

Research Article

Involvement of P2X₇ Receptor in Proliferation and Migration of Human Glioma Cells

Zhenhua Ji,^{1,2} Yuting Xie,³ Yu Guan,³ Yujian Zhang,³ Kin-Sang Cho,⁴
Min Ji ,³ and Yongping You ¹

¹Department of Neurosurgery, The First Affiliated Hospital with Nanjing Medical University, Nanjing 210029, China

²Department of Head and Neck Surgery, Affiliated Tumor Hospital of Nantong University, Nantong 226001, China

³Department of Ophthalmology, Affiliated Hospital of Nantong University, Nantong 226001, China

⁴Schepens Eye Research Institute, Massachusetts Eye and Ear, Department of Ophthalmology, Harvard Medical School, Boston, MA 02114, USA

Correspondence should be addressed to Min Ji; amyji1234@hotmail.com and Yongping You; yypl9@njmu.edu.cn

Received 5 September 2017; Revised 22 November 2017; Accepted 29 November 2017; Published 9 January 2018

Academic Editor: Jens Schittenhelm

Copyright © 2018 Zhenhua Ji et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Previous studies have demonstrated that activation of P2X₇ receptors (P2X₇R) results in the proliferation and migration of some types of tumor. Here, we asked whether and how the activated P2X₇R contribute to proliferation and migration of human glioma cells. Results showed that the number of P2X₇R positive cells was increasing with grade of tumor. In U87 and U251 human glioma cell lines, both expressed P2X₇R and the expression was enhanced by 3'-O-(4-benzoylbenzoyl) ATP (BzATP), the agonist of P2X₇R, and siRNA. Our results also showed that 10 μM BzATP was sufficient to induce the proliferation of glioma cell significantly, while the cell proliferation reached the peak with 100 μM BzATP. Also, the migration of U87 and U251 cells was significantly increased upon BzATP treatment. However, the number of apoptotic cells of U87 and U251 was not significantly changed by BzATP. In addition, the expression of ERK, p-ERK, and proliferating cell nuclear antigen (PCNA) protein was increased in BzATP-treated U87 and U251 glioma cells. PD98059, an inhibitor of the MEK/ERK pathway, blocked the increased proliferation and migration of glioma cells activated by BzATP. These results suggest that ERK pathway is involved in the proliferation and migration of glioma cells induced by P2X₇R activation.

1. Introduction

Malignant glioma is the most common malignant tumor of the central nervous system [1]. The invasive performance and easy transformation from lower grades to higher grades were the main causes of the poor prognosis of glioma [2, 3]. Low apoptosis and aggressive cell proliferation, invasion, and angiogenesis of glioma make it very challenging to be treated [4, 5]. Therefore, investigating the underlying mechanisms of glioma malignant proliferation and invasive growth was essential to glioma treatment.

Microenvironment of solid tumors is characterized by a strikingly features of high concentration of adenosine and adenosine triphosphate (ATP) [6]. Activated purine receptor has been shown in many kinds of tumors [7]. However, its role in tumorigenesis is not fully elucidated. Purinergic

receptors have been studied extensively in inflammation and degeneration of the central nervous system in recent years [8–10]. P2X₇R is a nonselective cation channel receptor [11]. Brief exposure to P2X₇R agonist such as ATP or 3'-O-(4-benzoylbenzoyl) ATP (BzATP) leads to the opening of cation channel allowing K⁺ efflux and Ca²⁺ and Na⁺ influx into the cells [12]. However, prolonged activation of P2X₇R will be resulted in formation of large aqueous pores and finally leads to cell death [13]. Compared to all other members of P2X family and other ligand-gated ionotropic receptors, the most striking feature of P2X₇R mediated currents is the absence of current desensitization with agonist treatment. Instead, P2X₇R mediated currents are incredibly increasing in amplitude upon repeated brief applications or sustained application of agonists [14, 15].

Although a growing number of studies show the involvement of P2X₇R in the tumorigenesis, its role is still controversial. Previous studies have shown that activation of P2X₇R can induce angiogenesis, increase vascular endothelial growth factor production, accelerate cell invasion and migration, and hence promote tumor growth [16, 17]. Recent evidences show a possible direct antiangiogenic role of P2X₇R on tumor-derived endothelial cells [18]. However, other studies showed that inhibiting the activation of P2X₇R induced tumor growth and accelerated tumor cell death [19, 20]. Thus, the role of P2X₇R in glioma cell proliferation and migration remains unclear.

Herein, we examined the role of P2X₇R on the migration, cell proliferation, and downstream signaling pathways using human glioma cell lines and human glioma tissues.

2. Materials and Methods

2.1. Human Glioma Samples. Twenty primary glioma and adjacent normal tissues were obtained from patients with glioma grades I to IV (5 samples per grade), who underwent surgical resection at the Affiliated Hospital of Nantong University. The study was approved by the Ethics Committee of the Affiliated People's Hospital of Jiangsu University and Affiliated Hospital of Nantong University. The clinical and pathological features of the patients were independently diagnosed by two independent pathologists. Every sample were collected and fixed in 10% formalin overnight and then dehydrated through gradient alcohol and xylenes. The samples were imbedded in paraffin and sectioned in 5 μ m thickness.

2.2. Culture of Human Glioma Cell Lines. The immortalized human malignant glioma cell lines U87 and U251 were purchased from Chinese Academy of Sciences Cell Bank (Shanghai, China) and were incubated in Dulbecco's modified Eagle's medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin). The cell lines were maintained in a CO₂ incubator containing 5% CO₂ at 37°C and were used no more than F10.

2.3. Immunofluorescent Staining. Paraffin sections of human glioma tissue were deparaffinized, rehydrated, and blocked by 5% BSA in phosphate buffer saline (PBS) for 2 hours in room temperature. Sections were incubated with the primary antibody against P2X₇R (1:400, Abcam, Cambridge, MA) at 4°C overnight. Then a second antibody (1:500, Jackson ImmunoResearch, West Grove, PA) and 4,6-diaminodiphenyl-2-phenylindole (DAPI, Sigma- Aldrich) were added in a dark room and incubated for 2-3 h. After washing, sections were mounted in 50% glycerol in PBS. The immunofluorescent signal of P2X₇R was visualized under a fluorescent microscope (Leica, Germany).

U87 and U251 glioma cells were washed in PBS and then fixed in 4% paraformaldehyde solution for 20 min at room temperature. Cells were rinsed in PBS and incubated in PBS containing 0.1% Triton X-100 and 3% bovine serum albumin (BSA) for 1 hour to block the nonspecific binding sites. Then

cells were incubated with antibody against P2X₇R (1:400, Abcam, Cambridge, MA) at 4°C overnight. On the following morning, the appropriate secondary antibodies and DAPI were added in a dark room and incubated for 2-3 h. Each immunolabeling experiment was triplicates. After washing, cells were mounted, and the immunofluorescent signal was visualized under a fluorescent microscope (Leica, Germany).

2.4. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay. Proliferation of glioma cell lines was determined by MTT method. U87 and U251 glioma cells were seeded at a density of 1×10^5 per well of 24-well plate and cultured overnight. The medium was replaced with fresh medium supplemented with different concentrations (5, 10, 50, 100, 500, and 1000 μ M) of BzATP. Before the end of treatment, 25 μ L of MTT solution (5 mg/ml) was added to cell cultures and incubated at 37°C for 3 hours. Then the medium was collected and formazan crystals were dissolved in 150 μ L dimethyl sulfoxide. Absorbance was measured at 570 nm with a Microplate Reader (Model 680, Bio-Rad, Hercules, CA). The value is shown as mean \pm standard error (SE) from three independent experiments. Each experiment was performed in triplicate.

2.5. Western Blot Analysis. Western blot analysis was conducted as previously described [21]. Briefly, the lysates were boiled for 15 min followed by centrifugation at 12,000 rpm for 5 min, and the supernatant was collected. Protein concentrations were measured by Bio-Rad protein assay (Bio-Rad Laboratories, Segrate, Milan, Italy). Aliquots of lysates containing an equal amount of protein were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% skimmed milk at room temperature for 1 h followed by incubation with primary antibodies against P2X₇R (1:1000, Abcam, Cambridge, MA), ERK (1:200, Cell Signaling Technology, Danvers, MA), p-ERK (1:200, Cell Signaling Technology, Danvers, MA), PCNA (1:200, Cell Signaling Technology, Danvers, MA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:1000, Sigma-Aldrich) at 4°C overnight. The PVDF membranes were then incubated with horseradish peroxidase- (HRP-) conjugated goat anti-rabbit or goat anti-mouse secondary antibody (1:2000, Thermo Scientific, Rockford, IL, USA) at room temperature for 2 hours. The membranes were incubated with chemifluorescent reagent ECL (Thermo Scientific, Rockford, IL, USA) and then exposed to X-ray film in the dark room. The protein bands were quantitatively analyzed with ImageJ software.

2.6. In Vitro Cell Migration Assay. U87 and U251 cell lines were cultured in 6-well plates till 70–80% confluency. The monolayer of cells was wounded with a sterile 200 μ l pipette tip in a straight line along the diameter of the well and then washed three times with PBS. The cells were cultured for further 24 h allowing cell migration into the open scratched area. Images of cells were captured at 0 and 24 hour after wounding, using a Leica microscope (Leica, Germany). The

absolute value of distance migrated by cells was quantified as the change in the perpendicular distance between the edge of the gap after 24 hours. The value was then normalized to the 0 hour starting measurement, which represents “migration.”

2.7. siRNA Transfection. siRNA targeting specific sequences of P2X₇R and a negative control (scrambled 1 siRNA) were synthesized by Gene Pharma Co. Ltd. (Shanghai, China). The siRNA sequences directed against P2X₇R were sense: 5'-GGAUCCAGAGCAUGAAUUAUU-3', antisense: 5'-UAA-UUCAUGCUCUGGAUCCUU-3'. Transfections of control and P2X₇R-siRNA were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.8. In Situ Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Staining. The malignant glioma cell lines U87 and U251 were seeded on cover glass which were placed in 24-well plates. TUNEL assay was performed at 24 hours after treatment with the BzATP using a fluorescein in situ cell death detection kit (Roche Applied Science, Germany) according to the manufacturer's instructions. Nuclei were stained with DAPI at room temperature for 15 min. The double-stained positive cells with DAPI and fluorescein were visualized under fluorescence microscope (Leica, Germany) and were quantified with Image J software.

2.9. Statistical Analysis. All experiments were independently repeated in triplicate. The value is presented as mean \pm standard error. Statistical significance between groups was analyzed using *t*-test (two-sided, nonparametric) or one-way-ANOVA by GraphPad Prism. *P* value of less than 0.05 was considered statistically significant.

3. Results

3.1. P2X₇R Expression in Human Glioma Cell. Paraffin sections of human glioma tissue with different stages of diagnosis or adjacent normal tissue were stained for P2X₇R. We found that P2X₇R positive glial cells were rarely seen in normal tissues. However, the P2X₇R positive cell was detected to be increased in higher stage of glioma. The percentage of positive cell in normal tissue was $3.5 \pm 0.6\%$, while the percentage of positive cell was $58.2 \pm 2.1\%$ in grade I ($P < 0.01$), $60.8 \pm 1.9\%$ in grade II ($P < 0.01$), $77.0 \pm 1.9\%$ in grade III ($P < 0.01$), and $89.3 \pm 1.3\%$ in grade IV ($P < 0.01$) (Figures 1(a) and 1(b)).

Other studies have also shown that most glioma cell lines expressed P2X₇R [20, 22]. Here, we selected U87 and U251 glioma cell lines to check if these cell lines also express P2X₇R. The immunofluorescence staining showed that P2X₇R expressed in almost all cells in these two cell lines (Figure 1(c)).

3.2. BzATP Promotes the Proliferation and Migration of U87 and U251 Glioma Cells. MTT assay was performed to detect the effect of BzATP (5 to 1000 μ M) on glioma cell proliferation. We found that the proliferation of U87 and U251 glioma cell lines was significantly increased in the presence of 10–1000 μ M and 100–1000 μ M BzATP, respectively. In

addition, the peak of cell proliferation of both U87 and U251 cell lines was at 100 μ M BzATP (Figures 2(a) and 2(c)). To investigate the optimal incubation time of BzATP, both glioma cell lines were incubated with 100 μ M BzATP for 2 to 72 hours. We found that the optimal incubation time is 24 hours in both U87 and U251 cell lines (Figures 2(b) and 2(d)). The cell proliferation induced by BzATP could be blocked by brilliant blue G (BBG), the specific antagonist of P2X₇R (Figures 2(a)–2(d)). These results suggest that BzATP leads to proliferation of glioma cell lines mediated by activation of P2X₇R.

Next, we examined the effect of BzATP on the migration of glioma cells scratch injury. The migration rate of U87 cells in the untreated group was $39.7 \pm 2.3\%$ while BzATP (100 μ M, 24 h) treated group was $73.0 \pm 2.1\%$ ($P < 0.05$, *t*-test). The effect of BzATP was abolished by P2X₇ antagonist BBG and P2X₇R-siRNA with the migration rate $53.0 \pm 6.6\%$ (compare to BzATP group, $P < 0.05$, *t*-test) and $43 \pm 5.7\%$ (compare to BzATP group, $P < 0.05$, *t*-test). In addition, our results also showed that PD98059, the blocker of MEK/ERK pathway, partially blocked the BzATP-induced migration of U87 glioma cell lines (Figures 3(a) and 3(b)). Similar results were observed in the U251 glioma cell line (Figures 3(c) and 3(d)). P2X₇R-siRNA almost completely blocked the expression of P2X₇R in Western blot (Figure 3(e)). PD98059 had a high efficiency in suppressing the expression of p-ERK in Western blot (Figure 3(f)). We also checked the effect of P2X₇R-siRNA on BzATP mediated cell proliferation. MTT data showed that the 24 hours incubation of 100 μ M BzATP increased the U87 and U251 cell proliferation to 1.6-fold compared to control group, while P2X₇R-siRNA totally blocked the effect (Figures 3(g) and 3(h)). It suggests that activation of P2X₇R enhances the migration and proliferation of human glioma cell lines.

To investigate if BzATP affect the cell survival of glioma cell lines, we determine the number of apoptotic cells of U87 and U251 cell lines following 24-hour incubation of 100 μ M BzATP. TUNEL assay was used to detect the apoptosis of glioma cell lines. Compared to the control/untreated group, the number of TUNEL positive cells in the BzATP-treated group had no significant change (Figure 4).

The cytotoxicity and necrosis of malignant glioma cells can increase the release of ATP and also its accumulation in the microenvironment of glioma tissue, while the capability of ATP degradation during the pathological condition was decreased [23]. Excessive extracellular ATP might lead to an activation and expression of P2X₇R in human and rat glioma cells [22]. To investigate if P2X₇ agonist BzATP could increase the expression of P2X₇R in U87 and U251 glioma cell lines, immunocytochemical staining and Western blot were used to determine the changes of P2X₇R expression. We found that BzATP induced the upregulation of P2X₇R in U87 and U251 glioma cells (Figure 5).

3.3. Involvement of MEK/ERK Pathway in BzATP Mediated Proliferation of U87 and U251 Glioma Cells. MEK/ERK pathway is a common intracellular signaling pathway related to glioma cell proliferation [24]. Our study also demonstrated the role of MEK/ERK pathway in the proliferation and

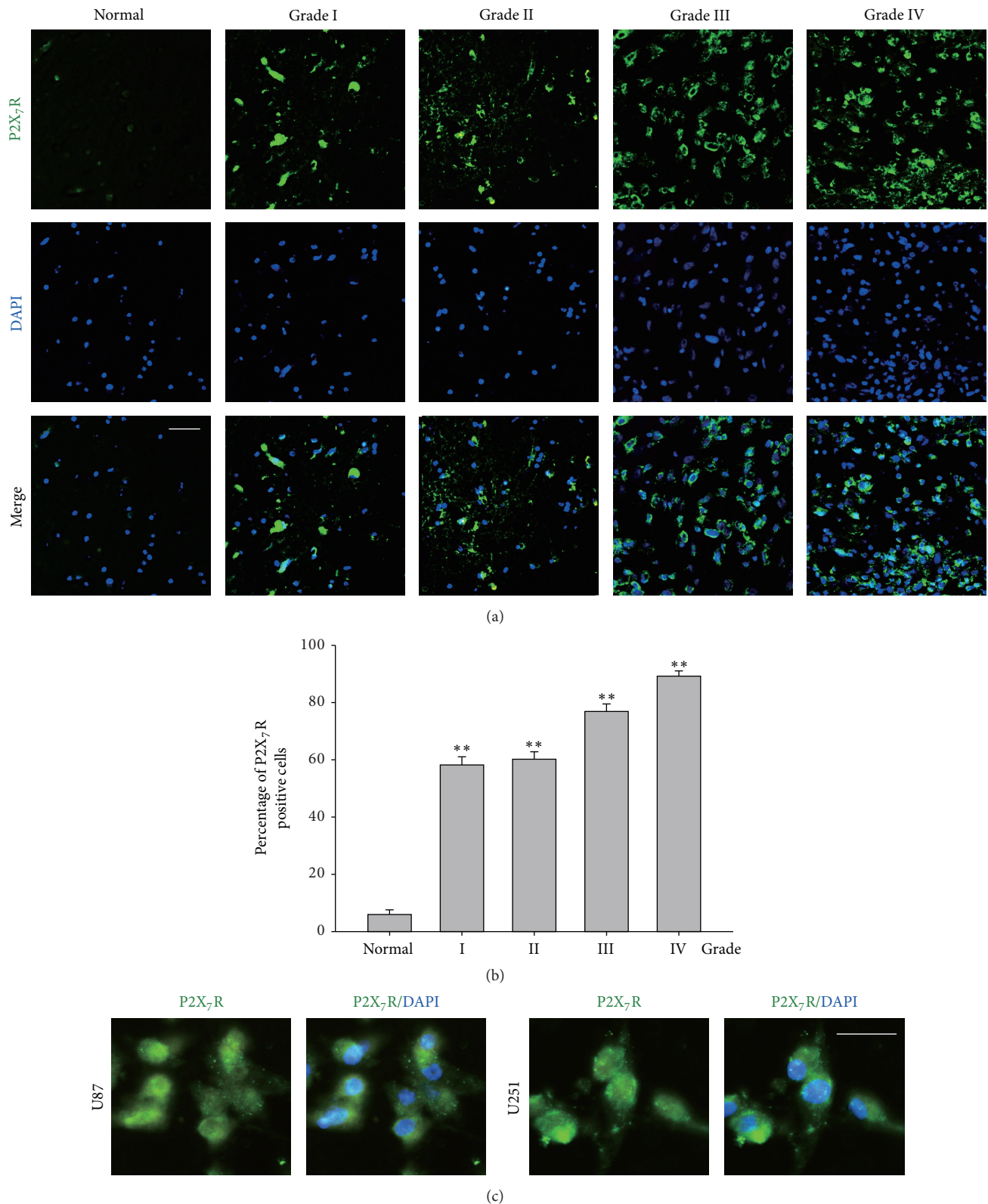


FIGURE 1: *P2X₇R* expression in different grades of human glioma and human glioma cells. (a) Immunofluorescence labeling of *P2X₇R* (green) in different grades of human glioma showed that *P2X₇R* positive cells were rarely seen in normal brain tissue. As the glioma becomes more advanced, the percentage of *P2X₇R* positive cells dramatically increased, especially in grade III and grade IV glioma. Scale bar = 50 μ m. (b) Bar charts showing the percentage of *P2X₇R* positive cells in normal and different grades of glioma tissue ($n = 5$ for each group). ** $P < 0.01$ versus Ctr. Data analyzed by ANOVA test. (c) Immunofluorescence labeling showing *P2X₇R* protein expression in U87 cells and U251 cells. Scale bar = 20 μ m.

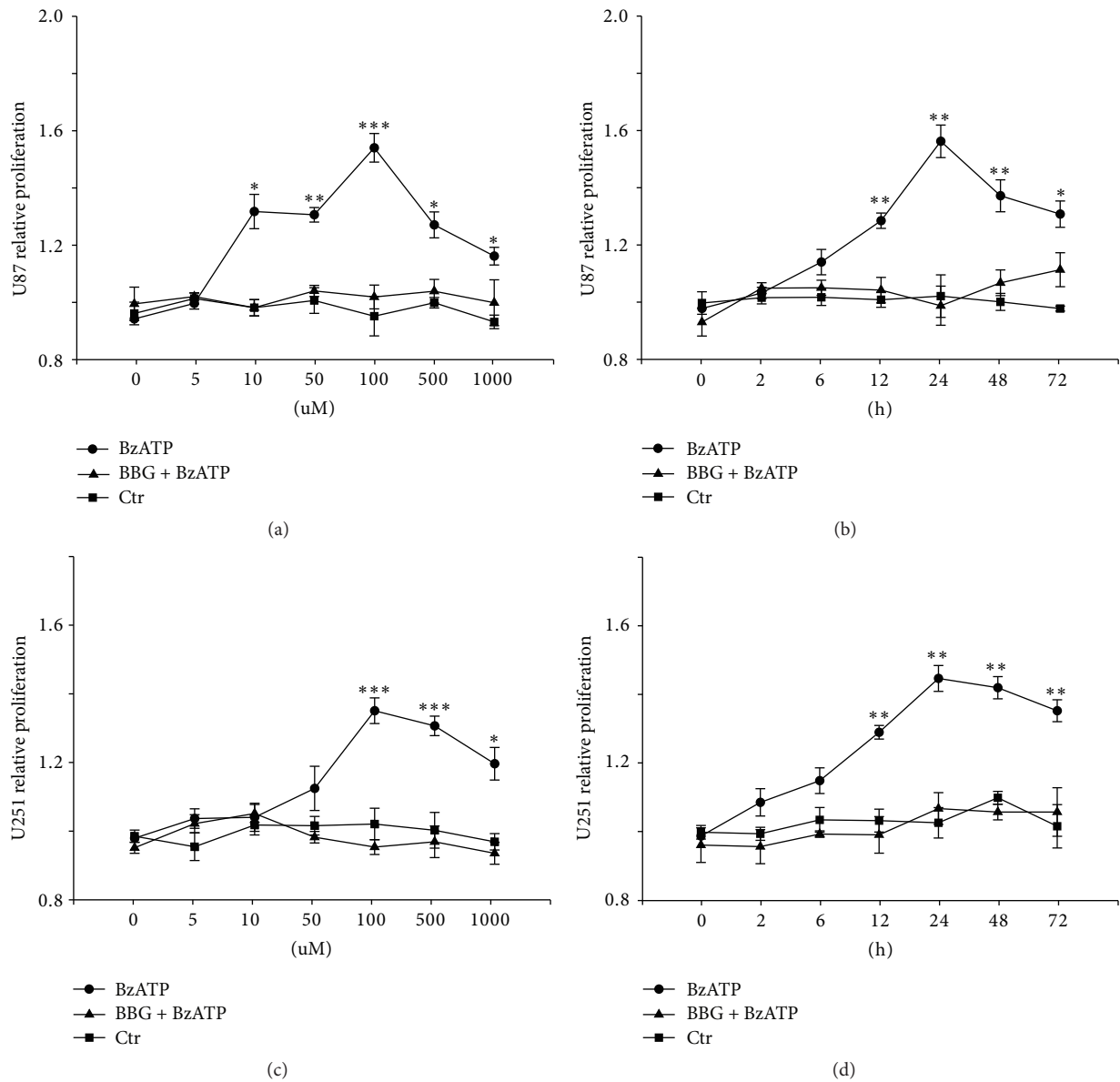


FIGURE 2: BzATP promotes the proliferation of U87 and U251 glioma cells. (a) The proliferation of U87 glioma cell with treatment of BzATP/BBG at different concentrations (5, 10, 50, 100, 500, and 1000 μM). (b) The proliferation of U87 glioma cell with treatment of BzATP/BBG at different time points (2, 6, 12, 24, 48, and 72 h). (c) The proliferation of U251 glioma cell with treatment of BzATP/BBG at different concentrations (5, 10, 50, 100, 500, and 1000 μM). (d) The proliferation of U251 glioma cell with treatment of BzATP/BBG at different time points (2, 6, 12, 24, 48, and 72 h). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared to the control groups at the same time point.

migration of glioma cells induced by P2X₇R activation. Proliferating cell nuclear antigen (PCNA) is only found in normal proliferating cells and tumor cells. In general, the expression level of PCNA in tumor is correlated with the degree of malignancy. Here, we first detected the expression of ERK/p-ERK protein with activation of P2X₇R. The results showed that BzATP significantly increased of ERK, p-ERK, and PCNA protein expression in both U87 and U251 cell lines. This effect was completely abolished in the presence of BBG (Figure 6).

We further investigated if BzATP induced glioma cell proliferation and migration are mediated by ERK

pathway. Results showed that PD98059, the specific inhibitor of MEK/ERK pathway, completely inhibited the BzATP-induced proliferation of glioma cells in U87 and U251 cell lines (Figures 7(a) and 7(b)). Overall, these results suggest that the MEK/ERK pathway plays an important role in glioma cell proliferation and migration mediated by the activation of P2X₇R.

4. Discussion

4.1. Activation of P2X₇R Induces Proliferation and Migration of Glioma Cells. Microenvironment of tumors including

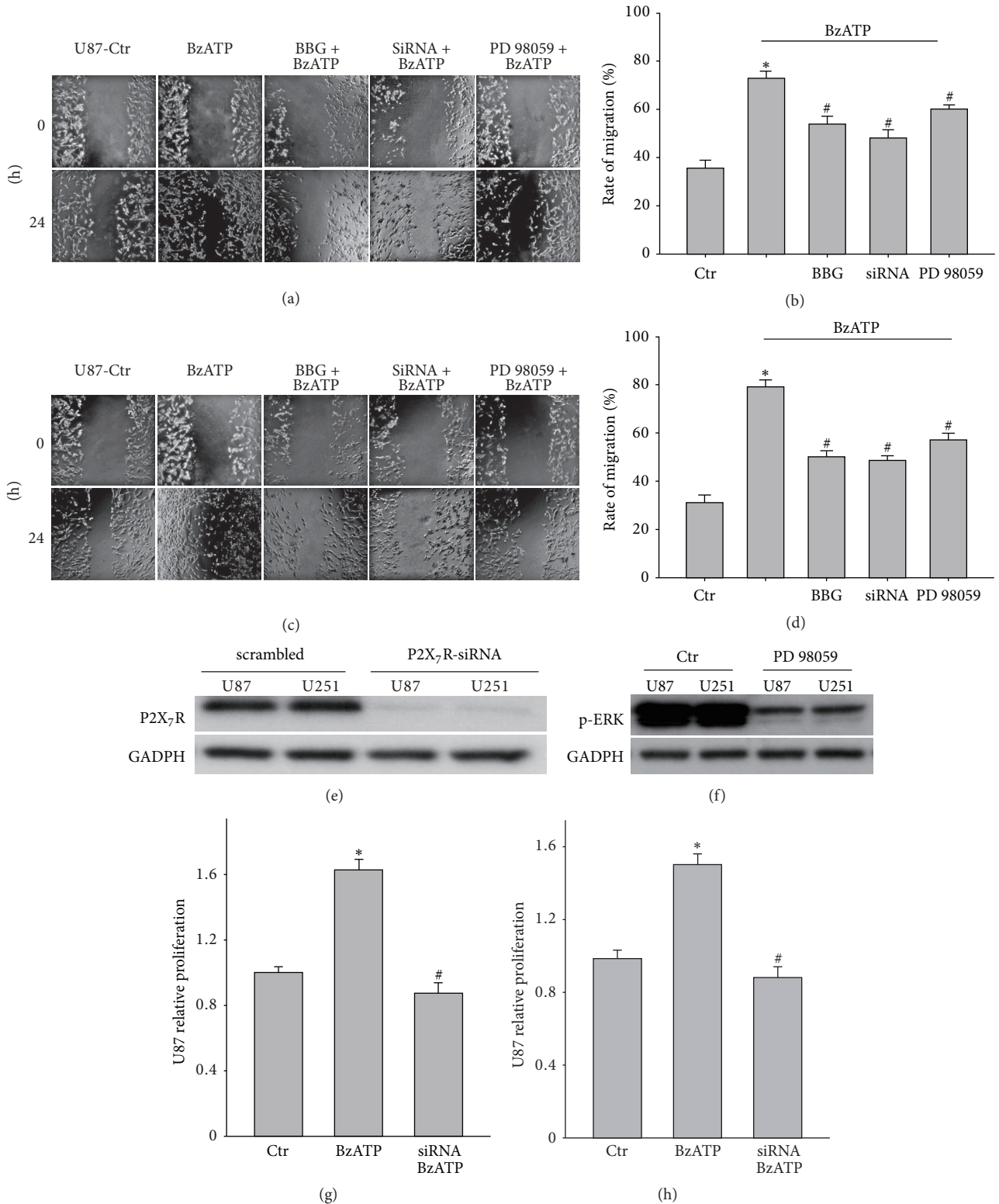
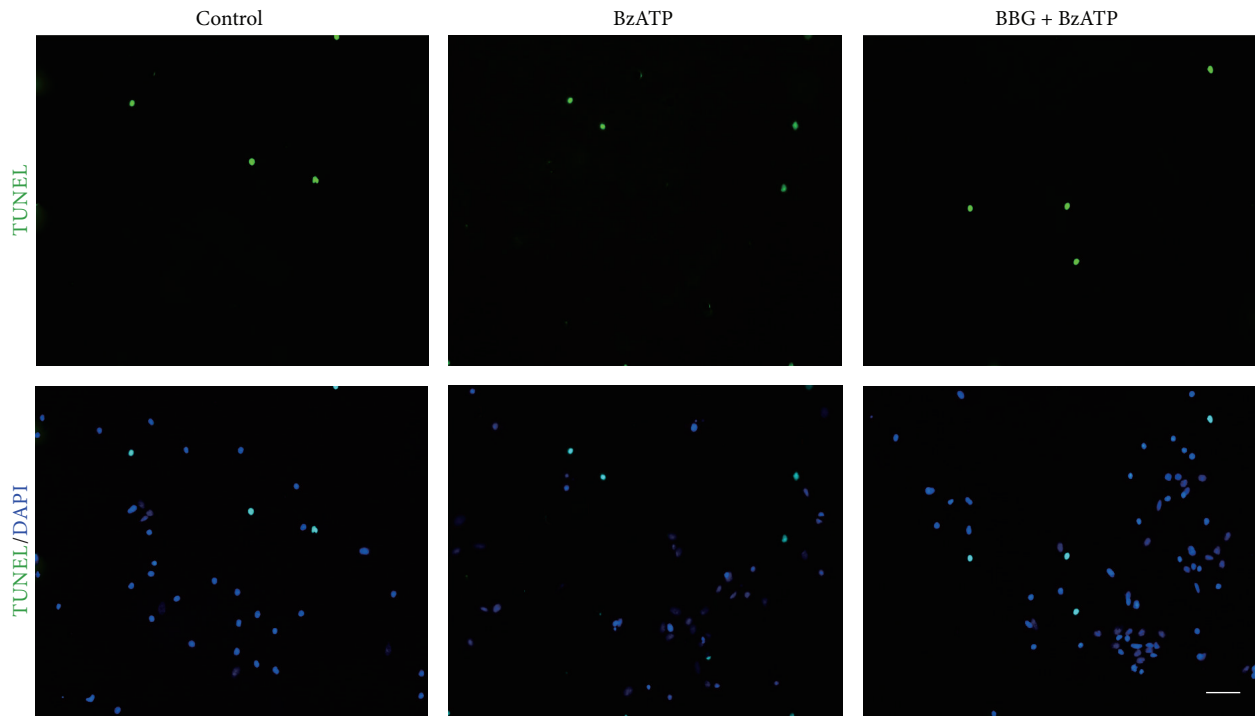
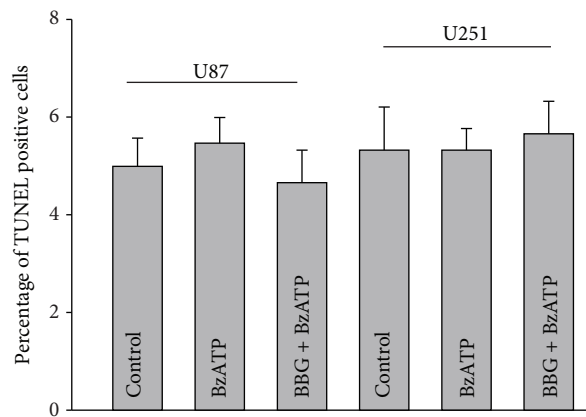


FIGURE 3: BzATP enhances migration and proliferation of U87 and U251 glioma cells. (a) Increased migration of the U87 cells with the treatment of BzATP and blocked by BBG (antagonist of P2X₇R), P2X₇R-siRNA, and PD98059 (ERK inhibitor) for 24 h. (b) Bar charts summarizing the migration of U87 cells under different conditions. (c) Increased migration of the U251 cells with the treatment of BzATP and blocked by BBG, P2X₇R-siRNA and PD98059 for 24 h. (d) Bar charts summarizing the migration of U251 cells under different conditions. (e) A strong expression of P2X₇R was detected in the scrambled siRNA treated groups and its expression was almost absent in the P2X₇R-siRNA treated groups. (f) The expression of p-ERK was suppressed by PD98059. (g) The proliferation mediated by BzATP of U87 glioma cell was blocked by P2X₇R-siRNA. (h) The proliferation mediated by BzATP of U251 glioma cell was blocked by P2X₇R-siRNA. * $P < 0.05$ compared with the control; # $P < 0.05$ compared with the BzATP group.



(a)



(b)

FIGURE 4: BzATP does not enhance apoptosis in U87 and U251 glioma cells. (a) Microphotographs show representative confocal images of TUNEL signals (green) and DAPI (blue) in U87 glioma cells treated by BzATP and blocked by BBG. (b) Bar charts summarizing the percentage of TUNEL positive cell in U87 and U251 cells treated by BzATP and blocked by BBG.

glioma is characterized by a strikingly high concentration of adenosine and ATP [6]. P2X₇R is an ATP-gated cation channel that regulates cell proliferation and apoptosis [25–28] and it is widely expressed in the immune system and nervous system [28, 29]. P2X₇R expression would increase in various inflammatory diseases, neurodegenerative diseases, neuropathic pain, and trauma [29–31]. In addition, it is also expressed in different types of tumors such as leukemia, prostate cancer, breast cancer, neuroblastoma, and thyroid papillary carcinoma [32–34]. Some studies have reported that P2X₇R activation correlated with tumor severity, prognosis, and survival. For example, in breast tumor, P2X₇R activation promoted tumor cell proliferation, while KN62, the P2X₇R

antagonist, or shRNA of P2X₇R inhibited the proliferation and even promoted the apoptosis of tumor cells [34]. The metastatic ability of lymphoma cells decreased significantly while P2X₇R was silenced by shRNA [34]. Giannuzzo et al. found that P2X₇R were expressed in human pancreatic cancer cells, and AZ10606120, a specific inhibitor of P2X₇R, inhibited the metastasis and invasion of pancreatic tumor cells [35]. In neuroblastoma, increased expression and activation of P2X₇R accelerate the proliferation and metastasis of tumor cells. Higher percentage of P2X₇R positive tumor cells makes poor prognosis of neuroblastoma [36]. In present study, P2X₇R expression increased with a higher grade of gliomas, suggesting that P2X₇R expression was associated with the

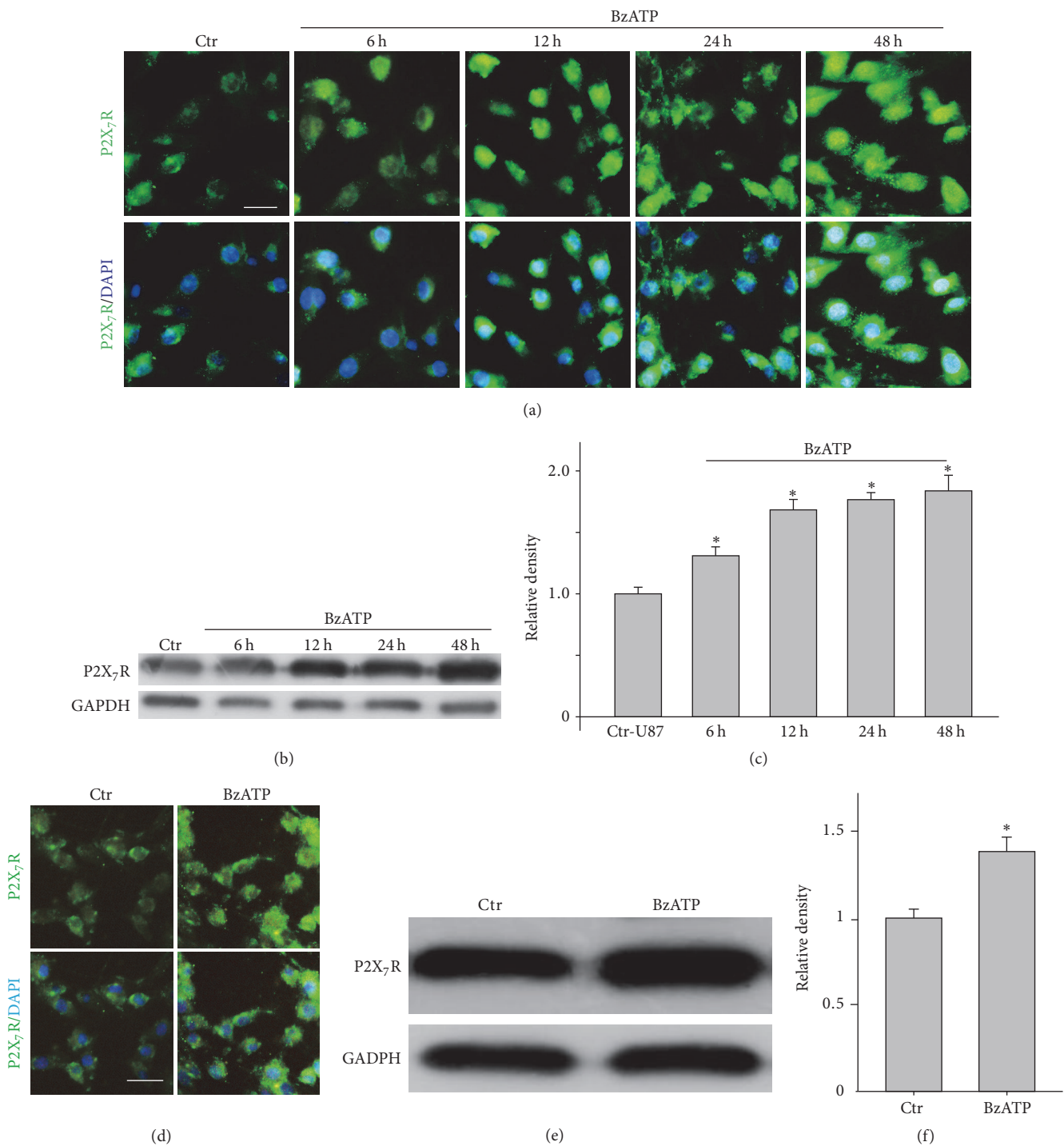


FIGURE 5: *P2X₇R* protein expression was induced by BzATP in human glioma cells. (a) Immunofluorescence labeling showing *P2X₇R* protein expression in control (Ctr) and BzATP (100 μ M)-treated U87 cells for different time (6–48 h). Scale bar = 20 μ m. (b) Representative immunoblots showing *P2X₇R* protein expression in Ctr and BzATP (100 μ M)-treated U87 cells for different time (6–48 h). (c) Bar charts summarizing the average densitometric quantification of immunoreactive bands of *P2X₇R* proteins of U87 cells under different conditions, $n = 6$ for each group. * $P < 0.05$ versus Ctr. (d) Immunofluorescence labeling showing the changes in *P2X₇R* protein expression in Ctr and BzATP (100 μ M)-treated U251 cells for 24 h. Scale bar, 20 μ m for all images. (e) Representative immunoblots showing the changes in *P2X₇R* protein expression in Ctr and BzATP (100 μ M)-treated U251 cells for 24 h. (f) Bar charts summarizing the average densitometric quantification of immunoreactive bands of *P2X₇R* proteins of U 251 cells in Ctr and BzATP (100 μ M)-treated groups, $n = 6$ for each group. * $P < 0.05$ versus Ctr.

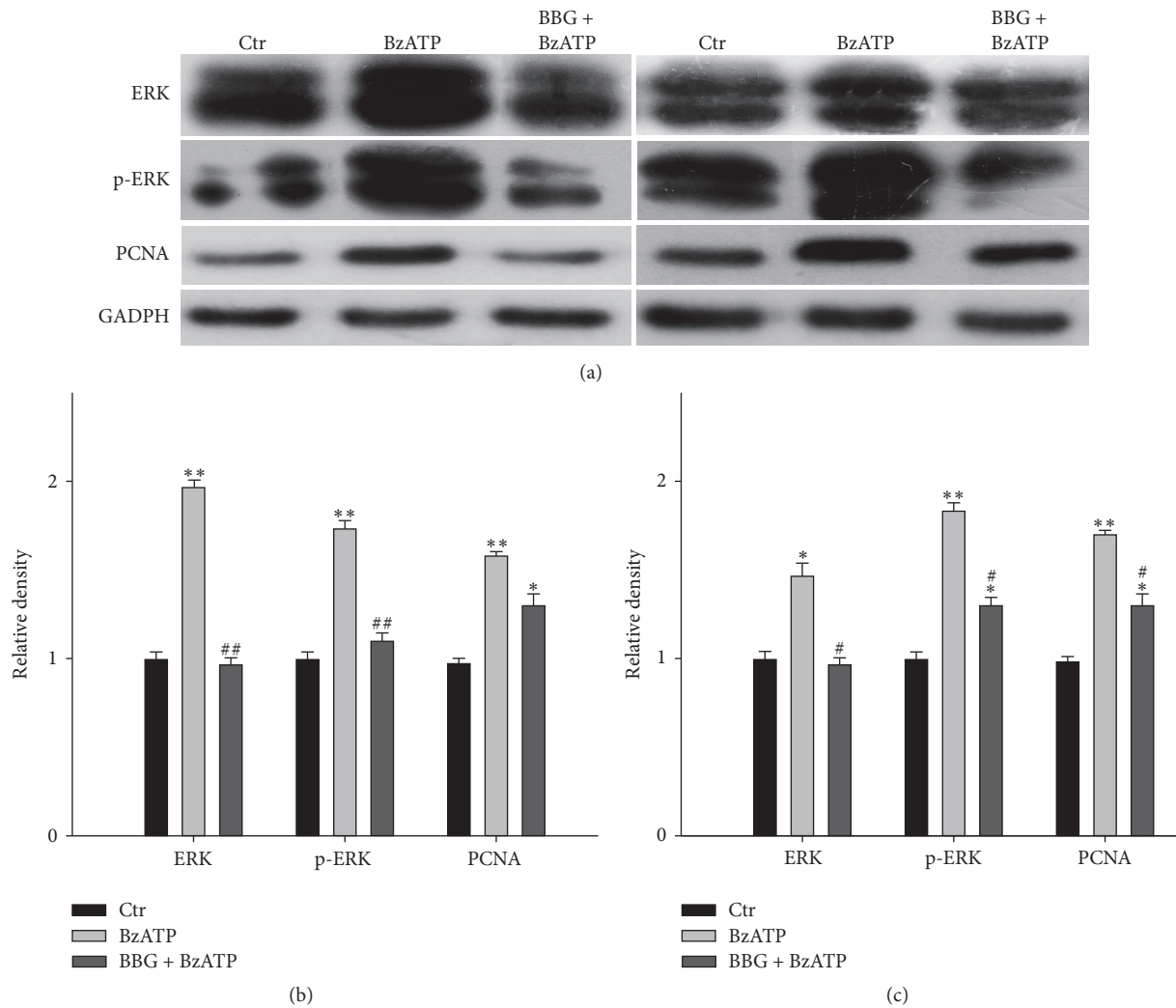


FIGURE 6: BzATP-induced activation of ERK pathway in U87 and U251 glioma cells. (a) Representative immunoblots showing the changes in ERK, p- ERK, and PCNA protein expression in control (Ctr), BzATP (100 μ M), and BzATP + BBG-treated U87 (left) and U251 (right) cells. (b, c) Bar charts summarizing the average densitometric quantification of immunoreactive bands of ERK, p-ERK, and PCNA proteins of U87 (b) and U251. (c) Cells under different conditions, $n = 6$ for each group. * $P < 0.05$ and ** $P < 0.01$ versus Ctr; # $P < 0.05$ and ## $P < 0.01$ versus BzATP group.

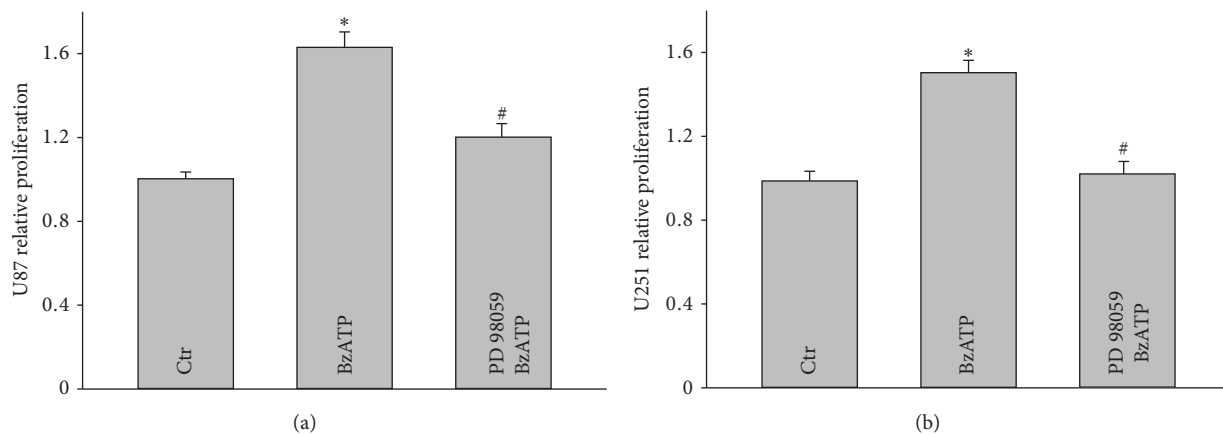


FIGURE 7: Involvement of ERK pathway in the BzATP mediated proliferation of U87 and U251 glioma cells. (a) The proliferation of U87 glioma cell upon treatment of BzATP (100 μ M) alone or together with PD98059 for 24 h. (b) The proliferation of U251 glioma cell upon treatment of BzATP (100 μ M) alone or together with PD98059 for 24 h; * $P < 0.05$ compared to the control groups; # $P < 0.05$ compared to the BzATP groups.

tumor prognosis. The increasing expression of P2X₇R in highest grades of glioma tissues could be a secondary effect of the tumor progression and not necessarily a causal factor. Our in vitro study showed that activation of P2X₇R promoted proliferation and migration of human glioma cells, and the effects were blocked by an antagonist of the receptor. Targeting P2X₇R seems to have a suppression effect on glioma progression.

4.2. P2X₇R Activation Does Not Induce Apoptosis of Glioma Cells. Brief stimulation of P2X₇R leads to increase of intracellular calcium influx whereas repeated or prolonged stimulation of P2X₇R induces the formation of a nonselective pore allowing the entry of solutes up to 900 Da in size, which leads to membrane blebbing, release of cytokines, and eventually cell death [14, 15]. Thus, overactivation of P2X₇R can lead to different kinds of cell death [9]. In some neurons, activation of P2X₇R leading to cell death is one of the key mechanisms causing neurodegeneration in Alzheimer's disease and multiple sclerosis [37, 38]. Our previous data also showed activation of P2X₇R increased the death of retinal ganglion cell in glaucoma [21, 39]. However, in tumor tissues, P2X₇R activation inhibited the apoptosis of tumor cells and promoted cell proliferation [40]. A number of studies have reported that elevated levels of extracellular ATP contribute to the progression of brain tumor growth that may be correlated with the activation of P2X₇R [41–43]. Despite considerable technical difficulties of measuring extracellular ATP concentration, it is estimated that extracellular ATP in millimolar level could be present in pathological condition of inflammation and tumor [44]. In human glioma cell lines U87 and U251, it was reported that ATP in millimolar concentration promoted the release of cytokines such as IL-8 without cells death [22]. In our present study, BzATP which has a higher affinity than ATP to P2X₇R was used to activate P2X₇R. Although other studies showed BzATP could cause the formation of large pore size and cell death in human and rat central nervous system [9], the present study showed no significant increase in apoptosis of BzATP-treated human glioma cells lines.

4.3. The MEK/ERK Pathway Was Involved in P2X₇R Mediated Proliferation and Migration of Glioma Cells. The highly conserved RAS-mitogen activated protein kinase (MAPK) signaling pathway is involved in a wide range of cellular processes, including cell survival, differentiation, and proliferation [45]. MEK/ERK pathway is one of the major pathways in MAPK pathway. In tumor cells, members of the MEK/ERK pathway encoding genes are often mutated and become overactivated, which causes this pathway to be important for the development of many human tumors such as breast cancer, thyroid carcinoma, and squamous cell carcinoma [46, 47]. The MEK/ERK pathway also plays a critical role in the development of gliomas [24, 48, 49]. The MEK/ERK pathway is involved in purinergic receptor, such as P2X₇R, mediated excitotoxic neuronal injury, and neuroprotection [50]. Our results showed that the ERK/p-ERK and PCNA protein expressions were increased in BzATP-treated glioma cell lines. As P2X₇R is a nonspecific cation channel

receptor, its activation will increase the intracellular calcium concentration and finally cause the expression of ERK and its phosphorylation. We further found that P2X₇R mediated glioma cell proliferation and migration dramatically decreased by blocking the MEK/ERK pathway. Therefore, our results suggest that MEK/ERK pathway is involved in P2X₇R mediated proliferation and migration of human glioma cells.

In conclusion, we found that the number of P2X₇R positive glioma cells increased with the grade of human tumor. Activation of P2X₇R in human glioma cells in vitro promoted the proliferation and migration of glioma cells but has no significant effect on apoptosis. Using a specific MEK blocker PD98059, we revealed that MEK/ERK pathway was involved in the P2X₇R mediated the proliferation and migration of glioma cells. As P2X₇R inhibitors have been tested in clinical trials, our results supports the idea that blocking the P2X₇R activation may be a feasible approach to prevent the progression of glioma.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (81200680; 81670852).

References

- [1] C. Ma, C. Zheng, E. Bai, and K. Yang, "miR-101 inhibits glioma cell invasion via the downregulation of COX-2," *Oncology Letters*, vol. 12, no. 4, pp. 2538–2544, 2016.
- [2] S. Mittal, M. C. Szlachzky, and G. R. Barger, "Low-grade gliomas in adults," *Current Treatment Options in Neurology*, vol. 10, no. 4, pp. 271–284, 2008.
- [3] J. C. L. Alfonso, A. Köhn-Luque, T. Stylianopoulos, F. Feuerhake, A. Deutsch, and H. Hatzikirou, "Why one-size-fits-all vaso-modulatory interventions fail to control glioma invasion: In silico insights," *Scientific Reports*, vol. 6, Article ID 37283, 2016.
- [4] B. Grobbsen, P. P. De Deyn, and H. Slegers, "Rat C6 glioma as experimental model system for the study of glioblastoma growth and invasion," *Cell and Tissue Research*, vol. 310, no. 3, pp. 257–270, 2002.
- [5] G. Zhang, L. Chen, K. Sun et al., "Neuropilin-1 (NRP-1)/GIPC1 pathway mediates glioma progression," *Tumour Biology: The Journal of the International Society for Oncodevelopmental Biology and Medicine*, vol. 37, no. 10, pp. 13777–13788, 2016.
- [6] G. Bianchi, M. Vuerich, P. Pellegatti et al., "ATP/P2X₇ axis modulates myeloid-derived suppressor cell functions in neuroblastoma microenvironment," *Cell Death & Disease*, vol. 5, no. 3, Article ID e1135, 2014.
- [7] S. TrabANELLI, D. Očadlíková, S. Gulinelli et al., "Extracellular ATP exerts opposite effects on activated and regulatory CD4⁺ T cells via purinergic P2 receptor activation," *The Journal of Immunology*, vol. 189, no. 3, pp. 1303–1310, 2012.
- [8] H. Franke, A. Verkhatsky, G. Burnstock, and P. Illes, "Pathophysiology of astroglial purinergic signalling," *Purinergic Signalling*, vol. 8, no. 3, pp. 629–657, 2012.

- [9] M. Tewari and P. Seth, "Emerging role of P2X₇ receptors in CNS health and disease," *Ageing Research Reviews*, vol. 24, pp. 328–342, 2015.
- [10] P. Pevarello, S. Bovolenta, P. Tarroni et al., "P2X₇ antagonists for CNS indications: recent patent disclosures," *Pharmaceutical patent analyst*, vol. 6, no. 2, pp. 61–76, 2017.
- [11] L. Stokes, S. J. Spencer, and T. A. Jenkins, "Understanding the role of P2X₇ in affective disorders—are glial cells the major players?" *Frontiers in Cellular Neuroscience*, vol. 9, 258 pages, 2015.
- [12] A. Baroja-Mazo, M. Barberà-Cremades, and P. Pelegrín, "The participation of plasma membrane hemichannels to purinergic signaling," *Biochimica et Biophysica Acta (BBA) - Biomembranes*, vol. 1828, no. 1, pp. 79–93, 2013.
- [13] R. Jiang, A. Taly, and T. Grutter, "Moving through the gate in ATP-activated P2X receptors," *Trends in Biochemical Sciences*, vol. 38, no. 1, pp. 20–29, 2013.
- [14] P. A. Verhoef, M. Estacion, W. Schilling, and G. R. Dubyak, "P2X₇ receptor-dependent blebbing and the activation of Rho-effector kinases, caspases, and IL-1 β release," *The Journal of Immunology*, vol. 170, no. 11, pp. 5728–5738, 2003.
- [15] S. Roger, P. Pelegrin, and A. Surprenant, "Facilitation of P2X₇ receptor currents and membrane blebbing via constitutive and dynamic calmodulin binding," *The Journal of Neuroscience*, vol. 28, no. 25, pp. 6393–6401, 2008.
- [16] W. Wei, J. K. Ryu, H. B. Choi, and J. G. McLarnon, "Expression and function of the P2X₇ receptor in rat C6 glioma cells," *Cancer Letters*, vol. 260, no. 1-2, pp. 79–87, 2008.
- [17] J. K. Ryu, N. Jantarotnotai, M. C. Serrano-Perez, P. L. McGeer, and J. G. McLarnon, "Block of purinergic P2X₇R inhibits tumor growth in a c6 glioma brain tumor animal model," *Journal of Neuropathology & Experimental Neurology*, vol. 70, no. 1, pp. 13–22, 2011.
- [18] D. Avanzato, T. Genova, A. Fiorio Pla et al., "Activation of P2X₇ and P2Y₁₁ purinergic receptors inhibits migration and normalizes tumor-derived endothelial cells via cAMP signaling," *Scientific Reports*, vol. 6, Article ID 32602, 2016.
- [19] J. Fang, X. Chen, L. Zhang et al., "P2X₇R suppression promotes glioma growth through epidermal growth factor receptor signal pathway," *The International Journal of Biochemistry & Cell Biology*, vol. 45, no. 6, pp. 1109–1120, 2013.
- [20] M. P. Gehring, T. C. B. Pereira, R. F. Zanin et al., "P2X₇ receptor activation leads to increased cell death in a radiosensitive human glioma cell line," *Purinergic Signalling*, vol. 8, no. 4, pp. 729–739, 2012.
- [21] B. Xue, Y. Xie, Y. Xue et al., "Involvement of P2X₇ receptors in retinal ganglion cell apoptosis induced by activated Müller cells," *Experimental Eye Research*, vol. 153, pp. 42–50, 2016.
- [22] E. Braganhol, F. Kukulski, S. A. Lévesque et al., "Nucleotide receptors control IL-8/CXCL8 and MCP-1/CCL2 secretions as well as proliferation in human glioma cells," *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, vol. 1852, no. 1, pp. 120–130, 2015.
- [23] F. B. Morrone, A. P. Horn, J. Stella et al., "Increased resistance of glioma cell lines to extracellular ATP cytotoxicity," *Journal of Neuro-Oncology*, vol. 71, no. 2, pp. 135–140, 2005.
- [24] X. Li, Y. Cen, Y. Cai et al., "TLR9-ERK-mTOR signaling is critical for autophagic cell death induced by CpG oligodeoxynucleotide 107 combined with irradiation in glioma cells," *Scientific Reports*, vol. 6, Article ID 27104, 2016.
- [25] J. Wang, S. Liu, Y. Nie et al., "Activation of P2X₇ receptors decreases the proliferation of murine luteal cells," *Reproduction, Fertility and Development*, vol. 27, no. 8, pp. 1262–1271, 2015.
- [26] Á. Oliveira, P. Illes, and H. Ulrich, "Purinergic receptors in embryonic and adult neurogenesis," *Neuropharmacology*, vol. 104, pp. 272–281, 2016.
- [27] F. Amoroso, E. Salaro, S. Falzoni et al., "P2X₇ targeting inhibits growth of human mesothelioma," *Oncotarget*, vol. 7, no. 31, pp. 49664–49676, 2016.
- [28] A. Agrawal, Z. Henriksen, S. Syberg et al., "P2X₇Rs are involved in cell death, growth and cellular signaling in primary human osteoblasts," *Bone*, vol. 95, pp. 91–101, 2017.
- [29] R. Le Feuvre, D. Brough, and N. Rothwell, "Extracellular ATP and P2X₇ receptors in neurodegeneration," *European Journal of Pharmacology*, vol. 447, no. 2-3, pp. 261–269, 2002.
- [30] G. Burnstock, "Physiopathological roles of P2X receptors in the central nervous system," *Current Medicinal Chemistry*, vol. 22, no. 7, pp. 819–844, 2015.
- [31] C. Volonté, S. Apolloni, S. D. Skaper, and G. Burnstock, "P2X₇ Receptors: channels, pores and more," *CNS & Neurological Disorders - Drug Targets*, vol. 11, no. 6, pp. 705–721, 2012.
- [32] G. Burnstock and F. Di Virgilio, "Purinergic signalling and cancer," *Purinergic Signalling*, vol. 9, no. 4, pp. 491–540, 2013.
- [33] F. Di Virgilio, D. Ferrari, and E. Adinolfi, "P2X₇: A growth-promoting receptor - Implications for cancer," *Purinergic Signalling*, vol. 5, no. 2, pp. 251–256, 2009.
- [34] C. Tan, L. Han, L. Zou et al., "Expression of P2X₇R in breast cancer tissue and the induction of apoptosis by the gene-specific shRNA in MCF-7 cells," *Experimental and Therapeutic Medicine*, vol. 10, no. 4, pp. 1472–1478, 2015.
- [35] A. Giannuzzo, M. Saccomano, J. Napp, M. Ellegaard, F. Alves, and I. Novak, "Targeting of the P2X₇ receptor in pancreatic cancer and stellate cells," *International Journal of Cancer*, vol. 139, no. 11, pp. 2540–2552, 2016.
- [36] R. Gómez-Villafuertes, P. García-Huerta, J. I. Díaz-Hernández, and M. T. Miras-Portugal, "PI3K/Akt signaling pathway triggers P2X₇ receptor expression as a pro-survival factor of neuroblastoma cells under limiting growth conditions," *Scientific Reports*, vol. 5, Article ID 18417, 2015.
- [37] A. D. Sadovnick, B. J. Gu, A. L. Trabousee et al., "Purinergic receptors," *Human Mutation*, vol. 38, no. 6, pp. 736–744, 2017.
- [38] H. G. Lee, S. M. Won, B. J. Gwag, and Y. B. Lee, "Microglial P2X₇ receptor expression is accompanied by neuronal damage in the cerebral cortex of the APP^{swe}/PS1^{dE9} mouse model of alzheimer's disease," *Experimental & Molecular Medicine*, vol. 43, no. 1, pp. 7–14, 2011.
- [39] Y. Xue, Y. Xie, B. Xue et al., "Activated Müller Cells Involved in ATP-Induced Upregulation of P2X₇ Receptor Expression and Retinal Ganglion Cell Death," *BioMed Research International*, vol. 2016, Article ID 9020715, 2016.
- [40] E. Adinolfi, M. G. Callegari, D. Ferrari et al., "Basal activation of the P2X₇ ATP receptor elevates mitochondrial calcium and potential, increases cellular ATP levels, and promotes serum-independent growth," *Molecular Biology of the Cell (MBoC)*, vol. 16, no. 7, pp. 3260–3272, 2005.
- [41] S. H. Sun, "Roles of P2X₇ receptor in glial and neuroblastoma cells: The therapeutic potential of P2X₇ receptor antagonists," *Molecular Neurobiology*, vol. 41, no. 2-3, pp. 351–355, 2010.
- [42] F. Di Virgilio, "Purines, purinergic receptors, and cancer," *Cancer Research*, vol. 72, no. 21, pp. 5441–5447, 2012.

- [43] F. Di Virgilio and E. Adinolfi, "Extracellular purines, purinergic receptors and tumor growth," *Oncogene*, vol. 36, no. 3, pp. 293–303, 2017.
- [44] S. Falzoni, G. Donvito, and F. Di Virgilio, "Detecting adenosine triphosphate in the pericellular space," *Interface Focus*, vol. 3, no. 3, 2013.
- [45] J. Masliah-Planchon, S. Garinet, and E. Pasmant, "RAS-MAPK pathway epigenetic activation in cancer: MiRNAs in action," *Oncotarget*, vol. 7, no. 25, pp. 38892–38907, 2016.
- [46] T. De Raedt, E. Beert, E. Pasmant et al., "PRC2 loss amplifies Ras-driven transcription and confers sensitivity to BRD4-based therapies," *Nature*, vol. 514, no. 7521, pp. 247–251, 2014.
- [47] K. Blagotinšek and D. Rozman, "Targeting signalling pathways in Hepatocellular carcinoma," *Current Pharmaceutical Design*, vol. 23, no. 1, pp. 170–175, 2017.
- [48] L. K. Yang, J. Zhu, Y. H. Chen et al., "Knockdown of angiopoietin-like protein 2 inhibits proliferation and invasion in glioma cells via suppressing the ERK/MAPK signaling pathway," *Oncology Research: Featuring Preclinical and Clinical Cancer Therapeutics*, vol. 25, no. 8, pp. 1349–1355, 2017.
- [49] J. W. Ma, Y. Zhang, J. C. Ye et al., "Tetrandrine exerts a radiosensitization effect on human glioma through inhibiting proliferation by attenuating ERK phosphorylation," *Biomolecules & Therapeutics*, vol. 25, no. 2, pp. 186–193, 2017.
- [50] F. Ortega, R. Pérez-Sen, E. G. Delicado, and M. Teresa Miras-Portugal, "ERK1/2 activation is involved in the neuroprotective action of P2Y₁₃ and P2X₇ receptors against glutamate excitotoxicity in cerebellar granule neurons," *Neuropharmacology*, vol. 61, no. 8, pp. 1210–1221, 2011.