Genome-Wide Promoter Analysis UnCOVERS Portions of the Cancer Methylome

Mohammad Obaidul Hoque,¹ Myoung Sook Kim,¹ Kimberly Laskie Ostrow,¹ Junwei Liu,¹ G. Bea A. Wisman,¹ Hannah Lui Park,¹ Maria Luana Poeta,¹,² Carmen Jeronimo,³ Rui Henrique,⁵ Ágnes Lendvai,¹ Ed Schuuring,¹ Shahnaz Begum,¹ Eli Rosenbaum,¹ Maté Ongenaert,⁵ Keishi Yamashita,¹ Joseph Califano,¹ William Westra,² Ate G.J. van der Zee,³ Wim Van Criekinge,⁶ and David Sidransky¹

¹Department of Otolaryngology–Head and Neck Surgery, The Johns Hopkins School of Medicine and ²Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, Maryland; Departments of ³Gynecologic Oncology and ⁴Pathology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands; ⁵Department of Pathology, Portuguese Oncology Institute, University of Porto, Porto, Portugal; ⁶OncoMethylome Sciences S.A, CHU Niveau +4, Tour 4 dePharmacie (bâtiment 36), Liege, Belgium; Laboratory of Molecular Medicine and Biotechnology, University Campus Bio-Medico School of Medicine, Rome, Italy; and ⁷Bioinformatics and Computational Genomics (Biobix), Faculty of Agricultural and Applied Biological Sciences, University of Ghent, Ghent, Belgium

Abstract

DNA methylation has a role in mediating epigenetic silencing of CpG island genes in cancer and other diseases. Identification of all gene promoters methylated in cancer cells “the cancer methylome” would greatly advance our understanding of gene regulatory networks in tumorigenesis. We previously described a new method of identifying methylated tumor suppressor genes based on pharmacologic unmasking of the promoter region and detection of re-expression on microarray analysis. In this study, we modified and greatly improved the selection of candidates based on new promoter structure algorithm and microarray data generated from 20 cancer cell lines of 5 major cancer types. We identified a set of 200 candidate genes that cluster throughout the genome of which 25 were previously reported as harboring cancer-specific promoter methylation. The remaining 175 genes were tested for promoter methylation by bisulfi te sequencing or methylation-specific PCR (MSP). Eighty-two of 175 (47%) genes were found to be methylated in cell lines, and 53 of these 82 genes (65%) were methylated in primary tumor tissues. From these 53 genes, cancer-specific methylation was identified in 28 genes (28 of 53; 53%). Furthermore, we tested 8 of the 28 newly identified cancer-specific methylated genes with quantitative MSP in a panel of 300 primary tumors representing 13 types of cancer. We found cancer-specific methylation of at least one gene with high frequency in all cancer types. Identification of a large number of genes with cancer-specific methylation provides new targets for diagnostic and therapeutic intervention, and opens fertile avenues for basic research in tumor biology. [Cancer Res 2008;68(8):2661–70]

Introduction

Solid human tumors arise and progress through aberrant function of various genes that positively and negatively regulate many aspects of cell function, including proliferation, apoptosis, genome stability, angiogenesis, invasion, and metastasis (1). Discovery and functional assessment of these genes is essential for understanding the biology of cancer and for clinical applications, including identification of therapeutic targets, early cancer detection, and improved prediction of cancer risk and disease course. Many factors can affect gene function, including genetic alterations as well as epigenetic modifications.

Epigenetic modifications are defined as all meiotically and mitotically heritable changes in gene expression that are not coded in the DNA sequence itself. Methylation of the C₅ positions of cytosine residues in DNA has long been recognized as an epigenetic silencing mechanism of fundamental importance (2, 3). DNA methylation alters chromosome structure, inhibits the binding of proteins, such as CTCF, and defines regions of transcriptional regulation (4). DNA methylation can also promote the binding of proteins, such as MECP2, MBD1, MBD2, MBD3, and MBD4, which induce histone modification (5).

CpG dinucleotides are found at increased frequency in the promoter region of many genes, and methylation in the promoter region is frequency associated with “gene silencing”; i.e., the gene is not expressed in the presence of methylation but is expressed in its absence (6). Both global hypomethylation and gene-specific promoter hypermethylation are associated with malignancy (7, 8). Several studies have shown that these epigenetic changes are an early event in carcinogenesis and are present in the precursor lesions of a variety of cancers including lung (9), head and neck (10), and colon (11).

Challenges in analyzing CpG Island (CGI) methylation include distinguishing islands from repetitive DNA sequences, which are usually heavily methylated, and identifying those that regulate gene expression. In an effort to identify important tumor suppressor genes (TSG) silenced by promoter methylation, genome-wide screening techniques to detect differences in DNA methylation were developed. Many of these studies documented that when CGI methylation in promoter regions is appropriately validated, expression of downstream genes is almost always found to be severely repressed or absent (12, 13).

In this study, we used advanced bioinformatics tools and robust data sets from cancer cell lines treated with demethylating agents
to identify novel cancer-specific methylated genes. We then used bisulfite DNA sequencing, methylation-specific PCR (MSP), and quantitative MSP (QMSP) to confirm cancer-specific methylation in a large number of novel genes. Our results confirm computational prediction of methylated CpG sites in cancer through extensive experimentation. Moreover, this approach has greatly expanded our knowledge of methylated promoters in cancer cell lines and primary tumors, has led to the discovery of a substantial portion of “the cancer methylome”, sets the stage for rapid and full elucidation of methylated gene targets and pathways in human cancer.

Materials and Methods

Cell lines. We used 20 different human cancer cell lines in this study. Cell lines were propagated in accordance with the instructions from American Type Culture Collection. Details of the cell lines and their cell of origin are given in Supplementary Table S1 online.

5-aza-2’-deoxycytidine treatment of cells. We seeded all cell lines (1 x 10^6) in their respective culture medium and maintained them for 24 h before treating them with 5 mol/L 5-aza-2’-deoxycytidine (5-aza-dC; Sigma) for 3 d. We renewed medium containing 5-aza-dC every 24 h during the treatment. We handled control cells the same way, without adding 5-aza-dC. Stock solutions of 5-aza-dC were dissolved in phosphate buffer saline PBS (pH 7.5). We prepared total RNA using the RNeasy Mini kit (Qiagen).

Biotinylated RNA probe preparation and hybridization. Several versions of Affymetrix arrays were used for gene expression profiling per the manufacturer's instruction. Hu95A.V2 arrays containing 12,500 human genes were used for the 2 lung squamous cancer cell lines. HGU 133 plus 2 arrays with >55,000 probes for analysis of >47,000 human transcripts were used for profiling the 4 cervical cancer cell lines. For the remaining 14 cell lines, we used GeneChip Human Genome U133A Arrays containing >22,000 probesets for analysis of >18,400 transcripts, which include >14,500 well-characterized human genes. Probe preparation and hybridization were performed following manufacturer’s instructions. Digitized image data were processed using the GeneChip software (version 3.1) available from Affymetrix.

Analysis of expression data. We computed gene expression summary values for Affymetrix GeneChip data using the bioconductor package (which uses background adjustment, quantile normalization, and summarization; ref. 14). Raw data quality was assessed using intensity plots and RNA degradation plots (data not shown). In a second stage, the retained data sets for each cell line of each cancer type were normalized using the MAS5 algorithm (Affymetrix software). We also normalized among the cell lines of each cancer type and among cell lines of all cancer types analyzed (data not shown).

We performed at least two replicates for each cell lines. The expression calls “P” (present), “M” (marginal), and “A” (absent) were determined according to the Affymetrix Array Suite software package. P in the 5-aza-dC treatment data sets was assigned a score of 1 (P-score), and A in the nontreatment data sets was assigned a score of 1 (A-score). For each probe, the expression score was calculated as the sum of the P-score and A-score. Only genes represented by probes with at least one reactivation event (A before treatment to P after treatment) are selected. We then used the previously published algorithm to select candidate genes (12) modified by further selection of promoters with structural and sequence similarities to genes empirically found to be methylated. Brief descriptions of this approach are described below.

BROAD analysis: genome-wide promoter alignment. The Database of Transcription Start Sites (DBTSS)9 mapped each sequence on the human draft genome sequence to identify its transcriptional start site, which provides us with more detailed information on distribution patterns of transcriptional start sites (TSS) and adjacent regulatory regions. From −14,500 well-characterized human genes present in the Affymetrix GeneChip Human Genome U133A Arrays, we extracted 8,793 sequences from the DBTSS (version 3.0 based on human assembly build 31; refs. 15, 16). The remaining genes (14,500 − 8,793 = 5,707) on the Affymetrix array contained no reported TSS according to DBTSS. Subsequently, Newcgrepport (17) was used to identify CGIs [a CGI is defined as a region of minimal 200 bp, a GC content larger than 50%, and the CpG\_adjacent/CpG\_expected (O/E) ratio is >0.60; ref. 18]. These conditions are slightly less stringent than the one proposed by Jones et al. (19). We justified these approaches because we worked using experimentally established and verified gene promoter regions (regions that are closely associated with gene expression) instead of applying the criteria to a genome-wide scan. This resulted in a sequence set of 4,728 genes that were complemented with a set of 56 reported/known cancer-specifically methylated genes chosen from published articles or our data10 (Supplementary Table S2). Of the 4,728 sequences used for clustal alignment, 245 were found to show a given minimal homology to the 56 known genes methylated in cancer but not in normal tissues. We then excluded 132 genes that did not pass the reactivation filter or were already reported to be cancer-specifically methylated, leaving 113 genes (245−132), which we validated by laboratory experimentation.

DEEP analysis: specific binding patterns. Apart from a broad promoter alignment, we sought to determine if there were shorter patterns lost in global alignment (BROAD) associated with known cancer-specific methylation. Therefore, the second (DEEP) part of the computational promoter analysis focused on identification of a discriminating sequence feature between two different functional classes (A and B) of CGI-containing promoters. Class A lists genes that are only methylated in cancer and not in normal, whereas class B enumerates genes that are at least partially methylated in normal (predominantly imprinted genes) tissues (Supplementary Table S3). For each of these genes, we extracted a symmetrical region of 1 kb around the predicted TSS using the DBTSS database (15, 16), and the same definition for CGI was used as for the BROAD analysis. No significant differences in either starting position, GC content, length, or O/E ratio were found for CGIs of genes belonging to class A and class B.

We looked exhaustively for patterns using the Teiresias algorithm (20, 21) with a minimum of 7 nonwild card nucleotides (L) and a maximal length between two nonwild cards of 9 nucleotides (W) that are present in at least 25% of the sequences for each class (A and B). In the next step, we applied different machine learning techniques (22) to extract those patterns for which the frequencies allowed for a discrimination between classes A and B. The following seven motifs (GGGC*GC*C, GCC*GCAC, CTGGG*GA, GCC*GGGGC, AGCTG*CT, A*GGG*GGG, and A*CCG*GCC) were found to be overrepresented in class A versus class B. Using this set of 7 motifs, we identified 261 genes from 8,793 genes extracted from DBTSS. Finally, we ruled out 191 genes (70 remaining) that did not pass the reactivation filter or were already reported cancer-specifically methylated genes.

A total of 10 genes passes both (BROAD and DEEP) sequence filter. Excluding the 25 known cancer-specific methylated genes, a total of 175 genes were tested by laboratory experimentation that passes both sequence and reactivation filters. The list of 25 previously reported methylated genes details in Supplementary Table S4.

Tissue samples and DNA extraction. We evaluated tissue samples from 13 different types of primary cancers (a total of 300 human samples). Tissue samples from 106 age-matched individuals without a history of malignancy were used as controls.

Tissue samples were microdissected to isolate >70% epithelial cells in both neoplastic and nonneoplastic tissues. DNA was prepared as described previously (23).

Bisulfite genomic sequence analysis, conventional MSP, and QMSP. Bisulfite sequence analysis was performed to determine the methylation status in cell lines and a limited number of tissues including primary tumors and age-matched normal controls from the same organ. Bisulfite modification of genomic DNA was carried out as described previously (24).

9 http://elm0.imus.u-tokyo.ac.jp/dbtss/

10 Unpublished data.
and was amplified for the 5' region that included at least a portion of the CGI within 1 kb of the proposed TSS using primer sets (Supplementary Table S5). PCR products were gel purified using the QIAquick Gel Extraction kit (Qiagen) according to the manufacturer's instructions. Each amplified DNA sample was sequenced by the Applied Biosystems 3700 DNA analyzer using nested, forward, or reverse primers and BD terminator dye (Applied Biosystems). When necessary, MSP primers were designed to amplify methylated or unmethylated DNA. Bisulfite-modified DNA was used as template for fluorescence-based QMSP, as previously described (24, 25). Primers and probes were designed to specifically amplify the promoters of the eight genes of interest and the promoter of a reference gene, actin B (ACTB). Primer and probe sequences and annealing temperatures are provided in Supplementary Table S6. The relative level of methylated DNA for each gene in each sample was determined as a ratio of MSP for the amplified gene to ACTB and then multiplied by 1,000 for easier tabulation (average value of triplicates of gene of interest/average value of triplicates of ACTB × 1,000). The samples were categorized as unmethylated or methylated based on detection of methylation above a threshold set for each gene. This threshold was determined by analyzing the levels and distribution of methylation (if any) in normal (nonneoplastic) age-matched tissues and by maximizing the sensitivity and specificity.

Reverse transcription-PCR and real-time reverse transcription-PCR. Reverse transcription-PCR (RT-PCR) was performed as described previously (26). One microliter of each cDNA was used for real-time RT-PCR using QuantiFast SYBR Green PCR kit (Promega). Amplifications were carried out in 384-well plates in a 7900 Sequence Detector System (Perkin-Elmer Applied Biosystems). Expression of genes relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was calculated based on the threshold cycle (Ct) as 2^(-ΔΔCt), where 2^ΔCt = Ct,GENE - Ct,GAPDH and 2^ΔΔCt = 2^ΔCt,M - 2^ΔCt,Aza (M, mock treatment; Aza, 5-Aza-dC treatment). Detailed PCR conditions and primer sequences are available upon request.

Results

We modified the methylated gene discovery algorithm that were applied in our previous studies (12, 26, 27) that required excessive experimental effort and time for a relatively small yield through a process of inclusion of only those targets with similar promoter pattern with known cancer-specific methylated genes. Briefly, we used two selection rules to identify candidate methylated genes in the Human Genome. From the DBTSS, we identified genes with well-characterized TSS included on Affymetrix expression microarrays. We then developed a bioinformatics approach based on two criteria to predict cancer-specific methylated genes. In one part of the analysis, we assumed sequence homology in the promoter regions of known methylation-prone genes and the estimated sequence length containing CGIs. In a further analysis, we identified seven overrepresented sequence patterns in a learning set of known cancer-specific methylated genes versus
Figure 2. Distribution of the predicted 200 methylated genes along the chromosomal map by computational approach. Most of the genes are clustered in limited chromosomal regions. No genes were found on chromosome Y.
tissue-specific methylated genes. We then applied these patterns to real data sets generated in 20 cancer cell lines from the most common types of cancer treated with 5 μmol/L 5-aza-dC to reactivate gene transcripts silenced by promoter methylation. The gene filtering approach and data analysis are depicted in Fig. 1.

We considered a gene to be reactivated if re-expression occurred in at least one cell line of any particular cancer type. The 200 methylation-prone genes identified from this computational approach are shown on a chromosomal map in Fig. 2.

Validation of modified approach in cell lines. Out of the 200 genes predicted to be methylated by our modified approach, 25 genes were identified as reported to harbor cancer-specific methylation after a literature search [Pubmed search words, (particular gene name) and (methylation)]. To validate the reidentification of genes previously shown to be methylated in human cancers represents a critical validation of our modified approach in this study; (b) Genes in which no previous methylation in human cancers was discovered but had been linked to cancer through functional studies (e.g., APC, SFRP1, FHIT, and TWIST). The reidentification of genes previously shown to be methylated in human cancers and absent or lower level/frequency in normal tissues as a control. If the frequency of methylation is higher in cancer and absent or lower level/frequency in normal tissue at an optimal cutoff, we considered it as a cancer-specific methylation. Thus, 28 of 175 (16%) new cancer-specific methylated genes were identified through our combination of a computational approach and empirical studies. We used age-matched normal tissues and cancer cell lines of different cancer types. The cell lines examined showed methylation of target gene and exhibited silencing of mRNA expression (Fig. 3B). This suggests that mRNA expression of these genes were regulated by promoter hypermethylation.

Candidate cancer genes. The cancer-specific methylated genes identified in this study are listed in Table 2. By modified approach, we selected 200 genes, and after empirical testing, 28 were newly identified candidate cancer genes (methylated in primary tumors but not in progenitor cells). Overall, between 2 and 12 newly methylated cancer genes were identified in lung, breast, colon, prostate, and cervical cancer.

The 200 candidate cancer genes identified in this study fell into three categories: (a) genes previously observed to be altered in human cancer by methylation (e.g., APC, SFRP1, FHIT, and TWIST). The reidentification of genes previously shown to be methylated in human cancers is 25 (30–5).

New targets of aberrant methylation in major types of cancer by QMSP. We noted that some of the cancer-specific methylated genes were reactivated and methylated in more than one type of cell line. To determine the frequency of methylation in a larger set of samples and in multiple cancer types, we selected 8 of the most frequently cancer-specific methylated genes from our list of newly identified 28 genes and developed a QMSP assay. We found cancer-specific methylation at various frequencies for each

Table 1. Summary of findings on 200 candidate methylated

<table>
<thead>
<tr>
<th>Tissue types</th>
<th>Potential methylated genes*</th>
<th>Reported methylation in cancer</th>
<th>Number of genes tested</th>
<th>Methylated in cell lines</th>
<th>Methylated in tumor tissues</th>
<th>Methylated in normal tissue</th>
<th>Cancer-specific methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung Squamous</td>
<td>36</td>
<td>7</td>
<td>21</td>
<td>9/21</td>
<td>6/9</td>
<td>3/6</td>
<td>3/6</td>
</tr>
<tr>
<td>Lung adeno</td>
<td>18</td>
<td>5</td>
<td>13</td>
<td>6/13</td>
<td>2/6</td>
<td>1/2</td>
<td>1/2</td>
</tr>
<tr>
<td>Breast</td>
<td>31</td>
<td>1</td>
<td>28</td>
<td>6/28</td>
<td>5/6</td>
<td>3/5</td>
<td>2/5</td>
</tr>
<tr>
<td>Prostate</td>
<td>45</td>
<td>9</td>
<td>36</td>
<td>16/36</td>
<td>8/16</td>
<td>5/8</td>
<td>3/8</td>
</tr>
<tr>
<td>Colorectal</td>
<td>48</td>
<td>4</td>
<td>44</td>
<td>23/44</td>
<td>16/23</td>
<td>10/16</td>
<td>6/16</td>
</tr>
<tr>
<td>Cervical</td>
<td>45</td>
<td>4</td>
<td>33</td>
<td>22/33</td>
<td>16/22</td>
<td>3/16</td>
<td>13/16</td>
</tr>
<tr>
<td>Total</td>
<td>233</td>
<td>30†</td>
<td>175</td>
<td>82/175</td>
<td>53/82</td>
<td>25/53</td>
<td>28/53</td>
</tr>
</tbody>
</table>

NOTE: Total experimentally tested genes: 200 = 25 = 175.

*aNumber of genes selected by modified approach.

†33 overlapping genes across all the cancer types.

5 overlapping in different types of cancer. Thus, number of known cancer-specific methylated genes is 25 (30–5).
gene in multiple types of cancer (Table 3). A high frequency of cancer-specific methylation for at least one gene was identified in every cancer type, supporting the notion that methylated genes are likely to play a role across multiple cancer types.

**Discussion**

Most studies on DNA methylation in cancer have focused on a candidate gene approach where a tumor suppressor or previously reported methylated gene is tested in another type of cancer.

**Figure 3.** A. promoter methylation of representative candidate genes. a, methylation of KIF1A and OSMR by conventional methylation-specific PCR in cancer cell lines and primary tissues; M, methylated; U, unmethylated; NBT, normal breast tissues from noncancer patients; BTT, breast tumor tissues; NN, normal colon epithelium from noncancer patients; PN, paired normal colon tissues from colon cancer patients; PT, paired colon cancer tissues. b, representative sequencing results of the PAK3 and NISCH in cancer cell lines, normal, and cancer tissues. Normal tissues were taken from noncancer patients. Arrows, all guanines present after sequencing are complementary to methylcytosines on the opposite DNA strand. B, re-expression of representative genes analyzed by semiquantitative RT-PCR or real-time RT-PCR. a and b, reactivated PAK3 and OGDHL were observed by the 5-Aza-dC treatment in H23 and Siha cell lines by semiquantitative RT-PCR. c, overexpression of OSMR was observed by the 5-Aza-dC treatment analyzed by real-time quantitative RT-PCR. Relative fold was calculated by the expression of OSMR mRNA to GAPDH (an internal control). Fold increase of OSMR ranged from 5.2 (HCT116) to 2,868 (DLD-1). Experiments were performed in duplicate; columns, mean; bars, SD. *, P < 0.05.
Cancer-specific methylated genes (12, 26, 27) were methylated by bisulfite sequencing and/or MSP, and 65% of these genes were methylated in primary tumors. Our approach. Pancreas, gastric, thyroid, and ovary cancers displayed relatively low levels of methylation. Colon, prostate, esophagus, and kidney tumors, however, displayed a much higher frequency of methylation overall. Some tumors within a type displayed high inherent levels of methylation, whereas others within the same tumor type displayed low levels (data not shown). The data are not consistent with chance variation from tumor to tumor because in the absence of heterogeneity, the variance of the methylation frequency of methylation of any particular gene in primary tumors is generally less than that observed in cell lines. The discrepancy between the computationally and pharmacologically predicted (175) and experimentally (82) identified methylated genes in cell lines may be partially due to the analysis of limited regions (~200–300 bp for most of the genes) by bisulfite sequencing or MSP.

To compare the overall pattern of methylated CGIs among tumors, we tested 300 primary tumors of 13 different types with 8 frequently cancer-specific methylated genes identified from our approach. Pancreas, gastric, thyroid, and ovary cancers displayed relatively low levels of methylation. Colon, prostate, esophagus, and kidney tumors, however, displayed a much higher frequency of methylation overall. Some tumors within a type displayed high inherent levels of methylation, whereas others within the same tumor type displayed low levels (data not shown). The data are not consistent with chance variation from tumor to tumor because in the absence of heterogeneity, the variance of the methylation frequency of methylation of any particular gene in primary tumors is generally less than that observed in cell lines. The discrepancy between the computationally and pharmacologically predicted (175) and experimentally (82) identified methylated genes in cell lines may be partially due to the analysis of limited regions (~200–300 bp for most of the genes) by bisulfite sequencing or MSP.

To compare the overall pattern of methylated CGIs among tumors, we tested 300 primary tumors of 13 different types with 8 frequently cancer-specific methylated genes identified from our approach. Pancreas, gastric, thyroid, and ovary cancers displayed relatively low levels of methylation. Colon, prostate, esophagus, and kidney tumors, however, displayed a much higher frequency of methylation overall. Some tumors within a type displayed high inherent levels of methylation, whereas others within the same tumor type displayed low levels (data not shown). The data are not consistent with chance variation from tumor to tumor because in the absence of heterogeneity, the variance of the methylation frequency of methylation of any particular gene in primary tumors is generally less than that observed in cell lines. The discrepancy between the computationally and pharmacologically predicted (175) and experimentally (82) identified methylated genes in cell lines may be partially due to the analysis of limited regions (~200–300 bp for most of the genes) by bisulfite sequencing or MSP.

To compare the overall pattern of methylated CGIs among tumors, we tested 300 primary tumors of 13 different types with 8 frequently cancer-specific methylated genes identified from our approach. Pancreas, gastric, thyroid, and ovary cancers displayed relatively low levels of methylation. Colon, prostate, esophagus, and kidney tumors, however, displayed a much higher frequency of methylation overall. Some tumors within a type displayed high inherent levels of methylation, whereas others within the same tumor type displayed low levels (data not shown). The data are not consistent with chance variation from tumor to tumor because in the absence of heterogeneity, the variance of the methylation frequency of methylation of any particular gene in primary tumors is generally less than that observed in cell lines. The discrepancy between the computationally and pharmacologically predicted (175) and experimentally (82) identified methylated genes in cell lines may be partially due to the analysis of limited regions (~200–300 bp for most of the genes) by bisulfite sequencing or MSP.

To compare the overall pattern of methylated CGIs among tumors, we tested 300 primary tumors of 13 different types with 8 frequently cancer-specific methylated genes identified from our approach. Pancreas, gastric, thyroid, and ovary cancers displayed relatively low levels of methylation. Colon, prostate, esophagus, and kidney tumors, however, displayed a much higher frequency of methylation overall. Some tumors within a type displayed high inherent levels of methylation, whereas others within the same tumor type displayed low levels (data not shown). The data are not consistent with chance variation from tumor to tumor because in the absence of heterogeneity, the variance of the methylation frequency of methylation of any particular gene in primary tumors is generally less than that observed in cell lines. The discrepancy between the computationally and pharmacologically predicted (175) and experimentally (82) identified methylated genes in cell lines may be partially due to the analysis of limited regions (~200–300 bp for most of the genes) by bisulfite sequencing or MSP.

Table 2. Cancer-specific methylated genes and their proposed functions

<table>
<thead>
<tr>
<th>Genes</th>
<th>Loci</th>
<th>Gene name</th>
<th>Proposed function</th>
</tr>
</thead>
<tbody>
<tr>
<td>NISCH</td>
<td>3p21.1</td>
<td>Nischarin</td>
<td>Human II(i)-imidazidine receptor candidate gene, IRAS</td>
</tr>
<tr>
<td>PIGH</td>
<td>14q11-q24</td>
<td>Phosphatidylinositol glycan, class H cyclin-dependent kinase inhibitor 1A (p21, Cip1) activated</td>
<td>Encodes an endoplasmic reticulum associated-protein that is involved in GPI-anchor biosynthesis</td>
</tr>
<tr>
<td>PAK3</td>
<td>Xq22.3-q23</td>
<td>Kinase 3</td>
<td>Critical effectors that link Rho GTases to cytoskeleton reorganization and nuclear signaling</td>
</tr>
<tr>
<td>TUBB4</td>
<td>19p13.3</td>
<td>Tubulin, β4</td>
<td>β-tubulin</td>
</tr>
<tr>
<td>KIF1A</td>
<td>2q37.3</td>
<td>Kineas family member 1A</td>
<td>Anterograde motor protein that transports membranous organelles along axonal microtubules</td>
</tr>
<tr>
<td>MAL</td>
<td>2cen-q13</td>
<td>T-cell proliferation protein</td>
<td>A candidate linker protein in T-cell signal transduction</td>
</tr>
<tr>
<td>ENPEP</td>
<td>4q25</td>
<td>Glutamyl aminopeptidase</td>
<td>Type II integral membrane protein</td>
</tr>
<tr>
<td>MCAM</td>
<td>11q23.3</td>
<td>Melanoma cell adhesion molecule</td>
<td>Cell adhesion</td>
</tr>
<tr>
<td>SSBP2</td>
<td>5q13.3</td>
<td>ssDNA-binding protein 2</td>
<td>DNA binding</td>
</tr>
<tr>
<td>B4GALT1</td>
<td>9p21</td>
<td>β 1,4-galactosyltransferase, polypeptide 1</td>
<td>Elevated level in highly metastatic human lung cancer cells</td>
</tr>
<tr>
<td>OSMR</td>
<td>5p12</td>
<td>Oncostatin M receptor</td>
<td>Induces apoptosis in adrenocortical Y-1 tumor cells</td>
</tr>
<tr>
<td>NTRK2</td>
<td>9q22.1</td>
<td>Neurotrophic tyrosine kinase, receptor, type 2</td>
<td>Receptor for brain-derived neurotrophin, neurotrophin-3</td>
</tr>
<tr>
<td>PAPSS2</td>
<td>10q23.1-q23.2</td>
<td>3′-phosphoadenosine</td>
<td>Bifunctional enzyme with both ATP sulfurylase and aPS kinase activity</td>
</tr>
<tr>
<td>SERP4</td>
<td>7p14.1</td>
<td>Secreted frizzled-related protein 4</td>
<td>Modulators of Wnt signaling and Wnt signaling</td>
</tr>
<tr>
<td>TUBG2</td>
<td>17q21</td>
<td>β-Tubulin gene</td>
<td>Ostomalacia-associated tumors and endometrial and breast carcinomas</td>
</tr>
<tr>
<td>PTGS2</td>
<td>1q25.2-q25.3</td>
<td>Prostaglandin-endoperoxide synthase 2</td>
<td>Prostaglandin biosynthesis</td>
</tr>
<tr>
<td>NPTX1</td>
<td>17q25.1-q25.2</td>
<td>Neuronal pentraxin I</td>
<td>Calcium ion binding, transport, and synaptic transmission</td>
</tr>
<tr>
<td>CCNA1</td>
<td>13q12.3-q13</td>
<td>Cyclin A1</td>
<td>Cell cycle</td>
</tr>
<tr>
<td>ASMTL</td>
<td>Xp22.3: Yp11.3</td>
<td>Acetylserotonin O-methyltransferase like</td>
<td>O-methyltransferase activity, S-adenosylmethionine-dependent methyltransferase activity</td>
</tr>
<tr>
<td>TFP12</td>
<td>7q22</td>
<td>Tissue factor pathway inhibitor 2</td>
<td>Serine-type endopeptidase inhibitor activity, extracellular matrix structural constituent</td>
</tr>
<tr>
<td>OGDHL</td>
<td>10q11.23</td>
<td>Oxoglutarate dehydrogenase like</td>
<td>Plays a major role in the citric acid cycle, converting α-ketoglutarate to succinyl CoA</td>
</tr>
<tr>
<td>DLA1</td>
<td>15q14</td>
<td>ß- like 4 (Drosophila)</td>
<td>Notch binding, calcium ion binding</td>
</tr>
<tr>
<td>GDAPIL1</td>
<td>20q12</td>
<td>Ganglioside-induced differentiation-associated protein 1-like 1</td>
<td>Mutation in human GDAP1 are associated with Charcot-Marie-Tooth-type 4A disease</td>
</tr>
<tr>
<td>C10orf166</td>
<td>1p36.12</td>
<td>Hypothetical protein LOC79594</td>
<td>Involved in mediating protein-protein interactions</td>
</tr>
<tr>
<td>ARMC7</td>
<td>17q25.1</td>
<td>Amadillo repeat containing 7</td>
<td>Binding</td>
</tr>
<tr>
<td>C1orf18</td>
<td>13q14.12</td>
<td>Protein chromosome 13 open reading frame 18</td>
<td>Protein phosphatase inhibitor activity</td>
</tr>
<tr>
<td>C9orf19</td>
<td>9p13-p12</td>
<td>Golgi-associated plant pathogenesis-related protein 1</td>
<td>Extracellular region</td>
</tr>
<tr>
<td>HCP1</td>
<td>17q11.2</td>
<td>Home carrier protein 1</td>
<td>Transporter activity</td>
</tr>
</tbody>
</table>

Although a number of studies have attempted to detect additional gene targets, in general, the gene selection methodologies have not been sensitive enough to identify target genes with comparatively less time and labor. By developing a new tool to analyze gene promoters in combination with a relatively large expression microarray data set, it has been possible for the first time to identify a large number of target genes. In our experience, this is a major advance over previous empirical techniques that required excessive experimental effort and yielded only a few (~0.5%) cancer-specific methylated genes (12, 27). Our yield, based on a combination of re-expression arrays and promoter sequence pattern, provided a nearly 500-fold higher yield of genes harboring promoter methylation.

We found that 47% (82 of 175) of the genes tested in cell lines were methylated by bisulfite sequencing and/or MSP, and 65% (53 of 82) of these genes were methylated in primary tumors. Our results are consistent with previous studies (12, 26, 27), where the
We found cancer-specific and tissue-specific methylation events in different tissue types. For example, *PAK3* cancer-specific methylation was found in esophagus, lung, cervix, and bladder cancers with high frequency. *PAK3* was also occasionally methylated in other normal tissues. *PAK3* is located in the X chromosome; thus, it is likely that there will always be methylated signal in samples from female patients. However, we consider *PAK3* as cancer-specific methylation as we also found high frequency of methylation in samples from male cancer patients. Like *PAK3*, some other genes showed either cancer-specific or tissue-specific methylation in multiple organs (Table 3). Although there have been reports of *MCAM* overexpression in melanoma, we found a high frequency of *MCAM* promoter methylation in prostate cancer. Oncostatin M receptor (*OSMR*) showed cancer-specific methylation only in colon cancer and was previously shown to have a major functional role in breast and other cancers (30, 31). Liang et al. (32) reported loss of expression in 50% of myeloid leukemia cell lines and concluded that loss of expression of *SSBP2* in 50% of myeloid leukemia cell lines and concluded that loss of expression may underlie the impaired differentiation seen in human myeloid leukemia. However, before this report, there was no reported mechanism for loss of expression of this gene in lung, head and neck, and gastric cancer. *KIF1A* is a member of the KIF1/Unc104 family, and targeted deletion of the *KIF1A* gene in mice causes accumulation of clear small vesicles in the cell body of neurons as well as marked neuronal death (39). We report for the first time a high frequency of cancer-specific methylation of *KIF1A* in majority of human tumors.

The frequency of methylation within a tumor type of the individual CGIs affected in at least three different tumor types is shown (Table 3). Some targets were methylated at a high frequency in one tumor type but infrequently in others (e.g., *OSMR*; Table 3), whereas other targets (e.g., *KIF1A*) were methylated at relatively high frequencies in the majority of tumor types. Thus, whereas some CGIs targets are shared by multiple tumor types, others are methylated in a tumor-type–specific manner. It has been documented that virtually all biochemical, biological, and clinical attributes are heterogeneous within human cancers of the same histologic subtypes (40). Our data suggest that differences in the methylated genes in various tumors could account for a major part of this heterogeneity.

Like any global genomic and epigenomic approach, our study has limitations. First, we were not able to test all the known and newly discovered methylated genes in all the 13 types of cancer included in this study. Second, although mosaic methylation occurred in most of the cases, focal methylation for some genes was also reported, and methylation in 5’ untranslated regions would not be detectable by the methods we used. Future studies using a combination of different technologies will be able to address these issues.

The results of this study inform future cancer methylome discovery effort in several important ways:

- A major technical challenge of such studies will be discerning cancer-specific methylation from the large number of tissue-specific methylated genes. In our study, using modified gene

### Table 3. Methylation frequency in different cancer types

<table>
<thead>
<tr>
<th>Tissue types</th>
<th>PAK3</th>
<th>NISCH</th>
<th>KIF1A</th>
<th>OGDHL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T</td>
<td>N</td>
<td>T</td>
<td>N</td>
</tr>
<tr>
<td>Breast</td>
<td>13/29 (45)</td>
<td>1/10 (10)</td>
<td>8/24 (33)</td>
<td>1/10 (10)</td>
</tr>
<tr>
<td>Esophagus</td>
<td>5/19 (26)</td>
<td>0/10 (0)</td>
<td>4/19 (21)</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>Lung</td>
<td>16/34 (47)</td>
<td>1/17 (6)</td>
<td>12/34 (35)</td>
<td>1/6 (6)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>7/32 (22)</td>
<td>0/2 (0)</td>
<td>0/32 (0)</td>
<td>2/2 (100)</td>
</tr>
<tr>
<td>Colon</td>
<td>9/20 (45)</td>
<td>2/13 (15)</td>
<td>1/20 (5)</td>
<td>0/13 (0)</td>
</tr>
<tr>
<td>Prostate</td>
<td>10/20 (50)</td>
<td>2/20 (10)</td>
<td>1/20 (5)</td>
<td>2/20 (10)</td>
</tr>
<tr>
<td>Gastric</td>
<td>4/14 (29)</td>
<td>10/10 (100)</td>
<td>6/14 (43)</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>Cervix</td>
<td>13/24 (54)</td>
<td>0/10 (0)</td>
<td>3/24 (13)</td>
<td>1/10 (10)</td>
</tr>
<tr>
<td>Thyroid</td>
<td>8/20 (40)</td>
<td>ND</td>
<td>4/20 (20)</td>
<td>ND</td>
</tr>
<tr>
<td>Kidney</td>
<td>7/24 (29)</td>
<td>ND</td>
<td>13/20 (65)</td>
<td>ND</td>
</tr>
<tr>
<td>Head &amp; Neck</td>
<td>13/25 (52)</td>
<td>0/8 (0)</td>
<td>9/25 (36)</td>
<td>0/8 (0)</td>
</tr>
<tr>
<td>Ovary</td>
<td>10/19 (53)</td>
<td>ND</td>
<td>9/19 (47)</td>
<td>ND</td>
</tr>
<tr>
<td>Bladder</td>
<td>3/20 (15)</td>
<td>0/6 (0)</td>
<td>5/20 (25)</td>
<td>1/6 (17)</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not done.

frequency would not be expected to be greater than the mean. Therefore, aberrant methylation of CGIs can be quantitatively different in individual tumors within a tumor type and more pronounced in particular tumor types.

The results of this study inform future cancer methylome discovery effort in several important ways:

- A major technical challenge of such studies will be discerning cancer-specific methylation from the large number of tissue-specific methylated genes. In our study, using modified gene

*α5* integrin and shown to inhibit cell migration by inhibiting the ability of PAK1 to phosphorylate substrates (37, 38). We found a high frequency of cancer-specific methylation of this gene in lung, head and neck, and gastric cancer. *KIF1A* is a member of the KIF1/Unc104 family, and targeted deletion of the *KIF1A* gene in mice causes accumulation of clear small vesicles in the cell body of neurons as well as marked neuronal death (39). We report for the first time a high frequency of cancer-specific methylation of *KIF1A* in majority of human tumors.

The frequency of methylation within a tumor type of the individual CGIs affected in at least three different tumor types is shown (Table 3). Some targets were methylated at a high frequency in one tumor type but infrequently in others (e.g., *OSMR*; Table 3), whereas other targets (e.g., *KIF1A*) were methylated at relatively high frequencies in the majority of tumor types. Thus, whereas some CGIs targets are shared by multiple tumor types, others are methylated in a tumor-type–specific manner. It has been documented that virtually all biochemical, biological, and clinical attributes are heterogeneous within human cancers of the same histologic subtypes (40). Our data suggest that differences in the methylated genes in various tumors could account for a major part of this heterogeneity.

Like any global genomic and epigenomic approach, our study has limitations. First, we were not able to test all the known and newly discovered methylated genes in all the 13 types of cancer included in this study. Second, although mosaic methylation occurred in most of the cases, focal methylation for some genes was also reported, and methylation in 5’ untranslated regions would not be detectable by the methods we used. Future studies using a combination of different technologies will be able to address these issues.

The results of this study inform future cancer methylome discovery effort in several important ways:

- A major technical challenge of such studies will be discerning cancer-specific methylation from the large number of tissue-specific methylated genes. In our study, using modified gene
Table 3. Methylation frequency in different cancer types (Cont’d)

<table>
<thead>
<tr>
<th>OSMR</th>
<th>B4GALT1</th>
<th>MCAM</th>
<th>SSBP2</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>N</td>
<td>T</td>
<td>N</td>
</tr>
<tr>
<td>1/28 (0)</td>
<td>0/10 (0)</td>
<td>11/28 (39)</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>1/20 (5)</td>
<td>0/10 (0)</td>
<td>12/20 (60)</td>
<td>1/10 (10)</td>
</tr>
<tr>
<td>1/34 (0)</td>
<td>0/17 (0)</td>
<td>21/34 (60)</td>
<td>0/17 (0)</td>
</tr>
<tr>
<td>2/32 (6)</td>
<td>0/2 (0)</td>
<td>9/32 (28)</td>
<td>0/2 (0)</td>
</tr>
<tr>
<td>16/20 (75)</td>
<td>0/13 (0)</td>
<td>18/20 (90)</td>
<td>0/13 (0)</td>
</tr>
<tr>
<td>0/20 (0)</td>
<td>0/20 (0)</td>
<td>20/20 (100)</td>
<td>0/20 (0)</td>
</tr>
<tr>
<td>0/14 (0)</td>
<td>12/12 (100)</td>
<td>11/14 (79)</td>
<td>8/12 (67)</td>
</tr>
<tr>
<td>1/24 (4)</td>
<td>0/10 (0)</td>
<td>4/24 (17)</td>
<td>0/10 (0)</td>
</tr>
<tr>
<td>0/19 (0)</td>
<td>ND</td>
<td>5/19 (26)</td>
<td>ND</td>
</tr>
<tr>
<td>0/23 (0)</td>
<td>ND</td>
<td>10/23 (43)</td>
<td>ND</td>
</tr>
<tr>
<td>0/26 (0)</td>
<td>0/8 (0)</td>
<td>3/26 (12)</td>
<td>2/8 (25)</td>
</tr>
<tr>
<td>2/19 (11)</td>
<td>ND</td>
<td>9/19 (47)</td>
<td>ND</td>
</tr>
<tr>
<td>0/20 (0)</td>
<td>0/6 (0)</td>
<td>9/20 (45)</td>
<td>0/6 (0)</td>
</tr>
</tbody>
</table>

selection criteria in pharmological unmasking strategy, we identified 47% methylated genes in contrast to 10% to 20% by previous criteria. In the future, improvements in gene selection strategy for prediction of methylation-prone gene should result in less labor and less empirical experimentation.

Another technical issue is the development of high throughput assays for the analysis of large numbers of samples. In this study, we developed QMSP assay for eight novel cancer-specific methylated genes and similar real-time assays could be developed individually for newly identified methylated targets. Once a methylation target set is known for a particular cancer, or even if the entire cancer “methylome” is discovered, other genomic approaches such as chip arrays may facilitate large scale research and clinical efforts.

Although it is likely that studies of other solid tumor types will also identify a large number of methylated genes, it will be important to apply rigorous approaches to identify the specific methylated genes that have been selected for during tumorigenesis. Our modified approach can predict for cancer-specific methylated genes and reduce empirical testing.

There has been much discussion about which genes should be the focus of future efforts for methylation analysis. Our results suggest that many genes not previously implicated in cancer are methylated at significant levels and may provide novel clues to cancer pathogenesis.

Adding these data to previous reports, perhaps up to one third (~300 genes total) of the cancer methylome has now been discovered, compared with the identification of perhaps 200 mutated genes over the past 2 decades and recent genome-wide mutation analysis in primary tumors (41). An emerging picture of genetic and epigenetic changes and their relationship is unraveling the biological networks responsible for human cancer. The genetic and epigenetic alterations in different cancer types are diverse (42, 43), and we and others previously found unique inverse relationships between genetic/epigenetic changes (27, 44, 45). However, 26 genes obtained in the Vogelstein’s last mutation screening are also methylated in our study (41, 46). Ultimately, the epigenome of all cancer tissues will be mapped out even as we now approach a total molecular signature of cancer. According to Dr. Peter Jones (as reviewed in ref. 47), each differentiated cell has a different epigenome. Our comprehensive analysis contributes greatly to the emerging epigenomic map of DNA methylation in the human genome. Additional studies using similar and complementary genomic strategies should yield further insights into the dynamics and hierarchy of epigenetic regulation during tumorigenesis. These data define the epigenetic landscape of major human cancer types, provide new targets for diagnostic and therapeutic intervention, and open fertile avenues for basic research in tumor biology.

Acknowledgments

Received 10/17/2007; revised 1/16/2008; accepted 2/7/2008.

Grant support: National Cancer Institute U01-CA84986, Oncomethylome Sciences, SA, and Dutch Cancer Society (project number RUG 2004-3161). Under a licensing agreement between Oncomethylome Sciences, SA and the Johns Hopkins University, D. Sidransky is entitled to a share of royalty received by the University upon sales of any products described in this article. D. Sidransky owns Oncomethylome Sciences, SA stock, which is subject to certain restrictions under University policy. D. Sidransky is a paid consultant to Oncomethylome Sciences, SA and is a paid member of the company’s Scientific Advisory Board. The Johns Hopkins University in accordance with its conflict of interest policies is managing the terms of this agreement. A. van der Zee is a paid consultant for OncoMethylome Sciences, SA, Læge, Belgium. M.D. Hogue is a recipient of the FAMRI Young Clinical Scientist Award and a Young Investigator Award from the International Association for the Study of Lung Cancer.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References


8. Ehrlich M. DNA hypomethylation, cancer, the immuno
deficiency, centromeric region instability, facial anomalies syndrome and chromosomal rearrangements.

methylation of p16 (INK4a) is an early event in lung cancer and a potential biomarker for early diagnosis.


cologic unmasking of epigenetically silenced tumor suppressor genes in esophageal squamous cell carcino-


Database of Transcriptional Start Sites and full-

16. Olson SA. EMBOS opens up sequence analysis.
European Molecular Biology Open Software Suite. Brief


18. Takai D, Jones PA. Comprehensive analysis of CpG


20. Rigoutsos I, Floratos A. Combinatorial pattern

mining in bioinformatics using Weka. Bioinformatics

22. Hoque MO, Lee CC, Cairns P, Schoenberg M, Sidransky D. Genome-wide genetic characterization of
bladder cancer: a comparison of high-density single
nucleotide polymorphism arrays and PCR-based micro-

of promoter methylation of multiple genes in urine DNA
and bladder cancer detection. J Natl Cancer Inst 2006;98:
996–1004.

methylation-specific polymerase chain reaction gene
patterns in urine sediment distinguish prostate cancer
patients from control subjects. J Clin Oncol 2005;23:
6569–75.

25. Kim MS, Yamashita K, Baek JH, et al. N-methyl-d-
appartate receptor type 2B is epigenetically inactivated and exhibits tumor-suppressive activity in human

correlation between cyclin A1 hypermethylation and
p53 mutation in head and neck cancer identified by
reversal of epigenetic silencing. Cancer Res 2004;64:
5982–7.

27. Vogelstein B, Kinzler KW. Cancer genes and the

2006;312:1162–5.

Vestal RE. Oncostatin M-specific receptor expression
and function in regulating cell proliferation of normal
and malignant mammary epithelial cells. Cytokine 1998;

30. Savarese TM, Campbell CL, McQuain C, et al. Coexpression of oncostatin M and its receptors and

31. Liang H, Samanta N, Nagarajan L. SSBP2, a candidate
tumor suppressor gene, induces growth arrest and
differentiation of myeloid leukemia cells. Oncogene

32. Lo NW, Shaper JH, Pevsner J, Shaper NL. The
expanding β 4-galacctosyltransferase gene family: mes-

33. Wang Y, Zhou Y, Szabo K, Haft CR, Trejo J. Down-
regulation of protease-activated receptor-1 is regulated

receptor antisera-selected (IRAS) cDNA: cloning and charac-

Insulin receptor substrate 4 associates with the protein

36. Dontenwill M, Pascal G, Piletz JE, et al. IRAS,
the human homologue of Nischarin, prolongs survival of

37. Alahari SK, Nasrallah H. A membrane proximal
region of the integrin α5 subunit is important for its

synaptic vesicle precursor transport and neuronal cell
death in KIF1A motor protein-deficient mice. J Cell Biol

39. Shapiro JR, Shapiro WR. Clonal tumor cell heteroge-

coding sequences of human breast and colorectal

41. Costello JF, Fruhwald MC, Smiraglia DJ, et al. A
berrant CpG-island methylation has non-random and

42. Esteller M, Corn PG, Baylin SB, Herman JG. A gene
hypermethylation profile of human cancer. Cancer Res

43. Xing M, Cohen Y, Mambto E, et al. Early occurrence of
BRAF mutation in thyroid tumors and its potential
association with BRAF mutation in thyroid carcinoma.

44. Toyooka S, Tokumo M, Shigematsu H, et al.
Mutational and epigenetic evidence for independent
pathways for lung adenocarcinomas arising in smokers

45. Wood LD, Parsons DW, Jones S, et al. The genomic
landscapes of human breast and colorectal cancers.

46. Garber K. Momentum building for human epige-
Genome-Wide Promoter Analysis Uncovers Portions of the Cancer Methylome

Mohammad Obaidul Hoque, Myoung Sook Kim, Kimberly Laskie Ostrow, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/68/8/2661

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2008/04/15/68.8.2661.DC1

Cited articles
This article cites 47 articles, 17 of which you can access for free at:
http://cancerres.aacrjournals.org/content/68/8/2661.full#ref-list-1

Citing articles
This article has been cited by 14 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/68/8/2661.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/68/8/2661. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.