

DNA Methylation Changes after 5-Aza-2'-Deoxycytidine Therapy in Patients with Leukemia

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Abstract

5-Aza-2'-deoxycytidine (decitabine) is postulated to have clinical activity in myeloid leukemias via its ability to inhibit DNA methylation. To study this, we examined DNA methylation in patients with leukemia treated with decitabine. Five days after the treatment, total genomic 5-methylcytosine/cytosine decreased on average by 14% (from 4.3% to 3.7%), whereas methylation of repetitive DNA elements showed a mean decrease of 9% and 16% for *Alu* and long interspersed nucleotide elements, respectively. Methylation decreased linearly with increasing doses between 5 and 20 mg/m²/d ($r = 0.88$; $P = 0.05$) but showed a plateau above that. Hypomethylation correlated with response in patients with acute myelogenous leukemia treated with low doses (5-20 mg/m²/d), but patients with chronic myelogenous leukemia treated with high doses (100-180 mg/m²/d) showed no such correlation. Aberrant methylation of *p15* (>10%) was found in 27% of patients, and 80% of these showed a decrease by at least one third, but this did not correlate with response. The imprinted gene *H19* showed little change in methylation after decitabine. In conclusion, we show dose-dependent hypomethylation after decitabine at low doses. Increasing the dose, which has been shown previously to result in a reduced response rate, was not accompanied by further hypomethylation. (Cancer Res 2006; 66(10): 5495-503)

Introduction

5-Aza-2'-deoxycytidine (decitabine) is a pyrimidine analogue first synthesized ~40 years ago, and early clinical trials showed that it had consistent activity in patients with myeloid leukemia. There is considerable experience in the use of decitabine and a similar drug, 5-azacytidine, in several clinical trials in patients with chronic myelogenous leukemia (CML), acute myelogenous leukemia (AML), and myelodysplastic syndrome (MDS; refs. 1-4).

Decitabine once incorporated into DNA in place of cytosine can covalently trap DNA methyltransferase to DNA causing irreversible inhibition of the enzyme (5). This covalent enzyme-DNA adduct can be cytotoxic at high doses (6). By contrast, low-dose schedules

of decitabine are believed to capitalize on the ability of the drug to inhibit DNA methylation and to reactivate gene expression (7, 8). Recent efforts have focused on administering lower doses of decitabine to minimize toxicity and exploit the unique property of decitabine to inhibit DNA methylation (8, 9). Interestingly, in a phase I study of low-dose decitabine, 15 mg/m² administered >10 days (5 days on, 2 days off, and 5 days on), which is significantly lower than the maximally tolerated dose, seemed to be the optimal clinically effective dose with a response rate of ~50% (8).

Methylation of cytosine residues in gene promoters is associated with gene silencing, and aberrant DNA methylation patterns are associated with tumorigenesis (10). It is believed that altered DNA methylation patterns and epigenetics play a crucial role in cancer and differentiation. Aberrant DNA methylation patterns of multiple genes have been described in leukemia as well as other tumor types (11-13). Altered methylation of certain gene loci is associated with prognosis, and methylation changes may define different subtypes of leukemia. Decitabine has been shown to reverse the aberrant hypermethylation of the *p15* gene in some patients with AML and MDS (8, 14).

In this study, we examined global and gene-specific DNA methylation changes in the peripheral blood of patients with leukemia treated with decitabine. We found a dose-dependent hypomethylation response that remarkably parallels *in vitro* studies and a relationship between hypomethylation-dependent responses at low but not high doses of decitabine.

Materials and Methods

Patient samples. Samples were collected from patients treated as part of two clinical studies using decitabine as a single agent. The first study was a phase II study in CML conducted from 1993 to 2000 (before the availability of imatinib mesylate) and treated patients with 50 to 90 mg/m² decitabine twice daily for 5 consecutive days (Fig. 1A; Table 1; ref. 15). Pretreatment and post-treatment samples were available from 18 patients in this study. The second study was a phase I study, which attempted to capitalize on the demethylating properties of decitabine by using a lower dose of decitabine, 5 to 20 mg/m² once daily, for a longer period, 10 days over a 2-week period (Fig. 1A; ref. 8). Some patients in this lower dose of decitabine trial received decitabine for up to 20 days; however, no samples were analyzed beyond week 2 of the treatment. Samples were available from 23 patients in this study. As controls, we used samples from patients with solid tumor treated with another nucleoside analogue gemcitabine and patients with acute lymphocytic leukemia treated with multiagent cytotoxic chemotherapy. Peripheral blood samples were collected before, during, and after the treatment. Donation of blood samples for laboratory studies was voluntary, and patients gave informed consent for sample collection according to institutional guidelines.

DNA isolation and bisulfite treatment. All peripheral blood samples were collected by venipuncture, and mononuclear cells were isolated by

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Ficoll separation, immediately frozen (nonvially), and stored at -70°C . DNA was isolated using standard phenol-chloroform extraction methods. DNA was treated with bisulfite, which selectively deaminates cytosine but not 5-methylcytosine to uracil. This leads to a primary sequence change, as unmethylated cytosines are converted to uracil and then thymidine after PCR; however, 5-methylcytosine is not converted by bisulfite and remains as

a cytosine after PCR (16). This primary sequence change can be quantitated using direct sequencing, restriction digestion, or pyrosequencing. In brief, 1.5 μg DNA was denatured in 50 μL of 0.2 mol/L NaOH for 10 minutes at 37°C . Then, 30 μL of freshly prepared 10 mmol/L hydroquinone (Sigma, St. Louis, MO) and 520 μL of 3 mol/L sodium bisulfite (Sigma; pH 5) were added and mixed. The samples were overlaid with mineral oil to prevent evaporation and incubated at 50°C for 16 hours. Bisulfite-treated DNA was isolated using the Wizard DNA Clean-Up System (Promega, Madison, WI). DNA was eluted by 50 μL warm water, and 5.5 μL of 3 mol/L NaOH were added for 5 minutes. DNA was ethanol precipitated with glycogen as a carrier and resuspended in 20 μL water. Bisulfite-treated DNA was stored at -20°C until ready for PCR amplification and analyzed by cloning followed by direct sequencing, restriction digestion [combined bisulfite restriction analysis (COBRA); ref. 17], or pyrosequencing (18).

Analysis of *Alu* repetitive element and long interspersed nucleotide element-1 methylation. A previously described COBRA of *Alu* repetitive DNA elements was used as a surrogate for global DNA methylation changes (19). PCR primers directed to an *Alu* consensus sequence (Supplementary Table S1) were used in a low-stringency PCR to amplify a pool of $\sim 15,000$ *Alu* elements. Then, a restriction digest was used to distinguish methylated from unmethylated sequences. The digested PCR products were then run on an Agilent Biosystems (Santa Clara, CA) DNA analyzer to quantitate the cut (methylated) and uncut (methylated) DNA. The methylation changes observed for the *Alu* repetitive element were small; therefore, each sample was done in triplicate with an average being used for analysis. The mean of each assay was 1.5% with a coefficient of variation of 6%.

The long interspersed nucleotide elements (LINE)-1 assay was based on a similar principle to the *Alu* element COBRA assay but used nonselective PCR of LINE and pyrosequencing to quantitate methylation. A 50 μL PCR was carried out in 60 mmol/L Tris-HCl (pH 8.8), 15 mmol/L ammonium sulfate, 0.5 mmol/L MgCl_2 , 1 mmol/L deoxynucleotide triphosphate (dNTP) mix, 1 unit Taq polymerase, 10 pmol forward primer (primer sequences are in Supplementary Table S1), 1 pmol reverse primer, and 10 pmol universal biotinylated primer. PCR cycling conditions were 95°C , 50°C , and 72°C for 30 seconds for 35 cycles. The PCR product was purified and quantitated using the PSQ HS 96 Pyrosequencing System (Pyrosequencing, Inc., Westborough, MA) and the sequencing primer listed in Supplementary Table S1. Methylation was quantitated using the provided pyrosequencing software, and methylation of the CpG site immediately adjacent to the sequencing primer was used for analysis.

COBRA of *HoxA5* and *H19*. COBRA methylation analysis was done for two gene loci. *HoxA5* is a gene heavily methylated in normal and leukemic cells (20). *H19* is an imprinted gene locus that is methylated on the paternal

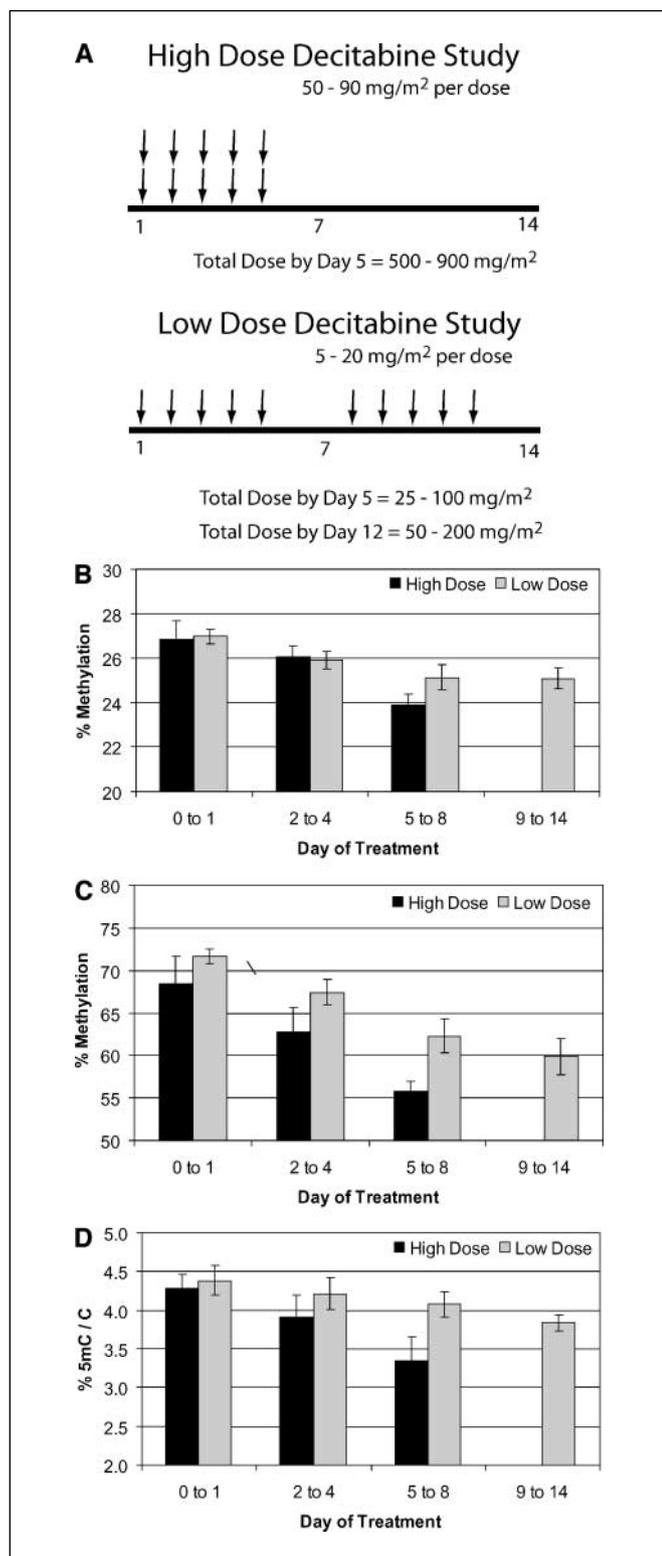


Figure 1. Treatment schema and methylation results. A, treatments. Patients were treated as part of a phase II study of decitabine that was given initially at a dose of 90 mg/m² twice daily for 5 consecutive days. This dose was later decreased to 50 mg/m² twice daily due to toxicity. In this high-dose study, a total dose of 500 to 900 mg/m² of decitabine was given over a 5-day period. In the second study, a phase I strategy of low-dose decitabine was used. This study was designed to take advantage of hypomethylating properties of decitabine. Escalating doses of decitabine of 5, 10, 15, and 20 mg/m² were given as 10 daily doses over a 2-week period. The total dose delivered was only 50 to 200 mg/m² over a 12-day period. B, *Alu* methylation. Methylation of peripheral blood from leukemia patients was assessed for sequential days of decitabine treatment. A pool of *Alu* elements was amplified by bisulfite PCR. Methylation was analyzed by restriction digestion with *Mbol*, which cuts only if the original DNA was methylated followed by quantitation by capillary electrophoresis. Mean *Alu* methylation for leukemia patients in the high-dose (black columns) and low-dose (gray columns) studies is shown. Methylation decreases are seen for both studies during the treatment. Note that no samples were collected on days 9 to 14 for patients in the high-dose study. Columns, mean; bars, SE. C, *LINE* methylation. A pool of *LINE* was amplified by bisulfite PCR and quantitated by pyrosequencing. Mean *LINE* methylation for leukemia patients in the high-dose (black columns) and low-dose (gray columns) studies is shown. Methylation decreases are seen for both studies during the treatment. D, 5-mC content. Methylation was quantitated by digestion of total genomic DNA into single nucleotides and quantification of 5-methylcytosine by LC-MS. Total 5-methylcytosine is reported as a percentage of total cytosine measured. Mean methylation for all patients treated on the high-dose (black columns) and low-dose (gray columns) studies is shown.

Table 1. Patient characteristics

	High-dose study	Low-dose study
No. patients	18	23
Sex		
Male	11	14
Female	7	9
Age (y)		
Mean	68	58
Range	52-79	27-83
Diagnosis		
AML	3	20
CML	2	0
CML chronic	3	0
CML accelerated	5	1
CML blast	7	0
Peripheral blasts		
Mean (%)	22.5	14
Range	0-94	0-81
BM blasts		
Mean (%)	26.3	38
Range	1-78	9-75
WBC		
Mean (1,000/mL ³)	27.9	10.9
Range	1.2-124.4	0.8-16.1
Response		
Complete response + partial response, <i>n</i> (%)	7 (39)	7 (30)
Complete response, <i>n</i> (%)	5 (28)	4 (17)
Partial response, <i>n</i> (%)	2 (11)	3 (13)
No response	11	16

chromosome and unmethylated on the maternal chromosome (see Supplementary Table S1 for PCR primer sequences, PCR cycling conditions, and restriction enzymes used; ref. 21). Digested PCR products were quantitated by densitometry after separation by PAGE.

Methylation analysis of the *p15* gene. The *p15* tumor suppressor gene, which is normally unmethylated or lightly methylated but becomes aberrantly methylated in cancer, was also examined. Methylation of the *p15* tumor suppressor gene was difficult to analyze consistently by COBRA due to the relatively low level of aberrant methylation at this locus. Therefore, pyrosequencing was used to analyze methylation of the *p15* gene. A 50 μ L PCR was carried out in 60 mmol/L Tris-HCl (pH 8.8), 15 mmol/L ammonium sulfate, 2 mmol/L MgCl₂, 1 mmol/L dNTP mix, 1 unit Taq polymerase, 10 pmol forward primer (primer sequences are in Supplementary Table S1), 1 pmol reverse primer, and 10 pmol universal biotinylated primer. PCR cycling conditions were melting temperature of 95°C for 30 seconds, annealing for 45 seconds at a temperature of 50°C for 3 cycles, 48°C, 46°C, and 44°C for 4 cycles, and 42°C for 43 cycles, and extension temperature of 72°C for 45 seconds.

For selected cases, PCR products were cloned using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, and multiple different clones were sequenced. Minipreps were prepared using QIAprep Spin Miniprep kit (Qiagen, Valencia, CA). DNA sequencing was done at The M.D. Anderson Cancer Center Core Sequencing Facility.

Liquid chromatography-mass spectrometry. Total 5-methylcytosine content was quantitated in genomic DNA using a previously described highly specific assay using liquid chromatography (LC)-mass spectrometry (MS; ref. 22). In brief, 0.5 μ g genomic DNA was hydrolyzed by sequential digestion with three enzymes, nuclease P1 (Roche Molecular Biochemicals, Mannheim, Germany), venom phosphodiesterase I (Sigma), and alkaline phosphatase (Sigma). The hydrolyzed DNA solution was directly delivered onto the analytical column (Supelco, Bellefonte, PA) in isocratic mode. This allowed the

separation of the four DNA bases as well as the identification of 5-methylcytosine. Identification of cytosine and 5-methylcytosine was obtained by MS analysis of chromatographic peaks. The isotopomers [¹⁵N₃]-2'-deoxycytidine and methyl-D3, ring-6-D1 5-methyl-2'-deoxycytidine (Cambridge Isotope Laboratories, Cambridge, MA) were used as internal standards, allowing the quantitation of absolute amounts of the deoxycytidine and methylated cytosine residues in genomic DNA. DNA methylation status is presented as percentage of 5-methylcytosine in total cytosine.

p53 sequencing. Mutation analysis of the *p53* gene was done by direct sequencing of exons 5 to 9 of the *p53* gene. Genomic DNA was amplified using PCR (Supplementary Table S1), and the PCR product was purified. DNA sequencing was done using primers used for PCR amplification with the ABI PRISM BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and the ABI PRISM 3730 DNA Analyzer (Applied Biosystems). Samples containing mutations were confirmed by reamplification of genomic DNA and sequencing.

Statistical methods. Microsoft Excel was used to analyze the data. To determine the relationship of DNA methylation changes before and after the treatment with decitabine, a two-sided *t* test was used to compare methylation levels before and after the treatment. Paired *t* tests were also used to confirm these results. In parallel, methylation was also expressed as a percentage change from baseline and analyzed by two-sided *t* tests. To determine the relationship of DNA methylation changes and response to decitabine therapy, a two-sided *t* test was used to compare methylation levels of the patients whose disease responded with methylation levels of the patients whose disease did not respond. Two-sided *P*s are reported, and *P* < 0.05 was considered significant.

Results

Decitabine induces hypomethylation *in vivo*. To see if decitabine could inhibit methylation over the entire genome, we measured quantitatively the methylation of *Alu* repetitive elements, LINE repetitive elements and total 5-methylcytosine content of patient samples collected before, during, and after the treatment with decitabine. The mean (SE) WBC count, for all the patients in both studies, decreased slightly from 13,800 (3.3) to 10,700 (2.5) on days 5 to 8. The mean (SE) blast percentage slightly increased from 20.7% (4.5%) to 25.4% (6.2%) on days 5 to 8 of the treatment. Therefore, samples collected during a course of treatment were very similar in terms of WBC and percentage of leukemic blasts for any given patient. Bisulfite treatment of DNA followed by a PCR that amplifies a pool of *Alu* LINE can be used as a surrogate marker for genome-wide DNA methylation changes (19). Because repetitive elements are very abundant and usually heavily methylated in the genome, PCRs are robust, and very small decreases in methylation can be detected when using a sensitive quantitation system (Supplementary Fig. S1A).

We used two-sided *t* tests to compare methylation levels before and after the treatment. Analysis of the data using alternate methods (paired *t* tests, analysis of relative change) gave similar results (data not shown). Methylation of *Alu* repetitive elements in peripheral blood samples of leukemia patients ranged from 23.6% to 29.3% with a mean (SE) methylation of 26.9% (0.27%) before treatment with decitabine (Fig. 1B). As reported previously, this assay underestimates the amount of methylation because mutations of *Alu* elements will appear as unmethylated CpG sites. Obvious decreases in methylation could be detected after decitabine treatment with a decrease in mean methylation to 26% (SE, 0.35%; range, 18-29.3%; *P* = 0.036, compared with baseline) by days 2 to 4 and 24.5% (SE, 0.51%; range, 16.9-29.1%; *P* < 0.001) by days 5 to 8 in both studies combined. Samples were not collected from days 9 to 14 for the high-dose study, but in the low-dose study DNA methylation did not significantly decrease beyond day 8 with

a mean methylation plateau of 25.1% (SE, 0.47%; range, 19-28.2%; $P = 0.002$) on days 9 to 14 (Fig. 1B). The mean decrease in absolute *Alu* methylation was 2.3%, which corresponds to a relative decrease of 9% (range, 0-31.5%). The maximum relative methylation decrease was 31.5% in one patient.

Similar results were obtained for *LINE* methylation. Baseline methylation of *LINE* before the treatment was 70.2% (SE, 0.9%; range, 55.1-77.7%). After decitabine treatment, *LINE* methylation decreased to 65% (SE, 1.65%; range, 26.2-86%; $P = 0.008$) by days 2 to 4 and 59.2% (SE, 1.9%; range, 35-76.2%; $P < 0.001$) by days 5 to 8 in both studies combined. Again, a plateau was seen with mean methylation of 59.9% (SE, 2.11%; range, 35.7-74.4%; $P < 0.001$) on days 9 to 14 of decitabine treatment (Fig. 1C; Supplementary Fig. S1B).

These data were confirmed by direct measurement of 5-methylcytosine in the genome using LC-MS. This required more DNA, and data were obtained from only 103 of the original 143 samples. Before the treatment, 5-methylcytosine made up on average 4.3% of cytosine in the genome of patients. After decitabine treatment, mean 5-methylcytosine decreased to 4.1% (SE, 0.12%; range, 2.9-5.4%; $P = 0.10$) by days 2 to 4 and 3.7% (SE, 0.19%; range, 1.7-5.3%; $P = 0.009$) by days 5 to 8 in both studies combined. In the low-dose study, mean (SE) 5-methylcytosine content decreased to 3.8% (SE, 0.11%; range, 2.5-4.8%; $P = 0.003$) by days 9 to 14 (Fig. 1D).

To confirm that the observed methylation changes are specific to decitabine therapy, we examined the peripheral blood of three patients with renal cancer treated with cytotoxic chemotherapy (capecitabine and gemcitabine in combination). The mean (SE) *Alu* methylation before the treatment was 26.1% (1%) and 25.9% (0.4%) on day 7 of the treatment ($P = 0.8$). The mean (SE) *LINE* methylation was 64.5% (0.7%) before the treatment and did not change significantly by day 7 [62.2% (1.1%); $P = 0.17$]. To determine whether this was also true when studying neoplastic cells, we examined seven patients with acute lymphocytic leukemia treated with multiagent cytotoxic chemotherapy. In these patients, mean (SE) *LINE* methylation was 75.1% (2.3%) before chemotherapy and 76.1% (1.8%) 14 days after chemotherapy ($P = \text{NS}$). Finally, we have shown previously that repetitive element methylation is stable for several days to months in patients with leukemias (23). These data confirm that repetitive element methylation is stable over time and is not affected by cytotoxic chemotherapy, suggesting that the decreases in DNA methylation observed in this study are unique to decitabine.

Although changes in DNA methylation can clearly be observed after decitabine treatment, it is unclear in which cells the DNA methylation changes are occurring. The peripheral blood studied represents a mixture of cell types. The decrease in DNA methylation is occurring in all peripheral blood cells, but, ideally, cell sorting and isolation of leukemic blasts would be of interest. Unfortunately, cell-sorted samples are not available for this study. Therefore, to assure that the changes in DNA methylation were not greatly influenced by the proportion of neoplastic cells in the samples, we compared DNA methylation changes in those patients who had a blast percentage below the mean (22.5%) with those patients who had a blast percentage greater than the mean. In patients who had a blast percentage of <22.5%, *LINE* methylation decreased from 69.8% (SE, 1.1%) to 58.8% (SE, 1.9%) by days 5 to 8 of decitabine treatment. In patients with blast percentage of >22.5%, *LINE* methylation decreased from 70.9% (SE, 1.5%) to 60.3% (SE, 4.9%). The change in DNA methylation was identical for the two groups and therefore independent of leukemic blasts.

Dose dependence of decitabine inhibition of DNA methylation. In this study, 5 patients were treated at 5 mg/m²/d, 5 patients at 10 mg/m²/d, 10 patients at 15 mg/m²/d, 3 patients at 20 mg/m²/d, and 15 patients at 100 mg/m²/d. To examine the dose dependence of decitabine-induced hypomethylation, we pooled the patients treated in both studies and compared days 5 to 6 values across different doses. Only one patient sample was available at the 20 mg/m²/d dose level for days 5 to 6. Mean methylation of *Alu* elements before the treatment was 26.8%, and by day 5 to 6, mean methylation decreased to 25.7%, 26.5%, 24.5%, 23.4%, and 23.9% for 5, 10, 15, 20, and 100 mg/m²/d decitabine, respectively (Fig. 2A). Similar results were obtained for *LINE* with mean *LINE* methylation decreasing from 70.2% to 71.4%, 62.8%, 60.1%, 59.4%, and 55.7% for 5, 10, 15, 20, and 100 mg/m²/d decitabine, respectively (Fig. 2B). Thus, there was a dose-dependent linear decrease in methylation at low doses of 5 to 20 mg/m²/d ($r = 0.88$; $P = 0.046$) with no significant increase in hypomethylation beyond 20 mg/m²/d in the *Alu* and *LINE* assays, suggesting a plateau effect. Assessment of 5-methylcytosine by LC showed greater decreases at higher doses, but a plateau phenomenon was not seen (Fig. 2C). It is unclear whether this is due to fewer samples being studied, differences in the assays, or differences in the methylation dynamics of repetitive elements compared with the entire genome. Alternatively, this may be due to the fact that the majority of patients treated at <20 mg/m²/d had AML, whereas the majority of patients treated with >20 mg/m²/d had CML.

Hypomethylation and response to decitabine. A critical question is whether decitabine has clinical activity in cancer through its ability to induce hypomethylation. We initially examined this issue using the *Alu* assay. In the high-dose study comprising mainly CML patients, both responders and non-responders to decitabine showed a decrease in *Alu* element methylation (Fig. 3A) with a nonsignificant trend to greater decreases in nonresponders (mean decrease of 1.3% in responders and 4.1% in nonresponders; $P = 0.23$).

By contrast, in the low-dose study (mostly AML patients), decreases in methylation were seen in both nonresponders and responders, but the responders showed a statistically significant larger decrease by days 5 to 8 and days 9 to 14 [2.6% ($P = 0.04$) and 3.4% ($P = 0.02$), respectively] when compared with non-responders (1.1% and 1.2%, respectively; Fig. 3B). Similar trends were observed using analysis of *LINE* and global methylation, with greater decreases in methylation being observed in the non-responders in the high-dose study (Fig. 3C and E), and in the low-dose study, responders again showed a greater decrease in methylation when compared with the nonresponders (Fig. 3D and F). These differences, however, did not reach statistical significance.

The low-dose study examined escalating dose levels from 5 to 20 mg/m²/d; therefore, it is possible that the correlation observed between response and change in DNA methylation may be confounded by more responders being treated in the higher dose levels. However, the mean (SE) dose used to treat patients whose leukemia responded was 11.4 mg/m²/d (1.8) and the mean dose used to treat patients whose leukemia did not respond was 12.9 mg/m²/d (1.2), with no significant difference between the two groups ($P = 0.50$).

Methylation of *HoxA5* and *H19* in patients treated with decitabine. In addition to studying global changes in DNA methylation, we examined two gene-specific loci that are normally methylated. *HoxA5* is a gene that is normally hypermethylated in

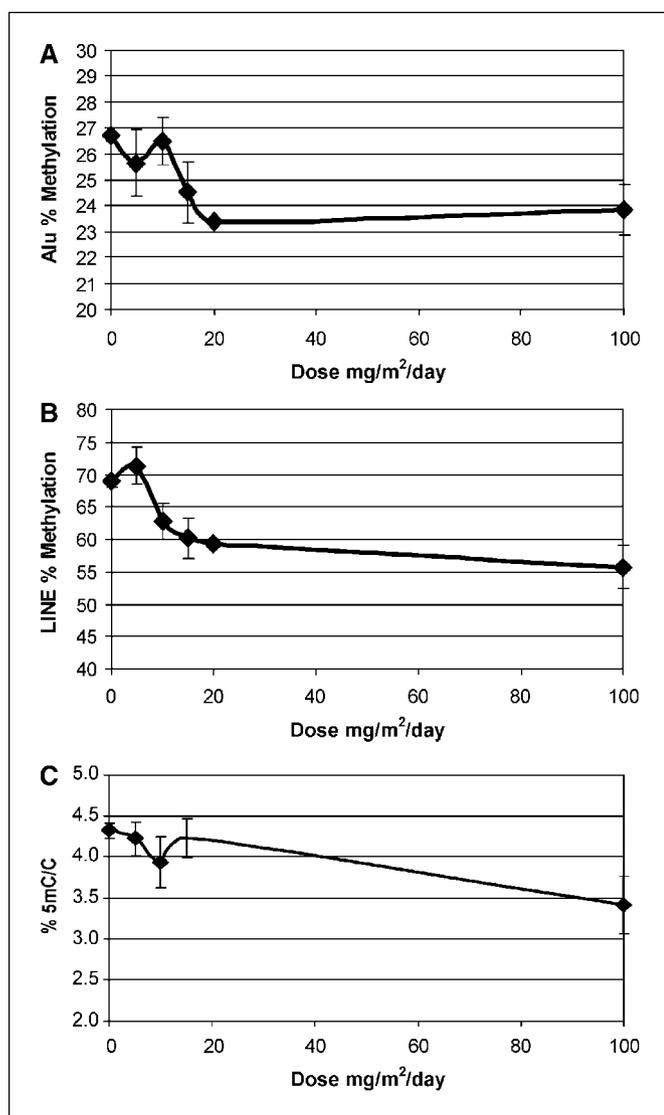


Figure 2. Hypomethylation dose response of decitabine. Data from both low-dose (5, 10, 15, and 20 mg/m²/d) and high-dose (100 mg/m²/d) decitabine studies were combined. DNA methylation of *Alu* elements (A), *LINE* (B), and 5-methylcytosine by LC-MS (C) at days 5 to 6 was compared with the dose given. The measurement of methylation inhibition in *Alu* elements (A) and *LINE* (B) showed that ~15 to 20 mg/m²/d seemed to be the optimal demethylating dose with no significant decrease in methylation with higher doses of decitabine. Note only one patient sample was available for the patients treated at 20 mg/m²/d. Similar data were obtained for quantification of methylation by LC-MS (C), but data were obtained from fewer samples.

peripheral blood mononuclear cells (Fig. 4), and *H19* is an imprinted gene that is normally methylated only on the paternal allele and unmethylated on the maternal allele (Fig. 4).

The *HoxA5* locus was heavily methylated in all patients examined (mean, 56.5%; SD, 25.7%; range, 11-95%; Fig. 4). The mean methylation of *HoxA5* did not change significantly in either high-dose or low-dose study. Mean methylation in the high-dose study at baseline was 58.8% (SE, 4.3%), 53.3% (SE, 4.1%; $P = 0.37$) by days 2 to 4 of the treatment and 55.1% (SE, 5.1%; $P = 0.52$) by days 5 to 8 of the treatment. Overall, the patients in the low-dose study showed less *HoxA5* methylation at baseline 38.2% (SE, 4.3%), decreasing to 34.7% (SE, 5.2%; $P = 0.60$) on days 2 to 4, 40% (SE, 8.5%; $P = 0.85$) on days 5 to 8, and 36.5% (SE, 5.6%; $P = 0.81$) on days

9 to 14. Taking the maximum change in methylation observed, overall, 9 of 24 (38%) patients analyzed showed an absolute decrease in methylation of >10% (range, 31-43.1%; SD, 14.8%). Only 1 of 24 (4%) patients showed an increase of methylation of >10%. The majority of patients (14 of 24, 58%) showed no change or a change in methylation of <10%.

H19, an imprinted gene that is only methylated on the paternal allele, was found to have ~46.9% (range, 30-58%; SD, 7.7%) methylation before treatment with decitabine (Fig. 4). This is close to the expected 50% methylation for an imprinted gene, and baseline methylation was consistent between the two studies. A decrease in absolute methylation of >10% could be detected in only 4 of 33 (12%) patients treated with decitabine with a mean decrease in methylation of only 1.1% (range, -16.9-32.9%; SD, 13.6%). An increase in methylation of >10% was seen in 4 of 33 (12%) patients, and the remaining 25 of 33 (76%) patients showed changes of <10%. Although methylation changes were observed for both *HoxA5* and *H19* after decitabine treatment, a dose-dependent response could not be shown, and there was no correlation between response and hypomethylation and at either locus.

Methylation of the *p15* gene in patients treated with decitabine. We quantitated methylation of the *p15* gene using bisulfite pyrosequencing. Methylation at the start of the treatment ranged broadly from 0% to 74% with a mean (SE) methylation of 11.1% (4.5%) and 12.9% (3.7%) for the high- and low-dose studies, respectively (Fig. 5A). Combining both studies, the median amount of *p15* methylation was only 4% (mean, 12.1%; range, 0-73.8%), showing that aberrant methylation of *p15* was infrequent. Substantial methylation (>10%) could be seen in 10 of 37 (27%) patients at the start of the treatment. Overall, decitabine decreased mean *p15* methylation by 5.1% (mean after treatment, 7%; range, 0-34.3%; $P = 0.14$) by days 5 to 8. Of the 10 patients who had >10% methylation before treatment (mean, 33.7%; range, 13.9-73.8%), there was a 53% relative decrease in methylation by days 5 to 8 (mean, 15.9%; range, 1-34.3%; $P = 0.05$). To confirm these results, bisulfite sequencing was done on samples from one patient with CML blast crisis and high level of methylation. The data shown in Fig. 5B show directly that methylation can be inhibited from days 0 to 5 of the treatment and are in general agreement with the pyrosequencing data obtained for this patient. It is worth noting that the percentage blasts in this patient on days 0, 2, and 5 were 49%, 59%, and 41%, respectively; therefore, the decrease in DNA methylation in this patient cannot be attributed to replacement of the leukemic clone. This very strong and rapid decrease in DNA methylation with no significant change in the blast percentage is surprising and raises the possibility of active demethylation but could still conceivably be related to passive demethylation in a patient with rapidly cycling cells.

Although methylation changes could be shown for *p15* during the treatment, no correlation between induction of hypomethylation and decitabine dose or response could be found. However, *p15* methylation status before the treatment paradoxically predicted a lower response to the drug. Mean methylation of patients who did not respond to *p15* was 15.9% compared with 5% for patients who responded ($P = 0.02$). No patient who had >25% methylation of *p15* before the treatment responded to decitabine, and 11 of 13 (85%) patients who had a complete or partial response following decitabine treatment had <10% methylation of *p15* at baseline. There were no pretreatment differences in *Alu*, *LINE*, *HoxA5*, or *H19* methylation levels in responders compared with non-responders (data not shown).

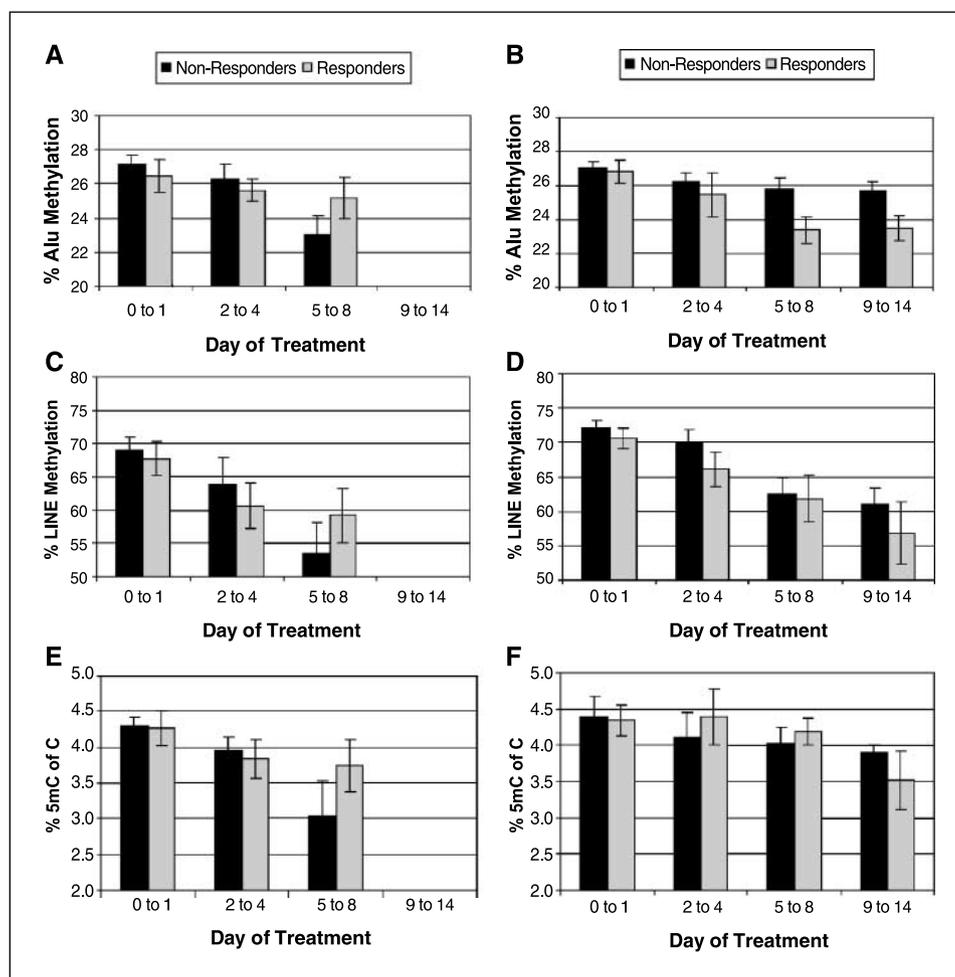


Figure 3. Hypomethylation of *Alu* elements was compared in those patients who responded to decitabine with those patients who did not respond to decitabine for both high-dose (A, C, and E) and low-dose (B, D, and F) studies. In the high-dose study, methylation inhibition did not correlate with response; surprisingly, there was a trend for nonresponders to have a greater decrease in methylation in *Alu* elements (A; $P = 0.23$). In the low-dose study, methylation inhibition at *Alu* elements correlated with response [B; days 5-8 ($P = 0.04$); days 9-14 ($P = 0.02$)]. Similar results were obtained for the *LINE* and LC-MS assays with a trend to greater decrease in methylation in the nonresponders (C and E) in the high-dose study and more methylation inhibition in the responders in the low-dose study (D and F), but these trends did not reach statistical significance.

***p53* mutations and response to decitabine.** Previous reports suggested that *p53* is important for induction of apoptosis by hypomethylation (24). To determine the clinical relevance of this finding, we sequenced exons 5 to 9 of *p53* in patients treated with decitabine. Exons 5 to 9 of *p53* are conserved domains, in which the majority of *p53* mutations occur. We identified only 3 of 41 patients studied with *p53* mutations that change the amino acid composition. This low frequency of mutation (7%) is consistent with previous reports in hematologic malignancies (25). Of the three patients with *p53* mutations, all had AML, one had a complete response, and two did not respond to decitabine. Therefore, no correlation was found between *p53* mutation and response to decitabine.

Discussion

Several recent studies have shown that decitabine can inhibit DNA methylation *in vivo*. We have shown global hypomethylation following decitabine therapy in CML (23). Global DNA methylation and *p21* DNA methylation were shown to decrease in serial bone marrow samples from seven patients with MDS (26). In that study, hypomethylation induction was markedly delayed in some patients, which should be contrasted with the rapid hypomethylation we describe here (and earlier in ref. 23). The reason for the differences may be related to the timing of sample collection vis-à-vis DNA

methylation analysis. Another study showed that global DNA methylation and *MAGE-1* promoter DNA methylation decreased in the peripheral blood of nine patients with refractory solid tumors who were treated with decitabine (27). These studies clearly show that decitabine can induce global and gene-specific changes in patients treated with decitabine but are smaller studies that could not link the change in DNA methylation with dose or response.

In this article, we have shown using multiple assays that methylation clearly decreases rapidly both globally and in a gene-specific manner following decitabine treatment. Global changes in DNA methylation were dose dependent with a plateau and correlated with response to decitabine at low but not high doses. We chose to study peripheral blood specimens in this study due to the ease of collection and ability to follow frequent serial samples. Leukemic blasts were not purified from the samples, and the DNA isolated in our study is a mixture of normal-appearing leukocytes and blasts. Our data reflect methylation changes in a mixture of blasts and normal-appearing leukocytes, and it is possible that the methylation changes observed are occurring preferentially in one population. However, previous work has shown that, in patients with leukemia, normal-appearing cells have cytogenetic and methylation changes identical to those of the leukemic clone (28). In addition, the therapeutic effect of decitabine is delayed with responses occurring several weeks after treatment without significant changes in the peripheral blood (8). In the short period

from which samples were collected, the WBC count and blast percentage did not change significantly. It is also possible that decitabine treatment leads to a selective toxicity of hypermethylated cells and a selection and expansion of hypomethylated clones instead of induction of true hypomethylation. To account for this possibility, the study was designed to assay frequent serial samples from very early (days) after the start of the treatment and not after completing the therapy (weeks). This minimizes the possibility that a selection phenomenon is being observed, and true inhibition of methylation is being observed. For these reasons, we believe that our data truly reflect dynamic changes in methylation in leukemic cells.

We observed a linear decrease in methylation with increasing decitabine doses from 5 to 20 mg/m²/d; interestingly, in DNA repetitive elements, there was no significant decrease in methyl-

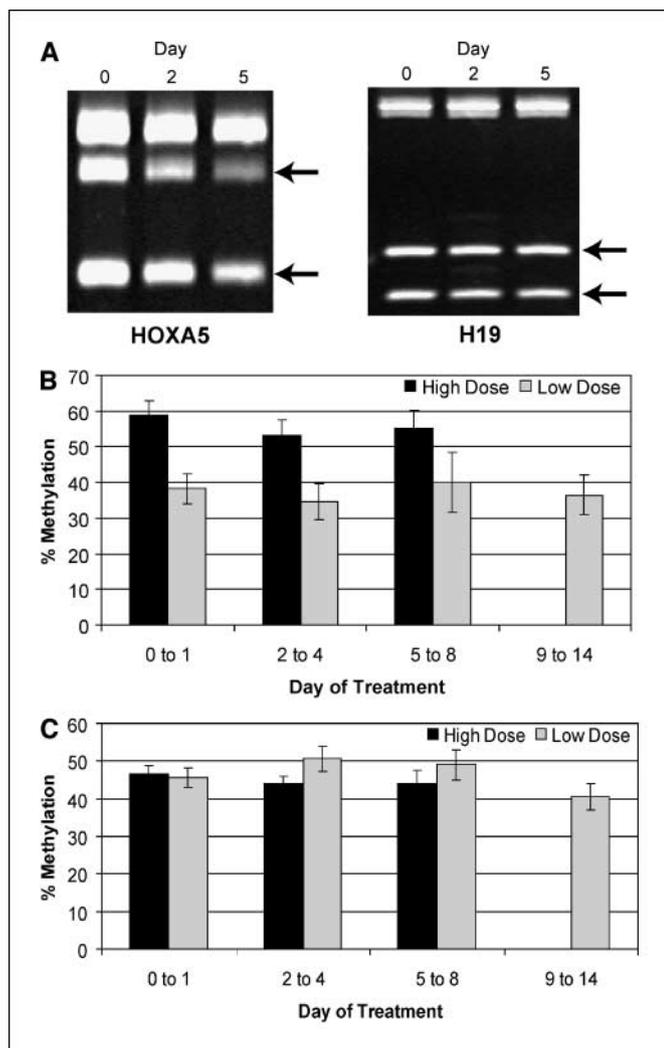


Figure 4. Gene-specific methylation analysis of leukemia patients treated with decitabine. Methylation of peripheral blood from leukemia patients was assessed for sequential days of decitabine treatment. Methylation was quantitated by bisulfite PCR followed by restriction enzyme digestion (COBRA). Analysis of *HoxA5* and *H19* was quantitated by densitometry of bands on polyacrylamide gel. Representative data are shown. Arrows, methylated bands/peaks. *B*, mean methylation of *HoxA5* for the high-dose (black columns) and low-dose (gray columns) studies on sequential days of treatment. *C*, mean methylation of *H19* for the high-dose (black columns) and low-dose (gray columns) studies. No trends in decreasing methylation can be shown for the *HoxA5* or *H19* genes with decitabine treatment.

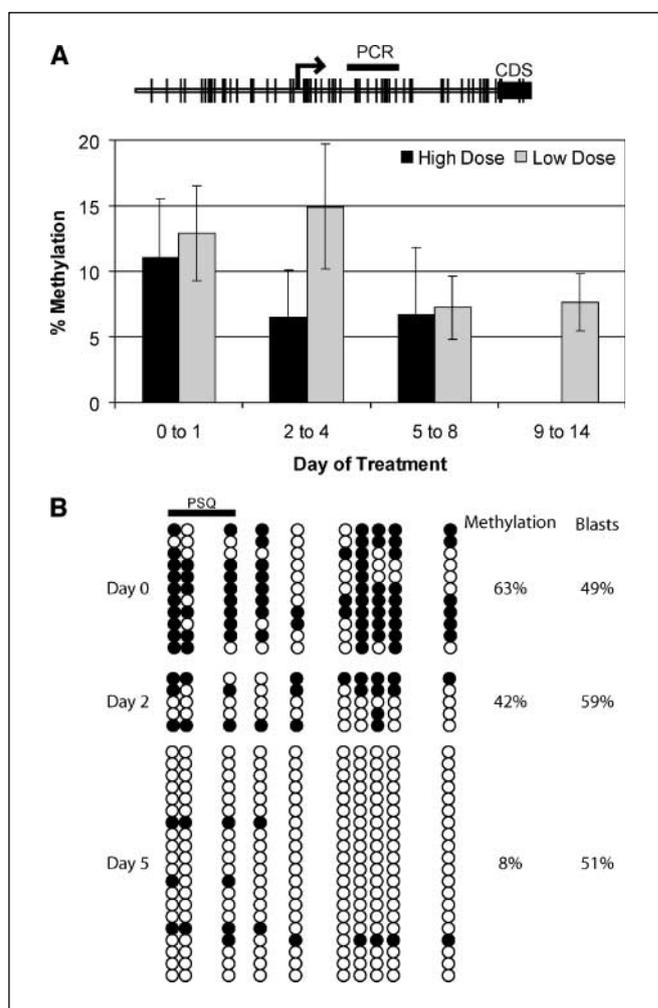


Figure 5. Methylation changes in *p15* induced by decitabine. *A*, hatch marks, map of the *p15* gene with CpG sites. Arrow, transcription start site and beginning of the coding sequence (CDS). PCR product used for both pyrosequencing and cloning-sequencing. DNA methylation of *p15* was quantitated using bisulfite PCR and pyrosequencing. Mean methylation of *p15* for the high-dose (black columns) and low-dose (gray columns) studies. *B*, analysis of *p15* methylation for a single patient treated with decitabine by bisulfite sequencing. PSQ, bases assayed by pyrosequencing. ●, methylated CpG sites; ○, unmethylated sites. By pyrosequencing, methylation before treatment with decitabine was 63% with a decrease to 42% by day 2 and a further decrease to 8% by day 5, whereas the percentage blasts were 49%, 59%, and 41%, respectively, showing the persistence of leukemia during the change in DNA methylation.

ation at higher doses. The mechanism of this plateau warrants further study. It may reflect a simple pharmacokinetic phenomenon, in which the metabolism and incorporation of decitabine into DNA is saturated at higher doses. A given level of hypomethylation could conceivably lead to inhibition of further decitabine incorporation, or this threshold level of hypomethylation may lead to cell death and therefore a selection of cells with a fixed “maximum” hypomethylation. This also may reflect biological differences in AML versus CML, as most of the patients treated at lower doses suffered from AML, whereas the majority of patients treated at higher doses had CML. It is possible the methylation changes at higher doses can be explained by the predominance of malignant cells in CML and is independent of the decitabine dose. Alternatively, this dose plateau may reflect the rapid induction of remethylation past a given threshold, and *in vitro* work provides

evidence for such remethylation (29). Although we did not have samples at later time points available on this study, a previous study indeed shows recovery of methylation back to baseline within a few weeks of the treatment (23). Overall, our data suggest that higher doses of decitabine may add toxicity but no further ability to induce DNA hypomethylation. This biochemical evidence supports the use of low-dose decitabine in clinical practice. Due to the small number of patients being studied and the heterogeneity of the patients being treated, the dose response data must be viewed cautiously, and further studies will be needed to confirm these results.

The finding that decreases in global methylation correlate with clinical benefit of decitabine only in the low-dose study may be attributable to the disease, predominately CML in the high-dose study versus AML in the low-dose study. Indeed, the findings in CML of a paradoxical greater decrease in methylation in responders were also seen in a different group of patients (23). Another possibility is that the dosing regimens may be important to the clinical activity of decitabine. In the high-dose study, decitabine was given at high doses and may act as a cytotoxic pyrimidine analogue, whereas in the low-dose study decitabine was given at lower doses for longer periods to take advantage of its demethylating properties. This agrees with previous studies *in vitro* that have shown that the ability of hypomethylating agents to induce cellular differentiation is optimal at lower doses (7). In addition, the proliferative rate of the tumor under study may also influence to relative cytotoxicity versus hypomethylation effects of the drug. It has been argued that in CML the inverse correlation between hypomethylation and response can be attributed to rapid death of sensitive cells, whereas resistant cells hypomethylate but do not die (23). Overall, it is important to note that the number of patients studied here with a substantial response is small and that our results have to be interpreted with caution.

Comparison of all the loci examined showed variable decreases in mean methylation after treatment with decitabine. Although the methods used to measure DNA methylation varied, the relative decrease of methylation was *LINE* (15.6%) > total 5-methylcytosine (14.1%) > *Alu* elements (9%) > *HoxA5* and *H19* (minimal change). Interestingly, the mean decrease of *p15* methylation in patients who were hypermethylated at that gene was 53%, showing that genes aberrantly methylated in cancer may be particularly susceptible to the drug. These observations need confirmation using a larger number of samples and genes. The mechanisms of this gene-specific effect and whether it contributes to the therapeutic index remain unknown. Nevertheless, it is reassuring that normally methylated genes do not show large decreases in methylation, which had been a concern with this therapy (30, 31).

It is important to remember that methylation of repetitive elements and total 5-methylcytosine serve as indicators for

changes in DNA methylation, but changes in DNA methylation can have varied downstream effects. We did not study samples for markers of differentiation, which is an important potential therapeutic mechanism of agents that inhibit DNA methylation. Possibly, the simplest explanation about how decitabine has activity in cancer is that it reactivates expression of tumor suppressor genes that have been silenced by aberrant DNA methylation (10). One would expect tumor suppressor genes, such as *p15*, which are aberrantly methylated and silenced in leukemia to be reactivated by treatment with decitabine. We have shown a preferential susceptibility of the hypermethylated *p15* gene to inhibition of methylation by decitabine, which may help explain the cancer selective toxicity of decitabine and therefore, in part, its therapeutic index. However, methylation of *p15* at baseline inversely correlated with response. This finding may be due to other confounding factors, such as increased bone marrow blasts, which correlate with *p15* methylation. Alternatively, hypomethylation of *p15* may not be a crucial target gene for decitabine treatment, and methylation changes of other tumor suppressor genes and genomic loci need to be evaluated. It is also important to note that we have not examined the expression of *p15* or other genes in this study, and such an analysis may yield results quite different from those obtained with DNA. Previous studies have shown that decitabine can induce expression of tumor antigens that could theoretically induce an antitumor immune response (32). If this were true, sequential therapy of decitabine followed immunotherapy could prove valuable.

Our work expands on previous studies showing the ability of decitabine to inhibit DNA methylation in patients. It highlights the difficulty in using gene-specific assays to measure DNA methylation changes but shows that global methylation changes occur and correlate with both dose and response to decitabine. Ironically, we show that patients whose leukemia was hypermethylated at one tumor suppressor gene, *p15*, were less likely to respond to decitabine. Nevertheless, our work supports *in vitro* work that lower doses of decitabine are optimal for hypomethylation and encourages pursuit of low-dose clinical strategies and the study of other genomic loci, in which hypomethylation is induced by decitabine.

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