Original Article
TUCR.454 inhabits metastasis in lung cancer cells

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Abstract: Transcribed ultraconserved regions (TUCRs) belong to long non-coding RNAs (lncRNAs) are transcripts longer than 200 base pair RNA with no protein-coding capacity. Previous studies showed that TUCRs serve as oncogenes or tumor suppressor genes are involved in tumorigenesis and cancer progressive. However, little is known about the expression level and biological role of TUCR.454 in lung carcinoma. Our data showed TUCR.454 is significantly down-regulated in NSCLC tissue and lung cancer cell lines, and the down-regulated TUCR.454 is associated with lymph node metastasis. Transfection with TUCR.454 markedly inhibited cell migration and invasion in A549 and H460 lung cancer cell lines. K-Ras was demonstrated to be negatively regulated by TUCR.454 at the posttranscriptional level by dual-luciferase reporter assay. Down-expression of K-Ras via siRNA inhibited NSCLC cell migration and down-regulates P63 and MMP9 in protein level, resembling that of overexpression of TUCR.454. In conclusion, these findings suggested that TUCR.454 acts as a novel tumor suppressor by targeting the K-Ras gene thus inhabiting lung cancer cell migration and invasion.

Keywords: Lung carcinoma, TUCR.454, K-Ras, P63, MMP9, metastasis

Introduction

Lung cancer (LC) is currently one of the most common malignancies with the highest incidence and mortality in the world. It ranks first in the global cancer because of numerous new cases and deaths each year [1]. Early detection of molecular markers is the most effective method for LC prevention; however the molecular mechanisms underlying LC progression are not well characterized.

Transcribed ultraconserved regions (TUCRs) belong to long non-coding RNAs (lncRNAs) are transcripts longer than 200 base pair RNA with no protein-coding capacity [2]. Previous studies showed that UCRs can regulate gene expression over long distances and associated with the presence of the tumor [3-5]. However, little is known about the expression level and biological role of TUCR.454 in lung carcinoma.

In the current study, the differential expression of TUCR.454 in human NSCLC samples and adjacent normal lung tissues was identifiedd using quantitative Real time polymerase chain reaction (qRT-PCR), and its role was also analyzed in migration and invasion of lung cancer cells. In addition, to understand the metastasis mechanism of lung cancer, the target of TUCR.454 was further investigated.

Materials and methods

NSCLC tissue collection

A total of 72 paired tissue specimens from NSCLC and matched normal lung tissue were used in this study and obtained from 72 NSCLC patients (age range, 34-74 years; 44 males and 28 females) at the Departments of Thoracic Surgery of the Affiliated Hospital of Yangzhou University (Yangzhou, China). The NSCLC diagnosis and the absence of tumor cells in the matched normal tissues were confirmed by two pathologists. All tissues were obtained during surgery and immediately stored in liquid nitrogen prior to use. Approval for this study was granted by the Institute Research Medical Ethics Committee of the Affiliated Hospital of Yangzhou University. Patients provided written informed consent.

Cell line culture

Lung cancer cell lines (A549 and H460) and normal Lung cell line (BEAC-2B) were purchased from the Chinese Peking Union Medical College Cell Bank (Beijing, China). All cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) (HyClone Victoria, Australia), 100 IU/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2.

Plasmids

The TUCR.454-murine stem cell virus (MSCV) plasmid and K-Ras-siRNA plasmid were chemically synthesized at Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China).

qRT-PCR analysis

Total RNAs were isolated from cells using TRIzol reagent (Invitrogen Life Technologies, SanDiego, CA, USA). One microgram of RNA was reverse-transcribed to cDNA using the PrimeScript First Strand cDNA synthesis kit (Takara Bio, Inc., Dalian, China) according to the instructions. qRT-PCR was performed on an Applied Biosystems 7500 Real Time PCR system (Applied Biosystems, White Plains, NY, USA). The U6 small RNA was used as internal controls. All the reactions were run in triplicate and the following primers were used: Forward, 5'-CTGCAATT-ACCTGTGGTG-3' and reverse, 5'-GTGAACTACC-TCGGTAGT-3' for TUCR.454; forward, 5'-CGCT-TCGGCAGCACATATAC-3' and reverse, 5'-TTCAC-GAATTTGCGTGTCAT-3' for U6; and forward, 5'-CGTAGGCAAGAGTGCCTT-3' and 5'-TGCACTGTA-CTCCTCTTG-3' for K-Ras mRNA.

Transfection of lung cancer cells

A549 and H460 cells were transfected with TUCR.454-MSCV plasmid, K-Ras-siRNA plasmid and control for 48 h before further experiments. The transfected cells were incubated at 37°C with 5% CO_2. The TUCR.454 and K-Ras RNA level in the transfected lung cancer cells were identified by qRT-PCR.

Dual luciferase reporter assay

The full length 3'-UTR of K-Ras was amplified by PCR from genomic DNA and cloned into the EcoRI and XhaI sites of pGL3-BS vector (Promega, WI, USA). The primers for K-Ras 3'-UTR were as follows: 5'-TTCCTGCTCCATGCAGACTGT-3' and 5'-GACTGGCACTGAAGATGGTGTA-3'. The mutant construct of K-Ras 3'UTR was generated using a Quick Change mutagenesis kit (Stratagene, Heidelberg, Germany). Co-transfection of reporter vectors and TUCR.454 mimics or negative control was performed using Lipofectamine 2000 (Invitrogen, San Diego, USA). After 48 h, dual luciferase activity was measured using a dual luciferase reporter assay system according to the instructions (Promega, WI, USA).

Transwell invasion assay

Cells were respectively harvested after trypsin digestion, and resuspended into serum-free medium, and inoculated into the upper chamber for transwell at 5×103 per well in 200 μL of serum-free medium, and 500 μl DMEM containing 10% FBS was added into the lower chamber. After 24 h incubation, the chambers were rinsed twice in PBS, and cells were fixed using 4% paraformaldehyde, and subsequently stained with 0.1% crystal violet. Gently wipe the upper chamber membrane with a cotton swab, the image shoot under microscope.

Transwell migration assay

After digested by pancreatic enzymes and washed in PBS twice, 5×10^4 cells were cultured in 1% FBS medium and then placed into Transwell chamber. 24 h later, cells were soaked in calcium free PBS twice, fixed and stained using methyl alcohol and with 0.1% crystal violet respectively. Cells in the upper chambers were gently wiped out by a cotton swab. Finally, we observed under microscope and then took pictures.

Western blot assay

Proteins were extracted by Cell lysis for western kit (Beyotime, Shanghai, China) according to the instructions. Protein concentration was quantified using Enhanced BCA protein assay kit (Beyotime). For western blot analysis, equal amounts of total protein were boiled, and separated by SDS-PAGE. After electrophoresis, protein was blotted onto a PVDF membrane and blocked for 2 h at room temperature. Membranes were incubated with first antibody (K-Ras, P63, MMP9) (Cell Signaling Technologies, Boston, MA) at 1:1000 dilutions for overnight at
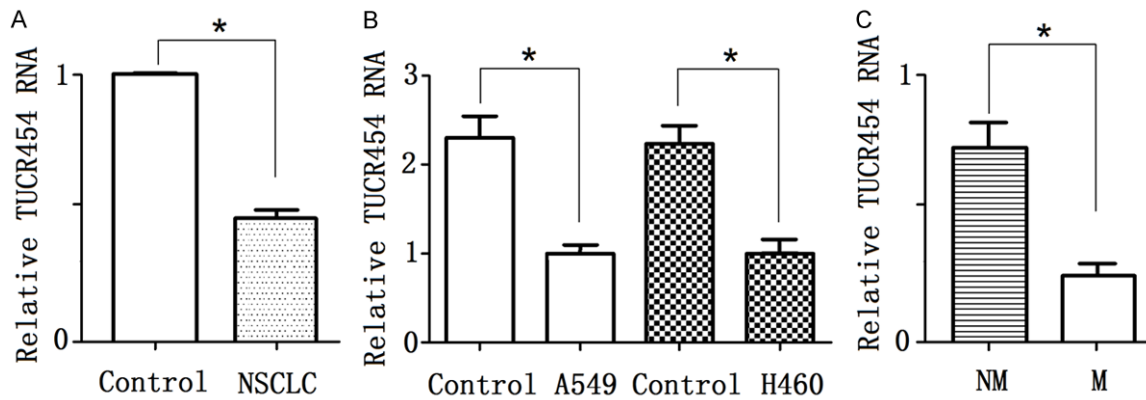
Figure 1. TUCR.454 is downregulated in NSCLC. A. The TUCR.454 relative expression levels were determined by qRT-PCR in 72 paired human NSCLC tissues and their corresponding normal samples and normalized against an endogenous U6 RNA control. Student's t test was used to analyze the significant differences between the tumor and normal tissues. B. The relative expression levels of TUCR.454 in the two NSCLC cell lines and human lung cell BEAC-2B. C. The relative expression levels of TUCR.454 in NSCLC with lymph-node metastasis (M; n = 37) and non-metastasis (NM; n = 35). Data are presented as mean ± SD. Two-tailed Student's t test was used to analyze the significant differences, *P < 0.05.
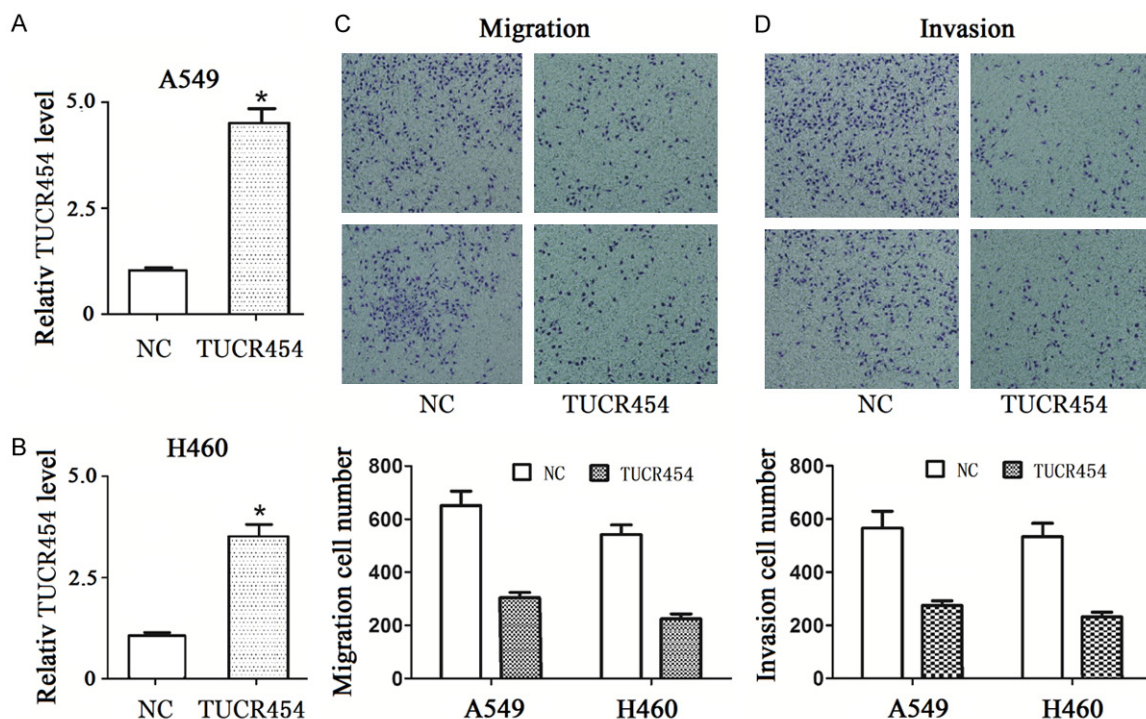

Figure 2. Overexpression of TUCR.454 inhibits migration and invasion of NSCLC cell lines. A, B. Transfection of TUCR.454-MSCV to A549 and H460 increases the expression of TUCR.454 detected by qRT-PCR. C, D. TUCR.454 inhibits invasion and migration in A549 and H460 cells. Data are presented as mean ± SD. Two-tailed Student's t test was used to analyze the significant differences, *P < 0.05.

4°C. K-Ras protein level was detected by horseradish peroxidase (HRP) conjugated mouse and rabbit secondary antibody (Beyotime) for 2 h at room temperature. Protein bands were detected on FluorChem FC2 Imaging System (Alpha Innotech, San Leandro, CA).

Statistical analysis

Statistical analyses were performed using SPSS 16.0 software package (SPSS, Chicago, USA). All data from three independent experiments were expressed as mean ± SD. Differen-

A

B

C
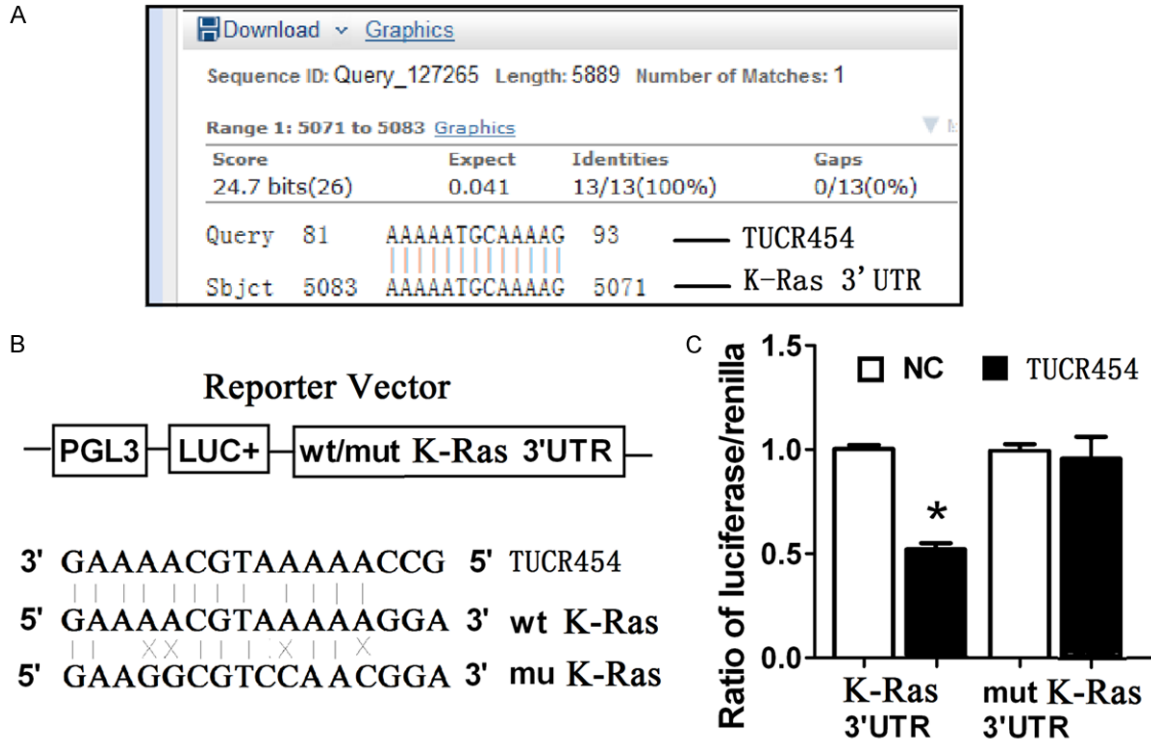
Figure 3. K-Ras 3'UTR is a target of TUCR.454. A. K-Ras was the target gene of TUCR.454 by bioinformatic prediction. B. Diagram of the luciferase reporter plasmids with the wild-type or mutant K-Ras 3'UTR. C. The relative luciferase activity in A549 cells was determined after the plasmid with wild-type or mutant K-Ras 3'UTR was co-transfected with TUCR.454. Data are presented as mean ± SD. Two-tailed Student's t test was used to analyze the significant differences, *P < 0.05.

ces were assessed by two-tailed Student's t test. The relationship between K-Ras and TUCR.454 expressions was tested with two-tailed Pearson's correlation. P < 0.05 was considered statistically significant.

Results

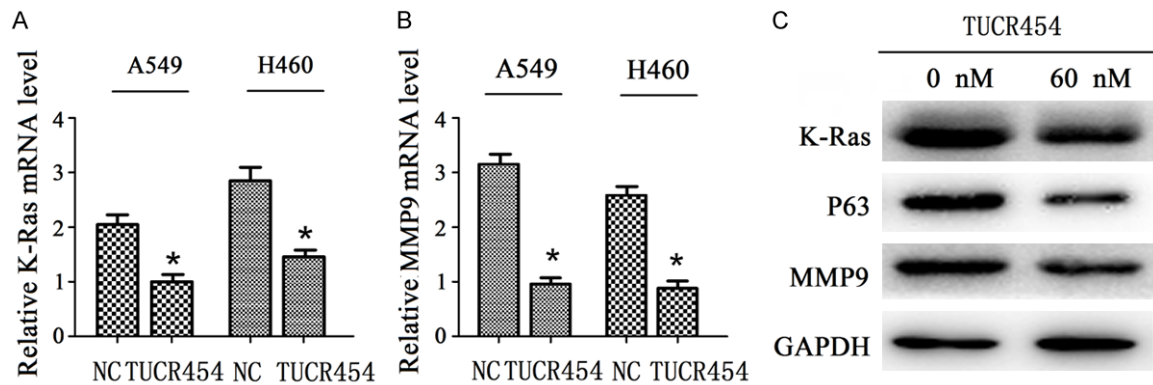*Expression of TUCR.454 is down-expression in NSCLC tissues and cell lines*

Since TUCR.454 was down-regulated in prostate cancer [6], we investigated whether TUCR.454 expression is altered in human NSCLC tissues. We compared the endogenous expression of TUCR.454 in human NSCLC tissues and adjacent normal lung tissues by qRT-PCR. As shown in **Figure 1A**, expression of TUCR.454 was decreased in 87.5% (63 out of 72) of NSCLC tissues compared with the corresponding adjacent normal lung tissues. Similarly, we found that expression of TUCR.454 was much lower in two lung cancer cell lines than that of normal human lung cell BEAS-2B (**Figure 1B**). We also found that TUCR.454 expression was

further significantly down-expression in NSCLC with lymph-node metastasis (n = 51) compared those without lymph-node metastasis (P < 0.05) (**Figure 1C**). Collectively, the above findings suggest that low-expression of TUCR.454 expression may play an important role in lung cancer metastasis.

Over expression of TUCR.454 inhibits NSCLC cell migration and invasion

Based on the above results, we checked whether TUCR.454 could suppress migration and invasion of lung cancer cells. We transfected A549 and H460 cells with TUCR.454 or negative control (NC), and then evaluated by cell invasion and migration assays. As expected, transfection of TUCR.454 increased TUCR.454 expression compared with negative control in A549 and H460 cells (**Figure 2A**, **2B**). Moreover, the cell migration and invasion assay showed that over expression of TUCR.454 resulted in reduced migration rate and invasion rate both in A549 and H460 cells compared with the control (**Figure 2C**, **2D**). Our results indicate that

Figure 4. TUCR.454 negatively regulates expression of K-Ras and its downstream gene expression. A, B. Expression of K-Ras and MMP9 was significantly lower at the mRNA level after transfection of TUCR.454-MSCV compared with control. C. Expression of K-Ras, P63 and MMP9 was significantly down-regulated at the protein level after transfection of TUCR.454-MSCV compared with control. Data are presented as mean ± SD. Two-tailed Student's t test was used to analyze the significant differences, *P < 0.05.
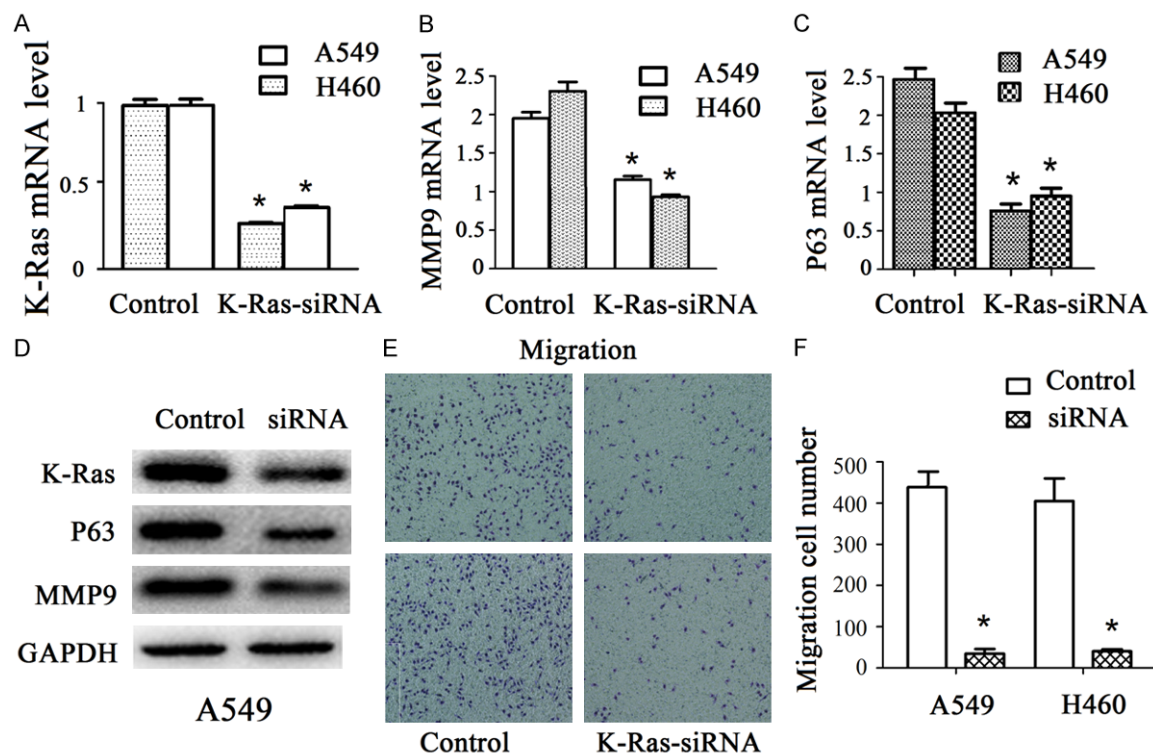
Figure 5. Knockdown of K-Ras reduces migration of NSCLC cells and upregulates MMP9. A-C. The mRNA levels of K-Ras, P63, and MMP9 were lower in A549 and H460 after K-Ras-siRNA transfection. D. The protein levels of K-Ras, P63, and MMP9 were also significantly down-regulated in A549 and H460 after K-Ras-siRNA transfection. E, F. Down-regulation of K-Ras strongly inhibited migration in NSCLC cells.

TUCR.454 worked as a tumor suppressor and contributed to inhibition of migration and invasion of lung cancer cells.

K-Ras is a direct target of TUCR.454

To investigate TUCR.454 signaling pathways regulating lung cancer migration, we tried iden-

tifying potential targets for TUCR.454 by prediction of bioinformatics (http://blast.ncbi.nlm.nih.gov, https://cancergenome.nih.gov/). We found TUCR.454 and K-Ras have complementary gene sequences in the posttranscriptional region, and then K-Ras gene is served as the putative target gene of TUCR.454. To verify whether TUCR.454 directly targeted K-Ras, Duel-

luciferase reporter assays were conducted. As shown in **Figure 3**, Co-transfection of A549 and H460 cells with K-Ras-3'UTR/pGL3-BS and TUCR.454 caused significant decrease in the luciferase activity compared with the negative control (P < 0.05). This repressive effect disappeared by point mutations in the binding sites of the K-Ras 3'-UTR. This result indicated that TUCR.454 exerts inhibitory effects on K-Ras expression via interaction with the 3'UTR of K-Ras.

TUCR.454 negatively regulates expression of K-Ras, P63 and MMP9

To further confirm that K-Ras is a target gene for TUCR.454 and K-Ras promoted P63 led to over-expression of MMP9 by previous data [7, 8], qRT-PCR and Western blot analysis were used to detect the expression of K-Ras, P63 and MMP9 regulated by TUCR.454 both in A549 and H460 cells. Our data show that expression of K-Ras, P63 and MMP9 was notably downregulated at the mRNA (**Figure 4A**, **4B**) and protein level (**Figure 4C**) after transfection of TUCR.454 compared with control. Taken together, our results suggest that TUCR.454 negatively regulates K-Ras gene expression at posttranscriptional level, and K-Ras is a potential target gene of TUCR.454, TUCR.454 also negatively regulates expression of P63 and MMP9.

Downregulation of K-Ras reduces migration potential of NSCLC cells

K-Ras was downregulated in NSCLC cells by transfection with K-Ras-siRNA plasmids (**Figure 5A**). Meanwhile, the mRNAs of P63 and MMP9, which are downstream genes of K-Ras, were significantly decreased in A549 and H460 after K-Ras-siRNA transfection (**Figure 5B**, **5C**). It was also confirmed that the expression of P63 and MMP9 was significantly downregulated at protein levels (**Figure 5D**) after K-Ras siRNA. Furthermore, downregulation of K-Ras strongly inhibited migration in NSCLC cells (**Figure 5E**, **5F**), which resembled the inhibitory effects of TUCR.454 overexpression. These data suggested that K-Ras siRNA could inhabit migration of NSCLC cells and positively promote gene expression of P63 and MMP9.

Discussion

Lung squamous cell carcinoma is a most common cancers and a leading cause of cancer-related death all over the world [9]. By far the biological mechanisms underlying NSCLC metastasis are not well characterized, which will provide diagnostic and prognostic markers and potential targets for the therapeutic intervention of NSCLC.

Ultraconserved regions (UCRs) are noncoding protein gene sequences that are strictly conserved among mice, rats, humans and other mammal. Transcribed UCRs could regulate expression and translation of other RNAs. Previous studies showed that TUCRs serve as oncogenes or tumor suppressor genes in development of tumor. Over-expression of uc.63 suppressed apoptosis of B lymphoma cells and might be predict poor prognosis [10]. Uc.73 modulates cell proliferation and apoptosis in colorectal cancer cell lines [11]. High expression of uc.8 was associated with poor prognosis in bladder cancer tissues [12], but silence of uc.8 inhibited the proliferation, invasion and migration of cancer cells [13]. Uc.338 was served as novel oncogene which increased expression of MMP9 to improve invasion and migration of cancer cells in colorectal carcinoma [14]. TUC.454 located in 20p12 is a member of the UCRs, it was low-expression in prostate cancer [6], but little is known about the expression level and biological role of TUCR.454 in NSCLC.

Our data showed that TUCR.454 was lower expression in NSCLC than in matched normal lung tissues. We also found that overexpression of TUCR.454 inhibited two NSCLC cell lines invasion and migration. These results suggest that TUCR.454 as a novel tumor suppressor plays a role in the infiltration and metastasis of lung carcinoma. Moreover, we found K-Ras is potential target of TUCR.454 by theoretical prediction.

The mutation status of the k-RAS gene in the tumor tissues involved in cell proliferation, migration, matrix remodeling, cell survival and matrix degradation and participates in the most of the physiological and pathological processes [15-18]. MMP9 is a member of MMPs family. It has been reported that MMP9 is overexpressed in lung and lung tumor and related to metastasis and invasion in breast, upper urinary tract, and oral squamous cell tumor [19-22].

In this study, we found that K-Ras was negatively regulated by TUCR.454 at the posttran-

scriptional level, via a specific target site within the 3'UTR, and TUCR.454 inhibited NSCLC cells migration and invasion through the K-Ras/P63/MMP9 pathway. These results suggest that that low-expressed TUCR.454 increases K-Ras thus participating in progression and metastasis of lung cancer. These data suggest that dysregulation of K-Ras signaling pathway by TUCR.454 is an important mechanism underlying lung cancer cell migration and invasion, the identification of TUCR.454 as an important regulator of tumor metastasis in vitro emphasizes an essential role of this UCR in mediating lung carcinoma invasion and migration.

In summary, our results demonstrate that TUCR.454 is down-regulated in lung carcinoma, the down-regulated TUCR.454 was significantly associated with lymph-node metastasis, and TUCR.454 played a suppressive role in lung cancer metastasis. Furthermore, overexpression of TUCR.454 inhibits invasion and migration in lung cancer cells, TUCR.454 directly inhibited K-Ras expression by targeting its 3'UTR. In addition, low-expression of TUCR.454 could result in overexpression of K-Ras, P63 and MMP9 in lung carcinoma. TUCR.454/K-Ras signaling pathway further our understanding of the molecular mechanisms involved in lung cancer metastasis, which may potentially serve as a therapeutic target for lung cancer.

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Disclosure of conflict of interest

None.

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