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ORIGINAL RESEARCH

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Knockdown of PPFIA4 inhibits hepatocellular carcinoma growth and glycolysis via PFKFB3

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Abstract

Background: Protein tyrosine phosphatase receptor type F polypeptide interacting protein alpha 4 (PPFIA4) has been implicated in the growth and dissemination of tumor cells and is known to be elevated in several malignancies. However, the biological function and regulatory mechanisms of PPFIA4 in hepatocellular carcinoma (HCC) remain poorly understood. **Methods**: The Ualcan database was used to analyze PPFIA4 expression levels and survival outcomes in patients with HCC. HCC cell lines were transfected with siRNAs targeting PPFIA4 or with PPFIA4 overexpression plasmids. Cell viability at multiple time points was assessed using the Cell Counting Kit-8 (CCK8) assay. A colony formation assay was performed to quantify the number of crystal violet-stained colonies. Commercial kits were used to measure lactate production, glucose uptake, and Adenosine Triphosphate (ATP) content. The protein expression of the glycolytic enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) was evaluated by western blotting. To further investigate the mechanistic relationship, HCC cells were co-transfected with si-PPFIA4 and PFKFB3 plasmids, followed by assessment of lactate production, glucose uptake, ATP levels, and cell viability. All experiments were conducted in triplicate. **Results**: Analysis of the Ualcan database revealed that patients with HCC exhibited significantly elevated PPFIA4 expression ($p < 0.05$), which was associated with poorer survival outcomes. Functional assays demonstrated that PPFIA4 knockdown reduced HCC cell viability and colony formation, and significantly decreased ATP levels, glucose uptake, lactate production, and PFKFB3 protein expression ($p < 0.05$). Importantly, overexpression of PFKFB3 partially reversed the inhibitory effects of si-PPFIA4 on HCC cell proliferation and glycolysis ($p < 0.05$). **Conclusions**: This study demonstrates that PPFIA4 is highly expressed in HCC and promotes tumor progression by positively regulating PFKFB3 expression, thereby enhancing glycolytic activity and cell proliferation. These findings highlight the PPFIA4/PFKFB3 axis as a potential regulatory pathway in HCC metabolism and suggest that PPFIA4 may represent a promising therapeutic target.

Keywords

Hepatocellular cancer; PPFIA4; Proliferation; Glycolysis; PFKFB3

1. Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and is associated with a high mortality rate, making it one of the most prevalent malignant tumors worldwide. The major risk factors for the development of HCC include chronic hepatitis virus infection, aflatoxin exposure, and epigenetic modifications, and due to the high incidence of metastasis and recurrence, the prognosis of liver cancer remains poor [1]. Given that the molecular mechanisms underlying HCC progression are not yet fully elucidated, identifying novel therapeutic targets and clarifying the regulatory pathways involved are of great importance.

Aerobic glycolysis, a hallmark of tumor metabolism, has been extensively investigated in HCC. In addition to providing ATP to sustain cellular metabolism, glycolysis also creates a microenvironment that favors tumor cell proliferation and dissemination [2]. During tumorigenesis, aerobic glycolysis is associated with altered expression of numerous genes, among which 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) plays a pivotal role. PFKFB3 is highly expressed in various human malignancies and in rapidly proliferating cells, where it markedly accelerates glycolytic flux [3].

PPFIA4, which encodes liprin-α4, has recently emerged as a regulator of tumor progression and metabolic reprogramming, and growing evidence suggests that PPFIA4 promotes glycolysis in several cancer types [4]. For instance, knockdown of

PPFIA4 in ovarian cancer significantly reduced cell viability, migration, and invasion by suppressing hypoxia-induced glucose metabolic reprogramming [5]. Similarly, in colon cancer, silencing of PPFIA4 decreased glycolytic activity and inhibited proliferation, migration, and invasion, an effect mechanistically linked to the regulation of PFKFB3 [6]. These findings collectively suggest a potential connection between PPFIA4 and the Warburg effect across multiple malignancies.

Despite these advances, the expression profile, biological function, and mechanistic role of PPFIA4 in HCC remain poorly defined, and it remains unknown whether PPFIA4 exerts similar effects in HCC progression by modulating cellular metabolism. In this study, we demonstrate that PPFIA4 is markedly overexpressed in HCC and that its knockdown suppresses glycolysis and tumor progression, at least in part, through the regulation of PFKFB3.

2. Methods

2.1 Biological analysis

Differences in gene expression between malignant and normal tissues were analyzed using the Ualcan database (http://ualcan.path.uab.edu), which was then used to examine the expression of PPFIA4 in HCC and evaluate its association with overall survival in patients.

2.2 Cell culture and transfection

Human hepatoma cell lines (Huh-7 and Hep3B) and normal human liver cells (LO2), purchased from the American Type Culture Collection (ATCC), were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were then seeded into 24-well plates and incubated under the same conditions. All cell lines were authenticated by the supplier through short tandem repeat (STR) profiling and were confirmed to be free of mycoplasma contamination before use. Transfection was performed when cells reached approximately 50% confluence. Entranster-R4000 was used as the transfection reagent to introduce either si-*PPFIA4* (sequence: UUCUCUUCCUGAAUCAUCCUG), small interfering RNA negative control (si-NC), sequence: UUCUCCGAACGUGUCACGUTT) or *PPFIA4* overexpression plasmids (National Center for Biotechnology Information gene ID: 5209, pcDNA 3.1). Protein lysates were collected 48 hours after transfection to evaluate transfection efficiency.

2.3 CCK8 assay

To assess cell viability, HCC cells were seeded in 96-well plates at a density of 5×10^3 cells per well and transfected with plasmid or siRNA, as previously described. Each experimental group included three to six replicate wells. After transfection, 10 μL of CCK-8 reagent was added to each well, the plates were incubated for 2 hours at 37 °C in the dark, and absorbance was measured at 450 nm using a microplate reader.

2.4 Colony formation assay

Cells were seeded in 6-well plates at a density of 200 cells per well and maintained for 14 days at 37 °C in a humidified incubator with 5% CO_2. At the end of the culture period, the medium was removed, and the wells were rinsed once with Phosphate Buffered Saline (PBS) to eliminate residual dead cell debris. Colonies were then fixed with 1 mL of 4% paraformaldehyde per well for 15 minutes at room temperature. After removal of the fixative, crystal violet staining solution was added and incubated at room temperature for 20 minutes. Excess dye was removed by rinsing the wells gently three times with PBS until the background appeared clear and dry. Colonies containing at least 50 cells were counted as valid.

2.5 Glucose, lactate and ATP determination

Glucose consumption, lactate production, and ATP content were quantified using commercially available glucose, lactate, and ATP assay kits according to the manufacturers' protocols. Briefly, the cells were seeded in 12-well plates at a density of 5×10^4 cells per well. For each assay, 50 μL of sample or standard solution was added to the designated wells, followed by 50 μL of working solution. The plates were then incubated for 30 minutes at 37 °C in the dark, absorbance was measured using a microplate reader, and the values were used to calculate the concentrations of glucose, lactate, and ATP.

2.6 Western-blot

The cells were lysed in RIPA buffer supplemented with 1% protease inhibitor and incubated on ice for 20 minutes. The lysates were collected into centrifuge tubes and centrifuged at 12,000 rpm for 15 minutes at 4 °C, after which the supernatants were harvested. Protein concentrations were determined using the Bicinchoninic Acid Assay (BCA) protein assay kit following the manufacturer's instructions. Equal amounts of protein were mixed with Sodium Dodecyl Sulfate (SDS) loading buffer, adjusted to uniform concentrations with lysis buffer, and boiled for 5 minutes at 95 °C. The proteins (20 μg per lane) were separated by SDS-Polyacrylamide Gel Electrophoresis (PAGE) and transferred onto Polyvinylidene Fluoride (PVDF) membranes. After transfer, membranes were blocked with 5% skim milk in Tris Buffered Saline with Tween-20 (TBST) for 1 hour at room temperature and then incubated overnight at 4 °C with primary antibodies. The membranes were washed three times in TBST (10 minutes each wash) and incubated for 1 hour at room temperature with Horseradish Peroxidase (HRP)-conjugated secondary antibodies. Following three additional washes with TBST, membranes were exposed to Enhanced Chemiluminescence (ECL) reagent, and chemiluminescence signals were captured using an imaging system. Band intensities were quantified using the ImageJ software. The following primary antibodies were used: anti-PPFIA4 (Sigma-Aldrich, WH0008497M10, 5 μg/mL, St. Louis, MO, USA), anti-PFKFB3 (Sigma-Aldrich, SAB4301856, 1:1000, St. Louis, MO, USA), and anti-β-actin (Abcam, ab8227, 1:1000, Cambridge, UK).

2.7 Statistical analysis

Statistical analyses were performed using Statistical Product and Service Solutions (SPSS version 22.0, IBM Corporation, Chicago, IL, USA). Data are expressed as mean ± standard deviation (SD). Normality of data distribution was assessed with the Shapiro-Wilk test. Comparisons between two groups were conducted using the Student's *t*-test. For comparisons among multiple groups, one-way analysis of variance (ANOVA) was performed, followed by Tukey's *post hoc* test for multiple comparisons. The Kaplan-Meier survival analysis method was applied to evaluate the association between PPFIA4 expression and overall survival in HCC patients using data from the Ualcan database. All experiments were conducted with at least three independent biological replicates.

3. Results

3.1 PPFIA4 expression is elevated in hepatocellular cancer

Analysis of the Ualcan database revealed that PPFIA4 expression was significantly higher in HCC tissues compared with normal liver tissues (Fig. 1A). Survival analysis demonstrated that patients with HCC exhibiting high levels of PPFIA4 expression had significantly poorer overall survival compared with those with low PPFIA4 expression (Fig. 1B). In addition, western blotting experiments showed that PPFIA4 expression was significantly upregulated in HCC cell lines compared with normal human hepatocytes (Fig. 1C).

3.2 PPFIA4 knockdown inhibits the proliferation of hepatocellular carcinoma cells

To investigate the functional role of PPFIA4 in hepatocellular carcinoma cell proliferation, Hep3B and Huh7 cells were transfected with si-PPFIA4, and transfection efficiency was confirmed by western blotting. The results showed a significant reduction in PPFIA4 protein levels in the si-PPFIA4 group, confirming successful knockdown (Fig. 2A). Cell viability, measured by the CCK8 assay at multiple time points, was significantly reduced in cells transfected with si-PPFIA4 compared with controls (Fig. 2B). Consistently, colony formation assays demonstrated that the number of crystal violet-stained colonies was substantially decreased in the si-PPFIA4 group (Fig. 2C), indicating that knockdown of PPFIA4 suppresses the proliferative capacity of Huh7 and Hep3B cells. In contrast, overexpression of PPFIA4 in Huh7 and Hep3B cells resulted in significantly increased PPFIA4 protein levels, as confirmed by western blotting (Fig. 2D). Functional assays demonstrated that PPFIA4 overexpression enhanced cell viability (Fig. 2E) and promoted colony formation, as shown by an increased number of crystal violet-stained colonies (Fig. 2F). These findings indicate that PPFIA4 overexpression stimulates the proliferation of hepatocellular carcinoma cells, whereas PPFIA4 knockdown exerts an inhibitory effect.

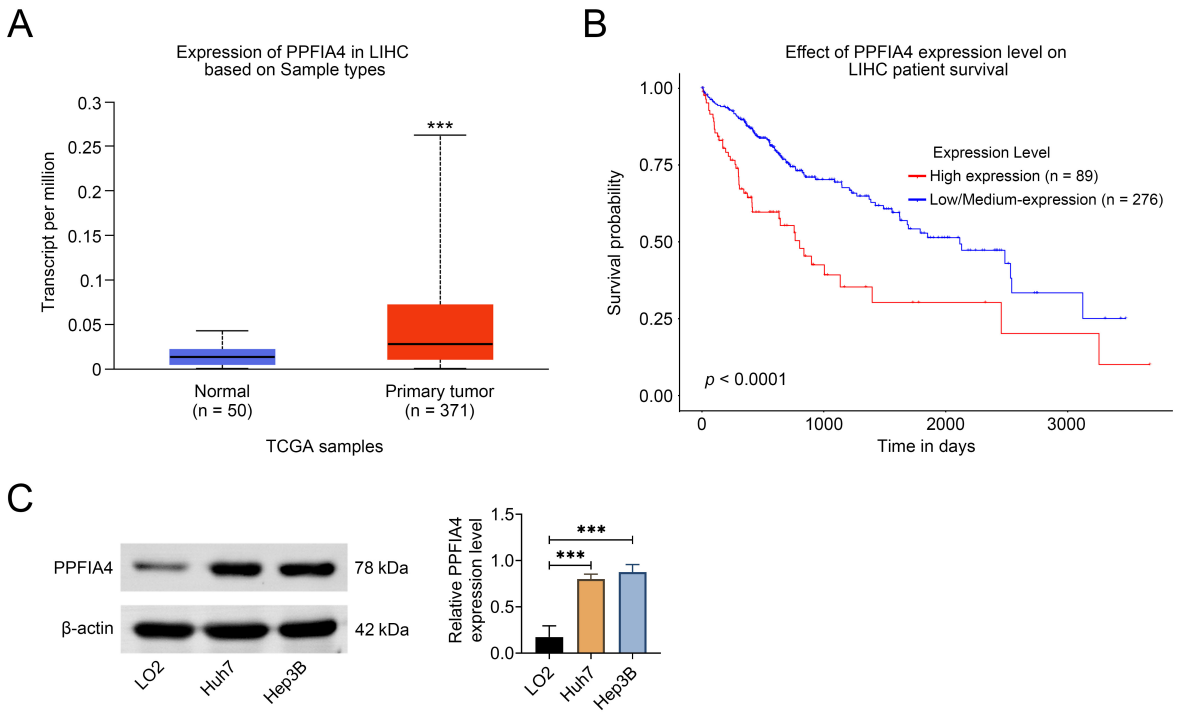

FIGURE 1. The expression of PPFIA4 is elevated in hepatocellular cancer. (A) Expression of PPFIA4 in HCC compared with normal tissues based on Ualcan database analysis. (B) Kaplan-Meier survival analysis showing the association between PPFIA4 expression and overall survival in HCC patients, as obtained from the Ualcan database. (C) PPFIA4 expression in HCC cell lines compared with normal human hepatocytes. ***$p < 0.001$ versus Nromal; ***$p < 0.001$ versus LO2. LIHC: Liver Hepatocellular Carcinoma; TCGA: The Cancer Genome Atlas; PPFIA4: Protein tyrosine phosphatase receptor type F polypeptide interacting protein alpha 4; LO2: normal human liver cells; Huh7: human hepatoma cell lines; Hep3B: human hepatoma cell lines.

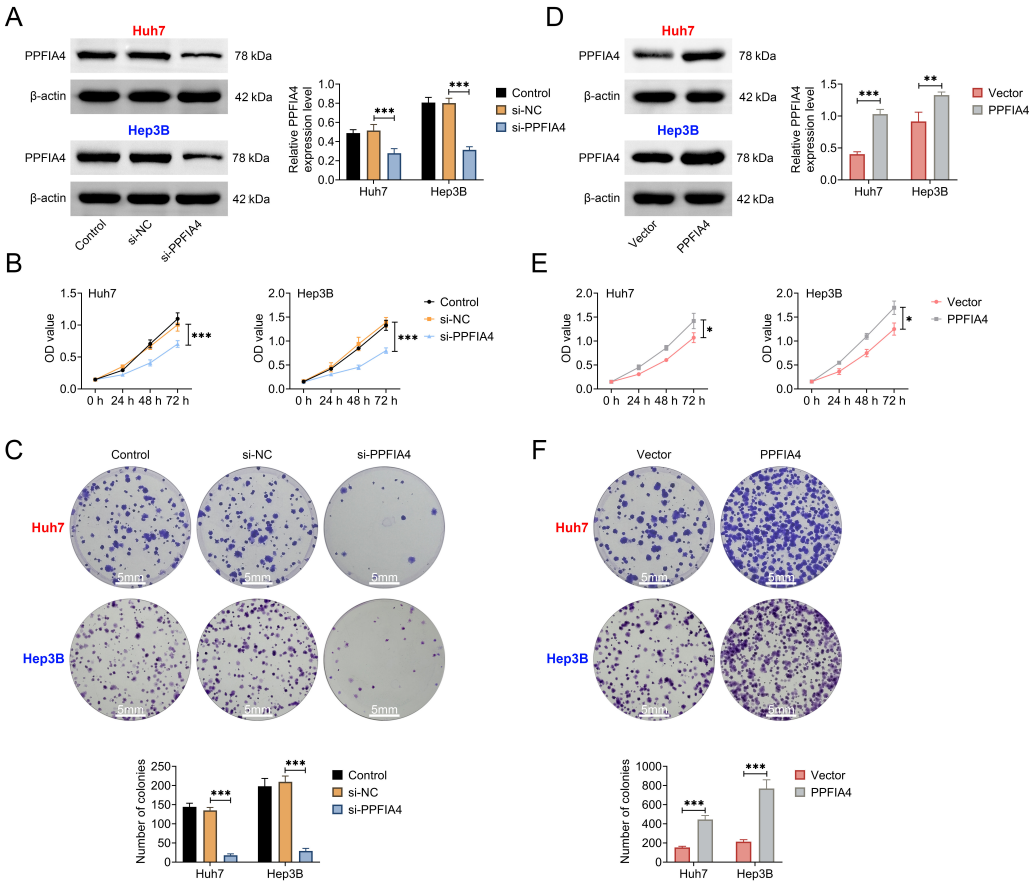

FIGURE 2. PPFIA4 knockdown inhibits hepatocellular carcinoma cell proliferation. (A) Western blot analysis of PPFIA4 protein expression in cells transfected with si-PPFIA4. (B) Cell viability of si-PPFIA4-transfected cells measured by CCK8 assay at different time points. (C) Colony formation assay showing the number of crystal violet-stained colonies in si-PPFIA4-transfected cells. (D) Western blot analysis of PPFIA4 protein expression in cells transfected with PPFIA4 overexpression plasmid. (E) Cell viability of PPFIA4-overexpressing cells assessed by CCK8 assay at different time points. (F) Colony formation assay showing the number of crystal violet-stained colonies in PPFIA4-overexpressing cells. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. PPFIA4: Protein tyrosine phosphatase receptor type F polypeptide interacting protein alpha 4; si-NC: small interfering RNA negative control; OD: Optical Density; Huh7: human hepatoma cell lines; Hep3B: human hepatoma cell lines.

3.3 PPFIA4 knockdown inhibits glycolysis in hepatocellular carcinoma cells

To determine whether PPFIA4 regulates glycolytic activity in hepatocellular carcinoma cells, intracellular ATP levels, glucose consumption, and lactate production were measured using commercial assay kits, while the protein expression of PFKFB3 was evaluated by western blotting. Cells transfected with si-PPFIA4 exhibited significantly decreased ATP content, reduced glucose consumption, and lower lactate levels compared with controls (Fig. 3A). In addition, western blot analysis demonstrated that PFKFB3 protein expression was markedly downregulated in si-PPFIA4-transfected cells (Fig. 3B).

Conversely, cells transfected with the PPFIA4 overexpression plasmid displayed significantly increased ATP levels, elevated glucose consumption, and higher lactate production compared with controls (Fig. 3C). Western blot analysis further revealed that PFKFB3 protein expression was upregulated in PPFIA4-overexpressing cells (Fig. 3D). These findings indicate that PPFIA4 promotes glycolysis in Hep3B and Huh7 cells by enhancing ATP generation, glucose utilization, lactate production, and PFKFB3 protein expression.

3.4 PPFIA4 knockdown inhibits hepatocellular carcinoma growth and glycolysis via PFKFB3

To further investigate whether PPFIA4 promotes hepatocellular carcinoma cell proliferation and glycolysis through regulation of PFKFB3, rescue experiments were performed in which Huh7 and Hep3B cells were co-transfected with si-PPFIA4 and PFKFB3. Cell viability, assessed using the CCK8 assay, demonstrated that PFKFB3 overexpression partially reversed the inhibitory effect of PPFIA4 knockdown on tumor cell growth (Fig. 4A). In parallel, measurements of ATP levels, glucose consumption, and lactate production revealed that restoration of PFKFB3 expression attenuated the reduction caused by PPFIA4 silencing (Fig. 4B). These results indicate that PPFIA4 promotes tumor cell proliferation and glycolytic activity in hepatocellular carcinoma cells, at least in part, through positive regulation of PFKFB3.

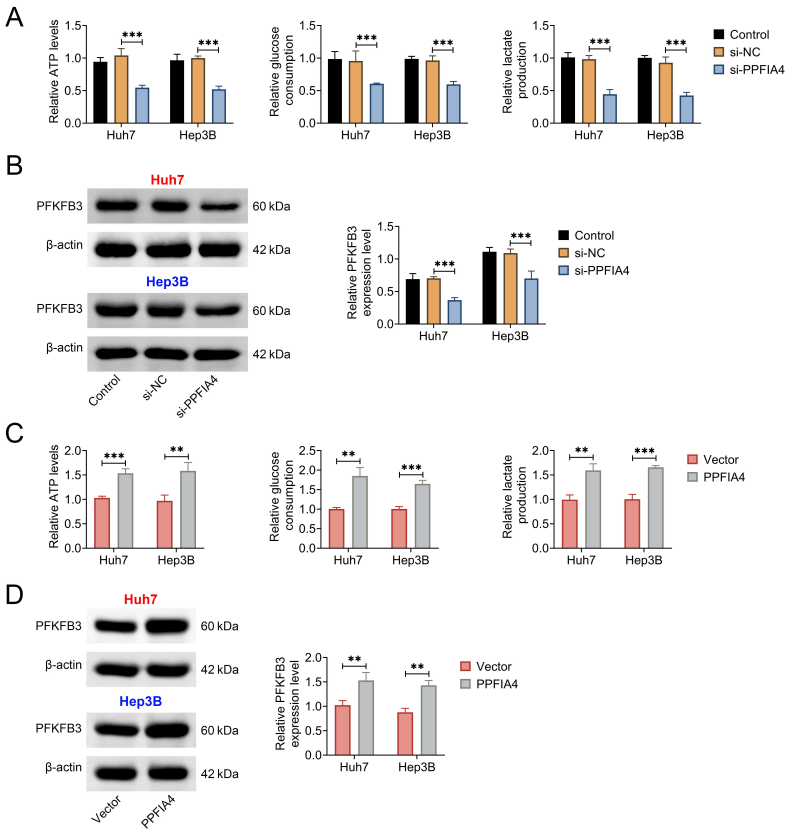

FIGURE 3. PPFIA4 knockdown inhibits glycolysis in hepatocellular carcinoma cells. (A) ATP content, glucose consumption, and lactate production in si-PPFIA4-transfected cells measured by commercial kits. (B) Western blot analysis of PFKFB3 protein expression in si-PPFIA4-transfected cells. (C) ATP content, glucose consumption, and lactate production in PPFIA4-overexpressing cells measured by commercial kits. (D) Western blot analysis of PFKFB3 protein expression in PPFIA4-overexpressing cells. $^{**}p < 0.01$, $^{***}p < 0.001$. PPFIA4: Protein tyrosine phosphatase receptor type F polypeptide interacting protein alpha 4; si-NC: small interfering RNA negative control; Huh7: human hepatoma cell lines; Hep3B: human hepatoma cell lines; ATP: Adenosine Triphosphate; PFKFB3: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3.

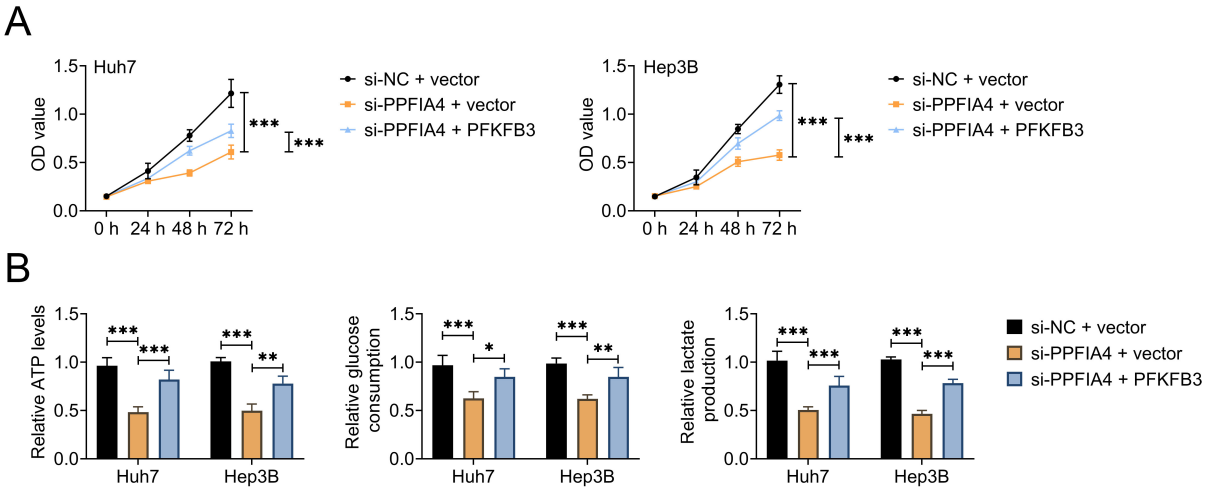

FIGURE 4. PPFIA4 knockdown inhibits hepatocellular carcinoma growth and glycolysis via PFKFB3. (A) CCK8 assay showing cell viability in cells co-transfected with si-PPFIA4 and PFKFB3 at different time points. (B) ATP content, glucose consumption, and lactate production in cells co-transfected with si-PPFIA4 and PFKFB3 measured by commercial kits. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. PPFIA4: Protein tyrosine phosphatase receptor type F polypeptide interacting protein alpha 4; si-NC: small interfering RNA negative control; Huh7: human hepatoma cell lines; Hep3B: human hepatoma cell lines; ATP: Adenosine Triphosphate; PFKFB3: 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3; OD: Optical Density.

4. Discussion

Primary liver cancer is the sixth most common malignant tumor worldwide and is responsible for the fourth-highest cancer-related mortality, with HCC accounting for approximately 80% of all cases [7]. HCC continues to represent a major global health burden, as it remains the leading cause of cancer-related death. Among the defining features of malignant transformation, metabolic reprogramming has been recognized as a central hallmark, with aerobic glycolysis playing a particularly important role by supplying both the energy and biosynthetic intermediates required to sustain rapid tumor cell proliferation and survival [8, 9]. In this context, the identification of novel molecular markers that contribute to these processes carries considerable clinical significance, as they may provide opportunities to improve patient prognosis and enhance quality of life, particularly in individuals with advanced-stage HCC. The present study demonstrated that HCC patients with high PPFIA4 expression levels had a significantly poorer overall survival rate. Functional analyses revealed that knockdown of PPFIA4 reduced both proliferation and glycolysis in HCC cells, whereas PPFIA4 overexpression enhanced these processes, effects that were mediated through the regulation of PFKFB3 expression.

Normal human cells primarily rely on mitochondrial oxidative phosphorylation to generate energy in aerobic environments; however, under conditions of oxygen deprivation, they switch to glycolysis as an alternative pathway [10]. Tumor cells differ markedly from normal cells in this respect, and a defining feature of tumor cell metabolic reprogramming is the adoption of aerobic glycolysis, whereby cancer cells preferentially metabolize glucose through the glycolytic pathway to produce lactate, even in the presence of sufficient oxygen supply [11]. This phenomenon, first described by Nobel laureate Otto Warburg in 1956 [12], is closely associated with tumor microenvironment remodeling, resistance to therapy, and malignant progression.

Among the key enzymes regulating this process, PFKFB3 plays a critical role in the control of glycolysis. Beyond its metabolic function, PFKFB3 has been implicated in tumor angiogenesis, chemoresistance, and cell proliferation [13]. Clinical studies have reported that patients with ovarian cancer exhibiting elevated PFKFB3 expression had significantly reduced survival, as PFKFB3 promoted cancer cell dissemination and enhanced proliferative activity [14]. Similarly, in HCC, high PFKFB3 expression was associated with poor prognosis, whereas pharmacological inhibition of PFKFB3 suppressed the proliferation of both tumor endothelial cells and HCC cell lines [15]. These observations support the concept that sustained PFKFB3 expression drives glycolysis and accelerates HCC cell proliferation [16].

The role of PPFIA4 has been shown to include the promotion of tumor growth, migration, and glycolysis. High expression of PPFIA4 has been observed in esophageal squamous cell carcinoma, where its inhibition reduces ATP and lactate levels in tumor cells and prevents invasion and migration [17]. In pancreatic cancer, inhibition of PPFIA4 suppresses invasion, epithelial-mesenchymal transition, and proliferation both *in vitro* and *in vivo* [18].

The clinical significance of our findings is further emphasized by the observation that elevated PPFIA4 expression is associated with poor prognosis in HCC patients according to the Ualcan analysis of TCGA data, indicating that PPFIA4 could serve as both a prognostic biomarker and a potential therapeutic target. Dependence of HCC cells on PPFIA4-driven glycolysis makes them metabolically vulnerable. Although direct targeting of scaffold proteins such as PPFIA4 remains difficult, the downstream effector PFKFB3 represents a druggable enzyme. Several small-molecule inhibitors of PFKFB3, including PFK15, have already demonstrated promising antitumor activity in preclinical studies [19, 20].

The identification of the PPFIA4-PFKFB3 axis as a central regulator of glycolysis in HCC represents a key innovation of this study. The inhibitory effects of PPFIA4 knockdown on cell proliferation and glycolysis were partially rescued by PFKFB3 overexpression, confirming that PFKFB3 acts downstream of PPFIA4. This observation is consistent with findings in other malignancies but represents the first demonstration in HCC of a direct regulatory effect of PPFIA4 on the PFKFB3-driven glycolytic pathway.

The ability of PPFIA4 to enhance ATP production and anaerobic glycolysis highlights its importance in maintaining energy homeostasis in rapidly dividing tumor cells. These findings expand the current understanding of HCC pathogenesis and support the potential of PPFIA4 as a therapeutic target for disrupting tumor metabolic adaptability.

Several limitations of this study should be considered. First, all experiments were conducted *in vitro*, and the absence of *in vivo* validation restricts the ability to fully assess the role of PPFIA4 in tumor growth within the physiological context. Second, the study did not explore the contribution of PPFIA4 to therapeutic resistance or metastatic progression, which are clinically important aspects of HCC biology. These limitations highlight the need for future investigations incorporating animal models and broader experimental approaches to provide a more comprehensive understanding of the functions of PPFIA4 in hepatocellular carcinoma.

5. Conclusions

This study demonstrates that PPFIA4 is overexpressed in HCC and contributes to tumor progression. Our experiments indicate that PPFIA4 promotes glycolysis and cellular proliferation through regulation of PFKFB3, thereby suggesting that the PPFIA4-PFKFB3 axis may represent a potential therapeutic target in HCC. Although these findings provide novel insights into the metabolic reprogramming of HCC, further validation in *in vivo* models and clinical samples will be essential. Overall, this work not only broadens the understanding of metabolic regulation in HCC but also establishes a rationale for targeting PPFIA4-PFKFB3 signaling in future mechanistic and translational studies.

AVAILABILITY OF DATA AND MATERIALS

The original data of the experiments in this study are available from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

CHJ—designed the study and carried them out. CHJ, YLG, ZZX, JYZ, HWL, CXS—supervised the data collection; analyzed the data; interpreted the data. CHJ, YLG, ZZX, BZ—prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This article does not involve any studies with human participants or animals performed by the authors.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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