Azacytidine causes complex DNA methylation responses in myeloid leukemia

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Abstract

Aberrant DNA methylation patterns play an important role in the pathogenesis of hematologic malignancies. The DNA methyltransferase inhibitors azacytidine and decitabine have shown significant clinical benefits in the treatment of myelodysplastic syndrome (MDS), but their precise mode of action remains to be established. Both drugs have been shown the ability to deplete DNA methyltransferase enzymes and to induce DNA demethylation and epigenetic reprogramming *in vitro*. However, drug-induced methylation changes have remained poorly characterized in patients and therapy-related models. We have now analyzed azacytidine-induced demethylation responses in myeloid leukemia cell lines. These cells showed remarkable differences in the drug-induced depletion of DNA methyltransferases that coincided with their demethylation responses. In agreement with these data, DNA methylation analysis of blood and bone marrow samples from MDS patients undergoing azacytidine therapy also revealed substantial differences in the epigenetic responses of individual patients. Significant, transient demethylation could be observed in 3 of 6 patients and affected many hypermethylated loci in a complex pattern. Our results provide important proof-of-mechanism data for the demethylating activity of azacytidine in MDS patients and provide detailed insight into drug-induced demethylation responses. [Mol Cancer Ther 2008;7(9):2998 – 3005]

Introduction

The azanucleosides 5-azacytidine (azacytidine) and 2′-deoxy-5-azacytidine (decitabine) are two closely related compounds that have found increasing clinical use for the treatment of myeloid leukemias (1, 2). Azanucleosides are cytosine analogues that can become incorporated into nucleic acids and thereby exert a wide range of molecular effects. Although the precise mode of action of these drugs remains to be established, both azacytidine and decitabine have been found to be particularly effective in inducing DNA demethylation and epigenetic gene reactivation *in vitro* (3, 4). These effects are caused by covalent trapping of DNA methyltransferases through DNA-incorporated azacytosine bases and subsequent degradation of the trapped enzymes (5, 6).

Azacytidine and decitabine have shown significant clinical benefits in the treatment of myelodysplastic syndrome (MDS; refs. 7, 8) and have subsequently obtained Food and Drug Administration approval for this indication. In addition, decitabine has also been used successfully for the treatment of acute and chronic myelogenous leukemias (2). For these indications, several studies have shown a significant demethylating activity of decitabine. It has been shown that genomic cytosine methylation levels can decrease from 4% to 2% during therapy in a subset of MDS patients (9). Similarly, it has been shown that the methylation levels of genomic LINE-1 elements can decrease from 70% to 60% during decitabine therapy (10).

More recently, methylation analyses have also been used to characterize epigenetic changes in azacytidine-treated MDS/AML patients. It was shown that azacytidine treatment can result in the demethylation of the p15 tumor suppressor gene (11). In addition, azacytidine treatment also caused minor demethylation of LINE-1 elements from 63% to 58% (12). However, formal proof-of-mechanism data (the demonstration of genome-wide demethylation) has not been established yet. In addition, the effects of azacytidine on genomic methylation patterns remain poorly characterized both in cellular models and in patients.

We have used various technologies to comprehensively characterize azacytidine-induced DNA methylation changes in myeloid leukemia cell lines and in patients. Our results show differential demethylation responses in myeloid leukemia cell lines and significant but transient demethylation in a subset of patients. Array-based analysis of genomic DNA methylation patterns revealed widespread demethylation of numerous markers, which provides substantial potential for drug-induced epigenetic reprogramming.

Materials and Methods

Cell Culture

HEL, HL-60, and K562 cells were cultured in RPMI 1640 supplemented with 5% L-glutamine, 10% FCS (Invitrogen),

100 units/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate (Invitrogen). Cell viability was determined with the CellTiter-Blue Viability Assay (Promega) as described in the instruction manual (15,000 cells per well). Cells were incubated with azacytidine (Sigma) for 72 h, unless indicated otherwise.

Patients

All patients fulfilled the WHO criteria for MDS and informed consent of each patient was obtained (Department of Hematology/Oncology, University of Heidelberg Medical Center). 5-Azacytidine (Vidaza; Pharmion) was given s.c. for 7 consecutive days at a dosage of 75 mg/m^2. Treatment cycles were repeated every 4 weeks. Blood samples were collected immediately before the first treatment cycle and every 7 days during therapy. Bone marrow aspirates were obtained during various time points, as indicated. Peripheral blood mononuclear cells were isolated by density-gradient centrifugation (Biocoll Separating Solution; Biochrom), washed in PBS without calcium and magnesium, and lysed in TRIZOL reagent (Invitrogen).

Flow Cytometry

For cell cycle analysis, azacytidine was added to a total of 1×10^6 cells growing in regular medium for 24 h. Cells were washed once with $1\times$ PBS, fixed with 100% ethanol at 4°C for 45 min, and incubated with propidium iodide solution (50 μg/mL) containing RNase (50 μg/mL) at 37°C for 30 min. DNA fluorescence was measured using a Becton Dickinson FACSCanto II flow cytometer.

DNA Methyltransferase 1 Trapping Assay

Cells were treated with their cell line-specific IC$_{50}$ concentration for the indicated period. Protein extracts were prepared as described previously (13) and analyzed by Western blotting using antibodies against DNA methyltransferase 1 (DNMT1) and β-actin (Santa Cruz Technology).

DNA Methylation Analysis

Genomic DNA was purified using the DNeasy Blood and Tissue Kit (Qiagen) or TRIZOL (Invitrogen). Global methylation levels were determined by capillary electrophoresis as described previously (14). For gene-specific methylation analysis, genomic DNA was deaminated using the EpiTect Bisulfite Kit (Qiagen) and analyzed by combined bisulfite restriction analysis (15). Primers were used as described previously (9, 11, 16–18). PCR products were digested as follows: C1S2 and LINE-1, *Hin*fI; p15, *Bst*UI; HoxA4, *Taq*I; and RIL, *Hha*I. Digested PCR products were separated on agarose gels. Primers for DAPK1 and MLF1 bisulfite sequencing were DAPK1_for (AGTTTTAA-TAAAGGGTGTTTTTTTT), DAPK1_rev (ACAT-CAATCTCCAATCCTTTTAAAA), MGMT_for (TGGTAAATTAAGGTATAGAGTTTTAGG), MGMT_rev (AAAACCTAAAAAAAAACAAAAAAAC), MLF1_for (TTTGGTTTAAATTTTGATTTTTTTT), and MLF1_rev (AACTACTATTCAACATCCTAAACAT). PCR products were gel extracted and cloned using the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. Array-based methylation analysis was done on

universal bead arrays (19) using the GoldenGate Methylation Cancer Panel I (Illumina) according to the manufacturer's instructions. Briefly, bisulfite-converted DNA was hybridized to methylation-specific oligonucleotides and washed. The hybridized oligonucleotides were then extended and ligated to create templates for PCR amplification with fluorescently labeled primers. Amplification, labeling, hybridization, and array imaging were provided by the DKFZ Genomics and Proteomics Core Facility.

Results

Characterization of Azacytidine Responses in Myeloid Leukemia Cell Lines

The increasing clinical use of azacytidine has raised numerous questions regarding the epigenetic changes induced by this drug. As an initial step toward characterizing the effects of azacytidine in a therapy-related model, we used three human myeloid leukemia cell lines (HEL, HL-60, and K562). We determined the sensitivity of these cell lines to azacytidine in viability assays and found that all cell lines had IC$_{50}$ concentrations in the low micromolar range (1-7 μmol/L; Fig. 1A). Thus, none of the myeloid leukemia cell lines used in this study appeared resistant to azacytidine. Cells were then treated with their respective
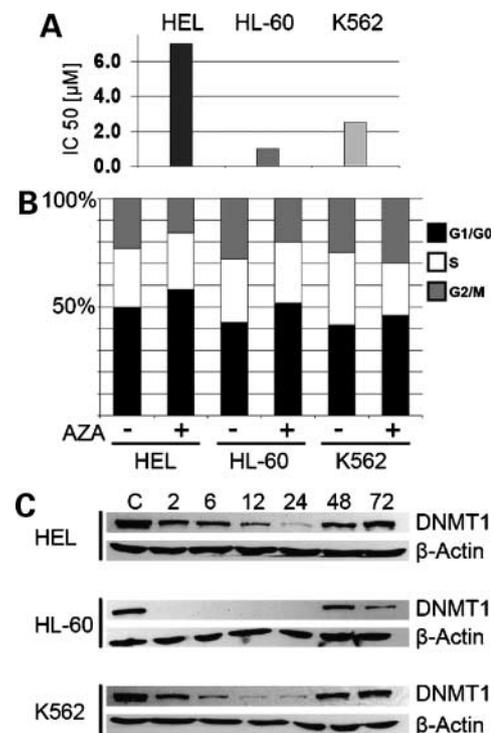
Figure 1. Myeloid leukemia cells as therapy-related models for azacytidine treatment. **A,** determination of IC$_{50}$ drug concentrations for three different human myeloid leukemia cell lines. **B,** flow cytometric cell cycle analysis. Cells were treated with their respective cell line-specific IC$_{50}$ drug concentrations for 24 h. DNA content was measured by staining with propidium iodide and flow cytometry. Each experiment was repeated at least three times. **C,** DNMT1 trapping assay. Cells were treated with the cell line-specific IC$_{50}$ drug concentration for the time indicated and DNMT1 protein was detected by Western blotting of protein extracts.

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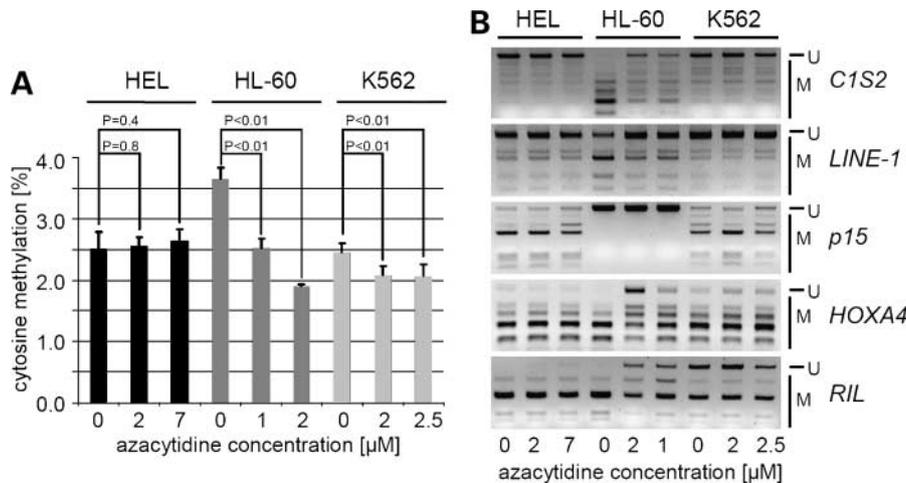
Figure 2. Myeloid leukemia cell lines show differential demethylation at the total cytosine methylation level and at marker loci. **A,** determination of genomic cytosine methylation levels in cells treated with azacytidine at the concentrations indicated. All measurements were repeated at least three times. *Bars,* SD. **B,** combined bisulfite restriction analysis of the methylation pattern of two repetitive elements (LINE-1 and C1S2) and three tumor suppressor genes (p15, RIL, and HOXA4). *U,* unmethylated epialleles; *M,* methylated epialleles.

cell line-specific IC_{50} concentrations and their cell cycle phase distributions were analyzed after 24 h by flow cytometry. All three cell lines showed similar overall cell cycle profiles and a minor drug-dependent increase (4-9%) in the G_1 fraction (Fig. 1B).

Azacytidine-mediated demethylation is generally considered to be the consequence of covalent trapping and depletion of the DNA methyltransferases. For this reason, we analyzed the ability of azacytidine to covalently trap the DNMT1 enzyme in the myeloid leukemia cell lines. Cells were treated at equitoxic (IC_{50}) drug concentrations and protein extracts were prepared at various time points ranging from 2 to 72 h. Western analysis revealed evidence for DNMT1 trapping in all three cell lines (Fig. 1C). However, there were pronounced differences in the strength and duration of DNMT1 depletion (Fig. 1C). DNMT1 was undetectable in HL-60 cells after 2 h of drug incubation. In K562 cells, DNMT1 became strongly reduced after 6 h, but low amounts of protein could be observed at all time points. HEL cells appeared relatively resistant to DNMT1 depletion and the protein remained detectable in all samples (Fig. 1C). The rapid depletion of DNMT1 in HL-60 cells might indicate that metabolic activation of azacytidine (20) is particularly effective in this cell line. In addition, the results predicted that HL-60 cells should be relatively sensitive to azacytidine-mediated demethylation, whereas HEL cells should be relatively resistant.

Characterization of Azacytidine-Induced Demethylation in Myeloid Leukemia Cell Lines

We then sought to characterize azacytidine-induced demethylation in human myeloid leukemia cell lines. Cells were treated for 72 h with 2 μmol/L azacytidine to allow DNA replication after DNMT1 depletion (passive demethylation) and total cytosine methylation levels were determined. This revealed substantial demethylation in HL-60 cells, minor but significant demethylation in K562 cells, and no significant methylation changes in HEL cells (Fig. 2A). To minimize the effects of cell line-specific differences in drug toxicity, we repeated the methylation analysis under equitoxic conditions and determined ge-

nomic methylation levels following incubation at the respective cell line-specific IC_{50} drug concentrations (Fig. 2A). The results were very similar to those obtained with 2 μmol/L azacytidine. Thus, demethylation responses between cell lines varied greatly under conditions that induce similar cellular responses (IC_{50} drug concentration). This indicated that cellular and epigenetic responses to azacytidine can be independent.

To further characterize azacytidine-induced demethylation, we used combined bisulfite restriction analysis and determined methylation patterns of five epigenetic markers that have been linked previously to myeloid leukemias: C1S2 and LINE-1 elements are heavily methylated in normal tissues but have been shown be demethylated following decitabine treatment (9, 16). p15 and RIL represent two tumor-suppressor genes that have been described to be hypermethylated in MDS (17, 21) and aberrant methylation of HOXA4 has been described in myeloid and lymphoid leukemias (18). No methylation changes were detectable in HEL cells, which is consistent with the results from the analysis of total cytosine methylation levels in these cells. HL-60 cells showed detectable demethylation of C1S2, LINE-1, RIL, and HOXA4 (Fig. 2B). However, with the exception of C1S2, the effects appeared rather weak, which indicated that the methylation changes of the LINE-1, RIL, and HOXA4 might not adequately reflect the genome-wide methylation changes. Similarly, none of the gene-specific methylation assays detected any changes in K562 cells, although this cell line had shown a significant azacytidine-dependent reduction in the genomic cytosine methylation level.

Array-Based Analysis of DNA Methylation Patterns in Myeloid Leukemia Cell Lines

To further expand our characterization of azacytidine-induced demethylation, we used an array-based methylation profiling platform that allows the analysis of 1,505 CpG dinucleotides representing 807 cancer-associated genes (see Materials and Methods for details). In a first set of experiments, we validated the technology by analyzing the methylation patterns of untreated myeloid

leukemia cell lines. The comparison of the methylation patterns of two independent batches of HL-60 cells produced highly similar results, with a correlation coefficient of 0.96 (Fig. 3A). In contrast, the comparison of methylation patterns between HL-60 and K562 cells indicated different methylation patterns, with a correlation coefficient of 0.16 (Fig. 3A). For further validation, we used bisulfite sequencing to determine the methylation patterns of two leukemia-associated genes, DAPK1 (22) and MLF1 (23), which the array predicted to be strongly methylated in HL-60 cells and to be unmethylated in K562 cells (Fig. 3B). This prediction was accurately confirmed, showing 100% (DAPK1) and 98% (MLF1) methylation in HL-60 cells and 3% (DAPK1) and 0% (MLF1) methylation in K562 cells (Fig. 3B). We also confirmed the ability of the array to identify intermediate methylation changes. Bisulfite sequencing of the MGMT promoter showed heterogeneous methylation patterns in both cell lines with methylation levels of 71% in K562 cells and 33% in HL-60 cells, which corresponded well to the array results (Fig. 3B). Together, these results suggest that the array-based analysis provides an accurate representation of cellular DNA methylation patterns.

In subsequent experiments, we used the array to characterize azacytidine-dependent demethylation in mye-
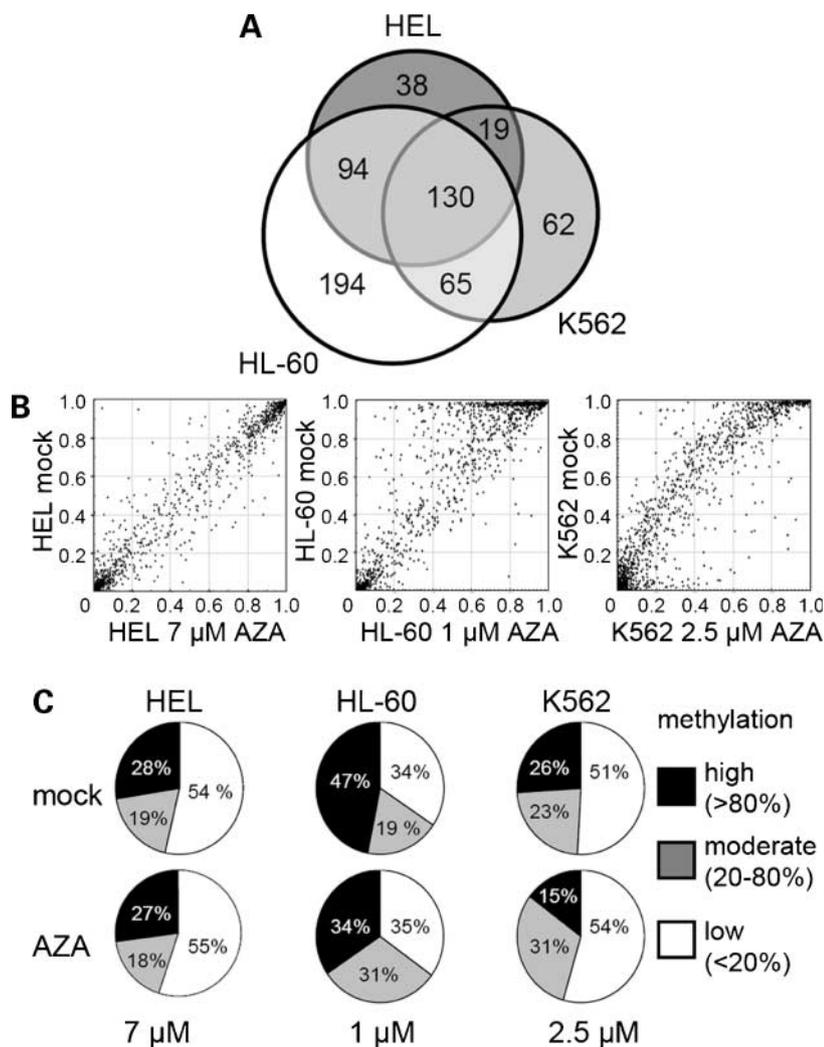
loid leukemia cell lines. Untreated cell lines revealed a high number of hypermethylated (>80% methylation) CpG island associated markers and a substantial overlap between the cell lines tested (Fig. 4A). Cells were then drug-treated under equitoxic conditions, at their respective cell line-specific IC_{50} concentrations, and genomic DNA was isolated after 72 h. DNA methylation patterns were determined and the resultant data sets were compared between azacytidine-treated cells and untreated control cells. The results revealed demethylation of numerous hypermethylated (>80% methylation) markers in HL-60 and K562 cells, and no consistent demethylation trend in HEL cells (Fig. 4B and C). These data confirm and complement the results obtained by capillary electrophoresis and combined bisulfite restriction analysis (Fig. 2). In conclusion, our data show heterogeneous demethylation patterns in myeloid leukemia cell lines and suggest that demethylation responses might be complex in patients.

Characterization of Demethylation Responses in Azacytidine-Treated MDS Patients

To characterize the demethylation responses in MDS patients undergoing azacytidine therapy, we treated 10 MDS patients with azacytidine using standard recommended dosage and administration (Fig. 5A). Of these 10
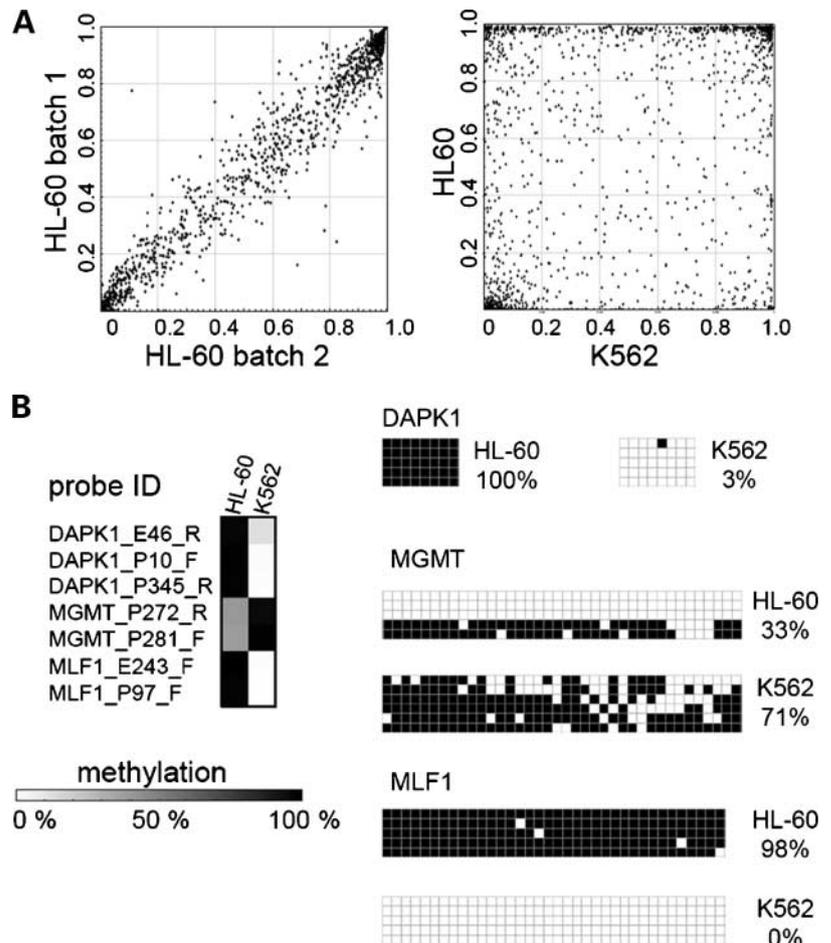
Figure 3. Array-based analysis of DNA methylation patterns. **A,** reproducibility of array-based methylation profiles. Two independent batches of HL-60 cells show highly similar methylation patterns ($r = 0.96$). In contrast, the methylation pattern of HL-60 cells was distinct from that of K562 cells ($r = 0.16$). Scatter plots show methylation values of individual markers before and after drug treatment; methylation values range from 1 (100%) to 0 (0%). **B,** validation of array-predicted methylation patterns of DAPK1, MGMT, and MLF1 promoter methylation by bisulfite sequencing.

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Figure 4. Myeloid leukemia cell lines show differential genomic demethylation patterns. **A,** Venn diagram of highly methylated (>80% methylation) CpG island associated markers in the three cell lines. **B,** array-based analysis of locus-specific DNA methylation patterns in myeloid leukemia cell lines treated with azacytidine. Scatter plots show methylation values of individual markers before and after drug treatment; methylation values range from 1 (100%) to 0 (0%). **C,** diagrams show drug-dependent changes in methylation levels of all 1,505 markers on the array.

patients, 6 showed hematologic improvement or stable disease and could complete at least one treatment course consisting of four treatment cycles.[3] Blood samples were obtained at weekly intervals and total cytosine methylation levels were quantified by micellar electrokinetic chromatography of genomic DNA. This revealed significantly different demethylation responses between the patients analyzed. Methylation levels remained unchanged in patients 2, 3, and 6 (Fig. 5B; data not shown), whereas significant therapy-dependent demethylation could be detected in patients 1, 4, and 5 (Fig. 5C). In patient 1, methylation levels decreased from 4.1% before treatment to 2.0% ($P < 0.01$) in week 3, became restored to 3.6% ($P < 0.01$) in week 4, and again decreased to 1.9% ($P < 0.01$) in week 7. In patient 4, methylation levels decreased from 4.0% before treatment to 2.8% ($P < 0.01$) in week 7 and showed cyclic changes that coincided with the drug

administration schedule. Patient 5 also showed cyclic demethylation ($P < 0.01$), and the lowest methylation levels were detected 1 week after drug administration. Methylation levels of bone marrow aspirates were close to baseline methylation levels, when the samples were obtained at the beginning of a treatment cycle (Fig. 5B and C). In one patient (patient 4), we were able to obtain bone marrow aspirates at the end of every treatment week, and the methylation levels were significantly reduced during later cycles (Fig. 5C), which is consistent with earlier observations from decitabine-treated MDS patients (9). These results provide definite confirmation for a significant demethylating activity of azacytidine in a subset of MDS patients. Importantly, demethylation was transient and methylation levels were restored to baseline levels within individual treatment cycles.

For a detailed analysis of demethylation responses in patients, we used the methylation array to analyze samples from the first treatment cycle of patient 1, where capillary electrophoresis has shown a pronounced reduction in the genomic cytosine methylation level. The results indicated

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[3] U. Mahlknecht, in preparation.

widespread demethylation of many loci during weeks 1 to 3, and partial restoration of methylation patterns in week 4 (Fig. 6A). Eighty-one of the 297 CpG island-associated markers that were methylated (>20% methylation) before therapy were demethylated by ≥20% in weeks 1 to 3. The fraction of highly methylated (>80% methylation) gene markers was substantially reduced from 22% to 10% and increased again to 16% in week 4 (Fig. 6B). These results provided an important confirmation of our biochemical cytosine methylation analysis and suggested that many genes can become demethylated by azacytidine treatment.

We then compared the methylation responses of patients 1, 4, and 5 and analyzed pretreatment samples to samples obtained after week 1. The results indicated a substantial similarity of the methylation patterns at highly methylated (>80% methylation) CpG island markers. Of the 123 markers methylated before therapy in at least one patient, 56 (46%) were consistently methylated in all three patients (Fig. 6C). A comparison of the 56 markers hypermethylated in all three patients indicated demethylation of various markers in all three patients, but no consistent demethylation pattern was conserved between all three patients (Fig. 6D). These data provide further confirmation for the demethylating activity of azacytidine in patients and also illustrate the complexity of demethylation responses. The identification of epigenetic biomarkers for drug-induced demethylation will be an important aspect of future research and will require the analysis of substantially higher patient numbers.

Discussion

Based on the ability of azacytidine to inhibit DNA methyltransferases *in vitro*, it is generally assumed that epigenetic changes play an important role in mediating the drug's effects. Our results provide definite evidence for a large-scale demethylating activity of azacytidine in a subset of MDS patients and thus provide important proof-of-mechanism data in the approved indication.

Earlier studies have suggested that methylation of the p15 tumor suppressor gene might be frequent in MDS (21) and that demethylation and reactivation of p15 might contribute to clinical drug responses to decitabine and azacytidine (11, 24). These findings established p15 as a candidate biomarker for epigenetic therapy responses. Somewhat surprisingly, we could not detect any evidence for p15 methylation in our patient samples and p15 was unmethylated in the demethylation-sensitive HL-60 cells. This is in agreement with other studies that suggest a comparatively low incidence of p15 methylation in MDS/AML patients (25) and the lack of correlations between p15 methylation changes and patient responses (26).

The demonstration of widespread demethylation of numerous markers represents a significant advance over previous studies. It will now be important to analyze complex demethylation patterns in larger patient numbers to identify candidate biomarkers for epigenetic responses. The demethylation of many markers also suggests a considerable potential for epigenetic reprogramming (27)
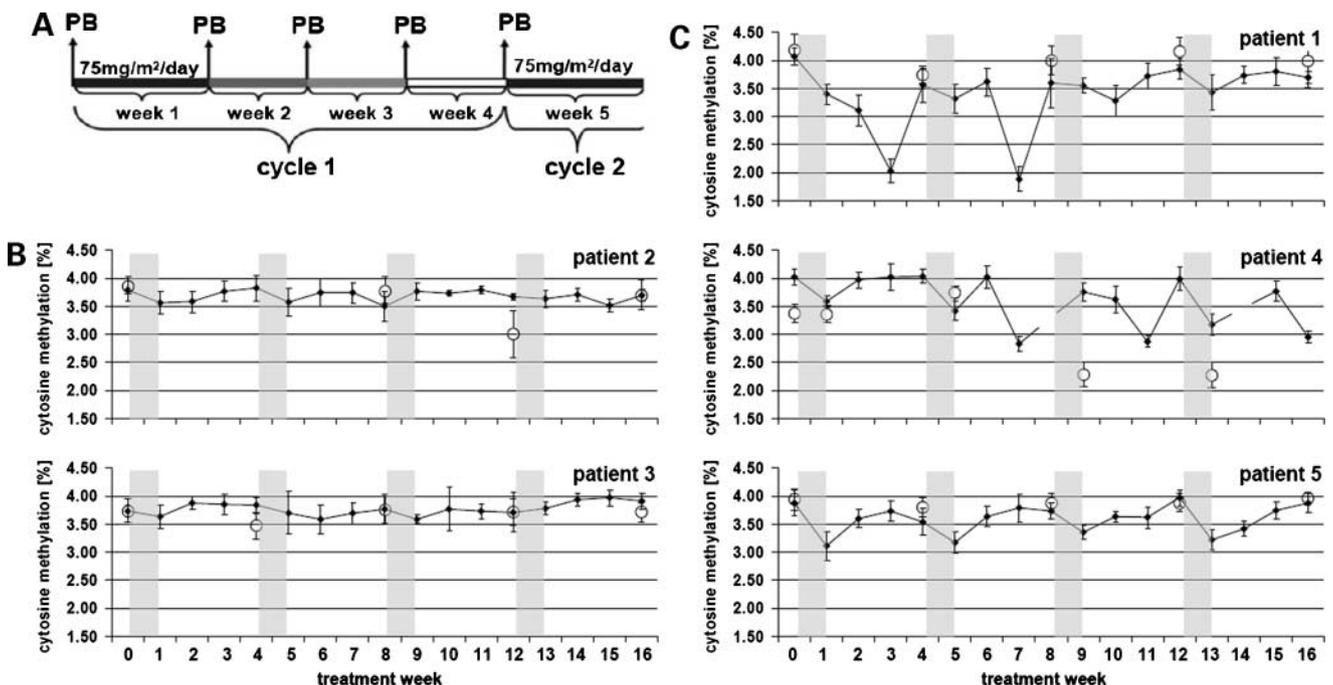
Figure 5. Transient reductions in the genomic cytosine level in a subgroup of MDS patients. **A,** schematic overview about the treatment protocol. **B, C,** high-resolution analysis of genomic DNA methylation levels in peripheral blood mononuclear cells (*black diamonds*) or bone marrow aspirates (*white circles*) over a complete treatment course of four cycles. *Gray columns,* times of treatment. All measurements were repeated at least three times. *Bars,* SD. Significant ($P < 0.01$) drug-induced DNA demethylation was detectable in patients 1, 4, and 5.
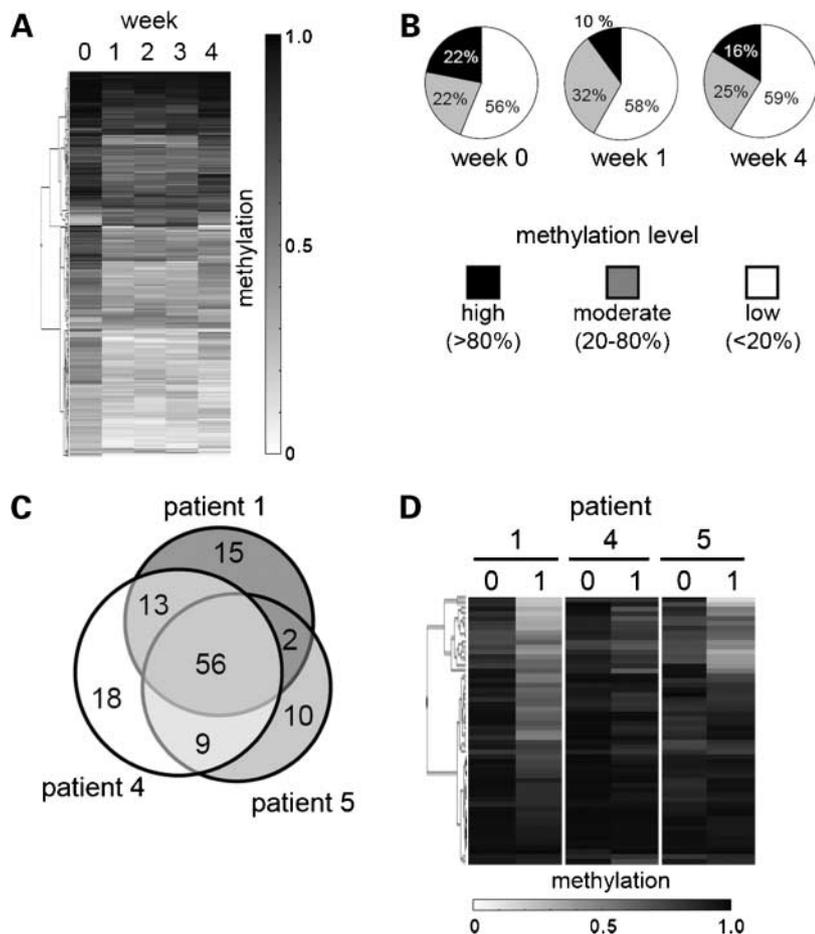
Figure 6. Widespread DNA demethylation in a subgroup of MDS patients. **A,** demethylation of methylated (>20% methylation before therapy) CpG islands. The heatmap shows methylation changes of CpG island-associated markers during the first treatment cycle (weeks 0-4) of patient 1. **B,** diagrams show drug-dependent changes in methylation levels of all 1,505 markers on the array. **C,** Venn diagram of highly methylated (>80% methylation) CpG island associated markers in patients 1, 4, and 5. **D,** comparative analysis of demethylation patterns in patients 1, 4, and 5. The heatmap indicates the methylation status of the 56 markers identified in **C** before therapy (*0*) and after the first treatment week (*1*).

and it will be important to elucidate the role of epigenetic changes in clinical responses. We observed similar clinical responses in all 6 patients that completed at least one treatment course (stable disease, minor hematologic improvement), but only a subset of these patients (3 of 6) showed detectable demethylation responses. This suggests that DNA demethylation is not required for clinical responses to azacytidine.

Potentially independent cellular and epigenetic responses were also observed in myeloid leukemia cell lines. It is noteworthy that azacytidine showed substantial growth inhibition at low micromolar concentrations in all myeloid leukemia cell lines tested but that demethylation responses differed strongly between the cell lines. Both HL-60 and K562 cells showed significant demethylation, whereas HEL cells were resistant to the azacytidine-mediated demethylation, even under conditions with substantial cellular drug activity (at the cell line-specific IC_{50} drug concentration). This confirms that cellular and epigenetic responses to azacytidine can be independent and indicates that factors involved in metabolic drug activation play an important role in mediating demethylation responses. Similar factors could conceivably also play a role in determining epigenetic responses in patients and might

explain why only half (3 of 6) of the patients showed detectable drug-dependent demethylation.

In cell lines, DNA demethylation responses seemed to be associated with the strength and duration of DNMT1 depletion. It has been shown that drug-dependent depletion of DNMT1 also occurs in patients (28), but the kinetics of this process and its relation to therapy responses have not been analyzed yet. It is conceivable that DNMT1 depletion can be influenced by patient-specific factors that modulate the metabolization and DNA incorporation of azacytidine (20). The identification of drug-dependent demethylation signatures might thus require more comprehensive approaches and could also be facilitated by the analysis of genetically more homogeneous subpopulations of MDS patients that have been stratified according to their karyotype or other clinicopathologic variables (1).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Mol Cancer Ther 2008;7:2998-3005.

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