Clinicopathological features of *in situ* follicular neoplasm and relations with follicular lymphoma in Japan

(In situ follicular neoplasia in Japan)

Key words: in situ follicular neoplasm, *BCL-2*, pathology, immunohistochemistry

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Abstract

Objectives: To investigate the clinicopathological features of *in situ* follicular neoplasm (ISFN) in Japan. ISFN is a rare condition formerly considered as an early precursor of follicular lymphoma (FL). This is a first original report of ISFN from Asian country.

Methods: We reviewed 19 biopsy samples of ISFN. ISFNs were categorized into two groups: (1) ISFN, consisting of ISFN with strong positivity for BCL-2 immunohistochemical staining (IHC), and obvious translocation of *BCL-2*, and (2) ISFN-like FL, featuring cases without obvious translocation but having morphological features and characteristic IHC findings of ISFN. As control, we adopted obvious FL. For some cases showing coexisting ISFN and FL lesions in the same lymph node, we could conduct further clonality analysis for each lesion.

Results: Nine of the 19 cases of ISFN coexisted with FL or had a history of overt B or T-cell lymphoma including FL. Statistical comparison among ISFN-like FL and FL showed no significant differences in pathological features. Molecular analysis suggested that ISFN lesion and FL lesion in the same lymph node having each different clonality.

Conclusions: ISFN coexist or associate with other overt lymphomas frequently.

INTRODUCTION

Follicular lymphoma (FL) is a common lymphoid neoplasm, especially in the United States of America and western Europe. It accounts for about 20% of malignant lymphoma. In Asia, eastern Europe, and developing countries, its incidence is much lower.[1] In situ follicular neoplasia (ISFN), formerly referred as follicular lymphoma *in situ*, is defined as partial or total colonization of germinal centers (GCs) by clonal B cells carrying the *BCL-2* translocation that are characteristic of FL but are confined to the germinal centers of apparently-reactive lymph nodes.[2] Three previous studies conducted immunohistochemical (IHC) screening using the *BCL-2* antibody for ISFN in surgically removed, otherwise reactive lymph nodes, and identified ISFN in 2.3–3.2% of cases.[3-5] Moreover, progression of ISFN within 3–5 years is low (<5%) in the absence of prior or concurrent lymphoma.[1]

Highly sensitive PCR assays have identified t(14;18) cells (FL like B-cells) in the peripheral blood of healthy individuals, and the prevalence of FL like B-cells has been demonstrated to increase with age, smoking, and pesticide exposure.[6] The prevalence of FL like B-cells in Asian (Japanese) individuals (10% to 20%) seems to be lower than

that in Caucasians (50–70%).[7-9] Although the clinical significance of FL like B-cells in the peripheral blood of healthy individuals is not thoroughly understood, one study reported that high frequency of FL like B-cells in peripheral blood is associated with increased risk of developing overt FL.[10] Both FL like B-cells and ISFN have been described in the same patient.[11] This finding suggests that ISFN may originate from FL like B-cells.

Although there are several studies on ISFN, few studies clarify the correlation between the number of follicles involved within a single lymph node showing ISFN and the subsequent risk of lymphoma [12,13]. Moreover, there are few reports on ISFN in Asian countries. In this study, we retrospectively investigated the number of affected follicles within a single lymph node, along with *BCL-2* translocation using chromogenic in situ hybridization (CISH), in 19 cases of ISFN or ISFN-like FL evaluated the clinical significance.

MATERIALS AND METHODS

Patients

We reviewed 19 biopsy samples from patients diagnosed with ISFN.

Additionally, 10 biopsy samples of FL were included in this study for comparison. Nineteen cases of ISFN exhibiting lymphadenopathy were found to be those in which malignant lymphoma had been clinically suspected. These cases were extracted from approximately 40,000 diagnosed lymphoma cases among patients admitted for diagnosis to the Department of Pathology, Kurume University, Japan from 2006 to 2016. The use of patient materials and clinical information was approved by the Research Ethics Committee of Kurume University and was in accordance with the Declaration of Helsinki.

Definition and Classification of ISFN and ISFN-like FL patients

The definition of ISFN based on the morphological and clinical findings in this study is as follows: focal GCs strongly positive for BCL-2 by IHC staining, which is stronger than that of reactive T cells or mantle-zone B cells; strongly positive cells for BCL-2, which are uniformly centrocytes, confined to GCs, and not always replacing the entire follicle center; no evidence of interfollicular infiltration; *BCL-2* translocation confirmed by FISH or CISH; and limited lesions clinically.

Cases without obvious translocation but having morphological features and characteristic IHC findings of ISFN were classified as ISFN-like FL as previously reported.[12,14-19] This group included 3 subgroups; (i) ISFN in a broad sense, which comprised cases fulfilling the definition of ISFN without confirmed *BCL-2*

translocation,[14,15] (ii) Histological ISFN, which were cases of ISFN without confirmation of *BCL-2* translocation or with swollen lymph nodes other than at the biopsy sites;[14,16] and (iii) Clinical ISFN, which were cases of ISFN without confirmation of *BCL-2* translocation or with pathological findings of concomitant lymphoma including both ISFN and FL lesion in the same lymph node.[12,17-19]

Immunohistochemical staining for BCL-2

IHC for BCL-2 was performed on 3-μm-thick, formalin-fixed, paraffin-embedded (FFPE) tissue sections. After being deparaffinized and rehydrated, antigen retrieval was performed using Tris-EDTA buffer in an autoclave. After cooling and rinsing with 0.05 M Tris-HCl buffer, incubated with 3% hydrogen peroxide for 5 min. The anti-BCL-2 mouse monoclonal antibodies (Clone 124; 1:100 dilution, Agilent Technologies Japan) was used as the primary antibody. Subsequently, the section were incubated with peroxide-labeled anti-mouse goat polyclonal IgG antibodies (K1492; Agilent Technologies Japan). BCL-2-protein was visualized with diaminobenzidine. Slides were then dehydrated with ethanol, permeated with xylene, and mounted under coverslips.

***BCL-2* CISH**

Chromogenic *in situ* hybridization (CISH) was performed on 3-μm-thick FFPE

tissue sections. The Zyto Dot 2C CISH Implementation Kit (Zyto Vision; Bremerhaven, Germany) was used only for the visualization steps. In brief, these sections were de-paraffinized and incubated in a citrate acid buffer solution, in a water bath at 98°C for 20 min, and subsequently incubated in 2× saline sodium citrate (SSC) buffer at 75°C for 40 min. Enzymatic digestion was conducted by Pepsin solution (Ready to use, Invitrogen, Tokyo, Japan), incubated at 37°C for 10 min. ZytoDot 2C SPEC *BCL-2* Break Apart Probe (Zyto Vision; Bremerhaven, Germany) was applied to slides covered with coverslips. These were denatured with a Hybridizer (Agilent Technologies Japan) 75°C for 5 min, and hybridization was conducted overnight at 35°C. Subsequently, washed with 2× SSC/0.3% NP40 for 2 min, at 72°C. The ZytoDot 2C SPEC *BCL-2* Break Apart Probe is designed to detect translocations involving the chromosomal region 18q21.33 harboring the *BCL-2* gene. This probe is a mixture of a digoxigenin (DIG)-labeled and a dinitrophenyl (DNP)-labeled probe hybridizing to the 18q21.33 band. The probes were detected by sequential incubation with anti-DIG and DNP mix-solution, according to the manufacturer's instructions. CISH was evaluated with using a microscope (Olympus, Japan) equipped with a ×100 objective lens.

DNA extraction

Genomic DNA samples from each tumor region were analyzed via immunostaining. Those which were positive for areas containing *BCL-2* were extracted using a commercial kit (KAPA Express Extract kit, KAPA BIOSYSTEMS, Wilmington, MA).

PCR for detection of IgH rearrangements

Amplification of *IgH* from the framework 2 region of segment V to the J region was carried out in genomic DNA using consensus primers complementary to both the framework 2 portion of the VH region (FR2B) and the JH region (CFW1) in genomic DNA. Sequences of primers used were as follows: FR2B, 5'-GTCCTGCAGGC(C/T)(C/T)CC-GG(A/G)AA(A/G)(A/G)GTCTGGAGTGG-3'; CFW1, 5'-ACCTGAGGAGACGGT-GACCAGGGT-3'. The PCR conditions were as follows: initial denaturation at 95℃ for 10 min, 5 cycles (95℃ for 30 s, 63℃ for 30 s and 72℃ for 30 s) followed by 45 cycles (95℃ for 30 s, 60℃ for 30 s and 72℃ for 30 s) with a final extension at 72℃ for 10 min. Analysis of PCR products were performed according to the procedures described above for TCRγ amplification. The sizes of *IgH* rearrangement fragments ranged between 250 bp and 300 bp. Amplified PCR products were electrophoresed in 3% agarose gels and visualized via ethidium bromide staining under ultraviolet light. *β-actin* was used as an

internal control. The expected size of genomic *β-actin* for nested PCR was 148 bp.

Sequencing

Following electrophoresis, the resulting PCR bands were extracted from 3% agarose gel. DNA from amplified PCR products was purified using a MinElute Gel Extraction kit (QIAGEN, Germantown, MD, USA). Sanger sequencing (Direct sequence) was used to confirm the *IgH* rearrangement region. The sequencing reaction was carried out using an ABI BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems) and cycled at 96°C for 1 min; 25 cycles of 96°C for 10 s; 50°C for 5 s, and 60°C for 4 min. The primers FR2B and SJHb were used for the sequencing reaction. The resulting products were run on an Applied Biosystems SeqStudio Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). For overlapping samples that were impossible to sequence directly, cloning was performed using a pT7-T vector (Novagen, Madison, WI, USA). Plasmid DNA was purified using a QIAGEN Plasmid Mini kit (QIAGEN). Sequence analyses were performed on 7 to 16 plasmid DNAs per sample. Sequencing operations were carried out in the same manner as that described above, using T7 primers present in the vector. Homology analysis of the IgVH region was conducted using **IMGT/V-QUEST(http://www.imgt.org/IMGT_vquest/vquest)**.

Statistical analysis

Clinical characteristics of patients among ISFN-like FL and FL were compared using the chi-square test or Fisher's 2-sided exact test. The Wilcoxon rank sum test was used to analyze the number of erythrocytes, leukocytes, platelets, and affected follicles as well as their proportions. Statistical significance was set at $P<0.05$. JMP version 14.0 (SAS Institute Inc., Cary, NC) was used for all analyses.

RESULTS

Clinical characteristics of ISFN patients

The clinicopathological features are summarized in Table 1. There were 11 males (58%) and 8 females (42%), who ranged in age from 49 to 75 years, with a median age of 62.2years.

In case 2 and 5, there was no other swollen LN clinically, and case 5 had a history of non-Hodgkin's lymphoma. Cases 1, 3, 4, and 6–14 had other swollen lymph nodes clinically. Of these, 2 cases (case 6, case 7) had a history of FL, and case 8 had a history of adult T-cell lymphoma/leukemia. The 5 cases (case 15-19) of ISFN coexisting with FL in a same lymph node showed no swollen lymph node other than the biopsied sample.

Histopathological observation of ISFN

All 19 cases of ISFN lesions showed very strong positivity for BCL-2 IHC,

even stronger than that of reactive T cells or mantle-zone B cells. The degree of involvement by ISFN cells within the biopsy tissue was evaluated [Table 2].

Histologically, 13 (case 1-6, 8-14) of the 19 cases were diagnosed as ISFN in an otherwise reactive lymph node [Figure 1]. Case 7 was a composite of metastatic carcinoma and ISFN lesion. The remaining 5 cases (15-19) of ISFN coexisted with FL lesions in the same lymph node. Case 17 showed BCL-2 negative FL with the ISFN lesion [Figure 3]. Other 4 cases showed ordinary BCL-2 positive FL coexistence with the ISFN lesion [Figure 2].

***BCL-2* CISH results and ISFN grouping**

We conducted CISH for all 19 cases. Of these, 12 were evaluated. An apparent translocation was seen in case 1 [Fig. 4], whereas case 13 appeared to demonstrate *BCL-2* amplification [Fig. 5]. We were not able to prove a significant *BCL2* translocation for the remaining 10 cases [Table 1].

From these results and ISFN definition, we concluded that case 1 was ISFN, and the other 18 cases were ISFN-like FL. The subgrouping is provided in Table 1.

Statistical comparison of clinicopathological features among the ISFN-like FL and FL

We conducted a statistical comparison of clinicopathological features including the absolute number of follicles within a given lymph node, absolute number of BCL2++ follicles and their proportion (%), and the degree of involvement within individual follicles [Table2] as previously reported.[13] There were no significant differences among the 2 groups [Table 3]. In clinical features, the number of erythrocytes in the peripheral blood of FL patients was significantly lower than that of patients with ISFN like FL ($p = 0.0454$).

PCR and Sequence analysis

For cases 15 and 16, PCR, which was performed to detect *IgVH* rearrangement, was followed by sequence analysis. ISFN lesions and FL lesions in the same LN represented different clonalities [Fig. 6-1, Table 4]. In case 15, sizes of the *IgVH* PCR bands derived for in FL and ISFN via agarose electrophoresis, were different [Fig. 6-1 a-2]. On the other hand, in case 16, the sequences of main *IGVH* clones of FL and ISFN were different [Figs. 6-2 and 6-3].

Homology analysis of the *IgVH* region, using IMGT/V-QUEST, indicated that each IgVH was oligoclonal [Table 4]. The IgVH clones in ISFN like FL and FL were also different. Several abnormal B cells were evident in lesions where ISFN like FL and early FL

occurred simultaneously.

DISCUSSION

In this study, approximately half of ISFN cases showed coexistence or association with overt B-cell lymphoma or T-cell lymphoma. The number of erythrocytes in the peripheral blood of FL patients tended to be lower than that in ISFN-like FL.

We have summarized the previous major studies on ISFN in Table 5. Based on ISFN incidence, no obvious sexual specificity was observed. In previous reports, 8 cases of ISFN occurred in the twenties and thirties even though ISFN has been regarded as occurring mainly in the forties and fifties as with FL.[12,20] Previous reports and this study suggest that at least 15–50% of ISFN cases coexist or associate with overt lymphoma [Table 5]. Besides, the rate of ISFN progression to overt lymphoma within 3–5 years is 2–5% if there is no prior or concurrent lymphoma. Some cases of ISFN prior to or coexisting with T-cell lymphomas have been reported including our study, even though ISFN mainly coexists with B-cell lymphoid neoplasms. ISFN might thus represent something other than merely being a precursor of common FL.[17] These associations with lymphomas other than FL suggest that ISFN might be a sign of an increased tendency

to develop lymphoid neoplasms due to their genetic instability involving the IGH rearrangement process and B-cell development.[12]

In this study, 5 cases (cases 15–19) presented both ISFN lesions and FL lesions within a same lymph node. ISFN and overt FL, which are morphologically separate, differ in BCL-2 expression. ISFN neoplastic cells showed very strong positivity for BCL-2 IHC as well as atypical cells confined to germinal centers. FL lesions exhibited neoplastic follicles, most of which were poorly defined and lacked mantle zones as well as polarization. The neoplastic cells of FL were positive for BCL-2 IHC, but did not display expression as strong as that displayed by ISFN cells in the same node. There are several reports of ISFN with BCL-2 negative FL.[12,17] Although the mechanism of changing from neoplastic cells with strong BCL-2 expression into those without BCL-2 could not be determined, mutations in *BCL-2* gene might cause this change.[16,21] Even though there are not many reports of ISFN coexisting with FL in the same lymph node.[14,17,18]

Previous studies substantiated the results of the current study indicating that ISFN was associated with overt FL in the same lymph node.[14,17,18] Differentiation between partial involvement of follicular lymphoma (PFL) and ISFN is vital. Jegalian et al., declared that 53% of PFL cases progressed to FL, whereas only 1 case of ISFN (5%) progressed to FL. [12] According to a WHO report, the presence of ISFN at another site,

or in patients with overt FL is uncommon.[1] PFL can usually be suspected based on morphological features of H&E-stained sections, whereas ISFN can only be detected via subsequent IHC stains. PFL is represented by atypical cells outside the germinal center, and follicle sizes which are often expanded, whereas ISFN is represented by normal follicle size, atypical cells confined to the germinal center and very strong positivity for IHC of BCL-2 and CD10.[1] Based on these criteria, we concluded that 19 of our cases were ISFN. CISH was performed for all ISFN cases and Case 13 seemed to show *BCL-2* amplification [Fig.5]. In WHO classification [1], there is no description about ISFN showing *BCL-2* amplification. However, there is a previous report of ISFN with *BCL-2* amplification [14]. In addition, in many past reports, BCL-2 translocation was proven via FISH in some or all cases. However, in other cases, demonstration of t(14;18) was impossible. FISH detected *BCL-2* gene rearrangements only in 1 of 2 cases.[15] Only 1 out of 4 cases showed a possible t(14;18) [13]. The data suggest that a case with *BCL-2* translocation in the minor cluster region is included in the t(14;18) negative group. Therefore, we surmised that findings of *BCL-2* amplification and that the translocation was not confirmed do not amount to exclusion of ISFN. Further molecular studies are useful for analyzing the relationship between ISFN and FL. Some molecular studies were performed using spare, extra specimens from cases 15 and 16. The results illustrated that

ISFN lesions and FL lesions in the same LN showed different clonalities. It proved that PFL could be excluded in these 2 cases, and that neoplastic B-cells in ISFN lesions are possibly clonally heterogeneous.

According to previous reports and this study, patients with ISFN should undergo scrutinous clinical examination. It is important to rule out the presence of overt lymphoma in other regions of the body. Moreover, careful staging should be recommended, including a biopsy of an additional nodal or extranodal suspicious tissue, blood flow cytometry, and computed tomography [Table 6].[22] In cases with localized ISFN without any evidence of overt lymphoma, a watchful waiting policy after diagnosis is strongly suggested. Treatment should be started according to overt lymphoma, independently of ISFN findings in cases of ISFN with overt lymphoma.[22,23]

In this study, more than half of the cases represented fewer than half of the total GCs with strong positivity for BCL-2, and these GCs tended to be adjacent to one another. Cong. et al also showed that in more than half of ISFN cases, BCL-2 strongly positive follicles were a minority of the GCs within a lymph node, and that strongly positive follicles tended to be adjacent to one another.[20] Although the reason for this is not determined, this finding could be observed in ISFN lesions as well.

This study could not demonstrate statistically different pathological parameters

among the ISFN and FL groups. Pillai et al. examined the absolute number of affected follicles, their proportion, and the degree of involvement within individual follicles in ISFN, but found that none of these parameters could predict the association with overt FL.[13] Thus, information regarding clinical features might be needed to predict the possibility of progression from ISFN to FL.

This study has some limitations. First, the clinical information that we could obtain was only that submitted for the pathological diagnosis. There might thus be a lack of enough information including clinical follow-up. Second, this study included a relatively small number of cases and additional studies with more ISFN cases are desirable. Third, it is necessary to thoroughly investigate ISFN by including molecular studies in the future, even though we could analyze the clinicopathological features in this study.

In conclusion, although ISFN lesions could represent the early phase of FL lesions, coexistence or association with overt lymphomas is sometimes observed. Because this disease entity is not sufficiently investigated, future studies including molecular examination are desirable.

REFERENCES

1. Jaffe ES, Harris NL, Swerdlow SH, et al. Follicular Lymphoma. In: Swerdlow SH,

Campo E, Harris NL. eds. (2017) *WHO classification of tumours of haematopoietic and lymphoid tissues. Revised.* 4th ed. Lyon: International Agency for Research on Cancer; 266-277.

2. Swerdlow SH, Campo E, Pileri SA, et al. (2016) The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood.* 127:2375-2390.

3. Bermudez G, Gonzalez de Villambrosia S, Martinez-Lopez A, et al.(2016) Incidental and isolated follicular lymphoma *in situ* and mantle cell lymphoma *in situ* lack clinical significance. *Am J Surg Pathol.* 40:943-949.

4. Henopp T, Quintanilla-Martinez L. Prevalence of follicular lymphoma *in situ* in consecutively analysed reactive lymph nodes.(2011) *Histopathology.* 59:139-142.

5. Carvajal-Cuenca A, Sua LF, Silva NM, et al. *In situ* mantle cell lymphoma: clinical implications of an incidental finding with indolent clinical behavior. (2012) *Haematologica.* 97:270-278.

6. Scheler F, Hirt C. Chromosomal translocation t(14;18) in healthy individuals. (2003) *Semin Cancer Biol.* 13:203-209.

7. Ohshima K, Kikuchi M. Amplified bcl-2/JH rearrangements in reactive lymphadenopathy. (1993) *Virchows Arch B Cell Pathol Incl Mol Pathol.* 63:197-198.

8. Levy D, Bertoldi ERM. Presence of t(14;18) translocation in healthy individuals varies

according to ethnic background in the Brazilian population. (2017) *Braz J Med Biol Res.* 50:e6172.

9. Yasukawa M, Bando S, Dolken G, et al. Low frequency of BCL-2/J(H) translocation in peripheral blood lymphocytes of healthy Japanese individuals. (2001) *Blood.* 98:486-488.

10. Roulland S, Kelly RS, Morgado E, et al. t(14;18) translocation: a predictive blood biomarker for follicular lymphoma. (2014) *J Clin Oncol.* 32:1347-1355.

11. Cheung MC, Bailey D, Pennell N, et al. In situ localization of follicular lymphoma: evidence for subclinical systemic disease with detection of an identical BCL-2/IGH fusion gene in blood and lymph node. (2009) *Leukemia.* 23:1176-1179.

12. Jegalian AG, Eberle FC, Pack SD, et al. Follicular lymphoma in situ: clinical implications and comparisons with partial involvement by follicular lymphoma. (2011) *Blood.* 118:2976-2984.

13. Pillai RK, Surti U. Follicular lymphoma-like B cells of uncertain significance (in situ follicular lymphoma) may infrequently progress, but precedes follicular lymphoma, is associated with other overt lymphomas and mimics follicular lymphoma in flow cytometric studies. (2013) *Haematologica.* 98:1571-1580.

14. Montes-Moreno S, Castro Y. Intrafollicular neoplasia/*in situ* follicular lymphoma:

review of a series of 13 cases. (2010) *Histopathology.* 56:658-661.

15. Carbone A, Tibiletti MG, Canzonieri V, et al. In situ follicular lymphoma associated with nonlymphoid malignancies. (2012) *Leuk Lymphoma.* 53(4):603-608.

16. 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. (2011) *Blood.* 118:3442-3444.

17. Carbone A, Della LD, Zannier L, et al. *In situ* follicular lymphoma associated with overt B- or T-cell lymphomas in the same lymph node. (2011) *Am J Hematol.* 86: E66-E70.

18. Torlakovic E, Torlakovic G. Follicular colonization by follicular lymphoma. (2002) *Arch Pathol Lab Med.* 126:1136-1137.

19. Schmidt J, Salaverria 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. (2014) *Leukemia.* 28:1103-1112.

20. Cong P, Raffeld M. In situ localization of follicular lymphoma: description and analysis by laser capture microdissection. (2002) *Blood.* 99:3376-3383.

21. Masir N, Campbell LJ. Pseudonegative BCL2 protein expression in a t(14;18) translocation positive lymphoma cell line: a need for an alternative BCL2 antibody.

(2010) *Pathology.* 42:3:212-216.

22. Carbone A, Gloghini A. Emerging issues after the recognition of *in situ* follicular lymphoma. (2014) *Leuk Lymphoma.* 55:3:482-490.

23. Oishi N, Montes-Moreno S. *In situ* neoplasia in lymph node pathology. (2018) *Seminars in Diagnostic Pathology.* 35:76-83.

Figure legends

Fig. 1

In situ follicular neoplasia(ISFN). (a-c) Some ISFN components are shown. (b,c) ISFN components(red arrows) show very strong positivity for *BCL2* and CD10 with a higher intensity than that in adjacent T cells. (a) Hematoxylin and eosin (HE) staining, (b)*BCL-2* immunohistochemical staining (IHC), (c)CD10 IHC. original magnification ×100.

Fig. 2

ISFN coexists with FL within a lymph node. (a-d) Some ISFN components (arrow heads) proliferate with large FL follicles (arrows) lacking polarization. (a,c)Hematoxylin and eosin (HE) staining, (b,d) *BCL-2* immunohistochemical staining (IHC), original magnification ×40.

Fig. 3

BCL-2 negative FL with some ISFN components. (a,b) Some ISFN components (arrow heads) coexist with FL in many *BCL-2* negative large follicles (arrows) lacking polarization. (a) HE, (b) *BCL-2* IHC, (c) CD10, original magnification ×40.

Fig. 4

BCL-2 chromogenic in situ hybridization (CISH). *BCL-2* CISH displayed isolated red and green signals, indicating the translocation of *BCL-2*. A cell with no *BCL-2* translocation was used as a control (red circle). Original magnification ×1000.

Fig. 5

(a),(b) *BCL-2* IHC of ISFN, (c) *BCL-2* CISH of ISFN.

(a) Low-power image of *BCL-2* stained sections showing an overall intact architecture with a minority of follicle centers involved in ISFN(arrow heads).

(b) *BCL-2* staining highlighted intensely stained ISFN components.

(c) *BCL-2* CISH indicates *BCL-2* amplification (arrows). A cell with no translocation or amplification of *BCL-2* was used as a control (red circle).

(a)*BCL-2* IHC, original magnification ×40. (b) *BCL-2* IHC, original magnification ×100.

(c) *BCL-2* CISH, original magnification ×1000.

Fig. 6-1 Immunostaining of BCL-2 （A-1 and B-1) and agarose gel electrophoresis of IgVH PCR (A-2 and B-2).

Case 15 and 16 are show in A and B, respectively.

In Fig. A-1 and B-1 (BCL-2), Areas of ISFN and FL from which DNA was extracted are surrounded by blue and red lines, respectively. Fig. A-2 and B-2 : M; 50 bp DNA ladder marker,

lane 1;ISFN IgVH, lane 2; FL IgVH, lane 3; ISFN b-actin, lane 4; FL b-actin.

In all cases, IgVH PCR band of about 250 bp was obtained (A-2 and B-2).

IgVH band sizes of ISFN and FL in case 15, were different (A-2 ; lane 1 and 2).

Fig. 6-2 IMGT/QUEST homology of case 16 ISFN (clone 3-10) IgVH (250 bp) Group A (6/16 clones)

Sequence primers (FR2B and SJHb) are shown in red and blue letters, respectively.

V, D, and J regions are represented by a green upper case letter.

The IMGT/ junction analysis was determined to have one out of frame region (#) in the D-J junction nucleotide sequence.

Fig. 6-3 IMGT/QUEST homology of case 16 FL (clone 4-1) IgVH (248 bp)

main (6/16 clones)

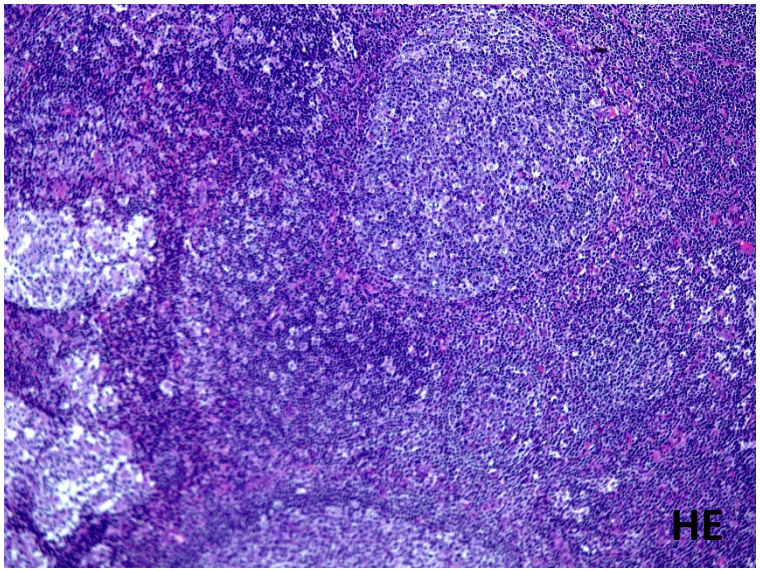
Sequence primers (FR2B and SJHb) are shown in red and blue letters,

respectively.

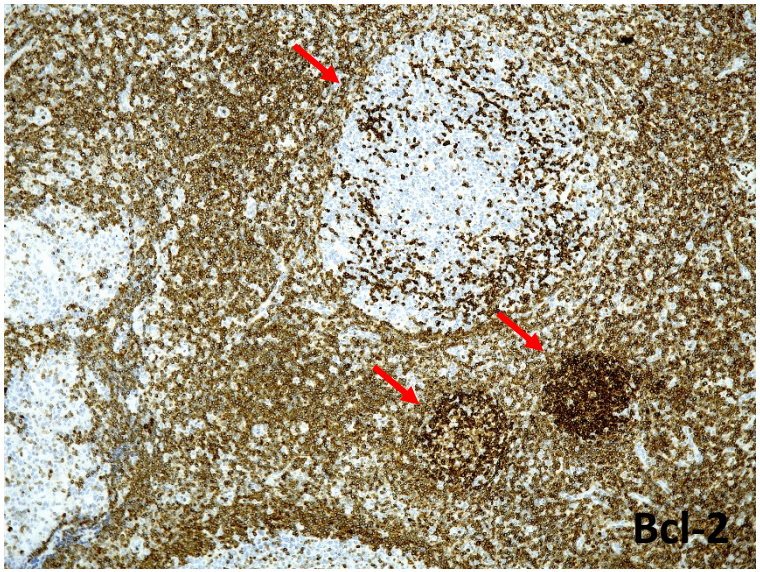
V, D, and J regions are represented by a green upper case letter.

Fig. 1

(a)



(b)



(c)

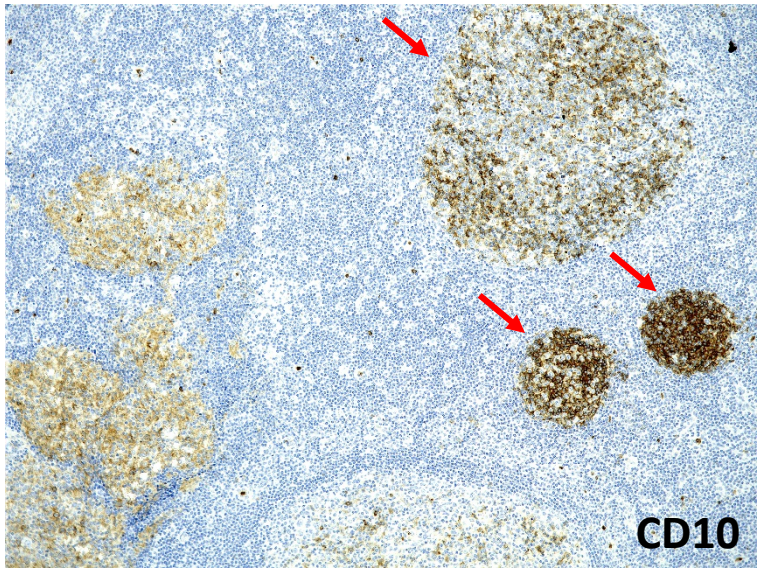
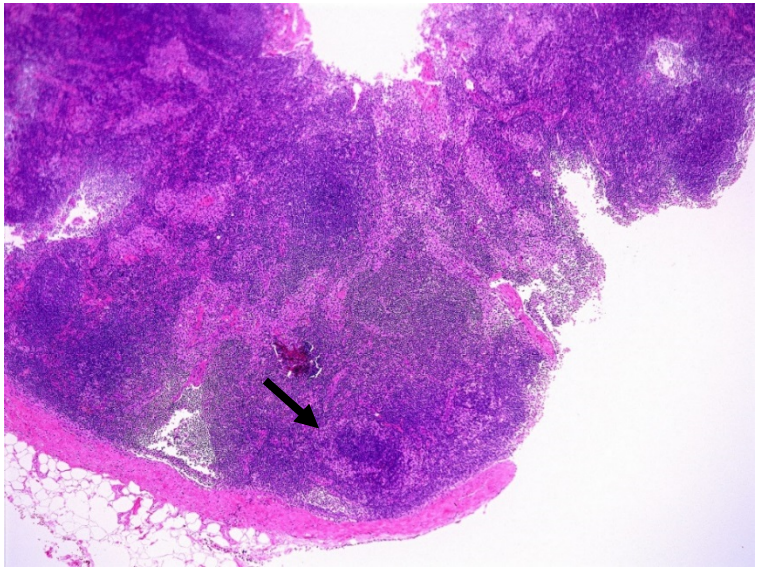
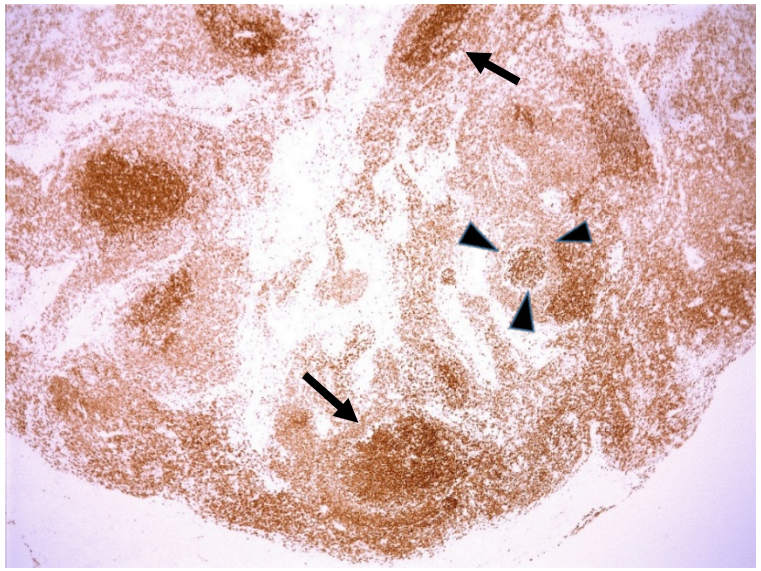


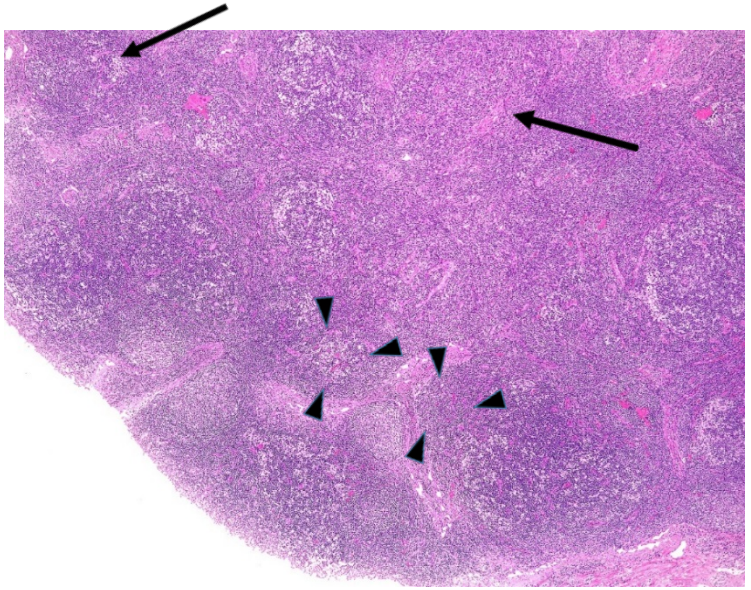
Fig. 2

(a)

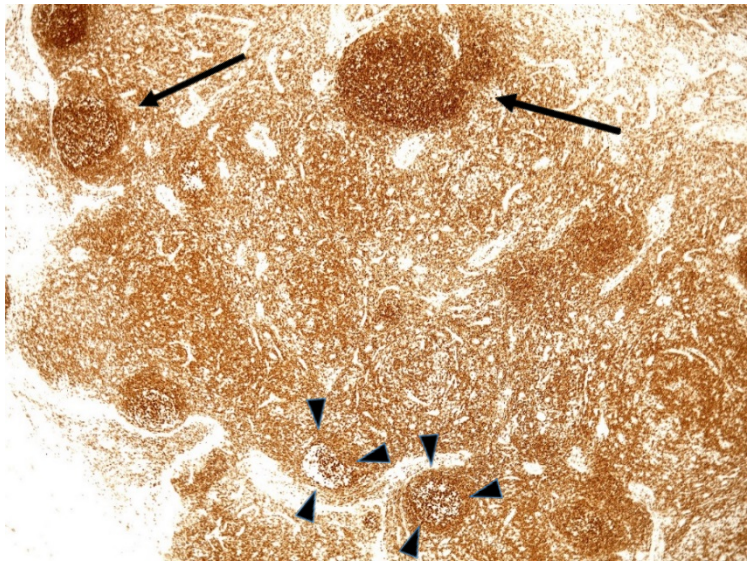


(b)





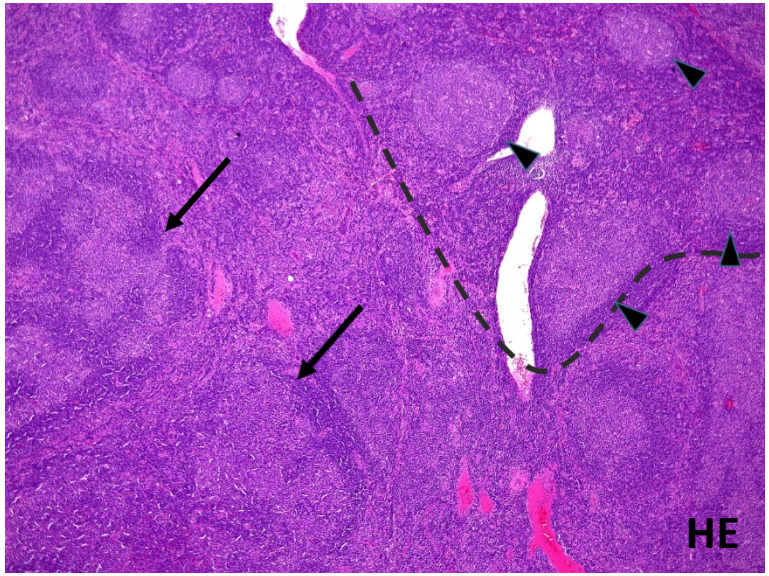
(c)



(d)

Fig.3

(a)



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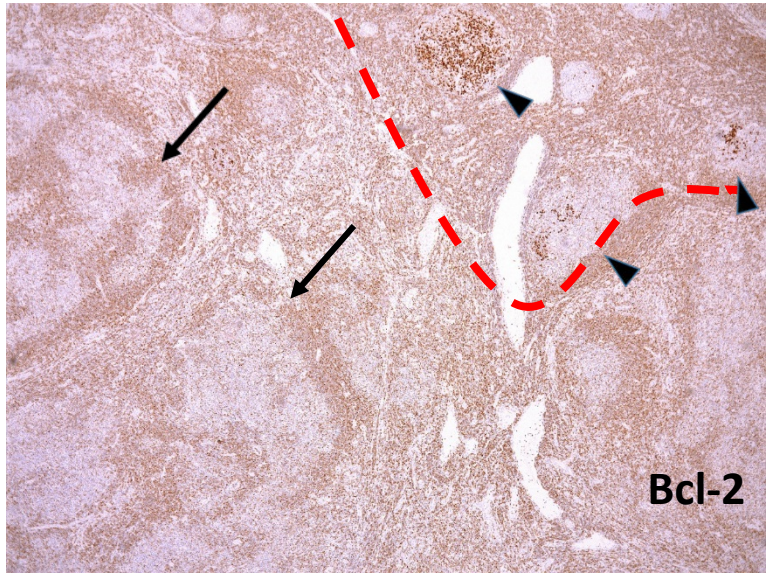


(c)

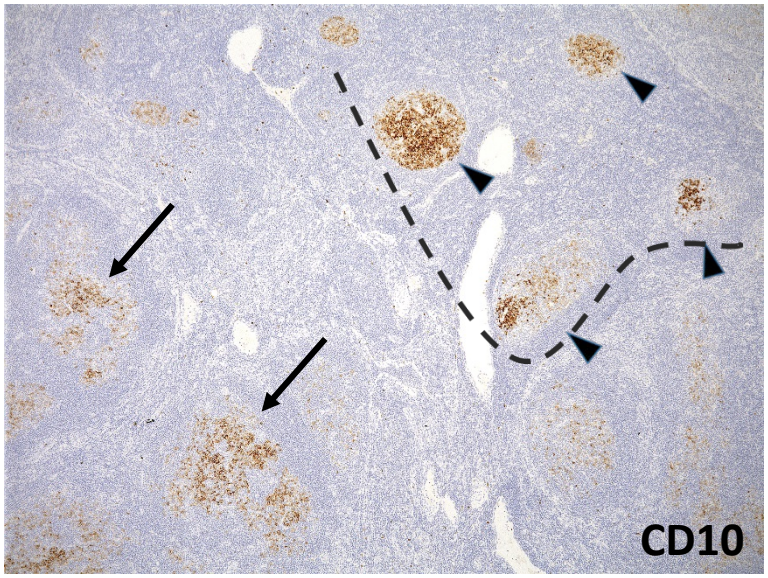


Fig. 4

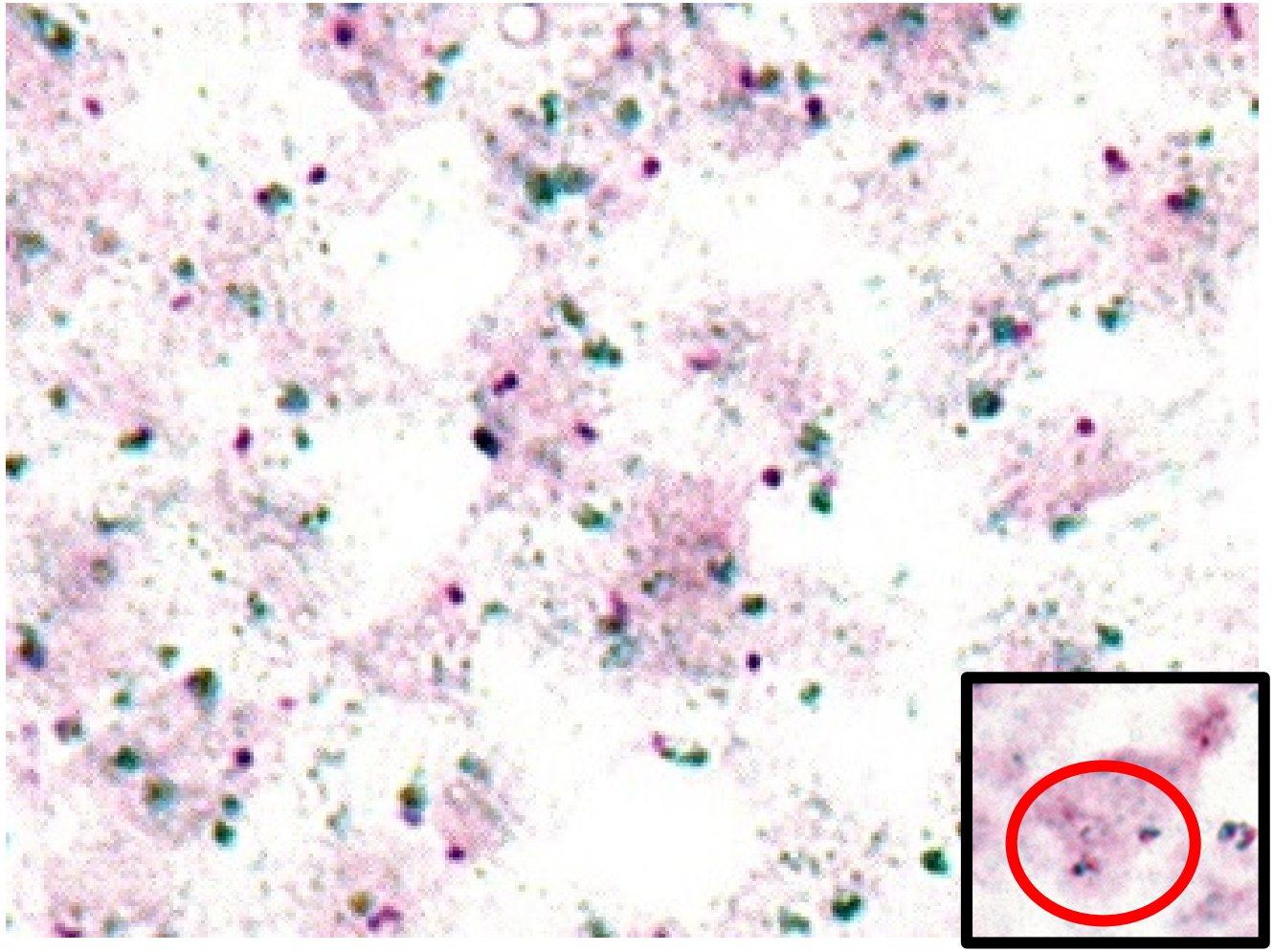
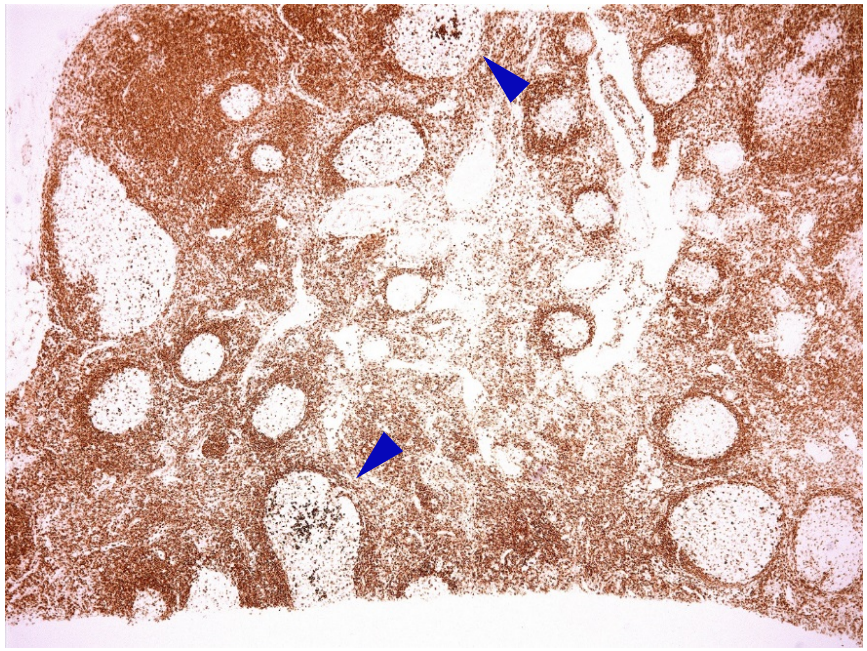


Fig. 5

(a)



(b)

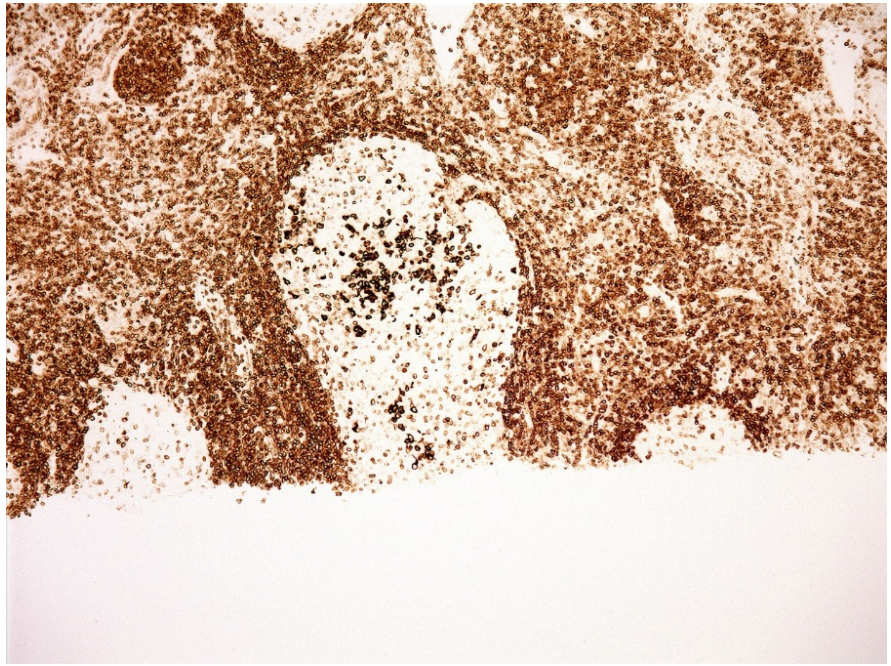


Fig. 5

(c)

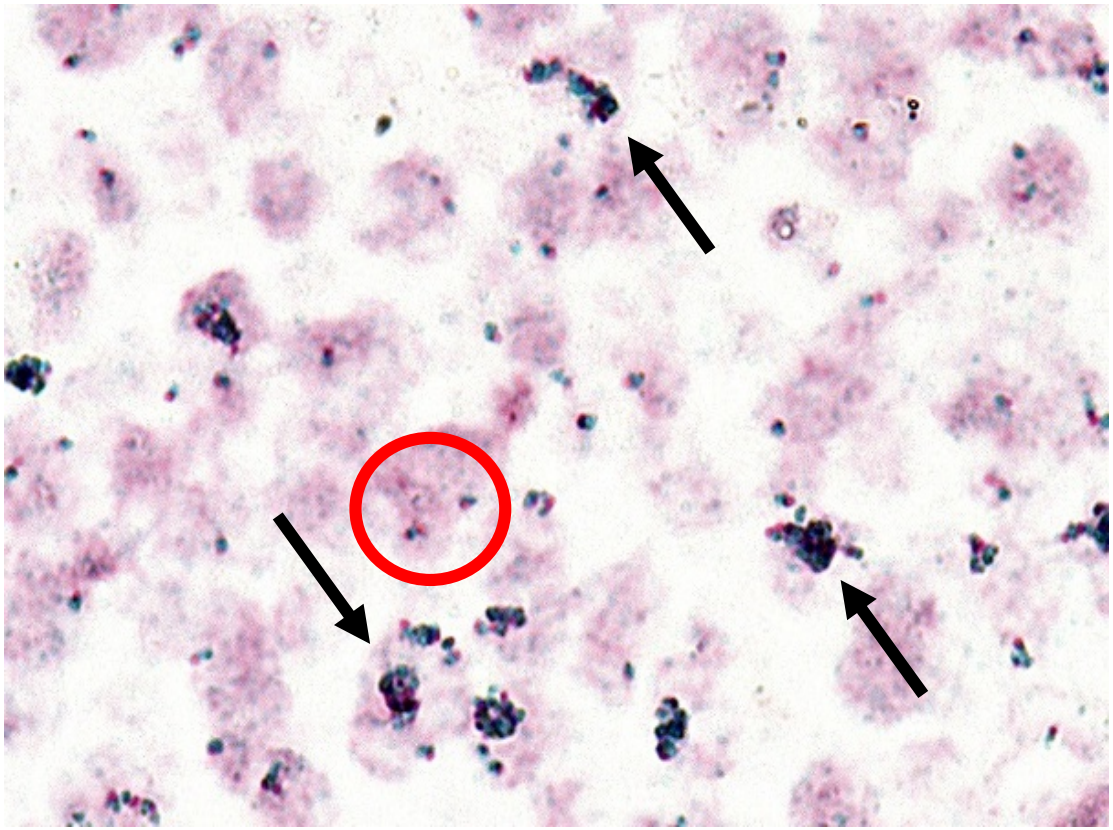


Fig.6-1

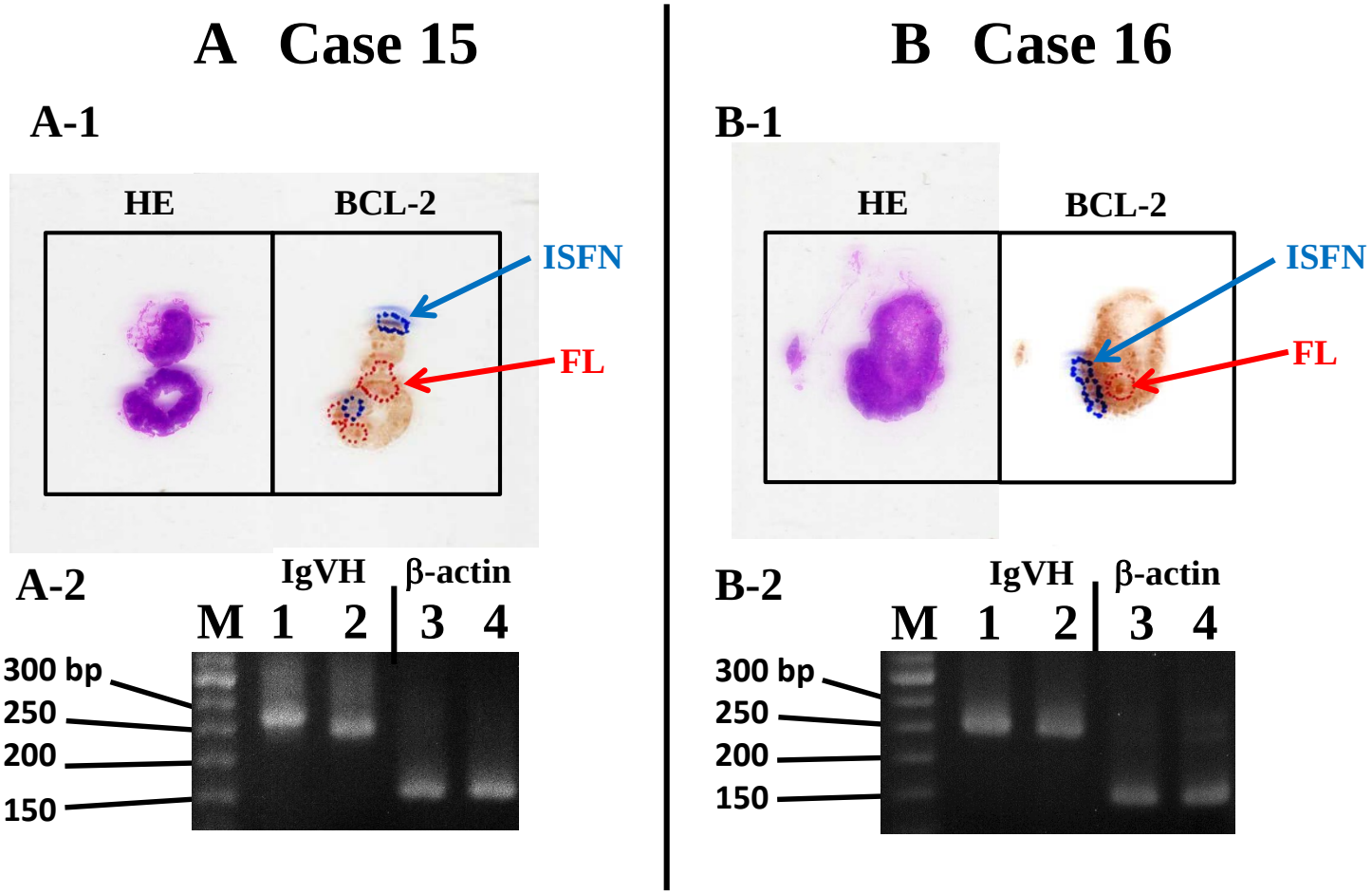
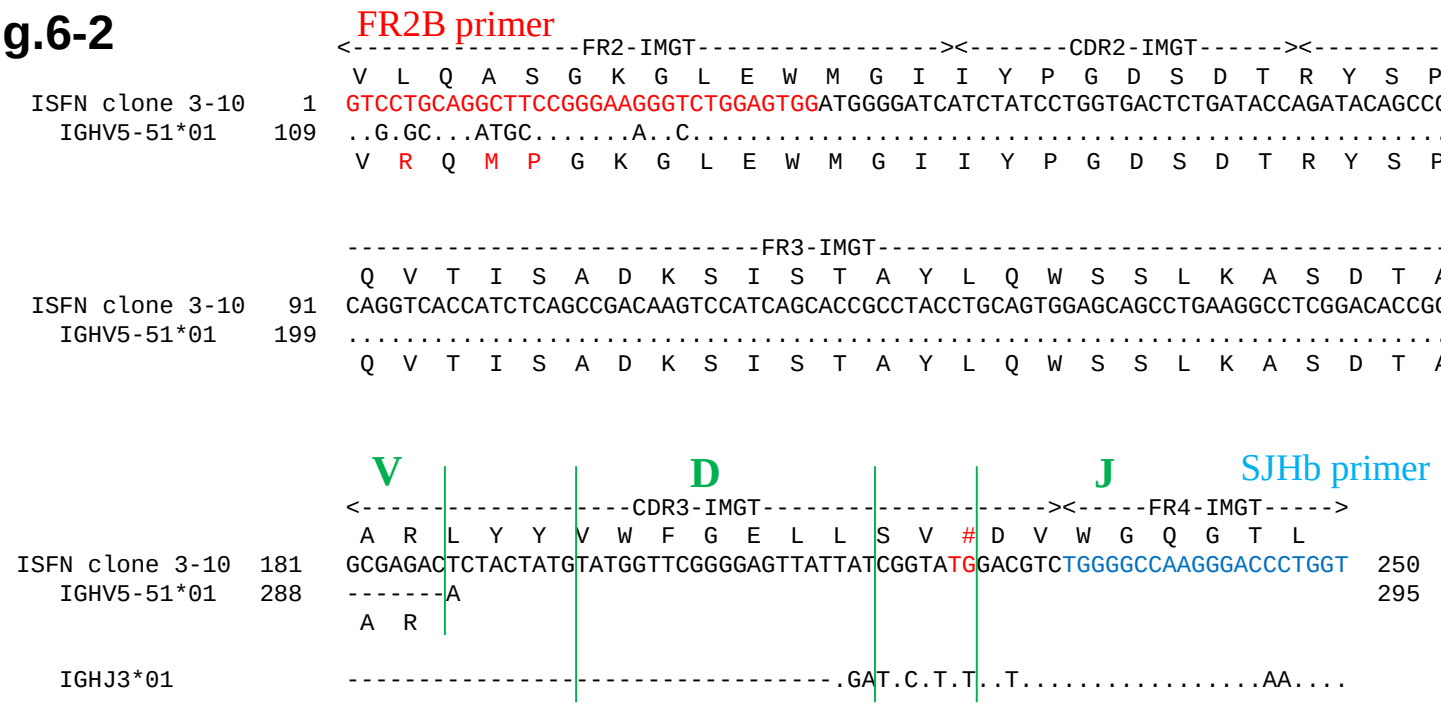

A Case 15
A-1
HE
BCL-2
ISFN
FL
A-2
IgVH
β-actin
M 1 2 3 4
300 bp
250
200
150
B Case 16
B-1
HE
BCL-2
ISFN
FL
B-2
IgVH
β-actin
M 1 2 3 4
300 bp
250
200
150

Fig.6-2



IMGT/V-QUEST homology search

V-gene IGHV5-51*01F or IGHV5-51*03F 95.0%

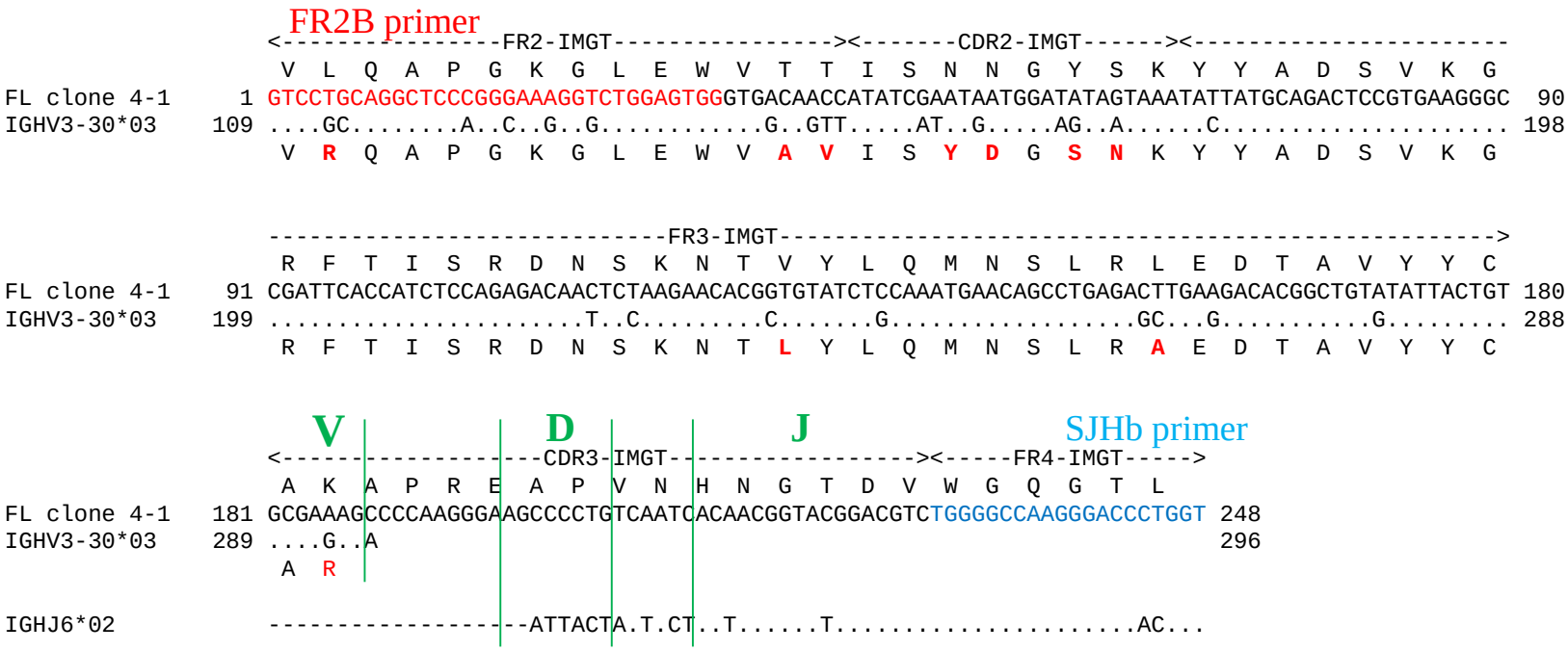
D-gene IGHD3-10*01F

J-gene IGHJ3*01F 75.0%

Case 16 ISFN Group A (clone 3-10)

unproductive, rearranged, IGH sequence

Fig.6-3



IMGT/V-QUEST homology search

V-gene IGHV3-30*03F, 18F , IGHV3-30-5*01F, or V3-33*03F 86.1%

D-gene IGHD6-13*01F

J-gene IGHJ6*02F 71.4%

Case 16 FL main (clone 4-1) productive, rearranged, IGH sequence

Table 1. Clinical features of 19 cases of ISFN and 10 cases of FL

Case	Age	Sex	Site of biopsy	swollen lymph node in other region	Past history of lymphoma	BCL-2 CISH	Group	Fever	Weight loss	Performance status	Ann Arbor Stage	Hepatomegaly	Splenomegaly	Skin rash	Bone marrow infiltration	peripheral blood data		
																RBC ($\times 10^{12}$/L)	WBC ($\times 10^{9}$/L)	PLT ($\times 10^{9}$/L)
1	72	F	LN, Laxillary	cervical	No	translocation	ISFN	−	−	N.D.	N.D.	−	−	−	−	4.68	7.8	290
2	52	M	Asceding colon ulcer	−	No	unclear	ISFN-like FL (i)	−	−	N.D.	N.D.	−	−	−	−	4.91	6.3	300
3	58	F	LN, paraaortic	abdominal	No	unclear	ISFN-like FL (ii)	N.D.	N.D.	N.D.	Ⅱ	−	−	N.D.	N.D.	N.D.	6.51	236
4	72	M	LN, inguinal	general	No	unclear	ISFN-like FL (ii)	−	−	0	Ⅲ	−	−	−	−	4.72	4.8	186
5	66	F	LN, L cervical	−	non-Hodgkin's lymphoma	unclear	ISFN-like FL (i)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	4.28	4.9	177
6	61	M	L.tonsil	cervical	FL	failed	ISFN-like FL (ii)	−	−	N.D.	N.D.	−	−	−	−	4.60	6.61	260
7	51	M	LN, supraclavicular	mediastinum	FL	unclear	ISFN-like FL (ii)	−	−	N.D.	Ⅱ	−	−	−	−	4.34	8.9	390
8	69	M	LN, abdominal	deep abdominal	adult T-cell lymphoma/leukemia	unclear	ISFN-like FL (ii)	N.D.	N.D.	N.D.	N.D.	−	−	N.D.	N.D.	3.97	8.8	223
9	49	F	LN, L.submandibular	supraclavicular, deep cervical	No	unclear	ISFN-like FL (ii)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	4.59	6.68	298
10	62	M	LN, Linguinal	abdominal,multiple	No	failed	ISFN-like FL (ii)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
11	74	M	LN, abdominal	deep abdominal, paraaortic	No	failed	ISFN-like FL (ii)	−	−	N.D.	Ⅱ	−	−	−	N.D.	5.43	4.63	189
12	52	M	LN, R.inguinal	deep abdominal	No	failed	ISFN-like FL (ii)	−	+	N.D.	N.D.	−	−	−	N.D.	N.D.	N.D.	N.D.
13	75	F	LN, abdominal	abdominal,multiple, axilla	No	amplification	ISFN-like FL (ii)	+	−	0	N.D.	−	−	−	−	N.D.	N.D.	N.D.
14	54	M	N.D.	general,inflammation suspected	No	unclear	ISFN-like FL (ii)	−	−	0	Ⅲa	+	+	+	−	3.39	7.7	116
15	56	M	LN, Linguinal	−	No	failed	ISFN-like FL (iii)	−	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	5	167
16	61	F	LN, Linguinal	−	No	failed	ISFN-like FL (iii)	−	−	0	N.D.	−	−	−	N.D.	4.82	7.2	299
17	64	M	LN, para-submandibular gland	−	No	unclear	ISFN-like FL (iii)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
18	66	F	retroperitoneal	−	No	failed	ISFN-like FL (iii)	−	−	N.D.	N.D.	−	−	−	−	N.D.	N.D.	N.D.
19	68	F	LN, cervical	−	No	unclear	ISFN-like FL (iii)	−	−	N.D.	N.D.	−	−	−	−	N.D.	N.D.	N.D.
20	77	M	LN, abdominal	mediastinum, deep abdominal	No	failed	FL	−	−	2	Ⅳ	−	−	−	N.D.	4.03	6.43	112
21	62	M	LN, inguinal	axilla	No	failed	FL	+	−	N.D.	N.D.	−	+	−	N.D.	3.22	7.3	102
22	74	M	LN, mesenteric	deep abdominal	No	failed	FL	−	−	2	N.D.	−	−	−	−	4.18	4.5	127
23	80	F	LN, Linguinal	−	No	failed	FL	−	−	N.D.	N.D.	−	Infiltration of FL	−	−	2.90	7.4	348
24	65	F	LN, L.submandibular	cervical	No	translocation	FL	−	−	N.D.	N.D.		N.D.	−	−	4.13	5.5	230
25	71	F	LN, Linguinal	−	No	translocation	FL	−	−	0	Ⅰ	−	−	−	−	3.65	3.4	197
26	37	F	N.D.	N.D.	No	−	FL	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
27	53	M	LN, mesenteric	N.D.	No	−	FL	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
28	78	F	LN, R.cervical	N.D.	No	failed	FL	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
29	54	F	LN, mesenteric	N.D.	No	failed	FL	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	4.80	5.2	198

ISFN, in situ follicular neopolasm; FL,follicular lymphoma; LN, lymph node; RBC, red blood cell; WBC, white blood cell; PLT,platelete; N.D.,no data

Table 6. ISFN staging procedures* [18)]

Biopsy of additional nodal or suspected extranodal involvement
Physical examination
Blood flow cytometry
CT scan with i.v. contrast of neck, chest, abdomen, and pelvis
Unilateral bone marrow biopsy†

ISFN, in situ follicular neoplasm; CT, computed tomography.

*In the presence of signs/symptoms related to bone marrow involvement as well as flow cytometry abnormalities.

Table 2. Pathological findings of 19 cases of ISFN and 10 cases of FL

Case	Age	Sex	Histopathological diagnosis	absolute number of follicles	degree of involvement within individual follicles by BCL2++ cells										
					0	5	10	20	30	40	50	60	70	80-100	total number of 5-100 follicle(%)
1	72	F	ISFN	28	4	2	1	0	1	0	0	0	0	10	14(50.0)
2	52	M	ISFN-like FL	140	5	6	10	11	10	6	0	4	3	10	60(42.9)
3	58	F	ISFN-like FL	225	160	4	9	13	7	0	0	1	1	1	36(16.0)
4	72	M	ISFN-like FL	135	73	6	7	5	3	9	3	2	1	9	45(33.3)
5	66	F	ISFN-like FL	64	59	1	1	0	0	0	0	1	0	1	4(6.3)
6	61	M	ISFN-like FL	57	17	0	17	14	2	0	0	0	0	1	34(59.6)
7	51	M	ISFN-like FL + metastatic carcinoma	48	12	11	9	10	2	0	0	0	3	1	36(75.0)
8	69	M	ISFN-like FL	60	4	4	5	2	1	4	2	5	2	0	25(41.7)
9	49	F	ISFN-like FL	40	19	4	2	2	0	1	0	0	1	7	17(42.5)
10	62	M	ISFN-like FL	50	9	2	3	2	5	6	2	2	1	0	23(46.0)
11	74	M	ISFN-like FL	75	8	1	6	5	3	1	0	0	1	1	18(24.0)
12	52	M	ISFN-like FL	17	5	0	2	0	0	1	0	1	1	3	8(47.1)
13	75	F	ISFN-like FL	85	36	41	7	1	0	0	0	0	0	0	49(57.6)
14	54	M	ISFN-like FL	159	13	6	5	2	0	1	0	0	0	0	14(8.8)
15	56	M	FL(G1)+ISFN-like FL	50	0	1	0	0	0	0	0	0	5	15	21(42.0)
16	61	F	FL(G1)+ISFN-like FL	53	0	0	0	0	1	1	1	4	6	19	32(60.4)
17	64	M	FL(G1)+ISFN-like FL	140	129	2	0	5	3	0	0	0	1	0	11(7.9)
18	66	F	FL(G1)+ISFN-like FL	37	0	1	4	4	7	4	0	1	3	4	28(75.7)
19	68	F	FL(G1)+ISFN-like FL	22	1	4	5	6	0	0	0	0	2	0	17(77.3)
20	77	M	FL	124	38	0	0	0	0	0	0	0	0	0	0
21	62	M	FL	10	3	0	0	0	0	0	0	0	0	0	0
22	74	M	FL	20	2	0	0	0	0	0	0	0	0	0	0
23	80	F	FL	18	2	0	0	0	0	0	0	0	0	0	0
24	65	F	FL	62	0	0	0	0	0	0	0	0	0	0	0
25	71	F	FL	22	6	0	0	0	0	0	0	0	0	0	0
26	37	F	FL	150	10	0	0	0	0	0	0	0	0	0	0
27	53	M	FL	185	0	0	0	0	0	0	0	0	0	143	143(77.3)※
28	78	F	FL	160	0	0	0	0	0	0	0	0	0	0	0
29	54	F	FL	6	1	0	0	0	0	0	0	0	0	0	0

ISFN, in situ follicular neoplasm; FL, follicular lymphoma; G1, Grade 1

※BCL-2 strongly positive cells were mainly observed in GCs.

Table 3. Statistical analysis of differences in clinicopathological characteristics between ISFN like FL and FL

	ISFN (n=1)	ISFN like FL (n=18)		FL (n=10)		ISFN like FL *vs*. FL
Characteristic	No.	No.	%	No.	%	*P*
Number of follicles						
total follicles (range)	28	81(17-225)		76(6-185)		0.5329
Abs.n. of BCL-2(++)5-100% GC	14	27	-	14	-	0.0005
% of GC with BCL-2(++)5-100% in total follicle	50	-	42	-	18	0.0004
Sex (male/female)		11/7	61/39	4/6	40/60	0.4328
Age, y median(range)	72	62 (51-75)		65(37-80)		0.2495
fever	0	1	8	1	17	1.0000
loss of weight	0	1	9	0	0	1.0000
bleeding tendency	0	0	0	0	0	-
hepatomegaly	0	1	8	0	0	1.0000
splenomegaly	0	1	8	0	0	-
bone marrow involvement	0	0	0	0	0	-
Peripheral blood cell count						
RBC, $\times 10^{12}$/L, median(range)	4.68	4.50(3.39-5.43)		3.77(2.90-4.28)		0.0454
WBC, $\times 10^{9}$/L, median(range)	7.80	6.50(4.63-8.9)		5.7(3.4-7.4)		0.3749
Platelets, $\times 10^{9}$/L, median(range)	290	237(116-390)		188(102-348)		0.3749

ISFN, *in situ* follicular neoplasm; FL, follicular lymphoma

Table 4-1 Summary of IgVH clonality (IMGT/V-QUEST)-Case 15

lesion	clone (number)	PCR size (number)	IGHV	IGHV homology	IGHD	IGHJ	IGHJ homology
ISFN	main (9/12)	266 bp (8/12)	V1-3*01F	96.1%	D5-12*01F	J6*02F	87.8%
		325 bp (1/12)	V1-3*01F	77.8%	D5-12*01F	J6*02F	87.8%
	minor (3/12)	266 bp (2/12)	V3-7*02F	92.2%	D3-22*01F	J6*02F	79.6%
		323 bp (1/12)	V3-7*02F	72.6 %	D3-22*01F	J6*02F	79.6%
FL	sub A (4/12)	242 bp (4/12)	V3-11*06F V3-48*03F, 04F	82.2%	D3-9*01F	J4*03F	88.2%
	sub B (3/12)	242 bp (2/12)	V3-21*01F, 02F	96.1%	D3-22*01F	J4*03F	82.4%
		239 bp (-3 base, 1/12)	V3-21*01F, 02F	96.6%	D3-22*01F	J4*03F	82.4%
	sub C (2/12)	248 bp (2/12)	V3-13*02F	91.0%	D3-10*01F	J4*03F	88.2%
	others	254 bp (1/12)	V3-30*01F, 02F	91.7%	D3-16*02F	J1*01F	84.2%
		233 bp (1/12)	V3-48*03F	93.9%	D4-23*01 ORF	J4*03F	85.3%
		227 bp (1/12)	V4-59*01F, 02F, 07F V4-61*01F, 07F	88.1%	D3-16*02F	J4*01F, 03F	76.5%

Table 4-2 Summary of IgVH clonality (IMGT/V-QUEST)-Case 16

lesion	clone (number)	PCR size (number)	IGHV	IGHV homology	IGHD	IGHJ	IGHJ homology
ISFN	sub A (6/16)	250 bp (6/16)	V5-51*01, 03F	95.0%	D3-10*01F	J3*01F	75.0%
	sub B (6/16)	239 bp (4/16)	V4-59*01F, 02F, 07F V4-61*01F, 07F	93.2%	D3-10*01F	J6*03F	83.7%
		236 bp (-3 base, 2/16)	V4-59*01F, 02F, 07F V4-61*01F, 07F	93.2%	D3-10*01F	J6*03F	81.6%
	sub C (3/16)	263 bp (1/16)	V3-21*01F, 02F	96.1 %	D4-11*01 ORF	J6*03F	91.8%
		260 bp (-3 base, 1/16)	V3-21*01F, 02F	95.6%	D4-11*01 ORF	J6*03F	89.8%
		257 bp (-6 base, 1/16)	V3-21*01F, 02F	95.6%	D4-11*01 ORF	J6*03F	83.7%
	other (1/16)	257 bp (1/16)	V3-21*01F, 02F	93.3%	D4-17*01F	J6*02F	83.7%
FL	main (2/7)	248 bp (2/7, Direct)	V3-30*03F, 18F V3-30-5*01F V3-33*03F	86.1%	D6-13*01F	J6*02F	71.4%
	minor (2/7)	242 bp (2/7)	V3-73*01F, 02F	93.0%	D7-27*01F	J5*01F, 02F	89.2%
	others	269 bp (1/7)	V5-51*01F, 03F	90.0%	D3-10*01F	J5*01F, 02F	83.8%
		254 bp (1/7)	V4-59*03F	89.3%	D2-21*01F	J6*03F	83.7%
		245 bp (1/7)	V4-59*01F, 02F, 07F V4-61*01F, 07F	95.3%	D3-10*01F	J5*01F, 02F	89.2%

Table 5. Proportion of ISFNs associated with other overt lymphomas

	Published Year	All ISFN cases	Sex (male/female)	Age; median (range)	Without overt lymphoma	Prior FL	Concurrent FL	Subsequent FL	Prior/concurrent other B- or T-cell lymphoma
Cong et al.	2002	25	11/14	52 (23-76)	10	0	6	2	2
Torlakovic et al.	2002	4	N.D.	N.D.	0	1	3	N.D.	0
Montes-Moreno et al.	2010	13	6/7	55 (40-85)	4	N.D	3	4	3
Jegalian et al.	2011	34	15/19	52.2 (23-76)	20	2	4	1	5
Pillai et al.	2013	31	21/10	72 (38-88)	14	3	4	0	7
Schmidt et al.	2014	10	3/4	63.8 (42-76)	4	2	6	1	0
Bermudez et al.	2016	27	11/16	62.5 (40-84)	16	1	2	2	9
Present study	2019	19	11/8	62.2 (49-75)	10	2	5	N.D.	2

ISFN, *in situ* follicular neoplasm; FL, follicular lymphoma