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Original Article

MiR-514a-3p inhibits cell proliferation and epithelial-mesenchymal transition by targeting EGFR in clear cell renal cell carcinoma

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Abstract: Purpose: Dysregulation of miR-514a-3p has been reported in multiple human malignancies. However, its biological function and molecular mechanisms in renal cell cancer (RCC) remain unclear. The aims of this study were to explore the role of miR-514a-3p and its potential mechanism in human RCC. Methods: The expression level of miR-514a-3p was quantified by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) in 20 cases of paired ccRCC and adjacent normal tissues and RCC cell lines. The role of miR-514a-3p in RCC cells was further evaluated by functional analyses. Western blot was applied to probe into the biological mechanism of miR-514a-3p in RCC cells. Results: The qRT-PCR results confirmed that miR-514a-3p was dramatically down-regulated in ccRCC specimens. Restoration of miR-514a-3p expression might distinctively suppress cell proliferation, viability, migration and invasion in comparison with negative control in RCC cells and negatively regulate the proteins related to epithelial-mesenchymal transition (EMT), such as E-Cadherin, N-Cadherin and Vimentin. Results of luciferase reporter assay and Western blot analysis identified that miR-514a-3p might inversely regulate the expression of epidermal growth factor receptor (EGFR) directly by binding to its 3'-untranslated region (3'-UTR) at the translational level. Further studies showed that the phenotypic changes of RCC cells caused by miR-514a-3p occurred through EGFR/MAPK/ERK pathway rather than PI3K/AKT signaling. Moreover, the inhibitory effect of miR-514a-3p was also confirmed in vivo study. Conclusions: MiR-514a-3p is a novel tumor suppressor in ccRCC and potentially functions through EGFR/MAPK/ERK pathway.

Keywords: miR-514a-3p, renal cell carcinoma, EGFR, proliferation, EMT

Introduction

Renal cell carcinoma (RCC) is the third most common urological cancer after prostate and bladder cancer, representing approximately 3% of all cancer in adults [1]. An estimated 65,150 new cases and 13,680 deaths from kidney and renal pelvis cancer in the United States, compared with 67,100 new cases and 24,100 deaths in China, occurred in 2013 with the reported incidence rate increasing by 2.5% annually [2-4]. Clear cell renal cell carcinoma (ccRCC) is the most common histological subtype of RCC, representing 75~80% of RCC, which is characteristic of great potential of local invasion and distant metastasis with resistance to radiotherapy and chemotherapy [5]. Despite increased early detection and more minimally invasive surgery, nearly 30% of RCC patients develop metastases at initial diagnosis and additional metastases occur in roughly one-third of patients following radical resection of primary tumor [6, 7]. Recent advances in ccRCC treatment, including immunotherapy and targeted therapy, have changed the landscape to a certain extent for locally advanced and metastatic RCC [8]. Unfortunately, the overall 5-year survival rate of RCC is reported to be about 55%, and that of metastatic RCC is nearly 10% [9]. Therefore, it is of utmost importance to develop a better understanding of the pathogenesis underlying the RCC, which can facilitate the development of more effective targeted therapeutic strategies and new diagnostic and prognostic biomarkers.

MicroRNAs (miRNAs) are an abundant class of endogenous, small, noncoding, single stranded RNA of 19~22 oligonucleotides in length, which may bind to 3'-untranslated region of target mRNAs via imperfect complementarily base pairing and induce mRNA cleavage or translational repression [10]. There is increasing evidence that miRNAs play a vital role in multiple physiological and pathological processes, including carcinogenesis. It is reported that miRNAs can regulate approximately 30% of all human genes, and a single miRNA can bind to one or more target mRNA while one mRNA also can be regulated by more than one miRNA [11]. Notably, miRNAs can act as potential oncogenes or tumor suppressor genes during tumor initiation, development and progression. Accumulating evidence indicates that miRNAs play roles in the pathogenesis of RCC [12-15]. MiR-514a-3p is a member of a cluster of miRNAs on human chromosome Xq27.3, which is observed to be involved in the pathogenesis of some tumors, including melanoma, testicular germ cell tumor, and ovarian cancer [16-18]. However, the biological role of miR-514a-3p in ccRCC has not yet been clearly elucidated.

EGFR is a membrane glycoprotein that belongs to the ErbB family of tyrosine kinase receptors, which plays a pivotal role in governing multiple cellular processes, including cell survival, proliferation, and migration [19, 20]. EGFR can activate several signaling pathways, mainly the MAPK/ERK and PI3K/AKT pathways [21]. EGFR-dependent signal overactivation usually accompanies tumor development and generally present with a more aggressive disease, including RCC [22]. Previously published study has demonstrated that miR-27a functions as a tumor suppressor in RCC by targeting EGFR [23]. However, the relationship of miR-514a-3p with EGFR implicated in RCC remains elusive and needs to be further explored.

The objectives of our study were to analyze the expression difference of miR-514a-3p in clinical pairing ccRCC specimens and different RCC cell lines, and investigated the effects of miR-514a-3p on the proliferation, migration and invasion in two renal cell lines. Then, preliminary exploration was conducted to analyze the mechanism underlying miR-514a-3p as a tumor suppressor in ccRCC targeting EGFR.

Materials and methods

Patient samples

A total of 20 primary ccRCC tissues and corresponding adjacent normal tissues were obtained from patients who underwent radical nephrectomy in the Department of Urology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology between March 2016 and September 2016. None of the patients had received radiotherapy or chemotherapy before surgery. Matched normal tissues were acquired at least 5 cm away from the primary tumor. After surgical resection, clinical specimens harvested from excised kidney were rinsed with saline solution before immersed in freezing tube supplemented with moderate RNAwait (Solarbio, Beijing, China) and stored at -80°C until use. Histopathological diagnoses of all specimens were validated by a pathologist. These samples were staged according to the American Joint Committee on Cancer (AJCC)-Union Internationale Contre le Cancer (UICC) tumour-node-metastasis (TNM) classification and histologically graded. Use of clinical sample cohorts in this study was authorized by all paricipants and was approved by the Ethical Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, and this study complied with the Declaration of Helsinki.

Cell cultures

Four RCC cell lines (786-O, ACHN, Caki-1 and Caki-2) and one immortalized normal renal proximal tubule epithelial cell (HK-2) were obtained from American Type Culture Collection (ATCC) and employed in this study. Among the all cell lines, 786-O, ACHN and HK-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; HyClone, Utah, USA), and Caki-1 and Caki-2 were cultured in McCoy's 5A (HyClone, Utah, USA), all supplemented with 10% fetal bovine serum (FBS; Gibco, NY, USA) and 1% penicillin–streptomycin (Solarbio, Beijing, China) within a humidified atmosphere containing 5% CO_2 at 37°C. All cell lines regularly passaged to maintain exponential growth.

Bioinformatics analysis and target prediction

The miRNA microarray analysis was undertaken at online small RNA-sequencing (smRNA-

seq) database (ngs.ym.edu.tw/ym500v2/index.php) supported by professor Wang's Lab, National Yang-Ming University in Taiwan. In the "Meta-Analysis Sample Comparison" module, researcher could select sample type in human ccRCC dataset for Group One and Group Two respectively, then sequentially manipulated subsequent processes according to the online tips, and the results of differential expressed miRNAs were obtained by E-mail. Then, the online target prediction algorithms such as miRDB (www.mirdb.org/miRDB), TargetScan (www.targetscan.org/vert_71), and miRNA Target Visualization (cm.jefferson.edu/rna22/Precomputed) were exploited to predict a most promising mRNA which might be a direct target of screened out miRNA.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from RCC tissues and cells using TRIzol reagent (Invitrogen, CA, USA). The concentration and purity of RNA were measured by ultraviolet absorbance at 260 nm and 280 nm. Complementary DNA of mRNA and miRNA were synthesized using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Biomedical Technology, Dalian, China) and the SYBR® PrimeScript™ miRNA RT-PCR Kit (Takara Biomedical Technology, Dalian, China) respectively according to the manufacturer's instructions. Quantitative real-time PCR was performed using SYBR® Premix Ex Taq™ (Tli RNaseH Plus) kit (Takara Biomedical Technology, Dalian, China) and ABI ViiA7 QPCR System (Applied Biosystems, CA, USA). GAPDH and U6 snRNA were used as internal controls of EGFR mRNA and miR-514a-3p, respectively. The $2^{-\Delta\Delta Ct}$ method was used to assess the relative expression of mRNA or miRNA. All primers were purchased from Sangon Biotech Co., Ltd. (Shanghai, China) and the sequences were: EGFR forward: 5'-GAGCCTCTGGATGGTGCAAT-3', reverse: 5'-GAGCCTCTGGATGGTGCAAT-3'; GAPDH forward: 5'-AGAAGGCTGGGGCTCATTTG-3', reverse: 5'-AGGGGCCATCCACAGTCTTC-3'. All experiments were repeated three times.

Oligonucleotides synthesis and transient transfection

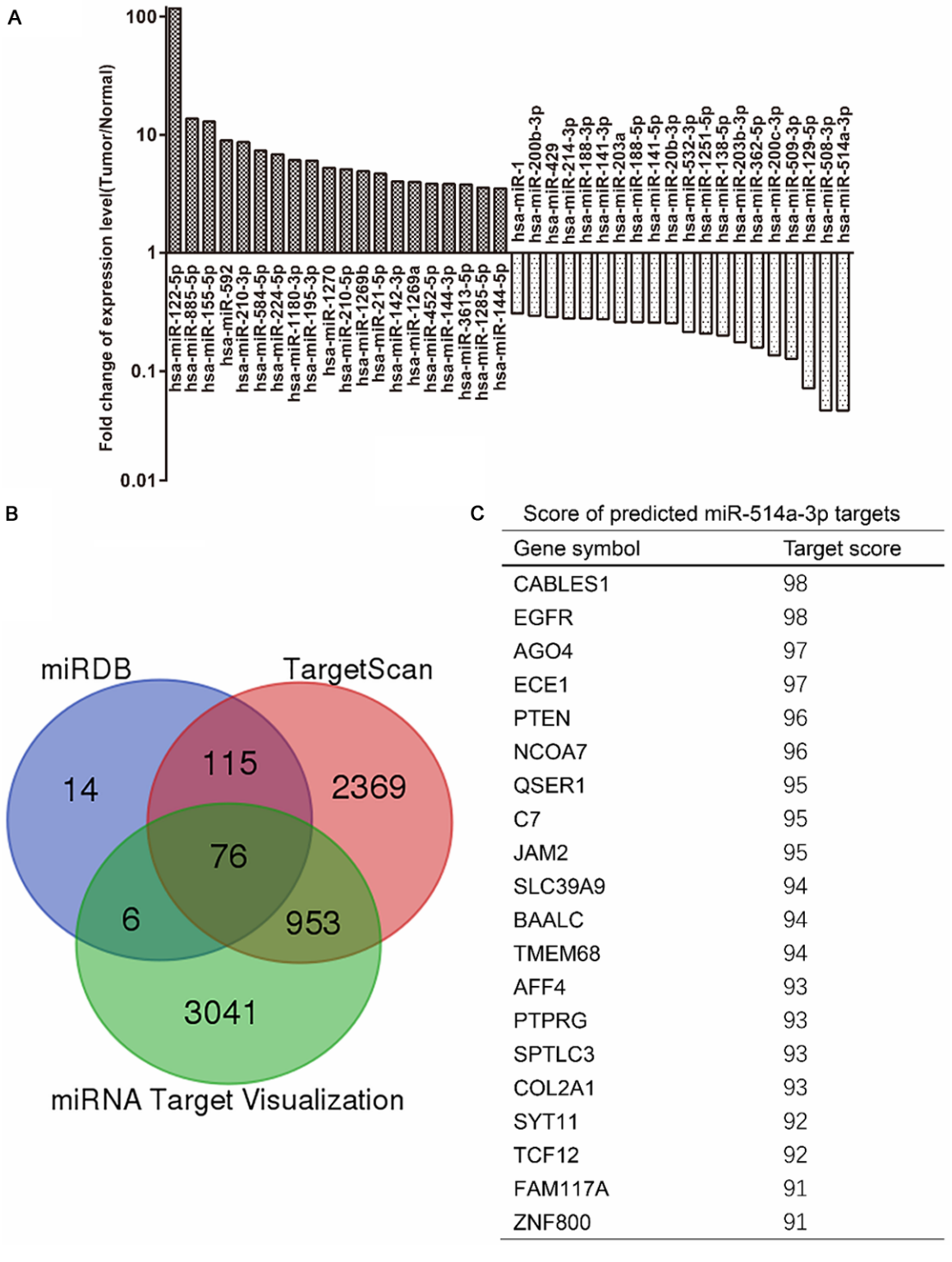
Six short, single or double stranded RNA oligonucleotides such as miR-514a-3p mimic, mimic negative control (mimic NC), miR-514a-3p inhibitor, inhibitor negative control (inhibitor NC), miR-514a-3p agomir and agomir negative control (agomir NC) were designed and synthesized by RiboBio Co., Ltd. (Guangzhou, China). Cells, ACHN and 786-O, were seeded in six-well plates at 70%~80% confluence 24 hours before transfection. MiR-514a-3p mimic and miR-514a-3p inhibitor were transfected at a final concentration of 50 nM and 100 nM respectively using Lipofectamine 2000 reagent (Invitrogen, CA, USA) in accordance with manufacturer's protocols. The cells were harvested for further assays 48 hours after transfection.

Plasmid construction and luciferase reporter assay

The 3'-UTR of the EGFR gene was amplified from human genomic DNA using PCR, and cloned into the XhoI and NotI site of the pmiR-RB-REPORT™ Luciferase reporter vector (RiboBio, Guangzhou, China). A mutation of seed region in the 3'-UTR of EGFR mRNA was generated using site-directed mutagenesis by the megaprimer PCR method. Similarly, the mutated PCR fragment was also inserted into the pmiR-RB-REPORT™ Luciferase reporter vector at the same XhoI/NotI site of wild type EGFR. All cloned products were confirmed by final sequencing. The reporter vector contains hRluc encoding Renilla luciferase as the reporter and hluc encoding firefly luciferase as the internal control. For Luciferase reporter assay, ACHN and 786-O cells were co-transfected with wild or mutant type reporter plasmid and miR-514a-3p mimic/inhibitor or corresponding negative control using Lipofectamine 2000 (Invitrogen, CA, USA), and Luciferase activity was measured 24 hours post-transfection using the Dual-Luciferase Reporter Assay system (Promega, USA) according to manufacturer's suggestion. Every experiment was carried out in triplicate.

Cell viability and colony formation assays

To assess cell viability, 786-O and ACHN cells were seeded at 96-well plates lasting for 24 hours and transfected with miR-506 mimic or miR-506 inhibitor or corresponding negative control. Then, the assessment of cell viability was implemented at 0, 24, 48, 72 and 96 h after transfection by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) method



C Score of predicted miR-514a-3p targets

Gene symbol	Target score
CABLES1	98
EGFR	98
AGO4	97
ECE1	97
PTEN	96
NCOA7	96
QSER1	95
C7	95
JAM2	95
SLC39A9	94
BAALC	94
TMEM68	94
AFF4	93
PTPRG	93
SPTLC3	93
COL2A1	93
SYT11	92
TCF12	92
FAM117A	91
ZNF800	91

Figure 1. Identification of differentiated expressed miRNAs and putative miR-514a-3p target genes in ccRCC. A. Differentiated expressed miRNAs panel with fold change more than three in primary ccRCC tissues compared with corresponding normal renal tissues ($P < 0.05$). B. Common targets of three online databases such as miRDB, TargetScan and miRNA Target Visualization were filtered out by getting intersection elements, as demonstrated in Venn Diagram. C. Top 20 genes of predicted miR-514a-3p targets score were listed in table.

(Sigma, USA) according to the manufacturer's protocol. The absorbance of samples was measured at a wavelength of 492 nm with a microplate reader (BioTek, USA). For colony formation

assay, exponentially growing cells post-transfection were plated at nearly 1×10^3 cells per well in 6-well plates and incubated for 10 days. Then, the number of colonies was counted using an inverted microscope (Olympus, Japan) after staining cells with 0.1% crystal violet solution (servicebio, Wuhan, China) for 20 min. All experiments in this section were conducted three times.

Cell migration and invasion assays

Cell migration and invasion capacity were evaluated using 24-well transwell plate with 8-μm pore polycarbonate membrane inserts, according to the manufacturer's instruction (Corning, New York, USA). For migration assay, 4×10^4 cells transfected with miR-514a-3p mimic or inhibitor or corresponding negative control in 200 μl serum-free media were plated in the upper chambers. For invasion assay, the upper chamber was pre-coated with Matrigel (BD Biosciences, Bedford, MA) diluted with serum-free DMEM medium (1:4) and 4×10^4 successfully transfected cells in 200 μl serum-free medium were seeded in it. The lower chamber was filled with 600 μl DMEM complete medium containing 10% FBS. After incubation for 24 h, cells were fixed with 1 ml 100% methanol for 15 min, followed by staining with 0.1% crystal violet (servicebio, Wuhan, China) for 30 min. Invaded or migrated cells were photographed and quantified in five random fields operating an inverted microscope (Olympus, Japan). Every experiment was performed independently three times.

Protein extraction and Western blot analysis

Total cellular proteins were extracted using radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Shanghai, China) containing a cocktail of protease inhibitors and phosphatase inhibitors (Beyotime Bio technology, Shanghai, China) and the concentration of various protein samples was quantified using the BCA Protein Assay kit (Beyotime Biotechnology, Shanghai, China). Equal quantities of protein were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA). The monoclonal EGFR (#4405), E-Cadherin (#3195), N-Cadherin (#13116), Vimentin (#5741), AKT (#4685), phosphorylated (p)-AKT (Ser473) (#4058), ERK1/2 (#4695) and p-ERK1/2 (Thr202/Tyr204) (#4370) antibodies were purchased from Cell Signaling Technology, Inc. (MA, USA). Rabbit polyclonal GAPDH antibody (ab9485; Abcam, MA, USA) and rat monoclonal β-actin antibody (BM0627, Boster, Wuhan, China) served as loading control respectively. Goat anti-rabbit (BA1054) and anti-mouse IgG (BA1051) secondary antibodies were supplied by Boster Bioengineering Limited Company (Wuhan, China). The protein signals were detected by the enhanced chemiluminescence (ECL) kit (Beyotime Biotechnology, Shanghai, China) exposing to the autoradiography films (X-OMAT BT; Eastman Kodak, NY, USA) and blots were analyzed with the ImageJ software. All experiments were conducted three times.

Immunohistochemistry (IHC)

Paraffin-embedded specimens which had been previously confirmed for the pathological type by a pathologist were serially sectioned at 4 μm thicknesses. IHC staining of ccRCC tissue and matched normal renal tissue sections were performed according to standard procedure. The primary monoclonal rabbit antibody against human EGFR (GB11084; servicebio, Wuhan, China) was applied at a dilution of 1:200 and goat anti-rabbit HRP-labeled antibody (BA1054; Boster Bio, Wuhan, China) was served as the secondary antibody. The visualization and scan of slides were completed by XSP-C204 microscope (COIC industrial, Chongqing, China) and Panoramic MIDI II scanning system (3D HISTECH Ltd., Budapest, Hungary), respectively.

Xenograft subcutaneously implantations

Total 12 BALB/c athymic nude mice (male, between 4 and 5 weeks old) in the present study were kept in specific pathogen free (SPF) condition. All animal experiments were carried out in accordance with the guidelines for the care and use of laboratory animals and institutional ethical guidelines for animal experiments of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, China. To establish RCC xenograft model, 5×10^6 prepared ACHN cells were suspended in 100 μl sterilized saline and inoculated subcutaneously into the right flank of nude mice. Tumor formation at the site of injection was validated

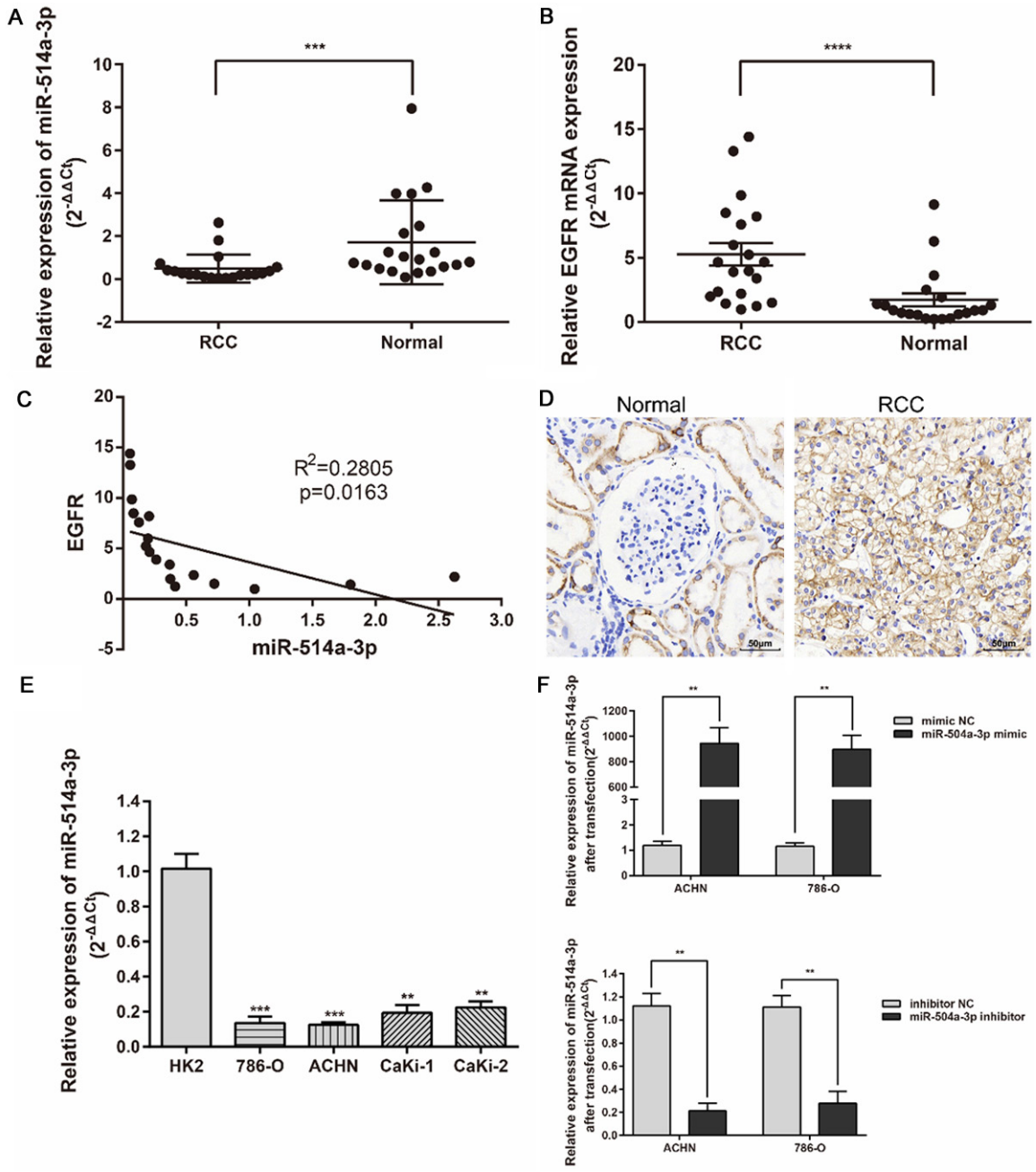


Figure 2. Expression of miR-514a-3p in clinical samples and various cell lines and its relationship with EGFR expression. A. MiR-514a-3p expression levels were significantly down-regulated in ccRCC tissues compared with corresponding normal tissues. B. Upregulation of EGFR mRNA in ccRCC tissues compared with their normal counterpart. C. Negative correlation of EGFR mRNA levels and miR-514a-3p levels in matched ccRCC tissues (R^2 = 0.2805, p = 0.0163). D. Representative images of EGFR IHC in ccRCC tissues and their paired normal tissues. E. MiR-514a-3p expressions in various RCC cell lines compared with that in HK2. F. MiR-514a-3p expressions after mimic/inhibitor transfection were detected by qPCR. Data were presented as the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

with approximate 5×10 mm^3 on day 14. These mice were randomly divided into two groups (n=6 each) and intratumorally injected with 1 nmol (in 20 μl phosphate-buffered saline) miR-514a-3p agomir (group agomir) or agomir NC (group agomir NC) per mouse at the site of tumor by the same trained experimenter. The administrations were replicated by every other

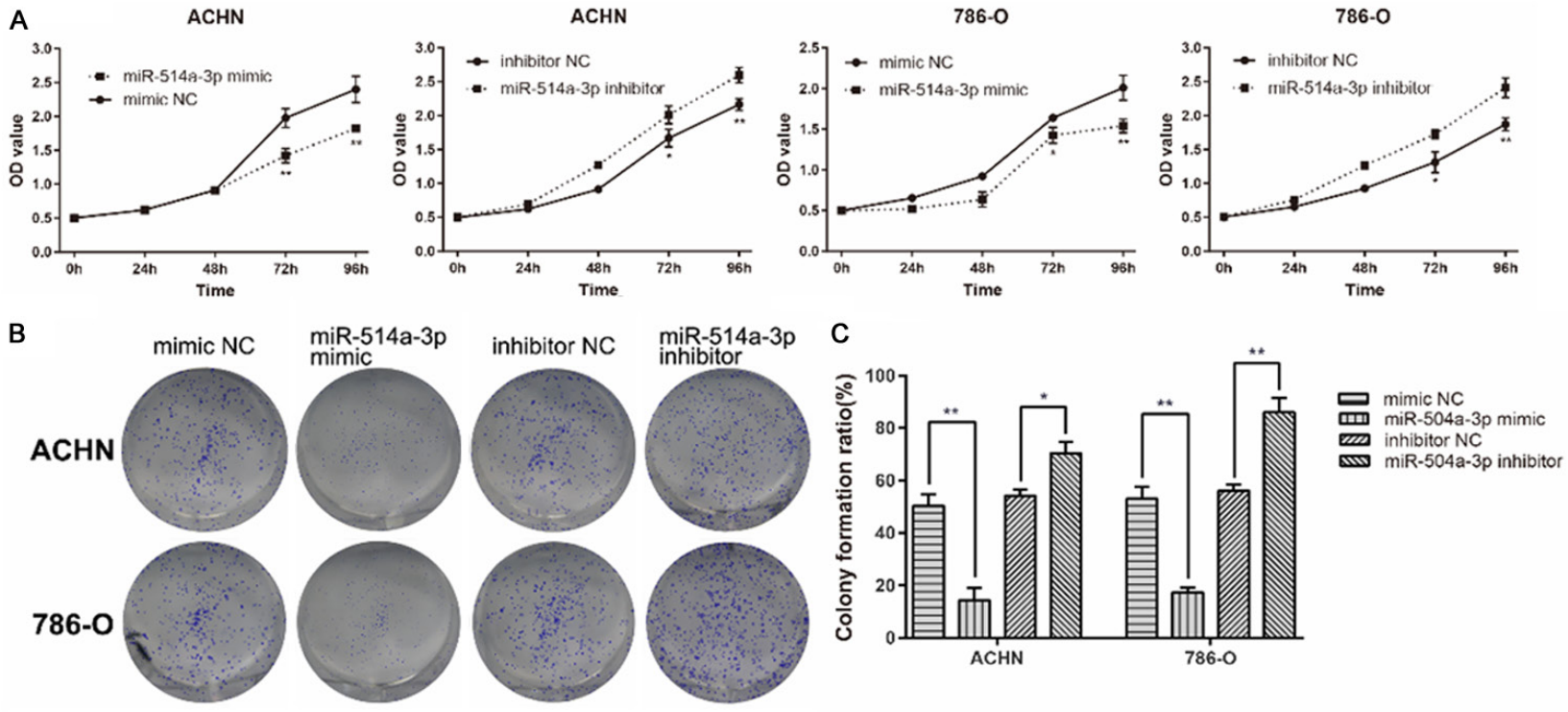


Figure 3. MiR-514a-3p attenuates the viability and colony formation in RCC cells. (A) MTS assay revealed the growth curves of indicated cells at different time intervals. (B, C) Representative micrographs (B) and relative colony formation ratio (C) of crystal violet-stained cell colonies examined by colony formation assay. Results were plotted as the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01.

3 days for seven consecutive times. Meanwhile, the tumor volume (V) was calculated by measuring the larger axe (L) and smaller axe (S) with calipers every 4 days by two trained laboratory staffs at different times, $V= (L \times S^2)/2$. Mice were executed by cervical dislocation after anaesthetized with 10% chloral hydrate in day 42, and the tumors were excised and weighed.

Statistical analysis

Student's t-test or Pearson correlation was performed by SPSS software version 19.0 to compare difference or correlation between groups and the results are described as mean ± standard deviation (SD) of three independent experiments. Two-sided $P < 0.05$ was regarded as statistically significant.

Results

Screening for the new tumor-suppressing miRNA in ccRCC

To find novel tumor inhibiting miRNA in ccRCC, we performed an online meta-analysis of miRNA expression profiles at [http://ngs.ym.edu.tw/ym500v2/index.php] and identified the differentiated expression miRNAs panel including top 20 significantly down-regulated and top 20 significantly upregulated miRNAs with fold change more than three (**Figure 1A**). Compared with 71 normal renal tissues, miR-514a-3p was the most significant down-expression in 496 primary ccRCC tissues. Then, a verifying experiment was fulfilled by collecting three pairs of ccRCC specimens to confirm the down-regulated miR-514a-3p. Built on the intersection results of miRNA target prediction of three afore-mentioned online websites, the Venn Diagram was outlined and 76 common target mRNAs were selected (**Figure 1B**). Among the top 20 targets, EGFR gets the highest target score in miRDB database and is the most-studied molecule (**Figure 1C**). Finally, miR-514a-3p and EGFR were chosen to be investigated in our study.

Distinguished miR-514a-3p and EGFR expressions in matched tumor and normal tissues of ccRCC

To explore the roles of miR-514a-3p and EGFR in ccRCC, miR-514a-3p and EGFR expressions were examined by qRT-PCR in several specimens (tumors and their normal counterpart). **Figure 2A**, **2B** reveal that miR-514a-3p was significantly down-regulated ($P < 0.001$) and EGFR remarkably upregulated ($P < 0.0001$) in ccRCC tumors compared with their corresponding normal tissues. To understand the relation between miR-514a-3p and EGFR at the RNA level, the Pearson correlation was performed and the analysis result showed that miR-514a-3p expression inversely related to EGFR expression ($R^2 = 0.2805$, $P < 0.05$) (**Figure 2C**). In order to examine further the expression of EGFR protein in ccRCC, the IHC assay was carried out and the result showed that the percentage of EGFR positivity in ccRCC tissues was markedly higher than matched normal tissues (**Figure 2D**).

MiR-514a-3p down-regulated in RCC cell lines

To investigated the role of miR-514a-3p in RCC cell lines, we evaluated miR-514a-3p expression in the four RCC cell lines such as 786-0, ACHN, Caki-1 and Caki-2, and the non-tumorigenic renal cell line HK-2 by qRT-PCR. Compared with HK-2 cell, all four RCC cell lines showed significantly lower miR-514a-3p expression ($P < 0.01$) and the ACHN and 786-0 cells were the lowest expression among the four RCC cell lines ($P < 0.001$) (**Figure 2E**). What's more, miR-514a-3p expression was greatly increased or decreased respectively after miR-514a-3p mimic or inhibitor transfection ($P < 0.01$) (**Figure 2F**).

MiR-514a-3p inhibits viability and cloning potential of RCC cells in vitro

To investigate the effect of miR-514a-3p on phenotypic changes in RCC cell lines, the overexpression and knockdown experiments were performed in two RCC cell lines, 786-0 and ACHN, and were validated by qRT-PCR (**Figure 2F**). The cell viability and cloning potential were measured by MTS and colony formation assay, respectively. As shown in **Figure 3A-C**, the proliferation rate and colony formation ratio of both RCC cell lines transfected with miR-514a-3p mimic were dramatically reduced in comparison with cells transfected with mimic NC and vice versa ($P < 0.05$).

MiR-514a-3p inhibited EMT of RCC cells

Metastasis is one of the most hazardous properties of most cancers, and EMT is the pre-req-

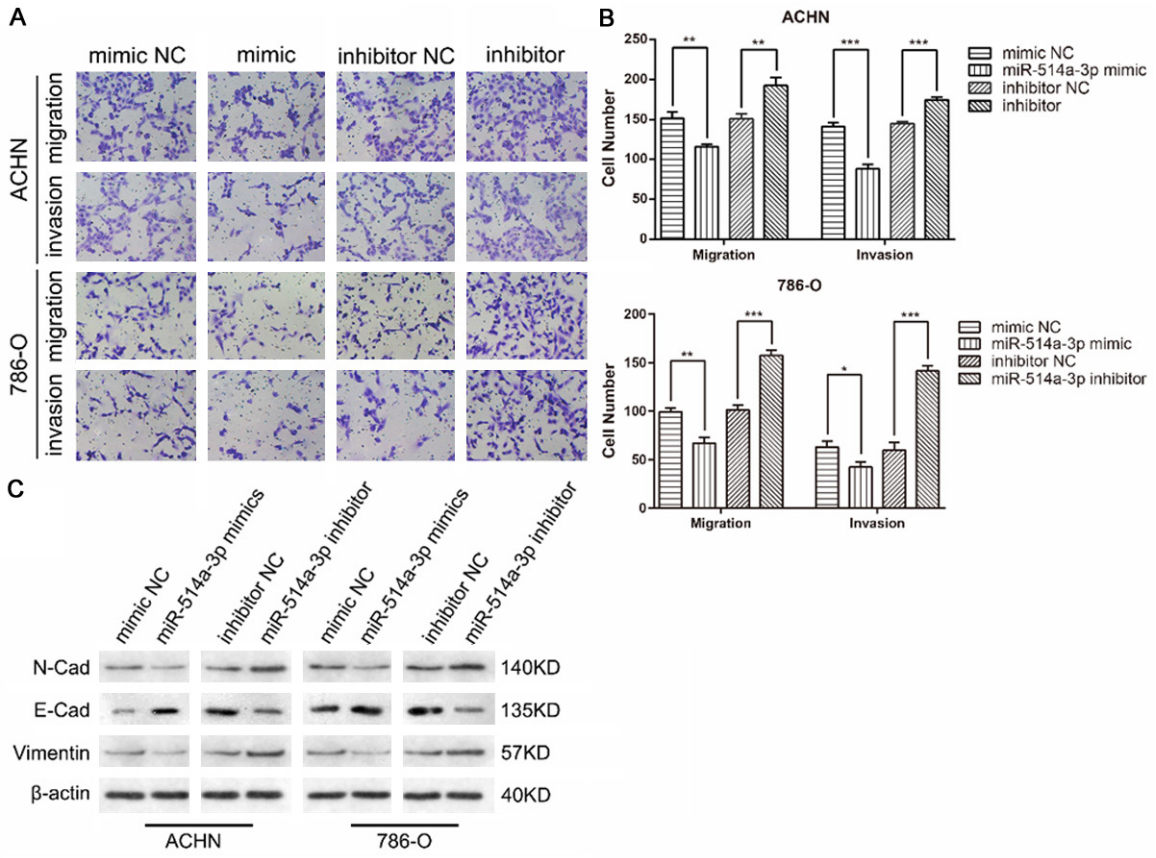
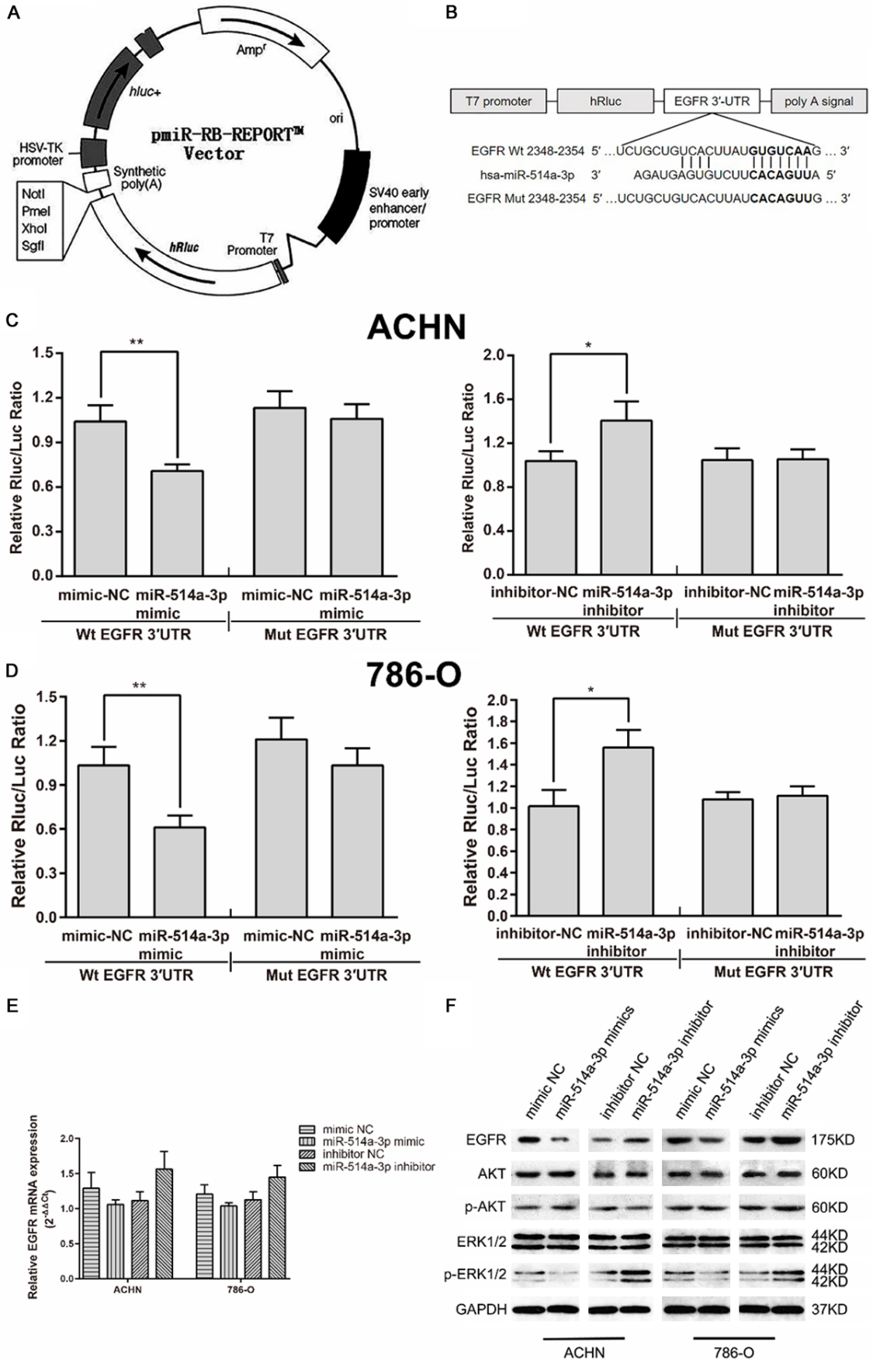


Figure 4. MiR-514a-3p inhibits epithelial-mesenchymal transition (EMT) in RCC cells. A. Representative photographs of transwell migration and invasion assays were taken at ×200 magnification and the number of migrated or invaded cells was quantified in five random images from each treatment group. B. Migrating cells number after transfecting with miR-514a-3p mimic or inhibitor relative to mimic NC or inhibitor NC. Results were presented as the mean ± SD from three independent experiments. C. Western blot analysis was used to detect the changes in EMT markers in ACHN and 786-O cells treated with miR-514a-3p mimic/inhibitor or corresponding NC. *P < 0.05, **P < 0.01, ***P < 0.001.

uisite of metastasis. To further investigated whether miR-514a-3p was involved in EMT of RCC cells, the transwell migration and invasion assays were completed and revealed that miR-514a-3p mimic markedly inhibited the migration and invasion in ACHN and 786-O cells ($P < 0.05$) (**Figure 4A** and **4B**). In contrast, silence of miR-514a-3p with miR-514a-3p specific inhibitor led to a significant increase in RCC cell migration and invasion abilities ($P < 0.01$) (**Figure 4A** and **4B**). Then, we examined the effect of miR-514a-3p on the EMT relative markers' expression at the protein level. The Western's results revealed that ectopic expression of miR-514a-3p triggered alterations in EMT markers' expression with a gain in E-cadherin expression and loss of N-Cadherin and Vimentin, while the down-regulation of miR-514a-3p decreased the E-cadherin's expression and increased the expressions of N-Cadherin and Vimentin (**Figure 4C**). Put together, these results indicate that miR-514a-3p may contribute to inhibition of EMT in RCC cells and play an important role in RCC metastasis.

MiR-514a-3p negatively regulated EGFR gene expression by directly targeting its 3'-UTR

To validate whether EGFR was a direct target of miR-514a-3p, luciferase reporter assay was done in RCC cells. The seed region sequence of containing wild type 3'-UTR (Wt 3'-UTR) or mutant type (Mut 3'-UTR) of EGFR was cloned into luciferase reporter vector (**Figure 5A** and **5B**). These constructed reporter vectors were co-transfected with miR-514a-3p mimic/inhibitor or corresponding negative control into ACHN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Synthetic poly(A)
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SgfI
hRluc
T7 Promoter
SV40 early enhancer/ promoter
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T7 promoter
hRluc
EGFR 3′-UTR
poly A signal
EGFR Wt 2348-2354 5′ …UCUGCUGUCACUUAUGUGUCAAG … 3′
hsa-miR-514a-3p 3′ AGAUGAGUGUCUUCACAGUUA 5′
EGFR Mut 2348-2354 5′ …UCUGCUGUCACUUAUCACAGUUG … 3′
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inhibitor-NC
miR-514a-3p inhibitor
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E
Relative EGFR mRNA expression
mimic NC
miR-514a-3p mimic
inhibitor NC
miR-514a-3p inhibitor
ACHN
786-O
F
mimic NC
miR-514a-3p mimics
inhibitor NC
miR-514a-3p inhibitor
EGFR
AKT
p-AKT
ERK1/2
p-ERK1/2
GAPDH
175KD
60KD
44KD
42KD
37KD

Figure 5. EGFR mRNA is a target of miR-514a-3p at specific 3'-UTR site and miR-514a-3p suppresses the malignant behavior of RCC cells through EGFR/MAPK/ERK pathway. (A) Image of pmiR-RB-REPORT™ dual-luciferase reporter vector provided by RiboBio Co., Ltd. (Guangzhou, China). (B) The binding site for miR-514a-3p in the EGFR 3'-UTR and several mutated nucleotides within the binding site are shown. The Wt and Mut 3'-UTR sequence were cloned into the vector. (C, D) Relative luciferase activity of reporter vector carrying Wt or Mut EGFR 3'-UTR in ACHN (C) and 786-O (D) cells co-transfected with miR-514a-3p mimic/inhibitor or corresponding NC. (E) EGFR mRNA expression didn't differ significantly between groups after transfection. (F) Western blot analysis of the MAPK/ERK and PI3K/AKT pathways. p-ERK was discovered to decrease with forced expression of miR-514a-3p and a low expression of EGFR, and the situation was inverted by suppression of miR-514a-3p and the restoration of EGFR expression, but no changes were found in the expression of p-Akt. All assays were performed in triplicate and the results were plotted as mean ± SD. *P < 0.05, **P < 0.01.

and 786-O cell lines. In both two cells, the results showed that the luciferase activity of the Wt EGFR 3'-UTR construct transfected with miR-514a-3p mimic was strikingly inhibited in comparison with that of mimic NC (P < 0.01) (**Figure 5C** and **5D**), while the luciferase activity of the Wt EGFR 3'-UTR construct significantly increased with miR-514a-3p inhibitor transfection compared with that of inhibitor NC (P < 0.01) (**Figure 5C** and **5D**). However, there were no obvious differences in the luciferase activity of reporter vector uploaded with Mut EGFR 3'-UTR between groups (**Figure 5C** and **5D**). To ascertain whether miR-514a-3p functions as an inhibitory molecule through directly inducing EGFR mRNA cleavage or translational repression, we examined the expressions of EGFR mRNA and protein by qRT-PCR and Western blot after transfection of miR-514a-3p mimic/inhibitor or corresponding negative control into ACHN and 786-O cells. The results indicated that there were no significant differences in EGFR mRNA expression between groups (**Figure 5E**), but EGFR expression at the protein level was effectively down-regulated in miR-514a-3p mimic-transfected RCC cells and vice versa (**Figure 5F**), confirming that miR-514a-3p inhibited the expression of EGFR by translational repression, not by mRNA cleavage.

MiR-514a-3p suppresses EMT through the MAPK/ERK pathway

To understand the potential molecular mechanism by which miR-514a-3p might suppress cell proliferation and EMT, we further exploited the effects of transient miR-514a-3p upregulation or down-regulation on the downstream MAPK/ERK and PI3K/AKT signaling pathways of EGFR using Western blot. The results uncovered that p-ERK expression was significantly decreased with loss of EGFR in miR-514a-3p mimic-transfected RCC cells (**Figure 5F**), while opposite results manifested in miR-514a-3p inhibitor-transfected RCC cells. However, no differences were observed in AKT, p-AKT and total ERK1/2 expression among all treated cells (**Figure 5F**). All these imply that miR-514a-3p may potentially suppress cell proliferation and EMT through the MAPK/ERK pathway instead of PI3K/AKT signaling.

MiR-514a-3p attenuates ccRCC tumor growth in vivo

To investigate the effect of miR-514a-3p on tumorigenicity in vivo, an animal experiment for ACHN xenograft model was employed to verify the role of miR-514a-3p in ccRCC aggressiveness. As demonstrated in **Figure 6A**, when the mean tumor volume of the mice in both groups was approximately 50 mm^3 two weeks after subcutaneous inoculation of ACHN cells, the subcutaneous xenograft tumors were periodically exposed to miR-514a-3p agomir or agomir NC and obtained four weeks after interventions. The results showed that forced expression of miR-514a-3p in ACHN cell dramatically suppressed tumor growth such as tumor volume and weight compared with agomir NC (P < 0.01) (**Figure 6B** and **6C**).

Discussion

Numerous studies have demonstrated that miRNAs as regulatory molecules are involved in cell proliferation, apoptosis and metastasis in human cancer and are associated with survival in tumor patients [24-27]. Recently, many aberrant miRNAs have been revealed by microarray in urologic cancers, including RCC [28-31]. According to the Meta-analysis result of ccRCC smRNA-seq datasets from YM500v2 database, miR-514a-3p was dramatically down-regulated in ccRCC (**Figure 1A**), which is consistent with the research results of Jung et al and Weng et al [13, 32].

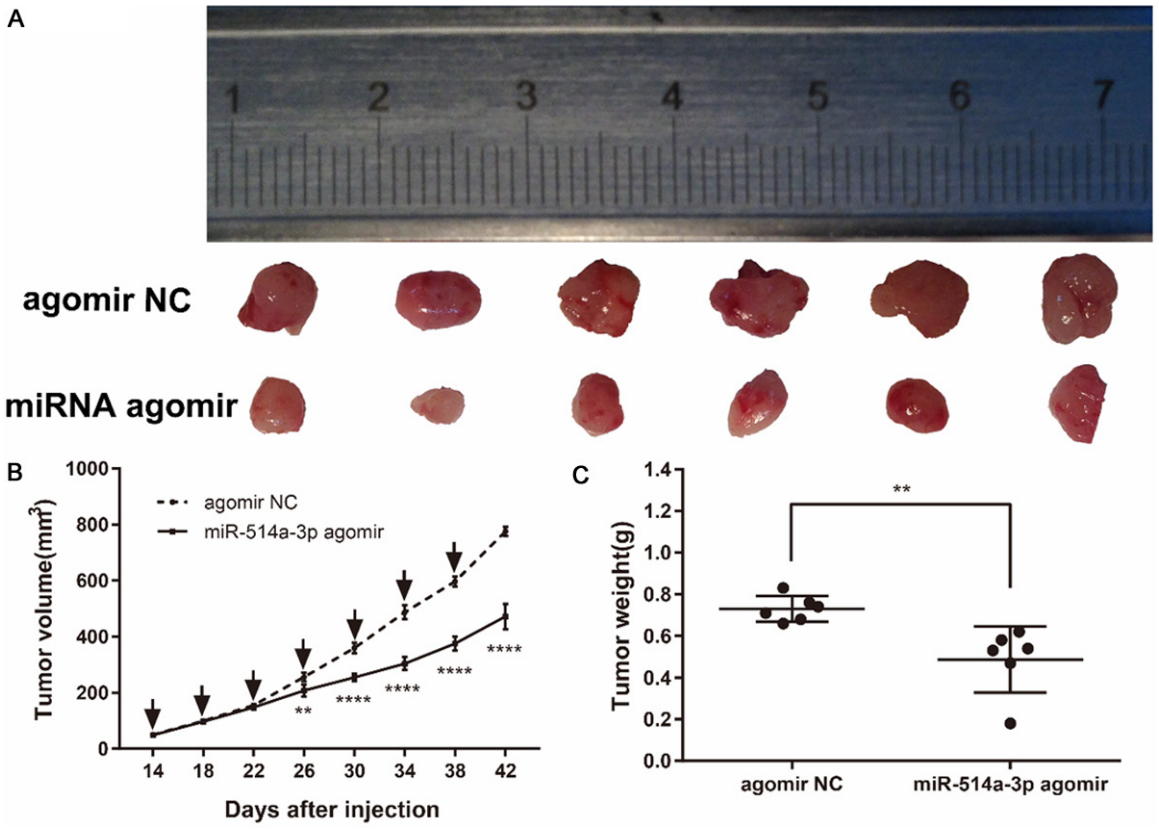

Figure 6. MiR-514a-3p inhibits the growth of subcutaneous xenograft tumor. (A) Photographs of tumors excised 42 days after inoculation of ACHN cell into nude mice. Black arrow signifies the administrations of miR-514a-3p agomir or agomir NC timing. (B, C). Tumor volume on the indicated days (B) and excised tumor weight (C) were presented as mean ± SD. **P < 0.01, ****P < 0.0001.

To confirm the result, we examined the expression of miR-514a-3p in ccRCC samples in Chinese population and demonstrated that miR-514a-3p was indeed significantly down-regulated in ccRCC tissues compared with adjacent normal tissues. Meanwhile, we also tested the expression of miR-514a-3p in human RCC cell lines and found that there was less miR-514a-3p in RCC cells than that in normal renal proximal tubule cell. These results indicate that miR-514a-3p is a promising tumor suppressor in RCC. It was reported that deregulation of miR-514a-3p also took place in other types of cancers [16-18]. Among these cancers, down-regulation of miR-514a-3p was detected in testicular germ cell tumor [17] and ovarian high-grade serous carcinoma [18], but expression of miR-514a-3p increased in melanoma [16]. This inconsistency may be explained by the various tumor types and specific cellular context.

Accumulating evidence indicates that aberrant miRNAs can either suppress or promote the differentiation, proliferation, apoptosis, and metastasis of RCC [29]. To explore the effect of miR-514a-3p on the biological behaviors in RCC cell lines, MTS, colony formation assay, transwell migration and invasion assays were performed. The results showed that miR-514a-3p might trigger the phenotypic changes in RCC cells, for instance, forced expression of miR-514a-3p by transfecting with miR-514a-3p mimic into ACHN and 786-O cells distinctively inhibited cell proliferation, viability, migration and invasion in comparison with mimic NC. In addition, restoration of miR-514a-3p expression might also negatively regulate the proteins associated with EMT, such as E-Cadherin, N-Cadherin and Vimentin. However, RCC cells exhibited opposite effects after transfection with miR-514a-3p inhibitor. Therefore, we conclude that miR-514a-3p would be a novel tumor

suppressor in RCC due to its negative effect on tumor growth and metastasis.

It is well known that miRNA could regulate the expression of target genes by interacting with the 3'-UTR of their target mRNAs [33]. In our study, three computational algorithms (miRDB, TargetScan, and miRNA Target Visualization) were selected to help screen out the potential target gene of miR-514a-3p and we found that the EGFR oncogene, a member of the well-known ErbB family, is the most promising target of miR-514a-3p. It is reported in literatures that EGFR overexpression is found in approximately 30% of primary tumors and has been related to advanced disease, poor prognosis, overall survival and therapy response [34]. The ErbB family is very important in the etiology and progression of various carcinomas, including RCC [34, 35]. EGFR demonstrates numerous oncogenic effects, including the initiation of DNA synthesis, regulation of the cell cycle, enhancement of cell growth, invasion and migration [36].

In the present study, we observed that because of the down-regulation of miR-514a-3p in ccRCC tissues, EGFR expression decreased remarkably, and a negative association was identified between the expressions of EGFR and miR-514a-3p at the RNA level, indicating that EGFR may be a direct target gene of miR-514a-3p. Results of the luciferase reporter assay further confirm the conclusion. Furthermore, restoration of miR-514a-3p expression markedly suppressed EGFR protein but not mRNA expression, suggesting potential translational control of EGFR expression caused by miR-514a-3p. A study organized by Li et al [23] showed that miR-27a significantly inhibited proliferation, migration and invasion in 786-O cell; in addition, Western blot analysis revealed that ectopic expression of miR-27a distinctly suppressed the expression of EGFR protein, signifying that miR-27a might function as a tumor suppressor in RCC by targeting EGFR. An additional research was performed by Li et al [37] to discover that the elevated miR-520b/e expression inhibited cell proliferation and migration by directly binding with the 3'-UTR of EGFR in gastric cancer, while down-regulation of miR-520b/e promoted these properties. The phenomena prove the theory that several miRNAs can target the same gene and one single miRNA can target multiple genes [11].

With the results of the present study showing a potential role of miR-514a-3p in RCC pathogenesis, we continued investigations to understand the biological mechanism of miR-514a-3p in tumor carcinogenesis. EGFR is reported to play a vital role in cellular signal transduction and its mutation appears to be crucial in gastric cancer, colorectal cancer, lung cancer, and other cancers [37-39]. Activation of EGFR signaling could stimulate multiple downstream pathways, including the MAPK/ERK and PI3K/AKT, thus promoting tumorigenesis [34]. Western's results in the current study presented that a low expression of EGFR associated with forced expression of miR-514a-3p in RCC cells induced a decrease of p-ERK1/2, but no change was found in the expression of p-AKT. All these results imply that miR-514a-3p could suppress the proliferation and EMT in RCC cells mainly through the downstream MAPK/ERK pathway of EGFR rather than the PI3K/AKT pathway. Admittedly, there are other targets of miR-514a-3p, which may contribute to the inhibition of cell proliferation and EMT in ccRCC; therefore, further studies are required to determine if other signaling or mechanisms are collaboratively implicated in the development and progression of RCC as well.

In addition to the function of miR-514a-3p in vitro study, a RCC xenograft model in vivo was established and the initial results of interventions displayed that intratumorally administration of miR-514a-3p agomir triggered regression of tumor growth, embodied by the volume and weight of tumor. Consequently, the result implies that miR-514a-3p is a tumor suppressor and may be of therapeutic use in RCC.

In summary, our results show that miR-514a-3p is frequently down-regulated in ccRCC and may function as a potential tumor suppressor in ccRCC by targeting EGFR. Overexpression of miR-514a-3p inhibited the EGFR/MAPK/ERK signaling pathway in ACHN and 786-O cells, which in turn regulated the proliferation, migration and invasion. However, there are some limitations in the present study. Firstly, the number of samples is little small with being lack of stage T4 tumor and is not enough to analyze the relationship between miR-514a-3p and clinicopathological features. Secondly, the interventions in experiments are limited to miR-514a-3p such as mimic, inhibitor, and agomir, being short of focus on EGFR. Finally, our data

are mainly derived from cell lines and may aid in the understanding of the molecular role of miR-514a-3p in ccRCC tumorigenesis, but cannot be regarded as accurate representation of clinical ccRCC study. Further studies are still needed to evaluate the roles of miR-514a-3p in in vivo and clinical context.

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Disclosure of conflict of interest

None.

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