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haematologica
Journal of The Ferrata Storti Foundation

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Haematologica 2019 [Epub ahead of print]

Citation: Aurore Touzart, Nicolas Boissel, Mohamed Belhocine, Charlotte Smith, Carlos Graux, Mehdi Latiri, Ludovic Lhermitte, Eve-Lyne Mathieu, Françoise Huguet, Laurence Lamant, Pierre Ferrier, Norbert Ifrah, Elizabeth Macintyre, Hervé Dombret, Vahid Asnafi, and Salvatore Spicuglia. Low level CpG island promoter methylation predicts a poor outcome in adult T-cell acute lymphoblastic leukemia.
Haematologica. 2019; 104:xxx
doi:10.3324/haematol.2019.223677

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

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Research support: MeDIP experiments were supported by the INCA-project PAIR-Lymphomes. Work in SS lab was supported by Ligue contre le Cancer (Equipe labellisée). Work in the Necker hematology department and SS lab was supported by grants from ITMO (Institut Thématique Multi-Organisme) épigénétique et cancer. The GRAALL was supported by grants P0200701 and P030425/AOM03081 from Le Programme Hospitalier de Recherche Clinique, Ministère de l'Emploi et de la Solidarité, France and the Swiss Federal Government in Switzerland. AT was supported by a grant from the French program CARPEM (CAncer Research for PErsonalized Medicine).

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Running head: *DNA* methylation in T-ALL

Disclaimer: The authors report no conflict of interest

Word count: Abstract: 193 words, Full text: 2755 words

4 Figures, 2 Tables

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 is poorly defined. Here, we performed methylated DNA immunoprecipitation to obtain a comprehensive genome-wide analysis of promoter methylation in adult T-cell Acute Lymphoblastic Leukemia (n=24) compared to normal thymi (n=3). We identified a CpG hypermethylator phenotype that distinguishes two T-cell acute lymphoblastic leukemia subgroups and further validate it in an independent series of 17 T-Lymphoblastic Lymphoma. Next, we identified a methylation classifier based on 9 promoters which accurately predict the methylation phenotype. This classifier was applied to an independent series of 168 primary adult T-cell Acute Lymphoblastic Leukemias treated accordingly to the GRAALL03/05 trial using methylation-specific multiplex ligation-dependent probe amplification. Importantly hypomethylation correlated with specific oncogenic subtypes of T-cell Acute Lymphoblastic Leukemias 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-cell Acute Lymphoblastic Leukemias 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 ALL (1). T-ALL is associated with wide range of acquired genetic abnormalities that contribute to developmental arrest and abnormal proliferation of malignant lymphoid progenitors (2, 3). Despite the diversity of observed mutations and deletions, genome wide expression (4-6) assays led to the identification of few oncogenic T-ALL subgroups, namely the immature/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 (7). Epigenetics is defined as changes in gene expression that are not due to changes in gene sequence, and include DNA methylation, histone modifications, microRNAs (miRNAs) 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 BCP-ALL describing promoter hypermethylation and specific methylation signatures according to the cytogenetic subgroup (8). In pediatric T-ALL, DNA methylation was analyzed by Infinium 27K

and 450K 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 (9, 10). Furthermore, it has more recently been shown in a pediatric series that CIMP status correlated with known oncogenic subgroups, for instance, with higher expression of *TAL1* in 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 methylated DNA immunoprecipitation (MeDIP) in a cohort of adult T-ALLs. 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 clinical outcome. Thus, promoter-methylation profiling is a potential candidate for risk stratification of adult T-ALLs and could provide important information in treatment decision making and therapeutic targeting.

Methods

Patients and treatments

Adult patients (15-60 years old) included in 2 successive French ALL cooperative group trials (GRAALL-2003 and GRAALL-2005) with T-ALL, and defined according to the 2008 WHO classification, were analyzed. The GRAALL-2003 protocol was a multicenter Phase II trial, which enrolled 76 adults with T-ALL between November 2003 and November 2005 of whom 50 had sufficient diagnostic tumor material available (12). The multicenter randomized GRAALL-2005 Phase III 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 (13). 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 (14-16). 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.

Methylation-dependent Immunoprecipitation (MeDIP)

Global DNA methylation was assessed by Methylated DNA Immunoprecipitation (MeDIP) assay on an initial series of 24 T-ALLs and 3 human thymi and a second (confirmatory series) of 17 T-Lymphoblastic Lymphomas (T-LBL) and 3 human thymi. Briefly, methylated DNA was immunoprecipitated as described previously (17) 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, USA) covering either 17,970 promoters (17) (T-ALL series) or 25,490 promoters (18) (T-LBL series), following the manufacturer's

instructions. The median-normalized log2 enrichment ratios (MeDIP/Input) were calculated for each probe using the CoCAS software (19) 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 **Supplementary Tables S1** and **S2**, 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 (20), 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 (21). The list of differentially methylated promoters in T-ALL and T-LBL is provided in **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 (**Supplementary Table S4**) and, SALSA® MLPA® 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). In addition, the promoter methylation

patterns were verified by 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 (FRD<0.121) between these two groups resulted in 300 unique differentially methylated gene promoters with a vast majority of hypermethylated promoters (297/300) in the so-called hyperMethylated (hyperM) group. The second group displayed an intermediate methylation profile (interM) compared to the normal thymic tissue (**Figure 1B and Figure S1**). Interestingly, all the TLX+ cases without exception (including 6 TLX1+ and 2 TLX3+ cases) clustered in the hyperM group. Conversely, the two SIL-TAL1+ cases belonged to the interM group; suggesting a role of oncogenetic abnormalities in the observed methylation profiles.

A very similar differential methylation signature (**Figure 2A and 2B**) was observed in an independent series of 17 T-LBLs (T-Lymphoblastic Lymphoma). One TLX1+ and five TLX3+ T-LBLs, as in T-ALL, were clustered in the group with a hypermethylated promoter profile (253/255 hypermethylated 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 **Supplementary table S2**). Among them, the differential methylation of *MEIS1* promoter was confirmed with two different targeted methods, MeDIP-QPCR (**Figure S2A**) and bisulfite sequencing (**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 9 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 9 gene promoters and a methylation ratio corresponding to the methylation average of these 9 DMR (Differentially Methylated Regions) was calculated. As expected, this predictor allowed the separation of "hyperM" and "interM" T-ALLs from the training cohort (p=0.0016) (**Figure 3B-C**). We then performed this analysis on a series of 168 primary adult T-ALLs 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+ and TLX3+ T-ALLs displayed significantly hypermethylated 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-ALLs (p<0.0001). Conversely, SIL-TAL1+ cases and others T-ALLs expressing high level of TAL1 had significantly hypomethylated promoters (p<0.0001) compared to TLX1/3+, HOXA+ or others T-ALLs. Unlike oncogenetic status, immature ETP-ALL (Early Thymic Progenitor) lacked a significant distinct methylation signature compared to non-ETP-ALLs (**Figure 3E**).

Low level of promoter methylation predicted a poor outcome subgroup of adult T-ALLs.

T-ALL patients with the lowest methylation level (Q1, N=42/168) were more significantly men, were younger, and had a higher WBC at diagnosis than patients with higher methylation levels (Table 1). Moreover, hypomethylated T-ALLs demonstrated a significantly

more frequent mature phenotype (TCR$\alpha\beta$+) 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 (22). 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 hypomethylated subgroup (CIMP-neg) as compared to the Int/High methylated cases (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, patients with low methylation levels had higher CIR (SHR 1.87, 95%CI [1.03-3.38], p=0.04, **Table 2**, **Figure 4A**) and a shorter OS (HR 1.78, 95%CI [1.06-2.98], p=0.03, **Table 2**, **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, 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-ALLs. As previously reported in pediatric T-ALLs (9-11), we showed that DNA methylation status is also a prognostic factor in adult T-ALL. Similarly, patients with a hypomethylated profile display an unfavorable outcome compared to hypermethylated patients. Importantly, even if hypomethylated status is associated with the molecular High-Risk classifier (22), 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 glucosteroids and initial chemotherapy responses [chemosensitivity or MRD (Minimal Residual Disease)] between hypo- and hypermethylated 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 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 (23), rectal

(24), breast (25), bladder cancer (26), prostate cancer (27), and adrenocortical cancer (28). 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 should 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, hypomethylated 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.

Acknowledgments: We thank all French, Swiss and Belgian participants, clinicians, biologists and clinical research assistants, in the LALA-94 and GRAALL 2003-2005 trials, for collecting and providing data and samples.

Authorship contributions: AT, ML, MB, EVM and SS performed diagnostic and experimental analyses. AT, NB and VA analyzed clinical data. All authors contributed to supervision of clinical research and data collection. VA and SS designed and oversaw conceptual development of the project. AT, NB, VA and SS wrote the manuscript.

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	Low methylation N=42 (Q1)	Int/High methylation N= 126 (Q2-Q4)	p-value†
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%)	**<0.0001**
TCRγδ+	1/36 (3%)	14/111 (13%)	0.12
ETP immunophenotype	4/37 (11%)	26/110 (24%)	0.1
NOTCH1/FBXW7^{mutated}	18/42 (43%)	99/126 (79%)	**<0.0001**
High Risk Classifier*	29/42 (69%)	43/125 (34%)	**0.0001**
Oncogenetic Category			
TLX1	0/41 (0%)	35/120 (29%)	**<0.0001**
TLX3	0/41 (0%)	21/120 (18%)	**0.0022**
SIL-TAL1	16/41 (39%)	2/120 (2%)	**<0.0001**
CALM-AF10	0/41 (0%)	8/120 (7%)	0.2
None of the above	25/41 (61%)	54/120 (45%)	0.1
HOXA deregulation	3/39 (8%)	40/112 (36%)	**0.0008**
Clinical Subsets Analyzed			
Age, median (range)	23.2 (16.6-56.2)	33.4 (16.3-59.1)	**<0.001**
Sex ratio, M/F	35/7	85/41	0.05
WBC (G/L), median (range)	80 (4-604)	30 (1-645)	**0.003**
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) <10-4	16/19 (84%)	48/73 (66%)	0.164
Long-term outcome			
5-year CIR (95% CI)	45% (31-62)	27% (20-36)	**0.04**
5-year OS (95% CI)	50% (34-64)	68% (59-76)	**0.03**

Table 1. Patients' characteristics and outcome according to methylation status. Abbreviations: 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: T-cell receptor; ETP: early thymic precursor. * The unfavorable classifier includes NOTCH1, FBXW7, RAS and PTEN (Trinquand, *et al* 2013). †Chi-square or Mann-Whitney tests were used where appropriate.

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

Table 2. Univariate and multivariate analysis for CIR and OS. Abbreviations: 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

Figures legends

Figure 1: MeDIP-array hierarchical clustering in T-ALL. (A) Unsupervised hierarchical clustering of 24 adult T-ALLs based on the genome-wide promoter methylation (MeDIP-array). The hypermethylated (hyperM; Group 1) and intermediate (interM; Groupe 2) methylated 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).

Figure 2 : MeDIP-array hierarchical clustering in T-LBL. A) Unsupervised hierarchical clustering of 17 T-LBLs based on genome-wide promoter methylation (MeDIP-array). The hypermethylated (hyperM; Group 1) and intermediate (interM; Groupe 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.

Figure 3: Targeted promoter methylation analysis in GRAALL 03/05 T-ALL series. (A) List of the nine gene promoters classifier allowing methylation status prediction. (B) Representative ratio charts of MS-MLPA analysis for one normal thymus and two T-ALLs from the training series belonging to the interM subgroup and the 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-ALLs 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-ALLs

included in GRAALL03/05 trial and according to the driver oncogene involved (TLX1, TLX3, HOXA, SIL-TAL1). (E) Methylation ratio according to the ETP phenotype.

Figure 4: Outcome of patients according to the methylation ratio. (A) and (B) Kaplan-Meyer graphs according to methylation status (hypomethylated cases Q1 *vs* the others (Q2-Q4) for CIR and OS, respectively, for patients included in the GRAALL03-05 trial**.**

Figure 1

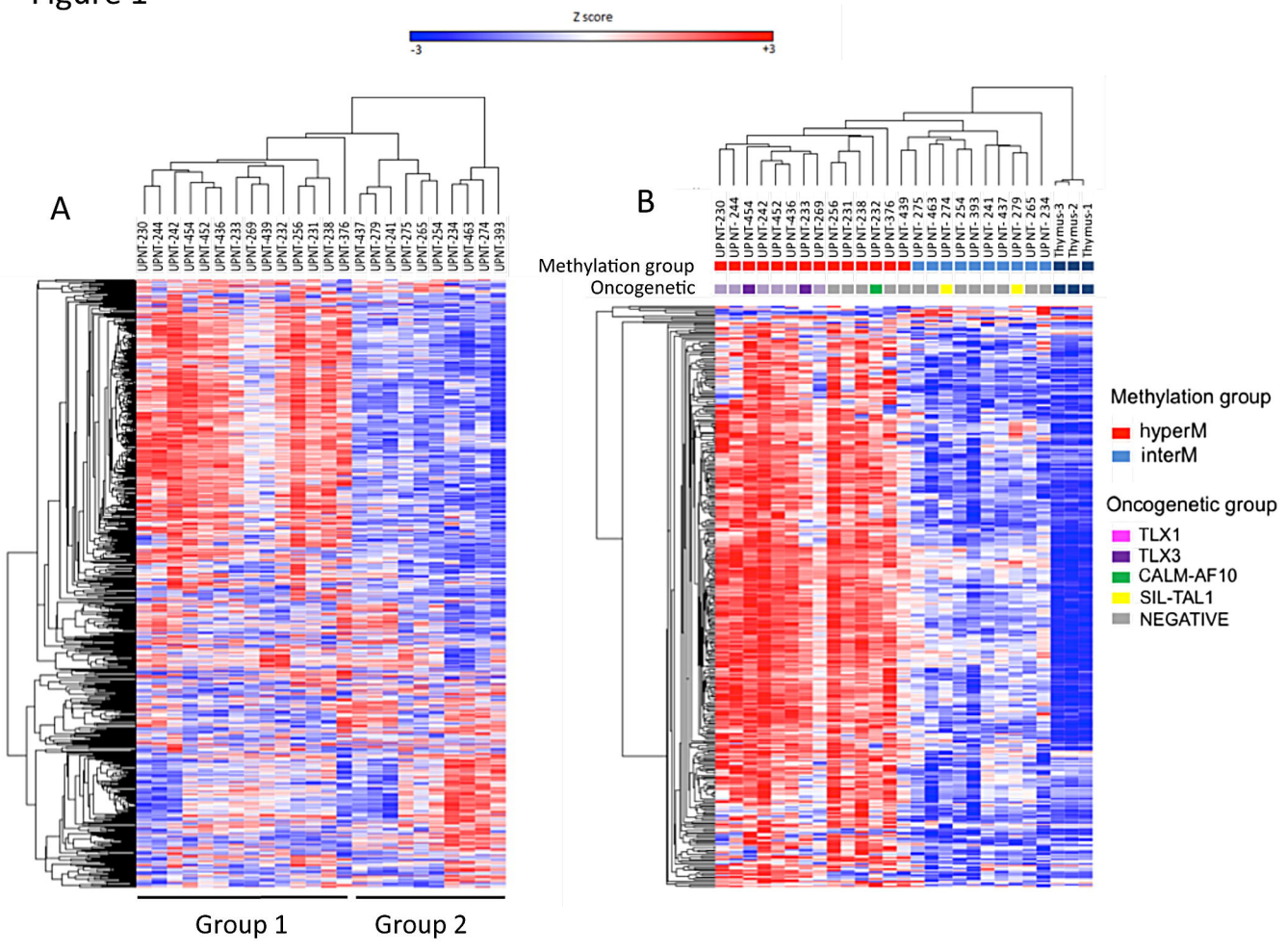
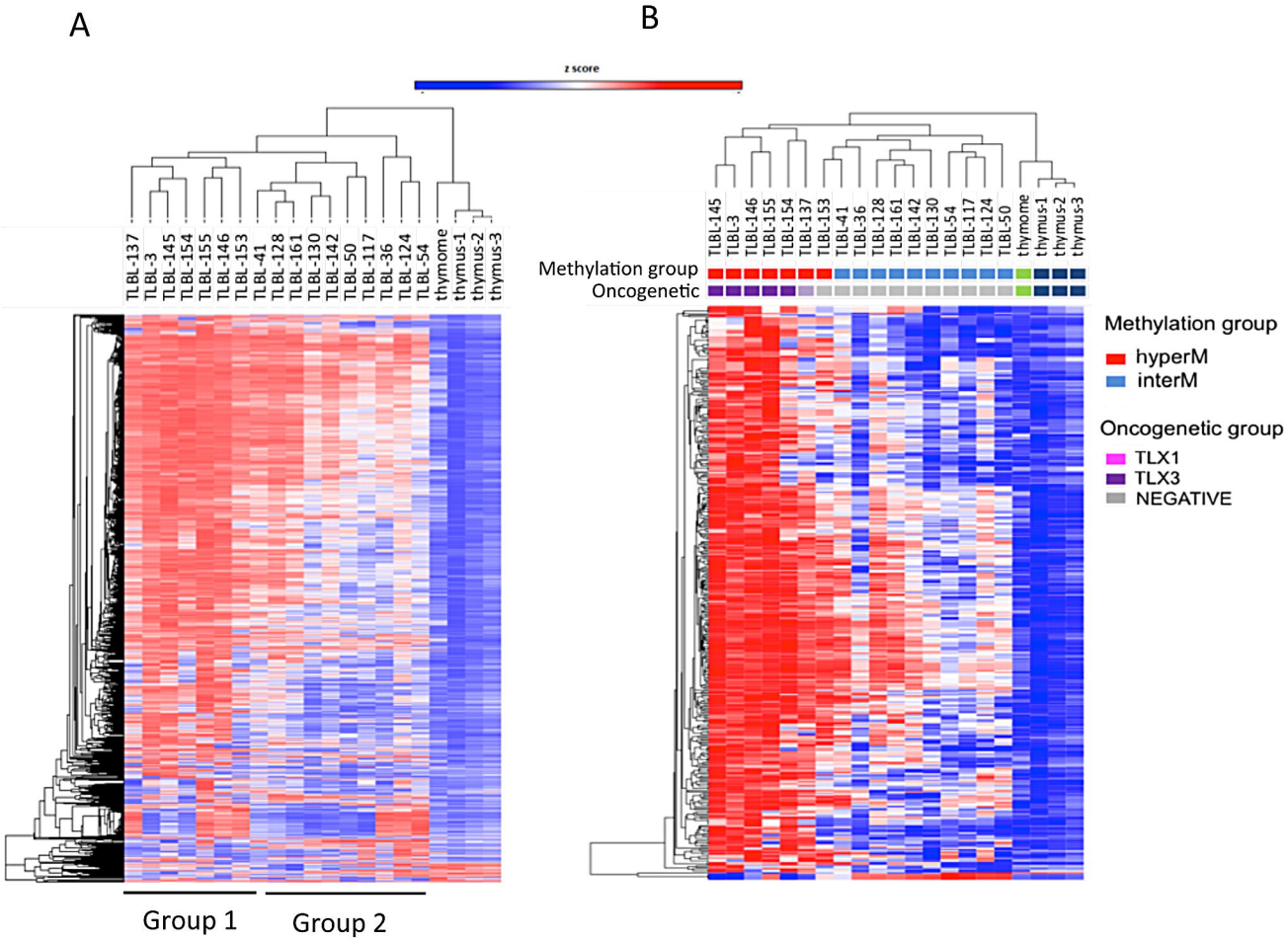

Z score
-3
+3
A
UPNT-230
UPNT-244
UPNT-242
UPNT-454
UPNT-452
UPNT-436
UPNT-233
UPNT-269
UPNT-439
UPNT-232
UPNT-256
UPNT-231
UPNT-238
UPNT-376
UPNT-437
UPNT-279
UPNT-241
UPNT-275
UPNT-265
UPNT-254
UPNT-234
UPNT-463
UPNT-274
UPNT-393
Group 1
Group 2
B
UPNT-230
UPNT- 244
UPNT-454
UPNT-242
UPNT-452
UPNT-436
UPNT-233
UPNT-269
UPNT-256
UPNT-231
UPNT-238
UPNT-232
UPNT-376
UPNT- 439
UPNT- 275
UPNT- 463
UPNT- 274
UPNT- 254
UPNT- 393
UPNT- 241
UPNT- 437
UPNT- 279
UPNT- 265
UPNT- 234
Thymus-3
Thymus-2
Thymus-1
Methylation group
Oncogenetic
Methylation group
hyperM
interM
Oncogenetic group
TLX1
TLX3
CALM-AF10
SIL-TAL1
NEGATIVE

Figure 2


A
B
z score
TLBL-137
TLBL-3
TLBL-145
TLBL-154
TLBL-155
TLBL-146
TLBL-153
TLBL-41
TLBL-128
TLBL-161
TLBL-130
TLBL-142
TLBL-50
TLBL-117
TLBL-36
TLBL-124
TLBL-54
thymome
thymus-1
thymus-2
thymus-3
Group 1
Group 2
Methylation group
Oncogenetic
Methylation group
hyperM
interM
Oncogenetic group
TLX1
TLX3
NEGATIVE

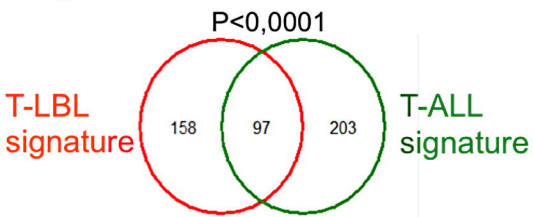
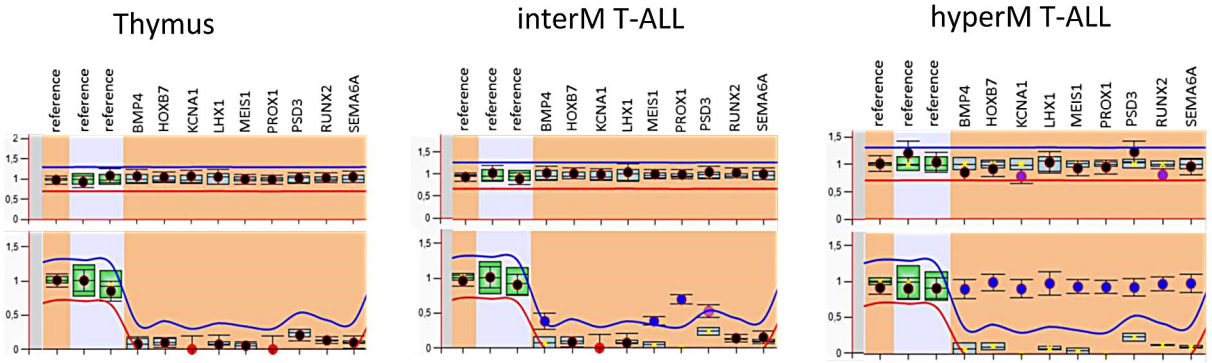

C
P<0,0001
T-LBL signature
158
97
203
T-ALL signature

Figure 3

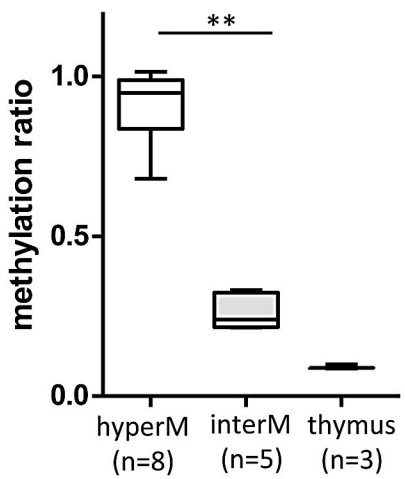
A

gene	weight
BMP4	4.663165
HOXB7	4.680117
KCNA1	4.6555853
LHX1	4.854362
MEIS1	4.8830695
PROX1	4.84076
PSD3	4.743963
RUNX2	3.737859
SEMA6A	4.7371855

B



C



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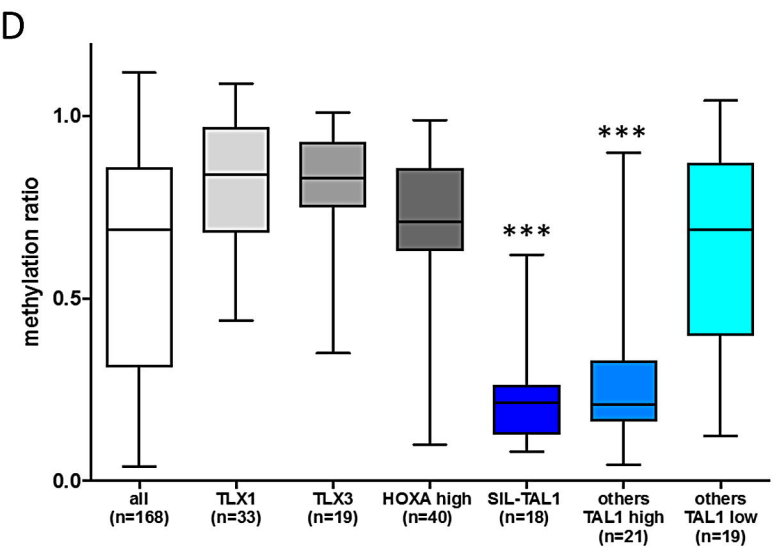


E

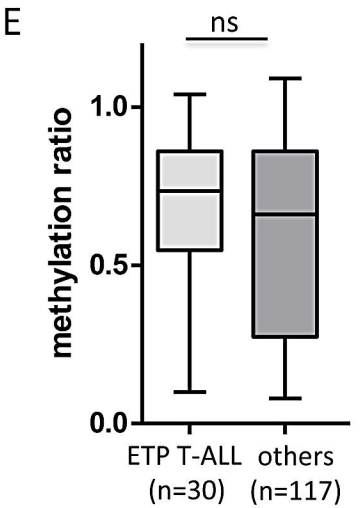
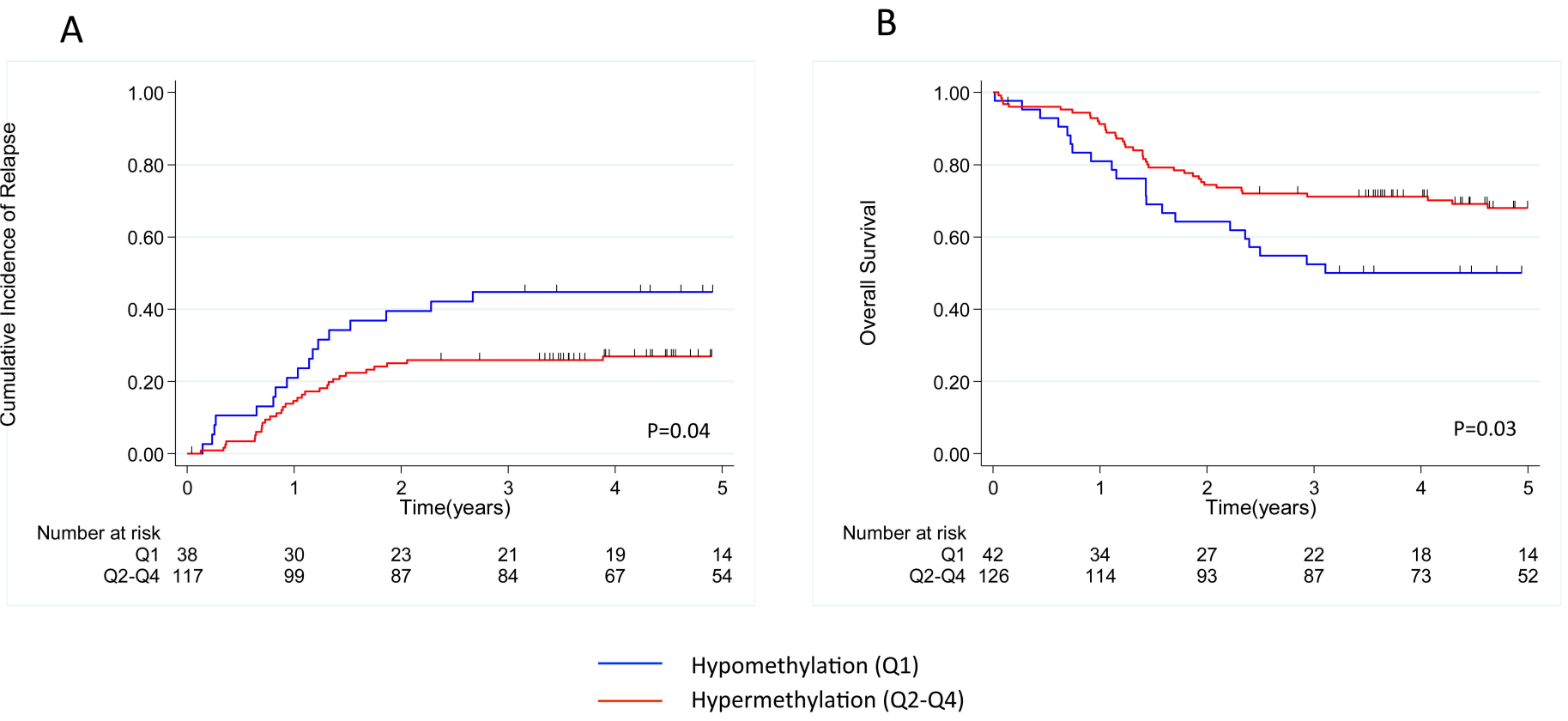


Figure 4


A
Cumulative Incidence of Relapse
1.00
0.80
0.60
0.40
0.20
0.00
P=0.04
0
1
2
3
4
5
Time(years)
Number at risk
Q1 38 30 23 21 19 14
Q2-Q4 117 99 87 84 67 54
B
Overall Survival
1.00
0.80
0.60
0.40
0.20
0.00
P=0.03
0
1
2
3
4
5
Time(years)
Number at risk
Q1 42 34 27 22 18 14
Q2-Q4 126 114 93 87 73 52
Hypomethylation (Q1)
Hypermethylation (Q2-Q4)

Supplementary methods

Methylation classifier

In order to develop a classifier of gene promoter methylation markers which is predictive of the hyper-methylated group, we computed a correlation coefficient for each of the 17,970 promoter regions using "neighborhood analysis". Regions with an absolute coefficient greater than 3.5 were retained for subsequent training and testing. Eleven gene promoters were selected and used their degree of correlation (Wi) as weights between the two distinct classes (1). We randomly created 10 subsets of 12 samples issued from the original training data set (25 samples). The prediction score for each patient in the 10 randomly training sets was then computed according to the established weights, following the procedure described in (2):

1) Center the methylation level of each feature to the corresponding feature mean of the subset;

2) Multiply the centered methylation level by the corresponding feature weight;

3) Sum the weighted methylation levels as single predictive score for each patient.

4) Compute the best Threshold Score of Prediction (TSP) (~1.48) with these random subsets.

Next, we made a prediction model to challenge the training and test data sets. We computed the prediction score as described above and correlated them with the TPS to assign each sample to either the hypermethylated (TSP > 1.48) or the hypomethylated (TSP < 1.48) group.

Statistical analysis

Comparison of continuous and categorical variables between subgroups was performed by Mann-Whitney test and Fisher's exact test, respectively. The Overall Survival (OS) was calculated from the date of prephase initiation to death date censoring patients alive at last follow-up. The cumulative incidence of relapse (CIR) corresponds to the time from complete remission date to relapse date censoring patients alive without relapse at last follow-up date and considering death in complete remission (CR) as a competitive event. Univariate and multivariate analyses were performed with a Cox model for OS and a Fine-Gray model for CIR. Proportional-hazards assumption was checked before conducting multivariate analyses.

Variables associated with OS or CIR in univariate analyses with a $p<0.1$ were considered to be included as covariates in multivariable models. Statistical analyses were performed with the STATA software (STATA 12.0 Corporation, College Station, TX). All tests were two-sided with a significance level of 0.05.

Supplementary references

1. Golub TR, Slonim DK, Tamayo P, Huard C, Gaasenbeek M, Mesirov JP, et al. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science (New York, NY. 1999 Oct 15;286(5439):531-7.
2. Figueroa ME, Lugthart S, Li Y, Erpelinck-Verschueren C, Deng X, Christos PJ, et al. DNA methylation signatures identify biologically distinct subtypes in acute myeloid leukemia. Cancer cell. 2010 Jan 19;17(1):13-27.

Supplementary figures.

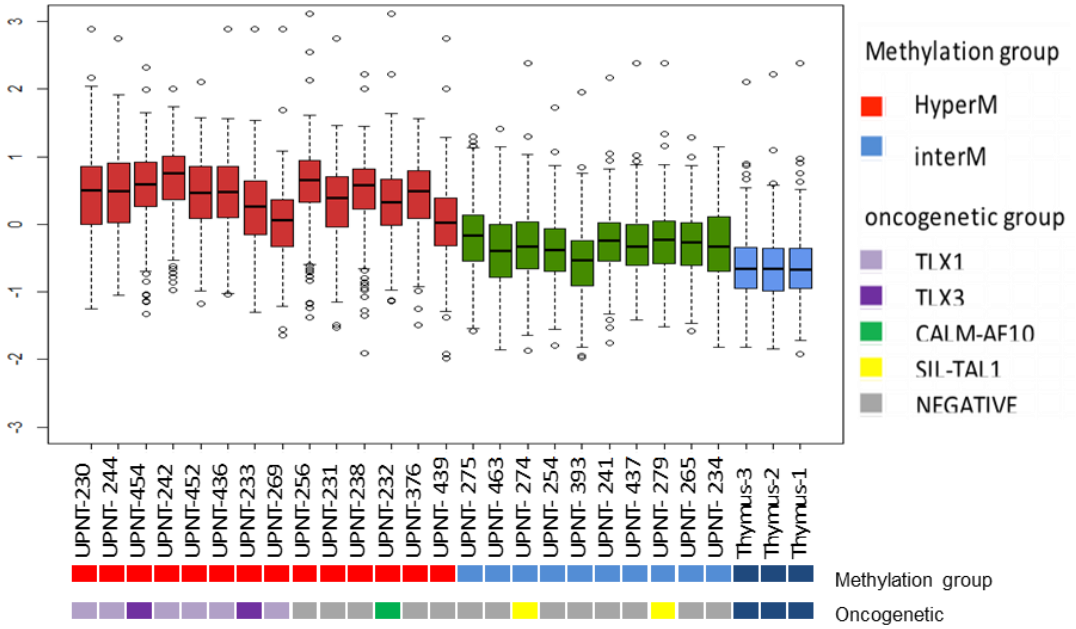


Figure S1: Global promoter methylation in T-ALL and normal thymic samples. The methylation score is calculated from the median enrichment ratio of overlapping promoter probes.

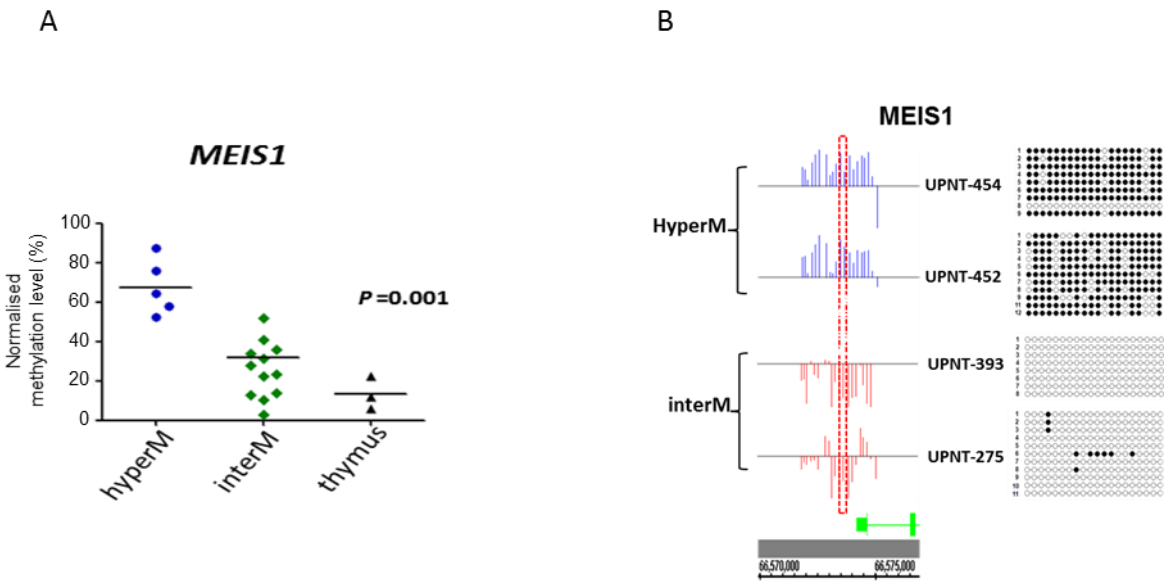


Figure S2: Validation of the methylation levels. A) Methylation of the *MEIS1* promoter was assessed by qPCR analyses of MeDIP on a subset of hyperM and interM T-ALL samples, along with human thymus. The methylation levels were normalized with respect to an *in vitro* methylated genomic DNA. Statistical significance between the hyperM and interM subgroups was assessed by unpaired T test. B) Methylation of the highlighted region (dashed box) of the *MEIS1* promoter was assessed by bisulfite sequencing of two hyperM and two interM T-ALL samples. Each line represents a sequenced clone, where black and white circles indicate methylated and unmethylated CpG.

Supplementary Table S1 on Excel file only. MeDIP Methylation scores per promoter in T-ALL samples

Supplementary Table S2 on Excel file only. MeDIP Methylation scores per promoter in T-LBL samples

Supplementary Table S3 on Excel file only. Differentially methylated promoters in T-ALL and T-LBL

Gene	LHS	RHS
BMP4	CCTCTCGGTTTTAGAACCGCGCTCTCC	CGCCCCAGGAGATTCCTTGGGGCCGAG
HOXB7	TTCCTTCAACATGCACTGCGCGCCCTTTGAG	CAGAACCTCTCCGGGGTGTGTCCCGGCGACT
KCNA1	TATTCCAGGCGCTTTCTCAGGTTTCTGCTGATCTT	GCAGCGCCCAGAAATGGACCGAGCGGACCCGCCGCC
LHX1	TCCTTCCCCCGCGCCGGCGCCGCGCCTCCGGTCTCCC	CGCCCCCATCAGGAAACCGCCCGAATCAACTTTGCAAG
MEIS1	GAGGAAAGTCATGAAGTCTATGCGCGGAGCCCTGTGCAA	AATAACTCCCGCTGCTGCCTGCCCGGCGTTGATTCCCAAT
PROX1	GTCCTGGAAGAGCTAGTGTGAGCCGGGCGCCGCTCGCGCCG	TCTCCCGCTTTGCATAGTGCCCGCAGATGGCTCGCTCCGGCC
PSD3	GCCCCGGAGCGCCCGGCGGCGGTTTCGGCGCGCGGCCGGGCTGGC	GATGGAAGATGGAAGGAAGGAGCGCAGCGGTGAGCTCCGGGGCCGG
RUNX2	GAGTAGTATCCCCTGAACTCCATCCTTACCCCTCGAGAGCGCACACC	TGGCTACCCCGCACCCCCACCTCTGCTCCCGCGGTCTGGCAGACCCTC
SEMA6A	CTGTGCCTGCCATTCTTCATGTGATCATAACAATAGCGCCTTGGAAGTG	GTTGCGATTTTCTTCTTCCATAAACCTTTTGGGTTCTCACTGGAATTGTA

Supplementary Table S4. Methylation-specific multiplex ligation-dependent probes on the 9 promoter regions forming the methylation classifier. LHS: left hybridizing sequence, RHS: right hybridizing sequence.

	Low methylation N=42 (Q1)	Int/High methylation N=126 (Q2-Q4)	p-value†
oncogenetic classifier			
NOTCH1/FBXW7 mutation	18/42 (43%)	99/126 (79%)	**<0.0001**
RAS mutation	3/42 (7%)	13/126 (10%)	0.76
PTEN alteration	14/42 (33%)	8/125 (6%)	**<0.0001**

Supplementary Table S5. The incidence of NOTCH-activating mutations (NOTCH/FBXW7), RAS mutations and PTEN alterations (mutations or deletions) found in the low methylated subgroup (CIMP-negative) compared to the Intermediate/ High methylated subgroup.