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Long noncoding RNA UFC1 acts as an oncogene *via* stimulating EZH2-induced inhibition of APC expression in renal cell carcinoma

Jinteng Wang[1], Guanlin Liu[2,*]

[1] Health Science Center, Ningbo University, Ningbo, China
[2] Ningbo First Hospital, Ningbo, China

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ABSTRACT

This study aimed to illustrate the biological functions of long noncoding RNA (lncRNA) UFC1 in the carcinogenesis and cancer development of renal cell carcinoma (RCC), and the potential molecular mechanism. UFC1 levels in RCC tissues and cell lines were detected by quantitative real-time polymerase chain reaction (qRT-PCR). Diagnostic and prognostic potentials of UFC1 in RCC were assessed by depicting receiver operating characteristic (ROC) curves and Kaplan-Meier curves, respectively. After transfection with si-UFC1, proliferative and migratory changes in ACHN and A498 cells were detected by cell counting kit-8 (CCK-8) and transwell assay, respectively. Subsequently, chromatin immunoprecipitation (ChIP) was conducted to examine the enrichments of EZH2 (enhancer of zeste homolog 2) and H3K27me3 in the APC promoter region. Finally, rescue experiments were carried out to identify the co-regulation of UFC1 and APC on RCC cell behaviors. The results showed that UFC1 was highly expressed in RCC tissues and cell lines. ROC curves revealed the diagnostic potential of UFC1 in RCC. Besides, survival analysis showed that highly expressed UFC1 predicted poor prognosis in RCC patients. Knockdown of UFC1 in ACHN and A498 cells attenuated cell proliferative and migratory abilities. UFC1 was able to interact with EZH2, and the knockdown of UFC1 could upregulate APC. In addition, both EZH2 and H3K27me3 were enriched in the APC promoter region, which could be blocked by the knockdown of UFC1. Moreover, rescue experiments demonstrated that the silence of APC was able to abolish the inhibited proliferative and migratory abilities in RCC cells with UFC1 knockdown. LncRNA UFC1 inhibits APC level through upregulating EZH2, thus aggravating the carcinogenesis and cancer development of RCC.

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Introduction

Renal cell carcinoma (RCC) is one of the most common malignant tumors of the urinary system (1). Due to the hidden anatomic location of the kidneys, symptoms of RCC in the early phase are atypical or inconspicuous. As a result, the detective rate of RCC in the middle or advanced stage is relatively high. Clear-cell renal cell carcinoma (ccRCC) is the major histological subtype of RCC, and its clinical prognosis is poor due to the high metastasis rate in the advanced stage (2). About 50-60% of RCC cases are detected in physical examinations (3). Seriously, nearly 30% of confirmed RCC patients develop metastases (4). RCC is insensitive to radiotherapy and/or chemotherapy (5). In addition, RCC patients are poorly responded to immunotherapy and some patients are intolerable. Targeted therapy easily causes drug resistance and many other complications in RCC patients, so the screening and diagnosis of RCC in the early phase are of great significance. It is urgent to clarify the mechanisms of RCC and to develop effective biomarkers for diagnosis and treatment.

Long noncoding RNAs (lncRNAs) with more than 200 nucleotides are research highlights after microRNAs (miRNAs) (6,7). Powerful lncRNAs exert an irreplaceable role in various aspects of biological processes (8), and they are responsible for histone modification (9), transcriptional (10) and post-transcription regulation (11). LncRNAs are extensively involved in carcinogenesis and cancer progression (12). It is reported that lncRNA HOTAIR is upregulated in RCC samples than normal ones. Silence of HOTAIR effectively weakens proliferative, migratory and invasive capacities, and induces apoptosis in RCC (13).

LncRNA UFC1 (2,791 bp) is an intronic transcript, which is initially reported as the target of miRNA-34a. UFC1 is upregulated in hepatocellular carcinoma (HCC) profiling through microarray analysis, and it triggers HCC proliferation and inhibits apoptosis by upregulating β-catenin *via* interacting with the mRNA-stabilizing protein HuR (14). Yu et al. (15) detected that UFC1 is upregulated in colorectal cancer (CRC) tissues compared with adjacent non-tumoral tissues. Knockdown of UFC1 inhibits proliferative capacity and stimulates apoptosis in CRC by regulating β-catenin and p38 signaling. The potential influences of UFC1 on RCC progression are unclear. In this paper, the diagnostic and prognostic potentials of UFC1 in RCC were analyzed, and its potential role in regulating RCC cell behaviors and molecular mechanisms were identified.

* Corresponding author. Email: kennynb2023@163.com

Materials and Methods

Clinical Samples and Cell Culture

A total of 24 paired RCC and adjacent non-tumoral tissues were collected from diagnosed RCC patients in Ningbo First Hospital from 2017 December to 2019 December. Inclusion criteria: None of recruited RCC patients were treated by preoperative chemotherapy, radiotherapy or targeted therapy. RCC tissues were confirmed by pathology. Exclusion criteria: RCC patients combined with other tumors. RCC patients underwent preoperative chemoradiotherapy or other treatment. There are other causes of death. Clinical samples were stored in liquid nitrogen. Written informed consent was obtained from all subjects. This study obtained the approval from Ningbo First Hospital Ethics Committee in accordance with the Helsinki Declaration.

RCC cell lines (786-O, A498, ACHN and OS-RC-2) and the human renal tubular epithelial cell line (HK-2) were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). They were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (Biochrom AG, Berlin, Germany) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) in a 5% CO_2 incubator at 37°C.

Cell transfection

UFC1 siRNA, APC siRNA and negative controls were synthesized by GenePharma (Shanghai, China). When cells were cultured to 60-70% density in 6-well plates, serum-free medium was replaced. Transfection was conducted using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Six hours later, complete medium was replaced. Thereafter, the transfected cells were collected for cell function experiments.

Cell Counting Kit-8 (CCK-8)

Transfected cells were collected at 80% confluence and prepared to suspension at 1×10^5 cells/mL. The suspension was inoculated in a 96-well plate with 100 μL per well, and six replicates were set. Then 10 μL of CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) was applied per well for 1 h incubation and optical density at 450 nm was measured.

Transwell assay

Crystal violet solution (0.5%) was prepared by dissolving 0.05 g crystal violet in methanol, which was used in the cell experiment after dilution in phosphate-buffered saline (PBS) at 1: 5 to 1: 8. Cells were prepared into suspension at 2.5×10^5 cells/mL. Transwell chambers containing 200 μL of suspension were inserted in the 24-well plate where 500 μL of medium containing 20% FBS was applied in each well. After 24 h cell culture, transwell chambers were taken out. Cells in the bottom were subjected to methanol fixation for 15 min, and crystal violet staining for 20 min. Finally, migratory cells were counted in 5 randomly selected fields per sample.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Tissue and cellular RNAs harvested using TRIzol (Invitrogen, Carlsbad, CA, USA) were stored at -80°C. qRT-PCR system was prepared, including 1 μg RNA, primers, and reverse transcriptase, and the RNA was transcribed at 37°C for 20 min and 95°C for 5 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was the internal reference. The relative level of the specifically amplified target was calculated by $2^{-\Delta\Delta ct}$. Target gene sequences were searched from NCBI and synthesized to primers by ABI, USA as follows. UFC1: 5'-GATCGTGAGTTGTGGGTGCA-3' (forward) and 5'-TGTTGGATGACGCCCTTCTG-3' (reverse); GAPDH: 5'-AATCCCATCACCATCTTCC-3' (forward) and 5'-GTCCTTCCACGATACCAA-3' (reverse); EZH2: 5'-TTCGTTTTGCTAATCATTCAGTAA-3' (forward) and 5'-TTCGTTTTGCTAATCATTCAGTAA-3' (reverse); APC: 5'-TGCTGCCGAGAGTCCGTC-3' (forward) and 5'-TGGCCGTACTAGCGACGAA-3' (reverse); U6: 5'-ATACAGAGAAAGTTAGCACGG-3' (forward) and 5'-GGAATGCTTCAAAGAGTTGTG-3' (reverse).

RNA binding protein immunoprecipitation (RIP)

EZMagna RIP kit (Millipore, Billerica, MA, USA) was used for examining the interaction between UFC1 and EZH2. Cells were lysed in RIPA and incubated at 4°C for 6 hours with magnetic beads conjugated with anti-EZH2 or anti-IgG. Subsequently, magnetic beads were washed and incubated with Protease K for clearing proteins. The purified RNA was subjected to qRT-PCR.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was conducted according to a previous study (16).

Subcellular distribution analysis

At least 1×10^6 cells were lysed in 200 μL of Lysis Buffer J and centrifuged for obtaining the supernatant, which was the cytoplasmic fraction. The remaining was incubated with Buffer SK and absolute ethanol. Nuclear RNAs were obtained by column centrifugation, and cytoplasmic and nuclear RNA levels were determined by qRT-PCR.

Western Blotting

Cells were lysed in radioimmunoprecipitation assay (RIPA) containing 1% cocktail (Sigma-Aldrich, St. Louis, MO, USA). The protein samples were separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and loaded on polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). Next, the membranes were incubated in 5% skim milk for 2 h and primary and secondary antibodies. At last, band exposure was conducted using the Bio-Rad (Hercules, CA, USA) image system.

Statistical analysis

Data processing was conducted using Statistical Product and Service Solutions (SPSS) 20.0 (IBM, Armonk, NY, USA) and they were expressed as mean ± standard deviation. Differences between groups were analyzed by the Student's *t*-test. $P<0.05$ was considered as statistically significant.

Results

Expression Levels of UFC1 In RCC Tissues and Cell Lines

UFC1 levels in RCC tissues and adjacent non-tumoral

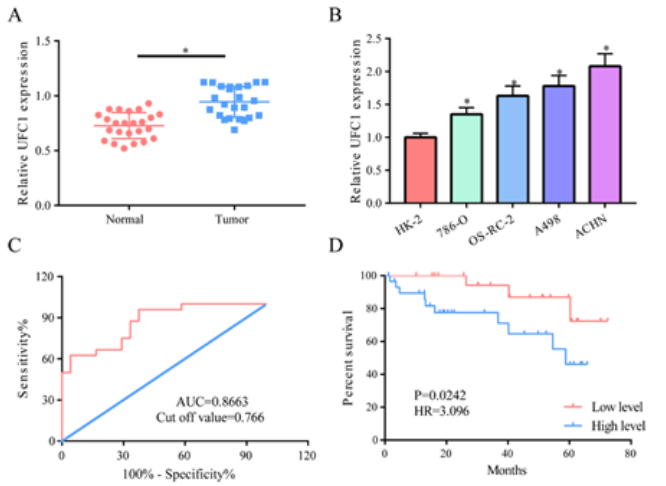

Figure 1. Expression levels of UFC1 in RCC tissues and cell lines. (A) UFC1 was upregulated in RCC tissues (n=24) than non-tumoral tissues; (B) UFC1 was upregulated in RCC cell lines; (C) ROC curves demonstrated the diagnostic potential of UFC1 in RCC (AUC = 0.8663, cut-off value = 0.766); (D) Kaplan-Meier curves demonstrated the prognostic potential of UFC1 in RCC (P=0.0242, HR = 3.096). *P<0.05.

tissues were first detected. It was shown that UFC1 was upregulated in RCC tissues more than in normal tissues (Figure 1A). Subsequently, *in vitro* level of UFC1 was assessed. UFC1 was highly expressed in RCC cell lines compared with HK-2 cells (Figure 1B). ROC analysis showed the diagnostic potential of UFC1 in RCC (AUC = 0.8663, cut-off value = 0.766, Figure 1C). In addition, depicted Kaplan-Meier curves showed that the high level of UFC1 was related to poor prognosis in RCC (P=0.0242, HR=3.096, Figure 1D).

Knockdown of UFC1 Attenuated Proliferative and Migratory Abilities In RCC

To elucidate the influences of UFC1 on RCC cell functions, the UFC1 level was downregulated by transfection of si-UFC1 in ACHN and A498 cells (Figure 2A). CCK-8 assay showed that the knockdown of UFC1 markedly inhibited proliferative ability in ACHN and A498 cells (Figure 2B). Meanwhile, the transwell assay revealed that compared with those transfected with si-NC, RCC cells transfected with si-UFC1 displayed a lower number of migratory cells (Figure 2C). It was indicated that UFC1 stimulated RCC cells to proliferate and migrate.

UFC1 Downregulated APC By Interacting With EZH2

To elucidate the regulatory mechanism of UFC1 on RCC cell functions, the subcellular distribution of UFC1 was first analyzed. In ACHN and A498 cells, UFC1 was mainly distributed in the cytoplasm, suggesting its potential role in transcriptional regulation (Figure 3A). A recent study has demonstrated that lncRNAs silence target genes by interacting with PRC2. Here, it was verified that UCF1 could interact with EZH2 (Figure 3B). It is previously reported that the APC level can be reduced by EZH2 (17). Thus it was speculated that the interaction between UFC1 and EZH2 may result in APC downregulation. As qRT-PCR and Western blotting results uncovered, the knockdown of UFC1 upregulated both mRNA and protein levels of APC in RCC cells (Figure 3C-3E). Additionally, the ChIP assay showed that EZH2 could bind to the

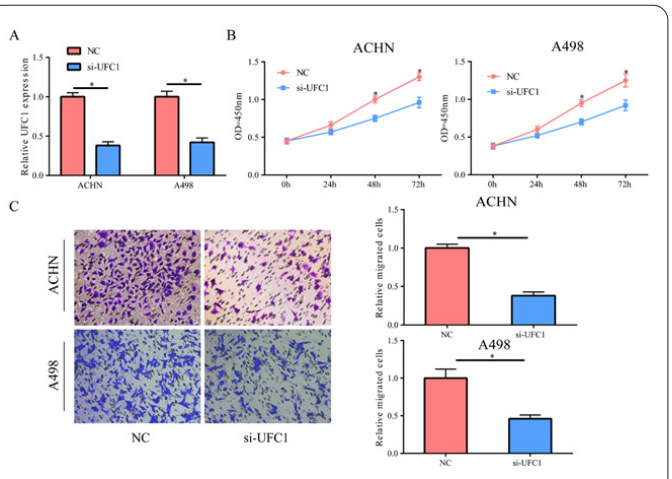

Figure 2. Knockdown of UFC1 attenuated proliferative and migratory abilities in RCC. (A) UFC1 was downregulated in ACHN and A498 cells transfected with si-UFC1; (B) Knockdown of UFC1 inhibited the proliferation of ACHN and A498 cells; C, Knockdown of UFC1 inhibited the migration of ACHN and A498 cells. (magnification: 200×) *P < 0.05.

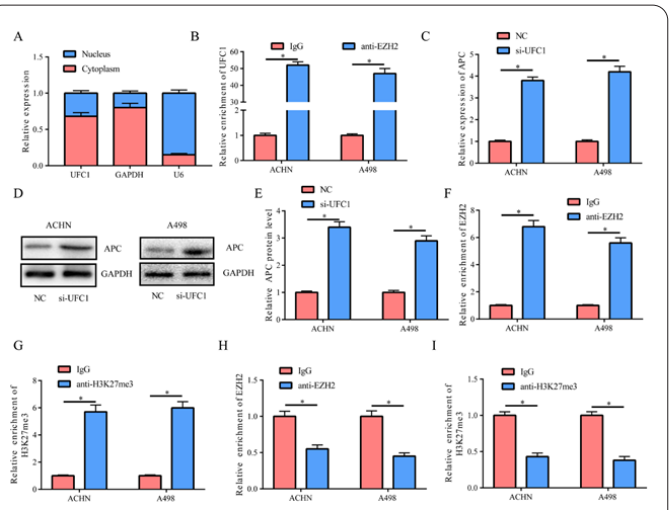

Figure 3. UFC1 downregulated APC by interacting with EZH2. (A) UFC1 was mainly distributed in the cytoplasm of ACHN and A498 cells; (B) RIP assay uncovered the interaction between UFC1 and EZH2; (C) The mRNA level of APC was upregulated in ACHN and A498 cells with UFC1 knockdown; (D, E) Protein level of APC was upregulated in ACHN and A498 cells with UFC1 knockdown; (F, G) ChIP-qRT-PCR analysis showed that EZH2 and H3K27me3 were enriched in APC promoters in ACHN and A498 cells; (H, I) ChIP-qRT-PCR analysis revealed that knockdown of UFC1 in ACHN and A498 cells reduced the enrichments of EZH2 and H3K27me3 in APC promoter. *P<0.05.

APC promoter (Figure 3F), leading to H3K27me3 enrichment and transcriptional repression of APC (Figure 3G). Notably, the enrichments of EZH2 and H3K27me3 in the APC promoter region were reduced by the knockdown of UFC1 (Figure 3H, 3I).

Knockdown of APC Could Abolish the Role of Lowly Expressed UFC1 In RCC Cell Functions

Subsequently, the involvement of APC in UFC1-regulated RCC cell functions was examined. A lower APC level was detected in ACHN and A498 cells co-transfected with si-UFC1 and si-APC than those transfected with solely si-UFC1, confirming the transfection efficacy of si-APC (Figure 4A). Furthermore, the knockdown of APC

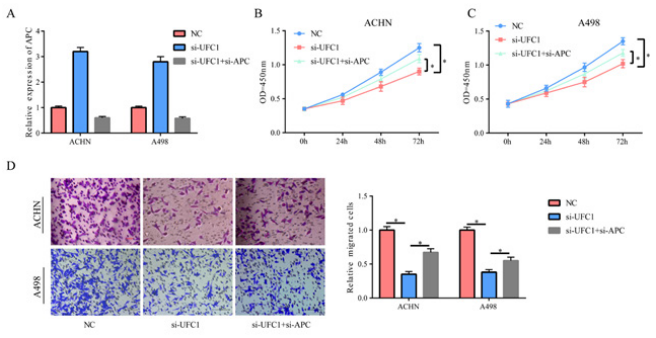

Figure 4. Knockdown of APC could abolish the role of lowly expressed UFC1 in RCC cell functions. (A) The mRNA level of APC in ACHN and A498 cells transfected with NC, si-UFC1 or co-transfected with si-UFC1 and si-APC was detected by qRT-PCR. (B, C) Co-silence of APC partially abolished the inhibitory effect of silenced UFC1 on proliferation in ACHN and A498 cells; (D) Co-silence of APC partially abolished the inhibitory effect of silenced UFC1 on migration in ACHN and A498 cells. (magnification: 200×) *P<0.05.

could abolish the inhibitory effect of silenced UFC1 on RCC cell viability to some extent (Figure 4B, 4C). Similarly, the Transwell assay revealed that the inhibition of APC level partially alleviated the inhibited migratory ability in RCC cells with UFC1 knockdown (Figure 4D).

Discussion

LncRNAs exert an important role in cancer progression. It is reported that lncRNA XIST aggravates CRC by regulating the HIF-1A/AXL signaling (18). LncRNA TUG1 is responsible for mediating proliferative and migratory potentials of prostate cancer cells *via* activating the Nrf2 signaling. So far, the pathogenesis and etiology of RCC are still required to be largely explored. The findings of this study showed that lncRNA UFC1 was upregulated in RCC tissues and cell lines. A series of cell function experiments demonstrated that the knockdown of UFC1 remarkably weakened proliferative and migratory abilities in ACHN and A498 cells. Therefore, it was speculated that UFC1 may drive the aggravation of RCC as an oncogene.

Compared with miRNAs, the biological functions of lncRNAs are more complicated and diverse. Serving as ceRNAs, lncRNAs are able to mediate various aspects of tumor cell behaviors (19,20), and they transcriptionally regulate target gene expressions, thus influencing cancer progression. The malignant progression of HCC is accelerated by lncRNA SHRG by stimulating the transcription of HES6 (21).

EZH2 (enhancer of zeste homolog 2), located on human chromosome 7q35, is a vital member of the PcG (Polycomb group) family, and it is related to cell proliferation and highly expressed in many types of malignant tumors (22,23). PcG proteins constitute different PRCs (Polycomb repressive complex), mainly including PRC1 and PRC2. EZH2 is a catalytic subunit of PRC2 that functions as a histone methyltransferase. Silence of EZH2 markedly inhibits malignant phenotypes of tumors and induces apoptosis (24). The findings of this study verified an interaction between UFC1 and EZH2, indicating that EZH2 may be involved in the biological regulations of UFC1 on RCC progression.

A previous study illustrated that EZH2 can regulate APC levels in HCC samples (17). APC is a tumor suppressor that weakens tumor cell behaviors by inactivating the Wnt signaling (17). As a result, it was speculated that APC may be involved in the co-regulation of UFC1 and EZH2 on RCC progression. Here, the knockdown of UFC1 remarkably upregulated mRNA and protein levels of APC in ACHN and A498 cells. Furthermore, UFC1 knockdown dramatically blocked the enrichment of EZH2 and H3K27me3 in the APC promoter region. Furthermore, rescue experiments suggested that the silence of APC was able to abolish the inhibited proliferative and migratory abilities in RCC cells transfected with si-UFC1. To sum up, UFC1 was capable of regulating APC level by interacting with EZH2, thus triggering RCC cells to proliferate and migrate.

Briefly, this study first discovered that lncRNA UFC1 inhibited APC level through upregulating EZH2, thus aggravating the carcinogenesis and cancer development of RCC. This research not only enriches the theoretical evidences underlying RCC progression but also provides a new way for the clinical diagnosis and treatment of RCC.

Data Availability

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

Acknowledgments

Not applicable.

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Authors' contributions

Jinteng Wang and Guanlin Liu designed the study and performed the experiments, Jinteng Wang collected the data, Guanlin Liu analyzed the data, Jinteng Wang and Guanlin Liu prepared the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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