DNA Methylation of Multiple Promoter-associated CpG Islands in Adult Acute Lymphocytic Leukemia[1]

Guillermo Garcia-Manero,[2] Jerry Daniel, Terry L. Smith, Steven M. Kornblau, Ming-Sheng Lee, Hagop M. Kantarjian, and Jean-Pierre J. Issa

Departments of Leukemia [G. G-M., J. D., H. M. K., J-P. J. I.], Biostatistics [T. L. S.], Bone Marrow Transplantation [S. M. K.], and Hematopathology [M-S. L.], University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

ABSTRACT

Purpose: **Aberrant methylation of promoter-associated CpG islands is an epigenetic oncogenic mechanism. The objective of this study was to define the methylation characteristics of patients with acute lymphocytic leukemia (ALL).**

Experimental Design: **Using bisulfite-PCR followed by restriction enzyme digestion (COBRA), we have analyzed the methylation status of 10 promoter-associated CpG islands in 80 untreated adult patients with ALL.**

Results: **Mean methylation density of *MDR1, THBS2, MYF3, ER, p15, THBS1, CD10, C-ABL,* and *p16* was 24.5%, 20.8%, 17.6%, 16.1%, 11.3%, 8.9%, 4.5%, 3.7%, and 1.3% respectively. *p73* was methylated in 17 of 80 cases (21.2%). A total of 86.2% of the cases had methylation of at least one gene, and 42.5% of the cases had methylation of three or more genes. *MDR1* methylation was inversely correlated with age ($P = 0.01$). *CD10* methylation inversely correlated with CD10 expression ($P = 0.0001$). Methylation of *MDR1* and *THBS1* was inversely associated with the presence of the Philadelphia chromosome, whereas *C-ABL* methylation correlated with the presence of the p210 variant of the Philadelphia chromosome. In univariate analysis, methylation of *THBS1* was associated with a favorable outcome ($P = 0.02$), whereas methylation of *p73, p15,* and *C-ABL* was associated with a trend toward worse prognosis.**

Conclusions: **Aberrant DNA methylation of promoter-associated CpG islands is very common in adult ALL and potentially defines subgroups with distinct clinical and biological characteristics.**

INTRODUCTION

Aberrant methylation of promoter-associated CpG islands has emerged as a distinct molecular pathway leading to cellular malignant transformation. By silencing key regulatory genes, such as tumor suppressor genes (1, 2), DNA methylation is the epigenetic equivalent of mutation/deletion in oncogenesis. The causes of aberrant methylation are unknown. In particular, it is not clear whether methylation represents rare random events selected for during carcinogenesis or results from underlying factors, including defects in the methylation machinery. It has recently been proposed that human malignancies can be classified according to their methylation characteristics in two different phenotypes: (*a*) tumors characterized by the simultaneous methylation of multiple CpG islands; and (*b*) tumors in which this phenomenon is not observed (3). Of importance, differences in methylation phenotype are associated with distinct clinical and genetic characteristics (4), suggesting that aberrant methylation of multiple promoter-associated CpG islands is not a random process but the result of as yet unidentified molecular defects that promote increased methylation of multiple CpG islands. This phenomenon has also been observed in gastric cancer (5), pancreatic cancer (6), and AML[3] (3, 7, 8).

ALL includes a heterogeneous group of malignant lymphoid disorders with different biological and clinical features (9). Prognosis and therapy in ALL are based on age, immunophenotype, karyotype, and predicted risk of central nervous system involvement (9) (10). Several genes are known to be abnormally methylated in ALL (11), including *calcitonin* (12, 13), *ER* (14), *p15* (15–17), *p16* (18, 19), *HIC-1* (20), *p73* (21, 22), and *E-cadherin* (23), among others. No study has evaluated the characteristics of multiple promoter-associated CpG islands in ALL.

To study the methylation characteristics of patients with ALL and its possible clinical/biological associations, we have analyzed the methylation status of 10 genes in 80 untreated adult patients with ALL. Our results suggest that aberrant methylation of multiple promoter-associated CpG islands is a common event in ALL, suggesting that methylation profiling could be used to develop a molecular epigenetic classification of ALL.

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[2] To whom requests for reprints should be addressed, at Department of Leukemia, Box 428, University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 745-3428; Fax: (713) 794-4297; E-mail: ggarciam@mdanderson.org.

[3] The abbreviations used are: AML, acute myelocytic leukemia; ALL, acute lymphocytic leukemia; ER, estrogen receptor; Ph, Philadelphia chromosome; MSP, methylation-specific PCR; CR, complete remission; DFS, disease-free survival; OS, overall survival; CALLA, common acute lymphocytic leukemia antigen.

Table 1 Genes studied

Gene/accesion no.	Primers (coordinates)[a]	Restriction enzyme/restriction sites	Annealing temperatures (no. of cycles)
ER	GGTTTTTGAGTTTTTTGTTTTG(300)	*Bst*UI	60 (3), 57 (4), 54 (5), and 51 (25)
X03635	AACTTACTACTATCCAAATACACCTC(505)	1	
p15	GGAGTTTAAGGGGGTGGG (24747)	*Bst*UI	59 (37)
AC000049	CCTAAATTACTTCTAAAAAAAAAC (24918)	2	
p16	GGTTTTGGYGAGGGTTGTTT (917)	*Taq*I	58 (3), 56 (4), 54(5), and 52 (23)
X94154	ACCCTATCCCTCAAATCCTCTAAAA (1078)	1	
MDR1	GTTATAGGAAGTTTGAGTTT (140829)	*Taq*I	54 (3), 51 (4), 48 (5), and 45 (26)
AC002457	AAAAACTATCCCATAATAAC (141001)	1	
C-ABL	TTAATAAAGGGTTYGGAGAG (37356)	*Taq*I	58 (34)
U07563	CTAAAATAAAATAAAACAAACCTACA (37633)	2	
THBS1	GGAGAGAGGAGTTTAGATTGGTT (46325)	*Bst*UI	54 (34)
AC037198	AATAAAAATTACTCCTAAAAAAC (46489)	1	
THBS2	ATTAGAAGGGGGATGATGTT (5571)	*TaqI*	58 (37)
U79410	ACCCTACAATACAAAATACTCC 5708)	1	
Myf3	TGTTGGAGAGGTTTGGAAAGG (9952)	*Rsa*I	60 (3), 57 (4), 54(5), and 51(25)
AF027148	AAAATCCRAAACCAATAAAAACAC (10141)	1	
CD10	TTYGGTTTTAGTTTGGAGTT (3665)	*Bst*UI	58 (3), 56(4), 54(5), and 52(26)
X79438	CCCTTTAAACCTTTCTCCCT (3843)	1	
p73 methylated (Ref. 21)	ACCCCGAACATCGACGTCCG GGACGTAGCGAAATCGGGGTTC		60 (34)
p73 unmethylated (Ref. 21)	ATCACAACCCCAAACATCAACATCCA AGGGGATGTAGTGAAATTGGGGTTT		60 (34)

[a] Y indicates C or T.

MATERIALS AND METHODS

Samples. Stored frozen mononuclear cells from bone marrow from 80 untreated adult patients with ALL were used in this study. Patients were selected solely based on sample availability. All patients were treated with the hyper-CVAD chemotherapy program (24) at the University of Texas M. D. Anderson Cancer Center. Patient characteristics and results of this program have been reported elsewhere (24). Consent for sample drawing and storage was obtained from all patients following institutional guidelines.

Bisulfite Modification of DNA. Methods for bisulfite modification of DNA and subsequent PCR techniques used in this study are described in detail on the World Wide Web.[4] Bisulfite treatment of DNA converts unmethylated CpG sites to UpG without modifying methylated sites, thus allowing their differentiation by allele-specific PCR, restriction digestion, or sequencing. Bisulfite treatment of genomic DNA was performed as described previously (25). In summary, DNA was extracted from bone marrow mononuclear cells using standard phenol-chloroform methods. After extraction, 2 μg of DNA were used for bisulfite treatment. This quantity of bisulfite-treated DNA can be used to analyze at least 10 genes by PCR. 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 H_2O.

Primer Design. To minimize overestimation of methylated alleles, the following points were considered: (*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 alleles equally (with a mixture of C or T used for the sense strand or a mixture of G or A used 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 multiple restriction enzymes to confirm the methylation status and sequence of the fragment analyzed and performed mixing experiments (using methylated and unmethylated templates mixed at a known ratios) to exclude any potential amplification bias. Primer sequences, coordinates, GenBank accession numbers, the number of expected restriction fragments, and PCR conditions are shown in Table 1.

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 triphosphate, 6.7 mM $MgCl_2$, 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, PCR products (except for p73) were digested with specific restriction enzymes that digest alleles that were methylated before bisulfite treatment and separated in nondenaturing polyacrylamide gels. Gels were stained with ethidium bromide. The proportion of methylated *versus* unmethylated product (digested

[4] http://www.mdanderson.org/leukemia/methylation.

versus undigested) was quantitated by densitometric analysis, determining the density of methylation. Densitometric analysis was performed using a Bio-Rad Geldoc 2000 digital analyzer equipped with Quantity One version 4.0.3 software. Due to the small size of the reported sequence in the promoter region of p73, we used MSP (21) to analyze the methylation status of this gene. This technique only allows a qualitative analysis of methylation. A list of primer sequences and restriction enzymes used is shown in Table 1.

Statistical Analyses. Correlation coefficients were computed between individual gene methylation values and the patient characteristics age, gender, percentage of blasts, WBC and platelet counts at presentation, albumin, cytogenetic abnormalities, immunophenotype and number of courses to achieve remission, and the risk of relapse as determined by a previously reported model (24). Extent of methylation for the 10 individual genes was compared between patients with and without the Ph chromosome, by means of the Wilcoxon rank-sum test. Agreement between two categorical variables was represented by a κ statistic. Correspondence of extent of methylation for the 10 genes considered was assessed by Spearman rank correlation coefficient. CR and DFS have been defined elsewhere (24). Estimates of 5-year DFS and OS rates were based on the Kaplan-Meier method, and differences were tested using the log-rank test. For some of the analyses below, for which a qualitative assessment of methylation was required, we used a threshold of $\geq$15% to define a sample as methylated. This threshold allows for factoring in methylation density when defining a sample as methylated and is similar to the method we used in previous studies. The use of a 10% threshold instead of a 15% threshold affects only 5% of the methylation measurements and does not appreciably change the results. All *P*s reported are two-sided.

RESULTS

Patient Population. We have analyzed the methylation characteristics of pretreatment bone marrow samples from 80 adult patients with ALL uniformly treated with the hyper-CVAD chemotherapy program at a single institution (24). The clinicopathological features of these patients are described in Table 2. The group studied here differed from the total hyper-CVAD cohort in several clinical features. These included a higher WBC count ($P = 0.001$) and lactate dehydrogenase level ($P = 0.001$) at presentation. These differences did not translate into prognostic differences in terms of CR rate (91% for both groups), DFS (32% *versus* 38% at 5 years), or OS (34% *versus* 39% at 5 years).

Methylation Status of 10 Selected Genes. Genes studied included *ER, p15, p16, MDR1, THBS1, THBS2, C-ABL, p73, MYF3,* and *CD10*. Different criteria were used for gene selection. *ER* (14), *p15* (17), *p16* (19), *p73* (21), and *MYF3* (26) were selected because of their frequent methylation in ALL. *MDR1* (27), *THBS1* (28), and its homologue, *THBS2*,[5] were studied because they have been found to be methylated in other malignancies, and their abnormal expression could have potentially important roles in ALL. We have also studied *C-ABL*, which is hypermethylated in Ph-positive chronic myelogenous leukemia (29, 30) and in Ph-positive ALL (31). *CD10* (CALLA) has recently been shown to be methylated and silenced in prostate cancer and is frequently expressed in ALL (32). For all these genes, with the exception of *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 bone marrows by other investigators (21, 22).

Table 2 Patient Characteristics

Characteristic	Study cohort	%
Median age (yrs)	39	
Male	48	60
WBC count >30 × 10^9/liter	37	46
Platelet count >100 × 10^9/liter	61	76
Hemoglobin level <10 g/dl	51	63
LDH[a] level >600 units/liter	73	91
Karyotype		
Diploid	19	23
Ph positive	19	23
T (8;14), t(8;2);t(8;22)	3	4
6q−, 14q+	7	9
Insufficient metaphases	9	11
Hyperdiploid	4	5
Hypodiploid	2	2.5
Immunophenotype:		
Mature B cell	6	7.5
T cell	9	11
T-CALLA	3	3.75
Precursor B	7	8.75
CALLA	45	56
Null	5	6.25
CR rate	73	91
DFS[b]	32%	
OS[b]	34%	

[a] LDH, lactate dehydrogenase.
[b] At 5 years.

Fig. 1 shows representative examples of methylation of each gene. Table 3 summarizes the distribution of methylation densities for each individual gene. Methylation (>2%) was lowest for the *p16* gene (6 of 80 cases) and highest for the *THBS2* gene (74 of 80 cases). Because aberrant hypermethylation will likely not only influence the number of CpG islands methylated but also influence the density of methylation of each particular island, we have quantitated the median methylation density of cases considered methylated ($\geq$15%). These ranged from 30.2% for *THBS1* to 46.6% for *C-ABL*. To assess the reproducibility of the methylation assay used here, we repeated 150 of the 720 non-MSP reactions. The Spearman rank order correlation coefficient between different measurements was $r = 0.811$ ($P = 0.000000$). The methylation status of *p73* was analyzed using MSP (21); therefore, no quantitative analysis could be performed. Seventeen (21%) cases were methylated at *p73*.

Simultaneous Methylation of Multiple CpG Islands in ALL. Using a methylation density cutoff point of 15% and considering MSP-positive reactions as indicative of significant

[5] X-Q. Chen, L. L. Shen, N. Ahuja, G. Garcia-Manero, and J-P. Issa. THBS2 promoter methylation and silencing in cancer, submitted for publication.

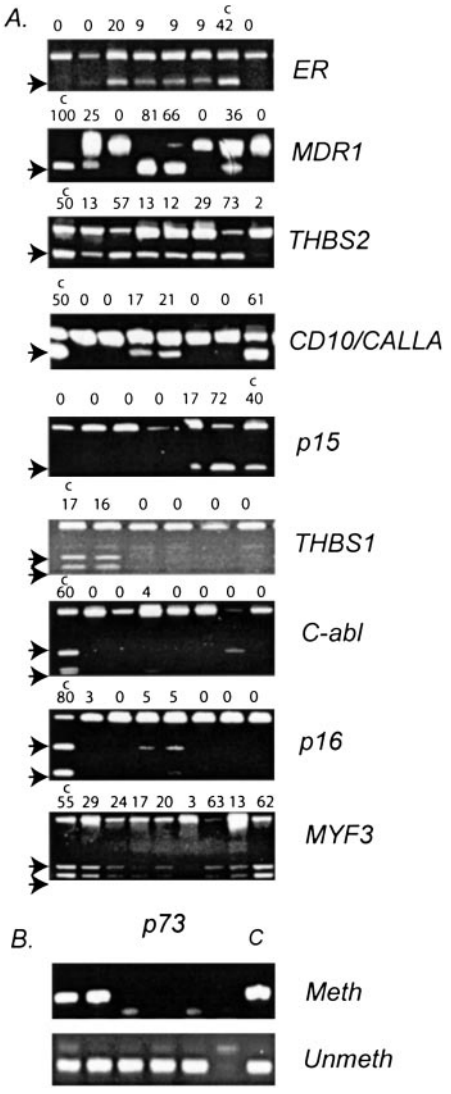

Fig. 1 Examples of methylation analysis of multiple CpG islands in ALL. *A*, aberrant methylation was detected by bisulfite-PCR followed by restriction enzyme digestion. Unmethylated (*top rows*) and methylated (*bottom rows, arrows*) bands were quantitated by densitometry. Methylation density (percentage of restricted *versus* unrestricted fragment) is shown *above* each lane. *B*, for analysis of aberrant methylation of *p73*, MSP was used. Two different PCR reactions were carried out, one with primers specific for the methylated sequence (*Meth*), and one with primers for the unmethylated sequence (*Unmeth*). This technique does not allow quantitative analysis. A sample was considered methylated if a band was visualized in the methylated reaction. Appropriate controls (*C*) consisted of cell lines with known methylation of the gene studied.

methylation, 11 patients (13.75%) had methylation of 0 genes, 17 patients (21.25%) had methylation of 1 or 2 genes, 8 patients (10%) had methylation of 3 genes, 11 patients (13.75%) had methylation of 4 genes, 8 patients (10%) had methylation of 5 genes, 5 patients (6.25%) had methylation of 6 genes, and 1 patient (1.25%) had methylation of 7 or 8 genes. No case was found to have methylation of more than 8 genes (Fig. 2). By analyzing the methylation status of 10 genes simultaneously, we were able to study correlations between their methylation status. Positive correlations among pairs of individual genes are summarized in Table 4. Methylation of *ER* was associated with methylation of *MDR1*, *THBS2*, and *MYF3* (all r values = 0.51), as well as *THBS1* and *p73* ($r = 0.36$ and $\kappa = 0.23$, respectively). *CD10* was weakly associated with *THBS1* and *THBS2* but not with *ER*, *MDR*, *MYF3*, or *p73*. By contrast, methylation of *p15* and *C-ABL* showed no significant correlation with each other or with any of the above-mentioned group of genes. *p16* was only methylated (≥15%) in two cases, precluding correlation analyses.

Table 3 Distribution of methylation densities

Gene	Methylation density			
	<2% *N* (%)	2–14.9% *N* (%)	15–49.9% *N* (%)	>50% *N* (%)
ER	39 (49)	12 (15)	20 (25)	9 (11)
p15	41 (51)	20 (25)	14 (18)	5 (6)
MDR1	37 (46)	7 (9)	19 (24)	17 (21)
THBS1	60 (75)	4 (5)	12 (15)	4 (5)
p16	74 (93)	4 (5)	1 (1)	1 (1)
C-ABL	73 (91)	1 (1)	3 (4)	3 (4)
THBS2	7 (9)	31 (39)	37 (46)	5 (6)
MYOD	21 (26)	28 (35)	23 (29)	8 (10)
CD10	64 (80)	8 (10)	5 (6)	3 (4)
p73 methylated[a]	17 (21.2)			

[a] For *p73*, MSP was used.

Clinical and Pathological Correlates. Using univariate analyses, we have explored possible correlations between methylation of a particular locus/loci with clinical characteristics and known prognostic factors in ALL. Factors analyzed included age, gender, percentage of blasts, WBC and platelet counts at presentation, albumin, risk model (24), Ph chromosome and other cytogenetic abnormalities, immunophenotype, and courses to achieve CR. An inverse association was found between *MDR1* methylation and age ($r = -0.25$), but no associations with gender, blasts, WBC and platelet count at presentation, albumin, or risk model were detected for any individual gene or cluster of genes. Immunophenotype was associated with several distinct abnormalities. First, *CD10* methylation was almost completely restricted to CD10-negative cases (CD10 expression <20% by flow cytometry): only one of eight cases with *CD10* methylation was considered CD10 positive (CD10 expression = 54%); the rest (seven of eight) were CD10 negative with a range of CD10 expression of 0–4% ($r = -0.43$; $P < 0.0001$). *THBS1* methylation was less frequent in CALLA-positive cases compared with those with B- or T-cell phenotype ($P < 0.01$). No association was found between T-cell immunophenotype and methylation of any of the genes studied, including *p15*.

We next studied associations between chromosomal changes and aberrant methylation in ALL. *C-ABL* methylation (≥15%) was associated with the presence of the Ph chromosome: *C-ABL* was methylated in 5 of 19 Ph-positive cases *versus* 1 of 61 Ph-negative cases ($P = 0.01$). The only Ph-negative case with methylation of *C-ABL* had multiple chromosomal abnormalities. $p210^{BCR/ABL}$ and $p190^{BCR/ABL}$ status was known for 14 of the 19 Ph-positive cases. None of the 10 $p190^{BCR/ABL}$ cases was methylated at C-*ABL*, whereas all 4

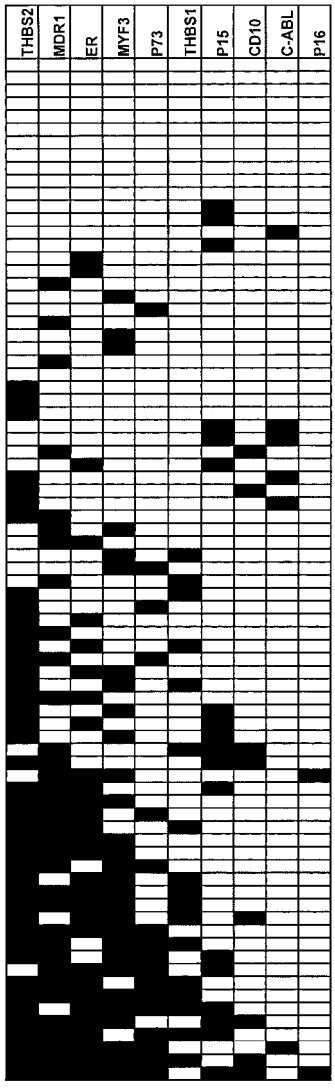

Fig. 2 Methylation profile of the cohort studied. We have analyzed the methylation status of 10 genes in 80 patients with ALL. A sample was considered methylated for a particular gene if the density of methylation was ≥15%, or if a band was present in the methylated reaction for p73 (*black boxes*). *White boxes* indicate nonmethylated cases.

$p210^{BCR/ABL}$ cases were methylated at c-*abl* (density of methylation, 28–80%). Differences were also observed between Ph-positive and Ph-negative cases for *THBS1* (0 of 19 Ph-positive cases methylated *versus* 16 of 61 Ph-negative cases, $P = 0.01$) and *MDR1* (2 of 19 Ph-positive cases methylated *versus* 34 of 61 Ph-negative cases, $P = 0.005$). *p15* methylation was associated with a diploid karyotype. No other associations could be found with other chromosomal abnormalities. Table 5 summarizes associations between clinical/biological characteristics and methylation.

Next we studied the impact of methylation of each individual gene in CR rate, DFS, and OS. *THBS1* methylation was associated with a better OS (66% *versus* 28% at 5 years; $P = 0.02$) and DFS (37% *versus* 29% at 5 years; $P = 0.04$). Methylation of *MDR1* was associated with a trend toward better OS (47% *versus* 22% at 5 years; $P = 0.09$). In contrast, methylation of *p73* and *p15* were associated with a poorer prognosis (Table 6). As expected, *c-abl* methylation was associated with a worse outcome: OS was 36% *versus* 0% at 5 years ($P = 0.26$). These results were reproduced when only the patients with Ph-negative disease were evaluated (Table 6).

DISCUSSION

The aim of this study was to determine the methylation characteristics of patients with adult ALL and explore possible associations between aberrant methylation and clinical/biological characteristics. This study is the first to analyze the methylation status of multiple promoter-associated CpG islands from a large cohort of uniformly treated adult patients with ALL. Results indicate that methylation of multiple genes is a common phenomenon in ALL; 87% of cases had at least 1 gene methylated, whereas 42.5% of cases had 3 or more genes methylated. In addition, methylation of several of the genes analyzed here was strongly correlated, suggesting that a molecular defect inducing hypermethylation of multiple CpG islands may occur in a subgroup of patients with ALL.

We have found several important clinical/biological associations with gene-specific methylation. CD10 methylation is inversely associated with CD10 expression, indicating that methylation of the CD10 promoter is associated with its silencing, as has been reported in prostate cancer (33). We have also found a striking association between methylation of *C-ABL* and the presence of the Ph chromosome. *C-ABL* methylation was detected only in those cases with the $p210^{BCR/ABL}$ variant as opposed to the $p190^{BCR/ABL}$ form, as has been reported by Shteper *et al.* (31). Other important findings include the inverse association between *MDR* and *THBS1* methylation and the presence of the Ph. In particular, the inverse association between *MDR1* methylation and the Ph abnormality suggests that MDR1-mediated chemotherapy resistance mechanisms may be involved in the poor prognosis of patients with Ph-positive acute leukemias. An inverse association between *MDR1* methylation and age was also observed. No other relevant clinical/biological features were found to be associated with abnormal methylation patterns. In particular, we could not find any of the previously described (17) associations between *p15* methylation and cytogenetic abnormalities, despite the larger number of patients analyzed in this study. This may be related to the specific patient populations studied or to the fact that we used a different technique to study *p15* methylation than other studies. In preliminary univariate analysis, we found correlates between methylation and outcome, including methylation of *THBS1, MDR1,* and *ER* associated with a favorable outcome, and methylation of *p15, p73,* and c-*abl* associated with a worse outcome. Such correlates have been described previously for *ER* methylation in AML (34). These studies need to be confirmed and extended in a larger group, where multivariate analysis will be possible.

This study has several limitations. Our results are partially limited by the number of genes studied. By increasing the number of genes profiled, one could refine the concept of the hypermethylator phenotype and expand our knowledge about the clinical and biological implications of the hypermethylator phenotype in ALL.

Table 4 Correlations between methylation densities for selected genes

r values between −0.2 and 2 were omitted.

Gene	*MDR*	*THBS1*	*p73*[a]	*THBS2*	*MYOD*	*CD10*
ER	0.51	0.36	0.23	0.51	0.51	
MDR1		0.26	0.28	0.34	0.24	
THBS1				0.45	0.38	0.24
p73				0.25	0.25	
THBS2					0.41	0.25

[a] κ statistic substituted for *r* value for p73.

Table 5 Correlations between DNA methylation and clinical biological characteristics

	Methylation density (%)									
	ER	*p15*	*p16*	*MDR1*	*C-ABL*	*THBS1*	*p73*	*THBS2*	*MYF3*	*CD10*
Age (yrs)										
>39	14.1	10	1.2	19.6	5.6	6.7	23%	20.5	14.3	3.3
<40	17.9	12.5	1.2	29.6	2	11.2	17%	21.3	19.5	5.7
				$P = 0.02$						
Sex										
Male	18.3	11.3	0.1	24.6	3	12	20.8%	22	18.5	4.6
Female	12.8	11.2	2.9	24.3	4.7	4.3	21.8%	18.9	16.3	4.3
CG[a]										
Diploid	15	16.6	0.4	28.8	0.1	2	23.5%	16.4	13.9	4.4
Ph+	9.2	9.5	0.5	6.6	13.5	0	11.7%	16.4	11.9	0.12
Others	21.1	3.1	1.5	35.8	1.7	9	64.7%	22.6	18.7	3.8
		$P = 0.02$		$P = 0.03$	$P = 0.03$					
Phenotype										
T cell	29.5	16.4	1.2	29.3	0.4	28.8	20%	27.5	37.3	7.4
B cell	14.9	5.1	0	5.7	0	18.9	0%	25.6	15.4	1.3
CALLA	13.1	8.8	0.1	28.1	5.7	3.6	29%	18.5	14.7	0.6
Others	17.1	16.2	4	19.7	1.9	8.8	10%	21.5	16.1	13.3
										$P = 0.04$

[a] CG, cytogenetics.

Table 6 Impact of methylation on 5-year OS

Summary of the impact of methylation (first figure) *versus* unmethylation (second figure) on 5-year OS. Second row summarizes results for patients with Ph=negative disease.

	ER	*p15*	*p16*	*p73*	*MDR1*	*c-abl*	*MYF3*	*CD10*	*THBS1*	*THBS2*
All patients ($n = 80$)	43%	28%	50%	27%	51%	0%	35%	33%	66%	37%
	vs.	*vs.*	*vs.*	*vs.*	*vs.*	*vs.*	*vs.*	*vs.*	*vs.*	*vs.*
	28%	37%	43%	36%	33%	36%	35%	32%	28%	34%
	$P = 0.4$	$P = 0.4$	$P = 0.8$	$P = 0.5$	0.09	$P = 0.2$	$P = 0.7$	$P = 0.8$	$P = 0.02$	$P = 0.2$
Ph=negative patients ($n = 57$)	50%	26%	50%	30%	48%	0%	40%	63%	66%	45%
	vs.	*vs.*	*vs.*	*vs.*	*vs.*	*vs.*	*vs.*	*vs.*	*vs.*	*vs.*
	35%	46%	42%	46%	49%	51%	44%	41%	36%	42%
	$P = 0.5$	$P = 0.09$	$P = 0.8$	$P = 0.1$	$P = 0.4$	$P = 0.01$	$P = 0.9$	$P = 0.9$	$P = 0.1$	$P = 0.3$

The development of microarray technologies to study methylation in the near future will help clarify these issues (35). In turn, the number of cases studied limits the clinical implications of our results. Given the heterogeneity of patients with ALL, a larger study of uniformly treated patients might allow multivariate analyses that are needed to address these issues. Finally, aberrant methylation of promoter-associated CpG islands is characterized by transcriptional inactivation and subsequent loss of function of the gene regulated in this fashion without structural modifications. In the present study, we have not directly analyzed the silencing of the genes studied. However, evidence reported by other investigators, including us, demonstrates that methylation of all genes studied here is usually associated with transcriptional silencing (14, 16, 21, 27, 28, 32). The strong inverse association found between *CD10* methylation and expression serves as a surrogate marker that methylation, at least of *CD10*, is indeed associated with gene silencing, as studied here. Finally, in view of the number of genes and variables analyzed in this study, these results should be considered as hypothesis-generating and need to be confirmed in future studies.

The study has several important implications. Methylation profiling in ALL could provide useful markers complementing standard immunophenotypic and cytogenetic studies. It may also provide clues related to mechanisms of disease resistance, in particular those related to the presence of the Ph. By studying methylation patterns during remission and at relapse, methylation profiling could be developed to detect minimal residual disease. In addition, demethylating agents, such as 5-aza-2′-deoxycytidine, have shown promising activity in myelodysplasia (36) and AML (37). Hypomethylation of target genes such as *p15* has been shown to correlate with clinical responses in patients with chronic myelogenous leukemia (38). In view of the extent of aberrant methylation demonstrated by our analysis, clinical trials using hypomethylating agents are indicated in patients with relapsed ALL. Prospective knowledge of pretreatment methylation patterns may help determine candidate patients for these therapies.

In summary, our results demonstrate that aberrant methylation in ALL is a common phenomenon in ALL. This knowledge may have implications in developing a molecular classification of ALL and may help develop new markers of minimal residual disease and determine candidate patients for demethylating therapies.

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