

IDH1 gene mutation activates Smad signaling molecules to regulate the expression levels of cell cycle and biological rhythm genes in human glioma U87-MG cells

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Abstract. Isocitrate dehydrogenase 1 (IDH1) mutation is the most important genetic change in glioma. The most common IDH1 mutation results in the amino acid substitution of arginine 132 (Arg/R132), which is located at the active site of the enzyme. IDH1 Arg132His (R132H) mutation can reduce the proliferative rate of glioma cells. Numerous diseases follow circadian rhythms, and there is growing evidence that circadian disruption may be a risk factor for cancer in humans. Dysregulation of the circadian clock serves an important role in the development of malignant tumors, including glioma. Brain-Muscle Arnt-Like protein 1 (BMAL1) and Circadian Locomotor Output Cycles Kaput (CLOCK) are the main biological rhythm genes. The present study aimed to further study whether there is an association between IDH1 R132H mutation and biological rhythm in glioma, and whether this affects the occurrence of glioma. The Cancer Genome Atlas (TCGA) database was used to detect the expression levels of the biological rhythm genes BMAL1 and CLOCK in various types of tumor. Additionally, U87-MG cells were infected with wild-type and mutant IDH1 lentiviruses. Colony formation experiments were used to detect cell proliferation in each group, cell cycle distribution was detected by flow cytometry

and western blotting was used to detect the expression levels of wild-type and mutant IDH1, cyclins, biological rhythm genes and Smad signaling pathway-associated genes in U87-MG cells. TCGA database results suggested that BMAL1 and CLOCK were abnormally expressed in glioma. Cells were successfully infected with wild-type and mutant IDH1 lentiviruses. Colony formation assay revealed decreased cell proliferation in the IDH1 R132H mutant group. The cell cycle distribution detected by flow cytometry indicated that IDH1 gene mutation increased the G₁ phase ratio and decreased the S phase ratio in U87-MG cells. The western blotting results demonstrated that IDH1 R132H mutation decreased the expression levels of the S phase-associated proteins Cyclin A and CDK2, and increased the expression levels of the G₁ phase-associated proteins Cyclin D3 and CDK4, but did not significantly change the expression levels of the G₂/M phase-associated protein Cyclin B1. The expression levels of the positive and negative rhythm regulation genes BMAL1, CLOCK, period (PER) 1, 2 and 3 and cryptochrom (CRY) 1 and 2 were significantly decreased, those of the Smad signaling pathway-associated genes Smad2, Smad3 and Smad2-3 were decreased, and those of phosphorylated (p)-Smad2, p-Smad3 and Smad4 were increased. Therefore, the present results suggested that the IDH1 R132H mutation may alter the cell cycle and biological rhythm genes in U87-MG cells through the TGF- β /Smad signaling pathway.

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Introduction

Isocitrate dehydrogenase (IDH) genes are mutated in multiple types of tumor, including glioma, chondrogenic tumors, leukemia and other bone marrow proliferative tumors. In glioma and leukemia, IDH1 and IDH2 mutations occur in >70% of low-grade tumors (level II and III) (1,2). IDH mutation is the most important genetic change in glioma. The mutation is located at the isocitric acid binding site (Arg/R132 of IDH1 and R172 of IDH2) of a single amino acid (3,4). IDH1/2 mutations are common in World Health Organization (WHO)

grade II and III gliomas and secondary glioblastoma (GBM; 70-80%), while primary GBM (WHO grade IV) IDH1/2 mutations are rare (<5%) (5,6). IDH1 mutations account for >90% of glioma IDH1/2 mutations (7). IDH1 Arg132His (R132H) mutation can affect the proliferation of glioma cells, which is slower than the corresponding wild-type IDH1 cells (8,9). Clinical studies (10,11) have shown that mutations in IDH1 were found to be associated with younger age, secondary GBMs (grade IV tumors that arise from biopsy-proven lower-grade predecessors), and increased overall survival (OS) (12). Further studies (13,14) have revealed that IDH1/2 mutations as good prognostic markers are universally present in grade II and III glioma and secondary glioblastoma, and serve an important role in the occurrence, development and evolution of glioma (15). Therefore, studying the role of the IDH1 R132H mutation in the occurrence of glioma may provide new ideas for clinical treatment.

There is growing evidence that dysregulation of the circadian clock serves an important role in the development of malignant tumors, including glioma (16,17). Circadian timing is a basic biological process that affects most aspects of eukaryotic and prokaryotic physiology. Circadian dysrhythmia may lead to an increased risk of cancer, as well as affect the response of patients with cancer to treatment (18). In a circadian rhythm, the oscillator is coordinated by a set of interlocked transcription-translation feedback loops. Brain-Muscle Arnt-Like protein 1 (BMAL1) and Circadian Locomotor Output Cycles Kaput (CLOCK) are the main biological rhythm genes (19). CLOCK and BMAL1 heterogeneous dimers combined with Period (PER) and Cryptochrom (CRY) proteins in E-box device drive rhythmic transcription (20-22). The PER and CRY proteins inhibit CLOCK-BMAL1 complexes, which inhibit PER and CRY protein degradation after release (23-25). Upon epigenetic modification and increase post-translational modifications, the core CLOCK proteins in the suprachiasmatic nucleus can maintain peripheral CLOCK oscillation and rhythmic expression (26), and the downstream targets are similar to those in the suprachiasmatic nucleus; the molecular biological clock of the peripheral tissues and organs of the body is also composed of a transcription-translation feedback loop regulated by clock genes (27-32). Disruption of circadian rhythms may negatively affect normal cellular function and may lead to an increased incidence of multiple types of cancer, such as colorectal cancer (33), breast cancer (34), prostate cancer (35), pancreatic cancer (36), osteosarcoma (37) and others (38,39). Therefore, it is very important to study the role of the circadian clock in the development and progression of cancer. Mutations in circadian clock components can increase the proliferation rate of cells through the general dysregulation of the cell cycle, thereby causing the cell to become cancerous (40). Several studies have revealed an interaction between the biological clock and the cell cycle. For example, a previous study has indicated that light-induced phase shifts in mouse behavior lead to corresponding changes in the time of intestinal cell proliferation (41). Another study has suggested that clock genes are involved in the regulation of important cell cycle checkpoints (42).

Understanding the mechanism of circadian clock changes in tumors is of great importance for the improvement of tumor therapy. However, to the best of our knowledge, the mechanism of biorhythm change in glioma with IDH1 R132H mutation has not been previously reported. In the present study, the

IDH1 R132H mutant gene was introduced into human glioma U87-MG cells to observe the effect of IDH1 on biological rhythm genes and to analyze its relevant mechanism, laying a theoretical foundation for the study of the effect of biological rhythm on the biological function of malignant tumors.

Materials and methods

Cell line and culture. The full name of the cell line used is U87-MG human brain astroblastoma, which is a GBM of unknown origin. Cells were cultured in Minimum Essential Medium (MEM; Shanghai Zhongqiao Xinzhou Biotechnology Co., Ltd.) containing 10% fetal bovine serum (FBS; HyClone; Cytiva) at 37°C with 5% CO₂. The cell line was purchased from Shanghai Zhongqiao Xinzhou Biotechnology Co., Ltd.

Reagents and instruments. pLVX-IDH1-mCMV-ZsGreen-PGK-Puro and pLVX-IDH1(MUT)-mCMV-ZsGreen-PGK-Puro lentiviruses were purchased from Beijing Xibei Hongcheng Biotechnology Co., Ltd. (<http://www.xbhcbio.com/>). Trypsin (0.25%)-EDTA was purchased from Invitrogen (Thermo Fisher Scientific, Inc.) and 0.45- μ m PVDF membranes and a chemiluminescence kit (Immobilon Western Chemiluminescent HRP Substrate, eCl@ss cat. no. 42029053) were purchased from EMD Millipore. A total protein extraction kit (Whole protein extraction kit, cat. no. KGP250), a BCA assay protein content detection kit (BCA Protein Quantitation Assay, cat. no. KGPBCA), an SDS-PAGE gel preparation kit (KGI SDS-PAGE Gel Preparation kit, cat. no. KGP113) and a flow cytometry cell cycle analysis kit (Cell Cycle Detection kit, cat. no. KGA511) were purchased from Jiangsu Kaiji Biotechnology Co., Ltd. (<http://keygentec.com.cn/index.php?cid=1>). The BSA (BSA-V, cat. no. A8020) used to dilute the antibody was purchased from Beijing Solarbio Science & Technology Co., Ltd. Mouse anti-IDH1 (R132H; cat. no. SAB4200548) was purchased from Sigma-Aldrich (Merck KGaA). Rabbit anti-IDH1 (cat. no. 8137S), rabbit anti-BMAL1 (cat. no. 14020S), rabbit anti-CLOCK (cat. no. 5157S), rabbit anti-phosphorylated (p)-Smad2 (cat. no. 18338S), rabbit anti-p-Smad3 (cat. no. 9520S), rabbit anti-Smad2 (cat. no. 5339S), rabbit anti-Smad2-3 (cat. no. 86855S), rabbit anti-Smad3 (cat. no. 9513S), rabbit anti-Smad4 (cat. no. 46535S), mouse anti- β -actin (cat. no. 3700S) and mouse anti-GAPDH (cat. no. 51332S) primary antibodies, as well as HRP-labelled goat anti-rabbit IgG (cat. no. 7074S) and goat anti-mouse IgG (cat. no. 7076S) secondary antibodies, were purchased from Cell Signaling Technology, Inc. Rabbit anti-Cyclin A (cat. no. AF0142), rabbit anti-CyclinB1 (cat. no. DF6786), rabbit anti-CyclinD3 (cat. no. DF6229), rabbit anti-CDK2 (cat. no. AF6237), rabbit anti-CDK4 (cat. no. DF6102), rabbit anti-P18 (cat. no. AF0620), rabbit anti-P21 (cat. no. AF6290), rabbit anti-P27 (cat. no. AF6324), rabbit anti-PER1 (cat. no. DF9080), rabbit anti-PER2 (cat. no. DF12304), rabbit anti-PER3 (cat. no. DF7349), rabbit anti-Cry1 (cat. no. DF8932) and rabbit anti-Cry2 (cat. no. DF12919) primary antibodies were purchased from Affinity Biosciences. An IX73 inverted fluorescence microscope was purchased from Olympus Corporation, and an Amersham Imager 600 gel imaging analysis system was purchased from General Electric.

The cancer genome atlas (TCGA) database analysis. BMAL1 and CLOCK expression data of all types of tumor analyzed in the present study were derived from TCGA database (<https://portal.gdc.cancer.gov/>) and the online analysis software GEPIA2 (<http://gepia2.cancer-pku.cn/#index>).

U87-MG human glioma cell culture and lentiviral infection. Construction of recombinant lentiviral vector: 1 μ g Plasmid and pLVX/IDH1, 1 μ l EcoRI, 1 μ l BamHI, 2 μ l pLVX, 6 μ l target gene, 1 μ l 10X DNA Ligase Buffer and 1 μ l T4 DNA Ligase, were used to construct lentiviral vectors. The 239T cell line (Beijing Xibei Hongcheng Biotechnology Co., Ltd.) was used for virus purification. After culturing for 12 to 16 h, the medium was discarded (500 μ l HET Buffer A + 10 μ l recombinant lentiviral vector + 15 μ l lentiviral packaging vector + HET Buffer B 50 μ l + ddH₂O 425 μ l), and replaced with 10 ml fresh complete medium solution (DMEM + 10% FBS + P/S). The cells were placed in a 37°C, 5% CO₂ incubator for 48 h, the supernatant was collected after centrifugation at 500 x g and room temperature for 5 min and the cell debris was discarded. The supernatant was filtered with a 45 μ m PVDF filter into a 50 ml round bottom centrifuge tube. This was centrifugated at a high speed of 6,000 x g at 4°C for 2 h, and purified recombinant lentivirus was obtained after centrifugation. Lentivirus can be introduced into U87-MG cells after 48 h of infection with MOI value (20:1) (43). U87-MG cells were inoculated in MEM containing 10% FBS at a density of 2x10⁵ cells/100-mm culture dish, incubated at 37°C and then subjected to lentivirus infection 6-8 h after becoming adherent. The titre of the treatment and control lentiviruses was set to an MOI of 20:1, and MEM with 2% FBS was used to prepare viral titre gradient solutions for subsequent experiments. The experimental groups were the IDH1 wild-type (WT) and the IDH1 R132H mutant (MUT) groups, and the corresponding lentiviral vectors were pLVX-IDH1-mCMV-ZsGreen-PGK-Puro and pLVX-IDH1(MUT)-mCMV-ZsGreen-PGK-Puro, respectively. Subsequent experiments were performed 72 h after lentiviral infection.

Western blot analysis. Following lentiviral transfection, U87-MG cells in each group were collected for extraction of total protein using the aforementioned whole protein extraction kit. The BCA assay kit was used to quantify the protein, and equal amounts of protein (40 μ g/10 μ l) were separated via 8% SDS-PAGE. Subsequently, the proteins were transferred to PVDF membranes, which were blocked at room temperature in 5% skimmed milk for 1 h and then incubated at 4°C overnight with the following primary antibodies diluted with 3% BSA: Anti-IDH1 (R132H; 1:1,000), anti-IDH1 (1:1,000), anti-GAPDH (1:2,000), anti- β -actin (1:2,000), anti-BMAL1 (1:1,000), anti-CLOCK (1:1,000), anti-PER1/2/3 (1:1,000), anti-CRY1/2 (1:1,000), anti-CyclinA (1:1,000), anti-CyclinB1 (1:1,000), anti-Cyclin D3 (1:1,000), anti-CDK2 (1:1,000), anti-CDK4 (1:1,000), anti-P18 (1:1,000), anti-P21 (1:1,000), anti-P27 (1:1,000), anti-p-Smad2 (1:1,000), anti-p-Smad3 (1:1,000), anti-Smad2 (1:1,000), anti-Smad2-3 (1:1,000), anti-Smad3 (1:1,000) and anti-Smad4 (1:1,000). The membranes were incubated with secondary antibodies (HRP-conjugated goat anti-rabbit IgG and goat anti-rat IgG; both 1:2,000) at room temperature for 1 h. The chemiluminescence reagent was used for color development for 1 min, and the membranes were exposed for greyscale

measurement. The protein expression level was semi-quantified using Image Pro Plus 6.0 (Media Cybernetics, Inc.).

Colony formation experiments. After seeding 1,000 lentivirus-infected U87-MG cells into 100-mm culture dishes, the cells were divided into the WT and MUT groups. After 5 days of culture, the medium was changed once, and whether the cells formed clumps or sheets was observed under a 10x light microscope. The medium was then discarded. The cells were fixed in 4% paraformaldehyde at room temperature for 20 min. Methylene violet solution (4 ml) was added to each dish, and the cells were stained at room temperature for 30 min before rinsing with pure water. After drying at room temperature, images were captured, and the colonies, which are clumps or flakes formed by cells, were counted using Image-Pro Plus 6.0 (Media Cybernetics, Inc.) for statistical analysis.

Cell cycle distribution detection by flow cytometry. A cell cycle analysis kit (Cell Cycle Detection kit, cat. no. KGA511) was used for cell cycle detection. The cells in each group were digested with trypsin at 37°C for 1 min, collected by centrifugation at 1,500 x g for 5 min at normal temperature and rinsed with PBS to form a cell suspension. The concentration was adjusted to 1x10⁶ cells/ml. After removing the supernatant by centrifugation at 1,500 x g for 5 min at normal temperature, 500 μ l precooled 75% ethanol was used to fix the cells overnight at 4°C. The fixation solution was removed by centrifugation at 1,500 x g for 5 min at 4°C, 100 μ l RNase A was added and the cells were incubated in a water bath at 37°C for 30 min. A 400 μ m mesh screen was used for filtration. Then, 400 μ l PI dye was added and the solution was gently mixed and incubated at 4°C for 1 h in the dark. Analysis was performed using a flow cytometer (FACSCalibur, BD Biosciences; FlowJo, Version 10, FlowJo LLC).

Statistical analysis. SPSS 21.0 (IBM Corp.) statistical software was used for statistical analysis. The data are expressed as the mean \pm SD. Each independent experiment was repeated three times. Independent sample t-test was used to analyze differences between two groups of continuous data. One-way ANOVA was used for the comparison among multiple groups followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Biological rhythm genes are abnormally expressed in glioma. BMAL1 and CLOCK expression was analyzed in TCGA database. The expression levels of BMAL1 and CLOCK appeared to differ among the 33 types of tumor. BMAL1 expression in acute myeloid leukemia was higher than that in normal tissues (Fig. 1A). By contrast, BMAL1 expression was lower in diffuse large adrenocortical carcinoma (ACC), cervical squamous cell carcinoma, endocervical adenocarcinoma (CESC), colon adenocarcinoma (COAD), lung adenocarcinoma, lung squamous cell carcinoma, ovarian serous cystadenocarcinoma, prostate adenocarcinoma, rectum adenocarcinoma, skin cutaneous melanoma, testicular germ cell tumors (TGCT), uterine corpus endometrial carcinoma and uterine carcinosarcoma compared with that in normal tissues (Fig. 1A). Additionally, BMAL1 expression in brain lower grade glioma (LGG) tissues was lower than that in

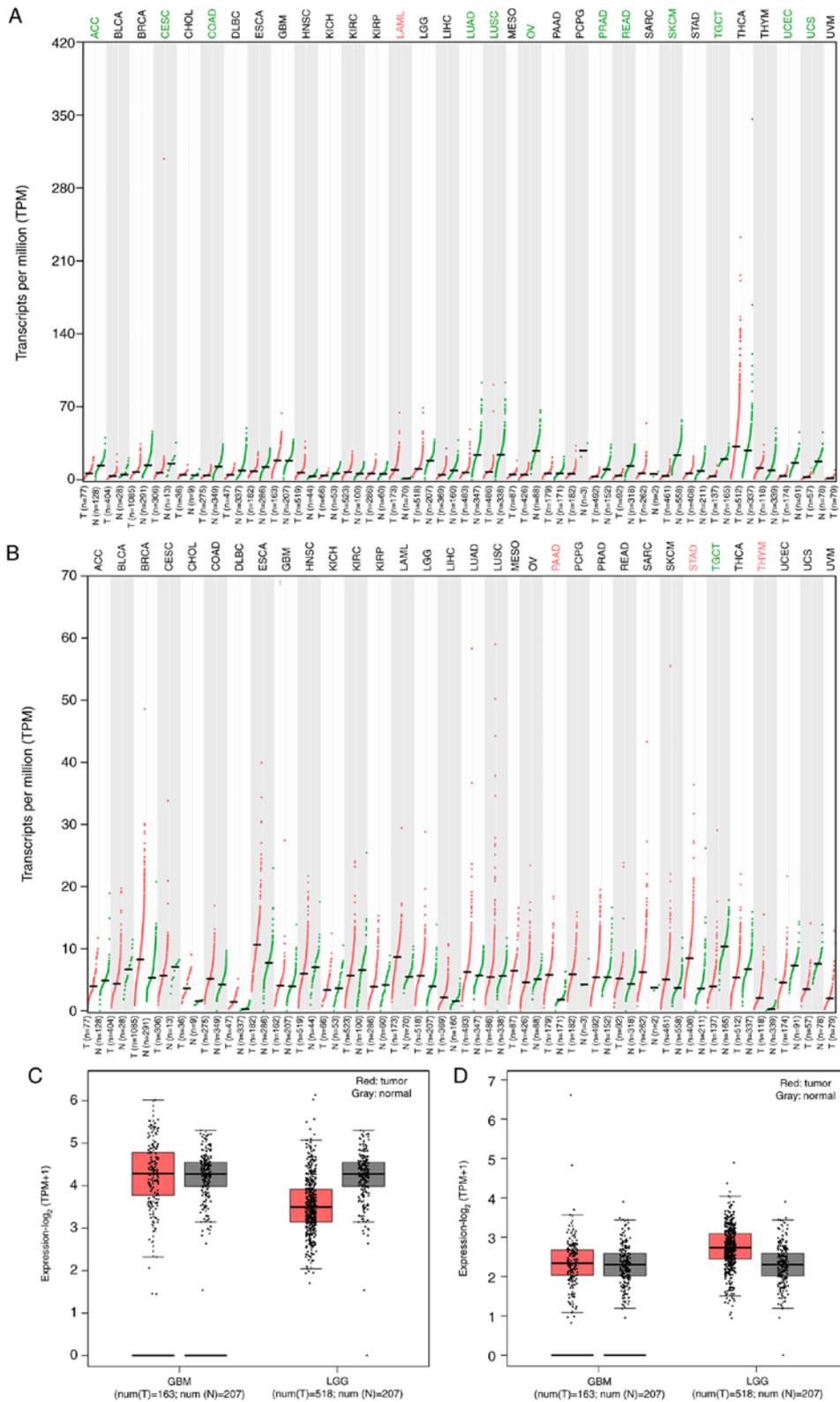


Figure 1. Expression of BMAL1 and CLOCK in different tumors in TCGA database. (A) BMAL1 and (B) CLOCK expression in 33 types of tumor from The Cancer Genome Atlas database. Red represents tumor samples, while green represents normal tissue samples. (C) BMAL1 and (D) CLOCK expression in GBM and LGG tumor and normal tissues. The red box represents tumor samples, while the gray box represents normal tissue samples. T, tumor; N, normal; TPM, transcripts per million; BMAL1, Brain-Muscle Arnt-Like protein 1; CLOCK, Circadian Locomotor Output Cycles Kaput; ACC, Adrenocortical carcinoma; BLCA, Bladder Urothelial Carcinoma; BRCA, Breast invasive carcinoma; CESC, Cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, Cholangiocarcinoma; COAD, Colon adenocarcinoma; DLBC, Lymphoid Neoplasm Diffuse Large B-cell Lymphoma; ESCA, Esophageal carcinoma; GBM, Glioblastoma multiforme; HNSC, Head and Neck squamous cell carcinoma; KICH, Kidney Chromophobe; KIRC, Kidney renal clear cell carcinoma; KIRP, Kidney renal papillary cell carcinoma; LAML, Acute Myeloid Leukemia; LGG, Brain Lower Grade Glioma; LIHC, Liver hepatocellular carcinoma; LUAD, Lung adenocarcinoma; LUSC, Lung squamous cell carcinoma; MESO, Mesothelioma; OV, Ovarian serous cystadenocarcinoma; PAAD, Pancreatic adenocarcinoma; PCPG, Pheochromocytoma and Paraganglioma; PRAD, Prostate adenocarcinoma; READ, Rectum adenocarcinoma; SARC, Sarcoma; SKCM, Skin Cutaneous Melanoma; STAD, Stomach adenocarcinoma; TGCT, Testicular Germ Cell Tumors; THCA, Thyroid carcinoma; THYM, Thymoma; UCEC, Uterine Corpus Endometrial Carcinoma; UCS, Uterine Carcinosarcoma; UVM, Uveal Melanoma.

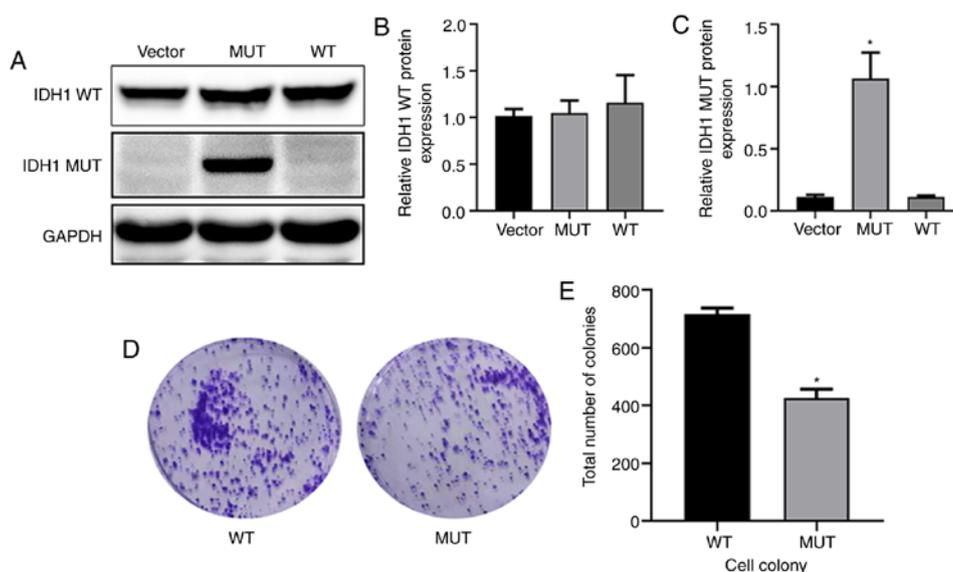


Figure 2. Establishment of a cell model transfected with IDH1 lentivirus, and detection of the IDH1 R132H mutation affects the proliferation of glioma cells. (A) Detection of lentiviral transfection in U87-MG cells. The protein expression levels of IDH1 WT and MUT in each group of U87-MG cells was detected by western blot analysis. (B) Relative IDH1 WT protein expression. (C) Relative IDH1 MUT protein expression. (D and E) Colony formation experiment was used to detect the number of colonies in the two groups. * $P < 0.05$ vs. WT. MUT, mutant; WT, wild-type; IDH1, isocitrate dehydrogenase 1.

normal tissues (Fig. 1C). The expression of BMAL1 in GBM tissue was not significantly different from that in normal tissue (Fig. 1C). CLOCK expression in TGCT tissues was lower than that in normal tissues (Fig. 1B). By contrast, CLOCK expression was higher in diffuse large pancreatic adenocarcinoma (PAAD), stomach adenocarcinoma (STAD) and thymoma (THYM) tissues compared with that in normal tissues (Fig. 1B). Additionally, CLOCK expression in brain LGG was higher than that in normal tissues (Fig. 1D). The expression of CLOCK in GBM tissues was slightly higher compared with that in normal tissues (Fig. 1D). However, how the rhythm genes are expressed in IDH1 R132H mutated glioma is unclear. Therefore, further experiments were performed to investigate this.

Introduction and expression analysis of WT and MUT IDH1 genes in U87-MG cells. U87-MG cells were transfected with lentivirus. Western blot analysis (Fig. 2A) indicated that after 72 h of lentiviral infection, IDH1 WT protein levels in WT U87-MG cells were slightly higher than those in the MUT U87-MG cells (Fig. 2B). The IDH1 MUT protein was highly expressed in the MUT group (Fig. 2C). The effect of IDH1 gene mutation on U87-MG cell proliferation was detected through colony formation experiments, and the results indicated that significantly fewer new colonies were formed in the MUT group than in the WT group (Fig. 2D and E). The current results indicated that a cell model of IDH1 lentivirus transfection was successfully established and that IDH1 R132H mutation influenced the colony formation of glioma cells.

Effects of IDH1 gene mutation on the cell cycle of U87-MG cells. Cell cycle distribution was detected by flow cytometry. In the WT group, 34.41% of cells were in S phase, while in the MUT group, 19.39% of cells were in S phase (Fig. 3A-C and G). The proportion of cells in S phase in the MUT group was markedly lower than those in the WT and Vector groups (Fig. 3A-C and G). The proportion of cells in

G₁ phase in the MUT group was 72.83%, which was higher than that in the WT group (57.83%); additionally, there was no marked difference in the proportion of cells in G₂ phase among the groups (Fig. 3A-C and G), suggesting that the IDH1 R132H mutation may inhibit cells from entering S phase. To further describe the changes in the cell cycle, the expression levels of S phase-associated proteins (Cyclin A and CDK2), G₂/M phase-associated protein (Cyclin B1), G₁ phase-associated proteins (Cyclin D3 and CDK4), inhibition of early G₁ phase P18 protein via inhibiting Cyclin D-CDK4/6 (44,45) and inhibition of late G₁ phase P27 and P21 proteins via Cyclin E-CDK2 (46) were analyzed by western blot analysis (Fig. 3D). The results revealed that the protein expression levels of Cyclin A and CDK2 were significantly lower in the MUT group than in the WT and vector groups (Fig. 3E and F). There were no significant differences in Cyclin B1 expression among the groups (Fig. 3H). The expression levels of Cyclin D3 and CDK4 were significantly higher in the MUT group (Fig. 3I and J). The protein expression levels of P18 protein in the MUT group were significantly lower than in the WT group, and the expression levels of P27 and P21 protein in the MUT group were significantly higher than those in the WT and vector groups (Fig. 3K-M). Therefore, the flow cytometry results were verified by detecting the protein expression levels of relevant cyclins, indicating that the IDH1 R132H mutation may affect the regulation of the cell cycle.

Effect of IDH1 gene mutation on biological rhythm genes in U87-MG cells. Abnormal clock gene expression and circadian rhythm disorders are closely associated with the occurrence and development of tumors (47). Western blot analysis revealed that the expression levels of the biological rhythm genes BMAL1 and CLOCK in the IDH1 MUT group were significantly lower than those in the WT group (Fig. 4A-C). Transcriptionally, the clock is driven by positive regulators of the loop. Basic helix-loop-helix heterodimeric transcription

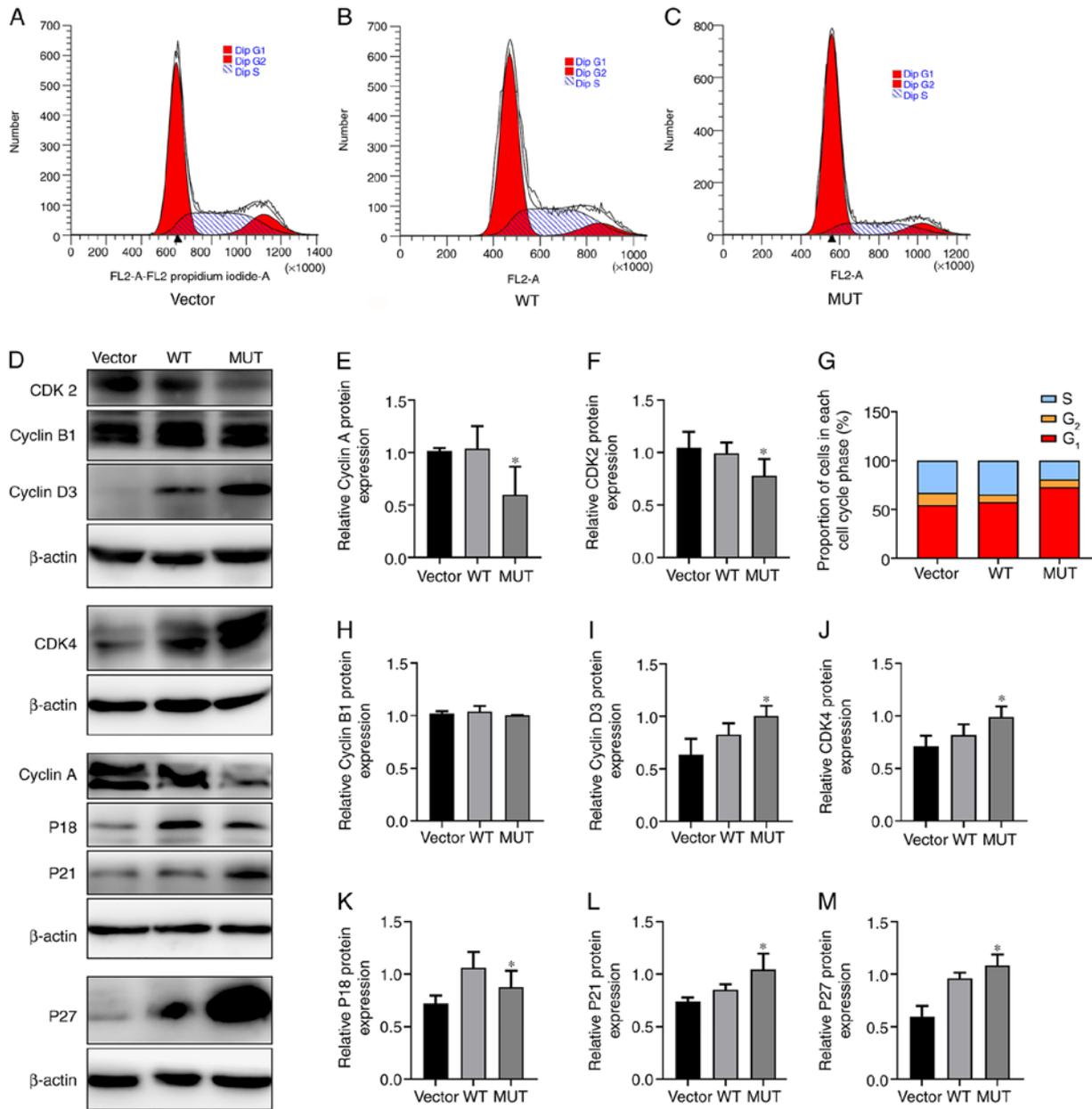


Figure 3. Effect of IDH1 gene mutation on cell cycle. Changes in (A) vector, (B) WT and (C) MUT U87-MG cells after transfection with lentivirus. The cell cycle changes in each group were detected by flow cytometry. (D) Protein expression levels of CyclinA, CyclinB1, CyclinD3, CDK2, CDK4, P18, P21 and P27 in each group of U87-MG cells were detected by western blot analysis. Quantification of relative protein expression levels of (E) CyclinA and (F) CDK2. (G) Proportion of cells in each cell cycle phase in each group. Semi-quantification of relative protein expression levels of (H) CyclinB1, (I) CyclinD3, (J) CDK4, (K) P18, (L) P21 and (M) P27. *P<0.05 vs. WT. MUT, mutant; WT, wild-type; IDH1, isocitrate dehydrogenase 1.

factors (CLOCK/BMAL1 or BMAL1/NPAS2) regulate the expression levels of key circadian genes, including CRY genes (Cry1 and Cry2) and PER genes (PER1, PER2, and PER3), which are negative regulators of the circadian loop (48,49). CRY and PER form a transcriptional repressor complex that enters the nucleus to repress CLOCK/BMAL1 activity, thus creating a negative feedback loop to control the clock (50). Therefore, the expression levels of negative regulators of clock genes were also examined. The negative regulatory factors of biological rhythm PER1, PER2, PER3, Cry1 and Cry2 were significantly downregulated compared with the WT group (Fig. 4D-I). These results indicated that the IDH1 R132H mutation may change the expression levels of biological rhythm genes and may participate in the regulation of the cell biological rhythm.

Effects of IDH1 gene mutation on Smad signaling molecules. IDH1 regulates the TGF- β signaling feedback loop through α -ketoglutarate (α -KG) and TGF- β R-IDH1-Canine adenovirus 1 (Cav1) to enhance TGF- β signaling in a regulatory network between cellular signaling and cell metabolism (51). Additionally, TGF- β is an important regulator of the physiological clock (52,53). Therefore, the present study further investigated any changes in the TGF- β /Smad signaling pathway in IDH1 R132H mutated glioma cells. Western blot assays were used to detect the effects of MUT and WT IDH1 on the levels of p-Smad2, p-Smad3, Smad2, Smad2-3, Smad3 and Smad4 in U87-MG cells (Fig. 5A). Compared with the WT group, the MUT group exhibited significantly decreased protein expression levels of Smad2, Smad3 and Smad2-3

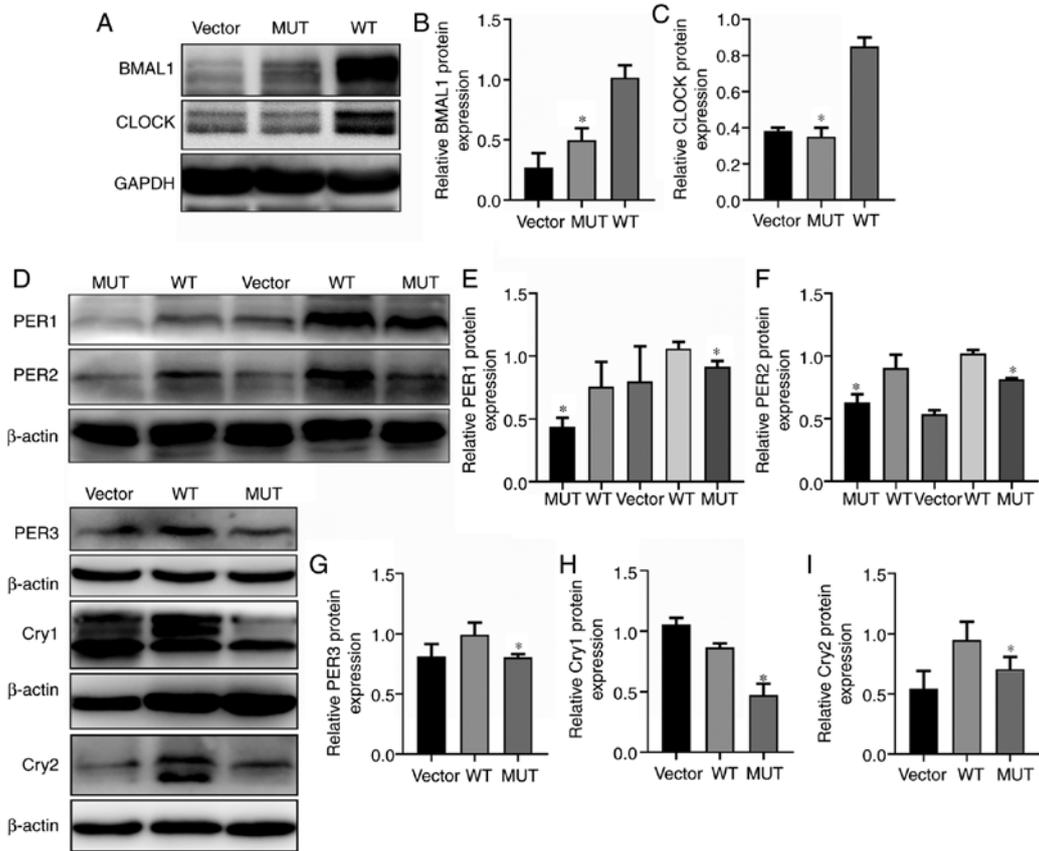


Figure 4. Effects of IDH1 gene mutation on biological rhythm genes. (A) Protein expression levels of BMAL1 and CLOCK in the WT, MUT and vector groups. Quantification of relative protein expression levels of (B) BMAL1 and (C) CLOCK. (D) Protein expression levels of PER1, PER2, PER3, CRY1 and CRY2 in the WT, MUT and vector groups. Quantification of relative protein expression levels of (E) PER1, (F) PER2, (G) PER3, (H) Cry1 and (I) Cry2. *P<0.05 vs. WT. MUT, mutant; WT, wild-type; IDH1, isocitrate dehydrogenase 1; BMAL1, Brain-Muscle Arnt-Like protein 1; CLOCK, Circadian Locomotor Output Cycles Kaput; PER, Period; Cry, Cryptochrom.

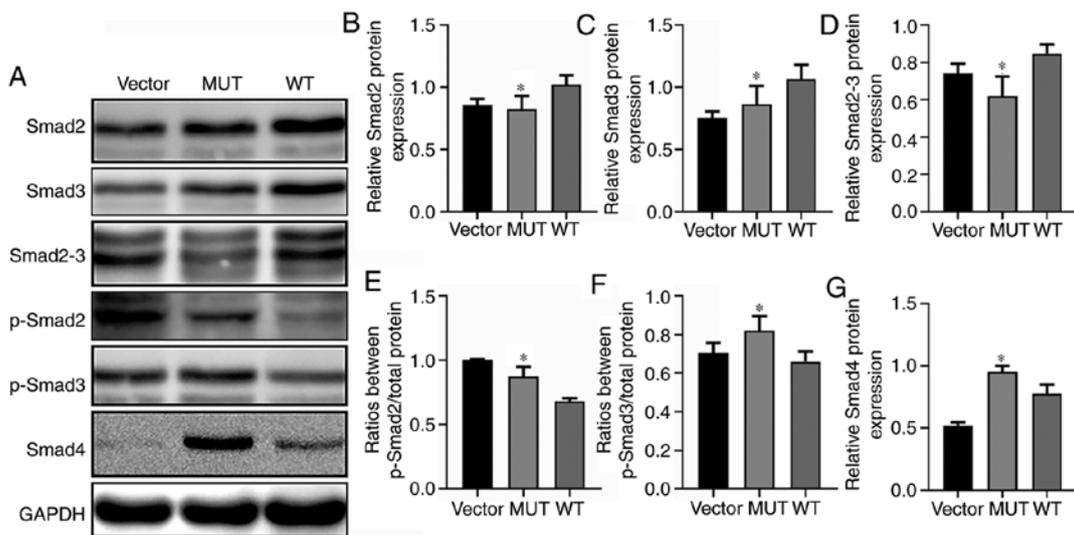


Figure 5. Changes in TGF- β /Smad signaling pathway-associated proteins in the IDH1 WT and MUT groups. (A) Expression levels of proteins associated with the Smad signaling pathway were detected by western blot analysis. Quantification of relative protein expression levels of (B) Smad2, (C) Smad3, (D) Smad2-3, (E) p-Smad2/total protein, (F) p-Smad3/total protein and (G) Smad4. *P<0.05 vs. WT. MUT, mutant; WT, wild-type; IDH1, isocitrate dehydrogenase 1; p, phosphorylated.

(Fig. 5B-D) and significantly increased levels of p-Smad2 and p-Smad3 (Fig. 5E and F). Furthermore, Smad4 expression was significantly upregulated in the MUT group compared with in

the WT group (Fig. 5G). TGF- β initiates signaling pathways by phosphorylating Smad2 and Smad3, and phosphorylated Smad2 and Smad3 bind to Smad4 to form complexes that

are transferred to the nucleus to interact with multiple transcription factors in order to induce cellular responses (54,55). Thus, it was hypothesized that the IDH1 R132H mutation may affect biological rhythm genes of glioma cells through the TGF- β /Smad signaling pathway.

Discussion

Glioma arises from glial cells and most often occurs in the brain (56). Glioma accounts for ~30% of central nervous system tumors and 80% of malignant brain tumors (57). It is characterized with highly infiltrative growth and a poor prognosis. The most common genetic changes in glioma include IDH1/2 mutations, TP53 mutations and 1p/19q heterozygous deletion, which are present in most cases (>90%) of glioma (58,59). The most common and early genetic change in glioma is the IDH1/2 mutation. The most common IDH1 mutation results in the amino acid substitution of R132, which is located at the active site of the enzyme (60). IDH1 mutated glioma cells have a reduced proliferative rate (61). Numerous diseases follow circadian rhythms, and there is growing evidence that circadian disruption may be a risk factor for cancer in humans (62). A previous study has reported that the BMAL1 gene serves a role as a potential tumor suppressor gene in pancreatic cancer by activating the P53 tumor suppressor signaling pathway (63). Circadian rhythm genes activate certain pathways in tumors or are activated by certain pathways to affect the development of tumors. β -catenin showed an increased expression in NIH-3T3 cells after BMAL1 overexpression, indicating that activation of the canonical Wnt pathway may be the mechanism underlying the effect of the circadian clock gene BMAL on promoting cell proliferation (64). BMAL1 suppresses cancer cell invasion by blocking the PI3K-Akt-MMP-2 signaling pathway (65).

However, to the best of our knowledge, there are no studies on the mechanism between IDH1 R132H mutation and biological rhythm genes. Therefore, the present study aimed to describe the role of biological rhythm genes in glioma cells with IDH1 R132H mutation to determine how the IDH1 R132H mutation affects biological rhythm genes and the proliferation of glioma cells.

The current study revealed that the IDH1 R132H mutation in U87-MG cells decreased the number of new colonies formed by tumor cells. The effect of the IDH1 R132H mutation on the cell cycle was further investigated, leading to increased cells in G₁ phase and decreased cells in S phase. The effect of the mutation on cyclins was also examined. The expression levels of various cyclically related proteins was altered, including the S phase-associated proteins Cyclin A and CDK2. No change in Cyclin B1 expression in G₂/M phase was observed. The expression levels of the G₁ phase-associated proteins Cyclin D3 and CDK4 were increased in the MUT group compared with in the WT group. The protein expression levels of P18 were decreased, while those of P21 and P27 were increased in the MUT group compared with in the WT group. Subsequently, the effect of the IDH1 R132H mutation was examined on biological rhythm genes. To the best of our knowledge, the current study was the first to analyze the effect of the IDH1 R132H mutation on biological rhythm. The IDH1 R132H mutation significantly decreased the protein expression levels of both BMAL1 and CLOCK compared with in the WT

group, as well as the expression levels of the negative regulation factors PER1, PER2, PER3, Cry1 and Cry2. A previous study has demonstrated that the disturbance of the circadian clock has a strong influence on tumor transformation and growth by affecting various cancer regulatory signaling pathways, including cell cycle, apoptosis and metabolism (40). The detection of biological rhythm genes in the present study revealed that IDH1 R132H mutation inhibited the protein expression levels of CLOCK and BMAL1, and further inhibited PER and CRY proteins. Therefore, it was shown that the IDH1 R132H mutation has an effect on the expression of biological rhythm genes and has been confirmed in *in vitro* experiments.

Furthermore, how the IDH1 R132H mutation may affect biological rhythm genes was further investigated. Previous studies have shown that the TGF- β signaling pathway is closely associated with the occurrence of glioma (66,67). TGF- β is a factor that strongly inhibits the proliferation of epithelial, astrocyte and immune cells, and is considered a tumor suppressor (68). Some tumors acquire mutations in components of the TGF- β signaling pathway to evade the TGF- β cellular inhibitory response (69). On the other hand, in some malignant tumors, including glioma, the ability of TGF- β to inhibit cell proliferation and maintain the integrity of the TGF- β signaling pathway is selectively lost (70). TGF- β signaling decreases differences in IDH1 expression by normalizing the Smad signaling pathway, and inhibition of Cav1 expression by IDH1 is regulated by α -KG epigenetic regulation. Finally, downregulation of Cav1 expression interrupts the degradation of TGFBR and enhances the Smad signal. IDH1 regulates the TGF- β signaling feedback loop through α -KG and TGFBR-IDH1-Cav1 to enhance TGF- β signaling in a regulatory network between cellular signaling and cell metabolism (51). In the present study, it was observed that the IDH1 R132H mutation significantly decreased the protein expression levels of Smad2, Smad3 and Smad2-3, and significantly upregulated the levels of p-Smad2, p-Smad3 and Smad4. A previous study has suggested that TGF- β is a multi-potent cytokine that controls tissue homeostasis and embryonic development. TGF- β binds and activates a membrane receptor serine threonine kinase complex to phosphorylate Smad2 and Smad3; after phosphorylation, Smad proteins accumulate in the nucleus and form complexes with transcription factors, such as Smad4, to regulate transcription (54). The present study revealed that the IDH1 R132H mutation affected the TGF- β /Smad signaling pathway. Additionally, TGF- β is an important regulator of the physiological clock. A previous study has demonstrated that TGF- β , by regulating the expression of positive and negative regulators of circadian rhythm oscillation, serves a vital role in regulating circadian rhythm. Adenovirus-mediated TGF- β expression can significantly induce BMAL1 and NPAS2 expression (71). It has been demonstrated that the expression levels of the TGF- β -activated transcription factor Smad3 display similar expression patterns with BMAL1, and that Smad3 functions as an upstream molecule of BMAL1, explaining how TGF- β induces BMAL1 expression (53). However, TGF- β strongly inhibits the expression levels of PER1, PER2, PER3, Rev-erba, retinoic acid receptor-related orphan receptor α and D-site albumin promoter-binding protein (71). Therefore, increased expression levels of TGF- β and negative regulators of the circadian

clock prolong the arousal cycle (52). The impact of CLOCK and BMAL1 on cancer pathogenesis is highly context- and disease-dependent (50). For instance, CLOCK or BMAL1 provide tumor suppressor-like functions in prostate, breast, ovarian and pancreatic cancer, but exhibit tumor-promoting roles in colorectal cancer and acute myeloid leukemia (50,72). In glioma, CLOCK or BMAL1 are tumor-promoting factors that regulate glioma cell proliferation and migration via regulating the NF- κ B signaling pathway (73), and can support glioma stem cell function via regulation of anabolic metabolism (74). Therefore, the current study hypothesized that the IDH1 R132H mutation may affect biological rhythm genes through the TGF- β /Smad signaling pathway, thus affecting the proliferation of glioma cells.

In summary, the IDH1 gene serves crucial roles in the occurrence and development of numerous types of tumor (75), and imbalance in the circadian clock plays an important role in the development of malignant tumors, including glioma (76). The present study revealed that the IDH1 R132H mutation affected the expression levels of cyclin and biological rhythm genes, and thus may affect the occurrence of glioma. Based on the current results, it was hypothesized that IDH1 mutation may affect the expression levels of biological rhythm genes of cells through the TGF- β /Smad signaling pathway. To the best of our knowledge, the present results are the first to report the association between IDH1 R132H mutation and biological rhythm genes, as well as the effect on glioma cell proliferation and the possible underlying mechanisms. However, further studies are required to confirm the current results.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XC and SH designed the experiments, guided the study and revised the draft. YW performed the experiments and wrote the manuscript with support from all the other authors. YG and NZ performed the data analysis and contributed to the writing of the manuscript. HY, JY and JM participated in some of the experiments and in data collection. FW and HX participated in some of the data analyses. XC, YW and YG confirmed the authenticity of the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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