Early lesions of follicular lymphoma: a genetic perspective

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ABSTRACT

The pathogenesis of follicular lymphoma is a multi-hit process progressing over many years through the accumulation of numerous genetic alterations. Besides the hallmark t(14;18), it is still unclear which other oncogenic hits contribute to the early steps of transformation and in which precursor stages these occur. To address this issue, we performed high-resolution comparative genomic hybridization microarrays on laser-capture micro-dissected cases of follicular lymphoma *in situ* (n=4), partial involvement by follicular lymphoma (n=4), and duodenal follicular lymphoma (n=4), assumed to represent, potentially, the earliest stages in the evolution of follicular lymphoma. Cases of reactive follicular hyperplasia (n=2), uninvolved areas from follicular lymphoma *in situ* lymph nodes, follicular lymphoma grade 1-2 (n=5) and follicular lymphoma grade 3A (n=5) were used as controls. Surprisingly, alterations involving several relevant (onco)genes were found in all entities, but at significantly lower proportions than in overt follicular lymphoma. While the number of alterations clearly assigns all these entities as precursors, the pattern of partial involvement by follicular lymphoma alterations was quantitatively and qualitatively closer to that of follicular lymphoma, indicating significant selective pressure in line with its faster rate of progression. Among the most notable alterations, we observed and validated deletions of 1p36 and gains of the 7p and 12q chromosomes and related oncogenes, which include some of the most recurrent oncogenic alterations in overt follicular lymphoma (*TNFRSF14*, *EZH2*, *MLL2*). By further delineating distinctive and hierarchical molecular and genetic features of early follicular lymphoma entities, our analysis underlines the importance of applying appropriate criteria for the differential diagnosis. It also provides a first set of candidates likely to be involved in the cascade of hits that pave the path of the various progression phases to follicular lymphoma development.

Introduction

Follicular lymphoma (FL) has a widely variable clinical course, with most patients displaying an indolent form of the disease, associated with slow progression over many years. This progression can be followed through histological modifications, in both pattern and cell type, a fact that is reflected in the World Health Organization (WHO) grading system for FL.[1] Genomic studies of FL have shown an accumulation of genetic aberrations correlating with histological transformation during the clinical course of the disorder.[2,3]

One of the earliest events in follicular lymphomagenesis is thought to occur in B-cell precursors in the bone marrow, giving rise to B cells carrying the t(14;18)(q32;q21) translocation.[4] However, this translocation is also detected, albeit at extremely low frequencies, in B cells from most healthy adults.[5] Furthermore, studies tracking these cells over more than 10 years have demonstrated the persistence and evolution of a major, dominant proliferating (14;18)-carrying B-cell clone within a given individual.[6,7] Two anecdotal reports also described that the t(14;18)-B-cell clone represented in the tumor at diagnosis was already present several years before

the diagnosis of FL.[8,9] Many aspects regarding the fate and site(s) of these FL-like B cells remain open, particularly those regarding their role as precursors of FL.

The phenomenon of localization of FL cells to isolated germinal centers (GC) within a lymph node has been termed FL *in situ* (FLIS)[10] or intrafollicular neoplasia.[1] This pattern may be seen with FL at other sites of disease, or as the only manifestation of disease in some patients. In the true "*in situ*" lesion, clusters of B cells strongly positive for CD10 and BCL2 are localized to GC in an otherwise reactive lymph node. FLIS is often an incidental finding in a lymph node biopsied for other reasons, and in such patients, if there is no other evidence of disease, the risk of progression is very low, in the order of 5% or less. A clinically similar phenomenon is duodenal FL (DFL), which usually presents as isolated mucosal polyps with a low risk of dissemination.[11] Both FLIS and DFL have been shown to have the *BCL2/IGH* translocation, but the presence of other genetic aberrations associated with FL is largely unexplored in these lesions. Given the very low rate of progression, it has been postulated that the *BCL2* translocation might be the sole genetic aberration.[12,13]

Histological and immunophenotypic criteria have been pro-

posed to distinguish FLIS from partial involvement by FL (PFL). In PFL there is greater architectural distortion, and the diagnosis of lymphoma is more evident histologically. However, interestingly, patients with PFL appear to present with low-stage disease, with a relatively low risk of progression and often a very prolonged disease-free interval with limited therapy.[12] Thus, PFL may represent a *bona fide* early stage in the biological evolution of FL, and not just a limited state of lymph node replacement by a fully developed neoplasm.[14]

Advances in laser capture microdissection now enable the isolation of even small numbers of cells representative of the clonotypic population,[15] and improvements in genomic methods allow the analysis of genetic aberrations in DNA derived from formalin-fixed, paraffin-embedded tissues. These new technologies now provide the unique opportunity to explore the genetic landscape of these early lesions, and to understand their relationship to overt FL better.

Methods

Samples

Most cases were selected from formalin-fixed, paraffin-embedded archive specimens submitted to the Hematopathology Section at the National Cancer Institute (NCI) or the Institute of Pathology, at the Medical University of Vienna. Seven cases of FLIS, five cases of PFL and five cases of DFL were identified based on previously published criteria.[11,12] Patients with FLIS and PFL had no other evidence of disease during the period of follow-up.[12] These samples were compared to five cases of FL grade 1-2 and five of FL grade 3A, included as controls of the most frequent alterations occurring in FL. Finally, sections of reactive follicular hyperplasia (RFH, n=2) and laser micro-dissected lymphoid cells from uninvolved areas of FLIS#3 (FLIS background) were also hybridized and used as references for normal cells (*Online Supplementary Table S1*). Only cases with confirmed t(14;18) were included in the study. The Institutional Review Boards of the NCI and the Medical University of Vienna approved the study.

Laser capture microdissection, DNA extraction and quality control

To obtain sufficient enrichment of lesional cells positive for t(14;18), which was necessary for the genome-wide array analysis, BCL2+ GC were microdissected from the selected FLIS/PFL/DFL/FL grade 1-2 cases. Laser capture microdissection was performed using a Leica LMD6000 (Leica Microsystems, Germany) on hematoxylin-stained slides with BCL2+ immuno-stained slides as a guide for involved follicles.[15] BCL2-negative GC were microdissected from RFH, without evidence of FLIS. Tumor cell DNA was isolated from FL grade 3A without microdissection, given the high tumor cell content (>75%). DNA was extracted using a QIAamp® DNA FFPE Kit. The amount of DNA collected from the different cases ranged from 506 to 2820 ng. With a calculation of 6.6 pg of DNA/cell this amounts to a minimum of ~77,000 cells per case needed for appropriate hybridization. The integrity of the DNA was controlled with an Agilent DNA1000 Kit®. The sizes of amplifiable fragments (from 100 to 800 bp) were evaluated with a multiplex-polymerase chain reaction. Only samples with amplicon sizes >300 bp passed the quality control for array comparative genomic hybridization (CGH). Using these selective criteria, four cases of FLIS, four PFL, four DFL, ten FL, two RFH and one FLIS#3 background provided enough material and had sufficient DNA quality to undergo array CGH analysis.

Array comparative genomic hybridization

Genomic imbalances were analyzed using 244K array CGH microarrays (HU-244A); 2 μg of sample and reference DNA were labeled with the ULS-labeling kit (Agilent Technologies) and hybridized on a molecular oncology platform (CRCM, Marseille), as previously described.[16] Briefly, data extraction (log$_2$ ratio) was performed from CGH-analytics, whereas normalized and filtered log$_2$ ratios were obtained from the Feature Extraction™ software (version10.3; Agilent Technologies). Raw data were visualized as graphical figures within the Genomic Workbench v5.0 software. The frequency of alterations was computed for each probe locus as the proportion of samples showing an aberration. The gain and loss status for each probe was assigned using a conservative approach with a threshold of |0.4| on at least five consecutive probes across the genome.

Alterations found by array CGH were further validated by fluorescence *in situ* hybridization (FISH) on cases with sufficient material (*Online Supplementary Figure S1*).

Further details of the samples, FISH analyses,[17] transcription profiles, public datasets, Sanger-sequencing and statistics are provided in the *Online Supplementary Methods*.

Results

Genomic alterations accumulate in the pathogenesis of early follicular lymphoma

To gain insights into the kinetics of genomic aberrations accumulating during the early steps of the pathogenesis of FL, high-resolution array CGH was performed on a retrospective collection of biopsies representing putative early stages of FL evolution.[12]

Genomic alterations were found in all samples from each early-FL group (FLIS: 4/4; PFL: 4/4; DFL: 4/4). Typical hybridization profiles are presented in Figure 1 (*in extenso* data are summarized in Table 1). As expected, no major genomic alterations (>700 kb) were found in normal cells (RFH and FLIS background); in contrast, numerous lesions could be found in positive controls (FL grade 1-2 and 3A). Despite the low sampling, the alterations in FL grade 1-2 and 3A (Table 1 and *Online Supplementary Figure S2*) were overall comparable to those previously reported,[18-20] further validating our technological approach. Likewise, chromosomes most frequently affected in early-FL groups were overall similar to those recurrently observed in overt FL, including chromosomes 1 and 18 (accounting for 17% and 33% of alterations, respectively). Gain of the *BCL2* region on chromosome 18/der14 was particularly apparent (33.5%), and confirmed by FISH (representative examples are shown in *Online Supplementary Figure S1A*), further highlighting the crucial role of BCL2 over-expression in early steps of FL development/maintenance.[20] Although the mean number of alterations per sample increased with disease progression (from 2.5 in FLIS to 24.4 in FL3A, Figure 2A), alteration sizes were not significantly different in DFL, PFL and FL (Figure 2B), suggesting the occurrence of similar mechanisms of genomic instability. Interestingly the acquisition of very large chromosomal alterations (>10 Mb) was rare in FLIS samples. As previously reported in FL,[19] gains were more frequently observed than losses in all groups, but only gains were identified in FLIS (*Online Supplementary Figure S3*).

Altogether these data indicate that most early FL precursors, including FLIS, already stand in a phase of progression in which increased genomic instability is at work.

Increased selective pressure with follicular lymphoma progression on a background of established genomic instability

We next sought to determine whether and, if so, how alterations observed in the various early-FL entities related to FL progression and maintenance.

Follicular lymphoma in situ

A total of 259 annotated genes (listed in *Online Supplementary Table S2*) were spread over ~52 Mb in ten amplified regions from the four FLIS samples. Among these, only a few could be identified as relevant hits recurrently observed in FL. Twelve were known oncogenes (including *BCL2*,[4] *RUNX1*,[21] and *KDSR*,[22] and were also found translocated and/or amplified in 100%, 70% and

30% of our series of FL samples, respectively *(Online Supplementary Table S3)*. Four amplified genes (*TOX, BACH2*,[23] *AFF3*,[24] and *EBF1*,[25] *Online Supplementary Figure S4*) were functionally related to the GC reaction, and were also found amplified in 100%, 90%, 80%, and 70% of FL, respectively. Notably, gain of *AFF3* was recurrently found in two distinct FLIS samples. Although their functional relevance in FL lymphomagenesis remains to be demonstrated directly, some of the amplified genes were also found to be up-regulated in FL as compared to their levels in normal B-cell subsets (public microarray dataset GSE12195)(*Online Supplementary Figure S5* and *Online Supplementary Table S3*). However, only 12 (4%) of the amplified genes were identified oncogenes, while 32 (13%) were gains of genes with tumor suppressive func-
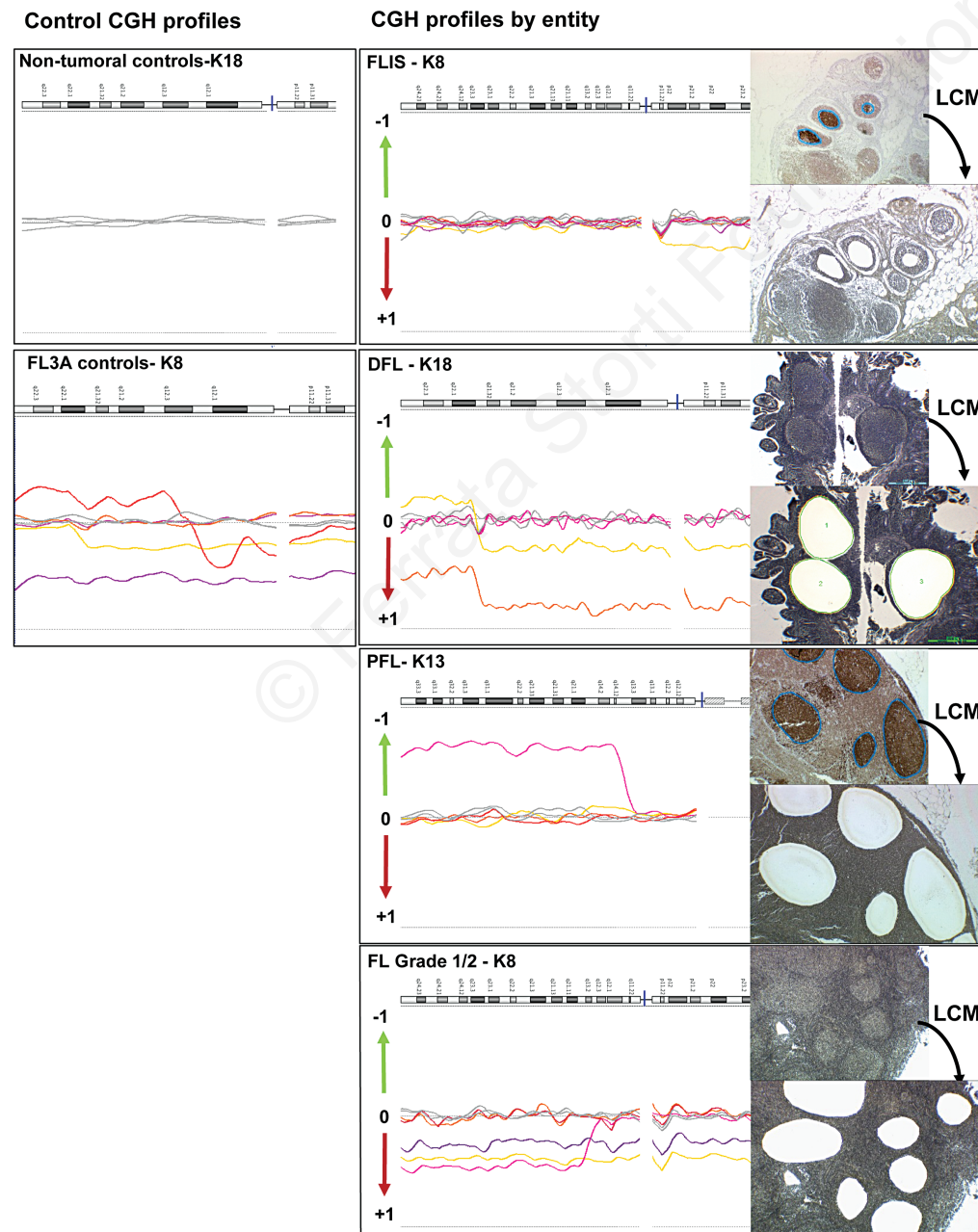

Figure 1. Array CGH profiles by entities from laser capture microdissection (LCM) of germinal center (gc) regions. To enrich for cells of interest, LCM of BCL2+ GC from the selected FLIS/PFL/DFL cases was performed on hematoxylin-stained slides with BCL2+ immunostained slides as a guide for involved follicles. LCM was also performed on FL grade 1-2 biopsies to select for neoplastic follicles. Lymphoid cells from cases of FL grade 3A containing >75% tumor cells were utilized without microdissection, given the high tumor cell content. Finally, reactive GC were captured by LCM from cases of RFH and utilized as negative controls. DNA from all of the above lesions was hybridized on 244k array CGH chips. Representative profiles of alterations found in the different entities are shown. Controls samples (RFH) are in gray, early-FL and FL samples are in color).

tions (Figure 3A and *Online Supplementary Tables S2* and *S3*). Furthermore, and unlike other entities, no loss of genes with tumor suppressive function could be identified in the absence of loss in FLIS. This suggests that a significant fraction of the amplified genes in FLIS might be passenger alterations, rather than selected alterations as observed in more advanced entities.

Partial involvement by follicular lymphoma

A total of 1128 genes were spread over ~268 Mb in 13 amplified regions from the four PFL samples; 370 genes were spread over a total of ~85 Mb in three losses (listed in *Online Supplementary Table S2*). A subset of the alterations was validated by FISH in our cohort (loss of 1p36, 7p, 12q, and *BCL2* amplifications, *Online Supplementary Figure S1A-D*). The major fraction of amplified genes (99%) was shared with genes also found to be amplified in FL grade 1-2 and/or FL grade 3A (Figure 3B), suggesting selective pressure for their maintenance during the progression of FL. In agreement with this, most of the shared genes were functionally involved in cell proliferation, expression, transcription, and differentiation. Sixty-seven of them (~6%) were known oncogenes, six of which (*BCL2*, *RUNX1*, *NACA*,[26] *CARD11*,[27] *ELN*[28] and *IKZF1*[29]) were previously identified as major players in the oncogenesis of leukemia/lymphoma (*Online Supplementary Table S3*). In line with the functional relevance of these candidate hits in FL development, *RUNX1*, *AFF3*, and *BACH2* were recurrently amplified in FLIS, and/or PFL, and *RUNX1*, *NACA*, and *IKZF1* were transcriptionally highly up-regulated in overt FL compared to their normal B-cell counterparts (*Online Supplementary Figure S5* and *Online Supplementary Table S3*). Shared amplified genes in PFL also comprised histone modifiers such as *MLL2*, *EZH2*,[30] *ARID2*,[31] and *HDAC7*[32] recently identified as some of the most frequent altered function events of FL pathogenesis.[3,33] As for FL grade 1-2 and 3A, less annotated genes (n=370) were found in the deleted areas of PFL. Losses included 27 tumor suppressors (but also 18 oncogenes), and the corresponding functional pathways (cell death, apoptosis, necrosis, cell migration) appeared coherent with loss-of-function in lymphomagenesis. However, and in contrast to amplified regions, only a few of these genes were shared with FL grade 1-2 and/or 3A (60/370 ~16%, Figure 3B). Among these, shared losses comprised deleted genes on chromosome 1p, a chromosome classically affected in FL.[34] Several genes related to cytokines and cytokine receptors appeared as potent early loss-of-function hits (*Online Supplementary Tables S2* and *S3*). This included tumor necrosis factor-family members such as *TNFRSF9* and *TNFRSF25*, and more particularly *TNFRSF14*, which has recently been identified as one of the most frequent secondary genetic abnormalities in FL (>65%).[35] In this regard, we confirmed that TNFRSF14

Table 1. Results from array CGH analysis in FL grade 3A, FL grade 1-2, PFL, DFL and FLIS samples compared to negative controls (2 RFH and 1 FLIS background).

Samples	N. of samples	Samples with alterations	Total n. of alterations	Recurrency* (%)	Mean alteration N. / sample	Mean size of alterations
FL grade 3A	5	5	122	47.0	24.4	2088 kbp
FL grade 1/2	5	5	61	49.0	12.2	3366 kbp
PFL	4	4	16	25.0	4.0	3004 kbp
DFL	4	4	16	33.0	4.0	2242 kbp
FLIS	4	4	10	27.0	2.5	845 kbp
Controls	3	0	0	0.0	0.0	0 kbp

* Recurrency represents the percentage of alterations that are found in at least two samples.
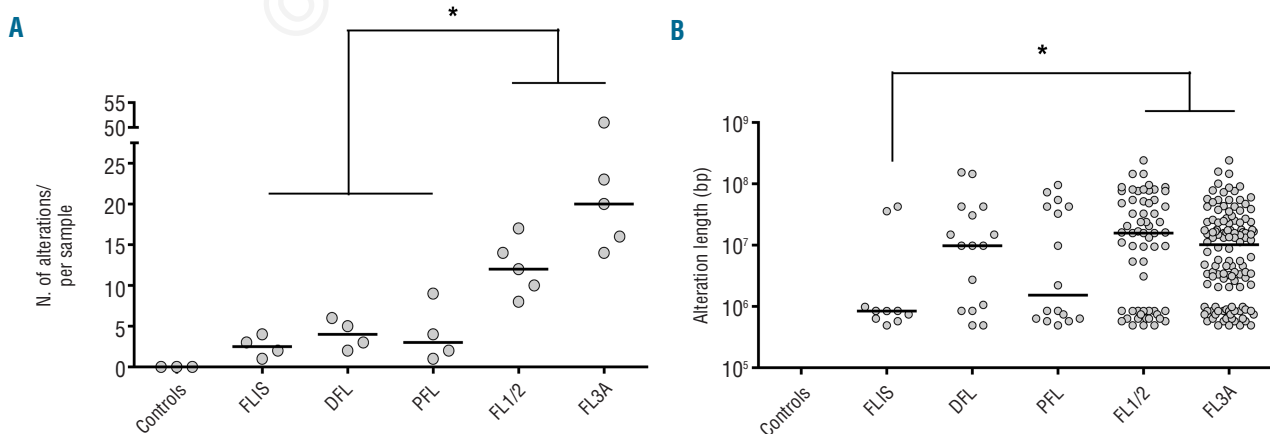

Figure 2. Quantitative and qualitative characteristics of alterations found in the various stages of progression of follicular lymphomagenesis. (A) Number of alterations observed in each sample, classified by entity. (B) Length (in base pairs) of each alteration observed in the various subtypes. *P<0.05. Controls were reactive GC isolated by laser capture microdissection from RFH, and FLIS background as defined in the *Methods*.

was a site of frequent somatic mutations even in early FL samples (*Online Supplementary Table S4*), suggesting early involvement of this molecule in the pathogenesis of FL. Ten genes in shared losses were recognized tumor suppressor genes (*Online Supplementary Tables S2* and *S3*), including *TP73*,[36] also deleted in DFL, 50% of FL, and transcriptionally down-regulated in large cohorts of FL (*Online Supplementary Figure S6*). While several of the amplified oncogenes have been previously associated with FL, to our knowledge only few of the deleted tumor suppressors have yet been reported as recurrent events or functionally involved in FL pathogenesis and/or lymphomagenesis, and we cannot exclude that some might correspond to passenger alterations.

Overall, although the global number of alterations remains quantitatively and qualitatively much lower than in FL (Figure 3A), the large proportion of gains maintained throughout FL progression and the occurrence of functionally relevant gains and losses corresponding to frequent FL hits indicate that PFL already goes through significant selective pressure, in agreement with its reported high rate of progression to FL.

Duodenal follicular lymphoma

A total of 1021 genes were localized in 12 amplified regions (~390 Mb) from DFL samples (listed in *Online Supplementary Table S2*). While the biological functions of these genes were globally relevant to oncogenesis, tumor
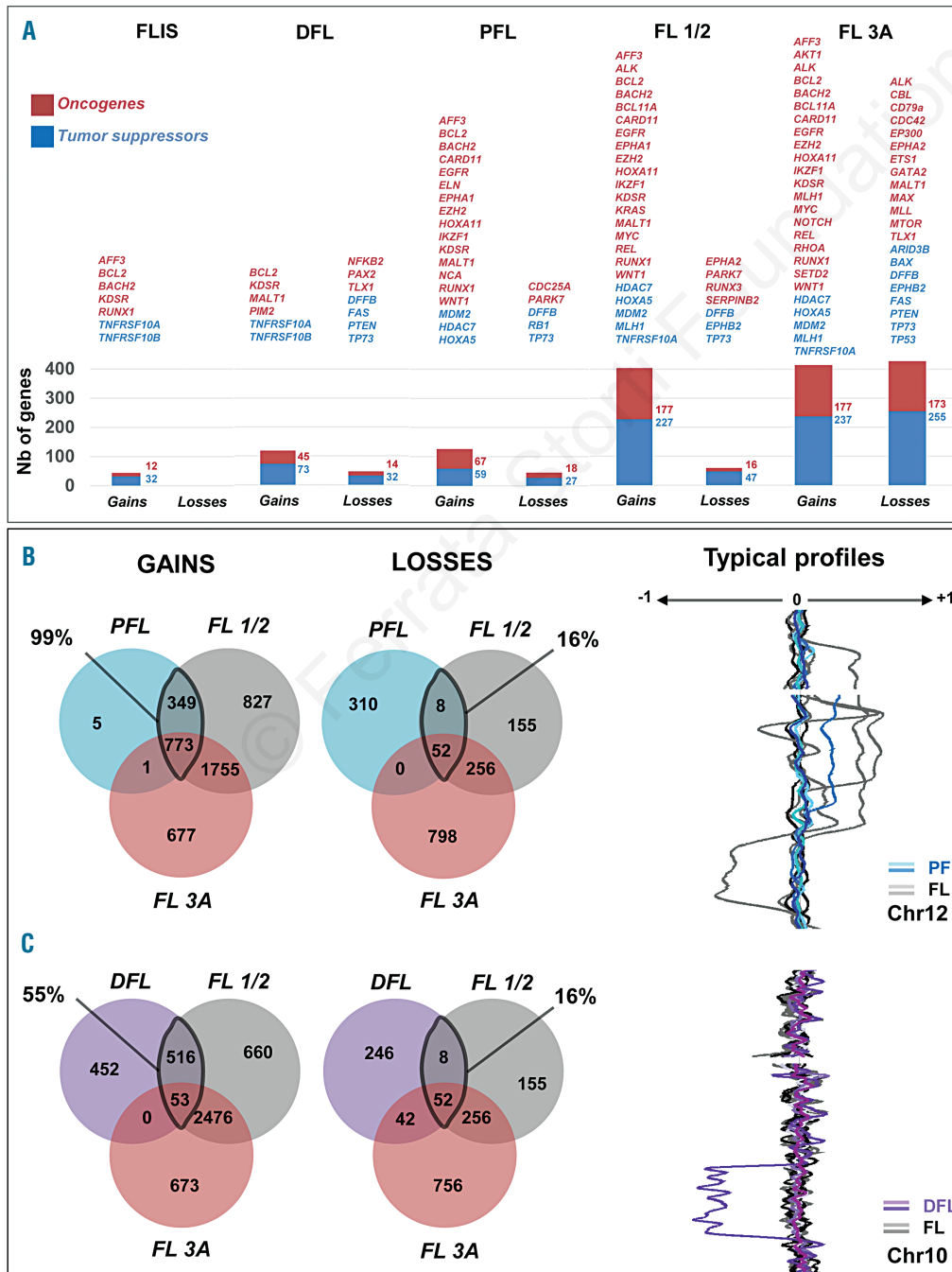

Figure 3. Summary of alterations observed in early FL and FL grade 1-2 and FL grade 3A. (A) Oncogene (red) and tumor suppressor (blue) genes located in the gains and losses, presented by entity. The total number of altered genes in each category is indicated at the right of the bars, and some of the most relevant genes are listed above the bars. (B) Venn diagram showing the number of genes found in the gained and lost regions of samples of PFL, FL grade 1-2 and FL grade 3A. The dotted area indicates the percentage of genes located in amplified or lost regions of the PFL samples, which are also amplified or lost in FL grade 1-2 samples. An example of a region (gain) shared by one PFL (in blue) and several FL samples (in gray), containing several genes (notably the *NACA* oncogene) is shown. RFH controls are in black. (C) Venn diagram showing the number of genes found in the gained and lost regions of samples of DFL, FL grade 1-2 and FL grade 3A. The dotted area indicates the percentage of genes located in amplified or lost regions of the DFL samples, which are also amplified or lost in FL grade 1-2 samples. An example of one alteration observed in a DFL sample. DFL samples are in purple, FL grade 1-2 and FL grade 3A are in gray, RFH in black.

suppressors (n=73) were more frequently found than oncogenes (n=45) in amplified regions (Figure 3A). A similar trend was observed for the 348 genes located in six deleted regions (~40 Mb), with 32 tumor suppressors and 14 oncogenes. In contrast to the situation in PFL, large fractions of both gains and losses were not found in FL grades 1-2 and/or 3A (45 to 83%, respectively, Figure 3C). Among the shared genes, a limited set of relevant amplified oncogenes (*BCL2, BCL6, FGFR1, EIF4A2* and *TFRC*) and deleted tumor suppressors (*PTEN, FAS,* and *TP73*) could nevertheless be identified; some of them were also shared with the other FLIS/PFL entities (*BCL2, BCL6, TP73, Online Supplementary Table S3*), previously reported to be altered in non-Hodgkin lymphoma (*BCL2, BCL6, TFRC,* and *TP73*)[33,37] and/or highly increased transcriptionally in FL samples compared to in normal B-cell subsets (*EIF4A2, TFRC, Online Supplementary Figure S5C* and *Online Supplementary Table S3*). Furthermore, recurrent deletion of chromosome 1p was again observed, encompassing the same TNFR family members previously identified in PFL and FL, as well as a high mutation load of *TNFRSF14* exons (*Online Supplementary Figure S6B* and *Online Supplementary Table S4*).

Altogether, this suggests that while DFL displays increased genomic instability and bears some of the classical hits of FL genesis (*Online Supplementary Tables S4* and *S5*), the absence of strong selective forces might have (yet?) limited their evolution to malignant transformation.

Discussion

Early FL entities have thus far been defined histologically and, besides t(14;18), very little information is available on their molecular status.[13,38] Using high-resolution array CGH analysis, we provide here the first comprehensive genome-wide genetic viewpoint of FLIS, PFL and DFL in otherwise "healthy" individuals. We first clearly show the presence of genomic alterations in all three entities. Considering the extensive subclonal nature of FL and pre-FL,[39,40] the early events uncovered here probably vastly underestimate the multitude of other genetic lesions present in minor and/or non-selected subclones. Furthermore, considering that next-generation sequencing data have recently revealed that smaller alterations – including point mutations – are the most frequent events in cancer cells,[33] it is highly likely that such events have also extensively accumulated in FLIS, PFL and DFL. In line with this possibility, we found that small copy number variations (between 100 and 700 kb) also tended to increase significantly between pre-FL and FL entities, with a dramatic change between FL grade 1-2 and 3A (*data not shown* and *Online Supplementary Figures S1A* and *S7*). Next-generation sequencing on a set of fresh prospective FLIS, PFL, and DFL samples will be necessary to uncover the extent of genomic instability precisely in these entities.

Nonetheless, our array CGH analysis first revealed that FLIS exhibited much greater genetic complexity than anticipated, suggesting that increased genomic instability is already at work in these cells, and confirming their status of "early lesions". However, the diverse functional relevance of the altered genes, and the low proportion of those as recurrent FL hits, suggest that only a few could be major actors in the malignant progression. As such, FLIS seemingly stands at a crossroads between increased

genomic instability and low selective pressure for malignant transformation, consistent with the observation that it rarely progresses to overt FL. This adds to the notion that the oncogenic network required for full malignant transformation proceeds through a complex Darwinian-like process of stepwise, multi-hit events of selection/counter-selection.

From both the genetic and functional perspectives, FLIS clearly appears as a less advanced entity than PFL, supporting the validity of the recently proposed criteria for refined histological stratification.[12] The mean number and size of alterations were greater in PFL than in FLIS. The major fraction of amplified genes in PFL (99%) was also found to be amplified in FL grade 1-2 and/or 3A and comprised several known FL oncogenes, suggesting selective pressure for their maintenance during FL progression. Nevertheless, it still appears that PFL are genetically not equivalent to overt FL, both in terms of the overall lower number of alterations (3-fold and 6–fold lower compared to FL grades 1-2 and 3A, respectively) and of the small overlap of shared genes in losses (~16%), showing (in contrast to gains) incomplete functional evolution to FL. The observed dichotomy between gains and losses is intriguing, and might suggest a more deleterious/irreversible effect of large chromosomal losses in early stages of the progression of FL (deletion of amplified regions/sub-regions being possible, but not the reverse), leading to their late acquisition in FL.[41] Overall, our data clearly demonstrate that when rigorously applying histological criteria for their identification,[12] cases of PFL generally do not constitute partial colonization of the lymph node by overt FL, but rather represent an earlier stage of tumor evolution.[40] Our definition of PFL used for case selection excluded patients with known FL at another site. In the presence of concurrent FL and/or FL by history, we cannot rule out that partial colonization of lymph nodes by overt FL might histologically resemble/be undistinguishable from PFL.

Collectively, our results add to the hierarchical model in which FLIS, PFL, FL grade 1-2, and FL grade 3A constitute a continuum (*Online Supplementary Figure S8*).[42] At the beginning of this spectrum, the relationship between FLIS and FL-like cells is still unclear. The report of a circulating t(14;18)[+] counterpart in a FLIS case provided the first direct evidence of such an affiliation.[9,43] The extensive dissemination of FL-like clones in lymphoid organs (including bone marrow) and blood, the important dynamics of clonal evolution in these cells, the presence of FL hallmarks of selective pressure (such as the allelic-paradox allowing sIgM to be maintained in spite of AID activity), and the frequent imprints of AID-mediated genomic instability further argue for a large overlap between these two entities.[39] (Sungalee *et al. unpublished data*).

Our data also revealed that a number of candidate-hits, some of which are frequently seen in FL and/or assumed to play a major role in FL pathogenesis, may be acquired very early in the genesis of FL. These include further gain of *BCL2* on chromosome 18 or der14, stressing the crucial role of *BCL2* overexpression in early steps of FL development/maintenance. Histone modifiers such as CREBBP, EP300, and EZH2 also recently emerged as some of the most frequent alterations in FL, and it has been proposed that such alterations occur early on in the natural history of FL.[3,40] In line with this proposal, we show here recurrent gains of *MLL2* and *EZH2* in both PFL and DFL, and an

increased mutation rate in *CREBBP* (*Online Supplementary Table S5*). Regarding the recent description of EZH2 as a major player in GC formation, and of the role of *EZH2* gain-of-function mutations in enhancing lymphomagenesis,[44] we found that some of the alterations in FLIS and PFL were functionally related to GC reaction biology, suggesting that selective forces in FLIS might initially converge on events contributing to the GC reaction and/or GC retention. Consistent with the dependency of FL on the microenvironment, members of the TNFR family located on the frequently deleted 1p36 region also emerged among tangible early candidate hits and were lost in both PFL and DFL. *TNFRSF14*, in particular, has recently been identified as one of the most frequent secondary genetic abnormalities in FL (>65%),[35] and *TNFRSF9* (*4-1BB*) deficient mice are predisposed to develop GC-derived (follicular-like) B-cell lymphoma.[45] Other members of the TNFR family located on chromosomes 8 and 18 (notably *TNFRSF11A*,[46] a major activator of the NF-κB and MAPK8/JNK pathways) were amplified in one FLIS sample, further adding to the potential relevance of this gene family. Interestingly, some of the affected regions in PFL and DFL, and particularly the deletion of the 1p region, were previously identified as being significantly associated with poor survival and/or transformation to diffuse large B-cell lymphoma.[34] This might suggest that gene deregulations associated with poor prognostic factors may also be acquired early on, but that their adverse effects might be unleashed only at more advanced stages and/or under therapeutic pressure.

With confinement to the duodenum and little evidence for FL progression, DFL appears histologically and clinically as a separate entity[11,47] and it is as yet unclear how it integrates in the FLIS-PFL-early FL scheme. DFL share the t(14;18) with other variants of nodal FL. The propensity to remain localized to the mucosa may relate to the site in which the relevant antigen is first encountered, a feature that these lesions may share with mucosa-associated lymphoid tissue (MALT) lymphoma. DFL might initiate from the same early founder t(14;18)-only precursor exiting the bone marrow, or from a genetically more advanced one (i.e. one that has already experienced antigenic challenge and GC transit before settling in the specific extra-nodal environment).[11,48] From a genetic viewpoint, DFL appear to be at increased risk of genomic instability with a large number of already acquired genomic alterations (comparable to PFL), including some of the classical hits of FL genesis. In contrast to PFL, however, only half of the amplified genes were shared with FL (or PFL), suggesting still limited selection pressure to malignant pathways. It has previously been shown that DFL are devoid of AID expression.[49] This suggests either that the genomic instability observed is not AID-dependent or that the observed alterations have been acquired elsewhere in the presence of AID (potentially in the context of a classical GC reaction) before colonization of duodenal GC. By limiting further genomic instability and selection potential, this scenario might partly explain why this entity rarely evolves to overt malignancy. Interestingly, the low propensity of DFL to progress to overt FL, despite the presence and combination of several oncogenic alterations, adds to the evidence that such hits are not sufficient for transformation, and that extrinsic factors (such as successive immunological challenges in GC) might play a key role in the selection process.[39]

DFL, FLIS and PFL show very distinct progression rates when left untreated (~5%, ~5%, and ~53%, respectively). In the current debate on the clinical management of early and asymptomatic FL patients with low tumor mass, none from a small series of PFL patients treated with rituximab or radiotherapy progressed to overt FL after 7 years of follow-up.[12] By further delineating distinctive and hierarchical molecular and genetic features of FLIS, DFL and PFL, our analysis reinforces the critical importance of applying appropriate criteria for differential diagnosis. It also provides a first set of candidate gene alterations likely involved in the early phases of progression of FL, and which could be explored in prospective studies as innovative therapeutic targets.

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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