Epigenetic dysregulation in follicular lymphoma

The adoption of next-generation sequencing technologies has led to a remarkable shift in our understanding of the genetic landscape of follicular lymphoma. While the disease has been synonymous with the t(14;18), the prevalence of alterations in genes that regulate the epigenome has been established as a pivotal hallmark of these lymphomas. Giant strides are being made in unraveling the biological consequences of these alterations in tumorigenesis opening up new opportunities for directed therapies.

First draft submitted: 5 July 2015; **Accepted for publication:** 22 September 2015; **Published online:** 23 December 2015

Keywords: CREBBP • EP300 • epigenetics • EZH2 • follicular lymphoma • histone acetylation • histone methylation • KMT2D • noncoding RNA

Background

Follicular lymphoma (FL) is one of the most common non-Hodgkins' lymphoma (NHL), with the tumor cells resembling germinal center (GC) B cells. Although the clinical course is typically considered indolent, it is punctuated by multiple relapses, demonstrates eventual refractoriness to standard therapies and about 30% of patients transform to aggressive lymphomas, usually diffuse large B-cell lymphoma (DLBCL). While the addition of the anti-CD20 monoclonal antibody, rituximab, to treatment regimens has markedly improved outcomes, it remains an incurable cancer [1,2] where patients with chemorefractory or transformed disease remain an area of unmet clinical need for which novel treatment strategies are warranted.

The primary genetic event in the classical model of FL pathogenesis is the reciprocal translocation t(14;18)(q32;q21) that leads to aberrant constitutive overexpression of the antiapoptotic BCL2 protein, occurring in 85–90% of patients [3,4]. While this translocation confers a survival advantage, several bodies of evidence suggest that it is

insufficient on its own for lymphoma development [5–7] and secondary genetic hits are required for cellular transformation to FL [8,9]. Indeed, we now have a near complete catalogue of coding mutations and recognize that genetic alterations of several pathways, including multiple components of the epigenetic machinery, are a defining feature of FL pathogenesis (Table 1) [10–19].

Epigenetic mechanisms of gene regulation play a critical role in normal cellular processes. These epigenetic modifications display dynamic features that involve the addition or removal of chemical groups to DNA or histones and are catalyzed by families of enzymes known as 'writers' or 'erasers' of the epigenetic code. Another group of proteins ('readers') are recruited to specific regions of the genome through recognition of the modifications created by 'writers' and 'erasers', and include proteins that are able to further alter the chromatin state and subsequently regulate the expression of target genes. A more detailed account of the mechanisms through which epigenetic regulators and modifications regulate gene

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expression is beyond the scope of this review, and have been extensively discussed elsewhere [23–25]. In this review, we focus attention on FL, the potential functional consequences of alterations of these regulators of the epigenome and discuss the latest progress in harnessing this knowledge for successful epigenetic targeting.

Epigenetic landscape of FL

In FL, epigenomic mutations predominantly target the histone methyltransferases *KMT2D* (90%) and *EZH2* (25%), as well as the histone acetyltransferases *CREBBP* (30–60%) and *EP300* (9%), with almost 80% of cases having co-occurring mutations [10–19]. These somatic mutations are predominantly inactivating, with the exception of *EZH2* [10–11,20–21,26] where mutations cluster to 3 amino acid positions (Y641, A682 and A692) located within its catalytic SET domain. These ‘writers’ of the histone code play a central role in regulating gene expression by modifying two key lysine residues; the histone 3 lysine position 4 (H3K4; *KMT2D*) and histone 3 lysine position 27 (H3K27; *EZH2*, *CREBBP* and *EP300*) marks, while seemingly sparing other residues (Figure 1) [25]. These aberrations are complemented by mutations in a plethora of additional genes that have received less attention, including mutations of linker and core histones which reduce their binding affinity for chromatin and truncating mutations of the chromatin remodeling complex *ARID1A* which increases genomic instability, all of which lead to some form of epigenetic chaos in support of malignant transformation [12,22].

Although the precise genes and cellular pathways dysregulated by these alterations are not fully characterized, we can make several assumptions from the evidence currently available. Firstly, the prevalence of co-occurring ‘epimutations’ suggests a combinatorial, rather than truly independent or additive mechanism of action. Secondly, the temporal sequencing of sequential FL tumors has highlighted that mutations in *KMT2D* and *CREBBP* represent early oncogenic events, providing compelling evidence for their role in forming the founder ‘common progenitor cell’ population (CPC) [12–13,19]. This putative population is postulated to evade chemotherapy and act as a reservoir that seeds each relapse and transformation event, with effective targeting of this population a potential route to a cure [27]. In addition, the incorporation of these epigenetic mutations has led to improved prognostication tools with a recent model termed the m7-FLIPI combining clinical factors with mutations in seven genes including *CREBBP*, *ARID1A*, *EP300* and *EZH2*, where *EZH2* mutations were strongly associated with good risk disease [28].

From a therapeutic perspective, the opportunities to exploit the inherent plasticity of epigenetic changes are manifold. While epigenetic therapies such as histone deacetylase inhibitors (HDACi) have previously been evaluated in FL, results were overall disappointing and predated our current knowledge of the epigenomic landscape of this malignancy [29], which may provide a rationale to guide therapy in accordance to patients’ underlying (epi)genetic profiles. It is also important to recognize that epigenomic alterations are not restricted to FL but occur in almost every type of cancer, high-

Table 1. The frequency and function of commonly mutated epigenetic genes in follicular lymphoma.					
Symbol	Gene name	Biological function	Oncogenic alteration	Mutation frequency (%)	Ref.
<i>KMT2D</i>	Lysine (K)-specific methyltransferase 2D	H3K4 methylase: activator	Loss of function	76–89	[10,12,14]
<i>CREBBP</i>	CREB binding protein	H3K27 and H3K18 acetylase: activator	Mutations cluster in histone acetyltransferase domain. Loss of function [†]	33–68	[10–12,14]
<i>EP300</i>	E1A binding protein p300	H3K27 and H3K18 acetylase: activator	Loss of function [†]	9	[10–12,14]
<i>EZH2</i>	Enhancer of zeste 2 polycomb repressive complex 2 subunit	H3K27 methylase: repressor	Hotspot mutations at Y641, A682, and A692. Gain of function [†]	7–27	[15–16,20–21]
<i>HIST1H1B-E</i>	Histone H1 family genes B-E	Linker histones	To be elucidated	28	[12,14,22]
<i>ARID1A</i>	AT rich interactive domain 1A (SWI-like)	Nucleosome remodeling	To be elucidated	9–11	[12,14,22]
[†] Also subject to copy number changes.

Figure 1. A model for how mutations of epigenetic regulators can lead to dysregulation of H3K4 and H3K27 modification in follicular lymphoma. Loss of function (-) and gain of function (+) mutations confer a repressed chromatin state and alongside promoter hypermethylation (CpG-me) may act to repress gene expression.

lighting their emerging role as a hallmark of cancer [30]. Interestingly, the (epi)genetic landscape of FL overlaps significantly with the related aggressive subtype, germinal center B-cell DLBCL (GCB-DLBCL), with the epigenetic basis of DLBCL the subject of a recent review [31]. However, FL appears to be a rather unique malignancy with epimutations arising in nearly every patient, and therefore may represent a valuable model to examine how epigenetic perturbations drive cancer in general.

Histone modifications

In FL, one postulates that the prevailing consequence of the mutations in the histone-modifying enzymes is a shift in the equilibrium towards aberrant repression of gene transcription brought about by loss of active marks of transcription, catalyzed by the ‘writers’ KMT2D (H3K4me3) and CREBBP/EP300 (H3K27ac), and increase in the repressive mark H3K27me3 through mutations in *EZH2* (Figure 1). It seems implausible that these mutations are acting alone but must do so in a co-ordinated manner, working in concert to impact expression of key regulators of B-cell homeostasis. Mechanistic insight as to how these aberrations promote lymphomagenesis is the subject of ongoing work, with an initial focus on the role of individual components, with *EZH2* the best characterized presently.

EZH2 is the SET domain-containing catalytic subunit of PRC2, which catalyses methylation of the H3K27 residue. It has been recognized for some time that *EZH2* plays a controlling role in co-ordinating gene expression in stem cells through the formation of ‘bivalent’ or ‘poised’ genes, which are marked by both the active H3K4me3 and the repressive H3K27me3 mark, allowing for exquisite control of gene expression during differentiation [32,33]. Morin *et al.* were the first to report mutations of this histone methyltransferase in GC lymphomas, leading to an increase in H3K27me3 [15,20–21]. The expression of *EZH2* is undetectable in resting B cells, but rapidly increases as

cells enter the GC reaction, and falls following exit of the GC [34,35]. It has also been implicated in control of B-cell proliferation since silencing of *EZH2* leads to cell cycle arrest at the G1/S transition and expression of tumor suppressor genes including *CDKN1A*, [35]. Critically, *EZH2* was found to be a cardinal regulator of the GC phenotype by repressing the expression of known regulators of B cell differentiation such as *PRDM1* and *IRF4* through the deposition of H3K27me3 upon entry into the GC [36,37]. Furthermore, mutant *Ezh2* reinforces the formation of bivalent genes within the GC and permanently suppresses certain *Ezh2* target genes, ‘locking’ cells into the GC reaction and preventing terminal differentiation [37]. Similarly, conditional expression of mutant *Ezh2* within lymphocytes induced GC hyperplasia, albeit the mutation was insufficient to induce lymphomagenesis on its own. However, conditional expression of mutant *Ezh2* accentuated lymphoma development in Eμ-Myc and vav-Bcl2 mice, suggesting that while epigenetic mutations might be insufficient to induce lymphomagenesis in isolation, they co-operate with additional oncogenic lesions to promote malignant transformation [37,38].

In parallel with these genetic discoveries, there has been a rapid development of inhibitors targeting *EZH2* histone methyltransferase activity, which are demonstrating promising results in both *in vitro* and *in vivo* models [39,40]. Moreover, there is encouraging new data supporting a role for these inhibitors in solid cancers, where oncogenic and synthetic lethal dependencies on *EZH2* can be exploited [41–43]. At present, three compounds have proceeded to early phase clinical trials in patients; Epizyme-E7438 (NCT01897571) [44], Constellation Pharmaceuticals-CPI-1205 (NCT02395601) [45] and the GlaxoSmithKline compound GSK2816126 (NCT02082977) [46], and although it is too early to draw firm conclusions regarding efficacy, emerging Phase I data suggest a potential role in *EZH2* wild-type tumors [47].

KMT2D is a member of the KMT2 family of methyltransferases, which promote transcription and chromatin accessibility through methylation of H3K4, and have been implicated in a wide range of malignancies [48]. The majority of genetic alterations are predicted to lead to truncation of the protein and presumably repress gene expression through decreased methylation of H3K4. Recent studies support a role for KMT2D in regulating cell type-specific enhancer activity through monomethylation of H3K4 [48–50] and it is, therefore, reasonable to speculate that these inactivating *KMT2D* mutations in FL may well disrupt enhancer activation impacting on key B-cell networks. Suitable *in vitro* and *in vivo* conditional models will be required to address the question of the mechanistic role of KMT2D in FL, since homozygous deletions of *Kmt2d* are embryonically lethal [49], while heterozygous germline mutations are associated with the childhood developmental disorder Kabuki syndrome [51]. There is no compelling evidence to suggest that these patients are predisposed to lymphoma, suggesting that the most informative experiments will be those where KMT2D function is explored within the context of other recurring gene mutations.

Although research into the function of CREBBP and EP300 in normal GC B cells and FL is still in its infancy, inactivating genetic aberrations are predicted to contribute to a repressed gene expression state through decreased acetylation of H3K27 (H3K27ac) and other target proteins. Homozygous null mutations in either *Crebbp* or *Ep300*, or compound heterozygosity, result in early embryonic death [52,53]. Recent *in vitro* studies reveal that *CREBBP* mutants have reduced ability to acetylate the proteins, p53 and BCL6, with acetylation enhancing the action of p53 and repressing that of BCL6, in keeping with its presumed role as a tumor suppressor [11]. More recently, Green *et al.* demonstrated that *CREBBP*-mutated FL tumors were linked to a diminished antigen presentation gene signature and downregulation of MHC Class II surface expression on tumor cells where corresponding tumors profoundly reduced the proliferation capacity of tumor-infiltrating T cells, collectively suggesting that *CREBBP* mutations may in some way promote immune-surveillance escape mechanisms [14].

DNA methylation

While aberrant DNA methylation is a feature of all cancers, it is only in myeloid malignancies where the use of DNA methyltransferase inhibitors (DNMTi) has demonstrated efficacy [54,55]. Despite this, hypermethylation of tumor suppressor genes has been demonstrated in numerous studies including FL [56,57], with a significant overlap noted between hypermethyl-

ated CpGs and PRC2 target genes in embryonic stem cells [58]. Although initial reports suggested conservation of methylation profiles between paired pre- and post-transformation samples [58], the introduction of higher resolution platforms, suggests measurable differences in relapsed and more aggressive tumors [59,60]. Recent studies by Clozel *et al.* propose a paradigm shifting change in how epigenetic therapies may be used to greatest effect; in a study of DLBCL, low-dose DNMTi exposure was used as a means of sensitizing chemoresistant tumors to doxorubicin through increased expression of a restricted number of genes including *SMADI* and demonstrated that combination of pretreatment with 5-azacitidine followed by chemoimmunotherapy recapitulated this observation in a Phase I clinical trial [61].

Noncoding regulatory regions

Genetic studies of FL, along with the vast majority of cancer studies to date, have focused on the protein-coding region constituting less than 2% of the entire human genome. Functional noncoding elements including promoters and enhancers, located proximal or distal to protein-coding genes respectively and which regulate gene expression through transcription factor (TF) binding, have for the most part been ignored. Additionally, noncoding RNAs including miRNAs and long noncoding RNAs (lncRNAs) represent equally important new players responsible for fine-tuning gene expression alongside DNA- and histone-based epigenetic modifications. Indeed, the availability of whole genome sequencing has opened the door to a more rigorous exploration of the noncoding genome for the first time highlighted with the discovery of *TERT* promoter mutations in melanoma and other solid cancers, resulting in increased expression of the oncogenic *TERT* [62–64]. The first major effort to examine the FL noncoding genome, alongside several other B-cell lymphomas, was recently performed by Mathelier *et al.* where they focused on overlaying somatic noncoding mutations with publically available TF-binding sites and testing the expression of proximal genes in order to identify *cis*-regulatory mutations within promoters [65]. This approach led to the identification of genes with altered expression as a consequence of disrupted TF binding including several with known key roles in lymphomagenesis. Enhancer profiling by Koues *et al.* in FL demonstrated that FL tumors not only suppress specific enhancers so as to shed nonessential components of the GC transcriptional circuitry but additionally hijacked critical enhancers from other lineages to acquire specific advantageous tumorigenic functions [66]. Taken together, these recent studies highlight the contribution of the noncoding genome

and earmark the importance of this exciting new chapter in FL pathogenesis.

Conclusion & future perspective

Advances in the molecular characterization of FL have yielded important insights into the genetic basis of this malignancy, categorically placing epigenetic dysregulation at the center of germinal center lymphoma pathogenesis. While detailed functional studies are underway to decipher how each of these genetic attributes contributes independently to the development of FL, combinatorial experimental systems are essential to model the human tumors and delineate how the complex interplay between these alterations leads to lymphomagenesis and establishment of the CPC in FL. In parallel, the supporting role of the noncoding genome in gene regulation represents a new area of exploration requiring systematic integrated genomic, epigenomic and transcriptomic approaches to further enhance our understanding of the personality of this disease.

Altogether, opportunities are now presenting themselves to translate knowledge of the molecular landscape into novel epigenetically targeted therapies that directly or indirectly abrogate the epigenetic alterations in FL and this has been exemplified by early phase

clinical trials with EZH2 inhibitors. On a cautious note, as epigenetic changes can lead to broad global gene expression changes, we must carefully devise therapies that limit their effects to the malignant tumor population and restrict off-target effects. Ultimately, it is anticipated that the future clinical management of patients will move away from our current 'one size fits all' approach and towards a much more stratified strategy where we would treat patients on the basis of each of their underlying tumor's (epi)genetic signature. The ability to target the very essence of the tumor may invariably make FL a potentially curable malignancy or at least improve the outcomes for the poorer risk patients.

Financial & competing interests disclosure

J Fitzgibbon is supported by a Cancer Research UK Programme Grant (15968). S Araf is a recipient of a Cancer Research UK Clinical Research Fellowship. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Executive summary

Epigenetic landscape of follicular lymphoma

- Epigenetic dysregulation has emerged as a key feature in the pathogenesis of follicular lymphoma (FL).
- Mutations that modify histones are the most frequently mutated in FL, present in over 90% of cases.
- Clonal evolution studies suggest that these mutations occur early in lymphomagenesis.
- The mutations are predicted to result in a global pattern of gene repression and center on two key lysine residues, H3K4 and H3K27.

Histone modifications

- *EZH2* mutations occur in the catalytic SET domain (Y641, A682, A692) and are gain-of-function mutations, resulting in an increase of H3K27me₃, a repressive gene transcription mark.
- *EZH2* is a key regulator of the GC reaction and acts to repress terminal B-cell differentiation genes.
- The function of *KMT2D*, *CREBBP* and *EP300* mutations in FL has yet to be fully elucidated but are predicted to decrease H3K4 methylation and H3K27 acetylation respectively.

DNA methylation

- Tumor suppressor genes are frequently hypermethylated in FL, many of which are also targets for repression by the PRC2 complex.
- DNA methyltransferase inhibitors have been shown to sensitize chemoresistant diffuse large B-cell lymphoma to chemotherapy, highlighting the need for similar studies in FL.

Noncoding regulatory regions

- There is emerging evidence to suggest that mutations targeting transcription factor binding and enhancer dysregulation contribute to the development of FL.

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