Genome-Wide Transcriptional Response to 5-Aza-2′-Deoxycytidine and Trichostatin A in Multiple Myeloma Cells

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Abstract

To identify epigenetically silenced cancer-related genes and to determine molecular effects of 5-aza-2′-deoxycytidine (Aza-dC) and/or trichostatin A (TSA) in multiple myeloma (MM), we analyzed global changes in gene expression profiles of three MM cell lines by microarray analysis. We identified up-regulation of several genes whose epigenetic silencing in MM is well known. However, much more importantly, we identified a large number of epigenetically inactivated cancer-related genes that are involved in various physiologic processes and whose epigenetic regulation in MM was unknown thus far. In addition, drug treatment of MM cell lines resulted in down-regulation of several MM proliferation-associated factors (i.e., *MAF, CCND1/2, MYC, FGFR3, MMSET*). Ten Aza-dC and/or TSA up-regulated genes (*CPEB1, CD9, GJA1, BCL7c, GADD45G, AKAP12, TFPI2, CCNA1, SPARC*, and *BNIP3*) were selected for methylation analysis in six MM cell lines, 24 samples from patients with monoclonal gammopathy of undetermined significance (MGUS), and 111 samples from patients with MM. Methylation frequencies of these genes ranged between 0% and 17% in MGUS samples and between 5% and 50% in MM samples. Interestingly, methylation of *SPARC* and *BNIP3* was statistically significantly associated with a poor overall survival of MM patients ($P = 0.003$ and $P = 0.017$, respectively). Moreover, *SPARC* methylation was associated with loss of SPARC protein expression by immunostaining in a subset of MM patients. In conclusion, we identified new targets for aberrant methylation in monoclonal gammopathies, and our results suggest that DNA methyltransferase and histone deacetylase inhibition might play an important role in the future treatment of patients with MM. [Cancer Res 2008;68(1):44–54]

Introduction

Previous molecular studies have shown that multiple myeloma (MM) cells from the majority of patients harbor cytogenetic aberrations, particularly illegitimate rearrangements of the immunoglobulin heavy chain gene (*IGH*) on 14q32, monosomy 13, and deletions of 13q (1–3). In addition to genetic aberrations, there is increasing evidence that epigenetic changes play an important role in the pathogenesis of MM (4, 5). Aberrant methylation (referred to as methylation) of gene promoter regions leading to gene silencing is to date the most widely studied epigenetic abnormality in human malignancies (6). Costello et al. (7) reported that, on average, 600 CpG islands are targets for methylation in malignant diseases. To date, ~20 cancer-related genes have been identified that are frequently silenced by methylation in MM (5, 8, 9). Thus, identification of unknown epigenetically inactivated cancer-related genes in MM is of tremendous importance for a better understanding of the pathogenesis of this disease. In addition to DNA methylation, histone hypoacetylation plays a critical role in epigenetic gene silencing (10). Chromatin inactivated by histone H3 and/or H4 deacetylation is associated with silencing of several cancer-related genes in cancer cells (11–13).

In contrast to genetic alterations, epigenetic changes are potentially reversible. DNA methyltransferase inhibitors 5-azacytidine and 5-aza-2′-deoxycytidine (Aza-dC) are analogues of deoxycytidine, which is the target nucleoside for methylation; these drugs have been extensively studied in reactivating by methylation-silenced genes (6, 14). Trichostatin A (TSA) is a potent histone deacetylase inhibitor. Moreover, Xiong et al. (15) reported that besides HDAC activity, TSA has DNMT3b down-regulating properties, and several studies have shown that TSA is capable of activating the expression of methylation-silenced genes (16–18) and of genes that are silenced by aberrant histone deacetylation in the absence of DNA methylation (19). Aza-dC and TSA have been shown to act synergistically in the reexpression of methylated cancer-related genes (16). In addition, several studies have shown that Aza-dC and TSA inhibit cancer cell growth (20–22).

Here, we describe the results of a microarray-based genome-wide screen for genes responding to DNA methyltransferase inhibition and HDAC inhibition in MM cell lines. Our study presents a large number of genes whose epigenetic silencing in MM was unknown thus far. Based on the results of our microarray assay, we selected several cancer-related genes whose epigenetic silencing in monoclonal gammopathies has been unknown thus far and analyzed their methylation status in a large number of monoclonal gammopathies of undetermined significance (MGUS) and MM samples. Moreover, the methylation results were compared with certain clinicopathologic variables of the patients. Some of them may be of clinical relevance for MM patients.

Materials and Methods

Clinical specimens. Bone marrow aspirates and clinical data were collected from 24 patients with MGUS and from 111 patients with MM as reported recently (5). For control experiments, bone marrow specimens from a healthy bone marrow donor and from nine patients with localized non–Hodgkin's lymphoma without bone marrow infiltration were analyzed.

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

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Mononuclear cells were isolated using Histopaque-1077 (Sigma) according to the manufacturer's instructions and stored at −80°C.

Cell culture and treatment. The human MM cell lines MM1, U266, and NCI-H929 were grown in RPMI 1640 supplemented with 10% fetal bovine serum. Cells (2×10^5/mL) were treated either with 0.5 μmol/L Aza-dC for 7 days or with 100 ng/mL TSA for 24 h or with the combination of 0.5 μmol/L Aza-dC for 7 days and 100 ng/mL TSA for additional 24 h. Control cells received no drug treatment.

RNA extraction and cRNA preparation. Total cellular RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instruction. First- and second-strand cDNA synthesis was performed using SuperScript double-stranded cDNA synthesis kit (Invitrogen) and 10 μg of purified RNA according to the manufacturer's protocol with the use of an oligo-dT primer containing a T7 RNA polymerase promoter site. IVT was performed using the BioArray High Yield RNA Transcript Labeling kit (Enzo Life Sciences). Fifteen micrograms of cRNA were fragmented at 94°C for 35 min in a fragmentation buffer (Affymetrix).

Array hybridization and scanning. The microarray assay was carried out using the Affymetrix GeneChip HG-U133A. The hybridization, scanning, washing, and staining procedure was performed as recommended by the manufacturer. The readings from the quantitative scanning were analyzed by the Affymetrix Gene Expression Analysis Software. Hybridization quality control variables were attended according to Affymetrix guidelines for efficient hybridization. All microarray analyses were done in duplicates starting from the same independently treated cell population.

Statistical analysis of microarray data. Affymetrix Microarray Analysis Suite version 5 (MAS5) was used to process the scanned chip images and to generate a cell intensity file for each chip. Statistical analysis was performed using Bioconductor's affy package.[6] The significance of changes in the expression level of each gene was evaluated with the CyberT algorithm.[7] Differentially expressed genes between treated and untreated groups were selected using following criteria: (*a*) results derived from drug-treated cells had to be at least 4-fold higher than those derived from control cells in each pairwise comparison; (*b*) differences in gene expression between drug-treated and control cells had to be statistically significant ($P < 0.001$); and (*c*) the detection call had to be present (*P*) in minimum 1 sample.

Gene Ontology analysis. To classify up-regulated genes into statistically significant overrepresented functional categories Gene Ontology analysis was performed using GoMiner (23) comparing the total set of genes represented on the HG-U133A GeneChip and the subset of genes that was up-regulated after drug treatment.

Real-time reverse transcription-PCR. For validation of microarray results, real-time reverse transcription-PCR (RT-PCR) was performed using Taqman Gene Expression Assays (Applied Biosystems) as recommended by the manufacturer.

Nucleic acid isolation and methylation-specific PCR. Genomic DNA was isolated from MM cell lines and from mononuclear cells by digestion with Proteinase K, followed by standard phenol-chloroform extraction and ethanol precipitation (24). One microgram of genomic DNA was modified by treatment with sodium bisulfite as reported previously (25). Using methylation-specific PCR (MSP) analysis, the methylation status of the genes *CPEB1, CD9, GJA1, BCL7c, GADD45G, AKAP12, TFPI2, CCNA1, SPARC*, and *BNIP3* was analyzed. Primer sequences and PCR conditions of these genes are available upon request.

Immunostaining. Paraffin-embedded bone marrow biopsies were cut in 5-μm-thick sections, dewaxed, and immunostained for SPARC using rabbit anti-SPARC polyclonal antibody at a 1:1,000 dilution (Abcam). Labeling was detected with the Envision Plus Detection Kit (DAKO) as recommended by the manufacturer, and all sections were counterstained with hematoxylin.

Statistical analysis of methylation results. The methylation status of the genes *CPEB1, CD9, GJA1, BCL7c, GADD45G, AKAP12, TFPI2, CCNA1, SPARC*, and *BNIP3* was compared with certain clinicopathologic characteristics from the MM patients including age, gender, β_2-microglobulin, lactate dehydrogenase (LDH), hemoglobin, serum creatinine and calcium levels, type of paraprotein, type of light chain, tumor stage, tumor grade, and deletion of chromosome 13q14. Statistical analysis was performed using χ^2 test and Fisher's exact test for differences between groups and *t* tests between means. Overall survival was calculated using Kaplan-Meier log rank testing.

Results

Analysis of Global Gene Expression Pattern of MM Cell Lines after Drug Treatment

Using the DNA microarray technique, we analyzed global changes in gene expression after treatment of three MM cell lines (MM1, U266, and NCI-H929) with Aza-dC, TSA, and a combination of Aza-dC and TSA. The gene expression profile was compared before and after treatment using Affymetrix HG-U133A GeneChip, which contains 22,283 probe sets. Replicate array analysis using RNA from independently treated cell lines revealed a mean correlation coefficient of 0.96 (range 0.93–0.98), indicating high comparability between the arrays.

Identification of genes induced by Aza-dC in MM cell lines. Aza-dC treatment resulted in up-regulation of 92 probe sets (0.4% of 22,283 transcripts analyzed) in the cell line MM1, 65 probe sets (0.2%) in U266, and 220 probe sets (0.9%) in NCI-H929, respectively. The level of induced expression varied from 4.2-fold to a maximum of 147.03-fold in MM1, from 5.04-fold to 131.42-fold in U266 and from 4.28-fold to 137.32-fold in NCI-H929 Aza-dC–treated cells compared with untreated cells. As multiple probe sets for the same gene are present on Affymetrix microarrays, up-regulated probe sets represent 78 unique genes in the case of MM1, 60 unique genes in the case of U266 and 194 unique genes in case of NCI-H929. Overall, 284 unique genes were up-regulated in at least one of the three MM cell lines after exposure to Aza-dC (Supplementary Table S1). ENSEMBL database was used to obtain the genomic sequence, including 5′ region, exons and introns of each of the 284 up-regulated genes. The MethPrimer program (26) and the CpG Island Searcher (27) were used to determine whether the sequences contain CpG islands. The data showed that 73% of up-regulated genes contained CpG islands within the analyzed regions.

Identification of genes induced by TSA in MM cell lines. We also determined the gene expression profile of the three MM cell lines after exposure to the HDAC inhibitor TSA. TSA treatment resulted in up-regulation of 87 probe sets (0.4% of 22,283 transcripts analyzed) in the cell line MM1, 174 probe sets (0.8%) in U266, and 195 probe sets (0.9%) in NCI-H929, respectively. The level of induced expression varied from 4.39-fold to a maximum of 52.38-fold in MM1, from 4.3-fold to 199.33-fold in U266 and from 4.36-fold to 112.51-fold in NCI-H929 TSA-treated cells compared with untreated cells. Up-regulated probe sets represent 70 unique genes in the case of MM1, 146 unique genes in the case of U266, and 169 unique genes in case of NCI-H929. Overall, 324 unique genes of which 94% contain CpG islands were induced in one or more of the three MM cell lines after exposure to TSA (Supplementary Table S2).

Identification of genes induced by treatment of MM cell lines with both drugs. At last, we determined the gene expression profile of the three MM cell lines after exposure to the combination of Aza-dC/TSA, which resulted in up-regulation of 215 probe sets (1% of 22,283 transcripts analyzed) in the cell line MM1, 276 probe sets (1.2%) in U266, and 254 probe sets (1.1%) in NCI-H929, respectively. The level of induced expression varied from 4.26-fold to a maximum of 147.44-fold in MM1, from 4.35-fold to 295.04-fold

[6] http://www.bioconductor.org
[7] http://cybert.microarray.ics.uci.edu/

in U266, and from 4.43-fold to 209.97-fold in NCI-H929 Aza-dC/TSA-treated cells compared with untreated cells. Up-regulated probe sets represent 180 unique genes in the case of MM1, 242 unique genes in the case of U266, and 216 unique genes in case of NCI-H929. Overall, 470 unique genes of which 88% contain CpG islands were induced in one or more of the three MM cell lines after exposure to the combination of Aza-dC and TSA (Supplementary Table S3).

In addition, the distribution of up-regulated genes was relatively even; however, the highest proportions of induced genes were located at chromosome 6 where a 7 Mb cluster with high density of up-regulated genes was identified (Supplementary Fig. S1*A* and S1*B*). This cluster contains genes encoding for MHC class II proteins that are normally constitutively expressed in antigen-presenting cells.

Overlap of up-regulated genes between MM cell lines and drug treatment. To evaluate overlaps of Aza-dC and TSA up-regulated genes between MM cell lines, Venn diagrams were generated which show that there is only a limited overlap of Aza-dC and/or TSA-induced genes in the three MM cell lines (Fig. 1*A*). Four genes were found to be up-regulated in all 3 MM cell lines after Aza-dC treatment, 11 genes after TSA treatment, and 35 genes after Aza-dC/TSA treatment, respectively. By comparing genes up-regulated in two cell lines after Aza-dC, TSA, or Aza-dC/TSA treatment, it is noteworthy that in MM1 and NCI-H929 more commonly up-regulated genes were found than in U266 when compared with MM1 and NCI-H929. Next, we identified genes whose expression was up-regulated as response to a particular drug in each cell line individually (Fig. 1*B*). Interestingly, only 2, 1, and 2 genes were commonly up-regulated after Aza-dC and TSA treatment in MM1, U266, and NCI-H929, respectively, indicating that Aza-dC and TSA affect different groups of genes and pathways. In addition, a high percentage (57% in MM1, 67% in U266, and 41% in NCI-H929, respectively) of genes induced by TSA were also found to be up-regulated after Aza-dC/TSA treatment in each cell line. However, the percentage of commonly Aza-dC and Aza-dC/TSA up-regulated genes was much lower (14% in MM1, 28% in U266, and 19% in NCI-H929, respectively; Fig. 1*B*).

Identification of genes down-regulated after drug treatment. Although at a lower number we also identified genes whose expression was down-regulated in MM cell lines. Treatment of the cell lines with Aza-dC resulted in down-regulation of 76 genes in MM1, of 20 genes in U266, and of 15 genes in NCI-H929, respectively. After TSA treatment, 79 genes were found to be down-regulated in MM1, 107 in U266, and 148 in NCI-H929, respectively. Aza-dC/TSA treatment resulted in down-regulated expression of 149 genes in MM1, 70 genes in U266, and 141 genes in NCI-H929, respectively. Interestingly, by Aza-dC and TSA, down-regulated genes included several proto-oncogenes that have been found to be substantially overexpressed in MM (i.e., *cyclin D1, cyclin D2, MAF, MAFF, MAFG, FGFR3, MMSET*; members of the myc protein family and *PIM2*; Table 1). Of note, as shown in Table 1, down-regulation of these genes was more a response to TSA and Aza-dC/TSA than to Aza-dC alone. Results from microarray analysis from certain cancer-related genes were validated by real-time RT-PCR (Tables 1 and 3). Detailed results are available online (Supplementary Table S4).

Functional Analysis by GoMiner

To identify biological processes statistically significantly affected by Aza-dC and TSA genes whose expression was up-regulated >4-fold in at least one MM cell line after treatment with Aza-dC, TSA or Aza-dC/TSA were analyzed using the GoMiner program (23). GoMiner dereplicates total and changed input files so that only one instance of a gene name is processed. This resulted in 13,018 reference genes to which 284 genes whose expression was up-regulated after Aza-dC treatment, 324 genes whose expression was up-regulated after TSA treatment, and 470 genes whose expression was up-regulated after Aza-dC/TSA treatment were compared. Detailed results are shown in Table 2. Aza-dC and TSA were found to up-regulate different groups of genes. Although Aza-dC treatment resulted in statistically highly significant up-regulation of genes involved in response to stimulus and immune response TSA treatment up-regulated genes involved in cell organization and biogenesis. In addition, statistically significant up-regulation of genes involved in apoptosis, cell cycle, cell adhesion, proliferation, and cell migration was observed (Table 2).

Aza-dC and TSA Affected Cancer-Related Genes

Our approach identified several Aza-dC and/or TSA up-regulated genes that are involved in important cancer-related pathways, including cell cycle, proliferation, apoptosis, and cell adhesion (Table 3). Aza-dC up-regulated genes previously reported to be methylated in MM include *CDH1* (4, 5), *DAPK* (5), *TIMP3* (5), and *SOCS1* (5). More important, cancer-related genes, hitherto unknown to be subject to epigenetic alterations in MM, have been identified (i.e., *JUP, BIK, CD9, BCL7c, AKAP12, TFPI2, CCNA1, SPARC*, and *BNIP3*). A summary of these genes is shown in Table 3. Additionally, we found several cancer-related genes induced after combined treatment of MM cell lines with Aza-dC/TSA (i.e., *CDKN1A/p21, WIG1, BIK, CGREF1, JUP,* and *IGFBP3*). Interestingly, treatment of MM cell lines with TSA alone also resulted in up-regulation of important cancer-related genes including *ING1*^{p33}, *TIMP3, CDKN2D, PTENP1, CDKN1C/p57*^{KIP2}, *DAPK*, and *WIG1*, suggesting that aberrant histone deacetylation is an important mechanism for inactivation of cancer-related genes in MM.

Methylation Analysis of 10 Cancer-Related Genes in MM Cell Lines by MSP

Based on our microarray data, we selected 10 genes whose expression was up-regulated after drug treatment of MM cell lines and analyzed their methylation status in six MM cell lines. The genes analyzed were *CPEB1, CD9, GJA1, BCL7c, GADD45G, AKAP12, TFPI2, CCNA1, SPARC*, and *BNIP3*. All of these genes were identified as having a CpG island in their 5′ regions. We found methylation of all of these genes in the six MM cell lines at varying frequencies (Fig. 1*C*). The most frequently methylated gene was *BCL7c* (methylated in 100%) followed by *AKAP12, SPARC*, and *TFPI2* (83%, respectively); *CPEB1* (67%); *GJA1, CCNA1*, and *BNIP3* (50%, respectively); *CD9* (33%); and *GADD45G* (17%). Aberrant methylation of up to 9 of the 10 genes per cell line was observed.

Methylation Analysis of 10 Cancer-Related Genes in Samples from Patients with MGUS and MM

To investigate if methylation of the 10 genes was not only a phenomenon in MM cell lines, we performed MSP analysis of the 10 genes also in samples from 24 MGUS and 111 MM patients. As shown in Fig. 1*E*, the frequency of methylation of the 10 genes was lower in MGUS samples (17% for *CPEB1*, 16% for *CD9*, 13% for *GJA1*, 8% for *BCL7c*, 5% for *AKAP12*, and 4% for *BNIP3*; no methylation was found for *GADD45G, TFPI2, CCNA1*, and *SPARC*) compared with MM samples (50% for *CPEB1*, 28% for *CD9*, 23% for *GJA1*, 21% for *BCL7c*, 19% for *GADD45G*, 13% for *AKAP12*, 10% for *TFPI2*, 8% for *CCNA1*, 8% for *SPARC*, and 5% for *BNIP3*). This

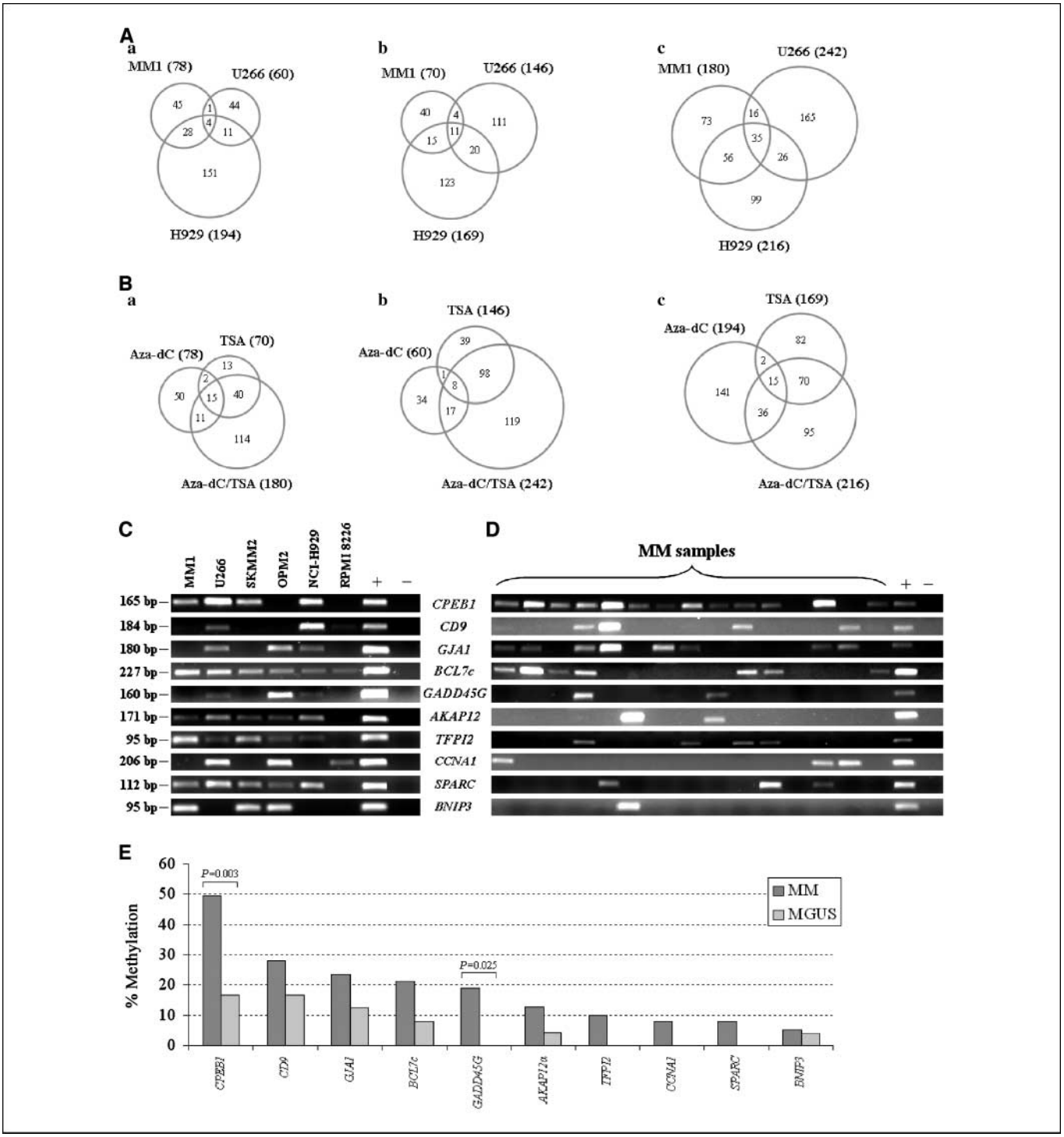

Figure 1. *A,* Venn diagrams that show the overlap of up-regulated genes in the MM cell lines U266, MM1, and NCI-H929 after (*a*) Aza-dC, (*b*) TSA, and (*c*) Aza-dC/TSA treatment. The region of overlap between all circles indicates the number of genes up-regulated in all three MM cell lines. Regions of overlap between two cell lines indicate up-regulated genes common between two of the three MM cell lines. Regions that do not overlap between circles indicate genes up-regulated in particular MM cell lines. *B,* Venn diagrams showing overlap of up-regulated genes after Aza-dC, TSA, and Aza-dC/TSA treatment in the cell lines (*a*) MM1, (*b*) U266, and (*c*) NCI-H929. *C,* methylation analysis of the genes *CPEB1, CD9, GJA1, BCL7c, GADD45G, AKAP12, TFPI2, CCNA1, SPARC*, and *BNIP3* in six MM cell lines. *D,* representative results of MSP assay of the 10 genes in MM samples. *E,* comparison of methylation frequencies of the 10 genes in 24 samples from patients with MGUS and in 111 samples from patients with MM.

difference was statistically significant in the cases of *CPEB1* (P = 0.003, χ^2 test) and *GADD45G* (P = 0.025, Fisher's exact test). Examples of MSP results are shown in Fig. 1*D*. In addition, bone marrow specimens from 10 control persons were analyzed as negative controls for methylation of the 10 genes. Methylation of at least one gene was detected in 73% of MM samples including methylation of one gene in 31% of MM samples, methylation of two genes in 16% of MM samples, methylation of three genes in 6% of

Table 1. Selected proto-oncogenes down-regulated by Aza-dC, TSA, or Aza-dC/TSA in MM cell lines

Accession*	Gene name	Symbol	Cytoband	Fold induction†			Cell line
				Aza-dC	TSA	Aza-dC/TSA	
AF054183	*RAN, member RAS oncogene family*	RAN	12q24.3	−1.9	**−5.6**	−3.2	H929
BF508646	*V-maf musculoaponeurotic fibrosarcoma oncogene homologue (avian)*	MAF	16q22-q23	1.2	−5.27 (−19.0)	**−8.4 (−2.8)**	MM1
				−6.6	**−34.3**	**−32.9**	U266
				−1.6	**−36.4 (−16.0)**	**−15.0**	H929
AL021977	*V-maf musculoaponeurotic fibrosarcoma oncogene homologue F (avian)*	MAFF	22q13.1	**−8.1**	**−13.0**	**−20.3**	MM1
NM_002359	*V-maf musculoaponeurotic fibrosarcoma oncogene homologue G (avian)*	MAFG	17q25.3	−2.1	−1.6	**−4.5**	MM1
BC000076	*Cyclin D1*	CCND1	11q13	−2.3	**−17.2**	**−11.0**	U266
AW026491	*Cyclin D2*	CCND2	12p13	1.6	**−6.4**	**−4.6**	MM1
				−3.3	**−5.5**	**−5.6**	U266
				1.2	−5.0	**−6.0**	H929
NM_001674	*Activating transcription factor 3*	ATF3	1q32.3	**−13.5**	**−10.0**	**−6.9**	MM1
NM_000142	*Fibroblast growth factor receptor 3*	FGFR3	4p16.3	1.6	−6.2	**−12.6**	MM1
				−1.7	**−24.6**	**−37.1**	H929
AF083389	*Wolf-Hirschhorn syndrome candidate 1*	WHSC1 (MMSET)	4p16.3	−1.8	−3.9	**−41.8 (−17.8)**	MM1
				−1.5	**−7.7**	−5.2 (−2.3)	U266
				−4.7	**−15.2 (−18.1)**	**−59.5**	H929
NM_002467	*V-myc myelocytomatosis viral oncogene homologue (avian)*	MYC	8q24.12	−1.3	−2.9	**−19.5 (−11.6)**	MM1
				−8.1 (−7.5)	**−24.4**	**−23.4**	H929
AI273812	*V-myc myelocytomatosis viral oncogene homologue 1, lung carcinoma derived*	MYCL1	1p34.2	−1.1	**−10.0**	**−11.0**	U266
BC002712	*V-myc myelocytomatosis viral-related oncogene, neuroblastoma derived*	MYCN	2p24.1	−1.6	**−6.8**	**−9.4**	H929
NM_005375	*V-myb myeloblastosis viral oncogene homologue (avian)*	MYB	6q22-q23	1.3	**−6.2**	**−7.3**	H929
NM_002466	*V-myb myeloblastosis viral oncogene homologue (avian)-like 2*	MYBL2	20q13.1	−4.2	**−6.6**	**−6.3**	U266
NM_000633	*B-cell CLL/lymphoma 2*	BCL2	18q21.33	−1.2	−2.4	**−5.2**	MM1
				−1.8	**−6.9**	**−7.7**	H929
U72398	*BCL2-like 1*	BCL2L1	20q11.21	−1.7	**−6.5**	**−13.6**	U266
NM_005178	*B-cell CLL/lymphoma 3*	BCL3	19q13.1-q13.2	2.4	−2.6	**−5.4**	H929
NM_006875	*Pim-2 oncogene*	PIM2	Xp11.23	−2.8	−3.1	**−5.8**	H929
S72848	*Interleukin 6 receptor*	IL6R	1q21	−1.3	**−13.5**	**−9.1**	U266

NOTE: Bold data indicate that genes passed filtering criteria described in Materials and Methods; bracketed numbers indicate fold changes obtained by real-time RT-PCR.
*Genbank accession number.
†Mean fold change.

MM samples, methylation of four genes in 8% of MM samples, methylation of five genes in 5% of MM samples, methylation of six genes in 2% of MM samples, methylation of seven genes in 3% of MM samples, and methylation of eight genes in 2% of MM samples.

Comparison of Clinicopathologic Characteristics from MM Patients with Results of Methylation Analysis

We next analyzed possible correlations between methylation data and clinicopathologic variables of the MM patients. Factors analyzed included age and gender of the patients, β_2-microglobulin, LDH, hemoglobin, serum creatinine and calcium levels, type of paraprotein, type of light chain, tumor stage, tumor grade, and chromosome 13q14 deletion at the time of diagnosis. Factors that have been found to statistically significantly correlate with methylation of a certain gene include age, type of paraprotein and light chain, and deletion of chromosome 13q14 (Table 4). In addition, 105 patients with MM were used for overall survival analysis. We observed that methylation of *BNIP3* and *SPARC* correlated with a poor overall survival of the patients ($P = 0.003$, $P = 0.017$, respectively; log-rank test; Fig. 2*A*). Moreover, *BNIP3* and/or *SPARC* methylation that has been observed in 12% of MM patients statistically significantly correlated with a poor overall survival of these patients ($P = 0.0004$). Additionally, immunostaining of bone marrow biopsies of MM patients whose bone marrow specimen were *SPARC* methylated ($n = 4$) and of patients whose bone marrow specimen were *SPARC* unmethylated ($n = 3$) was performed. Interestingly, in all samples that were found to be methylated myeloma cells did not express SPARC. In two of three samples that were found to be *SPARC* unmethylated, myeloma cells showed homogeneous SPARC expression. In the remaining sample, myeloma cells did not express SPARC (Fig. 2*B*). The

Table 2. Gene Ontology functions of genes whose expression is up-regulated >4-fold in at least one MM cell line after treatment with Aza-dC, TSA, or Aza-dC/TSA

Biological process	Reference genes (n = 8,649)	Aza-dC (n = 211)	P	TSA (n = 201)	P	Aza-dC/TSA (n = 333)	P
Immune response	674	63	<0.0001	15		27	
Antigen presentation	31	8	<0.0001	2		4	
Antigen processing	22	8	<0.0001	2		3	
Cellular defense response	88	9	0.0002	0		2	
Response to stimulus	1,624	86	<0.0001	30		57	
Defense response	746	66	<0.0001	15		28	
Response to biotic stimulus	773	70	<0.0001	16		29	
Response to external stimulus	427	25	<0.0001	8		17	
Response to stress	886	48	<0.0001	22		37	
Cell proliferation	464	24	0.0003	15		26	0.022
Cell recognition	12	3	0.0025	0		1	
Mitosis	124	9	0.0029	6		8	
Negative regulation of signal transduction	50	5	0.0067	1		2	
Apoptosis	407	18	0.0091	9		17	
Cell organization and biogenesis	1,124	22		40	0.0041	64	0.0002
Nucleosome assembly	51	1		8	<0.0001	11	<0.0001
Chromatin assembly	60	1		8	0.0001	11	<0.0001
Actin cytoskeleton organization	205	4		12	0.0031	10	
Actin filament bundle formation	7	1		3	0.0004	3	0.0016
Actin filament organization	20	1		4	0.0011	4	0.0055
Cell cycle	572	15		23	0.0075	23	
Mitotic cell cycle	166	9		11	0.0017	9	
Cell cycle arrest	53	1		5	0.0077	4	
Development	1,522	43		40		78	<0.0001
Cell differentiation	389	15		16		32	<0.0001
System development	378	10		16		28	0.0003
Cell development	81	5		5		10	0.0007
Cell-cell signaling	473	16		12		30	0.0026
Cell growth	116	4		4		11	0.0036
Cell motility	186	7		7		15	0.0037
Cell migration	77	2		2		8	0.0073

NOTE: Representation of functions defined by Gene Ontology "Biological Process" categories analyzed by GoMiner. Seven hundred eighty-eight Gene Ontology categories were found for Aza-dC up-regulated genes, 700 for genes up-regulated by TSA, and 911 for genes induced by Aza-dC/TSA. Categories for which statistically significant results ($P < 0.01$) were obtained are shown.

methylation status of any of the other 10 genes had no prognostic effect. As reported previously, deletion of chromosome 13q is associated with a poor prognosis of MM patients (1, 3). This finding was also observed in our cohort of patients ($P = 0.002$).

Discussion

Epigenetic silencing of cancer-related genes is a frequently occurring event in carcinogenesis (6). To date, only a few genes are known to be aberrantly methylated in MM. In an effort to identify additional epigenetically silenced genes in MM cells and to better understand the effects of Aza-dC and TSA on gene expression of MM cells, we examined expression profiles of three MM cell lines treated with Aza-dC and TSA, either alone or in combination using the microarray technique. Moreover, we compared these results with expression results from untreated cells. Additionally, using MSP, we investigated aberrant methylation patterns of 10 selected genes in MM cell lines and samples from patients with MGUS and MM.

Our microarray data revealed that expression of many genes was up-regulated after Aza-dC and/or TSA treatment of MM cells. The number of induced genes was very similar as reported for other cell types (28–30). Using the online tools MethPrimer (26) and CpG Island Searcher (27), we found that 73% of Aza-dC up-regulated genes contained a CpG island. This is in agreement to other reports that showed that inhibiting DNA methylation has both direct and indirect effects on gene expression (30, 31). Interestingly, we observed that 94% of genes whose expression was induced by TSA contained a CpG island, suggesting that TSA more specifically induces expression of CpG island containing genes than Aza-dC. The difference between Aza-dC and TSA up-regulated CpG island positive genes was statistically significant ($P = 0.0000000007$, χ^2 test).

We observed that a few genes show an overlap in the up-regulation between the three cell lines. In agreement to previous reports demonstrating that each tumor contains up to 600 methylated genes, we also observed a high number of epigenetically regulated genes in each cell line (7). We hypothesize that this

Table 3. Selected cancer-related genes up-regulated by Aza-dC, TSA, or Aza-dC/TSA in MM cell lines

Accession*	Gene name	Symbol	Cytoband	Fold induction †			Cell line
				Aza-dC	TSA	Aza-dC/TSA	
Induction of apoptosis (GO:0006917)							
NM_000041	*Apolipoprotein E*	APOE	19q13.2	**35.1**	1.5	**43.6**	U266
				58.5	3.4	**41.4**	H929
NM_004765	*B-cell CLL/lymphoma 7C*	BCL7C	16p11	1.5	4.1	2.6	H929
NM_003790	*Tumor necrosis factor receptor superfamily, member 25*	TNFRSF25	1p36.2	**12.1**	3.9	5.5	U266
NM_004052	*BCL2/adenovirus E1B 19 kDa interacting protein 3*	BNIP3	10q26.3	2.0	3.8	3.2	MM1
NM_000389	*Cyclin-dependent kinase inhibitor 1A (p21, Cip1)*	CDKN1A	6p21.2	**29.3**	**13.9**	**27.3**	H929
BF686824	*Death-associated protein kinase 3*	DAPK3 ‡	19p13.3	1.9	**6.3**	5.6	H929
NM_000362	*Tissue inhibitor of metalloproteinase 3*	TIMP3 ‡	22q12.3	1.7	**8.5**	**17.1**	U266
Regulation of cell growth (GO:0001558)							
AB005043	*Suppressor of cytokine signaling 1*	SOCS1 ‡	16p13.13	**10.6**	1.1	2.4	MM1
				28.4	2.1	2.2	H929
BG035761	*Suppressor of cytokine signaling 3*	SOCS3	17q25.3	**8.1**	1.3	5.8	MM1
				5.5	8.7	2.8	H929
Negative regulation of cell proliferation (GO:0008285)							
AF184174	*Somatostatin receptor 2*	SSTR2	17q24	**5.9**	3.8	3.6	H929
NM_006569	*Cell growth regulator with EF-hand domain 1*	CGREF1	2p23.3	−3.0	**7.5**	**14.0**	U266
AL535380	*B-cell translocation gene 1*	BTG1	12q22	2.6	2.3	**5.0**	H929
Cell adhesion (GO:0007155)							
AI424797	*Agrin*	AGRN	1p36.33	2.8	4.3	**5.2**	MM1
				2.0	**11.2**	**12.0**	U266
				9.0	**19.6**	**13.4**	H929
NM_004360	*Cadherin 1, type 1, E-cadherin (epithelial)*	CDH1 ‡	16q22.1	−1.1	−2.0	**38.7**	U266
				87.2	1.3	1.2	H929
NM_001769	*CD9 antigen (p24)*	CD9	12p13.3	−2.0	**9.3 (122.4)**	**26.1 (156.0)**	U266
NM_021991	*Junction plakoglobin*	JUP	17q21	3.3	**7.9**	**13.1**	U266
Cell cycle (GO:0007049)							
NM_003914	*Cyclin A1*	CCNA1	13q12.3-q13	1.1	2.0	**23.4**	U266
				2.4	1.7	**6.2**	H929
NM_000076	*Cyclin-dependent kinase inhibitor 1C (p57, Kip2)*	CDKN1C	11p15.5	7.5	**27.2**	**82.5**	U266
				6.3	**8.2**	6.1	H929
NM_005537	*Inhibitor of growth family, member 1*	ING1	13q34	2.1	**5.5**	**10.9**	U266
NM_000059	*Breast cancer 2, early onset*	BRCA2	13q12.3	4.1	7.1	**11.2**	MM1
Signal transduction (GO:0007165)							
NM_004528	*Microsomal glutathione S-transferase 3*	MGST3	1q23	1.1	**6.9**	**9.1**	MM1
				1.5	**14.2**	**16.6**	H929
AB003476	*A kinase (PRKA) anchor protein (gravin) 12*	AKAP12	6q24-q25	2.8	**7.7 (5.0)**	**11.2 (8.9)**	MM1
				3.9	**11.2**	**44.8**	U266
				6.4	**9.1 (9.6)**	**12.6**	H929
AF143684	*Myosin IXB*	MYO9B	19p13.1	**9.9**	3.0	**11.2**	MM1
				7.2	5.3	**10.3**	U266
				8.2	7.4	9.0	H929
NM_003118	*Secreted protein, acidic, cysteine-rich (osteonectin)*	SPARC	5q31.3-q32	**6.1**	−1.5	2.9	U266
Regulation of translation (GO:0006417)							
NM_030594	*Cytoplasmic polyadenylation element binding protein 1*	CPEB1	15q25.2	3.5 (6.8)	1.7	2.2	U266
				7.6	1.0	1.4	H929
Cell communication (GO:0007154)							
NM_000165	*Gap junction protein, α1*	GJA1	6q21-q23.2	3.9 (3.76)	2.0	1.0	MM1
NM_014737	*Ras association (RalGDS/AF-6) domain family 2*	RASSF2	20pter-p12.1	**10.6**	−1.0	1.7	H929
DNA repair (GO:0008152)							
NM_006705	*Growth arrest and DNA-damage inducible γ*	GADD45G	9q22.1-q22.2	1.1	2.3	10.6 (160.0)	U266
Blood coagulation (GO:0007596)							
L27624	*Tissue factor pathway inhibitor 2*	TFPI2	7q22	1.5	1.6	**16.0**	U266

NOTE: Bold data indicate that genes passed filtering criteria described in Materials and Methods; bracketed numbers indicate fold changes obtained by real-time RT-PCR.

*Genbank accession number.

† Mean fold change.

‡ Genes that have already been analyzed for methylation in MM.

pattern represents the individual character of each cell line; however, we believe that the commonly altered genes may be "key players" in the pathogenesis of MM.

Increased expression of proto-oncogenes has been described for many different malignancies. One of the novel findings of our present study is that several proto-oncogenes that are involved in the pathogenesis of MM were down-regulated after treatment of MM cells with Aza-dC and TSA (Table 1). Down-regulation may be explained either by a direct inhibitory effect of Aza-dC and TSA or by an indirect down-regulation by Aza-dC and TSA affected genes. Proto-oncogenes that are frequently overexpressed in MM include *CCND1, MAF, FGFR*, and *MMSET* (32–34). In our microarray assay, *MAF* is substantially down-regulated by TSA and Aza-dC/TSA in all three MM cell lines analyzed. In addition, expression of target genes (*CCND2, CCR1*, and *ITGB7*) of the MAF transcription factor was down-regulated in MM cell lines after TSA and Aza-dC/TSA treatment, suggesting that TSA primarily inhibits *MAF* and secondarily down-regulates *CCND2, CCR1*, and *ITGB7*. Similar results were found for FGFR3, which promotes MM cell proliferation and antiapoptosis and MMSET. We found expression of both genes strongly down-regulated in response to TSA and Aza-dC/TSA in all three MM cell lines analyzed. In addition, we observed that components of the IL-6 signaling pathway are affected by Aza-dC and/or TSA providing an additional mechanism to inhibit proliferation and induce apoptosis of MM cells. The IL-6/IL-6R complex associates with gp130 (IL-6ST), which was found to be up-regulated in myeloma cells (35), resulting in promotion of cell proliferation and survival. We found that TSA and Aza-dC/TSA treatment of U266 cells, which are known to produce IL-6 (36), resulted in marked down-regulation of IL-6R. Moreover, although down-regulation did not pass our filtering criteria, gp130 down-regulation was observed. Interestingly, Mitsiades et al. (37) reported that treatment of MM cells using the HDAC inhibitor SAHA resulted in down-regulation of both IL-6R and gp130, suggesting that inhibition of IL-6 pathway using HDAC inhibitors might be a new strategy in the future treatment of MM patients.

By comparing expression profiles of Aza-dC– and/or TSA-treated cells and untreated cells, we identified a large number of up-regulated cancer-related genes that are involved in several cancer-associated pathways, including cell cycle, cell growth, and apoptosis (Tables 2 and 3). Thus far, epigenetic silencing was not known for most of these genes. We selected 10 genes (*CPEB1, CD9, GJA1, BCL7c, GADD45G, AKAP12, TFPI2, CCNA1, SPARC*, and *BNIP3*) whose expression was up-regulated after drug treatment and determined their methylation status in 6 MM cell lines, 24 samples from patients with MGUS, and 111 samples from patients with MM by MSP. Our methylation results support previous studies that showed that methylation of multiple cancer-related genes is a frequently occurring event in the pathogenesis of MM. The most frequently methylated gene in MM samples was *CBEP1*, a gene whose epigenetic silencing in malignant diseases has not been reported thus far. CPEB1 belongs to the cytoplasmic polyadenylation element binding protein family. Besides its function as translational activator, it has been reported that CPEB1 has also translational repressor properties by inducing stress granules (38). By comparing our methylation data with clinicopathologic characteristics from the MM patients, we observed that *CPEB1* methylation was already present in MGUS samples but at a statistically significantly higher frequency in MM samples (P = 0.003), suggesting that *CPEB1* methylation may be an early

Table 4. Clinical characteristics of MM patients and correlation with methylation results of nine methylated genes

Characteristic	Patients (n)	% Methylation												
		CPEB1	P	*CD9*	P	*GJA1*	P	*BCL7c*	*GADD45G*	*AKAP12*	P	*TFPI2*	P	*SPARC*	*BNIP3*	*SPARC/BNIP3*
Sex	111															
Female	47	40.4		27.7		29.8		21.3	17.0	8.5		10.6		8.5	6.4	10.6
Male	64	56.3		28.1		18.8		20.3	20.3	15.6		9.4		7.8	4.7	12.5
Age (y)	111															
<60	40	35.0		27.5		17.5		17.5	10.0	12.5		12.5		2.5	5.0	7.5
>60	71	57.7	0.021	28.2		26.8		22.5	23.9	12.7		8.5		11.3	5.6	14.1
Ig subtype	111															
IgA	31	58.1		19.4		38.7		16.1	29.0	12.9		12.9		6.5	6.5	9.7
Non-IgA	80	46.3		31.3		17.5	0.018	22.5	15.0	12.5		8.8		8.8	5.0	12.5
Light chain	111															
λ	38	44.7		34.2		23.7		28.9	18.4	23.7		18.4		7.9	7.9	13.2
κ	73	52.1		24.7		23.3		16.4	19.2	6.8	0.016	5.5	0.04	8.2	4.1	11.0
Disease stage	109															
I, II	19	68.4		15.8		15.8		15.8	10.5	10.5		15.8		5.3	0	5.3
III	90	45.6		31.1		25.6		22.2	21.1	13.3		8.9		8.9	6.7	13.3
Del. of 13q14*	75															
No	42	38.1		21.4		16.7		14.3	19.0	9.5		7.1		4.8	2.4	4.8
Yes	33	63.6	0.028	45.5	0.027	36.4		30.3	22.2	12.1		9.1		12.1	9.1	18.2

NOTE: P values are two sided and were obtained either by χ^2 or by Fisher's exact test.
Abbreviation: Ig, immunoglobulin.
*Results from fluorescence *in situ* hybridization analysis have been reported previously (3).

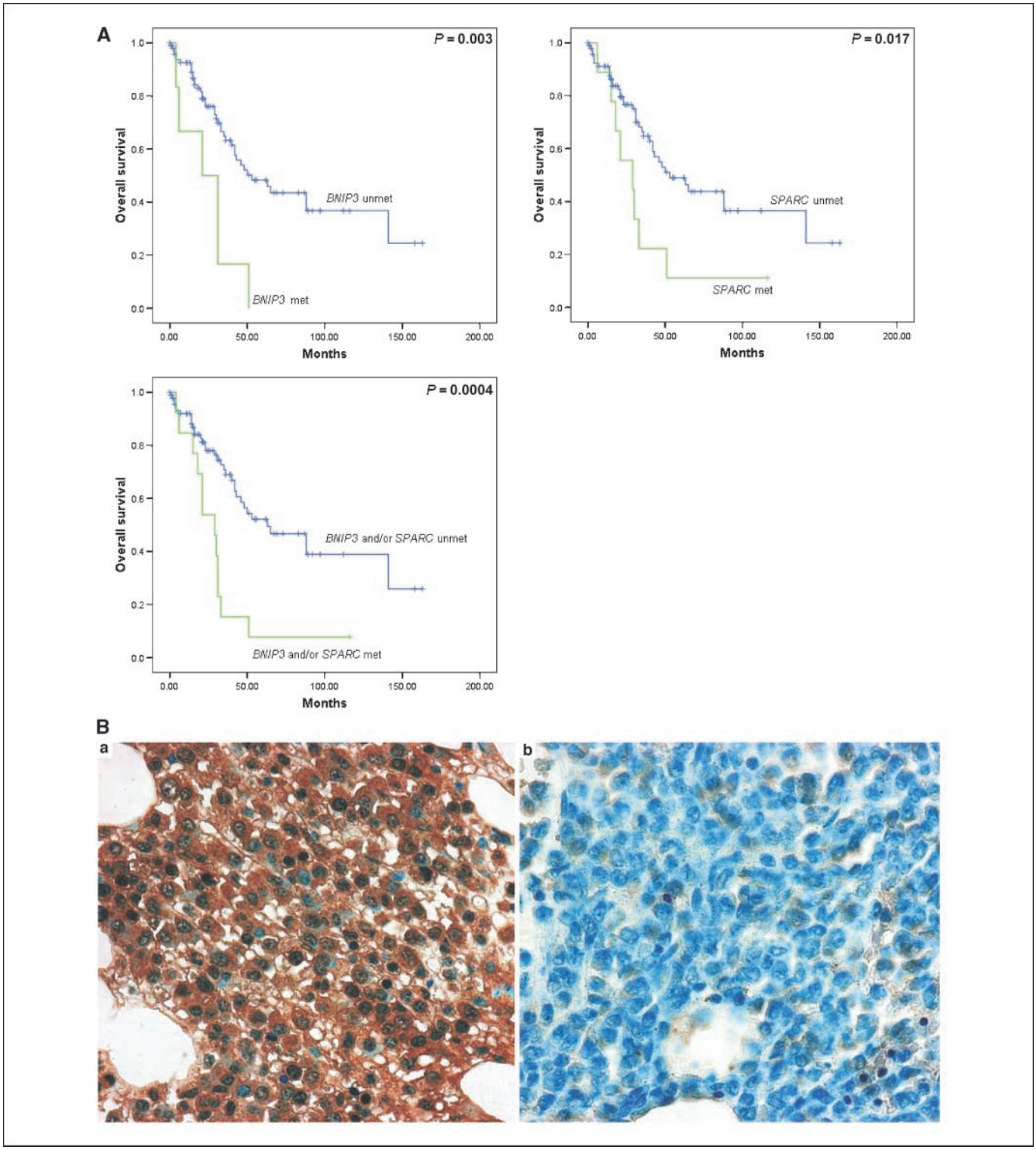

Figure 2. *A,* overall survival and methylation of *BNIP3, SPARC*, and *BNIP3* and/or *SPARC* in 105 patients with MM. Kaplan-Maier log rank testing was used for statistical analysis. *B,* examples of SPARC immunostaining of myeloma cells in bone marrow biopsies. *a*, positive SPARC staining; *b*, no SPARC staining.

event in the development of monoclonal gammopathies. In addition, although the difference in overall survival between patients with and without *CPEB1* methylation was statistically not significant, we observed a trend toward a poor prognosis for MM patients with *CPEB1* methylation. Additional studies are necessary to elucidate the role of *CPEB1* in the pathogenesis of MM.

Interestingly, we observed that methylation of two genes, *SPARC* and *BNIP3*, was associated with a poor overall survival of MM patients (Fig. 2*A*), suggesting that methylation of these genes may

be a potential biomarker for prognosis in patients with MM. SPARC is a matrix-associated protein that modulates cellular interaction with the extracellular matrix and inhibits cellular proliferation (39). Its role in tumorigenesis is complex, and expression is often being down-regulated in tumor cells accompanied by up-regulation in juxtatumoral stromal cells. It has been suggested that acting of SPARC either as tumor suppressor or oncogene may depend on the tumor stage (40). Recent reports showed that *SPARC* methylation is associated with loss of SPARC expression in lung, pancreatic, and colon cancer (40–42). We investigated SPARC protein expression in bone marrow biopsies of a small group of MM patients by immunostaining. Whereas all *SPARC* methylated samples did not express SPARC in the myeloma cells, all except one unmethylated sample showed homogeneous SPARC protein expression in the myeloma cells. Although the number of samples investigated was very small, our results show that *SPARC* methylation is associated with loss of SPARC protein expression, suggesting the hypothesis that SPARC acts as a tumor suppressor in MM. BNIP3 is a proapoptotic member of the Bcl-2 protein family involved in hypoxia-induced cell death. Loss of *BNIP3* expression due to DNA methylation has been reported recently in colorectal and pancreatic cancer (43, 44). In addition, Murai et al. (45) found *BNIP3* methylation in 21% (3 of 14) of MM samples. However, the frequency of *BNIP3* methylation in our study was much lower (5%). This discrepancy may be explained by the different sample sizes analyzed. Our results suggest that MM cells of some patients escape hypoxia-induced apoptosis through down-regulating *BNIP3* by methylation and that these cells are more aggressive than *BNIP3* unmethylated MM cells.

In conclusion, we show that expression of a large number of genes that are involved in important cancer-related pathways is affected by Aza-dC and TSA. Methylation of certain genes whose epigenetic silencing in MM has not been reported thus far might be important in the development and progression of MM. In addition, our results suggest that inhibition of DNA methyltransferases and HDACs might be new therapeutic strategies in the future treatment of MM patients.

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References

1. Kaufmann H, Kromer E, Nosslinger T, et al. Both chromosome 13 abnormalities by metaphase cytogenetics and deletion of 13q by interphase FISH only are prognostically relevant in multiple myeloma. Eur J Haematol 2003;71:179–83.
2. Tricot G, Barlogie B, Jagannath S, et al. Poor prognosis in multiple myeloma is associated only with partial or complete deletions of chromosome 13 or abnormalities involving 11q and not with other karyotype abnormalities. Blood 1995;86:4250–6.
3. Zojer N, Konigsberg R, Ackermann J, et al. Deletion of 13q14 remains an independent adverse prognostic variable in multiple myeloma despite its frequent detection by interphase fluorescence *in situ* hybridization. Blood 2000;95:1925–30.
4. Galm O, Wilop S, Reichelt J, et al. DNA methylation changes in multiple myeloma. Leukemia 2004;18:1687–92.
5. Seidl S, Ackermann J, Kaufmann H, et al. DNA-methylation analysis identifies the E-cadherin gene as a potential marker of disease progression in patients with monoclonal gammopathies. Cancer 2004;100:2598–606.
6. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet 2002;3:415–28.
7. Costello JF, Fruhwald MC, Smiraglia DJ, et al. Aberrant CpG-island methylation has non-random and tumour-type-specific patterns. Nat Genet 2000;24:132–8.
8. Ng MH, To KW, Lo KW, et al. Frequent death-associated protein kinase promoter hypermethylation in multiple myeloma. Clin Cancer Res 2001;7:1724–9.
9. Ng MH, Lau KM, Wong WS, et al. Alterations of RAS signalling in Chinese multiple myeloma patients: absent BRAF and rare RAS mutations, but frequent inactivation of RASSF1A by transcriptional silencing or expression of a non-functional variant transcript. Br J Haematol 2003;123:637–45.
10. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet 2003;33:245–54.
11. Tani M, Ito J, Nishioka M, et al. Correlation between histone acetylation and expression of the MYO18B gene in human lung cancer cells. Genes Chromosomes Cancer 2004;40:146–51.
12. Mitani Y, Oue N, Hamai Y, et al. Histone H3 acetylation is associated with reduced p21(WAF1/CIP1) expression by gastric carcinoma. J Pathol 2005;205:65–73.
13. Danam RP, Howell SR, Brent TP, Harris LC. Epigenetic regulation of O6-methylguanine-DNA methyltransferase gene expression by histone acetylation and methyl-CpG binding proteins. Mol Cancer Ther 2005;4:61–9.
14. Baylin SB, Herman JG. DNA hypermethylation in tumorigenesis: epigenetics joins genetics. Trends Genet 2000;16:168–74.
15. Xiong Y, Dowdy SC, Podratz KC, et al. Histone deacetylase inhibitors decrease DNA methyltransferase-3B messenger RNA stability and down-regulate *de novo* DNA methyltransferase activity in human endometrial cells. Cancer Res 2005;65:2684–9.
16. Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB. Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. Nat Genet 1999;21:103–7.
17. Jones PL, Veenstra GJ, Wade PA, et al. Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat Genet 1998;19:187–91.
18. Bovenzi V, Momparler RL. Antineoplastic action of 5-aza-2′-deoxycytidine and histone deacetylase inhibitor and their effect on the expression of retinoic acid receptor β and estrogen receptor α genes in breast carcinoma cells. Cancer Chemother Pharmacol 2001;48:71–6.
19. Choi MC, Jong HS, Kim TY, et al. AKAP12/Gravin is inactivated by epigenetic mechanism in human gastric carcinoma and shows growth suppressor activity. Oncogene 2004;23:7095–103.
20. Missiaglia E, Donadelli M, Palmieri M, Crnogorac-Jurcevic T, Scarpa A, Lemoine NR. Growth delay of human pancreatic cancer cells by methylase inhibitor 5-aza-2′-deoxycytidine treatment is associated with activation of the interferon signalling pathway. Oncogene 2005;24:199–211.
21. Donadelli M, Costanzo C, Faggioli L, et al. Trichostatin A, an inhibitor of histone deacetylases, strongly suppresses growth of pancreatic adenocarcinoma cells. Mol Carcinog 2003;38:59–69.
22. Shaker S, Bernstein M, Momparler LF, Momparler RL. Preclinical evaluation of antineoplastic activity of inhibitors of DNA methylation (5-aza-2′-deoxycytidine) and histone deacetylation (trichostatin A, depsipeptide) in combination against myeloid leukemic cells. Leuk Res 2003;27:437–44.
23. Zeeberg BR, Feng W, Wang G, et al. GoMiner: a resource for biological interpretation of genomic and proteomic data. Genome Biol 2003;4:R28.
24. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning—a laboratory manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press; 1989.
25. Herman JG, Graff JR, Myöhänen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A 1996;93:9821–6.
26. Li LC, Dahiya R. MethPrimer: designing primers for methylation PCRs. Bioinformatics 2002;18:1427–31.
27. Takai D, Jones PA. The CpG island searcher: a new WWW resource. In Silico Biol 2003;3:235–40.
28. Liang G, Gonzales FA, Jones PA, Orntoft TF, Thykjaer T. Analysis of gene induction in human fibroblasts and bladder cancer cells exposed to the methylation inhibitor 5-aza-2′-deoxycytidine. Cancer Res 2002;62:961–6.
29. Ibanez de Caceres I, Dulaimi E, Hoffman AM, Al-Saleem T, Uzzo RG, Cairns P. Identification of novel target genes by an epigenetic reactivation screen of renal cancer. Cancer Res 2006;66:5021–8.
30. Suzuki H, Gabrielson E, Chen W, et al. A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer. Nat Genet 2002;31:141–9.
31. Karpf AR, Peterson PW, Rawlins JT, et al. Inhibition of DNA methyltransferase stimulates the expression of signal transducer and activator of transcription 1, 2, and 3 genes in colon tumor cells. Proc Natl Acad Sci U S A 1999;96:14007–12.
32. Chesi M, Nardini E, Brents LA, et al. Frequent translocation t(4;14)(p16.3;q32.3) in multiple myeloma is associated with increased expression and activating mutations of fibroblast growth factor receptor 3. Nat Genet 1997;16:260–4.
33. Chesi M, Brents LA, Ely SA, et al. Activated fibroblast growth factor receptor 3 is an oncogene that contributes to tumor progression in multiple myeloma. Blood 2001;97:729–36.
34. Kienast J, Berdel WE. c-maf in multiple myeloma: an oncogene enhancing tumor-stroma interactions. Cancer Cell 2004;5:109–10.
35. Barille S, Thabard W, Robillard N, et al. CD130 rather

than CD126 expression is associated with disease activity in multiple myeloma. Br J Haematol 1999;106:532–5.
36. Schwab G, Siegall CB, Aarden LA, Neckers LM, Nordan RP. Characterization of an interleukin-6-mediated autocrine growth loop in the human multiple myeloma cell line, U266. Blood 1991;77:587–93.
37. Mitsiades CS, Mitsiades NS, McMullan CJ, et al. Transcriptional signature of histone deacetylase inhibition in multiple myeloma: biological and clinical implications. Proc Natl Acad Sci U S A 2004;101:540–5.
38. Wilczynska A, Aigueperse C, Kress M, Dautry F, Weil D. The translational regulator CPEB1 provides a link between dcp1 bodies and stress granules. J Cell Sci 2005;118:981–92.
39. Brekken RA, Sage EH. SPARC, a matricellular protein: at the crossroads of cell-matrix communication. Matrix Biol 2001;19:816–27.
40. Suzuki M, Hao C, Takahashi T, et al. Aberrant methylation of SPARC in human lung cancers. Br J Cancer 2005;92:942–8.
41. Yang E, Kang HJ, Koh KH, Rhee H, Kim NK, Kim H. Frequent inactivation of SPARC by promoter hypermethylation in colon cancers. Int J Cancer 2007;121:567–75.
42. Sato N, Fukushima N, Maehara N, et al. SPARC/osteonectin is a frequent target for aberrant methylation in pancreatic adenocarcinoma and a mediator of tumor-stromal interactions. Oncogene 2003;22:5021–30.
43. Murai M, Toyota M, Suzuki H, et al. Aberrant methylation and silencing of the BNIP3 gene in colorectal and gastric cancer. Clin Cancer Res 2005;11:1021–7.
44. Okami J, Simeone DM, Logsdon CD. Silencing of the hypoxia-inducible cell death protein BNIP3 in pancreatic cancer. Cancer Res 2004;64:5338–46.
45. Murai M, Toyota M, Satoh A, et al. Aberrant DNA methylation associated with silencing BNIP3 gene expression in haematopoietic tumours. Br J Cancer 2005;92:1165–72.

Genome-Wide Transcriptional Response to 5-Aza-2′-Deoxycytidine and Trichostatin A in Multiple Myeloma Cells

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