Overexpression of C1QTNF6 Predicts a Poor Prognosis in Bladder Cancer

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Research

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Abstract

Background: Bladder cancer is one of the most common urinary cancer. This study aimed to provide promising molecular biomarkers for bladder cancer by investigating the correlations between C1QTNF6 expressions and clinical characteristics as well as prognosis in patients with bladder cancer.

Methods: C1QTNF6 RNA sequencing profiles of bladder cancer patients were collected to evaluate different expressions between normal bladder mucosa and bladder cancer from TCGA database and GEO database. The associations between C1QTNF6 expression and clinical characteristics as well as prognosis were evaluated by two independent cohorts. The cell expression of C1QTNF6 expression between normal bladder cell and bladder cancer cells were tested in western blot and PCR, and the underlying molecular mechanism was investigated.

Results: RNA levels of C1QTNF6 were found to be differentially expressed in two independent public cohorts including TCGA database and GSE13507 from GEO database. The protein and RNA expressions C1QTNF6 in bladder cell lines were both significantly elevated than normal bladder cell line. High C1QTNF6 expressions were found in advanced T status, M status, pathological grade, AJCC stage compared with low C1QTNF6 expression group. The underlying mechanism may be explained by cell migration and invasion assays that bladder cancer cells 5637 and T24 were significantly reduced migration and invasion ability with the knock-down C1QTNF6 expressions. The low RNA expression group of C1QTNF6 demonstrated OS advantage over high-expression group in both TCGA and GSE13507 cohorts. Besides, protein expressions in tissues were further validated in HPA database and TMA. Survival analysis also indicated that the high expression of C1QTNF6 indicated unfavorable OS compared with low expressions group.

Conclusions: High expression of C1QTNF6 predicted poor prognosis for bladder cancer patients, and the underlying mechanism is associated with cancer cell migration and invasion ability.

Introduction

Bladder cancer is calculated as the fourth most common male cancer, with 61,700 estimated new cases and 12,870 estimated deaths of male patients in the United States in 2019[1]. Compared with men, women have relatively fewer morbidity and mortality, with 18770 estimated new cases and 4800 estimated deaths[1]. Clinical and histopathological characteristics including tumor stage, lymph node metastasis, and molecular markers such as epidermal growth factor receptor 3 play significant roles in predicting prognosis of bladder cancer. Therefore, deep exploration of molecular mechanism on carcinogenesis as well as biomarkers of bladder cancer may provide new strategies for the diagnosis, therapies and prognosis of bladder cancer[2].

C1QTNF6, as a member of the superfamily of C1q and tumor necrosis factor (C1QTNF), was found to be involved in the carcinoma of digestive cancers. Among 30 liver cancer samples, 21 were positive for C1QTNF6, while normal liver tissues were negative, moreover the recombinant protein C1QTNF6 could

effectively increase the expression of Akt and promote liver cancer angiogenesis[3]. Further study indicated C1QTNF6 suppression reduced liver cancer cell invasion and migration through inactivating the AKT signaling pathway[4]. C1QTNF6 was also overexpressed in gastric cancer, and the proliferation and metastatic ability of gastric cancer cells after C1QTNF6 knockout was significantly decreased[5]. The increased C1QTNF6 secretion may contribute to mitochondrial DNA loss, which closely involved in tumorigenesis[6].

Considering the scarcity of investigation of C1QTNF6 in bladder cancer, in this study, we aimed to investigate the different expressions of C1QTNF6 in bladder cancer and normal tissues, and explore the underlying correlations between C1QTNF6 expressions and clinical characteristics as well as prognosis, which may shed light in understanding the biological functions of C1QTNF6 in bladder cancer.

Materials And Methods

Data collection

We obtained the RNA sequencing profiles and clinical data of bladder cancer from The Cancer Genome Atlas (TCGA) data portal (https://portal.gdc.cancer.gov/) and the cBio Cancer Genomics Portal (http://cbioportal.org) in August 2019. Another independent dataset GSE13507 from GEO database was also retrieved to validate the differential expressions of C1QTNF6 in bladder cancer and normal tissues, as well as characteristics and prognosis.

Cell culture

The human bladder cancer cell lines (T24, 5637, UMUC3, RT4, and BIU87) and normal urothelial bladder cell line (SV-HUCL) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). T24, BIU87 and 5637 cells were grown in RPMI 1640 medium (Corning Incorporated, Corning, NY, USA) containing 10% fetal bovine serum (FBS, Gibco, Thermo Scientific, Waltham, MA, USA). UM-UC-3 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Corning Incorporated, Corning, NY, USA) containing 10% FBS. RT4 cells were grown in McCoy’s 5A (modified) medium (Boster Biological Technology, Wuhan, China) containing 10% FBS, SV-HUCL cells were grown in F-12K medium (Thermo Scientific, Waltham, MA, USA) containing 10% FBS. All cells were maintained in medium supplemented with 1% penicillin/streptomycin and were then cultured at 37°C with 5% CO2.

The small interfering RNAs (siRNAs) against C1QTNF6 (siC1QTNF6; siRNA1, GCAACGACUUCGACACCUATT UAGGUGUCGAAGUCGUUGCTT; siRNA2, CCUGAUGUGUGAGAUCCCUTT AGGGAUCUCACACAUCAGGTT) were synthesized by Genepharma (Shanghai, China). Lipofectamine 3000 (Invitrogen, Thermo Scientific, Waltham, MA, USA) was utilized for cell transfection under the manufacturer's instructions.

Western blot

Cells were lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China) containing 1% protease inhibitor (Sigma Aldrich, Saint Louis, MO, USA). The protein concentrations were determined using a BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, China). Total proteins (30-40ug) were separated by 10% SDS-PAGE gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Darmstadt, Germany), followed by blocking with 5% non-fat milk in Tris-buffer saline with 0.1% Tween 20 (TBST) for 1–2 h at room temperature. Then, membranes were incubated overnight at 4 °Cwith primary antibody against C1QTNF6 (ab36900, 1:1000 dilution, Abcam, USA); GAPDH (AB0037, 1:5000 dilution, Abways, China). After washing with TBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (#7074, Cell Signaling Technology, USA). The protein signals were visualized by enhanced chemiluminescence (ECL) chromogenic substrate (Bio-Rad Laboratories, USA). GAPDH was used as an internal control.

Quantitative reverse transcription PCR

Total RNA was extracted using a Trizol reagent (Takara Biotechnology Co., Ltd., China), and cDNA reverse transcription, and qRT-PCR was conducted under the guidance of Reverse Transcription PrimeScript 1st Stand cDNA Synthesis kit (Takara Biotechnology Co., Ltd., China) as well as quantitative PCR reagents SYBR PremixExTaqTM (Takara Biotechnology Co., Ltd., China). β-action was applied as the corresponding internal control. All mRNA levels were evaluated by the 2-ΔΔCT method. Each reaction was performed in triplicate.

In vitro migration and invasion assays

For the migration and invasion analysis, cells were seeded in serum-free medium in the upper chambers of Transwells (Corning Incorporated, Corning, NY, USA). For the migration assay, cells were harvested at 48 h post-transfection and then resuspended in serum-free RPMI 1640; cells (5 × 104/100 μL) were loaded into the upper chamber. The lower chambers contained 600 μL of medium with 10% FBS. The incubation time was 12 h for the T24 cell line and 24 h for the 5637 cell line. After washing with PBS, the cells on the upper surface of the chamber were removed with a cotton swab. The cells on the lower surface of the membrane were fixed with 4%paraformaldehyde for 15 min and stained with a 0.1% crystal violet solution for 15 min, five Transwells fields were photographed with an inverted optical microscope. For the invasion assay, it was performed almost identically to the migration assay except that a Transwells chamber with Matrigel was used instead.

Immunohistochemistry

Rabbit polyclonal antibody against C1QTNF6 was obtained from Sigma (College Park, MD, USA). Tissue microarray (TMA) consisting of 54 bladder cancer cases was obtained from OUTDO BIOTECH (Shanghai, China). After deparaffination, rehydration, and antigen retrieval, the TMA slide was incubated with primary rabbit polyclonal anti-human CTRP6 (dilution 1:200; Sigma Antibody; SAB487P) overnight at 4℃. The slide was then incubated with anti-rabbit secondary antibody (ready-to-use solution; Cell Signaling Technology; # 8114), followed by chromogen diaminobenzidine staining. We quantitatively scored the TMA slide using a microscope based on the staining intensity and proportion. Staining intensity of each specimen was scored as follows (negative = 0, weakly positive = 1, moderate positive = 2, and strong positive = 3), and staining percentage as follows (0–10% = 1, 11%–50% = 2, 50–75% = 3, and 75–100% = 4). According to the immunohistochemical scores of C1QTNF6 protein expression, specimens were divided into two groups of high expression group with score great than or equal to 4 and low expression group with score less than 4.

Human Protein Atlas

The Human Pathology Atlas (HPA) project (https://www.proteinatlas.org) included immunohistochemistry data using a tissue microarray-based analysis of 44 different normal tissue types, and proteome analysis of 17 major cancer types[7, 8]. Immunostaining intensity and patient information with the corresponding cancer types were available online. In this study, representative images of protein expression using immunohistochemistry for C1QTNF6 were captured and compared in bladder cancer tissues and normal bladder tissues in the HPA.

Gene set enrichment analysis

Gene set enrichment analysis (GSEA) was applied to evaluate the correlations between C1QTNF6 expression and relevant pathways, using the bladder cancer dataset from TCGA. The detailed protocol for GSEA is available on the Broad Institute Gene Set Enrichment Analysis website (http://www.broad.mit.edu/gsea). Datasets and phenotype label files were created and loaded onto GSEA software (v 4.0.1; Broad Institute, Cambridge, USA). The samples were separated into a high and a low group based on median C1QTNF6 level. The analysis was randomly repeated 1,000 times. A meaningful gene set was defined as an adjusted p-value < 0.05 and a false discovery rate (FDR) < 0.25. Statistical analysis and graphical plotting were conducted using R software (Version 3.3.2).

Statistical analysis

The statistical differences between groups were analyzed by Student’s t-test or one-way analysis of variance (ANOVA) using GraphPad Prism (GraphPad Software, CA, USA). Survival curves were determined by Kaplan-Meier method. P value < 0.05 was considered significant.

Results

RNA Expressions of C1QTNF6 in bladder cancer and normal tissues

To compare C1QTNF6 mRNA expression levels between bladder cancer and normal tissues, we initially analyzed the RNA-Seq data on C1QTNF6 in the TCGA BLCA cohort. A total of 19 normal bladder tissues and 411 bladder cancer were included to compare the RNA expressions of C1QTNF6, as shown in Fig. 1A, the expressions of C1QTNF6 was significantly elevated in bladder cancer than in bladder normal tissues. A paired comparison on 19 bladder cancer and corresponding normal tissues also demonstrated the higher expressions of C1QTNF6 in bladder cancer with statistical significance (Fig. 1B). Moreover, to further evaluate the differential expressions of C1QTNF6 in other cohorts, another independent cohort was applied. Firstly, 9 normal bladder tissues and 188 bladder cancer tissues were compared, with elevated C1QTNF6 expressions in bladder cancer as shown in Fig. 1C. Secondly, 58 adjacent normal bladder tissue from bladder cancer patients were also compared the 188 bladder cancer tissues, suggesting the higher expressions of C1QTNF6 in bladder cancer(Fig. 1D, $P < 0.001$). Additionally, comparison of C1QTNF6 between 52 bladder cancer tissues and paired adjacent normal tissues was also conducted and showed the elevated C1QTNF6 expression in bladder cancer(Figure 1E, $P = 0.002$).

Expressions of C1QTNF6 in cells

Although RNA expressions of C1QTNF6 in tissues were explored in 2 different cohorts, it is still elusive of the expression of C1QTNF6 in cell levels. The proteins coded by mRNA C1QTNF6 was examined between normal bladder cell line SV-HUCL and bladder cancer cell lines including UMUC3, RT4, 5637, T24 and BIU87(Fig. 2A). A further exploration of RNA expression was conducted on RNA expressions, and compared with normal bladder cell, the relative expressions of UMUC3, T24, BIU 87 and 5637 were all elevated with statistical significance (Fig. 2B).

Correlations between C1QTNF6 RNA expressions and clinical characteristics

To explore the underlying correlations between C1QTNF6 RNA expressions and clinical characteristics, we retrieved the clinical information of 413 bladder cancer patients. By combining the T status, M status, N status, presence of papillary growth type, histological grade and stage were used to evaluate the potential association between C1QTNF6 expressions and clinical characteristics. T status, M status, N status, presence of papillary growth type, histological grade and stage were used to evaluate the potential association between C1QTNF6 expressions and clinical characteristics. The higher C1QTNF6 expressions were found in advanced T status (Fig. 3A), Non-papillary growth type (Fig. 3D), pathological grade (Fig. 3E) and AJCC stage (Fig. 3F), however not associated with positive lymph nodes (Fig. 3B), and M status (Fig. 3C). A validation exploration in GSE also indicated the tight association between higher C1QTNF6 expressions and advanced T status (Fig. 4A), M status (Fig. 4C), pathological grade (Fig. 4D)

and AJCC stage (Fig. 4E). No positive associations were found in lymph node and C1QTNF6 expressions (Fig. 4B).

Correlations between C1QTNF6 RNA expressions and cell migrations as well as invasion

Considering the tight associations between RNA expressions and T status and M status, the underlying correlations between C1QTNF6 RNA expressions were evaluated in 5627 and T24 cells. Transwell cell migration and invasion assays were performed. In the migration assays, the number of migrating 5637siRNA1 and T24siRNA1 were significantly decreased compared with the control groups of 5637 and T24 NC groups (Fig. 5A and 5B). Moreover, the invasion assays also indicated the significantly reduced invading cells in 5637siRNA1 and T24siRNA1 groups, compared with 5637 and T24 NC groups (Fig. 5C and 5D).

Gene set enrichment analysis (GSEA)

In this study, the RNAseq data of bladder cancer patients with different levels of C1QTNF6 expression were compared, and the results showed that the genes that varied significantly in patients with high C1QTNF6 expression were enriched in the bladder cancer, cytokine-cytokine receptor interaction, ECM receptor interaction, ERBB signaling pathway and melanoma (Fig. 6). While the low C1QTNF6 expression was enriched in mTOR signaling pathway, non-small cell lung cancer, Notch signaling pathway, TGF-β signaling pathway and ubiquitin mediated proteolysis (Fig. 6).

High expression predicts poor OS and PFS

A total of 395 bladder cancer patients with detailed follow-up information were included for survival analysis, and were divided into low expression (n = 198) and high expression90two groups based on the median expression of C1QTNF6. A total of 395 bladder cancer patients with detailed follow-up information were included for survival analysis, and were divided into low expression (n = 198) and high expression groups based on the median expression of C1QTNF6. The overall survival (OS) of low expression group of C1QTNF6 was more favorable than high expression group with statistical significance (Fig. 7A). Due to dropouts or missing data for progression-free survival (PFS), only 310 patients with detailed follow-up information were included to compare PFS differences between low and high expression groups. Although the low expression group of C1QTNF6 demonstrated PFS advantage over high-expression group, however without statistical significance (Fig. 7B, P = 0.183). In the validation cohort from GSE, the median expression of C1QTNF6 was set as the cutoff for both OS and PFS, the low expression group of C1QTNF6 showed significant OS and PFS advantages over the high expression group (Fig. 7C for OS, P = 0.002; Fig. 7D for PFS, P = 0.005).

Protein expression of C1QTNF6 and characteristics

The information of protein expressions of C1QTNF6 were retrieved from the HPA database, as demonstrated in Fig. 8A and 8B, the normal bladder mucosa was negative while the bladder cancer was positive. To further investigate the protein expressions of C1QTNF6, we conducted the TMA IHC and retrieved the 54 bladder cases with detailed follow-up information. Based on the IHC scores, the total group was divided into Low-C1QTNF6 and High-C1QTNF6 expression groups. The clinical characteristics were summarized in the supplementary Table 1. The typical IHC results were demonstrated in Fig. 9, divided by the multiplying of staining and areas. The survival analysis also indicated that the high expression of C1QTNF6 indicated unfavorable OS compared with low expressions of C1QTNF6 group (Fig. 8C).

Discussions

Approximately 70% of the newly diagnosed bladder cancer cases were non-muscle invasive type, and transurethral resection of bladder tumor was usually adopted with intravesical chemotherapy or Bacillus Calmette–Guerin immunotherapy. With respect to muscle-invasive bladder cancer, radical cystectomy remains the most effective treatment, however short- and long- term complications following radical cystectomy are tightly related to patient prognosis[9]. The modified Charlson Comorbidity Index is widely used to predict the perioperative mortality and long-term overall survival of patients with muscle-invasive bladder cancer undergoing radical cystectomy[10]. Although the surgical methods of bladder cancer have been improved in recent years, combined with chemotherapy, immunotherapy and other methods, the treatment effect of bladder cancer has been improved to a certain extent, but postoperative recurrence and metastasis are still common with no significant improvement of 5-year survival rate[11].

The superfamily of C1QTNF, adiponectin, leptin and cerebellar peptide are collectively referred to as the adipose factor superfamily[12]. The C1QTNF family has a highly conserved c-terminal complementary C1q domain consisting of 16 CTRP members, including C1QTNF 1–9,9b, 10–15[13]. All C1QTNF members are secreted proteins that are widely expressed in a variety of tissues and cell types and are involved in a variety of biological processes, including host defense, inflammation, apoptosis, autoimmunity, cell differentiation, organogenesis, hibernation, and insulin-resistant obesity[14–19]. C1QTNF6, as a member of C1QTNF family, was demonstrated to be associated with carcinogenesis in digestive cancers, however, the biological functions in bladder cancer still required investigating.

This study firstly explored the differential expressed RNA levels of C1QTNF6 in two independent public cohorts including TCGA database and GSE13507 from GEO database. Compared with normal bladder mucosa and paired adjacent bladder mucosa, the bladder cancer was demonstrated to be associated with C1QTNF6 overexpression. Regarding expression levels in cells, the protein and RNA expressions C1QTNF6 in bladder cell lines were both significantly elevated than normal bladder cell line. Further exploration of correlations between C1QTNF6 RNA expressions and clinical characteristics indicated high C1QTNF6 expressions were found in advanced T status, M status, pathological grade, AJCC stage

compared with low C1QTNF6 expression group. The underlying mechanism may be explained by cell migration and invasion assays that bladder cancer cells 5637 and T24 were significantly reduced migration and invasion ability with the knock-down C1QTNF6 expressions. The possible cancer-related pathways included cytokine-cytokine receptor interaction, ECM receptor interaction, ERBB signaling pathway, mTOR signaling pathway, Notch signaling pathway, TGF-β signaling pathway and ubiquitin mediated proteolysis. Moreover, this study investigated the predictive values of C1QTNF6 expression in prognosis of bladder cancer patients. The low RNA expression group of C1QTNF6 demonstrated OS advantage over high-expression group in both TCGA and GSE13507 cohorts, however statistically significant PFS advantages was only found in GSE13507 cohort. Besides, protein expressions in tissues were further validated in HPA database and TMA. Based on the IHC scores of TMA, survival analysis also indicated that the high expression of C1QTNF6 indicated unfavorable OS compared with low expressions group.

However, there were still some limitations in this study. Firstly, the expression difference and prognosis evaluation were analyzed using retrospective data. Besides, the molelar mechanism for high expression of C1QTNF6 required to be further investigated for the related cancer pathway and in vivo studies. Finally, the limited sample number of bladder cancer patients and diffenct datasets may lead to heterogeneity of data analysis.

Conclusions

In conclusion, this study demonstrated the high expressions of C1QTNF6 in proteins and RNA from samples of bladder cancer cells and tissues, compared with normal bladder mucosa. Moreover, High expression of C1QTNF6 predicted poor prognosis for bladder cancer patients, and the underlying mechanism may be explained by increased cancer cell invasion and migration ability associated with high C1QTNF6 expressions.

Abbreviations

C1QTNF: C1q and tumor necrosis factor

TCGA: The Cancer Genome Atlas

FBS: Fetal bovine serum (

DMEM: Dulbecco's modified Eagle's medium

siRNAs: Small interfering RNAs ()

TBST: Tris-buffer saline with 0.1 Tween 20 ()

HRP: Horseradish peroxidase

ECL: Enhanced chemiluminescence

TMA: Tissue microarray

HPA: Human Pathology Atlas

GSEA: Gene set enrichment analysis

FDR: false discovery rate

ANOVA: Analysis of variance

OS: Overall survival

PFS: Progression-free survival

Declarations

Authors' contributions

Xin Gou and Weiyang He designed this study; Xin Zhu, Hang Tong, Shun Gao, Hubin Yin, Gongmin Zhu and Xinyuan Li conducted experiments and collected the data; Xin Zhu performed the statistical analysis; Xin Zhu wrote the paper. All authors read and approved the final version of the paper.

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Not applicable.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The authors declare that the data supporting the findings of this study is available within the article.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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References

1. Siegel RLA-Ohoo, Miller KDA-Ohoo, Jemal AA-Ohoo: **Cancer statistics, 2019.**
2. Antoni S, Ferlay J, Soerjomataram I, Znaor A, Jemal A, Bray F: **Bladder Cancer Incidence and Mortality: A Global Overview and Recent Trends.** *Eur Urol* 2017, **71:**96-108.
3. Takeuchi T, Adachi Y, Nagayama T: **Expression of a secretory protein C1qTNF6, a C1qTNF family member, in hepatocellular carcinoma.** *Anal Cell Pathol (Amst)* 2011, **34:**113-121.
4. Wan X, Zheng C, Dong L: **Inhibition of CTRP6 prevented survival and migration in hepatocellular carcinoma through inactivating the AKT signaling pathway.** *J Cell Biochem* 2019.
5. Qu HX, Cui L, Meng XY, Wang ZJ, Cui YX, Yu YP, Wang D, Jiang XJ: **C1QTNF6 is overexpressed in gastric carcinoma and contributes to the proliferation and migration of gastric carcinoma cells.** *Int J Mol Med* 2019, **43:**621-629.
6. Kim MJ, Lee W, Park EJ, Park SY: **Depletion of mitochondrial DNA stabilizes C1qTNF-related protein 6 mRNA in muscle cells.** *J Korean Med Sci* 2012, **27:**465-470.
7. Thul PJ, Lindskog C: **The human protein atlas: A spatial map of the human proteome.** *Protein Sci* 2018, **27:**233-244.
8. Pontén F, Schwenk JM, Asplund A, Edqvist PH: **The Human Protein Atlas as a proteomic resource for biomarker discovery.** *J Intern Med* 2011, **270:**428-446.
9. Alfred Witjes J, Lebret T, Compérat EM, Cowan NC, De Santis M, Bruins HM, Hernández V, Espinós EL, Dunn J, Rouanne M, et al: **Updated 2016 EAU Guidelines on Muscle-invasive and Metastatic Bladder Cancer.** *Eur Urol* 2017, **71:**462-475.
10. Mayr R, May M, Martini T, Lodde M, Comploj E, Pycha A, Strobel J, Denzinger S, Otto W, Wieland W, et al: **Comorbidity and performance indices as predictors of cancer-independent mortality but not of cancer-specific mortality after radical cystectomy for urothelial carcinoma of the bladder.** *Eur Urol* 2012, **62:**662-670.
11. Abdollah F, Gandaglia G, Thuret R, Schmitges J, Tian Z, Jeldres C, Passoni NM, Briganti A, Shariat SF, Perrotte P, et al: **Incidence, survival and mortality rates of stage-specific bladder cancer in United States: a trend analysis.** *Cancer Epidemiol* 2013, **37:**219-225.
12. Wong GW, Wang J, Hug C, Tsao TS, Lodish HF: **A family of Acrp30/adiponectin structural and functional paralogs.** *Proc Natl Acad Sci U S A* 2004, **101:**10302-10307.

13. Schaffler A, Scholmerich J, Salzberger B: **Adipose tissue as an immunological organ: Toll-like receptors, C1q/TNFs and CTRPs.** *Trends Immunol* 2007, **28:**393-399.
14. Cirillo F, Catellani C, Sartori C, Lazzeroni P, Amarri S, Street ME: **Obesity, Insulin Resistance, and Colorectal Cancer: Could miRNA Dysregulation Play A Role?** *Int J Mol Sci* 2019, **20**.
15. Thanasupawat T, Glogowska A, Burg M, Krcek J, Beiko J, Pitz M, Zhang GJ, Hombach-Klonisch S, Klonisch T: **C1q/TNF-related peptide 8 (CTRP8) promotes temozolomide resistance in human glioblastoma.** *Mol Oncol* 2018, **12:**1464-1479.
16. Xin Y, Zhang D, Fu Y, Wang C, Li Q, Tian C, Zhang S, Lyu X: **C1qTNF-related protein 1 improve insulin resistance by reducing phosphorylation of serine 1101 in insulin receptor substrate 1.** *Endocr J* 2017, **64:**787-796.
17. Ma ZG, Yuan YP, Xu SC, Wei WY, Xu CR, Zhang X, Wu QQ, Liao HH, Ni J, Tang QZ: **CTRP3 attenuates cardiac dysfunction, inflammation, oxidative stress and cell death in diabetic cardiomyopathy in rats.** *Diabetologia* 2017, **60:**1126-1137.
18. Liu ZH, Li C, Chen JW, Shen Y, Gao J, Shen WF, Zhang RY, Wang XQ, Lu L: **C1q/TNF-related protein 1 promotes endothelial barrier dysfunction under disturbed flow.** *Biochem Biophys Res Commun* 2017, **490:**580-586.
19. Li Y, Wright GL, Peterson JM: **C1q/TNF-Related Protein 3 (CTRP3) Function and Regulation.** *Compr Physiol* 2017, **7:**863-878.

Figures

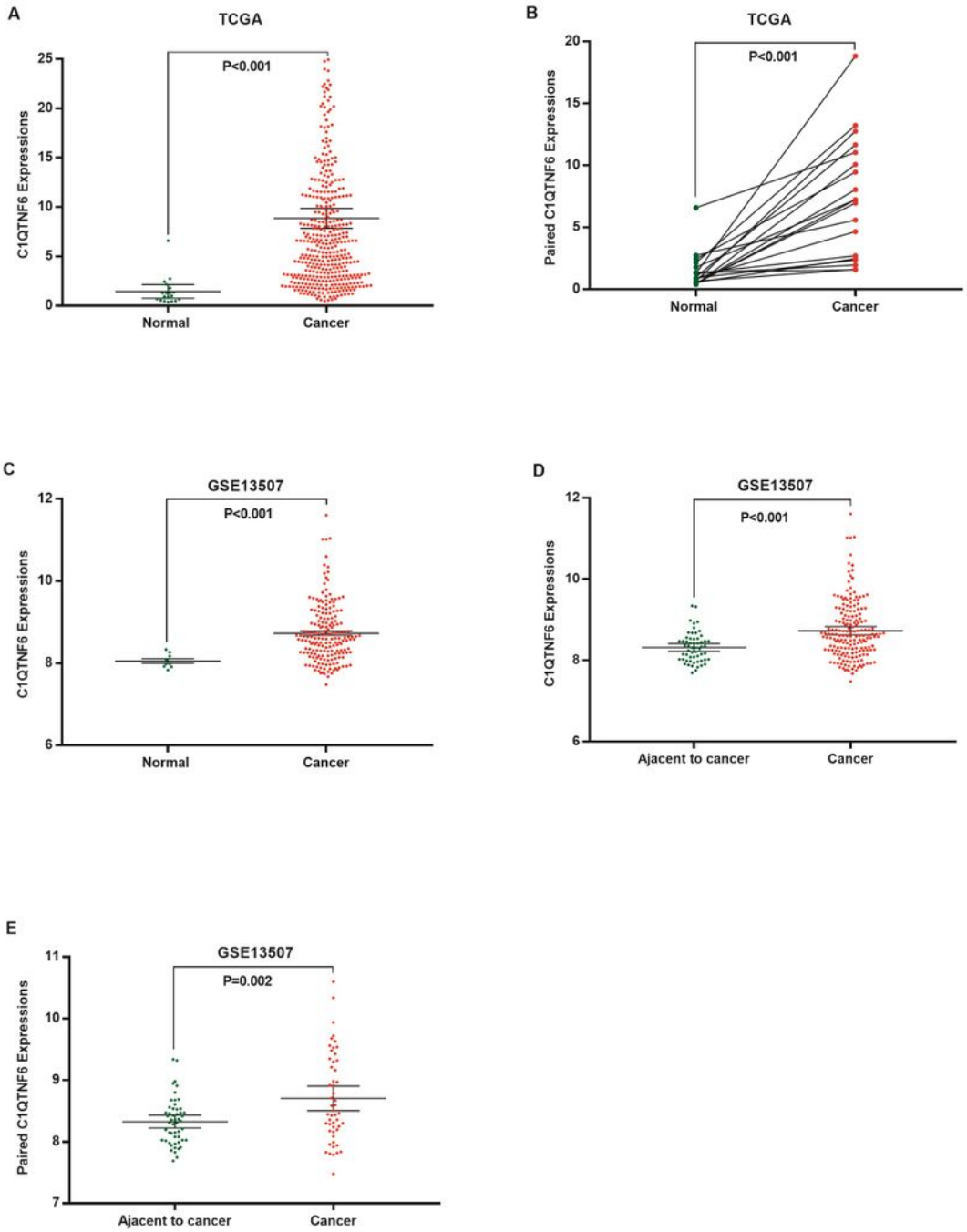

Figure 1

C1QTNF6 expressions between normal and bladder cancer tissues in TCGA and GSE13507 cohorts. (A) Scatter plot indicated higher expression of bladder cancer tissues than normal tissues in TCGA cohort (P < 0.001). (B) Paired C1QTNF6 expressions between normal and bladder cancer tissues in TCGA cohort (P<0.001). (C) Scatter plot indicated higher expression of bladder cancer tissues than normal tissues in GSE13507 cohort (P < 0.001). (D) Scatter plot indicated higher expression of bladder cancer tissues than

adjacent normal tissues in GSE13507 cohort (P < 0.001). (E) Paired C1QTNF6 expressions between adjacent normal and bladder cancer tissues in GSE13507 cohort (P=0.002).

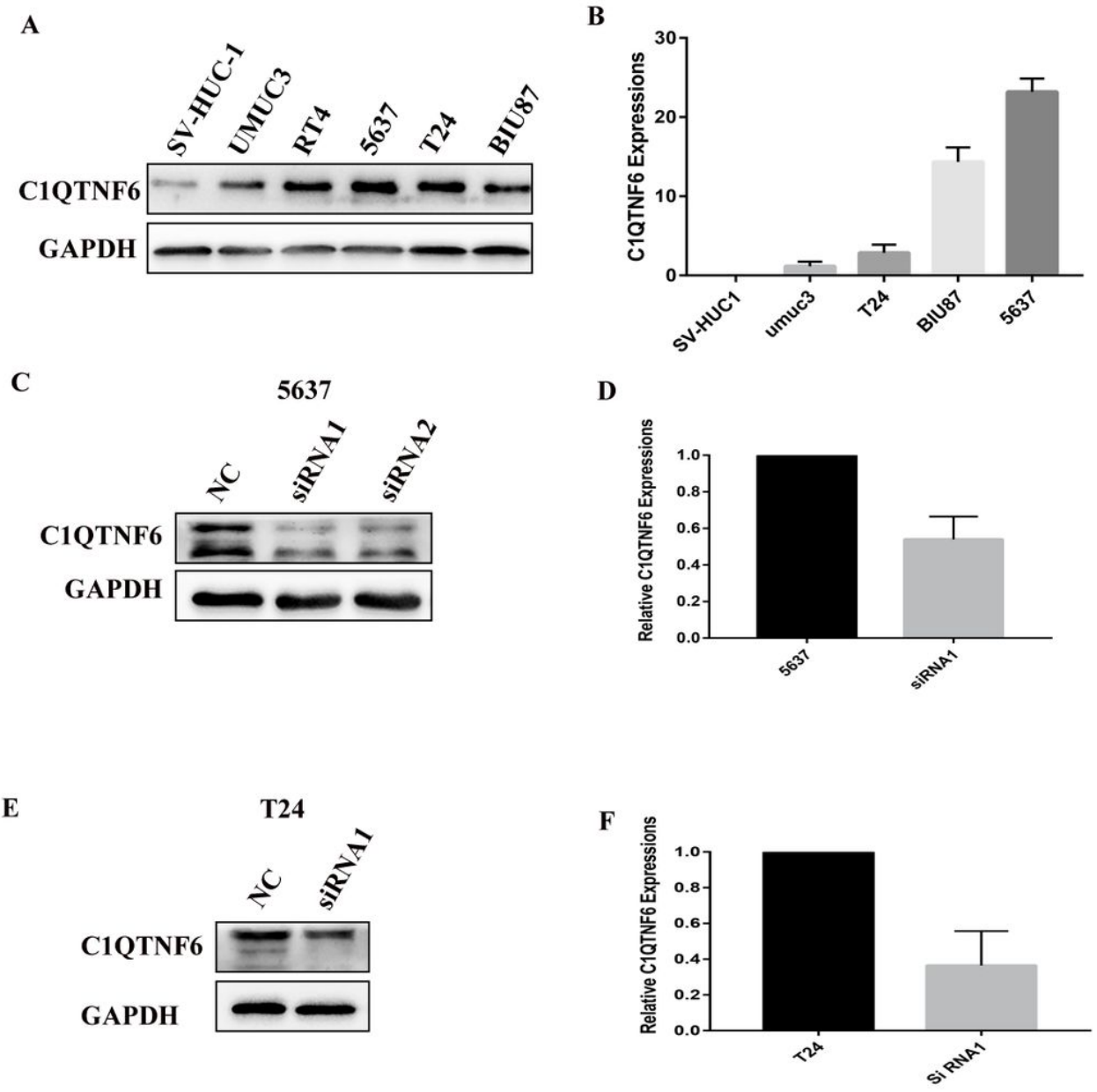

Figure 2

Protein and RNA expressions of C1QTNF6 in cell levels. (A) The protein expression of C1QTNF6 between normal bladder cell line SV-HUC1 and bladder cancer cell lines including UMUC3, RT4, 5637, T24 and BIU87. (B) RNA expression of C1QTNF6 between normal bladder cell line SV-HUC1 and bladder cancer cell lines including UMUC3, T24, BIU 87 and 5637. (C)Down-regulation effects of siRNAs against C1QTNF6 on protein expression of C1QTNF6 in bladder cancer cell line 5637. (D)Down-regulation effects of siRNAs against C1QTNF6 on RNA expression of C1QTNF6 in bladder cancer cell line 5637. (E)Down-regulation effects of siRNA1 against C1QTNF6 on protein expression of C1QTNF6 in bladder cancer cell

line T24. (D)Down-regulation effects of siRNA1 against C1QTNF6 on RNA expression of C1QTNF6 in bladder cancer cell line T24.

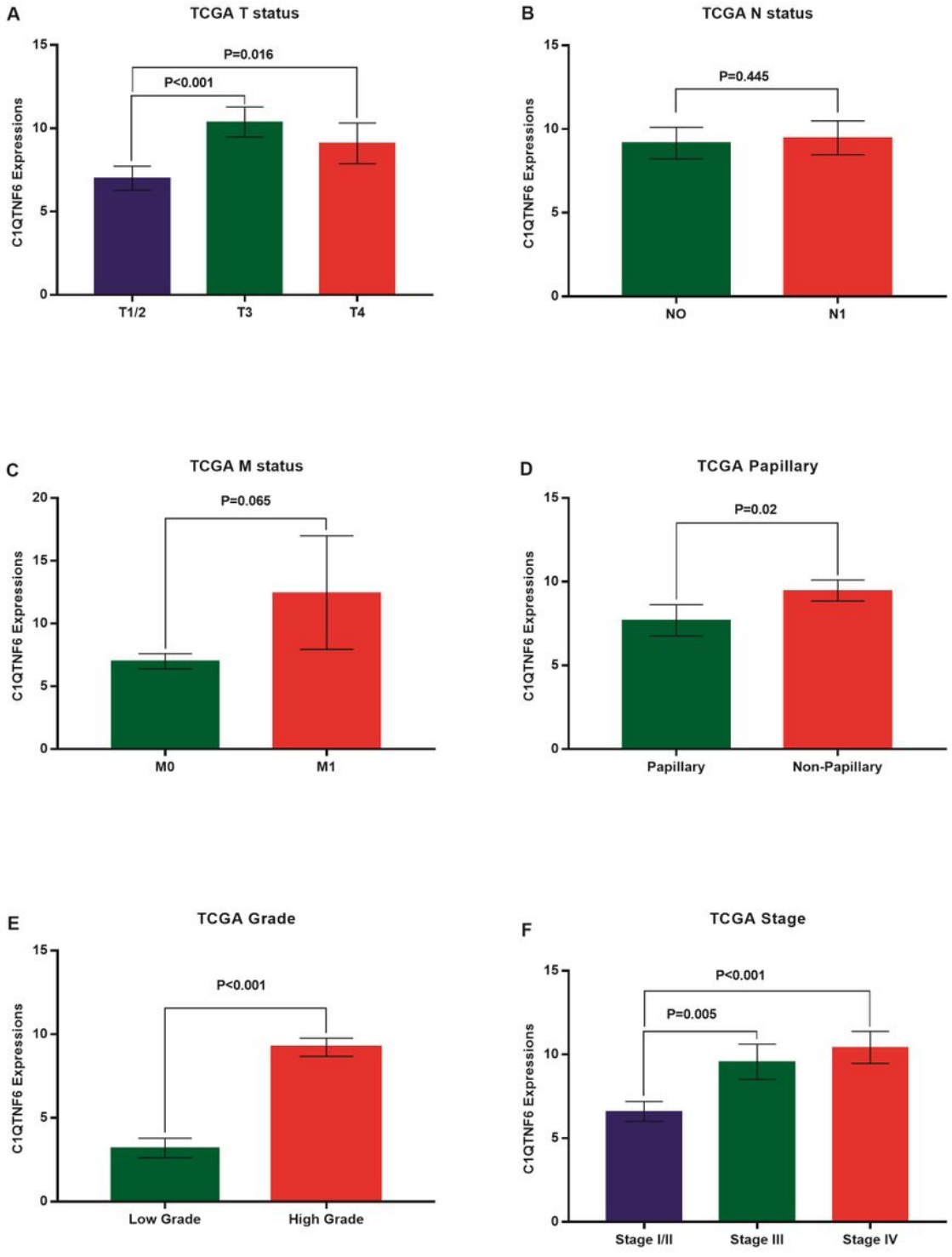

Figure 3

Associations between C1QTNF6 expressions and clinical characteristics in TCGA cohort. (A) Distribution of C1QTNF6 expressions stratified by T status, and bar graph indicated higher C1QTNF6 expression was associated with T3 (vs. T1/2, P< 0.001), and T4 (vs. T1/2, P=0.016). (B) Bar graph indicated no

significant associations between C1QTNF6 expressions and N1 (vs. N0, P = 0.445). (C) Bar graph indicated a trend for higher C1QTNF6 expression in M1, without statistic significance (vs. M0, P = 0.065). (D) Bar graph indicated higher C1QTNF6 expression associated with non-papillary morphological type (vs. Papillary type, P = 0.02). (E) Bar graph indicated higher C1QTNF6 expression in high grade, compared with low grade (P< 0.001). (F) Bar graph indicated higher C1QTNF6 expression in Stage III (vs. Stage I/II, P= 0.005) and Stage IV (vs. Stage I/II, P< 0.001).

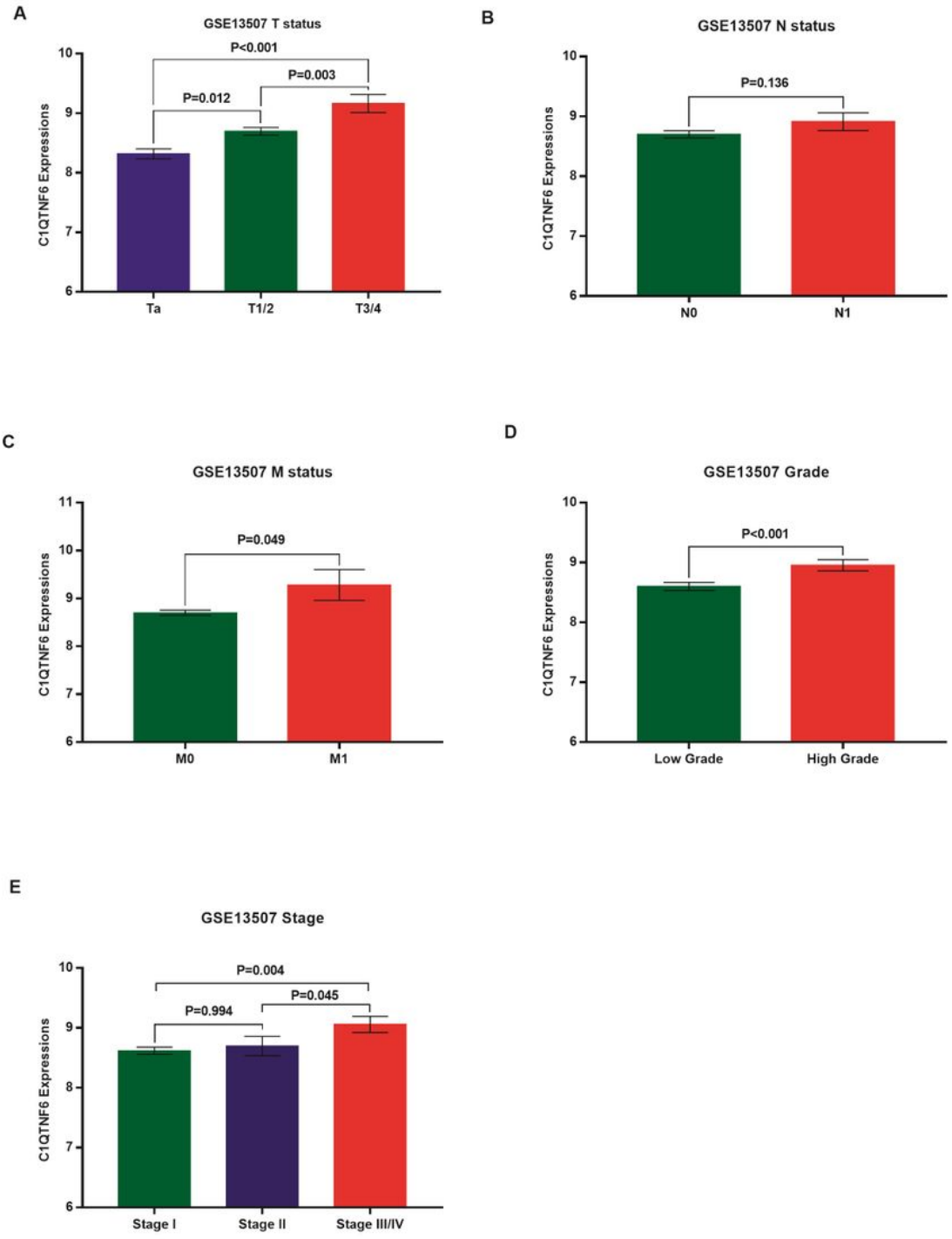

Figure 4

Associations between C1QTNF6 expressions and clinical characteristics in GSE13507 cohort. (A) Distribution of C1QTNF6 expressions stratified by T status, and bar graph indicated higher C1QTNF6 expression was associated with T1/2 (vs. Ta, P=0.012), with T3/4 (vs. Ta, P< 0.001; vs. T1/2, P=0.003). (B) Bar graph indicated no significant associations between C1QTNF6 expressions and N1 (vs. N0, P = 0.136). (C) Bar graph indicated higher C1QTNF6 expression in M1 (vs. M0, P = 0.049). (D) Bar graph indicated higher C1QTNF6 expression was associated with high grade, compared with low grade (P< 0.001). (E) Bar graph indicated higher C1QTNF6 expression in Stage III/IV (vs. Stage I, P= 0.004; vs. Stage II, P=0.045).

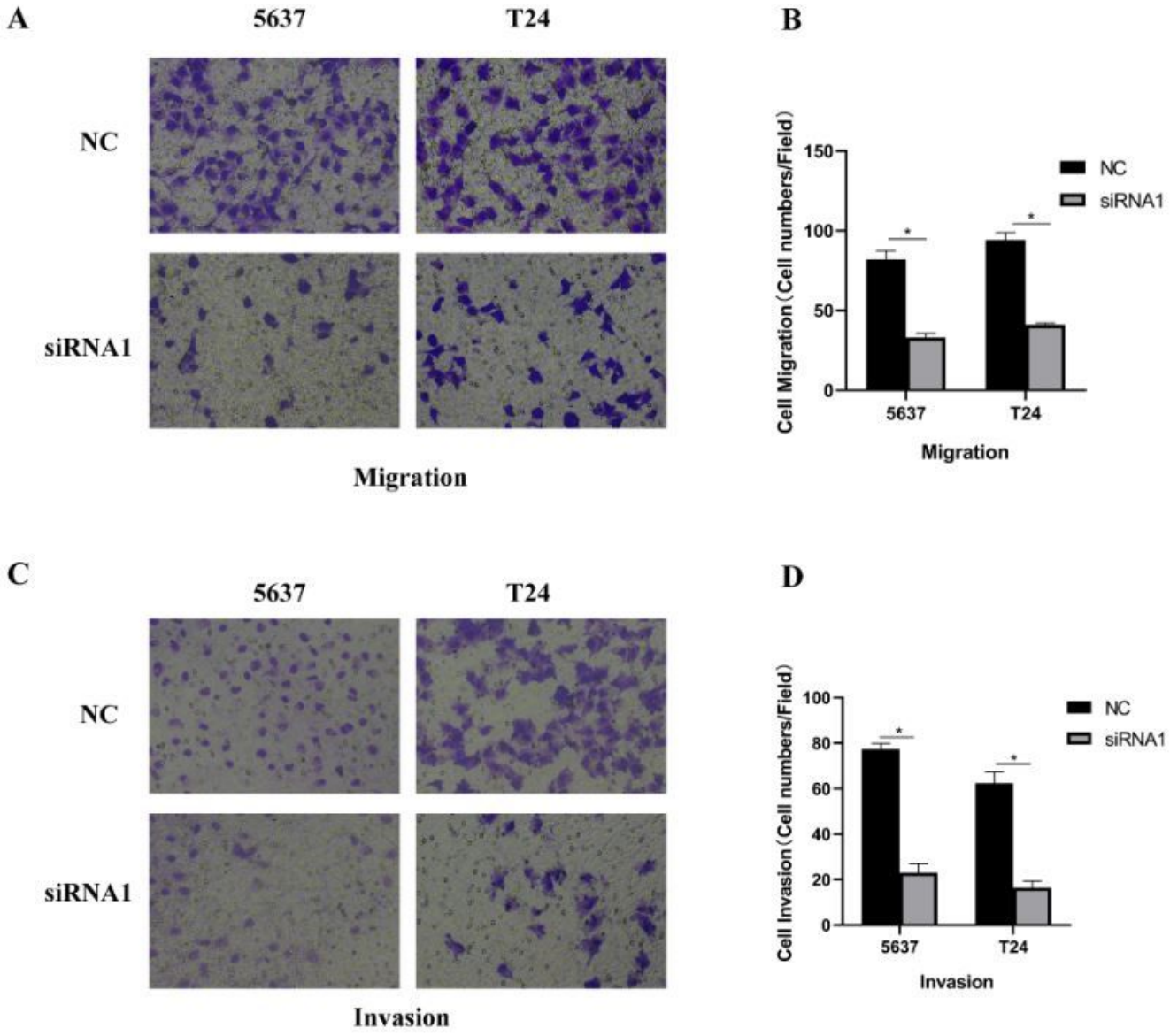

Figure 5

Inhibitions of C1QTNF6 RNA expressions decreased the invasion and migration of 5637 and T24 cells. (A) Migration assays in 5637 and T24 cells between NC and SiRNA1.*P <0.05;**P<0.01. (B)Cell migration

activity evaluated by the migrating cells between two groups in 5637 and T24 cells. (C) Invasion assays in 5637 and T24 cells between NC and SiRNA1.*P <0.05;**P<0.01. (D)Cell invasion activity evaluated by invading cells between NC and SiRNA1 groups in 5637 and T24 cells.

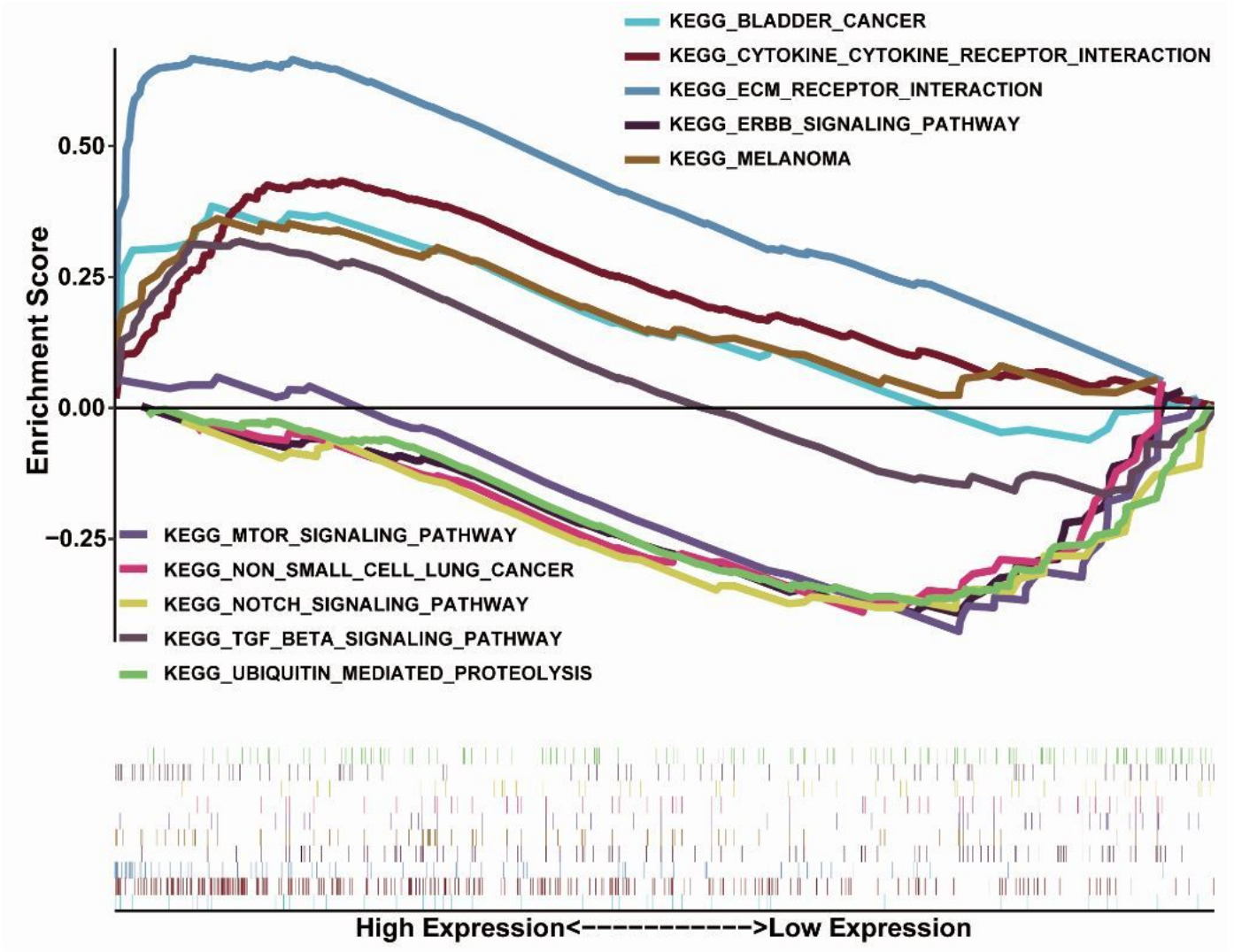

Figure 6

GSEA of C1QTNF6 RNA expression in TCGA cohort. High expression group was enriched in the bladder cancer, cytokine-cytokine receptor interaction, ECM receptor interaction, ERBB signaling pathway and melanoma; Low expression group was enriched in mTOR signaling pathway, non-small cell lung cancer, Notch signaling pathway, TGF-β signaling pathway and ubiquitin mediated proteolysis

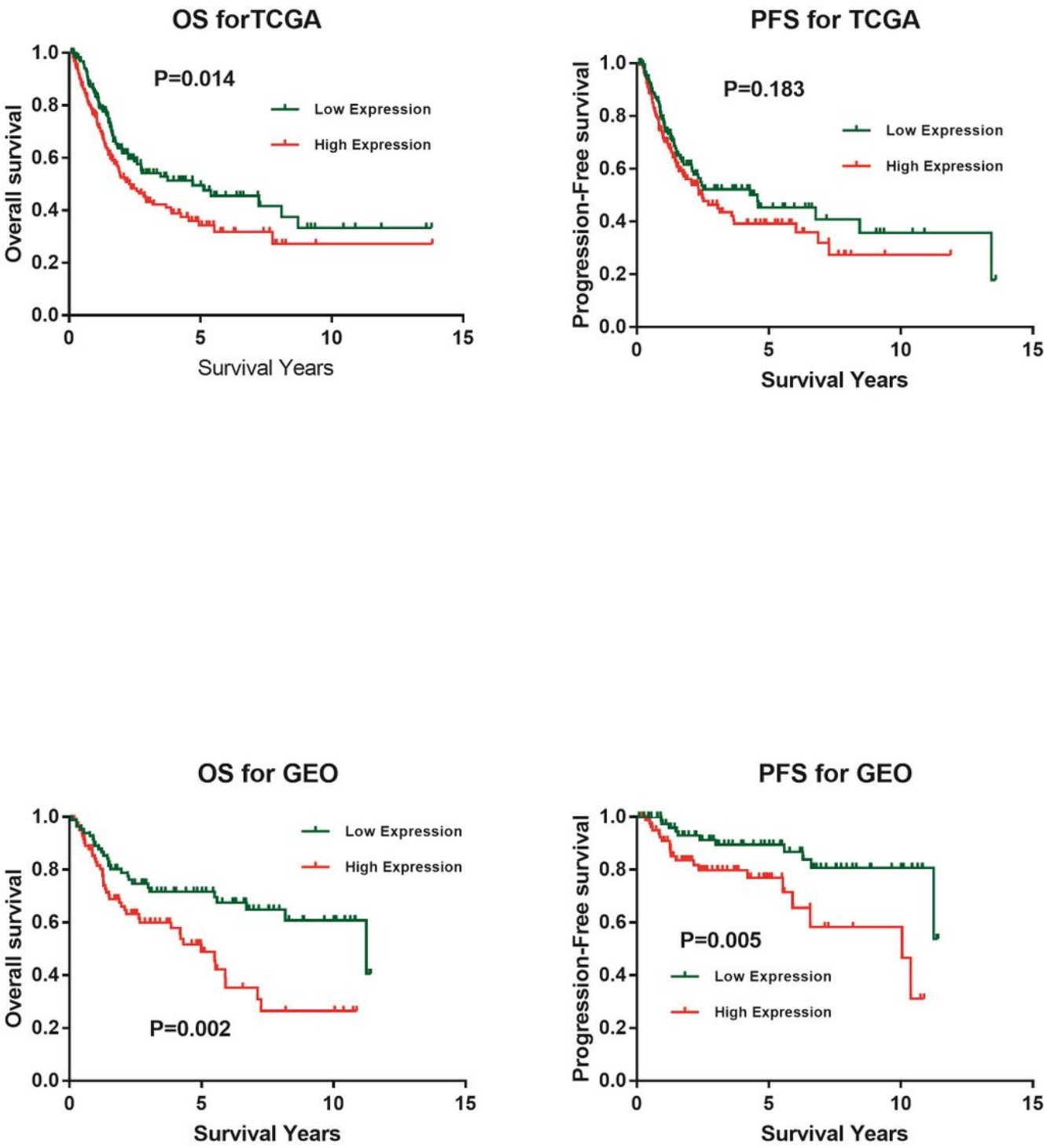

Figure 7

Kaplan-Meier survival curves for C1QTNF6 in TCGA and GSE13507 cohorts. (A) Kaplan-Meier survival curve of overall survival for C1QTNF6 in TCGA cohort (P=0.014); (B) Kaplan-Meier survival curve of progression-free survival for C1QTNF6 in TCGA cohort (P=0.183); (C) Kaplan-Meier survival curve of overall survival for C1QTNF6 in GSE13507 cohort (P=0.002); (D) Kaplan-Meier survival curve of progression-free survival for C1QTNF6 in GSE13507 cohort (P=0.005).

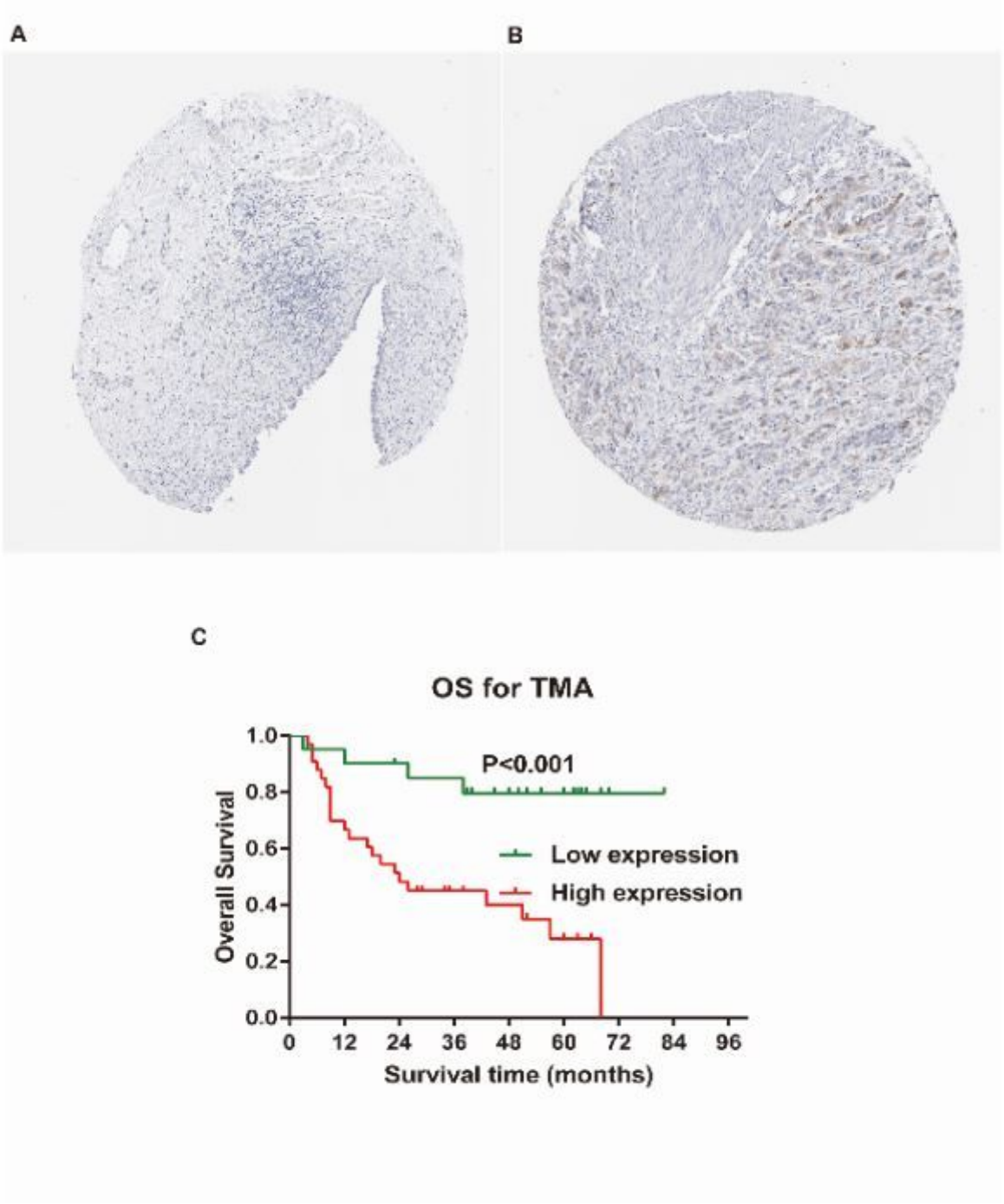

Figure 8

(A) Protein expressions of C1QTNF6 in normal bladder mucosa from the HPA database; (B) Protein expressions of C1QTNF6 in bladder cancer from the HPA database. (C) Survival analysis indicated that high expression group of C1QTNF6 was associated with unfavorable OS compared with low expression group.

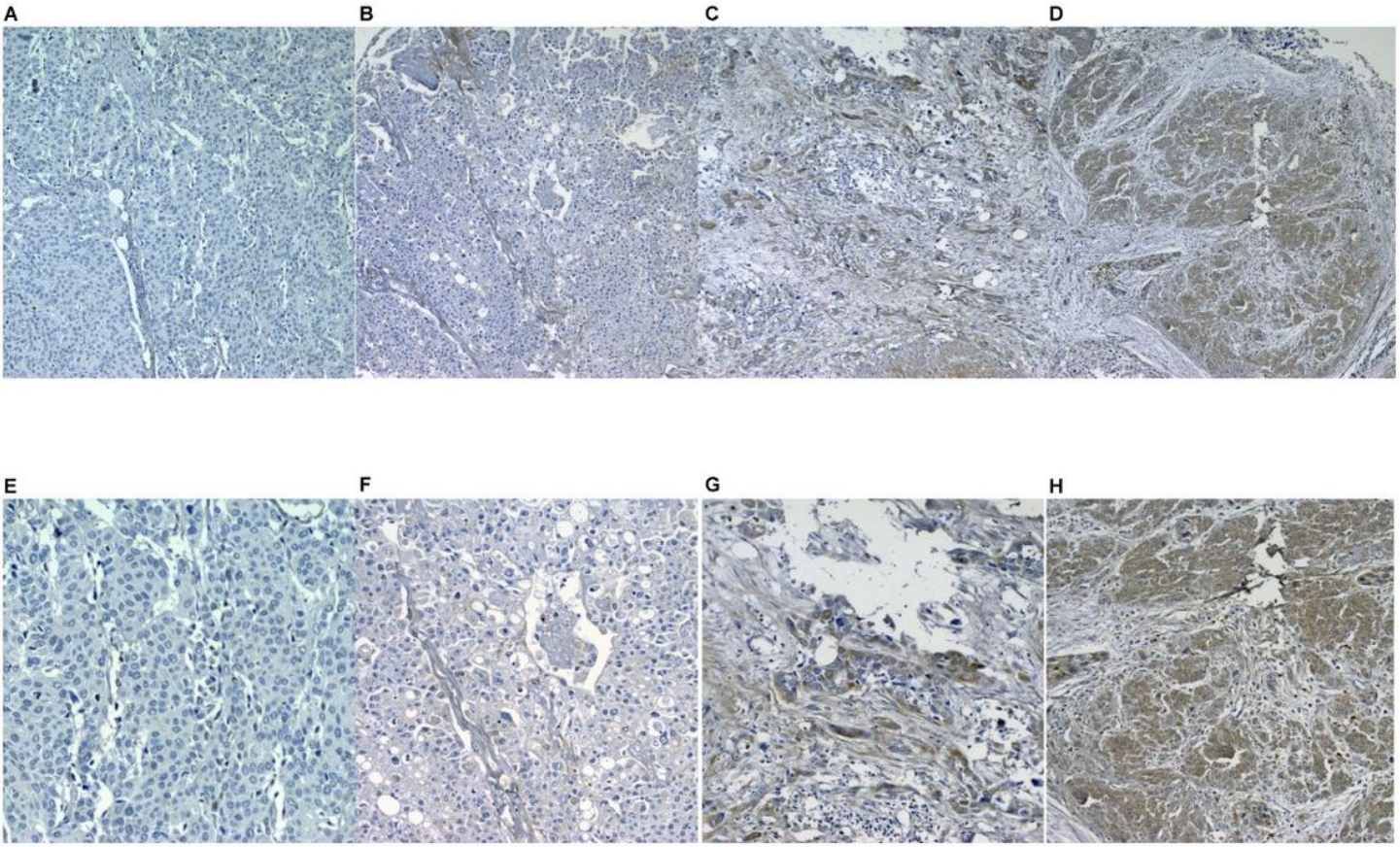

Figure 9

Representative immunohistochemical staining for C1QTNF6 expressions. (A) (E) Negative staining (score 0) in bladder cancer tissues. (B) (F) Weakly positive staining in bladder cancer tissues. (C) (G) Positive staining in bladder cancer tissues. (D) (H) Strongly positive staining (score 2) in bladder cancer tissues. Magnification, ×100 and ×200.

Supplementary Files

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