Cooperating leukemogenic events in MLL-r rearranged (MLL-r) infant acute lymphoblastic leukemia (ALL) are largely unknown. We explored the role of promoter CpG island hypermethylation in the biology and therapeutic targeting of MLL-r infant ALL. The HELP (HpaII tiny fragment enrichment by ligase-mediated polymerase chain reaction [PCR]) assay was used to examine genome-wide methylation of a cohort of MLL-r infant leukemia samples (n = 5), other common childhood ALLs (n = 5), and normals (n = 5). Unsupervised analysis showed tight clustering of samples into their known biologic groups, indicating large differences in methylation patterns. Global hypermethylation was seen in the MLL-r cohort compared with both the normals and the others, with ratios of significantly (P < .001) hypermethylated to hypomethylated CpGs of 1.7 and 2.9, respectively. A subset of 7 differentially hypermethylated genes was assayed by quantitative reverse-transcription (qRT)-PCR, confirming relative silencing in 5 of 7. In cell line treatment assays with the DNA methyltransferase inhibitor (DNMTi) decitabine, MLL-r (but not MLL wild-type cell lines) showed dose- and time-dependent cytotoxicity and re-expression of 4 of the 5 silenced genes. Methylation-specific PCR (MSP) confirmed promoter hypermethylation at baseline, and a relative decrease in methylation after treatment. DNMTi may represent a novel molecularly targeted therapy for MLL-r infant ALL. (Blood. 2010;115(23):4798-4809)

Introduction

The 5-year event-free survival of childhood acute lymphocytic leukemia (ALL) now exceeds 80%.1 However, the outcome for infants with ALL is substantially worse. Despite intensified treatment, the event-free survival for children younger than 12 months with ALL is only 30% to 40%.2 Because of this, distinct mechanisms of leukemogenesis need to be explored to develop a rational basis for the design of novel therapies in this disease.

Infant ALL has long been known to be an outlier from other childhood lymphocytic leukemias. It is clinically distinct in presentation and is less sensitive to effective ALL chemotherapy.3-5 In addition, although morphologically and histochemically lymphoblastic, it is immunophenotypically unique, as it usually lacks the early lymphocyte antigen CD10 and has a propensity to coexpress myeloid antigens including CD15 and CD65.6 Finally, it is characterized by an extremely high incidence of reciprocal translocations involving the mixed-lineage leukemia (MLL, ALL1, HRX) gene.7 In comparison with ALL in older children, where MLL rearrangements (MLL-r’s) are demonstrated in only 8% of cases,8 most studies demonstrate the rate in infants to be 70% to 80%, particularly for younger infants.8,9 Considered together, although there are now more than 60 characterized fusion partners of MLL,10 these findings have established MLL-r ALL to be a unique leukemia. This has been corroborated by gene expression profiling, which has shown MLL-r ALL to be easily differentiated from MLL wild-type (MLL-wt) ALL and acute myeloid leukemia (AML).11-14

The origin of MLL-r leukemia is an area of active investigation but several well-established mechanisms of leukemogenesis in other leukemias have been ruled out as contributing to the disease in infants. For example, a recent large genome-wide, high-density, single nucleotide polymorphism study of childhood ALL demonstrated a striking paucity of copy number alterations in MLL-r cases in comparison with other leukemias.15 In addition, although activating JAK mutations have been demonstrated in 10% of MLL-wt high-risk pediatric leukemias, they have not been discovered in MLL-r infant leukemia.16 Furthermore, a survey of the kinome in MLL-r infant leukemia samples found no activating tyrosine kinase mutations, other than a low rate of FLT3 mutations, in 30 samples (Melissa Wright, P.B., D.S., unpublished data, December 2008).

Several theories have been proposed to explain the leukemogenic potential of the MLL oncprotein. These have included transcriptional activation, chromatin structure changes, association with signal transduction, dimerization or oligomerization leading to altered DNA binding, recruitment of transcriptional effector molecules, and sequestration of cofactors resulting in dominant-negative effects on target gene expression.17 Because an alteration of gene expression profiles is the common result of all of these hypothesized functions, it has been suggested that a unifying mechanism of MLL-r oncogenesis may be epigenetic regulation.17 Several groups have started examining the influence that histone modification may play in MLL-r oncogenesis.18 DNA methylation has yet to receive as much attention. However, it is known that 1 of the 2 essentially retained N-terminal domains necessary to produce an activated MLL oncprotein with leukemic potential is a 100-amino acid area that displays homology to the regulatory portion of eukaryotic DNA methyltransferase 1 (DNMT1) and has
be termed the MT domain. In addition, the MLL MT domain specifically recognizes unmethylated CpG dinucleotide sequences and has been shown to be transcriptionally repressive.

The epigenetic phenomenon of CpG island hypermethylation in tumor suppressor gene promoters, leading to repression and silencing of expression, is an important contributor to oncogenesis. To test the hypothesis that CpG island hypermethylation may contribute to oncogenic transformation in MLL-r infant leukemia, we explored the role of global gene promoter hypermethylation in this disease. Here we show that MLL-r infant leukemia is globally hypermethylated in promoter CpG islands in comparison with other forms of childhood leukemia and normal controls, that the genes regulated by these promoters are largely repressed or silenced, and that in many cases these genes can be re-expressed when cells are treated with the demethylating agent decitabine. Thus, decitabine might be an effective antileukemic agent in infant ALL.

### Methods

#### Cells

Primary patient samples were collected from patients treated at the Johns Hopkins Hospital, The Children’s Hospital of Philadelphia, or Children’s Mercy Hospital and healthy donors between 2004 and 2008. Samples were collected under the respective center’s institutional review board–approved cell procurement protocols for newly diagnosed infants and children with ALL. Informed consent was obtained in accordance with the Helsinki protocol. Samples were enriched from diagnostic bone marrow collections by Ficoll-Hypaque centrifugation and stored in 5 hours of collection in 90% fetal bovine serum (FBS) and 10% dimethyl sulfoxide at 80°C until use. The diagnosis of ALL was based on morphology and flow cytometric analysis of immunophenotype. Cytogenticics were determined by standard procedures.

The NALM6, KOPN8, 380, and TANOUE cell lines were originally obtained from German Collection of Microorganisms and Cell Cultures (DSMZ), whereas the HB1119 cell line was provided from the laboratory of Dr Michael Cleary (Stanford University) and the SEMK2 cell line was provided from the laboratory of Dr Carolyn Felix (University of Pennsylvania). Cell lines were maintained in RPMI-1640 plus 10% FBS and 1% penicillin streptomycin at 37°C and 5% CO2.

### DNA methylation analysis by HELP

High-molecular-weight DNA was isolated from Ficoll-Hypaque centrifugation–enriched diagnostic patient bone marrow samples using the PureGene kit (QIAGEN) and following the manufacturer’s instructions and the HELP (HpaII fragment) assay was carried out as previously described. All samples for microarray hybridization were processed by the Microarray Facility at the Cornell University Life Sciences Laboratories Center. Samples were labeled using cyanin-labeled random primers (9 mers) and then hybridized onto a human HG17 custom-designed oligonucleotide array (50-mer) covering 25 626 HpaII-amplifiable fragments located at gene promoters and imprinted regions. HpaII-amplifiable fragments are defined as genomic sequences contained between 2 flanking HpaII sites found within 200 to 2000 bp from each other. Annotation was performed using the University of California Santa Cruz (UCSC) Genome Browser 2006 human assembly to identify genes located within 2000 bp 5’ to 2000 bp 3’ of the HpaII fragment. Each HpaII-amplifiable fragment on the array is represented by 15 individual probes distributed randomly across the microarray slide. Scanning was performed using a GenePix 4000B scanner (Molecular Devices) as previously described. Quality control was performed as previously described. DNA methylation was measured as the log(MspII/HpII) ratio, where HpaII reflects the hypomethylated fraction of the genome (as HpaII is methylation-sensitive and therefore recognizes 5’-CCCG-3’ only when both cytosines are unmethylated) and MspI represents the whole genome reference (as MspI is methylation insensitive and recognizes 5’-CCGG-3’ regardless of cytosine methylation status). The raw data were processed using the Bioconductor oligo package (http://www.bioconductor.org/packages/release/bioc/html/oligo.html). The red and green channels were quantile normalized.
separately. For each channel, we summarized intensities in each probe set (representing HpaII fragments) using median polish as done by Robust Multi-array Average (RMA). Then, $M$ values were calculated using the log (base 2) ratio of the green divided by the red channel intensity. Note that the higher values of $M$ represent more methylation for the region associated with the respective probe set. For the unsupervised clustering, we first computed the standard deviation (SD) of the $M$ values for each probe set. We filtered probe sets with low SD values. Specifically, we required the SD to be larger than the median SD across all probe sets. Note that this filtering step is necessary because most regions do not exhibit biologic variation, but rather, vary because of measurement error. Hierarchic clustering was applied to the $M$ values of the probe sets surviving this filtering step. Note that class label information was not used in any of these steps. To find differentially methylated genes among the 3 groups (MLL-r, MLL-wild-type [MLL-wt], normal), we processed the raw data with RMA and computed a moderated $t$ statistic and $P$ value (adjusted for multiple comparison) using limma for each probe for each pairwise comparison (MLL-r vs MLL-wt, MLL-r vs normal, MLL-wt vs normal). All microarray data have been submitted to the Gene Expression Omnibus repository (accession no. GSE19671).

Figure 2. MLL-r primary samples show global promoter hypermethylation compared with MLL-wt ALLs and normal controls in the HELP assay. Analysis of global methylation differences between the study groups is shown. Hierarchic clustering using a subset of probes, which demonstrated highly significant differences between groups ($P < .001$), was used to generate heat maps. Heat maps are shown for comparisons between (A) MLL-r ALL versus MLL-wt ALL, (B) MLL-r versus normal controls, and (C) MLL-wt ALL versus normal controls. Individual samples are represented on the heat map as columns, whereas individual probe sets are represented as rows. It is again evident that the groups cluster together and that within each group of samples there are certain probes that are differentially hypermethylated (red) or hypomethylated (blue). (D) Numeric representation of the heat maps shows that for the MLL-r group, there were 1.7- and 2.9-fold more probes, respectively, demonstrating relative hypermethylation than hypomethylation compared with either the other ALL samples or the normal marrow controls. This difference was not evident when MLL-wt ALL samples were compared with normal controls.
Cytotoxicity assay

Cytotoxicity assays were performed on cell lines grown in RPMI-1640 plus 10% FBS plus 1% penicillin streptomycin with media refreshed for optimal survival. Each cell line was split into 5 flasks containing 250 mL of media and 150 million total cells just before treatment. 5-Aza-2’-deoxycytidine (decitabine; Sigma-Aldrich) was dissolved in diethylpyrocarbonate (DEPC)–treated water at a working stock concentration of 1mM. It was then filtered through a 0.22-μm syringe membrane before use. Decitabine was introduced into each culture flask within 1 hour of being dissolved, so that final concentrations of the drug were 0, 0.5, 1, 2, and 4μM on day 1 of the experiment. MTI (3,4,5-dimethylthiazol-2,5-diphenyltetrazolium) assays were performed on cells extracted under sterile conditions from the treatment flasks and introduced into a 96-well plate. Extractions for each drug concentration were performed in triplicate. The MTI plate was prepared from the flasks at 8, 24, 48, and 72 hours from introduction of drug, per the manufacturer’s instructions (Roche) and read using a Bio-Rad model 680 plate reader.

DNA and RNA extraction

Ten million cells were removed from the cytotoxicity assay flasks at time points 0, 2, 4, 8, 24, 48, and 72 hours of treatment from which DNA and RNA were extracted for further analysis. DNA was extracted using the QiAamp DNA Mini Kit (QIAGEN) and RNA was extracted using the RNeasy Mini Kit (QIAGEN) per the manufacturer’s instructions.

Gene expression assay

Gene expression assays were performed on both primary samples and cell lines treated in the cytotoxicity assay. Complementary DNA was produced from RNA recovered, as described in “DNA and RNA extraction,” by treatment with reverse transcriptase by standard procedures. Quantitative reverse-transcriptase PCR (qRT-PCR) was performed using materials from TaqMan Gene Expression Assays (Applied Biosystems) and carried out per the manufacturer’s instructions. PCR was conducted using a Bio-Rad real-time iCycler and threshold cycle (Ct) results were calculated using the manufacturer’s instructions. PCR was conducted following the protocol written for the EZ DNA Methylation Kit (Zymo Research) per the manufacturer’s instructions. PCR was carried out following the protocol written for methylation-specific PCR (MSP) by Licchesi and Herman.31 MSP primers for DAPK1 and p7332 were designed using MethPrimer (University of California, San Francisco).36 MSP primers for CCR6 were designed using the UCSC Genome Browser human assembly (March 2006). The DNA sequence was explored from 1500 bp 5’ to 200 bp 3’ of the CCR6 start codon, assumed to be the gene’s promoter region,35 for CpG islands and appropriate MSP primers using MethPrimer (University of California, San Francisco).36 MSP primers for CCR6 were as follows: forward methylated

\[
\text{test. Copy number fold change was calculated by } \Delta \Delta C_t \ (\Delta C_t \text{ at time } 0 - \Delta C_t \text{ at time } 0 + n).
\]

Data mining from previously published microarrays

Three large-sample gene expression arrays have been published comparing MLL-r ALL samples to MLL-wt ALL samples.31,32,34 Raw data from the arrays are available in public databases (Armstrong et al, http://www.broadinstitute.org/cgi-bin/cancer/publications/pub_paper.cgi?mode=view&paper_id=63; Yeoh et al, http://www.stjuderesearch.org/data/ALL1/index.html; and Ross et al, http://www.stjuderesearch.org/data/ALL3/dataFiles.html, all accessed in December 2009). Datasets were mined for our “genes of interest” using the Affymetrix probe numbers for the appropriate gene chip as published by http://www.gene_cards.org/. When greater than one probe was annotated to a particular gene of interest, all probes were given equal weight in our analysis. Average relative fluorescence was calculated for each relevant probe. For the Armstrong et al data, MLL-r ALL samples were compared with MLL-wt ALL samples. For the Yeoh et al and Ross et al data, MLL-r ALL samples were compared with a combination of TEL-AML1-r ALL and hyperdiploid ALL samples. Statistical significance for each gene was calculated using a 2-tailed distribution, 2-sample unequal Student t test.

Table 2. Genes of interest from HELP analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene full name</th>
<th>Location</th>
<th>Comparison</th>
<th>ΔM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIFR</td>
<td>Leukemia inhibitory factor receptor-α</td>
<td>Chr 5p13-p12</td>
<td>MLL vs hyperdiploid</td>
<td>3.60</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>CCR6</td>
<td>Chemokine (C-C motif) receptor 6</td>
<td>Chr 6p27</td>
<td>MLL vs normal controls</td>
<td>3.76</td>
<td>&lt; .001</td>
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<tr>
<td>DAXX</td>
<td>Death-associated protein 6</td>
<td>Chr 6p21.3</td>
<td>MLL vs normal controls</td>
<td>3.43</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>DAKP1</td>
<td>Death-associated protein kinase 1</td>
<td>Chr 9q34.1</td>
<td>MLL vs normal controls</td>
<td>6.22</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>CASP9</td>
<td>Caspase 9</td>
<td>Chr 1p36.3-36.1</td>
<td>MLL vs MLL-wt ALL</td>
<td>3.84</td>
<td>.005</td>
</tr>
<tr>
<td>HRK</td>
<td>Harakiri</td>
<td>Chr 12q24.22</td>
<td>MLL vs MLL-wt ALL</td>
<td>4.17</td>
<td>.005</td>
</tr>
</tbody>
</table>

To complete the list of genes for qRT-PCR analysis from Table 2, 6 additional genes were selected. One gene, FHT, was selected based on known reports of it being hypermethylated in MLL-r infant leukemia; 3 genes (FLT3, HOXA9, and MEIS1) were selected based on their known importance in MLL-r infant leukemia; and 2 housekeeping genes, ABL and GAPDH, were also selected.

Table 3. Genes of interest from literature

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene full name</th>
<th>Chromosome</th>
<th>Rationale for selection</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLT3</td>
<td>FMS-like tyrosine kinase 3</td>
<td>Chr 13q12.2</td>
<td>Known to be up-regulated and of biologic importance in MLL-r infant ALL29</td>
</tr>
<tr>
<td>HOXA9</td>
<td>Homeobox A9</td>
<td>Chr 7p15-p14</td>
<td>Known to be up-regulated and of biologic importance in MLL-r infant ALL11</td>
</tr>
<tr>
<td>MEIS1</td>
<td>MEIS-homebox 1</td>
<td>Chr 2p14-p13</td>
<td>Known to be up-regulated and of biologic importance in MLL-r infant ALL13</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Chr 12p13.31</td>
<td>Housekeeping gene</td>
</tr>
<tr>
<td>ABL</td>
<td>C-ABL oncogene, receptor tyrosine kinase</td>
<td>Chr 9q34.1</td>
<td>Housekeeping gene</td>
</tr>
</tbody>
</table>
Figure 3.

A

<table>
<thead>
<tr>
<th>Known to be over-expressed in MLL-r ALL</th>
<th>Hypoxia</th>
<th>TEL/AML1</th>
<th>Normal</th>
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<tbody>
<tr>
<td>FLT3</td>
<td>5</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>MEIS1</td>
<td>6</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>HOXa9</td>
<td>14</td>
<td>3</td>
<td>14</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Found CpG island hypermethylated in MLL-r ALL by HELP*</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLT3</td>
</tr>
<tr>
<td>MEIS1</td>
</tr>
<tr>
<td>DAPK1</td>
</tr>
<tr>
<td>CCND1</td>
</tr>
<tr>
<td>HLR</td>
</tr>
<tr>
<td>Hoxa9</td>
</tr>
<tr>
<td>CSE1P9</td>
</tr>
</tbody>
</table>

*No FHIT probe is present on current permutation of HELP microarray; FHIT previously shown to have hypermethylated promoter region CpG island

B

M-H vs. TEL/AML, P<.05
M-H vs. Normal, P<.02

C

M-H vs. MLL-r, P<.03
M-H vs. Normal, P<.03

D

FHIT

E

DAPK1

F

Predicted Up-regulated

Predicted Equivalent

Predicted Down-regulated

G

<table>
<thead>
<tr>
<th>Array-Gene</th>
<th>MLL-r</th>
<th>MLL-wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLT3</td>
<td>4002.275</td>
<td>1584.761</td>
</tr>
<tr>
<td>MEIS1</td>
<td>2042.192</td>
<td>1096.005</td>
</tr>
<tr>
<td>HOXa9</td>
<td>2156.660</td>
<td>1096.005</td>
</tr>
<tr>
<td>FHIT</td>
<td>791.15</td>
<td>2474.666</td>
</tr>
<tr>
<td>DAPK1</td>
<td>213.65</td>
<td>814.154</td>
</tr>
<tr>
<td>CCND1</td>
<td>2647.123</td>
<td>3367.368</td>
</tr>
<tr>
<td>LIFR</td>
<td>559.05</td>
<td>1999.071</td>
</tr>
<tr>
<td>HRK</td>
<td>406.7</td>
<td>796.195</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Yeoh et al. (2002) - U95A</th>
<th>MLL-r</th>
<th>MLL-wt</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLT3</td>
<td>4002.275</td>
<td>1584.761</td>
<td>0.008</td>
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<tr>
<td>MEIS1</td>
<td>2042.192</td>
<td>1096.005</td>
<td>0.003</td>
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<td>HOXa9</td>
<td>2156.660</td>
<td>1096.005</td>
<td>0.001</td>
</tr>
<tr>
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<td>791.15</td>
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<td>0.007</td>
</tr>
<tr>
<td>DAPK1</td>
<td>213.65</td>
<td>814.154</td>
<td>0.016</td>
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<td>CCND1</td>
<td>2647.123</td>
<td>3367.368</td>
<td>0.076</td>
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<td>LIFR</td>
<td>559.05</td>
<td>1999.071</td>
<td>0.032</td>
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<tr>
<td>HRK</td>
<td>406.7</td>
<td>796.195</td>
<td>0.003</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ross et al. (2003) - U133A</th>
<th>MLL-r</th>
<th>MLL-wt</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLT3</td>
<td>5125.405</td>
<td>859.375</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MEIS1</td>
<td>5269.008</td>
<td>90.851</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HOXa9</td>
<td>1795.635</td>
<td>40.888</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FHIT</td>
<td>180.23</td>
<td>1227.448</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DAPK1</td>
<td>288.452</td>
<td>810.254</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CCND1</td>
<td>452.87</td>
<td>512.790</td>
<td>0.006</td>
</tr>
<tr>
<td>LIFR</td>
<td>93.245</td>
<td>186.588</td>
<td>0.02</td>
</tr>
<tr>
<td>HRK</td>
<td>603.575</td>
<td>513.172</td>
<td>0.048</td>
</tr>
</tbody>
</table>

*Where applicable, for FLT3 analysis, MLL-r samples were combined with hyperdiploid samples and compared to "other ALL" secondary to the fact that with MLL-r ALL, Hyperdiploid ALL is known to show overexpression of FLT3
*Where applicable, negative relative fluorescence array data was normalized to zero with value added to corresponding samples
Results

Childhood ALL subtypes and MLL-r infant ALL samples have differential methylation patterns; MLL-r infant samples are characterized by several hypermethylated gene promoter regions.

We hypothesized that gene promoter hypermethylation might be a characteristic of MLL-r infant ALL. Therefore, we carried out a genome-wide DNA methylation study using the HpaII tiny fragment enrichment by ligation-mediated PCR (HELP) assay. This technique has been shown to accurately identify the DNA methylation levels of CpG dinucleotides throughout the genome.23,37,38 We processed 15 samples, which included precursor B-cell ALL from infants carrying an MLL-r (n = 5), MLL-wild-type (wt) childhood leukemias including precursor B-cell ALL with a TEL-AML-r (n = 3), and precursor B-cell ALL with a hyperdiploid karyotype (n = 2) and normal controls of CD19-r-enriched cord blood (n = 3) and CD34-r-enriched cord blood (n = 2). The HELP assay was performed in this case using a long-oligonucleotide microarray representing the DNA methylation level of greater than 50,000 CpGs corresponding to 14,000 promoter regions.

The HELP data were initially processed using an unsupervised analysis, in which the samples largely clustered into their biologic subtypes (Figure 1). All of the MLL-wt samples clustered together and separately from the MLL-r and normal samples. Furthermore, within the MLL-wt cohort, the TEL-AML-r samples and hyperdiploid samples further separated into subgroups. Although the MLL-r and normal samples were all contained within the same branch of the dendrogram, there was tight clustering of 4 of the 5 MLL-r samples and 4 of the 5 normal samples. This suggests that there are intragroup similarities and intergroup differences in the promoter methylation patterns of infant ALL and childhood ALL. These patterns may be reflective of each leukemia’s unique underlying biology.

We extended the analysis of the HELP data by creating a supervised list of the most differentially (P < .001) methylated HpaII sites for each of 3 pairwise comparisons of the biologic groups (MLL-r vs MLL-wt, MLL-r vs normal, MLL-wt vs normal). The heat map for each of these comparisons is shown in Figure 2A, B, and C, respectively. The number of significantly hypermethylated and significantly hypomethylated CpG sites for each comparison and the ratio of these numbers are shown in Figure 2D. It is evident that the MLL-r cohort is globally hypermethylated compared with both the MLL-wt leukemia cohort (ratio, 2.9) and the normal cohort (ratio, 1.7). The MLL-wt and the normal cohorts were similarly methylated (ratio, 1.0). Thus, global CpG island hypermethylation is comparatively specific to MLL-r infant ALL within this set of childhood lymphoid leukemias.

Results of qRT-PCR assay of gene expression strongly correlate with HELP data and are consistent with known leukemia biology.

We selected a set of 12 genes to further analyze using qRT-PCR. Six genes were selected by correlating HpaII sites that were differentially hypermethylated in MLL-r samples (P < .001) with their list of annotated genes (DAPK1, DAXX, CCR6, CASP9, LIFR, and HRK). We focused on these genes because they had previously been shown in the literature to be silenced, hypermethylated, epigenetically influenced, or related to tumor suppression or apoptosis in cancer. Four further genes were selected on the basis of known importance in MLL-r leukemia biology (FLT3, HoxA9, MEIS1, and FHIT). FLT3 is known to be highly expressed in MLL-r infant leukemia as well as precursor B-cell ALL with hyperdiploid cytogenetics.39 MEIS1 and HoxA9 have also been shown to be highly expressed in MLL-r leukemia.40 FHIT, on the other hand, has been demonstrated to be silenced due to promoter CpG methylation in MLL-r leukemia.41 Finally, 2 genes were selected as housekeeping genes (GAPDH and ABL; Tables 2-3). Reverse-transcriptase TaqMan qRT-PCR for all selected genes was run on each primary sample and normal controls. Results are shown in heat map format (Figure 3A). All 3 of the genes for which expression in MLL-r cases was expected to be relatively high were confirmed by qRT-PCR (supplemental Table 1, available on the Blood Web site; see the Supplemental Materials link at the top of the online article). This difference was particularly notable for FLT3 (Figure 3B) and MEIS1 (Figure 3C), in which these differences were highly significant. Of the 7 genes for which expression in MLL-r cases was expected to be relatively low because of differential promoter hypermethylation, 5 showed either statistical significance or a trend toward statistical significance in down-regulation or silencing. The only exceptions were LIFR, which was silenced in all, and CASP9, which was expressed at similar levels in all. The most striking differences were seen for FHIT (Figure 3D) and DAPK1 (Figure 3E).

Comparison with published gene expression microarrays validates TaqMan qRT-PCR results.

To further validate that our TaqMan qRT-PCR results were generalizable to both the MLL-r and MLL-wt populations, we compared our gene expression data with 3 published large-sample arrays comparing MLL-r ALL to MLL-wt ALL.31,13,14 Based on our

Figure 3. TaqMan qRT-PCR on genes of interest shows preferentially silenced or decreased expression in MLL-r primary samples in comparison with MLL-wt ALL primary samples and in normal controls in comparison with published gene expression microarrays validating results. The 12 genes described in Tables 2 and 3 were analyzed by qRT-PCR on the HELP primary samples and 2 peripheral blood samples from healthy donors (PB). The results are summarized in the heat map (A), which has the genes of interest represented by rows and individual samples, by columns. Each box shows the ΔCt (gene Ct – GAPDH Ct), such that higher numbers indicate lower RNA expression. In addition, the heat map is color-coded based on its biology as described in the key. The color code was determined by natural peaks in a histogram of all Cts in the cohort. This histogram is also shown in the key. The MLL-r samples show a greater number of silenced genes and more underexpressed genes than do the other leukemias or normals. Ct values were then converted to relative gene expression using the 2(-ΔΔCt) method, and MLL-r expression levels were compared with MLL-wt and normal control levels (raw data in supplemental Table 1). By dot plot, many of the genes show statistically significant differences in gene expression between groups. In analysis, FLT3 (B) was statistically significantly up-regulated in the combination of MLL-r infant leukemia (M) and hyperdiploid (H) leukemia compared with normals; in addition, MEIS1 (C) was shown to be up-regulated in MLL-r leukemia; both of these phenomena are consistent with published literature. Finally, there was statistically significant down-regulation on FHIT (D) and DAPK1 (E) in MLL-r leukemia compared with “other leukemias” and normal controls. (F) To further validate our qRT-PCR results, we compared our data to 3 published gene expression microarrays. Based on our qRT-PCR results (A-E and supplemental Table 1), we predicted that the genes FLT3, MEIS1, and HoxA9 were up-regulated in MLL-r ALL in comparison with MLL-wt ALL, that the genes DAXX, LIFR, and CASP9 had equivalent expression in MLL-r ALL and MLL-wt ALL, and that the genes FHIT, DAPK1, CCR6, and HRK were down-regulated in MLL-r ALL in comparison with MLL-wt ALL. (G) Based on 3 independent gene expression microarrays using 2 different Affymetrix platforms (HG-U95A and HG-U133A) published by Armstrong et al (A),11 Yeoh et al (Y),14 and Ross et al (R),13 we confirmed the predictions.
qRT-PCR results (Figure 3A-E and supplemental Table 1), we predicted that the genes FLT3, MEIS1, and HOXa9 were up-regulated in MLL-r ALL in comparison with MLL-wt ALL, that the genes DAXX, LIFR, and CASP9 had equivalent expression in MLL-r ALL and MLL-wt ALL, and that the genes FHIT, DAPK1, CCR6, and HRK were down-regulated in MLL-r ALL in comparison with MLL-wt ALL. When our results were compared with the 3 independent gene expression microarrays, which used 2 different Affymetrix platforms (HG-U95A and HG-U133A), we confirmed the predictions (Figure 3F). Both predicted up-regulated genes and predicted down-regulated genes were shown differentially expressed between MLL-r and MLL-wt ALL with robust statistical significance (Figure 3G).

**Treatment with the demethylating agent 5-Aza-2’-deoxycytidine (decitabine) selectively kills MLL-r precursor B-cell ALL cell lines and can be correlated with the re-expression of several silenced genes**

Having shown that the MLL-r infant ALL samples contained an increased number of hypermethylated gene promoters in comparison with childhood MLL-wt ALL and normal controls, and that 5 of the 7 genes with hypermethylated promoters were silenced or down-regulated, we investigated whether demethylating agents might serve to reverse aberrant methylation signaling and allow gene re-expression. In addition, if MLL-r infant ALL promoter hypermethylation follows the epigenetic paradigm of several other cancers, reversal of silencing of key tumor suppressor genes may lead to cytotoxicity of leukemia cells. Thus, ALL cell lines carrying the MLL-AF4 translocation (SEMK2), MLL-ENL translocation (KOPN8 and HB1119), and wild-type MLL (NALM6, 380 and TANOUE) were treated with the DNA methyltransferase inhibitor 5-aza-2’-deoxycytidine (decitabine) at concentrations of 0, 0.5, 1, 2, and 4 μM for 72 hours. We performed MTT assays at 8, 24, 48, and 72 hours and isolated RNA from each sample at 0, 2, 4, 8, 24, 48, and 72 hours. MLL-wt cell lines all showed robust time- and dose-dependent cell kill by 72 hours (Figure 4A-C,G). Although decitabine was clearly cytotoxic to the MLL-r cell lines, there was little response in the MLL-wt cell line (Figure 4D-G).

Biologic correlates to the MTT assay results were seen in a parallel examination of the treated cells using TaqMan gene
expression assays. As noted, the genes FHIT, DAPK1, CCR6, and HRK were shown to be underexpressed or silent in our primary samples. This was also seen in our MLL-r cell lines (supplemental Figure 1). When treated with decitabine, there was re-expression of FHIT, DAPK1, and CCR6 in the SEMK2 cells (Figure 5A), of DAPK1 and CCR6 in the KOPN8 cells (Figure 5B), and of FHIT, DAPK1, CCR6, and HRK in the HB1119 cells (Figure 5C) but no change in expression in the NALM6, 380, and TANOUE cells (Figure 5D-F). The re-expression occurred relatively quickly, with several genes responding in MLL-r cell lines treated with 4 μM decitabine within 24 hours (supplemental Figure 2).

MSP validates HELP assay results and provides evidence that the mechanism of gene re-expression with decitabine treatment is reversal of promoter methylation

To both validate the findings from the HELP assay and verify that the re-expression of the genes DAPK1, HRK, and CCR6 was linked to gene promoter hypermethylation and not a secondary effect of decitabine, we performed methylation-specific PCR (MSP) on primary samples and cell lines both before and after treatment with decitabine. Post–bisulfite-treated methylation-specific primers for the promoter regions of the genes DAPK1 and HRK were based on previously published MSP data, whereas primers for CCR6 were custom designed. Complete bisulfite conversion was assured by treating REH genomic DNA (gDNA) under the same conditions and running MSP for the gene promoters DAPK1 and p73, as it is known that the DAPK1 promoter region is completely unmethylated whereas the p73 promoter is completely methylated in REH cells (Figure 6A).

After assurance of complete unmethylated cytosine to uracil conversion, MSP was carried out on gDNA from all primary samples (Figure 6B) and cell lines (supplemental Figure 3) and densitometry was performed on each amplicon. From densitometry results, the average unmethylated amplicon–methylated amplicon (U:M) ratios were calculated for each biologic subtype, with lower ratios indicating a higher degree of methylation. The DAPK1 (1.08 in MLL-r vs 1.19 in TEL-AML-r, 1.37 in hyperdiploid, and 1.12 in normal controls) and HRK (1.40 in MLL-r vs 1.57 in TEL-AML-r, 2.19 in hyperdiploid, and 1.71 in normal controls) promoters were preferentially methylated in MLL-r ALL cells in comparison with other leukemias and normals. CCR6 promoters had some methylation in all leukemias, but were preferentially methylated in MLL-r ALL and preferentially unmethylated in normals. With the exception of the CCR6 gene, where hyperdiploid leukemias showed greater methylation than MLL-r leukemias, all genes and groups compared either reached or trended toward statistical significance, with MLL-r leukemias having a lower U:M ratio (Figure 6C-D). In cell line analysis, MSP U:M amplicon ratios of genes of interest increased during treatment with decitabine in MLL-r cell lines, indicating that methylation was reversed during the course of treatment (Figure 7). In summary, we found that for the genes identified by the HELP assay and shown in the cell lines to be re-expressed after decitabine treatment, the promoter regions were reversibly methylated. This serves both as a validation of the HELP data and implies that reversal of methylation is the likely mechanism of gene re-expression and cell kill during treatment with decitabine.

Discussion

This study revealed several interesting findings. First, in an unsupervised analysis using HELP data, each of the 3 sample
cohorts (MLL-r leukemias, MLL-wt leukemias, and normal controls) tended to cluster together. Genome-wide methylation studies have been previously used to classify cancers, but unique genome-wide “methylome signatures” have not been previously described in pediatric leukemias. The findings suggest that there are remarkable intragroup similarities and intergroup differences in promoter methylation patterns among the study populations and that these patterns are driven by the underlying biology of each group (primarily determined by recurrent cytogenetic abnormalities in the leukemias). In addition, this lends further evidence suggesting that infant MLL-r ALL is a unique leukemia.

Second, it also demonstrated that MLL-r infant ALL demonstrates global promoter CpG island hypermethylation in comparison with the groups studied here, which included other common childhood ALLs and normal controls. This shows that promoter CpG island hypermethylation is comparatively specific to infant ALL within the subset of childhood lymphoid leukemias, suggesting that therapeutic agents that reverse DNA hypermethylation may have selective antileukemic efficacy in infants with MLL-r ALL. Further studies will be needed to determine whether this finding is specific to infants with MLL-r ALL, or whether it also applies to older children and adults with MLL-r ALL or to patients of any age.
Third, the DNA methyltransferase inhibitor (DNMTi) 5-aza-2'-deoxycytidine (decitabine) was preferentially cytotoxic to MLL-r ALL cell lines, and the mechanism of action of this selective cytotoxicity is likely reversal of promoter methylation and up-regulation of silenced tumor suppressor genes. It is known that DNA methyltransferase (DNMT) is required for the maintenance of promoter hypermethylation and several DNA methyltransferase inhibitors (DNMTIs) have attained Food and Drug Administration approval for myelodysplastic syndromes and are undergoing phase II trials in adult AML and chronic myeloid leukemia. We approval for myelodysplastic syndromes and are undergoing phase II trials in adult AML and chronic myeloid leukemia.44,45 We previously correlated with cancer and tumor progression. However, only DAPK1 has been specifically linked to MLL-r ALL in past studies. The death-associated protein kinase 1 (DAPK1) is part of a 5-member family of proapoptotic serine/threonine kinases that are ubiquitously expressed and are capable of inducing apoptosis.49 DAPK1 is a well-described member of the BH3 family of proapoptotic serine/threonine kinases that are ubiquitously expressed and are capable of inducing apoptosis. DAPK1 is a well-described member of the BH3 family (Harakiri) of proapoptotic serine/threonine kinases that are ubiquitously expressed and are capable of inducing apoptosis.
hypermethylation and silencing have been described previously in solid tumors but not in leukemia.34,60 However, in a recent gene expression array, it was shown to be down-regulated in L-asparaginase resistant B-lineage ALL cells.61 This finding correlates well with the studies cited in this section, noting that infant leukemia blasts in vitro are L-asparaginase resistant.3,4 It is not yet clear how the epigenetic modifications of DAPK1, CCR6, and HRK might influence infant ALL. However, their known role in other cancers and their known importance in cell survival, along with the results from our study, support the concept that their silencing, and reactivation after treatment with decitabine, may well play a role in the biology of MLL-r leukemias.

Limitations of the study include the small number of samples analyzed within each group and the fact that several important subsets of childhood ALL were not represented (eg, Philadelphia chromosome-positive ALL). Definitive conclusions will require further studies with larger samples sizes. It will be of interest, for example, to determine methylation patterns in a wider spectrum of MLL-r infant ALL cases, including samples with other MLL fusion partners (eg, AF9, AF6) and samples from various age groups (eg, <90 days vs >90 days). It will also be of considerable interest to extend this analysis to cohorts of other MLL-r leukemias such as MLL-r ALL in older children and adults and MLL-r AML.

The epigenetic phenomenon of CpG island hypermethylation in tumor suppressor gene promoters is known to be an important contributor to oncogenesis.22 The MLL gene, which is rearranged in 80% of infant leukemia cases, is known to harbor domains with epigenetic activity, and silencing of several tumor suppressor genes in MLL-r infant leukemia has been described in the literature.3,21

In this study, we have demonstrated that silencing of many genes through global promoter region CpG island hypermethylation is a characteristic of MLL-r infant leukemia samples compared with other childhood leukemias and normal controls. In addition, we have demonstrated that the HELP assay can identify individual genes whose promoters are hypermethylated in MLL-r infant ALL and that many of the genes identified are transcriptionally silenced. Finally, we showed not only that the demethylating agent decitabine preferentially kills MLL-r lymphoblastic leukemia cell lines but also that this response correlates with the up-regulation of several of the identified silenced genes. Future studies using increased numbers of primary samples will be important to generalize these findings and to better correlate methylated promoter CpG islands with genome-wide gene expression. This should enable us to better identify a set of biologically important genes that are both hypermethylated and down-regulated in an effort to increase our understanding of leukemogenesis in infant ALL and to determine the ultimate gene set whose re-expression can be targeted with new treatment strategies.

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Authorship

Contribution: E.S. designed and performed research, performed data analysis, and wrote the paper; R.I. performed data analysis and edited the paper; S.N. and E.M. performed research; D.S. contributed to data analysis and edited the paper; M.E.F. performed research; contributed to data analysis, and edited the paper; and A.M. designed research, contributed to data analysis, and edited the paper; P.B. designed research, performed data analysis, and wrote and edited the paper.

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