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Original Article

Altered expression of connexin43 and phosphorylation connexin43 in glioma tumors

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Abstract: In this study, we aim to evaluate the connexin (Cx43) and phosphorylation Cx43 (p-Cx43) expression of human glioma tumors and correlate their expression with degrees of malignancy and proliferation, apoptosis, and migration activity of tumors. Cx43 and p-Cx43 expression were examined by Western blot analysis and immunohistochemical staining. The U251 cell viability was measured by MTT analysis. The apoptosis and migration were also evaluated by flow cytometric analysis and fluoroblok transwell chambers, respectively. We found that the Cx43 expression were significantly downregulated in in malignant glioma (WHO grade III and IV), compared to the malignant glioma (WHO grade I and II) and the p-Cx43 expression levels of malignant glioma (WHO grade III and IV) were significantly increased ($P<0.05$), compared to the malignant glioma (WHO grade I and II) at immunohistochemical analysis. After treatment of cells with a specific inhibitor of PKC, MAPK, and PTK inhibitors, the cell viability and migration were significantly decreased, while the apoptosis was slightly induced. In conclusion, the Cx43 expression level is inversely correlated with the tumor grade and proliferation and migration activity of tumor. Higher p-Cx43 expression level in high tumor grade suggests that a complex mechanism is involved in the suppression of tumor growth by connexins.

Keyword: Connexin43, phosphorylation, malignant glioma, U251

Introduction

Tumors of the central nervous system (CNS) are relatively rare compared to other tumor types, accounting for less than 2% of all malignancies [1]. However, brain cancer is often lethal and the average survival rate of patients is about 1 year after diagnosis. Current treatment consisting of surgery, radiation and chemotherapy has not been very effective due to the heterogeneity of the tumor and the seamless integration of tumor mass with the normal brain parenchyma [2]. Gliomas are the most common neoplasm of the CNS. The most aggressive subtype is glioblastoma multiforme (GBM) and has been traditionally classified by the World Health Organization (WHO) grade IV, which is associated more than 3 years [3]. With a median survival of approximately 14 months, GBM is considered the most lethal type of primary brain tumors in adults [4].

As one kind of connexins, there has been a remarkable and purposeful focus on Connexin43 (Cx43) for a number of valid reasons. Firstly, it is by far the most broadly and ubiquitously expressed member of the connexin family being expressed in nearly all vital organs that include the brain, heart, lungs, bone, stomach and intestine [5]. Second, Cx43 is probably the most extensively phosphorylated connexin and distinct phosphorylation processes regulate events related to connexin assembly, disassembly, turnover and channel function [6, 7]. Third, Cx43 has an extraordinarily rapid half-life of only 2-4 h [8, 9]. Gap junction mediated communication has been detected in both astrocytes and neural progenitors [10, 11]. Previous studies showed that Cx43 was highly expressed in astrocytes [12] and was the pre-dominant connexin proteins in neural progenitors [13].

Many connexins (e.g., Cx31, 32, 37, 40, 43, 45, 46, and 50) have multiple phosphorylation sites-with Cx43 being the most prevalent and well studied [14]. A variety of protein kinases (PKC, PKA, PKG, MAPK and PTK) play important roles in regulating phosphorylation level of GJ protein and changing conformation of the protein, which will due to function change by depolymerization and distortion of matching proteins [15]. The phosphorylation levels of different kinases are varied depending on different tissues and even diseases and kinases interactions, forming a complex regulatory network. Under conditions of injury or growth factor treatment, which find that Cx43 becomes sequentially phosphorylated by PKA, MAPK and PTK coincident with sequential changes in gap junctions, including increased gap junction size followed by inhibition of gap junctional communication and internalization of gap junctions from the plasma membrane [14]. Additionally, activation of protein kinase C (PKC) can lead to phosphorylation of Cx43, which affects trafficking, assembly, degradation, and channel gating of Cx43 gap junction channels [16].

Mis-expression of Cx43 in primary human gliomas has been investigated in a few studies with a limited number of samples. In general, decreasing Cx43 expression is associated with increasing proliferation and higher tumor grades. A screen of 18 primary tumor samples revealed low expression of Cx43 protein in grade III and IV tumors [17]. However, a recent study with 32 human samples reported high levels of Cx43 mRNA but low levels of Cx43 protein in high-grade gliomas [18], suggesting a post-transcriptional regulation of Cx43 protein. Indeed, it appears that the phosphorylation state and localization of Cx43 vary with tumor grades [19]. While low-grade gliomas show increased levels of Cx43 protein that are localized to the membrane and therefore presumably functional, Cx43, if present in high-grade tumors, is often non-phosphorylated and exhibits a predominantly cytoplasmic localization [19].

The objective of the present investigation was to determine the Cx43 expression and phosphorylation in human gliomas. Further functional analysis revealed the effects of Cx43 phosphorylation on cell viability, apoptosis and invasion of gliomas cells, providing new ideas and targets for the treatment of gliomas.

Materials and methods

Reagents and antibodies

RPMI 1640 and Dulbecco's modified Eagle's medium (DMEM) were supplied by GIBCO (Gaithersburg, MD), and FBS was supplied by Invitrogen (Grand Island, NY). Penicillin, streptomycin and DMSO were purchased from Invitrogen (Carlsbad, CA). PKC inhibitor (Staurosporine), MAPK inhibitor (SB 203580), PTK inhibitor (Vatalanib 2HCl PTK787) were bought from Sigma (St. Louis, MO). Anti-Cx43 and anti-phospho-Cx43 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Unless otherwise stated, all other reagents and buffers were obtained at biotechnology grade from ThermoFisher Scientific (Nepean, ON).

Tissue samples and cell lines

Eighty freshly resected glioma specimens were collected during the operations in the Department of Neurosurgery at Hsiang-ya Hospital (Changsha, China) from 2012 to 2013 and classified according to WHO categories. The specimens included 20 cases of pilocytic astrocytoma (tumor grade I), 20 cases of protoplasmic and fibrillary astrocytoma (tumor grade 2), 20 cases of anaplastic astrocytoma (tumor grade 3) and 20 cases of glioblastoma multiforme (tumor grade 4). Twenty samples of normal brain tissues were obtained from the internal de-compression of patients with gliomas, cerebral injury and epilepsy as controls. The tissue samples were snap frozen in the liquid nitrogen after resection and stored at -80°C. The U251 cell lines were cultured in DMEM supplemented with 10% FBS and subcultured every other day.

Immunohistochemistry

For immunostaining of Cx43 and p-Cx43, the ABC-peroxidase method was used. The tissue specimens and cells samples were prepared as frozen sections and cultured cells were plated on coverslips until confluency. After fixation with acetone and washing with PBS, the sections or coverslips were incubated with a primary Cx43 and p-Cx43 antibody (1:1000 dilution, Santa Cruz, USA) overnight at 4°C, then incubated with a biotinylated secondary antibody (1:200 dilution) at room temperature for 1 h followed by the incubation with ABC-peroxidase reagent (Vector, USA) for an additional 1

h, washed with PBS and stained with 3, 3-diaminobenzidine (30 mg dissolved in 100 mL Tris buffer containing 0.03% H_2O_2) for 5 min, rinsed in water and counterstained with hematoxylin.

For evaluation of Cx43 and p-Cx43 expression in various grades of tumors, 10 visual fields were examined randomly in each section or coverslip under 200× magnification of light microscope and the positive stained cells were counted in a total number of 500-1000 cells. The expression levels were scored according to the percentage of positive staining cells: (0) no positive cells; (1) positive cells < 25%; (2) positive cells between 25 and 50%; (3) positive cells between 50 and 75%; (4) positive cells >75%.

Western blotting

The tissue specimens cells samples were homogenized on ice in lysis buffer (8 mol/L urea, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 50 mmol/L Tris-HCl, pH = 8.0), supplemented with a proteinase inhibitor cocktail (Sigma) and sonicated three times for 10 s on ice. The protein in the samples was quantified by Bradford's method. Western blotting was performed as described previously [20]. A 10 μg sample of each protein was loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel and the band was transferred to the PVDF membrane (Millipore). The membranes were pre-incubated with 5% skim milk in PBS containing 0.05% Tween 20 for 1 h, then incubated with 1:10,000 anti-Cx43 and anti-p-Cx43 (Santa Cruz, USA) and 1:1000 anti-GAPDH (Epitomics) antibody overnight. Afterwards the membranes were washed in PBS containing 0.05% Tween 20, followed by incubation with anti-rabbit immunoglobulin-conjugated peroxidase-labeled dextran polymer (1:1000, Jackson) for 1 h at room temperature. The immune-reactivity was visualized by use of an ECL plus western blotting detection system (Millipore). Relative intensity of interest bands were measured with the Li-Cor Biosciences Odyssey infrared imaging system and associated software.

MTT test

Cell viability was assessed by the uptake of MTT (Sigma, St. Louis, MO). GMCs were seeded into 96-well plates (1500-2000 cells and 200 μL medium per well) in RPMI 1640 containing 15% FBS. On the next day, the cells were treated with 100 mL of serum-free medium for 16 h and then incubated under the specified conditions. After specific periods, 3- (4, 5-dimethylthiazol 2-yl)-2, 5- (diphenyltetrazolium bromide) (MTT) (5 mg/mL, 10 μL/well) was added into the wells, and the samples were incubated at 37°C for another 4 h. Subsequently, the medium was carefully removed in such a way that no loosely adherent cells were removed. Cells containing the trapped MTT crystals were then solubilized in 100 μL DMSO at 37°C for 10 min. The optical density (OD) at 490 nm was determined on a Labsystems Multiscan RC plate reader (Fischer Scientific, Pittsburgh, PA, USA).

Flow cytometric (FCM) analysis of apoptosis

After treatment, cells were trypsinized, washed with PBS and suspended with 500 μL of binding buffer containing 5 μL of Annexin V-FITC and 5 μL of propidium iodide (PI) (BD Biosciences, San Jose, CA, USA). After incubation for 15 min at room temperature in the dark, cells were subjected to flow cytometry assay. Flow cytometry was performed using a FACSCanto 6-color flow cytometer (BD Biosciences, San Jose CA, USA).

For migration assays, transwell chambers (Falcon HTS FluoroBlok Inserts; 8 μm pore size) combined with 24-well cell culture companion plates (Becton Dickinson) were used. Before migration, the cells were serum-starved over night in DMEM containing 0.5% NBCS. The cells were trypsinized, resuspended in DMEM-0.5% NBCS and seeded onto the filter membrane of the insert (5×10^4 cells in 400 μL). The bottom compartment of the chambers was filled either with 0.8 mL DMEM-0.5% NBCS only (unstimulated controls) or with 0.8 mL DMEM with serum added as stimulus. Cells were allowed to migrate through the filter membrane as indicated for 8 or 16 h at 37°C in a CO_2 incubator. All treatments were performed in triplicate. Following incubation, media were aspirated and the migrated cells, now at the bottom surface of the light-impermeable FluoroBlok filter membranes, were stained with calcein AM (4 μg/mL; Invitrogen) for 90 min at 37°C in a CO_2 incubator. Migration was then quantified as fluorescence units using a fluorescence reader with bottom reading capability at 485/535 nm (TECAN). Stimulated cell migration was expressed as fold migration (increase) over the con-

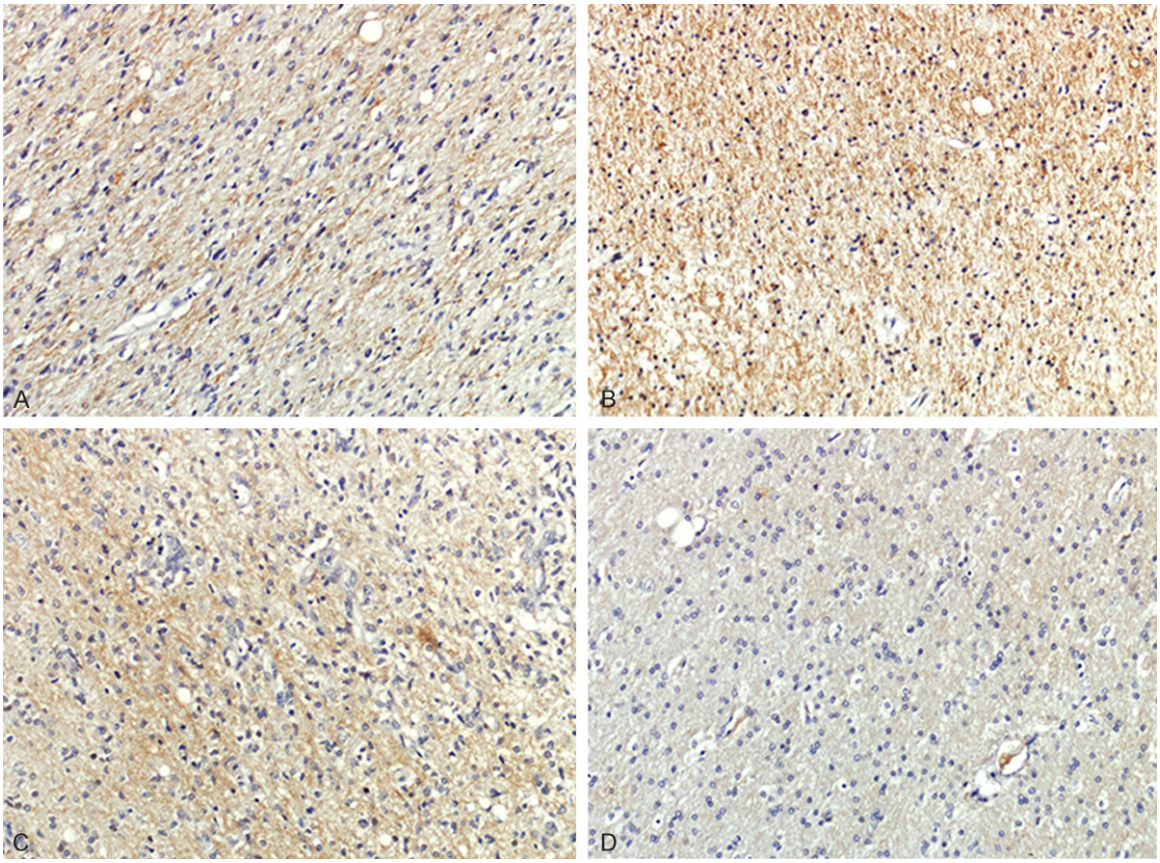

Figure 1. Immunohistochemistry analysis of the Cx43 expression in tumor and normal tissues. A. Normal tissues (200×); B. I-II grade gliomas tissues (200×); C. III grade gliomas tissues (200×); D. IV grade gliomas tissues (200×).

trol values obtained in unstimulated cells (migrated cells through membrane towards stimulus/migrated cells through membrane without stimulus). Results are represented as mean ± SD of fold migration as normalised to unstimulated controls.

Statistical analysis

Values are expressed as the means ± SD. All statistical calculations were performed with SPSS 17.0 (SPSS Inc., Chicago, IL). The data that fit a normal distribution were analyzed with ANOVA, while those that did not meet the normal distribution were analyzed with a nonparametric Mann-Whitney test. A level of $P < 0.05$ was considered statistically significant.

Results

The Cx43 and p-Cx43 expression of normal and malignant glioma tissues

Immunohistochemical staining showed that all of the 20 normal brain tissue expressed Cx43 protein (100%, 20/20). However, the percentage of positive Cx43 expression in 80 astrocytic tumors was 57.5% (46/80) and the percentage was decreased with the ascending of tumor grade (**Figure 1**). For the low level of malignant glioma (WHO grade I and II), the percentage of positive Cx43 expression was 100% (20/20) and 80% (16/20), respectively. For the high level of malignant glioma (WHO grade III and IV), the percentage of positive Cx43 expression was 25% (5/20) and 10% (2/20), respectively. In addition, the results of western blotting were the same as the immunohistochemical staining (**Figure 3**). The Cx43 expression levels of malignant glioma (WHO grade III and IV) were significantly lower than the normal brain tissue ($P < 0.05$). However, the Cx43 expression levels of malignant glioma (WHO grade I and II) were no significant difference ($P > 0.05$), compared to the normal brain tissue.

Phosphorylation of Connexins (Cxs) at different sites controls protein conformation, and further

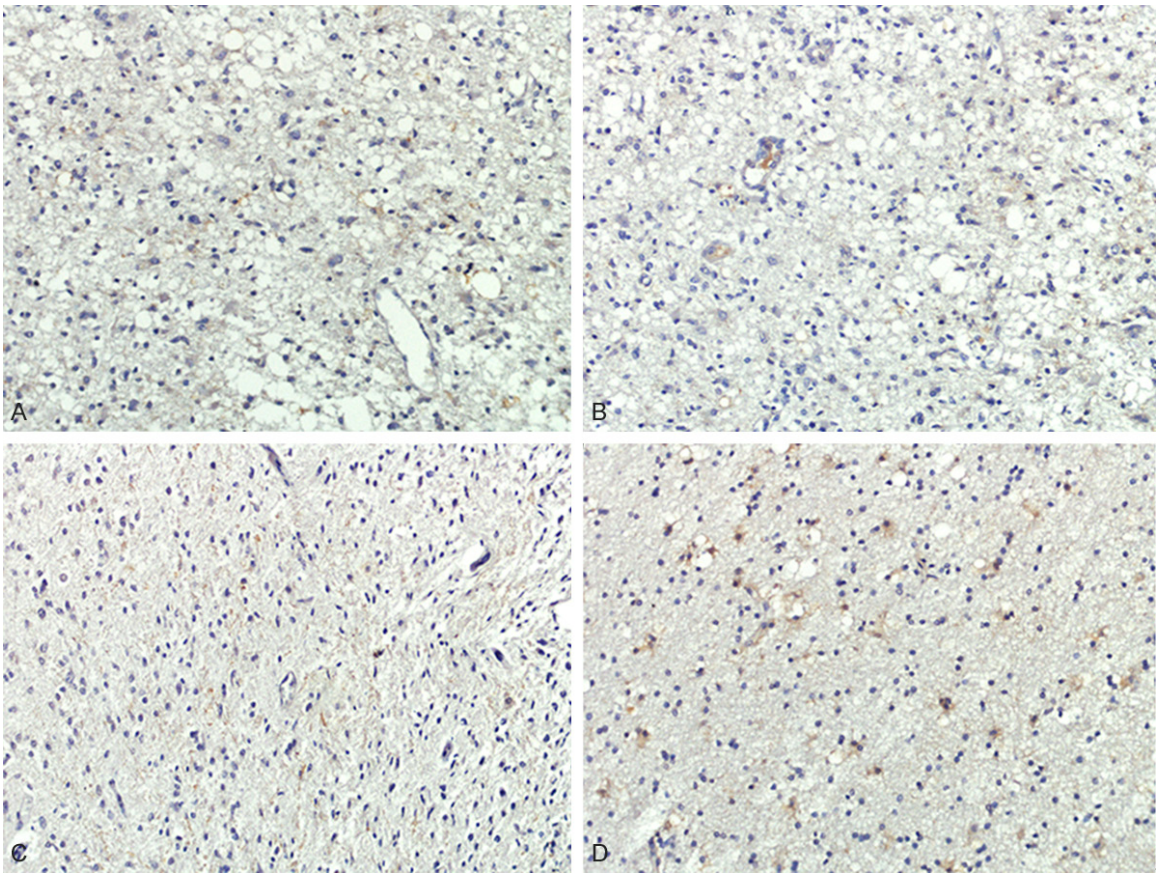

Figure 2. Immunohistochemistry analysis of the p-Cx43 expression in tumor and normal tissues. A. Normal tissues (200×); B. I-II grade gliomas tissues (200×); C. III grade gliomas tissues (200×); D. IV grade gliomas tissues (200×).

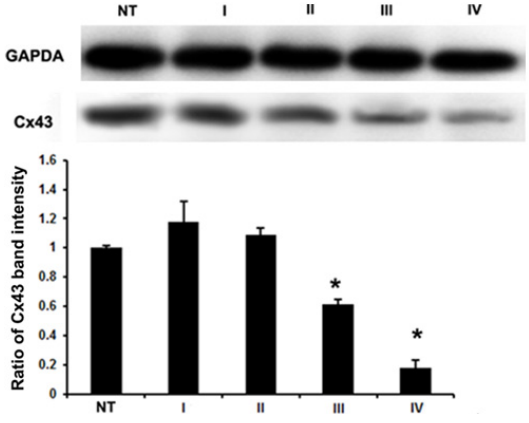

Figure 3. Western blot analysis of the Cx43 expression in tumor and normal tissues. *vs control, P < 0.05.

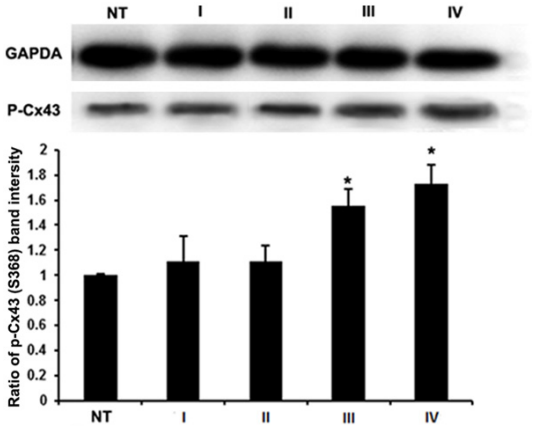

Figure 4. Western blot analysis of the p-Cx43 expression in tumor and normal tissues. *vs control, P < 0.05.

controls gap junction assembly, gap junction size and gap junction turnover which determines the intercellular communication. In present study, the p-Cx43 expression levels of malignant glioma (WHO grade III and IV) were significantly increased (P < 0.05), compared to the malignant glioma (WHO grade I and II) at immunohistochemical analysis (**Figure 2**). The western blotting results indicated that p-Cx43 expression levels of malignant glioma (WHO

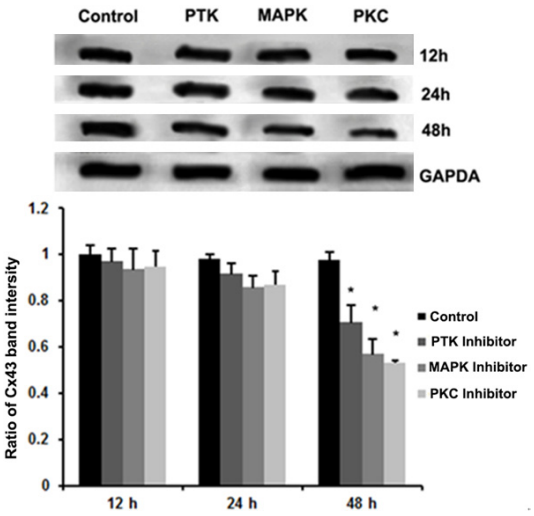

Figure 5. The PKC, MAPK, and PTK inhibitors affect Cx43 expression. U251 cells treated with PKC, MAPK, and PTK inhibitors for 12, 24, and 48 h. *vs control, $P < 0.05$.

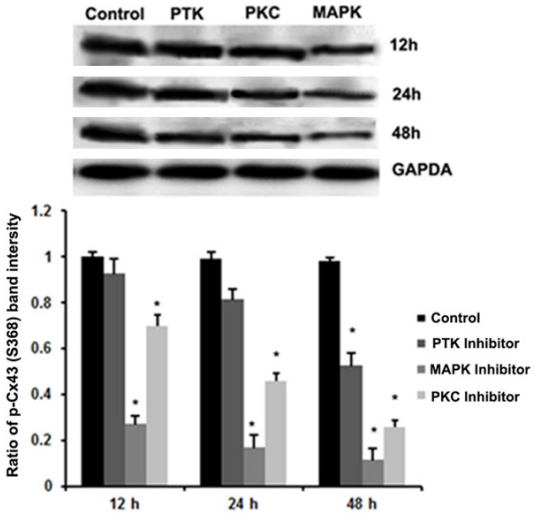

Figure 6. The PKC, MAPK, and PTK inhibitors affect Cx43 expression. U251 cells treated with PKC, MAPK, and PTK inhibitors for 12, 24, and 48 h. *vs control, $P < 0.05$.

grade III and IV) were significantly higher than the normal brain tissue ($P < 0.05$). However, the p-Cx43 expression levels of malignant glioma (WHO grade I and II) were no significant difference ($P > 0.05$), compared to the normal brain tissue (**Figure 4**).

Downregulation of Cx43 by PKC, MAPK, and PTK inhibitors

As shown in **Figure 5**, treatment of cells for 12 h with a specific inhibitor of PKC, MAPK, PTK respectively resulted in no obvious influences of Cx43 expression in U251 cells. Treatment of cells for 24 h with a specific inhibitor of PKC, MAPK, PTK respectively resulted in a slightly decreased Cx43 expression (92%, 85%, 87%, respectively) in U251 cells. After treatment of cells for 48 h with a specific inhibitor of PKC, MAPK, and PTK respectively, the Cx43 expression were strongly decreased and the effect of inhibitions of MAPK (57%) and PTK (53%) inhibitors were higher than the PKC (71%) inhibitor.

Downregulation of p-Cx43 by PKC, MAPK, and PTK inhibitors

As shown in **Figure 6**, compared to the control group, treatment of cells for 12 h with a specific inhibitor of MAPK resulted in a strongly decreased p-Cx43 expression (27.2%) in U251 cells, while treatment of cells for 12 h with a specific inhibitor of PTK resulted in a obviously decreased p-Cx43 expression (70.1%) in U251 cells. However, there was no obviously inhibition effect of p-Cx43 expression after treatment of cells for 12 h with a specific inhibitor of PKC. Treatment of cells for 24 h with a specific inhibitor of PKC, MAPK, PTK respectively resulted in a slightly decreased p-Cx43 expression (81.4%) and strongly decreased p-Cx43 expression (17.1% and 45.7%), respectively. After treatment of cells for 48 h with a specific inhibitor of PKC, MAPK, and PTK respectively, the p-Cx43 expression were strongly decreased and the effect of inhibitions of MAPK (11.4%) and PTK (25.7%) inhibitors were higher than the PKC (52.8%) inhibitor.

Proliferation of U252 cells is reduced by PKC, MAPK, and PTK inhibitors

After treatment of cells with a specific inhibitor of PKC for 12, 24, 48 h respectively, the cell viability was significantly decreased and the viability of 12 and 24 h was lower than the 48 h treatment group. After treatment of cells with a specific inhibitor of MAPK for 12, 24, 48 h respectively, the cell viability was significantly decreased and the inhibition effect of MAPK was higher than the PKC's. However, the U251 cell viability has not been influenced by the PTK inhibitor, obviously (**Figure 7**).

Apoptosis of U251 cells is induced by PKC, MAPK, and PTK inhibitors

After treatment of cells with a specific inhibitor of PKC for 12, 24, 48 h respectively, the cell

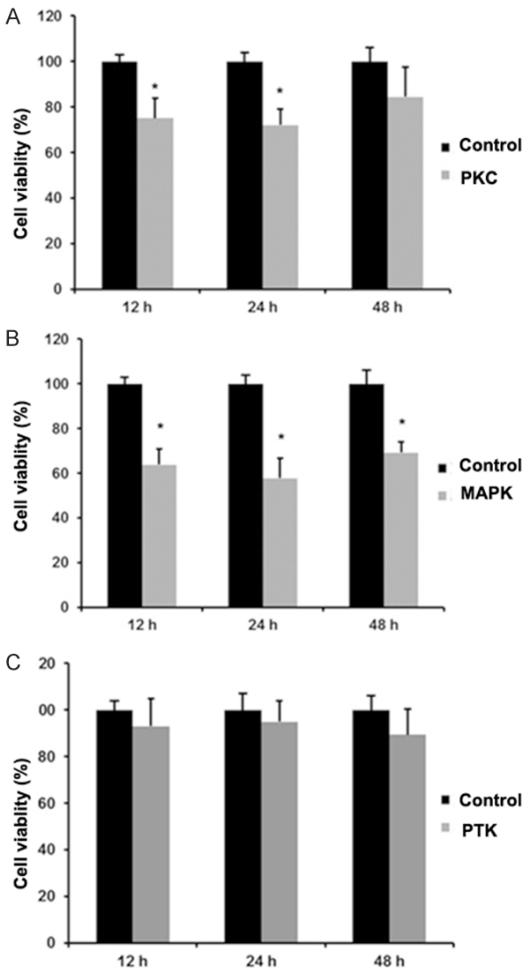

Figure 7. MTT analysis the effect of PKC (A), MAPK (B), and PTK (C) inhibitors on cell activity. U251 cells treated with PKC, MAPK, and PTK inhibitors for 12, 24, 48 h. *vs control, $P < 0.05$.

apoptosis was slightly induced compared to the control group. After treatment of cells with a specific inhibitor of MAPK for 12, 24, 48 h respectively, the cell apoptosis was obviously induced compared to the control group. However, the U251 cell apoptosis has not been influenced by the PTK inhibitor, obviously (**Figure 8**).

Migration of U251 cells is reduced by PKC, MAPK, and PTK inhibitors

Compared to the control group, treatment of cells with a specific inhibitor of MAPK and PKC for 12 and 24 h resulted a obviously decreased migration. However, after treatment of cells with a specific inhibitor of PTK for 12 and 24 h, the migration of U251 cells had no significantly decreased compared to the control group. In addition, the after 48 h treatment of PKC, MAPK, PTK inhibitors, the migration of U251 cells had no significantly decreased compared to the control group (**Figure 9**).

Discussions

Gap junctions, composed of proteins from the connexin gene family, are highly dynamic structures that are regulated by kinase-mediated signaling pathways and interactions with other proteins. Phosphorylation of Cx43 at different sites controls gap junction assembly, gap junction size and gap junction turnover [14]. Various stimuli can lead to sequential activation of multiple kinases and changes in Cx43 phosphorylation over time. For example, S365 phosphorylation plays a “gatekeeper” role by preventing downregulation of gap junctional communication by subsequent Cx43 phosphorylation at S368 [21]. Src activation subsequently leads to Src, MAPK and PKC phosphorylation of Cx43 at 6 serine and 2 tyrosine [22] also decreasing gap junction function. Cx43 phosphorylation at casein kinase 1 (CK1) or PKA-related sites cause changes in Cx43 structure that increase gap junction assembly [23]. These findings all argue that an understanding of how coordinated kinase activation and Cx43 phosphorylation regulates gap junction formation and disassembly under homeostatic and pathological conditions could lead to important insights that are translatable to therapeutic intervention.

To further elucidate the signaling molecules that those phosphorylate Cx43 in human gliomas, we studied the role of PKC, MAPK, and PTK. Our data indicated that inhibition of PKC, MAPK, PTK pathways by their specific inhibitors treatment 48 h resulted in the reduces Cx43 expression in gliomas, which the MAPK pathway played a most significant role in reducing Cx43 expression among the three pathways. After evaluated the phosphorylation of Cx43 affected by PKC, MAPK, and PTK inhibitors, we found that p-Cx43 expression was reduced by MAPK and PKC inhibitors which treated for 12, 24, and 48 h in gliomas cells. However, the PTK inhibitor had rare reduced the p-Cx43 expression in gliomas cells. Those suggested that the MAPK and PKC inhibitors may influence the gap junctions which resulted in the invasion and migration of gliomas through mediated the p-Cx43 expression. And those results were similar to previous studies [24, 25].

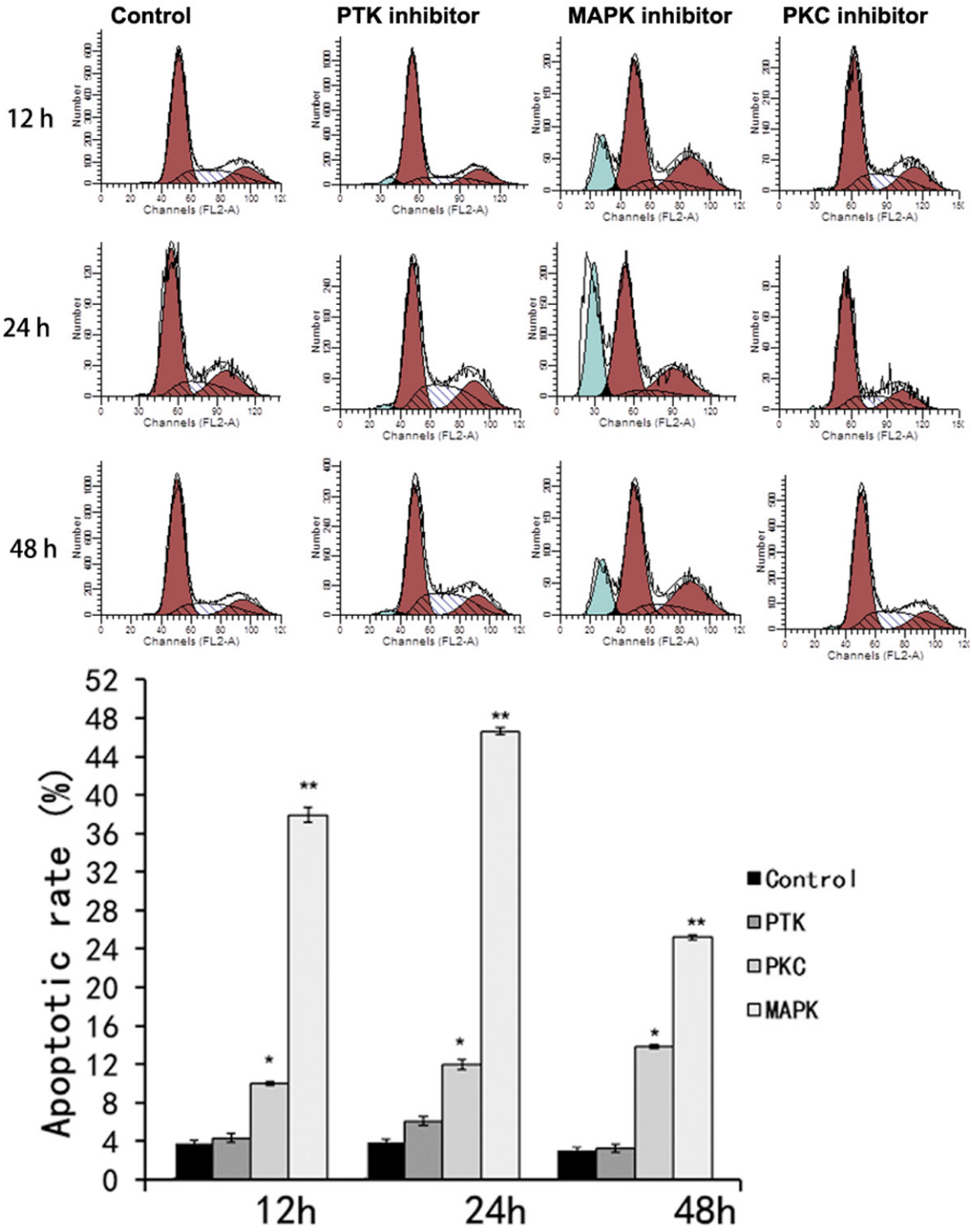

Figure 8. Flow cytometry analysis the effect of PKC, MAPK, and PTK inhibitors on cell apoptosis. U251 cells treated with PTK inhibitors for 12, 24 and 48 h. *vs control, $P<0.05$; **vs control, $P<0.01$.

Various studies in gliomas have demonstrated a role of Cx43 in glioma growth control and migration [2]. The reduction of GJIC in tumor cells is often attributed to reduced Cx43 expression [26]. The role of Cx43 in apoptosis is less well studied and it remains controversial whether GJIC is pro- or anti-apoptotic [2]. Recent evidence suggested the hemichannel activity of Cx43 may have a more significant role than intercellular channel activity in affect-

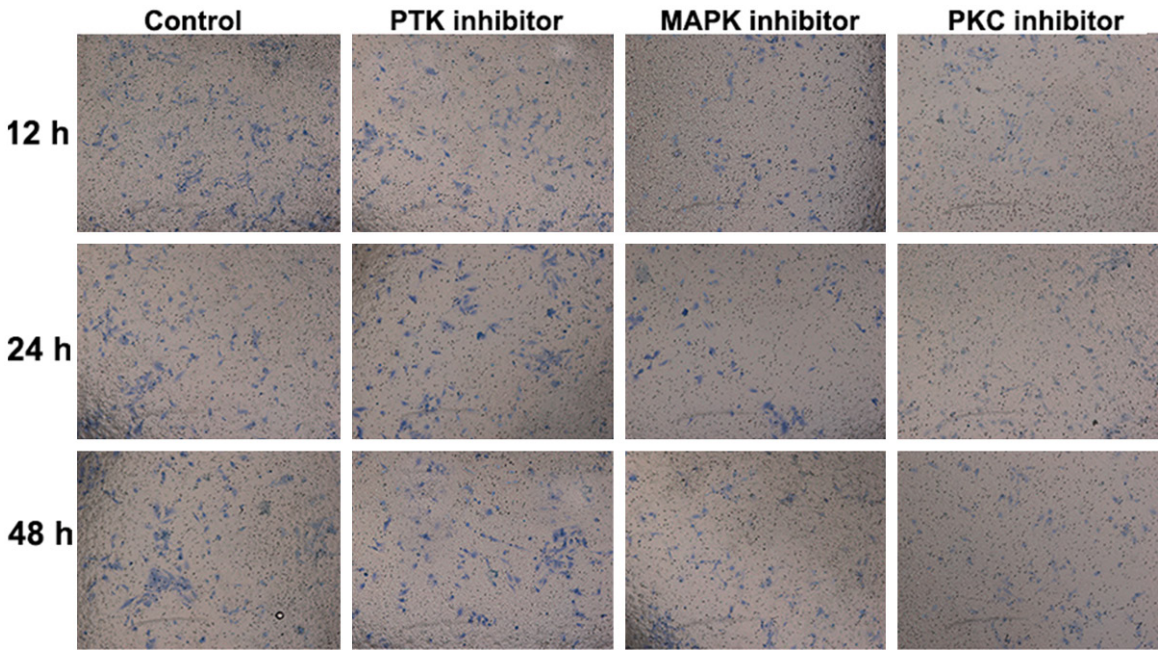

Figure 9. Transwell analysis the effect of PKC, MAPK, and PTK inhibitors on cell invasion. U251 cells treated with PKC, MAPK, and PTK inhibitors for 12, 24, and 48 h.

ing cell death [27-29]. In present study, we showed that the viability and migration were reduced and apoptosis was induced by the MAPK and PKC inhibitors through mediated the Cx43 and/or p-Cx43 expression.

Cell migration was assessed by two different methods, which in spite of different experimental conditions yielded remarkably similar results. Migration of cells through a membrane towards a stimulus in a modified Boyden chamber was increased by 37% in the presence of Cx43. However, when using this assay it cannot be excluded that enhanced migration was primarily due to less firm cell-cell contacts in the presence of Cx expression. Loosening of cell-cell contacts and disaggregation is required for single cells to migrate through the small pores of the membrane. This possibility could be excluded using the second migration assay which allows direct observation of cell migration by time lapse video microscopy. Increases in cell proliferation could also misinterpreted as increases in cell migration when the transmigration through the filter is used. However, Cx43 substantially promoted cell migration. There are three possible explanations for this effect: (i) Cx43 might form hemichannels that allow the passage of migration-stimulating humoral factors from the cytosol across the cell membrane and/or (ii) expression of Cx43 might lead to synthesis and release of humoral factors that enhance migration or (iii) Cx43 might be part of a signaling pathway that does not include communication across the membrane and would therefore be independent of "channel functions" of Cx43 [30].

Recent identification of Cx43 in mitochondria [31, 32] suggests Cx43 may participate in mitochondrial signaling, including apoptosis. Overexpression of Cx43 in human glioma cells leads to increased Bcl-2 expression [33]. Similarly, transcriptome analysis of Cx43-null astrocytes identified alteration in levels of expression of common apoptotic genes [34]. Cx43 has been demonstrated to inhibit caspase-3 activation [35]. In the mitochondria, Cx43 is known targets of PKC activation during cardioprotection against ischemia/hypoxia. The role of Cx43 in protecting cell from mitochondria mediated and hypoxia induced cell death was finally proved by cell apoptosis assay and mitochondrial release of cytochrome C [24].

In conclusion, a complex mechanism is involved in the suppression of tumor growth by connexins. The exact roles of Cx43 and GJIC in differ-

ent tumors and stage of carcinogenesis is far from elucidated and that should be studied further. In the present study, the evidences show that the high grade glioma tumors exhibit significantly reduced Cx43 and induced p-Cx43 expression, not only indicating the role of decreased Cx43 and increased p-Cx43 expression in the progression of glioma tumors, but also suggesting that Cx43 can be the potential target of therapy for malignant gliomas.

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Disclosure of conflict of interest

None.

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