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Original Article

Effect of CCL2 siRNA on the cell migration and adhesion in human glioma cell lines U251 and U373

Bin Lu[1], Yue Zhou[1], Ai Yan[1], Zhongzhou Su[1], Peng Ding[2]

[1]*Department of Neurosurgery, Huzhou Central Hospital, Huzhou, Zhejiang, China;* [2]*Department of Neurosurgery, The First Affiliated Hospital of Kunming Medical College, Kunming, Yunnan, China*

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Abstract: Glioma is one of the most common tumors in the central nervous system. High expression of CCL2 was detected in various cancers. The effect of CCL2 siRNA on the cell proliferation, invasion, migration and adhesion of glioma cell line U251 and U373 cells was investigated in this study. The glioma cell lines were selected from four glioma patients enrolled in the study. Cell proliferation was measured using MTT assay, while cell invasion and migration were detected by Transwell assay. Western blot and real-time PCR were used to measure the expression levels of biologic pathway related proteins FN1, Vimentin, E-cadherin, β-catenin, Twist and Snail. As a result, CCL2 highly expressed in the glioma cell lines U251 and U373 cells and effectively inhibited by CCL2 siRNA. Cells transfected with CCL2 siRNA exhibited inhibited cell proliferation, invasion, migration and adhesion. The down-regulated expression levels of cell migration related proteins FN1 and Vimentin indicated the inhibition effect of CCL2 siRNA on the cell invasion and migration, as well as the declined CTNNB1 signaling pathway protein β-catenin. In conclusion, CCL2 siRNA exhibited effective inhibition ability against the cell proliferation, invasion, migration and adhesion of glioma cell lines U251 and U373 cells, which might provide theoretical basis for *in vitro* research and clinical treatment as a new anti-cancer candidate.

Keywords: Glioma, CCL2, RNA interference, cell migration, cell adhesion

Introduction

Glioma is one of the most common tumors with the percentage of 44.6% approximately among the central nervous system cancer [1]. The incidence of glioma was reported at 0.003-0.01%, among which 70-80% is diagnosed as malignant glioma, accounting for 1-3% in total malignant tumors [2]. Every year 180,000 to 600,000 middle-young people were died for malignant glioma, making it the second cause of death in the patients with cancer under 34 years old and the third from 35 to 54 [2]. Besides, patients received surgery with radiotherapy and chemotherapy have a recurrence rate at about 98% with the median overall survival less than one year at average [2]. Glioma can hardly be removed completely through surgery due to the infiltrative growth and incomplete differentiation of glioma in deep brain tissue, while chemical drugs and traditional Chinese medicine with anti-cancer effect also present poor curative effect because of the blood-brain barrier [3]. Therefore, great significance in glioma related studies faced by current neurosurgery is involved in the investigation of basic characteristics, biological behavior and relevant treatment of glioma.

CCL2, also known as MCP-1 which was firstly named as tumor derived chemokine is one of β-chemokines family [4]. Human CCL2 located in the chromosome 17q11 was first cloned in 1989 from the supernatant of glioma culture [5]. The abnormal expression of CCL2 was observed in various cancers including breast cancer, gastric cancer, prostate cancer, ovarian cancer, cervical cancer, esophageal cancer, and nasopharynx cancer [6]. CCL2 was reported to gather monocyte and macrophage to participate in the inflammatory response. Besides, its chemotaxis and activation on basophils to release histamine involved in immune regulation were also reported [6]. Functional CCL2 specifically combined with surface receptor CCR2 can activate phospholipase C and kinase C, leading to the release of calcium ions, resulting in the action of target cells [7]. It was dem-

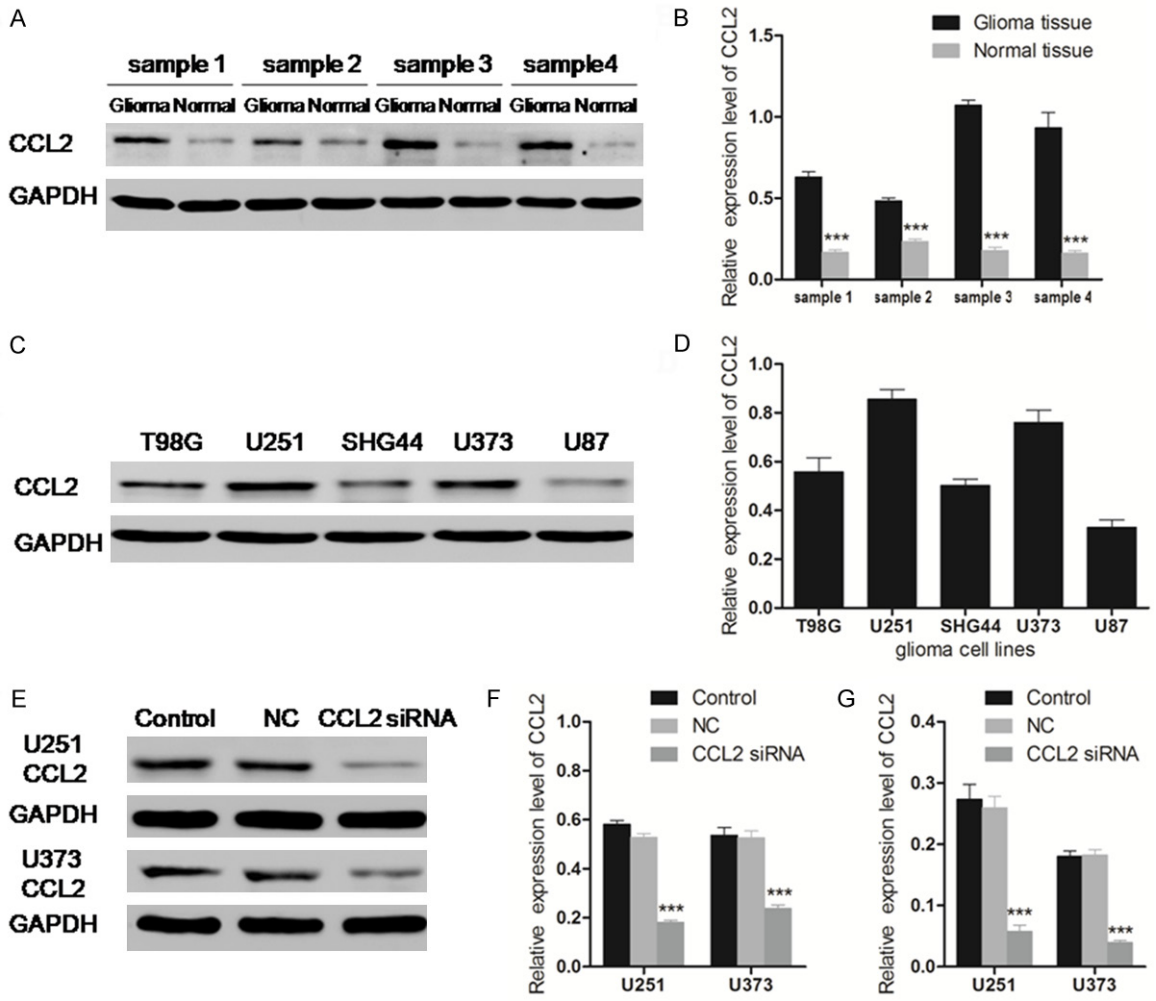

Figure 1. The selection of CCL2 with high expression level from human glioma tissues. A, B. The expression level of CCL2 in four glioma samples was measured using Western blot. ***$P < 0.001$, compared with the normal tissue. C, D. Western blot was also used to detect the expression level of CCL2 in five human glioma cell lines. E, F. CCL2 siRNA exhibited significant inhibition ability against the expression levels of CCL2 in human glioma cell lines U251 and U373. ***$P < 0.001$, compared with the control group (n=3). G. The effect of CCL2 siRNA on the gene expression of CCL2 was also detected by real-time PCR. ***$P < 0.001$, compared with the control group (n=3).

onstrated that CCL2/CCR2 axis plays a critical role in the occurrence and development of tumor, however, the biological function of CCL2 in glioma was barely reported. Obstacle is existed in the improvement of the recurrence rate, death rate and survival time of patients with glioma in clinical. Therefore, molecular biology and cell biology methods were used to explore the function of CCL2/CCR2 axis and the mechanism involved in the invasion, migration, adhesion and apoptosis of glioma cells in this study. Glioma cell line U251 and U373 cells transfected with CCL2 siRNA were detected for the cell proliferation using MTT assay. Transwell assay was used to determine the invasion and migration of glioma cells, as well as the cell adhesion. In addition, the expression levels of FN1, Vimentin, E-cadherin, β-catenin, Twist and Snail were measured through Western blot and real-time PCR in order to further investigate biologic function of CCL2 involved in the cell migration and adhesion.

Materials and methods

Screen of CCL2 expression cell lines

Four tissue samples from glioma patients with complete clinical and pathological follow-up data were detected for the expression level of CCL2. Each sample contains glioma tissue and non-neoplastic tissue obtained from the same patient. Western blot was used to measure the expression levels of CCL2 in the four tissue

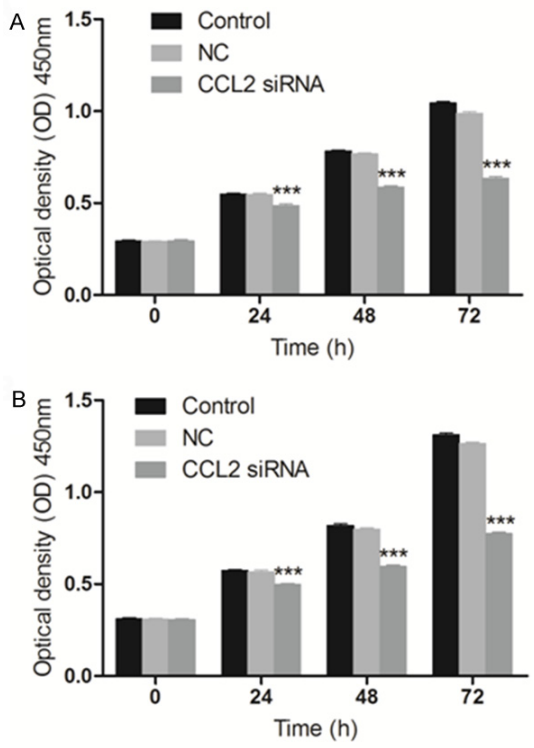

Figure 2. MTT assay exhibits the effect of CCL2 siRNA on the cell proliferation of glioma cell line U251 and U373 cells. A. The optical density of U251 cells decreased with a time-dependent manner, indicating the inhibited cell proliferation by CCL2 siRNA. ***P < 0.001, compared with the control group (n=3). B. The cell proliferation of U373 cells was also inhibited by CCL2 time-dependently. ***P < 0.001, compared with the control group (n=3).

sample. In addition, in order to obtain further insight into the mechanism involved in the effect of CCL2 on glioma, five glioma cell lines including T98G, U251, SHG44, U373 and U87 obtained from human glioma tissues were measured for the expression of CCL2 by Western blot. The study protocol was approved by the local independently. Ethical approval for the study was provided by the independent ethics committee, Shanghai Tongren Hospital. Informed and written consent was obtained from all patients or their advisers according to the ethics committee guidelines.

Transfection

Cultured U251 and U373 cells were inoculated in antibiotic-free medium and incubated for 24 h. Cells were transfected with 50 nmol/L of CCL2 siRNA or empty plasmid as negative control using lipofectamine™ 2000 (Invitrogen, Shanghai, China) according to the instructions provided by the manufacturer. The sequence of CCL2 siRNA was shown as blow: 5'-CTCGCGAGCTATAGAAGAA-3'.

After 48 hours, transfected cells were collected and processed for proliferation, invasion, migration, adhesion, Western blot and real-time PCR assay.

Cell proliferation assay using MTT

Golima cell lines U251 and U373 purchased from Shanghai cell bank of Chinese academy of sciences were cultured in DMEM (Hyclone Co., Ltd) with 10% FBS (JRDUN Biotechnology Co., LTd) and antibiotics (Gibco Co., Ltd) at 37°C. Cultured cells were digested and diluted with 1~5×10^4 cells/mL. Then cells were inoculated in 96-well plates (1~5×10^3 cells/well) and incubated at 37°C for 12 h. Negative control group and interference group were tranfected with 0.1 μL empty plasmid and CCL2 siRNA, respectively. 0.5% MMT solution was added into each well at 0, 24, 48 and 72 h after the transfection and then incubated for 4 h. Then the cell viability was determined by the OD_{450nm} measured using a microplate reader.

Cell invasion and migration assay using transwell

Transfected cells were cultured in serum-free medium for 24 h before inoculation. Then cells were digested by trypsin and diluted to 1×10^5 cells/mL using DMEM containing 1% FBS. In cell invasion assay, the upper chamber was coated with 80 μL matrigel before the transfer of cells. 0.5 mL cell suspension was added into each Transwell chamber and 24-well plates were filled with 0.75 mL DMEM containing 10% FBS in each well. After the incubation for 48 h, cells were fixed by 1 mL 4% methyl alcohol in each well for 10 min, and then stained by 1 mL 0.5% crystal violet for 30 min. The 24-well plates were washed using 1× PBS for three times and counted for the invaded cells in three randomly selected vision using a microscope (OLYMPUS, Shenzhen, China) under 200 magnified visual field.

Adhesion assay

Cultured U251 and U373 cells were inoculated in 6-well plates (3×10^5 cells/well) and incubated for 24 h. Transfected cells were digested by 0.25% trypsin (Gibco, Shanghai, China) and

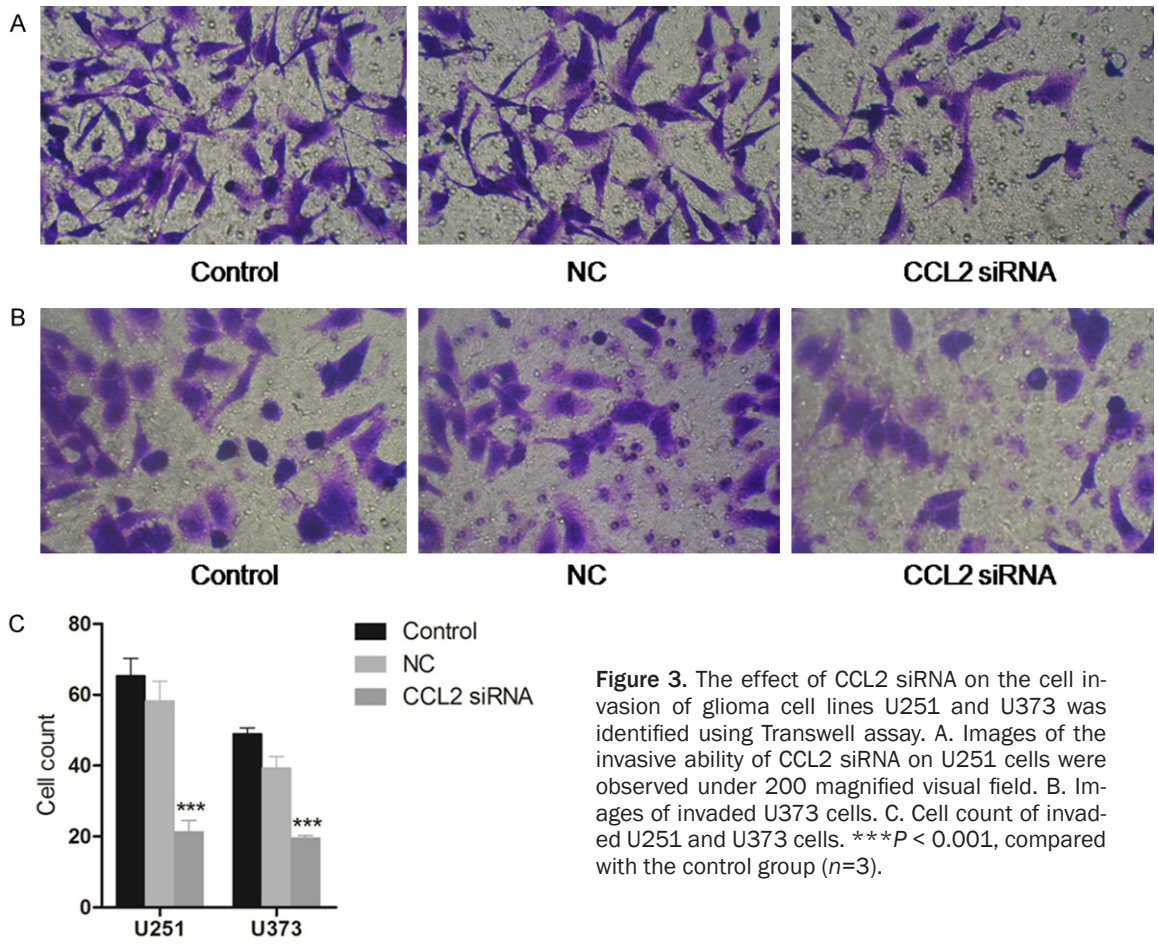

Figure 3. The effect of CCL2 siRNA on the cell invasion of glioma cell lines U251 and U373 was identified using Transwell assay. A. Images of the invasive ability of CCL2 siRNA on U251 cells were observed under 200 magnified visual field. B. Images of invaded U373 cells. C. Cell count of invaded U251 and U373 cells. ***$P < 0.001$, compared with the control group (n=3).

then suspended in DMEM (Hyclone) medium containing 10% FBS (Gibco). Cells were diluted and inoculated in FN coated 12-well plates (1×10^5 cells/well). After 1-hour incubation, the supernatant was discarded and cells were washed by PBS (Gibco) for 2 times. Cells were fixed by 4% paraformaldehyde (JRDUN biotech, Shanghai, China) for 15 min, and then stained by Giemsa (JRDUN) for 30 min. After washing for several times, cell count was calculated using a microscope (OLYMPUS).

Western blot assay

Glioma cell line cells and CCL2 siRNA transfected U251 and U373 cells were washed with PBS and fully lysed in RIPA buffer at 4°C for 10 min and centrifuged at 12000 RCF for another 10 min. 35 μg of supernatant was loaded onto sodium dodecyl sulfate-polyacrylamide electrophoresis gels (SDS-PAGE) after the protein content was quantified using BCA protein assay reagent (Sangon Biotech). Nitrocellulose filter membrane (Millipore) was used to transfer the protein after soaked in the transfer buffer for 10 min. Anti-CCL2, FN1, Vimentin, E-cadherin, β-catenin, Twist, Snail and GAPDH antibodies (Santa Cruz Bio-technology) were diluted and added into blocking buffer (Millipore), followed by the incubation at 4°C for 12 h. Then the membrane was incubated with the secondary antibodies tagged with HRP (Beyotime Co., Ltd) at 37°C for 1 h and developed using ECL-detecting reagents (Millipore).

Real-time PCR assay

The gene expression levels of migration related proteins and CTNNB1 signaling proteins were measured using real-time PCR. TRIzol Reagent (Gibco-BRL, Gaithersburg, MD) was used to extract and quantify total mRNA from U251 and U373 cells. Then reverse transcription was performed to synthesis cDNA through Thermoscript RT-PCR System at a total volume of 25 μL (12 μl RNA-primer Mix, 5 μl 5×RT reaction buffer, 1 μl 25 mM dNTPs, 1 μl 25 U/μl RNase inhibitor, 1 μl 200 U/μl M-MLV Rtase, 1 μl $Oligo(dt)_{18}$ and 4 μl DNase-free ddH_2O). A total PCR system of 25 μL (12.5 μl SYBR Green Mix, 0.5 μl forward

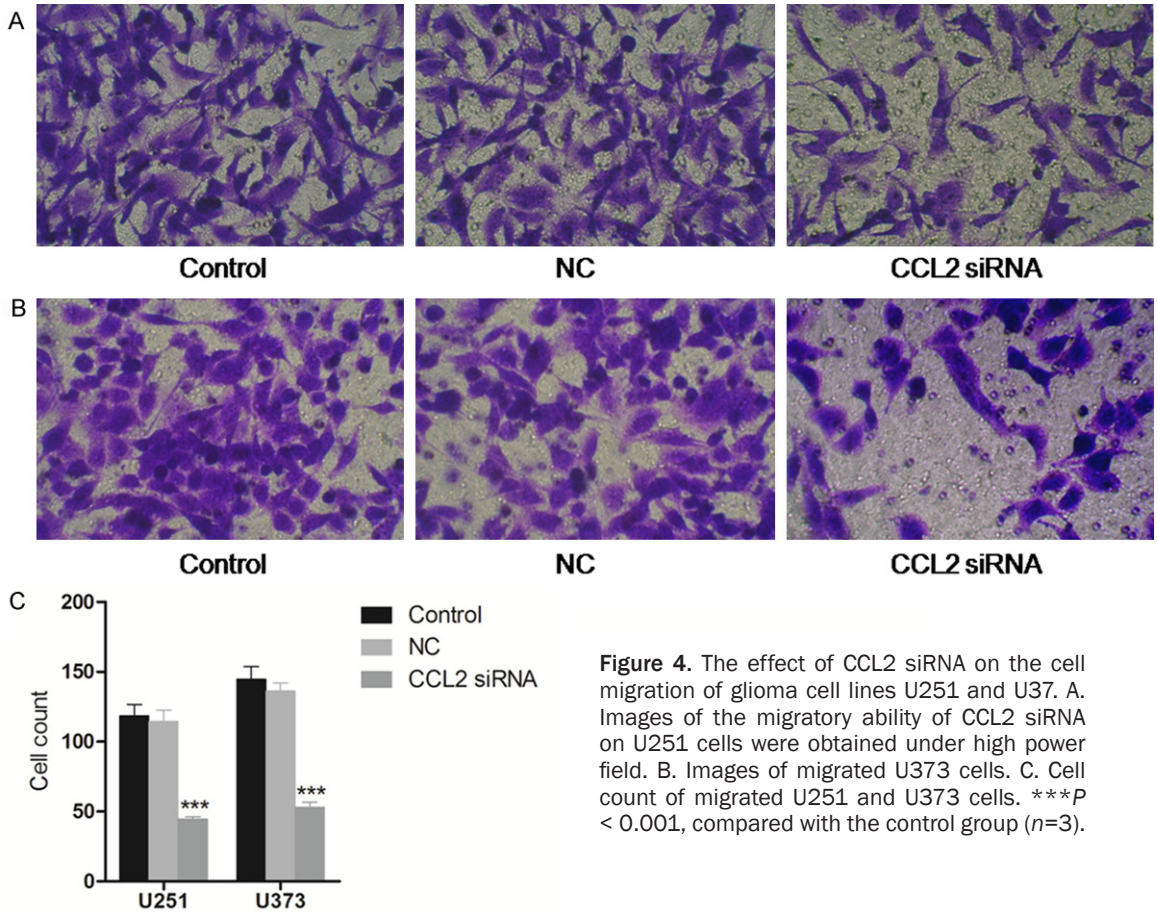

Figure 4. The effect of CCL2 siRNA on the cell migration of glioma cell lines U251 and U37. A. Images of the migratory ability of CCL2 siRNA on U251 cells were obtained under high power field. B. Images of migrated U373 cells. C. Cell count of migrated U251 and U373 cells. ***P < 0.001, compared with the control group (n=3).

primer, 0.5 μl reverse primer, 9.5 μl ddH_2O and 2 μl cDNA template) was amplified through 40 cycles of denaturing at 95°C for 15 s, annealing at 60°C for 30 s and elongation at 60°C for 45 s. Amplification kinetic curves were obtained and the data was analyzed using ABI Prism 7300 SDS Software.

Statistical analysis

Data were expressed as mean ± standard deviation and analyzed using *t*-test and analysis of variance. GraphPad Prism 5.0 software was used to perform and analyze the data.

Results

Screen of glioma cell line with high expression level of CCL2

Compared with the expression of CCL2 in normal tissue, that of glioma tissue from the same sample exhibited significantly high level (**Figure 1A** and **1B**), suggesting the potential relationships between the expression of CCL2 and glioma. In order to screen out the glioma cell line with high expression level of CCL2, five human glioma cell lines including T98G, U251, SHG44, U373 and U87 were detected using Western blot. As shown in **Figure 1C**, U251 and U373 cells presented obviously high expression levels of CCL2 among the five glioma cell lines. Besides, U251 and U373 cells transfected with CCL2 siRNA showed significant decrease in the expression level of CCL2 through both Western blot and real-time PCR (**Figure 1E-G**, P < 0.001), indicating the inhibitive ability of CCL2 siRNA against the glioma cell lines. Therefore, human glioma cell lines U251 and U373 were selected in the following research of the possible mechanism involved in the siRNA interference against glioma.

CCL2 siRNA inhibited cell proliferation

The cell viability of glioma cell lines U251 and U373 interfered with CCL2 siRNA was detected using MTT assay. As shown in **Figure 2**, the cell proliferation of both U251 and U373 was declined dramatically with a time-dependent manner compared with the control group (P < 0.001), indicating the inhibitive ability of CCL2

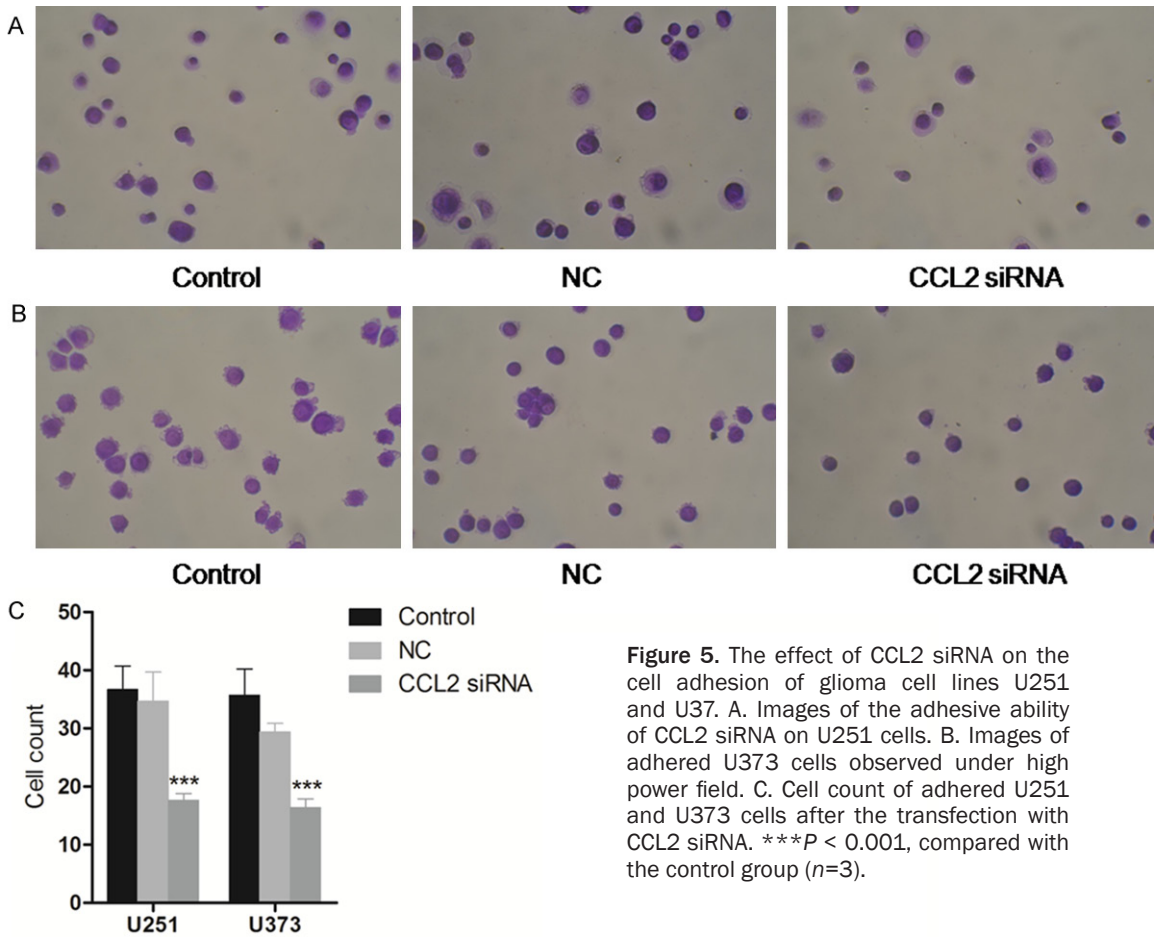

Figure 5. The effect of CCL2 siRNA on the cell adhesion of glioma cell lines U251 and U37. A. Images of the adhesive ability of CCL2 siRNA on U251 cells. B. Images of adhered U373 cells observed under high power field. C. Cell count of adhered U251 and U373 cells after the transfection with CCL2 siRNA. ***$P < 0.001$, compared with the control group (n=3).

siRNA against the cell viability of glioma cells lines U251 and U373.

CCL2 siRNA inhibited cell invasion and migration

The invasion and migration ability of U251 and U373 cells was evaluated using the Transwell assay. As the result shown in **Figure 3**, the invasion rate of both U251 and U373 cells treated with CCL2 siRNA was suppressed dramatically compared with the control group, as well as the migration rate which was shown in **Figure 4** ($P < 0.001$). In addition, the expression levels of cell migration proteins FN1 and Vimentin were measured using Western blot and the result was shown in **Figure 6**. Compared with the control group, the expression of FN1 and Vimentin exhibited significant decrease in CCL2 siRNA transfected U251 and U373 cells ($P < 0.001$). Similar to the results obtained in the Western blot assay, there was decrease in the gene expression levels of cell migration related proteins observed through real-time PRC (**Figure 7**, $P < 0.001$), indicating the down-regulation of CCL2 siRNA on the expression of cell migration related proteins.

CCL2 siRNA inhibited the cell adhesion

Cellular adhesion capability of U251 and U373 cells after the transfection with CCL2 siRNA was determined using adhesion assay. As the result shown in **Figure 5C**, the cell count of adhered U251 and U373 cells declined significantly when compared with the control group ($P < 0.001$), suggesting the decreased adhesion ability after the treatment with CCL2 siRNA. In addition, Western blot and real-time PCR were used to explore the mechanism further. Adhesion related proteins were measured the expression levels using Western blot. As shown in **Figure 6**, declined expression of β-catenin, Twist and Snail corresponded to the results obtained in the adhesion assay ($P < 0.001$), indicating the inhibited adhesion ability due to the interference of CCL2. Oppositely, the expression level of E-cadherin which contributes to the inhibition of metastasis showed significantly increase ($P < 0.001$), further verifying

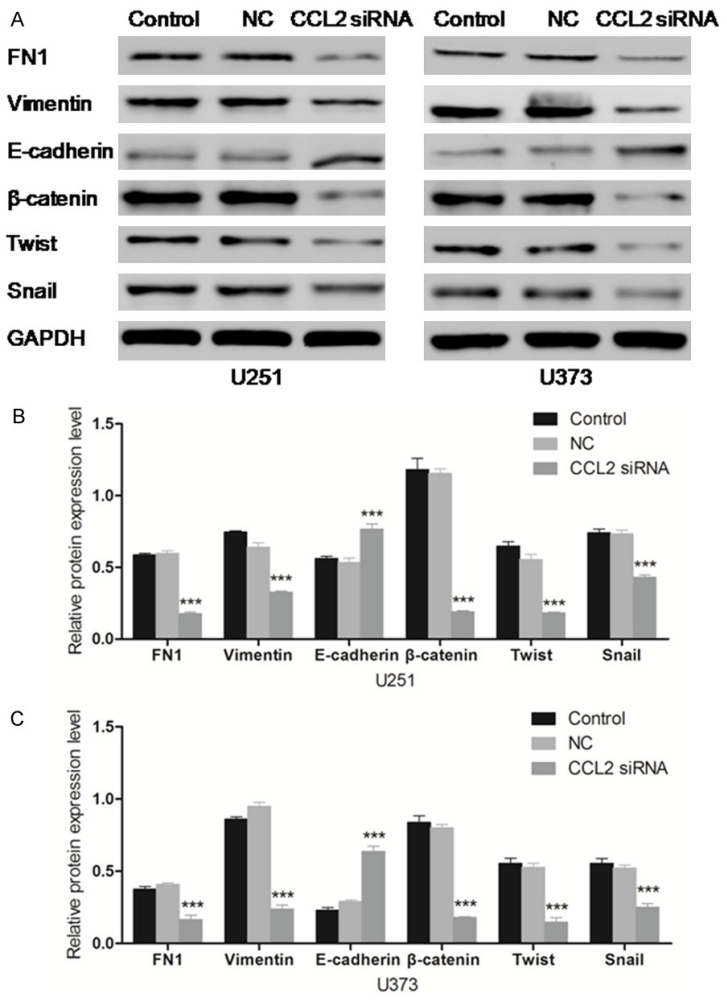

Figure 6. The expression levels of cell invasion related proteins regulated by CCL2 siRNA were measured using Western blot. A. The expression of FN1, Vimentin, E-cadherin, β-catenin, Twist and Snail was exhibited by SDS-PAGE. B. Western blot was used to measure the expression levels of cell invasion related proteins in U251 cells. ***$P < 0.001$, compared with the control group (n=3). C. The protein expression levels were also measured by Western blot in U373 cells. ***$P < 0.001$, compared with the control group (n=3).

the inhibition effect of CCL2 siRNA against the adhesion capability of glioma cell lines U251 an U373.

Discussion

Chemokine with chemotaxis containing 70 to 100 amino acids belongs to small molecular weight secreted protein superfamily. Its G-protein coupled receptors are mainly located in the cell membrane of endothelial, immune and tumor cells [8]. Studies have revealed the effect of chemokine and its receptors on the cell growth, differentiation, apoptosis and tissue damage in body cells, as well as on the cell proliferation and migration in tumor cells [9]. It was reported that they played a dual role in biological behavior of tumor cells. On one hand, chemokine actives immune cells or inhibits angiogenesis so as to exhibit anti-cancer capability. On the other hand, it induces the growth, invasion and migration of tumor through the chemotaxis [10]. Therefore, great significance exists in the research of related chemokine and their receptors in order to explore the genesis, progression and therapies of tumor [11, 12].

Tumor cells secret proteolytic degradation of the cellular basement membrane so as to invade and migrate to distant organs, which is considered as the main cause of cancer death [13]. Fibronectin1 (FN1) is a kind of macromolecule glycoprotein which distributed in the surface of human cells and plasma widely [14]. FN1 involves in the process of wound healing, tissue inflammation, fibrosis and hardening. It possesses the functions of promoting the phagocytosis of macrophage and the connection between cells and fiber matrix [15]. Besides, FN1 has great value in the diagnosis of severe disease as a kind of important opsonin. In addition, as the cell migration related protein, Vimentin contributes to the flexibility of cells [15]. It provides elasticity which does not existed in microtubules and actin. Therefore, Vimentin is responsible for the maintaining the integrity of the cytoskeleton [16]. Furthermore, cells without Vimentin received serious damage after the treatment of acupuncture [17]. Although normal function was maintained in Vimentin gene knockout mice, the microtubules-network was damaged due to the loss of Vimentin, revealing the interaction between microtubules and Vimentin [18]. Moreover, restruction of Vimentin was observed when microtubules were depolymerized, further indicating the relationship between the two systems. Vimentin were also responsible for controlling the transfer of cholesterol

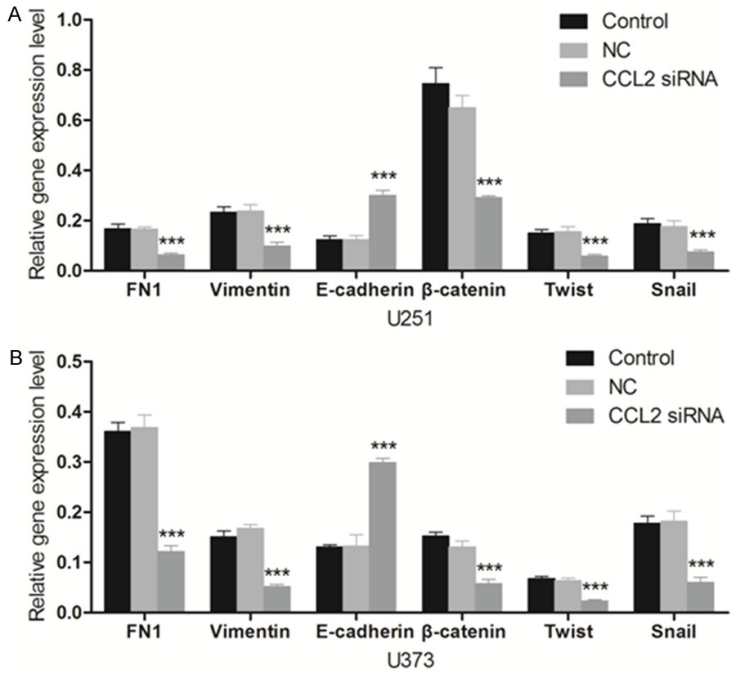

Figure 7. The gene expression levels of cell invasion related proteins regulated by CCL2 siRNA were measured using real-time PCR. A. The gene expression levels of FN1, Vimentin, E-cadherin, β-catenin, Twist and Snail in U3737 cells were measured by real-time PCR. ***$P < 0.001$, compared with the control group (n=3). B. Real-time PCR was also used to measure the gene expression levels in U251 cells. ***$P < 0.001$, compared with the control group (n=3).

derived by low density lipoprotein (LDL) from lysosome to esterification site. Less lipoprotein will be accumulated in cells when the transport is blocked, which relies on the cholesterol ester generated from LDL [17]. As the results shown in **Figure 4**, both of Western blot and real-time PCR presented the declined expression levels of FN1 and Vimentin, in view of the inhibited cell invasion and migration, indicating the down-regulation effect of CCL2 siRNA on the tumor metastasis.

Cell adhesion signaling via CTNNB1 was also known as β-catenin which mainly located in cell membrane. It responds for the mediation of the adhesion between cells and the expression of related genes. β-catenin was found as an adhesion factor at first, and then it was demonstrated as a kind of multifunctional proteins involved in the regulation of cell proliferation, differentiation and apoptosis in various types of cells including endothelial cells, fibroblasts and osteoblasts [19]. Interaction between β-catenin and calcium element forms adhesive tape in the junctions between cells, while free β-catenin regulates gene expression in nucleus [19]. The accumulation of intracellular β-catenin could be induced by Wnt signaling pathways, the abnormal expression or activation of which can cause tumors, corresponding to the inhibited cell adhesion caused by the down-regulated β-catenin (**Figure 5**). E-cadherin mainly distributed in epithelial tissue is a kind of calcium dependent transmembrane glycoprotein calcium which mediates cell homogeneity adhesion between cells [20]. As an inhibitor of cell metastasis in cancer, its expression level is negatively related to the tumor differentiation, cell invasion and metastasis, while positively related to the prognosis [21]. The low expression or unstable expression of E-cadherin could promote the cell metastasis of tumor cells. However, it is not the only factor which regulates the differentiation, invasion and metastasis in a malignant tumor [22]. Twist, as well as Snail, is a highly conserved transcription factor which belongs to alkaline spiral-ring-spiral protein family [23]. It plays an important role in the occurrence and development of embryos as a key regulation factor in the process of epithelium-interstitial change [24]. Twist was demonstrated as a potential cancer gene protein which promotes the occurrence, invasion, metastasis and tolerance, and inhibits the apoptosis of tumor cells [24]. The down-regulation of Twist and Snail indicated the inhibition effect of CCL2 siRNA on the cell adhesion in glioma cell lines U251 and U373 cells, which corresponded to the decreased expression level of β-catenin.

Conclusion

In conclusion, we demonstrated the regulation effect of CCL2 on the cell proliferation, invasion, migration and adhesion of glioma cell lines U251 and U373 through blocking the expression of CCL2 with CCL2 siRNA. Our work might provide theoretical basis for *in vitro* research and clinical treatment against glioma.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Peng Ding, Department of Neurosurgery, The First Affiliated Hospital of Kunming Medical College, 295 Xichang Road, Kunming 650032, Yunnan, China. Tel: +86-572-2555756; Fax: +86-572-2555762; E-mail: dingp1210@163.com

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