Bcl-2-negative *IGH-BCL2* translocation-negative follicular lymphoma of the thyroid differs genetically and epigenetically from Bcl-2-positive *IGH-BCL2* translocation-positive follicular lymphoma

Running title: Bcl-2-negative follicular lymphoma of the thyroid

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Abstract

Aims

Follicular lymphoma (FL), comprising a minor subset of primary thyroid lymphomas, is divided into two groups based on Bcl-2 expression and *IGH-BCL2* translocation. The clinicopathological features exhibited by Bcl-2-negative *IGH-BCL2* translocation-negative FL of the thyroid (Bcl-2$^-$/*IGH-BCL2*$^-$ tFL) are different from those of conventional FL; however, its lymphomagenesis remains unclear. Here, we collected samples from seven patients with Bcl-2$^-$/*IGH-BCL2*$^-$ tFL to investigate their epigenetic and genetic aberrations.

Methods and Results

The immunohistochemical profiles of epigenetic modifiers and the methylation status of histones were examined, including EZH2, MLL2/KMT2D, CBP/CREBBP, EP300, H3K27me3, and H3K4me3, in Bcl-2$^-$/*IGH-BCL2*$^-$ tFL and Bcl-2-positive *IGH-BCL2* translocation-positive FL of the thyroid (Bcl-2$^+$/*IGH-BCL2*$^+$ tFL). Most Bcl-2$^-$/*IGH-BCL2*$^-$ tFLs retained the positivity of epigenetic modifiers and lower expression of H3K27me3, although Bcl-2$^+$/*IGH-BCL2*$^+$ tFLs exhibited aberrant immunohistochemical patterns of EZH2 and CBP/CREBBP and overexpression of H3K27me3. Samples from seven cases were further analysed using targeted sequencing, focusing on the exons of 409 key tumour suppressor genes and oncogenes. Bcl-2$^-$/*IGH-BCL2*$^-$ tFLs do not have pathogenic mutations of epigenetic modifiers, such as *EZH2, MLL2/KMT2D, MLL3/KMT2C, EP300*, and *ARID1A*, which have been reported in FLs in the literature, whereas Bcl-2$^+$/*IGH-BCL2*$^+$ tFLs are likely pathogenic/pathogenic missense mutations or frameshift mutations of these genes. Additionally, novel mutations in *TET2* and *EP400* were detected in Bcl-2$^-$/*IGH-BCL2*$^-$ tFLs.

Conclusions

Different genetic and epigenetic abnormalities might be involved in the oncogenesis of Bcl-2$^-$/*IGH-BCL2*$^-$ tFLs from Bcl-2$^+$/*IGH-BCL2*$^+$ tFLs and other FLs.

Keywords: Thyroid; Follicular lymphoma; Bcl-2; Epigenetics

Introduction

Primary thyroid lymphoma (PTL) is a relatively rare neoplasm that accounts for 1%–5% of thyroid malignancies.[1] B-cell PTLs include diffuse large B-cell lymphoma, extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue type (MALT lymphoma), follicular lymphoma (FL), small lymphocytic lymphoma, and Burkitt lymphoma. Diffuse large B-cell and MALT lymphomas are the most frequently observed B-cell PTLs, followed by FL.[2] Most PTLs are closely associated with preceding autoimmune lymphocytic thyroiditis (Hashimoto's disease).[3]

Bacon *et al*.[3] demonstrated that thyroid follicular lymphoma can be clearly divided into two groups according to their clinical, morphological, immunophenotypic, and genetic features. The features of the first group are the presence of t(14;18)/*IGH-BCL2* translocation and/or Bcl-2 expression, high CD10 positivity, and World Health Organization (WHO) grade 1-2, whereas those of the second group are the absence of t(14;18)/*IGH-BCL2* translocation and Bcl-2 expression, high CD10 negativity, and WHO grade 3. The molecular pathogenesis of this disease has not been fully investigated.

Recently, frequent mutations of epigenetic modifiers, including *EZH2*, *MLL2/KMT2D*, *CBP/CREBBP*, *EP300*, and *MEF2B*, have been described in FL.[4] Gain-of-function mutations in *EZH2* promote methylation at lysine 27 of histone H3, thus suppressing the transcription of anti-oncogenic genes (ex. *CDKN1A, CDKN2A,* and *PRDM1*) and promoting the expression of oncogenic genes (ex. *IRF4*). Loss-of-function mutations in *MLL2/KMT2D* result in demethylation of the lysine located at position 4 of H3 and subsequent gene suppression (ex. *TNFAIP3, SOCS3*, and *TNFRSF14*). Loss-of-function mutations in both *CBP/CREBBP* and *EP300* (*CBP/EP300* complex) leads to deacetylation of the lysine of H3, and subsequent suppression of *MHC class II* and acetylation of non-histone proteins (p53 and Bcl-6).

We compared the clinicopathological characteristics of Bcl-2-negative *IGH-BCL2* translocation-negative FL of the thyroid (Bcl-2$^-$/*IGH-BCL2*$^-$ tFL) and Bcl-2-positive *IGH-BCL2* translocation-positive FL of the thyroid (Bcl-2$^+$/*IGH-BCL2*$^+$ tFL) and examined the differences in the expression of epigenetic modifiers and methylation status. We also analysed genetic aberrations to elucidate the differences in oncogenesis between these two diseases.

Materials and methods

Patients

We selected seven patients with Bcl-2^{-}/*IGH-BCL2*$^{-}$ tFL and four patients with Bcl-2^{+}/*IGH-BCL2*$^{+}$ tFL from the pathological database (from 2013 to 2019) of the Osaka International Cancer Institute (OICI), Osaka University Hospital, Kindai University Nara Hospital, and Kuma Hospital. Additionally, as control samples, five cases with nodal Bcl-2^{+} FL and three cases with reactive lymphoid hyperplasia from OICI were included in our study. This study was approved by the Ethical Review Boards of OICI (No. 19031), Osaka University Hospital (No. 20050 and No. 20305), Kindai University Nara Hospital (No. 603), and Kuma Hospital (No. 20201008-3). All experiments were performed in accordance with the guidelines and regulations of all the committees.

Immunohistochemistry

All tissue samples were fixed in 10% formalin, embedded in paraffin, cut into 4-μm-thick serial sections, and used for haematoxylin and eosin staining and immunohistochemical staining. The latter was performed using the Roche BenchMark ULTRA IHC/ISH Staining Module (Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer's instructions. The primary antibodies used in this study and their dilution ratios are listed in Table 1. Immunohistochemical staining was scored by two independent pathologists (Y.H. and S.N.). Weak positivity was defined as weaker staining than that of the positive control cells or internal control cells (Table 1). Focal positivity was defined as a positivity rate of <50%. Cases which fulfilled the following criteria were classified as "negative": (1) less than 10% of the positivity rate and (2) weak-to-null intensity. Regarding Bcl-2, we estimated the net positivity rate in neoplastic B cells in germinal centres.

Analyses for *IGH-BCL2* rearrangement and *IGH* clonal rearrangement using polymerase chain reaction (PCR) and fluorescent *in situ* hybridization (FISH)

To analyse the *IGH-BCL2* rearrangement status, we performed PCR-based studies and FISH analyses using tumour tissues. Briefly, for PCR, sections cut from paraffin-embedded specimens were processed by deparaffinization, and DNA was extracted. We assessed *IGH-BCL2* rearrangement according to modified BIOMED-2 protocols.[5, 6] For FISH, analyses were performed on paraffin sections using the BOND FISH Kit (Leica Biosystems Newcastle Ltd., Newcastle upon Tyne, UK), Kreatech XL probe (BCL2 (18q21) Break), BOND Enzyme Pretreatment Kit, BOND

Hybridisation Solution, and BOND-III, according to the manufacturer's instructions. We also performed *IGH* clonal rearrangement analyses on the extracted DNA according to the modified BIOMED-2 protocols.

Targeted sequencing

Informed consent was obtained from four of the seven patients diagnosed with Bcl-2^{-}/*IGH-BCL2*$^{-}$ tFL and from three of four patients diagnosed with Bcl-2^{+}/*IGH-BCL2*$^{+}$ tFL for targeted sequencing studies. DNA extraction from paraffin-embedded specimens and targeted sequencing were performed by Macrogen Japan Corp. (Kyoto, Japan). The Ion AmpliSeq Comprehensive Cancer Panel (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to analyse the exons of 409 key tumour suppressor genes and oncogenes (Supporting data, Table S1).

Variant data analysis

We used the following filtering parameters for variant selection: (1) total coverage ≥ 20, (2) variant allele coverage ≥ 10, (3) variant allele fraction ≥ 5%, (4) variants with both forward and reverse reads, and (5) nonsynonymous or splice-site variants. To remove likely germline variants, we used allele frequency information available from public polymorphic variant databases and excluded any variants that possessed an observed allele frequency ≥ 0.5% in any of the ethnic populations in gnomAD exome (v2.0.1), gnomAD genome (v2.0.1),[7] 1000 genomes (August 2015 collection),[8] NHLBI Exome Sequencing Project with 6500 exomes,[9] 4.7KJPN Allele Frequency Panel (v20190826) from the Tohoku Medical Megabank Organization (ToMMo),[10] and HGVD (v2.30) from Kyoto University[11] using ANNOVAR.[12] Datasets for ToMMo[13] and HGVD[14] were used. Other datasets were downloaded from the ANNOVAR site.[15] Additionally, variants exhibiting greater than 95% variant allele fraction were excluded from the analysis, as seven patients had no family history of cancer.

Results

Clinical findings

The clinical findings of the seven cases with Bcl-2$^-$/*IGH-BCL2*$^-$ tFL and four cases with Bcl-2$^+$/*IGH-BCL2*$^+$ tFL are summarised in Table 2. Both groups showed a female predilection. The mean age of the patients with Bcl-2$^-$/*IGH-BCL2*$^-$ tFL and Bcl-2$^+$/*IGH-BCL2*$^+$ tFL were 71 and 63 years, respectively. Five of seven patients with Bcl-2$^-$/*IGH-BCL2*$^-$ tFL had antecedent autoimmune thyroiditis, whereas only one of four patients with Bcl-2$^+$/*IGH-BCL2*$^+$ tFL had it. All thyroid FLs were localised regardless of the Bcl-2/*IGH-BCL2* status. None of the patients died because of tumour progression. In Case 3, a recurrent tumour was detected and resected repeatedly.

Histological findings

All Bcl-2$^-$/*IGH-BCL2*$^-$ tFLs and Bcl-2$^+$/*IGH-BCL2*$^+$ tFLs exhibited enlarged neoplastic follicles with attenuated mantle zones. Moderate- to large-sized lymphoid cells that consisted of centrocyte-like or centroblast-like cells proliferated in the absence of tingible body macrophages in expanding germinal centres. In Bcl-2$^-$/*IGH-BCL2*$^-$ tFLs mitotic figures were frequent and all seven cases were classified as WHO grade 2 or 3A, whereas in Bcl-2$^+$/*IGH-BCL2*$^+$ tFLs, mitotic figures were rare and the WHO grade varied among the cases (Table 3 and Figure 1).

Immunohistochemistry

The immunohistochemical findings for tFLs are shown in Tables 3 and 4. In patients with Bcl-2$^-$/*IGH-BCL2*$^-$ tFL (Cases 1–7), neoplastic lymphoid cells were positive for CD20 and Bcl-6 and negative for CD3 and Bcl-2. The expression of CD10 was decreased; however, the expression levels of Bcl-6, LMO2, and HGAL were retained (Figure 2A–F). The MIB-1 labelling index ranged from 60% to 90%. In patients with Bcl-2$^+$/*IGH-BCL2*$^+$ tFLs (Cases 8–11), neoplastic lymphoid cells were positive for CD10 and Bcl-2, and the MIB-1 labelling index ranged from 20% to 40%.

The comparison of the immunohistochemical analyses for epigenetic modifiers and the methylation status of histones in Bcl-2$^-$/*IGH-BCL2*$^-$ tFL (Cases 1–7, Figure 3A–D) and Bcl-2$^+$/*IGH-BCL2*$^+$ tFL (Cases 8–11, Figure 3E–H) are summarised in Table 4.

In Bcl-2$^-$/*IGH-BCL2*$^-$ tFL, positivity for EZH2, MLL2/KMT2D, CBP/CREBBP, and EP300 were retained except in one case (Case 1) that exhibited a lower positivity of EZH2, whereas in Bcl-2$^+$/*IGH-BCL2*$^+$ tFL, EZH2 positivity was decreased in all cases and CBP/CREBBP was negative in two of four cases (Cases 9 and 11). In Bcl-2$^+$/*IGH-BCL2*$^+$ tFL, three of four cases exhibited an increased expression of H3K27me3, whereas in Bcl-2$^-$/*IGH-BCL2*$^-$ tFL, all cases exhibited no or

low-level expression (Cases 3, 4, and 7). All but one case (Case 1) retained H3K4me3 expression in both groups of thyroid FL regardless of Bcl-2 status.

Analyses for *IGH-BCL2* rearrangement and *IGH* clonal rearrangement using PCR and FISH

Clonal *IGH* rearrangement was detected in all but one case with Bcl-2$^-$/*IGH-BCL2*$^-$ tFL and Bcl-2$^+$/*IGH-BCL2*$^+$ tFL (Table 5). The *BCL2* rearrangement status examined using the PCR-based study and FISH analysis is shown in Table 5.

Targeted sequencing

Recurrently mutated genes ranked in the top 15 and *EZH2* and *EP300* are presented in Figure 4. Bcl-2$^-$/*IGH-BCL2*$^-$ tFL cases had no likely pathogenic/pathogenic mutations of epigenetic modifiers and linker histones including *EZH2*, *MLL2/KMT2D*, *MLL3/KMT2C*, *EP300*, and *ARID1A*, although pathogenic mutations of these genes have been previously reported in FLs. Likely pathogenic/pathogenic missense mutation (p.Pro494Thr, Case 8) and frameshift insertion (p.Ser1587fs and p.Arg1636fs, Case 9) of *ARID1A,* nonsense mutation (p.Gln4322Ter, Case 10) and frameshift deletion (p.Glu1957fs, Case 9) of *MLL2/KMT2D,* missense mutation (p.Tyr646Phe, Case 9) of *EZH2,* nonsense mutation (p.Tyr816Ter, Case 8; p.Tyr816Ter, Case 9; p.Gln2430Ter, Case 10; p.Tyr816Ter, Case 10) of *MLL3/KMT2C* were observed in our Bcl-2$^+$/*IGH-BCL2*$^+$ tFL cases. Likely pathogenic/pathogenic missense mutation (p.Lys1299Gln, Case 4), nonsense mutations (p.Gln1632Ter, Case 2; p.Glu1909Ter Case 3), splice site mutation (Case 10), frameshift insertion (p.Tyr1255fs, Case 3; p.Thr1183fs Case 1), and frameshift deletion (p.Ter2003fs, Case 2) of *TET2* were observed in Bcl-2$^-$/*IGH-BCL2*$^-$ tFLs, but not in Bcl-2$^+$/*IGH-BCL2*$^+$ tFLs, except in Case 10. Likely pathogenic/pathogenic missense mutation (p.Thr1452Met, Case 1) and frameshift deletion (p.Gln2727fs, Case 3; p.Gln2727fs, Case 10) of *EP400* were also observed in Bcl-2$^-$/*IGH-BCL2*$^-$ tFLs, but not in Bcl-2$^+$/*IGH-BCL2*$^+$ tFLs, except in Case 10. These mutations have not been previously reported as pathogenic mutations in FLs. Likely pathogenic/pathogenic missense mutations of *CBP/CREBBP* were detected in one case in each of Bcl-2$^-$/*IGH-BCL2*$^-$ and Bcl-2$^+$/*IGH-BCL2*$^+$ tFLs (p.Ser2382Phe, Case 1 and p.Ser2382Phe, Case 10, respectively). Mutations in *FGFR3* (p.Val684Ile) and *GATA2* (p.His323Tyr) were recurrently observed in seven specimens of Bcl-2-/*IGH-BCL2*- and Bcl-2+/*IGH-BCL2*+ tFLs (Cases 1–4, 8–11). These mutations were reported to be pathogenic according to OncoKB[16] and COSMIC v91,[17] but we reserved the conclusion regarding their

pathogenicity because the judgments of these databases were based on only one reference.[18]

Discussion

IGH-BCL2$^-$ FLs account for 10%–15% of FLs.[4] The prognosis for *IGH-BCL2*$^-$ FL was reported to be better than that for common FL.[4] Bcl-2$^-$/*IGH-BCL2*$^-$ tFL often presents with WHO grade 3 histology and CD10-negative immunohistochemistry, and a similar variant of FL has been described at other extranodal sites such as the skin or testes.[3] Epigenetic mutations are commonly reported in FL; however, these mutations are infrequent in *IGH-BCL2* translocation-negative FL.[4]

Recent studies have demonstrated that B-cell lymphomas originating from germinal centre cells, such as FL, often harbour epigenetic alterations, particularly chromatin remodelling. In our study, Bcl-2$^+$/*IGH-BCL2*$^+$ tFL exhibited low or no immunohistochemical positivity of molecules related to epigenetic modification, likely owing to mutation and overexpression of H3K27me3. Nodal Bcl-2$^+$ FL also exhibited aberrant expression of epigenetic modifiers and abnormal methylation status of histones similar to Bcl-2$^+$/*IGH-BCL2*$^+$ tFL (Supporting data, Table S4), suggesting common oncogenic mechanisms. However, Bcl-2$^-$/*IGH-BCL2*$^-$ tFL did not exhibit H3K27me3 overexpression. In addition, Bcl-2$^-$/*IGH-BCL2*$^-$ tFL retained immunohistochemical positivity for EZH2; therefore, methylation of H3K27 induced by mutated EZH2 is not involved in the oncogenesis of Bcl-2$^-$/*IGH-BCL2*$^-$ tFL. Immunohistochemical positivity for other epigenetic modifiers, MLL2/KMT2D, CBP/CREBBP, and EP300 was also retained in Bcl-2$^-$/*IGH-BCL2*$^-$ tFL. These immunohistochemical findings suggest that epigenetic abnormalities common to Bcl-2$^+$/*IGH-BCL2*$^+$ tFL and nodal Bcl-2$^+$ FL may not play a role in the development of Bcl-2$^-$/*IGH-BCL2*$^-$ tFL.

Targeted sequencing analysis demonstrated that Bcl-2$^-$/*IGH-BCL2*$^-$ tFLs do not have pathogenic mutations of epigenetic modifiers, such as *EZH2, MLL2/KMT2D, MLL3/KMT2C, EP300*, and *ARID1A*, which have been reported in FLs in the literature[4], whereas Bcl-2$^+$/*IGH-BCL2*$^+$ tFLs exhibit likely pathogenic/pathogenic missense mutations or frameshift mutations of these genes. However, Bcl-2$^-$/*IGH-BCL2*$^-$ tFLs are likely pathogenic mutations of other epigenetic modifiers, *TET2* and *EP400*, which have never been observed previously in FLs. These findings suggest that Bcl-2$^-$/*IGH-BCL2*$^-$ tFLs play an oncogenic role in the different genetic and/or epigenetic mechanisms of Bcl-2$^+$/*IGH-BCL2*$^+$ tFLs and other FLs.

TET2 promotes DNA demethylation and modulates the epigenetic status. Somatic mutations in *TET2* have been commonly observed in leukaemias and lymphomas, including

angioimmunoblastic T-cell lymphomas, mantle cell lymphomas, and diffuse large B-cell lymphomas.[19] We detected p.Gln1632Ter (nonsense mutation) and p.Lys1299Gln (missense mutation) in Cases 2 and 4 as likely pathogenic mutations. These mutations are located within the oxygenase domains of the 2-oxoglutarate and iron (II)-dependent dioxygenase (2OGFeDO) superfamily, which modifies nucleic acids.[20] These variants have been previously reported in polycythaemia vera (p.Gln1632Ter) and myelodysplastic-myeloproliferative neoplasm (p.Lys1299Gln), and classified as "pathogenic" in COSMIC v91.[17]

EP400 is a chromatin remodelling protein that promotes histone acetylation to control the epigenetic status.[21] Somatic mutations in *EP400* have been observed in bladder cancer and lymphoblastic leukaemia.[22] We detected p.Thr1452Met (missense mutation) in Case 1. This mutation has been previously reported in a gastric adenocarcinoma study[23] and classified as "pathogenic" in COSMIC v91.[17]

Missense mutations in *FGFR3* (p.Val684Ile) and *GATA2* (p.His323Tyr) are frequently detected in tFLs regardless of *IGH-BCL2* translocation. The specific pathogenicity of tFL in these genes is unknown, although *GATA2* regulates haematopoietic proliferation and differentiation,[24] and somatic mutations in *GATA2* have been observed in leukaemias[25].

Different genetic and epigenetic abnormalities in Bcl-2$^-$/*IGH-BCL2*$^-$ FLs from Bcl-2$^+$/*IGH-BCL2*$^+$ FLs might be resulted from suppression of Bcl-2, alternatively these abnormalities might be specific spots that were susceptible to mutagenic events in Bcl-2$^-$/*IGH-BCL2*$^-$ FLs, but we could not show decisive evidences for these possibilities. Zamo *et al.* reported that mutations of epigenetic modifiers, such as *CBP/CREBBP*, *EZH2*, and *EP300*, were found in *IGH-BCL2*$^-$ FL,[26] although the localisation of FLs was not specified in their study. They observed that enrichment of mutations in the genes associated with N-glycosylation, inflammation, and NF-κB signalling and speculated that increased crosstalk with the microenvironment that may compensate for the lack of N-glycosylation may contribute to oncogenesis of t(14;18)-negative FLs. To the best of our knowledge, these mutations have not been previously linked to thyroiditis or other autoimmune diseases. It should be noted that our study had some limitations. We analysed only exons, and based on this, we could not assess mutations in promoters and introns.

Conclusion

Our study demonstrated differences in aberrations of the expression of epigenetic modifiers and methylation status of histones between Bcl-2$^-$/*IGH-BCL2*$^-$ tFL and Bcl-2$^+$/*IGH-BCL2*$^+$ tFL. Different

genetic and/or epigenetic abnormalities might be involved in the oncogenesis of Bcl-2^{-}/*IGH-BCL2*$^{-}$ tFLs from those associated with Bcl-2^{+}/*IGH-BCL2*$^{+}$ tFLs and other FLs.

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Author contributions

Yuichiro Hamamoto performed data analysis and wrote the manuscript. Yoji Kukita performed the data analysis of targeted sequencing and wrote the manuscript. Masanori Kitamura, Keiichiro Honma, Tomoko Wakasa, Mitsuyoshi Hirokawa, and Eiichi Morii confirmed the diagnoses of the cases and approved the submitted manuscript. Masako Kurashige and Ayana Suzuki contributed to collecting cases and clinical information. Hiroaki Masaie, Shigeo Fuji, Jun Ishikawa, and Hitoshi Hanamoto helped the patients to provide informed consent. Shin-ichi Nakatsuka designed the research study and revised the manuscript.

Supporting information

The list of all the 409 genes targeted in the Ion AmpliSeq Comprehensive Cancer Panel is shown in Table S1. The list of all the extracted mutations is shown in Table S2. Clinical findings of nodal Bcl-2^+ FLs are shown in Table S3. Results of immunohistochemical analyses for epigenetic modifiers and methylation status of histone in nodal Bcl-2^+ FLs are shown in Table S4.

Data Availability Statement

The data that supports the findings of this study are available in the supplementary material of this article.

References

1. Noble VV, Ermann DA, Griffin EK, Silberstein PT. Primary Thyroid Lymphoma: An Analysis of the National Cancer Database. Cureus 2019; 11(2): e4088.
2. Hirokawa M, Suzuki A, Hashimoto Y et al. Prevalence and diagnostic challenges of thyroid lymphoma: a multi-institutional study in non-Western countries. Endocr J 2020; 67(11): 1085–1091.
3. Bacon CM, Diss TC, Ye H et al. Follicular lymphoma of the thyroid gland. Am J Surg Pathol 2009; 33(1): 22–34.
4. Korfi K, Ali S, Heward JA, Fitzgibbon J. Follicular lymphoma, a B cell malignancy addicted to epigenetic mutations. Epigenetics 2017; 12(5): 370–7.
5. Dongen JJM van, Langerak AW, Bruggemann 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(12): 2257–317.
6. Langerak AW, Groenen P, Bruggemann M et al. EuroClonality / BIOMED-2 guidelines for interpretation and reporting of Ig / TCR clonality testing in suspected lymphoproliferations. Leukemia 2012; 26(10): 2159–71.
7. Karczewski KJ, Francioli LC, Tiao G et al. The mutational constraint spectrum quantified from variation in 141,456 humans. Nature 2020; 581(7809): 434–43.
8. Auton A, Brooks LD, Durbin RM et al. A global reference for human genetic variation. Nature 2015; 526(7571): 68–74.
9. NHLBI Exome Sequencing Project with 6500 exomes [Internet]. [Accessed July 17, 2020]: Available from: https://evs.gs.washington.edu/EVS/
10. Tadaka S, Saigusa D, Motoike IN et al. jMorp: Japanese Multi Omics Reference Panel. Nucleic Acids Res 2018; 46(D1): D551–7.
11. Higasa K, Miyake N, Yoshimura J et al. Human genetic variation database, a reference database of genetic variations in the Japanese population. J Hum Genet 2016; 61(6): 547–53.

12. Wang K, Li M, Hakonarson H. ANNOVAR: Functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res 2010; 38(16): e164.
13. ToMMo [Internet]. [Accessed July 17, 2020]: Available from: https://jmorp.megabank.tohoku.ac.jp/201909/downloads/
14. HGVD [Internet]. [Accessed July 17, 2020]: Available from: http://www.hgvd.genome.med.kyoto-u.ac.jp/download.html
15. ANNOVAR [Internet]. [Accessed July 17, 2020]: Available from: https://doc-openbio.readthedocs.io/projects/annovar/en/latest/user-guide/download/
16. OncoKB [Internet]. [Accessed July 17, 2020]: Available from: https://www.oncokb.org/
17. COSMIC v91 [Internet]. [Accessed July 17, 2020]: Available from: https://cancer.sanger.ac.uk/cosmic
18. Chakrabarty S, Varghese VK, Sahu P et al. Targeted sequencing-based analyses of candidate gene variants in ulcerative colitis-associated colorectal neoplasia. Br J Cancer 2017; 117(1): 136–43.
19. Solary E, Bernard OA, Tefferi A, Fuks F, Vainchenker W. The Ten-Eleven Translocation-2 (TET2) gene in hematopoiesis and hematopoietic diseases. Leukemia 2014; 28(3): 485–96.
20. Deepa A, Naveena K, Anindya R. DNA repair activity of Fe(II)/2OG-dependent dioxygenases affected by low iron level in Saccharomyces cerevisiae. FEMS Yeast Res 2018; 18(2): 1–8.
21. Fazzio TG, Huff JT, Panning B. An RNAi screen of chromatin proteins identifies Tip60-p400 as a regulator of embryonic stem cell identity. Cell 2008; 134(1): 162-74.
22. Chen C, Bartenhagen C, Gombert M et al. Next-generation-sequencing-based risk stratification and identification of new genes involved in structural and sequence variations in near haploid lymphoblastic leukemia. Genes Chromosomes Cancer 2013; 52(6): 564-79.
23. Study ICGC(STAD-US), COSU541
24. Coma S, Allard-Ratick M, Akino T, van Meeteren LA, Mammoto A, Klagsbrun M. GATA2 and Lmo2 control angiogenesis and lymphangiogenesis via direct transcriptional regulation of neuropilin-2. Angiogenesis 2013; 16(4): 939–52.
25. Zhang SJ, Ma LY, Huang QH et al. Gain-of-function mutation of GATA-2 in acute myeloid transformation of chronic myeloid leukemia. Proc Natl Acad Sci USA 2008; 105(6): 2076–81.
26. Zamò A, Pischimarov J, Schlesner M et al. Differences between BCL2-break positive and negative follicular lymphoma unraveled by whole-exome sequencing. Leukemia 2018; 32(3): 685–693.

Table 1. Primary antibodies used in this study

Antibody	Clone	Dilution	Manufacturer	Control cells
CD20	L26	RTU	Roche	B-cells
CD3	2GV6	RTU	Roche	T-cells
CD5	SP19	RTU	Roche	T-cells
CD10	56C6	RTU	Leica (Novocastra)	Germinal centre B-cells
Bcl-2	SP66	RTU	Roche	Mantle zone B-cells
Bcl-6	GI191E/A8	RTU	Roche	Germinal centre B-cells
LMO2	SP51	RTU	Roche	Germinal centre B-cells
HGAL	MRQ-49	RTU	Roche	Germinal centre B-cells
MUM1	MRQ-43	RTU	Roche	DLBCL (ABC)
IRTA1 (FCRL4)	EPR21961	1:100	abcam	MALT lymphoma
CD23	SP23	RTU	Roche	Follicular dendritic cells
C-myc	Y69	RTU	Roche	Burkitt lymphoma
MIB-1	Mib-1	1:500	agillent (DAKO)	Germinal centre B-cells
EZH2	D2C9	1:200	Cell signaling TECHNOLOGY	Germinal centre B-cells
MLL2/KMT2D	Polyclonal	1:200	Sigma-Aldrich	Germinal centre B-cells
CBP/CREBBP	C-1	1:100	SANTA CRUZ BIOTECHNOLOGY	Germinal centre B-cells
EP300	D8Z4E	1:1000	Cell signaling TECHNOLOGY	Germinal centre B-cells
H3K27me3	C36B11	1:200	Cell signaling TECHNOLOGY	Vascular endothelial cells
H3K4me3	Polyclonal	1:100	Cell signaling TECHNOLOGY	Germinal centre B-cells

RTU, ready to use; DLBCL (ABC), diffuse large B-cell lymphoma (activated B-cell)

Table 2. Clinical findings

No.	Age (y)	Sex	Tumour size (mm)	Autoimmune thyroiditis	sIL-2R (U/ml)	LDH (U/l)	BM involvement	Leukemic picture in PB	Clinical stage	B symptoms	Treatment	Survival
Bcl-2$^-$/*IGH-BCL2*$^-$ tFL												
1	79	F	50×40	+	482	202	−	−	IE	−	RT	alive at 2 y 4 mo, NED
2	69	F	39×33×23	+	472	250	−	−	IE	ND	R-CHOP	alive at 1 y 5 mo, NED
3	73	F	12×11×7	+	220	184	ND	−	ND	ND	observation	alive at 5 y 2 mo, recurrence
4	77	F	36×35×18	−	432	197	−	−	IIE	−	RT	alive at 3 y 0 mo, NED
5	47	M	35×18×14	+	663	270	ND	−	ND	ND	R-CHOP	ND
6	81	F	32×22×13	−	568	237	ND	−	ND	−	R-CVP	alive at 3 y 5 mo, NED
7	55	F	68×53	+	419	157	ND	−	ND	−	observation	alive at 1 y 0 mo, NED

Bcl-2^{+}/*IGH-BCL2*$^{+}$ tFL												
8	58	F	21×18×14	+	224	156	ND	−	I	−	resection and observation	alive at 3 y 2 mo, NED
9	62	F	28×18×9	−	476	249	ND	−	III at least	−	R-B	alive at 2 y 5 mo, CMR
10	66	F	19×8×13	−	238	159	ND	−	IE	−	resection	alive at 6 y 7 mo, NED
11	66	F	24×10×18	−	441	208	ND	−	IIE	−	resection and R-CHOP	alive at 7 y 0 mo, NED

Bcl-2^{-}/*IGH-BCL2*$^{-}$ tFL, Bcl-2-negative *IGH-BCL2* translocation-negative follicular lymphoma of the thyroid; Bcl-2^{+}/*IGH-BCL2*$^{+}$ tFL, Bcl-2-positive *IGH-BCL2* translocation-positive follicular lymphoma of the thyroid; y, year; sIL-2R, soluble interleukin-2 receptor; BM, bone marrow; ND, no data; PB, peripheral blood; RT, radiation therapy; R-CHOP, chemotherapy with rituximab, cyclophosphamide, doxorubicin hydrochloride (hydroxydaunorubicin), vincristine (oncovin), and prednisone; R-CVP, chemotherapy with rituximab, cyclophosphamide, vincristine (oncovin), and prednisone; R-B, chemotherapy with rituximab and bendamustine; mo, month; NED, no evidence of disease; CMR, complete metabolic response

Table 3. Results of histological and immunohistochemical analyses

No.	Grade	LEL	Mitosis	CD20	CD3	CD5	CD10	Bcl-2	Bcl-6	Cyclin D1	LMO2	HGAL	MUM1	IRTA1	CD23	C-myc index	MIB-1 index
Bcl-2^{-}/*IGH-BCL2*$^{-}$ tFL																	
1	3A	+	frequent	+	−	−	w+	−	+	−	+	+	−	ND	−	1-2%	80%
2	3A	+	frequent	+	−	−	w+	−	+	−	+	+	w+	−	w+	10-30%	80%
3	2	+	frequent	+	−	−	w+	−	+	−	+	+	−	f, w+	−	<1%	70%
4	3A	+	frequent	+	−	−	w+	−	w+	−	+	w+	−	w+	−	<1%	70%
5	3A	+	frequent	+	−	−	w+	−	+	−	+	+	−	NA	−	<1%	>90%
6	2	+	frequent	+	−	−	w+	−	+	−	+	+	w+	f+	−	1-5%	90%
7	2	+	frequent	+	−	−	w+	−	+	−	+	+	−	f+	−	5-10%	60%

Bcl-2^{+}/*IGH-BCL2*$^{+}$ tFL																	
8	3A	+	rare	+	−	−	+	+	+	−	+	+	−	−	−	1-5%	40%
9	2	+	rare	+	−	−	+	+	+	−	+	w+	−	−	−	5%	40%
10	1	+	rare	+	−	−	+	+	+	−	+	+	−	−	−	5%	20-30%
11	3A	+	rare	+	−	−	+	+	+	−	+	+	−	−	−	1-10%	ND

Bcl-2^{-}/*IGH-BCL2*$^{-}$ tFL, Bcl-2-negative *IGH-BCL2* translocation-negative follicular lymphoma of the thyroid; Bcl-2^{+}/*IGH-BCL2*$^{+}$ tFL, Bcl-2-positive *IGH-BCL2* translocation-positive follicular lymphoma of the thyroid; LEL, lymphoepithelial lesion; w, weak; ND, no data; f, focal; NA, not analysable

Table 4. Results of immunohistochemical analyses for epigenetic modifiers and methylation status of histone

	Epigenetic modifiers				Methylation status of histone	
No.	EZH2	MLL2/KMT2D	CBP/CREBBP	EP300	H3K27me3	H3K4me3
Bcl-2^{-}/*IGH-BCL2*$^{-}$ tFL						
1	w+	+	+	+	−	−
2	+	+	+	+	−	+
3	+	+	+	+	w+	+
4	+	+	+	+	w+	+
5	+	+	+	+	−	+
6	+	+	+	+	−	+
7	+	+	+	+	w+	+
Bcl-2^{+}/*IGH-BCL2*$^{+}$ tFL						
8	w+	+	+	+	w+	+
9	w+	+	−	+	+	+
10	w+	+	+	+	+	+
11	w+	+	−	+	+	+
RLH						
12	+	+	+	+	−	+
13	+	+	+	+	−	+
14	+	+	+	+	−	+

Bcl-2^{-}/*IGH-BCL2*$^{-}$ tFL, Bcl-2-negative *IGH-BCL2* translocation-negative follicular lymphoma of the thyroid; Bcl-2^{+}/*IGH-BCL2*$^{+}$ tFL, Bcl-2-positive *IGH-BCL2* translocation-positive follicular lymphoma of the thyroid; RLH, nodal reactive lymphoid hyperplasia; w, weak

Table 5. Results of the PCR-based gene rearrangement study and FISH analyses

No.	PCR		FISH
	IGH clonal rearrangement	*IGH-BCL2* translocation	*BCL2* rearrangement
Bcl-2$^-$/*IGH-BCL2*$^-$ tFL			
1	+	−	ND
2	+	−	−
3	+	−	−
4	+	−	−
5	+	−	ND
6	+	−	ND
7	−	−	ND
Bcl-2$^+$/*IGH-BCL2*$^+$ tFL			
8	+	+	+
9	+	−	+
10	NA	NA	+
11	NA	NA	+

Bcl-2$^-$/*IGH-BCL2*$^-$ tFL, Bcl-2-negative *IGH-BCL2* translocation-negative follicular lymphoma of the thyroid; Bcl-2$^+$/*IGH-BCL2*$^+$ tFL, Bcl-2-positive *IGH-BCL2* translocation-positive follicular lymphoma of the thyroid; NA, not analysable; ND, no data

Figure legends

Figure 1 Histology of Bcl-2^{-}/*IGH-BCL2*$^{-}$ follicular lymphoma of the thyroid (tFL) (Case 1, haematoxylin and eosin staining).
Numerous enlarged lymph follicles were observed in the thyroid tissue (original magnification 20×) (A). Centrocyte-like or centroblast-like cells proliferated in expanding germinal centres. Mitosis was frequent (one or more mitotic figures were apparent in one high-power field) (original magnification 400×) (B).

Figure 2 Representative images of immunohistochemical analyses of Bcl-2^{-}/*IGH-BCL2*$^{-}$ follicular lymphoma of the thyroid (tFL).
Tumour cells were CD20 (+) (A), CD3 (－) (B), CD10 (weak+) (C), Bcl-2 (－) (Bcl-2-positive T-cells were included in germinal centre) (D), and Bcl-6 (+) (E) (Case 7, original magnification 100×). The MIB-1 labelling index was 80% (Case 2, original magnification 400×) (F).

Figure 3 Representative images of immunohistochemical analyses for epigenetic modifiers and methylation status of histones (original magnification 100×).
Bcl-2^{-}/*IGH-BCL2*$^{-}$ follicular lymphoma of the thyroid (tFL) exhibited negative staining for H3K27me3 (Case 1) (A), positive staining for EZH2 (Case 2) (B), positive staining for CBP/CREBBP (Case 1) (C), and positive staining for MLL2/KMT2D (Case 1) (D). Bcl-2^{+}/*IGH-BCL2*$^{+}$ tFL of the thyroid exhibited positive staining for H3K27me3 (Case 9) (E), weakly positive staining for EZH2 (Case 10) (F), negative staining for CBP/CREBBP (Case 9) (G), and positive staining for MLL2/KMT2D (Case 9) (H).

Figure 4 Oncoplot in targeted sequencing.
Recurrently mutated genes ranked in the top fifteen and *EZH2* and *EP300* are shown. Mutations which were considered likely pathogenic/pathogenic based on databases (OncoKB, COSMIC v91, and ClinVar), frameshift mutations, nonsense mutations, and splice site mutation are emphasized with a white frame.