EZH2 mutations in follicular lymphoma from different ethnic groups and associated gene expression alterations

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Translational Relevance

Follicular lymphoma (FL) is an indolent, but incurable subtype of non-Hodgkin lymphoma. These tumor harbor t (14;18) translocation in at least 90% of patients. Recently, activating EZH2 mutations have been found in a significant number of patients with FL. However, the reported incidence has been quite variable and has come almost exclusively in Western countries. We have developed a sensitive pyrosequencing assay for all mutation variants affecting codon 641. Our study has shown that Chinese and western FL patients exhibit EZH2 Y641 mutation at similar frequencies, and the mutation is far more frequently associated with the BCL2 rearranged group in both populations. We have also demonstrated by gene expression profiling (GEP) that FL cases with this mutation showed a set of significantly down-regulated genes in EZH2 mutant cases. The gene signature provided some insight on the mechanisms of action of abnormally enhanced EZH2 function. EZH2 mutations may play a similar role in lymphomagenesis in different ethnic groups and can serve as a biomarker for potential EZH2 targeted therapy.
Abstract

**Purpose:** Gain-of-function mutations of **EZH2** occur frequently in diffuse large B-cell lymphomas and in follicular lymphomas (FLs). However, the frequency of **EZH2** mutation in Chinese FLs and the potential targets affected by this mutation are unknown.

**Experimental Design:** We determined **EZH2** codon 641 mutations in Chinese FLs (n=124) and compared with Western FLs (n=70) using a sensitive pyrosequencing assay. Gene expression profiling (GEP) was performed to determine differential gene-expression between the mutated vs unmutated subgroups, and selected genes were validated using immunohistochemistry (IHC).

**Results:** Our results showed similar frequencies of **EZH2** codon 641 mutations in Chinese and Western FL cohorts (16.9% vs 18.6%, χ²-test, p=0.773), including all five reported mutation variants. We observed significant association of **EZH2** mutation with low morphologic grade FLs (Grade 1-2, 23.6% vs Grade 3, 7.7%, χ²-test, p=0.02). **EZH2** mutations also showed significant association with **BCL2** rearrangement in the Chinese cohort (26.8% vs 8.8% χ²-test, p=0.008) and combined cohorts (26.3% vs 9.1%, χ²-test, p=0.002). GEP analysis identified several genes including **TCF4**, **FOXP1**, **TCL1A**, **BIK** and **RASSF6P** with significantly lower mRNA expression (p<0.01) in mutated cases and the potential target **TCL1A** showed consistent results at the protein level.

**Conclusion:** Similar prevalence of **EZH2** mutation in two ethnic groups suggests shared pathogenetic mechanisms. The much lower frequency of **EZH2** mutation in cases without **BCL2** translocation suggests a different pattern of evolution of this subtype of FL. GEP studies showed a set of differentially expressed genes and suggested that **EZH2** mutation may help to lock the tumor cells at the GC stage of differentiation.

**Keywords:** follicular lymphoma; **EZH2** gene mutation; pyrosequencing, Chinese lymphoma; GEP
Introduction

Follicular lymphoma is a common germinal center (GC) B cell-derived non-Hodgkin lymphoma (NHL) exhibiting a spectrum of morphologies, immunophenotypes, and genetic aberrations. It is the second most common type of NHL representing about 30% of NHL in western countries (1, 2); however, the incidence of FL is lower in Asian countries (3,4). A recent study of 98 Chinese FL cases revealed a lower frequency of BCL2 rearrangement in Chinese FL (58.5%) than in Western FL (80% to 90%) (5). The BCL2 rearrangement results in constitutive expression of the antiapoptotic BCL2 protein, which plays an important role in tumorigenesis of FL; however, BCL2 rearrangement alone is not sufficient for malignant transformation of B cells. Multiple genetic abnormalities, including mutation of Enhancer of Zeste homolog 2 (EZH2), have recently been reported in FL (6-9). The EZH2 gene encodes a histone methyltransferase that constitutes the catalytic component of the polycomb repressive complex-2 (PRC2) and is involved in repressing gene expression through methylation of histone H3 on lysine 27 (H3K27). EZH2 codon 641 mutation, the most common mutation hot-spot, is a gain-of-function mutation leading to enhanced trimethylation of histone H3K27 and plays an important role in the tumorigenesis of GCB-type DLBCL and FL (6-11). Interestingly, highly selective EZH2 inhibitors have now been developed which is a potential therapeutic strategy for lymphomas with EZH2 activating mutations (12). However, it is unclear whether Chinese FL utilizes similar pathogenetic pathways as their Western counterpart and the frequency of EZH2 mutation in Chinese FL is not known. The aim of the present study is to identify the frequency of EZH2 gene codon 641 mutation in Chinese FL and the association with the status of BCL2 expression and rearrangement. We also analyzed the gene expression profiling (GEP) data of FLs with and without EZH2 gene mutation to identify unique associations with EZH2 mutation in FL.
Materials and methods

Patient materials

124 Chinese FL diagnosed between January 1999 and May 2010 were obtained from Hong Kong and provinces of Shandong, Tianjing and Shaan Xi, China, among which 109 cases had BCL2 rearrangement data detected by interphase fluorescence in situ hybridization (FISH). We also included 70 Western FL with BCL2 rearrangement data detected by FISH from the University of Nebraska Medical Center for comparison. As FL without or without t(14;18) may evolve through different molecular pathways, our initial hypothesis is that EZH2 mutation may be present at different frequencies in FL with different t(14;18) status. We enriched both cohorts with a substantial number of FL cases with no BCL2 rearrangement (From previous cytogenetics and FISH studies) to test the hypothesis as such cases are uncommon particularly in Western FL. The cases were reviewed by expert hematopathologists (J.K.C.C., W.C.C. and B.M.) and re-reviewed by W.C.C. and S.P.G, and classified based on the World Health Organization criteria. These cases showed at least 30%-40% of the sectional areas consisting of malignant follicles on hematoxylin and eosin (H&E) examination. This study was approved by the institution review boards of the respective institutions.

Construction of tissue microarray and immunohistochemistry

Representative areas of FL tissue blocks were selected to prepare the tissue microarray (TMA) by examination of the corresponding H&E stained slides. To ensure accurate representation, three areas of each tumor were selected for core preparation. Each target area on the selected blocks was punched to obtain a 1 mm diameter tissue core to be placed consecutively on the recipient master blocks. TMA containing representative tumor sections from 124 Chinese and 70 Western FLs were used for this retrospective analysis. Sections from the TMAs were stained with anti-human BCL2 (clone 124), CD20 (clone
L26), BCL6 (clone PG-B6p), (Dako Corp, Denmark), and CD10 (clone 56C6 ), CD21 (clone EP3093), CD3 (clone SP7), (Abcam, MA), and anti-TCL1A antibody (clone 1-21, Santa Cruz Biotechnology Inc, Texas). 5 µm thick tissue sections were deparaffinized with xylene, washed with ethanol, and blocked with methanol with 3% hydrogen peroxide. Sections were incubated with the above antibodies using Bond polymer refine detection kit and bond IHC Stainer (Leica Microsystems Inc. Buffalo Grove, IL). For TCL1A staining, GC B-cells and Naïve B-cells in the mantle zone of reactive tonsil were used as positive controls. The percentage of positive tumor cells and the staining intensity were graded from 0-3, and the two scores were added to form a final score to divide the cases from negative to strong TCL1A expression as described in supplementary Table 1A.

**Fluorescence in-situ hybridization**

TMA of 124 Chinese FLs and 70 Western FLs were assessed for the presence of *BCL2* rearrangement on 4µm FFPE sections using the Vysis LSI *BCL2* dual color break-apart rearrangement probe (Abbott Molecular, Des Plaines, IL). Cases were scored as *BCL2* rearranged on the basis of detecting unambiguous probe separation above the set threshold.

**DNA extraction, PCR and Pyrosequencing**

5-10 µm thick tissue sections of representative formalin-fixed, paraffin-embedded (FFPE) tissue blocks were prepared. Genomic DNA was extracted using a Qiagen DNA FFPE Tissue Kit (Valencia, CA). Genomic DNA encompassing *EZH2* codon 641 was PCR amplified using forward primer 5'-GGCTGGGGGATTTTTATCAA-3' and biotinylated reverse primer 5'- GTTATCAGTGCCTTACCTCTCCA-3'. The PCR products were sequenced on a Qiagen Pyromark Q24 instrument (Valencia, CA) using a forward sequencing primer 5'-GAAAAATGAATTCATCTCAG-3'. Genomic DNA of cell line SU-DHL-6,
harboring an EZH2 codon Y641N mutation, and normal tonsil lacking EZH2 mutation, was mixed with SU-DHL-6 DNA constituting 5%, 10%, 20%, 30%, 50% and 70% of the total DNA respectively. The mixture was used as template to determine the sensitivity of pyrosequencing to detect the mutant. The sensitivity of the assay was determined to be at 5% of the genomic DNA of the cell line SU-DHL-6. According to the principle of pyrosequencing, the light emission in the process of sequencing is proportional to the quantity of dNTPs incorporated into the template. dNTPs were dispensed in a novel order of G, A, A, T, C, G, A, T and C, based on the sequence of wild type EZH2 codon 641 and 5 mutation variants. With this order of addition, at least one addition results in nucleotide incorporation for the mutant but not the wild type allele, allowing all 5 mutations to be identified and detected with maximal sensitivity (Table 1). All detected mutations were validated by repeating the assay.

**Gene expression profiling data analysis**

The gene expression profiling (GEP) data from 68 cases of UNMC FL were analyzed to find genes differentially expressed between FLs with or without EZH2 mutation. We used HG-U133 plus 2 arrays (Affymetrix Inc., Santa Clara, CA) for our analysis, and the methods for isolation and processing of RNA and acquisition of GEP raw data have been described previously (13). The raw data were uploaded in BRB-Array Tools (version 3.7.0) for normalization, and the analysis was supervised by the EZH2 gene mutation status. Genes were selected at significance of p<0.01 and >1.5 fold difference in expression level between the two groups. To enhance the identification of differential gene expression in FL tumor cells, only FL cases with a high B-cell content (enriched B-cell signature of >2-fold above the mean of the B-cell signature (pan B-cell markers) in the entire FL data set) were chosen for analysis.

**Statistical analysis**
Statistical significance of differences in EZH2 mutation frequency between lymphoma groups with or without BCL2 rearrangement and expression was assessed by chi-squared ($\chi^2$) test. Statistical significance was defined as $p<0.05$. 


Results

Patient characteristics

124 Chinese FL patients were included in this study. The median age of these patients was 59.5 years (range, 26-89 years), and included 64 (51.6%) males and 60 (48.4%) females. These cases were enriched in BCL2 translocation negative cases so that only 56 harbored a BCL2 rearrangement and 68 lacked a BCL2 rearrangement. Among the 70 western FLs, the median age was 60 (range, 35-88 years) and there were 33 (47.1%) male and 37 (52.9%) female patients. This cohort was also enriched in BCL2 wild type cases, 39 harbored a BCL2 rearrangement and 31 lacked a BCL2 rearrangement. There were no significant differences in clinical features between Chinese and Western FLs. On histological morphological assessment, 72 of 124 (58%) in Chinese cohort and 39 of 70 cases (55.7%) in the Western cohort were of grade 1-2.

Frequencies of EZH2 codon 641 mutations

We observed EZH2 codon 641 mutations in 16.9% (21 of 124) Chinese FL and 18.6% (13 of 70) Western FLs using pyrosequencing and the results are summarized in Table 2. No significant difference in the frequency of EZH2 mutation in FLs between Chinese and the western patients was observed (16.9% (21/124) vs 18.6% (13/70), \( \chi^2 \)-test, \( p=0.773 \)). Representative results of pyrosequencing of wild type EZH2 codon 641 and 5 mutant variants are shown in Figure 1. The five mutation variants resulted in the amino acid change from tyrosine (Y) 641 to phenylalanine (F), serine (S), cysteine (C), asparagine (N) and histidine (H) as shown in Table 1. These five variants were detected in Chinese FL with frequencies similar to those of Western FL. Y641F was the most common mutation, accounting for 38% of mutants, with decreasing frequencies from Y641S (24%), Y641H (14%), Y641C (14%) to Y641N (10%).
Association between EZH2 mutation and morphologic FL grades

The EZH2 mutation frequency was related to the morphologic FL grades, with higher frequency in low morphologic grade FLs in both Chinese (Grade 1-2, 23.6% vs Grade 3, 7.7%, χ²-test, p=0.02), and Western population (Grade 1-2, 25.6% vs Grade 3, 9.7%, χ²-test, p=0.09 ) (Table 2). The distribution of EZH2 mutation in different morphological grades of Western FL was similar to that of Chinese FL. The percentage of EZH2 mutation in grade 1 (7/33; 21.2%), and grade 2 (10/39; 25.6%) FL was quite similar in Chinese FLs, and similar trend was observed in Western FLs.

Association between EZH2 mutation and BCL2 translocation

As expected from our case selection, BCL2 rearrangements was detected in 56/124 (45.2%) and 39/70 (55.7%) in Chinese and Western FL respectively. BCL2 expression were observed in 76/124 (61.3%) and 51/70 (72.9%) in Chinese and Western FL respectively. The positivity of BCL2 rearrangement and expression was higher in grade 1-2 than in grade 3 FLs in the two cohorts (Table 2). The frequency of EZH2 mutation was significantly higher in FLs with a BCL2 rearrangement than in those lacking a BCL2 rearrangement in Chinese FL (26.8% vs 8.8%, χ²-test, p=0.008). However, the difference observed in Western FL did not reach statistical significance (25.6% vs 9.7%, χ²-test, p=0.163). EZH2 mutation was significantly associated with BCL2 rearrangement in the combined Chinese and Western cases (26.3% vs 9.1%, χ²-test, p=0.002) (Figure 2). EZH2 mutation was also significantly associated with the BCL2 protein expression in Chinese (22.4% vs 8.3%, χ²-test, p=0.042) and the combined FL cohorts (22.8% vs 7.5%, χ²-test, p=0.007, respectively). The percentage of EZH2 mutation was enriched in BCL2 protein positive cases in Western cohort, however it did not reach statistical significance (23.5% vs 5.3%, χ²-test, p=0.155) (Figure 2).
Differential gene expression analysis

Our analysis focused on FL cases with high B-cell contents to facilitate the detection of changes related to the tumor cells. A set of genes was significantly down-regulated in mutated cases. Among these down-regulated genes, a number of them, such as TCF4, FOXP1, TCL1A, BIK and RASSF6P were also marked repressed compared with normal centroblasts (Figure 3). There was also a set of genes such as PTPN22, which was up-regulated in mutant cases probably as secondary changes. We also compared our signature with the specific gene signature derived previously using an EZH2-specific inhibitor (GSK126) on GCB cell lines with EZH2 mutation (12). Notably, this signature was significantly (p<0.02) down-regulated in cases with EZH2 mutation compared with wild type cases (Figure 3).

Down-regulation of TCL1A in EZH2 mutated FLs

One of the target genes that were reported to be up-regulated upon treatment of EZH2 mutant cell lines by a specific inhibitor of EZH2 mutant protein (GSK12) is TCL1A (ref 12). Consistent with this observation, we observed that this gene was down-regulated in FL cases carrying EZH2 Y641 mutation compared to our wild type. There have been reports suggesting variable expression of TCL1A in FL. We evaluated TCL1A expression at the protein level and observed that B cells in the mantle zone of reactive tonsil showed strong expression of TCL1A, whereas GC B-cells showed moderate expression in both cytoplasm and nucleus. Of the FL cases without EZH2 Y641 mutation, the majority of the FL cases (67.2%, 39 of 58) showed strong homogenous expression of TCL1A, whereas 10.3 % (6 of 58) were moderate, and 22.4% (13 of 58) were either weak or negative. Interestingly, none of the FL case with EZH2 Y641 mutation showed strong expression; it was generally variable from moderate (6 cases) to weak (1 case), or negative (3 cases) in 10 mutant cases. The results are summarized in supplementary Table 1B, and representative images are shown in Figure 4.
Discussion

EZH2 is the catalytic subunit of the polycomb repressive complex 2 (PRC2) and is involved in repressing gene expression through methylation of lysine 27 of histone H3. The EZH2 codon 641 mutation is a gain-of-function mutation leading to enhanced trimethylation of histone H3K27 (6). EZH2-mediated epigenetic silencing in GC-B cells contributes to proliferation and lymphomagenesis (14).

The frequency of EZH2 mutations varied from 7.2% to 22% (Supplementary Table 2) in prior studies (7,9-10); the variation is probably due to the different methods used. Pyrosequencing was used in the present study because it is more sensitive than conventional Sanger sequencing and is widely used clinically to detect point mutations in FFPE tissues. The assay was able to detect 5% of the genomic DNA of the cell line SU-DHL-6, which harbors an EZH2 codon Y641N mutation. Assays with lower sensitivity may miss the mutation in cases with low tumor content, which are not uncommon in FLs. We detected a similar frequency of EZH2 codon 641 mutations in Chinese FL [21 of 124 (16.9%)], and in Western FL [13 of 70 (18.6%)]. All of the five reported mutations, Y641F, Y641S, Y641C, Y641H and Y641N, were detected in Chinese FL with frequencies similar to those previously reported in Western FL (7,9-11). Y641F was the most common mutation, accounting for 38% of mutants. It has been reported that all of the five mutant proteins confer a gain-of-function phenotype and that Y641N is the most potent at generating H3K27me3, whereas Y641H is the least potent (8).

The BCL2 rearrangement is a genetic hallmark of FLs, but a small percentage of FL cases lack this rearrangement; the latter are much more frequent in the Chinese population (5). It is possible that cases with or without BCL2 rearrangement may have different pathogenetic mechanisms that may be reflected in different profiles of genetic mutations. A recent study in Western patients indeed found a marked difference in EZH2 mutation frequency between the two subgroups (7). We investigated the association of EZH2 mutation and the status of BCL2 rearrangement in FL including a substantial number of cases.
with no BCL2 rearrangement. The frequency of EZH2 mutation was significantly associated with BCL2 rearrangement in Chinese FL, but did not reach statistical significance in western FL, probably due to the small number of cases. However overall (combining both cohorts) EZH2 mutation was significantly associated with BCL2 rearrangement. The frequency of EZH2 codon 641 mutation in Chinese FL with BCL2 rearrangement was very close to that reported in a previously study (28%), using a sensitive detection technique (7). The mutation frequency in Chinese FL lacking BCL2 rearrangement was low and comparable to similar patients in Western countries but far higher than reported previously (7). As EZH2 codon 641 mutation was first identified in a grade I FL lacking BCL2 rearrangement (9), the reported lack of EZH2 mutation in cases negative for BCL2 rearrangement (7) is probably spurious and due to the low number of cases investigated. The low frequency of EZH2 mutation in FLs without BCL2 rearrangement and in grade 3 FL suggests the preferential use of different pathways to promote the malignant transformation of GC B-lymphocytes to FL.

Although it has been demonstrated that the EZH2 codon 641 mutation enhances trimethylation of histone H3K27, the genomic targets that are affected by the mutation and thus promote lymphomagenesis have not been identified. Our GEP data analysis revealed a set of differentially expressed genes; among them TCF4, FOXP1, RASSF6, TCL1A and BIK were down-regulated, compared with both wild type FL and normal CB. Both TCF4 and FOXP1 were consistently up-regulated in ABC-DLBCL compared to GBC-DLBCL (15). This observation suggests that one of the functions of EZH2 mutation may be locking the cells at the GCB cell stage of differentiation by down-regulating the expression of crucial genes for further differentiation. TCL1A is also of particularly interest; it was initially identified as an oncogene in T-cell prolymphocytic leukemias, but may also be expressed in B-cell lymphomas including FL (16). TCL1A serves as a coactivator of the AKT/mTOR pathway and has also been reported as an inhibitor of de novo DNA methylation in B-cell chronic lymphocytic leukemia (CLL).
(17), so down-regulation of TCL1A may have diverse effects including epigenetic alterations. We further confirmed the frequent expression of TCL1A protein in FLs. It was revealed that FLs displayed striking variability in the intensity of TCL1A staining and there is a correlation with EZH2 Y641 mutation. 39 cases showing strong positivity for TCL1A harbored no EZH2 Y641 mutation, whereas the expression of TCL1A in the mutant cases varied from moderate to weak, or negative, and none showed strong positivity. The IHC results are in keeping with the GEP analysis and may explain the reported variability of TCL1A expression in FL. Interestingly, it was also noted that the expression of TCL1A increased in DLBCL cell lines harboring EZH2 mutation when treated with the EZH2 specific inhibitor GSK126 (12). This observation is consistent with our finding and suggests that TCL1A is likely gene targeted by EZH2 mutation in FL. We also observed that specific gene signature generated by EZH2 mutant inhibitor (GSK1206) (12) showed expected correlation in mutant FL cases, suggesting that such inhibitors may provide a rationale and viable therapeutic drug for these patients. Down-regulation of BIK (Bcl2-interacting killer), a pro-apoptotic gene, is also of interest as 40% of cases of multiple myeloma were reported to have a methylated BIK CpG island (18). Another down-regulated gene is RASSF6, a Ras-association family (RASSF) member that has been shown to be frequently epigenetically inactivated by promoter CpG island hypermethylation in childhood leukemias (19).

Among the up-regulated genes, PTPN22 is intriguing. The expression of PTPN22 in FLs with EZH2 mutation is significantly higher than in normal B-cells (naïve B cells, centrocytes, and centroblasts) and in FLs without EZH2 mutation. PTPN22 is considered to be one of the principal negative regulators of antigen-receptor signaling in normal B and T lymphocytes, but it can also positively regulate the kinase AKT, providing a powerful survival signal. B-cell receptor signaling in FL may need to be finely tuned to avoid further differentiation and simultaneous activating the AKT pathway may optimize cell survival, perhaps compensating for the effects of TCL1 down-regulation on the AKT/mTOR pathway (20).
In conclusion, *EZH2* codon 641 mutations occurs at similar frequency in Western and Chinese FL and more often associated with low grade cases. The mutations are far more frequent in the *BCL2* rearranged group. This mutation, probably plays a similar role in the pathogenesis of FLs in both Chinese and Western patients and may serve as a marker for potential *EZH2* targeted therapy.

**Disclosure/conflict of interest**

The authors declare no conflict of interest.

**Authors’ Contributions**

Conception and design: W.C.Chan
Development of methodology: T.McKeithan, T.C.Greiner
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W.C.Chan, J.Iqbal, T.C.Greiner
Analysis and interpretation of data (e.g., statistical analysis): W. Zhang
Writing, review, and/or revision of the manuscript: S.P.Guo, J.Iqbal, R. Wang, W. C.Chan
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.P.Guo, J.K.C.Chan, K.Fu, B.Meng, Y.Pan, W. Cheu, D. Luo
Study supervision: W.C.Chan

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References


Table 1. EZH2 codon 641 mutation variants by adding dNTPs in the designated order (G,A,A,T,C,G,A,T,C) during pyrosequencing

<table>
<thead>
<tr>
<th>Amino Acid Change</th>
<th>Nucleotide Change</th>
<th>Signal strength by nucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y641</td>
<td>TAC</td>
<td>W-AATACT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>W-AATACT___A4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T2  C0  G0  A2  T0  C2</td>
</tr>
<tr>
<td>Y641F</td>
<td>TAC&gt;TTC</td>
<td>M-AATTCT__A4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T3  C1  G0  A1  T1  C1</td>
</tr>
<tr>
<td>Y641S</td>
<td>TAC&gt;TCC</td>
<td>M-AATCCT__A4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T2  C2  G0  A1  T1  C1</td>
</tr>
<tr>
<td>Y641C</td>
<td>TAC&gt;TGC</td>
<td>M-AATGCT__A4</td>
</tr>
<tr>
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<td></td>
<td>T2  C0  G1  A1  T0  C2</td>
</tr>
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<td>TAC&gt;AAC</td>
<td>M-AAAACT___A6</td>
</tr>
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<td></td>
<td></td>
<td>T1  C1  G0  A1  T1  C1</td>
</tr>
<tr>
<td>Y641H</td>
<td>TAC&gt;CAC</td>
<td>M-AACACT__A4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T1  C1  G0  A2  T0  C2</td>
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</tbody>
</table>

Abbreviation: W-wild type, M-Mutant, Y-Tyrosine, F-Phenylalanine, S-Serine, C-Cysteine, N-Asparagine, H-Histidine.
Table 2. EZH2 mutation and BCL2 rearrangement and expression in FLs

<table>
<thead>
<tr>
<th>Classification</th>
<th>EZH2 mutation</th>
<th>BCL2 rearranged</th>
<th>BCL2 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Chinese FL (n=124)</td>
<td>21 (16.9%)</td>
<td>56 (45.2%)</td>
<td>76 (61.3%)</td>
</tr>
<tr>
<td>Grade 1-2 (n=72)*</td>
<td>17 (23.6%)</td>
<td>47 (65.3%)</td>
<td>57 (79.2%)</td>
</tr>
<tr>
<td>Grade 3 (n=52)</td>
<td>4 (7.7%)</td>
<td>9 (17.3%)</td>
<td>19 (36.5%)</td>
</tr>
<tr>
<td>All Western FL (n=70)</td>
<td>13 (18.6%)</td>
<td>39 (55.7%)</td>
<td>51 (72.9%)</td>
</tr>
<tr>
<td>Grade 1-2 (n=39)**</td>
<td>10 (25.6%)</td>
<td>30 (76.9%)</td>
<td>33 (84.6%)</td>
</tr>
<tr>
<td>Grade 3 (n=31)</td>
<td>3 (9.7%)</td>
<td>9 (29%)</td>
<td>18 (58.1%)</td>
</tr>
</tbody>
</table>

*Includes grades 1 (n=33) and 2 (n=39).
**Includes grades 1 (n=17) and 2 (n=22).
Figure Legend

Figure 1. Pyrosequencing of *EZH2* codon 641 mutation in representative FLs:

(A) Normal tonsil, wild type *EZH2* codon Y641 (A4, T2, C0, G0, A2, T0, C2). (B-F). Dotted red lines show the wild type pattern. Filled arrows show dNTP additions that yield incorporation into the mutant but not the wild type allele.

(B) FL60, *EZH2* codon Y641N (A6, T1, C1, G0, A1, T1, C1).

(C) FL51, *EZH2* codon Y641F (A4, T3, C1, G0, A1, T1, C1).

(D) FL66, *EZH2* codon Y641S (A4, T2, C2, G0, A1, T1, C1).

(E) FL45, *EZH2* codon Y641C (A4, T2, C0, G1, A1, T0, C2).

(F) FL62, *EZH2* codon Y641H (A4, T1, C1, G0, A2, T0, C2).

Figure 2. *EZH2* mutation in FLs by BCL2 status:

*EZH2* mutations were significantly more frequent in cases with rearrangements and expression of BCL2 in Chinese FL and combined Chinese and Western FL.

Figure 3. Differential gene expression between *EZH2* mutant vs wild type FL cases (p<0.01; >1.5 fold change). Only cases with high B-cell content were included in the analysis.

* Gene signature was derived in GCB cell lines with mutant EZH2 gene EZH2 specific inhibitor (GSK126). The authors have included only up regulated genes (35 probe set) in at least 4 of 5 cell lines after inhibition of mutant EZH2 (Ref 12). CB: centroblast, CC: centrocyte.

Figure 4. Expression of TCL1A in FLs:

(A) Expression of TCL1A in GC and mantle zone cells in a reactive tonsil (Magnification 40×).

(B) A FL case without *EZH2* Y641 mutation showing strong TCL1A expression (Magnification 40×).

(C) FL cases with *EZH2* Y641 mutation showing moderate expression of TCL1A (Magnification 40×).

(D) Mutant with weak TCL1A expression (Magnification 20×).

(E) Mutant negative for TCL1A, scattered T-cell and mantle zone B-cells were strongly positive (Magnification 40×).
Figure 2

EZH2 mutation frequency

- BCL2 negative
- BCL2 rearranged
- BCL2 wild type

Chinese Fls
Western Fls
Chinese and Western Fls

p = 0.008
p = 0.163
p = 0.002
p = 0.007
Figure 3

Relative Level of Expression (as median value)

EZH2 target gene signature (Mean)*

Pan B-cell signature (mean)

Normal B-cell subsets

- Mean
- Mut
- WT

Mutant EZH2

Wild-type EZH2

EZH2 mRNA
Clinical Cancer Research

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