European Review for Medical and Pharmacological Sciences 2020; 24: 6195-6203

UCHL1 enhances the malignant development of glioma *via* targeting GAS2

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Abstract. – OBJECTIVE: To detect the expression pattern of UCHL1 in glioma samples and its influence on the metastasis of glioma, as well as the underlying mechanism.

PATIENTS AND METHODS: UCHL1 levels in 42 paired glioma tissues and paracancerous ones were detected. The relationship between UCHL1 level and pathological indexes in glioma patients was analyzed. After establishing UCHL1 knockdown model in U251 and T98-G cells, their migratory ability was assessed by transwell and wound healing assay. At last, Luciferase assay, Western blot and rescue experiments were conducted to explore the role of UCHL1 in aggravating the development of glioma through targeting GAS2.

RESULTS: UCHL1 was upregulated in glioma samples than paracancerous ones. High level of UCHL1 indicated high rates of lymphatic metastasis and distant metastasis, as well as low rates of overall survival and progression-free survival in glioma. Knockdown of UCHL1 markedly inhibited migratory ability in glioma cells. GAS2 was the downstream gene of UCHL1. A positive correlation was found between expression levels of UCHL1 and GAS2 in glioma tissues. Overexpression of GAS2 could reverse the inhibitory effects of silenced UCHL1 on migratory ability in glioma cells.

CONCLUSIONS: UCHL1 level is linked to lymphatic metastasis, distant metastasis and prognosis in glioma patients. It stimulates migratory ability in glioma by positively regulating GAS2 level.

Key Words:
UCHL1, GAS2, Glioma, Migration.

Introduction

Human glioma is a popular tumor in the nervous system, accounting for 50-60% of intracranial tumor cases. The incidence of adult glioma is 6/100,000, and the 5-year survival rate is only 20-30%[1-3]. Currently, the rapid development of modern science, in-depth researches on the etiology and pathogenesis of gliomas, and the great advances on surgical resection contribute to improve the diagnostic and therapeutic efficacies in glioma[3-5]. Nevertheless, clinical prognosis of glioma is far away from satisfy[6,7]. High metastasis rate of glioma is the leading cause of its high mortality[8,9]. Therefore, molecular mechanisms underlying metastasis of glioma require to be analyzed[10-12]. How to prevent and fight against its metastasis should be well concerned.

UCHL1 is a member of the ubiquitin carboxy-terminal esterase family[13,14]. Protein degradation first requires ubiquitin through a series of biochemical reactions, followed by proteasome degradation[14,15]. Members in the ubiquitin carboxyl terminal esterase family can hydrolyze the ubiquitin linked to a specific protein, thus destroying the process of protein ubiquitination[15]. UCHL1 is abundantly expressed in testis, ovary and brain tissues. Mutations in the UCHL1 gene result in a variety of neurodegenerative diseases, including Parkinson's disease, Alzheimer's disease, etc.[13,15]. At present, abnormally expressed UCHL1 is found in different types of tumor tissues[16,17]. Serving as an oncogene, UCHL1 is closely related to tumor development[17,18]. Bioinformatics analysis uncovered an interaction between UCHL1 and GAS2. Protein sequences of GAS2 have been analyzed in humans, dogs, mice, cattle, and Xenopus species. It is suggested that GAS2 is highly conserved during the evolution, manifesting as similar sequences in different species[19,20]. Structurally, GAS2 contains the CH domain (calponin homology domain) in the N-terminal and the GAS2 domain in the C-terminal[20,21]. As a conserved microfilament and microtubule-associated protein, GAS2 is involved in the regulation of cell morphology, apoptosis and division[20,21].

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In this paper, we aim to uncover the role of UCHL1 in the development of glioma and its underlying mechanism. Our findings may provide a new idea in therapeutic strategy of glioma.

Patients and Methods

Glioma Samples

A total of 42 glioma and paracancerous tissues were surgically resected. All tissues were pathologically confirmed and standardly graded. Tumor node metastasis (TNM) staging and histological classification of glioma were defined according to the criteria proposed by UICC/AJCC. Inclusion criteria: patients with no severe diseases in other organs, and none of patients had preoperative chemotherapy/radiotherapy, endocrine or molecular targeted therapy. Exclusion criteria: patients with distant metastasis, those complicated with other malignancies, those with mental disease, those complicated with myocardial infarction, heart failure or other chronic diseases, or those previously exposed to radioactive rays. This investigation was approved by the Ethics Committee of Cancer Hospital of China Medical University, Capital Medical University and it was conducted after informed consent of each subject.

Cell Lines and Reagents

The human glioma cell lines (U251, U87, T98-G, A172) and a human brain normal glial cells (HEB) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) in a 5% CO_2 incubator at 37°C. 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin were applied in culture medium. Cell passage was conducted when cells were grown to 80-90% confluence.

Transfection

Cells were cultured to 30-50% confluence in 6-well plates and transfected with plasmids constructed by GenePharma (Shanghai, China), using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). 48 hours later, cells were collected for determination of transfection efficacy and functional experiments.

Transwell Migration Assay

Transwell chambers (Millipore, Billerica, MA, USA) were inserted in each well of a 24-well plate. 200 μL of suspension (5×10^5 cells/mL) was applied in the upper layer of the chamber with 700 μL of medium containing 20% FBS at the bottom. After 48-h incubation, bottom cells were reacted with 15-min methanol, 20-min crystal violet and captured using a microscope. Migratory cells were counted in 10 randomly selected fields per sample.

Wound Healing Assay

Cells were inoculated in 6-well plates and grown to 90% confluence. After creation of an artificial wound in cell monolayer, medium with 1% FBS was replaced. 24 hours later, wound closure was captured for calculating the percentage of wound healing.

Quantitative Real Time-PCR (qRT-PCR)

Extracted RNAs by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNAs underwent qRT-PCR using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Each sample was performed in triplicate. Relative level was calculated by $2^{-\Delta\Delta Ct}$ and normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). UCHL1: Forward: 5'-AAGGCCAATGTCGGGTAGATG-3', Reverse: 5'-GACTTCTCCTTGCTCACGCT-3'; GAS2: Forward: 5'-GTATGAAGAGGGGTAGTGCGG-3', Reverse: 5'-CACTTGTAATACCCCCGCGT-3'; GAPDH: Forward: 5'-AAGGTGAAGGTCGGAGTCAA-3', Reverse: 5'-AATGAAGGGGTCATTGATGG-3'.

Western Blot

Cells or tissues were lysed for isolating proteins and electrophoresed. Protein samples were loaded on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 hours. Membranes were reacted with primary and secondary antibodies for indicated time. Band exposure and analyses were finally conducted.

Luciferase Assay

Wild-type or mutant-type UCHL1 vector was constructed by inserting corresponding sequences into the pmiR vector. HEK293 cells were pre-seeded in a 24-well plate. They were

co-transfected with NC/pcDNA3.1-GAS2 and UCHL1-WT/UCHL1-MUT, respectively. After 48 h cell culture, they were lysed for measuring Luciferase activity (Promega, Madison, WI, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM Corp., Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Differences between groups were analyzed by the *t*-test. Chi-square test was conducted for analyzing the relationship between UCHL1 level and pathological indexes of glioma patients. Pearson correlation test was applied for evaluating the relationship between two genes. Kaplan-Meier curves were depicted for survival analysis. $p<0.05$ was considered as statistically significant.

Results

UCHL1 Was Highly Expressed in Glioma Samples

Compared with paracancerous tissues, UCHL1 was highly expressed in glioma tissues (Figure 1A). Similarly, UCHL1 was upregulated in glioma cell lines (Figure 1B). Among the four tested glioma cell lines, U251 and T98-G cells expressed the highest abundance of UCHL1, and they were selected to establish the *in vitro* UCHL1 knockdown model.

UCHL1 Expression Was Correlated with Metastasis and Prognosis in Glioma

42 included glioma patients were classified into two groups based on their median level of UCHL1 in glioma tissues. It is shown that UCHL1 level was positively correlated to rates of lymphatic metastasis and distant metastasis, while it was unrelated to age, gender and T stage in glioma patients (Table I). Meanwhile, higher level of UCHL1 was observed in glioma patients with lymphatic metastasis or distant metastasis than those without (Figure 1C, 1D). We collected their follow-up data and analyzed them. Kaplan-Meier curves showed poor overall survival and progression-free survival in glioma patients expressing high level of UCHL1 (Figure 1E, 1F).

Knockdown of UCHL1 Inhibited Migratory Ability in Glioma

Transfection efficacy of sh-UCHL1 was tested in U251 and T98-G cells as Western blot and qRT-PCR results revealed (Figure 2A). Transwell assay showed that knockdown of UCHL1 markedly decreased migratory cell number in glioma, indicating the suppressed metastasis (Figure 2B). Decreased percentage of wound closure in U251 and T98-G cells transfected with sh-UCHL1 also suggested that UCHL1 promoted migratory ability in glioma (Figure 2C).

GAS2 Was the Downstream Target of UCHL1

Potential downstream targets of UCHL1 were predicted by bioinformatics analysis, and

Table I. Association of UCHL1 expression with clinicopathologic characteristics of glioma.

Parameters	No. of cases	UCHL1 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.768
< 60	22	12	10	
≥ 60	20	10	10	
Gender				0.768
Male	20	10	10	
Female	22	12	10	
T stage				0.808
T1-T2	26	14	12	
T3-T4	16	8	8	
Lymph node metastasis				0.013
No	27	18	9	
Yes	15	4	11	
Distance metastasis				0.031
No	26	17	9	
Yes	16	5	11	

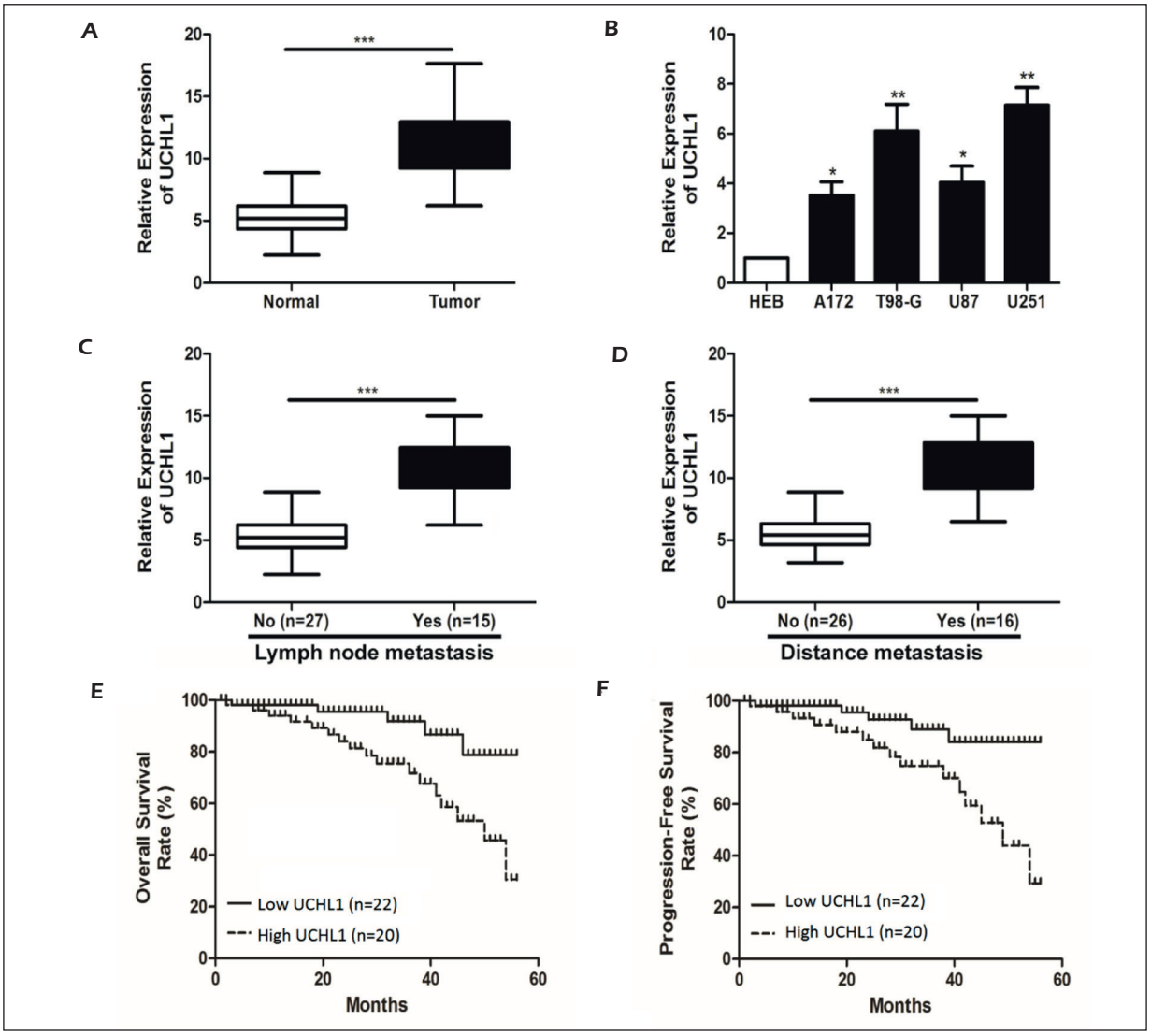

Figure 1. UCHL1 was highly expressed in glioma samples. **A,** Differential expression of UCHL1 in glioma tissues and paracancerous ones. **B,** UCHL1 level in glioma cell lines. **C,** UCHL1 level in glioma patients either with lymphatic metastasis or not. **D,** UCHL1 level in glioma patients either with distant metastasis or not. **E,** Overall survival in glioma patients based on their UCHL1 level. **F,** Progression-free survival in glioma patients based on their UCHL1 level. Data were expressed as mean±SD. *p<0.05, **p<0.01, ***p<0.001.

GAS2 shared a binding sequence with that in the 3'UTR of UCHL1 (Figure 3A). Overexpression of GAS2 decreased Luciferase activity in wild-type UCHL1 vector, confirming the binding relationship between UCHL1 and GAS2 (Figure 3B). It is found that GAS2 was upregulated in glioma tissues and cell lines (Figure 3C, 3E). In glioma tissues, GAS2 level was positively correlated to that of UCHL1 (Figure 3D). Protein level of GAS2 was downregulated in U251 and T98-G cells transfected with sh-UCHL1, further demonstrating their positive correlation (Figure 3F).

GAS2 Was Involved in UCHL1-Regulated Migratory Ability in Glioma

To uncover the interaction between UCHL1 and GAS2, as well as their involvement in the development of glioma, rescue experiments were conducted by cell co-transfection. Higher level of GAS2 was observed in glioma cells co-transfected with sh-UCHL1 and pcDNA3.1-GAS2 than those with solely UCHL1 knockdown (Figure 4A). Interestingly, overexpression of GAS2 increased migratory ability in glioma cells with UCHL1 knockdown (Figure 4B).

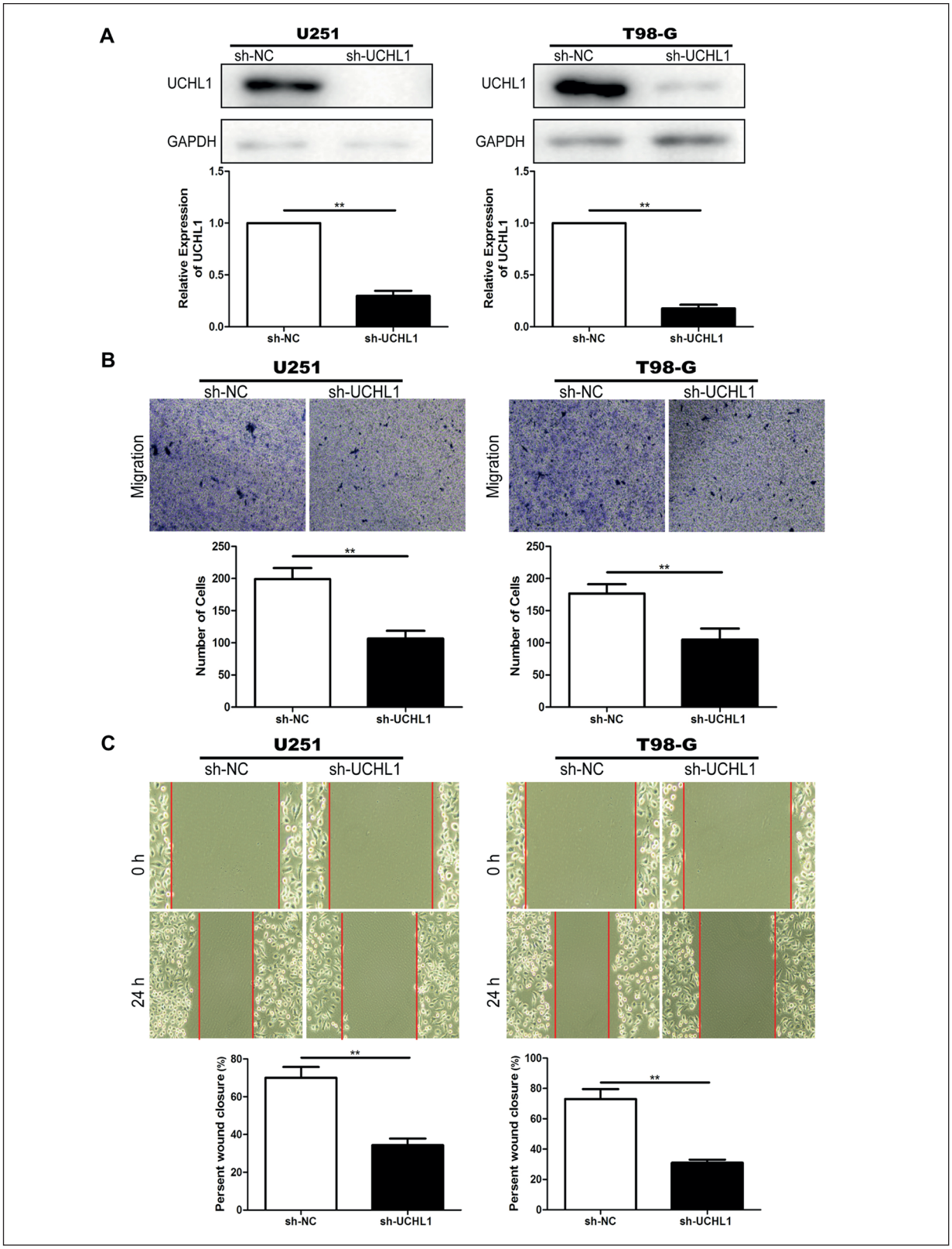

Figure 2. Knockdown of UCHL1 inhibited migratory ability in glioma. **A,** Transfection efficacy of sh-UCHL1 in U251 and T98-G cells. **B,** Migration in U251 and T98-G cells transfected with sh-NC or sh-UCHL1 (magnification: 40×). **C,** Percentage of wound closure in U251 and T98-G cells transfected with sh-NC or sh-UCHL1 (magnification: 40×). Data were expressed as mean±SD. ***p*<0.01.

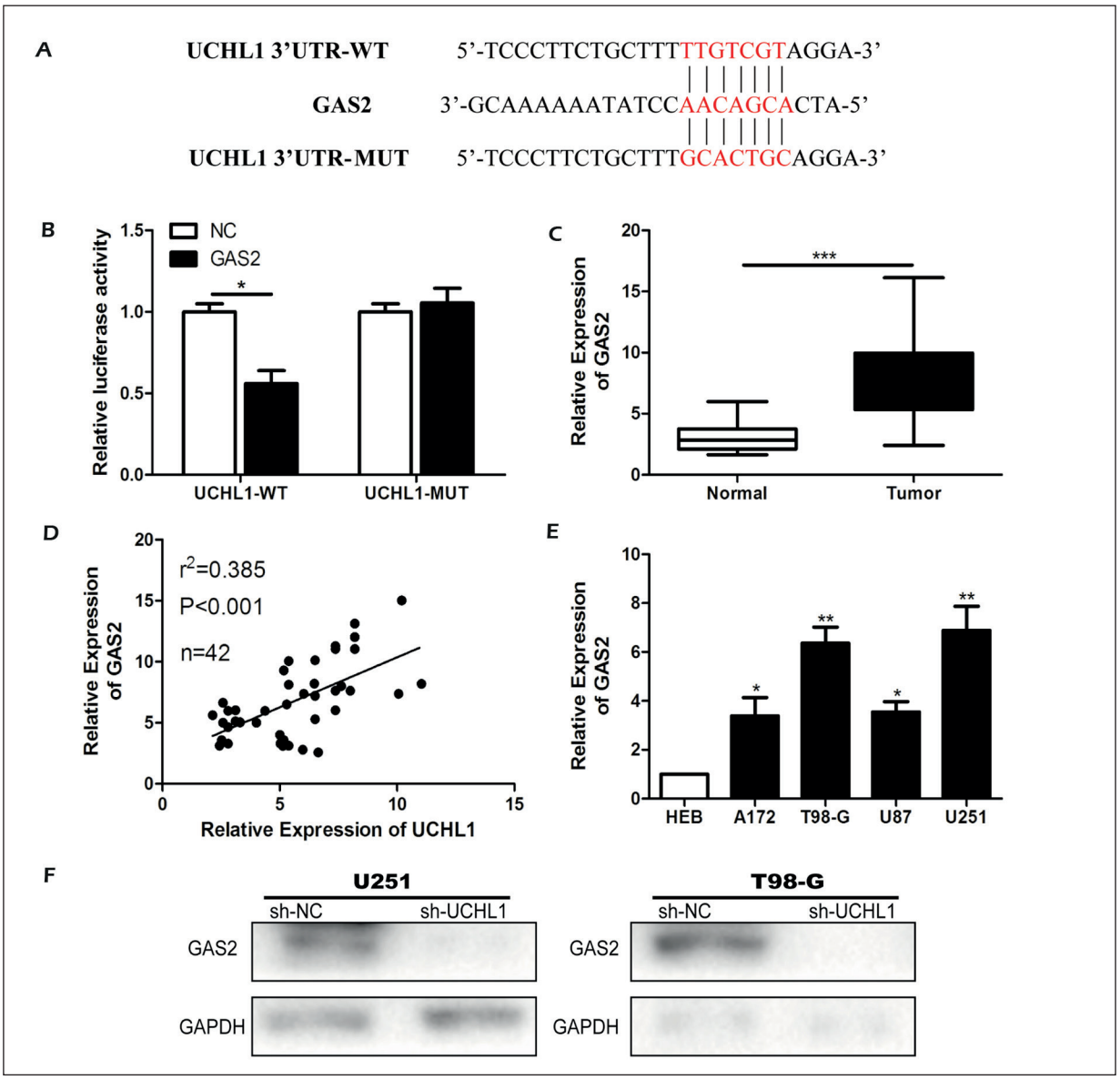

Figure 3. GAS2 was the downstream target of UCHL1. **A,** Binding sequences in the 3'UTR of UCHL1 and GAS2. **B,** Luciferase activity in HEK293 cells co-transfected with NC/pcDNA3.1-GAS2 and UCHL1-WT/UCHL1-MUT. **C,** Differential expression of GAS2 in glioma tissues and paracancerous ones. **D,** A positive correlation between expression levels of UCHL1 and GAS2 in glioma samples. **E,** GAS2 level in glioma cell lines. **F,** Protein level of GAS2 in U251 and T98-G cells transfected with sh-NC or sh-UCHL1. Data were expressed as mean±SD. *p<0.05, **p<0.01, ***p<0.001.

Discussion

Glioma has an extremely poor prognosis[1-3]. In recent years, the onset of glioma has become younger and its incidence has increased as well. Glioma cells are rapidly proliferated. They can infiltrate alongside white matter fibers and invasively metastasize throughout the body, leading to a high recurrence rate even after surgery combined active postoperative chemotherapy/radiotherapy[4-6]. With the research deepening on the genetic level, the molecular mechanism of glioma has been well explored[5,6]. Tumor development is featured by six characteristics, including persistent activation of growth signaling, uncontrolled growth, apoptosis suppression, unlimited proliferative capacity, angiogenesis promotion and tumor metastasis[7-9]. Particularly, metastasis is an essential event during tumor development[10-12]. As a result, clarifying molecular mechanisms of

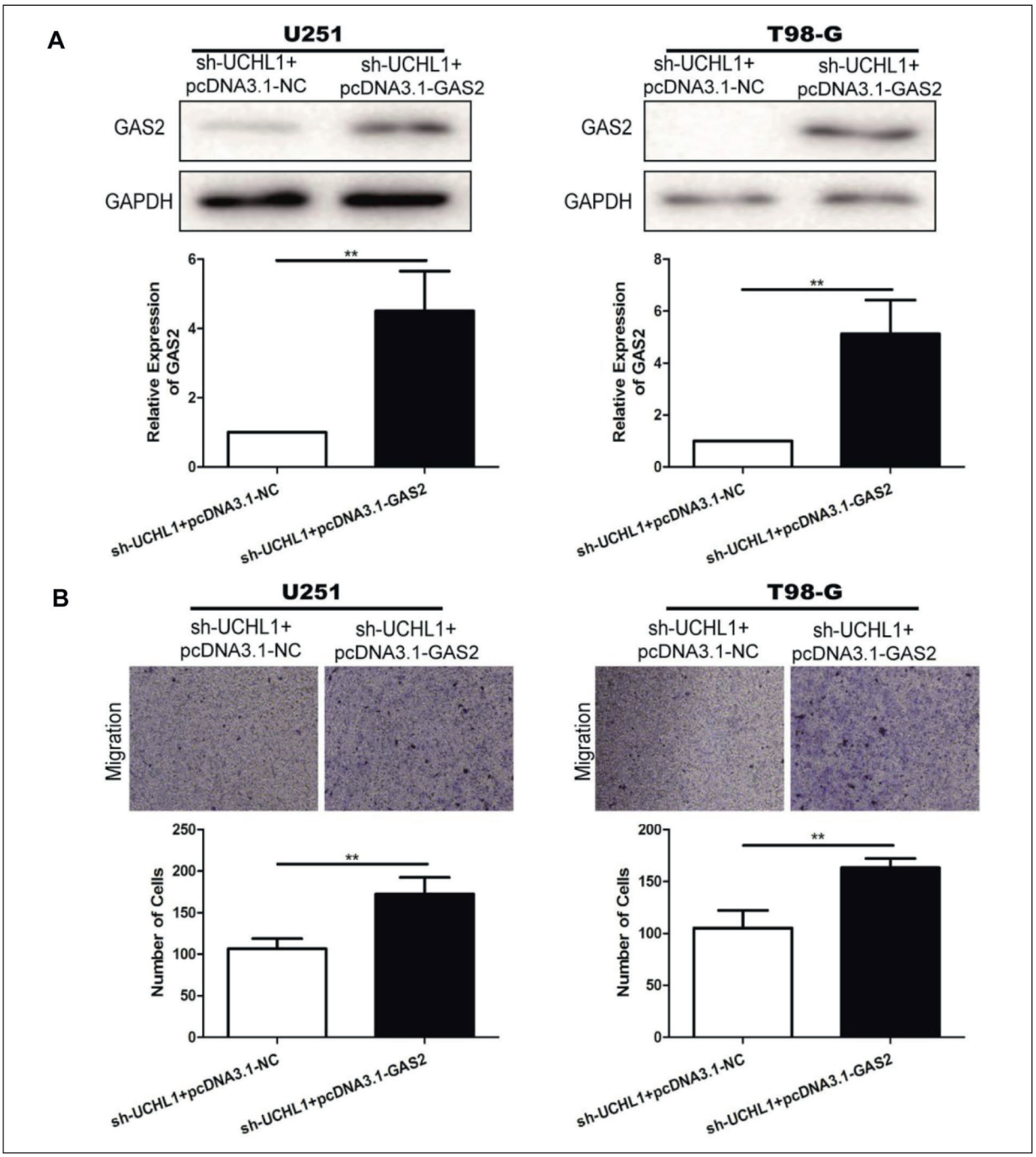

Figure 4. GAS2 was involved in UCHL1-regulated migratory ability in glioma. **A,** GAS2 level in U251 and T98-G cells co-transfected with sh-UCHL1+pcDNA3.1-NC or sh-UCHL1+pcDNA3.1-GAS2. **B,** Migration in U251 and T98-G cells co-transfected with sh-UCHL1+pcDNA3.1-NC or sh-UCHL1+pcDNA3.1-GAS2 (magnification: 40×). Data were expressed as mean±SD. $^{**}p<0.01$.

glioma are of both theoretical and clinical significances. Previous studies[17,18] have shown the involvement of UCHL1 in many types of tumors. Its specific role in glioma, however, remains largely unclear.

It is reported that UCHL1 is upregulated in several types of tumor tissues and cell lines, indicating an oncogenic role. Here, we also demonstrated the highly expressed UCHL1 in glioma samples. Its level was positively correlated to

rates of lymphatic metastasis and distant metastasis in glioma. By analyzing follow-up data of included glioma patients, it is concluded that UCHL1 was unfavorable to the prognosis in glioma. Subsequently, *in vitro* UCHL1 knockdown model was established in U251 and T98-G cells. Both transwell and wound healing assay uncovered the promotive effect of UCHL1 on migratory ability in glioma.

To further illustrate how UCHL1 influences the malignant development of glioma, we predicted potential downstream targets of UCHL1 by bioinformatics analysis. The results showed that the sequence coincidence between the 3'UTR of UCHL1 and GAS2 was the largest. GAS2 is involved in tumor cell growth and metastasis[19-21]. Luciferase assay proved the binding relationship between UCHL1 and GAS2. After transfection of sh-UCHL1, protein level of GAS2 was markedly downregulated in glioma cells. A positive correlation was then discovered between expression levels of UCHL1 and GAS2 in glioma samples. Compared with glioma cells with UCHL1 knockdown, migratory cell number was much higher in those co-transfected with sh-UCHL1 and pcDNA3.1-GAS2. It is suggested that overexpression of GAS2 could abolish the inhibitory effect of silenced UCHL1 on migratory ability in glioma.

Conclusions

We detected that UCHL1 level is linked to lymphatic metastasis, distant metastasis and prognosis in glioma patients. It stimulates migratory ability in glioma by positively regulating GAS2 level.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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