

# Low level CpG island promoter methylation predicts a poor outcome in adult T-cell acute lymphoblastic leukemia

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## ABSTRACT

Cancer cells undergo massive alterations in their DNA methylation patterns which result in aberrant gene expression and malignant phenotypes. Abnormal DNA methylation is a prognostic marker in several malignancies, but its potential prognostic significance in adult T-cell acute lymphoblastic leukemia (T-ALL) is poorly defined. Here, we performed methylated DNA immunoprecipitation to obtain a comprehensive genome-wide analysis of promoter methylation in adult T-ALL (n=24) compared to normal thymi (n=3). We identified a CpG hypermethylator phenotype that distinguishes two T-ALL subgroups and further validated it in an independent series of 17 T-lymphoblastic lymphoma. Next, we identified a methylation classifier based on nine promoters which accurately predict the methylation phenotype. This classifier was applied to an independent series of 168 primary adult T-ALL treated accordingly to the GRAALL03/05 trial using methylation-specific multiplex ligation-dependent probe amplification. Importantly hypomethylation correlated with specific oncogenic subtypes of T-ALL and identified patients associated with a poor clinical outcome. This methylation-specific multiplex ligation-dependent probe amplification based methylation profiling could be useful for therapeutic stratification of adult T-ALL in routine practice. The GRAALL-2003 and -2005 studies were registered at <http://www.clinicaltrials.gov> as #NCT00222027 and #NCT00327678, respectively.

## Introduction

T-cell acute lymphoblastic leukemias (T-ALL) are aggressive and heterogeneous malignancies which are predominated by the 10-39-year age group where they account for 20% of acute lymphoblastic leukemias (ALL).<sup>1</sup> T-ALL is associated with a wide range of acquired genetic abnormalities that contribute to developmental arrest and abnormal proliferation of malignant lymphoid progenitors.<sup>2,3</sup> Despite the diversity of observed mutations and deletions, genome wide expression<sup>4,6</sup> assays led to the identification of few oncogenic T-ALL subgroups, namely the imma-



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ture/early thymic precursor (ETP) (Lyl1, MEF2C), late cortical (TAL1), early cortical (TLX1/3 and NKX2.1) and HOXA clusters. Although cancer is typically considered a genetic disease, epigenetic aberrations also play important roles in tumor potentiation, initiation, and progression.<sup>7</sup> Epigenetics is defined as changes in gene expression that are not due to changes in gene sequence, and include DNA methylation, histone modifications, microRNA (miRNA) and nucleosome positioning. Unlike genetic alterations, epigenetic changes are reversible by enzymatic activity and pharmacological treatment with small molecule inhibitors, like those targeting enzymes involved in DNA methylation or chromatin modifications. Altered epigenetic states are a common feature of all cancer types and the most studied epigenetic modification in primary cancer samples is DNA methylation, which is known to display characteristic changes in malignant cells compared to normal tissue. These include diffuse hypomethylation and focal hypermethylation changes at discrete loci potentially associated with repression of specific genes related to cancer pathogenesis.

In the field of ALL, DNA methylation studies have mostly focused on pediatric B-cell precursor ALL (BCP-ALL) describing promoter hypermethylation and specific methylation signatures according to the cytogenetic subgroup.<sup>8</sup> In pediatric T-ALL, DNA methylation was analyzed by Infinium 27 K and 450 K arrays and two distinct CpG island methylator phenotype (CIMP) groups were identified. Patients with a CIMP-negative profile displayed a significantly higher cumulative incidence of relapse (CIR) compared to CIMP-positive patients suggesting a prognostic relevance of aberrant DNA methylation profiles in T-ALL.<sup>9,10</sup> Furthermore, it has more recently been shown in a pediatric series that CIMP status correlates with known oncogenic subgroups, for instance, with higher expression of TAL1 in a CIMP-negative subgroup (11). However such data for adult T-ALL are still lacking. In this work, we report genome-wide promoter methylation profiling by methylation-dependent immunoprecipitation (MeDIP) in a cohort of adult T-ALL. Subsequently, a nine-promoter classifier was applied to a large series of 168 adult T-ALL included in the GRAALL 03/05 trial that distinguished two subgroups with highly significant differences in the clinical outcome. Thus, MeDIP profiling is a potential candidate for risk stratification of adult T-ALL and could provide important information in treatment decision making and therapeutic targeting.

## Methods

### Patients and treatments

Adult patients (15-60 years old) included in two successive French ALL cooperative group trials (GRAALL-2003 and GRAALL-2005) with T-ALL, and defined according to the 2008 World Health Organisation classification, were analyzed. The GRAALL-2003 protocol was a multicenter phase 2 trial, which enrolled 76 adults with T-ALL between November 2003 and November 2005 of whom 50 had sufficient diagnostic tumor material available.<sup>12</sup> The multicenter randomized GRAALL-2005 phase 3 trial was very similar to the GRAALL-2003 trial, with the addition of a randomized evaluation of an intensified sequence of hyperfractionated cyclophosphamide during induction and late intensification.<sup>13</sup> Between May 2006 and May 2010, 337 adults with T-ALL were randomized in the GRAALL-2005, of which 185

had available diagnostic material. All samples contained >80% blasts. Phenotypic and oncogenetic characteristics were as described.<sup>14-16</sup> Informed consent was obtained from all patients at enrollment. All trials were conducted in accordance with the Declaration of Helsinki and approved by local and multicenter research ethical committees.

### MeDIP-assay

Global DNA methylation was assessed by a MeDIP assay on an initial series of 24 T-ALL and three human thymi and a second (confirmatory series) of 17 T-lymphoblastic lymphomas (T-LBL) and three human thymi. Briefly, methylated DNA was immunoprecipitated as described previously<sup>17</sup> using 2 µg of sonicated genomic DNA. MeDIP samples were directly subjected to labeling and hybridization to previously described custom human promoter arrays (Agilent, Santa Clara, CA, USA) covering either 17,970 promoters<sup>17</sup> (T-ALL series) or 25,490 promoters<sup>18</sup> (T-LBL series), following the manufacturer's instructions. The median-normalized log<sub>2</sub> enrichment ratios (MeDIP/Input) were calculated for each probe using the CoCAS software<sup>19</sup> and visualized using the IGB tool (<http://bioviz.org/igb>). Finally, a methylation score was computed for each promoter by calculating the median enrichment ratio of overlapping probes. A summary of the methylation scores per promoter in T-ALL and T-LBL samples is provided in the *Online Supplementary Tables S1-2*, respectively.

### Clustering of methylation profiles

Hierarchical clustering analysis (Average Linkage) based on the methylation signal of the top 5% genes with highest variance was performed with the TIGR MeV v. 4.9.0 program,<sup>20</sup> using the -1 Spearman rank correlation method. Analysis of the differential methylation signal between the groups was performed using the significant analysis of microarrays (SAM) algorithm (threshold value: FDR<0.121 and delta=2.144). The graphical clustering representation of the clustering was done with the GenePattern software.<sup>21</sup> The list of differentially methylated promoters in T-ALL and T-LBL is provided in *Online Supplementary Table S3*.

### Validation of DNA methylation signature

Direct methylation levels were analyzed by methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) with custom probes (*Online Supplementary Table S4*) and, SALSA<sup>®</sup> MLPA<sup>®</sup> P200 Reference-1 probemix and EK1 reagent kits from MRC-Holland (Amsterdam, the Netherlands), according to manufacturer's recommendations. Data were analyzed with the Coffalyser software (MRC-Holland, Amsterdam, the Netherlands). In addition, the promoter methylation patterns were verified by quantitative PCR (qPCR) analysis of MeDIP samples and by bisulfite sequencing using specific primers for the MEIS1 gene promoter.

## Results

### DNA methylation signatures in T-ALL/T-LBL

Global promoter regions DNA methylation by MeDIP-array was performed in a training series of 24 adult T-ALL. Unsupervised hierarchical clustering defined two major groups (group 1 and group 2) with distinct methylation profiles (Figure 1A). The supervised signature of differential methylation (FDR<0.121) between these two groups resulted in 300 unique differentially methylated gene promoters with a vast majority of hypermethylated (hyperM) promoters (297 of 300) in the so-called hyperM group. The second group displayed an intermediate methylation

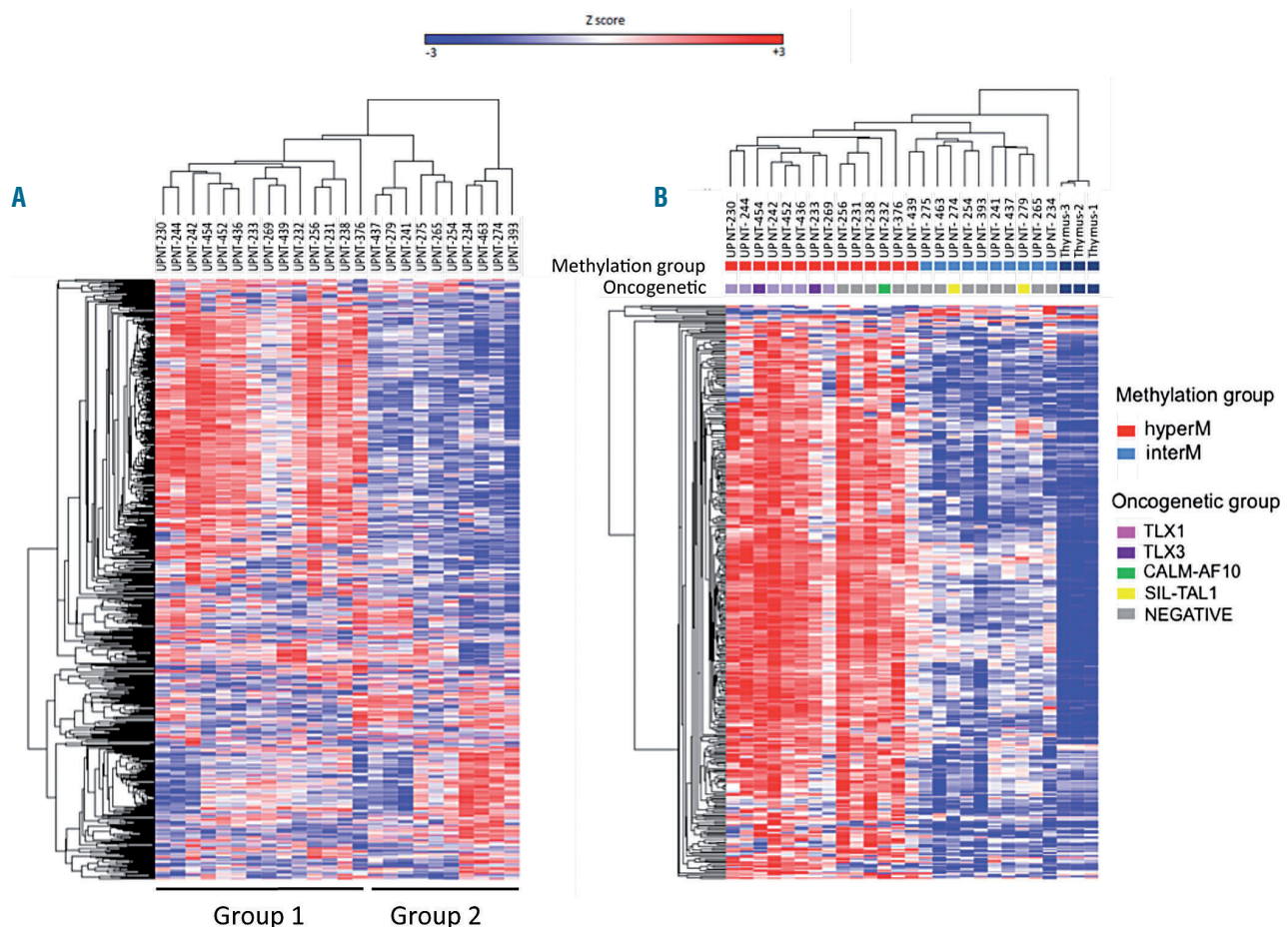
(interM) profile compared to the normal thymic tissue (Figure 1B and *Online Supplementary Figure S1*). Interestingly, all the TLX<sup>+</sup> cases without exception (including six TLX1<sup>+</sup> and two TLX3<sup>+</sup> cases) clustered in the hyperM group. Conversely, the two SIL-TAL1<sup>+</sup> cases belonged to the interM group; suggesting a role of oncogenic abnormalities in the observed methylation profiles.

A very similar differential methylation signature (Figure 2A-B) was observed in an independent series of 17 T-LBL. One TLX1<sup>+</sup> and five TLX3<sup>+</sup> T-LBL, as in T-ALL, were clustered in the group with a hyperM promoter profile (253 of 255 hyperM gene promoters). T-ALL and T-LBL promoter methylation signatures displayed a highly significant overlap ( $P < 0.0001$ ) with 97 common gene promoters differentially methylated (Figure 2C and *Online Supplementary Table S2*). Among them, the differential methylation of MEIS1 promoter was confirmed with two different targeted methods, MeDIP-QPCR (*Online Supplementary Figure S2A*) and bisulfite sequencing (*Online Supplementary Figure S2B*).

### Driver oncogenes defined distinct aberrant methylation profiles

In an effort to explore the DNA methylation profiles in a larger T-ALL series, a minimal robust signature able to

predict the methylation state was defined with a remaining error risk inferior to 0.05. This predictor contained the following nine gene promoters: *BMP4*, *HOXB7*, *KCNA1*, *LHX1*, *MEIS1*, *PROX1*, *PSD3*, *RUNX2*, *SEMA6A* (Figure 3A). A MS-MLPA panel was designed to explore the methylation status of these nine gene promoters and a methylation ratio corresponding to the methylation average of these nine differentially methylated regions (DMR) was calculated. As expected, this predictor allowed the separation of hyperM and interM T-ALL from the training cohort ( $P = 0.0016$ ) (Figure 3B-C). We then performed this analysis on a series of 168 primary adult T-ALL uniformly treated according to the GRAALL03-05 trial (Figure 3D). The methylation ratio was widely ranged (mean=0.62, min=0.04, max=1.1) and major oncogenetic drivers (TLX1, TLX3, SIL-TAL1, HOXA overexpression) defined distinct methylation profiles. TLX1<sup>+</sup> and TLX3<sup>+</sup> T-ALL displayed significantly hyperM promoters compared to the HOXA subgroup ( $P = 0.03$  and  $P = 0.02$  respectively), to the SIL-TAL1 subgroup ( $P < 0.0001$ ) or the others T-ALL ( $P < 0.0001$ ). Conversely, SIL-TAL1<sup>+</sup> cases and others T-ALL expressing high level of TAL1 had significantly hypomethylated (hypoM) promoters ( $P < 0.0001$ ) compared to TLX1/3<sup>+</sup>, HOXA<sup>+</sup> or others T-ALL. Unlike oncogenic status, immature early thymic progenitor-ALL



**Figure 1. Genome-wide promoter methylation-array hierarchical clustering in T-cell acute lymphoblastic leukemias.** (A) Unsupervised hierarchical clustering of 24 adult T-cell acute lymphoblastic leukemias (T-ALL) based on the genome-wide promoter methylation (MeDIP-array). The hypermethylated (hyperM; group 1) and intermediate methylated (interM; group 2) clusters are indicated. (B) Supervised clustering of T-ALL samples along with three human thymi using the differentially methylated signature obtained between groups 1 and 2 (panel A).

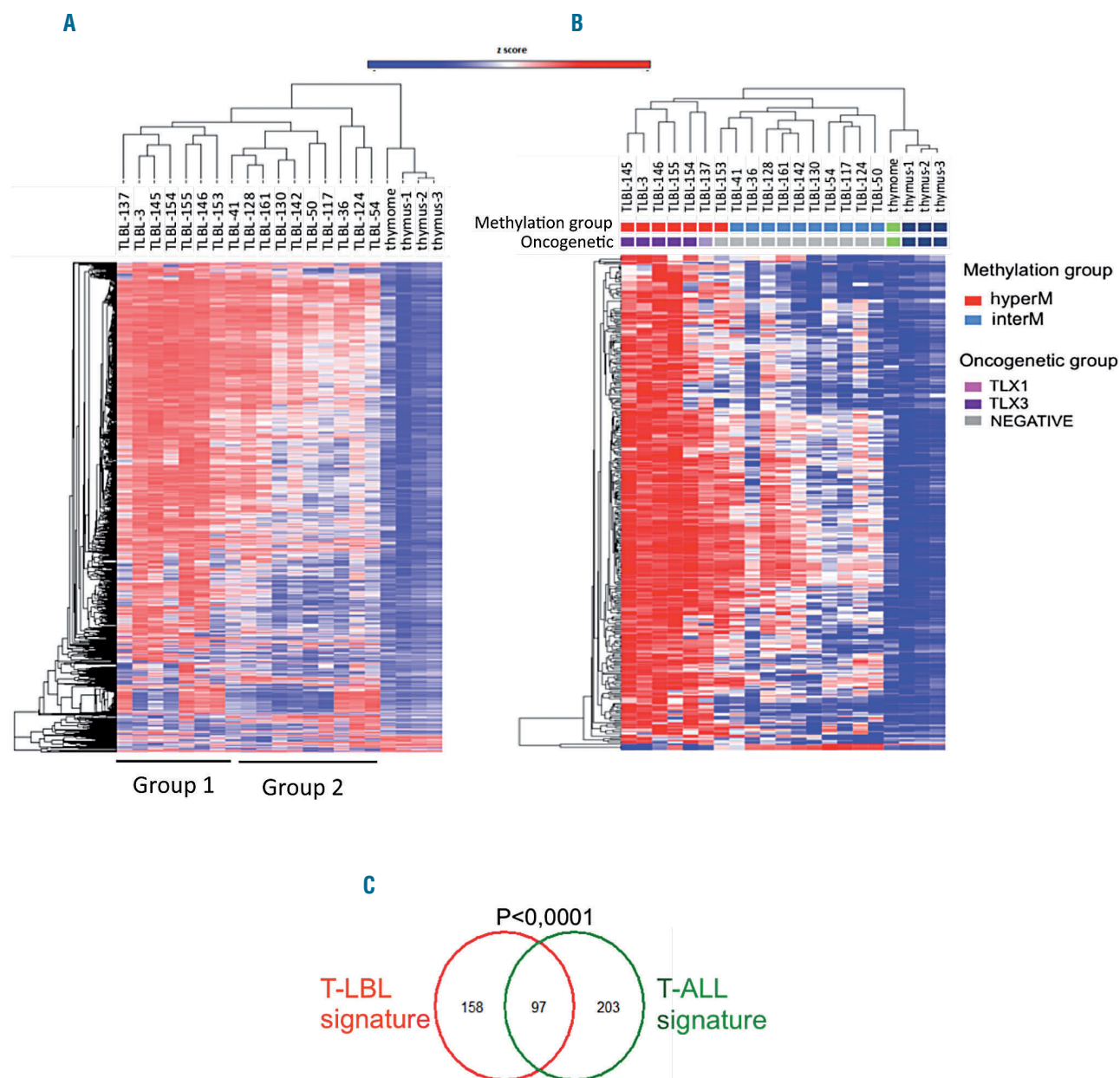


(ETP-ALL) lacked a significant distinct methylation signature compared to non-ETP-ALL (Figure 3E).

**Low level of promoter methylation predicted a poor outcome subgroup of adult T-ALL**

T-ALL patients with the lowest methylation level (Q1, n=42/168) were significantly more men, were younger, and had a higher white blood cell (WBC) count at diagnosis than patients with higher methylation levels (Table 1). Moreover, hypoM T-ALL demonstrated a significantly more frequent mature phenotype (TCRαβ<sup>+</sup>) and were associated with SIL-TAL1 rearrangement. They were also

significantly associated with a low rate of NOTCH1 pathway mutations and a high risk NOTCH1/FBXW7/RAS/PTEN molecular classifier.<sup>22</sup> In detail, we observed a significantly lower incidence of *NOTCH1/FBXW7* mutations and also a greater incidence of *PTEN* alterations (mutation and/or deletion) in the hypoM subgroup (CIMP-neg) as compared to the Int/High methylated cases (*Online Supplementary Table S5*). Despite a better bone marrow response at D8 (M1 status) in patients with low methylation, we did not observe any impact of methylation on complete remission (CR) rate or post-induction minimal residual disease (MRD) level. In univariate analysis,



**Figure 2. Genome-wide promoter methylation-array hierarchical clustering in T-lymphoblastic lymphomas.** (A) Unsupervised hierarchical clustering of 17 T-lymphoblastic lymphomas (T-LBL) based on genome-wide promoter methylation (MeDIP-array). The hypermethylated (hyperM; group 1) and intermediate methylated (interM; group 2) methylated clusters are indicated. (B) Supervised clustering of T-LBL samples, one thymoma and three thymi, using the differentially methylated signature obtained between groups 1 and 2 (panel A). (C) Venn diagram representing the overlap between the differentially methylated promoters between hyperM and interM subgroups found in T-ALL and T-LBL samples. Statistical significance was assessed by a Hypergeometric test.

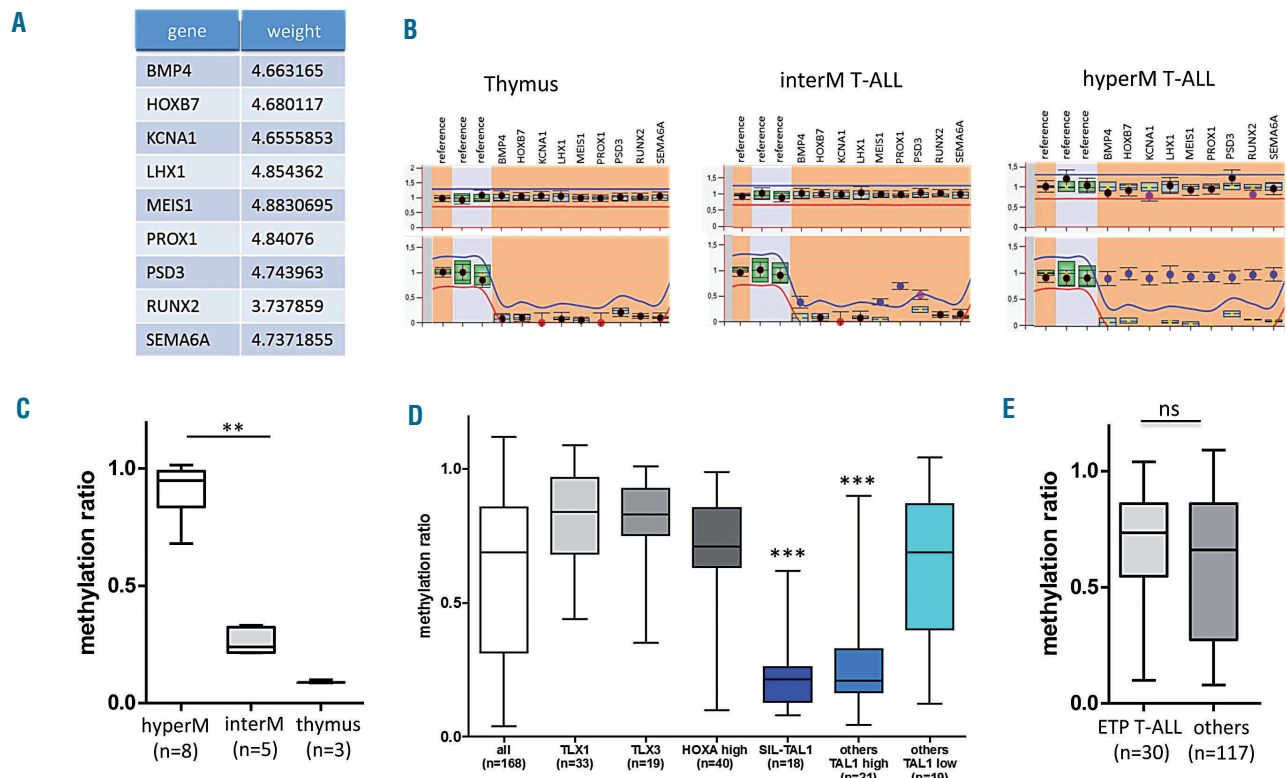
patients with low methylation levels had higher CIR (sub-distribution hazard ratio [SHR] 1.87, 95% CI: 1.03-3.38,  $P=0.04$ ; Table 2 and Figure 4A) and a shorter overall survival (OS) (hazard ratio [HR] 1.78, 95% CI: 1.06-2.98,  $P=0.03$ ; Table 2 and Figure 4B). In multivariate analysis for CIR, the only prognostic factor to be significantly associated with a reduced CIR was the NOTCH1/FBXW7/RAS/PTEN molecular classifier. However, in multivariate analysis for OS, including age, WBC at diagnosis, central nervous system (CNS) involvement, prednisone response, the molecular classifier, and the methylation level as covariates, a low methylation was still independently associated with a higher risk of death (HR 1.79, 95% CI: 1.00-3.19,  $P=0.05$ ; Table 2).

## Discussion

Despite recent insights into the molecular and cellular mechanisms responsible for T-ALL onset and progression, survival rates remain around 50% in adults, justifying the search for novel therapeutic options or more adapted/personalized regimens. The present study focused on promoter DNA methylation in a large series of adult T-ALL. As previously reported in pediatric T-ALL,<sup>9-11</sup> we showed that DNA methylation status is also a prognostic factor in adult T-ALL. Similarly, patients with a hypoM profile dis-

play an unfavorable outcome compared to hyperM patients. Importantly, even if hypoM status is associated with the molecular high-risk classifier,<sup>22</sup> methylation level remains an independent prognostic factor. Moreover, methylation status does not seem to influence the initial clinical response to therapy since there were no significant differences regarding the glucocorticoids and initial chemotherapy responses (chemosensitivity or MRD) between hypoM and hyperM patients. Methylation status could therefore represent a relevant additional prognostic factor for adult T-ALL. Nevertheless, further validation by another independent series is needed. Moreover, it would be interesting to study the prognostic impact of this methylation signature in T-LBL, which displayed similar methylation distortion patterns.

We used the relatively new methodology of methylation specific-multiplex ligation-dependent probe amplification (MS-MLPA) to evaluate the promoter DNA methylation level. MS-MLPA is a powerful and easy-to-perform PCR-based technique and we demonstrated that MS-MLPA could provide an attractive alternative way to assess methylation classification compared to array analysis. This approach permits methylation analysis of multiple targets in a single experiment and has been successfully used to evaluate the diagnostic relevance of different markers in several tumor types including lung,<sup>23</sup> rectal,<sup>24</sup> breast,<sup>25</sup> bladder,<sup>26</sup> prostate,<sup>27</sup> and adrenocortical cancer.<sup>28</sup>



**Figure 3. Targeted promoter methylation analysis in GRAALL 03/05 T-cell acute lymphoblastic leukemias series.** (A) List of the nine gene promoters classifier allowing methylation status prediction. (B) Representative ratio charts of methylation specific-multiplex ligation-dependent probe amplification (MS-MLPA) analysis for one normal thymus and two T-cell acute lymphoblastic leukemias (T-ALL) from the training series belonging to the intermediate methylated (interM) subgroup and the hypermethylated (hyperM) subgroup respectively. Top panels refer to the MLPA (undigested) reference panel and the bottom panel the MS-MLPA (digested with HhaI restriction enzyme) panel. (C) Methylation ratio was assessed by MS-MLPA for T-ALL from the training series and according to their methylation subgroup and for three normal thymi. (D) Methylation ratio assessed by MS-MLPA for 168 adult T-ALL included in GRAALL03/05 trial and according to the driver oncogene involved (TLX1, TLX3, HOXA, SIL-TAL1). (E) Methylation ratio according to the early thymic precursor (ETP) phenotype.

**Table 1. Patients' characteristics and outcome according to methylation status.**

|  | Low methylation<br>N=42 (Q1) | Int/High methylation<br>N= 126 (Q2-Q4) | P†                |
|--|------------------------------|--|-------------------|
| TCR subsets analyzed                   |                              |  |                   |
| Immature (IM0, IMδ, IMγ)               | 4/36 (11%)                   | 32/111 (29%)                           | 0.04              |
| IMβ/pre-αβ                             | 20/36 (56%)                  | 60/111 (54%)                           | 0.99              |
| TCRαβ+                                 | 11/36 (31%)                  | 5/111 (5%)                             | <b>&lt;0.0001</b> |
| TCRγδ+                                 | 1/36 (3%)                    | 14/111 (13%)                           | 0.12              |
| ETP immunophenotype                    |                              |  |                   |
| <i>NOTCH1/FBXW7</i> <sup>mutated</sup> | 18/42 (43%)                  | 99/126 (79%)                           | <b>&lt;0.0001</b> |
| <i>High Risk Classifier</i> *          | 29/42 (69%)                  | 43/125 (34%)                           | <b>0.0001</b>     |
| Oncogenetic Category                   |                              |  |                   |
| <i>TLX1</i>                            | 0/41 (0%)                    | 35/120 (29%)                           | <b>&lt;0.0001</b> |
| <i>TLX3</i>                            | 0/41 (0%)                    | 21/120 (18%)                           | <b>0.0022</b>     |
| <i>SIL-TALI</i>                        | 16/41 (39%)                  | 2/120 (2%)                             | <b>&lt;0.0001</b> |
| <i>CALM-AF10</i>                       | 0/41 (0%)                    | 8/120 (7%)                             | 0.2               |
| <i>None of the above</i>               | 25/41 (61%)                  | 54/120 (45%)                           | 0.1               |
| <i>HOXA</i> deregulation               | 3/39 (8%)                    | 40/112 (36%)                           | <b>0.0008</b>     |
| Clinical Subsets Analyzed              |                              |  |                   |
| Age, median (range)                    | 23.2 (16.6-56.2)             | 33.4 (16.3-59.1)                       | <b>&lt;0.001</b>  |
| Sex ratio, M/F                         | 35/7                         | 85/41                                  | 0.05              |
| WBC (G/L), median (range)              | 80 (4-604)                   | 30 (1-645)                             | <b>0.003</b>      |
| CNS involvement                        | 7/42 (17%)                   | 17/126 (13%)                           | 0.616             |
| Early Response                         |                              |  |                   |
| Prednisone response                    | 23/42 (55%)                  | 68/126 (55%)                           | 1                 |
| Bone marrow response                   | 29/39 (74%)                  | 66/126 (52%)                           | 0.02              |
| Complete remission                     | 38/42 (90%)                  | 117/126 (93%)                          | 0.739             |
| MRD (TP1) <10 <sup>-4</sup>            | 16/19 (84%)                  | 48/73 (66%)                            | 0.164             |
| Long-term outcome                      |                              |  |                   |
| 5-year CIR (95% CI)                    | 45% (31-62)                  | 27% (20-36)                            | <b>0.04</b>       |
| 5-year OS (95% CI)                     | 50% (34-64)                  | 68% (59-76)                            | <b>0.03</b>       |

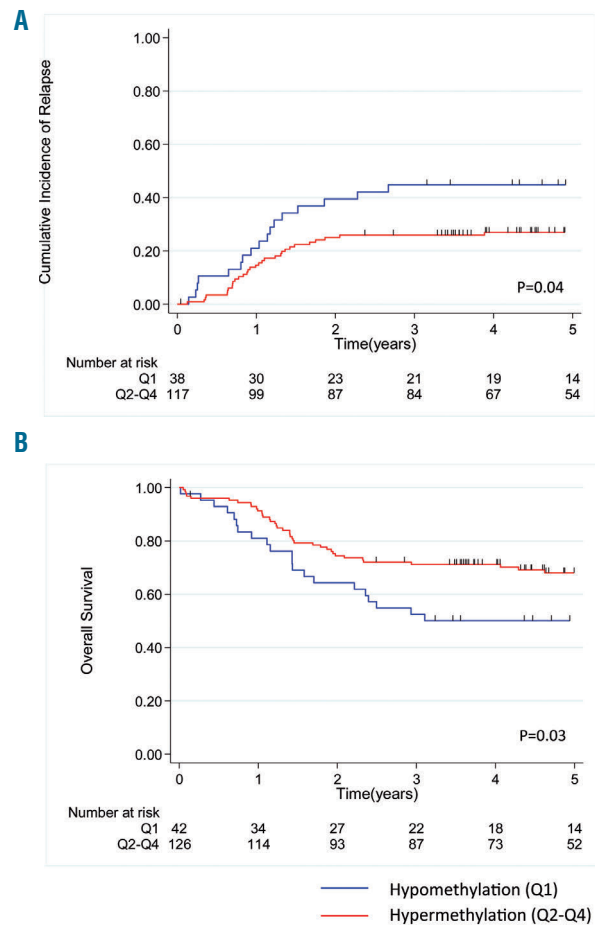
IM: immature; WBC (g/L): white blood cells; CNS: central nervous system; MRD (TP1): post-induction minimal residual disease; CIR: cumulative incidence of relapse; OS: overall survival; 95%CI: 95% confidence interval; TCR: Tcell receptor; ETP: early thymic precursor. \* The unfavorable classifier includes NOTCH1, FBXW7, RAS and PTEN (Trinquand, et al. 2013). †χ<sup>2</sup> or Mann-Whitney tests were used where appropriate.

**Table 2. Univariate and multivariate analysis for cumulative incidence of relapse and overall survival.**

| CIR                         | Univariate |             |        | Multivariate |             |        |
|-----------------------------|------------|-------------|--------|--------------|-------------|--------|
|                             | SHR        | 95%CI       | P      | SHR          | 95%CI       | P      |
| Age*                        | 1.00       | 0.97 – 1.03 | 0.90   | –            | –           | –      |
| WBC**                       | 1.00       | 0.98 – 1.04 | 0.29   | –            | –           | –      |
| CNS involvement             | 1.55       | 0.75 – 3.21 | 0.23   | –            | –           | –      |
| Unfavorable risk classifier | 3.77       | 2.04 – 6.98 | <0.001 | 3.53         | 1.85 – 6.73 | <0.001 |
| Prednisone responder        | 0.71       | 0.40 – 1.25 | 0.24   | –            | –           | –      |
| Bone marrow responder       | 0.76       | 0.43 – 1.35 | 0.35   | –            | –           | –      |
| Low methylation (Q1)        | 1.87       | 1.03 – 3.38 | 0.04   | 1.25         | 0.67 – 2.34 | 0.49   |

| OS                        | Univariate |             |        | Multivariate |             |        |
|---------------------------|------------|-------------|--------|--------------|-------------|--------|
|                           | HR         | 95%CI       | P      | HR           | 95%CI       | P      |
| Age*                      | 1.03       | 1.01 – 1.06 | 0.01   | 1.04         | 1.02 – 1.06 | 0.001  |
| WBC**                     | 1.01       | 1.00 – 1.03 | 0.12   | –            | –           | –      |
| CNS involvement           | 2.14       | 1.18 – 3.88 | 0.01   | 2.32         | 1.24 – 4.35 | 0.01   |
| Favorable risk classifier | 3.81       | 2.24 – 6.50 | <0.001 | 2.93         | 1.65 – 5.21 | <0.001 |
| Prednisone responder      | 0.64       | 0.39 – 1.05 | 0.08   | 0.69         | 0.41 – 1.16 | 0.16   |
| Bone marrow responder     | 0.78       | 0.47 – 1.27 | 0.31   | –            | –           | –      |
| Low methylation (Q1)      | 1.78       | 1.06 – 2.98 | 0.03   | 1.79         | 1.00 – 3.19 | 0.05   |

WBC: white blood cell; CNS: central nervous system; CIR: cumulative incidence of relapse; OS: overall survival; HR: hazard ratio; SHR: sub-distribution hazard ratio; 95% CI: 95% confidence interval. \*Age as continuous variable, SHR/HR for 1-year increment. \*\* WBC as continuous variable, SHR/HR for 10 g/L increment





Additionally, MS-MLPA has the advantage of requiring little DNA and does not require DNA bisulfite conversion or immunoprecipitation. MS-MLPA is readily compatible with clinical routine and could enhance prognostication and precision medicine.

However, array analysis or methylation analysis at the whole genome level would be relevant in T-ALL to gain information and investigate how aberrant methylation patterns are involved in leukemogenesis. We have observed that aberrant methylation profiles were mostly associated with the driver oncogene involved. In particular, a hypoM subgroup with unfavorable outcome is mainly enriched in SIL-TAL1+ cases and also in cases negative for the main oncogenes TLX1, TLX3, SIL-TAL1 and HOXA. Deciphering the molecular mechanism of aberrant methylation and the relationship with driver oncogenes could identify new deregulated pathways for adapted-therapy.

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