

RNA binding protein PUM2 promotes hepatocellular carcinoma proliferation and apoptosis via binding to the 3'UTR of BTG3

ZHENHUA LIU¹ and CHUNYE LV²

¹Department of General Surgery, The First People's Hospital of Lin Ping District, Hangzhou, Zhejiang 311100;

²Department of General Surgery, The Affiliated Jiangning Hospital with Nanjing Medical University, Nanjing, Jiangsu 211100, P.R. China

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Abstract. Pumilio homolog 2 (PUM2) is an RNA-binding protein that functions as an oncogene in various types of cancer. However, its role in hepatocellular carcinoma (HCC) has remained to be fully elucidated. In the present study, the role of PUM2 was investigated in HCC and its regulation was assessed by examining its binding to the 3'-untranslated region (UTR) of B-cell translocation gene 3 (BTG3). The expression levels of PUM2 were determined in datasets from the UALCAN and Cancer Cell Line Encyclopedia databases. Furthermore, Gene Expression Profiling Interactive Analysis was used to analyze overall survival in patients with HCC. Reverse transcription-quantitative PCR (RT-qPCR) and western blot analyses were then performed to detect the expression levels of PUM2 and BTG3 in HCC cells. Cell proliferation was assessed using Cell Counting Kit-8 and colony-formation assays. The induction of cell apoptosis was evaluated using TUNEL and western blotting assays. StarBase and RNA-Protein Interaction Prediction were used to determine the possible direct interaction between PUM2 and BTG3. The interaction between PUM2 and BTG3 was then verified by luciferase reporter and RNA-binding protein immunoprecipitation assays. The results indicated that PUM2 expression was upregulated in HCC tissues and cells and that it was associated with the prognosis of patients with HCC. PUM2 silencing inhibited the proliferation and promoted the apoptosis of Huh-7 cells. In addition, PUM2 was confirmed to directly bind to the 3'UTR of BTG3. Downregulation of BTG3 reversed the effects of PUM2 silencing on cell proliferation and apoptosis in Huh-7 cells. Collectively, the results suggested

that PUM2 regulated HCC cell proliferation and apoptosis via interacting with BTG3, which may provide a novel therapeutic strategy for the treatment of human HCC.

Introduction

Hepatocellular carcinoma (HCC) is a common type of malignant tumor of the digestive system, accounting for ~90% of liver cancer cases (1). The incidence rate of HCC has increased in recent years worldwide, with an estimated incidence of >1 million cases by 2025 (2). It is generally accepted that multiple risk factors may result in HCC development, such as hepatitis B and C viral infection, cirrhosis, non-alcoholic steatohepatitis, dietary toxins, aflatoxins and aristolochic acid (3,4). To date, the mainstay curative treatments for patients with HCC remain surgery, including hepatic resection and liver transplantation (5). In addition, transarterial chemoembolization has been the most widely used method in standard therapy for intermediate-stage HCC over the past two decades (6). Although significant advances have been made in the therapeutic methods for HCC, the discovery of useful molecular markers for HCC therapy is an urgent requirement due to the current lack thereof (7). It is also necessary to develop novel therapeutic targets for the treatment of patients with HCC.

Pumilio homolog 2 (PUM2) is an RNA-binding protein that serves as a translation repressor (8). PUM2 regulates the translation or stability of certain mRNAs by binding directly to their 3' untranslated region (UTR) (9). A previous study reported that PUM2 was able to bind to the 3'UTR of sirtuin 1 (SIRT1) mRNA to inhibit SIRT1 expression in a model of hypoxia/reoxygenation-induced cardiomyocyte injury (10). Furthermore, PUM2 suppressed kinesin family member 18A to affect proliferation, apoptosis and the cell cycle of human male germ cell lines (11). In addition, PUM2 expression was indicated to be upregulated in several types of human tumor, such as osteosarcoma (12). It was reported that PUM2 overexpression significantly promoted the degradation of insulinoma-associated protein 1 mRNA and inhibited its protein expression in MCF-7 and MDA-MB-231 cells (13). In addition, Wang *et al* (14) revealed that PUM2 accelerated cell proliferation and migration by targeting the 3'UTR of B-cell translocation gene (BTG)1 mRNA in glioblastoma cells. However, the roles of PUM2 in HCC development have

Correspondence to: Dr Chunye Lv, Department of General Surgery, The Affiliated Jiangning Hospital with Nanjing Medical University, 168 Hushan Road, Jiangning, Nanjing, Jiangsu 211100, P.R. China

E-mail: lvchunye311@163.com

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remained elusive. In the present study, the biological roles of PUM2 and its potential mechanism of action were investigated in HCC.

Materials and methods

Bioinformatics analysis. The mRNA levels of PUM2 were analyzed in the UALCAN database (<http://ualcan.path.uab.edu>) and the significance of differences was estimated by using Student's unpaired t-test (15). Gene Expression Profiling Interactive Analysis (GEPIA; <http://gepia.cancer-pku.cn>) was used to analyze the overall survival of patients with HCC (method: Overall survival; Group cutoff: Median; Cut-off high value and low value was set to 50%; hazard ratio: Yes; confidence interval: 95%; axis units: Months) (16). StarBase (<http://starbase.sysu.edu.cn/>) was used to predict the interactions between PUM2 and targeting RNAs (17). RNA-Protein Interaction Prediction (RPISeq; <http://pridb.gdc.b.iastate.edu/RPISeq/index.html>) was used to confirm the interaction between PUM2 and the 3'-UTR of BTG3 and analyze the interaction probability (RPISeq predictions are based on Random Forest or Support Vector Machine classifiers trained and tested on 2 non-redundant benchmark datasets of RNA-protein interactions, RPI2241 and RPI369, extracted from PRIDB, a comprehensive database of RNA-protein complexes extracted from the PDB) (18).

Cell culture. The HCC cell lines HCC36, HCC-T, HCC-M and HHS-89 were purchased from the American Type Culture Collection. The HCC cell line Huh-7 and human the hepatocyte cell line HHL5 (used as the control cell line) were obtained from the Health Science Research Resources Bank. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Hyclone; Cytiva), 100 U/ml penicillin and 100 μ g/ml streptomycin (both Gibco; Thermo Fisher Scientific, Inc.) in a humidified atmosphere with 5% CO₂ at 37°C.

Cell transfection. The specific short hairpin RNA (shRNA) targeting PUM2 (shRNA-PUM2-1: 5'-GCA ATATAG TGTTGTATAA-3'; shRNA-PUM2-2: 5'-CATAGTTGT TGACTGTAA-3'), the specific shRNA targeting BTG3 (shRNA-BTG3-1: 5'-GATTATGTATGGAGAGAAA-3'; shRNA-BTG3-2: 5'-GATTAATCCTCACATGTTA-3') and the corresponding control shRNA (shRNA-NC: 5'-CCGGCA ACAAGATGAAGAGCACCAACTC-3') were synthesized with pRNA-U6.1/Hygro vector as the plasmid backbone by Shanghai Integrated Biotech Solutions. These recombinant nucleotides were transfected into Huh-7 cells using Lipofectamine[®] 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Following 48 h of incubation, the cells were collected for subsequent experiments.

Cell counting kit-8 (CCK-8) assay. Following transfection, Huh-7 cells were placed in 96-well plates (2x10⁴ cells/well) and cultured in DMEM with 10% FBS at 37°C. Following incubation for 24, 48 and 72 h, 10 μ l CCK-8 solution (Beyotime Institute of Biotechnology) was added to each well and the cells

were incubated for 2 h. Finally, the absorbance of each well was detected at 450 nm with a microplate reader (RT-3001; Thermo Fisher Scientific, Inc.).

Colony-formation assay. The cells were seeded in 6-well plates at 500 cells/well and incubated in DMEM with 10% FBS at 37°C. Following incubation for two weeks, the plates were fixed with 4% paraformaldehyde for 15 min at a room temperature and stained with 0.5% crystal violet (Wako Pure Chemical Industries, Ltd.) for 30 min at room temperature. The colonies were imaged and counted by light microscopy (Olympus Corporation). The number of colonies, defined as >50 cells/colony, was counted.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNAs were extracted from Huh-7 cells by TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocol (19). The concentrations of the RNA samples were detected using NanoDrop[®] 3000 (Thermo Fisher Scientific, Inc.). Subsequently, a cDNA synthesis kit (PrimeScript RT Master Mix; Takara Bio, Inc.) was used to reverse transcribe 2 μ g RNA into cDNA following the manufacturer's protocol. The reaction mixture was incubated at 25°C for 5 min, 42°C for 30 min and 85°C for 5 min, and then kept at 4°C for 5 min. Amplification of the cDNA was performed by real-time qPCR using the SYBR Premix Ex Taq[™] II kit (Takara Bio, Inc.) following the manufacturer's protocol in an ABI PRISM 7900 Real-Time system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR program was 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min. A final extension step at 72°C for 7 min was performed in each PCR assay. The primer sequences for PCR were as follows: PUM2 forward, 5'-GGGAATGGGAGAGACCATTCAA-3' and reverse, 5'-AGG ATTAGGAAGAGGCCCA-3'; BTG3 forward, 5'-TCCACC TCTCCAATGTGGC-3' and reverse, 5'-TCCGGTCACAAT GCATTCCA-3'; GAPDH forward, 5'-GGGAACTGTGGCGT GAT-3' and reverse, 5'-GAGTGGGTGTCGCTGTTGA-3'. The relative expression levels of the target gene were calculated by the relative quantification (2^{- $\Delta\Delta$ C_q}) method (20) and GAPDH mRNA levels were used for normalization.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. A TUNEL assay was performed to evaluate Huh-7 cell apoptosis using an apoptosis detection kit (cat. no. 11684795910; Roche Diagnostics) in accordance with the manufacturer's guidelines. The transfected cells were fixed with 4% paraformaldehyde for 10 min at 4°C and incubated with proteinase K (Beijing Solarbio Science & Technology Co., Ltd.) for 15 min at 37°C. Subsequently, the cells were placed in 3% H₂O₂ for 15 min at room temperature and stained with the reagents from the TUNEL kit, followed by counterstaining with DAPI for 10 min at room temperature. The labeled cells were observed using fluorescence microscopy (Olympus Corporation; magnification, x200). The number of TUNEL-positive (green) and DAPI-positive cells (blue nuclear stain) was visually counted and at least 10 fields per section were examined. The percentage of apoptotic cells was calculated as (number of TUNEL-positive cells/total number of cells) x100%.

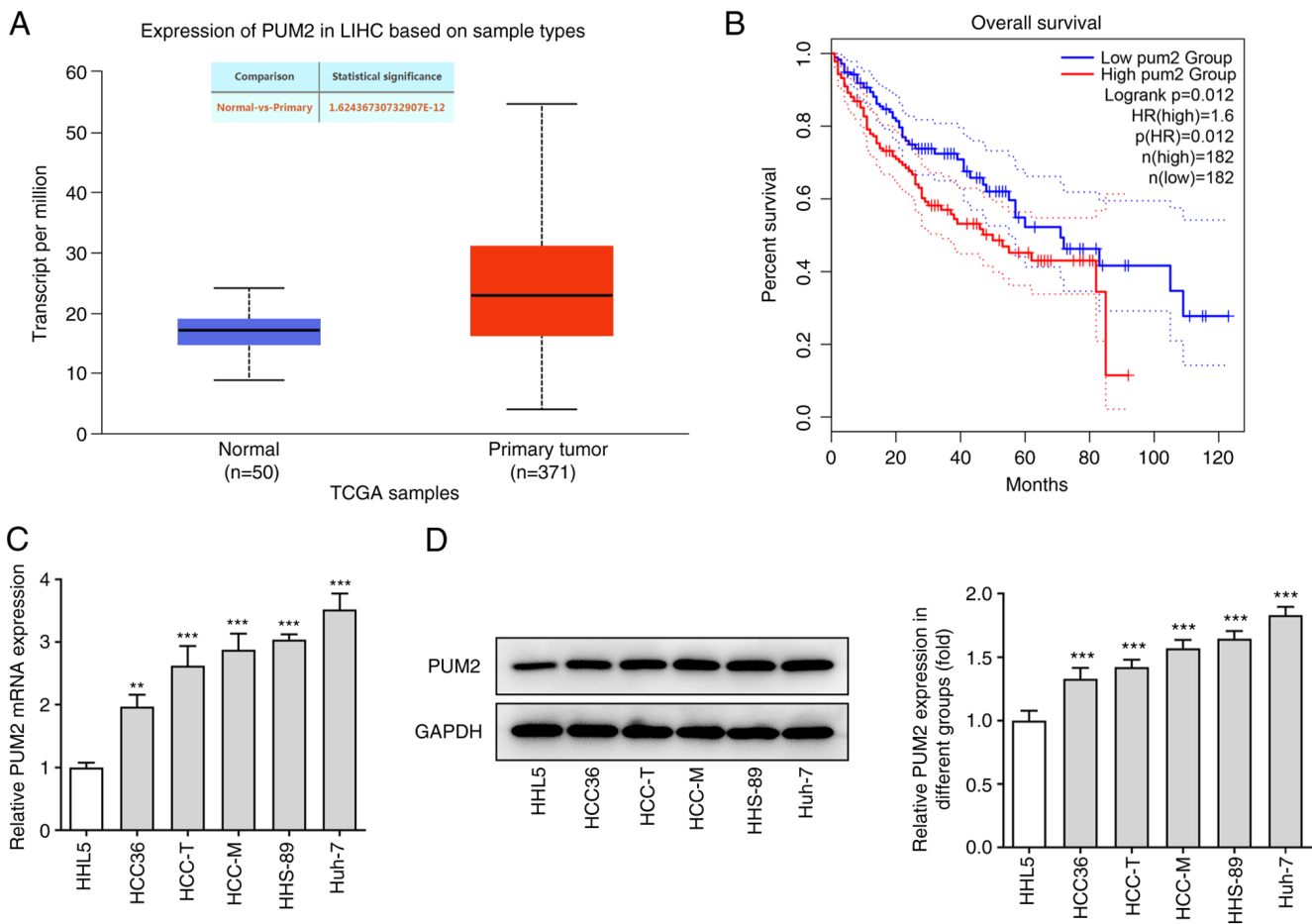


Figure 1. PUM2 is upregulated in HCC tissues and cells. (A) Analysis of PUM2 expression in HCC determined using the UALCAN database. (B) Association between PUM2 expression and overall survival determined using the GEPIA database. (C) mRNA expression and (D) protein level of PUM2 in several HCC cell lines and the human hepatocyte cell line HHL5 (as the control cell line) were detected by reverse transcription-quantitative PCR and western blot assays, respectively. The PUM2 expression in HHL5 was normalized to 1 and the relative PUM2 expression in all other groups was normalized to that in HHL5 cells. GAPDH was used as an internal reference. Values are expressed as the mean \pm standard deviation. ** $P < 0.01$, *** $P < 0.001$ vs. HHL5. LIHC, liver hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; HR, hazard ratio; PUM2, Pumilio homolog 2.

Luciferase reporter assay. To obtain a 3'-UTR-luciferase reporter plasmid, the 3'UTR of BTG3 was amplified using PCR from genomic DNA of the human HCC cell line Huh-7 (Invitrogen; Thermo Fisher Scientific, Inc.) and cloned into the *XhoI/NotI* sites of the psiCHECK-2 vector (Promega Corporation) following digestion with *XhoI* and *NotI* (Beyotime Institute of Biotechnology). Amplification of the cDNA was performed by real-time qPCR using the SYBR Premix Ex Taq™ II kit (Takara Bio, Inc.) following the manufacturer's protocol in an ABI PRISM 7900 Real-Time system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The amplification condition was 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min, prior to a final extension step at 72°C for 7 min. The 3'-UTR-luciferase reporter plasmids and the short hairpin (sh)RNA-PUM2 or negative control (NC) sequences were co-transfected into Huh-7 cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Following transfection, the cells were incubated for 48 h and the dual-luciferase assay system (Promega Corporation) was used to measure firefly and *Renilla* luciferase activity levels.

RNA-binding protein immunoprecipitation (RIP) assay. The interaction between PUM2 and BTG3 was identified by an RIP assay using the EZ-Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (cat. no. 17-701; MilliporeSigma) according to the manufacturer's instructions. The cells were lysed in complete RIP lysis buffer and the protein extract was then prepared. Anti-PUM2 antibody (cat. no. ab92390; 1:10 dilution; Abcam) and NC normal rabbit IgG (cat. no. NI01; 1/200 dilution; MilliporeSigma) were incubated with the protein extract from the lysed cells at 37°C overnight. The co-precipitated RNAs were detected by RT-qPCR as specified above.

Western blot analysis. Total protein was extracted from cells using RIPA buffer (Bio-Rad Laboratories, Inc.). Total protein was quantified using a BCA assay (Beyotime Institute of Biotechnology), according to the manufacturer's protocol. A total of 30 μ g protein per lane was loaded on 10% SDS-polyacrylamide gels and after electrophoresis, proteins were transferred to polyvinylidene membranes (MilliporeSigma). The membranes were blocked in 5% non-fat milk (Beyotime Institute of Biotechnology) at room temperature for 2 h and incubated with primary antibodies against PUM2 (1:1,000 dilution; cat. no. ab92390), Bcl-2 (1:1,000 dilution; cat. no. ab32124),

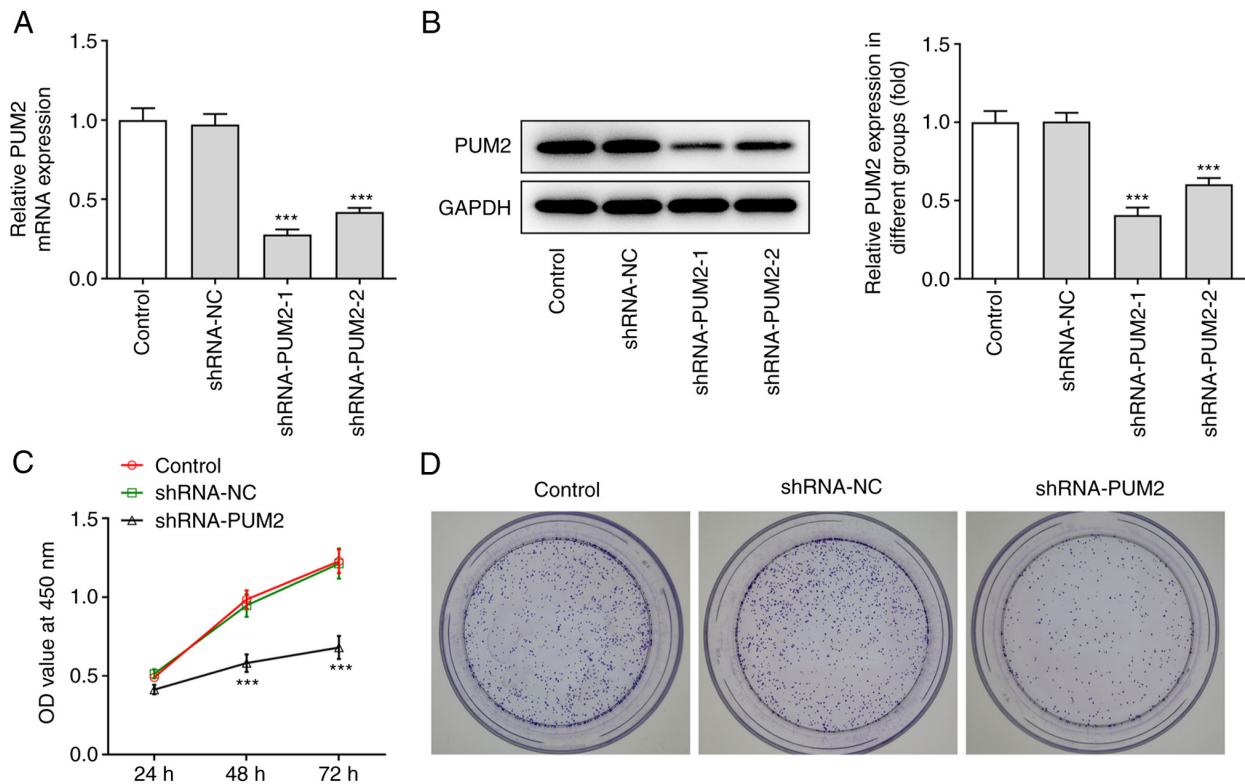


Figure 2. PUM2 silencing inhibits cell proliferation of Huh-7 cells. (A) mRNA expression and (B) protein levels of PUM2 in Huh-7 cells after PUM2 was silenced were detected by reverse transcription-quantitative PCR and western blot assays, respectively. (C) A Cell Counting Kit-8 assay and (D) colony-formation assay were used to assess cell proliferation. Values are expressed as the mean \pm standard deviation. *** $P < 0.001$ vs. shRNA-NC. PUM2, Pumilio homolog 2; NC, negative control; shRNA, short hairpin RNA; OD, optical density.

Bax (1:1,000 dilution; cat. no. ab32503), cleaved caspase 3 (1:500 dilution; cat. no. ab32042), caspase 3 (1:1,000 dilution; cat. no. ab32351), cleaved poly(ADP-ribose) polymerase (PARP; 1:1,000 dilution; cat. no. ab32561), PARP (1:1,000 dilution; cat. no. ab32138), BTG3 (1:1,000 dilution; cat. no. ab112938) and GAPDH (1:1,000 dilution; cat. no. ab8245; all from Abcam) overnight at 4°C. Finally, the membranes were incubated with horseradish peroxidase-labeled anti-rabbit IgG (cat. no. 7074; 1:1,000 dilution; Cell Signaling Technology, Inc.) or anti-mouse IgG (cat. no. 14709; 1:1,000 dilution; Cell Signaling Technology, Inc.) at room temperature for 1 h. The protein bands were visualized using an enhanced chemiluminescence detection system (Amersham; Cytiva) according to the manufacturer's instructions. The density of each band was quantified by ImageJ software (v.1.8.0; National Institutes of Health).

Statistical analysis. Statistical analysis was performed using SPSS 22.0 software (IBM Corporation). Values are expressed as the mean \pm standard deviation. Significant differences between two groups were analyzed by Student's unpaired t-test, while differences among multiple groups were analyzed using one-way analysis of variance followed by Bonferroni's post-hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

PUM2 expression is upregulated in HCC tissues and cells. To explore the role of PUM2 in HCC development, the expression

levels of PUM2 were initially detected in a public dataset of patients with HCC and in HCC cell lines. According to the data obtained from the UALCAN database, PUM2 was highly expressed in HCC tissues compared with those in the control subjects (Fig. 1A). In addition, analysis with the GEPIA database indicated that upregulation of PUM2 expression was associated with poor prognosis of patients with HCC (Fig. 1B). In addition, RT-qPCR and western blot assays indicated significantly higher mRNA and protein expression levels of PUM2 in HCC cell lines compared with those in the non-cancerous control cell line. Huh-7 cells exhibited the highest PUM2 expression level; therefore, Huh-7 cells were selected for the subsequent experiments (Fig. 1C and D).

Inhibition of PUM2 expression reduces cell proliferation and facilitates apoptosis in Huh-7 cells. To investigate the effect of PUM2 on HCC cell proliferation and apoptosis, specific shRNA sequences targeting PUM2 were transfected into Huh-7 cells. The transfection efficiency was evaluated by RT-qPCR and western blot analyses (Fig. 2A and B). The knockdown efficiency of shRNA-PUM2-1 was better than that of shRNA-PUM2-2 and thus, shRNA-PUM2-1 was used for the further experiments (named as shRNA-PUM2 from here onwards). Subsequently, the CCK-8 assay was used to assess cell proliferation. The results indicated that PUM2 silencing significantly suppressed Huh-7 cell proliferation compared with that noted in the NC group (Fig. 2C). Furthermore, the colony-formation assay demonstrated that the number of colonies in PUM2-silenced cells was decreased compared

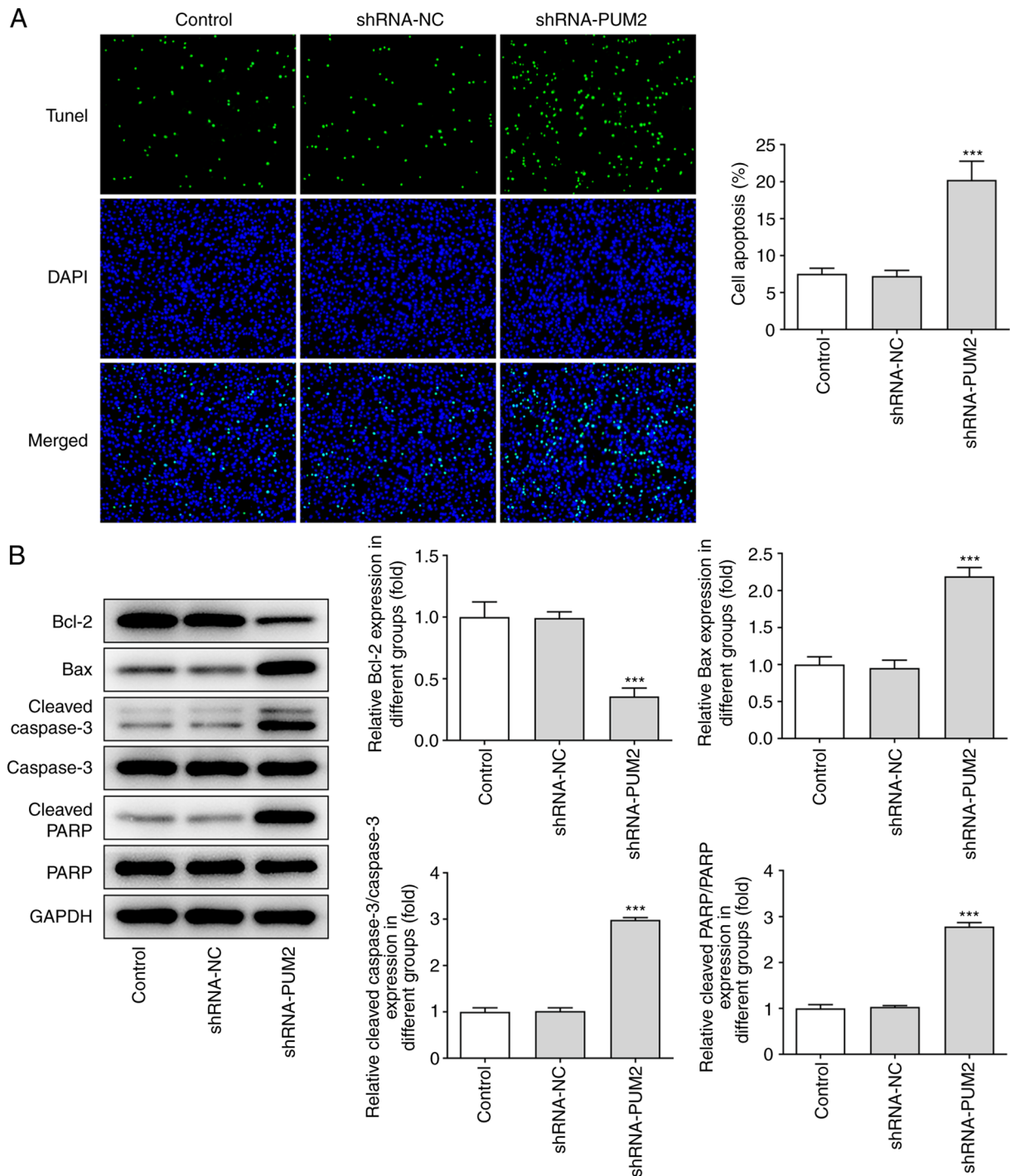


Figure 3. PUM2 silencing represses apoptosis in Huh-7 cells. (A) A TUNEL assay was carried out to quantify apoptosis of Huh-7 cells transfected with shRNA-PUM2 (magnification, x200). (B) Western blot analysis was performed to detect the protein levels of Bcl-2, Bax, caspase3, cleaved caspase3, PARP and cleaved PARP. Values are expressed as the mean \pm standard deviation. *** $P < 0.001$ vs. shRNA-NC. PUM2, Pumilio homolog 2; NC, negative control; shRNA, short hairpin RNA; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; PARP, poly(ADP ribose) polymerase.

with that in the NC group (Fig. 2D). Furthermore, it was observed that the apoptotic rate of Huh-7 cells transfected with shRNA-PUM2 was considerably elevated. Induction of apoptosis was accompanied with decreased Bcl-2 levels and increased levels of Bax, cleaved caspase 3 and cleaved PARP, as determined by western blot analysis (Fig. 3).

PUM2 binds directly to the 3'UTR of BTG3. The mechanisms underlying the regulatory role of PUM2 were further

explored in HCC cells. By using the starBase database, PUM2 was predicted to bind to several RNAs. Among these RNAs, PUM2 protein was predicted to bind to the 3'-UTR of BTG3 and the interaction probability was 0.99 according to the RPISeq website. Furthermore, the binding sequence of the PUM2 and BTG3 3'UTR was predicted by Ensembl (Fig. 4A). Furthermore, the mRNA and protein levels of BTG3 were both increased following shRNA-mediated inhibition of PUM2 expression in Huh-7 cells (Fig. 4B and C). A luciferase reporter

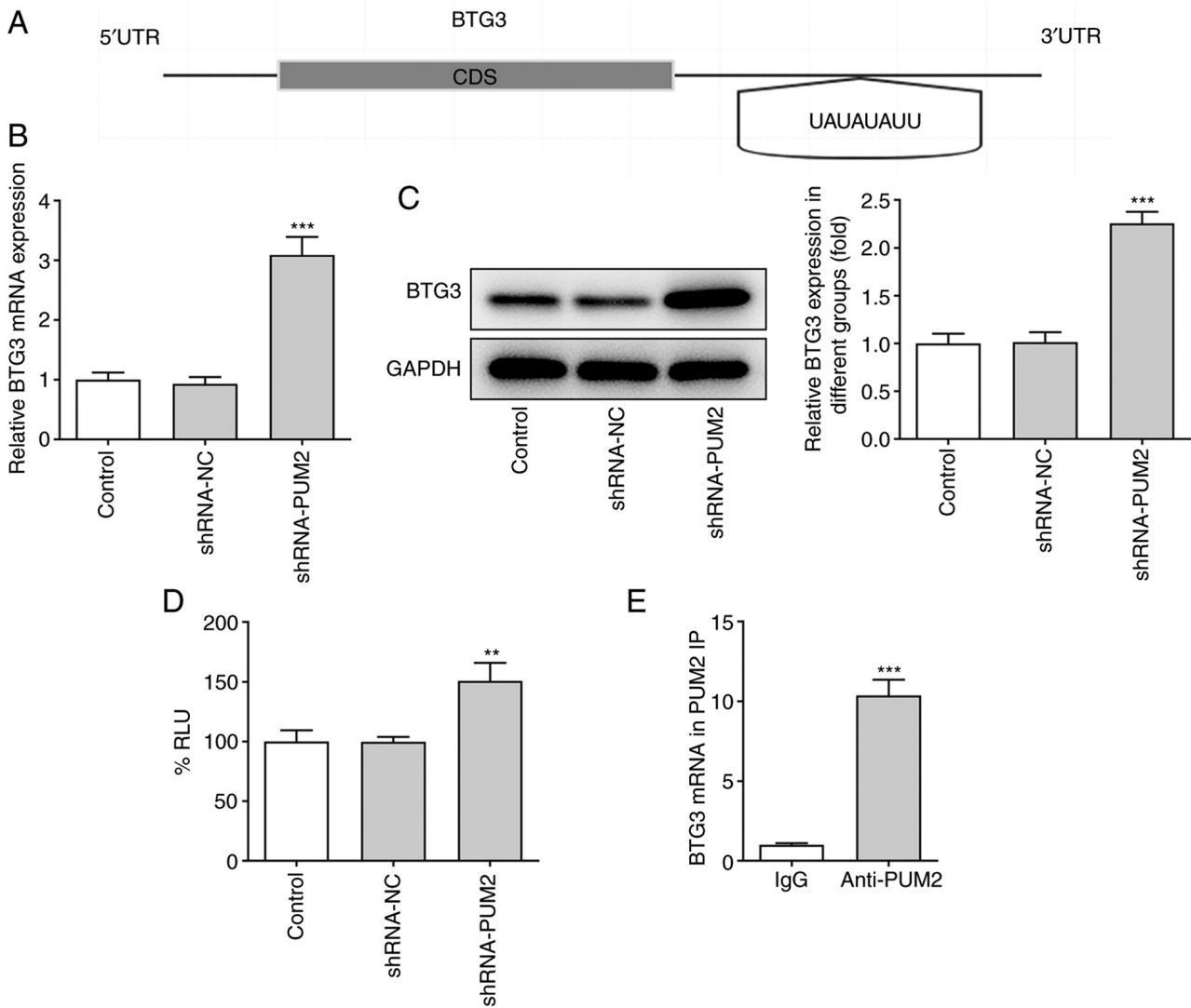


Figure 4. PUM2 binds to 3'UTR of BTG3. (A) The binding site of PUM2 and 3'UTR of BTG3. (B) mRNA expression and (C) protein levels of BTG3 in Huh-7 cells after PUM2 was silenced were detected by reverse transcription-quantitative PCR and western blot assay, respectively. (D) Luciferase activity of the 3'UTR of BTG3 was measured by a luciferase reporter assay. (E) RIP assay was conducted to verify the binding of PUM2 to the 3'UTR of BTG3. Values are expressed as the mean \pm standard deviation. ** $P < 0.01$, *** $P < 0.001$ vs. shRNA-NC or IgG. CDS, coding sequence; PUM2, Pumilio homolog 2; NC, negative control; shRNA, short hairpin RNA; RLU, relative luciferase activity; RIP, RNA-binding protein immunoprecipitation; BTG3, B-cell translocation gene 3.

assay was performed and the data indicated that the luciferase activity of the 3'UTR of BTG3 was significantly increased by PUM2 silencing, while no apparent changes were noted in the luciferase activity in the shRNA-NC group as compared with the control group (Fig. 4D). In addition, the results of the RIP assay were able to verify the combination of PUM2 and BTG3 (Fig. 4E).

PUM2 silencing represses cell proliferation and promotes apoptosis of Huh-7 cells by targeting BTG3. To assess the role of BTG3 in PUM2-mediated HCC progression, BTG3 expression was silenced in Huh-7 cells. RT-qPCR analysis indicated a significant decrease in BTG3 expression following transfection with shRNA-BTG3-1 or -2. shRNA-BTG3-1 exhibited improved knockdown efficiency; therefore, shRNA-BTG3-1 (denoted as shRNA-BTG3 from here onwards) was selected for the subsequent assays (Fig. 5A). The CCK-8 assay results indicated that BTG3 silencing reduced the optical density values of Huh-7 cells transfected with shRNA-PUM2

(Fig. 5B). In addition, depletion of BTG3 inhibited the reduction of the number of colonies by downregulation of PUM2 (Fig. 5C). Furthermore, the TUNEL assay indicated a marked decrease in the apoptotic rate of Huh-7 cells co-transfected with shRNA-PUM2 and shRNA-BTG3 compared with that in cells transfected with shRNA-PUM2 (Fig. 5D and E). In addition, western blot analysis indicated that BTG3 silencing reversed the effects of PUM2 silencing on the protein levels of Bcl-2, Bax, cleaved caspase 3 and cleaved PARP in transfected Huh-7 cells (Fig. 5F and G).

Discussion

HCC is a common malignant tumor type of the digestive system with high morbidity and mortality (21). A high degree of malignancy and distal metastasis is observed in the majority of patients with HCC at diagnosis, leading to poor prognosis (22). In recent years, the development of diagnostic and therapeutic applications for HCC has improved the prognosis

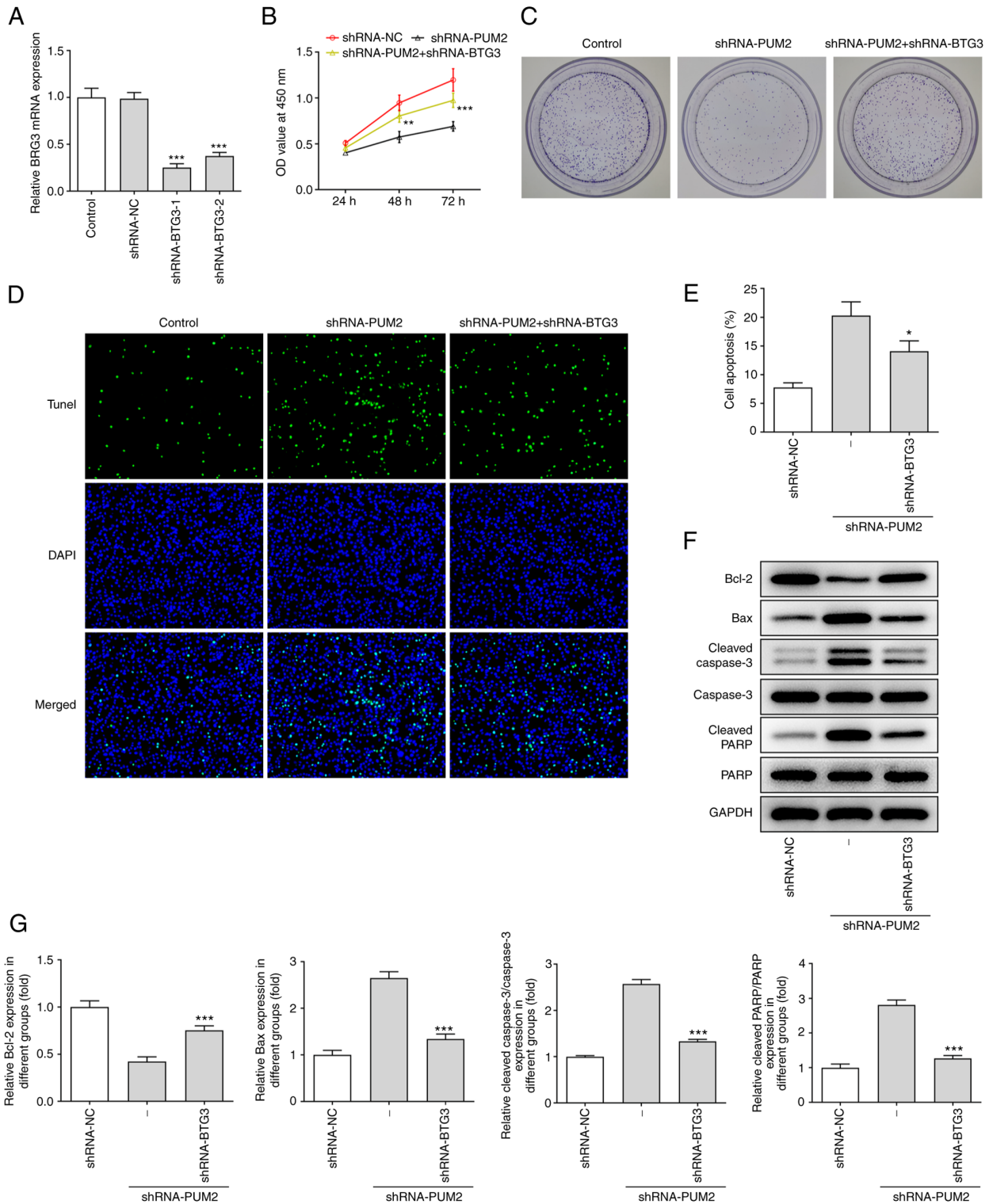


Figure 5. PUM2 silencing inhibits Huh-7 cell proliferation and promotes apoptosis by targeting BTG3. (A) mRNA expression of BTG3 in Huh-7 cells after BTG3 was silenced were detected by reverse transcription-quantitative PCR. (B) A Cell Counting Kit-8 assay and (C) colony-formation assay were used to examine cell proliferation. (D and E) A TUNEL assay was employed to determine apoptosis in Huh-7 cells transfected with shRNA-BTG3. (D) Representative images of TUNEL staining (magnification, x200) and (E) quantified results. (F and G) Western blot analysis was utilized to evaluate the protein level of Bcl-2, Bax, caspase3, cleaved caspase3, PARP and cleaved PARP. (F) Representative western blots and (G) quantified protein levels. Values are expressed as the mean \pm standard deviation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. shRNA-NC in A or vs. shRNA-PUM2 in B-G. PUM2, Pumilio homolog 2; NC, negative control; shRNA, short hairpin RNA; PARP, poly(ADP ribose) polymerase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; OD, optical density; BTG3, B-cell translocation gene 3.

of HCC to a certain extent; however, the 5-year survival rate of patients with HCC remains $< 26\%$ (23,24). Therefore, it is important to further explore the pathogenesis of HCC and

identify biomarkers for evaluation of the prognosis of the disease (25,26). In the present study, the expression levels of the RNA binding protein PUM2 were elevated in HCC

tumor tissues and cell lines. Silencing of PUM2 inhibited HCC cell proliferation and induced cell apoptosis. In addition, PUM2 was demonstrated to bind to the 3'UTR of BTG3 and regulate BTG3 expression in Huh-7 cells. Downregulation of BTG3 expression reversed the effects of PUM2 knockdown on Huh-7 cell proliferation and apoptosis. Taken together, the present study demonstrated for the first time, to the best of our knowledge, that PUM2 may be a therapeutic target for HCC, which has a regulatory role by binding to the 3'UTR of BTG3.

The RNA-binding proteins of the Pumilio and FBF (PUF family) have crucial roles in the occurrence and development of multiple diseases (27-29). The PUF family proteins have regulatory roles by combining with Pumilio binding elements located on the 3'UTR of specific mRNAs (30). PUM2 is the mammalian member of the PUF family that has been extensively investigated in germ and stem cells; however, its involvement in the development of various cancer types remains to be fully elucidated (31-33). A recent study reported that PUM2 expression was upregulated in cervical cancer (CC) tissues, while its silencing inhibited the viability of CC cells (34). An additional study revealed that knockdown of PUM2 expression significantly suppressed cell proliferation and migration, while promoting apoptosis of the glioblastoma cell lines U87 and U251 (14). In the present study, PUM2 expression was upregulated in HCC tumor tissues and cell lines. Furthermore, PUM2 knockdown reduced cell proliferation and induced apoptosis of Huh-7 cells, which is consistent with the previously published reports.

BTG3 belongs to the B-cell translocation gene/transducer of the Erb-B2 receptor tyrosine kinase 2 protein family (35). It has been indicated that BTG3 serves as a tumor suppressor gene in various cancer types, including lung adenocarcinoma (36), oral squamous cell cancer (37) and renal cell carcinoma (38). StarBase was used to predict whether PUM2 binds to BTG3. Bioinformatics analysis also revealed a reaction element responsible for the binding of PUM2 with the 3'UTR of BTG3. The RPISeq website predicted that the index of PUM2 required to bind to the 3'UTR of BTG3 (positive) was 0.99. In addition, the mRNA and protein levels of BTG3 were upregulated in Huh-7 cells following knockdown of PUM2. The binding of PUM2 to the 3'UTR of BTG3 was confirmed by luciferase reporter and RIP assays. Ren *et al* (39) demonstrated that knockdown of BTG3 expression accelerated the proliferation, migration and invasion of gastric cancer cells. Wang *et al* (40) further reported that BTG3 reversed the effects of miR-519c-3p overexpression to promote Hep3B cell proliferation, migration and invasion in HCC. The results of the present study are consistent with these findings and indicate that BTG3 silencing reverses the suppressive effects of shRNA-PUM2 in HCC cells by promoting the shRNA-PUM2-mediated inhibition of cell proliferation and by reducing the cell apoptosis rate. Collectively, these findings indicated that BTG3 acts as a tumor suppressor in HCC. The findings also indicated that BTG3 was involved in the regulatory roles of PUM2 in HCC.

In conclusion, the results of the present study demonstrated that PUM2 expression was upregulated in HCC and that it was associated with poor prognosis. PUM2 regulated cell proliferation and apoptosis of HCC cells by directly binding to the 3'UTR of BTG3. Rescue experiments indicated that BTG3 silencing reversed the effects of PUM2 expression knockdown

on cell proliferation and apoptosis, which implies the potential for this protein to be used as a novel therapeutic target for the treatment of HCC.

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Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author on reasonable request.

Authors' contributions

ZL and CL designed the study, performed the experiments and drafted and revised the manuscript. ZL analyzed the data and performed the literature search. ZL and CL confirmed the authenticity of all the raw data. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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