Genomic Screening for Genes Silenced by DNA Methylation Revealed an Association between RASD1 Inactivation and Dexamethasone Resistance in Multiple Myeloma

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Abstract Purpose: Epigenetic changes such as DNA methylation play a key role in the development and progression of multiple myeloma. Our aim in the present study was to use genomic screening to identify genes targeted for epigenetic inactivation in multiple myeloma and assess their role in the development of resistance to dexamethasone.

Experimental Design: Gene expression was examined using microarray screening, reverse transcription-PCR, and real-time quantitative PCR. DNA methylation was examined using bisulfite PCR, bisulfite sequencing, and bisulfite pyrosequencing in 14 multiple myeloma cell lines, 87 multiple myeloma specimens, and 12 control bone marrow samples. WST-8 assays were used to assess cell viability after treatment with 5-aza-2'-deoxycytidine and/or dexamethasone.

Results: Microarray analysis was done to screen for genes up-regulated by 5-aza-2'-deoxycytidine. In RPMI8226 cells, 128 genes were up-regulated, whereas 83 genes were up-regulated in KMS12PE cells. Methylation of 22 genes with CpG islands in their 5' regions, including RASD1, was confirmed. Methylation of RASD1 was associated with its inactivation, which correlated with resistance to dexamethasone. Treating multiple myeloma cells with 5-aza-2'-deoxycytidine restored sensitivity to dexamethasone. Methylation of RASD1 was also detected in a subset of primary multiple myeloma specimens, and the levels of methylation were increased after repeated antitumor treatments. Gene signature analysis revealed various genes to be synergistically induced by treatment with a combination of 5-aza-2'-deoxycytidine plus dexamethasone.

Conclusion: Our findings indicate that epigenetic inactivation of genes, including RASD1, plays a key role in the development of dexamethasone resistance in multiple myeloma. Moreover, they show the utility of demethylation therapy in cases of advanced multiple myeloma.

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Multiple myeloma remains a lethal malignancy despite the development of treatments such as high-dose chemotherapy combined with stem cell transplantation (1). Until recently, the standard initial treatment for multiple myeloma was conventional drug therapy, with dexamethasone serving as a first-line drug because of its efficacy for killing multiple myeloma cells (2, 3). In fact, dexamethasone continues to be used even in very new regimens with bortezomib or lenalidomide (4–7). The main difficulties when treating multiple myeloma are drug resistance and opportunistic infections related to the long-term use of high doses of dexamethasone. To overcome these problems, new agents are being sought that enhance the cytotoxicity of dexamethasone toward multiple myeloma cells (8–10).

Multiple myeloma arises through the accumulation of multiple genetic changes that include immunoglobulin gene rearrangements involving in cyclin D (11, 12). In addition, recent studies have shown that epigenetic changes such as DNA methylation play a role by silencing various cancer-related genes in multiple myeloma (13–17), and DNA methyltransferase inhibitors such as decitabine (5-aza-2'-deoxycytidine) are
Translational Relevance

Multiple myeloma remains a lethal malignancy despite the development of treatments such as high-dose chemotherapy combined with stem cell transplantation. The main difficulties when treating multiple myeloma are drug resistance and opportunistic infections related to the long-term use of high doses of dexamethasone. However, molecular mechanisms involved in drug resistance to dexamethasone in multiple myeloma remain to be determined. Epigenetic changes such as DNA methylation play a role by silencing various cancer-related genes in multiple myeloma. In this study, we found that epigenetic inactivation of RASD1 is closely correlated with resistance to dexamethasone in multiple myeloma cells. Restoration of RASD1 expression in multiple myeloma cells using a DNA methyltransferase inhibitor restored sensitivity to dexamethasone. Although further study is needed to determine how important RASD1 hypermethylation is in the clinical course of multiple myeloma, our results indicate the potential utility of demethylation therapy in cases of advanced multiple myeloma.

Materials and Methods

Cell lines and specimens. Fourteen multiple myeloma cell lines (ARH77, Ho3328, Hs-sultan, KHM1B, KMs5, KMS12BM, KMS12PE, MM.15, MM.1R, NCI-H929, OPM1, RPMI8226, RPMI8226/Dox40, and U266) were cultured in the appropriate medium. DNA was extracted using the phenol-chloroform method, and total RNA was extracted using Trizol (Invitrogen) or a RNeasy kit (Qiagen) according to the manufacturer's instructions. To assess changes in RASD1 expression or cytotoxicity, cell lines were incubated for 24 or 48 h with 0.1, 1, or 10 μmol/L dexamethasone. In addition, to assess restoration of RASD1 expression or cytotoxicity, cell lines were incubated for 72 h with 0.1, 1, or 2 μmol/L 5-aza-2’-deoxycytidine (Sigma), a methyltransferase inhibitor. The cells were then harvested, and total RNA was extracted for further analysis. The primary bone marrow samples from 116 multiple myeloma patients and 12 patients without tumors used in this study are described in detail in the Supporting Information.

cDNA microarray analysis. RNA samples extracted from cells were quantified using NanoDrop ND-100, after which the quality was assessed using an Agilent Technologies 2100 Bioanalyzer. The RNA concentration in the samples was >100 ng/μL, with a RNA integrity score of 8 to 10 (10 was the highest possible score). Sample amplification and labeling were done using a Low RNA Input Fluorescent Linear Amplification kit (Agilent Technologies) according to the manufacturer's instructions. Samples (1.65 μg) labeled with Cy3 were hybridized and processed on a 4x44K Whole Human Genome Oligo Microarray. Scanning was done with an Agilent G2565BA microarray scanner using the settings recommended by Agilent Technologies. All raw data were normalized and analyzed using GeneSpring GX 10.0 (Agilent Technologies). The Gene Expression Omnibus accession numbers of the microarray data are GSE356990, GSE356991, GSE356992, and GSE356993, and the accession number of the series entry is GSE14261.

Reverse transcription-PCR. First-strand cDNA was prepared by reverse transcription of 5 μg samples of total RNA using SuperScript III reverse transcriptase (Invitrogen). Real-time quantitative PCR was carried out using Taqman Gene Expression Assays (Applied Biosystems) with a 7900HT Fast Real-time PCR System (Applied Biosystems). SDS2.1 software (Applied Biosystems) was used to do comparative ΔΔCt analyses. Glyceraldehyde-3-phosphate dehydrogenase served as an endogenous control.

Methylation analysis. Genomic DNA (2 μg) was modified with sodium bisulfite using an EpiTect bisulfite kit (Qiagen). Bisulfite sequencing analysis was then done as described previously (28, 29). Amplified bisulfite sequencing PCR products were cloned into pCR2.1-TOPO vector (Invitrogen), and 10 to 12 clones from each sample were sequenced using an ABI3130x automated sequencer (Applied Biosystems). Primer sequences used in this study, methods for combined bisulfite restriction analysis, and bisulfite pyrosequencing were described in Supplementary Information and Supplementary Table S1.

Cytotoxicity and cell viability assay. Cell lines cultured to log phase were harvested and plated in a 96-well microplate to a density of 5,000 to 20,000 per well. After incubation with drugs (as described above), cell viability assays were carried out using a Cell Counting Kit-8 (Dojindo). Briefly, WST-8 reagent solution was added to each well, after which the microplate was incubated for 2 h at 37°C. The absorbance at 450 nm was then measured using a microplate reader (Multiskan; Labsystems).

Statistics. Methods for statistical analysis are shown in Supplementary Information.

Results

Genomic screening of genes silenced by DNA methylation in multiple myeloma cell lines. To identify genes up-regulated by 5-aza-2’-deoxycytidine, we carried out cDNA microarray analyses with and without 5-aza-2’-deoxycytidine using two multiple myeloma cell lines (RPMI8226 and KMS12PE). After treatment with 5-aza-2’-deoxycytidine, 128 genes were up-regulated >10-fold in RPMI8226 cells and 83 were similarly up-regulated in KMS12-PE cells (Fig. 1A and B; Supplementary Tables S2 and S3). When we then used real-time PCR to examine the expression status of the
Fig. 1. Genomic screening of genes inactivated by DNA methylation in multiple myeloma cells. A, summary of genes up-regulated by 5-aza-2′-deoxycytidine in two multiple myeloma cell lines. RPMI8226 and KMS12PE cells were incubated with or without 1 μmol/L 5-aza-2′-deoxycytidine, after which cDNA microarray analysis was carried out. B, dot plot showing the expression levels of genes altered by 5-aza-2′-deoxycytidine. Red squares, genes showing at least a 10-fold increase in expression with 5-aza-2′-deoxycytidine. C, methylation profiles of 22 selected genes identified by microarray: 11 multiple myeloma cell lines, 29 primary samples (bone marrow), and 3 non-multiple myeloma samples were examined. RASD1 was selected from among genes methylated only in multiple myeloma samples (red square). D, representative results of combined bisulfite restriction analysis. Left, genes examined; top, cell lines used. U, unmethylated alleles; M, methylated alleles.
genes, we found that 32 were down-regulated in at least one of the multiple myeloma cell lines and then up-regulated by 5-aza-2'-deoxycytidine (Supplementary Figs. S1-S4). Of those, 22 had CpG islands in their 5' regions and were further analyzed for DNA methylation. We initially assessed the methylation status of the 22 selected genes in a panel of multiple myeloma cell lines, primary multiple myeloma specimens, and control tissues, which included myelodysplastic

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**Fig. 2.** Levels of RASD1 expression measured using real-time quantitative PCR were inversely correlated with methylation levels determined by pyrosequencing in multiple myeloma cell lines. A, regions analyzed by combined bisulfite restriction analysis, pyrosequencing, and bisulfite sequencing. Vertical bars, CpG sites. B, representative pyrogram for the indicated multiple myeloma cell lines. Percentages on peaks indicate the methylation levels in each CpG site calculated from the C/T ratio in samples after bisulfite treatment. C, examples of bisulfite sequencing in multiple myeloma in the indicated cell lines. Ten clones were sequenced in each cell line. White and black circles, unmethylated and methylated alleles, respectively. D, gene expression levels relative to normal bone marrow: top, cell lines/normal bone marrow; middle, fold increase in the presence of 1 μmol/L 5-aza-2'-deoxycytidine/mock; bottom, levels of RASD1 methylation (%) determined by pyrosequencing.
syndrome, lymphoma, and normal bone marrow specimens. Methylation of 11 genes was frequently detected even in control tissues (Fig. 1C and D). On the other hand, there were 9 genes whose methylation was only detected in a subset of multiple myeloma specimens. The remaining 2 genes showed no methylation, indicating that they were silenced by mechanisms other than DNA methylation.

**Epigenetic inactivation of RASD1 in multiple myeloma cell lines.** Among the 9 genes showing multiple myeloma-specific methylation, we selected RASD1 for further analysis because it was originally detected based on its induction by dexamethasone (26). We initially confirmed the methylation status of RASD1 using bisulfite sequencing and bisulfite pyrosequencing. The results of the bisulfite sequencing were consistent with the methylation levels determined by pyrosequencing (Fig. 2B and C), and there was a significant correlation between the results of pyrosequencing and those of the combined bisulfite restriction analysis ($r = 0.905, P = 0.001; rs = 0.854, P = 0.003$). Moreover, levels of RASD1 expression determined by real-time quantitative reverse transcription-PCR were inversely correlated with the methylation levels determined by pyrosequencing ($r = -0.766, P = 0.010; rs = -0.842, P = 0.002$; Fig. 2D). All cell lines with methylated RASD1 exhibited >5-fold increase in RASD1 expression after 5-aza-2'-deoxycytidine treatment, and the induction of RASD1 following 5-aza-2'-deoxycytidine treatment was correlated with the methylation level before treatment ($r = 0.645, P = 0.044; rs = 0.733, P = 0.16$).

We next carried out real-time PCR with and without dexamethasone treatment to assess the degree to which RASD1 is induced by dexamethasone in multiple myeloma cell lines (Fig. 3A and B). RPMI8226 cells, in which RASD1 is unmethylated, showed the greatest increase in expression (100 nmol/L dexamethasone versus mock). When we then tested whether 5-aza-2'-deoxycytidine would enhance the induction of RASD1 by dexamethasone in multiple myeloma cell lines showing RASD1 methylation, we found that 5-aza-2'-deoxycytidine acted synergistically with dexamethasone to induce RASD1 expression in all of the cell lines tested (Fig. 3B).

**5-Aza-2-deoxycytidine acts synergistically with dexamethasone to suppress dexamethasone-resistant OPM1 cell viability.** Although multiple myeloma cells are generally sensitive to dexamethasone treatment, tolerance appears during the end stage of the disease. Given that RASD1 reportedly suppresses cell growth (27), we hypothesized that dexamethasone acts through activation of RASD1. To test that idea, we first carried out a set of WST-8 assays to determine the dexamethasone sensitivity of each multiple myeloma cell line. Two cell lines (RPMI8226 and MM.1S) with unmethylated RASD1 were clearly sensitive to dexamethasone, which dose-dependently suppressed their viability (Fig. 4A and B). Consistent with their lack of RASD1 methylation, 5-aza-2'-deoxycytidine had no effect on the viability of these two cell lines (Fig. 4B). By contrast, cell lines with methylated RASD1 showed no sensitivity to dexamethasone treatment (Fig. 4A), suggesting that dexamethasone resistance is associated with RASD1 methylation. That finding prompted us to test whether demethylating RASD1 using 5-aza-2'-deoxycytidine would alter the dexamethasone sensitivity of RASD1-methylated cell lines. We found that, in the absence of 5-aza-2'-deoxycytidine, OPM1 cells were insensitive to dexamethasone treatment as reported previously (ref. 8; Fig. 4A). In the presence of 5-aza-2'-deoxycytidine, however, dexamethasone suppressed OPM1 cell viability in a time- and dose-dependent manner (Fig. 4C). Thereafter, we compared the sensitivities to dexamethasone and 5-aza-2'-deoxycytidine treatment of two drug-resistant cell lines derived from the dexamethasone-sensitive cell lines shown in Fig. 4B, taking into consideration the differences in their methylation status. RPMI8226/Dox40 cells are

![Fig. 3. RASD1 expression was synergistically induced by 5-aza-2'-deoxycytidine + dexamethasone in cell lines with methylated RASD1 alleles. A, fold expression of RASD1 (100 nmol/L dexamethasone/mock) with or without dexamethasone treatment in cell lines with unmethylated RASD1. B, fold RASD1 expression with dexamethasone, 5-aza-2'-deoxycytidine, or 5-aza-2'-deoxycytidine + dexamethasone versus control in multiple myeloma cell lines exhibiting RASD1 methylation.](image-url)
doxorubicin-resistant cells derived from the RPMI8226 line; RASD1 is highly methylated (95%; data not shown) in these cells, and like OPM1 cells, their viability was suppressed by treatment with 5-aza-2-deoxycytidine + dexamethasone (Fig. 4D). By contrast, MM.1R cells, which are derived from MM.1S cells, are known to be dexamethasone-resistant due to glucocorticoid receptor truncation, not RASD1 methylation (30, 31). Consistent with the absence of RASD1 methylation (<5%; data not shown), 5-aza-2-deoxycytidine had no ability to enhance dexamethasone cytotoxicity toward MM.1R cells (Fig. 4D).

Increased RASD1 methylation after repeated treatments in multiple myeloma patients. To assess the levels of RASD1 methylation in primary multiple myeloma cells, we performed pyrosequencing using CD138+ cells from 87 multiple myeloma patients and 12 control subjects without tumors (Fig. 5A and B). We selected 10% as the cutoff for methylation based on our findings that it represents the 75th percentile among the control samples and that cell lines with methylation of >10% showed down-regulated RASD1 expression that was restored by 5-aza-2-deoxycytidine (Fig. 2B). Methylation of RASD1 was observed in 8 of the 87 (8%) primary multiple myeloma samples (Fig. 5B). Moreover, levels of RASD1 methylation were elevated in all multiple myeloma cases (5 cases) in which there was repeated administration of antitumor therapy, including dexamethasone (P < 0.001; Fig. 5C).

Identification of the genes involved in the synergistic effect of 5-aza-2-deoxycytidine with dexamethasone. To identify genes responsible for the synergistic effect of 5-aza-2-deoxycytidine with dexamethasone, we performed cDNA microarray analyses using cDNA prepared from dexamethasone-resistant OPM1
cells treated with or without dexamethasone, 5-aza-2'-deoxycytidine, or 5-aza-2'-deoxycytidine + dexamethasone. We then selected genes whose expression was up-regulated or down-regulated >3-fold by 5-aza-2'-deoxycytidine + dexamethasone for analysis of gene tree clustering (Fig. 6A; Supplementary Table S4). One cluster exhibited an expression pattern similar to that of RASD1 shown in Fig. 3B. This cluster included RASD1 as well as other known cancer-related genes (Supplementary Table S4). To validate the results of the microarray analysis, we then carried out quantitative real-time PCR for four genes chosen from this cluster (Fig. 6B), and the results were consistent with those obtained with the microarray. We also examined the expression of SOCS3, which plays a role in the regulation of the interleukin-6 signaling pathway. Like that of RASD1, expression of SOCS3 was synergistically up-regulated in cells treated with 5-aza-2'-deoxycytidine + dexamethasone (Fig. 6B).

Finally, we performed bisulfate sequencing analysis to examine the role of DNA methylation in silencing genes up-regulated by 5-aza-2'-deoxycytidine + dexamethasone (Supplementary Fig. S5). Significant methylation of BNIP3 was detected, although methylation of SOCS3 gene was not, and ROS1 does not contain a CpG island in its 5' region. Thus 5-aza-2'-deoxycytidine + dexamethasone appears to suppress cell growth in both DNA methylation-dependent and DNA methylation-independent manners.

Discussion

Although genomic screening of genes silenced by DNA methylation has been reported previously (14, 16), the epigenetic alterations involved in tumorogenesis of multiple myeloma are still not fully understood. In the present study, genomic screening revealed that 128 genes were up-regulated >10-fold by 5-aza-2'-deoxycytidine in RPMI8226 cells, and 83 were similarly up-regulated in KMS12-PE cells, which is comparable with earlier findings (14). In addition, we found that 5-aza-2'-deoxycytidine up-regulated 424 genes >10-fold in OPM1 cells. This suggests that different numbers of genes are targeted for methylation in different multiple myeloma cells. This implies that the utility of the expression-based approach to finding target genes silenced by DNA methylation is limited because (a) 5-aza-2'-deoxycytidine induces expression of genes silenced via multiple mechanisms (e.g., DNA damage and growth inhibition) and (b) the sensitivity of microarrays is limited. We therefore focused on genes that have CpG islands in their 5' regions using real-time PCR to confirm restoration of their expression by 5-aza-2'-deoxycytidine and bisulfite PCR to assess their methylation status. We found specific methylation of 9 genes in multiple myeloma cells and tissue specimens but not in normal bone marrow cells, suggesting that these 9 genes are involved in multiple myeloma tumorigenesis.

RASD1 was originally identified as a dexamethasone-inducible gene (26), and its product was shown to be a receptor-independent activator of G-protein signaling (32, 33). RASD1 protein belongs to the Ras family (e.g., RIG, ARH1/NOEY2, and RRP22), which was recently shown to suppress cell growth (34–36). Located on chromosome 17p11.2, loss of RASD1 heterozygosity is frequently detected in human tumors, and Furuta et al. reported epigenetic inactivation of RASD1 in a melanoma cell line (37), which suggests that inactivation of RASD1 provides a growth advantage to tumor cells.
present study, we showed that RASD1 methylation is closely associated with dexamethasone resistance in multiple myeloma cells. Levels of RASD1 methylation were well correlated with the silencing of RASD1 and impaired the sensitivity to dexamethasone in multiple myeloma cell lines. Although RASD1 hypermethylation was less frequently observed in primary multiple myeloma samples than multiple myeloma cell lines, methylation levels were always elevated after repeated antitumor therapy, suggesting that RASD1 methylation is pivotal for disease progression and the development of drug resistance in multiple myeloma.

We found that expression of RASD1 was not induced by dexamethasone in multiple myeloma cells showing RASD1 methylation, but treating these cell lines with 5-aza-2'-deoxycytidine restored dexamethasone-mediated gene expression. This indicates that the impaired response to dexamethasone need not be caused by the absence of a transcription factor but can be caused by methylation of the RASD1 promoter. Our findings are consistent with earlier reports that inhibition of DNA methylation induces demethylation-sensitive clones in lymphoid cell lines (38) and that B-cell proliferation and activity are negatively regulated by RASD1 (39). We also found that 5-aza-2'-deoxycytidine had no significant effect on MM.R1 cells, a dexamethasone-resistant cell line that does not show RASD1 methylation. Taken together, our findings indicate that combined treatment with 5-aza-2'-deoxycytidine and dexamethasone is a potentially effective therapy for dexamethasone-resistant multiple myeloma.

Microarray analysis identified several genes that were synergistically up-regulated by 5-aza-2'-deoxycytidine + dexamethasone. Among the affected genes, BNIP3 expression has been shown to be up-regulated when cells undergoing apoptosis induced by hypoxia or glucocorticoids (40, 41). It has also been suggested that SOCS3, a negative regulator of JAK-STAT signaling, is altered in a variety of tumors (42). Although SOCS3 is reportedly methylated in various tumor types (43, 44), we did not detect SOCS3 methylation in multiple myeloma, which suggests that the antitumor effect of 5-aza-2'-deoxycytidine + dexamethasone involves both demethylation-dependent and demethylation-independent mechanisms, and the synergistic effect of 5-aza-2'-deoxycytidine with dexamethasone works through multiple mechanisms in multiple myeloma cells. Further study will be necessary to clarify...
the molecular mechanisms underlying the synergistic effect of 5-aza-2′-deoxycytidine with dexamethasone.

In conclusion, we have shown that epigenetic inactivation of RASD1 is closely correlated with resistance to dexamethasone in multiple myeloma cells. Restoration of RASD1 expression in multiple myeloma cells using 5-aza-2′-deoxycytidine also restored sensitivity to dexamethasone. Although further study is needed to determine how important RASD1 hypermethylation is in the clinical course of multiple myeloma, our results are indicative of the potential utility of demethylation therapy in cases of advanced multiple myeloma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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