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
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 PRC2 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 PRC2, 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 (attached 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 3H-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 was 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 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 3H-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 Kcat and kcat/Km values were determined; these data are tabulated in Table 1. The peptides displayed sigmoidal behavior and the data were fit using a sigmoidal
The A687V mutation is unique. In Fig. 3, the Y641 and A677 mutations, the pattern of substrate usage by the lysine 27 of histone H3. While this is akin to the effect seen with EZH2, mutation at A687V changes the orientation of the residue to adopt a similar conformation, but leaves the hydrogen-bonding ability intact, creating a mutant enzyme that is proficient in transition-state recognition with respect to the H3K27me1 and H3K27me2 peptides, observed by inspection of $k_{cat}$ plots. Overall, in comparison to the wild-type enzyme, the catalytic efficiency of the A687V EZH2 is drastically reduced on the H3K27me0 peptide, but is greatly enhanced on the H3K27me1 peptide, and is virtually the same on the H3K27me2 peptide. This is in contrast to the Y641 mutations that see their catalytic efficiency increase with increasing methylation state [17–19] or the A677G mutation which is catalytically proficient on all H3K27 substrates [17].

Relating these mutations to the overall architecture of the SET domain, modeled in Fig. 1, we see that the A687V residue could be part of the putative lysine binding channel and hydrophobic core located near the Y641 residue. The A677 residue, in contrast, is behind the Y641 residue. Mutation of the A687 residue to valine could alter the size of the lysine binding channel directly or enlarge the hydrophobic core in turn to shift the location of the Y641 residue. It is hypothesized that the Y641 residue and analogous residues in other PKMTs are steric gatekeepers that restrict the ability to perform trimethylation [17–19,23,24]. It is believed that mutation of Y641 to another residue changes the orientation of the Y641 residue further away from the lysine binding pocket [17,18,23]. This change presumably causes the lysine binding channel to become more permissive to substrate lysines bearing H3K27me2, but disrupts the ability to interact with the unmethylated lysine due to loss of hydrogen bond between the tyrosine hydroxyl and the amino group of lysine. In the same vein, it is predicted that the A677G mutation also encourages the Y641 residue to adopt a similar conformation, but leaves the hydrogen-bonding ability intact, creating a mutant enzyme that is proficient on all methylation states [17]. We propose that the A687V mutation may be acting by either mechanism: 1) the additional two hydrophobic methyl groups of valine directly affect the steric interactions with H3K27, or 2) the additional two hydrophobic methyl groups indirectly affect the lysine binding site by causing the Y641 residue to adopt a conformation that increases the physical space within the lysine binding channel. However, in the absence of an X-ray crystal structure for EZH2, we are unable to make a definitive conclusion as to which, if either, of these two mechanisms may be utilized. While the A687V mutation greatly enhances the utilization of monomethylated substrate, we propose that it does not alleviate the steric crowding sufficiently to cause an improvement in the use of the dimethylated substrate.

H3K27 methylation is an important epigenetic mark that is altered in many solid and hematologic cancers [3–9]. This mark is methylated by PRC2 complexes containing either EZH1 or EZH2 and it is demethylated by the enzymes UTX and JMJD3. Among these enzymes, several studies have shown consistently and unambiguously that EZH2 knockdown attenuates both hypertrimethylation.

Table 1

<table>
<thead>
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<th>Enzyme</th>
<th>H3K27 methylation status</th>
<th>$K_{1/2}$ (nM)</th>
<th>$k_{cat}$ (h⁻¹)</th>
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<tr>
<td>Wild-typea</td>
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<td>0 157 ± 12</td>
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<td></td>
<td>1</td>
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<tr>
<td>A687 V</td>
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<td>0.21 ± 0.04</td>
<td>17 ± 25</td>
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<tr>
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The binding model rather than classic Michaelis–Menten fits to determine the concentration of peptide resulting in half-maximal velocity [18,22]. In addition, the catalytic efficiency of the mutant and wild-type enzymes is compared by analysis of the ratio of $k_{cat}/K_{1/2}$ for each peptide substrate. The enzymes showed no activity on a peptide of the same length and sequence containing trimethylated lysine 27 (data not shown), ensuring that there was no methylation at a lysine other than H3K27 occurring.

4 Discussion

Mutational analysis of EZH2 in Non-Hodgkin lymphomas, culled from two separate large sequencing efforts, revealed that between 10 and 24% of these cases were characterized as having EZH2 SET domain mutations at Y641 (Y641F, N, H and S). Another mutation at A677G was found to occur in about 1% of the cases. These mutations have been well-characterized in both cell-based and enzymatic assays to change the substrate specificity of EZH2 such that, when paired with a wild-type enzyme, they lead to hypertrimethylation of H3K27, and implicate this mutation as a driver of lymphomagenesis [17,18,20]. Also of interest, is another potential heterozygous SET domain mutation of EZH2 at A687V [17,18,20].

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The $k_{cat}/K_{1/2}$ plots. Overall, in comparison to the wild-type enzyme, the catalytic efficiency of the A687V EZH2 is drastically reduced on the H3K27me0 peptide, but is greatly enhanced on the H3K27me1 peptide, and is virtually the same on the H3K27me2 peptide. This is in contrast to the Y641 mutations that see their catalytic efficiency increase with increasing methylation state [17–19] or the A677G mutation which is catalytically proficient on all H3K27 substrates [17].

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Fig. 3. Steady-state kinetic parameters for methylation reactions catalyzed by PRC2 containing wild-type or A687V EZH2. Illustrating how different methylation states of H3K27 influences the (A) $K_{1/2}$, (B) $k_{cat}$ and (C) $k_{cat}/K_{1/2}$ ratio. The mean and standard error of 3 experiments is plotted.
of H3K27 and the attendant tumorigenic phenotype of specific cancer cells.

Recent studies in lymphoma have shown that mutations of specific residues in the EZH2 SET domain, at Y641 and A677, alter the substrate utilization of the enzyme in such a way that, in concert with the wild-type enzyme, leads to hypertrimethylation of H3K27 and resultant lymphomagenesis. In the present study we have shown that the A687V mutation in EZH2 also alters the pattern of substrate utilization, in this case greatly favoring methylation of the H3K27me1 state over other H3K27 forms. This observation leaves open the question of whether cells containing the A687V mutation would display hypertrimethylation of H3K27, as a result of enzymatic coupling with wild-type EZH2 activity. The current lack of cultured cell lines containing this mutation has precluded our ability to experimentally address this important question. However, this inference seems reasonable in that the altered activity of the A687V mutant would be expected to result in a disproportionate build-up of the H3K27me2 state, the substrate for enzymes performing the H3K27me2 to H3K27me3 reaction (i.e., both wild-type and A687V mutant EZH2). Hence, based simply on Michaelis–Menten kinetics one would expect the flux through the H3K27me2 to 1 to 2 to 3 system to increase in A687V mutant-bearing cells, resulting in elevated levels of the lymphomagenetic H3K27me3 state. Thus, the A687V EZH2 mutant deserves further in vitro and in vivo study, as it is likely to be an addition to the known repertoire of EZH2 genetic alterations that lead to tumorigenesis in human cancers.

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References