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Knockdown of Long Noncoding RNA uc.338 by siRNA Inhibits Cellular Migration and Invasion in Human Lung Cancer Cells

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Lung cancer remains a critical health concern worldwide. Long noncoding RNAs with ultraconserved elements have recently been implicated in human tumorigenesis. The present study investigated the role of ultraconserved element 338 (uc.338) in the regulation of cell proliferation and metastasis in human lung cancer. Our data showed that the expression of uc.338 in lung cancer was remarkably increased *in vivo* and *in vitro*. Depletion of uc.338 with specific siRNA interference retarded the cell proliferative rate in lung cancer cell lines NCI-H929 and H1688. Furthermore, knockdown of uc.338 caused cell cycle arrest in the G₀/G₁ phase in both cell lines. Transwell assays showed that inhibition of uc.338 notably decreased migration and invasion in NCI-H929 and H1688 cells. Moreover, uc.338 depletion decreased the expression of cyclin B1, Cdc25C, Snail, vimentin, and N-cadherin while increasing the protein level of E-cadherin, shown with Western blot analysis. These results suggested the pro-oncogenic potential of uc.338 in lung cancer, which might provide novel clues for the diagnosis and treatment of lung cancer in the clinic.

Key words: Ultraconserved element 338 (uc.338); Lung cancer; Proliferation; Metastasis; Epithelial–mesenchymal transition (EMT)

INTRODUCTION

Lung cancer remains a critical health concern worldwide. In a large consortium of prospective cohorts in Europe, lung cancer was responsible for the largest number of lost disability-adjusted life-years (DALYs) (1). In an area in China with heavy air pollution, the lung cancer burden has been increasing over the last 40 years (2). The great burden of lung cancer is attributed to a wide range of factors, including the environment, genetic background, aberrant expression of critical molecules, and so on. Despite the great progress that has been made as to the treatment of lung cancer over the last few decades, the prognosis for lung cancer remains dim due to the difficulties with early diagnosis. Hence, there is always an urgency to find novel markers that are critically involved in the regulation of growth and metastasis in lung cancer.

Long noncoding RNAs (lncRNAs) are a category of transcripts that contain more than 200 nucleotides and do not have the potential for protein coding. lncRNAs can be classified into intergenic, intronic, antisense, and

enhancer lncRNAs according to their genomic locations (3). Recently, lncRNAs have been increasingly recognized for their role in malignant transformation and tumor growth (4). However, only a handful of lncRNAs have been identified in human tumorigenesis (5–8). For the most part, their function remains unclear (9,10). One interesting observation regarding lncRNAs is that there may be sequence conservation across species, which indicates that a given lncRNA may have a cellular function (11). A pioneer genome-wide survey identified hundreds of lncRNAs that lacked natural variation across the human, mouse, and rat genomes (12). These highly conserved lncRNAs have been named ultraconserved elements and have been shown to also be conserved across other species (12). The property of high conservation and wide distribution in the genome suggested that these ultraconserved lncRNAs have biological functions in the pathophysiology of human diseases (13,14).

It has recently been observed that specific ultraconserved RNAs have been aberrantly expressed in leukemia

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and other solid tumors (15). The ultraconserved element 73 (uc.73) has been shown to functionally modulate cell apoptosis and proliferation in colorectal cancer (16,17). Ultraconserved RNAs have also been related to clinical prognostic factors (18). A more recent custom microarray found that ultraconserved element 338 (uc.338) was dramatically increased in hepatocellular carcinoma, and its expression regulated cell proliferation in this malignancy (4). However, the role of lncRNA uc.338 in other solid tumors remains to be elucidated, and our aim was to evaluate their expression and potential involvement in the regulation of cell growth and metastasis in human lung cancer.

MATERIALS AND METHOD

Human Tissues

This study was approved by the Ethics Committee of Shandong University of Technology. A total of 100 patients diagnosed with lung cancer and admitted to the Department of Thoracic Surgery, Central Hospital of Tai'an were collected. The tumor tissues and their adjacent noncancerous tissues were obtained from each patient. All patients showed their full intentions to participate in our study, and written informed consents from each patient were also obtained.

Total RNA Extraction and cDNA Synthesis

The total RNAs from both clinical patients and cultured cells were isolated by TRIzol Reagent (TaKaRa, Dalian, China) according to the manufacturer's instructions. The quality and concentration of each sample were identified by measuring the absorbance at 260 and 280 nm with Nanodrop 2000 (Thermo Scientific, Pittsburgh, PA, USA). First-strand cDNAs were then generated with the PrimeScript RT Master Mix Perfect Real Time (TaKaRa).

Relative Real-Time PCR

All real-time polymerase chain reactions (PCRs) were performed in an ABI PRISM 7900 Real-Time System with the SYBR Premix Ex Taq Kit (TaKaRa) following the manufacturer's protocols. The primers are listed below, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also included as the internal control. Each experiment was performed at least in triplicate. uc.338: 5'-AGCGACAGTGCGAGCTTT-3' (forward) and 5'-TTCCGAGTGAGTTAGGAAGG-3' (reverse); GAPDH: 5'-GTGGACATCCGCAAAGAC-3' (forward) and 5'-AAAGGGTGTAAACGCAACTA-3' (reverse).

Cell Culture and Western Blot Analysis

Human lung cancer cell lines H-125, NCI-H292, H1688, NCI-H446, and H1975 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and

cultured in recommended media (Gibco, Los Angeles, CA, USA) supplied with 10% fetal bovine serum (FBS) (Gibco). Lung cancer cells 95D and A549, and normal lung cell line MRC-5 were obtained from the Cell Bank of the Chinese Academy of Sciences and cultured at a 37°C incubator supplied with 5% CO₂. Proteins from NCI-H929 and H1688 cells were extracted for subsequent immunoblot analysis when the growth confluence of siuc.338-treated cells was above 80%. Primary antibodies against cyclin B1, Cdc25C, and vimentin were commercially from Abcam (Cambridge, UK). Primary antibodies against Snail, N-cadherin, E-cadherin, GAPDH, and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Immunoreactivities were determined with enhanced chemiluminescent autoradiography (Thermo Scientific) with the machine Las3000.

siRNA Transfection

Specific siRNAs against human uc.338 were designed and synthesized by Gama Co. (Shanghai, China). The sequences are as follows: siuc.338-1, 5'-UGACAGCC CUGGAGACUGA-3'; siuc.338-2, 5'-CCACAGGACA GGUACAGCA-3'. Both NCI-H929 and H1688 cells were transfected with siuc.338 or scramble siRNA with transfection reagent Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA). Six hours posttransfection the media were replaced with normal culture media supplied with 10% FBS.

Cell Proliferation Assays

Cell proliferation assays were performed with the MTT kit (Sigma-Aldrich, St. Louis, MO, USA). Briefly, both NCI-H929 and H1688 cells were cultured in the presence or absence of siRNAs against uc.338 for 5 days, and afterward a total of 100 µl of CellTiter-Glo solution was added into the cells. Cells were then incubated at room temperature for 10 min, and the luminescence of each well was collected at an absorbance of 450 nm.

Cell Cycle Assay

For evaluation of cell cycle, scramble and uc.338 siRNA-treated NCI-H929 and H1688 cells were trypsinized and fixed in precool methanol. After RNase incubation, cells were treated with protease inhibitor (PI) for 30 min, and the fluorescence intensities were measured by the FC500 flow cytometer (Beckman, USA) fitted with FL-1H filter. Afterward, Modfit 2.0 software was used to quantify the percentage of cells in the G₀/G₁, S, and G₂/M phases, respectively.

Cell Migration and Invasion Assays

An amount of 3 × 10⁴ NCI-H929 or H1688 cells in serum-free medium was seeded into the upper chamber

(pore size: 8 μm ; Corning, Corning, NY, USA) for migration assays and Matrigel invasion assays. The lower chambers were filled with 600 μl of recommended media containing 10% FBS. After 12 h of incubation at 37°C, cells that migrated to the lower surface of the membrane were fixed in paraformaldehyde and stained with 0.1% crystal violet (Sigma-Aldrich) and afterward were photographed. Five random fields were selected and counted manually under a microscope at a magnification of 200 \times (Nikon). Three independent experiments were performed for both cell lines.

Statistical Analysis

Results were presented as means \pm standard deviation (SD). The Student's *t*-test was used to evaluate the significance of intergroup differences. A value of $p < 0.05$ was considered as statistically significant.

RESULTS

lncRNA uc.338 Was Upregulated in Clinical Lung Cancer Patients and Cultured Cells

We first examined the expression of uc.338 in clinical lung cancer patients. Thus, a total of 100 patient samples were collected for RT-PCR analysis. As shown in Figure 1A, the relative transcript levels of uc.338 in cancerous tissues were significantly increased when compared with their adjacent noncancerous counterparts. H-125 and 95D are two cell lines of lung cancer, whereas NCI-H292 is derived from lung cancer patients with lymphatic metastasis; H1688 and NCI-H446 are two mature cell lines of small-cell lung cancer, while A549 and H1975 are isolated from patients with lung adenocarcinoma. Furthermore, MRC-5 is a normal lung epithelial cell line and included here as a control. It was shown that the transcript levels of uc.338 in all of the lung cancer cell lines were significantly higher than that of control cells (Fig. 1B). Of note, NCI-H292 and H1688 cell lines showed the highest expression of uc.338, up to 3.5-fold and 3.2-fold when compared with MRC-5. Thus, these two cell lines were selected for the subsequent analysis. Our data suggested that the relative expression of uc.338 was upregulated in human lung cancer in vivo and in vitro.

Knockdown of uc.338 Inhibited Cell Proliferative Rate in Lung Cancer Cell Lines

We next examined the detailed role of uc.338 in human lung cancer. To this end, two specific siRNAs (siuc.338-1 and siuc.338-2) were included here to elucidate the effects of uc.338 depletion on cell proliferation. When cells were transfected with siuc.338-1 or siuc.338-2, the relative expression of uc.338 was notably decreased in both cell lines, indicating the high transfection efficiency of the

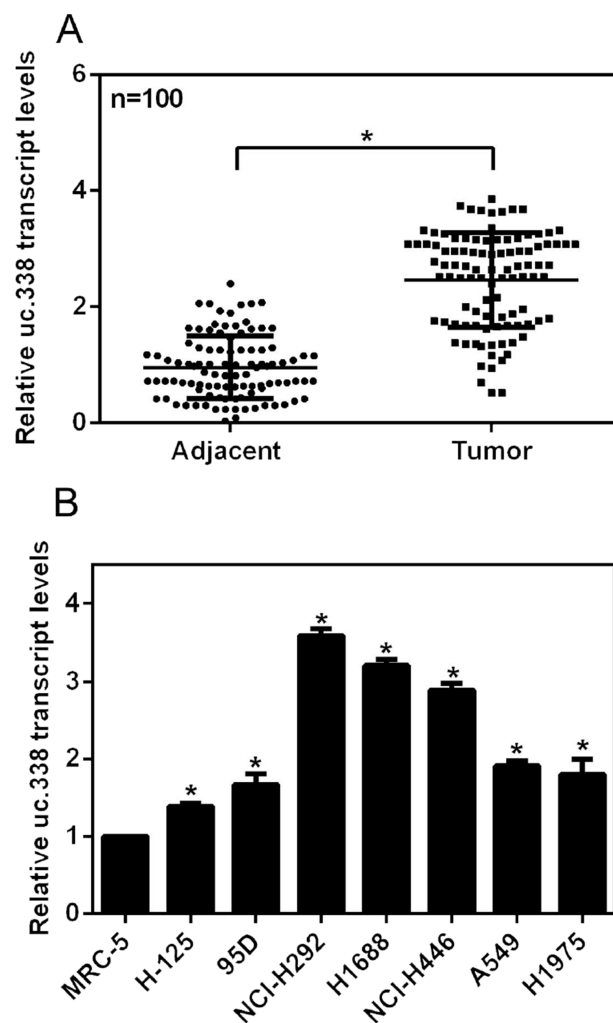


Figure 1. Long noncoding RNA uc.338 was upregulated in clinical lung cancer patients and cultured cells. (A) RT-PCR analysis revealed the relative transcript levels of uc.338 in tumor tissues and their adjacent noncancerous tissues from 100 patients who underwent surgeries. $*p < 0.05$, Tumor versus Adjacent. (B) Relative expression of uc.338 in normal lung cell MRC-5 and lung cancer cells H-125, 95D, NCI-H292, H1688, NCI-H446, A549, and H1975. $*p < 0.05$ versus MRC-5.

synthesized siRNAs (Fig. 2A). Cell proliferation assays were performed in the presence or absence of siuc.338. As shown in Figure 2B, knockdown of uc.338 in NCI-H929 cells caused no notable difference in the first 3 days; however, on the fourth day, the cell proliferative rate was decreased by 18% in siuc.338-1-treated cells and by 19.3% in siuc.338-2-transfected cells, respectively. The inhibitory effects of uc.338 depletion were further increased on the fifth day compared to the controls in NCI-H929 cells. A similar phenomenon was also observed in H1688 cells. Our data revealed that knockdown of uc.338 inhibited cell proliferation in human lung cancer cells.

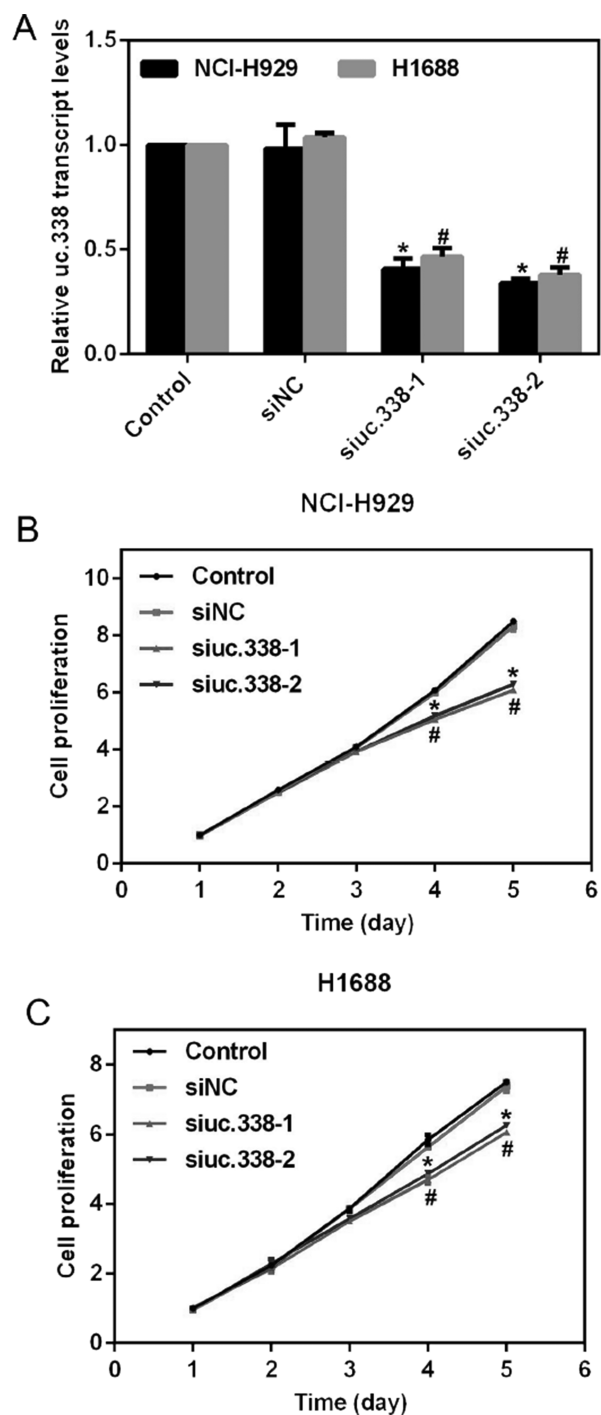


Figure 2. Knockdown of uc.338 inhibited cell proliferative rate in lung cancer cell lines. (A) Transfection of siuc.338-1 or siuc.338-2 in NCI-H929 and H1688 cells successfully decreased the expression of uc.338. siNC, scramble siRNA. * $p < 0.05$ versus Control in NCI-H929 cells. # $p < 0.05$ versus Control in H1688 cells. (B) Knockdown of uc.338 in NCI-H929 cells inhibited cell proliferation on the fourth and fifth day after transfection. (C) Knockdown of uc.338 in H1688 cells inhibited cell proliferation on the fourth and fifth day after transfection. * $p < 0.05$ versus Control in siuc.338-1-transfected cells. # $p < 0.05$ versus Control in siuc.338-2-transfected cells.

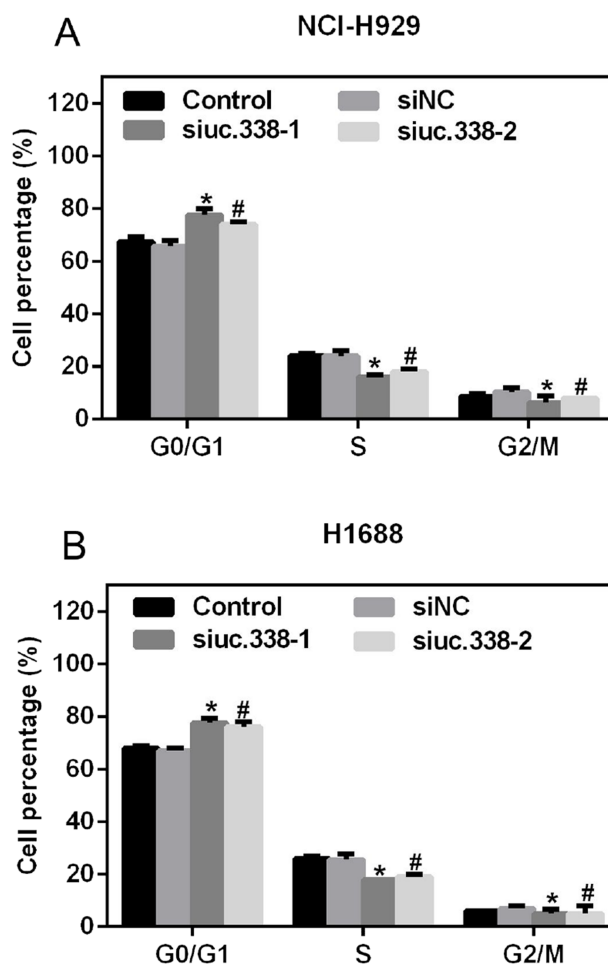


Figure 3. Depletion of uc.338 caused cell cycle arrest in the G₀/G₁ phases in NCI-H929 and H1688 cells. (A) Cell cycle was arrested in the G₀/G₁ phase when NCI-H929 cells were transfected with either siuc.338-1 or siuc.338-2. (B) Cell cycle was arrested in the G₀/G₁ phase when H1688 cells were transfected with either siuc.338-1 or siuc.338-2. * $p < 0.05$ versus Control in siuc.338-1-transfected cells. # $p < 0.05$ versus Control in siuc.338-2-transfected cells.

Depletion of uc.338 Caused Cell Cycle Arrest in G₀/G₁ Phases in NCI-H929 and H1688 Cells

We further examined the role of uc.338 in cell cycle of lung cancer cells. As shown in Figure 3A, transfection of siuc.338-1 in NCI-H929 cells caused cells in the G₀/G₁ phase to increase by 10%. Meanwhile, it decreased the percentage of cells in the S and G₂/M phase. Knockdown of uc.338 with siuc.338-2 also led the cell cycle to shift to the G₀/G₁ phase, although it was not as effective as siuc.338-1. Likewise, depletion of uc.338 in H1688 cells shifted the cell cycle from the S and G₂/M phase to the G₀/G₁ phase by approximately 10% (Fig. 3B). These results, together with Figure 2, indicated that knockdown of uc.338 in lung cancer cells caused cell cycle arrest in

the G₀/G₁ phase, implying that uc.338 promoted cell proliferation in NCI-H929 and H1688 cells.

Knockdown of uc.338 Inhibited Cell Migration and Invasion in Lung Cancer Cells

Cell metastasis is always associated with tumor progression and leads to disappointing outcomes for patients. Therefore, we also explored the role of uc.338 in cell metastasis in NCI-H929 and H1688 cells. As shown in Figure 4, scramble siRNA caused no difference in the ability for metastasis in both cell lines. For the migration assay, approximately 580 NCI-H929 cells and 500 H1688 cells migrated through the membrane, while only 160 (siuc.338-1-treated group) and 180 (siuc.338-2-treated group) NCI-H929 cells as well as 165 (siuc.338-1-treated group) and 160 (siuc.338-2-treated group) H1688 cells were observed on the lower surface of the membrane (Fig. 4A and B). Similar results were also observed in the cell invasion assays (Fig. 4C and D). All observations suggested that knockdown of uc.338 in lung cancer cells inhibited cell migration and invasion.

Knockdown of uc.338 Inhibited the Expression of Cell Cycle Regulators and Interrupted the Epithelial–Mesenchymal Transition (EMT) in Lung Cancer

On the basis of the above observations, key cell cycle regulators, including cyclin B1 and Cdc25C, as well as EMT markers such as Snail, vimentin, mesenchymal N-cadherin, and epithelial E-cadherin, were examined in NCI-H929 cells with siuc.338 transfection. As shown in Figure 5, when uc.338 was knocked down by either siuc.338-1 or siuc.338-2, the protein levels of cyclin B1, Cdc25C, Snail, vimentin, and N-cadherin were all decreased, whereas the expression of E-cadherin was notably increased after uc.338 depletion. These data were supportive of the above functional observations.

DISCUSSION

Although great efforts have been made to diagnose cancer in an early stage, lung cancer still remains the leading cause of cancer-related deaths worldwide (19). Lung cancer is considered to be a silent killer since the

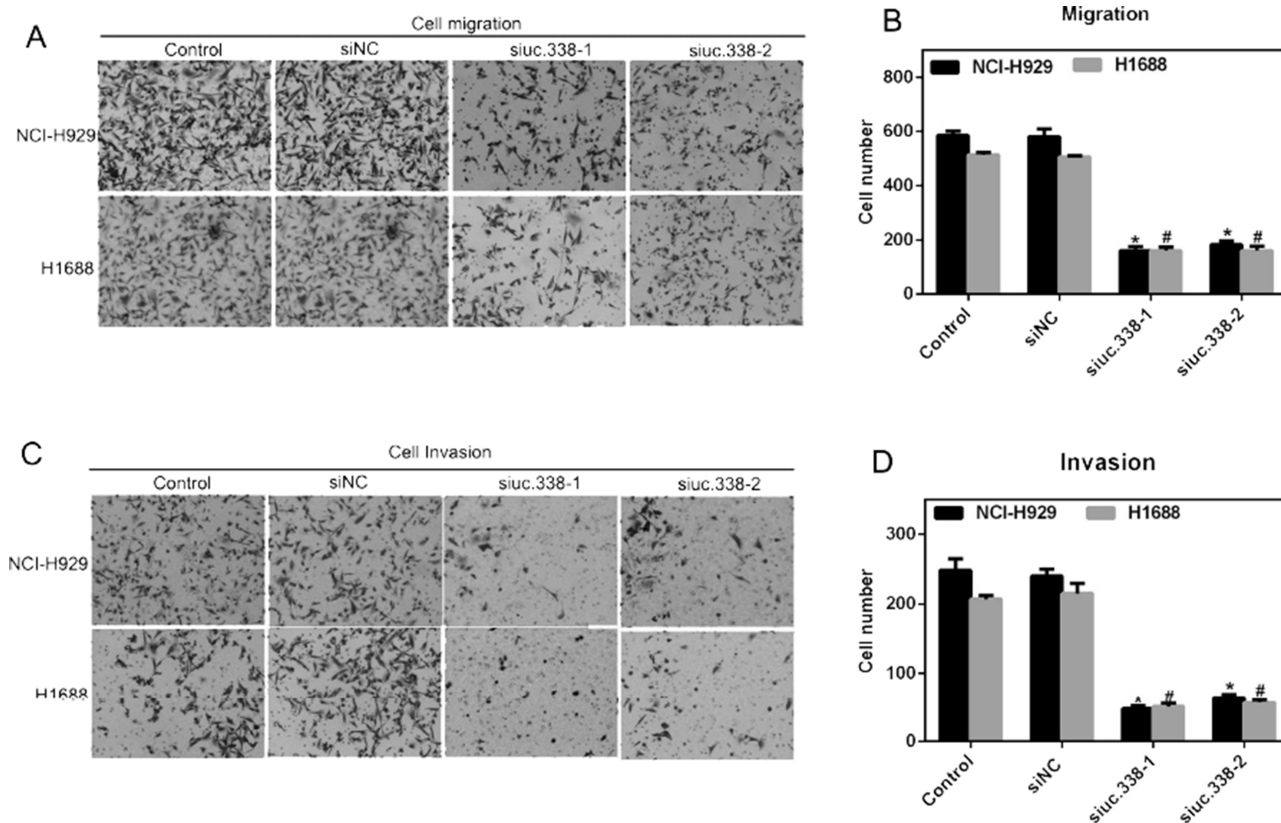


Figure 4. Knockdown of uc.338 inhibited cell migration and invasion in lung cancer cells. (A) Representative images of cell migration assays for both NCI-H929 and H1688 cells when siuc.338 was added to the cells. (B) Quantification of cell migration assay revealed that knockdown of uc.338 inhibited cell migration potential for both cell lines. (C) Representative images of cell invasion assays for both NCI-H929 and H1688 cells when siRNAs were transfected. (D) Quantification of cell invasion assay revealed that knockdown of uc.338 suppressed cell invasive abilities for both cell lines. **p* < 0.05 versus Control in siuc.338-1-transfected cells. #*p* < 0.05 versus Control in siuc.338-2-transfected cells.

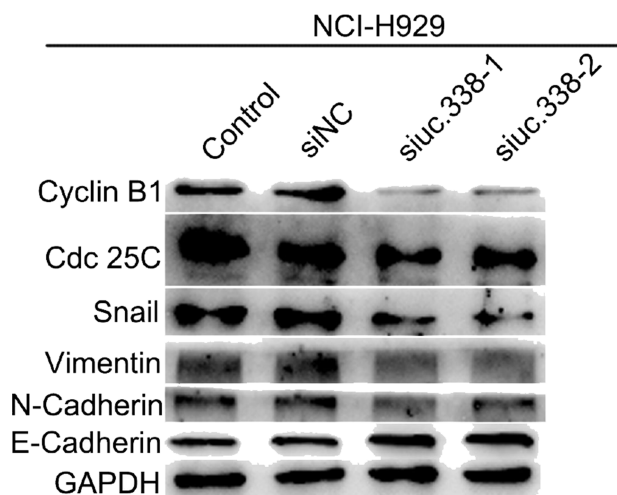


Figure 5. Knockdown of uc.338 inhibited the expression of cell cycle regulators and interrupted the epithelial–mesenchymal transition (EMT) in lung cancer. When NCI-H929 cells were transfected with siuc.338, the protein levels of cyclin B1, Cdc25C, Snail, vimentin, and N-cadherin were decreased, while the expression of E-cadherin was upregulated. GAPDH was included here as an internal control.

majority of lung cancer patients are asymptomatic until the tumor has metastasized at an advanced stage (20). Aggregative evidence has shown that the aberrant expressions of lncRNAs are tightly associated with the tumorigenesis of lung cancer (21–23). Our study indicated that upregulated uc.338 in lung cancer promoted cell proliferation and metastasis, thus accelerating the progression of the disease. Moreover, knockdown of uc.338 inhibited EMT process in lung cancer cell lines (Fig. 5).

EMT is a critical process during which epithelial cells lose cell–cell adhesion and polarity, and further gain their migrated and invasive potential to form mesenchymal stem cells, which is essential for the initiation of metastasis. Multiple regulatory molecules are involved in the process of EMT, such as the Zeb family and the Snail family (24). EMT is always manifested by various procedures, including increased expression of mesenchymal markers (vimentin and N-cadherin), decreased protein level of epithelial markers (E-cadherin), and altered location of specific transcription factors (Snail) (25,26). It was shown that EMT activation in lung cancer is associated with chemoresistance (27), the latter of which causes medicine to be ineffective and cancer recurrence and metastasis after traditional surgical treatment against lung cancer. Our results showed that uc.338 depletion in lung cancer cell line NCI-H929 inhibited EMT-related proteins. Thus, uc.338 might also work as a potential target for the diagnosis and treatment of lung cancer in clinics.

The limitation of this study was the lack of detailed mechanisms of how uc.338 promoted cell proliferation

and metastasis. We did not include an in vivo study in a mouse model either. In situ RNA hybridization could be used to examine the expression of uc.338 in the tumor section of mouse models. We will keep working on this project to finally elucidate this mystery.

In all, our results suggested that the transcript level of uc.338 was remarkably increased in lung cancer in vivo and in vitro. Knockdown of uc.338 in NCI-H929 or H1688 cells significantly inhibited cell proliferation by arresting cell cycle in the G₀/G₁ phase. Moreover, uc.338 depletion notably suppressed cell metastasis and interrupted the EMT process in lung cancer. On the basis of the above observations, uc.338 might serve as a clinical diagnostic and treatment agent for lung cancer.

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