

# Highly Expressed Antisense Noncoding RNA in the INK4 Locus Promotes Growth and Invasion of Renal Clear Carcinoma Cells via the $\beta$ -Catenin Pathway

Qingchun Li,\* Yuan Tian,† Guangrui Hu,† Yun Liang,† Wei Bai,† and Hongjun Li†

\*Department of Gastrointestinal Colorectal and Anal Surgery, China–Japan Union Hospital of Jilin University, Changchun, P.R. China

†Center of Physical Examination, China–Japan Union Hospital of Jilin University, Changchun, P.R. China

Long noncoding RNA (lncRNA) antisense noncoding RNA in the INK4 locus (ANRIL) is involved in several human cancers. However, the role of ANRIL in renal cell carcinoma (RCC) remains unclear. This study aimed to explore whether, and how, ANRIL affects the progression of RCC. First, the expression of ANRIL in clinical tumor tissues and four kinds of RCC cell lines was evaluated. After transfection, cell viability, colony number, apoptosis, migration, and invasion were assessed. The expression of proteins related to apoptosis, epithelial-to-mesenchymal transition (EMT), and the  $\beta$ -catenin signaling pathway was then assessed. In addition, the effect of IWR-endo ( $\beta$ -catenin inhibitor) on cell viability, migration, and invasion, as well as  $\beta$ -catenin expression, was also evaluated. The results showed that ANRIL was highly expressed in RCC tissues and RCC cell lines. ANRIL significantly promoted cell proliferation, migration, invasion, and EMT but inhibited cell apoptosis. Additionally, the expression levels of  $\beta$ -catenin, Ki-67, glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ), phosphorylated GSK-3 $\beta$ , T-cell transcription factor 4 (TCF-4), and leukemia enhancer factor 1 (LEF-1) were all markedly upregulated by ANRIL. The effect of ANRIL silencing was opposite to that of ANRIL overexpression. The effect of ANRIL on proliferation, migration, and invasion of RCC cells was found to be reversed by IWR-endo. In conclusion, ANRIL, which is highly expressed in RCC, acted as a carcinogen in RCC cells through the activation of the  $\beta$ -catenin pathway.

**Key words:** Long noncoding RNA ANRIL; Renal clear cell carcinoma; Invasion;  $\beta$ -Catenin signaling pathway

## INTRODUCTION

Renal cell carcinoma (RCC) is the second most common urological malignancy, accounting for approximately 90% of kidney cancers<sup>1,2</sup>. Based on pathology, RCC is classified into four categories, including clear cell RCC, papillary disease, chromophobe, collecting duct, and unclassified carcinomas<sup>3</sup>. Among them, clear cell RCC is the most common type, representing approximately 75% of primary kidney neoplasms<sup>4</sup>. Owing to the fact that RCC has a high resistance to conventional chemotherapy and radiotherapy, immunotherapy performed with interleukin-2 or interferon- $\alpha$  (IFN- $\alpha$ ) is used as the first-line treatment, but the outcome is very poor<sup>5</sup>. Although radical nephrectomy acts as an effective treatment for RCC, the rate of metastasis and local recurrence after treatment is still around 20%–40%. If patients are untreated, the median survival is 6 to 12 months, and the 5-year survival is lower than 9%<sup>6</sup>. Thus, it is urgent to explore promising therapeutic treatments for RCC.

Long noncoding RNA (lncRNA), a kind of noncoding RNA whose length is more than 200 nucleotides, has recently been studied for its involvement in multiple biological processes including growth, development, apoptosis, and differentiation<sup>7,8</sup>. Additionally, an array of lncRNAs was interestingly investigated for their participation in various pathological processes, for example, acting as a tumor suppressor or oncogene in tumor progression<sup>9</sup>. In particular, lncRNAs play a crucial role in nasopharyngeal<sup>10</sup>, thyroid<sup>11</sup>, and colorectal carcinomas<sup>12</sup>. To the best of our knowledge, the lncRNAs related to RCC have been poorly investigated.

Antisense noncoding RNA in the INK4 locus (ANRIL), a kind of lncRNA, was originally found in patients with familial melanoma<sup>13</sup>. After this discovery, accumulating evidence has identified the effect of ANRIL on substantial cancer progression<sup>14,15</sup>. ANRIL is encoded in the chromosome 9p21 region and has been proven to modulate its neighbor, cyclin-dependent kinase inhibitor (CDKN)

2A/2B, which acts as a tumor suppressor through epigenetic mechanisms<sup>16</sup>. More recently, a study indicated that CDKN2B mutation might be a novel cause of familial RCC<sup>17</sup>. Therefore, we hypothesized that ANRIL might be involved in the development of RCC. Considering that little is known about the role of ANRIL in RCC progression, uncovering the regulatory role of ANRIL in RCC is greatly significant.

In our study, we first identified the expression level of ANRIL in RCC tissues and cell lines. We then focused on the effects of ANRIL overexpression or silencing on cell proliferation, apoptosis, migration, and invasion in RCC cells. The alteration of key kinases involved in possible signaling pathways was also explored.

## MATERIALS AND METHODS

### *Tumor Sample Collection*

Patients (10 males and 10 females, average age: 62.25 ± 8.54) who were pathologically diagnosed with primary RCC between August 2012 and December 2015 signed informed consents before sample collection. Patients with two or more different malignancies and patients who had received therapy were excluded. The tumor and adjacent nontumor tissues (≥10 cm from the carcinoma) were surgically resected and collected for further studies. The experimental process was approved by the medical ethics committee of the China–Japan Union Hospital of Jilin University.

### *Cell Culture*

Human RCC cell lines, including 786-O, A498, caki-1, and caki-2, and the normal HEK-293T cell line, which acted as the control, were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The 786-O cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA), A498 cells were cultured in Eagle's minimum essential medium (EMEM, Gibco), and caki-1 and caki-2 cells were cultured in McCoy's 5A medium (Gibco). HEK-293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco). Media for the cells were all supplemented with 10% fetal bovine serum (FBS; Gibco). All cells were maintained in a humidified incubator with 5% CO<sub>2</sub> at 37°C.

### *Cell Transfection*

The lentiviral vector with overexpression of ANRIL was constructed by Genechem (Shanghai, P.R. China). The lentiviral vector was transfected into cells, acting as a control. In brief, 786-O and A498 cells were seeded into six-well plates. After 24 h, the lentiviral vectors with ANRIL and empty vectors were infected into 786-O or A498 cells, respectively, at a multiplicity of infection (MOI) of 10. The infection efficiency was evaluated by

GFP expression at 3 days after infection. Small interfering (si) RNA targeting ANRIL (si-ANRIL) and scrambled negative control (si-NC) were both synthesized by GenePharma (Shanghai, P.R. China). These siRNAs were transfected into cells with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the protocol of the supplier. Furthermore, IWR-endo (10 μM; Santa Cruz Biotechnology, Santa Cruz, CA, USA), a kind of β-catenin inhibitor, was added into cells to verify the effect of ANRIL on carcinogenesis.

### *CCK-8 Assay*

Cell viability was assessed by cell counting kit-8 (CCK-8) reagent (Dojindo, Japan). In brief, 100 μl of cells with a density of 4 × 10<sup>3</sup> cells/well was seeded into a 96-well plate and maintained at 37°C with 5% CO<sub>2</sub>. At 24, 48, and 72 h after transfection, 10 μl of CCK-8 reagent was added into each well. After 1.5 h of incubation at 37°C, absorbance of plates at 450 nm was detected by a microplate reader (BioTek, Winooski, VT, USA).

### *Colony Formation Assay*

At 24 h after transfection, cells with a density of 300 cells/well were seeded into a 12-well plate. Cells were then maintained at 37°C for 7 days, along with medium change every 3 days. Cells were stained with 12% crystal violet, and the colonies that contained more than 50 cells were counted. Each group consisted of three wells.

### *Cell Apoptosis*

Annexin-V-FITC/PI apoptosis detection kit (BestBio, Shanghai, P.R. China) was used to evaluate cell apoptosis. In brief, 48 h after transfection, cells were collected and washed with prechilled phosphate-buffered saline (PBS). Resuspended in binding buffer, cells were stained in turn with 5 μl of annexin V-FITC for 10 min and 10 μl of propidium for 5 min in the dark. Finally, the cell apoptosis was detected by FACScan cytofluorometer (Mansfield, Boston, MA, USA) and analyzed by FlowJo software (Tree Star, San Carlos, CA, USA).

### *Quantitative Reverse Transcription (qRT)-PCR*

Total RNA of tissues and cultured cells was isolated by TRIzol reagent (Invitrogen) in line with the supplier's instructions. RNA that was obtained was reversely transcribed into cDNA using High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA) in accordance with the supplier's protocol. Quantitative PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) in accordance with the supplier's instructions. The relative expressions of ANRIL and mRNAs were calculated according to the 2<sup>-ΔΔCt</sup>

method<sup>18</sup>. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) acted as an internal control gene.

#### *Cell Migration and Invasion Assay*

The migration assay was examined by Transwell assay. Complete medium (600  $\mu$ l) was placed into the lower chamber of Transwell assay inserts (Millipore, Billerica, MA, USA) with an uncoated membrane containing 8- $\mu$ m pores. Meanwhile, transfected cells in a serum-free medium (200  $\mu$ l) were filled into the upper chamber at an amount of  $1 \times 10^5$ . Forty-eight hours after incubation at 37°C, the cells remaining on the top layer of the insert were removed carefully with a cotton swab. Cells that had migrated to the bottom surface of the insert were stained with 0.1% crystal violet, followed by photographing using a digital inverted microscopy (Nikon, Mississauga, ON, Canada). Five fields were randomly selected for counting the apoptotic cells. Experiments were repeated three times. The invasion assay was performed in the same way as the migration assay except that the Transwell membrane was precoated with Matrigel (BD Biosciences, San Jose, CA, USA).

#### *Western Blot Analysis*

Proteins were extracted from transfected cells in radioimmunoprecipitation assay (RIPA) buffer (KeyGene Biotech, Nanjing, P.R. China) supplemented with protease inhibitors (Applygen Technologies Inc., Beijing, P.R. China) at 4°C for 30 min. After quantifying with BCA<sup>TM</sup> Protein Assay Kit (Pierce, Appleton, WI, USA), equal amounts of protein were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Boston, MA, USA). The membranes were then blocked for 1 h with 5% nonfat milk (Nestlé, Shuangcheng, P.R. China) at room temperature, followed by incubation at 4°C overnight with primary antibodies against mammalian B-cell lymphoma-2 (Bcl-2; ab32124), Bcl-2-associated X protein (Bax; ab77566), GAPDH (ab128915), E-cadherin (ab15148), vimentin (ab137321),  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA; ab5694),  $\beta$ -catenin (ab6302), Ki-67 (ab92742), glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ; ab131356), phosphorylated GSK-3 $\beta$  (p-GSK-3 $\beta$ ; ab131097), T-cell transcription factor 4 (TCF-4; ab185736), or leukemia enhancer factor 1 (LEF-1; ab52017) (all from Abcam, Cambridge, MA, USA). After rinsing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. Finally, the PVDF membranes were washed and transferred into the ChemiDoc<sup>TM</sup> XRS system (Bio-Rad, Hercules, CA, USA), followed by soaking in 200  $\mu$ l of Immobilon Western Chemiluminescent HRP Substrate (Millipore).

The signals were captured, and the intensity of the bands was quantified using Image Lab<sup>TM</sup> software (Bio-Rad). Protein levels were normalized to GAPDH expression.

#### *Statistical Analysis*

All the experiments were repeated three times. The results were presented as mean  $\pm$  standard deviation (SD). Statistical analysis was performed using GraphPad Prism 5 software (GraphPad, San Diego, CA, USA). The *p* values were calculated using the two-way analysis of variance (ANOVA) with Tukey's correction for comparison between three groups or two-tailed unpaired *t*-test for comparison between two groups. Values of *p* < 0.05 were considered significant.

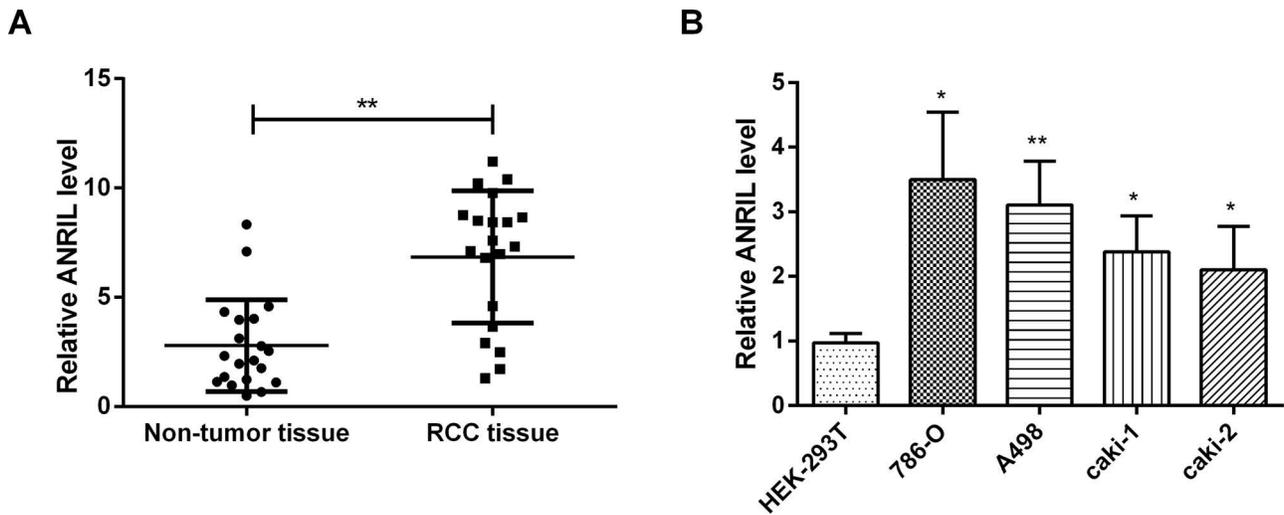
## RESULTS

#### *ANRIL Is Highly Expressed in RCC Tissues and Cell Lines*

To investigate the expression of ANRIL in RCC tissues and cell lines, qRT-PCR was performed. Compared with adjacent nontumor tissues, the expression of ANRIL in RCC tissues was significantly upregulated (*p* < 0.01) (Fig. 1A). When compared to HEK-293T cells, the expression of ANRIL was obviously upregulated in RCC cells, including 786-O cells (*p* < 0.05), A498 cells (*p* < 0.01), caki-1 cells (*p* < 0.05), and caki-2 cells (*p* < 0.05) (Fig. 1B). Hence, we drew the conclusion that ANRIL was highly expressed in RCC tissues and cells.

#### *ANRIL Promotes Proliferation but Inhibits Apoptosis of RCC Cells*

CCK-8 and colony formation assays were performed to assess RCC cell proliferation, and an apoptosis assay was performed to evaluate RCC cell apoptosis. ANRIL was overexpressed by infection of a lentiviral vector carrying ANRIL while being silenced by transfection of si-ANRIL. Cell viabilities of 786-O and A498 cells were markedly increased by ANRIL overexpression compared with the control group at 24, 48, and 72 h after transfection (*p* < 0.05) (Fig. 2A). Conversely, cell viabilities of 786-O and A498 cells were markedly decreased by ANRIL silencing when compared with the si-NC group at 24, 48, and 72 h after transfection (*p* < 0.05 or *p* < 0.01). The relative colony formations of 786-O and A498 cells were both markedly enhanced by ANRIL overexpression (*p* < 0.05 or *p* < 0.01), while both were markedly reduced by ANRIL silencing (*p* < 0.05 or *p* < 0.01) when compared with their respective controls (Fig. 2B). The effect of ANRIL on cell apoptosis was opposite to that of cell viability, resulting in a significant decrease (*p* < 0.05) in apoptotic rate by ANRIL overexpression but a significant increase (*p* < 0.05) in apoptotic rate by ANRIL silencing when compared to their respective controls (Fig. 2C).



**Figure 1.** ANRIL is highly expressed in RCC tissues and cells. The expression of ANRIL was significantly enhanced in RCC tissues (A) compared with adjacent nontumor tissues and RCC cells and (B) compared with normal cells. Data are expressed as mean  $\pm$  standard deviation (SD) of at least three independent experiments. \* $p < 0.05$ ; \*\* $p < 0.01$ . ANRIL, antisense noncoding RNA in the INK4 locus; RCC, renal cell carcinoma.

The expression level of apoptosis-associated proteins is shown in Figure 2D. The proapoptotic Bax was obviously downregulated by ANRIL overexpression while being upregulated by ANRIL silencing. The alteration of anti-apoptotic Bcl-2 was just the opposite. Therefore, we proposed that ANRIL could promote proliferation but inhibit apoptosis of RCC cells.

#### *ANRIL Promotes RCC Cell Migration and Invasion*

The influence of ANRIL on migration and invasion was tested by Transwell assay in 786-O and A498 cells. Cell migration and invasion were remarkably increased by ANRIL overexpression compared with the control group ( $p < 0.05$ ) while being remarkably decreased by ANRIL silencing when compared with the si-NC group ( $p < 0.05$  or  $p < 0.01$ ) (Fig. 3A and B). Subsequently, the expression of epithelial-to-mesenchymal transition (EMT)-related proteins was assessed by qRT-PCR and Western blot analysis. Both mRNA and protein expression levels of E-cadherin were obviously downregulated by ANRIL overexpression when compared with the control group ( $p < 0.05$ ), whereas the expressions of vimentin and  $\alpha$ -SMA were obviously increased by ANRIL overexpression compared with the control group ( $p < 0.001$ ) (Fig. 3C and D). The influence of ANRIL silencing on the expression level of EMT-related proteins was opposite to that of ANRIL overexpression. Hence, we concluded that ANRIL could promote RCC cell migration and invasion by enhancing EMT.

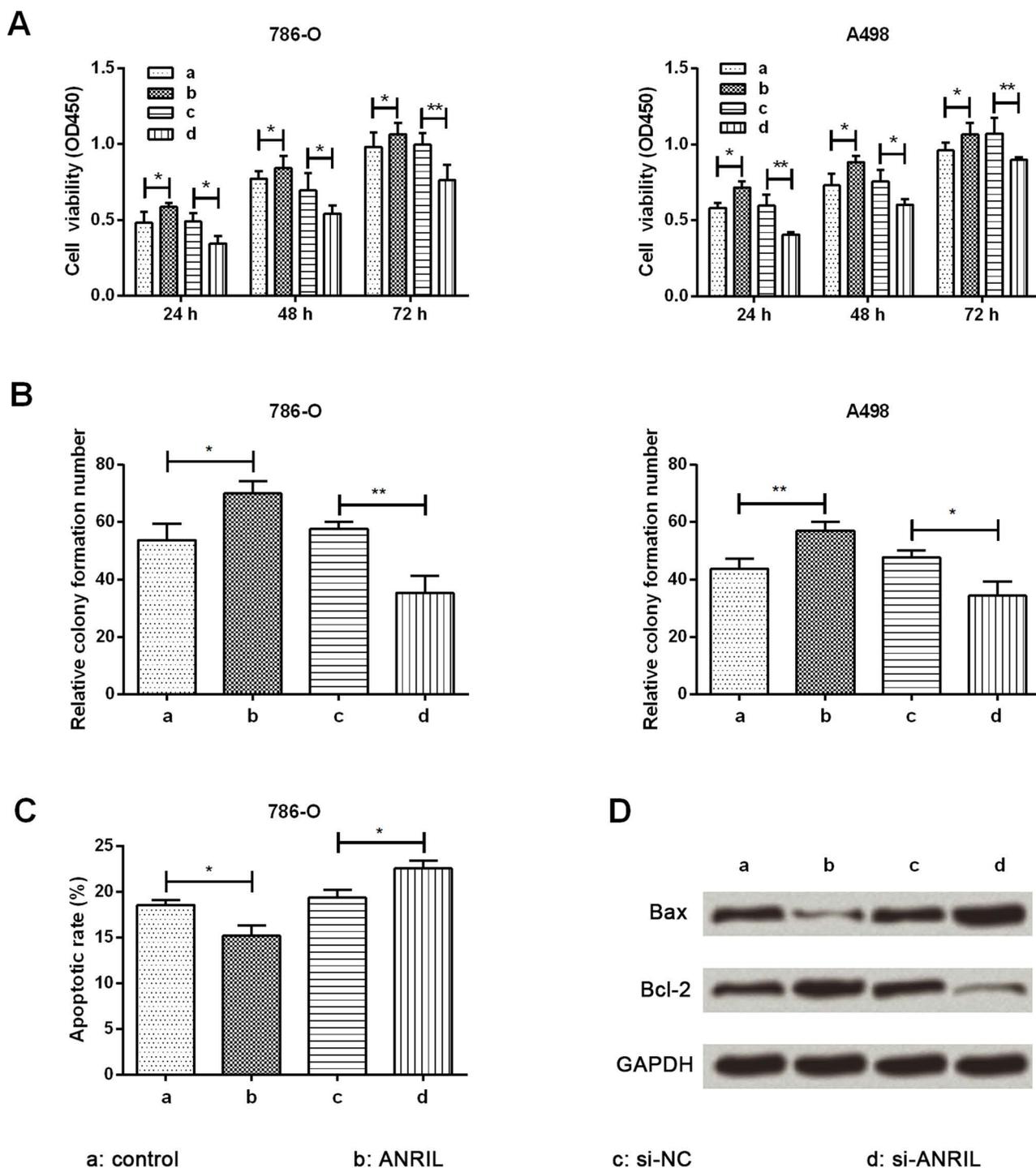
#### *ANRIL Activates the $\beta$ -Catenin Pathway*

Protein expression levels of growth-related Ki-67 and key kinases involved in the  $\beta$ -catenin signaling pathway

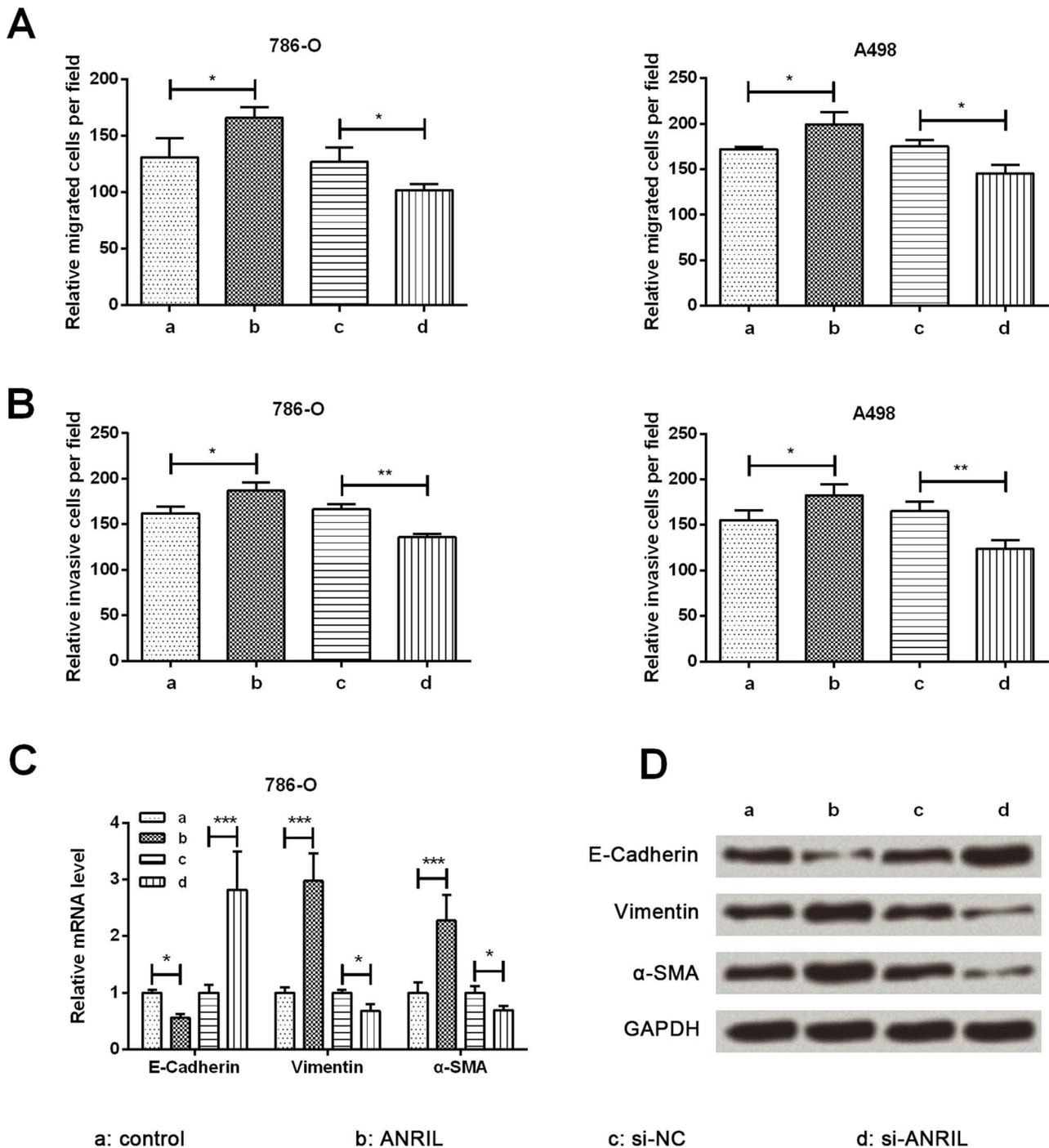
were evaluated using Western blot analysis. The expression levels of Ki-67,  $\beta$ -catenin, GSK-3 $\beta$ , and p-GSK-3 $\beta$  were upregulated by ANRIL overexpression while being downregulated by ANRIL silencing (Fig. 4A and B). Thereafter, the mRNA and protein expression of vital transcription factors were detected by qRT-PCR and Western blot analysis. mRNA and protein expression levels of TCF-4 and LEF-1 were obviously upregulated by ANRIL overexpression ( $p < 0.001$ ) while being downregulated by ANRIL silencing ( $p < 0.05$ ) (Fig. 4C and D) when compared to their respective controls. Taken together, this indicates that ANRIL could activate the  $\beta$ -catenin signaling pathway.

#### *Inhibitor of $\beta$ -Catenin Represses Carcinogenesis Induced by ANRIL*

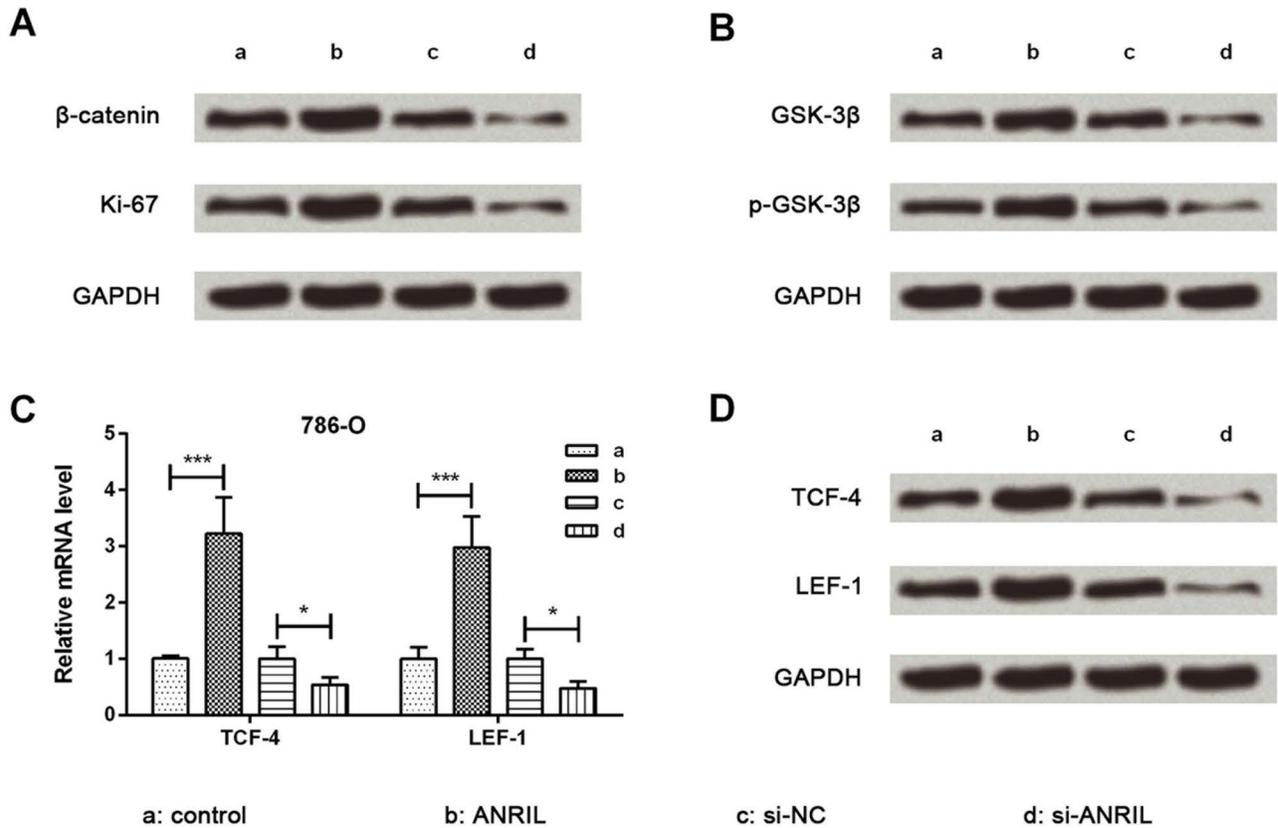
To verify the role of the  $\beta$ -catenin pathway in the carcinogenesis of ANRIL, the proliferation, migration, and invasion of 786-O cells were assessed by CCK-8 and Transwell assays. Cells were divided into three groups by transfection and IWR-endo stimulation. The expression of  $\beta$ -catenin was significantly upregulated by ANRIL, whereas upregulation was obviously reduced by IWR-endo induction (Fig. 5A). Cell viability results in Figure 5B show that at 24, 48, and 72 h after treatment, the increase in cell viability induced by ANRIL overexpression was remarkably reduced by IWR-endo stimulation when compared to the ANRIL group ( $p < 0.05$  or  $p < 0.01$ ). Increases in cell migration and invasion induced by ANRIL overexpression were remarkably reduced by IWR-endo stimulation when compared to the ANRIL group ( $p < 0.05$ ) (Fig. 5C and D). Thus, we suggest that inhibition of  $\beta$ -catenin represses the carcinogenesis induced by ANRIL.



**Figure 2.** ANRIL promotes cell proliferation while repressing cell apoptosis in RCC cells. (A) Cell viability by cell counting kit-8 (CCK-8) assay. The proliferation of 786-O and A498 cells was obviously promoted by ANRIL overexpression. (B) The number of colonies was markedly enhanced by ANRIL overexpression in 786-O and A498 cells. (C) Flow cytometry analysis showed cell apoptosis of 786-O cells. (D) Western blot analysis showed expression of apoptosis-associated proteins in 786-O cells. Data are expressed as mean  $\pm$  SD of at least three independent experiments. \* $p$  < 0.05; \*\* $p$  < 0.01. Bcl-2, mammalian B-cell lymphoma-2; Bax, Bcl-2-associated X protein; si-ANRIL, small interfering RNA targeting ANRIL; si-NC, negative control of si-ANRIL.



**Figure 3.** ANRIL promotes cell migration, invasion, and epithelial-to-mesenchymal transition (EMT) in RCC cells. Overexpression of ANRIL promoted migration (A) and invasion (B) in 786-O and A498 cells. Overexpression of ANRIL promoted EMT in 786-O cells at the mRNA level (C) and the protein level (D). Loss of E-cadherin and gain in vimentin and  $\alpha$ -SMA were observed in the ANRIL group cells. Data are expressed as mean  $\pm$  SD of at least three independent experiments. \* $p$ <0.05; \*\* $p$ <0.01; \*\*\* $p$ <0.001.  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin.



**Figure 4.** ANRIL activates the  $\beta$ -catenin signaling pathway. Western blot analysis showed that protein expression levels of  $\beta$ -catenin, Ki-67 (A), GSK-3 $\beta$ , p-GSK-3 $\beta$  (B), TCF-4, and LEF-1 (D) were all increased with the high expression of ANRIL in 786-O cells. Quantitative reverse transcription (qRT)-PCR showed that mRNA expression levels of TCF-4 and LEF-1 (C) were increased with the high expression of ANRIL in 786-O cells. Data are expressed as mean  $\pm$  SD of at least three independent experiments. \* $p < 0.05$ ; \*\*\* $p < 0.001$ . GSK-3 $\beta$ , glycogen synthase kinase 3 $\beta$ ; TCF-4, T-cell transcription factor 4; LEF-1, leukemia enhancer factor 1.

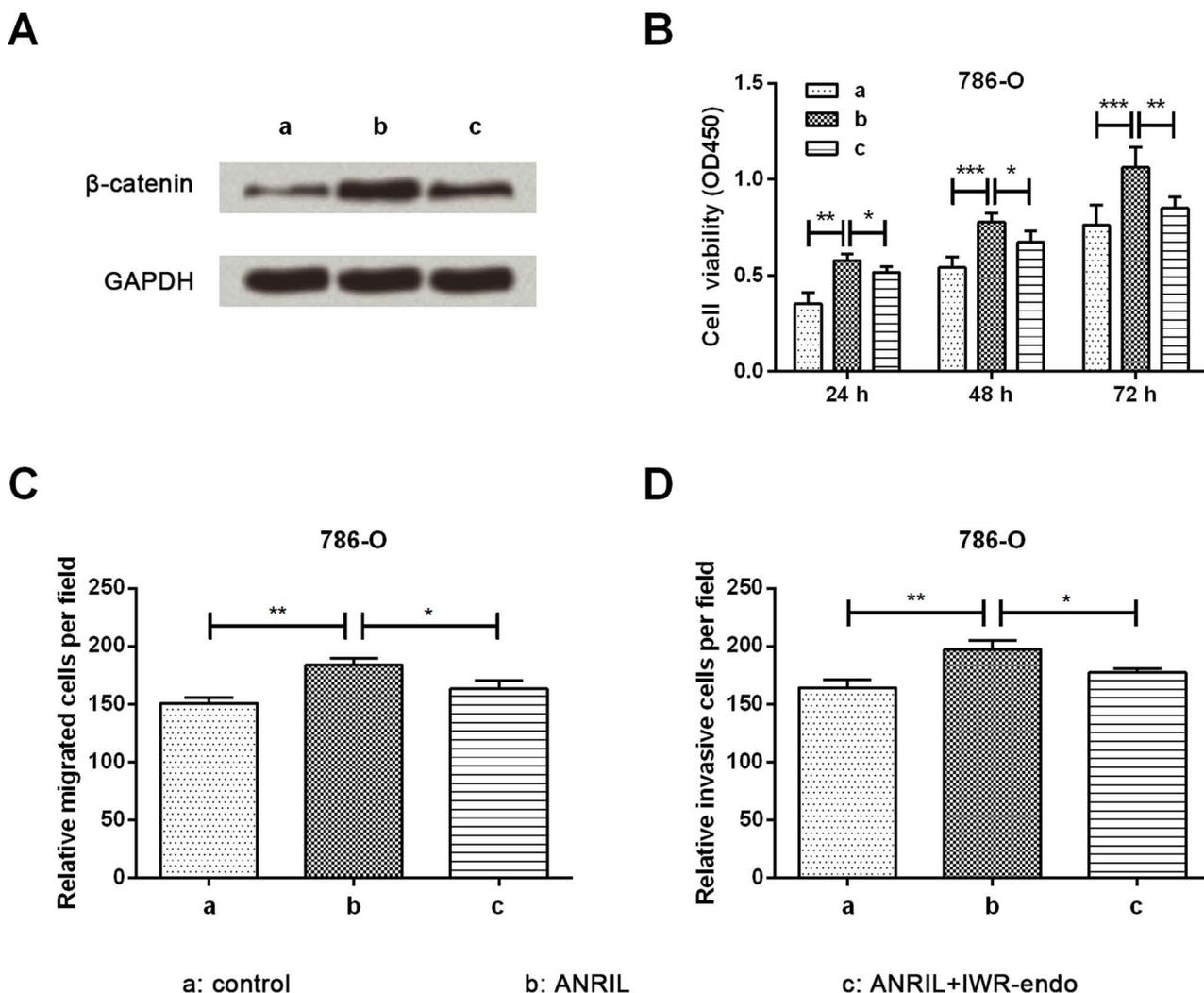
## DISCUSSION

RCC is a malignant tumor that accounts for about 2% of all new cancer cases worldwide, resulting in 38,890 new cases and 12,840 deaths in the US in 2006<sup>19</sup>. Except for the modern development of therapeutic approaches, the outcome of metastatic RCC remains unsatisfactory<sup>20</sup>. In our study, we first illustrated that ANRIL was markedly upregulated in both RCC tissues and RCC cells. By means of transfection, we subsequently identified that ANRIL overexpression obviously promoted cell proliferation, migration, invasion, and EMT but suppressed cell apoptosis. Moreover, the  $\beta$ -catenin pathway was activated by ANRIL overexpression. As proof, the effect of ANRIL overexpression was reversed by a  $\beta$ -catenin inhibitor.

ANRIL is a recently identified lncRNA that acts as an oncogene in multiple types of cancer. When compared to adjacent nontumor tissues, ANRIL has been reported to be remarkably upregulated in gastric cancer, lung cancer, hepatocellular carcinoma, esophageal squamous cell carcinoma, bladder cancer, and ovarian cancer<sup>21</sup>. However, ANRIL expression in RCC remains unclear. In our study,

we explored ANRIL expression not only in clinical RCC tissues but also in RCC cells. The results were in accordance with other types of cancers described previously, indicating an obvious upregulation of ANRIL in RCC. Thus, we hypothesized that ANRIL might be involved in RCC progression.

To better understand the modulation of ANRIL in RCC, we overexpressed and silenced ANRIL in 786-O and A498 cells through infection of a lentiviral vector with ANRIL or transfection of si-ANRIL. Subsequently, we investigated the differences in cell viability, colony formation, and cell apoptosis among the transfected cells. A previous study once reported that ANRIL promoted non-small cell lung cancer cell proliferation but inhibited apoptosis<sup>22</sup>. Meanwhile, another study indicated that ANRIL silencing inhibited the cell proliferation of gastric cancer cells<sup>23</sup>. In epithelial ovarian cancer, cell proliferation was promoted and apoptosis was inhibited by ANRIL<sup>24</sup>. As in previous investigations, proliferation was promoted and apoptosis was suppressed by ANRIL in RCC cells in our study. In addition, the proapoptotic



**Figure 5.** Observation of cell proliferation, migration, and invasion after IWR-endo stimulation. (A) Expression of  $\beta$ -catenin in 786-O cells by Western blot analysis. The upregulated expression of  $\beta$ -catenin induced by ANRIL was attenuated after IWR-endo stimulation. (B) Cell proliferation by CCK-8 assay. The promoting effect of ANRIL on cell proliferation of 786-O cells was attenuated after IWR-endo stimulation. Cell migration (C) and cell invasion (D) by Transwell assay. The promoting effects of ANRIL on cell migration and invasion of 786-O cells were both attenuated after IWR-endo stimulation. Data are expressed as mean  $\pm$  SD of at least three independent experiments. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

Bax was increased while the antiapoptotic Bcl-2 was decreased in ANRIL-overexpressed RCC cells, leading to a lower Bax/Bcl-2, which might be an explanation for alteration of RCC cell apoptosis.

As characteristics of cancer cells, migration and invasion have been proven to be promoted by ANRIL in various cancers, such as colorectal cancer<sup>25</sup> and ovarian cancer<sup>26</sup>. However, to our knowledge, there are only a few studies about the modulation of ANRIL on cell migration and invasion of RCC cells. Not surprisingly, migration and invasion were both markedly enhanced by ANRIL in 786-O and A498 cells. EMT is a process in which epithelial cells transdifferentiate into motile mesenchymal cells<sup>27,28</sup>. During EMT, E-cadherin is downregulated

along with the upregulation of N-cadherin, resulting in the alteration of cell adhesion and facilitation of cell migration and invasion<sup>29</sup>. Vimentin, which is known as the major intermediate filament protein of mesenchymal cells, has been reported to be obviously upregulated during EMT<sup>30</sup>. Similarly,  $\alpha$ -SMA is reported to be a marker of mesenchymal cells<sup>31</sup>. In our study, E-cadherin was significantly downregulated, and vimentin and  $\alpha$ -SMA were both upregulated, by ANRIL in RCC cells, indicating that EMT was promoted by ANRIL. This might be an explanation for ANRIL modulation on cell migration and invasion in RCC cells.

Ki-67 is a large nuclear protein and acts as a cellular marker for proliferation due to its presence at all active

phases of the cell cycle, but not in resting cells<sup>32</sup>. Recently, Ki-67 has been considered to be a potential target for cancer therapy. Moreover, activation of the  $\beta$ -catenin/TCF-4 complex could promote progression of RCC<sup>33</sup>. In addition, GSK-3 $\beta$  is a serine/threonine protein kinase whose inhibition leads to inhibition of cell proliferation and survival in RCC cells<sup>34</sup>. Thus, we subsequently explored the regulation of ANRIL on the expressions of Ki-67,  $\beta$ -catenin, GSK-3 $\beta$ , and p-GSK-3 $\beta$ , and we interestingly found that the expressions were all significantly enhanced by ANRIL.  $\beta$ -Catenin is considered to induce cancer through the activation of TCF/LEF family transcriptional factors, including TCF-4 and LEF-1<sup>35</sup>. LEF-1 expression is associated with RCC progression<sup>36</sup>. Therefore, we also detected the effect of ANRIL on the expression of TCF-4 and LEF-1 and, unsurprisingly, found these were both upregulated by ANRIL. Taken together, we made a hypothesis about the modulation of ANRIL in RCC<sup>37</sup>. Normally, the destruction complex, which is composed of Axin, adenomatous polyposis coli (APC), and GSK-3 $\beta$ , binds and phosphorylates  $\beta$ -catenin, followed by ubiquitination and degradation of  $\beta$ -catenin. When ANRIL is overexpressed, the  $\beta$ -catenin pathway is activated, resulting in the separation of the destruction complex. As a consequence, GSK-3 $\beta$  and  $\beta$ -catenin are both accumulated.  $\beta$ -Catenin then translocates to the nucleus to activate target genes, including Ki-67, TCF-4, and LEF-1, leading to the promotion of cell proliferation, migration, invasion, and EMT. Experiments that followed IWR-endo stimulation partially validated our hypothesis. Specifically, IWR-endo could increase the Axin level and then destabilize  $\beta$ -catenin to inhibit the Wnt/ $\beta$ -catenin signaling pathway<sup>38</sup>.

In conclusion, we first reported that ANRIL promoted cell proliferation, migration, invasion, and EMT but inhibited cell apoptosis in RCC cells. Moreover, the underlying mechanism might be connected with the activation of the  $\beta$ -catenin signaling pathway. The carcinogenesis of ANRIL provides a novel strategy for clinical diagnosis and treatment of RCC.

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## REFERENCES

- Lewis G, Maxwell AP. Early diagnosis improves survival in kidney cancer. *Practitioner* 2012;256(1748):13–62.
- Yun SA, Koo HC, Ji WJ, Kim A, Yong SK, Jung SH, Kim SH, Sung WK. A case of metastatic renal cell carcinoma bleeding of the pancreas manifesting as cholangitis. *Korean J Pancreas Biliary Tract* 2014;19(2):101–4.
- Renshaw AA. Subclassification of renal cell neoplasms: An update for the practising pathologist. *Histopathology* 2002;41(4):283–300.
- Fu L, Wang E, Shevchuk MM, Nanus DM, Gudas LJ. Expression of mutated, constitutively active HIF1 $\alpha$  in the mouse kidney results in early stage clear cell renal cell carcinoma [abstract 2857]. *Cancer Res.* 2011;71(8 Suppl).
- Mcdermott DF, Regan MM, Clark JI, Flaherty LE, Weiss GR, Logan TF, Kirkwood JM, Gordon MS, Sosman JA, Ernstoff MS. Randomized phase III trial of high-dose interleukin-2 versus subcutaneous interleukin-2 and interferon in patients with metastatic renal cell carcinoma. *J Clin Oncol.* 2005;23(1):133–41.
- Thomas AZ, Adibi M, Borregales LD, Hoang LN, Tamboli P, Jonasch E, Tannir NM, Matin SF, Wood CG, Karam JA. Surgical management of local retroperitoneal recurrence of renal cell carcinoma after radical nephrectomy. *J Urol.* 2015;194(2):316–22.
- Achawanantakun R, Chen J, Sun Y, Zhang Y. LncRNA-ID: Long non-coding RNA Identification using balanced random forests. *Bioinformatics* 2015;31(24):3897–905.
- Bozgeyik E, Igci YZ, Jacksi MFS, Arman K, Gurses SA, Bozgeyik I, Pala E, Yumurtas O, Temiz E, Igci M. A novel variable exonic region and differential expression of LINC00663 non-coding RNA in various cancer cell lines and normal human tissue samples. *Tumor Biol.* 2016(7):1–8.
- Berrondo C, Flax J, Messing EM, Beckham C. The long non-coding RNA HOTAIR affects exosome-mediated bladder cancer progression [abstract 152]. *Cancer Res.* 2015; 75(15 Suppl).
- Peng S, Ye LF, Cen Z, Tao P, Zhou XH. Long non-coding RNA XIST exerts oncogenic functions in human nasopharyngeal carcinoma by targeting miR-34a-5p. *Gene* 2016; 592(1):8–14.
- Zhang R, Hardin H, Chen J, Guo Z, Lloyd RV. Non-coding RNAs in thyroid cancer. *Endocr Pathol.* 2016;27(1):12–20.
- Qiu JJ, Yan JB. Long non-coding RNA LINC01296 is a potential prognostic biomarker in patients with colorectal cancer. *Tumor Biol.* 2015;36(9):7175–83.
- Pasmant E, Laurendeau I, Héron D, Vidaud M, Vidaud D, Bièche I. Characterization of a germ-line deletion, including the entire INK4/ARF locus, in a melanoma-neural system tumor family: Identification of ANRIL, an antisense noncoding RNA whose expression coclusters with ARF. *Cancer Res.* 2007;67(8):3963–9.
- Yi L, Zhou X, Ling X, Rong C, Shen C, Wei B. Long non-coding RNA ANRIL could be transactivated by c-Myc and promote tumor progression of non-small-cell lung cancer. *Oncotargets Ther.* 2015;9:3077–84.
- Xu Z, Chen J, Luk JM, Wei D. LncRNA ANRIL indicates a potential prognostic biomarker in gastric cancer and promotes tumor growth by silencing of miR-99a/miR-449a [abstract 157]. *Cancer Res.* 2015;75(15 Suppl).
- Congrains A, Kamide K, Ohishi M, Rakugi H. ANRIL: Molecular mechanisms and implications in human health. *Int J Mol Sci.* 2013;14(1):1278–92.
- Jafri M, Wake NC, Ascher DB, Pires DE, Gentle D, Morris MR, Rattenberry E, Simpson MA, Trembath RC, Weber A. Germline mutations in the CDKN2B tumor suppressor gene predispose to renal cell carcinoma. *Cancer Discov.* 2015; 5(7):723–9.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001;25(4):402–8.
- Ljungberg B, Campbell SC, Choi HY, Jacqmin D, Lee JE, Weikert S, Kiemeny LA. The epidemiology of renal cell carcinoma. *Eur Urol.* 2006;176(1):2353–8.
- Krishna VM, Noronha V, Prabhaskar K, Joshi A, Patil V, Bhosale B, Ravi T, Menon H, Gupta S, Banavali SD.

- Sunitinib in metastatic renal cell carcinoma: A single-center experience. *Indian J Cancer* 2013;50(5):268–73.
21. Li Z, Yu X, Shen J. ANRIL: A pivotal tumor suppressor long non-coding RNA in human cancers. *Tumor Biol.* 2016; 11(5):1–5.
  22. Nie FQ, Sun M, Yang JS, Xie M, Xu TP, Xia R, Liu YW, Liu XH, Zhang EB, Lu KH. Long noncoding RNA ANRIL promotes non small cell lung cancer cells proliferation and inhibits apoptosis by silencing KLF2 and P21 expression. *Mol Cancer Ther.* 2014;14(1):268–77.
  23. Zhang EB, Kong R, Yin DD, You LH, Sun M, Han L, Xu TP, Xia R, Yang JS, De W. Long noncoding RNA ANRIL indicates a poor prognosis of gastric cancer and promotes tumor growth by epigenetically silencing of miR-99a/miR-449a. *Oncotarget* 2014;5(8):2276–92.
  24. Qiu JJ, Wang Y, Liu YL, Zhang Y, Ding JX, Hua KQ. The long non-coding RNA ANRIL promotes proliferation and cell cycle progression and inhibits apoptosis and senescence in epithelial ovarian cancer. *Oncotarget* 2016;7(22):32478–92.
  25. Sun Y, Zheng ZP, Li H, Zhang HQ, Ma FQ. ANRIL is associated with the survival rate of patients with colorectal cancer, and affects cell migration and invasion in vitro. *Mol Med Rep.* 2016;14(2):1714–20.
  26. Qiu JJ, Lin YY, Ding JX, Feng WW, Jin HY, Hua KQ. Long non-coding RNA ANRIL predicts poor prognosis and promotes invasion/metastasis in serous ovarian cancer. *Int J Oncol.* 2015;46(6):2497–505.
  27. Marcucci F, Stassi G, De MR. Epithelial-mesenchymal transition: A new target in anticancer drug discovery. *Nat Rev Drug Discov.* 2016;15(5):311–25.
  28. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol.* 2014;15(3):178–96.
  29. Wheelock MJ, Shintani Y, Maeda M, Fukumoto Y, Johnson KR. Cadherin switching. *J Cell Sci.* 2008;121(Pt 6):727–35.
  30. Wunderlich KA, Tanimoto N, Grosche A, Zrenner E, Pekny M, Reichenbach A, Seeliger MW, Pannicke T, Perez MT. Retinal functional alterations in mice lacking intermediate filament proteins glial fibrillary acidic protein and vimentin. *FASEB J.* 2015;29(12):4815–28.
  31. Peter B, Jürgen F. The renal (myo-)fibroblast: A heterogeneous group of cells. *Nephrol Dialysis Transplant* 2012; 27(8):3027–36.
  32. Li XQ, Pei DS, Qian GW, Yin XX, Cheng Q, Li LT, Li HZ, Zheng JN. The effect of methylated oligonucleotide targeting Ki-67 gene in human 786-0 renal carcinoma cells. *Tumor Biol.* 2011;32(5):863–72.
  33. Zhao W, Zhou J, Deng Z, Gao Y, Cheng Y. SPOP promotes tumor progression via activation of  $\beta$ -catenin/TCF4 complex in clear cell renal cell carcinoma. *Int J Oncol.* 2016; 49(3):1001–8.
  34. Tsukigi M, Bilim V, Yuuki K, Ugolkov A, Naito S, Nagaoka A, Kato T, Motoyama T, Tomita Y. Re-expression of miR-199a suppresses renal cancer cell proliferation and survival by targeting GSK-3 $\beta$ . *Cancer Lett.* 2012;315(2):189–97.
  35. Idogawa M, Sato S, Shinomura Y, Imai K, Tokino T, Hirohashi S, Yamada T. Proteomic analysis of a nuclear complex containing  $\beta$ -Catenin and TCF-4. *Cancer Res.* 2006; 66:621.
  36. Shang D, Bi R, Han T, Wang D, Ye T, Liu Y. Expression and proliferation-promoting role of lymphoid enhancer-binding factor 1 in human clear cell renal carcinoma. *Cancer Invest.* 2014;32(7):368–74.
  37. Clevers H, Nusse R. Wnt/ $\beta$ -catenin signaling and disease. *Cell* 2012;149(6):1192–205.
  38. Lu J, Ma Z, Hsieh JC, Fan CW, Chen B, Longgood JC, Williams NS, Amatruda JF, Lum L, Chen C. Structure-activity relationship studies of small-molecule inhibitors of Wnt response. *Bioorg Med Chem Lett.* 2009;19(14):3825–7.