

Effects of miR-455 on PIK3R1 gene expression regulation and kidney cancer cell functions

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Abstract. – **OBJECTIVE:** Migration and proliferation of kidney cancer cells are critical factors affecting effective treatment. Abnormal gene expression exists during cancer pathogenesis. The study showed certain effects of PIK3R1 gene on migration and proliferation of kidney cancer cells, although a few studies have been performed regulatory mechanism of microRNA on PIK3R1 gene. Previous sequencing of PIK3R1 gene found the existence of mir-455 binding site. This study confirmed the regulation of PIK3R1 gene expression in kidney cancer cells of mir-455 by *in vitro* assay, and analyzed its effects on migration or proliferation of kidney cancer cells.

MATERIALS AND METHODS: mir-455 agonist and inhibitor sequences were designed and synthesized to transfect kidney cancer cell line CAKI-1, which were further divided into agonist, inhibitor, and control group. qRT-PCR was used to test expression of mir-455 and PIK3R1 at 12 h, 24 h and 48 h after transfection. PIK3R1 protein expression was measured by Western blot. MTT and cell scratch assay were employed to measure cell proliferation and migration potency.

RESULTS: 12 h after transfection with mir-455, no significant difference of the level of PIK3R1 was found among the three groups ($p>0.05$). However, at 24 h and 48 h post-transfection, PIK3R1 expression in agonist group was significantly elevated, along with weakened cell proliferation or migration potency ($p<0.05$ with significant between-group comparison). By contrast, the level of PIK3R1 was statistically decreased in inhibitor group, in which cell proliferation and migration were enhanced ($p<0.05$).

CONCLUSIONS: In kidney carcinoma cell CAKI-1, mir-455 expression regulation can positively alter PIK3R1 gene expression. Over-expression of PIK3R1 gene could reduce the proliferation or migration potency of CAKI-1 cells.

Key Words:

Kidney carcinoma cells, MiR-155, PIK3R1 gene, Cell proliferation, Cell migration.

Introduction

Kidney cancer, usually called renal cell carcinoma, is the major form of renal malignant tumor and occupies about 2% of all kinds of tumors, thus severely affecting body or mental health of patients¹. Statistics showed increasing trends of incidence of kidney cancer at 2% annual rate. Currently, surgery is still the major approach in the therapy of kidney cancer, but the results were frequently unsatisfactory. Cancer metastasis also causes frequent post-op recurrence. Therefore, the development of effective drugs targeting kidney cancer is of critical importance. With the research of molecular biology technique and gene therapy application in clinics, the discovery of biological targets for a potential medicine become the research focus^{2,3}. Cumulative studies demonstrated abnormal alternation of PI3K/AKT signal pathway during the occurrence and progression of various tumors including breast cancer, ovary carcinoma and colon cancer, indicating its effect in tumor invasion or metastasis potency⁴⁻⁶. It has been estimated that about 29% kidney cancer is caused by the dysregulation of PI3K/AKT signal pathway. Current studies showed that type I PI3K mainly comprised catalytic subunit p110 α and regulatory subunit p85 α , presenting dual enzymatic activity including lipid kinase and protein kinase, the latter of which was mainly coded by PIK3R1 gene⁷⁻⁹. MicroRNA is widely distributed in cells and tissues, and exerts gene regulation function. MicroRNA can directly lead to the degradation of target gene mRNA, and can impede gene translation at post-transcriptional stage to degrade target gene mRNA or to decrease the protein expression level. During the occurrence and development of tumor, it functions as either oncogene or tumor suppressor gene to regulate cell proliferation or apoptosis^{10,11}. Evidence unraveled

that miRNAs were developed as specific markers for early tumor pathogenesis, and also became distinctive targets for clinical drug treatment^{12,13}. Increasing studies showed the over-expression of miR-455 in cervical carcinoma cells. In pathogenesis of cervical cancer, mir-455 represented onco-gene to facilitate the tumorigenesis^{14,15}. Our previous work analyzed PIK3R1 gene sequence and found functional targets of mir-455 towards this gene. However, the functions of mir-455 in the regulation of kidney cancer, as well as its effects on PIK3R1 gene expression, require further assays. In this study, therefore, kidney cancer cell line CAKI-1 was recruited as *in vitro* cell model, which was transfected with mir-455 agonist or inhibitor designed based on its sequence, for the measurement of mir-455 and PIK3R1 expression in those cells.

Materials and Methods

Materials

Human kidney cancer cell line CAKI-1 was purchased from Beino Bio (Shanghai, China) and was cryo-preserved in liquid nitrogen. Dulbecco's Modified Eagle Medium (DMEM)/F12 culture medium, fetal bovine serum (FBS) and trypsin were produced by Hyclone (South Logan, UT, USA). PBS was purchased from Beyotime (Shanghai, China). Dual antibiotics for culture were produced by Tiangen Bio (Beijing, China). Cell transfection equipment was purchased from Loza. Electro-transfection reagent was provided by Gibco (Rockville, MD, USA). RNA extraction kit (Trizol) was produced by Invitrogen (Carlsbad, CA, USA). cDNA synthesis kit was produced by Baitaike Bio (Beijing, China). RT-PCR reagent was obtained by Sigma-Aldrich (St. Louis, MO, USA). Fluorescent quantitative PCR cyclers were a product of ABI. Human PIK3R1 protein assay antibody (goat anti-human for primary, mouse anti-goat for secondary) was purchased from Xinlebio (Shanghai, China). Microplate reader was produced by Bio-Rad (Hercules, CA, USA). Mir-455 agonist (5'-GUCCAUGGGCAUAUACAC-3') and inhibitor (5'-UAUGUGCCUUUGGACUACAUCG-3') were synthesized by Baolige Bio (Shanghai, China).

CAKI-1 cell Culture and Cell Transfection

CAKI-1 cells at 3rd generation were thawed following routine method. After complete thawing, cells were firstly resuscitated in DMEM/F12 medium containing 12% FBS for 24 h, followed by amplifying culture for further experiments.

Before each passage, 10 μ l cell suspensions were collected for cell counting. Each well contained about 1×10^4 cells. After 18 h continuous incubation, cells were transfected by complete mixture. In brief, each transfection cassette contained 1.5×10^6 cells, which were mixed with 500 μ l transfection buffer. Transfection was performed at 48 V in 35 μ s pulse. Final concentration for mir-455 agonist and inhibitor was 3 μ g/ml. After electrical transfection, cells were placed in complete medium for 800 g centrifugation for 2 min. The supernatant was removed, and cell precipitation was re-suspended into 6-well plate for continuous incubation. Control group was introduced using normal cultured cells.

qRT-PCR for mRNA Levels After Cell Transfection

Total cell RNA was extracted before transfection, and at 12 h, 24 h and 48 h after transfection from all groups for cDNA synthesis following manual instruction of test kit. The mixture was placed on ice for 5-10 min, followed by the addition of 4 μ l reverse transcriptase buffer, 2 μ l DTT and 1 μ l dNTPs. The mixture was then incubated at 42°C for 2 min. 1 μ l reverse transcriptase was then added for 1 h incubation at 42°C. Reverse transcriptase activity was quenched at 65°C. Using mir-455 gene sequence as the template, primer was designed and synthesized. Using GAPDH as the internal reference, primer and related sequence were shown in Table I. RT-qPCR system consisted of 10 μ l master mix, 2 μ l cDNA, 0.4 μ l Taq polymerase, 0.08 μ M forward/reverse primers, and ddH₂O till 20 μ l. The mixture was centrifuged at 200 g. RT-qPCR program was: 94°C 3 min, followed by 45 cycles each containing 95°C 13 s, 63°C for 40 s. Each sample was set in triplicated wells to minimize errors.

Western Blot for PIK3R1 Expression

Cell proteins were collected at 12 h, 24 h and 48 h after transfection. Culture medium was firstly removed from culture plate, which was rinsed by pre-cold 1 $\times$ PBS. When all liquids were removed, the plate was placed on ice. Protein lysis buffer was added at 50 μ l per well. Phosphate-buffered saline (PBS) was used to rinse well bottom and wall repeatedly. Cells were transferred to enzyme-free tubes for repeated mixture on ice. After complete lysis, cell debris was centrifuged at 12000 g for 15 min under 4°C. The supernatant was collected into pre-cold tubes. 1 μ l protein solution was collected for quantification. Using β -actin as internal

Table 1. Primers using in this study.

Primer name	Primer sequence (5'-3')
PIK3R1 forward	CATCCCAGGACCTCTCT
PIK3R1 reverse	CGGGGGACTGGCGA
mir-455 forward	CTAAGACCTGTGGAATGGC
mir-455 reverse	CTCAAAGATGTCATTGCC
GAPDH forward	CGGAGTCAACGGATTTGGTCGTA
GAPDH reverse	AGCCTTCTCCATGGTGGTGAA

reference, primary antibody was diluted at 1:200 for 8-10 h incubation at 4°C. By TBST rinsing, 500 × diluted secondary antibody was added for 2 h room temperature incubation. Following 3-5 times rinsing, chromogenic substrate and development buffer were added for assay on Bio-Rad fully automatic analyzer to observe gray values of each group. Relative expression level of PIK3R1 protein was calculated.

MTT Assay for the Effect of PIK3R1 Expression Change on CAKI-1 Cell Proliferation

Cells after transfection were seeded into 96-well plate at 1×10^4 cells per well. At 12 h, 24 h and 48 h after transfection, serum-free culture medium was used to rinse cells. Each well was added with 20 µl methylsulfonyl tetrazolium bromide (MTT) solution for 4 h continuous incubation. Culture medium was removed, followed by adding 150 µl dimethyl sulfoxide (DMSO) for 10 min room temperature incubation. OD values of each well were measured by UV spectrometry. Each group was tested in triplicates for calculating average values. Cell proliferation was calculated as OD of experimental group/OD of control group.

Scratch Assay for Cell Migration Potency After Transfection

Sterilized marker pen was used to draw parallel lines with 1 cm distance on the surface of 6-well plate using sterilized rulers. Cell concentration was adjusted and added into the plate at 5×10^3 per well. Each well was filled with 2 mL culture medium for overnight incubation. Each group was tested in triplicates. After 24 h incubation and when cells covered the dish bottom, 100 µl pipette were used to draw scratches vertical against marker lines on the back of culture plate. PBS was used to rinse cell colony on the scratch line. After 3-5 times of rinsing, complete culture medium was added for continuous incubation for 12 h, 24 h or 48 h. Images were captured under

inverted microscope for analyzing scratch line width using ImageJ software. Three lines were randomly selected from all scratch lines to record cell migration distance and average values.

Statistical Analysis

Data analysis and statistics were performed using SPSS11.3 (SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) was used to compare differences between group. LSD and S-N-K tests were assumed and provided as equal variances while Tamhane's T2 test was used as equal variances were not assumed. Significant difference was defined when $p < 0.01$.

Results

qRT-PCR for mir-455 mRNA Expression in Cells

Total RNA was extracted from cells after transfection. Using GAPDH as the internal reference, qRT-PCR was used to detect mir-455 expression. As shown in Figure 1, mir-455 mRNA levels at 24 h and 48 h after miR agonist transfection were significantly elevated compared to that of control group ($p < 0.05$). Of note, after cells being transfected with inhibitor for 24 h or 48 h, mir-455 mRNA expression was significantly decreased ($p < 0.01$ compared to control group). At 12 h post transfection, mir-455 expression was not significantly changed among different groups ($p > 0.05$). These *in vitro* results indicated that agonist or inhibitor effectively regulated mir-455 expression in cultured CAKI-1 cells, in a time dependent manner.

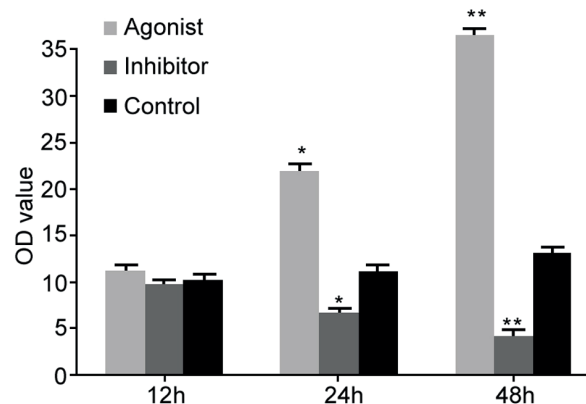


Figure 1. Expressional assay of mir-455 at 12 h, 24 h and 48 h post transfection. *, $p < 0.05$ compared to control group; **, $p < 0.01$ compared to control group.

qRT-PCR for Cellular Expression of PIK3R1

Using extracted cDNA as the template, qRT-PCR was used to quantify PIK3R1 expression. As shown in Figure 2, PIK3R1 expression was positively correlated with mir-455. PIK3R1 expression was significantly increased or decreased at 24 h and 48 h after transfecting mir-455 agonist or inhibitor, respectively, as consistent with the change pattern of mir-455 expression ($p < 0.05$ compared to control group), suggesting that mir-455 could positively regulate PIK3R1 expression in cultured CAKI-1 cells.

Western Blot for PIK3R1 Expression in Cell Culture Supernatant

Total proteins were extracted at 12 h, 24 h and 48 h post transfection. Western blot result was shown in Figure 3. PIK3R1 expression was increased in agonist group as transfection time was longer, and was decreased in inhibitor group ($p < 0.05$ compared to control group).

MTT for the Effect of PIK3R1 Expression on CAKI-1 cell Proliferation

Cell suspension after transfection was transferred into 96-well plate. MTT approach was used to measure the effect of PIK3R1 expression on proliferation of CAKI-1 cells at 12 h, 24 h and 48 h post transfection. As shown in Figure 4, negative correlation was found between the PIK3R1 expression and CAKI-1 cell proliferation in a time dependent manner.

Cell Scratch Assay to Measure Cell Migration Ability

Cell scratch assay was used to determine cell migration potency across different treatment

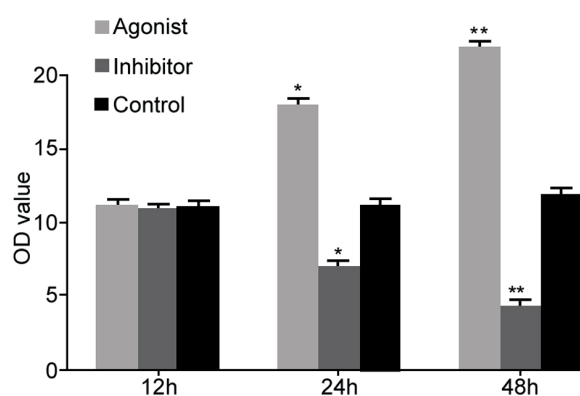


Figure 2. PIK3R1 expression assay at 12 h, 24 h and 48 h post transfection. *, $p < 0.05$ compared to control group; **, $p < 0.01$ compared to control group.

groups. As shown in Figure 5, cell migration among diverse groups showed no significance at 12 h and 24 h after transfection. However, at 48 h post transfection, cell migration distances were $23 \pm 2.13 \mu\text{m}$, $11.32 \pm 8.13 \mu\text{m}$ and $16.85 \pm 3.12 \mu\text{m}$ in inhibitor, agonist and control group, manifesting significant difference among groups ($p < 0.05$). These results indicated that the over-expression of PIK3R1 could suppress migration ability of *in vitro* cultured CAKI-1 cells, whilst the reduction of PIK3R1 could facilitate cell migration in turn.

Discussion

The occurrence of kidney cancer is an extremely complicated process, during which cancer cell proliferation and apoptosis exert critical regulatory roles. Proliferation and apoptosis of cancer

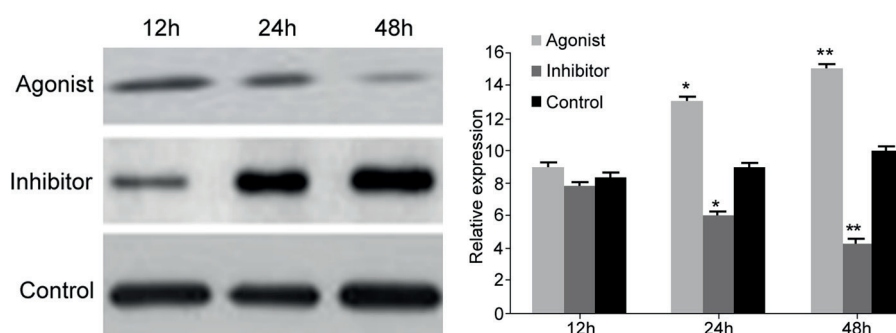


Figure 3. Western blot for PIK3R1 expression after agonist or inhibitor transfection. *, $p < 0.05$ compared to control group; **, $p < 0.01$ compared to control group.

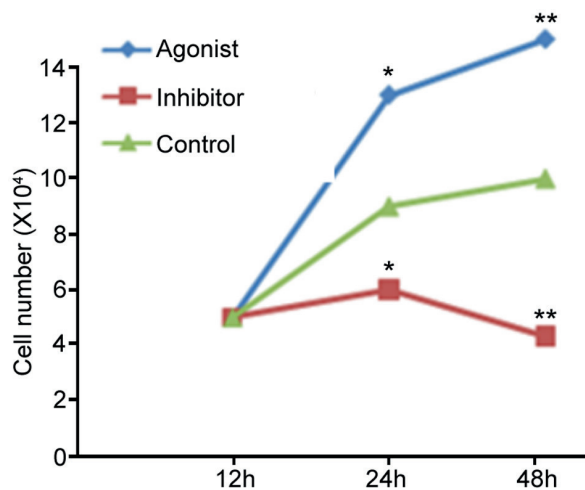


Figure 4. Effects of PIK3R1 expression on CAKI-1 cell proliferation. *, $p < 0.05$ compared to control group; **, $p < 0.01$ compared to control group.

cells are under the regulation of multiple signal pathways such as p53/p16-18. Several studies¹⁹⁻²¹ revealed the PI3K/AKT signal pathway participated in the regulation of multiple tumors, as about cases concerning 1/3 of tumors showed that gene mutation of PI3K/AKT occurred during pathogenesis. Structural change of key encoding genes, such as PIK3CA, AKT, and PTEN in PI3K/AKT signal pathway, facilitated PI3K activation and cell transformation via relieving negative regulation of normal tyrosine kinase receptor RTK signal on p85/p110 complex^{22,23}. Previous report revealed close correlation between microRNA and various human diseases. For instance, microRNA can regulate biological activities of cancer cells including proliferation, apoptosis and migration as tumor suppressor gene^{10,11}. In various tumor researches, mir-455 was reported to exert both anti-cancer gene function and oncogenic functions.

For example, mir-455 significantly decreased viability in cervical carcinoma cell line SiHa and facilitated cell apoptosis, along with significantly higher mir-455 expression in cervical carcinoma tissues compared to normal cervical tissues²⁴. However, other paper showed down-regulation of mir-455 during nasopharyngeal carcinoma occurrence, and that the decrease of mir-455 could facilitate proliferation and migration of nasopharyngeal carcinoma cells²⁵. In this work, we demonstrated that mir-455 played an oncogene role during kidney cancer pathogenesis. As mentioned above, PI3K/AKT signal pathway greatly affected cancer pathogenesis. We found the existence of mir-455 functioning targets in PIK3R1 gene by bioinformatics analysis. We thus speculated the possible role of mir-455 in kidney cancer pathogenesis via mediating PIK3R1 gene expression and PI3K/AKT signal pathway. Our data showed that mir-455 agonist and inhibitor effectively changed mir-455 expression level of *in vitro* cultured CAKI-1 cells. When mir-455 expression level was changed, PIK3R1 gene expression was altered correspondingly. Notably, these results were inconsistent with most studies showing gene expression inhibition by microRNA, suggesting positive regulating effect of mir-455 on PIK3R1. In the study of proliferation and migration potency of kidney cancer cells across different groups, we found that the up-regulation of PIK3R1 gene could suppress proliferation of CAKI-1 cells, and decreased migration potency, as consistent with the role of PIK3R1 gene in other tumors such as cervical carcinoma, ovary cancer and melanoma²⁶. Our preliminary work demonstrated regulatory effects between mir-455 and PIK3R1 at *in vitro* level; however, *in vivo* research remains to be further investigated with certain clinical implications.

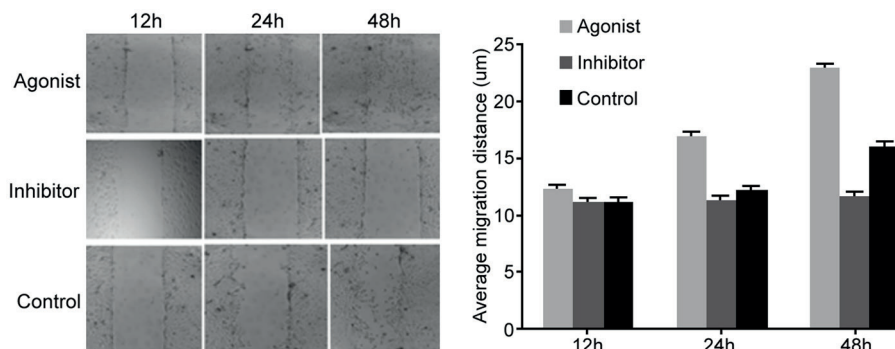


Figure 5. Cell scratch assay images and average migration distance. *, $p < 0.05$ compared to normal group.

Conclusions

Our *in vitro* research with cultured kidney cancer cell line CAKI-1 illustrated that, using mir-455 agonist or inhibitor, proliferation and migration potency of CAKI-1 cells was further altered. Mir-455 played an oncogene role during kidney cancer pathogenesis via positive modulation of PIK3R1 gene expression, which provided evidence for identifying novel gene therapy for clinical treatment of kidney cancer.

Acknowledgments

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Conflict of interest

The authors declare no conflicts of interest.

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