The chronic lymphocytic leukemia epigenome: biological and clinical insights

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A B S T R A C T

Transcriptional deregulation through genetic alterations is a well-established phenomenon in chronic lymphocytic leukemia. In contrast, the role and implications of epigenetics, which studies the molecular mechanisms underlying transcriptional control without simultaneous genetic changes, are just now beginning to be understood in CLL. The recent development of unbiased high-throughput approaches is providing an exceptional opportunity to study the DNA methylome of CLL and provide new insights into its clinico-pathological features. These new studies have revealed that different CLL subtypes maintain epigenetic imprints of B cells at distinct maturation stages and that CLLs are characterized by widespread genome-wide hypomethylation and local hypermethylation. Furthermore, it is becoming increasingly evident that the widely accepted relationship between gene expression and DNA methylation is more nuanced than previously appreciated, and that the roles of DNA methylation are multiple and dependent on the genomic and chromatin context. At the clinical level, multiple genes have been associated with prognosis, and recent reports indicate that epigenetic patterns can define three CLL subgroups with different biological features and clinical behavior. The goal of this review is to present an overview of the role and clinical impact of DNA methylation in CLL using the most recent literature in the field.

Learning goals

At the conclusion of this activity, participants should:
- be aware that the roles of DNA methylation are multiple and (epi)genomic-context dependent;
- know that the DNA methylome is widely modulated during B-cell differentiation and that chronic lymphocytic leukemia (CLL) cells seem to maintain an epigenetic imprint of the differentiation stage from which they may originate;
- know that the clinical behavior of CLL patients is influenced by the putative cell of origin identified by DNA methylation patterns, and that 3 major clinico-biological subgroups of the disease can be identified using five epigenetic biomarkers.

Introduction

Chronic lymphocytic leukemia (CLL) is the most frequent leukemia of adults in Western countries. CLL is characterized by the gradual accumulation of monoclonal CD5-positive B cells and a variable clinical evolution.[1,2] The different clinical behavior has been mainly associated with two molecular subtypes defined by the mutational status of the variable region of the immunoglobulin heavy chain locus (*IGHV*).[2] Cases lacking or with a low proportion (<2%) of *IGHV* somatic hypermutation (uCLL) have a worse clinical behavior than those showing more than 2% somatic hypermutation (mCLL). The acquisition of particular chromosomal changes has been also related to the clinical course of the disease, with deletions of 11q22-q23 and 17p13 associated with adverse outcome, whereas deletion of 13q14 is frequently found in cases with a favorable prognosis.[3] The recent application of next generation sequencing technologies has allowed us to obtain a high-resolution map of the mutational landscape of CLL.[4,5] These analyses have led to the identification of a very heterogeneous pattern, with few driver genes mutated in 10%-15% of the patients and a large number of genes affecting 1%-5% of the cases.[6-9] The most frequently mutated genes in CLL can be included within few different pathways, such as *NOTCH1* (NOTCH1 signaling), *SF3B1* and *XPO1* (mRNA splicing, processing and transport), *ATM*, *TP53* and *POT1* (DNA damage response pathway), as well as *MYD88* and *TLR2* (innate inflammatory pathway). In addition to genomic alterations, cancer cells also display alterations in the epigenetic code.[10] Similarly to solid tumors and other hematologic neoplasms, CLL cells also show epigenetic changes. The next section will provide an introduction to the epigenetic mechanisms, and in particular to DNA methylation, which will be the main focus of this review.

Introduction to epigenetic mechanisms and DNA methylation

Epigenetics is classically defined as the study of changes in gene expression that occur independently of changes in the DNA sequence. The epigenetic language comprises

various regulatory layers, such as DNA methylation, chromatin marks (histone modifications or variants), nucleosome positioning and nucleosome accessibility.[11] By far the most widely studied epigenetic mark in the context of human diseases is DNA methylation.[12] However, recent studies have started to show that multiple epigenetic marks are essential to obtain a more precise characterization of epigenome in the context of normal differentiation and disease.[13,14] Chromatin does not only plan the structural role of packing DNA into the cell nucleus, but it also functions as a highly dynamic scaffold essential in regulating gene expression. Multiple post-translational modifications of the N-terminal tails of histones are involved in this process, such as methylation, acetylation and phosphorylation, among others, and the number of modifications keeps on increasing.[15] These modifications are not only associated to gene activation or repression, but also allow us to classify different segments of the genome according to their function. Different combinations of these histone marks are called "chromatin states", and are able to define active, weak or poised promoters, active or weak enhancers, insulators (mainly sites of CTCF binding), transcribed regions, repressed regions (Polycomb-repressed) or heterochromatic regions.[16] These chromatin states vary among different cell types, and therefore chromatin modulation and activity of distinct regions of the genome are cell-type specific.

DNA methylation is the most intensely studied epigenetic modification in mammals. According to the first studies in the field, and multiple reports published afterwards, gene silencing seems to be the main function of DNA methylation.[17] Mostly due to this regulatory role, DNA methylation has been reported to be involved in multiple physiological processes such as organismal development and cell differentiation, genomic imprinting, chromosome X-inactivation, suppression of repetitive elements and genomic stability.[18] At the biochemical level, DNA methylation consists of the covalent addition of a methyl group (-CH_3) to cytosine, generally within the context of CpG dinucleotides. Typically, these dinucleotides are concentrated in clusters, called CpG islands (CGIs) that are enriched in promoter and first exon regions. In the human genome, nearly 60% of all human promoters contain CGIs.[19] Cytosine methylation is mediated by a class of enzymes called DNA methyltransferases; DNMT1, DNMT3A and DNMT3B are the best characterized.[17] DNA demethylation may occur passively through lack of maintenance during cell division or actively though the function of Ten-Eleven Translocation (TET) family of proteins or Activation-Induced Cytidine Deaminase (AID) followed by base-excision repair that introduces an unmethylated cytosine.[20]

As DNA methylation is associated with processes essential for cell physiology, it is not surprising that alterations in DNA methylation levels or patterns are linked to various diseases, most notably in cancer.[10,12,18,21] Historically, the two main epigenetic features of virtually all tumors studied reveal that they show a disrupted DNA methylome, characterized by global hypomethylation and local hypermethylation of CGIs in the promoter region of tumor suppressor genes. Thus, gene hypermethylation can serve as an alternative to genetic mechanisms (mutation or deletion) in the process of tumor suppressor gene inactivation. So far, DNA methylation has been shown to inactivate multiple individual genes and non-coding transcripts in cancer.[12]

Recent technological advances, such as the development of next generation sequencing and high-density microarrays applied to epigenetics, are now paving the way for a deeper understanding of the role of DNA methylation in normal differentiation and neoplastic transformation. In fact, the latest reports using unbiased DNA methylation techniques invite us to revise the classical roles of DNA methylation in cell (patho)physiology.[22-28] It is becoming increasingly clear from these studies that the changes in the role of DNA methylation in cancer are more complex than initially thought,[29] and the integration of different layers of epigenetic information are important to interpret those data in a biologically and clinically meaningful way. These new concepts derived from genome-wide approaches in the context of CLL biology and clinical behavior will be further addressed in more detail in this review.

The DNA methylome of normal B cells

DNA methylation is not only tissue- and cell type-specific but is also widely modulated during the maturation program of a single cell lineage. The relevance of describing the epigenetic changes taking place during B-cell development in the context of this review is because the DNA methylation pattern of different CLL subtypes can be linked to B cells at different maturation stages.[24] The differentiation process starts in the bone marrow (BM), where hematopoietic stem cells differentiate into common lymphoid progenitors. Then, such progenitor cells commit to the B-cell lineage and give rise to precursor B cells, which differentiate into mature naive B cells that leave the BM to enter the blood stream. Eventually, naive B cells are activated by specific antigens via activation of the B-cell receptor, which induces the germinal center reaction in the lymph node. Germinal center B cells further rearrange and mutate their immunoglobulin genes, and rapidly differentiate and proliferate. Finally, the germinal center reaction gives rise to plasma cells and memory B cells that provide the basis for adaptive immunity. Several recent studies have analyzed the DNA methylome of various sorted B-cell subpopulations, including precursor B cells, naive B cells, germinal center B cells, memory B cells, and plasma cells.[30-32] In the context of mature B cells, the study by Kulis *et al*. performed a deep analysis of the DNA methylome of the two most prevalent B-cell subtypes in peripheral blood, i.e. naive B cells and memory B cells.[24] Using whole-genome bisulfite sequencing (WGBS) and 450k methylation arrays, this study identified 1.7 million differentially methylated CpGs between these two cell types and 97% of them were hypomethylated in memory B cells. Part of the massive hypomethylation observed in memory B cells as compared to naive B cells is most likely acquired in the germinal center and remains in memory B cells as an epigenetic imprint of the germinal center reaction.[30,32]

Insights into the cellular origins of CLL through DNA methylation profiling

The putative cellular origin of CLL has been controver-

sial. The differences in the *IGHV* mutational status, *IGHV* sequence repertoire and BCR reactivity suggest that uCLL and mCLL derive from B cells at different maturation stages.[33] In contrast, gene expression profiling studies have identified only minor differences between uCLL and mCLL, suggesting that both subtypes may derive from a single cell of origin, such as an antigen-experienced, memory-like B cell.[34] Two recent studies have provided new insights into this controversy. In a genome-wide DNA methylation study, Kulis *et al*. identified that both uCLL and mCLL globally have a DNA methylome more similar to memory B cells, which is in line with their functional features of antigen-experienced cells. However, the differences in the DNA methylation profile of uCLL and mCLL can mostly be attributed to similarities with different B-cell subpopulations.[24] In particular, this analysis revealed that uCLL resembles both naïve B cells (IgD+, CD27-) and CD5+ pre-germinal center mature B cells (CD5+, IgD+, CD27-), whereas mCLL is more similar to non-class-switched and class-switched memory B cells (IgM/D+ or IgA/G+, CD27+). Thus, although overall both uCLL and mCLL are more similar to antigen-experienced B cells, uCLLs seem to maintain an epigenetic imprint of naive, pre-germinal center B cells whereas mCLLs are epigenetically more similar to memory B cells. Interestingly, this study identified a third group of CLL with an intermediate DNA methylation pattern and enriched for mCLLs with a moderate level of somatic *IGHV* mutations. Thus, this group might be derived from a third B-cell type, e.g. an antigen-experienced, germinal center-independent B cell that has acquired low levels of somatic hypermutation.

An additional study by Seifert *et al*. represents a further step forward towards the identification of the cellular origin of uCLL and mCLL by using a detailed transcriptional profiling of CLL cells and multiple B-cell subpopulations from peripheral blood and spleen.[35] In line with the epigenomics study, this report also detected CD5+ pre-germinal center mature B cells (CD5+, CD27-) as the origin of uCLL, whereas a previously unrecognized CD5+ post-germinal center B-cell subset (CD5+, CD27+) was postulated as the most likely origin for mCLL.

DNA methylome of CLL: insights from genome-wide studies

During the last decades, multiple reports have identified individual genes, microRNAs and pathways epigenetically deregulated in CLL as well as different prognostic subtypes of CLL.[36-64] Some of the genes known to be differentially expressed in uCLL and mCLL, such as *LPL*, *ZAP70*, *CRY1*, *SPG20*, *CLLU1* or *LAG1*, show differential DNA methylation patterns in the two subtypes of the disease that seem to be related to their putative cell of origin.[24,36,38] In the last few years, however, several studies have characterized the CLL epigenome, particularly the DNA methylome, using unbiased approaches. These studies include sequencing-based methods such as whole-genome bisulfite (WGBS) and reduced representation bisulfite sequencing (RRBS), as well as microarray-based techniques (Table 1).[24,65-73] Besides individual genes, this section will focus on the global characterization of the DNA methylome of CLL based on these new epigenomic reports. These studies have revealed a large number of DNA methylation changes at CpG sites. In particular, the two samples studied by WGBS by Kulis *et al*. reveled 3.79 million individual CpGs differentially methylated between uCLL and naive B cells, and 1.84 million between mCLL and memory B cells.[24] Most of these changes involved hypomethylation of low CpG content areas but also, although to a lower extent, local hypermethylation of CGIs was observed. The cancer epigenetics literature shows abundant examples of genes whose aberrant hypermethylation in tumor cells leads to gene silencing.[12] The great majority of these published studies were biased to the analysis of few candidate tumor suppressor genes. Based on these findings, the inverse association between aberrant methylation and gene expression in cancer became a widely accepted phenomenon. However, virtually all new studies analyzing the DNA methylome and transcriptome of cancer cells in an unbiased manner demonstrate that this association needs to be revisited. A comprehensive analysis of DNA methylation and gene expression in a large series of CLLs has detected that only 5%-10% of the genes show a significant correlation.[24] Interestingly, as well as confirming the negative association between

Table 1. Overview of the most recent studies analyzing the DNA methylation profile of chronic lymphoid leukemia using high-throughput techniques (ordered chronologically).

Title of the study	Technique applied	N. cases analyzed	Reference
Locally disordered methylation forms the basis of intratumor methylome variation in chronic lymphocytic leukemia.	RRBS	104	Landau *et al.*[68]
Evolution of DNA methylation is linked to genetic aberrations in chronic lymphocytic leukemia.	450k arrays	68	Oakes *et al.*[69]
Promoter methylation patterns in Richter syndrome affect stem-cell maintenance and cell cycle regulation and differ from de novo diffuse large B-cell lymphoma.	27k arrays	19	Rinaldi *et al.*[72]
450K-array analysis of chronic lymphocytic leukemia cells reveals global DNA methylation to be relatively stable over time and similar in resting and proliferative compartments.	450k arrays	56	Cahill *et al.*[36]
Distinct transcriptional control in major immunogenetic subsets of chronic lymphocytic leukemia exhibiting subset-biased lobal DNA methylation profiles.	27k arrays	39	Kanduri *et al.*[67]
Epigenomic analysis detects widespread gene-body DNA hypomethylation in chronic lymphocytic leukemia.	WGBS 450k arrays	139	Kulis *et al.*[24]
Genome-wide DNA methylation analysis reveals novel epigenetic changes in chronic lymphocytic leukemia.	RRBS	11	Pei *et al.*[70]
Differential genome-wide array-based methylation profiles in prognostic subsets of chronic lymphocytic leukemia.	27k arrays	23	Kanduri *et al.*[66]

RRBS: reduced representation bisulfite sequencing; 450k arrays: Infinium Human Methylation450 BeadChip (Illumina); 27k arrays: Infinium Human Methylation27 BeadChip (Illumina); WGBS: whole-genome bisulfite sequencing.

DNA methylation and gene expression levels in a limited fraction of promoter regions, it was observed that the association between gene-body methylation and gene expression can be both positive and negative. Furthermore, methylation in the gene body is also associated with the use of different promoters, as exemplified by the *RB1* gene, and alternative splicing of a subset of genes, including *CD45* and *BCL2L1*[24] (Figure 1). Therefore, the functional impact of DNA methylation on gene expression is clearly dependent on the genomic context.[29]

A still unsolved question is why CLL cells, and cancer cells in general, show a large amount of hypermethylated CGIs in promoter regions in the absence of *de novo* silencing in tumor cells as compared to normal cells.[74] In general, hypermethylation frequently targets genes already silenced in non-tumoral cells by repressive histone modifications such as H3K27me3[75-78] (Figure 1). Thus, although it is true that some tumor suppressor genes become *de novo* methylated and *de novo* silenced in cancer, hypermethylation affects mostly genes already silenced in normal cells.[75,79] This can suggest that DNA methylation at promoter regions could be a secondary event playing a role in achieving stable gene inactivation. Hence, in many cases, CGI methylation may be more a consequence rather than a cause of gene repression.

CLL hypomethylation has been reported to frequently affect DNA repeats.[80] However, linking the results obtained by WGBS to chromatin states in normal B cells indicates that DNA hypomethylation in CLL, in addition to target heterochromatic regions, is highly enriched in enhancer elements, in particular in the gene body (Figure 1).[24] Enhancers are regulatory elements distant to the transcriptional start site, and epigenomic studies are now revealing that the DNA methylation pattern of enhancers is more dynamic than that of promoter regions.[81,82] Enhancer methylation has been shown to inversely correlate with enhancer activity and, therefore, can affect the expression of their target genes, even without DNA methylation changes in their promoter regions.[25]

An elegant study published in 2014 has analyzed the CLL methylomes from a different perspective. Landau and co-workers describe that CLLs display higher intra-sample variability of DNA methylation patterns across the genome, and that such heterogeneity seems to arise from a stochastic process leading to disordered methylation patterns in malignant cells. This disordered methylation was further associated with adverse clinical outcome.[68]

Collectively, these studies reveal that the DNA methylation landscape of CLL is more complex than anticipated, and not only include alterations in promoter regions but also alternative intragenic promoters, alternative splicing and frequent changes in the DNA methylation pattern of enhancer regions. It is also worth underlining the fact that only a small fraction of the genes show significant correlation between DNA methylation and gene expression levels. Therefore, this finding apparently suggests that DNA methylation has more roles than gene regulation. As recently proposed by Landau *et al*.,[68] global patterns of disordered methylation may play a similar role to that of genetic instability, enhancing the ability of cancer cells to search for superior evolutionary trajectories.

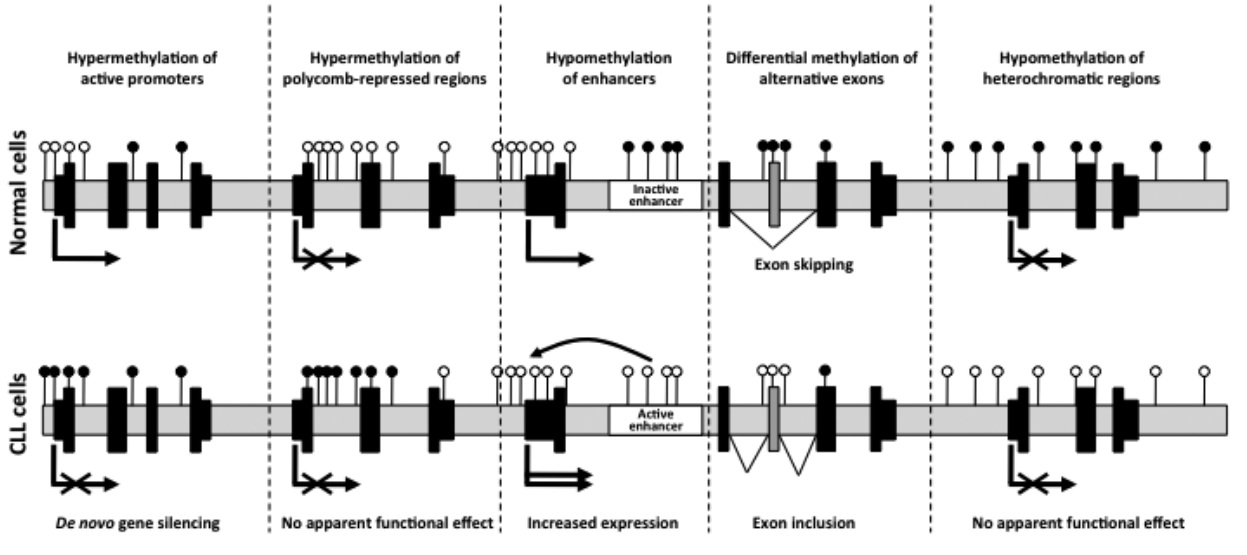

Figure 1. Schematic representation of the most relevant DNA methylation changes observed in chronic lymphoid leukemia cells as compared to normal B cells. Promoter hypermethylation (left) can rarely lead to *de novo* gene repression and in most instances affects genes already repressed in normal cells by histone modifications (e.g. H3K27me3 mediated by the polycomb complex). Hypomethylation (right) is extensive both in gene bodies and large blocks of heterochromatic regions. In the gene body, hypomethylation frequently affects enhancer elements but can also affect weak exons leading to alternative splicing. Black lollipops represent methylated sites whereas white lollipops point to unmethylated sites.

Clinical impact of DNA methylation patterns

Recent epigenomic studies in CLL have shown a massive modulation of the DNA methylome in the disease including more than 1 million differentially methylated sites per case and affecting at least one CpG of a large proportion of all human genes. The altered DNA methylome of CLL samples is quite stable over the course of the disease,[65] although patients transforming to Richter syndrome seem to acquire additional DNA methylation changes.[72] Within the extensive reconfiguration of the DNA methylome in CLL, multiple studies have identified individual genes whose altered DNA methylation pattern is related to the clinical behavior of the patients.[38,40,43,47,50,51,57,59] Many of these genes are related to the mutational status of IGHV, such as *ZAP70*. Two studies have identified and confirmed that the methylation status of a single CpG in *ZAP70* is able to distinguish between patients with a different prognosis.[83,84] Instead of focusing on individual genes, Kulis and co-workers followed a different approach. They described a signature of 1649 CpGs differentially methylated between uCLL and mCLL that could be assigned to different methylation patterns between naive and memory B cells. A consensus clustering analysis of this signature revealed three robust groups instead of the expected two, and these three groups had different clinical behavior, i.e. those with epigenetic imprints of naive B cells show a poor prognosis, those epigenetically more similar to memory B cells have a favorable prognosis and those with an intermediate methylation profile present a moderate clinical behavior.[24] In a follow-up study, Queirós *et al*. performed a complexity-reduction step and extracted a signature of 5 CpGs whose methylation levels could accurately identify each of the three subgroups. Using a bisulfite pyrosequencing approach, they studied two independent series with a total sample size of 308 patients and the prognostic impact of the 3-group classification was confirmed. A multivariate model further indicated that the epigenetic variable was the most significant independent variable associated with prognosis in CLL, beyond clinical staging, expression of ZAP70 or CD38, or the presence of specific genetic alterations. Interestingly, epigenetic subgrouping seemed to replace the *IGHV* mutational status in the multivariate model. This finding indicates that, although both classifications reflect the cellular origin of CLL, the categorization in three epigenetic categories is more significantly associated with prognosis than the separation into two groups based on *IGHV* status.[85]

Conclusions and future directions

The initial reports describing the DNA methylome of CLL underline the fact that the roles and implications of this epigenetic mark in the disease are multiple and context-dependent. These studies reveal that only a small fraction of the DNA methylation changes seem to correlate with gene expression, and that they not only affect classical promoter regions, but also intragenic alternative promoters, alternative splicing and enhancer elements. The majority of DNA methylation patterns in CLL, however, do not seem to be directly associated with gene expression. Part of them, especially those differentially methylated between uCLL and mCLL, seem to be related to an epigenetic imprint of B cells at distinct maturation stages. CLL cells undergo other changes in a stochastic way and these changes affect repressed or lowly expressed regions, suggesting that they may not be actively induced but rather may represent an epigenetic drift associated with leukemogenesis and disease progression.

In the near future, the DNA methylomes of CLL will be complemented by genome-wide patterns of additional epigenetic marks, such as histone modifications and chromatin accessibility maps, in the context of large international consortia, such as the Blueprint Project and the International Cancer Genome Consortium.[86,87] These reference epigenomes will then be analyzed in the context of genetic changes, and this combined genetic and epigenetic approach should provide new insights into the molecular mechanisms that govern CLL pathogenesis and progression. This increased knowledge can then be translated into clinical practice, allowing an individualized molecular diagnosis, a better stratification of the patients according to risk groups, as well as the development and application of more effective therapies.

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