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

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G rowing evidence has implicated aberrant promoter methylation in the molecular pathogenesis of several human cancers.1-3 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.4-6

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 p16.1 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.3,4 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 (O6-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.8-11

MGMT is a DNA repair gene that removes mutagenic and cytotoxic adducts introduced
into the DNA from environmental and therapeutic alkylating agents. In particular, MGMT inactivation increases cell sensitivity to the genotoxic effect of alkylating agents both in vitro and in vivo. 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.

Aberrant promoter methylation of DAP-\(k\) has been suggested to have a potential role in lymphomagenesis. 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. In addition, DAP-\(k\) also participates in counter-acting c-MYC-induced transformation by activating the p53 checkpoint and favoring c-MYC-induced apoptosis. 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. Its loss of expression is a risk factor for the development of cancer in null mice. Although epigenetic alterations of GSTP1 are recurrent in a wide range of solid tumors, its methylation pattern in B-cell neoplasia is not known. Caspase-8 (CASP8) and p73 are also potential targets of aberrant promoter methylation in lymphoma. 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. 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.

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. 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, 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.

Bisulfite treatment

DNA from tumor specimens was subjected to chemical treatment with sodium bisulfite as previously reported. 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. 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 MgCl2, 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 TTT GTG 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 TCT TTC CGA AAA CGA AAC G-3’ (antisense) for the methylated reaction. 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 GAT AAT GTT-3’ (sense) and 5’-CAA ATC CCT CCC AAA AAC CAC CA-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. The annealing temperature for both the unmethylated and the methylated reactions was 60°C. Primers for p73 were: 5’-AGG GGA TGG GAT 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’-CCA CCC CAA TAC TAA ATC ACA ACA -3’ (antisense) for the methylated reaction. The annealing temperature for both the unmethylated and the methylated reactions was 58°C. Primers for GSTP1 were: 5’-TAG GGG ATT TGG AGA TTT GGA TTC-3’ (sense) and 5’-CAT ATA TCT ACA AAA ACA A-3’ (antisense) for the unmethylated reaction; and 5’-GGA ATT CCA ATG CCA TTC GC-3’ (sense) and 5’-GCA TTT CCT CCC AAA CAC CAA-3’ (antisense) for the methylated reaction. The annealing temperature was 54°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 expression 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. This antibody had been previously demonstrated to be useful for immunohistochemistry and to correlate with MGMT activity. 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 10³ 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/TBS for 2 hours and then incubated with an anti-DAP-k goat polyclonal antibody (1:1,000 dilution in 1% BSA/TBS; 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. 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). Non 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
Aberrant promoter methylation 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 clinical-pathologic spectrum of B-cell neoplasia, and preferentially targeted MLBCL (7/10; 70.0%) and DLBCL (47/132; 35.6%) 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 lymphoma cell lines. This analysis showed that i) MGMT protein is physiologically expressed by normal peripheral B lymphocytes and in selected lymphoma samples. Immunohistochemistry experiments showed that MGMT protein was absent in lymphoma cells harboring aberrant MGMT promoter expression in lymphoma cells. In fact, all (n = 17) lymphoma samples carrying aberrant MGMT 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 analysis 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 more frequently observed in mature B-cell neoplasms (132/281; 46.9%). Among mature B-cell neoplasms, aberrant methylation of DAP-k did not occur randomly, but rather clustered with specific clinical-pathologic categories. In particular, FL and MALT lymphomas 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%), FR (5/20; 25.0%) and FL (7/20; 35.0%) among the indolent lymphoid malignancies (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

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

<table>
<thead>
<tr>
<th>Histology</th>
<th>DAP-k</th>
<th>MGMT</th>
<th>GSTP1</th>
<th>p73</th>
<th>CASP8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precursor B-cell neoplasms</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute lymphoblastic leukemia</td>
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<td>5/21</td>
<td>5/11</td>
<td>3/22</td>
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<td>Peripheral B-cell neoplasms</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-cell chronic lymphocytic leukemia</td>
<td>8/30</td>
<td>3/30</td>
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<td></td>
</tr>
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<td>0/8</td>
<td>0/7</td>
</tr>
<tr>
<td>Mantle cell lymphoma</td>
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<td>1/18</td>
<td>2/8</td>
<td>0/2</td>
<td>0/9</td>
</tr>
<tr>
<td>Follicular lymphoma</td>
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<td>5/20</td>
<td>10/18</td>
<td>1/10</td>
<td>0/9</td>
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<td>MALT lymphoma</td>
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<td>3/11</td>
<td>5/10</td>
<td>1/11</td>
<td>0/9</td>
</tr>
<tr>
<td>Hairy cell leukemia</td>
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<td>4/10</td>
<td>6/8</td>
<td>0/9</td>
<td>0/5</td>
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<td>0/2</td>
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<td>2/16</td>
<td>0/10</td>
<td>0/11</td>
<td>0/10</td>
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</table>

*nd: not done.
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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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**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.
Aberrant promoter methylation in B-cell neoplasia

samples. 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.
DAP-k aberrant methylation is the commonest epigenetic alteration identified to date in FL and MALT lymphoma, further confirming the role of apoptosis 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. 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, 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. 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. Remarkably, BL, which have c-MYC deregulation in all cases, also carry DAP-k inactivation in 50% of samples.

Our results demonstrate that MGMT inactivation
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. The pathogenetic role of MGMT inactivation is further supported by the fact that MGMT knockout mice develop lymphoma at high frequency.\(^\text{15}\) 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.\(^\text{18}\) 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.\(^\text{33-35}\) 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).

<table>
<thead>
<tr>
<th>Case</th>
<th>DAP-k</th>
<th>MGMT</th>
<th>GSTP1</th>
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U: unmethylated; M: methylated; –: not available.

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.
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. 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, 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. 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. 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. Polymorphisms in the GSTP1 gene may affect the enzyme’s function and have been associated with lower survival in patients with breast cancer, while high GSTP1 protein expression, leading to increased detoxification of chemotherapeutic agents, correlates with a worse outcome in DLBCL.

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. 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, MV, and BB contributed to data collection and interpretation. The authors indicated no potential conflict of interest.

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