

LncRNA HOTAIR regulates HIF-1 α /AXL signaling through inhibition of miR-217 in renal cell carcinoma

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Long non-coding RNA HOTAIR was regarded as an oncogene in multiple cancers. Previous studies have shown that HOTAIR is involved in the proliferation and tumorigenesis of renal carcinoma cells, while microRNA (miR)-217 functions as a tumor suppressor in renal cell carcinoma (Rcc). However, the underlying molecular mechanism of HOTAIR in Rcc, especially in association with miR-217, has not been studied. In this study, we first demonstrated that HOTAIR expression was upregulated, which was correlated with tumor progression, and miR-217 downregulated in Rcc tissues and cells. Importantly, HOTAIR expression was negatively correlated with miR-217 expression in Rcc tissues. Gain- and loss-of-function of HOTAIR revealed that HOTAIR functioned as a ceRNA for miR-217 to facilitate HIF-1 α expression and then upregulated AXL level promoting Rcc proliferation, migration, and EMT process, and inhibiting apoptosis. Furthermore, HOTAIR knockdown suppressed tumor growth and reduced the expression of proliferation antigen ki-67, HIF-1 α , and AXL, but upregulated the expression of miR-217 *in vivo*. Finally, with AXL inhibitor BGB324, we confirmed that HOTAIR promoted Rcc activity through AXL signaling both *in vitro* and *in vivo*. In conclusion, these results suggest that HOTAIR promotes Rcc tumorigenesis via miR-217/HIF-1 α /AXL signaling, which may provide a new target for the diagnosis and therapy of Rcc disease.

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Renal cell carcinoma (Rcc) represents more than 90% of cases of kidney cancer, which is currently the 9th most common cancer in men and the 14th in women all over the world.¹ Rcc is mainly classified into three histological subtypes: clear cell (70–80%), papillary (10–15%), and chromophobe (5–10%) Rcc.² The incidence of Rcc varies between countries and is still increased worldwide.¹ Despite advances in imaging techniques, the diagnosis of early stage kidney cancer is very poor.³ In addition, the resistance to traditional therapies results in poor prognosis in Rcc patients.^{4,5} Therefore, it is urgent to find an effective method for early diagnosis and treatment of Rcc.

Long non-coding RNAs (lncRNAs), a heterogeneous class of transcripts longer than 200 nucleotides, are pervasively transcribed in the genome and associated with physiological and pathological processes.⁶ lncRNAs cannot code protein but can regulate gene expression at the transcriptional and post-transcriptional levels.^{5,6} Increasing evidence indicates that lncRNAs are involved in the modulation of cancer cell behavior, such as proliferation, metastasis, epithelial-mesenchymal transition (EMT), apoptosis, and drug resistance.⁵ HOX transcript antisense intergenic RNA (HOTAIR) is a lncRNA encoded by the human HOXC locus on chromosome 12q13.13.⁷ Increased expression of HOTAIR has been involved in the increased metastasis of breast, cervical, and colorectal cancer.^{8,9} Furthermore, HOTAIR is associated with the poor prognosis of pancreatic, breast, liver, and cervical cancer.^{8,10} Recent studies reported that HOTAIR expression is elevated in Rcc cells, and

silencing HOTAIR expression inhibits the proliferation and tumorigenicity of Rcc cells.¹¹ However, the underlying molecular mechanism of HOTAIR in Rcc has not been fully understood.

Recent reports have suggested that lncRNAs function as competing endogenous RNAs (ceRNAs) for microRNA (miRNA) to mediate mRNAs expression at post-transcriptional level.¹² HOTAIR regulates cyclin J expression via inhibition of miR-205 expression in bladder cancer.¹³ HOTAIR modulates HER2 expression through sponging miR-331-3p to promote the tumorigenesis of gastric cancer.¹⁴ miR-217 is a tumor suppressor in many cancers including Rcc.¹⁵ Recently, studies showed that the interaction between HOTAIR and PIK3R3 is mediated through miR-217 in ovarian cancer cells,¹⁶ but the interaction between HOTAIR and miR-217 in Rcc has not been reported before.

In this study, we hypothesized that HOTAIR might promote Rcc progression through inhibition of miR-217 expression. In the present study, we first detected the expression of HOTAIR and miR-217 in tumor tissues from clear cell Rcc patients as well as in Rcc cells. Furthermore, the underlying mechanism of HOTAIR in the development of Rcc was analyzed both *in vitro* and *in vivo*. This study may provide a new target for Rcc treatment.

Results

HOTAIR expression is negatively correlated with miR-217 in human renal cancer tissues. To verify whether HOTAIR is differently expressed in Rcc tissues, HOTAIR expression

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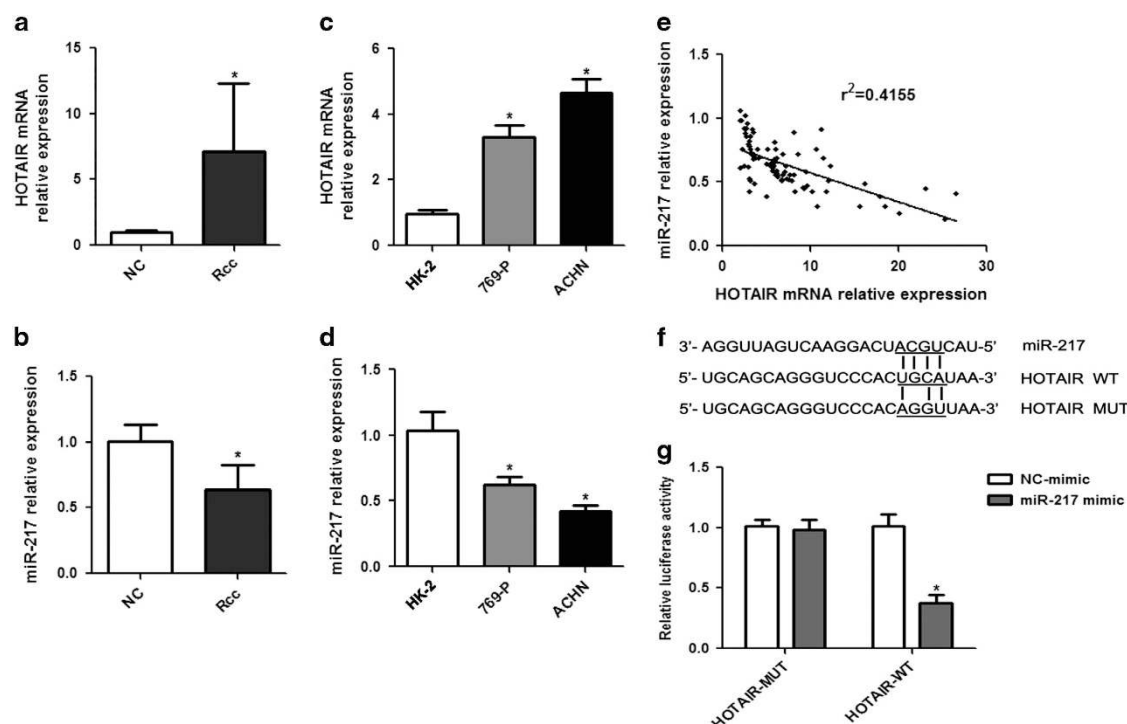


Figure 1 The expression of HOTAIR and miR-217 in Rcc tissues and cells and the relationship between HOTAIR and miR-217. qRT-PCR for the expression of HOTAIR (a) and miR-217 (b) in Rcc tissues and adjacent histological normal tissues. Data were analyzed by paired Student's *t*-test. The expression levels of HOTAIR (c) and miR-217 (d) were assayed in Rcc cells (769-P and ACHN) and normal kidney cells (HK-2). The expression of HOTAIR and miR-217 was normalized to that in HK-2. The differences between groups were analyzed by unpaired Student's *t*-test. (e) Bivariate correlation analysis of the relationship between HOTAIR expression and miR-217 level. (f) The putative miR-217 binding sequence of the wild type and mutation sequence of HOTAIR. (g) Relative luciferase assays. Statistical analysis was conducted using unpaired Student's *t*-test. **P* < 0.05

was determined in 86 paired Rcc samples and adjacent histological normal tissues. As shown in Figure 1a, compared with normal control, HOTAIR expression was notably upregulated in Rcc tissues. Interestingly, the elevated HOTAIR expression was positively correlated with the TNM stage (Table 1). Also, we detected miR-217 expression and found it was reduced in Rcc tissues (Figure 1b). Surprisingly, miR-217 expression was inversely correlated with HOTAIR levels in 86 Rcc tissue samples ($r^2=0.4155$, $P<0.0001$) (Figure 1e). Moreover, HOTAIR level was higher and miR-217 was lower in Rcc cell lines, including 769-P and ACHN cells, than in normal renal cells (HK-2; Figures 1c and d).

HOTAIR is a target of miR-217. To clarify the underlying relationship between HOTAIR and miR-217, we performed bioinformatics analysis by miRcode (<http://www.mircode.org>). The data showed that HOTAIR contains one conserved target site of miR-217 (Figure 1f), which is consistent with previous study.¹⁶ Moreover, miR-217 reduced the luciferase activity of pGL3-HOTAIR but not pGL3-HOTAIR-MUTANT (Figure 1g). These results indicate that miR-217 could bind directly to HOTAIR at the miRNA recognition sites.

HOTAIR modulates Rcc proliferation, migration, apoptosis, and EMT via negative regulation of miR-217. To further investigate whether HOTAIR functions through miR-217, HOTAIR expression was upregulated/downregulated and intervened with miR-217 mimic or anti-217 in Rcc cells. HOTAIR overexpression increased the proliferation,

Table 1 Correlation between HOTAIR expression and patient characteristics

Characteristic	Number (%)	HOTAIR expression	P-value
<i>Age</i>			0.544
≤ 60	53 (61.6)	6.86 ± 4.96	
> 60	33 (38.4)	7.56 ± 5.58	
<i>Gender</i>			0.796
Male	61 (70.9)	7.22 ± 5.12	
Female	25 (29.1)	6.90 ± 5.43	
<i>Tumor stage</i>			<0.001
T1	36 (41.9)	5.30 ± 3.97	
T2	25 (29.0)	8.12 ± 5.08	
T3	17 (19.8)	10.45 ± 3.02	
T4	8 (9.3)	19.13 ± 5.59	
<i>Lymph nodes metastasis</i>			<0.001
N0	65	5.56 ± 3.28	
N1	21	12.56 ± 6.79	
<i>Metastasis</i>			<0.001
M0	63	5.89 ± 4.13	
M1	23	11.69 ± 7.56	

migration, and the expression of Vimentin and Snail, but reduced E-cadherin expression in ACHN cells; however, this effect could be ablated by miR-217 mimic (Figures 2a, c, f, and h). In addition, HOTAIR downregulation significantly reduced the proliferation, migration, and the expression of Vimentin and Snail induced by TGF-β1, but facilitated

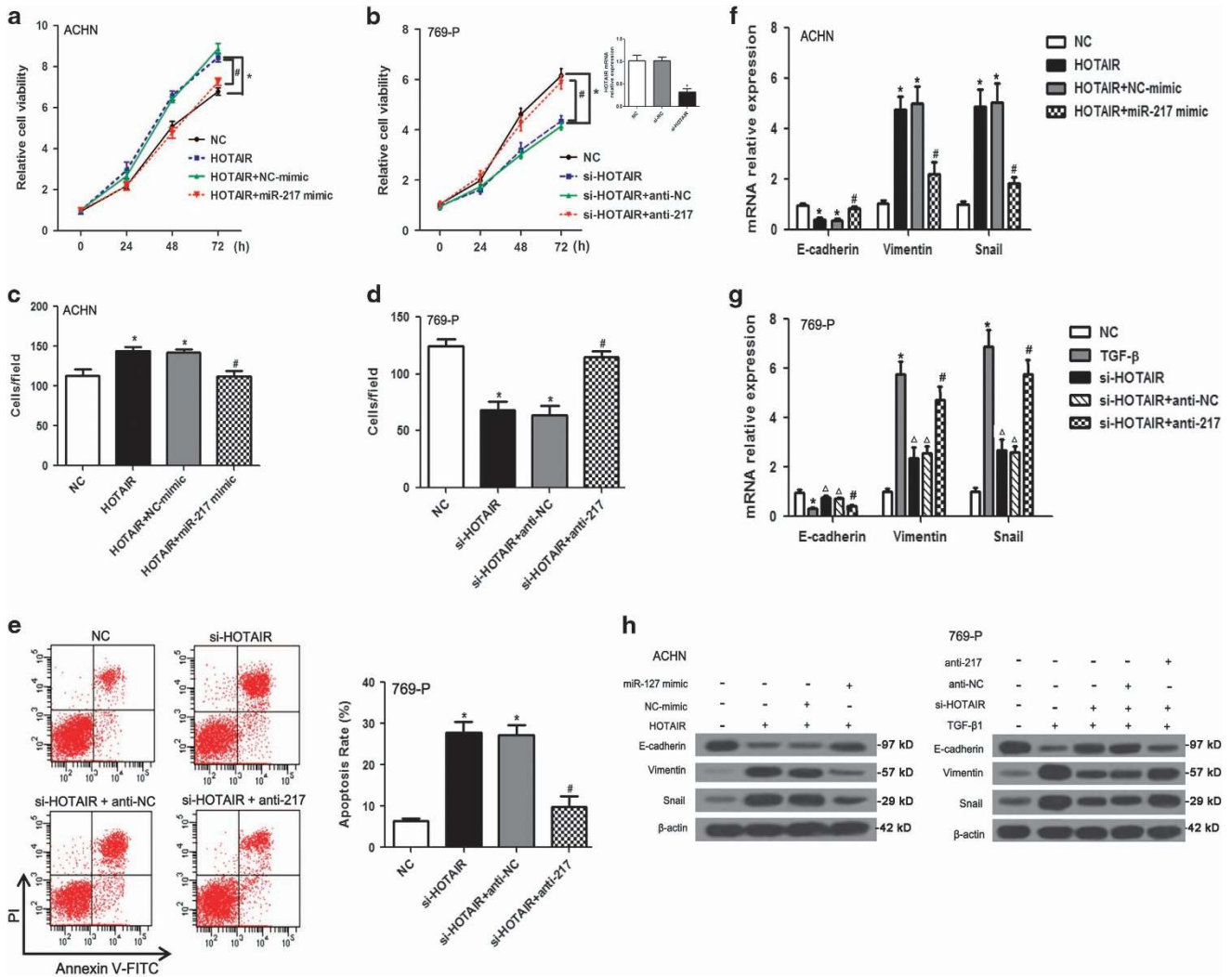


Figure 2 HOTAIR modulates Rcc proliferation, migration, apoptosis, and EMT via negative regulation of miR-217. Cell viability was determined by MTT assay in ACHN (a) and 769-P (b) cells. The inset graph illustrates the efficiency of si-HOTAIR on HOTAIR expression in 769-P cells. Transwell assays were used to analyze the migration of ACHN (c) and 769-P (d) cells. (e) Flow cytometry for apoptosis in 769-P cells. (f) qRT-PCR assay for the mRNA expression of E-cadherin, Vimentin, and Snail in ACHN cells. (g) The mRNA levels of EMT-related genes in 769-P cells. (h) The protein levels of EMT-related genes in Rcc cells. * $P < 0.05$ versus NC group, # $P < 0.05$ versus HOTAIR or si-HOTAIR group, $\Delta P < 0.05$ versus TGF- β group. Data were analyzed using One-way ANOVA

E-cadherin expression and the apoptosis of 769-P cells, which could be counteracted by anti-217 (Figures 2b, d, e, g, and h). These data suggest that HOTAIR promotes Rcc cell proliferation, migration, EMT, and inhibits apoptosis via downregulation of miR-217.

HOTAIR functions as a ceRNA for miR-217 to facilitate HIF-1 α expression. Through searching TargetScan (<http://www.targetscan.org>), we found that miR-217 could bind to the 3'UTR of HIF-1 α (Figure 3a). Moreover, miR-217 mimic significantly suppressed the mRNA and protein level of HIF-1 α in ACHN cells, while downregulation of miR-217 increased HIF-1 α expression in 769-P cells (Figures 3b and c). In addition, miR-217 remarkably dampened the luciferase activity of cells transfected with pGL3-HIF-1 α but not pGL3-HIF-1 α -MUTANT, suggesting a direct interaction between miR-217 and the 3'UTR of HIF-1 α (Figure 3d).

HOTAIR and HIF-1 α share the same response elements of miR-217 (Figures 1f and 3a). Hence, HOTAIR may act as a ceRNA for miR-217 to mediate HIF-1 α expression in Rcc development. To test this hypothesis, we first determined miR-217 expression after downregulation of HOTAIR and found si-HOTAIR significantly upregulated miR-217 levels in Rcc cells (Figure 3e). Then, we performed a RIP assay on Ago2, which is the vital component of the RNA-induced silencing complex (RISC).¹⁷ As shown in Figure 3f, overexpression of HOTAIR led to the increased enrichment of Ago2 on HOTAIR but substantially decreased enrichment on HIF-1 α transcripts. In parallel, knockdown of HOTAIR had the contrary effects. These results indicate that HOTAIR could compete with HIF-1 α transcripts for the Ago2-based RISC.

Moreover, we evaluated whether the HOTAIR-mediated sequestration of miR-217 was responsible for the upregulation

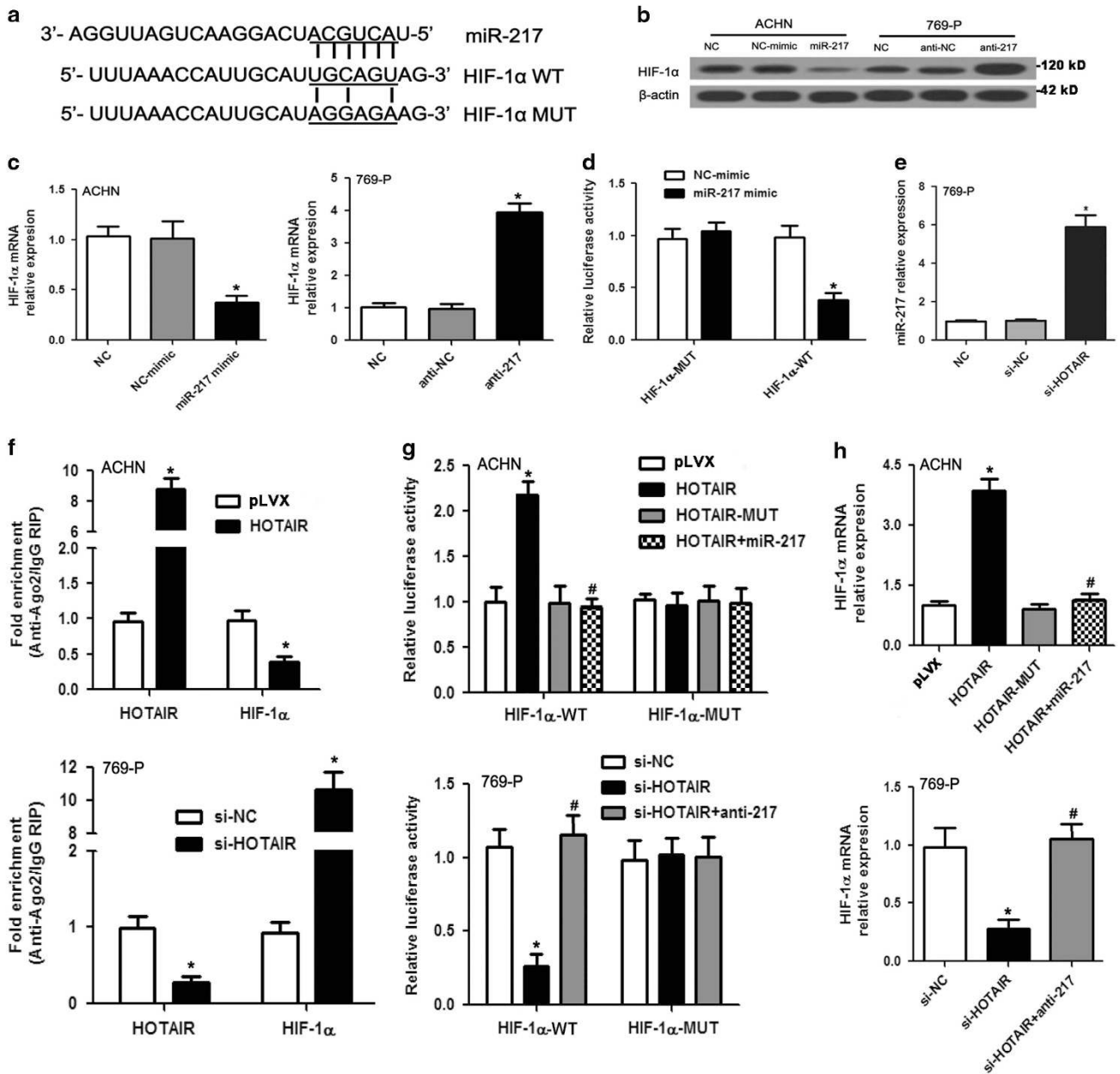


Figure 3 HOTAIR functions as a ceRNA for miR-217 to facilitate HIF-1 α expression. (a) The putative miR-217 binding 3'UTR of HIF-1 α (HIF-1 α -WT) and HIF-1 α mutation sequence (HIF-1 α -MUT). The protein (b) and mRNA (c) levels of HIF-1 α in ACHN and 769-P cells. * P < 0.05 versus NC group. (d) Luciferase activity assay. * P < 0.05 versus NC-mimic group. (e) The expression of miR-217 in 769-P cell transfected with si-NC or si-HOTAIR. * P < 0.05 versus NC group. Data were analyzed by unpaired Student's t -test. (f) RIP assay of the enrichment of Ago2 on HOTAIR and HIF-1 α transcripts relative to IgG in cells transfected with pLVX-HOTAIR or si-HOTAIR. (g) Luciferase activity of pGL3 reporters which contained wild type or mutant HIF-1 α 3'UTR with indicated treatment in Rcc cells. (h) qRT-PCR analysis for HIF-1 α mRNA expression in ACHN and 769-P cells. * P < 0.05 versus pLVX group, # P < 0.05 versus HOTAIR group in ACHN cells; * P < 0.05 versus si-NC group, # P < 0.05 versus si-HOTAIR group in 769-P cells. Statistical analysis was conducted using One-way ANOVA

of HIF-1 α . The luciferase activity of HIF-1 α wild-type reporters but not the mutant one was significantly increased after HOTAIR upregulation, whereas miR-217 mimic abolished this effect. And HOTAIR siRNA showed the reversed effect on the luciferase activity of HIF-1 α , which was rescued by anti-217 (Figure 3g). In addition, these results were further confirmed at HIF-1 α mRNA level (Figure 3h). Collectively, these results suggest that HOTAIR functions as a molecular sponge for miR-217 to facilitate HIF-1 α expression.

HOTAIR promotes Rcc tumorigenesis through HIF-1 α /AXL signaling both *in vitro* and *in vivo*. Studies have indicated that HIF-1 could bind directly to AXL and activate its expression,¹⁸ hence, to further investigate the mechanisms of HOTAIR, we detected AXL expression in Rcc cells. HOTAIR overexpression increased the mRNA and protein levels of AXL, whereas downregulation of HOTAIR notably decreased their expression (Figures 4a and b). To determine whether HOTAIR promotes Rcc activity through AXL signaling, an

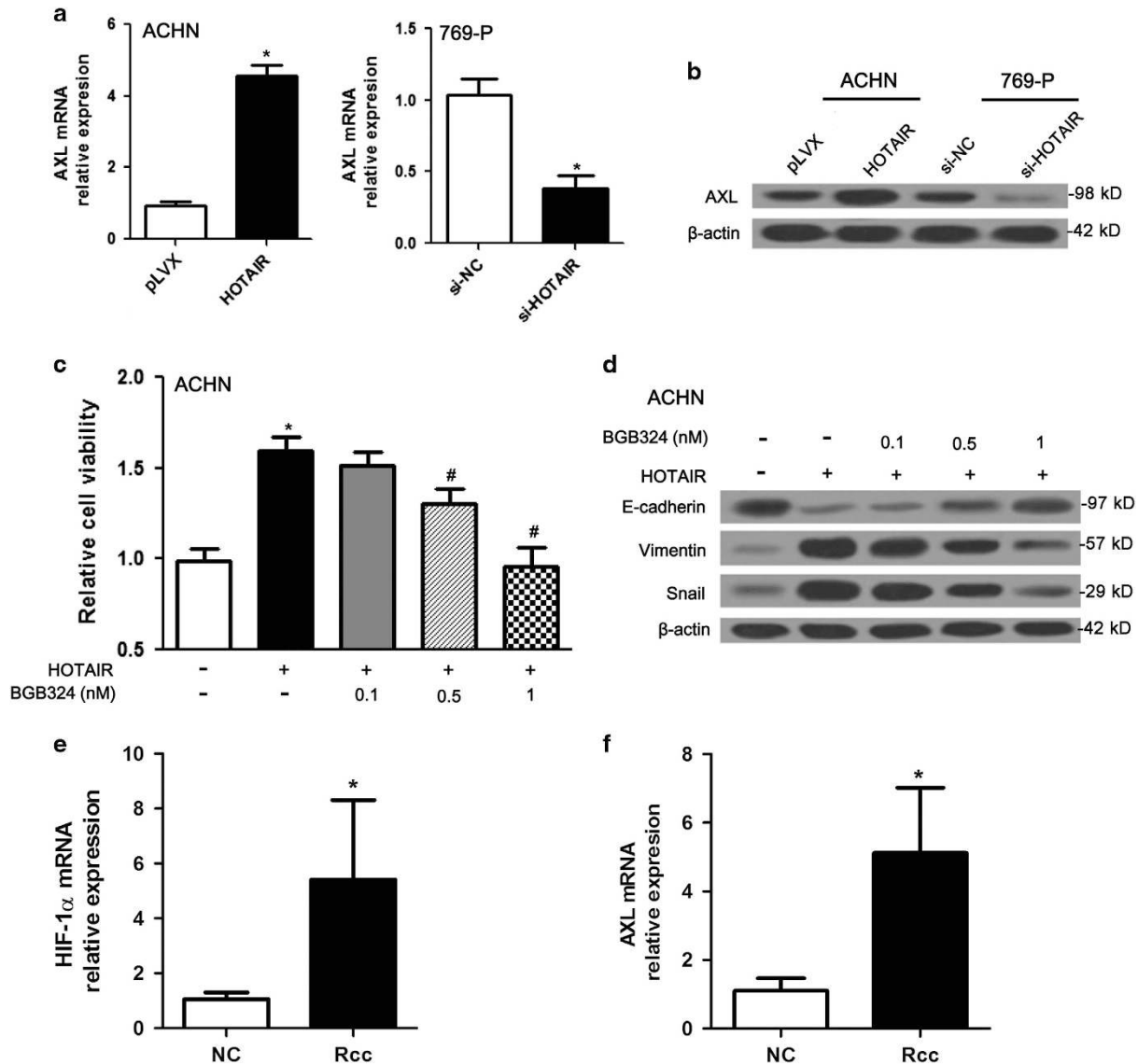


Figure 4 HOTAIR regulates Rcc activity through AXL signaling *in vitro*. The mRNA (a) and protein (b) expression of AXL in ACHN and 769-P cells. Statistical analysis was conducted by unpaired Student's *t*-test. (c) ACHN cells stably transfected with HOTAIR were treated with BGB324 (0.1, 0.5, 1 nM) for 72 h to examine the cell viability. Data were analyzed using One-way ANOVA. (d) The protein expression of E-cadherin, Vimentin, and Snail in ACHN cells stably transfected with HOTAIR and treated with different concentrations of BGB324 were assayed by western blot. The mRNA expression of HIF-1 α (e) and AXL (f) in 86 paired Rcc tissues and adjacent histological normal tissues. Data were analyzed paired Student's *t*-test. **P*<0.05 versus control group, #*P*<0.05 versus HOTAIR treated only group

AXL inhibitor BGB324 was used. The results showed that BGB324 suppressed the effect of HOTAIR upregulation on the proliferation and EMT process of ACHN cells in a dose-dependent manner (Figures 4c and d). Also, the mRNA levels of HIF-1 α and AXL were detected in 86 Rcc samples and adjacent histological normal tissues. As shown in Figures 4e and f, the mRNA expression of HIF-1 α and AXL in Rcc tissues was dramatically upregulated compared with normal control tissues. Furthermore, the role of HOTAIR in Rcc tumorigenesis was analyzed *in vivo* and the results showed that knockdown of HOTAIR significantly inhibited tumor growth and the staining intensity of proliferation antigen ki-67 (Figures 5a and b). HOTAIR downregulation reduced the expression of HIF-1 α and AXL, but elevated miR-217

levels (Figures 5c–f). In addition, the role of AXL in HOTAIR-mediated Rcc tumorigenesis was further confirmed by oral administration of BGB324. BGB324 notably decreased HOTAIR-induced tumor growth and EMT process (Figures 5g and h). Collectively, these data show that HOTAIR promotes Rcc tumorigenesis via activating HIF-1/AXL signaling.

Discussion

Studies have revealed that lncRNAs play vital roles in Rcc pathogenesis.¹⁹ However, the role and mechanism of HOTAIR in Rcc has not been fully understood. In this study, we found that HOTAIR expression was significantly increased in Rcc tissue samples compared with their corresponding non-tumor

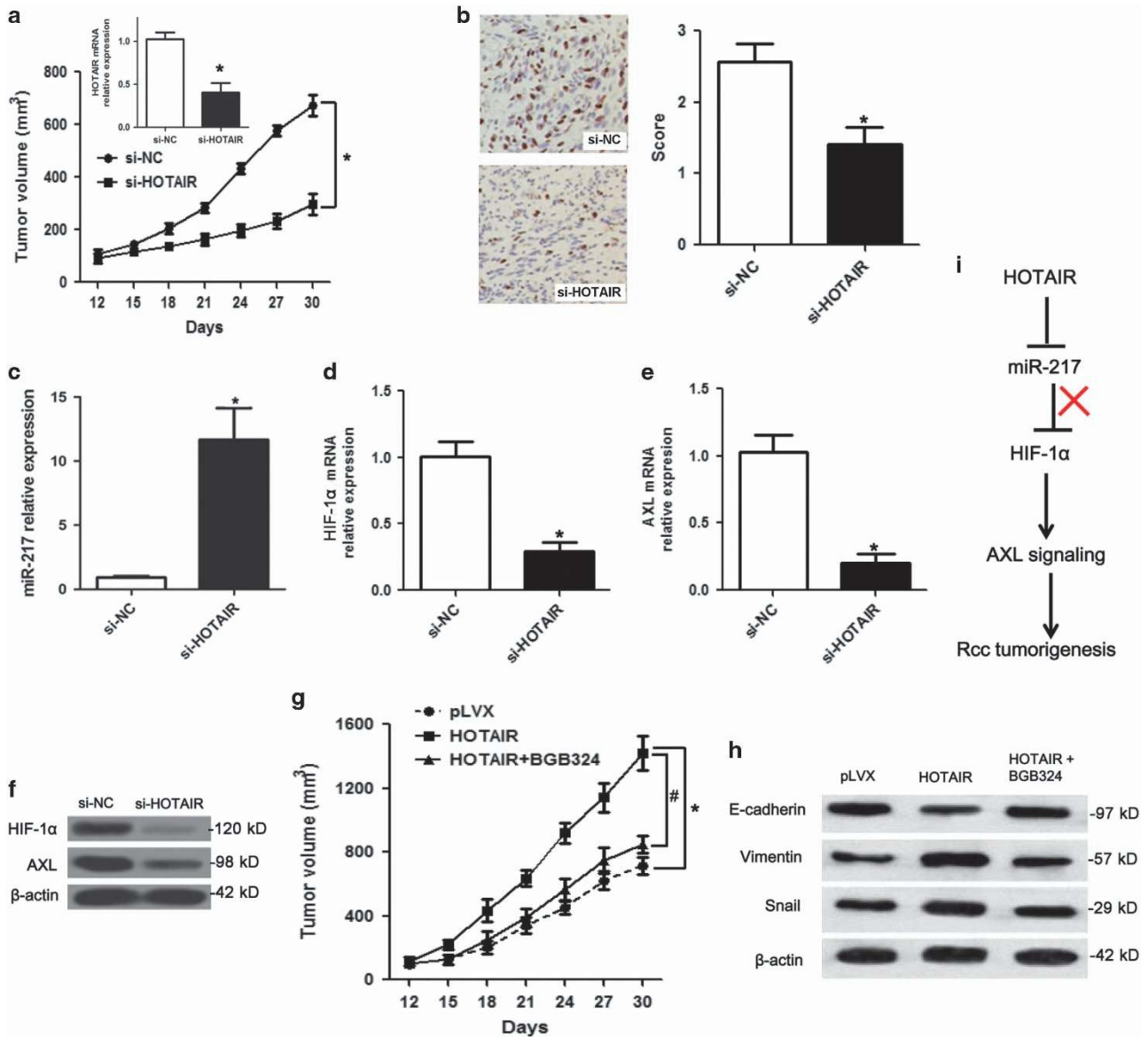


Figure 5 HOTAIR promotes Rcc cell growth via AXL signaling *in vivo*. (a) Tumor volume was evaluated for 12–30 days. The *inset* graph illustrates the efficiency of si-HOTAIR on HOTAIR expression. (b) Representative ki-67 staining (x100). mRNA expression of miR-217 (c), HIF-1 α (d), and AXL (e) in tumor tissues. Statistical analysis was conducted by unpaired Student's *t*-test. * $P < 0.05$ versus si-NC group (f) Western blot analysis for the protein levels of HIF-1 α and AXL in tumor tissues. (g) Mice were subcutaneously injected with ACHN cells stably transfected with HOTAIR and orally administrated with BGB324 twice daily. Tumor volume was examined. (h) The expression of E-cadherin, Vimentin, and Snail was determined by Western blot. Data were analyzed using One-way ANOVA. $n = 6$ per group, * $P < 0.05$ versus pLVX group, # $P < 0.05$ versus HOTAIR group. (i) Schematic of the proposed mechanism of HOTAIR in Rcc. HOTAIR functions as a ceRNA to 'sponge' miR-217 and upregulates the expression of HIF-1 α as well as the HIF-1 α downstream effector that promotes Rcc progression

tissues, and the elevated expression was positively correlated with the TNM stage (Figure 1). Also, HOTAIR expression was higher in Rcc cells. Studies have shown that HOTAIR acts as a ceRNA to sponge miRNAs to modulate the de-repression of miRNA targets in bladder and gastric cancer.^{13,14} Hence, we speculated that HOTAIR acts as a ceRNA in Rcc. miR-217 plays a suppression role in the growth of various types of tumors including Rcc.¹⁵ We further investigated miR-217 expression and found it was remarkably downregulated in Rcc tissues and cells. Furthermore, miR-217 expression was negatively correlated with HOTAIR level in Rcc tissues (Figure 1).

The results of bioinformatics analysis showed HOTAIR possesses one conserved target site for miR-217 binding. And dual-luciferase reporter assay demonstrated that miR-217 could reduce the luciferase activity of pGL3-HOTAIR (Figure 1). In addition, HOTAIR overexpression markedly increased the proliferation, migration, and EMT process, which were ablated by miR-217 mimic. Contrarily, knockdown of HOTAIR remarkably reduced the proliferation, migration, EMT, and promoted the apoptosis of 769-P cells, which could be counteracted by anti-217 (Figure 2). Taken together, these results indicate that HOTAIR promotes Rcc development via negative regulation of miR-217.

HIF-1 α is a transcription factor that is frequently stabilized and active in human clear cell Rcc.²⁰ It is largely responsible for activating the transcription of target genes that are widely involved in the malignant features of tumors.^{20,21} High levels of HIF-1 α are associated with the poor prognosis of clear cell Rcc.²² It has previously been reported that miR-217 increased HIF-1 α levels by targeting SIRT1 in rat glomerular mesangial cells cultured with high glucose.²³ However, in our study, the result of bioinformatics analysis revealed HIF-1 α is a potential target of miR-217. And miR-217 decreased HIF-1 α expression, which could be upregulated by anti-217. Also, miR-217 notably dampened the luciferase activity of pGL3-HIF-1 α . These results indicated that HIF-1 α was a direct target of miR-217. An RIP assay on Ago2 showed HOTAIR overexpression increased the enrichment on HOTAIR but significantly decreased the enrichment on HIF-1 α transcripts and knockdown of HOTAIR had the reversed effect. These results indicated that HOTAIR could compete with HIF-1 α transcripts for the Ago2-based RISC. Furthermore, the luciferase activity of HIF-1 α reporters and HIF-1 α mRNA expression were increased after HOTAIR overexpression, but reversed by miR-217 mimic; however, they were reduced when HOTAIR was downregulated, which could be rescued by anti-217 (Figure 3). Taken together, these results suggest that HOTAIR functions as a ceRNA for miR-217 to facilitate the expression of HIF-1 α .

Rankin *et al.*¹⁸ demonstrated that HIF-1 bound directly to AXL to activate its expression. AXL, a member of the TAM family of receptor tyrosine kinases (RTKs), has been identified as an essential mediator of cancer metastasis including Rcc.¹⁸ Binding of its ligand, growth arrest-specific protein 6 (Gas6), AXL receptor is dimerized and autophosphorylated at its tyrosine residues, which subsequently triggers a cascade of intracellular signaling, involving PI3K/Akt, ERK, NF- κ B, p38, Rho family proteins, JAK-STAT, and Src family kinases.²⁴ AXL oncogenic signaling promotes cancer cell survival, proliferation, migration, invasion, and the EMT phenotype, as well as resisting tumor cell apoptosis.^{25,26} In addition, AXL mediates a resistance to sunitinib treatment in Rcc.²⁷ AXL is highly expressed in Rcc tissues and cells, and knockdown of AXL reduces cell viability.²⁸ To evaluate whether HOTAIR regulates Rcc activity through HIF-1 α /AXL signaling, we detected AXL expression after gain- and loss-of-function of HOTAIR in Rcc cells. Our results revealed that overexpression of HOTAIR notably increased while downregulation of HOTAIR decreased AXL expression. Similarly, the expression of HIF-1 α and AXL in Rcc tissues was higher than in normal tissues in the clinical specimens. Moreover, BGB324 inhibited the effect of HOTAIR upregulation on the proliferation and EMT process of ACHN cells in a dose-dependent manner (Figure 4). In addition, HOTAIR knockdown significantly suppressed tumor growth and ki-67 expression, increased miR-217 levels and decreased the expression of HIF-1 α and AXL, and BGB324 abrogated the effect of HOTAIR on tumor growth and EMT process *in vivo* (Figure 5). However, si-HOTAIR treatment resulted in a > 50% decrease in tumor volume after 30 days, whereas treatment with the AXL inhibitor resulted in a much more modest reduction of < 20%, strongly implying that other AXL-independent pathways make important contributions. Previous study has reported that the PI3K/Akt

pathway can be regulated directly by miR-217 targeting PTEN (independent of AXL),²⁹ which may account for the role of HOTAIR in our study. Taken together, these results indicate that HOTAIR promotes Rcc tumorigenesis partly by activating HIF-1 α /AXL signaling.

In conclusion, our study revealed that HOTAIR is an oncogene in Rcc. Elevated level of HOTAIR is positively correlated with tumor progression. HOTAIR functions as a ceRNA for miR-217 to facilitate Rcc progression partly via HIF-1 α /AXL signaling (Figure 5). Thus, our study provides further insight into the molecular mechanism of HOTAIR in Rcc tumorigenesis, which may promote the development of lncRNA-directed diagnosis and therapy for this disease.

Material and Methods

Patients and samples. 86 clear cell Rcc tissue samples and neighboring non-cancerous tissues (collected postoperatively from June 2011 to January 2014) used in this study were obtained from Chinese PLA General Hospital. All patients provided informed consent and had not undergone any previous therapy. Tumors were classified according to the tumor-node-metastasis (TNM) system of classification (2010 version). This study was approved by the Human Research Ethics Committee of Chinese PLA General Hospital (Beijing, China). The clinical data of patients were shown in Table 1.

Cell lines. The human Rcc cell lines (769-P and ACHN) and normal kidney cell line (kidney proximal tubular cells, HK-2) were obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China) and cultured in a humidified incubator at 37 °C with an atmosphere of 5% CO₂. 769-P, ACHN, and HK-2 cells were maintained in RPMI-1640, MEM, and K-SFM medium, respectively.

Plasmid constructs and transfection. HOTAIR cDNA was amplified from human Rcc tissue with primers: 5'-CCGCTCGAGACATTCTGCCCTGATTCCGGAACC-3' (forward) and 5'-CGCGGATCCCCACCACACACACAACCTACAC-3' (reverse). cDNA was then cloned into the XhoI and BamHI sites of pLVX-IRES-Neo vector (Invitrogen) to construct the pLVX-HOTAIR vector. The vector was then transfected into HEK293T cells to package lentivirus using Lipofectamine 2000 (Invitrogen). ACHN cells were infected by the lentivirus and stably transfected cells were selected by G418 (0.5 mg/ml, Sigma-Aldrich, St Louis, MO, USA). To construct luciferase reporter vectors, HIF-1 α 3'-untranslated regions (UTR) and HOTAIR cDNA fragment containing the predicted potential miR-217 binding sites or mutant sites were amplified by PCR, and then cloned into the XhoI and KpnI sites of pGL3 luciferase reporter vector (Promega, Madison, WI, USA).

HOTAIR siRNA, control siRNA, miR-217 mimic, control mimic (NC-mimic), miR-217 antagonists (anti-217), and control antagonists (anti-NC) were synthesized by GenePharma Co. (Shanghai, China). The sequences were listed in Supplementary Table S1. To construct si-HOTAIR vector, the self-complementary hairpin DNA oligonucleotides were annealed and subcloned into the pEGFP-N1 plasmid vector. A negative control was named as si-NC vector. The vectors were transfected into 769-P cells with Lipofectamine 2000 and stable cell lines were established by G418 selection. Stable transfected cells were grown in 6-well plates and transfected using Lipofectamine 2000 according to manufacturer's instructions. Cells were collected for real-time PCR or western blot 48 h after transfection. The final concentrations of miRNAs or plasmids used in this study were as follows: HOTAIR/negative control 50 nM/ml, HOTAIR siRNA/control siRNA 30 nM/ml, miR-217 mimic/NC mimic 120 nM/ml, and anti-217/anti-NC 200 nM/ml.

Proliferation assay. Proliferation of cells transfected with indicated vector was measured by MTT assay kit at 24, 48, and 72 h, respectively. In addition, ACHN cells with stable HOTAIR upregulation were treated with BGB324 (0.1, 0.5, 1 nM), which also known as R428 (Selleck, Houston, TX, USA), for 72 h and the cell viability was detected.

Transwell analysis. Cells, transfected with the designated vector for 24 h, were seeded in the upper chambers in 200 μ l serum-free medium at a density of 2×10^4 /well. The lower chambers were filled with 500 μ l medium containing 10%

FBS for inducing cell migration. After 48 h incubation, the cells attached to the lower surface were fixed with 4% paraformaldehyde, stained with crystal violet, and then examined under a microscope.

Flow cytometry. Apoptosis was assayed through dual staining with annexin V-FITC and propidium iodide (PI; BD Biosciences, Franklin Lakes, NJ, USA). In brief, cells were harvested 48 h after transfection; Annexin V-FITC and PI were added to the cellular suspension according to the manufacturer's instructions. Then, samples were analyzed by a FACSCalibur flow cytometer (BD, San Jose, CA, USA).

Luciferase assay. 0.3 μ g of pGL3-HOTAIR (HOTAIR-WT), pGL3-HIF-1 α (HIF-1 α -WT), pGL3-HOTAIR-MUTANT (HOTAIR-MUT), or pGL3-HIF-1 α -MUTANT (HIF-1 α -MUT) was cotransfected with 40 nM miR-217 mimic or NC-mimic into HEK293T cells using Lipofectamine 2000. The luciferase activities were measured 48 h after transfection using the Dual-luciferase assay kit (Promega) according to the manufacturer's protocol.

RNA immunoprecipitation assay. RNA immunoprecipitation (RIP) was implemented by the EZ-Magna RIP RNA-binding protein immunoprecipitation kit (Millipore, Billerica, MA, USA). Cells were lysed into complete RIP lysis buffer. 100 μ l of cell lysate was incubated with RIP buffer containing magnetic beads conjugated with human anti-Ago2 antibody (1:50 dilution, Millipore) and negative control normal mouse IgG. Samples were incubated with Proteinase K buffer and then target RNA was extracted for further study.

Establishment of tumorigenicity models. Five-week-old female BALB/C athymic nude mice were obtained from the National Laboratory Animal Center (Beijing, China). The animals were acclimated for 7 days before the experiment. All animal protocols were approved by the Animal Ethics Committee of the Chinese PLA General Hospital and Military Medical College. To establish Rcc xenograft models, 4 \times 10⁶ 769-P cells stably transfected with si-HOTAIR or si-NC were inoculated subcutaneously into the dorsal right flank of each mouse. To validate the mechanism of HOTAIR *in vivo*, 4 \times 10⁶ ACHN cells with stable HOTAIR upregulation were injected into the mice. Mice were treated with BGB324 twice daily (oral administration) at a dose of 25 mg/kg 48 h after inoculation. Tumor diameter (mm) was measured every 3 days starting the 12th day after inoculation. Tumor volume (mm³) was calculated using the formula $V = (\text{shortest diameter})^2 \times (\text{longest diameter}) \times 0.5$. Tumor tissues were excised for further study in day 30.

Real-time PCR (qRT-PCR). Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA) and reversely transcribed into cDNA. Then, qRT-PCR was performed with Power SYBR Green (Takara, Kusatsu, Shiga, Japan). Data were normalized to GAPDH expression. The amplification and detection of miR-217 were using a TaqMan Human MiRNA Assay Kit (Applied Biosystems, Foster City, CA, USA) with normalization to U6. PCR primers were listed in Supplementary Table S2 and the relative expression levels were calculated using the 2^{- $\Delta\Delta$ Ct} method.

Western blot. Proteins were extracted by RIPA buffer. After determination of the protein concentrations, 30 μ g of proteins per lane were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% fat-free milk powder in TBST buffer and then exposed to antibodies against E-cadherin (Abcam, Cambridge, MA, USA), Vimentin (Abcam), Snail (Abcam), AXL (Abcam), and HIF-1 α (Cell signaling, Danvers, MA, USA) at 4 $^{\circ}$ C overnight. β -actin (Abcam) was used as an internal control.

Immunohistochemistry. The Rcc tissues were fixed with 10% neutral formalin, embedded in paraffin, and sectioned into 4- μ m thick slices. After dewaxing, rehydration, and antigen retrieval, the slides were then incubated with anti-Ki-67 antibody (Abcam), a HRP-conjugated secondary antibody, and DAB. Finally, the nuclei were counterstained with hematoxylin. Images were obtained under a light microscopy. Staining intensity in positive cases was graded on a scale of 0–3 (0, negative; 1, weakly positive; 2, moderately positive; 3, strongly positive).³⁰

Statistical analysis. All *in vitro* experiments were performed at least three times, independently. The results are expressed as mean \pm S.D. Student's *t*-test and One-way ANOVA were performed using SPSS 13.0 (NY, USA) to analyze the data including *in vivo* and *in vitro*. *P* < 0.05 was considered statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

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