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*Original Article*

# CTHRC1 mediates multiple pathways regulating cell invasion, migration and adhesion in glioma

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**Abstract:** Recently, collagen triple helix repeat containing-1 (CTHRC1) has been reported to be increased in several types of human solid cancers and to be associated with tumor invasion and metastasis. However, the expression and function of CTHRC1 in glioma have not yet been reported. In the present study, we investigated whether CTHRC1 plays a role in glioma pathogenesis. Using the tissue microarray technology, we found that CTHRC1 expression is significantly increased in glioma compared with tumor adjacent normal brain tissue ($P<0.01$, $\chi^2$ test) and increased CTHRC1 staining was associated with WHO stages ($P<0.05$, $\chi^2$ test). The mRNA and protein levels of CTHRC1 were significantly upregulated in human primary glioma tissues ($P<0.001$, $\chi^2$ test). We also found that CTHRC1 was significantly increased in glioma cell lines compared to normal human astrocytes ($P<0.01$, $\chi^2$ test). Furthermore, Knockdown of CTHRC1 suppressed glioma cell invasion and inhibited enzyme activity of MMP-2. Moreover, our data showed that knockdown of CTHRC1 inhibited glioma cell migration and adhesion capacity when compared with the control cells, and CTHRC1-siRNA reduced the levels of phosphorylated Src and FAK protein expression. Taken together, this study suggests that CTHRC1 plays a role in glioma development and progression by regulating invasion, migration and adhesion capabilities of cancer cells.

**Keywords:** CTHRC1, glioma, invasion, migration, adhesion

## Introduction

Glioma are the most common primary central nervous system (CNS) tumors, and are classified into grades I to IV according to the WHO criteria [1, 2]. Glioblastoma multiforme (GBM) (grade IV) is the most common and aggressive type of malignant glioma, with a current median survival of approximately 15 months in patients with newly diagnosed disease following treatment with surgery, chemotherapy and radiotherapy [3]. The invasive nature of GBM has been frequently implicated as a key feature of GBM's resistance to therapy. Many studies showed that many factors were involved in the invasion of GBM, including adhesion molecules, extracellular matrix (ECM), protease system and angiogenesis, yet the exact molecular mechanism and process of the invasion growth of GBM remain poorly understood [4, 5]. Therefore, there is an urgent need to clarify the molecular mechanisms underlying invasion and migration behavior of human glioma.

The mammalian Collagen triple helix repeat containing-1 (CTHRC1) gene was first found in balloon-injured rat arteries where it is expressed by fibroblasts of the remodeling adventitia and by smooth muscle cells of the neointima [6]. Increased expression of CTHRC1 was found to promote cell migration and inhibited collagen synthesis in rat fibroblasts. In transgenic mice, Kimura et al. found that CTHRC1 increased the bone mass and functioned as a positive regulator of osteoblastic bone formation [7]. These results have been suggested that CTHRC1 contribute to tissue repair in vascular remodeling in response to injury by limiting collagen matrix deposition and promoting cell migration.

Some evidence suggests that tissue repair and carcinogenesis are tightly linked [8, 9]. Thus,

CTHRC1 may potentially contribute to carcinogenesis and influence the activity of cancer. Recently, some studies have found CTHRC1 expression was increased in malignant melanoma [10], breast cancer [11], pancreatic cancer [12], gastric cancer and colorectal cancer [13, 14] and seemed to be associated with cancer tissue invasion and metastasis. According to expression of analysis of CTHRC1, it plays a crucial role in the differential diagnosis of dermatofibrosarcoma protuberans and dermatofibroma [15]. CTHRC1 has been shown to be high active and potent degrading ECM proteins and promoting cancer cell migration and invasion [16]. Park et al. reported CTHRC1 promoted pancreatic cancer growth and metastatic spread of cancer cells to distant organs in orthotopic xenograft tumor mouse models [12]. Although numerous studies have investigated CTHRC1 expression in various forms of cancer, little is known of the expression of CTHRC1 in glioma and its effects on tumor cell behavior.

In this study, we used a tissue microarray technology (TMA) of human glioma patients and immunohistochemistry to evaluate the expression of CTHRC1 in relation to clinicopathologic features. We found that CTHRC1 expression was increased in glioma compared with tumor adjacent normal brain tissue and this increase was associated with WHO stage. We also report that CTHRC1 was significantly upregulated in glioma cells compared to normal human astrocytes (NHA). Moreover, our data demonstrated that silencing of CTHRC1 in human glioma cell lines reduced the cell invasion, migration and adhesion abilities. In addition, we investigated the mechanisms by which CTHRC1 expression was regulated.

## Materials and methods

### *Ethics statement*

This study was performed under a protocol approved by the Institutional Review Boards of The Affiliated Hospital of Xuzhou Medical University and all examinations were performed after obtaining written informed consents.

### *Patients and samples*

A glioma TMA was purchased from Shanxi Alenabio Biotechnology (Xi'an, China). Pathologic grades of tumors were defined according to the WHO criteria as follows: 133 cases of benign tumor (Grade I and II), 57 cases of malignant tumor (Grade III and IV), 8 cases of tumor adjacent normal brain tissue and 8 cases of normal brain tissue. The array dot diameter was 1.0 mm, and each dot represented a tissue spot from one individual specimen that was selected and pathologically confirmed. Five human glioma tissues and five nontumorous brain tissues (internal decompression in cerebral trauma) were obtained from the Department of Neurosurgery, The Affiliated Hospital of Xuzhou Medical University.

### *Immunohistochemistry of TMA*

The TMA slides were dewaxed by heating at 55°C for 30 min and by three washes, 5 min each, with xylene. Tissues were rehydrated by a series of 5 min washes in 100, 90, and 70% ethanol and PBS. Antigen retrieval was performed by microwaving the samples for 4 min, 20 sec at full power in 250 ml of 10 mM sodium citrate (pH 6.0). Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 20 min. Nonspecific binding was blocked with goat serum for 30 min. The primary monoclonal rabbit anti-CTHRC1 antibody (Abcam, Cambridge, MA, USA) was diluted 1:400 using goat serum and incubated at room temperature for 1 hour. After three washes, 2 min each with PBS, the sections were incubated with a biotinylated goat anti-rabbit secondary antibody for 30 min (Santa Cruz Biotechnology, Santa Cruz, CA), followed by the incubation with streptavidin-peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) for an additional 30 min. After rinsing with PBS 3 times for 2 min, the sections were stained using DAB (Zhongshan Biotech, Beijing, China) for 15 min, rinsed in distilled water and counterstained with hematoxylin. Dehydration was then performed following a standard procedure, and the sections were sealed with cover slips. Negative controls were performed by omitting CTHRC1 antibody during the primary antibody incubation.

### *Evaluation of immunostaining*

The CTHRC1 staining was examined double-blinded by two independent pathologists, and a consensus was reached for each core. The expression of CTHRC1 was graded as positive when over 5% of tumor cells showed immunopositivity. Biopsies with less than 5% tumor

cells showing immunostaining were considered as negative.

*RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)*

Total RNA of brain samples and glioma cell lines were extracted using the Trizol reagent (Tiangen Biotech) according to the manufacturer's instructions. RT-PCR was performed using a Takara RNA PCR Kit (AMV) version 3.0 (Takara, Shiga, Japan) according to the manufacture's protocol. Actin served as an internal control. The primers used were: forward, 5'-TCATCGCACTTCTTCTGTGGA-3'; reverse, 5'-GCCAACCCAGATAGCAACATC-3' (for CTHRC1); forward, 5'-GCGCGGCTACAGCTTCAC-3'; reverse, 5'-GGGGCCGGACTCGTCATA-3' (for Actin). Relative and intensities were determined using image J software.

*Cell culture and transfection*

Primary normal human astrocytes (NHA) were purchased from the KeyGEN Biotech Company (Nanjing, China) and cultured under the conditions as instructed by the manufacturer. Human glioma cell lines (U251, U87, T98G, SHG44, A172) and Rat glioma cell C6 were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Science. Cells were cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Shanghai, China). All cells were maintained in 5% $CO_2$ atmosphere at 37°C. Cells were grown to 50% confluency before small interfering RNA (siRNA) transfection. Nonspecific control siRNA or CTHRC1 siRNA (GenePharma, Shanghai, China) was transfected by siLentFect Lipid Reagent (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The target short interfering RNA (siRNA) sequences of CTHRC1 is as follows: 5'-CCCATTGAAGCTATAATTTAT-3'. 12 hours after transfection, the medium containing transfection reagents was removed. The cells were rinsed twice with PBS and incubated in fresh medium. Subsequent, cells were either lysed for Western blot assay, or subjected to cell proliferation assay, cell matrigel invasion assay, migration assay and adhesion assay after transfection.

*Western blot analysis*

Cells were harvested and washed thrice with PBS. Whole cell proteins were extracted as described previously [17]. Protein concentrations were determined by protein assay (Bio-Rad, Hercules, CA, USA). Western blot analysis was done as described previously [18]. The following antibodies were used for Western blot: rabbit anti-CTHRC1 (Abcam, Cambridge, MA), rabbit anti-FAK, Src, MMP-2 (all from Cell Signaling Technology, Beverly, MA, USA) and mouse anti-β-actin (Zhongshan Biotech, Beijing, China). Infrared IR dye-labeled secondary antibody was applied to the blot for 1 hour at room temperature. The signals were detected with Odyssey IR Imaging system (LI-COR, Lincoln, NE, USA).

*Cell proliferation assay*

Cellular proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-diphen-yltetra-zolium bromide (MTT) assay. In brief, cells ($5 \times 10^3$ cells/well) were incubated in a 96-well plate, at 37°C in a humidified atmosphere containing 5% $CO_2$. At the end of the experiment, 20 µl of 5 mg/ml MTT (Sigma, St. Louis, USA) was added to each well. 4 hours later, 100 µl of DMSO was added to each well and the absorption at 570 nm was determined using an ELX-800 spectrometer reader (Bio-Tek Instruments, Winooski, USA).

*Invasion assay*

Cell invasion was assessed by matrigel precoated Transwell inserts (8.0 µm pore size with polyethylene tetraphthalate membrane) according to the manufacturer's protocol. To assess invasion, filters were precoated with 30 µl of 5 mg/ml matrigel (BD Biosciences, NJ, USA). $1 \times 10^5$ cells were seeded in serum-free medium in the upper chamber. After 24 hours incubation at 37°C, cells in the upper chamber were carefully removed with a cotton swab and the cells that had traversed the membrane were fixed in methanol, stained with Giemsa and photographed in five independent × 100 fields for each well. Three independent experiments were done and used to calculate fold invasion relative to control.

*Wound healing assay*

After U251 and U87 glioma cells were transfected with siRNA, cells were cultured in fresh medium for 24 hours and treated with 10 µg/ml mitomycin C for 2 hours. After washing with PBS, a standard 200 µl pipette tip was drawn

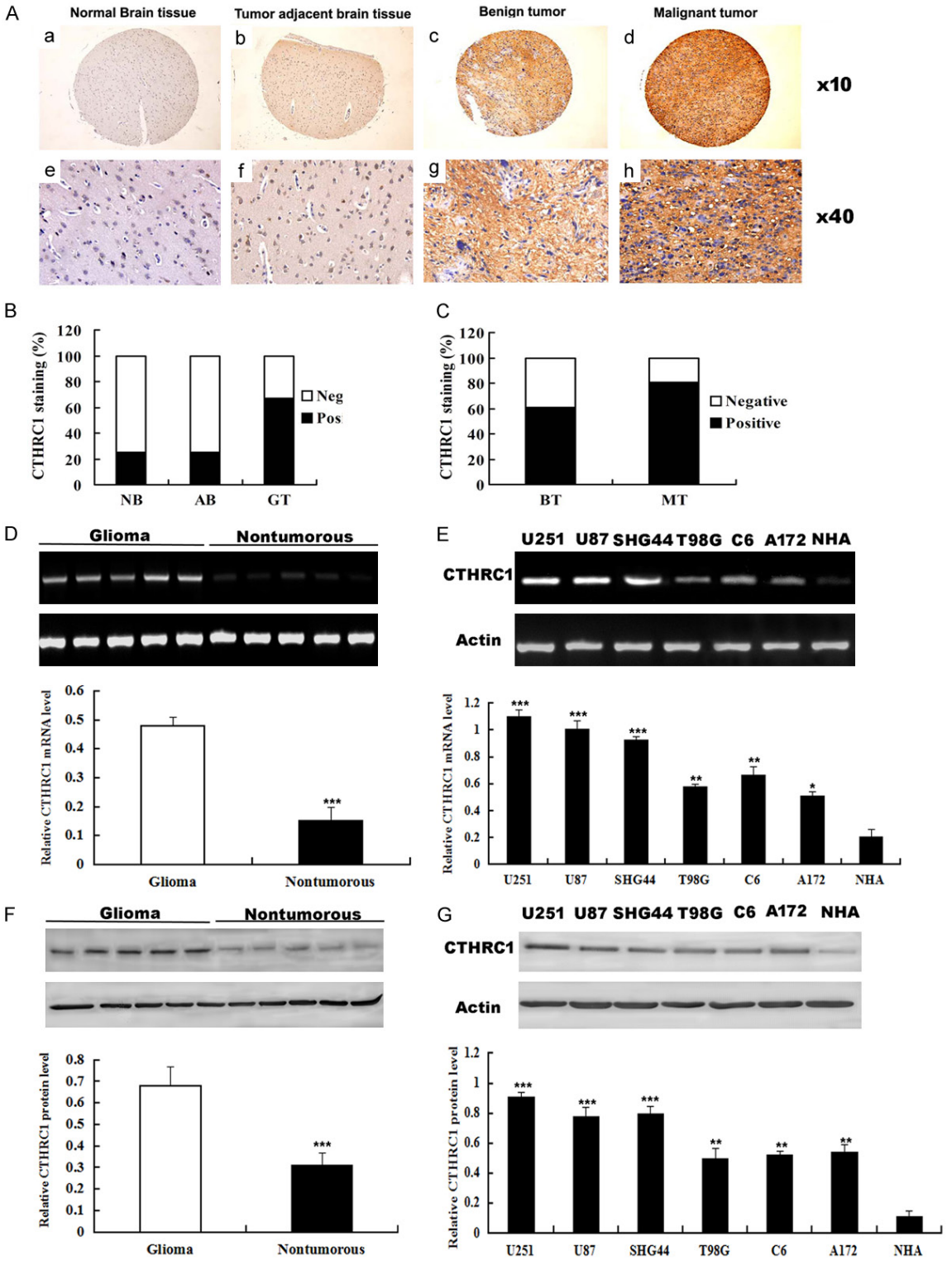


**Figure 1.** Expression of CTHRC1 in human glioma samples and glioma cell lines. A. Representative images depict CTHRC1 immunohistochemical staining. (a, e) Negative CTHRC1 staining in normal brain tissue (NB); (b, f) Negative CTHRC1 staining in adjacent normal brain tissue (AB); (c, g) Positive CTHRC1 staining in benign tumor (BT); (d, h) Positive CTHRC1 staining in malignant tumor (MT). B. A significant difference in CTHRC1 staining was observed between normal brain tissue and glioma tissue (GT) ($P<0.01$, $\chi^2$ test) and between tumor adjacent normal brain tissue and glioma tumor ($P<0.01$, $\chi^2$ test). C. CTHRC1 staining was dramatically increased in malignant tumor compared with benign tumor ($P<0.05$). D. Representative RT-PCR analysis of total. RNA extracted from human glioma

(five samples shown) and nontumorous brain (five samples shown). Actin served as the internal control. E. RT-PCR analysis of CTHRC1 expression in normal human astrocytes NHA and glioma cell lines, including U251, U87, SHG44, T98G, C6, A172. F. Whole-cell protein extracts were further prepared from five glioma tissues and five nontumorous brain. The CTHRC1 protein level was determined by Western blot analysis. G. Western blot analysis of CTHRC1 expression in normal human astrocytes NHA and glioma cell lines, including U251, U87, SHG44, T98G, C6, A172. All experiments were carried out in triplicate. Data are shown as mean ± SD. **P*<0.05, ***P*<0.01, ****P*<0.001. Original magnification (a-d) × 100; (e-h) × 400.

across the center of each well to produce a wound of ~0.5 mm in width. The wounded monolayers were washed twice to remove nonadherent cells, and fresh medium was added. The photo-graphs were taken at the same position of the wound at various time intervals. The starting wound edges were defined in each photo by white lines according to the scratch at 0 hour time point. The wound-healing percentage was determined by the ratio of healing width at each time point to the wound width at 0 hour. Experiments were carried out in triplicate, and three random fields of each well were recorded.

*Migration assay*

Cell migration was determined by using a modified two chamber migration assay with a pore size of 8 μm. For migration assay, $1 \times 10^5$ cells were seeded in serum-free medium in the upper chamber. After 12 hours incubation at 37°C, cells in the upper chamber were carefully removed with a cotton swab and the cells that had traversed the membrane were fixed in methanol, stained with Giemsa and photographed in five independent × 100 fields for each well. Three independent experiments were done and used to calculate fold migration relative to control.

*Cell attachment assay*

96-well plates were coated with 1.25 μg/ml collagen type IV (Sigma) in 100 μl PBS overnight at 4°C. Wells coated with bovine serum albumin (BSA) served as negative control. The plates were blocked with 2.5 mg/ml BSA for 2 hours in DMEM at 37°C. Cells were trypsinized and $2 \times 10^4$ cells were seeded in each well for 1 hour at 37°C, and then the cell adhesion assay was performed as previously described [19].

*Gelatin zymography*

$2 \times 10^6$ cells were seeded in 100 mm plate for 24 hours, cells were transfected with nonspecific control siRNA or CTHRC1 siRNA. 48 hours after transfection, serum-free medium was applied to the cells overnight and the proteins in the conditioned medium were concentrated with Ultracel-30k centrifugal filters (Millipore, Billerica, MA) at 5,000 × *g* for 20 min at 4°C. Proteins (50 μg) were loaded on a 10% polyacrylamide gel containing 0.1% gelatin (Sigma). After electrophoresis, gel was incubated in Triton X-100 exchange buffer (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 5 mM $CaCl_2$ and 2.5% Triton X-100) for 30 min followed by 10 min wash with incubation buffer (same buffer without Triton X-100) thrice. The gel was then incubated in incubation buffer overnight at 37°C, stained with 0.5% Coomassie blue R250 (Sigma) for 4 hours and destained with 30% methanol and 10% glacial acetic acid for 2 hours. Gelatinolytic activity was shown as clear areas in the gel.

*Statistical analysis*

Statistical analysis was performed with SPSS 16.0 software (SPSS, Chicago, IL), Data are expressed as the means ± SD. The association between CTHRC1 staining and the clinicopathologic parameters of the glioma patients, including age, gender, WHO grade and histologic type, was evaluated by $\chi^2$ test. For MTT cell proliferation assays, Student's *t* test was used. Differences were considered significant when P<0.05.

## Results

*CTHRC1 expression is up-regulated in glioma tissues and glioma cell lines*

In order to investigate whether CTHRC1 expression is changed in glioma, we utilized a TMA to evaluate the CTHRC1 expression in normal brain tissue, tumor adjacent normal brain tissue, benign tumor (Grade I and II) and malignant (Grade III and IV). The representative pictures presented in **Figure 1A** showed that CTHRC1 protein in cytoplasm was stained in brown. CTHRC1 positive staining was observed

**Table 1.** CTHRC1 staining and clinicopathological characteristics of 190 glioma patients

| Variables | CTHRC1 staining | | | |
|---|---|---|---|---|
| | Negative, No. (%) | Positive, No. (%) | Total | *P** |
| Age | | | | |
| <45 years | 17 (18.9%) | 73 (81.1%) | 90 | 0.719 |
| ≥45 years | 22 (22.0%) | 78 (78.0%) | 100 | |
| Gender | | | | |
| Male | 25 (21.7%) | 90 (78.3%) | 115 | 0.487 |
| Female | 20 (26.7%) | 55 (73.3%) | 75 | |
| WHO Grade | | | | |
| Benign (I-II) | 52 (39%) | 81 (61%) | 133 | 0.011 |
| Malignant (III-IV) | 11 (19.3%) | 46 (80.7%) | 57 | |
| Histologic type | | | | |
| Astrocytoma | 32 (23.9%) | 102 (76.1%) | 134 | 0.980 |
| Glioblastoma | 9 (25.7%) | 26 (74.3%) | 35 | |
| Oligoastrocytoma | 3 (21.4%) | 11 (78.6%) | 14 | |
| Ependymoma | 2 (28.6%) | 5 (71.4%) | 7 | |

*$x^2$ test.

in 2 of 8 (25%) normal brain tissues, 2 of 8 (25%) tumor adjacent normal brain tissues, 127 of 190 (67%) glioma tissues. A significant difference in CTHRC1 staining was observed between normal brain tissues and glioma tissues ($P$<0.01, $\chi^2$ test) and between tumor adjacent normal brain tissues and glioma tissues ($P$<0.01, $\chi^2$ test) (**Figure 1B**). To further confirm these observations, RT-PCR and Western blot assay were done using five human glioma tissues and five nontumorous brain tissues. It was clear that the glioma tissue had a drastic increase of CTHRC1 expression as compared with the nontumorous tissues (**Figure 1D**, **1F**), which was consistent with the level of CTHRC1 protein expression determined by immunohistochemical staining. In addition, RT-PCR and Western blot analyses showed that expression of CTHRC1 was markedly higher in all 6 analyzed glioma cell lines, including SHG44, C6, U251, T98G, U87, A172 as compared with that in normal human astrocytes (NHA) (**Figure 1E**, **1G**). Collectively, our results suggest that CTHRC1 is up-regulated in gliomas.

*Correlation of CTHRC1 expression with clinicopathological parameters*

The clinicopathologic features of 190 glioma biopsies were summarized in **Table 1**. WHO grade and histologic type are known to be important prognostic markers for patients with glioma. We studied whether CTHRC1 expression correlates with these markers. We found CTHRC1 positive staining in 81 of 133 (61%) benign tumor and 46 of 57 (80.7%) malignant tumor. Therefore, CTHRC1 staining was dramatically increased in WHO stages III-IV compared with stages I-II ($P$< 0.05, $\chi^2$ test, **Figure 1C**). However, we did not find significant correlations between CTHRC1 expression and histologic type (**Table 1**). There is also no significant correlations between CTHRC1 expression with other clinicopathologic variables, including patient age and gender (**Table 1**).

*Knockdown of CTHRC1 in glioma cell lines inhibits cell invasion and MMP2 activity in vitro*

Since CTHRC1 expression is drastically increased in glioma compared with tumor adjacent normal brain tissue, we investigated the involvement of CTHRC1 in glioma cells proliferation and invasion. We first transiently transfected U251 and U87 glioma cells with CTHRC1 siRNA or control siRNA. Western blot indicated at least 60% knockdown of CTHRC1 protein expression in both U251 and U87 cells transfected with CTHRC1 siRNA compared with those transfected with control siRNA (**Figure 2A**, **2B**). 36 hours after transfection, cells were subjected to cell proliferation and invasion assay. Our data suggested that the cell proliferation rates were similar between control group and the CTHRC1-siRNA group in both U251 and U87 cells (**Figure 2C**, **2D**). However, in cell invasion assay, CTHRC1-siRNA inhibits cell invasive ability of U251 and U87 cells in matrigel-coated Boyden chamber by 60 and 65%, respectively (**Figure 2E**, **2F**).

Since MMPs play a crucial role in cell invasion, we carried out the western blotting and gelatin zymography to measure the MMPs expression and activities in glioma cells. As shown in **Figure 2G**, MMP-2 gelatinolytic activity was dramatically decreased by 47% and 53% in CTHRC1-siRNA U251 and U87 cells compared with the control cells, respectively. Then, we performed western blot to examine the MMP-2 expression in glioma cells. Western blot results showed that MMP-2 protein level was sharply

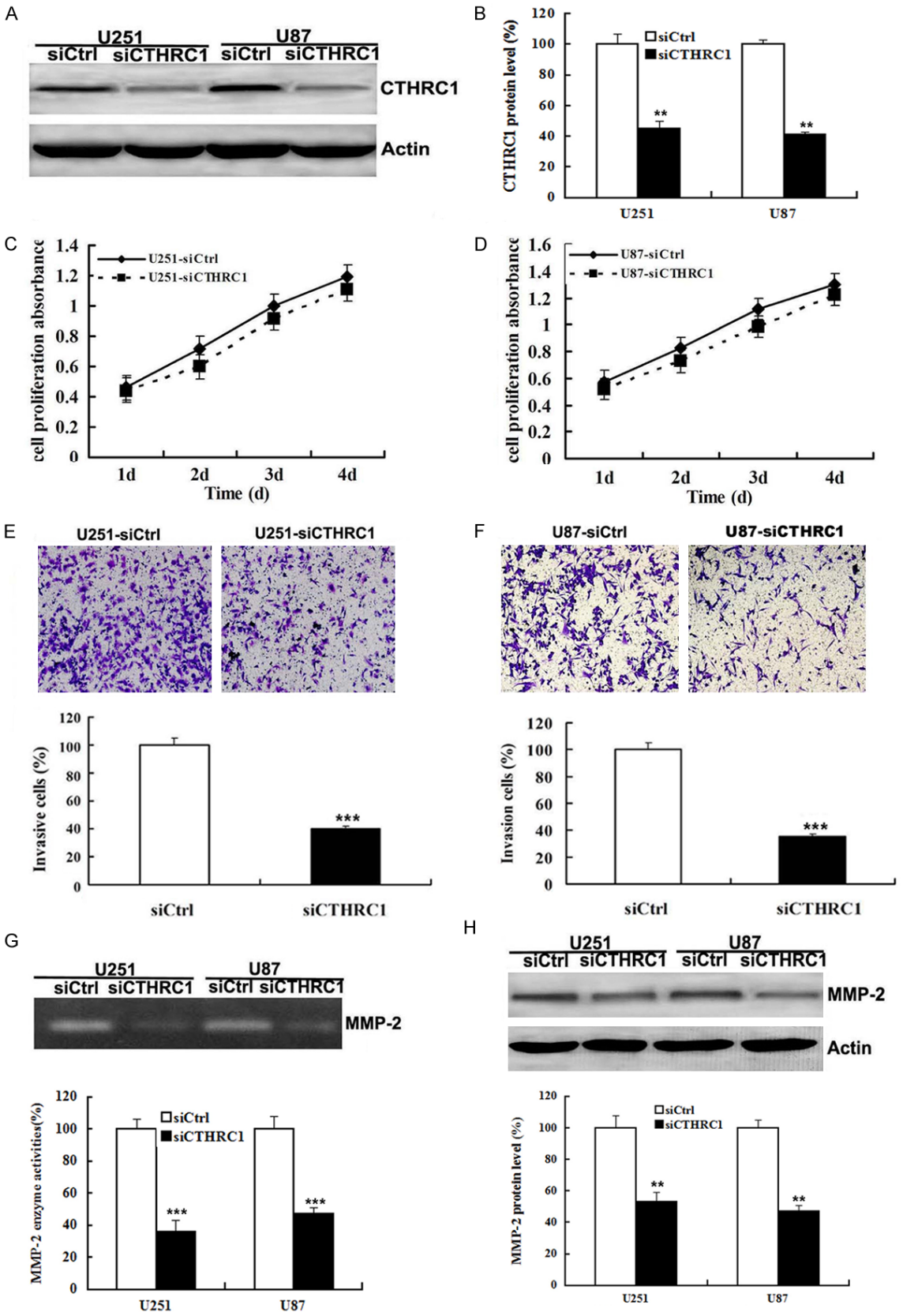


**Figure 2.** Knockdown of CTHRC1 suppresses cell invasion but not cell proliferation in glioma cells. A, B. 36 hours after transfection, the expression of CTHRC1 in U251 and U87 glioma cells was evaluated by western blot. C, D.

MTT cell proliferation assay was performed following knockdown of CTHRC1 in U251 and U87cells. E, F. Matrigel cell invasion assay was performed following knockdown of CTHRC1 in U251 and U87 cells. G. Gelatin zymography analysis of the relative enzyme activities of MMP-2 in CTHRC1 knockdown and siRNA control for both U251 and U87 cells. H. Western blot analysis of the relative protein levels of MMP-2 in CTHRC1 knockdown and control group of U251 and U87 cells. All experiments were carried out in triplicate. Data are shown as mean ± SD. $**P<0.01$; $***P<0.001$.

decreased after CTHRC1-siRNA in U251 and U87 cells (**Figure 2H**).

*Knockdown of CTHRC1 in glioma cell lines inhibits cell migration, adhesion and Src, FAK expression in vitro*

Tumor cell adhesion to the extracellular matrix is implicated in tumor cell motility, invasion and metastasis [20]. We then used the migration and adhesion assay to detect if CTHRC1 affect cell motility and adhesiveness. First, we investigated the role of CTHRC1 in migration of glioma cells by wound-healing assay and migration assays. We found that there was significant delay in wound closure following knockdown of CTHRC1 (**Figure 3A**, **3B**). In addition, the cell migration assay revealed that U251-siCTHRC1 and U87-siCTHRC1 cells exhibited the ability to migrate through the Boyden chamber by 70 and 72%, respectively, compared with the control cells (**Figure 3C**, **3D**). Moreover, CTHRC1 knockdown decreased cell attachment ability of glioma cells by 70 and 62%, respectively (**Figure 4A**, **4B**).

Convincing evidence exists that the activities of Src family kinases and focal adhesion kinase (FAK) control cell migration and adhesion processes, and increased expression of these kinases has been associated with more adhesive and aggressive phenotypes [21, 22]. In order to elucidate the molecular mechanisms responsible for the induction of cell migration and adhesion by CTHRC1, we examined the protein levels of total Src and FAK, phosphorylated Src and FAK by western blotting. Our results showed that CTHRC1 knockdown downregulated phosphorylated Src and FAK expression in U251 and U87 glioma cell lines, but not affect total Src and FAK expression (**Figure 4C**, **4D**).

## Discussion

The possible involvement of CTHRC1 in human carcinogenesis was first suggested by a report that some stromal cells in breast cancer expressed CTHRC1 mRNA as determined by cDNA microarray analysis and in situ hybridization [23]. Tang et al. then observed that CTHRC1 expression was upregulated in invasive and metastatic melanoma compared with non-metastatic melanoma [16]. Furthermore, in a survey of 310 human tumor-derived tissues representing 19 types of human solid cancers, CTHRC1 was found to be increased within 16 of these 19 cancer types [16]. Immunohistochemical analysis also revealed that CTHRC1 is highly overexpressed in pancreatic cancer [12, 24], gastric cancer [13] and dermatofibrosarcoma protuberans (a locally aggressive neoplasm that frequently recurs and metastasize [15]. The relevance of CTHRC1 in cancer was reinforced by the identification that CTHRC1 upregulation was correlated with poor patient survival in metastatic breast [11] and colorectal cancer [25]. Therefore, CTHRC1 may play a role in general human cancer pathogenesis. However, the role of CTHRC1 in glioma has not been clearly studied. In this study, our data first demonstrated that CTHRC1 was significantly increased in glioma compared with tumor adjacent normal brain tissue by TMA and Immunohistochemical technique (**Figure 1A**, **1B**). In addition, CTHRC1 expression is upregulated in high grade (III-IV) glioma in comparison to low grade (I-II) glioma (**Figure 1C**). To further confirm these observations, RT-PCR and Western blot analyses showed that expression of CTHRC1 was markedly higher in 5 clinical glioma tissues and all 6 glioma cell lines, (**Figure 1D-G**). Our results are consistent with previous findings in other tumors [16]. This indicated that CTHRC1 may play an important role in the glioma development and progression.

In the in vitro assays, we found that knockdown of CTHRC1 did not significantly regulate cell proliferation (**Figure 2C**, **2D**), but the cell invasion ability is dramatically suppressed in glioma cell lines (**Figure 3E**, **3F**). Invasion is the most characteristic biological phenotype of glioblastoma, which is thought to involve aberrant interactions between tumor cells and ECM [26]. Previous studies found that CTHRC1 has

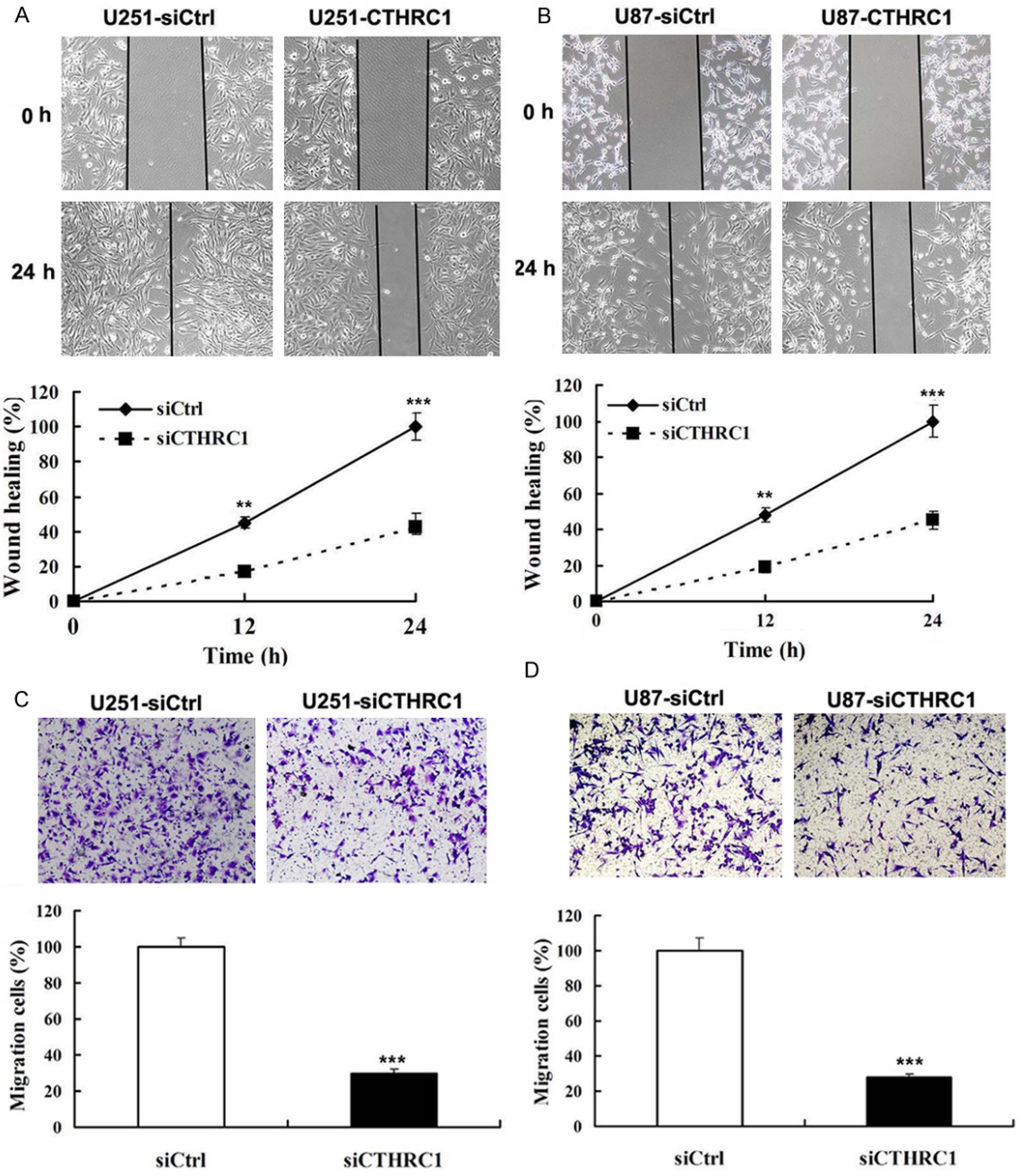


**Figure 3.** Knockdown of CTHRC1 inhibits glioma cell migration. A, B. Wound-healing assay was done on monolayers of U251 and U87 glioma cells after 36 hours of transfection. The photographs were taken at 0, 12 and 24 hours after wounds were made. C, D. Cell migration assay was performed after CTHRC1 knockdown in glioma U251 and U87 cells. All experiments were carried out in triplicate. Data are shown as mean ± SD. $^{**}P<0.01$; $^{***}P<0.001$.

been shown to be high active and potent degrading ECM proteins in melanoma cell lines [16]. High levels of MMP2 in tissues are associated with tumor cell invasion [27, 28]. MMP2 is thought to be key enzymes involved in the degradation of type IV collagen, which is a component of the ECM [27]. In this study, our data suggested that down-regulation of CTHRC1 expression by short interfering RNA significantly inhibited MMP-2 protein expression and enzyme activity in glioma cell lines (**Figure 2G**, **2H**). Therefore, knockdown of CTHRC1 expression can suppress glioma cell invasiveness through MMP2 down-regulation.

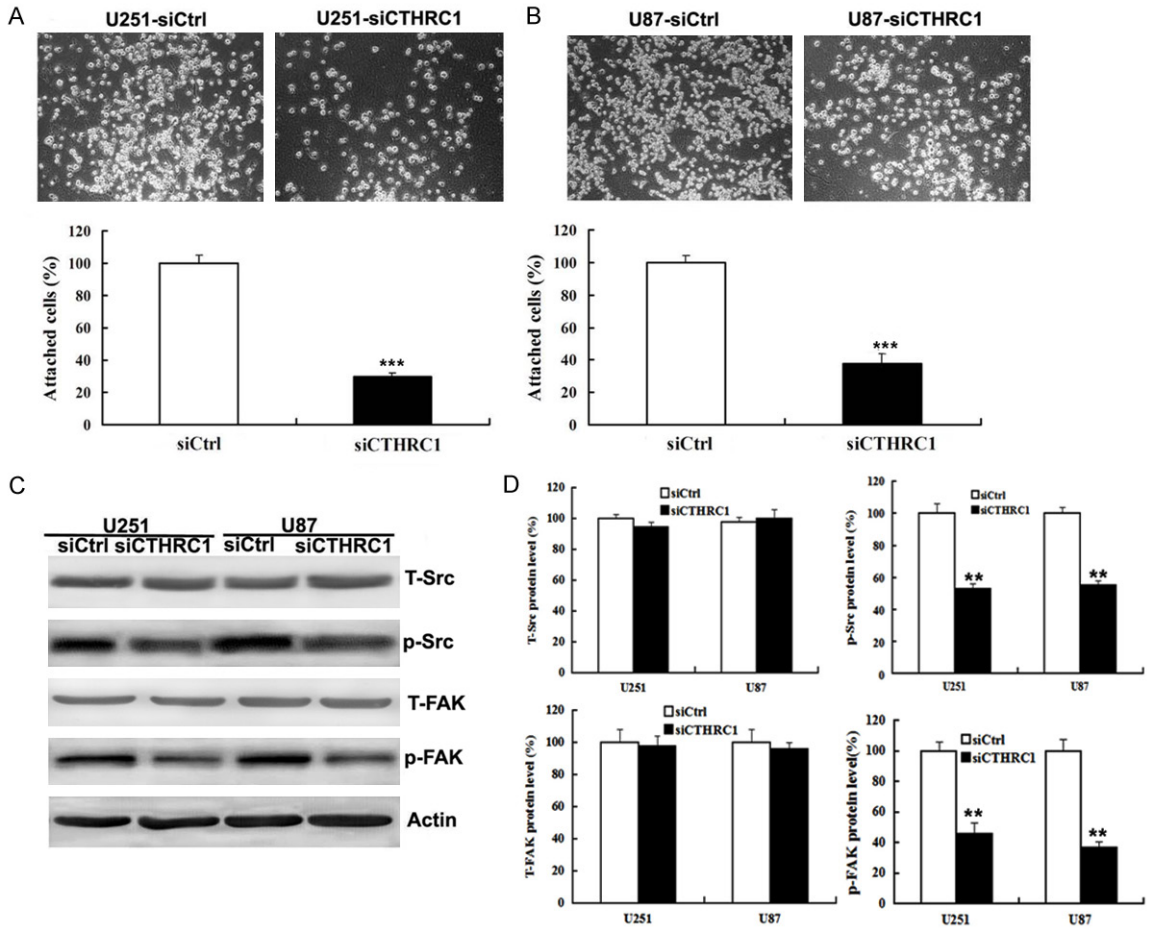


**Figure 4.** Knockdown of CTHRC1 inhibits glioma cells adhesion ability. A, B. Cell attachment assay after CTHRC1 knockdown in U251 and U87 cells. C. Western blot analysis of the relative protein levels of total Src, total FAK, phosphorylated Src and phosphorylated FAK in CTHRC1 knockdown and control group for both U251 and U87 cell lines. D. Quantitative analysis of relative protein level of total Src, total FAK, phosphorylated Src and phosphorylated FAK in glioma U251 and U87 cells. All experiments were carried out in triplicate. Data are shown as mean ± SD. ***$P$<0.001.

A multistep model of invasion suggests that cancer cells must first adhere to the ECM, proteolytically degrade the matrix, and finally migrate through this barrier to surrounding tissue [29, 30]. Consequently, it is possible that CTHRC1 contributes to glioma tissue invasion by increasing tumor cell migration and adhesion. A previous study suggested that increased CTHRC1 expression caused the fibroblast cells to have increased migration in rat cells [6]. Thus, it might be possible that CTHRC1 has a promigratory role in glioma cells. Indeed, we found that knockdown of CTHRC1 decreased cell migration and adhesion abilities of glioma cells through down-regulating both phosphorylated Src and FAK (**Figures 3** and **4**). Our finding is consistent with another study in pancreatic cancer cell lines [12]. The Src-FAK signaling cascade has multiple cellular functions, and modulation of their activities can alter cellular responses that are often perturbed in cancer cells, such as adhesion, migration, and invasion [31-33]. FAK promotes cancer cell migration and adhesion by regulating focal adhesion formation and turnover, which involve activation of Src [34]. The constitutively activated Src-FAK pathway is capable of inducing malignant transformation of a variety of cell types, including glioma [35]. Inhibition of Src-FAK pathway decreased glioma migration and invasion [36]. Park et al. found that CTHRC1 promote pancreatic cancer cell migration through activation of Wnt5a signaling cascade, which induce the activation of phosphorylated Src

and FAK [12]. However, the more exact molecular mechanism of how CTHRC1 regulates Src and FAK expression need us further investigation.

In summary, we found that CTHRC1 expression is significantly increased in glioma compared with tumor adjacent normal brain tissue and increased CTHRC1 staining is associated with WHO stages. The mRNA and protein levels of CTHRC1 were significantly upregulated in human primary glioma tissues. We also found that CTHRC1 was significantly upregulated in glioma cell lines compared to normal human astrocytes. Furthermore, we found that knockdown of CTHRC1 inhibited glioma cell invasion by suppressing MMP-2 protein expression and enzyme activity. Moreover, our data showed that knockdown of CTHRC1 inhibited glioma cell migration and adhesion capacity compared with the control and CTHRC1-siRNA reduced the levels of phosphorylated Src and FAK protein expression. Our results imply that targeting of the CTHRC1 pathway may constitute a potential treatment modality for glioma.

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## Disclosure of conflict of interest

None.

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