Epigenetic Profiling in Chronic Lymphocytic Leukemia Reveals Novel Methylation Targets

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ABSTRACT

CpG island methylation is an epigenetic alteration that contributes to tumorogenesis by transcriptional inactivation of genes. Little is known about the overall levels of CpG island methylation in chronic lymphocytic leukemia (CLL). To provide a baseline estimate of global aberrant methylation and identify target sequences for additional investigation, we performed Restriction Landmark Genomic Scanning on 10 CLL samples. Two methylation-sensitive landmark enzymes were used (NotI and Ascl), allowing assessment of over 3000 CpG islands in each sample. Tumor-derived Restriction Landmark Genomic Scanning profiles were compared with profiles from CD19-selected B cells from normal volunteers and matched normal neutrophils from 4 CLL patients. We found 2.5–8.1% (mean 4.8%) of the CpG islands in CLL samples were aberrantly methylated compared with controls, and the methylation events had a nonrandom distribution (P < 0.0001). Furthermore, we identified 193 aberrantly methylated sequences, of which 93% have CpG island characteristics and 90% have homology to genes or expressed sequences. One such gene, the G protein-coupled metabotropic glutamate receptor 7 (GRM7), possibly inhibits cyclic AMP signaling in the induction of apoptosis. Bisulfite sequencing of GRM7 confirmed extensive CpG island methylation, and treatment with 5-aza-2′-deoxycytidine (decitabine) resulted in up-regulated expression of several genes in vitro with concurrent cellular depletion of DNMT1 protein. Our dual-enzyme global methylation study shows that CLL is characterized by widespread nonrandom CpG island methylation similar to other tumors and provides a panel of novel methylation targets that can be used in larger studies designed to assess impact on disease progression and survival.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a clonal malignancy of mature neoplastic B cells characterized by both a low proliferation rate and disrupted apoptosis. It is estimated that there are 7000 new cases and 4500 deaths from CLL in the United States each year (1). Chromosomal abnormalities occur in as many as 80% of CLL cases, and frequently involve deletions at 13q14, 11q23, 17p13, and 6q, as well as trisomy 12 (2). In addition, CLL can be classified by the presence or absence of somatic mutations in the immunoglobulin variable region (3). Whereas many of these genetic changes provide evidence of tumor progression, CLL becomes symptomatic it is an incurable disease that diminishes quality of life and is fatal in most cases.

DNA cytosine methylation is a normal process in development, X chromosome inactivation, parent-of-origin allele-specific imprinting, and tissue-specific gene regulation, and is also observed in some tissues as a function of aging (reviewed in Costello and Plass; Ref. 8). DNA methyltransferase enzymes catalyze the addition of a methyl group to the number 5 carbon of a cytosine that is immediately 5′ to a guanine (CG dinucleotide). There is a strong association between DNA promoter methylation and transcriptional inactivation, which results in the functional equivalence of a genomic deletion or inactivating mutation (9). Therefore, it is apparent that aberrant DNA methylation plays an important role in tumor progression. These aberrations include both genome-wide hypomethylation and promoter CpG island hypermethylation. Promoter methylation has been shown to occur to some extent in most tumor types that have been investigated (10–12).

CpG island methylation can be assessed on previously identified candidate genes by various techniques (13). However, this gene-by-gene approach can lead to an underestimation of the overall methylation level in a tumor genome and does not identify novel methylation targets. To address this, our group has used a genome-wide methylation scanning method called Restriction Landmark Genomic Scanning (RLGS; Ref. 14). We reported previously hypermethylation in both solid tumors and acute myeloid leukemia (10, 15). We found a marked variation in the amount of aberrant methylation in acute myeloid leukemia blasts compared with normal bone marrow, and these methylation events occurred in a nonrandom distribution (16).

Studies of aberrant methylation in CLL have mostly demonstrated hypomethylation events (reviewed in Refs. 16, 17). Specific hypo-methylated genes include BCL2 (18), ornithine decarboxylase and Erb-A1 (19), and TCL1 (20). Stach et al. (21), using high-throughput capillary electrophoresis, studied overall genomewide hypomethylation in CLL patients recently and found a high degree of interindividual variation between total genomic 5-methylcytosine levels. There are few reports of hypermethylation in CLL, although a recent study by Melki et al. (22) showed methylation of multiple CpG islands in CLL cells when 8 candidate genes were examined by bisulfite sequencing (23, 24). To our knowledge, no genome-wide analysis of CpG island methylation has been reported. Here we present the results of using RLGS to interrogate the promoter methylation status in 10 primary CLL samples using two methylation-sensitive restriction enzymes, with direct comparison to normal B lymphocytes. We determined that CLL is characterized by substantial CpG island methylation relative to normal B lymphocytes. In addition, we identified a panel of 193 genes or sequences that are novel targets for methylation in CLL and warrant future investigation in larger studies designed to assess patient impact.

MATERIALS AND METHODS

Patient Selection and Sample Collection. Blood was obtained from patients with B-cell CLL through the CLL Research Consortium Tissue Core
Table 1 Patient samples and clinico pathological data

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Status</th>
<th>Age</th>
<th>Sex</th>
<th>VIF ( ^{%} ) Status</th>
<th>FISH abnormalities</th>
<th>Cytogenetics</th>
<th>Prior Rx</th>
<th>% Meth</th>
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<tr>
<td>4</td>
<td>CLL</td>
<td>37</td>
<td>F</td>
<td>Unmutated</td>
<td>del 17p13.1, del 6q21</td>
<td>NA</td>
<td>3</td>
<td>2.5</td>
</tr>
<tr>
<td>6</td>
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<td>52</td>
<td>F</td>
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<td>del 11q</td>
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<tr>
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<td>40</td>
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<td>1</td>
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<tr>
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<td>M</td>
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<td>del 5q15q23</td>
<td>0</td>
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</table>

\( ^{*} \) VIF, immunoglobulin heavy chain mutation result; NA, not available; FISH, fluorescence in situ hybridization; Prior Rx, number of previous chemotherapy regimens received; % Meth, percent of spot loss on NotI and Ascl RLGS profiles combined; CLL, chronic lymphocytic leukemia.

Bank, and also from healthy volunteers after obtaining informed consent under a protocol approved by the Ohio State University Institutional Review Board. Clinical characteristics of the patients and fluorescence in situ hybridization data are shown in Table 1. All of the patients examined in this series had immunophenotypically defined CLL as outlined by the modified 96 National Cancer Institute criteria (25). Patients from whom blood was obtained had not received therapy in the 2 months before sample acquisition. CLL cells were isolated immediately after donation using Ficoll density gradient centrifugation (Ficoll-Paque Plus; Pharmacia Biotech, Piscataway, NJ). CD19 cells were isolated after the density gradient separation procedure using CD19 microbeads and MACS separation columns according to the manufacturer’s instructions (Miltenyi Biotech, Auburn, CA). The neutrophil isolate from paired CLL samples was obtained by ficoll-hypaque density gradient centrifugation specific for neutrophils (Histopaque 1119 and 1077; Sigma-Aldrich). Cell purity of >95% was verified using a FITC-conjugated CD13 antibody (Becton Dickinson, Franklin Lakes, NJ). The CLL cell line, WaC3CD5, was provided by the investigators. In the past we have shown that a decrease of 30% in the relative spot loss on NotI and Ascl RLGS profiles combined was reliably detectable as a methylation event (10). The lack of labeling with radioactive nucleotides. The neutrophils available from 4 of the CLL samples (25% at an intensity level of 1) were used to confirm that we could detect a 30% decrease in the relative spot loss on NotI and Ascl RLGS profiles combined. 

The loci on the normal RLGS profile has a unique identifier as described previously (10). Loci of interest were cloned using NotI-EcoRV or Ascl-I-EcoRV arrayed plasmid libraries as described previously, and candidate plasmid clones were confirmed in RLGS mixing gels (29).

**Sequence Analysis of Cloned Loci.** Once the candidate plasmid was confirmed on a mixing gel (29) and sequenced from the NotI or Ascl end, the sequence was submitted to the BLAT search4 July 2003 freeze to determine chromosomal location and presence or absence of a CpG island. The genomic sequence extending from 2.5 kb upstream and 2.5 kb downstream of the NotI or Ascl site was used to determine homology to a gene, mRNA, or expressed sequence tag. A CpG island was classified as 5′ if it spanned the region upstream of the transcription start site and/or exon 1, 3′ if it occurred in the last exon only, and body if it spanned internal exons but did not include the first or last exon.

**Southern Hybridization Analysis.** DNA was either single-digested with EcoRV (New England Biolabs, Beverly, MA) for 4 h or double-digested with EcoRV and 40 units of EcoRV for 4 h followed by 20 units of Ascl (New England Biolabs) for 4 h. Three and a half μg of each DNA sample were electrophoresed on a 0.8% agarose gel for 16 h at 35 V and transferred by vacuum to a nylon membrane (Zeta Probe; Bio-Rad, Hercules, CA). Probes were prepared by purifying restriction fragments from the Ascl-I-EcoRV plasmid clone, random primed with Prime-It II kit (Strategene, La Jolla, CA) according to the manufacturer’s instructions, hybridized overnight, and exposed for 24 h on a Storm PhosphorImager (Molecular Dynamics, Amersham Biosciences, Piscataway, NJ).

**Sodium Bisulfite Genomic Sequencing.** One μg of genomic DNA from CLL samples and normal controls was treated with sodium bisulfite according to published protocols (30) with the minor modification that the DNA purification steps were done with the Gel Extraction kit (Qiagen, Chatsworth, CA). Primers were designed so that they did not contain CG dinucleotides but did contain several non-CpG cytosines that were converted to thymine, thus making them specific for bisulfite-treated DNA only. The GRM7 forward primer was 5′-GGAAGATTTAGGGGTGTTTGTAGG-3′ and the reverse primer was 5′-ATCCCTACCTCTTCCCAAT-3′. PCR was carried out in a 50-μl reaction using 1 μl of sodium bisulfite-treated DNA, 60 pm of each primer, 2.5 mM of each deoxynucleoside triphosphate, 2.5 μl of Platinum Taq polymerase (Invitrogen), and 5 μl PCR buffer. Reaction conditions were 95°C × 10 min, 35 cycles of 96°C × 30 s, 60°C × 30 s, 72°C × 30 s, and final extension of 72°C × 10 min. Ten μl of the PCR products were visualized on an 8% polyacrylamide gel. The remaining 40 μl were purified from a 1.5% agarose gel using the Qiagen Gel Extraction kit according to manufacturer’s protocol. Purified PCR products were cloned using the TOPO TA-Cloning kit (Invitrogen), and 8–10 clones were randomly chosen for sequencing. Com-

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4 Internet address: http://genome.ucsc.edu/cgi-bin/hgBlat?command=start.
plete bisulfite conversion was assured by having $<0.01\%$ of the cytosines in non-CC dinucleotides unconverted in the final sequence.

Reverse Transcription-PCR (RT-PCR). RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer’s directions and converted to cDNA with SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Semi-quantitative SYBR Green hot start PCR was performed with the LC FastStart DNA Master SYBR Green 1 kit (Roche Diagnostics, Indianapolis, IN) in a Bio-Rad iCycler. The 20-$\mu$l reaction contained 1X LC FastStart DNA Master SYBR Green, 1.2 mM MgCl$_{2}$, 10 $\mu$M each primer, and 1 $\mu$l template. GRM7 forward primer was 5’-CTCTGGCGGTATGACTCCTTT-3’ and the reverse primer 5’-CAATGCGGCTGTCATTGAG-3’. Reaction conditions were 95°C × 10 min, 35 cycles of 96°C × 20 s, 58°C × 20 s, and a final extension at 70°C × 10 min. Regular RT-PCR reactions for additional genes were: IFI1: forward primer CTCTTGCCATGAGAGCTGC, reverse primer TTCATCGAGAGATGACAC. Reaction conditions were 95°C × 10 min, 35 cycles of 96°C × 30 s, 59°C × 20 s, and a final extension at 70°C × 5 min. DLX5: forward primer TCTCCCTACCTCCGGCTTCCT, reverse primer TTGCCATTTCACCTTCTCA, 95°C × 10 min, 35 cycles of 96°C × 30 s, 62°C × 20 s, 72°C × 30 s, and final extension 70°C × 10 min. TBX18: forward primer GGGTTGGGAAGCCTTGGTGG, reverse primer GCCAGAATAGTCAGCAGGGG, 94°C × 10 min, 35 cycles of 96°C × 30 s, 60°C × 30 s, 72°C × 30 s, and final extension 70°C × 7 min. TBR1: forward primer ACTCGTTCATCCGGCTAC, reverse primer GAATCCAGGGGTGGTGTTG, 95°C × 10 min, 35 cycles of 96°C × 30 s, 60°C × 30 s, 72°C × 30 s, and final extension 70°C × 10 min. The housekeeping gene GPI was used as a control for RNA integrity in the RT-PCR reactions: forward primer GACCCCAGATTCCAGAGCTG, reverse primer GCATCACGTCCTCCGTCACC, 95°C × 10 min, 35 cycles of 96°C × 15 s, 60°C × 15 s, 72°C × 60 s, and final extension 72°C × 10 min. Universal Human Reference RNA (Strategene) was used as a positive control.

Vi$_{3}$GEAR Analyses. Analysis for Vi$_{3}$GEAR somatic mutation was performed by the CLL Research Consortium Tissue Bank described previously (31). Sequences were compared with those deposited in the V BASE and GenBank databases. Somatic mutations were identified by comparison with the most homologous germ-line Vi$_{3}$GEAR gene sequences with $<$98% homology to germline sequence were considered mutated (32).

SDS-PAGE/Immuno blotting. Whole cellular lysates were prepared as described previously (33). For a limited number of experiments, lysates were made as described previously (33) with the addition of 0.1% SDS or 450 mM NaCl to either increase nuclear membrane lysis or diminish protein-protein interactions, respectively. Additionally, aggressive sonication to break up noncovalent protein DNA bonds was performed. Total protein in each sample was quantified, and samples were separated along with molecular weight markers (Bio-Rad) by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Blots were incubated with antibodies to DNA Methyltransferase 1 (DNMT1; New England Biolabs) and with Glyceraldehyde 3-Phosphate Dehydrogenase in each lane. Blots were developed with X-OMAT film (Kodak). Protein bands were digitally quantified using a Bio-Rad ChemiDoc instrument, and DNMT1 levels were calculated relative to Glyceraldehyde 3-Phosphate Dehydrogenase in each lane.

Fluorescence in Situ Hybridization. Cells from 10 CLL patients were thawed rapidly, washed twice in PBS, diluted to $1 \times 10^6$ cells/ml, and treated with 0.075 M KCl for 15 min at 37°C. The cells were fixed in 3:1 methanol:acetic acid. Hybridization with probes for del (17; p13.1), del (13; q14.3), del (11; q22.3), del (6; q21), del (6; q16), and centromere 12 (Vysis, Inc., Downers Grove, IL) was done according to the manufacturer’s specifications and as described (34).

Loss of Heterozygosity (LOH) Analysis. DNAs from the four matched pairs of CLL cells and non-neoplastic neutrophils (patients 7–10) were amplified with fluorescently labeled microsatellite markers from ABI Prism Linkage Mapping Set Version 2 (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol. Sixteen markers on 2q (D2S160, D2S347, D2S112, D2S151, D2S142, D2S230, D2S335, D2S364, D2S117, D2S325, D2S2382, D2S126, D2S396, D2S206, D2S338, and D2S125), 1 on 3p26.1 (D3S1304), 4 on 8p22–23.2 (D8S549, D8S550, D8S277, and D8S264), and 2 on 22q11 (D22S420 and D22S359) were examined in each of the four pairs of matched samples. PCR products were loaded on an ABI 3700 DNA Analyzer.

Fluorescence intensity and allele size were determined with the Genescan and Genotyper software (Applied Biosystems). The ratio of the two alleles (when both were present) was determined for each CLL and neutrophil sample. Because of the high purity (>95%) of both the tumor and control tissues, samples were scored as having LOH if the ratio of the tumor alleles divided by the ratio of the neutrophil alleles was >2.0 or <0.5.

RESULTS

CLL Cells Have Nonrandom Methylation. RLGS profiles were generated with CLL cells from 10 patients, matching non-neoplastic neutrophils if available (patients 7–10) and CD19+ B cells from two normal donors. Both the Not1-EcoRV-Hinfl and AcrI-EcoRV-Hinfl enzyme combinations were used for all of the samples. CLL profiles were compared with matched neutrophil profiles or normal CD19+ profiles to determine differences in spot intensity. Studies from other malignancies have demonstrated that the absence of an RLGS locus on the tumor profile coupled with the presence of the same locus on the control profile is highly suggestive of methylation due to the failure of the methylation-sensitive restriction enzyme (Not1 or AcrI) to digest the genomic DNA at that site (10). Fig. 1A shows an RLGS profile prepared from CLL cells using the AcrI-EcoRV-Hinfl enzyme combination.

RLGS loci that were present in control tissue profiles but absent in CLL profiles were recorded for each CLL profile using our “master profile” recording system published previously (10, 28). Each locus on the Not1-EcoRV-Hinfl and AcrI-EcoRV-Hinfl profiles has been assigned a unique address consisting of a row and column (delimiting a section on the profile) in addition to a unique number within that section. Fig. 1B shows decreased intensity at locus A-5G07 (spot 7 in row 5 and column G) in CLL patient 10 as compared with the same locus in matching neutrophils and control CD19+ cells. Because some methylation events can be tissue-specific (i.e., methylated in B cells but unmethylated in normal neutrophils), an RLGS locus was consid-

\[ \text{A} \]
\[ \text{B} \]

Fig. 1. Restriction Landmark Genomic Scanning (RLGS) profile using AcrI-EcoRV-Hinfl restriction enzymes in chronic lymphocytic leukemia (CLL). A, an AcrI-EcoRV-Hinfl RLGS profile prepared from the CLL cells of patient 10. The directions of the first and second dimensions are shown (arrows) along with the approximate molecular sizes in kb. B, inset of RLGS profile showing presence of spot A-5G07 (arrow), the GRM7 5' region, in normal CD19 cells (left), normal neutrophils (center), and CLL cells (right) of patient 10. The spot in the CLL profile has a reduced intensity relative to that of the surrounding spots due to methylation at the AcrI site.
ered to have tumor-specific methylation if it was absent in the CLL profile and present in both of the CD19 controls. The total number of loci that can be clearly analyzed on each profile varies slightly. The percentage of methylation on the CLL profiles ranged from a low of 2.5% to a high of 8.1% (mean 4.8%).

Some loci were methylated in multiple patients. However, this is not evidence that these loci are preferentially methylated because it is possible this could occur merely by chance. Therefore, using a set of 1204 Not-I EcoRV loci that were determined previously to be non-polymorphic (10), we tested this hypothesis with a standard goodness-of-fit test and accompanying simulation-based methods. The results were highly significant (P < 0.0001), indicating that the aberrant patterns of methylation are not random.

**RLGS Spot Cloning and Sequence Analysis.** One of the advantages of using RLGS for methylation analysis is the ability to clone loci of interest using our arrayed plasmid libraries. Loci were chosen for cloning based on frequency of methylation in CLL and availability in the plasmid libraries. Our libraries provide ~75% coverage of the RLGS profiles, so not every locus can be readily cloned. Thus, we also used a direct cloning approach described recently (35). Additionally, some loci cloned previously from other studies were also included in this analysis (10, 11, 15, 36).

After confirmation by RLGS mixing gels that a plasmid corresponds to the correct RLGS locus, the plasmid sequences were analyzed for gene or expressed sequence tag homology, chromosomal location, and CpG island characteristics. The data for each of the 193

<table>
<thead>
<tr>
<th>Table 2. Methylated sequences in CLLa</th>
</tr>
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</table>

### Notes
- Table 2 provides a detailed list of methylated sequences in CLL patients, including their chromosomal location, CpG island characteristics, and their potential role in cancer research.
- The table highlights the significance of these findings in understanding the epigenetic landscape of CLL, which could have implications for both diagnosis and treatment strategies.
cloned loci are shown in Table 2. For sequences with homology to known genes and CpG island characteristics, the location of the CpG island within the gene is noted. Importantly, the majority (93%) of the clones have CpG island characteristics (H022 200 bp long, H1350 50% GC content, observed/expected CG H1022 0.6), confirming our previous observations that RLGS is strongly biased toward identification of CpG island regions (10).

Of the 193 sequences, 173 (90%) had homology to known genes or expressed sequence tags. The location of the CpG island in the gene or mRNA was determined for 137 clones. Of these, the CpG island was in the 5 region for 102 loci (74.5%), in the body of the gene for 25 loci (18.2%), and in the 3 region for 10 loci (7.3%). These data additionally demonstrate that RLGS is an effective tool for identifying aberrant promoter methylation.

Confirmation of Methylation of Selected Loci. We have determined previously that loss of an RLGS locus is due to methylation in 95% of the loci analyzed by alternative methods (10). However, the possibility that a locus is absent or has reduced intensity due to homozygous or hemizygous deletion cannot be ruled out. Therefore, we analyzed loci by Southern hybridization to rule out deletion as a cause of the reduced intensity. Genomic DNA from CLL cells, CD19+ normal controls, and normal donor peripheral blood was digested with EcoRV alone and in combination with AscI, and used for preparation of Southern blots. The membrane was hybridized with a suitable probe from RLGS loci A-5G07 (GRM7). In all of the cases methylation of the AscI site was confirmed, and there was no evidence of homozygous or hemizygous deletion of the genomic region under investigation. Fig. 2 shows a representative Southern blot using a
probe from RLGS locus A-5G07. The presence of the higher molecular weight band in the double-digested CLL DNA demonstrates resistance to digestion by the methylation-sensitive *Asc*I restriction enzyme.

Novak et al. (37) recently performed a comprehensive allelotyping analysis on 46 CLL patients and showed that CLL cells may have frequent submicroscopic allelic imbalances not detected by conventional cytogenetics. Therefore, we sought additional evidence that the decreased intensity and spot loss observed on the RLGS profiles was indeed attributable to DNA methylation and not to LOH. We reasoned that chromosomal regions with clusters of RLGS spot loss could possibly represent combinations of methylation and LOH, or alternatively, homozygous deletion. We chose four such regions for genotyping, 2q, 3p26, 8p22–23, and 22q11 (see Table 2 and “Materials and Methods”). We had matched normal tissue (neutrophils) from patients 7–10. Twenty-three markers were examined, and we found only a single LOH at marker D3S1304 (3p26.1) in patient 9 (data not shown). These results are consistent with recent observations by Zardo et al. (35) that concurrent methylation and deletion is infrequent in brain tumors.

**Bisulfite Genomic Sequencing of GRM7.** RLGS yields information about the methylation status of only the landmark enzyme restriction site (*Not*I or *Asc*I). To obtain a more extensive assessment we selected *GRM7* and examined promoter methylation with bisulfite genomic sequencing. This gene was chosen based on: (a) the presence of a CpG island spanning the 5′ regulatory region; and (b) the fact that *GRM7* has been linked to inhibition of cyclic AMP (cAMP) signaling (38). Treatment of DNA with sodium bisulfite results in the selective conversion of unmethylated cytosines to uracil although leaving methylated cytosines unchanged. During subsequent PCR the uracil is converted to thymine, and sequencing the PCR product allows determination of the original methylation status in the native DNA. Primer pairs located 5′ to the transcription start site did not contain any CG dinucleotides, which gives an amplification product regardless of the original methylation status (39). The results of the *GRM7* bisulfite sequencing are shown in Fig. 3.
The Ascl landmark site encompasses CGs 13 and 14. We detected an additional CG dinucleotide (position 15 in Fig. 3) in all of our samples that was not present in the published sequence. Three patients with decreased RLGS spot intensity (patients 7, 8, and 10) showed extensive methylation of the GRM7 5′ region, although patients 4 and 9 who did not have reduced spot intensity showed much less methylation. There appear to be certain CGs in the region that are more susceptible to methylation. For example, CGs 2–5 seem relatively resistant to methylation, although CGs 7–19 have moderate to marked methylation density in those patients with RLGS spot loss. Patients 4 and 9 also show a similar pattern, although to a much lesser degree.

Both of the CD19/H11001 controls show a low level of methylation in a mosaic pattern. The bisulfite sequencing data from GRM7 confirm that reduced or absent RLGS spot intensity can be considered a reliable surrogate for more extensive methylation within the CpG island.

Methylation Regulates Expression in CLL Cell Line (WaC3CD5). To determine whether expression of methylated genes could be induced by a hypomethylating agent we treated the CLL cell line, WaC3CD5, with 5-aza-2\(^{-}\)deoxycytidine. Whereas DNA methyltransferases (DNMTs) have a brief covalent adherence to the DNA target CpG island, the incorporation of 5-aza-2\(^{-}\)deoxycytidine into DNA leads to a permanent covalent DNA-protein bond and depletion of DNMT. Loss of DNMT1 from the soluble protein fraction after 5-aza-2\(^{-}\)deoxycytidine treatment was found previously for human breast cancer cells (40). Here we demonstrate in our WaC3CD5 cell line that 5-aza-2\(^{-}\)deoxycytidine decreases the whole cellular content of noncovalently bound DNMT1 (Fig. 4, A and B). Maximum depletion was achieved after 0.5 µM treatment (Fig. 4A). This loss was not processing-specific, as three additional preparative procedures including lysozyme procedures with addition of 0.1% SDS, 450 mM NaCl, and aggressive sonication that reduced DNA fragment sizes to between 200 and

Fig. 2. GRM7 gene structure and Southern blot. A. GRM7 gene structure and location of Ascl-EcoRV fragment detected by Restriction Landmark Genomic Scanning. The location of the CpG island and the region analyzed by bisulfite sequencing are indicated. B. Southern blot confirms methylation in GRM7 5′ region. Normal peripheral blood lymphocytes (PBL), CD19\(^{+}\) cells, and chronic lymphocytic leukemia samples were digested with EcoRV only (PBL, Lane 3), or double digested with Ascl and EcoRV (Lanes 4–11) and hybridized with a restriction fragment from the Ascl-EcoRV plasmid corresponding to the GRM7 5′ region (RLGS spot A-5G07). The large EcoRV-EcoRV fragment in Lane 3 indicates the size of a methylated fragment (M) when DNA fails to digest the internal Ascl restriction site. The smaller size band (U) shows the size of the fragment produced when the unmethylated DNA is digested by Ascl. Low-level methylation is present in normal PBLs and CD19\(^{+}\) cells. Patients 7 and 10 exhibit almost complete methylation. Patients 4, 8, and 9 have partial methylation, but to a larger degree than the normal controls.

Fig. 3. Bisulfite sequencing of the GRM7 5′ region. Bisulfite sequencing was performed to examine 20 CGs in the 5′ region of GRM7. The □ indicate unmethylated CGs and □ indicate methylated CGs. X indicates that the sequence in that area could not be evaluated. The location of this region relative to the transcription start site is shown in Fig. 2A. Each horizontal line represents the analysis of one randomly selected clone.
DISCUSSION

To our knowledge this is the first report of a genome-wide scan for aberrant promoter methylation in CLL, and the first study on any type of tumor in which two different methylation-sensitive landmark restriction enzymes (NotI and AciI) were used, thereby allowing the examination of several thousand CG dinucleotides in each tumor genome. We found RLGS to be an effective tool to identify novel methylation targets in CLL that would warrant additionally characterization in a larger sample set. Our data demonstrate that CLL exhibits extensive CpG island methylation similar to many other solid tumors and acute myeloid leukemia. Importantly, the loci we identified have a high frequency of occurrence within CpG islands rather than within noncoding DNA regions, thus making RLGS a potent tool for the identification of gene-associated methylation events. The non-random occurrence of some of the methylation events is similar to what we have observed in other tumors (10, 15). This could indicate a selection advantage for those cells that harbor methylation of specific loci or it could indicate that certain loci are more susceptible to methylation during tumor progression.

CLL is characterized by numerous genetic alterations including frequent deletions of chromosomal segments 13q14, 11q22-23, 6q21, 17p13, and trisomy of chromosome 12 (2). It is intriguing to speculate that hypermethylation and chromosomal instability are somehow correlated; however evidence from the literature linking these two events is rare. More convincing at this time is the finding of hypomethylation of centromeric and satellite repeat sequences, a phenomenon leading to chromatin decondensation and chromosomal fragility (8, 41). The methylation events that we have identified occur throughout the genome without preferential clustering and in line with a previous report on brain tumors demonstrating that aberrant methylation and genetic alterations do not coincide (35). It is important, however, to note that some of the methylation events that we identified are within or close to chromosomal segments that show frequent genetic alterations (e.g., N-3C57 on 13q14.11 or N-5E14 on 11q22) possibly targeting candidate CLL genes. This seems to be a rare event

600 bp did not alter the dose-dependent decrease in DNMT1 protein expression observed after treatment with 5-aza-2'-deoxycytidine (data not shown). Depletion of DNMT1 after 5-aza-2'-deoxycytidine treatment correlated with increased expression of GRM7, measured by semiquantitative RT-PCR (Fig. 4C). The increase in GRM7 mRNA was detected even at low dosage (0.01 μM) of 5-aza-2'-deoxycytidine and continued to increase, although the level of DNMT1 appeared to change very little at the higher concentrations. Similar results were obtained for randomly selected genes IPF1, TBX18, DLX5, and TBR1. These genes were methylated in the WaC3CD5 cell line and not expressed. However, expression was restored after treatment of WaC3CD5 with 5-aza-2'-deoxycytidine at 0.5 μM and 5 μM concentrations (Fig. 5).

Our results indicate that expression of these genes is regulated, at least in part, by DNA methylation, and that expression levels can be modulated by methyltransferase inhibitors. Furthermore, these data suggest that measurement of whole cellular DNMT1 may serve as a surrogate marker for effective decitabine levels in vivo where one could expect to observe re-expression of genes of which the promoter region is regulated by methylation.

![Diagram of DNMT1 and GRM7 expression](image)

Fig. 4. Depletion of DNMT1 protein correlates with increased GRM7 expression in chronic lymphocytic leukemia cell line WaC3CD5 after 5-aza-2'-deoxycytidine treatment. Chronic lymphocytic leukemia cell line WaC3CD5 was treated with 5-aza-2'-deoxycytidine at the indicated concentrations for 72 h. A. DNMT1 levels were depleted with increasing doses of 5-aza-2'-deoxycytidine as shown by Western blot and compared with Glyceraldehyde 3-Phosphate Dehydrogenase control. B. graphical representation of data in A; bars, ±SD. C; semiquantitative reverse transcription-PCR shows increased expression of GRM7 with increasing doses of 5-aza-2'-deoxycytidine.

![Diagram of re-expression of genes](image)

Fig. 5. Re-expression of genes silenced by DNA methylation. Reverse transcription-PCR reactions for IPF1, TBX18, DLX5, and TBR1 in chronic lymphocytic leukemia cell line WaC3CD5, treated with 0, 0.5 and 5.0 μM of 5-aza-2'-deoxycytidine for 72 h. Expression of glucose phosphate isomerase (GPI), a housekeeping gene, was used to assess RNA integrity. Universal human reference RNA was used as a positive control for reverse transcription-PCR reactions.
because it was shown previously that down-regulation of candidate tumor suppressor genes at 13q14 does not involve promoter methylation (42).

The ability to clone methylated loci is one of the strengths of RLGS, and accordingly, we identified 193 sequences, 173 (90%) of which have homology to known genes or expressed sequence tags. A number of these genes are transcription factors (DERMO1, FOXE1, TBX3, and IPF1). TBX3 was shown recently to be differentially expressed between high-risk and standard-risk childhood acute lymphocytic leukemia by gene expression profiling (43). Other loci (TBR1, GLRB, and PAK5) are associated with genes known to play a role in the nervous system, and any role they may have in CLL is unclear.

CpG islands in somatic cells are usually considered to be maintained in an unmethylated state. However, bisulfite sequencing of the GRM7 5′ region revealed that CD19+ cells from normal donors have a considerable amount of methylation. These cells showed equivalent levels of methylation between the two donors (10.5–11% of the total 190 CGs examined), as well as similar mosaic patterns. The significance of this degree of methylation is uncertain; however, regional DNA methylation can be influenced by Alu (44) or cis-acting sequences (45). Once de novo methylation has been “seeded,” spreading of methylation may be possible (46).

Here we have demonstrated by RLGS, bisulfite sequencing, and Southern hybridization that GRM7 is methylated in the majority of CLL patients examined. Treatment of a CLL cell line with 5-aza-2′-deoxycytidine resulted in up-regulated expression of several genes, including GRM7, suggesting that these loci are regulated to some extent by promoter methylation. GRM7 can inhibit cAMP signaling in the induction of apoptosis (47). Investigators have shown that in B-CLL, cAMP phosphodiesterases catalyze CAMP to 5′ AMP, thus inhibiting apoptosis (48), and this inhibition can be reversed by inactivation of phosphodiesterases (49). Thus, GRM7 is an interesting candidate gene, and its potential role in the pathogenesis of CLL is under investigation.

Some of the methylation events that we found occurred in regions that were discovered recently to have LOH or allelic imbalance in CLL (37). However, we found only one LOH event out of the 23 markers that we examined in 4 patients. These data, combined with the fluorescence in situ hybridization analysis of markers known to be involved in CLL, showed that methylation occurred independently of LOH in the vast majority of instances. This confirms recent data by Zardo et al. (35) who performed a similar integrated analysis in gliomas. It is also in accordance with the absence of concurrent methylation of the micro-RNA genes mir15 and mir16 in the minimally deleted region of 13q14 in CLL (50), and the absence of methylation differences between CLL cells and normal B cells in several other down-regulated genes at 13q14.3. Thus, simultaneous methylation and deletion appear to be rare events in CLL. An alternative explanation for clusters of methylated loci is that they represent repressive heterochromatin domains, as demonstrated recently by Nguyen et al. (51). Additional investigation is needed to determine the relative contribution of histone alterations to the methylation events at these loci.

In summary, we have performed the first dual-enzyme genome scan for methylation in CLL. Before this study, there was little known about the extent of promoter methylation in this disease. We have identified many aberrantly methylated genes and demonstrated that CLL exhibits widespread and nonrandom epigenetic lesions that are attractive targets for additional analysis. It is unlikely that all of these methylation events have pathological significance in the development of CLL. Instead, we suggest that a portion of these events confer a selective advantage to the malignant cell, although others may reflect global deregulation of methyltransferase activity and/or other epigenetic processes, such as histone deacetylase activity. Analysis of a larger sample set may identify methylation patterns with prognostic or therapeutic significance, as well as provide insight into the pathogenesis of this disease. The current use of demethylating agents such as decitabine, and histone deacetylase inhibitors, such as depsipeptide, in clinical trials (52) provides a potential mechanism to alter this disordered gene expression in CLL. The promising preclinical activity of these drugs makes it imperative that we continue to investigate the contribution of epigenetic alterations in this disease.

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Epigenetic Profiling in Chronic Lymphocytic Leukemia Reveals Novel Methylation Targets


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