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Suppression of long non-coding RNA UCA1 inhibits proliferation and invasion and induces apoptosis in human lung cancer cells

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Abstract. – OBJECTIVE: Lung cancer is one of the deadliest cancers responsible for significant mortality and morbidity across the globe. The unavailability of efficient treatments, lack of reliable biomarkers and potent therapeutic targets, limit the treatment of lung cancer. In this study, we explored the potential of long non-coding RNA (lncRNA) urothelial carcinoma-associated 1 (UCA1) as the therapeutic target for lung cancer.

MATERIALS AND METHODS: The expression analysis was carried out by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Cell viability was monitored by cell counting kit 8 (CCK-8) assay. The 4′,6-diamidino-2-phenylindole (DAPI), annexin-V/Propidium iodide staining and comet assays were used to detect apoptosis. Boyden chamber and wound heal assays were used for cell to asses cell invasion and migration respectively. Protein expression was determined by immunoblotting.

RESULTS: The expression of lncRNA UCA1 was determined by qRT-PCR in six different types of lung cancer cell lines. It was observed that lncRNA UCA1 was significantly ($p < 0.05$) upregulated in all the lung cancer cell lines. To investigate the role of lncRNA UCA1 in lung cancer, its expression was suppressed by transfection of the lung cancer NCI-H23 cells by si-UCA1. The results showed that suppression of lncRNA UCA1 significantly ($p < 0.05$) reduced the viability of NCI-H23 cancer cells via induction of the apoptosis. Furthermore, the lncRNA UCA1 suppression ($p < 0.05$) significantly inhibited the migration and invasion of the NCI-H23 lung cancer at least in part via inhibition of mitogen-activated protein kinase 1 (MAPK1). Additionally, the suppression of MAPK1 exhibited similar effects on the proliferation, migration, and invasion of the NCI-H23 cells as that of UCA1 silencing. Finally, the co-suppression of lncRNA UCA1 and MAPK1 exhibited synergistic effects on cell proliferation, migration, and invasion.

CONCLUSIONS: We demonstrated that lncRNA UCA1 could be an important therapeutic target for curbing lung cancer.

Key Words:
Long non-coding RNA, Apoptosis, Cell migration, Invasion, Lung cancer.

Introduction

Lung cancer is one of the major causes of cancer-related mortality across the globe[1]. Around 8590% of cases of lung cancer are categorized as nonsmall cell lung cancer (NSCLC), and the majority of patients with lung cancer are diagnosed at advanced cancer[1]. The treatment strategies for lung cancer are still not sufficient and are associated with a lot of side effects. Therefore, to look for novel drug options or to identify novel therapeutic targets for the treatment of lung cancer is pivotal. Although the human genome is very large, only a small percent of human genome codes for proteins. The rest of the genome is mostly inactive except for some genes that code for non-coding RNAs[2]. Non-coding RNAs are grouped into two categories depending on the length of their transcripts: short-chain non-coding RNA (small ncRNAs, sncRNAs) and long non-coding RNA (lncRNA). Short-range non-coding RNAs include a wide range of known and newly discovered RNAs, such as tiny transcription initiation RNAs (tiRNA), small interfering RNAs (siRNAs), microRNAs (miRNAs), and so on[3]. Over the years, the researches directed on miRNAs have shown that they play vital roles in a wide array of cellular and physiological processes which also include

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cancer development, diagnosis, and prognosis[4]. However, comparatively less research has been carried out on the long-chain non-coding RNAs (lncRNAs). Long-chain non-coding RNA is generally more than 200 nucleotides and may even be thousands of nucleotides in length. With advancements in science, lncRNAs have been shown to be involved in the controlling of many biological processes, such as abnormal transcription in the tumor, cell proliferation, cell cycle regulation, and apoptosis[5]. Human urothelial carcinoma associated 1 (UCA1) is an important long non-coding RNA that was shown to be upregulated in bladder cancers[6]. Thereafter, it was also reported to be upregulated in breast cancer, gastric cancer, and several other types[7,8]. Given these observations, it is believed that UCA1 might be playing a common role in human cancers. Herein, we examined the expression of lncRNA UCA1 in six different lung cancer cells lines and attempted to delimit its role in lung cancer. The results revealed that lncRNA UCA1 is overexpressed in all the studied lung cancer cell lines, and the suppression of UCA1 inhibits cell proliferation, migration, and invasion of lung cancer cells by targeting mitogen-activated protein kinase 1 (MAPK1). Taken together, these results indicate that lncRNA might act as a potential target for the treatment of lung cancer.

Materials and Methods

Cell Lines, Culture Conditions and Transfection

Human lung cancer cell lines (HCC827, HCC2935, SK-LU-1, NCI-H23, NCI-H1563, NCI-H1651), and normal human lung cell line (RL-65) were procured from American Type Culture Collection. They were maintained under standard conditions. Briefly, the cells were cultured continuously in Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 10% fetal bovine serum albumin (FBS) containing specific antibiotics (Invitrogen, Carlsbad, CA, USA) and maintained at 37°C. The siRNAs construct for MAPK1 were procured from Genepharma (Shanghai, China). Transfection of si-NC or si-MAPK1 was carried out with the help of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The study has been approved by the Ethics Committee of Shengjing Hospital of China Medical University under approval number CHM/A22C502/2018.

Isolation of RNA, cDNA Synthesis and Expression Analysis

The RNA from all the lung cancer and normal cell lines was extracted with the help of RNeasy RNA isolation kit (Qiagen, Hilden Germany) and the whole procedure was carried out as per the instructions of the manufacturer. Thereafter, cDNA was synthesized with the help of RevertAid cDNA synthesis kit (Fermentas, Waltham, MA, USA) with manufacturer's protocol. To carry out the RT-PCR, the cDNA was diluted 20 times, and qRT-PCR was carried out in three replicates in ABI StepOne Real time using SYBR Green Master Mix (Fermentas, Waltham, MA, USA). The relative quantification method ($^{\Delta\Delta}$-CT) was employed to determine the quantitative difference between the replicates examined. The GAPDH was used as positive control.

CCK-8 Assay for Cell Viability

The cell viability of the lung NCI-H23 cancer cells was evaluated by cell counting kit 8 (CCK-8) assay. The cells were collected at 24, 48, 72, and 96 h after transfection and subjected to CCK-8 assay. The cell viability was performed later by cell counting kit-8 (Dojindo, Kumamoto, Japan) following the manufacturer's guidelines.

Apoptosis Assay

For detection lung NCI-H23 cancer were cultured (2×10^5 cells/well) in 6-well plates. The cells were then transfected and incubated for 24 h. DAPI was carried by treating the cells in 6-well plates with DAPI or AO/EB (Acridine orange/ethidium bromide). The cells were then washed with phosphate buffered saline (PBS) and then fixed with formaldehyde (10%). The DAPI or AO/EB stained cells were then subjected to fluorescence microscopy. For estimation of apoptotic cell populations, a similar procedure was carried out except for the cells were stained with annexin V/PI staining and analyzed by flow cytometer.

Comet Assay

DNA damage triggered by suppression of lncRNA UCA1 in lung cancer cells was assessed by the comet assay. Briefly, lung NCI-H23 cancer cells transfected with Si-NC and Si-UCA1 were harvested and suspended in cold PBS. The cells in the 0.5% low melting point agarose were kept on a slide precoated with a layer of 1% regular agarose. Afterwards, the-

se two layers were allowed to solidify at 4ºC, and then, suspended in a cold lysis buffer for 50 min at 4ºC. Then, the gel slides were allowed to dry and the dried slides were soaked in fresh electrophoresis solution for 25 min. Then, electrophoresis was carried out at 300 mA, 25 V for 25 min at 4ºC. This was followed by staining with ethidium bromide (20 µg/ml) for 12 min, and neutralization of the slides with 0.4 M Tris-HCl (pH 7.5). Finally, the slides were washed and observed under a fluorescent microscope (BX51; Olympus, Tokyo, Japan).

Wound Healing Assay

This assay was employed to determine the cell migration potential of lung NCI-H23 cancer cells. Briefly, 5×10^4 cells/well were seeded in 96-well plates. Afterwards, the plates were incubated for 12 h at 37°C to allow the cells to adhere. Then, a wound was scratched using a sterile pipette tip after the cells reached confluence. The cells were then washed with PBS to clear the detached cells. The cells were monitored after 20 h interval and photographed.

Boyden Chamber

The cell invasion was performed by employing Boyden chamber assay. Briefly, cells with the density of 3×10^4 cells in each well were added in 12% FBS medium and put in the upper chamber of transwells (8 µm) with Matrigel. Thereafter, medium containing with 5% FBS was supplied to the lower chamber. Then, the cells were incubated for 24 h. The unmigrated cells on the membrane's upper surface were removed. However, the cells that migrated to lower surface cells were fixed in (100%) methanol and finally stained with Giemsa. The cell migration expressed the total number of cells that migrated to lower surface after observation under a microscope (Olympus, Tokyo, Japan) at 200× magnification.

Western Blotting

After lysis of the lung cancer cells in RIPA lysis buffer, the protein content of each lysate was estimated by bicinchoninic acid assay (BCA) assay. The samples were then loaded on the SDS-PAGE. The gels were then transferred to nitrocellulose membranes and subjected to treatment with primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C for 24 h. After this, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:1000) for 50 min at 25°C. Enhanced chemiluminescence reagent was used to visualize the protein bands.

Statistical Analysis

The experiments were repeated thrice and the values are mean +SD. One way ANOVA followed by Tukey's post-hoc test was used for statistical analysis with the help of GraphPad prism 7 software (La Jolla, CA, USA). Significance was tested at $p < 0.05$.

Results

The lncRNA UCA1 is Overexpressed in Lung Cancer Cells

The therapeutic potential of lncRNA UCA1 in lung cancer was determined by examining its expression in six lung cancer cell lines (HCC827, HCC2935, SK-LU-1, NCI-H23, NCI-H1563, NCI-H1651) and one normal human lung cell line (RL-65) by quantitative RT-PCR. The results revealed that the transcripts of lncRNA UCA1 were significantly overexpressed ($p < 0.05$) in all the lung cancer cell lines in comparison to the normal lung cell line (Figure 1). Additionally, the expression of lncRNA UCA1 was around 2.7 to 5.6 fold higher in the lung cancer cells in comparison to normal cells. These outcomes of the study indicated that lncRNA UCA1 could be a potential therapeutic target for the treatment of lung cancer.

Suppression of lncRNA UCA1 Inhibits Lung Cancer Proliferation, Invasion, and Migration

To unveil the role of lncRNA UCA1, one lung cancer cell line (NCI-H23) was transfected with si-NC or si-UCA1 which lead to the inhibition of the expression of UCA1 (Figure 2A). Thereafter, the cells were subjected to the CCK-8 assay for assessment of cell viability. The results indicated that the suppression of lncRNA UCA1 in lung NCI-H23 cancer cells significantly inhibited ($p < 0.05$) their proliferation time dependently (Figure 2B). These results further prompted us to examine the effect of lncRNA UCA1 suppression on the cell invasion and migration of lung cancer cells by Boyden chamber and wound healing assays. We found that the suppression of IncRNA UCA1 in NCI-H23 cancer cell lines caused significant decrease ($p < 0.05$) in cell the cell invasion capacity of the lung cancer cells (Figure 2C). Similar effects were observed for cell migration wherein

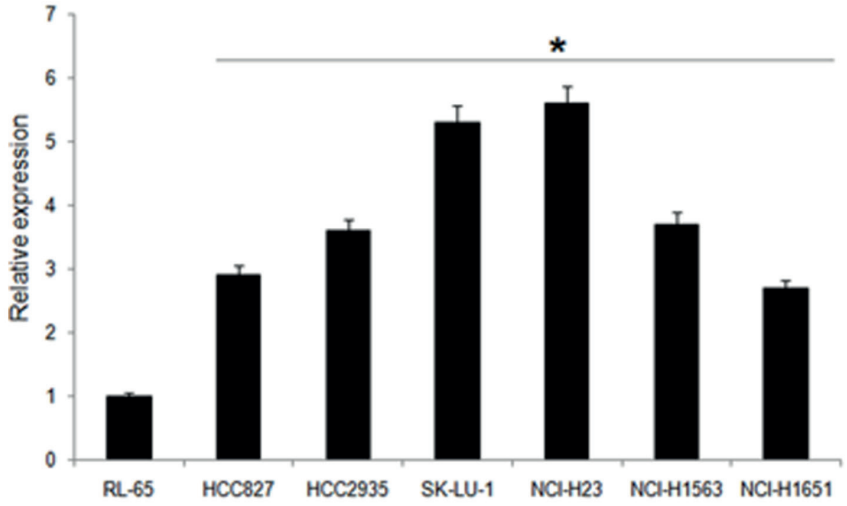

Figure 1. Expression of LncRNA UCA1 in different human lung cancer cell lines. The experiments were carried out in triplicates and expressed as mean ± SD (*p<0.05).

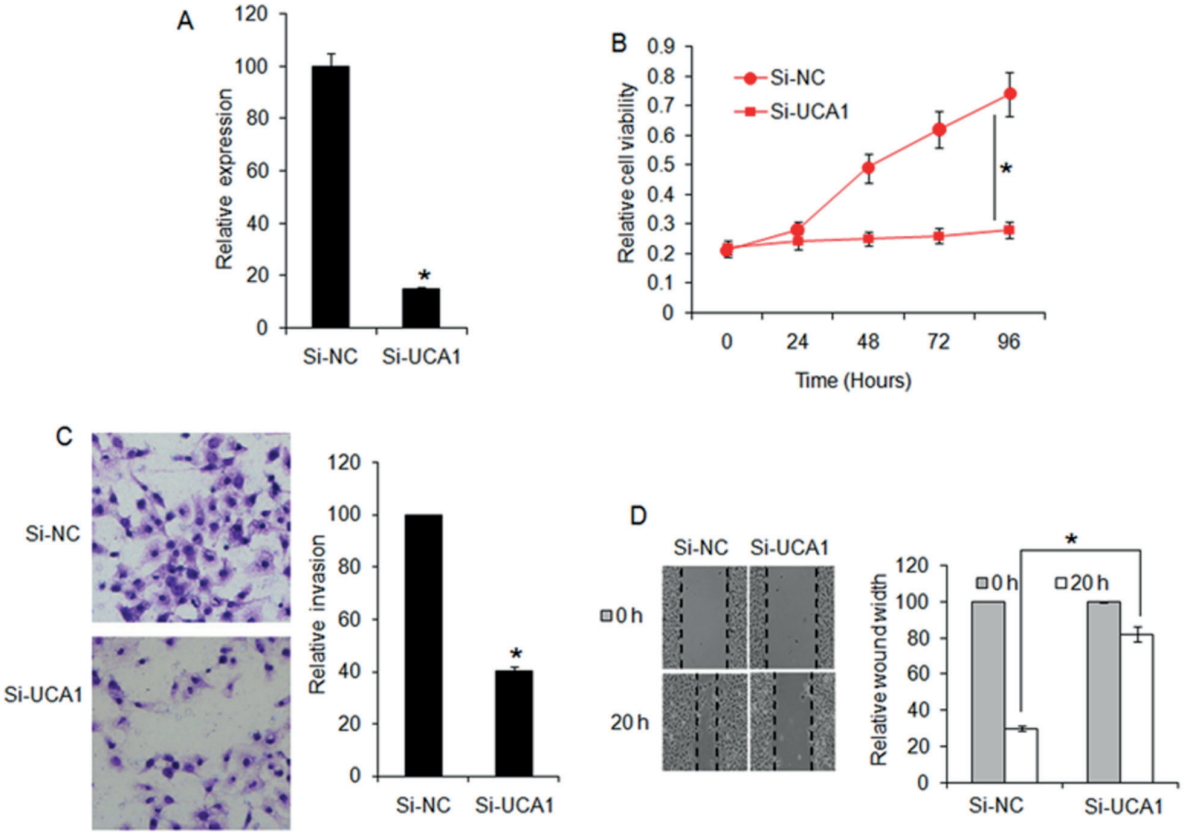

Figure 2. ***(A)*** Expression of lncRNA UCA1 in Si-NC and Si-UCA1 transfected NCI-H23 lung cancer cells ***(B)*** Relative cell viability of Si-NC and Si-UCA1 transfected NCI-H23 lung cancer cells ***(C)*** Effect of suppression of lncRNA UCA1 on cell invasion ***(D)*** Effect of suppression of lncRNA UCA1 on cell migration. The experiments were carried out in triplicates and expressed as mean ± SD (*p<0.05).

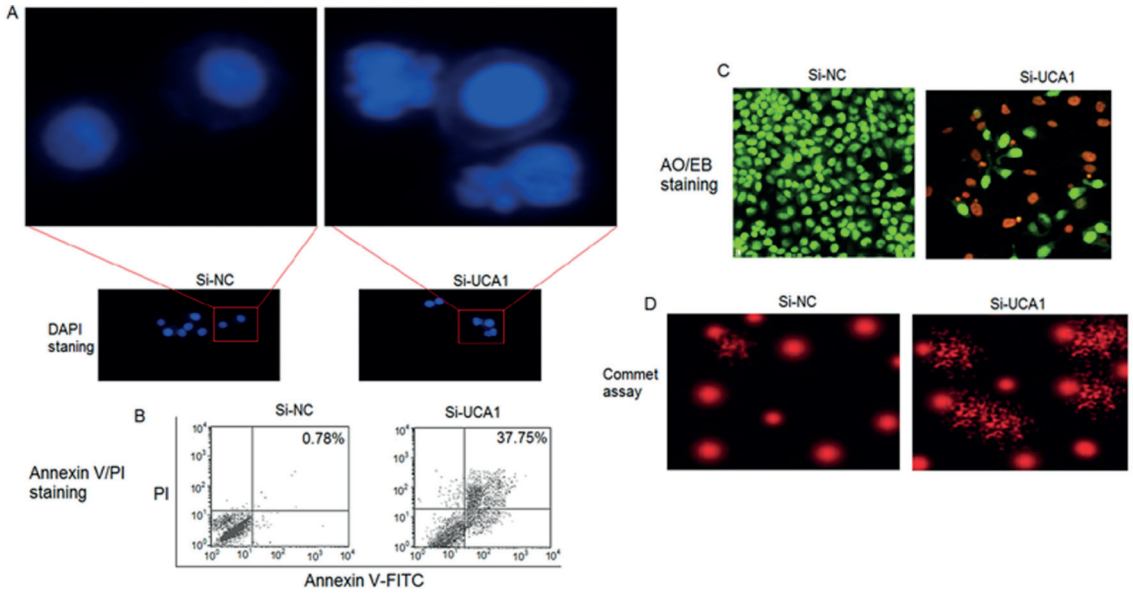

Figure 3. Suppression of lncRNA UCA triggers apoptosis in NCI-H23 lung cancer cells ***(A)*** DAPI staining ***(B)*** annexin V/PI staining ***(C)*** AO/EB staining ***(D)*** Comet assay. The experiments were carried out in triplicates.

the suppression of IncRNA UCA1 expression was associated with reduced cell migration (Figure 2D). These outcomes of the study undoubtedly point towards the role of lncRNA UCA1 in the proliferation, migration, and invasion of the lung cancer cells.

Suppression of lncRNA UCA1 in Lung Cancer Cells Triggers Apoptosis

To understand the mechanism behind the decrease in the cell viability of NCI-H23, we carried out DAPI staining. The results showed that suppression of lncRNA UCA1 triggered apoptosis in lung NCI-H23 cancer cells (Figure 3A). Further annexin V/PI staining revealed that the apoptotic cell populations increased from 0.78% in Si-NC to 37.75% in Si-UCA1 transfected NCI-H23 cancer cells (Figure 3B). Comment assay and AO/EB staining (Figure 3C and D) further showed that suppression of lncRNA UCA1 triggers apoptosis in NCI-H23 cancer cells.

The lncRNA UCA1 Exerts its Effects Via MAPK1

In a previous study[9], it was observed that the lncRNA UCA overexpression in bladder cancer cells is associated with the upregulation of MAPK. Therefore, we investigated the effect of lncRNA UCA1 suppression in lung NCI-H23 cancer cells on the expression of MAPK1. The results showed that suppression of UCA1 caused a significant decrease in the expression of MAPK1 (Figure 4A and B), indicating that lncRNA UCA1 might exert its effects at least in part via MAPK1. Further, silencing of MAPK1 inhibited the proliferation, migration, and invasion of the lung cancer NCI-H23 cells (Figure 4C-E).

MAPK1 and lncRNA UCA1 Exhibit Synergistic Effects on Lung Cancer Proliferation, Invasion, and Migration

To elucidate if MAPK1 and lncRNA UCA1 control the proliferation, invasion, and migration of the lung cancer cells synergistically, the lung cancer cells were co-transfected with si-UCA1 and si-MAPK1. The outcomes of this research showed that the inhibition of MAPK1 and lncRNA UCA1 together had more profound effects on cell proliferation, invasion, and migration than lncRNA UCA1 or MAPK1 individually (Figure 5A-C). These results suggest that MAPK1 and UCA1 exhibit synergistic effects on the proliferation, migration, and invasion of lung cancer cells.

Discussion

Lung cancer, being one of the deadliest cancers, is responsible for considerable mortality and morbidity world over[10]. However, there are

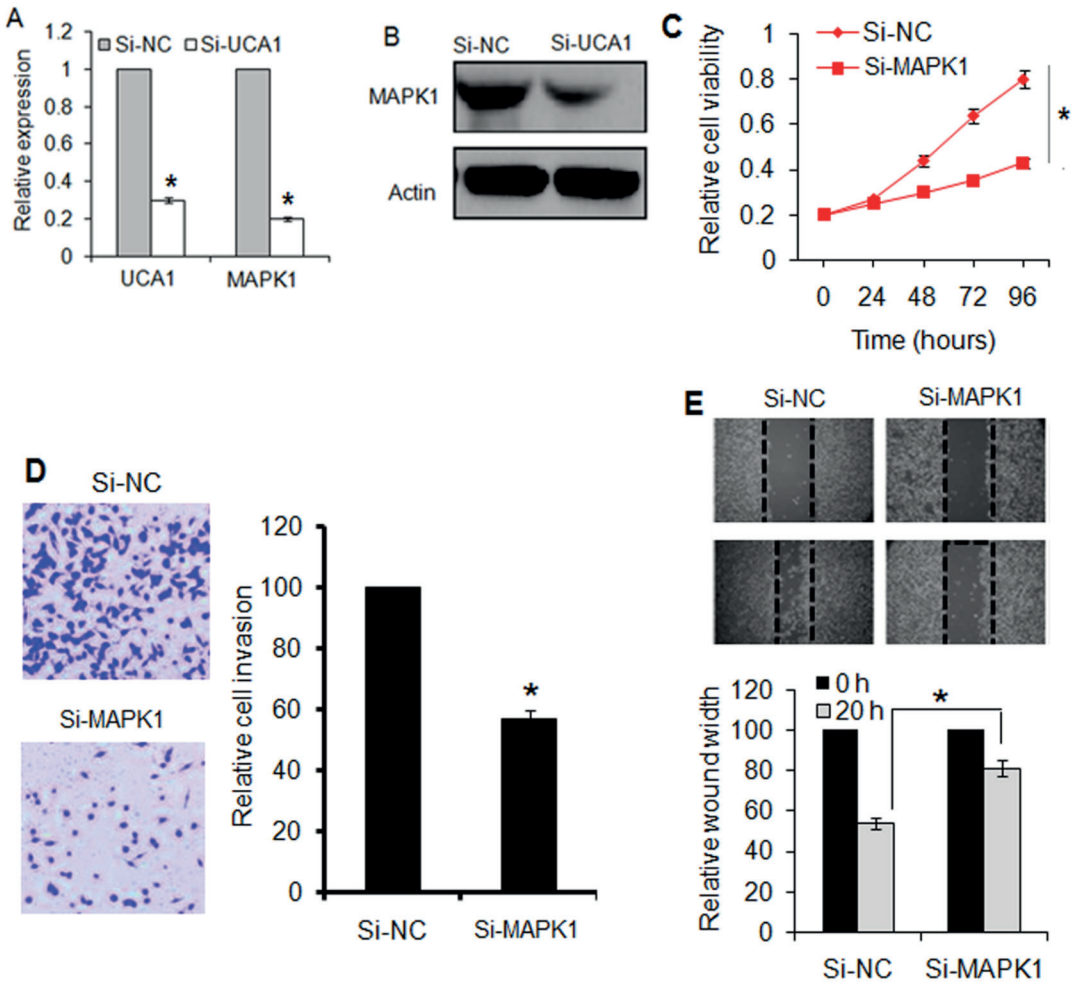

Figure 4. ***(A)*** Expression of MAPK1 in Si-NC and Si-UCA1 transfected NCI-H23 lung cancer cells determined by RT-PCR analysis ***(B)*** Protein expression of MAPK1 in Si-NC and Si-UCA1 transfected NCI-H23 lung cancer cells ***(C)*** Effect of MAPK1 silencing on NCI-H23 proliferation ***(D)*** Effect of MAPK1 silencing on NCI-H23 invasion ***(E)*** Effect of MAPK1 silencing on NCI-H23 migration. The experiments were carried out in triplicates and expressed as mean ± SD (*p<0.05).

limited treatment options for lung cancer, and hence, there is a pressing need to explore novel drug options and/or to look for novel therapeutic drug targets. We explored the potential of lncRNA UCA1 as a therapeutic target in lung cancer. The results indicated that the expression of UCAI was overexpressed in all the lung cancer cells studied. Our findings are all supported by previous studies wherein the expression of lncRNA UCA1 was found to be dysregulated in several types of cancer. For instance, a work carried out on the bladder cancer showed that UCA1 is considerably overexpressed in bladder cancer cells and could prove an important predictive biomarker for bladder cancer[9]. To find out the functional role of lncRNA UCA1 in lung cancer, its expression was suppressed by transfection of the NCI-H23 lung cancers with si-UCA1. Notably, it was observed that suppression of UCA1 in lung cancer cells was associated with a decrease in the cell viability of NCI-H23 cells. The decrease in the cell viability was found to be mainly due to the induction of apoptosis in NCI-H23 lung cancer cells. In a previous study[11], lncRNA UCAI has been shown to affect the cell viability of the gastric cancer cells by induction of apoptosis further confirming our results. Apoptosis is an important mechanism that involves the elimination of the harmful and unwanted cell from the body of the organism. Induction of apoptosis also prevents the development of drug resistance in cancer cells[12,13]. The migration and invasion potential of cancer cells is an important feature that determines their metastatic nature[14]. Herein it was observed that

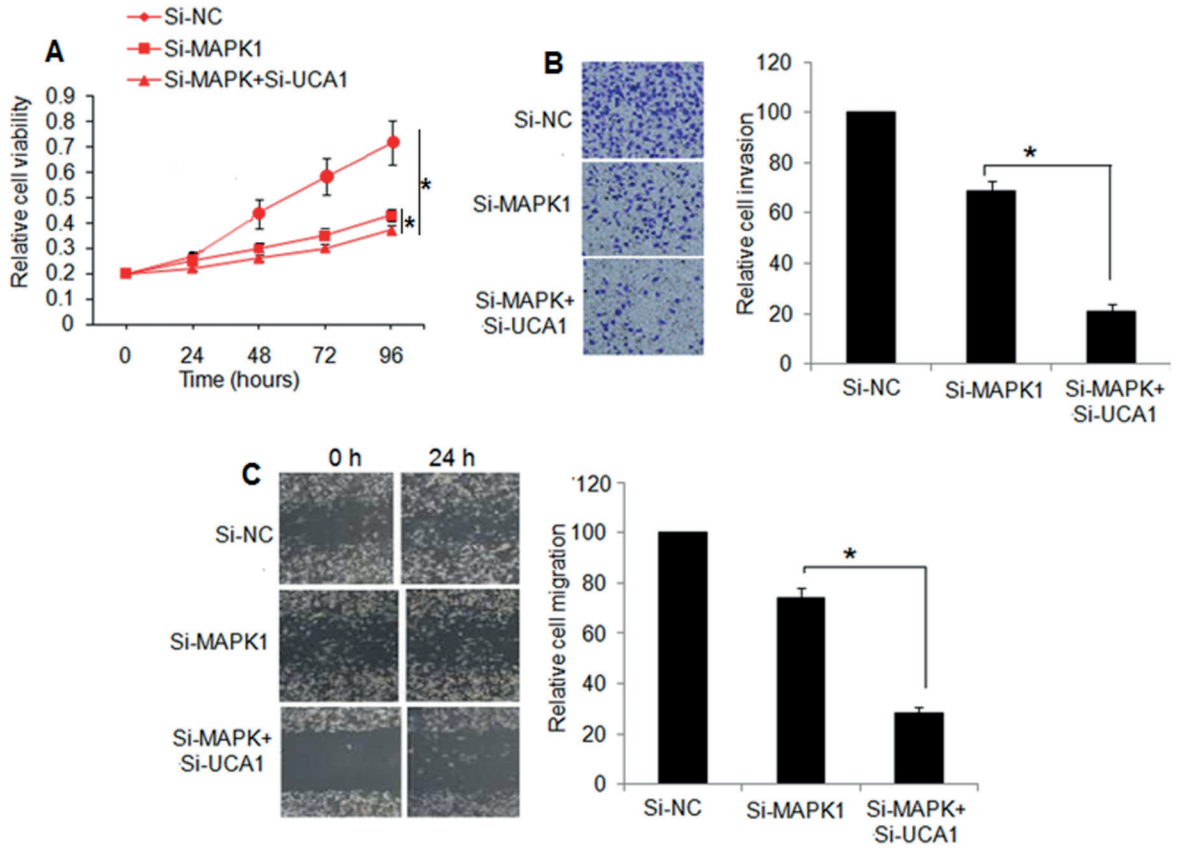

Figure 5. ***(A)*** Cell viability of Si-NC, Si-MAPK1 and Si-MAPK1 + Si-UCA1 transfected NCI-H23 lung cancer cells ***(B)*** Cell invasion of Si-NC, Si-MAPK1 and Si-MAPK1 + Si-UCA1 transfected NCI-H23 lung cancer cells ***(C)*** Cell migration of Si-NC, Si-MAPK1 and Si-MAPK1 + Si-UCA1 transfected NCI-H23 lung cancer cells. The experiments were carried out in triplicates and expressed as mean ± SD (*p<0.05).

suppression of lncRNA UCA1 could inhibit the migration and proliferation of the cancer cells. Our results agree with previous studies wherein UCAI was reported to regulate the cancer cell migration and invasion in the esophageal squamous cell carcinoma[15]. In another study, it was observed that the enhanced expression of lncRNA UCA1 was positively associated with the expression of MAPK1. Therefore, we investigated the effect of UCA1 suppression on MAPK1 expression. Interestingly, the results showed that suppression of lncRNA UCAI caused a significant reduction in the expression of MAPK1, indicating that UCA1 might exert its effects at least in part via MAPK1 signaling. This was also confirmed by silencing of MAPK1 in NCI-H23 cells which lead to the inhibition of cell proliferation, migration, and invasion. Furthermore, suppression of both si-MAPK1 and si-UCA1 in breast cancer cells exhibited synergistic effects on the cell migration and invasion of the lung NCI-H23 cancer cells. These results suggest that UCAI may also have other targets except MAPK1 and hence the synergistic effect. Taken together, our findings reveal that lncRNA could be a potential candidate for the management of lung cancer.

Conclusions

Wey revealed that lncRNA UCA1 was overexpressed in lung cancer cells and its suppression inhibited proliferation through induction of apoptosis. Besides lncRNA UCA1 suppression inhibited migration and invasion of lung cancers cells at least in part via regulation MAPK1 expression.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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