Widespread in situ follicular neoplasia in patients who subsequently developed follicular lymphoma

Rachel Dobson1, Andrew Wotherspoon2, Shizhang Alexander Liu1, Francesco Cucco1, Zi Chen1, Yuan Tang1,3 and Ming-Qing Du1,4*

1 Division of Cellular and Molecular Pathology, Department of Pathology, University of Cambridge, Cambridge, UK
2 Histopathology Department, The Royal Marsden Hospital, London, UK
3 Department of Pathology, West China Hospital, Sichuan University, Chengdu, PR China
4 Department of Histopathology, Addenbrooke’s Hospital, Cambridge University Hospitals NHS Foundation Trust, Cambridge, UK

*Correspondence to: M-Q Du, Division of Cellular and Molecular Pathology, Department of Pathology, University of Cambridge, Level 3 Lab Block, Box 231, Addenbrooke’s Hospital, Hills Road, Cambridge, CB2 0QQ, UK. E-mail: mqd20@cam.ac.uk

Abstract

In situ follicular neoplasia (ISFN) is usually an occasional incidental finding in lymph nodes by BCL2 immunohistochemistry, and its true scale is unknown. We have identified six cases of follicular lymphoma (FL) with a history of solid neoplasm 4–16 years ago, from which ISFN was identified widely in the surgically cleared lymph nodes (LNs). Using clone-specific PCR and BaseScope in situ hybridisation with primers or probes specific to the VDJ or BCL2–IGHJ junction sequence, we confirmed the clonal identity among different ISFNs and overt-FL in each of the four cases successfully investigated. Mutation analyses of overt-FL by targeted next-generation sequencing identified multiple potential pathogenic changes involving CREBBP, EZH2, KMT2D, TNFRSF14, and STAT6. Further investigations of these mutations in paired ISFNs using Fluidigm PCR and Illumina sequencing showed the presence of the FL-associated mutations in early lesions for two of the six cases investigated (CREBBP and KMT2D in one case and STAT6 in the other), with one case displaying stepwise accumulation of its observed mutations. Remarkably, there were considerable divergences in BCL2 variants among different ISFN-involved lymph nodes in all four cases successfully investigated, indicating ongoing intraclonal diversification by somatic hypermutation machinery. Our findings demonstrate widespread distribution of ISFN lesions, further implicating their dynamic nature with the neoplastic cells undergoing active trafficking and clonal evolution.

Keywords: in situ follicular neoplasia; follicular lymphoma; clonal evolution; somatic mutation

Received 15 June 2021; Revised 7 December 2021; Accepted 23 December 2021

Conflict of interest statement: M-QD is an Associate Editor of The Journal of Pathology. No other conflicts of interest were declared.

Introduction

Follicular lymphoma (FL) is characterised by t(14;18) (q32;q21)/IGH::BCL2 and expansion of the transformed translocation-positive cells in B-cell follicles. The translocation juxtaposes the BCL2 gene to the immunoglobulin heavy chain joining region (IGHJ) and causes BCL2 overexpression. The translocation is caused by erroneous genomic rearrangements during VDJ recombination at the pre-B stage of B-cell development in the bone marrow. Most breakpoints in the BCL2 genes are clustered, thus allowing detection of the BCL2–IGHJ fusion by PCR [1].

Apart from lymphoma, the translocation is found in peripheral blood lymphocytes in more than 50% of healthy adults by PCR [2]. These circulating translocation-positive cells are most likely equivalent to those of in situ follicular neoplasia (ISFN) in lymph nodes (LNs) identified incidentally by BCL2 immunohistochemistry [3,4]. The B-cell follicles involved by ISFN usually display only subtle changes that are not readily identifiable using conventional H&E staining but are recognised by strong BCL2 staining in germinal centre B-cells [5]. Generally, few ISFNs progress into an overt B-cell lymphoma. However, its true incidence remains unclear [3,6].

There are limited studies documenting the evolution of ISFN to subsequent FL or other B-cell lymphomas, and their underlying genetic changes [7–9]. Several FL-associated somatic genetic changes have been found previously in ISFN, including CREBBP, KMT2D, EZH2, and TNFRSF14 mutations [7–9]. However, most previous studies involve analysis of a single ISFN tissue specimen. Therefore, the true extent of ISFN lesions and
their intraclonal genetic changes are unknown. We have identified six cases of FL with a history of solid neoplasia, from which ISFN was retrospectively identified widely in the surgically removed LNs. The present study reports the extent of ISFN lesions, their clonal relationship, and underlying genetic changes.

### Table 1. Case information and number of LNs showing prominent ISFN involvement with at least one follicle displaying clustered or diffuse BCL2 positivity.

| Case | Original diagnosis | Original therapy applied | ISFN in cleared lymph nodes | Follicular lymphoma (FL) | FL treatment |
|------|--------------------|-------------------------|-----------------------------|--------------------------|--------------|
| A    | 52 years/female: Left breast ductal carcinoma | Chemotherapy, followed by mastectomy and axillary lymph node clearance | Seen in 4/17 lymph nodes cleared | 6 years later | Right axillary lymph node biopsy, FL, grade 2, stage IV |
| B    | 45 years/female: Right breast ductal carcinoma | Lumpectomy and axillary lymph node clearance, followed by chemotherapy | Seen in 3/21 lymph nodes cleared | 16 years later | Left groin lymph node biopsy, FL, grade 1–2, stage III |
| C    | 48 years/female: Left lower leg melanoma | Surgical excision and ilio-inguinal lymph node clearance | Seen in 14/19 lymph nodes cleared | 6 years later | Axillary lymph node biopsy, FL, grade 1, stage IIIa |
| D    | 57 years/male: Gastric adenocarcinoma | Chemotherapy, followed by total gastoectomy | Seen in 4/30 lymph nodes cleared | 4 years later | Left level II neck lymph node, FL, grade 1, stage I |
| E    | 49 years/male: Metastatic moderately differentiated squamous cell carcinoma in a neck lymph node | Radiotherapy (50 Gy) | Seen in 3/4 lymph nodes cleared | 4 years later | Right neck lymph node, FL, grade 3a, stage I |
| F    | 48 years/male: Pleomorphic salivary gland adenoma | Surgical excision | Seen in 1/2 lymph nodes cleared | 7.5 years later | Tonsil, FL, grade 1–2, stage III |

DLBCL, diffuse large B-cell lymphoma; R, rituximab; PMitCEBO, prednisolone, mitoxantrone, cyclophosphamide, etoposide, bleomycin, and vincristine; CODOX-M–IVAC, cyclophosphamide, vincristine, doxorubicin, methotrexate, ifosfamide, etoposide, and cytarabine; CVP, cyclophosphamide, vincristine, and prednisone; Gy, Gray.

Figure 1. The extent of ISFN involvement in lymph nodes (LNs) removed from solid neoplasia surgery. Variations of BCL2 staining in various LNs that show prominent ISFN involvement as defined above (see Figure 3 for examples). The mean and range number of follicles involved by ISFN in each case are indicated. Case F has been excluded from the analysis as the slides available for research were not evaluable. Data includes only the LNs that show prominent ISFN involvement with at least one follicle displaying clustered or diffuse BCL2 positivity, and those with minimal involvement (few scattered strong BCL2-positive cells in a germinal centre) are not included.
Case and tissue materials

Local ethical guidelines were followed for the use of archival tissues for research with the approval of the ethics committee (05-Q1604-10). The study included six FL cases with a history of solid neoplasia 4–16 years ago, from which ISFN was identified widely in the surgically removed LNs by BCL2 immunohistochemistry (Table 1).
Methodology outline
Detailed methods are provided in Supplementary materials and methods.

In brief, DNA was extracted from whole LN tissue sections of FL or microdissected BCL2-positive follicles from LNs involved by ISFN. The rearranged IG genes

A  BCL2 immunohistochemistry

B  BCL2-JH PCR & clone-specific PCR

C  Overt follicular lymphoma  In situ follicular neoplasia

Figure 3  Legend on next page.
and BCL2–IGHJ fusion were amplified from the FL samples using the respective BIOMED-2 assays [1] and sequenced using the Illumina MiSeq method [10,11].

In four cases, a clone-specific PCR (CS-PCR) was designed using a primer targeting the unique V(D)J sequence of the clonal IGH/IGK rearrangement, or the BCL2–IGHJ fusion. CS-PCR was then used to screen the FL clonally related cells in ISFNs.

In addition, BaseScope in situ hybridisation (BS-ISH) (Advanced Cell Diagnostics, Newark, CA, USA) using DNA probes specific to the unique VDJ or BCL2–IGHJ junctional sequence was performed to depict the localisation of FL clonally related cells.

Somatic mutations in FL were investigated by targeted sequencing of 70 genes (Agilent Technologies, Santa Clara, CA, USA) [12,13]. These mutations were then investigated in ISFN samples using Fluidigm PCR (Fluidigm Access Array System, South San Francisco, CA, USA) and Illumina sequencing (Illumina, San Diego, CA, USA) [11,12].

Results

Widespread ISFNs in LNs removed from solid neoplasia surgery

Several LNs cleared from the solid neoplasia surgery in cases A–E showed clear evidence of ISFN involvement by BCL2 immunohistochemistry, with one or more follicles displaying clustered or diffuse BCL2 positivity (Table 1 and Figure 1). Most of the remaining LNs in each of these cases also displayed some scattered strong BCL2-positive cells in follicle centres, with staining intensity much higher than that of germinal centre T-cells, suggesting potential involvement by ISFN cells (data not included in Figure 1 and Table 1).

Clonal relationship between ISFNs and overt-FL by CS-PCR

Since the DNA quality from ISFN samples was poor (only amenable for optimal amplification of up to 200 bp genomic fragments), inadequate for conventional PCR of the rearranged IG genes and BCL2–IGHJ fusion, we first investigated the overt-FL by PCR and sequencing of the clonally rearranged IG genes and BCL2–IGHJ fusion. We then designed CS-PCR using a primer targeting the unique V(D)J (cases A and B: IGH and IGK, respectively) or BCL2–IGHJ (cases C and D) junction sequence. CS-PCR sensitivity and specificity were attested using serial dilutions of the corresponding overt-FL DNA and a range of unrelated lymphoid specimens (supplementary material, Figures S1 and S3).

Next, we used CS-PCR to investigate whether the FL clone was present in the ISFN LNs removed from the solid neoplasia surgery (Figures 2 and 3 and supplementary material, Figures S1–S3). We demonstrated the presence of the FL clone in almost all the LNs involved by ISFN in cases A (4/4), C (13/14), and D (4/4). In case B, we showed the presence of the FL clone in one involved LN but not in the two remaining nodes where ISFN was inconspicuous in the remaining tissue available for research.

The CDR3 sequencing analysis of case A also revealed an N-glycosylation site.

Clonal relationship between ISFN and overt-FL by BS-ISH

To prove and depict the clonal relationship among ISFNs and FL, we performed BS-ISH in three cases. The probes were designed to bind the unique V-D (case A) or BCL2–IGHJ (cases C and D) junction sequence. In each case, the specificity of BS-ISH was ascertained by the expected hybridisation signals in the corresponding overt-FL but not in unrelated lymphoid tissues. BS-ISH identified the clonally related cells essentially in the follicle centre involved by ISFN in all LNs examined in each case (4/4 in case A, 6/6 in case C, and 3/3 in case D) (Figures 2 and 3 and supplementary material, Figure S2 and Table S1).

Comparison of mutations between paired overt-FL and ISFNs

We first investigated overt-FL by targeted sequencing of 70 genes [12,13]. The mutations identified were then screened in the ISFN LNs using PCR and Illumina sequencing. We identified potential pathogenic mutations in CREBBP, EZH2, KMT2D, TNFRS14, and STAT6 in overt-FL, and demonstrated their variable presence in the corresponding ISFNs (Figure 4A and supplementary material, Figure S4 and Table S2). In case A, there is evidence of stepwise accumulation of the observed mutations in the ISFN–overt-FL sequence (Figure 4A).
Figure 4  Legend on next page.
As BCL2 translocation causes its high transcriptional activities, hence predisposition to mutation by the somatic hypermutation machinery [14,15], we examined BCL2 sequence changes regardless of their impact on protein coding (Figure 4B). Remarkably, there were considerable divergences in BCL2 variants among different ISFN-involved LNs in the four cases successfully investigated and among different ISFN lesions within the same LN, indicating ongoing intraclonal diversifications.

Discussion

By comprehensive investigation of the LNs cleared from the solid neoplasia surgery in patients who subsequently developed an overt-FL, our present study uncovers the widespread distribution of ISFN lesions, involving many of the cleared LNs. Despite such extensive multifocal ISFN lesions in the cleared LNs, these ISFNs in each case are clonally related and linked to the subsequent overt-FL. Remarkably, such widespread ISFN lesions occurred in middle-aged individuals (45–57 years), and they remain dormant for a considerable period (4–16 years) before overt-FL diagnosis.

Although the overall risk of t(14:18)/IGH::BCL2 cells undergoing malignant transformation is low, a high level of their presence in the peripheral blood of healthy individuals is a predictive marker for FL development [16]. The widespread ISFNs in the cases investigated in the present study may also represent a relatively high clonal burden of t(14:18)/IGH::BCL2-positive cells. However, the retrospective nature of the present study does not allow direct comparison with previous observations based on peripheral blood samples. Assessing the risk of FL development based on the extent of ISFN involvement would be a formidable challenge, given the large number of cases required.

Our findings indicate that the neoplastic cells of ISFN are actively trafficking, transiting from one B-cell follicle to another and spreading widely among LNs. Like germinal centre B-cells, the ISFN cells show a high level of intraclonal variations in their rearranged IG genes, particularly among different LNs, as shown by previous case studies [17,18]. In line with this, our present study further demonstrates considerable sequence variations in the BCL2 gene among different ISFN lesions including those from the same LN, most likely caused by the somatic hypermutation machinery [14,15]. In keeping with the above observations, dynamic trafficking of BCL2-overexpressing B-cells and their multiple germinal centre transits have been elegantly documented in a mouse model study [18].

Comparison of the observed pathogenic mutations and BCL2 variants reveals evidence of the co-existence of different subclones in the same LN involved by ISFN (Figure 4). For example, the majority of the BCL2 variants detected in the ISFN-involved LNs for cases A and C were not found in overt-FL, despite sharing CREBBP/KMT2D or STAT6 mutations. There are three possible explanations: (1) these BCL2 variants are artefacts from the experimental system. However, this is unlikely as they are reproducible in two experimental replicates, with some being common to different ISFN lesions; (2) these variants are from reactive B-cells. Again, this is unlikely as BCL2 is not expressed in germinal centre B-cells, thus not targeted by the hypermutation machinery [14,19,20]; (3) these variants are present in a subclone(s) of ISFN but not in those that eventually progressed into FL. The third possibility is the most pertinent, and this further underscores the dynamic nature of ISFN.

The protracted clonal expansion of ISFN cells in a germinal centre microenvironment may predispose them to a high risk of acquiring genetic changes that confer oncogenic potential, which are not efficiently repaired or eliminated due to apoptosis evasion by BCL2 overexpression. As expected, we confirm the variable presence of the lymphoma-associated mutations in the ISFN lesions, although not to the extent reported previously [8,9,21]. Interestingly, the CREBBP mutation (p. S1680del) identified in case A, previously reported in a case with synchronous ISFN/DLBCL [9], and the STAT6 mutation (p.D419I) identified in cases C and E are the known hotspot changes in FL [22]. Nonetheless, ISFN lesions generally show no or few concurrent secondary mutations, in keeping with their insidious clinical and histological presentations.

Although broadly similar to the observations of previous studies [8,9,21], the present study showed a lesser extent of mutations in ISFN, notably by lack of
the mutations associated with paired FL in four cases. Such variations are perhaps expected, due to the small number of cases investigated in each of these studies, and absence of detectable pathogenic mutations in ISFN was also seen in previous studies [8,9,21,23], particularly in cases where paired FL/high-grade B-cell lymphoma lacked the mutations seen in ISFNs [8,9]. Of note, the FL of cases D and E in the present study showed a paucity of typical FL mutations. In addition, a high proportion of ISFNs investigated in previous studies were identified synchronously with FL or high-grade B-cell lymphoma [7–9,19], and these lesions are at a relatively ‘late phase’ of ISFN evolution. As t(14;18)/IGH::BCL2-positive cells are actively trafficking, transiting from one follicle to another and in the meanwhile accumulating genetic changes, as evidenced by BCL2 variants, the extent of genetic changes in ISFN may depend on the number of follicles that the t(14;18)/IGH::BCL2 neoplastic cells have gone through, hence their phase during ISFN evolution. In line with this, Mamessier et al have shown progressive acquisition and accumulation of genetic changes from ISFNs to partial involvement by FL and overt-FL, although based on non-paired cases [21].

The quality of DNA samples from ISFNs in the present study is suboptimal, not permitting a large scale of mutation screening. Consequently, we only focused on analysis of the mutations identified in their paired FL. This would underestimate any potential mutations exclusively associated with ISFNs but not their paired FL. Nonetheless, the sequence regions containing mutations found in paired FL were adequately covered in ISFNs by PCR and next-generation sequencing (NGS). The detection of FL-associated mutations in paired ISFNs in cases A and C, and BCL2 variants in ISFNs of all the cases ascertain the adequacy of the methodology used in the study.

In summary, our findings demonstrate the widespread distribution of ISFN lesions, further implicating their dynamic and fluidic nature with the neoplastic cells undergoing active trafficking and clonal evolution.

Acknowledgements

We would like to thank Wen-Qing Yao and Fangtian Wu for their advice and support for clonality analysis and assistance preparing samples for MiSeq. We would also like to thank Sarah Moody for her support and advice on the Fluidigm technique and Hongxiang Liu for providing control samples for clonality PCR. In addition, we thank Graeme Clark and colleagues for their assistance with Illumina sequencing and the Human Research Tissue Bank for assistance with preparing FFPE tissue sections. This research was supported by grants from Cancer Research UK (C8333/A29707) and Blood Cancer UK (13006, 19010).

Author contributions statement

RD, SL, FC, ZC and YT designed the experiments, collected data, and carried out the analysis. AW was responsible for case contribution and pathology. MQD and RD wrote and prepared the manuscript. MQD and AW designed and coordinated the study. All the authors commented on the manuscript and approved its submission for publication.

References

1. van Dongen JJ, Langerak AW, Brüggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia 2003; 17: 2257–2317.
2. Dölk G, Dölk L, Hirt C, et al. Age-dependent prevalence and frequency of circulating (t(14;18)-positive cells in the peripheral blood of healthy individuals. J Natl Cancer Inst Monogr 2008; 39: 44–47.
3. Henopf T, Quintanilla-Martinez L, Fend F, et al. Prevalence of follicular lymphoma in situ in consecutively analysed reactive lymph nodes. Histopathology 2011; 59: 139–142.
4. In situ follicular neoplasia. In WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, Swerdlow SH, Campo E, Harris NL, et al. (eds), Revised 4th edn. IARC: Lyon, 2017; 274–275.
5. Cong F, Raffeld M, Teruya-Feldstein J, et al. In situ localization of follicular lymphoma: description and analysis by laser capture micro-dissection. Blood 2002; 99: 3376–3382.
6. Roulland S, Navarro JM, Grenot P, et al. Follicular lymphoma-like B cells in healthy individuals: a novel intermediate step in early lymphomagenesis. J Exp Med 2006; 202: 2425–2431.
7. Schmidt I, Salaverría I, Haake A, et al. Increasing genomic and epigenomic complexity in the clonal evolution from in situ to manifest t(14;18)-positive follicular lymphoma. Leukemia 2014; 28: 1103–1112.
8. Schmidt J, Ramis-Zaldívar JE, Bonzheim I, et al. CREBBP gene mutations are frequently detected in in situ follicular neoplasia. Blood 2018; 132: 2687–2690.
9. Vogelsberg A, Steinhilber J, Mankel B, et al. Genetic evolution of in situ follicular neoplasia to aggressive B-cell lymphoma of germinal center subtype. Haematologica 2021; 106: 2673–2681.
10. Yao WQ, Wu F, Zhang W, et al. Angioimmunoblastic T-cell lymphoma contains multiple clonal T-cell populations derived from a common TET2 mutant progenitor cell. J Pathol 2020; 250: 346–357.
11. Wang M, Escudero-Ibarz L, Moody S, et al. Somatic mutation screening using archival formalin-fixed, paraffin-embedded tissues by Fluidigm multiplex PCR and Illumina sequencing. J Mol Diagn 2015; 17: 521–532.
12. Cucco F, Clipson A, Kennedy H, et al. Mutation screening using formalin-fixed paraffin-embedded tissues: a stratified approach according to DNA quality. Lab Invest 2018; 98: 1084–1092.
13. Cucco F, Barrans S, Sha C, et al. Distinct genetic changes reveal evolutionary history and heterogeneous molecular grade of DLBCL with MYC/BCL2 double-hit. Leukemia 2020; 34: 1329–1341.
14. Schuetz JM, Johnson NA, Morin RD, et al. BCL2 mutations in diffuse large B-cell lymphoma. Leukemia 2012; 26: 1383–1390.
15. Huet S, Szafer-GLusman E, Tesson B, et al. BCL2 mutations do not confer adverse prognosis in follicular lymphoma patients treated with rituximab. Am J Hematol 2017; 92: 515–519.
16. Roulard S, Kelly RS, Morgado E, et al. t(14;18) Translocation: a predictive blood biomarker for follicular lymphoma. J Clin Oncol 2014; 32: 1347–1355.
17. Kosmidis P, Bonzheim I, Dufke C, et al. Next generation sequencing of the clonal IGH rearrangement detects ongoing mutations and interfollicular trafficking in in situ follicular neoplasia. PLoS One 2017; 12: e0178503.

18. Sungalee S, Mamessier E, Morgado E, et al. Germinal center reentries of BCL2-overexpressing B cells drive follicular lymphoma progression. J Clin Invest 2014; 124: 5337–5351.

19. Bonzheim I, Salaverria I, Haake A, et al. A unique case of follicular lymphoma provides insights to the clonal evolution from follicular lymphoma in situ to manifest follicular lymphoma. Blood 2011; 118: 3442–3444.

20. Liu M, Duke JL, Richter DJ, et al. Two levels of protection for the B cell genome during somatic hypermutation. Nature 2008; 451: 841–845.

21. Mamessier E, Song JY, Eberle FC, et al. Early lesions of follicular lymphoma: a genetic perspective. Haematologica 2014; 99: 481–488.

22. Nann D, Ramis-Zaldivar JE, Müller I, et al. Follicular lymphoma t(14;18)-negative is genetically a heterogeneous disease. Blood Adv 2020; 4: 5652–5665.

23. Hellmuth JC, Louissaint A Jr, Szczepanowski M, et al. Duodenal-type and nodal follicular lymphomas differ by their immune microenvironment rather than their mutation profiles. Blood 2018; 132: 1695–1702.

24. Duez M, Giraud M, Herbert R, et al. Vidjil: a web platform for analysis of high-throughput repertoire sequencing. PLoS One 2016; 11: e0166126.

Reference 24 is cited only in the supplementary material.

SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods

Figure S1. Confirmation of clonal identity between the early in situ follicular neoplasias (ISFNs) and late overt follicular lymphoma (FL) in case B

Figure S2. Confirmation of clonal identity between the early in situ follicular neoplasias (ISFNs) and late overt follicular lymphoma (FL) in case D

Figure S3. Analysis of the sensitivity and specificity of clone-specific (CS) PCR

Figure S4 Examples of somatic mutations identified by PCR and Illumina MiSeq sequencing in overt-FL and matched ISFN lesion in case A (panels A–C) and case C (panel D)

Table S1. Summary of clonal identity results between lymph nodes from multiple ISFNs and subsequent matched overt-FL

Table S2. Summary of the pathogenic variants identified in overt-FL samples and matched ISFN samples

Table S3. Summary of unique clone-specific (CS) PCR primers and conditions for cases A–D (referred to in Supplementary materials and methods)

Table S4. Summary of common sequence tagged primers used for targeted PCR sequencing in overt-FL and matched ISFN for Illumina MiSeq