DNA Methylation Patterns at Relapse in Adult Acute Lymphocytic Leukemia

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ABSTRACT

Purpose: Aberrant DNA methylation of promoter-associated CpG islands is an epigenetic DNA modification observed in acute leukemias that in certain cases has been associated with a poor prognosis and increased relapse rates. To study the role of DNA methylation in relapse mechanisms in acute lymphocytic leukemia (ALL), we have compared the methylation status of five genes at the time of initial presentation and at first relapse in 25 adult patients with ALL.

Experimental Design: Genes studied included the estrogen receptor (ER), multidrug resistance gene 1 (MDR1), p73, p15, and p16. DNA was extracted from paraffin-embedded bone marrow biopsies. DNA methylation was analyzed using PCR of bisulfite-modified DNA.

Results: Results indicate that methylation at the time of relapse was stable in 92% of patients for p73, 88% for ER, 80% for p16, 72% for MDR1, and 60% for p15. Only one case had p16 methylation at initial presentation, whereas 6 patients (P = 0.0001) had methylation at relapse. Three cases had concomitant methylation of p15 and p16 at relapse. The degree of MDR1 methylation inversely correlated with the presence of MDR1 expression as detected by immunohistochemistry. Eighteen patients (72%) had acquired no or one methylation change, whereas the rest (28%) had methylation changes in two or three genes. No clinical-biological correlations were found between methylation of any particular gene or pattern.

Conclusions: In summary, DNA methylation patterns are stable in a majority of patients with relapsed ALL, but a subset of patients acquire new methylation changes, in particular affecting cell cycle regulatory genes.

INTRODUCTION

DNA methylation refers to the addition of a methyl group to the cytosine in a cytosine-guanosine pair (CpG). CpG pairs are underrepresented in eukaryotic genomes but can be found at near-expected frequency in intergenic repetitive sequences such as Alu repeats or in the proximity of gene promoters. Methylation of these promoter-associated areas has been associated with gene silencing and is a physiological compensatory dosage mechanism in X-linked and imprinted genes. By silencing functionally relevant genes, aberrant DNA methylation has been linked to diverse human pathologies (1), including cancer (2) and aging (3). Aberrant methylation of multiple promoter-associated CpG islands has been found in acute leukemias (4–6). These studies have revealed that subgroups of patients with acute leukemia are characterized by a hypermethylator phenotype defined by the simultaneous methylation of multiple genes. This is similar to the CpG island methylator phenotype described in colon cancer (7) and suggests that aberrant methylation is an important oncogenic mechanism in acute leukemias.

Methylation of several genes has been associated with poor prognosis in patients with acute leukemia. Hypermethylation of the calcitonin gene has been associated with poor prognosis and disease progression in ALL (8, 9). This phenomenon has also been observed for HIC1 (11) and WT-1 (12) in AML. In contrast, hypermethylation of ER has been associated with a better prognosis in AML (13), although abnormal methylation of this gene is also found at the time of relapse (14). In bladder cancer, hypomethylation of the MDR1 gene has been associated with protein reexpression and a chemoresistant phenotype at relapse (15), p15 and p16, two cell cycle regulatory genes, are considered tumor suppressor genes. Diverse abnormalities of these two genes, including p15 methylation, have been shown to be acquired at the time of relapse in pediatric ALL (16). Methylation of p15 has been shown to confer poor prognosis and to predict the risk of relapse in patients with acute leukemias (17, 18). Methylation of p16, a rare event in human leukemias (6, 19), has been associated with disease progression in adult T-cell leukemia (20). Other lymphoid neoplasms, such as non-Hodgkin’s lymphomas, also are characterized by abnormal methylation of these two genes, a phenomenon also associated with relapse.

1 The abbreviations used are: ALL, acute lymphocytic leukemia; AML, acute myeloid leukemia; MRD, minimal residual disease; CR, complete remission; MDR1, multidrug resistance 1; CR, complete remission; MSP, methylation-specific PCR; COBRA, combined bisulfite restriction analysis.
These findings suggest that aberrant methylation may have a role in leukemic relapse. Knowledge of the dynamics of methylation patterns at the time of relapse may have important implications, including the understanding of cellular clonal changes during disease progression, the development of assays to detect MRD, and the development of targeted therapies using hypomethylating agents for high-risk patients.

We have previously studied the methylation status of ER, MDR1, p73, p15, p16, THBS1, THBS2, CD10, c-abl, and Myod in 80 patients with ALL (6). We have selected five of them, ER, MDR1, p73, p15, and p16, to study the methylation patterns both at the time of initial presentation and at relapse in 25 patients with relapsed ALL who had achieved complete remission after initial induction chemotherapy. Our results indicate that the methylation patterns of patients with relapsed ALL are stable in a majority of patients, but that a subgroup of patients acquire new methylation changes, in particular of genes involved in cell cycle regulation.

**MATERIALS AND METHODS**

**Samples.** Paraffin-embedded bone marrow biopsies from 25 adult patients with ALL obtained at the time of original diagnosis and at first relapse have been analyzed in this study. Patients were selected from a cohort of 80 patients studied previously (6). Selection was based on evidence of relapse and at relapse in 25 patients with relapsed ALL who had achieved complete remission after initial induction chemotherapy. Our results indicate that the methylation patterns of patients with relapsed ALL are stable in a majority of patients, but that a subgroup of patients acquire new methylation changes, in particular of genes involved in cell cycle regulation.

**Bisulfite Modification of DNA.** Methods for bisulfite modification of DNA and subsequent PCR techniques used in this study are described in detail. Bisulfite induces deamination of unmethylated cytosines, converting unmethylated CpG sites to UpG without modifying methylated sites. This allows their differentiation by allele specific PCR (MSP), restriction digestion (COBRA) or sequencing. Bisulfite treatment of genomic DNA was performed as described (23). DNA was extracted using standard phenol-chloroform methods after samples had been deparaffinized using xylene and ethanol followed by digestion with proteinase K. After extraction, 2 μg of DNA were used for bisulfite treatment. DNA was denatured in 0.2 N NaOH at 37°C for 10 min and incubated with 3 M sodium bisulfite at 50°C for 16 h. DNA was then purified using the Wizard cleanup system (Promega) and desulfonated with 0.3 N NaOH at 25°C for 5 min. DNA was then precipitated with ammonium acetate and ethanol, washed with 70% ethanol, dried, and resuspended in H2O.

**Primer Design.** Primer sequences, coordinates, GenBank accession numbers, number of expected restriction fragments, and PCR conditions are shown in Table 1. For p73 and p16, we used MSP as described elsewhere (19, 24). For p15, MDR1, and ER, we used COBRA (25). To minimize overestimation of methylated alleles when using this method, the following points were followed: (a) primers were designed to contain a minimum number of CpG dinucleotides in their sequence to avoid biased amplification of methylated alleles. If primers contained CpG sites, they were designed to amplify methylated and unmethylated equally (with a mixture of C or T used for the sense strand or G or A for antisense primers); (b) primers were designed to contain a maximum number of thymidines converted from cytosines to avoid amplification of the nonconverted genomic sequence; (c) amplification of genomic DNA not treated with bisulfite was always carried out to monitor lack of nonspecific amplification; (d) primers were designed to be within 300 bp of known transcription start sites; and (e) for each set of primers, we tested: (i) multiple restriction enzymes to confirm the methylation status and sequence of the fragment analyzed; and (ii) performed mixing experiments (using methylated and unmethylated templates mixed at a known ratios) to exclude any potential amplification bias.

**PCR and Restriction Digestion.** PCR reactions were carried in 50-μl reactions. In each reaction, 2 μl of bisulfite-treated DNA were used, as well as 1.25 mM deoxynucleotide

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**Table 1 Genes studied**

<table>
<thead>
<tr>
<th>Gene accession number</th>
<th>Primers (coordinates)</th>
<th>Restriction enzyme</th>
<th>Restriction sites</th>
<th>Annealing temperatures (no. of cycles)</th>
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</thead>
<tbody>
<tr>
<td>ER X03635</td>
<td>GGTTTTTGAGTTTTTTGTTTTG (300)</td>
<td>BstUI</td>
<td>1</td>
<td>60 (3), 57 (4), 54 (5), 51 (25)</td>
</tr>
<tr>
<td>p15 AC000049</td>
<td>GGAGTTTAAGGGGTTGGG (24747)</td>
<td>BstUI</td>
<td>2</td>
<td>60 (3), 57 (4), 54 (5), 51 (25)</td>
</tr>
<tr>
<td>MDR1 AC002457</td>
<td>GTTATAAGGATTGAGTTT (140829)</td>
<td>TaqI</td>
<td>1</td>
<td>60 (3), 57 (4), 54 (5), 51 (25)</td>
</tr>
<tr>
<td>p73 methylated (Ref. 24)</td>
<td>ACCCCGAACATCGACGTCCG</td>
<td>60 (30)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p73 unmethylated (Ref. 24)</td>
<td>GACGTAGCGAACTCGGGGTTC</td>
<td>60 (30)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p16 methylated (Ref. 19)</td>
<td>ATCCACACCCCCAACATCAACATCCA</td>
<td>60 (30)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p16 unmethylated (Ref. 19)</td>
<td>AGGGGATGATATTGGGTTT</td>
<td>60 (30)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

triphosphate, 6.7 mM MgCl₂, 5 μl of PCR buffer, 1 nmol of primers and 1 unit of Taq polymerase. All PCR reactions were performed using a hot start at 95°C for 5 min. After amplification, except for p73 and p16, PCR products were digested with specific restriction enzymes that digest alleles that were methylated prior to bisulfite treatment (25) and separated in non-denaturing polyacrylamide gels. Gels were stained with ethidium bromide. The proportion of methylated versus unmethylated product (digested versus undigested) was quantitated by densitometric analysis, determining the density of methylation. Den- sitometric analysis was performed using a Bio-Rad Geldoc 2000 digital analyzer equipped with the Quantity One version 4.0.3 software. For both p73 and p16, we used MSP as described (19, 24).

Immunocytochemistry. Immunohistochemical staining for MDR was performed on formalin-fixed, paraffin-embedded tissue sections of bone marrow biopsy or aspiration clot sections, an avidin-biotin-peroxidase complex method, and an automated immunostainer (Dako, Carpinteria, CA). All tissue sections underwent heat-induced epitope retrieval using citrate buffer (pH 6.0). After rinsing in PBS, an anti-MDR1 antibody (1:25 dilution of C-19 goat polyclonal IgG; Santa Cruz Biotechnology, Santa Cruz, CA) was applied as primary antibody for 1 h at room temperature. The detection system used was LSAB2 detection kit (Dako). Negative and positive controls were also run.

Statistical Analyses. Criteria for stable, increased, or decreased methylation were defined based on the technique used. For MSP (used for p73 and p16), stable methylation was considered if the samples at original presentation and relapse were either methylated or unmethylated at both endpoints, increased methylation if the bone marrow at presentation showed no methylation but there was evidence of methylation at relapse, and decreased methylation if only the initial presentation sample showed evidence of methylation. For COBRA (used in ER, p15, and MDR1), methylation was stable if the samples had a density of methylation of 0–2% at both endpoints or more than that at both endpoints, increased methylation if the initial sample had a density of 0–2% and >5% at relapse, and decreased methylation if the sample had a density of methylation >5% at initial presentation and <2% at relapse. Methylation patterns refer to the number of methylated or unmethylated genes in a single patient. For those genes for which COBRA was used to measure methylation, and thus a qualitative analysis could be performed, we used a t test for dependent samples to compare the methylation status at initial presentation and at relapse. For those two genes where MSP was used (p73 and p16) and only a qualitative analysis could be performed, we used the McNemar χ² test. Correlation coefficients were computed using Spearman rank correlation coefficients. Variables analyzed include individual gene methylation values and the patient characteristics age, gender, WBC, hemoglobin, platelet counts, albumin, β2-microglobulin, cytogenetics, immunophenotype, and systemic and central nervous system risk at presentation (22). Definition of CR and disease-free survival have been described elsewhere (22). Estimated 5-year disease-free survival and overall survival were based on the Kaplan-Meier method, and differences were tested using the log-rank test.

### RESULTS

**Patient Population.** We have analyzed the methylation status of ER, MDR1, p73, p15, and p16 from paraffin-embedded biopsies from 25 patients with ALL obtained at the time of initial presentation and at relapse. All patients were enrolled in the hyper-CVAD program at the University of Texas M. D. Anderson Cancer Center (22). All patients had achieved CR prior to relapse. The clinical-pathological features of these patients are summarized in Table 2.

**Methylation Status of Five Selected Genes.** To study methylation patterns at the time of initial presentation and relapse in ALL, we have analyzed the methylation status of five genes in 25 adult patients with adult ALL. Genes studied included ER, MDR1, p73, p15, and p16. Different criteria were used for gene selection. ER, MDR1, p15, and p16 are known to be methylated in ALL (6, 14, 17, 24, 26). Methylation of ER and p15 have also been shown to correlate with disease progression (17, 27). Expression of MDR1 has been correlated with a chemoresistant phenotype in bladder cancer (15). MDR1 expression has been associated with a poor prognosis in elderly patients with AML (28). p16 is infrequently methylated in ALL (6, 19), but it has been found to be deleted at higher frequency in relapse pediatric ALL (16).

For all these genes, except for p73, we have analyzed four to five normal bone marrow specimens, none of which showed significant (>2%) methylation (data not shown). p73 has been shown not to be methylated in normal marrow (24). Fig. 1 shows representative examples of methylation for each gene. Table 3 summarizes the distribution of methylation densities for each individual gene both at initial presentation and at relapse. Definitions for stable, increased, and decreased methylation are given above. ER methylation remained stable in 22 cases (88%) of cases, increased in 2 (8%), and decreased in 1 (4%). MDR1

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**Table 2** Patient characteristics

<table>
<thead>
<tr>
<th>Study cohort (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Median age</strong></td>
<td>26</td>
</tr>
<tr>
<td><strong>Male sex</strong></td>
<td>15 (60)</td>
</tr>
<tr>
<td><strong>WBC count &gt;30 × 10⁹/liter</strong></td>
<td>14 (56)</td>
</tr>
<tr>
<td><strong>Platelet count &gt;100 × 10⁹/liter</strong></td>
<td>7 (28)</td>
</tr>
<tr>
<td><strong>Hemoglobin level &lt;10 g/dl</strong></td>
<td>7 (28)</td>
</tr>
<tr>
<td><strong>Lactate dehydrogenase level &gt;600 units/liter</strong></td>
<td>24 (96)</td>
</tr>
<tr>
<td><strong>Karyotype</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Diploid</strong></td>
<td>3 (12)</td>
</tr>
<tr>
<td><strong>Ph-positive</strong></td>
<td>7 (28)</td>
</tr>
<tr>
<td><strong>Hyperdiploid</strong></td>
<td>1 (4)</td>
</tr>
<tr>
<td><strong>Miscellaneous</strong></td>
<td>8 (32)</td>
</tr>
<tr>
<td><strong>Insufficient metaphases</strong></td>
<td>6 (24)</td>
</tr>
<tr>
<td><strong>Immunophenotype</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Mature B</strong></td>
<td>2 (8)</td>
</tr>
<tr>
<td><strong>T</strong></td>
<td>1 (4)</td>
</tr>
<tr>
<td><strong>CALLA</strong></td>
<td>19 (76)</td>
</tr>
<tr>
<td><strong>Null</strong></td>
<td>1 (4)</td>
</tr>
<tr>
<td><strong>First CR rate</strong></td>
<td>25 (100)</td>
</tr>
<tr>
<td><strong>Second CR rate</strong></td>
<td>10 (43)</td>
</tr>
<tr>
<td><strong>Median duration 1 CR</strong></td>
<td>52 wk</td>
</tr>
<tr>
<td><strong>Median duration 2 CR</strong></td>
<td>85 wk</td>
</tr>
<tr>
<td><strong>Overall survival</strong></td>
<td>107 wk</td>
</tr>
</tbody>
</table>

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"Only 23 patients evaluable."
methylated in 18 cases (72%), increased in 4 (16%), and decreased in 3 (12%), and p15 remained stable in 15 (60%), increased in 7 (28%), and decreased in 3 (12%). The methylation status of p73 and p16 was analyzed using MSP; therefore, no quantitative analysis could be performed. For p73, 23 cases remained stable (92%), whereas 1 increased and 1 decreased (4% each). p16 remained stable in 20 cases (80%) but increased in 5 (20%; \( P = 0.0001 \); Fig. 2). No case of p16 demethylation was observed.

The analysis of multiple genes at presentation and relapse allows the evaluation and comparison of methylation patterns at relapse (Table 4). Eighteen patients (72%) had changes (increased or decreased methylation) of 0 or 1 genes. Three patients had methylation changes of two genes (12%) and 4 patients (16%) had changes in three genes, including 1 patient with complete demethylation of all three previously methylated genes. Of importance, of the 5 cases with increased p16 methylation at relapse, 3 had concomitant increase in p15 methylation and 1 was methylated at p15 at initial presentation.

**DNA Methylation Status Correlates with Protein Expression.** DNA methylation has been associated with transcriptional inactivation and gene silencing. To demonstrate that the changes in DNA methylation observed in this study correlated with the level of gene activation, we performed immunohistochemical analysis of MDR1 in cases with known methylation densities. Because of the sample source, we could not study RNA levels. Three paired samples with known methylation levels were blindly evaluated for MDR1 expression by a hematopathologist. None of the samples studied with methylation of MDR1 had detectable levels of the corresponding protein by immunostaining, whereas all unmethylated samples had evidence of protein expression, representing an excellent correlation between DNA methylation and MDR1 protein expression.

**Clinical and Pathological Correlates.** Using Spearman Rank correlation analysis, we have explored possible correlations between methylation of a particular locus or methylation pattern with clinical and pathological characteristics and known prognostic factors in ALL. Methylation patterns analyzed included patients with 0–1 methylation changes versus more than one gene and patients with concomitant p15 and p16 methylation. Factors analyzed included age, gender, WBC, hemoglobin, platelet counts, albumin, \( \beta2 \)-microglobulin, cytogenetics, immunophenotype, and systemic and central nervous system risk at presentation. No significant association was found between methylation of any of the genes studied, any of the above patterns, and any of the studied clinical characteristics. Next, we
studied the impact of gene-specific methylation and methylation patterns on second CR rate, duration of first and second remission, and overall survival. No correlation with second CR rate, duration of first and second remission, or overall survival was observed.

**DISCUSSION**

In the present study, we have analyzed the methylation patterns at the time of initial presentation and relapse in 25 adult patients with ALL. Results indicate that the methylation status of the five genes analyzed remained stable at relapse in 60–92% of the cases, depending on the gene studied. As a consequence, methylation patterns are not altered at the time of relapse in a majority of patients (72%), although a significant minority (28%) acquires changes at relapse. These changes most frequently involved *MDR1*, *p15*, and *p16*. It is important to note that *p16* methylation is significantly increased at relapse, and that this is usually accompanied by concomitant *p15* methylation. No associations were observed between methylation at initial presentation and any of the clinical-biological characteristics analyzed or with CR rates, disease-free survival, or overall survival.

This is the first study to simultaneously analyze the methylation status of multiple genes both at initial presentation and relapse in a homogeneously treated cohort of patients. ER is frequently methylated in acute leukemias and has been shown to track with disease activity in ALL (14). Although ER methylation has been shown to confer good prognosis to patients with AML (13), we were unable to demonstrate such observation in a large series of ALL patients (6). ER methylation remains stable at relapse in close to 90% of the cases studied. Unfortunately, its role in disease prognostication and in MRD evaluation is limited because ER methylation is observed in nonneoplastic tissues (27).

*p73* is a p53 homologue that has been shown to be selectively methylated in lymphoid malignancies but not in normal tissues (24, 26). No prior information exists regarding its methylation frequency in relapsed ALL. In our series, this gene remains stable in 90% of cases, suggesting that evaluation of *p73* methylation could have a role in studies of MRD in ALL.

Expression of *MDR1* has been shown to correlate with a poorer prognosis in elderly patients with AML (28). Methylation of *MDR1* has been shown to correlate with the level of expression and resistance in leukemia cell lines and in chronic lymphocytic leukemia (29). In bladder cancer, methylation of *MDR1* has been shown to inversely correlate with *MDR1* overexpression, an effect promoted by exposure to chemotherapy. In our series, *MDR1* methylation remained stable in 72% of cases, the rest of cases affected both by demethylation and hypermethylation of the gene. These changes in methylation density correlated with the level of MDR1 protein expression. Several studies have
analyzed the role of \textit{p15} and \textit{p16} abnormalities in relapse ALL. In a study of pediatric ALL (16), \textit{p16} was shown to be deleted at relapse in 5 of 18 samples (30\%). The same study also evaluated \textit{p15} deletion and methylation at relapse; 3 of 18 (16\%) cases acquired \textit{p15} deletion at relapse. All 3 cases had also acquired concomitant \textit{p16} deletion. Four of these cases were evaluated for \textit{p15} methylation both at initial presentation and at relapse; in 2 cases, methylation appeared at relapse; 1 case remained unmethylated; and 1 case was demethylated.

There are several limitations to our study. The first one is related to the number of patients and genes studied. Obviously, by increasing the number of patients, we could have observed relevant clinical-biological associations not found presently. Also, by studying more genes, we could have found other important genes aberrantly methylated at relapse. The second limitation is related to the techniques used to study methylation. In this study, we used archival paraffin-embedded biopsies, the only relapse material available to us. The source and small quantity of DNA obtained precluded the use Southern blot as done in prior studies. Bisulfite sequencing of PCR fragments, the current gold standard for methylation analyses, would have required close to 2500 sequencing reactions to conduct a comprehensive analysis of the cohort studied here. To circumvent these issues, we have used two different methods, depending on the genes studied. Both techniques, COBRA and MSP, exploit the modification of DNA by bisulfite. By specifically deaminating nonmethylated cytosines, it allows the design of specific primers for unmethylated and methylated sequences (MSP) or the use of specific restriction enzymes (COBRA). Because we are comparing the methylation patterns at both endpoints, the results obtained should be representative of the methylation changes occurring in the promoter areas of the genes analyzed.

It is important to emphasize that in the present study, we have not attempted to evaluate the role of \textit{p15} and \textit{p16} deletions in relapse ALL, nor have we attempted to clarify whether the methylation changes are acquired in the initial dominant malignant cell or whether they represent a shift toward a selected cellular clone at relapse.

To our knowledge, this is the most extensive study of DNA methylation at the time of relapse in ALL. It is important to note that all patients studied here were treated homogeneously with the same chemotherapy program. Although these results need to be confirmed in prospective larger scale studies, this work has important implications in the understanding of relapse mechanisms in leukemia. The results suggest that relapse in ALL is dominated by the original cellular malignant clone in a majority of patients, but a subgroup of patients acquires methylation changes at relapse. The frequency of concomitant methylation of \textit{p16} and \textit{p15}, together with the high rate of deletions reported by other groups (30), confirms the important role of these two genes in relapsed leukemia. The fact that we have not found any defining clinical or biological characteristic of this subgroup of patients is probably a result of the relatively few patients studied. These results have implications in the development of methylation techniques to study MRD in ALL. Genes such as \textit{p73} and \textit{p16} could be used to monitor disease progression in patients with ALL. The information obtained could be used to treat patients still in morphological remission with hypomethylating agents. The use of 5-aza-2-deoxycytidine could also be explored for remission maintenance in patients with known methylation patterns.

In summary, our results indicate that methylation patterns at relapse in ALL are stable in a majority of patients and confirm the importance of \textit{p15} and \textit{p16} in relapsed leukemia.

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