survival (OS) was defined as time interval from diagnosis to death or, for patients remaining alive, time interval from diagnosis to the last follow-up. Progression-free survival (PFS) was defined as time interval from achievement of treatment to date of disease progression or recurrence, the last follow-up, or death occurrence. Statistical tests were two sided, and a $P < 0.05$ was considered to be statistically significant. All statistical analyses were performed using the SPSS statistical software program (SPSS, Chicago, IL, USA).

Results

Patients' characteristics. The patients included 52 men and 29 women with a median age of 60 years (range, 3–85 years). Forty-one patients (50.5%) were diagnosed with an advanced Ann-Arbor clinical stage (stages III or IV). Twenty-four patients (60%) presented a good performance status (<2). An elevated level of serum LDH was observed in 35 patients (87.5%). Thirty patients (75%) exhibited low or low-intermediate IPI scores.

Nevertheless, 11 patients (27.5%) showed B symptoms and four patients (10%) showed a bulky tumor. The number of extranodal sites of the disease at more than one site was observed in five patients (12.5%). Primary site of tumors was lymph nodes in 61 patients and extranodal organs in 20 patients (stomach [eight cases], intestine [five cases], skin [four cases], pharynx [two cases], and lung [one case]). Twenty-three patients (57.5%) achieved a complete remission at the end of the treatment procedure, while four (10%) were in partial remission and 13 (32.5%) had stable or progressive disease. Besides, 57 (70%) of cases were assigned to the GCB lymphoma group and 24 (30%) cases were assigned to the non-GCB lymphoma group and 22 (7%) cases showed the presence of the chromosomal translocation t(14;18). Moreover, the expression of P53, BCL2, and Ki67 was observed in 42 (52%), 51 (63%), and 51 (63%) of the investigated cases, respectively.

Follow-up data were available for only 40 patients and the median follow-up period was 16 months (range, 0–96 months). Among the clinical parameters only IPI scores ($P < 0.0001$ and $P = 0.0003$; log-rank test), clinical stages ($P = 0.003$ and $P = 0.009$; log-rank test), B symptoms ($P = 0.013$ and $P = 0.043$, respectively; log-rank test), WHO performance scores ($P = 0.0002$ and $P = 0.007$; log-rank test), and bulky tumor ($P = 0.013$ and $P = 0.034$; log-rank test) demonstrated a significant prognostic effect on OS and PFS survival rates, respectively. In contrast, univariate analysis of P53, BCL2, and Ki67 expression as well as the presence of the translocation t(14;18) in tumor cells revealed no significant effect on patient prognosis. With regard to the immunophenotype profile, GCB center phenotype displayed a significantly better OS ($P = 0.015$; log-rank test) and PFS ($P = 0.035$; log-rank test).

DNA methyltransferase 1 (DNMT1), DNMT3a, and DNMT3b protein expression in diffuse large B-cell lymphomas. Overexpression of DNMT1, DNMT3a, and DNMT3b was observed in 48 (59%), 11 (13%) and 37 (45%) of the 81 investigated cases, respectively. In addition, significant correlations were found between the co-expression of DNMT1 and DNMT3b ($P < 0.0001$) and between DNMT1 and DNMT3a ($P = 0.021$). However, no significant correlation between DNMT3a and DNMT3b was observed ($P = 0.531$). Representative results for DNMT1, DNMT3a, and DNMT3b expressions are shown in Figure 1.

Correlation between DNMT expression status and clinicopathological parameters. DNA methyltransferase 1 (DNMT1) overexpression was significantly correlated with advanced clinical stages ($P = 0.028$). In addition, DNMT1 overexpression was significantly more frequent in older patients (age > 60 years) than younger patients (age ≤ 60 years) ($P = 0.004$) (Table 1). With regard to DNMT3b expression status, a significant correlation was also observed between advanced clinical stages and DNMT3b overexpression ($P = 0.016$) (Table 1). However, no significant correlation was observed between DNMT3a overexpression and any of the clinicopathological parameters analyzed, including the Ann-Arbor clinical stage, the LDH level, and the IPI scores (Table 1). On the other hand, taking the immunophenotype profile into account, no significant correlations were found between the expression of DNMT1, DNMT3a, or DNMT3b and the GCB immunophenotype ($P = 0.259$, $P = 0.378$, and $P = 0.215$ respectively) (Table 2). Likewise, no significant correlations were observed between the expression of DNMT1, DNMT3a, or DNMT3b and the presence of the chromosomal translocation t(14;18) ($P = 0.457$, $P = 0.623$, and $P = 0.821$ respectively) (Table 2). Moreover, no significant
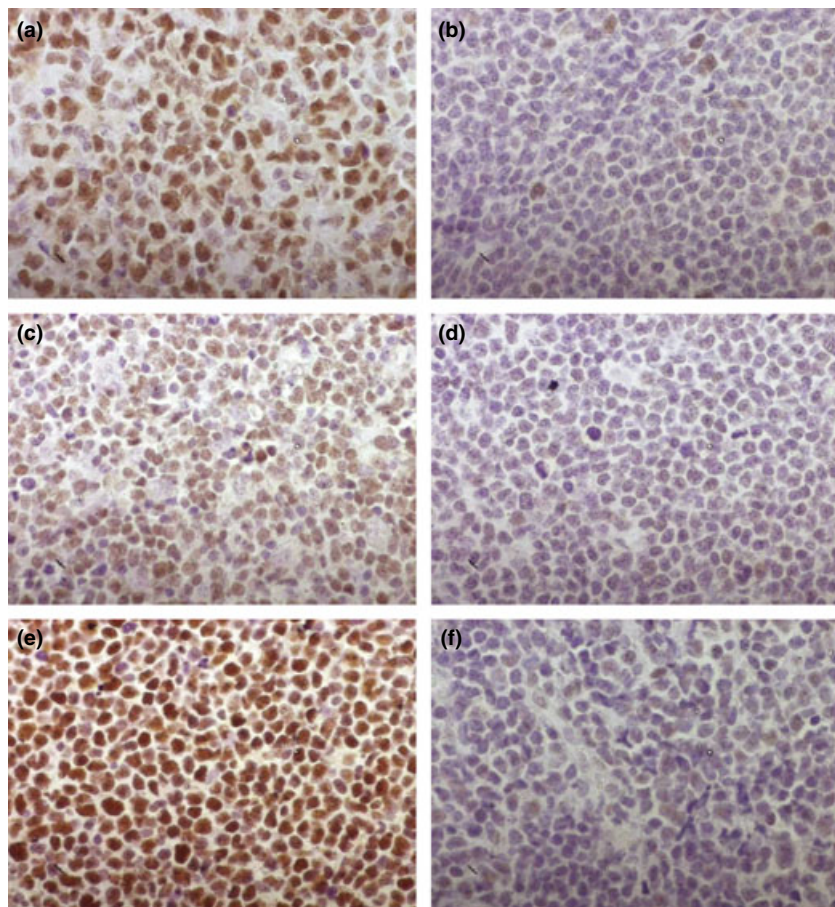

Fig. 1. Immunohistochemical staining patterns of DNA methyltransferase 1 (DNMT1), DNMT3a, and DNMT3b proteins expression in diffuse large B-cell lymphomas. (a,c,e) Representative examples of positive immunostaining for DNMT1, DNMT3a, and DNMT3b expression, respectively. (b,d,f) Representative examples of negative immunostaining for DNMT1, DNMT3a, and DNMT3a expression, respectively.

Table 1. Association between DNMT1, DNMT3a, and DNMT3b protein overexpression and clinicopathological parameters in diffuse large B-cell lymphomas

Parameters		Total	DNMT1	DNMT3a	DNMT3b	DNMT1 + 3b	DNMT1 + 3a
		n†	n (%)*	n (%)	n (%)*	n (%)*	n (%)
Gender							
Male		52	30 (60)	7 (13.5)	24 (46)	19 (36.5)	6 (11.5)
Female		29	18 (62)	4 (14)	13 (45)	12 (41.5)	4 (14)
	P-value		0.701	0.607	0.909	0.667	0.512
Age (years)							
≤60		46	21 (46)	7 (15)	17 (37)	12 (26)	6 (13)
>60		35	27 (77)	4 (11.5)	20 (57)	19 (54)	4 (11.5)
	P-value		**0.004**	0.439	0.057	**0.010**	0.553
Tumor location							
Nodal		61	37 (61)	11 (18)	28 (46)	23 (38)	10 (16.5)
Extranodal		20	11 (55)	1 (5)	9 (45)	8 (40)	0 (0)
	P-value		0.655	0.142	0.944	0.855	0.123
Stage (Ann Arbor)							
I/II		40	19 (47.5)	3 (7.5)	13 (32.5)	10 (25)	3 (7.5)
III/IV		41	29 (71)	8 (19.5)	24 (58.5)	21 (51)	7 (17)
	P-value		**0.028**	0.115	**0.016**	**0.015**	0.312
Serum LDH							
Normal		5	2 (40)	0 (0)	4 (80)	2 (40)	0 (0)
Elevated		35	26 (47.5)	5 (14.5)	15 (43)	15 (43)	5 (14)
	P-value		0.149	0.493	0.141	0.646	0.493
Performance status							
<2		24	15 (62.5)	4 (17)	10 (42)	8 (33)	4 (17)
≥2		16	13 (81.5)	1 (6.5)	9 (56)	9 (56)	1 (6.5)
	P-value		0.181	0.323	0.366	0.151	0.323
No. of extranodal sites							
<2		35	25 (71.5)	5 (14.5)	18 (51.5)	16 (46)	5 (14.5)
≥2		5	3 (60)	0 (0)	1 (20)	1 (20)	0 (0)
	P-value		0.477	0.493	0.345	0.116	0.493
IPI scores‡							
Low		30	19 (63.5)	4 (13.5)	12 (40)	10 (33)	4 (13)
High		10	9 (90)	1 (10)	7 (70)	7 (70)	1 (10)
	P-value		0.113	0.633	0.100	**0.040**	0.633
B symptoms							
Absent		29	19 (65.5)	5 (17.5)	13 (45)	11 (38)	5 (17)
Present		11	9 (82)	0 (0)	6 (54.5)	6 (54.5)	0 (0)
	P-value		0.275	0.180	0.583	0.276	0.180
Bulky tumor (>10 cm)							
Absent		36	25 (69)	5 (14)	16 (44.5)	14 (40)	5 (14)
Present		4	3 (75)	0 (0)	3 (75)	3 (75)	0 (0)
	P-value		0.654	0.537	0.365	0.197	0.537
Response to treatment							
CR		23	14 (61)	3 (13)	8 (35)	6 (26)	3 (13)
PR/SD/PD		17	14 (82.5)	2 (12)	11 (65)	11 (65)	2 (12)
	P-value		0.143	0.646	0.112	**0.015**	0.646

*Bold numbers indicate significant correlation (P < 0.05). †Total number of samples in some categories is less than the overall number analyzed because clinical data was not available for these samples. ‡Low scores included low and low-intermediate scores; high scores included intermediate-high and high scores. CR, Complete response; DNMT, DNA methyltransferase; IPI, International Prognostic Index; LDH, lactate dehydrogenase; PD, Progressive disease; PR, Partial response; SD, Stable disease.

correlations were observed between the expression status of the three DNMTs investigated and P53, BCL2, or Ki-67 expression (Table 2).

On the basis of our initial findings, showing positive correlations between simultaneous expressions of DNMT1 and DNMT3a as well as between DNMT1 and DNMT3b expressions, we analyzed the effect of different combinations of DNMTs co-expression on patients' outcomes (Table 1). Significant correlations were found between concomitant expressions of DNMT1 and DNMT3b and advanced clinical stages ($P = 0.015$), high IPI scores ($P = 0.040$), and age ($P = 0.010$). More interestingly, concomitant expression of DNMT1 and DNMT3b was significantly correlated with worse therapy response (Table 1). In fact, only 26% of patients who achieved complete response showed simultaneous expression of DNMT1 and DNMT3b, whereas 65% of patients who failed to give a complete response showed overexpression of DNMT1 and DNMT3b ($P = 0.015$) (Table 1). Moreover, concomitant expression of DNMT1 and DNMT3b was notably correlated with non-GCB immunophenotype although it was not statistically significant ($P = 0.097$) (Table 2).

Correlation between DNMT expression status and DNA hypermethylation. Promoter hypermethylation of *GSTP1*, *SHP1*, *DAPK*, *RB1*, *P14*, *P15*, *P16*, *VHL*, *CDH1*, *RASSFA1*, and

Table 2. Association between DNMT1, DNMT3a, and DNMT3b protein overexpression and pathological parameters in diffuse large B-cell lymphomas

Parameters		Total	DNMT1	DNMT3a	DNMT3b	DNMT1 + 3b	DNMT1 + 3a
		n	n (%)	n (%)	n (%)	n (%)	n (%)
P53 immunoexpression							
Present		42	24 (57)	7 (17)	21 (50)	15 (36)	6 (14)
Absent		39	24 (61.5)	4 (10.5)	16 (41)	16 (41)	4 (10.5)
	P-value		0.860	0.605	0.557	0.793	0.739
BCL2 immunoexpression							
Present		51	32 (63)	5 (10)	26 (51)	22 (43)	5 (10)
Absent		30	16 (53.5)	6 (20)	11 (37)	9 (30)	5 (17)
	P-value		0.550	0.313	0.309	0.348	0.487
Ki-67 immunoexpression							
Present		51	31 (61)	8 (16)	22 (44)	18 (35)	7 (14)
Absent		30	17 (57)	3 (10)	15 (50)	13 (43)	3 (10)
	P-value		0.897	0.738	0.713	0.630	0.737
GCB immunophenotype status							
GCB phenotype		57	31 (54.5)	6 (10.5)	23 (40.5)	18 (32)	5 (9)
Non-GCB phenotype		24	17 (71)	5 (21)	14 (58)	13 (54)	5 (21)
	P-value		0.259	0.288	0.215	0.097	0.152
t(14;18)							
Positive		22	15 (68)	3 (14)	11 (50)	9 (41)	3 (14)
Negative		59	33 (56)	8 (13.5)	26 (44)	22 (37)	7 (12)
	P-value		0.457	0.623	0.821	0.967	0.547

DNMT, DNA methyltransferase; GCB, germinal center B-cell like.

TIMP3 genes was detected in 53 (65%), 52 (64%), 58 (71%), 12 (15%), 41 (51%), 34 (42%), 44 (54%), 30 (37%), 57 (70%), 21 (30%), and 26 (32%) of the 81 DLBCL cases investigated, respectively. With regard to survival rates, promoter hypermethylation of *DAPK* and *P16* were significantly associated with shortened OS ($P = 0.001$, and $P = 0.002$, respectively; log-rank test) and PFS ($P = 0.005$ and $P = 0.006$, respectively; log-rank test).

The analyses of the relation between promoter hypermethylation of the 11 investigated genes and DNMTs expression status revealed significant correlations between DNMT3b overexpression and hypermethylation of *GSTP1* ($P = 0.025$), *SHP1* ($P = 0.025$), *TIMP3* ($P = 0.042$), and *P16* ($P = 0.009$) genes (Table 2). In addition, we noted a significant correlation between DNMT3b overexpression and hypermethylation of multiple genes ($P = 0.005$; Table 2). DNMT1 overexpression was significantly correlated with the hypermethylation of *VHL* ($P = 0.040$) and *RB1* ($P = 0.038$) genes (Table 3). However, no significant correlations were observed between DNA hypermethylation of any of the 11 genes analyzed and DNMT3a overexpression (Table 3).

Prognostic significance of DNMT expression status in diffuse large B-cell lymphomas. Data concerning survival was available only for 40 patients. In univariate survival analysis, DNMT3b overexpression was significantly correlated with shorter OS ($P = 0.006$, log-rank test; Fig. 2e) and shorter PFS ($P = 0.016$, log-rank test; Fig. 2f). DNMT1 overexpression was notably correlated with shorter OS although it was not statistically significant ($P = 0.175$, log-rank test) (Fig. 2a,b). With regard to DNMT3a overexpression, no significant differences in OS and PFS were noted between patients groups (Fig. 2c,d). Most importantly, when simultaneous DNMT1 and DNMT3b expression were taken into consideration we found that overexpression of both DNMTs was associated with a statistically significant decrease in OS ($P = 0.0006$, log-rang test) and PFS rates ($P = 0.001$, log-rank test). However, when simultaneous DNMT1 and DNMT3a expression were taken into consideration no significant differences were observed in OS and PFS rates. Furthermore, a multivariate progression analysis based on

Cox's proportional hazard model was performed, using backward stepwise selection, to test the independent value of potential parameters predicting patients' survival. The variables analyzed were IPI scores, B symptoms, GCB immunophenotype, *DAPK*, *P16*, and *VHL* hypermethylation, and DNMT3b expression status. We did not include in this analysis clinical stages, WHO performance scores, and the bulky tumor because all these parameters are part of the IPI scores. Interestingly, multivariate analysis showed that DNMT3b overexpression was identified as an independent prognostic factor in predicting shortened OS ($P = 0.004$) and PFS ($P = 0.017$) of DLBCL patients (Table 4).

Discussion

In the present study, we investigated the expression of DNMT1, DNMT3a, and DNMT3b in 81 DLBCL cases using immunohistochemistry and evaluated their pathological and prognostic values in those lymphomas. Our results indicate that DNMTs are commonly overexpressed in DLBCL and may be an important way for induction of hypermethylation of multiple tumor-related genes in those lymphomas. With regard to survival data, we found, although survival data was available only for 40 patients, that DNMT3b overexpression was strongly correlated with advanced clinical stages and worse patient survival.

DNA methyltransferase 1 (DNMT1) is recognized as the "maintenance" DNMT that copies methylation patterns after DNA replication as its preference for hemi-methylated, rather than unmethylated, substrates *in vitro* and targets replication foci by binding to proliferating cell nuclear antigen (PCNA).[12,37] However, it has been proposed that DNMT1 possesses both maintenance and de novo DNA methylation activity *in vivo*, regardless of its preference for substrates *in vitro*. Moreover, recent studies showed that DNMT1 could interact with DNMT1-associated protein (DMAP) 1, histone deacetylase (HDAC) 1, HDAC2, and Rb, and repress gene transcription. DNMT1 overexpression has been reported in several human cancers.[13,17,19,20,24,26,38,39] In the present study, DNMT1 overexpression was detected by immunohistochemistry in 48%

Table 3. Association between DNMT1, DNMT3a, and DNMT3b protein overexpression and DNA promoter hypermethylation in diffuse large B-cell lymphomas

Genes		Total	DNMT1	DNMT3a	DNMT3b	DNMT1 + 3b	DNMT1 + 3a
		n	n (%)*	n (%)	n (%)*	n (%)*	n (%)
GSTP1							
Unmethylated		28	18 (62.5)	3 (11)	8 (28.5)	7 (25)	3 (11)
Methylated		53	30 (57)	8 (15)	29 (55)	24 (77.5)	7 (13)
	P-value		0.503	0.429	**0.025**	**0.044**	0.524
DAPK							
Unmethylated		23	16 (69.5)	3 (13)	10 (43.5)	5 (22)	3 (13)
Methylated		58	32 (55.5)	8 (14)	27 (46.5)	26 (45)	7 (12)
	P-value		0.235	0.620	0.802	0.061	0.584
SHP1							
Unmethylated		28	12 (43)	2 (7)	8 (28.5)	5 (18)	2 (7)
Methylated		53	36 (68)	9 (17)	29 (55)	26 (49)	8 (15)
	P-value		0.133	0.189	**0.025**	**0.006**	0.255
RASSFA1							
Unmethylated		60	37 (62)	10 (16.5)	26 (43.5)	22 (37)	9 (15)
Methylated		21	11 (52.5)	1 (5)	11 (52.5)	9 (43)	1 (5)
	P-value		0.456	0.159	0.474	0.615	0.205
TIMP3							
Unmethylated		55	32 (58.5)	5 (9)	21 (38.5)	18 (33)	5 (9)
Methylated		26	16 (61.5)	6 (23)	16 (61.5)	13 (50)	5 (19)
	P-value		0.774	0.161	**0.042**	0.135	0.174
CDH1							
Unmethylated		24	15 (62.5)	3 (12.5)	12 (50)	10 (42)	3 (12.5)
Methylated		57	33 (58)	8 (14)	25 (44)	21 (37)	7 (12)
	P-value		0.700	0.581	0.612	0.683	0.619
VHL							
Unmethylated		51	26 (51)	7 (14)	21 (41)	14 (27.5)	6 (12)
Methylated		30	22 (73.5)	4 (13.5)	16 (53.5)	17 (57)	4 (13.5)
	P-value		**0.040**	0.620	0.289	**0.038**	0.547
RB1							
Unmethylated		68	37 (54.5)	8 (12)	29 (43)	25 (37)	8 (12)
Methylated		13	11 (85)	3 (23)	8 (62)	6 (46)	2 (15.5)
	P-value		**0.032**	0.245	0.210	0.367	0.504
P14							
Unmethylated		40	28 (70)	4 (10)	21 (52.5)	19 (47.5)	4 (10)
Methylated		41	20 (49)	7 (17)	16 (39)	12 (29.5)	6 (14.5)
	P-value		0.114	0.353	0.224	0.145	0.385
P15							
Unmethylated		47	29 (62)	7 (15)	23 (49)	20 (43)	7 (15)
Methylated		34	19 (56)	4 (12)	14 (41)	11 (32.5)	3 (9)
	P-value		0.599	0.475	0.489	0.351	0.322
P16							
Unmethylated		37	19 (51.5)	5 (13.5)	10 (27)	9 (24)	5(13.5)
Methylated		44	29 (66)	6 (14)	26 (59)	22 (50)	5 (11.5)
	P-value		0.184	0.987	**0.009**	**0.018**	0.515
TSG methylation†							
≤3 genes		15	8 (53)	3 (20)	2 (13.5)	2 (13.5)	3 (20)
>3 genes		66	40 (61)	8 (12)	35 (46)	29 (44)	7 (10.5)
	P-value		0.605	0.330	**0.005**	**0.028**	0.271

*Bold numbers indicate significant correlation ($P < 0.05$). †TSG methylation was defined as the hypermethylation of more than three genes. DNMT, DNA methyltransferase; TSG, tumor suppressor gene.

of the DLBCL cases investigated. Moreover, the overexpression of DNMT1 correlated significantly with advanced clinical stages. The above evidence indicates that increased DNMT1 overexpression may play a role in the malignant progression of DLBCL.

DNA methyltransferase 3a (DNMT3a) and DNMT3b are essential for early embryonic development and responsible for de novo methylation.[14] In fact, previous studies reported that aberrant DNA methylation of many tumor suppressor genes is frequently found in human cancers. In addition, overexpression of DNMT3a and DNMT3b has been reported in various human cancer tissues, while other cancer tissues do not show altered expression of the enzyme, suggesting that DNMT3a and DNMT3b play an important role in the development of aberrant promoter methylation during tumorigenesis.[13,16,18,19,22,25,26,40] In the present study, DNMT3a and DNMT3b overexpression was detected by immunohistochemistry in 13% and 45% of the DLBCL cases investigated, respectively. The overexpression of DNMT3b was significantly correlated with advanced clinical stages which also indicates that increase of DNMT3b expression
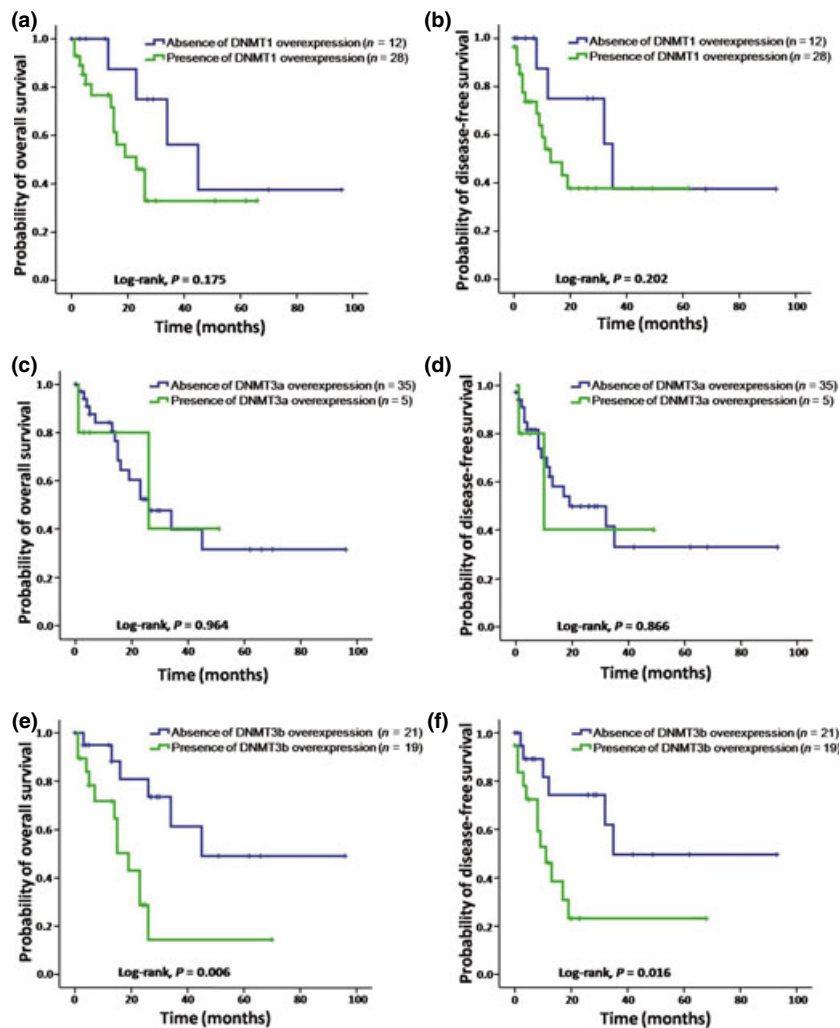
Fig. 2. Kaplan–Meier analyses of overall survival and disease-free survival in diffuse large B-cell lymphomas with respect to DNA methyltransferase 1 (DNMT1), DNMT3a, and DNMT3b protein overexpression. Survival curves show that DNMT3b overexpression was significantly correlated with poorer overall survival (P = 0.006; [e]) and progression-free survival (P = 0.016; [f]), whereas no significant difference was seen in overall survival or disease-free survival between patients showing normal or overexpression of DNMT1 (a,b) or DNMT3a (c,d).

Table 4. Multivariate analysis: Cox regression analysis for prognostic factors affecting patient survival by means of backward selection

Variables	Overall survival		Progression-free survival	
	Hazard ratio (95% CI)	P-value	Hazard ratio (95% CI)	P-value
IPI scores				
Low vs high scores†	275.474 (22.961–3304.952)	0.0002	22.750 (5.099–101.496)	0.0004
GCB immunophenotype				
GCB vs non-GCB phenotype	7.897 (1.790–34.832)	0.006	3.993 (1.128–14.135)	0.032
DAPK methylation				
Unmethylated vs methylated	11.840 (2.485–56.411)	0.002	4.797 (1.347–17.083)	0.016
DNMT3b expression				
Normal vs overexpression	6.814 (1.821–25.493)	0.004	4.168 (1.284–13.285)	0.017

†Low scores included low and low-intermediate scores; high scores included intermediate-high and high scores. CI, confidence interval; DNMT, DNA methyltransferase; GCB, germinal center B-cell like; IPI, International Prognostic Index.

may play a role in the malignant progression of DLBCLs. Furthermore, previous studies have showed that DNMT overexpression was correlated with unfavorable prognosis in various human malignancies.[13,16,19,27–30,38] In our study we showed significant correlations between DNMT3b overexpression and poor OS and PFS (Fig. 2). Interestingly, in multivariate analysis, DNMT3b overexpression was identified as an independent prognostic factor for predicting shortened overall and disease-free survival in patients with DLBCL. Our results are similar to those of Girault *et al.*,[23] who found that DNMT3b overexpression was associated with a short relapse-free survival duration in a

subgroup of breast cancer patients and Wang *et al.*,[16] who have demonstrated that increased DNMT3b expression led to lower survival duration in patients with head and neck cancers. Moreover, in this study, DNMT1 overexpression was notably correlated with shorter OS although it was not statistically significant. Our results are consistent with those of Vallbohmer *et al.*,[28] who reported similar results in a study of 91 non-small-cell lung cancer patients. However, our results are contradictory with those reported by Lin *et al.*,[13] and Xing *et al.*,[27] who showed that DNMT1 overexpression was significantly associated with poor OS in non-small-cell lung cancer patients. However,

because our study is the first to report evidence suggesting that DNMT3b overexpression contributes to poorer patient survival in DLBCL, further studies with a larger number of patients are needed to confirm our results.

The functional mechanism of DNMT1 and DNMT3b expression in cancer development and prognosis is still unclear. However, it has also been suggested that both DNMT1 and DNMT3b overexpression was associated with cancer cell survival by inhibiting apoptosis;[38,41] and this finding suggests that a common regulatory pathway may exist for these three enzymes. In the current study, we demonstrated significant positive correlations between concomitant expression of DNMT1 and DNMT3a or between DNMT1 and DNMT3b. Moreover, significant correlations were found between simultaneous expression of DNMT1 and DNMT3b and advanced clinical stages and high IPI scores. Interestingly, in our present study, we found a significant correlation between DNMT1 and DNMT3b overexpression and the response to treatment (Table 1). These data indicate that the block of apoptosis in relation to DNMT1 and DNMT3b overexpression could be a mechanism of resistance to chemotherapy-induced apoptosis in DLBCLs, probably through the induction of the hypermethylation of multiple tumor-related genes. However, because our study is the first reporting the value of these two DNMTs for predicting resistance to treatment in DLBCL, and because of the limited number of samples, additional studies with a larger number of cases are mandatory to confirm these observations. On other hand, in the present study, we found significant correlations between DNMT3b and DNMT1 overexpression and the hypermethylation of multiple TSGs. Similar results have been observed in other human cancers even though reports in which multiple methylated target genes of DNMTs were analyzed are quite limited.[13,20,42,43] These findings are in agreement with the idea that DNMT3b alone has limited effect in promoter methylation because the maintenance of the methylated promoters of tumor suppressor genes could only be effectively disrupted when both DNMT3b and DNMT1 genes are knocked out.[44,45]

These results imply, therefore, that the association between DNMT overexpression and the promoter hypermethylation of tumor-related genes may be clinically relevant. In conclusion, this study represents the first research investigating the prognostic impact of DNMT1, DNMT3a, and DNMT3b overexpression DLBCL. Patients with DNMT overexpression were commonly characterized by aggressive disease and poor prognosis, probably in relation to the hypermethylation of multiple cancer-related genes. Interestingly, DNMT3b overexpression has been shown to be an independent new prognostic factor that could be helpful for predicting shortened survival and resistance to treatment, and therefore could be useful in identifying patients who would benefit from aggressive therapy.

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Disclosure Statement

The authors have no conflict of interest.

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