Original Article
Long non-coding RNA UCA1 modulates the glycolysis of cervical cancer cells by miR-493-5p/HK2

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Abstract: Long noncoding RNAs (lncRNAs) are associated with tumor development and progression. LncRNA UCA1 (UCA1) recently has been reported to take part in cancer cell proliferation. However, the expression and underlying molecular mechanism of UCA1 in cervical cancer cell glycolysis is unclear. This study aimed to investigate the role of UCA1 in cervical cancer. In order to explore the role of UCA1 in cervical cancer, first, the expression levels of UCA1 in cervical cancer tissues were measured, and the results showed that UCA1 levels were higher in cancer tissues compared to matched adjacent normal tissues. The inhibition of UCA1 expression suppressed human cervical cancer cell proliferation and glycolysis. Additionally, our experimental results indicated that UCA1 could directly bind to miR-493-5p and regulate miR-493-5p expression in an inverse manner. Namely, UCA1 could reverse the inhibitory effect of miR-493-5p on cervical cancer cells' proliferation and glycolysis. Moreover, we revealed that HK2 is a target gene of miR-493-5p through a Targetscan prediction. It was verified that miR-493-5p downregulated HK2 mRNA and protein levels using real time RT-PCR and Western blotting. In a summary, this study demonstrated that UCA1 functioned as an oncogene by UCA1/miR-493-5p/HK2 axis in cervical cancer.

Keywords: Cervical cancer, UCA1, miR-493-5p, HK2

Introduction

Cervical cancer is one of the leading causes of lethal malignancies among gynecological malignant tumors worldwide and accounts for a great proportion of cancer fatalities among women [1, 2]. It is a horrific that the incidence of cervical cancer is still increasing. Although current medical technologies continue to advance and various treatments are used for cervical cancer, such as surgery, chemotherapy and radiotherapy [3], cervical cancer remains a major health problem. Therefore, it is necessary to reveal the underlying mechanism of cervical cancer in order to find new therapeutic strategies to treat cervical cancer.

Long noncoding RNAs (lncRNAs) have a length of more than 200 nucleotides and no protein-coding capability potential, are in the nuclear or cytoplasmic fractions of cells and have a critical role in gene expression through transcription regulation, post-transcription regulation, chromatin modification, and genomic imprinting [4, 5]. Many lncRNAs are closely linked to tumorigenesis and development, especially in lung cancer [6], kidney cancer [7], breast cancer [8] and liver cancer [9]. Moreover, a series of studies has demonstrated that lncRNAs exert essential functions in the biological activities of tumor cells, including cycle, differentiation and apoptosis [10-12].

At present, many lncRNAs have been found in cervical cancer. For example, Cao et al. found that GAS5 was downregulated in cervical cancer and correlated with cervical cancer progression [13, 14]. Kim et al. showed that HOTAIR promoted cervical cancer aggressiveness through the upregulation of VEGF and MMP-9 and EMT-related genes [14]. Zhang et al. reported that MEG3 could decrease tumor growth in cervical cancer and acted as a tumor suppressor by regulating miR-21-5p [15]. Therefore, it is urgent to know the pathological mechanism of lncRNAs in cervical cancer and identify the function of lncRNAs.

In the current study, we found that lncRNA urothelial cancer associated 1 (UCA1) was signifi-

cantly overexpressed in cervical cancer tissues compared to adjacent normal tissues. Furthermore, we found UCA1 negatively regulated miR-493-5p targeting HK2. These findings strongly clarified that UCA1 is involved in the progression and development of cervical cancer. Thus, we hypothesized that UCA1 can be a remarkable biomarker in the diagnosis of cervical cancer. MiR-493-5p may be a potential prognostic factor and therapeutic target in patients with cervical cancer.

Materials and methods

Patients and tissue specimens

In this study, cervical cancer tissues and adjacent normal tissues were collected from Department of Gynecology and Obstetrics, Beijing Hospital, National Center of Gerontology (Beijing, China). Written informed consent was obtained from all patients prior to participation in the study. All tissue specimens were frozen at -80°C.

Cell lines and cell culture

In this study, the human cervical cancer cells included HEC251, HEC-1B, Hela, N3CA, HEC-1A, RL95-2, and Ishikawa3h12. The cell lines were obtained from ATCC and Jing Kang Biotech (Shanghai, China). Respectively, cells were cultured in RPMI1640 growth medium supplemented with 10% fetal bovine serum (Gibco), 50 U/ml of penicillin and 50 μg/ml of streptomycin. All cells were cultured in a sterile incubator maintained at 37°C with 5% CO_2.

Quantitative real-time PCR

Total RNA was extracted from cells and tissues using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's specification. cDNA was reversely transcribed from total RNA using the PrimeScriptTM RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). As described, ssynthesis of cDNA was amplified with the SYBR- Green PCR Master Mix kit (Takara, Japan) to measure the mRNA expression levels of UCA1, miR-493-5p and HK2 using the ABI 7500 System (Applied Biosystems, Foster City, CA, USA). Relative expression levels were calculated as ratios normalized against GAPDH. The quantification of gene expression was performed using the $2^{-\Delta\Delta Ct}$ method.

Cell proliferation assay

To complete a ccell proliferation assay in this study, we used the Cell Counting Kit-8 (Byotime, Haimen, China) according to the manufacturer's protocol. $5×10^3$ cells were seeded in each well of the 96-well plates. The absorbance of each well was detected at a wavelength of 450 nm using a microplate reader (BioTek) from day 0 to day five. All experiments were done at least three times independently.

Western blot analysis

Cells were lysed in a cold RIPA lysis buffer including a protease inhibitor cocktail (Sigma-Aldrich, USA). Then, according to the manufacturer's protocol, the protein concentrations were measured by a bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Equal protein concentrations were loaded and separated by SDS-PAGE and then electrophoretically transferred to PVDF membranes (Pall, New York, NY, USA). After blocking in 5% skimmed milk powder in Tris-based saline with Tween20 for 1 hour at room temperature, the primary antibodies were incubated in the PVDF membranes at 4°C overnight. The membranes were further incubated with a secondary antibody for 2 hours. Finally, the membranes were scanned with the Odyssey infrared imaging system.

Glucose consumption

The cells were planted on 10 cm dishes at about $5×10^6$ per milliliter on the first day. The next day, cells were washed with a glucose-free medium and incubated in a fresh glucose-free RPMI-1640 medium for 3 h, and then incubated with 0.2 Ci/ml3H-2-deoxyglucose for 1 h. The glucose consumption represented by 3H radioactivity was determined by liquid scintillation counting and normalized by cell number.

Measurements of extracellular acidification rate

The extracellular acidification rate (ECAR), a product of glycolysis, was largely determined by lactic acid release. The ECAR approximated the glycolysis flux rate [16]. To measure ECAR, the Lactate Acid Assay kit (Biovison) was used after we collected and concentrated the cellar cul-
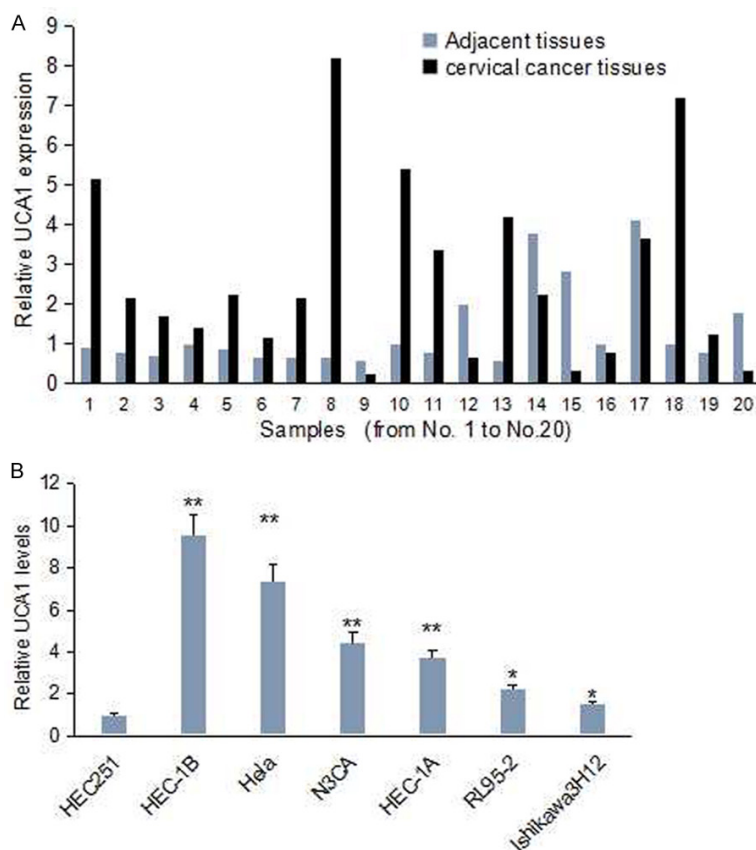
Figure 1. UCA1 expression was upregulated in cervical cancer samples. A. UCA1 expression in cervical cancer tissues and normal tissues. Real time RT-PCR was used to detect UCA1 expression. B. UCA1 expression in cervical cancer cells. Real time RT-PCR was used to detect UCA1 expression. **P<0.01, *P<0.05.

ture medium which cultured fresh glucose-free RPMI-1640 medium for 3 h.

MicroArray analysis

A DNA microarray assay was performed using the Affymetrix GeneChip System (Affymetrix). The Affymetrix Human Exon 1.0 ST Array containing 1.4 million probe sets was used. Total cellular RNA was extracted from HEK293 cells transfected with negative miRNA mimic and mimic miRNA-6852 using a phenol-chloroform based extraction using Qiazol (Qiagen) and quantitated following the manufacturer's protocols (Affymetrix). Terminal labeling and hybridization, array wash, stain, and scan were processed according to the Affymetrix recommended standard protocol. Intensity data were processed and summarized to gene level with Partek (Partek). Differentially expressed gene candidates were selected for verification with

an absolute fold change difference >2.0.

Statistical analysis

All experimental data were showed as mean (SD). All graphs were plotted using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). The statistical significance of the differences was determined by ANOVA followed by Student-Newman-Keuls (S-N-K) test or unpaired two-tailed t-tests. P<0.05 was considered to indicate statistically significant differences. The sstatistical analysis was performed using SPSS 17.0 statistical software.

Results

lncRNAs UCA1 expression was up-regulated in cervical cancer samples

To explore the expression levels of UCA1 in human cervical cancer tissues, we detected UCA1 levels in 20 pairs of human cervical cancer tissues and matched adjacent normal tissues by real time RT-PCR. Our results revealed that lncRNA UCA1 was up-regulated in 65% (13/20) of cervical cancer tissues (**Figure 1A**). To verify the UCA1 expression in cervical cancer cells, HEC-1B, Hela, N3CA, HEC-1A, RL95-2, Ishikawa3H12 cancer cell lines and the HEC251 normal endometrium cell line were used for UCA1 expression analysis by real time RT-PCR. The results showed that the relative expression of UCA1 was significantly higher in HEC-1B, Hela, N3CA, HEC-1A, RL95-2, Ishikawa3H12 cells compared to the UCA1 expression in HEC251 (**Figure 1B**). These findings show that UCA1 might act as an oncogene in cervical cancer.

Inhibition of UCA1 suppressed cell proliferation and glycolysis

The data in **Figure 1B** show the UCA1 expression in HEC-1B cells was the highest, and the
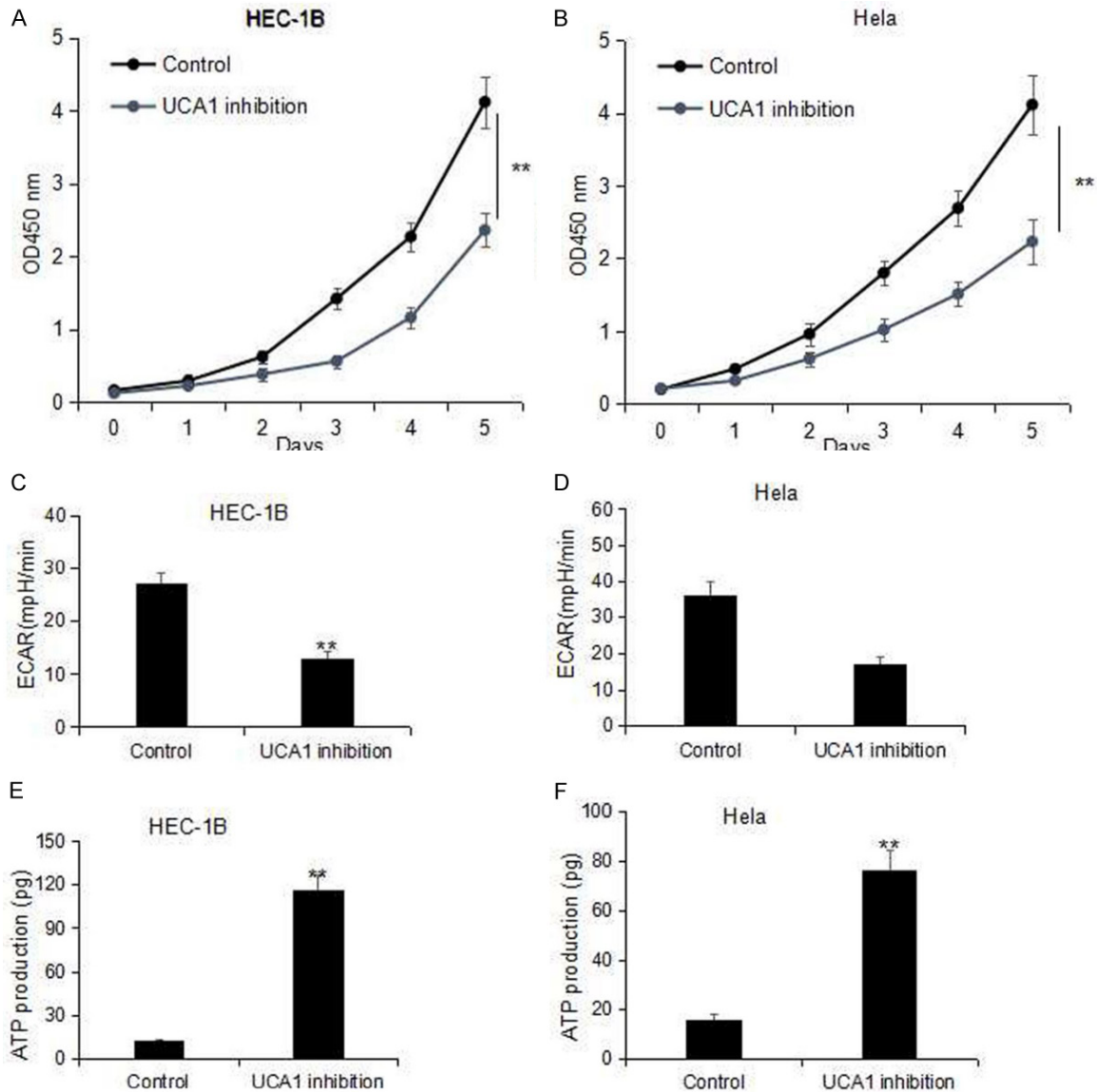
Figure 2. Inhibition of UCA1 suppressed cell proliferation and glycolysis. A, B. Inhibition of UCA1 suppressed cervical cancer cell proliferation. HEC-1B and HeLa cells were transfected the inhibitors of UCA1 for 24 h and cell survival ability was measured by CCK8. C, D. Inhibition of UCA1 suppressed cervical cancer cell ECAR. HEC-1B and HeLa cells were transfected the inhibitors of UCA1 for 24 h and ECAR was measured. E, F. Inhibition of UCA1 suppressed cervical cancer cell OCR. HEC-1B and HeLa cells were transfected with the inhibitors of UCA1 for 24 h and OCR was measured. $*P<0.05$, $**P<0.01$.

expression in the HeLa cells was the second highest. HEC-1B and HeLa cells were selected for further investigation. To study the function of UCA1 in cervical cancer proliferation, we transfected the inhibitor of UCA1 into HEC-1B and HeLa cells. The resulting data indicated that the suppression of UCA1 significantly inhibited cell proliferation of HEC-1B and HeLa cells compared to the control through the CCK-8 assay (**Figure 2A**, **2B**). Afterwards, we

probed the effect of UCA1 on the glycolysis in HEC-1B and HeLa cells. UCA1 was transfected to HEC-1B and HeLa cells and the cellular glycolysis state was determined by measuring the cellular glucose consumption and extracellular acidification rate (ECAR). The results showed that the inhibition of UCA1 suppressed the ECAR of HEC-1B and HeLa cells (**Figure 2C**, **2D**). The iinhibition of UCA1 suppressed cervical cancer cell OCR (**Figure 2E**, **2F**). Thus, the
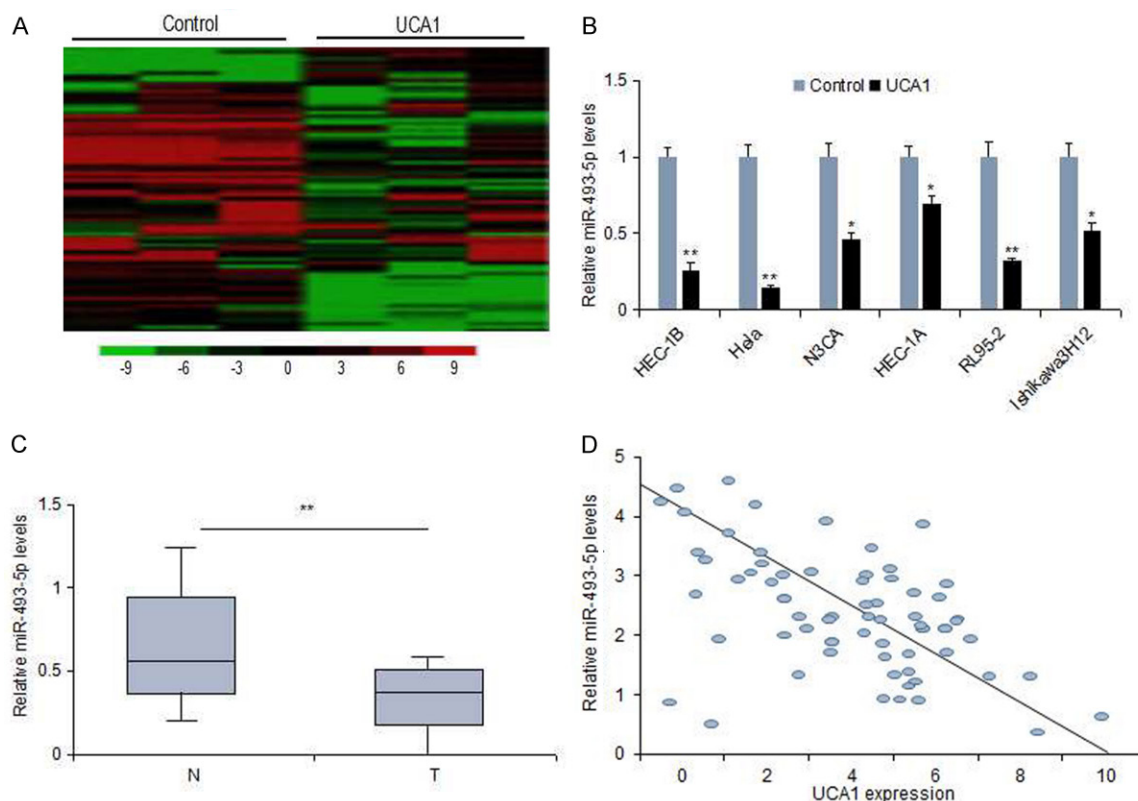
Figure 3. MiRNA profile in cervical cancer cells with the up-regulation of UCA1 expression. A. The miRNA assay in HeLa cells with UCA1 transfection. HeLa cells were transfected with UCA1 and performed for miRNA array analysis. B. MiR-493-5p expression in the cervical cancer cells with UCA1 transfection. Cells were transfected with UCA1 and performed for a real time RT-PCR analysis. C. miR-493-5p levels in cervical cancer tissues. Real time RT-PCR was used for miR-493-5p expression in the cervical cancer tissues. D. The relationship between UCA1 levels and miR-493-5pexpression in cervical cancer tissues. *P<0.05, **P<0.01.

results notably clarified that downregulation of UCA1 suppressed cell proliferation and glycolysis.

MiRNA profile in cervical cancer cells with the up-regulation of UCA1 expression

To explore the mechanism of UCA1 in regulating the glycolysis of cervical cancer cells, HeLa cells were transfected with UCA1 and carried out for an miRNA array to screen out the significant miRNAs. The data showed that UCA1 significantly decreased the level of miR-493-5p (**Figure 3A**). A real-time RT-PCR was used to detect the relative expression level of miR-493-5p in cervical cancer cells. As expected, the expression level of miR-493-5p in cells with UCA1 overexpression was lower than the control (**Figure 3B**). Furthermore, we tested the relative expression levels of miR-493-5p in cervical cancer tissues. The result indicated a similar trend with cervical cancer cells, namely

the relative expression level of miR-493-5p in normal tissues was lower in cervical cancer tissues (**Figure 3C**). The relationship analysis indicated that miR-493-5p expression was inversely correlated with UCA1 expression in cervical cancer tissues (**Figure 3D**). The data indicated that UCA1 might regulate miR-493-5p expression in cervical cancer cells.

MiR-493-5p suppressed cell proliferation and glycolysis

To learn the role of miR-493-5p in cervical cancer cells, HEC-1B and HeLa cells were transfected with miR-493-5p mimics for 24 h and cell survival ability was measured by the MTT method. As the results show, miR-493-5p can efficiently suppress the proliferation of cervical cancer cells (**Figure 4A**, **4B**). Because of the reverse relationship of UCA1 and miR-493-5p, we further studied the glycolysis of HEC-1B and HeLa cells transfected with inhibitors of miR-
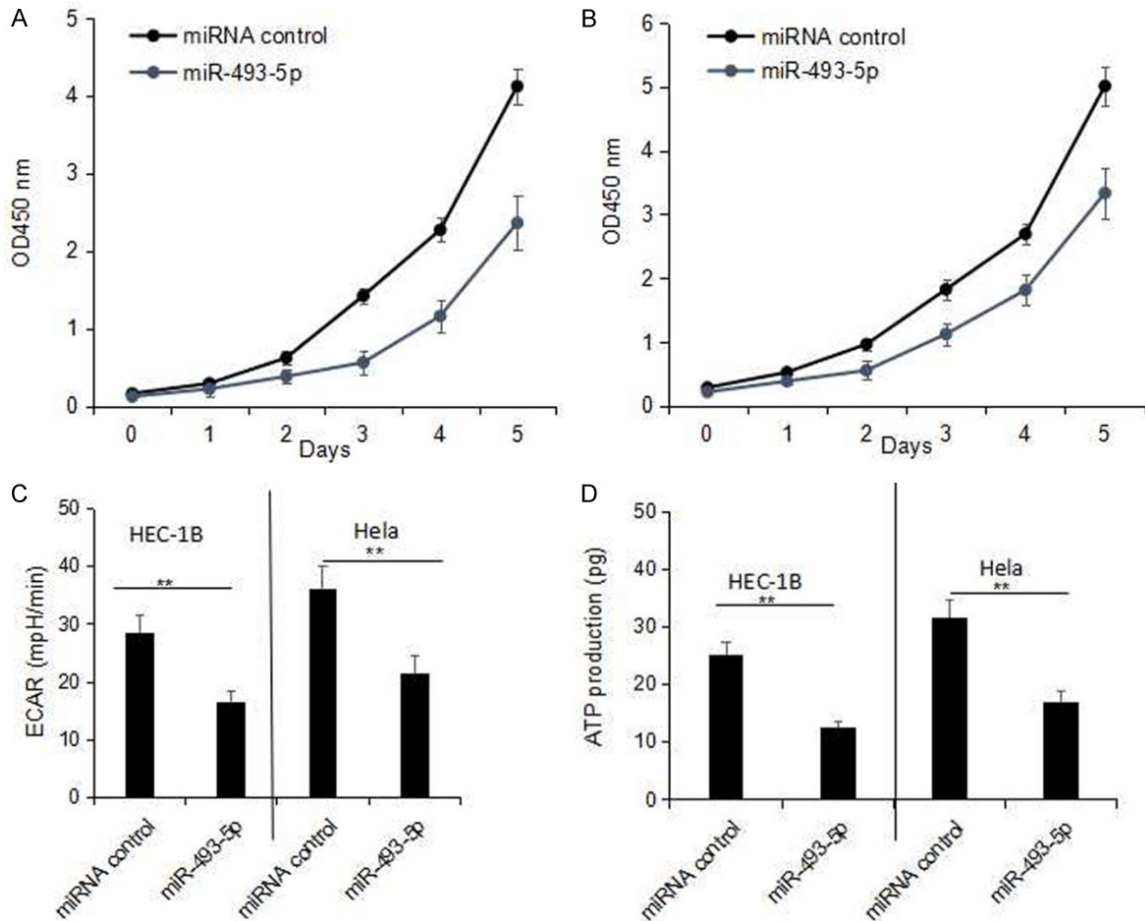
Figure 4. MiR-493-5p suppressed cell proliferation and glycolysis. A, B. miR-493-5p suppressed cervical cancer cell proliferation. HEC-1B and HeLa cells were transfected miR-493-5p for 24 h and cell survival ability was measured by MTT method. C. miR-493-5p suppressed cervical cancer cell ECAR. HEC-1B and HeLa cells were transfected the inhibitors of miR-493-5p mimics for 24 h and ECAR was measured. D. MiR-493-5p suppressed cervical cancer cell ATP production. HEC-1B and Hela cell were transfected the inhibitors of miR-493-5p mimics for 24 h and ATP production was measured. *P<0.05, **P<0.01.

493-5p mimics for 24 h. The resulting data demonstrated that miR-493-5p suppressed cervical cancer cell ECAR (**Figure 4C**) and ATP production (**Figure 4D**) by measuring the levels of ECAR and ATP. So, the results suggested that miR-493-5p suppressed cell proliferation and glycolysis.

HK2 is a target gene of miR-493-5p in cervical cancer cells

Now, based on the above result, we know that miR-493-5p was a target miRNA of UCA1. To investigate the potential target gene regulated by miR-493-5p, the online software Targetscan was used to predict miR-493-5p target genes. The result of bioinformatics manifested that

HK2 was a putative target gene with miR-493-5p (**Figure 5A**). To uncover the interaction between HK2 with miR-493-5p, HeLa cells were co-transfected with miR-493-5p mimics and HK2 3'UTR using the dual luciferase system to measure the luciferase activity. The data showed that miR-493-5p mimics reduced the luciferase activity of HK2 3'UTR WT rather than HK2 3'UTR MUT (**Figure 5B**). To know whether miR-493-5p regulates HK2 expression, HeLa cells were transfected with miR-493-5p mimics, and HK2 mRNA expression levels were analyzed by qRT-PCR, and it was found that miR-493-5p down-regulated HK2 mRNA levels in cells (**Figure 5C**). A Western blotting assay was used to verify that the HK2 protein levels were down-regulated in HeLa cells transfected with

Figure 5. HK2 was a target gene of miR-493-5p in cervical cancer cells. A. HK2 was predicted as a potential target gene of miR-493-5p. The prediction result was from Targetscan. B. A luciferase assay was used to analyze the luciferase activity of HK2 3'UTR in HeLa cells. The cells were co-transfected with miR-493-5p mimics and HK2 3'UTR and then the dual luciferase system was used to measure the luciferase activity. C. MiR-493-5p down-regulated HK2 mRNA levels in cells. The cells were transfected with miR-493-5p mimics and then HK2 mRNA was analyzed by real time RT-PCR. D. MiR-493-5p down-regulated HK2 protein levels in cells. The cells were transfected with miR-493-5p mimics and then HK2 was immunoblotted by Western blotting. *$P<0.05$, **$P<0.01$.

miR-493-5p mimics (**Figure 5D**). Therefore, we demonstrated that HK2 was a target gene of miR-493-5p in cervical cancer cells.

Discussion

Cervical cancer threatens women's health and life worldwide. Efficient early diagnosis and treatment could largely increase the chances of survival [17, 18]. Recently, lncRNAs were found to play a crucial role in tumorigenesis, metastasis, prognosis, and diagnosis, and acted as oncogenes or cancer suppressor genes [19, 20]. Therefore, it's urgent to know the underlying molecular mechanisms of lnc-

RNAs in cervical cancer and identify the function of lncRNA.

Urothelial cancer associated 1 (UCA1) locates at 19p13.12 and can regulate several genes, such as the CREB (cAMP responsive element binding protein) gene, which encodes a transcriptional factor and affects oncogenesis. Currently, UCA1 plays significant roles in many cancers. For example, Tuo et al. discovered that UCA1 was up-regulated in breast cancer and directly interacts with miR-143 to modulate breast cancer cell growth and apoptosis [21]. Fan et al. investigated whether UCA1 was over-expressed in cisplatin-resistant bladder cancer

cells and could increase the cell viability by enhancing the expression of Wnt6 [22]. Zheng et al. reported that UCA1 expression was remarkably increased in gastric cancer tissues and cell lines compared with expressions in the normal controls. Clinical pathologic analysis revealed that a high UCA1 expression was correlated with worse differentiation, tumor size, invasion depth and TNM stage in gastric cancer [23]. However, the function of UCA1 in cervical cancer is still unclear. In our study, we revealed that UCA1 was up-regulated in cervical cancer tissues compared to adjacent normal tissues and UCA1 inhibition suppressed the progression of cervical cell proliferation and glycolysis. Hence, we concluded that UCA1 plays a role in the progress and development of cervical cancer.

MicroRNAs (miRNAs), are a kind of highly conserved noncoding RNAs with about 21-23 nucleotides. Studies have been documented that miRNAs play an effective role in the disease progression of various cancers [24, 25]. MiRNAs participate in biological processes, such as proliferation, metastasis, and invasion by targeting mRNAs in numerous cancers [26, 27]. MiR-493-5p exerts significant functional roles in many cancers, such as breast cancer [28], hepatocellular carcinoma [29] and lung cancer [30]. However, the function and mechanisms of miR-493-5p are still unclear in cervical cancer.

HK2 (hexokinase2), is a molecule that's in the first step in most glucose metabolism pathways and catalyzing the phosphorylation of hexose [31]. It localizes to the outer membrane of mitochondria. Additional research suggests that HK2 is involved in increasing the rate of glycolysis in rapidly growing cancer cells. Our results showed that miR-493-5p efficiently suppressed cell proliferation and glycolysis. Afterwards, bioinformatics Targetscan software was used to predict that HK2 was a target gene of miR-493-5p. A lluciferase assay was used to analyze the luciferase activity of HK2 3'UTR in HeLa cells. Our data showed that miR-493-5p mimics reduced the luciferase activity of HK2 3'UTR WT rather than HK2 3'UTR MUT. Those results further confirmed that HK2 was directly targeted by miR-493-5p. The mRNA and protein expression of HK2 was also decreased in the cervical cancer cells transfected with miR-493-5p mimics.

To summarize, we found the following: (1) the miRNA assay manifested that long non-coding RNA UCA1 directly binds miRNA-493-5p; (2) the expression of miR-493-5p was downregulated in cervical cancer tissues and cells and had an inverse correlation with UCA1 expression; (3) UCA1 modulated the glycolysis of cervical cancer cells by miR-493-5p/HK2. Our study revealed that lncRNA UCA1 might serve as an oncogene by modulating the glycolysis of cervical cancer. Notably, our results provide a therapeutic method by modulating the UCA1-miR-493-5p-HK2 pathway.

Acknowledgements

Disclosure of conflict of interest

None.

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