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Original Article

Silencing CCT6A suppresses cell migration and invasion in glioblastoma in vitro

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Abstract: Glioblastoma (GBM) (also described as grade IV glioma) is the most common type of primary intracranial malignant glioma in adults. Most GBM patients have a high morbidity and recurrence rate, with a median survival time of only 12-14 months. The tumour cells that infiltrate normal brain tissue are the main obstacle for surgical treatments and may serve as the origin of cancer relapse. However, the epithelial-mesenchymal transition (EMT) process is involved in initiating cell invasion and migration. Herein, we demonstrate that a chaperonin containing TCP1, subunit 6A (CCT6A), is overexpressed in GBM tissue. Likewise, silencing of CCT6A decreased the invasion and migration of the GBM cell lines U87 and U251. CCT6A knockdown increased the expression of the epithelial marker E-cadherin but repressed the expression of mesenchymal markers including N-cadherin, Vimentin, Snail, Slug, ZEB-1 and Metalloprotease 9 and 2 (MMP9 and MMP2). Taken together, our study indicates that CCT6A is critical for the EMT of GBM cells and provides evidence for the development of CCT6A as a potential therapeutic target for GBM.

Keywords: CCT6A, epithelial-mesenchymal transition, glioblastoma, invasion

Introduction

GBM accounts for more than 50% of all gliomas [1] and is the most common primary brain tumour in adults. GBM usually leads to rapid death, displaying unlimited proliferation, resistance to apoptosis and profuse infiltration into the brain [2]. Even if surgery is combined with radiation and chemotherapy, the overall median survival time of patients with GBM is only 15 months.

Epithelial to mesenchymal transition (EMT), a cellular process characterized by a loss of polarized epithelial features towards a more motile mesenchymal (MES) phenotype, is frequently observed in malignant and invasive human cancers. EMT has been shown to promote mesenchymal differentiation and migration in a number of human tumours, including colorectal cancer [3], prostate cancer [4] and glioma [5].

EMT was considered a crucial mechanism for the increased motility of GBM cells and is believed to foster migration and invasion [5]. During this process, cells change their cell-cell and cell-matrix interactions and increase their motility and invasiveness. EMT is a process in which epithelial-type cells decrease epithelial cell surface markers and cytoskeletal components (*e.g.,* E-cadherin and cytokeratins) while increasing mesenchymal markers and extracellular matrix components (*e.g.*, N-cadherin and fibronectin) [6].

CCT6A (chaperonin containing TCP1, subunit 6A (zeta 1)) encodes the zeta subunit of the molecular chaperone containing TCP1 complex (CCT), also known as the TCP1 ring complex. CCT6A is the only cytosolic chaperonin in eukaryotes, and this complex can assist in the folding of proteins in an ATP-dependent manner [7]. CCT6A participates in the repair of damaged proteins and helps proteins translocate to the membrane. Differential modulation of chaperone activity might alter the half-life of several proteins that, in turn, may affect the sensitivity of tumour cells to chemotherapy. Tanic *et al* found that CCT6A expression was upregulated in drug-resistant variants of a line of human melanoma cells compared with parental cells, which indicated that CCT6A was probably

involved in the sensitivity to therapy of melanoma cancer [8]. Previous studies have shown that CCT6A was upregulated in testicular germ cell tumours [9]. CCT6A is also involved in colorectal cancer invasion and correlated with proliferation of HCT116 cells [10].

Here, we investigate the role of CCT6A in cell invasion and migration in GBM. Our results show that siRNA knockdown of CCT6A decreases the migration and invasion of GBM cells and disrupts the EMT process. Analysis of the prognostic value of CCT6A demonstrates that CCT6A expression is inversely correlated with GBM overall survival (OS). Together, our results indicate that CCT6A contributes to GBM progression by regulating cell invasion and migration, thus highlighting its prognostic and therapeutic potential in GBM.

Materials and methods

Cell culture and tissue specimens

The human GBM cell lines U87, U251 and HEB were obtained from the American Type Culture Collection (ATCC) (Vienna, VA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Gibco, NY, USA) containing 10% foetal bovine serum (FBS). All cells were incubated at 37°C in a humidified incubator with 5% CO_2.

The tissue specimens from patients who received neither chemotherapy nor radiotherapy before surgery. Pathological diagnoses were made independently by three experienced senior pathologists. Our study was conducted according to the principles of the Helsinki Declaration.

Gene expression analyses based on cluster validation

Ten non-tumour specimens and GBM samples from 525 cases in the Cancer Genome Atlas (TCGA) were used as a genomic discovery set. Raw Affymetrix Genome-wide Human Gene Expression Array data and clinical data were retrieved from the Open Access and Controlled Access Data Tiers Portal (https://tcga-data.nci.nih.gov/tcga/) [11]. An independent cohort of 181 GBM and 14 non-tumour specimens were included from the Rembrandt database acquired public data depository (https://caintegrator.nci.nih.gov/rembrandt/) [12]. Eighty GBM samples and four non-neoplastic brain tissue samples were analysed by Murat A *et al.* The microarray data is deposited in the Gene Expression Omnibus (GEO) database at (http://www.ncbi.nlm.nih.gov/geo/ (accession number GSE7696)) [13]. Eight non-neoplastic brain tissue samples, 117 low grade glioma samples and 159 GBM samples were analysed by Gravendeel, *et al* and deposited in the GEO database (http://www.ncbi.nlm.nih.gov/geo/ (accession number GSE16011)) [14]. An independent cohort of 148 low grade glioma samples and 21 GBM samples from Kamoun database (http://www.ebi.ac.uk/arrayexpress) [15].

Transient transfection

The CCT6A-siRNA and control-siRNA were purchased from RiboBio (Guangzhou China). CCT6A-siRNA#1 target sequence: 5'-TCACGAAATGGGCCTTCAT-3', CCT6A-siRNA#2 target sequence: 5'-GCATACATCCTCACTTCGTA-3', and the control-siRNA target sequence: 5'-TTCTCCGAACGTGTCACGT-3'. The glioma cells were transfected with CCT6A-siRNA or negative-control siRNA according to the manufacturer's instructions.

Real-time qRT-PCR

Real-time qRT-PCR was performed as previously described [16]. Specific primer sets for CCT6A (Forward, 5'-TCATGAAGTGTGACGTTGACA-3' and reverse, 5'-CCTAGAAGCATTTGCGGTGCAC-3') and β-actin (Forward, 5'-TCATGAAGTGTGACGTTGACA-3' and reverse, 5'-CCTAGAAGCATTTGCGGTGCAC-3') were purchased from RiboBio (Guangzhou China). The expression of CCT6A was normalized to β-actin.

Western blot

Western blot analyses were conducted as previously described [16]. The primary antibodies were as follows: rabbit anti-CCT6A (Polyclonal #AB35092, Absci, Baltimore, MD), rabbit anti-human vimentin (mAb #5741, Cell Signaling Technology, Danvers, MA), rabbit anti-human Snail (mAb #3879, Cell Signaling Technology), rabbit anti-human N-cadherin (mAb #13116, Cell Signaling Technology), rabbit anti-human Slug (mAb #9585, Cell Signaling Technology), mouse anti-human E-cadherin (mAb ab76055, Cambridge, UK), rabbit anti-human TCF8/ZEB1 (mAb #3396, Cell Signaling Technology), rabbit anti-human MMP-2 (mAb #87809, Cell Sig-

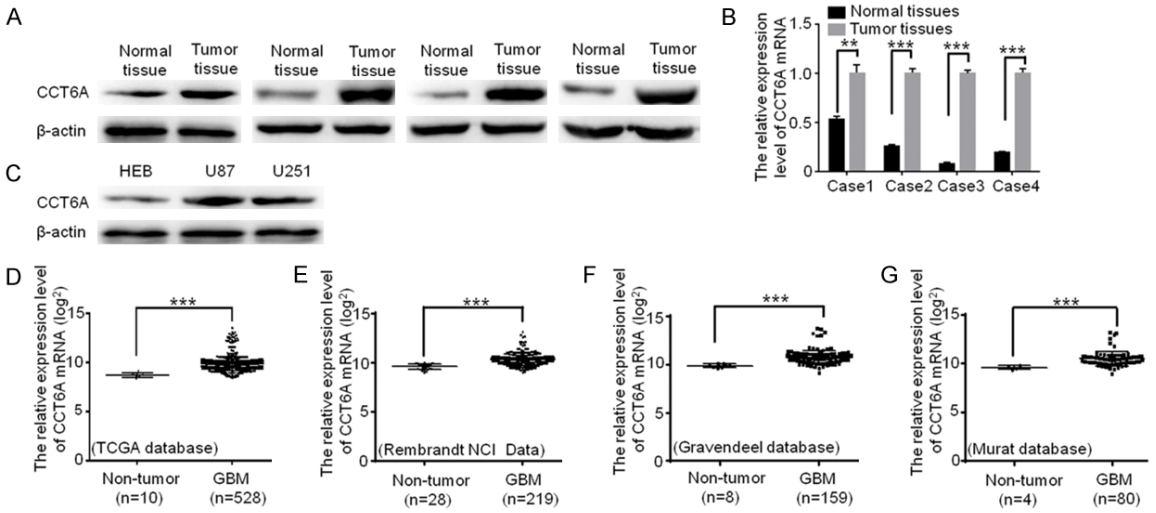

Figure 1. CCT6A overexpression in GBM tissues and cells. (A) Western blot of CCT6A in tumour and normal brain tissues. (B) RT-PCR analysis of the CCT6A mRNA expression levels in tumour and normal brain tissues. (** indicates significant difference $p<0.01$, *** indicates $p<0.001$). (C-G) Public database analysis of CCT6A expression levels in the TCGA database, the Rembrandt Database, the Gravendeel cohort, and the Murat cohort, respectively (*** indicates p<0.001).

naling Technology), rabbit anti-human β-Actin (mAb #5125, Cell Signaling Technology), and rabbit anti-human MMP-9 (Polyclonal #2270, Cell Signaling Technology). Secondary antibodies were HRP-labelled goat anti-rabbit IgG (H+L) (A0208, Beyotime Shanghai China), and HRP-labelled goat anti-mouse IgG (H+L) (A0216, Beyotime).

Migration and invasion assay

Transwell chambers pores sized 8 μm (MCEP-24H48, Millipore, Darmstadt Germany) were used to determine the migratory and invasion capabilities of cells. U87MG and U251MG cells were transfected with CCT6A-siRNA or negative-control siRNA, respectively, for 48 hours, then cells were and seeded in the upper chamber. The lower chamber was added with DMEM containing 10% FBS. After incubating at 37°C for 24 h, non-migratory cells on the upper surface of chambers were wiped with a cotton swab. The cells that migrated to the bottom chambers were fixed with 4% paraformaldehyde and stained with Crystal Violet Staining Solution. Migrated or invaded cells were counted in five randomly selected fields under a light microscope at 400× magnification. Chambers pre-coated with Matrigel (#356234, Corning) were used for evaluation of cell invasion capability. Experiments were repeated three times.

Statistical analysis

Statistical evaluation was conducted with SPSS 22.0 (SPSS Inc., Chicago, IL, USA), GraphPad Prism v.6 (La Jolla, CA, USA), and X-tile 3.6.1 software (Yale University, New Haven, CT, USA) [17]. The Kaplan-Meier method was used to analyse patient survival, and comparisons were made using the log-rank test. X-tile software was used to determine the optimal cut-off values for CCT6A expression in the Kaplan-Meier survival probability estimates. mRNA expression values less than the "cut-off" value is defined as "low" and expression greater than the cut-off value is defined as "high". The unpaired Student's t-test was used to determine the significant differences in the transwell assay and RT-PCR analysis. Data were presented as the mean ± SD. $p<0.05$ was considered statistically significant.

Results

CCT6A is overexpressed in GBM

Since CCT6A has been shown to be upregulated in multiple cancers while the expression pattern of CCT6A in GBM is still unclear, we first analysed CCT6A expression levels in GBM and the corresponding normal tissues via western blot. The results showed that CCT6A expression was significantly higher in the GBM tissues

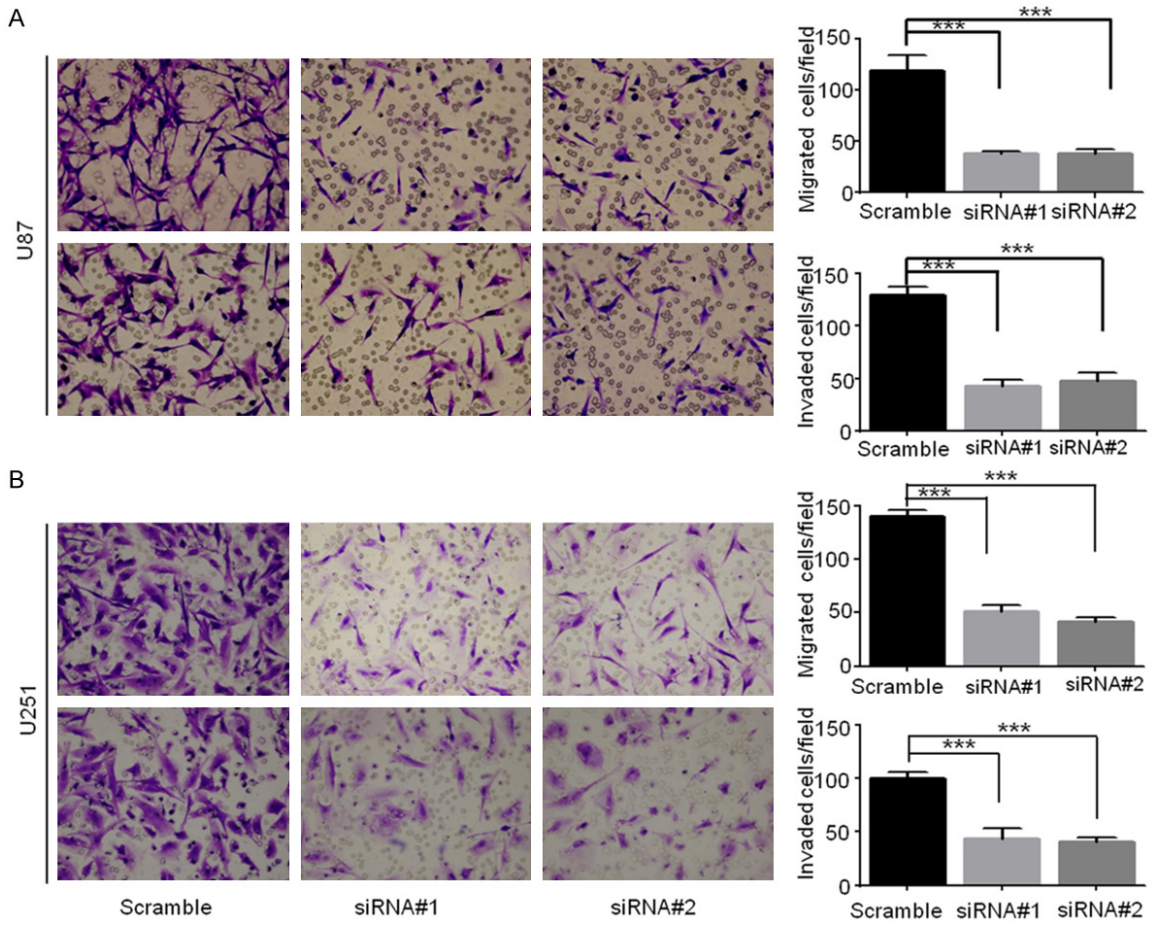

Figure 2. Silencing of CCT6A expression suppresses GBM cell migration and invasion. (A) Migration and invasion analysis of U87 cells transfected with negative-control siRNA or CCT6A-siRNA. (B) Migration and invasion assay of U251 cells transfected with negative-control siRNA or CCT6A-siRNA. Representative images from one experiment are shown (*** indicates significant difference $p<0.001$).

than that in the corresponding normal tissues (**Figure 1A**). Furthermore, quantitative real-time (qPCR) analysis showed that CCT6A mRNA was also overexpressed in GBM tissues, which was consistent with the western blot results (**Figure 1B**). We further performed western blot analysis on CCT6A levels in U251 and U87 GBM cell lines as well as the HEB cell line (**Figure 1C**), which suggested that CCT6A expression is higher in glioma cell lines compared to that in the human glial HEB cells. These results were further confirmed by analysing CCT6A expression in four public databases (**Figure 1D** and **1E**) (TCGA and REMBRANDT project). We analysed previously published human glioma data by Gravendeel *et al* and another cohort by Murat *et al* (**Figure 1F** and **1G**). These data demonstrate that CCT6A is increased in GBM tissues and cell lines compared with normal tissue and cells.

Silencing of CCT6A suppresses GBM cell migration and invasion

To determine whether CCT6A exerts its biologic actions on GBM cells, we sought to identify whether CCT6A could promote GBM malignancy. To investigate whether CCT6A is involved in migration and invasion of GBM, we assessed the invasive and migrative capacity of U87 and U251 on membranes with and without Matrigel coating using CCT6A siRNA-mediated gene silencing *in vitro*. As expected, these findings demonstrated that CCT6A knockdown reduced the invasion and migration of U87 and U251 cells after its transfection for 48 h compared with those in the control siRNA-treated group (**Figure 2A** and **2B**). These results indicate that CCT6A facilitates the migration and invasion of GBM cells. CCT6A may function as a master switch gene for the infiltration of GBM cells.

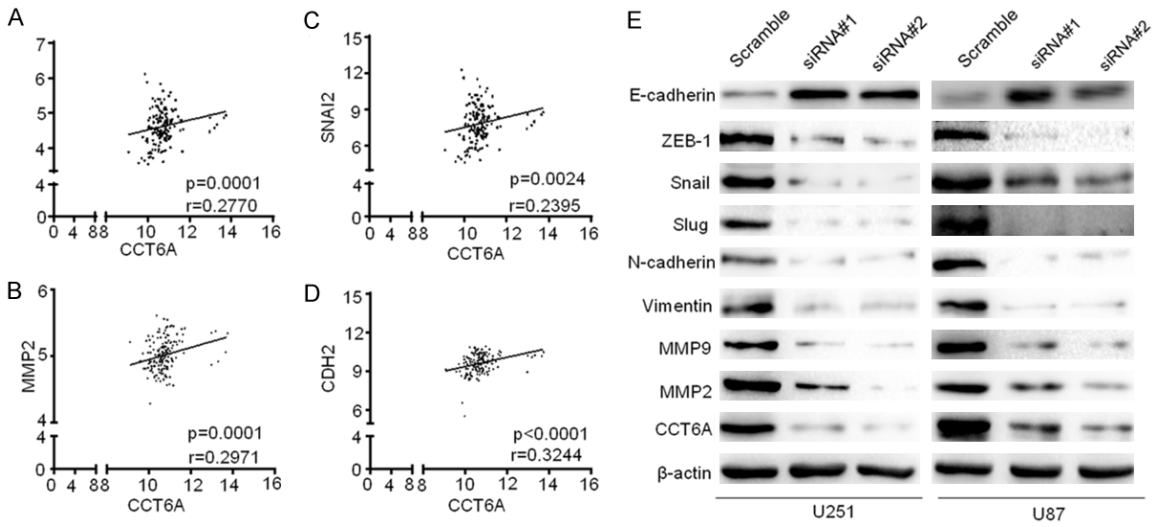

Figure 3. Silencing of CCT6A disrupts EMT-related markers. (A-D) The relationship between CCT6A and SNAI1, MMP2, SNAI2, and CDH2, respectively, in GBM. (E) Western blot analysis confirmed the upregulation of E-cadherin and downregulation of N-cadherin, Vimentin, Snail, Slug, MMP9, MMP2, and ZEB1 by CCT6A-siRNA in U87 and U251 cells. Experiments were repeated three times.

Knockdown of CCT6A suppresses the EMT process in GBM cells

EMT is a crucial process involved in cell motility. We analysed microarray data from the Grevandeel *et al* cohort of EMT-related genes and the CCT6A expression in GBM. The results indicated that CCT6A was weakly correlated with CDH2 ($p<0.0001$, r=0.3244), MMP2 ($p=0.0001$, r=0.2971), SNAI1 ($p=0.0001$, r=0.2770) and SNAI2 ($p=0.0024$, r=0.2395) (**Figure 3A-D**). We further investigated the effects of CCT6A on the EMT process. The western blot results showed that E-cadherin, a marker of the epithelial phenotype, was upregulated in the siCCT6A group compared to scrambled siRNA. In contrast, N-cadherin and vimentin, markers of the mesenchymal phenotype, were downregulated in siCCT6A-transfected glioma cells compared with those in the control siRNA-treated group. MMP2 and MMP9, which have been shown to facilitate cancer infiltration and be involved in the EMT process, were also decreased in the siCCT6A group. Snail, Slug, and ZEB1, which are vital EMT-related genes, were significantly decreased in siCCT6A glioma cells (**Figure 3E**). These results demonstrate that CCT6A is involved in promoting GBM cell migration and invasion in an EMT-dependent manner.

CCT6A levels correlate with glioma grade and higher levels of predicted poor prognosis in GBM patients

We further determined the association between CCT6A expression and the histological grades of glioma patients. Analysis of 620 glioma samples from the TCGA database showed that CCT6A expression was positively associated with glioma grade (grade II *vs.* grade III, $p<0.0001$; grade II *vs.* grade IV, $p<0.0001$; grade III *vs.* grade IV, $p<0.0001$) (**Figure 4A**). The results from the Kamoun cohort (grade II *vs.* grade III, $p<0.001$; grade II *vs.* grade IV, $p<0.001$; grade III *vs.* grade IV, $p=0.003$), Rembrandt database (grade II *vs.* grade III, $p<0.0001$; grade II *vs.* grade IV, $p<0.0001$; grade III *vs.* grade IV, $p=0.672$), and Gravendeel's cohort (grade II *vs.* grade III, $p=0.026$; grade II *vs.* grade IV, $p<0.001$; grade III *vs.* grade IV, $p=0.061$) also suggested a similar conclusion (**Figure 4B-D**).

We further confirmed the relationship between CCT6A expression on survival using Kaplan-Meier survival probability estimates in glioma patients. Survival analysis based on the CCT6A expression levels of 767 glioma patients from the TCGA database indicated that patients who harboured higher CCT6A levels were more likely to have a poor outcome (p<0.0001). The data

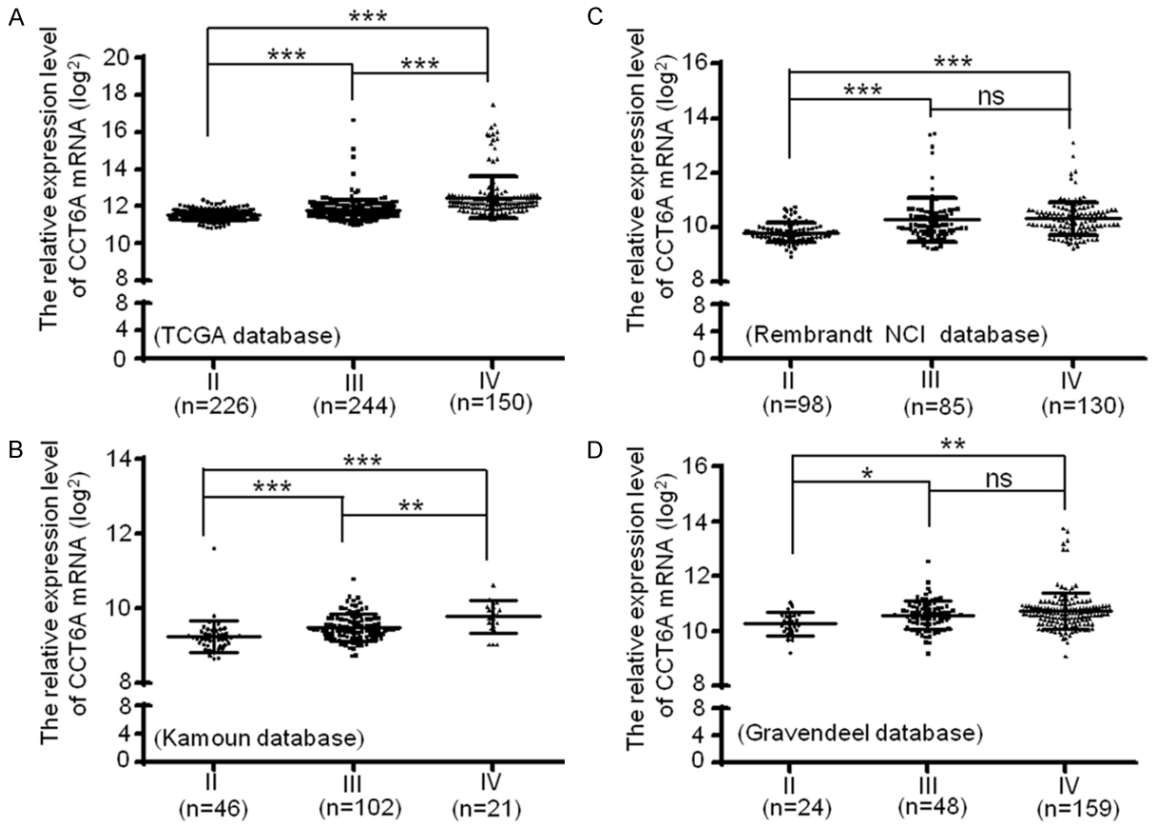

Figure 4. CCT6A expression was positively correlated with glioma grade. (A) Analysis of the expression levels of CCT6A in different grades of glioma. Raw data were acquired from TCGA database (*** indicates $p<0.001$). (B) Analysis of the expression levels of CCT6A in different grades of glioma. Raw data acquired from the Kamoun cohort (** indicates $p<0.01$, *** indicates $p<0.001$). (C) Analysis of the expression levels of CCT6A in different grades of glioma. Raw data acquired from REMBRANDT cohort (*** indicates $p<0.001$, ns indicates non-significant difference). (D) Analysis of the expression levels of CCT6A in different grades of glioma. Raw data acquired from the Gravendeel cohort (* indicates $p<0.05$, ** indicates $p<0.01$, ns indicates non-significant difference).

from the cohorts from the Rembrandt database, the Kamoun database and Gravendeel *et al* were also consistent with these results ($p<0.0001$, $p=0.002$ and $p<0.0001$, respectively) (**Figure 5A-D**).

GBM is the major primary and lethal factor in brain tumours. Data from 525 GBM patients in the TCGA database analysed by Kaplan-Meier survival probability estimates demonstrated that the expression levels of CCT6A indicated a significant trend towards a modest decrease in median survival for patients with high CCT6A expressing tumours compared with low CCT6A expressing tumours ($p=0.008$) with a median patient survival time of 12.4 and 14.7 months, respectively (**Figure 5E**). The data from the Rembrandt database and Gravendeel's cohorts also suggested the same conclusion (**Figure 5F** and **5G**). These results indicate that CCT6A serves as a prognostic marker for GBM and all grade glioma patients.

Discussion

GBM is the most common malignant intracranial tumour. Tumour infiltration into adjacent tissue is a crucial characteristic and acts as an obstacle to surgical treatment, contributing to tumour recurrence. CCT6A is overexpressed in several cancers and takes part in the post-transcriptional modification of tubulin and cyclin E1 [18, 19].

In this study, we investigated the potential role of CCT6A in promoting the migration and invasion of human GBM. EMT is a vital process involved in various cancers (including colorectal cancer and gastric adenocarcinoma), and recent literature suggests that EMT also plays

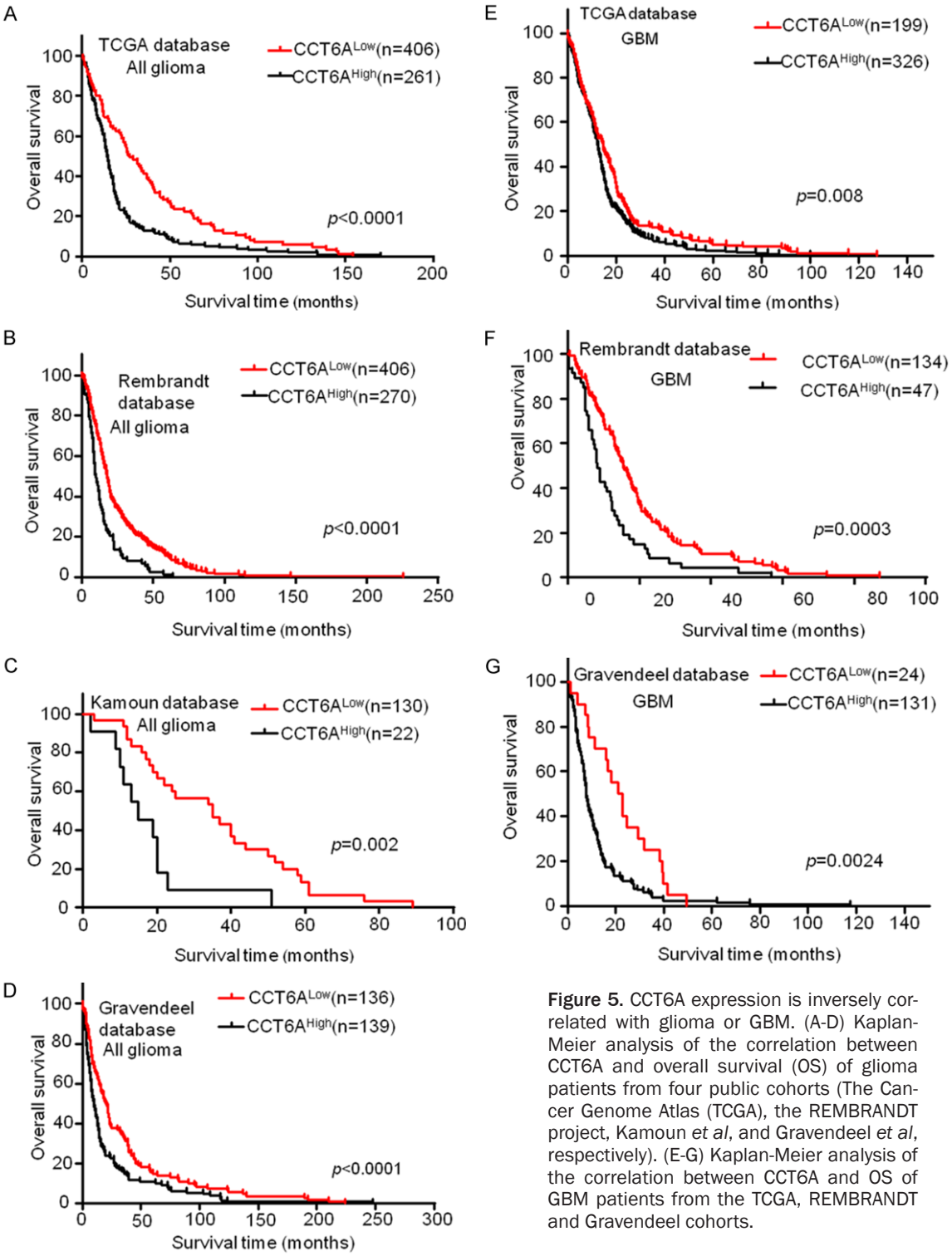

Figure 5. CCT6A expression is inversely correlated with glioma or GBM. (A-D) Kaplan-Meier analysis of the correlation between CCT6A and overall survival (OS) of glioma patients from four public cohorts (The Cancer Genome Atlas (TCGA), the REMBRANDT project, Kamoun *et al*, and Gravendeel *et al*, respectively). (E-G) Kaplan-Meier analysis of the correlation between CCT6A and OS of GBM patients from the TCGA, REMBRANDT and Gravendeel cohorts.

an indispensable role in fostering GBM cell migration and invasion. Our results indicate that CCT6A knockdown could inhibit the expression of mesenchymal markers N-cadherin and vimentin. E-cadherin is an epithelial marker, and elevated E-cadherin levels are considered to be a marker for reduced of EMT. As expected, E-cadherin is significantly increased in the CCT6A-silenced group. EMT-related markers, such as Slug, Snail, and ZEB1, also decreased

in the CCT6A-silenced GBM cells compared to control cells. On the other hand, extracellular matrix (ECM) degradation and remodelling was regarded as a crucial step for tumour cells to enhance their migration and invasion through EMT [20]. MMPs such as MMP2 and MMP9 are a class of important proteases that are involved in extracellular matrix degradation, have been proven to contribute to the invasion of glioma cells [21, 22] and are also degraded in the siCCT6A GBM cells.

These results indicate that CCT6A promotes the motor abilities of GBM cells in an EMT-dependent manner. Previous studies indicated that CCT8 promotes the invasion and migration of the GBM cell lines U87MG and U251MG by regulating the cytoskeleton, and in doing so is a predictor of poor outcome [23].

Accumulating evidence suggests that Stat3 binds to CCT3 and induces the activation of STAT3 in breast cancer HS-578T cells and hepatocellular carcinoma HepG2 cells [24, 25]. IL-6/STAT3 signalling has been proven to promote EMT processes in several cancers [26, 27]. We speculate that CCT6A may also be involved in the EMT process of GBM cells by mediating the IL-6/STAT3 pathway.

CCT6A was upregulated in GBM tissues compared to normal tissues, indicating its oncogenic function. By analysing the association between CCT6A and glioma grade, we showed that CCT6A expression was significantly increased in cases with higher grades of CRC glioma. Kaplan-Meier survival analysis revealed that CCT6A acts as a vital prognostic factor for glioma and GBM. In other words, patients with glioma or GBM who have higher levels of CCT6A tended to exhibit shorter overall survival. These findings indicate that CCT6A has an oncogenic function in glioma progression.

In summary, we demonstrate that CCT6A is overexpressed in GBM, while high levels of CCT6A indicate a poor outcome for patients with GBM. CCT6A has a tumour supportive effect on the EMT process of GBM, which may serve as a novel molecular target for GBM treatment.

Acknowledgements

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Disclosure of conflict of interest

None.

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