Characterization of DNA Demethylation Effects Induced by 5-Aza-2′-Deoxycytidine in Patients with Myelodysplastic Syndrome

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Abstract

Azanucleoside drugs such as 5-azacytidine (Vidaza) and 5-aza-2′-deoxycytidine (decitabine, Dacogen) function as DNA methyltransferase inhibitors *in vitro* and represent promising new drugs for the treatment of myelodysplastic syndrome (MDS) and acute myeloid leukemia. In this study, we aimed to determine the effect of decitabine on the genomic methylation level in MDS patients. Comparison of different assays established micellar electrokinetic chromatography as a reliable method for the analysis of genomic methylation levels. When used for the determination of DNA methylation levels in bone marrow DNA from MDS patients during various time points of decitabine treatment, the results revealed a significant (up to 70%) demethylation in five of seven patients. Interestingly, genome-wide demethylation appeared after karyotype normalization, which suggests demethylation of nonclonal cells. Drug-induced demethylation dynamics were also confirmed by bisulfite sequencing of pericentromeric satellite elements. Our results are the first to show a genome-wide demethylating activity of decitabine in tumor material. In addition, our data uncovers novel targets of decitabine-mediated demethylation that are important for the refinement of treatment schedules with demethylating drugs. (Cancer Res 2005; 65(16): 7086-90)

Introduction

5-Azacytidine (Vidaza) and 5-aza-2′-deoxycytidine (DAC, decitabine) represent the two most prominent DNA methyltransferase inhibitors that are being used in clinical practice (1). 5-Azacytidine has shown antileukemic effects in several clinical trials (2) and was recently approved for the treatment of myelodysplastic syndrome (MDS). The closely related compound decitabine has shown similar efficacy in the treatment of leukemias (3, 4) and is currently awaiting approval for the treatment of MDS. When given at low doses, both inhibitors are characterized by promising response rates in high-risk MDS patients that are not eligible for aggressive chemotherapy due to their age and comorbidity (2, 3). This has established the drugs as an attractive treatment option for MDS and possibly for other leukemias as well. However, DAC and 5-azacytidine at higher doses are also characterized by a substantial cytotoxicity, which has been the driving force behind the establishment of the currently used low-dosage treatment regimens (2–4). Under these conditions, the estimated drug serum level decreases below the concentration that is normally used for *in vitro* experiments and the observed demethylating effect at two isolated genomic loci (5, 6) could potentially have been influenced by the cytotoxic activity of the drugs. Thus, an important molecular end point for treatment with demethylating agents, the unambiguous demethylation of genomic DNA has not yet been established. In this study, we show that genomic DNA from bone marrow aspirates becomes strongly demethylated in the majority of decitabine-treated MDS patients. The global demethylation effect was transient and appeared later than the karyotypic normalization, which confirms that the drug demethylates nonclonal bone marrow cells. In addition, our results reveal novel targets for decitabine-mediated demethylation, the *p21* tumor suppressor gene and the chromosome 1 satellite 2 (C1S2) repeats, with different implications for the further development of epigenetic therapies.

Materials and Methods

Cell culture. KG-1 and Jurkat cells (American Type Culture Collection, Rockville, MD) were cultured in RPMI 1640 supplemented with 5% L-glutamine and 10% FCS (Invitrogen, Karlsruhe, Germany). HCT116 cells and HCT116 DNMT1;DNMT3B double knock-out (DKO) cells (7) were cultured in McCoy's 5a medium supplemented with 10% FCS. For the analysis of LINE-1 and Alu methylation, 5 μmol/L 5-aza-2′-deoxycytidine (Sigma, Heidelberg, Germany) was added after a 24-hour seeding period and cells were harvested 72 hours later (8). For the analysis of C1S2 methylation, cells were incubated with DNA methyltransferase inhibitors, as indicated and harvested after 72 hours. Genomic DNA was prepared with the DNeasy tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Patient samples. Bone marrow mononuclear cells from MDS patients (median age, 70 years) were collected before and during treatment with low-dose decitabine (kindly provided by Pharmachemie B.V., Haarlem, the Netherlands) within a phase 2 study (3), approved by the local ethics committee after obtaining informed written consent. As control samples, bone marrow mononuclear cells were derived from three healthy donors (aged 29, 30, and 36 years, respectively). Genomic DNA was prepared with the DNeasy tissue kit (Qiagen) according to the manufacturer's instructions.

DNA methylation analysis. A detailed protocol for whole-genome methylation analyses by capillary electrophoresis has been published previously (9). Bisulfite PCR methylation analysis of Alu and LINE-1 elements was done essentially as described (8). For methylation analysis of *p21* and C1S2, genomic DNA was treated with sodium bisulfite (10) and analyzed by COBRA (11) using the following primers: p21_forward 5′-TGAGAGAGATTTTTTGGTAGGAAGA-3′, p21_reverse 5′-AAACAAATCAACCCTTAAACCATAA-3′. PCR cycling conditions were 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 40 seconds for 35 cycles. The PCR product (644 bp) was digested with *Bst*UI (New England Biolabs). For C1S2, the following primers were used: C1S2_forward 5′-ATGGAATTTTTATGAAATTGAAATG-3′, C1S2_reverse 5′-CATTCCATTAAATAATTCCATTC-3′. DNA was amplified in 35 cycles of 95°C for 30 seconds, 51°C for 30 seconds, and 72°C for 30 seconds. The PCR product (210 bp) was digested with *Hin*fI (New England Biolabs). Digested PCR products were separated on agarose gels and quantified by densitometry. For bisulfite sequencing, PCR products were gel-extracted and cloned using the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions.

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Results and Discussion

The global DNA methylation level of decitabine-treated leukemia patients has been previously estimated by bisulfite PCR of euchromatic repetitive DNA elements and the results suggested only minimal demethylation in peripheral blood cells from drug-treated patients (12). However, it remained unclear whether the methylation status of these elements is representative of the global genome methylation level, especially because a large fraction of methylated DNA is present in the context of centromeric and pericentromeric repeats. We have previously established a sensitive method for the precise determination of whole-genome methylation levels in clinical samples. This procedure uses capillary electrophoresis for the separation of chemically derivatized nucleotides to determine cytosine methylation levels in genomic DNA (9). The validity of the results obtained by this kind of analysis has been confirmed in a number of investigations with diverse experimental backgrounds (see for example, refs. 13, 14).

In order to determine the relationship between the methylation status of euchromatic repetitive elements and the genomic cytosine methylation level, we treated HCT116 cells with 5 μmol/L DAC, as described previously (8), and analyzed genomic DNA with both methods (bisulfite PCR and capillary electrophoresis). For controls, we also included untreated HCT116 cells and a genetically engineered HCT116 cell line that lacks the activity of the major DNA methyltransferases DNMT1 and DNMT3B (7). The results obtained with bisulfite PCR showed a relatively weak demethylation of Alu and LINE-1 elements in DAC-treated cells (Fig. 1*A* and *B*). However, both elements were almost completely demethylated in DNMT1;DNMT3B DKO cells (Fig. 1*A* and *B*), which is in agreement with published data (7) and represents an important control for the PCR assay. We then analyzed the same DNA samples by capillary electrophoresis, which provides quantitative data about the whole-genome cytosine methylation level. Analysis of DNA from DAC-treated HCT116 cells revealed a strong reduction of genomic cytosine methylation (Fig. 1*C*), which confirmed the pharmacological activity of the inhibitor in these cells. The genome methylation level of DKO cells was also reduced (Fig. 1*C*), albeit less strongly. The significant genome methylation in DKO cells could be due to DNMT3A activity and was also observed in the originally published DKO8 cell line (7). In summary, DAC induced a relatively weak (20%) demethylation of euchromatic repetitive elements, whereas it caused a much stronger (60%) reduction in the genomic cytosine methylation level. These discrepancies indicate that global methylation changes can be substantially underestimated by the analysis of euchromatic repetitive elements.

Until now, MDS represents the only form of cancer for which the use of an azanucleoside inhibitor has gained Food and Drug Administration approval. The disease is characterized by bone marrow failure and we therefore focused our subsequent

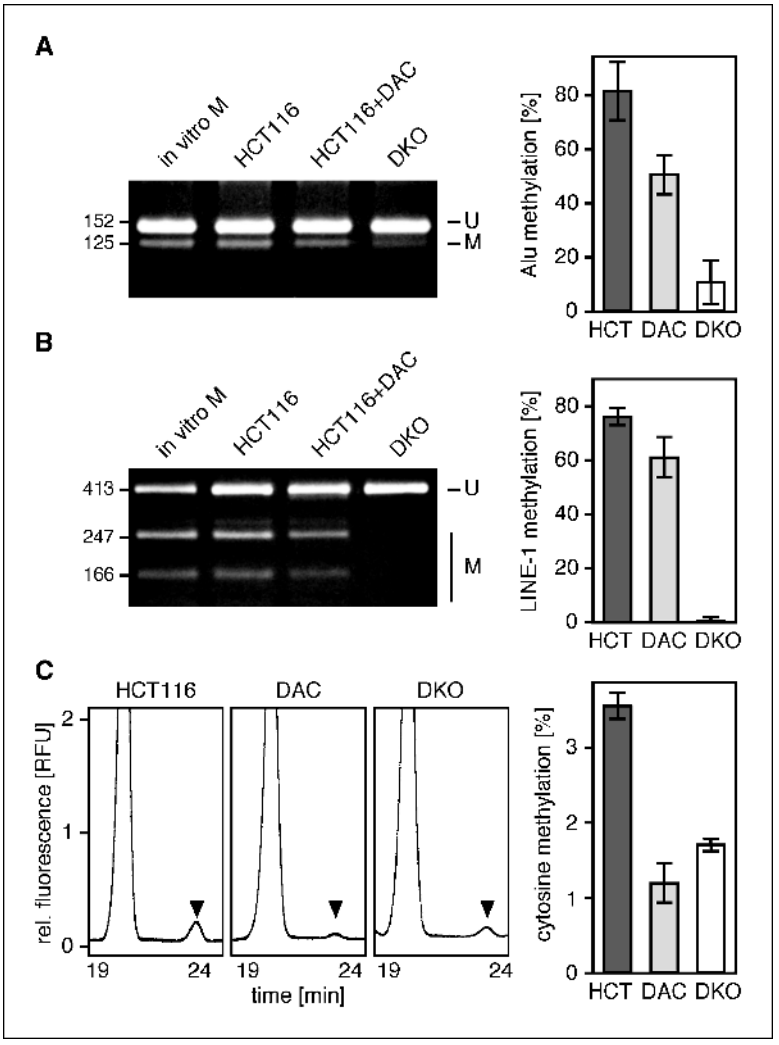

Figure 1. Comparison of bisulfite PCR and capillary electrophoresis assays for the determination of whole genome methylation levels. *A,* bisulfite PCR assay for Alu methylation. *B,* bisulfite PCR assay for LINE-1 methylation. HCT116 cells were cultured in the presence (+DAC) or absence of 5-aza-2′-deoxycytidine. "*In vitro* M", *in vitro* methylated DNA from HCT116 cells; DKO, DNA from DNMT1;DNMT3B double-knock-out HCT116 cells; "U" bands, unmethylated epialleles; "M" bands represent methylated epialleles. Quantitative results were derived by densitometric analysis of ethidium bromide stained agarose gels. *C,* representative electropherograms from a whole-genome methylation analysis of the same DNA samples. The left peak represents cytosine, the smaller, right peak (arrowhead) represents 5-methylcytosine. *Columns,* means; *bars,* SD.

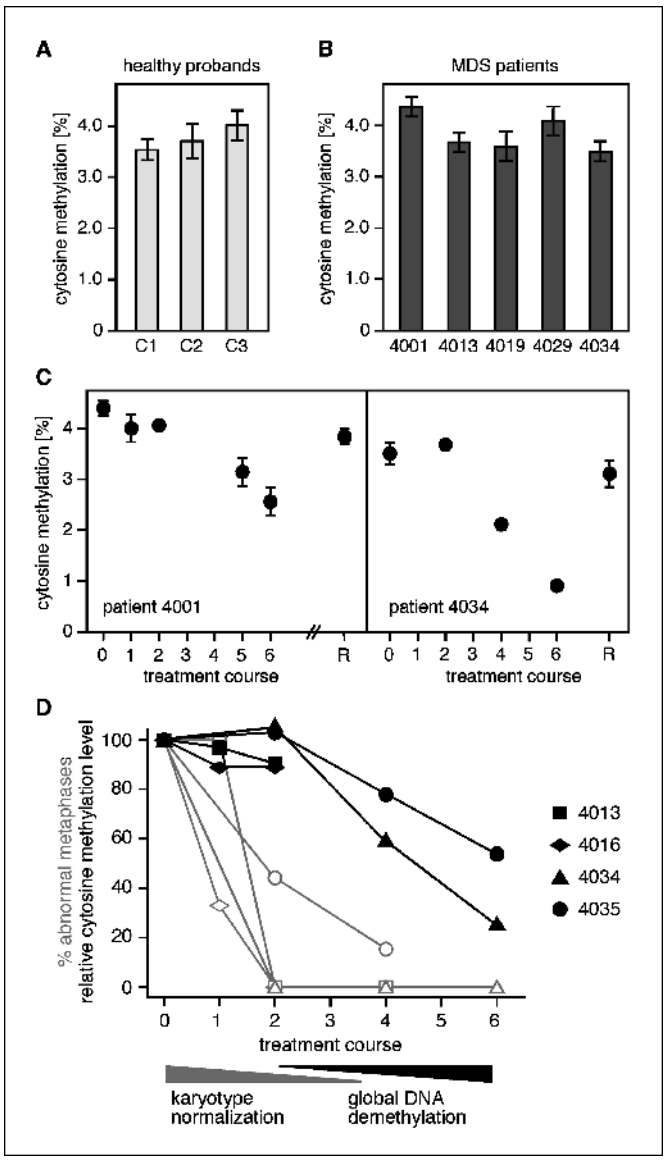

Figure 2. Determination of whole-genome methylation levels in bone marrow aspirates. *A,* genome methylation levels of bone marrow aspirates from healthy individuals. *B,* genome methylation levels of bone marrow aspirates from MDS patients at the time of first diagnosis. *C,* demethylation dynamics of bone marrow cells from patients treated with decitabine. "R", samples collected after relapse. *Points,* means; *bars,* SD. *D,* the effect of decitabine treatment on the karyotype and DNA methylation level of bone marrow cells from MDS patients. Gray open symbols, fraction of abnormal metaphases; black closed symbols, level of genomic DNA methylation (relative to the pretreatment level). Karyotype normalization preceded DNA demethylation in all cases.

experiments on bone marrow aspirates. In a first set of experiments, we determined the cytosine methylation level of normal bone marrow cells from three healthy individuals aged 29, 30, and 36 years, respectively. This revealed methylation levels between 3.5% and 4.0% (Fig. 2*A*), which is in agreement with cytosine methylation levels reported for other human tissues (15). We then sought to determine the methylation level in MDS patients at the time of first diagnosis. Capillary electrophoretic analysis of bone marrow DNA from five MDS patients revealed cytosine methylation levels between 3.5% and 4.4% (Fig. 2*B*). This indicates similar levels of genomic DNA methylation in normal bone marrow and clonal MDS cells.

In order to evaluate the effect of decitabine on the genome methylation level, we analyzed DNA from MDS patients that had undergone at least one single-agent treatment cycle consisting of six to eight courses (3). We focused our analysis on patients from which material had been collected at the time of first diagnosis, during treatment and after treatment course 6. In total, we determined the methylation level of 34 bone marrow aspirates from seven patients using capillary electrophoresis. This provided detailed insights into the drug-induced demethylation dynamics (Table 1; Fig. 2*C*). In five of seven patients, we observed a substantial demethylation of 20% to 70% when compared with the time of first diagnosis. In two samples, we observed methylation levels of 0.9% and 1.3%, respectively, which is far below the reference range for human tissues (15) and provided

Table 1. Cytosine methylation levels of bone marrow mononuclear cells from patients treated with decitabine

Patient no.	Sample collection	% 5mC	SD
4001	pre-DAC	4.4	0.2
	post-DAC1	4.0	0.3
	post-DAC2	4.1	0.1
	post-DAC5	3.2	0.3
	post-DAC6	2.7	0.3
	12 months post-DAC6	3.8	0.2
	post-2 DAC1	3.6	0.1
	post-2 DAC2	3.4	0.2
4013	pre-DAC	3.7	0.2
	post-DAC1	3.6	0.1
	post-DAC2	3.3	0.3
	post-2 DAC2	2.7	0.2
	post-2 DAC5	1.3	0.2
4016	pre-DAC	n.d.	n.d.
	post-DAC1	3.7	0.2
	post-DAC2	3.7	0.1
	6 months post-DAC6	4.2	0.3
	15 months post-DAC6	3.9	0.2
	post-2 DAC2	3.4	0.2
	post-2 DAC6	3.4	0.2
	6 months post-2 DAC6	4.2	0.1
4019	pre-DAC	3.6	0.3
	post-DAC6	3.6	0.3
4029	pre-DAC	4.1	0.3
	post-DAC6	3.3	0.3
	5 months post-DAC6	4.1	0.3
4034	pre-DAC	3.5	0.2
	post-DAC2	3.7	0.1
	post-DAC4	2.1	0.1
	post-DAC6	0.9	0.1
	2 months post-DAC6	3.0	0.2
4035	pre-DAC	n.d.	n.d.
	post-DAC2	3.8	0.3
	post-DAC4	2.9	0.1
	post-DAC6	2.0	0.1
	6 months post-DAC8	3.5	0.2

NOTE: Numbers before DAC indicate treatment cycles, numbers after DAC indicate treatment courses. Follow-up samples (months post-DAC6) were collected from relapsed or remitted patients. Cytosine methylation levels (% 5mC) were determined by capillary electrophoretic analysis and calculated by dividing the fluorescence signal of 5-methylcytosine through the sum of fluorescence signals for cytosine plus 5-methylcytosine. n.d., not determined.

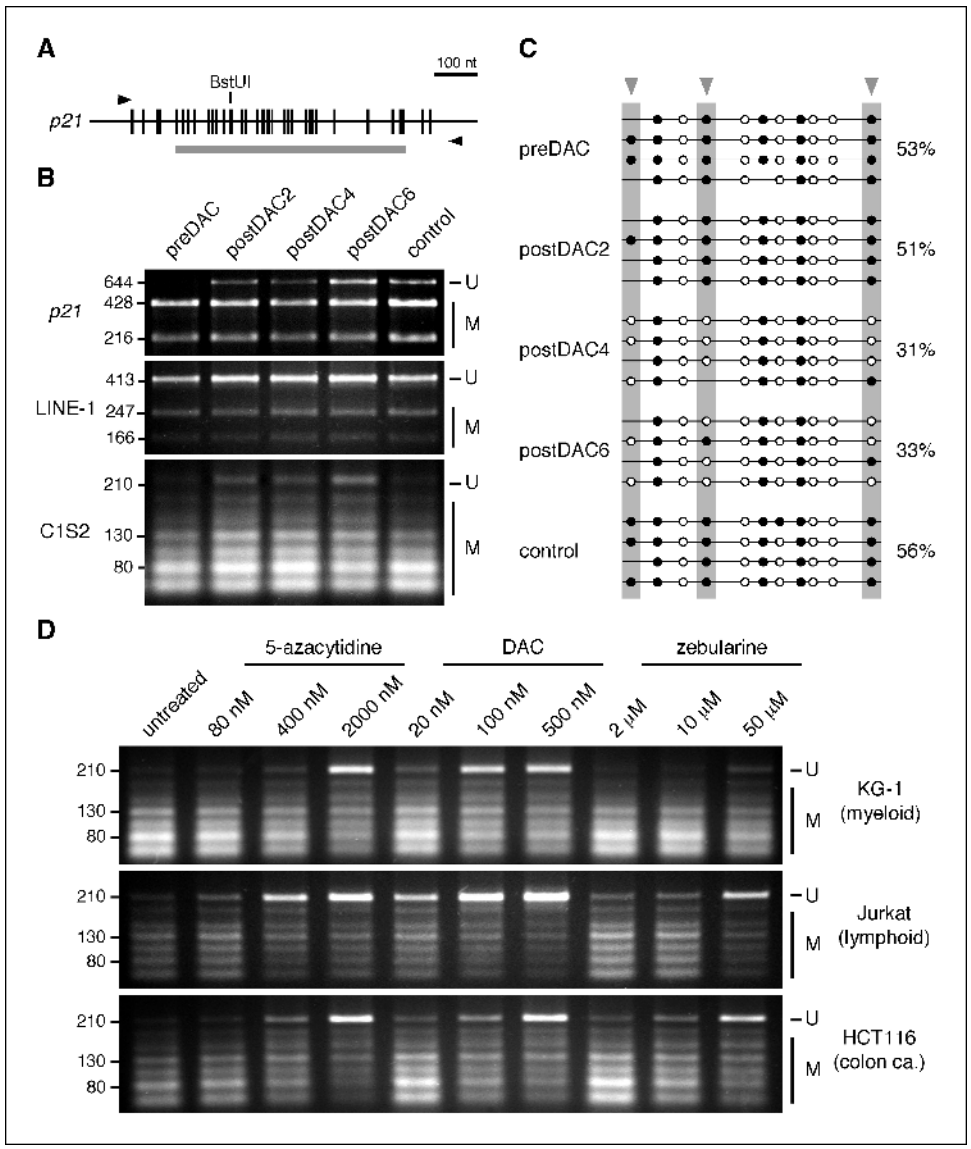

Figure 3. Sequence-specific DNA methylation analysis. *A,* structure of the CpG island in the *p21* exon 2 region. CpG dinucleotides are shown as dashes, the position of the exon is indicated by a gray bar. Black arrowheads represent the bisulfite primers used for PCR amplification. *B,* determination of locus-specific DNA methylation for *p21*, LINE-1 and C1S2 in sequential bone marrow samples from patient 4034 by bisulfite PCR. "U" bands, unmethylated epialleles; "M" bands, methylated epialleles. *C,* high-resolution DNA methylation analysis of C1S2 elements by bisulfite sequencing. ○, unmethylated CpG dinucleotides; ●, methylated CpG dinucleotides. Gray arrowheads and shading, position with CpG dinucleotides with strong differential methylation. The cytosine methylation level is shown on the right side, the difference between the methylation level of normally methylated (preDAC, postDAC2, control) and demethylated (post-DAC4 and post-DAC6) samples was highly significant ($P < 0.01$), as determined by a standard *t* test. *D,* induction of C1S2 satellite demethylation in cancer cell lines with different DNA methyltransferase inhibitors. Cell lines of various tissue origin were incubated with pharmacologically effective concentrations of the DNA methyltransferase inhibitors 5-azacytidine, decitabine (DAC) and zebularine, respectively. The methylation status of satellite sequences was determined by bisulfite PCR of genomic DNA.

another indication for the demethylating activity of decitabine. In two of seven patients (4016 and 4019), we failed to detect a significant demethylation. Thus, the response in the genome methylation level varied between individual patients.

It has been suggested that the demethylating effects of decitabine treatment could be mediated by two distinct mechanisms (or a combination of both; ref. 5): the drug could selectively suppress malignant cells in the bone marrow and thereby facilitate the expansion of normal (and potentially less methylated) bone marrow progenitor cells. Alternatively, the drug could directly demethylate malignant bone marrow cells and thereby allow their epigenetic reprogramming. To distinguish between these two possibilities, we made use of the fact that bone marrow cells from a large fraction of MDS patients are characterized by an abnormal karyotype. These chromosome abnormalities become strongly reduced after the first or second treatment course with decitabine and provide an important marker for drug efficacy (5). When we compared the changes in the genomic DNA methylation level to the cytogenetic changes (in the limited number of patients where both data sets were available) we observed that the karyotype normalization consistently preceded the DNA demethylation in four of four patients (Fig. 2*D*). Our results thus show that the drug is able to transiently demethylate nonclonal bone marrow cells and suggest that a strong demethylation of genomic DNA is not required for karyotypic normalization.

A recent analysis of transcriptional profiles of DAC-treated myeloid cells identified a comparatively small group of 81 genes that showed decitabine-dependent up-regulation in myeloid cells (16). However, the majority of genes analyzed was found to be unmethylated in untreated control cells. Indications for drug-induced demethylation could only be obtained for the myeloperoxidase (*MPO*) gene and the significance of this finding for the therapeutic response remained unclear. We noted that the *p21* gene, which was also found to be up-regulated in a decitabine-dependent manner (16), contained a well-defined CpG island in its exon 2 region (Fig. 3*A*). We therefore established a bisulfite PCR methylation assay and analyzed serial bone marrow aspirates from a patient that had shown genome-wide demethylation in previous experiments (see Materials and Methods). Our results indicated strong hypermethylation of the *p21* exon 2 region before drug treatment and a weak demethylation after treatment cycles 2, 4, and 6 (Fig. 3*B*). When we

analyzed the same DNA for LINE-1 methylation, we again observed only minimal demethylation (Fig. 3*B*), which is consistent with the results published by others (12), and suggests that the methylation of euchromatic repeat elements is comparatively resistant to decitabine treatment. However, most of the genomic 5-methylcytosine resides in centromeric and pericentromeric repeat elements. Because the methylation status of chromosome 1 satellite 2 (C1S2) elements has been extensively analyzed in previous studies (17, 18), we designed C1S2-specific bisulfite primers by using the published DNA sequence (Genbank accession no. X72623). Examination of sequential DNA samples from patient 4034 by combined bisulfite restriction analysis revealed high levels of methylation before decitabine treatment and in untreated control individuals, but detectable demethylation after treatment courses 2, 4, and 6 (Fig. 3*B*). Similar results were also obtained with DNA samples from other drug-treated patients (data not shown). These observations were confirmed by bisulfite sequencing, which also revealed a substantial diversity of the amplified satellite repeats (Fig. 3*C*). CpG dinucleotides in C1S2 sequences (53%) were methylated before drug treatment, whereas 51% were methylated after treatment course 2, 31% after course 4, 33% after course 6, and 56% in a healthy control individual. This demethylation was particularly pronounced in a subset of CpG dinucleotides (Fig. 3*C*). Our results thus uncover an independent and novel target of DAC-mediated demethylation and provide additional confirmation for the demethylation dynamics established in earlier experiments. Because demethylation of satellite sequences could be observed in diverse cell lines and with different nucleoside DNA methyltransferase inhibitors (Fig. 3*D*), it is likely a general characteristic of these compounds.

Until now, it has been unclear whether the observed demethylation effects after DAC treatment were due to the expansion of the less methylated cell population or to the inhibition of DNA methyltransferase activity. Even though it has been shown previously that demethylation of the *p15* tumor suppressor gene coincided with karyotype normalization (5), it remained possible that the methylation changes were a consequence of the proapoptotic activity of the drug, followed by the expansion of nonclonal myeloid progenitor cells. In this respect, the time-lag between karyotype normalization and global demethylation observed in our experiments provides an important confirmation for a direct demethylating activity of DAC because it shows the demethylation in nonclonal cells. At the same time, these data also suggest that the karyotype normalization is not dependent on the global demethylating activity of the drug and that restricted gene-specific demethylation and/or cytotoxicity might play an important role in the early patient response.

In some of the patient samples, DNA methylation levels decreased to >50% of the pretreatment level, which is similar to the reduction observed after drug treatment of cultured cell lines. Under these *in vitro* conditions, myeloid cells up-regulate a relatively small number of genes, including the *p21* tumor suppressor (16). Our results indicate that *p21* might be a target gene for decitabine-mediated gene demethylation and suggest that epigenetic reactivation of this locus might contribute to the therapeutic effects observed after drug treatment. Further analyses will be required to substantiate the demethylation effect and to clarify the role of *p21* in the patient response. In addition, our results also indicate the presence of potentially adverse demethylation effects in decitabine-treated MDS patients. Demethylation of C1S2 sequences has been observed in numerous forms of cancer and might represent a molecular marker with considerable prognostic significance (17, 18). It has been suggested that demethylation of satellite sequences might promote chromosome instability (19), which is supported by the observation that strong demethylation of genomic DNA causes genomic instability and concomitant tumorigenesis in transgenic mice (20). Although the transient inhibition of DNA methyltransferases in drug-treated patients is not directly comparable to the permanent reduction of methyltransferase activity in the transgenic mouse model, the demethylation of satellite sequences raises a cautionary note about the use of demethylating drugs that should be addressed in the future development of epigenetic cancer therapies.

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References

1. Egger G, Liang G, Aparicio A, Jones PA. Epigenetics in human disease and prospects for epigenetic therapy. Nature 2004;429:457–63.
2. Silverman LR, Demakos EP, Peterson BL, et al. Randomized controlled trial of azacitidine in patients with the myelodysplastic syndrome: a study of the cancer and leukemia group B. J Clin Oncol 2002;20:2429–40.
3. Wijermans P, Lübbert M, Verhoef G, et al. Low-dose 5-aza-2′-deoxycytidine, a DNA hypomethylating agent, for the treatment of high-risk myelodysplastic syndrome: a multicenter phase II study in elderly patients. J Clin Oncol 2000;18:956–62.
4. Issa JP, Garcia-Manero G, Giles FJ, et al. Phase 1 study of low-dose prolonged exposure schedules of the hypomethylating agent 5-aza-2′-deoxycytidine (decitabine) in hematopoietic malignancies. Blood 2004;103:1635–40.
5. Daskalakis M, Nguyen TT, Nguyen C, et al. Demethylation of a hypermethylated P15/INK4B gene in patients with myelodysplastic syndrome by 5-Aza-2′-deoxycytidine (decitabine) treatment. Blood 2002;100:2957–64.
6. Chan AT, Tao Q, Robertson KD, et al. Azacitidine induces demethylation of the Epstein-Barr virus genome in tumors. J Clin Oncol 2004;22:1373–81.
7. Rhee I, Bachman KE, Park BH, et al. DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. Nature 2002;416:552–6.
8. Yang AS, Estecio MR, Doshi K, Kondo Y, Tajara EH, Issa JP. A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. Nucleic Acids Res 2004;32:e38.
9. Stach D, Schmitz OJ, Stilgenbauer S, et al. Capillary electrophoretic analysis of genomic DNA methylation levels. Nucleic Acids Res 2003;31:e2.
10. Frommer M, McDonald LE, Millar DS, et al. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci U S A 1992;89:1827–31.
11. Xiong Z, Laird PW. COBRA: a sensitive and quantitative DNA methylation assay. Nucleic Acids Res 1997;25:2532–4.
12. Yang AS, Estecio MR, Garcia-Manero G, Kantarjian HM, Issa JP. Comment on "Chromosomal instability and tumors promoted by DNA hypomethylation" and "Induction of tumors in mice by genomic hypomethylation". Science 2003;302:1153b.
13. Hiendleder S, Mund C, Reichenbach HD, et al. Tissue-specific elevated genomic cytosine methylation levels are associated with an overgrowth phenotype of bovine fetuses derived by *in vitro* techniques. Biol Reprod 2004;71:217–23.
14. Lyko F, Stach D, Brenner A, et al. Quantitative analysis of DNA methylation in chronic lymphocytic leukemia patients. Electrophoresis 2004;25:1530–5.
15. Gama-Sosa MA, Midgett RM, Slagel VA, et al. Tissue-specific differences in DNA methylation in various mammals. Biochim Biophys Acta 1983;740:212–9.
16. Schmelz K, Sattler N, Wagner M, Lübbert M, Dörken B, Tamm I. Induction of gene expression by 5-Aza-2′-deoxycytidine in acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) but not epithelial cells by DNA-methylation-dependent and -independent mechanisms. Leukemia 2005;19:103–11.
17. Narayan A, Ji W, Zhang XY, et al. Hypomethylation of pericentromeric DNA in breast adenocarcinomas. Int J Cancer 1998;77:833–8.
18. Widschwendter M, Jiang G, Woods C, et al. DNA hypomethylation and ovarian cancer biology. Cancer Res 2004;64:4472–80.
19. Ehrlich M. DNA methylation in cancer: too much, but also too little. Oncogene 2002;21:5400–13.
20. Gaudet F, Hodgson JG, Eden A, et al. Induction of tumors in mice by genomic hypomethylation. Science 2003;300:489–92.

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