

MiR-125b promotes migration and invasion by targeting vitamin D receptor in renal cell carcinoma

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Research

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Abstract

Background To investigate the expression of miR-125b and vitamin D receptor (VDR) in renal cell carcinoma (RCC) and assess the possible association between them. Then, to elucidate whether miR-125b can regulate the expression of VDR and affect proliferation and metastasis in RCC.

Methods The expression of miR-125b was detected by quantitative real-time polymerase chain reaction (RT-PCR) in RCC cell lines. MiR-125b mimic and inhibitor were employed to measure the function and behavior of miR-125b in RCC cell lines. The relationship between miR-125 and VDR was verified using luciferase assays, and their expression was also examined in primary tumor and normal peritumoral kidney tissues in 20 clear cell RCC (ccRCC) samples.

Results Overexpression of miR-125b promoted migration and invasion and reduced cell apoptosis in ACHN cells, while inhibition of miR-125b suppressed migration and invasion and induced cell apoptosis in 786-O cells. Overexpression of miR-125b decreased VDR expression via targeting VDR. Expression of miR-125b mRNA was significantly higher in ccRCC tissues than in normal adjacent tissues, and the expression of miR-125b mRNA negatively correlated with that of VDR ($r=-0.444$, $p=0.04$).

Conclusion Overexpression of miR-125b decreased the expression of VDR and the promoted migration and invasion of RCC cells; in addition, there was a negative correlation between miR-125b and VDR expression in ccRCC.

Background

Renal cell carcinoma (RCC) accounts for approximately 3% of all human cancers and is one of the most lethal urological malignancies¹. Despite improvements in available treatment, both incidence and mortality rates increase each year. It is estimated that there were 73,820 new diagnoses and 14,770 deaths related to RCC in 2019 in the United States². Therefore, it is crucial to identify novel biomarkers and effective therapeutic targets in RCC.

Vitamin D receptor (VDR) is a nuclear class II receptor family that includes androgen receptor and estrogen receptor³. The ligand for VDR is 1,25-dihydroxyvitamin D₃, and several recent lines of evidence have shown that this metabolite could reduce the risk of RCC⁴⁻⁶. On the other hand, VDR expression was significantly reduced in RCC compared to normal kidney tissue and was associated with RCC prognosis⁷⁻¹⁰. Specifically, our previous studies showed that VDR overexpression could significantly inhibit RCC cell proliferation, migration, and invasion, and promote apoptosis and confirmed that VDR expression was associated with RCC pathological types^{10,11}. These results indicate that VDR may play a key role in RCC development and progression.

MicroRNAs (miRNAs) are a class of small noncoding single-stranded RNAs that consist of ~ 22 nucleotides and can play important regulatory roles in human cancer by targeting mRNAs for cleavage or

translational repression¹². In the present study, we identified miRNAs potentially targeting VDR. By a computational search via TargetScan (<http://www.targetscan.org/>), VDR is predicted as a target of miR-125b. Several lines of evidence have shown that mi-125b plays a critical role in regulating biological processes of human cancer via degradation or translational inhibition of specific target mRNAs¹³⁻¹⁷. In recent studies, correlations have been observed between miR-125b expression and VDR in breast cancer^{3, 18, 19}. This has led to speculation that the development of breast cancer may be the result of miR-125b changes that control VDR expression and activity. However, there are currently no studies regarding the correlation between miR-125b expression and VDR in RCC.

Therefore, the aim of the present study was to investigate the correlation of miR-125b and VDR expression in RCC and to further analyze the possible mechanism by which miR-125b controls VDR expression in RCC. We confirmed that overexpression of miR-125b could decrease the expression of VDR and promote migration and invasion in RCC cells, and there was a negative correlation between miR-125b and VDR expression in clear cell RCC (ccRCC).

Methods

Cell culture

Human embryonic kidney 293T cells and four RCC cell lines, ACHN, A498, Caki-1 and 786-O, were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 100 µl/ml penicillin and 100 mg/ml streptomycin. All the cells were incubated at 37 °C in a 5% CO₂ atmosphere.

Clinical sample collection

A total of 20 pairs of ccRCC and adjacent normal tissues were collected from Affiliated Sanming First Hospital of Fujian Medical University (Sanming, China) between May 2016 and May 2018. The study was approved by the Institutional Ethics Committee of Affiliated Sanming First Hospital of Fujian Medical University, and written informed consent was obtained from all patients. The tumor samples were histopathologically diagnosed as ccRCC and were stored in liquid nitrogen immediately after surgical resection until further use.

Cell Transfection

ACHN or 786-O cells were seeded into 6-well plates at a density of 60%. Synthesized miR-125b mimic or inhibitor was transiently transfected into cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). MiR-125b inhibitors (sequence: 5'-UCACAAGUUAGGGUCUCAGGGA-3'), NC inhibitor (sequence: 5'-UCUACUCUUUCUAGGAGGUUGUGA-3'), miR-125b mimics (sequence: 5'-UCCCUGAGACCCUAACUUGUGA-3'), and NC mimic (sequence: 5'-UCACAACCUCCUAGAAAGAGUAGA-3') were purchased from GenePharma (Shanghai, China). The efficiency of miR-125b expression regulation was confirmed using qRT-PCR.

RNA Isolation and qRT-PCR

Total RNA was extracted from the tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. For miR-125b detection, Taqman assays were employed, and U6 was used as an internal control. For mRNA analysis, total RNAs were reverse transcribed into cDNA using the RT MasterMix Kit (Abm). Then, qRT-PCR was performed using SYBR Green-I as the fluorogenic dye. The relative expression fold changes were calculated by the $2^{-\Delta\Delta CT}$ method.

Cell proliferation assay

Cell proliferation was measured using the CCK-8 (Dojindo, Kumamoto, Japan) assay. Cells (786-O and ACHN) transfected with miR-125b mimic and inhibitor were seeded into 96-well culture plates at a density of 1×10^4 cells/well. The proliferation assay was carried out for 4 days, and cell growth was measured every 24 hours. Briefly, 10 μ l of CCK-8 solution was added to each well, and the plate was incubated at 37 °C for 24 hours. Then, the absorbance of each sample was measured at 450 nm using a microplate reader. All experiments were performed in triplicate.

Cell apoptosis assay

Cell apoptosis analysis was performed by flow cytometry using a fluorescein isothiocyanate (FITC) and propidium iodide (PI) kit (Vazyme, Nanjing, China). Cells were harvested after treatment with miR-125b mimic or inhibitor and washed with precooled PBS. The cells were mixed with 500 μ L of binding buffer and 5 μ L of FITC and PI and incubated for 10 minutes at room temperature (20 to 25 °C). Apoptosis rates were then measured by flow cytometry (FACS, Partec AG, Arlesheim, Switzerland), and the percentage of apoptotic cells was calculated. Each experiment contained three replicates for each condition.

Cell migration and invasion assays

Cell migration was measured using a Transwell chamber plate (Corning, Bedford, MA, USA). Briefly, 1×10^5 cells were seeded onto the Transwell insert 48 hours after transient transfection. Twenty percent fetal bovine serum was used as a chemoattractant. After 48 hours of incubation at 37 °C, cells that did not migrate through the pores of the Transwell insert were manually removed with a cotton swab. The cells present at the bottom of the membrane were fixed in 4% polymethanol for 15 minutes and then visualized by incubation with 0.1% crystal violet for 20 minutes. Independent experiments were repeated three times. The invasion assay was performed in a similar manner to the migration assay with the only difference being that the upper layer of the Transwell chamber membrane uniformly covered the Matrigel. Two independent experiments were performed in triplicate for each experiment.

miRNA target prediction and luciferase reporter assay

TargetScan was used to predict miRNAs that could potentially target VDR and identify possible binding regions. The VDR 3'-untranslated region (UTR) contained miR-125b binding sites. The dual luciferase reporter system (Beyotime, Shanghai, China) was then used to verify luciferase activity. The VDR 3'-UTR

cDNA sequence, including the mutant or wild-type miR-125b binding region, was amplified and cloned into the pGL3 luciferase vector (Promega, Madison, WI, USA). Next, 786-O cells were transfected with luciferase reporter plasmids and either miR-125b mimics or NC using Lipofectamine 2000, according to the manuscript protocol. Then, the activity of luciferase was determined using a luminometer (Promega, Madison, WI, USA) and measured based on that of the empty pGL3 vector.

Western blotting

VDR expression was assessed by Western blotting using standard protocols. Briefly, cells were harvested 48 hours after transfection, and lysates were prepared in radioimmunoprecipitation assay buffer. Equal amounts of protein, as determined by the Bradford Protein Assay (Beyotime, Shanghai, China), were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Beyotime, Shanghai, China). Five percent skim milk powder was added for 2 hours at room temperature, and after incubation with a peroxidase-conjugated secondary antibody (Beyotime, Shanghai, China), the blot was developed using an enhanced chemiluminescence reagent and exposed to X-ray film to detect the labeled protein. β -actin served as a reference standard for protein expression.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 7 and SPSS 22, and all the data are presented as the mean \pm SD. $P < 0.05$ was considered to indicate a significant difference.

Results

Expression levels of miR-125b in human RCC cell lines

To examine the expression levels of miR-125b in the RCC cell line and HEK-293T, miR-125b expression levels were measured by RT-PCR. The expression of miR-125b in the RCC cells was higher than that in HEK-293T cells (Fig 1A). To further characterize the function of miR-125b, we used a model in which ACHN cells were treated with miR-125b mimics and 786-O cells were treated with miR-125b inhibitors. The treatment efficiency was measured by qRT-PCR (Fig 1B-1C).

Effects of miR-125b on RCC cell proliferation

To verify the effect of miR-125b on cell proliferation, an MTT assay was performed. Overexpression or inhibition of miR-125 regulated cell proliferation in 786-O and ACHN RCC cells. The results of the MTT assay showed that there was no significant difference between miR-125b mimics and NC mimic, and there was no significant difference between inhibitor and NC inhibitor (Fig 2).

Effects of miR-125b on RCC cell apoptosis

Cell apoptosis of miR-125b was assessed using flow cytometry. ACHN cells overexpressing miR-125b exhibited reduced cell apoptosis, while 786-O cells with inhibited miR-125b showed increased cell apoptosis compared to the control groups (Fig 3). These results suggest that overexpression of miR-125b reduced RCC cell apoptosis and that inhibition of miR-125b induced RCC cell apoptosis.

Effects of miR-125b on RCC cell migration and invasion

Cell migration and invasion play an important role in the development of cancer. The migration and invasion of miR-125b in RCC cells was evaluated by the Transwell assay. The Transwell assay results indicated that overexpression of miR-125b in ACHN cells increased cell migration and invasion ability; in contrast, inhibition of miR-125b in 786-O cells obviously reduced cell migration and invasion activity when compared with that in the NC group (Fig 4). These findings suggest that overexpression of miR-125b could promote cell migration and invasion in RCC cells.

Effects of miR-125b on VDR expression

By a computational search via TargetScan, VDR was predicted to be a target gene of miR-125b (Fig 5A). To verify whether miR-125b directly targets VDR, dual-luciferase reporter assays were performed. We found that miR-125b significantly decreased luciferase activity of the wild-type but not the mutant VDR (Fig 5B). In addition, Western blotting was also conducted to determine whether VDR was altered posttransfection of miR-125b mimic in ACHN cells and miR-125b inhibitor in 786-O cells. Compared to each control group, miR-125b mimics decreased the expression of VDR in ACHN cells (Fig 5C, 5D), and miR-125b inhibitors increased the expression of VDR (Fig 5C, 5F). These results indicate that VDR may be a direct target gene of miR-125b.

Increased miR-125b expression in ccRCC was attenuated by VDR.

To investigate the relationship between the expression levels of miR-125b and VDR in ccRCC samples, 20 human ccRCC and normal adjacent tissues were analyzed. We found that the expression level of miR-125b was significantly higher in RCC than in normal adjacent tissues, while the expression level of VDR was significantly lower in RCC than in normal adjacent tissues. We analyzed the correlation among the quantified expression levels of miR-125b and VDR mRNA in ccRCC. MiR-125b mRNA expression was significantly negatively correlated with that of VDR ($r=-0.444$, $p=0.04$) (Fig 6). These results suggest that the expression of miR-125b was negatively correlated with the presence of VDR in ccRCC.

Discussion

The expression of miR-125b has been reported to be altered in certain types of cancer. miR-125b expression was low in tumor tissue compared normal tissue in ccRCC²⁰ and expression of miR-125b is increased during malignant hematopoiesis, suggesting involvement in carcinogenesis²¹. MiR-125b has decreased expression in bladder cancer and inhibits migration and invasion in vitro^{22, 23}. However, overexpression of miR-125b promoted migration, invasion and metastasis in gastric cancer²⁴, and high

levels of miR-125 expression were associated with the risk of metastasis in non-small-cell lung cancer²⁵. Expression of miR-125b is strongly increased in chronic myeloid leukemia (CML), and inhibition of miR-125b decreases proliferation and promotes apoptosis with cell cycle arrest at the G0/G1 phase in CML cells²⁶. All of these findings suggest that the role of miR-125b is essential in the tumorigenesis of certain tumors, but the expression of miR-125b in different tumors varies.

In this study, we found that miR-125b was significantly increased in RCC compared with normal tissue and that overexpression of miR-125b promoted migration and invasion in RCC cell lines in vitro. Numerous studies have already proven the role of miR-125b in proliferation, apoptosis and cellular differentiation in carcinogenesis²⁴⁻²⁶. For RCC, the mechanism by which miR-125b upregulation regulates migration and invasion is still unknown.

TargetScan, which can predict potential target genes of miRNAs, found that the seed sequence of miR-125b was perfectly matched with that of the VDR mRNA 3'-UTR and that VDR could be a target of miR-125b. Furthermore, we used a luciferase activity assay to verify that miR-125b could directly target the VDR 3'-UTR, as predicted by bioinformatics. Interestingly, it has been reported that the 3'-UTR of VDR mRNA has a miR-125b recognition element (MRE125b) containing 8 nucleotide elements and that miR-125b could functionally recognize MRE125b^{3, 18}. In breast cancer cells, it was demonstrated that the overexpression of miR-125b significantly decreased the endogenous VDR protein level¹⁸. In melanoma cell lines, the endogenous VDR mRNA level is inversely associated with the expression of miRNA-125b²⁷. Our work showed that miR-125b upregulation also led to downregulation of VDR protein in RCC cells, and the expression of miR-125b was negatively correlated with that of VDR in ccRCC samples. These findings revealed that miR-125b might regulate VDR expression and promote the development and metastasis of RCC.

VDR is one of the most studied tumor suppressor genes and acts as a transcription factor and regulates numerous physiologic processes, such as serum calcium regulation, cell proliferation and differentiation, and immunomodulatory functions³. We previously demonstrated that VDR overexpression significantly suppresses RCC cell proliferation, migration and invasion¹¹. VDR could inactivate mutations, deletions, and promoter methylation to regulate cell proliferation and differentiation^{4, 28}. The results of the present study revealed that miR-125b could promote RCC cell migration and invasion by targeting VDR. It is worth noting that here we did not show that miR-125b could regulate cell proliferation by targeting VDR, possibly due to the fact that multiple epigenetic interactions with VDR counteract the effects on cell proliferation, and the specific mechanisms require further study.

Conclusion

This work is the first to indicate that VDR is a target gene of miR-125b in RCC. Moreover, miR-125b expression was negatively correlated with VDR level, and overexpression of miR-125b promoted migration and invasion in RCC by targeting VDR. A better understanding of the role and relationship of

miR-125b and VDR in tumors might be beneficial for the development of new gene therapy strategies against RCC.

Declarations

Disclosure

The author reports no conflicts of interest in this work

MiR-125b promotes migration and invasion by targeting vitamin D receptor in renal cell carcinoma

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Ethics approval and consent to participate: The study was approved by the Institutional Ethics Committee of Affiliated Sanming First Hospital of Fujian Medical University, and informed consent was obtained from all patients.

Consent for publication: All authors have read and comply with the requirements set forth in Information for Authors, and consent for publication.

Availability of data and material: During data collection, the principles of randomness, control, and repetition were used. T-test and analysis of variance were used for the count data, and X2 test was used for the measurement data.

Competing interests: The author reports no conflicts of interest in this work.

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Authors' contributions: **XiYuan He**: Data curation, Writing-Original draft preparation. **ShangFan Liao**: Visualization, Investigation. **DongMing Lu**: Supervision. **FaBiao Zhang**: Methodology, Software. **YongYang Wu**: Conceptualization, Writing-Reviewing and Editing, Funding acquisition.

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Figures

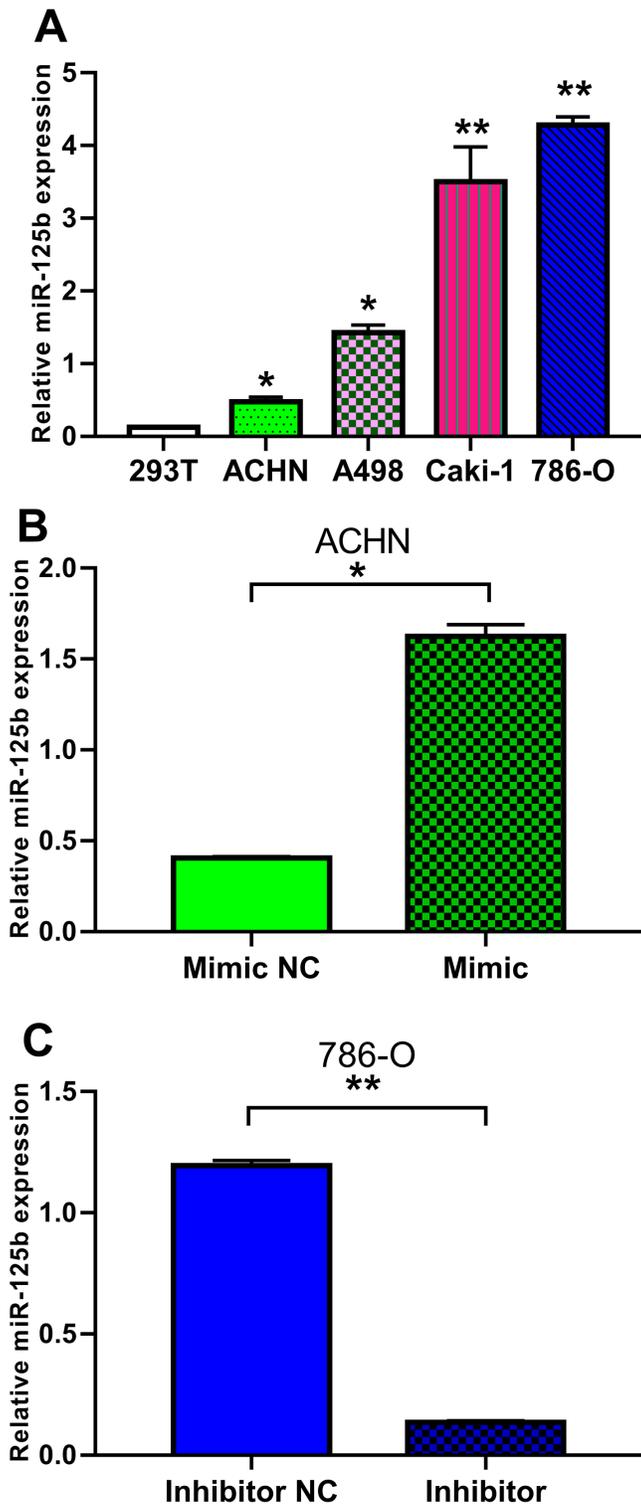


Figure 1

MiR-125b is upregulated in renal cell carcinoma (RCC) cell lines. (A) MiR-125b expression level in human embryonic kidney 293T cell and RCC cell lines (ACHN, A498, Caki-1, 786-O). (B) Level of miR-125b in miR-125b mimic treated ACHN cells. (C) Expression of miR-125b in miR-125b inhibitor treated 786-O cells. U6 was used as an internal control. * $p < 0.05$, ** $p < 0.01$. NC, negative control.

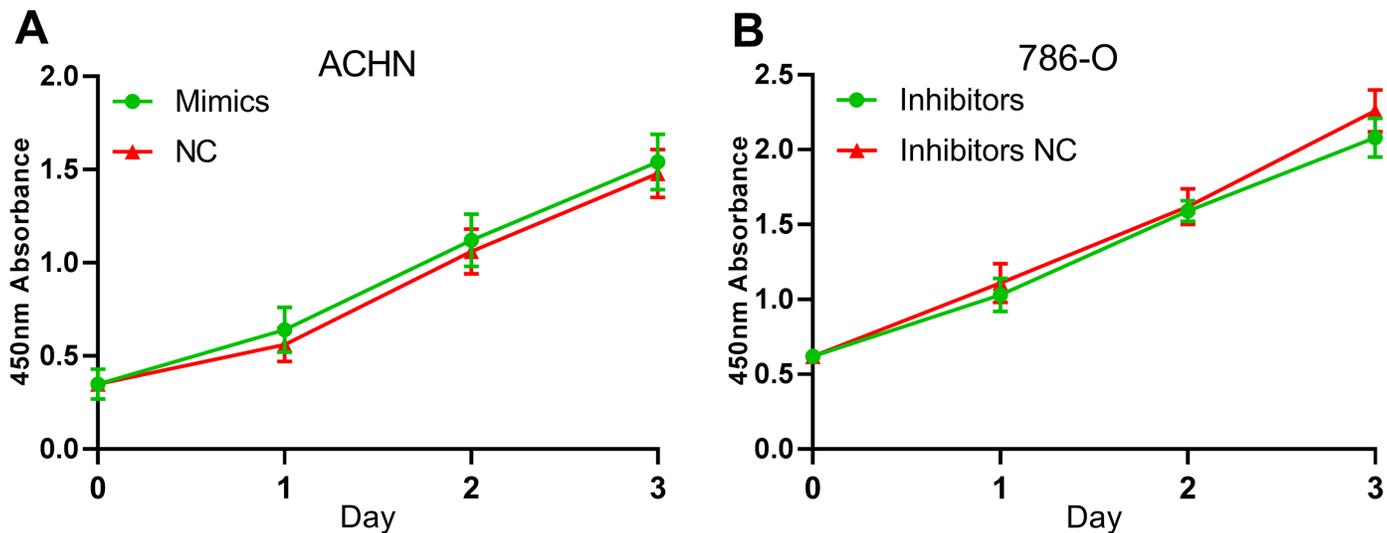


Figure 2

Cell proliferation of MTT assay in ACHN and 786-O. The results revealed that miR-125b had no effect on cell proliferation. Data are presented as the mean \pm standard deviation of absorbance values. NC, negative control.

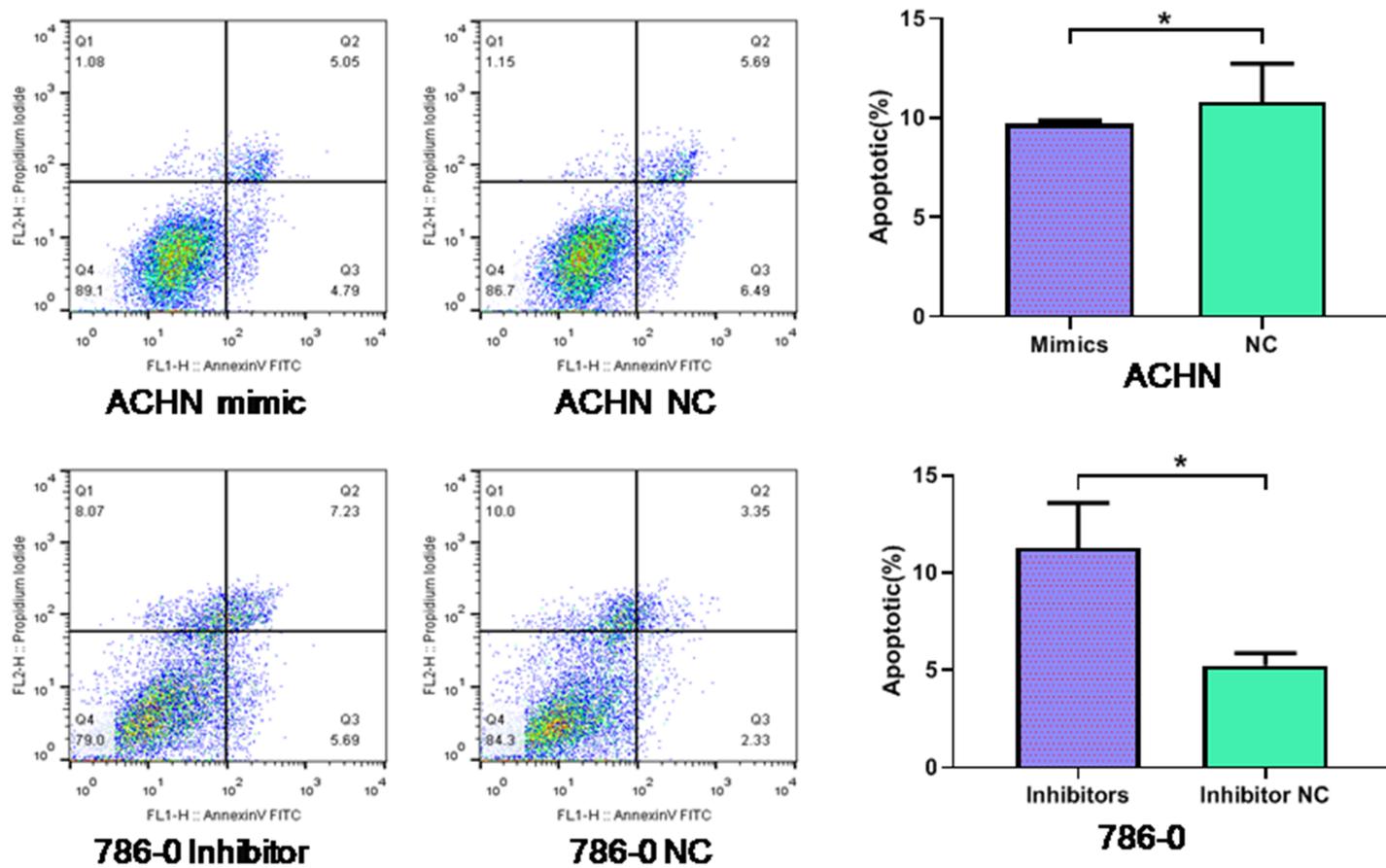


Figure 3

MiR-125 effect apoptosis of RCC cells. Flow cytometry indicated that miR-125b mimics reduced cell apoptosis, and miR-125b inhibitors induced cell apoptosis. The data were expressed as the mean \pm SD of three independent experiments. * p <0.05, ** p <0.01.

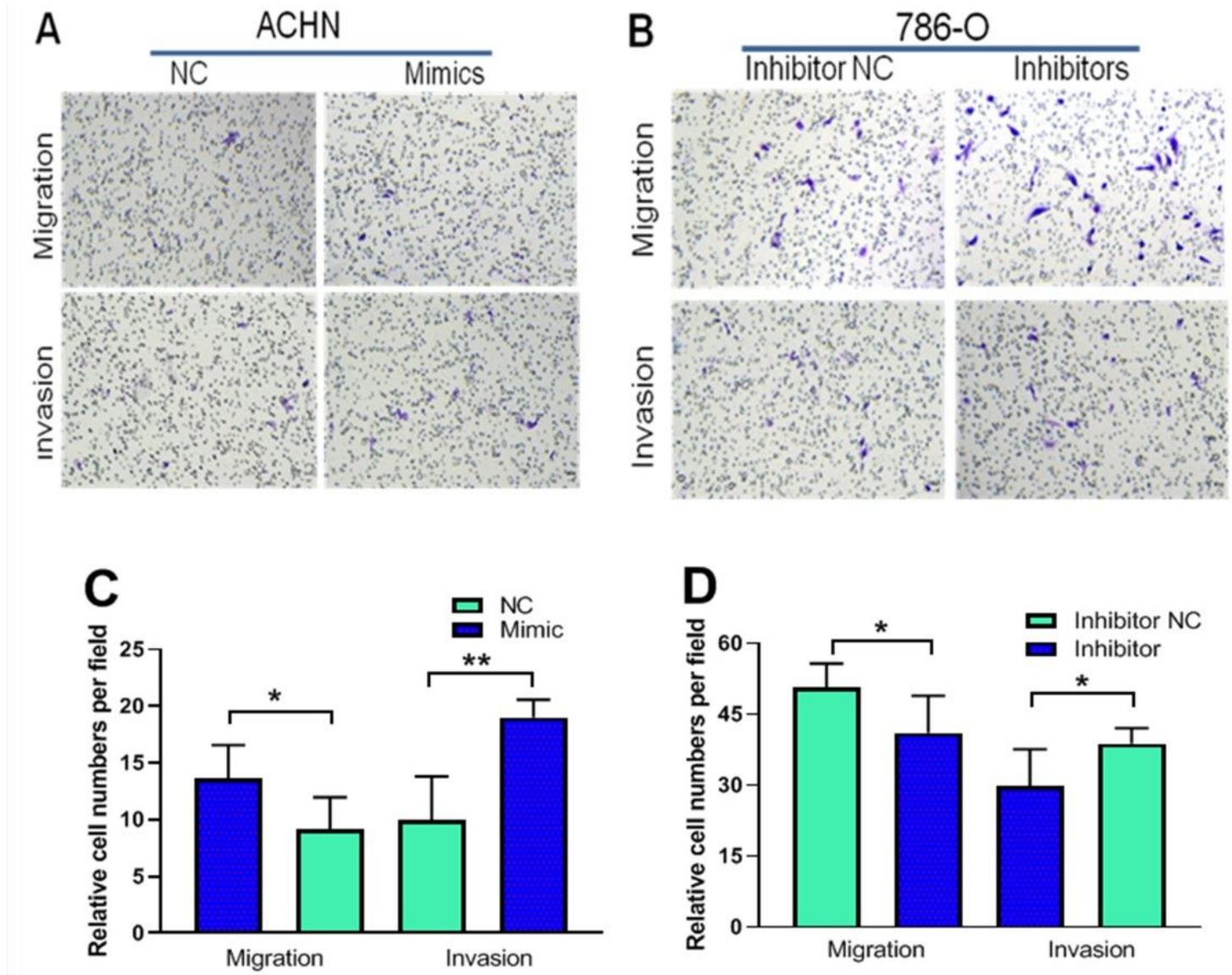


Figure 4

MiR-125b promote the migration and invasion of RCC cells, as shown by representative images of migration and invasion assays of (A) ACHN and (B) 786-O, and quantification of the migration and invasion of (C) ACHN and (D) 786-O cells. Data are presented as the mean \pm SD of five random vision. * p <0.05, ** P <0.01. NC, negative control.

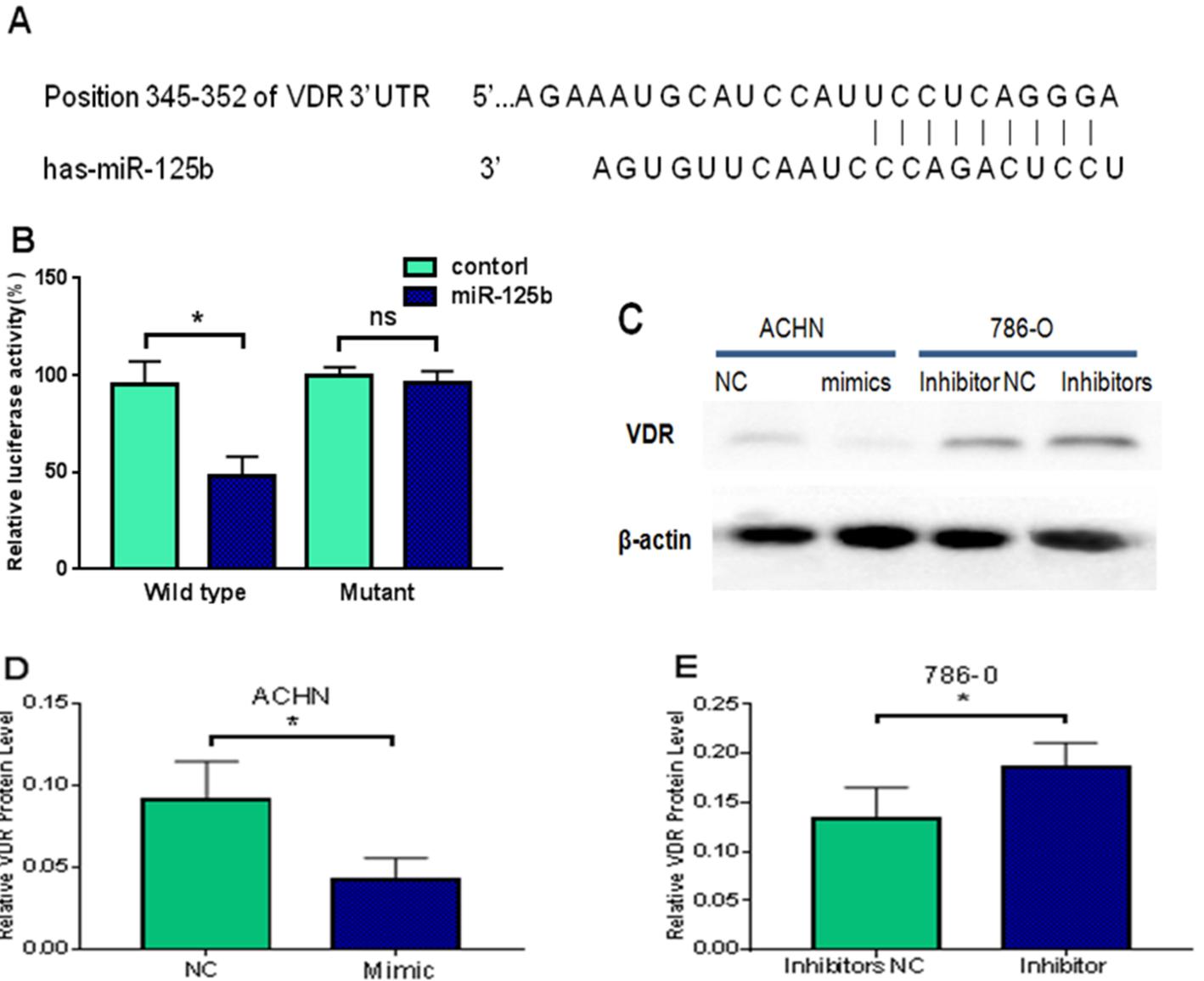


Figure 5

VDR was direct target of miR-125b. (A) The predicted binding region of miR-125b in the 3'-UTR of VDR. (B) Luciferase reporter assay showed that VDR may be a direct target of miR-125b in 786-O cells. (C, D) VDR was significantly downregulated in miR-125b mimics ACHN cells. (C, E) MiR-125b inhibitors promote VDR upregulated in 786-O cells. The protein levels were moralized to that of β -actin. * $p < 0.05$, ns, not significant.

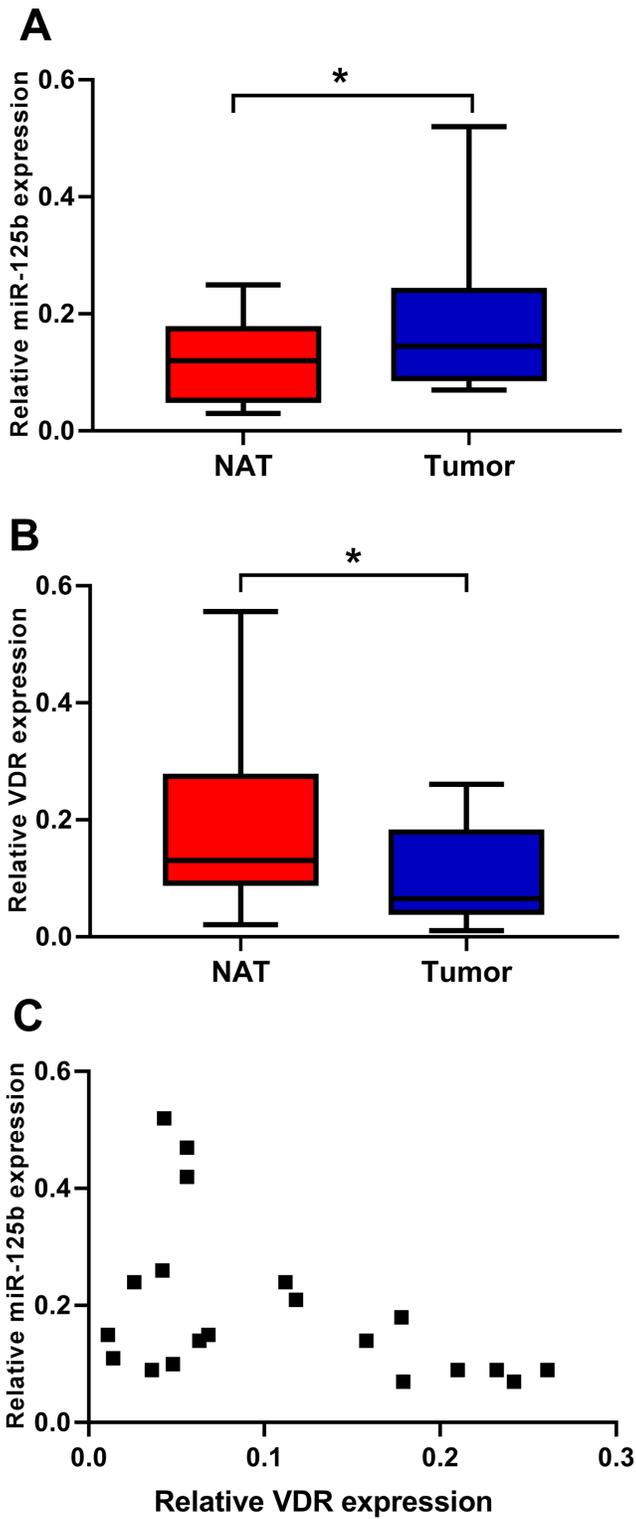


Figure 6

Expression of miR-125b and VDR in RCC tumor and normal adjacent tissues (NAT). (A) MiR-125b was higher in RCC patients. (B) VDR was lower in RCC patients. (C) Pearson correlation analysis of the association between miR-125b and VDR in RCC patients showed $R = -0.44$ ($p = 0.04$). $*p < 0.05$.