Ribonucleic acid-binding protein CPSF6 promotes glycolysis and suppresses apoptosis in hepatocellular carcinoma cells by inhibiting the BTG2 expression

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Abstract
Hepatocellular carcinoma (HCC) is currently the sixth most common malignancy and the second major cause of tumor-related deaths in the world. This study aimed to investigate the role of cleavage and polyadenylation factor-6 (CPSF6) and B-cell translocation gene 2 (BTG2) in regulating the glycolysis and apoptosis in HCC cells. The RNA and protein expression of CPSF6 and BTG2 in normal hepatocyte and HCC were, respectively, detected by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) analysis and Western blot analysis. The viability and apoptosis of transfected Huh-7 cells were, respectively, analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay. The expression of apoptosis-related proteins and HK-2 in transfected Huh-7 cells was also detected by Western blot analysis. The levels of glucose and lactate in the culture supernatant of transfected Huh-7 cells were, respectively, detected with the glucose assay kit and lactate assay kit. The interaction of CPSF6 and BTG2 was confirmed by RNA binding protein immunoprecipitation (RIP) assay. As a result, CPSF6 expression was increased while BTG2 expression was decreased in Huh-7 cells. Interference with CPSF6 suppressed the viability and glycolysis, and promoted the apoptosis of Huh-7 cells. Furthermore, CPSF6 interacted with BTG2 and interference with CPSF6 upregulated the BTG2 expression and inhibited the protein kinase B (AKT)/extracellular signal-regulated kinase (ERK)/nuclear factor (NF)-κB pathway. Interference with BTG2 could partially reverse the above cell changes caused by interference with CPSF6. In conclusion, CPSF6 inhibited the BTG2 expression to promote glycolysis and suppress apoptosis in HCC cells by activating AKT/ERK/NF-κB pathway.

Keywords: CPSF6, BTG2, Glycolysis, Hepatocellular carcinoma cells, AKT/ERK/NFκB pathway
Introduction
Hepatocellular carcinoma (HCC) was a pathological type with the highest incidence in liver malignant tumor, accounting for about 85% of primary liver cancer. The incidence of HCC was the sixth highest among all malignancies and the second highest among all tumor-associated causes of death [1, 2]. Despite the improvement and optimization of HCC treatment protocols in recent years, the poor prognosis of HCC patients remains unchanged. The high recurrence rate after treatment and the invasion and metastasis inside and outside the liver are the main reasons for the failure of HCC therapy [3–5]. Therefore, it is necessary to screen new and effective diagnostic and therapeutic targets for HCC to open up new approaches for early diagnosis and treatment of HCC.

Cleavage and polyadenylation factor-6 (CPSF6), as one of the cleavage factor Im (CFIm) subunits during alternative polyadenylation (APA) of mRNA, has been thought to be related to the occurrence and development of cancer in recent years [6]. HER2-overexpressing and triple-negative subtypes depend on CPSF6 for viability and tumorigenic capacity in aggressive breast cancer cells of luminal B, and CPSF6 is overexpressed in human breast cancer cases, which is associated with poor prognosis [7]. In breast cancer, downregulation of CPSF6 leaded to the decrease of proliferation, migration and invasion of cells [8]. In the colon cancer cells expressing Snail1, expression of CPSF6 and splicing factor proline/glutamine-rich (SFPQ) is higher than that of the control group, suggesting the pro-oncogenic effect of CPSF6 [9]. ENCORI (http://starbase.sysu.edu.cn/index.php) shows that CPSF6 is upregulated in patients with HCC and is associated with poor prognosis (Additional file 1: Figure S1A, B).

According to the prediction of ENCORI, CPSF6 as an RNA binding protein can bind and regulate B-cell translocation gene 2 (BTG2). BTG2, a member of the BTG/TOB family, was the first gene to be identified as a recognized tumor suppressor gene with anti-proliferation properties. BTG2 was identified as a direct and functional target of miR-6875-3p. miR-6875-3p promoted the epithelial–mesenchymal transition (EMT) and promotes proliferation and metastasis of HCC cells by down-regulating BTG2 expression [10]. Moreover, upregulated expression of BTG2 inhibited the migration, invasion, EMT and glycolysis in lung adenocarcinoma (LUAC) cells and tumor growth [11]. In addition, BTG2 expression in liver cancer cells was obviously declined and upregulation of BTG2 expression suppressed the proliferation and invasion while enhanced the apoptosis of HepG2 cells [12]. Kaplan–Meier curves indicate that liver cancer patients with high BTG2 expression have significantly longer survival time than those with low BTG2 expression (Additional file 2: Figure S2).

Therefore, this study was to explore the role of CPSF6 and BTG2 in regulating the glycolysis and apoptosis in HCC cells and the underlying mechanism.

Results
Expression level of CPSF6 in HCC cells
The mRNA expression of CPSF6 was obviously upregulated in hepatocellular carcinoma cells compared with THLE-3 cells and the mRNA expression of CPSF6 in Huh-7 cells was the highest (Fig. 1A). The protein expression of CPSF6 in those cells was consistent with mRNA expression (Fig. 1B). Therefore, Huh-7 cell line was selected.
Interference with CPSF6 promotes apoptosis of HCC cells

After Huh-7 cells were transfected with sh-CPSF6-1/2, CPSF6 mRNA expression was markedly decreased and lower in sh-CPSF6-2 group (Fig. 2A), which was consistent with the results of western blot analysis (Fig. 2B), thereby sh-CPSF6-2 was selected for subsequent experiment. Interference with CPSF6 significantly suppressed the viability (Fig. 2C) and obviously promoted the apoptosis (Fig. 2D) of Huh-7 cells. Interference with CPSF6 suppressed the Bcl-2 expression while enhanced the expression of Bax, cleaved-caspase3 and cleaved-caspase9 (Fig. 2E).

Interference with CPSF6 inhibits glycolysis of HCC cells

After Huh-7 cells were transfected with sh-CPSF6-2, the ability of glucose uptake (Fig. 3A) and lactate production (Fig. 3B) was obviously declined. The level of glycolytic enzyme HK-2 was also lowly expressed in sh-CPSF6-2 transfected Huh-7 cells (Fig. 3C).

CPSF6 as an RNA binding protein can bind and regulate BTG2 expression

The mRNA expression of BTG2 was remarkably declined in Huh-7 cells compared with THLE-3 cells (Fig. 4A), which was consistent with the protein expression of BTG2 (Fig. 4B). RIP assay indicated that CPSF6 was interacted with BTG2 (Fig. 4C). The mRNA expression of BTG2 was obviously reduced in Huh-7 cells transfected with shRNA-BTG2-1/2 (Fig. 4D), which was also observed in protein expression of BTG2 (Fig. 4E), thereby shRNA-BTG2-1 was chosen. Interference with CPSF6 significantly upregulated the mRNA and protein expression of BTG2, which was partially reversed by interference with BTG2 (Fig. 4F, G).

Interference with BTG2 can partially reverse the effects of interference with CPSF6 on apoptosis and glycolysis of HCC cells

Interference with BTG2 markedly promoted the viability (Fig. 5A) and suppressed the apoptosis (Fig. 5B, C) of Huh-7 cells transfected with sh-CPSF6-2. Interference with BTG2 significantly increased the Bcl-2 expression and obviously decreased the expression of Bax, cleaved-caspase3 and cleaved-caspase9 in Huh-7 cells transfected with sh-CPSF6-2 (Fig. 5D). Interference with BTG2 notably improved the
ability of glucose uptake (Fig. 5E) and lactate production (Fig. 5E), and also markedly increased the glycolytic enzyme HK-2 (Fig. 5F) in Huh-7 cells transfected with sh-CPSF6-2.

**Interference with CPSF6 inhibits AKT/ERK/NF-κB signaling by upregulating BTG2 expression**

Interference with CPSF6 notably suppressed the expression of p-AKT, p-ERK, p-IKKβ and p-p65 in Huh-7 cells, which was partially increased by interference with BTG2 (Fig. 6).
Discussion

Treatment for HCC has been less than satisfactory, and incidence has increased over the last 20 years due to viral infections such as hepatitis B virus (HBV) and hepatitis C virus (HCV), as well as multiple factors such as alcoholism and aflatoxin [13–15]. Therefore, it is particularly important to further explore the molecular mechanism and new targets in the occurrence and development of HCC.

CPSF6 is localized on human chromosome 12 with a molecular weight of 68 kDa. Previous studies have shown that CPSF6 is associated with a variety of human diseases, including acute myeloid leukemia [16, 17], myeloproliferative neoplasms [18], and human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) [19]. Studies have shown that CPSF6 promotes the development of colon cancer [9] and CPSF6 and all core paraspeckles proteins have found to be highly expressed in human breast cancer, which is correlated with poor prognosis [7]. In the present study, CPSF6 expression was confirmed to be upregulated and interference with CPSF6 suppressed the viability and promoted the apoptosis of Huh-7 cells.

BTG1 and BTG2 have been considered as regulators of genotoxic and cellular stress signaling pathways, which can modulate cell apoptosis or survival [20]. In breast carcinoma, low BTG2 expression is related to increased tumor grade, disease progression

Fig. 3 Interference with CPSF6 inhibits glycolysis of hepatocellular carcinoma cells. A The glucose uptake activity of Huh-7 cells transfected with sh-CPSF6-2 was analyzed by glucose assay kit. B The lactate production of Huh-7 cells transfected with sh-CPSF6-2 was analyzed by lactate assay kit. C HK-2 expression in Huh-7 cells transfected with sh-CPSF6-2 was detected by Western blot analysis. **P < 0.01 and ***P < 0.001 vs. Control group. #P < 0.01 and ###P < 0.001 vs. sh-NC group. N = 3
and poor overall survival. Knockdown of BTG2 causes increased cyclin D1 expression and elevated AKT phosphorylation [21–24]. The increased level of cyclin D1/cyclin E in liver cancer leading to the decreased BTG2 expression causes the increase of tumor grade [25]. In prostate cancer, BTG2 suppression promotes disease progression, therapy resistance, and metastasis [26, 27]. These findings indicate that BTG2 has inhibitory action on tumorigenesis, which is also confirmed in knockout and overexpression of BTG2 in medulloblastoma mice [28, 29]. In this study, BTG2 expression was also decreased in HCC cells. In addition, interference with BTG2 could partially reverse the effects of interference with CPSF6 on the proliferation and apoptosis of HCC cells.

Warburg has found a special phenomenon that in the condition of adequate oxygen supply, tumor cells still show a high level of glycolysis, which produces ATP to supply energy for the metabolic activities of cells, known as aerobic glycolysis or Warburg effect [30, 31]. Tumor cells are more sensitive to glycolysis inhibition [32] and targeting metabolic pathways provides a new strategy for the treatment of tumors.
IGF-1R is a transmembrane receptor widely present on the surface of human cells. The expression of IGF-1R is upregulated in HCC, and inhibition of IGF-1R can significantly inhibit the proliferation, migration and invasion of HCC [34]. A study has shown that miR-342-3p directly acts on IGF-1R 3′UTR and reduces the expression of IGF-1R, thereby lowering GLUT1 level, inhibiting glucose uptake of lactic acid to produce ATP, and inducing glycolysis to mitochondrial respiration [35]. miR-199a-5p directly acts on the 3′UTR of HK2, thus inhibiting the generation of glucose consumption lactic acid, reducing cell glucose-6-phosphate (G6P) and ATP levels, affecting cell proliferation and leading to the occurrence of HCC cells [36]. The above
findings have confirmed that glycolysis is very important in HCC. Here, we found that interference with CPSF6 inhibited glycolysis of HCC cells, which could partially increase glycolysis by the interference with BTG2.

The tumor suppressor BTG2 served as a regulator of myocardial necrosis by inhibiting AKT/ERK while activating glycogen synthase kinase 3 (GSK3) and cyclophilic protein D [37]. miR-650 binds directly to the 3’UTR of ras-related estrogen-regulated growth inhibitor (RERG) and activates the AKT/ERK/NF-κB pathway mediated by the RERG/PH Domain Leucine-rich Repeat Protein Phosphatase (PHLPP) 2 complex to promote the growth of glioblastoma multiforme [38]. The inhibiting effect of licorice flavone A on glycolysis is mainly due to the blockage of AKT signaling pathway, and the inhibition of glycolysis is greatly weakened when AKT is overexpressed [39]. In uncoupling protein 2 (UCP2) knockdown gallbladder cancer cells, proliferation and glycolysis is suppressed and IKKβ and downstream signaling molecules NF-κB/FAK/β-catenin was also downregulated [40]. Of course, interference with CPSF6 blocked the NF-κB/FAK/β-catenin pathway to inhibit the glycolysis of HCC cells by upregulating BTG2 expression.

In conclusion, CPSF6 expression was increased and BTG2 expression was decreased in Huh-7 cells. CPSF6 could bind with BTG2. Interference with CPSF6 promoted apoptosis and inhibited glycolysis of Huh-7 cells by suppressing the NF-κB/FAK/β-catenin pathway, which was partially reversed by BTG2 expression. However, this study is limited for cell experiment. Animal experiment will be used to further confirm the present conclusion in our future study.
Materials and methods

Cell culture
Normal hepatocyte (THLE-3) and HCC cells (SK-Hep-1, Huh-7, SNU-449, HCCLM3 and MHCC97H) were obtained from American Type Culture Collection (ATCC). The whole cells were placed in DMEM medium (Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.) for routine culture and passage in a biochemical incubator at 37 °C and 5% CO2.

Cell transfection
Synthetic sequences of short hairpin RNA (shRNA) targeting CPSF6 (sh-CPSF6; Shanghai GenePharma co., ltd.), shRNA targeting BTG2 (sh-BTG2) and non-targeting shRNA (shRNA-NC) were inserted into pGPU6/Neo vector (Shanghai GenePharma Co., Ltd.). Trypsin was used to digest the Huh-7 cells to prepare the cell suspension and Huh-7 cells were seeded into a 96-well plate (1 × 10^4 cells/well). Then, Huh-7 cells were transfected with sh-NC, sh-CPSF6-1/2 and shRNA-BTG2-1/2 in different combinations using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). Transfected Huh-7 cells were collected 24 h later for subsequent detection.

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) analysis
The cells in each group were collected and added with 20 times of the volume of TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for lysis. Cells were repeatedly beaten with a disposable syringe to make full lysis. RNA isolation kit (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract the total RNA, which was reverse transcribed into cDNA, and fluorescence quantitative PCR instrument was used for detection. CPSF6 forward, 5'-GGAGCAGCACCACAGGGTGTGTC-3' and reverse, 5'-CTCCCAAGAATGAACGTCTTC-3'; BTG2 forward, 5'-CATCACGAGCGTGC-3' and reverse 5'-CCCAGTGCGTGAGC-3'; GAPDH forward, 5'-GTCGCCCACCACTGTATGCG-3' and reverse 5'-GGCATGGACTGTGGTGTATGAG-3'. The 2−ΔΔCt method is used to standardize the relative expression of CPSF6 and BTG2.

Western blot analysis
The cells were lysed with RIPA lysis (Beyotime) and centrifuged to extract the proteins which were quantified by BCA Protein Assay kit (Beyotime). The proteins were denatured by boiling for 10 min and 60 μg proteins were subjected to 12% SDS-PAGE electrophoresis (Beyotime) per lane. After proteins were transferred to PVDF membranes (EMD Millipore), PVDF membranes were placed in TBST containing 5% skimmed milk powder, which was slowly shaken and sealed for 3 h at room temperature. The primary antibodies [CPSF6 (ab175237; dilution, 1:10000; Abcam), Bcl-2 (ab32124; dilution, 1:1000; Abcam), Bax (ab32503; dilution, 1:1000; Abcam), cleaved-caspase3 (#9664; dilution, 1:1000; Cell signaling technology), cleaved-caspase9 (#20750; dilution, 1:1000; Cell signaling technology), caspase3 (#9662; dilution, 1:1000; Cell signaling technology), caspase9 (#9502; dilution, 1:1000; Cell signaling technology), HK-2 (#2867; dilution, 1:1000; Cell signaling technology), BTG2
(ab197362; dilution, 1:500; Abcam), p-AKT (#4060; dilution, 1:2000; Cell signaling technology), t-AKT (#9272; dilution, 1:1000; Cell signaling technology), p-ERK (ab201015; dilution, 1:1000; Abcam), t-ERK (ab184699; dilution, 1:10000; Abcam), p-IKKβ (#2697; dilution, 1:1000; Cell signaling technology), t-IKKβ (ab97406; dilution, 1:1000; Abcam), p-p65 (ab76302; dilution, 1:1000; Abcam), t-p65 (ab32536; dilution, 1:1000; Abcam) and GAPDH (ab9485; dilution, 1:2500; Abcam) were added to the membranes for culture at 4 °C overnight and then membranes were washed with TBST for three times. The horseradish peroxidase-linked IgG second antibody (#7074; dilution, 1:1000; Cell Signaling Technology, Inc.) was added to the membranes, which were slowly shaken and cultured for 1 h at room temperature. After washing the membrane, the protein expression was detected by ECL chemiluminescence method, and the expression levels of above proteins were reflected by the ratio of the gray values of above protein bands to that of GAPDH band.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay
Huh-7 cells were digested with 0.25% trypsin (1 mL) for 3 min, inoculated into a 96-well plate with density of 1 × 10⁴ cells/well, and cultured at 37 °C for 24 h. Then, Huh-7 cells were transfected and, respectively, cultured for 24 h, 48 h and 72 h. MTT solution (5 mg/mL) (Beyotime) was added to the plate with 0.02 mL per well. After further culture for 4 h under the original culture conditions, the supernatant in the wells was poured out, and 0.15 mL DMSO was added to each well, which was placed on the oscillator for 10 min to completely dissolve the crystals. The absorbance value of each well with the wavelength of 490 nm was determined by a Multiskan™ Go microplate spectrophotometer (Thermo Fisher Scientific, Inc.).

Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assay
Huh-7 cells were seeded into a 6-well plate with 1 × 10⁵ cells/well. After cell fusion, cells were transfected accordingly for 24 h, followed by the abandonment of medium. The transfected Huh-7 cells were fixed with 4% formaldehyde for 15 min, dehydrated with 50%, 75%, 95% and 100% ethanol for 5 min, washed with phosphate buffer twice, and treated with 0.5% Triton x-100 for 20 min. TUNEL working solution (Beyotime) was added for incubation at 37 °C for 1 h. Apoptotic cells (green fluorescence staining) were detected by fluorescence microscope (Olympus corporation).

Detection of glucose and lactate
Huh-7 cells at logarithmic growth stage were inoculated into a 96-well plate with density of 1 × 10⁴ cells/well. After indicated transfection for 24 h, the supernatant of the cells in each group was taken, and the levels of glucose and lactate in the culture supernatant was, respectively, detected with the glucose assay kit (Elabscience) and lactate assay kit (Elabscience).

RNA binding protein immunoprecipitation (RIP)
Transfected Huh-7 cells were collected and treated according to the RIP kit instructions. Rabbit IgG antibody (#3900; dilution, 1:100); or anti-CPSF6 antibody (ab175237; dilution, 1:100; Cell Signaling Technology, Inc.) was added to cell lysate and incubated
at 4 °C overnight to obtain RNA–protein complex RIP-IgG or RIP-CPSF6. The RNA was extracted and qPCR assay was conducted to detect the enrichment of BTG2 in the RNA–protein complex.

Statistical analysis
GraphPad Prism 8.0 software (GraphPad Software) was used for statistical analysis of experimental data in the form of mean ± standard deviation (SD) and preparation of figures. One-way analysis of variance (ANOVA) followed by Tukey’s post hoc test and unpaired t-test, respectively, analyzed the differences in the multiple groups and two groups. Statistical significance was considered to be P < 0.05.

Supplementary Information
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Authors’ contributions
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Availability of data and materials
The experimental data will be available on the request.

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Ethics approval and consent to participate
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Competing interests
The authors declare they have no competing interests.

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