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Impact of aberrant DNA methylation patterns including CYP1B1 methylation in adolescents and young adults with acute lymphocytic leukemia

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Abstract

Introduction—Aberrant promoter DNA methylation is a well-described mechanism of leukemogenesis within hematologic malignancies, including acute lymphoblastic leukemia (ALL). However, the importance of methylation patterns among the adolescent and young adult (AYA) ALL population has not been well established.

Methods—DNA methylation of 18 candidate genes in 33 AYA ALL patients was analyzed at diagnosis and during treatment, to evaluate the frequency and clinical relevance of aberrant methylation in an AYA population treated on a uniform therapeutic regimen.

Results—Of 16 informative genes, there was a median of 6 methylated genes per AYA ALL patient. Correlations were identified between increasing number of methylated genes with male sex (p=0.04), increased white blood cell (WBC) count (p=0.04) and increased bone-marrow blast percentage (p=0.04). Increasing age was associated with EPHA5 methylation (p=0.05). Overall, patients experienced favorable outcomes with median survival that was not reached. On univariate analysis, methylation of CYP1B1 was associated with worse overall survival (HR 10.7, 95% CI 1.3–87.6, p=0.03), disease-free survival (HR 3.7, 95% CI 1.1–9.2, p=0.04) and correlated with decreased CYP1B1 gene expression.

Conclusions—A significant incidence of methylation within the AYA ALL population was identified, with increased methylation associated with distinct clinicopathologic features including male gender and elevated WBC count. Our results suggest aberrant methylation among AYA patients is frequent, and may provide a common pathogenic mechanism. The inferior outcome identified with methylation of the cytochrome p450 gene CYP1B1, an enzyme involved in drug metabolism and steroid synthesis, warrants further investigation.

Keywords

acute lymphoblastic leukemia; DNA methylation; adolescent young adult

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Introduction

Acute lymphoblastic leukemia (ALL) is a heterogeneous disease affecting both pediatric and adult patient populations. With the advent of intensified combination chemotherapy regimens, children with ALL now enjoy a greater than 80% disease-free long-term survival[1–3], whereas adults unfortunately continue to experience long-term survival rates closer to 40–50%[4–6]. In addition to age, several factors have been associated with ALL clinical outcome such as cytogenetic aberrations including mixed lineage leukemia (MLL) rearrangements and the Philadelphia (Ph) chromosome, specific leukemia-associated immunophenotypes, patient performance status, and presenting white blood cell count[37–10].

In ALL, the adolescent and young adult (AYA) population comprises a unique cohort of patients. These patients may be treated by either pediatric or adult oncologists, and treatment regimens have historically varied based on the training background of the treating physician. The AYA cohort has received much recent attention, as retrospective reviews have suggested that AYA patients have intermediate survival compared to children or adults, but that AYA patients treated on pediatric regimens have improved outcome compared to patients treated on adult protocols[11–18]. Many different factors for the differences in outcome in AYA patients have been proposed, including treatment intensity, psychosocial factors, and variations in the disease biology itself including cytogenetic and immunophenotypic variations.[19–22]

In addition to standard prognostic factors in ALL, the importance of recurrent abnormalities within epigenetic patterning, such as aberrant promoter DNA methylation, is also of interest with respect to prognostication within ALL and within the AYA cohort in particular. DNA methylation at promoter cytosine residues within conserved CpG islands is an essential component of regulated gene expression, and aberrant methylation can lead to gene silencing of critical genes and has been a well described phenomenon in hematologic malignancies including ALL[23–26]. Within ALL, abnormal methylation of various genes appears to cluster within specific molecular functional pathways, including Wnt-related genes, the glucocorticoid pathway, Ephrin family kinases, and mitogen-activated protein kinase (MAPK) pathways[27–29].

Correlations between the presence and number of detectable methylated genes in ALL have been associated with various clinicopathologic features and in some studies have been linked with poor prognosis. For example, increased methylation of the p73/p15/p57KIP2 cell cycle regulatory pathway members was found to occur rarely in pediatrics but frequently in adults, and was associated with worse outcome[30]. Within the pediatric population, published reports suggest a correlation between methylation and prognosis[3132], with increased methylation frequencies particularly identified in T-cell phenotype ALL patients[33], and a globally hyper-methylated phenotype associated with infants with mixed lineage leukemia (MLL) rearrangements, a high-risk pediatric group with particularly poor outcome[34].

There is preliminary information to suggest that prognostic differences between and within age groups could be related in part to differences in methylation patterns, and that identification of aberrantly methylated subsets may improve risk-based treatment decisions. In light of the differences in ALL survival between children, AYAs and adults, we have investigated DNA methylation patterns of 16 informative genes in ALL patients treated on a uniform treatment regimen, to evaluate the frequency and clinical relevance of aberrant methylation in an AYA population.

Methods

Patients and Treatment

Primary DNA samples from 33 adolescent and young adult patients with newly diagnosed ALL, age 13 to 37, who received treatment on our augmented Berlin-Frankfurt-Munster (BFM) therapeutic protocol at the University of Texas M.D. Anderson Cancer Center were enrolled from 2006 to 2009. Clinical information of all patients in this study is provided in Table 1, including patient age, sex, ALL subtype, white blood cell (WBC) count at diagnosis, platelet (PLT) count at diagnosis, karyotype, LDH, bone marrow percentage, and overall patient outcome. All patients were negative for the Philadelphia chromosome rearrangement by virtue of a negative BCR/ABL quantitative PCR. In addition to the pretreatment samples, there were 24 patients with at least one repeat sample obtained during induction treatment that were analyzed for changes in methylation over time. All samples were collected following institutional guidelines with informed consent in accordance with the Declaration of Helsinki. Evaluation of promoter methylation levels was a pre-specified aim of the augmented BFM protocol.

Gene Selection, DNA Extraction and Methylation Analysis

A total of 18 genes, including those within the Notch pathway (Notch3, Jag1), Ephrin pathway (EPHA2, EPHA4 – EPHA7, EPHA10), and glucocorticoid pathway (HSPAL4, PTPRM, CYP1B1) were analyzed. The selected genes were chosen based on our results of a previous genome-wide analysis, identifying 26 candidate genes that were subsequently validated and confirmed as hyper-methylated in ALL cell lines.[27] Genes with a high likelihood of importance in ALL pathogenesis by their location in top canonical pathways such as cellular growth and proliferation, gene expression and cell death, using Ingenuity Pathway Analysis (IPA) software were analyzed. Candidate genes from top canonical pathways such as the Ephrin receptor signaling pathway were similarly chosen. Our methods of DNA extraction, bisulfite modification and methylation analysis have been previously described[263536] and the specific primers used are available in Supplementary Table 1. GAPDH was used as the reference control gene. Evaluation of promoter methylation was performed by PCR amplification of peripheral blood followed by pyrosequencing of CpG sites within target genes. A candidate gene was considered methylated if the methylation density was ≥10% as previously established[37].

Statistical Analysis

Associations between methylation of single genes or groups of genes were assessed using the Spearman rank correlation coefficient. Categorical variables were compared using the χ^2 or Fisher's exact test, and continuous variables using the Wilcoxon rank-sum test. The number of methylated genes was analyzed as both a continuous and categorical variable. When indicated, *p*-values to indicate statistical significance were adjusted for testing of multiple comparisons using the Bonferonni adjustment.

Disease-free survival (DFS) and overall survival (OS) were based on the Kaplan-Meier method, with differences tested using the log-rank test. OS was measured as the time from ALL diagnosis to death or date of last follow-up (censored). Disease-free survival was defined as the time from complete remission (CR) to treatment failure including relapse, death, or date at last follow-up (censored). All analyses were performed using STATA software, version 12.0 (College Station, TX).

Results

Clinical and disease-specific patient characteristics of samples are detailed in Table 1. Methylation density results of each of the 18 genes analyzed (TP73, SKP2, HOXD13, Olig2, Jag1, Notch3, EPHA2, EPHA4, EPHA5, EPHA6, EPHA7, EPHA10, EPHB3, ENFA5, GNA14, HSPAL4, PTPRM and CYP1B1) are presented in Table 2. Of note, SKP2 was methylated in all analyzed samples and TP73 in all but three samples, with minimal variation in methylation levels of these two genes, and thus TP73 and SKP2 were not evaluated for further correlations given the near-universal methylation observed in our AYA cohort. All other genes were methylated in a proportion of patients; the frequency of methylated genes ranged from EPHB3 which was methylated in only 2 samples (6%), to EPHA5 which was methylated in 30 (91%) of samples (Table 2). Seven genes (Olig2, EPHA5, EPHA10, ENFA5, GNA14, HSPAL4 and PTPRM) were methylated in more than 50% of the samples analyzed.

Using a standard methylation cut-point of 10% or more, only one sample (3%) was without any observed methylation of the n=16 genes. 9 (27%) had methylation of 1–4 genes, 11 samples (33%) had methylation of 5–9 genes, and 12 (36%) had methylation of 10 or more genes (Table 3) suggestive of a “hypermethylated phenotype”. In a Spearman’s correlation matrix assessment using the Bonferroni adjustment for multiple comparisons, methylation of certain genes was found to be significantly associated with the presence of methylation of other analyzed genes (Table 4). For instance, methylation of Notch3 was associated with methylation of Jag1 (p=0.009), EPHA10 (p=0.006) and GNA14 (p=0.02).

There was no association with the number of methylated genes and either ALL subtype, EGIL classification or patient karyotype. Specifically, when analyzed by ALL subtype, the 27 patients with precursor-B (pre-B) ALL had a median of 7 methylated genes, and the 4 patients with pre-T ALL had a median number of 6 methylated genes. The one patient with pre-B lymphoblastic lymphoma (LL) had 5 methylated genes, and the one patient with pre-T LL had one methylated gene.

There was no relationship identified between the number of methylated genes with patient age, platelet count at diagnosis, or presenting LDH count. Significant correlations were identified between increasing number of methylated genes and male sex (p=0.02), increased white blood cell count at diagnosis (p=0.04), and an increased BM blast percentage at diagnosis (p=0.04) (Figure 2).

All 33 patients achieved an initial CR on therapy. Overall, patients experienced favorable outcomes with a median survival that was not reached. On univariate survival analysis, the presence of methylated CYP1B1 was associated with worse OS (HR 10.7, 95% CI 1.3 – 87.6, p=0.03) and DFS (HR 3.7, 95% CI 1.1 – 9.2, p=0.04) which retained significance in multivariate analysis (HR 4.4, 95% CI 1.1–18.3, p=0.04). To evaluate CYP1B1 further, we additionally performed quantitative PCR of CYP1B1 in n=27 AYA subjects with remaining samples, as well as in six normal controls. Compared to normal controls, 21 of 27 AYA ALL patients had lower relative CYP1B1 gene expression. We furthermore identified a significant inverse association (Spearman’s rho = −0.54, p = 0.005) between increased CYP1B1 methylation and decreased CYP1B1 expression (Figure 5). In addition, a non-significant trend towards worse survival was identified in patients with methylated ENFA5 (HR 5.5, 95% CI 0.67 – 44.5, p=0.06). No other relationships were identified between single genes or methylation patterns on patient outcome. Specifically, the number of methylated genes did not correlate with prognosis.

There were 24 patients who had repeat samples obtained for at least one additional time point in addition to the pretreatment baseline sample (repeat samples were obtained from day 28 – to day 110 of therapy). In samples without gene methylation at diagnosis, no significant increase in methylation over time was noted with the exception of Jag1, which experienced increased methylation during the first thirty days of therapy (Figure 3). Importantly, in samples with methylated pretreatment genes, the average methylation score was noted to decrease during the first thirty days of therapy. The change in methylation at the time of relapse was not able to be determined, as only one patient relapsed during the first 150 days; this patient had 10 genes methylated at diagnosis (HOXD13, Olig2, Jag1, EPHA2, EPHA5, EPHA6, EPHA10, GNA14, PTPRM, and CYP1B1).

Finally, we divided our AYA cohort into a “pediatric cohort” aged 13–19 and an “adult cohort” aged 20–37, to determine whether methylation characteristics were different within these two groups. There were no identified differences in important clinical or biologic characteristics between these age groups (Supplementary Table 2). The adult cohort was more likely to be methylated at EPHA4 (p=0.05); this did not impact patient outcome. Otherwise, no statistically significant differences were observed in terms of gene specific methylation between both groups.

Discussion

The adolescent and young adult population comprises a unique cohort among ALL patients, with outcomes that historically fall in-between the published pediatric and adult ALL outcomes. Multiple different factors have been purported to influence outcomes in AYA patients, however the role of DNA promoter methylation within this population has not been systematically investigated. In our study, we evaluate the methylation profile of 33 AYA patients treated on a uniform protocol, the change in methylation during initial treatment, and investigate the role of methylation on various clinical and biologic ALL characteristics, as well as overall patient outcome.

Previous studies have identified that increased methylation is associated with age and worse overall survival[31323839], while other studies have not identified these correlations. Our analysis identified an increased rate of methylation of EPHA4 in the adult cohort compared to our pediatric cohort within our AYA population, but otherwise we were unable to detect differences between gene-specific methylation based on patient age. However, it is important to note that some of the genes analyzed in our study are different than those previously evaluated, with inclusion of several novel genes in our study due to their relative significance using IPA software, and exclusion of other genes found to be methylated in ALL in prior publications. Interestingly, the p53 homologue p73 and the MYC target gene SKP2 were methylated in nearly all and all AYA patients within our cohort, respectively. Previous studies have identified a low incidence of p73 methylation in pediatric ALL, with an increased frequency of p73 methylation (>20% incidence) in adults with ALL.[2638] While absent or limited expression of p73 as a result of promoter methylation has been a well described phenomena in hematologic malignancies,[4041] universal methylation has not been previously described in other leukemia cohorts to date.

A significant adverse prognosis was identified in patients with methylated CYP1B1, a gene which belongs to the cytochrome p450 enzymatic superfamily and is thought to catalyze reactions involved in drug metabolism and steroid synthesis.[42] This novel finding was supported by the additional recognition that increased CYP1B1 methylation inversely correlates with CYP1B1 gene expression, suggesting down-regulation of this gene may decrease glucocorticoid efficacy. The association of CYP1B1 with patient response to glucocorticoids during leukemia treatment, as well as drug metabolism and therapy-related

toxicity will be essential future relationships to investigate. While a relationship between CYP1B1 and acute lymphoblastic leukemia has not been previously identified and will require validation in an independent cohort, overexpression of other genes involved in glucocorticoid resistance (i.e. the S100 family members including S100A8 and S100A9) have been recently identified to predict for steroid-resistance in ALL patients, and therapeutic inhibition of this pathway is currently under evaluation.[43] In the methylation correlation matrix analysis, methylation of CYP1B1 was significantly associated with EPHA10 methylation. The significance of this correlation is not clear, however the prognostic significance of CYP1B1 was not dependent on methylation status of EPHA10.

We additionally identified several clinical-biological associations that have not been previously well described, including a correlation between increased methylation scores with male gender, elevated white blood cell count at diagnosis, and an increased bone marrow blast percentage at diagnosis. Whether these findings are due to chance or represent true findings will require additional testing of larger datasets. Given that our study utilized peripheral blood of patients for methylation analysis, it is also possible that these correlations of “tumor burden” with methylation may be related to the dilution of methylated DNA in the periphery. Of note, the majority of the published literature on methylation patterns within ALL exclusively use samples with >80–90% bone marrow blasts, so this relationship may have been masked in previous studies[3244].

The augmented BFM combination chemotherapy regimen in our AYA population provided excellent outcomes, with a median OS and DFS that were not reached. This and additional preliminary data from our ongoing trial helps support the notion that AYA patients treated with intensive pediatric regimens can achieve similarly favorable outcomes as younger pediatric patients. The exceptional survival in this cohort could actually have proven to be a limitation in our study, as the lack of “events” may have prevented the detection of differences in patient outcome, based on relevant methylation characteristics.

Importantly, as opposed to other recurrent genetic changes in leukemias such as mutations and cytogenetic abnormalities, DNA methylation is an epigenetic event which does not alter the underlying genetic code, and is thus potentially reversible with therapy. There is only limited experience with epigenetic therapy in ALL, but preliminary studies have identified both histone deacetylase inhibitors and DNA methyltransferase inhibitors can lead to re-expression of genes shown to be methylated and silenced in ALL[45]. Further evaluation of epigenetic therapy for ALL is warranted, although it is important to note that in our analysis of methylation levels over time, we observed that the majority of patients treated with intensive ALL therapy obtain “reversal” of methylation with standard ALL therapy.

In summary, we identified a significant presence of methylated genes within the AYA ALL population, suggesting a potential epigenetic role of promoter DNA methylation in leukemogenesis. Increased methylation of evaluated genes were associated with distinct clinical and pathologic ALL features including male gender and elevated white blood cell count and bone marrow blast percentage at diagnosis. Finally, given the prognostic importance of the cytochrome p450 gene CYP1B1 in our cohort, further evaluation of CYP1B1 as a prognostic and/or therapeutic marker is justified.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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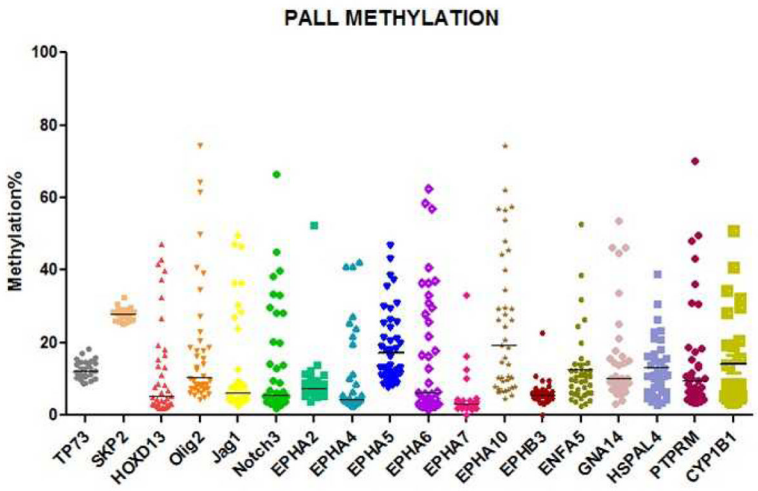

Figure 1.
Baseline methylation levels of selected genes (as %) in patient samples (n=33)

Figure 2a

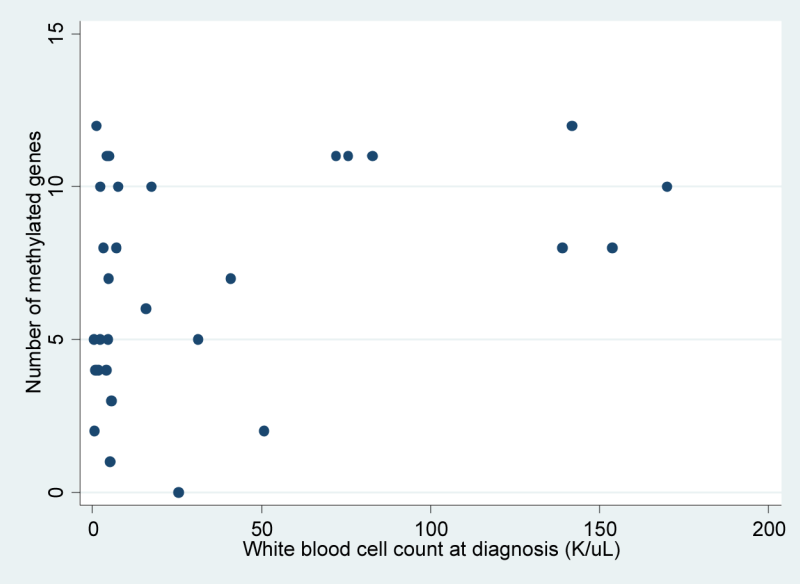

Figure 2b

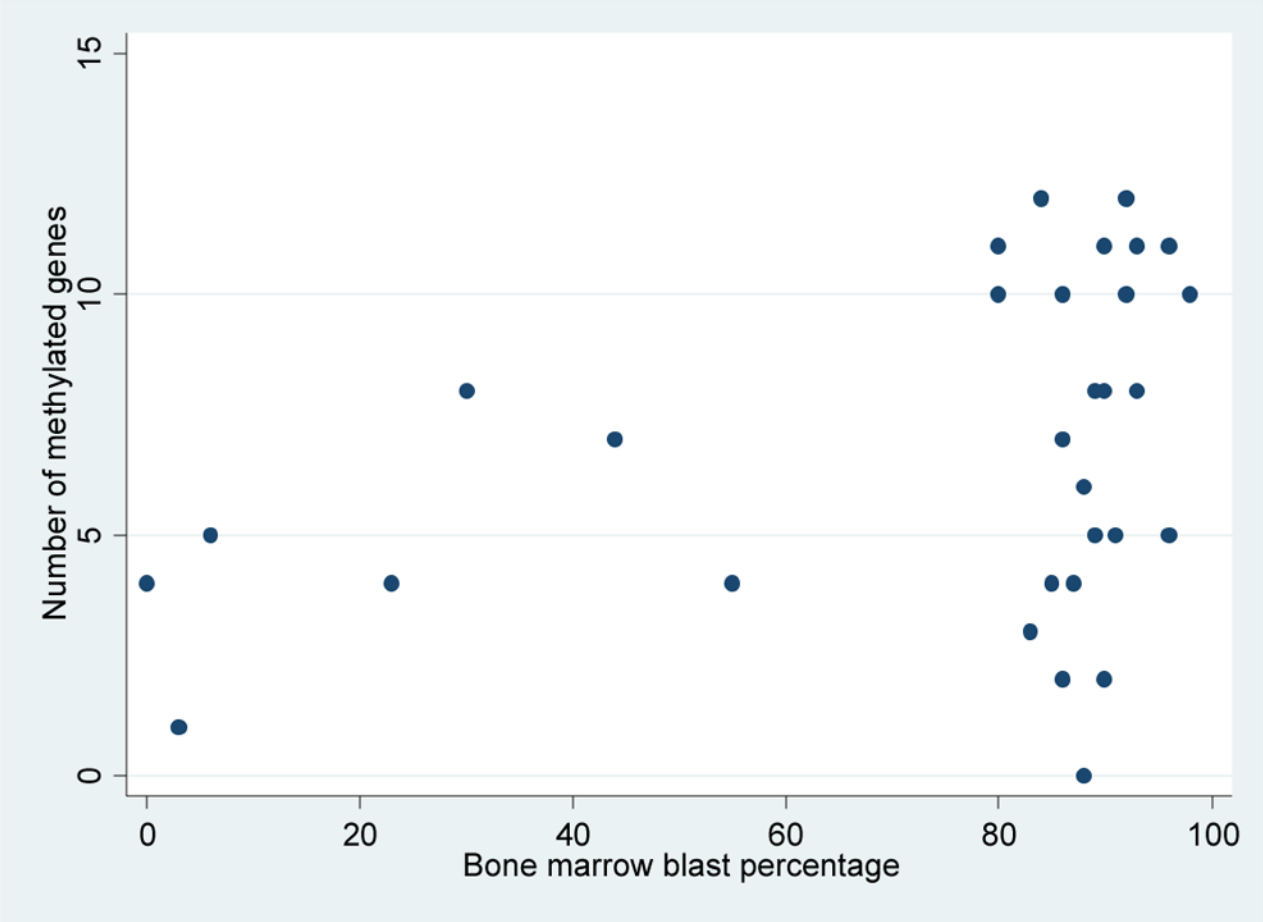

Figure 2.

Figure 2a. Relationship of white blood cell count at diagnosis and number of methylated genes per sample at diagnosis.

Figure 2b. Relationship of bone marrow blast percentage at diagnosis and number of methylated genes per sample at diagnosis.

Figure 3a

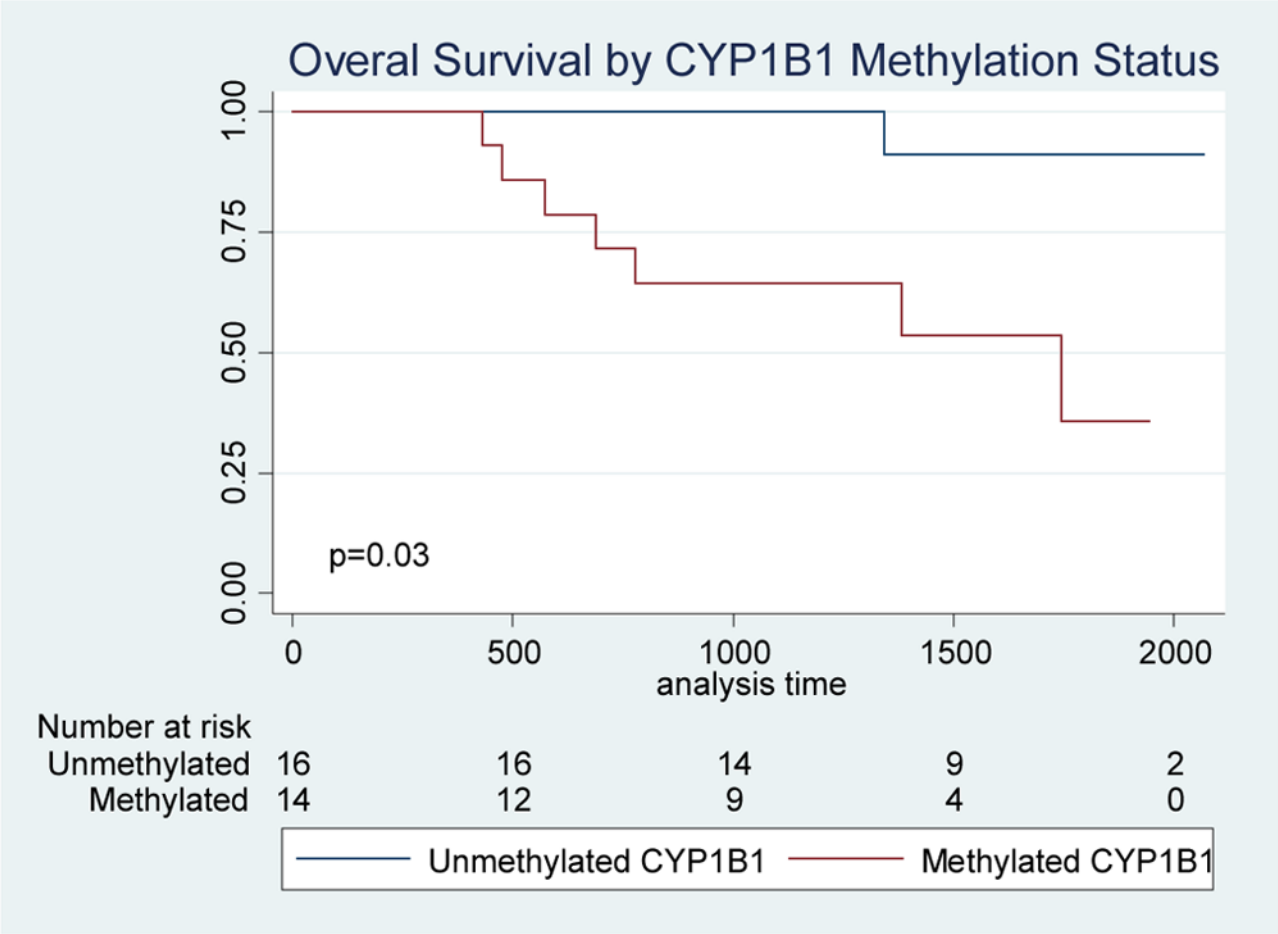

Figure 3b

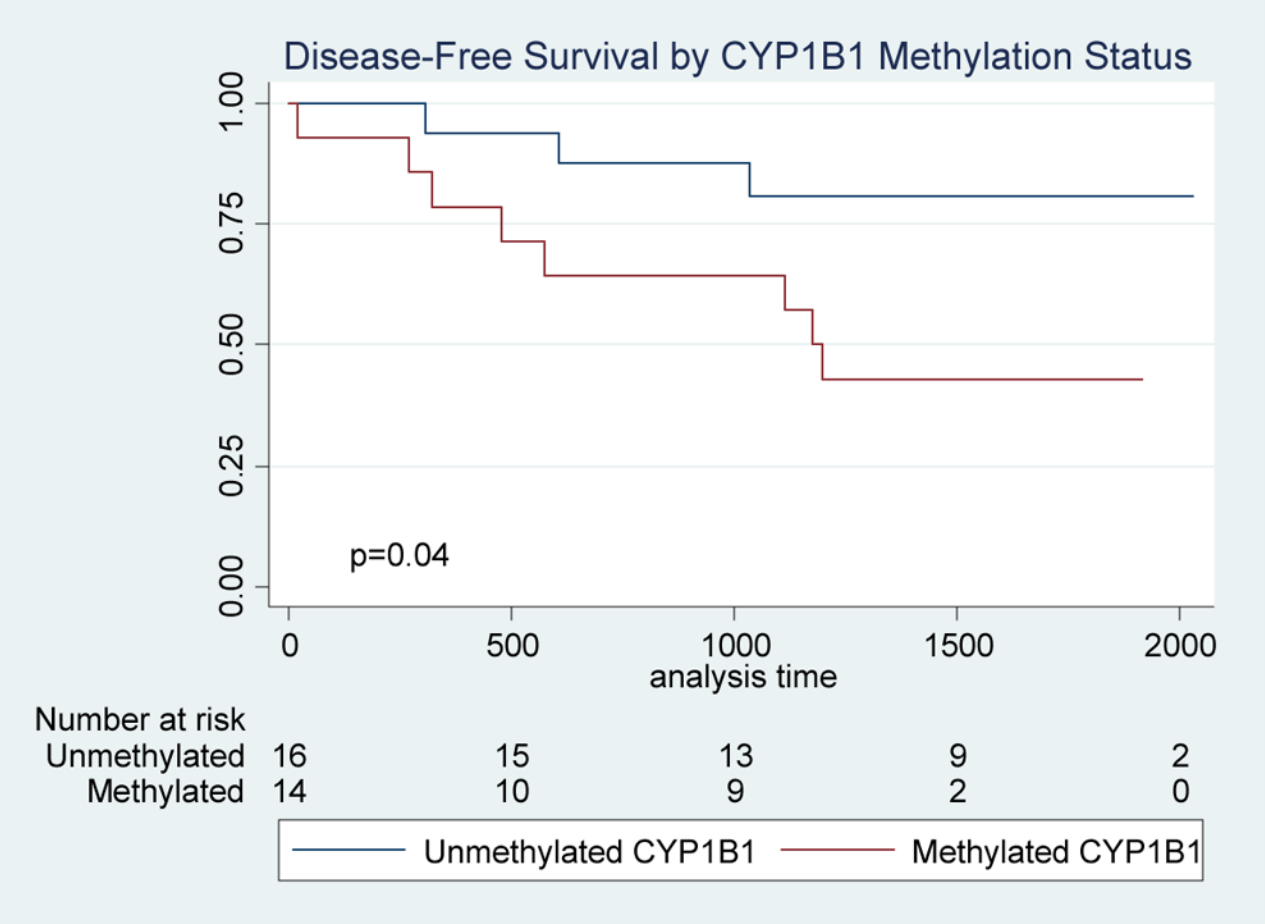

Figure 3.
Figure 3a. Overall Survival by CYP1B1 Methylation Status
Figure 3b. Disease-Free Survival by CYP1B1 Methylation Status

Figure 4a

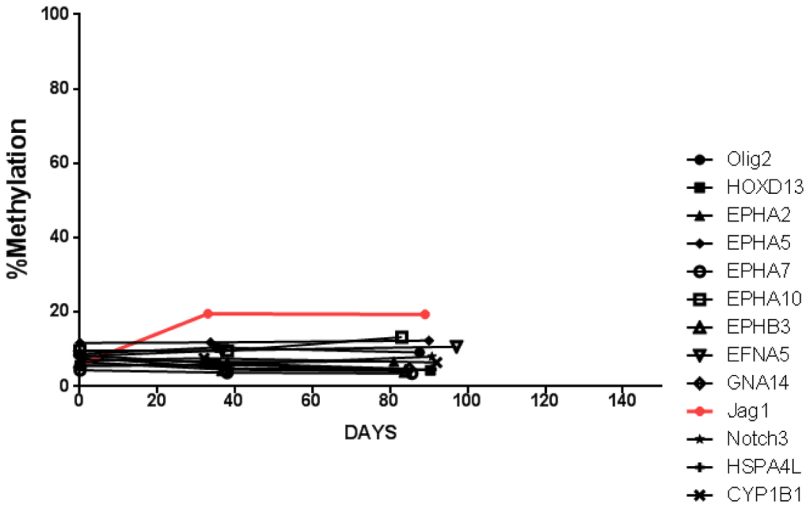

Figure 4b

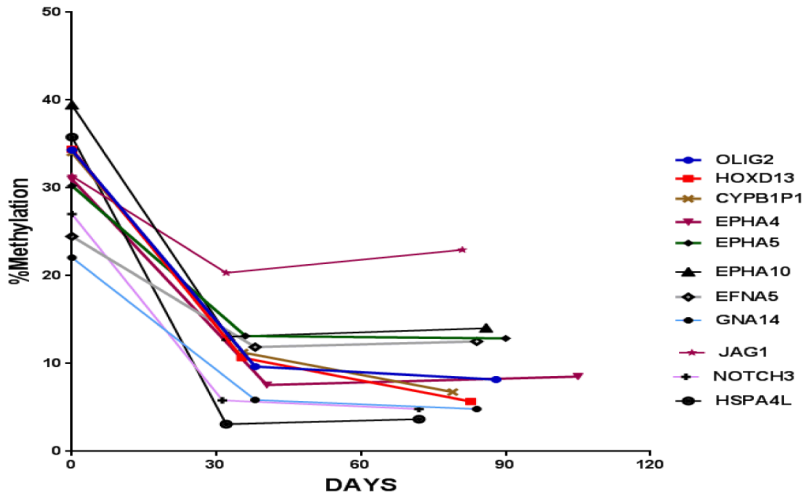

Figure 4.

Figure 4a. Change in methylation over time in samples with unmethylated gene status at diagnosis

Figure 4b. Change in methylation over time in samples with methylated gene status at diagnosis

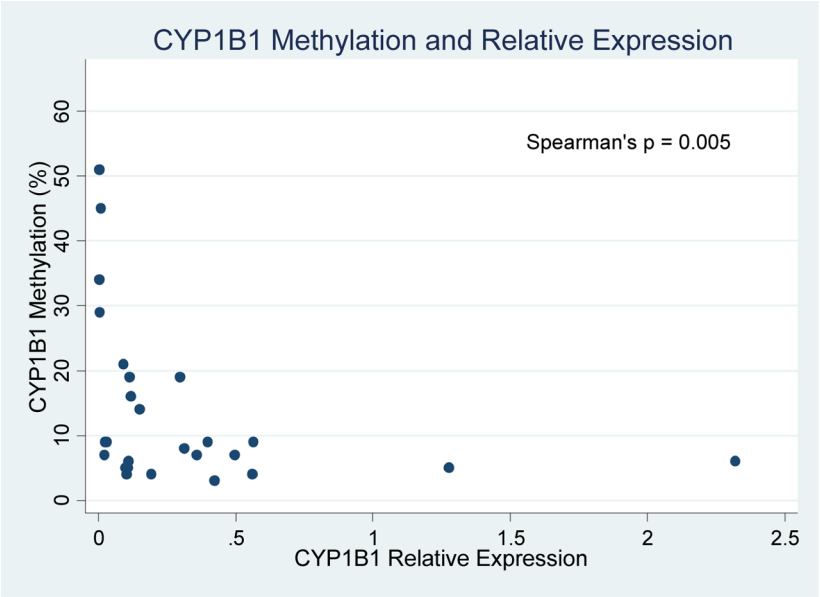

Figure 5.
Inverse relation of CYP1B1 methylation % and relative gene expression

Table 1

Cohort Patient Characteristics

	Total Cohort (n=33)
Age (yrs)	
Median	20
Range	13–37
Male Sex	23 (70%)
ALL Subtype	
Pre-B ALL	27 (82%)
Pre-T ALL	4 (12%)
Pre-B LL	1 (3%)
Pre-T LL	1 (3%)
WBC count	
Median	5.2
Range	0.4 – 170.1
PLT count	
Median	61
Range	4 – 274
BM Blast %	
Median	88
Range	0–98
LDH	
Median	1040
Range	284 – 9784
Karyotype	
Diploid	17 (40%)
Hyperdiploid	7 (17%)
Hypodiploid	4 (10%)
Other	6 (14%)
Unavailable	8 (19%)
CR Rate	33 (100%)
Relapse	12 (36%)
Survival	24 (73%)

Table 2

Methylation Densities of Analyzed Genes

	# Samples with gene promoter ≥10% methylated	Median methylation % (range)
TP73	25 (89%)	12% (7–18%)
SKP2	29 (100%)	28% (25–32%)
HOXD13	14 (42%)	8% (2–47%)
Olig2	22 (67%)	13% (4–74%)
Jag1	12 (41%)	8% (3–50%)
Notch3	14 (44%)	8% (3–66%)
EPHA2	6 (18%)	7% (4–52%)
EPHA4	5 (16%)	4% (2–41%)
EPHA5	30 (91%)	18% (8–47%)
EPHA6	12 (41%)	6% (2–58%)
EPHA7	2 (6%)	3% (2–33%)
EPHA10	25 (76%)	21% (5–74%)
EPHB3	2 (6%)	6% (0–11%)
ENFA5	18 (62%)	11% (4–39%)
GNA14	18 (62%)	10% (3–54%)
HSPAL4	19 (70%)	11% (3–39%)
PTPRM	16 (59%)	10% (4–70%)
CYP1B1	14 (47%)	9% (3–66%)

* note not all columns have n=33 samples

Table 3

Number of Methylated Genes per Patient at Diagnosis

# Methylated Genes	Frequency
0	1 (3%)
1	1 (3%)
2	2 (6%)
3	1 (3%)
4	5 (15%)
5	4 (12%)
6	1 (3%)
7	2 (6%)
8	4 (12%)
10	5 (15%)
11	5 (15%)
12	2 (6%)

Table 4

Methylation Correlation Matrix

	HOXD13	Olig2	Jag1	Notch3	EPHA2	EPHA4	EPHA5	EPHA6	EPHA7	EPHA10	EPHB3	ENFA5	GNA14	HSPAL4	PTPRM
HOXD13															
Olig2	0.08														
Jag1	0.29	1.0													
Notch3	0.19	1.0	**0.009**												
EPHA2	1.0	**0.002**	1.0	1.0											
EPHA4	1.0	1.0	1.0	1.0	1.0										
EPHA5	0.54	**0.002**	1.0	1.0	0.36	1.0									
EPHA6	1.0	1.0	1.0	1.0	1.0	**0.004**	1.0								
EPHA7	1.0	**0.04**	1.0	1.0	1.0	1.0	0.8	1.0							
EPHA10	0.21	**0.02**	1.0	**0.006**	1.0	1.0	**0.0001**	1.0	1.0						
EPHB3	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0					
ENFA5	**0.05**	1.0	**0.04**	0.87	1.0	1.0	0.5	1.0	1.0	1.0	1.0				
GNA14	0.91	1.0	1.0	**0.02**	1.0	1.0	1.0	1.0	1.0	**0.01**	1.0	0.20			
HSPAL4	1.0	1.0	1.0	0.32	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.75		
PTPRM	0.86	**0.04**	1.0	1.0	**0.03**	1.0	**0.002**	1.0	0.17	**0.0001**	1.0	1.0	**0.02**	1.0	
CYP1B1	1.0	1.0	1.0	0.09	1.0	1.0	0.16	1.0	1.0	**0.009**	1.0	0.07	0.08	1.0	1.0

* reported values are level of significance using Bonferroni adjustment for multiple comparisons