Brief Report

LYMPHOID NEOPLASIA

EZH2 mutations are frequent and represent an early event in follicular lymphoma

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Key Points

- *EZH2* mutations occur in more than 25% of follicular lymphoma patients.
- Mutations predominantly represent an early/clonal event in the pathogenesis of the disease.

Gain of function mutations in the H3K27 methyltransferase EZH2 represent a promising therapeutic target in germinal center lymphomas. In this study, we assessed the frequency and distribution of *EZH2* mutations in a large cohort of patients with follicular lymphoma (FL) (n = 366) and performed a longitudinal analysis of mutation during the disease progression from FL to transformed FL (tFL) (n = 33). Mutations were detected at 3 recurrent mutation hot spots (Y646, A682, and A692) in 27% of FL cases with variant allele frequencies (VAF) ranging from 2% to 61%. By comparing VAF of *EZH2* with other mutation targets (*CREBBP*, *MLL2*, *TNFRSF14*, and *MEF2B*), we were able to distinguish patients harboring clonal *EZH2* mutation from rarer cases with subclonal mutations. Overall, the high incidence of *EZH2* mutations in FL and their stability during disease progression makes FL an appropriate disease to evaluate EZH2 targeted therapy. (*Blood*. 2013; 122(18):3165-3168)

Introduction

Next-generation sequencing (NGS) studies have shown frequent mutations in epigenetic regulators in almost all cases of follicular lymphoma (FL).[1,2] These include EZH2, the catalytic subunit of PRC2, which catalyzes trimethylation of lysine 27 on histone H3 (H3K27me3), a repressive chromatin mark.[3] Somatic gain-of-function mutations of *EZH2* at codon Y646 (previously Y641) were identified in 7% to 22% of FLs and germinal center B-cell type diffuse large B-cell lymphomas leading to elevated H3K27 trimethylation[4-8] with mutations at codons A682 and A692 described in isolated cases of diffuse large B-cell lymphomas.[2,9-11] As highly selective EZH2 inhibitors have now been developed,[12-14] we set out to assess *EZH2* mutation status, the effect of mutations on global gene expression, and the clonal representation of *EZH2* mutations as the disease progresses.

Study design

Patient samples

Genomic DNA from 181 diagnostic FL patients with accompanying clinical and gene expression data[15] were obtained through the Lymphoma/Leukemia Molecular Profiling Project consortium. DNA from 185 additional FL patients (56 obtained at diagnosis and 129 at relapse) and 33 paired FL and transformed FL (tFL) samples were sourced from the tissue archive at the Barts Cancer

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Institute. The study was approved by the London Research Ethical Committee (05/Q0605/140) and was conducted in accordance with the Declaration of Helsinki.

Mutation analysis

NGS was performed on all 432 samples, with the entire coding region (n = 19 exons) of *EZH2* screened in 46 FLs with the remaining 320 samples and the 33 paired FL-tFL cases restricted to exon 16 (Y646) and 18 (A682 and A692). The mutation analysis was performed by an NGS amplicon deep-sequencing assay using the Titanium amplicon chemistry (454 Life Sciences, Branford, CT)[16,17] achieving at least a 200-fold coverage (sensitivity <5%). Exons 16 and 18 of *EZH2* were also analyzed by bidirectional Sanger sequencing, as described previously (supplemental Table 1 on the *Blood* Web site).[4,18] Subsequently, targeted resequencing of *CREBBP, MLL2, TNFRSF14, and MEF2B* was performed using the multiplex Access Array platform (Fluidigm) as per the manufacturer's recommendations in selected FL cases with *EZH2* mutation (variant allele frequencies [VAF] range: 3.1% to 49.1%). Corrected VAF of *EZH2* for the sequential FL-tFL cases were determined using tumor cell content estimates calculated by the ASCAT algorithm[19] using previously generated SNP6.0 array data.[20]

Gene expression data analysis

Existing gene expression profiling data[15] from 181 FL samples from the Lymphoma/Leukemia Molecular Profiling Project FL cohort were analyzed as described in the supplemental Methods.

Results and discussion

Incidence of *EZH2* mutations in FL is higher than previously reported

The incidence and distribution of *EZH2* mutations were investigated in 366 FL patients (237 at diagnosis, 129 at relapse) using NGS and Sanger sequencing. Sequence analysis of the entire coding region of *EZH2* in 46 FL cases confirmed recurrent mutations at codons Y646, A682, and A692, previously reported by Morin and colleagues,[2,5] and the absence of additional mutational hotspots. We subsequently restricted our targeted resequencing to exons 16 and 18.

Sanger sequencing showed 63 *EZH2* mutations in 62 patients (17%) (Table 1). Using the more sensitive NGS approach (≥200-fold coverage), *EZH2* mutations were detected in 39 additional patients, increasing the total number of mutated patients to 101 (27.5%). Multiple mutations were observed in 4 patients; these were monoallelic (n = 2) or located to different reads (n = 2), suggesting either biallelic *EZH2* mutation or the presence of mutations in different FL clones (supplemental Table 2). In total, 106 mutations were detected in 101 patients, which included 87 Y646, 9 A682G, and 7 A692 mutations at a mean VAF of 21.6%, significantly lower in comparison with a VAF of 29.8% for mutations detected by both sequencing methods. The remainder corresponded to 3 novel variants K634E (VAF: 3.5%), V637A (VAF: 25%), and V679M (VAF: 2.5%), all located within the catalytic SET domain of EZH2 (Figure 1A). The somatic origin of the K634E mutation was confirmed using matched remission DNA. There was no difference in the mutation frequency at diagnosis (29%; n = 70/237) and relapse (24%; n = 31/129) with detailed distribution and frequencies of the *EZH2* mutations summarized in Figure 1A and Table 1. Mutation status was not associated with overall survival of FL in the 2 cohorts studied (supplemental Figure 1).

The majority of *EZH2* mutations represent clonal events

Although novel EZH2 inhibitors hold great promise, it is critical for the success of these therapies that the actionable mutations are clonally present within the tumor population. To decide whether *EZH2* mutations were clonal or subclonal events in FL, we compared *EZH2* VAFs with those of other mutation targets (*CREBBP, MLL2, TNFRSF14,* and *MEF2B*) in 43 FLs with *EZH2* mutations (VAF range: 3.1% to 49.1%; supplemental Table 3). Although the direct comparison may often be complicated by presence of acquired uniparental disomy or changes in copy number leading to VAFs of >50%,[21,22] we were able to discriminate 3 different patterns for *EZH2* mutations (Figure 1B). The majority of *EZH2* variants (81%; 35/43) represented true clonal events with similar VAFs for other genes mutated in the same sample. These included rare cases (4/43) characterized by low VAFs across all the mutational targets, which is probably a reflection of low tumor content within these biopsy samples. True subclonal *EZH2* mutations, with lower *EZH2* VAFs compared with the other genes, represented 19% (8/43) of all the *EZH2* variants tested. The dominance of clonal *EZH2* variants was also supported using our previous array-based methylation profiling data,[23] which allowed us to rank the samples based on their tumor content as described in the supplemental Information (supplemental Figures 2 and 3).

We next tested whether *EZH2* mutation status defined a particular subgroup of FL patients, based on global gene expression profiles. Although we failed to identify an *EZH2* gene expression signature using the entire cohort of 181 cases (125 wt vs 56 *EZH2* mutated), we were able to define a weak *EZH2* signature of 106 differentially expressed genes (Figure 1C; supplemental Table 4) by restricting the analysis to cases with estimated high tumor content ($\Delta\beta > 0.1657$, supplemental Figure 2) and *EZH2* VAF (>17%) (*EZH2* mutated; n = 18 and *EZH2* wt; n = 51). The relatively small number of differentially expressed genes and the low fold changes observed in our signature are consistent with the findings of McCabe et al, reporting only 35 common loci reactivated in 4 cell lines on treatment with the EZH2 inhibitor GSK126 highlighting the complexity and diversity of the EZH2 mediated epigenetic deregulation in individual lymphoma samples.[13]

EZH2 mutations are maintained during transformation of FL and represent an early event in FL

To determine the clonal representation of *EZH2* mutations during the disease progression, 33 sequential FL-tFL cases were screened for *EZH2* mutations using NGS. Of the 33 cases, 8 carried *EZH2* Y646 mutations (24.2%) with an average VAF of 27.6%. *EZH2* mutation was detected in both the FL and tFL biopsy in 6 patients, and in 2 additional individuals the *EZH2* mutation was restricted

Table 1. Numbers of *EZH2* mutations detected using the different sequencing approaches

Mutations	Sanger sequencing and NGS	Additional mutations by NGS only	Total
K634E	0	1	1
V637A	1	0	1
Y646N	18	18	36
Y646F	18	9	27
Y646S	8	4	12
Y646H	6	3	9
Y646C	0	3	3
V679M	0	1	1
A682G	7	2	9
A692V	5	2	7
In total	63	43	106
Mean VAF (range)	29.78% (4-61)	9.71% (2-31)	21.64% (2-61)

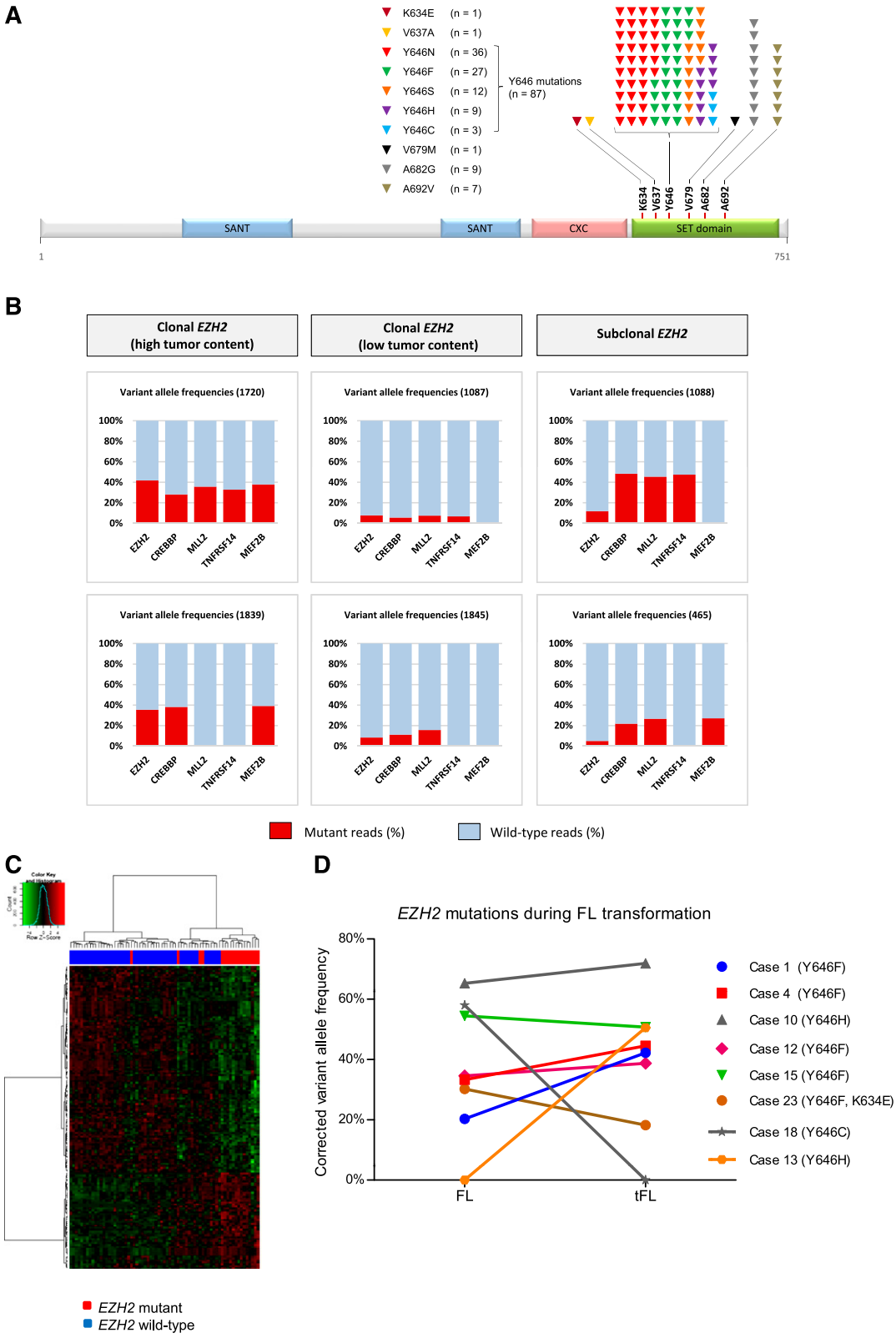

Figure 1. Detailed distribution and frequencies of the *EZH2* mutations. (A) Distribution and frequency of *EZH2* SET domain mutations detected in 366 diagnostic and relapsed FL cases. Altogether, 106 mutations were detected in 101 FL patients. The most prevalent variants were the mutations resulting in replacement of the tyrosine at codon 646 (Y646), followed by A682 and A692 mutations. Of note, we also detected 3 previously unreported mutations in B-cell lymphomas: K634E, V637A, and V679M of unknown significance. (B) Comparison of VAF for *EZH2, CREBBP, MLL2, TNFRSF14,* and *MEF2B* mutations identified by NGS-based targeted resequencing demonstrating *i,* true clonal variants with similar VAFs (less than 20% difference between VAFs for *EZH2* compared with the other genes excluding VAFs of >50%) across the genes analyzed (cases 1720 and 1839), *ii,* clonal variants with similar, but low VAFs across the mutation targets reflecting the low tumor content within these biopsy samples (cases 1087 and 1845), and *iii,* true subclonal *EZH2* variants with lower *EZH2* VAFs as compared with the other genes (cases 1088 and 465). (C) Hierarchical clustering and heatmap of 69 FL cases (51 wild type vs 18 *EZH2* mutated) with estimated high tumor content ($\Delta\beta > 0.1657$) and *EZH2* VAF (>17%) showing the gene expression signature of 106 differentially expressed genes. (D) Clonal representation of *EZH2* mutations during transformation of FL. Illustrated are the corrected VAFs observed in sequential FL and tFL samples. The *EZH2* mutations were maintained during transformation in 6 cases with relatively stable VAFs, whereas it was restricted to the FL and tFL samples in single patients (cases 13 and 18). Case 23 harbored 2 *EZH2* mutations (Y646F and K634E) in monoallelic configuration.

to either the FL or tFL biopsy (supplemental Table 5). Green and colleagues reported significant clonal diversity in genes that are recurrently mutated in FL highlighting *CREBBP* mutations as an early driver event in the disease evolution, based on their clonal nature at diagnosis and their maintained presence between diagnosis and relapses.[24] Our data demonstrate that *EZH2* mutations are also present at relatively high allelic frequencies and in the majority of cases are maintained through transformation of the disease (Figure 1D), implying that they may also represent early mutations in this lymphoma.

In conclusion, our observations demonstrate a higher frequency of *EZH2* mutations in FL than previously reported.[4,5] Mutations cluster to 3 codons, Y646, A682, and A692, are clonal in the majority of cases, and are stable during disease progression. The variable tumor content in FL biopsies supports the use of more sensitive and quantitative approaches during routine screening of FL to select patients with clonal *EZH2* mutations, as these will be better suited for treatment with EZH2 inhibitors.

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Authorship

Contribution: C.B. and J.F. designed the study, performed research, analyzed data, and wrote the manuscript; C.B., V.G., A.K., and T.H. performed the mutation analysis; N.P., J.O., C.O., K.T., S.A., A.M.L., A.C., and H.R. performed research and analyzed data; S.M., T.A.L., and J.G. selected patients for the study; S.I. and J.M. provided clinical information; A.R., G.O., E.C., L.M.R., E.B.S., W.C.C., R.M.B., L.M.S., G.W., A.M., and R.D.G. provided samples; J.M., J.W., C.C., O.E., and R.H. performed bioinformatical analyses; and all other authors read and approved the final manuscript.

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