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A687V EZH2 is a gain-of-function mutation found in lymphoma patients

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ABSTRACT

Heterozygous point mutations at Y641 and A677 in the EZH2 SET domain are prevalent in about 10–24% of Non-Hodgkin lymphomas (NHL). Previous studies indicate that these are gain-of-function mutations leading to the hypertrimethylation of H3K27. These EZH2 mutations may drive the proliferation of lymphoma and make EZH2 a molecular target for patients harboring these mutations. Here, another EZH2 SET domain point mutation, A687V, occurring in about 1–2% of lymphoma patients, is also shown to be a gain-of-function mutation that greatly enhances its ability to perform dimethylation relative to wild-type EZH2 and is equally proficient at catalyzing trimethylation. We propose that A687V EZH2 also leads to hypertrimethylation of H3K27 and may thus be a driver mutation in NHL.

1. Introduction

The EZH2 gene encodes a SET domain-containing lysine methyltransferase that forms a complex with 3 to 4 other partners (Suz12, EED, RbAp48 and AEPB2) known as the Polycomb Repressive Complex 2 (PRC2). PRC2 methylates lysine 27 on histone H3 (H3K27) up to three times; trimethylated H3K27 represses the transcription of proximal genes [1], playing an important role in X-inactivation, stem cell pluripotency and germline development [2]. Many studies have implicated EZH2 overexpression in the development, aggressiveness and metastatic potential of an array of solid tumors [3–7] and hematopoietic malignancies [8,9]. Additionally, loss-of-function mutations in UTX, an H3K27 demethylase, are frequently seen in a number of cancers [10–12], emphasizing the importance of the methylation status of H3K27 in oncogenesis.

Several whole-genome sequencing efforts of cell lines and primary tumor samples have identified genes involved in chromatin and transcriptional regulation that are commonly mutated in Non-Hodgkin Lymphomas (NHL). These include EZH2, MLL2, MEF2B and CREBBP among others [13–17]. Of particular interest, EZH2 mutations occur in a unique and mechanistically clear pattern; roughly 10–24% of Non-Hodgkin lymphoma patients are heterozygous for point mutations at two specific residues, Y641 and A677, in the catalytic SET domain of EZH2. Additionally, cells and tumor tissue harboring these heterozygous mutations display a clear reduction in dimethylated H3K27, but have elevated trimethylated H3K27. Biochemical studies using recombinant enzymes containing all of these mutations (Y641F, Y641N, Y641H, Y641S, Y641C and A677G) have revealed stark changes in enzymatic function [17–20]. In the case of the Y641 mutants, the substrate specificity is reversed from the wild-type enzyme such that the catalytic efficiency is weakest for the unmethylated H3K27 state but gets progressively greater with increasing methylation status [18–20], while the A677G mutation enhances EZH2 activity on substrates of all H3K27 methylation states [17]. It is hypothesized that in lymphomas with heterozygous EZH2 mutations, the pairing of wild-type and gain-of-function EZH2 mutants creates an accelerated pathway towards trimethylated H3K27, thus promoting the malignant transformation of B-cells [17,18].

Due to the important status of EZH2 and dysregulation of H3K27 trimethylation in cancer research, we were inspired to examine additional mutations in the EZH2 SET domain. The mutational analysis of EZH2 from two massive whole genome sequencing efforts [15,16] of Non-Hodgkin lymphoma were analyzed, and the A687V

Abbreviations: DLBCL, diffuse large B-cell lymphoma; CPM, counts per minute; GCB, germinal Center B-cell-like; H3K27, histone H3 lysine 27; NHL, non-Hogdkin lymphoma; PKMT, protein lysine methyltransferase; PRC2, Polycomb Repressive Complex 2; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; ^{3}H-SAM, SAM bearing tritiated methyl group

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mutation of EZH2 appeared to be a candidate for causing a gain-of-function. To investigate the biochemical effect of this point mutation within proximity to the Y641 and A677 residues, we purified PRC2 containing A687V EZH2 and characterized its ability to methylate histone H3 peptides with zero-, mono- or dimethyl lysine 27. Our findings indicate that, indeed, A687V is another example of a gain-of-function EZH2 mutation that widens the scope and importance of EZH2 alterations in Non-Hodgkin lymphomas.

2. Materials and methods

Recombinant 4-component PCR2 was produced in *Spodoptera frugiperda* (Sf9) cells using a baculovirus expression system. Wild-type EZH2 (NM_004456) was coexpressed with wild-type FLAG-EED (NM_003797), SUZ12 (NM_015355), and RbAp48 (NM_005610). An N-terminal FLAG tag on the EED was used to purify active PRC2 complex from cell lysates. For production of mutant 4-component PCR2, EZH2 carrying the A687V mutation was co-expressed with wild-type FLAG-tagged EED (NM_003797), SUZ12 (NM_015355) and RbAp48 (NM_005610), again using the baculovirus system. The FLAG-tag was used for purification. The purity and stoichiometry of the subunits of the final PRC2 preparations was assessed by SDS–PAGE with Coomassie blue staining and microfluidic capillary electrophoresis (Agilent Bioanalyzer). Peptide substrates representative of histone H3 residues 21–44 containing either zero-, mono-, di- or trimethylated lysine 27 and a C-terminal biotin (appended to a C-terminal amide-capped lysine) were HPLC-purified to greater than 95% purity. The enzyme assay was performed with 4 nM wild-type enzyme or 2 nM A687V enzyme as previously described [18,19] using 0.8 μM unlabeled SAM and 0.2 μM ^{3}H-SAM and a peptide titration.

3. Results

Recent whole-genome sequencing efforts in B-cell lymphoma were analyzed for the mutation status of EZH2. A study by Morin et al. comparing normal samples to malignant samples that included 13 diffuse large B-cell lymphomas, 1 follicular lymphoma and 113 other non-Hodgkin lymphomas identified 109 mutated genes. Out of 127 total cases, EZH2 was mutated at Y641 (Y641F, N, S, H) 31 times (24.4% frequency), and was found to also have one A677G (0.8% frequency) and one A687V (0.8% frequency) mutation [15]. Each of these EZH2 point mutations was confirmed to be heterozygous. In another study, whole-genome exome sequencing of diffuse large B-cell lymphoma was performed by Lohr et al., and revealed that 5/49 patients (10.2% frequency) carried a Y641 mutation (Y641F or N) and 1/49 (2% frequency) had the A687V mutation [16]. In the Morin et al. study, the subtype classification of the lymphoma is not available, however in the Lohr et al. study, the lymphoma with the A687V mutation is classified as diffuse large B-cell lymphoma.

A 3-dimensional model of the EZH2 SET domain was constructed based on the crystal structure of the MLL1 SET domain (PDB code: 2W5Z) using SWISS-MODEL [21] to visualize the location of the A687V mutation relative to the known gain-of-function mutations at Y641 and A677 (Fig. 1). In this model, the A687 residue is located near a bound molecule of the product SAH. The side chain of the A687 residue is forming a hydrophobic core with residues I631, I689, I715 and F729 in proximity to the Y641 residue and may be part of the putative lysine binding channel. The side chain of the Y641 residue is pointing towards the lysine binding channel and the A677 residue is situated behind this putative channel. From this observation it could be rationalized that an A687V substitution could potentially alter the substrate specificity or catalytic efficiency of EZH2 based on the methylation state of H3K27. To test this hypothesis, A687V EZH2 was cloned and the

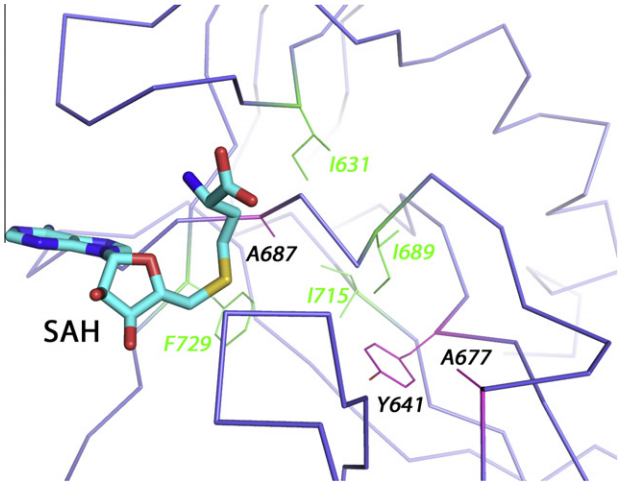

Fig. 1. An EZH2 SET domain model showing the location of the several mutation sites discussed in the text. The model and the location of SAH are based on the crystal structure of the MLL1 SET domain (PDB code 2W5Z). The C_α trace of the EZH2 SET domain is presented and the side chains of A687, Y641 and A677 are colored in magenta, while the residues forming the hydrophobic core with A687 are colored in green.

recombinant 4-component PRC2 complex (EZH2 A687V, Suz12, RbAp48 and FLAG-EED) was expressed with and purified by the FLAG affinity tag, analogous to the wild-type enzyme purification [19]. The PRC2 complex was judged to be 93% pure, having near 1:1 stoichiometry between all the subunits. The activity of this complex was initially interrogated in parallel with the wild-type enzyme by examining the ability to transfer tritiated methyl groups from ^{3}H-SAM to a series of biotinylated peptides spanning residues 21–44 of histone H3 and representing all states of H3K27 methylation (0, 1, 2 and 3). A full kinetic analysis was performed on the A687V EZH2-containing PRC2 complex as illustrated in Fig. 2 and compared to data previously obtained for the wild-type enzyme [19]. The peptide substrates containing zero-, mono- and dimethyl lysine 27 were titrated and $K_{1/2}$ and k_{cat} values were determined; these data are tabulated in Table 1. The peptides displayed sigmoidal behavior and the data were fit using a sigmoidal

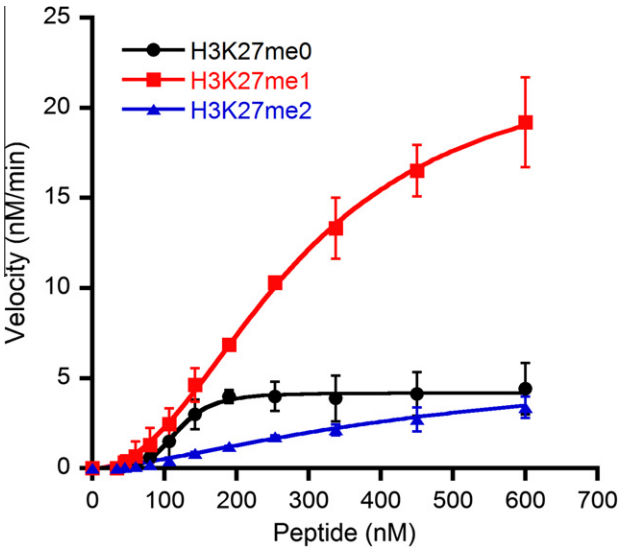

Fig. 2. Steady-state kinetics of peptide substrate utilization by recombinant PRC2 complex containing A687V EZH2. The peptide substrates used here all represented histone H3, residues 21–44, with lysine 27 present in the zero-, mono- or dimethylated state. The mean and standard error of 3 experiments is plotted.