Article

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Protective Role of Protocatechuic Acid in Glioma: Modulation of Cell Growth, Migration, and Pyroptosis via NLRP3/Caspase-1/GSDMD Axis

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Background: Glioblastoma is a common primary malignant tumor posing a serious threat to human life and health. Protocatechuic acid (PCA) is a natural phenolic compound with good anti-tumor activity. The study aimed to investigate whether pyroptosis can be activated by PCA in glioma cell.
Methods: Different concentrations of PCA were used to treat glioma cell lines U87 and U251 for varying durations. Cell proliferation was quantified using the Cell Counting Kit-8 (CCK-8) assay. The Transwell chamber assay was employed to evaluate cell invasion, while cell migration was assessed via the scratch assay. Pyroptosis levels were determined through immunofluorescence staining. Additionally, the protein and mRNA expression levels of nucleotide-binding and oligomerization domain-like receptor thermal protein domain-associated protein 3 (NLRP3), cysteinyl aspartate specific proteinase (caspase-1), and gasdermin D (GSDMD) were analyzed using Western blotting and quantitative reverse-transcription polymerase chain reaction (qRT-PCR).
Results: Intervention with PCA resulted in a significant suppression of viability, invasion and migration of glioma cells in a dose-dependent manner ($p < 0.05$). Additionally, the GSDMD positivity rate, as well as the protein and mRNA expression levels of NLRP3, caspase-1, and GSDMD showed significant increases in glioma cells ($p < 0.05$). Further intervention with NLRP3-specific inhibitor MCC950 reversed the effects of PCA and resulted in a significant increase in cell viability and number of invading cells ($p < 0.01$), a significant decrease in GSDMD positivity ($p < 0.01$), and a significant decrease in the protein and mRNA expression levels of NLRP3, caspase-1, and GSDMD in glioma cells ($p < 0.01$).
Conclusion: PCA mediates pyroptosis in glioma cells by regulating the NLRP3/caspase-1/GSDMD signaling pathway.

Keywords: protocatechuic acid; glioma cells; U87; U251; NLRP3/caspase-1/GSDMD pathway; pyroptosis

Introduction

Glioblastoma (GBM) is the most common type of brain tumor [1]. According to statistics, individuals with GBM have an average survival period of 12 to 15 months, with a 5-year survival of merely around 5% [2]. Unfortunately, the global incidence of GBM presents an increasing trend on an annual basis, posing a significant health threat to patients and encumbering the current healthcare system.

At present, the main clinical approach for treating GBM is surgical resection, followed by radiotherapy and chemotherapy. This combination aims to prevent tumor recurrence and metastasis, thereby extending patient survival [3]. However, the invasive growth of GBM cells causes potential damage to surrounding normal tissues, making complete surgical resection challenging. Additionally, radiotherapy and chemotherapy have significant side effects on patients [4]. Owing to its unique theoretical system and efficacy, traditional Chinese medicine (TCM) is gradually gaining recognition in the realm of modern medicine. TCM therapies offer the advantages of multi-pathway, multi-target, and multi-functional effects, garnering widespread attention by virtue of minimal side effects, high relative safety, sustained inhibitory action, and favorable results in GMB treatment [5]. Thus, TCM can play a more important role as a part of the comprehensive GMB therapy [6].

Protocatechuic acid (PCA) is a naturally existing phenolic compound abundant in fruits and vegetables [7], showcasing multiple beneficial biological activities, including anti-inflammatory, neuroprotective, antitumor, antibacterial, antidiabetic, and anti-apoptotic properties [8,9]. It has been reported that PCA effectively inhibits the activity of human glioma cell line U251MG. Furthermore, PCA can inhibit cell proliferation by inducing apoptosis in glioma cells [10], indicating its potential as a therapeutic agent for GMB.

Pyroptosis is a type of regulated cell death that is both lytic and pro-inflammatory. This process is characterized by cell swelling, the formation of membrane pores, and ultimately the rupture of the plasma membrane [11]. Pyroptosis is involved in tumor immunoregulation, influencing tumor progression and treatment [12]. In the es-

tablished pathway of pyroptosis, the nucleotide-binding and oligomerization domain-like receptor thermal protein domain-associated protein 3 (NLRP3) inflammasome recruits and binds the apoptosis-associated speck-like protein containing a Caspase Recruitment Domain (Apoptosis-associated Speck-like protein containing a CARD), leading to the aggregation of ASC. This aggregation then recruits and activates the cleaved caspase-1. Caspase-1 is responsible for inducing the production of interleukin (IL)-18/IL-1β and cleaving the cleaved N-terminal-gasdermin D (cleaved N-terminal-GSDMD). Upon released, the cleaved N-terminal-GSDMD induces the formation of membrane pores. As a consequence, IL-18 and IL-1β are released, leading to the accumulation of fluid outside the cells, which causes cell swelling, membrane breakdown, and ultimately cell demise [13,14]. Drugs or compounds have been reported to cause tumor cell damage by inducing cellular pyroptosis [11,15]. Therefore, we hypothesize that PCA plays a role in activating the NLRP3/caspase-1/GSDMD pathway, triggering pyroptosis in glioma cells.

Materials and Methods

Cells

Human glioma cell lines U87 (Cat. No.: CL-0238) and U251 (Cat. No.: CL-0237) were procured from Wuhan Pricella Biotechnology Co., Ltd. (Wuhan, China). All cell lines were authenticated by means of short tandem repeat (STR) analysis and confirmed to be mycoplasma-free after testing. The cells were then cultivated in Dulbecco's Modified Eagle Medium (DMEM; A4192002, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution (V900929, Sigma-Aldrich, St. Louis, MO, USA). The cells were maintained in an incubator set at 5% CO_2 and 37 °C.

Cell Counting Kit-8 (CCK-8) Assay

After being resuspended in a culture medium, the cell suspension with a density of 6×10^3 cells/mL was seeded in 96-well plate. Subsequently, the cells were exposed to varying concentrations of PCA (1.25 µM, 2.5 µM, 5 µM, 10 µM, 20 µM) (2954-52-1, J&K Scientific, Beijing, China) [10] or PCA+MCC950 (NLRP3-specific inhibitor, 50 µM; 256373-96-3, Sigma-Aldrich, St. Louis, MO, USA) for 24 or 48 h [16]. After removing the supernatant, 10 µL of CCK-8 solution (CK04, Dojindo, Shanghai, China) was added to each well prior to incubation for 2 h. Cell viability was determined by measuring absorbance at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA) and calculated with the formula in the following:

Cell survival rate (%) = $A_{PCA}/A_{control} \times 100$ (A refers to the absorbance values obtained from the microplate reader)

Calculating the cell survival rate was performed to determine the appropriate dosage and treatment duration.

Transwell Assay

In the Transwell assay, 600 µL of culture medium supplemented with 10% fetal bovine serum (FBS) was added into each well on a 24-well plate, followed by the addition of PCA (2.5 µM, 5 µM, 10 µM) or a combination of PCA and MCC950 (50 µM). In the Transwell upper chamber (3395, Corning, New York, NY, USA), Matrigel glue (50 µL; 354234, Corning, New York, NY, USA) was spread and sterilized by ultraviolet radiation. The medium was then transferred to Transwell chambers and 200 µL of cell suspension with a cell density of 4×10^4 cells/well was added, followed by incubation for 24 h at 37 °C. Next, the cells were treated with a 4% paraformaldehyde solution (30525-89-4, Macklin Biochemical Co., Ltd., Shanghai, China) and fixed for 15 min, followed by 8-min staining with crystal violet (548-62-9, Macklin Biochemical Co., Ltd., Shanghai, China). Afterward, the cells were rinsed three times with Phosphate-Buffered Saline (PBS), and the liquid in the chamber was removed by aspiration. The invasion of cells to the bottom of the chamber was observed under an inverted microscope (DMI4000 B, Leica, Wetzlar, Germany).

Scratch Test

Three parallel lines were drawn on the bottom side, directly beneath each well, of a 6-well plate using a marker pen. Cell suspension (2.5×10^5 cells/well) was seeded into a 6-well plate and incubated until reaching 80% confluence. Three parallel scratches perpendicular to the marker lines were made in each well. After washing the dead cells, the cultures were replenished with fresh medium containing PCA (2.5 µM, 5 µM, 10 µM) or PCA+MCC950 (50 µM). After making scratches, 24 and 48 h later, the cells were photographed using an inverted microscope (DMI4000 B, Leica, Wetzlar, Germany). ImageJ software (version: 1.53k, National Institutes of Health, Bethesda, MD, USA) was used to evaluate the scratch healing. The migration rate was calculated as follows:

Migration rate = (0-hour cell gap – observed time cell gap)/0-hour cell gap

Immunofluorescence Staining

Cells (2.5×10^5 cells/well) were plated onto a 6-well dish and cultured until reaching 80% confluence. The cells were then treated with different concentrations of PCA (2.5 µM, 5 µM, 10 µM) or PCA+MCC950 (50 µM) and then incubated for 24 h. Next, the cells were harvested and treated with 4% paraformaldehyde for a duration of 25 min. Subsequently, the cells were blocked using goat serum (C0265, Beyotime, Shanghai, China) for 25 min before being exposed to the primary antibody targeting GSDMD (1:200; ab219800, Abcam, Cambridge, MA, USA) overnight. In low-light conditions, the cells underwent a 45-min incubation with the Cy3-labeled fluorescent secondary antibody

Table 1. Primer sequences.

Gene	Accession no.	Primer sequences
NLRP3	NM_004895	F: 5′-GGACTGAAGCACCTGTTGTGCA-3′ R: 5′-TCCTGAGTCTCCCAAGGCATTC-3′
Caspase-1	NM_033292	F: 5′-GCTGAGGTTGACATCACAGGCA-3′ R: 5′-TGCTGTCAGAGGTCTTGTGCTC-3′
GSDMD	NM_024736	F: 5′-ATGAGGTGCCTCCACAACTTCC-3′ R: 5′-CCAGTTCCTTGGAGATGGTCTC-3′
GAPDH	NM_002046	F: 5′-GTCTCCTCTGACTTCAACAGCG-3′ R: 5′-ACCACCCTGTTGCTGTAGCCAA-3′

NLRP3, nucleotide-binding and oligomerization domain-like receptor thermal protein domain-associated protein 3; *caspase-1*, cysteinyl aspartate specific proteinase; *GSDMD*, gasdermin D; *GAPDH*, Glyceraldehyde 3-Phosphate Dehydrogenase.

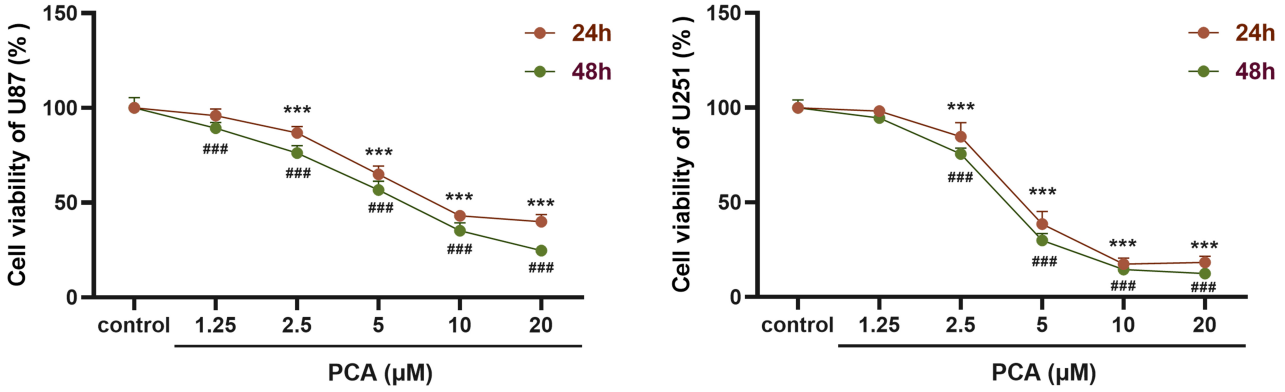

Fig. 1. Detection of cell viability by Cell Counting Kit-8 (CCK-8) assay in U87 and U251 cells (n = 6). $^{***}p < 0.001$ compared with the control group at 24 h, $^{###}p < 0.001$ compared with the control group at 48 h.

(1:500; P0183, Beyotime, Shanghai, China) and were observed by utilizing a fluorescence microscopee (ECLIPSE Ts2, Nikon, Tokyo, Japan).

Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)

Cells were seeded into 6-well dish and incubated until reaching 80% confluence. They were then treated with different concentrations of PCA (2.5 μM, 5 μM, 10 μM) or PCA+MCC950 (50 μM) for 24 h. Afterward, the cells were collected for total RNA extraction using Trizol reagent (R0016, Beyotime, Shanghai, China). cDNA was synthesized from the RNA samples using a reverse transcription kit (RR037Q, TaKaRa, Tokyo, Japan). Next, qRT-PCR was performed. mRNA expression levels were analyzed using $2^{-\Delta\Delta CT}$ method. The primers used in the experiment are listed in Table 1.

Western Blotting

Cells were seeded into 10 cm petri dishes and incubated until reaching 80% confluence. Treatment with different concentrations of PCA (2.5 μM, 5 μM, 10 μM) or PCA+MCC950 (50 μM) was conducted on the cells for 24 h. Then, the cells were collected for protein isolation using a total protein extraction kit (89901, Thermo Fisher Scientific, Waltham, MA, USA). Protein quantification was performed using a Bicinchoninic Acid Assay (BCA) Protein Assay Kit (P0012, Beyotime, Shanghai, China). After that, the proteins were separated through a 12% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) process and were subsequently transferred onto Polyvinylidene Fluoride (PVDF) membranes. The membrane was then blocked using 5% skim milk for 1 h, followed by an overnight incubation at 4 °C with primary antibodies targeting caspase-1 (1:1000; ab207802, Abcam, Cambridge, MA, USA), GSDMD (1:1000; ab219800, Abcam, Cambridge, MA, USA), NLRP3 (1:1000; ab263899, Abcam, Cambridge, MA, USA), and Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) (1:1000; ab8245, Abcam, Cambridge, MA, USA). Subsequently, the membrane was exposed to secondary antibodies: goat anti-rabbit IgG H & L (1:10,000; ab150077, Abcam, Cambridge, MA, USA) and goat anti-mouse IgG H & L (1:10,000; ab150113, Abcam, Cambridge, MA, USA) at room temperature for an hour. This was followed by the addition of enhanced chemiluminescence (ECL) substrate (P0018S, Beyotime, Shanghai,

China), enabling visualization of the bands, whose intensity was then analyzed using ImageJ software. The grayscale values of all protein bands were normalized using GAPDH as the internal control.

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). Quantitative data are presented as mean ± standard deviation. To compare multiple groups, one-way analysis of variance (ANOVA) was employed, with Tukey's test used for post-hoc analysis. A significance level of $p < 0.05$ was deemed statistically significant.

Results

PCA Inhibited the Viability of U87 and U251 Cells

The viability of U87 and U251 cells was assessed using the CCK-8 assay after being treated with PCA (0–20 μM) for 24/48 h. The U87 and U251 cell viability was reduced with the increasing concentrations of PCA ($p < 0.001$, Fig. 1). The results suggested that PCA could suppress the growth of U87 and U251 cells in a manner correlated with the dosage administered. Interestingly, PCA at a concentration of only 1.25 μM had a significant inhibitory effect on U87 cells after a 24-hour treatment ($p < 0.05$). A higher concentration of PCA, at 2.5 μM, exhibited a more conspicuous inhibitory effect on U251 cell proliferation ($p < 0.01$). Therefore, PCA concentrations of 2.5 μM, 5 μM, and 10 μM were selected for subsequent experiments.

PCA Inhibited the Invasion and Migration of U87 and U251 Cells

Subsequently, we assessed the impact of PCA on cellular invasion and migration. Following a 24-hour period of treatment, a notable decrease in the number of cells penetrating the membrane was observed in the PCA-treated cells in comparison to the control group ($p < 0.05$) (Fig. 2A). Among the PCA-treated groups, the 10 μM PCA treatment group exhibited the most pronounced inhibitory effect on cell invasion ($p < 0.001$). The scratch test demonstrated that the wound healing rates in the PCA-treated groups were lower than that in the control group for both U87 and U251 cells ($p < 0.05$) (Fig. 2B,C), with the 10 μM PCA treatment group showing the largest reduction in migration ($p < 0.001$). These findings indicate that PCA exhibits significant suppressive effects on both the invasion and migration capabilities of glioma cells.

PCA Induced Pyroptosis in U87 and U251 Cells

We explored whether PCA could affect the pyroptosis of U87 and U251 cells using immunofluorescence staining to detect the expression of pyroptosis-related GSDMD. In this study, GSDMD fluorescence intensity was enhanced with increasing concentration of PCA treatment ($p < 0.05$, Fig. 3). Without PCA intervention, GSDMD fluorescence could barely be observed in cell fluorescence images. At 5 μM PCA, the GSDMD positive rate in PCA-treated group was significantly increased ($p < 0.05$), while the 10 μM PCA treatment group showed the highest GSDMD positive rate ($p < 0.001$). The results indicate that PCA promotes pyroptosis in U87 and U251 by facilitating the production of GSDMD.

PCA Promoted mRNA and Protein Expression Levels of NLRP3, Caspase-1 and GSDMD in U87 and U251 Cells

We employed qRT-PCR and Western blotting to investigate whether PCA promotes pyroptosis through the NLPR3/caspase-1/GSDMD canonical pathway. PCA intervention led to an increase in the mRNA and protein expression levels of the pyroptosis-related proteins NLRP3, caspase-1 and GSDMD in U87 and U251 cells ($p < 0.05$), with the 10 μM PCA treatment group showing particularly higher expression levels of these proteins ($p < 0.01$, Fig. 4). This suggests that the intervention of PCA could promote NLPR3/caspase-1/GSDMD pathway in glioma cells.

PCA Regulated Cellular Activity of U87 and U251 by Regulating NLRP3/Caspase-1/GSDMD-Mediated Pyroptosis

The NLRP3-specific inhibitor MCC950 was employed in the further investigation of the effect of PCA on the activity of U87 and U251 cells. Compared to the 10 μM PCA treatment group, the 10 μM PCA+MCC950 group exhibited significantly increased rates of cell viability and invasion ($p < 0.01$, Fig. 5A–C), and a significant reduction in GSDMD positive rate ($p < 0.05$, Fig. 5D). Moreover, the application of MCC950 led to a notable decrease in the protein and mRNA expression levels of NLRP3, caspase-1 and GSDMD in U87 and U251 cells, which were originally augmented by PCA ($p < 0.05$) (Fig. 5E,F).

Discussion

GBM is the most common malignant tumor that affects the central nervous system, featuring aggressive tumor cell growth, an invasive nature, and notable resistance to medications [17]. In recent years, the application of multimodal treatment strategy involving surgical resection, chemoradiotherapy, and temozolomide adjuvant therapy has obviously favorable impact on improving patient survival rates, but such strategy does not seem to significantly improve the prognosis for GBM patients [18,19]. Therefore, finding effective treatments for GMB holds significant clinical value. Owing to the highly invasive nature of GBM, it is extremely challenging to completely eliminate the tumor by surgical means, leaving behind residual surviving GBM cells that potentially lead to recurrence and

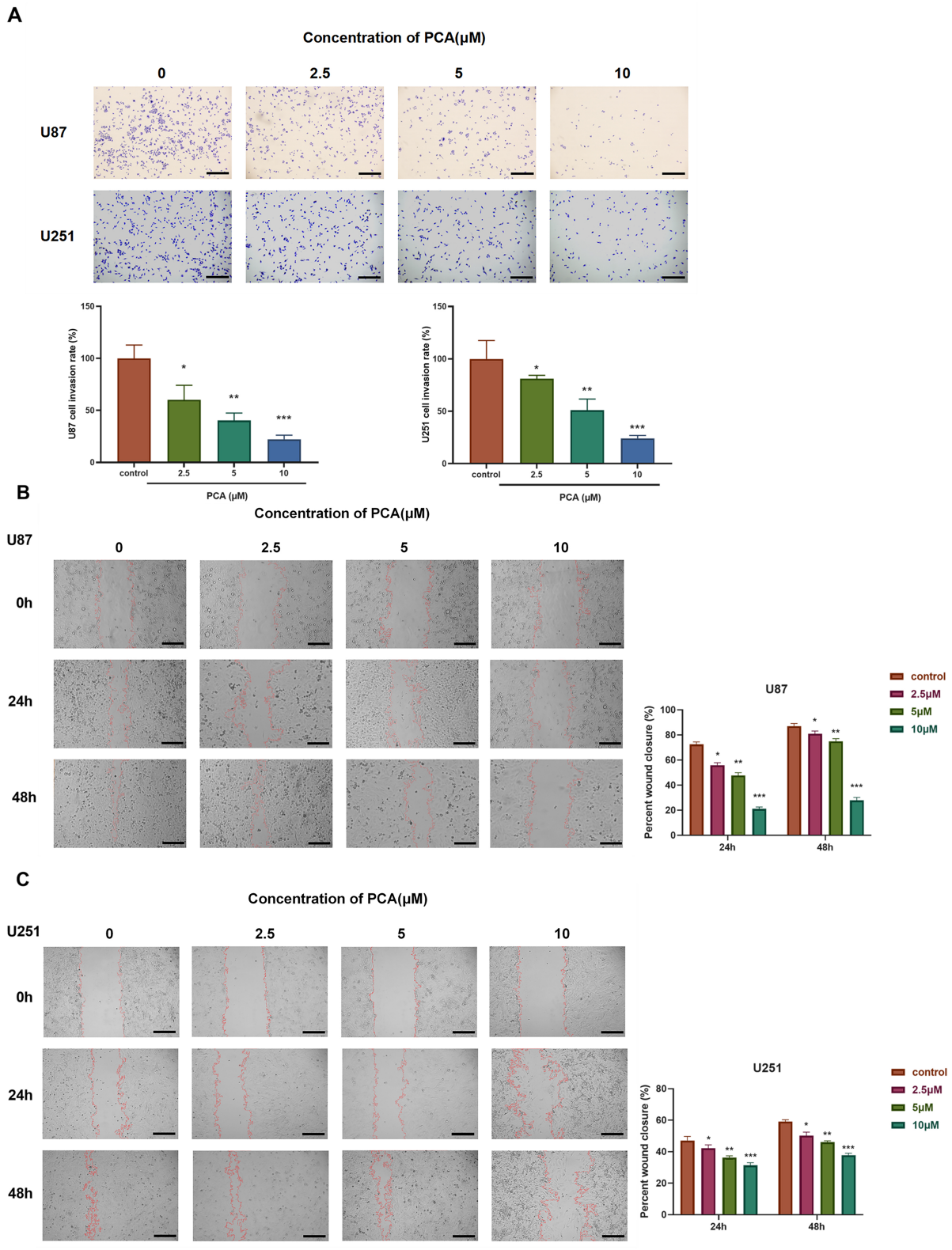

Fig. 2. Protocatechuic acid (PCA) inhibits the invasion and migration of U87 and U251 cells (n = 3). (A) Detection of cell invasion using Transwell assay in U87 and U251 cells. Scale bars: 50 μm (crystal violet staining, 200× magnification). Detection of cell migration using scratch test in (B) U87 and (C) U251 cells. Scale bars: 100 μm (100× magnification). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the control group.

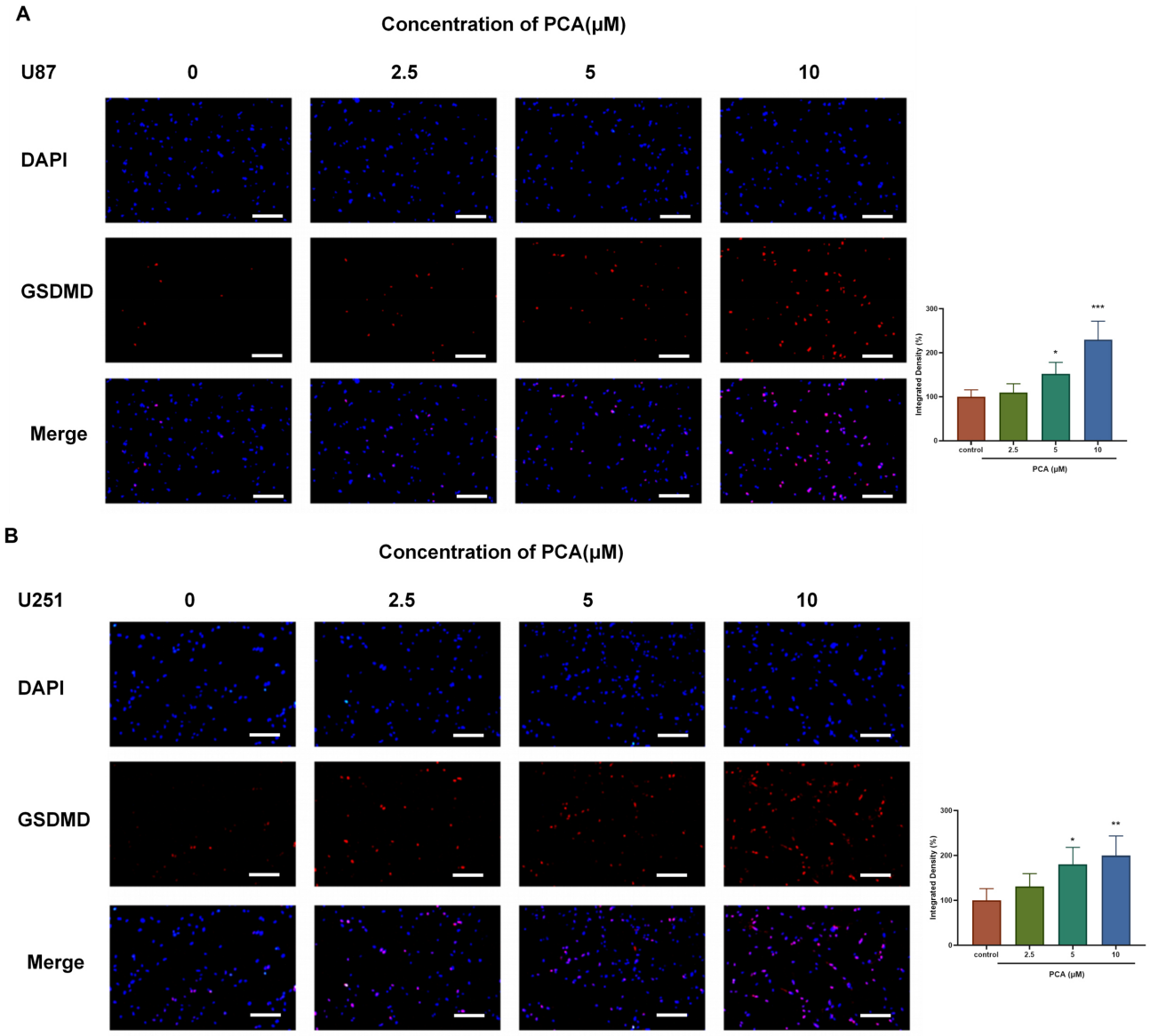

Fig. 3. PCA promoted pyroptosis in U87 and U251 cells (n = 3). (A) Immunofluorescence staining to detect expression of GSDMD in U87 cells. (B) Immunofluorescence staining to detect expression of GSDMD in U251 cells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the control group. Scale bars: 50 μm (200× magnification).

treatment resistance [20]. Thus, identifying effective therapies to inhibit the invasiveness of GBM cells is crucial for treating GBM and improving patient outcomes.

A recent study has indicated that PCA exhibits the ability to suppress the growth, migration, invasiveness, and formation of capillary structures in human umbilical vein endothelial cells (HUVECs), exhibiting strong anti-angiogenic activity. This effect may be crucial in preventing tumor metastasis and recurrence [21]. Xie *et al.* [22] showed that PCA can influence apoptosis and autophagy, consequently impeding the proliferation of ovarian cancer cells. These findings lend credence to the hypothesized role of PCA in exerting significant inhibitory impacts on the growth and spread of tumors. Our results revealed that PCA significantly reduced cell viability, invasion, and migration in a concentration-dependent manner. Specifically, higher concentrations of PCA led to a marked decrease in these cellular activities, underscoring its potent anti-tumor effects. These findings align with the previously reported mechanisms of PCA, suggesting that in addition to impairing glioma cell proliferation, PCA can inhibit their invasion and migration capabilities. With this wide spectrum of capabilities, PCA holds immense promise as a therapeutic agent for glioma. The findings of this study provide a basis for further investigations into its mechanisms and potential clinical applications.

Pyroptosis, a type of orchestrated cell demise, has been identified as intricately linked to the progression of gliomas, alongside other modes of programmed cell death such as apoptosis [23]. The canonical pathway of pyrop-

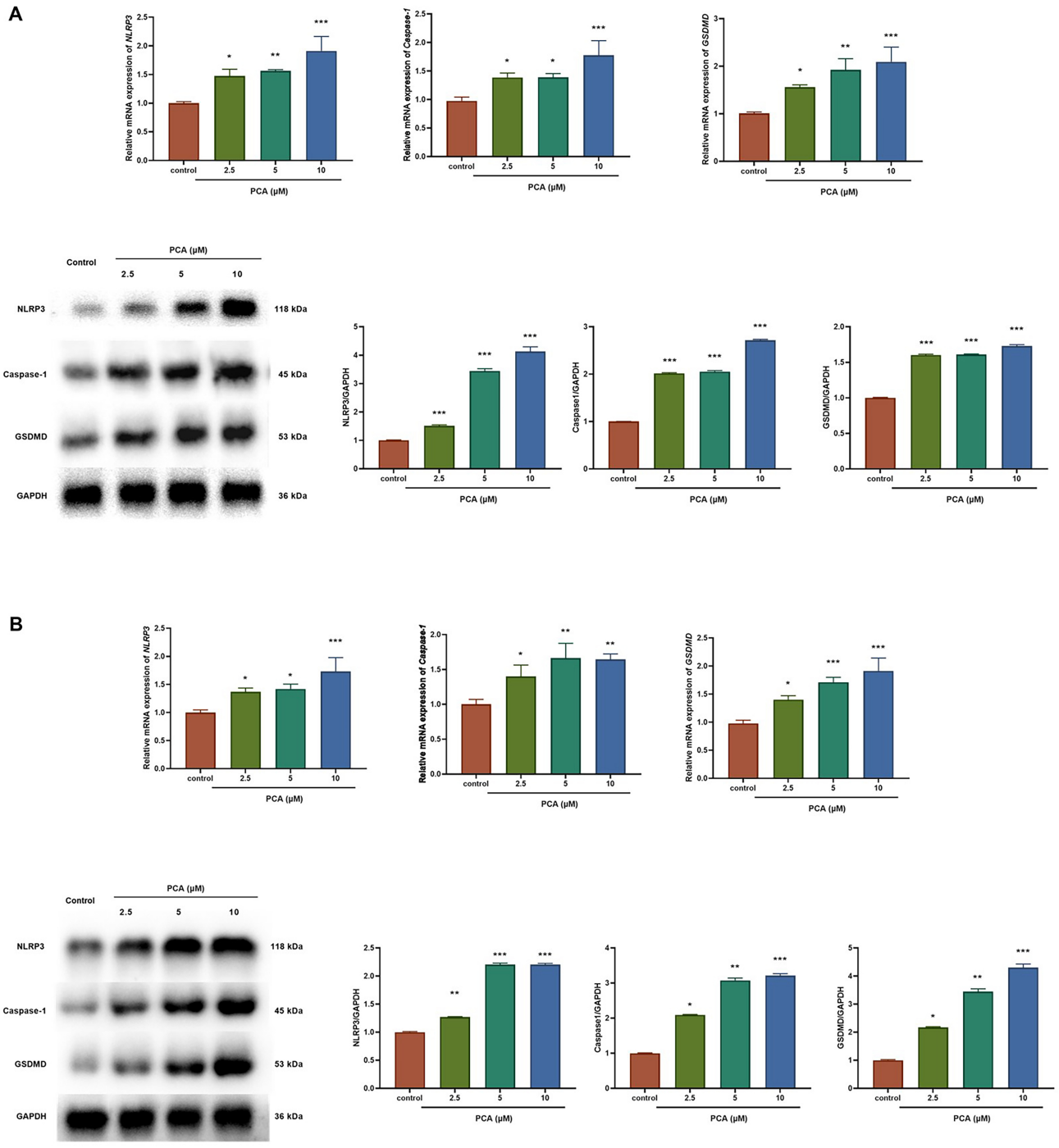

Fig. 4. PCA promoted mRNA and protein expression levels of NLRP3, caspase-1 and GSDMD in (A) U87 and (B) U251 cells (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the control group.

tosis, predominantly encompassing caspase-1, involves the activation of inflammasomes such as NLRP3, which subsequently leads to the cleavage of GSDMD, ultimately resulting in cell lysis. As one of the inflammasomes associated with pyroptosis, NLRP3 inflammasome consists of NLRP3 protein, ASC and the effector protein precursor caspase-1 (pro-caspase-1). The activation of pro-caspase-1 in response to non-microbial triggers promotes the maturation of inactive IL-1β and IL-18 precursors, which pave the way for cleaving GSDMD protein to expose its N-terminal active domain, ultimately leading to cell rupture. In this process, GSDMD is regarded as the executor of pyroptosis [16]. Research has revealed that GBM cells treated with benzimidazole exhibit pyroptotic characteristics such as cell membrane swelling and rupture. The markers for pyroptosis (NLRP3, GSDMD, caspase-

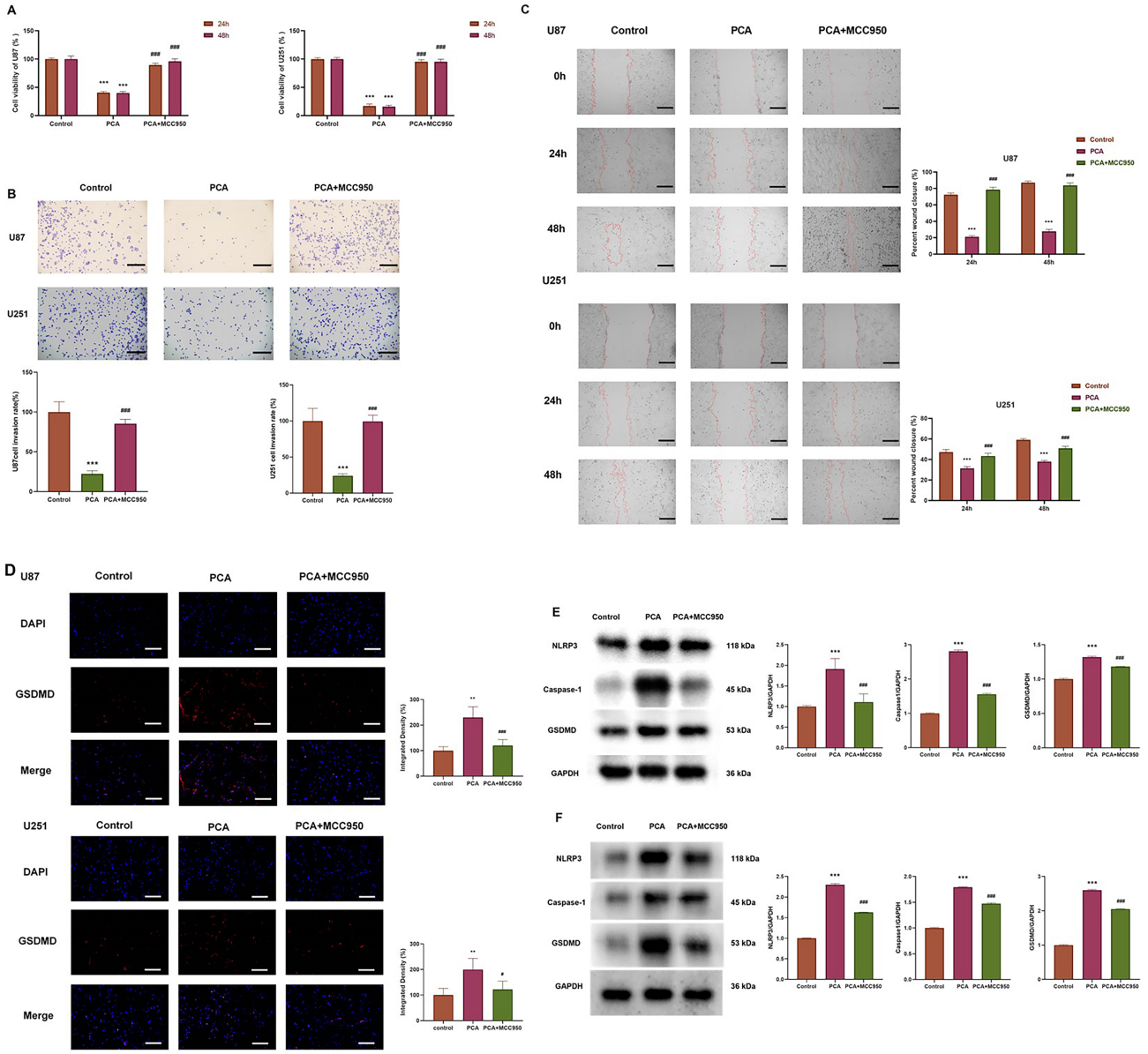

Fig. 5. PCA regulated NLRP3/caspase-1/GSDMD-mediated pyroptosis in U87 and U251 cells (n = 3). (A) Cell viability, (B) rate of cell invasion (scale bars: 50 μm; crystal violet staining, 200× magnification), (C) scratch healing in cell layers (scale bars: 100 μm; 100× magnification) of U87 and U251 cells. (D) GSDMD positive rate of cells detected by immunofluorescence staining (scale bars: 50 μm; 200× magnification). The mRNA and protein expression levels of NLRP3, caspase-1, and GSDMD in (E) U87 cells or (F) U251 cells. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the control group; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ compared with the 10 μM PCA treatment group.

1) are notably increased in expression levels [24]. Furthermore, Kaempferol, an anti-tumor drug, raises reactive oxygen species (ROS) levels in glioma cells, and subsequently leads to pyroptosis. ROS has the potential to trigger caspase-3 activation, which subsequently causes the fragmentation of GSDMD, ultimately leading to the occurrence of pyroptosis in glioma cells [25]. The current study demonstrated that activation of the NLRP3/caspase-1/GSDMD signaling pathway by PCA boosted the protein and mRNA expression levels of NLRP3, caspase-1, and GSDMD in glioma cells, thereby inducing pyroptosis. Treatment of the glioma cells with MCC950, an NLRP3-specific inhibitor, resulted in the suppression of their viability, invasion, migration and the expression of PCA-induced pyroptosis-related protein. The findings revealed that MCC950 counteracted the pyroptosis promoted by PCA in glioma cells, indicating that the activation of the NLRP3/caspase-1/GSDMD signaling pathway plays a role in PCA-mediated pyroptosis. These findings provide strong evidence that the anti-glioma effects of PCA are mediated

through its influence on the pyroptosis pathways, thus establishing PCA and pyroptosis pathways as potential therapeutic strategies for glioma treatment.

Conclusion

In conclusion, this study substantiates that PCA suppresses GBM by inducing pyroptosis through the regulating of the NLRP3/caspase-1/GSDMD signaling pathway.

Availability of Data and Materials

Data involved in the present work are available from the corresponding author upon request.

Author Contributions

HZ designed the research study. WZ performed the research. HZ, YC, and WZ were involved in analyzing the data, drafting and critical revision of the manuscript. All authors contributed significantly to editorial changes of important content. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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