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Specific promoter methylation identifies different subgroups of *MLL*-rearranged infant Acute Lymphoblastic Leukemia, influences clinical outcome and provides therapeutic options

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SPECIFIC PROMOTER METHYLATION IDENTIFIES DIFFERENT SUBGROUPS OF *MLL*-REARRANGED INFANT ACUTE LYMPHOBLASTIC LEUKEMIA, INFLUENCES CLINICAL OUTCOME AND PROVIDES THERAPEUTIC OPTIONS

Abstract

MLL-rearranged infant Acute Lymphoblastic Leukemia (ALL) remains the most aggressive type of childhood leukemia, displaying a unique gene-expression profile. Here we hypothesized that this characteristic gene-expression signature may have been established by potentially reversible epigenetic modifications. To test this hypothesis, we used Differential Methylation Hybridization (DMH) to explore the DNA methylation patterns underlying *MLL*-rearranged ALL in infants. The obtained results were correlated with gene-expression data to confirm gene silencing as a result of promoter hypermethylation. Distinct promoter CpG island methylation patterns separated different genetic subtypes of *MLL*-rearranged ALL in infants. *MLL* translocations t(4;11) and t(11;19) characterized extensively hypermethylated leukemias, whereas t(9;11)-positive infant ALL and infant ALL carrying wild-type *MLL* genes epigenetically resembled normal bone marrow. Furthermore, the degree of promoter hypermethylation among infant ALL patients carrying t(4;11) or t(11;19) appeared to influence relapse-free survival, with patients displaying accentuated methylation being at high relapse risk. Finally, we show that the demethylating agent zebularine reverses aberrant DNA methylation, and effectively induces apoptosis in *MLL*-rearranged ALL cells. Collectively these data suggest that aberrant DNA methylation occurs in the majority of *MLL*-rearranged infant ALL cases and guides clinical outcome. Therefore inhibition of aberrant DNA methylation may be an important novel therapeutic strategy for *MLL*-rearranged ALL in infants.

Introduction

While long-term survival rates in childhood Acute Lymphoblastic Leukemia (ALL) exceed 80%¹, the survival chances of infants (<1 year of age) still range between 20-50%². Approximately 80% of infants with ALL carry chromosomal translocations involving the *MLL* gene³, fusing the N-terminal portion of the *MLL* gene to the C-terminal region of one of its translocation partner genes. The most frequent *MLL* translocations among infant ALL patients are t(4;11), t(11;19) and t(9;11)^{2,4}, giving rise to the fusion proteins MLL-AF4, MLL-ENL and MLL-AF9. These chimeric *MLL* fusion proteins exhibit pronounced transforming capacities⁵, and independently contribute to an unfavorable prognosis.^{2,6}

As a member of the trithorax gene family, *MLL* is involved in transcriptional regulation⁷. Therefore, structural alterations of this gene may be expected to affect its function, presumably leading to transcriptional deregulation. Not surprisingly, recent gene expression profiling studies characterized *MLL*-rearranged ALL as a unique type of leukemia that is genetically clearly separable from other ALL subtypes^{8,9}. As epigenetic modifications affect gene expression patterns¹⁰, we hypothesized that the specific gene expression profiles associated with *MLL*-rearranged infant ALL may well be driven by

epigenetic changes, which recently have been established to play important roles in the development and progression of leukemia¹¹. The most widely studied epigenetic event in hematological malignancies constitutes transcriptional gene silencing by promoter CpG island hypermethylation^{11,12}. This phenomenon either leads directly to the silencing of tumor suppressor genes, or indirectly to up-regulation of other genes, when silencing of certain regulatory genes relaxes the suppression on their target genes. Hence, genome-wide promoter hypermethylation potentially results in abnormal gene expression profiles that favor malignant transformation. For example, we recently demonstrated that *FHIT*, a putative tumor suppressor gene, is characteristically silenced in *MLL*-rearranged infant ALL cells by CpG hypermethylation, and that re-expression of this gene induced apoptosis in these cells¹³.

Here we applied Differential Methylation Hybridization (DMH), an array-based technique that allows genome-wide screening of DNA methylation, using two different microarray platforms to explore the DNA methylation patterns underlying *MLL*-rearranged infant ALL. We show that different types of *MLL* translocations are associated with distinct patterns of DNA methylation, and we found that the degree of DNA methylation influences clinical outcome, identifying subgroups of *MLL*-rearranged infant ALL patients that may particularly benefit from therapeutic strategies containing demethylating agents.

Material and Methods

Patient samples

We studied 57 newly diagnosed infant ALL patients, enrolled in the international INTERFANT-99 treatment protocol² (patient characteristics listed in Supplemental table 1). Forty-four patients (77%) carried *MLL* translocations, and thirteen (23%) harbored untranslocated (wild-type) *MLL* genes. Among the *MLL* translocated patients, twenty one were positive for t(4;11), seventeen for t(11;19) and six patients carried translocation t(9;11). Written informed consent and institutional review-board approval were obtained for all patients. Whole normal bone marrow samples obtained from eight non-leukemic pediatric patients were included as controls. Leukemic cell isolation and enrichment to achieve more than 90% leukemic blasts, as well as DNA and RNA extractions were performed as described before¹⁴.

Leukemia cell lines

RS4;11, SEMK2 and BEL-1 represent t(4;11)-positive precursor B-cell ALL cell lines. SEMK2 was originally derived from a 5-year-old girl at relapse¹⁵ and was kindly provided by Dr. Scott Armstrong (Dana Farber Cancer Institute, Boston, Massachusetts, USA). BEL-1 was a generous gift from Dr. Ruoping Tang (University Laboratory, Paris, France).¹⁶ RS4;11 was established from the bone marrow of a 32-year-old woman¹⁷, and was, like all other cell lines used in this study, purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). KOPN-8 harbors translocation t(11;19) and was derived from a 3-month-old infant girl with B-cell precursor ALL. REH and TOM-1 represent precursor B-lineage ALL cells exhibiting a TEL-AML1 fusion, and a Philadelphia chromosome, respectively. JURKAT and HSB2 both are T-lineage ALL cell lines, and Kasumi-1 and MV4;11 are AML cell lines. Kasumi-1 carries the t(8;21) AML1-ETO fusion gene, and MV4;11 harbours *MLL* translocation t(4;11). All cell lines were maintained as suspension cultures in RPMI 1640 with L-Alanyl-L-Glutamine (Invitrogen) supplemented

with 10% FCS (Integro), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.125 µg/ml fungizone (Invitrogen) at 37°C in humidified air containing 5% CO₂.

Differential Methylation Hybridization using CpG island microarrays

Differential Methylation Hybridization (DMH) was performed essentially as described by Yan et al. (Supplemental Methods)^{18,19}. DMH was applied on two different CpG island microarray platforms with limited overlap in CpG island probes. The first was the custom spotted 9K microarray chip developed by Huang and co-workers, containing 8,640 *MseI* fragment probes¹⁸. In addition, we also used the first commercially available genome-wide CpG island microarrays (Agilent Technologies, Santa Clara, USA). These high-resolution microarrays contain 243,497 60-mer oligonucleotide probes, including 67,487 CpG island probes located in or near gene promoters. For the present study, only these probes located in gene promoters were used. Due to restricted availability of patient material, DNA methylation profiling using the Agilent microarrays was performed in 49 of the 57 infant ALL patients.

Gene expression profiling using Affymetrix GeneChips

Gene expression profiles were generated for t(4;11)-positive (n=15) and t(11;19)-positive (n=14) infant ALL cases, using the same samples for which DNA methylation profiles were already produced on Agilent microarray chips. Expression profiles were also generated for whole healthy pediatric bone marrow samples, however, these did not correspond to the samples in which the DNA methylation patterns were determined. RNA processing, microarray hybridization (HU133 plus 2.0 Affymetrix GeneChips), and washing steps were performed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA, USA). The infant ALL gene expression data and DNA methylation data presented in this study have been deposited in the NCBI Gene Expression Omnibus and are accessible via the GEO Series accession number GSE18400.

In vitro cytotoxicity assay and exposure to zebularine

In vitro sensitivity of leukemia cell lines to the demethylating agent zebularine^{20,21} was determined by four-day MTT-assays as described previously²². Zebularine was a generous gift from Dr. Victor E. Marquez (National Cancer Institute of Frederick, Frederick, Maryland, USA). To study the effects of demethylation on *MLL*-rearranged ALL cells, the cell lines SEMK2 and RS4;11 were cultured for 10 days in the presence or absence of 100 µM zebularine.

Statistical analyses

Normalization of the CpG island microarray data was performed using global locally weighted scatterplot smoothing (loess) normalization²³, and differentially methylated CpG islands were identified using the linear models for microarray data (limma) package in the R statistical environment (R Development Core Team, 2007) (Supplemental Methods)²⁴. The resulting list of p-values was corrected for multiple testing by the false discovery rate (FDR) step-up procedure of Benjamini & Hochberg²⁵. An FDR-adjusted p-value <0.01 was regarded significant. As a measure of internal validation for the subtype-specific methylation signatures, permutation testing (global test)²⁶ was applied to evaluate whether genes were significantly associated with a certain type of *MLL* translocation. For this, the tendency of repeated re-assignment of individual samples to their original cluster

was assessed. (Supplemental Methods)

Relapse-free survival was computed with the Kaplan Meier estimator. The duration of relapse-free survival was defined as the time from diagnosis until the date of leukemia relapse or the last follow-up. The probability of relapse in complete remission was estimated by applying the cumulative incidence estimator. The log-rank test was used to compare outcomes between different patient groups, and a one-step Cox model was applied to estimate the hazard of relapse for these patients, adjusting for already established risk stratification according to the international INTERFANT-06 treatment protocol. (Supplemental Methods).

Results

Unsupervised analysis based on DNA methylation patterns separates different infant ALL subtypes

Using Differential Methylation Hybridization (DMH) on two different microarray platforms, genome-wide promoter DNA methylation profiles were generated for infant ALL patients carrying *MLL* translocations t(4;11), t(11;19) or t(9;11), and infant ALL patients bearing wild-type *MLL* genes. To explore whether these samples showed leukemia-specific increases in promoter CpG island methylation, these profiles were compared with DNA methylation patterns obtained from bone marrow samples derived from healthy children. Initially, we performed a principal component analysis (PCA), using all CpG island probes present on each array without any selection. Based on the first three components of the PCA, which explain 41.8% (9K chip) and 32.2% (244K chip) of the total variance, the patient samples were visualized (Figure 1). Interestingly, for both microarray platforms, this unsupervised analysis separated two major groups. Infant ALL samples that carry t(9;11) or wild-type *MLL* genes clustered together with normal bone marrow samples, whereas infant ALL samples carrying t(4;11) or t(11;19) clustered tightly together separately from the other samples. Although the cluster comprising t(9;11)-positive, untranslocated infant ALL samples and normal bone marrow samples appeared more heterogeneous, it has to be taken into account that this cluster consists of three different types of samples. Moreover epigenetic heterogeneity is already present among the normal bone marrow samples. Finally, we emphasize that this analysis is completely unguided.

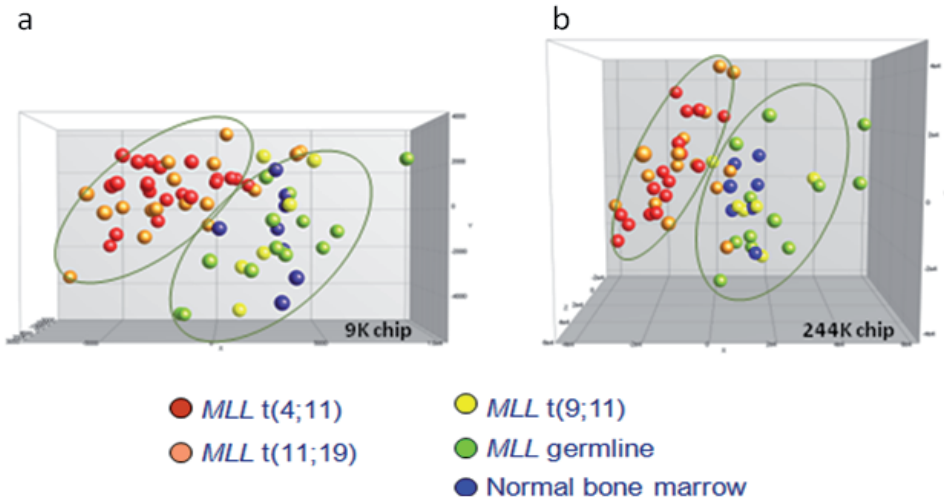


Figure 1 - Unsupervised clustering analysis of DNA methylation in infant ALL. Principal Component Analyses (PCA) of the CpG island methylation data from infant ALL patients and normal bone marrows using all probes present on each microarray platform. Each case is color-coded indicating the specific infant ALL subgroups. **a-1.** shows data from the custom spotted 9K CpG island microarray. t(4;11) (n=21; red), t(11;19) (n=17; orange), t(9;11) (n=6; yellow), *MLL* wild-type ALL (n=13, green) and normal bone marrow (n=8; blue). **a-2.** shows data from the commercially available 244K CpG island microarray (Agilent). t(4;11) (n=16; red), t(11;19) (n=15; orange), t(9;11) (n=6; yellow), *MLL* wild-type ALL (n=12; green) and normal bone marrow (n=7; blue). Due to restricted availability of patient material, Agilent DNA methylation profiles were generated for 49 infant ALL patients and 7 normal bone marrow samples.

Specific DNA methylation patterns further separate the different infant ALL subtypes

Subsequently, to explore whether specific DNA methylation profiles could define the genetic subgroups of infant ALL more accurately, the 20 most discriminative hypermethylated genes for each group (as compared with all other relevant subgroups combined) were selected. For both microarray platforms the gene names, log-fold changes, and p-values are listed in the Supplemental data (tables 2S and 3S). Permutation testing validated the robustness of the subtype-specific methylation signatures. Using the selected genes, we generated heatmaps in which both the genes and samples were clustered hierarchically (Euclidean distance and complete linkage) (Figure 2a). This semi-supervised analysis revealed that *MLL* t(4;11) and t(11;19)-positive patients could clearly be separated from one another and from the other samples. In contrast, hypermethylated genes that unambiguously separate t(9;11)-positive samples from *MLL* wild-type (untranslocated) infant ALL samples, could not be identified. Moreover, the most significantly hypermethylated genes shared by t(9;11)-positive and wild-type *MLL* samples were also methylated in healthy bone marrow samples (Supplemental figure 1S), and therefore likely reflect normal methylation in healthy hematopoietic cells. Importantly, these genes are hypomethylated in infant ALL harbouring translocation t(4;11) or t(11;19).

Next, PCA was used to better visualize these different clusters, emphasizing the separation of the samples into the three expected groups characterized by t(4;11), t(11;19) or t(9;11) together with translocation-negative infant ALL (Figure 2b). When included, the normal

bone marrow samples remained within the cluster comprising samples carrying t(9;11) or wild-type *MLL* genes (Figure 2c). In concordance with this, no significant aberrant DNA methylation could be detected in t(9;11)-positive or untranslocated (wild-type *MLL*) infant ALL, when separately compared with normal bone marrow.

Correlation between promoter methylation and gene expression

Given the aberrant methylation patterns in t(4;11)- and t(11;19)-positive infant ALL samples, we investigated the effects of promoter hypermethylation (Agilent platform) on gene expression (Affymetrix platform) of corresponding genes. Compared with normal bone marrow samples, infant ALL cells carrying t(4;11) displayed a total of 794 hypermethylated CpG island probes (FDR<0.01), and 75 probes were significantly hypermethylated in t(11;19)-positive infant ALL (FDR<0.01). From these analyses, the most significantly hypermethylated genes were selected. Gene names, log-fold changes in methylation, and p-values for these genes are listed in the Supplemental data (tables 4S and 5S). Next, DNA methylation array data was compared with gene expression profiles from the same samples, and visualized as heatmaps and PCA plots (Figure 3). Promoter hypermethylation and down-regulated gene expression correlated for ~90-95% of the genes in both t(4;11)-positive and t(11;19)-positive infant ALL. However, for the remaining genes we observed the opposite; despite extensive hypermethylation, these genes were higher expressed in leukemic samples than in normal bone marrow.

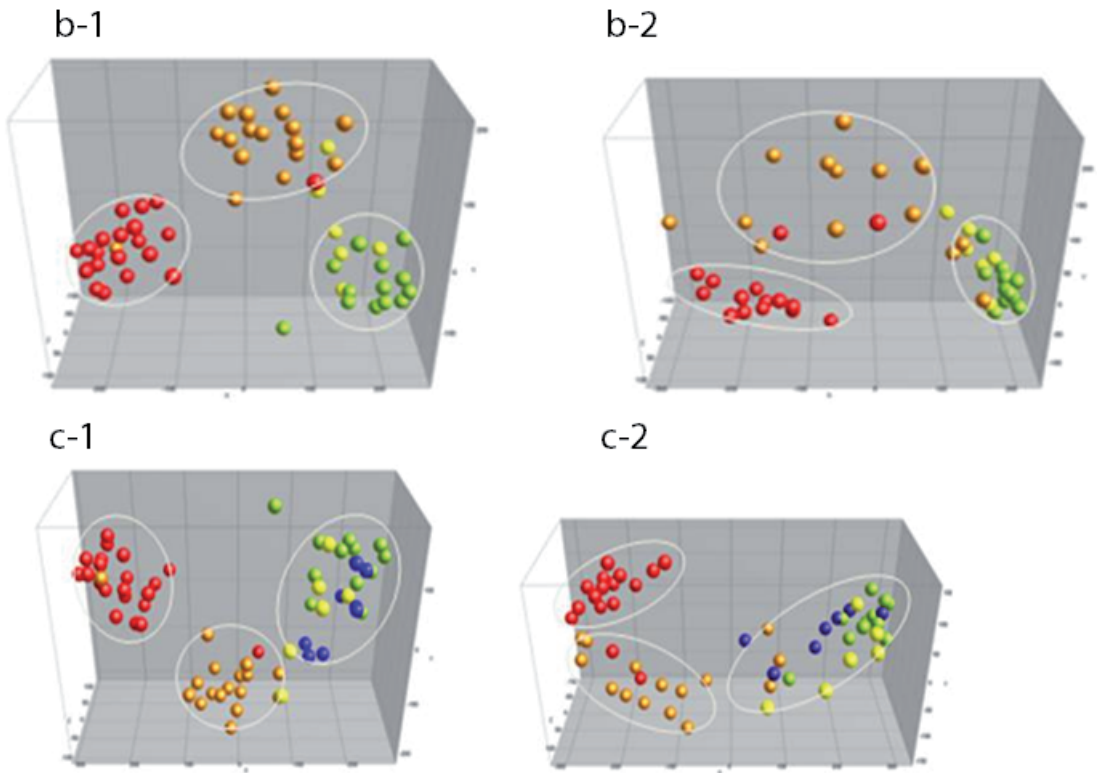


Figure 2 - Infant ALL subtype-specific CpG island hypermethylation. **a1-2.** Heatmaps showing the 20 most significantly hypermethylated probes for each infant ALL subtype. Columns represent patient samples and rows represent genes. Relative DNA methylation levels are shown in red (high) and blue (low). Genes and samples were ordered using hierarchical cluster analysis (Euclidean distance, complete linkage), and gene identifiers are listed at the right. **b1-2.** Principal component analyses (PCA) separating t(4;11) (red), t(11;19) (orange), t(9;11) (yellow) and *MLL* wild-type infant ALL (green). **c1-2.** shows the PCA when normal pediatric bone marrow samples (blue) are included in the analysis. **2-1.** shows data from the custom 9K CpG island microarray. **2-2.** shows data from the commercially available 244K CpG island microarray (Agilent).

The degree of methylation influences clinical outcome in MLL-rearranged infant ALL

In both the heatmaps and PCA plots that represent the most significantly hypermethylated genes among t(4;11)-positive and t(11;19)-positive infant ALL, a clustering of patient samples into two subgroups appeared. Ostensibly, one cluster represents patient samples that, at least for the selected genes, seem to be more densely hypermethylated than the samples in the other cluster. (Figure 3) To better visualize this difference in degree of methylation we plotted the normalized methylation log-ratios of the genes. This semi-quantitative representation of the data indeed confirmed differences in the degree of methylation between both clusters (Figure 2S). To explore the clinical relevance of these subgroups, we computed risk of relapse statistics for these patient groups (Figure 3S). Four patients received bone marrow transplantation (BMT) in complete remission. For these patients data were censored at BMT. One patient died before the start of treatment (referred to as early death), and was excluded from further analyses. Twelve out of 16 (75%) patients from the “heavily” methylated subgroup had a relapse after achieving complete remission, whereas among the “lightly” methylated patients, relapses occurred in 5/12 (42%) of the cases. The cumulative incidence of relapse at 1 year after diagnosis was significantly ($p < 0.05$) different for the “heavily” and “lightly” methylated subgroups with incidences of 52.5 (SE: 13.7) and 35.7 (SE: 15.5) respectively. The number of patients that could be included in these analyses is not sufficient to evaluate the impact of the degree of DNA methylation adjusted for known prognostic factors (like age, white blood cell count and the *in vivo* response to prednisone) separately. Therefore we used the INTERFANT-06 risk stratification which represents a combination of these factors (see: Supplemental Methods)². Although these results must be interpreted with caution, the Cox regression model indicates that heavy methylation confers an increased risk of relapse (hazard ratio 5.77, 95% CI 1.57-21.2, $p = 0.01$) (table 6S). The separate clustering of these two patient groups, however, did not appear in the gene expression profiles (Figure 3). This implies that the grouping of these patients and the observed variance in relapse-free survival rather reflects progressive accumulation of genome-wide methylation, than direct differences in gene expression. In line with this hypothesis we show that the division into “heavy” and “light” methylation remains present when all significantly hypermethylated probes are used in a semi-quantitative representation (Figure 2S).

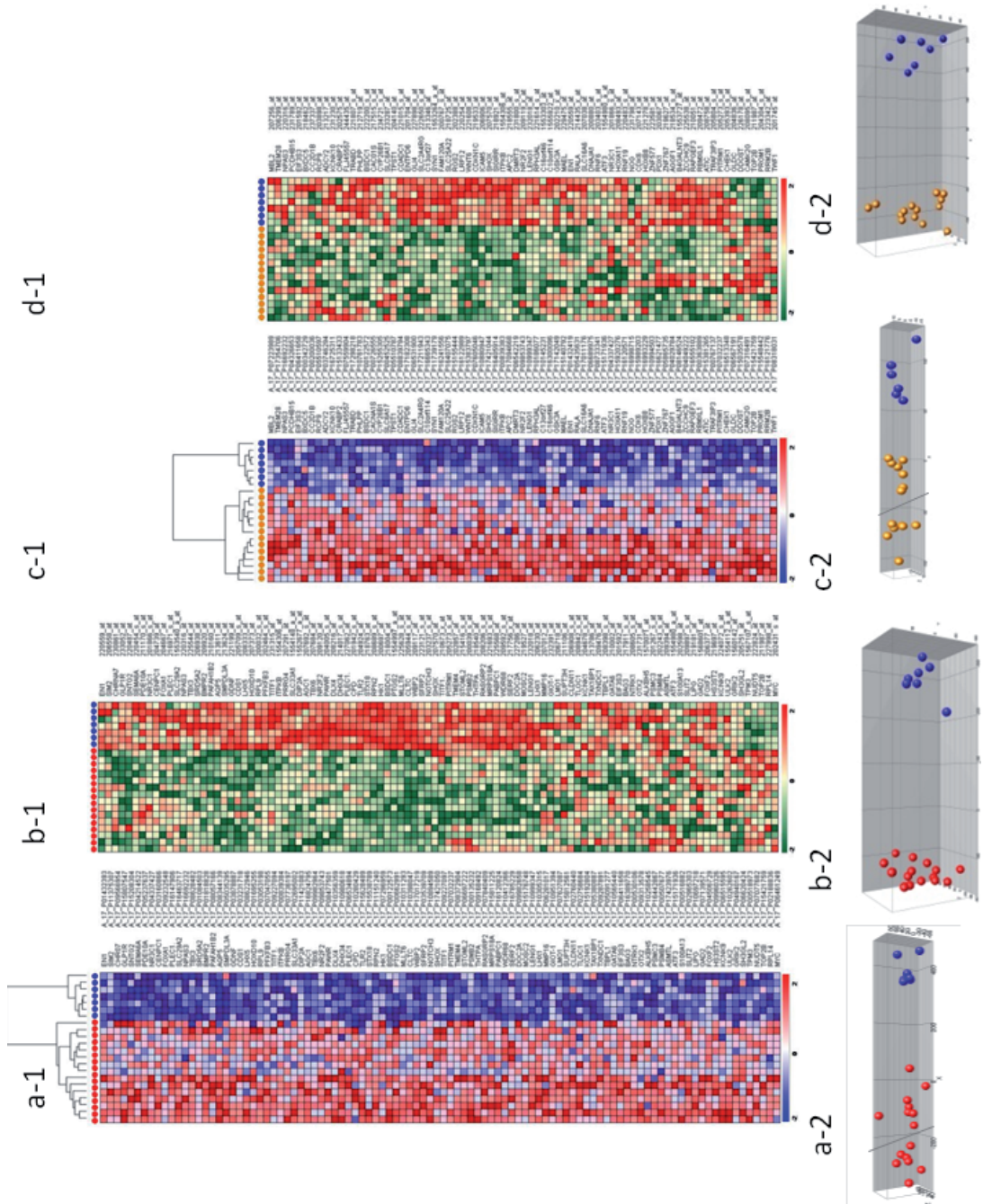


Figure 3 - Correlation between CpG island methylation and gene expression. **a.** Heatmap and PCA showing the most significantly hypermethylated genes in t(4;11)-positive infant ALL (red dots) compared with normal bone marrows (blue dots). Relative DNA methylation levels are shown in red (high) and blue (low). **b.** Heatmap and PCA showing the corresponding gene expression levels from the same genes and samples as presented in Figure 3A. Relative gene expression values are shown in red (high) and green (low). Similarly, the **c.** CpG methylation data and **d.** gene expression data are presented for t(11;19)-positive infant ALL samples (orange dots) as compared with normal bone marrows (blue dots).

MLL t(4;11)-positive cell lines as models for demethylation

DNA methylation patterns of two t(4;11)-positive precursor B-cell ALL cell lines (i.e. RS4;11 and SEMK2) were compared with the profiles from the t(4;11)-positive infant ALL samples. Nearly 50% of the 100 most significantly hypermethylated genes in t(4;11)-positive infant ALL were also hypermethylated in these cell lines (Figure 4a). Representing reasonable models for t(4;11)-positive infant ALL samples, we next studied the effects of demethylation on these genes by comparing DNA methylation profiles of these cell lines before and after a 10-day exposure to 100 μ M of the demethylating agent zebularine. In the SEMK2 and RS4;11 cell lines, respectively 72% (33/46) and 59% (27/46) of the hypermethylated genes showed notable decreases in methylation upon exposure to zebularine. The genes display varying degrees of drug-induced demethylation (Figures 4b-c). For some of the genes the methylation status could be restored to nearly normal levels as observed in healthy hematopoietic cells.

Specific zebularine sensitivity in MLL-rearranged ALL cells

To further investigate the sensitivity of ALL cells to *in vitro* demethylation, cytotoxicity assays were performed using escalating dosages of zebularine. Also two AML cell lines were added to the data set. AML cells (with or without an *MLL* translocation) seem to be less sensitive to the demethylating agent zebularine than *MLL*-rearranged ALL cells, but the *MLL*-rearranged AML cell line MV4-11 does appear more sensitive than the t(8;21)-positive AML cell line Kasumi-1. Clearly, *MLL*-rearranged ALL cells were significantly more sensitive to zebularine than the other cell lines ($p < 0.01$) (Figures 5A-B). As shown in figure 5B, on average the IC_{50} value (i.e. the concentration inhibitory to 50% of the cells) in *MLL*-rearranged ALL cells was $\sim 50 \mu$ M, whereas zebularine failed to reach an IC_{50} value in other types of ALL cell lines.

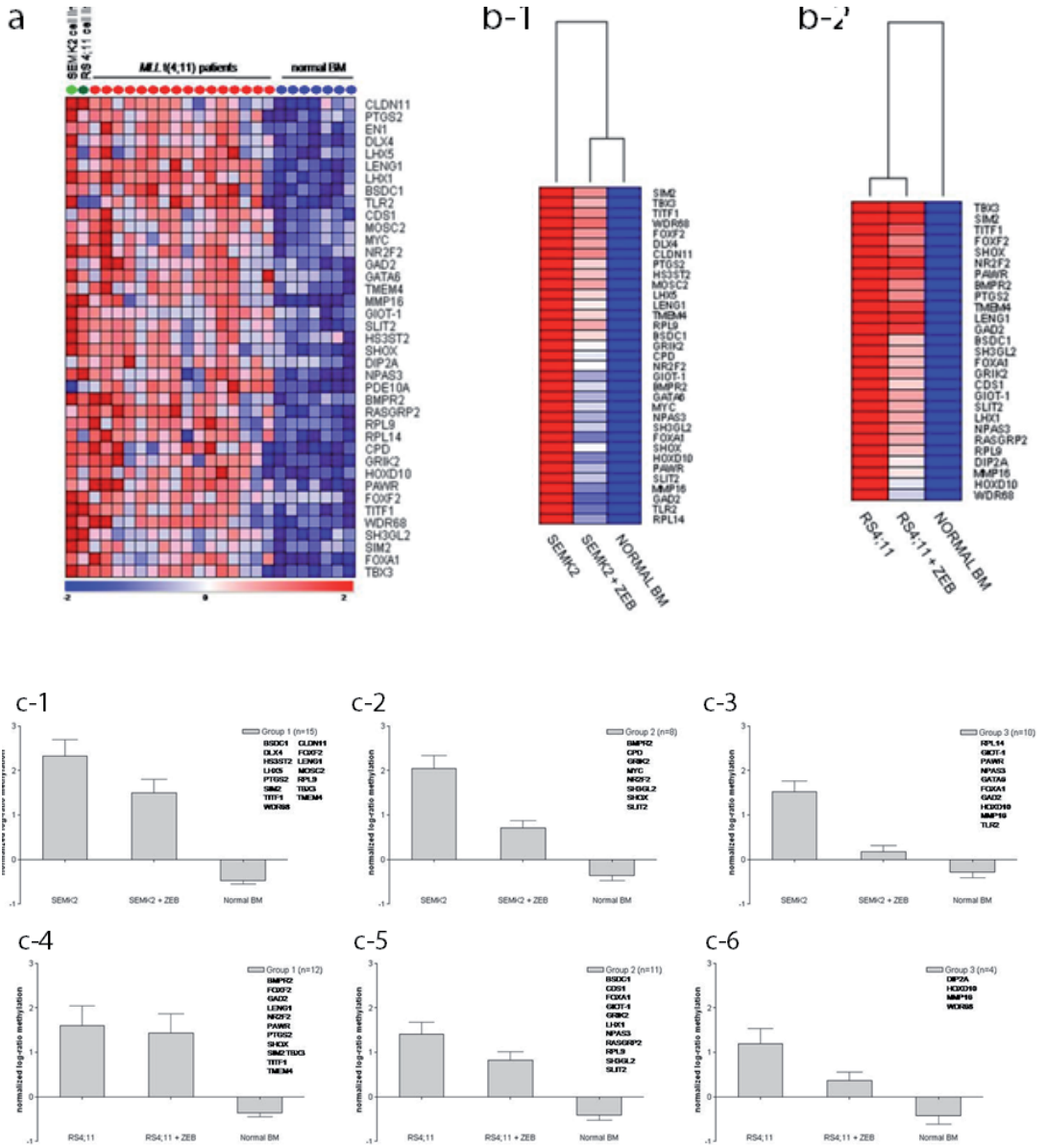


Figure 4 - ALL cell lines as models for (de)methylation **a**. Heatmap showing methylation levels in the t(4;11)-positive B-ALL cell lines SEMK2 (light green dot) and RS4;11 (dark green dot) of genes most significantly methylated in t(4;11)-positive infant ALL patients (red dots) as compared with normal bone marrows (blue dots). **b**. Heatmaps showing methylation levels of these genes after exposure to zebularine. These methylation levels were compared with the average methylation levels as determined from normal bone marrow samples (n=7). **b1** SEMK2 cell line and **b2**. RS4;11 cell line **c1-6**. Graphs displaying the mean and the standard error of the mean (SEM) of changes in methylation levels after zebularine exposure. Genes were divided into three groups for each cell line according to the degree of responsiveness to zebularine. **c1-3**. SEMK2 cell line and **c4-6**. RS4;11 cell line.

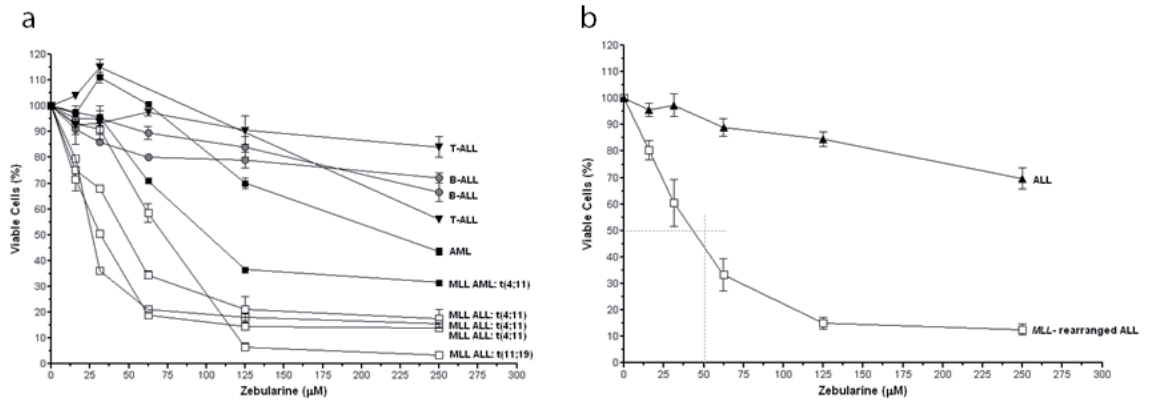


Figure 5 - *In vitro* cytotoxicity to zebularine. **a**. Dose-response curves showing the *in vitro* cytotoxic response to zebularine in individual leukemia cell lines with or without *MLL* rearrangements, or **b**. the mean cytotoxic response for *MLL*-rearranged ALL cell lines (n=4) and for the other ALL cell lines (n=4). Error bars represent standard error of the mean (SEM). The differences between the means of the groups were statistically analyzed using the 2-tailed Student *t* test ($p < 0.01$ for each concentration used).

Discussion

We here present the first global view of the DNA methylome in infant *MLL*-rearranged Acute Lymphoblastic Leukemia (ALL). *MLL*-rearranged infant ALL represents an aggressive and difficult to treat type of leukemia characterized by a unique gene expression profile, that clearly separates this malignancy from other ALL subtypes^{8, 9}. Since epigenetic modifications directly influence gene expression patterns¹⁰, we hypothesized that specific DNA methylation patterns may underlie the characteristic gene signature as observed for *MLL*-rearranged infant ALL. Our data largely support this hypothesis, as the majority of *MLL*-rearranged infant ALL cases (i.e. those characterized by t(4;11) or t(11;19)) represent hypermethylated leukemias, whereas t(9;11)-positive and *MLL* translocation-negative (wild-type *MLL*) infant ALL display DNA methylation patterns that closely resemble that of normal bone marrow. Moreover, distinct leukemia-specific DNA methylation patterns could be identified for the different *MLL*-rearranged infant ALL subtypes as defined by the type of *MLL* translocation or absence of such translocations. Interference of non-leukemia related epigenetic differences in DNA methylation (such as age, sex-specific differences in methylation and differences related to B-cell maturation stages of leukemic cells) with our results could be excluded (Supplemental Results, tables 7S and 8S).

Thus, the presence as well as the patterns of aberrant DNA methylation in infant ALL appear, at least to some extent, dependent on the presence and type of *MLL* fusion, which may reflect a mechanism proposed recently²⁷. Apart from DNA methylation, a second component of the epigenetic code involves histone modifications²⁸, shaping the chromatin in an open (transcriptionally active) or closed (inactive) conformation. An inactive chromatin state usually is associated with hypermethylated CpG promoter regions, whereas active chromatin marks, such as H3K4 trimethylation and H3K79 dimethylation, denote unmethylated promoters, allowing transcription. Interestingly, the *MLL* gene itself has specific histone methyltransferase activity^{29,30}, which is lost during fusion of the *MLL* gene to one of its translocation partners. Therefore, *MLL* fusions can be expected to result in altered chromatin structures due to aberrant histone modifications. Recently,

Krivtsov and Armstrong (2007) proposed that the recruitment of different histone methyltransferases by different *MLL* fusion proteins, may indeed result in inappropriate histone modifications directed by the *MLL* fusion partner²⁷. Given the sound interplay between histone modification and CpG island methylation, this proposed influence of different *MLL* fusion genes on histone modifications, and the apparent influence of the *MLL* fusion partner on DNA methylation as shown in the present study, are presumably linked. In addition, the paper by Krivtsov et al.(2007)²⁷, as well as the study by Mueller and co-workers (2007)³¹ demonstrated the recruitment of a transcriptional elongation complex to *MLL* target genes, resulting in gene activation (i.e. expression). These studies suggest that *MLL* fusion proteins trigger or maintain the leukemia by the activation of specific target genes. In contrast, our present study shows that, apart from specific gene activation, *MLL*-rearranged ALL is also characterized by severe gene inactivation, which may well be driven by the same *MLL* fusion. *MLL*-rearranged ALL cells typically mirror highly immature B-cells. Possibly, the *MLL* fusion ignores the activation of many genes that should have been activated (by wild-type *MLL*) at this stage of B-cell development, and necessary for proper differentiation towards mature and functional B-cells. This would suggest that our observed patterns of gross genome-wide DNA methylation is in favor of blocking B-cell differentiation, while simultaneously the *MLL* fusion activates several (proto-onco)genes in favor of uncontrolled cell proliferation and survival. Alternatively (or additionally), inappropriate activation of certain genes by the *MLL* fusion, may in turn induce abnormal inactivation (silencing) of several other genes. However, these proposed mechanisms are highly speculative and remain to be confirmed.

Nonetheless, we can conclude from our data that *MLL*-AF4 and *MLL*-ENL represent *MLL* fusion proteins that both alter histone modifications that result in strongly altered DNA methylation patterns. The differences found in DNA methylation patterns between *MLL*-AF9 and *MLL*-ENL may then seem surprising given the apparent common mechanism of transformation involving the recruitment of *DOT1L* as put forward by others^{27,31}. Surprisingly, the *MLL*-AF9 fusion did not lead to significant aberrant DNA methylation in infant ALL. This suggests that oncogenic transformation in t(4;11)- and t(11;19)-positive infant ALL patients may be facilitated or largely driven by gross epigenetic changes, whereas t(9;11)-positive infant ALL cells presumably transform via alternative mechanisms. In concordance with this is that t(9;11)-positive ALL patients characteristically seem to be different from other *MLL*-rearranged infant ALL patients. For example, t(9;11)-positive infant ALL is typically diagnosed at a later stage during infancy and usually is characterized by a more mature immunoglobulin gene rearrangement pattern (immunophenotype) than t(4;11)- and t(11;19)-positive infant ALL^{2,4}. On the other hand, no significant differences in survival exist between infant ALL patients carrying either t(4;11) or t(11;19) and patients with t(9;11).²

Studying the genes most significantly hypermethylated in t(4;11)- and t(11;19)- positive infant ALL samples, we found that the expression of the vast majority of these genes (~90-95%) was indeed down-regulated. Among the hypermethylated genes we found genes that were previously described to be silenced due to DNA hypermethylation in *MLL*-rearranged ALL, such as the tumor suppressor gene *FHIT*¹³ and the *DLX3* gene³², demonstrating the integrity of our data. Moreover, most of these genes responded well to exposure to the demethylating agent zebularine in t(4;11)-positive cell line models. Among the most significantly hypermethylated genes for either t(4;11)-positive or t(11;19)-

positive infant ALL, a limited overlap was observed. Nevertheless, global gene ontology analysis showed that most of the down-regulated genes in both subgroups are involved in transcriptional regulation (table 9S). This pronounced epigenetic deregulation of the transcriptional machinery may indeed have contributed to the unique gene expression profile characteristic for *MLL*-rearranged ALL^{8,9}. Yet, this would not be true for t(9;11)-positive infant ALL, as no aberrant promoter hypermethylation was observed in these samples. This apparent contradiction, however, is easily explained by the fact that most of the published *MLL*-specific gene expression signatures, including the signatures reported by Armstrong et al. (2002) are predominantly based on t(4;11)- and t(11;19)-positive samples⁸. Therefore gene expression profiling studies including t(9;11)-positive infant ALL samples may well come to demonstrate that profiles associated with t(9;11) are different from those obtained in t(4;11)- and t(11;19)-positive samples.

Remarkably, about 5% of the most significantly hypermethylated genes in t(4;11)- and t(11;19)-positive infant ALL remained highly expressed. This observation controverts the dogma that promoter methylation per definition induces suppression of gene expression. However, Weber and co-workers recently nuanced this dogma by elegantly demonstrating the influence of promoter CpG density on the ability to induce transcriptional repression³³. Therefore, these methylated but highly expressed genes may well exhibit promoters containing weak CpG islands (i.e. a low or intermediate CpG density), unable to repress transcription even when methylated. Another possible explanation for this would again be the involvement of the *MLL* fusion protein, which may have induced activating histone modifications on otherwise inactive regions in the chromatin associated with promoter methylation. In turn, this newly acquired open chromatin state may have overruled the relatively weak DNA methylation, allowing transcription despite earlier established epigenetic silencing. If so, this group of genes may well represent potential therapeutic targets directly influenced by the *MLL* fusion itself.

Most of the genes that were methylated in t(9;11)-positive infant ALL and infant ALL carrying wild-type *MLL* genes were also methylated in normal bone marrow. Presumably, these represent genes that were already silenced in normal hematopoietic cells, but became hypomethylated in t(4;11) and t(11;19) positive infant ALL cells. Interestingly, among these were several genes with oncogenic potential, such as *CDH3*, *TBX2*, *ERCC1* and *NPR2* (figures 2A-B), that have been reported to be involved in proliferation, tumor aggressiveness and prognosis in a wide range of human cancers^{34,35}. Interestingly, among these hypomethylated genes also appeared the *HOXA9* gene which was previously described to be protected from methylation by the *MLL*-fusion itself³⁶. Thus, the present study not only characterizes epigenetically down-regulated genes, but also identifies proto-oncogenes that may be inappropriately expressed in t(4;11)- and t(11;19)-positive *MLL*-rearranged ALL in infants. Obviously, such genes represent yet another set of candidate target genes for future therapeutic intervention.

Of main therapeutic interest is our finding that the degree of DNA methylation among t(4;11)- and t(11;19)-positive infant ALL patients is related to relapse-free survival, with patients presumably carrying heavily methylated genomes being at an increased risk of relapse. Therefore, these children in particular should be considered candidates for therapies including inhibitors of DNA methylation, especially since we here show that *MLL*-rearranged ALL cells are highly sensitive to zebularine *in vitro*. The authors believe that this increased sensitivity to demethylation is rather based on the presence of a general

methylator phenotype (i.e. globally deregulated DNA methylation) than on the actual re-expression of a fixed number of hypermethylated genes. Apparently, genome-wide demethylation is sufficient to cause *MLL*-rearranged ALL cells to undergo apoptosis. This is in concordance with the identification of a heavily and a lightly methylated subgroup of *MLL*-rearranged infant ALL, which is also based on a widespread phenotype with more or less pronounced levels of DNA methylation that are in fact not visible at the gene-expression level. In conclusion, the findings presented here urgently require gene per gene validation studies and mandate additional studies using demethylating agents in the currently only available genuine mouse model for *MLL*-rearranged ALL, recently described by Krivtsov et al.³⁷

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Supplementary data

Supplemental Results

Exclusion of non-leukemia-related epigenetic differences between subtypes of infant ALL

To test whether non-leukemia-related epigenetic differences were present among the sample population that may compromise our findings, we performed several comparisons deducting this possibility. Since leukemic samples were either obtained as peripheral blood or as bone marrow, we first compared DNA methylation patterns in bone marrow samples with samples derived from peripheral blood. No significant differences in promoter CpG island methylation were observed. From this, we concluded that samples of both sources could legitimately be used together in our analyses.

Next, and similarly, sex differences were assessed. A female predominance (62% vs. 38%) was identified among the *MLL*-rearranged infant ALL subgroups, whereas a male predominance (61% vs. 39%) was present in the translocation-negative (wild-type *MLL*) infant ALL group. 176 probes were differentially methylated between male and female patients (FDR<0.01) according to the Agilent microarray platform (Tables S7 and S8). The 121 probes corresponding to 75 genes, hypermethylated in females were all located on the X chromosome. Conversely, most of the 55 probes, corresponding to only 5 genes hypermethylated in male individuals were located on the Y chromosome. None of these genes appeared to interfere with the infant ALL subtype-specific methylation signatures.

In general, infant ALL represents a highly immature type of precursor B-cell leukemia with t(4;11)- and t(11;19)-positive infant ALL most often

displaying the most immature phenotypes.¹ We therefore investigated whether the identified DNA methylation profiles to some extent reflected different maturation stages of early B-cell development. For this, we compared samples with a pro-B-cell (CD34⁺, CD19⁺, CD10⁻) leukemia with the more mature pre-B-cell (CD34⁻, CD19⁺, Cylgμ⁺) leukemia within the group of samples carrying t(11;19), t(9;11) or wild-type *MLL* genes. Infant ALL samples carrying t(4;11) were excluded because they nearly all represented pro-B-cell leukemias and thus would strongly bias these analyses. No significant differences were found between pro-B and pre-B infant ALLs.

Finally, no significant differences in promoter CpG island methylation could be observed when DNA methylation patterns from infants diagnosed below 6 months of age were compared with those from infants diagnosed between 6 months and 1 year or age.

Taken together these comparisons indicate that the DNA methylation patterns as presented in this study represent leukemia-specific profiles that are unlikely to be influenced by differential CpG island methylation induced by non-leukemic factors.

Supplemental Methods

Differential methylation hybridization using CpG island microarrays

Differential methylation hybridization (DMH) was performed as described by Yan et al.² Briefly, 0.5 ug of high-quality genomic DNA was digested using the restriction enzyme *MseI* (New England Biolabs). Next, unphosphorylated linkers were ligated to the digested fragments, which were then sequentially digested with two methylation-sensitive restriction enzymes (*BstUI* and *HpaII*, New England Biolabs). These second digestions eliminate unmethylated fragments, enriching the samples for methylated sequences. The digested linker-ligated DNA was then used as a template for polymerase chain reaction (PCR) amplification (20 cycles of 97°C for 1 min and 72°C for 3 min, final extension at 72°C for 10 min), generating methylated amplicons. Using the BioPrime Array-CGH Genomic Labeling kit (Invitrogen, Carlsbad, USA), amino-allyl dUTPs were incorporated into the amplicons, allowing the amplicons to be labeled with the fluorescent dyes Cy5 (patient samples) and Cy3 (common reference samples). The common reference for all samples was a commercially available genomic DNA pool derived from five healthy males and five healthy females (Promega Benelux BV, Leiden, the Netherlands). Hybridization and washing were performed according to DeRisi³ for the custom 9K microarray chips, and for the 244K microarray chips (Agilent), the Agilent ChIP-on-chip protocol version 9.0 was used. Hybridized slides were scanned with a 2565 AA DNA microarray scanner (Agilent Technologies), and the acquired images were analyzed using the GenePix Pro 6.0 software or the Agilent Feature Extraction 9.5.3 software.

Statistical analyses and software

Analyses of differential methylation

Differentially methylated CpG islands were identified using linear models for microarray data (limma).⁴ These models use an empirical Bayes approach to moderate the standard errors of the estimated standardized log-fold changes by borrowing information across genes. This results in more stable assumptions and enhanced power, especially when group sizes are small.⁵

Permutation testing

As a measure of internal validation for the subtype-specific methylation signatures, permutation testing (global test)⁶ was applied to evaluate whether genes were significantly associated with a certain type of *MLL* translocation. Therefore the tendency of repeated re-assignment of individual samples to their original cluster was assessed. As a default 10.000 permutations were used to calculate p-values.

Gene expression

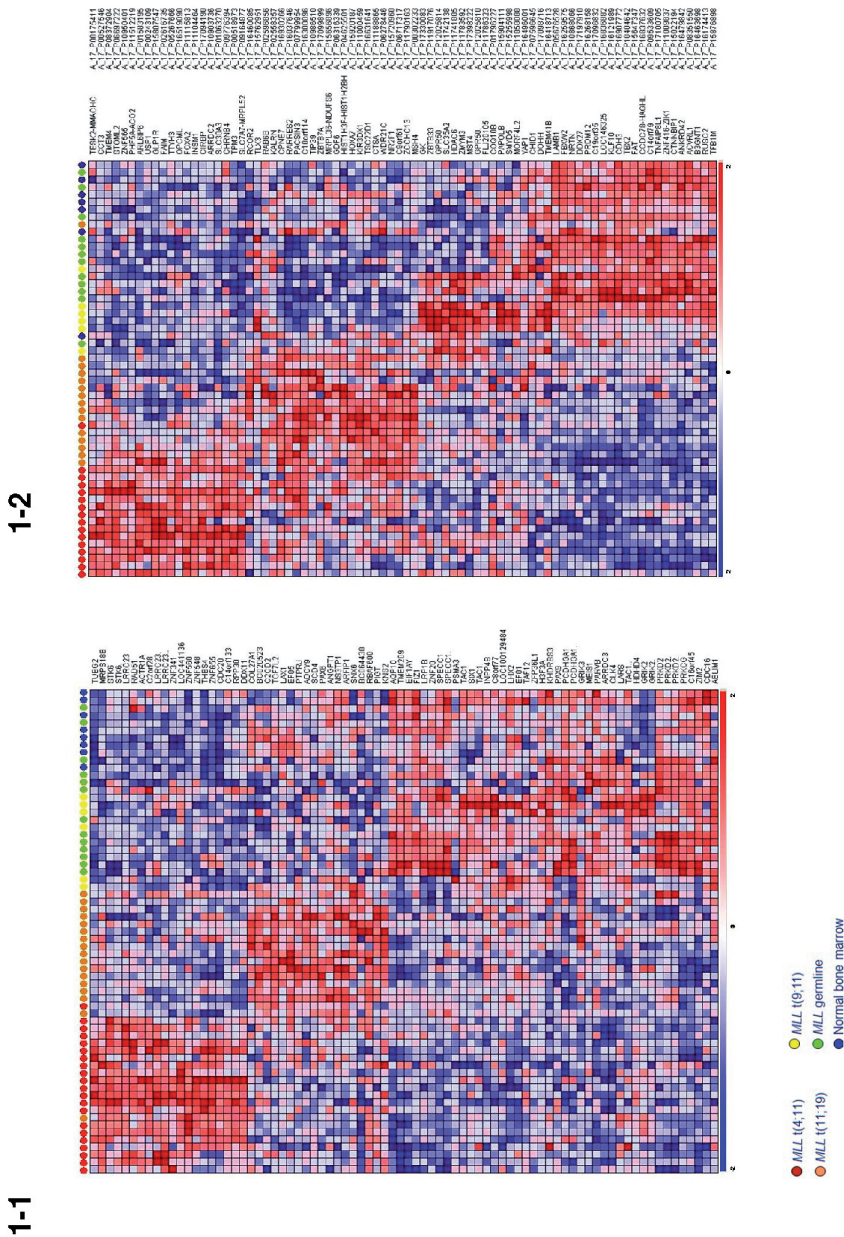
Gene expression values were calculated using Affymetrix Microarray Analysis Suite® (MAS) 5.0.2 software. Unscaled expression signals were normalized using variance stabilization and normalization (vs_n).⁷

Risk stratification according to the INTERFANT-06 treatment protocol

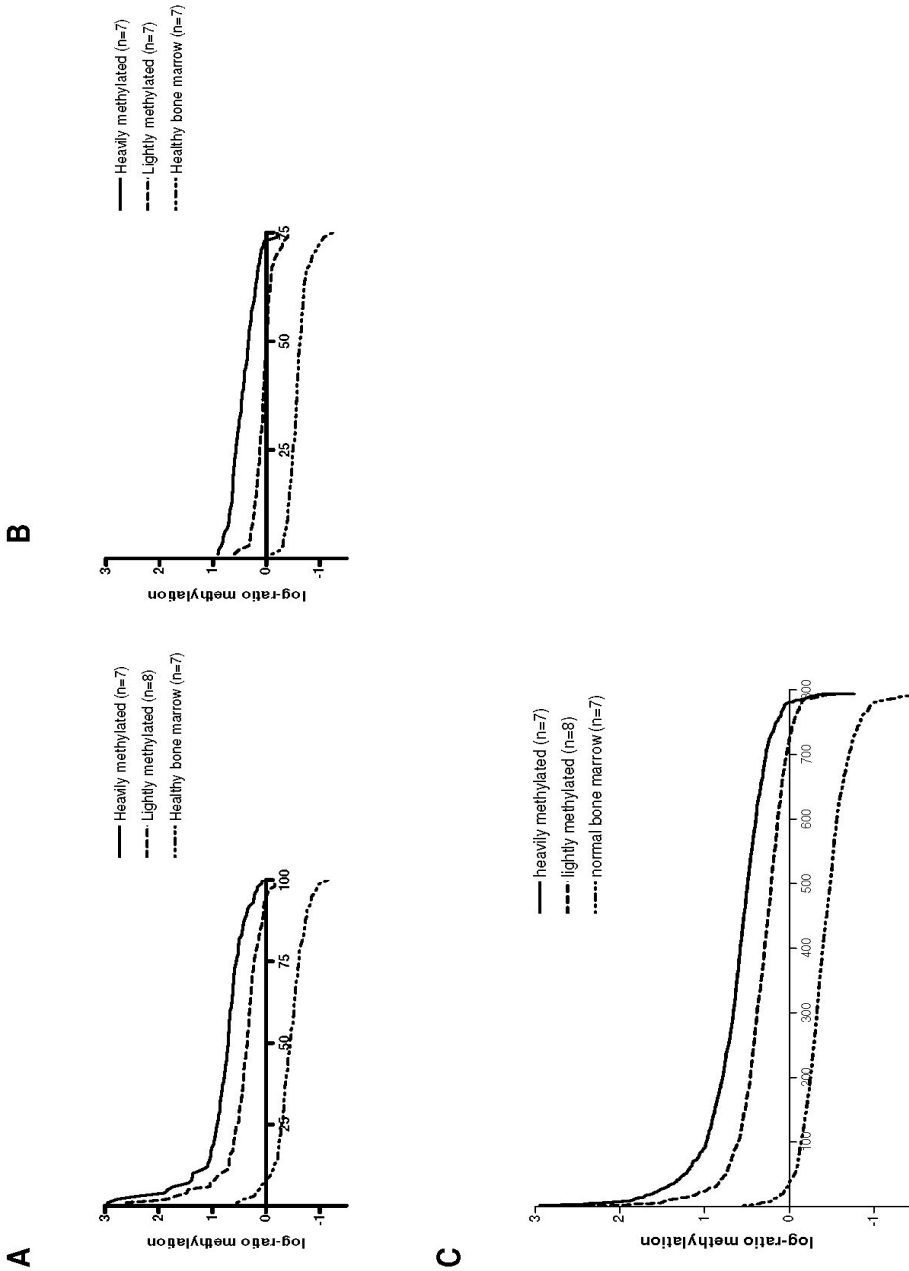
For the one-step Cox model patients were stratified as high-risk when aged <6 months (183 days) and displaying white blood cell counts (WBC) >300 × 10⁹/L at diagnosis. Alternatively, patients were classified as medium-risk when aged >6 months at presentation, or aged <6 months with WBC <300 × 10⁹ / L.

(Statistical) software

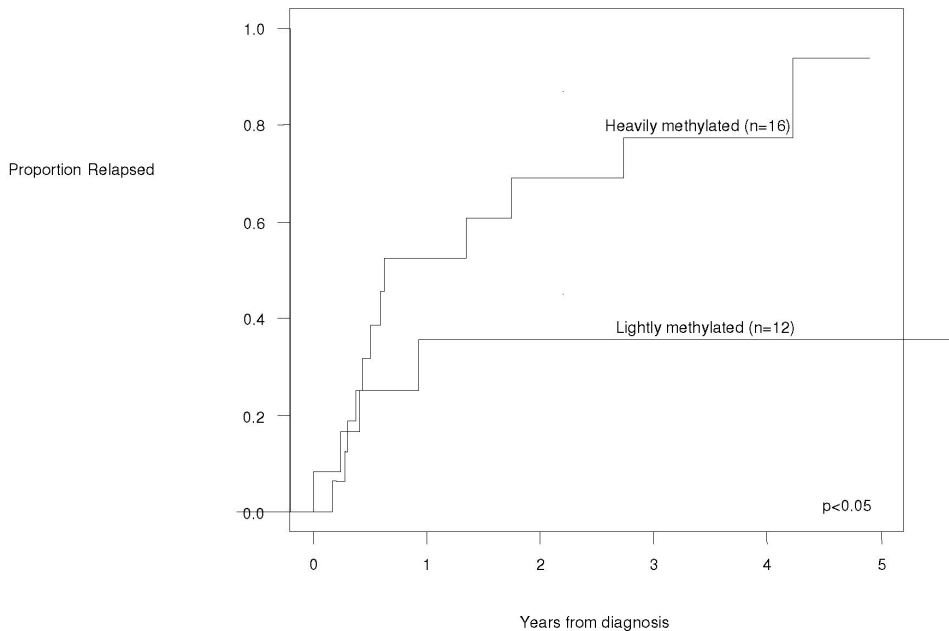
We used the statistical environment R (R Development Core Team, 2007) version 2.6.1 for the microarray analyses, including packages limma,⁴ global test,⁶ and vs_n.⁷ Heatmaps were generated in GenePattern version 3.1.1 (Broad Institute, MIT, <http://genepattern.broad.mit.edu>), and PCA plots were produced using Genemath XT 1.6.1. software (Applied Maths, Inc., Austin TX, USA). SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for computation of survival statistics.



Supplementary Figure S1 - Heatmaps showing the top 20 most significantly hypermethylated probes for each infant ALL subtype (compared with the other subgroups). Additionally, normal bone marrow samples were added to the heatmaps. Columns represent patient samples and rows represent genes. Relative DNA methylation levels are then shown in red (high) and blue (low). Genes and samples were ordered using hierarchical cluster analysis (Euclidean distance, complete linkage) and gene identifiers are listed at the right. Samples are color-coded to indicate the genetic subtype of infant ALL: t(4;11) (red), t(11;19) (orange), t(9;11) (yellow), infant ALL with wild-type *MLL* genes (green) and normal bone marrow (blue). **1-1**. Data are shown for the 9K chip and **1-2**. the 244K chip (Agilent). Due to restricted availability of patient material, Agilent DNA methylation profiles were generated for 49 infant ALL patients (t(4;11)-positive (n=16), t(11;19)-positive (n=15), t(9;11)-positive (n=6), and *MLL* wild-type (n=12)) and 7 normal bone marrows. The 20 most significantly hypermethylated genes shared by t(9;11)-positive and wild-type *MLL* samples were also methylated in normal bone marrow samples, implying that these genes are normally methylated in healthy hematopoietic cells.



Supplementary Figure S2 - Semi-quantitative representation of the degree of methylation. Visualization of the normalized and sorted log-ratios (patient signal divided by common reference signal) of methylation (Y-axis) for the most significantly hypermethylated genes (X-axis) in A. t(4;11) and B. t(11;19)-positive infant ALL patients. Different patient groups represent either heavily or lightly methylated clusters (figure 3). C. Visualization of the normalized and sorted log-ratios of methylation for all significantly hypermethylated genes in t(4;11)-positive infant ALL patients, compared with normal bone marrow samples. These data demonstrate that the observed separation of two patient groups with varying degrees of methylation as observed among both t(4;11) and t(11;19)-positive infant ALL, is not restricted to the most significantly methylated genes. As shown here, these differences remain present when all hypermethylated probes (n=794) are used for t(4;11) positive patients.



Supplementary Figure S3 - Relapse-free survival in t(4;11)-positive and t(11;19)-positive infant ALL patients divided by the degree of DNA methylation (figure 2S), based on hierarchical clustering as shown in figure 3. t(4;11)-positive and t(11;19)-positive patients were combined. Risk of relapse is presented on the Y-axis and the time of follow-up (in years) is presented on the X-axis. The p-value is from a log-rank test. Risk of relapse is significantly increased in the heavily methylated subgroup of infant ALL.

Supplementary Table S1. Patient characteristics. Available at: <http://bloodjournal.hematologylibrary.org/content/114/27/5490/suppl/DC1>

Supplementary Table S2 - The top 20 of genes most significantly hypermethylated in each genetic subtype of infant ALL (compared with the other subgroups) are listed in order of decreasing statistical significance. Data are shown for the 9K CpG island microarray chip. The Gene ID, Gene Name, log- fold change (logFC), p-value, and p-value adjusted for multiple testing (adj.P.Val) are listed (limma analyses). Results of permutation testing are added for each subgroup (global test). This 9K chip is a custom spotted microarray, therefore no official probe IDs are included. Available at: <http://bloodjournal.hematologylibrary.org/content/114/27/5490/suppl/DC1>

Supplementary Table S3 - The top 20 of genes most significantly hypermethylated in each genetic subtype of infant ALL (compared with the other subgroups) are listed in order of decreasing statistical significance. Data are shown for the 244K CpG island microarray chip. The probe ID (Agilent ProbeName), Entrez Gene ID, Gene Name, log- fold change (logFC), p-value, and p-value adjusted for multiple testing (adj.P.Val) are shown (limma analyses). Results of permutation testing are added for each subgroup (global test). Available at: <http://bloodjournal.hematologylibrary.org/content/114/27/5490/suppl/DC1>

Supplementary Table S4. The top 100 of genes most significantly hypermethylated in t(4;11)-positive infant ALL (compared with normal bone marrow) are listed in order of decreasing statistical significance. The probe ID (Agilent ProbeName), Entrez Gene ID, Gene Name, log- fold change (logFC), p-value, and p-value adjusted for multiple testing (adj.P.Val) are shown (limma analyses). The corresponding Affymetrix probe ID from the gene expression platform (Affymetrix ProbeName) is added for these genes as well as the adjusted p-value for differential gene expression (adj.P.Val expression) (limma model). Available at: <http://bloodjournal.hematologylibrary.org/content/114/27/5490/suppl/DC1>

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