Submitted: 11 April, 2024 Accepted: 29 May, 2024 Published: 30 June, 2024 DOI:10.22514/jomh.2024.099

ORIGINAL RESEARCH

JOMH Journal of Men's Health

ZNF677, a potential target in liver cancer, inhibits growth and stemness in hepatoma cells

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Abstract

Liver cancer is a common type of cancer worldwide, with over 90% of cases being hepatocellular carcinoma (HCC). The discovery of new targets holds promise for improving the prognosis of HCC patients. Zinc finger protein 677 (ZNF677), a member of the Kruppel C2H type 2 zinc finger family, has been implicated in the progression of several tumors. However, its specific role in HCC remains unclear. In this study, we investigated the role of ZNF677 in HCC progression. Our analysis revealed low expression of ZNF677 in HCC, which correlated with patient prognosis. Functionally, ZNF677 suppressed the growth, migration, invasion, and stemness of HCC cells. Additional data confirmed its ability to block the stemness of HCC cells. Mechanistically, ZNF677 achieved these effects by targeting the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) axis. In summary, ZNF677 inhibits both growth and stemness in hepatoma cells *via* modulation of the PI3K/Akt axis.

Keywords

Hepatocellular carcinoma (HCC); ZNF677; Prognosis; Stemness; Migration; Invasion; PI3K/Akt pathway

1. Introduction

Liver cancer is a common type of cancer worldwide, with over 90% of cases being hepatocellular carcinoma (HCC) [1]. Over the past few decades, the mortality rate associated with HCC has significantly increased [2], and patient prognosis remains poor, with a 5-year survival rate below 20% [3]. Due to the absence of reliable biomarkers for HCC, many patients are diagnosed at advanced stages. The key genes driving the progression of this cancer are potential targets for treatment, and identifying and characterizing new targets is essential for improving patient outcomes. Thus, there is an urgent need to discover novel targets to improve HCC patient prognosis.

Zinc finger (ZNF) proteins are transcription factors characterized by a "fingerlike" domain, playing important roles in various biological processes, including cell growth and maintaining homeostasis [4]. Studies have shown that ZNF proteins can act as cofactors in cell motility [5] and are implicated in cancer progression [6]. ZNF proteins are involved in all major pathways of carcinogenesis [7]. Furthermore, the role of ZNF proteins in tumorigenesis and their prognostic significance have been demonstrated in several cancers. ZNF677, a member of the Kruppel C2H2 type ZNF protein family, is notably absent in several tumor types [8] and acts by inhibiting the Akt/Forkhead box O3a (FOXO3a) pathway, which leads to the suppression of cell growth, migration, invasion, and tumorigenesis in oral squamous cell carcinoma (OSCC) [4]. ZNF677 is frequently silenced by promoter methylation in thyroid cancer [9] and has been shown to inhibit cell proliferation and induce apoptosis in renal cell cancer cells [8]. However, the specific role and mechanism of ZNF677 in HCC are not yet fully understood.

The PI3K/Akt axis is a crucial pathway that regulates apoptosis, autophagy, and cell growth [10–12]. Additionally, it plays a significant role in tumorigenesis and development, closely interacting with other pathways that govern tumor-related biological processes [12]. PI3K is also involved in the activation of substrates in HCC.

In this study, we investigated the role of ZNF677 in HCC progression, and our findings indicate that ZNF677 suppresses both the growth and stemness of HCC cells by modulating the PI3K/Akt axis.

2. Materials and methods

2.1 Bioinformatics

Transcriptome and survival rate data were obtained from The Cancer Genome Atlas (TCGA) of the Ualcan (https://ualcan.path.uab.edu/) and Prognoscan database (http://dna00.bio.kyutech.ac.jp/PrognoScan/index.html).

2.2 Cell culture

All cell lines, including HCC cell lines Huh7 and MHCC-97H, were purchased from the American Type Culture Collection (ATCC, Virginia, USA) and cultured using Eagle's Minimum Essential Medium (11965092, Gibco, CA, USA)

supplemented with 10% fetal bovine serum (FBS) (10099-133, Gibco, CA, USA) in 5% carbon dioxide (CO_2).

2.3 Cell transfection

The vectors designed to target ZNF677 (pcDNA3.1-ZNF677) and their respective control vectors (pcDNA3.1-vector) were developed by GenePharma (Shanghai, China). Briefly, 600 ng of each vector were transfected into Huh7 and MHCC-97H cells using Lipofectamine 3000 (L3000-015, Thermo, Waltham, Massachusetts, USA) and incubated for 24 h. After transfection, several *in vitro* assays were conducted for further experiments.

2.4 Immunoblot

Radio-Immunoprecipitation Assay (RIPA) lysate was used to lyse cells completely and extract proteins, which were quantified using a Bovine serum albumin (BCA) reagent. The proteins were then separated by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a polyvinylidene difluoride (PVDF) membrane, and blocked with Tris-buffered saline with Tween 20 (TBST) containing 5% milk for 1 h. The membrane was then incubated with the following primary antibodies: ZNF677 (Abnova, PAB21748; 1:500), Oct4 (Abcam, ab181557; 1:2000), Sox2 (Abcam, ab92494; 1:500), Akt (Abcam, ab8805; 1:1000), phosphorylated Akt (Abcam, ab38449; 1:1000, T308), and β-actin (Abcam, ab8226; 1:3000) for 1 hour, followed by secondary antibodies. Chemiluminescence was performed to detect the protein bands.

2.5 CCK-8 assay

Cell viability assays were performed using a CCK-8 kit (C0038, Beyotime, Beijing, China). Briefly, the cells were seeded in 96-well plates at approximately 1000 cells per well and incubated with CCK-8 solution for 3 hours, after which absorbance was measured at 450 nm using a microplate reader.

2.6 Colony formation assay

For this assay, 1000 cells were plated in 6-well plates and cultured in media containing 10% FBS for 14 days at 37 °C. Then, the cells were fixed with paraformaldehyde (PFA) for 15 minutes and stained with 0.1% crystal violet for 20 minutes. Lastly, the cells were photographed to assess colony formation.

2.7 Transwell assay

Migration analysis was conducted by allowing the cells to move into a transwell without Matrigel (for migration assays) or with 20% Matrigel (for invasion assays) for 24 hours. Following this, the cells were fixed with 4% paraformaldehyde, stained with 0.2% crystal violet, and subsequently imaged.

2.8 Formation of tumor spheroids

HCC cells were sorted and cultured in a medium containing 20 ng/mL of basic fibroblast growth factor (FGF) and epidermal growth factor (EGF). This culturing process lasted for 1–2 weeks, and the medium was replaced every three days. At the end of the culture period, the spheroids that had formed were detached using 0.25% trypsin and were then examined under a microscope.

2.9 Statistical analysis

Statistical analysis was conducted using GraphPad 5.0 software (GraphPad company, San Diego, CA, USA). Data are presented as mean $\pm$ standard deviation (SD), and a p-value of less than 0.05 was considered statistically significant.

3. Results

3.1 Downregulation of ZNF677 in human HCC samples and association with poor prognosis

To investigate the potential effects of ZNF677 on HCC progression, we analyzed its expression levels in HCC tissues using data from the TCGA database. The results indicated that ZNF677 transcripts per million were significantly reduced in HCC tissues (Fig. 1a). Additionally, we examined the relationship between ZNF677 expression and patient prognosis using bioinformatic analysis and observed that lower ZNF677 expression was associated with poorer prognosis in HCC patients (Fig. 1b). These results suggest that ZNF677 is expressed at low levels in human HCC tissues and is inversely correlated with patient prognosis.

3.2 ZNF677 suppressed HCC cell growth

Next, we investigated the effects of ZNF677 on HCC cell growth *in vitro*. Initially, pcDNA3.1-ZNF677 plasmids were transfected into two HCC cell lines, Huh7 and MHCC-97H, to enhance ZNF677 expression. CCK-8 assays demonstrated that ZNF677 overexpression significantly inhibited the growth of these cells, as indicated by reduced optical density at 450 nm (OD450) compared to control or vector-transfected cells (Fig. 2a). Additionally, ZNF677 overexpression led to reduced colony formation in both Huh7 and MHCC-97H cells, as reflected by fewer colonies (Fig. 2b), thus confirming that ZNF677 could suppress the growth of HCC cells.

3.3 ZNF677 restrained the migration and invasion of HCC cells

Then, we examined the effects of ZNF677 on the motility of HCC cells. Using transwell migration assays, we observed that ZNF677 overexpression significantly reduced the migration of both Huh7 and MHCC-97H cells compared to control or vector-transfected cells (Fig. 3a). Similarly, transwell invasion assays revealed that ZNF677 overexpression inhibited the invasion of these cell lines, as indicated by a decrease in the number of invasive cells (Fig. 3b). These results collectively demonstrate that ZNF677 effectively restrains the motility of HCC cells.

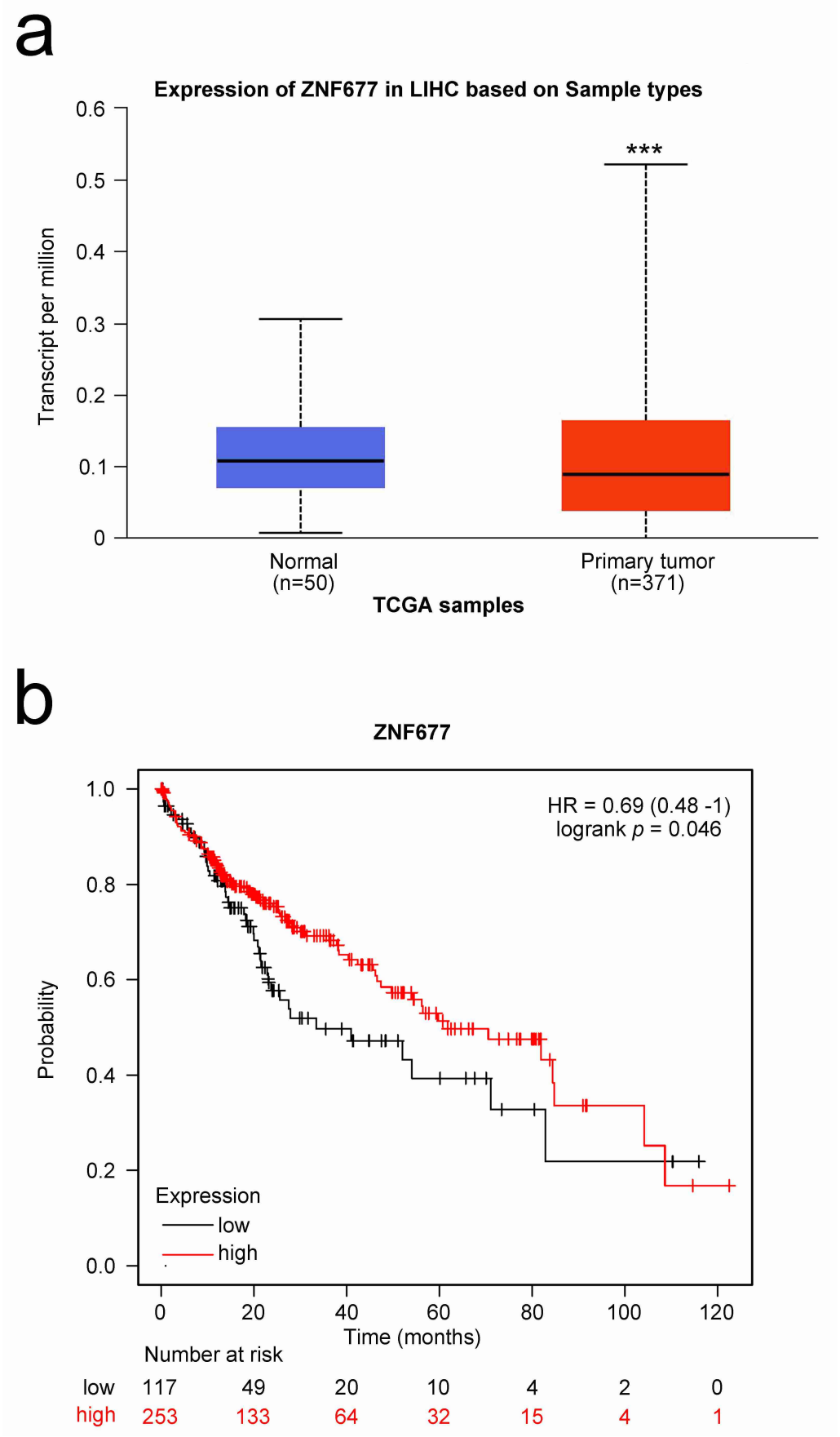

FIGURE 1. Low ZNF677 expression in human HCC tissues and correlation with prognosis. (a) Analysis from the TCGA database indicates low ZNF677 expression in 371 HCC tissues compared to 50 normal tissues. (b) Data from the PrognoScan database demonstrate a correlation between low ZNF677 expression and poor prognosis in HCC patients ($p = 0.046$). ZNF: Zinc finger; TCGA: The Cancer Genome Atlas; LIHC: Liver Hepatocellular Carcinoma; HR: Hazard Ratio; ***$p < 0.001$.

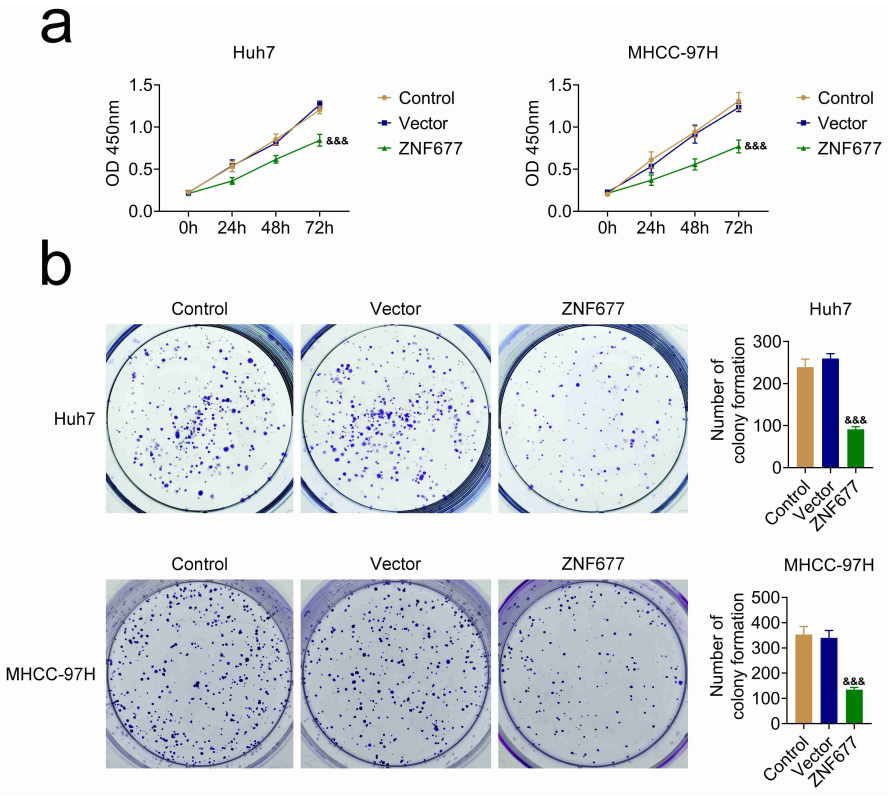

FIGURE 2. ZNF677 suppressed HCC cell growth. (a) CCK-8 assays evaluating the viability of Huh7 and MHCC-97H cells following transfection with control and ZNF677 overexpression plasmids for 24 hours. The optical density at 450 nm (OD450) was measured to assess cell viability. (b) Colony formation assays demonstrating the growth of Huh7 and MHCC-97H cells after 24 hours of transfection with control vectors and ZNF677 overexpression plasmids. The number of colonies was counted. $^{\&\&\&}p < 0.001$, ZNF677 *vs.* vector. ZNF: Zinc finger.

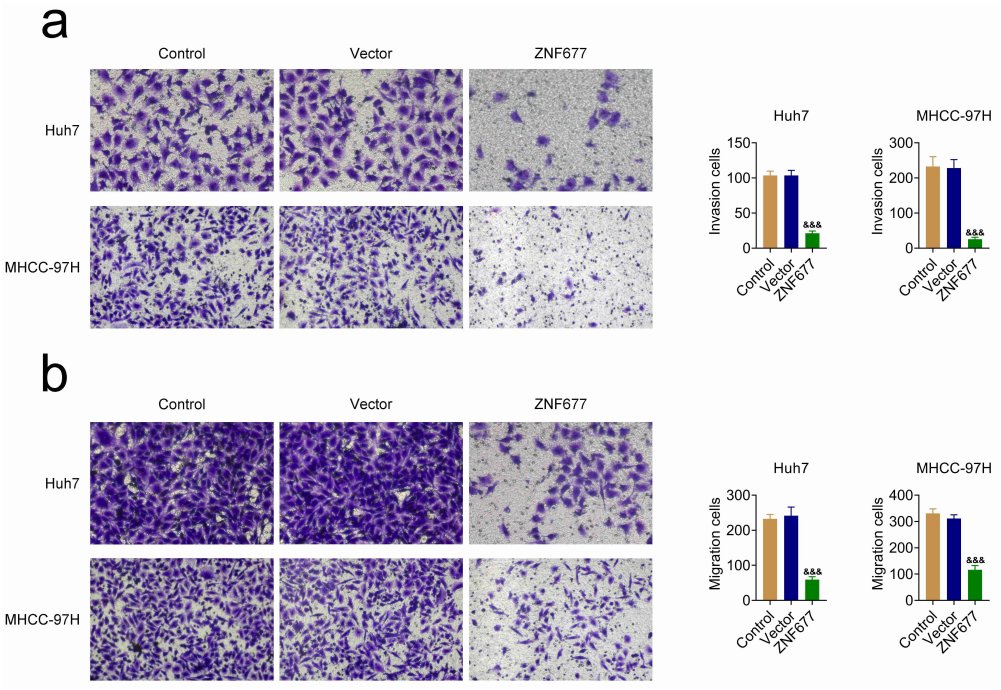

FIGURE 3. ZNF677 inhibits HCC cell migration and invasion. (a) Transwell migration assays displaying the migration of Huh7 and MHCC-97H cells after 24 hours of transfection with control vectors and ZNF677 overexpression plasmids. The number of migrating cells was counted. (b) Transwell invasion assays illustrating the invasion of Huh7 and MHCC-97H cells following 24 hours of transfection with control vectors and ZNF677 overexpression plasmids. The number of invading cells was counted. $^{\&\&\&}p < 0.001$, ZNF677 *vs.* vector. ZNF: Zinc finger.

3.4 ZNF677 inhibited the stemness of HCC cells

To assess the impact of ZNF677 on HCC cell stemness, an important process essential for HCC progression, sphere-forming assays were conducted. The results showed that overexpression of ZNF677 significantly reduced the number of spheres formed by Huh7 and MHCC-97H cells, indicating suppression of stemness (Fig. 4a). Further, immunoblot assays validated that ZNF677 overexpression led to a decrease in the expression levels of two key stemness markers, Sox2 and Oct4, in these cell lines (Fig. 4b). Collectively, these findings demonstrate that ZNF677 effectively inhibits the stemness of HCC cells.

3.5 ZNF677 inhibits growth, motility as well as stemness in HCC cells *via* PI3K/Akt pathway

Lastly, we investigated the mechanism by which ZNF677 suppresses HCC progression *in vitro*. Immunoblot assays revealed that ZNF677 overexpression decreased the phosphorylation levels of Akt in Huh7 and MHCC-97H cells, suggesting an inhibition of the PI3K/Akt axis (Fig. 5a). To explore this further, we treated these cells with SC79, an activator of Akt, upon ZNF677 overexpression and observed that SC79 treatment reversed the suppression of Akt phosphorylation induced by ZNF677 (Fig. 5b). Additionally, CCK-8 assays showed that while ZNF677 overexpression reduced HCC cell growth, SC79 treatment alleviated this growth inhibition in both Huh7 and MHCC-97H cells (Fig. 5c). Moreover, SC79 also counteracted the reduction in Sox2 and Oct4 expression caused by ZNF677 overexpression (Fig. 5d). Taken together, these results suggest that ZNF677 inhibits the growth, motility, and stemness of HCC cells primarily through the PI3K/Akt pathway.

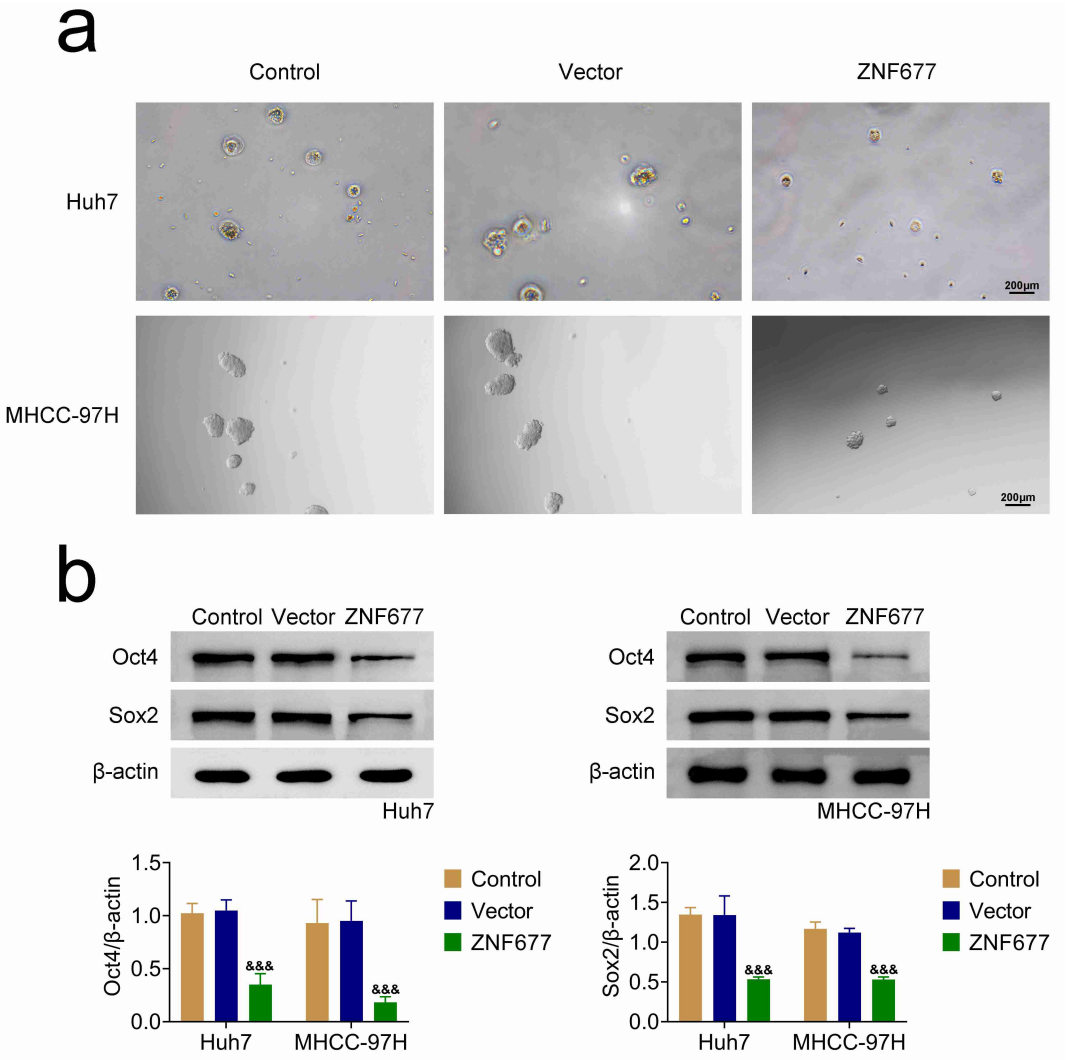

FIGURE 4. ZNF677 reduces stemness in HCC cells. (a) Sphere-forming assays assessing the stemness capacity of Huh7 and MHCC-97H cells following 24 hours of transfection with control vectors and ZNF677 overexpression plasmids. The number of spheres was quantified. Scale bar: 200 μm. (b) Immunoblot assays evaluating the expression of stemness markers Oct4 and Sox2 in Huh7 and MHCC-97H cells post-transfection with control vector and ZNF677 overexpression plasmids for 24 hours. Relative protein expression levels were compared. $^{\&\&\&}p < 0.001$, ZNF677 *vs.* vector. ZNF: Zinc finger.

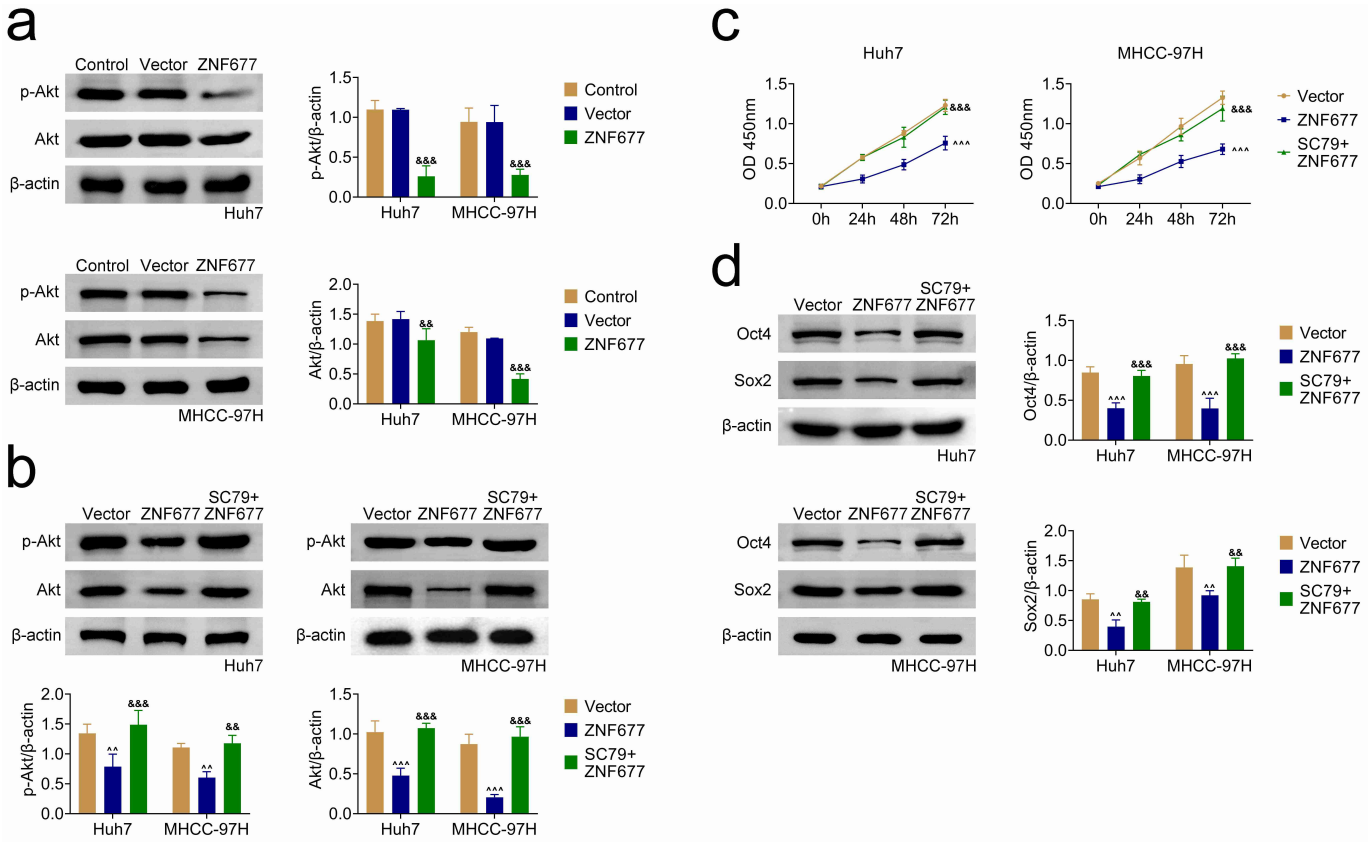

FIGURE 5. ZNF677 inhibits the growth, motility and stemness of HCC cells *via* the PI3K/Akt pathway. (a) Immunoblot assays showing the expression and phosphorylation levels of Akt in Huh7 and MHCC-97H cells transfected with control and ZNF677 overexpression plasmids for 24 hours. $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$, ZNF677 *vs*. vector. (b) Immunoblot assays evaluating the expression and phosphorylation levels of Akt in Huh7 and MHCC-97H cells after specified treatments. $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$, ZNF677 *vs*. vector. $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$, ZNF677 + SC79 *vs*. ZNF677. (c) CCK-8 assays evaluating cell viability in Huh7 and MHCC-97H cells under indicated treatments for 24 hours. Optical density was measured at 450 nm (OD450). $^{\wedge\wedge\wedge}p < 0.001$, ZNF677 *vs*. vector. $^{\&\&\&}p < 0.001$, ZNF677 + SC79 *vs*. ZNF677. (d) Immunoblot assays displaying the expression of stemness markers Oct4 and Sox2 in Huh7 and MHCC-97H cells following specified treatments. $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$, ZNF677 *vs*. vector. $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$, ZNF677 + SC79 *vs*. ZNF677. ZNF: Zinc finger; Akt: protein kinase B.

4. Discussion

Liver cancer ranks as the sixth most prevalent cancer globally [13], and for patients with advanced HCC, targeted drug therapy has become the standard of treatment [14]. Although sorafenib (Dorgemet) is among the most commonly prescribed targeted drugs for HCC [15], targeted therapy is not the principal treatment for all liver cancers, and HCC treatment primarily relies on early detection and intervention [16]. There remains an unmet need to identify new targets to combat this disease. In our study, we found low levels of ZNF677 in HCC tissues, which were associated with poor prognosis. Further analyses demonstrated that ZNF677 not only inhibited the growth but also the stemness of HCC cells. Therefore, ZNF677 presents a promising therapeutic target for HCC.

Our results demonstrated that ZNF677 suppresses both the growth and motility of HCC cells. The role of ZNF677 in cancer progression has been well-established in literature [8]. For instance, ZNF677 has been shown to inhibit growth and stemness in OSCC by modulating FOXO3a expression [4]. Similarly, our findings indicate that ZNF677 also suppresses growth and stemness in HCC cells. Additionally, ZNF677 has been reported to hinder the progression of renal cell carcinoma by affecting N6-methyladenosine and transcriptionally repressing Cyclin Dependent Kinase Inhibitor 3 (CDKN3) expression [7]. In gastric cancer, the suppression of ZNF677 through promoter hypermethylation has been suggested as a key driver of cancer progression [5], indicating a potential avenue for investigation in HCC. Moreover, ZNF677 was found to restrain Akt phosphorylation and tumorigenesis in thyroid cancer [8], aligning with our observation that ZNF677 inhibits HCC *via* the PI3K/Akt pathway. Another study showed that DNA methylation transcriptionally regulates ZNF677 in non-small cell lung cancer (NSCLC), further underlining the broad implications of epigenetic modifications in ZNF677's function across different cancers.

Numerous studies have highlighted the role of a small subset of cells in HCC, known as cancer stem cells (CSCs), in cancer initiation, progression, treatment resistance, and recurrence [13]. As HCC progresses, these CSCs tend to abnormally upregulate various stem cell-related factors, which contributes to increased treatment resistance [17]. Therefore, elucidating the mechanisms underlying hepatic CSCs is essential for a deeper understanding of HCC pathogenesis and could signif-

icantly enhance the development of more effective targeted therapies. Interestingly, our findings demonstrate that ZNF677 suppresses the stemness of HCC cells, suggesting its potential to inhibit HCC progression by targeting these cancer stem cells.

The Akt signaling pathway plays an essential role in the regulation of various cellular processes in response to extracellular signals [18]. In over 40% of human HCC cases, the Akt pathway is activated, promoting survival and growth. This includes inhibition of Transforming growth factor (TGF)-β-dependent apoptosis and negation of the growth-suppressive effects of C/enhancer-binding protein alpha (EBP-α) through its dephosphorylation at Ser193 by protein phosphatase 2A (PP2A) [19]. Both mechanisms contribute to tumor formation, particularly at the stage of cirrhosis. Additionally, the PI3K/Akt pathway plays a significant role in various aspects of HCC growth and metastasis [20]. Herein, our research demonstrated that ZNF677 inhibited HCC progression by targeting this pathway, confirming the potential of the PI3K/Akt pathway as a therapeutic target in HCC management.

Despite these promising results, our study had several limitations. The experiments conducted *in vitro* remain to be validated through *in vivo* investigations to confirm the therapeutic potential of targeting ZNF677 in HCC. In addition, experiments using clinical samples should be conducted to further validate our findings and enhance our understanding of ZNF677's role in tumor growth and metastasis. Furthermore, a detailed investigation is required to elucidate the precise molecular mechanisms *via* which ZNF677 regulates the PI3K/Akt pathway.

5. Conclusions

In conclusion, ZNF677 inhibits growth, motility and stemness in HCC cells *via* the PI3K/Akt pathway, suggesting its potential as a therapeutic target in HCC.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this published article.

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

AUTHOR CONTRIBUTIONS

JW—perform material preparation and the experiments. SW and HLY—perform data collection and analysis. XPF—write the first draft of the manuscript. All authors contributed to the study conception and design. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

ACKNOWLEDGMENT

Not applicable.

FUNDING

This research received no external funding.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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How to cite this article: Jun Wang, Shi Wang, Hailin Ye, Xiaopeng Fan. ZNF677, a potential target in liver cancer, inhibits growth and stemness in hepatoma cells. Journal of Men's Health. 2024; 20(6): 116-123. doi: 10.22514/jomh.2024.099.