GLOBAL AND GENE-SPECIFIC DNA METHYLATION ANALYSIS IN HUMAN LEUKEMIA

DISSERTATION

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By
Laura J. Rush, B.S., D.V.M.

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The Ohio State University
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Dissertation Committee: Approved by
Dr. Christoph Plass, Co-Advisor __________________________ Co-Advisor
Dr. Michael A. Caligiuri, Co-Advisor __________________________ Co-Advisor
Dr. Gary J. Kociba
Dr. Michael D. Lairmore Department of Veterinary Biosciences
Leukemia is a malignant neoplasm of hematopoietic cells characterized by a block in differentiation and a failure to respond to signals that govern normal proliferation and apoptosis. For clinical purposes, leukemias are classified according to the stage of development (acute or chronic) and by cell lineage (lymphoid or myeloid). The pathogenesis of the various forms of leukemia differs, but one thing leukemias have in common with all other cancers is an underlying genetic defect such as chromosomal translocations and other rearrangements, small deletions, amplifications, mutations, or loss or gain of entire chromosomes. Some of these abnormalities are apparent at the cytogenetic level, while others are detected only with molecular techniques.

Recurrent chromosomal changes have been known for quite some time. However, in the past decade there has been increasing awareness of the importance of epigenetic alterations in the initiation and progression of cancer. Epigenetic lesions are heritable changes in the DNA that are not caused by changes in the DNA sequence. The field of epigenetics currently involves the study of DNA cytosine methylation and chromatin structure. Chromatin structure is largely dictated by the acetylation state of histones, which affects the ability of transcription factors and DNA binding proteins to access the DNA. DNA methylation is a modification of the number 5 carbon of cytosine, and occurs most often in the context of a CG dinucleotide (CG). While most CGs in the human genome are methylated, dense stretches of unmethylated CGs are congregated in CpG islands, which are frequently located in the 5’region of genes, encompassing the promoter, exon 1, and sometimes extending into intron 1. Aberrant methylation of CpG islands in cancer cells correlates with transcriptional inactivation of the gene, thus resulting in the functional equivalent of a deletion or inactivating mutation.

We hypothesized that aberrant methylation plays a role in hematologic malignancies by contributing to the silencing of genes involved in normal growth and differentiation. We tested this by employing a novel methylation analysis technique, Restriction Landmark Genomic Scanning (RLGS), to identify methylated genes in acute myeloid leukemia (AML) and chronic lymphocytic leukemia (CLL). Chapter 1 presents an overview of what is currently known about aberrant
methylation in hematologic malignancies. The RLGS technique is described in detail in Chapter 2, along with background information on past use of RLGS to identify methylation patterns in tumors, spot cloning, and future applications to answer more mechanistic questions in the field of cancer epigenetics.

Chapter 3 describes the results of a genome scan for methylation in diagnostic AML blood and bone marrow samples, where we demonstrated a wide range of methylation levels in AML patients. AML was already known to be quite heterogeneous at the cytogenetic and molecular levels, and is now recognized as being heterogeneous at the epigenetic level as well. In addition to providing information on the degree of CpG island methylation, our scan revealed a number of novel methylation targets that had not been previously identified in cancer, including the Cytochrome P450 gene CYP1B1.

To determine if the methylation patterns we observed in AML were also present in CLL, we initiated a genome methylation scan in ten CLL patients. In Chapter 4 we show that CLL does indeed have a methylator phenotype, but with less variability than AML. Dozens of RLGS loci were cloned, and several of these were tested for methylation along a larger region of their promoters. Analysis of methylation fingerprints in conjunction with relevant clinical data failed to reveal any significant correlations, likely due to the small sample size used in our study.

In Chapter 5 the results of a detailed gene-specific methylation analysis of CYP1B1 are presented. Extensive bisulfite sequencing analysis of 59 CG sites in this gene demonstrated increased methylation within the body of the gene in AML patients. In vitro experiments with a leukemia cell line confirmed that transcription of CYP1B1 is regulated, at least in part, by DNA methylation and chromatin modification. Male patients whose RLGS profiles showed an unmethylated NotI site had a significantly worse outcome than those who were methylated.

Finally, in Chapter 6, the future directions of our methylation analysis in leukemia are discussed, along with experiments designed to elucidate the mechanisms of global and gene-specific methylation in the pathogenesis of leukemia.
Dedicated to my human and animal patients
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VITA

September 3, 1958………………………………………Born – Marion, Ohio

1985……………………………………………………….AD, Nursing
Marion Technical College

1985-1995………………………………………………..Registered Nurse, Oncology

1993……………………………………………………….BS, Biology
University of North Carolina
Chapel Hill, NC

1997……………………………………………………….DVM
North Carolina State University
Raleigh, NC

1997-present……………………………………………..Graduate Research Associate
Department of Veterinary Biosciences
Division of Human Cancer Genetics
The Ohio State University

PUBLICATIONS

Research Publications


Invited Reviews


FIELDS OF STUDY

Major Field: Veterinary Biosciences
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CHAPTER 1

INTRODUCTION

ALTERATIONS OF DNA METHYLATION

IN HEMATOLOGIC MALIGNANCIES

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1.1 Introduction

Without question genetic alterations underlie the pathogenesis of cancer. In recent years, however, epigenetic alterations have gained increasing recognition as important participants in tumor development and progression. Epigenetic changes include DNA methylation and histone modifications (acetylation and methylation), which influence chromatin structure or modify the DNA without altering the native nucleotide sequence. This may be particularly relevant in the leukemias, as mouse models created by insertion of an oncogenic chromosomal translocation fusion protein sometimes fail to produce overt leukemia, thus indicating that additional genetic or epigenetic events are required for malignancy \(^1\).\(^2\). Because epigenetic changes are potentially reversible, they make attractive targets for therapeutic intervention. Therefore, a thorough understanding of epigenetic regulation and the identification of loci involved in dysregulation are critical for the rational use of demethylating agents and histone deacetylase inhibitors in cancer
patients. In addition, DNA methylation alterations can also be exploited as biomarkers for monitoring treatment efficacy and minimal residual disease. This review will focus on the current understanding of DNA methylation abnormalities in hematologic malignancies and discuss how this knowledge contributes to our understanding of the pathogenesis of these diseases.

1.2 Methylation and transcriptional regulation

1.2.1 CpG islands

CG dinucleotides are unevenly distributed in the genome. The vast majority are found in repetitive elements and heterochromatin, and in this context they are normally methylated. CpG islands, however, are stretches of DNA 200-2000 basepair long, with a higher frequency of CG dinucleotides than the bulk of the genome. When CpG islands are present in the promoter or 5' region of a gene they are usually unmethylated, regardless of the transcriptional state of the gene. Methylation patterns are established during normal embryonic development, and participate in X chromosome inactivation and genomic imprinting. Tissue-specific methylation also occurs, and methylation has been shown to increase in some tissues with aging.

Cytosine methylation usually only occurs in the context of a CG dinucleotide. The transfer of a methyl group to the cytosine base is catalyzed by various DNA methyltransferase (DNMT) enzymes. DNMT1, known as the maintenance methyltransferase, acts at the replication fork during DNA synthesis to convert hemi-methylated DNA to a fully methylated state. On the other hand, DNMT3a and DNMT3b possess de novo methyltransferase activity and methylate previously unmethylated loci. Recent evidence, however, suggests that DNMT1 and DNMT3b work together to establish and maintain a hypermethylated phenotype in tumor cells.

1.2.2 Methylation and transcriptional regulation

Methylation of promoter CpG islands is correlated with transcriptional inactivation of the associated gene, and this inactivation is equivalent to a loss-of-function genetic alteration such as a deletion or mutation. If the gene has tumor suppressor properties or is involved in growth regulation, differentiation, or apoptosis, aberrant methylation can play a central role in
tumorigenesis (reviewed in \cite{14,15}). Methylation can be biallelic or monoallelic. For example, monoallelic methylation with a concomitant loss-of-function genetic alteration on the other allele results in complete lack of a normal gene product \cite{16}. Alternatively, biallelic methylation can also cause complete transcriptional inactivation \cite{17}.

Concurrent with CpG island hypermethylation in tumor cells there is an overall decrease in 5-methylcytosine levels arising from hypomethylation of normally methylated repetitive elements \cite{18,19}. The biologic consequences of this global hypomethylation remain unclear. However, hypomethylation of a normally methylated gene promoter can lead to activation of that gene, as will be discussed later. Some of the biologic processes that are subject to altered methylation in cancer are shown in Figure 1.1.

Figure 1.1: Biological processes that could be subject to disruption by aberrant methylation during lineage development of a multipotent stem cell. At the top of the arrow are processes that could be influenced by hypomethylation events. At the bottom are processes that could be influenced by hypermethylation events.
Exactly how methylation abrogates transcription remains unclear, but involves recruitment of methyl-binding proteins and co-repressor complexes \(^{20}\). These, in turn, may inhibit transcription factor binding and permit adoption of a closed chromatin configuration mediated by histone deacetylation. Transcription from methylated promoters can be induced by treatment with 5-azacytidine or 5-aza-2'-deoxycytidine \(^{21}\). These compounds covalently bind DNMT1, resulting in depletion of enzymatic activity. Histone deacetylase inhibitors allow an “open” chromatin configuration and have been shown to be synergistic when used with demethylating drugs in some systems \(^{22,23}\). Unraveling the complexities of the interactions between DNA methylation and chromatin modification is critical for understanding their roles in tumor pathogenesis and for designing rational approaches for the use of epigenetic modifiers in cancer patients.

1.3 **Laboratory methods for detecting methylation**

Standard nucleotide sequencing does not discriminate between cytosine and 5-methylcytosine, so alternative methods must be employed to assess the presence or absence of methylation. Methylation-sensitive restriction enzymes are frequently used, often in conjunction with their methylation-insensitive isoschizomers (e.g. \(Hpa\) II and \(Msp\) I), followed by hybridization with a gene-specific probe. Differential digestion when using these two enzymes provides a quantitative measure of the amount of methylation in the restriction site being examined. A more recent innovation is the use of sodium bisulfite to convert unmethylated cytosines to uracil (and then to thymine in subsequent PCR reactions), while leaving methylated cytosines unchanged \(^{24}\). This conversion is the cornerstone of bisulfite genomic sequencing, which allows one to examine each CG dinucleotide within a PCR amplicon, and methylation-specific polymerase chain reaction (MS-PCR), which provides a readout of the presence or absence of methylated alleles depending on the specificity of the primers used \(^{25}\).

While these techniques provide important information on selected candidate genes, they do not allow the assessment of global levels of promoter methylation, nor can they be used to identify novel methylated sequences. Our laboratory uses restriction landmark genomic scanning
RLGS to assay up to 2000 methylation-sensitive restriction sites in a single tumor profile \(^{26}\). RLGS is not dependent on hybridization kinetics, and prior knowledge of specific sequences is not needed in order to detect methylation changes (reviewed in \(^{27}\)). A detailed discussion of techniques utilized in methylation analysis is outside the scope of this review, and the reader is referred to a number of excellent articles on this subject \(^{28-30}\).

1.4 Methylation in normal hematopoiesis

As mentioned previously, not all promoter methylation is abnormal or pathogenic. In fact, dynamic changes in promoter methylation and chromatin structure appear to be important for expression of growth factors, growth factor receptors, cytokines, and other molecules during normal myeloid development \(^{31}\). In B cells, demethylation of one kappa light-chain allele precedes somatic rearrangement of that allele, and retention of methylation on the germ line allele may be one mechanism for preventing two somatic rearrangements in the same cell \(^{32}\). Differential methylation patterns have been correlated with regulation of expression of PU.1 in several blood cell lineages \(^{33}\). Intron 2 of the M-CSF gene is unmethylated and the gene is expressed in monocytes and macrophages, while non-expressing granulocytes and lymphocytes are methylated at this locus \(^{34}\). Similarly, the G-CSF receptor is unmethylated in normal granulocytes and monocytes but methylated in lymphocytes \(^{35}\). Methylation also plays a key role in regulating gene expression in T cells (reviewed in \(^{36}\)). Demethylation of IFN-gamma is associated with increased expression in activated mouse CD8+ T cells \(^{37}\). Pestano and colleagues examined CD8+ T cells that fail to recognize the MHC class I molecule and inadvertently escape the thymus. Once in the periphery, these cells have progressive methylation of the CD8 gene, followed by decreased CD8 expression and upregulation of Fas and FasL, leading them to succumb to apoptosis \(^{38}\). In another study, clones derived from single T cells of an individual showed marked variability in the methylation patterns, indicating that there might be more heterogeneity in methylation patterns than previously appreciated \(^{39}\).

It is important to understand methylation patterns in normal tissues in order to accurately interpret abnormalities in cancer. This can be problematic when using highly sensitive techniques...
such as MS-PCR to evaluate the presence of methylated alleles in a tumor sample. MS-PCR can
detect methylation in one cell out of 1000 \(^{25}\). Because tumors samples inevitably contain some
normal tissue contamination, the presence of low-level methylation could reflect normally
methylated alleles in noncancerous blood cells or stroma. Cancer-related genes that show some
degree of methylation in normal hematopoietic cells include \(p15^{40,42}\), \(p21^{43}\), and \(IGF-2^{44,45}\).

1.5 Hypermethylation in myeloid leukemia and myelodysplastic syndrome

1.5.1 Acute myeloid leukemia

Several groups have shown that acute myeloid leukemia (AML) cells possess a number
of methylation lesions. Melki et al. analyzed promoter methylation of eight genes by bisulfite
genomic sequencing \(^{46}\). They found that 19 of 20 (95\%) of AML patients were hypermethylated
for at least one gene, and 15 patients (75\%) had hypermethylation of at least two genes. There
was no correlation between the degree of methylation and methyltransferase levels in these
patients, although previous work by this group had shown increased DMNT1 expression in some
AML patients \(^{47}\). In a somewhat larger study, Toyota and colleagues examined 15 promoters in
36 AML patients and found high levels of methylation for a subset of these genes \(^{48}\). Interestingly,
there was an inverse correlation between the number of methylated promoters and the age of the
patient, i.e. older patients had statistically significant fewer methylated genes. Our group used
RLGS to examine 16 paired AML diagnostic and remission samples and discovered a wide
variation in the amount of aberrant methylation among different patients \(^{49}\). These changes occur
in a non-random fashion, and sequence analysis of methylated loci allowed us to identify many
novel targets of methylation. We also demonstrated an increased number of methylated loci
located on chromosome 11 \(^{49}\). De Bustros et al. had previously identified 11p as a methylation
“hot spot” \(^{50}\) in multiple types of neoplasms, and our study allowed us to extend this phenomenon
to the entire chromosome. Taken together, these studies suggest that AML frequently
demonstrates a hypermethylated phenotype, and underscore the marked heterogeneity that
exists between patients.
Some of the most frequently examined genes in AML have been the p15 and p16 cyclin-dependent kinase inhibitors. Cameron et al. found variable levels of p15 methylation. In this study transcriptional repression was correlated more strongly with the overall density of methylated sites rather than with any specific sites in the promoter. Other groups have also shown that p15 methylation is quite variable between individual patients and even among different cells within the same patient. But while p15 is often methylated in AML, p16 methylation is much less frequently detected. Indeed, Herman et al. reported that AML is characterized by p15 methylation in the absence of p16 methylation, while high-grade non-Hodgkin’s lymphomas often display methylation of p16 but not p15, thus setting the stage for classification of hematologic malignancies according to their methylation profiles. Other genes with a high frequency of methylation in AML include the estrogen receptor (ER), E-cadherin, and HIC1.

But what do these methylation patterns tell us about the biology of AML? HIC1 methylation appears to occur in late-stage AML, and WIT1 methylation is associated with chemoresistant AML. Elegant work by Di Croce et al. has provided new insight into how oncogenic fusion proteins, a hallmark of AML, might interact with the methylation machinery to disrupt transcription. They showed that the PML-RAR fusion protein could recruit methyltransferases to the RARβ2 promoter, inducing methylation-mediated transcriptional repression. Although they examined only one target promoter, this work has important implications for leukemogenesis. Do other fusion proteins act in a similar fashion to achieve transcriptional repression? If so, identification of these targets will provide clues to the pathogenesis of these diseases. Importantly, methylation of specific, rather than random, targets may underlie the observation that different hematologic malignancies harbor distinct methylation signatures.

1.5.2 Chronic myeloid leukemia

Chronic myeloid leukemia (CML) can be divided into three stages: chronic phase, accelerated phase, and a terminal blast crisis. Therefore, several investigators have examined...
stage-specific methylation events in order to elucidate the molecular mechanisms responsible for disease progression. Increasing levels of methylation of the calcitonin 63,64, HIC1 58, ER 54, and ABL1 65 genes have all been found during evolution from chronic phase to blast crisis. Other stage-specific events include loss-of-imprinting of IGF2 (see later). Aberrant methylation has also been shown to occur around the major breakpoint cluster region in CML patients both with and without the Philadelphia chromosome 66.

1.5.3 Myelodysplastic syndrome

Myelodysplastic syndrome (MDS) is included here because of the tendency for MDS to progress to AML. As was reported for AML, MDS showed a lack of p16 methylation, but p15 methylation was present in 16 of 32 patients (50%) 67. Furthermore, the frequency of p15 methylation was greater in high-risk MDS and increased in some patients as they progressed to overt leukemia. Similar results were reported in another study in which p15 methylation was followed serially as patients progressed through their disease 68.

1.6 Hypermethylation in lymphoproliferative disorders

1.6.1 Acute lymphocytic leukemia

Many of the same genes that are methylated in AML are also methylated in acute lymphocytic leukemia (ALL). For example, investigators have shown variable levels of p15 41, ER 54, HIC1 58 and E-cadherin 57 methylation in primary ALL samples of both B and T cell lineages. In addition, hypermethylation of p73, a homologue of p53, is methylated in a number of ALL cell lines 69,70.

Recently, Roman-Gomez et al. examined p21 methylation in 124 adult and pediatric ALL cases, including both B-ALL and T-ALL 43. p21 is a cyclin-dependent kinase inhibitor and candidate tumor suppressor gene that is frequently down-regulated in ALL. They found that promoter methylation was highly correlated with transcriptional repression. Furthermore, patients with p21 methylation had significantly reduced disease-free survival and overall survival times,
and the methylation status was an independent prognostic factor for prediction of disease-free survival. Thus, this study supports the notion that methylation can be a useful biomarker in leukemia.

1.6.2 Chronic lymphocytic leukemia

Few studies have examined hypermethylation events in chronic lymphocytic leukemia (CLL). Bechter et al. reported that methylation of hTERT, the catalytic subunit of telomerase, is correlated with decreased levels of telomerase activity in CLL. Biallelic methylation of the DXS255 polymorphic locus on the X chromosome was found in one study of female CLL patients, and methylation of E-cadherin was described in three of five patients with CLL. Hypomethylation events have also been examined and these may play an important role in the pathogenesis of CLL (see later).

1.6.3 Lymphoma

Esteller and colleagues have recently correlated MGMT promoter methylation and improved survival in diffuse large B-cell lymphoma. MGMT repairs DNA damage caused by alkylating agents such as cyclophosphamide, which is frequently used in chemotherapeutic regimens for the treatment of lymphoma. Therefore, the authors hypothesize that methylation-induced silencing of MGMT may inhibit the ability of the neoplastic cells to repair damage caused by alkylating agents.

Sui et al. have examined the methylation status of a panel of genes in 33 cases of natural killer cell lymphoma and found high levels of p73 methylation (94%) as well as MLH1 (63%), p16 (63%), p15 (48%), and RARβ (47%). Of note, they described methylation of two or more genes in 88% of these cases, and described differential methylation between the primary and metastatic lesions in 2 cases.

Methylation of DAP-kinase, a regulator of apoptosis, has been reported in Burkitt's lymphoma and B-cell lymphoma. In this study, down-regulation of DAP-kinase transcription
was associated with increased resistance to IFNγ mediated apoptosis. Other genes hypermethylated in lymphoma include p15 and p16 \(^{76}\), ER \(^{54}\), HIC1 \(^{58}\), and Myf-3 \(^{77}\).

### 1.6.4 Multiple myeloma

Cyclin-dependent kinase genes p16 and p15 have been shown to be targets of methylation in both multiple myeloma (MM) and monoclonal gammopathy of undetermined significance (MGUS) \(^{78-80}\). Mateos et al. found p16 methylation in 41 of 98 cases of MM and 4 of 5 cases of plasma cell leukemia, but not in MGUS, concluding that p16 methylation was associated with advanced or aggressive disease \(^{78}\). On the other hand, Guillerm and colleagues examined p15 and p16 methylation in 33 cases each of MM and MGUS and found few differences between the two groups \(^{79}\). DAP-kinase methylation has also been reported in MM and MGUS \(^{81}\). Clearly, more work is needed to determine the role that methylation plays in the pathogenesis and progression of MM and MGUS.

### 1.7 Imprinting and leukemia

Genomic imprinting is a normal process that results in parent-of-origin allele-specific transcription, mediated to a large extent by DNA methylation. In most tissues IGF2 is expressed only from the paternal allele. However, several groups have observed demethylation and biallelic expression from neoplastic cells in MDS \(^{82}\), CML \(^{83}\), and AML \(^{84}\). Originally it was thought that this change represented a specific alteration in tumor cells. However, it has now been shown by several groups that loss of imprinting of IGF2 and subsequent biallelic expression is a feature of some normal hematopoietic cells when they are in a proliferative state \(^{44,82}\). Methylation of the CTCF-binding site in the H19/IGF2 locus can also be responsible for disruption of normal imprinting in cancer \(^{85,86}\). Recently it was reported that the normally imprinted neuronatin gene, NNAT, undergoes biallelic methylation in childhood leukemias \(^{87}\). Since imprinted genes are frequently involved in growth regulatory functions, a more complete understanding of both normal and tumor-associated methylation changes is needed. The readers are referred to a recent review of imprinting and cancer for further details \(^{88}\).
1.8 Hypomethylation in hematologic malignancies

Just as hypermethylation events can lead to transcriptional repression of tumor suppressor genes, hypomethylation, or demethylation, can activate potential oncogenes. The TCL1 gene is oncogenic in T-cell prolymphocytic leukemia following translocation to the T-cell receptor and subsequent over-expression. However, some cases of Burkitt’s lymphoma (BL) and CLL exhibit TCL1 over-expression without rearrangement of TCL1. Yuille et al. reported biallelic promoter demethylation in BL cell lines and primary CLL samples which correlated with over-expression of TCL1, suggesting that hypomethylation events in these neoplasms could be functionally equivalent to oncogenic translocations 89.

Similarly, the HOX11 gene is translocated and over-expressed in some cases of T-ALL, but over-expression also occurs in the absence of translocations. Demethylation of the HOX11 promoter was then discovered to be an alternative to rearrangement for activation of this oncogene 90. Several groups have investigated hypomethylation events in CLL, including demethylation of the multidrug resistance gene, MRD1 91, ornithine decarboxylase 92, Erb-A1 92, and the anti-apoptotic gene BCL2 93.

In contrast to localized, site-specific CpG island hypermethylation events that occur in tumors, many cancers have an overall decrease of 5-methylcytosine 18,94. Wahlfors et al. confirmed this observation in CLL and reported that this disease is characterized by global hypomethylation 95. The significance of tumor-associated global hypomethylation and its relationship to CpG island hypermethylation remains unclear, however it is another indication of a generalized defect in the regulation of the methylation machinery.

1.9 Therapeutic considerations

Methylation and chromatin modifying agents are currently being evaluated in clinical trials. Therefore, it is critical that we more fully understand the spectrum of epigenetic features in both normal and tumor cells. 5-aza-2'-deoxycytidine has been used to treat various tumors and appears to exert both demethylating and cytotoxic effects (reviewed in 96). Patients with MDS
have shown overall response rates of 49%\textsuperscript{97}, and in one trial 5-aza-2'-deoxycytidine was associated with major cytogenetic responses and improved survival\textsuperscript{98}. Depsipeptide (FR901228), a histone deacetylase inhibitor, has demonstrated efficacy against cutaneous and peripheral T-cell lymphomas\textsuperscript{99}. Combined use of demethylating agents and histone deacetylase inhibitors may result in even more promising results. Suzuki et al. recently reported a number of genes whose regulation in colorectal cancer cell lines can be modulated by 5-aza-2'-deoxycytidine, trichostatin A (a histone deacetylase inhibitor) or a combination of both drugs\textsuperscript{23}. This work increases our understanding of the widespread effects of these drugs and provides the groundwork for mechanistic investigation into the regulation these genes.

In addition to demethylating agents and histone deacetylase inhibitors, antisense oligonucleotides directed against DNA methyltransferases are also in clinical trials. One such compound, MG-98, is currently being used in Phase I trials for patients with AML and Phase II trials for patients with epithelial tumors\textsuperscript{100}.

Importantly, we need to develop appropriate markers and standardized methods for evaluating treatment efficacy and monitoring minimal residual disease in patients who receive epigenetic modifying drugs. Given the plethora of hypermethylation targets in hematologic malignancies, how does one choose which promoters to evaluate to determine the efficacy of a demethylating agent? How will we quantify the level of demethylation of a target gene so that data from different investigators can be reasonably compared? Will demethylation of tumor suppressor CpG islands and re-establishment of transcription result in inadvertent activation of oncogenes and loss of imprinting? These questions and others will need to be addressed in order to make the best use of these therapeutic agents and optimize their use in targeting epigenetic lesions.

Figure 1.2 illustrates some of the possible consequences of using a demethylating agent in a leukemic patient. This therapy can result in cytotoxicity, differentiation, and/or demethylation of the leukemic cells. Investigators must assess the level of demethylation, determine if global or gene-specific hypomethylation has detrimental effects on the patient, and monitor for re-methylation of demethylated genes. One must consider which genes to evaluate in order to
document that sufficient demethylation has occurred. Ideally, these genes would be completely unmethylated in normal tissues in order to avoid a positive signal obtained by very sensitive PCR methods. As noted earlier, some genes exhibit considerable amounts of methylation in normal tissues, thus rendering them unsuitable for monitoring treatment efficacy unless assayed in a quantitative fashion.

1.10 Conclusions

Most hematologic malignancies appear to have some degree of epigenetic dysregulation. While lesions such as p15 methylation are found in the majority of the diseases examined, others, such as p16 methylation, seem to be more specific for a certain type of disease. Our group has described a number of leukemia-specific methylation targets that are not found in solid tumors. However, with the increasing number of methylated genes being reported, the biologic and clinical significance of many of these events remains undetermined. It is unlikely that each of these methylation alterations has a functional role in the pathogenesis of the particular tumor in which it was found. The challenge for molecular biologists and clinicians is to tease out the relevant targets that can be exploited for diagnostic and therapeutic purposes. The analysis of larger sample sets and the further development of high-throughput methylation scans should greatly aid in deciphering the molecular pathogenesis of leukemias and lymphomas, as well as provide useful tools for subclassification and prognostication.
Figure 1.2: Potential outcomes after treatment of leukemia with a demethylating agent. At the left is a schematic representation of a leukemic bone marrow. The large cells represent leukemic blasts; the small cells represent normal marrow elements. For the promoter of interest, “U” represents an unmethylated promoter; “M” represents a methylated promoter. Following treatment with a demethylating agent, various outcomes are depicted. The expected results of a methylation-sensitive PCR reaction (MS-PCR) for promoter methylation are shown in cartoon form as the presence or absence of a band on a gel.
2.1 Introduction

Tumor genomes are known to have multiple genetic aberrations. These include gains and losses of whole chromosomes, chromosomal rearrangements, deletions of segments encompassing tumor suppressor genes [loss of heterozygosity (LOH)], gains of segments encompassing oncogenes, and single nucleotide mutations. Chromosomal rearrangements can be identified with standard cytogenetics and chromosome painting, while comparative genomic hybridization and representation difference analysis are useful for determination of gross copy number alterations. Locations of potential tumor suppressor genes can be pinpointed with genome-wide LOH screens. Genome scanning techniques have played a critical role in identifying recurrent aberrations which then guide more focused investigation to elucidate the
target genes involved in cancer development and progression. The readers are referred to an excellent review on genome-wide analyses by Gray and Collins \textsuperscript{103}.

It is now clear that epigenetic changes, including DNA methylation, also play a role in tumor development. The scanning methods mentioned above cannot detect methylation changes between normal and tumor DNA. However, Restriction Landmark Genomic Scanning (RLGS) is a technique well-suited for interrogating the tumor genome for aberrant methylation. The use of methylation-sensitive restriction enzymes (such as \textit{NotI} or \textit{AscI}) with a bias for CpG islands allows one to quantitatively assess methylation differences in tumor DNA versus that of normal tissue. Hypomethylation of repetitive sequences can also be demonstrated on RLGS profiles, as well as copy number changes due to genomic amplification. In this review we will discuss technical aspects of generating RLGS profiles and cloning target loci, and highlight important contributions to the field of cancer biology that have been made from RLGS studies. We will conclude with our views of the future directions, goals, and utility of RLGS in cancer-related methylation analysis.

\subsection*{2.2 Restriction Landmark Genomic Scanning}

Changes in DNA methylation cannot be analyzed by standard sequencing methods; therefore, alternative techniques must be used in assessing normal and aberrant methylation. RLGS was modified from earlier developments in two-dimensional electrophoresis systems and uses radiolabeled restriction endonuclease sites to create the “landmarks” seen on the final autoradiograph \textsuperscript{26,104-106}. A schematic outline of the RLGS procedure is shown in Figure 2.1. First, high molecular weight DNA is isolated from fresh or fresh-frozen tissue \textsuperscript{107}. High quality, high molecular weight DNA is absolutely necessary to insure the integrity of large pieces of DNA and to prevent non-specific labeling of degraded fragments. Three restriction enzymes are used during processing, the first of which creates the landmarks. If the landmark enzyme is methylation-sensitive (i.e., will cut an unmethylated site but will not cut a methylated site), then the resulting profile provides a display of radiolabeled unmethylated restriction sites. The landmark methylation-sensitive enzyme should be a rare-cutter with at least two CpG sites in the
recognition sequence, such as NotI (GC↓GGCCGC) or AscI (GG↓CGCGCC). This increases the likelihood of the recognition site occurring in CG rich sequences called CpG islands (see later). Most CpG islands encompass gene promoters and first exons, making them very informative targets for methylation analyses.

Following the restriction digestion with a methylation-sensitive enzyme, the DNA is then subjected to a second restriction digestion to produce smaller pieces and allow adequate separation in the first dimension electrophoresis. For human DNA, the most common second enzyme is EcoRV, but PstI or PvuII have also been used. NotI-PstI and NotI-PvuII combinations are used more extensively in rodent studies.

Once the second digestion is complete, the DNA is electrophoresed in a 60 cm. 0.8% agarose tube gel overnight. The gel is extruded from the tube, placed in an equilibration buffer, and then drawn into a flexible tube that has a slightly larger diameter than the gel. Here the in-gel third restriction enzyme digest takes place to cut the DNA into smaller pieces in situ, allowing adequate separation in the second dimension. The choice of this enzyme varies, but Hinfl, Mbol, and DpnII are common selections. Following the third enzyme digest, the tube gel is again equilibrated in buffer, placed perpendicularly on top of a large 5% acrylamide slab gel, and electrophoresed overnight. The gel is dried and exposed to autoradiography film for 4-7 days. Alternatively, the gel can be exposed to a phosphorimager screen for 24 hours, followed by scanning. A complete step-by-step protocol can be found in either of two excellent publications\textsuperscript{107} 108.
Random breaks in high-molecular weight DNA are blocked with dideoxy-[α-thio] nucleotide analogues to prevent non-specific labeling and reduce background.

First digest with landmark enzyme NotI, recognition site GC↓GGCCGC

NotI ends filled in with $^{32}$P-dCTP and $^{32}$P-dGTP

Second digestion with EcoRV

1st dimension electrophoresis in 60 cm tube gel, 0.8% agarose, overnight

Third digestion, in gel, with Hinfl

2nd dimension electrophoresis in 5% polyacrylamide slab gel overnight

Figure 2.1: Schematic diagram of RLGS procedure. See text for details.
The end result is a highly reproducible RLGS profile that displays over 2000 radiolabeled NotI sites (Figure 2.2). The profile can be compared to other profiles from different tissues, such as comparing a tumor profile to one from adjacent normal tissue from the same patient. When methylation-sensitive restriction enzymes are used for the landmark, comparison of two profiles will demonstrate methylation differences by the presence of a radiolabeled locus in one profile coupled with the absence of the locus in the other profile. The absence of an RLGS fragment due to methylation results from the failure of the methylation-sensitive enzyme to digest the DNA, preventing endlabeling of that genomic sequence. One can verify that the locus loss is not due to homozygous or hemizygous deletion by other techniques, such as Southern hybridization or genomic PCR.

The majority of the landmark NotI sites on the profile are derived from diploid DNA segments (termed “single copy spots”) that provide an internal control for quantitation. Methylation is detected by the absence or decrease in signal intensity, while amplification or hypomethylation results in increased signal intensity or the addition of a new spot. Using the surrounding single copy spots as an internal control, the loss or gain of intensity of a locus can be visually estimated or quantified by densitometry. Examples of complete and partial spot losses are shown in Figure 2.1.

2.3 Analysis of RLGS profiles

Once an RLGS profile is obtained, it must be analyzed for alterations. The highly reproducible nature of RLGS lends itself well to the comparison of one profile to another. As RLGS has become more adapted to high-throughput systems and the ability to clone RLGS loci has improved (see later), methods to standardize and increase the efficiency of analysis has also improved.

The standard method of profile analysis has been visual inspection of the autoradiographs. The two profiles to be compared are superimposed, and the differences in spot intensities are recorded on a clear acetate, such as bleached radiograph film. In order to standardize data collection and facilitate data exchange between collaborators, our group has
developed a system whereby each locus has been assigned a unique address. A “master profile” generated from normal peripheral blood lymphocytes was divided into 9 horizontal sections (A-I) and 7 vertical sections (1-7), forming a grid with 63 sections. Within each section, each locus was sequentially numbered, resulting in a unique identifier for each locus. For example, locus 3B36 is the 36th spot in section 3B. Alterations are then recorded in a spreadsheet and large data sets can be easily manipulated and exchanged with other researchers. Our master profile and sequence data for selected RLGS loci can be viewed at http://pandora.med.ohio-state.edu/masterRLGS.

Other methods of profile analysis include computerized scanning. Takahashi and colleagues have developed an automated process that uses complex algorithms to compare locus intensities among different profiles. They have used this system to identify recurrent methylation alterations in colon cancer. Though it was used with the enzyme combination of NotI-EcoRV-Hinfl, this system has the advantage of being easily adapted to other enzyme combinations, thereby increasing the amount of data one can obtain by using other methylation-sensitive landmark enzymes.

An automated RLGS analysis tool (RAT) was also developed by Sugahara et al. In this system an RLGS profile is converted to a binary image, with each pixel scored as zero or one. Their program was able to compensate for the inevitable low-level background signal and warping that occurs during profile preparation. RAT was then used to construct a genetic linkage map of the recombinant inbred mouse strain SMXA.

2.4 Spot cloning and identification

The information derived from the analysis of RLGS profiles provides invaluable descriptive data on DNA copy number changes and methylation alterations. However, a major advantage of RLGS as a genome scanning tool lies in the accessibility of sequence data which can be obtained from the altered loci. This capability provides us with tools for a more complete analysis of each target locus, including determination of CpG island characteristics, chromosomal localization, identification of sequence context (e.g. promoter or exonic region), and access to
information for designing probes for Southern hybridization and primers for PCR. The ability to provide sequence data makes RLGS a powerful tool for the identification of novel genes involved in the biologic process being investigated, whether it is tumorigenesis, genomic imprinting, or pathogenesis of developmental diseases.

All of the above require that one is able to determine the genomic sequence corresponding to an RLGS locus of interest. One of the first attempts to link RLGS loci with specific chromosomal regions was the development of a Chromosomal-Assigned RLGS profile by Yoshikawa et al.\textsuperscript{112} Using a series of RLGS profiles made with flow-sorted human chromosomes, they mapped RLGS loci to specific chromosomes. They determined that the \textit{NotI-EcoRV-Hinfl} profile consists of 2676 monochromosomal loci, 82 dichromosomal loci, 101 multichromosomal loci, and 12 highly repetitive loci corresponding to ribosomal DNA.

### 2.4.1 PCR-based methods

Initial attempts at cloning individual loci were carried out with PCR-based methods using DNA extracted from the actual gels. Once a locus of interest was identified, a small piece of the acrylamide gel corresponding to the locus identified on the autoradiograph was cut out, and DNA eluted either by crushing or electroelution. Then adaptors would be ligated to the \textit{NotI} and \textit{Hinfl} ends, and these adaptors would be used for PCR amplification\textsuperscript{113}.

This method was applied by Ohsumi and colleagues to clone sequences that underwent methylation changes during development of the mouse brain\textsuperscript{113}. The technique had limited success though, due to the high background (up to 1500 times) of unlabeled DNA (fragments without a \textit{NotI} site) that surrounds each radiolabeled \textit{NotI} site, the very small amount of target DNA present at each single copy locus (attomole amounts), and the inherent difficulties in amplifying GC-rich sequences. A similar technique was used by Nagai \textit{et al.} with some modifications, such as using biotinylated \textit{NotI} linkers to increase capture of the ligated fragments\textsuperscript{114}. Thoraval \textit{et al.} increased the yield of DNA from RLGS gels by adding unlabeled genomic DNA\textsuperscript{115}. With this modification they were able to clone several demethylated repetitive elements from neuroblastomas.
A major advance in RLGS spot cloning was made by Hayashizaki’s group with the use of restriction trapper DNA to prepare the gels used for cloning. Genomic DNA is digested with the landmark enzyme (NotI) and also with the second restriction enzyme (EcoRV). The DNA is then passed through the restriction trapper which has NotI adaptors attached to latex beads. The DNA applied to the column is ligated to the adaptors, and the remaining unligated DNA passes through the column. The DNA retained in the column is released from the NotI adaptors by a second NotI digestion, yielding only NotI-EcoRV fragments that can then be subjected to the usual RLGS protocol. In this manner, the high background of uninformative DNA in the gel is eliminated, and the resulting profile is enriched for the informative DNA. This greatly increases the chances of successful PCR amplification using the method described above. The major drawbacks of the restriction trapper are its expense and the large amount of starting material (hundreds of micrograms of DNA) needed to prepare the cloning gel.

### 2.4.2 Arrayed library of NotI-EcoRV fragments

Our group has circumvented these difficulties with the construction of an arrayed NotI-EcoRV plasmid library made from human peripheral blood lymphocyte DNA. This library was subsequently used to establish a series of RLGS “mixing gels”. Thirty-two 384-well microtiter plates containing NotI-EcoRV plasmid clones were selected from the library. The pooled plasmid DNA from each plate was radiolabeled at the NotI site and added to normal peripheral blood lymphocyte genomic DNA, followed by the standard RLGS protocol. The same procedure was carried out using pooled plasmid DNA from each of the 24 columns and each of the 16 rows in the 32 plates. The end result is a collection of 72 RLGS mixing gels with the cloned NotI-EcoRV-HinfI fragments appearing as high intensity spots on a genomic background of low intensity spots.

By analyzing the full set of mixing gels, one can identify the unique plasmid library address for a locus of interest. For example, enhancement of a target locus on the mixing gels from Plate 4, Column 12, and Row 20 would allow one to return to the arrayed library and select the corresponding plasmid clone. Verification that the correct clone has been chosen is
accomplished by adding the plasmid DNA to normal genomic DNA and observing an enhancement of the locus of interest. This method has been used to clone over 130 loci, and has the additional advantages of immediate access to the clone for use as a hybridization probe and for sequence analysis. While the majority of loci on the RLGS profile can be accessed with this tool, a portion (approximately 30%) cannot, due to the existence of NotI-NotI genomic fragments that were excluded by our cloning vector, failure of plasmid growth, polymorphisms in the restriction enzyme recognition sites, and tissue-specific methylation differences. We have established that there are approximately 900 NotI-NotI fragments on an RLGS profile prepared with the enzymes NotI-EcoRV-Hinfl, and are currently developing a NotI-NotI library, analogous to the NotI-EcoRV library, to aid in the retrieval of these loci. Additionally, an arrayed Ascl-EcoRV plasmid library has also been completed.

2.4.3 Computer-based approaches

Two web-based RLGS cloning tools have been developed. The Riken Hayashizaki group has done extensive mapping of the mouse genome and correlated RLGS loci with this mapping data. Their user-friendly website at [http://genome.gsc.riken.go.jp/RLGS/RLGShome.html](http://genome.gsc.riken.go.jp/RLGS/RLGShome.html) allows one to access mapping information for selected loci on RLGS profiles derived from mouse DNA (Japanese wild mouse *Mus. musculus molossinus*) using the restriction enzyme combinations NotI-PstI-PvuII and NotI-PvuII-PstI. Clicking on a locus of interest provides information on chromosomal location and MIT markers. With the increasing use of mouse models, resources such as this will aid in the understanding of the pathogenesis of disease.

Rouillard and colleagues in the Hanash laboratory have taken advantage of the abundant human sequence data in the public domain to create a Virtual RLGS by using bioinformatics tools to analyze the finished and draft genome for sequences that would fall within RLGS profiles prepared with NotI-EcoRV-Hinfl and NotI-EcoRV-DpnII. They determined the first and second dimension sizes of the restriction fragments in the genome and predicted where these fragments would fall on the actual profiles. By comparing 29 sequences from known RLGS loci with the predictions, they report a 75% success rate, limited only by the availability of the complete
genome sequence and by restriction site polymorphisms in the deposited sequence. Their virtual profile can be viewed at http://dot.ped.med.umich.edu:2000/VGS/index.html. The predicted loci do not always correspond to the actual loci shown on the profile. As noted in their publication, there is tight precision in the first dimension predictions, but less precision in the second dimension. Nevertheless, the Virtual RLGS will prove to be a useful tool for linking RLGS loci with genomic sequence. Periodic updates with more current sequence information (last updated November 23, 2001) will continue to improve the success rate of locus linking.

2.5 RLGS vs. candidate gene approach

There are a number of methodologies used in the detection of promoter methylation, each with its own advantages and inherent disadvantages, and no single technique can provide a complete assessment of the full complement of changes in a tumor or tissue. Early studies relied on methylation-sensitive restriction enzymes such as *NotI*, *HpaII* and *SmaI* in conjunction with Southern hybridization. While this provides information on the specific restriction enzyme site, it uses large quantities of DNA and is not useful for determining global methylation patterns.

As stated previously, standard sequencing methods cannot distinguish 5-methylcytosine from unmodified cytosine, thus hindering the analysis of multiple CpG sites within a CpG island. In 1994, Clark and colleagues described genomic sequencing of bisulfite-treated DNA as a way to distinguish methylated from unmethylated cytosines. When DNA is treated with sodium bisulfite, unmethylated cytosines are converted to uracil, leaving 5-methycytosines unchanged. Using primers that surround a CpG-rich area but do not contain CpGs themselves, a PCR reaction can be carried out. The product can then be cloned and sequenced, or subjected to restriction enzyme analysis to determine which of the original CpGs were converted and which were not. This type of analysis has become very important in the investigation of methylation of cancer-related genes, and has been used in the analysis of novel methylation targets identified by RLGS. Table 2.1 lists differences that must be considered between the RLGS genome scanning approach and a candidate gene approach for methylation analysis.
### Table 2.1: Comparison of methylation analysis between RLGS and candidate gene approach.

<table>
<thead>
<tr>
<th>Consideration</th>
<th>RLGS</th>
<th>Candidate Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prior knowledge of sequence</td>
<td>Not necessary</td>
<td>Necessary</td>
</tr>
<tr>
<td>Use of methylation-sensitive</td>
<td>Common</td>
<td>Common</td>
</tr>
<tr>
<td>restriction endonucleases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ability to identify new methylation</td>
<td>Well-suited</td>
<td>Uns suited</td>
</tr>
<tr>
<td>targets</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Assess many genes simultaneously</td>
<td>Well-suited</td>
<td>Uns suited</td>
</tr>
<tr>
<td>Ability to target CpG islands</td>
<td>Well-suited</td>
<td>Possible, depending on technique</td>
</tr>
<tr>
<td>DNA needed</td>
<td>High quality, µg amounts</td>
<td>Lesser quality, possibly ng amounts</td>
</tr>
<tr>
<td>Ability to identify amplifications</td>
<td>Well-suited</td>
<td>Possible, depending on technique</td>
</tr>
<tr>
<td>Ability to identify hypomethylation</td>
<td>Well-suited</td>
<td>Possible, depending on technique</td>
</tr>
<tr>
<td>events</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ability to identify mutations</td>
<td>Uns suited</td>
<td>Possible, depending on technique</td>
</tr>
<tr>
<td>Quantitative ability</td>
<td>Quantitative</td>
<td>Possible, depending on technique</td>
</tr>
<tr>
<td>Number of &quot;CpG sites detected</td>
<td>Landmark site only</td>
<td>Possibly more than one, depending on technique</td>
</tr>
<tr>
<td>Assessment of global level of methylation</td>
<td>Well-suited</td>
<td>Uns suited</td>
</tr>
<tr>
<td>Reliance on hybridization kinetics</td>
<td>None</td>
<td>Possible, depending on technique</td>
</tr>
<tr>
<td>Reproducibility</td>
<td>High</td>
<td>High</td>
</tr>
</tbody>
</table>

2.6 DNA Methylation

DNA methylation involves the addition of the methyl group to the number 5 carbon of a cytosine in a CG dinucleotide (CpG). Thus, DNA methylation is one type of epigenetic change; that is, it modifies the DNA without changing the DNA sequence. The methyl groups are added by a family of DNA methyltransferase enzymes. DNMT1 catalyzes the majority of maintenance methylation (addition of methyl groups to hemimethylated DNA strands immediately following DNA replication), while DNMT3a and DNMT3b are believed to be more involved in de novo methylation.

Methylation is a normal phenomenon in a number of processes. For example, embryos undergo a wave of demethylation followed by de novo methylation. Genomic imprinting with
parent-of-origin allele-specific transcription is controlled in part by methylation \(^7\), as is X-chromosome inactivation \(^6\). Approximately 40% of tissue-specific genes contain CpG islands \(^123\), and methylation may play a role in tissue-specific expression of some of these genes \(^124\). CpG island methylation has also been shown to increase with age in some tissues, such as the colonic mucosa \(^10,125,126\).

The CpG dinucleotides that are subject to methylation are unevenly distributed in the human genome. The majority of the genome is depleted of CpGs, and those found scattered in the bulk of the genome are usually heavily methylated. However, clusters of CpGs, called CpG islands, are frequently found in the 5’ regulatory regions of genes (spanning promoters and extending into exon 1), and these are usually unmethylated \(^3\). The most widely used definition of a CpG island is that provided by Gardiner-Garden and Frommer, who determined that a CpG island is greater than 200 base pair in length, with a GC content > 50% and an observed/expected CpG ratio \(\geq 0.6\) \(^4\). Our group has reported on 65 cloned RLGS loci, and due to the strong bias of NotI sites for CpG islands, over 95% of these sequences fulfill the criteria for CpG islands \(^49,101,118,127,128\). Not all CpG islands are in the 5’ regions, however, and some are found within exonic or intronic sequences. Recent work by the Jones laboratory indicates that tumor-related methylation may actually begin in exonic CpG islands and subsequently spread into promoter regions \(^129\). It is currently estimated that there are 29,000 CpG islands in the human genome \(^130\).

Methylation of CpG islands in the 5’ regulatory regions of genes has received the most scrutiny because methylation here has been convincingly associated with transcriptional inactivation. However, it is now clear that DNA methylation is but one facet of a complex, multilayered system involving histone methylation, histone acetylation and deacetylation, as well as recruitment of co-repressor complexes and overall changes in chromatin configuration \(^131-133\). Thus, our understanding of the role of DNA methylation and its impact on transcriptional regulation has important implications in normal development, diseases associated with imprinting disorders, and cancer biology.
2.7 DNA methylation and cancer

It is well known that tumor suppressor genes can be inactivated by deletions or mutations. A third mechanism, transcriptional inactivation due to promoter methylation, has recently gained considerable attention. Initially, aberrant methylation in cancer was investigated on a gene-by-gene basis, and this showed that a number of genes were methylated and inactivated in multiple types of cancers [reviewed in 14]. However, even with this growing number of genes, the overall contribution of methylation in cancer was underestimated.

In the first study of its kind, we analyzed aberrant methylation in seven different tumor types using RLGS 101. Comparing 1184 nonpolymorphic loci on the tumor profiles and normal tissues, we found that different types of tumors exhibit varying degrees of methylation changes. Head and neck squamous cell carcinomas, testicular tumors and breast tumors had relatively low levels of aberrant methylation, while pediatric and adult brain tumors, colon tumors and adult acute myeloid leukemias showed high levels of methylation. Our analysis also revealed that certain loci were methylated frequently in multiple tumor types while other loci were methylated in only one type of cancer (tumor-type specific). These findings are important for two reasons. First, they provide an overall estimate of the extent of global methylation in cancers that cannot be obtained on a gene-by-gene scale. Second, it allows the discovery of novel methylation targets that can be further investigated to discern disrupted pathways in cancer development and progression.

Other investigators have also begun to analyze larger panels of methylation targets with similar results. Huang and colleagues have developed a novel technique called Differential Methylation Hybridization, in which tumor and normal DNA is hybridized to GC-rich sequences spotted on a slide. They demonstrated increased methylation in poorly differentiated breast tumors 134. Using a larger set of GC-rich sequences they were able to correlate methylation patterns with hormone receptor status in 17 breast tumors 135. Recently, Maruyama et al. investigated the methylation of 10 genes in bladder cancer and determined that the methylation status of several genes could be correlated with poor survival 136. Overall, these results indicate
that global methylation analysis is a valid method of identifying cancer-related biomarkers, and that delineation of methylation signatures can provide insights into the pathogenesis and biologic behavior of many types of tumors.

2.8 From descriptive to mechanistic studies

Before the refinement of cloning techniques, investigators using RLGS to study tumor biology often presented descriptive data on the number, character (increase or decrease in intensity) and recurrence of locus alterations in various tumor types. While these reports provided important information, their usefulness was limited by the inability to carry out further analysis due to lack of corresponding sequence data. With the recent advancements in spot cloning and completion of the draft version of the human genome, we have now moved into an era where abnormalities identified with RLGS can be translated into more mechanistic studies.

A prime example of this is the work of Yoshikawa and colleagues. In 1994 they reported that one locus exhibited decreased intensity (presumed methylation) in 14 of 16 (88%) of hepatocellular carcinomas analyzed by RLGS. Eventual cloning of this locus demonstrated that it corresponded to a CpG island in the 5' region of Suppressor of Cytokine Signaling (SOCS1). SOCS1 directly interacts with Janus kinase to downregulate cytokine signaling. Furthermore, it was demonstrated that restoration of SOCS1 expression in non-expressing cell lines results in growth suppression and apoptosis.

In a similar study, Dai et al. reported methylation of the 5' CpG island of BMP3B in primary non-small cell lung cancers. Methylation was strongly correlated with down-regulation of transcription, and the role of BMP3B in lung cancer development is under investigation. Thus, the emphasis has shifted from descriptions of locus alterations to identification and detailed analysis of the particular genes subject to aberrant methylation. Table 2.2 lists numerous genes that fall into this category.
<table>
<thead>
<tr>
<th>Alteration</th>
<th>Tumor Type</th>
<th>Gene or Locus</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylation</td>
<td>Acute myeloid leukemia</td>
<td>WIT1 11p13</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Multiple genes, many on chr 11</td>
<td>49</td>
</tr>
<tr>
<td>Non-small cell lung cancer</td>
<td>Multiple genes, incl. BMP3B 10q11.21-11.23</td>
<td>118</td>
<td></td>
</tr>
<tr>
<td>98 primary tumors of 7 different types</td>
<td>Multiple genes</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td>Testicular</td>
<td>Head and neck squamous cell carcinoma</td>
<td>Multiple genes</td>
<td>Our unpublished data</td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>SOCS1</td>
<td>141, 121</td>
<td></td>
</tr>
<tr>
<td>Medulloblastoma</td>
<td></td>
<td>Breakpoint cluster region in 17p11.2</td>
<td>142</td>
</tr>
<tr>
<td></td>
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<td>Repeat sequences chr 3, 4, 9, 13, 14, 15, 21, 22</td>
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Table 2.2. Cancer-related genes identified by RLGS.
The future—RLGS-derived methylation signatures

The completion of the Human Genome Sequencing project, the widespread use of computer-assisted RLGS profile analysis, and the assignment of genomic sequences to each RLGS locus will soon culminate in the ability to obtain a comprehensive methylation signature for each tumor. It is envisioned that in the near future we will be able to classify tumors and their biological behavior based, in part, on their epigenetic composition, similar to what has been accomplished with microarray expression profiling. It has already been demonstrated that methylation of certain loci are tumor-specific, and we should now exploit this phenomenon to gain insight into the pathogenesis of that type of cancer.

In addition, the overall complement of methylation alterations can be combined with clinical and pathologic data to develop biomarkers that will enhance diagnostic and prognostic capabilities. Indeed, Itano et al. found that the number of RLGS alterations was an independent predictor of poor prognosis in hepatocellular carcinomas, and Frühwald et al. demonstrated a correlation between methylation of seven RLGS loci and poor survival in a series of medulloblastomas. One can predict a scenario in which a panel of biomarkers identified through RLGS analysis is used as a screening tool in clinical samples to provide data that will enhance molecular classification and treatment decisions. As DNA methylation is a potentially reversible process, clinical trials are already underway using methyltransferase inhibitors such as 5-deoxy-azacytidine, and a panel of predictive biomarkers can be used to help track treatment progress and monitor for residual disease. Combined with histone deacetylase inhibitors, these new therapies target epigenetic lesions in the tumor genome, and RLGS provides an excellent avenue for defining the exact nature of those lesions.
CHAPTER 3

NOVEL METHYLATION TARGETS IN DE NOVO ACUTE MYELOID LEUKEMIA WITH PREVALENCE OF CHROMOSOME 11 LOCI


3.1 Introduction

Acute myeloid leukemia (AML) is a clonal malignancy of hematopoietic cells of the myeloid lineage. It is a heterogeneous disease at the molecular and cytogenetic levels, as evidenced by the mixed clinical response to standard therapy. Over half of de novo AML cases exhibit cytogenetic abnormalities, including numerous recurring chromosome translocations and inversions that result in gene fusions. Some of the resultant fusion proteins have been shown to be involved in leukemogenesis, but it is thought that additional molecular changes are needed for the development of frank leukemia.

DNA methylation is one mechanism that is believed to contribute to cancer initiation and progression by causing the inactivation of gene expression. This can have important consequences if the inactivated genes are essential for the control of normal cell growth, differentiation, or apoptosis. Aberrant DNA methylation can be responsible for one or both hits of Knudson's two-hit hypothesis of carcinogenesis. The mechanisms that regulate normal and aberrant methylation are not fully understood, nor are the mechanisms by which methylation
interferes with transcription. These processes likely involve complex interactions between methyltransferase and demethylase enzymes, histone acetylases and deacetylases, transcription factors, and chromatin structure. (reviewed in\textsuperscript{15,172,173})

Studies of selected tumor suppressor genes such as \textit{p15} and \textit{p16},\textsuperscript{53,41} as well as candidate tumor suppressor genes including the estrogen receptor\textsuperscript{54} and hypermethylated in cancer 1 (\textit{HIC1})\textsuperscript{58} indicate that aberrant DNA methylation is involved in AML. Melki \textit{et al.} found that 15 of 20 AML patients showed aberrant methylation in two or more cancer-related promoters, suggesting that AML might be characterized by a general deregulation of CpG island methylation.\textsuperscript{46}

In contrast to techniques that allow assessment of the methylation status of individual genes, Restriction Landmark Genomic Scanning (RLGS) is a tool for the identification of genome-wide methylation changes in CpG islands regardless of whether the genomic sequence is known.\textsuperscript{143} This technique makes use of the rare-cutting methylation-sensitive restriction enzyme \textit{NotI} (recognition sequence GC\textsuperscript{↓}GGCCGC) which provides the landmarks comprising an RLGS profile—a display of up to 2000 CpG islands in a single assay. The majority of these sequences are located in the 5' regions of genes, thus providing a global portrait of the methylation status of hundreds of promoter sequences within a tumor sample. Some of the methylation changes within a single tumor type are unique to that tumor type, while other changes are common to several different tumor types.\textsuperscript{101} Therefore, it is possible that the identification of a unique set of methylation changes within a tumor type can establish a methylation signature or fingerprint, allow molecular classifications, and identify novel genes important in pathogenesis and treatment.

Previously we reported a genome-wide study of aberrant methylation in multiple tumor types, including AML. Our results showed that aberrant methylation in adult \textit{de novo} AML is variable and nonrandom.\textsuperscript{101} We have expanded our analysis using 16 of the original 17 pairs of diagnostic and remission samples. This expanded analysis includes assessment of a larger number of CpG islands (1740 vs. 1184) and a detailed description of 33 methylated loci from
diagnostic AML samples. Using this strategy we have identified novel methylation targets, epigenetic changes that appear specific for AML, and a trend toward over-representation of methylation of chromosome 11.

3.2 Materials and Methods

Restriction Landmark Genomic Scanning (RLGS)

RLGS profiles established from our previous study were used for the current analysis.

RLGS was performed as previously described using the enzyme combination NotI (methylation-sensitive), EcoRV, and HinfI.  

RLGS gel analysis

Gels are analyzed by overlying the tumor sample (AML at diagnosis) and matching normal control (remission sample), marking the changes on a clear acetate, and recording the master profile address of the altered fragments. Previously, we had reported the analysis of 1184 fragments in multiple tumor types. Since the AML samples all have matched normal controls (i.e. remission samples), this allows us to eliminate the possibility of spot changes being due to polymorphisms, rather than methylation events. Therefore, we were able to analyze 1740 fragments per sample in the current study.

Cloning of Methylated Loci

RLGS fragments were cloned with the use of a previously constructed arrayed plasmid library derived from NotI/EcoRV fragments from peripheral blood (PB) genomic DNA from a healthy female donor. Confirmation of the clone of interest was accomplished by running a set of four RLGS mixing gels in which 5.2, 10.4, 20.8, and 66.3 (or in some cases 83.2) pg of 32P-labeled NotI-EcoRV fragments of the plasmid DNA were added to normal PB DNA for the first dimension gel electrophoresis. The standard RLGS procedure was then followed. Progressive enhancement of the locus of interest on the RLGS profile confirms the validity of the clone (Figure 3.3).
**Determination Of CpG Island Characteristics**

RLGS clone sequences (either the entire *Not*I-*Eco*RV clone or available sequence from the *Not*I side) were submitted to the CpG island prediction program at WebGene http://www.itba.mi.cnr.it/webgene/. This program uses the CpG island criteria developed by Gardiner-Garden and Frommer.4

**Southern hybridization**

Southern hybridization was performed as previously described.117

**Tissues and Cell Lines**

Patient samples were obtained from the Cancer and Leukemia Group B Tissue Bank, Chicago, IL. The first 16 cases with the same available matched tissue (bone marrow [BM] (n=3) or peripheral blood [PB] (n=13) from diagnosis and remission with *de novo* AML were chosen. Samples were collected in anticoagulant (heparin or EDTA) and shipped overnight to the procurement laboratory for processing. Mononuclear cells were separated on a Histopaque 1077 (Sigma) gradient by centrifugation at 1500 rpm for 30 min. Buffy coats were collected in freezing medium (Sigma) and stored in liquid nitrogen at -80°C until DNA isolation. Isolation of high molecular weight DNA: High molecular weight DNA (approximately 1µg/µl) from patient samples and cell lines was isolated according to published protocol.107 Isolation of RNA: Total RNA was extracted using Trizol (GibcoBRL) according to manufacturer’s directions. Cell lines (HL-60, Kasumi-1, ML-1, and TI-1) were obtained from the laboratory of one of the coauthors (MAC). Cells were cultured in RPMI-1640 medium with 20% fetal bovine serum (GibcoBRL), 1% Antibiotic-Antifungal agent (GibcoBRL), and 1% Anti-PPLO agent (GibroBRL) at 37°C in a 5% CO₂ humidified atmosphere. 5-aza-2’-deoxycytidine (Sigma) was diluted with sterile water, filtered, and added directly to the culture medium in the concentrations and durations indicated.
cDNA Synthesis

RNA was converted to cDNA using the Superscript Preamplification System for First Strand cDNA Synthesis (GibcoBRL) following manufacturer's instructions, using 2-3µg of total RNA and 0.5µg Oligo(dT)_{12-18}.

PCR

PCR reactions were carried out in a total volume of 50µl. Reaction mixtures contained 2µl of cDNA, 5µl of 10X PCR buffer (GibcoBRL), 200µM of each dNTP, 1.5mM MgCl₂, 10 pmol of each primer, and 2.5 units Platinum Taq DNA polymerase (GibcoBRL), except for the GPI reactions which used AmpliTaq Gold (Perkin Elmer). Some reactions contained 5% dimethyl sulfoxide (DRD4, CYP1B1, GPI). All reactions were run in a GeneAmp PCR System 9700 (Perkin Elmer Applied Biosystems) thermal cycler. Ten microliters of each PCR reaction was loaded onto a nondenaturing 8% polyacrylamide gel, electrophoresed at 250 V for 50-60 minutes, stained with ethidium bromide and visualized under UV light. Primers for RT-PCR reactions: DRD4 forward 5' TCGTCTACTCCGAGGTCCAG and reverse 5' AGCACACGGACGAGTAGACC; CYP1B1 forward 5' ACAGCATGATGCAGCAACTTC and reverse 5' TGGTCAGGTCTTTGATG; GaOlf forward 5' CGGAAGACCAGGGCGTCGATGAA and reverse 5' CCCATTGACGTGCAGGATCCTCA; EST AI433656 forward 5' CCCAGAGCGAGGCCGTG and reverse 5' CGCGGGGCCACCAGTTTG; WIT1 forward 5' CCTCAGTTGGCCTCTCTTA and reverse 5' TACCAGGCCACCCCTCTCACC; GPI forward 5' GACCCCGAGGCAGGTG and reverse 5' GCATCAGTCTCCCGTACC. All reactions were performed with an initial denaturing step of 95°C x 10 min, followed by 35 cycles of denaturing at 96°C for 30 sec (GPI 15 sec). Annealing was 58°C x 30 sec for DRD4; 57°C x 30 sec for CYP1B1; 66°C x 60 sec for GaOlf; 62°C for 60 sec for EST AI433656; 58°C x 30 sec for WIT1; and 60°C x 15 sec for GPI. Extension was performed at 72°C x 60 sec (GaOlf for 30 sec), and final extension 72°C x 10 min (GPI 5 min).
Mapping of Clone 2D74

This clone was mapped as previously described. Primers used for mapping were: #1 forward TTAACTCCCTCTGCTGCTCTCG and reverse CAATCCCTTGACGTTTTGC; #2 forward TGGGTTTGCTCTAGGTGTTGGC and reverse TTTAGGTTGTTGAAGGGACC.

Statistical Analysis

Heterogeneity of fragment losses across samples was determined by a chi-square test comparing the loss distribution with the expected (Poisson) distribution under the homogeneity hypothesis. Similarly, chi-square goodness-of-fit tests were devised, comparing the observed vs. expected frequencies of fragments exhibiting multiple loss across samples, and comparing the observed vs. expected frequencies of chromosome specific loss events. For these analyses standard asymptotic p-value approximations did not apply, or were inaccurate. For each analysis 10,000 simulations from the permutation distribution of the data were performed to obtain empirical p-values. For the fixed observed number of methylation events on each chromosome we calculated the null expected number of events as proportional to chromosome length or gene number. The variance of the observed number of events per chromosome, however, is sensitive to high methylation frequencies of individual fragments, and may not reflect a propensity of an individual chromosome. We performed a permutation test in which the observed loss at each fragment was fixed, but the fragment was assigned randomly to each chromosome according to the null expectation. For each of 100,000 random permutations, we calculated the maximum chi-square cell contribution \((\text{observed-expected})^2/\text{expected}\) to obtain an overall empirical distribution. This approach properly considers the varying loss rates of individual fragments, and accounts for multiple statistical comparisons across several chromosomes.

3.3 Results

3.3.1 Comparison of RLGS profiles from AML diagnostic and remission samples

To assess the extent of hypermethylation in AML, RLGS profiles were obtained for 16 pairs of BM \((n=3)\) or PB \((n=13)\) samples from adult patients at the time of original diagnosis with
AML (Figure 3.1) and again at complete remission (defined according to standard criteria with <5% blast cells on bone marrow aspirate\textsuperscript{176}) following induction chemotherapy. Profiles were also obtained for 4 AML cell lines: Kasumi-1\textsuperscript{177}, HL-60\textsuperscript{178}, ML-1\textsuperscript{179} and Tl-1\textsuperscript{180}. Altered RLGS fragments in the patient samples were identified by comparing the diagnostic and remission profiles from the same patient. In each case, BM was compared to BM, and PB to PB. We have previously shown that the absence of a fragment on the diagnostic profile coupled with the presence of the fragment on the remission profile from the same patient is indicative of methylation in the diagnostic tissue\textsuperscript{60,101} (Figure 3.2). Each fragment on the RLGS profile has a unique address (http://pandora.med.ohio-state.edu/masterRLGS/) to allow for consistent recording of alterations. A total of 1740 fragments were assessed for each pair of diagnostic and remission profiles. The total number of methylation events (i.e., loss of a fragment) in the diagnostic profiles when compared to the remission profiles ranged from 0 to 145 (0 to 8.3% of the profile; mean 1.9%), indicating a wide range in the degree of aberrant methylation in these samples (Table 3.1). This range demonstrates heterogeneity across samples, indicating that some samples demonstrated an inherently higher methylation event rate than others (p<0.001, see Statistical Methods). Two hundred eight (16%) of the 1740 loci examined were methylated in at least one patient.
Figure 3.1: RLGS profile from a bone marrow aspirate at initial diagnosis with AML. Directions of the first and second dimension electrophoresis are shown, as well as molecular sizes of the fragments.

Figure 3.2: RLGS spot loss. Insets of two RLGS profiles demonstrate methylation of fragment 3B36 (arrow) in diagnostic sample (panel A) compared with remission sample (panel B) from same patient. Fragment 3B36, the CYP1B1 gene, is absent in the AML diagnostic sample due to methylation.
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"+" indicates that remission and/or survival status is ongoing at time of last follow-up.
FAB indicates French-American-British classification system; CR, complete remission; PB, peripheral blood; BM, bone marrow
* Patient died of treatment-related complications without evidence of leukemia
^Patient has at least one methylation event on chromosome 11

Table 3.1. Clinical data and extent of methylation for 16 patients with acute myeloid leukemia. (sorted by number of methylation events)
3.3.2 Cloning, sequencing, and characterization of altered RLGS loci

In order to further characterize the target sequences that become aberrantly methylated in AML we selected RLGS loci with both a high and a low frequency of loss in our diagnostic AML samples and were available in our cloning library. The cloning was accomplished by using an arrayed NotI-EcoRV plasmid library and RLGS mixing gels as previously described.\textsuperscript{117,116} Figure 3.3 shows a portion of an RLGS profile demonstrating positive identification of clone 3B36 in a representative mixing gel. A total of 33 RLGS loci that were altered in diagnostic AML samples have been cloned and either partially (single pass from the NotI end) or fully sequenced (Table 3.2). Previously we reported that certain RLGS fragments are lost exclusively in certain tumor types.\textsuperscript{101} Here we report the cloning of five loci (2C40, 2D14, 3F16, 3F72, and 4E53) methylated exclusively in AML and not in the breast (n=14), colon (n=8), head and neck (n=14), testicular (n=9), adult (n=14) or pediatric (n=22) brain tumors in our original analysis (Table 3.2). All clone sequences were analyzed for CpG island characteristics with the WebGene program (Consiglio Nazionale dello Ricerche Istituto Teonologic Biomediche Avanxate) (http://www.itba.mi.cnr.it/webgene/). Thirty-one of the 33 clones (94%) have CpG island characteristics. BLAST searches show that 11 clones have homology to known genes, 10 to EST sequences, 11 to bacterial artificial chromosomes (BACs) or P1 artificial chromosomes (PACs), and one has no homology. The results of the database searches are listed in Table 3.2. The data demonstrate the strong bias of RLGS for display of CpG islands and sequences associated with genes, rather than random genomic sequences.
Figure 3.3: RLGS mixing gels used in cloning procedure. Normal PB genomic DNA (panel A) with no additional NotI-EcoRV clone. PB with 5.2 pg (panel B) and 20.8 pg (panel C) of the radiolabeled candidate clone added to the genomic DNA. Progressive enhancement of the RLGS fragment of interest confirms identification of the correct NotI-EcoRV clone.
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Dash indicates data not available; EST, expressed sequence tag; PAC, P1 artificial chromosome; BAC, bacterial artificial chromosome

Table 3.2. Characteristics of 33 cloned RLGS loci methylated in leukemia.
To assess the methylation status of selected genes in AML cell lines, Southern blots were prepared and hybridized with six NotI-EcoRV clones (2C40, 3B36, 3D35, 3C01, 5C08, and 5E34) using either the entire clone or a suitable restriction fragment. Normal donor peripheral blood mononuclear cell DNA served as a control. DNA was digested with EcoRV alone (PB) or EcoRV plus the methylation-sensitive enzyme NotI (PB and cell lines). Failure of NotI to cut the cell line DNA was observed in most cases, strongly supportive of methylation of these genes in the cell lines (Figure 3.4). Previous work in our laboratory has confirmed that loss of a fragment on the RLGS profile corresponds to methylation in >99% of the samples analyzed by Southern hybridization. (60,101 and unpublished data)

Figure 3.4: Southern blots showing methylation of RLGS clones in AML cell lines. Lane 1: Control normal donor peripheral blood (PB) is digested with EcoRV only (RV only) to show size of unmethylated band. Lanes 2-6: PB and AML cell line DNA is double-digested with EcoRV and methylation-sensitive NotI (RV-NotI). The presence of an uncut band in these lanes is indicative of methylation at the NotI site. Blots are probed with the RLGS clones 2C40 (panel A), 5C08 (panel B), and 3B36 (panel C). The 1.7 kilobase (kb) band in panel B is due to partial methylation of an internal NotI site within the larger 3 kb EcoRV-NotI fragment. Kas-1 indicates Kasumi-1 cell line.
3.3.3 RT-PCR analysis using AML cell lines

As promoter hypermethylation has been linked to transcriptional silencing, we assessed the expression status of five sequences (four genes and one EST) that were aberrantly methylated in our diagnostic AML samples. Whereas previously we had examined expression of three of these loci (GaOlf, WIT1, and 3D41) in brain tumor cell lines, the current studies were carried out using three AML cell lines: HL-60, Kasumi, and TI-1. RLGS profiles for each cell line showed all five sequences were methylated in the Kasumi-1 line, while four out of five were methylated in the HL-60 (exception: 3B36) and in the TI-1 (exception: 5E34) cell lines (Table 3.3). Reverse transcription-polymerase chain reaction (RT-PCR) was performed on RNA from these cell lines before and after the cells were treated with the demethylating agent 5-aza-2′-deoxycytidine (Table 3.3). Expression status of three sequences, CYP1B1, GaOlf and EST AI433656, was changed in at least one of the cell lines after treatment. DRD4 expression was not detectable regardless of treatment, and expression of WIT1 remained unchanged.

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<td>M</td>
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The location of the CpG island in the gene is indicated. The methylation status of the gene on the RLGS profile is indicated by “M” if methylated and “U” if unmethylated. Cells were cultured with (AzaC) and without (No tx) 5-aza-2′-deoxycytidine at 2.5 micromolar for 72 hours. The absence of a band in RT-PCR reactions is indicated by a dash, and its presence by a plus. Shaded areas indicate that expression was restored with demethylation.

Table 3.3. Expression of methylated genes in AML cell lines. Expression of 5 genes was examined in AML cell lines by reverse transcription-polymerase chain reaction following treatment with 5-aza-2′-deoxycytidine.
3.3.4 Methylation changes in AML are nonrandom, with a trend toward representation of chromosome 11 loci

To determine if there were recurrent methylation events among diagnostic AML samples in our expanded set of CpG islands, we compared the analyses of fragment losses across our 16 patients. This comparison revealed that certain fragments (e.g. 2C40, Table 3.2) were altered in the majority of diagnostic AML samples (11/16), whereas other fragments were altered much less frequently (e.g. 3D41; 3/16) or not at all (e.g. 4B2; 0/16, not listed in Table 3.2 because not methylated in this sample set). A standard “goodness-of-fit” test was applied to test the hypothesis of a constant rate of alteration among the fragments. This hypothesis was clearly rejected (p<0.001, see Statistical Methods), with many more fragments showing repeated alteration than would be expected by chance. The expanded analysis of a larger set of CpG islands reported here upholds our previous observation of nonrandom methylation patterns in AML.

We also examined the BLAST search data for the chromosomal location of our 33 cloned methylated loci. Interestingly, of the 30 clones for which the chromosomal location was available, five (17%) map to chromosome 11 (three to the long arm and two to the short arm) (Table 3.2). We plotted the total number of loss events (i.e. the number of patients in which a specific locus was methylated) for each clone with a chromosomal assignment against the chromosome number (Figure 3.5). We calculated an “expected” rate of methylation for the chromosomes using two criteria: the relative physical length of the chromosomes\(^{181}\), and the estimated number of genes on each chromosome.\(^{182}\) We compared the observed vs. expected frequency of methylation on the chromosomes using a specially designed chi-square test with a permutation-based p-value (see Statistical Methods). While there appeared to be a clear trend for over-representation of methylation events on chromosome 11, the test did not reach statistical significance (maximum \(\chi^2 = 110.09\), overall p=.056).
Figure 3.5. Observed vs. expected numbers of methylation events per chromosome. Chromosomal assignment was available for 30 RLGS loci. The total number of methylation events per chromosome was determined by the number of times each RLGS locus with a chromosomal assignment was methylated in the 16 diagnostic samples. The data show a trend for over-representation of methylation events on chromosome 11.

3.4 Discussion

In this study we provide a detailed analysis of a genome-wide survey of aberrant methylation in adult de novo AML, extending our previous work with characterization of novel methylated CpG islands, identification of leukemia-specific methylation, and suggestion of preferential methylation events on chromosome 11. There is a remarkable variability in the degree of methylation in our sample set, indicating a much larger degree of methylation in primary patient AML samples than was previously known. Indeed, some AML samples exhibit
very little or no aberrant methylation while others exhibit a much larger degree of methylation (up to 8.3% of the RLGS profile, and thus presumably a similar proportion of the 45,000 CpG islands in the genome). The methylation events we have identified do not correlate with specific translocations associated with AML, and in addition, they occur in AMLs with normal cytogenetics. This finding suggests that the aberrant methylation could represent an independent epigenetic change in addition to the genetic changes. The use of complete remission samples for each patient as our normal control eliminates any differences in the profiles due to polymorphisms. Restoration of unmethylated CpG islands in the profiles obtained from remission samples is attributed to eradication of the leukemic blasts and repopulation of the bone marrow with normal hematopoietic cells.

The nonrandom nature of the methylation found in AML is particularly intriguing and can be explained by two scenarios. First, aberrant methylation may preferentially target specific genes. Perhaps there are intrinsic features of those sequences such as repetitive elements, sequence motifs, or chromatin structure that predispose them to undergo methylation at a high frequency in AML when the regulatory mechanisms of methylation have gone awry. A second explanation is that the methylation itself is completely random but cells with a certain methylation pattern achieve a selective advantage based on the altered gene expression profile that results from the methylation. This explanation is supported by documentation of methylation-induced biallelic inactivation of p15, p16 and MLH1 in various cancers.183,17

In addition to providing a comprehensive assessment of overall methylation changes, RLGS allows the identification of novel methylated genes. As shown in Table 3.2, one third (11/33) of the methylated fragments correspond to known genes, while almost another third (10/33) have homology to ESTs. Many of the genes identified in our samples were not previously known to be methylated in cancer. This underscores the utility of RLGS in identifying methylation in novel promoter and gene sequences and provides a rational approach for using these sequences in the study of leukemogenesis.

There are overwhelming data that promoter methylation is associated with transcriptional inactivation.184 In addition, there is evidence that methylation of 3' CpG islands could have an
activating effect on transcription. Interestingly, of the 11 genes that we identified, three (POU3F1, \( \text{TBX18} \), and insulin promoter factor) are known transcription factors. A fourth gene, cold shock domain A, is a member of a family of DNA- and RNA-binding genes involved in transcriptional and translational regulation. These observations are particularly relevant in light of the fact that genetic changes in AML frequently involve transcription factors. However, since some of the methylated genes that we identified are not expressed in normal blood (e.g. \( \text{DRD4} \), Table 3.3), the significance of the methylation of those genes in the pathogenesis of AML is unclear. It is possible, if not likely, that a fraction of the methylated genes are unrelated to the leukemic process, and the methylation is a reflection of a global deregulation of control mechanisms including methyltransferase or demethylase enzymes, or endogenous stimuli.

Our RT-PCR analyses showed that methylation of the RLGS landmark NotI restriction site does not always correlate with complete transcriptional inactivation. This finding agrees with other reports in the literature. Cameron and colleagues have previously shown that the density of methylated CpG dinucleotides is important for inactivation of the \( p15 \) gene, and Ganderton and Briggs showed that methylation of the 5’ region of \( \text{PTHrP} \) did not correlate with inactivation. In addition, the location of the CpG island within the gene may play a role in determining the effects of methylation on transcription. In our study, methylation of the CpG island in the middle of \( \text{CYP1B1} \) seemed to have a variable effect on transcription.

Importantly, our approach has identified five loci which appear to be methylated only in leukemia and not in the other tumor types we have investigated. Thus, we have the means to investigate genes whose effects may be specific to leukemia, as well as genes whose effects may be important in many different types of tumors.

As shown in Table 3.2, 30 of the 33 cloned sequences have been mapped to a specific chromosome, and of these 30, five (17%) reside on chromosome 11. As chromosome 11 accounts for only 4.7% of relative length of the autosomes, methylation may be over-represented on this chromosome. However, the trend for over-representation does not meet statistical significance. This will need to be further investigated with continuing elucidation of the
human genome and a clearer understanding of CpG island and NotI site distribution. In a previous report, de Bustros et al. postulated that the short arm of chromosome 11 is a “hot spot” for methylation. Our data with AML support these previous results and show that the methylation is not restricted to 11p but also involves 11q.

Chromosome 11 is already known to be involved in AML by virtue of translocations, partial tandem duplications, and trisomy 11. The association of methylation in these areas of chromosomal instability requires further investigation, but one hypothesis is that methylation occurs in these regions as a cause or consequence of genetic instability. Chromosome 11 is also known to harbor several cancer-related genes, including oncogenes and tumor suppressor genes such as HRAS, FGF3, cyclin D, MEN1, ATM, TSG101, and WT1. Aberrant methylation of any of these regions may lead to a selective advantage by activation or inactivation of these genes and an in vivo expansion of a malignant clone. In addition, there are several imprinted genes on chromosome 11, such as IGF2 and H19 at 11p15.5. Altered methylation of these genes is associated with Beckwith-Wiedemann syndrome. It is possible that the methylation associated with imprinting of these regions becomes deregulated and spreads over a larger region, resulting in the methylation of nearby genes. Together, these data indicate that not only genetic events but also methylation changes on chromosome 11 may play an important role in leukemogenesis.

Our findings provide a genome-wide characterization of a highly variable methylation pattern in AML, along with the identification of novel methylated sequences, leukemia-specific methylation, and a trend for hypermethylation on chromosome 11. As AML is known to be a heterogeneous disease at both the cytogenetic and genetic levels, this may now also be extended to the epigenetic level since no single fragment is methylated in 100% of the profiles from diagnostic samples. Our data provide new insights into the molecular biology of AML and the role of epigenetic changes in this disease. Further characterization of these events should enhance our understanding of the pathogenesis of AML and could potentially result in the identification of novel cancer related genes as well as molecular markers for the classification of AML.
CHAPTER 4

WIDESPREAD CpG ISLAND METHYLATION AND EPIGENETIC
TRANSCRIPTIONAL GENE REGULATION
IN CHRONIC LYMPHOCYTIC LEUKEMIA

4.1 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. 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. In addition, CLL can be classified by the presence or absence of somatic mutations in the immunoglobulin variable region. While many of these genetic changes provide important prognostic information, our basic understanding of the pathogenesis of this malignancy is still obscure. In addition, when 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, tissue-specific gene regulation, and is also observed in some tissues as a function of aging (reviewed in Costello and Plass). 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). Although most of the genome has been depleted of CGs, clusters of CGs called CpG islands are found in the 5’ regions (promoter and exon 1) of most housekeeping genes and almost half of tissue-specific genes. There is a
strong association between promoter methylation and transcriptional inactivation, which results in the functional equivalence of a genomic deletion or inactivating mutation.\textsuperscript{15} It has therefore become increasingly 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.\textsuperscript{101,118,216}

CpG island methylation can be assessed on previously identified candidate genes by various techniques.\textsuperscript{217} 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 circumvent these obstacles, our group has used a genome-wide methylation scanning method called Restriction Landmark Genomic Scanning (RLGS)\textsuperscript{218}. Previously we have reported hypermethylation in both solid tumors and acute myeloid leukemia (AML).\textsuperscript{60,101} In AML we found a marked variation in the amount of aberrant methylation in AML blasts compared to normal bone marrow, and these methylation events occurred in a nonrandom distribution.\textsuperscript{49}

Studies of aberrant methylation in CLL have mostly demonstrated hypomethylation events (reviewed in Rush and Plass\textsuperscript{219}). Genome-wide hypomethylation in CLL was reported a decade ago\textsuperscript{65}. Subsequent studies have demonstrated gene-specific hypomethylation for \textit{BCL2}\textsuperscript{93}, ornithine decarboxylase and \textit{Erb-A1}\textsuperscript{92}, and \textit{TCL1}.\textsuperscript{89} Recently Stach et al, using high-throughput capillary electrophoresis, studied overall genomic hypomethylation in CLL patients and found a high degree of interindividual variation between total genomic 5-methylcytosine levels.\textsuperscript{220} There are few reports of hypermethylation in CLL, although a recent study by Melki et al showed methylation of multiple CpG islands in CLL cells when 8 candidate genes were examined by bisulfite sequencing.\textsuperscript{56,72,91,221} 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 of 10 primary CLL samples using two methylation-sensitive restriction enzymes, with direct comparison to normal B-lymphocytes. We determined that CLL, like AML, is characterized by a hypermethylation phenotype as compared to normal B-
lymphocytes. In addition, we identified a panel of 94 genes or sequences that are novel targets for methylation in CLL and warrant future investigation in larger studies designed to assess impact on disease progression and survival.

4.2 Materials and Methods

Patient selection and sample collection

Blood was obtained from patients with B-cell CLL 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 (FISH) data are shown in Table 1. All patients examined in this series had immunophenotypically defined CLL as outlined by the modified 96 NCI criteria. Patients from whom blood was obtained had not received therapy in the two months prior to sample acquisition. CLL cells were isolated immediately following donation using ficoll density gradient centrifugation (Ficoll-Paque Plus, Pharmacia Biotech, Piscataway, NJ). CD19\(^+\) cells were isolated following the density gradient separation procedure using CD19 microbeads and MACS separation columns according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). The neutrophil isolate from paired CLL samples was obtained by ficoll-hypaque density gradient centrifugation specific for neutrophils (Histopaque\textsuperscript{®} 1119 and 1077, Sigma-Aldrich). Cell purity of greater than 95\% was verified utilizing a FITC conjugated CD13 antibody (Becton Dickinson, Franklin Lakes, NJ). Two CLL cell lines, 183E95 and WAC3CD5, were provided by one of the co-authors (JCB). The features of these two cell lines have been described. Cells were incubated (37°C and 5\% CO\(_2\)) in RPMI 1640 media supplemented with 10\% heat inactivated FBS (HyClone Laboratories, Logan, UT), 2 mM L-glutamine (Invitrogen, Carlsbad, CA), penicillin (100 U/ml), and streptomycin (100 µg/ml), and 5-aza-2’-deoxycytidine (Sigma-Aldrich) for up to 48 hours.
Restriction landmark genomic scanning

RLGS was performed according to published protocols.224,225 All CLL samples (n=10), control neutrophils (n=4) from CLL patients, and control CD19+ normal B cells (n=2) from healthy volunteers were analyzed with two restriction enzyme combinations: NotI-EcoRV-Hinfl and Ascl-EcoRV-Hinfl. Briefly, high molecular weight DNA was extracted according to published protocols.107 For the NotI-EcoRV-Hinfl profiles, the DNA was digested with the methylation-sensitive restriction enzyme NotI (restriction site GC↓GGCCGC) and the restriction sites were filled in with [α-32P]-dCTP and [α-32P]-dGTP. Methylated NotI sites are resistant to digestion and will not be radioactively labeled. Following the NotI digest, the DNA is further fragmented with EcoRV, electrophoresed overnight in a 0.8% agarose gel, digested in situ with Hinfl, and electrophoresed overnight on a 5% nondenaturing acrylamide slab gel. The gel is dried and exposed to X-Omat film (Kodak, Rochester, NY) for 2-7 days before autoradiography. The procedure for the Ascl-EcoRV-Hinfl enzyme combination is essentially the same, except that DNA is first digested with EcoRV followed by the methylation-sensitive enzyme Ascl (recognition site GG↓CGCGCC).225

RLGS profile analysis and spot cloning

Paired RLGS profiles from CLL cells and normal tissue (either neutrophils from the same CLL patient or normal donor CD19+ cells) were overlaid and differences between the two profiles were detected by visual inspection. The presence of a locus on the normal tissue coupled with the absence of the same locus on the tumor profile is highly suggestive of methylation due to failure of the methylation-sensitive enzyme to cut the DNA and subsequent lack of labeling with radioactive nucleotides. Each locus on the normal RLGS profile has a unique identifier as previously described101 and the changes in each tumor profile were recorded in an Excel spreadsheet. Loci of interest were cloned using a NotI-EcoRV or Ascl-EcoRV arrayed plasmid library as previously described.116,225 Candidate plasmid clones were confirmed in RLGS mixing gels.117
Sequence analysis of cloned loci

Once the candidate plasmid was confirmed on the mixing gel it was subjected to single pass sequencing from the NotI or Ascl end. This sequence was submitted to BLAT search (http://genome.ucsc.edu/cgi-bin/hgBlat?command=start) using the June 2002 freeze to determine chromosomal location and presence or absence of a CpG island. The genomic sequence extending from 2.5 kilobase (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 (EST). CpG islands were also identified on the BLAT search using previously established criteria. 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 40 units of EcoRV (New England Biolabs, Beverly, MA) for 4 hours or double-digested with 40 units of EcoRV (New England Biolabs) for 4 hours followed by 20 units of Ascl for 4 hours. Three and a half micrograms of each DNA sample were electrophoresed on a 0.8% agarose gel for 16 hours at 35 volts and transferred by vacuum to a nylon membrane (Zeta Probe, Biorad, Hercules, CA). Probes were prepared by purifying restriction fragments from the Ascl-EcoRV plasmid clones, random primed with Prime-It II kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions, hybridized overnight, and exposed for 24 hours on a Storm Phosphoimager (Molecular Dynamics, Amersham Biosciences, Piscataway, NJ).

Sodium bisulfite genomic sequencing

One microgram of genomic DNA from CLL samples and normal controls was treated with sodium bisulfite according to published protocols with the minor modification that the DNA purification steps were done with the Qiagen 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 DERM01 forward primer was 5’-AAGGGGGAGGTAAAATTGAAA-3’ and the reverse primer was 5’-CTAAACTAAATTACTAAATTACTC-3’. The GRM7 forward primer was 5’-GGAAAGTTTAGGGGTTTTTGTAGG-3’ and the reverse primer was 5’-ATCCCTACCTCTCCCCAAAT-3’. The PCR was carried out in a 50 µl reaction using 1 µl sodium bisulfite treated DNA, 60 picomole of each primer, 1.25 mM of each dNTP, 2.5 units of Platinum Taq polymerase (Invitrogen), and 5 µl PCR buffer as previously described. Reaction conditions had an initial denaturation step for 10 minutes at 95°C, 35 cycles of 96°C x 30 sec, 52°C (DERMO1) or 60°C (GRM7) x 30 sec, 72°C x 30 sec, and a final extension step at 72°C x 10 minutes. Ten µl of the PCR products were visualized on an 8% polyacrylamide gel. The remaining 40 µl was purified from a 1.5% agarose gel using the Qiagen Gel Extraction kit (Qiagen) according to manufacturer’s protocol. Purified PCR products were cloned using the TOPO TA-Cloning kit (Invitrogen) and 8 to 10 clones were randomly chosen for sequencing. Complete bisulfite conversion was assured by having less than 0.01% of the cytosines in non-CG dinucleotides nonconverted in the final sequence.

Semi-quantitative reverse transcriptase 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) in a BioRad iCycler. The 20 µl reaction contained 1x LC FastStart DNA Master SYBR Green, 1.2 mM MgCl2, 10 µM each primer, and 1µl template. The DERM01 forward primer was 5’-GCCGCCAGGTACATAGACTT-3’ and the reverse primer 5’-CCCCAAACATAAGACCCAGA-3’. Reaction conditions were denaturation at 95°C x 10 min, 35 cycles of 96°C x 30 sec, 58°C x 30sec, 72°C x 30 sec, and a final extension at 70°C x 10 min. The GRM7 forward primer was 5’-CCTGGGCGTTATGACATCTT-3’ and the reverse primer
5’-CAATGGGCGTGTCATTGTAG-3’. Reaction conditions were denaturation at 95°C x 10 min, 35 cycles of 96°C x 20 sec, 58°C x 20 sec, 72°C x 20 sec, and a final extension at 70°C x 10 min.

**VH gene analyses**

Analysis for VH gene somatic mutation was performed by the CLL Research Consortium Tissue Bank as previously described. Sequences were compared to those deposited in the V BASE and GenBank databases. Somatic mutations were identified by comparison with the most homologous germ line VH gene. Sequences with less than 98% homology to germ line were considered mutated.

**Fluorescence in situ hybridization**

Cells from 10 CLL patients were thawed rapidly, washed twice in phosphate buffered saline (PBS), diluted to 1 x 10⁶ cells/ml and treated with 0.075 M KCl for 15 minutes 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), and centromere 12 (Vysis, Inc, Downers Grove, IL) was done according to the manufacturer’s specifications. The probes for del(6)(q21) and del(6)(q16) were provided by Vysis, Inc. The probe for (6)(q16), approximately 730 kb, and the probe for (6)(q21), approximately 725 kb, were labeled with SpectrumOrange® (Vysis, Inc) and SpectrumGreen® (Vysis, Inc) respectively. The slides were viewed with a Zeiss Axioskop fluorescence microscope with the appropriate filters and imaging software (Perspective System Instrumentation, Santa Clara, CA). The number of signals was evaluated in 200 cells for each probe. Standard quality control procedures were used and a control sample was run concurrently with each test run. Prior to testing patient samples, appropriate specificity and sensitivity were established as specified on cells isolated and cryopreserved in a similar manner as described for the CLL cells above. The established mean ± 3 standard deviations, considered positive for a cytogenetic abnormality in these CLL samples were 4% for trisomy 12, 10% del(13)(q14.3), 9% del(17)(p13.1), 10% del(11)(q22.3), 4% del(6)(q21), and 9% del(6)(q16).
Loss of heterozygosity 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. Twelve markers on 2q (D2S160, D2S347, D2S112, D2S151, D2S142, D2S2330, D2S335, D2S364, D2S117, D2S325, D2S2382, D2S126, D2S396, D2S206, D2S338, D2S125), one on 3p26.1 (D3S1304), four on 8p22-23.2 (D8S549, D8S550, D8S277, D8S264), and two on 22q11 (D22S420, D22S539) 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 loss of heterozygosity (LOH) if the ratio of the tumor alleles divided by the ratio of the neutrophil alleles was >2.0 or < 0.5.229

4.3 Results

4.3.1 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 from CD19+ B cells from two normal donors. Both the NotI-EcoRV-HindIII and Ascl-EcoRV-HindIII enzyme combinations were used for all samples. CLL profiles were compared to 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 (NotI or Ascl) to digest the genomic DNA at that site.101 Figure 4.1 (A) shows an RLGS profile prepared from CLL cells, using the Ascl-Eco-RV-HindIII enzyme combination.
RLGS loci that were present in control tissue profiles but absent in CLL profiles were recorded for each CLL profile using our previously published “master profile” recording system. Each locus on the Notl-EcoRV-HinfI and Ascl-EcoRV-HinfI profiles has been assigned a unique address consisting of a row and column (delineating a section on the profile) in addition to a unique number within that section. Figure 4.1 (B) shows decreased intensity at locus 5G07 (spot 7 in row 5 and column G) in CLL Patient 10 as compared to 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 considered 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 percent of methylation on the CLL profiles ranged from a low of 2.5% to a high of 8.1% (mean 4.8%). Furthermore, computer simulations101 showed there were numerous loci that were methylated more frequently than would be expected to occur by chance alone (p < 0.001).

4.3.2 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.116,225 Loci were chosen for cloning based on frequency of methylation in CLL and availability in the plasmid libraries. These libraries provide approximately 75% coverage of the RLGS profiles; therefore, not every locus can be readily cloned. Additionally, some loci previously cloned from other studies were also included in this analysis.49,101,118,225,230

After confirmation by RLGS mixing gels that a plasmid corresponds to the correct RLGS locus, the plasmid sequences were analyzed for gene or EST homology, chromosomal location, and CpG island characteristics. The data for each of the 94 cloned loci are shown in Table 4.2. 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 (84%) of the clones have CpG
island characteristics (> 200 base pair long, ≥ 50% GC content, observed/expected CG > 0.64), confirming our previous observations that RLGS is strongly biased toward identification of CpG island regions.101

Eighty-four of the 94 sequences (89%) had homology to genes, ESTs, mRNAs, or hypothetical proteins. The location of the CpG island in the gene or mRNA was determined for 56 clones. Of these, the CpG island was in the 5’ region for 43 loci (77%), in the body of the gene for 9 loci (16%), and in the 3’ region for 4 loci (7%). These data further demonstrate that RLGS is an effective tool for identifying aberrant promoter methylation.

4.3.3 Confirmation of methylation of selected loci

We have previously determined that loss of an RLGS locus is due to methylation in greater than 95% of the loci analyzed by alternative methods.101 However, the possibility that a locus is absent or has reduced intensity due to homozygous or heterozygous deletion cannot be ruled out. We therefore analyzed two 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 Ascl and used for preparation of Southern blots. The membranes were hybridized with suitable probes from RLGS loci A-5G07 (GRM7) and A-2G15 (DERMO1). In all cases methylation of the Ascl site was confirmed, and there was no evidence of homozygous or hemizygous deletion of the genomic region under investigation. Figure 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 Ascl restriction enzyme. Similar results were obtained for DERMO1 (data not shown).

Novak et al 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.231 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 4.2 and Materials and Methods). Because we had only four patients with matched normal tissue (neutrophils), genotyping was limited to Patients 7-10. Nineteen markers were examined, and we found only a single LOH event at marker D3S1304 (3p26.1) in Patient 9. These results are consistent with recent observations by Zardo et al that concurrent methylation and deletion is infrequent in brain tumors.232

4.3.4 Bisulfite genomic sequencing of DERMO1 and GRM7

RLGS yields information about the methylation status of only the landmark enzyme restriction site, in our case NotI or AscI. To obtain a more extensive assessment we selected two genes, DERMO1 and GRM7, and examined promoter methylation of each gene with bisulfite genomic sequencing. These genes were chosen based on a) the presence of CpG islands spanning the 5’ regulatory regions and b) the reported functions of these genes. DERMO1 is a basic helix-loop-helix transcription factor involved in cell type determination and differentiation,233 and GRM7 is involved in signal transduction.234 Treatment of DNA with sodium bisulfite results in the selective conversion of unmethylated cytosines to uracil while leaving methylated cytosines unchanged. During subsequent PCR reactions, the uracil is converted to thymine, and sequencing the PCR product allows determination of the original methylation status in the native DNA. Primer pairs for both genes were located 5’ to the transcription start site did not contain any CG dinucleotides, which gives an amplification product regardless of the original methylation status.24

DERMO1 showed dense methylation of CGs 9-12 in the CLL samples that had reduced intensity of the nearby RLGS landmark (Patients 3 and 5), while CGs 1-6 and 13 remain unmethylated for the most part (Figure 4.3 (A)). Interestingly, Patients 3 and 5 were two patients with more advanced CLL that had required prior therapy. There is a rare methylated CG in the
CLL sample that did not show methylation by RLGS (Patient 4), similar to the levels seen in the CD19⁺ control. No methylated CGs were detected in the normal peripheral blood from a healthy volunteer.

Results of a similar analysis for the GRM7 promoter is shown in Figure 4.3 (B). The Ascl landmark site encompasses CGs 13 and 14. Three patients with decreased RLGS spot intensity (Patients 7, 8 and 10) showed extensive methylation, while Patients 4 and 9 who did not have reduced spot intensity, show 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, while 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, though to a much lesser degree. Interestingly, both of the CD19⁺ controls show a low level of methylation in a mosaic pattern. The bisulfite sequencing data from the DERMO1 and GRM7 promoters confirm that reduced or absent RLGS spot intensity can be considered a reliable surrogate for more extensive methylation within the CpG island.

4.3.5 Methylation regulates expression of DERMO1 and GRM7

Next we determined the levels of expression of DERMO1 and GRM7 in CLL cells compared to CD19⁺ cells by semi-quantitative RT-PCR using SYBR Green technology. As shown in Figure 4, most CLL patients had a marked reduction in transcript levels of DERMO1 relative to that of a housekeeping gene, Glucose Phosphate Isomerase (GPI). Two patients (5 and 10) showed up-regulation. GRM7 was not expressed in the normal control CD19+ cells, normal peripheral blood, or CLL cells.

To see if expression of these genes could be induced by a demethylating agent, we treated two CLL cell lines (138E95 and WAC3CD5) with 5-aza-2’-deoxycytidine, which inhibits the DNA maintenance methyltransferase enzyme, DNA methyltransferase-1 (DNMT1). Expression of DERMO1 was undetectable in the untreated cell lines, but restored following 48 hours of treatment (Figure 4.5), indicating that DNA methylation plays a role in regulation of this gene. Similarly, GRM7 levels were increased in both cell lines following 5-aza-2’-deoxycytidine
treatment. These 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.

4.4 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 the two different methylation-sensitive landmark restriction enzymes, NotI and Ascl, 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 further characterization in a larger sample set. Our data demonstrate that CLL exhibits a CpG island hypermethylation phenotype similar to many other solid tumors and AML. 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 nonrandom occurrence of some of the methylation events is similar to what we have observed in other tumors. 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.

The ability to clone methylated loci is one of the strengths of RLGS, and accordingly, we identified 94 sequences, 84 (89%) of which have homology to genes, ESTs, mRNAs, or hypothetical proteins. A number of these genes are transcription factors (DERMO1, FOXE1, TBX3, IPF1). TBX3 was recently shown to be differentially expressed between high risk and standard risk childhood acute lymphocytic leukemia by gene expression profiling. Other loci (TBR1, GLRB, 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 promoter 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\textsuperscript{235} or cis acting sequences.\textsuperscript{236} Once de novo methylation has been “seeded”, spreading of methylation may be possible.\textsuperscript{237}

To determine the relationship between promoter methylation and transcription, we assessed the expression of *DERMO1* by semi-quantitative RT-PCR. Compared to CD19\textsuperscript{+} cells, expression in CLL cells was reduced in eight of ten patients but increased in two patients (Patients 5 and 10). The reason for the enhanced expression is unclear. We know from our bisulfite sequencing results that Patient 5 has incomplete methylation, including two of the ten alleles being completely unmethylated. Therefore, it is possible that upstream events are mediating transcription from these unmethylated and/or partially unmethylated alleles in this case.

*GRM7* was not expressed in either CLL cells or CD19\textsuperscript{+} controls. Treatment of two CLL cell lines with 5-aza-2'-deoxycytidine resulted in up-regulated *DERMO1* and *GRM7* expression, suggesting that these loci are regulated to some extent by promoter methylation.

Methylation of *DERMO1* and *GRM7* was confirmed by bisulfite sequencing and Southern hybridization. Both of these genes could be involved, directly or indirectly, with apoptosis. *DERMO1*, a basic helix-loop-helix transcription factor, plays a role in cell type determination and differentiation, Myc and p53-induced apoptosis, and promotion of colony formation in E1A/ras-transformed mouse embryonic fibroblasts.\textsuperscript{233} *GRM7*, on the other hand, can inhibit cyclic AMP (cAMP) signaling in the induction of apoptosis.\textsuperscript{238} Investigators have shown that in B-CLL, cAMP phosphodiesterases (PDEs) catabolize cAMP to 5' AMP, thus inhibiting apoptosis.\textsuperscript{239} This inhibition can be reversed by inactivation of PDEs.\textsuperscript{240,241} Thus, both *DERMO1* and *GRM7* are interesting candidate genes, and their potential role in the pathogenesis of CLL is under further investigation.

Some of the methylation events that we found occurred in regions that were recently discovered to have LOH or allelic imbalance in CLL.\textsuperscript{231} However, we found only one LOH event in the 19 markers that we examined in four patients. These data, combined with the FISH 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 who performed a similar
integrated analysis in gliomas.\textsuperscript{232} 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,\textsuperscript{242} and the absence of methylation differences between CLL cells and normal B cells in several other down-regulated genes at 13q14.3.\textsuperscript{243} 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 recently demonstrated by Nguyen et al.\textsuperscript{244} Further 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. Prior to this study, there was little known about the extent of promoter methylation in this disease. We have identified a large number of aberrantly methylated genes and demonstrated that CLL exhibits widespread and nonrandom epigenetic lesions that are attractive targets for further analysis. However, 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, while 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\textsuperscript{98,245} provides a potential mechanism to alter this disordered gene expression in CLL. This potential, combined with the promising pre-clinical activity of the histone deacetylase inhibitor depsipeptide,\textsuperscript{246} makes it imperative that we continue to investigate the contribution of epigenetic alterations in this disease.
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CD19-1 normal control
CD19-2 normal control

PBL, peripheral blood lymphocytes
NA, not available
FISH, fluorescent in situ hybridization results
Prior Rx, number of previous chemotherapy regimens received
% Meth, percent of spot loss on NotI and Ascl RLGS profiles combined

Table 4.1. Patient samples and clinicopathological data.
Table 4.2. Methylated RLGS loci in CLL patients.
Figure 4.1. RLGS profile and spot loss in CLL. (A) An Ascl-EcoRV-HinfI 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 kilobase pair (kb). (B) Inset of RLGS profile showing presence of spot 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 Ascl site.
Figure 4.2. Southern blot confirms methylation in GRM7 5' region. Normal peripheral blood (PBL), CD19+ cells, and CLL 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 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 PBL 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.
Figure 4.3. Bisulfite sequencing of DERMO1 and GRM7 5' regions. Bisulfite sequencing was performed to examine 13 CGs in the 5' region of DERMO1 (A) and 19 CGs in the 5' region of GRM7 (B, following page). The white rectangles indicate unmethylated CGs and black rectangles indicate methylated CGs. Primer locations are described in Materials and Methods.
Figure 4.3 (B) See legend on preceding page.
Figure 4.4. DERMO1 expression is down regulated in many CLL patients. Semi-quantitative RT-PCR was performed on cDNA from 10 CLL patients (see Materials and Methods for quantitation analysis). The relative fold increase or decrease of DERMO1 expression compared to expression of Glucose Phosphate Isomerase (GPI), a housekeeping gene, is shown.
Figure 4.5. DERMO1 and GRM7 expression is increased after 5-aza-2'-deoxycytidine treatment. Two CLL cell lines 183E95 (183) and WAC3CD5 (WAC) were treated with 1uM and 5uM of 5-aza-2'-deoxycytidine for 48 hours. RT-PCR shows no DERMO1 expression in the untreated cells. Expression was restored following 5-aza-2'-deoxycytidine treatment. Normal lung and PBL were positive controls. RT-PCR for GRM7 shows lower expression in untreated cells than in cells treated with 5-aza-2'-deoxycytidine.
CHAPTER 5

METHYLATION STATUS OF CYTOCHROME P450 1B1 (CYP1B1) IN ACUTE MYELOID LEUKEMIA MAY PREDICT POOR PROGNOSIS

5.1 Introduction

Acute myeloid leukemia (AML) is known to have cytogenetic and molecular heterogeneity (reviewed in 166). Recently it has been observed that AML exhibits considerable epigenetic heterogeneity, most notably widespread CpG island methylation. 46,49,53 DNA methylation is associated with transcriptional repression in both normal and tumor cells (reviewed in 14), often in conjunction with histone modifications and a closed chromatin configuration. 247 In cancer cells, silencing of genes involved in growth regulation, differentiation, or apoptosis may confer a growth advantage to the neoplasic cell. 248,249 In order to identify targets of aberrant methylation in AML, we performed a genome scan for methylation using restriction landmark genomic scanning (RLGS). We discovered that CYP1B1, a member of the cytochrome P450 family, was methylated in 21 of 36 AML diagnostic samples examined.

CYP1B1 is a nonhepatic Cytochrome P450 that was cloned and characterized by Sutter and colleagues upon discovery of its induction in keratinocytes following treatment with dioxin. 250-252 CYP1B1 mRNA is transcribed in normal tissues in both humans and rodents, including steroidogenic tissues (adrenal, breast, ovary and prostate), as well as non-steroidogenic tissues such as kidney, colon and spleen. 253 CYP1B1 is a major isoform in peripheral blood monocytes
and macrophages, and is also highly expressed in fetal tissues. Expression of CYP1B1 is induced when polycyclic aromatic hydrocarbons (PAHs) bind the cytoplasmic aryl hydrocarbon receptor, which results in its translocation to the nucleus and heterodimerization with the aryl hydrocarbon receptor nuclear transporter (ARNT) (reviewed in). The ArH/ARNT complex then binds to dioxin responsive elements (DREs) in the 5' regulatory region of CYP1B1 causing transcription to be up-regulated.

Several lines of evidence implicate CYP1B1 in tumorigenesis. First, this enzyme activates procarcinogenic PAHs, such as benzo[a]pyrene and 7,12-dimethylbenz[a]anthracene (DMBA), to their carcinogenic metabolites. It also participates in the conversion of 17β-estradiol to the carcinogenic 4-hydroxyestradiol. Secondly, CYP1B1 protein is selectively over-expressed in many types of epithelial tumors, with concurrent lack of detectable protein in the adjacent normal tissue. Thirdly, mice with homozygous deletion of Cyp1b1 are resistant to DMBA-induced bone marrow cytotoxicity, preleukemia, and lymphoma.

Here we present the results of a detailed methylation analysis of CYP1B1 in AML and show that there is marked variation in the extent of methylation throughout a large CpG that extends from the 5' regulatory region through exon 2. Treatment with a methyltransferase inhibitor +/- a histone deacetylase inhibitor resulted in re-expression of CYP1B1 in a non-expressing leukemia cell line, with significant synergism when both drugs were used together. Importantly, we found that males with AML who were unmethylated at the RLGS NotI landmark site had significantly worse outcomes than males who were methylated, or females of either methylation state. Our data indicate that expression of CYP1B1 is controlled, at least in part, by epigenetic factors, and that the methylation status of this gene at the time of initial diagnosis with AML may be a novel prognostic indicator.

5.2 Materials and Methods

Patient selection and characteristics

Samples from patients diagnosed with de novo AML were obtained from the Cancer and Leukemia Group B (CALGB) Tissue Bank (Chicago, IL) and were selected based on the criteria
of having >50% blasts in the diagnostic samples with having matched tissue (blood or bone marrow) available for the remission sample, where applicable. The first 16 cases were previously studied.\textsuperscript{49,101} Cytogenetics were centrally reviewed. Morphologic classification was performed according to the FAB Cooperative Group criteria.\textsuperscript{266} All patients gave informed consent for treatment and cryopreservation of blood and bone marrow. Eighteen patients were treated on CALGB protocol 9621 for patients ≤ 60 years of age with normal cytogenetics. The remaining patients were treated on other protocols.

**Tissues and cell lines**

Bone marrow or peripheral blood samples were processed as previously described.\textsuperscript{49} Kasumi-1 cells\textsuperscript{177} were cultured in RPMI medium supplemented with 20% fetal bovine serum. Cells were treated with 5-aza-2'-deoxycytidine (Sigma, St. Louis, MO) and/or depsipeptide. Untreated cells served as controls. When both drugs were used, 5-aza-2'-deoxycytidine was added to the culture medium for 48 hours, after which cells were spun down, resuspended in fresh medium, and treated with depsipeptide for 24 hours. Depsipeptide was obtained from the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). High molecular weight DNA was isolated according to published protocol.\textsuperscript{107} Total RNA extraction was carried out using the Ambion RNAqueous kit (Ambion, Austin, TX) according to the manufacturer’s protocol.

**Restriction landmark genomic scanning**

RLGS profiles were prepared as previously described, using the enzyme combination \textit{NotI}-\textit{EcoRV}-\textit{Hinfl}.\textsuperscript{218}

**Sodium bisulfite treatment of DNA**

One microgram of genomic DNA was treated with sodium bisulfite according to published protocol\textsuperscript{25} with the exception that the DNA purification steps were carried out with the Qiagen Gel Extraction kit (Qiagen, Chatsworth, CA). Treatment of DNA with sodium bisulfite converts
unmethylated cytosines to uracil and leaves methylated cytosines unaltered. During subsequent PCR reactions the uracil from unmethylated cytosines is converted to thymine. Primers and conditions for bisulfite sequencing and methylation-specific PCR (MS-PCR) are provided in Table 1. For bisulfite sequencing, 10 ul of the PCR product was visualized on an 8% polyacrylamide gel. The remaining 40 ul was electrophoresed on a 1.5% agarose gel, purified with the Qiagen Gel Extraction kit (Qiagen), and cloned with the TOPO TA-Cloning kit (Invitrogen, Carlsbad, CA). Clones were randomly selected for sequencing. MS-PCR products were visualized on an 8% polyacrylamide gel stained with ethidium bromide.

Less than 0.01% of the non-CpG cytosines remained unconverted following bisulfite treatment. Primers for bisulfite sequencing contained several non-CG cytosines that had been converted to thymine, and did not contain CG dinucleotides. Additional controls included 100% \textit{in vitro} methylated DNA to ensure that the reactions conditions will amplify template that is GC-rich. This also serves as a positive control for MS-PCR. DNA that has not undergone bisulfite treatment is used as a negative control to ensure the specificity of the primers for bisulfite treated DNA only.

\textbf{Polymerase chain reactions for CYP1B1}

Primers and conditions for reverse transcription PCR (RT-PCR) and TaqMan® Real-Time PCR are provided in Table 5.1. RT-PCR primers spanned intron 2 to avoid genomic DNA contamination. Primers for Real-Time Taq-Man PCR were previously reported.\textsuperscript{267} cDNA samples from treated and untreated Kasumi-1 cells were used as template for Real-Time PCR, along with primers and a FAM-labeled probe for CYP1B1, and primers and a VIC-labeled probe for the 18S (housekeeping) gene. Quantification of CYP1B1 expression was performed as previously described\textsuperscript{268} with standards for CYP1B1 and 18S expression. Expression was calculated as the number of CYP1B1 copies per $10^6$ 18S copies or per microgram of RNA.
**Statistical analysis**

Demographic and clinical data were obtained from the CALGB. Patients were divided into two groups according to the methylation status of the RLGS \textit{NotI} landmark site. Those patients with full intensity of the \textit{NotI} site on the RLGS profile were classified as unmethylated, and those with at least 50\% decreased intensity were classified as methylated. Categorical variables such as sex and FAB were compared using Fisher’s exact test. Continuous variables such as age, white blood cell count and blast count were compared using the Kruskal-Wallis test. Disease-free survival (DFS) for patients who achieved a complete remission (CR) was measured from the time the CR was first documented until death or relapse, with censoring for patients still alive in CR. Overall survival (OS) was determined from the date of enrollment in the CALGB protocol until death, censoring for patients remaining alive. Kaplan-Meier curves were constructed for DFS and OS to compare the methylation status and sex, and the log-rank test was performed to evaluate significant difference between the survival curves.

5.3 Results

5.3.1 RLGS shows frequent and variable methylation of \textit{CYP1B1} in AML diagnostic samples

RLGS profiles were prepared from 36 AML pairs of AML diagnostic and complete remission samples. Seventeen pairs were obtained from bone marrow and 19 from peripheral blood. Previously we reported the identification of RLGS spot 3B36 as the \textit{CYP1B1} gene.\textsuperscript{49} Complete methylation on the RLGS profile was determined by complete loss of the 3B36 locus, while partial methylation was determined by a decrease in 3B36 intensity without complete loss. Locus 3B36 was partially to fully methylated in 21 of 36 (58\%) diagnostic samples. The 3B36 locus was present at full intensity in all of the remission samples.

GenBank accession number HSU56438 provides 12,177 base pair of genomic sequence that encompasses the entire \textit{CYP1B1} gene. The genomic structure, location of the CpG island (determined using established criteria\textsuperscript{5}), and location of the RLGS landmark site are shown in
Figure 5.1. The locations of the promoter and exons were determined by Greenlee and colleagues.\textsuperscript{262,269} The 3B36 RLGS \textit{NotI} landmark lies within a large CpG island that extends from 5’ of the promoter into intron 2. There are 436 CG dinucleotides in the entire 12 kb sequence.

Because RLGS provides information only about the methylation status of the landmark restriction site, we used sodium bisulfite modification of DNA to assess the methylation status of a larger number of CG dinucleotides throughout the surrounding sequence. Bisulfite sequencing reactions were designed for 5 regions in and around \textit{CYP1B1}. This provides an overview of the methylation status throughout the CpG island and results in the interrogation of a total of 59 CG dinucleotides. Due to the constraints of primer specifications for bisulfite sequencing, we were not able to design primers that flanked the landmark \textit{NotI} site. The bisulfite sequencing reactions amplified both methylated and unmethylated sequences (Figure 5.2 (A)). The methylation status of the original sequence was determined by cloning the PCR products and sequencing randomly selected clones (Figure 5.2 (B)).

Methylation of the CpG island was initially assessed by bisulfite genomic sequencing using DNA from AML paired diagnostic and remission samples when available, and peripheral blood and bone marrow from normal volunteers. Five to six clones per region for each sample were randomly chosen for sequencing. See Figure 5.1 for locations of the bisulfite sequencing regions and numbering of CG dinucleotides. Examples of the PCR products and controls are presented in Figure 5.2 (A). Figure 5.2 (B) shows representative chromatograms that demonstrate differential methylation in a diagnostic and remission sample from the same patient.

Region 1 was heavily methylated in all samples, as were the first 3 CGs of Region 2 (CGs 5-7). These results for Region 1 are not surprising, as this section lies outside of the CpG island, and most non-CpG island CGs are thought to be methylated. CGs 8-11 of Region 2 were mostly unmethylated, with a mosaic pattern of methylated sites admixed in an apparently random distribution. A similar mosaic pattern was found throughout Regions 4 and 5 (CGs 36-49 and 50-59, respectively). However, even though both diagnostic and remission samples exhibited mosaicism, there was considerably less methylation in the remission samples.
The bisulfite sequencing reaction for Region 3 interrogates 24 CG dinucleotides (CGs 12-35) in the area that was previously demonstrated to be the promoter by Wo et al.\textsuperscript{269} Interestingly, most of the alleles showed either complete methylation or complete lack of methylation of the 24 CGs. This is in striking contrast to the mosaic pattern that was prevalent throughout the remainder of the CpG island.

The results of bisulfite sequencing for eight pairs of AML diagnostic and remission samples are shown in Figure 5.3. Blood and bone marrow from healthy volunteers was also examined. The total percentage of methylated CG dinucleotides in diagnostic and remission samples is shown in Figure 5.4. The overall levels of methylation in Region 1 and the first part of Region 2 are high in both stages of disease. Promoter methylation is somewhat higher at diagnosis than relapse, but this is mostly due to a higher level of methylation in one patient (Patient 416). On the other hand, there is a striking divergence in methylation frequencies that begins in Region 4 and continues through Region 5. The widest variations occur in Region 5. The RLGS NotI site is located between Regions 4 and 5.

Since the 24 CGs in the promoter (Region 3) appear to be either completely methylated or completely unmethylated throughout the region, this area is ideal for analysis by MS-PCR. MS-PCR was performed on AML diagnostic and remission samples and the results correlated closely with those obtained with bisulfite sequencing (data not shown).

5.3.2 **CYP1B1 expression is regulated by epigenetic mechanisms**

To see if epigenetic mechanisms might play a role in the transcriptional regulation of CYP1B1, expression in leukemia cell lines was tested by RT-PCR. Initially four cell lines were tested: ML-1, Ti-1, Kasumi-1, and K562. Of these, only Kasumi-1 showed little to no expression (data not shown). Kasumi-1 cells were then treated with 5-aza-2'-deoxycytidine (decitabine) which inhibits the maintenance DNA methyltransferase enzyme, DNMT1. 5-aza-2'-deoxycytidine treatment resulted in up-regulation of gene expression (Figure 5.5), indicating that expression is controlled, at least in part, by DNA methylation.
Since DNA methylation is sometimes associated with histone modifications\textsuperscript{20,270}, we wanted to investigate if this might be the case in our Kasumi-1 cell line system. Depsipeptide, a histone deacetylase inhibitor, has previously been shown to be synergistic in re-activating gene expression when used in conjunction with 5-aza-2'-deoxycytidine in vitro\textsuperscript{271,272}. We treated Kasumi-1 cells with 5-aza-2'-deoxycytidine and depsipeptide as single agents and in combination, and measured CYP1B1 expression by TaqMan\textsuperscript{®} Real-Time PCR.

There was no expression in untreated cells or cells treated with depsipeptide alone. Incubation with 2.5 µM of 5-aza-2'-deoxycytidine for 48 hours resulted in an increase of CYP1B1 expression to 27 copies per 1 µg of RNA. Combination treatments of 2.5 µM 5-aza-2'-deoxycytidine plus 3 nM or 5 nM depsipeptide resulted in 89 and 339 copies of CYP1B1 per 1 µg RNA respectively (Figure 5.6). These data confirm our previous observation that there is a component of epigenetic regulation in CYP1B1 expression. They also demonstrate a marked synergism between a demethylating agent and a histone deacetylase inhibitor. Bisulfite sequencing results for Regions 1-5 in the Kasumi-1 cells are shown in Figure 5.7.

**5.3.3 CYP1B1 methylation as a prognostic indicator in AML**

To determine if the methylation status of the RLGS locus for CYP1B1 was correlated with clinical data, we first examined 18 patients on CALGB protocol 9621. These patients were all \(\leq 60\) years of age with normal cytogenetics, and represented the most uniform group in our sample set. Nine patients were unmethylated at the NotI site and nine were partially or fully methylated. The Kalpan-Meier plot in Figure 5.8 (A) shows a trend toward poorer survival for those patients who were unmethylated at time of diagnosis (\(p = 0.05\)). When the analysis was performed with the patients subdivided according to sex, there was a significantly poorer survival for the four unmethylated males (\(p = 0.0002\), Figure 5.8 (B)). A similar analysis performed using all of the 36 patients revealed that unmethylated males (\(n = 9\)) had a significantly shorter overall survival (\(p < 0.0001\), Figure 5.8(C)) and disease-free survival (\(p = 0.0003\), Figure 5.8 (D)). These results
indicate that methylation of the RLGS NotI landmark site is a potential predictor of poor outcome in males, and justifies further analysis of this locus in a larger sample set.

5.4 Discussion

In the past few years AML has been recognized as having a hypermethylation phenotype.\textsuperscript{46,48,101} In a previous study we used RLGS to demonstrate widespread methylation of multiple CpG islands in AML diagnostic samples.\textsuperscript{49} One of the strengths of this technique is the ability to identify novel methylation targets that might go undetected in a candidate gene approach to methylation analysis. Our initial screen identified CYP1B1 as one such methylation target in \textit{de novo} AML, and we have now extended our analysis to include more patients and to perform a detailed examination of the CpG island associated with this gene.

Bisulfite genomic sequencing allowed the interrogation of 59 CGs in five areas of the gene and the 5’ regulatory region, including the promoter. We showed extensive methylation outside of the CpG island in both normal cells and AML blasts, while methylation of the promoter showed interindividual heterogeneity. Overall, the levels of methylation between diagnostic and remission samples in the promoter region were similar. Methylation in the body of the gene, however, was strikingly different between diagnostic and remission samples, with a much higher percentage of methylation present at diagnosis (Figure 5.4). The RLGS NotI site is located just 3’ to Region 4, in an area that we were unable to incorporate in our bisulfite reactions.

\textit{In vitro} experiments with the Kasumi-1 AML cell line confirmed that CYP1B1 expression is epigenetically regulated. Untreated cells did not express the gene, but expression was activated with the use of 5-aza-2’-deoxycytidine, an inhibitor of DNMT1. Treatment with a histone deacetylase (HDAC) inhibitor alone did not restore expression, but the combined use of the two agents resulted in a marked increase in expression as measured by Real-Time PCR (Figure 5.6). While there was demethylation occurring in the CYP1B1 promoter (Region 3) following the various treatments, the majority of demethylation, as assessed by bisulfite sequencing, appeared to take place in Regions 1 and 2 (Figure 5.7). The synergy observed in our experimental system supports work by others who have also reported synergistic effects with combined demethylating
and histone modification treatments.\textsuperscript{22,23,271} These observations have important therapeutic implications for the combined use of these agents in clinical trials.

As stated earlier, several investigators have identified methylation of multiple genes in AML. However, the relationship of these methylation events to the pathogenesis of AML remains unclear. The finding that an unmethylated RLGS NotI site appears to correlate with poor outcome for males with AML requires further investigation with larger sample numbers and precise quantitation of the degree of methylation. Given the function of \textit{CYP1B1} in estrogen metabolism, perhaps males who are unmethylated at this site have more CYP1B1 activity and enhanced conversion of 17\(\beta\)-estradiol to the carcinogenic 4-hydroxyestradiol.\textsuperscript{260,261} There may also be unidentified xenobiotics whose metabolism to carcinogens is increased in males, and an unmethylated NotI site could be a surrogate marker for such enhanced activity. \textit{CYP1B1} may also play a role inactivating certain chemotherapeutic agents\textsuperscript{273,274}, and this potential mechanism for drug resistance warrants investigation in unmethylated males.

Data from mouse models show that disruption of \textit{Cyp1b1} is protective for chemically-induced hematopoietic neoplasms\textsuperscript{264,265}, and over-expression of human CYP1B1 protein has been noted in several types of human cancers.\textsuperscript{275} Since expression of \textit{CYP1B1} is driven by binding of the AhR/ARNT complex to the DREs in the 5’ region, gel shift experiments are underway to determine if methylation of the CG dinucleotide within the DREs core sequence (GCGTG)\textsuperscript{252} interferes with binding of the complex. We hypothesize that methylation at the DREs precludes AhR/ARNT binding, which would result in decreased expression of \textit{CYP1B1} and potentially less conversion of procarcinogens to carcinogens in normal and leukemic blast cells. The exact relationship between methylation at the DREs and the NotI site is uncertain at this time.

\subsection{Conclusion}

In conclusion, through our genome scan we identified differential methylation of \textit{CYP1B1} in primary AML samples. We demonstrated that expression of this gene is under epigenetic control, and can be re-activated by the use of drugs that inhibit methylation and histone
deacetylation. Analysis of outcome data for our 36 patients showed a strikingly poor overall survival and disease-free survival for males without methylation at the RLGS NotI landmark site. Taken together, these data provide evidence that methylation of CYP1B1 warrants further study as a potential biomarker in AML.
<table>
<thead>
<tr>
<th>Region</th>
<th>Sequence</th>
<th>HSU56438</th>
<th>Product Size bp</th>
<th>Annealing Temp C</th>
<th># CGs</th>
</tr>
</thead>
</table>
| 1      | F-TGGATATGGGAAGGTTGTTATGAA  
R-CAACACCTAAACCTCCAAAA | 307-560 | 254            | 62               | 4     |
| 2      | F-GGATTTGGAGTGAGGTTGTTG  
R-TTCACACACACACACCGTACATC | 1478-1654 | 177            | 60               | 7     |
| 3      | F-GATGGAGGTGGTTGTGATGGA  
R-CCACACTCCACTCCAAAATC | 2806-3062 | 257            | 63               | 24    |
| 4      | F-GAGAGTGTTGGGTTGATTGATG  
R-AACCCACATACACAAATAACCAACA | 3635-3914 | 280            | 60               | 14    |
| 5      | F-GGGTATGTGTGTAGTGAAGGAA  
R-CCTCCTAAATATCCCCCATCTCATTTC | 5333-5548 | 217            | 62               | 10    |

Bisulfite Sequencing

All CYP-BS rxns with 95 x 10m denat; 35 cycles of 96 x 30s, A x 30s, 72 x 30s, and final 72 x 10m extension

All rxns 1 ul DNA, 6 ul each primer (10 pmol/ul), 2.5 ul dNTP (25 mM), 5 ul 10x H's buffer, 2.5U Taq, 29.25 ul H2O

RT-PCR

F-ACAGCATGATGCAGCAACTTC  
R-TGGTCAGGCTTGTTGATG | 4251-8210 | 927 | 57 |
spans intron 2

Taq-Man

F-CGTACCACCGCCACTACATCTG  
R-TCACCACATACAGGCCAGACG | 4762-7939 | See reference 416 |
probe CTCCTCCTCCCACAGGTATCTCTG | probe spans junction of exons 2 and 3 |

Table 5.1. CYP1B1 PCR primers and conditions.
Figure 5.1. Genomic structure of CYP1B1. Twelve kb of genomic sequence that encompasses the CYP1B1 gene on 2p21 is shown. CG dinucleotides are depicted as hashmarks. The locations of the CpG island, RLGS NotI-EcoRV fragment, dioxin-responsive elements (triangles) and promoter (P) are shown. Exons 1-3 are designated by raised boxes. Regions 1-5 which were analyzed by bisulfite sequencing are shown at the bottom.
Figure 5.2. Bisulfite PCR products and chromatograms for CYP1B1. (A) Representative PCR products of bisulfite-treated DNA from AML diagnostic (Dx) and complete remission (CR) samples for Region 3. M: marker. Un: DNA that has not been treated with sodium bisulfite. (B) Portions of chromatograms showing differential methylation (arrows) of 2 CGs between the diagnostic and remission sample of the same patient.
Figure 5.3. Bisulfite sequencing of Regions 1-5 in paired AML samples. Bisulfite sequencing was performed on eight paired AML diagnostic (DX) and complete remission (CR) samples for Regions 1-5, to assess a total of 59 CGs. Peripheral blood and bone marrow from healthy volunteers are also shown. Patient numbers and disease status are at the far left. Each horizontal row in each region represents the sequencing results of one randomly selected cloned PCR product. Blue rectangles indicate a methylated CG. White rectangles indicate an unmethylated CG. An “X” indicates that the sequence could not be analyzed. Regions are numbered as in Figure 5.1.
Figure 5.4. Percentage of methylation of 59 CGs across CYP1B1. The frequency of methylation (expressed as percent) for each of the 59 CGs represented in Figure 5.3 was calculated for the AML diagnostic (blue diamonds) and remission (pink squares) samples. The boundaries between Regions 1-5 are marked by vertical lines.
Figure 5.5. RT-PCR of CYP1B1 in Kasumi-1 cell line. Treatment of the Kasumi-1 AML cell line with 2.5 µM 5-aza-2'-deoxycytidine resulted in enhanced expression compared to the non-treated control. GPI, glucose phosphate isomerase, was used as a control for RNA integrity.
Figure 5.6. Real-Time PCR shows synergistic effects of 5-aza-2'-deoxycytidine and depsipeptide. Kasumi-1 cells were treated with 5-aza-2'-deoxycytidine and depsipeptide as described in Materials and Methods. Real-Time PCR shows increased expression with 5-aza-2'-deoxycytidine alone, and a marked synergistic effect with the combined treatment.
Figure 5.7. Progressive demethylation of Kasumi-1 cells with 5-aza-2'-deoxycytidine +/- depsipeptide. Bisulfite sequencing on Kasumi-1 cells treated with 5-aza-2'-deoxycytidine +/- depsipeptide shows demethylation throughout the five regions, with concomitant increase in expression as shown in Figure 5.6. Blue rectangles indicated a methylated CG. White rectangles indicated an unmethylated CG.
Figure 5.8. Absence of methylation at the CYP1B1 RLGS NotI site is a predictor of poor outcome for males with AML. The methylation state of the RLGS NotI site was used to construct Kaplan-Meier plots of overall survival in 18 patients treated on CALGB protocol 9621 (A), and further subdivided by sex (B). The complete set of 36 patients was subdivided by sex and Kaplan-Meier plots were constructed for overall survival (C) and disease-free survival (D).
CHAPTER 6

FUTURE DIRECTIONS

6.1 Mechanisms of aberrant DNA methylation in cancer

Undoubtedly, DNA methylation plays an important role in tumor progression. But what mechanisms underlie the simultaneous global hypomethylation and CpG island hypermethylation that are frequently observed in cancer? How do these relate to changes in histone acetylation patterns and transcriptional control, and how amenable are these changes to pharmacologic manipulation? These are important questions in the field of epigenetics, and finding the answers will require careful dissection of the molecular events as they occur in vitro and in vivo.

Cell lines will provide an invaluable resource for investigating the interplay between methylation and histone modifications. This is already leading to important discoveries that will help us understand the relative contributions of each of these epigenetic changes in tumor cells. In addition, in vitro systems also allow the study of the pharmacologic capacity of epigenetic modifying drugs, such as 5-aza-2'-deoxycytidine to elicit demethylation, and trichostatin A, to inhibit histone deacetylation. These studies will lead to greater insight into the breadth and nature of transcriptional changes that occur in response to such interventions, as well as provide important information regarding effects on cellular proliferation, differentiation, and apoptosis. This research will be enhanced by new technologies such as cDNA microarrays and methylation arrays, which should provide important information not only about individual genes, but also about entire pathways that are affected by epigenetic lesions.

In vivo analyses will be critical for a complete understanding of these cellular processes. Genetically engineered mice will allow the evaluation of whole organisms, and help determine the
effects of epigenetic changes on both normal tissues and tumors. These model systems will also permit the investigation of pharmacologic interventions as chemopreventive or chemotherapeutic strategies.

Perhaps the most significant advances will be made using naturally occurring tumors in humans. Studies on colonic adenomas that progress to carcinomas (the classic “Vogelgram”\textsuperscript{277}) have given us great insight into sequential genetic changes that take place in cancer, and should be exploited to determine concomitant epigenetic changes. Other examples would include analysis of carcinoma \textit{in situ} of the cervix, Barrett’s esophagus, and bronchial epithelial dysplasia in smokers. Studies of this nature would provide relevant data on the role of epigenetic lesions during tumor initiation and progression, and increase our knowledge of the timing of these alterations during each of these phases.

6.2 Future studies in hematologic malignancies

The potential role of \textit{CYP1B1} in AML warrants further investigation. Biochemical studies to determine the level of activity in AML blasts and normal bone marrow are needed. In addition, the analysis of more male patients is essential to confirm our preliminary observation that methylation status predicts poor outcome. The potential involvement of chromosome 11 methylation in the pathogenesis of AML also deserves further study. It is well established that chromosome 11 harbors a host of cancer-related genes,\textsuperscript{200,204} as well as imprinted genes\textsuperscript{206} and leukemia-related genes.\textsuperscript{196,198} Future studies should focus on loci with a high susceptibility to methylation in these regions and should also investigate the phenomenon of methylation “spreading” in these areas.\textsuperscript{237} In CLL we have identified a panel of novel methylation targets that, for the most part, differ in frequency from AML. Ongoing investigation with a number of these genes will hopefully elucidate their role in the pathogenesis of this disease. More patient samples are required to determine if methylation of certain loci correlates with immunoglobulin variable region mutational status, a known predictor of outcome in CLL.\textsuperscript{210,227}

Our work on methylation in hematologic malignancies has provided preliminary evidence that overall DNA methylation patterns can be used to construct a “methylation signature”. We
showed that certain tumors, including AML, exhibit tumor-specific methylation patterns.\textsuperscript{101} We also showed that certain methylation events, such as those described for \textit{CYP1B1}, have potential use as a prognostic indicator. Clearly, larger sample sizes are needed to make any significant clinical correlations. We anticipate that identification of these methylation events will define critical pathways that are altered in leukemogenesis and will lead to improved treatment of patients with leukemia.

Prior to our global methylation analysis in leukemia, the extent of aberrant methylation was underestimated. This takes on clinical significance as patients undergo clinical trials using demethylating agents\textsuperscript{97} and histone deacetylase inhibitors.\textsuperscript{245} It is imperative that the complete spectrum of gene activation and deactivation in response to these agents is fully appreciated.

6.3 Closing remarks

The research presented in this thesis was performed to identify aberrantly methylated genes in leukemia and to gain insight into the role of methylation in the natural biology of these diseases. Our data have established that methylation is far more prevalent than previously recognized, and have provided the groundwork for ongoing studies into how specific genes may be involved in leukemogenesis.

It is my hope that the outcome of this work will truly be translational in nature, and that it will result in improved patient care. My exposure to research began at the bedside of leukemia patients, and I have not lost sight of the fact that each vial of cells obtained from the CALGB Leukemia Tissue Bank came from a patient with hopes, dreams, and anxious family members. This work is dedicated to them.
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