Invasion of Human Glioma Cells Is Regulated by Multiple Chloride Channels Including ClC-3

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Abstract. *Background: Glioblastoma is a type of highly malignant primary brain tumour. By means of ion excretion and the associated obligatory water loss, glioma cells can change shapes and undergo extensive migration and invasion. This study investigated the effects of inhibition of ion excretion in glioma cells. Materials and Methods: The expression of chloride channels (ClCs) and metalloproteinase-2 (MMP-2) was studied in two human glioma cell lines (STTG1 and U251-MG). The effects of ClC inhibition with chlorotoxin (a ClC-3 inhibitor), 5-nitro-2-3-phenylpropylamino benzoic acid (NPPB) (a non-specific ClC inhibitor), and ClC-3 siRNA knockdown were studied. Results: Both STTG1 and U251-MG cells expressed ClC family members ClC-2, -3, -4, -5, -6 and -7, as well as MMP-2. Glioma cell invasion was markedly but not completely inhibited by ClC-3 and MMP-2 siRNA knockdown, and by chlorotoxin treatment. Addition of chlorotoxin to siRNA-treated glioma cells only slightly increased the suppression of invasion. In contrast, invasion was completely blocked by the non-specific ClC blocker NPPB. Conclusion: ClCs are crucial in glioma cell migration and invasion. Blockade of a single ClC, however, is not sufficient to achieve complete inhibition of glioma cell invasion, suggesting that any future therapy should be targeted at pharmacological blockade of multiple ClCs.*

Glioma is one of the most common type of primary brain tumours in human adults, and mainly consists of astrocytic and oligodendrocytic tumours of different grades of malignancy. Glioblastoma multiforme (GBM), representing 60-70% of malignant gliomas, is the most lethal type of primary malignant brain tumour. Despite surgical resection, radiotherapy and chemotherapy, the prognosis of patients with GBM is poor. Malignant glioma cells are characterized by active migration through the narrow extracellular space of the brain along brain vasculature (1). To achieve this, glioma cells excrete osmotically active ions through the Ca^{2+}-activated potassium channel BK (2-4) and the voltage-gated chloride channel (ClC), leading to the release of cytoplasmic water and subsequently cell shrinkage (3, 5-7).

The ClC protein family consists of nine members, namely ClC-1 through ClC-7, ClC-Ka and ClC-Kb (8). ClC-2, ClC-3, ClC-5, ClC-6 and ClC-7 have been shown to be expressed in glioma cell lines, as well as in clinical biopsy specimens (9). Mammalian ClC proteins function as homodimers in which each monomer has its own pore (*i.e.* two-pore channels) (10, 11). Each monomer in the homodimer preserves its own ion selectivity and single-channel conductance characteristics (11). ClC-2 and ClC-3 are up-regulated in glioma membranes, and may play an important role in cell migration and invasion by means of the excretion of chloride ions and the associated obligatory movement of water (7, 12-14). ClC-3, in particular, has been suggested to be the main ClC contributing to the efflux of chloride ions and invasion in glioma cells (15). ClC-3 forms protein complexes with MMP-2, TIMP-2, MT1-MMP, and $\alpha v\beta 3$ integrin, co-localising with BK channel and AQP-4 to lipid raft domain of invadipodia (16).

The importance of ClC-3 in glioma cell invasion is further supported by the finding that the scorpion toxin, chlorotoxin (Cltx), bound specifically to the ClC-3/MMP-2 membrane complex, may cause endocytosis of ClC-3/MMP-2 and a reduction of glioma invasiveness (16). Cltx is a 36 amino acid peptide found in the venome of the giant yellow Israeli scorpion *Leiurus quinquestriatus* (17). TM-601, a synthetic version of Cltx, has been shown to inhibit glioma cell invasion in glioma-bearing mice (18, 19). Intracavitary administration of iodine-131-TM-601 in adults with

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Table I. *Primer sets used in RT-PCR for ClCs and MMP-2 in glioma cells. PCR reactions were performed for 30 cycles except for ClC-4, which required 35 cycles.*

Gene	Sequence (5'-3') *F: forward; R: reverse*	PCR product (bp)	Annealing temperature
ClC-1	*F: GCATCTGTGCTGCTGTCCTC* *R: GACACCGAGCATGACTTGGC*	410	57˚C
ClC-2	*F: GGGGGCCCAGTGTCACCAGGAAC* *R: CGGGGGAGGCCATGACGGGAGTG*	556	55˚C
ClC-3	*F: CCTCTTTCCAAAGTATAGCAC* *R: TTACTGGCATTCATGTCATTTC*	552	50˚C
ClC-4	*F: GCGGGCAGGATGGTGGGAATTG* *R: GCGCCGCAGCTTCAGGGGATGT*	650	55˚C
ClC-5	*F: GGAACATCCTGTGCCACTG* *R: AATCACAGAGCTTGGAGGAG*	543	50˚C
ClC-6	*F: GTTTAACTTCCCCTATTTCC* *R: GCATTCTCCTAACACCATCG*	519	50˚C
ClC-7	*F: GGGCGTGGTGGGCGGTGTG* *R: CGCCCCCGTGAGGTAGGACAGG*	472	50˚C
MMP-2	*F: CAAAAACAAGAAGACATACATCTT* *R: GCTTCCAAACTTCACGCTC*	233	50˚C
β-actin	*F: ACTCTTCCAGCCTTCCTTCC* *R: CGTCATACTCCTGCTTGCTG*	348	55˚C

recurrent high-grade glioma was recently shown to be well-tolerated, with some anti-tumour effect in a phase I single-dose study (20). However, other ClCs have also been suggested to contribute to membrane transport in glioma cells, and, indeed, a combination of ClC blockers may be needed for the complete blockage of chloride ion transport across glioma cell membrane (7).

The present study investigated the expressions and functions of ClCs in human malignant glioma cells. The study indicated that ClC-3 is the primary ClC associated with invasiveness, but pharmacological blockade of ClC-3 alone is not sufficient to inhibit glioma cell invasion completely.

Materials and Methods

Cell cultures. All experiments were performed on human glioma cell lines: STTG1 (anaplastic astrocytoma, WHO grade IV; American Type Tissue Collection, Manassas, VA, USA); U251-MG (GBM, a gift from Dr. Darell D. Bigner, Duke University Medical Center, USA). Cells were cultured in DMEM and F-12 medium (1:1) (Gibco; Life Technologies, Inc., USA) supplemented with 7% heat inactivated foetal bovine serum (Gibco), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco).

Reverse transcription-polymerase chain reaction (RT-PCR). Expressions of ClCs and *MMP-2* in glioma cells were analysed by RT-PCR. RNA was extracted from cultured cells using TRIzol (Invitrogen Corp., CA, USA). First-strand cDNA was synthesised using AMV Reverse Transcriptase (HC) (Promega Corp., WI, USA). PCR amplification was carried out in a 25 μl reaction mixture using AmpliTaq® Polymerase (Applied Biosystems, CA, USA) as follow: after initial denaturation (94˚C, 10 minutes), PCR was performed for 30 or 35 cycles (94˚C for 1 minute denaturation; annealing at primer-specific temperature for one minute (Table I); 72˚C for 1 minute extension). A final 10-minute extension at 72˚C was added. PCR products were analysed by electrophoresis in a 2% (w/v in TBE) agarose gel.

Western blot analysis. Whole-cell protein was extracted from cultured cells using lysis buffer (Cell Signaling Technology, Inc., MA, USA) supplemented with proteinase inhibitor (Roche Diagnostics, Mannheim, Germany) and 100 mM phenylmethylsulfonyl fluoride. Protein extracts (10 μg) were separated by SDS-PAGE electrophoresis and electro-transferred onto a polyvinylidine difluoride membrane (Immobilon; Millipore). After blocking (2 hours in TBS-T plus 5% non-fat milk), membrane was incubated with anti-ClC-3 or anti-MMP-2 (1:10000; Santa Cruz, CA, USA) for two hours at room temperature. The membrane was washed in TBS-T (3×10 minutes) before being incubated with HRP-conjugated secondary antibody (1 hour, room temperature, 1:10000, Zymed Laboratories, CA, USA). Protein bands were detected by ECL Plus Western Blotting Detection System (GE Healthcare, Amersham, UK). For re-probing, the membrane was stripped in buffer (7 μl/ml β-mercaptoethanol, 2% (w/v) SDS, 62.5 mM Tris-HCl) for 30 minutes at 60˚C. After TBS-T wash, the membrane was incubated with anti-β-actin (1 hour, room temperature, 1:25000, Sigma-Aldrich, MO, USA) followed by washing and incubation with HRP-conjugated secondary antibody (1 hour, room temperature, 1:25000, Zymed). β-Actin band was visualised by chemiluminescence.

Matrigel invasion assay. BD BioCoat Matrigel Invasion Chamber (24 wells, 8μm pore size; BD Biosciences, CA, USA) was rehydrated with serum-free DMEM/F12 at 37˚C in 5% CO_2 incubator for two hours. After rehydration, the medium was removed, and 0.05 ml of DMEM/F12/FBS (DMEM/F12 plus

5%FBS) with or without Cltx or NPPB was added to each well. DMEM/F12/FBS medium (0.5 ml with or without channel blocker) containing 1×10^5 STTGI cells or 4×10^4 U251-MG cells was added to each insert. The invasion chamber was incubated for six hours for U251-MG cells or for 24 hours for STTGI cells. After incubation, chamber inserts were fixed (4% paraformaldehyde, 10 minutes) before being stained with 1% crystal violet (Sigma-Aldrich) for two minutes. The total number of migrated cells in each treatment was calculated from ten random fields for each insert counted at ×100 magnification. The number of migrated cells per well in each treatment was averaged from triplicate samples, and the number was expressed as mean±standard deviation. The percentage inhibition of invasion in each treatment was calculated relative to that of the untreated well that was arbitrarily assigned as zero inhibition.

Transfection of small-interfering RNA (siRNA). Stealth *ClC-3* siRNA (5'-UGA GGU CCA UCA AUC CAU UUG GUA A-3'; 5'-UUA CCA AAU GGA UUG AUG GAC CUC A-3'), and Stealth MMP-2 siRNA (5'-CCC UUC UUG UUC AAU GGC AAG GAG U-3'; 5'-ACU CCU UGC CAU UGA ACA AGA AGG G-3') (Invitrogen) were used to knockdown *ClC-3* and *MMP-2* in cultured glioma cells. Stealth RNAi Negative Control Duplex (-ve siRNA) was included in the RNA interference (RNAi) experiments as a negative control. Two days before transfection, 5×10^5 of the glioma cells were seeded in 6-well plates without antibiotics. Transfection was performed using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. The siRNA:Lipofectamine transfection mix was composed of siRNA mix (10 μl siRNA plus 175 μl serum-free DMEM/F12) and lipofectamine mix (10 μl Lipofectamine 2000 plus 60 μl serum-free DMEM/F12). After 4.5 hours of transfection, the transfection medium was replaced with serum containing DMEM/F12 medium. The cells were further incubated for 24 hours until processed for RT-PCR, Western blot or migration assay.

Immunocytochemistry. Glioma cells cultured on glass slides were fixed with 4% paraformaldehyde (Sigma-Aldrich) at room temperature for 20 minutes. Fixed cells were washed with PBS (2×10 minutes) and blocked with 1% BSA (30 minutes, room temperature). Then, cells were incubated with either anti-ClC-3 or anti-MMP-2 antibody (1:100, Santa Cruz) for 16 hours at 4˚C. After primary antibody incubation, the cells were washed with PBS (3×10 minutes) before being incubated with appropriate FITC-conjugated secondary antibodies (1:400, Zymed; 1 hour, room temperature). After PBS wash (3×10 minutes), cells were dehydrated and mounted with DAPI containing anti-fade mounting fluid (Vector Laboratories, Inc., CA, USA). Photographs were taken on a Nikon Eclipse E600 (Nikon Corp. Japan) fluorescence microscope with a Sony digital camera DSM1200F (Sony Corp., Japan).

Statistics. Standard deviations, standard errors and *p*-values were calculated by Excel 2003 (Microsoft Corp, Redmont, WA, USA). Differences between experimental samples were considered significant at $p<0.05$.

Chemicals. Cltx was obtained from Sigma-Aldrich and was reconstituted in PBS to give a final concentration of 20 μM. 5-Nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) was reconstituted in dimethyl sulfoxide (DMSO) to give 0.4 M stock solution.

Results

Glioma cells expressed ClCs and MMP-2. The expression patterns of ClCs and MMP-2 in STTGI and U251-MG glioma cells were determined by RT-PCR analysis. As shown in Figure 1A, RT-PCR products of the expected length for *MMP-2* and *ClC-2*, -3, -4, -5, -6 and -7 were detected in STTGI and U251-MG glioma cells. However, *ClC-1* was undetectable in these cells. STTGI and U251-MG cells expressed comparable levels of transcripts of *MMP-2, ClC-3, ClC-4* and *ClC-7*. In contrast, STTGI cells expressed higher levels of *ClC-5, ClC-6* and *ClC-2* than did U251-MG cells. No PCR product was amplified from samples that lacked reverse transcriptase (RT-) indicating that the amplified bands were derived from mRNA. Expression of ClC-3 and MMP-2 protein on STTGI and U251MG cells were further confirmed by Western blot analysis (Figure 1B).

The cellular distributions of ClC-3 and MMP-2 in glioma cells were investigated by immunofluorescence. Immunoreactivity of CIC-3 and MMP-2 was localised at the cell surface and the cytoplasm of STTGI and U251-MG cells (Figure 1C). Furthermore, ClC-3 and MMP-2 appeared to associate strongly with cellular protrusions at the leading edges of the STTGI and U251-MG cells (arrows; Figure 1C).

Cltx has been shown to inhibit the invasion of glioma cells. However, it is not known whether Cltx affects the expression of CICs in glioma cells. RT-PCR analysis revealed that the mRNA levels of *ClC-2, -3, -4, -5, -7* and *MMP-2* did not show any noticeable changes in all these cell lines before and after Cltx treatment, except for a slight down-regulation of *ClC-6* (Figure 1D).

Cltx inhibits migration of glioma cell. The inhibitory effect of Cltx on STTGI and U251-MG cells displayed a sigmoid curve (Figure 2A, B). Inhibitions of invasion of Cltx on these two glioma cell lines reached a plateau at approximately 5 μM, with maximum percentage inhibition of approximately 60% in STTGI cells (Figure 2C) and 52% in U251-MG cells (Figure 2D). Cltx is a specific ClC blocker, blocking ClC-3 indirectly, and Cltx has been demonstrated to inhibit glioma cell invasion by induction of the endocytosis of the MMP-2/ClC-3 protein complex (21), suggesting that the expression levels of CIC-3 and MMP-2 are critical for glioma cell invasion.

Inhibition of glioma cell migration by CIC-3 and MMP-2 knockdown. The expression levels of CIC-3 and MMP-2 were knocked down in STTGI and U251-MG cells, and the effects of knockdown on the invasion of glioma cells were studied. Transfection of *ClC-3* siRNA and *MMP-2* siRNA effectively reduced the mRNA and protein levels of CIC-3 and MMP-2 in STTGI and U251-MG cells (Figure 3A, B). The number of glioma cells that migrated across the chamber membrane was

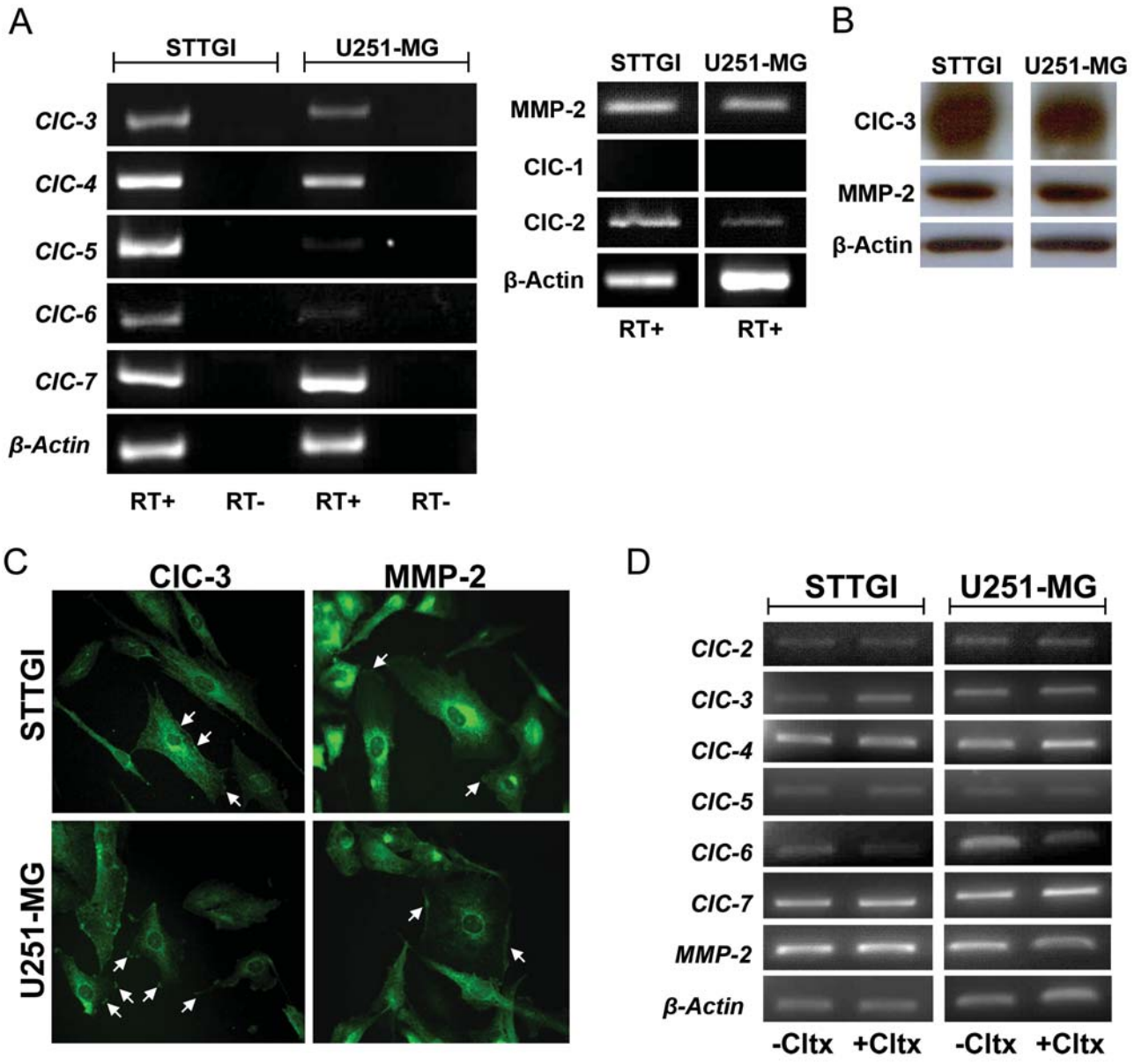

Figure 1. *Glioma cells express ClCs. A: Expression of transcript of ClCs in human glioma cells STTGI and U251-MG was examined by RT-PCR analysis in the presence (RT+) or absence (RT-) of reverse transcriptase. B: Expression of ClC-3 and MMP-2 protein by STTGI and U251-MG was confirmed by Western blotting. C: Using immunofluorescence analysis, ClC-3 (green) and MMP-2 (green) were detected at the cell membrane and the cytoplasm of STTGI and U251MG cells. Intense ClC-3 and MMP-2 immunoreactivity (arrows) was localised at cellular protrusions at the leading edges of the STTGI and U251-MG cells. D: Expression of ClCs and MMP-2 in glioma cells with (+Cltx) and without (-Cltx) Cltx treatment was examined by RT-PCR. RT-PCR for β-actin was included as a control to check the integrity of the mRNA.*

markedly reduced in siRNA-transfected STTGI and U251-MG cells compared to stealth RNAi control (-ve siRNA; Figure 3C, D). However, the number of cells that migrated across the chamber membrane was similar between siRNA-transfected cultures with or without the addition of Cltx. The number of cells that migrated across the chamber membrane in different treatments was counted and the percentage inhibition of each treatment was determined relative to stealth RNAi control (-ve siRNA) that was assigned 0% inhibition (Figure 3E, F). Statistical significance of the inhibition in each treatment was determined and is shown in Table II.

ClC-3 siRNA and *MMP-2* siRNA suppressed invasion by 67.3±3.5% and 86.1±4.7% in STTGI cells and by 68.4±3.4% and 87.2±5.6% in U251MG cells (Figure 3). Cltx treatment at

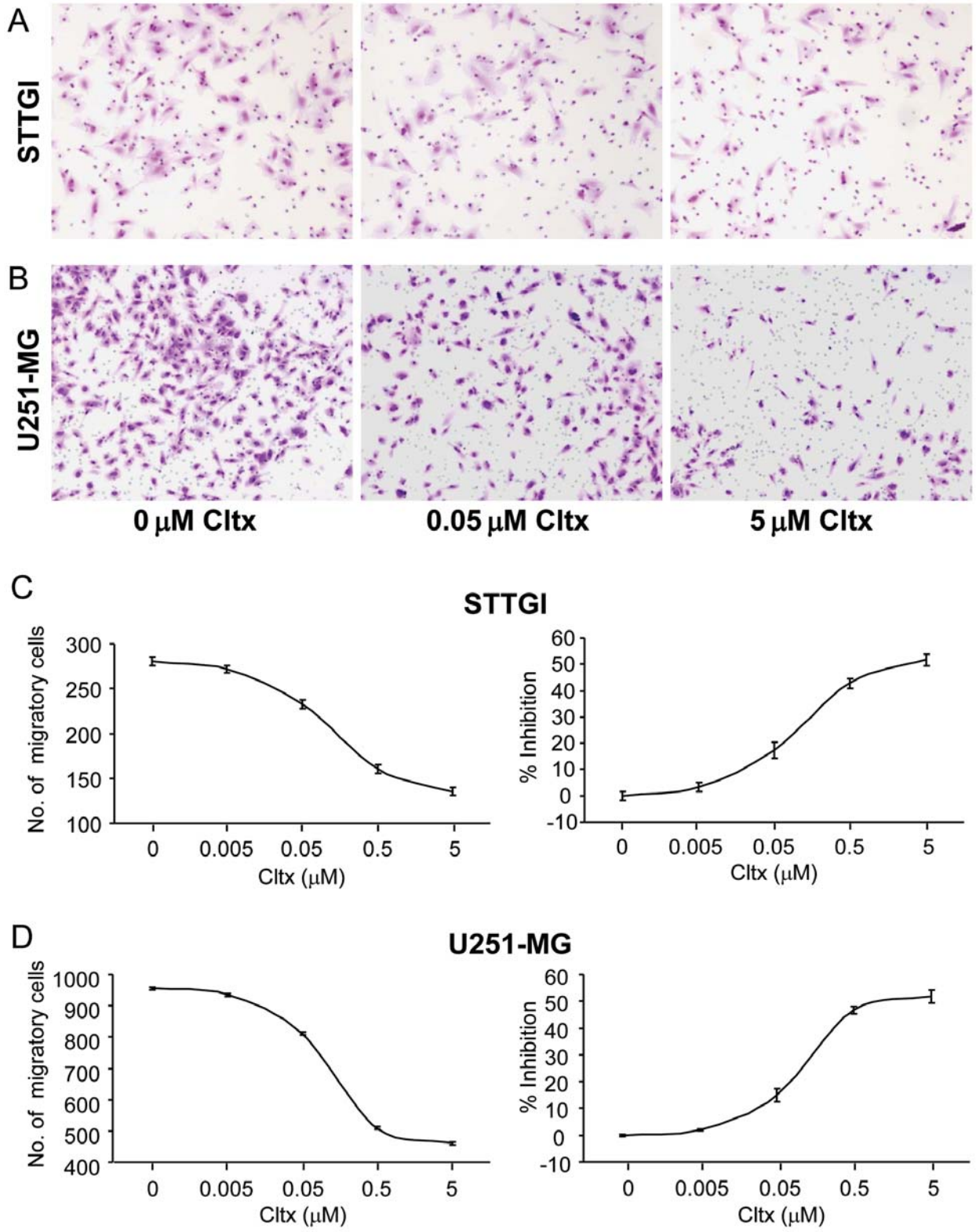

Figure 2. *Cltx reduces glioma cell invasion. The invasion of STTGI and U251-MG cells was examined in the absence and presence of Cltx by matrigel invasion assay. After incubation, cells which had migrated to the bottom side of the chamber inserts were fixed and stained with crystal violet (A, B). The number of migrated cells per well at different concentrations of Cltx was determined and was expressed as mean±standard deviation. Percentage inhibition of invasion in each treatment was calculated relative to that of the untreated well that was assigned zero inhibition (C, D).*

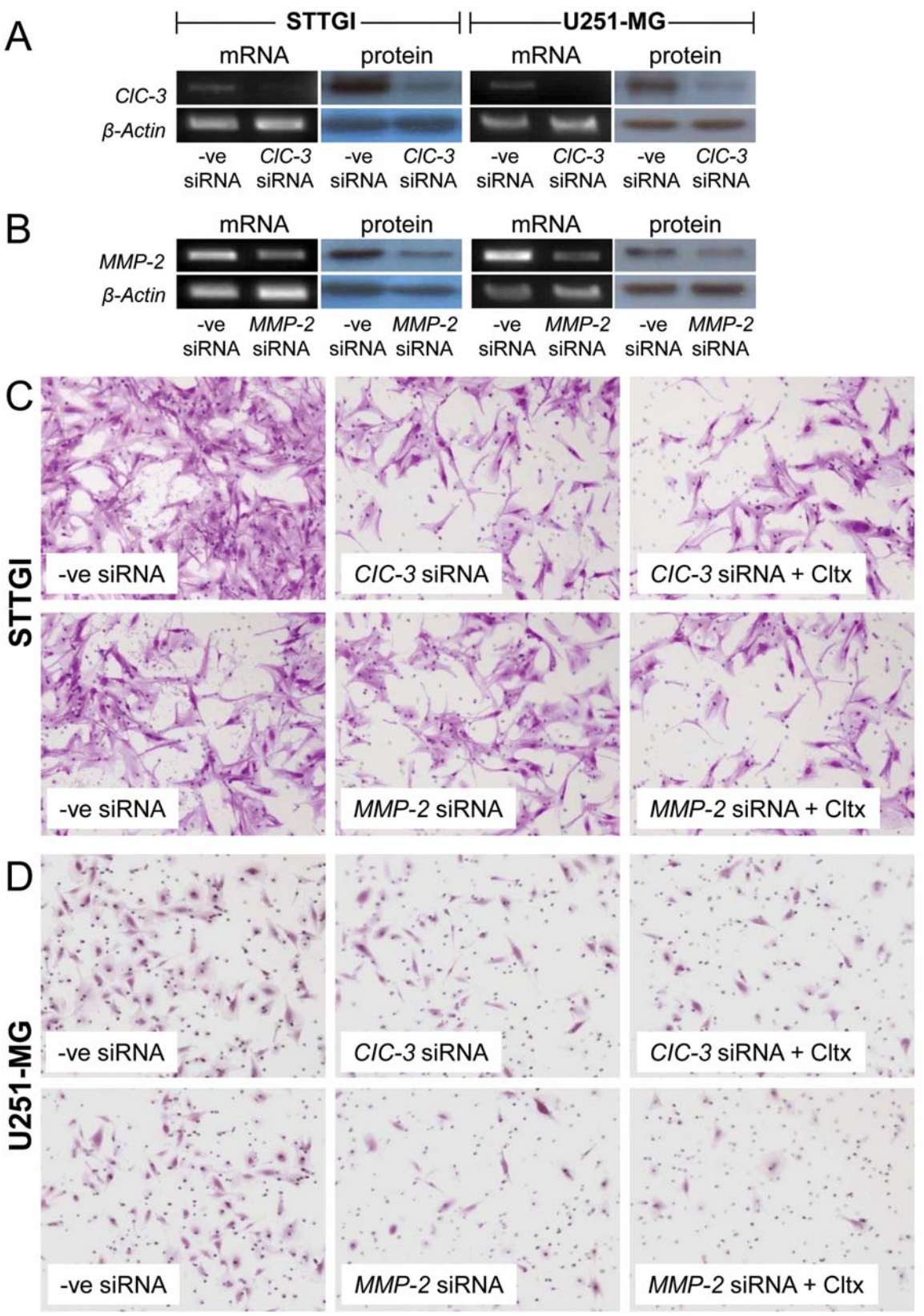

Figure 3. *Continued overleaf*

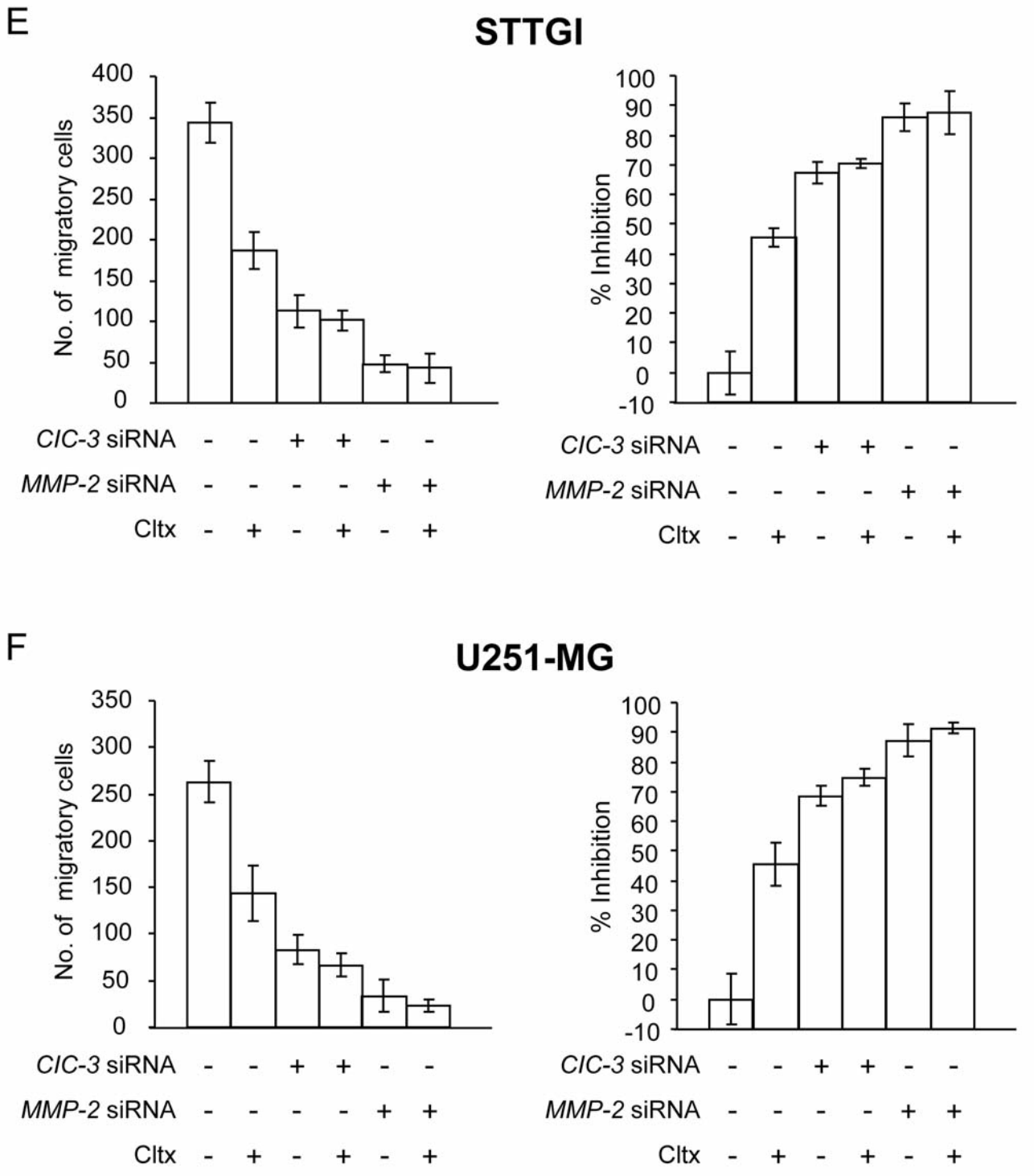

Figure 3. *Knockdown of ClC-3 and MMP-2 reduce glioma cell invasion. Expressions of ClC-3 and MMP-2 mRNA and protein in STTGI and U251-MG cells were examined by RT-PCR and Western blotting. Lower levels of mRNA and protein of ClC-3 (A) and MMP-2 (B) were detected in Stealth siRNA (ClC-3 siRNA and MMP-2 siRNA)-transfected STTGI and U251-MG cells than in Stealth RNAi Negative Control Duplex (-ve siRNA)-transfected cultures. Invasion of Stealth siRNA-transfected STTGI and U251-MG cells was examined in the absence and presence of Cltx by matrigel invasion assay. Cells which had migrated to the bottom side of the chamber inserts in different treatments were fixed and stained with crystal violet (C, D). The number of migrated cells per well under different treatments was determined and was expressed as mean±standard deviation. Percentage inhibition of invasion in each treatment was calculated relative to that of the untreated well that was assigned zero inhibition (E, F).*

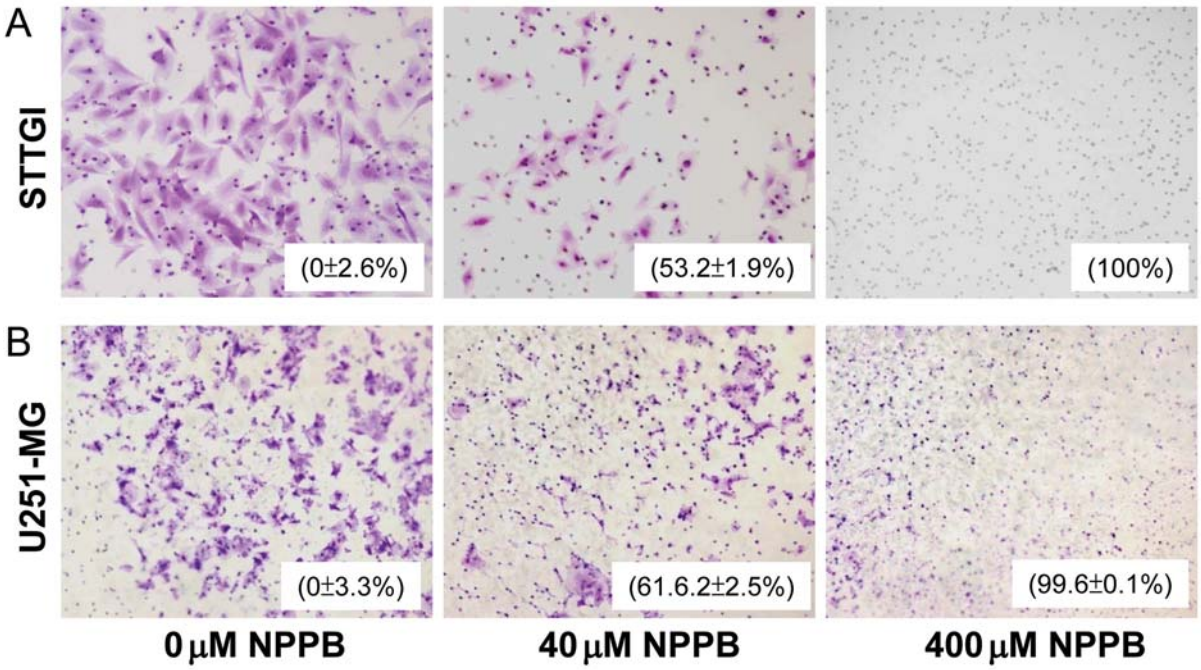

Figure 4. *Inhibition of glioma cell invasion by non-specific ClC blocker. The invasion of STTGI and U251-MG cells in the absence and presence of NPPB at different concentrations was examined by matrigel invasion assay. Cells which had migrated to the bottom side of the chamber inserts under different treatments were fixed and stained with crystal violet (A, B). Percentage inhibition of invasion in different concentrations of NPPB was calculated relative to that in the absence of NPPB that was assigned zero inhibition and shown in parenthesis.*

1 μM suppressed invasion by 45.4±3.1% and 45.6±7.4% in STTGI and U251-MG, respectively. Suppression of invasions in STTGI and U251-MG cells by siRNA and Cltx treatment reached statistical significance when compared to those in stealth RNAi controls (Table II). The addition of Cltx to siRNA-treated STTGI and U251-MG cells only slightly increased the suppression of invasion compared to treatment with siRNA alone (Figure 3E, F) and the increases were statistically insignificant (Table II). *ClC-3* and *MMP-2* knockdown displayed similar invasion inhibition on glioma cells (STTGI: *ClC-3* siRNA *vs*. *MMP-2* siRNA, *p*-value=0.141; U251-MG: *ClC-3* siRNA *vs*. *MMP-2* siRNA, *p*-value=0.101).

Complete blockage of glioma cell invasion by non-specific ClC blocker. The data of the present study indicated that blocking ClC-3 and MMP-2 activities either by siRNA or Cltx, a ClC-3 specific blocker, strongly inhibits glioma cell invasion, but fails to completely block the invasion, suggesting that other ClCs are also involved. To examine the contribution of other ClCs in glioma cell invasion, the non-specific ClC blocker NPPB was tested for the inhibition of glioma cell invasion. The migration of both STTG1 and U251-MG cells was blocked by 50% to 60% at 40 μM NPPB, and complete migration blockage was achieved at 400 μM NPPB (Figure 4).

Discussion

Glioma cells STTGI and U251-MG expressed ClC-2, -3, -4, -5, -6, -7 and MMP-2. These glioma cells expressed MMP-2 and the same set of ClCs as astrocytes, apart from ClC-6, which was only expressed by glioma cells and not by astrocytes (22). In astrocytes, ClC-2, -3, -4, -5 and -7 remain inactive in the resting state (13). In contrast, ClC-2 and ClC-3 are localised to the plasma membrane and active in glioma cells in the resting state (7). Furthermore, it was found that ClC-2 and ClC-3 were up-regulated at the glioma cell surface. High expression and elevated activities of ClC-2 and ClC-3 may facilitate glioma cells to regulate their cell shapes to invade through tortuous extracellular brain spaces (7).

Immunofluorescence staining showed that ClC-3 and MMP-2 were localised to the cell surface, and predominantly to the cellular protrusions at the leading edges of the STTGI and U251-MG cells. Cellular protrusions, also known as lamellipodia, are a characteristic feature at the front of motile cells and function as the actual motor, pulling the cell forward during the process of cell migration (23). ClC-3 was reported to associate with MMP-2 to these 'invadipodia' of glioma cells (7, 15, 16). Invadipodia are protrusions in the cell membrane of some cells that extend into the extracellular matrix (24, 25). They are associated with high levels of proteolysis and cell

Table II. *Statistical analysis for the invasion inhibition of STTGI and 251-MG cells in different treatments.*

Comparison of invasion inhibition between treatments	*p*-Value (STTGI)	*p*-Value (U251-MG)
-ve siRNA *vs*. -ve siRNA plus 1 μM Cltx	0.048	0.036
-ve siRNA *vs*. *ClC-3* siRNA	0.030	0.005
-ve siRNA *vs*. *ClC-3* siRNA plus 1 μM Cltx	0.040	0.005
-ve siRNA *vs*. *MMP-2* siRNA	0.030	0.002
-ve siRNA *vs*. *MMP-2* siRNA plus 1 μM Cltx	0.018	0.006
ClC-3 siRNA *vs*. *MMP-2* siRNA	0.141	0.101
ClC-3 siRNA *vs*. *ClC-3* siRNA plus 1 μM Cltx	0.696	0.447
MMP-2 siRNA *vs*. *MMP-2* siRNA plus 1 μM Cltx	0.842	0.612
-ve siRNA plus 1 μM Cltx *vs*. *ClC-3* siRNA	0.095	0.171
-ve siRNA plus 1 μM Cltx *vs*. *MMP-2* siRNA	0.017	0.045

signalling and are frequently seen in metastatic cancer cells that are invading surrounding tissues (4). The clustering of ClC-3 and MMP-2 to cellular protrusions was closely associated with the migration of glioma cells, suggesting that ClCs and MMPs function together to regulate the motility of glioma cells. *ClC-3* and *MMP-2* siRNA knockdown markedly inhibited the migration of STTGI and U251-MG glioma cells, which corroborated with the regulatory roles of ClC-3 and MMP-2 in the migration of glioma cells.

Cltx, unlike the related scorpion peptides, does not bind directly to a ClC; instead, it binds to the cell surface protein complex containing MMP-2 and MT1-MMP (16). Binding of Cltx causes the endocytosis of this complex along with ClC-3, leading to the depletion of cell-surface ClCs. Cltx effectively blocked the migration of STTGI and U251-MG glioma cells in the present study. However, addition of Cltx did not enhance the migratory inhibition of ClC-3 and MMP-2 siRNA treatment on STTGI and U251-MG cells. Taken together these data suggest that ClC-3 is the major ClC forming complex with MMP-2 and MT1-MMP on the glioma cell surface. Depletion of ClC-3 either by siRNA or Cltx inhibited the migration of glioma cells effectively, but ClC-3 depletion alone was not able to block glioma cell invasion completely. In contrast, the non-specific ClC blocker NPPB inhibited glioma cell migration completely. NPPB has been shown to inhibit several types of ClCs including ClC-3 (15). The conductance of ClC-3 is regulated through phosphorylation *via* Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (5). Inhibition of CaMKII reduced glioma invasion as effectively as direct inhibition of ClC-3. These data suggested that drugs reducing CaMKII activity in glioma cells may be used as potential therapeutic agents to decrease glioma invasiveness.

In conclusion, the present study provided further evidence for the important roles of ClCs, in particular ClC-3, in the regulation of glioma cell migration and invasion. The findings of this study also indicated that blockade of a single ClC is not sufficient to achieve complete inhibition of glioma cell invasion. Therefore, future therapy for gliomas should aim at pharmacologic blockade targeting multiple ClCs, as well as mixing ClC-3 and CaMKII activities.

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