

Expressions and Significances of CTSL, The Target of COVID-19 on GBM

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Abstract

CTSL is one of the SARS-entry-associated CoV-2's proteases and plays a key role in the virus's entry into the cell and subsequent infection. We investigated the association between the expression level of CTSL and overall survival in TCGA and CGGA databases, in order to better understand the possible route and risks of new coronavirus infection for patients with GBM. Meanwhile, the relationship between CTSL and immune infiltration levels was analyzed by means of the TIMER database. The impact of CTSL inhibitors on GBM biological activity was tested. The findings revealed that GBM tissues had higher CTSL expression levels than that of normal brain tissues, which was associated with a significantly lower survival rate in GBM patients. Meanwhile, CTSL was very negatively correlated with purity, B cell and CD8⁺ T cell in GBM. CTSL inhibitor significantly reduced U251 cell growth and invasion in vitro and induced mitochondrial apoptosis. According to the findings of this study, CTSL acts as an independent prognostic factor and can be considered as promising therapeutic target for GBM.

Introduction

Coronavirus disease 2019 (COVID-19) is highly infectious and transmitted mainly by inhaling droplets or aerosols released by an infected individual and possibly by a feco-oral transmission route, has accumulated 160,317,950 confirmed cases globally until mid-May 2021 (<https://www.worldometers.info/coronavirus/>). Understanding the mechanism of action of the virus is an important step in determining the best treatment. It has been reported that SARS-CoV2 enters host cells via spike proteins that bind to the angiotensin-converting enzyme 2 (ACE2) membrane bound protein(1). After binding to target cells, the S protein is cleaved into two subunits: S1 and S2 by TMPRSS2 and host cell proteases such as cathepsin L (CTSL), which promotes viral entry into the cell by inducing membrane fusion and endocytosis of several coronaviruses. This cleavage of S protein by host proteases is critical for viral activation and subsequent infection (2, 3). Infection by SARS-CoV-2 results in significant morbidity and mortality. While the lung is the major organ of infection, some studies implied that SARS-CoV-2 might invade the CNS, causing neurological disorders (4). Neurological symptoms, such as headache, dizziness, and impaired consciousness as well as symptoms involving the cranial nerves have been reported in COVID-19 patients (5).

CTSL is a member of the lysosomal cysteine protease family and plays a role in lysosomal protein degradation in most cell types (6). CTSL expressing on the surface of cancer cells and secreted into the extracellular matrix to degrade extracellular matrix components. CTSL preferentially cleaves the peptide bond between the aromatic residue at the p2 position and the hydrophobic residue at the P3 position (7). The acidic environment in lysosomes promotes the activation of proteolytic enzymes. In addition, the locally acidic environment caused by anaerobic glycolysis also promotes the activation of extracellular CTSL in the tumor microenvironment (8). This extracellular proteolytic activity encourages the invasion of cancer cells into surrounding tissues, blood and lymphatic vessels, and the metastasis of tumor tissue to distant tissues. Studies have shown that the expression of CTSL significantly increases in lung cancer, gastric cancer, ovarian cancer, breast cancer, glioma and other malignant tumors, and it promotes the

proliferation, invasion, migration, drug resistance and angiogenesis of tumor cells and other malignant biological behaviors (9, 10). Yu et al found that the over-expressed transcription factor Forkhead box O3a (FOXO3a) in gastric cancer cells can increase the activation of the CTSL promoter, inhibit the expression of E-cadherin, and promote gastric cancer (11). CTSL inhibitors can reduce the tumor model's ability to form blood vessels (12).

The pathogenesis of tumors is relatively complex, involves a variety of pathophysiological processes, and is affected by a variety of factors, among which the immune status of the body cannot be ignored. In the process of tumor occurrence and development, the immune response of the organism is often weakened (13). Due to the low immune function of patients with malignant tumors and the suppression of the systemic immune system caused by anti-tumor treatments such as radiotherapy and chemotherapy or surgery, tumor patients are more susceptible to SARS-CoV2 than non-tumor patients (14). In addition, the expression of SARS-CoV2 receptors (ACE2, CTSL and TMPRSS2) of is significantly increased in many kinds of tumors, such as gastric cancer, esophageal cancer and lung cancer, which makes viral entry into the cell and cancer patients more susceptible to SARS-CoV2 (15).

Analyses of CTSL expression in GBM and related biological processes can aid in the understanding of COVID-19 pathogenesis and the development of therapeutic strategies. In the present study, CTSL expression was identified by using TCGA and GEPIA databases and GBM tissues. Furthermore, the diagnostic and prognostic values of CTSL in GBM were determined. Subsequently, we evaluated whether CTSL can be regarded as a therapeutic target for GBM.

Materials And Methods

Reagents

CTSL were purchased from Selleck Chemicals (Shanghai, china). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies (CCK-8, Dojindo Molecular Technologies, Kumamoto, Japan). PE Annexin V apoptosis detection commercial kit were purchased from BD biosciences (Shanghai, China). Cell-Light EdU Apollo567 In Vitro Kit was purchased from Guangzhou Ruibo Biotechnology Co. LTD (Guangzhou, china). CTSL, Bax, Bcl-2 and MMP-9 antibodies were obtained from proteintech (Wuhan, china). GAPDH was purchased from abcam (Cambridge, UK).

Data collection

The Cancer Genome Atlas (TCGA; <https://www.cancer.gov/tcga>) was used to obtain GBM RNA-seq data. A total of 35 GBM and 16 normal brain tissue samples were acquired with signed informed consent from Lanzhou University Second Hospital. Normal brain tissue samples were obtained from patients without glioma who underwent surgery for other reasons, including cerebral trauma. All procedures involving human samples were approved by the Ethics Committee of Lanzhou University Second Hospital.

Survival analysis

The prognostic value of CTSL was analyzed using CGGA (<http://www.cgga.org.cn/>). The overall survival (OS) rates of the patients in the high-level and low-level GBM groups were evaluated using Kaplan-Meier analysis.

TIMER database analysis

TIMER is a database (<https://cistrome.shinyapps.io/timer/>) for systematic analysis of immune information of various types of tumors (16). The TIMER database contains data from a total of 10897 samples of 32 cancers from TCGA, and can assess the abundance of tumor immune infiltration. The "Gene" module was used to analyze the correlation between the expression level of CTSL and the abundance of immune cell infiltration in GBM.

Quantitative Real-Time PCR

Total RNA was extracted from tissues using TRIzol reagent (Invitrogen). Then, reverse transcribed with the reverse transcription kit (RR037A, Takara Bio, Japan). The expression of mRNAs of CTSL were determined with a LightCycler (RR390Q, Takara Bio, Japan) using TB SYBR green Premix Ex Taq II (Takara Bio, Japan). And the results were analyzed by $2^{-\Delta\Delta CT}$ method. The following primers were used: CTSL forward, 5'-GAAAGGCTACGTGACTCCTGTG-3' and reverse, 5'-CCAGATTCTGCTCACTCAGTGAG-3'; GAPDH, forward 5'-GGACCTGACCTGCCGTCTAG-3' and reverse, 5'-TAG CCCAGGAGGATGCCCTTGAG-3'.

CCK8 and Edu assays

U251 cells were inoculated in 96-well plates and treated with BCA for 24h, 48h and 72 h. Each well is added 10 μ l of CCK8, incubated for 2 hours at 37 °C incubator, and then detected absorbance with a microplate reader. U251 cells were seeded in 96-well plates, each well is added 100 μ l of 50uM Edu solution, incubated for 2 hours at 37 °C incubator, and then 4% Paraformaldehyde fixation. Wash with PBS for 3 times, and each well is added 100 μ l 1X Hoechst33342 solution, incubated at 37 °C in the dark for 30 min, then observed and analyzed under a fluorescence microscope.

Apoptosis analysis

U251 cells were treated with 0, 50, and 100 μ mol/L BCA for 48 h. Then, U251 cells 1×10^6 were collected, after which 5 μ l of PE Annexin V and 5 μ l of 7-AAD were added. Gently vortex the cells and incubate for 15 min at RT (25°C) in the dark and the suspension was analyzed by flow cytometry (BD FACSCanto™ flow cytometry, USA).

Wound healing assay

BCA-treated U251 cells were inoculated into a 6-well plate. When the cells reach a confluence of 70–80%, were gently and slowly scratched with a new 200 ml pipette tip. The relative distance of the cells migrating was monitored and measure using a bright-field microscope at 0, 12 and 24 h.

Transwell cell migration assay

Transwell chambers membrane was pre-coated with diluted Matrigel (1:8 BD biosciences). About 1×10^6 cells in 100 μ l serum-free medium were added into the top chambers, and 600 μ l DMEM medium was added into the lower chamber. After 24 h. the chambers were washed and cells were fixed in 4% paraformaldehyde, and subsequently stained with 0.1 % crystal violet. Cell invasion assay was performed as above except used the cell culture inserts coated with Matrigel (BD Biosciences).

Western blot

Cells (2×10^6) were lysed using RIPA buffer (Solarbio). The lysed cells were centrifuged at $12,000 \times g$ for 10 min at 4°C . Proteins were loaded on 10% SDS-PAGE gels and transferred onto PVDF membranes. The band was visualized by enhanced chemiluminescence with imageQuant LAS 500 system.

Statistical analyses

SPSS version 23.0 (IBM, Armonk, NY, USA), GraphPad Prism 8.0 (GraphPad, San Diego, CA, USA) and R software v4.0.3 (R Foundation for Statistical Computing, Vienna, Austria) were used to perform statistical analyses and graphing. Survival curves were estimated using the Kaplan-Meier method with the log-rank test. $P < 0.05$ was considered significant.

Results

Expressions of Cathepsin L in GBM

In order to elucidate the expression of CTSL in GBM, we analyzed the TCGA and GEPIA database. The level of CTSL expression was markedly upregulated in GBM ($p < 0.05$; Fig. 1A and B). The expression level of CTSL was calculated using RT-qPCR and western blotting in GBM tissue samples ($n=35$) and normal brain tissues ($n=16$) to confirm the GEPIA database findings. The results showed that the level of CTSL was significantly increase in GBM. ($p < 0.05$; Fig. 1C and D). Meanwhile, the data were consistent with TCGA and GEPIA database analysis. The correlation between clinicopathological and the expression of CTSL was evaluated in the cases of 35 GBM patients. The median mRNA level of CTSL was used as a cut-off value. However, the CTSL expression levels was not significantly associated with any of the other parameters assessed, including age, sex and Karnofsky Performance Status ($P > 0.05$) (Table I).

The effect of the expression of CTSL on prognosis in GBM

To determine whether CTSL is an independent prognostic factor for GBM patients, the correlation between OS and the expressions of CTSL was evaluated in the cases of GBM patients using Kaplan-Meier analysis and log-rank tests. The results indicated that patients with high expression of CTSL had a lower OS rate than patients with low expression of CTSL ($P = 0.035$; Fig. 2D). In addition, we further verified the results with TCGA and CGGA databases (Fig. 2B and 2C). To furtherly identify the diagnostic value of CTSL in GBM patients, ROC curve analysis was performed. The results showed (Fig. 2E) that the

AUC for 1, 3, and 5 years were 0.698(95% confidence interval [CI]=0.622–0.733), 0.627(95% confidence interval [CI]=0.494–0.76), and 0.337(95% confidence interval [CI] =0.259–0.416), respectively (Fig. 2E). It is suggested that the CTSL has higher prognostic value in the GBM. Furthermore, the correlations between CTSL expression and major clinic pathological factors were determined using Cox regression analysis. Univariate Cox regression analysis revealed that CTSL expression was an independent prognostic factor for overall survival in patients with GBM (hazard ratio, 3.599; 95% CI, 1.260-10.278; P = 0.017; Fig. 2F).

Relationship between CTSL expression and tumor immune infiltration

To have a better understanding of CTSL and the tumor immune microenvironment, we analysis the relationship between CTSL expression and tumor immune infiltration by using TIMER database. Purity of the tumor means the tumor cell proportion in the tissue. The results showed that CTSL was very negatively correlated with purity ($r = -0.375$, $p < 0.001$), B cell ($r = -0.138$, $p < 0.01$) and CD8⁺ T cell ($r = -0.246$, $p < 0.001$). However, the expression of CTSL was positively correlated with the level of immune infiltration of dendritic cell neutrophil ($r = 0.211$, $p < 0.001$) and dendritic cell ($r = 0.63$, $p < 0.001$) in GBM.

Cathepsin inhibitor 1 (CTSL-1) inhibitors proliferation and invasion of U251 cells

To evaluate the cytotoxic effect of CTSL-1 to GBM, U251 cells were seed in 96-well plates and treated with different concentration of BCA for 24, 48 and 72 h, and cell viability was measured using the CCK-8. As shown in (Fig. 1A), cell viability decreased following treatment with CTSL-1 in a concentration-dependent manner. In addition, the Edu assay was performed to determine the effect of CTSL-1 on GBM cell proliferation. CTSL-1 treatment significantly increased the percentage of Edu-positive cells compared with the control (Fig. 1 B). Taken together, these data indicated that CTSL-1 inhibited the growth of U251 cells in a concentration-dependent manner. To evaluate the effects of CTSL-1 on GBM cells migration, we performed wound healing assay. For this experiment, U251 cells were cultured and then co-incubated with different doses (0, 50 and 100 μM) of CTSL-1 for various time intervals (0, 12 and 24 h). Treatment with various doses of CTSL-1 for 12 and 24 h significantly decreased cell migration rates in U251 cells (Fig. 1C and 1E). We also examined cell migration and invasion capacity using a transwell chambers system after the indicated cell lines were treated with different doses (0, 50 and 100 μM) of CTSL-1 (Fig. 2D, 2F and 2G). U251 cells significantly decreased migration and invasion rates significantly in a dose-dependent manner. Consistent with the cell wound healing assay. These results showed that CTSL-1 inhibits U251 cell migration and invasion in vitro.

Cathepsin inhibitor 1 induced apoptosis of U251 cells

To verify whether CTSL-1 induced proliferation in an apoptosis-related manner, the effect of CTSL-1 on the apoptosis of U251 cells was examined using flow cytometry. We found that CTSL-1 treatment significantly increased the apoptosis of U251 cells (Fig. 4A and 4B). Meanwhile, we further detect molecular markers related to apoptosis. After treatment of CTSL-1, the expression of CTSL-1 and anti-apoptosis protein BCL-2 expression markedly decrease, and pro-apoptosis protein Bax expression increase

(Fig. 4C, 4D, 4E and 4F). Thus, these findings show that cytotoxic effects of CTSL-1 on U251 cells were partly caused by activation mitochondria-mediated intrinsic apoptotic pathway.

Discussion

In December 2019, a novel coronavirus (2019-nCov) has caused large-scale pandemics, which has caused a severe challenge for public health and the economy(17). A large number of studies have described the mechanism by which the SARS-CoV-2 virus enters the cell. Although, it has been widely reported that SARS-CoV2 promotes its entry into host cells through the spike protein that binds to the angiotensin-converting enzyme 2 (ACE-2) membrane-bound protein (18). CTSL seems to be responsible for the cleavage of S protein under different circumstances (19). Therefore, understanding the regulation of viral entry in a comorbid state will also require understanding the expression of these genes. In this study, we analysis the CTSL mRNA level of GBM in GEPIA and TCGA database. We evaluated the relationship between CTSL expression and the prognosis of patients with GBM and their susceptibility to COVID-19. In addition, TIMER database was also used to analyze the correlation between CTSL and immune infiltration in GBM. Moreover, we used CTSL inhibitor to observe the effect of inhibition of CTSL expression on the proliferation, invasion, migration and apoptosis of GBM cells.

Recently studies demonstrated that cancer patients are confirmed to have a higher incidence of COVID-19 and more severe symptoms and has a poor prognosis (20). SARS-CoV-2 can undergo endocytosis, endosomal maturation followed by cleavage with pH-dependent cysteine protease CTSL to promote SARS-CoV-2 entry in cells. Recently study demonstrated that down-regulation CTSL expression may be a potential mechanism that might reduce the ability of the virus to enter host cell, and regulation lysosome PH that would further interfere with proteolytic Spike protein activation may offer protection from the virus to enter (21). So, CTSL plays a vital role in for SARS-CoV-2 to enter cells. Here, our data demonstrated that CTSL upregulates in the GBM. In addition, SARS-CoV-2 might invade the CNS, causing neurological disorders. There is currently no effective targeted drug to treat COVID-19. Although several countries have made great progress in researching COVID-19 vaccines, the vaccines are only effective for some person. A large number of studies have shown that some small molecule drugs have a good effect on the treatment of COVID-19, for example, melatonin (22), hydroxychloroquine and chloroquine (23). The main mechanism of action of these small molecule drugs is to inhibit virus entry by targeting the endocytic pathway (24). Hydroxychloroquine and chloroquine increase the pH of the endosome, thereby inhibiting membrane fusion, which is a necessary mechanism for the virus to enter the cell (25). In addition, the glycosylation of ACE2 and S protein can be inhibited to inhibit virus infection of cells.

Cancer is a disorder with immune dysfunction, and cancer patients are more susceptible to infections(26). Both humoral and cellular immunity are involved in the resistance to SARS-CoV-2 infection in the body. There is evidence that the abnormal regulation of immune response, especially T cells, may be highly related to the pathological process of COVID-19 (27). At the same time, others have also proved that abnormal and excessive immune cells (such as monocytes and macrophages) play a role in immune damage in COVID-19 (24). We use the TIMER database to further explore its correlation with immune cell

infiltration. The result showed that CTSL was very negatively correlated with purity, B cell and CD8⁺ T cell. However, the expression of CTSL was positively correlated with the level of immune infiltration of dendritic cell neutrophil and dendritic cell in GBM. CTSL has the strongest correlation with dendritic cell.

In addition, Kaplan-Meier curves and the log-rank test result showed that low expression levels of CTSL had a favorable prognosis in GBM patients. Therefore, we could evaluate the prognosis of GBM infected with SARS-CoV-2 according to the expression level of CTSL. Previous studies have found that CTSL highly expressed not only in gliomas but also in a variety of tumors, such as lung cancer, gastric cancer, ovarian cancer, breast cancer and other malignant tumors. So, cancer patients may be more susceptible to the SARS-CoV-2 infectious disease.

CTSL plays an essential role in tumor cell detachment, degradation of extracellular and interstitial matrices and basement membranes, intravasation and extravasation across the capillary/lymphatic system. Previous studies have showed that CTSL expression is associated with the prognosis of tumor patients. For example, CTSL is over expression in nasopharyngeal carcinoma, breast cancer and pediatric acute myeloid leukemia, and is associated with poor disease-free survival (9, 10). In this study, Kaplan-Meier survival analysis showed that patients with high CTSL levels had shorter OS and worse prognosis. Interestingly, evidence indicates that CTSL expression may be linked to endometrial cancer patients grade and stage. In addition, CTSL ablation can block angiogenesis in breast cancer by affecting cell cycle-associated genes, including cyclin D1-D3, E2, A2, B2 and H (28). Wang et al demonstrated that Knockdown of Cathepsin L can inhibit GSC growth and increase tumor radio-sensitivity by decreasing CD133 expression and decrease DNA repair checkpoint proteins (ATM and DNA-PKcs) (29). Qin et al (30) demonstrated that CTSL can promote proliferation of breast cancer and downregulation of CTSL significantly inhibited the proliferation of MCF-7 cells. Moreover, CTSL can inhibited A549 and MCF-7 cells migration by regulating PI3K-AKT and Wnt signaling pathways.

Importantly, evidence indicates that CTSL proteolytic activity is significantly higher in tumor tissue compared to normal tissue. In normal tissues, the extracellular matrix is protected by endogenous cathepsin inhibitors (such as cysteine and stefins) from undesirable proteolytic activity. However, studies have shown that the expression of these CTSL inhibitors declines sharply in various tumors and CTSL proteolytic activity increase. Therefore, the use of some exogenous CTSL inhibitors can reduce the expression and proteolytic activity of CTSL. For example, CTSL inhibitor KGP94 have anti-angiogenic efficacy, and can inhibited angiogenesis of breast cancer(12). Dhivya R Sudhan et al found that KGP94 treatment significantly attenuation of tumor cell invasion and migration that it may have significant utility as an anti-metastatic agent. This study has found that CTSL inhibitor 1 significantly reduced the growth and invasion/transwell of U251 cells in vitro and triggered mitochondrial apoptosis. Taken together, these findings suggest CTSL as a promising therapeutic target for clinical therapy in GBM patients. Therefore, because CTSL plays an important role in the pathogenesis of COVID-19, the targeted inhibition of CTSL may be an effective treatment option for this infectious disease.

In conclusion, higher expression levels of CTSL were found in GBM tissues compared with that of normal brain tissues, which resulted in significantly a poor survival rate in GBM patients. We considered the possible role of CTSL in the pathogenesis of COVID-19. Therefore, assessment of CTSL gene expression can be used to predict the prognosis and susceptibility to COVID-19 in these patient groups. CTSL inhibitors may be considered as promising treatments for GBM and SARS-CoV-2 infection.

Declarations

Conflicts of interest

The authors have no conflicts of interest to declare.

Data availability statement

The datasets generated for this study are available on request to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Ethics Committee of the Lanzhou university Second hospital.

Author contributions

YP, GY and QD conceived the project. QD, QL, LD, HY, BW and LN performed the experiments. QD, QL, HW, YY, HZ and LN analyzed the data. QD, GY, QL, YY, BW and LD interpreted the data and revised the manuscript. YP, GY, QL, HW and QD wrote the manuscript. All authors read and approved the final manuscript.

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Figures

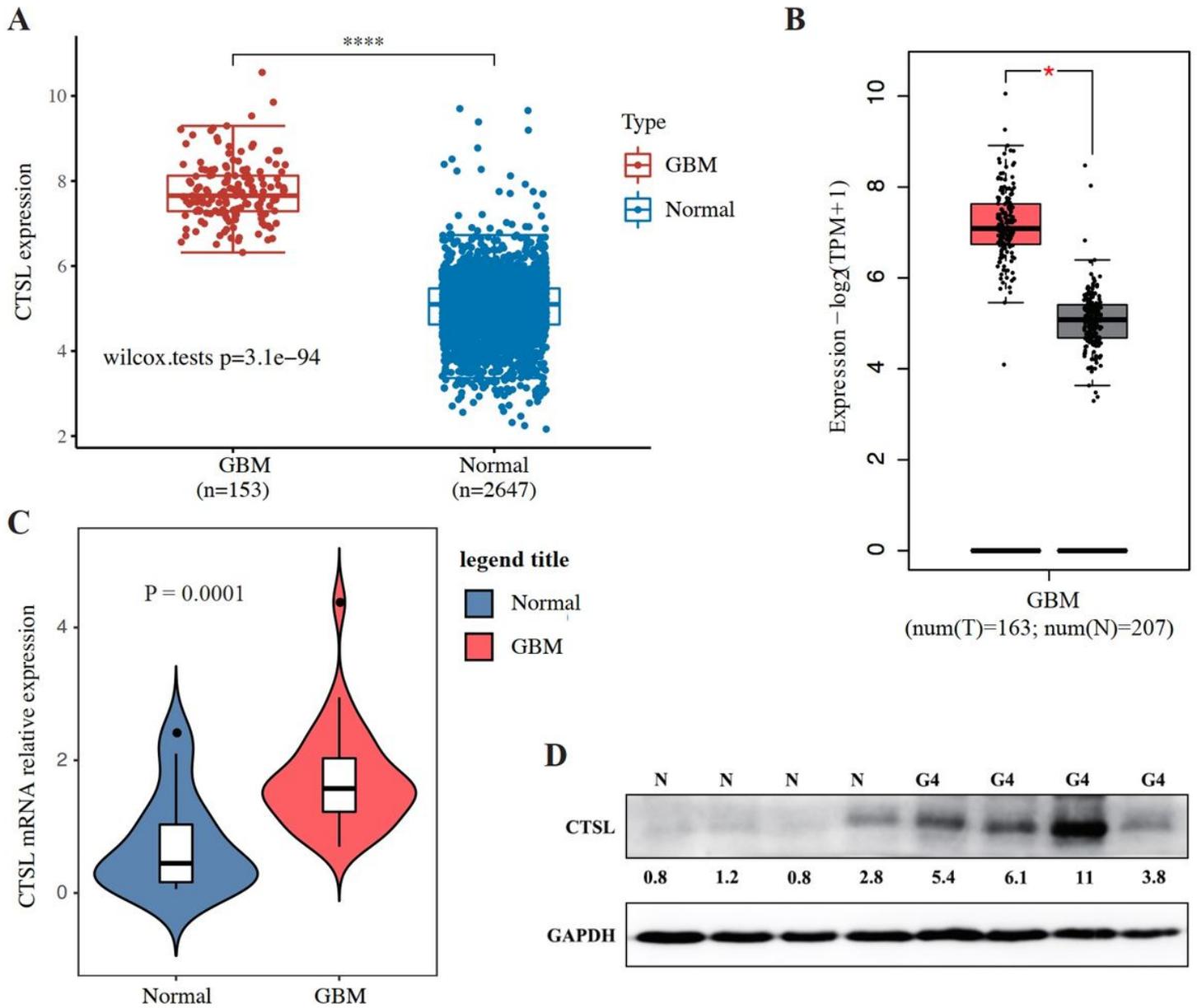


Figure 1

CTSL expression level was evaluated using TCGA and GEPIA databases and GBM tissues. (A) The CTSL mRNA expression level was analysis in TCGA database. (B) The CTSL mRNA expression level was analyzed in GEPIA database. (C) Expression of CTSL mRNA in GBM tissues were tested by RT-PCR. (D) The protein expression levels of CTSL in patients with GBM were analyzed by western blotting. *P < 0.05.

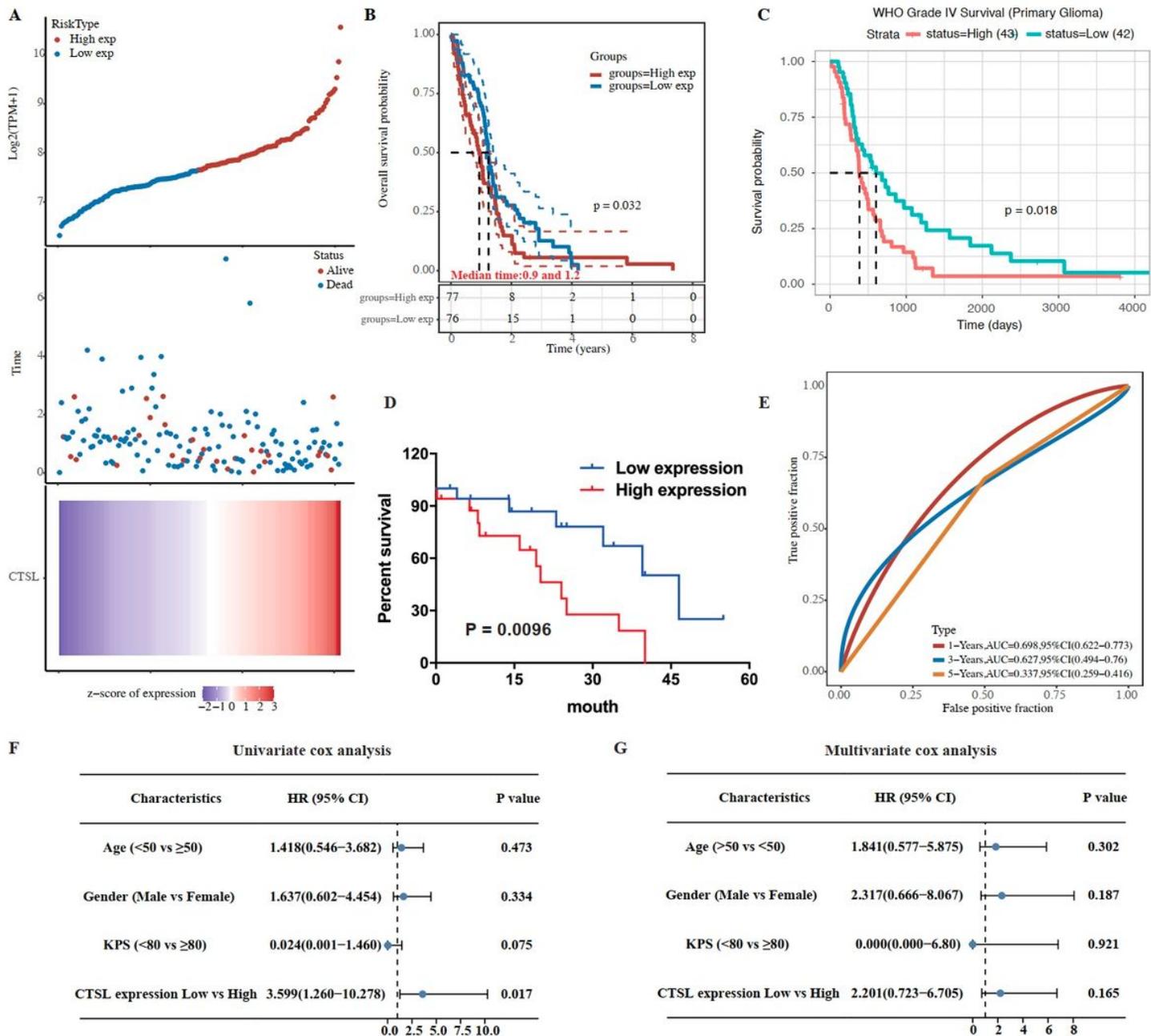


Figure 2

Evaluation of the relationship between the CTSL and patient prognosis. (A) The curve of risk score. Survival status of the patients. More dead patients corresponding to the higher risk score. Heatmap of the expression profiles of CTSL in low- and high-expression group. (B) Kaplan-Meier survival analysis of CTSL in the TCGA set. (C) Kaplan-Meier survival analysis of CTSL in the CGGA dataset. (D) Kaplan-Meier survival analysis of CTSL in the GBM tissue. (E) Time-dependent ROC analysis of the CTSL in the TCGA set. (F) Univariate Cox regression forest map. (G) multivariate Cox regression forest map.

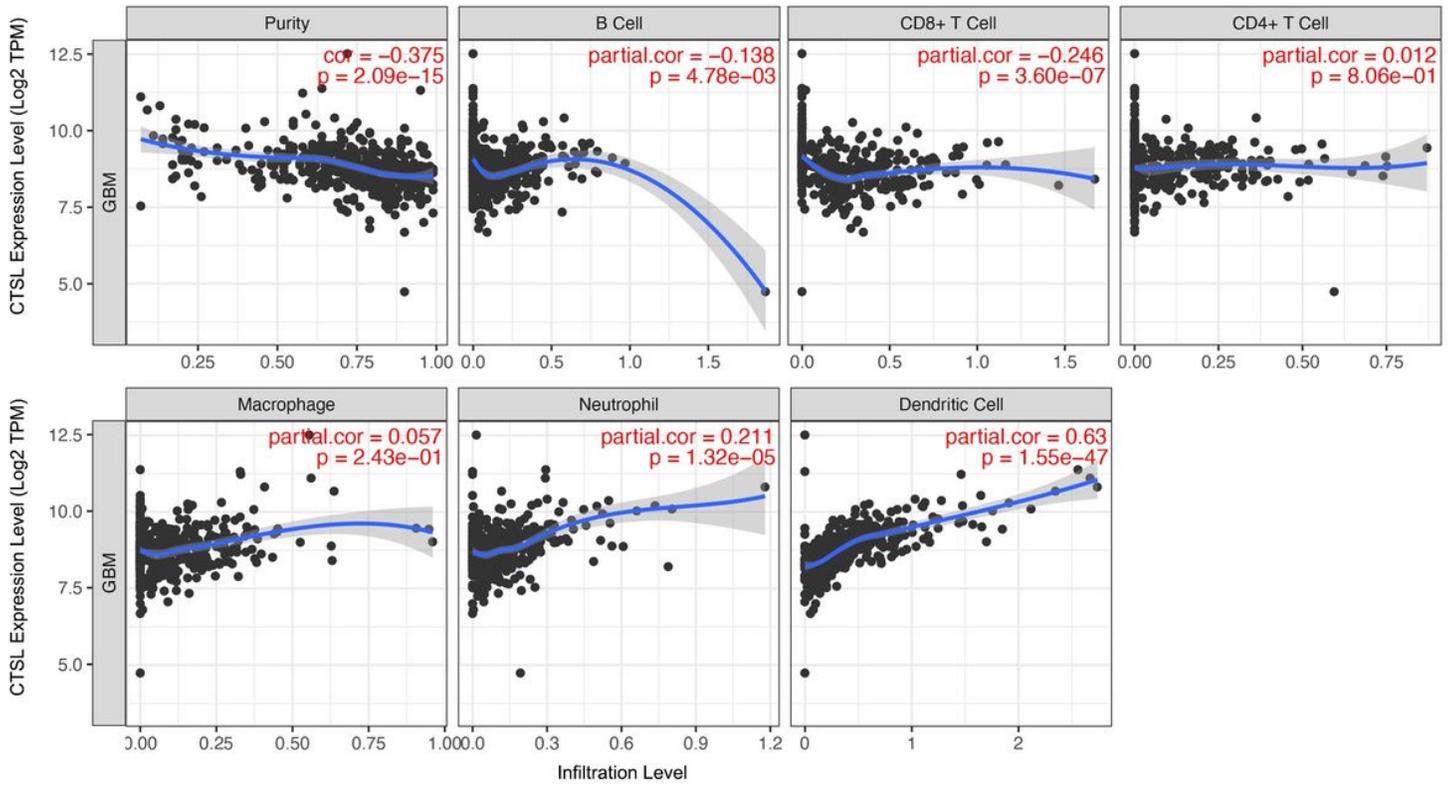


Figure 3

Correlation between CTSL expression and tumor immune infiltration levels in GBM through TIMER database analysis.

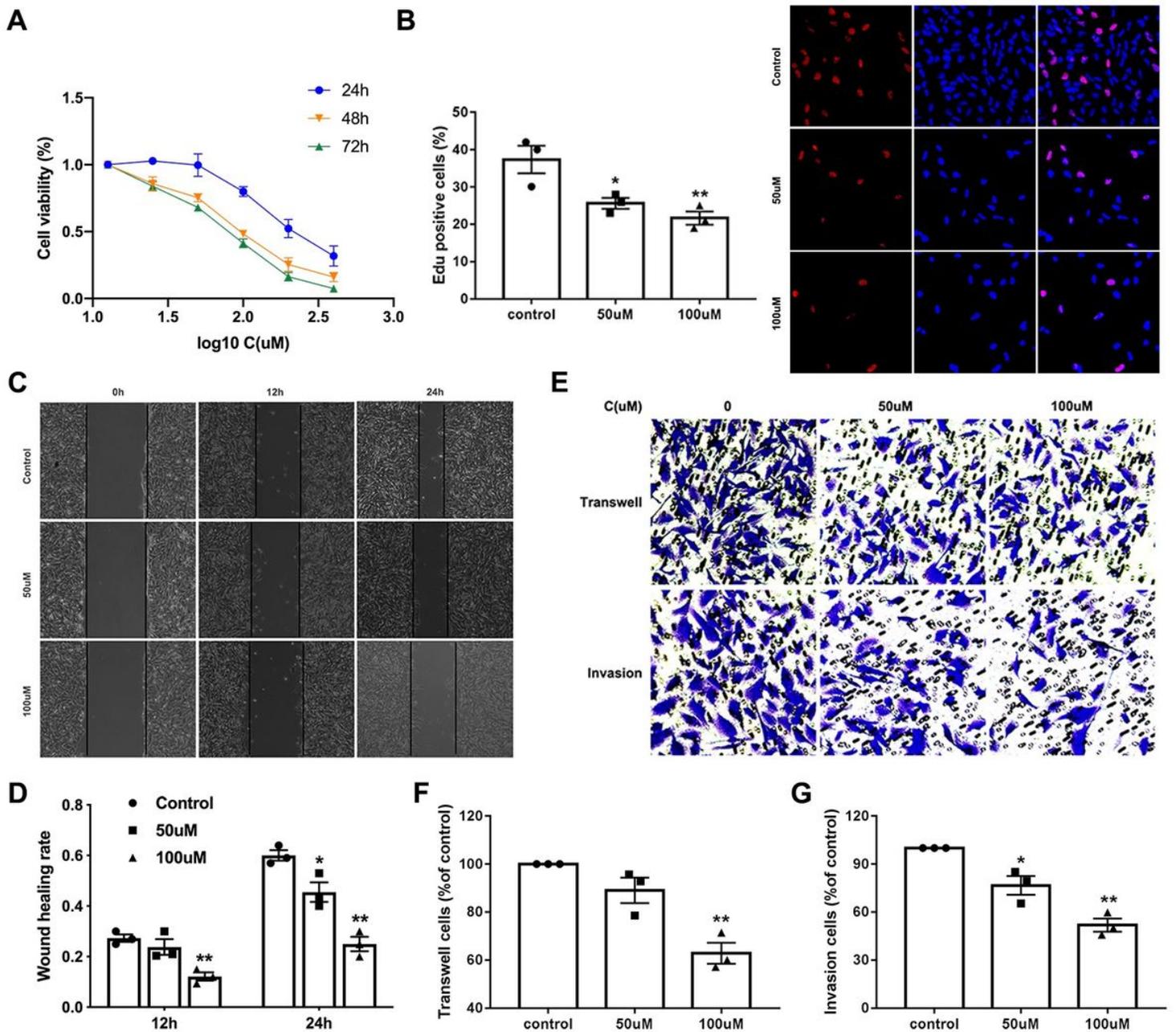


Figure 4

CTSL-1 inhibited the proliferation, migration and invasion of U251 cells. (A) U251 cells were treated with various concentrations BCA for 24, 48 and 72h. Cell proliferation was measured by CCK8 assay. (B) Cellular proliferation was measured via an Edu assay. (C) Wound healing assay shows the migrated cells at 0, 12 and 24 h after treatment with CTSL-1 (0, 50 and 100 μ M). (D) Quantification of the wound healing rate in A after treatment with BCA. (E) After treatment, transwell assay showed that the migration and invasion cells at 24h. (F-G) Quantification of the migration and invasion cells. * $p < 0.05$ and ** $p < 0.01$ for Student's t test.

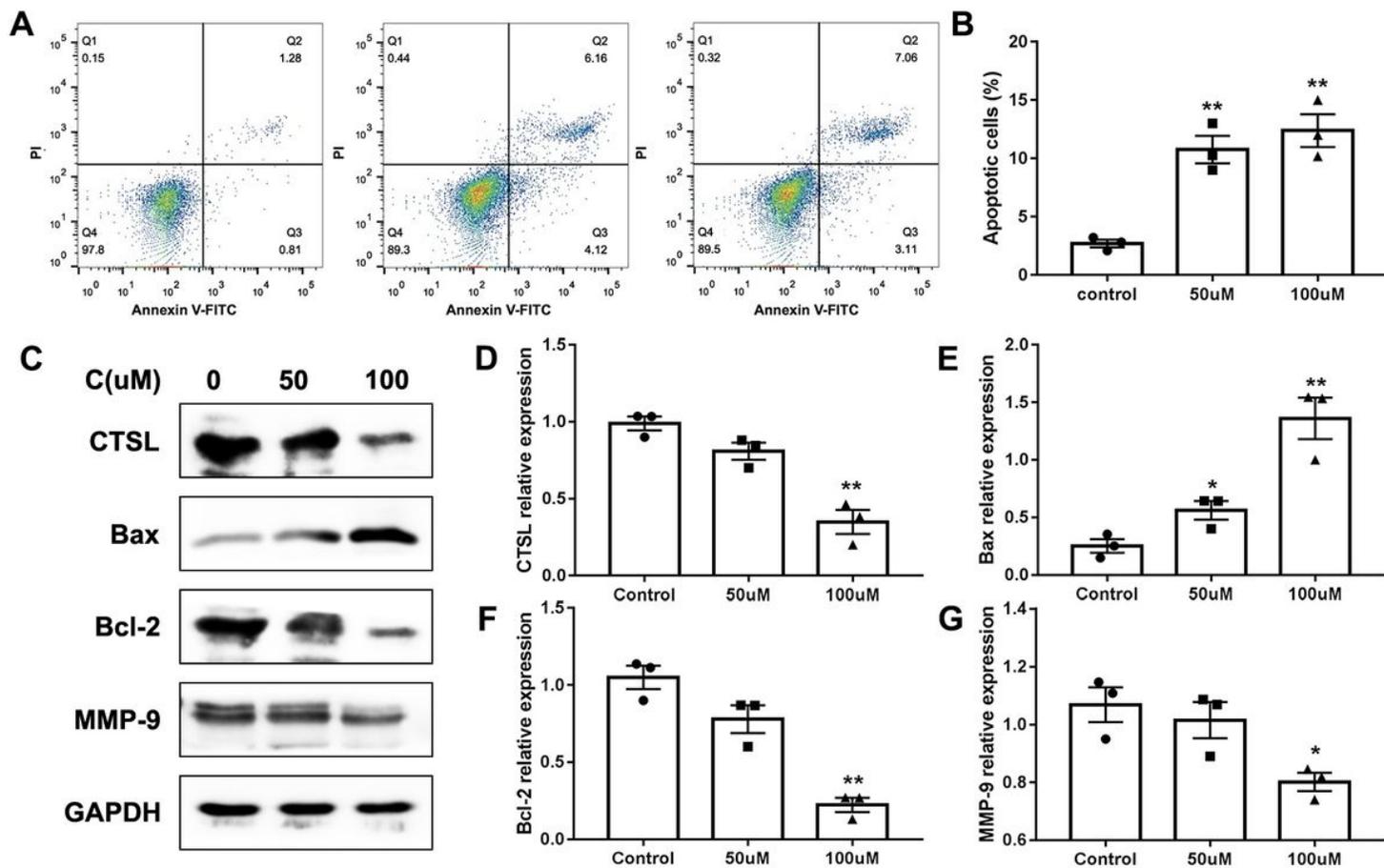


Figure 5

CTSL-1 promoted the apoptosis of U251 cells. (A) The apoptosis was measured by flow cytometry after treatment with 0, 50 and 100μM CTSL-1. (B) The percentage of cell apoptosis ratio in A. (C) Western blot analysis CTSL, MMP-9 Bax and Bcl-2 expression in U251 cells treated with 0, 50 and 100μM CTSL-1. (D-G) Analysis of relative expression levels of CTSL, MMP-9 Bax and Bcl-2 in F *p < 0.05 vs. Control group, **p < 0.01 vs. Control group.