DNA Methylation Analysis Determines the High Frequency of Genic Hypomethylation and Low Frequency of Hypermethylation Events in Plasma Cell Tumors

Bodour Salhia¹, Angela Baker¹, Gregory Ahmann², Daniel Auclair³, Rafael Fonseca², and John Carpten¹

Abstract

Multiple myeloma (MM) is a plasma cell malignancy of the bone marrow, which evolves from a premalignant stage called monoclonal gammopathy of undetermined significance (MGUS). In some patients, an intermediate stage referred to as smoldering multiple myeloma (SMM) is clinically recognized, with the full-bone malignancy termed MM. We conducted a study to assess differential CpG methylation at 1,500 genic loci during MM progression and profiled CD138⁺ plasma cells from MGUS, SMM, and MM specimens; human myeloma cell lines; and normal plasma cell (NPC) samples. We showed that the number of differentially methylated loci (DML) increased with tumor grade, and the vast majority were due to hypomethylation. Hierarchical clustering analysis revealed samples that coclustered tightly with NPC. These cases, referred to as “normal-like,” contained significantly fewer DML when compared with their non–normal-like counterparts and displayed overall methylation levels resembling NPC. This study represents one of the first methylome interrogation studies in MM and points toward global hypomethylation at genic CpG loci as an important and early mechanism driving myelomagenesis. Determining the set of critical genes and pathways based on the myeloma methylome is expected to lead to an improved understanding of biological mechanisms involved in myelomagenesis. Cancer Res; 70(17): 6934–44. © 2010 AACR.

Introduction

Multiple myeloma (MM) is characterized by proliferation of a population of clonal malignant plasma cells in the bone marrow and accounts for slightly more than 10% of all hematologic cancers (1). The annual age-adjusted incidence of myeloma in the United States is 4.3 per 100,000. MM evolves from an asymptomatic premalignant stage of clonal plasma cell proliferation termed monoclonal gammopathy of undetermined significance (MGUS; ref. 1). MGUS is present in more than 3% of the population above the age of 50 years and progresses to myeloma or related malignancy at a rate of 1% per year (1). In some patients, an intermediate asymptomatic but more advanced premalignant stage, referred to as smoldering multiple myeloma (SMM), is clinically recognized, with the full-bone malignancy termed MM (1).

Several genetic mechanisms underlying the pathogenesis of MM have been elucidated in the past decade especially due to the advent of modern technologies for genetic analysis, global gene expression, and proteomic profiling (1–3). Increasing evidence is pointing toward the importance of epigenetic mechanisms in the pathogenesis of MM (4–6). In a recent report, investigators performed a microarray-based genome-wide screen to identify methylated genes after treatment with a demethylating agent, 5-azacytidine, in human myeloma cell lines (HMC1; ref. 5). Additional studies have assessed DNA methylation in MM but have focused on single gene analyses using techniques such as bisulfite sequencing and methylation-specific PCR (MSP). Microarray-based DNA methylation profiling methods have been recently developed to access the epigenetic information for a large number of genes or the entire genome. The purpose of our study is to examine CpG methylation at different stages of myelomagenesis using a high-throughput universal bead array technology at loci spanning >800 genes, including known tumor suppressor genes, oncogenes, and genes involved in cancer-related cellular processes.

Materials and Methods

Sample acquisition and DNA isolation

Bone marrow aspirates were collected from patients diagnosed with MGUS (n = 13), SMM (n = 26), or MM (n = 140). Additionally, we included six normal CD138⁺ plasma cell samples (NPC, pooled from 20 individuals) obtained from the bone marrow of individuals who underwent hip or knee replacement surgeries. Sample characteristics are presented...
in Supplementary Table S1. All patients provided written informed consent under institutional review board approval. Bone marrow aspirates were treated with ACK lysis buffer to remove red blood cells. Plasma cells were subsequently isolated using immunomagnetic sorting on a Miltenyi AutoMacs or StemCell Robocept with anti-CD138 antibodies. We enumerated 100 nucleated cells for κ and λ staining to determine the purity of the CD138 sorts. We found that the mean plasma cell purity (κ and λ) was 95.5% for MM, 96.5% for SMM, and 96.2% for MGUS samples, whereas the mean clonal purity (clinical light chain) was 92.7% for all samples. Isolated cells were suspended in TRIZol (Invitrogen) and kept at −80°C for long-term storage. Nucleic acids were isolated from TRIZol following a standard protocol. Genomic DNA (gDNA) was extracted with proteinase K followed by RNase and phenol-chloroform treatments (7). HMCLs were maintained as previously described (8). We used four HMCLs, including MM-1, MY5, JMW, and JNJ3 (kindly provided by Dr. P.L. Bersagel [Comprehensive Cancer Center, Mayo Clinic Arizona, Scottsdale, AZ]). gDNA was isolated from HMCLs as described above. To rule out a CD138 selection bias, the JNJ3 and MM-1 cell lines underwent positive and negative (no antibodies or beads) CD138 selection.

Methylation-specific PCR

We performed MSP to examine the methylation status of four commonly methylated cancer genes in four HMCLs. Briefly, 500 ng of gDNA were subjected to sodium bisulfite conversion (EZ DNA Methylation Kit, Zymo Research) followed by PCR for CDKN2A, E-cadherin, MGMT, and hMLH1 (Chemicon CpG WIZ kits) with U-primers (recognize unmethylated CpG) and M-primers (recognize methylated CpG). Amplified PCR products were separated and visualized by agarose gel electrophoresis.

Methylation analysis using universal bead arrays

We used GoldenGate Methylation Cancer Panel I (Illumina) for direct measurement of DNA methylation at a specific set of CpG loci (9). The panel was developed to assay 1,505 CpG sites selected from 807 genes. Of the 1,505 loci on the array, 1,044 (69%) are probes designed to loci in CpG islands (CPI) and 461 (31%) are probes designed in non-CpG islands (NGC). Bisulfite conversion of DNA (500 ng) was performed as described above. Technical replicates of each bisulfite-converted sample were assayed. After bisulfite treatment, the remaining assay steps were identical to the GoldenGate genotyping assay (10), using Illumina-supplied reagents and conditions. Briefly, bisulfite-converted DNAs were biotinylated, hybridized to query oligos, and washed. The hybridized oligos were then extended and ligated to create amplifiable templates. The PCR that followed uses fluorescently labeled universal PCR primers. Image processing and intensity data extraction were performed using an Illumina BeadArray Reader.

Differential methylation and statistical analyses

The BeadStudio Methylation software from Illumina was used for data assembly and acquisition. All array data points were represented by fluorescent signals from the methylated (M) and unmethylated (U) alleles. Background intensity computed from a set of negative controls was subtracted from each analytic data point. The ratio of fluorescent signals was then computed from the two alleles: \( \beta = \frac{[\text{max}(M,0)]}{|U| + |M| + 100} \) (ref. 9). An average methylation (\( \beta \)) value was derived from ~30 replicate methylation (\( \beta \)) values and a M/U ratio (9). The average (\( \beta \)) value reports a methylation signal ranging from 0 to 1 representing completely unmethylated to completely methylated, respectively, and reflects the fractional methylation level of each CpG site.

Subsequent analyses were carried out by importing raw data for each sample into Genespring GX10 (Agilent). For exploratory and visualization purposes, hierarchical clustering was performed using Euclidian metric and centroid linkage. We used the Mann-Whitney nonparametric statistical test and applied a Benjamini-Hochberg correction to identify differentially methylated loci (DML) between each tumor group and NPC. To select the sites that had the largest differences between groups, we applied an additional filter that required a minimum average \( \beta / \) difference of 0.2 between the means of tumor and NPC groups. Finally, to avoid gender-specific bias, all loci on the X chromosome were dropped.

Array-based comparative genomic hybridization

Array-based comparative genomic hybridization (aCGH) was performed using the Human Genome CGH 244A microarrays (Agilent Technologies). The procedure used was an adaptation of the suggested protocol by the manufacturer (7). Briefly, 1 μg of patient gDNA and normal female reference (Promega) were digested with bovine DNase I and directly labeled with either Cy5 or Cy3 using the BioPrime Array CGH Genomic Labeling Module (Invitrogen). Labeled DNA was purified using Vivaspin 500 spin columns (Sartorius). The hybridization reactions containing equal amounts of test and reference DNA were prepared using the Oligo aCGH/ChIP-on-chip Hybridization Kit (Agilent) and were hybridized to the microarray at 65°C for 40 hours at a rotation speed of 20 rpm. The slides were washed using Agilent Oligo CGH Wash Buffer and scanned using the Agilent G2505B DNA microarray scanner. The microarray images were analyzed using Feature Extraction software v.10.5.5.1 (Agilent), and log2-transformed ratios were analyzed with DNA Analytics v.4.0.81 (Agilent). Data quality was assessed using the metrics value for DLRSpread, signal intensity, and signal-to-noise ratio (DNA Analytics).

Pyrosequencing

For DNA methylation analysis using Pyrosequencing technology (Pyrosequencing, Qiagen; refs. 11 and 12). 500 ng of DNA were bisulfite treated, as described above, by EpigenDx. The PCR was performed with 0.2 μmol/L of each primer. One of the PCR primers was biotinylated to purify the final PCR product using Sepharose beads (Amersham Biosciences). The Sepharose beads containing the immobilized PCR product were washed and denatured using a 0.2 mol/L NaOH solution as recommended by the manufacturer. Subsequently, 0.2 μmol/L Pyrosequencing primer was annealed to the purified single-stranded PCR product. Then, 10 μL of the PCR products were sequenced by the Pyrosequencing PSQ96 HS
System (Biotage AB) following the manufacturer’s instructions. The methylation status of each locus was analyzed individually as a T/C SNP using QCpG software. The methylation percentage of each locus was analyzed automatically by the QCpG software.

**Functional and pathway annotation**

Pathway enrichment analysis was performed using the MetaCore Analytical suite (GeneGo, Inc.; ref. 13). Enrichment analysis consisted of mapping gene IDs of the data set onto gene IDs in entities of built-in functional ontologies represented in MetaCore by pathway maps and networks. GeneGo ontologies were the result of in-house extensive classification effort and public ontologies such as Gene Ontology (GO; http://www.geneontology.org). GeneGo ontologies represented by canonical pathway maps, cellular process networks, and protein classification were queried. The statistical relevance of matched ontologies was calculated as the \( P \) value, or a probability of a match to occur by chance, given the size of the database.

**Results**

**Pattern of differential methylation between MGUS, SM, MM, HMCL, and NPC**

Overall, we obtained highly reproducible DNA methylation profiles between technical replicates. One MGUS, one SMM, and nine MM samples were excluded from the study due to correlation coefficients of less than 0.8 or poor performance according to GoldenGate assay metrics. The average correlation coefficient \( (R^2) \) of the remaining samples was greater than 0.90 when technical replicates were assessed. MSP data of \( CDKN2A \) (p16), \( CDH1 \), \( MGMT \), and \( MLH1 \) genes in four HMCLs perfectly correlated with the results of the methylation array (Fig. 1A). The number of DML that passed statistical and filtering criteria was 143 in MGUS, 165 in SMM, 226 in MM, and 343 in HMCLs (Supplementary Table S2a–d). The DML in each group corresponded to 110, 127, 159, and 249 unique genes, respectively. A union of all DML from each tumor group yielded 245 unique loci across 176 genes (Fig. 1B). The intersection of this Venn analysis identified 120 DML common to the groups (Fig. 1B; Supplementary Table S3). Intersections of this Venn analysis included 120 DML common to the groups (Fig. 1B; Supplementary Table S3). Interestingly, only 153 loci were common between HMCL and MM. The number of DML accounted for 9.5%, 10.96%, 15.01%, and 22.79% of all array probes in MGUS, SMM, MM, and HMCLs, respectively (Fig. 1C).

When the median methylation level of all 245 DML was calculated, NPC had the highest methylation level (0.68) of all groups (\( P < 0.0001 \)), whereas the median methylation level in MM (0.34) was the lowest (\( P < 0.0001 \); Fig. 1D). The median methylation levels of MGUS and SMM were 0.44 and 0.44, respectively \( (P = 0.47) \), but each differed from MM \( (P < 0.0001) \). Taken together, these data illustrate a phenomenon of global genic hypomethylation during the initiation and progression of MM.

Clinical samples used in this study represented those from both newly diagnosed and treated patients. To rule out treatment bias, we plotted the 245 DML according to treatment status, and found no difference between both groups \( (P = 0.44; \) Fig. 2A). To determine the effect of large genomic abnormalities on the methylation of genes in MM, we compared the methylation levels of hyperdiploid and nonhyperdiploid tumors as well as patients with or without 1q gain, 17p deletion, and 13 monosomy using our 245 DML list. As cytogenetic data were not available for the majority of our sample set, we extrapolated the genomic status from corresponding aCGH data available for 129 MM samples. Samples were classified as hyperdiploid \( (H, n = 66) \) if they had whole chromosome gains at minimally three odd numbered chromosomes (excluding chromosome no. 13). The remaining samples were deemed nonhyperdiploid \( (NH, n = 63) \) (Supplementary Table S1). There was no statistical significance between \( H \) and \( NH \) tumors \( (P = 0.978; \) Fig. 2B) or between patients with or without 17p deletions \( (P = 0.53; \) Fig. 2B). However, there seemed to be a slight trend toward hypomethylation in patients without 13 monosomy \( (P = 0.0198) \) and with 1q gain \( (P = 0.0052) \), although the medians differed by less than 1% (Fig. 2B). However, when individual probes were examined, there were no CpG loci that passed DML criteria because none of the loci differed by greater than 20% between the two groups in the case of 13 monosomy. When individual loci were examined in the case of 1q gain, only one locus of 245 \( (SFTPA1_E340_R) \) was deemed a DML; however, it was hypomethylated in patients with 1q gain by only 23%. Independent analyses of each cytogenetic marker yielded no significant DML (data not shown). Overall, our data suggest that methylation differences are not associated with specific common genomic alterations in MM.

To rule out a CD138 selection bias, we isolated DNA from JJN3 and MM-1 cells that underwent positive selection for CD138 and concurrently from cells that underwent negative selection (no beads or antibody). After unsupervised clustering analysis of the four conditions, the data show that the JJN3 and MM-1 cluster together tightly regardless of selection and that there is no significant difference between the means of the sorted and unsorted cells as seen in box plots (Fig. 2C).

**Genic hypomethylation occurs during the development and progression of MM**

Most DML in each group, 96.5% (MGUS), 90.3% (SMM), and 93.7% (MM), were hypomethylated compared with NPC (Supplementary Table S2a–c). Interestingly, significantly fewer DML (56.8%) were hypomethylated in HCMCLs (Supplementary Table S2d). HMCL also had larger degrees of change, up to 80% compared with NPC, whereas MM DML differed by no more than 68%. To examine the effect of CpG density on hypomethylated loci, we separately plotted the probes located in CPI and NCG. In each tumor group, the majority of all hypomethylated loci were located in NCG: 84% in MGUS, 81.2% in SMM, and 79.6% in MM (Fig. 3A). However, only 46.6% of DML were located in NCG in HCMCLs (Fig. 3A). To account for the fact that CGI islands represent nearly 70% of all array probes, CGI and NCG methylation values are also represented as a percentage of total NCG and CGI probes represented on the array (Supplementary Table S4). The data suggest that although there is a dramatic preference for NCG island hypomethylation, CGI probes are hypomethylated to a similar degree in these tumors (Fig. 3B).
Hypermethylation is extremely rare in plasma cell neoplasia

Only 22 loci were hypermethylated in the entire data set. There were five hypermethylated DML in MGUS, resulting in \( \sim 3.5\% \) of MGUS-specific DML (Figs. 1C and 3A; Supplementary Table S2a). Hypermethylation occurred in \( \sim 9\% \) of SMM-specific DML and represented \( \sim 6\% \) of MM-specific DML in MM (Figs. 1C and 3A; Supplementary Table S2c). Two genes (\( TRIP6 \) and \( ALOX12 \)) were hypermethylated in all three groups. Hypermethylation in HMCL differed significantly in number, degree of methylation, and genes involved, and accounted for 43.1\% of total DML, nearly 12-fold higher than that seen in clinical MM samples (Supplementary Table S2d). In addition, the degree of change in DML of patient samples differed by no more than 45\%, whereas HMCL had loci that differed by up to 81\% when compared with NPC.

When CpG density was examined, NCG hypermethylation occurred in 1.4\% of all NCG loci in MGUS, 2.42\% in SMM, and 0.9\% in MM (Fig. 3A). Interestingly, HMCL had relatively few NCG hypermethylation events occurring in only 2.91\% of HMCL-specific DML. When CGI hypermethylation was examined, 0.9\% of MGUS DML, 6.66\% of SMM DML, and 5.31\% of MM DML were due to CGI hypermethylation (Fig. 3A). These data indicate that hypermethylation is a relatively rare event in patients with plasma cell tumors.

Differential methylation profiling identifies normal-like subgroup

We used unsupervised hierarchical Euclidean clustering with centroid linkage to visualize DML identified in MGUS, SMM, and MM. Overall, tumor samples were clearly distinguished from NPC; however, a subset of samples in each group co-clustered with NPC and these were referred to as “normal-like” (NL) samples (Fig. 3C). All of the NL samples differed by less than 15\% overall when compared with NPC. There were two SMM and one MM that differed by less than 15\% but did not cluster with other NLs. However, these...
Figure 2. A, box plot demonstrating no difference in overall methylation levels according to whether a patient was treated or newly diagnosed. B, hyperdiploid status, 1q amplification, 17p deletion, or 13 monosomy were unrelated to overall methylation levels. C, heatmap and box plots demonstrating that methylation levels are unchanged in JNJ3 and MM-1 cells after positive and negative CD138 selection.
were included in the NL category. In total, 3 MGUS (25%), 4 SMM (16%), and 13 MM (9.9%) samples were categorized as NL. NL samples were then separated from the rest, and differential methylation analysis was performed as described above. Indeed, there was a dramatic reduction in the number of statistically significant DML: NL-MGUS (14 DML), NL-SMM (30 DML), and NL-MM (29 DML; Supplementary Table S5a–c). NL DML were mostly common with their non-NL counterparts. The median of DML in NL samples was significantly higher ($P < 0.001$) than in non-NL samples (Fig. 3D), and there was no statistical significance between NL samples and NPC. Interestingly, none of the NL samples had 17p deletions but there was no correlation to hyperdiploid status, 13 monosomy, 1q amplification, or treatment status. Median survival could not be compared between groups given that only two deaths occurred in NL cases. However, the median time since diagnosis for NL-MM samples was 40 months for 7 NL samples whereas it was 25.5 months for 77 non-NL-MM samples ($P = 0.03$).

**Validation of GoldenGate DML by Pyrosequencing**

We used Pyrosequencing technology to confirm the methylation status of genes identified as DML by the GoldenGate on 16 MM samples and 4 NPC that were also tested by GoldenGate. For a direct comparison, primers for Pyrosequencing were specifically designed to span the CpG locus/loci captured by GoldenGate although other CpGs were also included in the NL category. In total, 3 MGUS (25%), 4 SMM (16%), and 13 MM (9.9%) samples were categorized as NL. NL samples were then separated from the rest, and differential methylation analysis was performed as described above. Indeed, there was a dramatic reduction in the number of statistically significant DML: NL-MGUS (14 DML), NL-SMM (30 DML), and NL-MM (29 DML; Supplementary Table S5a–c). NL DML were mostly common with their non-NL counterparts. The median of DML in NL samples was significantly higher ($P < 0.001$) than in non-NL samples (Fig. 3D), and there was no statistical significance between NL samples and NPC. Interestingly, none of the NL samples had 17p deletions but there was no correlation to hyperdiploid status, 13 monosomy, 1q amplification, or treatment status. Median survival could not be compared between groups given that only two deaths occurred in NL cases. However, the median time since diagnosis for NL-MM samples was 40 months for 7 NL samples whereas it was 25.5 months for 77 non-NL-MM samples ($P = 0.03$).

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interrogated. The following GoldenGate probes that showed hypomethylation in patient samples were validated: IGF1_E394_F, MMP3_P55_F, MMP7_P613_F, MMP9_E88_R, MMP19_E274_R, MMP19_P306_F, and TRIM29_P261_F. In addition, one hypermethylated DML in MM, RARA_P1076_R, was also examined.

To test for PCR bias, unmethylated DNA and in vitro methylated DNA were mixed at different ratios for each primer pair being tested followed by bisulfite modification, PCR, and Pyrosequencing analysis (EpigenDX). The percentage of methylation obtained from the mixing study was highly correlated, with expected methylation percentages with \( R^2 \) values of 0.8 or higher (data not shown). Pyrosequencing of all eight probes recapitulated hypomethylation (Fig. 4A) and hypermethylation (Fig. 4B) as seen by GoldenGate. Histograms display the percentage of methylation of the same CpG locus/loci as measured by GoldenGate or Pyrosequencing. When additional CpG loci were captured by Pyrosequencing, the mean percentage methylation of all loci is also shown.

**Pathway and gene ontology enrichment**

GeneGo ontologies represented by canonical pathway maps, cellular processes, and enrichment by protein function were queried for differentially methylated genes in MGUS, SMM, and MM samples. Genes were represented indirectly through the proteins they encode and referred to as network objects. For enrichment of the data set by objects from different protein classes, a total of 177 network objects were analyzed, representing the union of all genes in the data set. The number of network objects from the data set for a given protein class is given by \( r \), and the probability to have the given value of \( r \) or higher not occur by chance is given by the \( P \) value (Table 1). The most highly enriched protein class was ligands \( (r = 26) \) followed by proteases \( (r = 13) \), which was followed by receptors \( (r = 20); \) Table 1). There were 23 ligands, 13 proteases, and 19 receptors enriched if MM data were analyzed separately.

Enrichment of canonical signaling pathways and cellular processes resulted in 136, 149, and 188 network objects.
DNA methylation profiling analysis comparing CD138+ sorted genes (6, 16 tides. Relatively little is still known about the degree to which epigenetic modifications involve methylation of CpG dinucleotides. Relatively little is still known about the degree to which CpG methylation contributes to myelomagenesis, and to date most studies have focused on the analyses of single genes (6, 16–27). Thus, the goal of this study is to perform a DNA methylation profiling analysis comparing CD138+ sorted MM and its precursor stages, MGUS and SMM, to NPC to identify important DNA methylation events associated with each tumor stage and tumor progression. This is the first study to show that genic hypomethylation occurs early in the development of MM and increases through disease progression, whereas, surprisingly, CpG hypermethylation is quite rare during plasma cell oncogenesis.

We now know that loss and gain of DNA methylation are observed concurrently and are frequently linked with altered chromatin structure, changes in DNA methyltransferase activity, loss of imprinting, and inactivation/activation of gene expression (15, 28–30). Among solid tumors, global hypomethylation in repetitive DNA elements has been most evident in breast, colon, and hepatocellular carcinomas, among other tumor types, and is known to destabilize the chromosomes and increase the rate of genomic instability and rearrangements (31). With hematologic malignancies, hypomethylation has been reported in chronic lymphocytic leukemia (32). Recently, Bollati and colleagues showed global hypomethylation in repetitive DNA elements in MM (4). Gene-specific demethylation is also known to occur frequently in a range of cancers, including those of the colon, pancreas, and breast (31).

Pathway enrichment of our data using GeneGO ranked the cell adhesion through ECM remodeling pathway as the top-ranking pathway in the data set. According to GO Cellular Processes, proteolysis during ECM remodeling and connective tissue degradation suggests that hypomethylation during myelomagenesis may favor invasion by increasing interactions with bone marrow ECM, leading to the necessary adhesive forces underlying bone invasion and consequently lytic bone lesions.

Surprisingly, we observed an overall low rate of CpG hypermethylation. Several studies have reported hypermethylation in MM for specific genes, including SPARC, BANP3, and TGFBR2 (5, 20). Other genes with reported hypermethylation in MM are VHL, XAF1, EGLN1, EGLN3, CDKN2A, CCND2, TP53, DCC ESRI, CDH1, and CDKN2B, among others (6, 19, 24, 25, 27, 33–37). A number of reasons might explain the discordance in ours versus these other studies. First, a previously reported gene may not have been represented on the array. Second, many previous studies report hypermethylation without the use of NPC as controls, in which the methylated state of the gene is possibly indicative of the normal methylation state. Third, many studies have not used CD138+ sorting, which could result in sample heterogeneity from nonplasma cells. Also, differences can arise depending on which CpG locus was interrogated and how data were analyzed. Last, the bead array technology is a quantitative assay measuring degree of methylation, which is in contrast to reporting the frequency of events by making present/absent calls. There were several instances in our data set in which a locus was considered a DML in MM but for which only a subset of patients had altered methylation. For example, SPARC was not selected as a DML in MM; however, 8% and 25% (MGUS), 28% and 66% (SMM), and 17.3% and 27.5% (MM) had hypermethylation of SPARC as indicated by the SPARC_E50_R and SPARC_P195_F probes, respectively. This is in contrast to the study by Heller and colleagues, which reported no SPARC methylation in MGUS and in only 8% of MM samples examined (5).

Furthermore, the use of HMCLs to identify differentially methylated genes without validation in clinical samples and normal controls may be misleading given the discordance found between HMCL and patient samples in our

![Table 1. Enrichment by protein function for 177 network objects (n) identified as DML](image)

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NOTE: The column denoted by r is the number of network objects from the data set for a given protein class, whereas R is the number of network objects for a given protein class in the complete data set. N represents the total number of network objects in the complete data set. P values <0.05 are significant.
Figure 5. The top-ranked GeneGO pathway maps (A) and cellular processes (B) for MGUS, SMM, and MM. The larger the $-\log(P)$ number, the more significant the data. Eleven of 52 total network objects belong to the data set analyzed. Thermometer bars represent hypomethylation (blue) or hypermethylation (red). Bars are filled relative to the degree of change and numbered according to disease state: MGUS (1), SMM (2), and MM (3). MM is indicated in pink where a pathway member has been previously implicated to MM.
study. Several factors could potentially change methylation patterns in human cell lines including Epstein-Barr virus infection and subsequent immortalization, cell culture conditions, and freezing cycles (12). A recent study corroborates this finding as it also reported that methylation marks are not retained in lymphoblastoid cell lines when compared with white blood cells from which they were derived and are prone to DNA methylation changes in random genomic locations especially after high passage number (12).

The recognition that epigenetic deregulation is important in MM is leading to clinical trials combining demethylating agents with chemotherapy. Agents such as 5-aza-cytidine have not only shown reactivation of putatively silenced genes in HMCL (5) but have also yielded anti-myeloma activity in vitro and led to promising results in clinical trials for other hematologic malignancies (6, 38–40). The presence of a significant degree of global and gene-specific hypomethylation, coupled with relatively little overall hypermethylation in MM, suggests that methylation-independent mechanisms, such as the cytotoxic effects these agents are known to have (41), may explain initial patient response. Thus, one could speculate that demethylation of the genome in the clinic is likely to select for clones with growth advantage due to severe hypermethylation and should be used with caution until further studies are delivered. Nevertheless, there may still be tremendous clinical benefit for detection of methylation changes for diagnostic and prognostic purposes using individual or a group of CpG genic loci. Given increasing hypomethylation with grade and the identification of NL samples, our data suggest that the overall degree of methylation may have some prognostic value and warrants further investigation.

The compendium of data arising from gene expression profiling, aCGH, and other cytogenetic analyses in MM have led to the identification of several genetic subtypes with unique clinicopathologic features and varying disease outcomes (3). In comparison, MGUS and SMM are still poorly understood. Although this gain in understanding genetic underpinnings is leading to some clinical advances in MM (3), the elucidation of epigenetic mechanisms during myeloma genesis is only just beginning. Our study has shown genomic hypomethylation as a mechanism of disease progression in MM. Recent data demonstrating hypomethylation in repetitive DNA elements in MM (4) are consistent with our findings and point toward the importance of DNA demethylation in MM. Although we report that hypermethylation in comparison occurs rarely, further studies are needed to determine the functional and clinical significance of our findings.

Disclosure of Potential Conflicts of Interest

The authors have no potential conflicts of interest to disclose.

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