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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 $\geq 20$, (2) variant allele coverage $\geq 10$, (3) variant allele fraction $\geq 5\%$, (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 $\geq 0.5\%$ 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 $IGH\text{-}BCL2$ 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$^-$/$IGH\text{-}BCL2^-$ FLs from Bcl-2$^+$/$IGH\text{-}BCL2^+$ 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$^-$/$IGH\text{-}BCL2^-$ 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 $IGH\text{-}BCL2^-$ 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$^-$/$IGH\text{-}BCL2^-$ tFL and Bcl-2$^+$/$IGH\text{-}BCL2^+$ 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.

**Acknowledgements**

**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.

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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<sup>+</sup>/*IGH-BCL2*<sup>+</sup> tFL

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

| No. | Epigenetic modifiers | | | | Methylation status of histone | |
|-----|------|-----------|-----------|-------|---------|---------|
|     | 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.