Table 1 Distribution of the MIF-173 genotypes in controls and in 151 patients with childhood acute lymphoblastic leukemia, overall and according to steroid response

MIF-173	*Controls N (%)*	*Acute lymphoblastic leukemia N (%)*	*Acute lymphoblastic leukemia & PPR N (%)*	*Acute lymphoblastic leukemia & PGR N (%)*
G/G	277 (78.4)	117 (77.5)	59 (76.6)	58 (78.4)
C/G	76 (21.4)	34 (22.5)	18 (23.4)	16 (21.6)
C/C	2 (0.6)	0	0	0
Total	355 (100)	151 (100)	77 (100)	74 (100)

the proliferation of leukemic blasts, nor in the protection from apoptosis, despite several observations that have involved MIF in protecting a variety of cell types from apoptosis. From a more practical point of view, the MIF-173 G/C polymorphism, as well as other previously investigated genetic variants, does not contribute to prednisone poor response *in vivo* in childhood acute lymphoblastic leukemia. Alternative biologic markers are worth to be investigated in such patients.

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Downregulation of the large tumor suppressor 2 *(LATS2/KPM)* gene is associated with poor prognosis in acute lymphoblastic leukemia

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TO THE EDITOR

The *human large tumor suppressor 2* (*LATS2/KPM*) gene, which encodes a novel serine/threonine kinase, has been mapped onto chromosome 13q11–12, a hot spot region for loss of heterozygosity in primary cancers, suggesting that *LATS2* might function as a tumor suppressor gene.[1] Although chromosome aberrations involving 13q12 have been described in a minority (3–6%) of acute lymphoblastic leukemia (ALL) patients and frequently occur as secondary events after relapse, these patients show an extremely poor outcome.[2] DNA methylation is an essential mechanism for the regulation of gene expression in mammalian cells. The promoter of the *LATS2* gene contains a defined CpG island and its hypermethylation has been recently associated to an aggressive phenotype in breast cancers.[3] All these facts led us to analyze the potential role of the inappropriate expression/promoter hypermethylation of the *LATS2* gene in a number of cell lines and primary cells from ALL patients.

Four human precursor-B (MY, TOM-1, NALM-20 and TANOUE) and three T cell (JURKAT, LOUCY and MOLT-4) ALL cell lines

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were used for experiments. Bone marrow samples were collected after an acquisition of informed consent from 101 consecutive patients (65 male; 36 female), who were diagnosed with *de novo* ALL between December 1996 and August 2004. The median age at diagnosis in the study population as a whole was 20 years (range, 0.7–72 years). Of these patients, 54 were children (median age, 7 years; range, 0.7–14) and 47 presented adult ALL (median age, 35 years; range, 15–72). Patients were studied at the time of initial diagnosis and were risk-stratified according to the therapeutic protocols of the PETHEMA Spanish Study Group. For statistical analyses, children were also grouped according to the National Cancer Institute (NCI) risk-classification criteria. In all, 24 patients relapsed. Eight patients received stem-cell transplantation (two autologous, six allogeneic). There are 68 patients currently alive.

Aberrant promoter methylation of *LATS2* gene (GeneBank: 26524) was determined by methylation specific PCR (MSP) method. Primer sequences of *LATS2* for the unmethylated reaction were: forward (5′-GGTGTTTTAGATTTGAAAGGTTGT AGT-3′) and reverse (5′-AAAAAACTAATTAACCCATAAAACA AT-3′). Primer sequences for the methylated reaction were: forward (5′-GTTTTAGATTCGAAAGGTCGTAGC-3′) and reverse (5′-AAAACTAATTAACCCGTAAAACGAT-3′). DNA from mononuclear marrow cells ($n=20$) from healthy donors were used as negative controls for methylation-specific assays.

RNA from mononuclear marrow cells was used for quantitative real-time PCR (qrt-PCR) for *LATS2* mRNA expression. Reverse transcription was performed on 1 μg total RNA with random hexamers as reaction primer. Qrt-PCR was performed in a rapid fluorescent thermal cycler (LightCycler2.0, Roche). Primer set was specific for the *LATS2* gene (GeneBank: NM_014572; forward *LATS2* exon 5, 5′- GTAGGACGCAAACG AAT-3′; reverse *LATS2* exon 7, 5′-CAGAAGTGAACCGGCA-3′). Amplification of *Abelson* (*ABL1*) gene transcripts was performed to assess RNA integrity and as reference gene. In order to reduce the variation between different assays and samples, a procedure based on the relative quantification of target genes *vs* their controls in relation to the reference gene was used. Calculations were automatically performed by LightCycler software (Real-Quant, version 1.0, Roche). The selected control was the mononuclear cells of bone marrow specimen from a healthy donor. It was considered as 100% expression.

To determine the cutoff point for altered *LATS2* expression in ALL patients and cell lines, the value for *LATS2* was firstly determined in 15 bone marrow samples from healthy donors. In these individuals, *LATS2* expression fell between 149 and 78% (mean: 104 ± 21%). A *LATS2* value equal or below 40% (determined as the mean minor 3 s.d.) was chosen to define underexpression of *LATS2* in ALL mRNA samples. We found a strong reduction of *LATS2* mRNA in all the B/T-cell precursor ALL cell lines tested (mean, 1.4%; range, 0–6%) and also in 35% of diagnostic ALL samples (mean, 15.7%; range, 0–40%).

CpG island of the *LATS2* promoter was revealed to be highly methylated in five ALL cell lines lacking *LATS2* expression (TOM-1, NALM-20, MY, TANOUE and LOUCY), whereas JURKAT and MOLT-4 cell lines showed an unmethylated pattern despite expressing low *LATS2* transcript levels (Figure 1a). Exposure to 4 μM concentration of the demethylating agent 5-Aza-2′-deoxycytidine restored the expression of *LATS2* mRNA in the MY, NALM-20, TOM-1, TANOUE and LOUCY ALL cell lines indicating that hypermethylation is a major mechanism by which *LATS2* expression is silenced in ALL cells (Figure 1b).

LATS2 methylation status was also studied in 66 ALL patients enrolled in this study in which DNA samples were available. A methylated promoter was observed in 16 of them (24%) and in

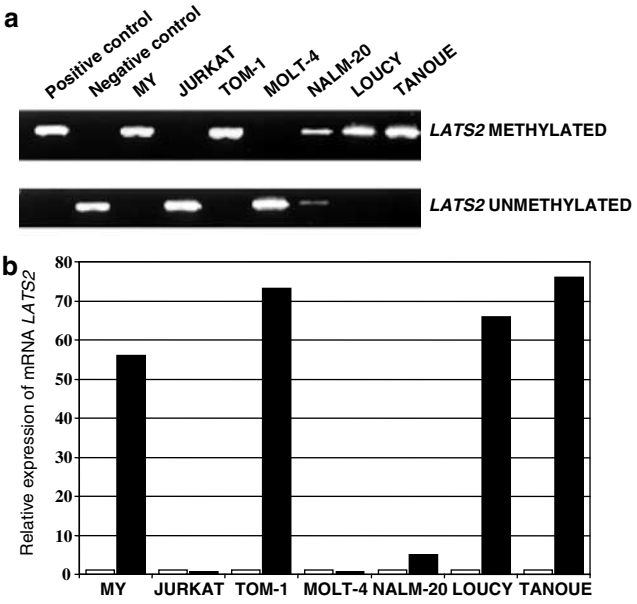

Figure 1 Methylation status and expression of *LATS2* in ALL cell lines. (a) MSP analysis of CpG island within *LATS2* promoter in seven ALL cell lines. Positive control represents methylated genomic DNA; negative control indicates unmethylated control (bone marrow mononuclear cells from a healthy donor). Promoter methylation is observed in MY, TOM-1, NALM-20, LOUCY and TANOUE cell lines. (b) Qrt-PCR analysis of *LATS2* mRNA before (white bar) and after (black bar) treatment with 5-Aza-2′-deoxycytidine in the ALL cell lines shown above. Expression of *LATS2* in all the methylated cell lines (MY, TOM-1, NALM-20, LOUCY and TANOUE) is reverted by 5-Aza-2′-deoxycytidine. Conversely, JURKAT and MOLT-4 cell lines, which were not methylated at *LATS2* promoter, show no changes in *LATS2* expression after the demethylating treatment.

13 of these cases (81.2%), methylation was associated with decreased *LATS2* expression levels. In contrast, low expression was detected in only 11 (8 B-ALL and 3 T-ALL) of 50 ALL with unmethylated pattern (22%). This result indicated that CpG methylation within *LATS2* promoter strongly correlated with decreased constitutive expression of *LATS2* in ALL cells ($P<0.001$).

Low expression of *LATS2* was detected at diagnosis in 35% (35 out of 101) of ALL patients with adult or childhood ALL belonging to all the FAB subtypes. Reduced levels of *LATS2* mRNA were more frequently observed among adult ALL patients (22 out of 47, 47%) than in children (13 out of 54, 24%) ($P=0.01$). Moreover, correlating *LATS2* expression with pre-treatment risk groups, we detected a significant association between reduced *LATS2* expression and high-risk ALL children assessed by the NCI scoring system ($P=0.004$) and also with two poor-risk subgroups defined by the presence of *BCR-ABL* fusion gene ($P=0.05$) and T-cell lineage ALL ($P<0.001$). Complete remission (CR) rates of patients with normal and low *LATS2* gene expression were 97 and 91%, respectively, suggesting that expression of the *LATS2* gene did not correlate with response to remission induction therapy.

As show in Table 1 patients with low expression had higher relapse (53 *vs* 12%, $P<0.001$) and mortality (69 *vs* 14%, $P<0.001$) rates than normal expressing patients. The adverse prognostic impact of low *LATS-2* expression on relapse and mortality remained after adjustment for age, WBC count, immunophenotype, NCI risk groups and cytogenetics.

Table 1 Relapse and mortality for patients who exhibited low levels of *LATS2* expression and the equivalent data for patients with normal *LATS2* gene according to different characteristics

	Relapse			*Exitus*		
	Low expression % (N = 30)	*Normal expression % (N = 64)*	*P*	*Low expression % (N = 35)*	*Normal expression % (N = 66)*	*P*
All patients (*N* = 101)	53	12	<**0.001**	69	14	<**0.001**
Age						
Younger than 15 years (*N* = 54)	50	7	**0.001**	46	7	**0.001**
Older than 15 years (*N* = 47)	56	22	**0.02**	82	24	<**0.001**
WBC						
Below 50×10^9/l (*N* = 73)	40	10	**0.004**	65	10	<**0.001**
Above 50×10^9/l (*N* = 28)	80	20	**0.003**	75	25	**0.009**
Blast lineage						
B lineage (*N* = 81)	56	12	<**0.001**	70	13	<**0.001**
T lineage (*N* = 20)	50	20	0.2	67	20	**0.06**
NCI risk groups (children)						
Standard (*N* = 31)	33	7	0.1	33	7	0.1
Poor (*N* = 23)	56	8	**0.01**	50	8	**0.02**
Cytogenetics/molecular abnormalities						
BCR-ABL (*N* = 14)	57	75	NS	100	83	NS
TEL-AML1 (*N* = 15)	50	0	**0.008**	0	0	NS

WBC = indicates white blood count;
NCI = National Cancer Institute.

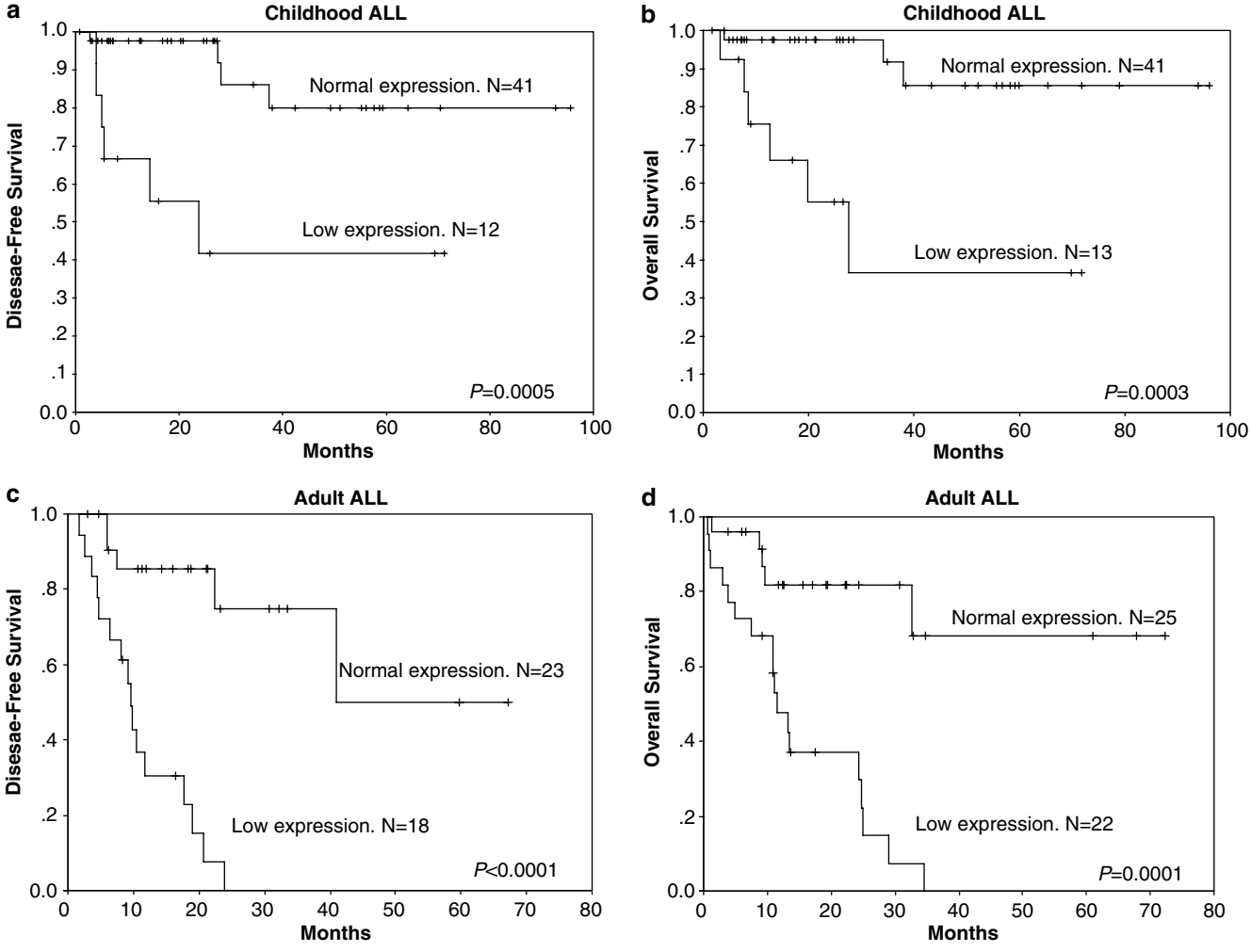

Figure 2 Kaplan–Meier survival function according to *LATS2* expression levels. DFS (panel a) and OS (panel b) curves for childhood. Adult DFS (panel c) and OS (panel d).

We analyzed the disease-free survival (DFS) and overall survival (OS) according to *LATS2* expression. Among children, the 6-year DFS was 80% for normal expressing group and 41.6% for low expressing group ($P=0.0005$) (Figure 2a). Among adult ALL patients, the 2-year DFS was 76% for patients with normal *LATS-2* levels and 0% for patients with low *LATS-2* levels ($P<0.0001$) (Figure 2c). Significant differences were observed in the actuarial OS among patients with normal and low levels of *LATS-2* in the separate analyses of children (85.6 *vs* 36.7%, respectively, $P=0.0003$; Figure 2b) and adults (63.3 *vs* 0%, respectively, $P=0.0001$; Figure 2d).

A multivariate analysis of potential prognostic factors demonstrated that expression of the *LATS2* gene was the most important prognostic factor in predicting DFS ($P<0.0001$) and OS ($P<0.0001$) in the global series and also in both childhood ($P=0.002$ in DFS and OS) and adult ALL ($P<0.0001$ in DFS and $P=0.003$ in OS). Only factors like BCR-ABL in adults ($P=0.02$ in DFS and $P=0.04$ in OS) and hyperleucocytosis in children ($P=0.05$ in DFS) reached also statistical significance in the multivariate analysis.

In this study we have identified, for the first time, the *LATS2* gene as a target gene for epigenetic regulation in ALL. Low expression of *LATS2* gene was associated with methylation of the *LATS2* promoter region in leukemic cells. Although methylation of the *LATS2* promoter was significantly associated with decrease of *LATS2* expression, some discordant values were recorded. A minority of patients ($n=3$) who expressed normal levels of *LATS2* also showed methylation of the gene. All these patients showed levels (42, 48 and 68%) near to the cutoff point (40%) chosen to define underexpression in this study. Although the presence of contaminating RNA from normal bone marrow cells cannot be ruled out in these cases, they also may indicate a partial methylation state of the promoter region. Moreover, our study cannot exclude other potential mechanisms of *LATS2* downregulation since in 11 of our ALL patients and also in JURKAT and MOLT-4 cell lines there were no apparent methylation despite loss of *LATS2* expression.

What is the functional significance underlying the downregulation of *LATS2* in ALL? *LATS2* is a putative tumor suppressor gene with three key functions: it negatively regulates the cell cycle by controlling G1-S and/or G2-M transition,[4,5] induces apoptosis through downregulating antiapoptotic proteins, Bcl-2 and Bcl-x(L),[6] and plays an essential role in the integrity of processes that govern centrosome duplication, maintenance of mitotic fidelity and genomic stability.[7] In this study, decrease of *LATS2* expression was found to be correlated with several dismal prognostic features in ALL patients: low expression of *LATS2* gene was more frequently observed among classical high-risk ALL groups (adults, T-cell phenotype, NCI poor risk and the presence of *BCR-ABL* fusion gene) and was also significatively and independently associated with a shorter DFS and OS in both adult and childhood ALL. These findings, together with the general expression of *LATS2* in normal bone marrow, the emerging role of the *LATS2* gene in other types of human cancer (ie, prostate and breast carcinoma),[3,8] and the confirmed role for *LATS2* gene in mechanisms related to cellular homeostasis, such as apoptosis, genomic integrity and cell cycle regulation are supportive of *LATS2* inactivation contributing directly to the clinical behavior of ALL

In conclusion, our results strongly suggest that downregulation of *LATS2* expression mainly by aberrant promoter methylation is a frequent event in ALL and plays a role in the clinical outcome of the disease.

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