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EZH2 Expression in Follicular Lymphoma Is Variable and Independent from the Progression of Disease Within 24 Months of First Treatment

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Abstract. *Background/Aim: Follicular lymphoma (FL) relapse within 24 months of the first immunochemotherapy (POD24) indicates more precisely poor overall survival and high risk of death. The aim of the study was to assess the potential value of POD24 in FL and describe the enhancer of zeste homolog 2 (EZH2) expression profile, in correlation with clinical/ histopathological/immunophenotypical characteristics. Materials and Methods: This retrospective single-center study included 75 patients with FL treated under watch and wait (W&W) and immunochemotherapy regimens. All cases were immunohistochemically assessed: assays were performed for EZH2, CD10, BCL6, BCL2, MUM1, MYC and p53. Results: POD24 was independent of clinical/histopathological/ immunohistochemical features and separated patients with inferior outcomes. EZH2 high expression was observed in high/low grade and follicular/diffuse FL patterns. BCL2-negative (p=0.042) and MUM1 (p=0.039), MYC (p<0.001), p53 (p<0.001) - positive cases had significantly higher EZH2 expression. Conclusion: POD24 is currently the most useful tool for the identification of poor outlook patients. EZH2 is crucial in FL biology, but the value of its protein expression is limited as a prognostic factor.*

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Key Words: Follicular lymphoma, EZH2, POD24, time to first systemic therapy, prognostic factors, expression profile.

Follicular lymphoma (FL) is an indolent B-cell lymphoma, but early relapse after first-line therapy occurs in up to 20% of patients (1-3). Different prognostic markers have been evaluated; however, the follicular International Prognostic Index (FLIPI) is still the only reproducible tool (4, 5). Its molecular modifications have not been yet widely accessible, while the next-generation sequencing-based genetic evaluation of FL is not a routine part of a diagnostic examination (6-8). Recently, the progression of disease within 24 months of first treatment (POD24) was applied for the identification of FL high-risk patients (3, 9). Early treatment failure is predictive of poor overall survival (10) and seems to be independent of initial treatment modality (11). Available results indicating the association of POD24 with prognostic markers, *i.e.*, reduced intratumoral immune infiltration, pre-treatment positron-emission tomography-based staging, and molecular status, are still inconsistent and unsatisfactory (12, 13).

Enhancer of zeste homolog 2 (EZH2) is a histone-lysine N-methyltransferase enzyme encoded by the *EZH2* gene. It is a functional component of polycomb repressive complex 2 (PRC2) and catalyzes the methylation of histone H3 lysine 27

(H3Kme27). EZH2 has a crucial role in transcription repression, development, and differentiation of healthy tissues. Conversely, in neoplastic cells, it activates transcription and stimulates proliferation. EZH2 alterations, both protein overexpression and mutations, were described in both solid tumors, *i.e.*, breast (14-18), hepatocellular (19, 20), gastric (21), urothelial (22, 23), prostate carcinomas (24, 25), melanoma (26, 27) and in non-Hodgkin's lymphomas (NHL) *i.e.*, diffuse large B-cell lymphoma (DLBCL), FL, Burkitt lymphoma and other small cell and aggressive B-cell NHL (28, 29). The inferior impact of EZH2 overexpression on overall survival has been outlined independently (30).

EZH2 pathway includes the repression of cyclin-dependent kinase inhibitor CDKN1A, which is fundamental in the germinal center (GC) formation (31). In FL, which is GC-derived lymphoma, the EZH2 is constitutively activated and imprints repressive marks on both proliferation-checkpoint and terminal-differentiation genes (32, 33). One of the possible explanations of EZH2 protein loss is gain-of-function mutation related to tyrosine 646 *EZH2* mutations have been reported in around 25% of FL (34, 35) and are believed to improve the clinical outcome. The *EZH* wild-type FL cases are associated with significantly increased risk of the early event. Therefore, the *EZH2* gene status was included in the revised m7-FLIPI (7, 36). The recent results have shown that the prognostic value of the m7-FLIPI clinical and genetic model seems dependent on a therapeutic regimen (37). The results of EZH2 protein expression in FL are conflicting. Some studies have shown that a high level of EZH2 protein correlates with FL aggressiveness (29), but its microscopical visualization remains highly limited. Additionally, the latest observations have shown that EZH2 expression and distribution is far more complicated and related to many non-genetic causes (38, 39).

Moreover, the EZH2 pathways include close interactions with other essential genes, *i.e.*, *MYC*, *BCL-2*, *BCL-6,* and *TP53*. Lately, the importance of cross-talk between MYC and TP53 (40, 41) as well as MYC and EZH2 (42, 43) is being discussed. The influence of MYC, BCL2, BCL6, or p53 protein expression in FL, on POD24, has not been yet thoroughly investigated.

Our study aims at EZH2 expression profiling in the FL, including its clinical impact. We focus on POD24 and time to first treatment within 24 months (TTFT24) analysis. The study illustrates the histopathological pattern of EZH2 expression concerning FL immunophenotype characteristics.

Materials and Methods

Study population. All FL cases were diagnosed at Pathology Laboratory, Department of Pathology and Laboratory Diagnostics, Maria Sklodowska-Curie National Research Institute of Oncology, Warsaw, Poland. The study protocol was approved by the Ethical Committee of Maria Sklodowska-Curie National Research Institute of Oncology. The study was conducted in accordance with the provisions of the Declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice. All cases were reviewed and reclassified according to the revised 4th edition of the World Health Organization (WHO) diagnostic recommendations (6). The inclusion criteria were: pathologically proven diagnosis of FL grade 1, 2, or 3A, available formalin-fixed paraffin-embedded (FFPE) material, and clinical observation at least 5 years. Asymptomatic patients were observed according to the watch and wait (W&W) strategy until progression. Treatment was initiated in the presence of High Tumor Criteria in FL by Groupe d'Etude des Lymphomes Folliculaires (GELF). Patients received RCHOP or RCVP by individual physician decision (44, 45).

Immunohistochemistry. Tissue microarrays (TMA) were constructed; 3 cores of 1mm diameter from representative samples of lymphoma were incorporated. The positive (tonsil, testis, placenta) and negative (liver, kidney) controls were introduced in each TMA. Slides were stained with monoclonal anti-EZH2 antibody (clone: SP129, RTU, Ventana, Roche) reactive in paraffin-embedded tissue according to the manufacturer recommendation (Benchmark, Ventana, Roche, Tuscon, AZ, USA). The positive nuclear, strong (3+), and moderate (2+) EZH2 expression within the total area were evaluated via the semi-quantitative method by experienced hematopathologists (A.S.-C., K.S., M.P.-S.). The two categories, as previously described (46), included <70% (low EZH2) and ≥70% (high EZH2) of positive cells. The panel of antibodies for assessment of FL immunophenotype was used: CD10 (clone: 56C6, RTU, pH 9.0, Dako Omnis), BCL2 (clone: 124, RTU, pH 9.0, Dako Omnis), BCL6 (clone: PG-B6p, RTU, pH 9.0, Dako Omnis), MUM1 (clone: MUM1p, RTU, pH 9.0, Dako Omnis), MYC (clone: Y69, 1:100, pH 9.0, Abcam), and p53 (clone: DO-7, RTU, pH 9.0, Dako Omnis) were investigated onto FL cells. The evaluation cut-offs were defined as below: MUM1 – nuclear staining, high (>30%) or low (10-30%) expression, negative (<10% positive nuclei); MYC - nuclear staining, high (>40%) or low (10-40%) expression, negative (<10% positive nuclei); p53 - nuclear staining, high (>30%) or low (10-30%) expression, negative (<10% positive nuclei). All photographs were taken using the microscope camera DP72 Olympus BX53 (Olympus, Japan) with a spectrum of 2× – 400× magnification.

Statistical analysis. All statistical analyses were performed using R version 3.6.3 software (R Foundation for Statistical Computing, Vienna, Austria). Overall survival (OS) was defined as the time from diagnosis to the date of death or last contact date if the patient was still alive (censored observation). On treatment, progression-free survival (PFS) was defined as the time from treatment onset to progression or last contact date if progression did not occur (censored observation). Before treatment PFS for patients on W&W strategy was defined as the time from diagnosis to progression or last contact date if progression did not occur. POD24 was defined as one if the time from first therapy to the first documented progression was not greater than 24 months (high-risk POD24 group) and 0 otherwise (low-risk POD24 group). Introduction of first treatment within 24 months since diagnosis (TTF24) was defined analogously to POD24, but only for patients on W&W strategy. The Wilcoxon rank-sum and Pearson's chi-square tests were used respectively to compare continuous and categorical factors between stratified cohorts. Multivariate Cox proportional hazard models were used to estimate hazard ratios (HRs).

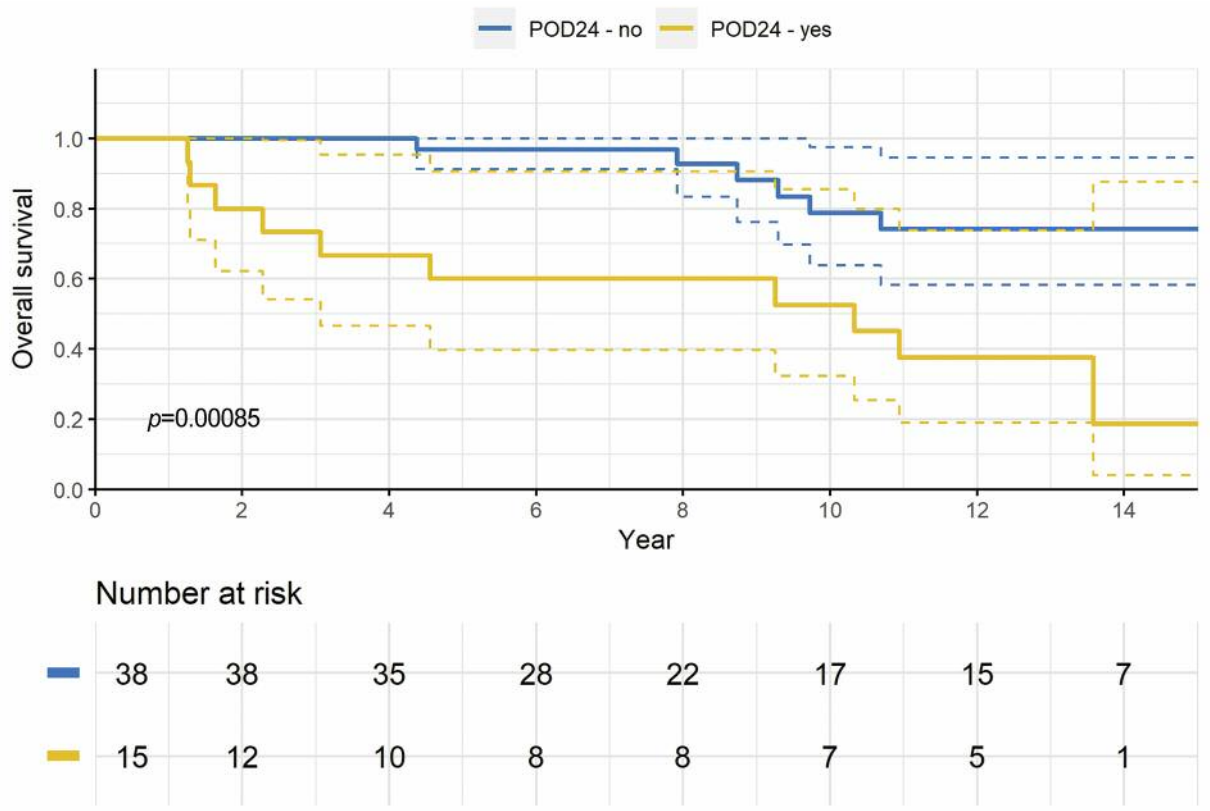

Figure 1. *Overall survival curve for patients who did or did not have progression of treatment within the first 24 months (POD24=yes and POD24=no, respectively). The dashed line indicates a 95% confidence interval.*

Results

POD24 high-risk FL patients have a significantly worse overall survival. We included 75 patients diagnosed with FL with available tissue material. W&W and immunochemotherapy groups included respectively 31 and 44 patients. Patients who progressed on treatment within the first 24 months (POD24 – yes) had significantly worse overall survival compared to those that did not have progression within the first two years (Figure 1). HRs for patients with POD24, including clinical staging: age, gender, LDH and Hemoglobin levels, Ann Arbor Stage, treatment options, FLIPI1, and FLIPI2, were not significant. HRs for FL on treatment progression are shown in Figure 2.

EZH2 expression in FL is variable and non-homogenous. EZH2 expression was identified as high and low in 33.3% (25/75) and 66.7% (50/75) of FL cases, respectively. The expression was moderate to strong which was comparable to positive controls (Figure 3, compare upper photos with A, B, and C). Strong staining was identified within cells with centroblast-like morphology (Figure 3, arrows). The EZH2 expression loss was seen in cases of each histopathological grade (1 – 46.0%, 23/50; 2 – 40.0%, 20/50; and 3a – 14.0%, 7/50) including low and high-grade distribution (86.0%, 43/50 and 14.0%, 7/50, respectively) (Figure 3). The FL histopathological patterns (follicular, follicular/diffuse and diffuse) were similar in EZH high and low groups.

The only clinical correlation between the EZH2 expression profile was age; older patients had higher EZH levels (60.5 *vs*. 52.0 years). Comparing EZH high to low groups and FL immunophenotype shows weak statistical differences in BCL2 and MUM1 protein profile. "Loss" of BCL2 and "gain" of MUM1 is observed in EZH2 high group (BCL2 negative: 48.0%, 12/25 *vs*. 22.0%, 11/50; MUM1 positive: 44.0%, 11/25 *vs*. 20.0%, 10/50). Expressions of MYC and p53 are strongly significant for the EZH high group (MYC positive: 76.0%, 19/25 *vs*. 8.0%, 4/50; p53 positive: 56.0%, 14/25 *vs*. 6.0%, 3/50). Eventually, FLs with high EZH expression exhibited lower BCL2 and higher MUM1, MYC, and p53 levels. The characteristic immunohistochemical profile of FL with EZH2 high expression is depicted in Figure 4. The clinical and histopathological characteristics according stratified EZH2 expression profile are summarized in Table I.

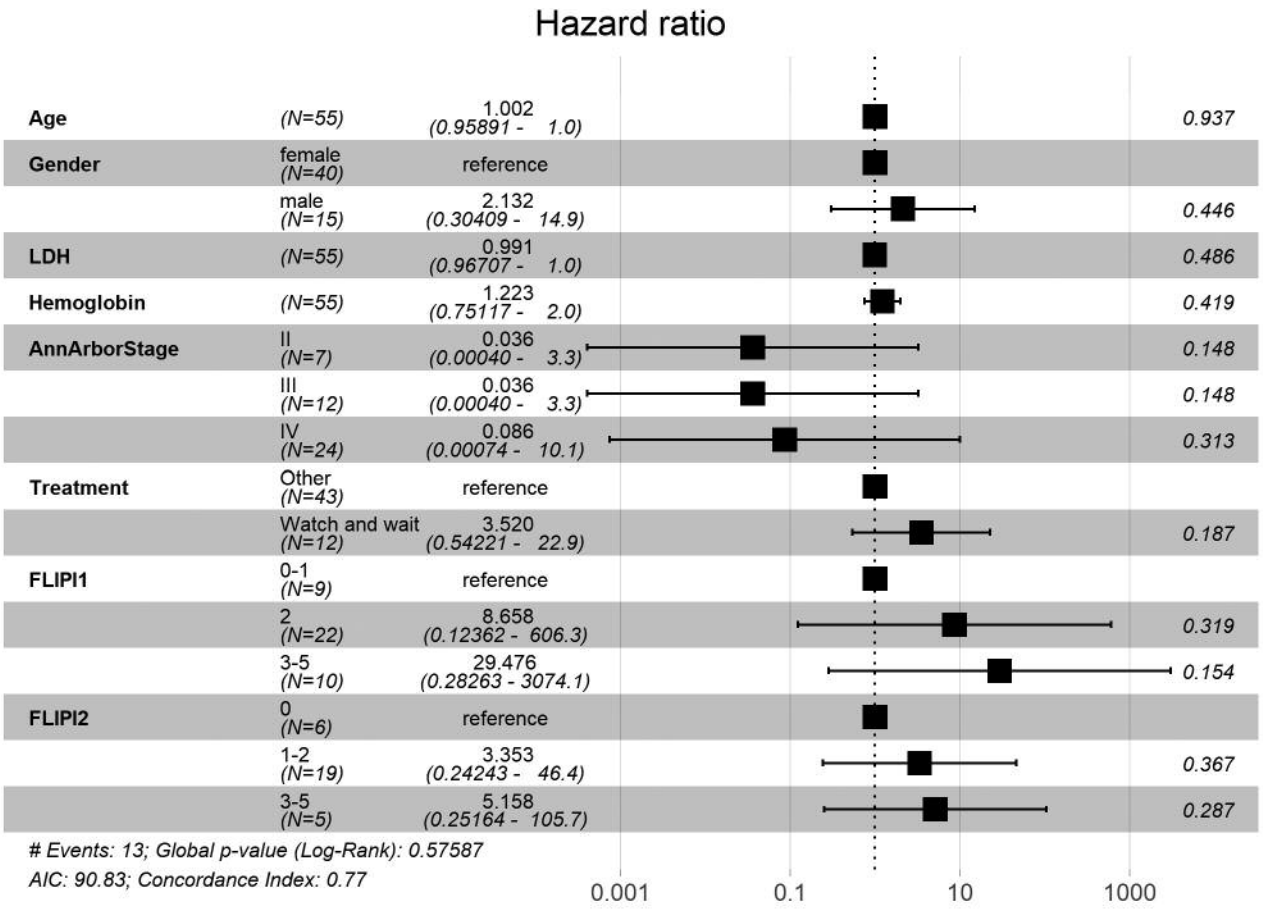

Figure 2. *Treatment progression hazard ratios (HRs) for the immunochemotherapy cohort from a multivariate Cox proportional hazards model.*

POD24 is not related to EZH2 expression profile or FL immunophenotype. The stratification with POD24 shows a similar clinical profile of patients. The EZH high expression profile is nearly the same for patients with and without POD24 (43.8%, 7/16 *vs*. 36.8%, 14/38). The FL histological grading and patterns do not influence POD24. The FL immunophenotype is not predictive for the identification of POD24 high-risk patients. The characteristics of the stratified POD24 cohorts are depicted in Table II.

Discussion

Regardless of the overall survival improvement in FL still, the leading cause of death is lymphoma and its transformation. In 10-year observation studies, the worst outcome concerns patients with high FLIPI1 score, with transformed disease to DLBCL and those who did not achieve event free-survival within 12 and 24 months of diagnosis (9, 10, 13, 47, 48). The prediction of POD24 and risk of relapse and progression reflects the need for a revised investigation of predictive and prognostic markers.

Our analysis revealed that EZH2 immunohistochemical expression was independent of clinical status. In the POD24 subgroups, age, gender, LDH, hemoglobin levels, Ann Arbor, FLIPI1, and FLIPI2 were not significant. The EZH2 high or low protein levels have no impact on POD24 and TTFT24. However, patients who progressed on treatment within the first 24 months had significantly worse overall survival. Our data confirm that POD24 is the most potent factor for outcome monitoring.

The clinical impact of EZH2 status is thought to be a leading predictive marker for targeted treatment selection (36). The GALLIUM trial within the m7-FLIPI, mutations in *EZH2* revealed the highest impact and were associated with longer PFS (HR 0.25, p=0.0036) in CHOP/CVP-treated patients, but not in Bendamustine-treated patients (HR 1.11, p=0.71) (36). Morschhauser *et al*. were the first who showed results of tazemetostat for high-risk patients whose disease progresses within 24 months of diagnosis (48). The open-label,

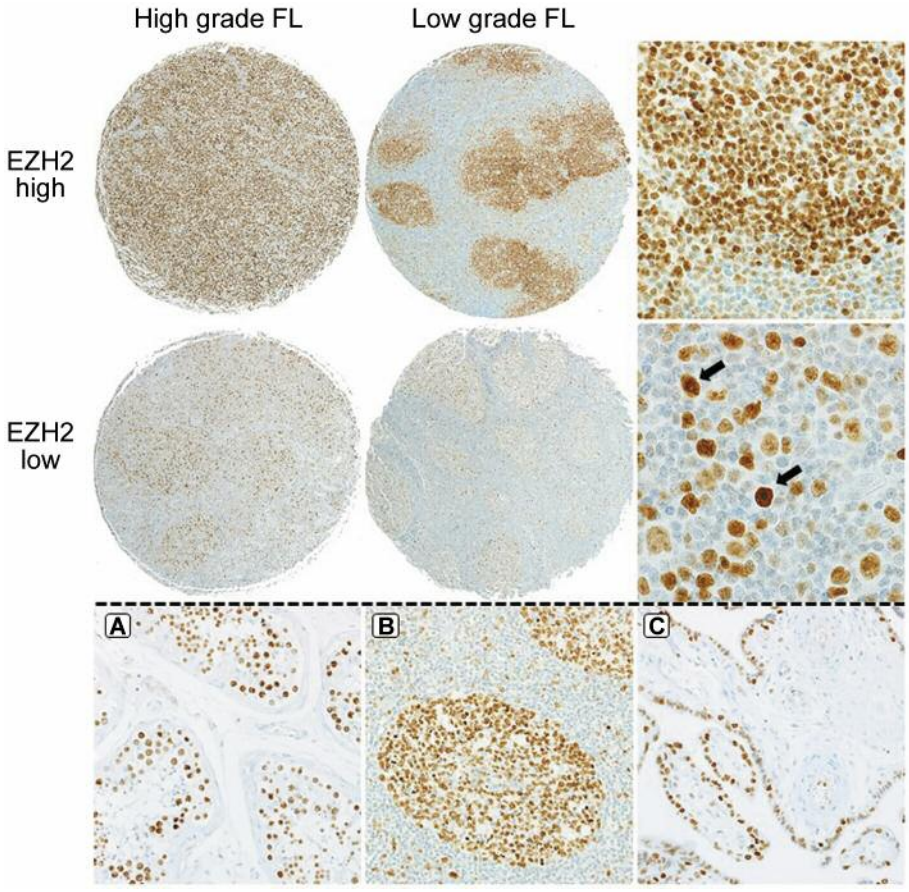

Figure 3. *EZH2 expression in follicular lymphoma (FL). The high and low-grade FL presented high and low EZH2 expression; the EZH2 was strongly expressed within centroblasts (arrows) (2× – 400×); the positive controls included (A) – testis (100×), (B) – reactive germinal centers of the tonsil (100×), (C) – placenta (100×).*

multicenter, phase 2 study (NCT01897571) included patients with relapsed/refractory FL with or without *EZH2* mutation (MT *EZH2*, n=45, POD24, n=17, 38%; WT *EZH2,* n=54, POD24, n=30, 56%, respectively). The objective response rate, partial response, progression-free survival, and the median duration of the response for POD24 groups MT *vs*. WT were: 65% *vs*. 30%, 59% *vs*. 30%, 13.8 months *vs*. 5.6 months, 8.2 months *vs*. 7.3 months, respectively (48). That has confirmed the influence of *EZH2* mutation on treatment results, but the expectations were much greater (39, 49). The latest disappointing result of *EZH2*-mutant DLBCL treatment with tazemetostat monotherapy has stressed the need to develop other biomarkers and further explore the translational mechanisms related to EZH2 (50). Akpa *et al*. established the sensitivity of lymphoma cell lines to indirect EZH2 inhibitor - 3-deazaneplanocin A (DZNep); the apoptosis was not *EZH2* mutation-dependent at all. Moreover, *MYC, BCL2*, and *BCL6* gene status did not influence the efficacy of DZNep in lymphoma cell line selective apoptosis (51). Recently, Huang *et al*. showed that in solid tumors, EZH2 intervention could influence numerous epigenetic histone modifications. In preclinical models, the role of oncogenic transcriptional reprogramming mediated by MLL1 interaction with the p300/CBP complex has been described; it is responsible for H3K27me loss and H3K27ac gain, which restricts the response for EZH2 inhibitors (52). Moreover, blockade of both H3K27me and H3K27ac is related to MAPK pathway repression. The authors presented a model based on EZH2 overexpression stratified into three treatment groups: EZH2-monotherapy or with BRD4 inhibitors/p300 inhibitors (double-combo) or a triple combination plus MAPK pathway inhibitors (triple-combo). The combination of agents targeting the epigenome seems to be more efficient. Initial results on DLBCL cell lines with BCL2 and EZH2 inhibition with venetoclax and tazemetostat showed a synergistic antitumor effect as well (53). In the future, personalized anti-EZH2

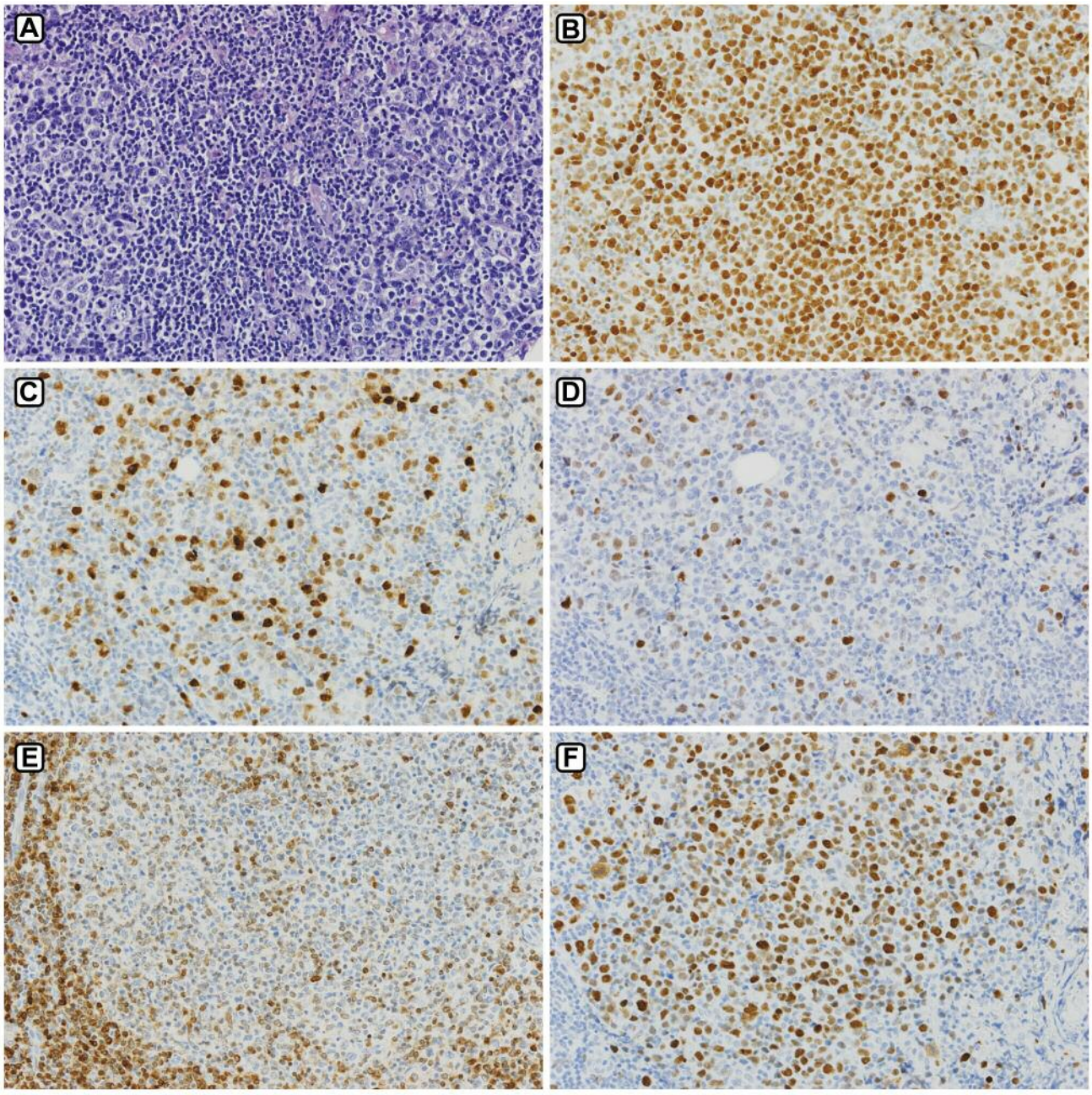

Figure 4. *Immunohistochemical profile of follicular lymphoma (FL): (A) Hematoxylin & eosin staining of FL, follicular type, high grade, grade 3a (200×); (B) EZH2 high expression profile (200×); (C) MUM1 high expression profile (200×); (D) MYC higher expression (200×); (E) BCL2 low expression (compare with a strong expression on reactive T-cells in the left bottom corner) (200×); (F) p53 high expression (compare control tissue – negative reactive T-cells in the left bottom corner) (200×).*

therapy in relapsed/refractory FL will probably mostly emphasize incorporating different agents to increase response duration.

The biology of EZH2 in the non-hematological and lymphoid malignancies is not fully understood. In solid tumors. *i.e.*, urothelial, prostate, breast, ovarian, endometrial colorectal cancers and melanoma, the aberrant EZH2 protein overexpression assessed immunohistochemically was associated with aggressive clinical behavior and inferior follow-up including shorter time to recurrence or death as

Table I. *The clinical and histopathological characteristics of the stratified EZH2 expression.*

	EZH2 High	EZH2 Low	*p*-Value
N	25	50	
Age (years) [median (IQR)]	60.50 [48.75, 68.25]	52.00 [43.00, 59.00]	**0.028**
Gender (%)			
0.066			
Female	21 (84.0)	30 (60.0)	
Male	4 (16.0)	20 (40.0)	
LDH [median (IQR)]	176.50 [148.50, 198.75]	177.00 [153.50, 200.50]	0.989
Hemoglobin (g/dl) [median (IQR)]	13.55 [12.75, 13.97]	13.80 [12.35, 14.80]	0.413
Ann Arbor stage (%)			0.183
I	3 (12.0)	2 (4.0)	
II	3 (12.0)	9 (18.0)	
III	7 (28.0)	9 (18.0)	
IV	6 (24.0)	23 (46.0)	
Missing	6 (24.0)	7 (14.0)	
FLIPI1 score (%)			0.395
0-1	4 (16.0)	16 (32.0)	
2	11 (44.0)	18 (36.0)	
3-5	3 (12.0)	8 (16.0)	
Missing	7 (28.0)	8 (16.0)	
FLIPI2 score (%)			0.347
0	6 (24.0)	7 (14.0)	
1-2	8 (32.0)	21 (42.0)	
3-5	1 (4.0)	5 (10.0)	
Missing	10 (40.0)	17 (34.0)	
Median on treatment PFS, (years)	7.26	5.46	0.79
Median OS (years)	13.6	Not reached	0.06
Treatment			0.159
W&W	7 (28.0)	24 (48.0)	
Immunochemotherapy	18 (72.0)	26 (52.0)	
Grade (%)			0.249
High	7 (28.0)	7 (14.0)	
Low	18 (72.0)	43 (86.0)	
Grade FL (%)			0.206
1	7 (28.0)	23 (46.0)	
2	11 (44.0)	20 (40.0)	
3a	7 (28.0)	7 (14.0)	
Pattern (%)			0.513
Follicular	13 (52.0)	28 (56.0)	
Follicular/diffuse	6 (24.0)	16 (32.0)	
Diffuse	6 (24.0)	6 (12.0)	
BCL2 (%)			**0.042**
Negative	12 (48.0)	11 (22.0)	
Positive	13 (52.0)	39 (78.0)	
CD10 (%)			0.195
Negative	8 (32.0)	8 (16.0)	
Positive	17 (68.0)	42 (84.0)	
BCL6 (%)			0.922
Negative	5 (20.0)	12 (24.0)	
Positive	20 (80.0)	38 (76.0)	
MUM1 (%)			**0.039**
High expression	5 (20.0)	2 (4.0)	
Low expression%	6 (24.0)	8 (16.0)	
Negative	14 (56.0)	40 (80.0)	
MYC (%)			**<0.001**
High expression	2 (8.0)	3 (6.0)	
Low expression	17 (68.0)	1 (2.0)	
Negative	6 (24.0)	46 (92.0)	
p53 (%)			**<0.001**
High expression	2 (8.0)	0 (0.0)	
Low expression	12 (48.0)	3 (6.0)	
Negative	11 (44.0)	46 (92.0)	
Missing	0 (0.0)	1 (2.0)	

N: Number of cases; LDH: lactate dehydrate; FLIPI: Follicular International Prognostic Index; PFS: progression-free survival; OS: overall survival; W&W: watch and wait. Bold values indicate statistical significance.

endpoints (15, 22, 23, 25, 27, 30, 54-58). Interestingly, EZH2 showed polarization of expression in some tumors, *i.e.*, colorectal cancer tumor invasion front showed EZH2 loss and correlated with poor clinical outcome (55). The latest promising results in myelodysplastic syndromes displayed that both *EZH2* mutation and protein loss was related to poor survival, independently of the Revised International Prognostic Scoring System. The authors indicated that EZH2 protein expression demonstrated even more statistical significance with survival than mutational status (59).

In FL, the EZH2 complex regulates normal hematopoietic stem-cell and B-cell renewal and differentiation. There are only a few publications in which EZH2 expression profile has been presented (29, 60). Some studies are convincing that high EZH2 protein levels, *i.e.*, in Burkitt lymphoma, DLBCL, high-grade FL, correlate with increased proliferation, aggressiveness, and poor prognosis (29, 39, 41, 43). Those observations are practically ignoring the role of EZH2 in germinal center B cell development. During lymphopoiesis, EZH2 protein is strongly expressed in proliferating germinal center B cells; it can be observed on control tissues like reactive germinal centers in lymph nodes or tonsils (61). The FL is GC-derived lymphoma, and restoration of EZH2 on protein level is a reminiscence of the cell of origin. There is strong evidence that EZH2 cooperates with BCL2 in the generation of GC-derived lymphomas (62). The loss of EZH2 might signal the more in-depth biological remodeling of the FL; the down-regulation during B-cell differentiation and maturation might be based on changing the mechanism which controls the pro-B to pre-B cell transition (33). Still, the role

Table II. *The characteristics of the stratified POD24 cohorts.*

	POD24 No	POD24 Yes	*p*-Value
N	38	16	
Age (years)	53.00	52.00	0.429
[median (IQR)]	[44.50, 64.00]	[51.50, 59.50]	
Gender (%)			0.482
Female	29 (76.3)	10 (62.5)	
Male	9 (23.7)	6 (37.5)	
LDH	175.50	178.00	0.445
[median (IQR)]	[143.50, 189.00]	[153.00, 235.00]	
Hemoglobin (g/dl)	13.45	13.50	0.745
[median (IQR)]	[12.57, 14.40]	[12.20, 13.90]	
Ann Arbor stage (%)			0.455
I	0 (0.0)	0 (0.0)	
II	3 (7.9)	3 (18.8)	
III	9 (23.7)	3 (18.8)	
IV	18 (47.4)	6 (37.5)	
Missing	8 (21.1)	4 (25.0)	
FLIPI1 score (%)			0.864
0-1	5 (13.2)	3 (18.8)	
2	16 (42.1)	6 (37.5)	
3-5	7 (18.4)	3 (18.8)	
Missing	10 (26.3)	4 (25.0)	
FLIPI2 score (%)			0.256
0	6 (15.8)	0 (0.0)	
1-2	13 (34.2)	5 (31.2)	
3-5	3 (7.9)	2 (12.5)	
Missing	16 (42.1)	9 (56.2)	
Grade (%)			0.251
High	7 (18.4)	6 (37.5)	
Low	31 (81.6)	10 (62.5)	
Grade FL (%)			0.298
1	18 (47.4)	5 (31.2)	
2	13 (34.2)	5 (31.2)	
3a	7 (18.4)	6 (37.5)	
Pattern (%)			0.356
Follicular	24 (63.2)	7 (43.8)	
Follicular/diffuse	9 (23.7)	5 (31.2)	
Diffuse	5 (13.2)	4 (25.0)	
EZH2 (%)			0.865
≥70%	14 (36.8)	7 (43.8)	
<70%	24 (63.2)	9 (56.2)	
BCL2 (%)			0.971
Negative	10 (26.3)	5 (31.2)	
Positive	28 (73.7)	11 (68.8)	
CD10 (%)			0.651
Negative	8 (21.1)	5 (31.2)	
Positive	30 (78.9)	11 (68.8)	
BCL6 (%)			1.000
Negative	8 (21.1)	4 (25.0)	
Positive	30 (78.9)	12 (75.0)	
MUM1 (%)			0.991
High expression	5 (13.2)	2 (12.5)	
Low expression	10 (26.3)	4 (25.0)	
Negative	23 (60.5)	10 (62.5)	
MYC (%)			0.851
High expression	4 (10.5)	1 (6.2)	
Low expression	10 (26.3)	5 (31.2)	
Negative	24 (63.2)	10 (62.5)	
p53 (%)			0.056
High expression	0 (0.0)	2 (12.5)	
Low expression	11 (28.9)	4 (25.0)	
Negative	27 (71.1)	9 (56.2)	
Missing	0 (0.0)	1 (6.2)	

N: Number of cases; LDH: lactate dehydrate; FLIPI: Follicular International Prognostic Index.

of EZH2 gain or loss function mutations are observed, but that is only one mechanism of redirecting its tumor-suppressive role in malignancy development. Recent studies show EZH2 in light of post-transcriptional, post-translational, and immunomodulating levels (63).

We showed that EZH2 protein loss *in vivo* is independent of histopathological features. EZH2 expression seems to be decreased in follicular and diffuse FL patterns as well as low and high-grade subtypes. We showed that EZH2 restoration is more visible on cells with centroblasts morphology. B cells transiting the GC reaction manifest phenotypic features that mimic many of the canonical biological hallmarks of lymphoma. Van Galen at al. showed in the hyperplastic, non-malignant tonsils that the EZH2 was expressed in the dividing follicular cells with the staining profile of centroblasts (64). The EZH2-mediating epigenetic silencing promotes GC B cell proliferation and prevents differentiation, which are two essential features of the so-called "dark zone" program. The "dark zone" is a histologically and functionally compartment of the GC in which B cells proliferate extensively and undergo immunoglobulin somatic hypermutation (65, 66). The EZH2 protein profile is not fully characterized among patients with FL. On the one hand, immunohistochemically, it is supposed to be overexpressed according to the well-known EZH2 role in GC B cell lymphoma formation; on the contrary, it is "reserved" for more aggressive B-cell lymphomas; however, in low-grade FL the centroblasts are highly EZH2 positive cells (29).

We observed the differences in FL cell immunohistochemical characteristics, including BCL2, MUM1, MYC, and p53. The FL with high EZH2 expression showed "loss" of BCL2 and "gain" of MUM1, MYC, and p53. The mechanisms of EZH2 expression models *in vitro* studies and mice showed close cooperation with BCL2 and BCL6 (31, 67). The earliest known oncogenic event is the t(14;18)(q32;q21), which covers the

BCL2 gene and results in BCL2 protein expression. FL cases with aberrant loss of *BCL2* translocation and protein expression are presenting as activated B cell-like (ABC) lymphoma with NFκB activation (68). The absence of BCL2 at the time of diagnosis is associated with transformation to ABC-like large B cell lymphoma, which occurs in a minority of FL progressing cases (69). In FL, MUM1 positivity could be a hallmark of early transformation to DLBCL. Moreover, MUM1 higher expression was an independent predictive factor for progression (69) and was associated with poor OS and PFS (70). In DLBCL groups, EZH2 positivity was related to MUM1 expression, which favored a non-germinal center-like phenotype (41). MYC is responsible for EZH2 up-regulation through EZH2-targeting miRNAs, *i.e*., miR-26a, miR-26b, or miR-101 (71, 72). On the contrary, EZH2 induces MYC expression by miR-494 and serine biosynthesis pathway (42); those interactions seem to be fundamental in the maintenance of metabolic and epigenetic reprogramming of lymphoma cells. Additionally, the cell-cycle progression of GC B cells is down-regulated by tumor suppressors such as *TP53*. Lately, Kridel *et al*. described that FL patients harboring relatively uncommon gene mutations associated with early progression, including *TP53* (69). Previously, the presence of *TP53* mutation at diagnosis of FL was identified as a high-risk group of patients with shortened time to disease progression and more reduced OS. However, the *TP53* was described only in approximately 6% of all cases (73, 74). Zhao *et al*. has classified the EZH2 as a p53 mRNA-binding protein (75). *In vitro* studies showed that EZH2 could "boost" p53 gain-of-function mutant-mediated cancer growth and metastasis (75, 76). In cultured human cancer cells, mouse tumor xenografts, and cancer patient specimens (brain tumor, colorectal and pancreatic cancer, sarcoma), EZH2 increased p53 protein levels, enhancing mRNA stability and protein translation (75, 77).

Conclusion

We present, for the first time, the EZH2 expression profile in FL according to POD24 and TTFT24 grouping. We did not observe any statistically significant influence of EZH2 on clinical outcomes. None of the clinical or histopathological features had a high significance onto POD24 and TTFT24. However, POD24 stratification of patients allowed us to confirm its crucial clinical impact in the retrieval of patients who should stay under intensive observation. EZH2 protein is mainly restored within centroblasts-like cells, but EZH2 loss was seen independently of histopathological grade and pattern. We found that EZH2 high levels are more frequently identified in cases with lower BCL2 and higher MUM1, MYC, p53 protein expression. We are pointing out that not only genetic and epigenetic abnormalities of EZH2 should be evaluated since recent studies have shown that targeted therapy seems to remain beneficial regardless of *EZH2* status. The role of EZH2 in lymphoid oncogenesis, mediation, and tumor transformation is still one of the fundamental fields for further investigation.

Conflicts of Interest

The Authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Authors' Contributions

Study concepts and study design, data analysis and interpretation, quality control of data and algorithms: A.S.-C., E.P.-K., J.P; data acquisition: A.S.-C., J.P., E.P.-K., G.R., K.S., M.Ko., D.O., M.Ka., B.P.; investigation: A.S.-C., J.P., E.P.-K., K.S., D.O., M.Ka., B.P., M.P.-S.; statistical analysis, visualization of the results: A.S.-C., J.P; manuscript preparation: A.S.-C., manuscript editing and review: A.S.-C., J.P., E.P.-K., G.R., K.S., M.Ko., D.O., M.Ka. B.P., J.W., M.P.-S. All Authors approved the manuscript.

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