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LETTER TO THE EDITOR

Promoter hypermethylation and global hypomethylation are independent epigenetic events in lymphoid leukemogenesis with opposing effects on clinical outcome

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Deregulation of the DNA-methylating machinery is associated with neoplastic transformation of many types of cells in humans. Both general genomic hypomethylation and regional hypermethylation coexist in DNA extracted from different types of neoplastic tissues. A number of studies have sought to reconcile this apparent paradox, suggesting that in solid cancers, the high frequency of DNA hypomethylation, the nature of the affected sequences and the absence of associations with DNA hypermethylation are consistent with an independent role of DNA undermethylation in the development of these solid neoplasms.[1,2]

We have recently shown that the methylation of cytosine nucleotides in acute lymphoblastic leukemia (ALL) cells is the most important way to inactivate cancer-related genes in this disease. In fact, this epigenetic event can help to inactivate tumor-suppressive apoptotic or growth-arresting responses and has prognostic impact in B- and T-ALL.[3,4] The presence in individual tumors of multiple genes simultaneously methylated (a condition termed CpG island methylator phenotype or CIMP+) is an independent factor of poor prognosis in both childhood and adult ALL in terms of disease-free survival (DFS) and overall survival (OS). However, no studies have addressed the role of hypomethylation in lymphoid leukemogenesis and therefore, it is unclear whether these epigenetic changes are causally linked, or are distinct and biologically unrelated phenomena.

In the present study, we analyzed the extent of both DNA hypomethylation and hypermethylation in 307 consecutive patients (179 men; 128 women) who were diagnosed with *de novo* ALL between January 1989 and December 2004 (median age at diagnosis in the study population as a whole was 14 years). Methylation-specific PCR (MSP) method was used to analyze 39 genes belonging to all of the molecular pathways involved in cell immortalization and transformation: cell cycle (*FHIT*, *LATS2*, *p15*, *p16*, *p57*, *REPRIMO* and *RIZ*), cell adherence and metastasis process (*ADAMTS1*, *ADAMTS5*, *CDH1* and *CDH13*), *p53* network (*ASPP1*, *p14* and *p73*), apoptosis (*APAF1*, *ARTS*, *DAPK*, *DBC1*, *DIABLO* and *TMS1*,), inhibitors of the oncogenic WNT signaling pathway (*DKK3*, *HDPR1*, *sFRP1*, *sFRP2*, *sFRP4*, *sFRP5* and *WIF1*), differentiation regulation (*LHX2* and *NES1*), folate carrier (*hRFC*), hormone receptor superfamily (*PGR*), ubiquitination (*PACRG and PARK2*), DNA repair (*SMC1L1* and *SMC1L2*), tyrosine kinase with an essential role in signal transduction (*SYK*), negative regulator of the Jak/STAT signaling pathway (*SHP1*) and main tumor-suppressor genes (*LATS1* and *PTEN*). Hypomethylation of the LINE1 (L1) retrotransposons (as indicator of genomic global hypomethylation) was assessed by means of a quantitative real-time MSP as previously described by our group.[5]

Gene methylation frequencies varied from 8 to 59%. Twenty-three genes demonstrated a relatively high frequency of aberrant methylation: *SMC1L2* (59%), *NES1* (56%), *ADAMTS1* (45%), *PGR* (40%), *sFRP1* (38%), *CDH1* (37%), *ADAMTS5* (36%), *CDH13* (35%), *LATS1* (34%), *DKK3* (33%), *WIF1* (30%), *LATS2* (28%), *REPRIMO* (28%), *sFRP5* (28%), *PARK2* (27%), *PACRG* (27%), *HDPR1* (26%), *RIZ* (26%), *APAF1* (23%), *ARTS* (22%), *ASPP1* (22%), *DIABLO* (22%) and *sFRP4* (21%). The other 16 genes studied showed a low frequency (8–19%) of methylation. No methylated genes were found in 45 of 307 patients (15%), whereas most ALLs (262 (85%) of 307) had methylation of at least one gene, ranging from one to 25 methylated genes. According to the number of methylated genes observed in each individual sample, 106 patients (35%) were included in the CIMP− group (0–2 methylated genes) and 201 (65%) in the CIMP+ group (more than two methylated genes). As shown in Table 1, clinical and laboratory characteristics did not differ significantly between methylation groups. Table 1 also details the relapse history, complete remission (CR) rates and mortality for patients included in the different methylation groups. CR rates of patients in the CIMP− and CIMP+ groups were 91 and 87%, respectively, accounting for 89% of the overall CR rate. This suggests that methylation profile did not correlate with response to remission-induction therapy. However, patients in the CIMP− group had a lower relapse rate than patients in the CIMP+ group (26 versus 58%, $P<0.0001$). Mortality rate was also lower for CIMP− group compared with CIMP+ group (34 versus 58%, $P<0.001$). Similar results were obtained in the separate analyses of children (relapse rate, 14% for CIMP− group versus 45% for CIMP+ group, $P<0.001$; mortality rate, 12% for CIMP− group versus 31% for CIMP+ group, $P=0.01$) and adults (relapse rate, 42% for CIMP− group versus 72% for CIMP+ group, $P=0.002$; mortality rate, 58% for CIMP− group versus 82% for CIMP+ group, $P=0.004$).

We analyzed the DFS among patients who achieved CR according to the methylation profile. Estimated DFS at 14 years were 68 and 32% for CIMP− and CIMP+ groups, respectively ($P<0.0001$; Figure 1a). The actuarial OS at 14 years calculated for all leukemic patients was 63% for CIMP− patients and 32% for CIMP+ patients ($P=0.0002$; Figure 1b). A multivariate analysis of potential prognostic factors demonstrated that hypermethylation profile was an independent prognostic factor in predicting DFS in the global series ($P<0.0001$) as well as in both childhood ($P=0.001$) and adult ALLs ($P=0.001$). Methylation status was also independently associated with OS in the global series ($P=0.003$), adult ALL ($P=0.03$) and childhood ALL ($P=0.05$).

L1 promoter was hypomethylated in 73/307 (24%) of ALL patients. We found no significant associations between the methylation status of any of the 39 studied CpG-rich regions and L1 hypomethylation levels. Moreover, L1 hypomethylation was similarly distributed among CIMP− and CIMP+ groups (26 versus 23%, Table 1).

Hypomethylated patients had lower relapse (33 versus 55%, $P=0.01$) and mortality (35 versus 48%, $P=0.04$) rates than normally methylated patients. Estimated DFS rates at 12 years were 63 and 38% for hypomethylated and normal patients,

Table 1 Clinical characteristics and outcome of 307 ALL patients according to gene methylation status

Feature	*CIMP–* (n = *106) %*	*CIMP+* (n = *201) %*	P-*value*
Age (years)			NS
<15	83	17	
>15	78	22	
Sex (male/female)	64/36	72/28	NS
WBC			NS
$<50 \times 10^9$/l	78	70	
$>50 \times 10^9$/l	22	30	
FAB classification			NS
L1	38	25	
L2	50	64	
L3	12	11	
Blast lineage			NS
B cell	91	89	
T cell	9	11	
NCI risk groups			NS
Standard	80	65	
Poor	20	35	
PETHEMA risk groups			NS
Standard	40	34	
Poor	60	66	
Treatment			NS
PETHEMA 89	25	27	
PETHEMA 93	75	73	
BMT	19	10	NS
Best response			
CR	91	87	NS
Cytogenetic/molecular abnormalities			NS
BCR-ABL	17	14	
t(1;19)	4	2	
11q23	3	3	
c-myc	6	8	
7q35-14q11	6	8	
Hyperdiploidy	9	5	
TEL-AML1	5	3	
Normal	40	48	
Others	3	3	
NT	7	6	
L1 hypomethylated	26	23	NS
Relapse	26	58	<0.0001
Death	34	58	0.001

Abbreviations: BMT, bone marrow transplantation; CR, complete remission; FAB, French-American-British; L1, Line 1; NCI, National Cancer Institute; NS, not significant; NT, nontested; PETHEMA, 'Programa para el estudio y tratamiento de las hemopatias malignas'; WBC, white blood count.
Data are expressed as percentages; CIMP–, patients with 0–2 methylated genes; CIMP+, patients with more than two methylated genes.

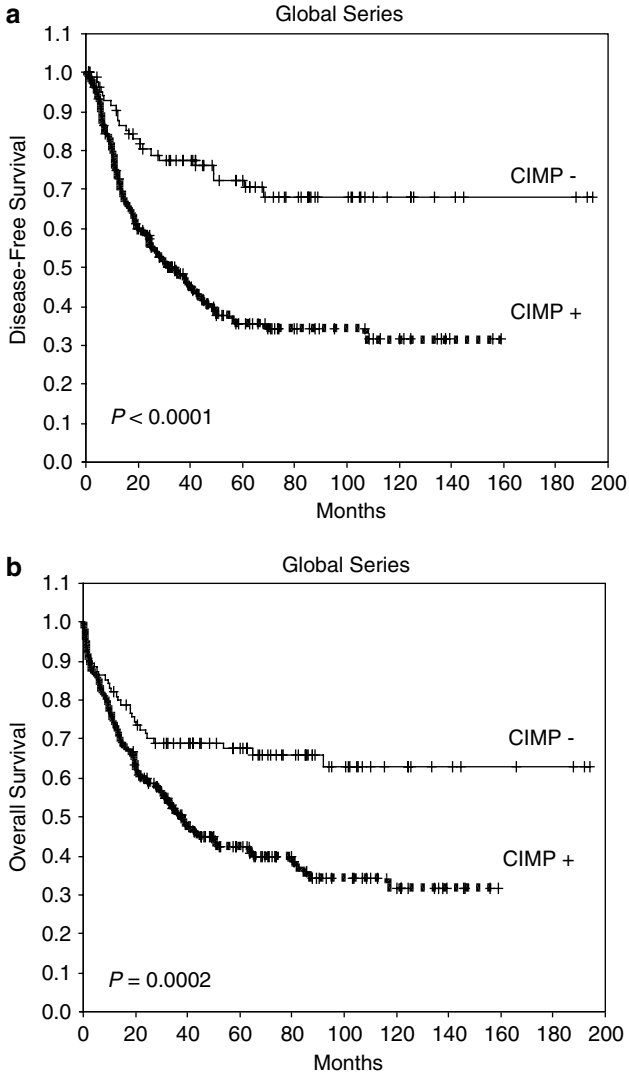

Figure 1 Kaplan–Meier survivor function for ALL patients according to the methylation profile. DFS (**a**) and OS (**b**) curves for all the patients enrolled in this study according to the methylation profile. CIMP– (patients with 0–2 methylated genes); CIMP+ (patients with more than two methylated genes).

respectively ($P = 0.02$, Figure 2a). The actuarial OS calculated for all leukemic patients was 61 and 43% at 12 years for cases with hypomethylated and normal L1, respectively ($P = 0.03$, Figure 2b). A multivariate analysis demonstrated that L1 hypomethylation was an independent prognostic factor in predicting DFS ($P = 0.05$) and OS ($P = 0.05$) in the global series. Interestingly, L1 hypomethylation was able to redefine the prognosis of the CIMP– patients, establishing a very low-risk ALL group. Among CIMP– patients, the 12–year DFS was 100% for L1-hypomethylated group and 50% for L1-methylated group ($P = 0.004$; Figure 2c). The actuarial OS at 12 years for the same patients was 81% for hypomethylated patients and 58% for methylated patients ($P = 0.08$; Figure 2d).

Our analysis of the abnormal DNA methylation patterns reveals the ubiquity of the epigenetic alterations in ALL. Hypermethylation of multiple genes is a common phenomenon; 85% of cases had at least one gene methylated, whereas 65% of cases had three or more genes methylated. In addition, we also report for the first time that most ALL tumors display global genomic hypomethylation, as has been described for some other types of cancer. Importantly, we found no association between the extent of methylation of any of the 39 CpG-rich regions or the CIMP status in the 307 ALL samples analyzed and their global DNA methylation levels. The absence of any such relationship suggests that tumor-associated DNA hypomethylation contributes to lymphoid leukemogenesis separately from

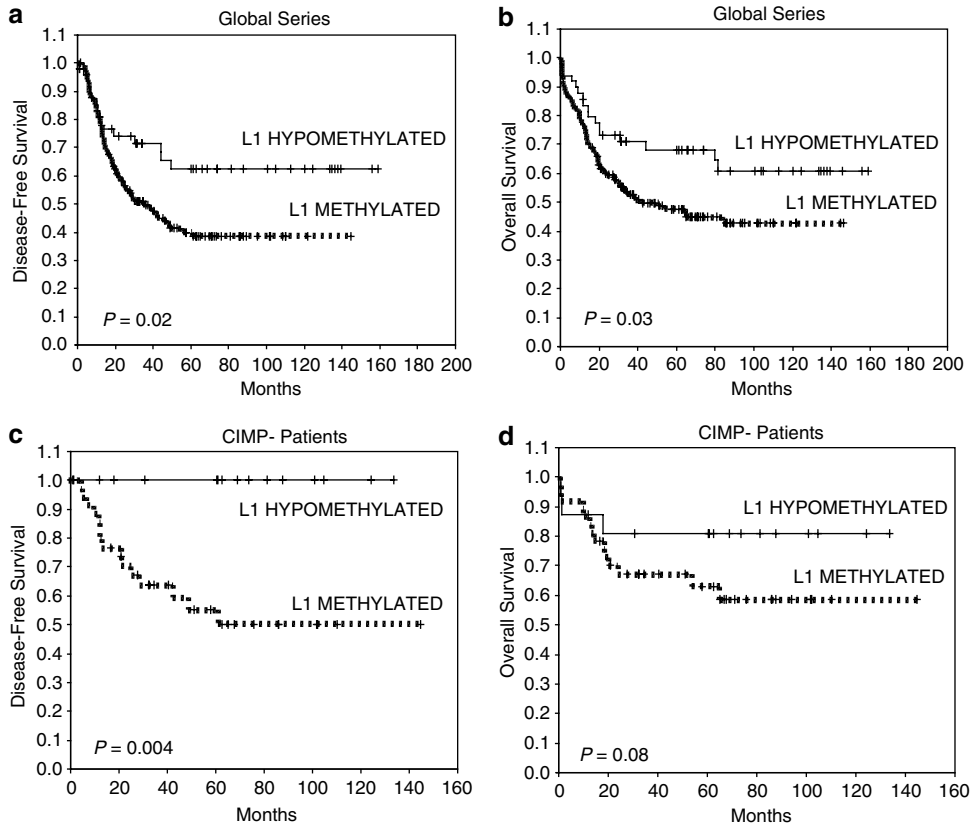

Figure 2 Kaplan–Meier survivor function for ALL patients. DFS (**a**) and OS (**b**) curves for all the patients enrolled in this study according to the methylation status of the L1 retrotransposon. DFS (**c**) and OS (**d**) curves for CIMP− patients (patients with 0–2 methylated genes) according to the methylation status of the L1 retrotransposon.

aberrant DNA hypermethylation and its attendant silencing of tumor suppressor genes.

In this paper, analyzing a larger series of patients with a longer follow-up and a more extensive number of genetic loci, we have confirmed our previous observation that aberrant methylation of CpG islands provides important prognostic information in ALL patients.[3,4] The presence in individual tumors of multiple genes simultaneously methylated is a factor of poor prognosis in ALL. In contrast, DNA hypomethylation was an indicator of good prognosis in both univariate and multivariate analyses and was able to define a very low-risk ALL group among CIMP− patients. This opposite association of the hyper- and hypomethylation with patient outcome is also consistent with the independent roles of both abnormalities in tumor initation and progression described above.

Although genome-wide DNA hypomethylation has been observed in a wide variety of human cancers, the functional significance of this alteration is still unclear. In human leukemogenesis, we have recently demonstrated that L1 promoter hypomethylation is an important feature in chronic myeloid leukemia (CML).[5] In fact, hypomethylation increased from chronic phase CML toward advanced phase CML, this hypomethylation being the most common molecular abnormality associated with blastic crisis reported to date and suggesting that hypomethylation has a limited role in the genesis of CML but is important in the progression of the disease. These data seem to be in disagreement with our current results in ALL in which DNA hypomethylation can promote early events in leukemogenesis while blocking progression and dismal prognosis. These dual effects of DNA hypomethylation on neoplastic cells have been previously reported. Several experiments have demonstrated that global DNA hypomethylation significantly suppresses intestinal tumorigenesis in mice.[6] In contrast, global DNA hypomethylation promotes chromosomal instability in mice, which results in the development of T-cell lymphomas and also accelerates tumor formation in murine sarcoma model.[7] Moreover, hypomethylation promotes early-stage tumor formation in the colon and liver tumorigenesis but strongly suppresses overall tumorigenesis in the intestine of $APC^{Min/+}$ mice.[8] Taken together, all these studies and our present report suggest that the reduction of the DNA methylation levels results in suppression or promotion of tumor progression depending on the tumor cell type, including the hematopoietic cell origin of the leukemia.

In conclusion, promoter hypermethylation and global hypomethylation contribute separately to the process of lymphoid leukemogenesis and have opposing effects on clinical outcome.

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