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 = 20), 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 marrow, and lymph nodes) from hematological patients were low (0.05%), 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.
Technologies, Inc.) supplemented with 5% fetal collection (Manassas, VA). They were grown in RPMI 1640 (Life and NCI-H929) or obtained from American Type Culture Collections (Table 2) were either initiated by us (HCC3234, Hut 78, and NCI-H929) to determine the methylation profile of multiple TSGs in the three major hematological malignancy types. Genes involved in the occurrence of methylation profile of the major forms of hematological malignancies and (b) to determine the methylation profile of MGUS and compare it with that of MM.

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 Southwest Medical Center or from the University Hospital, Vienna, Austria (Table 3). Control samples included peripheral blood

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Chromosomal location</th>
<th>Hallmark category</th>
<th>Previous studies</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</td>
<td>17, 37, 38</td>
</tr>
<tr>
<td>CDH1</td>
<td>E-cadherin</td>
<td>16q22</td>
<td>Tissue invasion and metastasis</td>
<td>Le</td>
<td>26</td>
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<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>
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<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>p16INK4A</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>p15INK4B</td>
<td>Cyclin-dependent kinase inhibitor 2B</td>
<td>9p21</td>
<td>Limitless replicative potential</td>
<td>MM, MGUS (29, 46)</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>
<tr>
<td>RARβ</td>
<td>Retinoic acid receptor β gene</td>
<td>3p24</td>
<td>Limitless replicative potential</td>
<td>Ly (37)</td>
<td>52</td>
</tr>
<tr>
<td>DcR1</td>
<td>Decoy receptor 1</td>
<td>8p22</td>
<td>Apoptosis</td>
<td>None</td>
<td>15</td>
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<tr>
<td>DcR2</td>
<td>Decoy receptor 2</td>
<td>8p22</td>
<td>Apoptosis</td>
<td>None</td>
<td>15</td>
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<tr>
<td>DAPK</td>
<td>Death-associated protein kinase</td>
<td>9q34</td>
<td>Apoptosis</td>
<td>Ly (19)</td>
<td>21</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>RAS association domain family protein 1A</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>
</tbody>
</table>

* Hallmark categories are defined by Hanahan and Weinberg (12), and the category selection was from Widschwendter and Jones (13).

* Le, leukemia; Ly, lymphoma; MM, multiple myeloma; MGUS, monoclonal gammopathy of undetermined significance.

Table 2

<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-2206</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>Raji</td>
<td>CCL-86</td>
<td>Lymphoma</td>
<td>Burkitt</td>
</tr>
<tr>
<td>HCC3234</td>
<td>CCL-213</td>
<td>Lymphoma</td>
<td>Burkitt</td>
</tr>
<tr>
<td>Jurkat</td>
<td>CRL-2264</td>
<td>Leukemia</td>
<td>ALL</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>MC/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>

* ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; MM, multiple myeloma.
Methylation Profile of Hematological Malignancies

Gender, and tumor types with multivariate logistic regression

methylation in individual genes and covariates such as age, flexibly

analyzed on 2% agarose gels containing ethidium bromide. DNA were included for each set of PCR. PCR products were

we used a combined restriction analysis for death-associated protein kinase (DAPK; Ref. 21). Negative control samples with-

resuspended in water. Modified DNA was stored at -80°C until use. References for methodology and gene information are

ulforated with 0.3M NaOH, precipitated with ethanol, and resuspended in water. Modified DNA was stored at -80°C until use. References for methodology and gene information are summarized in Table 1. The methylation status of 13 genes was determined by methylated-specific PCR. For technical reasons, we used a combined restriction analysis for death-associated protein kinase (DAPK; Ref. 21). Negative control samples without DNA were included for each set of PCR. PCR products were analyzed on 2% agarose gels containing ethidium bromide.

Data Analysis. Frequencies of methylation of two groups were compared using \( \chi^2 \) test or Fisher’s exact test. The methylation index (MI), a reflection of the methylation status of all of the genes tested, is defined as the total number of genes methylated divided by the total number of genes analyzed. To compare the extent of methylation for the panel of genes examined, we calculated the MI for each case (22) and then determined the mean for the different groups. Statistical analysis of MI between two variables was performed using the Mann-Whitney U nonparametric test. For all tests, probability values of \( P < 0.05 \) were regarded as statistically significant. We investigated the relationship between the absence or presence of methylation in individual genes and covariates such as age, gender, and tumor types with multivariate logistic regression models.

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

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

\( ^b \) 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 gammapathy of undetermined significance specimens consisted of bone marrows.

\( ^c \) ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia; CLL, chronic lymphocytic leukemias; MM, multiple myeloma; MGUS, monoclonal gammapathy of undetermined significance.

Table 3: Tumors

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 or 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 Nonmalignant Hematological Tissues. We examined the methylation status of 14 genes in control tissues 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 nonmalignant 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

| Results |

Table 4: Controls

<table>
<thead>
<tr>
<th>Normal lymphocytes</th>
<th>( n = 14 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients in remission ( n = 29 )</td>
<td>( n = 24 )</td>
</tr>
<tr>
<td>Peripheral blood</td>
<td>( n = 2 )</td>
</tr>
<tr>
<td>Lymph node</td>
<td>( n = 3 )</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>( n = 4 )</td>
</tr>
<tr>
<td>Patients without Ly(^a)</td>
<td>Le, MM ( n = 12 )</td>
</tr>
<tr>
<td>Peripheral blood</td>
<td>( n = 4 )</td>
</tr>
<tr>
<td>Lymph node</td>
<td>( n = 1 )</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>( n = 7 )</td>
</tr>
</tbody>
</table>

\( ^a \) Ly, lymphoma; Le, leukemia; MM, multiple myeloma.
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 (CDH1, 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 MM cases had MIs in the range of MGUS cases (0.29–0.36).

Tumor Patterns of Methylation. We used logistic regression models to ask whether the presence or absence 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 \( 10^3-10^4 \) nonmethylated 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 (\( P < 0.0001 \)).
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 of a cell that 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 cell lines derived from hematological malignancies.

We studied appropriate control tissues (peripheral blood mononuclear cells, bone marrow, and lymph nodes) from three groups of patients: healthy volunteers; hematological patients without cancer and hematological cancer patients in remission. Methylation of all of the genes in these tissues was absent or present at very low frequencies. In some tissues, especially those of the gastrointestinal tract, methylation of certain genes may be age related (24). However, we detected no relationship between methylation and age in either tumor or control tissues. These studies confirmed the tumor-specific nature of the methylation assays used. Because of the relative sensitivity of the methylation assays, our results were not biased in samples containing a low percentage of tumor cells.

There were statistically significant differences between individual types of malignancies and nonmalignant tissues (P < 0.0001). Thirteen genes showed tumor specificity of methylation, and 10 of the genes were methylated at frequencies of 29–68% in one or more tumor types. The mean MI of leukemias was lower than those of the other two tumor types. With two exceptions (DAPK and CRBP1), the methylation patterns of leukemias and lymphomas were similar. However, the pattern of MMIs varied from that of the other tumor types for six genes. Somewhat surprisingly, the major subtypes of leukemias and lymphomas had similar patterns, except that acute leukemias tended to have more methylation than chronic cases.

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 no tight correlation between the cell adhesion molecules CDH1 and CDH13 and between the decoy receptors for tumor necrosis factor-related apoptosis-inducing ligand DcR1 and DcR2.

A finding of considerable interest was the finding that 85% of MGUS cases had one or more genes methylated. Methylation in MGUS cases was significantly higher than that in control tissues but significantly lower than that in MM cases. Three MGUS cases (15%) had MIs similar to that of MMs. Currently, there is no method for determining which MGUS cases are at increased risk of progression to MM or other malignancy. Our findings suggest that methylation of certain genes (CDH1, CDH13, DAPK, and DcR1) may aid in this prediction. However, because of the limited number of cases studied and the lengthy observation period required, further work will be necessary to test this hypothesis.

Of the 14 genes we studied, 8 (CDH1, CDH13, 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 RASSFIA) we studied have not previously been investigated in detail in hematological malignancies. APC is a well-studied molecule involved in 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). *Ps* are shown when there was a significant difference between two groups.
Methylation Profile of Hematological Malignancies

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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Reference


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