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EPIGENETICS AND EPIGENOMICS (C BELL, SECTION EDITOR)

Advances in Epigenetics and Epigenomics in Chronic Lymphocytic Leukemia

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Abstract

Purpose of Review The development and progression of chronic lymphocytic leukemia (CLL), a highly heterogenous B cell malignancy, are influenced by both genetic and environmental factors. Environmental factors, including pharmacological interventions, can affect the epigenetic landscape of CLL and thereby determine the CLL phenotype, clonal evolution, and clinical outcome. In this review, we critically present the latest advances in the field of CLL epigenomics/epigenetics in order to provide a systematic overview of to-date achievements and highlight the potential of epigenomics approaches in light of novel treatment therapies.

Recent Findings Recent technological advances have enabled broad and precise mapping of the CLL epigenome. The identification of CLL-specific DNA methylation patterns has allowed for accurate CLL subtype definition, a better understanding of clonal origin and evolution, and the discovery of reliable biomarkers. More recently, studies have started to unravel the prognostic, predictive, and therapeutic potential of mapping chromatin dynamics and histone modifications in CLL. Finally, analysis of non-coding RNA expression has indicated their contribution to disease pathogenesis and helped to define prognostic subsets in CLL.

Summary Overall, the potential of CLL epigenomics for predicting treatment response and resistance is mounting, especially with the advent of novel targeted CLL therapies.

Keywords Chronic lymphocytic leukemia (CLL) · Epigenetics · Epigenomics · DNA methylation · Histone modifications · Chromatin · Non-coding RNAs

Introduction

Chronic lymphocytic leukemia (CLL), the most common adult leukemia in the Western world, is viewed as a disease with immense heterogeneity at the clinical, cellular, and molecular levels. A multitude of studies have provided insight on how CLL clinical heterogeneity can be reflected on epigenetic signatures at the DNA methylation, histone modifications, and non-coding RNAs levels. Epigenetic patterns can alter as a response to changes in the (micro)environment, including antigenic stimulus, cross-talk with other cells, exposure to soluble factors at CLL niche, or as a result of pharmacological interventions. Investigating epigenetics and epigenomics can offer not only valuable insights into cell ontogeny and disease pathogenesis but into underlying mechanisms for clinical and molecular heterogeneity. Furthermore, identification of epigenetic signatures via epigenome-wide approaches can assist the shift towards a precision-medicine model for disease management [1, 2]. Previous research summaries have documented the key scientific approaches around the CLL epigenome [3, 4]. In this review, we highlight recent advances in CLL epigenetics and epigenomics with a focus on translational potential of key genes/regions, as biomarkers or drug targets (Fig. 1).

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DNA Methylation

Over the last decade, a large body of experimental data has documented that DNA methylation plays variable functional roles linked to CLL pathogenesis and disease outcome. Our

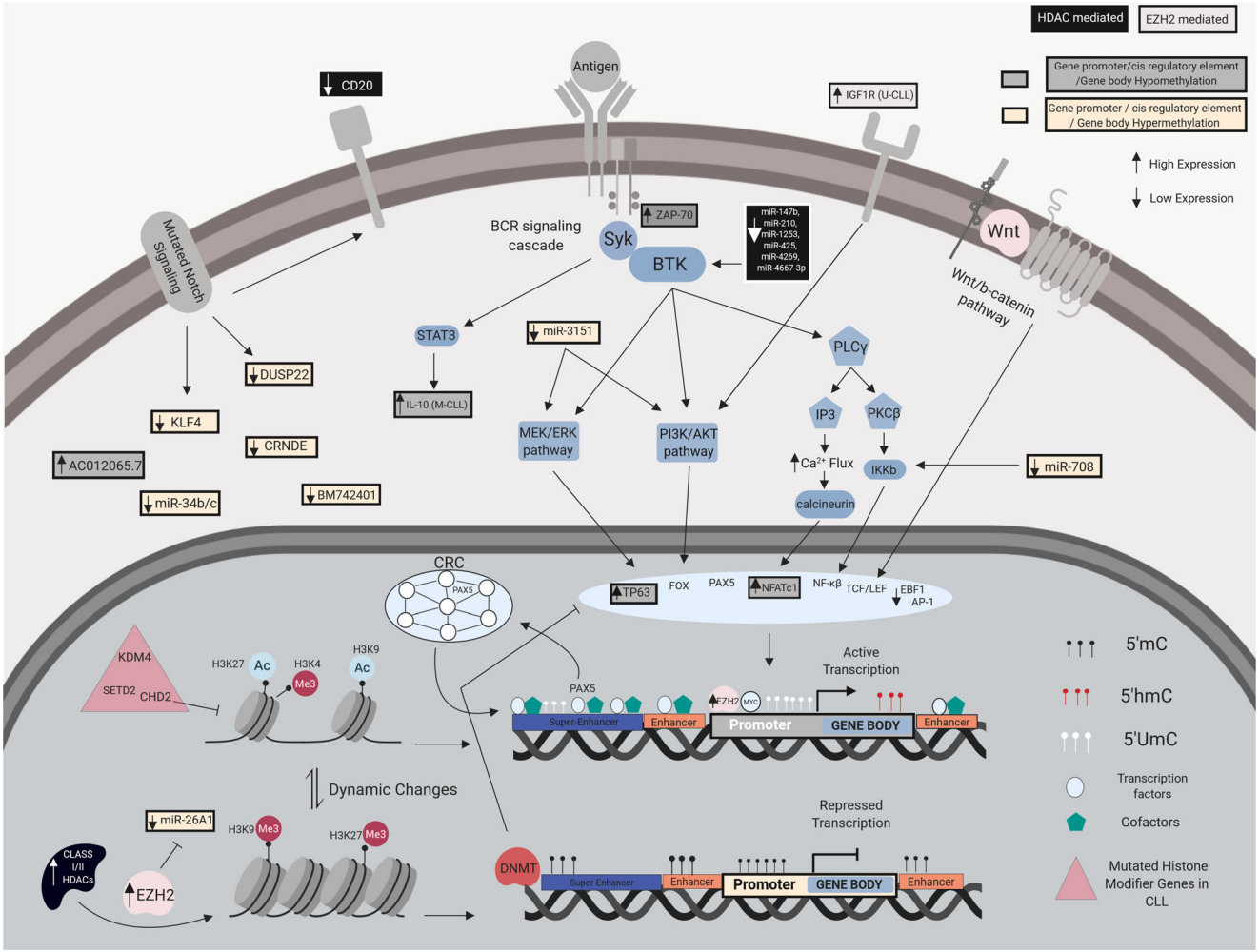

Fig. 1 Overview of CLL epigenetics. Microenvironmental signals shape to a critical point CLL B cell fate and B cell receptor (BCR) possess a central role into this translational process. A significant number of proteins have been reported to be regulated by DNA methylation in CLL. For instance, ZAP70, TP63, NFATc1, and others (dark grey boxes) have been found to be upregulated by DNA hypomethylation (promoter/gene body/cis-regulation) either in CLL as a whole or only in specific CLL subsets with diverse prognosis. Other molecules such as KLF4, DUSP22, and various miRs were downregulated and epigenetically silenced via DNA promoter hypermethylation or gene body/cis-regulatory element DNA methylation changes (light yellow boxes). Furthermore, molecules expressed either in the cytoplasm or in the nucleus such as tumor suppressive microRNAs (i.e., miR-708), long non-coding RNAs (i.e., CRNDE) and histone-modifying enzymes (HME) (i.e., EZH2) have been found to be epigenetically dysregulated in CLL. These molecules act as epigenetic regulators and are either downregulated or aberrantly overexpressed affecting the downstream signaling cascade, the epigenome and transcriptome. Interestingly, aberrant expression and function of various factors have been found to be actively mediated by HDACs (black boxes) or EZH2 enzymes (light grey boxes). Through a dynamic process, HMEs (HDAC, EZH2, SETD2, CHD2 and KDM4) can alter chromatin configuration and TF dependencies for regulatory areas (i.e., promoters, enhancers). These epigenetic changes are the catalytic switch for enabling or blocking gene transcription and not surprisingly, HME inhibition has shown to be the most promising strategy of epigenetic therapy in CLL. The figure was created with BioRender.com. AP-1, activator protein 1; BTK, Bruton's tyrosine kinase; BCR, B cell receptor; CHD2, chromodomain helicase DNA-binding protein 2; CLL, chronic lymphocytic leukemia; CRNDE, colorectal neoplasia differentially expressed; CD20, B-lymphocyte antigen CD20; CRC, core regulatory circuit; DNMT, DNA methyltransferase; DUSP22, dual specificity phosphatase 22; EBF1, early B cell factor 1; EZH2, enhancer of zeste homolog 2; FOX, forkhead box; HDAC, histone deacetylase; H3K27me3, histone H3 trimethylation at lysine 27; H3K4me3, histone H3 trimethylation at lysine 4; H3K9ac, histone H3 acetylation at lysine 9; H3K9me3 histone H3 trimethylation at lysine 9; H3K27ac, histone H3 acetylation at lysine 27; IL10, interleukin 10; IGF1R, insulin-like growth factor 1 receptor; IKKb, IκB-kinase β; IP3, inositol 1,4,5-triphosphate; KDM4, histone lysine demethylase subfamily 4; LEF, lymphoid enhancer-binding factor; M-CLL, IGHV-mutated CLL subset; MCPH1, microcephalin; MEK/ERK, mitogen-activated protein kinase/extracellular receptor kinase pathway; miR, microRNA; MYC, oncogene carried by the Avian virus, myelocytomatosis; NFATC1, nuclear factor of activated T cells, cytoplasmic 1; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NOTCH, neurogenic locus notch homolog protein; PAX5, paired box protein Pax-5; PI3K/AKT pathway, phosphoinositide 3-kinase/AKT or protein kinase B (PKB) pathway; PLCγ, phospholipase C gamma; PKCβ, protein kinase C beta; SETD2, SET domain containing 2; SYK, spleen associated tyrosine kinase; TP63, tumor protein 63; TCF, T cell factor; Wnt, wingless-related integration site; U-CLL, IGHV-unmutated CLL subset; ZAP70, zeta-chain-associated protein kinase 70; 5′-hmC, 5′-hydroxymethylcytosine; 5′-mC, 5′-methylcytosine; 5′-UmC, 5′-unmethylcytosine

knowledge has expanded from gene promoter DNA methylation to global high-resolution DNA methylation profiles, reflecting its role as a pivotal epigenetic alteration and a hallmark of malignant cells [5]. Eventually, these mediated methylation changes shape to a critical point the formation of the transcriptome and proteome of leukemic cells, thereby affecting their function and fate.

DNA Methylation Patterns Predict Cell of Origin, Clonal Evolution, and Clinical Outcome

Examining the normal DNA methylation patterns and understanding how this stable epigenetic mark works during development and ageing processes can provide valuable knowledge in terms of DNA methylation dynamics and their involvement at tumorgenesis. DNA methylation tends to increase with age at some CpG islands, particularly at Polycomb target genes and at promoters of tumor suppressor genes (TSG), while loss of DNA methylation occurs primarily in constitutive-heterochromatin repeat regions. There is a great deal of evidence that the DNA methylation seen in cancer and aging may stem from a small population of cells. It is therefore likely that the general de novo methylation seen in cancer already exists in a subpopulation of "normal" cells prior to their transformation by gene mutation [6, 7]. In line with this, it was shown that a higher degree of DNA methylation changes are more commonly found in *IGHV*-unmutated progressive cases of CLL [8, 9]. Mainly these changes affect PRC2 target genes and possibly involve developmental processes shared in common with the generation of normal memory B cells [9].

Although DNA methylation has been studied in CLL for almost two decades, only recently epigenetic studies have illuminated aspects of CLL ontogeny and clonal evolution [8]. First, when Kulis et al. proposed a classification of CLL cases into 3 groups, based on their DNA methylation profiles. Groups were reported as naive B cell–like, memory B cell–like, and intermediate CLL (n-, m-, and i-CLL, respectively), and DNA methylation patterns were associated with disease outcome and putative normal counterparts [10]. Subsequently, Martín-Subero's team expanded this work with quantitative DNA methylation assays in two independent CLL cohorts [8]. An epigenetic signature of five CpGs (Table 1) located in promoter, gene body, and intergenic regions was identified as a robust biomarker for CLL classification in three distinct subgroups [10, 32].

Intraclonal heterogeneity and clonal evolution was the major focus of Oakes and colleagues, in which DNA methylation was assessed by 450 K arrays and next-generation sequencing [33]. It was shown that only a fraction of CLL cases with unmutated *IGHV* genes (U-CLL) and other poor prognostic markers are characterized by heterogeneity and clonal evolution at DNA methylation level and correlated with high subclonal genetic complexity. Their work suggests that the selection pressure at epigenetic level for high-risk CLL cases may be linked to the wide variation of driver mutations [33].

Further emphasis on CLL intraclonal heterogeneity and contribution of the DNA methylome to clonal evolution has been placed by a whole-genome bisulfite sequencing (WGBS) and reduced-representation bisulfite sequencing (RRBS) analysis on a large cohort of primary samples. Landau et al. showed that intraclonal diversification arises from stochastically disordered DNA methylation and was linked to low-level expression and poor clinical outcome, suggesting a role for DNA methylation in promoting genetic instability and the survival of the adverse clone [34].

The importance of investigating DNA methylation status in specific genomic regions or single CpG sites has been underscored by a significant number of studies showing the immense potential for reliable biomarkers which could be employed for patient stratification. By quantitative methylation analysis (MassARRAY), Claus and colleagues identified a single non-promoter CpG (CpG +223) as critical for *ZAP70* expression which can serve as a reliable prognostic biomarker for OS and TFS [35]. Importantly, *ZAP70*_CpG+233 methylation was a superior biomarker as opposed to IGHV mutation status, *CD38*, or *ZAP70* expression levels (Table 1) [35].

In a subsequent study, Oakes et al. identified an epigenetic signature (methylation maturation score—MMS) of 18 loci, which confirmed the existence of three distinct CLL subtypes. The three subtypes—termed high-, intermediate-, low-programmed CLL (HP-, IP-, LP-CLL)—were suggested to have arisen from the malignant transformation of normal counterparts with diverse maturation levels, and thereby different marks per se, prior to clonal evolution [11•]. For instance, the LP-CLL subtype was associated with poor disease outcome, less maturity, and a germinal-center independent clonal history. Furthermore, they reported CLL-specific DNA methylation patterns linked to (i) hypermethylation as a result of blockage in hypomethylation and highly enriched for *EBF1*, *BATF/AP-1*, *RUNX3* binding sites (B cell–development TFs) and (ii) hypomethylation in regions associated with B cell–activation TFs, such as *NFAT* and *EGR* [11•].

By using MassARRAY for the 18 loci epigenetic signature, D'Avola and colleagues investigated associations between the MMS and sIgM expression/function in 57 cases [12]. They reported an inverse correlation between sIgM levels and MMS, and showed that the reduced sIgM and the high MMS within mutated IGHV CLL cases indicated an indolent disease subtype [12].

A later study employed pyrosequencing for the 5 CpG—epigenetic signature discussed above in 135 CLL cases in order to validate strong prognostic significance by classifying patients into novel CLL epigenetic subgroups. Furthermore, stereotyped subset #2 (IGHV3-21/IGLV3-21 expressing cases) were included in this study and they were classified

Table 1 An overview of molecules epigenetically dysregulated in CLL, their mechanisms of action and biomarker potential

Epigenetically regulations in CLL and biomarker discovery

Gene/region	Epigenetic mechanism	Clinical impact	References
5 single CpG – epigenetic signature	DNA methylation in single CpGs	Biomarker CLL classification in three subgroups (n-, m-, and i-CLL)	Kulis et al. 2012 [10] Queiros et al. 2015 [32] Bhoi et al. 2016 [13]
ZAP70	CpG+223 promoter hypomethylation	↓ TFS/OS	Claus et al. 2014 [35]
18-loci signature	DNA methylation in single CpGs ↓ s-IgM MMS↑	Biomarker CLL classification in three subgroups HP-, IP-, LP-CLL	D'Avola et al. 2016 [12] Oakes et al. 2016 [11•]
IL10	Distinct epigenetic reprogramming between CLL subsets M-CLL: variably methylated regions' (CLL-VMRs1/2) in gene body < U-CLL	U-CLL: poor outcome M-CLL: stable course of disease/anergic cell status	Drennan et al. 2017 [17]
PD-1	↓ DNA methylation ↑ PD-1 expression		Wu et al. 2016 [18]
MCPH1	M-CLL: binding to ANGPT2–DNMTs recruitment - epigenetic silencing of ANGPT2 U-CLL: No binding to ANGPT2—unmethylated promoter—expression of ANGPT2	M-CLL: good prognosis U-CLL: poor prognosis	Kopparapu et al. 2015 [20]
CRY1 and PAX9	Aberrant DNA methylation and mRNA expression	High risk to treatment initiation ↓TFS/OS	Rani et al. 2017 [21]
DUSP22	Promoter hypermethylation through NOTCH mutated signaling	Growth and homing of CLL cells	Arruga et al. 2017 [24]
LPL	2 distinct promoter methylation profiles: promoter hypermethylation promoter hypomethylation	↑ TFS ↓ 75% risk in CLL samples with hypermethylated LPL promoters	Daugaard et al. 2018 [25]
TP63	↓ DNA methylation ↑ Expression	Pro-survival factor Poor prognostic CLL subset #8	Papakonstantinou et al. 2019 [27]
CHD2	Mutated in M-CLL	Defective association with active chromatin	Rodriguez et al. 2015 [38]
NFAT, FOX, and TCF/LEF TF families	Extensive chromatin rewiring at H3K27ac active regulatory regions-enhancers	Essential for CLL progression	Beekman et al. 2018 [41] Mallm et al. 2019 [43•] Pastore et al. 2019 [44]
SETD2	Recurrent deletions and mutations (TP53 associated) Dysregulated enzyme related induction of Histone Modifications	↓ PFS and OS	Parker et al. 2016 [48]
HDAC	Increased global levels of enzymatic activity	↓ TFS/OS	Van Damme et al. 2014 [46]
KDM4 (A,B,C)	Dysregulated enzyme related induction of histone modifications KDM4A ↑ KDMB ↓ KDMC—not a significant change	Poor prognosis Adverse cytogenetics	Filiu-Braga et al. 2018 [55]
miR-708 (TSG)	Epigenetically silenced via enhancer hypermethylation	High-risk characteristics in patients	Baer et al. 2015 [66]
CRNDE (lncRNA) AC012065.7 (lncRNA)	Long non-coding RNA hypermethylation Long non-coding RNA hypomethylation	Inferior outcome	Subhash et al. 2016 [71]

↑: upregulation of gene expression /enhanced/increased levels of […]; ↓: downregulation of gene expression levels/enhanced/increased levels of each condition described, *ANGPT*, angiopoietin 2; *CHD2,* chromodomain helicase DNA-binding protein 2; *CLL*, chronic lymphocytic leukemia; *CpG*, cytosine and guanine separated by only one phosphate group; *CRY1*, cryptochrome circadian regulator 1; *CRNDE*, colorectal neoplasia differentially expressed; *DNMT*, DNA methyltransferase; *DUSP22*, dual specificity phosphatase 22; *FOX*, forkhead box; *HDAC*, histone deacetylase; *H3K27ac*, histone H3 acetylation at lysine 27; *HP-CLL*, high-programmed CLL; *i-CLL*, intermediate CLL; *IL10*, interleukin 10; *IP-CLL*, intermediate-programmed CLL; *KDM4*, histone lysine demethylase subfamily 4; *lncRNA*, long non-coding RNA; *LP-CLL*, low-programmed CLL; *LPL*, lipoprotein lipase; *m-CLL*, memory B cell–like CLL; *M-CLL*, IGHV-mutated CLL subset; *MCPH1*, microcephalin; *MMS*, methylation maturation status; *miR*, microRNA; *n-CLL*, naive B cell–like CLL; *NFAT*, nuclear factor of activated T cells; *NOTCH*, neurogenic locus notch homolog protein; *OS*, overall survival; *PAX9*, paired box gene 9; *PD-1*, programmed cell death protein 1; *PFS*, progression free survival; *SETD2*, SET domain containing 2; *TF*, transcription factor; *TFS*, treatment-free survival; *TP53*, tumor protein p53; *TSG*, tumor suppressor gene; *U-CLL*, IGHV-unmutated CLL subset; *VMR*, variably methylated region; *ZAP70*, zeta-chain-associated protein kinase 70

by methylation score as i-CLL subgroups, whereas their clinical classification resembled the n-CLL group [13].

Georgiadis and colleagues provided evidence that CLL-like changes at DNA methylation and transcriptome profiles existed more than 10 years before CLL diagnosis [14]. These findings suggest that the path from normal counterpart to malignant transformation and further clonal evolution is a long, multi-step process, in which DNA methylation can be viewed not only as a contributor to disease pathogenesis but also as a tool for understanding pre-malignant evolutionary trajectory.

Recent work by Tomasz K. Wojdacz and colleagues has further strengthened our knowledge on the clinical significance of DNA methylation in CLL [15]. High-throughput analysis on UK clinical trials' CLL samples identified DNA methylation signatures which can be used to divide *IGHV*-mutated CLL into clinically relevant subgroups. Last but not least, investigators reported that the memory-like DNA methylation subgroup (m-CLL) can work as an independent marker of prolonged survival for patients treated with chemoimmunotherapy [15].

Overall, independent groups have discovered DNA methylation signatures which have been confirmed in follow-up studies and have significantly enhanced our understanding of CLL heterogeneity, clonal history, and evolution.

DNA Methylation and Transcriptional Regulation in CLL

Recent studies have advanced our understanding of the interplay between hypomethylation and hypermethylation in CLL, and highlighted their role in altering gene regulation to support malignant transformation and/or disease progression. In some cases, DNA methylation-mediated altered gene expression seems to be linked directly to key CLL pathways, whereas in other cases, DNA methylation patterns were identified as potential biomarkers without clear pathogenetic implications (Table 1).

In a metanalysis of publically deposited DNA methylation data, Kushwaha et al. reported regions that were consistently differentially methylated (hypo- or hyper-C-DMRs) in CLL [16]. Hypo-C-DMRs were found enriched in regions harboring TF binding sites related to key signaling pathways (BCR, p53), whereas hyper- C-DMRs were linked to homeobox- and TATA box-containing regions [16].

In another study, it was shown that although the core *IL10* promoter was largely unmethylated in CLL cases, two gene body regions (VMRs) exhibited variable methylation in association with IGHV mutation status. *IL10* transcript levels were inversely correlated with DNA methylation of VMRs, but not the core promoter. VMR2 was located within transcriptional activation-associated H3K27ac peak regions, and harbored a functional *STAT3* motif. This work underscores the importance of DNA methylation for gene regulation in regions outside the promoters [17]. Hypomethylation in a putative enhancer for *PD-1* harboring *NFAT* and *STAT* motifs was reported to increase *PD-1* expression in CLL-derived CD8+ T cells. These T cells also exhibited disease-specific DNA methylation patterns, and top differentially methylated regions were included in *CCR6* and *KLRG* [18].

In the list of genes (Table 1) regulated by DNA methylation, one should add *ET-1*, *MCPH1*, *CRY1*, and *PAX9*. DNA methylation levels of an *ET-1* intron inversely correlate with expression [19]. The *MCPH1* was shown to have a tumor-suppressive function in M-CLL cases via binding to *ANGPT2* promoter, recruitment of DNMTs, and subsequent silencing of *ANGPT2* [20]. In an array-based study, *CRY1* and *PAX9* expression was significantly higher in U-CLL, whereas a single CpG island of *PAX9* was linked to *IGHV* mutation status. Although validation in external cohorts with quantitative approaches is required, these data suggest that DNA methylation is important for *CRY1* and *PAX9* gene regulation, and may have a prognostic value in CLL [21].

In a different setting, Hanney and colleagues worked with a *Dnmt3a*Δ/Δ model. They comparatively assessed global DNA methylation and gene expression in induced CLL and peripheral T cell lymphoma (PTCL) tumors, including B-1a and CD8+ T cells as controls. Interestingly, their analysis revealed a significant overlap between human and mouse transcriptomes and DNA methylomes [22].

Filarsky et al. reported aberrant DNA methylation for *KLF4* and *LILRA4* and provided evidence for a tumor-suppressive function for *KLF4*, which can be exerted upon NOTCH signaling inhibition [23]. Another link between Notch signaling and aberrant DNA methylation is suggested by Arruga and colleagues, who reported promoter hypermethylation of *DUSP22* in *NOTCH1*-mutated CLL [24]. Exploiting the CRISPR/Cas9 system in a CLL cell line, they showed that methylation of DUSP22 is regulated by NICD through a circuit including *DNMT3a*, *RBPJk*, and *HDAC1* [24]. Interesting work on a cohort of 112 patients revealed that the two promoters of *LPL* show heterogeneous DNA methylation levels across CLL cases correlated to TFS [25]. Wolf and colleagues showed that aberrant *NFATC1* promoter hypomethylation was inversely correlated with expression levels, and that inhibition of *NFAT* activity increased apoptotic cell rate [26].

Finally, in a recent study by Papakonstantinou and colleagues, *TP63* was reported to be hypomethylated and overexpressed in stereotyped subset #8 CLL cases [27]. By array-based genome-wide DNA methylation profiling in poor prognostic CLL cases, the researchers showed that stereotyped subset #8 exhibits distinct DNA methylation patterns as opposed to non-subset *IGHV*-unmutated CLL cases and subset #6 cases [27].

Overall, the list of genes that are differentially methylated at their promoter or non-promoter regulatory regions is

constantly expanding. However, observations require further investigation with state-of-the art technologies, validation in independent cohorts, or proof-of-concept experimental evidence to establish a clear mechanistic view of each gene's role in CLL molecular pathogenesis (Table 2).

DNA Hydroxymethylation in CLL

DNA hydroxymethylation was recently discovered as another important mark with active role in epigenetic reprogramming. It is the first product from TET-related oxidation in the process of active demethylation as described in different biological processes including primordial germ cells and zygotic development [28, 29]. Interestingly, several studies have associated 5-hmC with transcriptional activation mediated by enhancers, both in embryonic and differentiated cells [28, 30]. Like DNA methylation, hydroxymethylation has also been observed to be deregulated in various human pathologies such as degenerative diseases like Parkinson's, Alhzeimer's, and different cancer types [31, 36]. In the first thorough study exploring 5 hydroxymethylcytosine (5-hmC) in CLL pathogenesis, Wernig-Zorc et al. reported global loss of 5-hmC and identified aberrant 5-hmC and 5-mC patterns in gene body regions [37]. Interestingly, they reported TET enzyme-mediated 5-hmC enrichment in regulating the differential expression of three oncogenes: *NSMCE1*, *TUBGCP3*, and *TUBGCP6* [37].

Chromatin Dynamics and Histone Modifications

Advances in technologies for epigenome-wide screening provide insights and competent knowledge to the research community related to the complex mechanisms of chromatin configuration and dynamic modification of histones. A small number of studies in that direction—which will now be discussed—provide the first chromatin accessibility maps, report links to transcription factor networks and affected gene expression profiles, and above all, underscore the prognostic, predictive, and therapeutic potential value of chromatin dynamics and histone marks in CLL.

Profiling the Regulatory Chromatin Landscape in CLL

The seed for the emerging interest on chromatin in CLL has been shown by a study reporting a role for *CHD2*, an important chromatin remodeler in CLL pathogenesis. CHD2 was recurrently mutated in M-CLL and integrative analysis on a genomic, transcriptomic, and epigenomic level suggested that CDH2 mutation affects its DNA-binding properties and localization, leading to altered chromatin states [38]. In another early study, the identification of an SNP (rs539846) located within a super-enhancer (H3K27ac marks) of *BCL2*-modifying factor *(BMF*) linked it with disruption of a RELA binding site and decreased BMF expression [39].

The first effort towards large-scale analysis and mapping of chromatin accessibility in CLL was reported by Rendeiro et al., who profiled 88 CLL cases by ATAC-seq [40•]. Interestingly, ATAC-seq profiles were distinct between U-CLL and M-CLL, whereas one or two intermediate subtypes were revealed, based on chromatin accessibility patterns. Furthermore, they performed an RNA-seq/ChIPmentation integrative analysis, including H3K4me1, H3K27ac, and H3K27me3 histone marks and developed a bioinformatics pipeline for linking integrated profiles to clinical and molecular sample annotations. Their analysis revealed a pan-CLL gene regulatory network which included *SP1/2/3*, *CTCF*, *EGR*, *E2F*, and *PAX5* factors, as well as subtype-specific networks [40•].

A recent pioneer analysis of the reference CLL epigenome, together with extensive analysis of regulatory chromatin landscape, sheds light on important knowledge gaps in CLL epigenomics. Interestingly, it showed that chromatin dynamics mirrored normal B cell maturation and regulatory elements were de novo reprogrammed in CLL or associated with CLL subtypes. In general, U-CLL harbored more active and open chromatin than M-CLL. Among de novo-active regions in CLL, an enrichment for *NFAT*, *FOX*, and *TCF/LEF* binding sites was reported, highlighting their functional and therapeutic potential. Finally, a link between genetic alterations and chromatin configuration was observed for *MYD88* mutations and trisomy 12 [41].

Another recent important study delved further into the CLL epigenome and provided novel insights for CLL enhancer landscape and transcription factor dependencies. Ott and colleagues reported CLL-specific enhancer-related core regulatory circuits (CRCs) [42•]. Super enhancers were found to mediate CRCs, including "usual suspects"such as *CXCR4*, *CD74*, *PAX5*, *CD5*, *KRAS*, and *BCL2*. Further functional work showed that *PAX5* is a core regulator of CLL super enhancers, which regulates cell survival, whereas BET inhibitors block the super enhancer CRCs and promote apoptosis in vitro and in vivo [42•].

A significant advance in our knowledge of CLL epigenomics has arisen from a comprehensive study which dissected the aberrant epigenetic circuitry in CLL [43•]. Mallm and colleagues reported that CLL-specific chromatin features included (i) localized changes at regulatory enhancer and promoter elements and (ii) large-scale chromatin reorganization (> 1 Mb). In analogy to other cancers, partially methylated domains (PMDs) linked to repressive chromatin and gene silencing was a CLL trait demarcated by *CTCF*. Authors further reported a CLL-specific TF network based on aberrant chromatin features, which included key epigenetic molecules (i.e., *SIN3*, *NuRD*, *SWI/SNF*). H3K4me3 marks were found to be redistributed in CLL alongside nucleosome

Table 2 An overview of treatment-related epigenetic studies in CLL

Epigenetics and treatment in CLL

Molecule	Description	Regulation	Epigenetic mechanism	Effect on CLL cell/clinical outcome	Treatment	Outcome	Reference/s
KLF4	Tumor suppressor gene	↓ - Epi-silenced	Promoter hypermethylation	CLL progression	GSI Notch signaling inhibition	Modulation of BCR signaling components	Filarsky et al. 2016 [23]
NFATC1	Transcription factor	↑	Promoter Hypomethylation	Correlation with clinical stage	tacrolimus cyclosporin A Ibr (BTKi) in vitro	↑ Apoptosis (primary CLL cells)	Wolf et al. 2018 [26]
EZH2	Histone methyltransferase	↑ (U-CLL)	Canonical—PRC2-dependent way (H3K27me3 induction)—gene repression	↑ Viability ↑ Proliferation	EPZ6438, GSK343 (EZH2i)	↑ Apoptosis	Papakonstantinou et al. 2016 [52]
EZH2	Histone Methyltransferase	↑ (U-CLL)	Non-canonical—PRC2-independent manner—oncogenic function	Constitutive overactivation of pro-survival PI3K/AKT pathway	- DZNep (EZH2i) - JQ1 (MYCi)	↓ pAKT ↓ EZH2 and H3K27me3 ↑ inhibitor = > ↓IGF1R ↓EZH2	Kosalai et al. 2019 [53]
PAX5	Transcription factor	↑	Super enhancer	Mediates the CLL TF CRC ↑CLL survival	JQ1i in vitro	CRC expression ↑ Apoptosis in vitro and in vivo	Ott et al. 2018 [42•]
CD20	B-lymphocyte antigen CD20	↓	HDAC-mediated epigenetic dysregulation (notch signaling mutation driven)	anti-CD20 resistance in vitro	Valproic acid (HDACi) In vitro	↑ CD20	Pozzo et al. 2016 [49]
CD20	B-lymphocyte antigen CD20	↓	HDAC-mediated epigenetic dysregulation	CLL progression	Valproic acid (HDACi) In vivo	- No change in CD20 expression - Induction of H*3K9ac* and *EZH2*-mediated *H3K27me3* in CD20 promoter	Scialdone et al. 2017 [50]
Class I/II HDACs	Histone deacetylase	↑	↓ H3K27ac	Weak intensity of enhancer levels in CLL	Panobinostat (HDACi)	H3K9ac ↑ H3K27ac ↑ - Altered BCR signaling - Histone modifications changes	Mallm et al. 2019 [43•]
HDAC6	Histone deacetylase 6	↑	HDAC activity and transcription repression	CLL progression	ACY738 (HDAC6i) ACY738 and Ibr	↑ Apoptosis , ↓ Proliferation in vivo and in vitro Synergistic treatment higher efficiency	Maharaj et al. 2018 [51]
miR-34b/c	MicroRNA-tumor suppressor genes	↓ - Epi-silenced	Promoter hypermethylation	CLL progression	Decitabine and TSA (HDACi)	↑ Apoptosis (HG-3 cell line)	Deneberg et al. 2014 [65]
miR-3151	miRNA- tumor suppressor gene	↓ - Epi-silenced	Promoter hypermethylation	Protection of from apoptosis through constitutive activation of MEK/ERK and PI3K/AKT signaling pathways	Decitabine	↓ Proliferation ↑ Apoptosis	Wang et al. 2015 [67]
miR-26A1	miRNA- tumor suppressor gene	↓ - Epi-silenced	Differential methylation	↑methylation in poor prognostic, U-CLL Inverse correlation with EZH2	Decitabine	↑miR26A1 ↓EZH2	Kopparapu et al. 2016 [20]
miR-147b miR-210 miR-1253 miR-425	Set of miRNAs-TSG	↓ - Epi-silenced	HDAC-mediated promoter hypermethylation	CLL progression	-Panobinostat (HDACi) - Ibr and abexinostat	- H3K4me3 levels at the promoters of the BTK-targeting miRs- cell death - ↑BTK-targeting miRs - ↓Ibr-resistance	Bottoni et al. 2016 [64]

Table 2 (continued)

Epigenetics and treatment in CLL						
Molecule	Description	Regulation	Epigenetic mechanism	Effect on CLL cell/clinical outcome	Treatment	Outcome	Reference/s
miR-4269 miR-4667-3p BM742401	lncRNA- TSG	↓ - Epi-silenced	Promoter hypermethylation	CLL progression - BM742401 methylation cor	Decitabine	Promoter demethylation and re-expression of BM742401	Wang et al. 2016 [72]

↑: upregulation of gene expression /enhanced/increased levels of [.]; ↓: downregulation of gene expression levels/enhanced/increased levels of each condition described; *BTKi*, Bruton's tyrosine kinase inhibitor: *CD20*, B-lymphocyte antigen CD20; *CLL*, chronic lymphocytic leukemia; *CRC*, core regulatory circuit; *Decitabine*, 5-aza-2'-deoxycytidine; *Epi-silenced*, epigenetically silenced; *EZH2*, enhancer of zeste homolog 2; *GSI*, γ-Secretase inhibitor; *HDAC*, histone deacetylase; *HDACi*, histone deacetylase inhibitor or inhibition; *H3K27me3*, histone H3 trimethylation at lysine 27; *H3K4me3*, histone H3 trimethylation at lysine 4; *HP-CLL*, high-programmed CLL; *Ibr*, ibrutinib (BTK inhibitor); *i-CLL*, intermediate CLL; *IGF1R*, insulin-like growth factor 1 receptor; *IP-CLL*, intermediate-programmed CLL; *JQ1*, thienotriazolodiazepine (inhibitor of the BET family of bromodomain proteins); *KLF4*, Kruppel-like factor 4; *LncRNA*, long non-coding RNAs LP-CLL: low-programmed CLL; *m-CLL*, memory B cell–like CLL; *MEK/ERK*, mitogen-activated protein kinase/extracellular receptor kinase pathway; *miR*, microRNA; *n-CLL*, naive B cell–like CLL; *NFATC1*, nuclear factor of activated T cells, Cytoplasmic 1; *OS*, overall survival; *pAKT*, phosphorylated AKT; *PAX5*, paired box protein Pax-5; *PI3K/AKT pathway*, phosphoinositide 3-kinase/AKT or protein kinase B (PKB) pathway; *PFS*, progression free survival; *TF*, transcription factor; *TFS*, treatment-free survival; *TSA*, trichostatin A; *TSG*, tumor suppressor gene

gain at promoters and extensive changes of enhancer activity. Finally, a CLL-specific network centered around TFs targeting 17 central binding motifs was presented, describing gaining for *NFAT*, *TCF4*, and *LEF1* motifs, and loss for *EBF1* and *AP-1* motifs in CLL.

A deeper understanding of the relationship between intra-leukemic epigenetic and transcriptional diversity has been achieved via recent integrative analysis of CLL's epigenetic landscape. Pastore and colleagues reported corrupted coherence across different layers of the CLL genome and epigenome. An extensive chromatin rewiring at H3K27ac marks, mediated in particular by *NFAT* and *TCF/LEF*, was observed. These H3K27ac peaks showed hypomethylation at super enhancers regions, resulting preferentially in intermediate DNAme levels. H3K27me3-marked genes were incompletely silenced through the formation of PRC2 complex, reflecting to a set of dynamic changes on chromatin states tested. Interestingly, mutually exclusive (H3K27ac/ H3K27me3) histone marks were co-mapped. This work suggests intra-leukemic diversity is linked to stochastic cell activation of alternate gene programs, and leads to an admixture of cells with diverging identities [44].

All in all, the last few years' high-impact research studies have provided valuable resources related to the CLL chromatin landscape, including the until recently understudied histone modifications. These studies have significantly strengthened our knowledge of links between CLL phenotype, clinical outcome, and aberrant features of the CLL epigenome beyond the DNA methylome.

Histone-Modifying Enzymes: Targets for Therapy

Since the dysfunction of histone-modifying enzymes (HME) has been linked to human cancer initiation and progression, the CLL research community has placed significant emphasis on investigating HME. While initial studies have focused on canonical HME functions (catalyzing histone modifications), a few recent studies have explored the nonenzymatic roles of HME in transcriptional regulation [45].

An early study reported that the global levels of histone deacetylase (HDAC) can be an independent prognostic marker for treatment-free (TFS) and overall survival (OS) [46]. Later, Zhou and colleagues reported global histone H3/H4 hypoacetylation, H3K9 hypermethylation, and overexpression of *SIRT1* and *EZH2* in CLL patients [47], whereas Parker et al. discovered recurrent deletions and mutations (~ 7%) of the histone methyltransferase *SETD2* [48].

In another study, the association between *NOTCH1* mutations and low *CD20* expression was suggested to be HDAC-mediated. Interestingly, HDAC inhibition by Valproic acid was shown to upregulate both *MS4A1* and *CD20* [49]. Later work tested the in vivo efficancy of valproate in del13q/ *NOTCH1*wt CLL patients. Although in vivo treatment

resulted in global H3K9ac, it did not induce CD20 expression. In contrast, in vitro treatment of E95 CLL cell line resulted in CD20 upregulation. This finding was attributed to simultaneous induction of H3K9ac and EZH2-mediated H3K27me3 by valproate at the CD20 promoter in vivo, highlighting the need for combinatorial therapies [50].

Independent studies showed that expression of HDAC6 is upregulated in patient samples, cell lines, and euTCL1 transgenic mice. These findings, as well as functional in vivo HDAC6 inhibition, underlined the potential therapeutic value of HDAC6 inhibition for CLL treatment. Furthermore, it was shown that coadministration of an *HDAC6* inhibitor (ACY738) with BTK inhibitor Ibrutinib displayed synergistic cell death and improved overall survival compared with either single agent in vivo [51].

EZH2 appears to be an HME of great interest. An early study showed that *EZH2* is overexpressed in poor prognostic CLL cases and correlated with H3K27me3, whereas *EZH2* inhibition in vitro promoted apoptosis [52]. Recently, inhibition of EZH2 in combination with *PI3K* inhibition (Idelalisib) has been reported to effectively minimize both canonical and non-canonical *EZH2* functions [53]. Furthermore, the oncogenic potential of EZH2 via its antiapoptotic effect was shown to be regulated by microenvironmental signals [54].

Finally, a recent study by Filiu-Braga et al. explored the role of other HMEs, namely the *KDM4* histone demethylase family. Interestingly authors linked *KDM4B* and *KDM4C* expression to poor prognostic CLL patient subgroups [55].

Although all aforementioned studies have indicated an important role of HME in CLL, there is a therapeutic potential in light of combinatorial therapies (i.e., BCR signaling inhibitors and HME inhibitors) which is not yet systematically explored. Given the significant number and diversity of HME, together with canonical and recently emerging non-canonical functions [45], it is now evident that in-depth understanding of HME-specific functions is perquisite for designing a successful epigenetically relevant combinatorial therapy.

BCR Targeted Therapies and the Epigenome

B cell receptor signaling pathway inhibitors (BCRi) have shifted the treatment paradigm in CLL over the last 5 years [56]. Dissection of the epigenomic and transcriptomic landscape of CLL in parallel with the advent of new therapies targeting key biological pathways has led to an opportunity for a more pharmaco-epigenomics based approach in CLL research, which has recently emerged as a field with great potential for understanding treatment resistance.

To that end, a pioneering study by Schmidl et al. shed light on the epigenetic remodeling profiles in association with BTK inhibition. The team employed a systematic and innovative approach from matched CLL patients pre- and post-Ibrutinib, to identify treatment-specific vulnerabilities for drug combinations to overcome Ibrutinib resistance. They scanned the epigenetic cell state and regulatory profiles by ATAC-seq and combined the data with single-cell chemosensivity analysis, which included 131 drugs. Interestingly, Ibrutinib induced a significant loss (92%) of accessible regions, while fewer cases (8%) gained chromatin accessibility upon treatment. At a pathway level, downregulation of NF-κB and BCR signaling as well as specific enrichment for proteasome regulation and autophagy was reported. The study reported an ibrutinib-induced gain of CLL cell selectivity for proteasome inhibitors, *PLK1* inhibitors, and *mTOR* inhibitors, and sets the ground for novel combinatorial therapies [57•].

Another recent work by Holmes et al. elucidates the ibrutinib-induced changes in chromatin configuration of CLL. Authors report a remarkable loss of both H3K27ac and H3K27me3 marks together with EZH2 expression after 14 days treatment [58]. The IR-induced lymphocytosis tended to be quicker in treatment-naïve (TN) and delayed in relapsed-refractory (RR) patients. Interestingly, this delay was linked to H3K4me3 maintenance at PRC2 targets only in RR patients arguing for a mechanism of partial reprogramming that takes place during the process of relapse. Overall, these findings suggest a mechanism that CLL cells deploy to adaptively respond to Ibrutinib by histone hypomethylation and therefore passage from a proliferative state into quiescence [58].

Non-coding RNAS

The emerging role of non-coding RNAs (ncRNAs) in gene regulation and pathogenesis of numerous malignancies has been underscored by previous key reviews [59–61]. Studies exploring the molecular background and mechanisms mediated either through microRNAs (miRs) or long, non-coding RNAs (lncRNAs) showed that these molecules possess significant regulatory roles and influence to a critical point the epigenetic portrait of CLL. From a clinical standing, the clinical significance of microRNAs (miRs) in CLL has been well documented [62, 63], whereas more recent studies shed light on other types of non-coding RNAs.

In 2016, Bottoni et al. identified a set of miRNAs which directly downregulate *BTK* expression in CLL [64]. In a group of 83 patients, the HDAC complex was recruited to miR promoters to silence their expression. HDAC inhibition increased miR promoter H3K4me3 and decreased BTK protein levels. This work suggests that HDACi therapies may target Ibrutinib resistant CLL via miR-based epigenetic silencing of BTK-associated miRNAs [64].

The *miR-34b/c* (part of the *TP53* network) has been shown to be epigenetically silenced in cancer. In CLL, *miR-34b/c* shared promoter (with *BTG4*) is aberrantly hypermethylated in almost half of CLL cases. Promoter DNA methylation levels inversely correlated with expression and transfection

of *microRNA-34b/c* genes into HG3 CLL cells increased apoptosis underlying their tumor-suppressive role, which is epigenetically silenced in CLL [65].

Based on previous findings for aberrant promoter DNA methylation of *miR-708* in CLL, Baer C et al. explored the effects and pathway of downstream targets related to its tumor-suppressor function [66]. Remarkably, they found that miR-708 strongly interacted and inhibited NF-κB signaling, and therefore expression of target genes through endogenous repression of *IKKβ*. Overall, this tumor-suppressive function was found to be reversed in CLL and mostly in U-CLL subset as hypermethylation of a distinct enhancer located downstream of *miR-708* promoter drove the CLL cell to an epigenetic-mediated silenced state. Hence, this effect led to lower *miR-708* expression, and was associated with high-risk characteristics in CLL patients [66].

Another example for miR transcriptional inhibition is *miR-3151*. A tumor-suppressor role has been proposed for this miR. In CLL specifically, *miR-3151* promoter was mostly hypermethylated and demethylation treatment resulted in *miR-3151* expression, subsequent tumor suppression and enhanced apoptosis via downregulation of a MEK/ERK PI3K/AKT signaling [67].

In another study, *miR-26A1* was epigenetically repressed in CLL via promoter hypermethylation. Higher *miR-26A1* methylation was linked to poor prognosis; therefore, a tumor-suppressive role was suggested for *miR-26A1*. *miR-26A1* expression inversely correlated with the oncogenic driver *EZH2* in vitro, further supporting a tumor-suppressive function of this miR [68]. Also, a later study reports an inverse correlation between *EZH2* and *miR-26A1* expression in CLL lymph node samples [69].

A large-scale study by Rochetti and colleagues investigated lncRNA expression in a cohort of 217 CLL patients and reported 24 lncRNAs as being deregulated in CLL [70]. Concurrently, a significant percentage of CLL-specific differentially methylated genes were mapped to lncRNAs. Two novel lncRNAs (hypermethylated *CRNDE* and hypomethylated *AC012065.7*) were further validated and correlated with an inferior outcome [71]. Finally, another study suggested a tumor-suppressive role for lncRNA *BM742401* in CLL via enhanced apoptosis. Interestingly, B*M742401* expression was regulated by promoter DNA methylation [72].

Conclusion

CLL epigenomics has witnessed a rapid growth, very much linked to recent technological advances. The transition from array-based methods to whole-genome sequencing and single-cell approaches has been followed by an exponential growth of high-throughput data generation and high-impact publication in the field. This growth has had a large impact on the understanding of epigenetic mechanisms and shed plenty of light onto CLL ontogeny and subset characterization, including novel prognostic considerations.

From the global DNA methylome studies to single CpG biomarker discoveries, and from histone modification mapping to non-coding RNA contribution to leukemogenesis, epigenetics has emerged beyond doubt as a key element of current CLL translational research.

In the near future, CLL epigenomics could involve more single-cell approaches, as those may help us to better tackle intraclonal epigenomic diversification and understand emerging treatment resistance. The dissection of the epigenome of non-leukemic cells is yet another important direction, along with the characterization of the epigenomes of the various cellular components of the CLL niche. The impact of epigenetic treatments on non-CLL cells and how these affect the cross-talk between CLL cells and the microenvironment is a question that also remains to be systematically explored by epigenomic analyses.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest associated with this manuscript.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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