Review Article

Genetic heterogeneity in follicular lymphoma

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Abstract: The genetic underpinnings of follicular lymphoma (FL) are now better understood through sequencing efforts of the last decade. Epigenetic deregulation, particularly through mutations in chromatin-modifying enzymes, is recognised as a pivotal hallmark that occurs alongside the t(14;18) chromosomal translocation, together with mutations in genes that affect a number of secondary biological pathways including mTORC1, JAK-STAT, NF-kB signalling and immune evasion. In recent years, the functional relevance of these genetic aberrations has been independently deciphered. The protracted nature of FL has provided an excellent model to chart the heterogeneity and evolution of the genetic features of the lymphomas both temporally and spatially. These studies have pointed to the early and late genetic drivers of the disease and the existence of a putative reservoir population that is difficult to eradicate with conventional treatment and most likely contributes to the relapsing-remitting nature of FL. Additionally, these sequencing studies have identified similarities and distinct differences in the genetic profiles of FL compared to related histological entities. In this review, we aim to summarise the current state of our understanding of the genetic landscape and heterogeneity, its contribution to the spectrum of clinical phenotypes in FL and related entities and finally, the ongoing efforts to utilise biology to provide lines of sight to the clinic.

Keywords: Follicular lymphoma (FL); heterogeneity; genetics; epigenetics; clonal evolution

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Introduction

Follicular lymphoma (FL) is driven by the accumulation of clonal neoplastic cells that closely mimic several phenotypic and functional attributes of their normal counterparts, germinal centre (GC) B-cells. There is a recognised heterogeneity in both clinical phenotype and disease outcome. On one end of the scale, there are patients with early-stage disease, half of which have long durable remissions with therapy, those that can be managed expectantly for many years and a rare minority with spontaneous remission. At the other end, there are patients who follow the prototypical relapsing-remitting disease course and additional high risk cohorts that progress rapidly within 2 years (POD24) (1,2) or histologically transform to high grade lymphoma, usually diffuse large B-cell lymphoma (DLBCL) (3). Whilst the introduction of anti-CD20 monoclonal antibodies, such as rituximab since the late 1990s, has significantly improved prognosis in FL, there is a recognition that high-risk patients who progress during or after therapy and those that undergo transformation require a different and perhaps more aggressive treatment strategy (2). Hypothetically, accurate identification of these high-risk subsets at diagnosis could enable risk-adapted therapeutic intervention, providing the most effective, targeted therapies to high-risk patients whilst also identifying those with low-risk clinical phenotypes might mean that therapy could be de-escalated to avoid over-treatment in a similar vein to the strategies employed in Hodgkin's lymphoma. Current prognostic tools, based on clinical information (4,5)

and those that incorporate mutations or gene expression data (6,7), can stratify patients into different risk groups but have not demonstrated a high enough prognostic accuracy to be routinely implemented in clinical practice (8,9).

This variability in clinical trajectories has perhaps hinted for decades at the underlying biological heterogeneity. The advent of next generation sequencing (NGS) has enabled more powerful elucidation of the breadth of accumulating genetic events that occur alongside the pathognomonic t(14;18) translocation as well as a better understanding of the extent of the genetic diversity, not only among FL from different patients (inter-tumour heterogeneity) but also within individual patient's tumours (intra-tumour heterogeneity). This added insight at the intra-tumoral level permits the tracing of the lymphoma's life history and patterns of clonal evolution.

This review will focus on the recent developments in our understanding of the mutational landscape and how it contributes to FL pathogenesis and the genetic complexities and tumour dynamics in FL uncovered by spatial and temporal genomic profiling with NGS-based technologies. We also discuss the potential and promise of these insights in terms of patient stratification and targeted therapeutic strategies.

Pre-NGS era: t(14;18) and broad genomic changes

The t(14;18) somatic translocation (10) is considered an early genetic event, occurring in approximately 85% of patients (11) and leads to BCL2 overexpression by bringing the anti-apoptotic BCL2 protein under the control of the immunoglobulin heavy chain (IGH) enhancer. In the majority of cases, these translocations occur at the major breakpoint region (mbr), although other breakpoint sites such as the minor cluster region (mcr) downstream of the mbr, the intermediate cluster region (icr), 5'mcr and 3'BCL2 are also observed at lower frequencies (12-17). Evidence over the years demonstrate that the t(14;18) translocation alone is insufficient for malignant transformation such as the 10–15% cohort of patients with t(14;18)-negative FL and the detection of low levels of t(14;18)-positivity reported in the peripheral blood of healthy individuals (18-20). Notably much elevated levels of circulating t(14;18)-positive B-cells may represent a reservoir of pre-malignant FL precursors as these individuals have a higher propensity of developing overt FL (21). Whilst the t(14;18) translocation is clearly a critical early event in conferring risk and initiating lymphomagenesis, further genetic events are required for the development of overt FL.

Genomic copy-number heterogeneity occurs extensively within FL tumours. These were initially identified by lower resolution approaches including cytogenetics, array CGH and DNA microarrays which led to the identification of recurrent copy number aberrations (CNAs) and copy neutral loss of heterozygosity (cnLOH) in FL. Commonly observed CNAs include gains in 1q, 2p, 7, 8, 12q, 18q, X and deletions of 1p36, 6q, 10q, 13p, 17p (22-26). Due to the large genomic regions encompassed by these chromosomal changes, it was not always possible to identify the precise target genes affected within these regions that contributed to FL pathogenesis. Some exceptions include the identification of *TNFRSF14* within the frequently deleted 1p36 region (27), *EPHA7* uncovered as one of the tumour suppressor genes within the commonly deleted 6q region (28) and the amplification of *REL* and *MYC* oncogenes within the 2p and 8q24 regions, respectively (22,24,25). The degree of chromosomal and CN heterogeneity varies between patients, inter-tumour heterogeneity, as well as between diagnostic, relapsed and transformed biopsies within the same patient, intra-tumour heterogeneity. Cytogenetic analysis of paired FL and transformed FL (tFL) showed increased complexity of genomic aberrations associated with transformation (22,24,25). Although some specific CNAs have been associated with inferior prognosis [such as 1p36 and 6q (23,25)] and/or increased risk of transformation [such as 3q27, 9p21, 11 and 15q (22)], there has been considerable variability and lack of reproducibility across studies due to the heterogeneous cohorts studied across different treatment eras. As CNAs in FL can disrupt hundreds of genes compared to single gene mutations, they likely contribute significantly to the genomic instability that acts as a fuel for tumour evolution and the many alternate evolutionary trajectories. However, CNAs represent only one of the factors that contribute to FL genomic heterogeneity, its initiation and tumour evolution.

Inter-tumour heterogeneity—mutational landscape of FL

NGS has been instrumental in the last decade in providing a finalised mutational catalogue of coding genes in FL. A number of biological pathways including epigenetic regulation and key signalling networks are dysregulated by the acquisition of recurrent gene mutations in FL. *Figure 1* provides a summary of the main biological pathways

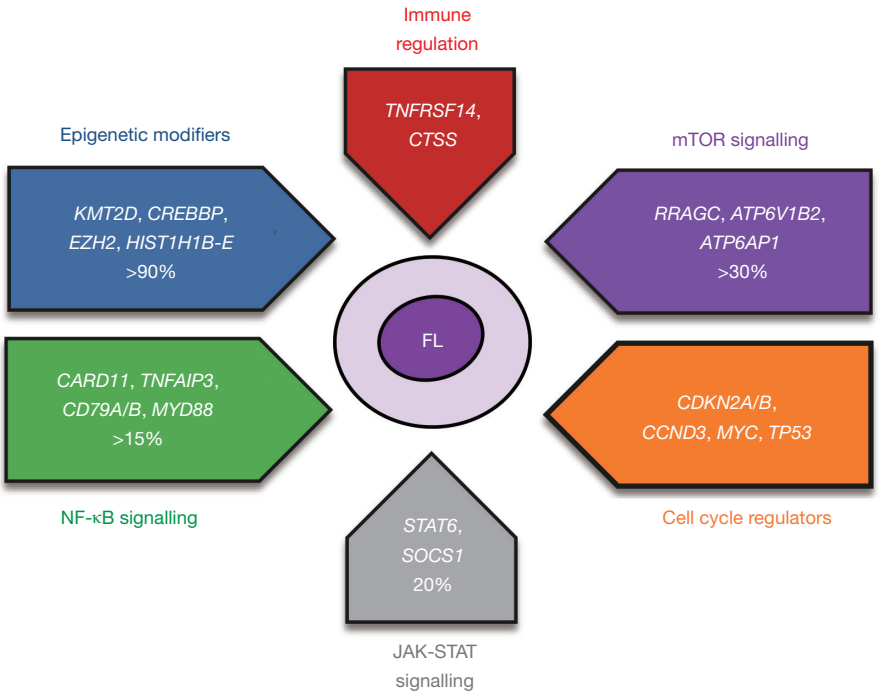

Figure 1 Main biological pathways affected in FL. Frequent genetic alterations in FL can affect several pathways in FL. Each FL tumour can harbour several gene mutations/alterations within the same and across multiple pathways. FL, follicular lymphoma.

affected in FL.

Mutations in epigenetic regulators—a defining hallmark of FL

Epigenetic regulatory mechanisms are commonly hijacked in tumorigenesis as they form an important part of a carefully choreographed gene regulation circuitry that permits the required sets of genes to be switched 'on' and 'off' within a particular cell at a specific time. A key functional unit of this circuitry, chromatin, a complex of DNA and histone proteins, exists in two main states: condensed transcriptionally repressed heterochromatin and transcriptionally active euchromatin. Epigenetic regulation between these two chromatin states occurs through a number of mechanisms including DNA methylation, histone post-translational modifications (PTMs) and chromatin remodelling.

A rather surprising finding from early NGS studies in FL was the high prevalence of mutations in epigenetic regulators, particularly those involved in histone PTMs. Approximately 90% of FLs carry one or more mutations in genes involved in epigenetic regulation through histone modifications (*KMT2D*, *CREBBP* and *EZH2*) and chromatin remodelling (*HIST1H1B-E*, *ARID1A*) (29).

KMT2D (also known as *MLL2*), a lysine-specific histone (H3K4) methyltransferase, is the most common histone-modifying enzyme mutated in FL, occurring in approximately 60–80% of patients. The mutations are typically inactivating in nature, leading to loss of the protein. Loss of *KMT2D* in conditional knock-out mouse models lead to a reduction in global H3K4 methylation, promoting a block at the GC stage of development and enhanced tumour suppressor gene expression (30,31).

Inactivating mutations in the histone acetyltransferase (HAT) enzymes *CREBBP* and, less commonly, *EP300* (a structural paralog to *CREBBP*) occur in up to 70% and 15% of cases, respectively. The mutations affecting *CREBBP* are either missense (mostly clustered within the catalytic HAT domain), truncating or deleterious in nature. CREBBP and EP300 regulate gene expression by catalysing the acetylation of lysine residues in both histone and non-histone proteins. Transcriptionally, *CREBBP*-mutant mice and human tumours display focal depletion of H3K27 acetylation at gene enhancers central to GC development, B-cell receptor signalling and antigen presentation (29,32,33). Decreased MHC II expression resulting from loss-of-function *CREBBP* mutations limits antigen presentation by tumour B-cells with reduction in infiltrating T-cells, altogether

promoting immune evasion (29). Notably, *CREBBP* mutations affecting the HAT domain confer a more severe functional phenotype (34). Functional genetic screens uncovered a synthetic lethal relationship between CREBBP and EP300, with *CREBBP*-mutant cancer cell lines showing a dependency on EP300 for their growth and survival by downregulating MYC expression (35,36).

The SET-domain histone methyltransferase, EZH2, is the enzymatic component of the polycomb repressor complex 2 (PRC2) and silences gene transcription by trimethylating histone 3 lysine 27 (H3K27me3). *EZH2* mutations occur in approximately 20–25% of FL patients, with the majority of mutations clustered at 3 hotspot amino acid residues, Y641, A682 and A692 in the SET domain (37,38). The gain-of-function *EZH2* mutations are heterozygous and promote an increase in H3K27 trimethylation. EZH2 appears essential for normal GC formation with mutant EZH2 regulating the GC phenotype-by suppressing cell cycle checkpoint genes such as *CDKN1A* and transcription factors that prevent GC exit such as *IRF4* (39). Interestingly, recent studies have found *EZH2* mutations also alter the tumour microenvironment (TME) by reducing tumour dependence on T-follicular helper (T_{FH}) cells whilst shifting to follicular dendritic cell (FDC) interactions. By remodelling interactions with the microenvironment, B-cells no longer need to compete for T_{FH}-dependent stimulation (which normally limits B-cell proliferation), enabling the large numbers of malignant-cells to persist in the GC (40). In DLBCL, MHC-I and MHC-II negative lymphomas are also strongly enriched for *EZH2* mutations (41), supporting the additional role of *EZH2* aberrations in immune evasion mechanisms.

In addition to mutations in histone-modifying genes, genes encoding components of chromatin remodelling complexes are also a feature of FL. Isoforms of the linker histones (*HIST1H1B-E*; also, H1B-E) are mutated in over 30% of FL tumours (42-44). These are predominantly heterozygous missense mutations clustered within the highly conserved globular domain, with the majority affecting the *H1C* and *H1E* isoforms. In normal cellular processes, these linker histones facilitate the folding of higher-order chromatin structures and regulate access of histone-modifying enzymes and chromatin remodelling complexes to their target genes (45). Aberrant *H1C* and *H1E* genes contribute to epigenetic reprogramming and gene silencing by impairing chromatin compaction and the 3D genome organisation thereby establishing H1 genes as tumour suppressors (42).

Most studies indicate that the mutations in epigenetic regulators, particularly *CREBBP* and *KMT2D* have high variant allelic fractions (VAFs) implying these mutations represent clonal genetic events. More remarkable is the co-existence of multiple 'epimutations' within a single FL tumour, at least 50% of cases harbour both *KMT2D* and *CREBBP* mutations, highlighting the importance of the convergence on H3K4 and H3K27, along the histone tail. The implications of this epigenetic intra-tumour heterogeneity and its phenotypic consequences have yet to be fully elucidated, although the nature of the histone marks suggests that the overall transcriptional effect is tipped preferentially towards a repressive gene expression state.

Mutations in immune modulators

Outside of epigenetic dysregulation, immune modulation is a mechanism frequently employed by tumours to evade the host's natural immune responses. TNFRSF14 (also known as HVEM) is a bidirectional signalling molecule that interacts with its ligand, B and T lymphocyte attenuator (BTLA), to modulate T-cell activation. Mutations in *TNFRSF14* occur in approximately 40% FLs, with the majority leading to loss of HVEM expression through gene deletions or truncations (27). *TNFRSF14* aberrations disrupt the binding to its signalling partner, BTLA, and contributes to the generation of a tumour-supportive TME by increasing cytokines that promote T_{FH} infiltration and activation of the tumour stroma (46-48). *TNFRSF14* alterations have been linked with clinical outcome and increased GvHD risk following allogeneic stem cell transplant, although the data is somewhat conflicting and requires validation in larger series (27,47).

CTSS (Cathepsin S) encodes for a cysteine protease involved in MHC-II antigen presentation by antigen presenting cells (APCs) and malignant B-cells, regulating proteolytic cleavage of antigenic peptides and CD74. Mutations in *CTSS* occur in around 6% FLs, mainly by Y132D mutation resulting in *CTSS* overactivation, whilst *CTSS* overexpression is found in approximately 13% FLs (49,50). Enhanced *CTSS* activation in lymphomas increases antigen specific $CD4^+$ T-cell activation and infiltration to garner tumour support and promote immune evasion through the exclusion of cytotoxic $CD8^+$ T-cells (49,50). *CTSS* mutations and overexpression, which are mutually exclusive, also seem favourable when in patients treated with immunotherapies (50). Interestingly, *CTSS* Y132D mutations are mutually exclusive with *TNFRSF14* and *RRAGC* (see

below) mutations which alter $CD4^+$ T-cell interactions (49).

Aberrations in mTORC1 signalling

Mutations converging on components of the amino-acid sensing arm of the mTORC1 signalling pathway have recently been reported (29,51,52). Recurrent mutations in the gene *RRAGC*, that encodes a Ras-related GTP-binding protein, Rag C, are enriched in FL, occurring in approximately 10–15% of patients, rarely present in other B-cell lymphomas. *In vitro*, *RRAGC* mutants can constitutively activate mTORC1 signalling even in amino acid deprived conditions suggesting that the mutant tumours have the capability of bypassing the normal metabolic checkpoint (51). *RRAGC*-mutant mice also have decreased tumour dependence on the microenvironment (53). Notably, *RRAGC* mutations frequently co-occur with mutations in *ATP6V1B2* and *ATP6AP1*, subunits of the vacuolar ATPase proton pump, v-ATPase, a multimeric complex also needed for mTORC1 signalling (51). Another observation is the mutual exclusivity of *RRAGC* mutations with deletions in the gene *Sestrin1* that encodes an upstream negative regulator of mTORC1. In aggregate, there are multiple genetic mechanisms that converge on mTORC1 signalling promoting aberrant metabolic reprogramming (54), emphasising its significance in FL pathogenesis.

Mutations in other signalling pathway components

Components of both the NF-κB and JAK-STAT pathway are subject to recurrent mutations in FL (43). Constitutive activation of the anti-apoptotic NF-κB signalling pathway, caused by mutations affecting positive and negative regulators, is an established feature of a number of B-cell lymphomas, particularly activated B-cell DLBCL (ABC-DLBCL). *CARD11* encodes a key scaffolding protein in the CBM (CARD11-BCL10-MALT1) signalosome complex, which promotes NF-κB activation upon antigen receptor ligation in B-cells and is mutated in just over 10% of cases. *TNFAIP3*, encoding the enzyme A20 that acts as a negative regulator of canonical NF-κB signalling, is mutated at a similar frequency. *CARD11* mutations occur within the coiled-coil domain and are activating aberrations (55), whilst the majority of the *TNFAIP3* mutations are inactivating (56), usually combined with deletions of the second allele. Mutations in other components of the BCR-NF-κB signalling pathways including *CD79A*, *CD79B* and *MYD88* are infrequently mutated in FL (52) compared to ABC-DLBCL or the newly recognised MCD/C5 DLBCL molecular subtypes (57,58).

In addition, mutations in the JAK-STAT signalling pathway, that is perhaps more synonymous with other lymphoma subtypes like Hodgkin lymphoma and primary mediastinal B-cell lymphoma are also recurrent in FL, leading to constitutive activation of the pathway and promoting B-cell survival (59). Activating mutations in *STAT6* and inactivating mutations in *SOCS1*, a negative regulator of JAK-STAT signalling each occur in approximately 10% of FL cases. The activating *STAT6* mutations induces a number of STAT6 target genes with the effects more pronounced in the presence of IL-4, a cytokine that independently drives STAT6 responsive genes, suggesting that both the mutations and the cytokine-driven signals from the microenvironment could activate the IL4-JAK-STAT axis supporting proliferation and survival of tumour cells (59).

Alterations affecting genes involved in proliferation and cell cycle regulation

Tumour cells co-opt oncogenes and circumvent tumour suppressors in order to proliferate uncontrollably and self-autonomously. In FL, several genes involved in the regulatory mechanisms of these processes are altered to give the tumour population a proliferative and survival advantage and are especially enriched at relapse and/or transformation (22,51,60-62). The proto-oncogene, *MYC*, can be mutated, amplified, or translocated in FL. Mutations and deletions of the genomic loci encompassing *TP53*, a tumour suppressor gene, occur in about 10–15% of FL, typically associated with adverse outcome. As alluded to earlier, certain cell cycle components and their regulators are recurrently perturbed by CNAs. The genomic region 12q13–15 is subject to frequent copy number gains and unsurprisingly this region encompasses several cell cycle regulatory components including MDM2, a ubiquitin-protein ligase that degrades TP53 and cyclin-dependent kinases (CDKs), CDK2 and CDK4, essential for G1/S transition of the cell cycle. CDK activity is negatively regulated by the inhibitor CDKN2A/p16, preventing their interaction with cyclin D and subsequent phosphorylation of the retinoblastoma (RB) proteins. The gene locus of CDKN2A/p16 has recurrent heterozygous and homozygous deletions, suggesting the removal of its inhibitory effect allows the potentially uncontrolled phosphorylation of RB proteins leading to the release of E2F transcription factors and continuous cell cycle progression. Cyclin D3 (*CCND3*), which encodes a

binding partner of the CDKs, is mutated in about 5–10% of FL cases, co-occurring with CDK4 amplifications. Collectively, these alterations mostly occur in a mutually exclusive manner (63) and the perturbation of this set of genes converge on both the p53 and RB axis, decreasing their activity, deregulating the cell cycle and ultimately promoting tumour proliferation and survival.

In summary, there is a much clearer picture of the genetic, especially the mutational, landscape of FL and how these gene mutations deregulate specific biological pathways relevant for its pathogenesis. Whilst we have a better idea as to the degree of FL interpatient genetic heterogeneity, this has primarily been derived from, at times, small single centre studies on patient samples from a range of clinical phenotypes, as well as different modalities in genetic profiling from targeted gene panel to whole genome sequencing. From these studies, there are early insights into patterns of genetic co-dependencies and mutual exclusivity, although we are unaware if there are distinct genetic subtypes, as has been identified for example in DLBCL (57,58,64) which might explain the different FL clinical phenotypes. To address this, there is a need for much larger scale, statistically powered studies (65), to not only understand the contemporary pre-treatment genetic profiles but also allow us to capture underlying genetic subtypes and define how this may in turn influence disease evolution and clinical outcome.

Intra-tumour heterogeneity—temporal and spatial

There is increased recognition that tumours within individual patients consist of multiple genetically distinct subclones and that this intra-patient or intra-tumour heterogeneity can indeed act as the substrate for (sub)clonal evolution, treatment resistance and disease progression (66). The extent of this heterogeneity has really come into prominence by genetic profiling of tumours both temporally (where tumours at different clinical time points from an individual are examined, for example diagnostic versus relapse) and also spatially (tumours from different sites of disease at approximately the same time point).

In FL, temporal genetic analysis of sequential tumour samples (at diagnosis, relapse and/or transformed disease) have identified the patterns of clonal evolution (29,43,60-62). These studies demonstrated that the predominant pattern of clonal evolution was for relapsed or tFL tumours to arise via branched divergent clonal evolution. Here, the key observation also was that by using the genetic data to reconstruct the clonal phylogenies, sequential tumours appear to arise and diverge from a presumed ancestral population referred to as the common progenitor cell (CPC) that is shared across all the tumours from the same individual. The persistence of this ancestral CPC population through the patient's clinical journey implies that this population is difficult to eradicate completely, especially with patients with recurrent disease (*Figure 2*). Further supporting evidence of the existence of a long-lived ancestral CPC population comes from two distinct cases of donor-derived FL (67,68). Here, both the donor and recipient develop FL many years after an allogeneic stem cell transplant with the tumours shown to be clonally related with identical t(14;18) translocations and other genetic events implying that an ancestral CPC must have been seeded at the time of the transplant and can exist for several years prior to overt FL development. Precise analyses of the genetic composition of temporally profiled tumours have brought into focus the early, initiating genetic events that must reside within this CPC population versus progression- or transformation-specific genetic aberrations. Mutations in the epigenetic regulators (*CREBBP* and *KMT2D*) together with the t(14;18) translocations represent initiating genetic events that drive lymphomagenesis, that must occur within the ancestral population and are predominantly conserved during the progression of the disease. Notably, some patients exhibited different mutations in *CREBBP* and *KMT2D* between FL and tFL, signifying convergent evolution and the importance of these mutations in lymphomagenesis (43,60).

There is also an emphasis to understand the latter genetic events that contribute to progression and transformation. Early progression of FL after conventional chemotherapy and transformation to DLBCL (tFL) are both associated with inferior outcomes and represent one of the major causes of patient deaths from their lymphoma (2,3,69). Early progressed FLs appear to arise from an expansion of pre-existing subclones already present at diagnosis, indicating these subclones were to a degree resistant to initial therapy (61). Mutations in a number of genes including *TP53*, *SOCS1*, *B2M* and *MYD88* were enriched in the diagnostic tumours of early progressed patients. Unsurprisingly, the genetic drivers of transformation are broad and heterogeneous with overlaps in gene mutations identified in both diagnostic and early progressed tumours, albeit seen at lower frequencies in pre-transformed biopsies. In addition to mutations in epigenetic

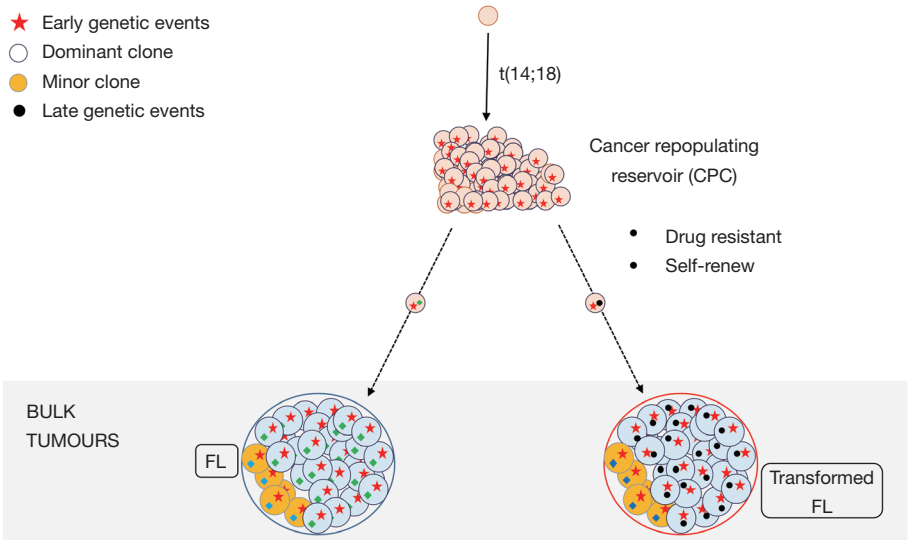

Figure 2 Origins and clonal evolution of FL. This schema highlights the acquisition of initiating genetic events that generates the reservoir CPC population and the divergent evolution with the accumulation of 'progressor' events leading to overt FL or tFL. FL, follicular lymphoma; CPC, common progenitor cell; tFL, transformed FL.

regulators and the t(14;18) translocation that serve as early drivers in putative CPC populations, there are increased aberrant somatic hypermutation and aberrations in genes involved with cell cycle progression, proliferation and DNA damage response such as *TP53*, *MYC*, *CDKN2A* and *REL* in transformed samples (29,43,60,61). Increased immune escape mechanisms are also a feature of transformed disease with more frequent *B2M* mutations and deletions in parallel occurring with a reduced $CD8^+$ T-cell infiltrate (61). The majority of tFLs retain a GCB-cell-of-origin gene expression signature however approximately 16% are classified as ABC-like (70). Interestingly, ABC-classified tFLs commonly evolve from t(14;18)-negative FL tumours and are associated with acquisition of NF-κβ mutations that are normally over-represented in ABC-DLBCLs (70). This coincides with a study demonstrating that an NF-κβ gene expression signature was associated with increased risk of transformation (71). This perhaps signals that NF-κβ biology may cultivate a more aggressive, fitter, subclonal phenotype in FL, as in DLBCL. The difference in GCB-like versus ABC-like tFLs did not appear to impact overall survival; although bearing in mind the caveats of the small heterogeneously-treated cohorts (70). Collectively, these longitudinal studies highlight the absence of a single (epi)genetic driver but instead, multifactorial genetic mechanisms enriched at transformation that contribute to the outgrowth and survival of more genomically complex subclones.

Spatial intra-tumour heterogeneity has been much less studied in FL but well reported in solid tumours (66). The majority of FL patients present with disease in multiple lymph nodes and other extra-nodal sites such as the bone marrow. By exome sequencing tumours from different sites of disease (spatially separated), there are expectedly varying degrees of spatial intra-tumour heterogeneity in FL (72). The mutations in *CREBBP* and *KMT2D* together with the t(14;18) translocation occur concordantly across spatially separated biopsies further emphasising their driver status in FL. However, the existence of inter-site heterogeneity is reminiscent of findings in other cancers and has important clinical implications for future biomarker-led therapeutic strategies. This is exemplified in a case that harbours an *EZH2* mutation in a larger proportion of the tumour population in the lymph node compared to a small subclonal fraction in the bone marrow. Hypothetically, if this patient were treated with an EZH2 inhibitor such as tazemetostat, one might predict differential clearance of the tumour population at the two different sites of disease. There is a suggestion that this spatial heterogeneity increases at transformation (72). Recently, transcriptional heterogeneity across spatially-separated lymph nodes at the single cell level in FL patients has been demonstrated (73), suggesting that genetic diversity is just one of a multitude of layers that contribute to intra-tumour heterogeneity and that a single biopsy

oversimplifies the molecular complexities of patients' tumours.

Analysis of cell-free tumour DNA (ctDNA), fragments released into the blood, a means of liquid biopsy, may capture and provide a better representation of the patient's intra-tumoral heterogeneity. Liquid biopsy analyses has been best studied in DLBCL with ctDNA able to capture the mutational landscape and clonal evolution as well as demonstrating prognostic relevance, with pre-treatment and interim ctDNA levels associated with outcome (74,75). Studies in FL are emerging with Delfau-Lareu and colleagues showing that ctDNA levels correlated with tumour burden and prognosis (76). Of even greater interest is if ctDNA has the utility to predict FL transformation. Scherer and colleagues showed, using CAPP-seq, that genetic events associated with transformation could be detected in the diagnostic ctDNA time point that was several months earlier (77). This minimally-invasive modality might offer the opportunity to capture heterogeneity whilst dynamically monitoring disease response to treatment and the ability to forecast progression. Further evaluations in this area are eagerly awaited.

Genetic heterogeneity between other FL-related entities

While it is clear that FL, tFL and DLBCL genetically overlap, an understanding of the trajectory from a normal B-cell to overt malignancy has been further improved by insights from a number of closely related entities, some recently described in the WHO classification 2016 update, with distinct clinico-pathological patterns compared to classical FL (78). This histological spectrum ranges from putative pre-malignant lesions like *in situ* follicular neoplasia (ISFN) to established malignancies such as t(14;18)-negative FL, paediatric-type FL (PTFL) and duodenal FL (DFL) (*Table 1*).

ISFN and partial involvement by FL (PFL)

ISFN, first identified in 2002, represents hyperplastic GCs colonised by $CD10^+$ $BCL2^+$ non-neoplastic B-cells (78-80). The true incidence of ISFN is unclear as they are typically detected inadvertently following biopsies for suspicious lymphadenopathy. It is reported to occur in approximately 2% of all individuals in which lymph nodes are removed for reasons other than lymphoma diagnostics (81). The risk of progression to overt classical FL is low (<5%) suggesting these presumed precursor lesions are yet to have acquired the full complement of genetic changes for lymphomagenesis. Unlike ISFN, PFL more closely resembles overt FL with altered lymph node architecture and higher rates of progression, possibly representing a more advanced pre-malignant stage (80,82). However half of patients with PFL still do not undergo malignant transformation (80,82,83). Notably, common genetic alterations seen in overt FL including mutations in *EZH2*, *KMT2D* and *TNFRSF14*, were identified in both ISFN and PFL, further supporting evidence for their early driver status (84). The degree of genomic complexity (CNAs and gene mutations) increased comparatively from ISFN to PFL to overt FL, suggesting a potential evolutionary hierarchy within these entities toward FL development (84).

T(14;18)-negative FL

T(14;18)-negative FL represents 10–15% of FLs and is more prevalent in early stage FL, which has a superior prognosis (85). Although lacking the BCL2 translocation, t(14;18)-negative FL patients exhibit similar clinical features to their t(14;18)-positive counterparts and the majority still express BCL2 protein (86,87). Whilst t(14;18)-negative FLs share much of the CNA profile of conventional FL, these occur at lower frequencies (85). Interestingly, the gene expression profile in t(14;18)-negative FLs are more reminiscent of ABC-like B-cell tumours with a particular enrichment for NF-κB signatures (87) and perhaps explains our earlier description that ABC-like tFLs more often evolve from a preceding t(14;18)-negative FL. *STAT6* mutations are also more prevalent compared to conventional FL (57% *vs.* 12%) (85) and frequently co-occur with *CREBBP* or *TNFRSF14* mutations, emphasising the alternative oncogenic potential even in the absence of the BCL2 translocation. There is no suggestion that t(14;18)-negative FLs need to be managed any differently to conventional FL.

DFL

DFL is a recognised variant anatomically restricted to the duodenum that follows a relatively benign clinical course and is morphologically similar to FL. Genetically, DFLs harbour mutations in genes seen in classical FL including *TNFRSF14*, *EZH2* and *CREBBP*, however have a significantly lower frequency of *KMT2D* mutations and extremely rare progression to overt FL (84,88-91). Unlike overt FL where activation-induced cytidine deaminase (AID) is highly expressed, consistent with its GC origin, DFLs rarely express AID—hinting DFLs originate from

Table 1 Pathological, genetic and clinical features of FL compared with related entities

Features	Classical FL	t(14;18)$^{-}$ FL	ISFN	PFL	DFL	PTFL
Disease course	Indolent with relapse-remitting course, high risk subsets progress rapidly and/or histologically transform	Enriched in early-stage compared to advanced-stage FLs	FL precursor lesion, low rate of progression to FL (<5%)	FL precursor lesion, higher rate of progression to FL (approx. 50%)	Low risk of dissemination, very low risk of progression to FL or histological transformation	Curable, low rates of recurrence
GC phenotype	GC B cell	Late GC/NF-κB	BCL2+ B cells in the GC	BCL2+ B cells in the GC	Non-GC B cell	BCL6+ B cells
Histology	Follicular proliferation of GC B cells	Reduced CD10 expression	BCL2+ cells colonising GC (GC restricted)	Greater architectural distortion, more similar histology to FL	Typical morphology of low-grade FL, restricted to intestinal mucosa (commonly the duodenum)	Localized lymphadenopathy in the head and neck with high-grade FL histological presentation
Age	Older adult	Older adult	Older adult	Older adult	Older adult	Child, young adult
M:F	M = F	M = F	M = F	M = F	M = F	M > F (10:1)
Grade at diagnosis	Grade 1/2	Grade 1/2	Low-stage disease	Low-stage disease	Low-stage disease	Grade 3
BCL2 status	BCL2-IGH rearrangement	BCL2-IGH negative but most express BCL2	BCL2-IGH rearrangement	BCL2-IGH rearrangement	BCL2-IGH rearrangement	BCL2 negative
Genetic events	t(14;18) translocation, frequent mutations in epigenetic modifiers, immune modulators, MTOR pathway, cell signalling pathways etc.	Similar to classical FL, enriched in proliferation and post-GC B-cell signatures	Similar to classical FL but at lower frequencies	Similar to classical FL, closest pre-cursor entity to FL	Similar to classic FL but fewer KMT2D mutations, chronic inflammation signature of TME	Enriched in TNFRSF14 and MAPK pathway mutations, lack mutations in epigenetic modifiers
Copy number alterations	Recurrent copy number gains and loses	Similar to classical FL	Similar CNAs to classical FL but at lower frequencies	Similar CNAs to classical FL, at closer frequencies to FL than ISFN	Recurrent deletion of chromosome 1p (TNFR family)	Low genomic complexity: very few CNAs

FL, follicular lymphoma; ISFN, *in situ* follicular neoplasia; PFL, partial involvement by FL; DFL, duodenal FL; PTFL, paediatric-type FL; GC, germinal centre; IGH, immunoglobulin heavy chain; TME, tumour microenvironment; CNA, copy number aberration.

a non-GC B-cell that migrated to the duodenum (89). A particularly differentiating feature of DFL is their distinct immune microenvironment compared to classical FL, with a gene expression signature of chronic inflammation that may also contribute to the clinical and anatomical differences between the other FL entities (91).

PTFL

PTFL is a variant of FL presenting as localized lymphadenopathy in the head and neck with a male preponderance that occurs mostly in children but also in adults. PTFLs lack the t(14;18) signature lesion seen in classical FL but have a surprisingly high proliferation index (>30%) for a disease with a typically excellent prognosis and low rates of recurrence (92). The genomic complexity of PTFLs is low with few CNA, an enrichment in mutations in *TNFRSF14* and genes involved in the MAPK pathway (such as *MAP2K1*, as high as 40%) but a paucity of mutations in epigenetic regulators (93,94), altogether supporting different routes of disease initiation compared to both classical and t(14;18)-negative FL.

Translational relevance of FL genetics

We now stand at a crossroads with the deluge of genomic information in FL and how best to prioritise and meaningfully translate this knowledge into patient benefit. Given the areas of unmet need in FL, potential avenues include development of targeted therapies directed to aberrant genomic profiles and improved biomarkers for risk stratification, disease monitoring and therapeutic response.

New therapeutic targets

As mutations in the epigenetic machinery are frequent and early driver events, there is much focus in developing therapeutic opportunities to reverse the impact of these mutations. The gain-of-function nature of *EZH2* mutations have made them particularly attractive targets with several direct small-molecule *EZH2* inhibitors already developed and being evaluated in clinical trials, including tazmetostat (95). In a phase II study of relapsed/refractory FL treated with tazmetostat, patients with *EZH2*-mutant lymphomas had superior overall response (96) compared to EZH2 wild-type. Interestingly, tazmetostat also rescues MHC expression and restores T-cell infiltration in *EZH2*-mutant-cell lines and mouse models (41), abrogating the immune evasion effects of the mutation. This could open the door to evaluating combinations of EZH2 inhibition with immunotherapies to enhance immune recognition and synergistically potentiate the efficacy of these therapies.

Potential therapeutics specifically targeting *CREBBP* mutant lymphomas are gathering momentum. *CREBBP* loss-of-function in FL leads to HDAC3-mediated suppression of gene enhancers. Although, pan-HDAC inhibitors have shown limited activity in B-cell lymphomas in early-phase studies (97,98), selective HDAC3-inhibitors may offer a more direct approach to counteracting *CREBBP* mutations (32,34,99). The synthetic lethality between *CREBBP* and *EP300* may also be exploited as *CREBBP*-mutant cell lines showed more susceptibility to deletion of *EP300* or pharmacological inhibition with HAT or bromodomain inhibitors (35,36). CCS1477, a first-in-class small molecule inhibitor of the p300/CBP conserved bromodomain (100) is currently being evaluated in early phase clinical trials in haematological malignancies.

Genomics informing predictive and prognostic markers

Identifying patients with high-risk FL is an area of unmet clinical need. Historically clinical information has been the bedrock for prognostic tools, although did not influence treatment decisions (4,5). Genomic information is now being integrated into prognostic tools such as the m7-FLIPI index, which assesses the mutation status of seven genes (*EZH2*, *ARID1A*, *MEF2B*, *EP300*, *FOXO1*, *CREBBP*, *CARD11*) together with clinical characteristics to risk stratify patients (101). Whilst the prognostic accuracy of the m7-FLIPI model may be treatment-dependent and not necessarily capture all high-risk patients, suggesting that different mutations may be implicated in response and resistance to different therapies. Nonetheless, it is an important stepping-stone to incorporating molecular parameters into risk stratification tools (8,102). A more recent prognostic iteration developed by Huet and colleagues (7) uses the expression of 23 genes, encompassing B-cell biology and the TME, to identify patients at increased risk of progression.

With increasing therapeutic options in FL in the first line and relapsed settings, defining biological predictors of both therapeutic response and resistance will be invaluable to select therapy. *EZH2* mutations already serve as a good predictive biomarker of response to the EZH2 inhibitor, tazemetostat. A recent, retrospective analysis of the phase III GALLIUM trial (96) identified a predictive link between

EZH2 mutations, and the chemotherapy backbone used to treat FL patients in the first line setting. *EZH2*-mutated FL patients had better clinical outcomes with a CHOP/CVP backbone compared to *EZH2* wild-type patients, irrespective of anti-CD20 therapy, suggesting that genetic mutations could also influence how we use conventional therapies, although this needs further evaluation (102). One anticipates that we will start to see many such studies to identify appropriate and accurate predictive biomarkers of response to conventional and novel therapies including immunotherapies such as CAR-T.

Future perspective and conclusions

The last decade has illuminated the breadth of genomic complexity and an appreciation of the heterogeneity in FL that a single biopsy, whilst informative, inadequately captures. The spatial and temporal heterogeneity may undermine accurate prognostication and impact on mutation-specific treatments. The residual CPC population potentiates the recurrent relapse-remitting nature of FL and could represent the Achilles' heel of these tumours. It is unclear if such CPC cells reside within the minimal residual disease (MRD) population that persist after treatment and the next focus is to understand the nature of the FL CPC: what are its characteristics, is there heterogeneity within this CPC and does this relate to the clinical phenotype and evolution of the disease.

Each FL tumour is a compendium of several genetically distinct subclones dependent on different epigenetic and signalling pathways, therefore therapeutically targeting a single genetic aberration is unlikely to be a successful long-term strategy in every patient as it would enhance subclonal competition and promote the outgrowth of resistant clones. Although the genetic and epigenetic signatures and heterogeneity are key drivers of this disease, other components such as the TME also play a supporting role. Combinatorial therapies targeting multiple tumour vulnerabilities coupled with means of measuring the response and clonal dynamics, for example with ctDNA assays, may prove to be the most effective strategy.

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