Stigmasterol exerts antiglioma effects by regulating lipid metabolism

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Abstract. Stigmasterol is a sterol compound found in various traditional Chinese medicines; however, its effects on glioma remain unclear. The present study aimed to investigate the effects of stigmasterol on the biological behaviors of glioblastoma (GBM) cells and to explore the underlying mechanisms. *In vitro* experiments assessed its effects on GBM cell proliferation, apoptosis, cell cycle progression, invasion, migration and vasculogenic mimicry (VM). The potential targets for stigmasterol in treating GBM were identified using databases and Venn diagram analysis, followed by enrichment analysis using R language. A prognostic model related to the target genes of stigmasterol was developed through univariate Cox regression and least absolute shrinkage and selection operator analyses. Stigmasterol was found to suppress the proliferation of GBM cells in a dose- and time-dependent manner, to induce apoptosis, and to inhibit invasion, migration and VM formation. Additionally, 31 potential targets of stigmasterol were identified, linked to lipid metabolism and the G protein-coupled receptor signaling pathway. Lipid metabolism assays revealed that stigmasterol significantly reduced free fatty acids and total cholesterol levels. Furthermore, two prognosis-related target genes, fatty acid binding protein 5 and α-1B adrenergic receptor, were selected, and the prognostic model effectively predicted GBM outcomes. Moreover, molecular docking revealed strong binding affinities between stigmasterol and the target proteins. Overall, these findings suggested that stigmasterol may exert anti-glioma effects, which could be potentially mediated through the regulation of lipid metabolism.

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Introduction

Glioblastoma (GBM) is the most prevalent primary malignant brain tumor in humans (1). Despite global scientific and neurosurgical endeavors to advance GBM therapies, patient prognosis remains unfavorable (1); therefore, there is a critical need to discover more effective treatments to improve the outlook for patients with GBM. Natural medicines have demonstrated efficacy in treating GBM in the past few years (2,3). A number of active ingredients in these natural medicines, such as luteolin (4), quercetin (5) and kaempferol (6), have been proven to have antitumor effects. Stigmasterol, a widely occurring plant sterol, is present in numerous vegetable oils, cereals, seeds and some medicinal plants (7). In addition, it is the main ingredient of certain Chinese medicinal herbs (8). Currently, the main function of stigmasterol is considered to be maintenance of the structure and function of cell membranes (9). Stigmasterol has also been proven to have a range of pharmacological effects, including anticancer, antiosteoarthritis, anti-inflammatory, antidiabetic, immunomodulatory, antiparasitic, antibacterial, antioxidant and neuroprotective activities (7). Regarding the nervous system, stigmasterol has been revealed to have protective effects on nerve cells. It is able to maintain intracellular levels of reactive oxygen species in nerve cells and can prevent cell death induced by oxidative stress, alleviating mitochondrial dysfunction and DNA damage (10,11). Notably, there is a lack of research on the effects of stigmasterol on glioma; however, one study did indicate that stigmasterol could exhibit cytotoxic effects on glioma cells (12). In addition, previous research by the authors confirmed that stigmasterol could inhibit glioma cell proliferation and invasion (8). However, the precise mechanism by which stigmasterol operates in glioma remains elusive.

Lipid metabolic reprogramming is an important characteristic of glioma and regulating lipid metabolism in glioma can inhibit tumor growth (13). Therefore, lipid metabolism has emerged as a promising therapeutic target for glioma. Stigmasterol has long been considered to regulate lipid metabolism (14,15); however, the regulatory effect of stigmasterol on lipid metabolism in glioma has not been previously reported.

The primary objective of the present study was to assess the impact of stigmasterol on the biological behaviors of GBM cells, such as proliferation, apoptosis, migration, invasion,

vasculogenic mimicry (VM) and lipid metabolism and to elucidate the underlying mechanisms associated with these effects.

Materials and methods

Cell lines. The U87 American Type Culture Collection (ATCC) human GBM cell line of unknown origin (cat. no. CL-0238), U251 human GBM cell line (cat. no. CL-0237) and U118 MG human GBM cell line (cat. no. CL-0458) were purchased from Procell Life Science & Technology Co., Ltd. All of the human GBM cell lines (U87, U251 and U118 MG) were authenticated by short tandem repeat profiling.

Cell culture. All GBM cell lines were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (HyClone; Cytiva) at 37˚C in a 5% CO_2 atmosphere.

Cell Counting Kit-8 (CCK-8) cell proliferation assay. CCK-8 reagents (cat. no. QS-S321; Keycell Biotechnology, Inc.) were utilized to assess the impact of stigmasterol on the proliferation of GBM cells. GBM cells were seeded in 96-well plates at a density of $3x10^3$ cells/well. Various concentrations (10, 20, 40, 80, 120, 160, 200 and 240 μmol/l) of stigmasterol (cat. no. HY-N0131; MedChemExpress, Inc.) were separately added to the culture medium. The cells were then maintained in a 5% CO_2 atmosphere at 37˚C. GBM cell proliferation was evaluated using the CCK-8 assay at 24 and 48 h post-stigmasterol treatment. Specifically, 10 μl CCK-8 solution was added to each well, followed by incubation at 37˚C for 2 h. The absorbance was measured at 450 nm using a microplate reader (Flexstation 3; Molecular Devices, LLC). The proliferation rate was determined using the following equation: Proliferation rate (%)=($OD450_{\text{experimental group}}$ - $OD450_{\text{blank control}}$)/($OD450_{\text{control group}}$ - $OD450_{\text{blank control}}$) x100.

Flow cytometry for cell apoptosis and cell cycle detection. The U87 cells were seeded in 6-well plates at a density of $5x10^5$ cells/well and were treated with 240 μmol/l stigmasterol at 37˚C for 24 h. Cell apoptosis was evaluated using an Annexin V-fluorescein isothiocyanate kit (cat. no. QS-S306; Keycell Biotechnology, Inc.), whereas propidium iodide (cat. no. P4170; Sigma-Aldrich) was employed to assess cell cycle distribution, as per the manufacturers' guidelines. A CytoFLEX flow cytometer and CytExpert 2.4 software (both from Beckman Coulter, Inc.) were used for detection and analysis. Additionally, the proliferation index (PI) was calculated to indicate the proliferation level of each group using the formula: PI (%)=(S + G2/M)/(G0/G1 + S + G2/M) x100.

In vitro cell invasion assay. To investigate the impact of stigmasterol on the invasion of GBM cells *in vitro*, a cell invasion assay was conducted. Transwell chambers (pore size, 8 μm; polyethylene terephthalate membrane; cat. no. 353097; Falcon; Corning, Inc.) were positioned in 24-well plates. Matrigel (cat. no. 356234; Corning, Inc.) was precooled at 4˚C for 24 h, after which, 0.1 ml Matrigel was thinly spread onto the Transwell chambers that had been prechilled at -20˚C for ≥10 min, followed by an incubation at 37˚C for 30 min. U87 cells resuspended in serum-free medium ($3x10^5$ cells/ml) were added to the upper chamber (200 μl/well) along with 240 μmol/l stigmasterol, while medium containing 10% fetal bovine serum (800 μl/well) was added to the lower chamber, and cultured overnight in a 5% CO_2 atmosphere at 37˚C. The invasive cells that had attached to the lower surface of the membranes were fixed with 70% ethanol at 4˚C for 1 h, stained with 0.5% crystal violet (cat. no. C0121; Beyotime Institute of Biotechnology) at room temperature for 10 min and were then counted using an inverted fluorescence microscope (IX51; Olympus Corporation) at a magnification of x200.

Wound healing assay. To assess the influence of stigmasterol on the migration of GBM cells, a wound healing assay was employed. U87 cells were seeded in 6-well plates at a density of $1x10^6$ cells/well. After 24 h, when the cell confluence reached >90%, a straight line was created across the cell monolayer using a 20-μl pipette tip, and the cells were rinsed three times with PBS. Subsequently, the cells were cultured in serum-free medium containing 240 μmol/l stigmasterol in a 5% CO_2 atmosphere at 37˚C for 24 h. Images of the plates were captured using an inverted fluorescence microscope (IX51; Olympus Corporation) at a magnification of x40 at 0 and 24 h. ImageJ 1.8 software (National Institutes of Health) was utilized to measure the migration area, and the percentage of wound healing was computed using the formula: ($Area_{0\,h}$-$Area_{24\,h}$)/$Area_{0\,h}$.

In vitro VM assay. To evaluate the effects of stigmasterol on VM formation in GBM cells *in vitro*, a Matrigel-based tube formation assay was performed. Matrigel was maintained at 4˚C for 24 h, and then 0.1 ml of Matrigel was evenly spread in 12-well plates previously prechilled at -20˚C for ≥10 min, followed by a 30-min incubation at 37˚C. Subsequently, U87 cells ($3x10^4$ cells/well) were seeded onto the Matrigel and incubated in serum-free medium containing 240 μmol/l stigmasterol for 24 h in a 5% CO_2 atmosphere at 37˚C. The quantification of VM formation was conducted by counting the number of tubes in an entire well directly under an optical microscope (IX51; Olympus Corporation) at a magnification of x40.

In vitro lipid metabolism assay. U87 cells were seeded in 6-well plates at a density of $5x10^5$ cells/well and were treated with 240 μmol/l stigmasterol at 37˚C for 24 h. Free fatty acids (FFAs) were detected using an FFA assay kit (cat. no. A042; Nanjing Jiancheng Bioengineering Institute), and total cholesterol (T-CHO) levels were measured using a T-CHO assay kit (cat. no. A111-1; Nanjing Jiancheng Bioengineering Institute) in U87 cells, according to the manufacturer's protocols.

Prediction of potential targets of stigmasterol in the treatment of GBM. In a previous study (8), 3,100 differentially expressed genes (DEGs) of GBM were identified through the analysis of transcriptome sequencing data sourced from The Cancer Genome Atlas (TCGA)-GBM cohort (www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga). This analysis was conducted using R 4.3.2 software (The R Foundation for Statistical Computing). TGCA-GBM dataset included 174 samples, of which 159 samples were

from patients with GBM with complete survival data and five samples were normal control tissues (10 samples lacking complete survival data were excluded). The target genes of stigmasterol were identified using the SwissTargetPrediction database (www.swisstargetprediction.ch) and the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (16). The target genes of stigmasterol and the DEGs associated with GBM were compared using a Venn diagram analysis with Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/). The genes that overlapped between the target genes of stigmasterol and the DEGs of GBM were considered potential target genes for stigmasterol in the context of GBM treatment.

Enrichment analysis. The Gene Ontology (GO; https://geneontology.org/) enrichment, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (17) and WikiPathways (WP; https://www.wikipathways.org/) signaling pathway enrichment analyses for the identified target genes of stigmasterol in the treatment of GBM were conducted using the 'clusterProfiler', 'org.Hs.eg.db', 'enrichplot' and 'pathview' packages in R 4.3.2 software. A P-value cutoff of 0.05 and q-value cutoff of 0.05 were utilized for the analysis, and the results were visualized using the 'ggplot2' (version 3.5.1; https://github.com/tidyverse/ggplot2) package in R 4.3.2 software.

Screening of prognosis-related genes in GBM. Univariate Cox regression analysis was employed to identify mRNAs associated with overall survival (OS) in TCGA-GBM cohort with a significance level of $P<0.05$. This analysis was conducted using the 'survival' package in R software, as previously described (18).

Prediction of the prognosis-related target genes of stigmasterol for GBM treatment. In order to assess whether stigmasterol could enhance the prognosis of GBM, prognosis-related target genes for GBM treatment associated with stigmasterol were identified. This was achieved by intersecting genes linked to prognosis determined through univariate Cox regression analysis with the potential targets of stigmasterol in GBM treatment using a Venn diagram. Additionally, to verify whether these genes were related to lipid metabolism, a total of 14,568 lipid metabolism-related genes were acquired from the human gene database GeneCards (19) and were analyzed using a Venn diagram.

Construction and validation of a prognostic model associated with stigmasterol target genes. To verify the association between the selected target genes and the clinical prognosis of GBM, an mRNA signature was constructed as a prognostic model. As mentioned in a previous study (18), samples from GBM in TCGA-GBM cohort were randomly split into a training set (n=109) and an internal validation set (n=50). Additionally, transcriptome sequencing data of 236 GBM samples with complete survival information from the mRNAseq-693 dataset were obtained from the Chinese Glioma Genome Atlas database (20) and utilized as an external validation cohort. The least absolute shrinkage and selection operator (LASSO) algorithm (21) was employed, utilizing the 'glmnet' package (version 4.1; https://glmnet.stanford.edu/) in R 4.3.2, to prevent model overfitting. The risk score was computed based on LASSO regression coefficients using the following formula:

$$\text{Riskscore} = \sum_{i=1}^{n} coef(gene_i) * \exp r(gene_i)$$

The training set samples were categorized into high- and low-risk groups according to the median risk score, and Kaplan-Meier survival analysis was conducted to compare survival differences between these groups. The performance of the prognostic model was evaluated through the area under the curve (AUC) analysis of the receiver operating characteristic curve (ROC) using the 'timeROC' package (version 0.4; https://github.com/cran/timeROC/commit/c2c807f93b22a64643ed4682150ed90d278e9faa) in R 4.3.2. Furthermore, risk scores of patients with GBM in both the internal and external validation set were calculated following the same methodology as the training set to independently validate the performance of the prognostic model.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The U87 cells were seeded in 6-well plates at a density of $5x10^5$ cells/well and were treated with 240 μmol/l stigmasterol for 24 h. Subsequently, the cells were lysed, total RNA was isolated using TRIzol® reagent (cat. no. 15596-026; Invitrogen; Thermo Fisher Scientific, Inc.) and the RNA underwent RT utilizing the HiScript® II Q Select RT SuperMix kit (cat. no. R233; Vazyme Biotech Co., Ltd.). The temperature protocol for RT was 25˚C for 5 min, 50˚C for 15 min, 85˚C for 5 min and 4˚C for 10 min. Subsequently, qPCR was carried out using 2X Q3 SYBR qPCR Master Mix (cat. no. 22204; Tolo Biotech Co., Ltd.) on a ViiA™ 7 Real-Time PCR System, with the data analyzed using QuantStudio™ Real-Time PCR Software v1.6.1 (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: Initial denaturation at 95˚C for 10 min, followed by 40 cycles at 95˚C for 10 sec and 60˚C for 60 sec, and then one cycle at 95˚C for 15 sec, 60˚C for 1 min and 95˚C for 15 sec. Primers, which were designed and synthesized by Beijing Tsingke Biotech Co., Ltd. (Table I), were used for the analysis. The expression levels of target genes were normalized against the expression levels of β-actin and alterations in gene expression were assessed using the $2^{-\Delta\Delta Cq}$ method (22).

Western blotting. U87 cells were plated in 6-well plates at a density of $5x10^5$ cells/well and were treated with 240 μmol/l stigmasterol for 24 h. Subsequently, total cellular proteins were extracted, and western blotting was performed as previously described (23). The following primary antibodies were utilized for western blotting: Rabbit anti-fatty acid binding protein 5 (FABP5) antibody (1:2,000; cat. no. 12348-1-AP; Proteintech Group, Inc.), rabbit anti-α-1B adrenergic receptor (ADRA1B) antibody (1:1,000; cat. no. 22419-1-AP; Proteintech Group, Inc.) and mouse anti-β-actin antibody (1:5,000; cat. no. T0022; Affinity Biosciences). For detection, the following secondary antibodies were used: HRP-conjugated goat anti-rabbit immunoglobulin G (IgG) antibody (dilution, 1:5,000; cat. no. A0208; Beyotime Institute of Biotechnology) for FABP5 and ADRA1B detection, and HRP-conjugated goat anti-mouse IgG antibody (dilution, 1:5,000, cat. no. SA00001-1; Proteintech Group, Inc.) for β-actin detection.

Table I. Primers used for reverse transcription quantitative PCR.

Gene	Sequence	Size
FABP5	Forward, 5'-AATGGCCAAGCCAGATTGTA-3'	189 bp
	Reverse, 5'-AATGGCCAAGCCAGATTGTA-3'	
ADRA1B	Forward, 5'-TGGGGAGAGTTGAAAAATGC-3'	216 bp
	Reverse, 5'-GGCCAGGTTGACAATGAAGT-3'	
β-Actin	Forward, 5'-CCCTGGAGAAGAGCTACGAG-3'	180 bp
	Reverse, 5'-CGTACAGGTCTTTGCGGATG-3'	

FABP5, fatty acid binding protein 5; ADRA1B, α-1B adrenergic receptor.

Molecular docking. The 2D structure of stigmasterol was obtained from the DrugBank database (https://go.drugbank.com/). The 3D structures of the proteins corresponding to FABP5 and ADRA1B were retrieved from the RCSB Protein Data Bank (https://go.drugbank.com/). Molecular docking was performed using AutoDockTools software version 1.5.7 (Molecular Graphics Laboratory, The Scripps Research Institute). It is generally accepted that lower binding energy values indicate more stable docking interactions. A binding energy of <-5.0 kcal/mol suggests a favorable affinity between the receptor and the ligand, while a value <-7.0 kcal/mol denotes a very strong affinity. Visualization of the molecular docking results was conducted using PyMOL software version 2.5.4 (Schrödinger, LLC).

Statistical analysis. The data are expressed as the mean ± standard deviations. Statistical analysis was conducted using SPSS 25.0 software (IBM Corp.). One-way analysis of variance was employed to compare multiple groups, followed by the least significant difference post hoc test for intergroup comparisons. An unpaired Student's t-test was used for comparing two sets of independent sample data. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Stigmasterol inhibits the proliferation of GBM cells. As revealed in Fig. 1A, stigmasterol inhibited the proliferation of GBM cells in a dose-dependent manner. The proliferation rate of GBM cells significantly decreased with increasing stigmasterol concentration ($F=25.552$, $P=0.001$ for U87 cells; $F=12.824$, $P=0.001$ for U118 cells; $F=8.067$, $P=0.001$ for U251 cells). Moreover, stigmasterol inhibited the proliferation of GBM cells in a time-dependent manner. As revealed in Fig. 1B, under the same concentration of stigmasterol (240 μmol/l), the proliferation rate of GBM cells significantly decreased with increasing treatment time ($t=11.171$, $P=0.001$ for U87 cells; $t=3.034$, $P=0.039$ for U118 cells; $t=3.554$, $P=0.024$ for U251 cells). Flow cytometric analysis revealed that stigmasterol significantly increased the proportion of U87 GBM cells in the G_0/G_1 phase ($t=-31.038$, $P=0.001$), and reduced the proportion of U87 GBM cells in the S phase ($t=15.046$, $P=0.001$) and G_2/M phase ($t=16.542$, $P=0.001$) (Fig. 1E and F), thereby significantly reducing the PI of GBM cells ($t=30.991$, $P=0.001$; Fig. 1G).

Stigmasterol induces the apoptosis of GBM cells. As revealed in Fig. 1C and D, after treatment with stigmasterol, the proportion of apoptotic U87 GBM cells significantly increased compared with that in the control group ($t=-18.241$, $P=0.001$).

Stigmasterol inhibits the invasion, migration and VM of GBM cells. As revealed in Fig. 2A and D, in the *in vitro* cell invasion assay, stigmasterol significantly reduced the number of invasive U87 GBM cells ($t=26.833$, $P=0.001$). The wound healing assays revealed that the wound-healing ability of U87 GBM cells was significantly decreased following treatment with stigmasterol ($t=75.964$, $P=0.001$; Fig. 2C and F), suggesting that stigmasterol inhibited U87 GBM cell migration. Moreover, an *in vitro* VM assay revealed that stigmasterol significantly inhibited VM in U87 GBM cells *in vitro* ($t=8.328$, $P=0.001$; Fig. 2B and E).

Prediction of stigmasterol targets in the treatment of GBM. A comprehensive list of 112 target genes associated with stigmasterol was identified (Fig. 3A; Table SI). Subsequently, by identifying the overlap between potential stigmasterol target genes and DEGs associated with GBM, a total of 31 potential target genes for stigmasterol in GBM treatment were identified (Fig. 3A; Table SII).

Enrichment analysis of potential targets of stigmasterol in the treatment of GBM. A total of 207 GO terms were identified through GO enrichment analysis of 31 potential stigmasterol targets for treating GBM. This encompassed 152 enriched biological process (BP) terms, 18 enriched cellular component (CC) terms, and 37 enriched molecular function (MF) terms. The top 10 enriched terms across the three categories are displayed in Figs. 3B-D. In the figures, the length of the line indicates the number of genes enriched in the term, while the color reflects the significance of the enrichment. The outcomes suggested that the therapeutic mechanism of stigmasterol in GBM treatment may be linked to various BP terms, including the regulation of the 'G protein-coupled receptor signaling pathway' (GPCR) and certain neurotransmitter receptor signaling pathways, such as 'serotonin receptor signaling pathway' and 'acetylcholine receptor signaling pathway'. The associated MF terms comprised 'G protein-coupled neurotransmitter receptor activity', 'G protein-coupled amine receptor activity', 'neurotransmitter receptor activity' and 'transcription coactivator binding'. Furthermore, related

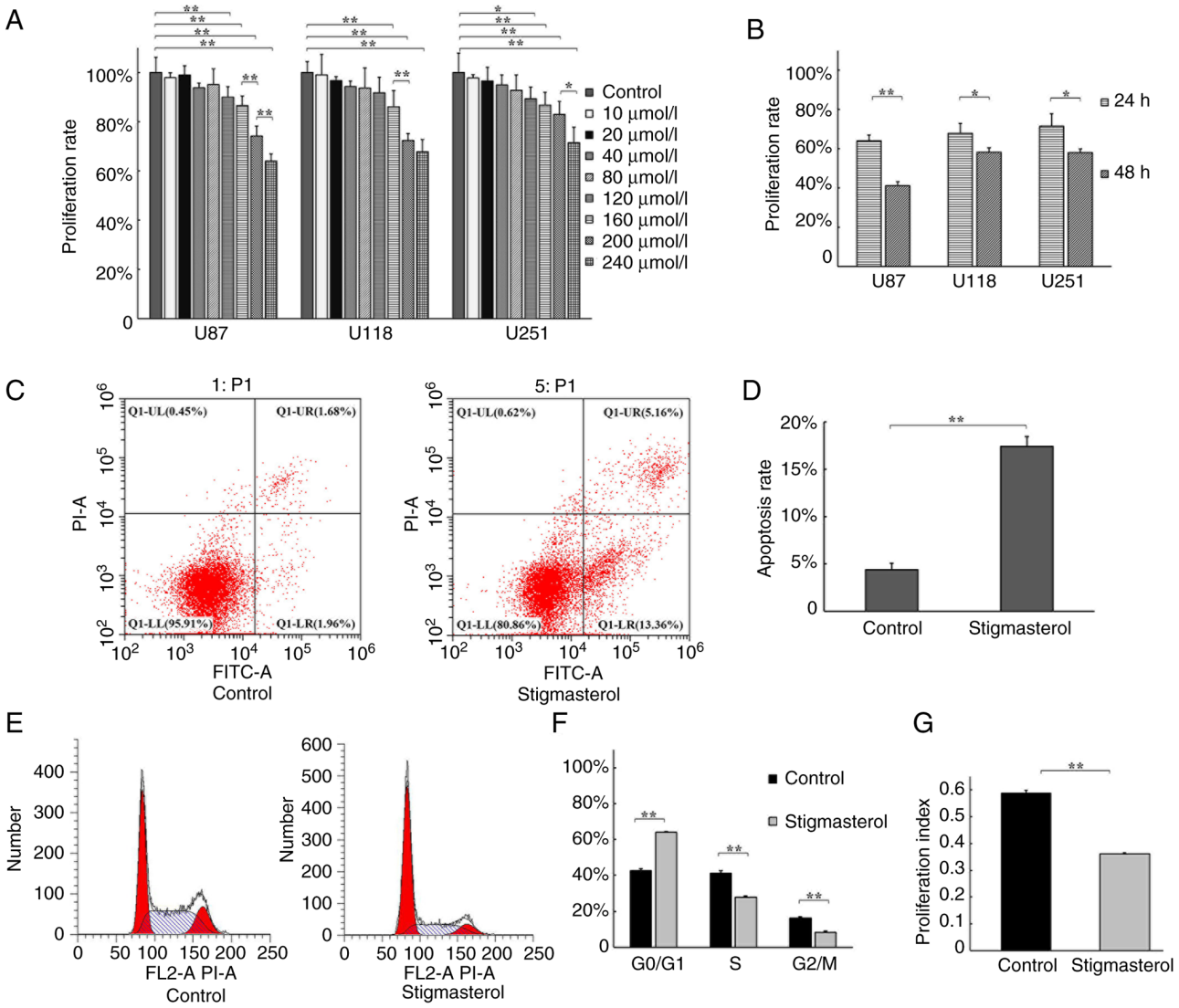


Figure 1. Stigmasterol inhibits the proliferation and induces the apoptosis of GBM cells. (A) Effect of different concentrations of stigmasterol on the proliferation of GBM cells. (B) Effect of different durations of stigmasterol intervention on the proliferation of GBM cells. (C) Apoptosis of GBM cells, as detected by flow cytometry. (D) Effect of stigmasterol on the apoptosis of GBM cells. (E) Cell cycle distribution of GBM cells, as detected by flow cytometry. (F) Effect of stigmasterol on the cell cycle distribution of GBM cells. (G) Effect of stigmasterol on the PI of GBM cells. *P<0.05 and **P<0.01. GBM, glioblastoma; PI, proliferation index.

CC terms included the 'postsynaptic membrane', 'synaptic membrane', and 'plasma membrane raft'.

Through KEGG pathway enrichment analysis (Fig. 3E), three pathways were identified, and 20 pathways were identified through WP signaling pathway analysis (Fig. 3F). In the figures, the length of the line corresponds to the number of genes enriched in the pathway, and the color reflects the significance of the enrichment. The outcomes indicated that the potential targets of stigmasterol in the treatment of GBM were associated with pathways involved in regulating 'neuroactive ligand-receptor interaction', 'regulation of lipolysis in adipocytes', 'small ligand GPCRs', 'calcium signaling pathway', 'head and neck squamous cell carcinoma' and 'fatty acid transporters'.

Prediction of prognosis-related targets of stigmasterol for GBM treatment. A Venn diagram predicted that all 31 potential target genes of stigmasterol were related to lipid metabolism (Fig. 4A). Univariate Cox regression analysis identified 325 mRNAs that were significantly associated with the OS of patients with GBM (P<0.05; Table SIII). After identifying the intersection of potential targets of stigmasterol in the treatment of GBM, prognosis-related mRNAs of patients with GBM and lipid metabolism-related genes, two prognosis-related target genes of stigmasterol for GBM treatment were identified, FABP5 and ADRA1B (Fig. 4A and B).

Establishment and validation of a prognostic model associated with stigmasterol target genes. The corresponding regression coefficients of FABP5 and ADRA1B were calculated by LASSO analysis (Fig. 4C and D). A prognostic model was developed using the expression levels of the two identified prognosis-related genes and their corresponding regression coefficients in the TCGA-GBM training set. The risk score calculation involved the formula: Risk score=0.14xFABP5 + 0.25xADRA1B. Patients were categorized into high- and

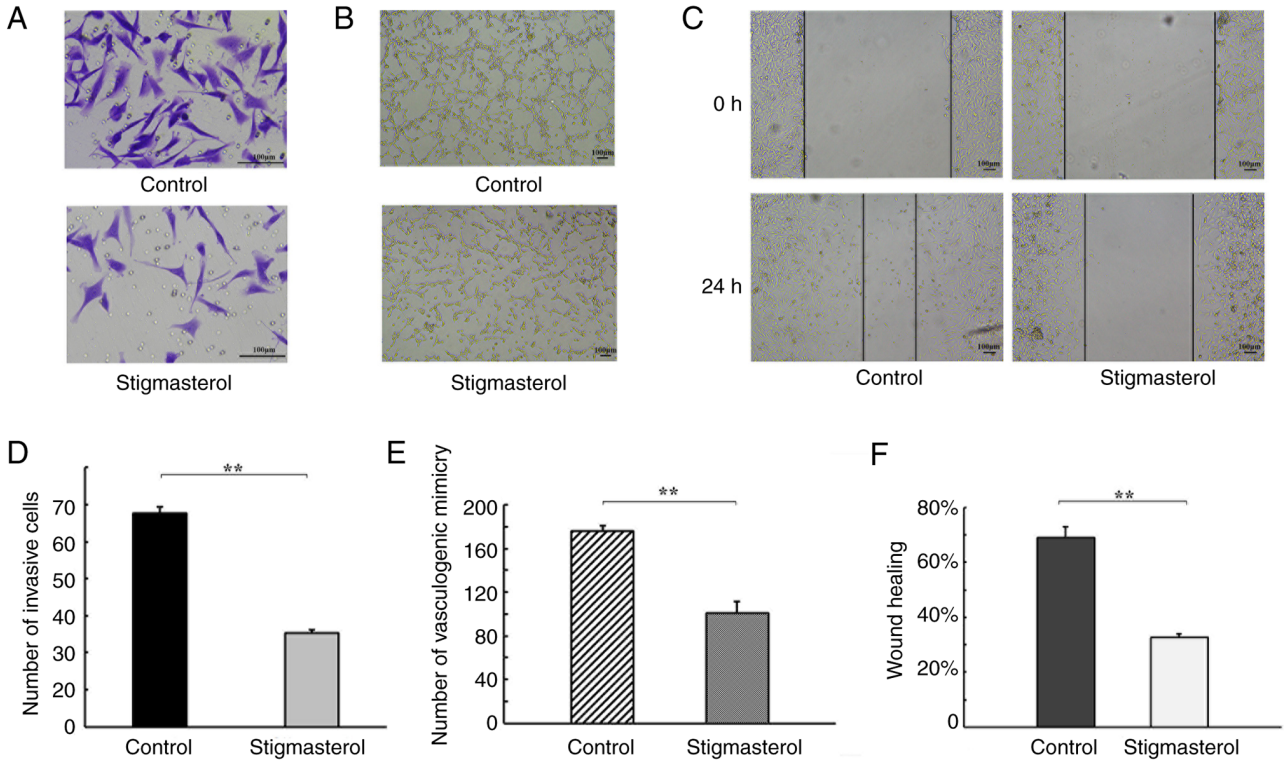


Figure 2. Stigmasterol inhibits the invasion, migration and VM of U87 GBM cells. (A and D) Effect of stigmasterol on the invasion of U87 GBM cells. (B and E) Effect of stigmasterol on VM in U87 GBM cells. (C and F) Effect of stigmasterol on the migration of U87 GBM cells. **P<0.01. GBM, glioblastoma; VM, vascular mimicry.

low-risk groups based on the median risk score of 1.038 in TCGA-GBM training set. The risk groups, survival status and risk score distribution of patients are illustrated in Fig. 4E. The expression patterns of the two prognostic genes demonstrated higher expression in patients with elevated risk scores (Fig. 4F). ROC curves were generated for patient survival at years 1, 3 and 5, with all AUC values being >0.6, indicating the effectiveness of the risk model (Fig. 4G). Survival analysis revealed a notably worse OS in the high-risk group compared with that in the low-risk group (P=0.036; Fig. 4H).

The validation sets also revealed good prediction accuracy of this prognostic model. In the internal validation set, patients were classified into high- and low-risk groups according to a median risk score of 0.98. The AUC values of the ROC curves were all >0.5 (Fig. 4I), and patients in the high-risk group had a worse prognosis (P=0.033; Fig. 4J). Consistent results were obtained in the external validation set (Fig. 4K and L).

Stigmasterol regulates lipid metabolism in GBM cells. Since the aforementioned results suggested that the potential mechanisms of stigmasterol in the treatment of GBM may be related to lipid metabolism, the effect of stigmasterol on lipid metabolism in U87 GBM cells was detected. As demonstrated in Fig. 5A and B, an *in vitro* lipid metabolism assay revealed that stigmasterol significantly reduced FFA and T-CHO levels in U87 GBM cells (t=4.097, P=0.015 for FFAs; t=4.930, P=0.008 for T-CHO).

Stigmasterol inhibits the expression of prognosis-related targets in GBM. Since FABP5 and ADRA1B are potential targets of stigmasterol related to GBM prognosis, the expression of these two genes were detected in U87 GBM cells after stigmasterol treatment. As revealed in Fig. 5C, stigmasterol significantly reduced the mRNA expression levels of FABP5 and ADRA1B in U87 cells (t=9.909, P=0.001 for FABP5; t=3.319, P=0.029 for ADRA1B). Moreover, stigmasterol also reduced the protein expression of FABP5 and ADRA1B in U87 cells (Fig. 5D). Molecular docking analysis revealed that the binding energies of stigmasterol with FABP5 (Fig. 5E) and ADRA1B (Fig. 5F) were -8.3 and -7.6 kcal/mol, respectively. These values suggested that there are favorable binding interactions between the target proteins and stigmasterol.

Discussion

A number of studies have confirmed that stigmasterol has antitumor effects on various types of malignant tumors, such as gastric cancer (24), breast cancer (25), liver cancer (26), skin cancer (27) and cholangiocarcinoma (28). However, there are currently few reports on whether stigmasterol has antitumor effects on glioma. In a previous study by the authors, stigmasterol was confirmed to inhibit the proliferation of U87 GBM cells (8). The present study further confirmed that stigmasterol inhibited the proliferation of GBM cells in a dose- and time-dependent manner. As the concentration of stigmasterol or the intervention time increased, its ability to inhibit the proliferation of GBM cells increased. The results of the proliferation assay revealed that among the three GBM cell lines tested, stigmasterol appeared to have a more significant inhibitory effect on the U87 GBM cell line. Therefore, the U87 cell line was selected for subsequent experiments.

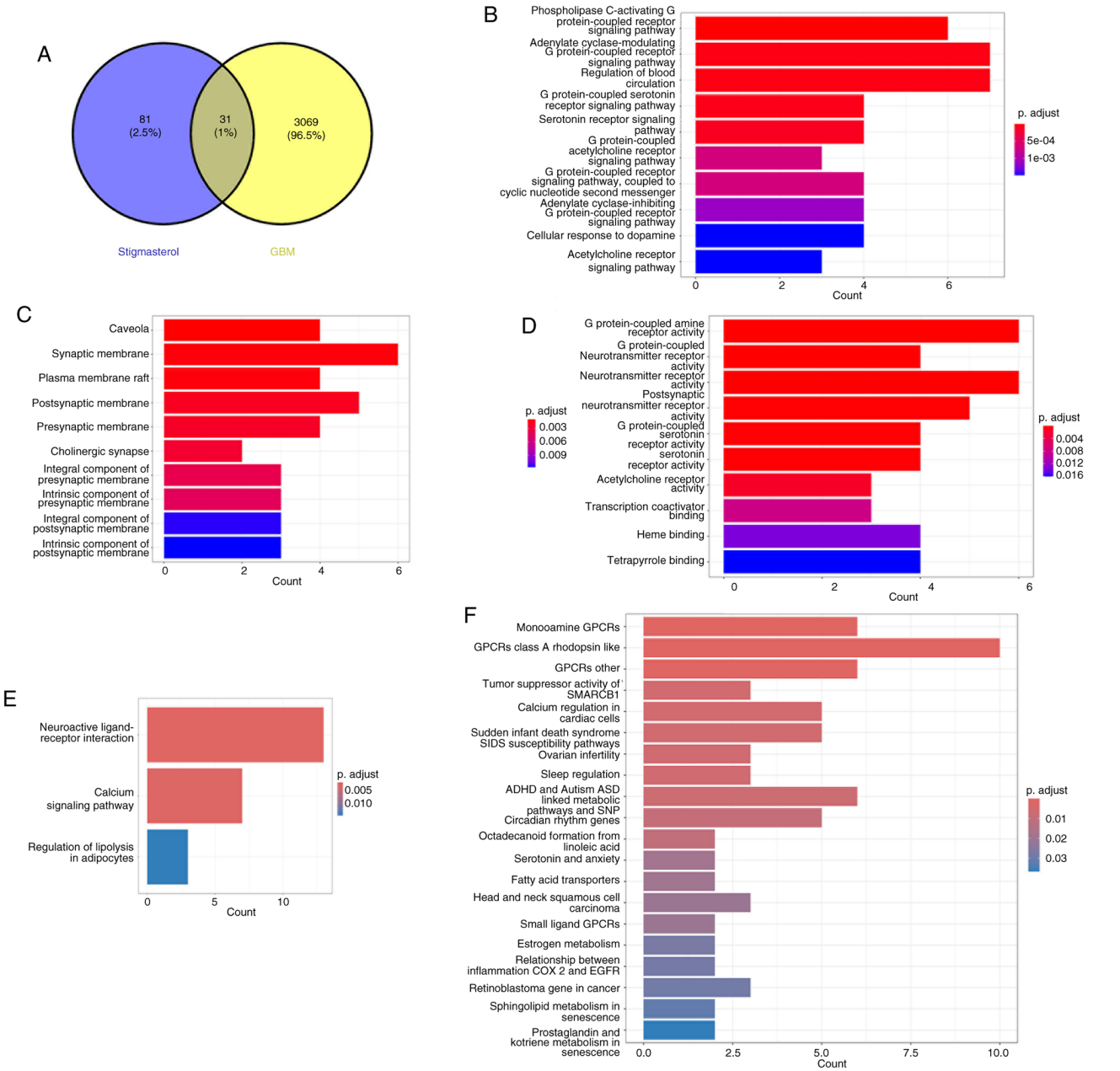


Figure 3. Enrichment analysis of potential targets of stigmasterol in the treatment of GBM. (A) Venn diagram of potential target genes of stigmasterol and differentially expressed genes in GBM. (B, C and D) Top 10 enriched terms from the Gene Ontology analysis. (B) Biological process terms. (C) Cellular component terms. (D) Molecular function terms. (E) Kyoto Encyclopedia of Genes and Genomes pathway analysis. (F) WikiPathways analysis. GBM, glioblastoma.

Flow cytometric analysis revealed that after treatment with stigmasterol, U87 GBM cells exhibited G_0/G_1 phase arrest and increased apoptosis, which may be important mechanisms by which stigmasterol inhibits the proliferation of GBM cells. In subsequent studies, stigmasterol significantly inhibited the invasion, migration and VM of U87 GBM cells *in vitro*. These biological behaviors are closely related to the growth of glioma (29,30); therefore, stigmasterol may be able to exert its anti-glioma effect through these mechanisms.

The present study further used enrichment analysis to explore the potential mechanism underlying the anti-glioma effect of stigmasterol. The results suggested that GPCR signaling pathways may serve important roles in this process. GPCRs are a large family of membrane receptors that influence various biological behaviors of tumors through canonical and noncanonical signaling pathways, making them important molecular components that control tumorigenesis (31). In canonical GPCR signaling pathways, receptor ligand binding leads to the binding and activation of G proteins, further activating downstream second messengers to elicit a cellular response. In addition to regulating canonical GPCR signaling pathways, GPCRs can regulate cellular biological behaviors through noncanonical signaling pathways, such as the transactivation of receptor tyrosine kinases (31). It has been reported that GPCRs are involved in regulating various biological behaviors of glioma, such as the cell cycle, proliferation, migration, invasion, metastasis, drug resistance, and angiogenesis (31-33). The expression of GPCR genes has also been

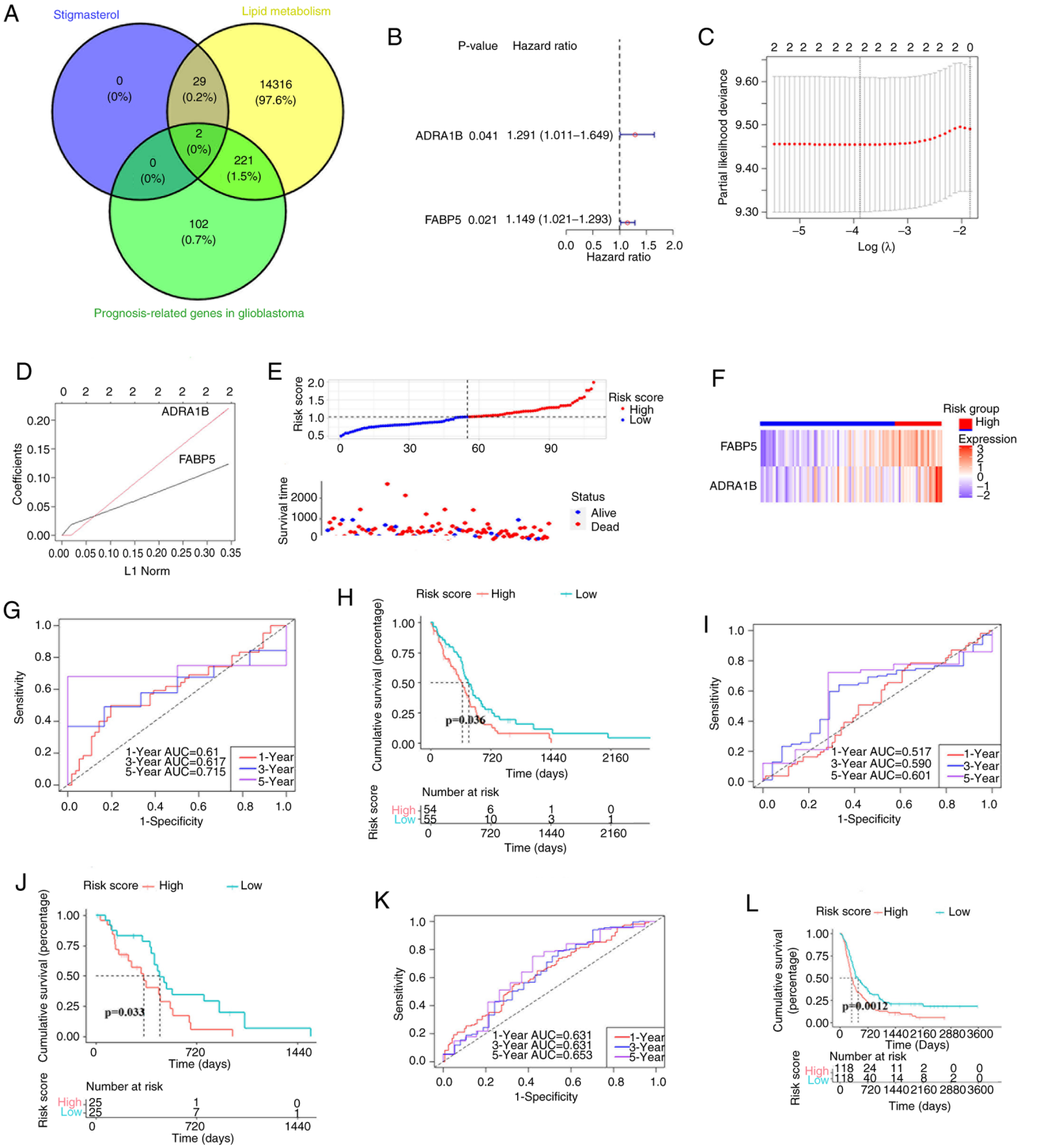


Figure 4. Construction and validation of a prognostic model associated with stigmasterol target genes. (A) Venn diagram of potential targets of stigmasterol in the treatment of GBM, prognosis-related genes of patients with GBM and lipid metabolism-related genes. (B) Two prognosis-related target mRNAs of stigmasterol for GBM treatment were selected. (C and D) Least absolute shrinkage and selection operator variable screening process. (E) Distribution of survival status and risk score of patients with GBM in the training set. (F) Expression characteristics of the two prognosis-related target mRNAs of stigmasterol for GBM treatment in the training set. (G) ROC curve for patient survival of the training set. (H) Survival analysis between the two risk subgroups in the training set. (I) ROC curve for patient survival of the internal validation set. (J) Survival analysis between the two risk subgroups in the internal validation set. (K) ROC curve for patient survival of the external validation set. (L) Survival analysis between the two risk subgroups in the external validation set. GBM, glioblastoma; ROC, receiver operating characteristic; FABP5, fatty acid binding protein 5; ADRA1B, α-1B adrenergic receptor; AUC, area under the curve.

reported to be closely related to the prognosis of glioma (34). Targeting GPCRs is considered a potential glioma treatment strategy (35,36).

Another notable mechanism identified by the enrichment analysis was that stigmasterol may exert anti-glioma effects by regulating lipid metabolism. Lipid metabolism has emerged as a crucial factor in the biology of GBM, significantly influencing tumor growth, invasion and therapeutic resistance. Recent studies have indicated that GBM cells exhibit altered lipid metabolism, characterized by increased fatty acid

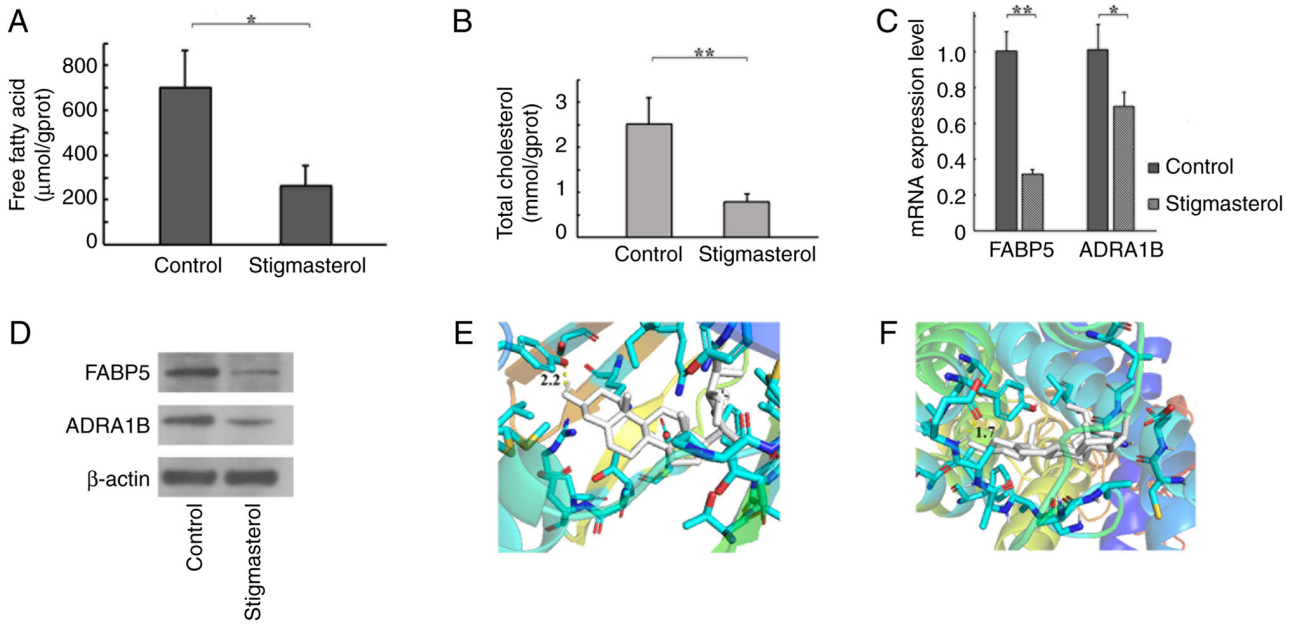


Figure 5. Effects of stigmasterol on lipid metabolism and the expression of related target genes in GBM cells. (A) Effect of stigmasterol on free fatty acid levels in GBM cells. (B) Effect of stigmasterol on total cholesterol levels in GBM cells. (C) Effect of stigmasterol on the mRNA expression of the two target genes (FABP5 and ADRA1B) in GBM cells. (D) Effect of stigmasterol on the expression of the proteins encoded by the two target genes in GBM cells. (E) Molecular docking of the protein encoded by FABP5 with stigmasterol. (F) Molecular docking of the protein encoded by ADRA1B with stigmasterol. *P<0.05 and **P<0.01. GBM, glioblastoma; FABP5, fatty acid binding protein 5; ADRA1B, α-1B adrenergic receptor.

synthesis and uptake, which supports rapid cell proliferation and survival in the adverse tumor microenvironment (37,38). This shift in metabolic pathways enables GBM cells to utilize lipids as an energy source, and contributes to the formation of membrane structures imperative for cell division and migration (38). In addition, the expression of genes involved in lipid metabolism has been associated with the prognosis of patients with GBM, highlighting the therapeutic potential of targeting lipid metabolic pathways (39). For specific lipids, some studies have highlighted the crucial role of FFAs and T-CHO levels in the proliferation and survival of GBM cells. Elevated levels of FFAs and cholesterol are commonly observed in tumor environments and significantly contribute to the aggressive nature of GBM (40,41). FFAs can serve as essential energy sources, supporting the rapid metabolism and energy demands of proliferating tumor cells (40), while high cholesterol levels can enhance membrane fluidity, and promote the formation of lipid rafts that facilitate signaling pathways crucial for cell survival and metastasis (41). In this context, the reduction of FFAs and T-CHO levels presents a potential therapeutic strategy for GBM suppression. By targeting lipid metabolism, interventions that lower these lipid levels may disrupt the metabolic flexibility of GBM cells, effectively hindering their growth and invasive capabilities (40,41). Therefore, strategies aimed at lowering FFAs and cholesterol may enhance the effectiveness of existing treatments and offer promising opportunities for GBM therapy. The results of the present study indicated that all 31 potential target genes of stigmasterol were related to lipid metabolism, and a lipid metabolism assay confirmed that stigmasterol reduced the content of FFAs and T-CHO in GBM cells, suggesting that the anti-glioma effect of stigmasterol may be related to its regulation of lipid metabolism.

Furthermore, in the present study, two lipid metabolism-related genes, FABP5 and ADRA1B, were selected as prognosis-related targets of stigmasterol for GBM treatment through univariate Cox regression analysis and Venn diagram analysis. The constructed prognostic model based on the expression levels of these two genes effectively predicted GBM prognosis, suggesting that targeting these two genes may improve GBM prognosis. In the present study, stigmasterol significantly reduced the expression of FABP5 and ADRA1B in GBM cells. FABP5, or epidermal fatty acid-binding protein, is recognized for its significant involvement in various biological processes, such as lipid metabolism, metabolic syndrome, cell growth, cell differentiation, immune response, neurite outgrowth, axon development, and inflammatory cytokine production (42). Previous studies have confirmed that FABP5 can promote tumor proliferation, progression and metastasis by regulating lipid metabolism (43-45). FABP5 can also act as an oncogene by promoting lipid droplet deposition, which may be achieved by activating the WNT/β-catenin signaling pathway (43). In glioma, FABP5 has been revealed to be associated with tumor malignancy (46). The knockdown of FABP5 resulted in a considerable reduction in the viability, proliferation, invasion and migration of glioma cells (47). Neurotransmitters and neuropeptides can stimulate tumor growth by acting on receptors expressed by tumor cells (48). As a neurotransmitter receptor, ADRA1B also has an important role in malignant tumor progression. Notably, ADRA1B can promote the growth of neuroblastoma (49). The expression levels of ADRA1B are considered to be negatively correlated with the prognosis of GBM (50) and breast tumors (51). In lipid metabolism, ADRA1B is involved in regulating the accumulation of fatty droplets within liver cells and fatty acid synthase (52). However, there are currently limited studies

on the relationship between ADRA1B and lipid metabolism in tumors. The results of the present study suggested that the potential anti-glioma effect of stigmasterol may be related to its inhibition of FABP5 and ADRA1B expression. Considering the association between the expression levels of these two genes and the prognosis of GBM revealed in the present study, it could be hypothesized that stigmasterol may improve the prognosis of GBM.

Notably, although the present study has provided valuable insights into the effects of stigmasterol on GBM, there are still some limitations. First, identifying potential target genes of stigmasterol from databases may have limitations, as some target genes might have been excluded due to constraints in the data sources. Second, the effects of stigmasterol on normal brain cells have not been studied. Comparative analyses with non-cancerous cell lines are essential to demonstrate the safety of stigmasterol in a therapeutic context. Furthermore, the present study lacks in-depth mechanistic insights regarding the specific downstream effects of stigmasterol and its potential interactions with other signaling pathways. This limitation hinders a comprehensive understanding of how stigmasterol affects GBM at the molecular level. Additionally, while the present study observed dose-dependent and time-dependent effects of stigmasterol on GBM cells, determining the optimal therapeutic dose, therapeutic window, potential side effects, chronic administration and potential resistance development in GBM will be pivotal for safely incorporating stigmasterol into treatment regimens. These are also shortcomings of the present study. Moreover, the present study predominantly relied on *in vitro* experiments, indicating an urgent need for robust *in vivo* studies using animal models to validate the anti-glioma effects of stigmasterol and to investigate its pharmacokinetics and pharmacodynamics.

To address the limitations in the present study, future research will be organized into several key areas, focusing on both *in vitro* and *in vivo* studies, as well as clinical trials. In the *in vitro* studies, the focus will be on investigating the effects of stigmasterol on GPCR signaling pathways, lipid metabolic processes, and apoptosis pathways (such as intrinsic and extrinsic pathways, caspase activation, and mitochondrial involvement), as well as elucidating the specific mechanisms of action, including the role of key regulators. Transcriptomics, proteomics, and metabolomics methods will be used to study these mechanisms. Furthermore, experimental validation of identified targets will include knockdown and overexpression studies for key genes such as FABP5 and ADRA1B to confirm their involvement in the action of stigmasterol. In addition to using established cell lines, primary cultured GBM cells will also be employed in these studies. Furthermore, the effects of stigmasterol on various cells in the tumor microenvironment, including normal neuronal cells and glial cells, will also be studied *in vitro*.

In terms of *in vivo* study, the effects of stigmasterol on the GBM tumor microenvironment, including its effects on normal brain cells, immune cells, and other components, will be assessed. Meanwhile, the therapeutic potential of stigmasterol, including its anti-glioma effects, safety, potential side effects, drug resistance, optimal administration route, and combination therapy strategies, will also be assessed in *in vivo* studies. In addition, pharmacological characteristics of stigmasterol will also be investigated to better understand its therapeutic profile. Concurrently, a comprehensive analysis of how stigmasterol affects the metabolism of GBM will be conducted using metabolomics approaches.

In terms of clinical research, studies will be conducted to investigate the pharmacological properties of stigmasterol in the treatment of GBM, the impact of patient genetic polymorphisms on the efficacy of stigmasterol, and the relationship between lipid metabolism markers and GBM progression and treatment response. Long-term studies on chronic administration and potential resistance development will also be taken into consideration. Furthermore, the interactions of stigmasterol with other GBM treatments, such as temozolomide, radiotherapy and immunotherapy, will be investigated to explore the potential for synergistic effects when used in combination therapies. In addition, the prognostic model established in the present study based on FABP5 and ADRA1B will also be validated in larger independent cohorts and prospective clinical trials to ensure its robustness and utility. Furthermore, the effects of stigmasterol on GBM stem-like cells will be analyzed both *in vitro* and *in vivo*, as these cells are often responsible for tumor recurrence and progression. Finally, based on the aforementioned *in vivo*, *in vitro*, and clinical trials, investigating nanoparticle-based drug delivery systems to enhance the bioavailability and targeted delivery of stigmasterol to GBM cells will also be key focuses of future research.

In conclusion, the present study confirmed that stigmasterol can promote the apoptosis of GBM cells, and inhibit their proliferation, migration, invasion and VM. Enrichment analysis suggested that the potential target genes and related signaling pathways affected by stigmasterol in the treatment of GBM are associated with lipid metabolism and GPCRs. In addition, stigmasterol inhibited the expression of target genes negatively associated with the prognosis of GBM and reduced lipid levels in GBM cells. These results suggested that stigmasterol may have potential effects on the treatment of glioma and improve the prognosis of GBM. The regulation of lipid metabolism by stigmasterol may be a potential mechanism underlying its anti-glioma effect.

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Availability of data and materials

Part of the data generated in the present study may be found in the TCGA database (www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga), TCMSP database (www.tcmsp-e.com/#/database), CGGA database (www.cgga.org.cn), GeneCards database (www.genecards.org), SwissTargetPrediction database (www.swisstargetprediction.ch), DrugBank database (https://go.drugbank.com/) and RCSB Protein Data Bank (https://go.drugbank.com/). The

data generated in the present study may be requested from the corresponding author.

Authors' contributions

CL designed the research. TW conducted the *in vitro* experiments. RL performed the bioinformatics analysis and SG handled statistical data analysis. CL and TW contributed to manuscript writing, and confirm the authenticity of all the raw data. All authors read and approved the final version of the 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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