

# lncRNA Ftx promotes aerobic glycolysis and tumor progression through the PPAR $\gamma$ pathway in hepatocellular carcinoma

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**Abstract.** Aerobic glycolysis is a phenomenon by which malignant cells preferentially metabolize glucose through the glycolytic pathway, rather than oxidative phosphorylation to proliferate efficiently. The present study aimed to investigate the expression and functional implications of long non-coding (lnc)RNA Ftx in the aerobic glycolysis and tumorigenesis of hepatocellular carcinoma (HCC). It was identified that lncRNA Ftx was upregulated in human HCC tissues and cell lines and, notably, was associated with aggressive clinicopathological features. lncRNA Ftx overexpression promoted the proliferation, invasion and migration of HCC cells, whereas lncRNA Ftx knockdown resulted in the opposite effects. Furthermore, lncRNA Ftx affected the activity and expression of key enzymes in carbohydrate metabolism, suggesting that lncRNA Ftx may be involved in aerobic glycolysis in HCC. The measurement of glucose consumption, lactate production and glucose transporter expression further supported this assumption. Mechanistically, peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) expression in human HCC tissues and cell lines was positively correlated with lncRNA Ftx. Inhibiting PPAR $\gamma$  in Huh7 cells partially abrogated the alterations in glucose uptake, lactate production and relative glycolytic enzyme expression induced by lncRNA Ftx; similarly, PPAR $\gamma$  activation in Bel-7402 cells partially rescued the lncRNA Ftx-mediated alterations. In conclusion, lncRNA Ftx is a promoter of the Warburg effect and tumor progression, partly via the PPAR $\gamma$  pathway, and may serve as a promising therapeutic target for HCC treatment.

## Introduction

Hepatocellular carcinoma (HCC) is the principal type of liver malignancy and the third leading cause of cancer-associated mortality worldwide; the morbidity and mortality rates of HCC are particularly high in China (1). The lack of reliable biomarkers for tumorigenesis and the unclear clarification of the heterogeneous genetic and epigenetic alterations contribute to the poor prognosis of HCC (2).

Aerobic glycolysis, also termed the Warburg effect, is a phenomenon by which highly proliferative malignant cells preferentially utilize glycolysis rather than oxidative phosphorylation, even in the presence of sufficient oxygen, to satisfy their high nutrient requirements (3,4). This effect is characterized by the consumption of glucose at a higher rate and the production of more lactate compared with normal differentiated cells. Accumulating evidence suggests that metabolic alteration, which is one of the most consistent hallmarks of cancer, exerts critical effects on tumor progression (5), and the aberrant expression of glycolysis-associated molecules contributes to tumorigenesis (6,7). Therefore, aerobic glycolysis is pivotal to producing energy in cancer cells, indicating that the molecules involved may be potential biomarkers and therapeutic targets for HCC (8) (Fig. 1A).

Long non-coding RNAs (lncRNAs), which do not have protein-coding ability, are a class of functional RNAs of >200 nucleotides in length and are involved in various biological processes (9). lncRNAs modulate target gene expression at the transcriptional and posttranscriptional levels (10). Recently, lncRNAs have emerged as important regulators of carbohydrate metabolism, lipid metabolism (11,12) and HCC development (13).

*Ftx* is a well-conserved noncoding gene encoded within the X-inactivation center on the X chromosome (14). *Ftx* encodes a highly conserved transcript of 2,300 nucleotides that is termed lncRNA Ftx (Fig. 1B). *Ftx* encodes nine introns, the second and seventh of which encode two clusters of microRNAs (miRs; miR-421/miR-374b and miR-545/miR-374a). RNA fragments transcribed from other introns compose lncRNA Ftx. Thus, there are no reduplicated sequences in lncRNA Ftx and the miRs. It has been demonstrated that lncRNA Ftx/miR-545 contributes significantly to the tumorigenesis of HCC through activation of phosphatidylinositol 3-kinase/RAC- $\alpha$

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serine/threonine-protein kinase by targeting DEXD/H-box helicase 58 (15). However, the specific association between lncRNA Ftx and aerobic glycolysis, and the underlying mechanism, remain unclear. The present study may provide a novel insight into therapeutic interventions for HCC.

Once activated by ligands, peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) heterodimerizes with the retinoid X receptor and combines with PPAR response elements to regulate the transcription of target genes. It has been demonstrated that PPAR $\gamma$  serves a vital role in steatosis-associated hepatic tumorigenesis (16), in addition to increasing cell sensitivity to insulin and reversing insulin resistance (17). PPAR $\gamma$  activation is additionally involved in the regulation of a number of crucial enzymes in carbohydrate metabolism; for example, PPAR $\gamma$  activation promotes insulin-responsive glucose transporter 4 (GLUT4) expression (18) and inhibits pyruvate dehydrogenase kinase 1 (PDK1) expression (19). Furthermore, PPAR $\gamma$  activation may reduce tumor necrosis factor (TNF) $\alpha$  and leptin production, thus facilitating glucose utilization and improving insulin sensitivity in liver cells (20). However, the role of lncRNA Ftx in PPAR $\gamma$ -mediated tumor metabolism remains poorly understood.

The present study investigated the aberrant status of lncRNA Ftx and its potential target gene PPAR $\gamma$  to examine the possible signaling pathway that regulates aerobic glycolysis, and to identify a novel therapeutic target for HCC treatment.

## Materials and methods

**Ethics statement.** Written informed consent was obtained from each patient recruited for the present study for the use of materials. The consent procedures and all experimental protocols were approved by the Medical Institutional Ethical Committee of Shandong Provincial Hospital Affiliated to Shandong University (Jinan, China; approval no. 2017-231), according to the Declaration of Helsinki.

**Tissue specimens.** A total of 73 patients with HCC were recruited between February 2012 and January 2013 at Shandong Provincial Hospital Affiliated to Shandong University. The inclusion criteria were as follows: i) Patients with pathologically confirmed HCC; ii) patients who underwent curative surgical resection; and iii) patients >18 years old. The exclusion criteria were as follows: i) Patients who received preoperative chemotherapy or radiotherapy; and ii) patients with two or more primary tumors, asynchronously or synchronously. For each patient, paired HCC tissues and adjacent non-tumor tissues (as a control) were fresh-frozen in liquid nitrogen immediately following surgical resection and stored at -80°C. Patients with HCC were divided into metastasis (n=24) and non-metastasis (n=49) groups, and complete capsule (n=45) and incomplete capsule (n=28) groups, according to their clinicopathological features.

**Cell culture and reagents.** The human immortalized normal hepatic cell line LO2 and HCC cell lines (Huh7, SMMC-7721 and Bel-7402) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The Bel-7402 cell line was derived from a surgical specimen obtained in 1974 from a 53-year-old male patient with HCC and a positive

serum  $\alpha$ -fetoprotein status (21). The Huh7 cell line is a well-differentiated hepatocyte-derived cellular carcinoma cell line, originally obtained from a liver tumor from a 57-year-old Japanese male in 1982 (22). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (both from Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA), penicillin G (100 U/ml) and streptomycin (100  $\mu$ g/ml), at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. The PPAR $\gamma$  antagonist GW9662 (10  $\mu$ M) and the PPAR $\gamma$  agonist pioglitazone (10  $\mu$ M) (MedChemExpress Co., Ltd., Monmouth Junction, NJ, USA) were dissolved in dimethyl sulfoxide (DMSO; as a vehicle) and added to the cell culture medium of Huh7 and Bel-7402 cells, respectively. Following lncRNA Ftx overexpression, Huh7 cells were treated with GW9662 or vehicle for 24 h, whereas following lncRNA Ftx downregulation, Bel-7402 cells were treated with pioglitazone or vehicle for 24 h.

**Lentiviral transfections and construction of stable cell lines.** Transfections of lentivirus (LV)-Ftx and its negative control LV-CON220 (Ubi-MCS-SV40-EGFP-IRES-puromycin) and LV-Ftx-RNA interference (RNAi) and its negative control LV-CON077 (hU6-MCS-Ubiquitin-EGFP-IRES-puromycin) were performed using a lentivirus (Shanghai GeneChem Co., Ltd., Shanghai, China), according to the manufacturer's protocol. To obtain cell lines stably expressing lncRNA Ftx, Huh7 cells were transfected with the LV-Ftx, polybrene and enhanced infection solution (Shanghai GeneChem Co., Ltd.) and selected with puromycin (2  $\mu$ g/ml) for 24 h. LV-CON220 was used as a control. To produce cell lines with stably interfered expression of lncRNA Ftx, Bel-7402 cells were transfected with LV-Ftx-RNAi, polybrene and enhanced infection solution (Shanghai GeneChem Co., Ltd.) and selected with puromycin (2  $\mu$ g/ml) for 24 h. LV-CON077 was used as a control. The stably overexpressing or interfered cell lines were validated by RT-qPCR. Huh7 clones with ~12 times increased lncRNA Ftx expression levels compared with the normal control were chosen as Ftx, and Bel-7402 clones with ~74.68% decreased lncRNA Ftx expression levels compared with the normal control were chosen as short hairpin (sh)-Ftx. Their negative controls were termed Ftx-NC and sh-NC, respectively.

**Cell proliferation assay.** The transfected Bel-7402 and Huh7 cells were seeded onto 96-well plates (Corning Incorporated, Corning, NY, USA) at densities of ~3,000 and 5,000 cells/well, respectively. Following an overnight incubation, 10  $\mu$ l Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) solution was added to each well and incubated at 37°C for 3 h. Subsequently, the optical density values were measured at 450 nm using a Multiskan Go spectrophotometer (Thermo Fisher Scientific, Inc.).

**Cell invasion and migration assays.** Cell invasion and migration were assayed using Transwell chambers (Corning Incorporated) with and without Matrigel (BD Biosciences, Franklin Lakes, NJ, USA), respectively. For the invasion assay, each Transwell chamber was coated with 60  $\mu$ l Matrigel and placed into a 24-well plate. Following lentiviral transfection, Bel-7402 cells and Huh7 cells (1 $\times$ 10<sup>5</sup>) were seeded into each chamber in serum-free medium, and the lower chambers

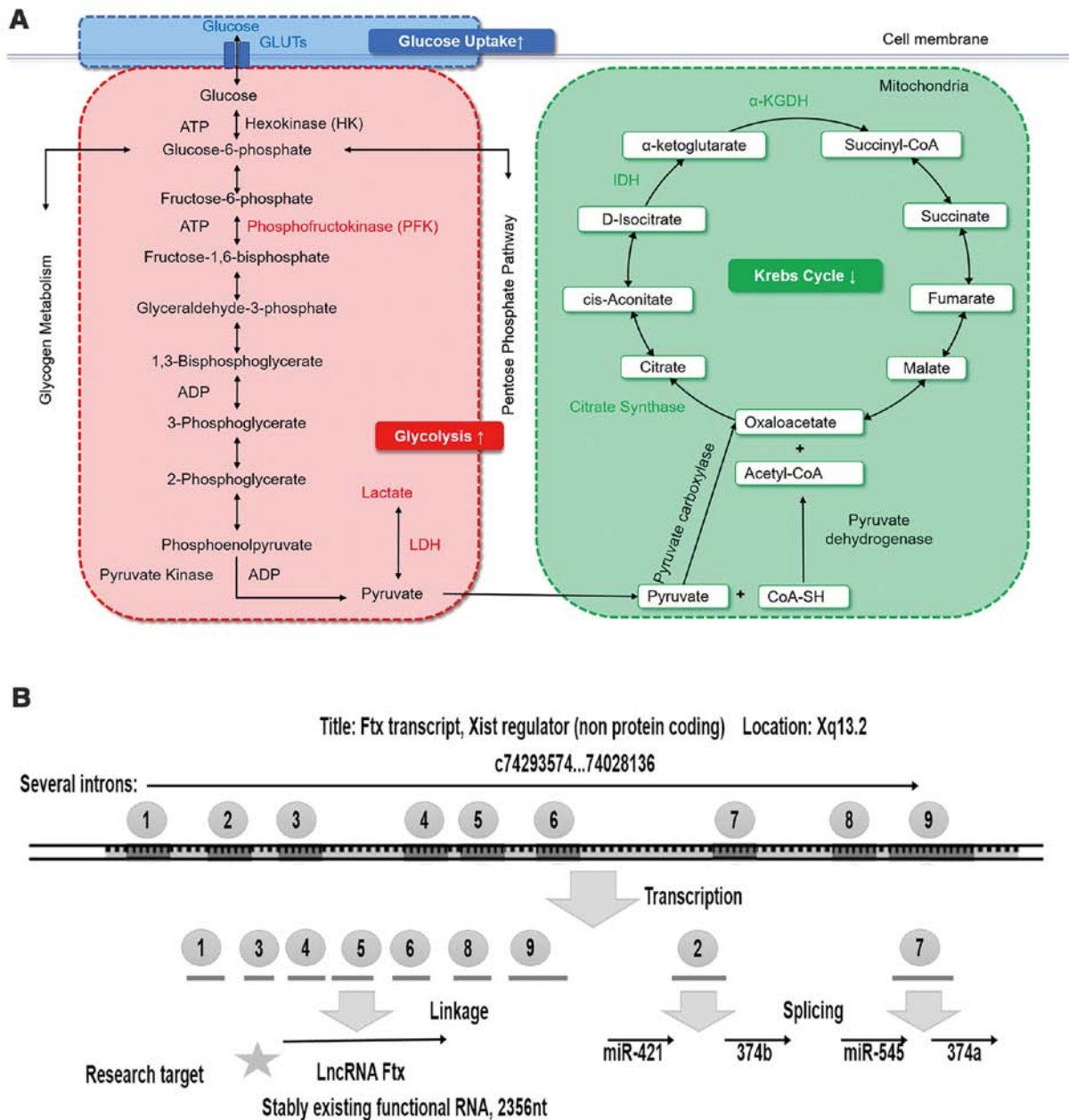


Figure 1. Schematic illustrations. (A) The Warburg effect; (B) the genomic location of lncRNA Ftx. lncRNA, long non-coding RNA; GLUT, glucose transporter; miR, microRNA; ATP, adenosine 5'-triphosphate; ADP, adenosine 5'-diphosphate.

were loaded with DMEM supplemented with 10% FBS. A total of 48 h subsequently, non-migrated cells in the upper chambers were removed with cotton swabs. For the migration assay, HCC cells ( $5 \times 10^4$ ) were seeded in the upper chambers in serum-free medium without a Matrigel membrane, and the lower chambers were loaded with DMEM supplemented with 10% FBS. A total of 36 h subsequently, HCC cells in the upper chambers that had not migrated were removed with cotton swabs, and the migrated cells were fixed in 100% methanol at room temperature for 30 min. The cells on the bottom surface of the membrane were stained with hematoxylin (Wuhan Servicebio Technology Co., Ltd., Wuhan, China) at room temperature for 20 min. Cell images were obtained in high-power (x400 magnification) fields using a phase-contrast microscope (Leica DM4000B; Leica Microsystems, GmbH, Wetzlar, Germany).

#### Measurement of aerobic glycolysis.

**Analysis of glucose consumption.** Following transfection, cells were seeded into 6-well plates, and after 6 h, the culture medium was changed to complete medium and incubated for a further 48 h. Subsequently, the medium was collected to measure the glucose concentrations, and the cells were harvested to obtain protein lysates. Glucose concentrations were detected with a glucose assay kit (cat. no. 361500; Shanghai Rongsheng Biotech Co., Ltd., Shanghai, China), according to the manufacturer's protocol. All values were normalized to the total protein levels determined using a Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Inc.).

**Measurement of lactate generation.** A total of  $\sim 1 \times 10^5$  cells were seeded onto 6-well plates and cultured for 48 h. Subsequently, the culture medium was used to determine the

lactate concentration using a lactate assay kit (cat. no. KGT023; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China), and the HCC cells were harvested to determine the protein concentration according to the manufacturer's protocol. All lactate concentration values were normalized to the corresponding protein concentration values.

**Detection of glycolytic enzymes.** The enzymic activity levels of isocitrate dehydrogenase 1 (IDH1),  $\alpha$ -ketoglutarate dehydrogenase (OGDH), citrate synthase (CS), phosphofructokinase, liver type (PFKL), and lactate dehydrogenase (LDH) were analyzed using an IDH1 mitochondrial assay (cat. no. BC2160), OGDH assay (cat. no. BC0710) (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), CS assay (cat. no. A108), PFKL assay (cat. no. A129) and LDH assay (cat. no. A020-1) (all from Nanjing Jiancheng Bioengineering Institute, Nanjing, China), respectively, according to the manufacturers' protocols. All values were normalized to the total protein levels.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was isolated from cultured Huh7 cells or Bel-7402 cells, or frozen tissues, using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). A total of 1  $\mu$ g RNA was reverse-transcribed into cDNA with an RT reagent kit (Takara Bio, Inc., Otsu, Japan), according to the manufacturer's protocol. The conditions were as follows: 37°C for 15 min, 85°C for 5 sec and 4°C for 10 min. The amplification was detected using a SYBR Premix Ex Taq kit (Takara Bio, Inc.) and a LightCycler® 480 Real-Time PCR system (Roche Diagnostics, Indianapolis, IN, USA). The thermocycling conditions were as follows: Pre-incubation at 95°C for 5 min; 45 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 10 sec, and extension at 72°C for 10 sec; a melting cycle at 95°C for 5 sec, 65°C for 1 min, and 97°C with continuous per 5°C acquisition of fluorescence; and finally cooling at 40°C for 30 sec. The primer sequences are listed in Table I. The relative gene expression values are presented according to the  $2^{-\Delta\Delta C_q}$  method (23), relative to  $\beta$ -actin.

**Western blotting.** Total protein was extracted from tumor cells with Radioimmunoprecipitation Assay Lysis Buffer (Beyotime Institute of Biotechnology, Haimen, China) and phenylmethanesulfonyl fluoride (Beijing Solarbio Science & Technology Co., Ltd.), and the concentrations were determined with a Pierce BCA protein assay kit (Thermo Fisher Scientific, Inc.). A total of 40  $\mu$ g protein was separated by 10 or 12% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes, following blocking with 10% skimmed milk at room temperature for 1 h, were probed with the following primary antibodies at 4°C overnight: Rabbit anti-PPAR $\gamma$  (1:1,000; cat. no. ab191407; Abcam, Cambridge, UK), rabbit anti-CS (1:1,000; cat. no. 16131-1-AP), rabbit anti-PFKL (1:1,000; cat. no. 15652-1-AP), mouse anti-TNF $\alpha$  (1:1,000; cat. no. 60291-1-Ig) (Wuhan Sanying Biotechnology, Wuhan, China), rabbit anti-OGDH (1:250; cat. no. bs-17710R; BIOSS, Beijing, China), rabbit anti-IDH1 (1:500; cat. no. PB0632), rabbit anti-GLUT1 (1:500; cat. no. PB0439), rabbit anti-PDK1 (1:200; cat. no. BA4499), rabbit anti-leptin (1:100; cat. no. BA1231) and rabbit anti-GLUT4 (1:500; cat. no. PB0143)

(Wuhan Boster Biological Technology, Ltd., Wuhan, China). Subsequently, the membranes were incubated with horse-radish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG secondary antibodies (cat. no. ZB-2301 or ZB-2305, respectively; OriGene Technologies, Inc., Beijing, China) at a dilution of 1:8,000 at room temperature for 1 h, followed by enhanced chemiluminescence reagent (EMD Millipore, Billerica, MA, USA). The protein bands were visualized using an Amersham Imager 600 (GE Healthcare, Chicago, IL, USA). Total protein levels were normalized to tubulin- $\beta$  (1:1,000; cat. no. BM1453; Wuhan Boster Biological Technology, Ltd.) expression on the same membrane, and the bands were quantified using ImageJ k 1.45 software (National Institutes of Health, Bethesda, MD, USA).

**lncRNA target prediction.** Bioinformatics analysis of predicted lncRNA targets was performed using the nucleotide BLASTn program (blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE\_TYPE=BlastSearch).

**Statistical analysis.** The data were obtained from at least three independent experiments and are presented as the mean  $\pm$  standard error of the mean (unless otherwise stated). Pearson's correlation ( $r$ ) was utilized to measure correlations and logarithmic regression was used to derive the equation of the slope. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS 22.0 (IBM Corporation, Armonk, NY, USA). The significance of differences was evaluated by Student's  $t$ -tests (two-tailed) for two-group comparisons, and the one-way analysis of variance and the Bonferroni post hoc test for multiple comparisons.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**lncRNA Ftx is upregulated in human HCC tissues and significantly associated with poor prognosis-associated clinicopathological features.** To examine the expression of lncRNA Ftx, RT-qPCR was performed to analyze 73 HCC and adjacent non-tumorous liver samples. Compared with the non-tumorous control tissues, lncRNA Ftx expression was markedly higher in the HCC tissues (Fig. 2A). The clinicopathological characteristics, including metastasis, tumor capsule, histological grade and tumor size, are summarized in Table II.

In addition, to determine the potential role of lncRNA Ftx in HCC, correlations between the expression status of lncRNA Ftx and important clinical features associated with tumor progression and disease prognosis were analyzed. Although there was no significant association between lncRNA Ftx expression and age, sex, tumor size or histological grade, high lncRNA Ftx expression levels were positively associated with incomplete capsules and HCC metastasis (Fig. 2A).

Taken together, the results indicated that the upregulation of lncRNA Ftx expression is associated with poor prognosis-associated clinicopathological features, suggesting that lncRNA Ftx is involved in HCC tumorigenesis.

**lncRNA Ftx promotes HCC cell proliferation, invasion and migration in vitro.** To choose suitable cell lines for functional

Table I. Sequences of primers.

Primer	Sequence (5'→3')
TNF $\alpha$	F CTGCCTGCTGCACTTTGGAG
	R ACATGGGCTACAGGCTTGTCACT
PDK1	F GTCACAGAGGAGCGTTTCTGG
	R TGCCGCCAGAAACATAAATGAGG
LEP	F CACCAGGATCAATGACATTTTCAACA
	R AGCCCAGGAATGAAGTCCAAAC
GLUT1	F TGTGGGCATGTGCTTCCAGTA
	R CGGCCTTTAGTCTCAGGAACCTTTG
GLUT4	F GGGCTGAGACAGGGACCATAAC
	R CATGAGCAATGGCATCCAGAA
IDH1	F AATCAGTGGCGGTTCTGTGGTA
	R ACTTGGTCGTTGGTGGCATC
PFKL	F GCATTTATGTGGGTGCCAAAGTC
	R AGCCAGTTGGCCTGCTTGA
OGDH	F GGCTACGTGTTGACGCCATA
	R CTCAACTTAGCAGCACAAGTCCTTA
CS	F GTCTGGCTAACACAGCTGCAGA
	R CATGGCCATAGCCTGGAACA
PPAR $\gamma$	F TTGTTCCAGGGAAATTCAGTGC
	R CGCCGTAAATTATTTCTAAACC
lncRNA Ftx	F GAATGTCCTTGTGAGGCAGTTG
	R TGGTCACTCACATGGATGATCTG
ACTB	F TGGCACCCAGCACAAATGAA
	R CTAAGTCATAGTCCGCCTAGAAGCA

TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; PDK1, pyruvate dehydrogenase kinase 1; LEP, leptin; GLUT, glucose transporter; IDH1, isocitrate dehydrogenase 1; PFKL, phosphofructokinase, liver type; OGDH,  $\alpha$ -ketoglutarate dehydrogenase; CS, citrate synthase; PPAR $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$ ; lncRNA Ftx, long non-coding RNA Ftx; ACTB,  $\beta$ -actin; F, forward; R, reverse.

research, lncRNA Ftx expression was screened in a panel of HCC cell lines and a non-neoplastic hepatic cell line (LO2). It was identified that the expression levels of lncRNA Ftx were significantly increased in the HCC cell lines compared with the LO2 cell line. Additionally, the expression level of lncRNA Ftx was the highest in Bel-7402 cells and the lowest in Huh7 cells (Fig. 2B).

The present study aimed to overexpress and knock down lncRNA Ftx and investigate the general role of lncRNA Ftx. Thus, lncRNA Ftx was overexpressed in Huh7 cells as the base level of lncRNA Ftx was low, and it was knocked down in Bel-7402 cells as the base level of lncRNA Ftx was high. Bel-7402 cells were transfected with LV-Ftx-RNAi (sh-Ftx) and its negative control LV-CON077 (sh-NC), while Huh7 cells were transfected with LV-Ftx (Ftx) and its negative control LV-CON220 (Ftx-NC). RT-qPCR was used to confirm the transfection efficiency. lncRNA Ftx expression in the overexpressing Huh7 cells was ~12 times higher compared with the

Table II. Clinicopathological characteristics of patients with hepatocellular carcinoma.

Characteristic	No. of patients (n=73)
Age, years	
<50	27
$\geq 50$	46
Sex	
Male	47
Female	26
Tumor size	
<5 cm	29
$\geq 5$ cm	44
Histological grade	
Good	14
Moderate	40
Poor	19
Metastasis	
With	24
Without	49
Tumor capsule	
Complete	45
Incomplete	28

normal control cells, while the inhibition rate of lncRNA Ftx in Bel-7402 cells was ~74.68% (Fig. 2C).

Tumor cell proliferation, invasion and migration are pivotal steps in tumorigenesis. First, CCK-8 assays were performed to measure cell viability and determine whether lncRNA Ftx has an impact on tumor cell proliferation. Cell proliferation was significantly increased in lncRNA Ftx-overexpressing Huh7 cells (via LV-Ftx transfection) compared with negative control cells at the 48, 72 and 96 h time-points, whereas decreased Bel-7402 cell viability occurred following endogenous lncRNA Ftx knockdown (via LV-Ftx-RNAi transfection), even at 24 h (Fig. 2D). These results suggested that lncRNA Ftx supports HCC cell proliferation.

In addition, to assess the effect of lncRNA Ftx on HCC cell invasion and migration ability, cell invasion and migration assays were performed using stably transfected cells. As presented in Fig. 2E and F, significant migration and invasion decreases were found in lncRNA Ftx-knockdown Bel-7402 cells compared with negative control cells, and cell migration and invasion increases were observed in lncRNA Ftx-overexpressing Huh7 cells; these findings suggested that lncRNA Ftx enhances the invasion and migration ability of HCC cell lines *in vitro*.

Collectively, the present data demonstrated that the overexpression of lncRNA Ftx exerts a promoting effect on HCC cell proliferation, migration and invasion.

**Measurement of the Warburg effect.** The measurement of the Warburg effect comprises three parts: The analysis of glucose consumption, the measurement of the glycolytic pathway, and



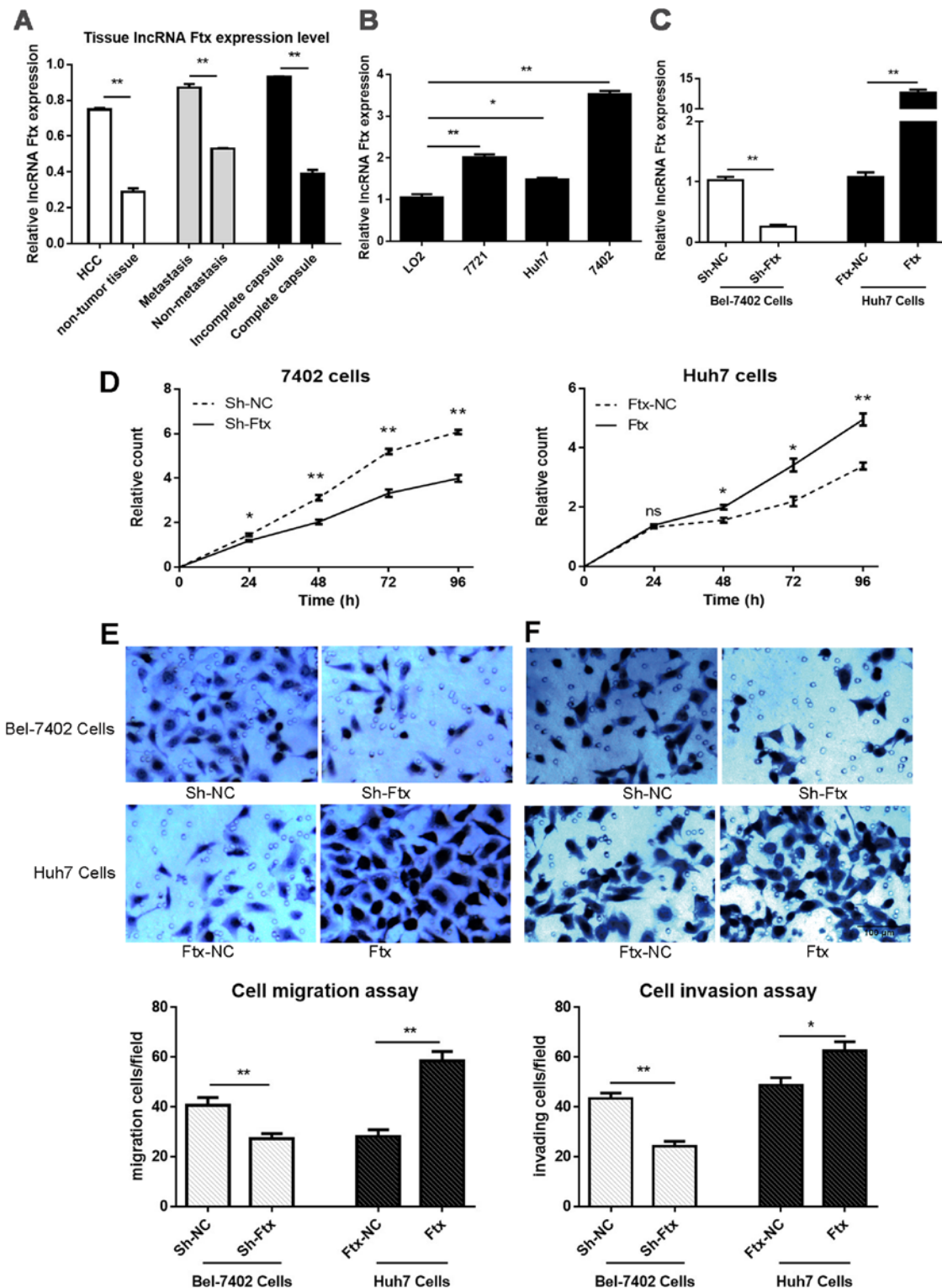


Figure 2. lncRNA Ftx is upregulated in HCC tissues and promotes HCC cell proliferation, invasion and migration *in vitro*. (A) Expression levels of lncRNA Ftx were increased in HCC tissues (n=73) compared with matched adjacent normal tissues (n=73), as determined by RT-qPCR. Patients with HCC were divided into metastasis (n=24) and non-metastasis (n=49) groups, and complete capsule (n=45) and incomplete capsule (n=28) groups, according to their clinicopathological features. lncRNA Ftx expression levels were increased in the metastasis group compared with the non-metastasis group, and decreased in the complete capsule group compared with the incomplete capsule group. (B) lncRNA Ftx expression levels in the three human HCC cell lines (Bel-7402, Huh7 and SMMC7721) compared with a non-transformed liver cell line (LO2). (C) Identification of lentiviral transfection efficiency. Bel-7402 cells were transfected with sh-Ftx or sh-NC, and Huh7 cells were transfected with Ftx or Ftx-NC. The transfection efficacy was determined by RT-qPCR. (D) Cell proliferation was markedly decreased in lncRNA Ftx-knockdown Bel-7402 cells compared with the negative control cells, according to the CCK-8 assay (left column). lncRNA Ftx-overexpressing Huh7 cells had significantly higher cell proliferation compared with the negative control cells, according to the CCK-8 assay (right column). (E) Bel-7402 cells and (F) Huh7 cells were transfected, and cell migration and invasion assays were performed using Transwell membranes and Matrigel-coated Transwell membranes, respectively (x400 magnification). Representative images of the migration and invasion chambers and the average counts from five random microscopic fields are shown. The experiments were repeated at least three times and yielded similar results; the error bars represent the mean  $\pm$  standard error of the mean. \* $P < 0.05$  and \*\* $P < 0.01$ . lncRNA, long non-coding RNA; HCC, hepatocellular carcinoma; sh, short hairpin; NC, negative control; CCK-8, cell counting kit-8; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

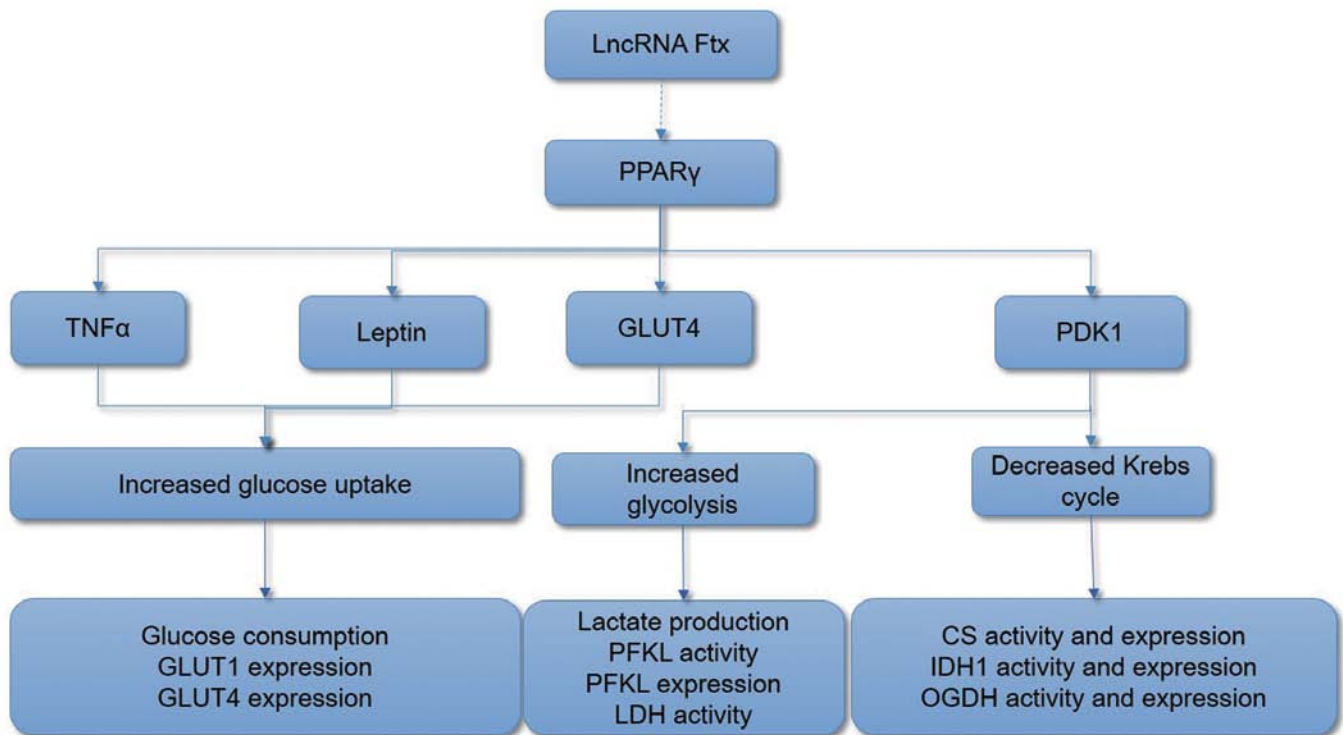


Figure 3. Illustrations of the aerobic glycolysis measurement methods. lncRNA, long non-coding RNA; PPAR $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$ ; TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; GLUT, glucose transporter; PFKL, phosphofructokinase, liver type; LDH, lactate dehydrogenase; CS, citrate synthase; IDH1, isocitrate dehydrogenase 1; OGDH,  $\alpha$ -ketoglutarate dehydrogenase.

the detection of the Krebs cycle by analyzing the activity and expression of the involved enzymes and the assessment of relative products in carbohydrate metabolism (Fig. 3). Bel-7402 cells exhibited increased GLUT1, PFKL and PPAR $\gamma$  mRNA expression levels and decreased IDH1, CS and OGDH mRNA expression levels compared with Huh7 cells (Fig. 4A), indicating that lncRNA Ftx may promote glycolytic metabolism in HCC cells.

*lncRNA Ftx facilitates glucose consumption through GLUTs in HCC cells.* The majority of cancer cells generate more ATP by increasing glucose utilization (24). To investigate whether lncRNA Ftx may affect aerobic glycolysis in HCC, a glucose assay kit was used. lncRNA Ftx-overexpressing Huh7 cells had significantly increased glucose consumption compared with negative control cells, while lncRNA Ftx-knockdown Bel-7402 cells had reduced glucose utilization (Fig. 4B); these findings suggested that lncRNA Ftx may be involved in aerobic glycolysis in HCC. GLUTs, as facilitative-type glucose transporters, are highly expressed in a number of types of cancer and are involved in cellular glucose consumption (25). To gain an understanding of the mechanisms through which lncRNA Ftx promotes glucose utilization in HCC cells, the GLUT1 and GLUT4 mRNA and protein expression levels were measured following lentiviral transfection. As presented in Fig. 4C and D, the mRNA and protein expression levels of GLUT1 and GLUT4 were significantly decreased following the downregulation of lncRNA Ftx in Bel-7402 cells. Consistently, the opposite results occurred with the upregulation of lncRNA Ftx in Huh7 cells. Taken together, the present findings suggested that GLUT1 and GLUT4 may be involved in lncRNA Ftx-mediated HCC glucose uptake.

*lncRNA Ftx favors lactate production by regulating glycolytic enzymes in vitro.* To evaluate glycolytic activity, a lactate assay kit was used to measure lactate production. lncRNA Ftx knockdown significantly decreased the lactate production in Bel-7402 cells, and lncRNA Ftx overexpression enhanced the lactate production in Huh7 cells (Fig. 5A). Glycolysis is regulated by slowing down or speeding up certain steps in the glycolytic pathway, and this regulation is accomplished by inhibiting or activating the involved enzymes. Furthermore, PFKL, a kinase enzyme that phosphorylates 6-phosphofructose, is a key regulatory enzyme in glycolytic metabolism. To elucidate the regulatory mechanism of lncRNA Ftx, the enzymic activity levels of LDH and PFKL and the mRNA and protein expression levels of PFKL were evaluated in lncRNA Ftx-overexpressing Huh7 cells and lncRNA Ftx-knockdown Bel-7402 cells, respectively. A positive association was observed between the lncRNA Ftx expression level and the enzymic activity levels of LDH and PFKL, and the mRNA and protein levels of PFKL (Fig. 5B-E), indicating that lncRNA Ftx enhances the activity and expression of glycolytic enzymes to increase lactate production in HCC cells.

*lncRNA Ftx weakens Krebs-cycle-associated molecules in HCC cells.* As the Krebs cycle is determined by the activity and expression of relative enzymes, the enzymatic activity and mRNA and protein expression levels of CS, IDH1 and OGDH were measured. As presented in Fig. 6, the activity, mRNA and protein expression levels of CS, IDH1 and OGDH were significantly enhanced with the downregulation of lncRNA Ftx in Bel-7402 cells, whereas the overexpression of lncRNA Ftx in Huh7 cells impaired the activity and expression levels. The results revealed that lncRNA Ftx promotes

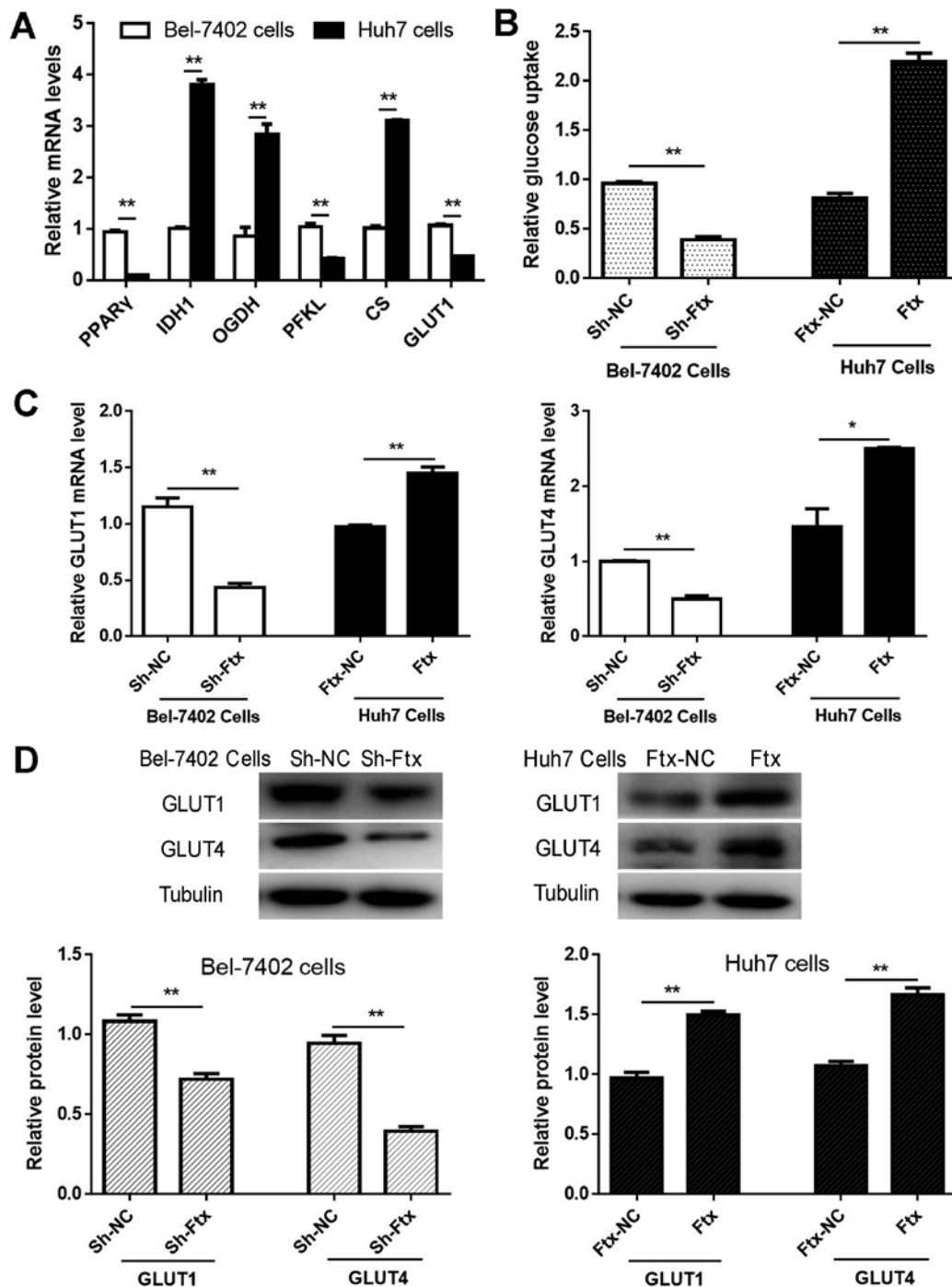


Figure 4. lncRNA Ftx facilitates glucose consumption via GLUTs in hepatocellular carcinoma cells. (A) Detection of GLUT1, PFKL, PPAR $\gamma$ , IDH1, CS and OGDH mRNA expression levels in Bel-7402 cells and Huh7 cells. (B) Measurement of glucose consumption levels in transfected HCC cells with a glucose assay kit. Bel-7402 cells and Huh7 cells were transfected, and the (C) mRNA and (D) protein expression levels of GLUT1 and GLUT4 were assayed by reverse transcription-quantitative polymerase chain reaction and western blotting, respectively. The mean and standard error of the mean of three independent experiments performed in triplicate are presented. \* $P < 0.05$  and \*\* $P < 0.01$ . lncRNA, long non-coding RNA; PPAR $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$ ; GLUT, glucose transporter; PFKL, phosphofructokinase, liver type; CS, citrate synthase; OGDH,  $\alpha$ -ketoglutarate dehydrogenase; IDH1, isocitrate dehydrogenase 1; sh, short hairpin.

the Warburg effect by impairing the activity and expression of Krebs-cycle-associated molecules in HCC cells.

*lncRNA Ftx expression is positively correlated with PPAR $\gamma$  expression in HCC tissues and cells.* To determine the underlying mechanism of the lncRNA Ftx-induced promotion of HCC cell tumorigenesis and aerobic glycolysis, bioinformatics

analysis was performed to predict the possible target genes or proteins of lncRNA Ftx. According to the nucleotide BLASTn program, the 940-1058 nt region of *Ftx* (a length of 118 nt) was highly homologous with PPAR $\gamma$  (Fig. 7A). The present results suggested that lncRNA Ftx may directly target the PPAR $\gamma$  gene and regulate the transcriptional and post-transcriptional expression of PPAR $\gamma$ .



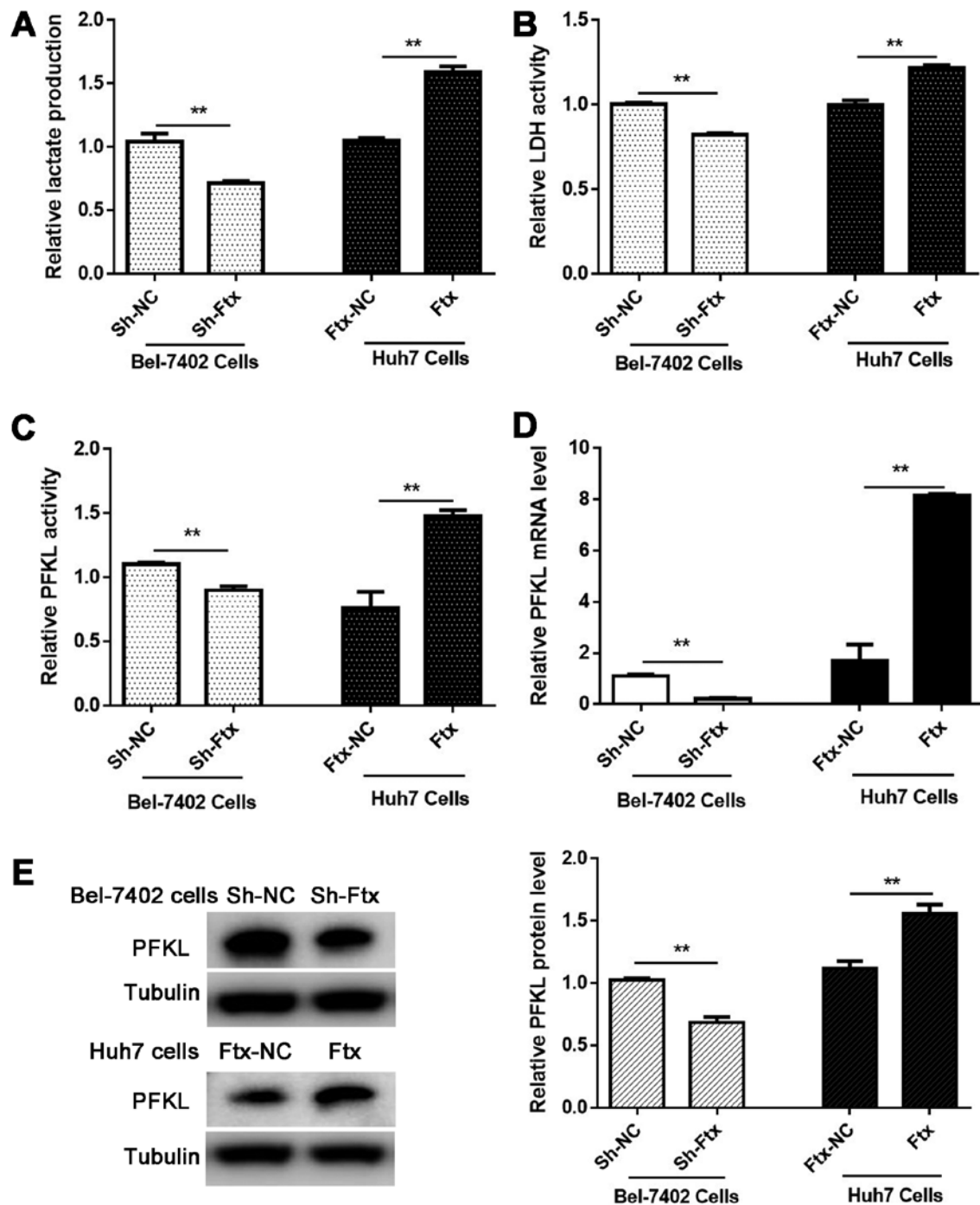


Figure 5. IncRNA Ftx favors lactate production through glycolytic enzymes *in vitro*. (A) Lactate production was determined with a lactate assay kit when hepatocellular carcinoma cells were transfected. Enzymatic activity levels of (B) LDH and (C) PFKL were determined with LDH and PFKL assay kits, respectively, following transfection. (D) mRNA and (E) protein expression levels of PFKL. Bel-7402 cells and Huh7 cells were transfected as described above. PFKL mRNA expression levels were identified by reverse transcription-quantitative polymerase chain reaction, and PFKL protein expression levels were detected by western blotting. The experiments were repeated at least three times and yielded similar results; the error bars represent the mean  $\pm$  standard error of the mean. \*\* $P < 0.01$ . IncRNA, long non-coding RNA; sh, short hairpin; NC, negative control; PFKL, phosphofructokinase, liver type; LDH, lactate dehydrogenase.

To assess PPAR $\gamma$  expression levels and their potential association with IncRNA Ftx in HCC tissues, the mRNA expression levels of PPAR $\gamma$  were compared in 73 pairs of HCC and adjacent normal tissue samples using RT-qPCR. The results demonstrated a positive correlation between the expression levels of IncRNA Ftx and PPAR $\gamma$  in HCC tissues (Fig. 7B). To identify the PPAR $\gamma$  expression status in HCC cells, mRNA and protein expression levels were assessed

by RT-qPCR and western blotting upon knocking down and overexpressing IncRNA Ftx in Bel-7402 cells and Huh7 cells, respectively. Consistent with the tissue results, PPAR $\gamma$  mRNA and protein expression levels were positively associated with IncRNA Ftx levels (Fig. 7C and D).

Together, these results suggested that PPAR $\gamma$  is a target gene of IncRNA Ftx and functions downstream of IncRNA Ftx in HCC cells.

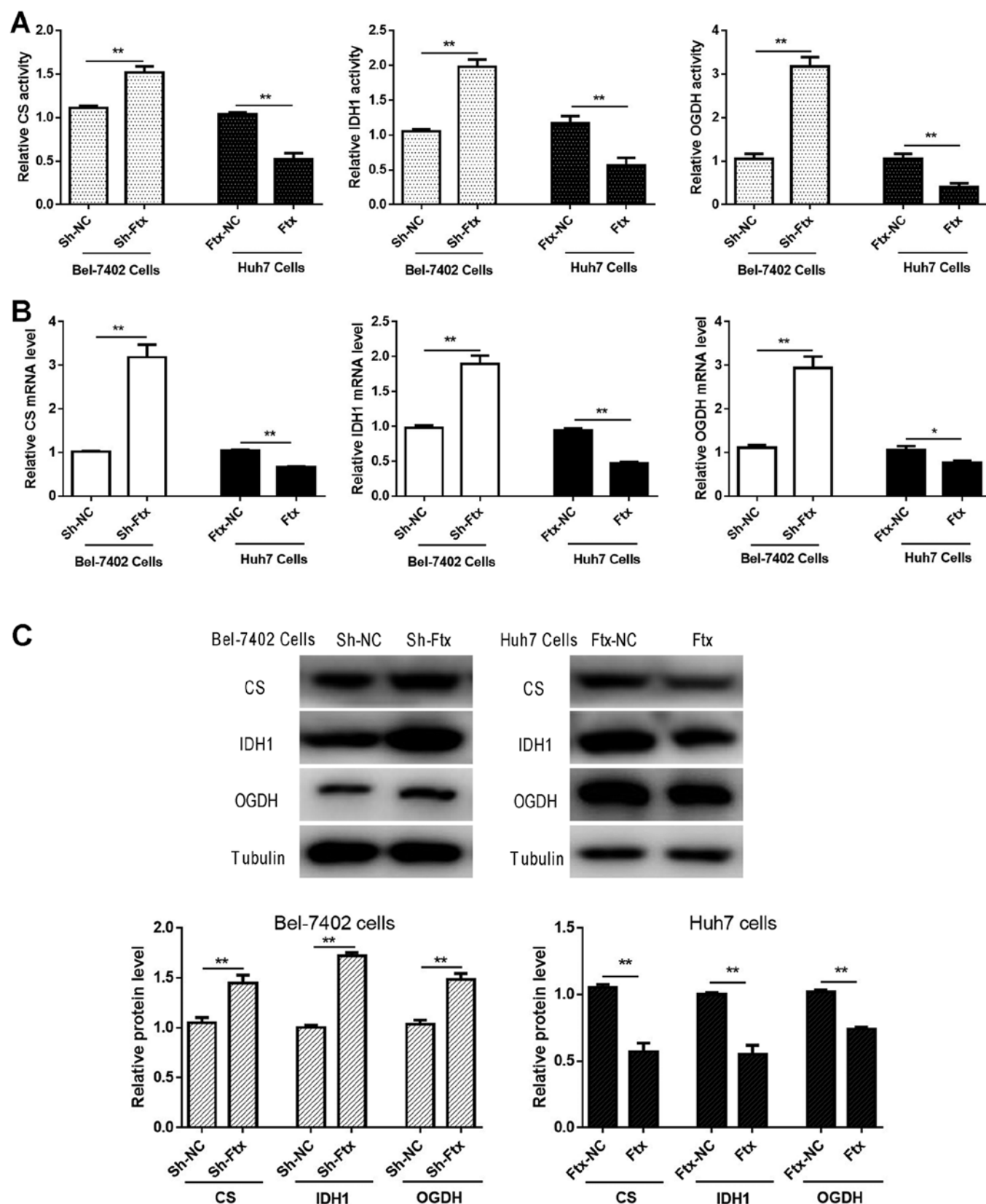


Figure 6. Long non-coding RNA Ftx downregulates Krebs-cycle-associated molecules in HCC cells. (A) Analysis of the activity levels of Krebs cycle-associated enzymes in HCC cells following lentiviral transfection. A total of three enzymes in the Krebs cycle were chosen for measurement via specific assays: CS, IDH1 and OGDH. (B) mRNA and (C) protein expression levels of Krebs cycle-associated enzymes (CS, IDH1 and OGDH). Bel-7402 cells and Huh7 cells were transfected; mRNA expression levels were identified by reverse transcription-quantitative polymerase chain reaction, and protein expression levels were detected by western blotting. The experiments were repeated at least three times and yielded similar results; the error bars represent the mean  $\pm$  standard error of the mean. \* $P < 0.05$  and \*\* $P < 0.01$ . HCC, hepatocellular carcinoma; sh, short hairpin; NC, negative control; CS, citrate synthase; IDH1, isocitrate dehydrogenase 1; OGDH,  $\alpha$ -ketoglutarate dehydrogenase.

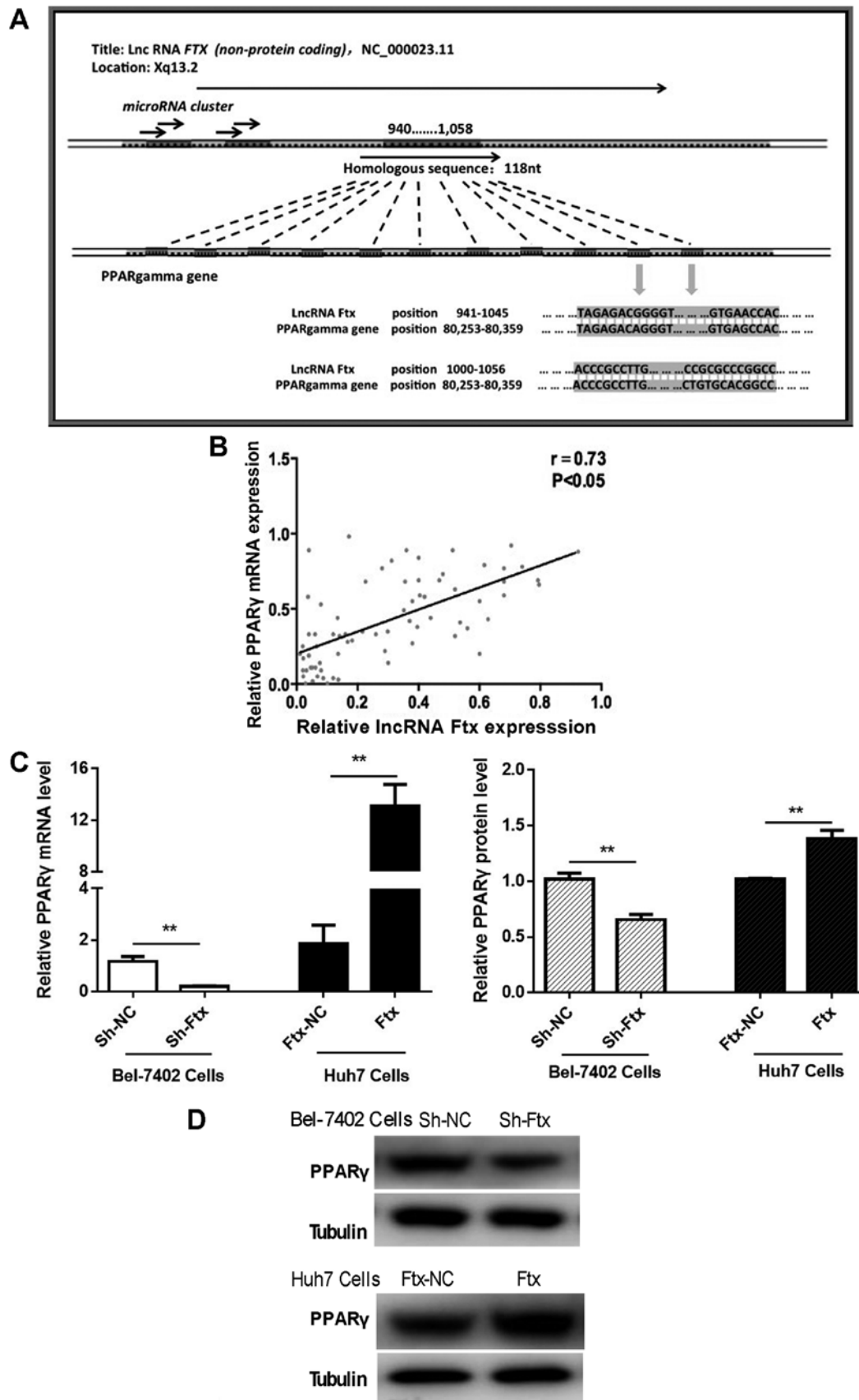


Figure 7. Analysis of PPAR $\gamma$  expression patterns in HCC tissues and cells. (A) Bioinformatics analysis of LncRNA Ftx. According to the National Center for Biotechnology Information databases, LncRNA Ftx and the PPAR $\gamma$  gene are highly homologous. (B) Correlation between LncRNA Ftx and PPAR $\gamma$  mRNA expression in HCC tissues. mRNA expression levels of LncRNA Ftx and PPAR $\gamma$  were detected in 73 pairs of HCC and adjacent normal tissue samples using the reverse transcription-quantitative polymerase chain reaction. Pearson's correlations were calculated to measure LncRNA Ftx expression and PPAR $\gamma$  expression. Analysis of PPAR $\gamma$  (C) mRNA and (D) protein expression levels in HCC cells following transfection. The experiments were repeated at least three times and yielded similar results; the error bars represent the mean  $\pm$  standard error of the mean. \*\* $P < 0.01$ . LncRNA, long non-coding RNA; PPAR $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$ ; NC, negative control; sh, short hairpin; HCC, hepatocellular carcinoma.

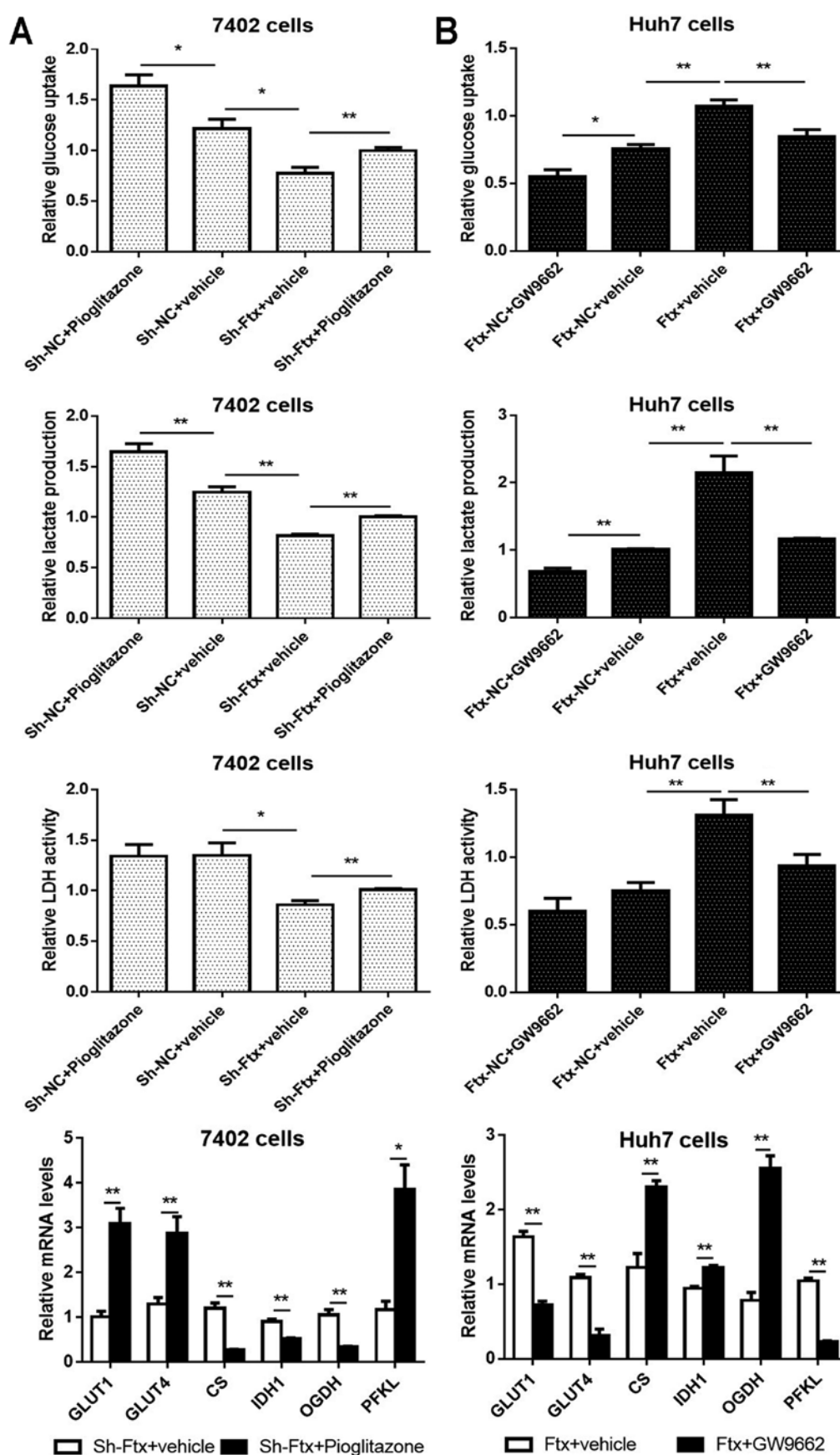


Figure 8. Alterations in PPAR $\gamma$  partially abolish lncRNA Ftx-mediated HCC aerobic glycolysis *in vitro*. (A) PPAR $\gamma$  activation induced effects opposite to those caused by lncRNA Ftx knockdown in Bel-7402 cells (left panel). (B) PPAR $\gamma$  inhibition partially abrogated the effects of lncRNA Ftx overexpression in Huh7 cells (right panel). The experiments were repeated at least three times and yielded similar results; the error bars represent the mean  $\pm$  standard error of the mean. \* $P < 0.05$  and \*\* $P < 0.01$ . PPAR $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$ ; sh, short hairpin; NC, negative control; lncRNA, long non-coding RNA; GLUT, glucose transporter; CS, citrate synthase; IDH1, isocitrate dehydrogenase 1; OGDH,  $\alpha$ -ketoglutarate; PFKL, phosphofructokinase, liver type.

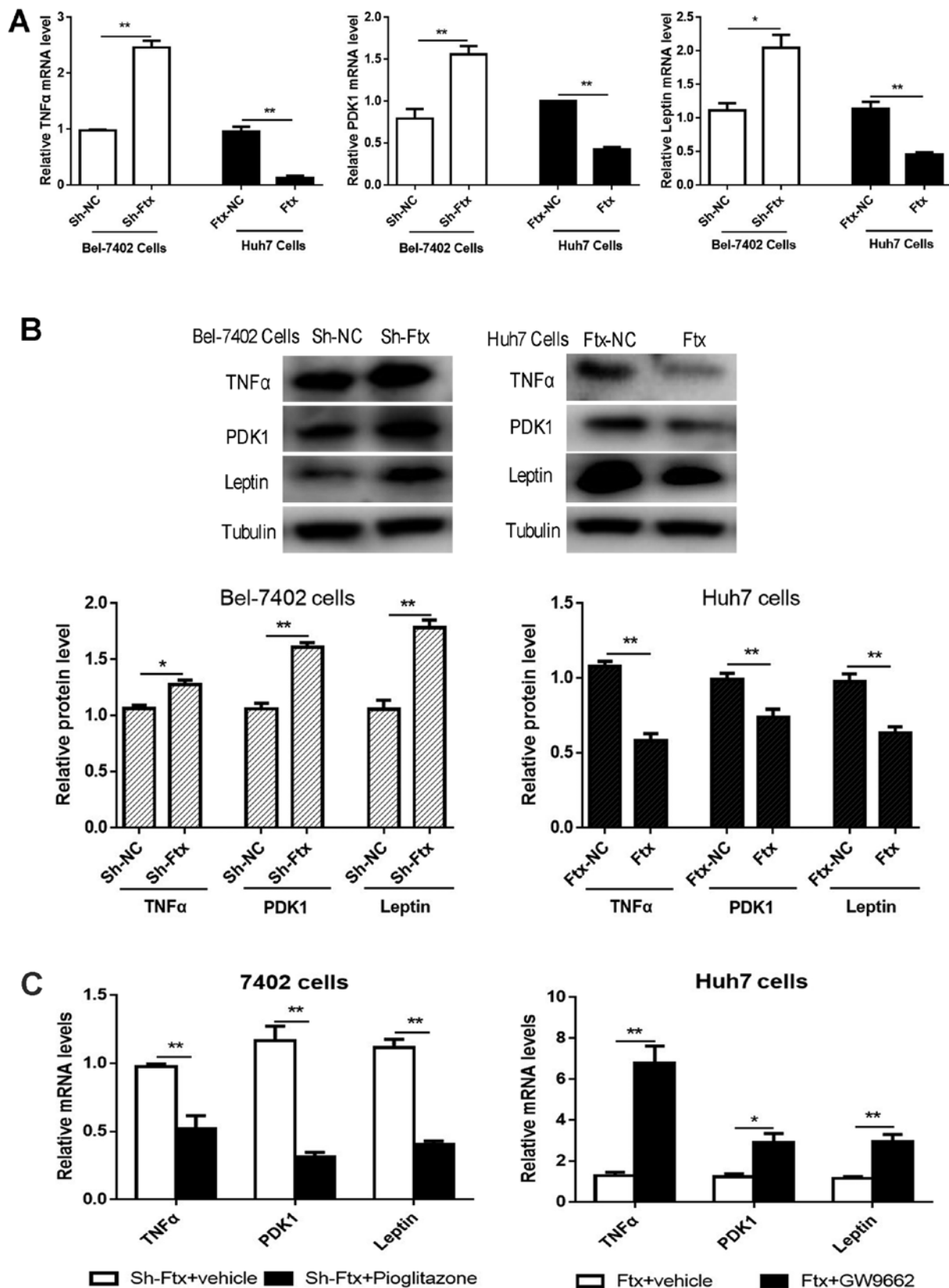


Figure 9. Analysis of PPAR $\gamma$  effectors: TNF $\alpha$ , PDK1 and leptin expression in HCC cells. Analysis of TNF $\alpha$ , PDK1 and leptin (A) mRNA and (B) protein expression levels in HCC cells following transfection. (C) Measurement of TNF $\alpha$ , PDK1 and leptin mRNA levels upon activating or inhibiting PPAR $\gamma$ . The experiments were repeated at least three times and yielded similar results; the error bars represent the mean  $\pm$  standard error of the mean. \*P<0.05 and \*\*P<0.01. PPAR $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$ ; TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; HCC, hepatocellular carcinoma; PDK1, pyruvate dehydrogenase kinase 1.

*Alterations in PPAR $\gamma$  partially abolish lncRNA Ftx-mediated HCC aerobic glycolysis in vitro.* To confirm whether the

promotion of aerobic glycolysis by lncRNA Ftx is mediated through the PPAR $\gamma$  pathway, PPAR $\gamma$  was suppressed with



its antagonist GW9662 in lncRNA Ftx-overexpressing Huh7 cells, and activated with its agonist pioglitazone in lncRNA Ftx-knockdown Bel-7402 cells. Subsequently, the glucose consumption, lactate production, LDH activity and mRNA expression levels of relative enzymes and molecules were analyzed. As presented in Fig. 8A, PPAR $\gamma$  activation enhanced glucose uptake, lactate production, LDH activity and the mRNA expression of GLUTs and PFKL, and suppressed the expression of CS, IDH1 and OGDH in lncRNA Ftx-knockdown Bel-7402 cells. The opposite results were observed in Huh7 cells (Fig. 8B). These results indicated that PPAR $\gamma$  may act as an important functional downstream mediator of lncRNA Ftx in HCC.

*lncRNA Ftx promotes the activation of the PPAR $\gamma$  pathway: TNF $\alpha$ , leptin and PDK1.* To further validate the underlying mechanisms, the protein and mRNA expression levels of PPAR $\gamma$  pathway effectors (TNF $\alpha$ , leptin and PDK1) were examined. It was observed that upon lncRNA Ftx overexpression in Huh7 cells, TNF $\alpha$ , leptin and PDK1 expression levels were decreased significantly, whereas in Bel-7402 cells, the opposite results were observed (Fig. 9A and B). As presented in Fig. 9C, pioglitazone downregulated the expression of TNF $\alpha$ , leptin and PDK1, while GW9662 upregulated their expression. Taken together, these data further support the hypothesis that lncRNA Ftx overexpression promotes the activation of the PPAR $\gamma$  pathway, thereby attenuating TNF $\alpha$ , leptin and PDK1 expression to maintain increased glycolysis in HCC.

## Discussion

lncRNAs have been increasingly recognized to serve principal functions in multiple biological processes, including the regulation of gene expression and chromatin conformation (26). Accumulating evidence has confirmed that a number of lncRNAs are abnormally expressed, and associated with tumor progression and prognosis in HCC (27). Therefore, the discovery of tumorigenesis-associated lncRNAs and the corresponding mechanisms may provide a novel insight into the diagnosis and treatment of HCC.

Recently, lncRNA Ftx was proposed to be a novel prognostic predictor and a prospective therapeutic target for HCC (28). Nevertheless, the molecular mechanisms through which lncRNA Ftx exerts its role in tumorigenesis remain largely unclear. In the present study, lncRNA Ftx was identified as an HCC tumor oncogene. First, lncRNA Ftx was significantly upregulated in HCC tissues and cell lines and associated with major clinicopathological features. Second, the ectopic expression of lncRNA Ftx accelerated proliferation, invasion, migration and aerobic glycolysis *in vitro*. Finally, it was identified that lncRNA Ftx exerted its tumor-promoting function, at least partially, via PPAR $\gamma$ . Taken together, these results support the further investigation of lncRNA Ftx as a therapeutic target for HCC.

The novel discovery of the present study was that the upregulation of lncRNA Ftx may facilitate HCC tumorigenesis through the PPAR $\gamma$  pathway, which is pivotal in lipid and carbohydrate metabolism. Based on the nucleotide BLASTn program, Ftx is highly homologous with PPAR $\gamma$ , including the promoter region of PPAR $\gamma$ . Thus, it was hypothesized that

lncRNA Ftx may directly interact with the promoter region or transcription factors of the PPAR $\gamma$  gene, thus influencing the transcriptional levels of PPAR $\gamma$ . Alternatively, lncRNA Ftx may regulate the expression of PPAR $\gamma$  by competitively sponging miRs; however, this hypothesis requires further investigation. In investigating the molecular mechanism of the promoting potential of the lncRNA Ftx/PPAR $\gamma$  axis, it was observed that lncRNA Ftx induced PPAR $\gamma$  overexpression to reduce the expression of downstream signaling proteins (TNF $\alpha$ , leptin and PDK1) in Huh7 cells; these proteins have been demonstrated to be downregulated by PPAR $\gamma$  (18-20). Furthermore, the PPAR $\gamma$  agonist and antagonist exerted similar effects on TNF $\alpha$ , leptin and PDK1. Therefore, the newly identified lncRNA Ftx/PPAR $\gamma$  axis provides an innovative approach to HCC tumorigenesis and indicates a potential target for HCC therapeutics.

The significance of the present study was also highlighted by the novel role of the lncRNA Ftx/PPAR $\gamma$  axis in promoting the glycolytic phenotype, which frequently correlates with HCC pathogenesis and worse clinical outcomes (29). Thus, future studies may focus on uncovering the essential role and underlying mechanism of aerobic glycolysis in HCC progression; this mechanism may be used for possible therapeutic interventions. The present data indicated that lncRNA Ftx facilitated glucose consumption, glucose transporter (GLUT1 and GLUT4) expression, lactate production, and glycolytic enzyme (LDH and PFKL) activity and expression. Conversely, lncRNA Ftx suppressed Krebs cycle-associated enzyme (CS, IDH1 and OGDH) activity and expression. Given that the PPAR $\gamma$  antagonist GW9662 partially rescued lncRNA Ftx overexpression-induced glycolysis increases, and the PPAR $\gamma$  agonist pioglitazone partially abolished lncRNA Ftx knockdown-mediated glycolysis decreases, it was hypothesized that there may be alternative targets of lncRNA Ftx that contribute to enhancing glycolysis.

Consistently, numerous lncRNAs have previously been reported to be associated with malignant carbohydrate metabolism (30,31). Therefore, targeting key metabolic enzymes is an important method for treating cancer (32). In the present study, it was observed that lncRNA Ftx, by activating the PPAR $\gamma$  pathway, regulated key glycolytic genes (including GLUT4, PDK1 and PFKL) and promoted glucose metabolism in HCC cells, indicating that lncRNA Ftx may serve as a novel metabolism-targeting therapeutic agent against HCC. In addition, recent studies have indicated that lncRNA Ftx inhibits cardiomyocyte apoptosis (33) and promotes glioma (34), colorectal cancer (35) and renal cancer (36) growth, suggesting that lncRNA Ftx is a general target for antitumor therapy.

Taken together, the present results provide the first evidence, to the best of our knowledge, that lncRNA Ftx promotes aerobic glycolysis and HCC cell progression by activating the PPAR $\gamma$  pathway. However, certain questions remain unclear and require further study. First, PPAR $\gamma$  may be proposed as the downstream effector of lncRNA Ftx, although direct binding was not proven in the present study and requires investigation. In addition, the alternative targets of lncRNA Ftx upon enhancing glycolysis merit investigation, in addition to *in vivo* experiments.

In conclusion, the present study recognized lncRNA Ftx to be a novel promoter of HCC progression and glycolysis by

targeting the PPAR $\gamma$  pathway. Therefore, lncRNA Ftx has the potential to be a promising diagnostic biomarker, a novel prognostic predictor and a therapeutic target for HCC. Targeting this aberrantly activated pathway may provide a novel approach for HCC therapy and merits further investigation.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

All authors involved helped to perform the research. CQ and QZ designed the experiment and revised the manuscript. XL and QZ performed the experiments. JQ and WW performed statistical data analysis. XL, DZ and ZL wrote the manuscript. All authors read and approved the content of the manuscript.

## Ethics approval and consent to participate

Written informed consent was obtained from each patient recruited for the present study for the use of materials. The consent procedures and all experimental protocols were approved by the Medical Institutional Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong University (Jinan, China; approval no. 2017-231), according to the Declaration of Helsinki.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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