Immunostaining of Increased Expression of Enhancer of Zeste Homolog 2 (EZH2) in Diffuse Midline Glioma H3K27M-Mutant Patients with Poor Survival

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Keywords

Diffuse midline glioma · H3K27M mutant · EZH2 · Immunohistochemical staining

Abstract

Introduction: The interaction of K27M mutation in histone H3 (H3K27M mutation) with polycomb repressive complex 2 (PRC2) is facilitated by the enhancer of zeste homolog 2 (EZH2). Subsequently, this interaction leads to the global reduction level of H3K27me3. We analyzed the EZH2 expression level in H3K27M mutation-positive tumors and revealed the association of high EZH2 expression with poor survival. **Methods:** Our study included 12 patients, with an age range of 6–56 years and treated between 2007 and 2016. All patients underwent MRI study for nonenhanced T1, T2, diffusion, gadolinium-enhanced T1-weighted imaging, and fluid-attenuated inversion recovery (FLAIR). Immunohistochemical staining was performed against H3K27M, H3K27me3, EZH2, EED, mutant isocitrate dehydrogenase 1 (IDH1), α -thalassemia X-linked intellectual disability (ATRX), p53, O6-methylguanine-DNA methyltransferase (MGMT), and Ki-67 antibodies. **Results:** All

patients were negative for IDH1R132H and H3K27me3, but H3K27M-positive. Staining against EZH2 was negative in all histological features of grade II cases (3/12) and positive in grade III and IV cases; EZH2 positivity is associated with poor prognosis ($p = 0.0082$). EZH2 positivity was not associated with EED positivity. Retained ATRX staining was found mostly in grade III and IV cases (6/12). P53 was predominantly positive in cases of astrocytoma and glioblastoma (8/12). The labeling index of Ki-67 was 1.2–31.4% for grade II and III histological features and 11.2–24.8% for grade IV. **Conclusion:** We suggest that the expression of EZH2 is not associated with the PRC2 pathway and increases in patients with H3K27M-mutant diffuse midline glioma and a poor prognosis. Further studies are necessary to understand the mechanism involved.

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Introduction

Histone H3 is 1 of the 5 main histone proteins in the structure of chromatin in eukaryotic cells [1]. Modification of histone influences transcription and other pro-

cesses in DNA, and one of these modifications is methylation [2]. Lys 27 histone H3 is methylated by polycomb repressive complex 2 (PRC2), an epigenetic regulator, through its functional enzymatic component, enhancer of zeste homolog 2 (EZH2). In this process, Lys 27 histone H3 is trimethylated into the stable mark of H3K27me3. H3K27me3 plays an important role in epigenetic gene silencing [3, 4].

The K27M mutation in histone H3 (i.e., the H3K27M mutation) is developed from methionine substitution at lysine 27 in histone H3F3A and the *HIST1H3B/C* gene [5, 6]. H3K27M also interacts with PRC2-EZH2 and causes inhibition of PRC2 function, disrupting the H3K27me3 and PRC2-EZH2 interaction. As a result, this mutation leads to a global reduction in H3K27me3 [7], which may further result in a loss of function of epigenetic gene silencing. Many studies have shown that EZH2 activation is involved in many kinds of cancer development and progression; EZH2 is therefore considered to be a therapeutic target in certain cancers [8–12].

Diffuse midline glioma is an infiltrative glioma located in the brain stem, thalamus, and spinal cord, with predominantly astrocytic differentiation. Sequencing studies of these gliomas have shown recurrent mutations in the histone H3.3. This tumor is defined as grade IV in the WHO 2016 update because of the poor prognosis [13]. Subsequently, pertaining to the detection of H3K27M mutation protein expression, immunohistochemical staining has been confirmed to have a reliable sensitivity and specificity in glioma even when compared to the whole-exome sequencing result [14, 15]. However, the role of EZH2 in diffuse midline glioma has not been determined yet.

In spite of the WHO 2016 update grading, some recent studies found that diffuse midline glioma H3K27M has more variety in its histomorphology, i.e., features of WHO grades I–IV, and that the prognosis of the patients is not always poor [16–19]. In this study, we focused on the H3K27M mutation immunopositive tumors. We analyzed the EZH2 expression level and revealed its association with the poor survival of patients with H3K27M mutant-positive tumors.

Patient and Methods

Patients were treated in our hospital between 2007 and 2016. Of the 12 patients, 8 were males and 4 were female, with age range of 6–56 years. Based on the histomorphological features, there were 6 cases of glioblastoma, 2 anaplastic astrocytomas, 1 anaplastic oligodendrogliomas, 2 diffuse astrocytomas, and 1 oligodendroglioma.

All patients underwent MRI study for nonenhanced T1-weighted imaging, T2-weighted imaging, fluid-attenuated inversion recovery (FLAIR), diffusion-weighted imaging (DWI), and gadolinium-enhanced T1-weighted imaging. Cystic components were differentiated as hyper- and hypointense areas on T2-weighted MRI and FLAIR images, respectively. Necrotic components were differentiated on contrast-enhanced T1-weighted images as the interior nonenhancing parts of enhanced lesions. Hemorrhagic lesions were differentiated on nonenhanced T1-weighted MR images as areas of hyperintensity.

Tissue samples were obtained from surgical procedures. All samples were fixed with 10% buffer formalin, processed as formalin-fixed paraffin-embedded (FFPE) tissue, and stained with hematoxylin-eosin for routine histopathological diagnosis.

Statistical analyses were done with PRISM v7.0 (GraphPad Software Inc, La Jolla, CA, USA). The survival time of H3 K27M-mutant immunopositive patients was measured from the time of diagnosis by MRI to the time of death or last follow-up (range 3.7–80.9 months; median 14.67 months). Factors analyzed as the potential prognostic markers included: hemorrhagic characteristics at diagnosis, gadolinium enhancement, DWI high intensity, or p53, ATRX, and EZH2 immunopositivity. Kaplan-Meier's survival analysis (log-rank test), incorporating each prognostic factor, was performed to evaluate prognostic value in H3K27M-mutant immunopositive patients. For all statistical results, significance was assigned when the *p* value was <0.05.

Immunohistochemical Staining

Immunostaining was performed with automated immunostainers (BenchMark GX; Ventana, Tucson, AZ, USA) using ultra-View Universal DAB detection kit (cat. No. 760–500, Ventana Medical Systems, Tucson, AZ, USA). Slides were deparaffinized and treated with heat-induced epitope retrieval (HIER) using cell conditioning (CC)-1 (cat. No. 950–124, Ventana Medical Systems). The antibodies were rabbit polyclonal anti-H3K27M (#ABE419, Millipore, Billerica, MA, USA; 1 µg/mL); rabbit monoclonal anti-H3K27me3 (#9733, CST, Danvers, MA, USA; 13.33 µg/mL); rabbit monoclonal anti-EZH2 (790–4651, Ventana Medical Systems; 15.9 µg/mL); mouse monoclonal anti-human IDH1R132H (DIA-H09, Dianova, Hamburg, Germany; 20 µg/mL); rabbit polyclonal anti-ATRX (HPA001906, Sigma-Aldrich, St. Louis, MO, USA; 5 µg/mL); monoclonal mouse anti-human p53 protein (NCL-L-p53-DO7, Leica, Newcastle, UK; 10 µg/mL); monoclonal mouse anti-human Ki-67 antigen clone MIB1 (F7268, Dako, Denmark; 40 µg/mL); monoclonal mouse anti-EED antibody (163C, Abcam, Cambridge; 10 µg/mL) and monoclonal rabbit anti-MG-MT (EPR4397, Abcam; 5 µg/mL).

H3K27M and H3K27me3 immunostaining was performed using the ultraView template protocol. Slides were treated with HIER for 30 min and incubated with antibody for 32 min at 37°C. EZH2 staining was performed according to the manufacturer's instructions. Immunostaining for IDH1R132H and ATRX used the ultra-View template with HIER for 60 min, and antibody incubation was for 40 and 32 min, respectively. Staining for p53 and Ki-67 (MIB1) used the following customized protocol: HIER treatment for 60 min, peroxidase blocking reagent (S2023, Dako) application for 10 min, and antibody incubation for 40 min. Secondary antibody (414151F, Nichirei Bioscience Inc., Tokyo, Japan) incubation was for 40 min, and was visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (425312F, Nichirei Bioscience Inc.) and

Table 1. Imaging characteristics of H3K27M immunopositive tumors

Case No.	Age, years	Sex	Main tumor location	T2/FLAIR	DWI	Gadolinium enhancement	ITH	Cyst formation	Operation (removal)	Local RT dose (Gray)	Chemotherapy	Dissemination + distant recurrence	OS	Dead/alive
1	56	F	Thalamus	High	High	None	–	–	Biopsy	50	None	–	80.90	Alive
2	48	M	Thalamus	High	High	Partly	–	–	Biopsy	54	TMZ	+	23.70	Dead
3	29	F	Thalamus	High	High	Partly	–	–	Partial	60	TMZ/BEV	+	13.43	Dead
4	31	M	Thalamus	High	High	Homogeneously	–	–	Biopsy	60	TMZ	+	14.67	Dead
5	43	M	Thalamus	High	High	Homogeneously	+	–	Partial	48	BEV	–	3.67	Dead
6	30	M	Thalamus	High	High	Heterogenously	+	–	Partial	60	TMZ/BEV	+	11.97	Dead
7	6	F	Thalamus	High	High	Heterogenously	+	–	Biopsy	60	BEV	+	14.87	Dead
8	14	F	Thalamus	High	High	Partly	–	+	Biopsy	60	ACNU/CDDP	+	14.53	Dead
9	21	M	Thalamus	High	High	Heterogenously	+	+	Biopsy	60	TMZ	+	11.03	Alive
10	30	M	Pons	High	Iso	None	–	–	Biopsy	54	TMZ	+	28.37	Dead
11	11	M	Pons-medulla	High	Low	Partly	–	–	Biopsy	54	TMZ/BEV	+	19.40	Dead
12	16	M	Pons-medulla	High	High	Partly	–	–	Biopsy	54	TMZ/BEV	–	10.40	Dead

F, female; M, male; DWI, diffusion-weighted imaging; ITH, intratumoral hemorrhage; RT, radiation therapy; OS, overall survival; TMZ, temozolomide; BEV, bevacizumab; ACNU, nimustine; CDDP, cisplatin.

copper solution for 8 min each. All counterstains were done manually with hematoxylin for 30 s and blueing reagent for 5 s.

Evaluation of immunostaining was done at 3 different sites, based on the previous criteria. H3K27M immunopositivity was defined by >80% of tumor cells with strong nuclear staining [14], and there was at least 71% positivity for H3K27me3 in the cell nuclei [15]. Positivity for EZH2 staining was determined based on strong nuclei staining [20]. Immunostaining was positive for IDH1R132H with strong cytoplasmic staining [21]. The ATRX and p53 staining positive cut-off value was 10% nuclear staining [22, 23]. Labeling index evaluation for Ki-67 was based on nuclear staining in tumor cells [24].

Results

MRI features are summarized in Table 1. Most of the 12 patients were adults, with a tumor located in the thalamus ($n = 9$) and features of glioblastoma WHO grade IV ($n = 6$). All tumors showed high intensity on T2/FLAIR images. Gadolinium enhancement was observed in 83% (10/12 patients) and showed high intensity on DWI (10/12 patients). Intratumoral hemorrhage is the most important characteristic of H3K27M mutant-positive thalamic glioma and was observed in 44.4% (4/9 patients) of the thalamic tumors, while no brain stem tumors showed hemorrhagic characteristics. Dissemination and distant/remote recurrence are characteristics of H3K27M mutant-positive tumors and were observed in 75% (9/12) of our series of patients. Prognosis was poor and median overall survival was 14.7 months. Median overall survival

of patients with thalamic and brain stem tumors was 14.7 and 19.4 months, respectively ($p > 0.05$).

Immunohistochemical staining is summarized in Table 2. All patients with H3K27M-positive staining revealed IDH1R132H and H3K27me3 negativity. Staining against EZH2 was negative in all histological features of grade II cases (3/12) and positive in grade III and IV cases. EED was positive in 8/11 tumors and was not associated with WHO grade or EZH2 positivity. MGMT expression was positive in 6/12 patients. Retained ATRX staining was found mostly in grade III and IV cases (6/12) and in 1 grade II case. P53 staining was predominantly positive in the cases of astrocytoma and glioblastoma (8/12). The labeling index of Ki-67 was 1.2–31.4% for grade II and III histological features and 11.2–24.8% for grade IV glioblastoma. Representative cases of our series are shown in Figures 1–3.

We compared the prognosis of patients with H3K27M mutant-positive tumors by taking into account the clinical factors. Between brain stem- and thalamus-located tumor groups, there was no statistical difference (Fig. 4a; $p > 0.05$). There is a poorer prognosis for patients with hemorrhagic tumors than for those with nonhemorrhagic tumors. However, our result did not show any statistical difference (Fig. 4b; $p = 0.1471$).

Based on the immunohistochemical staining, we found a significant difference between patients with high and low EZH2 expression (Fig. 4c; $p = 0.0082$). The median overall survival of these 2 groups was 14.5 and 28.4 months, respectively. Furthermore, the overall survival

Table 2. Immunohistochemical characteristics of H3K27M immunopositive tumors

Case No.	Age, years	Sex	Main tumor location	WHO 2007 diagnosis	mIDH1 R132H	ATRX	p53	MIB1	MGMT	H3K27M mutant	H3 K27me3	EED	EZH2
1	56	F	Thalamus	DA	–	–	+	4.5%	+	+	–	1+	–
2	48	M	Thalamus	O	–	–	–	3.3%	–	+	–	1+	–
3	29	F	Thalamus	AO	–	+	+	31.4%	+	+	–	2+	+
4	31	M	Thalamus	GB	–	+	+	27.8%	+	+	–	2+	+
5	43	M	Thalamus	GB	–	+	+	16.1%	–	+	–	1+	+
6	30	M	Thalamus	GB	–	–	+	28.8%	+	+	–	–	+
7	6	F	Thalamus	GB	–	–	+	21.9%	+	+	–	2+	+
8	14	F	Thalamus	AA	–	–	–	2.3%	–	+	–	–	+
9	21	M	Thalamus	GB	–	+	+	11.6%	–	+	–	1+	+
10	30	M	Pons	DA	–	–	+	3.3%	–	+	–	1+	–
11	11	M	Pons-medulla	AA	–	–	+	11.9%	+	+	–	2+	+
12	16	M	Pons-medulla	GB	–	–	+	24.8%	–	+	–	–	+

DA, diffuse astrocytoma; O, oligodendroglioma; AA, anaplastic astrocytoma; AO, anaplastic oligodendroglioma; GB, glioblastoma; F, female; M, male; –, negative; +, positive; 1+, partial positive; 2+, strong positive.

time of the patients with EZH2-positive thalamic glioma was significantly shorter than that of EZH2-negative thalamic glioma patients ($p = 0.0355$; 14.5 vs. 52.3 months; data not shown).

We also analyzed the relationship between tumor grade and staining of EZH2, EED, ATRX, and p53 expression. We found that only EZH2 was statistically associated with tumor grade, and high-grade tumors showed positive expression of EZH2 ($p = 0.0045$, Fisher's exact test). No other staining was associated with grade ($p > 0.05$). Compared to EZH2-negative tumors, the positive ones showed a tendency of a high MIB1 labeling index, but considering the small sample size, the result did not show statistical difference ($p = 0.0591$).

Regarding prognosis, EED expression status was not associated with prognosis ($p > 0.05$; data not shown). ATRX-positive and p53-positive staining as well as MGMT expression status, were not associated with prognosis ($p > 0.05$; data not shown). In addition, we could not find any relationship between immunohistochemical staining results and clinical or imaging characteristics, including surgical achievement, irradiation dose, chemotherapy, dissemination/distant recurrence, hemorrhage, enhancement, and DWI intensity.

Discussion

Histone modification can be associated with chromatin function and gene activity change in cancer [25]. Lysine 27 trimethylation on histone H3 (H3K27me3) func-

tion is important in lineage specification through developmental gene silencing [25, 26]. In the homeotic gene expression function, PRC2 methylates lysine 27 on histone H3 as a repressive chromatin [27]. This methyltransferase activity is mediated by multiple interactions of the PRC2 components. H3K27me3 acts as a stable marker bound to PRC2 as well as activating the PRC2 complex [27]. The cellular H3K27me3 level is determined by the PRC2-EZH2 site and a positive feedback interaction is maintained [3, 4]. Consequently, modification on H3K27me3 may result in aberrant gene expression and genomic instability [25].

In glioma, H3K27M mutation is associated with a global reduction in H3K27me3 level [7, 15, 20, 28]. Some studies found that the level of the H3K27me3 gene promoter is low or even lost in H3K27M glioma [15, 28, 29]. However, the mechanism of this effect remains unclear as the mutation is only present in some of the histones [27]. Mutation of H3K27M occurs in relation to mutation in histone H3 genes (H3F3A and HIST1H3B) resulting in lysine 27 substitution to methionine [5, 6, 30]. H3K27M mutation may influence tumorigenesis through the gene expression reprogramming linked to its interaction with epigenetic regulator PRC2 in the SET domain [27, 28].

PRC2 is susceptible to either gain- or loss-of-function alterations in malignancy [23]. According to the PRC2 structural basis described in Justin et al. [27] (the structural basis of the oncogenic H3K27M inhibition of PRC2), the binding of PRC2 and the nucleosome involves multiple interactions of PRC2 components. These components are SUZ12 and RbAp48 binding to H3, the SET

Fig. 1. Radiological and histological features of brain stem diffuse astrocytoma in a 30-year-old male. **a** Axial T1-weighted image. **b** Axial T1-weighted image after gadolinium infusion. **c** FLAIR image (pre-enhancement). **d** Diffusion-weighted image (pre-enhancement). **e** Biopsy of the mass revealed a predominantly low

cellularity astrocytic tumor. Mutant IDH1R132 staining was negative (**f**) and mutant H3K27M staining was positive (**g**). Staining was negative for H3K27me3 (**h**), EZH2 (**i**), and ATRX (**j**) antibodies. **k** P53 was positive. **l** Ki-67 labeling index was 3.3%.

Fig. 2. Radiological and histological features of brain stem anaplastic astrocytoma in an 11-year-old male. **a** Axial T1-weighted image. **b** Axial T1-weighted image after gadolinium infusion. **c** FLAIR image (pre-enhancement). **d** Diffusion-weighted image (pre-enhancement). **e** The tissue sample indicated a tumor composed of

moderate-to-high cellularity with atypical mitoses. **f** The tumor was mutant IDH1R132-negative. Staining for mutant H3K27M was positive (**g**) while H3K27me3 staining was negative (**h**). EZH2 staining was positive (**i**), as was ATRX staining (**j**). P53 was negative (**k**) with a Ki-67 labeling index of 11.9% (**l**).

Fig. 3. Radiological and histological features of a thalamic glioblastoma in a 6-year-old female. **a** Axial T1-weighted image. **b** Axial T1-weighted image after gadolinium infusion. **c** FLAIR image (pre-enhancement). **d** Diffusion-weighted image (pre-enhancement). **e** Tissue biopsy showed anaplastic tumor nuclei with fre-

quent mitoses and perivascular proliferation. **f** Mutant IDH1R132 staining was negative. Staining against mutant H3K27M antibody was positive (**g**) while H3K27me3 staining was negative (**h**). EZH2 (**i**), ATRX (**j**), and p53 (**k**) all showed immunopositivity. **l** The average Ki-67 labeling index was 21.9%.

Fig. 4. Kaplan-Meier survival curves illustrating cumulative survival rates for patients of H3 K27M-mutant immunopositive patients. Comparisons were made between thalamic tumors and brain stem tumors (a), hemorrhagic and nonhemorrhagic tumors (b), and EZH2 immunonegative and immunopositive tumors (c).

domain binding to H3K27, and EED binding to H3K-27me3. The EZH2 SET domain requires the other PRC2 components for activation and shows autoinhibition without them [27]. Referring to its natural role in gene silencing/repression, PRC2 maintains the determined transcriptional program to preserve cell identity and differentiation [3, 4, 31]. Accordingly, its role in cancer is also dependent on the context of the specific combinations of the tumorigenic alteration, including the regulated genes, the cell of origin, and the developmental history [31].

As one of the active sites of PRC2, EZH2 expression is increased or lost in malignancy [32, 33]. Overexpression of EZH2 has mostly been related to progressive behavior in solid tumors in breast, endometrial, lung, and prostate cancers [8–11]. Cancer with EZH2 overexpression revealed oncogenic activity via the transcriptional activation of EZH2 independent of PRC2 function; for this reason, EZH2 has become an appealing target in tumor therapy [12]. However, a deficiency of EZH2 has also been identified in malignancies like T cell acute leukemia and myelodysplastic disorders [34, 35]. Therefore, it is postulated that the role of EZH2 in malignancy is dependent on its affinity to histone and nonhistone substrates [33]. Increased expression of EZH2 has been found to be related to high-grade glioma and poor prognosis in glioblastoma [20, 36–39]. In diffuse midline glioma, the up-regulation of transcription activity is also related to the loss of PRC2 repression function [15, 29]. As indicated by Mohammad et al. [40] and Piunti et al. [41], H3K27M tumor also requires PRC2-EZH2 for tumor growth preservation and proliferation [40–42].

Recent studies on diffuse midline glioma have put forward a new understanding regarding the H3 mutant in

diffuse intrinsic pontine glioma. Piunti et al. [41] revealed that H3K27me3 reduction is due to the PRC2 exclusion from chromatin. Their data indicate that H3K27M does not involve EZH2, thus excluding PRC2 binding. Subsequently, H3K27M progresses through H3K27 acetylation and represses PRC2 activity [41, 42]. Mohammad et al. [40] added the evidence that H3K27M is excluded from some loci due to DNA methylation [42]. This methylation results in the incorporation of H3K27M at the weak polycomb target, but this is not enough to reduce the overall activity of PRC2. Thus, H3K27me3 may be retained at several loci in H3K27M-mutant tumors.

Consistent with other studies [15, 28, 29], all of the H3K27M immunostaining positive cases in our study were negative for H3K27me3. Furthermore, despite varying age and tumor grades, EZH2 expression was higher in high-grade glioma and in patients with a worse prognosis. Although it is still difficult to determine the connection between H3K27M and EZH2, our result correlates with studies that found that PRC2-EZH2 was a requirement for tumor growth and proliferation. Regarding the interaction between PRC2 components, we also found that this EZH2 positivity is not correlated with EED immunostaining expression. This indicates that EZH2 overexpression is independent of the PRC2 activity. Nonetheless, we could not determine whether this role of EZH2 is unique to diffuse midline glioma or also a feature of other gliomas and cancers in general.

With regard to other molecular characteristics in glioma, we also performed immunohistochemical staining against IDH1R132H, ATRX, p53, MGMT, and MIB1. Mutation of IDH1 is mutually exclusive with H3F3A mutation [30, 43] and was found to be negative on immunostaining in all H3K27M mutation gliomas [16].

Mutant IDH1R132H immunostaining has sensitivity of 83% and a specificity of 100% compared to direct sequencing [21]. All of our cases showed up as IDH1R132-negative, consistent with results of previous studies. TP53 mutation is found in roughly 50% of the cases of H3K27M mutation [43] and positive p53 expression in 40% of the cases [6, 44]. Less frequent than TP53, ATRX mutation is also found in a smaller percentage of H3K27M mutation cases; this mutation includes frameshift insertions/deletions, gains of a stop codon, and missense SNVs, which lead to a loss of expression on immunostaining [16, 26]. We also found a consistent result, although with slightly higher percentages of both p53-positive and ATRX-negative staining. This was presumably linked to the limited number and variety of cases. MGMT inactivation is correlated with clinical response to alkylating agents like temozolomide [45]. Half of our cases showed positivity. Furthermore, our labeling index of Ki-67 result was higher in glioblastoma and anaplastic glioma features, compared to the grade II feature. This result is consistent with the glioma profile in general [24].

We acknowledge that our study had limitations. We had only a small number of cases with little variety regarding the location, and we did not make any differentiation between adult and pediatric characteristics. The

study was also based on only a few components of PRC2 (EED2 and EZH2) with an immunostaining modality, and no DNA analysis was performed regarding the complex interaction among PRC2 components.

In conclusion, our immunostaining profile against H3K27M and H3K27me3 was consistent with previous studies on diffuse midline glioma. All tumors with positive staining against H3K27M showed a global reduction of H3K27me3 expression. Additionally, EZH2 staining increased expression as a clinical progressive factor is also consistent with the previous studies in glioma and other cancers. Accordingly, this finding is interesting with reference to the variety of prognoses despite the tumor grade of WHO IV as well as EZH2 inhibition as a potential target for therapy in diffuse midline glioma.

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Disclosure Statement

All authors have no conflicts of interest.

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