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Targeting Aurora kinase B attenuates chemoresistance in glioblastoma via a synergistic manner with temozolomide

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ABSTRACT

Background: Recent studies have demonstrated that aberrant expression or activation of kinases results in oncogenesis of a wide range of cancers including GBM. Inhibition of kinases expression induces a reduction of therapy resistance. In this study, we investigate the underlying mechanism by which glioblastoma (GBM) cells acquire resistance to Temozolomide (TMZ) through Aurora kinase B (AURKB) thus to identify novel therapeutic targets and prognostic biomarkers for GBM.

Methods: AURKB was identified as a key candidate kinase-encoding gene in chemoresistance regulation by using kinome-wide bioinformatic analysis. Afterwards, the potential biological functions of AURKB in oncogenesis and chemoresistance were investigated by lentivirus-dependent silencing of AURKB combined with qRT-PCR, western blot and *in vivo* intra-cranial xenograft mice models. Additionally, immunohistochemistry (IHC) assays were performed to explore the clinical significance of AURKB in glioma patients. Lastly, Chou-Talalay method was used to confirm the synergistic effect of TMZ combined with AURKB inhibitor.

Results: AURKB was among the most significantly up-regulated kinase-coding genes in TMZ resistant GBM cells according to database GSE68029, moreover, an increased expression of AURKB was closely associated with poor prognosis in glioma and GBM patients. AURKB knock-down resensitized U87 resistant cells to TMZ both *in vitro* and *in vivo*. Additionally, the combination of TMZ and Hesperadin, a specific AURKB inhibitor, significantly suppressed the proliferation of TMZ resistant GBM cells thus dramatically prolonged the survival of xenograft mice *via*a synergistic effect with TMZ.

Conclusion: Elevated AURKB expression was strongly correlated to TMZ resistant acquisition and poor prognosis, furthermore, targeting AURKB would be a potential therapeutic target for GBM patients.

1. Introduction

Glioblastoma multiforme (GBM) is the most frequent and lethal brain malignant tumor, with a median survival of less than 2 years [1]. In spite of the significant improvement driven by recent studies, clinical treatments for GBM have progressed relatively slow due to the therapy resistance and frequent recurrence caused by cellular heterogeneity of GBM [2]. Current standard treatment for GBM includes maximal safe surgical resection, temozolomide (TMZ) chemotherapy and radiation therapy [3]. As well known, TMZ inhibits GBM growth mainly through DNA alkylation/methylation, which occurs in guanine residues at the position of N-7 or O-6 (4). However, tumor recurrence was frequently observed in patients with higher expression of O^6-methylguanin-DNA-methyltransferase (MGMT) gene, which encodes a specific enzyme inducing chemoresistance in tumor [5]. Accordingly, finding a novel therapeutic target to enhance the sensitivity of TMZ in GBM becomes urgently needed.

As well known, kinases function extensively as signals transmission and processes regulation in cells [6]. Aberrant expression or activation of kinases results in oncogenesis of a wide range of cancers including GBM [7]. Accordingly, treatment targeting kinases has been proved to be an efficient way to reduce tumor proliferation in various subtypes of

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cancer [8–11]. Aurora kinases (AURK) are identified as a group of serine/threonine kinases which participate in cellular division through regulation of chromatid segregation [12]. AURK has three subclasses: AURKA, AURKB and AURKC [13]. Kleiton et al [14] reports that inhibition of AURK expression induces a reduction of therapy resistance of GBM, indicating that AURK contributes to therapy resistance and could be a potential therapeutic target in GBM. Recent studies demonstrated that Aurora kinase B (AURKB) contributes to the development and progression of various tumors including gastric cancer, esophageal squamous cell carcinoma and colorectal cancer [15–17]. Accumulating evidence suggests aberrant elevation of AURKB leads to unequal chromosomal separation in cell division then causes abnormal numbers of chromosomes in a single cell, which is an initial event during cancer development [18]. Additionally, Kumari et al [19] found that siRNA-loaded lactoferrin nanoparticles increased the toxicity of TMZ on GBM cells, implying that artificial suppression of AURKB through small molecule inhibitor could be used as adjuvant therapy to TMZ in purpose to enhance the anti-tumor effects of TMZ in GBM. To this end, a small molecule Hesperadin was picked for this study based on its specific inhibition of AURKB by efficient prevention of chromosome alignment and segregation [20]. Herein, our study indicated that AURKB was functionally required for tumorigenesis and acquired TMZ resistance in GBM. Clinically, AURKB expression was conversely correlated to poor prognosis for glioma/GBM patients. Moreover, inhibition of AURKB through either shRNAs or Hesperadin reduced TMZ resistance in GBM. Altogether, AURKB was a novel clinically relevant biomarker and could become a potential therapeutic strategy for GBM.

2. Materials and methods

2.1. Ethical statement

The usage of the experimental animals and human samples in this study were approved by the Ethics Committee of the School of Medicine, Xi'an Jiaotong University (approval no. 2016-085) and the Scientific Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University (approval no. 2016-18), respectively. All patients signed for the consent of the use for their tissue specimens, and the standard methodologies strictly followed the Declaration of Helsinki.

2.2. Identification of differentially expressed kinases in TMZ resistance through GEO database

Gene expression data was extracted from Gene Expression Omnibus (GSE68029, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE68029). Cluster 3.0 was used to analyze the Hierarchical bi-clustering of the targeting genes. Average linkage and Euclidean distance were used as clustering methods and similarity metric, respectively. Fold changes were used to present the comparison of relative gene expression among naive and TMZ resistant GBM cells. Afterwards, these differentially expressed genes were aligned with kinases database to find out differential kinase-encoding genes.

2.3. Analysis of expression and prognostic value of genes in glioma with the Cancer genome Atlas data (TCGA)

Analyses for the expression pattern and prognostic value of genes in glioma (low grade glioma and GBM) were performed based on TCGA RNA sequencing database [21] and Rembrandt [22] databases. The RNA sequencing expression data are analyzed according to the standard processing pipeline.

2.4. Reagents and antibodies

Dulbecco's modified Eagle 'medium-nutrient mixture F12 (DMEM-F12), alamarBlue reagent, fetal bovine serum (FBS), PageRuler plus prestained protein ladder and Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis kit was bought from Thermo Fisher Scientific (Waltham, MA, USA). Accutase solution, phosphatase inhibitor and protease inhibitor, radio immunoprecipitation assay (RIPA) lysis buffer were purchased from Merck KGaA (Darmstadt, Germany). iScript Reverse Transcription SuperMix and Bradford reagent were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Bovine serum albumin (BSA) was purchased from New England BioLabs (Ipswich, MA, USA). Hesperadin was obtained from Medchem Express (Cat log no. HY-12054, NJ, USA). TMZ was provided by Tasly Group Co, Ltd (Jiangsu province, China).

Antibodies used in this study were shown as below: anti-AURKB primary antibodies were bought from Abcam (Cat log no. ab2254) for western blot and immunohistochemistry. Anti-β-actin antibody was purchased from Abcam (Cat log no. ab115777).

2.5. Cell culturing and proliferation assay

GBM cell lines U251, A172 and NHA were provided by the First Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China). U87 cell line was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), catalog no. TCHu138. Cells were cultured in DMEM-F12 containing 10% vol FBS and antibiotics (1% penicillin and streptomycin). All these cells were cultured in a humidified condition containing 5% CO_2 at 37°C.

For *in vitro* cell proliferation assay, cells were collected and suspended adequately and then seeded into 96 wells plate at a density of 1×10^3 cells/well. After seeding, the cell number was counted by alamarBlue strictly followed the manufacturer's instructions at day 0, 2, 4, 6 and 8.

2.6. In vitro cell viability assay

Cell number was calculated by cell counter with trypan blue, then cells were seeded into 96 well plates after adequate suspension at a density of 2×10^3 cells/100 u L per well and cultured for 12 h. Afterwards different concentration of TMZ or Hesperadin containing fresh medium was added into these plates. Finally, cell number was counted by alamarBlue and IC50 was calculated by using SPSS 22.0. The synergism of TMZ and Hesperadin was quantified by Chou-Talalay method with COMPUSYN 2.0 as previously published [23].

2.7. Lentiviral shRNA construction and transfection

Lentivirus production and transduction was conducted as previously described [23]. The plasmid for shAURKB lentivirus targeting sequences were shown below:

shAURKB#1: GGAGGAGGATCTACTTGATTC
shAURKB#2: GCAGAAGAGCTGCACATTTGA

2.8. TMZ resistance induction in GBM cells

U87 GBM cells were seeded into the 6-well plates and incubated overnight with DMEM-F12 medium containing 10% FBM. Afterwards cells were cultured with TMZ containing medium at a starting dose of 100 u M. For the first 5 days, the medium with TMZ was replaced every 24 h. TMZ concentration was gradually increased every 2 weeks for 3 consecutive months and the dose limit was 500 u M.

2.9. Flow cytometry

Flow cytometry assays were conducted as mentioned previously [23]. U87 cell apoptosis was measured by the Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis kit according to the manufacturer's instructions.

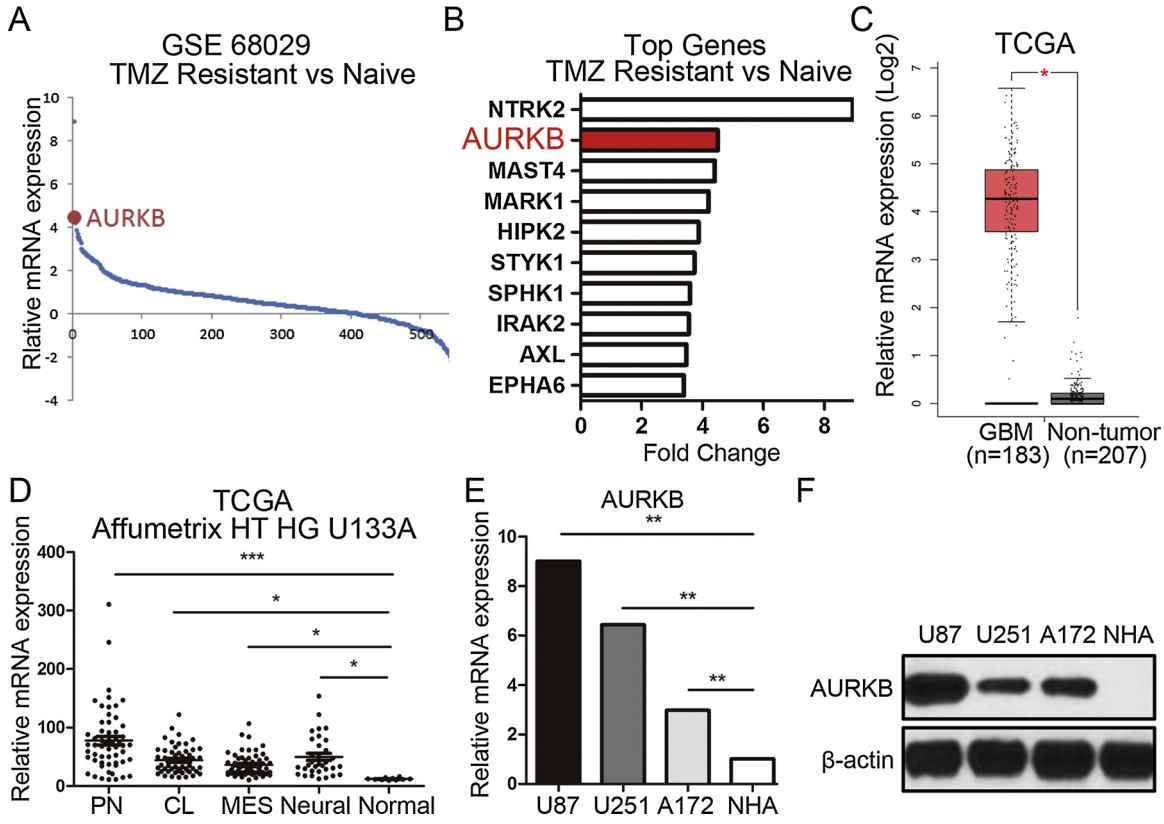

Fig. 1. AURKB was overexpressed in GBM.

A. Genome-wide transcriptome microarray analysis (GSE 68,029) indicated that AURKB was among the most enriched kinase-encoding genes in TMZ resistant group compared with naïve GBM cell lines. B. Top 10 kinase-encoding differentially expressed genes in TMZ resistant GBM cells to naïve GBM cell lines. C. Expression patterns of AURKB in TCGA database, red bar represents the glioma tissues and blue bar shows the normal brain tissues (with *t* test). D. Analysis of Rembrandt database shows AURKB expression in different glioma subtypes (*P < 0.05, **P < 0.01, with one-way ANOVA followed by Dunnett's posttest). E. AURKB mRNA expression in human glioma cell lines and NHA was measured by quantitative real-time RT-PCR (**P < 0.01, with one-way ANOVA followed by Dunnett's posttest). F. AURKB protein expression in human glioma cell lines and NHA were measured by Western blot analysis. β-actin was used as an internal control. All data were presented as the mean ± standard deviation of triplicate independent experiments. TMZ, Temozolomide; AURKB, Aurora Kinase B; RT-PCR, reverse transcription-polymerase chain reaction; mRNA, messenger RNA.

2.10. Quantitative real-time RT-PCR (qRT-PCR)

Total RNA of patients' samples and glioma cells were extracted using the RNeasy mini kit according to the protocol. Nanodrop 2000 was utilized to determine the concentration of RNA. iScript reverse transcription Supermix was used to synthesize cDNA according to the manufacturer's instructions. qRT-PCR analysis was performed using StepOnePlus real-time PCR system strictly followed the manufacturer's protocol with SYBR Select Master Mix (Applied Biosystems). GAPDH was utilized as internal controls. The primers sequences were shown as below:

AURKB, Forward: 5′-CTGCACCATCCCAACATCCT-3′; Reverse: 5′-TGCCAACTCCTCCATGATCG-3′;

GAPDH, Forward: 5′-GAAGGTGAAGGTCGGAGTCA-3′; Reverse: 5′-TTGAGGTCAATGAAGGGGTC-3′;

Relative gene expression was calculated by using the $2^{-\Delta\Delta Ct}$ method.

2.11. Western blot analysis

Western blot analysis was performed as previously diescribed [23]. After protein of cell lysates was distracted by RIPA buffer containing protease and phosphatase inhibitor, the concentrations of proteins were measured by Bradford method. 10ug/lane of protein was electrophoresed and transferred to PVDF membrane (Invitrogen) and then incubated with corresponding primary antibodies overnight at 4°C, afterwards, the secondary antibodies was used at room temperature for 1 h. Consequently, the immunocomplexes were detected by the enhanced chemiluminescence kit and AI600 Imager System (Amersham, GE Healthcare Life Sciences). β-actin was used as a internal control.

2.12. Immunohistochemistry (IHC)

IHC was performed as described previously [23]. German immunohistochemical scoring (GIS) was utilized to analyze the expression of AURKB immunoreactivity score. The positive cell score was counted as below: 0, negative; 1, < 10% positive; 2, 11-50% positive; 3, 51-80% positive; 4, > 80% positive. Staining intensity score was grouped as: 0, negative; 1, weakly positive; 2, moderately positive; 3, strongly positive. Immunoreactivity score > 3 was considered as positive staining.

2.13. In vivo intracranial xenograft model

In vivo xenograft model was performed by using 6-week-old female nude mice. Corresponding GBM cell (pre-transfected with shNT and shAURKB) was adequately suspended and diluted to the density of 1×10^5 cells in 2 u L PBS then injected into the nude mice brains as described previously [23]. Each group of treatment was consist of 5 mice and they were monitored unless the following symptoms came out: arched back, unsteady gait, more than 10% bodyweight loss or leg paralysis. TMZ (50 mg/kg/d) and Hesperadin (20 mg/kg/d) was injected through vein after 7 days of implantation. Moreover, Hesperadin

was injected into xenograft mice's tail right after TMZ injection when they were combined.

2.14. Statistical analysis

All the results in this study are exhibited as mean ± Standard Deviation. Number of independent replications is presented in the corresponding Figure legends. 2-tailed *t* tests were used to evaluate statistical differences. One-way ANOVA following Dunnett's posttest was utilized in comparisons more than 2 groups. Kaplan-Meier survival analysis was conducted using log-rank analysis. All statistical analysis was calculated by SPSS 22.0 or GraphPad Prism 7 software. Unless specifically indicated, statistical significance was considered as a two-sided P value < 0.05.

3. Results

3.1. AURKB was overexpressed in TMZ resistant GBM

To investigate the differentially expressed kinase-encoding genes in TMZ resistant GBMs and their original source, GSE68029 database was analyzed and we found out the differentially expressed genes in two groups, afterwards we aligned the results with the 668 known kinase-encoding gene symbols. As is shown in Fig. 1A, AURKB was among the most significantly elevated kinase-encoding genes in TMZ resistant group, together with other kinases including NTRK2, MAST4, MARK1, HIPK2, STYK1, SPHK1, IRAK2, AXL, EPHA6 (Fig. 1B). Based on the result derived from GEPIA [21], we found that AURKB was significantly upregulated in GBM tissues compared with normal tissues (Fig. 1C). To further explore whether the expression of AURKB had subtype differences in glioma, AURKB expression among 4 subtypes including proneural, classical, mesenchymal and neural glioma cell in TCGA GBM database were compared. As is shown in Fig. 1D, AURKB was significantly upregulated in these subtypes, proneural cell in particular. Additionally, AURKB expression is higher in GBM cell lines compared with the NHA (normal human astrocyte) (Fig. 1E and F). Altogether, these results suggested that AURKB expression was highly enriched in GBM and it might be essential for TMZ resistance of GBM.

3.2. Elevated AURKB expression was closely associated with poor prognosis in glioma/GBM patients

Results from the Rembrandt database [22] indicated that AURKB was highly expressed in GBM compared with astrocytoma (Fig. 2A). To gain more insight into the clinical characters of AURKB, IHC staining by using tumor samples derived from patients underwent surgical resection in the First Affiliated Hospital of Xi'an Jiaotong University was performed. The results showed that AURKB was more likely to be elevated in high grade glioma rather than low grade glioma (Fig. 2B and C). Furthermore, Kaplan-Meier analysis showed that higher AURKB expression was correlated to more severe overall survival than those with lower AURKB (Fig. 2D). In addition, similar results could be observed in when analyzing Rembrandt and TCGA databases (Fig. 2E–G). Notably, the expression level of AURKB not only correlated to overall survival but also strongly affected disease free survival (Fig. 2G). These data indicated that the upregulation AURKB might be a contributory factor in progression and poor prognosis of glioma, suggesting that AURKB could be considered as a novel diagnostic and prognostic marker in glioma.

3.3. Close Correlation between AURKB expression and TMZ resistance in GBM cell lines

To validate the potential function of AURKB as previously mentioned, we induced TMZ resistance U87 cell lines according to the previous studies [24,25]. As is shown in, After 3 months of culturing with TMZ-contained medium, U87 cells acquired stable TMZ resistance (Fig. 3A and B). Furthermore, mRNA and protein expression of AURKB were measured in TMZ resistance and naive U87 cells and the results showed that AURKB expression was markedly increased in TMZ resistant U87 cells while the naive cells exhibited a lower expression of AURKB expression (Fig. 3C and D). Altogether, these results suggested a possible correlation between AURKB expression and TMZ resistance in GBM.

3.4. AURKB knock-down enhanced TMZ sensitivity in GBM

To further investigate the biological functions of AURKB in GBM cells, AURKB specific lentiviral shRNA were introduced into U87 TMZ resistant cells. Afterwards, qRT-PCR and western blot analysis illustrated that up to 70% of AURKB expression was inhibited by lentiviral shAURKB (Fig. 4A and B). In addition, *in vitro* cell viability assays were conducted to explore the combined effect of TMZ and shAURKB on TMZ resistant cells. As is shown in Fig. 4C, TMZ combined with AURKB knock-down significantly downregulated the cell growth of TMZ resistant U87 cells. Similarly, flow cytometry analyses also indicated that shAURKB-transfected U87 TMZ resistant cells were more vulnerable to TMZ than control groups (Fig. 4D). We then tested the effect of shAURKB on acquired TMZ resistance by using mice xenograft models. Consistently, a prolonged survival was observed when xenograft mice were treated with the combination of shAURKB and TMZ (Fig. 4E). Altogether, our results illustrated that inhibition of AURKB enhanced the sensitivity to TMZ in GBM.

3.5. AURKB inhibitor reduced TMZ resistance of GBM viaa synergistic effect

As mentioned previously, AURKB functions as a key regulator of the acquisition of TMZ resistance in GBM. To further investigate the efficacy of AURKB inhibition in GBM, a small molecule Hesperadin was picked as a specific AURKB inhibitor (Fig. 5A) [20]. As is shown in Fig. 5B, both TMZ resistant U87 and its naive controls exhibited an undifferentiated sensitivity to Hesperadin, indicating that Hesperadin functioned equally to these two cell groups. Additionally, *in vitro* cell viability assays demonstrated that Hesperadin inhibited cell proliferation of TMZ resistant U87, notably, the acquired TMZ resistance was dramatically reversed by Hesperadin (Fig. 5C). Furthermore, flow cytometry analyses were conducted to measure the combined effect of Hesperadin and TMZ on TMZ resistant U87 cells. The results demonstrated that the combination of Hesperadin and TMZ significantly increased the ratio of apoptotic cells and strengthened the cytotoxicity of TMZ on resistant U87 cells (Fig. 5D). Next, we explored the effect of Hesperadin and TMZ combination on intracranial xenograft mice models. Similar to our prediction, although there were no significant differences on survival when the mice were treated with TMZ alone, the Hesperadin and TMZ combination dramatically increased the survival of xenograft mice models (Fig. 5E). Taken together, these results indicated that AURKB inhibitor reduced tumor proliferation as well as TMZ resistance of GBM both *in vitro* and *in vivo*.

To further evaluate the clinical prospect of AURKB inhibition, Chou-Talalay model aimed to detect and quantify the drug combination effects between TMZ and Hesperadin was used in this study [26]. TMZ resistance acquired U87 cells were exposed to different dose of either TMZ, Hesperadin or their combination for 3 days, then the inhibition of cell viability was investigated. As is shown in Fig. 5F, the combination index (CI) was less than 1.0, indicating that Hesperadin had synergistic effects with TMZ. Accordingly, our results suggested that TMZ treatment combined with AURKB inhibition overcame TMZ resistance *via*a synergistic effect.

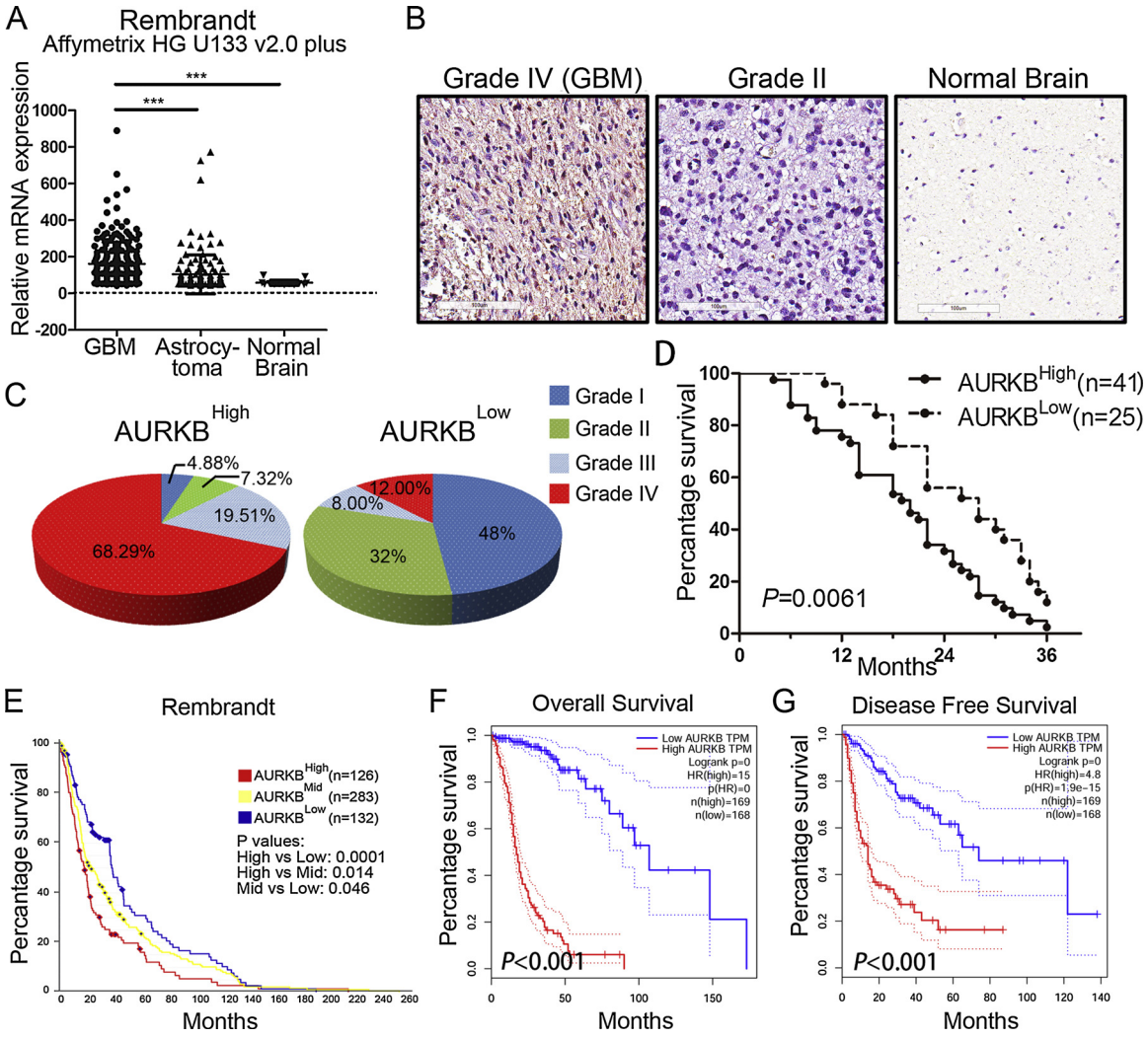

Fig. 2. Elevated AURKB expression was closely associated with poor prognosis in glioma/GBM patients.
A. The expression patterns of AURKB in Rembrandt database (*P < 0.05, **P < 0.01, ***P < 0.001 ns P > 0.05, with one-way ANOVA followed by Dunnett's posttest). B. Representative images of IHC results indicating AUKRB expression in different grades of glioma; C. pie chart shows the different proportion of glioma patients grouped by high or low level of AURKB expression; D. Kaplan-Meier plot indicates the overall survival in glioma patients grouped by high or low level of AURKB (P < 0.01, n = 41 for AURKB up-regulated group, n = 25 for AURKB down-regulated group, with log-rank test). E. Kaplan-Meier plot with log-rank test derived from Rembrandt database, different expression groups is shown as indicated; F, G the overall and disease-free Kaplan-Meier survival curve (with log-rank test) for AURKB expression in glioma patients. Red curve represents patients with a high level of AURKB expression (top 25%), while blue curve represents patients with low levels of AURKB expression (bottom 25%). AURKB, Aurora Kinase B; TPM, transcripts per Kilobase million.

4. Discussion

GBM is among one of the most common malignancies in human brain. Despite the evolution in therapy strategies during the past decades, the prognosis of GBM patients is still dismal [27]. Therefore, discovery of new therapeutic targets and screening survival-relevant biomarkers for early diagnosis are meaningful to improve the clinical outcomes of GBM patients [28]. Recent studies have clarified that kinase-dependent activation of treatment resistance is critical for mortality and recurrence in GBM patients [29]. Accumulating data pinpoints that AUKRB, a serine/threonine kinases participates in cellular division by controlling chromatid segregation, contributes to chemoresistance by promoting the cellular survival in response to chemotherapeutic drugs [30–32]. Additionally, AURKB has also been demonstrated to participate in the regulation of various biological behaviors such as cell proliferation and tumorigenesis as a pivotal oncogene in chromosomal separation [33]. Previous studies clarify that abnormal expression of AURKB results in tumorigenesis of astrocytoma and pediatric diffuse intrinsic pontine glioma [34–36]. However, the clinical significance of AURKB in glioma has not been fully elucidated.

In this study, we illustrated that AURKB was overexpressed in glioma and its elevation was closely correlated to poor prognosis. Moreover, our findings demonstrated that AURKB was highly expressed in TMZ resistant GBM cells and was responsible for TMZ resistance in GBM. Furthermore, our results showed that inhibition of AURKB through either lentiviral transfection or specific small molecule inhibitor caused a reduced TMZ resistance as well as decreased tumorigenesis of glioma cells *in vitro* and *in vivo*, indicating the potentials of AURKB to become a novel clinical therapeutic target for GBM. Additionally, the specific AURKB inhibitor Hesperadin exhibited significant reduction on tumor growth both *in vivo* and *in vitroviaa* synergistic effect with TMZ. Most intriguingly, Chou-Talalay model was used to detect and quantify the drug combination effects between TMZ and Hesperadin, and we found that Hesparadin suppressed glioma progression as well as chemoresistance in a synergistic manner with TMZ [26]. Taken together, these findings demonstrated that AURKB promoted TMZ resistance and tumorigenesis and could be used as a prognostic factor for GBM patients, moreover, small specific molecules

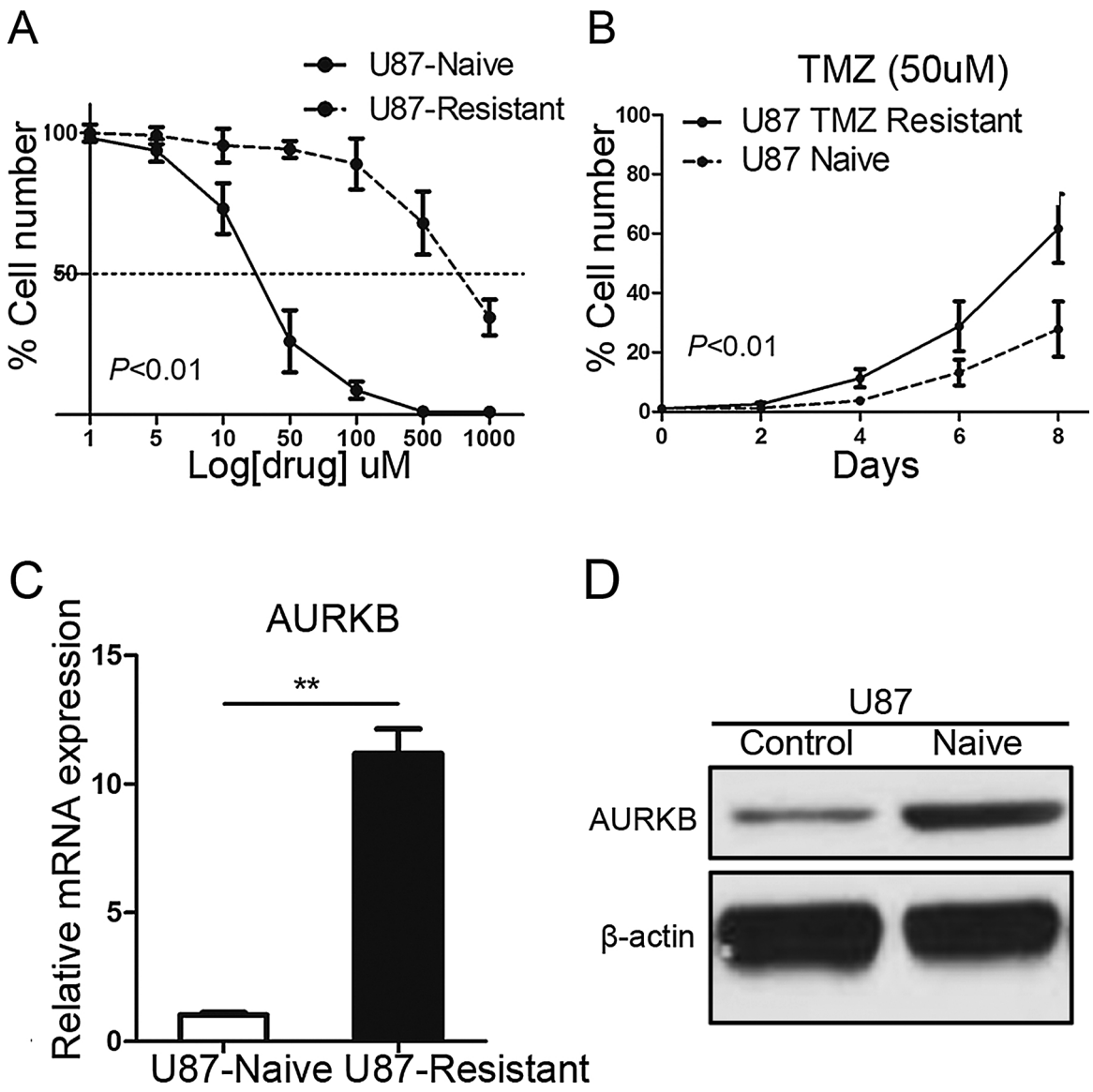

Fig. 3. Close Correlation between AURKB expression and TMZ resistance in GBM cell lines.
A. Cell viability assay indicates the different susceptibility of TMZ resistant U87 cells and naïve U87 cells to graded concentration of TMZ ($P < 0.01$, with *t* test). B. Time survival curve exposed to the same concentration of TMZ shows the different *in vitro* cell viability of TMZ resistant cells and naïve cells ($P < 0.01$, with *t* test). C. AURKB mRNA expression in TMZ resistant U87 cells and naïve cells (**$P < 0.01$, with *t* test). D. AURKB protein expression in TMZ resistant U87 cell line and naïve cell lines, β-actin served as a loading control. AURKB, Aurora Kinase B; mRNA, messenger RNA.

for AURKB could be synthesized and served as an ancillary drug to enhance the vulnerability of GBM cells to TMZ in order to improve the survival of GBM patients. However, due to the limitation of *in vitro* experiments and the small number of replicates, it is hardly to extend the conclusion to *in vivo* system which are more complicated and multivariate regulated. Furthermore, as the tumor microenvironment of xenograft intracranial mice model is more complicated than *in vitro*cell proliferation or flow cytometry assays, no significance is found in the survival of mice despite the proliferation or apoptotic rate has changed significantly in AURKB-KD cells. Therefore, new AURKB inhibitor with less side effect should be designed and synthesized, and further multi-central clinical trials need to be conducted to deeply testify the effect of AURKB inhibition.

Current standard therapeutic strategy for GBM includes surgery, radiotherapy and chemotherapy, besides, TMZ is the most commonly used agent for the chemotherapy of GBM [37]. However, the overall survival of GBM patients is still less than 15 months even the patients were treated with the maximum comprehensive treatment [38]. Herein, we found that abnormally enriched AURKB expression promotes TMZ resistance in GBM and was conversely correlated with poor prognosis in GBM, which is consistent with the previous studies in gastric cancer, lung cancer and breast cancer [16,39,40]. As is well known, cell proliferation is an exceedingly complex process that involves multiple coordinated and sequential steps, including the contribution of several kinases like cyclin-dependent kinases, polo-like kinases and Aurora kinases [41]. A previous study showed that the expression and activation of AURKB begin in S phase and peak in the G2/M phase, indicating that AURKB might promote cell proliferation in leukemia [18]. Meanwhile, He et al [42] reports that inhibition of AURK induced apoptosis and autophagy *via*AURKB/p70S6K/RPL15 axis through PI3K/Akt/mTOR, AMPK, and p38 MAPK signaling pathways in leukemia. Moreover, Borges et al [14]. Reports that suppressing Aurora kinases by unspecific inhibitor enhances the efficacy of radiotherapy in GBM, indicating that Aurora kinases might confer GBM cells with radioresistance. Additionally, AURKB have also been found to directly phosphorylate p53 and induce the degradation of p53 through Mdm2-

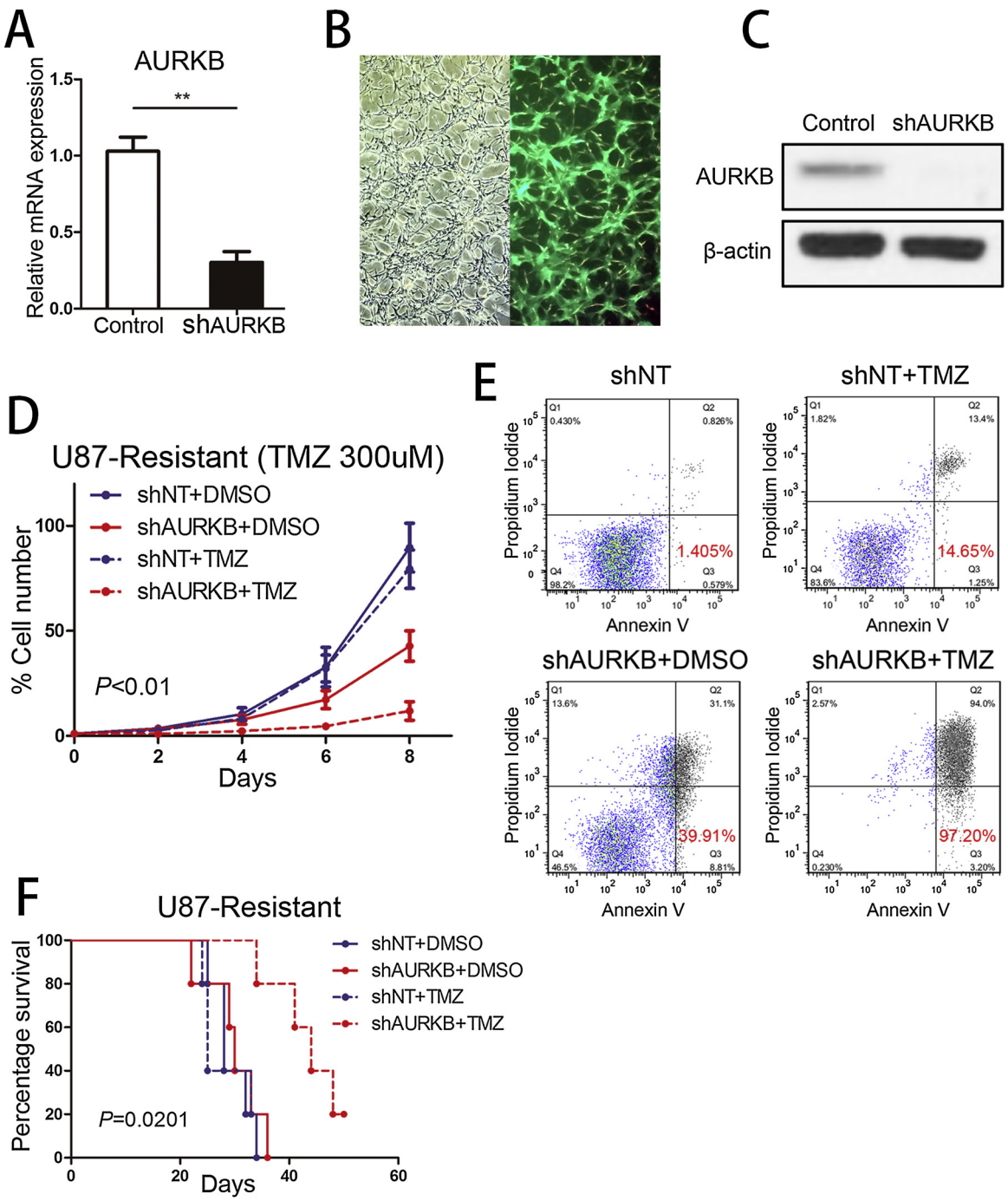

Fig. 4. AURKB knock-down enhanced TMZ sensitivity in GBM.
A. qRT-PCR analysis indicating AURKB mRNA expression in TMZ resistant U87 cells transfected with lentiviral shAURKB and negative control (**P < 0.01, with *t* test). B. Representative images of immunofluorescence showing the transfection efficiency of shAURKB. C. Western blot analysis showing AURKB protein expression in TMZ resistant U87 cells transfected with lentiviral shAURKB and its negative control, β-actin was used as an internal control. D. *In vitro* cell proliferation assays for AURKB knock-down combined with TMZ using resistant U87 cell line (P < 0.01, with one-way ANOVA followed by Dunnett's posttest). E. Flow cytometry analysis using Annexin V and Propidium Iodide for apoptotic ratio analysis in TMZ resistant U87 cells transfected with lentiviral shAURKB with or without TMZ treatment. F. Kaplan-Meier analysis for *in vivo* xenograft mice models using TMZ resistant U87 cells transfected with lentiviral shAURKB or shNT, afterwards these intracranial implanted mice received corresponding treatment (TMZ 50 mg/kg/d or DMSO *via*tail vein injection) as indicated for continuous 10 days through tail vein injection (P = 0.0201, with log-rank test).

mediated ubiquitination thus promoting carcinogenesis and cancer progression [43]. These studies suggest that AURKB regulates enormous functional proteins and crucial signaling pathways including cell cycle procession, apoptosis and autophagy. To this end, the exact molecular mechanism of the regulation of AURKB in malignancies still remained unclear. The activation mechanisms of AURKB and the related pathways were not deeply investigated in this study. Further studies focused on the transcriptional regulator and downstream targets of AURKB in TMZ resistance of GBM should be performed in the future.

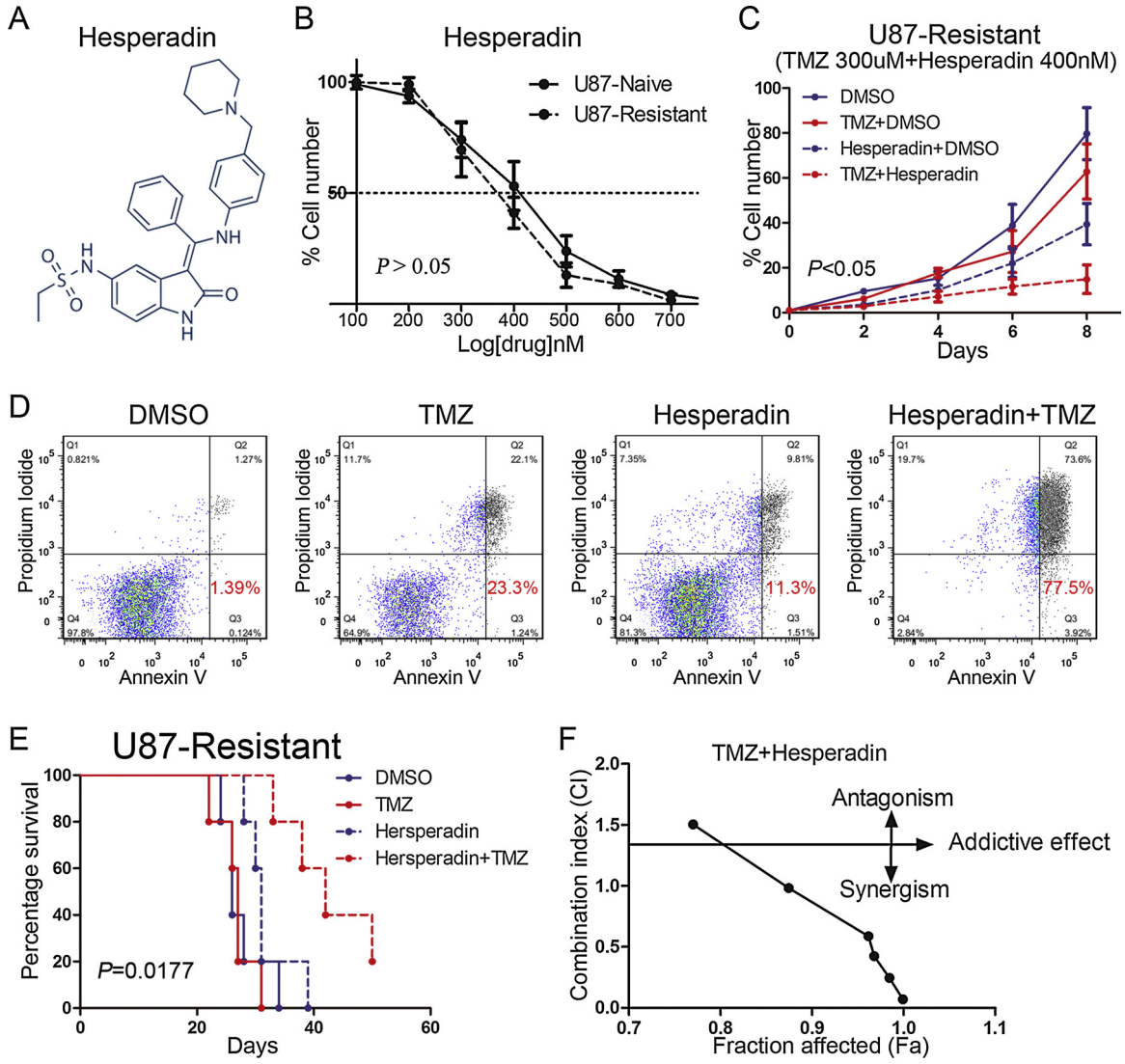

Fig. 5. AURKB inhibitor reduced TMZ resistance of GBM *via* asynergistic effect.
A. The chemical formula of Hesperadin, a specific AURKB inhibitor. B. *In vitro* cell viability assays of TMZ resistant U87 cells and naïve U87 cells treated with graded concentration of Hesperadin (P > 0.05, with *t* test). C. *In vitro* cell proliferation assays of TMZ resistant U87 cells received the indicated treatments (P < 0.05, with one-way ANOVA followed by Dunnett's posttest). D. Flow cytometry analysis showing the different apoptotic ratio of TMZ resistant U87 cells treated with TMZ, Hesperadin or their combination. E. Kaplan-Meier curve indicating the different survival of intracranial TMZ resistant U87 cells implanted mice models when received indicated treatments (TMZ 50 mg/kg/d, Hesperadin 20 mg/kg/d or DMSO through tail vein injection, P = 0.0177 with log-rank test). F. CI-Fa analysis was used to quantify the synergistic effect of Hesperadin combined with TMZ in resistant GBM cells.

Declaration of Competing Interest

No conflicts of interest exists in the submission of the manuscript and the manuscript is approved by all authors for publication.

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References

[1] A. Rape, B. Ananthanarayanan, S. Kumar, Engineering strategies to mimic the glioblastoma microenvironment, Adv. Drug Deliv. Rev. 79–80 (2014) 172–183, https://doi.org/10.1016/j.addr.2014.08.012.
[2] M.A. Qazi, P. Vora, C. Venugopal, S.S. Sidhu, J. Moffat, C. Swanton, et al., Intratumoral heterogeneity: pathways to treatment resistance and relapse in human glioblastoma, Ann. Oncol. 28 (7) (2017) 1448–1456, https://doi.org/10.1093/annonc/mdx169.
[3] B.M. Alexander, T.F. Cloughesy, Adult glioblastoma, J. Clin. Oncol. 35 (21) (2017) 2402–2409, https://doi.org/10.1200/JCO.2017.73.0119.
[4] F.V. Jacinto, M. Esteller, MGMT hypermethylation: a prognostic foe, a predictive friend, DNA Repair (Amst). 6 (8) (2007) 1155–1160, https://doi.org/10.1016/j.dnarep.2007.03.013.
[5] S. Hombach-Klonisch, M. Mehrpour, S. Shojaei, C. Harlos, M. Pitz, A. Hamai, et al., Glioblastoma and chemoresistance to alkylating agents: involvement of apoptosis, autophagy, and unfolded protein response, Pharmacol. Ther. 184 (2018) 13–41, https://doi.org/10.1016/j.pharmthera.2017.10.017.
[6] T. Otto, P. Sicinski, Cell cycle proteins as promising targets in cancer therapy, Nat. Rev. Cancer 17 (2) (2017) 93–115, https://doi.org/10.1038/nrc.2016.138.
[7] F.E. Bleeker, S. Lamba, C. Zanon, R.J. Molenaar, T.J. Hulsebos, D. Troost, et al., Mutational profiling of kinases in glioblastoma, BMC Cancer 14 (2014) 718, https://doi.org/10.1186/1471-2407-14-718.

[8] D.J. Slamon, B. Leyland-Jones, S. Shak, H. Fuchs, V. Paton, A. Bajamonde, et al., Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2, N. Engl. J. Med. 344 (11) (2001) 783–792, https://doi.org/10.1056/NEJM200103153441101.

[9] P.B. Chapman, A. Hauschild, C. Robert, J.B. Haanen, P. Ascierto, J. Larkin, et al., Improved survival with vemurafenib in melanoma with BRAF V600E mutation, N. Engl. J. Med. 364 (26) (2011) 2507–2516, https://doi.org/10.1056/NEJMoa1103782.

[10] V.A. Miller, V. Hirsh, J. Cadranel, Y.M. Chen, K. Park, S.W. Kim, et al., Afatinib versus placebo for patients with advanced, metastatic non-small-cell lung cancer after failure of erlotinib, gefitinib, or both, and one or two lines of chemotherapy (LUX-Lung 1): a phase 2b/3 randomised trial, Lancet Oncol. 13 (5) (2012) 528–538, https://doi.org/10.1016/S1470-2045(12)70087-6.

[11] J.E. Cortes, D.W. Kim, H.M. Kantarjian, T.H. Brummendorf, I. Dyagil, L. Griskevicius, et al., Bosutinib versus imatinib in newly diagnosed chronic-phase chronic myeloid leukemia: results from the BELA trial, J. Clin. Oncol. 30 (28) (2012) 3486–3492, https://doi.org/10.1200/JCO.2011.38.7522.

[12] A.L. Nguyen, K. Schindler, Specialize and divide (Twice): functions of three Aurora kinase homologs in mammalian oocyte meiotic maturation, Trends Genet. 33 (5) (2017) 349–363, https://doi.org/10.1016/j.tig.2017.03.005.

[13] A.C. Borisa, H.G. Bhatt, A comprehensive review on Aurora kinase: small molecule inhibitors and clinical trial studies, Eur. J. Med. Chem. 140 (2017) 1–19, https://doi.org/10.1016/j.ejmech.2017.08.045.

[14] K.S. Borges, A.M. Castro-Gamero, D.A. Moreno, V. da Silva Silveira, M.S. Brassesco, R.G. de Paula Queiroz, et al., Inhibition of Aurora kinases enhances chemosensitivity to temozolomide and causes radiosensitization in glioblastoma cells, J. Cancer Res. Clin. Oncol. 138 (3) (2012) 405–414, https://doi.org/10.1007/s00432-011-1111-0.

[15] X. Yu, Q. Liang, W. Liu, L. Zhou, W. Li, H. Liu, Deguelin, an Aurora b kinase inhibitor, exhibits potent anti-tumor effect in human esophageal squamous cell carcinoma, EBioMedicine. 26 (2017) 100–111, https://doi.org/10.1016/j.ebiom.2017.10.030.

[16] X. Han, J.J. Zhang, Z.Q. Han, H.B. Zhang, Z.A. Wang, Let-7b attenuates cisplatin resistance and tumor growth in gastric cancer by targeting AURKB, Cancer Gene Ther. 25 (11–12) (2018) 300–308, https://doi.org/10.1038/s41417-018-0048-8.

[17] B. Subramaniyan, V. Kumar, G. Mathan, Effect of sodium salt of Butrin, a novel compound isolated from Butea monosperma flowers on suppressing the expression of SIRT1 and Aurora B kinase-mediated apoptosis in colorectal cancer cells, Biomed. Pharmacother. 90 (2017) 402–413, https://doi.org/10.1016/j.biopha.2017.03.086.

[18] B. Goldenson, J.D. Crispino, The aurora kinases in cell cycle and leukemia, Oncogene. 34 (5) (2015) 537–545, https://doi.org/10.1038/onc.2014.14.

[19] S. Kumari, D. Bhattacharya, N. Rangaraj, S. Chakarvarty, A.K. Kondapi, N.M. Rao, Aurora kinase B siRNA-loaded lactoferrin nanoparticles potentiate the efficacy of temozolomide in treating glioblastoma, Nanomedicine Lond. (Lond) 13 (20) (2018) 2579–2596, https://doi.org/10.2217/nnm-2018-0110.

[20] S. Hauf, R.W. Cole, S. LaTerra, C. Zimmer, G. Schnapp, R. Walter, et al., The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint, J. Cell Biol. 161 (2) (2003) 281–294, https://doi.org/10.1083/jcb.200208092.

[21] Z. Zhang, Z. Tang, C. Li, B. Kang, G. Gao, C. Li, GEPIA: A web server for cancer and normal gene expression profiling and interactive analyses, Nucleic Acids Res. 45 (W1) (2017) W98–W102, https://doi.org/10.1093/nar/gkx247.

[22] S. Madhavan, J.C. Zenklusen, Y. Kotliarov, H. Sahni, H.A. Fine, B.K. Rembrandt, Helping personalized medicine become a reality through integrative translational research, Mol. Cancer Res. 7 (2) (2009) 157–167, https://doi.org/10.1158/1541-7786.MCR-08-0435.

[23] J. Wang, P. Cheng, M.S. Pavlyukov, H. Yu, Z. Zhang, S.H. Kim, et al., Targeting NEK2 attenuates glioblastoma growth and radioresistance by destabilizing histone methyltransferase EZH2, J. Clin. Invest. 127 (8) (2017) 3075–3089, https://doi.org/10.1172/JCI89092.

[24] J. Stritzelberger, L. Distel, R. Buslei, R. Fietkau, F. Putz, Acquired temozolomide resistance in human glioblastoma cell line U251 is caused by mismatch repair deficiency and can be overcome by lomustine, Clin. Transl. Oncol. 20 (4) (2018) 508–516, https://doi.org/10.1007/s12094-017-1743-x.

[25] S. Yip, J. Miao, D.P. Cahill, A.J. Iafrate, K. Aldape, C.L. Nutt, et al., MSH6 mutations arise in glioblastomas during temozolomide therapy and mediate temozolomide resistance, Clin. Cancer Res. 15 (14) (2009) 4622–4629, https://doi.org/10.1158/1078-0432.CCR-08-3012.

[26] T.C. Chou, Drug combination studies and their synergy quantification using the Chou-Talalay method, Cancer Res. 70 (2) (2010) 440–446, https://doi.org/10.1158/0008-5472.CAN-09-1947.

[27] A. Linkous, D. Balamatsias, M. Snuderl, L. Edwards, K. Miyaguchi, T. Milner, et al., Modeling patient-derived glioblastoma with cerebral organoids, Cell Rep. 26 (12) (2019) 3203–3211, https://doi.org/10.1016/j.celrep.2019.02.063 e5.

[28] D. Alexandru-Abrams, M.R. Jadus, F.P. Hsu, A. Stathopoulos, D.A. Bota, Therapeutic targeting of malignant glioma, Anticancer Agents Med. Chem. 14 (8) (2014) 1075–1084.

[29] M. Minata, A. Audia, J. Shi, S. Lu, J. Bernstock, M.S. Pavlyukov, et al., Phenotypic plasticity of invasive edge glioma stem-like cells in response to ionizing radiation, Cell Rep. 26 (7) (2019) 1893–1905, https://doi.org/10.1016/j.celrep.2019.01.076 e7.

[30] Y. Zhang, C. Jiang, H. Li, F. Lv, X. Li, X. Qian, et al., Elevated Aurora B expression contributes to chemoresistance and poor prognosis in breast cancer, Int. J. Clin. Exp. Pathol. 8 (1) (2015) 751–757.

[31] J. Yu, J. Zhou, F. Xu, W. Bai, W. Zhang, High expression of Aurora-B is correlated with poor prognosis and drug resistance in non-small cell lung cancer, Int. J. Biol. Markers 33 (2) (2018) 215–221, https://doi.org/10.1177/1724600817753098.

[32] A.M. Sparta, D. Bressanin, F. Chiarini, A. Lonetti, A. Cappellini, C. Evangelisti, et al., Therapeutic targeting of Polo-like kinase-1 and Aurora kinases in T-cell acute lymphoblastic leukemia, Cell Cycle 13 (14) (2014) 2237–2247, https://doi.org/10.4161/cc.29267.

[33] R. Sorrentino, S. Libertini, P.L. Pallante, G. Troncone, L. Palombini, V. Bavetsias, et al., Aurora B overexpression associates with the thyroid carcinoma undifferentiated phenotype and is required for thyroid carcinoma cell proliferation, J. Clin. Endocrinol. Metab. 90 (2) (2005) 928–935, https://doi.org/10.1210/jc.2004-1518.

[34] Z. Yu, Y. Sun, X. She, Z. Wang, S. Chen, Z. Deng, et al., SIX3, a tumor suppressor, inhibits astrocytoma tumorigenesis by transcriptional repression of AURKA/B, J. Hematol. Oncol. 10 (1) (2017) 115, https://doi.org/10.1186/s13045-017-0483-2.

[35] A. Chalil, A. Weeks, S. Agnihotri, J. Lymer, R. Diaz, S. Isik, et al., Epithelial cell transforming 2 and Aurora kinase B modulate formation of stress granule-containing transcripts from diverse cellular pathways in astrocytoma cells, Am. J. Pathol. 186 (6) (2016) 1674–1687, https://doi.org/10.1016/j.ajpath.2016.02.013.

[36] P. Buczkowicz, M. Zarghooni, U. Bartels, A. Morrison, K.L. Misuraca, T. Chan, et al., Aurora kinase B is a potential therapeutic target in pediatric diffuse intrinsic pontine glioma, Brain Pathol. 23 (3) (2013) 244–253, https://doi.org/10.1111/j.1750-3639.2012.00633.x.

[37] S.W. Lai, B.R. Huang, Y.S. Liu, H.Y. Lin, C.C. Chen, C.F. Tsai, et al., Differential characterization of temozolomide-resistant human glioma cells, Int. J. Mol. Sci. 19 (1) (2018), https://doi.org/10.3390/ijms19010127.

[38] A.A. Stavrovskaya, S.S. Shushanov, E.Y. Rybalkina, Problems of glioblastoma multiforme drug resistance, Biochemistry Mosc. 81 (2) (2016) 91–100, https://doi.org/10.1134/S0006297916020036.

[39] J. Bertran-Alamillo, V. Cattan, M. Schoumacher, J. Codony-Servat, A. Gimenez-Capitan, F. Cantero, et al., AURKB as a target in non-small cell lung cancer with acquired resistance to anti-EGFR therapy, Nat. Commun. 10 (1) (2019) 1812, https://doi.org/10.1038/s41467-019-09734-5.

[40] G.C. Bobustuc, A.B. Kassam, R.A. Rovin, S. Jeudy, J.S. Smith, B. Isley, et al., MGMT inhibition in ER positive breast cancer leads to CDC2, TOP2A, AURKB, CDC20, KIF20A, cyclin A2, cyclin B2, cyclin D1, ERalpha and survivin inhibition and enhances response to temozolomide, Oncotarget. 9 (51) (2018) 29727–29742, https://doi.org/10.18632/oncotarget.25696.

[41] D.S. Moura, I. Campillo-Marcos, M. Vazquez-Cedeira, P.A. Lazo, VRK1 and AURKB form a complex that cross inhibit their kinase activity and the phosphorylation of histone H3 in the progression of mitosis, Cell. Mol. Life Sci. 75 (14) (2018) 2591–2611, https://doi.org/10.1007/s00018-018-2746-7.

[42] S.J. He, L.P. Shu, Z.W. Zhou, T. Yang, W. Duan, X. Zhang, et al., Inhibition of Aurora kinases induces apoptosis and autophagy via AURKB/p70S6K/RPL15 axis in human leukemia cells, Cancer Lett. 382 (2) (2016) 215–230, https://doi.org/10.1016/j.canlet.2016.08.016.

[43] C.P. Gully, G. Velazquez-Torres, J.H. Shin, E. Fuentes-Mattei, E. Wang, C. Carlock, et al., Aurora B kinase phosphorylates and instigates degradation of p53, Proc Natl Acad Sci U S A. 109 (24) (2012) E1513–22, https://doi.org/10.1073/pnas.1110287109.