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TGF-β1 upregulates the expression of lncRNA UCA1 and its downstream HXK2 to promote the growth of hepatocellular carcinoma

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Abstract. – OBJECTIVE: TGF-β1 plays pivotal roles in the development of various malignancies such as hepatocellular carcinoma, while the mechanism of the TGF-β1 function in hepatocellular carcinoma remains unclear. Our study aimed to investigate the molecular mechanisms of the TGF-β1 function in hepatocellular carcinoma.

PATIENTS AND METHODS: Tumor tissues and adjacent healthy tissues were collected from hepatocellular carcinoma. Blood samples were collected from both hepatocellular carcinoma patients and healthy controls. Expression of TGF-β1, long non-coding RNA (lncRNA) UCA1 and hexokinase 2 (HXK2) in those tissues was detected by qRT-PCR. All patients were followed up for 5 years, and prognostic values of serum HOTAIR for hepatocellular carcinoma were investigated by survival curve analysis. TGF-β1, UCA1, and HXK2 overexpression hepatocellular carcinoma cell lines were established, and the effects on cell proliferation were detected by the CCK-8 assay. Interactions between TGF-β1, UCA1, and HXK2 were explored by Western blot. Effects of TGF-β1 on lactate production, glucose uptake, and ATP production were detected by lactate assay, glucose uptake assay, and ATP assay.

RESULTS: TGF-β1, UCA1, and HXK2 expression levels were upregulated in tumor tissues comparing with adjacent healthy tissues. Serum levels of TGF-β1, UCA1, and HXK2 increased with the increases of primary tumor stage. Patients that have high serum levels of TGF-β1, UCA1, and HXK2 showed lower overall survival rate compared with patients with low serum levels of TGF-β1, UCA1, and HXK2. TGF-β1, UCA1, and HXK2 overexpression promoted proliferation of hepatocellular carcinoma cell. TGF-β1 is a positive upstream regulator of UCA1, which is a positive upstream regulator of HXK2. TGF-β1 overexpression increased lactate production, glucose uptake and ATP production in hepatocellular carcinoma.

CONCLUSIONS: TGF-β1 may accelerate cancer cell energy metabolism to promote the growth of hepatocellular carcinoma by upregulating UCA1 and its downstream HXK2.

Key Words:
Hepatocellular carcinoma, TGF-β1, lncRNA UCA1, Hexokinase 2.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the liver, which affects about 700,000 new cases every year[1]. In spite of the achievements that have been made in the treatment and prevention of this disease, HCC now is still the third leading cause of cancer-related death and the fifth most common malignancies[2]. Rapid economic development and changes in people's lifestyle significantly cause an increased rate of hepadnavirus infection, which is directly correlated with the occurrence and development of hepatocellular carcinoma especially in developing countries such as China. Hepadnavirus infection rate and incidence of hepatocellular carcinoma are predicted to increase shortly[3]. Hepatocellular carcinoma in the early stage is asymptomatic. Most patients with hepatocellular carcinoma lost the chance of surgical resection by the time of diagnosis due to distant tumor metastasis[4,5]. Therefore, the development of radical treatment is urgently needed to improve the sur-

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vival rate of hepatocellular carcinoma patients, though the treatment relies on an understanding of the disease pathogenesis.

Transforming growth factor beta 1(TGF-β1) is a member of transforming growth factor beta (TGF-β) superfamily of cytokines that plays multiple roles in cell growth, proliferation, differentiation and apoptosis[6]. TGF-β1 is usually overexpressed in HCC and it participates in various aspects of tumor growth and development such as epithelial-to-mesenchymal transition[7,8]. TGF-β1 signaling transduction in HCC remains unclear.

Patients and Methods

Patients

From August 2011 to August 2012, a total of 88 patients with HCC were enrolled in Qingdao No. 6 People's Hospital. HCC was diagnosed by pathological and imaging examinations. Those patients included 49 males and 39 females, and the age ranged from 21 to 77 years with the average age of 47±13.1 years. Primary tumors were staged according to the following standards: solitary tumor without vascular invasion, T1; multiple tumors or solitary tumor with vascular invasion or, the greatest diameter < 5 cm, T2; branch of the portal vein or hepatic vein invaded by single tumor or multiple tumors or greatest diameter of multiple tumors > 5 cm, T3; adjacent organs other than the gallbladder were invaded, T4. There were 16 cases of stage 1, 18 cases of stage 2, 20 cases of stage 3 and 34 cases of stage 4. All patients received surgical resection of primary tumors, and tumor tissues, as well as adjacent healthy tissues, were collected and confirmed by pathological examinations. At the same time, 32 healthy volunteers with similar age and gender distributions were involved in this study to serve as control group. This study was approved by the Ethics Committee of Qingdao No. 6 People's Hospital (Date of approval: 5/25/2017). All patients signed informed consents. Those patients were followed up for 5 years to record their survival conditions.

Preparation of Serum Samples

Fasting blood (about 30 ml) was collected from all 88 patients and 32 healthy controls in the morning of the day just after admission. Blood samples were kept at room temperature for 1.5 h, followed by centrifugation at 2000 rpm for 15 min to obtain the supernatant, which is serum. Serum samples were stored at -80ºC before use.

Cell Lines and Cell Culture

Human HCC cell lines HepG2 and Huh7 were obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, P. R. China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% heat-activated fetal bovine serum (FBS; GE Healthcare Life Sciences, Logan, UT, USA), 2 mM L-glutamine (Invitrogen; Thermo Fisher Scientific, Inc. Waltham, MA, USA) and 1% penicillin/streptomycin (Sigma-Aldrich: Great Island, NY, USA; Merck KGaA, Darmstadt, Germany), at 37°C in a humidified incubator with 5% CO_2. Cells were harvested during logarithmic growth phase for subsequent experiments.

Establishment of TGF-β1, UCA1, and HXK2 Overexpression Cell Lines

TGF-β1, UCA1, and HXK2 expression vectors were established by inserting an EcoRI-EcoRI fragment containing full-length TGF-β1, UCA1 or HXK2 cDNA into pIRSE2-EGFP (Clontech, Palo Alto, CA, USA). Cells were cultured overnight to reach 80-90% confluent before transfection. Transfection was performed using Lipofectamine 2000 transfection reagent (11668-019, Invitrogen, Carlsbad, CA, USA) according to the instructions. Empty vector without TGF-β1, UCA1 or HXK2 cDNA was used as a negative control.

Cell Proliferation Assay

Cells were collected during the logarithmic growth phase and they were used to make cell suspension. Then, 100 μl cell suspension containing $4x10^4$ cells were added to each well of a 96-well plate. Cells were cultured in an incubator (37°C), and CCK-8 solution (10 μL) was added to each well 24, 48, 72 and 96 hours later. After the addition of CCK-8 solution, cells were cultured at 37°C for another 4 h, and Fisherbrand™ accuSkan™ GO UV/Vis Microplate Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to measure OD values at 450 nm.

Lactate Production, Glucose Uptake, and ATP Production Assay

ATP levels were measured using ATP assay kit (Celltiter-Glo Luminescent Cell Viability Assay, Promega, Madison, WI, USA) according to the manufacturer's instructions. In glucose uptake assay and lactate production assay, cells were cultured in 6-well plates with about 5×10^5 cells in

each well for 24 h. The supernatant was collected and OD values at 570 nm were measured using a microplate reader with fresh culture medium as blank control. The following measurement was used: [Glucose uptake] = [concentration of glucose in fresh culture medium] – [concentration of glucose in tested samples]. Lactate production was measured according to the standard curve. Lactate production and glucose uptake were normalized to cellular protein.

Real-Time Quantitative PCR

After extracting total RNA from tumor tissues, adjacent healthy tissues, serum and *in vitro* cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), RNA quality was tested by gel electrophoresis, followed by reverse transcription to synthesize cDNA. PCR system was prepared using SYBR® Green Real-Time PCR Master Mixes (Thermo Fisher Scientific, Waltham, MA, USA). The following primers were used in PCR reactions: 5'-GGCCTTTCCTGCTTCTCATGG-3' (forward) and 5'-CCTTGCTGTACTGCGTGTCC-3' (reverse) for TGF-β1; 5'-GCCCAAG GAACATCTCACCAATTT-3' (forward) and 5'-TTGAGGGGTCAGACTTTTGACAAGG-3' (reverse) for UCA1; 5'-GGGCATCTTGAAACAAG-3' (forward); 5'-GGTCTCAAGCCCTAAG-3' (reverse) for HK2; 5'-GACCTCTATGCCAACACAGT-3' (forward) and 5'-AGTACTTGCGCTCAGGAGGA-3' (reverse) for β-actin. PCR reaction conditions were: 95°C for 50 s to 1 min, followed by 40 cycles of 95°C for 12 s and 60°C for 30-45 s. Ct values were processed using the $2^{-\Delta\Delta CT}$ method. The relative expression level of each gene was normalized to endogenous control β-actin.

Western Blot

Total protein extraction from *in vitro* cultured cells was performed using RIPA solution (Thermo Fisher Scientific, Waltham, MA, USA), and protein quality was determined by BCA method. After that, protein samples (20 μg) were subjected to 10% SDS-PAGE gel electrophoresis, followed by transmembrane to PVDF membrane. After blocking with 5% skimmed milk for 2 h at room temperature, membranes were incubated with primary antibodies including rabbit anti-TGF-β1 (1:2000, ab92486, Abcam, Cambridge, MA, USA), anti-HK2 (1:2000, ab37593, Abcam, Cambridge, MA, USA), and anti-GAPDH primary antibody (1:1000, ab8245, Abcam, Cambridge, MA, USA) and were performed overnight at 4°C. After that, membranes were washed 3 times with PBS, 15 min for each time, and incubated with anti-rabbit IgG-horseradish peroxidase (HRP) secondary antibody (1:1000, MBS435036, MyBioSource) for 2 h at room temperature, followed by signal detection using ECL (Sigma-Aldrich, St. Louis, MO, USA) method. Signals were scanned by MYECL™ Imager (Thermo Fisher Scientific, Waltham, MA, USA), and Image J software was used to normalize relative expression level of TGF-β1 and HK2 to endogenous control GAPDH.

Statistical Analysis

SPSS19.0 (SPSS Inc., Chicago, IL, USA) was used to process all the data. Measurement data were expressed as ($\bar{x} \pm s$), and comparisons between two groups were performed by two-tailed *t*-test. Comparisons among multiple groups were performed by the ANOVA and LSD test. Count data were compared using x^2-test. $p < 0.05$ was considered to be statistically significant.

Results

Expression of TGF-β1, UCA1 and HXK2 in Tumor Tissues As Well As Adjacent Healthy Tissues of Patients With HCC

Expression of TGF-β1, UCA1 and HXK2 in tumor tissues, as well as adjacent healthy tissues of 88 patients with HCC, was detected by qRT-PCR. As shown in Figure 1a, expression of TGF-β1 was significantly upregulated in tumor tissues compared with adjacent healthy tissues in 71 out of 88 patients ($p < 0.05$), while lower expression level of HOTAIR in tumor tissues than that in adjacent healthy tissues was observed in 7 cases ($p < 0.05$). No significant difference was found in 10 cases. For UCA1, a significantly higher expression level in tumor tissues than that in adjacent healthy tissues was found in 79 out of 88 patients ($p < 0.05$). A significantly lower expression level in tumor tissues than that in adjacent healthy tissues was found in 6 out of 88 patients, and no significant difference was found in 3 cases. For HXK2, a significantly higher expression level in tumor tissues than that in adjacent healthy tissues was found in 73 out of 88 patients ($p < 0.05$). A significantly lower expression level in tumor tissues than that in adjacent healthy tissues was found in 4 out of 88 patients, and no significant difference was found in 11 cases.

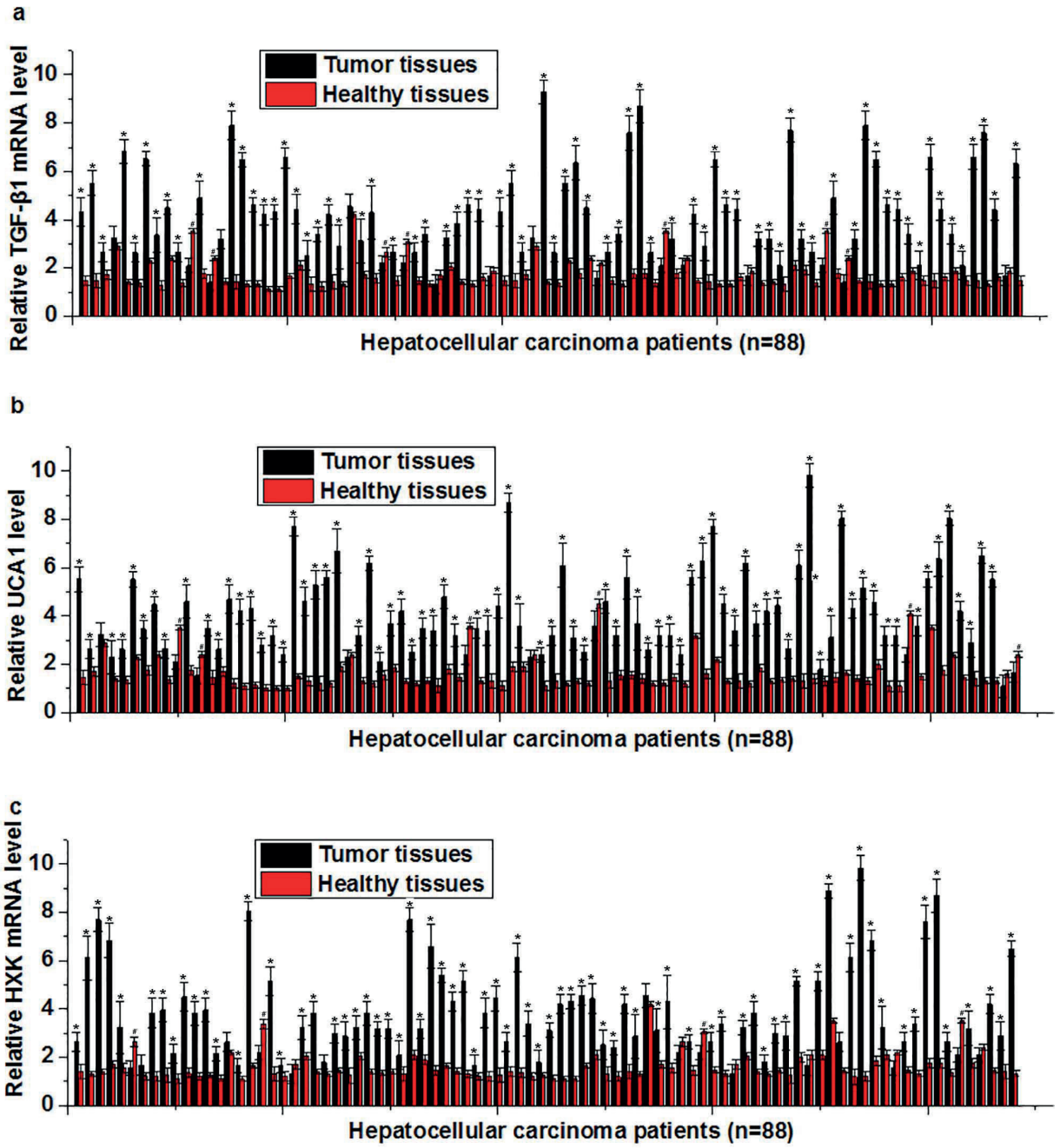

Figure 1. Expression of TGF-β1, UCA1 and HXK2 in tumor tissues and adjacent healthy tissues of patients with hepatocellular carcinoma. a. Expression of TGF-β1 in tumor tissues and adjacent healthy tissues; b. Expression of UCA1in tumor tissues and adjacent healthy tissues; c. Expression of HXK2 in tumor tissues and adjacent healthy tissues. Notes: *compared with tumor tissues, $p<0.05$; #compared with adjacent healthy tissues, $p<0.05$.

Expression of TGF-β1, UCA1 and HXK2 in Serum of Healthy Controls As Well As Patients With Different Stages of HCC

As shown in Figure 2a, the expression level of TGF-β1 mRNA from serum was significantly lower in healthy controls than that in patients with different stages of HCC ($p < 0.05$). In addition, the expression level of TGF-β1 mRNA was further increased with the increased stage of the primary tumor ($p < 0.05$). Similarly, serum levels of UCA1 and HXK2 were also significantly lower in healthy controls than those in patients with different stages of oral squamous

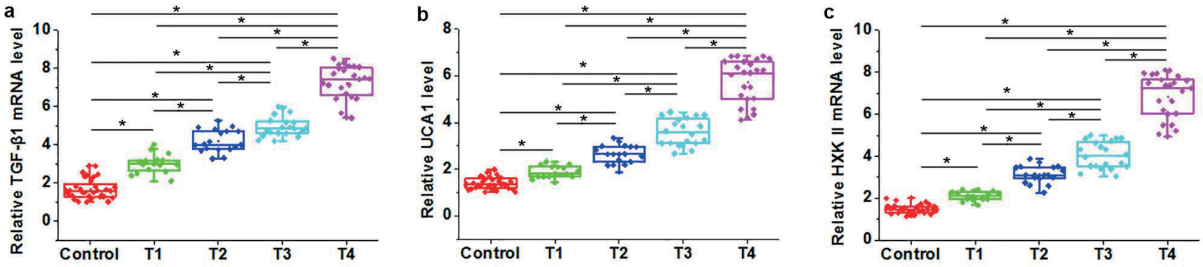

Figure 2. Expression of TGF-β1, UCA1, and HXK2 in serum of healthy controls and patients with different stages of HCC. a. Expression of TGF-β1 in serum of healthy controls and patients with different stages of HCC; b. Expression of UCA1 in serum of healthy controls and patients with different stages of HCC; c. Expression of HXK2 in serum of healthy controls and patients with different stages of HCC. Notes: *, $p<0.05$.

cell carcinoma (Figure 2b and c, $p < 0.05$). In addition, the expression level of UCA1 and HXK2 were significantly increased with the increased stage of the primary tumor (Figure 2b and c, $p < 0.05$). Those results suggest that upregulation of TGF-β1, UCA1 and HXK2 are very likely to be involved in the pathogenesis of HCC.

Prognostic Values of Serum TGF-β1 mRNA, UCA1, and HXK2 mRNA for HCC

All patients were followed-up for 5 years or until their death. Patients were divided into high expression group and low expression group according to the median serum level of TGF-β1 mRNA, UCA1, and HXK2 mRNA, respectively. Survival curve of each group of patients was plotted using Kaplan-Meier method and the curves were compared by log-rank *t*-test. As shown in Figure 3a, the overall survival rate of patients with the high expression level of TGF-β1 mRNA in serum was significantly lower than that of patients with the low expression level of TGF-β1 mRNA. Similarly, patients with high serum levels of UCA1 and HXK2 mRNA also showed lower overall survival rates compared with patients with low serum levels of UCA1 and HXK2 mRNA (Figure 3b and c).

TGF-β1, UCA1, and HXK2 Overexpression Promoted the Proliferation of HCC

TGF-β1, UCA1, and HXK2 overexpression were confirmed by detecting the expression levels of TGF-β1 mRNA, UCA1 and HXK2 mRNA by qRT-PCR (data not shown). Effects of TGF-β1, UCA1, and HXK2 overexpression on cell proliferation of HCC cell lines HepG2 and Huh7 were explored by the CCK-8 assay. As shown in Figure 4a and b, TGF-β1, UCA1 and HXK2 overexpression promoted the proliferation of HCC cell lines HepG2 and Huh7.

Interactions Between TGF-β1, UCA1,

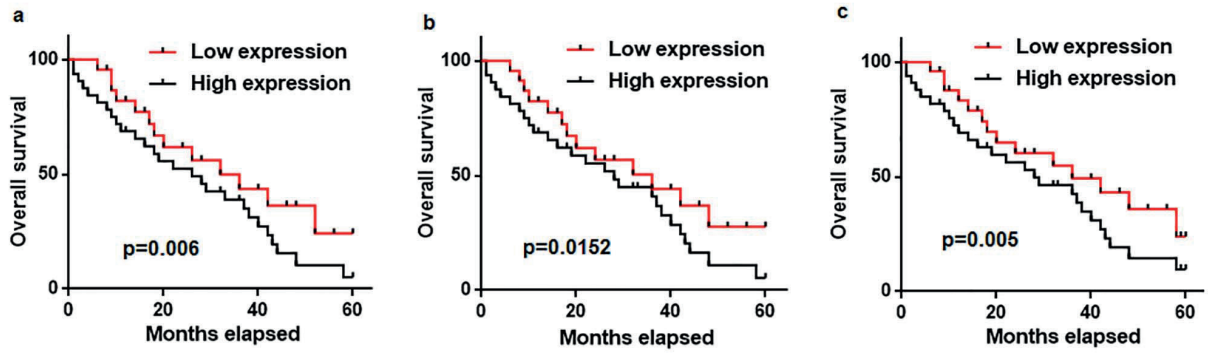

Figure 3. Prognostic values of serum TGF-β1 mRNA, UCA1, and HXK2 mRNA for HCC. a. Comparison of survival curves of patients with high and low serum level of TGF-β1 mRNA; b. Comparison of survival curves of patients with high and low serum level of UCA1; c. Comparison of survival curves of patients with high and low serum level of HXK2 mRNA.

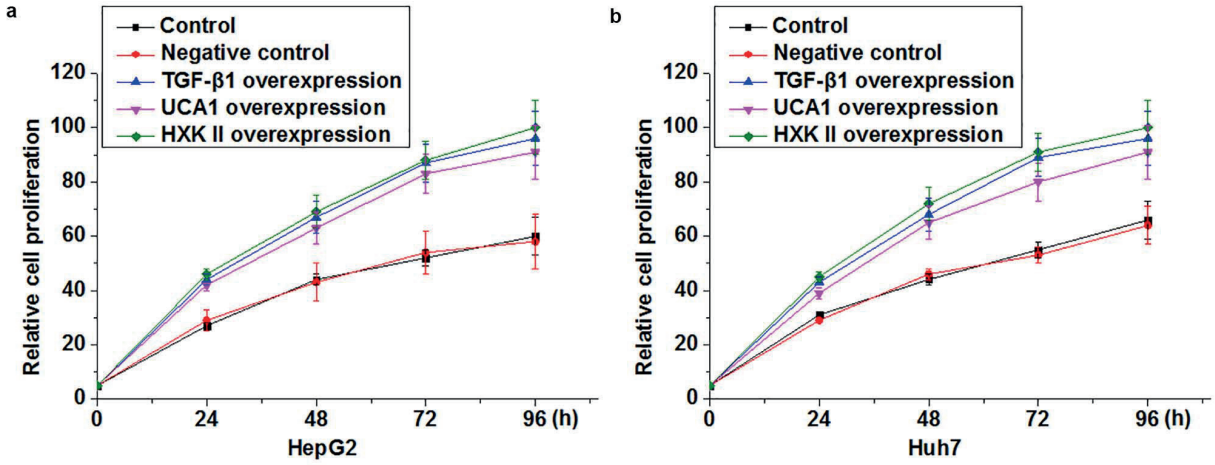

Figure 4. TGF-β1, UCA1 and HXK2 overexpression promoted the proliferation of HCC. a. TGF-β1, UCA1, and HXK2 overexpression promoted the proliferation of HCC cell line HepG2; b. TGF-β1, UCA1 and HXK2 overexpression promoted the proliferation of HCC cell line Huh7.

and HXK2

As shown in Figure 5a, TGF-β1 overexpression significantly increased the expression levels of UCA1 and HXK2 protein in the two-HCC cell lines ($p < 0.05$). UCA1 overexpression significantly promoted the expression of HXK2 protein but not that of TGF-β1 protein ($p < 0.05$, Figure 5b). In addition, HXK2 overexpression showed no significant effects on the expression of TGF-β1 or UCA1. Those data suggest that TGF-β1 is a positively upstream regulator of UCA1, which can positively regulate the expression of HXK2.

TGF-β1 Overexpression Promoted Lactate Production, Glucose Uptake and ATP Production in HCC Cells

As shown in Figure 6a, compared with the control cells, glucose uptake was significantly increased in cells of two HCC cell lines after TGF-β1 overexpression ($p < 0.05$). Similarly, lactate production (Figure 6b) and ATP production (Figure 6c) were also significantly increased in two cell lines after TGF-β1 overexpression. Those results suggest that TGF-β1 may accelerate cancer cell energy metabolism to promote the growth of HCC by upregulating UCA1 and its downstream HXK2.

Discussion

Upregulation of TGF-β1 expression is common in various types of human malignancies including HCC [7]. UCA1 is usually considered to be an oncogenic lncRNA in the development and progression of different human malignancies, such as non-small cell lung cancer[9], osteosarcoma[10] and so on. UCA1 is also upregulated during the progression of HCC, and the increased expression level of UCA1 has been proved to have diagnostic values for HCC [11]. Hexokinase II, or HXK2, is the key isozymes in aerobic glycolysis that is highly expressed in various types of cancer cells[12]. Consistent with previous studies, in the current study, expression levels of TGF-β1, UCA1, and HXK2 were found to be significantly higher in tumor tissues than those in adjacent healthy tissues for most of the patients with HCC. Besides, serum levels of TGF-β1 mRNA, UCA1, and HXK2 mRNA were significantly lower in healthy controls than those in patients with different stages of HCC . Expression levels of TGF-β1 mRNA, UCA1 and HXK2 mRNA were further increased with the increased stage of the primary tumor. Besides that, overall survival rate of patients with the high expression level of TGF-β1 mRNA, UCA1 and HXK2 mRNA in serum was significantly lower than that of patients with low expression levels. Those data suggest that upregulation of TGF-β1, UCA1 and HXK2 is very likely to be involved in the pathogenesis of HCC .

TGF-β1 plays different roles in the proliferation of various types of cells. It has been reported that TGF-β1 can induce the expression of basic fibroblast growth factor 2 to promote the proliferation of human renal fibroblasts[13]. In another study, it was found that downregulation of TGF-β1 expression by microRNA-663

inhibits the proliferation of glioblastoma cells[14]. In contrast, TGF-β1 is reported to be with an anti-proliferative function that can inhibit the growth of tumors[15]. UCA1 is usually considered

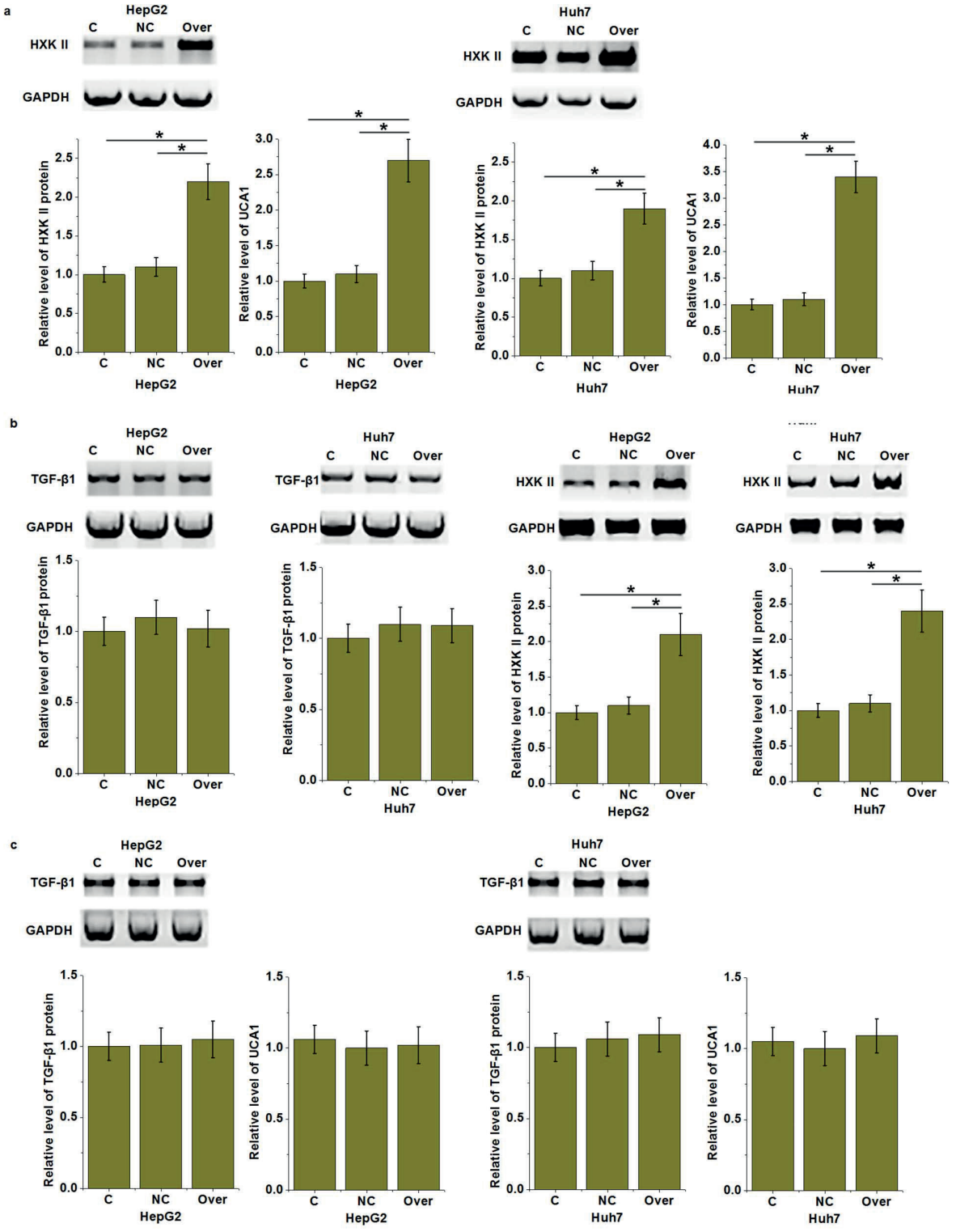

Figure 5. Interactions between TGF-β1, UCA1, and HXK2. a. Effects of TGF-β1 overexpression on expression of UCA1 and HXK2; b. Effects of UCA1 overexpression on expression of TGF-β1 and HXK2; c. Effects of HXK2 overexpression on expression of TGF-β1 and UCA1. Notes: *, $p<0.05$.

to be an oncogenic lncRNA that can promote the growth of different types of tumors by accelerating the proliferation rate of tumor cells[16]. HXK2 is the main isozyme in aerobic glycolysis, which is the energy resource of cancer cell proliferations[12]. In our study, TGF-β1, UCA1, and HXK2 overexpression promoted the proliferation of HCC cell lines HepG2 and Huh7. Those results suggest that upregulated expression of TGF-β1, UCA1, and HXK2 contributes to the proliferation of HCC cells.

A recent paper[17] reported that TGF-β1 induced the upregulation of lncRNA expression in gastric cancer, which in turn promoted invasion and migration of cancer cells. In another work, lncRNA UCA1 was reported to promote glycolysis by upregulating HXK2 in bladder cancer[18]. In our study, TGF-β1 overexpression significantly increased the expression levels of UCA1 and HXK2 protein in two HCC cell lines. In addition, UCA1 overexpression significantly promoted the expression of HXK2 protein but not the expression of the TGF-β1 protein. In contrast, HXK2 overexpression showed no significant effects on the expression of TGF-β1 and UCA1. Besides that, TGF-β1 overexpression significantly promoted glucose uptake, lactate production and ATP production in the two HCC cell lines. Those outputs suggest that TGF-β1 may accelerate cancer cell energy metabolism to promote the growth of HCC by upregulating UCA1 and its downstream HXK2.

Conclusions

We found that TGF-β1, UCA1 and HXK2 expression levels were upregulated in tumor tissues comparing with adjacent healthy tissues in most patients with HCC. Serum levels of TGF-β1, UCA1, and HXK2 increased with the increased primary tumor stage. Patients have high serum levels of TGF-β1, UCA1, and HXK2 showed lower overall survival rate than that of patients with low serum levels of TGF-β1, UCA1 and HXK2. TGF-β1, UCA1, and HXK2 overexpression promoted proliferation of HCC cell. TGF-β1 is a positive upstream regulator of UCA1, and UCA1 is a positive upstream regulator of HXK2. TGF-β1 overexpression increased lactate production, glucose uptake and ATP production in HCC cells. Therefore we may conclude that TGF-β1 could promote the growth of HCC by accelerating cancer cell energy metabolism through the upregulation of UCA1 and its downstream HXK2.

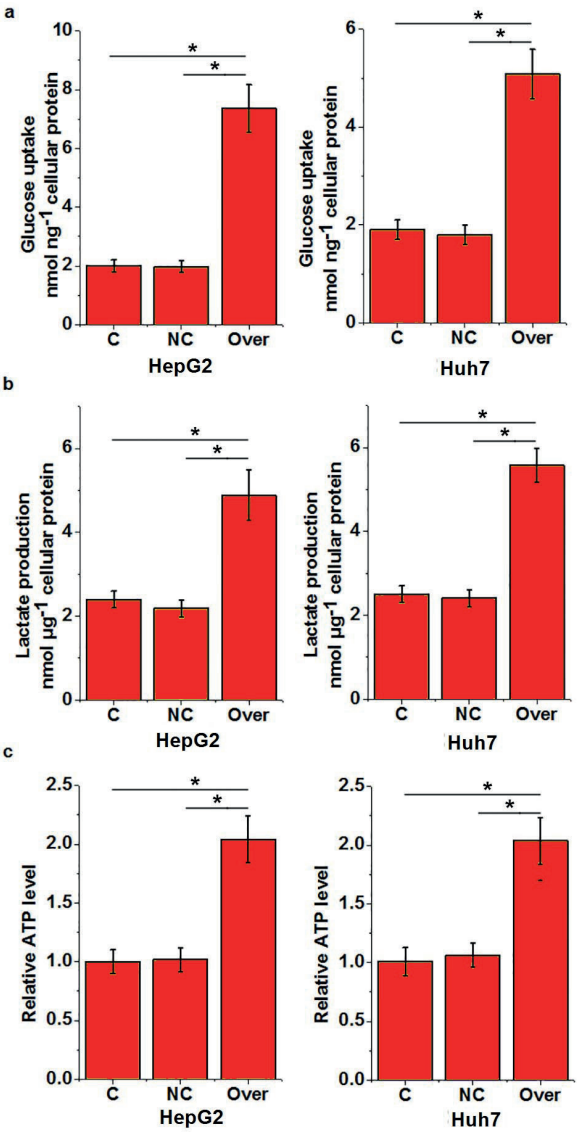

Figure 6. TGF-β1 overexpression increased lactate production, glucose uptake and ATP production in hepatocellular carcinoma cells. a. TGF-β1 overexpression increased glucose uptake in hepatocellular carcinoma cells; b. TGF-β1 overexpression increased lactate production in hepatocellular carcinoma cells; c. TGF-β1 overexpression increased ATP production in hepatocellular carcinoma cells. Notes: *, $p<0.05$.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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