H3.3K27M Mutation Promotes The Migration and Invasion of Glioma Cells By Activating β -Catenin/USP1 Signaling

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Research Article

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Abstract

H3.3K27M is a newly identified molecular pathology marker in glioma and is especially correlated with the malignancy of diffuse intrinsic pontine glioma (DIPG). In recent years, accumulating research has revealed that other types of glioma also contain the H3.3K27M mutation. However, the role of H3.3K27M in high-grade adult glioma, which is the most malignant glioma, has not been investigated. In this study, we focused on exploring the expression and function of H3.3K27M in high-grade adult glioma patients. We found that H3.3K27M is partly highly expressed in high-grade glioma tissues. Then, we introduced H3.3K27M into H3.3 wild-type glioma cells, U87 cells and LN229 cells. We found that H3.3K27M did not regulate the growth of glioma *in vitro* and *in vivo*; however, the survival of mice with transplanted tumors was significantly reduced. Further investigation revealed that H3.3K27M expression mainly promoted the migration and invasion of glioma cells. Moreover, we certified that H3.3K27M overexpression enhanced the protein levels of β -catenin and p- β -catenin, the protein and mRNA levels of ubiquitin-specific protease 1 (USP1), and the protein level of enhancer of zeste homolog 2 (EZH2). Importantly, the β -catenin inhibitor XAV-939 significantly attenuated the upregulation of the aforementioned proteins. Overall, the H3.3K27M mutation is present in a certain proportion of high-grade glioma patients and facilitates a poor prognosis by promoting the metastasis of glioma by regulating the β -catenin/USP1/EZH2 pathway.

Introduction

Gliomas are a primary type of tumor in the central nervous system. In the 2016 World Health Organization (WHO) classification, glioblastoma (GBM) was defined as grade IV glioma and makes up 45.2% of all malignant central nervous system (CNS) tumors and 80% of all primary malignant CNS tumors[1,2]. GBM and other high-grade gliomas are challenging for neurosurgeons, as no effective treatment exists. Surgical treatment, radiotherapy and chemotherapy are the main treatment methods, but their curative effect falls short of patients' expectations[3]. Therefore, many neuroscientists put much effort into studying malignant glioma to find effective ways to cure this disease.

Histones are the core components of the nucleosome subunit, forming an octamer containing four core histones (H3, H4, H2A, and H2B) surrounded by a 147-base pair DNA fragment. Histone tails are influenced by a wide range of covalent posttranslational modifications (PTMs) that jointly regulate chromatin status[4]. These PTMs can change the electronic charge and structures of these histone tails, which bind to the DNA, to alter the chromatin status and subsequent gene expression[5]. The PTM of histones is closely related to the occurrence of many tumors[6]. As histone mutations directly affect histone PTMs that are associated with gene activity, it is likely that these mutations contribute to tumor development through either the activation of oncogenes or the repression of essential tumor suppressor genes[7]. Thus, it is necessary to explore the role of H3.3 mutation in the mechanism of tumor development.

Peter et al first reported that human diffuse intrinsic pontine gliomas (DIPGs) have the K27M mutation[8]. In the 2016 WHO classification, the H3 K27M mutant was first added to the list of diffuse glioma for the

new classification[1]. In recent years, scientists have focused on H3.3K27M-mutant diffuse midline gliomas. However, an increasing number of scholars have reported that some other kinds of intracranial tumors, such as ependymomas and gangliogliomas, exhibit the H3.3K27M mutation[9,10]. Researchers found that K27M-mutant diffuse midline gliomas are associated with significantly worse survival across all midline tumor locations[11]. Accordingly, H3.3K27M plays an important role in the occurrence and progression of intracranial tumors; nevertheless, the potential molecular mechanism of H3.3K27M is not very clear and needs further investigation.

Enhancer of Zeste homolog 2 (EZH2) is a significant constituent of polycomb repressive complex 2 (PRC2). It plays an oncogenic role in the occurrence and progression of tumors by regulating epigenetic genes[12]. PRC2 is a chromatin-associated methyltransferase catalyzing the mono-, di-, and trimethylation of lysine 27 on histone H3 (H3K27)[13]. EZH2 has been reported as a potential therapeutic target for H3K27M-mutant pediatric gliomas[14]. However, the function of EZH2 in adult H3K27M-mutant glioma is not very clear.

In this study, we first assessed the expression of H3.3K27M in glioma patients and glioma cell lines by Western blotting. Then, we introduced H3.3K27M into glioma cells and investigated its effect on proliferation, migration and invasion. Finally, we investigated the underlying mechanisms by which the H3.3K27M mutation promotes the progression of glioma.

Materials And Methods

Glioma specimens

Glioma tissues and normal brain tissues were collected at the Affiliated Hospital of Xuzhou Medical University (Xuzhou, Jiangsu, China). All human glioma specimens were assessed to confirm the pathological diagnosis. The research was approved by the Research Ethics Committee of Xuzhou Medical University, and written informed consent was obtained from patients who underwent surgery at the Affiliated Hospital of Xuzhou Medical University.

Antibodies and reagents

Antibodies specific for AKT (#4685), P-AKT (#13038), MEK1/2 (#4694), P-MEK1/2 (#9154), STAT3 (#12640), P-STAT3 (#9145), β -Catenin (#8480), P- β -catenin (#4176), H3K27M (#74829), Myc-Tag (#2276), GAPDH (#5174), and β -actin (#8457) were purchased from Cell Signaling Technology (Danvers, MA, USA). USP1 (14346-1-AP), and EZH2 (21800-1-AP) antibodies were purchased from Proteintech (Wuhan, China). XAV-939 and puromycin were obtained from MedChemExpress (Shanghai, China).

Cell culture

All the cell lines used in the experiment (HEK-293T, U251, U87, LN229, U118, A172, T98G) were purchased from Shanghai Cell Bank, Chinese Academy of Sciences. Cells were cultured in Dulbecco's modified

Eagle's medium (DMEM) (Gibco, CA, USA) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and incubated in an incubator at 37°C with 5% CO₂.

Western blotting

The cells were collected on ice and lysed in RIPA buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and 1% NP-40). The protein was harvested after 12,000 rpm in the centrifuge. Equal amounts of samples were isolated by 8%, 10% or 12% SDS-PAGE and then transferred to 0.22 µm PVDF membranes (Roche Diagnostics GmbH, Mannheim, Germany). The membranes were blocked in 3% bovine serum albumin (BSA) for 2 h and then incubated overnight at 4°C with diluted primary antibodies (AKT, P-AKT, MEK1/2, P-MEK1/2, STAT3, P-STAT3, β -catenin, P- β -catenin, USP1, EZH2, H3K27M, Myc-Tag, GAPDH, and β -actin). The next day, the membranes were incubated with the secondary antibody for 2 h. Finally, the membranes were detected by an enhanced chemiluminescence detection system (Thermo Fisher, Waltham, MA, USA).

RNA isolation, cDNA synthesis and RT-PCR

RNA was extracted from stable cell lines by using TRIzol (Beyotime, Shanghai, China), and cDNA was synthesized by employing a Prime Script RT Reagent Kit (TIANGEN, Beijing, China) according to the manufacturer's instructions. The target gene was amplified in a final volume of 20 µl with SYBR Green PCR Master mix (TIANGEN, Beijing, China). Quantitative RT-PCR was carried out by an ABI7300 real-time PCR instrument (Applied Biosystems, Carlsbad, CA, USA) using SYBR Green. The primers for the amplification of USP1 and β -actin were as follows: USP1-F: 5-GCT GCT AGT GGT TTG GAG TTT-3, USP1-R: 5-GCA TCA CAA CCG CAA ATA ATC C-3; β -Actin-F: 5-CCA ACC GCG AGA AGA TGA-3, β -Actin-R: 5-CCA GAG GCG TAC AGG GAT AG-3.

Lentivirus packaging and stable cell lines

Wild-type Myc-tagged H3F3A or Myc-tagged H3.3K27M cDNA was subcloned into a lentiviral vector (pCDH-CMV-MCS-EF1-GFP, where the inserted gene and GFP were expressed independently. Lentiviruses were produced in HEK-293T cells by transfecting the core plasmid and two assistant plasmids (pMD2.G and psPAX2) with PolyJet reagent (SignaGen Laboratories, Frederick, MD, USA). We established stable cell lines by infecting U87 and LN229 cells with vector, Myc-H3F3A or Myc-H3.3K27M viruses and screening with puromycin (2.5 µg/ml) after 48 h of infection.

Cell Counting Kit-8 (CCK-8) assay

Two hundred microliters of medium containing 4000 cells was seeded in a 96-well plate. At the designated time points, the original medium was replaced with medium containing 10% CCK-8 reagent (Victimed, Xuzhou, China) and incubated at 37°C for 2 h. Afterward, the absorbance at 450 nm was detected with a microplate reader. Cell viability was calculated based on absorbance values.

Colony formation assay

Five hundred cells suspended in 5 mL of medium were inoculated into each 60 mm dish and cultured continuously until macroscopic cell colonies were formed. Then, the cells were fixed with 100% methanol and stained with 0.1% crystal violet solution for 15 min. After washing with PBS, the plates were photographed using a digital camera. Colonies containing more than 50 cells were counted manually.

Wound healing assay

Stable cell lines were inoculated in six-well plates under normal conditions for 24 h. On the second day, the best cell density to perform the scratch assay was when cells were close to confluent. After the scratch was completed by the pipette tip in the middle of the wells, the unattached cells were washed with PBS twice, and the culture medium was replaced with serum-free culture medium. Photographs were taken at 0 h, 24 h, 48 h or 0 h, 12 h, and 24 h by an inverted microscope (IX71; Olympus, Tokyo, Japan).

Transwell invasion and migration assays

Transwell assays were carried out with a polycarbonate filter membrane with a diameter of 6.5 mm and pore size of 8 μ m (Corning, Bedford, MA, USA) according to the manufacturer's protocol. Matrigel (BD, San Jose, CA, USA) was used to precoat the filters to analyze cell invasion. Trypsin-treated cells were resuspended in serum-free medium. A total of 100 μ l of cell suspension containing 3000 cells was added to the upper compartment, and 500 μ l of 3% FBS medium was added to the lower compartment as a chemoattractant. After incubation at 37 °C for 36 h, the chamber was washed twice with PBS to remove the noninvasive cells from the upper surface. Then, the filters were fixed with methanol for 30 min. After washing with PBS twice, crystal violet was added for 30 min. After drying, photographs of five randomly selected fields from each well were taken using an inverted microscope. The same experimental design was used for migration experiments except that the filters were not pretreated with Matrigel.

Orthotopic mouse model

Animal experiments were approved by the ethical committee and met the standards required by the guidelines of Xuzhou Medical University (Xuzhou, China). First, we constructed luciferase-mCherry-U87 cells, and then we used lentiviruses to construct cells that are stably expressed the vector Myc-H3F3A and Myc-H3.3K27M in luciferase-mCherry-U87 cells. After narcotizing all the nude mice, we incised the skin of the mouse head for approximately 1 cm and then drilled a small hole 3 mm deep at 1.8 mm right lateral to bregma. Then, the constructed U87 cells (5×10^5) were diluted in L15 medium and injected into the striatum of 7-week-old male nude mice by a microinjector. Finally, the incision was sutured, and all mice were rewarmed and put into the cage. A luciferase assay was performed to ensure the existence of glioma on day 7 posttransplantation, and the glioma growth rate and location of the tumors were determined using an IVIS kinetic imaging system. The glioma growth rate was assessed using fluorescence imaging analysis.

Statistical analysis

All experiments were performed three times, and data are presented as the mean $\pm$ SD. Data were analyzed with GraphPad Prism 7. One-way ANOVA, two-way ANOVA and Tukey's multiple comparisons

test were used to analyze differences in each three-group comparison. Overall survival curves were generated using the Kaplan-Meier method and compared using the log-rank test. $P < 0.05$ was considered to indicate statistical significance.

Results

The H3.3K27M mutation is present in a portion of human glioma patients

To assess the expression of H3.3K27M in glioma patients, we performed Western blot assays with the total protein from 6 nontumor tissues and 22 glioma tissues by using H3K27M antibody. Interestingly, the H3K27M mutation was found in three glioma patients, all of whom were diagnosed with high-grade glioma (one sample was grade III, and two samples were grade IV) (Fig. 1A). Subsequently, we performed DNA sequencing to confirm the mutation in these three tissues (Fig. 1B). All of them were mutated from the AAG codon at position 27 to the ATG codon. Finally, to confirm whether the mutation was present in the commonly used glioma cell lines, we assessed the H3.3K27M mutation status in six glioma cell lines (U87, U251, LN229, A172, U118 and A172). However, no H3.3K27M mutation was found in these cell lines (Fig. 1C and 1D).

H3.3K27M has no obvious effect on glioma cell proliferation but promotes glioma cell migration and invasion in vivo

Due to the lack of H3.3K27M in these cell lines, we introduced the mutation by constructing cell lines stably expressing H3.3K27M. At the same time, we established a cell line overexpressing wild-type H3.3 as a negative control and a cell line overexpressing empty vector as a blank control. The successful generation of these cell lines was confirmed by Western blotting assay (Fig. 2A). After constructing the cell lines, we focused on exploring the role of H3.3K27M in tumorigenesis. Colony formation and CCK-8 assays were used to determine the role of H3.3K27M in glioma cell proliferation. The expression of H3.3K27M had no effect on the proliferative ability of glioma cells U87 and LN229 (Fig. 2B, 2C and 2D). Further detection at the molecular level indicated that overexpression of H3.3K27M did not change the expression of three classical proliferation pathway-related kinases, including the AKT, MEK1/2, and STAT3 pathways (Fig. 2E). Finally, we established a tumor model to confirm the above results by transplanting the above three cell lines expressing luciferase into the right striatum of nude mice. Tumor size was measured by luciferase assay every seven days. The overexpression of H3.3K27M had no obvious effect on tumor growth (Fig. 3A and 3B). However, H3.3K27M expression significantly reduced the survival rate of mice (Fig. 3C). Moreover, by hematoxylin and eosin (HE) staining of mouse brain slices, we found that a large number of invasive tumor cells infiltrated into the surrounding tissues from primary tumors in the H3.3K27M group (Fig. 3D).

H3.3K27M promotes glioma cell migration and invasion in vitro

Based on the above results, we hypothesized that H3.3K27M mainly affects glioma cell motility. Thus, we explored its roles in glioma cell migration and invasion. The wound healing assay showed that the migration ability of U87 and LN229 cells stably expressing H3.3K27M was obviously enhanced compared with that of the control or WT group (Fig. 4A and 4B). The Transwell migration assay (without Matrigel) obtained similar results as the wound healing assay (Fig. 4C). Additionally, the Transwell invasion (with Matrigel) assay showed that the H3.3K27M group had a much stronger invasion ability than the control or WT group (Fig. 4D).

H3.3K27M positively regulates the β -catenin/USP1 signaling pathway

In 2019, researchers reported that EZH2 participates in regulating the TGF- β pathway via a novel pathway axis that could potentially be relevant in regulating the metastasis and aggressiveness of GBM[15]. Recent studies also showed that EZH2 is a potential therapeutic target for H3K27M-mutant pediatric gliomas[14]. We thus hypothesized that EZH2 expression is involved in H3K27M mutation-induced glioma cell migration and invasion. We therefore detected the protein expression of EZH2 by Western blotting. As expected, the EZH2 protein was significantly upregulated upon overexpression of the H3.3K27M mutant (Fig. 5A). Recent studies have proven that the EZH2 protein is mainly regulated by the ubiquitin-proteasome-dependent degradation pathway, which is directly stabilized by ubiquitin-specific protease 1 (USP1). Additionally, β -catenin is the main transcription factor that promotes the transcription of USP1[16]. Based on this knowledge, we assessed whether EZH2 upregulation is attributed to the β -catenin-USP1 axis. Then, we further detected the protein and mRNA levels of USP1. Western blotting and qRT-PCR results showed that overexpression of H3.3K27M upregulated USP1 levels at both the mRNA and protein levels in U87 and LN229 cell lines (Fig. 5B and 5C). Furthermore, USP1 upstream transcription factor β -catenin was found to be more significantly activated, which was reflected in the significant increase in the levels of both total β -catenin and phosphorylated β -catenin upon H3.3K27M overexpression. More importantly, to confirm that H3.3K27M-induced EZH2 upregulation was dependent on the β -catenin/USP1 pathway, the β -catenin inhibitor XAV-939 was used to perform a rescue experiment. Overexpression of H3.3K27M in U87 and LN229 cells significantly upregulated the levels of USP1 and EZH2, which could be significantly blocked by the β -catenin inhibitor XAV-939 (Fig. 5E and 5F). Taken together, these results indicate that H3.3K27M plays an important role in promoting glioma cell migration and invasion by activating the β -catenin/USP1/EZH2 signaling pathway.

Discussion

In our study, we assessed the expression of H3K27M in 28 human glioma tissues. We found that three high-grade glioma patients had H3K27M mutations. To confirm the existence of H3.3K27M in these three samples, we performed DNA sequencing and found that all of them mutated from AAG codon at position

27 to ATG codon. Then, we constructed stable cell lines expressing H3.3K27M from the H3F3A wild-type glioma cell lines U87 and LN229. In the animal experiment, we found that H3.3K27M had no obvious effect on the growth of glioma but promoted the migration and invasion of glioma cells. In parallel, H3.3K27M had no effect on classical kinase-related proliferation pathways, including the AKT, MEK1/2, and STAT3 pathways. This interesting phenomenon motivated us to investigate the role of H3.3K27M in the migration and invasion of cell lines *in vitro*. Wound healing and Transwell assays demonstrated that H3.3K27M could increase the migration and invasion of U87 and LN229 cells. Finally, we focused on studying the mechanism by which H3.3K27M promotes migration and invasion. We discovered that H3.3K27M significantly activated the β -catenin/USP1/EZH2 pathway in U87 and LN229 cells. Our study reveals the molecular mechanism by which the H3.3K27M mutation promotes the malignancy of mutated glioma and provides some direction for research on targeted drugs.

In 2015, it was pointed out that although H3K27M mutations are frequently observed in brainstem and thalamic gliomas, such mutations often lead to a poor prognosis for brainstem gliomas[17]. However, Karremann et al showed that anatomical midline location, histopathological grade and tumor resection range had no effect on survival in H3K27M-mutant glioma[11]. Although anatomic location is controversial for patient outcomes, the poor prognosis of patients with H3K27M mutated glioma and the lack of treatment are issues that need to be addressed. Hence, the specific molecular mechanism of H3.3K27M mutation-driven glioma has not been elucidated and should be further studied. The H3.3K27M mutant exists in children's and adults' glioma and may lead to a poor prognosis, so we wanted to investigate whether H3.3K27M has an effect on the malignant behaviors of glioma. We found that the expression of H3.3K27M is related to the migration and invasion of glioma and may explain the poor prognosis of H3.3K27M glioma. The finding of an active pathway may provide some inspiration for precision treatment of H3.3K27M-mutated glioma.

EZH2 is the methyltransferase component of PRC2. H3.3K27M histones bind to the SET domain of EZH2, causing a global reduction in H3K27 dimethylation and trimethylation (H3K27me_{2/3})[18]. However, researchers reported that the H3.3K27M mutant increased H3K27 methylation at unique loci in SF7761 cells due to the recruitment of EZH2. Their genome-wide sequencing data also suggest that the loss and gain of H3K27me₃ by the H3.3K27M mutation may drive tumor formation in pediatric DIPG[19]. Li et al uncovered a SOX4-dependent epithelial-mesenchymal transition (EMT)-inducing mechanism underlying MTA1-driven cancer metastasis and suggested a widespread TGF- β -MTA1-SOX4-EZH2 signaling axis that drives EMT in various cancers[20]. Chen et al identified a novel metastasis-promoting lncRNA, MRPL23-AS1, which mediates the transcriptional silencing of E-cadherin by forming an RNA-protein complex with EZH2[21]. EZH2 is also closely associated with the EMT of other tumors, such as pancreatic cancer, head and neck squamous cell carcinoma and esophageal cancer[22-24]. It has been reported that the EZH2 inhibitors MC4040 and MC404 reverse EMT and hamper cell migration and invasion, attenuating the glioma malignant phenotype[25]. Studies have also reported that EZH2 is involved in a novel miR-490-3p/TGIF2/TGFBR1 axis inducing migration and EMT in glioblastomas[15]. EZH2 is also connected with glioma proliferation and metastasis and has been regarded as a potential predictor and therapeutic target in glioma[26,27]. In general, EZH2 could be a marker associated with EMT in glioma. Our study revealed

that EZH2 is closely related to the migration and invasion of glioma and supports that EZH2 plays an important role in the EMT of glioma.

Researchers have put much effort into studying the mechanism of H3.3K27M glioma due to the poor prognosis of H3.3K27M-mutant glioma in children, and they have made some advances in clarifying the molecular mechanism of DIPG. Mechanistically, research has revealed that the JNK pathway, NOTCH pathway, RAS/MYC axis and Rb/E2F1 pathway participate in the development of H3.3K27M-positive gliomas[28-31]. In our research, we wanted to explore the mechanism of enhanced invasion and migration. As previously mentioned, EZH2 participates in the migration and invasion of glioma. Thus, we detected the expression of EZH2 after overexpressing H3.3K27M in U87 and LN229 cells. As a result, the expression of EZH2 increased with H3.3K27M overexpression. It has been reported that abnormal ubiquitination and degradation are the main reasons for EZH2 protein accumulation, which is primarily regulated by the deubiquitinase USP1[16]. We therefore focused on exploring the expression of USP1 and its upstream pathway. Our results showed that both the protein and mRNA levels of USP1 increased upon H3.3K27M overexpression. It is known that β -catenin acts as both a transcriptional coregulator and an adaptor protein for intracellular adhesion[32]. In the mechanism of glioma occurrence, β -catenin/TCF4 activates the transcription of the deubiquitinase USP1, which then directly interacts with and deubiquitinates EZH2. USP1-mediated stabilization of EZH2 promotes the occurrence and development of glioma[16]. Furthermore, we demonstrated that the upregulation of USP1 is attributed to the activation of β -catenin, a classical signal transduction protein related to malignant progression of glioma, and the corresponding signaling pathway. These results indicate that H3.3K27M promotes glioma invasion and migration through the β -catenin/USP1 signaling pathway. Nevertheless, β -catenin/USP1/EZH2 has no obvious effect on the proliferation of glioma. As reported, the age of the patient and the specific location of the H3K27M tumor lead to different biological behaviors[33]. H3.3K27M-mediated gliomagenesis was found to be dependent on PDGF signaling in a genetic mouse model[34]. Thus, we speculate that H3.3K27M has no direct effect on the proliferation of glioma, which might be due to the lack of special genetic mutations. On the basis of the above evidence, H3.3K27M promotes glioma malignant behavior by activating the β -catenin/USP1/EZH2 pathway.

Conclusions

In conclusion, we provided the first evidence that H3.3K27M is linked with the β -catenin/USP1 pathway. This signaling pathway is also involved in human glioma malignant activity. Our research results may provide some basis for studying the function of H3.3K27M in adult glioma. The β -catenin/USP1 pathway may be a potential target for H3.3K27M glioma. However, we should conduct more exhaustive studies to explore the detailed mechanisms of H3.3K27M in future projects, such as whether H3.3K27M directly binds to β -catenin to activate this pathway.

Declarations

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Authors' contributions

Study conception or design: Zhiyuan Sun. Acquisition, analysis, or interpretation of the data: Yufu Zhu, Xia Feng, Xiaoyun Liu, Kunlin Zhou. Writing and revision of the manuscript: Zhiyuan Sun, Hengliang Shi. All authors read and approved the final manuscript.

Data availability

All datasets supporting the conclusions contained in the present report are included in the manuscript.

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Ethical approval

All animal experimental protocols were approved by the Ethics Committee of Xuzhou Medical University.

Consent to Participate

Glioma patients signed an internal regulatory document, stating that residual samples used for diagnostic procedures can be used for retrospective academic studies, without any additional informed consent.

Consent for Publication

Not applicable.

Conflict of interest

The authors declare that they have no competing interests.

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Figures

Figure 1

H3K27M expression in glioma patients and glioma cell lines. (A) Analysis of total proteins isolated from cancer tissue specimens of 28 patients using Western blotting to assess H3K27M protein levels. (B) Gene sequence showing H3.3K27M mutation in three samples. (C) Six glioma cell lines were used to assess H3K27M protein levels using Western blotting. (D) H3.3K27M mutation in six glioma cell lines according to gene sequence analysis.

Figure 2

H3.3K27M has no obvious effect on the proliferation of glioma *in vitro*. (A) Overexpression efficiency in U87 and LN229 cells was verified by Western blotting. Representative images (B) and statistical analysis

(C) of colony formation showed no discernible difference among the vector group, Myc-H3F3A group and Myc-H3.3K27M group in U87 and LN229 cells. (D) The CCK-8 assay displayed no significant effect on the cell abilities of the three groups of U87 and LN229 cells. (E) Representative blots of AKT, P-AKT, MEK1/2, P-MEK1/2, STAT3 and P-STAT3 levels in the three groups of U87 and LN229 cells. ns means no statistical significance.

Figure 3

H3.3K27M overexpression has no effect on the proliferation of glioma cells but enhances their invasion and migration *in vivo*. Bioluminescent imaging (A) and quantification analysis (B) of nude mice showed that H3.3K27M had no distinct effect on the growth rate of tumors compared with the vector group and Myc-H3F3A group. (C) Kaplan-Meier survival curve of nude mice injected orthotopically. H3.3K27M impaired the survival ability of nude mice. (D) Representative images of HE staining in brain tissue. The Myc-H3.3K27M group showed stronger invasion ability than the other two groups. The red circle indicates the invasion site. * $P < 0.05$, ns means no statistical significance.

Figure 4

H3.3K27M overexpression promotes the invasion and migration of glioma *in vitro*. Representative images (A) and quantification (B) of wound healing results in U87 and LN229 cells. * $P < 0.05$. Representative images (C) and statistical graph (D) of Transwell migration and invasion results in U87 and LN229 cells. * $P < 0.05$, ns means no statistical significance.

Figure 5

H3.3K27M regulates the β -catenin/USP1/EZH2 signaling pathway. (A) Representative blots and statistical graph of H3.3K27M-induced increased EZH2 activity in U87 and LN229 cells. (B) Representative blots and histograms showing quantitative analysis of increased USP1 protein levels induced by H3.3K27M in U87 and LN229 cells. (C) Quantification of USP1 mRNA levels in U87 and LN229 cells after stable overexpression. (D) Representative blots and statistical graph of β -catenin and P- β -catenin in U87 and

LN229 cells. Representative blots (E) and histograms (F) of β -catenin, P- β -catenin, USP1 and EZH2 after XAV-939 addition in U87 and LN229 cells. XAV-939 blocks the β -catenin/USP1/EZH2 signaling pathway induced by H3.3K27M. The concentration of XAV-939 was 20 μ M for 48 h. * P < 0.05.