# Application of Circulating Tumor DNA Analysis for the Detection of *EZH2* Mutations in Follicular Lymphoma

**PhD thesis**

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## 1. Introduction

Follicular lymphoma (FL) is the most common indolent non-Hodgkin lymphoma, with an overall survival of 15-20 years. Despite the modern immunochemotherapeutic approaches the disease is still incurable.

Recent genomic studies conducted in the last decade elucidated the genetic background of the disease. Noteworthy, almost all FL cases carry at least one alteration in the epigenetic regulatory machinery, among these the third most common mutations are the gain of function mutations of the *EZH2* gene, which are present in around 25% of the cases. The gain of function mutations of the gene results in an aberrant trimethylation of the histone 3, leading to an aberrant silencing of the target genes. There are seven different types of *EZH2* mutations in this disease affecting the catalytic set domain of the protein, locating at exon16 (p.Y646N/F/C/H/S) and exon18 (p.A682G and p.A692V). These

alterations are considered early clonal events, however cases with *EZH2* mutation restricted to disease progression or relapse were also described. The gain of function nature of these alterations proved to be an attractive pharmaceutical target, consequently tazemetostat, a small molecular inhibitor of EZH2 has recently been approved in the US for the treatment of relapsed/refractory FL. The approval was based on a phase II trial, where patients with an *EZH2* mutation had an objective response rate (ORR) of 69%, meanwhile patients with wild-type *EZH2* had an ORR of only 35%.

FL is also characterized by a profound spatial heterogeneity, which means that the phenotypic and molecular characteristic of the disease may be different in distinct tumor sites. Moreover, the clonal architecture of the disease can substantially change throughout the disease course, which phenomenon can be referred to as temporal heterogeneity.

Liquid biopsy (LB) refers to the molecular analysis of cell-free DNA (cfDNA) biomolecules from the blood plasma. Healthy and tumor cells can either be the source of cfDNA. The tumor-derived fraction of cfDNA in patients suffering from malignancies is termed circulating tumor DNA (ctDNA). The molecular genetic alterations of the tumor can non-invasively be investigated in the ctDNA compartment of the blood plasma as well. ctDNA specimens also represent the systematic disease, hence DNA fragments are shed to the bloodstream from all affected disease sites, therefore tumors with a considerable spatial heterogeneity, like FL, can be investigated with this method. However, the amount of ctDNA molecules can be very low in a considerable portion of patients with malignancies, therefore sensitive molecular biology techniques are needed for LB-based molecular analysis, like droplet digital PCR (ddPCR) and new generation sequencing.

## 2. Objectives

In this work we aimed to:

1) Develop a cost-effective molecular biology test for the reliable and sensitive detection of *EZH2* mutations in FL.
2) Determine the *EZH2* mutation frequency in a Hungarian FL patient cohort.
3) Test *EZH2* mutations from LB samples.
4) Compare LB- and TB-biopsy based *EZH2* mutation analysis.
5) Investigate the temporal dynamics of *EZH2* mutations.
6) Test the extent spatial heterogeneity.
7) Correlate the result of *EZH2* mutation analysis with clinical parameters.
8) Monitor *EZH2* mutations.
9) Compare the cellular and acellular (cfDNA) component of the blood in terms of *EZH2* mutation analysis.

## 3. Methods

We collected paired LB and tumor biopsy (TB) samples from 117 FL patients. LB samples from 9 progressing/relapsed patients who lacked paired relapse TB samples were also included.

After DNA isolation from LB, TB and peripheral blood mononuclear cells (PBMNC), we determined the *EZH2* mutation status of the samples with an in-house designed, multiplex ddPCR assay.

## 4. Results

First, we designed a novel multiplex ddPCR approach for the simultaneous detection of distinct *EZH2* mutations. We compared the sensitivity of classical single vs multiplex ddPCR amplification for all *EZH2* alterations, and we found no statistical difference between the two approaches, proving that our novel multiplex assay maintains the sensitivity of the ddPCR, required for LB-based analyzes. Upon comparing LB-based to TB-based *EZH2*

analysis, we found that the LB-based approach slightly outperforms the TB-based one, in terms of sensitivity and negative predictive value (NPV).

We detected *EZH2* mutations in 42.1% (53/126) of the patients when considering all available sample types. Analyzing the paired TB-LB samples (n=117), the *EZH2* mutation frequency was 38.4% (45/117), importantly, in six patients (5.1%) the *EZH2* mutation was detected exclusively in the TB samples, while in nine (7.7%) patients, the *EZH2* mutation was only seen in the corresponding LB sample (Figure 1).

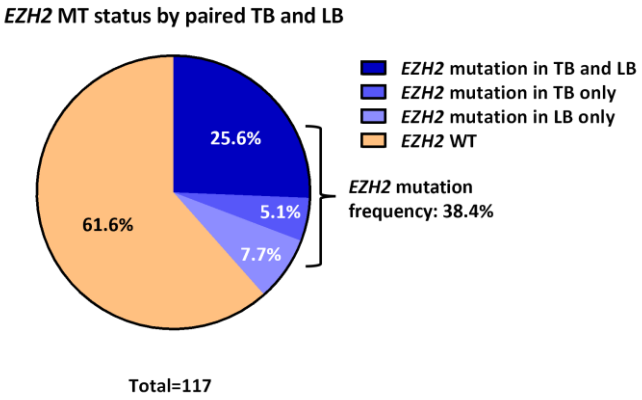

***Figure 1.*** *EZH2 MT status in patients with paired tissue (TB) and pre-treatment liquid biopsy (LB) samples.*

Altogether, 39 patients had an *EZH2* mutation analysis both at the time of diagnosis and relapse/progression from at least one sample. Here, we observed a switch in the *EZH2* mutation status during the disease course in 14 patients (35.9%) (Figure 2).

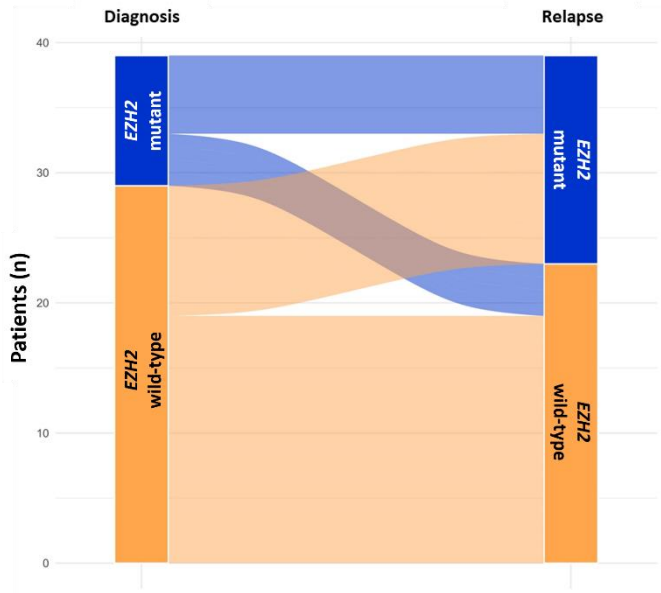

***Figure 2.*** *EZH2 mutation status switch phenomenon in patients with paired diagnostic and relapse sample.*

Multiple TB samples collected at the same time from distinct sites were available from 54 patients. Spatial heterogeneity was documented in 8 cases (15%), where different *EZH2* mutation types were detected in distinct sites, or in patients with at least one mutant and one wild-type tumor site. In Pt-7, two distinct *EZH2* mutations (p.Y646F and p.Y646N) were detected in the peripheral blood. The analysis of the TBs proved that the p.Y646N mutation originated from a low-grade FL in the inguinal lymph nodes, meanwhile the p.Y646F mutation was found in an extranodal transformed FL from the duodenum wall (Figure 3).

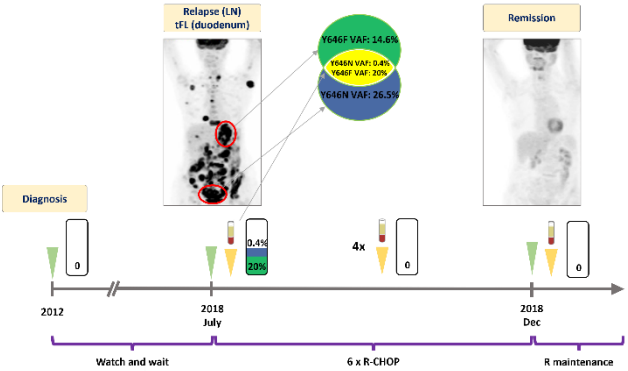


***Figure 3.*** *Illustration of spatial heterogeneity and longitudinal treatment monitoring with liquid biopsy in the case of Pt-7.*

Upon comparing the result of the *EZH2* mutation analysis and clinical parameters, we found that higher histological grade is significantly associated with mutant TB *EZH2* status. Importantly, we found a trend between LB *EZH2* VAF levels and histological grade, clinical stage, FLIPI (FL international prognostic index) score, LDH (lactate dehydrogenase) levels, presence of extranodal involvement and PET/CT SUV max (maximum 18-fluorodeoxy glucose standardized

uptake value) value, where the group(s) with higher mean VAFs was always associated with more aggressive clinical factors. The presence of B symptoms was also significantly (Chi-square test, p=0.03) associated with mutant *EZH2* status.

Interestingly, we found that wild-type *EZH2* status may be associated with increased risk of BM infiltration. Histologically confirmed bone marrow (BM) infiltrates were more frequently *EZH2* wild-type, regardless of the mutation status of the paired TB. Altogether, 16% (7/44) of infiltrated BM samples harbored the mutation, meanwhile 84% (37/44) proved to be *EZH2* wild-type.

Follow-up LB samples collected on the first day of each treatment cycle were available from 24 patients. Median *EZH2* VAF was 3.8% and 0.12% before treatment and at the time of C2D1 (cycle 2 day 1), respectively. Patients responding to therapy were characterized by a prompt decrease in the *EZH2* VAF level after the initiation of treatment.

This was registered in Pt-7, where both *EZH2* mutations quickly eliminated after only one cycle of ICT treatment, five months prior to the PET/CT examination, which eventually confirmed the complete metabolic response (Figure 3).

We tested the *EZH2* mutation status of PBMNC samples of patients whose paired LB sample carried an *EZH2* mutation. Fifty-five paired PBMNC and LB samples were available for analysis with only 17/55 (31%) PBMNC samples carrying the *EZH2* mutation detected in the corresponding LB sample.

## 5. Conclusions

1) We developed a unique multiplex ddPCR assay, which can detect all the seven *EZH2* hotspot mutations in FL.

2) In an unselected cohort of Hungarian FL patients, the *EZH2* mutation frequency proved to be 42.1%,

higher than the previously published literature data (25%).

3) The higher mutation frequency in FL may further expand the subset of patients who would most likely benefit from the recently approved targeted therapy.

4) With our multiplex ddPCR assay, *EZH2* mutations are directly detectable from LB samples without any prior knowledge of the TB mutation status.

5) The sensitivity and NPV of the LB-based *EZH2* mutation detection analysis slightly outperforms the TB-based one (87%, 92% vs. 80%, 89%).

6) Spatial heterogeneity affects at least 25% of the patients.

7) Spatial heterogeneity contributes to the higher *EZH2* mutation frequency found in our cohort.

8) *EZH2* mutations are an unstable biomarker, and the mutation status can change throughout the disease course.

9) Mutant *EZH2* status is associated with more aggressive histology, presence of B-symptoms and lack of BM infiltration.

10) ctDNA kinetics indicate treatment responses in FL.

11) The acellular component of the blood (ctDNA) is more reliable sample source for DNA-based biomarker analysis in FL than the cellular part (PBMNC).

## 6. Bibliography

Publications in connection with the thesis

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