Knockdown of CUEDC1 restrains the growth and migration of hepatocellular carcinoma cells through affecting the TβRI/Smad signaling pathway

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Abstract
Hepatocellular carcinoma (HCC), as one usual tumor, and owns the significant mortality rates. The involvement of the coupling of ubiquitin conjugation to endoplasmic reticulum degradation (CUE) domain containing 1 (CUEDC1) in cancer progression, either facilitating or inhibiting, has been documented in various cancers. Nevertheless, the regulatory role of CUEDC1 in HCC progression holds unclear. This work aims to elucidate the impact of CUEDC1 on HCC progression. Analysis using the Gene Expression Profiling Interactive Analysis (GEPIA) online database revealed elevated expression of CUEDC1 in liver hepatocellular carcinoma (LIHC) tissues. Subsequently, in HCC cell lines, the elevated CUEDC1 protein expression was confirmed. Silencing CUEDC1 was found to restrain tumor growth in HCC, alongside attenuating cell migration and invasion. Notably, knockdown of CUEDC1 was determined to activate the transforming growth factor-β receptor I (TβRI)/Smad signaling pathway. In conclusion, this study demonstrates that CUEDC1 knockdown restrained the growth and migration of HCC cells under affecting the TβRI/Smad signaling pathway. These findings suggest CUEDC1 could be a helpful target for HCC improvement.

Keywords
CUEDC1; Migration; Hepatocellular carcinoma; The TβRI/Smad signaling pathway

1. Introduction
Liver cancer is the dominating reason of cancer-associated mortality, ranking as the fifth deadliest cancer [1]. Besides, hepatocellular carcinoma (HCC) constitutes a substantial majority in primary liver cancers, comprising approximately 70%–90% of cases [2, 3]. The incidence of HCC shows notable gender disparity, with men being affected at a ratio of 3–6 to 1 compared to women, resulting in a higher overal burden among men [4]. Despite significant progress in surgical and drug therapies for HCC, their clinical efficacy is severely limited by adverse effects and drug resistance [5]. Thus, it is needful to elucidate the mechanisms and notarize novel targeted therapeutic molecules to enhance HCC clinical efficacy.

Numerous proteins have been identified to play a part in the progression of HCC. Among them, CUE domain-containing protein 1 (CUEDC1) has been ascertained to be a versatile regulator, exerting either tumor-promoting or suppressive effects. For instance, CUEDC1 has been elaborated to impede epithelial-mesenchymal transition (EMT) progression, thereby attenuating tumor advancement in non-small cell lung cancer [6]. Moreover, CUEDC1 is influenced by estrogen-receptor alpha (ERα) and influences tumor growth in breast cancer [7]. Furthermore, CUEDC1 has been found to enhance the proliferation of MOLT-4 cells in acute myeloid leukemia [8]. But, the precise impacts and underlying mechanisms of CUEDC1 in HCC remain elusive, necessitating further investigation for clarification.

In this study, we demonstrated that suppression of CUEDC1 can result in the attenuation of growth and migration of HCC cells, primarily under influencing the TβRI/Smad signaling pathway, providing important insights into understanding HCC pathogenesis and could pave the way for novel therapeutic interventions in HCC treatment.

2. Materials and methods

2.1 Cell lines and cell culture
The HCC cell lines (Hep3B, SMMC-7721, PLC/PRF/5, HC-CLM3) and normal hepatocyte cell line (L-02) were bought from the American Tissue Culture Collection (ATCC, USA), kept in Dulbecco’s Modified Eagle Medium (DMEM), and cultured at 37 °C, 5% carbon dioxide (CO₂), humidified atmosphere.

2.2 Cell transfection
Short hairpin RNAs targeting CUEDC1 (shCUEDC1) and negative control (sh-NC) were obtained from GenePharma
(Shanghai, China). Transfections were executed under Lipofectamine 2000 (11668019, Invitrogen, Carlsbad, CA, USA).

### 2.3 Western blot

The proteins extracted from HCC cells were divided using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto polyvinylidene fluoride (PVDF) membranes (Beyotime, Shanghai, China). Post sealing, the primary antibodies for overnight, and the secondary antibodies (1:2000; ab7090) for 2 h. Protein expressions were inspected under one chemiluminescence detection kit (89880, Thermo Fisher Scientific, Inc., Waltham, MA, USA).

The primary antibodies used were as follows: CUEDC1 (1:500; hz-8243R; Shanghai Huzhen Biotechnology Co., Ltd, Shanghai, China), mitochondrial membrane potential (MMP)-9 (1:1000; ab76003; Abcam, Shanghai, China), MMP-2 (1:1000; ab92536), p-Smad2 (1:1000; ab280888), Smad3 (1:1000; ab63403), Smad2 (1:1000; ab40854), p-Smad2 (1:1000; ab280888), Smad2 (1:2000; ab40855), TβRI (1:1000; ab235578), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:500; ab8245).

### 2.4 Cell counting kit-8 (CCK-8) assay

In the 96-well plate, HCCLM3 and SMMC-7721 cells were placed for 24 h. Then, CCK-8 solution (CK04, Dojindo Laboratories, Kumamoto, Japan) was appended (10 µL/each well). Post 2 h, cell viability was then assessed under a spectrophotometer (ND-ONE-W, Thermo Fisher Scientific, Waltham, MA, USA).

### 2.5 Colony formation

HCCLM3 and SMMC-7721 cells were placed into a 6-well plate. Post 14 days, fixation (4% paraformaldehyde) and dyeing (0.1% crystal violet) were made in cells. The number of colonies was then quantified.

### 2.6 Flow cytometry

After washing, HCC cells were treated in the dark with the staining solution containing Annexin V-fluoresceine isothiocyanate (FITC) (C1062S, Beyotime, Shanghai, China)/propidium iodide (PI). Then, cell apoptosis was ascertained under flow cytometry (BD FACSCalibur, BD Biosciences, San Jose, CA, USA).

### 2.7 Transwell assay

The upper chambers coated with or not Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) were appended with HCCLM3 or SMMC-7721 cells and serum-free medium (200 µL). Next, the lower chamber was appended with the medium containing 20% fetal bovine serum (FBS, 600 µL). After 48 h, cells that had migrated were treated with fixation (4% paraformaldehyde) and staining (0.1% crystal violet). Lastly, cell migration and invasion were inspected under a microscope (CX41, Olympus Optical Co., Ltd., Tokyo, Japan).

### 2.8 Statistical analysis

All data are exhibited as mean ± standard deviation (SD). Statistical analyses were executed through GraphPad Prism Software 9 (GraphPad Software, La Jolla, CA, USA). Each experiment was conducted in triplicate. The analysis of comparisons in groups was made through the Student’s t-test or one-way one-way analysis of variance (ANOVA). The p less than 0.05 was set as statistically significant.

### 3. Results

#### 3.1 CUEDC1 existed the elevated expression in HCC tissues

Using the GEPIA online database, we observed a higher expression of CUEDC1 in LIHC tissues (Fig. 1A). Additionally, the protein expression of CUEDC1 was confirmed to be elevated in HCC cell lines (Fig. 1B). In summary, CUEDC1 exhibited increased expression levels in HCC.

#### 3.2 Silencing of CUEDC1 restrained tumor growth in HCC cells

First, we confirmed the efficacy of CUEDC1 knockdown, which exhibited a significant decrease in CUEDC1 protein expression (Fig. 2A). Next, we observed a reduction in cell viability after CUEDC1 knockdown (Fig. 2B). Furthermore, results from colony formation assay uncovered that cell proliferation was attenuated after CUEDC1 suppression (Fig. 2C). Furthermore, inhibition of CUEDC1 led to enhanced cell apoptosis (Fig. 2D). Overall, our findings indicate that silencing CUEDC1 effectively impedes tumor growth in HCC.

#### 3.3 Suppression of CUEDC1 decreased cell migration and invasion capabilities of HCC cells

Following CUEDC1 suppression, the capabilities of cell migration and invasion were discovered to be diminished (Fig. 3A,B). Moreover, Western blot analysis revealed decreased protein expressions of MMP-9 and MMP-2 after CUEDC1 knockdown (Fig. 3C). Collectively, findings manifested that suppression of CUEDC1 could attenuate cell migration and invasion in HCC.

#### 3.4 Knockdown of CUEDC1 triggered the TβRI/Smad signaling pathway

The protein levels of p-Smad3/Smad3, p-Smad2/Smad2, and TβRI were all increased after CUEDC1 inhibition (Fig. 4), indicating that knockdown of CUEDC1 activates the TβRI/Smad signaling pathway.

### 4. Discussion

CUEDC1 has been recognized as either a facilitator or suppressor in certain cancers [6–8], but its regulatory roles in HCC progression remain unclear. In this work, under the GEPIA online database, the higher expression of CUEDC1 was testified in LIHC tissues. Subsequently, we affirmed that the protein expression of CUEDC1 is elevated in HCC cell
**Figure 1. Elevated expression of CUEDC1 in HCC.** (A) Analysis of CUEDC1 expression levels in liver hepatocellular carcinoma (LIHC) tissues compared to normal tissues from the GEPIA online database. (B) Verification of CUEDC1 protein expression in normal hepatocyte cell line (L-02) and HCC cell lines (Hep3B, SMMC-7721, PLC/PRF/5, HCCLM3) by western blot analysis. Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001. CUEDC1: CUE domain containing 1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

**Figure 2. Silencing of CUEDC1 suppresses HCC growth.** The study groups were separated into Control, shNC and shCUEDC1 groups. (A) The efficiency of CUEDC1 knockdown was made by western blot. (B) Cell viability was determined by CCK-8 assay. (C) Colony formation assay was to inspect cell proliferation. (D) Flow cytometry was used to examine cell apoptosis. Statistical significance: **p < 0.01, ***p < 0.001. CUEDC1: CUE domain containing 1; PI: propidium iodide; shNC: negative control. GAPDH: glyceraldehyde-3-phosphate dehydrogenase; FITC: fluoresceine isothiocyanate.
FIGURE 3. Suppression of CUEDC1 attenuated HCC cell migration and invasion. Groups were divided into Control, shNC, and shCUEDC1 groups. (A) Cell migration was evaluated using the Transwell assay. (B) Cell invasion was determined under the Transwell assay. (C) Protein expressions of MMP-9 and MMP-2 were inspected through western blot. Statistical significance: **p < 0.01, ***p < 0.001. CUEDC1: CUE domain containing 1; shNC: negative control; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; MMP: Matrix metalloproteinase.
FIGURE 4. Knockdown of CUEDC1 activated the TβRI/Smad signaling pathway. Groups were divided into Control, shNC, and shCUEDC1 groups. Protein expressions of p-Smad3, Smad3, p-Smad2, Smad2, and TβRI were examined through western blot. Statistical significance: **p < 0.01, ***p < 0.001. CUEDC1: CUE domain containing 1; shNC: negative control; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; TβRI: transforming growth factor-β receptor I.

lines. Furthermore, we demonstrated that silencing CUEDC1 effectively inhibited tumor growth in HCC.

Tumor metastasis involves the migration of malignant tumor cells to distant sites, where they continue to proliferate [9]. Metastasis is a primary factor contributing to treatment failure in cancer [10], prompting extensive research efforts to understand and address this phenomenon in cancer progression. For instance, previous studies have shown that schlafen 11 (SLFN11) targets ribosomal protein S4 X (RPS4X) to suppress tumorigenesis and metastasis through the mammalian target of rapamycin (mTOR) pathway in HCC [11]. Additionally, AT-rich interaction domain 2 (ARID2) modulates the DNA methyltransferase 1 (DNMT1)-Snail axis to reduce metastasis in HCC [12]. Moreover, ONECUT2 enhances fibroblast growth factor 2 (FGF2) and ATP citrate lyase (ACLY) expression, thereby promoting metastasis in HCC [13]. Consistent with these previous reports, this study clarified that suppression of CUEDC1 attenuated cell migration and invasion in HCC, aligning with previous reports on the role of various molecular pathways in HCC metastasis.

Transforming growth factor (TGF)-β has been implicated in tumorigenesis and metastasis, particularly in the context of HCC [14]. The TGF-β/Smad pathway has been extensively studied for its involvement in HCC regulation. For example, miR-181a-5p retards the Egr1/TGF-β/Smad pathway to restrain HCC progression [15]. Similarly, Six2 accelerates EMT progress by modulating the TGF-β/Smad pathway in HCC [16]. Additionally, p21-activated kinase 3 (PAK3) exacerbates tumor metastasis in HCC through affecting the TGF-β/Smad pathway [17]. Interestingly, in non-small cell lung cancer, CUEDC1 has been discovered to influence the TβRI/Smad signaling pathway, and then attenuate tumorigenesis [6]. However, the regulatory role of CUEDC1 on the TβRI/Smad signaling pathway in HCC progression holds unknown. In this work, we also observed that knockdown of CUEDC1 activated the TβRI/Smad signaling pathway.

5. Conclusions

This work firstly manifested that CUEDC1 knockdown restrained the growth and migration of HCC cells under affecting the TβRI/Smad signaling pathway. Nevertheless, certain limitations also exist, such as the absence of human samples and animal models. Therefore, further investigations are warranted to validate these findings and explore additional aspects of CUEDC1’s role in HCC progression.

AVAILABILITY OF DATA AND MATERIALS

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.
AUTHOR CONTRIBUTIONS

JW, ZLC—designed the study and carried them out, prepared the manuscript for publication; JW, KHC—supervised the data collection, analyzed the data, interpreted the data. KHC—reviewed the draft of the manuscript. All authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES
