Epigenetic Silencing of the Tumor Suppressor MicroRNA Hsa-miR-124a Regulates CDK6 Expression and Confers a Poor Prognosis in Acute Lymphoblastic Leukemia

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Abstract

Whereas transcriptional silencing of genes due to epigenetic mechanisms is one of the most important alterations in acute lymphoblastic leukemia (ALL), some recent studies indicate that DNA methylation contributes to down-regulation of miRNAs during tumorigenesis. To explore the epigenetic alterations of miRNAs in ALL, we analyzed the methylation and chromatin status of the miR-124a loci in ALL. Expression of miR-124a was down-regulated in ALL by hypermethylation of the promoter and histone modifications including decreased levels of 3mK9H3 and 3mK27H3 and increased levels of 2mK9H3, 3mK9H3, and 3mK27H3. Epigenetic down-regulation of miR-124a induced an up-regulation of its target, CDK6, and phosphorylation of retinoblastoma (Rb) and contributed to the abnormal proliferation of ALL cells both in vitro and in vivo. Cyclin-dependent kinase 6 (CDK6) inhibition by sodium butyrate or PD-0332991 decreased ALL cell growth in vitro, whereas overexpression of pre-miR124a led to decreased tumorigenicity in a xenograft in vivo Rag2−/−γc−/− mouse model. The clinical implications of these findings were analyzed in a group of 353 patients diagnosed with ALL. Methylation of hsa-miR-124a was observed in 59% of the patients, which correlated with down-regulation of miR-124a (P < 0.001). Furthermore, hypermethylation of hsa-miR-124a was associated with higher relapse rate (P = 0.001) and mortality rate (P < 0.001), being an independent prognostic factor for disease-free survival (P < 0.001) and overall survival (P = 0.005) in the multivariate analysis. These results provide the grounds for new therapeutic strategies in ALL either targeting the epigenetic regulation of microRNAs and/or directly targeting the CDK6-Rb pathway.

Introduction

MicroRNAs (miRNA) are small, non–protein-coding RNAs of 19 to 25 nucleotides that regulate gene expression by targeting miRNAs in a sequence-specific manner, inducing translational repression or miRNA degradation (1, 2) and playing important roles in cell processes such as proliferation, apoptosis, differentiation, or even in tumorigenesis (reviewed in ref. 3). A systematic search for a correlation between the genomic position of miRNAs and the location of cancer-associated regions revealed that more than half of the mapped miRNAs are located at fragile chromosome regions involved in human cancer (4). MiRNA expression profiles also indicate that most miRNAs have lower expression levels in tumors compared with normal tissues (5), suggesting that some of these miRNAs may act as putative tumor suppressor genes (6). Whereas hundreds of miRNAs are discovered and various functions of miRNAs have been intensively studied, the mechanisms that control their expression are largely unknown.

In cancer, transcriptional silencing due to epigenetic mechanisms is one of the most important alterations (7). DNA hypermethylation of tumor suppressor genes, global genomic hypomethylation, and aberrant histone modification signatures are the three most important epigenetic alterations associated with malignant transformation (8). The fact that miRNA expression occurs in a tissue-specific or developmental stage–specific manner and that some miRNAs are imprinted (9), supports the hypothesis that DNA methylation may regulate miRNA expression as recent studies have indicated (10–14). Therefore, it is conceivable that DNA methylation regulates miRNA expression during tumorigenesis (15).

We and others have extensively shown that hypermethylation of gene promoters is a frequent mechanism of gene silencing and a finding associated with the prognosis of the disease and response to therapy in patients with acute lymphoblastic leukemia (ALL; refs. 16–21). Methylation in human ALL cells participates in the inactivation of key cellular pathways such as cell cycle (21), apoptosis (22), and cell-cell adhesion (23). Moreover, aberrant methylation of CpG islands is quantitatively different in individual tumors within the same tumor type, and this patient-specific methylation profile provides important prognostic information in ALL patients (17–19). The presence in individual tumors of multiple epigenetic events that affect different pathways implicated in cell survival and proliferation, such as the WNT pathway, also provides important insights into the pathogenesis of ALL (16).
All these data suggest the putative presence of DNA methylation-associated silencing of miRNAs in ALL cells. To explore this hypothesis, we analyzed the epigenetic regulation of miRNAs in ALL by chromatin immunoprecipitation (ChIP)-on-Chip using histone marks of closed and open chromatin, identifying the mir-124a loci as a potential candidate for epigenetic silencing. Methylation and expression analysis showed that DNA methylation contributes to the transcriptional down-regulation of mir-124a in ALL-derived cell lines.

Figure 1. Expression of hsa-miR-124a is regulated epigenetically in ALL-derived cell lines. A, quantitative ChIP-PCR analysis for the histone marks 3mK4H3, ACH3, 2mK9H3, 3mK9H3, and 3mK27H3 in the hsa-miR-124a-1, 124a-2, and 124a-3 CpG islands. Values are expressed as the percentage of the corresponding histone mark in ALL-derived TOM-1 and NALM-20 cell lines in comparison with expression in bone marrow human lymphocytes (HL; 100%). B, bisulfite sequencing and MSP analysis of the hsa-miR-124a-1, 124a-2, and 124a-3 CpG island regions in positive methylated control (MC), HL, and ALL-derived cell lines. Each box indicates a CpG dinucleotide (white box, unmethylated; black box, methylated). U, unmethylated allele; M, methylated allele. C, hsa-miR-124a expression in TOM-1 and NALM-20 cell lines before and after treatment with 5-aza-2′-dCyd or trichostatin A. D, quantitative ChIP-PCR analysis of the histone mark ACH3 in the hsa-miR-124a-1, 124a-2, and 124a-3 CpG island regions in TOM-1 and NALM-20 cell lines before and after treatment with trichostatin A. Quantitative real-time MSP analysis of the hsa-miR-124a-1, 124a-2, and 124a-3 CpG island regions in TOM-1 and NALM-20 cell lines before and after treatment with 5-aza-2′-dCyd. TRI, trichostatin A; AZA, 5-aza-2′-dCyd. A, C, and D, columns, mean of three independent experiments; bars, SD. B, representative experiment of at least three different studies.
ALL, and that this epigenetic event affects the proliferative potential of the leukemic cells by modulating cyclin-dependent kinase 6 (CDK6). Abnormal methylation of miR-124a in ALL showed a negative prognostic effect in ALL patients. These results provide a rationale for the use of epigenetic drugs and CDK6 inhibitors in the treatment of ALL.

**Materials and Methods**

**Cell lines and patients.** Six ALL-derived cell lines (TOM-1, NALM-20, MY, LOUCY, JOURKAT, and TANOU) were purchased from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) and grown as described (16). We studied 353 patients (210 male and 143 female) with de novo ALL who were enrolled in successive multicenter studies of the Programa para el estudio y tratamiento de las hemopatias malignas (PETHEMA) Spanish study group. Clinical characteristics of the patients are listed in Supplementary Table S1 and Supplementary Materials and Methods.

**Chromatin immunoprecipitation.** Lymphocytes from peripheral blood of healthy donors, TOM-1, and NALM-20 cell lines, were subjected to ChIP. The ChIP assays were done as previously described (ref. 11; Supplementary Materials and Methods).

**DNA methylation analyses.** Methylation status was analyzed by methylation-specific PCR (MSP) using primers specific for either the methylated or bisulfite modified unmethylated DNA (Supplementary Table S2 and Supplementary Materials and Methods).

**Treatment of all cell lines.** ALL-derived TOM-1 and NALM-20 were treated with 4 μmol/L of 5-aza-2′-deoxycytidine (Sigma-Aldrich) for 4 d, 50 nmol/L of trichostatin A (Sigma-Aldrich) for 4 d, 50 nmol/L of 5-aza-2′-deoxycytidine (5-aza-2′-dCyd; Sigma-Aldrich) for 4 d, 50 nmol/L of trichostatin A for 4 d, and 10 mmol/L of PD-0332991 (Pfizer) for 24 h (Supplementary Materials and Methods).

**Quantitative expression analysis of hsa-miR-124a and CDK6 genes.** Expression of the different miRNAs and genes was analyzed with specific primers and TaqMan probe according to the TaqMan MicroRNA Assay protocol (Applied Biosystems; Supplementary Table S2 and Supplementary Materials and Methods).

**RNA and miRNA transfection.** The pre-miR-124a precursor molecule, negative precursor-miR control, siCDK6-select (43900824 ID: S53), and negative siRNA#1 control were purchased from Ambion and used for transfection studies (Supplementary Materials and Methods).

**Western blot analysis.** Proteins extracted from the ALL-derived cell lines TOM-1 and NALM-20 were analyzed by Western blot as previously described (ref. 16; Supplementary Materials and Methods).

**In vivo transplantation studies.** Animal studies were done according to institutional guidelines. The ALL-derived cell line TOM-1 was transfected in vivo with pre-miR-124a precursor molecule or precursor-miR control. Untransfected cells (mock) or cells transfected with the empty plasmid were used as controls. Twenty-four hours after transfection, 5 × 10⁶ viable cells were injected s.c. into the left flanks of 6-wk-old female BALB/cA-Rag2⁻/⁻ /γc⁻/⁻ mice (n = 6 per group). Mice were sacrificed after 20 d, and sizes of tumors were measured. Tumor volumes were calculated by using the equation $V = (A \times B^2)/2$, where $A$ is the largest diameter and $B$ is the perpendicular diameter. The tumor volume of mice injected with TOM-1 cells transfected with the precursor-miR control was considered as reference.

**Statistical analysis.** For statistical purposes, residual values of tumor volume were used to test for normal distribution using Shapiro-Wilk’s test. One-way ANOVA and Tamhane T2 multiple comparison test, after Levene’s test, were used to compare the mouse groups. $P < 0.05$ was considered significant in all statistical tests (detailed statistical analysis is provided in Supplementary Materials and Methods).

**Results**

**Expression of hsa-miR-124a in ALL cells is regulated by histone modification and CpG island hypermethylation.** Our ChIP-on-Chip analysis of 27,800 CpG islands using two ALL cell lines (TOM-1 and NALM-20) revealed that 11 of the 78 CpG islands that embedded or were close to known 13 miRNAs genes presented a decrease of 3mK4H3 and/or an increase of 2mK9H3 marks in ALL-derived cell lines in comparison with healthy donor samples (a pattern of histone modifications underlying a closed chromatin structure associated with repressive gene expression; Supplementary Table S3). Among the 13 miRNAs presumably regulated by histone modifications, we decided to focus on hsa-miR-124a miRNA family. Although hsa-miR-124a corresponds to a single mature miRNA, there are three genomic loci [hsa-miR-124a-1 (8p23.1), hsa-miR-124a-2 (8q13.2), and hsa-miR-124a-3 (20q13.33)] that encode for the same mature miRNA. Interestingly, the hsa-miR-124a-1 and hsa-miR-124a-3 genes are located within CpG islands whereas hsa-miR-124a-2 is 760 bp downstream of a CpG island. Recent studies have shown in colon cancer that the corresponding CpG islands of all three genes are methylated (11) whereas hsa-miR-124a-1 and hsa-miR-124a-3 are frequently methylated in acute myeloid leukemia (AML; ref. 24). The results obtained by the ChIP-on-Chip analysis were verified using quantitative PCR assay showing a decrease of 3mK4H3 and/or an increase of 2mK9H3 marks in ALL-derived TOM-1 and NALM-20 cell lines in comparison with healthy donor samples for all three miRNAs [hsa-miR-124a-1 (8p23.1), hsa-miR-124a-2 (8q13.2), and hsa-miR-124a-3 (20q13.33); Fig. 1A]. In addition, we analyzed the levels of Ach3, 3mK9H3, and 3mK27H3 in the same samples by quantitative PCR and observed a decrease of Ach3 and an increase of 3mK9H3 and 3mK27H3, all of these marks being associated with a closed chromatin structure (Fig. 1A).

Next, we analyzed the methylation of the CpG island–associated hsa-miR-124a (hsa-miR-124a-1, hsa-miR-124a-2, and hsa-miR-124a-3) in ALL-derived cell lines by bisulfite genomic sequencing and MSP (Fig. 1B). Hsa-miR124a1 and hsa-miR-124a3 were hypermethylated in every cell line (with the exception of MOLT-4 in which hsa-miR124a1 was not methylated) but not in healthy lymphocytes. However, nonneoplastic marrow lymphocytes or peripheral blood lymphocytes displayed hsa-miR-124a2 methylation (Fig. 1B). Therefore, a quantitative real-time MSP was done to quantify hsa-miR-124a2 methylation. Based on the background fluorescence intensity, a cutoff level was determined for specific fluorescence for both methylated hsa-miR-124a2 sequences and Alu control sequences in normal lymphocytes. This threshold was used to calculate the cycle threshold or crossing point ($C_T$) of each sample. The $C_T$ value was directly proportional to the amount of target sequence present in the sample. The normal sample that showed the lowest difference in $C_T$ between the target (methylated sequences) and the reference (Alu sequence) genes was used as a control sample for relative quantification of the hsa-miR-124a2 CpG island methylation in both healthy individuals and ALL samples and was considered as 100% normalized ratio. Based on these prerequisites and to determine the cutoff value for altered hsa-miR-124a2 methylation in ALL samples, we quantified by quantitative real-time MSP the methylation status of the hsa-miR-124a2 CpG island in 30 healthy donors. $N_{\text{hsa-miR-124a2}}$ ratios fell between 65% and 100% (85 ± 15%). A $N_{\text{hsa-miR-124a2}}$ ratio ≥130% (determined as the mean + 3 SD) was chosen to define hypermethylation of hsa-miR-124a2 in ALL DNA samples. By quantitative real-time MSP, the CpG island of hsa-miR-124a2 was revealed to be hypermethylated in all ALL-derived cell lines studied (median $N_{\text{hsa-miR-124a2}}$: 180 ± 38%).

Whereas expression of hsa-miR-124a was significantly down-regulated in ALL cell lines in comparison with peripheral blood...
lymphocytes from healthy donors (Supplementary Fig. S1), treatment of ALL cell lines with trichostatin A (HDAC inhibitor) or with the demethylating agent 5-aza-2'-dCyd induced an up-regulation of hsa-miR-124a expression (Fig. 1C), indicating that, indeed, expression of hsa-miR-124a is regulated epigenetically. Treatment with trichostatin A induced an increase of AcH3 levels in the three miRNA genomic regions, whereas demethylation of the three CpG island–associated hsa-miR-124a was observed after treatment with 5-aza-2'-dCyd in TOM-1 and NALM-20 cell lines (Fig. 1D). All these results indicate that methylation and chromatin modification are responsible, at least in part, for the abnormal down-regulation of hsa-miR-124a expression in ALL cells.

Up-regulation of hsa-miR-124a decreases ALL cell growth. To determine the role of hsa-miR-124a in ALL, expression of hsa-miR-124a was up-regulated in ALL-derived TOM-1 cells by nucleofection with pre-miR-124a, and cell growth was evaluated and compared with cells transfected with pre-miR scrambled or TOM-1 cells 20 d after s.c. injection of cells. Columns, mean tumor volume in BALB/c-A-Rag2−/− γc−/− mice (six different animals per condition and a representative example); bars, SD. *, P < 0.01; **, P < 0.001.

Figure 2. Hsa-miR-124a transfection decreases ALL cell growth in vitro and in vivo. A, expression analysis of hsa-miR-124a measured by quantitative reverse transcription-PCR (RT-PCR). Expression of hsa-miR-124a in TOM-1 and NALM-20 cells transfected with scrambled control was considered as 100% expression. B, cell viability and total cell counts of transfected TOM-1 cells were determined at various times by trypan blue exclusion. TOM-1 cells transfected with scrambled pre-miR were used as control. C, apoptosis was measured by Cell Death Detection ELISA kit. A to C, columns, mean from three independent experiments; bars, SD. D, comparison of tumor size in female BALB/c-A-Rag2−/− γc−/− mice transplanted with TOM-1 transfected with premiR-124a, TOM-1 transfected with scrambled, or TOM-1 cells 20 d after s.c. injection of cells. Columns, mean tumor volume in BALB/c-A-Rag2−/− γc−/− mice (six different animals per condition and a representative example); bars, SD.
decreased progressively throughout the culture. The level of expression remained above the control at day 8 to 10 and returned to control levels (no expression) by day 15 to 20 after transfection (Supplementary Fig. S2A). These results suggest that down-regulation of hsa-miR-124a could be associated with an increase in cell proliferation at least in vitro.

To further study the putative tumor-suppressive function of hsa-miR-124a in vivo, 5 x 10^6 viable TOM-1 untransfected cells, transfected in vitro with pre-miR scrambled control or pre-miR-124a precursor molecule, were injected s.c. into the left flanks of 6-week-old female BALB/cA-Rag2^-/-yc^-/- mice. Whereas animals transplanted with untransfected cells or with scrambled pre-miR control developed large tumors after 20 days, animals receiving TOM-1 cells transfected with pre-miR-124a showed a very significant decrease in tumor growth (Fig. 2D). At day 20, the median tumor volumes in the untransfected and empty vector–treated mice were 3,939.05 mm^3 (881.29) and 4,426.5 mm^3 (1,936.32; P = 0.932), respectively, whereas in mice inoculated with TOM-1 cells transfected with pre-miR-124a, the volume was 1,182.03 mm^3 (968.4), significantly lower than that in mice transplanted with cells treated with empty vector (P = 0.022; Fig. 2D). Histologic analysis of the tumors showed tumors infiltrated by lymphoblast, characteristic of ALL (Supplementary Fig. S3). These results together with the in vitro experiments indicate that down-regulation of hsa-miR-124a mediates cell growth in ALL and supports the role of hsa-miR-124a as a tumor suppressor gene.

Figure 3. Hsa-miR-124a expression mediates activation of CDK6-Rb in ALL cells. A, complementarity between hsa-miR-124a and CDK6 3’-UTR as predicted by PICTAR (http://pictar.bio.nyu.edu/). Green, hsa-miR-124a sequence; red, CDK6 3’-UTR sequence. B, CDK6 mRNA expression analysis by quantitative RT-PCR and protein level analysis of CDK6, Rb-P, and Rb-Total by Western blot in ALL-derived TOM-1 and NALM-20 cell lines transfected with pre-miR-124a molecule and scrambled precursor control. Expression of CDK6 in TOM-1 and NALM-20 cells transfected with scrambled pre-miR control was considered as 100% expression. Tubulin was used as a loading control in Western blot analysis. C, CDK6 mRNA expression analysis by quantitative RT-PCR in ALL-derived TOM-1 and NALM-20 transfected with negative siRNA#1 control and siCDK6. TOM scrambled and NALM scrambled (cells transfected with negative siRNA#1 control) were considered as 100% expression. h-Actin was used as a loading control in Western blot analysis. D, Western blot analysis of CDK6, Rb-P, and Rb-Total levels in ALL-derived TOM-1 and NALM-20 transfected with negative siRNA#1 control and siCDK6. Tubulin was used as a loading control. B and C, columns, mean of three experiments; bars, SD. B and D, representative study of three different experiments.
Hsa-miR-124a regulation of ALL cell growth is mediated through CDK6-Rb. It has been previously shown that CDK6 is a direct target of hsa-miR-124a (11), and as predicted by PICTAR, there is a complementarity between hsa-miR-124a and CDK6 3' untranslated region (UTR; Fig. 3A). Thus, to determine the potential role of hsa-miR-124a in the regulation of ALL cell growth in ALL cells and to experimentally validate if CDK6 is a target for hsa-miR-124a in ALL, we analyzed CDK6 mRNA and protein levels in human ALL-derived cell lines after reexpression.

7 http://pictar.bio.nyu.edu

Figure 4. Inhibition of the CDK6-Rb pathway by sodium butyrate and PD-0332991 leads to decreased growth and induction of apoptosis of ALL cells. TOM-1 and NALM-20 cells were treated with 1 and 10 mmol/L of sodium butyrate for 48 and 72 h (A and B) or 1 μmol/L PD-0332991 (C and D). A, CDK6 mRNA analysis by quantitative RT-PCR, protein level analysis by Western blot, and cell viability (determined at various times by trypan blue exclusion) in TOM-1 and NALM-20 cell lines treated with sodium butyrate. B, apoptosis of TOM-1 and NALM-20 cell lines treated with sodium butyrate measured by Cell Death Detection ELISA kit and Western blot of the 85-kDa fragment of poly(ADP-ribose) polymerase (PARP). C, Western blot analysis of CDK6, Rb-P, and Rb-Total levels in ALL-derived TOM-1 and NALM-20 treated with PD-0332991, and cell apoptosis measured by Cell Death Detection ELISA kit. β-Actin was used as a loading control. D, cell viability in TOM-1 and NALM-20 cell lines treated with PD-0332991 (determined at various times by trypan blue exclusion) and cell cycle analysis (propidium iodide only in NALM-20). Blue, control cells; green, cells treated with PD-0332991. A representative study of three different experiments or mean (± SD) of three independent experiments is shown. E.F., enrichment factor.
hsa-miR-124a. ALL-derived TOM-1 and NALM-20 cells nucleofected with pre-miR-124a showed an increase in expression of hsa-miR-124a (Fig. 2A) and a decrease in CDK6 mRNA and CDK6 and phosphorylated retinoblastoma (Rb-P) protein (Fig. 3B) levels in comparison with cells transfected with pre-miR scrambled control or empty vector. Inhibition of CDK6 expression in vitro was transitory and correlated inversely with expression of hsa-miR-124a, so that 8 days after transfection, it remained down-regulated but its expression was restored to normal levels by day 15 after transfection (Supplementary Fig. S2B). These results indicate that the epigenetic silencing of hsa-miR-124a in ALL leads to the activation of the CDK6-Rb oncogenic pathway. Interestingly, down-regulation of CDK6 expression induced by transfection of TOM-1 and NALM-20 cell lines with siRNA against CDK6 (Fig. 3C) was associated with a decrease in Rb phosphorylation (Fig. 3D), which further suggested the potential role of the CDK6-Rb pathway in ALL.

**Inhibition of the CDK6-Rb pathway leads to decreased ALL cell proliferation and growth.** The fact that CDK6 is involved in cell cycle progression and differentiation (11) and the existence of several molecules that inhibit CDK6 activity such as sodium butyrate (25) or PD-0332991 (26) establish CDK6 as an attractive target in ALL. To show the role of CDK6 in the pathogenesis of ALL, TOM-1 and NALM-20 cell lines were treated with sodium butyrate and PD-0332991. Incubation of ALL cells with 1 and 10 mmol/L of sodium butyrate for 48 and 72 hours induced down-regulation of CDK6 mRNA and protein expression levels, decreased cell growth (Fig. 4A), and increased apoptosis (Fig. 4B), in contrast to up-regulation of hsa-miR-124a, which did not induce apoptosis. This effect probably is due to the fact that sodium butyrate is not a specific inhibitor of CDK6 but can also inhibit other pathways involved in cell cycle and apoptosis, such as p53, cyclin D1, or histone deacetylase (27, 28).

![Figure 5](https://www.aacrjournals.org/doi/fig/10.1158/0008-5472.CAN-08-4025)

**Figure 5.** Methylation analysis of hsa-miR-124a in patients with ALL. A, methylation frequencies for hsa-miR-124a1, 124a2, and 124a3 in ALL patient samples and methylation frequencies for hsa-miR-124a in ALL patient samples. B, expression of hsa-miR-124a and CDK6 measured by quantitative RT-PCR in the nonmethylated and methylated groups of patients with ALL (**, P < 0.001). C, MSP analysis of hsa-miR-124a-1 and 124a-3 and expression of hsa-miR-124a measured by quantitative RT-PCR.
Next, we examined the effect in ALL cell lines of the small molecule PD-0332991 (26), a recently described specific inhibitor of CDK4/6. Treatment of TOM-1 and NALM-20 cells with 1 μmol/L PD-0332991 for 24 hours induced dephosphorylation of the Rb protein without changes in the protein levels of CDK6 or Rb, consistent with the mechanism of PD-0332991–mediated inhibition of CDK activity but with lack of transcription/translation inhibition (Fig. 4C). Treatment of ALL cell lines with 1 μmol/L PD-0332991 for 5 days induced a significant inhibition of cell growth and proliferation (Fig. 4D), but unlike sodium butyrate, it had no effect on cell apoptosis as shown by the analysis of oligonucleosomal fragments (Fig. 4A). This is in agreement with previous studies on multiple myeloma in which a combination of PD-0332991 with dexamethasone was required to induce cell apoptosis of myeloma cells, whereas treatment with PD-0332991 alone was able to induce inhibition of cell proliferation (29).

Expression of hsa-miR-124a in patients with ALL is regulated by CpG island hypermethylation. To determine the role of epigenetic regulation of hsa-miR-124a in a more clinically relevant model, we analyzed the methylation status of the three CpG islands associated with hsa-miR-124a in a group of 353 ALL patients (Supplementary Table S1). Methylation frequencies were as follows: 49% for hsa-miR-124a1, 46% for hsa-miR-124a2, and 42% for hsa-miR-124a3 (Fig. 5A). No methylated miRNAs were found in 145 of 353 patients (nonmethylated group, 41%) whereas most ALLs [208 of 353 (59%)] had methylation of at least one miRNA (methylated group, Fig. 5A). Methylation of the three hsa-miR-124a genes was significantly associated (Supplementary Table S4). Expression of hsa-miR-124a was also down-regulated in patients with methylation of hsa-miR-124a in comparison with nonmethylated patients (32 ± 36% versus 96 ± 43%; P < 0.001; Fig. 5B) with a correlation between methylation and expression (Fig. 5C), indicating that expression of hsa-miR-124a was regulated, at least in part, by methylation. In line with the studies in cell lines, patients with methylation of hsa-miR-124a showed a significant up-regulation of CDK6 expression compared with nonmethylated patients (332 ± 217% versus 167 ± 115%; P < 0.001; Fig. 5B).

Clinical outcome and hsa-miR-124a methylation profile. Finally, to determine whether methylation and expression of hsa-miR-124a was associated with prognosis in patients with ALL, we analyzed the outcome of a series of 353 patients diagnosed and treated uniformly within the PETHEMA study group. As shown in Supplementary Table S1, clinical and laboratory characteristics did not differ significantly between patients with methylated or unmethylated hsa-miR-124a, with the exception of higher WBC counts and the presence of more frequent BCR-ABL positivity among methylated patients. Other poor-risk cytogenetics or molecular events, risk groups according to both National Cancer
of cancer that may have significant consequences for cancer patients undergoing treatment with DNA-demethylating agents (31). Although our approach using ChIP-on-Chip yielded a number of miRNAs regulated by histone modifications (32), we decided to focus on hsa-miR-124a as a potential tumor suppressor gene with a role in the pathogenesis of ALL for several reasons: (a) The epigenetic regulation of hsa-miR-124a had been recently described in colon cancer and medulloblastoma. (b) Hypermethylation of gene promoters is a frequent mechanism of gene silencing and a finding associated with prognosis and response to therapy in ALL (19–22). (c) There was a known target gene of hsa-miR-124a, such as CDK6, a classic oncogene involved in cell proliferation and differentiation (33, 34). (d) CDK6 constitutes an attractive candidate for the use of small inhibitory molecules (26). The results of the study clearly support the approach, showing that epigenetic manipulation of hsa-miR-124a or pharmacologic inhibition of CDK6 results in inhibition of leukemic cell growth both in vitro and in vivo, and that methylation and down-regulation of hsa-miR-124a represents an independent prognostic factor in patients with ALL.

MiRNAs have been implicated in the pathogenesis of malignant and nonmalignant disease (35); however, information about the role of miRNA expression as prognostic factor in different tumors has only been recently described (36, 37). Using a miRNA summary value obtained from the analysis of 64 patients with cytogenetically normal AML and validated in another 55 patients, Marcucci and colleagues (36) recently showed the association between miRNA signature and clinical outcome, and more specifically, it was shown that the expression of miRNA-181 family members was associated with event-free survival. Using a similar approach in 84 patients with colon cancer, it was shown that high expression of miR-21 is associated with poor survival and patient outcome (37). In our study, using a large sample of patients with ALL (n = 553), we show that epigenetic regulation and expression of a specific miRNA defines a group of patients with ALL associated with poor outcome independently of other risk factors. Although the prognostic value of hypermethylation of cancer-related genes in patients with ALL has already been shown (16, 17), this is the first study to show that hypermethylation of a miRNA is independently associated with prognosis in ALL. The fact that methylation of hsa-miR-124a was also associated with other established risk factors such as WBC count or Ph positivity does not limit our conclusion because epigenetic regulation of hsa-miR-124a remains an independent prognostic factor in the multivariate analysis. This association should help in tailoring the treatment of patients with ALL.

An interesting and clinically relevant conclusion of our study is the role for CDK6 in the abnormal behavior of ALL cells. CDK6 is a member of a family of serine-threonine kinases involved in the control of cell cycle progression (38). CDK6 partners with cyclin D to phosphorylate the Rb protein during the G1-S cell cycle transition. Although CDKs are not essential for cell cycle progression (39), it has been well established that inappropriate regulation of CDKs is one of the most frequent alterations in human cancer, making CDKs an attractive target for the development of new inhibitors (40). Most of the information about the role of cyclin-dependent proteins in ALL is related to cyclins and cyclin-dependent quinase inhibitors such as p27Kip1 or p21 (41, 42). However, our in vitro results using ALL cell lines and a CDK6 inhibitor (PD-0332991) indicate that inhibition of this pathway maybe an effective therapy for ALL patients. A caveat to this conclusion is that a combination of PD-0332991 with other drugs
capable to induce apoptosis may be required, as suggested by the fact that PD-032991 was able to induce cell cycle inhibition but not cell apoptosis. Similar findings have been reported in a recent study using myeloma cells and a CDK4/6 inhibitor (43). PD-032991 potently inhibited CDK4/6-specific phosphorylation of Rb and cell cycle progression in myeloma cells, leading to induction of cell cycle arrest and increasing the sensitivity of myeloma cells to killing by bortezomib, but not inducing cell apoptosis on its own (43). Similar conclusions can be drawn from the in vitro studies with Rg2-“/yc”-/”: Although expression of pre-miR-124a induced a decrease in cell growth, ALL cells eventually were able to continue proliferating once the expression of pre-miR-124a was down-regulated and CDK6 expression was again up-regulated (as suggested by in vitro time course analysis of hsa-miR-124a and CDK6 expression; Supplementary Fig. S2). The capacity of the cells to eventually override the growth inhibition induced by hsa-miR-124a could also be related to the existence of other epigenetically regulated miRNAs that would affect the expression of CDK6. In fact, miRNA-34b/c has been described to regulate the expression of CDK6 and also to be epigenetically silenced in colorectal carcinoma (13), and we have preliminary evidence that miRNA-34b/c is hypermethylated in ALL.8 Taking all these considerations together, we believe inhibition of CDK6 to be more specific and clinically relevant for patients with ALL, inducing the overexpression of hsa-miR-124a.

In addition to the marks used in the ChIP-on-chip analysis, we examined other histone marks associated with chromatin state such as the repressive mark 3mK27H3 associated with the polycomb repressive complex 2 (PRC2; ref. 44). Recent studies in both malignant and normal stem cells have hypothesized that genes involved in pluripotency and malignant transformation are present in a “bivalent” chromatin state expressing both marks of open (3mK4H3) and closed (3mK27H3) chromatin, and that genes repressed by PRC2 proteins may be susceptible to hypermethylation, leading to tumor progression (45). Interestingly, the results of our work indicate that hsa-miR-124a as well as some of the other miRNAs identified in the ChIP-on-Chip analysis is regulated by PRC2 and by hypermethylation of their promoters. In that sense, it would be interesting to determine the chromatin state of these genes not only in the leukemic population but also in the population of leukemia-initiating cells to provide new evidence supporting the epigenetic progenitor origin of cancer hypothesis (46).

In summary, we show that the epigenetic regulation of hsa-miR-124a mediates, at least in part, the increased expression of CDK6 and contributes to the abnormal proliferation of ALL cells both in vitro and in vivo. Furthermore, hypermethylation of hsa-miR-124a is an independent prognostic factor for DFS and OS in patients with ALL. All these findings open the possibility of new therapeutic strategies for patients with ALL, either targeting epigenetically regulated genes using drugs that inhibit methylation and histone modifications and/or directly targeting the CDK6 protein.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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