Drug Sensitivity Prediction by CpG Island Methylation Profile in the NCI-60 Cancer Cell Line Panel

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Abstract

Aberrant promoter hypermethylation and associated gene silencing are epigenetic hallmarks of tumorigenesis. It has been suggested that aberrant DNA methylation can affect the sensitivity of cancers to antineoplastic agents by altering expression of genes critical to drug response. To study this issue, we used bisulfite PCR to assess DNA methylation of 32 promoter-associated CpG islands in human cancer cell lines from the National Cancer Institute (NCI) drug-screening panel (NCI-60 panel). The frequency of aberrant hypermethylation of these islands ranged from 2% to 81% in NCI-60 cancer cells, and provided a database that can be analyzed for the sensitivity to ≈30,000 drugs tested in this panel. By correlating drug activity with DNA methylation, we identified a list of methylation markers that predict sensitivity to chemotherapeutic drugs. Among them, hypermethylation of the *p53* homologue *p73* and associated gene silencing was strongly correlated with sensitivity to alkylating agents. We used small interfering RNA to down-regulate *p73* expression in multiple cell lines, including the resistant cell lines TK10 (renal cancer) and SKMEL28 (melanoma). Down-regulating *p73* substantially increased sensitivity to commonly used alkylating agents, including cisplatin, indicating that epigenetic silencing of *p73* directly modulates drug sensitivity. Our results confirm that epigenetic profiles are useful in identifying molecular mediators for cancer drug sensitivity (pharmaco-epigenomics).
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Introduction

The NCI has established a panel of cell lines (NCI-60) and used it to screen compounds for anticancer activity over the last decades (1). At this moment, the NCI-60 cells constitute the most extensively and diversely characterized cell panel anywhere, and several databases have been generated based on these cell lines, such as drug activity database for >30,000 chemically defined compounds, expression profile of >8,000 different genes, and selected genetic changes (2). Genome-wide gene expression profiling of the NCI-60 cell lines (3) identified strong associations between selected gene expression and chemosensitivity in this panel, suggesting that gene-drug interactions may be useful for predicting the responsiveness of cancer cells to anticancer agents.

CpG island methylation is now recognized as a common feature of many human neoplasms (4–6). Methylation changes mark-specific pathways in tumorigenesis that seem to result from distinct exposures and may have important prognostic and therapeutic implications (7–9). Methylation profiling may therefore provide valuable clinical information. Studies have suggested that DNA methylation could provide a good molecular marker to predict sensitivity to chemotherapy (10–12); for instance, O^6-methylguanine-DNA methyltransferase (*MGMT*) methylation indicates sensitivity to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) in gliomas, *CHFR* (checkpoint with FHA and RING finger) methylation indicates sensitivity to microtubule inhibitors in gastric and oral squamous cell cancers, and methylation of *WRN* (Werner syndrome gene) predicts good clinical response to a topoisomerase inhibitor irinotecan. Based on these, we hypothesized that DNA methylation could provide good molecular marker to indicate the sensitivity or resistance to chemotherapy. Here, we report a methylation profile of 32 CpG islands in the NCI-60 cell lines. As a proof of principle, by correlating DNA methylation with drug response, we show that *p73* methylation and silencing of this gene predicts sensitivity to alkylating agents, and down-regulation of *p73* gene expression by small interfering RNA (siRNA) sensitized the resistant cell lines to several alkylating agents tested.

Materials and Methods

Cell lines. A total of 58 cell lines were obtained from the NCI Anticancer Drug Screen Panel, including seven cell lines from breast cancer, six from the central nervous system, seven from colon cancer, six from leukemia, eight from melanoma, nine from non–small cell lung cancer, six from ovarian cancer, two from prostate cancer, and seven from renal cancer. Genomic DNA was extracted from these cell lines using a standard phenol-chloroform method, and total cellular RNA was extracted with TRIzol (Invitrogen) according to the manufacturer's protocol. For siRNA or short hairpin RNA (shRNA) knockdown experiment, two human renal carcinoma cells, TK10 and 786-0, and one human melanoma cell, SK-MEL28, were grown in RPMI medium (TK10 and 786-0) or DMEM (SK-MEL28) with 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 atmosphere.

Bisulfite-PCR methylation analysis. Bisulfite-based PCR method was used for methylation study as reported previously (13). We used both combined bisulfite restriction analysis (COBRA; ref. 14) and methylation-sensitive PCR (MSP; ref. 15) assay to analyze the methylation status. In brief, COBRA assay relies on bisulfite-induced RFLPs; that is, methylated bands are digested by restriction enzymes but unmethylated bands are not. Digested PCR products were separated by electrophoresis on 6% polyacrylamide gels. Gels were stained with ethidium bromide, imaged, and quantitated in a Bio-Rad Geldoc 2000 imager (Bio-Rad). The methylation density for each sample was computed as a ratio of the density of the digested band to the density of all bands in a given lane. MSP-PCR products also were visualized on acrylamide gels as described earlier, and quantitated by densitometric determination of the density of the band in the methylated lane divided by the sum of the bands in both methylated

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Table 1. The frequency of hypermethylation of each marker in NCI-60 cell lines in nine different tissue types

Gene	Frequency of hypermethylation (%)								
	Leukemia	NSCLC	Colon	CNS	Melanoma	Ovarian	Renal	Prostate	Breast
MINT1	50	67	86	67	38	50	100	100	57
MINT2	33	0	71	0	13	0	29	50	14
MINT31	17	44	71	67	75	83	43	100	71
MINT25	17	33	14	0	25	17	43	0	29
P16	50	40	86	0	13	0	14	50	25
P14	20	13	29	0	0	0	29	0	0
P15	60	0	0	0	0	0	0	0	17
P57	50	11	0	17	25	0	29	0	43
P73	50	22	14	0	50	0	57	100	14
MGMT	50	44	57	100	63	0	57	0	43
hMLH1	0	0	14	0	0	0	0	0	0
COX2	50	11	0	17	0	0	0	50	29
THBS1	100	0	14	0	38	0	0	0	0
RIZ1	33	0	71	67	0	17	71	0	14
MDR1	50	56	57	100	25	67	29	100	71
CD10	50	44	100	0	13	50	14	50	57
c-Abl	17	0	0	0	0	0	0	0	0
RASSF1A	33	78	43	100	75	67	100	100	71
RARβ	33	22	86	0	63	17	0	0	29
TIMP3	17	11	29	0	0	17	29	50	14
GSTP	0	11	14	0	0	0	43	0	14
ECAD	83	11	0	50	63	17	71	0	43
BNIP3	0	0	14	0	0	17	0	0	0
ER	67	22	100	83	63	50	43	50	43
TMS1	67	89	57	83	63	83	86	100	86
DAPK	33	22	43	17	13	0	0	50	71
Ril	100	44	86	50	75	67	57	50	57
KR18	17	0	14	0	13	17	14	0	0
THBS4	67	78	100	67	63	67	71	50	86
Megalin	33	0	100	0	25	17	14	0	43
p101	50	89	100	100	88	33	100	100	71
LPH3	50	33	100	0	0	17	29	100	57

NOTE: Homozygous deletion was found in 16 cases for *p16*, seven cases for *p14*, and eight cases for *p15* in NCI-60 cancer cells.
Abbreviations: NSCLC, non–small cell lung cancer; CNS, central nervous system.

and unmethylated lanes. Most of the assays used have previously been published (16–31), including validation of methylation for multiple sites within each island, correlation with other techniques (Southern blotting and bisulfite-pyrosequencing), and lack of PCR bias. Primer sequences, PCR conditions, and restriction enzymes used for COBRA are listed in Supplementary Table S1.

Clustering of human cancer cell lines according to CpG island promoter hypermethylation. Using Euclidean distances and average linkage algorithm, we applied an unsupervised hierarchical cluster analysis of NCI-60 cell lines on the basis of methylation measured as continuous variables. A color-coded cluster image map was generated using CIMminer (Cluster Image Map Program Package) software tool (3, 32).[3] Cell lines with correlated methylation profiles were identified using the Pearson's correlation coefficient (r), which was taken as a measure of similarity or distance between values. The output file was visualized as a binary tree. The scale above the dendrogram depicts the correlation coefficient represented by the branches connecting pairs of nodes.

Drug activity profiles. The drug activity profiles of anticancer agents are available online.[4] Growth inhibition was assessed from the changes in total cellular protein after 48 h of drug treatment using a sulforhodamine B assay. Drug activities ($\log_{10}$ GI_{50}) were recorded across the human cancer cell lines and GI_{50} is calculated by the concentration required to inhibit cell growth by 50% compared with untreated controls.

Chemosensitivity prediction. First, we used quantitative methylation level of each gene (continuous variable) as a seed for COMPARE analysis, which includes >30,000 chemical compounds tested. COMPARE was originally developed by Paull et al. (33) to analyze the pattern for a "seed" among the NCI-60 cancer cells; this method determines Pearson correlation coefficients for the seed against each of the compounds in the database and results in a list of the highest correlations. In this analysis, Bonferroni adjustment was used to determine the possible significance of two-tailed P values; for instance, standard agent database is made up of 170 compounds; therefore, only P values $<0.05/170 = 0.0003$ is considered as statistically significant. A positive correlation indicates that a greater abundance of the seed (methylation) may be associated with sensitivity to the drug, whereas a negative correlation is indicative of more methylation of target gene conferring cellular resistance to the given drug.

Next, we generated a drug response profile (sensitive, intermediate, and resistant) of the NCI-60 cell lines to 118 standard anticancer agents whose

[3] http://discover.nci.nih.gov/cimminer/
[4] http://dtp.nci.nih.gov/dtpstandard/dwindex/index.jsp

mechanisms of action have been defined (32, 34). For each drug, cell lines with $\log_{10}$ (GI_{50}) values at least 0.8 SD above the mean were defined as resistant to this drug; and cell lines with $\log_{10}$ (GI_{50}) at least 0.8 SD below the mean were defined as sensitive to the drug. The remaining cell lines with $\log_{10}$ (GI_{50}) within 0.8 SD were defined as intermediate to the range of drug responses.

Reverse transcription-PCR, quantitative real-time PCR, and Western blot for P73 expression analysis. Two micrograms of total RNA were used as a template to generate complementary DNA (cDNA) by random hexamers and M-MuLV reverse transcriptase (Roche). Reverse transcription samples without reverse transcriptase were also used in each case as negative controls. One thirtieth of cDNA product was used to amplify a 306-bp product of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene as an RNA quality control. The primers for *GAPDH* were: CGGAGTCAACGGATTTGGTCGTAT (sense) and AGCCTTCTCCATGGTGGTGAAGAC (antisense). One tenth of the cDNA was used to amplify a 181-bp product of the *p73* gene. The primers for *p73* were TTGAGCACCTCTGGAGCTCT (sense) and ATCTGGTCCATGGTGCTGC (antisense). PCR conditions were as follows (in 50 μL reaction volume): 15 min at 95°C for initial denaturation, followed by 30 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C, with a final extension at 72°C for 10 min. PCR products were visualized on 2% agarose gels stained with ethidium bromide.

Quantitative real-time PCR assay was carried out using ABI Prism 7700 sequence detector (Applied Biosystems) by the following parameters: 95°C (15 min) followed by 40 cycles of 94°C (15 s), and 60°C (30 s). Primers and probes for *p73* and *GAPDH* were purchased from Applied Biosystems (design no. HS00232088-m1 for *p73* to specifically amplify transcripts from TA promoter). A relative gene expression level was calculated by the ratio of the target *p73* gene to *GAPDH* gene expression using Sequence Detector Systems version 2.0 software (Applied Biosystems).

Western blots for TAp73 protein expression (Imgenex) were done as previously reported (35). Equal protein loading was confirmed by blotting with control antibody against GAPDH (Novus).

Down-regulation of *p73* expression by RNAi approaches. siRNAs targeting *p73* were designed and prepared as reported previously (36). The siRNA sequences were as follows: *p73*-siRNA: 5′-CGGAUUCCAGCAUGGACGUdTdT-3′ and 5′-dTdTGCCUAAGGUCGUACCUGCA-3′; scrambled siRNA: 5′-UAGCCACCACUGACGACCUdTdT-3′ and 5′-dTdTAUCGGUGGUGACUGCUGGA-3′.

RNA oligonucleotides were obtained from Dharmacon. One day before transfection, cells were seeded such as they were 30% to 50% confluent the next day. Cells were transfected with 100 nmol/L of siRNA using Oligofectamine transfection reagent (Invitrogen) in Opti-MEM I reduced serum medium (Invitrogen) for 5 h. The medium was removed and replaced with fresh RPMI 1640 supplemented with 5% FBS. Control cells were treated with scrambled siRNA. Down-regulation of *p73* expression in SK-MEL28 cells was achieved by retrovirus vector (RNAi-Ready pSIREN-RetroQ Vector)–mediated shRNA targeting the same region as *p73*-siRNA. After infection and selection, we obtained five single clones and bulk cells for further analysis.

Measurement of growth inhibition and apoptosis in the cell lines before and after cisplatin treatment. Dilutions of cisplatin (Sigma; concentration ranged from 1 to 50 μmol/L), BCNU (Sigma, concentration ranged from 12.5 to 200 μmol/L), and carboplatin (MP Biomedicals, concentration ranged from 12.5 to 200 μmol/L) were freshly prepared before each experiment. Cells were allowed to recover from siRNA treatment for 48 h before the treatment. After exposure to each compound for 48 h, cell growth inhibition was measured by hemocytometry with trypan blue dye exclusion. Detection and quantification of apoptotic cells by Annexin V staining were done by flow cytometric analysis at the core facility in M.D. Anderson Cancer Center. All the experiments were repeated at least three times.

Results

Methylation profile of NCI-60 cancer cell lines. The methylation profile of NCI-60 cell lines was determined by testing the methylation status of 32 CpG islands. All but one (*MINT2*) of the CpG islands we tested correspond to promoters of specific genes. These included four cell cycle regulatory genes ($p16^{INK4a}$, $p14^{ARF}$, $p15^{INK4b}$, and $p57^{KIP}$), two DNA repair genes (*MGMT* and *hMLH1*), five angiogenesis-related genes (*THBS1, THBS4, TIMP3, E-cadherin*, and *DAPK*), four mediator of apoptosis genes (*TMS1, RIL, p73*, and *BNIP3*), two drug metabolism genes (*GSTP1* and *MDR1*), eight signal transduction genes (*ERα, RARβ2, COX2, cABL, RASSF1A, p101, MINT31* corresponding to the calcium channel *CACNA1G* gene, and *MINT25* corresponding to the calcineurin binding protein 1, *CABIN1* gene), two transcription regulator genes (*RIZ1* and *KR18*), and others (*CD10, LPH3, Megalin*, and *MINT1* corresponding to synaptic vesicle glycoprotein 2C, *SV2C* gene).

We used COBRA as a quantitative test to study methylation of all the genes except for *p73, RIZ1, RASSF1A, RARβ2, TIMP3, GSTP1*,

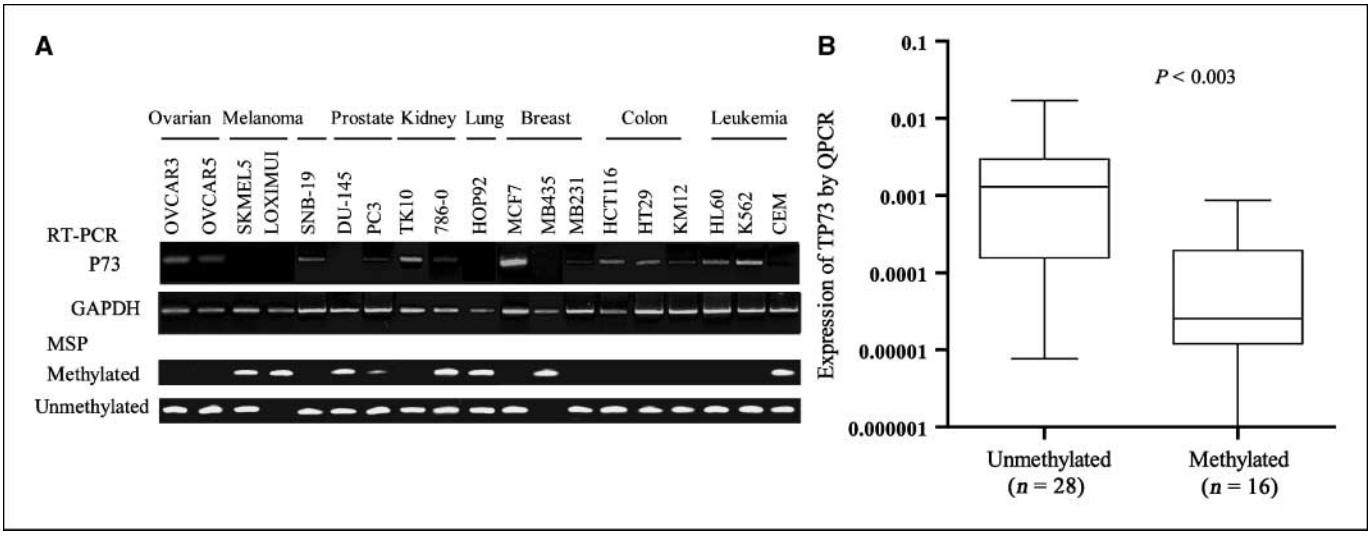

Figure 1. *p73* promoter methylation is correlated with gene expression. *A,* representative examples of *p73* expression by conventional RT-PCR and methylation status in NCI-60 cancer cell lines. *B,* comparison of *p73* gene expression by real-time RT-PCR in cell lines with or without promoter methylation.

Table 2. The correlation between gene methylation and drug sensitivity by COMPARE (170 standard agents)

Gene	Chemical name	MOA	Pearson correlation coefficient	*P* (two-tailed)
ECAD	BCNU	AC	0.58787	0.00000
	PCNU	AC	0.55098	0.00001
	Methyl CCNU	AC	0.50237	0.00006
	CCNU	AC	0.48644	0.00011
	Mitozolamide	AC	0.47533	0.00016
	Emofolin sodium		0.46926	0.0002
	Guanazole	DR	0.46138	0.00027
	IMPY	DR	0.45593	0.00032
DAPK	Merbarone		0.45246	0.00036
P73	Spirogermanium		0.55727	0.00001
	Merbarone		0.48347	0.00012
	Busulfan	A7	0.47271	0.00018
P57	Anguidine		0.505765	0.00005
	Neocarzinostatin		0.45411	0.00034
MINT1	Pyrazoloacridine	T2	0.48606	0.00011
GSTPi	Tamoxifen		0.47427	0.00017
THBS1	BCNU	AC	0.46173	0.00026
	Pentamethylmelamine		−0.45938	0.00029
	Pyrrolizine		0.45403	0.00034
	Anguidine		0.45267	0.00036
P14	Pyrrolizine		0.52037	0.00009
	Dichloroally lawsone	RO	0.50987	0.00013
P15	Melphalan	A7	0.70898	0.000000
	Ara AC		0.69365	0.00000
	Fluorodopan	A7	0.69059	0.00000
	Pipobroman	A7	0.67088	0.00000
	Chlorambucil	A7	0.63949	0.00000
	Yoshi-864	A7	0.61047	0.00000
	BCNU	AC	0.60178	0.00000
	Pentamethylmelamine		−0.59999	0.00000
	Carmethizole		0.59616	0.00000
	VM-26	T2	0.59304	0.00001
	Anguidine		0.58946	0.00001
	Mitindomide		0.57803	0.00001
	Thiotepa	A7	0.57522	0.00001
	Triethylenemelamine	A7	0.57330	0.00001
	Hydrazine sulfate		0.56765	0.00002
	Uracil nitrogen mustard	A7	0.56574	0.00002
	Thioguanine	DI	0.55405	0.00003
	L-Asparaginase		0.55337	0.00003
	Topo1A		0.55298	0.00003
	Topo1B		0.55079	0.00003
	Menogaril	T2	0.54534	0.00004
	Hepsulfam	A7	0.53888	0.00005
	Cytosine arabinoside	DP	0.54943	0.00005
	Piperazine alkylator	A7	0.53099	0.00007
	Dianhydrogalactitol	A7	0.52713	0.00008
	Teroxirone	A7	0.51836	0.00012
	Pyrrolizine		0.51834	0.00012
	Merarone		0.51474	0.00013
	Thymidine		0.51341	0.00014
	N-*N*-dibenzyldaunomycin	T2	0.55450	0.00021
	Deoxydoxorubicin	T2	0.50057	0.00022
	Nitrogen mustard	A7	0.50021	0.00024
	Pyrimidine-5-glycodialdehyde		0.49768	0.00031
	Asaley	A7	0.48982	0.00031
	Chip	A7	0.48945	0.00032
	5-Azadeoxycytidine	DI	0.48886	0.00034
	Pyrazine diazohydroxide		0.48644	0.00034

(*Continued on the following page*)

Table 2. The correlation between gene methylation and drug sensitivity by COMPARE (170 standard agents) (Cont'd)

Gene	Chemical name	MOA	Pearson correlation coefficient	*P* (two-tailed)
COX2	Pentamethylmelamine		−0.59720	0.00000
	Pyrrolizine		0.54451	0.00001
	Melphalan	A7	0.51615	0.00003
	Pipobroman	A7	0.51011	0.00004
	Merbarone		0.49971	0.00007
	Fluorodopan	A7	0.49507	0.00008
	Carmethizole		0.48595	0.00011
	Chlorambucil	A7	0.48280	0.00012
	Ara AC		0.47696	0.00015
	Yoshi-864	A7	0.47639	0.00016
RASSF1A	Trimethyltrimethylolmelam		−0.43239	0.00017
BNIP3	Rhizoxin	TU	−0.57941	0.00000
KR18	Rhizoxin	TU	−0.4536	0.00039

NOTE: Alkylating agents: A2, alkylating at N2 position of guanine; AC, alkyl transferase–dependent cross-linkers; A7, alkylating at N7 position of guanine; AI, DNA intercalators. Anti-DNA agents: DI, incorporated; DP, polymerase inhibitors; DR, RNase reductase inhibitors. Nucleotide synthesis inhibitors: RF, antifolates; RI, irreversible inhibitors; RO, anti–other precursors; R, unknown locus of inhibition. T1, topoisomerase I inhibitors; T2, topoisomerase II inhibitors; TU, tubulin-active antimitotic agents; MOA, mechanism of action.

and *CDH1* (*E-cadherin*), for which we used MSP. The data for *KR18, THBS4, Megalin, p101*, and *LPH3* genes were previously reported (24) and used here for analysis of drug sensitivity. All methylation data are shown in Supplementary Table S2. Representative examples of methylation analysis are shown in Supplementary Fig. S1, and the frequency of aberrant methylation according to tissue type is summarized in Table 1. At least one gene is hypermethylated in all cell lines, and >90% of cell lines have aberrant methylation of at least four genes.

Clustering of human cancer cell lines according to their CpG island methylation profile. To elucidate tissue specificity of methylation changes, we did cluster analyses on the basis of gene methylation patterns (Supplementary Fig. S2*A*). With average linkage clustering and a correlation metric, cell lines derived from leukemia and colon cancer showed tight clustering into independent terminal branches specific to their respective organ types (Supplementary Fig. S2*B*). Cell lines derived from other malignancies, however, were distributed in multiple different terminal branches, suggesting that their methylation patterns were more heterogeneous. This analysis may be biased by the inclusion of several genes initially identified as methylated in colon cancer.

Correlation between gene expression and methylation. Among 32 CpG island–associated genes we analyzed, we obtained gene expression results based on microarrays for 17 genes in the NCI-60 database. We therefore correlated methylation with expression and found that promoter methylation correlated well with gene expression in 12 genes (Supplementary Table S3). However, there were exceptions. For example, because there was only one cell line with *hMLH1* or *c-Abl* hypermethylation, the correlation between methylation and expression was weak for both genes ($R = -0.25$, $P = 0.185$ for *hMLH1* and $R = -0.22$, $P = 0.13$ for *c-Abl*, respectively); for *p57*, an imprinted gene, the correlation between methylation and expression was also weak ($R = -0.24$, $P = 0.07$). The correlation between methylation and expression for *MDR1* gene was not significant, possibly because the methylation assay was designed based on a CpG island located in the intron 1 region ($R = -0.22$, $P = 0.09$). Alternatively, nonsignificant correlation could occur when gene repression results from other mechanisms independent of DNA methylation and/or microarray gene expressions are prone to probe and background effects that may confound such correlation in the absence of statistical algorithms designed to account for these effects. We found that there was no significant correlation between *p73* gene methylation and expression by microarray, perhaps due to complicated gene structure with multiple alternate transcripts of this gene. To address this, we did reverse transcription-PCR (RT-PCR) designed specifically for the methylation-targeted promoter of *p73* (TA promoter), and found an excellent correlation between methylation and expression by both conventional RT-PCR and real-time RT-PCR (Fig. 1).

We then correlated methylation with the expression of epigenetic modifiers. For each cell line, we defined a methylation index (MI) as the ratio between the number of methylated genes and the number of analyzed genes and divided the cell lines into methylation low (MI <0.2), moderate (MI between 0.2 and 0.4), and high (MI >0.4). As shown in Supplementary Table S4, there was no correlation between methylation and the expression levels of *DNMT3a, DNMT3b,* or any other epigenetic modifiers examined.

Predicting anticancer drug sensitivity by methylation. We next tested whether DNA methylation profiling in the NCI-60 panel predicts drug sensitivity or resistance. First, we used COMPARE analysis to correlate methylation of each marker with drug response at the GI_{50} (50% growth inhibition) level of effect. The correlation coefficient and *P* values are provided in Table 2 for standard agents, in Supplementary Table S5 for compounds selected for evaluation by the Biological Evaluation Committee of the Developmental Therapeutics Program, and in Supplementary Table S6 for open database compounds including ≈30,000 open unrestricted compounds. Because of the large number of agents with data available (>30,000), a large number of spurious associations is expected. To begin reducing this complexity, we initially focused on the 170 standard compound databases. Using

Bonferroni-adjusted P values, we find ~70 significant correlation (Table 2), some of these can be explained by tissue-specific methylation and drug sensitivity; for example, aberrant methylation of $p15^{INK4b}$ was found in 60% of leukemia cell lines (among six leukemia cell lines, three are methylated, two are not methylated, and one has homozygous deletion) and correlated with sensitivity to antileukemia drug. Other associations, however, were true across tissues and were of interest, such as correlation between *CDH1* methylation and sensitivity to BCNU ($R = 0.6$, $P < 0.00001$) and methylation of *GSTP1* and sensitivity to tamoxifen ($R = 0.47$, $P = 0.0002$). Interestingly, we also found that methylation at multiple genes (*THBS1*, $p15^{INK4b}$, and *COX2*) was significantly correlated with resistance to pentamethylmelamine ($R = -0.46$, $P = 0.0003$ for *THBS*; $R = -0.60$, $P = 0.000001$ for $p15^{INK4b}$ and $R = -0.60$, $P = 0.000001$ for *COX2*).

Because any spurious correlations between methylation and drug sensitivity would not be explained mechanistically by effects on pathways that alter sensitivity to whole classes of agents, we therefore looked at correlations between methylation and sensitivity for groups of drugs based on their mechanisms of action. A subset of 118 compounds were classified into five groups: group 1, alkylating agents including A2, A7, and A6, alkylating at the N^2, N^7, and O^6 position of guanine, respectively; group 2, DNA synthesis inhibitors; group 3, tubulin-active antimitotic agents, group 4, topoisomerase I inhibitors; and group 5, topoisomerase II inhibitors. Based on GI_{50}, we defined drug activity for each agent in each cell line as sensitive, intermediate, and resistant (detailed in Materials and Methods). For each class of agents, cell lines were classified into sensitive cell line if the majority of agents were sensitive in that cell line, or resistant cell line if the majority of agents were resistant in that cell line. Methylation of each CpG islands was then compared with drug activity profiles in each of these five groups. The most striking association was between *p73* hypermethylation and increased sensitivity to alkylating agents in general (Fig. 2). Out of 17 cell lines with *p73* methylation, 15 (88%) were generally sensitive to alkylating agents; in contrast, out of 41 cell lines with no *p73* methylation, only 16 cell lines (39%) were sensitive to alkylating agents ($P < 0.01$ by χ^2 test). No association was found between *p73* methylation with the sensitivity to topoisomerase I or II inhibitor or to tubulin-active antimitotic agents.

To ask whether there is a direct relation between *p73* hypermethylation and associated silencing with increased sensitivity to alkylating agents, we transfected *p73* siRNA targeting the TA promoter or scrambled siRNA to two renal cancer cell lines TK10 and 786-0. As shown in Fig. 1, *p73* was methylated and weakly expressed in parental 786-0, whereas in parental TK10 *p73* was not

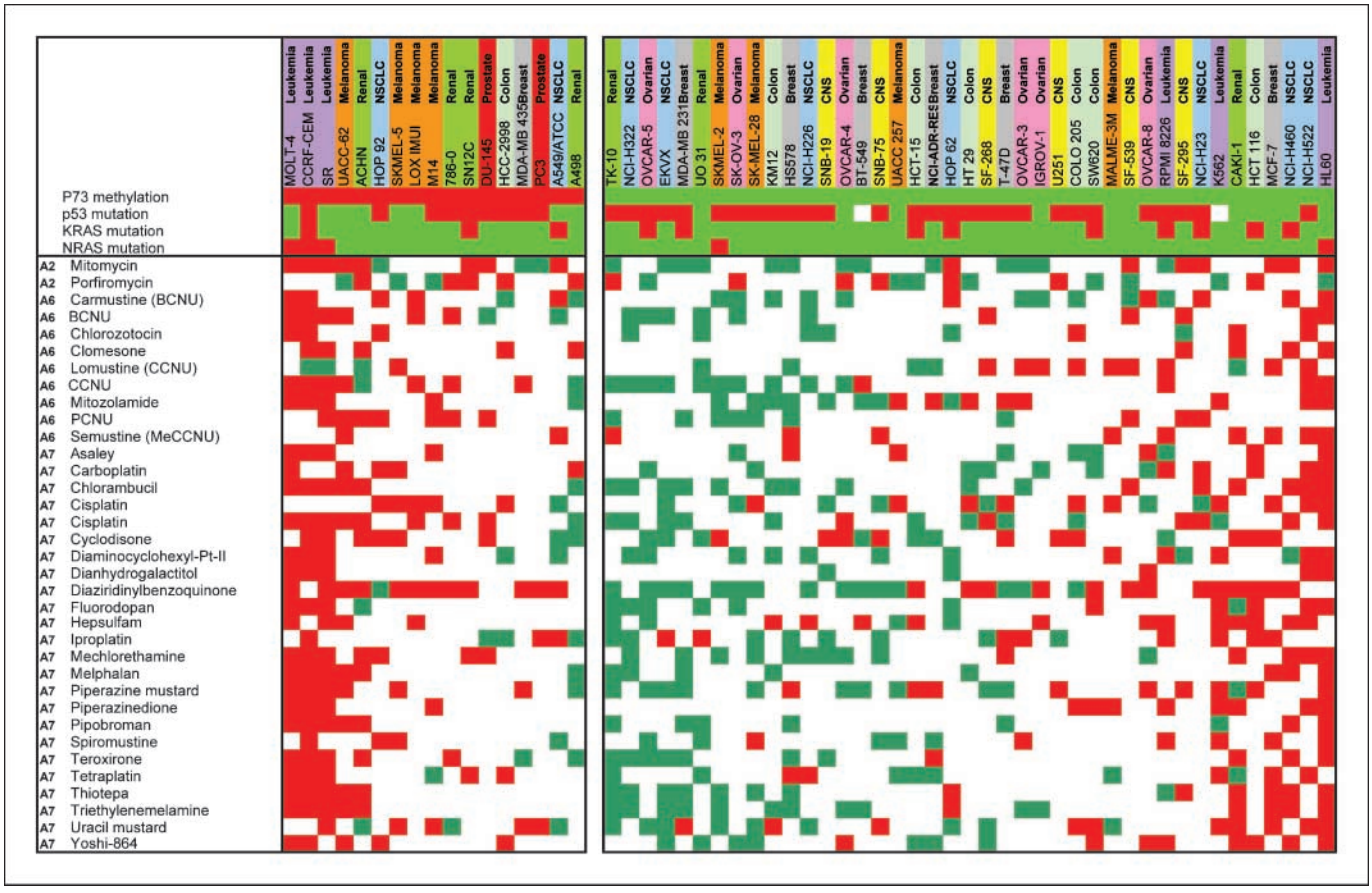

Figure 2. *p73* methylation is significantly associated with increased sensitivity to alkylating agents. Each column represents a cancer cell line. *Top,* methylation status of *p73*; *green,* no methylation; *red,* methylation. Next is mutation status of *p53, KRAS*, and *NRAS* genes; *green,* wild-type; *red,* mutant. *Bottom,* drug response to each of the 35 standard alkylating agents in each cell line; they are classified as sensitive (*red*): $\log_{10}(GI_{50}) <$ mean $-$ 0.8 SD; resistant (*green*): $\log_{10}(GI_{50}) >$ mean + 0.8 SD; and intermediate (*white*): mean $-$ 0.8 SD $< \log_{10}(GI_{50}) <$ mean + 0.8 SD. For BCNU, CCNU, and cisplatin, the NCI reports two separate data sets in their Web site (http://dtp.nci.nih.gov/dtpstandard/dwindex/index.jsp). Each data set was independently generated from various numbers of repeated tests, and we used both of them for the analysis.

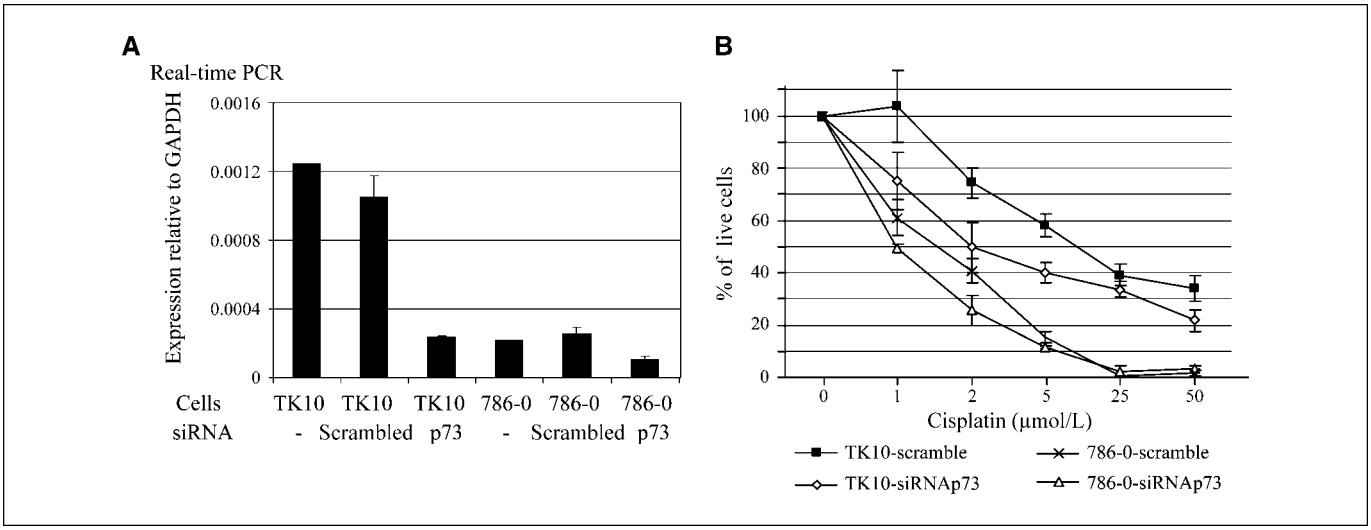

Figure 3. Down-regulating *p73* expression by siRNA increased sensitivity to cisplatin in two renal cancer cell lines. *A,* expression of *p73* gene analyzed by real-time PCR. *B,* percent of live cells after being cultured with increasing concentrations of cisplatin for 48 h. *X axis,* concentration of cisplatin used for the treatment. *Y axis,* percentage of live cells.

methylated and expressed well. Transfection of *p73* siRNA but not scrambled siRNA significantly decreased *p73* expression in TK10, and reduction of p73 expression was also observed in 786-0 after transfecting *p73* siRNA, although less dramatic compared with TK10 (Fig. 3*A*). Next, we selected cisplatin as the treatment drug because it is the most commonly used alkylating agent for cancer treatment. Compared with 786-0, TK10 is more resistant to cisplatin; the GI_{50} for cisplatin is 1 μmol/L for 786-0 and 12.5 μmol/L for TK10. We found that down-regulating p73 expression increased the sensitivity to cisplatin in both TK10 and 786-0 cells, but such an effect was not found in control scrambled siRNA (Fig. 3*B*). The effect of increased sensitivity was more dramatic in *p73*-expressing TK10 cells (GI_{50} decreased from 12.5 to 2.5 μmol/L ~5-fold), compared with the weakly expressing 786-0 cells.

To confirm these results, we used another approach by designing a retroviral vector encoding shRNA that specifically targets the *p73* TA promoter and infected this vector into the resistant melanoma cell line, SK-MEL28. Down-regulation of *p73* expression at both mRNA and protein levels was found in the cells infected with this vector but not in cells infected with the control vector, and the effect of reduction was very similar in multiple clones we analyzed as well as bulk cells (Fig. 4*A* and *B*). Consistent with the previous results, we found that *p73* down-regulation also significantly increased the sensitivity to cisplatin in SK-MEL28 cells (GI_{50} decreased from 5 to 1.5 μmol/L, up to 4-fold, in Fig. 4*C*), and the increased sensitivity was associated with an increased apoptosis (Fig. 4*D*). We also tested two other alkylating agents, BCNU and carboplatin, and observed similar results of increased sensitivity in the cells after knockdown of *p73* (Supplementary Fig. S3).

Discussion

The NCI has developed a panel of human cancer cell lines (NCI-60) as a drug discovery tool by profiling sensitivity to a broad range of chemical compounds. As an initial step to identify epigenetic-mediated drug sensitivity or resistance (pharmaco-epigenomics), we have determined DNA methylation status of 32 CpG islands in this panel of untreated cells and provided a database that can be correlated with drug responses (sensitivity or resistance) for >30,000 compounds tested.

By focusing on standard anticancer drugs whose mechanisms of action are known, we identified a list of significant correlations between gene methylation and drug sensitivity. Although most of them seem to be tissue-type specific, there are several associations that were present in multiple tumor types, such as methylation of *CDH1* predicts sensitivity to BCNU and methylation of *GSTP1* predict sensitivity to tamoxifen. *CDH1* is a major epithelial cell-cell adhesion molecule that functions as a tumor suppressor and plays a causative role in tumor invasion and metastasis (37). *GSTP1* is a member of the glutathione *S*-transferase superfamily that catalyzes the conjugation of the peptide glutathione with electrophilic compounds, resulting in less toxic and more readily excreted metabolites (38). Loss of function of these genes through DNA methylation at promoter CpG islands has been reported in both cancer cell lines and primary cancer patients; hypermethylation of *CDH1* has been found in multiple tumors, including brain, breast, and gastric cancers (39–41); and hypermethylation of *GSTP1* is a frequent event in prostate and breast cancers (23, 42). Interestingly, we also observed several significant correlations between methylation of *THBS1, p15^{INK4b}*, and *COX2* genes with resistance to pentamethylmelamine, an agent that alkylates DNA and forms DNA intrastrand and DNA-protein cross-links to prevent DNA replication. It would be worth to further validate these genes in the future. Our findings also raise the possibility that these epigenetic markers can be used to assist in selection of therapy for individual patients rather than the current empirical decision-making process. These hypotheses, however, need to be tested in the setting of clinical trials.

Next, we correlated methylation profiling with groups of drugs based on their mechanism of action, and identified a significant association between *p73* methylation and sensitivity to alkylating agents. We further confirmed the functional link between *p73* and drug sensitivity by showing that down-regulation of *p73* increases sensitivity to commonly used alkylating agents, including cisplatin, in several cancer cell lines tested. *p73*, as a *TP53* homologue, is located on chromosome 1p, a region frequently showing loss of heterozygosity in primary cancers. Interestingly, it has been

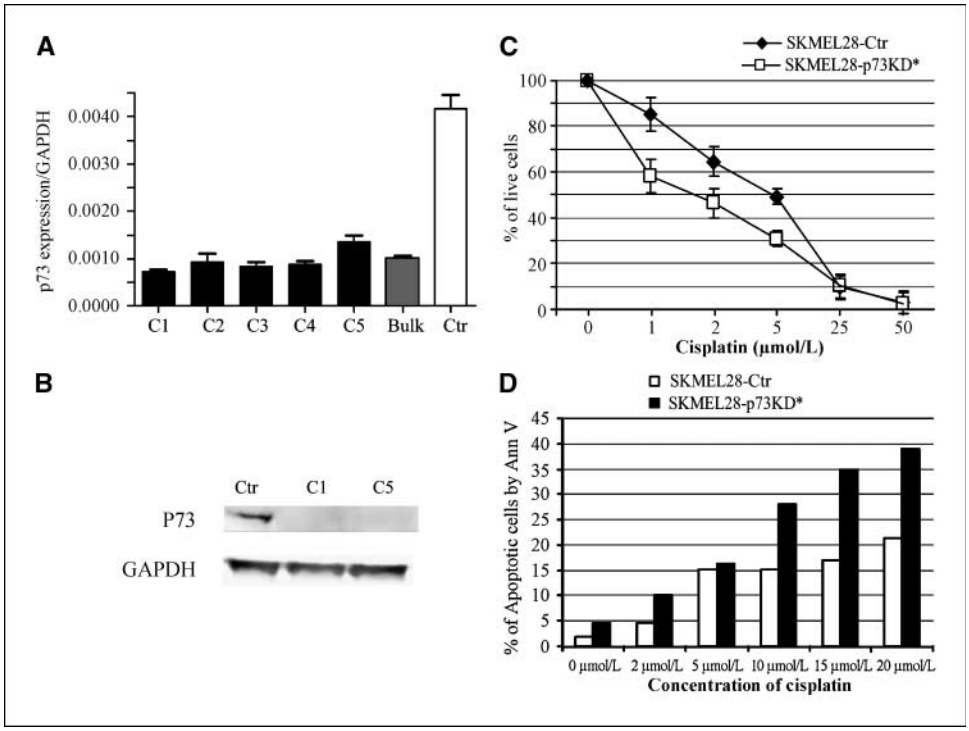

Figure 4. Down-regulating *p73* expression by shRNA increased sensitivity to cisplatin in SKMEL28. *A,* expression of *p73* gene analyzed by real-time RT-PCR. *B,* expression of *p73* gene analyzed by Western blot. *C,* percentage of live cells after being cultured with increasing concentrations of cisplatin for 48 h. *D,* inhibition of *p73* expression (*black column*) augments cisplatin-induced apoptosis.

reported that allelic loss of chromosome 1p predicts chemosensitivity in patients with oligodendroglial neoplasms (43). Functionally, *p73* has been associated with DNA damage response by regulating programmed cell death (44), mismatch repair (45), and transcriptional regulation (46, 47). However, *p73* shows much greater functional complexity than *p53* due to the alternative promoter usage and differential mRNA splicing (48). At least 13 different protein isoforms have been reported for *p73*. Overexpression of *TAp73* mRNA is commonly observed in primary human tumors, including neuroblastoma, bladder, hepatocellular, colorectal, lung, melanoma, breast, and ovarian cancers, and associated with increased expression of many genes, including members of the NER and MMR pathways (48, 49). In cancers, it has been suggested that inactivation of *p73* in cancer cell lines by siRNA or dominant negative mutation resulted drug resistance to cisplatin through reduced apoptosis (36). However, our results indicate that methylation of *p73* leads to loss of gene expression and results in sensitivity to alkylating agents in NCI-60 cancer cell lines. Moreover, down-regulating the expression of *p73* in resistant cell lines by siRNA targeting of the same region as in the previous report (36) can sensitize the resistant cells to cisplatin. Interestingly, we found that the increased sensitivity to cisplatin in cells transfected with *p73* shRNA was associated with increased apoptosis. One possible explanation is that the previous reports mainly focused on drug resistance of cisplatin in certain types of cancers (such as colon), whereas our study focused on *p73* methylation in a panel of cancer cells derived from different tissues and analyzed the drug sensitivity to alkylating agents in general. It will be interesting to determine whether *p73* overexpression could lead to chemoresistance in the cancer cells that are sensitive to alkylating agents. Previous studies had implicated epigenetic inactivation of *MGMT* and sensitivity to alkylating agents in glioma patients treated with BCNU (11). Here, we did not find that *MGMT* methylation had a general predictive significance to alkylating agents. However, there was an association between *MGMT* methylation and sensitivity to BCNU (R = 0.324, P = 0.01), which is consistent with the published data. In addition, our results show that *CDH1* methylation predicts sensitivity to BCNU (R = 0.59, P = 0.000001) and *p73* methylation predicts sensitivity to alkylating agents in general. It may be worth testing these markers in the same patient populations.

Previous studies in drug sensitivity investigated gene-drug correlation by transcription profiling (3, 32). By correlating DNA methylation with microarray expression analysis, we found that promoter methylation correlated with gene expression by microarray for most cases; however, unlike gene expression–based approaches that rely on differential expression levels of transcripts, epigenetic profiling such as DNA methylation analysis allows us to distinguish silenced states (accompanied by DNA methylation) from physiologic (or transient) decreased expression. In addition, epigenetic profiling is useful for genes with low baseline expression and genes with multiple alternate transcripts, two situations that are problematic in gene expression profiling. Although the candidate epigenetic markers identified in this study must be confirmed on a larger series of clinical patient studies, our results nonetheless suggest that it is feasible to predict chemotherapeutic responses by DNA methylation profiling, and future studies, including unbiased methods for DNA methylation analysis such as high-throughput methylation analysis and methylation microarray, will be needed to achieve a comprehensive study of pharmaco-epigenomics.

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