

P3H4 affects renal carcinoma through up-regulating miR-1/133a

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Abstract. – **OBJECTIVE:** P3H4, as a kind of quinone compounds, is a coenzyme Q10 (ubiquinone) analogues. A recent study investigated the role of P3H4 on cerebral ischemia-reperfusion and hypertension. It is well known that ischemia-reperfusion is closely associated with kidney disease. This study aims to investigate the impact of P3H4 in the occurrence and development of the tumor.

MATERIALS AND METHODS: A total of 40 rats at 8-week old were selected to establish renal cancer model by burying suture method for 4 months. The rats in the experimental group received P3H4 treatment at 100 mg/kg every 48 h for consecutive 4 weeks, while the rats in control received normal saline. H&E staining was applied to test the histological changes of tissue at 0, 1, 2 weeks after treatment. Gene microarray was adopted to screen miRNA expression to explore the function of miRNA in renal cancer cell line. Renal cancer cell migration and invasion were evaluated by wound healing and transwell assays after the treatment of P3H4, miR-1a, miR-133a, respectively.

RESULTS: The rats were randomly equally divided into experimental group and control. HE staining result showed renal interstitial fibroblasts hyperplasia, renal tubular necrosis, and glomerular reduction after modeling. P3H4 treatment significantly alleviated the lesion severity and inhibited the tumor cells invasion. Microarray demonstrated that miR-1a and miR-133a were significantly upregulated in rat renal cancer tissue after the treatment of P3H4. The overexpression of miR-1a and miR-133a markedly reduced renal cell invasion and migration, which was consistent with the effect of P3H4.

CONCLUSIONS: P3H4 suppressed the development of renal carcinoma through upregulating miR-1a and miR-133a, which provides fundamental leads for the future anti-cancer therapy.

Key Words:

P3H4, Renal cancer, miR-1a, miR-133a.

Introduction

The incidence of renal cancer in China accounts for the fifth among all kinds of tumors. Following air pollution and diet changes, there has been an increasing proportion of renal cancer patients in recent years. The death of renal cancer is mainly caused by the rapid growth and malignant invasion of tumor cells. Surgical resection is relatively the optimum treatment for renal cancer patients. However, about 80% of patients are accompanied with renal inflammation, leading to only 10-15% receiving surgical resection^{1,2}. Tumor size and other potential diseases restrict radical resection and cause recurrence. Therefore, appropriate medication is of necessity for the clinical application.

P3H4, also named Dub, is a kind of small molecular compounds belonging to quinone. Some studies^{3,4} mainly focused on its roles in cerebral ischemia-reperfusion and hypertension. However, the effect of P3H4 on cancer cells has not been well reported, especially in hypertension-related renal cancer. MiRNA is a type of small non-coding RNA that plays a significant role in regulating signaling pathway^{5,6}. Increasing evidence indicated that miR-133a was downregulated in renal cancer tissue, suggesting that miR-133a was a tumor suppressor factor. It was also revealed that miR-133a can restrain the invasion and metastasis of renal cancer cells^{7,8}. The inhibition of miR-133a markedly enhanced renal cancer cell motility. MiR-1a is a muscle-specific miRNA that closely related to cardiac development, arrhythmia, and myocardial hypertrophy, and it has been found that it can promote myoblast growth^{9,10}. Thus, we intend to discuss the impact of P3H4 on renal cancer as well as the mechanism via establish renal cancer model.

Materials and Methods

Experimental Rats and Cells

SD rats at 6-8 weeks old were selected for the investigations from Animal Monitoring Center of Central South University (Changsha, Hunan, China) and feed according to Specific Pathogen Free (SPF) grade. There were 20 male rats and 20 females. Renal cancer cell Renca was bought from Institute of Biochemistry and Cell Biology, SIBS, CAS (Beijing, China). The cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) medium (Thermo Fisher Scientific, Waltham, MA, USA) and cultured at 37°C and 5% CO₂.

Hematoxylin-Eosin (H&E) Staining

The rat tissue was fixed and dehydrated for embed. The tissue was cooled at 4°C and repaired, and was then cut into a slice at 4 µm. After baked at 65°C for 1 h, the slice was dewaxed and stained by hematoxylin for 1 min. It was stained by eosin for 10 s, dried and observed under the microscope. The slice was analyzed by three different pathologists.

RNA Extraction

The tissue was rinsed in liquid nitrogen and treated with 1 ml TRIzol (Invitrogen, Carlsbad, CA, USA). After 10 min at room temperature, the sample was added by 0.2 ml of chloroform at room temperature for 2-3 minutes. Then, the sample was centrifuged at 4°C and 12,000 g for 15 min, and the upper aqueous phase was moved to a new tube. Next, the sample was treated with 0.5 ml isopropanol at room temperature for 10 min. After centrifuged at 4°C and 12,000 g for 10 min, the RNA sediment could be observed on the bottom of the tube. Then, the sample was washed with 1 ml ethanol at 75% and centrifuged at 4°C and 7,500 g for 5 min. After drying, the RNA was solved in 20 µl DEPC water and tested on a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). At last, the RNA was reverse transcribed to cDNA or stored at -80°C.

MiRNA Array Detection

Total RNA was centrifuged in 96 well plate and reverse transcribed to cDNA. In brief, the reaction solution (Thermo Fisher Scientific, Waltham, MA, USA) was prepared according to the instruction, including 2 µg total RNA, 1 µl oligo primer (50 µM), 1 µl dNTP mix (10

µM), and ddH₂O. The solution was pre-degenerated at 65°C for 5 minutes. Next, cDNA first chain synthesis reaction system was prepared, including 2 µl 10 × RT buffer, 4 µl MgCl₂ (25 µM), 2 µl DTT (0.1 M), 1 µl RNAase OUT (40 U/µl), 1 µl SuperScript III RT (200 U/µl), and ddH₂O. The reaction condition was composed of 50°C for 50 min and 85°C for 5 min. Real-Time Polymerase Chain Reaction (RT-PCR) was then performed by Affymetrix (PAMM-015Z; Santa Clara, CA, USA) (7.5 µl 2 × premix, 10 mM forward and reverse primers, dH₂O to a final volume of 15 µl) in the following condition: 94°C denature for 30 sec, followed by 40 cycles each containing 94°C denature for 5 sec, and 60°C annealing for 30 sec with LightCycler 480 (Roche, Indianapolis, IN, USA).

MiR-133a and miR-1 Transfection

Renca cells in logarithmic phase were seeded in 6-well plate at 3-8 × 10⁵ cells/well. A total of 5 µl lipo2000 was mixed in 100 µl serum free medium for 5 min, while 12 µl miR-133a and miR-1 plasmids were mixed in 100 µl serum free medium. After well mixing, they were added to 1800 µl serum-free medium for the following experiments.

MTT Assay

Renca cells were seeded in 96 well plate at 5000 cells/well. A total of 20 µl MTT solution at 5 mg/ml (pH = 7.4). After 4 h incubation, the plate was added with 150 µl Dimethyl sulfoxide (DMSO) for 10 min. At last, the plate was detected on microplate reader to determine cell growth.

Wound Healing Assay

Renca cells in logarithmic phase were seeded in 6-well plate at 5 × 10⁵ cells/well. The cells were scratched to draw “+” and took a photo every 12 h.

Transwell Assay

The cells were resuspended and seeded in the upper chamber of the transwell at 37°C for 3 h. After washed by phosphate-buffered solution (PBS), the cells were maintained in 200 µl serum free DMEM, whereas the lower chamber was added by 600 serum-free medium at 37°C for 16 h. Next, the membrane was fixed by 4% paraformaldehyde for 40 min. At last, the cells were observed under the microscope for calculation after stained by DAPI for 3 min.

Statistical Analysis

All the data was presented as ($\bar{x} \pm s$), and the analysis was performed on Image-Pro-Plus6.0 and Graph Pad Prism 5. The *t*-test was used for the intergroup comparison. One-way ANOVA with the Tukey's post hoc-test was conducted for multiple groups comparison. *p*-values < 0.05 were considered statistically significant.

Results

HE Detection of Rat Renal Tissue

H&E staining was performed on the 7th day and 14th day after P3H4 treatment. On the 7th day, the renal medulla gradually faded away, and the inflammatory cells infiltration increased. Compared with control, the test group showed fewer expanded renal tubules, better glomerulus integrity, and lighter inflammation infiltration. On the 14th day, both test group and control presented the aggravation trend. A large amount of renal interstitial fibroblasts hyperplasia, renal tubular necrosis, and glomerular number reduction was found from the rats in control group. However, the lesion degree in the test group was lower than that in control (Figure 1).

P3H4 Inhibited Renal Cancer Cell Migration and Invasion

Previous results confirmed that P3H4 treatment affected gene expression in renal cancer tissue, whereas its impact on renal cancer cell

line was still controversial. Wound healing assay proved that cell migration ability was markedly blocked in the test group compared with that in the control (Figure 2A). Furthermore, transwell assay also revealed that cell invasion ability was restrained in the test group (Figure 2B).

P3H4 Upregulated miR-1a and miR-133a

MiRNA array analysis was performed to detect the changes of miRNAs expressions induced by P3H4. Of note, the data showed that both of miR-1a and miR-133a were remarkably upregulated compared with that in control, while the other miRNAs showed no statistical significance between the two groups (Figure 3A). Real-time PCR further certified the results that P3H4 significantly increased the level of miR-1a and miR-133a (*p* < 0.001) (Figure 3B).

MiR-1a/133a Cluster Restricted Renal Cancer Cell Migration and Invasion

Renca cells were transfected by miR-1a and miR-133a plasmids, respectively. After 6 and 12 h, the overexpression of miR-1a and miR-133a significantly declined Renca cell migration and invasion similarly to P3H4 (Figure 4).

Discussion

Renal cancer is a kind of solid tumor with high malignancy. Its etiology is relatively complex. Abnormal gene expression may give rise to the

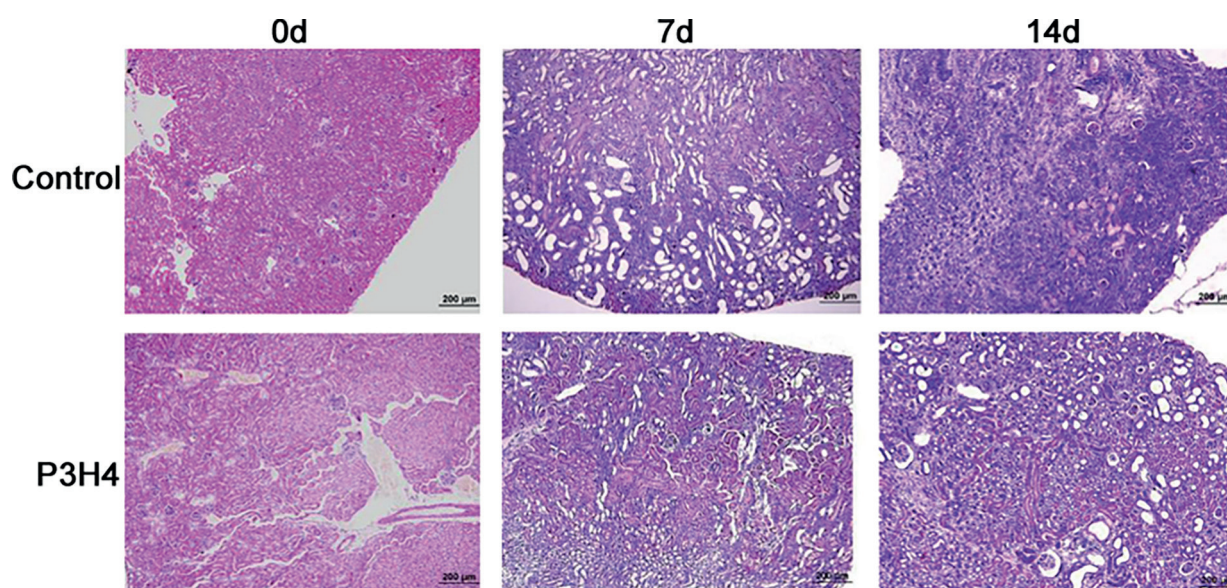


Figure 1. HE detection of renal tissue changes in rat model.

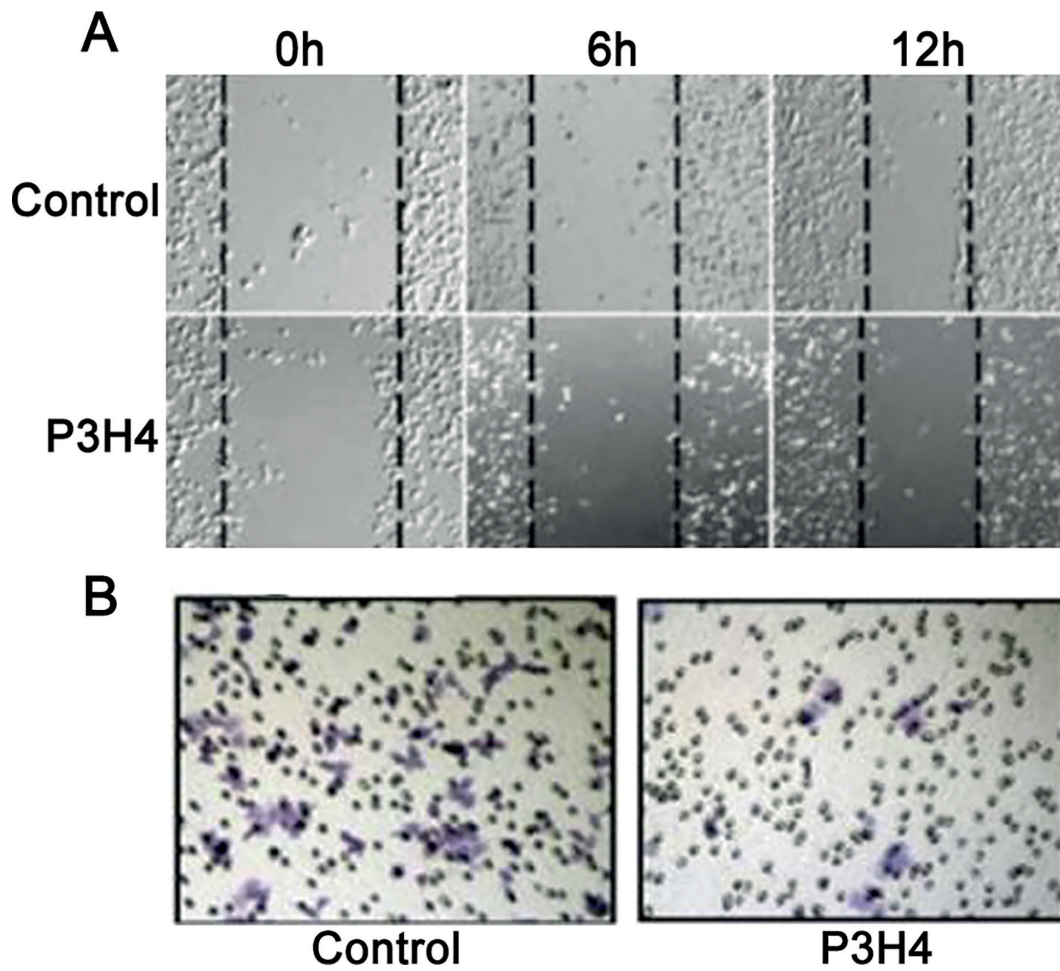


Figure 2. P3H4 affected renal cancer cells. **A**, Wound healing assay. **B**, Transwell assay.

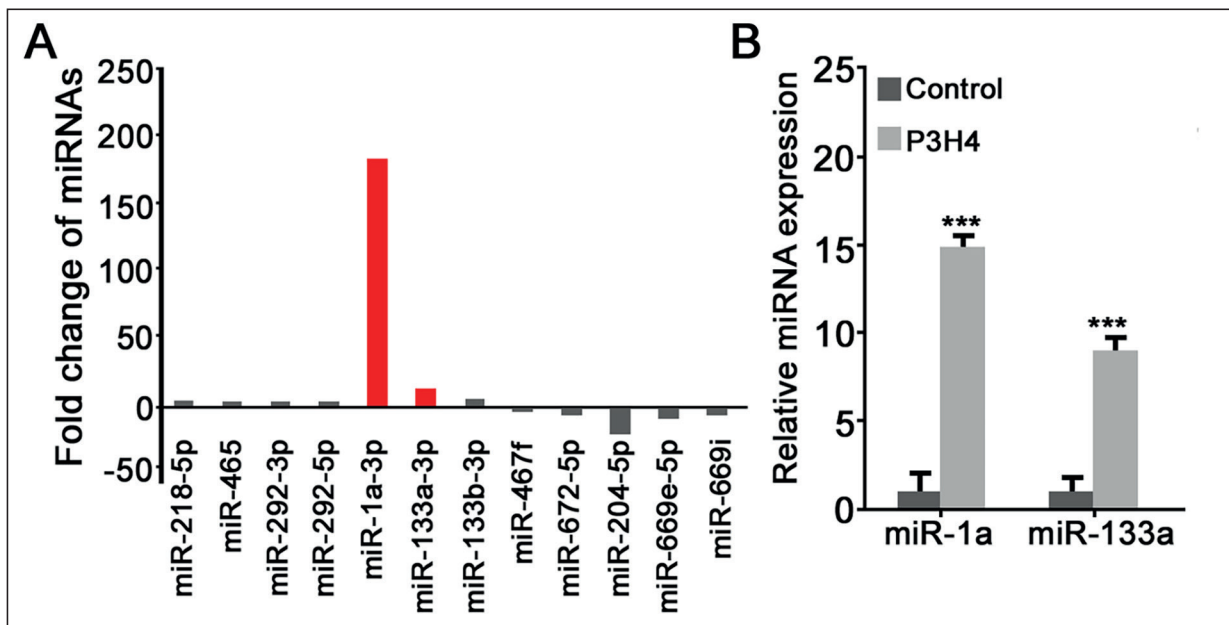


Figure 3. P3H4 upregulated miR-1a and miR-133a. **A**, Array screening. **B**, q-PCR. ***, $p < 0.001$.

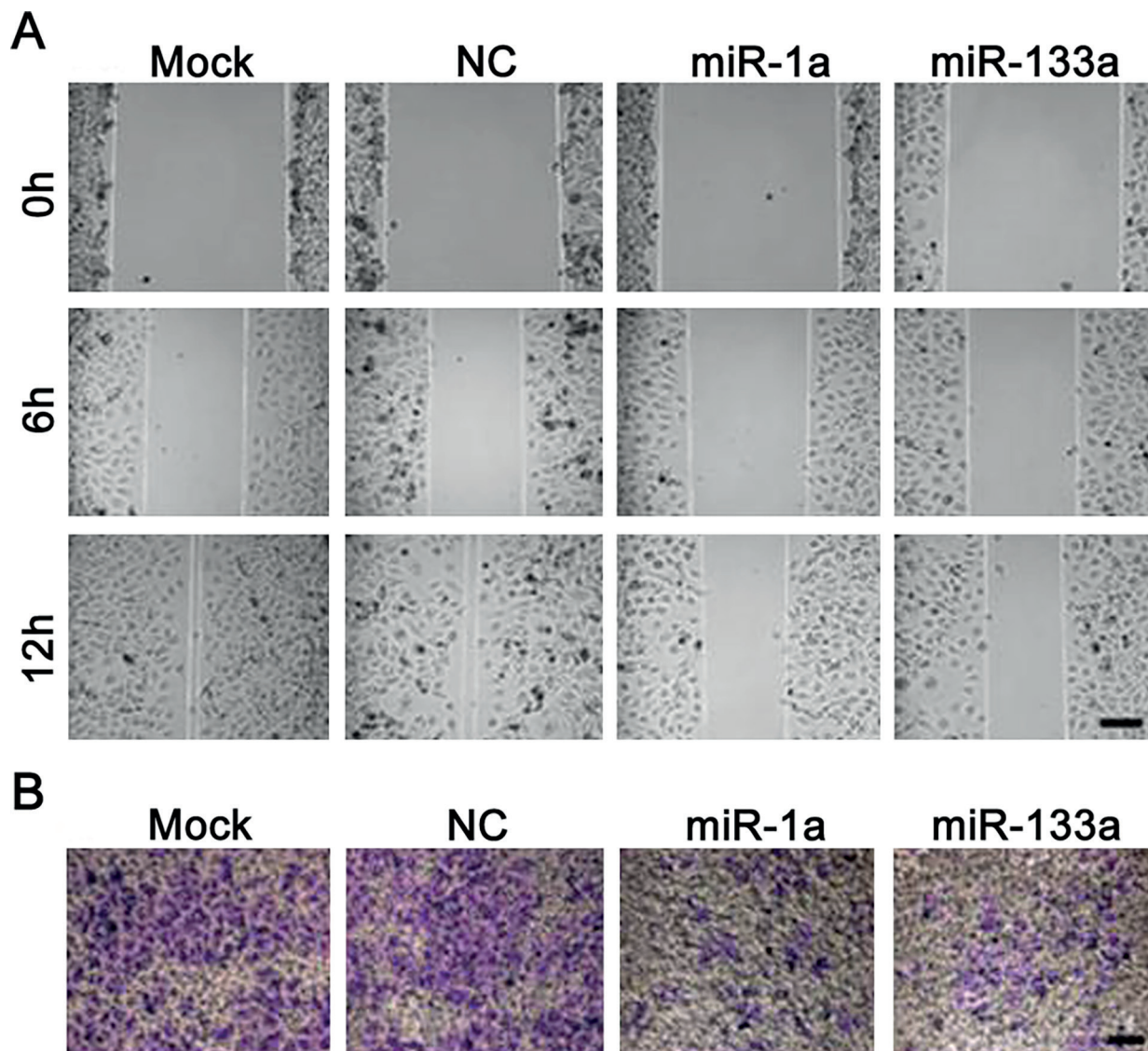


Figure 4. MiR-1a/133a cluster affected renal cancer cell migration and invasion. **A**, Wound healing assay. **B**, Transwell assay.

tumor. The occurrence and development of renal cancer are associated with abnormal cell proliferation, apoptosis, invasion, and metastasis. The function of miRNAs in renal cancer has been widely and deeply investigated. Numerous abnormal gene expressions were screened out in the early stage of renal cancer^{11,12}. Previous studies reported that miRNAs play both inhibitory and promoting function in the regulation of tumor, during the process of cell apoptosis, metabolic pathway, and signal transduction¹³.

There are various miRNAs treated as the targeting loci of small molecule drugs in clinic. It has been showed that drugs targeting miR-21 were developed for gastric cancer therapy. In

2015, ASCO meeting suggested precision medicine as the future direction for cancer therapy¹⁴⁻¹⁶. P3H4 has been studied as a small-molecule drug, while its role in cancer, especially renal cancer is still controversial. P3H4 may affect cells via the inducing gene differentiation into an oncogene. Our results showed that P3H4 can effectively impede the progression of renal cancer, delay the dissolution necrosis of renal tubular, and postpone glomeruli reduction. MiR-1a was explored for anti-angiogenesis by affecting cell polarity and changing cell movement state, such as the polarity protein Scrib¹⁷⁻¹⁹. As is known to all, cell polarity will affect cell movement and migration abilities²⁰. MiR-133a can regulate cell

proliferation through affecting G2/M phase. We then conducted miRNA array and found that P3H4 treatment significantly upregulated miR-1a and miR-133a, indicating their possible role in regulating renal cancer progression. By overexpression of miR-1a and miR-133a, cell invasion and migration were apparently decreased, which contributed to the same effect of P3H4. However, the in-depth study on the regulatory mechanism of P3H4/miR-1a/133a ought to be continued as the previous finding showed that miR-1a can regulate VEGFR2 and PDGFR β signaling pathway to affect prostate cancer, which is by impacting vascular generation^{21,22}. It was suggested that MET and LASP1 were the target genes of miR-133a²³. Moreover, cocktail therapy with new-discovered targets such as NudCD1²⁴, P3H4 requires further evaluation by *in vivo* assay with a large number of samples.

Conclusions

We revealed that P3H4 restricted the invasion and migration of renal carcinoma cells through upregulating miR-1a and miR-133a, which provides new insights for the therapy against renal cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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