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Research Article

A Pyrosequencing Assay for the Detection of *EZH2*Y641 Mutations in Diffuse Large B cell Lymphoma and Follicular Lymphoma

Timothy Taxter[1], Zheng Zhou[2], David Dittmann[3], Jonathan Licht[2], Yi-Hua Chen[1] and Juehua Gao[1]*

[1]*Department of Pathology, Northwestern University Feinberg School of Medicine, USA*
[2]*Department of Medicine, Northwestern University Feinberg School of Medicine, USA*
[3]*Molecular Biology Laboratories, Northwestern Memorial Hospital, Chicago, USA*

***Corresponding author**
Juehua Gao, Department of Pathology, Northwestern University Feinberg School of Medicine, Feinberg 7-209A, 251 E. Huron Street, Chicago, IL,USA 60611; Tel: 312-926-9319; Fax: 312-926-0560; Email: j-gao@northwestern.edu

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Abstract

Aims: Enhancer of Zeste Homolog 2 (EZH2) is a critical enzymatic subunit of Polycomb Repressive Complex 2 (PRC2), which provides inhibitory regulation of gene transcription through trimethylation of histone H3 on lysine 27 (H3K27me3). The somatic mutation *EZH2* Y641 in its highly conserved catalytic SET domain has been reported in diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL) by methods of Sanger sequencing and whole exome sequencing. To develop a clinically applicable assay uses pyrosequencing method for accurate detection of *EZH2*Y641 mutations in formalin- fixed-paraffin-embedded (FFPE) tissue.

Methods: We validated the pyrosequencing method for the detection of *EZH2* Y641 mutation in DLBCL cell lines. We also screened a cohort of 43 DLBCL and 12 FL cases by pyrosequencing, and correlated with the results of Sanger sequencing of EZH2 exon 15.

Results: We detected *EZH2* Y641 mutations in 3/22 (13.6%) germinal center B cell-like DLBCL, 2/12 (16.7%) FL and none of the 21 activated B cell-like DLBCL. The mutations detected by pyrosequencing were in complete agreement with Sanger sequencing. We have identified the most common 4 types of mutations of *EZH2* Y641.

Conclusion: The rapid and accurate detection of the *EZH2* mutation fromFFPE tissue sections in the clinical laboratory will facilitate screening patients with DLBCL and FL for enrollment of clinical trials using EZH2 inhibitors.

ABBREVIATIONS

EZH2: Enhancer of Zeste Homolog 2; **DLBCL:** Diffuse large B cell lymphoma; **FL:** Follicular lymphoma

INTRODUCTION

Enhancer of Zeste homolog 2 (EZH2) is a member of thepolycomb group proteins (PcG) that regulate key developmental genes [1]. EZH2 is the critical catalytic component of the polycomb repressive complex 2 (PRC2) of the PcG, and functions as a histone methyltransferase responsible for the sequential addition of methyl groups to Lysine 27 of histone H3 (H3K27me3) [2,3]. Accumulation of trimethylated H3K27 (H3K27me3) has been implicated to suppress downstream transcription [4]. The catalytic activity of EZH2 relies on the conserved SET domain of EZH2, which along with other members of the PRC2 complex confers its histone methyltransferase activity [5].

In normal hematopoietic tissue, EZH2 is expressed at high levels in the centroblasts of normal germinal centerof lymph nodes and plays an important role in the formation of lymphoid follicles [6]. Although the exact mechanism of EZH2 in this process is not entirely clear, animal models suggest that EZH2 methyltransferase activity is required for normal germinal center B cells to proliferate, undergo immunoglobulin heavy

chain (IgH) rearrangement and eventually exit GC [6-9]. EZH2 is also involved in the development and maintenance of stem cells since many genes involved in this process are targets of H3K27methylation [10-11]. In malignant processes,EZH2 appears capable of functioning as both an oncogene and tumor-suppressor depending on factors such as gene dosage and cellular context. Solid tumors have shown increased levels of EZH2 mRNA correlating with cancer progression in tissues which normally express low or undetectable levels of EZH2 such as breast and prostate [12,13], while in hematologic disease missense mutated forms of EZH2 have been associated with a myriad of processes from myelodysplasitic syndrome to T and B cell lymphomas [14-19]. This complex phenotype indicates EZH2 is associated with a broad range of target genes resulting in potentially opposing downstream effects [20].

Somatic mutations in the catalytic SET domain of *EZH2* occur in diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL), and the Tyrosine 641 (Y641) residue is the most frequently mutated residue [17,21]. The Y641 *EZH2* mutation appears to confer an enzymatic gain of function that alters substrate preference resulting in an overall increase in trimethylase activity of EZH2 leading to elevated global levels of the H3K27 tri-methylation (H3K27me3) [21]. Interestingly, the converse has been shown in myeloid neoplasms where *EZH2* mutations confer a loss of function from premature chain termination or direct abrogation of histone methyltransferase activity [14].

Recently highly selective small molecule inhibitors of EZH2methyltransferase activity have been developed and shown to effectively suppress global H3K27me3 levels resulting in restoration of downstream target gene expression and proliferation arrest in *EZH2* mutant cell lines [22]. Animal models with xenograftedKARPAS-422 cells (human follicular lymphoma cellwith *EZH2* mutation) showed significant reduction in tumor size in response to inhibitor treatment [22,23]. Interestingly in vitro models have shown significant differences in efficacy within mutational subtypes of Y641 which will require further investigation to reveal if this is of clinical relevance [24]. Clinical trials with EZH2 inhibitors are currently underway thus necessitating the clinical testing of *EZH2*Y641 mutation during initial work up of lymphoma patients.

The current methods for detection of Y641 vary the molecular testing spectrum depending on application. Recent studies evaluating for Y641 in patient derived samples have primarily utilized Sanger and next generation sequencing platformsin the research setting, and may not be applicable to many of the clinical laboratories. In this paper, we describe a clinical laboratory based assay using pyrosequencing followed by Sanger sequencing validation to accurately identify *EZH2* Y641 mutations in formalin-fixed-paraffin-embedded (FFPE) tissue sections.

MATERIALS AND METHODS

Case selection

Cases with a diagnosis of DLBCL and FL were randomly selected from Department of Pathology archives. Formalin fixed paraffin embedded (FFPE) tissue blocks with adequate materials were selected. The H&E- and the available immunohistochemical-stained slides as well as the results of ancillary studies were re-reviewed to confirm the diagnosis based on the current WHO classification. The subclassification of DLBCL into germinal center B cell and activated B cell types was routinely performed and interpreted based on immunohistochemistry algorithm previously described [25]. Immunohistochemical stains were performed onparaffin-embedded tissue sectionsusing Ventana Benchmark XT (Tucson, AZ, USA). The primary antibodiesused were as follows: Immunohistochemical stains of CD10 monoclonal antibody at a diluation of 1:10 (Novocastra, Leica Biosystems, Buffalo Grove, IL), predilutedBCL6 monoclonal antibody (Dako, Carpinteria, CA), MUM1 monoclonal antibody at a dilutionof 1:10 (Dako, Carpinteria, CA).This study is approved by Northwestern University Institutional Review Board.

DNA extraction

DNA extraction and purification was performed on automated nucleic extraction instrument QIAsymphony SP using QIAsymphony DNA Mini Kit (Qiagen, Valencia, CA). Two freshly cut sections of FFPE tissue, each with a thickness of 5 µm, were processed in one preparation following manufacturer's protocol for deparaffinization and then loaded on QIAsymphony SP for automatic DNA extraction. The final elution volume was 60uL.

Pyrosequencing

PCR amplification primers (Sigma) for Pyrosequencing were as follows: forward, 5′- TTTTTGATGATGTGATTGTGTTTT -3′; and reverse biotinylated primer, 5′- TGGCAATTCATTTCCAATCA -3′. Each reaction contained 2× PCR master mixincludingHotStarTaq DNA Polymerase, MgCl2 and dNTPs (Qiagen, Valencia, CA), 1µl of 10µM forward and reverse primers, 10 µl of template DNA, and dH_2O to 20µl final volume.PCR conditions consisted of initial denaturing at 95°C for 15 minute; 42 cycles of 95°C for 20 seconds, 56°C for 30 seconds, and 72°C for 20 seconds; and final extension at 72°C for 5 minute. The PCR products were electrophoresed in an agarose gel to confirm successful amplification of the 239-bp PCR product. The PCR products (each 10 µl) were sequenced by Pyrosequencing pyromark Q24 System (QIAGEN, Valencia, CA) following the manufacturer's instructions, using Pyrosequencing primers 5'-ATGAATTCATCTCAGA-3'. Nucleotide dispensation order was cyclic (ACTG from 5′ to 3′).In rare cases when mutation occurs causing an out of synchronized read, a second dispensation order GCTA order was also used for interpretation. Data analysis was performed using PyroMark Software. A minimum signal of 20 units for the single peak level of Pyrogram is required. The percentages of mutant alleles were calculated based on the height ratios between the mutant and normal peaks determined by PyroMark software. Negative dispensation signal strength limits were determined based on polyclonal control DNA (200 ug/ml) (Invivoscribe, San Diego, CA) control samples without EZH2 mutations. A minimum of 10% is considered for assessment for the variable positions.

Sanger sequencing

PCR amplification primers (Sigma,St. Louis, MO) for Sanger sequencingwereasfollows:forward,5'-ATGAATTCATCTCAGA-3'; and reverse 5'-TGGCAATTCATTTCCAATCA. PCR products were purified using QiaQuick reagents (Qiagen, Valencia, CA) and

were cycle sequenced using Big Dye v3.1 reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Automated sequencing was performed by capillary electrophoresis on an ABI3700 (Applied Biosystems,Foster City, CA). Sanger sequencing was performed at the Northwestern University Genomic Core facility. Sequences were aligned and examined by visual inspection of the electropherogram, using FinchTV software (Geospiza, Seattle, WA).

RESULTS AND DISCUSSION

Our study included 43 cases of DLBCL, among which 40 were de novo DLBCL. Three cases were those transformed from low grade FL, small lymphocytic lymphoma (SLL) or a low grade B cell lymphoma. We also included 12 cases of FL, including 9 cases of grade 1-2, and 3 cases of grade 3 with a grade 1-2 component. The H&E slides as well as the immunohistochemical stains were reviewed to confirm the diagnosis, classification and histologic grading. Based on Hans's classification, the 40 cases of de novo DLBCL were classified as 21 germinal centers B cell type and 19 activated B cell type. The 3 transformed DLBCL include 1 germinal center B cell type and 2 acticated B cell type. In addition, we include two DLBCL cell lines with known *EZH2* mutation (WSU-DLCL2 and OCI-LY1) and one DLBCL cell line of activated B lymphocytes phenotype without *EZH2* mutation (U2932).

For the initial test validation, pyrosequencing was performed on DNA extracted from the DLBCL cell lines with or without the *EZH2*Y641 mutation. We confirmed that the presence of A to T mutation resulted in the replacement of tyrosine with phenylalanine in WSU-DLCL2, and a T to A mutation resulted in tyrosine to asparagine substitution in OCI-LY1. Mutations in these cell lines were well studied and reported by other sequencing methods [17]. The wild-type cell line (U2932) had no Y641 mutation identified.

Table 1: EZH2 Tyr641 mutations detected by pyrosequencing.

Histologic Diagnosis	Classification or Grade	Mutation	Effect
De novo DLBCL	Germinal center B cell type	A>C	Y641S
De novo DLBCL	Germinal center B cell type	A>T	Y641F
De novo DLBCL	Germinal center B cell type	T>A	Y641N
FL	Grade 1-2	A>C	Y641S
FL	Grade 1-2 (20%), Grade 3 (80%)	T>C	Y641H

Abbreviations: DLBCL: diffuse large B cell lymphoma; FL: follicular lymphoma

We then completed pyrosequencing of Y641 on DNA extracted from FFPE tissue sections. We identified mutations resulting in Y641 substitutions in 3 of 22germinal center B type DLBCL samples (13.6%) and in 2 of the 12 follicular lymphoma samples (16.7%), and none of the 21 cases of activated B cell type DLBCL. The mutations in DLBCL included 1 case of Y641S, 1 case of Y641N and 1case of Y641F. Mutations in follicular lymphoma casesincluded 1 case of Y641S and 1 case of Y641H (Table 1). All mutations were interpreted as heterozygous based on the peaks in the pyrograms. All the 55 cases of DLBCL and FL were subjected to Sanger sequencing of the exon15 containing codon 641. Thepresence of Y641-altering mutations in all 3 DLBCL and 2 follicular lymphoma were confirmed, and were the sole mutation in exon 15. Sanger sequencing confirmed the negative

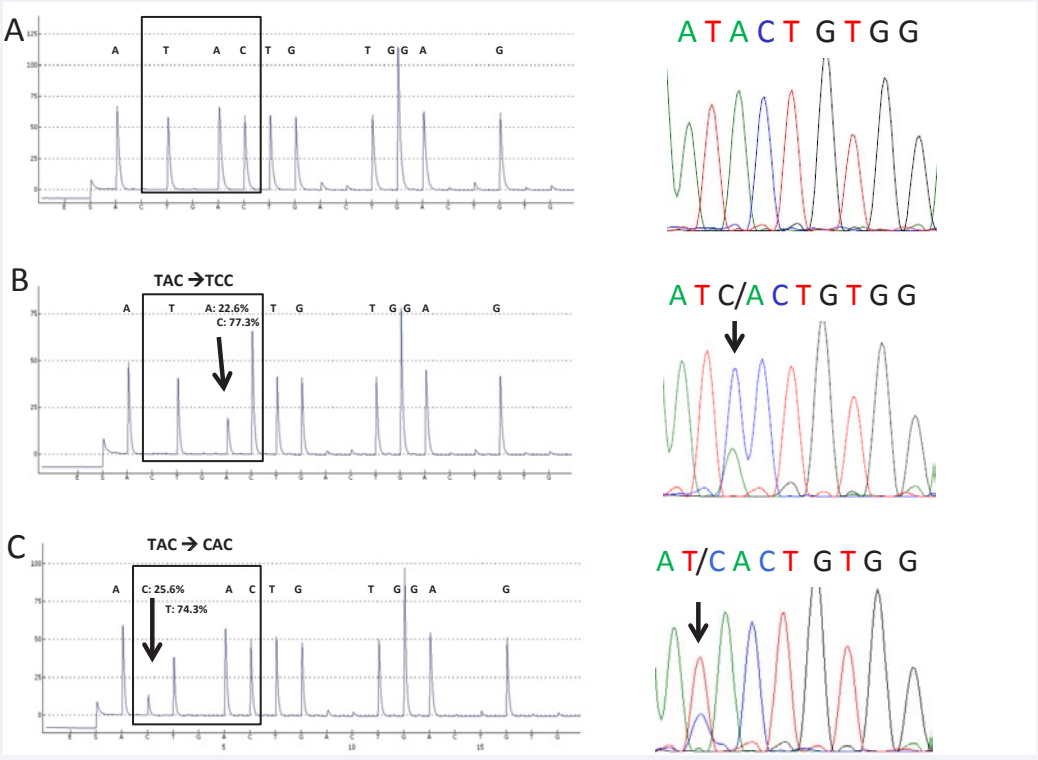

Figure 1 Pyrogram and Sanger sequencing electropherogram ofEZH2 Tyr641 mutation in lymphoma. (A). DLBCL without mutation (B).DLBCL with TAC-->TCC, p.Tyr641Ser (C).FL with TAC-->CAC,p.Tyr641His

cases determined by pyrosequencing. All theY641 mutations detected by Sanger sequencing showed clear evidence for the presence of both alleles, suggesting these samples containing heterozygous mutations. All of the DLBCL samples positive for *EZH2* mutations were of the germinal center B cell subtype (none in activated B cell subtype). This is consistent with previous studiesdemonstrating significant enrichment of Y641-altering mutations among the germinal center B cell subtype of DLBCLs.

CONCLUSION

The role of mutations involving epigenetic modulation in lymphomagenesis has recently been illustrated. Developments in integrated genomic analysis using whole genome or whole exome sequencing have identified mutations of histone-modifying genes as one of the early driver mutations in follicular lymphoma. Concurrent mutations in at least two of the histone-modifying enzymes were detected in over 70% of the follicular lymphoma cases [26]. *EZH2* mutation has been reported in 7~22% of FL and 14~22% of DLBCL [17,21]. Accumulating evidence support the concept thatmutant *EZH2* may induce expansion of the proliferative germinal center B cell compartmentin FL and DLBCL, and the cooperation with other oncogenic hits promotesthe transformation to overt lymphoma [9,18,27]. Mostly important, data from cell culture and animal models suggest EZH2-mediated epigenetic effects are reversible, which provides the basis for pharmacologic modulation of EZH2 in patients with lymphoma [28]. The high incidence of *EZH2* mutation and its role in disease progression makes FL and germinal center B cell type DLBCL appropriate candidates to evaluate EZH2 targeted therapy

Previously, the analysis of *EZH2* mutations reported in the literature has predominately beenperformed using Sanger sequencing and next generation deep sequencing. Sanger sequencing is also known as sequencing by termination, since DNA syntheses is terminated by the random incorporation of fluorophore labeled deoxynucleotidetriphosphate (ddNTP). The DNA fragments of variable length can be detected by size using capillary electrophoresis. Benefits of Sanger sequencing include detection of previously unexpected mutation and relatively long reads of up to 800bp. It may also detect rare single nucleotide polymorphic variants that have unknown clinical significance. However, it is labor intensive and has a relatively long turnaround time.Next generation sequencing is a powerful tool to analyze large sets of genetic data, but it requires relatively complex sample preparation, massive infrastructure and bioinformatics support, all of which have led to limited use in clinical diagnostic laboratories. Here we developed a rapid and reliable pyrosequencingassay to detect the Y641 mutation in *EZH2* from FFPE tissue. Pyrosequencing utilizes a different principle from Sanger sequencing as it allows sequencing of a single strand of DNA by synthesizing the complementary.When the template strand of DNA is being sequenced, deoxynucleotide triphosphates (dNTPs) are sequentially dispensedand removed from the reaction. When there is successful DNA extension, inorganic pyrophosphate is released and converted to a detectable chemiluminescent signal by an enzymatic reaction. The sequence of the dNTP which produces the signals allows the determination of the sequence of the template.And the intensity of the signaling reflected by the height of the peak is directly proportional to the number of nucleotides being incorporated into the growing sequence during each dispensation. The reads for pyrosequencing are shorter compared to Sanger sequencing and not suitable for identifying unknown mutations. However, it can provide quantitative information and allows testing of a large number of samples in a single reaction. The limit of detection for mutant alleles is ~5%, which is superior to the ~15% required for Sanger sequencing [29,30].

Here we take advantage of the *EZH2* mutation occurring in a very specific site for DLBCL and FL. Given the labor intensive nature of Sanger sequencing and thecost and current lack of availability of the next generation sequencing inmanyclinical laboratories, pyrosequencing can be a very economical and efficient method to detect *EZH2* mutational status.The major limitation of this assay is that due to pyrosequencing's generation of short read lengths.It does not detect other nearby mutations that may also contribute to the structure of the catalytic domain of EZH2. This is of most significance in follicular lymphoma where positions A677, A682, A692 have been shown to account for up to 16% of *EZH2* mutations [20]. However, in germinal center B cell type DLBCL and other non-Hodgkin lymphoma, prevalence of these mutations maybe as low as 1% [18]. The clinical significance of these low frequency mutations is unclear at this time, but they could be adapted into a panel of pyrosequencing assays if necessary. Particularly given the multiplexing capability of pyrosequencing, the mutations in close proximity could be detected on one amplicon.

The prevalence of *EZH2*Y641 in the literature has shown some degree of variation that can be attributed to methods of detection and study cohorts. To confirm the mutations initially detected in whole-transcriptome shotgun sequencing and to determine the prevalence of this mutation in lymphoma tissue samples, Morin et al Sanger sequenced lymphoma samples and reported *EZH2*Y641 occurred in 21.7% of germinal center B cell type DLBCLs and 7.2% of FLs [17]. Ryan et al reported 14% in diffuse large B cell lymphomas with a germinal center immunophenotype and 22% in follicular lymphomas using a sensitive SNaPshot single nucleotide extension technology [21]. [Using the pyrosequencing method, we detected the prevalence of *EZH2*Y641 in the similar range (~13.6% germinal center B cell type DLBCL samples and ~16.7% follicular lymphoma samples containing *EZH2* Y641). We have identified the most common 4 types of mutations described by Morin et al including 2 cases of Y641S, 1 case of Y641N, 1case of Y641F, 1 case of Y641H. We did not identify any cases with rare Y641C that was reported in only one case from Morin et al's large cohort of 251 follicular lymphomas and 320 diffuse large B cell lymphomas [17].

In summary, in this study, we have developed a clinically applicable pyrosequencing assay with Sanger sequencing validation for reliabledetection of *EZH2* codon 641 mutations in FFPE tissue. Our results confirm the previous observation that *EZH2* mutations are commonly present in a subset of diffuse large B cell lymphoma with germinal center origin and in follicular lymphoma. The rapid and accurate detection of the *EZH2* mutation using this method in the clinical laboratory setting will facilitate enrolling lymphoma patients possessing such mutations for clinical trials testingEZH2 inhibitors.

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