Effects of Connexin43 Overexpression on U251 Cell Growth, Migration, and Apoptosis

Authors' Contribution:
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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Glioblastoma multiforme (GBM) is a highly aggressive malignant brain tumor with a high incidence in adults. Connexin43 (Cx43) has general roles in tumorigenesis and is expressed in U251 glioma cells. Accordingly, the effects of Cx43 on the growth, migration, and apoptosis and the underlying mechanisms mediating Cx43-dependent migration and apoptosis were examined in U251 cells.

Material/Methods: A Cx43-overexpressing U251 cell line was generated to analyze the effects of Cx43 overexpression on cell growth, wound healing, and apoptosis-related protein expression after treatment with temozolomide.

Results: The growth rate of U251 cells overexpressing Cx43 was significantly lower than that of parental wild-type cells, and cell morphology was considerably altered. The expression level of Bcl-2 was higher and the expression levels of Bax and caspase-3 were lower in cells overexpressing Cx43 than in wild-type cells. Additionally, the Bax/Bcl-2 ratio decreased.

Conclusions: Cx43 inhibited the growth of U251 cells, promoted morphological changes and migration, and inhibited apoptosis via a mitochondria-associated pathway.

MeSH Keywords: Apoptosis • Connexin 43 • Glioma

Abbreviations: GBM – glioblastoma multiforme; Cx43 – Connexin43; TMZ – temozolomide; GJIC – gap junction intercellular communication

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Background

Glioblastoma multiforme (GBM) is a type of malignant brain tumor with a high incidence in adults. GBM is highly aggressive and has a poor prognosis; the average survival time of patients with GBM is less than 1 year [1]. The common DNA alkylating agent temozolomide (TMZ) is widely used as a treatment for glioma [2].

Connexin43 (Cx43) is a small protein involved in gap junction intercellular communication (GJIC). It has been studied extensively in recent years owing to its association with tumorigenesis and the proliferation, migration, and apoptosis of tumor cells [3]. Cx43 is expressed at low levels in U251 glioma cells, derived from a grade II neuroblastoma. We evaluated the role of Cx43 in glioma cell growth, migration, and apoptosis using U251 cells as a model. Our findings provide a foundation for further studies of the mechanisms of Cx43 in glioma.

Material and Methods

Material

The human glioma cell lines U251 and the GFP-Cx43 plasmid were obtained from our laboratory inventory. IMDM culture medium was purchased from Gibco (Grand Island, NY, USA). Streptomycin, Lipofectamine 2000, and TRIzol were purchased from Invitrogen (Carlsbad, CA, USA). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the ECL Light-emitting Substrate Kit was purchased from Thermo (Waltham, MA, USA). The Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Kit was purchased from Roche (Basel, Switzerland).

Cell culture and establishment of stable cell lines

U251 human glioma cells were cultured in standard culture conditions in IMDM medium supplemented with 10% fetal bovine serum. Culture medium was refreshed every 1–2 days, and cells were passaged using 0.25% trypsin.

Serum-free medium was prepared according to the instructions provided with the Lipofectamine 2000 reagent, and the GFP-Cx43 plasmid was transfected into U251 cells. The medium was refreshed 6–7 days after transfection. Stable GFP-Cx43-transfected U251 cells were established by selection with G418.

Cell growth assay

U251 and GFP-Cx43 U251 cells were seeded in 24-well plates in medium containing 10% fetal bovine serum, with 3 duplicate samples in each group. The cell growth curves were plotted using data obtained at 7 different time points from day 1 to day 7.

Wound healing assay

U251 and stably transfected GFP-Cx43 U251 cells were seeded in 6-well plates and grown until cells reached at least 80% confluence. We used a 200-µL pipette tip to produce a linear wound. The size of the wound was then measured on days 1, 3, 4, and 5.

Apoptosis assays in Cx43-overexpressing U251 cells treated with TMZ

U251 and GFP-Cx43 U251 cells were plated in 96-well plates. Cells were untreated or treated with 100, 200, 400, 800, or 1000 µM TMZ in triplicate. After 24 h, MTS assays were used to detect cell proliferation by determining the optical density (OD) at 490 nm. The TMZ concentration was plotted against the cell survival rate. The expression of Cx43 and apoptosis-related genes was analyzed by reverse transcription polymerase chain reaction (RT-PCR).

U251 and GFP-Cx43 U251 cells were seeded in 96-well plates and treated with various concentrations of TMZ. RNA was extracted from the cells using TRIzol reagent. Reverse transcription was performed in a volume of 20 µL with incubation at 42°C for 60 min and 70 min. PCR amplification was then performed using the cDNA generated by reverse transcription. The PCR protocol was as follows: 95°C for 90 s, 94°C for 30 s, 52–56°C for 30 s, 72°C for 40 s, and 72°C for 10 min. The PCR primers are shown in Table 1.

Western blot analysis of Cx43 and apoptosis-related protein expression

U251 and GFP-Cx43 U251 cells were treated with or without 1000 µM. After 24 h, cells were collected and lysed. The protein concentration was determined using the BCA method. Vertical electrophoresis was carried out using 12% concentrating gels (80 V for 20 min) and 4% separation gels (120 V for 50 min). Wet transfer to membranes was carried out at 100 V for 50 min. The blot was blocked at 25 °C for 1 h. After blocking non-specific binding, the membranes were incubated overnight at 4°C with different specific primary antibodies in blocking solution. Membranes were then incubated with secondary antibodies for 2 h at room temperature. The blots were developed using enhanced chemiluminescence and analyzed using a Tanon GIS Gel Image Processing System (Shanghai, China).

Table 1. List of PCR primers.

| Target	Primer	Sequences	Product size (bp)
GAPDH	Sense	5'-GATTGTTGCCATCAACGACC-3'	371
	Antisense	5'-GTGCAGGATGCATTGCTGAC-3'	
H-Cx43	Sense	5'-TTGCTGCGAACCTACATC-3'	473
	Antisense	5'-TTGCTCACTTGCTTGCTT-3'	
H-Bcl-2	Sense	5'-TTCTTTGAGTTCGGTGGG-3'	206
	Antisense	5'-TTCAGAGACAGCCAGGAG-3'	
H-Bax	Sense	5'-GATGCGTCCACCAAGAAG-3'	273
	Antisense	5'-GAAGTCCAATGTCCAGCC-3'	
H-Caspase-3	Sense	5'-CTGGACTGTGGCATTGAG-3'	187
	Antisense	5'-GCTTGTCGGCATACTGTT-3'	
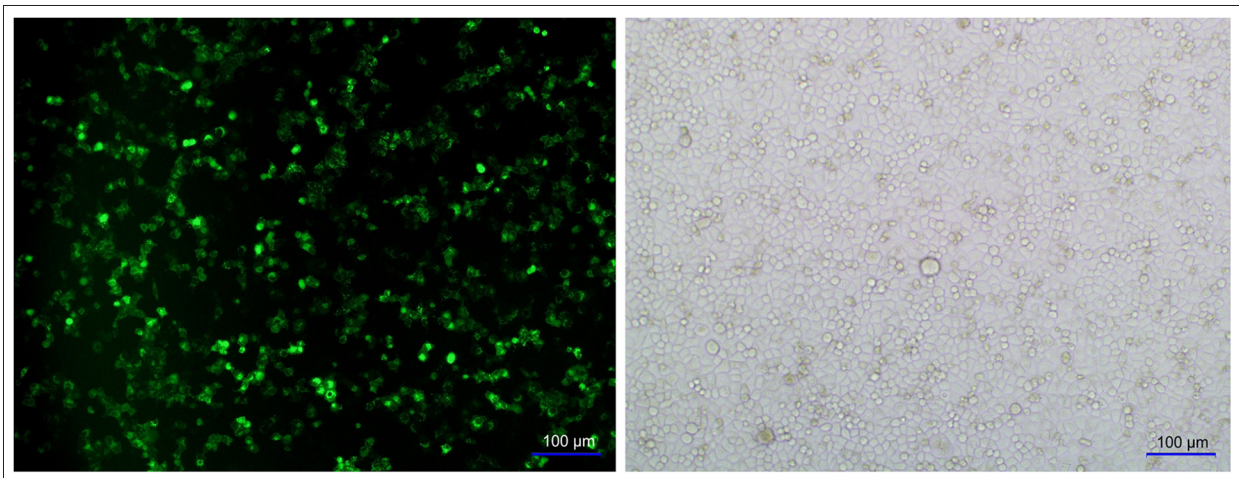
Figure 1. Stably transfected GFP-Cx43 U251 cells.

TUNEL assays

U251 and GFP-Cx43 U251 cells were plated in 24-well plates and treated with or without 400 or 1000 μM TMZ. Apoptosis was determined 24 h later using a TUNEL Apoptotic Cell Detection Kit. The cells were observed and counted under a fluorescence microscope.

Statistical analysis

Data were analyzed using SPSS 17.0. The statistical significance of differences between groups was determined using *t* tests. Data are expressed as means ± standard deviations. Results of the Western blot analysis were evaluated using Quantity One software. Differences with *p*-values of less than 0.05 were considered significant.

Results

Establishment of stably transfected GFP-Cx43 U251 cells

Stably transfected GFP-Cx43 U251 cells were established by transfection with the GFP-Cx43 plasmid and selection with G418, as shown in Figure 1.

Effects of Cx43 overexpression on U251 cell proliferation

There were minimal differences from days 1–4 between the numbers of U251 and Cx43-U251 cells (Figure 2). However, the number of U251 cells was significantly higher than the number of Cx43-U251 cells from days 5–7. These data suggested that there were significant differences in cell growth control between U251 and Cx43-U251 cells.
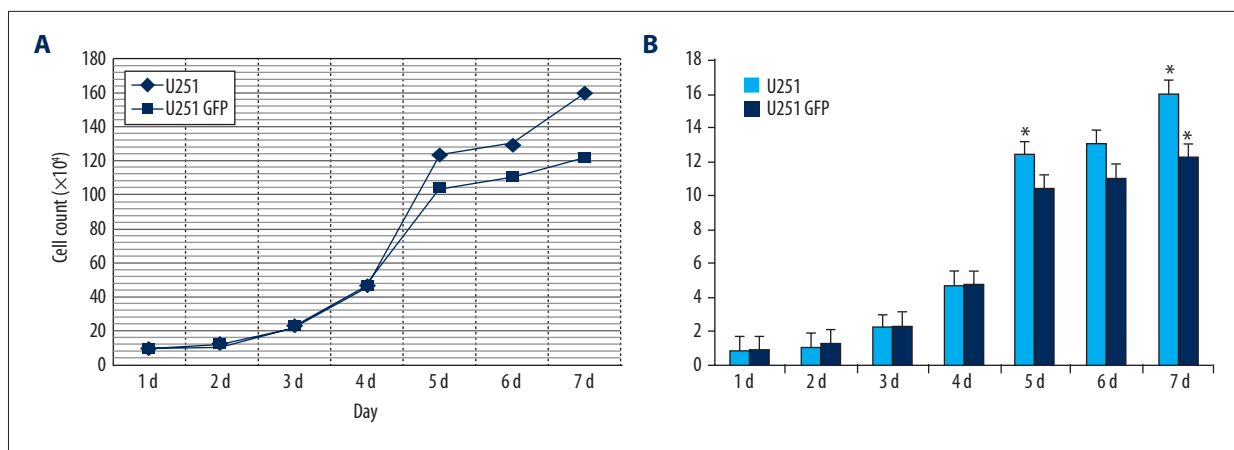
Figure 2. Changes in the numbers of U251 and Cx43-U251 cells over time. * *p*<0.05.

Effect of Cx43 overexpression on U251 cell migration

The migration abilities of U251 and GFP-Cx43 U251 cells were detected by wound healing assays. The morphology of GFP-Cx43 U251 changed from small polygon-shaped cells to long fusiform-shaped cells, suggesting that the recovery capacity of these cells was greater than that of U251 cells. Notably, the morphology of U251 cells did not change and significant recovery was not observed (Figure 3).

Effects of Cx43 overexpression on cell viability

The viability of U251 and GFP-Cx43 U251 cells treated with various concentrations of TMZ was detected by MTS assays. Different concentrations of TMZ inhibited cell viability depending on the cell line (Figure 4).

Expression levels of apoptosis-related factors in U251 and Cx43-U251 cells

The mRNA levels of apoptotic-related factors (*Bcl-2*, *Bax*, and caspase-3) and *Cx43* were significantly altered in both U251 and Cx43 U251 cells following TMZ treatment. Compared with the levels in U251 cells, *Cx43* and *Bcl-2* expression levels were significantly increased in GFP-Cx43 U251 cells, whereas caspase-3 expression was slightly decreased. Additionally, the *Bax/Bcl-2* ratio was significantly different between cell types (*p*<0.05; Figure 5).

Next, Western blotting was used to analyze protein expression levels. Compared with levels in U251 cells, Cx43-U251 cells treated with 400 µM TMZ showed increased expression of Cx43, caspase-3, and Bax and decreased expression of Bax. In U251 cells treated with 1000 µM TMZ, the ratio of Bax/Bcl-2 was significantly increased (*p*<0.05; Figure 6), suggesting that Cx43 inhibits apoptosis via a mitochondria-related pathway.

Analysis of apoptosis rates

Treatment with 400 µM TMZ induced apoptosis in nearly 50% of U251 cells, whereas treatment with 1000 µM TMZ caused most cells to become apoptotic. In contrast, the number of apoptotic cells was substantially lower in Cx43-U251 cells treated with 400 or 1000 µM TMZ (Figure 7).

Discussion

Effects of Cx43 overexpression on U251 cell proliferation

Cx43 expression is negatively correlated with glioma cell proliferation [4,5]. Moreover, Cx43 inhibits cell growth by blocking cell cycle progression to the S or M phase, as demonstrated by the accumulation of cells in G_1 or S phase. These changes are attributed to the effects of Cx43 on the expression of *loci* that regulate the cell cycle [6,7]. For example, the expression levels of cell cycle-related proteins are reduced and the relative levels of cell cycle inhibitors (e.g., p21 and p27) are increased [8]. Some studies have shown that Cx43 plays an important role in the regulation of these genes [9].

Consistent with these previous findings, we demonstrated that U251 and Cx43-U251 cells proliferated rapidly within the first 4–5 days of culture and then steadily for the next few days. Notably, although there were no significant differences in growth rates from days 1–4 and on days 5–7, compared with that of Cx43-overexpressing cells, the proliferation of U251 cells was significantly increased. The proliferation of U251 cells on days 5–7 was significantly higher than that of Cx43-overexpressing cells. Thus, our findings suggested that Cx43 can decrease cell proliferation.
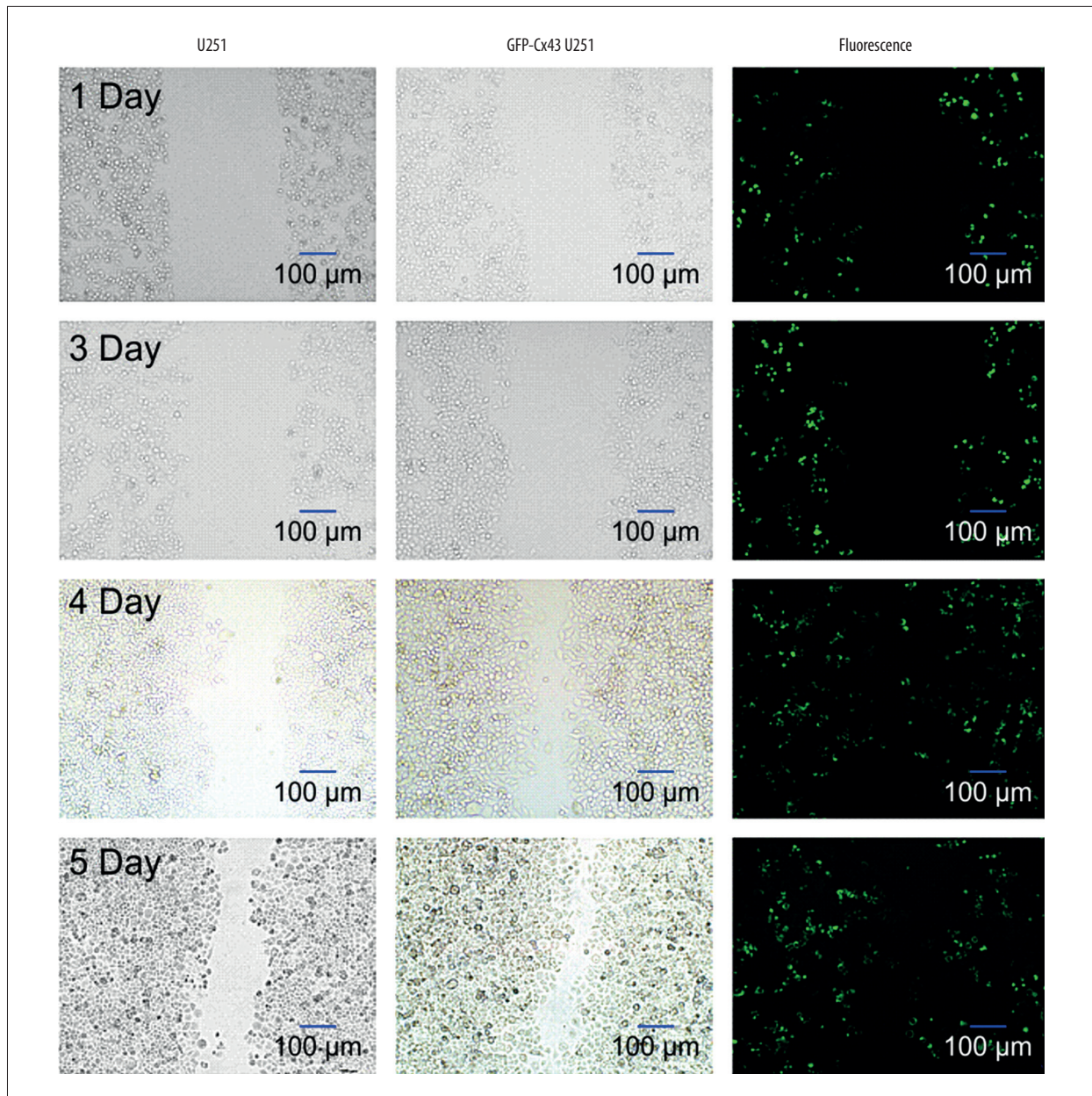
Figure 3. Wound healing assays using U251 and Cx43-U251 cells.

Effects of Cx43 overexpression on U251 cell migration

Studies have shown that the migration ability of glioma cells is related to the Cx43 carboxyl tail, which has multiple binding sites [10]. However, other studies have shown that truncation of the Cx43 tail influences cell migration, suggesting that Cx43 may affect cell migration by other mechanisms. In this study, we found that U251 cells had a greater wound healing ability than that of Cx43-U251 cells. Moreover, the Cx43-U251 cell cytoskeleton showed obvious changes during the migration process, resulting in dramatic changes in cell morphology. These data show that Cx43 may be involved in the regulation

of cytoskeletal proteins, which could affect the migration ability of U251 cells.

Effect of Cx43 overexpression on TMZ-induced apoptosis in U251 cells

TMZ is an effective drug for the treatment of malignant glioma. Importantly, the expression of Cx43 is significantly down-regulated in malignant gliomas compared with that in normal astrocytes [5]. Studies have shown that Cx43 is expressed in the mitochondria and may therefore be involved in the mitochondrial apoptotic pathway. In this study, we further explored
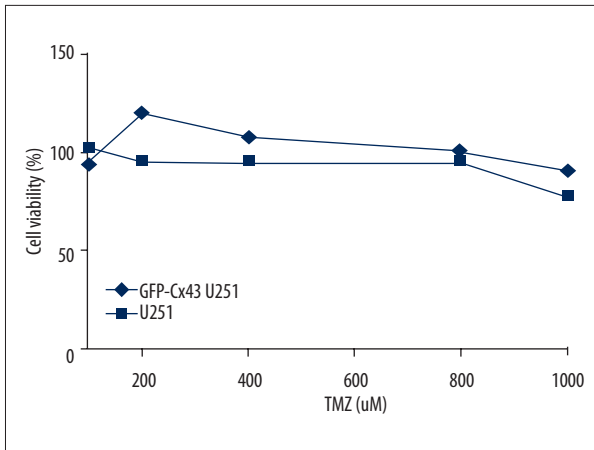
Figure 4. Cell proliferation analysis by MTS assays. (**A**) Differences in viabilities of the 2 cell lines after TMZ treatment. (**B**) Differences in OD values for U251 and Cx43 U251 cells treated with TMZ.
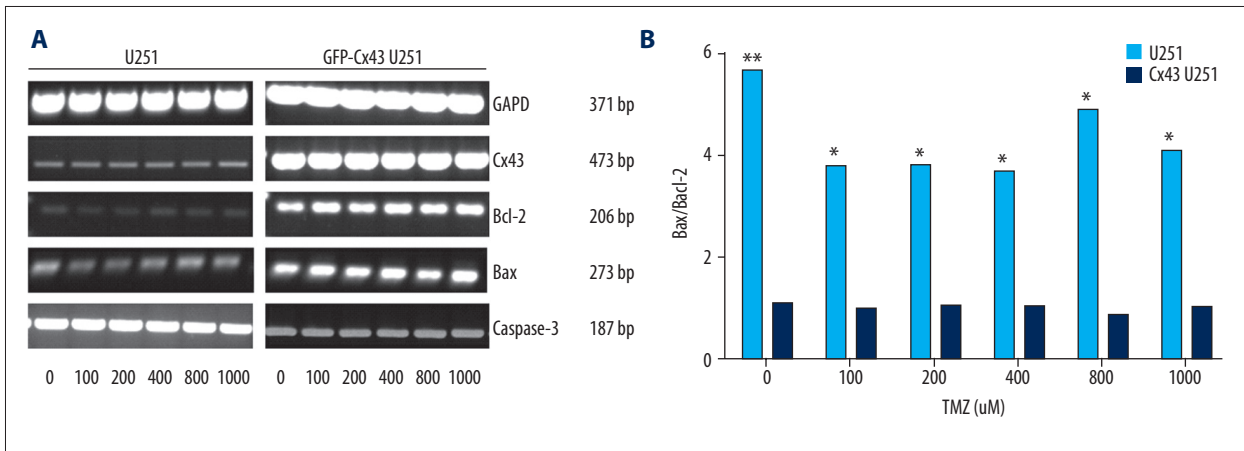

Figure 5. Expression of *Cx43*, *Bcl-2*, *Bax*, and caspase-3, and the *Bax/Bcl-2* ratio in U251 and Cx43-U251 cells. * *p*<0.05.
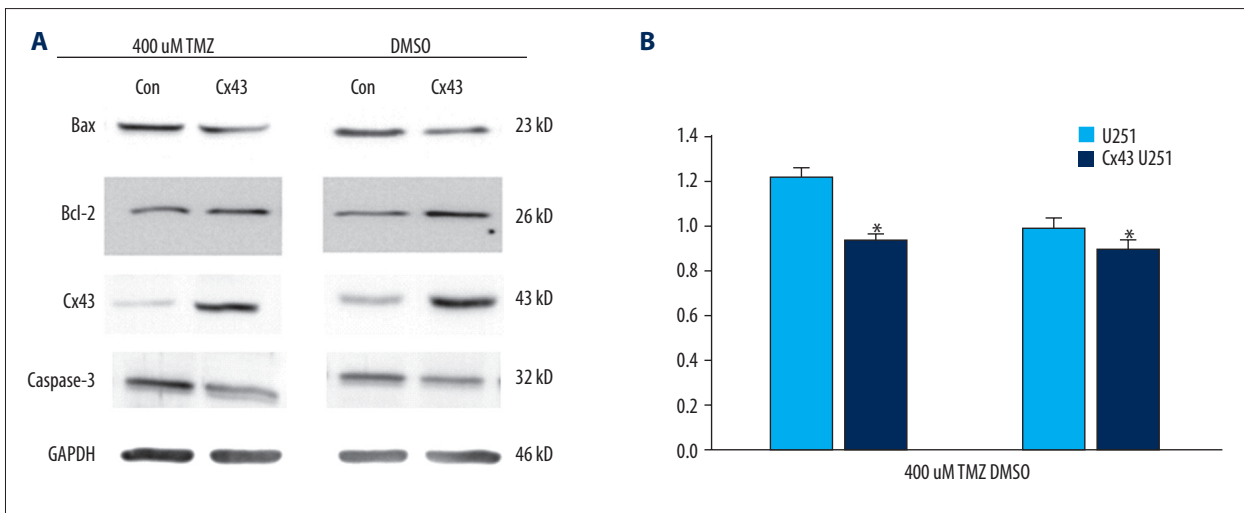

Figure 6. Differences in the expression levels of Cx43, Bcl-2, Bax, and caspase-3 proteins and the Bax/Bcl-2 ratio. (**A**) Expression levels of Cx43, Bcl-2, Bax, and caspase-3 were measured by Western blotting. (**B**) Changes in the Bax/Bcl-2 ratio after TMZ treatment. DMSO treatment was used as a control. * *p*<0.05.
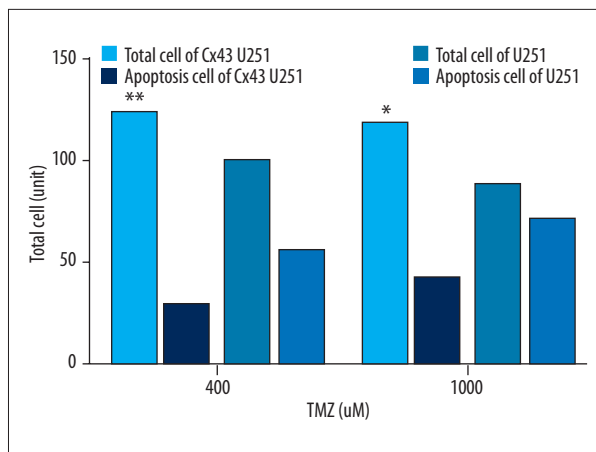
Figure 7. TMZ-induced apoptosis in U251 and Cx43-U251 cells, as determined using TUNEL assays.

the effects of TMZ on Cx43-U251 cells. Our results showed that there was a correlation between Cx43 and TMZ resistance. In Cx43-U251 cells, the expression of Bcl-2 increased and the expression of Bax decreased; thus, the Bax/Bcl-2 ratio was significantly decreased compared with that in wild-type cells. Moreover, caspase-3 expression decreased. Together, these

effects led to a net decrease in the rate of apoptosis. The factors affected by Cx43 expression are all involved in the mitochondrial apoptotic pathway; thus, our findings indicated that Cx43 may contribute to TMZ resistance by affecting the mitochondrial apoptotic pathway.

Conclusions

Cx43 inhibited U251 cell proliferation, enhanced migration, and inhibited apoptosis. Some studies have shown that GJIC regulation by Cx43 is related to pro-apoptotic and anti-apoptotic activities [11]. Additionally, Cx43 is thought to be activated by a half channel, rather than GJIC, functioning to protect cells from oxidative stress, metabolic inhibition, and other cellular damage [12–14]. Thus, although our results provide important insights into these pathways, the roles of Cx43 in U251 proliferation, migration, and apoptosis require further investigation.

Conflict of interest

None.

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2923

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