DNA Methylation of Tumor Suppressor Genes in Clinical Remission Predicts the Relapse Risk in Acute Myeloid Leukemia

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Abstract

Epigenetic changes play an important role in leukemia pathogenesis. DNA methylation is among the most common alterations in leukemia. The potential role of DNA methylation as a biomarker in leukemia is unknown. In addition, the lack of molecular markers precludes minimal residual disease (MRD) estimation for most patients with hematologic malignancies. We analyzed the potential of aberrant DNA promoter methylation as a biomarker for MRD in acute leukemias. Quantitative real-time PCR methods with bisulfite-modified DNA were established to quantify MRD based on estrogen receptor α (ERα) and/or p15INK4B methylation. Methylation analyses were done in >370 DNA specimens from 180 acute leukemia patients and controls. Methylation of ERα and/or p15INK4B occurred frequently and specifically in acute leukemia but not in healthy controls or in nonmalignant hematologic diseases. Aberrant DNA methylation was detectable in >20% of leukemia patients during clinical remission. In pediatric acute lymphoblastic leukemia, methylation levels during clinical remission correlated closely with T-cell receptor/immunoglobulin MRD levels (r = +0.7, P < 0.01) and were associated with subsequent relapse. In acute myelogenous leukemia patients in clinical remission, increased methylation levels were associated with a high relapse risk and significantly reduced relapse-free survival (P = 0.003). Many patients with acute leukemia in clinical remission harbor increased levels of aberrant DNA methylation. Analysis of methylation MRD might be used as a novel biomarker for leukemia patients’ relapse risk. [Cancer Res 2007;67(3):1370–7]

Introduction

Acute leukemias are caused by genetic and epigenetic mechanisms involving tumor suppressors and oncogenes (1–3). Intensive chemotherapy and often stem cell transplantation have emerged as the treatments of choice. However, current treatment is associated with significant short- and long-term toxicity and even treatment-related death. Cytogenetic aberrations play an important role for prognosis and treatment stratification in acute myelogenous leukemia (AML; refs. 4, 5). In some leukemias, molecular diagnostics based on specific genetic changes have successfully been used to quantify the residual number of leukemic cells and to predict the risk of leukemia relapse. In leukemias with balanced translocations such as t(9;22), t(15;17), inv(16), and t(8;21), the resulting chimeric fusion genes lead to expression of mRNAs that can be quantified by real-time reverse transcription-PCR analyses (6, 7). The results from these analyses correlate with patients’ outcome (8). In addition, in acute lymphoblastic leukemia (ALL), quantitative analysis of rearranged T-cell receptor (TCR) or immunoglobulin loci can be used to estimate the burden of clonal leukemia cells (9). Unfortunately, specific primers have to be developed for each patient rendering this method expensive and labor intensive. In addition, results remain inconclusive in some patients. Nevertheless, minimal residual disease (MRD) quantified with this method has been shown to be a strong predictor of relapse in ALL (10). Consequently, several pediatric and adult treatment study groups for ALL now use MRD quantitation for treatment stratification (11–13). However, this methodology is not suitable for AML and fails in some patients with ALL. In adult AML, >50% of the patients relapse after achieving a complete remission (14, 15). Unfortunately, no reliable prognostic variable is available for the majority of AML patients (16). In addition, in these patients, quantitation of MRD at the molecular level is not possible due to a lack of specific markers. The search for reliable molecular markers is therefore warranted.

Epigenetic changes, such as aberrant DNA methylation, are the most frequent alterations found in acute leukemia (1, 17–25). Promoter silencing by DNA methylation is an established mechanism to inhibit tumor suppressor genes (26). These mechanisms are thought to contribute to leukemia pathogenesis. Due to the high frequency of DNA methylation in acute leukemias and its involvement in leukemia pathogenesis, we investigated whether sensitive and specific analysis of DNA methylation patterns could serve as reliable markers for MRD quantitation and as a predictor for leukemia relapse in remission. Recently, we introduced a rapid and quantitative methylation detection methodology based on real-time PCR (27). Here, we show increased estrogen receptor α (ERα) and p15INK4B methylation levels in bone marrow from leukemia patients in clinical remission. Intriguingly, high ERα and p15INK4B methylation levels in clinical remission were associated with a high risk for leukemia relapse and poor relapse-free survival. These findings suggest that analysis of methylation levels in clinical remission can be used as a biomarker for patients’ relapse risk.
Materials and Methods

**Samples.** Genomic DNA was isolated by means of standard procedures from bone marrow samples obtained for diagnostic reasons from adult patients with different types of leukemia \((n = 114)\) and other malignancies \((n = 24)\); nonmalignant and normal bone marrow \((n = 21)\) samples were obtained for control purposes (Table 1). Sequential bone marrow aspirates were obtained when possible. AML patients were treated within protocols of the German AML Cooperative Group. More detailed clinical information about the AML and myelodysplasia patients is provided in Supplementary Tables S1 and S2.

Ten pediatric ALL patients with four time points of follow-up were selected for analysis (Table 2). These patients received treatment according to either the COALL-06-97 or COALL-07-03 study (28). MRD analyses were done as described (29). Genomic DNA was bisulfite treated and used for detecting methylation of \(p15^{INK4B}\) and \(ER\alpha\) as described below.

Informed consent was obtained from all patients and healthy subjects.

**Bisulfite treatment of genomic DNA.** Bisulfite treatment of DNA converts all unmethylated CpG sites to UpG, leaving methylated CpGs intact (30). A CpG genome kit for the bisulfite conversion of genomic DNA samples was used following manufacturer's instructions (Chemicon, Temecula, CA). Following bisulfite treatment, all DNA samples were stored at \(-80^\circ\)C.

**Primers and probe design.** Bisulfite-treated DNA sequence for \(p15^{INK4B}\) (NM_000125) and \(ER\alpha\) (NM_004936) were used for primers and probe design. A nested PCR approach was used to specifically amplify the region of interest. The outer primers amplified DNA irrespective of the methylation status, whereas the nested primers bound to methylated or non-methylated DNA (Fig. 1A). The outer primers amplified DNA irrespective of the methylation status, whereas the nested primers bound to methylated or non-methylated sequences within the region amplified by the first PCR. All primers were designed by the MethPrimer program V1.1 beta (31). Primers sequences for \(ER\alpha\) were TTAGGGTAAGGTAATAGTTTTTGGT (outer-F), AACTTACTACCGGGATGGG (outer-R), ATGCTTGTGTGGTG (MSP-F), and CCAAAATTAAAAACAACA-TCATCACTACCACCCCCACTA (USP-R). Primers sequences for \(p15^{INK4B}\) were ATGGTGTGTTGGT-TATTGTA (outer-F), ATCATCATAACCTAATACAC (outer-R), GAAAGGAGAGAGGTGCGAAGTATTACATG (MSP-F), and TATCTACTACCGCCCC-CACA (MSP-R), and TATCTACTACGGCCTTCT (USP-F), and ATCCATCATAACCTACCTAC (USP-R).

**Detection of CpG methylation.** Bisulfite-converted genomic DNA was amplified using the outer primers. Five units of GoTaq polymerase (Promega, Madison, WI), 150 nmol/L of each primer, and 2 \(\mu\)L of bisulfite-treated DNA were used in a total volume of 15 \(\mu\)L. Following 2 min of incubation at 95°C, 17 PCR cycles were done for 45 s at 95°C, 45 s at 55°C, and 60 s at 72°C. A total of 1 \(\mu\)L amplified product from the first PCR reaction was used as a template for the nested PCR reaction using methylation-specific-or non–methylation-specific primers mentioned above. Reactions were done in a total volume of 12 \(\mu\)L with 250 nmol/L of each primer, 125 nmol/L probe, and real-time PCR master mix (Eurogentec). Real-time PCR (HT7900, Applied Biosystems) was used for amplification and analyses.

**Quantitation and statistical analyses.** CpG methylation was quantitated according to the following formula: fraction of methylated molecules = \(2^{(\text{threshold non–methylation-specific primers} - \text{threshold methylation-specific primers})}\). All PCR reactions were independently done at least twice. The means of the threshold values were used for the final calculation.

A standard curve was established to determine the percentage of methylated molecules. For this purpose, completely methylated DNA (Chemicon) was diluted with genomic DNA from peripheral blood of a healthy person. In addition, genomic DNA from a leukemia patient with a high methylation level was exponentially diluted with healthy donor non-methylated genomic DNA. Similar amounts of total DNA \((1 \mu\)g\) were bisulfite treated and PCR amplified as described above. Statistical analyses were done with SPSS 12.0. Nonparametric tests were used to compare differences in methylation levels and relapse risk. The log-rank test was used to compare differences in methylation levels and relapse risk. The log-rank test was used to compare differences in methylation levels and relapse risk. The log-rank test was used to compare differences in methylation levels and relapse risk. The log-rank test was used to compare differences in methylation levels and relapse risk. The log-rank test was used to compare differences in methylation levels and relapse risk.
used to determine the statistical significance of the Kaplan-Meier plots. The α error was set at 5%. Frequencies in cross tables of relapse analyses were compared using the χ² test.

Results

Detection of aberrant DNA methylation patterns by real-time PCR. Aberrant methylation of CpG dinucleotides occurs frequently in acute leukemia. Recently, we introduced a real-time PCR method to quantitatively analyze DNA methylation patterns of specific gene promoters with high sensitivity (27).

We used this method to analyze DNA methylation patterns in bone marrow specimens from adult patients with AML and ALL at different stages of disease. Overall, 241 samples were analyzed from AML patients and 33 samples from patients with adult ALL (acute leukemia total: n = 274). Samples from patients with myelodysplasia (n = 11) and other malignant diseases (n = 15) and

<table>
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<tr>
<th>Patient no.</th>
<th>Sex</th>
<th>Age (y)</th>
<th>WBC at diagnosis</th>
<th>Immunophenotype</th>
<th>Time to relapse (d)</th>
<th>Time to BMT (d)</th>
<th>Phase</th>
<th>Time to death (d)</th>
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<tr>
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<td>7.2</td>
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<tr>
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<td>68.8</td>
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<tr>
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<td>C-ALL</td>
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<td>2.CCR</td>
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<td>Sept pneumonia in relapse</td>
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NOTE: CR1 to CR5 are five patients in remission. R1 to R5 are five relapsed patients. Abbreviation: BMT, bone marrow transplantation.

Figure 1. DNA methylation analysis by real-time PCR. A, primers and probes used for analyzing methylation in ERα (Genbank NM_000125) and p15 (Genbank NM_004936). A nested PCR approach was used for increased sensitivity and specificity. The outer primers bind irrespective of the methylation status to bisulfite-modified DNA. The nested primers are specific for methylated (MSP) or non-methylated (USP) sequences. The FAM-TAMRA–labeled probe was designed to bind irrespective of the methylation status. B and C, correlation of two independent experiments. To analyze the reproducibility of the method, the scatter plot indicates the correlation of Ct values, obtained from two independent experiments. B, MSP; C, USP. The correlation coefficient for p15 methylation (n = 321 patients) was highly significant (r = 0.93, P < 0.001). D, a standard curve was prepared by serially diluting completely methylated genomic DNA with healthy donor lymphocyte genomic DNA. Methylation status was analyzed for p15 and for ERα using primers and probe mentioned in (A). These standard curves were used to calculate the percentage of methylated DNA for individual patients.
specimens with nonmalignant/normal bone marrow (n = 21) were analyzed for comparison and control reasons. We chose to concentrate on p15INK4B and ERα promoter methylation because these have been shown to be frequently methylated in acute leukemias (32, 33). We developed real-time primers and probes that specifically detected methylated or nonmethylated sequences in bisulfite-treated DNA. To increase sensitivity and specificity, a nested PCR was done (Fig. 1A). All samples were independently analyzed for non-methylated and methylated p15INK4B and ERα sequences at least two times. Percentages of methylated sequences were calculated using the 2^{ΔCt–ΔCt} method. Results of independent analyses closely correlated well with each other (Fig. 1B and C). Experiments with serially diluted DNA indicated that the method was quantitative within a wide range of methylated DNA sequences (Fig. 1D and Fig. 3A).

Having established sensitivity and specificity of the method, we first analyzed the frequency of DNA methylation in different diseases (Fig. 2). For p15INK4B methylation, average methylation percentages indicated that DNA methylation was specific for malignant diseases and occurred preferentially in AML and ALL.

Nonhematologic malignant diseases without bone marrow involvement and bone marrow specimens from patients without malignant disease (n = 36) showed far lower levels of p15INK4B DNA methylation compared with patients with active leukemia (primary diagnostic relapse and refractory AML; n = 139; Fig. 2; P < 0.001, Mann-Whitney U test; Table 1; Supplementary Table S1).

The highest methylation level of p15 and ERα in nonmalignant samples excluding one obvious outlier were set as cutoff values (2.9 and 5.1, respectively), and methylation status in patients with AML or ALL was analyzed. We found 60% of AML patients (69 of 115) with high levels of p15 methylation compared with 6% (1 of 15) patients with nonmalignant diseases (Fig. 2B). The one patient with nonmalignant disease with high levels of p15 methylation actually suffered from autoimmune hepatitis.

Similar findings were obtained for the analysis of ERα methylation. Sixty-three percent of patients with AML (83 of 132) harbor high methylation of ERα compared with 0% patients with nonmalignant diseases having methylation level higher than the cutoff value (Fig. 2B). Samples from ALL patients also showed higher methylation of p15 and ERα (42% and 70%, respectively; Fig. 2A and B). The frequency and degree of p15 and ERα methylation in AML and ALL significantly exceeded the levels found in samples from controls and patients without leukemia (P < 0.001).

MRD quantitation using serial samples from individual patients. The use of MRD for clinical decision making is well established in pediatric ALL. Patient-specific primer and probe combinations allow the detection of residual leukemia cells at a sensitivity of ∼10^{-4}. Serial analyses of individual patients are routinely done. To analyze the potential of methylation patterns for MRD analysis (methylation MRD) in serial analyses, we exponentially diluted primary samples with high levels of methylation. Dilutions were done with genomic DNA from unmethylated primary sample in several logs up to 10^{-4}. The methylation level decreased accordingly for both genes ERα and p15. The methylation levels in normal healthy donors were at the dilution range 10^{-4}, which indicates the basal level of methylation in normals (ERα, 0.0009 ± SD 0.0001; p15, 0.023 ± SD 0.003; Fig. 3A).

MRD analyses were done for samples from 10 pediatric ALL patients treated within protocols of the Cooperative Study Group for Childhood Acute Lymphoblastic Leukemia (COALL) in Germany. MRD analyses by PCR for TCR/IgH/IgL were done as described (29). The samples were analyzed for ERα methylation without prior knowledge of either MRD status or clinical course of the disease. Methylation level for each patient was set as 1 at the time of primary diagnosis. The change in DNA methylation levels were calculated at several follow up time points for the individual patient. Relapses were correctly predicted in three patients (three of five), and five of five patients in continuous remission were correctly predicted. As shown in Fig. 3C, ERα methylation levels significantly decreased after induction therapy and remained low in five of five patients with continuous complete remission (Fig. 3C). These results correlated well with the data from TCR/immunoglobulin rearrangement analysis (Fig. 3D). At the same time, the levels of ERα methylation stayed higher in relapsing patients following induction therapy (three of five). In two patients, methylation decreased after induction therapy but increased again at the time of relapse (Fig. 3D). MRD level by TCR/immunoglobulin rearrangement analysis in these patients with relapse was higher compared with patients in remission (Fig. 3F). Methylation analysis in patients with relapse correlated closely with the TCR/immunoglobulin in the majority of patients (four of five: compare...
patients 1, 2, 4, and 5; Fig. 3E and F). Overall, a close correlation was observed between classic TCR/immunoglobulin–based MRD and methylation MRD analyses (Fig. 3B). The linear correlation was highly significant ($r = +0.75$, $P < 0.001$). Taken together, high methylation MRD levels during clinical remission in pediatric ALL correlate with classic MRD analyses and are associated with an increased risk of leukemia relapse. These findings provided the basis for further analyses in AML where established MRD markers are missing for the majority of patients.

**DNA methylation patterns in different stages of AML and risk of relapse.** Serial analyses of DNA methylation in individual patient samples allowed the identification of MRD levels. The detection of aberrant methylation in remission without analysis at the time of diagnosis could be another option for methylation MRD analysis. Several ALL patients that ultimately relapsed showed methylation levels in remission that were higher than $10^{-4}$ (1%). This indicated that even in remission, leukemia patients can harbor significant amounts of methylated sequences. We therefore analyzed methylation levels in AML samples without morphologically detectable blast counts (<5%). Overall, methylation levels in samples from leukemia patients in complete remission were significantly lower compared with patients at the time of initial diagnosis or relapse ($P < 0.001$, Mann-Whitney) for p15INK4B (median, 1% versus 4.7%) and ERT (median, 4.47% versus 8.1%; Fig. 4A and B). The percentages of the blasts in the bone marrow of AML patients during primary diagnosis were 80% (median) or 72% (mean) and during complete remission were 0% (median) or 0.2% (mean). Levels of methylation were stable or increased in samples obtained at the time of relapse compared with initial diagnosis (data not shown). We also analyzed the percentages of patients with AML at diagnosis or in complete remission having methylation levels higher than the cutoff value (highest methylation level in patients with nonmalignant diseases: 2.9 for p15 and 5.1 for ERT). In the case of p15, 73% of patients at primary diagnosis had high methylation compared with 20% of patients in complete remission. Similar results were observed for ERT, where 58% of patients showed high methylation, but only 20% of patients in complete remission.
This indicates that methylation levels decreased in most of the patients in complete remission.

However, elevated levels of DNA methylation were present in a significant fraction of patients compared with controls: 20% of patients for p15\textsuperscript{INK4B} (19 of 94) and 20% of patients for ER\textalpha{} (18 of 90) in complete remission still retained high methylation levels (Fig. 4A and B). To study the prognostic effect of methylation levels during complete remission, we analyzed the relapse-free survival in AML patients who seem to reach complete remission.

**Methylation MRD during clinical remission and relapse risk in AML.** Patients whose bone marrow showed substantial levels of DNA methylation in complete remission experienced a significantly increased rate of subsequent relapse compared with patients without detectable DNA methylation (Fig. 5). Patients with higher levels of ER\textalpha{} methylation (cutoff value = 4.9%) in the first remission of their disease experienced shortened relapse-free survival (mean, 410 versus 643 days) with borderline statistical significance ($P = 0.068$, log-rank test; Fig. 5A). Six of 10 patients (60%) of patients with high levels of ER\textalpha{} methylation in their first remission sample relapsed, whereas only 30% (12/40) of patients with low-level methylation relapsed ($P = 0.077$; Fig. 5B). Similar findings were obtained for p15\textsuperscript{INK4B} methylation levels (Fig. 5A and B): Patients with high levels of p15\textsuperscript{INK4B} methylation (cutoff = 1.4%) showed significantly decreased relapse-free survival (mean, 691 versus 427 days; $P = 0.003$). Accordingly, the majority of patients with high levels of p15\textsuperscript{INK4B} methylation in remission (10 of 17, 59%) relapsed, whereas patients with low levels of methylation did not (5 of 29, 17%; $P = 0.004$). When the patients were separated into three groups according to the methylation status (low for both genes, low for ER\textalpha{}, and high for p15\textsuperscript{INK4B} versus high for both genes), significant differences in relapse-free survival were observed (mean, 704 versus 551 versus 305 days; $P = 0.006$). Patients with methylation of neither gene relapse in 17% of the cases, whereas all four patients with both genes methylated relapsed ($P = 0.003$). These data provide evidence that aberrant DNA methylation patterns in hematologic remission are associated with leukemia relapse.

**Discussion**

In this study, we show increased levels of methylation in the bone marrow of patients with acute leukemia in clinical remission. Our analyses provide evidence for a close correlation of methylation MRD with established MRD markers in acute leukemia. The presence of aberrant DNA methylation in remission is a powerful indicator for a high risk of leukemia relapse.

Epigenetic regulation plays a key role in the pathogenesis of leukemia (34–37). Aberrant DNA methylation patterns are the most frequent molecular alterations detected in AML. Whereas the pathogenetic importance of these changes has begun to emerge, DNA methylation has thus far only played minor roles as biomarker in diagnosis, prognosis prediction, and treatment control.

There are several prerequisites of any genetic or epigenetic alteration to be used to monitor MRD. First, the underlying event should occur frequently. In AML, aberrant promoter methylation of p15\textsuperscript{INK4B} and/or ER\textalpha{} occurred in the majority of primary AML bone marrow samples (Fig. 2A and B). This is in line with reports from other authors who showed ER\textalpha{} methylation ranging from 50% to 90% (33) and aberrant methylation of p15\textsuperscript{INK4B} ranging from 50% to 80% in hematopoietic malignancies (32). Importantly, significant levels of DNA methylation were shown not only in patients with overt leukemia but also in samples that did not harbor morphologically detectable blasts. Our methodology does not allow to specify which individual cell was methylated during complete remission, but we speculate that these were the residual malignant cells that were not detectable by conventional morphologic analysis. In line with this, our data showed a significant correlation between the degree of ER\textalpha{} methylation and the TCR/immunoglobulin transcript that was specific for the malignant cell clone. Moreover, we were able to show a significant correlation of residual methylation with the time of disease-free survival. Patients with high methylation during complete remission had a higher rate of relapse, whereas low methylation during complete remission predicted lower rates of relapse during at least 12 months of follow-up.

PCR analyses of either the TCR or immunoglobulin heavy- or light-chain rearrangements as well as balanced translocations have
allowed identifying infrequent leukemic cells well below a threshold of 1%. These PCR-based methods represent the current gold standard of MRD detection in ALL. Importantly aberrant DNA methylation levels correlated closely with conventional TCR/immunoglobulin MRD levels in ALL, indicating the potential use of the easier methylation analysis for relapse prediction.

The combination of p15INK4B and ERα methylation levels was more powerful in predicting disease recurrence compared with either marker alone. In addition, in ALL (similar to TCR/immunoglobulin–based MRD analyses), we did not identify all relapsing patients. It seemed that methylation analysis was able to identify a high-risk group, whereas the TCR/immunoglobulin–based MRD analyses rather characterized a low-risk group with continuously decreasing MRD levels. Both techniques stratified patients with similar accuracy. It is possible that a combination of both techniques might improve the capabilities to correctly predict treatment outcome with conventional chemotherapy.

It should be emphasized that this study is a proof-of-principle study with regard to the use of ERα and p15INK4B as markers for methylation based MRD. It is currently unclear which genes show the best distinction in terms of leukemia-specific methylation compared with background methylation analyses. We used ERα and p15 for methylation analyses because both are known to be methylated in many patients with acute leukemias. Recently, novel high-throughput technologies have emerged that allow successful identification of methylated gene promoters. These technologies will be helpful to identify the set of genes with the highest predictive value for MRD assessment. The methylation MRD approach is not restricted to acute leukemia. Aberrant DNA methylation levels occur in most, if not all, hematologic and nonhematologic malignancies. Thus, methylation MRD could be widely applicable after further validation in prospective studies for individual diseases.

The effect of antileukemic treatment was associated with a decline of methylation as detected by PCR. This was most likely due to apoptosis of the malignant cells and recovery of normal hematopoiesis. Previous reports found that resistance of tumor cells to conventional chemotherapy may be associated with global hypermethylation, and that this resistance can be overcome by the use of demethylating agents (38). Interestingly, another antineoplastic agent (arsenic trioxide) has recently been shown to demethylate methylated tumor suppressor gene promoters and restore gene transcription through down-regulation of DNMT1 (39). Thus, future studies will show which therapeutic agents lead to demethylation and which agents do not. Thus far, there is no evidence that the chemotherapy given to the patients of our study had any direct effect on the methylation.

It is noteworthy that drugs with demethylating activity are increasingly used and studied in hematologic malignancies (40–42). One could speculate that the presence of increased DNA methylation levels in acute leukemias in clinical remission might provide a rationale for the use of these drugs in affected patients.

Figure 5. Increased methylation MRD levels are associated with a high relapse rate and decreased survival. A, Kaplan-Meier plots of relapse-free survival of AML patients in first complete remission after induction therapy. Methylation levels for ERα and p15INK4B were determined and regarded as low or high. For the combination of both markers, patients with one methylated promoter (either ERα or p15INK4B) are indicated as low/high. B, relapse risk at 12 mos after the sample was obtained in first complete remission according to the methylation status of the indicated gene. The actual numbers of patients in each category are shown as well.
patients. Further clinical studies are necessary to clarify this possibility and to define the accurate potential of methylation MRD estimation.

In conclusion, our findings provide evidence that aberrant DNA methylation is present in clinical remissions in leukemia patients. DNA methylation analyses can be used as molecular markers for MRD. These findings may offer the possibility of MRD estimation for many patients who currently lack adequate markers.

References


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