PFKFB3 is involved in breast cancer proliferation, migration, invasion and angiogenesis

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Abstract. 6-Phosphofructo 2-kinase/fructose 2, 6-bisphosphatase 3 (*PFKFB3*) has been reported to be overexpressed in human cancer tissues and to promote the proliferation and migration of cancer cells. However, the role of *PFKFB3* in the progression and prognosis of breast cancer is not yet fully understood. In the present study, we investigated the specific role of *PFKFB3* in breast cancer progression and its preliminary mechanisms of action. We first used an immunohistochemistry assay to determine that *PFKFB3* was highly expressed in breast cancer tissues and that this high level of expression was involved in the poor overall survival of patients with breast cancer. In addition, the suppression of *PFKFB3* by lentiviruses carrying shRNA against *PFKFB3* (sh*PFKFB3*) subsequently inhibited breast cancer cell (MDA-MB-231 and MDA-MB-468) proliferation, migration and invasion, and induced cell cycle G_1 and S phase arrest *in vitro*. Moreover, *PFKFB3* inhibition decreased p-AKT and increased p27 expression levels in breast cancer cells. Furthermore, *PFKFB3* suppression inhibited breast cancer cell tumor xenograft growth in nude mice. We also found that PFKFB3 inhibition suppressed vascular endothelial growth factor α (VEGFα) protein expression and inhibited the angiogenic activity of human umbilical vein endothelial cells (HUVECs). On the whole, our results indicate that *PFKFB3* is involved in the proliferation, migration, invasion and angiogenesis of breast cancer.

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Introduction

Breast cancer (BC) is thought to have the highest diagnostic rate in cancer and is the principal cause of cancer-related mortality in women worldwide (1). Thus far, the pathological diagnosis has long been recognized to be the gold criteria for the diagnosis and pathology-based classifications that are essential for guiding the therapy of patients with BC. However, currently, gene expression analyses have a considerable influence on our understanding of the biology and molecular analysis of BC, thereby providing clinically relevant information and targeted therapy (2).

The 6-phosphofructo 2-kinase/fructose 2, 6-bisphosphatase (*PFKFB*) family is composed of bifunctional enzymes that control the level of fructose 2,6-bisphosphate (Fru-2, 6-BP), an essential allosteric activator in the glycolytic flux (3). There are 4 isoforms of this enzyme encoded by distinct genes, *PFKFB1*, *PFKFB2*, *PFKFB3* and *PFKFB4*, and each isozyme has been found to be expressed in different tissues (4-6). The third PFKFB isozyme encoded by the *PFKFB3* gene has also been termed PGR1, inducible PFK-2 (iPFK-2), placental PFK-2 and ubiquitous PFK-2 (uPFK-2) (4). Compared with the other isozymes, the *PFKFB3* isozyme has a very high kinase activity due to its crystal structure and has been thoroughly researched (4,7). Previous findings have reported a close correlation between the aberrant expression of PFKFBs and the tumor aggressiveness grade, which demonstrates that *PFKFBs* may play a crucial role in carcinogenesis (3). *PFKFB* has been found to be overexpressed in various tumor types, particularly in cancers of the colon (8), prostate (9), ovaries (9) and thyroid (9). Upregulated *PFKFB3* levels have been correlated with poorer survival statistics in patients with human epidermal growth factor receptor 2 ($HER2^+$) BC (10), and

its inhibition seems to suppress glucose metabolism and the growth of $HER2^+$ BC (10). The overexpression of microRNA (miRNA)-206 has been demonstrated to regulate PFKFB3 expression, possibly suppressing the proliferation and migration of BC cells (11). Targeting *PFKFB3* regulates cell the proliferation and apoptosis of bladder cancer cells by altering the tumor microenvironment (12). Taken together, these results suggest that *PFKFB3* plays important roles in several biological processes and in the progression of human cancers.

Glycolysis is important in the initiation of angiogenesis in tumor vessels (13) and since *PFKFB3* is a key regulator of endothelial glycolysis metabolism, the inhibition or deletion of endothelial *PFKFB3* decreases angiogenesis (13). Primary tumor growth and metastasis is dependent on angiogenesis (14). Tumor cells preferentially metabolize glucose through aerobic glycolysis, a phenomenon known as the 'Warburg effect' (15). There are many genes, molecular signals and metabolic pathways involved in the movement and activity of angiogenesis in tumor cells. *PFKFB3* expression is upregulated by vascular endothelial growth factor (VEGF), thereby increasing the rate of glycolysis, and promoting the budding of blood vessels (16). Previous studies have determined that *PFKFB3* plays an important role in regulating tumor glycolysis, cell proliferation and survival. AKT is a serine-threonine-kinase that phosphorylates proteins in several pathways regulating metabolism and proliferation in cancer cells (17). Activated AKT regulates a number of targets implicated in tumor progression (18) and *PFKFB3* overexpression has been reported to increase phosphorylated AKT (p-AKT) expression, thereby preventing apoptosis in an osteoarthritic cartilage explant (19). p27 is a cell cycle inhibitor that regulates cell proliferation, motility and apoptosis (20) and its loss may precede tumor invasion (21). Increased levels of p27 protein have been demonstrated in the nucleus and cytoplasm after *PFKFB3* knockdown in HeLa cells (22). The inhibition of the cell cycle and the induction of apoptosis caused by the suppression of *PFKFB3* can be attenuated by p27 inhibition (23). Furthermore, since tumor vessel formation is important for tumor progression, the inhibition of VEGF, which is a main regulator of angiogenesis in vascular endothelial cells (ECs), has provided benefits to a large number of cancer patients (24,25).

In the present study, in order to gain insight into the molecular mechanisms behind the regulation of *PFKFB3* expression in BC, we identified the expression status of *PFKFB3* in BC tissues and the correlation between the *PFKFB3* expression level and the prognosis of patients with BC. We sequentially investigated the specific role of *PFKFB3* in BC cell proliferation, migration, invasion, cell cycle progression and angiogenesis, as well as whether the AKT-related pathway, p27, and VEGF participate in the above-mentioned process.

Materials and methods

Ethics statement. This study was approved by the Ethics Committee of Sun Yat-Sen University (Guangzhou, China). The collection and analysis of clinical specimens were sanctioned by the local Ethics Committee of Sun Yat-sen University Cancer Center. Written informed consent was obtained from each patient prior to participation. The animal experiments were approved by the Animal Ethics and Welfare Committee of Sun Yat-Sen University.

Clinical specimens and cell culture. Breast tissue specimens in this study were collected from 74 patients with BC who had undergone surgery at the Sun Yat-sen University Cancer Center from 2004 to 2012. These cases included 49 women aged ≤50 years and 25 women aged >50 years. The mean age of the study population was 47.83±10.37 years. None of the patients had received pre-operative treatment, such as chemotherapy or radiotherapy, prior to surgery. The normal breast tissues were obtained with at least 5 cm clearance around the tumor, and all samples were examined histologically. The clinical pathological characteristics of all 74 patients and their correlation with PFKFB3 expression are shown in Table I.

Table I. Correlation between *PFKFB3* expression and clinicopathological characteristics of patients with breast cancer.

	PFKFB3 expression score				
Characteristics	All	1	2	3	P-value
Age (years)					
≤50	49	13	22	14	0.928
>50	25	10	5	10	
ER status					
-	74	16	29	29	N/A
+	0	0	0	0	
PR status					
-	74	16	29	29	N/A
+	0	0	0	0	
HER-2 status					
-	74	16	29	29	N/A
+	0	0	0	0	
TNM stage					
I, II	34	6	16	12	0.428
III, IV	40	10	13	17	
Tumor size (cm)					
≤3	54	18	17	19	0.286
>3	20	5	10	5	
Distant metastasis					
0 (No)	55	21	19	15	**0.025**[a]
1 (Yes)	19	2	8	9	
Recurrence					
No	66	22	24	20	0.179
Yes	8	1	3	4	
Differentiation					
Moderate	66	20	24	22	0.608
Well	8	3	3	2	

[a]Bold font indicates statistical significance. ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor 2; N/A, not applicable.

The human BC cell lines, MDA-MB-231 and MDA-MB-468, and human umbilical vein endothelial cells (HUVECs) were all purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The MDA-MB-231 and MDA-MB-468 cells were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA), and the HUVECs were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS; Gibco). All cells were cultured in a humidified incubator at 37˚C in a 5% CO_2 atmosphere.

Immunohistochemistry. Subsequently, the tissues were embedded in paraffin and cut into 4-μm-thick sections, followed by treatment with xylene to remove the paraffin, then rehydrated and treated with 0.3% hydrogen peroxide for 10 min to eliminate endogenous peroxidase activity. The sections were blocked with 5% goat serum and incubated for 20 min at room temperature. After washing, the sections were treated with rabbit polyclonal anti-*PFKFB3* antibody raised to the recombinant protein for 30 min (1:200 dilution, ab96699; Abcam, Cambridge, MA, USA). The primary antibody was replaced with 1% non-immune rabbit serum (negative control), followed by incubation with an anti-*PFKFB3* antibody in the presence of excess recombinant PFKFB3. The sections were subsequently incubated with biotinylated goat anti-rabbit immunoglobulin (Vector Laboratories, Burlingame, CA, USA) and were developed with the Vectastain Elite BCA kit (Vector Laboratories) as chromogen, according to the manufacturer's recommendations. Following counterstaining with Mayer's hematoxylin (Sigma-Aldrich, St. Louis, MO, USA), the sections were dehydrated and coverslips were attached with neutral resins (Thermo Fisher Scientific, Pittsburgh, PA, USA). Each experiment was performed twice. The expression of *PFKFB3* protein was assessed by a proportion score of the tumor cells and was categorized into scores of 1 (<5% of tumor cells stained positive), 2 (5-25% of tumor cells stained positive) and 3 (>25% of tumor cells stained positive), as previously described (26), with higher scores indicating a greater proportion of positive cells. In order to investigate the association of *PFKFB3* expression with the overall survival (OS) of patients, the survival analysis was performed using the Kaplan-Meier method.

To further investigate the prognostic value of *PFKFB3* expression in BC, an online survival analysis was performed via the online database Kaplan-Meier Plotter (http://kmplot.com/analysis/), which contained expression data from 54,675 genes and survival information from 5,143 breast cancer patients. In order to analyze the OS (n=1,402), recurrence-free survival (RFS) (n=3,951), distant metastasis-free survival (DMFS) (n=1,746), and post-progression survival (PPS) (n=414) of the patients with BC, the patient samples were split into 2 groups by median expression (high or low expression), with a hazard ratios (HR), 95% confidence intervals (CI) and log-rank P-values. The gene symbol *PFKFB3* (202464_s_at) was selected to obtain Kaplan-Meier plots in which the number at risk was indicated below the main plot.

Lentiviral vector construction and transduction. The *PFKFB3* full-length cDNA was amplified by RT-PCR using mRNA from the MDA-MB-231 cells with specifically designed primers (forward primer, 5'-TAG GAT CCA TGG ACT ACA AGG ACG ACG ACG ACA AGT TGG AAC TGA CGC AGA GCC GA-3'; and reverse primer, 5'-TGA AGC TTG GAA ATG GAA TGG AAC CGA C-3'). The PCR products were then digested with *Bam*HI (R3136; New England BioLabs, Inc., Ipswich, MA, USA) and *Hin*dIII I (D6389; Beyotime Institute of Biotechnology, Shanghai, China) prior to insertion into a lentiviral vector. A lentiviral vector expressing enhanced green fuorescent protein (EGFP) was used as the control. Furthermore, we designed a short-hairpin RNA (shRNA) to target human *PFKFB3* (Guangzhou RiboBio Co., Ltd., Guangzhou, China), and cloned the shRNA into a human U6 promoter-containing pBluescript SK (+) plasmid (pU6). The U6-shRNA cassettes were then sub-cloned into a lentiviral vector. Lentivirus carrying shRNA targeting firefly luciferase (shNC) was used as the control. Lentiviral vectors carrying shRNA targeting *PFKFB3* were generated. A total of $5x10^4$ BC cells (MDA-MB-231 and MDA-MB-468) were then transduced with the lentiviruses (sh*PFKFB3* or NC) as previously described (27). Lentiviruses were harvested 3 days after purification and precipitation.

MTS cell proliferation assay. Cell proliferation was determined by MTS assay with the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). Briefly, 3,000 cells (100 μl/well) were seeded into 96-well plates at a density of $3x10^3$ cells/well and cultured at 37˚C for 1, 2 or 3 days. At these time-points, 20 μl of CellTiter 96® AQueous One Solution Reagent were added to each well, and the cells were then incubated for an additional 4 h at 37˚C in a 5% CO_2 atmosphere. An ELx800 microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) was subsequently used to measure the corresponding absorbance at 490 nm. Each condition was determined in quintuplicate, and all experiments were repeated thrice.

Quantitative PCR (qPCR). Total RNA was extracted from the BC cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and *PFKFB3* cDNA synthesis was performed with a random RT primer using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA). The *PFKFB3* expression level was evaluated by qPCR using SYBR-Green PCR Master Mix (Applied Biosystems, Warrington, UK) and an ABI 7500 real-time PCR system (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), an endogenous housekeeping gene, was used to normalize the relative mRNA expression level of *PFKFB3* with the following primers: *PFKFB3* forward, 5'-GG CCGCATCGGGGGCGACTC-3' and reverse, 5'-TTGCGTCT CAGCTCAGGGAC-3'; and GAPDH forward, 5'-CCGAGA ATGGGAAGCTTGTC-3' and reverse, 5'-AAGCACCAACA GAGGAGAA-3'. The results were normalized to GAPDH expression and RNA enrichments were calculated using the $2^{-\Delta\Delta Cq}$ method (28).

Cell cycle analysis. Cell cycle analysis was performed with propidium iodide (PI; Sigma-Aldrich) staining, as previously described (29). The cells were collected and fixed in 70% (v/v) ethanol on ice, then washed with phosphate-buffered saline (PBS) and suspended in the propidium iodide staining solution

(50 mg/l) supplemented with 0.1% Triton X-100 and RNase (100 mg/l). Following 30 min of incubation, cell fluorescence was measured using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA), and cell cycle analysis was performed with the ModFit LT 2.0 software (Verity Software House Inc., Topsham, ME, USA). Each experiment was performed in triplicate.

Cell migration and invasion assays. The cell migration and invasion assays were evaluated using Transwell cell culture chambers with 8 μm pores (Corning Costar Corp., Cambridge, MA, USA) according to the manufacturer's instructions. For the migration assay, the MDA-MB-231 and MDA-MB-468 cells incubated in serum-free medium (Gibco) were separately seeded into the upper chambers without Matrigel, and the complete medium was supplemented with 10% FBS and was used in the lower chambers as a chemoattractant. For invasion assays, Transwell membranes were pre-coated with 10 μl Matrigel (diluted 1:3; BD Biosciences). Following incubation at 37˚C for 24 h, the migratory and invasive cells on the bottom surface were fixed using 4% paraformaldehyde and stained with a 0.1% crystal violet solution (Sangon Biotech, Co., Ltd., Shanghai, China). The cells that did not migrate were removed from the upper membrane surface with a cotton swab. Five randomly selected fields from each membrane were counted using a light microscope (Nikon Eclipse E600; Nikon, Tokyo, Japan) with a X20 objective. Each experiment was performed in triplicate.

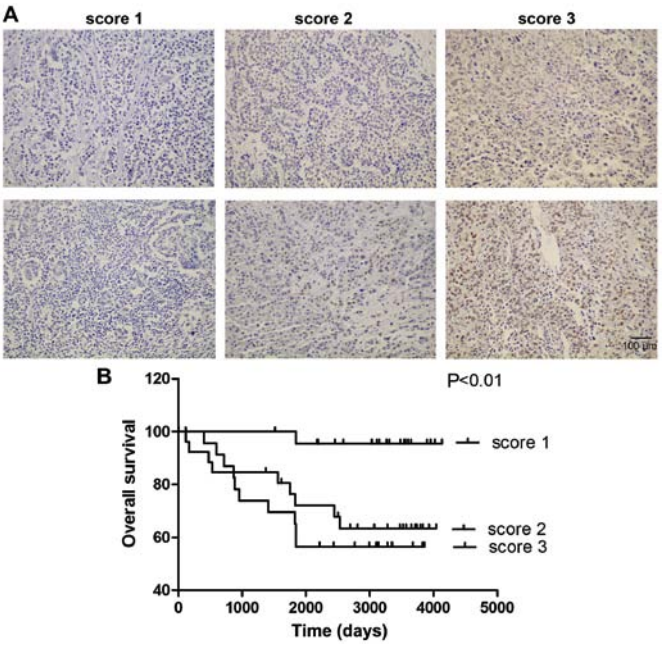

Figure 1. *PFKFB3* expression and prognosis in breast cancer tissues. (A) *PFKFB3* expression was detected by immunohistochemistry in tissues from 74 patients with BC. Nuclear expression was assessed by a proportion score of the positive tumor cells, and was categorized into scores of 1 (<5%), 2 (5-25%), or 3 (>25%) for *PFKFB3*. Scale bar, 100 μm. (B) Kaplan-Meier plots were used to evaluate the effect of *PFKFB3* on the prognosis of patients with breast cancer.

Tube formation assay. The effect of the silencing of *PFKFB3* using a lentiviral vector carrying sh*PFKFB3* on the angiogenesis of BC cells was evaluated *in vitro* with a tube formation assay. The cell supernatant was obtained from the MDA-MB-231 and MDA-MB-468 transfected with sh*PFKFB3* cells by centrifugation at 3,000 x g for 10 min at 4˚C. A total of 30,000 HUVECs were seeded in Matrigel (BD Biosciences) with serum-free medium (Gibco) in the 24-well plates, in triplicate, co-incubated with the above cell supernatant at 37˚C for 12 h, and fixed with 4% paraformaldehyde at room temperature. The tubules were visualized under light microscopy (Nikon Eclipse E600; Nikon) at low magnification (x40). Photomicrographs from each well were captured, and the number of tubules was analyzed using ImageJ software, version 2.02 (National Institutes of Health, Bethesda, MD, USA).

Western blot analysis. The cells were lysed in Radio-Immunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific) supplemented with protease inhibitor cocktail (Thermo Fisher Scientific). The BCA protein assay kit (Qcbio Science Technologies, Co., Ltd., Shanghai, China) was used for the detection of protein concentrations. Proteins (40 μg) were separated using 10% polyacrylamide gels, electrophoresed and transferred to a polyvinylidene fluoride membrane (PVDF; Millipore, Billerica, MA, USA). All the membranes were blocked with 5% skim milk for 2 h at room temperature, and then incubated overnight with the respective primary antibodies at 4˚C. The following day, the membranes were washed using TBST (Tris-buffered saline and Tween-20), and incubated with horseradish peroxidase (HRP)-labeled secondary anti-mouse (ab157532, 1:500) or anti-rabbit IgG antibody (ab6728, 1:500) at room temperature for 1 h. After washing again with TBST, all blots were visualized using an enhanced chemiluminescence substrate kit (Amersham Biosciences Inc., Piscataway, NY, USA). GAPDH was used as the loading control. The related primary antibodies were rabbit anti-GAPDH antibody (sc-47724, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-PFKFB3 antibody (ab96699, 1:100), rabbit anti-AKT antibody (ab8805, 1:100), rabbit anti-p-AKT antibody (ab38449, 1:100), mouse anti-p27 antibody (ab215434, 1:100) and mouse anti-VEGFα antibody (ab42228, 1:100) (all from Abcam, Cambridge, MA, USA).

Tumor xenograft growth in nude mice. A total of 10 BALB/c nude mice (male, 5-6 weeks old, weighing 18-22 g) were purchased from the Shanghai LAC Laboratory Animal Co., Ltd. (Shanghai, China). The nude mice were housed in a specific-pathogen-free (SPF) grade animal center. The housing environment was maintained at 25±2˚C, 45-55% humidity, and a standard 12-h dark/12-h light cycle, and the mice were fed an autoclaved diet with free access to water. At the beginning of the experiment, the mice were subcutaneously injected with $2.5x10^6$ MDA-MB-231 cells transduced with either NC or shPFKFB3 in the front right legs (n=5 mice per group). The tumor sizes were recorded on days 10, 20 and 30 after the injection and the tumor volume (mm^3) was calculated as follows: Volume = 0.5 x length x $width^2$. The maximum diameter of the tumor was approximately 1.0 cm. On the 30th day post-injection, all mice (weighing 25-30 g) were sacrificed by CO_2 inhalation and the tumor nodules were dissected and weighed.

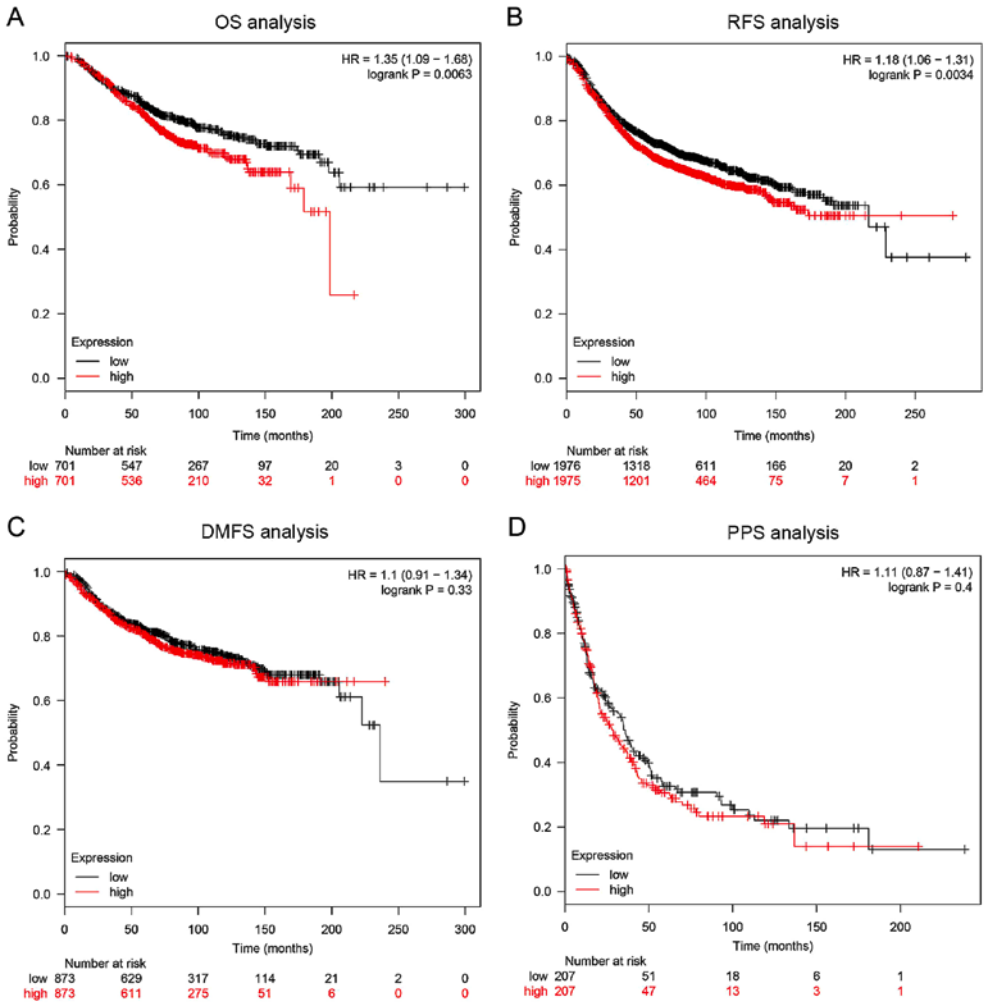

Figure 2. Prognostic value of the *PFKFB3* mRNA level in patients with breast cancer via an online survival analysis. (A) Overall survival (OS) in the Kaplan-Meier plotter (n=1,402). (B) Recurrence-free survival (RFS) in the Kaplan-Meier plotter (n=3,951). (C) Distant-metastasis free survival (DMFS) in the Kaplan-Meier plotter (n=1,746). (D) Post-progression survival (PPS) in the Kaplan-Meier plotter (n=414). The samples from patients with breast cancer were split into 2 groups by median expression (red line, high expression; black line, low expression), with the hazard ratio (HR) and 95% confidence intervals (CI) and log-rank P-value. The gene symbol *PFKFB3* (202464_s_at) was selected to obtain the Kaplan-Meier plots, in which the number at risk is indicated below the main plot. The patients with breast cancer with a higher *PFKFB3* mRNA expression were predicted to have a poor OS (P=0.0063) and RFS (P=0.0034).

Statistical analysis. All experiments were performed in triplicate, and data are presented as the means ± SD. Statistical analysis was performed using SPSS 19.0 (IBM, Armonk, NY, USA). The comparison of continuous values between 2 groups was performed by means of an independent-samples t-test. The association between *PFKFB3* expression and the clinicopathological characteristics was assessed using Chi-square tests. Survival was estimated by the Kaplan-Meier method, and differences between groups were assessed by the log-rank test. P-values of <0.05 were considered to indicate statistically significant differences.

Results

PFKFB3 expression and prognosis in BC tissues. In order to estimate the expression status of *PFKFB3* in BC, the expression level of *PFKFB3* was analyzed by immunohistochemistry in tissues from 74 patients with BC. The nuclear expression was analyzed by a proportion score of positive tumor cells and was categorized into scores of 1 (<5%), 2 (5-25%), or 3 (>25%) for PFKFB3 (Fig. 1A). The patients with BC were then separated according to the *PFKFB3* expression level (median split) and contrasted with different clinicopathological characteristics [age, tumor size, estrogen receptor (ER) status, progesterone receptor (PR) status, HER-2 status, TNM stage, distant metastasis, recurrence and differentiation] (Table I). Recurrence was defined as the return of cancer after a period of time during which it could not be detected. The recurrence could be in a different place or in the same location as the original tumor. Metastasis was defined as cancer spread from the original site to a different site of the body. The occurrence of distant metastasis was more frequent in groups with a higher *PFKFB3* expression (Chi-square test, P=0.025; Table I). Furthermore, Kaplan-Meier analysis revealed that a higher expression of *PFKFB3* was associated with a shorter OS time in patients with BC (Fig. 1B, P<0.01, log-rank test). In addition, online survival analysis revealed that a higher *PFKFB3* expression was associated with a poor OS (Fig. 2A, HR=1.35, P=0.0063, Kaplan-Meier) and RFS (Fig. 2B, P=0.0034). There was no difference, however, between DMFS (Fig. 2C, P=0.33) or PPS (Fig. 2D, P=0.4) in patients with BC with an altered *PFKFB3* expression. These results suggest that *PFKFB3* expression is

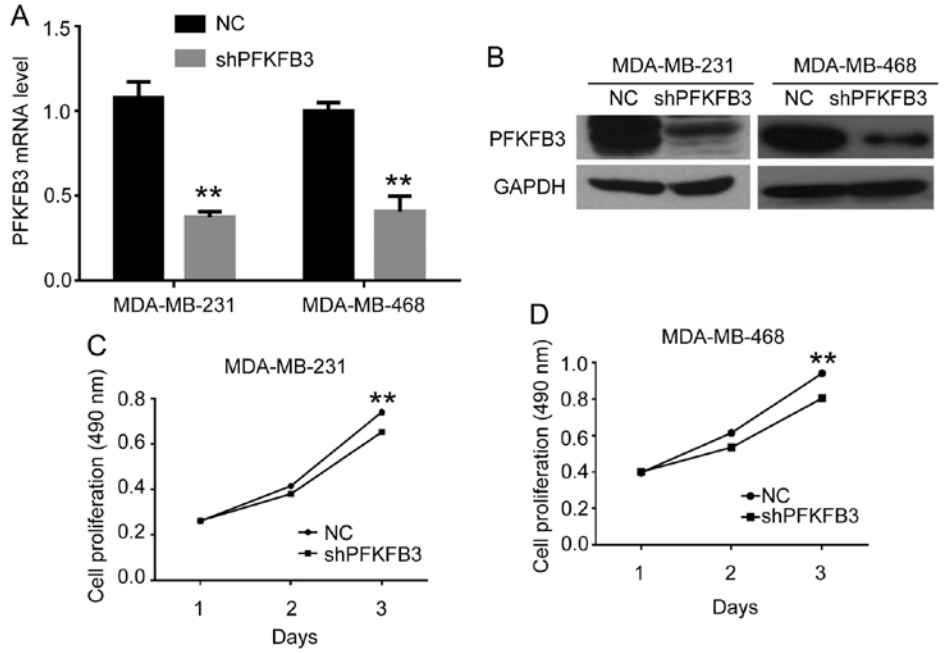

Figure 3. Suppression of *PFKFB3* inhibits breast cancer cell proliferation. (A) Quantitative PCR and (B) western blot analysis were used to confirm the suppression rate of *PFKFB3* in BC the cell lines, MDA-MB-231 and MDA-MB-468, that had been transduced with the shPFKFB3 lentivirus. (C and D) Effect of *PFKFB3* suppression on the proliferation of MDA-MB-231 and MDA-MB-468 cells. sh*PFKFB3*, lentiviral vector mediated shRNA against PFKFB3; NC, negative control; **P<0.01 vs. NC group.

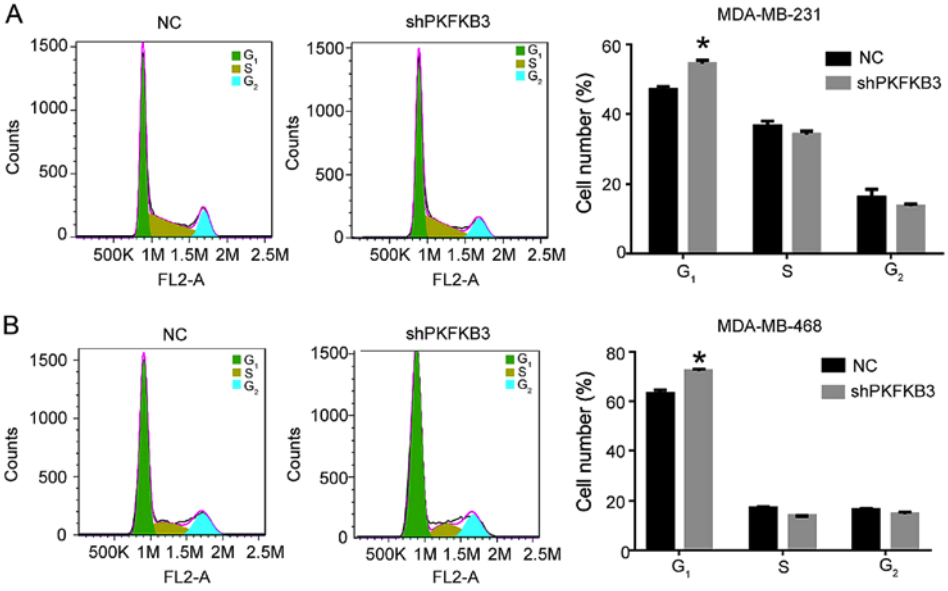

Figure 4. Suppression of *PFKFB3* induces breast cancer cell cycle arrest in the G_1 phase. The (A) MDA-MB-231 and (B) MDA-MB-468 cells were arrested in the G_1 phase of the cell cycle though transfection with sh*PFKFB3*. *P<0.05, compared with the NC group. The percentages of cells in G_1, M and G_2 phases were quantitatively analyzed.

upregulated in BC tissues and is associated with a poor patient prognosis.

Suppression of PFKFB3 inhibits BC cell growth, migration and invasion and induces cell cycle arrest. In order to investigate the biological roles of *PFKFB3* in the progression of BC, we constructed lentiviral vectors expressing shRNA targeting *PFKFB3*, and then infected the MDA-MB-231 and NDA-MB-468 cells with this shRNA lentivirus (sh*PFKFB3*). The expression of PFKFB3 in the cells following transfection with sh*PFKFB3* was confirmed by qPCR and western blot analysis in BC cells (Fig. 3A and B). We found that the mRNA and protein expression levels of *PFKFB3* were significantly inhibited by transfection with shRNA. We then performed an MTT assay to assess the effects of *PFKFB3* on BC cell proliferation. As shown in Fig. 3C and D, cell proliferation was significantly suppressed in both the MDA-MB-231 and MDA-MB-468 cells transfected with sh*PFKFB3* compared to the NC group at 3 days (P<0.01). The change in cell cycle distribution was observed by flow cytometry in the cells in

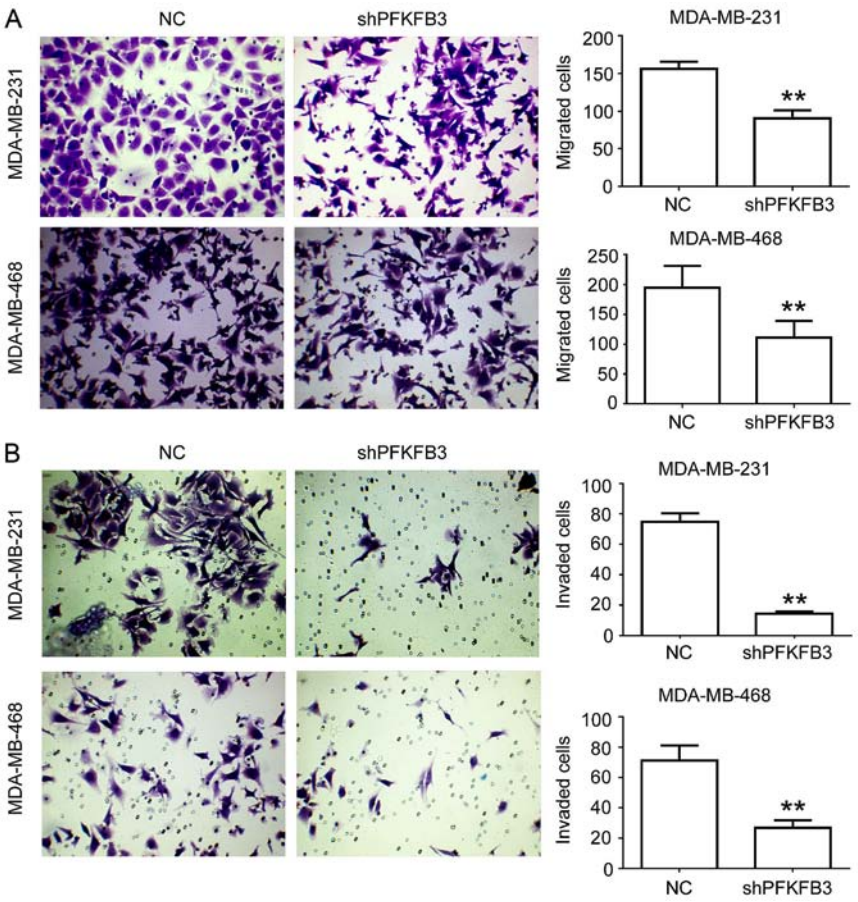

Figure 5. Effect of *PFKFB3* knockdown on breast cancer cell migratory and invasive abilities. (A) A migration assay was performed in MDA-MB-231 and MDA-MB-468 cells transduced with sh*PFKFB3* lentivirus. (B) The invasion assay was performed in MDA-MB-231 and MDA-MB-468 cells transduced with sh*PFKFB3* lentivirus. Magnification, x200; **P<0.01 vs. NC group.

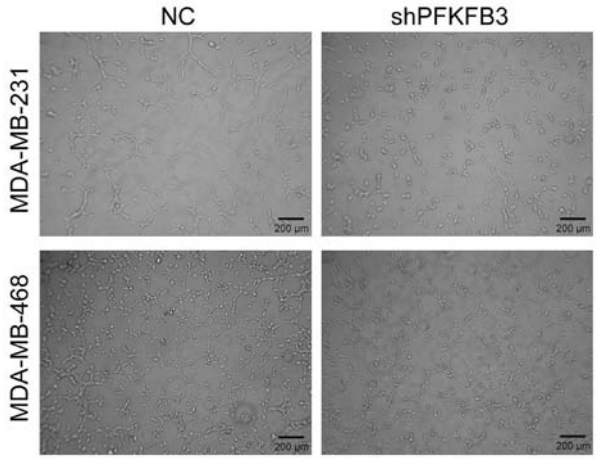

Figure 6. Suppression of *PFKFB3* prevents angiogenic activity in HUVECs. The MDA-MB-231 and MDA-MB-468 cells were transduced with sh*PFKFB3* or NC for 12 h. Following transfection, a cell supernatant was obtained and co-cultured with HUVECs. HUVEC tube formation in the Matrigel was observed and images were acquired under an optical microscope. Scale bar, 200 μm.

which *PFKFB3* was knocked down. We found that the cell cycle was arrested in the G_1 phase in the MDA-MB-231 and MDA-MB-468 cells transfected with the sh*PFKFB3* lentivirus (P<0.05; Fig. 4). The effect of PFKFB3 knockdown on cell migration and invasion was evaluated *in vitro* by a Transwell assay, revealing that the knockdown of *PFKFB3* significantly reduced cell migration (P<0.01; Fig. 5A) and invasion (P<0.01; Fig. 5B). These results suggest that *PFKFB3* suppression inhibits cell proliferation, migration and invasion, and induces cell cycle arrest in BC cells.

Suppression of PFKFB3 in BC cells prevents angiogenic activity in HUVECs. HUVECs have been a standard for cell-based assays in the field of *in vitro* angiogenesis research (30). Studies have shown that they can retain the ability to form tridimensional tubules in the Matrigel extract of the matrix-rich basement membrane (30-32). We further determined whether *PFKFB3* could stimulate angiogenesis. Therefore, the MDA-MB-231 and MDA-MB-468 cells were transfected with sh*PFKFB3* or NC for 12 h, the cell supernatant was collected and the HUVECs in Matrigel were cultured in the cell supernatant. Subsequently, tube formation was observed. We found that the suppression of *PFKFB3* significantly decreased HUVEC tube length and the number of MDA-MB-231 and NDA-MB-468 cells (Fig. 6). These findings suggest that *PFKFB3* is required for efficient tube formation in BC.

PFKFB3 is involved in the expression of p-AKT, p27 and VEGFα in BC cells. As shown above, we demonstrated the effects of *PFKFB3* knockdown on the proliferation and cell

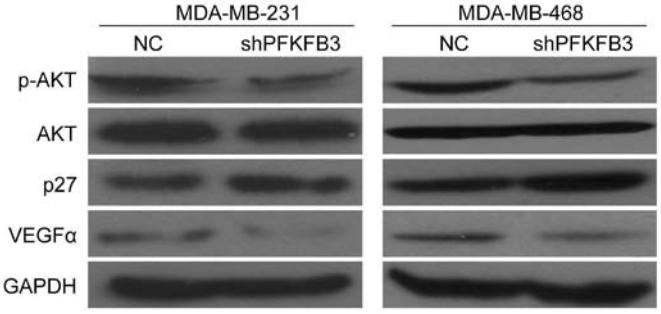

Figure 7. Silencing of *PFKFB3* regulates the expression levels of AKT, phosphorylated AKT (p-AKT), p27 and VEGFα. The expression levels of AKT, p-AKT, p27 and VEGFα were examined by western blot analysis in MDA-MB-231 and MDA-MB-468 cells transfected with sh*PFKFB3* or NC. GAPDH was used as loading control.

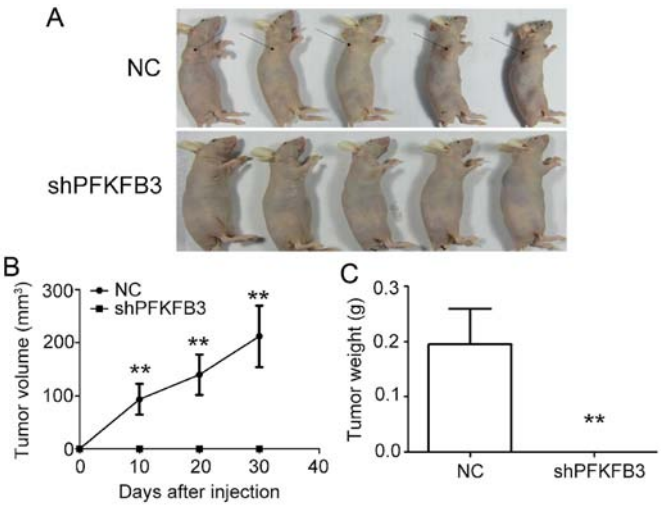

Figure 8. Suppression of *PFKFB3* inhibits xenograft tumor growth in nude mice. (A) MDA-MB-231 cells transduced with sh*PFKFB3* or NC were subcutaneously injected into the front right leg to establish the xenograft model. At the last time-point (30 days after first injection), tumors measured *in situ* were shown in both groups. Statistical analysis for (B) tumor volume and (C) weight in sh*PFKFB3* group revealed that the suppression of *PFKFB3* significantly suppressed tumor growth compared to the NC group. $^{**}P<0.01$ vs. NC group.

cycle arrest in BC cells. Therefore, we then wished to determine whether there was an association between *PFKFB3* expression and cell proliferation-related genes (AKT), cell cycle-related genes (p27), or angiogenesis-related genes (VEGF-α). The results revealed that the suppression of *PFKFB3* decreased p-AKT expression, but not AKT expression, while it increased p27 expression in the MDA-MB-231 and MDA-MB-468 cells (Fig. 7). Moreover, we also found that VEGFα expression was downregulated in the BC cells transduced with sh*PFKFB3* compared with the NC group (Fig. 7). These findings provide further evidence that PFKFB3 affects BC cell growth, migration and invasion abilities, and is involved in the cell cycle and angiogenesis of BC cells in this study.

Suppression of PFKFB3 inhibits BC cell growth in vivo. To examine the effect of *PFKFB3* on BC cell proliferation *in vivo*, we generated xenograft models by implanting MDA-MB-231 cells transduced with sh*PFKFB3* or NC into nude mice. No tumors formed in the mice injected with the BC cells transfected with sh*PFKFB3* (Fig. 8A). The results also revealed a marked decrease in the xenograft subcutaneous tumor volume (Fig. 8B) and weight (Fig. 8C) in the *PFKFB3* inhibition group compared with the control group. Taken together, these data confirm the inhibitory effect of *PFKFB3* knockdown on BC cell growth, both *in vivo* and *in vitro*.

Discussion

PFKFBs, a single homodimeric bifunctional enzyme family, are the master control point in the malignant cell glycolytic pathway (4,33). A number of studies have reported that *PFKFBs* play an important role in the Warburg effect (tumor cells exhibit a greater dependence on glycolysis for ATP generation than their origin tissue) and cancer growth (4,34,35). The overexpression of *PFKFB3* and other *PFKFB* isozymes has been observed in various tumor types, such as gastric, pancreatic, lung, breast and colon cancers (9,33,36,37).

At present, several studies have indicated that *PFKFB3* increases proliferation and regulates glycolysis and survival in response to mitophagy during mitotic arrest in MDA-MB-231 cells (38,39). The silencing of *PFKFB3* with siRNAs can lead to the decreased proliferation of MDA-MB-231 and MDA-MB-468 cells (40). Therefore, in this study, we selected the MDA-MB-231 and MDA-MB-468 cells as research targets. We examined the expression of *PFKFB3* in BC tissues and investigated the mechanisms responsible for the inhibition of *PFKFB3* expression in cell proliferation, migration, cell cycle and angiogenesis. Our results revealed that *PFKFB3* was highly expressed in BC tissues and was associated with a poor OS in patients with BC. We also found that the silencing of *PFKFB3* with shRNA significantly suppressed BC cell proliferation, migration, invasion and angiogenic abilities, and induced cell cycle arrest. In addition, we found that the silencing of *PFKFB3* caused a marked decrease in the rate of xenograft subcutaneous tumor growth. Therefore, we proved that *PFKFB3* was overexpressed in BC tissues, and that the suppression of *PFKFB3* inhibited BC cell growth both *in vivo* and *in vitro*. We confirmed that *PFKFB3* inhibition reduced the phosphorylation of AKT and causes a marked increase in p27 expression. Based on these results, we can therefore conclude that *PFKFB3* functions as a regulator of cell progression in BC cells.

Several previous studies have demonstrated that *PFKFB3* is overexpressed in cancer tissues and cells. Han *et al* (41) reported that *PFKFB3* was highly expressed in patients with gastric cancer and that it promoted the proliferation and migration of gastric cancer cells. *PFKFB3* expression was also found to be increased in pancreatic cancer cell lines (42) and BC cells (MCF-7RA and MCF-7RB) (43). Of note, in this study, survival analysis revealed that a high *PFKFB3* expression conferred a poor overall and recurrence-free survival in patients with BC, indicating that *PFKFB3* may be an essential downstream target for cancer therapies. *PFKFB3* inhibitors have been proven to function as metabolic regulators, which can be expected to suppress tumors both *in vivo* and *in vitro* (44). In addition, in this study, we found that *PFKFB3* inhibition triggered cell cycle arrest in BC cells in the G_1 phase, suggesting that this inhibition may prevent cancer cell proliferation. A previous

study demonstrated that *PFKFB3* inhibition suppressed glycolysis and induced G_2 phase cell cycle arrest in HeLa cells (22). Recent studies have also found that the role of *PFKFB3* in tumorigenesis was mainly dependent on, not only its glycolysis regulatory function, but also in regulating the cell cycle in the nucleus. For example, Yalcin *et al* (23) reported that PFKFB3 inhibition resulted in a G_1 block and caused a marked increase in p27 protein expression in HeLa cells. Our results revealed that *PFKFB3* inhibition decreased p-AKT expression and increased p27 expression in BC cells. p27 is a inhibitor of G_1 cyclin/cyclin-dependent kinase (Cdk) protein kinase activity, and plays an important role in the cancer cell cycle (45). One study indicated that p27 has an oncogenic potential to promote tumor progression through the induction of metastasis (46). We demonstrated that *PFKFB3* inhibition reduced AKT phosphorylation, causing a marked increase in p27 expression. *PFKFB3* is regulated by AKT and phosphatase and tensin homolog (PTEN), which is required for the survival and growth of multiple cancer types (47,48).

In addition, this study found that the suppression of *PFKFB3* weakened VEGFα expression and decreased angiogenic activity in BC cells. The overexpression of different *PFKFBs* has been reported to promote VEGF expression in gastric and pancreatic cancer cells under hypoxic conditions (33). Studies have also shown that vascularization is closely related to tumor development (49,50). Abnormal vascularization promotes metastasis in malignant tumors, and VEGF inhibitors have been used to treat tumors with a certain therapeutic effect. According to the latest research, the suppression of *PFKFB3* in endothelial cells (ECs) can improve tumor vessel maturation and perfusion, thereby inhibiting cancer cell invasion, intravasation and metastasis (51). *PFKFB3* inhibition reduces VE-cadherin endocytosis, thereby tightening the vascular barrier in ECs. Moreover, the suppression of *PFKFB3* in perithelial cells renders these cells more quiescent and adhesive through glycolysis reduction (51). Our data also indicated that *PFKFB3* suppression in BC cells significantly decreased HUVEC tube length and number. Since *PFKFB3* has powerful features of tumor cell metabolism, and since there is conclusive evidence that the expression of *PFKFB3* is involved in a poor prognosis in BC, it may serve as a potential target for the development of effective antineoplastic therapies.

In conclusion, this study revealed that *PFKFB3* was overexpressed in BC tissues. The lentivirus-mediated suppression of *PFKFB3* inhibited BC cell proliferation, migration and invasion, and induced cell cycle arrest *in vitro*. Moreover, we also found that the suppression of *PFKFB3* inhibited vascularization in BC cells by suppressing the VEGFα protein level and preventing HUVEC angiogenic behavior. In addition, p-AKT expression decreased, while the p27 level increased, in BC cells transduced with *PFKFB3* lentivirus carrying shPFKFB3. Furthermore, the suppression of *PFKFB3* inhibited BC cell xenograft growth in nude mice. Since no further evidence was found regarding the involvement of the AKT-related pathway in the suppression of cell cycle arrest, proliferation, migration and invasion induced by the inhibition of *PFKFB3*, further studies are required to confirm our findings. The association between *PFKFB3* expression and cancer types other than triple-negative BC remains unknown. Further unequivocal evidence for the regulatory role of *PFKFB3* in cell biological functions and glycolysis in different types of cancer is required.

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Competing interests

The authors declare that they have no competing interests.

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