Development of Gene Expression–Based Score to Predict Sensitivity of Multiple Myeloma Cells to DNA Methylation Inhibitors

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Abstract

Multiple myeloma is a plasma cell cancer with poor survival, characterized by the clonal expansion of multiple myeloma cells (MMC), primarily in the bone marrow. Novel compounds are currently tested in this disease, but partial or minor patients’ responses are observed for most compounds used as a single agent. The design of predictors for drug efficacy could be most useful to better understand basic mechanisms targeted by these drugs and design clinical trials. In the current study, we report the building of a DNA methylation score (DM score) predicting the efficacy of decitabine, an inhibitor of DNA methyltransferase (DNMT), targeting methylation-regulated gene expression. DM score was built by identifying 47 genes regulated by decitabine in human myeloma cell lines and the expression of which in primary MMCs of previously untreated patients is predictive for overall survival. A high DM score predicts patients’ poor survival, and, of major interest, high sensitivity of primary MMCs or human myeloma cell lines to decitabine in vitro. Thus, DM score could be useful to design novel treatments with DMNT inhibitor in multiple myeloma and has highlighted 47 genes, the gene products of which could be important for multiple myeloma disease development. Mol Cancer Ther; 11(12); 1–8. ©2012 AACR.

Introduction

Malignant transformation requires oncogenic activation and inactivation of tumor suppressor genes, which help cancer cells overriding the normal mechanisms controlling cellular survival and proliferation (1, 2). These molecular events are caused by genetic alterations (translocations, amplification, and mutations) and also by epigenetic modifications (3). Epigenetic modifications include methylation of DNA cytosine residues and histone modifications and have been shown to be critical in the initiation and progression of cancers (4). DNA methyltransferase inhibitors (DNMTi) and histone deacetylase (HDAC) inhibitors are now being used in the treatment of several hematologic malignancies including multiple myeloma (5–8).

Multiple myeloma is a plasma cell neoplasm characterized by the accumulation of malignant plasma cells, termed multiple myeloma cells (MMC) within the bone marrow. Despite the recent introduction of therapies such as lenalidomide and bortezomib, multiple myeloma remains an almost incurable disease. Multiple myeloma arises through the accumulation of multiple genetic changes that include an aberrant or overexpression of a D-type cyclin gene, cyclin D1 (CCND1) in the case of t(11;14) translocation or gain in 11q13, cyclin D3 (CCND3) in the case of the rare t(6;14) translocation, or cyclin D2 (CCND2) on the background of a translocation involving c-maf [t(14;16)] or MMSET/FGFR3 [t(4;14); refs. 9, 10]. Recent studies have shown that epigenetic changes such as DNA methylation play a role by silencing various cancer-related genes in multiple myeloma. Most of these studies have been conducted on limited number of genes using methylation-specific PCR (11–18). Among the genes identified with promoter hypermethylation in multiple myeloma, cyclin-dependent kinase inhibitor 2A (CDKN2A) and TGF-beta receptor 2 (TGFBR2) have been shown to be associated with a poor prognosis in patients with multiple myeloma with discrepant results for CDKN2A (12). Heller and colleagues have identified several cancer-related genes inactivated through methylation in 3 human myeloma cell lines (HMCL) and validated the relevance of 10 of these genes in 6 additional HMCLs, premalignant plasma cells from 24 patients with monoclonal gammopathy of undetermined significance.
(MGUS) and MMCs from 111 patients with multiple myeloma (19). A methylation of the promoter of the genes coding for secreted protein acidic and rich in cysteine (SPARC) or for Bcl2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3) promoters was associated with poor overall survival (OS) of patients with multiple myeloma (19). SOCS3 promoter methylation was found to be associated with extramedullary manifestations, plasma cell leukemia, and significant shortened survival in patients with multiple myeloma (20). More recently, Walker and colleagues have shown that the transition of normal plasma cells and MGUS stage to multiple myeloma stage is associated with DNA hypomethylation, but the transition of intramedullary multiple myeloma stage to plasma cell leukemia or HMCL stage is associated with DNA hypermethylation (21). They described 2 specific subgroups of hyperdiploid multiple myeloma on the basis of their methylation profile, which had a significantly different OS (21).

DNMT inhibitors can be subdivided into nucleoside analogue and non-nucleoside analogue families. 5-Azacytidine (azacytidine) or 5-aza-2'-deoxycytidine (decitabine) are both nucleoside analogues with approval for use in myelodysplastic syndrome by U.S. Food and Drug Administration. Clinical trials in myeloma combining these demethylating agents with chemotherapy or other agents are underway (8). An important objective for optimizing these clinical trials will be the identification of biomarkers predictive for sensitivity of MMCs to DNMTi.

In the present study, we used gene expression profiling to build a novel DNA methylation gene expression score that makes it possible identification of patients whose MMCs will be targeted by DNMT inhibition.

Materials and Methods

Human myeloma cell lines

XG-1, XG-2, XG-3, XG-4, XG-5, XG-6, XG-7, XG-10, XG-11, XG-12, XG-13, XG-14, XG-16, XG-19, XG-20, and XG-21 HMCLs were obtained as previously described (22-26). JJN3 was kindly provided by Dr. Ivan Van Riet (Academic Hospital, Free University Brussels, Bruxelles, Belgium), JIM3 by Dr. Ian MacLennan (University of Birmingham, Birmingham, UK), and MM1S by Dr. Steven Rosen (Northwestern University, Chicago, IL). AMO-1, LP1, L363, U266, OPM2, and SKMM2 were from DSMZ (and RPMI8226 from American Type Culture Collection. All HMCLs derived in our laboratory were cultured in the presence of recombinant interleukin (IL)-6. Gene expression profiling data from HMCLs have been deposited in the ArrayExpress public database under accession numbers E-TABM-937 and E-TABM-1088. The myeloma cell lines were authenticated in our laboratory.

Primary multiple myeloma cells

Bone marrow samples were collected after patients’ written informed consent in accordance with the Declaration of Helsinki and institutional research board approval from Heidelberg and Montpellier University Hospital (Montpellier, France). In particular, bone marrow were collected from 206 patients treated with high-dose melphalan (HDM) and autologous stem cell transplantation (ASCT; ref. 27), and this cohort is termed the following Heidelberg—Montpellier (HM) cohort (Supplementary Table S1). The .CEL files and MA55 files have been deposited in the ArrayExpress public database (E-MTAB-372). The structural chromosomal aberrations including t(4;14)(p16.3;q32.3) and t(11;14) (q13;q32.3), as well as numerical aberrations including 17p13 and 1q21 gain, were assayed by iFISH (28). We also used Affymetrix data of a cohort of 345 purified MMCs from previously untreated patients from the University of Arkansas for Medical Sciences (UAMS; Little Rock, AR). The patients were treated with total therapy 2 including HDM and ASCT (29) and termed in the following UAMS-TT2 cohort. These data are publicly available via the online Gene Expression Omnibus (Gene Expression Profile of Multiple Myeloma, accession number GSE2658). As iFISH data were not available for UAMS-TT2 patients, t(4;14) translocation was evaluated using MMSET spike expression (30) and del17p13 surrogated by TP53 probe set signal (31). After Ficoll density gradient centrifugation, plasma cells were purified using anti-CD138 MACS microbeads (Miltenyi Biotech).

Cell culture and treatment for gene expression profiling

The human multiple myeloma cell lines (HMCLs) XG-5, XG-6, XG-7, XG-20, and LP1 were grown in RPMI-1640 supplemented with 10% FBS. Around 2 ng/mL recombinant IL-6 was added to IL-6-dependent HMCLs (XG-5, XG-6, XG-7, and XG-20). Cells (2 × 10^5/mL) were treated without (control) or with 0.5 μM/L 5-aza-2'-deoxycytidine (decitabine, Sigma; Fig. 1) for 7 days as described by Heller and colleagues (19). At day 3, half the culture medium without (control) or with 0.5 μM/L decitabine was renewed. This decitabine concentration is the starting one inducing minor decrease in HMCL viability at day 7 of culture (Supplementary Table S2).

![Decitabine structure](Image)
Growth assay for myeloma cells
HMCLs were cultured for 4 days in 96-well flat-bottom microtiter plates in RPMI-1640 medium, 10% fetal calf serum (FCS), 2 ng/mL IL-6 culture medium (control), with graded decitabine concentrations. Cell growth was evaluated by quantifying intracellular ATP amount with a Cell Titer Glo Luminescent Assay (Promega) with a Centro LB 960 luminometer (Berthold Technologies).

Mononuclear cell culture
Mononuclear cells from tumor samples of 12 patients with multiple myeloma were cultured for 4 days at 2 × 10^5 cells/mL in RPMI-1640 medium, 10% FCS, 2 ng/mL IL-6, with or without graded concentrations of decitabine. In each culture group, viability and cell counts were assayed, and MMCs were stained with an anti-CD138-PE mAb (Immunotech) as previously described (32).

Preparation of complementary RNA and microarray hybridization
RNA was extracted using the RNeasy Kit (Qiagen) as previously described (33, 34). Biotinylated cRNA was amplified with a double in vitro transcription and hybridized to the human U133 2.0 plus GeneChips, according to the manufacturer’s instructions (Affymetrix). Fluorescence intensities were quantified and analyzed using the GECOS software (Affymetrix).

Gene expression profiling and statistical analyses
Gene expression data were normalized with the MAS5 algorithm and analyzed with our bioinformatics platforms—RAGE (35) and Amazonia (36)—or SAM (significance analysis of microarrays) software (37). The statistical significance of differences in OS between groups of patients was calculated by the log-rank test. Multivariate analysis was conducted using the Cox proportional hazards model. Survival curves were plotted using the Kaplan–Meier method. All these analyses have been done with R2.10.1 and bioconductor version 2.5. Gene annotation and networks were generated through the use of Ingenuity Pathways Analysis (Ingenuity Systems; ref. 38).

Results
Modulation of gene expression by decitabine in HMCLs: identification of prognostic genes
Five HMCLs were treated with 0.5 μmol/L of decitabine for 7 days. This was the starting concentration yielding to 10% loss in myeloma cell viability, with the aim to avoid conducting gene expression in apoptotic cells (Supplementary Table S2; ref. 19). Using SAM supervised paired analysis, the expression of 48 genes was found to be significantly upregulated and that of 79 genes downregulated by decitabine treatment [false discover rate (FDR) < 5%; Supplementary Tables S3 and S4]. Decitabine-regulated genes are significantly enriched in genes related to “cancer” and “cell death” pathways (FDR < 5%; Ingenuity Pathway Analysis, data not shown). Investigating the expression of these 127 decitabine-regulated genes in primary MMCS of a cohort of 206 newly diagnosed patients (HM cohort), 22 genes had bad prognostic value and 25 a good one after Benjamini–Hochberg multiple testing correction (Supplementary Table S5). These genes are enriched in genes encoding for IFN signaling pathway (Supplementary Fig. S1). The prognostic information of decitabine-regulated genes was gathered within a DNA methylation score (DM score), which is the sum of the beta coefficients of the Cox model for each prognostic gene, weighted by ±1 according to the patient MMC signal above or below the probe set maxstat value as previously described (38). The value of DM score in normal, premalignant, or malignant plasma cells is displayed in Fig. 2A. DM score was similar between normal bone marrow plasma cells (BMPC) and premalignant plasma cells from patients with MGUS. MMCS of patients had a significantly higher DM score than normal BMPCs or plasma cells from patients with MGUS (P < 0.01) and HMCLs the highest score (P < 0.001; Fig. 2A). Investigating the DM score in the 8 groups of the molecular classification of multiple myeloma (39), DM score was significantly higher in the proliferation, t(4;14) and MAF subgroups (P < 0.001) associated with a poor prognosis (39) and significantly lower in the low bone disease subgroup (P < 0.001; ref. 39; Fig. 2B).

Prognostic value of DM score compared to usual prognostic factors
Using patients’ HM cohort, DM score had prognostic value when used as a continuous variable (P < 10⁻⁴, results not shown) or by splitting patients into 2 groups using Maxstat R function (38). A maximum difference in OS was obtained with DM score = −15.8 splitting patients in a high-risk group of 34.5% patients (DM score > −15.8) with a 42.1-month median OS and a low-risk group of 65.5% patients (DM score ≤ −15.8) with not-reached median survival (Fig. 3). Using univariate Cox analysis, DM score, UAMS-HRS, IFM score, and GPI had prognostic value as well as t(4;14), del17p, β2m, albumin, and ISS (Supplementary Table S6). When compared 2 by 2, DM score tested with β2m remained significant. When these parameters were tested together, DM score, β2m and t(4;14) kept prognostic value. DM score was also prognostic for the UAMS-TT2 cohort of 345 patients treated with TT2 therapy (29). For each patient of UAMS-TT2 cohort, DM score was computed using parameters defined with patients’ HM cohort only. The median OS of patients within high score group (DM score > 15.8) was 53.7 months and not reached for patients with low DM score (P = 0.0008; Fig. 3). Using Cox univariate analysis, UAMS-HRS, IFM, and GPI scores as well as t(4;14) and del17p had prognostic value. Comparing these prognostic factors by 2 by 2, DM score remained significant compared with GPI, t(4;14), and del17p in the UAMS-TT2 cohort (Supplementary Table S6). When these parameters were tested together, UAMS-HRS, t(4;14), and del17p kept prognostic value.
DM score is predictive for sensitivity of HMCLs or patients’ primary MMCs to decitabine in vitro

We sought to determine whether DM score could predict for the sensitivity of 10 HMCLs to DNMTi. Starting from a large cohort of 40 HMCLs (22), the 10 HMCLs with the highest or lowest DM score were selected to assay decitabine sensitivity. The 5 HMCLs with the highest DM score exhibited a significant 11-fold higher decitabine sensitivity (median IC_50 = 0.68 μmol/L; range, 0.15–2.22 μmol/L) than the 5 HMCLs with low DM score (P = 0.01; median IC_50 = 7.94 μmol/L; range, 2.92–60.81 μmol/L; Fig. 4). Four of the 5 HMCLs with the highest DM score and higher decitabine sensitivity have ras mutations, contrary to the 5 HMCLs with the lowest DM score and poorly sensitive, which have no ras mutations (Table 1).

To determine whether DM score could predict the sensitivity of primary MMCs to DNMTi, we used the maxstat cutoff point (DM score = −15.8) defined in Fig. 3 to separate patients with multiple myeloma with high DM score from patients with low DM score. Primary MMCs from 12 patients were cultured together with their bone marrow environment, recombinant IL-6, and graded concentrations of decitabine for 4 days. Primary MMCs of patients with a DM score above maxstat cutoff point (> −15.8; Fig. 2A) exhibited a significant (P < 0.01) 2.2-fold higher decitabine sensitivity than MMCs with DM score below maxstat cutoff point (Fig. 5).

Discussion

In this study, we have identified a gene expression–based DM score, which is predictive for patients’ survival and for the in vitro sensitivity of HMCLs or patients’ primary myeloma cells to decitabine, a DNMTi. Given the clinical development of DNMTi in patients with multiple myeloma (8), it is of major interest to investigate whether this DM score could predict patients’ response...
Our group has shown that IFN-α is a survival factor for MMCs and protects MMCs from dexamethasone-induced apoptosis (42), whereas other groups found it inhibited multiple myeloma cell growth (43). IFN-α was used for several years as a maintenance therapy in patients with multiple myeloma (44) but its use was stopped in reason of lack of reproducible clinical efficacy (45, 46). It could be of interest to investigate whether IFN could control the methylation of some genes in MMCs.

All HMCLs but one with the highest DM score and higher decitabine sensitivity have ras mutations, contrary to the 5 HMCLs with the lowest DM score and poorly sensitive, which have no ras mutations (Table 1). The prevalence of activating mutations of K- and N-Ras in multiple myeloma ranges is approximately 15% each in a newly diagnosed multiple myeloma (47, 48) and is independent of clinical stage (49, 50). But the prevalence of RAS mutations increases with disease progression, in association with shorter survival (47, 48, 50), suggesting decitabine could be useful to treat these patients.

RECQ1 (ATP-dependent DNA helicase Q1) and KIF21B (kinesin family member 21B) are 2 of the 22 genes downregulated by decitabine treatment and associated with a poor prognosis. RECQ helicases constitute a ubiquitous family of DNA-unwinding enzymes involved in the maintenance of chromosome stability (51–53). Mutations in the RECQ genes are linked with genetic disorders associated with genomic instability, cancer predisposition, and

to these inhibitors. Besides a potential interest of DM score in selecting patients who could benefit from DNMTi therapies, the current study highlights pathways that could be involved in the development of multiple myeloma cells. Heller and colleagues have identified several cancer-related genes inactivated through methylation in 3 HMCLs (19). Among the 127 genes deregulated by decitabine treatment in our HMCL cohort, about one fifth (28 genes) were commonly identified by Heller and colleagues. (Supplementary Tables S7 and S8), including in particular some IFN-regulated genes. Indeed, decitabine treatment induced overexpression of some genes, the expression of which is regulated by IFN-α (OAS1, OAS2, IFI27, IFI35, G1P2, MX1, and STAT1) (Supplementary Fig. S1). Zhan and colleagues identified an overexpression of several IFN-induced genes found in that study, including OAS2, IFI27, and IFI35, as a characteristic of patients with hyperdiploid MMCs (39, 40). This observation indicates that the expression of these genes is repressed by promoter methylation and suggests IFN could activate them, partly by inducing demethylation of CpG islands as shown recently for IFITM3 gene (41). The biologic or clinical role of IFN in multiple myeloma is controversial. Our group has shown that IFN-α is a survival factor for MMCs and protects MMCs from dexamethasone-induced

Figure 3. Prognostic value of DM score in multiple myeloma. A, patients of HM cohort were ranked according to increased DM score and a maximum difference in OS was obtained with DM score = -15.8, optimally splitting patients into high-risk (65.5%) and low-risk (34.5%) groups. B, the prognostic value of DM score was tested on an independent UAMS-TT2 cohort of 345 patients treated with TT2 therapy. The parameters to compute DM score for patients of UAMS-TT2 cohort and the DM score cutoff point delineating the 2 prognostic groups were those defined with HM cohort only.

Figure 4. DM score predicts sensitivity of HMCLs to decitabine. HMCLs with high DM score (n = 5) exhibit significantly higher decitabine sensitivity than HMCLs with low DM score (n = 5). HMCLs were cultured for 4 days in 96-well flat-bottom microtiter plates in RPMI-1640 medium, 10% FCS, 2 ng/mL IL-6 culture medium (control), and graded decitabine concentrations. Data are mean values ± SD of 5 independent experiments.
features of premature ageing (52). Consistent with their ability to unwind DNA, several functions have been attributed to RECQ proteins, including roles in stabilization and repair of damaged DNA replication forks, telomere maintenance, homologous recombination, and DNA damage checkpoint signaling (51–53). Recent reports supported a role for RECQ1 in oncogenesis (54–56). RECQ1 silencing in cancer cells resulted in mitotic catastrophe and injection of siRNA targeting RECQ1 prevented tumor growth in murine models (54–56). More recently, it was shown that RECQ1 is highly expressed in various types of solid tumors including colon carcinoma, thyroid cancer, lung cancer, and brain glioblastoma tissues (57). In glioblastoma cell lines, depletion of RECQ1 by RNA interference results in a significant reduction of cellular proliferation, perturbation of S-phase progression, spontaneous γ-H2AX foci formation, and hypersensitivity to hydroxyurea and temozolomide treatments (57). KIF21B is a kinesin family member. Kinesins are a conserved class of microtubule-dependent molecular motor proteins that have adenosine triphosphatase activity and motion characteristics (58). KIF21B is a kinesin family member. Kinesins are a conserved class of microtubule-dependent molecular motor proteins that have adenosine triphosphatase activity and motion characteristics (58). Kinesins support several cellular functions, such as mitosis, meiosis, and the transport of macromolecules. In mitosis of eukaryotic cells, kinesins participate in spindle formation, chromosome congression and alignment, and cytokinesis (59). Abnormal expression and function of kinesins are involved in the development or progression of several kinds of human cancers (60, 61). Interestingly, KIF21B maps to chromosome 1q arm (1q32.1), which is amplified in MMCs of patients with high-risk multiple myeloma (62). More recently, KIF21B gene was found in a critical neighbor gene model associated with a poor prognosis across independent data sets of, respectively, 559, 247, and 264 patients with multiple myeloma (63). These data suggest that decitabine treatment could synergize with DNA-damaging agents, targeting genes involved in DNA repair and maintenance of chromosome stability in MMCs. In conclusion, we reported here the identification of genes regulated by a DNMTi in MMCs and predictive for patients’ survival, whose information could be

### Table 1. Characteristics of decitabine-sensitive and -resistant HMCLs

<table>
<thead>
<tr>
<th>HMCL name</th>
<th>IL-6 dependence</th>
<th>Origin</th>
<th>Disease</th>
<th>Patient sample</th>
<th>Gender</th>
<th>Isotype</th>
<th>t(14q32 or 22q11)</th>
<th>Target genes</th>
<th>Ras</th>
<th>TP53</th>
<th>CD45</th>
<th>HMCL classification</th>
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<td>Decitabine-resistant HMCLs</td>
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<td>XG-6 ++</td>
<td>MN MM PB F</td>
<td>Gl</td>
<td>t(16;22)</td>
<td>c-Maf</td>
<td>wt</td>
<td>wt</td>
<td>+</td>
<td>CTA/MS</td>
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<td>I</td>
<td>t(4;14)</td>
<td>MMSET</td>
<td>wt</td>
<td>abn</td>
<td>–</td>
<td>MS</td>
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<td>t(14;16)</td>
<td>c-Maf</td>
<td>wt</td>
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<td>+</td>
<td>MF</td>
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<td>SKMM2 –</td>
<td>CO PCL PB M</td>
<td>Gk</td>
<td>t(11;14)</td>
<td>CCND1</td>
<td>wt</td>
<td>abn</td>
<td>–</td>
<td>CD-1</td>
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<td>Gl</td>
<td>t(4;14)</td>
<td>MMSET/FGFR3</td>
<td>wt</td>
<td>abn</td>
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<td>MS</td>
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<td>Decitabine-sensitive HMCLs</td>
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<td>wt</td>
<td>+</td>
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<td>mut</td>
<td>abn</td>
<td>+</td>
<td>CTA/FRZB</td>
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<td>Al</td>
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<td>c-Maf</td>
<td>wt</td>
<td>wt</td>
<td>+</td>
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<td>Ak</td>
<td>t(14;16)</td>
<td>c-Maf</td>
<td>mut</td>
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<td>+/–</td>
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<td>abn</td>
<td>–</td>
<td>MF</td>
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Abbreviations: AF, ascitic fluid; BM, bone marrow; CO, collected; MM, Montpellier or Nantes; PB, peripheral blood; PCL, plasma cell leukemia; PCT, plasmacytoma; PE pleural effusion.

a ++, if growth is strictly dependent on adding exogenous IL-6; +, if dependent on adding exogenous IL-6; –, if not.
bOrigin of the HMCL.
cDisease at diagnosis.
dOrigin of the sample.

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**Figure 5.** DM score predicts decitabine sensitivity of primary myeloma cells of patients. Mononuclear cells from tumor samples of 12 patients with MM were cultured for 4 days in the presence of IL-6 (2 ng/mL) with or without graded decitabine concentrations. At day 4 of culture, the cell count and the viability were determined, and the percentage of CD138⁺ viable plasma cells was determined by flow cytometry. Black color represents patients with high DM score (n = 6; DM score > -15.8) and white represents patients with low DM score values (n = 6; DM score ≤ -15.8).
summed within a single DM score. This finding could help to better organize treatments with DNMTi in patients with multiple myeloma, to highlight proteins involved in multiple myeloma oncogenesis, and could be extended to other cancers.

Disclosure of Potential Conflicts of Interest
H. Goldschmidt has received commercial research support from and has honorary from speakers bureau from Jansen and Celgene and is a consultant/advisory board member of Millenium, Onyx, and Jansen. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Moreaux, T. Reme, W. Leonard, D. Hose, B. Klein
Writing, review, and/or revision of the manuscript: J. Moreaux, W. Leonard, J.-L. Veyrone, H. Goldschmidt, D. Hose, B. Klein

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Reme, G. Requirand
Study supervision: B. Klein

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References


Inhibitors

Sensitivity of Multiple Myeloma Cells to DNA Methylation


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