ARTICLE

Genetic evolution of *in situ* follicular neoplasia to aggressive B-cell lymphoma of germinal center subtype

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ABSTRACT

In situ follicular neoplasia (ISFN) is the earliest morphologically identifiable precursor of follicular lymphoma (FL). Although it is genetically less complex than FL and has low risk for progression, ISFN already harbors secondary genetic alterations, in addition to the defining t(14;18)(q32;q21) translocation. FL, in turn, frequently progresses to diffuse large B-cell lymphoma (DLBCL) or high-grade B-cell lymphoma (HGBL). By BCL2 staining of available reactive lymphoid tissue obtained at any time point in patients with aggressive B-cell lymphoma (BCL), we identified ten paired cases of ISFN and DLBCL/HGBL, including six *de novo* tumors and four tumors transformed from FL as an intermediate step, and investigated their clonal evolution using microdissection and next-generation sequencing. A clonal relationship between ISFN and aggressive BCL was established by immunoglobulin and/or *BCL2* rearrangements and/or the demonstration of shared somatic mutations for all ten cases. Targeted sequencing revealed *CREBBP*, *KMT2D*, *EZH2*, *TNFRSF14* and *BCL2* as the genes most frequently mutated already in ISFN. Based on the distribution of private and shared mutations, two patterns of clonal evolution were evident. In most cases, the aggressive lymphoma, ISFN and, when present, FL revealed divergent evolution from a common progenitor, whereas linear evolution with sequential accumulation of mutations was less frequent. In conclusion, we demonstrate for the first time that t(14;18)+ aggressive BCL can arise from ISFN without clinically evident FL as an intermediate step and that during this progression, branched evolution is common.

Introduction

B-cell lymphomas (BCL) are thought to arise from premalignant precursor cells by stepwise accumulation of mutations fostering survival and clonal expansion. Whereas some premalignant lesions such as monoclonal gammopathy of unknown significance (MGUS) and monoclonal B-cell lymphocytosis (MBL) have been known for many years, there are no known precursors for *de novo* aggressive BCL. Diffuse large B-cell lymphoma (DLBCL) not otherwise specified (NOS) is the most frequent form of BCL and represents 25-35% of adult BCL in the Western world. Based on gene expression profiling DLBCL can be sub-classified into activated B-cell-like (ABC) and germinal center B-cell-like (GCB) subtypes.[1,2] Approximately 20-30% of DLBCL, mostly of GCB subtype, exhibit the t(14;18)(q32;q21) translocation, the hallmark of follicular lymphoma (FL).[3] This translocation causes constitutive overexpression of the anti-apoptotic protein

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BCL2 and effectively abrogates negative selection in the germinal center (GC), leading to prolonged survival in the GC environment.[4-6] Another category of aggressive BCL harboring *BCL2* (18q21) translocations are high-grade B-cell lymphomas (HGBL) with an additional *MYC* rearrangement, so-called double-hit (DH) or triple-hit (TH) lymphomas, when also carrying *BCL6* translocations.[3] In addition to *de novo* presentation, both DLBCL and HGBL can arise from indolent BCL, most commonly FL.[3] Transformation of FL into an aggressive lymphoma occurs in 2-3% of patients per year and usually results in a GCB phenotype.[3,7]

The t(14;18)(q32;q21) occurs during early B-cell development and is considered a founding alteration.[4] However, this translocation alone is insufficient to cause the development of FL, as t(14;18)+ B cells can be identified at low frequencies in the peripheral blood of about half of otherwise healthy adults over the age of 50.[3,8] Distinct clones of these t(14;18)-carrying cells, termed follicular lymphoma like B cells (FLLC), have been shown to persist and even expand over years without progressing to manifest FL in most individuals.[3,9] The risk for progression depends on the clone size, rather than on the number of different t(14;18)+ clones.[10] The earliest identifiable tissue-based precursor of FL is *in situ* follicular neoplasia (ISFN), defined as colonization of GC by a monoclonal population of t(14;18)+ B cells in otherwise reactive lymphoid tissues.[3,11] Although by definition the normal lymphoid architecture is not altered, ISFN can be identified immunohistochemically by virtue of its strong staining for BCL2 and CD10 and a low proliferation index.[11] ISFN can occur syn- or metachronously with FL, as well as with other BCL, but can also be found in individuals without history of lymphoma.[12,13] The risk of progression seems to be low.[12] Although ISFN is considered a precursor lesion, it already demonstrates secondary genetic alterations typically associated with manifest FL, especially affecting chromatin modifier genes such as *CREBBP*, and less frequently *KMT2D* and *EZH2*, known as important factors in FL pathogenesis.[14-16] Importantly, both ISFN and FLLC also exhibit persistent expression of activation-induced cytidine deaminase (AID), which catalyzes class switch recombination and somatic hypermutation (SHM).[17,18] AID activity is responsible for the intraclonal heterogeneity of the re-arranged immunoglobulin heavy chain (IGH) genes and the acquisition of novel N-glycosylation sites in the IG variable regions.[18,19] Novel glycosylation motifs are a feature frequently observed in FL, but less so in normal B cells or other BCL, and are thought to enable the interaction with mannose-binding lectins, eliminating the need for conventional B-cell receptor signaling through antigen binding.[18,20] Furthermore, AID activity is believed to be an important driver for the genetic evolution of FL, leading to increased genomic instability and the accumulation of additional aberrations.[21,22]

Given the well-known role of ISFN as clonally related premalignant FL precursor and the frequent occurrence of the t(14;18) translocation in both *de novo* and secondary DLBCL and HGBL,[3,14,23,24] we aimed to identify syn- or metachronous ISFN in patients with DLBCL and HGBL, and used these paired samples to investigate the clonal relationship, clonal evolution and underlying genetic changes driving progression from ISFN to aggressive BCL.

Methods

Sample selection

Suitable cases were identified by searching the archives of the Institutes of Pathology of Tuebingen University Hospital and the Robert-Bosch-Krankenhaus (Stuttgart, Germany) for patients with a diagnosis of DLBCL or HGBL, with or without antecedent FL, for which reactive lymphoid tissue from any time point was available, and staining the lymphoid tissues for BCL2 to identify ISFN (as detailed in the *Online Supplementary Appendix*). The ISFN of case 8 has already been included in previous studies.[14,15] An additional case was provided by the Hospital Universitario Fundación Jiménez Díaz (Madrid, Spain). All diagnoses were made according to the criteria of the 2017 World Health Organization classification and reviewed by two experienced hematopathologists (LQ-M and FF).[3] This study was approved by the Ethics Committee of the University of Tuebingen (096/2016/B02).

Microdissection, immunohistochemistry and fluorescence *in situ* hybridization

Microdissection of ISFN samples was performed on 5 μm hematoxylin and eosin stained formalin-fixed, paraffin-embedded (FFPE) sections with an Axiovert 200M microscope (Zeiss, Oberkochen, Germany) and the P.A.L.M. system (Palm@Robo software 3.0; Zeiss). Fluorescence *in situ* hybridization (FISH) was performed on FFPE sections using Vysis LSI *BCL2*, *BCL6* and *MYC* Dual Color Break Apart Rearrangement Probes (Abbott Molecular, Wiesbaden, Germany). For additional information, including DNA extraction and immunohistochemistry, see the *Online Supplementary Appendix*.

Polymerase chain reaction and sequencing of the t(14;18) breakpoint region

The t(14;18) breakpoint regions were amplified by polymerase chain reaction (PCR) and sequenced using major breakpoint, minor cluster and intermediate cluster region primers together with a joining region consensus primer as previously described, as well as the IdentiClone BCL2/JH Translocation Assay (Invivoscribe, San Diego, CA, USA) (*Online Supplementary Appendix*).

Clonality analysis and immunoglobulin sequencing

Detection of monoclonal IGH and IGκ light chain (IGK) gene rearrangements was performed using BIOMED-2 primers as previously described.[25] Next-generation sequencing (NGS) of IGH genes was accomplished with the LymphoTrack Dx IGH Assay – PGM (Invivoscribe) on the Ion Torrent Personal Genome Machine (PGM; Thermo Fisher Scientific, Waltham, MA, USA). Data were analyzed with the LymphoTrack Dx Software – PGM (Invivoscribe). For a description of the IG sequence analysis and the construction of phylogenetic trees to illustrate the clonal evolution of the IGH, see the *Online Supplementary Appendix*.

Targeted next-generation sequencing analysis

Samples were subjected to NGS on the Ion Torrent PGM using AmpliSeq Custom Panels created with the Ion AmpliSeq Designer (Thermo Fisher Scientific). The panels target recurrent mutations of FL and DLBCL, covering 95.21% of the coding sequence of *BCL2*, *BCL6*, *BTG1/2*, *CARD11*, *CD79B*, *CREBBP*, *EP300*, *FOXO1*, *GNA13*, *HIST1H1B-E*, *IGLL5*, *KMT2D*, *IRF4*, *MEF2B*, *PIM1*, *PRDM1*, *TBL1XR1*, *TNFAIP3*, and *TNFRSF14* as well as exons 2-5 of *MYD88* and the Y646 *EZH2* hotspot (*Online Supplementary Table S1*). In addition, all aggressive BCL were analyzed with the Ion Ampliseq TP53 Panel (Thermo Fisher

Scientific). Variant validation was performed using either a bidirectional single amplicon resequencing approach or, for ISFN samples, a second targeted NGS analysis after microdissection of affected GC. For a detailed description of library preparation, sequencing, data analyses and validation, including a primer list, see the *Online Supplementary Appendix* and the *Online Supplementary Table S2*.

Results

Clinical features

Ten cases of aggressive BCL and paired ISFN were included (Table 1). In six cases, the aggressive lymphoma was considered *de novo*, whereas four cases also had an associated FL. In two of the four latter cases, the FL component was only detected during the screening of lymphoid tissues originally interpreted as reactive, while the two others had a history of FL. None of the six *de novo* cases developed or relapsed as FL during follow-up, which ranged from 7 to 54 months. In seven of ten cases, the ISFN lesions were present simultaneously in seemingly non-involved lymph nodes (LN) adjacent to the aggressive component. In cases 4, 5, and 7, the ISFN was identified retrospectively in LN resected for other reasons several years prior to the DLBCL diagnosis.

Histological and immunohistochemical findings

The ISFN samples exhibited the typical features with overall preserved LN architecture and involved GC showing strong staining for BCL2 and CD10 and a very low proliferation rate (Figure 1A and B). All aggressive BCL, including eight DLBCL (one case with two samples) and three DH/TH HGBL, were classified as GCB subtype according to the Hans algorithm and expressed BCL2 (Table 2; Figure 1D). The aggressive tumors of cases 1 and 3 exhibited strong and homogenous P53 expression, whereas the DLBCL of case 2 was completely negative. All of these samples showed *TP53* mutations (Figure 2; *Online Supplementary Table S4*). All FL were grade 1/2 and expressed BCL2. A summary of immunohistochemical findings is included in the *Online Supplementary Table S3*.

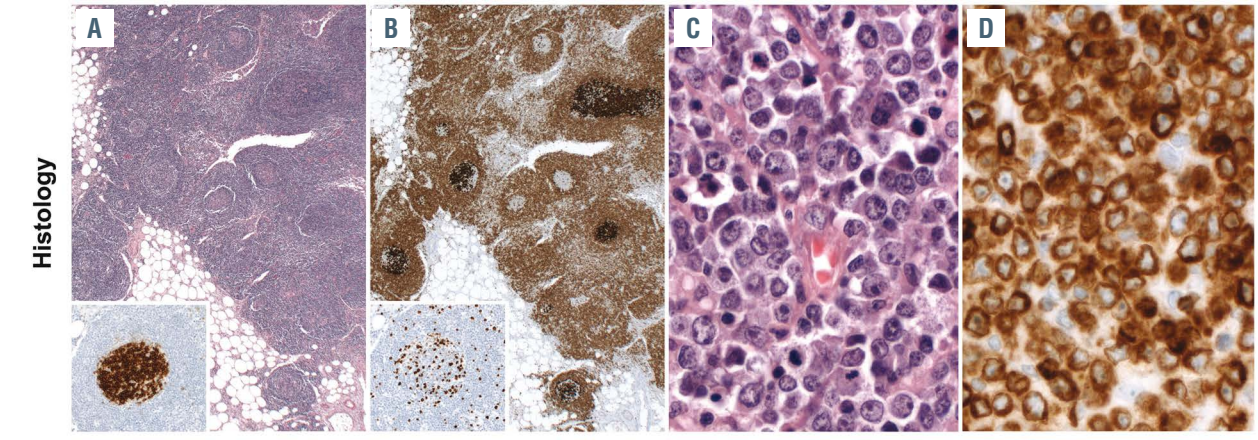
Chromosomal translocations

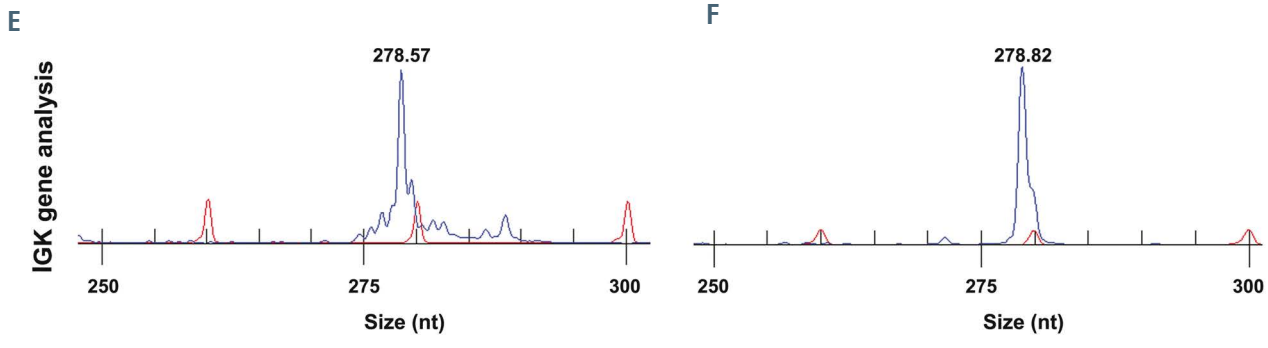
FISH analysis with a *BCL2* break-apart probe confirmed a break in *BCL2* for all samples of nine cases, indicative of

Table 1. Clinical data of patients with *in situ* follicular neoplasia and aggressive B-cell lymphoma.

Patient	Age* (years)	Sex	Diagnosis	Site	Stage	Additional information	Follow-up (Treatment)
De novo aggressive B-cell lymphoma							
1	59	M	ISFN	Mesenterial LN		Synchronous with HGBL	
			HGBL-TH	Mesenterial LN	IVA/IE		CR – 7 months (Sx + CT)
2	61	F	ISFN	Mesenterial LN		Synchronous with DLBCL	
			DLBCL	Mesenterial LN	N/A		N/A
3	64	M	ISFN	Axillary LN		Synchronous with primary DLBCL	
			DLBCL	Axillary LN	IIIA	Primary DLBCL	
			DLBCL	Cervical LN		7 months after primary DLBCL	DOD (CT+ RT)
4	76	F	ISFN	Mesenterial LN		Resected during surgery for colorectal cancer 159 months prior to DLBCL diagnosis	
			DLBCL	Paravertebral	IIA		CR – 54 months (CT)
5	67	M	ISFN	Mesenterial LN		Resected during surgery for bowel obstruction 42 months prior to DLBCL diagnosis	
			DLBCL	Paraaortic LN	IIIA		CR – 38 months (CT)
6	74	F	ISFN	Mesenterial LN		Synchronous with DLBCL	
			DLBCL	Small intestine	N/A		N/A
Transformed FL							
7	54	F	ISFN	Axillary LN		Sentinel node for melanoma 116 months prior to DLBCL diagnosis	
			FL	Cervical LN		Synchronous with DLBCL, Grade 1/2	
			DLBCL	Cervical LN	IIIS		CR – 54 months (CT)
8#	74	F	ISFN	Mesenterial LN		Synchronous with HGBL	
			FL	Mesenterial LN		Synchronous with HGBL, Grade 1/2	
			HGBL-DH	Small intestine	IIA		CR – 90 months (Sx + CT)
9	64	M	ISFN	Mesenterial LN		Synchronous with HGBL	
			FL	Mesenterial LN		Synchronous with HGBL, Grade 1/2	
			HGBL-DH	Large intestine	IVB		CR – 133 months (Sx + CT)
10	54	M	ISFN	Mesenterial LN		Synchronous with DLBCL	
			FL	Mesenterial LN		Synchronous with DLBCL, Grade 1/2	
			DLBCL	Mesenterial LN	IIA		CR – 158 months (Sx + CT)

CR: complete remission; CT: chemotherapy; DOD: dead of disease; F: female; M: male; N/A: information not available; RT: radiotherapy; Sx: surgery; ISFN: *in situ* follicular neoplasia; FL: follicular lymphoma; HGBL-TH: high-grade B-cell lymphoma triple-hit; HGBL-DH: HGBL double-hit; DLBCL: diffuse large B-cell lymphoma; LN: lymph node. *Age at diagnosis of aggressive B-cell lymphoma. #FL not available for analysis.





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BCL2 fusion

	BCL2 sequence (MBR)	*de novo* nucleotides	JH sequence (J6)
ISFN	...TCCTGCCCTCCTTCCGCGGGG	AGAACGGCGTCAAGGTTCGGTCC	CTAATACGGTATGGACGTCTGG...
HGBL	...TCCTGCCCTCCTTCCGCGGGG	AGAACGGCGTCAAGGTTCGGTCC	CTAATACGGTATGGACGTCTGG...

Figure 1. Morphology and molecular findings of the clonally related *in situ* follicular neoplasia and *de novo* high-grade B-cell lymphoma of case 1. (A) "Reactive" lymph node (LN) with *in situ* follicular neoplasia (ISFN) stained with hematoxylin and eosin (H&E). Note the intact lymphoid architecture. Inset: higher magnification of a strongly CD10 positive germinal center (GC). Original magnification 25x and 100x. (B) Strong BCL2 expression in GC colonized by ISFN. Inset: MIB1 stain with higher magnification demonstrating a low proliferation index. Original magnification 25x and 100x. (C and D) LN biopsy depicting high-grade B-cell lymphoma (HGBL) stained with H&E and BCL2. Original magnification 400x. (E and F) Immunoglobulin κ light chain (IGK) GeneScan analysis demonstrating a rearrangement involving the κ deleting element (Kde) with matching monoclonal peaks of 279 nucleotides (nt) in both lesions. (G) Sequencing of the *BCL2* breakpoint revealed an identical *BCL2*/JH junction in both samples, confirming their clonal relationship.

the t(14;18)(q32;q21) translocation (Table 2). In case 5, *BCL2* and IGH break-apart probes did not demonstrate re-arrangements in either sample. However, using an IGH/*BCL2* dual-color, double fusion probe, both ISFN and DLBCL showed an aberrant hybridization pattern with a single fusion signal, suggesting a cryptic *BCL2* translocation. Amplification of the *BCL2* breakpoint was successful for the samples of seven cases (cases 1, 3, 6, 7, 8, 9, and 10), with six breaks located in the major breakpoint region (MBR) and one (case 8) in the 3'MBR subcluster. Sequencing confirmed identical breakpoints for all paired samples (Figure 1G). *MYC* translocations were demonstrated in the aggressive component of three cases (cases 1, 8, and 9), with an additional break in *BCL6* in case 1, resulting in a diagnosis of HGBL with DH or TH, respectively (Figure 2).The corresponding ISFN and FL lesions showed no alterations of *MYC* or *BCL6*.

Clonality and immunoglobulin sequence analysis

The results of the IG analysis are summarized in Table 2 and Figure 2. A clonal relationship based on IG rearrangements was demonstrated for seven of ten paired samples (cases 1, 2, 4, 7, 8, 9, and 10) by NGS of the IGH and/or by an identical clonal peak in IGH or IGK GeneScan analysis (Figure 1E and F). In case 10, the presence of a clonal IGH rearrangement in the ISFN was demonstrated by the use of clone-specific primers, which produced the same peak of 127 base pairs in the paired ISFN, FL and DLBCL lesions, confirming their clonal relationship (see *Online Supplementary Appendix*). In case 6, NGS demonstrated a clonal rearrangement in the DLBCL, but not in the corresponding ISFN, although both samples were shown to be clonally related by sequencing of their BCL2 breakpoint. Clone-specific primers designed for the DLBCL rearrangement also failed to amplify a specific product in the paired ISFN (see *Online Supplementary Appendix*). In contrast, cases 3 and 5 did not exhibit amplifiable clonal IG rearrangements in any of the samples. Thus, together with the results of the *BCL2* breakpoint analysis, a clonal relationship between the ISFN and the corresponding lymphomas was firmly established for five of six *de novo* and four of four transformed cases.

Among samples successfully sequenced with the Lymphotrack Assay, we found novel N-glycosylation

Table 2. Cell of origin, *BCL2* translocation and immunoglobulin gene analysis.

Case	Diagnosis	Cell of origin based on Hans algorithm	BCL2 FISH	BCL2 breakpoint	Clonality*	IGH V/J usage	Glycosylation site Location	Glycosylation site Motif#	Status
De novo aggressive B-cell lymphoma									
1	ISFN	—	+	MBR-JH	Mono	V3/J4	CDR3	NLS	Clonally related
	HGBL-TH	GCB	+	MBR-JH	Mono	V3/J4	CDR3	NLS	
2	ISFN	—	+	Neg	Mono	V2/J4	CDR3	NDS	Clonally related
	DLBCL	GCB	+	Neg	Mono	V2/J4	CDR3	NTS	
3	ISFN	—	+	MBR-JH†	Poly	—	—		Clonally related
	DLBCL	GCB	+	MBR-JH	Poly	—	—		
	DLBCL	GCB	+	MBR-JH	Poly	—	—		
4	ISFN	—	+	Neg	Mono	V3/J6	CDR3	NAS	Clonally related
	DLBCL	GCB	+	Neg	Mono	V3/J6	CDR3	NAS	
5	ISFN	—	+‡	Neg	Poly	—	—		Clonally related§
	DLBCL	GCB	+‡	Neg	Poly	—	—		
6	ISFN	—	+	MBR-JH	Poly	—	—		Clonally related
	DLBCL	GCB	+	MBR-JH	Mono	V3/J3	FR3	NLT	
Transformed FL									
7	ISFN	—	+	MBR-JH	Mono	V3/J6	CDR3	NLT	Clonally related
	FL	—	+	MBR-JH	Mono	V3/J6	CDR3	NLT	
	DLBCL	GCB	+	MBR-JH	Mono	V3/J6	CDR3	NLT	
8	ISFN	—	+	3'MBR-JH	Mono	V3/J3	FR2/CDR2	NIT	Clonally related
	HGBL-DH	GCB	+	3'MBR-JH	Mono	V3/J3	None (FR1 PCR)		
9	ISFN	—	+	MBR-JH†	Mono	V3/J4	CDR3	NCS	Clonally related
	FL	—	+	MBR-JH	Mono	V3/J4	CDR3	NCS	
	HGBL-DH	GCB	+	MBR-JH	Mono	V3/J4	None (FR2 PCR)		
10	ISFN	—	+	MBR-JH	Mono"	V1/J6	CDR3	NFS	Clonally related
	FL	—	+	MBR-JH	Mono"	V1/J6	CDR3	NYS	
	DLBCL	GCB	+	MBR-JH	Mono"	V1/J6	CDR3	NYS	

CDR: complementarity-determining region; FR: framework region; Neg: negative (i.e., no *BCL2* rearrangement detected by polymerase chain reaction [PCR]); V/J: variable/joining gene segment; ISFN: *in situ* follicular neoplasia ; FL: follicular lymphoma; HGBL-TH: high-grade B-cell lymphoma triple-hit; HGBL-DH: HGBL double-hit; DLBCL: diffuse large B-cell lymphoma; LN: lymph node; MBR: major breakpoint region; GCB: germinal center B-cell-like; IGH: immunoglobulin H. *Based on Lymphotrack and/or GeneScan analysis. #Single-letter amino acid code. †Amplified with breakpoint-specific primers. ‡Demonstrated using an IGH/BCL2 dual-color, double fusion probe. §Based on the demonstration of shared mutations. "Demonstrated with clone-specific primers.

sites in seven of seven ISFN, three of three FL and six of eight aggressive BCL. In three cases (cases 1, 4, and 7), the ISFN and their transformed counterpart(s) demonstrated identical glycosylation sites, whereas two ISFN (cases 2 and 10) showed motifs at the same location, but with a different sequence than those exhibited by the clonally related manifest lymphomas. Moreover, two HGBL lacked N-glycosylation sites, although novel motifs were detected in the related ISFN (cases 8 and 9) and FL (case 9) lesions. Intraclonal heterogeneity of the clonal IGH rearrangement was present in all types of samples. However, heterogeneity was more pronounced in the precursor lesions, as evidenced by more evenly distributed subclones, whereas DLBCL and HGBL samples exhibited one or two subclones that were highly dominant. Phylogenetic trees constructed for five cases demonstrated separate clustering of ISFN and DLBCL/HGBL sequences indicative of divergent evolution (*Online Supplementary Figure S1*).

Mutational analysis reveals distinct clonal evolution patterns

BCL2 was the most frequently mutated gene, with all samples harboring at least one non-synonymous, synonymous or 5'UTR mutation, although most samples, including seven of ten ISFN lesions, demonstrated several BCL2 mutations (Figure 2; *Online Supplementary Tables S4* and *S5*). Other recurrently mutated genes were *CREBBP* (11 of 24 samples), *KMT2D* (11 samples), and *EZH2* (ten samples), as well as *TNFRSF14*, *IGLL5*, and *GNA13*. In ISFN lesions, mutations in chromatin modifying genes remained the most frequent alterations, with five samples showing a *CREBBP* mutation. In contrast, *TP53* (four samples), *CD79B* (one sample) and *HIST1H1B* (one sample) were exclusively altered in the aggressive components. All *TP53* mutations were located in the DNA binding domain, with variant allele frequencies ranging from 52% to 84%, indicating a loss of the second allele. For two samples (cases 1 and 2), this was confirmed by FISH.

Of the nine cases clonally related by IG and/or *BCL2* breakpoint sequence analysis, all but one (case 1) demonstrated shared mutations between the ISFN and the transformed counterpart(s), ranging from one to six shared mutations per paired samples. For example, the DLBCL of case 4 had four non-synonymous mutations of *BCL2*, *TNFRSF14*, *HIST1H1D* and *EP300* in common with the ISFN that was present 159 months prior. However, ISFN and DLBCL of case 5 also exhibited matching *KMT2D* p.(Q4473*) and *IGLL5* p.(C3S) mutations, demonstrating their clonal relationship despite the lack of a detectable clonal IG re-arrangement or *BCL2* translocation sequence. All investigated FL showed more than one mutation shared with both the ISFN and the aggressive BCL. Mutations only present in the clinically manifest lymphomas were observed in all cases with the exception of one FL (case 9). Nevertheless, six ISFN lesions also carried private variants that were not identified in their clonally related counterparts, indicating early divergence. The

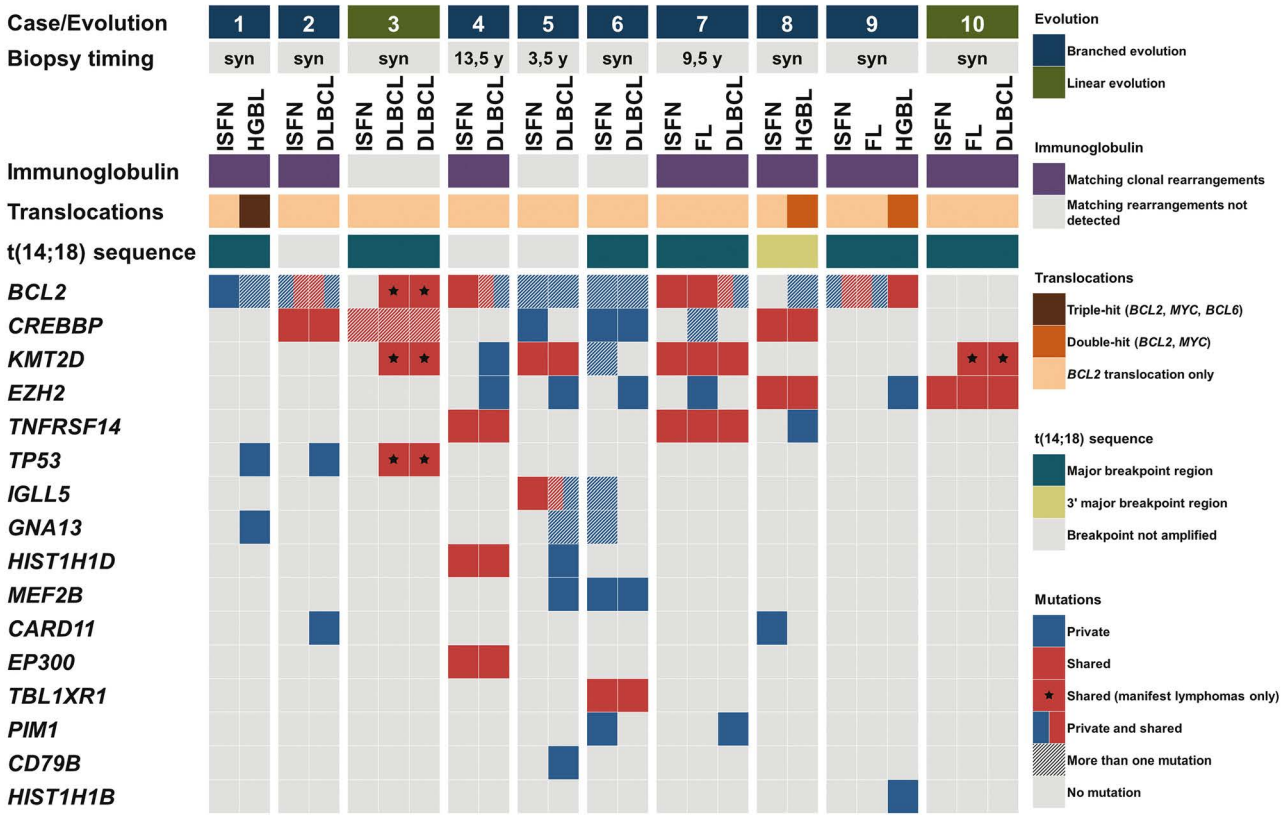

Figure 2. Overview of clonality results, translocations and non-synonymous variants in *in situ* follicular neoplasia, follicular lymphoma and aggressive B-cell lymphoma samples. Each column of the heatmap represents a sample with paired samples displayed next to each other. Rows represent an analysis or a gene. Genes are ordered from top to bottom according to the mutational frequency across all samples. Biopsy timing specifies the time period between the occurrence of the *in situ* follicular neoplasia (ISFN) and the diagnosis of aggressive B-cell lymphoma (BCL) in years ("y"), rounded to the nearest half year, with "syn" indicating a synchronous occurrence. In case 4, the assumption of branched evolution is based on a private 5'UTR mutation of *BCL2* in the ISFN (mutation not shown). Concerning case 9, "Private and shared mutations" of *BCL2* indicate that ISFN and follicular lymphoma (FL) exhibit a shared *BCL2* mutation not present in the high-grade B-cell lymphoma while a second *BCL2* mutation was shared between all three lesions.

ISFN of case 6 harbored the highest number of private mutations, with a total of 13 different non-synonymous alterations of *BCL2*, *KMT2D*, *CREBBP*, *GNA13*, *MEF2B*, *PIM1*, *TBL1XR1*, and *IGLL5*, as well as six synonymous and 5'UTR variants of *BCL2*, of which only a single *TBL1XR1* p.(L198*) mutation was shared with the clonally related aggressive BCL.

Based on the distribution of private and shared variants, two different patterns of clonal evolution from ISFN to aggressive BCL could be reconstructed (Figure 3; *Online Supplementary Figure S2*). The most frequent scenario (cases 1, 2, 4, 5, 6, 7, 8, and 9) was that of branched evolution, where aggressive lymphoma, ISFN and, when present, FL evolved from a common progenitor but gained distinct private mutations (Figure 3A and B). In contrast, the available data indicate a linear evolution in cases 3 and 10, where the DLBCL shared all ISFN mutations but gained additional alterations (Figure 3C).

Discussion

In this study, we analyzed the clonal evolution of t(14;18)+ aggressive BCL from the earliest morphologically identifiable putative precursor lesion - ISFN, using paired samples of ISFN and DLBCL or HGBL with DH/TH, with and without FL as an intermediate step. The clonal relationship of ISFN and aggressive BCL samples was confirmed by either identical IGH and/or *BCL2* rearrangements and/or the demonstration of shared somatic mutations in genes frequently affected in BCL of GC origin. This study demonstrates for the first time the evolution of "*de novo*" aggressive BCL from ISFN. Moreover, we identified different pathways of clonal evolution with an early branching pattern (early divergence) as the most frequent scenario.

The progression from ISFN to FL and the transformation of FL to DLBCL or HGBL are well-established. Our study expands these observations and suggests that a direct evolution of t(14;18)+ aggressive BCL from ISFN is possible. This finding is not surprising, given the common presence of discordant (i.e., low-grade) bone marrow infiltration in *de novo* DLBCL and the occasional recurrence of DLBCL as FL.[26-29] As for any other neoplasm with a stepwise evolution, we cannot entirely exclude the presence of a clinically and morphologically undetected FL. However, given the fact that approximately 30% of *de novo* DLBCL carry a *BCL2* translocation, t(14;18)+ DLBCL arising from ISFN without preceding FL could be a common phenomenon.[3,23] The more recent analyses of the molecular landscape of DLBCL also support this hypothesis of a shared progenitor population, since t(14;18)+ *de novo* DLBCL revealed a mutational signature very similar to FL.[30,31] In ISFN and *de novo* DLBCL of case 5, we were

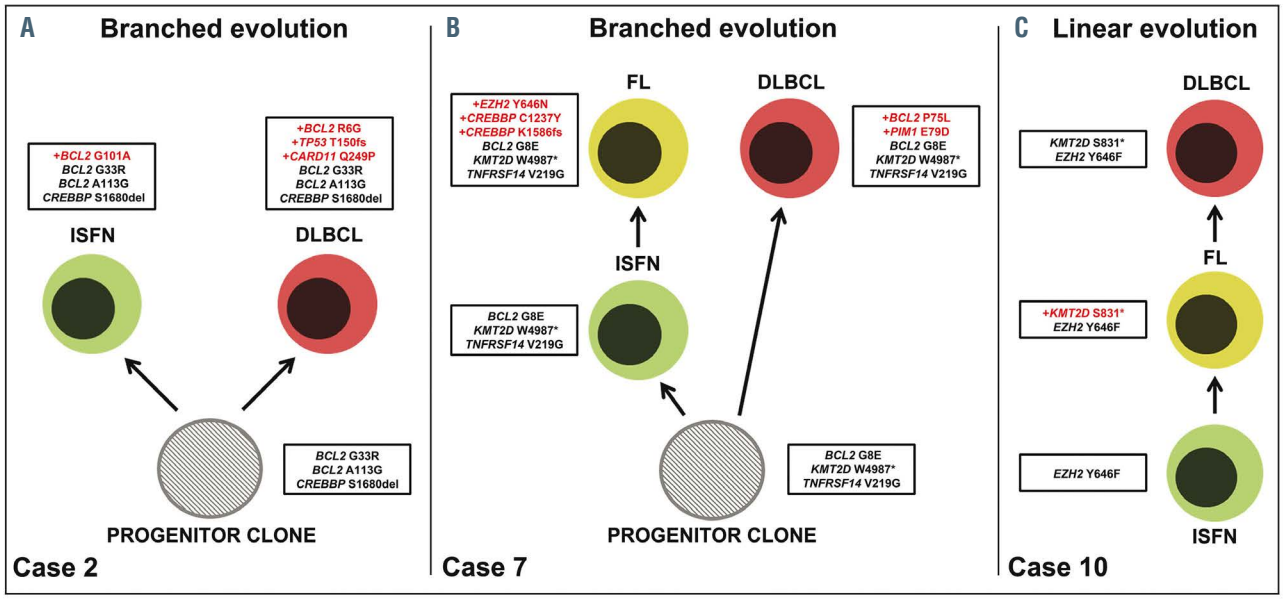

Figure 3. Different patterns of clonal evolution from *in situ* follicular neoplasia to aggressive B-cell lymphoma based on the distribution of mutations. Variants are depicted at protein level. Mutations highlighted in red were gained during the evolution. Synonymous and 5'UTR variants of *BCL2* are not shown, but were also taken into account for the construction. The existence of "Progenitor clones" was assumed on the basis of shared mutations. (A) *In situ* follicular neoplasia (ISFN) and diffuse large B-cell lymphoma (DLBCL) evolved divergently from a common progenitor clone. (B) ISFN and DLBCL evolved divergently from a common progenitor, with the ISFN subsequently progressing to follicular lymphoma (FL). (C) FL and DLBCL evolved directly from the ISFN and gained additional alterations.

unable to amplify a clonal B-cell rearrangement or the *BCL2* translocation sequence. Both samples, however, demonstrated a *BCL2* translocation only detectable by FISH using an IGH/*BCL2* dual-color, double fusion probe, possibly the result of a cryptic, non-canonical *BCL2* rearrangement.[32] This, in combination with shared mutations of *KMT2D* and *IGLL5*, serves as evidence of a common clonal origin.

Clonally related ISFN can be identified not only before or simultaneously with manifest lymphoma, but may also be present even years after the malignant transformation took place, most likely representing a subclone that diverged at an earlier stage of the disease.[14,33] Persisting precursors, presumably more resistant to chemotherapy, may therefore play a role in lymphoma relapse as well. Indeed, studies of FL and DLBCL relapses have shown that both the primary and the recurrent lymphoma often represent divergent subclones that arose independently from a common progenitor, which again supports our hypothesis that aggressive BCL can develop directly from a premalignant precursor.[22,34] The existence of such progenitor populations has been exemplified in two reports of clonally related FL and clonally related DLBCL arising in both donor and recipient after hematopoietic stem cell transplantation.[35,36] In both studies, the related lymphomas exhibited multiple shared alterations, which were therefore acquired prior to transplantation.[35,36] Likewise, FL and transformed FL have been shown to often arise by divergent evolution.[37,38] The complexity of this evolutionary process is also reflected in our paired ISFN and aggressive BCL cases, since the distribution of private and shared mutations suggests an early subclonal divergence for the majority of cases. This is supported further when IG data are taken into account, given the high frequency of different glycosylation sites in both components, and the phylogenetic trees based on SHM patterns of rearranged IGH sequences, which are compatible with a divergent evolution. Linear evolution from ISFN to aggressive BCL, at least based on the available mutational data, seems to be less frequent, but similar findings have been reported regarding the transformation of FL.[38] Our IGH data also provide insight into the process of early clonal selection. The more balanced distribution of subclones in the ISFN implies that at this point, no subclone has acquired a decisive selection advantage. In contrast, the aggressive BCL lesions demonstrated one or two highly dominant subclones, which possibly emerged after obtaining crucial secondary genetic alterations that improved clonal fitness and further paved the way towards high-grade malignancy.

The most commonly mutated gene in our study was *BCL2*, likely because *BCL2* juxtaposed to an IG promotor results in a significantly higher number of mutations compared to non-translocated counterparts, as a result of targeting by AID.[39,40] The abundance of *BCL2* mutations across ISFN lesions, as well as the intraclonal heterogeneity revealed by the IGH sequence analysis and the detection of novel N-glycosylation sites confirm that the process of SHM is ongoing in ISFN.[15,19] Prolonged AID activity is regarded as an important factor in the pathogenesis of GC-derived lymphomas and especially FL.[21,22,41] In a mouse model, multiple re-entries of long-lived BCL2+ B-cell clones into the GC environment resulted in the accumulation of secondary alterations with a mutational signature consistent with AID-mediated mutagenesis.[42] The same concept has been proposed for human FL development, where FLLC are subject to similar dynamics with an extensive dissemination throughout the body, leading to a multitude of subclones exhibiting different SHM signatures as evidence of their GC passage.[17,42] Since ISFN is considered the tissue-based counterpart of FLLC, our data also indicate that circulating t(14;18)+ FLLC clones might serve as precursors to aggressive BCL as well.[42,43]

The mutational spectrum identified in our study is consistent with published data obtained in FL and t(14;18)+ DLBCL. Both lymphomas were shown to be particularly enriched for mutations in the epigenetic regulators *CREBBP*, *EZH2*, *KMT2D*, and *EP300*, as well as for alterations of *TNFRSF14*, a gene encoding for a receptor of the tumor necrosis factor family.[23,30,31,37,38] *CREBBP* mutations have consistently been described as drivers of lymphomagenesis and as early genetic events in FL.[22,44-47] In agreement with this and in line with a recent report, we found *CREBBP* to be the most commonly affected gene in ISFN samples, with mutations being shared with the clonally related manifest lymphomas in three cases.[15] Moreover, ISFN and DLBCL of case 4, which lacked *CREBBP* alterations, exhibited a shared mutation of the closely related acetyltransferase *EP300*. *CREBBP* and *EP300* mutations have been suggested to play similar roles in the pathogenesis of FL and DLBCL and are therefore usually mutually exclusive.[23,47,48] Alterations of *EZH2*, *KMT2D*, and *TNFRSF14* have been described as both early driver and as accelerator mutations.[37,38,44-46,49] We confirm that these mutations can occur during the presumably earliest stages of the disease, evidently years before the diagnosis of malignant lymphoma.[14,15] Alterations of *KMT2D* and *EZH2* were, however, detected only in the manifest lymphomas of three of and five of seven cases respectively, suggesting they were often acquired later, possibly driving the clone towards the malignant transformation. Notably, *BCL2* mutations frequently occur at the earliest stages as well and are likely primarily an indicator of AID activity, rather than heralding aggressive behavior, as suggested by other authors.[50] *PIM1* and *IGLL5*, two additional genes known to be affected by aberrant SHM, were also mutated in one and two ISFN samples, respectively.[31] Re-arrangements of *MYC* and alterations of *TP53* are common drivers of FL transformation.[38] Accordingly, five of our cases carried these genetic alterations and as expected, they were only detected in the aggressive BCL and not in the clonally related ISFN lesions. However, due to technical limitations, the presence of these mutations in minor ISFN subclones cannot be excluded completely.

Although we were able to investigate the evolution of ISFN at multiple levels, this study has some limitations, in particular, the small sample size due to the rarity of identifiable ISFN lesions in patients with aggressive BCL, which warrants validation in further studies. The necessary restriction to FFPE tissue also narrowed the scope of feasible analyses and raised the detection threshold in our targeted NGS analysis because of low level sequencing artifacts. Nevertheless, systematic validation allowed us to delineate the clonal and genetic evolution of aggressive BCL starting from an early progenitor lesion.

In summary, our data extend previous studies and provide first evidence that t(14;18)+ DLBCL and HGBL can arise from clonally related ISFN without FL as an intermediate step. Moreover, during this progression, similar to the clonal evolution and transformation of FL, branched evolution with both private and shared alterations is common. Our results further confirm that ISFN is subject to persistent AID activity and frequently acquires secondary genetic alterations, in addition to the defining t(14;18) translocation.

Disclosures

No conflicts of interest to disclose.

Contributions

FF, IB and AV wrote the manuscript; FF conceived and designed the study, selected the cases, and supervised the experimental work and data analysis; LQ-M helped designing the study, reviewed the cases and helped writing the manuscript; AV performed the experimental work and analyzed the data; JuS, JaS, and IB supervised the experimental work and data analysis; BM performed FISH analysis; BF and IAM-M helped with case selection; PB and SN provided bioinformatics support and constructed the phylogenetic trees; MR-P, MAP, KH and GO contributed with cases and provided clinical information. All contributing authors revised the manuscript.

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