Beyond Genomics - Targeting the Epigenome in Diffuse Large B-Cell Lymphoma

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Abstract

After decades of intense research on genetic alterations in cancer and successful implementation of genetically-based targeted therapies, the field of cancer epigenetics is only beginning to be fully recognized. The discovery of frequent mutations in genes modifying the epigenome in diffuse large B-cell lymphoma (DLBCL) has highlighted the outstanding role of epigenetic deregulation in this disease. Identification of epigenetically-driven DLBCL subgroups and development of novel epigenetic drugs have rapidly advanced. However, further insights are needed into the biological consequences of epigenetic alterations and the possibility of restoring the aberrant epigenome with specific therapies to bring this treatment concept further into clinical practice. This review will summarize the main epigenetic changes found in DLBCL and their potential for precision medicine approaches.

Highlights

- The most frequent epigenetic alterations in DLBCL are described
- We review potential targets for epigenetic therapies in DLBCL
- The current clinical development of epigenetic drugs and future direction for combination therapies are outlined

Keywords

- Diffuse large B cell lymphoma
- Epigenome
- Epigenetic therapies

Background

Approximately one third of patients with diffuse large B-cell lymphoma (DLBCL) relapse after first-line therapy. Outcome of relapsed/refractory DLBCL remains poor and there is an unmet need for novel agents to improve treatment of this patient population. Remarkable progress has recently been made in the development of "epigenetic drugs" and in our understanding of the epigenetic basis of DLBCL. Epigenetics define mechanisms of regulating cellular functions without changing the genetic code. Key elements of the epigenome are chromatin modifications in form of DNA methylation as well as post-translational modifications of histones. This review will focus on chromatin modifications and their potential as therapeutic targets in DLBCL.

Chromatin is built of nucleosomes, a complex of DNA wrapped around an octamer of histone proteins, two of each histone H2A, H2B, H3 and H4 (Figure 1). Histone residues can be modified by methylation and acetylation, but also ubiquination, phosphorylation and sumoylation. These histone "marks" are predominantly found at arginine and lysine residues of histone tails. Similarly, DNA can be marked by methylation of cytosines that are part of CG dinucleotides (CpGs). Epigenetic marks modify the conformational structure of chromatin and thereby access and recruitment of proteins essential for key cellular functions such as transcription, DNA repair and replication.[1]

Several enzymes, so called epigenetic modifiers, are involved in concerting the epigenetic code, which are broadly categorized into "writers", "erasers" and "readers". Writers add epigenetic marks to the chromatin e.g. acetylate or methylate histone residues or CpGs [histone acetyltransferases (HATs), histone methyltransferases (HMTs), DNA methyltransferases (DNMTs)]. Accordingly, eraser proteins catalyze removal of these marks and comprise histone deacetylases (HDACs), histone demethylases (HDMTs) and DNA demethylases. Readers are effector proteins that recognize and bind epigenetic marks through specific domains (e.g. bromo- and chromo-domains) and induce downstream molecular changes such as activation/repression of gene expression or recruitment of DNA repair proteins.

The effect of epigenetic marks on gene transcription is complex and context-dependent.[1] DNA methylation in gene promoters is often associated with gene silencing, whereas gene body methylation is frequently linked to transcriptional activation. Histone methylation (mono-, di- and tri-methylation) is frequently found at histone H3, lysine residues 4, 9, 27, 36 and 79.[2] Methylation of H3 at lysine 4 (H3K4me) represents a histone mark associated with activation of gene expression. In contrast, H3K27me typically results in gene silencing.[2] Of note, promoter or enhancer regions controlling transcriptional activity can also be in a bivalent (poised) state, when both repressive and activating marks are present. Given the magnitude of epigenetic marks that co-occur at a specific genomic region, the functional implications of individual marks have to be interpreted in a broader context.[1]

Epigenetic modifications are key regulatory elements for normal cell development, controlling important cellular growth and survival pathways. Epigenetic patterns undergo substantial changes during cell differentiation, but also dynamic changes in response to various external stimuli.[1] This allows for a rapid on/off-switch of selected genes to adapt cells to particular requirements, such as changes during transition from naïve B-cell to plasma cell maturation.

Epigenetic changes in DLBCL

The significant role of epigenetics in the development of DLBCL and other germinal center (GC) lymphomas like follicular lymphoma (FL) was highlighted by the high incidence of somatic mutations in epigenetic modifiers discovered by next-generation sequencing studies. Genes most frequently affected encode for the HMTs EZH2 and KMT2D, as well as the HATs CREBBP and EP300.[3–6] Nearly all of these mutations are heterozygous. Allele frequencies in lymphoma cell subsets indicate that they are driver lesions occurring early during lymphomagenesis.[7,8] Mutations in epigenetic modifiers lead to aberrant patterns of epigenetic marks and thus perturbation of cellular activities. The shared biological consequence of the above mentioned mutations is transcriptional repression of specific target genes: through gain-of-function of transcriptional repressors like EZH2, or loss-of-function of transcriptional activators like KMT2D or CREBBP/EP300. Similar downstream effects can also be induced by mutations of transcription factors regulating epigenetic modifiers or by genes affecting metabolites that influence activity of these enzymes.

Perturbation of DNA methylation has also been implicated in the pathogenesis of DLBCL. In this regard, focal hypermethylation can lead to silencing of tumor suppressor genes, and global hypomethylation is believed to contribute to lymphomagenesis through genomic instability. Interestingly, recent studies have demonstrated that a number of transcription factors bind preferentially to hypermethylated DNA, demonstrating regulatory function of DNA methylation beyond established concepts.[9] In contrast to myeloid malignancies, DNA methylation changes in DLBCL are not linked to mutations of DNMTs and underlying mechanisms have yet to be defined.

On the basis of alterations in epigenetic modifiers and patterns of epigenetic marks, epigenetically driven subgroups of DLBCL are now increasingly being recognized and will help to identify suitable candidates for epigenetic therapy approaches.

Targeting the DLBCL epigenome

Histone lysine methylation

KMT2D

Mutations of *KMT2D* (also known as *MLL2*) are found in 20-35% of DLBCL, both in germinal center B-cell (GCB) and activated B-cell (ABC) subtypes.[4–6] Notably, the incidence of *KMT2D* mutations exceeds 80% in patients with FL.[4,10] The HMT KMT2D is a subunit of a protein complex that activates transcription through methylation of H3K4, predominantly at enhancer regions.[11] Mutations are widely distributed over the entire *KMT2D* gene and are mostly truncating and missense mutations leading to loss of its enzymatic function.[5] The majority of DLBCL cases harbor heterozygous mutations without evidence of inactivation of the second allele, suggesting KMT2D to be a haploinsufficient tumor suppressor.[5,12] First data on the functional role of KMT2D in lymphoma development have only recently been published, confirming tumor suppressor properties of the protein associated with loss of H3K4 methylation.[12,13] Target genes of KMT2D include regulators of immune signalling, toll-like receptor- and B-cell receptor signalling pathways.[13] Knockout of *KMT2D* promoted lymphoma development in FL/DLBCL mouse models.[12,13] Interestingly, KMT2D was shown to preferentially methylate bivalent promoters in embryonic stem cells, a unique feature among

KMT2 family members.[14] Thus, disruption of bivalency at poised promoters might play a role in the pathogenesis of *KMT2D*-deficient lymphomas.

So far, there is no specific treatment for *KMT2D* mutated DLBCL. One potential approach could be the development of inhibitors of histone demethylases that counteract KMT2D function. Another interesting area is the design of molecules that modulate reader proteins of KMT2D-induced marks to restore the defective KMT2D pathway.[15] In addition, there is evidence for cross-talk between histone methylation and acetylation networks and HDAC inhibitors were shown to increase methylation at H3K4.[16] In a study investigating Kabuki syndrome, a rare disorder associated with loss of *KMT2D*, HDAC inhibitor treatment restored the neurodevelopmental phenotype in a Kabuki syndrome mouse model.[17] It will be interesting to see whether DLBCL cases harboring *KMTD2* mutations show differential response to HDAC inhibitors with H3K4 methylation properties.

EZH2

EZH2 is a H3K27 methyltransferase which is mutated in 20-30% of GCB DLBCL and rarely in ABC subtypes.[3,18] EZH2 is the key enzymatic component of the Polycomb Repressor Complex 2 (PRC2). EZH2-mediated mono-, di- and trimethylation of H3K27 leads to silencing of a magnitude of genes,[19] the specific set of target genes being cell-type dependent. In B-cells, EZH2 plays an essential role during GC formation. EZH2 is transiently up-regulated in centroblasts promoting cell proliferation by suppressing the cell cycle inhibitor *CDKN1A* and blocking differentiation by silencing *IRF4* and *PRDM1*, genes essential for plasma cell maturation.[20]

Mutations of *EZH2* are predominantly heterozygous gain-of-function mutations affecting the catalytic SET domain, mostly at Y641.[3] Mutant EZH2 has improved di- and trimethylation efficiency, but is defective of H3K27 monomethylation, thus requiring presence of the wild-type enzyme.[21] EZH2-mediated gene silencing through H3K27 trimethylation mainly occurs at promoters that are activated by H3K4me3 marks, resulting in their transcriptional poising.[20] Presence of *EZH2* mutation locks cells in the GC state by maintaining repression of GC exit genes, facilitating malignant transformation.[20] Mutant EZH2 is not sufficient to induce lymphoma development, but was shown to accelerate BCL2- and MYC-driven lymphomagenesis.[20,22]

Several small inhibitors of EZH2 have been developed. EZH2 inhibitor treatment was shown to induce up-regulation of EZH2 targets and bivalent genes and suppressing EZH2-associated proliferation in GCB cells.[20] In ABC lymphoma cells, EZH2 inhibition leads to de-methylation of H3K27, but without significant impact on cellular growth.[20] Interestingly, anti-tumor activity was also seen in GCB DLBCL without *EZH2* mutation[20] and consequently clinical trials are enrolling both wild-type and mutated cases at present. Three compounds are currently tested in phase I/II trials – tazemetostat (Epizyme), GSK2816126 (GlaxoSmithKline), and CPI-1205 (Constellation Pharmaceuticals). A recent update on a phase II trial of single-agent tazemetostat in relapsed/refractory B-cell Non-Hodgkin lymphoma (NHL) showed remarkable efficacy, particularly in *EZH2* mutated FL with an ORR of 92%.[23] Response to tazemetostat in DLBCL seem to also depend on the *EZH2* mutational status with 5/17 (29%) responders in *EZH2* mutant compared to 18/119 (15%) in wildtype cases.[23] Further data of correlative biomarker studies in DLBCL will reveal how strongly activity of EZH2 inhibitors depends on the *EZH2* mutational status, cell-of-origin categories or other molecular characteristics of DLBCL.

Histone acetylation

CREBBP and EP300

The two interacting histone acetyltransferases CREBBP and EP300 are mutated in around 30% of DLBCL.[24] Both proteins are highly homologous and acetylate lysine residues of histones as well as non-histone substrates. Mutations are predominantly located in the catalytic HAT domain and lead to reduced acetylation activity and potentially dominant-negative effects.[24,25] Reduced acetylation levels of H3K4 and H3K18 have been shown in DLBCL cell lines after knockdown of wild-type p300 protein.[26] Current data indicate that *CREBBP* and *EP300* are haploinsufficient tumor suppressors, and several mechanisms have been described to account for this function, e.g. alteration of BCL6 and p53 activity through reduced acetylation of these proteins[24,27] or down-regulation of NfkB target genes.[26] Germline mutations of *CREBBP/EP300* are found in the autosomal dominant Rubinstein-Taybi syndrome which is associated with increased risk of lymphoma.[28] Interestingly, *CREBBP/EP300*-deficiency seems to have a unique role in B-cell malignancies and are rare in solid tumors.[29]

One could speculate that CREBBP/EP300 loss-of-function can be counteracted by treatment with HDAC inhibitors and there are pre-clinical data supporting this hypothesis.[30] However, another study indicated that *EP300* mutations confer HDAC inhibitor resistance in DLBCL cells.[25] No clear association of the *CREBBP/EP300* mutational status and response to HDAC inhibitors has been observed in patients so far,[31,32] but larger datasets should be awaited before drawing final conclusions.

HDAC inhibition

HDACs are involved in regulating a variety of cellular functions implicated in cancer development and have therefore been investigated as drug targets for many years.[33] HDAC inhibitors are currently the only class of epigenetic compounds that are approved for the treatment of lymphoma, namely for relapsed/refractory peripheral T-cell lymphoma. In DLBCL and other B-cell lymphomas, HDAC inhibitors seem to have limited single-agent activity, with overall response rates (ORR) of 5-10% for vorinostat and belinostat.[34,35] Combination of vorinostat with R-CHOP chemotherapy in the SWOG S0806 phase I/II trial did not indicate clinical benefit, but a high rate of infectious complications associated with vorinostat.[36] Results from two phase II trials suggest higher potency of panobinostat in DLBCL with an ORR of 20-29%, including durable responses.[32,37] Interestingly, presence of *MEF2B* mutations was associated with response to panobinostat in univariate analysis.[32] MEF2B recruits HDACs and HATs to target genes and is mutated in 12-15% of DLBCL.[4,32,38] Data from independent cohorts are needed to confirm whether *MEF2B* can serve as a biomarker of response to treatment with panobinostat or other HDAC inhibitors.

A major issue of current HDAC inhibitors is lack of specificity: they inhibit several different HDAC isotypes, but also modulate acetylation of a wide range of other proteins, such as growth factor receptor- and signalling proteins, transcription factors, and DNA repair proteins.[39] Accordingly, HDAC inhibitors have pleiotropic anti-cancer effects and it is entirely unclear whether histone acetylation or non-epigenetic off-target effects account for their anti-tumor activity. It will be essential to identify biomarkers of response to HDAC inhibitor treatment, but this is a challenging task with the mechanism of action being largely unknown. Development of selective isotype-specific HDAC

inhibitors might improve the therapeutic value of these agents.[40] In addition, exploiting synergies of HDAC inhibition with other cancer pathway inhibitors, like PI3K/mTOR inhibitors[41,42] or proteasome inhibitors[43] will be an important step to take this epigenetic therapy further in the treatment of DLBCL.

BET bromodomain inhibition

Bromodomains (BRDs) are found in reader proteins of histone lysine acetylation marks such as the bromodomain and extraterminal (BET) family of BRD proteins. The BET protein family comprises BRD2, BRD3, BRD4, and BRDT. They mediate recruitment of transcription factors and other proteins involved in gene transcription.[44] No genetic alteration affecting BET proteins is known in lymphoma or other hematological malignancies yet. However, critical oncogenes like c-MYC are regulated by BET proteins, making them attractive targets for specific therapies.[45] Several selective BET bromodomain inhibitors have been developed. Phase I data of the first-in-class small molecule BRD2, BRD3, and BRD4 inhibitor OTX015 (MK-8628) demonstrated promising efficacy in DLBCL and phase II studies are now underway.[46] In addition, synergistic effects between BET bromodomain inhibitors and other novel agents like HDAC inhibitors,[47] ATR inhibitors[48] or Bruton's tyrosine kinase inhibitors[49] warrant further clinical investigation.

DNA Methylation

Perturbation of DNA methylation is a hallmark of solid and hematologic malignancies including DLBCL.[50] The general assumption is that changes in *de novo* methylation in DLBCL are induced by genetic alterations. However, methylation changes could also be initiating events conferring a stem-like state in lymphoma precursors.[51] A high degree of aberrant methylation as well as high intra-tumor methylation heterogeneity were shown to be associated with poor outcome in DLBCL[52–54] and might contribute to clonal evolution and acquisition of chemoresistance. When comparing presentation and relapse samples of selected DLBCL cases, Pan *et al.* identified differentially methylated promoter and enhancer regions, further supporting a role of DNA methylation in disease evolution.[54] Intra-tumor methylation heterogeneity was reduced in relapse samples indicative of clonal selection. Differential methylation has also been described between ABC and GCB subtypes and may contribute to biological differences between these groups.[55] In addition to global methylation changes in DLBCL, focal hypermethylation of tumor suppressor genes such as *KLF4* or *p16(INK4a)* has been reported in several studies.[56,57] Despite these interesting novel data, the role of DNA methylation in DLBCL development and progression remains largely unclear.

DNA methylation is mediated by DNA methyltransferases DNMT1, DNMT3A, and DNMT3B, the two latter forms being mainly responsible for *de novo* methylation. Although DNMTs are frequently mutated in myeloid malignancies and peripheral T-cell lymphoma, such mutations are rare in B-cell lymphomas. Inhibition of DNMTs by cytosine analogs 5-azacytidine or decitabine is supposed to restore expression of epigenetically silenced genes. However, our understanding of how these drugs impact DNA methylation at specific loci is very limited. Both agents are successfully used in the treatment of acute myeloid leukemia and myelodysplastic syndrome, but have limited single-agent activity in lymphomas. However, the correct dosing and timing schedules of 5-azacytidine and decitabine seem to be crucial. Low-dose application of decitabine was demonstrated to prime DLBCL cells for subsequent chemotherapy and counteract mechanisms of resistance.[58] A phase I study

investigating 5-azacytidine priming before R-CHOP therapy in high risk DLBCL is currently ongoing. In addition, combination of DNMT and HDAC inhibitors revealed synergy in *in vitro* and xenograft models[59] and should be further explored. With an optimal dose, sequence and combination, hypomethylating agents might well play a role in future treatment approaches in DLBCL.

Conclusion and future directions

Next-generation sequencing studies have identified mutations of epigenetic modifiers as a hallmark event in DLBCL. In particular the discovery of recurrent, activating mutations of *EZH2* and insights into their biological function opened up avenues of stratified epigenetic therapies. Several early phase clinical trials with EZH2 inhibitors are expected to report soon and will hopefully encourage further exploration of epigenetic drugs in DLBCL. Besides EZH2, a plethora of other epigenetic alterations has been identified in DLBCL, but their role in disease biology and potential as therapeutic target remain elusive. Future research on the epigenetic structure of DLBCL and other lymphomas will further elucidate the functional consequences of epigenetic alterations and their unique role in specific biological subgroups. This will assist in the development of novel epigenetic therapies and in defining suitable patient groups for these agents.

However, with the exception of EZH2 inhibitors, identification of robust biomarkers for epigenetic therapies will likely continue to pose a challenge: in contrast to conventional drug targets like activated kinases, epigenetic changes are abundant, dynamic and complex to measure. In addition, epigenetic marks are cross-linked and their molecular functions are highly context-dependent. A better understanding of the molecular effects of epigenetic therapies, including non-epigenetic off-target effects, and ultimately the development of more specific new generations of epigenetic drugs, might facilitate successful biomarker discovery in the future.

Given the cross-talk of different epigenetic marks in normal and malignant cells, combination of epigenetic therapies are anticipated to act synergistically and should be more extensively studied. In addition, data on several interesting combinations of epigenetic drugs with various classes of targeted and chemotherapeutic agents are eagerly awaited. Finally, the increasing effort of integrating large genomic and epigenomic datasets will generate a more comprehensive picture of tumor-related alterations and their interaction in order to improve rational combination treatment trial design.

Contributors

A.K. wrote the manuscript, which was edited, reviewed and approved by D.C. and I.C.

Declaration of interest

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Figure 1: Epigenetic targets in DLBCL. The schematic figure illustrates DNA wrapped around a histone protein complex consisting of histones H2A, H2B, H3 and H4. DNA is methylated (grey) or unmethylated (white) at specific CpG sites. Lysine acetylation and methylation marks on histone tails can be altered through the presence of somatic mutations in *EZH2*, *KMT2D* and *CREBBP*/*EP300*, respectively.

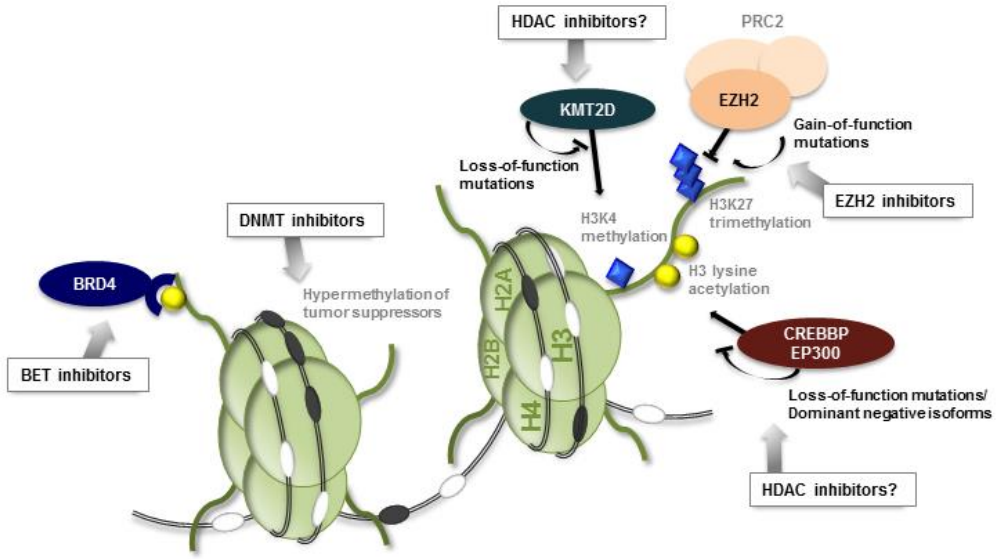

Table 1: Current clinical trials with epigenetic therapies in DLBCL.

Inhibitor	Phase	Combination	Indication	Trial number[#]
EZH2 inhibitors				
Tazemetostat	Phase 1/2	Monotherapy	Relapsed/refractory DLBCL	NCT01897571[23]
	Phase 1/2	R-CHOP	First-line DLBCL, high-risk	NCT02889523
GSK2816126	Phase 1	Monotherapy	Relapsed/refractory NHL	NCT02082977[60]
CPI-1205	Phase 1	Monotherapy	Relapsed/refractory lymphoma	NCT02395601
HDAC inhibitors				
Romidepsin	Phase 1	Alisertib	Relapsed/refractory lymphoma	NCT01897012
	Phase 1/2	Oral azacitidine (CC-486)	Relapsed/refractory NHL	NCT01998035
	Phase 1*	Gemcitabine/dexa-methasone, cisplatin	DLBCL and peripheral T-cell lymphoma	NCT01846390
Vorinostat	Phase 1	Pembrolizumab	Relapsed/refractory DLBCL, FL, Hodgkin lymphoma	NCT03150329
	Phase 1/2	Gemcitabine/busulfan/	Relapsed/refractory ABC	NCT02589145

		melphalan, lenalidomide	DLBCL	
	Phase 2*	Bortezomib	Relapsed/refractory DLBCL and MCL	NCT00703664
	Phase 1/2*	R-CHOP	First-line DLBCL	NCT00972478[36]
Belinostat	Phase 1	Carfilzomib	Relapsed/refractory NHL	NCT02142530
	Phase 2*	Yttrium Y 90 ibritumomab tiuxetan	Relapsed/refractory NHL	NCT01686165
Panobinostat	Phase 2*	Monotherapy	Relapsed/refractory NHL	NCT01261247
	Phase 1/2*	Everolimus	Relapsed/refractory lymphoma and myeloma	NCT00918333
	Phase 2*	Rituximab	Relapsed/refractory DLBCL	NCT01238692[32]
Mocetinostat	Phase 2*	Monotherapy	Relapsed/refractory DLBCL and FL with *CREBBP*/*EP300* mutation	NCT02282358
Hypomethylating agents				
Decitabine	Phase 1/2	R-CHOP	First-line DLBCL	NCT02951728
5-azacytidine	Phase 1b	Avelumab/utomilumab	Relapsed/refractory DLBCL	NCT02951156
Oral azacytidine (CC-486)	Phase 1	R-CHOP	First-line DLBCL, high-risk	NCT02343536
	Phase 1/2	Romidepsin	Relapsed/refractory NHL	NCT01998035
BET inhibitors				
MK-8628	Phase 1b*	Monotherapy	Relapsed/refractory DLBCL	NCT02698189
INCB057643	Phase 1	Monotherapy	Relapsed/refractory NHL	NCT02711137
INCB054329	Phase 1*	Monotherapy	Relapsed/refractory NHL	NCT02431260

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* Indicates active study, but not recruiting participants