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Aberrant promoter methylation of multiple genes throughout the clinico-pathologic spectrum of B-cell neoplasia

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A B S T R A C T

Background and Objectives. Aberrant promoter methylation targets CpG islands causing gene silencing. We explored aberrant promoter methylation of genes potentially involved in B-cell malignancies and encoding proteins implicated in DNA repair (O^6-methylguanine-DNA methyltransferase, *MGMT*), detoxification of environmental xenobiotics (glutathione S-transferase P1, *GSTP1*), apoptosis regulation (death associated protein kinase, *DAP-k* and caspase 8, *CASP8*) and cell cycle control (*p73*).

Design and Methods. Three hundred and seventeen B-cell malignancies were investigated by methylation-specific polymerase chain reaction (MSP) of *MGMT*, *GSTP1*, *DAP-k*, CASP8 and *p73* genes. In selected cases, MSP results were matched to protein expression studies by immunohistochemistry or Western blotting.

Results. *DAP-k* promoter methylation occurred at highest frequency in follicular lymphoma (85.0%) and MALT-lymphoma (72.2%). *MGMT* methylation targeted both precursor B-cell neoplasia (23.8%) and mature B-cell tumors (27.6%). *GSTP1* methylation was commonest in hairy cell leukemia (75.0%), follicular lymphoma (55.5%), Burkitt's lymphoma (52.0%), and MALT lymphoma (50.0%). Methylation of *p73* and *CASP8* was rare or absent. *DAP-k* and *MGMT* methylation caused absent protein expression.

Interpretation and Conclusions. Methylation of *MGMT*, *DAP-k* and *GSTP1* represents a major pathogenetic event in several B-cell malignancies. In follicular lymphoma and MALT lymphoma, frequent inactivation of the apoptosis extrinsic pathway through *DAP-k* methylation may reinforce the survival advantage already conferred by deregulation of the intrinsic apoptotic pathway. Inactivation of *GSTP1* in gastric MALT lymphoma represents an additional mechanism favoring accumulation of reactive oxygen species and lymphomagenesis. Finally, the frequency of *GSTP1* aberrant methylation in diffuse large B-cell lymphoma prompts studies aimed at verifying the prognostic impact of this epigenetic lesion in these lymphomas.

Key words: B-cell, lymphoma, leukemia, aberrant methylation, pathogenesis.

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Growing evidence has implicated aberrant promoter methylation in the molecular pathogenesis of several human cancers.[1-4] Aberrant promoter methylation is an epigenetic DNA modification targeting CpG islands located within the regulatory regions of human genes. As a consequence, aberrant methylation of CpG islands causes repression of gene transcription and represents a mechanism for tumor suppressor gene inactivation alternative to mutations/deletions of the locus.[1-4]

To date, the role of aberrant promoter methylation in B-cell lymphoid malignancies has not been investigated exhaustively, and extensive studies have been mainly restricted to the cyclin dependent kinase inhibitors *p15* and *p16*.[5] Although other genes have been found to be targeted by aberrant methylation in lymphoid neoplasia, their analysis has been limited to specific types of B-cell malignancies or to lymphoid tumor cell lines.[6-9] These observations prompted our comprehensive analysis aimed at exploring the prevalence of aberrant promoter methylation in a selected panel of genes potentially involved in the pathogenesis of B-cell malignancies and representative of genes implicated in DNA repair (O^6-methylguanine-DNA methyltransferase, *MGMT*), detoxification of environmental xenobiotics (glutathione S-transferase P1, *GSTP1*), apoptosis regulation (death associated protein kinase, *DAP-k* and caspase-8, *CASP8*) and cell cycle control (*p73*). Previous studies have shown that promoter hypermethylation of these genes represents the major mechanism of gene inactivation, whereas allelic loss or mutations are virtually absent.[10-11]

MGMT is a DNA repair gene that removes mutagenic and cytotoxic adducts introduced

into the DNA from environmental and therapeutic alkylating agents.[12] In particular, *MGMT* inactivation increases cell sensitivity to the genotoxic effect of alkylating agents both *in vitro* and *in vivo*.[13,14] The potential role of *MGMT* in lymphoma stems from the fact that *MGMT* inactivation favors lymphomagenesis in knockout mice and represents a favorable prognostic marker for diffuse large B-cell lymphoma (DLBCL) treated with regimens containing alkylating agents.[8,15]

Aberrant promoter methylation of *DAP-k* has been suggested to have a potential role in lymphomagenesis.[7] *DAP-k* is a pro-apoptotic serine-threonine kinase involved in the extrinsic pathway of apoptosis initiated by interferon γ, tumor necrosis factor-α and FAS ligand.[7,16] In addition, *DAP-k* also participates in counteracting *c-MYC*-induced transformation by activating the p53 checkpoint and favoring *c-MYC*-induced apoptosis.[17] Consequently, inactivation of *DAP-k* prevents apoptosis triggered by death receptors and weakens the apoptotic response secondary to *c-MYC* activation.

GSTP1 is an enzyme implicated in the detoxification of environmental carcinogens and chemotherapeutic agents.[18] Its loss of expression is a risk factor for the development of cancer in null mice.[19] Although epigenetic alterations of *GSTP1* are recurrent in a wide range of solid tumors, its methylation pattern in B-cell neoplasia is not known.[20] Caspase-8 (*CASP8*) and *p73* are also potential targets of aberrant promoter methylation in lymphoma.[6,21] Similarly to *DAP-k*, *CASP8* is involved in the extrinsic pathway of programmed cell death that transduces the apoptotic signal from a death receptor to the common pathway of apoptosis.[21,22] The *p73* gene is a candidate tumor suppressor gene sharing structural and functional similarities with *p53* and is involved in cell cycle control and apoptosis.[23]

In this study we investigated aberrant promoter methylation of multiple genes in a large panel of B-cell neoplasms representative of the clinico-pathologic spectrum of the disease.

Design and Methods

Tumor samples and DNA preparation

This study was based on 317 tumor samples representative of the clinico-pathologic spectrum of B-cell neoplasia recognized by the WHO classification.[24] Tumor samples were derived from lymph nodes, bone marrow, peripheral blood or other involved organs obtained during routine diagnostic procedures. In all instances, with the exception of DLBCL transformed from a follicular phase, the specimens were collected at diagnosis before specific therapy. Diagnosis was based on morphology and immunophenotypic analysis of cell surface markers and was complemented by immunogenotypic analysis of antigen receptor gene rearrangement and chromosomal translocations. In most cases, the fraction of malignant cells was > 70% and in all cases > 40%. According to the WHO classification,[24] B-cell neoplasia specimens were classified as precursor B-cell acute lymphoblastic leukemia (ALL; n = 21), B-cell chronic lymphocytic leukemia (B-CLL; n = 30), lymphoplasmacytic lymphoma (LPL; n = 9), mantle cell lymphoma (MCL; n = 19), follicular lymphoma (FL; n = 21), mucosa-associated lymphoid tissue (MALT) lymphoma (n= 11), hairy cell leukemia (HCL; n = 11), DLBCL (n = 140), mediastinal large B-cell lymphoma (MLBCL; n =10), Burkitt's lymphoma (BL; n = 29) and plasma cell myeloma (PCM; n = 16). The precursor B-cell ALL samples were representative of different molecular variants of the disease and included cases associated with hyperdiploidy (n = 5), *BCR/ABL* rearrangement (n = 3), *MLL* rearrangement (n = 5), *TEL/AML-1* rearrangement (n = 3), or not known genetic lesions (n = 5). MALT lymphomas originated from the gastrointestinal tract (n = 9) or the thyroid (n = 2). DLBCL were subdivided into DLBCL arising *de novo* without clinical evidence of previous lymphoma (*de novo* DLBCL; n = 129) and DLBCL transformed from a previous follicular lymphoma (transformed DLBCL; n = 11). The study was approved by the institutional review board and written consent was obtained from the patients.

Genomic DNA was purified by cell lysis followed by digestion with proteinase K, *salting out* extraction, and precipitation by ethanol.[25]

Bisulfite treatment

DNA from tumor specimens was subjected to chemical treatment with sodium bisulfite as previously reported.[26] Briefly, 1 μg of DNA was denatured by treatment with NaOH, and modified by sodium bisulfite treatment for 16 h at 50°C. DNA samples were then purified using Wizard DNA purification resin (Promega), desulphonated by incubating in a final concentration of 0.3 M NaOH, precipitated with ethanol, and resuspended in water.

Methylation-specific polymerase chain reaction (MSP)

The modified DNA was used as a template for MSP, a molecular technique that allows the distinction between methylated and unmethylated DNA.[26] MSP was performed on 50 ng of bisulfite-treated DNA under the following conditions: a denaturing step at 95°C for 7 min is followed by 35 cycles at 95°C (30 sec per cycle), the annealing temperature being specific for each reaction (30 sec per cycle), and 72°C (30 sec per cycle), in a Hybaid DNA thermal cycler. The PCR mixture contained 10×Gold buffer (Perkin Elmer), 6.7 mM $MgCl_2$, 10 μM of each primer, 1 mM dNTPs and 0.625 U of Taq Gold

(Perkin Elmer, 5 U/μL), in a final volume of 25 μL.

Primers for *MGMT* were: 5'-TTT GTG TTT TGA TGT TTG TAG GTT TTT GT-3' (sense) and 5'-AAC TCC ACA CTC TTC CAA AAA CAA AAC A-3' (antisense) for the unmethylated reaction; and 5'-TTT CGA CGT TCG TAG GTT TTC GC-3' (sense) and 5'-GCA CTC TTC CGA AAA CGA AAC G-3' (antisense) for the methylated reaction.[8] The annealing temperature for both the unmethylated and the methylated reactions was 59°C. Primers for *DAP-k* were: 5'-GGA GGA TAG TTG GAT TGA GTT AAT GTT-3' (sense) and 5'-CAA ATC CCT CCC AAA CAC CAA-3' (antisense) for the unmethylated reaction; and 5'-GGA TAG TCG GAT CGA GTT AAC GTC-3' (sense) and 5'-CCC TCC CAA ACG CCG A-3' (antisense) for the methylated reaction.[7] The annealing temperature for both the unmethylated and the methylated reactions was 60°C. Primers for *p73* were: 5'-AGG GGA TGT AGT GAA ATT GGG GTT T-3' (sense) and 5'-ATC ACA ACC CCA AAC ATC AAC ATC CA-3' (antisense) for the unmethylated reaction; and 5'- GGA CGT AGC GAA ATC GGG GTT C -3' (sense) and 5- ACC CCG AAC ATC GAC GTC CG -3' (antisense) for the methylated reaction.[6] The annealing temperature for both the unmethylated and the methylated reactions was 58°C. Primers for *CASP8* were: 5'-TAG GGG ATT TGG AGA TTG TGA-3' (sense) and 5'-CAT ATA TCT ACA TTC AAA ACA A-3' (antisense) for the unmethylated reaction; and 5'-TAG GGG ATT CGG AGA TTG CGA-3' (sense) and 5-CGT ATA TCT ACA TTC GAA ACG A-3' (antisense) for the methylated reaction.[21] The annealing temperature was 54°C for the unmethylated reaction and 52°C for the methylated reaction. Primers for *GSTP1* were: 5'-GAT GTT TGG GGT GTA GTG GTT GTT -3' (sense) and 5'-CCA CCC CAA TAC TAA ATC ACA ACA -3' (antisense) for the unmethylated reaction; and 5'-TTC GGG GTG TAG CGG TCG TC-3' (sense) and 5-GCC CCA ATA CTA AAT CAC GAC G-3' (antisense) for the methylated reaction.[20] The annealing temperature was 58°C for the unmethylated reaction and 52°C for the methylated reaction.

All MSP analyses were performed with positive and negative controls for both unmethylated and methylated alleles. Also, control experiments without DNA were performed for each set of PCR. Ten microliters of each PCR were directly loaded onto 2.5% agarose gels, stained with ethidium bromide, and visualized under UV illumination.

Immunohistochemical staining for MGMT

The correlation between *MGMT* methylation status and *MGMT* protein epression was assessed in a representative panel of 28 mature B-cell neoplasms. Paraffin-embedded tissue sections were deparaffinized with xylene, dehydrated by using a graded series of ethanol and treated for 30 minutes in TEC (Tris-EDTA-Citrate) solution (pH 7.8) in a microwave oven at 250 W. Immunohistochemistry was performed using the ABC method (ABC-Elite kit, Vector, Burlingame, CA, USA) with diaminobenzidine as the chromogen. Commercially available mouse anti-MGMT monoclonal antibody (clone MT3.1; Chemicon Intl., Temecula, CA, USA) at a 1:100 dilution was used.[27] This antibody had been previously demonstrated to be useful for immunohistochemistry and to correlate with MGMT activity.[11,28] Nuclear staining was determined by two authors (A.G. and A.C.) who did not have knowledge of the molecular analysis of the samples.

Protein preparation and Western blot analysis of DAP-kinase

Ice cold lysis buffer was used to lyse 10^7 cells. The composition of this lysis buffer was as follows: 10 mM phosphate buffer, pH 7.5, 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 1 μg/mL phenylmethyl sulphonyl fluoride (PMSF), and 1× complete protease inhibitor (Boehringer Mannheim). Subsequently, equal amounts of total protein (400 μg) from each sample were separated on 7.5% SDS-PAGE (Bio-Rad, Hercules, CA, USA). The proteins were transferred onto a nitrocellulose membrane, which was blocked with 5% BSA/TTBS for 2 hours and then incubated with an anti-*DAP-k* goat polyclonal antibody (1:1,000 dilution in 1% BSA/TTBS; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1-2 hours. After washing, filters were reacted with horseradish peroxidase-coupled secondary antibody, and revealed with an enhanced chemiluminescence (ECL) detection system (Amersham, Arlington Heights, IL, USA) after exposure to film.

Results

DAP-k, MGMT, GSTP1, p73 and CASP8 methylation in non-neoplastic samples

The sequences of *MGMT*, *DAP-k*, *GSTP1*, *p73* and *CASP8* analyzed in our study by MSP are localized in CpG islands spanning the 5' non-coding region of the corresponding genes. The aberrant methylation of these regions has been reported to be associated with transcriptional silencing.[6,7,11,20,21,28]

To verify that aberrant methylation of these genes is limited to neoplastic B-cells, and to exclude the possibility of non-specific reactions, we tested a panel of non-neoplastic lymphoid tissues represented by normal peripheral blood leukocytes (n = 5), EBV-immortalized B lymphocytes (n = 8), and samples of reactive polyclonal B-cell hyperplasia (n = 12). None of these non-neoplastic samples displayed methylation of *MGMT*, *DAP-k*, *GSTP1*, *p73* or *CASP8* CpG islands (*data not shown*), confirming that aberrant methylation is not

Table 1. Aberrant methylation of *DAP-k*, *MGMT*, *GSTP1*, *p73* and *CASP8* in B-cell neoplasia.

Histology	*DAP-k*	*MGMT*	*GSTP1*	*p73*	*CASP8*
Precursor B-cell neoplasms					
Acute lymphoblastic leukemia	3/22 (14.2 %)	5/21 (23.8 %)	3/11 (27.7%)	3/22 (22.7%)	0/10
Peripheral B-cell neoplasms					
B-cell chronic lymphocytic leukemia	8/30 (26.6 %)	3/30 (10.0 %)	1/10 (10.0%)	0/12	0/10
Lymphoplasmacytic lymphoma	0/9	1/9 (11.1 %)	0/7	0/8	0/7
Mantle cell lymphoma	5/19 (26.3 %)	1/18 (5.5 %)	2/8 (25.0%)	0/12	0/9
Follicular lymphoma	17/20 (85.0 %)	5/20 (25.0 %)	10/18 (55.5%)	1/10 (10.0%)	0/9
MALT lymphoma	8/11 (72.2 %)	3/11 (27.2 %)	5/10 (50.0%)	1/11 (9.1%)	0/9
Hairy cell leukemia	3/11 (27.2 %)	4/10 (40.0 %)	6/8 (75.0%)	0/9	0/5
Diffuse large B-cell lymphoma	71/126 (56.3 %)	47/132 (35.6 %)	38/99 (38.3%)	10/113 (8.8%)	0/100
Mediastinal large B-cell lympnoma	6/10 (60.0%)	7/10 (70.0%)	3/10 (30.0%)	0/4	0/10
Burkitt's lymphoma	14/28 (50.0 %)	5/28 (17.8%)	13/25 (52.0%)	1/9 (9.1%)	0/9
Primary effusion lymphoma	0/1	1/2	0/2	nd[a]	nd[a]
Plasma cell myeloma	0/16	2/16 (12.5%)	0/10	0/11	0/10

[a]*nd: not done.*

related to cell culture, viral transformation, cell activation or proliferation.

Aberrant methylation of MGMT promoter in B-cell neoplasia

The results of *MGMT* methylation analysis in B-cell neoplasia are detailed in Table 1 and representative examples are shown in Figure 1. Overall, *MGMT* aberrant methylation occurred in both precursor B-cell neoplasia (5/21 ALL; 23.8%) and mature B-cell tumors 79/286 (27.6%). *MGMT* aberrant methylation was heterogeneously distributed throughout the entire clinico-pathologic spectrum of B-cell neoplasia, and preferentially targeted MLBCL (7/10; 70.0%) and DLBCL (47/132; 38.0%) among the aggressive lymphomas, and HCL (4/10; 40.0%), MALT lymphoma (3/11; 27.2%) and FL (5/20; 25.0%) among the indolent lymphoid malignancies (Table 1).

In order to confirm the biological effect of MGMT promoter methylation, we performed expression studies by immunohistochemistry of the MGMT protein in normal germinal center B cells, which represent the putative normal counterpart of a large fraction of mature B-cell lymphoid tumors, and in selected lymphoma samples. Immunohistochemistry experiments showed that i) the MGMT protein is physiologically expressed by normal germinal center B cells (Figure 2), which are physiologically unmethylated at the *MGMT* promoter; and ii) *MGMT* promoter methylation correlated with absent MGMT protein expression in lymphoma cells. In fact, all (n = 17) lymphoma samples carrying MGMT aberrant methylation and tested by immunohistochemistry failed to express the protein in virtually all tumor cells (Figure 3); conversely, all (n = 11) lymphoma samples carrying unmethylated *MGMT* alleles and tested by immunohistohemistry expressed the *MGMT* protein (Figure 3).

Aberrant methylation of DAP-k promoter in B-cell neoplasia

The results of *DAP-k* promoter methylation in B-cell neoplasia are detailed in Table 1 and representative examples are shown in Figure 1. Overall, *DAP-k* promoter methylation was uncommon in ALL (3/22; 14.2%), while it was frequently observed in mature B-cell neoplasia (132/281; 46.9%). Among mature B-cell neoplasms, aberrant methylation of *DAP-k* did not occur randomly, but rather clustered with specific clinicopathologic categories. In particular, FL and MALT-lymphoma showed the highest prevalence of *DAP-k* aberrant methylation, since the aberrant methylation was detected in 17/20 (85.0%) FL and 8/11 (72.2%) MALT-lymphomas. *DAP-k* aberrant methylation was also common in aggressive NHL, occurring in DLBCL (71/126; 56.3%), MLBCL (6/10; 60.0%) and BL (14/28 50.0%) (Table 1).

In order to confirm the biological effect of *DAP-k* aberrant methylation, we performed expression studies by Western blot of the DAP-k protein in lymphoma cell lines. This analysis showed that DAP-k protein was expressed by lymphoma cell lines unmethylated at the *DAP-k* promoter, while DAP-k protein expression was absent in lymphoma cell lines harboring *DAP-k* promoter methylation (Figure 4).

Aberrant methylation of GSTP1 promoter in B-cell neoplasia

GSTP1 promoter hypermethylation was observed in both ALL (3/11; 27.7%) and mature B-cell neoplasia (78/207; 37.6%) (Table 1 and Figure 1). Among mature

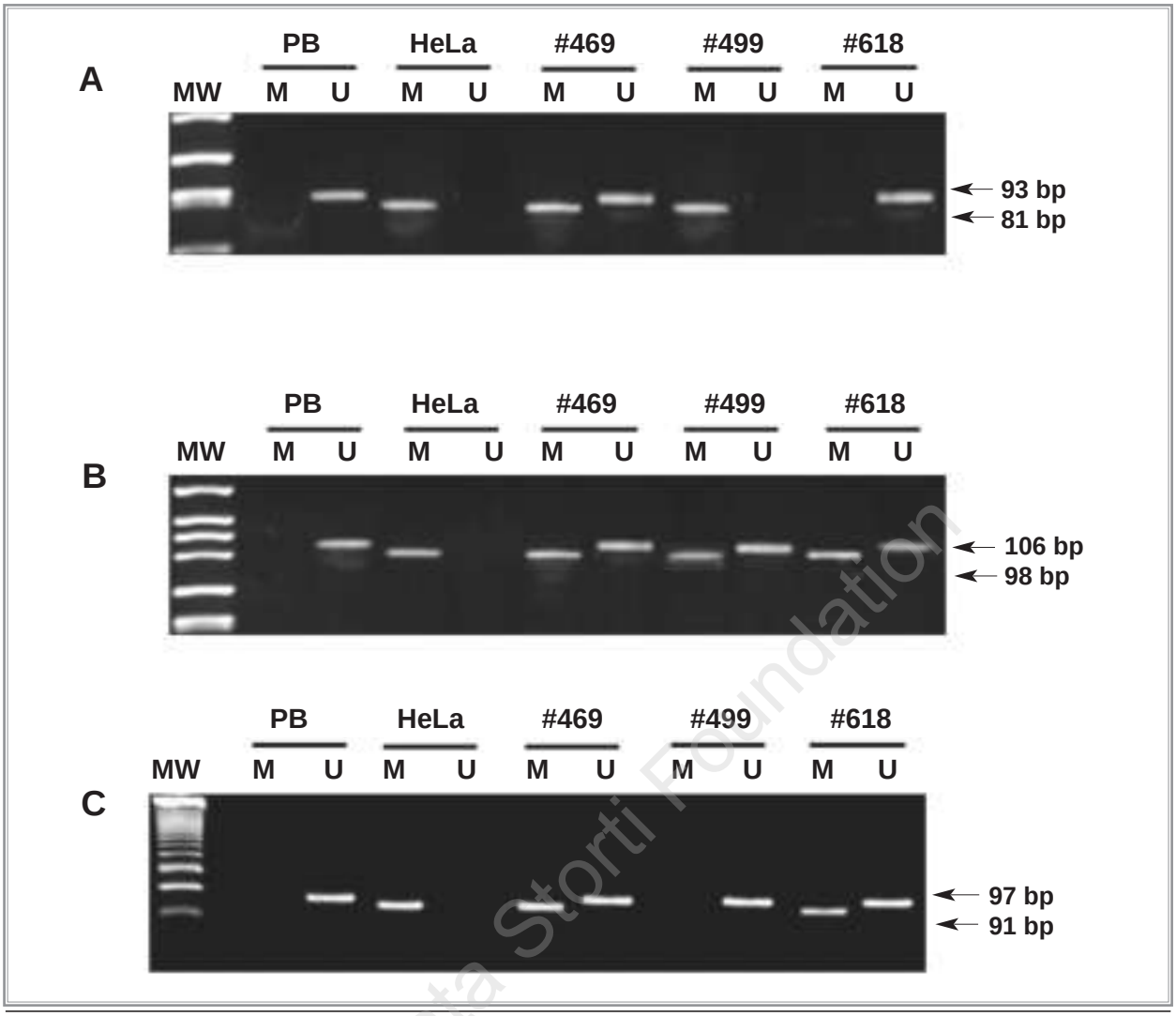


Figure 1. Methylation-specific PCR of the genes *MGMT* (panel A), *DAP-k* (panel B) and GSTP1 (panel C) in representative cases of DLBCL. The presence of a visible PCR product in lane M indicates the presence of a methylated allele (*MGMT*: 81 bp; *DAP-k*: 98 bp; *GSTP1*: 91 bp), while the presence of a product in lane U indicates the presence of unmethylated alleles (*MGMT*: 93 bp; *DAP-k*: 106 bp; *GSTP1*: 97 bp). In all experiments, the Hela cervical cancer cell line was used as the positive control for methylated alleles. Peripheral blood (PB) mononuclear cells were used as the control for unmethylated alleles. MW, molecular weight markers.

B-cell neoplasms, aberrant methylation of *GSTP1* was frequently detected in HCL (6/8; 75.0%) and also occurred in a fraction of FL (10/18; 55.5%), BL (13/25; 52.0%), MALT lymphoma (5/10; 50.0%) and DLBCL (38/99; 38.3%) (Table 1).

p73 promoter hypermethylation in B-cell neoplasia

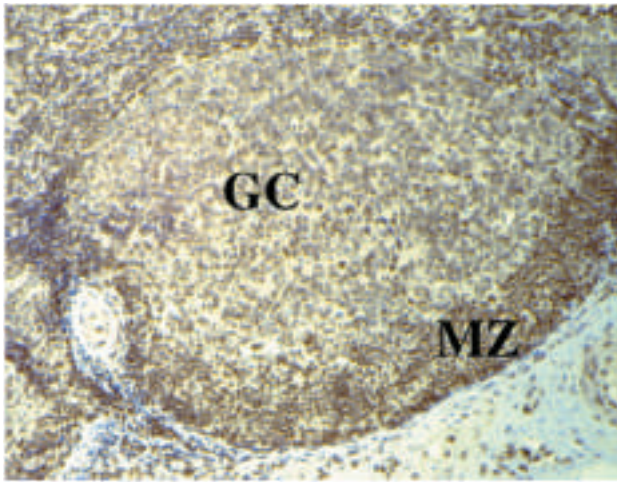
Aberrant methylation of *p73* occurred in a fraction of ALL (3/22; 22.7%), while it was uncommon in mature B-cell neoplasia (13/199; 6.5%) (Table 1). Notably, among BL cell lines, aberrant methylation of p73 occurred at a remarkably higher frequency (5/11; 45.4%) than among BL primary samples (1/9; 9.1%) (Table 1).

CASP8 promoter hypermethylation B-cell neoplasia

None of the B-cell neoplasms investigated in this study showed promoter methylation of the *CASP8* gene (Table 1).

Longitudinal follow-up of DAP-k, MGMT and GSTP1 aberrant methylation

In an attempt to clarify the timing of acquisition of aberrant methylation of *DAP-k*, *MGMT* and *GSTP1* in transformed cells, we studied 11 DLBCL transformed from a previous follicular phase before and after histologic progression (Table 2 and Figure 5). Aberrant methylation of *DAP-k* occurred in 7/9 (77.7%) patients in follicular phase and in 7/9 (77.7%) transformed sam-



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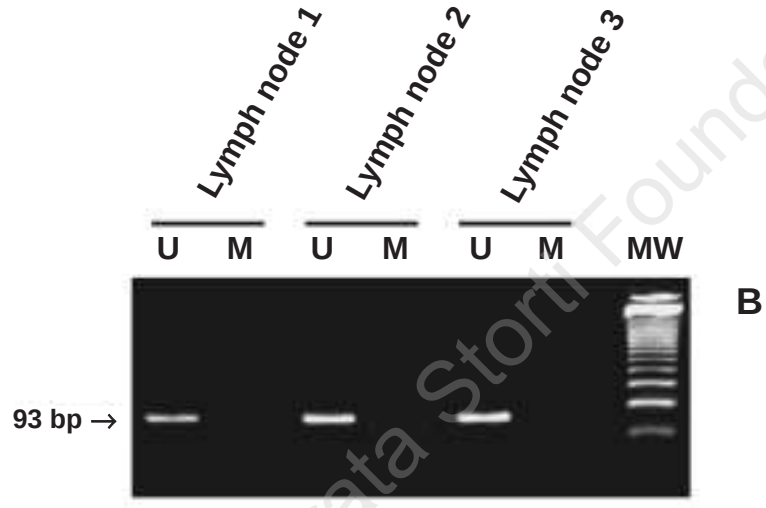

B

Figure 2. Panel A: MGMT protein expression in normal B cells. MGMT protein is expressed by normal mantle zone (MZ) and germinal center (GC) B cells. Paraffin-embedded tissue sections; ABC method, hematoxylin counterstain. Original magnification ×250. Panel B: Methylation-specific PCR of the *MGMT* gene in samples of normal lymph nodes. *MGMT* promoter is physiologically unmethylated in normal lymph nodes. MW, molecular weight markers.

ples. All cases displaying *DAP-k* methylation in the follicular phase retained this alteration during the progression to DLBCL. Aberrant methylation of *MGMT* occurred in 1/8 (12%) follicular phases and in 2/8 (25%) transformed samples. In particular, one patient displayed aberrant methylation of *MGMT* in the transformed, but not in the follicular phase, suggesting that aberrant methylation had been acquired at the time of histologic progression. Aberrant methylation of *GSTP1* occurred in 5/8 (62.5%) patients in follicular phase and in 4/8 (50.0%) transformed cases. Two patients acquired and 3 patients lost aberrant *GSTP1* methylation at the time of transformation.

Methylation status of B-cell neoplasia

We performed a comparative analysis of the methylation pattern of B-cell neoplasia. Overall, simultaneous promoter hypermethylation in ≥ 3/5 genes occurred in 3/10 (30.0%) MALT lymphoma, 2/8 (25.0%) HCL, 11/102 (10.7%) DLBCL, 1/18 (5.5%) FL and 1/24 (4.1%) BL.

Discussion

The aim of this study was a comprehensive investigation of aberrant promoter methylation of multiple genes throughout the clinico-pathologic spectrum of B-cell neoplasia. We report that i) aberrant promoter methylation of *MGMT*, *DAP-k* and *GSTP1* is involved in the molecular pathogenesis of B-cell neoplasia; and ii) *MGMT*, *DAP-k* and *GSTP1* promoter methylation is not randomly distributed among B-cell neoplasms, but preferentially targets specific clinico-pathologic categories of the disease. Overall, these data have multiple implications for the understanding of the molecular pathogenesis of B-cell neoplasia.

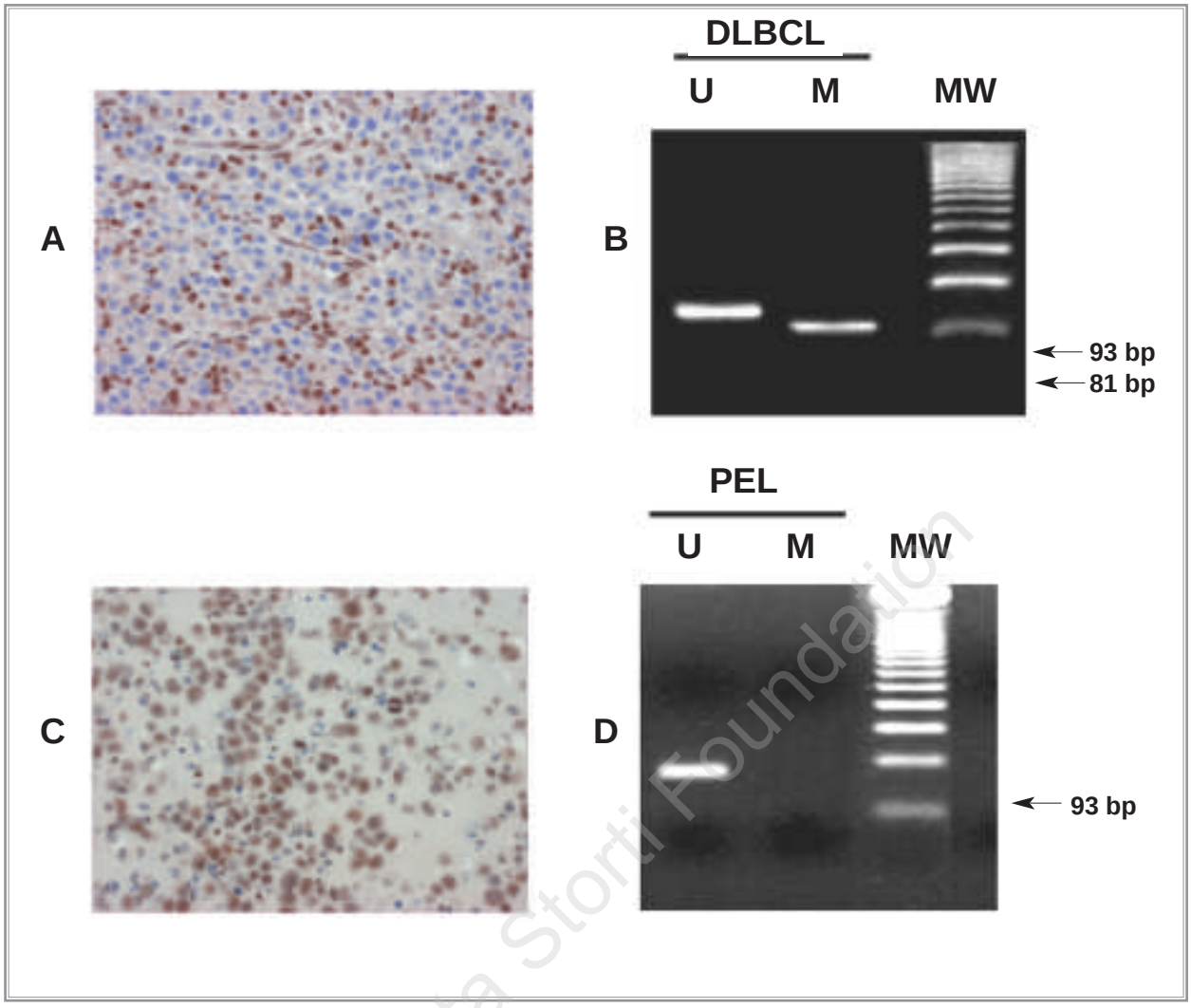

Figure 3. MGMT protein expression in B-cell neoplasia. Panels A-B: Loss of MGMT protein expression in lymphoma cells (panel A) is demonstrated in a representative case of DLBCL that displays *MGMT* promoter hypermethylation (panel B). The occurrence of an unmethylated signal in the methylation-specific PCR of the *MGMT* gene of this DLBCL case (panel B) may be ascribed to the presence of contaminating non-neoplastic cells staining positive for *MGMT* expression (panel A). Panel C-D: A case of primary effusion lymphoma (primary sample), unmethylated at the *MGMT* promoter (panel D), shows expression of the *MGMT* protein (panel C). A) Paraffin-embedded tissue section, C) Cell block; ABC method, hematoxylin counterstain. Original magnification × 400. MW, molecular weight markers.

DAP-k aberrant methylation is the commonest epigenetic alteration identified to date in FL and MALT lymphoma, further confirming the role of apopotosis deregulation in the molecular pathogenesis of these types of lymphoma. In fact, in lymphoma cells, *DAP-k* inactivation results in disruption of the extrinsic pathway of apoptosis initiated by interferon γ, tumor necrosis factor α and FAS ligand.[7,16,29] Because resistance to FAS-induced apoptosis is a common event in B-cell NHL pathogenesis and appears to occur independently of *FAS* gene mutations or FAS protein expression,[30] it is possible that *DAP-k* methylation may represent a major determinant of the FAS-resistant phenotype in lymphoma. Thus, inactivation of the extrinsic pathway of apoptosis through *DAP-k* methylation may reinforce and possibly co-operate with the survival advantage conferred to lymphoma cells by *BCL-2* deregulation in FL and *NK-κB* activation in MALT lymphoma.[31] On these bases, the concomitant disruption of both the intrinsic and the extrinsic pathways of apoptosis corroborate the view that FL and MALT lymphomas are diseases of cell death regulation. *DAP-k* inactivation may also play a synergistic role with *c-MYC* deregulation. In fact, loss of *DAP-k*, by downregulating *p53*, may favor *c-MYC*-induced transformation.[17] Remarkably, BL, which have *c-MYC* deregulation in all cases, also carry *DAP-k* inactivation in 50% of samples.[7]

Our results demonstrate that *MGMT* inactivation

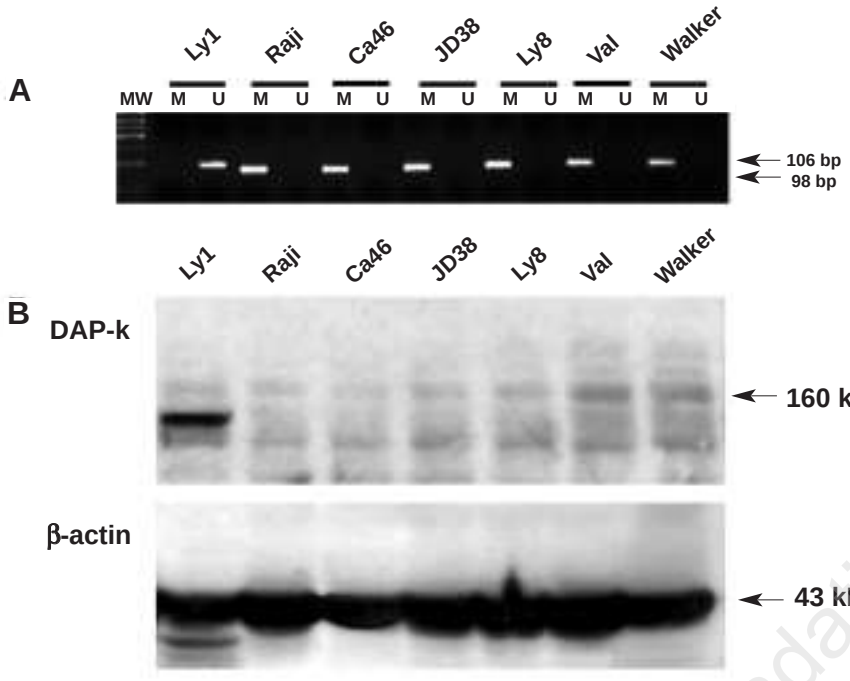

Figure 4.MSP analysis of *DAP-k* promoter hypermethylation (panel A) and Western Blot analysis of DAP-k protein and β–actin (as an unrelated protein reference) expression in various B-cell neoplasia cell lines (panel B). The diffuse large B-cell lymphoma cell line Ly1, unmethylated at the *DAP-k* gene promoter, maintains DAP-k protein expression. In contrast the Burkitt's lymphoma cell lines Raji, Ca46, JD38 and Walker and the diffuse large B-cell lymphoma cell lines Ly8 and Val, which carry *DAP-k* promoter hypermethylation, lack DAP-k protein expression. MW, molecular weight markers.

through promoter methylation occurs with variable frequencies throughout the entire spectrum of B-cell neoplasia. *MGMT* methylation is selectively restricted to neoplastic cells and is consistently absent in normal lymphoid cells, pointing to a pathogenetic role of this epigenetic lesion. Consistent with the DNA repair function of *MGMT* against spontaneous G to A transitions, *MGMT* inactivation may cause genetic instability favoring lymphomagenesis through the acquisition of DNA point mutations.[32] The pathogenetic role of *MGMT* inactivation is further supported by the fact that *MGMT* knockout mice develop lymphoma at high frequency.[15]

Aberrant methylation of *GSTP1* occurred frequently in several types of B-cell malignancies, including 50% MALT lymphomas. The potential pathogenetic role of *GSTP1* inactivation through promoter methylation is linked to its important role in scavenging reactive oxygen species and their metabolites and protecting cells from DNA damage produced by these agents.[18] Polymorphisms in the GST gene family, to which *GSTP1* belongs, may decrease enzyme activity and have been shown to be a risk factor for the development of NHL.[33-35] In particular, the risk of gastric *MALT* lymphoma, which develops in an inflammatory microenvironment rich in reac-

Table 2. Longitudinal follow-up of *DAP-k*, *MGMT* and *GSTP1* aberrant methylation in follicular lymphoma in follicular phase and after *transformation* to diffuse large B-cell lymphoma (transformed phase).

Case	*Follicular phase*			*Transformed phase*		
	DAP-k	*MGMT*	*GSTP1*	*DAP-k*	*MGMT*	*GSTP1*
2368	M	–	–	M	–	–
2700	–	M	–	–	M	–
2701	M	U	U	M	U	M
2702	–	U	–	–	U	–
2703	M	U	M	M	U	U
2704	M	U	M	M	U	M
2705	M	U	U	M	M	U
2706	U	U	U	U	U	M
2718	U	–	M	U	–	U
2721	M	U	M	M	U	M
2723	M	–	M	M	–	U

[a]*U: unmethylated; M: methylated; –: not available.*

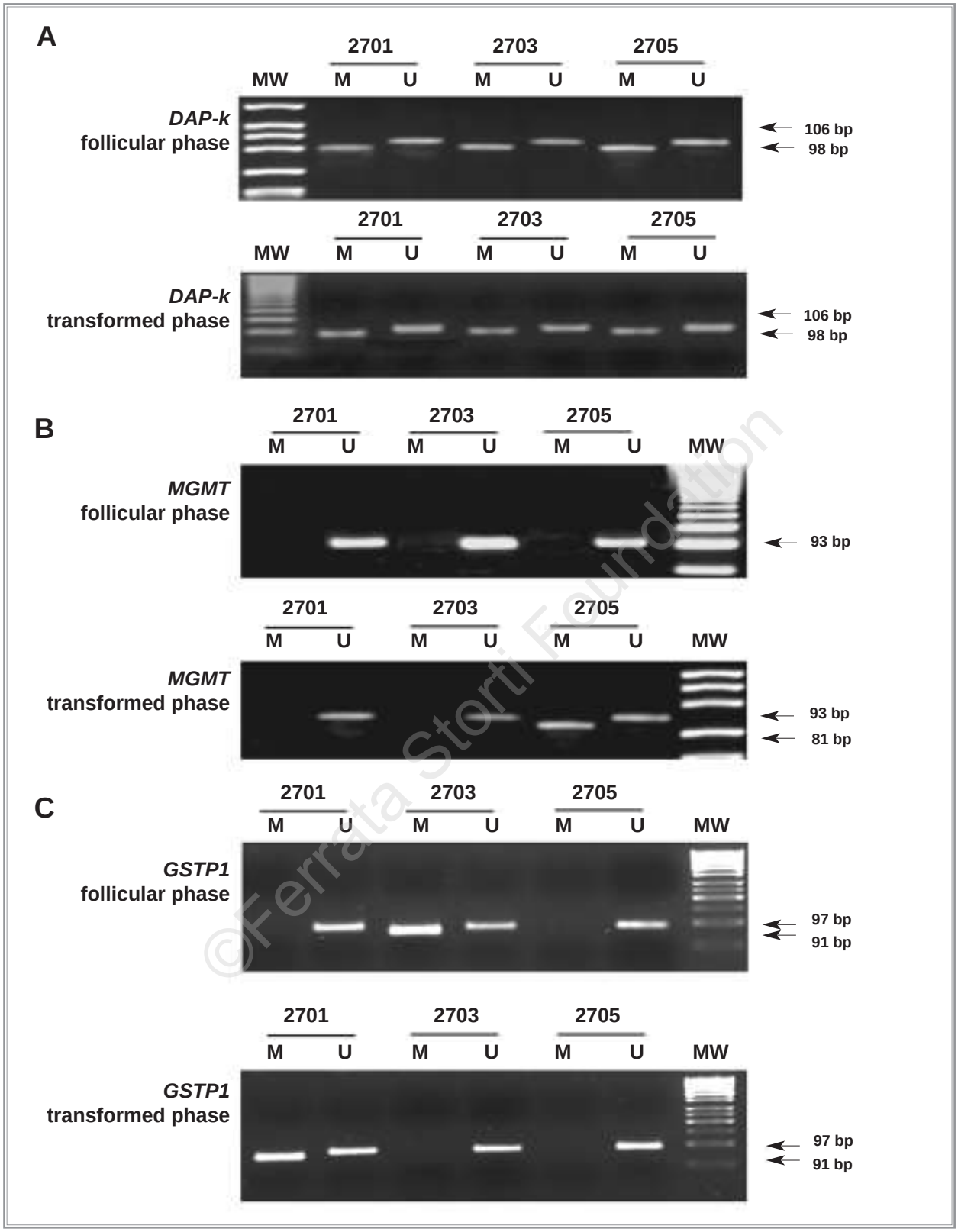

Figure 5. Longitudinal follow-up of *DAP-k*, *MGMT* and *GSTP1* aberrant methylation by MSP analysis in three representative lymphoma cases tested in the follicular phase and after transformation to diffuse large B-cell lymphoma. Panel A: All cases displaying *DAP-k* methylation in the follicular phase retain this alteration during progression to diffuse large B-cell lymphoma. Panel B: Case 2705, unmethylated at the *MGMT* promoter in the follicular phase, acquires *MGMT* methylation at the time of histologic progression to diffuse large B-cell lymphoma. Panel C: Case 2701 acquires and case 2703 loses aberrant *GSTP1* methylation at the time of transformation to diffuse large B-cell lymphoma. MW, molecular weight markers.

tive oxygen species induced by *Helicobacter pylori* infection, is strongly influenced by polymorphisms affecting the antioxidative capacity mediated by the GST enzyme family.[36] In this respect, the frequent epigenetic inactivation of *GSTP1* in MALT lymphoma may provide an alternative mechanism favoring accumulation of reactive oxygen species and lymphomagenesis in the context of chronic gastric inflammation.

In a longitudinal follow-up, three follicular lymphoma cases carrying *GSTP1* promoter hypermethylation during the follicular phase lost *GSTP1* methylation in the transformed phase. Since aberrant promoter hypermethylation is generally an irreversible epigenetic modification of DNA,[1] it is conceivable that loss of *GSTP1* methylation in these cases may have been caused by the emergence of tumor subclones unmethylated at the *GSTP1* promoter during transformation. Such subclones may have gained additional genetic lesions that rendered *GSTP1* inactivation no longer necessary for neoplastic cell survival.

Previous studies have reported a significant frequency of *p73* methylation in DLBCL and in BL.[6,10,37,38] In this report, *p73* aberrant methylation was restricted to a fraction of ALL, while it was virtually absent in all other primary tumor samples. Discrepancies may be related to the lower number of cases previously investigated or to the predominance of cell line samples in previous tumor panels. In fact, our results document that the prevalence of *p73* hypermethylation is remarkably higher in BL cell lines than in BL primary samples, suggesting that *p73* methylation may be selected for during *in vitro* establishment and/or growth of lymphoma cell lines.

The frequent involvement of *MGMT* and *GSTP1* inactivation in a fraction of B-cell neoplasms may also be of prognostic relevance. Indeed, *MGMT* hypermethylation is a major determinant of alkylator refractoriness in human tumors and has been shown to predict improved overall and progression-free survival in DLBCL patients treated with conventional cyclophosphamide-containing regimens.[8] Its prognostic impact in other B-cell malignancies has not been investigated to date. *GSTP1* is a phase 2 enzyme involved in detoxification from chemotherapeutic agents, including doxorubicin and alkylating agents.[18] Polymorphisms in the *GSTP1* gene may affect the enzyme's function and have been associated with lower survival in patients with breast cancer,[39] while high GSTP1 protein expression, leading to increased detoxification of chemotherapeutic agents, correlates with a worse outcome in DLBCL.[40] On these bases, the frequency of *GSTP1* aberrant methylation in DLBCL should prompt studies aimed at verifying the prognostic impact of this epigenetic lesion in these lymphomas.

Finally, the simultaneous inactivation of multiple genes in a lymphoma sample may be of potential significance for demethylating therapeutic strategies.[4] Because at least a fraction of MALT lymphoma, HCL and DLBCL display aberrant hypermethylation of ≥3 genes simultaneously, this study prompts future investigations aimed at analyzing the methylation status of large number of genes in B-cell neoplasia.

DR and DC were primarily responsible for collecting and interpreting the data of this work and DR prepared the first draft of the manuscript. SF and AG contributed to data collection and interpretation. AC, SAP and MP provided tumor samples and immunohistochemical data and participated in the study design. KB, GS and UV provided tumor samples, revised clinical files and contributed to the conception of the study. ME provided expertise with methylation analysis. GG was in charge of conceiving the study and revised the paper. All authors critically read the manuscript and gave final approval of the version submitted to the journal. Other authors: CD, EB, MC, AC, CV, MG, and BB contributed to data collection and interpretation. The authors indicated no potential conflict of interest.

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