Integrative Epigenomic Analysis Identifies Biomarkers and Therapeutic Targets in Adult B-Acute Lymphoblastic Leukemia

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ABSTRACT

Genetic lesions such as BCR-ABL1, E2A–PBX1, and MLL rearrangements (MLLr) are associated with unfavorable outcomes in adult B-cell precursor acute lymphoblastic leukemia (B-ALL). Leukemia oncogenes may directly or indirectly disrupt cytosine methylation patterns to mediate the malignant phenotype. We postulated that DNA methylation signatures in these aggressive B-ALLs would point toward disease mechanisms and useful biomarkers and therapeutic targets. We therefore conducted DNA methylation and gene expression profiling on a cohort of 215 adult patients with B-ALL enrolled in a single phase III clinical trial (ECOG E2993) and normal control B cells. In BCR-ABL1-positive B-ALLs, aberrant cytosine methylation patterning centered around a cytokine network defined by hypomethylation and overexpression of IL2RA (CD25). The E2993 trial clinical data showed that CD25 expression was strongly associated with a poor outcome in patients with ALL regardless of BCR-ABL1 status, suggesting CD25 as a novel prognostic biomarker for risk stratification in B-ALLs. In E2A–PBX1-positive B-ALLs, aberrant DNA methylation patterning was strongly associated with direct fusion protein binding as shown by the E2A–PBX1 chromatin immunoprecipitation (ChIP) sequencing (ChIP-seq), suggesting that E2A–PBX1 fusion protein directly remodels the epigenome to impose an aggressive B-ALL phenotype. MLLr B-ALL featured prominent cytosine hypomethylation, which was linked with MLL fusion protein binding, H3K79 dimethylation, and transcriptional upregulation, affecting a set of known and newly identified MLL fusion direct targets with oncogenic activity such as FLT3 and BCL6. Notably, BCL6 blockade or loss of function suppressed proliferation and survival of MLLr leukemia cells, suggesting BCL6-targeted therapy as a new therapeutic strategy for MLLr B-ALLs.

SIGNIFICANCE: We conducted the first integrative epigenomic study in adult B-ALLs, as a correlative study to the ECOG E2993 phase III clinical trial. This study links for the first time the direct actions of oncogenic fusion proteins with disruption of epigenetic regulation mediated by cytosine methylation. We identify a novel clinically actionable biomarker in B-ALLs: IL2RA (CD25), which is linked with BCR-ABL1 and an inflammatory signaling network associated with chemotherapy resistance. We show that BCL6 is a novel MLL fusion protein target that is required to maintain the proliferation and survival of primary human adult MLLr cells and provide the basis for a clinical trial with BCL6 inhibitors for patients with MLLr. Cancer Discov; 2(11): 1–20. © 2012 AACR.

INTRODUCTION

Adult B-cell precursor acute lymphoblastic leukemia (B-ALL) is an aggressive disease with a less than 40% long-term survival rate (1). This relatively poor outcome compared with childhood B-ALLs is partly explained by an increased frequency of high-risk molecular lesions such as BCR-ABL1 (20%–40% in adults vs. 2%–5% in children), MLL rearrangements (MLLr, 10%–20%), and E2A–PBX1 fusions (5%; refs. 1, 2). The molecular mechanisms underlying poor outcome in these adult B-ALLs are only partially understood. However, each of these B-ALL subtypes features distinct and perturbed gene expression profiles as compared with each other and to normal pre-B cells (3–6). A deeper understanding of the mechanisms driving aberrant gene expression as well as improved biomarkers and therapeutic targets are needed to improve risk stratification and therapy.

Transcriptional regulation and hence cellular phenotypes are increasingly understood to be programmed by epigenetic modifications of chromatin (7, 8). Epigenetic information is encoded, in large part, by patterns of cytosine methylation and histone modifications. An increased abundance of cytosine...
methyltransferases (DNMT) perturbs normal hematopoiesis (9). Accordingly, disruption of the function of DNA methylation patterning are epigenetically retained silencing, whereas decreased cytosine methylation may facilitate transcriptional activation. Importantly, perturbations in cytosine methylation patterning are epigenetically retained during cell division, enabling dividing cells to transmit transcriptional programming to their progeny (7, 8). This process allows cells within tissues including tumors to retain their specific phenotypes. Along these lines, DNA methylation patterning has been shown to shift during normal hematopoiesis and is believed to play an essential role in lineage specification (9). In acute leukemia, a number of genetic lesions encode for proteins that can potentially indirectly or directly alter epigenetic regulatory states (16, 17). Hence, combined genetic, transcriptional, and epigenetic profiling studies may provide a mechanistic link between genetically altered proteins, their impact on chromatin, and their associated transcriptional profiles.

Several lines of investigation suggest that epigenetic programming is globally disrupted in B-ALLs. For example, many of the common MLL fusion proteins can recruit the DOT1L histone methyltransferase which dimethylates H3K79, an event that is associated with transcriptional activation and elongation (18-23). Infants with MLL-rearranged ALLs were shown to feature prominent cytosine hypermethylation of many genes and to be susceptible to DNMT inhibitors in vivo (24, 25). Other studies generally in limited numbers of B-ALLs and CpGs identified aberrantly methylated genes in B-ALLs (26-29). Differential cytosine methylation of CpG islands was noted in subtypes of childhood B-ALLs such as those with high hyperdiploidy and t(12;21) (30). To better understand the contribution of aberrant epigenetic gene regulation to the pathogenesis of adult B-ALLs, we conducted integrative genome-wide cytosine methylation and transcriptional profiling of a large cohort of adult patients with B-ALL all enrolled in a single multicenter phase III clinical trial (ECOG E2993). We focused primarily on B-ALL subtypes with poor outcome. When coupled with chromatin immunoprecipitation (ChIP) sequencing (ChIP-seq) of leukemic fusion proteins and histone modifications and computational and functional assays, the resulting DNA methylation profiles provided insight into mechanisms driving aberrant gene expression in adult B-ALLs as well as new biomarkers and therapeutic targets.

RESULTS
Specific Promoter DNA Methylation Patterning in Genetically Defined B-ALL Subtypes

We reasoned that cytosine methylation patterning would provide biologically and clinically significant information about B-ALLs in adult patients. Therefore, we conducted HELP (HpaII tiny fragment enrichment by ligation-mediated PCR) DNA methylation assays (31) on a cohort of 215 newly diagnosed adult patients with B-ALLs with available diagnostic specimens enrolled in the ECOG E2993 multicenter phase III clinical trial (Supplementary Tables S1 and S2 for detailed patient description). Eighty-three of these patients featured BCR-ABL1 translocations, 7 had E2A-PBX1 fusions, and 28 harbored MLL rearrangements. We focused our attention principally on these B-ALLs because of their defined genetic background and association with poor clinical outcome. We also profiled normal pre-B cells (CD19+ and VpreB+) isolated from the bone marrows of 12 healthy adults as normal counterpart for our B-ALL tumor samples (Supplementary Table S2). HELP was conducted using a customized microarray design covering more than 50,000 CpGs annotated to 14,000 gene promoters and our standard quality control and normalization algorithms (31-33). We conducted technical validation using base pair resolution quantitative DNA methylation assessment by MassArray EpiTyper to confirm the accuracy of methylation values derived from HELP arrays. This procedure indicated that our HELP-derived DNA methylation values were highly concordant (correlation coefficient \( r = 0.87 \), Supplementary Fig. S1 and Supplementary Table S3) and that based on the regression line in Supplementary Fig. S1, a log ratio difference (dx) of 1 or 1.5 from HELP data corresponds to a methylation difference of 20% or 30%, respectively, by MassArray. Consistent with previous reports (14, 33), these data confirm that HELP allows DNA methylation to be accurately assessed as a continuous variable.

We next conducted a supervised analysis comparing each of these 3 cytogenetically defined B-ALL subtypes to normal pre-B cells. This procedure identified a total of 1,740 probesets differentially methylated in at least 1 of the 3 B-ALL subtypes as compared with pre-B cell controls with DNA methylation difference >30% (dx > 1.5 by HELP) and adjusted \( P < 0.01 \) [ANOVA followed by Dunnett’s test; (refs. 33, 34) and Benjamin-Hochberg correction (ref. 35)]. These probesets corresponded to 1,493 gene promoters, with a general trend toward genes being hypomethylated in these B-ALL subtypes compared with normal pre-B cells (Fig. 1A; Supplementary Table S4). Among hypomethylated probesets, 13.7% overlap with CpG islands, 55.3% with CpG shores (the 2 kb flanking CpG islands; ref. 36), and 31% were outside of CpG islands or shores. B-ALL DNA methylation signatures were queried for their association with gene sets relevant to lymphoid tumor biology (37). All 3 B-ALL subtypes were significantly enriched with MYC target genes (\( P < 1e-3, P < 1e-11, \) and \( P < 1e-7 \), respectively, for BCR-ABL1, E2A-PBX1, and MLLr; Fisher exact test with Benjamin-Hochberg correction; Fig. 1B; Supplementary Tables S5 and S6), suggesting that MYC transcriptional networks are disrupted epigenetically in all 3 leukemia subtypes consistent with the critical role of MYC in hematopoietic neoplasms. BCR-ABL1 and MLLr
Figure 1. B-ALL subtypes display aberrant DNA methylation patterns as compared with normal pre-B cells. A, heatmap representation of promoter DNA methylation levels of differentially methylated genes in BCR–ABL1 (n = 83), E2A–PBX1 (n = 7), and MLLr (n = 28) B-ALLs versus normal pre-B samples (n = 12). Probesets (1,740; 1,493 gene promoters) were identified with adjusted P < 0.01 and methylation difference >30% (i.e., dx > 1.5 on HELP).

Each row of the heatmap represents one probe set on the HELP array, and each column represents a patient with B-ALL or a healthy bone marrow. The color scale bar represents the methylation levels with purple for hypomethylation and yellow for hypermethylation.

B, the heatmap represents enrichment of B-cell and lymphoid-specific gene sets with the DNA methylation signatures of the 3 B-ALL subtypes versus normal pre-B cells. The statistical significance is provided in the color key. P values were calculated by Fisher exact test with Benjamini–Hochberg correction.
DNA methylation signatures were enriched in BCL6 target genes ($P = 0.002$ and $P = 0.049$, respectively). BCR–ABL1 B-ALLs also featured epigenetic deregulation of p53-induced genes ($P = 0.012$), interleukin (IL)10-responsive genes ($P = 0.013$), cell-cycle genes ($P = 0.022$), and others. E2A–PBX1 methylation signature involved genes with glutamine and glucose starvation ($P = 0.021$), SREBP-1 and 2 activity ($P = 0.016$), and T-cell cytokines ($P = 0.042$; Fig. 1B; Supplementary Tables S5 and S6). Detailed explanation and references for those signatures are provided in Supplementary Table S5. A similar analysis using Gene Ontology (GO) terms enriched for signal transduction, cell proliferation, and gene regulation in all 3 subtypes (Supplementary Tables S5 and S6). These data indicate that cytogenetically distinct forms of adult B-ALLs display specific and functionally defined DNA methylation signatures as compared with normal pre-B cells.

**BCR–ABL1 B-ALL DNA Methylation Signature Features Dysregulation of IL2RA**

To investigate epigenetic programming linked to BCR–ABL1 signaling, we sought to identify genes differentially methylated in BCR–ABL1-positive ALLs. We conducted a supervised analysis comparing DNA methylation profiles of the 83 BCR–ABL1-positive to the 132 BCR–ABL1-negative patients using the Student $t$ test with Benjamini–Hochberg correction for multiple testing. This yielded a set of 192 probesets (170 unique gene promoters) with methylation difference $>20\%$ (dx $>1$ by HELP) and adjusted $P < 0.01$ (Fig. 2A; Supplementary Table S7A). Gene expression profiling was also conducted in these E2993 patients to define potential links between DNA methylation and transcriptional regulation. Supervised analysis of gene expression profiling in the same patients yielded 605 probesets (417 unique genes) with an adjusted $P < 0.01$ and log$_2$ fold change $>1$ (Fig. 2B; Supplementary Table S7B). There was a tendency for DNA methylation to be inversely correlated with gene expression (i.e., the average expression fold changes (log$_2$) fold) of BCR–ABL1-positive versus -negative ALLs in the hypomethylated signature genes is higher than that in the hypermethylated signature genes, $P = 0.013$, one-tailed Wilcoxon test). More than 80% of the genes in the methylation signature were hypomethylated and 80% of the genes were overexpressed in BCR–ABL1-positive B-ALLs, suggesting strong gene activation functions downstream of the fusion protein. Using a Bayesian predictor (38) and leave-one-out cross-validation (LOOCV), the methylation and expression signatures reclassified 87% and 93%, respectively, of these B-ALL cases according to BCR–ABL1 status (Fig. 2A and B). To identify a core set of highly dysregulated genes, we examined the overlap between the DNA methylation and gene expression signatures. This analysis yielded 13 genes, 11 of which featured inversely correlated methylation and expression levels (Fig. 2C). Among the 11 core signature genes, the IL2RA gene (interleukin receptor 2 alpha chain), which encodes the CD25 protein, featured the highest overexpression and the second highest hypomethylation in BCR–ABL1 ALLs (Fig. 2C). Differential expression of some of these genes were also captured in a previous gene expression profiling effort conducted in S4 E2993 patients with B-ALLs (6). Differential expression and methylation was confirmed for all 11 genes by quantitative PCR (qPCR) and MASSArray EpiTyper (Supplementary Fig. S2A and S2B and Supplementary Tables S8 and S9). The DNA methylation and gene expression microarray data are available at GEO database (SuperSeries: GSE34941; gene expression: GSE34861; DNA methylation: GSE34937).

**Pathway analysis combining the full DNA methylation and gene expression signatures from Fig. 2A and B identified the heavy involvement of one particular gene network, centered around IL2RA (CD25) and other cytokines (Fig. 2D). Collectively, these analyses pointed toward IL2RA as a potentially significant gene in BCR–ABL1-positive B-ALLs. Indeed, we observed that that IL2RA is significantly more hypomethylated in BCR–ABL1-positive B-ALLs not only versus BCR–ABL1-negative B-ALLs but also versus normal pre-B cells ($P < 1e-6$ for both, $t$ test; Fig. 2E, top). IL2RA mRNA in gene expression profiling of the same patients was also significantly more abundant in BCR–ABL1-positive versus BCR–ABL1-negative B-ALLs or normal pre-B cells (Fig. 2E, middle). The same differential expression of IL2RA was detected upon examining an independent set of 132 B-ALL gene expression profiles (refs. 4, 39; Fig. 2E, bottom). Significant hypomethylation and upregulation of IL2RA in BCR–ABL1-positive ALLs was validated by MassArray EpiTyper and qPCR on randomly selected E2993 patient samples with available DNA ($P < 1e-6$ for all, one-tailed $t$-test; Fig. 2F and G).

**CD25 Positivity Confers Poor Clinical Outcomes**

BCR–ABL1 translocation is an indicator of unfavorable outcome in adults with B-ALLs (2). Examination of a total of 465 patients with B-ALLs enrolled in the E2993 trial including those profiled with microarrays plus additional patients with complete molecular and clinical annotation [median age, 37 years, interquartile range (IQR), 25–47 years; median follow-up, 7.9 years; IQR, 5–11 years] confirmed the inferior overall survival (OS) of BCR–ABL1-positive ($n = 113$) versus BCR–ABL1-negative B-ALL cases ($n = 352$, log-rank, $P < 0.0001$; Fig. 3A). The subset of imatinib-treated patients with B-ALLs was excluded from the survival analysis. Consistent with a previous report (40), CD25 positivity (measured by flow cytometry) was strongly associated with BCR–ABL1 (61.1% BCR–ABL1-positive patients are also CD25 positive, 69 of 113) but was rare in BCR–ABL1-negative patients (only 5.1% BCR–ABL1-negative patients are CD25 positive, 18 of 352). This difference was highly significant ($P < 1e-6$, Fisher exact test; Supplementary Fig. S3). When comparing expression profiles of CD25-positive versus CD25-negative cases among BCR–ABL1-positive B-ALLs, IL2RA was the most differentially expressed gene (>6-fold overexpressed in CD25-positive cases, $P = 0.005$, $t$ test) and also notably hypomethylated ($P = 0.01$, $t$ test; Supplementary Fig. S4A). The overexpression and hypomethylation of IL2RA was validated by qPCR and MassArray (Supplementary Fig. S4B). Those data suggest that IL2RA (CD25) might be an important factor in BCR–ABL1-positive ALLs. Indeed CD25 expression was clinically relevant, as BCR–ABL1-positive patients can be further stratified into 2 groups with significantly different clinical outcome according to CD25 positivity. CD25-positive patients have significantly worse...
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Figure 2. BCR-ABL1 B-ALLs feature DNA methylation and expression signatures centered around IL2RA(CD25). Methylation signature (A) and expression signature (B) of BCR-ABL1-positive versus BCR-ABL1-negative B-ALLs. Each row represents one probeset and each column one patient. The color scale bars represent the methylation or expression levels. The probability of signatures to reclassify cases according to BCR-ABL1 status is shown on the top. The actual case labels: red, BCR-ABL1-positive; blue, BCR-ABL1-negative. The numbers of correctly classified and misclassified cases are given below the heatmap. C, the difference of mean methylation or expression values in BCR-ABL1-positive versus -negative B-ALL is depicted by the color scale on the 11 core genes overlapping between methylation and expression signatures. D, the gene network most highly enriched by methylation and expression signatures in BCR-ABL1 B-ALLs is centered around IL2RA (CD25). E, the degree of IL2RA hypomethylation and expression from E2993 cases and from an independent cohort of 132 patients with St. Jude B-ALLs (4, 39) in BCR-ABL1-positive versus BCR-ABL1-negative and versus normal pre-B cells. MassArray (F) and qPCR (G) validation for IL2RA(CD25). The color key represents % methylation of each promoter CpG (rows). Columns represent individual cases. Location of HELP probesets and EpiTyper primers on IL2RA are shown (UCSC genome browser hg19). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
OS [median OS time, 0.8 years; 95% confidence interval (CI), 0.6–1.1, n = 69] than the CD25-negative patients (median OS time, 1.3 years; 95% CI, 0.90–2.5, n = 44, log-rank, P = 0.0064; Fig 3B). Although the CD25-negative BCR-ABL1-positive patients tended to display worse OS than the CD25-negative BCR-ABL1-negative patients, the difference did not reach statistical significance (log-rank, P = 0.08; Fig 3C).

Collectively, the data indicate that hypomethylation and corresponding overexpression of CD25 is strongly associated with BCR-ABL1 fusion protein and may be linked with processes that account, at least in part, for a particularly unfavorable clinical outcome in BCR-ABL1 B-ALLs. CD25 can serve as a new prognostic biomarker for risk stratification in B-ALLs.

Deregulated DNA Methylation Is Associated with Binding of the E2A–PBX1 Fusion Protein

We next examined whether E2A–PBX1 translocations were also associated with specific perturbations of DNA methylation patterning. Supervised analysis comparing 7 E2A–PBX1-positive versus 97 E2A–PBX1-negative ALLs identified 94 differentially methylated probesets (69 genes) with adjusted P < 0.01 and methylation difference more than 20% (Fig 4A; Supplementary Table S10A) and 1,312 differentially expressed probesets (863 genes) with adjusted P < 0.01 and log2 fold change > 1 (Fig 4B; Supplementary Table S10B). Most genes in the methylation signatures (58 of 69) were hypomethylated, and the hypomethylation was associated with overexpression [P < 0.01, one-tailed Wilcoxon test by comparing the means of the log2 expression fold changes (E2A–PBX1-positive vs. -negative cases) in the hypo- and hypermethylated gene groups]. When applied to a Bayesian predictor (38) using LOOCV, the DNA methylation and gene expression signatures reclassified E2A–PBX1 ALLs with 100% and 98% accuracy, respectively. Eight genes overlapped between methylation and expression signatures and 7 showed inverse correlation, including MLKL, SERINC5, and CTDSP2 (hypermethylated
Figure 4. Specific DNA methylation and expression signatures associated with binding of E2A–PBX1 fusion protein. Methylation signature (A), expression signature (B), and overlapping (C) between methylation and expression signatures of E2A–PBX1-positive versus E2A–PBX1-negative B-ALL. Rows are probesets and columns are patients. The color scale bars represent the methylation or expression levels. The probability of signatures to reclassify E2A–PBX1 cases is shown on the top. C, the difference of mean methylation and expression values in E2A–PBX1-positive versus E2A–PBX1-negative B-ALLs is depicted by the color scale. D, graphical representations of ChIP for E2A (orange), PBX1 (green), and E2A–PBX1 fusion proteins. ChIP-seq tracks (E) and qChIP (F) results for E2A (green), PBX1 (orange), and p300 (blue) antibodies versus input (black) in 697 cells on the 4 hypomethylation signature genes in (C). E, y-axis: number of reads for peak summits normalized by the total number of reads per track (set to 1 Gb for each track). Locations of CpG islands, HELP probesets, qChIP primers, and an enlarged view of binding peaks are shown. G, qPCR was carried out in 697 cells transfected with E2A-shRNA or scramble-shRNA. y-axis, relative expression normalized to the scramble-shRNA. Data represent means ± SEM (n = 3).
and underexpressed) and CALD1, ARL4C, ST6GALNAC3, and EXTL3 (hypomethylated and overexpressed; Fig. 4C). E2A–PBX1 is reported to act as an aberrant transcriptional activator that cooperates with the p300 histone acetyltransferase (41). We wondered whether the fusion protein itself might be linked to hypomethylation in these B-ALLs. We therefore conducted ChIP-seq using antibodies recognizing the N terminus of E2A (E2A\textsuperscript{N}), the C terminus of PBX1 (PBX1\textsuperscript{C}), as well as p300 in an E2A–PBX1-positive B-ALL cell line, 697 cells (Fig. 4D; Supplementary Table S11). Global analysis of these datasets identified 3,358 E2A\textsuperscript{N} peaks, located predominantly at intronic and upstream regulatory regions (83%: broken down into 41% intronic, 23% distal at 2–50 kb upstream, and 16% intergenic >50 kb from RefSeq TSS) versus only 11% at promoters (ChIPseeqer software; ref. 42). The 19,108 PBX1\textsuperscript{C} peaks were located at a much higher frequency at promoters (36%) but were also at putative regulatory regions (28% introns, 17% distal, and 13% intergenic; Supplementary Fig. S5A and Supplementary Table S14). Much of the intergenic/intronic PBX1\textsuperscript{C} sites may be accounted for by E2A–PBX1 fusion protein, as there was an 83% overlap between E2A\textsuperscript{N} sites with PBX1\textsuperscript{C} sites (2,971 peaks, z-score: 741.57, P < 1e-10, ChIPseeqer software; ref. 42; Supplementary Fig. S5B). p300 ChIP-seq identified 15,501 binding sites, which similar to E2A\textsuperscript{N} we were preferentially to regulatory regions (85%, with 43% in introns, 22% distal, and 20% intergenic). Remarkably, 96% of the putative PBX1–PBX1 fusion protein sites overlapped with p300 (2,684 peaks, z-score: 960.58, P < 1e-10, ChIPseeqer software; ref. 42), consistent with the known complex formation between these proteins (Supplementary Fig. S5B). Fully 87% of these joint binding sites were located at intronic/distal and intergenic regions and only 8% at promoters (Supplementary Fig. S5C). Altogether the data place a minority of E2A–PBX1–p300 complexes at traditional promoters and the majority at both intra- and intergenic regulatory regions.

Although promoter sites were the minority of fusion protein binding sites, we focused on these sites to more firmly link them to our studies on promoter DNA methylation. Focusing down on E2A–PBX1 signature genes, all 4 hypomethylated and upregulated genes in the core signature (Fig. 4C) showed enriched binding of E2A\textsuperscript{N}, PBX1\textsuperscript{C}, and p300 in their promoter regions (Fig. 4E). Ten additional hypomethylation signature genes were enriched by E2A\textsuperscript{N}, PBX1\textsuperscript{C}, and p300 antibodies, including ACOXL1, BLK, COMT, MAST4, MYBPH, SLAMF1, SMPD3, TERT, TNS5, and WAPAL. A total of 14 genes overlap between the E2A–PBX1 hypomethylation signature (n = 58 genes) and those identified as putative E2A–PBX1 and p300 direct targets (n = 2,684 overlapping peaks annotated to n = 1,642 genes). This level of overlap was indicative of overrepresentation of E2A/PBX1 target genes among hypomethylated genes (P = 0.006, 2-tailed \chi^2 test with Yates’ correction). We further validated these targets by conducting qChIP with E2A\textsuperscript{N}, PBX1\textsuperscript{C}, and p300 antibodies (as positive ChIP’s) and PBX1\textsuperscript{N} antibody (as a negative control ChIP) in 697 and Nalm6 cells (Nalm6 is an E2A–PBX1-negative B-ALL cell line). PBX1\textsuperscript{N} qChIP serves as a negative control, as PBX1\textsuperscript{N} binds to only wild-type PBX1 but not E2A–PBX1 fusion and wild-type PBX1 is not expressed in lymphoid precursor B cells (43), therefore we would expect no PBX1\textsuperscript{N} binding enrichment on those loci in neither 697 nor Nalm6 cells. This procedure confirmed binding enrichment of E2A\textsuperscript{N}, PBX1\textsuperscript{C}, and p300 to the 4 core hypomethylation signature genes in 697 but not Nalm6 cells and no binding enrichment of PBX1\textsuperscript{C} to those loci in either cell type (Fig. 4F; Supplementary Table S12). To determine whether these genes are directly controlled by E2A–PBX1, we conducted lentiviral short hairpin RNA (shRNA) knockdown in 697 cells which targeted the middle coding region (amino acids 340–348) of E2A (E12/E47) and therefore knockdown both wild-type E2A and E2A–PBX1 fusion. Figure 4G shows the shRNA effectively knocked down E2A, E2A–PBX1, as well as the 4 core signature genes. Collectively, these data identify a set of genes specifically deregulated epigenetically by E2A–PBX1 fusion, and moreover, directly implicate the fusion protein as driving this epigenetic and transcriptional signature.

**Adult MLLr B-ALL Displays a Unique DNA Methylation Signature with Predominant Hypomethylation**

Chromosomal translocations involving MLL are frequent in B-ALLs, can involve more than 50 partner genes, and are generally associated with a poor clinical outcome (44). Among the 28 MLLr cases in our cohort, there were 20 MLL–AF4, 6 MLL–ENL, 1 MLL–AF9, and 1 MLL–EPS1 cases. Unsupervised analysis of DNA methylation and gene expression profiles using hierarchical clustering showed that MLLr cases do not segregate according to fusion partners (data not shown). Moreover, a supervised analysis between the 20 MLL–AF4 and the other 8 MLLr cases showed no genes differentially methylated or expressed with adjusted P < 0.01 (data not shown). Many of the common MLL fusion partners such as AF9, ENL, and AF4 form part of transcriptional elongation complexes and directly or indirectly recruit the DOT1L histone 3 lysine 79 (H3K79) methyltransferase (21, 23). This biochemical commonality may explain why these MLLr DNA methylation profiles are highly similar to each other. Supervised analysis comparing the 28 MLLr versus the other 97 MLLr-negative ALLs identified 430 differentially methylated probe sets (379 genes) with adjusted P < 0.01 and methylation difference >20% (Fig. 5A; Supplementary Table S13A) and 1,581 differentially expressed probe sets (1,030 genes) with adjusted P < 0.01 and fold change >1 (Fig. 5B; Supplementary Table S13B). Sixty percent (231 of 379) differentially methylated genes were hypomethylated and displayed higher expression levels than the hypermethylated genes in MLLr B-ALL (P < 1e-6, one-tailed Wilcoxon test by comparing the means of the log2 expression fold changes of MLLr-positive vs. -negative cases in the hypo- and hypermethylated gene groups). When applying a Bayesian predictor (38) to these cases, the methylation and expression signatures each reclassified MLLr cases with 97% accuracy, (Fig. 5A and B). A core set of 32 genes overlapped between the methylation and expression signatures, among which 25 showed inverse correlation between methylation and expression. Those
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Figure 5. DNA methylation and expression signatures are linked to MLL fusion protein binding patterns. Methylation signature (A), expression signature (B), and overlapping genes between methylation and expression signatures (C) of MLLr versus non-MLLr B-ALLs. Rows for probesets and columns for patients. The probability of a Bayesian predictor to reclassify MLLr ALLs is shown on top. The numbers of correctly classified and misclassified cases are given below the heatmap. C, the color scale depicts the difference of mean methylation or expression values in MLLr versus non-MLLr cases. qPCR (D) and MassArray (E) validation of FLT3 in 5 MLLr, 5 non-MLLr B-ALLs, and 3 normal pre-B samples. Data present means ± SEM (n = 3). P values from one-tailed t-test. The heatmap represents the % methylation of CpGs (rows) in samples (columns). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. F, graphical representation of ChIP for MLL (red), AF4 (blue), and MLL-AF4 fusion. G, qChIP using MLLN, AF4C, H3K79me2, or H3 antibodies in RS4;11 and CCRF-CEM cells, enriching for 4 positive control and 7 MLLr hypomethylated and overexpressed loci. Data represent means ± SEM (n = 3). H, FLT3 locus ChIP-seq enrichment profiles in RS4;11 cells. Locations of TSS, CpG islands, HELP probesets, and MassArray EpiTyper primers are shown below the gene model in the UCSC genome browser view.

A B C

430 probesets, 379 genes, adjusted P < 0.01, dx > 1

5,811 probesets, 1,030 genes, adjusted P < 0.01, dx > 1

0.00 0.05 0.10 0.15 0.20 0.25 0.30

ITGA10 IMP-2 PCDHGB3 PCDHGA5 FUT4 PARPB MAP7 RBKS FLT3 ANXA5 MRPL33 TCP11 MAPT1 PRKCH LTB GAB1 AF33 LST1 ZAP70 CCR6 ITIH3 MLLX IFIT1 PIK3IP1
Aberrant DNA hypomethylation may thus contribute to potential therapeutic target in this B-ALL subtype (46, 47). FLT3 was previously core genes was from this list (Supplementary Fig. S6A and B). One of the Typer and qPCR confirmed the differential methylation, and pressred and , and MAP1A, RBKS, PARP8, FLT3, HGA5, ANXA5, MAP7, MEIS1, HOXA10, ITGAE, and histone H3 antibody also confirmed enriched binding at the MLLr fusion direct targets by ChIP-seq data (Supplementary Fig. S11). We assessed enrichment at promoters of (i) 4 positive control genes: HOX9A, HOX9B, HOX10, and MEIS1 (Fig. 5G; Supplementary Figs. S7 and S8A, and S8B); (ii) 4 genes from the core signature in Fig. 5B: FLT3, IGF2BP2/IMP2, PUF4, and IGF2; (iii) 2 randomly selected genes from among the top 100 most highly overexpressed genes (IGFBP7 and VAT1L/KIAA1576), which were also hypomethylated although did not reach the threshold for the signature in Fig. 5A: IGFBP7 (dx = 1.18, P = 0.091) and VAT1L (dx = 0.94, P = 0.0002); and (iv) BCL6, which was of interest because BCL6 target genes were one of the few pathways differentially methylated in patients with MLLr (Fig. 1B), and BCL6 was differentially methylated in MLLr B-ALLs versus normal pre-B cells in Fig. 1A; Supplementary Table S4 (dx = 1.59, P = 0.00001). We observed enrichment for MLLr and AF4 at all 7 of these loci and the 4 positive controls in RS4;11 but not CCRF-CEM cells, consistent with binding of the MLL-AF4 fusion protein (Fig. 5G). In accordance with this notion, we also noted increased H3K79me2 signal in RS4;11 but not CCRF-CEM cells at 9 of those 11 loci (except at IGFBAT and VAT1L), and histone 3 enrichment was relatively even in both cell lines (Fig. 5G). Similar enrichment for MLLr and AF4 was observed in a second MLL-AF4-positive B-ALL cell line, SEM, at all those 11 loci (Supplementary Fig. S8). We next conducted ChIP-seq with MLLr, AF4, and H3K79me2 antibodies in RS4;11 cells (GEO accession numbers: GSE38403). MLLr ChIP-seq identified 3,312 binding peaks, annotated to 1,928 promoters (promoter regions defined as 2 kb up- and downstream to TSS); AF4 ChIP-seq identified 6,253 peaks (1,387 promoters); and H3K79me2 ChIP-seq identified 28,476 peaks (5,395 promoters, Supplementary Tables S14 and S15). 603 gene promoters showed binding overlap with all 3 antibodies (MLLr, AF4, and H3K79me2) and were hence identified as MLL fusion direct targets by ChIP-seq data (Supplementary Table S15). These 603 MLL fusion target genes showed higher levels of hypomethylation and expression than the nontarget genes in the MLLr B-ALL cases (P < 1e-6 for both, one-tailed Wilcoxon test; Supplementary Fig. S9A and S9B), indicating MLL-AF4 binding is associated with DNA hypomethylation and overexpression of the target genes. Nine of the 11 loci queried by QChIP were also in this 603 gene list (except IGF2 and VAT1L). ChIP-seq read densities at the FLT3 locus are shown as an example in Fig. 5H. Our results showed that the hypomethylation signatures are linked with MLL fusion protein binding and H3K79me2, which induce overexpression of these genes. These are first examples of DNA methylation signatures directly mechanistically linked to binding patterns of aberrant fusion oncoproteins. Our data also revealed that FLT3 is in fact a direct target of MLL fusion proteins in B-ALLs, and consequent hypomethylation and H3K79 dimethylation of the FLT3 promoter in these tumors may contribute to direct transcriptional activation of this oncogenic tyrosine kinase.

**BCL6 is a Transcriptional Target of MLL Fusion Proteins**

Among novel MLLr fusion targets identified in this study, BCL6 captured our particular attention because of its known role as a potent oncoprotein in B-cell lymphomas. As noted above, direct binding of MLL-AF4 as well as increased H3K79me2 was observed at the BCL6 locus by qChIP (Fig. 5G). ChIP-seq by MLLr, AF4, and H3K79me2 antibodies also confirmed enriched binding at the BCL6 locus (Fig. 6A). We showed that hypomethylation of BCL6 is indeed an aberrant feature of MLLr by comparing the degree of cytosine methylation in MLLr B-ALLs with normal pre-B cells as captured by our HELP assays (P < 1e-6, t test; Fig. 6B). Hypomethylation of BCL6 in MLLr versus non-MLLr B-ALLs and normal pre-B cells was again confirmed by MasArray EpiTyper (P < 1e-3 and P < 1e-5, respectively, t test; Fig. 6C). Immunoblots using BCL6 antibody in a panel of 9 B-ALL cell lines, 11 primary B-ALL patient specimens, and 2 normal pre-B cells samples revealed that expression of BCL6 protein was elevated in MLLr as compared with other B-ALL subtypes and normal pre-B cells (Fig. 6D). BCL6 mRNA abundance was largely consistent with protein in these cells (Supplementary Fig. S10). Finally, to determine whether MLL fusion protein not only binds but also induces BCL6 expression, we conducted MLL-AF4 siRNA knockdown using fusion transcript-specific siRNA sequences (48) in RS4;11 and SEM cells. The siRNAs effectively downregulated MLL-AF4 transcripts and proteins within 48 hours, and this was accompanied by a reduction in expression of HOX9A (a known MLL fusion target) and BCL6 at both mRNA and protein levels (Fig. 6E and F). MLL fusion proteins thus bind
Figure 6. BCL6 is upregulated in MLL-AF4 ALL with its promoter hypomethylated and bound by MLL-AF4 fusion. A, ChIP-seq profiles at BCL6 enriched by MLL "red, AF4" (green), and H3K79me2 (blue) antibodies versus input (black) in RS4;11 cells. The locations of CpG islands, HELP probesets, and MassArray EpiTyper primers are shown. B, DNA methylation values on BCL6 derived from HELP assays in MLLr (n = 28) versus normal pre-B (n = 12) samples. P value from t test. C, heatmap representation of CpG methylation (rows) at the BCL6 locus measured by MassArray on 10 MLL-AF4-positive, 10 MLL-AF4-negative, and 10 normal pre-B samples (columns). The color key indicates percent DNA methylation. P values from t test comparing the average methylation level of CpGs in each group. D, BCL6 Western blot analyses conducted in ALL cell lines (black labels) and primary specimens (blue labels). E and F, RS4;11 and SEM cells were transduced with MLL-AF4 fusion-specific siRNA as described [48] or nontargeted control siRNA, followed 48 hours later by (E) qPCR or (F) immunoblots for MLL-AF4, HOXA9, and BCL6. D, E, and F, RS4;11 Western blot bands were quantified relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using ImageJ [80]. *Values from densitometry and normalized to GAPDH and control siRNA.
to the BCL6 promoter and contribute to its upregulation in MLLr B-ALLs.

**BCL6 Is a Therapeutic Target in MLLr B-ALL**

Constitutive expression of BCL6 is known to maintain the proliferation and survival of B-cell lymphomas (49). The fact that BCL6 is hypomethylated, overexpressed, and a direct transcriptional target of MLL fusion proteins suggests that it might contribute to the proliferation and survival of MLLr B-ALLs. To determine whether this is the case, we transduced MLL-AF4B-ALL cells (BEL-1) with a retrovirus expressing a 4-hydroxytamoxifen (4OHT)-inducible dominant-negative form of BCL6 consisting of its zinc finger domain and also expressing GFP through an internal ribosomal entry site (DNBCL6-ER72-ires-GFP; ref. 50) or ER72-ires-GFP control vectors. We observed that while the percentage of control ER-GFP–transduced cells remained constant after 3-day exposure to 4-OHT, there was a 55% decrease after 4-OHT induction in GFP-positive cells transduced with dominant negative BCL6 (15.9% vs. 35.00%; Fig. 7A), suggesting that BCL6 is important in maintaining cell proliferation in MLLr B-ALLs. Specific inhibitors of BCL6 were developed to block its transcriptional repressor activity (49, 51, 52) including the widely used peptidomimetic inhibitor RI-BPI, which specifically blocks BCL6 by preventing its binding to co-repressors (52). We tested the ability of 2 MLLrB-ALL cell lines (RS4;11 and SEM) to form colonies after exposure to 5 μmol/L RI-BPI or vehicle. RI-BPI resulted in approximately 90% reduction in colonies in both SEM and RS4;11 cells (Fig. 7B; P < 0.001 for both, t-test). In contrast, RI-BPI treatment of 2 MLLr wild-type ALL cell lines (Nalm6 and REH) did not suppress colony formation (Supplementary Fig. S11A and S11B), suggesting that BCL6 dependence is a feature of MLLr. Similarly, 5 μmol/L RI-BPI induced an approximately 40% reduction in viability of RS4;11 and an approximately 60% reduction in SEM cells after 48 hours as compared with vehicle (P = 0.003 and P < 0.001, respectively) or a control peptide (CP; P = 0.004 and P < 0.001, respectively, t-test; Fig. 7C).

Seven vialy frozen primary human MLLr B-ALL specimens from the E2993 clinical trial were thawed and placed in liquid culture. Expression of BCL6 was verified in each of these cases by qPCR (Supplementary Fig. S12). The cells were exposed to 5 μmol/L RI-BPI, 5 μmol/L CP, or vehicle for 48 hours and assayed for colony forming potential by plating cells in methylcellulose in the presence of IL7. RI-BPI caused a 30% to 70% reduction in colony formation of MLLr specimens (Fig. 7D).

Three patient specimens had sufficient cells to conduct dose–response viability assays by flow cytometry using Annexin V and 7-aminoactinomycin D (7AAD) co-staining. Cells were treated with RI-BPI or CP at doses ranging from 1.25 to 20 μmol/L to cover the range of IC50 values reported in BCL6-dependent lymphomas (52). In all 3 cases, RI-BPI, but not CP, induced apoptosis of these primary human MLLr B-ALL within a similar dose range as B-cell lymphomas (ref. 52; Fig. 7E). In contrast, when we ran cell viability experiments on 3 independent B-ALL primary samples (not from ECOG) with the phenotype labels blinded during the experiments, the RI-BPI suppression was only observed in the 2 MLLr cases but not the MLLr wild-type B-ALLs (Supplementary Fig. S13).

These data suggest that BCL6 plays a crucial role in maintaining the survival and proliferation of MLLr B-ALL cells and is a bona fide therapeutic target. Collectively, the integration of DNA methylation patterning studies in tandem with gene expression profiling and ChIP-seq provide an enhanced ability to understand the mechanism of action of oncogenic fusion proteins, establish putative prognostic biomarkers, and identify new therapeutic targets in adult patients with B-ALLs.

**DISCUSSION**

The focus of this study was to identify promoter DNA methylation profiles associated with unfavorable B-ALL subtypes in adult patients and determine their functional and clinical significance. This effort was facilitated by profiling a large and unique cohort of clinically well-annotated adult patients with B-ALL enrolled in a single multicenter phase III clinical trial (E2993). We observed that aberrant epigenetic regulation occurs universally and is distributed to specific gene sets in genetically defined B-ALL subtypes associated with poor outcomes. Integrative analysis of epigenetic and transcriptional profiles identified core gene signatures, associated with the mechanism of action of aberrant fusion proteins and pointing to key molecular mechanisms and potential therapeutic targets. We also conducted unsupervised hierarchical clustering of the 97 normal karyotype B-ALLs in our cohort, which yielded 2 robust clusters (1-Pearson, Ward method, ×1,000 bootstrapping, data not shown). A supervised analysis revealed 1,512 genes differentially methylated (methylation difference >20%, adjusted P < 0.01; Student t test) between the 2 clusters, enriched in hematopoietic cell quiescence genes (ref. 53; Benjamini–Hochberg adjusted P = 0.03) as well as MYC (Benjamini–Hochberg adjusted P = 0.02), and IKAROS target genes (ref. 54; Benjamini–Hochberg adjusted P = 0.03). These data suggest differences in the underlying pathogenesis of these cases and point toward a rationale for further study of mechanisms driving aberrant gene regulation in translocation-negative patients.

In the case of BCR–ABL1-positive B-ALLs, we identified the most deregulated gene network captured by integrative analysis of DNA methylation and gene expression profiles was centered around IL2RA, which is the most hypomethylated and overexpressed gene in BCR–ABL1-positive B-ALLs. In general, IL2RA (CD25) positivity was strongly associated with BCR–ABL1 translocation in this large cohort of patients with B-ALLs, which confirms a previous report on a smaller set of E2993 patients (40). BCR–ABL1-positive patients with ALLs experienced significantly worse outcome than other patients with B-ALLs in the E2993 clinical trial. However, much of the difference appears related to CD25 expression, as it was the subset of BCR–ABL1-positive CD25-positive B-ALLs that featured the lowest OS, whereas BCR–ABL1-positive CD25-negative cases were not statistically worse than BCR–ABL1-negative B-ALLs. Further research will be required to determine how and why IL2RA and BCR–ABL1 are functionally linked. It is notable that CD25 expression...
Figure 7. BCL6-targeted therapy kills MLLr B-ALL cells. A, BEL-1 cells (MLL-AF4-positive) were transduced with 4-OHT–inducible dominant-negative BCL6 (DN-BCL6;ER T2 ) or ER T2 control vectors (expressing GFP) and exposed or not exposed to 4-OHT for 3 days. Percentage of GFP-positive cells is indicated in the flow cytometric plots before and after 4-OHT for each construct. B, SEM and RS4;11 cells and (D) 7 E2993 MLL-AF4 patient samples were exposed to 5 μmol/L RI-BPI, 5 μmol/L CP, or vehicle for 48 hours, followed by colony formation assay. The y-axis shows percent colony formation of RI-BPI–treated cells versus vehicle. Experiments were carried out in triplicate. C, RS4;11 and SEM cells were exposed to 5 μmol/L RI-BPI, 5 μmol/L CP, or vehicle for 48 hours, followed by flow cytometry for Annexin V/7-AAD. Percent viability versus vehicle is shown on the y-axis. Experiments were carried out in triplicate. E, three E2993 MLL-AF4 patient samples were treated with increasing concentrations of RI-BPI or CP for 48 hours, and viability determined by Annexin V/7-AAD flow cytometry. Percent viability relative to CP is shown on the y-axis. Data represent means ± SEM (n = 3). Student t test was used to calculate P values.
signature in our B-ALLs (data not shown) was significantly associated with a leukemia stem cell (LSC) signature (ref. 55; Benjamini–Hochberg adjusted P < 1e-5) and a hematopoietic stem cell (HSC) signature (56) in leukemia (Benjamini–Hochberg adjusted P < 1e-6; Fisher exact test). Because LSCs are generally considered to be more chemoresistant (57), it is possible that CD25 positivity might be an indicator of an enlarged LSC-like fraction. The IL2RA network also featured aberrant regulation of numerous cytokines and interleukins. Notably, paracrine cytokine signaling by tumor cell microenvironment was recently shown to also confer chemoresistance (58). Along these lines, quiescent AML leukemia stem cells localized to the endosteal niche within the bone marrow strongly expressed CD25 (59). These endosteal localized AML stem cells were actively regulated by MLL fusion proteins (an illustration cartoon for a potential relationship between MLL-AF4 binding and DNA hypomethylation was shown in Supplementary Fig. S14A and S14B). Recent data from our group also suggest a link between HOXA9 and enhancer hypomethylation in myeloid MLLr leukemias (66). At the current time, there is no evidence for direct biochemical cross-talk between DNMTs and MLL fusion proteins. However, given the previously described cooperation between Polycomb complexes and DNMTs (67) and the generally antagonistic actions of MLL/trithorax proteins with Polycomb (68), it remains possible that competition between MLL and DNMTs might occur in at least an indirect manner (Supplementary Fig. S14). Additional studies will be needed to explore whether aberrant DNA hypomethylation of loci occurring during leukemogenesis facilitates the recruitment of MLL fusions and induces abnormal transcriptional activation or whether MLL fusion proteins in some way contribute or cause the initial aberrant hypomethylation of these loci. Whichever the case it seems clear that MLL binding to hypomethylated DNA is important to its leukemic functions and perhaps facilitates assembly of MLL fusion complexes with DOT1L, thus accounting for the association we observed between DNA hypomethylation, MLL fusion protein binding, and H3K79me2. Perhaps CXXC-induced DNA hypomethylation and DOT1L work together or even in a positive feedback loop to highly upregulate certain gene sets important for leukemogenesis in MLLr cells, including not only HOX genes but also factors such as FLT3 and BCL6. In fact, FLT3 was one of the most hypomethylated and highly expressed genes in MLLr B-ALLs. FLT3 expression in MLLr leukemia was previously reported (69) as functionally relevant, as FLT3 inhibitors could suppress the growth of MLLr cells (46). We show herein that FLT3 is in fact a bona fide direct target gene of MLL-AF4 and perhaps an example of a leukemogenic gene in which MLL fusion protein-associated DNA hypomethylation and H3K79me2 epigenetically deregulate its expression.

Integrative epigenomic analysis allowed us to identify the BCL6 transcriptional repressor as a therapeutic target in MLLr B-ALLs. The BCL6 locus displayed all the hallmarks of MLL fusion protein–mediated epigenetic deregulation including direct MLL-AF4 binding, DNA hypomethylation, H3K79 dimethylation, and transcriptional upregulation. BCL6 is required for germinal center B cells to undergo proliferation and immunoglobulin affinity maturation.
BCL6 mediates these effects by suppressing replication and DNA damage checkpoints (70–72). More recently, BCL6 was shown to play a similar role in maintaining survival of pre-B cells (73). Specifically, pre-B cells transiently express BCL6 during the transition from IL7-dependent to IL7-independent stages, whereas BCL6 protects cells from genotoxic stress during immunoglobulin light chain recombination (73). These actions link BCL6 to processes associated with development of B-ALLs. In the same way, that constitutive expression of BCL6 in germinal center cells contributes to formation of diffuse large B-cell lymphomas (74), it is reasonable to hypothesize that MLL fusion–driven constitutive expression of BCL6 might also contribute to B-ALL leukemogenesis. Importantly, our data indicate that MLLr B-ALLs are addicted to BCL6 and require its presence to maintain proliferation and survival. This scenario is quite different than the recently described role of BCL6 as an imatinib treatment–inducible factor that protects BCR–ABL1-positive B-ALL cells from the toxic effects of ABL kinase blockade (75). This current study expands the paradigm of BCL6-targeted therapy to an aggressive form of B-ALLs and supports the rationale for developing clinical trials of BCL6 inhibitors specifically for MLLr B-ALLs. Altogether, it appears that MLL fusion proteins drive expression of FLI1, BCL6, and other key downstream target genes through the dual epigenetic activating effects of inducing and maintaining aberrant DNA hypomethylation and aberrant H3K79 dimethylation.

A small previous study combining DNA methylation profiling, gene expression profiling, and ChIP-on-chip in 5 patients with leukemia illustrated the proof of principle that triangulating these platforms allowed more depth characterization of gene deregulation than single platforms alone (76). In manner similar to recent studies in AML showing that DNA methylation patterns can be used to identify new pathogenic mechanisms (14), this current analysis of adult patients with B-ALLs enrolled in ECOG E2993 phase III clinical study shows the power of the integrative epigenomic approach to identify disease mechanisms and presents prognostic biomarkers and therapeutic targets. These data point the way for future functional studies to explore in depth how BCR–ABL1, E2A–PBX1, and MLL fusions directly or indirectly alter DNA methylation during malignant transformation and to test the role of IL2RA signaling in mediating chemotherapy-resistant disease. Most importantly, we expect these studies to trigger therapeutic trials of BCL6 inhibitors in MLL leukemia and the application of CD25 as a putative biomarker for risk stratification in B-ALLs.

METHODS

Patient Samples, Human Cells, and Cell Lines

Pretreatment bone marrow or peripheral blood samples were obtained at diagnosis (before any treatment) from 215 patients enrolled in the Medical Research Council (MRC) UKALLXII/Eastern Cooperative Oncology Group (ECOG) E2993 phase III trial in adult B-lineage ALLs. Clinical details, including age, white blood cell count (WBC), and survival time, were collected by the Clinical Trial Service Unit (CTSU), University of Oxford, Oxford, UK. All patient specimens used in this study were accrued by ECOG. The diagnosis was established by central morphology review and confirmed by multiparameter flow cytometry and reverse transcription-PCR assays in ECOG’s Leukemia Translational Studies Laboratory. Cytogenetic information was reviewed by the ECOG Cytogenetics Committee. The study was approved by the Institutional Review Board of each treatment center. Normal human pre-B or pro-B cells (CD19+ and VpreB+) were sorted from the bone marrows of 12 healthy donors by flow cytometry using antibodies (BD Biosciences) and a FACSVantage SE cell sorter (BD Biosciences). The use of human tissue was in agreement with research ethics board of Weill Cornell Medical College (New York, NY). Patient characteristics are given in Supplementary Tables S1 and S2. The human ALL cell lines RS4-11, SEM, BEL-1, CCRF-CEM, 697, Nalm6, REH, BV173, NALM1, SUP-B15, TOM1, and MUTZ5 were obtained fresh from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany). The expression of aberrant fusion proteins was verified by Western blot analysis and qPCR.

Array-Based Methylation Analysis Using HELP

The HELP (HpaII tiny fragment enrichment by ligation-mediated PCR) assay was conducted as previously published (refs. 31, 33, 77; Supplementary Data). HELP data quality control (QC) and analysis were conducted as described previously (32) using R software (78). Basically, raw data (.pair) files were generated using NimbleScan software. Signal intensities at each HpaII-amplifiable fragment were calculated as a robust (25% trimmed) mean of their component probe-level signal intensities. Any fragments found within the level of background MspI signal intensity, measured as 2.5 mean absolute deviation (MAD) above the median of random probe signals, were considered as “failed” probes and removed. After QC processing, a median normalization was conducted on each array by subtracting the median log ratio (HpaII/MspI), resulting in median log ratio of 0 for each array. HELP Array Data Analysis

Identification of the aberrantly methylated genes for each B-ALL subtype compared with the normal pre-B cells was conducted using an ANOVA test followed by Dunnett post hoc test (34) using the normal pre-B samples as the reference group and multiple testing corrected by the Benjamini–Hochberg method (35). R Package “multcomp” Version 1.2-6 was used (34). The comparison between the positive and negative groups of BCR–ABL1, E2A–PBX1, and MLL was determined by the Student t test and the Benjamini–Hochberg adjusted P values. As the methylation difference between ALL and normal pre-B cells is more apparent than the methylation difference among the ALL subtypes, to capture and prioritize the top differentially methylated genes, we used a higher threshold (which corresponds to 30% methylation difference) in the ALL versus normal pre-B comparison, and a lower threshold (which corresponds to 20% methylation difference) in the ALL subtype comparison. On the basis of the linear regression line in MassArray EpiTyper validation (Supplementary Fig. S1, equation: y = −4.9484x + 3.3232, where x is MassArray readout and y is HELP readout), a log2 ratio difference (dx) of 1 and 1.5 from HELP data corresponds to methylation difference of 20% and 30%, respectively.

Single Locus Quantitative DNA Methylation Assays

MassArray EpiTyper assays (Sequenom) were conducted on bisulfite-converted DNA, as previously described (79). The primers were designed using Sequenom EpiDesigner beta software (Sequenom, Inc.), and data were analyzed using EpiTyper software version 1.0 (Sequenom, Inc.). Note that MassArray and qPCR validation studies were conducted in different sets of randomly selected specimens with available DNA and RNA, from among the cohort of 215 profiled E2993 patients.
Cell Culture

The human B-ALL cell lines were maintained in Roswell Park Memorial Institute medium (RPMI-1640, Invitrogen) with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Primary B-ALL cells were cultured in Iscove's modified Dulbecco's medium (IMDM, Invitrogen) with 10% FBS, LDL, 1× β-mercaptoethanol, 2 mmol/L l-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, recombinant human FLT3 ligand (rhFLT3) (20 ng/mL), rhSCF (50 ng/mL), IL3 (20 ng/mL), IL6 (20 ng/mL), and IL7 (10 ng/mL, Invitrogen). Cell cultures were kept at 37°C in a humidified incubator under a 5% CO2 atmosphere.

MLL-AF4 and E2A-PBX1 Knockdown

The MLL-AF4 knockdown in RS4;11 and SEM cells was carried out as described in the work of Thomas and colleagues (ref. 48; Supplementary Data). Synthetic sense and antisense oligonucleotides specific for the MLL-AF4 fusion sites were synthesized by Dharmacon RNAi Technologies. RNA was extracted 48 hours after transfection, and the levels of genes were determined by qPCR. The E2A-PBX1 knockdown in 697 cells was carried out using an E2A-shRNA that targets the middle coding region (AA 340–348) of E2A (E12/E47) and therefore affects both the E2A and E2A fusions (E2A–PBX1a and E2A–PBX1b). The shRNA vectors were obtained from Open Biosystems, clone ID: TRCN0000017534. The lentivirus was prepared as described in the Addgene website (Addgene). RNA was extracted 96 hours postinfection and transcript abundance was determined by qPCR.

Western Blot

For BCL6 Western blot analysis, rabbit antibody raised against BCL6-N3 (sc-838) and anti-actin-horseradish peroxidase (HRP) conjugated (sc-1615) was purchased from Santa Cruz Biotechnology. For the Western blot experiments in MLL-AF4 siRNA knockdown, SEM and RS4;11 cells were treated with control and MLL-AF4 siRNAs as described. A total of 4×10^6 cells were collected, and whole-cell extracts were made via resuspension in a modified radioimmunoprecipitation assay (RIPA) buffer (50 mmol/L HEPES-KOH, pH 7.6, 500 mmol/L LiCl, 1 mmol/L EDTA, 1% NP-40, 0.7% Na-Decosylcylolate, ddH2O) for 30 minutes at 4°C. Whole-cell extracts were split in half and loaded onto either a 3%-8% Tris-Acetate gel (NuPAGE, Life Sciences) or a 4%-12% Bis-Tris gel (NuPAGE, Life Sciences). gels were blotted onto PVDF membranes and probed with BCL6 antibodies (as described), anti-HOXA9 (Millipore, 07-178), anti-AF4-C (Abcam, ab31812), or anti-GAPDH (Bethyl, A300-641A). RS4;11 Western blot bands were quantitative relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using a standard protocol on ImageJ (80).

Cell Viability Assay

One hundred thousand MLL-AF4 B-ALL cells from the cell lines or 200,000 primary cells were seeded in a volume of 100 μL medium (as described in “Cell Culture”) per well. RI-BPI or CPs were diluted in medium and added at the indicated concentration in a total culture volume of 120 μL. After culturing for 48 hours, cell viability was measured by flow cytometry using Annexin V-fluorescein isothiocyanate (FITC; BD Biosciences) and 7-AAD (Molecular Probes-Invitrogen) to detect phosphatidylserine exposition and cell permeability, respectively. At least 30,000 events were recorded per condition on an LSR II flow cytometer (BD Biosciences). Data analysis was conducted using FlowJo 8.2 software for Mac OS X (TreeStar). Cells that were negative for Annexin V and 7-AAD were scored as viable. Fold changes were calculated using baseline values of untreated or the CP-treated cells as a reference.

ChIP and qChIP

The human B-ALL cell lines were maintained in Roswell Park Memorial Institute medium (RPMI-1640, Invitrogen) with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Primary B-ALL samples, 25,000 cells per sample were treated with RI-BPI, CP, or vehicle for 48 hours and plated in M3434 methylcellulose (Stem Cell Technologies) with IL7 (10 ng/mL, Invitrogen). Colonies were counted after 14 days.

ChIP-seq

See Supplementary Data for details. Peaks from ChIP-seq data were called using the ChiPseeker program (42) with parameters indicated in Supplementary Table S14.

Survival Analysis

Kaplan–Meier survival analysis was used to estimate OS, and log-rank test was used to compare survival differences between patient groups. R package “survival” version 2.35-8 (78) and “proc lifetest” in SAS was used for the survival analysis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Conception and design: H. Geng, W.-Y. Chen, R.G. Roeder, E. Paietta, O. Elemento, A.M. Melnick

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References

tion by DOT1L. Cancer Cell 2011;20:66–78.
24. Stumptel DJ, Schneider P, van Roon EH, Boer JM, de Lorenzo P, Valsecchi MG, et al. Specific promoter methylation identifies differ-
tent subgroups of MLL-rearranged infant acute lymphoblastic leu-
Integrative Epigenomics of Adult B-ALL

Integrative Epigenomic Analysis Identifies Biomarkers and Therapeutic Targets in Adult B-Acute Lymphoblastic Leukemia

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