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, transcatheter 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 a 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
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://geopia.cancer-pku.cn) was used to analyze the overall survival of patients with HCC (method: Overall survival; Group cutoff: Median; Cut-off 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.gdcb.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 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 µg/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'-GCAATATAGTGTTGTATATA-3'; shRNA-PUM2-2: 5'-CATAGTTGTGACTGTAAA-3'), the specific shRNA targeting BTG3 (shRNA-BTG3-1: 5'-GATATGTGAGACCATAA-3'; shRNA-BTG3-2: 5'-GATTACATCACCACATTTGA-3') and the corresponding control shRNA (shRNA-NC: 5'-CCGGCAAACAAGATGAAGAGACACACTC-3') were designed 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 µl 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 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 µg 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'-GGGATGGGAGAGCAGATTCA-3' and reverse, 5'-AGGATTAGGAAGAGCCC-3'; BTG3 forward, 5'-TCCACCTCTCTAATGTGC-3' and reverse, 5'-TCCGGTCACATCGATC-3'; GAPDH forward, 5'-GGGAAACTGTGGCCTGAT-3' and reverse, 5'-GATGCGGTTGCCTGTGTA-3'. The relative expression levels of the target gene were calculated by the relative quantification (ΔΔCT) 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%.
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 30µg 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),
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 ± 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 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 ± standard deviation. ***P<0.001 vs. shRNA-NC. PUM2, Pumilio homolog 2; NC, negative control; shRNA, short hairpin RNA; OD, optical density.
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

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**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 ± 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.
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).

**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.
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 BTG. The RPISeq website predicted that the index of PUM2 required for this protein to be used as a novel therapeutic target for the treatment of HCC.

Acknowledgements
Not applicable.

Funding
No funding was received.

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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