

Identification of Hypermethylated Genes Associated with Cisplatin Resistance in Human Cancers

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Abstract

Cisplatin is among the most widely used cytotoxic anticancer agents in solid tumors; however, the development of secondary resistance remains a major obstacle to clinical efficacy. Treatment-related DNA hypermethylation may play a role in creating drug-resistant phenotypes by inactivating genes that are required for cytotoxicity. We applied a pharmacologic unmasking approach to detect hypermethylated genes whose inactivation contributes to cisplatin resistance. Using three pairs of isogenic, cisplatin-sensitive, and cisplatin-resistant cell lines derived from two parental cell lines (KB-3-1 and SCC25), we identified several hundred genes that were downregulated in each resistant cell line and reactivated by the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine. Among them, 30 genes were common to two or more cell lines and/or reported to be downregulated in previous studies. Bisulfite sequencing confirmed that 14 genes were hypermethylated in resistant cell lines but not in the sensitive parental cell lines. Six of 14 genes (*SAT*, *C8orf4*, *LAMB3*, *TUBB*, *GOS2*, and *MCAM*) were cisplatin inducible in sensitive but not in resistant cell lines. Small interfering RNA knockdown of two genes, *SAT* and *S100P*, increased cell viability with cisplatin treatment in sensitive parental cell lines. *S100P* knockdown significantly decreased the S-phase fraction of parental sensitive cell lines and slowed cell proliferation, which was associated with decreased sensitivity to cisplatin. Based on these findings, we conclude that DNA methylation is a frequent event in cells that are chronically exposed to cisplatin and that methylation-induced gene silencing may play a role in the development of resistance to cytotoxic chemotherapeutic agents. *Cancer Res*; 70(7); 2870–9. ©2010 AACR.

Introduction

Drug resistance is a major obstacle in cancer chemotherapy. Although many solid tumors initially respond to chemotherapeutic drugs, most will recur or progress as a result of acquired drug resistance and the expansion of drug-resistant tumor cell populations (1, 2). Epigenetic alterations, including DNA and histone methylation, play an important role in the initiation and progression of cancer (3–5). Aberrant DNA methylation at CpG islands is associated with transcriptional repression and abnormal gene silencing (6). In recent years, emerging evidence has also linked epigenetic changes to the development of drug resistance. When compared with sensi-

tive, parental controls, MCF-7/Adriamycin-resistant tumor cells display DNA hypermethylation and drug resistance that can be reversed by downregulating DNA methyltransferase genes or by administration of a DNA methylation inhibitor (7). *In vivo*, 80% of patients enrolled in a phase II single-arm study in which the mild demethylating agents hydralazine and valproate were added to scheduled chemotherapy showed clinical benefit as well as a reduction in global DNA methylation (8).

Although these studies looked at changes in global methylation, there is also evidence that promoter hypermethylation of specific genes with known involvement in drug response may play a role in predicting clinical outcome. Promoter methylation of the DNA repair enzyme *O*⁶-methylguanine-DNA methyltransferase contributes to resistance to the DNA alkylating agent carmustine [1,3-bis(2-chloroethyl)-1-nitrosourea], as well as overall and disease-free survival (9). In primary ovarian tumors, promoter hypermethylation and inactivation of *FANCF* is associated with acquired cisplatin resistance (10). Finally, tumor DNA isolated from the plasma of patients with ovarian cancer before chemotherapy and at relapse showed that 25% of patients acquired methylation of the DNA mismatch repair protein hMLH1 and that this was associated with poor overall survival (11).

Despite all these efforts, to date, there has been no genome-wide study to systematically investigate methylation of individual genes following exposure to chemotherapy. Our

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laboratory has previously described an experimental algorithm that provides knowledge of methylation patterns across a large portion of the genome, which has succeeded in identifying novel methylated genes (12). In this study, we applied this algorithm to the study of cisplatin-sensitive and cisplatin-resistant tumor cell lines to identify epigenetically modified genes that are associated with acquired cisplatin resistance.

Materials and Methods

Cell lines. Two cell line pairs, KB-3-1/cisplatin sensitive and KB-3-1/cisplatin resistant (KB-3-1 and KB-CP), and BEL-7404 and 7404-CP20 were kindly provided by Dr. Michael Gottesman (NIH). Genetic fingerprinting of the four cell lines was consistent with the two parental cell lines being of identical origin and the resistant cell lines having been derived from a single parental strain; thus, the pairs were subsequently redesignated KB-3-1, KB-CP clone 1 and KB-3-1, KB-CP clone 2. All cell lines were reverified for their responsiveness to cisplatin treatment by cell viability as described below. The human squamous carcinoma cell lines SCC-25 and its cisplatin-resistant derivative SCC-25/CP were provided by Dr. A. J. Rainbow (McMaster University). All cell lines were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin. Medium for SCC-25 and SCC-25/CP cells was supplemented with hydrocortisone (0.04 $\mu\text{g}/\text{mL}$) as well. KB-CP-resistant cell lines were chronically maintained in medium supplemented with 5 $\mu\text{g}/\text{mL}$ cisplatin, whereas SCC-25/CP cells are stably cisplatin resistant.

5-Aza-2'-deoxycytidine treatment of cells. Cells were split to low density (10^6 per T-75 flask) 24 h before treatment. Cells were then treated with freshly prepared 5 $\mu\text{mol}/\text{L}$ 5-aza-2'-deoxycytidine (5-Aza-dC; Sigma-Aldrich) dissolved in 50% acetic acid/50% PBS or were mock treated with the same volume of vehicle (50% acetic acid/50% PBS) in the medium for 5 d (medium changed on day 3).

RNA extraction and microarray analysis. Total cellular RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer's instructions. We carried out oligonucleotide microarray analysis using the GeneChip U133A Affymetrix expression microarray containing 22,284 probes and 12K genes following the manufacturer's directions (Affymetrix). Signal intensity and statistical significance were established for each transcript using dChip version 2005.³ A 2-fold decrease in signal in each paired sensitive/resistant cell line in combination with 1.5-fold increase after 5-Aza-dC treatment (based on the 90% confidence interval of the result and expression minus baseline >50) was used as the statistical cutoff value to identify candidate genes.

Reverse transcription-PCR and real-time PCR. RNA was reverse transcribed to cDNA using SuperScript III (Invitrogen), which was then used as a template for PCR. PCR primers were designed to amplify cDNA fragments approximately 150 to 250 bp in length. Primer sequences are

shown in Supplementary Table S1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. For real-time PCR, iTaq SYBR Green Supermix with Rox dye (Bio-Rad) was used with the following amplification program for 40 cycles: 95°C for 15 sec and 60°C for 1 min. All reactions were performed in duplicate or triplicate, with water controls, and relative quantity was calculated after normalizing to GAPDH expression.

DNA extraction and bisulfite sequencing analysis. Genomic DNA was extracted using standard phenol/chloroform techniques, and bisulfite modification of genomic DNA was performed using the EpiTect kit (Qiagen) according to the manufacturer's instructions. The genomic sequence and sequence for 1,000 bases upstream of the transcription start site (TSS) were obtained from the University of California at Santa Cruz genome browser Web site.⁴ Primer sets were designed to recognize DNA alterations after bisulfite treatment. Bisulfite-treated DNA was amplified for the CpG islands noted to be approximately 500 to 1,000 bases 5' to the TSS. For genes without defined CpG islands, primers were chosen to cover the most CpG-rich sites including the first exon (6). Primer sequences are shown in Supplementary Table S1. All PCR products were gel extracted or PCR purified (Qiagen) before sequencing and were sequenced using both forward and reverse amplification primers and the ABI BigDye Cycle Sequencing kit (Applied Biosystems).

Methylation-specific PCR. Methylation-specific PCR (MSP) was performed as described previously (13). MSP primers were designed to match the bisulfite sequencing region. Primer sequences are displayed in Supplementary Table S1. Simultaneous reactions for both unmethylated and methylated primers were performed for 35 cycles using the following conditions: 95°C for 30 s, 58°C for 1 min, and 72°C for 1 min using platinum Taq (Invitrogen).

TA cloning of SAT promoter region. TA cloning was performed using a TA Cloning kit (Invitrogen) following the manufacturer's instructions. Briefly, bisulfite-treated DNA from KB-3-1 and KB-CP clone 2 cells was amplified using the same primer pairs previously used for bisulfite sequencing of the SAT promoter region. PCR amplicons were gel purified and immediately ligated into a linearized TA cloning vector using T4 DNA ligase at 14°C overnight. Ligation product (2 μL) was transformed into TOP10-competent cells, which were then plated on Luria-Bertoni agar plates coated with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside. Following an overnight incubation at 37°C, 10 white clones from each plate were selected for sequencing using T7 primer.

RNA interference transfection, cisplatin treatment, and viability assay. ON-TARGETplus SMARTpool control small interfering RNA (siRNA), SAT siRNA, and S100P siRNA were purchased from Dharmacon, and cells were transfected with RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. Briefly, cells were plated at a

³ <http://biosun1.harvard.edu/complab/dchip/>

⁴ <http://genome.ucsc.edu>

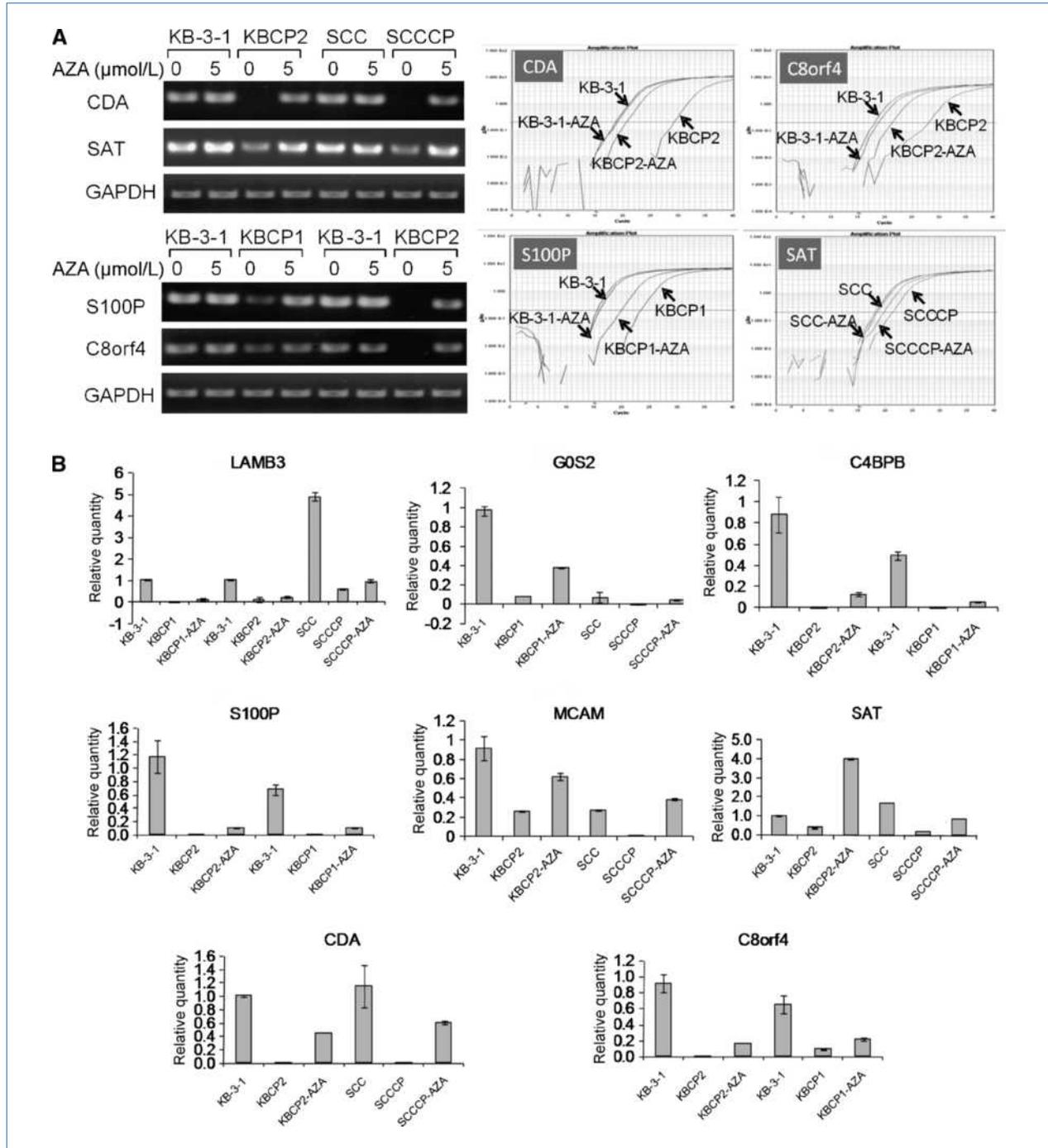


Figure 1. RT-PCR and real-time PCR analysis of candidate genes. A, RT was performed on all three cell line pairs. For select candidate genes, conventional PCR showed downregulation in the cisplatin-resistant daughter cell lines and reactivation after 5-Aza-dC (AZA) treatment. Representative real-time PCR data displaying amplification curves for the individual cell lines are shown. B, real-time PCR was performed on all 30 candidate genes identified by our algorithm. Each sample was normalized to GAPDH. Data are plotted as relative quantity compared with parental untreated control. Values are expressed as the mean \pm SD. Representative data from eight genes are displayed. KB-CP1, KB-CP clone 1; KB-CP2, KB-CP clone 2.

density of 3,000 per well in 96-well plates. The following day, a stock solution containing 4 μ L (20 μ mol/L) of siRNA (control, SAT, or S100P) and 6 μ L of transfection reagent per 1,000 μ L of Opti-MEM (Life Technologies) was prepared

and incubated at room temperature for 20 min. Medium was aspirated from each well and replaced with 80 μ L of antibiotic-free culture medium (DMEM/10% FBS) plus 20 μ L of transfection mixture. Following an overnight incubation, the

transfection mixture was replaced with complete culture medium. After an additional 24 h, cells were treated with cisplatin for 48 h. Cell viability was subsequently assayed using calcein AM (Invitrogen). Fluorescence signals generated as a result of calcein AM cleavage by viable cells were read by a plate reader (Molecular Devices) using an excitation frequency of 480 nm and an emission frequency of 535 nm. Each experiment was independently repeated thrice. A minimum of six-wells was tested for each cisplatin dose.

Cell growth curve and doubling time. Monolayer cultures were trypsinized to obtain single-cell suspensions. An equal number of ~1,000 cells were seeded in each well of 96-well plates, and a cell proliferation assay was performed for 5 d. Cell number was quantified by calcein AM assay as described above. Cell numbers on day 0 were performed 6 h after seeding when the cells had attached to the bottom of the plate, and transfection, when needed, was performed at this time point. Medium was changed on days 1 and 3, and the assay was completed on day 4, when untransfected cells had reached 90% to 100% confluency. Cell doubling times were calculated as described previously by the Patterson formula (14):

$$T_D = T \times \log 2 / (\log N_T - \log N_0)$$

where T_D is the population doubling time expressed as hours per doubling, T is days of assay, N_0 is the number of cells at time 0, and N_T is the cell number on the last day.

Cell cycle analysis. Cell cycle analysis was performed as previously described (15). Cells were plated in six-well plates and transfected as described above. Following replacement of transfection medium with complete culture medium, cells

were incubated for 48 h and then washed twice with cold PBS, harvested by scraping in PBS, pelleted, and resuspended in 1 mL of hypotonic staining solution containing 1 mg/mL sodium citrate, 3 μ L/mL Triton X-100, 20 μ g/mL RNase A, and 100 μ g/mL propidium iodide. Cell cycle analysis was carried out using a FACScan flow cytometer (Becton Dickinson). Data were analyzed with the ModFit LT software (Verity Software House, Inc.).

Statistical analysis. The paired Student's t test was used to determine statistical significance. Differences were considered significant at $P < 0.05$. SigmaStat 3.1 software was used for all statistics (Aspire Software). Results are expressed as the mean \pm SD, unless otherwise stated.

Results

Pharmacologic unmasking of transcriptionally repressed genes in cisplatin-resistant cell lines. Cells were treated with the demethylating agent 5-Aza-dC for 5 days to identify epigenetically inactivated genes associated with the drug-resistant phenotype in the isogenic cell line pairs KB-3-1 and KB-CP clones 1 and 2, and SCC-25 and SCC-25/CP. After treatment, changes in gene expression were measured using Affymetrix U133A microarray chips. The data discussed in this publication have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (GEO)⁵ and are accessible through GEO Series record number GSE19397.

We hypothesized that in these cell lines, methylation-dependent, sensitivity-associated genes would be expressed in the drug-sensitive parental cell lines, and expression would be silenced or diminished in the drug-resistant daughter cell lines. In addition, we hypothesized that pharmacologic

Table 1. Genes showing cell line-specific methylation in cisplatin-resistant cells

Genbank	Gene name	KBCP1		KBCP2		SCCCP	
		Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant
NM_001627	<i>ALCAM</i>			U	M	U	U
NM_001497	<i>B4galt1</i>			U	M		
NM_000716	<i>C4BPB</i>	U	M	U	M		
NM_020130	<i>C8orf4</i>	U	M	U	M		
NM_001785	<i>CDA</i>			U	M	U	M
NM_001311	<i>CRIP1</i>			M	M	U/M	M
NM_015714	<i>G0S2</i>			U	M	U	M
NM_000228	<i>LAMB3</i>	U	M	U	M	U	U
NM_006500	<i>MCAM</i>			U	M	U	M
NM_014322	<i>OPN3</i>	U	U/M	U	U/M	M	M
NM_005980	<i>S100P</i>	U	M	U	M		
NM_002970	<i>SAT1</i>			U	U/M	U	U/M
NM_014220	<i>TM4SF1</i>	U	U	U	M		
NM_001069	<i>TUBB2A</i>	M	M	M	M	U	M

Abbreviations: KBCP1, KB-CP clone 1; KBCP2, KB-CP clone 2; U, unmethylated; M, methylated; U/M, bisulfite sequencing showed mixed signal of methylated and unmethylated CpG.

unmasking of promoter hypermethylation would result in reexpression of these genes in the resistant cell lines following 5-Aza-dC treatment. Applying this algorithm, we identified 242 genes in the KB-3-1/KB-CP clone 1 cell lines, 368 genes in the KB-3-1/KB-CP clone 2 cell lines, and 168 genes in the SCC-25/SCC-25/CP cell lines, respectively (Supplementary Fig. S1).

We further screened for genes of interest by identifying those that had diminished expression in at least two of the resistant cell lines tested. Finally, we compared our candidate genes to those reported by other investigators to be downregulated in platinum-resistant cell lines (14, 16, 17).⁶ This approach identified 30 genes that were transcriptionally downregulated in a minimum of two cisplatin-resistant cell lines (Supplementary Fig. S1; Supplementary Table S2). Strikingly, eight genes from this final list are commonly downregulated in at least four cell lines (*C8orf4*, *CRIP1*, *CTSB*, *IGFBP4*, *MCAM*, *S100P*, *SAT1*, and *TUBB2A*), with eight additional genes common to at least three cisplatin-resistant cell lines (*ADM*, *CABBP*, *CES1*, *FSTL3*, *LAMB3*, *OPN3*, *PTGS1*, and *RHOB*), indicating that downregulation of these genes is frequent across cancer cell lines from different tissue types (Supplementary Table S2).

Reverse transcription-PCR confirmation of commonly identified genes. Changes in expression of candidate genes were examined by conventional reverse transcription-PCR (RT-PCR) and/or real-time quantitative RT-PCR (Fig. 1; Supplementary Fig. S2). RT-PCR confirmed observed microarray expression changes in at least one cell line screened for 87% of genes examined (25 genes; Fig. 1; Supplementary Table S2; Supplementary Fig. S2). Of note, CDA was highly expressed in KB-3-1 and SCC-25 parental cell lines but was barely detectable in the KB-CP clone 2 and SCC-25/CP-resistant cell lines. Treatment with 5-Aza-dC caused a robust reexpression of this gene. Likewise, S100P was highly expressed in KB-3-1 cells, virtually absent in both KB-CP clones, and reactivated after 5-Aza-dC treatment (Fig. 1A). Genes that were confirmed to be downregulated in resistant compared with sensitive cell lines and concomitantly upregulated after 5-Aza-dC treatment were selected for further study.

Promoter bisulfite sequencing and MSP. To confirm promoter hypermethylation in reactivated genes, DNA from parental and daughter cell lines was bisulfite treated, and candidate genes were sequenced using primers designed to encompass a portion of the CpG-rich gene promoter region. Additional regions were sequenced whenever a gene showed significant reactivation with 5-Aza-dC but failed to show differential methylation on initial sequencing. Using this approach, we determined that 14 of 25 genes (54%) were differentially methylated in at least one resistant cell line (Table 1), indicating that methylation occurs during chronic drug treatment and persists in the resistant phenotype.

To further confirm the methylation patterns seen with bisulfite sequencing, MSP was performed on select candidate

genes. Of note, *C8orf4* and *S100P* were unmethylated in parental KB-3-1 cells but displayed methylation in both resistant cell lines by promoter bisulfite sequencing (Fig. 2A). Consistent with these data, MSP showed unmethylated PCR products in the KB-3-1 parental cell line, whereas methylated PCR products were present in both KB-CP clone 1 and clone 2 (Fig. 2B). Interestingly, *SAT* did not display promoter methylation in KB-CP clone 2 cells by sequencing; however, a weak but consistently methylated PCR product was observed by MSP, which was absent in the parental KB-3-1 cells (Fig. 2B, arrow). To resolve this discrepancy, TA cloning of the promoter region from KB-CP clone 2 cells was performed and individual clones were sequenced. Of 10 clones sequenced, 2 displayed promoter methylation, indicating that in addition to the promoter methylation seen in SCC-25/CP cells, low-level *SAT* methylation is also present in KB-CP clone 2 cells.

Cisplatin treatment upregulated six genes in parental sensitive cells but not in their resistant counterparts. Because methylation and transcriptional silencing of these genes is associated with the development of cisplatin

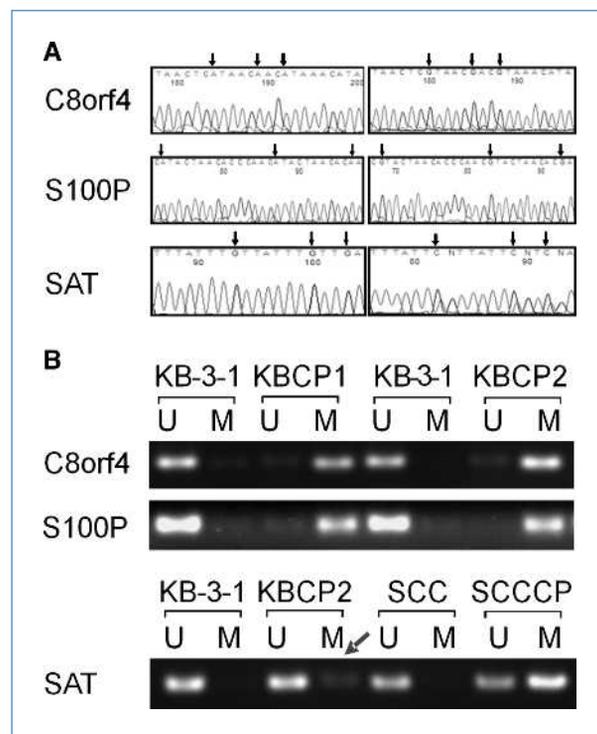
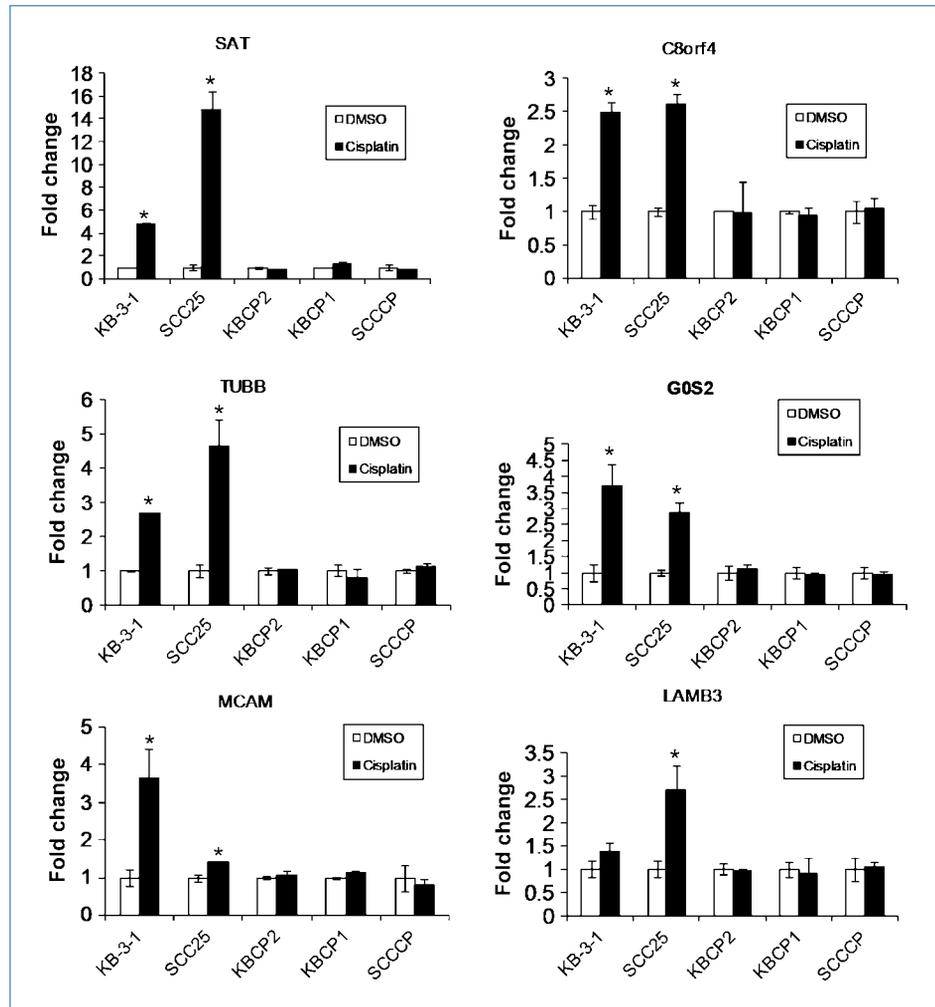


Figure 2. Bisulfite sequencing and MSP on candidate genes. A, direct bisulfite sequencing of gene promoters (*C8orf4*, *S100P*, and *SAT*). Representative sequencing data from either parental or resistant cell lines are shown. For *S100P* and *C8orf4*, all guanines present (arrows) after sequencing are derived from methylcytosines on the complementary strand. Sequence data for *SAT* are shown in the sense strand on which methylcytosines (arrows) remain cytosines after bisulfite treatment, whereas unmethylated cytosines are converted to thymidine. B, representative MSP is shown for *C8orf4*, *S100P*, and *SAT* in the cell lines indicated. U, PCR amplification with primers recognizing unmethylated DNA; M, PCR amplification with primers recognizing methylated DNA.

⁵ <http://www.ncbi.nlm.nih.gov/geo/>

⁶ S. Howell, personal communication.

Figure 3. Cisplatin treatment induces expression of six genes in cisplatin-sensitive but not cisplatin-resistant cell lines. Cells were treated with cisplatin (at a dose equivalent to the IC_{50} of the sensitive cell line) or vehicle (DMSO) for 24 h. RNA was collected and real-time RT-PCR was performed. Values are expressed as mean \pm SD. *, $P < 0.05$.



resistance, we hypothesized that expression of our candidate genes might be modulated by exposure to cisplatin. To test this theory, all five cell lines were exposed to cisplatin (IC_{50} of sensitive cell lines) or vehicle (DMSO), and RT-PCR was performed to examine the expression level of the 14 methylated genes. Interestingly, six genes (43%) were up-regulated by treatment in cisplatin-sensitive but not the corresponding cisplatin-resistant cell lines (Fig. 3). These six genes are *C8orf4*, *SAT*, *GOS2*, *LAMB3*, *TUBB*, and *MCAM*. Thus, nearly half of the methylated, downregulated candidate genes identified by our algorithm are induced by cisplatin in the sensitive, but not the resistant, cell lines tested.

***SAT* and *S100P* siRNA knockdown rendered cells more resistant to cisplatin.** To further examine the effect of candidate gene silencing on cisplatin resistance, siRNA duplexes were used to knock down gene expression of four genes (*C8orf4*, *CDA*, *SAT*, and *S100P*) in sensitive cell lines to determine if this altered cisplatin cytotoxicity. On initial screening, two genes (*CDA* and *C8orf4*) showed no protective effect (data not shown). However, two others (*SAT* and *S100P*)

did confer a survival advantage. When transfected cells were subsequently exposed to increasing concentrations of cisplatin for 48 hours, they displayed increased resistance to cisplatin, with better protection being conferred by *S100P* downregulation in KB-3-1 cells [Fig. 4A (KB-3-1) and B (SCC-25)]. Real-time PCR showed that siRNA transfection of KB-3-1 and SCC-25 cells resulted in a nearly 18- to 23-fold reduction in *SAT* expression and a 45-fold reduction in *S100P* (Fig. 4, right).

Pretreating cisplatin-resistant cells with 5 μ mol/L 5-Aza-dC for 4 days before cisplatin treatment, we found that 5-Aza-dC in the absence of cisplatin induced a significant but mild growth inhibition (13–19%) in all three cell lines by day 6 of treatment (Supplementary Fig. S3A). In part because of this effect, we observed decreased cell viability at all cisplatin doses in pretreated cells. However, after normalizing our results to eliminate the direct effects of 5-Aza-dC on cell growth/survival, we observed an additional synergistic effect of 5-Aza-dC pretreatment in combination with cisplatin on KB-CP clone 2 and SCC-25/CP cells at cisplatin doses near the IC_{50} of these cell lines (Supplementary Fig. S3B).

S100P knockdown slowed cell cycle progression and cell proliferation. Because the role of SAT in modulating drug resistance has been reported in detail previously (17, 18), we sought to further examine the protective effects of S100P. Platinum-based drugs are DNA-damaging agents, and as such, they generate cell cycle-sensitive toxicity. Hence, downregulation of cell cycling provides a survival advantage in resisting cisplatin. We had previously ascertained that both KB-CP clones showed significantly slower doubling times than their parental cell line. Thus, to test whether this slowing in growth was influenced by S100P expression, we transfected KB-3-1 cells with either control or S100P siRNA and performed a proliferation study for 4 days. Real-time PCR was performed to verify the S100P expression level of cells (Fig. 5A, bottom). Control siRNA transfection had negligible effects on gene expression and cell growth, but S100P knockdown significantly slowed cell growth. S100P knockdown increased doubling time from 1.58 to 2.12 days in KB-3-1 cells ($P < 0.05$). Both KB-CP clones displayed even lower S100P expression than the siRNA-transfected parental cells, and their growth rates were even lower (2.2 days for KB-CP clone 1 and 3 days for KB-CP clone 2; Fig. 5A). In keeping with these data, cell cycle analysis showed a significant drop in S-phase fraction (SPF) in S100P siRNA-transfected KB-3-1 cells compared with cells transfected with control siRNA ($17 \pm 1.6\%$ versus $12 \pm 0.5\%$; $P < 0.05$; Fig. 5B).

Discussion

In this genome-wide methylation study, we provide evidence that methylation of select genes occurs during chronic platinum exposure and can confer drug resistance. More than 50% of genes identified and studied were specifically methylated in at least one resistant cell line. Based on these data, and given the fact that there are hundreds of genes that fit our algorithm in each individual cell line pair, it is reasonable to assume that methylation-induced gene silencing is a frequent and relevant phenomenon during chronic drug exposure. It is also noteworthy that 6 of the 14 methylated genes identified by our selection algorithm are cisplatin inducible. This observation not only suggests their functional relevance to cisplatin cytotoxicity but also provides a rationale for the methylation-induced silencing of these genes in cells undergoing selection pressure from cisplatin.

Although these findings are in keeping with a temporal association between changes in cellular methylation patterns and the development of cisplatin resistance, we have furthered the causal relationship between the two as well by showing that the silencing of two specific genes identified by our algorithm results in increased drug resistance in our cisplatin-sensitive cell lines. One of these genes, SAT, has previously been associated with cisplatin-induced cytotoxicity

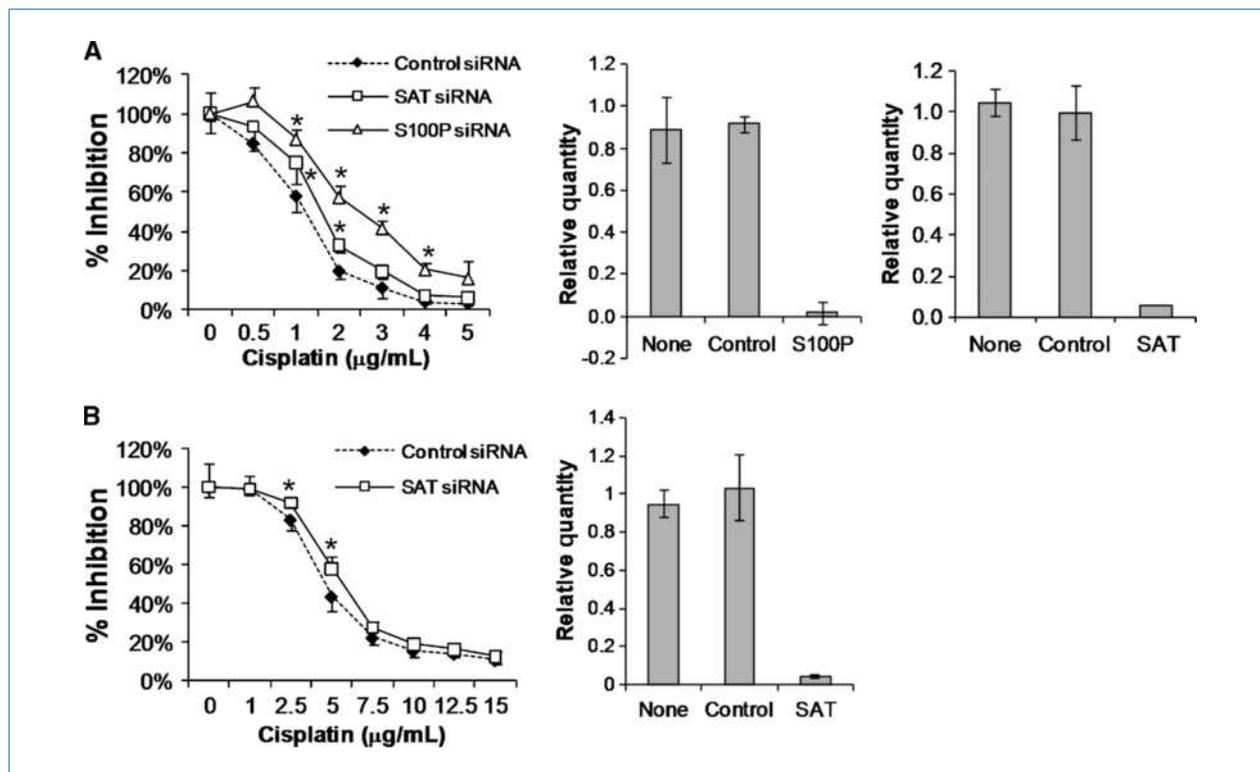


Figure 4. siRNA knockdown of SAT and S100P increases parental cell line resistance to cisplatin. KB-3-1 (A) and SCC-25 (B) cell lines were transfected with control, SAT, or S100P siRNA duplex nucleotides. Cells were treated with cisplatin for 48 h, and cell viability was assayed. Representative data are shown. Right, efficiency of SAT and S100P knockdown was confirmed by real-time RT-PCR. None and control indicate either untransfected or control-transfected cells. Representative data are shown. Values are expressed as mean \pm SD. *, $P < 0.05$.

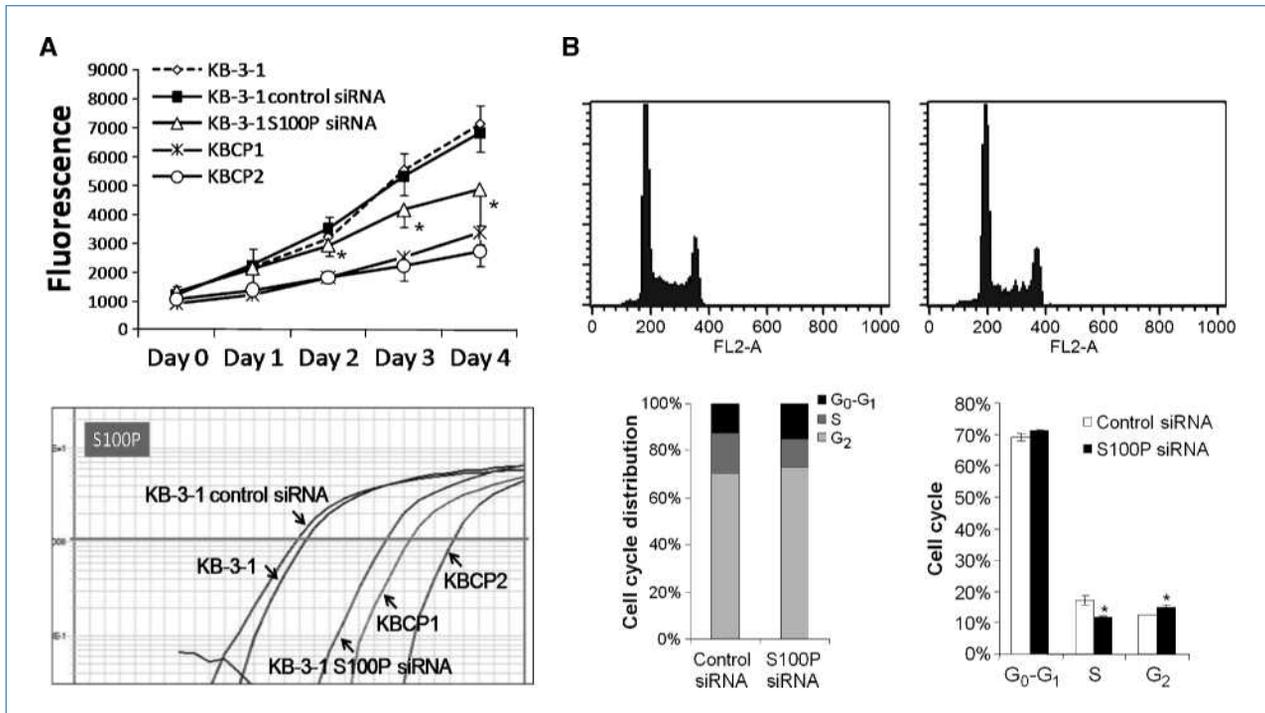


Figure 5. S100P knockdown slows cell growth and decreases the SPF of KB-3-1 cells. A, top, KB-3-1 cells were transfected with S100P siRNA and cell growth rate was assayed on indicated days. The growth rate of KB-CP cells (clones 1 and 2) was also assayed for comparison. Bottom, relative expression of S100P was examined by real-time PCR and amplification curves are shown. *, $P < 0.05$, significant difference between control siRNA-transfected and S100P siRNA-transfected cells. B, cell cycle analysis was performed on control siRNA-transfected and S100P siRNA-transfected cells (72 h after transfection). Cell cycle distribution and percentage are shown. Knockdown of S100P expression decreased SPF from 13% to 5%. Values are expressed as mean \pm SD of three independent experiments. *, $P < 0.05$.

(17, 19–21). In our studies, SAT expression decreased in two resistant cell lines, both with differential promoter methylation. It has been shown that overexpression of SAT makes cells more sensitive to cisplatin (18). Consistent with this finding, we have shown that siRNA-induced gene silencing of SAT in KB-3-1 cells is associated with increased drug resistance. Taken together, our data indicate that downregulation of SAT in resistant cell lines plays a role in drug resistance and that it can be modulated through promoter methylation.

Our data suggest a different role for S100P in the development of cisplatin resistance. S100P, a 95-amino acid protein, can act as an autocrine growth and survival factor (22). S100P displays minimal expression in normal tissue, but expression increases dramatically in some malignancies (23–27). This increased expression is associated with demethylation of the *S100P* gene (28). We and others have observed S100P expression to be drastically decreased (compared with parental) in at least five platinum-resistant cell lines, including two of our cell lines, two bladder cancer cell lines (16), and one colon cancer cell line (14). Thus, we provide the first evidence of S100P remethylation when cells that express S100P become cisplatin resistant. Although our findings are *in vitro*, a clinical study of lung cancer patients has shown similar results. Bartling and colleagues found S100P mRNA expression to be significantly elevated in lung adenocarcinomas compared with normal lung tissue. However, this association was not observed in

adenocarcinomas from patients who had received neoadjuvant chemotherapy before tumor resection (29).

The relationship between slower cell cycle progression and the responsiveness of tumors to chemotherapeutic agents has been widely documented elsewhere. Stewart and colleagues (30) suggest that the main factor driving failure of chemotherapy to cure advanced non-small cell lung carcinoma is deficiency or saturation of a factor required for cell killing, such as the presence of slow or noncycling cells. Cancer cells survive until therapy cessation by downregulating metabolism/cycling, thus becoming temporarily quiescent and resistant to chemotherapeutic agents (30–34). In a study of 184 breast cancer patients treated with primary radiotherapy or neoadjuvant chemotherapy, overall response to neoadjuvant chemotherapy was significantly lower for tumors with low SPF compared with tumors with high SPF (35). Likewise, a study of 81 breast cancer patients found that the response to chemotherapy was significantly better in patients with tumors with high SPF (36). We found in our study that S100P knockdown cells show slower cell cycle progression, less SPF, and increased resistance to cisplatin. Because the time course of downregulation/remethylation of S100P is within the time frame of the development of resistance, our data suggest that S100P, exerting its effects by regulating cellular proliferation, may be one of the factors that play a role in the failure of multiple cell cycle-sensitive

chemotherapy drugs. By using methylation and demethylation to modulate expression of S100P, tumor cells are able to provide themselves with both growth and survival advantages under different circumstances. This regulation through alternating methylation patterns is efficient, fast, and flexible.

It is clear, however, that cisplatin resistance has multiple mechanisms, and that changing S100P expression is not the only factor in the development of this resistance, even in the model described here. Gottesman has previously shown mislocalization of folate-binding protein (37, 38) and cytoskeletal defects, including downregulation of the carboplatin-binding proteins actin and filamin (39) in cisplatin-resistant cell lines. We similarly found downregulation of FOLR1, as well as transcripts for the structural proteins keratin8, laminin β 3, and tubulin β 2A. Interestingly, both laminin β 3 and tubulin β 2A displayed promoter methylation in the resistant cell lines. Although these genes may play a role in cisplatin resistance, S100P-induced cisplatin resistance is independent of these structural changes because it can be induced by silencing of the gene in parental cells.

In summary, this study provides evidence that epigenetic promoter methylation is a frequent event during chronic cisplatin exposure and that secondary changes in gene regulation can play an important role in generating drug-resistant phenotypes. Differentially methylated genes, including those identified in this study, may provide informative drug resistance markers as well as therapeutic targets, potentially lead-

ing to improved therapies for cancer patients with better and more durable clinical responses.

Disclosure of Potential Conflicts of Interest

Under a licensing agreement between Oncomethylome Sciences and the Johns Hopkins University, D. Sidransky is entitled to a share of royalties received by the University on 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.

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