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Guanylate-binding Protein 2 Expression Is Associated With Poor Survival and Malignancy in Clear-cell Renal Cell Carcinoma

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Abstract. *Background/Aim: Clear-cell renal cell carcinoma (ccRCC) is a common recalcitrant cancer. However, little is known about biomarkers of ccRCC. In the present study, we investigated the role of guanylate-binding protein 2 (GBP2), an interferon-induced GTPase, in ccRCC and its potential as a biomarker of this disease. Materials and Methods: GBP2 expression was analyzed using the Gene Expression Omnibus, The Cancer Genome Atlas, and Human Protein Atlas databases. Univariate and multivariate Cox-regression analyses were used to investigate the prognostic value of GBP2 in ccRCC. In addition, quantitative real-time polymerase chain reactions, western blotting, and immunohistochemistry were performed to confirm the expression of GBP2 in tissues from patients with ccRCC who had undergone radical nephrectomy at the Department of Urology, the First Affiliated Hospital of Chongqing Medical University (Chongqing, P. R. China). Cell-function assays were performed to investigate the effect of GBP2 on Caki-1 human kidney-cancer cells. Results: Bioinformatics and in vitro experiments showed that the expression of GBP2 was up-regulated in ccRCC tissues and cells and was positively correlated with the malignant clinicopathological parameters of the disease. Univariate and multivariate Cox-regression analyses showed that GBP2 expression was an independent risk factor for the prognosis of ccRCC. Cell-function assays showed that GBP2 expression promoted the proliferation, migration, and invasion of Caki-1 cells through the WNT/β-catenin signaling pathway. Conclusion: The present results indicate that GBP2 expression may serve as a prognostic biomarker for ccRCC.*

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Key Words: Guanylate-binding protein 2, expression, clear-cell renal cell carcinoma, prognostic biomarker.

Renal cell carcinoma (RCC) is the seventh most commonly diagnosed malignant tumor globally. In 2021, there were approximately 76,000 new cases of RCC in the USA alone (1). ccRCC accounts for 80% of primary RCC cases and is the most lethal pathological type of kidney cancer. Approximately 90,000 people worldwide die from ccRCC every year (2). Although the occurrence and development of ccRCC has been extensively studied, the specific mechanisms underlying this disease remains poorly understood. Therefore, there is an urgent need for further study of the molecular mechanisms of the origin and development of ccRCC in order to identify novel molecules for early diagnosis, prevention, and treatment.

Guanylate-binding protein 2 (GBP2) is a GTPase induced by interferon. GBP2 is very important for the immune defense against viruses and intracellular pathogens. Recently GBP2 has been implicated in cancer-cell survival and invasiveness (3, 4). However, the role of GBP2 in ccRCC is still not well understood. In the present study, the expression of GBP2 was investigated through bioinformatics analysis, and its prognostic value and related molecular function in ccRCC were elucidated. In addition, GBP2 expression was confirmed in the tissues of patients with ccRCC. Moreover, the effect of GBP2 expression on Caki-1 cell function *in vitro* and the related signaling pathways were studied. The present study provides more evidence regarding the prognostic value of GBP2 expression in ccRCC and its effect on the function of ccRCC cells.

Materials and Methods

Data collection. Gene-expression data related to ccRCC were collected from the Gene Expression Omnibus (GEO, https: //www.ncbi.nlm.nih.gov/geo/) and The Cancer Genome Atlas (TCGA, https: //portal.gdc.cancer.gov/) (5) databases. Representative GBP2 protein expression data from immunohistochemical images of ccRCC and normal kidney tissues were also collected from the Human Protein Atlas (HPA) database (https: //www.proteinatlas.org/) (6).

Survival analysis. RNA-seq data regarding patient overall survival (OS) from the TCGA-kidney renal clear-cell carcinoma (KIRC) cohort (n=533 with follow-up information) were used to investigate the prognostic value of GBP2 expression. The International Cancer Genome Consortium (ICGC, https: //dcc.icgc.org/) cohort (7) containing 474 patients with ccRCC, RNA-seq data, and clinical data were downloaded from the ICGC portal. Univariate and multivariate Cox-regression analyses were performed to determine whether GBP2 expression was an independent variable for OS prognosis in ccRCC. Patient characteristics and pathological stages were added to the multivariate model as covariates. A value of $p \leq 0.05$ was considered to indicate statistical significance.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis. Pearson-correlation analysis was used to determine the differentially-expressed genes (DEGs) which correlated with GBP2 expression in ccRCC. Gene pairs with Benjamini–Hochberg corrected *p*-value ≤0.05 and Pearson $|r| > 0.3$ were considered significant. The DEGs associated with GBP2 expression were used for function and pathway-enrichment analyses of GBP2 expression by GO and KEGG analysis. GO analysis includes cellular components, biological processes, and molecular functions. A value of $p \leq 0.05$ was considered as a threshold to determine significant GO functions and KEGG pathways.

TISIDB database analysis. The TISIDB database (8) (http: //cis.hku.hk/TISIDB) is a web portal for tumor and immune-system interaction, which integrates multiple heterogeneous data types. The correlation of GBP2 expression with tumor-infiltrating lymphocytes (TILs) and major histocompatibility complex (MHC) molecules was analyzed using the TISIDB database.

Patients and tissues. GBP2 expression in patients with ccRCC was analyzed by collecting 20 pairs of fresh ccRCC and adjacent normal tissues from the First Affiliated Hospital of Chongqing Medical University (Chongqing, PR China). All collected specimens were stored in liquid nitrogen immediately after surgery. A total of 79 paraffin-embedded ccRCC tumor sections and 34 adjacent normal tissue sections were retrieved from the Pathology Department of the First Affiliated Hospital of Chongqing Medical University. None of the patients enrolled in this study received targeted therapy before enrollment. The protocol was approved by the Institutional Ethics Committee of the First Hospital of Chongqing Medical University (approval number: 2021-465) and was in concordance with the Helsinki Declaration.

Quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was extracted from 20 pairs of fresh ccRCC and adjacent normal tissues using an Eastep™ Super Total RNA Extraction kit (Promega, Madison, WI, USA) and converted to cDNA using a PrimeScript RT reagent kit (TaKaRa, Shiga, Japan). qRT-PCR for GBP2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed using SYBR® Green (TaKaRa). The primers used were as follows. *GBP2*: Forward primer: CGAATCAAGGCAAACTCC; reverse primer: TTCCAGGGTGAAATCTCTGGAPDH. GADPH: forward primer: GGAGTCCACTGGCGTCTTCA; reverse primer: GTCATGAGT CCTTCCACGATACC. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative GBP2 mRNA expression.

Western blotting. Total protein was extracted from tissues (eight pairs of tumor and adjacent normal tissues) and the Caki-1 cell line using radioimmunoprecipitation assay buffer (Biosharp, Hefei, PR China) and mixed with 1 mM phenylmethylsulfonyl fluoride (Biosharp) and a phosphatase inhibitor mix (Biosharp). The protein sample was combined with a 5X protein-loading buffer (Beyotime Biotechnology, Shanghai, PR China) after determining the protein concentration and then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were transferred onto a polyvinylidene difluoride membrane, blocked using QuickBlock™ Blocking Buffer (Beyotime Biotechnology) for 15 min at 37˚C, and incubated with primary antibodies at 4˚C overnight. After washing the membrane thrice with Tris-buffered saline containing Tween, it was incubated with a secondary antibody for 1 h at room temperature, then visualized using the ECL reagent (Beyotime Biotechnology). The following antibodies were used: Rabbit monoclonal antibody against GBP2 (1: 1,000 dilution; Proteintech Group Inc., Rosement, IL, USA); rabbit monoclonal antibody against GAPDH (1:1,000 dilution; Proteintech Group Inc.); rabbit monoclonal antibody against β-catenin (1: 1,000 dilution; Cell Signaling Technology, Danvers, MA, USA); and rabbit monoclonal antibody against c-MYC proto-oncogene bHLH transcription factor (c-MYC; 1: 1,000 dilution; Proteintech Group Inc.) as the primary antibodies. Goat anti-rabbit IgG (1: 5,000 dilution; Proteintech Group Inc.) was the secondary antibody.

Immunohistochemistry. All specimens were stained with an immunohistochemistry (IHC) kit (ZSGB-BIO, Beijing, PR China). Briefly, the slides were first de-paraffinized using xylene and graded ethanol. Then antigen retrieval was conducted using sodium citrate buffer (0.01 M, pH 6.0) for 20 min at 100˚C, while non-specific antibody reactions were blocked using endogenous peroxidase blockers for 15 min at 37˚C. The sections were incubated with GBP2 primary antibody (1: 300, Proteintech Group) at 4˚C overnight and then with biotin-labeled goat anti-rabbit secondary antibodies for 20 min at 37˚C. After washing thrice with phosphate-buffered saline, all sections were visualized using diaminobenzidine and hematoxylin. Representative images were obtained at 100× and 400× to scan for positive staining in each slide. The IHC score was determined according to the staining range and intensity. The staining range was determined by the percentage of stained cells: 0: no stained cells; 1: 1-25%; 2: 26-50%; 3: 51-75%; and 4: 76-100%; Staining intensity was determined by the presence of brownish-yellow or deep yellow granules in cells as follows: 0: no staining; 1: pale brownish yellow; 2: brownish yellow; and 3: tan. The IHC score was obtained by multiplication of the above two scores: sections with 0-3 points were considered negative, and those with 4-12 points were considered positive (9, 10).

Cell lines and transfection. The ccRCC cell lines 786-O, ACHN, and Caki-1 were purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, PR China) and cultured at 37˚C in the presence of 5% CO_2. 786-O and ACHN cells were maintained in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (GIBCO). Caki-1 cells were

maintained in McCoy's 5A (GIBCO) with 10% fetal bovine serum (GIBCO). The normal kidney cell line HK-2 was purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in K-SFM (Hyclone, Logan, UT, USA). GBP2 siRNA and control siRNA were purchased from GenePharma (Shanghai, PR China). Caki-1 cells were transfected with negative control or GBP2 siRNA using lipofectamine RNAi MAX (Invitrogen, Carlsbad, CA, USA) according to the product specifications. GBP2 siRNA and control siRNA sequences were as follows. Negative control: Sense: 5'-UUCU CCGAA CGUGUCACGUTT-3'; antisense: 5'-ACGUGACACGUUCGGAG AATT-3'. siGBP2: sense: 5'-GGAU GUGGCUGAUGCA CUUTT-3'; antisense: 5'-AAGUGCAUCAGCC ACAUCCTT-3'. Efficacies of knockdown and overexpression of GBP2 were all determined by qRT-PCR and western blot. Cells were harvested 48 hours after transfection for further experiments.

Cell Counting Kit-8 (CCK-8) assay. The CCK-8 reagent (Promega, Madison, WI, USA) was used to measure cell proliferation. Forty-eight hours after transfection, Caki-1 or ACHN cells were plated in 96-well plates (2×10^3 cells per well). When cells were adherent to the well, CCK-8 reagents were added at a 1: 10 dilution to the cultures and plates were incubated for 1 h at 37˚C. The optical density was then measured using a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA) at 450 nm.

5-Ethynyl-20-deoxyuridine (EdU) staining assay. EdU was detected using a Cell-Light™ EdU Apollo® 488 kit (RioBio, Shanghai, PR China). In brief, successfully-transfected cells were seeded in 96-well plates (8×10^3 cells per well), incubated with 100 µl EdU for 2 h, fixed using 4% paraformaldehyde for 30 min, and then incubated with 100 µl 0.5% TritonX-100 for 10 min at room temperature. All the samples were finally stained with both Apollo® dye solution and Hoechst33342 dye solution. The EdU-positive cells were visualized under a fluorescence microscope (Olympus, Tokyo, Japan) and counted with ImageJ software (National Institutes of Health, Bethesda, MA, USA).

Wound-healing assay. A wound-healing assay was used to analyze the cell-migratory ability. Briefly, successfully-transfected cells were seeded in six-well plates. When the cells reached 90% confluence, an artificial scratch was created using a 200-µl sterile pipette tip. Then the cells were washed thrice gently with phosphate-buffered saline and cultured in a serum-free medium. Images were captured at 0, 24, and 48 h at the same place. ImageJ software was applied to measure the wound gap.

Transwell migration and invasion assay. Cell migration and invasion were investigated using 8-µm-pore non-coated and Matrigel-coated (BD Biosciences, San Jose, CA, USA) Transwell inserts (Corning, Tewksbury, MA, USA). Briefly, 2.5×10^4 cells, diluted in 200 µl serum-free Dulbecco's modified Eagle's medium, were added to the upper chamber while 600 µl complete medium containing 10% fetal bovine serum was added to the lower chamber. After 24 h of incubation, the cells in the chambers were fixed with methanol for 30 min and stained with 0.5% crystal violet. The cells in the upper chamber were removed and evaluated under a microscope. Images were obtained with a photo microscope (Olympus). The number of migrating and invading cells was quantified with ImageJ software.

Colony-formation assay. Briefly, 1×10^3 transfected cells were seeded in six-well plates and incubated for 2 weeks. Then cells were fixed with methanol for 30 min and stained with 0.5% crystal violet. Following washing and drying of the plates, the colonies were observed under a microscope (Olympus), and colonies containing >50 cells were counted.

Statistical analysis. R 4.1.0 (R Core Team, Vienna, Austria), SPSS 22.0 (IBM, Armonk, NY, USA), and GraphPad Prism 8.0 (GraphPad Software Company, San Diego, CA, USA) were used to analyze the statistical data and prepare graphs. The Student's *t*-test was used to determine significant differences between different groups. The Fisher's exact test and the chi-squared test were performed to analyze the association of GBP2 expression with clinicopathological characteristics. The Kaplan–Meier method was used to construct survival curves. The log-rank test was applied to show the effect of GBP2 expression on the patient's OS. The TCGA and ICGC data were analyzed by the Wilcoxon rank-sum test and logistic regression to estimate the relationship between clinical parameters and GBP2 expression. Univariate and multivariate Cox-regression analyses were used to evaluate the relationship between GBP2 expression and OS of patients. A value of $p\leq0.05$ was considered to indicate statistical significance.

Results

GBP2 is overexpressed in patients with ccRCC from multiple cohorts. The relative level of expression of GBP2 in patients with ccRCC was identified by comparing the expression between ccRCC and normal adjacent tissue based on RNA-seq data from TCGA and different cohorts from the GEO database. TCGA cohort (TCGA-KIRC) analysis showed that GBP2 expression was increased in ccRCC ($p<0.0001$) (Figure 1A). Similarly, in the three cohorts [GSE40435 (11), GSE46699 (12), and GSE53757 (13)] from the GEO database, GBP2 expression was up-regulated in renal-cancer compared to adjacent normal tissue ($p<0.0001$) (Figure 1B-D).

Immunohistochemistry data showed that GBP2 expression in ccRCC was significantly higher than in normal renal tissues using the same antibody (Figure 1E and F). Taken together, these results demonstrated that GBP2 expression is up-regulated in ccRCC tissues.

Correlation between GBP2 expression and clinicopathological features. The clinical significance of GBP2 expression in ccRCC was explored by studying the differences in GBP2 expression among different clinical subgroups using the TCGA-KIRC dataset. High *GBP2* expression was significantly positively correlated with clinical stage ($p<0.0001$) (Figure 2A), World Health Organization/International Society of Urological Pathology grade ($p<0.0001$) (Figure 2B), T-stage ($p<0.0001$) (Figure 2C), and M-stage ($p<0.05$) (Figure 2D), suggesting that high *GBP2* expression might be used as an indicator for poor prognosis of ccRCC.

GBP2 expression is an independent risk factor for OS. The prognostic value of GBP2 expression was investigated by downloading the RNA-seq data and clinical data from TCGA

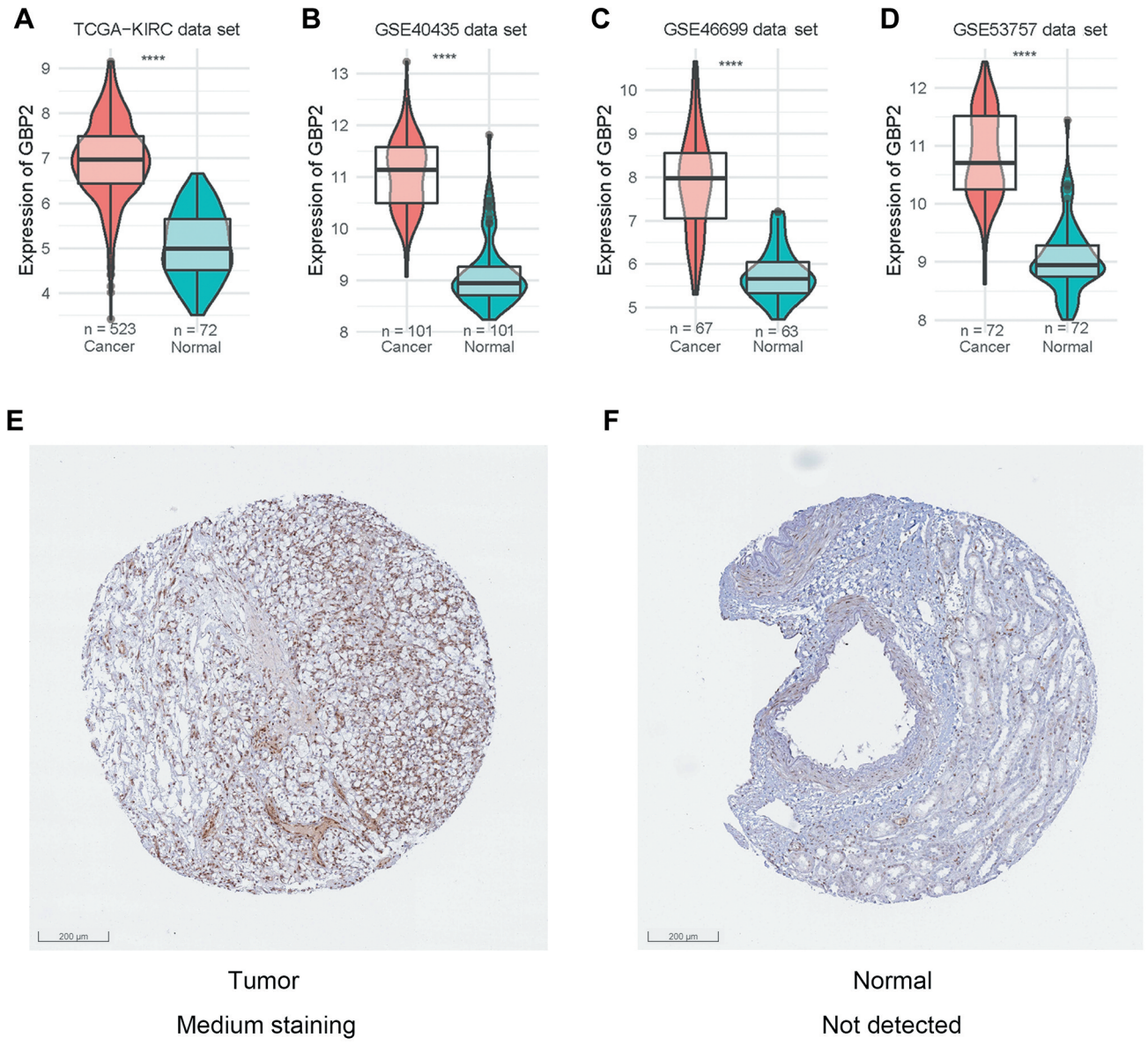

Figure 1. *Expression of guanylate-binding protein 2 (GBP2) in independent clear-cell renal cell carcinoma (ccRCC) cohorts. Compared with normal adjacent tissues, the expression of GBP2 was up-regulated in ccRCC tumor tissues in The Cancer Genome Atlas–Kidney renal clear- cell carcinoma (TCGA-KIRC) (A), Gene Expression Omnibus Series (GSE)40435 (B), GSE46699 (C) and GSE53757 (D) cohorts. Representative immunohistochemistry images of GBP2 expression in KIRC and normal kidney tissues from the Human Protein Atlas database. Immunohistochemical staining of GBP2 in KIRC was stronger than that in normal kidney using the same antibody (antibody number, CAB045975). ****Significantly different at p<0.0001.*

and ICGC databases. Univariate Cox analysis demonstrated that high GBP2 expression was significantly correlated with poor OS in both the TCGA [hazard ratio (HR)=1.56, 95% confidence interval (CI)=1.15-2.12, p=0.004] and ICGC (HR=1.54, 95% CI=1.39-1.73, p=0.017) cohorts (Table I and Figure 3). Multivariate Cox analysis confirmed that GBP2 expression is an independent risk factor for the OS of patients with ccRCC based on TCGA (HR=1.28, 95% CI=1.18-1.51, p=0.041) and ICGC cohorts (HR=1.20, 95% CI=1.03-1.82, p=0.046) (Table I).

GO and KEGG analyses of GBP2 expression. The biological functions and pathways related to GBP2 were studied by performing GO and KEGG analyses. The results of cellular components showed that molecules interacting with GBP2 were mainly located in the membrane-protein complex

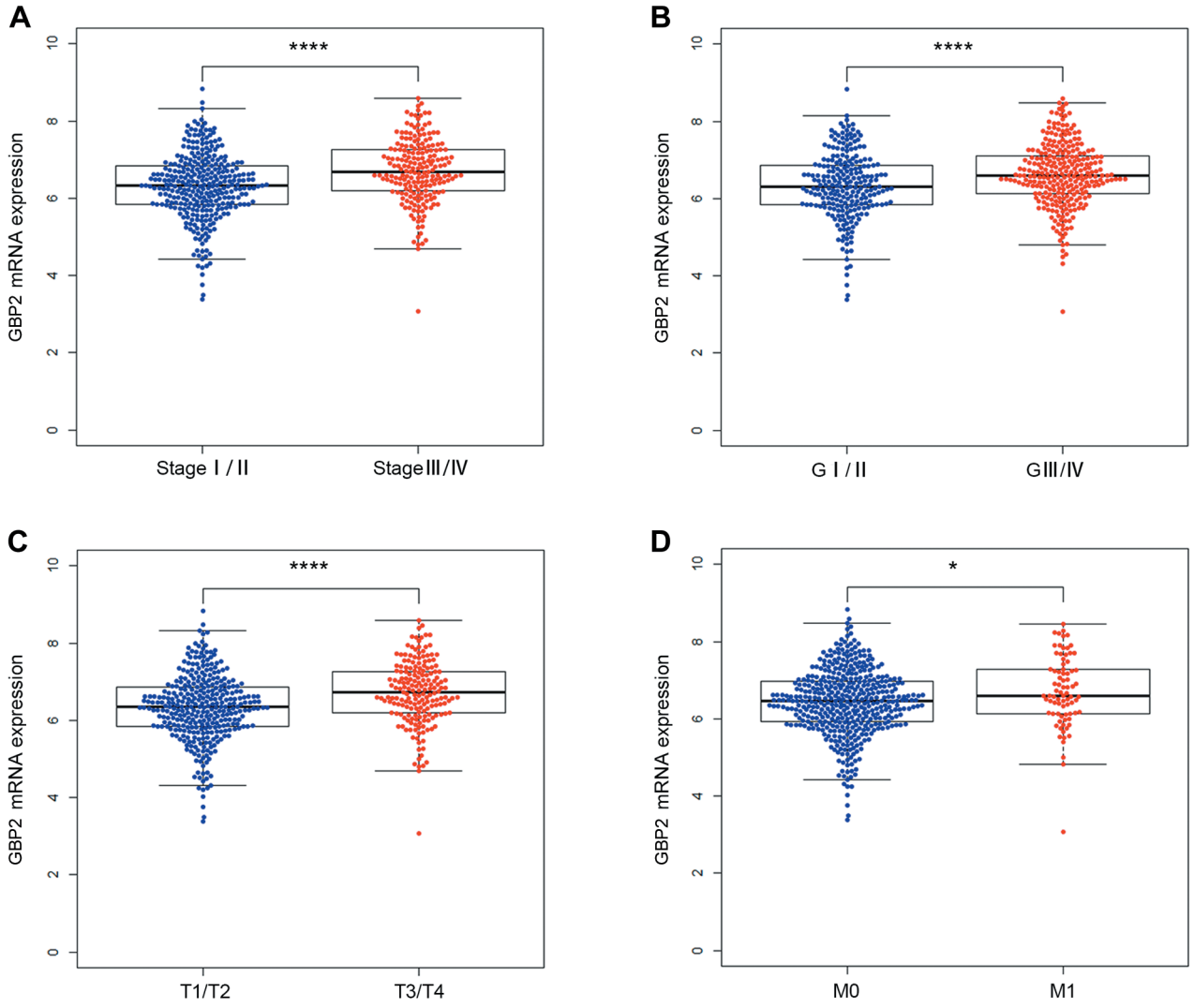

Figure 2. *Correlation between guanylate-binding protein 2 (GBP2) expression and clinicopathological parameters of clear-cell renal cell carcinoma (ccRCC) in the The Cancer Genome Atlas (TCGA) cohort. A: The expression of GBP2 was significantly increased in ccRCC at later clinical stages. B: The expression of GBP2 was up-regulated in ccRCC tissues with higher pathological grade. C: GBP2 expression was significantly increased in ccRCC at higher T stages. D: GBP2 expression was up-regulated in patients with metastatic ccRCC (M1). Significantly different at: *p<0.05 and ****p<0.0001.*

(Figure 4A). Biological-process analysis indicated that GBP2 expression was involved in immune-system development and cell adhesion (Figure 4B). Molecular function analysis demonstrated that GBP2 expression was related to G-protein-coupled receptor binding, protein serine-threonine kinase activity, and cytokine-receptor activity (Figure 4C). Furthermore, KEGG-pathway analysis showed that GBP2 expression may regulate the mitogen-activated protein kinase signaling pathway, WNT signaling pathway, and natural killer cell-mediated cytotoxicity (Figure 4D). These results suggest that GBP2 expression may be associated with tumor immunity and malignant progression in ccRCC.

Correlation between GBP2 expression and tumor immunity. For further clarification of the relationship between GBP2 expression and tumor immunity, the correlation of GBP2 expression with TILs and MHC molecules was analyzed. GBP2 expression was generally correlated with TILs and MHC molecules among heterogeneous human cancers (Figure 5A and C). In ccRCC, GBP2 expression was positively correlated with most tumor-infiltrating lymphocyte types, such as activated CD8 T-cells (Act_CD8; rho=0.635, $p<0.0001$); activated CD4 T cells (Act_CD4; rho=0.6, $p<0.0001$); effector memory CD8 T cells (Tem_CD8; rho=0.589, $p<0.0001$); and myeloid-derived suppressor cells (rho=0.588, $p<0.0001$) (Figure 5B). For MHC

Table I. *Univariate and multivariate Cox-regression analysis of factors associated with renal-cell carcinoma in The Cancer Genome Atlas (TCGA) and International Cancer Genome Consortium (ICGC) cohorts.*

		Univariate regression		Multivariate regression	
Variable	Subgroup comparison	Hazard ratio (95% CI)	*p*-Value	Hazard ratio (95% CI)	*p*-Value
TCGA cohort (n=533)					
GBP2 expression	High *vs.* low	1.56 (1.15-2.12)	0.004	1.28 (1.18-1.51)	0.041
Age	>61 *vs.* ≤61 Years	1.73 (1.27-2.34)	<0.001	1.58 (1.16-2.16)	0.004
Sex	Male *vs.* female	0.94 (0.69-1.28)	0.689	0.93 (0.67-1.29)	0.679
Stage	II *vs.* I	1.22 (0.65-2.26)	0.537	3.17 (0.83-12.12)	0.091
	III *vs.* I	2.60 (1.73-3.93)	<0.001	4.94 (1.71-14.31)	0.003
	IV *vs.* I	6.99 (4.78-10.23)	<0.001	16.79 (3.58-78.76)	<0.001
pM Stage	M1 *vs.* M0	4.63 (3.39-6.32)	<0.001	0.75 (0.21-2.75)	0.666
pN Stage	N1 *vs.* N0	3.61 (1.92-6.80)	<0.001	1.49 (0.65-3.44)	0.346
pT Stage	T2 *vs.* T1	1.57 (0.94-2.61)	0.085	0.33 (0.10-1.07)	0.064
	T3 *vs.* T1	3.31 (2.34-4.68)	<0.001	0.36 (0.13-0.97)	0.042
ICGC cohort (n=474)					
GBP2	High *vs.* low	1.54 (1.39-1.73)	0.017	1.20 (1.03-1.82)	0.046
Age	>61 *vs.* ≤61 Years	1.69 (1.43-2.32)	0.008	1.56 (1.14-2.12)	0.005
Sex	Male *vs.* female	0.90 (0.66-1.35)	0.689	0.99 (0.71-1.38)	0.959
Stage	II *vs.* I	1.32 (0.77-2.47)	0.645	3.75 (1.01-13.98)	0.049
	III *vs.* I	2.24 (1.77-3.53)	<0.001	5.26 (1.86-14.89)	0.002
	IV *vs.* I	6.34 (4.12-8.88)	<0.001	23.90 (4.81-118.73)	<0.001
pM Stage	M1 *vs.* M0	4.55 (3.42-7.12)	<0.001	0.56 (0.14-2.15)	0.395
pN Stage	N1 *vs.* N0	3.54 (1.91-6.32)	<0.001	1.54 (0.68-3.50)	0.300
pT Stage	T2 *vs.* T1	1.52 (0.97-3.14)	0.122	0.26 (0.08-0.83)	0.022
	T3 *vs.* T1	2.92 (2.13-5.00)	<0.001	0.33 (0.12-0.86)	0.023

CI: Confidence interval; HR: hazard ratio; GBP2: guanylate-binding protein; p: pathological.

molecules, GBP2 expression has a positive correlation with human leukocyte antigen (HLA)-class II histocompatibility antigen, DO beta chain (rho=0.697, $p<0.0001$), transporter associated with antigen processing 1 (TAP1) (rho=0.638, $p<0.0001$), HLA-class II histocompatibility antigen, DM alpha chain (rho=0.584, $p<0.0001$), and HLA-class II histocompatibility antigen, DP alpha 1 chain (rho=0.577, $p<0.0001$) (Figure 5D). These results indicate a close relationship between GBP2 expression and tumor immunity.

The relationship between GBP2 expression in ccRCC tissues and disease prognosis. The expression of GBP2 was analyzed by qRT-PCR in 20 paired cancer and adjacent normal tissues from patients with ccRCC. In addition, eight pairs of ccRCC and corresponding adjacent normal tissues were selected for western blotting to verify the expression of GBP2. Both qRT-PCR (Figure 6A) and western blotting (Figure 6B) showed that GBP2 expression was significantly higher in ccRCC tissues than adjacent normal tissues. The correlation between GBP2 expression and clinical characteristics was further verified by IHC staining analysis of 79 ccRCC tumor tissues and 34 adjacent normal tissues (Figure 6C). Among the 79 cases of ccRCC, 35 cases provided negative results, and 44 cases provided positive results (Table II). Combined with clinicopathological features and follow-up data, GBP2 expression was associated with T stage ($p<0.001$), World Health Organization/International Society of Urological Pathology grade ($p=0.031$), and distant metastasis ($p<0.001$) (Table III). High GBP2 expression was also significantly correlated with poor OS in patients with ccRCC (HR =4.70, 95% CI=2.37-9.31, $p<0.001$) (Figure 6D).

GBP2 silencing inhibited the proliferation, migration, and invasion of Caki-1 kidney-cancer cells in vitro. The expression of GBP2 in ccRCC cell lines and normal-kidney cell line HK2 was detected by qRT-PCR. The results showed that the mRNA expression of GBP2 in ccRCC cell lines was higher than that in HK2 cells (Figure 7A). Among the tested ccRCC cell lines, GBP2 expression was highest in the Caki-1 kidney-cancer cell line, therefore it was selected for GBP2 knockdown. The effect of GBP2 knockdown in Caki-1 cells is shown in Figure 7B and C. The results of EdU (Figure 7D) and CCK-8 (Figure 7E) assays show that GBP2 silencing inhibited the proliferation of Caki-1 cells. The colony-formation assay confirmed the inhibitory effect of GBP2 knockdown on cell proliferation (Figure 7F). The wound-healing experiment showed that the migratory ability of Caki-1 cells, after GBP2 knockdown, was inhibited compared to the control group (Figure 7G). Transwell

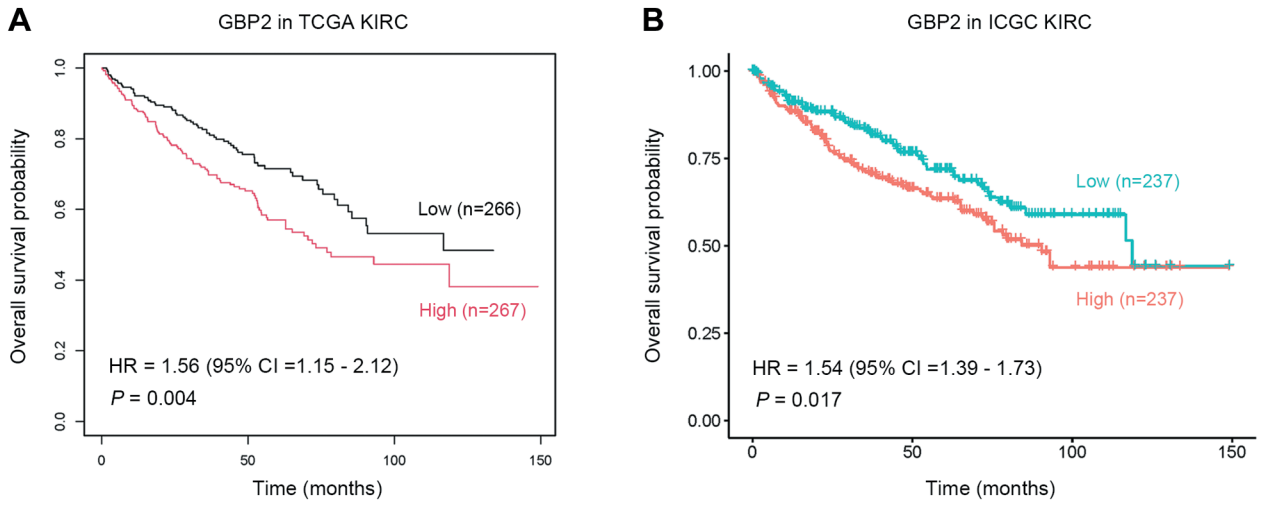

Figure 3. *Correlation between guanylate-binding protein 2 (GBP2) expression and overall survival of patients with clear-cell renal cell carcinoma (ccRCC). Survival analysis showed overexpression of GBP2 was associated with worse overall survival of patients with ccRCC in The Cancer Genome Atlas–kidney renal clear- cell carcinoma (TCGA KIRC) cohort (A), and International Cancer Genome Consortium kidney renal clear- cell carcinoma (ICGC KIRC) cohort (B). CI: Confidence interval; HR: hazard ratio.*

analysis further showed that GBP2 deficiency inhibited the migration and invasion of Caki-1 cells (Figure 7H). These results suggest that GBP2 expression promotes the proliferation, migration, and invasion of Caki-1 cells.

Wnt/β-catenin signaling protein was down-regulated after GBP2 knockdown in Caki-1 cells. According to KEGG analyses, GBP2 expression was significantly correlated with the WNT- signaling pathway. Western blotting was conducted to confirm the analyses. As indicated in Figure 8, GBP2 silencing in Caki-1 cells significantly reduced the level of β-catenin and c-MYC. Given these results, we hypothesize that GBP2 expression might regulate the migratory, invasive, and proliferative abilities of Caki-1 cells through the Wnt/β-catenin signaling pathway.

Discussion

ccRCC is the most lethal type of kidney cancer. For early-stage ccRCC, surgery is the preferred treatment. However, for advanced cases, there is still a lack of effective treatment (14). ccRCC is not sensitive to radiotherapy or chemotherapy; therefore targeted agents are the first choice for patients with advanced ccRCC (15). Although targeted agents can inhibit tumor proliferation to a certain extent, they have serious adverse effects, and the objective efficacy rate of treatment is less than 30% (16). Thus, there is an urgent need to find new treatments for advanced ccRCC.

With investigations into tumor immunology, more genes regulating immune responses have been identified (17). For ccRCC, its occurrence and development are immunogenic (18).

Table II. *Immunohistochemical analysis of expression of guanylate-binding protein 2 (GBP2) in clear-cell renal carcinoma and adjacent normal tissues.*

Tissue	Number of patients	Expression of GBP2		*p*-Value*
		Negative	Positive	
Cancer tissue	79	35 (31%)	44 (38.9%)	<0.001
Adjacent normal tissue	34	31 (27.4%)	3 (2.7%)	

*Fisher's exact test.

Recent studies have shown that immunotherapy is a promising treatment for patients with advanced ccRCC (19). Therefore, there is an urgent need to study further the role of immune-related genes in the occurrence and development of ccRCC.

GBP2 resides in a single cluster on chromosome 1q22.2 and has a conserved structure with an *N*-terminal globular GTPase domain and a *C*-terminal helical domain. The GBP family plays a central role in immune defense against viral and intracellular pathogens. To date, seven GBP family members (GBP1-7) have been identified (20).

Recent studies have shown that GBP2 plays an important role in the occurrence and development of malignant tumors. For example, GBP2 is highly expressed in human glioblastoma multiforme (GBM) tissues and cell lines. Its high expression promotes the migration and invasion of GBM cells through the signal-transducer and activator of transcription 3/fibronectin signaling cascade. GBP2 expression can also promote the proliferation and invasion of GBM tumors *in vivo* and reduce the survival of tumor-bearing mice (21).

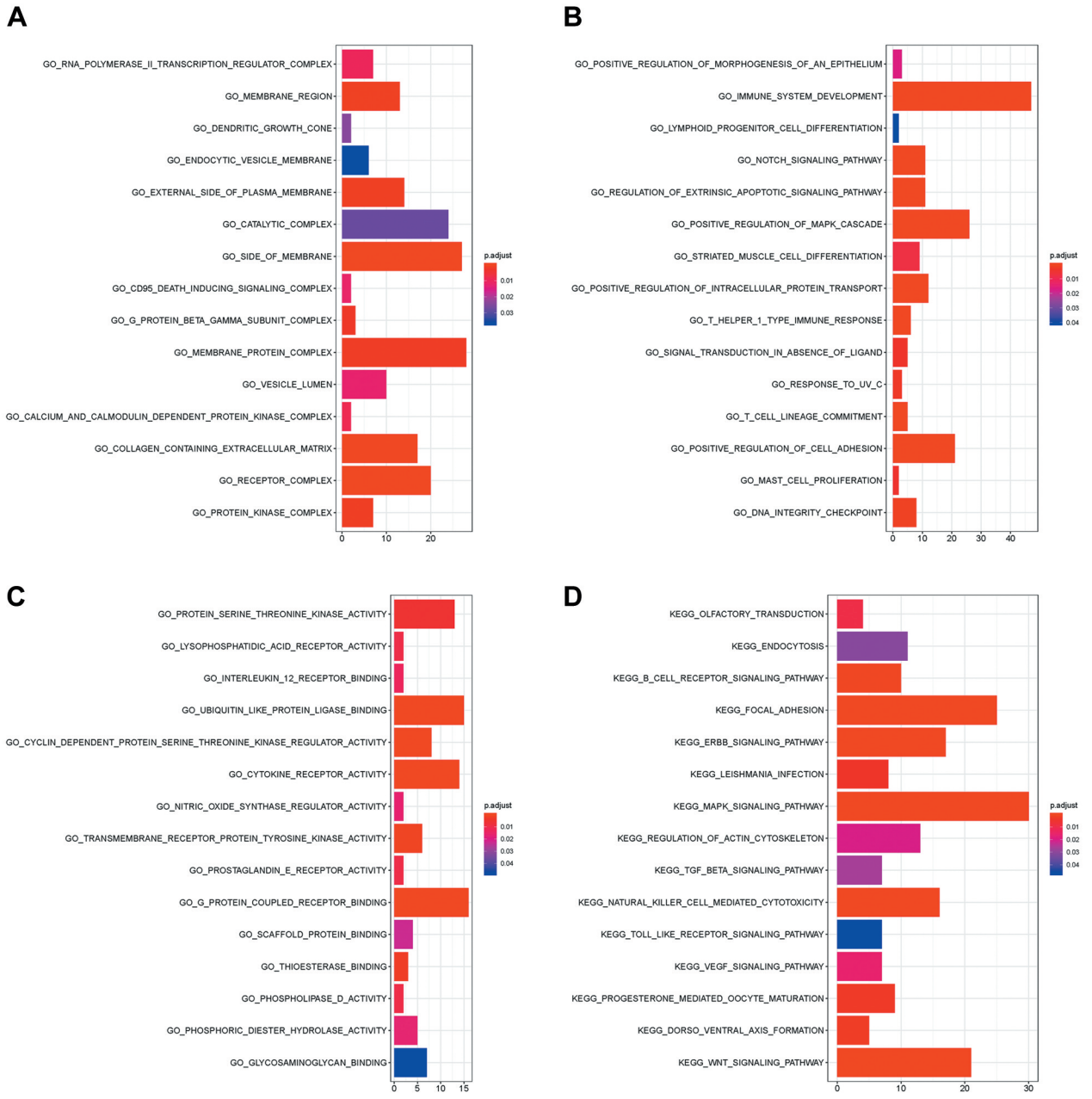

Figure 4. *Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses of guanylate-binding protein 2 (GBP2) in clear-cell renal-cell carcinoma (ccRCC). Cellular components (A), biological processes (B), molecular functions (C), and KEGG pathway analysis (D) for genes associated with GBP2. The length of the bar represents the number of genes associated with GBP2.*

GBP2 is also overexpressed in both esophageal squamous-cell carcinoma and pancreatic adenocarcinoma, which is associated with poor prognosis (22, 23). GBP2 was shown to promote the proliferation of murine fibroblasts and attenuate contact growth-inhibition (24). However, the function and mechanism of action of GBP2 in ccRCC are poorly understood.

In the present study, GBP2 expression was found to be up-regulated in ccRCC tissues through bioinformatics analysis, and it was related to tumor size, pathological grade, and metastasis of ccRCC. The prognostic value of GBP2 was investigated through univariate and multivariate Cox-regression analyses, and the results showed that GBP2 expression was an independent risk factor for poor OS of patients with ccRCC. In the analysis

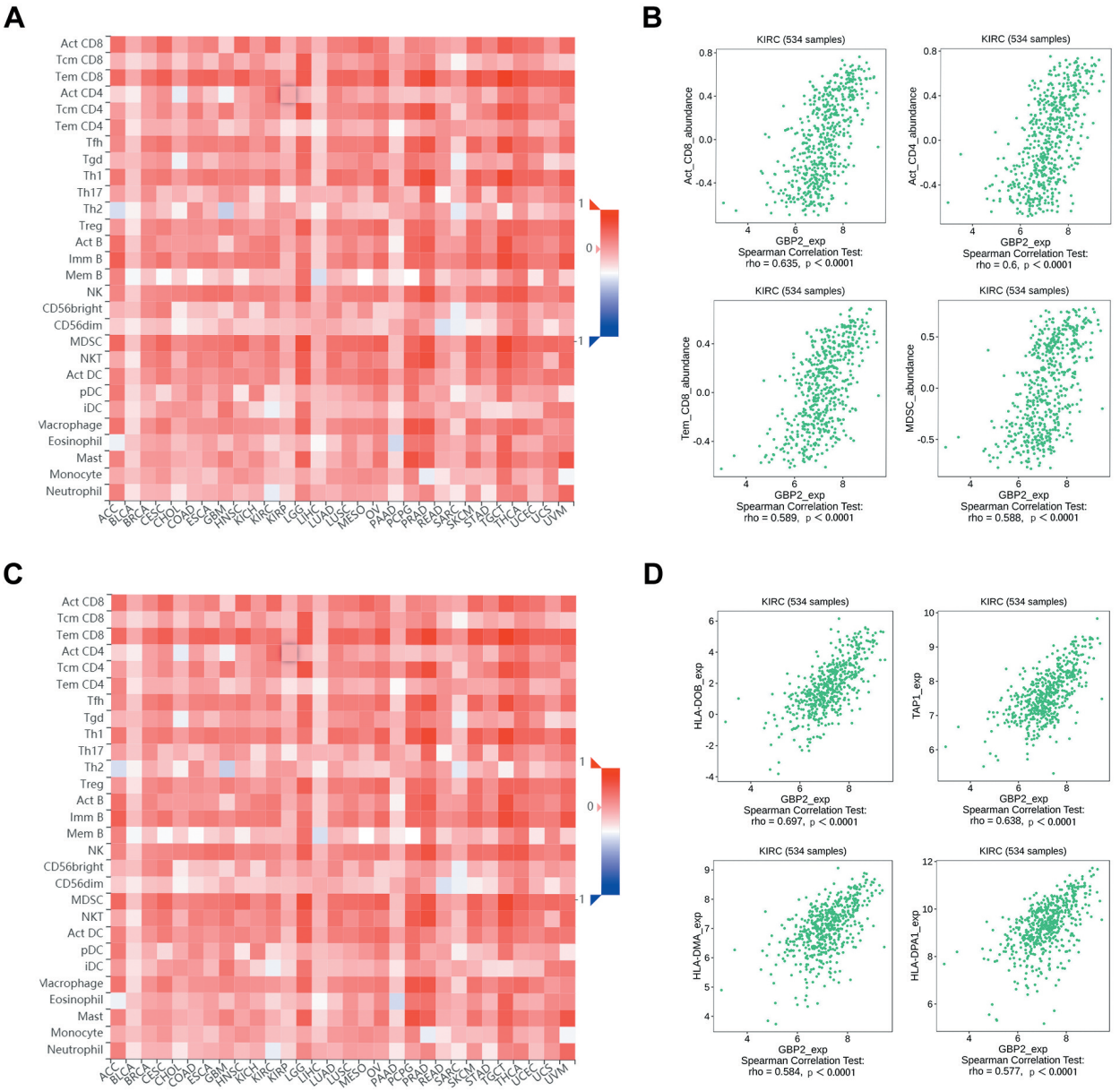

Figure 5. *Spearman correlation of guanylate-binding protein 2 (GBP2) expression with the extent of tumor-infiltrating lymphocytes (TILs) and major histocompatibility complex (MHC) molecules among human cancer types. A: Heatmap of correlation between GBP2 expression and TILs among human cancer types. Red represents positive correlation and blue represents negative correlation. B: Scatterplot of correlation analysis of GBP2 expression and TILs in kidney renal clear-cell carcinoma (KIRC). C: Heatmap of correlation between GBP2 expression and MHC molecules among human cancer types. Red represents positive correlation and blue represents negative correlation. D: Scatterplot of correlation analysis of GBP2 expression and MHC molecules in KIRC. Tumor types: ACC: Adrenocortical carcinoma; BLCA: bladder urothelial carcinoma; BRCA: breast invasive carcinoma; CESC: cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL: cholangiocarcinoma; COAD: colon adenocarcinoma; ESCA: esophageal carcinoma; GBM: glioblastoma multiforme; HNSC: head and neck squamous-cell carcinoma; KICH: kidney chromophobe; KIRP: kidney renal papillary-cell carcinoma; LGG: brain lower-grade glioma; LIHC: hepatocellular carcinoma; LUAD: lung adenocarcinoma; LUSC: lung squamous-cell carcinoma; MESO: mesothelioma; OV: ovarian serous cystadenocarcinoma; PAAD: pancreatic adenocarcinoma; PCPG: pheochromocytoma and paraganglioma; PRAD: prostate adenocarcinoma; READ: rectal adenocarcinoma; SARC: sarcoma; SKCM: Cutaneous melanoma; STAD: stomach adenocarcinoma; TGCT: testicular germ-cell tumors; THCA: thyroid carcinoma; UCEC: uterine-corpus endometrial carcinoma; UCS: uterine carcinosarcoma; UVM: uveal melanoma. Cell types: Act: Activated; B: B-cell; B2M: beta-2-microglobulin; CD4/CD8: $CD4^+$/$CD8^+$ T-cells; CD56: $CD56^+$ natural killer (NK) cells; DC: dendritic cells; HLA: human leukocyte antigen histocompatibility antigen; iDC: immature DC; Imm: immature; Mast: mast cells. HLA: human leukocyte antigen; MDSC: myeloid-derived suppressor cells; NKT: NK T-cells; pDC: plasmacytoid DC; TAP: transporter associated with antigen processing; TAPBP: TAP-binding protein; Tcm: central memory; Tem: effector memory; Tfh: follicular helper T-cells; Tgd: gamma delta T-cells; Th: T-helper cells; Treg: regulatory T-cells.*

Table III. *Relationship between guanylate-binding protein 2 (GBP2) expression and clinicopathological features of clear-cell renal carcinoma.*

Clinicopathological feature		Patients, n (%)	Expression of GBP2		*p*-Value
			Negative	Positive	
Sex	Female	26 (32.9%)	8 (10.1%)	18 (22.8%)	0.146
	Male	53 (67.1%)	27 (34.2%)	26 (32.9%)	
Age	<60 Years	37 (46.8%)	19 (24.1%)	18 (22.8%)	0.339
	≥60 Years	42 (53.2%)	16 (20.3%)	26 (32.9%)	
WHO/ISUP grade	I/II	47 (59.5%)	26 (32.9%)	21 (26.6%)	0.031
	III/IV	32 (40.5%)	9 (11.4%)	23 (29.1%)	
T-Stage	T1+2	33 (41.8%)	26 (32.9%)	7 (8.9%)	<0.001
	T3+4	46 (58.2%)	9 (11.4%)	37 (46.8%)	
M-Stage	M0	65 (82.3%)	35 (44.3%)	30 (38%)	<0.001
	M1	14 (17.7%)	0 (0%)	14 (17.7%)	

ISUP: International Society of Urological Pathology; WHO: World Health Organization.

of associated biological processes, GBP2 expression was found to be closely related to the regulation of the immune system and cell adhesion, which are key steps in tumorigenesis and metastasis. In the analysis of related signaling pathways, GBP2 expression was significantly related to the mitogen-activated protein kinase and WNT signaling pathways, both reported in various cancer studies to promote tumor occurrence and malignancy. The relationship between GBP2 expression and tumor immunity in ccRCC was analyzed based on the relationship of GBP2 expression and the extent of TILs and MHC molecules. GBP2 expression was found to be significantly related to effector-activated CD8 T-cells and activated CD4 T-cells. Recent studies have shown that activated CD8 T-cells and HLA-A molecules in ccRCC tumor tissues are related to immunotherapy efficacy (25). Thus, GBP2 is a prognostic marker in ccRCC and, to a certain extent, reflects the immune status of patients with ccRCC.

The expression of GBP2 was also studied in freshly-collected tissues and pathological sections of ccRCC. GBP2 was highly expressed in ccRCC tumor tissues, consistent with bioinformatics results in the present report. Knockdown of GBP2 inhibited the proliferation, migration, and invasion of ccRCC cells in culture. These results further illustrate that GBP2 overexpression is related to malignancy of patients with ccRCC and is consistent with GBP2 expression being correlated with poor OS.

The WNT/β-catenin pathway is a family of proteins involved in many important functions, such as stem-cell regeneration and organogenesis (26). In tumors, WNT/β-catenin signaling has been widely reported to facilitate tumor progression by altering a series of important physiological events such as cell proliferation, angiogenesis, and maintenance of stem cells (27, 28). Recent studies have reported that constitutive activation of the WNT/β-catenin signaling pathway promotes the proliferation of renal- carcinoma cells. In addition, clinical cases of renal cancer with a highly activated WNT pathway showed more malignant clinical features, such as a larger tumor diameter, more vascular invasion, and higher disease stage (29, 30). According to the results of the KEGG pathway analysis, GBP2 expression was significantly correlated with the WNT/β-catenin signaling pathway. We further confirmed this result by western blot analysis in Caki-1 cells treated with si-RNA to knock down GBP2 expression. Silencing GBP2 significantly reduced the protein expression of β-catenin and c-MYC. These results indicate that the regulatory mechanisms of GBP2 promoting proliferation, invasion, and migration of Caki-1 cells may be associated with the WNT/β-catenin signaling pathway.

In conclusion, the present study showed that GBP2 expression is up-regulated in ccRCC tissues. Patients with ccRCC with high GBP2 expression have a lower OS probability. GBP2 expression also promoted the proliferation, migration, and invasion of Caki-1 cells through the WNT/β-catenin signaling pathway. These results suggest that GBP2 expression can become a prognostic marker for ccRCC.

Conflicts of Interest

The Authors declare that they have no competing interests.

Authors' Contributions

DLW and DGD: Study design; RMH: article revision; QYL: qRT-PCR, IHC, western blotting, cell function experiments, article preparation and editing; JS and SQM: bioinformatics analysis and statistical analysis; JDZ: follow-up data collection. The Authors have all read through and approved the final article.

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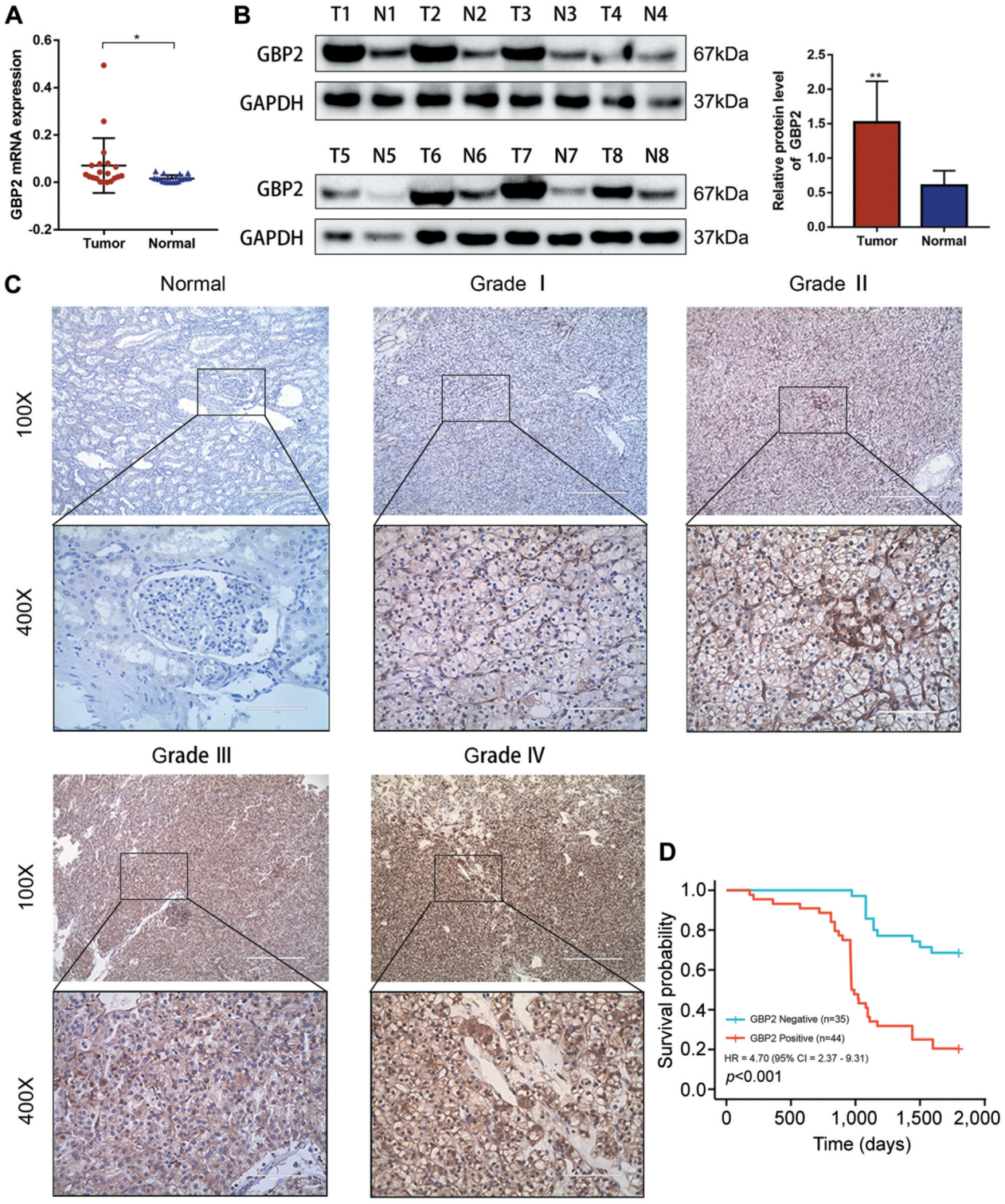

Figure 6. *Correlation of guanylate-binding protein 2 (GBP2) expression in clear-cell renal-cell carcinoma (ccRCC) tissues and patient prognosis. A: The mRNA level of GBP2 was detected in 20 paired cancer and adjacent normal tissues from patients with ccRCC. B: GBP2 protein was detected in eight pairs of ccRCC (T) and corresponding adjacent normal tissues (N) (left) and then quantified (right). C: Immunohistochemistry of GBP2 in normal tissue (negative) and in ccRCC of different World Health Organization/International Society of Urological Pathology grades. D: The correlation between GBP2 expression and overall survival in 79 patients with ccRCC. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase. Significantly different at: *p<0.05 and **p<0.01.*

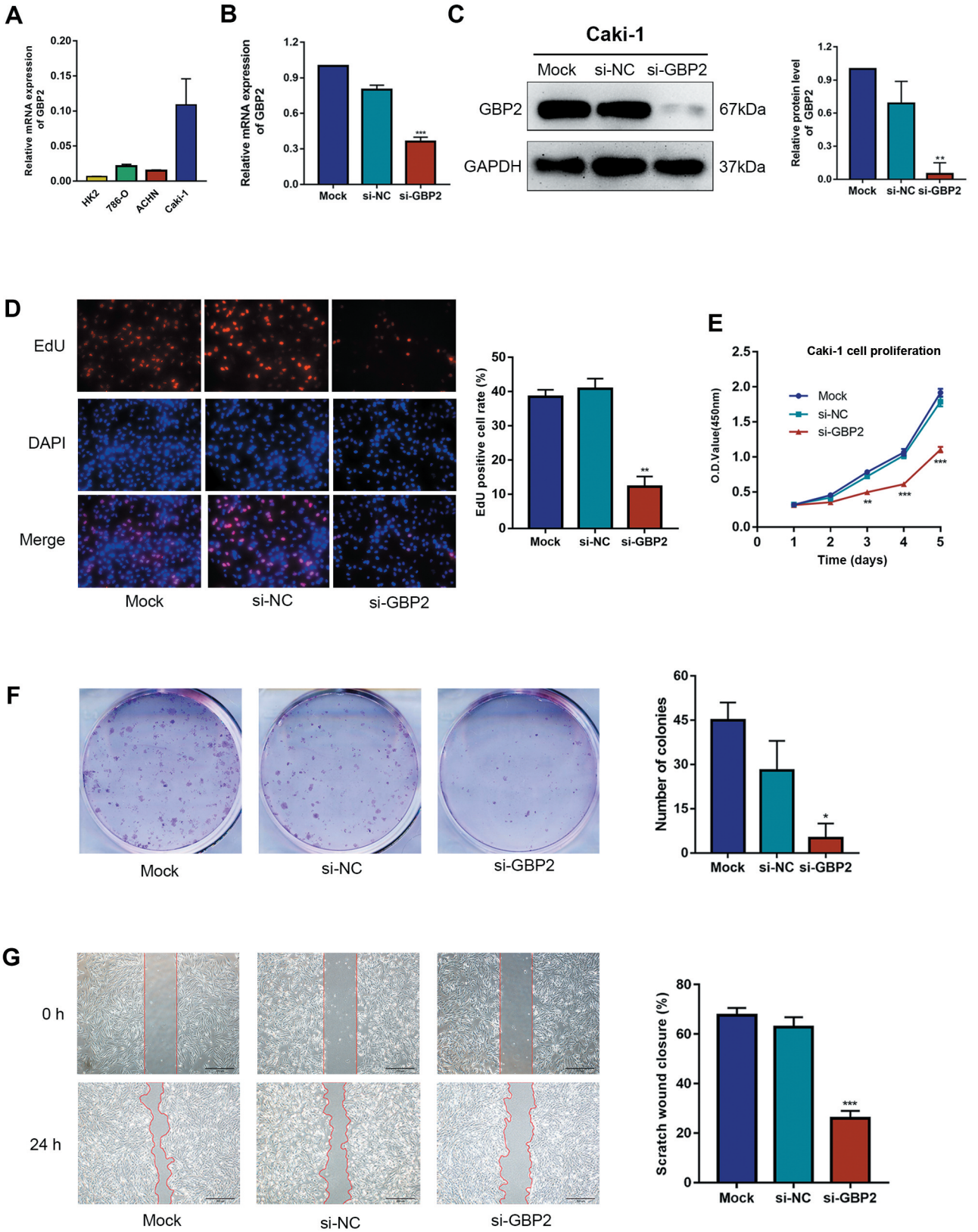

Figure 7. *Continued*

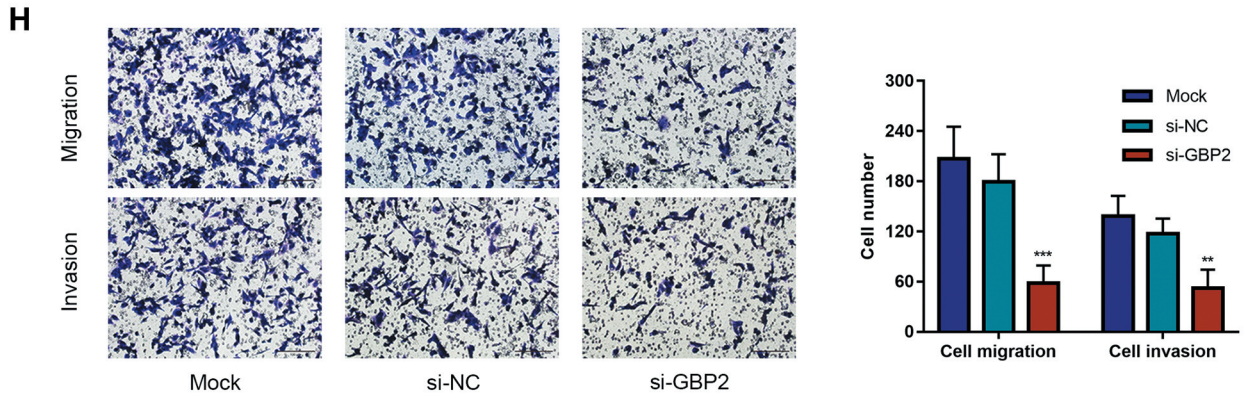

Figure 7. *Effects of guanylate-binding protein 2 (GBP2) silencing on the migration, invasion and proliferation of Caki-1 cells. A: The expression of GBP2 in ccRCC cell line Caki-1 and normal kidney cell line HK2 was detected by the quantitative real-time-polymerase chain reaction. The mRNA (B) and protein (C) levels of GBP2 in Caki-1 cells after silencing with GBP2 si-RNA. D: 5-Ethynyl-20-deoxyuridine (EdU) staining assay was used to analyze Caki-1 cell proliferation after GBP2 silencing. E: CCK-8 staining assay was applied to detect the viability of Caki-1 cells transfected with GBP2 siRNA. F: A colony-formation assay was used to detect Caki-1 cell proliferation after silencing with GBP2 si-RNA. G: A wound-healing assay was performed after Caki-1 cells were transfected with GBP2 si-RNA. H: The migration and invasion abilities of Caki-1 cells after silencing with GBP2 si-RNA were measured by a transwell assay. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase. Significantly different at: *p<0.05, **p<0.01 and ***p<0.001.*

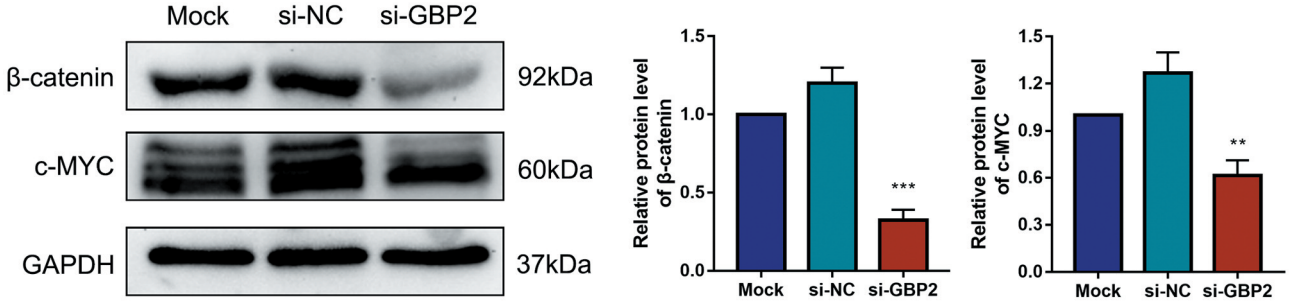

Figure 8. *Guanylate-binding protein 2 (GBP2) activates WNT/β-catenin signaling in Caki-1 cells. The expression of key proteins in WNT/β-catenin pathway in Caki-1 cells treated with and without GBP2 siRNA was detected by western blotting assay (left) and then quantified (right). Significantly different at: **p<0.01 and ***p<0.001. NC: Negative control.*

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