Epigenetics 8:2, 138–148; February 2013; © 2013 Landes Bioscience

Uncovering the DNA methylome in chronic lymphocytic leukemia

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Keywords: chronic lymphocytic leukemia, hypomethylation, hypermethylation, CpG islands, gene silencing, microarrays, next-generation sequencing

Over the past two decades, aberrant DNA methylation has emerged as a key player in the pathogenesis of chronic lymphocytic leukemia (CLL), and knowledge regarding its biological and clinical consequences in this disease has evolved rapidly. Since the initial studies relating DNA hypomethylation to genomic instability in CLL, a plethora of reports have followed showing the impact of DNA hypermethylation in silencing vital single gene promoters and the reversible nature of DNA methylation through inhibitor drugs. With the recognition that DNA hypermethylation events could potentially act as novel prognostic and treatment targets in CLL, the search for aberrantly methylated genes, gene families and pathways has ensued. Subsequently, the advent of microarray and next-generation sequencing technologies has supported the hunt for such targets, allowing exploration of the methylation landscape in CLL at an unprecedented scale. In light of these analyses, we now understand that different CLL prognostic subgroups are characterized by differential methylation profiles; we recognize DNA methylation of a number of signaling pathways genes to be altered in CLL, and acknowledge the role of DNA methylation outside of traditional CpG island promoters as fundamental players in the regulation of gene expression. Today, the significance and timing of altered DNA methylation within the complex epigenetic network of concomitant epigenetic messengers such as histones and miRNAs is an intensive area of research. In CLL, it appears that DNA methylation is a rather stable epigenetic mark occurring rather early in the disease pathogenesis. However, other consequences, such as how and why aberrant methylation marks occur, are less explored. In this review, we will not only provide a comprehensive summary of the current literature within the epigenetics field of CLL, but also highlight some of the novel findings relating to when, where, why and how altered DNA methylation materializes in CLL.

Introduction

Indisputably, genetic alterations are key players in chronic lymphocytic leukemia (CLL) leukemogenesis, yet these lesions only partially explain the pathobiology of this disease.[1] Today, growing evidence acknowledges the intricate interplay of genetic and epigenetic events shaping the complex molecular landscape in CLL. Unlike genomic lesions, epigenetic aberrations, such as anomalous histone and DNA methylation marks and dysregulated miRNAs, provoke changes to the chromatin/DNA without modifying the genomic sequence. DNA methylation is the most extensively studied epigenetic mark, involving the addition of a methyl group by DNA methyl-transferases (DNMTs) to the fifth position of the cytosine ring within CpG dinucleotides.[2-4] Repetitive regions of chromosomes carry the highest density of CpG dinucleotides and in normal cells remain heavily methylated. In contrast, small concentrated regions of CpGs, referred to as CpG islands located primarily in gene promoters, are normally unmethylated, with the exception of imprinted and tissue-specific genes. In cancer, the opposite scenario ensues, where DNA methylation engages primarily, but not exclusively, within CpG island promoters, whereas repetitive elements become increasingly unmethylated.[2,5] DNA methylation is also recognized as a relatively stable modification inducing transcriptional inactivation of both protein coding and non-coding regulatory miRNAs.[2,6,7] For this reason, DNA methylation is now considered one of the hallmark mechanisms of aberrant gene silencing in cancer. In this review, we will not only survey the literature of the evolving field of CLL epigenetics during the last two decades, from the finding of general hypomethylation in CLL to studies of single genes promoters and, more lately, the application of whole-genome technologies, but will also emphasize important findings, providing hints to when and how the epigenetic landscape takes form in CLL.

Hypomethylation Contributes to Genomic Instability and Gene Activation in CLL

Early DNA methylation studies implicated the importance of hypomethylation as a key tumorigenic event promoting genomic instability and proto-oncogene activation in CLL and other cancers (**Fig. 1**).[8-10] Over two decades ago, hypomethylation of *ornithine decarboxylase*, a vital downstream regulator of the *MYC* oncogene, was detected in CLL.[11] However, it was not until four years later that high-pressure liquid chromatography (HPLC) analysis revealed the DNA of CLL to be globally hypomethylated relative to healthy controls. As mentioned, it is the repetitive

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Submitted: 11/30/12; Revised: 12/20/12; Accepted: 12/28/12
http://dx.doi.org/10.4161/epi.23439

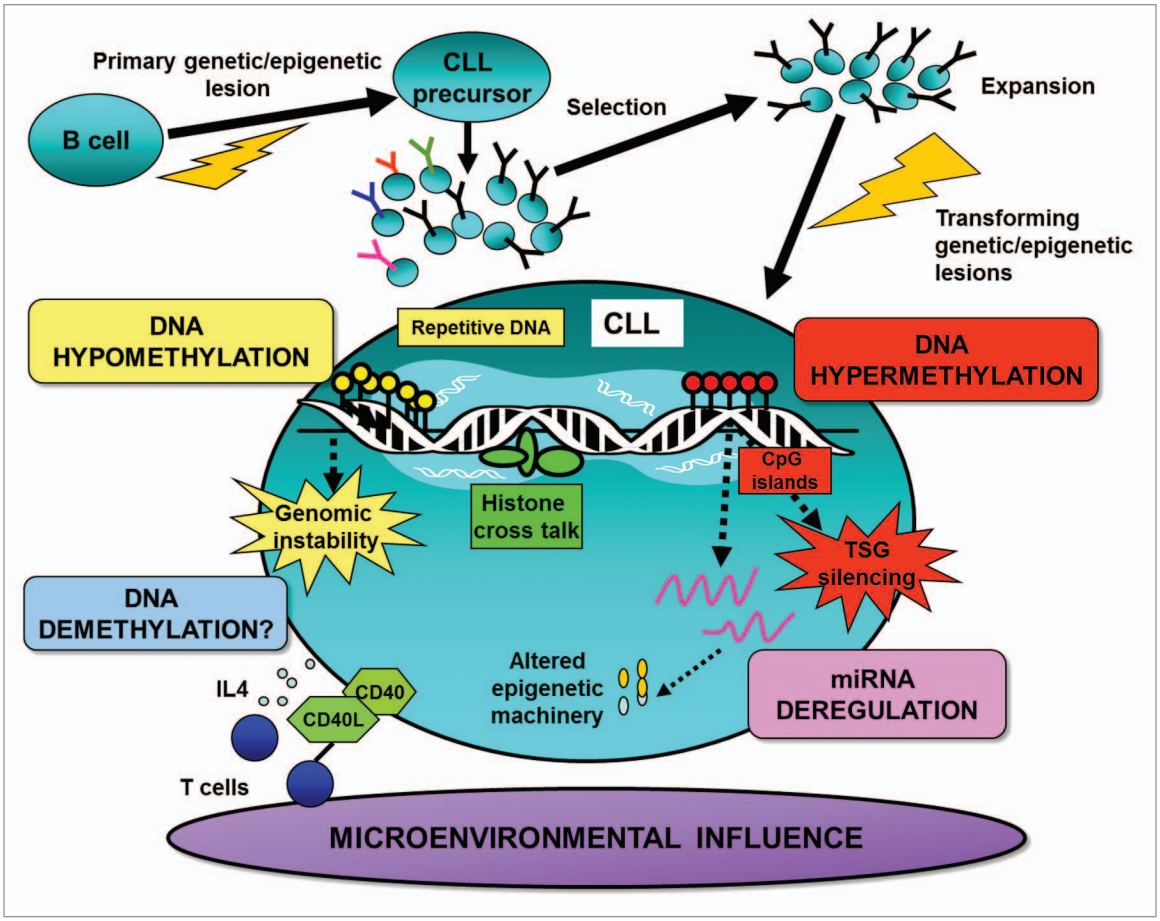

Figure 1. Illustration of epigenetic factors shaping the DNA methylome in CLL. This schematic details: (1) the possible timing and role of DNA methylation in CLL pathogenesis, (2) DNA hypermethylation silencing of vital tumor suppressor genes (TSGs), (3) DNA hypomethylation leading to genomic instability, (4) dysregulation of epigenetic regulators and machinery through aberrant methylation and (5) the interplay between DNA methylation and other epigenetic/microenvironmental factors.

sequences of the genome that mainly lend themselves to increased hypomethylation during tumorigenesis.[12] Independent analysis in CLL corroborates this finding showing aberrant hypomethylation of repetitive sequences, such as ALU, LINE and SATα, to be particularly marked in aggressive CLL cases with *TP53* aberrations.[13] Interestingly, low SATα methylation levels have been further shown to be an independent predictor of time to first treatment in CLL.[13] These latter findings are relevant since hypomethylation leading to genomic instability may be a contributing factor in the increased propensity of *TP53*-deleted/mutated cases to acquire genomic alterations. More recently, next-generation sequencing (NGS) of the DNA methylome has also noted gene body hypomethylation to be particularly widespread within enhancer regions in CLL patients.[14]

After the CLL genome was discovered to be hypomethylated, the rising pursuit for aberrantly methylated oncogene targets revealed hypomethylation of *BCL2,* a key anti-apoptotic gene, to correlate with increased protein expression of BCL2 in CLL.[15] Following this, *MDR1*, the multiple drug resistance gene,[16] and *TCL1*, an activator of NF-κB, were subsequently found to be hypomethylated and upregulated in CLL.[17] Through subsequent investigations, however, it became apparent that the activation of oncogenes through DNA hypomethylation was a rather infrequent lesion in CLL. Hence, with the landmark discovery of DNA hypermethylation silencing of tumor suppressor genes (TSGs) in cancer (**Fig. 1**),[2] the search for aberrantly methylated genes serving as candidate prognostic and treatment targets soared. As a result, the hunt for such markers has led to the rapid evolution of DNA methylation technologies from the single gene approach to more global explorative, high-resolution methodologies.

Clinical and Biological Consequences of Hypermethylated Genes in CLL: Lessons Learned from the Study of Single Gene Promoters

In CLL, a myriad of semi/fully quantitative DNA methylation studies of single gene promoters have identified a plethora of targets of potential clinical and biological interest. One of the first frequently hypermethylated promoters observed in CLL was E-Cadherin (*CHD1*),[18] a well-known suppressor of metastasis in solid tumors (as reviewed in ref. 19). Although a role for *CHD1* methylation in leukemia remains elusive, studies have noted a reduced or absent expression of E-cadherin in hypermethylated CLL cases relative to normal B cell.[18,20] Similarly, methylation

Table 1. Key genes inflicted with aberrant DNA methylation in CLL

Gene/pathway	Functional role	References
	CLL prognostic genes	
ZAP70	Involved in B cell/T cell signaling. Methylation related with decreased expression and *IGHV*-mutated CLL.	23, 42
LPL	Prognostic marker in CLL. Methylation associated with decreased expression and *IGHV*-mutated CLL.	42, 81
CLLU1	Prognostic marker in CLL. Methylation correlates with decreased expression and *IGHV*-mutated CLL.	42, 83
NOTCH1	Mutations of *NOTCH1* are associated progressive CLL disease and are a marker of poor prognosis.	42, 84
	Tumor suppressors	
WISP3	Involved in WNT signaling. Implicated in breast cancer. Preferentially methylated in *IGHV*-mutated CLL.	42, 85
VHL	Associated with von Hippel-Lindau syndrome, a hereditary cancer syndrome predisposing to cancer. Preferentially methylated in *IGHV*-unmutated CLL	44, 86
ABI3	ABI3 protein inhibits metastasis and tumor migration. Frequently methylated in *IGHV*-unmutated CLL	42, 44, 87
DAPK1	Positive mediator of apoptosis. Potential tumor suppressor. Commonly methylated in CLL, indicated in familial CLL.	31
	Survival/proliferation related genes	
MYB	Proto-oncogene regulates miRNA155 in CLL. Methylated in *IGHV*-mutated CLL	42, 88
LEF1	Involved in WNT signaling. Pro-survival factor in CLL, methylated in good-prognostic CLL.	42, 89
SNRP2 and *SNRP4*	WNT signaling inhibitors commonly methylated in CLL.	20
	Transcription factor genes	
TWIST2	Known silencer of p53 in hematological malignancies. Hypermethylation common in *IGHV*-mutated CLL.	22
HOXA4	Transcription factor important for cell development. Commonly methylated in poor-prognostic CLL.	26
	miRNAs*	
miR-34a, ***miR-129***, ***miR-708***, *miR-124–1*, *miR-203* and *miR-9*	Regulatory sequences thought to regulate gene expression of key genes involved in CLL pathogenesis such as ***TP53, XPO1*** and ***NOTCH1***	49–51, 58
Epigenetic regulator genes		
HDAC4/HDAC9	Histone deacetylases, differentitally methylated between prognostic CLL subgroups.	42, 90

*Anticipated targets of the bold marked miRNAs are correspondingly highlighted in bold.

of the telomerase enzyme *hTERT* promoter was found to be associated with low expression, low activity, shortened telomere length and poor overall survival in CLL.[21] Using one of the earliest genome-wide technologies, restriction landmark genome scanning (RLGS), *TWIST2*, a transcription factor and known silencer of p53, was shown be preferentially methylated in CLL.[22] Through subsequent analysis, methylation of *TWIST2* was demonstrated to be more frequent within favorable prognostic *IGHV*-mutated relative to poor-prognostic *IGHV*-unmutated CLL cases (**Table 1**).[22]

Around the same time, *ZAP70*, a known prognosticator in CLL and an intracellular tyrosine kinase involved in B cell (and T cell) signaling, was shown to be differentially methylated in CLL (**Fig. 2 and Table 1**).[23] Accordingly, several studies have found good-prognostic *IGHV*-mutated CLL to have low ZAP70 expression associated with DNA methylation silencing, whereas poor-prognostic, high ZAP70-expressing cases demonstrate less methylated promoters.[23-25] More specifically, Corcoran et al. described the methylation status of C-334, a CpG site 334 bp away from the transcription start site of *ZAP70*, to be predictive of prognosis and associated with expression and *IGHV* gene mutational status.[23] Similarly, Chantepie et al. have determined the methylation status of 4 CpG pairs in the first intron (C-223, C-243, C-254 and C-267) of *ZAP70* to correlate to clinical outcome and expression.[24] More recently, Claus and colleagues identified loss of methylation at a single CpG site within the 5' regulatory region to correlate not only to mRNA expression and prognosis but also to protein expression and ZAP70 activity.[25] Since the ZAP70 expression level in a particular sample, as measured by flow cytometry or real-time quantitative PCR, may be influenced by other immune cells expressing ZAP70, these latter studies endorse the use of quantitative *ZAP70* methylation measurement in clinical routine.[24,25]

Another recurrently methylated target, the *HOXA4* promoter, inversely correlates to *HOXA4* gene expression in CLL (**Table 1**).[26] *HOXA4* is part of the *HOXA* gene cluster, a family of transcription factors important for cell development whose expression is commonly altered in lymphoid malignancies.[27,28] Interestingly, hypermethylation of *HOXA4* is more commonly detected among poor-prognostic *IGHV*-unmutated CLL cases.[26] More recently, *HOXA4* was included in a panel of methylation markers that was proposed to improve the risk stratification in CLL. Relative to the individual loci, combining the methylation status of *HOXA4* along with *BTG4* and *CD38* to produce an

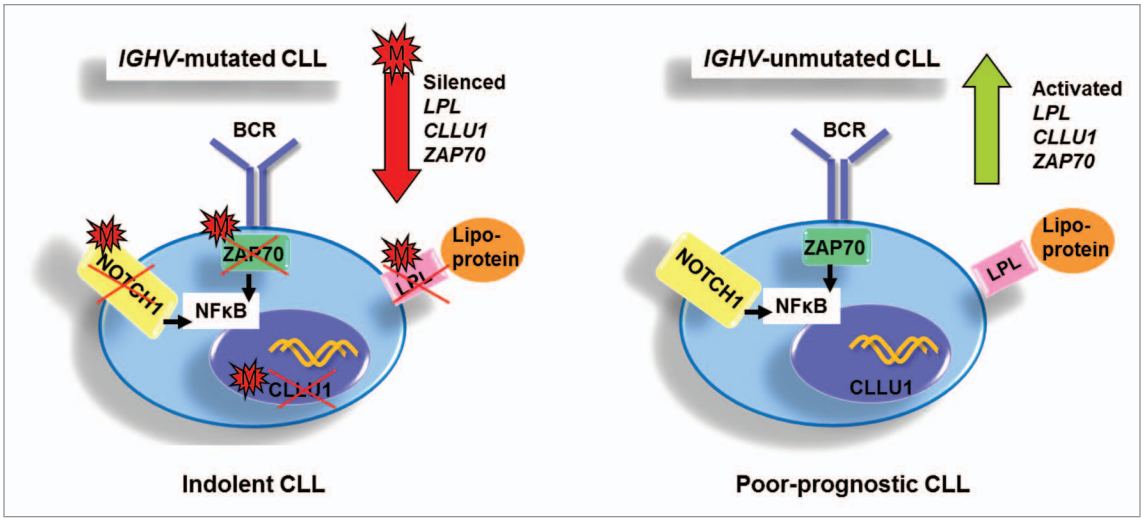

Figure 2. Illustration summarizing the differential methylation status of key prognostic genes, i.e., *LPL*, *CLLU1* and *ZAP70,* and their biological/clinical effects in favorable-prognostic *IGHV*-mutated vs. poor-prognostic *IGHV*-unmutated CLL. M, methylated.

overall methylation score was indicated to be a strong predictor of time to first treatment, independent of *IGHV* mutational and CD38 expression status. Notably, this panel could identify a subset of *IGHV*-mutated patients who had a greater risk of progressive disease.[29]

DNA methylation changes have also been associated with CLL transformation and familial CLL.[30,31] In 2007, a major breakthrough highlighting the importance of *DAPK1* DNA methylation in CLL came to light. Here, Raval and colleagues demonstrated DNA methylation silencing of *DAPK1*, a pro-apoptotic gene, to occur in almost all sporadic cases of CLL. Remarkably, they further showed *DAPK1* downregulation through promoter methylation to contribute to a heritable predisposition to CLL (**Table 1**).[30,31] More recent discoveries include hypermethylation of the *CRY1* gene in high risk CLL[32] and frequent promoter methylation of the *SLIT2* gene, a candidate tumor suppressor frequently inactivated in lung and breast cancer.[33] Since *CRY1* is a circadian gene involved in the expression of cell cycle and DNA damage response genes, and given the putative role of *SLIT2* in other cancers, it is interesting to speculate that deregulation of these genes is involved in CLL leukemogenesis.

As the single gene approach gave little insight into aberrantly methylated pathways at play in CLL, DNA methylation studies evolved to include more comprehensive investigations of signaling cascades and gene families. One of the most extensively studied is the WNT pathway, a key pathway in B cell development, constitutively activated in CLL.[34-36] Chim et al. found WNT signaling activation to be related to hypermethylation of WNT inhibitor genes.[35] In this study, a large proportion of the cohort showed methylation of all 7 WNT inhibitor genes studied, i.e., *WIF1, DKK3, APC, SFRP1, SFRP2, SFRP4* and *SFRP5* (**Table 1**). Interestingly, over half of CLL cases showed methylation of at least one inhibitor.[35] Later studies by Seeliger and Liu further corroborated the particularly frequent hypermethylation of the *SFRP1, SFRP2* and *SFRP4* genes in CLL and suggesting these as key players in leukemogenesis (**Table 1**).[20,34] More recently, another group of genes involved in the Salvador-Warts-Hippo (SWH) pathway, the RASF family gene members 1–10 and two upstream members of the SHW circuit, *KIBRA* and *CRB3,* have been studied at the DNA methylation level.[37] Here, the most frequently methylated genes were *RASF10*, followed by *RASF6*. The upstream regulator *KIBRA* was also found to be recurrently methylated and to be associated with poor-prognostic factors, such as unmutated *IGHV* genes and CD38 expression.[37]

Global Patterns of Aberrant DNA Methylation in CLL: The Genome-Wide Perspective

The characterization of methylation patterns of single genes in CLL, although limited, highlighted the ability of DNA methylation to influence a range of functionally diverse genes of clinical and biological importance in CLL. These initial findings have instigated a wave of genome-wide investigations searching to map the global DNA methylation landscape and determine the role of DNA methylation outside of traditional CpG island promoters. In the beginning, through capillary electrophoresis-laser induced detection, the overall DNA methylation level was found to be rather heterogeneous between CLL patients. Nonetheless, a high genomic methylation level was shown to be associated with poor-prognostic *IGHV*-unmutated CLL.[38] More recently, Yu and colleagues, through HPLC analysis, indicated that CLL patients with a higher methylation index (MI) relative to age-matched controls have an increased likelihood of requiring treatment, whereas a lower MI was observed in CLL without need of therapy.[39] In light of the fact that these latter methods lacked the capacity to identify target sequences influenced by DNA methylation, attention turned to the development of global methods facilitating the analysis of specific targets.

The advent of such technologies gave rise to RLGS, a 2D electrophoresis method interrogating ~3,000 CpG sites globally.

By applying RLGS to 10 CLL cases, Rush et al. demonstrated between 2.5–8.1% of CpG islands to be aberrantly methylated relative to healthy donors.[40] Of the 193 methylated sequences noted, 93% maintained CpG island characteristics and 90% had homology to expressed genes, such as known transcription factors (e.g., *FOXE1* and *TBX3*).[40] Since then, a number of microarray-based studies with an ever increasing resolution and rising number of CpG targets have come to light in CLL. Using two different microarrays, Rahmatpanah et al. identified over 100 genes to be hypermethylated in CLL relative to normal B cells.[41] Although the majority of genes maintained the same DNA methylation status across CLL samples with different CD38 expression levels, a panel of genes was shown to segregate according to high or low CD38 expression. For example, *NRP2*, *SFRP2* and *ADAM12* were preferentially methylated in "CD38 high" cases (poor-prognostic), whereas methylation of *DLEU7* was found in "CD38 low" cases (good-prognostic). Notably, an overrepresented number of WNT signaling genes, particularly the WNT inhibitory genes, were affected by methylation, a finding reported earlier using single gene promoter applications (see above).[41]

Other microarray studies have successfully identified aberrant methylation patterns in certain prognostic subgroups of CLL.[42-44] For instance, Tong et al. have revealed 280 aberrantly methylated targets in the poor-prognostic 17p-deleted CLL subgroup. These targets were shown to cover numerous functional networks and were more frequently found within chromosomes 11, 17 and 19. Interestingly, four aberrantly methylated genes identified on chromosome 17 where known to interact with p53.[43] At the global level, our research group has identified a differential methylation pattern distinguishing poor-prognostic *IGHV*-unmutated from favorable-prognostic *IGHV*-mutated CLL patients (**Fig. 3**).[42,44] In our first 27K microarray study, a number of TSGs, such as *ABI* and *VHL,* were observed to be preferentially methylated in *IGHV*-unmutated relative to *IGHV*-mutated CLL (**Table 1**). Furthermore, genes occupying MAPK and NF-κB pathways, involved in cell proliferation and progression, were found to be unmethylated in *IGHV*-unmutated cases compared with *IGHV*-mutated patients. Additionally, we also observed distinct methylation patterns deciphering poor-prognostic *IGHV3-21* CLL from *IGHV*-mutated and unmutated cases.[44] Using the same type of array, we also compared the methylation profiles in three major and paradigmatic CLL subsets with stereotyped B-cell receptors: the poor-prognostic subsets #1 (*IGHV1/5/7/IGKV1-39*) and #2 (*IGHV3-21/IGVL3-21*) and the favorable-prognostic subset #4 (*IGHV4-34/IGKV2-30*), which revealed distinct methylation profiles for each subset. Interestingly, gene ontology analysis of the differentially methylated genes showed a striking enrichment of genes involved in immune response, such as B-cell activation, which were generally methylated in subset #1 vs. subset #2 and, in particular, subset #4. As a prime example, the co-stimulatory molecules CD80 and CD86 were methylated and not expressed in subset #1, while these remained unmethylated and were expressed at high levels in subset #4, pointing to a key role for these molecules in the cross-talk with the microenvironment in subset #4 CLL cells.[45]

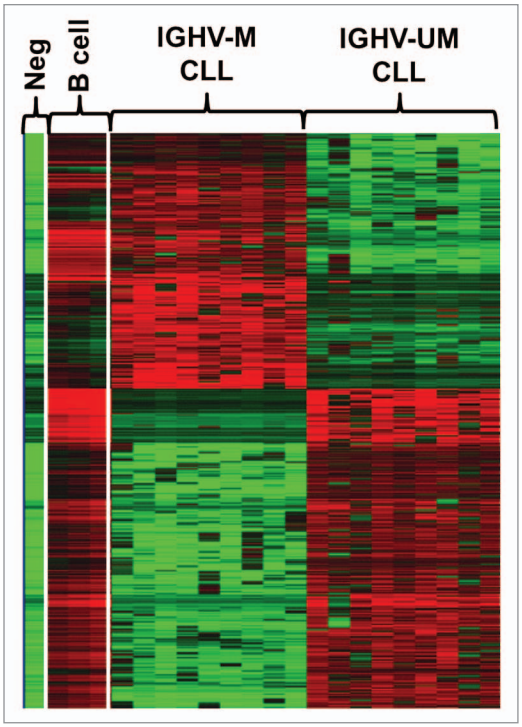

Figure 3. Heat-map showing the global differential DNA methylation profile distinguishing *IGHV*-mutated (IGHV-M) from *IGHV*-unmutated (IGHV-UM) CLL. Adapted from Cahill et al. 2012. Neg, whole genome amplified negative control; B-cell, aged-matched normal B cells.[42]

More recently, using 450K-array analysis, interrogating over 485,000 CpG sites, we revealed a set of CLL prognostic genes, i.e., *CLLU1*, *LPL*, *ZAP70* and *NOTCH1* (**Fig. 2 and Table 1**), as well as epigenetic regulators (i.e., *HDAC9*, *HDAC4)*, the B-cell signaling *IBTK* gene and numerous signaling targets involved in TGF-β and NF-κB/TNF pathways to be alternatively methylated between *IGHV*-mutated and unmutated CLL.[42] Furthermore, using these arrays, we for the first time noted DNA methylation in CLL to be relatively stable over time and similar in CLL cells derived from proliferative (lymph node) and resting (peripheral blood) microenvironments.[42] Unlike other array studies in CLL, we could characterize CpG sites outside of CpG islands and found a large proportion of the differentially methylated sites identified between *IGHV*-mutated and unmutated CLL to reside in CpG shores, regions positioned up to 2 kb away from the promoter (**Fig. 4**). Interestingly, Irizarry and colleagues have shown that the methylation status of these shore regions strongly correlates with gene expression.[46] In addition, on investigating the position of these CpG sites in relation to the gene orientation, we noted a large proportion of aberrantly methylated sites to occupy gene-bodies, a finding which has been also evidenced more recently using next-generation bisulfite sequencing in CLL.[14] As a proof of principle, we and others have also demonstrated the biological relevance and reversible nature of DNA methylation through reactivation of selected genes using methyl and HDAC inhibitors.[43,44,47] For instance, by treating primary CLL B cells with concomitant methyl and deacetyl inhibitors, we could induce a reduction in

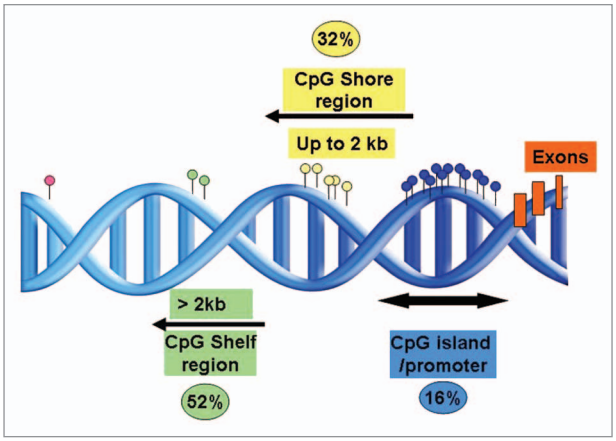

Figure 4. Representation of the proportion and positions of the annotated differentially methylated sites between *IGHV*-mutated and unmutated CLL in relation to the CpG island.[42]

DNA methylation and reinstate the expression of TSGs *ABI3* and *VHL* in CLL.[44]

Now, with the advent of NGS technologies, the limitations of microarray, such as restricted genome coverage, can be overcome. To date, only two NGS studies have been conducted in CLL, one employing the reduced representation bisulfite sequencing (RRBS) technique[48] and, the second, whole-genome bisulfite sequencing (WGBS).[14] Using RRBS, interrogation of 1.8–2.3 million CpGs were determined revealing ~45% of sites to be positioned in more than 23,000 CpG islands. However, global CpG methylation was determined to be rather similar between CLL and normal controls. That being said, 1,764 gene promoters were shown to be differential methylated in at least one CLL case relative to normal control. Almost 20% of the differentially methylated genes were implicated in transcription regulation. Of interest, aberrant methylation was found to be enriched in WNT signaling genes and all *HOX* gene clusters were subject to anomalous methylation. Using this technology, NFATc1 hypomethylation was identified and was further shown to be associated with increased mRNA and protein expression suggesting hypomethylation as a mechanism of constitutive activation of NFATc1 in CLL.[48] RRBS offers a rather high coverage of the genome; however, it is still limited by the fact that this technology is based on prior selection of the regions of interest. With sequencing costs becoming more affordable, WGBS, a complete genome-wide method, is set to revolutionize the DNA methylome mapping, providing unbiased coverage at single base resolution. Most recently, the validity of WGBS as a reliable method to characterize the CLL DNA methylome has been described. Using this technology, albeit on a rather small sample set, the methylation values retrieved using WGBS were found to be concordant to those given by the 450K microarray.[14] This conjoint sequencing/microarray study again revealed *IGHV*-mutated and unmutated CLL to differ at the global methylation level. Furthermore, they noted that extensive gene body DNA hypomethylation targeting mainly enhancer sites could distinguish these two molecular CLL subtypes relative to normal B cells and differentiate naive from memory B cells. Additionally, a relationship between gene body hypomethylation and gene expression was established.[14]

Undoubtedly, the aforementioned studies have broadened our knowledge of the DNA methylome in CLL providing us with numerous candidate aberrantly methylated targets and sites which maybe of biological and clinical significance. Nevertheless, further investigation, verification and careful interpretation of the above findings is needed, especially when considering whether the functional consequences are directly the result of aberrant methylation or due to other epigenetic regulatory marks. Additionally, it must be kept in mind that a number of biological and technical factors can influence the results. For instance, the type of technology used, batch bias, the cut-offs used to call the level of methylation, the type of cohort studied, the number of patients included and sample purity among others must be considered. Now with the abundance of aberrantly methylated marks identified, future challenges such as deciphering passenger from driver lesions, establishing the function of methylation outside of CpG islands and unraveling the role of 5-hydroxymethyl-cytosine in CLL awaits us.

Aberrant DNA Methylation Leads to Dysregulation of Non-Coding miRNAs in CLL

Most noteworthy, recent studies have found aberrant DNA methylation to be a key mechanism in the deregulation of miRNAs in CLL (**Fig. 1 and Table 1**).[49-51] These single stranded non-coding miRNAs, through their downregulation of target proteins, act either in an oncogenic or tumor suppressive manner. For this reason, aberrant miRNA expression is now accepted as one of the main signatures in CLL pathogenesis.[52-57] The mechanisms underlying altered miRNA expression are poorly known, however it is increasingly apparent that genetic and/or epigenetic manipulation may play a role at least in some CLL cases. As a prime example, the common 13q deletion, which includes miR15a and miR16-1, leads to upregulation of *BCL2* and deregulated apoptosis, a well-described mechanism in human and murine leukemogenesis.[52,56]

Until recently, the role of aberrant methylation in tumor suppressor miRNAs was rather undefined in CLL. Pallasch and colleagues noted a number of deregulated miRNA promoters associated with decreased miRNAs expression, to have gain of methylation in many CLL cases compared with normal B cells.[59] In particular, promoters of miR-139 and miR-582 showed a significant gain of methylation in CLL.[59] Furthermore, through combining DNA methylation and miRNAs promoter profiles, Baer et al. have defined a panel of 128 recurrent novel and known miRNAs targets subject to altered promoter DNA methylation in CLL.[51] For instance, hypomethylation of miR-21, miR-29a, miR-34a, miR-155, miR-574 and miR1204 was shown to correlate with an upregulated expression of these respective regulatory miRNAs. Conversely, hypermethylation of miR-124-2, miR-129-2, miR-9-2, miR-551 and miR-708 correlated with a reduced expression of these miRNAs. Interestingly, increased expression of *XPO1*, a predicted target of miR-129, *POT1* an inferred target of miR-9, and *NOTCH1,* an anticipated target of miR-708, correlated with a reduction in expression of their respective regulatory

miRNAs (**Table 1**).[51] Nevertheless, this is probably just the tip of the iceberg and further studies will hopefully clarify which of these aberrantly methylated miRNAs that are key to the pathobiology of CLL.

Complex Regulatory Mechanisms Shaping the Aberrant DNA Methylation Landscape in CLL

DNA methylation partakes in a hierarchal order of epigenetic events that concomitantly work together to regulate nuclear structure and gene activity. For instance, gene inactivation is preceded by repressive histone alterations and DNA methylation forming a condensed genomic architecture impeding the binding of transcriptional machinery and thus gene expression. Conversely, gene activation is permitted through the lack of repressive histone and DNA methylation marks. Through these finely tuned events, normal processes, such as cell differentiation, tissue specific expression, genomic imprinting and transposon silencing, are coordinated.[3,60] The ability of epigenetic marks to manipulate the DNA and chromatin relies on the stringent regulation of the epigenetic machinery such as the DNMTs, histone modification enzymes, methyl-binding proteins, polycomb complexes and miRNAs, among others.[60] In normal cells, the order and timing in which these regulators cross talk to synchronize epigenetic marks at specific targets is not fully elucidated. Nonetheless, evidence of when aberrant DNA methylation takes place, how altered DNA methylation occurs and where DNA methylation partakes in the sequence of epigenetic events at specific gene targets in CLL is slowly emerging. In the following sections, we will discuss some of these issues governing when and how altered DNA methylation takes place in CLL. A fully comprehensive discussion of this topic is not provided since it is beyond the scope of this review.

When Does Aberrant DNA Methylation Take Place in CLL Pathogenesis?

The Eμ-TCL1 transgenic mouse model, referred to as the *TCL1* model, has been instrumental in deciphering the timing of epigenetic events, particularly DNA methylation, in CLL leukemogenesis.[61,62] This model overexpresses *TCL1,* a known oncogene, leading to a CLL disease phenotype similar to that of human poor-prognostic *IGHV*-unmutated CLL. Normally, these mice develop a CLL-like disease at around 11 mo; however, these mice demonstrated aberrant methylation as early as 3 mo before disease indications appeared.[61] A genome-wide scan for promoter methylation during the course of disease observed methylation to steadily increase from 0.4% at 3 mo to 0.6%, 1.2% and 1.9% at 5, 7 and 9 mo, respectively. Finally, during advanced disease, a markedly increased methylation level of 3.9% was detected. Intriguingly, the majority of early silenced genes were found to be subsequently methylated in human CLL. Furthermore, hypermethylated genomic repeat sequences found in wild type mice were also found to be already hypomethylated in 7-mo-old *TCL1* mice. Similarly, in human CLL, hypomethylation of LINE repeat sequences were found to be more pronounced in late stage patients relative to early stage cases.[61,62] As previously mentioned, our 450K-array analysis, encompassing patient-paired diagnostic and follow-up samples, noted DNA methylation to relatively stable over time.[42] More specifically, we found no recurrent differences to occur in *IGHV*-mutated CLL and few recurrent changes in *IGHV*-unmutated over the course of disease.[42] That said, in light of the heterogeneous nature of CLL, we determined a variable number of non-recurrent changes in both prognostic subgroups of CLL and found the amount of changes to be more pronounced in *IGHV*-unmutated CLL.[42] As *IGHV*-unmutated cases are more prone to acquire genomic lesions over time,[63,64] perhaps the aggressive character of *IGHV*-unmutated CLL allow the acquisition of a higher number of "passenger" epimutations during disease evolution. All together, these studies indicate aberrant DNA methylation to be early initiating lesions in CLL leukemogenesis.[42]

How Does Altered DNA Methylation Occur in CLL?

There is growing evidence that the epigenetic machinery falls victim to deregulation. Genomic alteration of DNMT enzymes and histone alteration enzymes that catalyze the addition or removal of epigenetic marks, as well as altered expression and epigenetic modification of the epigenetic machinery have all contributed to aberrant epigenetic lesions observed in different cancers.[65-68] Additionally, even so called epi-miRNAs, in which the miRNA components of the epigenetic machinery themselves target further epigenetic modifiers have been described in lymphoma.[65]

In CLL, no difference in gene expression of the DNA methylation maintenance enzyme *DNMT1* has been noted relative to normal B cells.[69] However, downregulated expression of the de novo methylating *DNMT3B* gene has been evidenced in CLL.[69] Interestingly, expression of the histone methyltransferase 1 enzyme *HMT1* has also been associated with more advanced disease stages of CLL.[69] Given the functional redundancy of these enzymes and the fact that their relative expression maybe related to the proliferative rate of cells, expression analysis must be interpreted carefully.

As mentioned earlier, the *TCL1* mouse model, which resembles human *IGHV*-unmutated CLL, has shown increased methylation in CLL with disease progression and this has subsequently been shown to follow the pattern of de novo methylating DNMT activity.[61,62] Here, protein levels of DNMT3A/3B were found to be absent during transformation, yet levels were shown to rise at later stages of disease.[61,62] Reduced expression of miRNA29a and miRNA29c was also noted in the *TCL1* mouse at 5 and 7 mo. This reduction correlated with increasing DNMT3A and 3B protein expression at a later age, suggesting miRNA29s to be a direct regulator of DNMT3A/3B in CLL, an interaction also apparent in lung cancer.[61,62,70] Hence, increased activity of DNMT3A and 3B was proposed as a possible factor triggering increased GpG island hypermethylation at distinct promoters in CLL.[61,62]

In support of the above findings, and also illustrating the pivotal role of miRNA29 in CLL leukemogenesis, miRNA29 transgenic mice display expanding $CD5^+$ B cell populations with CLL

characteristics. This miRNA was also found to be preferentially expressed in indolent CLL relative to aggressive CLL cases.[71] Moreover, histone deacetylase (HDAC)-mediated elimination of acetyl groups, triggering compact chromatin formation, has been evidenced to mediate silencing of miRNA29b and other miRNAs in CLL.[72] All together, these studies highlight the important role aberrant regulatory miRNAs at play in CLL pathogenesis.

More recent studies of the *TCL1* transgenic mouse have found TCL1 to physically interact and inhibit de novo DNMT3A activity.[73] On analyzing the B-cells from 4–6 week old *TCL1* transgenic mice, who characteristically display no signs of disease, a significant decrease in DNA methylation was noted compared with wild type controls.[73] Similarly, human CLL cells with high TCL1 expression had a lower DNA methylation level relative to patients with low expression.[73] In a somewhat contradictory manner to the aforementioned study by Chen et al.,[61] which indicated increased DNA methylation on CLL progression, Palamarchuk et al. suggest that in these disease-free mice, inhibition of de novo DNA methylation maybe a common mechanism leading to DNA hypomethylation of distinctive CpG sites perhaps during early pathogenesis.[73] Nonetheless, these studies clearly implicate altered regulation of de novo methylation enzymes to play a key role in leukemogenesis.

Lessons from normal cell development may provide vital clues as to how DNA methylation may be elicited and where in the hierarchal order of coordinated epigenetic marks DNA methylation is placed. One scenario proposes that histone marks direct DNA methylation events by acting as platforms permitting the recruitment or inhibition of the DNA methylation machinery.[4,74] The opposing notion suggests DNA methylation to serve as messenger directing the assembly of histone alterations.[4,75] In CLL, the importance of cross-talk between DNA methylation and other chromatin modifications in defining the aberrant epigenetic landscape is slowly emerging. For instance, the *TCL1* mouse model shows that 70% of the early methylated promoters identified in preclinical disease were targets of the *FOXD3* transcription factor, a frequently methylated/silenced gene in the *TCL1* mice and human CLL with high *TCL1* expression.[61,62] Importantly, *FOXD3* expression was shown to be repressed in these mice prior to methylation through an NF-κB p50/p50:HDAC1 repressor complex, eventually leading to methylation of downstream targets.[61] In human CLL, DNA methylation and histone modification cross-talk has been described for *ZAP70*[75] and Aiolos,[76] a transcription factor regulating the *BCL2* family members. Additionally, the *ID4* transcription factor is known to have a relatively high but rather variable level of DNA methylation at its promoter.[77] Nevertheless, *ID4* mRNA and protein levels are shown to be universally silenced in CLL, implying that other silencing components are initially responsible for gene deregulation, with DNA methylation occurring at later stages.[77] Similarly, *PTPROt*, a protein tyrosine phosphatase primarily involved in lymphocyte survival, was initially demonstrated to be frequently methylated in CLL.[78] However, lessons from the *TCL1* mouse now describe that up to 60% of *PTPROt* gene inactivation is the consequence of other inactivating complexes working independently of DNA methylation.[79] Overall, these latter studies highlight the importance of coordinating regulatory modifications in the control of gene transcription. For the most part, these studies portray DNA methylation as a stabilizing secondary modification in a hierarchal order of epigenetic events.

Finally, even the cell microenvironment in which the cells reside is suggested to prompt epigenetic mechanisms in CLL (**Fig. 1**). Intriguingly, recent in vitro studies describe a demethylation process triggered by a set of stimulators typically found within the CLL microenvironment.[80] Here, Morena and colleagues observed that induction of the leukemic clone with CD40L/IL4 and anti-IgM resulted in demethylation of *LPL*,[80] a prominent prognostic gene also known to be differentially methylated in good and poor CLL subgroups.[42,81] On the global level, we ourselves have searched for DNA methylation differences in patient-matched samples derived from alternative microenvironments. However, we observed the global methylation profiles to be rather similar in CLL cells derived from the proliferative lymph node and resting peripheral blood compartments.[42,81]

Concluding Remarks

Compared with other epigenetic marks, DNA methylation is deemed to be a rather stable event, a property that has enabled this modification to be extensively studied for more than two decades. Today, we recognize DNA methylation to influence a number of signaling pathway genes and key tumor suppressors of biological and potential clinical importance to CLL. Furthermore, we are now beginning to understand the extent of global aberrant DNA methylation in different prognostic subsets, the fundamental role of DNA methylation in sites positioned outside of CpG islands and the collaboration of DNA methylation with other regulatory messengers in shaping the CLL methylome. In the wake of the increasing knowledge provided by genome-wide studies, it has also become apparent that similar to the genomic scenario, an abundance of "passenger" DNA methylation events likely accompany this disease. In the next coming years, we should focus our efforts to identifying key epigenetic lesions that act as 'driver' epimutations early during CLL pathogenesis, probably by investigating DNA methylation (1) in relation to potential CLL precursors, such as the novel $CD5^+/CD27^+$ post-germinal center B-cell subset, recently identified by Seifert et al.,[82] and (2) among clinically relevant and more homogenous subsets of CLL patients. Furthermore, we should also harmonize methodology to measure DNA methylation and investigate larger cohorts of samples in order to fully address the clinical impact of DNA methylation of certain key genes or pathways. Finally, deciphering the functional consequences of these lesions as well as determining the functional relationships between DNA methylation and other epigenetic messengers will be crucial in order to better understand the complex regulation of epigenetics in CLL, which ultimately has the potential to lead to tailored epigenetic-based therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This research was supported by the Nordic Cancer Union, the Swedish Cancer Society, the Swedish Research Council, and the Lion's Cancer Research Foundation, Uppsala.

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