DNA Methylation Profiles of Lymphoid and Hematopoietic Malignancies

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Abstract

Purpose: Aberrant methylation of the 5’ gene promoter regions is an epigenetic phenomenon that is the major mechanism for silencing of tumor suppressor genes in many cancer types. The aims of our study were (a) to compare the methylation profiles of the major forms of hematological malignancies and (b) to determine the methylation profile of monoclonal gammopathy of undetermined significance (MGUS) and compare it with that of multiple myeloma (MM).

Experimental Design: We compared the aberrant promoter methylation profile of 14 known or suspected tumor suppressor genes in leukemias (n = 48), lymphomas (n = 42), and MMs (n = 40). We also examined the methylation profile of MGUS (n = 40), a premalignant plasma cell dyscrasia. The genes studied represent five of the six “hallmarks of cancer.”

Results: Peripheral blood lymphocytes (n = 14) from healthy volunteers were negative for methylation of all genes, and methylation percentages in 41 nonmalignant tissues (peripheral blood mononuclear cells, bone marrows, and lymph nodes) from hematological patients were low (0–2%), for all 14 genes, confirming that methylation was tumor specific. Ten of the genes were methylated at frequencies of 29–68% in one or more tumor types, and the methylation indices (an indicator of overall methylation) varied from 0.25 to 0.34. With two exceptions, the methylation patterns of leukemias and lymphomas were similar. However, the pattern of MMs varied from the other tumor types for six genes. In general, the methylation pattern of MGUS was similar to that of MM, although the methylation frequencies were lower (the methylation index of MGUS was 0.15, and that of MM was 0.3). However, the methylation frequencies of six genes were significantly higher in MGUS than in control tissues. The relatively high frequencies of methylation in MGUS are consistent with it being a premalignant condition.

Conclusions: The three major forms of lymphoid/hematopoietic malignancies show overlapping but individual patterns of methylation.

Introduction

Lymphomas, leukemias, and multiple myelomas (MMs) are cancers that originate in the hematopoietic or lymphoid tissues (“hematological malignancies”). An estimated 106,200 people in the United States will be diagnosed with lymphoma (61,000), leukemia (30,600), or MM (14,600) in 2003 (1). New cases of these hematological malignancies account for nearly 8% of all estimated new cancer cases in 2003. Hematological cancers, the second most common cause of cancer deaths, are expected to kill over 57,000 Americans in 2003 (1). Lymphoid/hematopoietic disorders are divided primarily into lymphomas, leukemias, and plasma cell dyscrasias. Lymphomas are further classified into two major subtypes, Hodgkin’s lymphoma and non-Hodgkin’s lymphoma, whereas leukemias are divided into two major subtypes, acute and chronic leukemia. Plasma cell dyscrasias are classified into two major subtypes: (a) MM and related malignancies; and (b) monoclonal gammopathy of undetermined significance (MGUS). MGUS is characterized by the presence of a homogenous monoclonal protein (M-component) in the serum of persons without evidence of MM, macroglobulinemia, amyloidosis, or a related plasma cell proliferative disorder. Patients with MGUS are at increased risk for progression to MM or a related plasma cell cancer (2). The risk of progression of MGUS to MM or related disorders has been reported to be about 1%/year (3) or higher (4, 5).

DNA methylation of the promoter region of genes has emerged as the major mechanism of inactivation of tumor suppressor genes [TSGs; (6)]. In many cases, aberrant methylation of the CpG island genes has been correlated with loss of gene expression, and DNA methylation provides an alternative pathway to gene deletion or mutation for the loss of TSG function (6–8). Markers for aberrant methylation may represent a promising avenue for monitoring the onset and progression of cancer. Aberrant promoter methylation has been described for several genes in various malignant diseases, and each tumor type may have its own distinct pattern of methylation (7, 9, 10).

Whereas the methylation of multiple genes has been stud-
Materials and Methods

Cell Lines. Fourteen lymphoma, leukemia, and MM cell lines (Table 2) were either initiated by us (HCC3234, Hut 78, and NCI-H929) or obtained from American Type Culture Collection (Manassas, VA). They were grown in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 5% fetal bovine serum and incubated in 5% CO₂ at 37°C.

Reverse Transcription-PCR for Gene Expression. Expression of the genes was analyzed by reverse transcription-PCR. Total RNA was extracted from cell lines with Trizol (Life Technologies, Inc.) following the manufacturer’s instructions. The reverse transcription reaction was performed on 2 μg of total RNA with Superscript II First-Strand Synthesis using the oligo(dT) primer system (Life Technologies, Inc.). Primer sequences and conditions for reverse transcription-PCR product were as described previously (14–19). The housekeeping gene GAPDH was used as an internal control to confirm the success of the reverse transcription reaction. PCR products were analyzed on 2% agarose gels.

5-Aza-2′-Deoxycytidine Treatment. Cell lines with known gene promoter methylation were incubated in culture medium with methylation inhibitor 5-aza-2′-deoxycytidine at a concentration of 4 μM for 6 days, with medium changes on days 1, 3, and 5.

Clinical Samples. Forty-two lymphomas (36 B-cell lymphomas including 7 Burkitt’s lymphomas and 6 T-cell lymphomas), 48 leukemias (27 acute lymphocytic leukemias, 11 acute myelogenous leukemias, 9 chronic lymphocytic leukemias, and 1 chronic myelogenous leukemia), 40 MMs, and 20 MGUSs were obtained from the Flow Cytometry Facility or from the Department of Pathology at the University of Texas Southwestern Medical Center or from the University Hospital, Vienna, Austria (Table 3). Control samples included peripheral blood

Table 1 Summary data of genes tested

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Chromosomal location</th>
<th>Hallmark category&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Previous studies (ref. no.)</th>
<th>Ref. no. for methodology</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDH1</td>
<td>E-cadherin</td>
<td>16q22</td>
<td>Tissue invasion and metastasis</td>
<td>Le&lt;sup&gt;®&lt;/sup&gt; (17, 37, 38)</td>
<td>39</td>
</tr>
<tr>
<td>CDH1</td>
<td>E-cadherin</td>
<td>16q22</td>
<td>Tissue invasion and metastasis</td>
<td>Le (26)</td>
<td>14</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli gene</td>
<td>5q21</td>
<td>Tissue invasion and metastasis</td>
<td>None</td>
<td>40</td>
</tr>
<tr>
<td>TIMP3</td>
<td>Tissue inhibitor of metalloproteinase-3</td>
<td>22q12–13</td>
<td>Tissue invasion and metastasis</td>
<td>None</td>
<td>41</td>
</tr>
<tr>
<td>p16&lt;sup&gt;INK4A&lt;/sup&gt;</td>
<td>Cyclin-dependent kinase inhibitor 2A</td>
<td>9p21</td>
<td>Limitless replicative potential</td>
<td>Ly (27, 28, 42, 43)</td>
<td>70</td>
</tr>
<tr>
<td>p15&lt;sup&gt;INK4B&lt;/sup&gt;</td>
<td>Cyclin-dependent kinase inhibitor 2B</td>
<td>9p21</td>
<td>Limitless replicative potential</td>
<td>Ly (27, 28, 43, 42)</td>
<td>20</td>
</tr>
<tr>
<td>CRBP1</td>
<td>Cellular retinol-binding protein 1</td>
<td>3q21–22</td>
<td>Limitless replicative potential</td>
<td>Ly (16)</td>
<td>16</td>
</tr>
<tr>
<td>RIZ1</td>
<td>Rb-interacting zinc finger gene 1</td>
<td>1p36</td>
<td>Limitless replicative potential</td>
<td>None</td>
<td>51</td>
</tr>
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<td>RARβ</td>
<td>Retinoic acid receptor β gene</td>
<td>3p24</td>
<td>Limitless replicative potential</td>
<td>Ly (10)</td>
<td>52</td>
</tr>
<tr>
<td>DcR1</td>
<td>Decoy receptor 1</td>
<td>8p22</td>
<td>Apoptosis</td>
<td>Ly (19)</td>
<td>21</td>
</tr>
<tr>
<td>DcR2</td>
<td>Decoy receptor 2</td>
<td>8p22</td>
<td>Apoptosis</td>
<td>Le (19)</td>
<td>15</td>
</tr>
<tr>
<td>DAPK</td>
<td>Death-associated protein kinase</td>
<td>9q34</td>
<td>Apoptosis</td>
<td>Le (19)</td>
<td>15</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>RAS association domain family protein 1</td>
<td>3p21</td>
<td>Self-sufficiency in growth signals</td>
<td>None</td>
<td>34</td>
</tr>
<tr>
<td>p73</td>
<td>p53-related protein p73</td>
<td>1p36</td>
<td>Sustained angiogenesis</td>
<td>Ly (30, 31, 54)</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Le (31, 53, 55)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Hallmark categories are defined by Hanahan and Weinberg (12), and the category selection was from Widschwendter and Jones (13).

<sup>b</sup> Le, leukemia; Ly, lymphoma; MM, multiple myeloma; MGUS, monoclonal gammopathy of undetermined significance.

Table 2 Cell lines

<table>
<thead>
<tr>
<th>Name</th>
<th>ATCC#</th>
<th>Cell line type</th>
<th>Subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC-1</td>
<td>CRL-2230</td>
<td>Lymphoma</td>
<td>B cell</td>
</tr>
<tr>
<td>BC-3</td>
<td>CRL-2277</td>
<td>Lymphoma</td>
<td>B cell</td>
</tr>
<tr>
<td>RL</td>
<td>CRL-2261</td>
<td>Lymphoma</td>
<td>B cell</td>
</tr>
<tr>
<td>Hut 78</td>
<td>TIB-161</td>
<td>Lymphoma</td>
<td>T cell</td>
</tr>
<tr>
<td>Daudi</td>
<td>CCL-213</td>
<td>Lymphoma</td>
<td>B cell</td>
</tr>
<tr>
<td>Raji</td>
<td>CCL-86</td>
<td>Lymphoma</td>
<td>B cell</td>
</tr>
<tr>
<td>HCC3234</td>
<td>CCL-530</td>
<td>Lymphoma</td>
<td>B cell</td>
</tr>
<tr>
<td>CEM</td>
<td>CRL-2264</td>
<td>Leukemia</td>
<td>ALL&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Jurkat</td>
<td>TIB-152</td>
<td>Leukemia</td>
<td>ALL&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>KG-1</td>
<td>CCL-246</td>
<td>Leukemia</td>
<td>AML</td>
</tr>
<tr>
<td>K-562</td>
<td>CCL-243</td>
<td>Leukemia</td>
<td>CML</td>
</tr>
<tr>
<td>U266B1</td>
<td>TIB-196</td>
<td>MM</td>
<td>B cell</td>
</tr>
<tr>
<td>MCF/CAR</td>
<td>CRL-8083</td>
<td>MM</td>
<td>B cell</td>
</tr>
<tr>
<td>NCI-H929</td>
<td>CRL-9068</td>
<td>MM</td>
<td>B cell</td>
</tr>
</tbody>
</table>

<sup>a</sup> ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; MM, multiple myeloma.
Table 3  Tumors<sup>a,b</sup>

<table>
<thead>
<tr>
<th>Tumor</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphoma (n = 42)</td>
<td></td>
</tr>
<tr>
<td>B-cell</td>
<td>36</td>
</tr>
<tr>
<td>T-cell</td>
<td>6</td>
</tr>
<tr>
<td>Leukemia (n = 48)</td>
<td></td>
</tr>
<tr>
<td>ALL&lt;sup&gt;c&lt;/sup&gt;</td>
<td>27</td>
</tr>
<tr>
<td>AML</td>
<td>11</td>
</tr>
<tr>
<td>CML</td>
<td>9</td>
</tr>
<tr>
<td>CLL</td>
<td>1</td>
</tr>
<tr>
<td>MM</td>
<td>40</td>
</tr>
<tr>
<td>MGUS</td>
<td>25</td>
</tr>
</tbody>
</table>

<sup>a</sup> The male:female ratio of our tumor cases was 3:2, and their mean age was 49.6 years (range, 0–87 years).

<sup>b</sup> Lymphoma specimens consisted of 27 lymph nodes, 11 bone marrows, 3 peripheral blood mononuclear cells, and 1 pleural effusion. Leukemia specimens consisted of 22 bone marrows, 13 peripheral blood mononuclear cells and 13 lymph nodes. All of multiple myeloma and monoclonal gammopathy of undetermined significance specimens consisted of bone marrows.

<sup>c</sup> ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; CLL, chronic lymphocytic leukemia; MM, multiple myeloma; MGUS, monoclonal gammopathy of undetermined significance.

Results

Aberrant Promoter Methylation and Expression of CDH1, CDH13, DcR1, DcR2, TIMP3, CRBP1, and DAPK in Hematological Cancer Cell Lines. For seven genes (CDH1, CDH13, DcR1, DcR2, TIMP3, CRBP1, and DAPK) whose methylation status in hematopoietic tumors has not been previously studied in detail, we correlated aberrant promoter methylation with loss of gene expression (Fig. 1A) using a panel of 14 cell lines (Table 2). The overall concordance between loss or down-regulation of gene expression and aberrant methylation of these genes was 86% (range, 79–93%), confirming the relevance of our assay conditions.

5-Aza-2'-deoxycytidine Treatment. For the seven genes tested in cell lines, loss of down-regulation of gene expression in the 14 cell lines varied from 29% (DcR2) to 79% (CRBP1). Treatment with the methylation inhibitor 5-aza-2'-deoxycytidine restored expression in all methylated cell lines tested (Fig. 1B).

Frequency of Methylation of 14 TSGs in Lymphoma, Leukemia, and MM Samples and Nondiagnostic Hematological Tissues. We examined the methylation status of 14 genes in controls and in the three major types of hematological malignancies (Figs. 2 and 3A). The unmethylated form of p16, run as a control for DNA integrity, was detected in all of these samples. We examined the methylation status of 55 nondiagnostic tissue samples (peripheral blood mononuclear cells, bone marrow, and lymph node) derived from three groups (normal volunteers, hematological patients without malignancy, and hematological patients with malignancies in remission). Because methylation patterns of these three control groups were similar, we pooled our data. Most of the control tissues (44 of 55, 80%) had no methylation of any gene. None of the 14 genes was methylated at a frequency of >10%, and the mean MI was 0.02 (Fig. 3B).

In contrast, 90% of malignancies had at least one gene methylated, and the mean MIs were much higher (0.25–0.34). Ten of the genes were methylated at frequencies of 29–68% in one or more tumor types. In particular, five genes (CDH1, CDH13, DAPK, CRBP1, and RARβ) were frequently methylated (>30%) in all tumor types. In general, the methylation patterns of lymphomas and leukemias were similar, with eight genes methylated at frequencies of >20% (CDH1, CDH13, DAPK, CRBP1, p15, DcR1, RARβ, and TIMP3; Fig. 3A). Significant differences in frequencies between lymphomas and leukemias were present for only two genes (DAPK and CRBP1), although the mean MI for lymphomas (0.34) was higher than...
that for leukemias (0.25; P = 0.04; Fig. 3A). Whereas there was a tendency for more methylation in acute leukemia than in chronic leukemia and in B-cell lymphoma than in T-cell lymphoma, the mean MIs of these groups were not significantly different [except for acute leukemia compared with chronic leukemia (P = 0.04)]. Whereas the mean MI of MM (0.30) was not significantly different from that of lymphomas or leukemias, the methylation frequencies in MM of six genes were significantly different from those of one or both of the other two types of malignancies. In particular, DcR1 and p16 were methylated at greater frequencies in MM, whereas RASSF1A methylation was completely absent, and TIMP3 was infrequently methylated in MM (Fig. 3A).

Frequency of Methylation in MGUS Samples. Of the 20 MGUS samples, 17 (85%) had one or more genes methylated, and the mean MI and the frequencies for six genes were higher than those in control tissues (Fig. 3B). Whereas the percentage of MGUS cases with at least one gene methylated was similar to that of MM cases, the mean MI of MGUS cases (0.15) was lower than that of MMs (0.29; P = 0.0005). For most genes, the methylation frequencies were higher in MM cases, and for four genes (CDH13, DcR1, CDH13, and DAPK), these differences were significant. In particular, DAPK was methylated in 36% of MMs and in 0% of MGUS cases (Fig. 3B). Of interest, these MGUS cases had MIs in the range of MMs (0.29–0.36).

Tumor Patterns of Methylation. We used logistic regression to model the absence or presence of methylation in individual genes with different tumor types. These studies indicated that the methylation patterns of leukemias and lymphomas were similar, and in general, the methylation patterns of MMs and MGUS were similar, although MM tended to be a stronger predictor of methylation for many genes.

Effects of Age and Gender on Methylation Status. Because aging has been reported to be one factor in methylation and shows some degree of tissue type dependency, we examined the ages of our control (mean age, 46.3 years) and malignant (mean age, 49.6 years) groups. In the control group, there was no significant relationship between methylation frequencies of any gene or the MI and age. In addition, there was no relationship between methylation frequencies and tumor patient gender. Univariate and multivariate logistic regression analyses confirmed these findings.

Effects of Tumor Cell Percentage on Methylation Status. Because the number of tumor cells present in specimens of hematological malignancies varies greatly, the possibility of false negative methylation results has to be considered. The methylation-specific PCR assays we used were sufficiently sensitive to detect one methylated allele in the presence of 10^3–10^4 unmethylated alleles (20, 23). Because 87 (67%) of the tumor specimens were received from the flow cytometry laboratory, an accurate estimate was available of the number of tumor cells present. Whereas the percentage ranged from 0.1% to 91% (46.3%), there was no relationship between methylation frequencies and the tumor cell percentage.

Correlation between Individual TSGs in Hematological Malignancies. For the genes frequently methylated (>20%) in one or more malignancies, we determined whether there was any correlation between the methylation status of paired genes. We found a tight correlation between the cell adhesion molecules CDH1 and CDH13 (P = 0.0001), and between the decoy receptors DcR1 and DcR2 (P < 0.0001).

Fig. 1 A, representative examples of reverse transcription-PCR and methylation-specific PCR (CDH13 and DcR1) of combined restriction analysis (DAPK) of three genes in lymphoma, leukemia, and multiple myeloma cell lines. Expression of the housekeeping gene GAPDH was run as a control for RNA integrity. Positive control; N, negative control. The presence of a solid band indicates a positive reaction for reverse transcription-PCR and methylation-specific PCR assays. In most instances, there was concordance between the presence of methylation and loss of expression. In a few instances (open arrowheads), both methylated band and gene expression were present, indicating the presence of both methylated and unmethylated alleles. For the combined restriction analysis assay, the ampicilin (in black) is digested by two methylation-specific enzymes (shown) or BSTUI (data not shown). If methylation is present at one or more CpG sites in the ampicilin, the product is digested to yield multiple bands. Both enzymes yielded identical results, confirming their specificity. B, representative examples of the effect of 5-aza-2′-deoxycytidine treatment on restoring gene expression in methylated cell lines. Treatment with 5-aza-2′-deoxycytidine restored expression of the three genes (CDH13, DcR1, and DAPK) illustrated. Expression of the housekeeping gene GAPDH was run as a control for RNA integrity. +, with 5-aza-2′-deoxycytidine treatment; –, without 5-aza-2′-deoxycytidine treatment.
Discussion

Previous studies have described the importance of DNA methylation in human cancers and focused on regions of the genome that may have functional significance resulting from the extinction of gene activity. Whereas most individual cancers have several, perhaps hundreds, of methylated genes, the methylation profiles of individual tumor types are characteristic (7, 9, 23).

We studied the methylation profile of a panel of 14 known or potential TSGs in the three major tumor types of hematological malignancies. Recently, Hanahan and Weinberg (12) described six hallmarks that a cell has to acquire to become malignant: (a) limitless replicative potential; (b) self-sufficiency in growth signals; (c) insensitivity to growth-inhibitory signals; (d) evasion of programmed cell death; (e) sustained angiogenesis; and (f) tissue invasion and metastasis (12, 13). The 14 genes we studied were selected from one of these categories. For the seven genes whose methylation status in hematological malignancies had not been previously studied in detail, we confirmed that our methylation assay conditions were correlated with gene silencing in cells lines derived from hematological malignancies.

We studied appropriate control tissues (peripheral blood mononuclear cells, bone marrows, and lymph nodes) from three groups of controls: healthy volunteers; hematological patients with gene silencing in cells lines derived from hematological malignancies; and hematological cancer patients in remission. For the genes frequently methylated (20%) in one or more forms of hematological malignancies (CDH1, CDH13, DAPK, p16, p15, CRBP1, RARβ, DAPK, and p73) had previously been studied in one or more forms of hematological malignancies (Refs. 16, 17, 19, and 25–30; Table 1). Our findings, with one exception, fall within the published ranges. For DAPK, we have previously published that the original methylation-specific PCR method did not target the promoter region of the gene and that methylation did not correlate with loss of gene expression (21). Using a combined restriction analysis method we devised previously and validated (21), we found somewhat lower frequencies in MM/lymphomas (34–62%) than the figure reported by Katzenellenbogen et al. (19) and Ng et al. (32) using the methylation-specific PCR methodology.

To our knowledge, six of the genes (APC, TIMP3, RIZ1, DcR1, DcR2, and RASSF1A) we studied have not previously been investigated in detail in hematological malignancies. APC is a well-studied molecule involved wnt signaling, whereas TIMP3 is an inhibitor of matrix metalloproteinases. RIZ1 is a
retinoblastoma protein-interacting zinc finger gene and a member of the nuclear histone methyltransferase superfamily. DcR1 and DcR2 are decoy receptors for tumor necrosis factor-related apoptosis-inducing ligand and, apparently paradoxically, are methylated and silenced in many tumor type (15, 32). RASSF1A is an important new TSG frequently silenced via methylation in many tumor types (33–36). Of these six genes, DcR1, DcR2, and TIMP3 were methylated at frequencies of >20% in one or more hematological malignancies. Whereas RASSF1A is frequently methylated in many epithelial and some pediatric cancers, methylation frequencies in hematological malignancies were very low.

Fig. 3. Comparison of frequencies of aberrant methylation and mean methylation indices in tumor samples (A) or in multiple myeloma, monoclonal gammopathy of undetermined significance, and nonmalignant hematological tissues (B). P values are shown when there was a significant difference between two groups.
Our study represents the first comprehensive comparison of the profile of the three major forms of lymphopoietic/hematopoietic tumors. The profiles of these three tumor types showed important similarities and differences. Of particular interest, the majority of MGUS cases had methylation of one or multiple genes. Our findings are of biological and possible clinical interest.

References
Retraction: DNA Methylation Profiles of Lymphoid and Hematopoietic Malignancies

The authors wish to retract the article titled "DNA Methylation Profiles of Lymphoid and Hematopoietic Malignancies," which was published in the May 1, 2004, issue of Clinical Cancer Research (1).

After a thorough institutional investigation, it was concluded that there was incontrovertible evidence of improper manipulation of figures. It seems that the GAPDH loading controls of Fig. 1B—CDH13 and Fig. 1B—DcR1 (p. 2931) were created by manipulating (cropping of various lanes) Fig. 1A of the referenced article. Dr. Takao Takahashi has accepted the responsibility for Fig. 1B.

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