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Long noncoding RNA ZFPM2-AS1 promotes the tumorigenesis of renal cell cancer *via* targeting miR-137

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Abstract. – OBJECTIVE: Recently, long noncoding RNAs (lncRNAs) have attracted more attention for their roles in tumor progression. The aim of this study was to investigate the role of lncRNA ZFPM2 antisense RNA 1 (ZFPM2-AS1) in the progression of renal cell cancer (RCC), and to explore the possible underlying mechanism.

PATIENTS AND METHODS: Expression levels of ZFPM2-AS1 in both RCC cells and 50 paired tissue samples were detected by Real Time-quantitative Polymerase Chain Reaction (RT-qPCR). Moreover, the relationship between lncRNA ZFPM2-AS1 expression level and clinic-pathological characteristics as well as patients' disease-free survival rate was explored, respectively. Furthermore, cell proliferation assay, wound healing assay and transwell assay were performed to investigate the role of lncRNA ZFPM2-AS1 *in vitro*. Luciferase reporter gene assay and RNA immunoprecipitation assay were to explore the possible underlying mechanism.

RESULTS: The expression of ZFPM2-AS1 in tumor tissues was significantly higher than that of corresponding normal tissues. ZFPM2-AS1 expression was associated with lymph node metastasis, tumor size and survival time of RCC patients. Moreover, the overexpression of ZFPM2-AS1 significantly promoted the growth, invasion and migration of tumor cells, whereas remarkably inhibited cell apoptosis *in vitro*. Furthermore, experiments revealed that miR-137 was a direct target of ZFPM2-AS1. In addition, miR-137 expression in tumor tissues was negatively correlated with ZFPM2-AS1 expression.

CONCLUSIONS: Our findings indicated that ZFPM2-AS1 could promote metastasis and proliferation, whereas inhibiting the apoptosis of RCC *via* targeting miR-137. This study might provide a new vision for interpreting the mechanism of RCC development.

Key words:
Renal cell cancer (RCC), LncRNA, ZFPM2-AS1, MiR-137.

Introduction

Renal cell carcinoma (RCC), the third general genitourinary malignancy is becoming more and more common in clinic. More than 330,000 patients are diagnosed with RCC annually. Nearly more than 140,000 patients die from RCC each year worldwide[1]. Fortunately, the morbidity and mortality of RCC have greatly increased over the past decades. The etiology of RCC is multifactorial and complicated, involving genetic and environmental factors[3]. Although numerous studies have been performed to explore the mechanisms of RCC deeply, real causes of RCC remain unclear. Therefore, it is urgent to further explore the possible underlying mechanism of genomic changes in RCC.

Most genomic transcripts are non-coding RNAs, in which long non-coding RNAs (lncRNA) are particularly widely studied. LncRNAs are a type of non-protein encoding RNAs longer than 200 nucleotides in length. Recently, it has been found that lncRNAs play important roles in cellular functions and carcinogenesis. For example, lncRNA TUG1 affects the viability of osteosarcoma cells by regulating glycolysis[4]. SNHG1 can inhibit Treg cell differentiation, thereby impeding the immune escape of breast cancer[5]. Meanwhile, lnc-p23154 play a crucial role in Glut1-mediated glycolysis, eventually accelerating oral squamous cell carcinoma metastasis[6].

A recent study[7] have demonstrated that ZFPM2 antisense RNA 1 (ZFPM2-AS1) promotes the progression of gastric cancer by attenuating the p53 pathway. However, the exact role of ZFPM2-AS1 in RCC as well as the possible underlying mechanism remains unknown. In this work, we found that the expression of ZFPM2-AS1 was significantly increased in RCC tissues. Besides, it promoted the migration, invasion and proliferation of

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RCC *in vitro*. Furthermore, our findings elucidated the interaction between lncRNA ZFPM2-AS1 and miR-137, as well as the possible mechanism.

Patients and Methods

Clinical Samples and Cell Lines

Totally 50 RCC patients who received surgery at Dalian Municipal Friendship Hospital were enrolled in this research. The informed consent was obtained from each patient before the study. No patient received radiotherapy or chemotherapy before the operation. Tissue samples were resected from the surgery and stored immediately at -80°C for subsequent use. All collected tissues were confirmed by an experienced pathologist. This investigation was approved by the Ethics Committee of Dalian Municipal Friendship Hospital.

Human kidney epithelial cell lines, including Caki-1, 769-P, 786-O, ACHN, and HK-2, were used in this study. Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Thermo Fisher Scientific, Waltham, USA) containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and penic in a 37°C, 5% CO_2 incubator.

Cell Transfection

After synthesis, the lentiv targetin ZFPM2-AS1 was cloned i the pti-EF1a-EGFP-F2A-Puro vector (tia In CA, USA). 293T cells we pac ZFPM2-AS1 lentivi (ZF S1), viruses and empty vector tol). RCC re transfected with mi mics (Gene na Biotechnology C d., hai, China) according to the inst tions. Non c siRNA was also transfect to cells as a n control.

RN tract and Real Time-qu olym ase Chain Reacti -qPC

otal R was extracted in strict ac- nce wit structions of TRIzol reagent (In en, Ca bad, CA, USA). Subsequentl RNAs were reverse transcribed to NAs ac ding to the instructions of Reverse scription Kit (TaKaRa Biotechnology Co., alian, China). Thermal cycle was as follow 0 sec at 95°C, 5 sec at 95°C for a total of 40 cycles, and 35 sec at 60°C. MiR-137 expression was normalized to U6. Primers used in this study were as follows: miR-137, F: 5'-AGGTCA GGCAGCATCGGGAA-3', R: 5'-AGGCCCTGTGGATATCGTCCAG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTA R: 5'-CGCTTCAGAATTTGCGTGT .

Luciferase Reporter Gene Assay

In our study, pGL3 vecto omega on WI, USA) was used for th nstruction UTR of ZFPM2-AS1, ell as -type 3'-UTR. Quick-char -di ed mutagenesis kit (Stratagen Ce , TX,) for site-directed mu nesis 137 ng site of ZFPM2-A -UTR and MUT) 3'-UTR. WT- MUT-3'-U nd miR-ctrl or miR-9 mic o-transfected into cells. 48 h l Luciferas y was detected by the Du ciferase reporte y system (Promega, M on, WI, USA).

Immunop itation A RIP)

NA-Binding Protein Immunoprecipita Kit (Millipore, Billerica, MA, USA) s used to perform RIP assay. Co-precipitated re detected by RT-qPCR .

Cell Proliferation Assay

Transfected cells were first seeded into 96-well plates. The proliferation capacity of transfected cells was detected by Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) every 24 h. Finally, absorbance at 450 nm was detected by a microplate reader (Bio-Rad, Hercules, CA, USA).

Wound Healing Assay

Cells were first seeded into 6-well plates and cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium (Gibco, Rockville, MD, USA) overnight. After scratching with a plastic tip, the cells were cultured in serum-free DMEM. Finally, wound closure was monitored at different time points.

Matrigel Assay

Totally 5 $\times 10^4$ cells in 200 µL serum-free RPMI-1640 were transferred to the upper chamber of an 8-µm pore size insert (Millipore, Billerica, MA, USA) coated with 50 µg Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). Meanwhile, RPMI-1640 and FBS were added to the lower chamber. 48 h later, the upper surface of

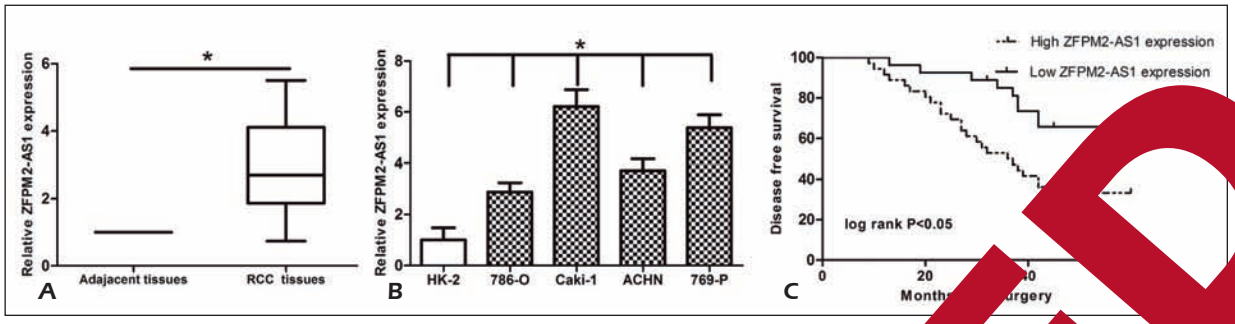


Figure 1. Expression level of ZFPM2-AS1 was significantly increased in RCC tissues and c[illegible]s. ZFP[illegible]S1 was as[illegible]ated with worse disease-free survival of RCC patients. **A**, ZFPM2-AS1 expression was sig[illegible]in[illegible]ed in RC[illegible] tissues when compared with adjacent normal tissues. **B**, The expression level of ZFPM2-AS1 [illegible]rma[illegible]ctin in [illegible]n RCC cell lines and HK-2 (human kidney epithelial cell line) was detected by RT-qPCR. **C**, [illegible]r level[illegible]2-A[illegible]s associated with worse disease-free survival of RCC patients. Data were presented as the [illegible] standard er[illegible]n. *p<0.05.

the chamber was wiped by a cotton swab and immersed in precooling methanol for 10 min. Subsequently, the cells were stained with crystal violet for 30 min. Three fields were randomly selected for each sample, and the number of invaded cells was counted.

Statistical Analysis

Statistical Product and Service Solu[illegible]s (SPSS) 17.0 (Chicago, IL, USA) was used[illegible] statistical analysis. Experimental data we[illegible]re-sented as mean ± SD. Chi-square test, St[illegible]t *t*-test, and Kaplan-Meier method were sele[illegible] when appropriate. p<0.05 was c[illegible]ered stat[illegible]tically significant.

[illegible]ults

ZF[illegible]-AS1 Level in [illegible]Tissues a[illegible]Cells

[illegible]e first detected the expression level of ZF-P[illegible]AS1 in 50 p[illegible]of RCC tissues and 4 RCC c[illegible]s by RT[illegible]CR. Results showed that ZF-PM[illegible]nificantly upregulated in tumor tissues ([illegible]1A). Meanwhile, ZFPM2-AS1 lev[illegible]l in RCC cells was remarkably higher than that [illegible]human kidney epithelial cells (Figure [illegible]). [illegible]alysis of clinic-pathological features in [illegible]CC patients demonstrated that upregulated ZF-PM2-AS1 was significantly correlated with lymph node metastasis and tumor stage (Table I). The

Table I. Correlation between li[illegible]F[illegible]ion and clinicopathological characteristics in RCC patients.

Characteristics	Pat[illegible]	Expression of lncRNA ZFPM2-AS1		*p*-value
		[illegible]ow-ZFPM2-AS1	High-ZFPM2-AS1	
Total	50	22	28	
Age (years				0.485
≤50		10	10	
>50		12	18	
Ge				0.124
	22	7	15	
Fe[illegible]	28	15	13	
[illegible]NM sta[illegible]				0.019
[illegible]I	27	16	11	
[illegible]-IV	23	6	17	
[illegible]				0.569
	25	12	13	
>3 cm	25	10	15	
[illegible]mphatic metastasis				0.006
	21	14	7	
	29	8	21	

50 patients were divided into two groups, lncRNA ZFPM2-AS1 high- and low-expression group, according to average number of the lncRNA ZFPM2-AS1 expression in tumor tissues. p<0.05 is considered as statistically significant.

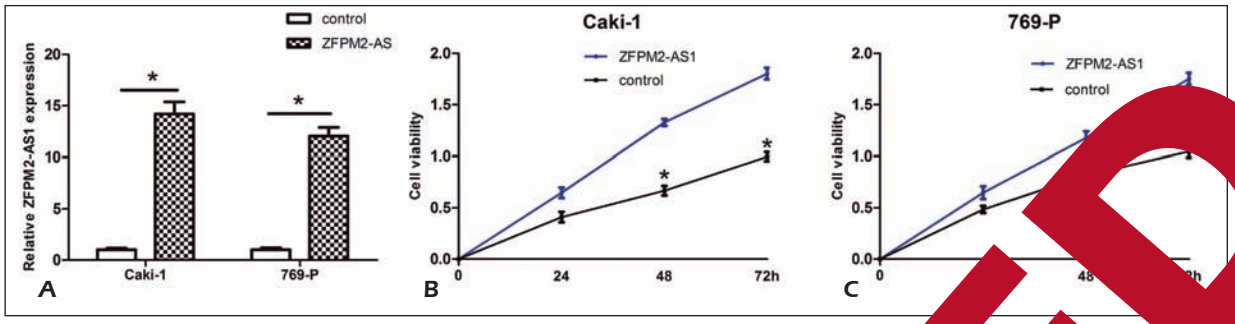


Figure 2. Overexpression of ZFPM2-AS1 increased the proliferation of RCC cells. **A**, ZFP S1 exp n in RCC s transfected with empty vector (control) or ZFPM2-AS1 virus (ZFPM2-AS1) was detected actin w used as an internal control. **B**, CCK-8 assay showed that overexpression of ZFPM2-AS1 signi antly the pro ion of Caki-1 cells. C, CCK-8 assay showed that the overexpression of ZFPM2-AS1 signific increas life of 769-P cells. The results represented the average of three independent experiments (mean dard error o p<0.05, as compared with the control cells. *p<0.05.

Kaplan-Meier analysis showed that RCC patients with higher ZFPM2-AS1 level had a significantly worse disease-free survival (Figure 1C).

Overexpression of ZFPM2-AS1 Promoted the Growth of RCC Cells

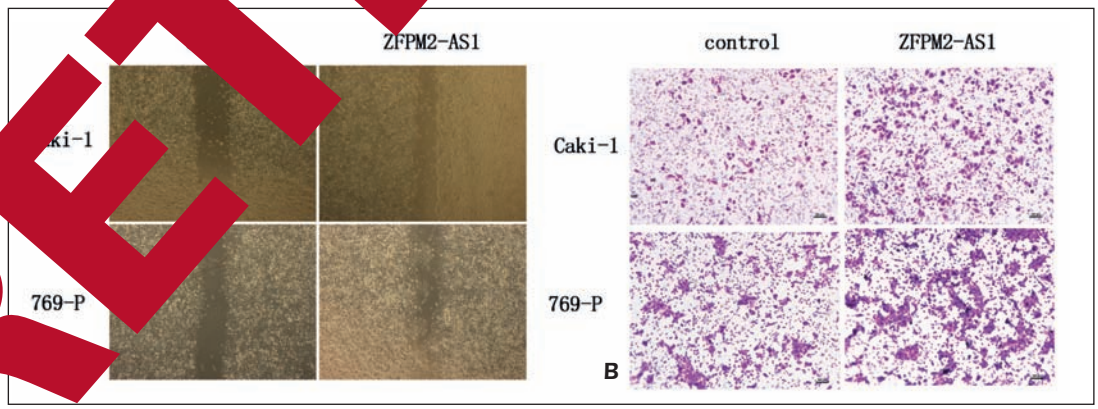
In our study, Caki-1, and 769-P RCC cell were chosen for ZFPM2-AS1 overexpress PM2-AS1 lentiviruses (ZFPM2-AS1) and ty vector (control) were synthesized and tran ed into these two cell lines. Then, RT-qPCR utilized to detect ZFPM2-AS1 ex ion (Fig 2A). Furthermore, CCK-8 ass d that th proliferation capacity of R cells s signifi- cantly promoted after Z -AS1 s- sion (Figure 2B and 2C).

Overexpression of ZFPM2-AS1 Promoted the Migration and Invasion of RCC Cells

Subsequent wound healing assay found that overexpressed ZF -AS1 remarkably promoted migration RCC cells (Figure 3A). Further Transwell assay indicated that the ZFPM2-AS overexpression remarkably promoted the invasion of RCC cells (Figure 3B).

Correlation Between miR-137 and ZFPM2-AS1 in RCC

DIANA LncBASE Predicted v.2 provided the miRNAs containing complementary bases with ZFPM2-AS1. Besides, RT-qPCR results showed that the expression level of miR-137 in RCC cells of the ZFPM2-AS1 group was significantly lower when



3. Overexpression of ZFPM2-AS1 increased the migration and invasion of RCC cells. **A**, Wound healing assay s at the migration ability in the ZFPM2-AS1 group was significantly promoted when compared with the control group. **B**, transwell assay showed that number of invaded cells in the ZFPM2-AS1 group was markedly increased when compared with the control group. The results represented the average of three independent experiments (mean ± standard error of the mean). *p<0.05, as compared with the control cells. *p<0.05.

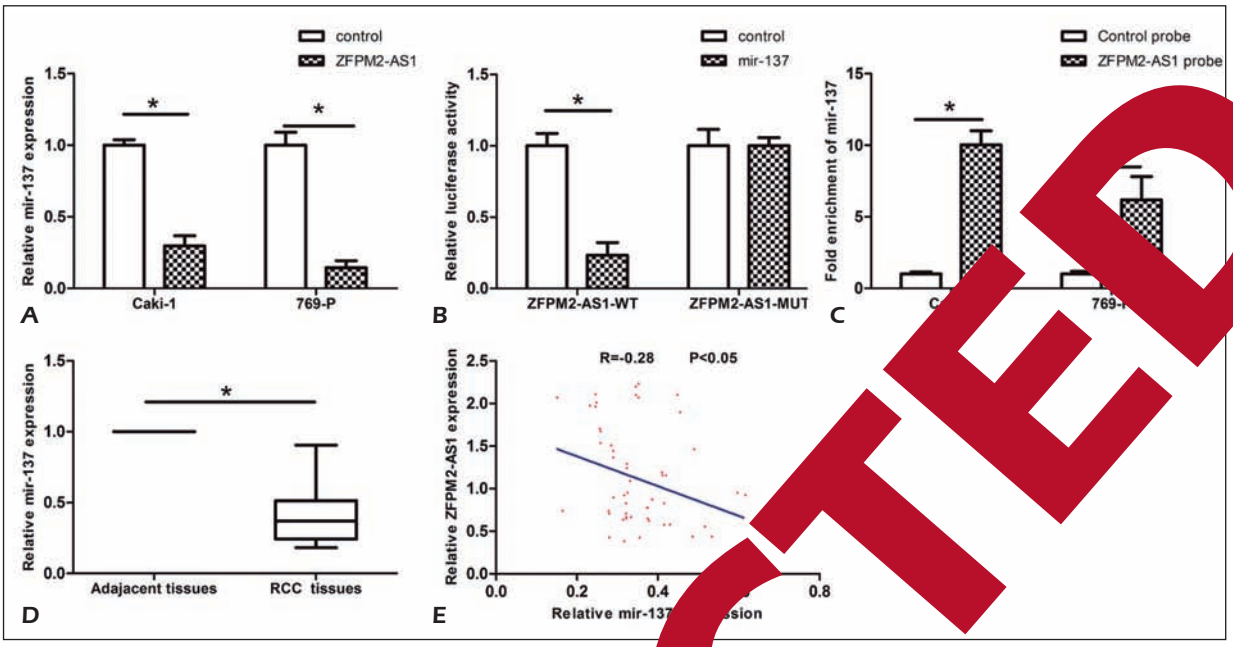


Figure 4. Interaction between ZFPM2-AS1 and miR-137. **A**, MiR[illegible]xpression in the [illegible]PM2-AS1 group was remarkably decreased when compared with the control group. **B**, Co-transfectio[illegible]R-137 and ZF[illegible]-AS1-WT in Caki-1 cells strongly decreased the Luciferase activity. However, co-transfection of miR-[illegible] ZFPM2[illegible]MUT did not change the Luciferase activity. **C**, RIP assay indicated that miR-137 was significantly enrich[illegible]-AS1 group when compared with the control group. **D**, MiR-137 was significantly downre[illegible]d in RCC tissu[illegible]ompared with adjacent normal tissues. **E**, The linear correlation between the expression level[illegible]d ZFPM2-AS1 in RCC tissues. The results represented the average of three independent experiments Data w[illegible]n ± standard error of the mean. *p<0.05.

compared with that of the control group (Figure [illegible] Furthermore, Luciferase assay rev[illegible] that the l[illegible] ciferase activity of ZFPM2-AS[illegible] transfec[illegible] ed with miR-137 mimics wa[illegible]nifica[illegible] reduced. However, no significant [illegible]s w[illegible] in the Luciferase activity of Z[illegible] M[illegible]s transfected with miR[illegible] (Figu[illegible] Meanwhile, RIP assay demons[illegible] that miR-[illegible]ld be remarkably enrich[illegible] ZFPM2-AS[illegible]p when compared wit[illegible] c[illegible]roup. This suggested that ZFPM2-AS1 might [illegible] a miR-137 sponge (Figure 4[illegible]We further fou[illegible] miR-137 expression in[illegible] tissu[illegible] was signific[illegible]ly lower than that of a[illegible]t nor[illegible]issues (Figure 4D). The correlation [illegible]onstrat[illegible] that miR-137 expression was neg[illegible]correla[illegible]with ZFPM2-AS1 expression in RC[illegible]gure 4E).

Discussion

Previous evidence has proved that lncRNAs p[illegible]te in tumorigenesis and development. Our stu[illegible] showed that ZFPM2-AS1 was significantly upregulated in RCC samples and cells. Meanwhile, ZFPM2-AS1 was correlated with tumor stage, lymph node metastasis and prognosis of RCC patients. Furthermore, after overexpression of ZFPM2-AS1, the growth, migration and invasion of RCC cells were significantly promoted. The above results indicated that ZFPM2-AS1 promoted tumorigenesis of RCC, which might act as an oncogene.

Latest studies have revealed that lncRNAs play a crucial function in cancer progression. By competing for shared miRNA response elements, lncRNAs participate in post-transcriptional regulation. This may further influence miRNA pathways[8,9]. In recent years, a new regulatory mechanism has been identified. Studies have found that, by competing for shared microRNAs (miRNAs) response elements, lncRNAs and mRNAs can interact with each other[8]. In this way, lncRNAs function as competing endogenous RNAs (ceRNAs) which can sponge miRNAs, thereby regulating the de-repression of miRNA targets. This reveals a new level of post-transcriptional regulation. For instance, lncRNA CHRF regulates Myd88 *via* sponging miR-489, further inducing cardiac hypertrophy[10]. By binding to HOTAIR in a sequence-specific manner, miR-141 has been found to downregulate HOTAIR expression in RCC cells. Furthermore, it inhibits cell prolifer-

ation and invasion[11]. LncRNA MALAT1 is remarkably upregulated in RCC cells and tissues. Hirata et al[12] have also observed the interaction between MALAT1 and miR-205. By sponging miR-335-5p, lncRNA RP11-436H11.5 promotes the proliferation and invasion of RCC cells[13].

MiR-137 has been reported to participate in multiple tumor processes. For example, miR-137 is downregulated in gastric carcinogenesis and acts as a tumor suppressor *via* targeting Cyclooxygenase-2 (Cox-2)[14]. Sequential miR-137 overexpression and DPN treatment can be used as a promising combination treatment to inhibit the growth of human glioblastoma cells[15]. Besides, miR-137 markedly inhibits cancer cell invasion and increases sensitivities to chemotherapy in pancreatic cancer[16]. Moreover, the knockdown of miR-137 can significantly promote the growth and metastasis of bladder cancer cells[17]. Furthermore, miR-137 is reported as a tumor suppressor in RCC[18,19].

In the present study, Luciferase reporter gene assay revealed that miR-137 was directly bound to ZFPM2-AS1. RIP assay indicated that miR-137 was significantly enriched by ZFPM2-AS1. In addition, miR-137 expression was signif ly downregulated after ZFPM2-AS1 ove sion. Moreover, miR-137 expression in R sues was negatively correlated with ZFPM2 expression. All the above results suggested ZFPM2-AS1 might promote the igenesis RCC *via* sponging miR-137.

Conclus

We showed th PM2-AS1 sion was remarkably up in RCC tiss nd was negatively re w ase-free survival of RCC patie s. Besides, 2-AS1 could promote the liferation of l s by sponging miR-1 hese findings sugg ted that lncRNA ZFP AS1 n contribute to RCC therapy as a ca t.

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Th thors declare no conflict of interest.

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