miR-142-5p promotes renal cell tumorigenesis by targeting TFAP2B

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Abstract. The transcription factor AP-2 β (TFAP2B) serves an important role in kidney development. MicroRNAs (miRNAs) regulate carcinogenic pathways and have gained increasing attention owing to their association with human clear cell renal cell carcinoma (ccRCC) tumorigenesis. However, whether miRNAs could affect renal cell tumorigenesis by regulating TFAP2B expression has not been identified. The aim of this study was to investigate the effects of miRNA on TFAP2B and its potential role in cell growth, invasion and migration. PCR, western blot and dual luciferase reporter assays were performed to analyze the effects of miR‑142‑5p on TFAP2B. Furthermore, MTT, flow cytometry, wound healing and Transwell migration assays were used to analyze the effect of miR‑142‑5p on cell proliferation and migration. The results demonstrated that miR‑142‑5p targeted TFAP2B and downregulated the expression of TFAP2B at the mRNA and protein levels, promoting cell proliferation and migration in two ccRCC cell lines, 786-O and A-498. This phenomenon supported the theory that miR‑142‑5p may function as an oncogene in ccRCC. The potential clinical significance of miR‑142‑5p as a biomarker and a therapeutic target provides rationale for further investigation into miR‑142‑5p‑mediated molecular pathways and how these may be associated with ccRCC development.

Introduction

Clear cell renal cell carcinoma (ccRCC) is a cancer in which malignant cells form in the tubules of the kidney (1,2). ccRCC constitutes >85% of kidney cancers, with an estimated 403,262 new cases (1,3,4), and 175,098 deaths in 2018, and the mortality rate is likely to continue to grow (5,6). Although there are several types of standard treatments, nearly half of the diagnosed patients succumb within five years (3,7). Therefore, a better understanding of the molecular mechanisms underlying this disease could help identify new therapeutic targets. In particular, insight into the networks that control signaling cascades associated with cell proliferation and migration may lead to the discovery of novel target genes for ccRCC treatment.

Transcription factor AP-2 β (TFAP2B) is a member of the TFAP2 protein family, which includes TFAP2-A, -B, -C, -D and -E (8). Previous studies have revealed that TFAP2B overexpression has been demonstrated to promote tumor growth, thus, contributing to a poor prognosis in human lung adenocarcinoma (13). In the present study, a systematic investigation of the effects of TFAP2B on ccRCC in vitro is described for the first time.

MicroRNAs (miRNAs) are a family of endogenous non-coding single-stranded RNA molecules with a length of 19-22 nucleotides that may act either as tumor suppressors or oncogenes, according to the function of their target genes (14). In the present study it was demonstrated that miRNA (miR)-142-5p serves as an onco-miRNA in ccRCC by targeting TFAP2B and downregulating its expression, promoting cell proliferation and migration. Thus, the findings of the present study revealed that the miR-142-5p/TFAP2B pathway may provide potential therapeutic targets for treatment of ccRCC.

Methods and materials

Cell lines and cell culture. 293T cells, the human kidney cell line HK-2 and the ccRCC cell lines 786-O and A-498 were obtained from ATCC. All cells were maintained in
DMEM containing 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin, and 2 mM L-glutamine at 37°C in a 5% CO₂ atmosphere and 21% oxygen.

Bioinformatic analysis. The expression of the TFAP2B was analyzed in The Cancer Genome Atlas (TCGA) ccRCC database (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga). The regulation network of the TFAP2B and the interaction partners of TFAP2B were identified using the STRING (https://string-db.org) software tool. TargetScan screen database (http://www.targetscan.org/vert_72/) was used online to screen the targets and binding sites of miR-142-5p. The miR-142-5p-associated survival was analyzed on the ONCOMIR website (http://www.oncomir.org) (15). The total number of patients for analysis was 517, which was divided into the low expression group (n=259) and the high expression group (n=258). The statistical difference in the survival rate was analyzed by the log-rank test.

Plasmid construction and lentiviral infection. The genomic DNA of 868-O cells was isolated using a FastPure Cell DNA Isolation Mini kit (cat. no. DC102; Vazyme Biotech Co., Ltd.). The 3’UTR of human TFAP2B gene was amplified from the genomic DNA by PCR using 2X Phanta Master Mix (cat. no. P511-01; Vazyme Biotech Co., Ltd.). The primers used were as follows: Forward, 5’-GAGCTCGTACGCCATGAGAAA TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT G-3’ and reverse, 5’-CATGGCC TGCAAGGCATCAAGAAGTTTAAACACAAGATCTC-3’. The thermocycling conditions were as follows: 95°C for 5 min, followed by 30 cycles of 95°C for 15 sec, 60°C for 15 sec and 72°C for 60 sec. The TFAP2B 3’UTR fragment was then cloned downstream to the firefly luciferase reporter gene in the pmirGLO vector (Promega Corporation), generating the pmirGLO-TFAP2B 3’UTR WT plasmid. The mutant TFAP2B 3’UTR was generated using the QuikChange II Site-Directed Mutagenesis kit (Agilent Technologies, Inc.) and was also cloned into the pmirGLO vector, generating the pmirGLO-TFAP2B 3’UTR Mutant plasmid. The lentivirus-based vector plv-EF1α-PGK-puro (Xiamen Anti-hela Biological Technology Tarde Co., Ltd.) was used to overexpress miR-142-5p. The precursor of miR-142-5p was amplified from the genomic DNA of A-498 cells by PCR using 2X Phanta Master Mix (cat. no. P511-01; Vazyme Biotech Co., Ltd.). The primers used were as follows: Forward, 5’-TCACGGCCGTCCGGG CGCAGCCGCTGAAGTAGCTACGCGCCG-3’ and reverse, 5’-CTAGGGATCCGGGCGGGCGGGCCGCACGAT GTGCCGTGTCG-3’. The thermocycling conditions were as follows: 95°C for 5 min, followed by 30 cycles of 95°C for 15 sec, 60°C for 15 sec and 72°C for 30 sec. To prepare lentiviral particles, 9 µg of plv-EF1α-miR-142-5p-PGK-puro or empty vector plasmid with packaging plasmids (3 µg pMD2G; 6 µg pspax2; Xiamen Anti-hela Biological Technology Tarde Co., Ltd.) were co-transfected into 293T cells in 10-cm dishes using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Lentivirus-containing medium was collected 48 h after transfection and used to infect A-498 and 786-O cells (1x10⁵ cells/well, in six-well plates) at a multiplicity of infection (MOI) of 30. Two days after lentiviral infection, A-498 and 786-O cells were maintained in the presence of 1.0 µg/ml puromycin (Sigma-Aldrich; Merck KGaA) for 5 days to generate stable miR-142-5p-overexpressing cells and empty vector negative control cells.

The plasmid pCDH-EF1α-TFAP2B-T2A-BSD for TFAP2B overexpression was constructed using the lentivirus-based vector pCDH-EF1α-MCS-T2A-BSD (Xiamen Anti-hela Biological Technology Tarde Co., Ltd.). Lentiviral particles carrying TFAP2B were produced as aforementioned. The cells (1x10⁶ cells/well, in six-well plates) stably overexpressing miR-142-5p were infected with the TFAP2B lentivirus at a MOI of 30. Two days after lentiviral infection, the cells were maintained in the presence of 0.4 µg/ml blasticidin S (Sigma-Aldrich; Merck KGaA) for 10 days to generate A-498 and 786-O cells stably overexpressing both miR-142-5p and TFAP2B (miR-142-5p + TFAP2B).

Transfection. 786-O and A-498 cells were plated into a 6-well plate (5x10³ cells/well) at 37°C. The next day, the pCDH-EF1α-MCS-T2A-BSD plasmid (3 µg/well) and the pCDH-EF1α-TFAP2B-T2A-BSD plasmid (4 µg/well) were transfected into the cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C. After a 24-h period of transfection, the cells were harvested for further experiments.

Reverse transcription-quantitative PCR (RT-qPCR) assay. RNA isolated from cells were subjected to RT using Superscript III Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific, Inc.) at 50°C for 30 min. TFAP2B and 18S were transcribed using random RT primers; the miR-142-5p RT primer sequence was 5’-GCTGTATCCGTGAGGG TCCGAGGTATTCCGCACTGGATACGACAGTGT-3’ and the U6 RT primer sequence was 5’-GGTCTACGAATTTGG CTGGTTCAT-3’. qPCR was conducted using a Bio-Rad CFX96 system with a ChamQ SYBR® qPCR Master Mix kit (Vazyme Biotech Co., Ltd.) to determine the mRNA or miRNA expression levels of the genes of interest. The method of quantification was as described previously (16). The thermocycling conditions were as follows: 94°C for 3 min, followed by 40 cycles of 94°C for 15 sec, 60°C for 20 sec and 72°C for 20 sec. Each detection was performed in triplicate. Expression levels were normalized to those of U6 or 18S ribosomal RNA.

The following primers were used for qPCR: miR-142-5p forward, 5’-GGCGGACACATAAGATAGAAGC-3’ and reverse, 5’-AGTGCGAGGTGGCCGAGATTT-3’; U6 forward, 5’-GGTCTACGAATTTGGCTGGTTCAT-3’; TFAP2B forward, 5’-GGTTGAGAGTGGCAATAAACAGCGG-3’ and reverse, 5’-GGACGGAGCAGAAACACCTCGC-3’; and 18S forward, 5’-CTCTCAGGAGCTTCCAAGC-3’.

Western blotting. Cells were lysed in ice-cold RIPA buffer (Sangon Biotech Co., Ltd.) and the protein was quantified using a BCA Protein Assay kit (Abcam). Lysates (20 µg/sample) were loaded on 8-12% denaturing SDS-PAGE gels and transferred to a PVDF membrane (Roche Diagnostics GmbH). Tris-HCl buffer containing 5% bovine serum albumin (BSA; Beijing Solarbio Science & Technology Co., Ltd.) was used to block the membranes at 2 h. The membrane was
probed with the primary antibodies prepared in Tris-HCl buffer containing 5% BSA at 4°C overnight. Subsequently, the membranes were washed three times with Tris-HCl buffer containing 0.1% Tween-20, followed by incubation with the appropriate secondary antibodies prepared in Tris-HCl buffer at 28°C for 1 h. Finally, the membranes were washed three times, detected and visualized by an enhanced chemiluminescence detection system (Thermo Fisher Scientific, Inc.). The antibodies used in this study included anti-TFAP2B (1:500; cat. no. 13183-1-AP; ProteinTech Group, Inc.), anti-GAPDH (1:5,000; cat. no. YM3029; ImmunoWay Biotechnology Company), HRP-conjugated goat anti-rabbit IgG (1:1,000; cat. no. 7074; Cell Signaling Technology, Inc.) and HRP-conjugated rabbit anti-mouse IgG (1:1,000; cat. no. 7076; Cell Signaling Technology, Inc.).

Dual luciferase reporter assay. miR-142-5p mimics (5'-CAU AAAGUAGAAAGCAGCUACU-3') and negative control mimic (mimics ctrl; 5'-UUGUACUACAAAGUACUG-3') were obtained from Guangzhou RiboBio Co., Ltd. 293T cells were seeded into a 6-well plate (10⁶ cells/well) and transfected with mimics ctrl or miR-142-5p mimics (200 pmol/well), along with the pmirGLO-WT TFAP2B 3'UTR (4 µg/well) or pmirGLO-Mutant TFAP2B 3'UTR plasmid (4 µg/well) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), and kept at 37 °C. The cells were lysed at 48 h post-transfection and luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega Corporation) according to the manufacturer's instructions. The firefly luciferase activity was calibrated to Renilla luciferase activity. Each treatment was carried out in triplicate.

Cell proliferation assay. A total of 3x10⁶ cells/well were seeded onto 96-well plates. Cell proliferation was measured using a MTT cell proliferation assay kit (cat. no. 11465007001; Shanghai Qbio Science & Technologies Co., Ltd.) at each time point (0, 24, 48 and 72 h) for 1 h at 37°C. The absorbance was measured at 450 nm. All experiments were performed in triplicate.

Cell cycle assay. A total of 1x10⁶ cells/well were seeded onto 6-well plates. Next day, 786-O and A-498 cells were harvested and fixed in 70% ethanol at 4°C overnight. The fixed cells were then incubated with PBS containing 10 µg/ml RNase A (Sangon Biotech Co., Ltd.) and 0.2% Triton X-100 for 30 min at 37°C, and then stained with 20 µg/ml propidium iodide for 30 min in the dark at room temperature. The stained cells were analyzed using the NovoCyte flow cytometer with NovoCyte 1.4.1 software (ACEA Biosciences, Inc.). All experiments were performed in triplicate.

Wound healing assay. 786-O and A-498 cells with stable overexpression of TFAP2B were seeded into 6-well plates to reach 100% confluence. Wound was generated using a 200 μl pipette tip with a straight scratch. Cells were maintained in serum free medium for 24 h and were observed at x40 magnification under a bright field microscope (Motic Incorporation, Ltd.). The percentage of the wound healing was quantified by ImageJ 1.8.0 software (National Institutes of Health).

Transwell assay. Migration assays were performed using Transwell plates with 8-µm pore size membranes. A total of 2.5x10⁵ 786-O and A498 cells with stable overexpression of TFAP2B were plated in the upper chambers of the Transwell plates. After a 24-h period of incubation at 37°C, the migrated cells were stained with 0.5% toluidine blue and photographed at x100 magnification under a bright field microscope (Motic Incorporation, Ltd.). The migrated cells were counted using ImageJ in three random fields.

Statistical analysis. All statistical analyses were conducted using SPSS version 19.0 (IBM Corp.) and GraphPad Prism version 8.0 (GraphPad Software, Inc.). Data are presented as the mean ± SD. Differences between two groups were analyzed with Student's t-test, whereas ANOVA followed by Tukey's post-hoc test was used for multiple comparisons of three or more experimental groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Database analysis of the expression profile of TFAP2B and its regulation. To identify genes that were lowly expressed in RCC, a screen was conducted using Oncomine software. The TFAP2B expression level in ccRCC was decreased compared with that in the normal tissue group; thus, TFAP2B was selected for further experiments based on the P-value and median expression rank (Fig. 1A). It is possible that TFAP2B protein levels may be regulated by miRNAs, as it contains a conserved miRNA target site in the 3' UTR region (Fig. 1B). To confirm this hypothesis and to determine the possible miRNAs that may target TFAP2B, a screen using the TargetScan software was performed, which lead to the identification of miR-142-5p as a putative miRNA that can bind to TFAP2B 3'UTR regulating its protein expression (Fig. 1C). Furthermore, TCGA ccRCC database analysis demonstrated that high levels of miR-142-5p is negatively associated with lower TFAP2B mRNA expression in patient samples (Fig. S1A and B). Moreover, the miR-142-5p expression was significantly associated (log-rank test) with the hazard ratio of death status in various cancer types in the TCGA public database using Kaplan-Meier analysis by long-rank tests between the miR-142-5p high expression group and the miR-142-5p low expression group (Fig. S1C). And the expression level of miR-142-5p was significantly associated with the survival rate in the kidney cancer (Fig. S1C). Furthermore, the STRING software tool was used to analyze the TFAP2B interaction network, which demonstrated that the Cbp/p300-interacting transactivator with ED-rich tail family, the TF2P family, the small ubiquitin-like modifier family and TP53 might be important regulators in the expression and functions of TFAP2B due to their direct and stronger interactions (Fig. S1D).

Comparison of TFAP2B expression levels in normal kidney cells and renal tumor cells. To identify suitable ccRCC cell lines for further study, RT-qPCR and western blot assays were used to analyze mRNA and protein expression levels of TFAP2B in ccRCC cell lines compared with the normal kidney cell line HK-2. As shown in Fig. 2A, the results indicated that the mRNA expression levels of TFAP2B was lower in both
miR142-5p targets TFAP2B. Based on the TargetScan screen database analysis, the interaction between miR-142-5p and its putative target TFAP2B was examined (Fig. 1B). The results demonstrated that the miR-142-5p mediated-decrease of TFAP2B protein level was rescued by the TFAP2B overexpression vector, compared with miR-142-5p group (Fig. S2A and B). In addition, as shown in Fig. S2C and D, TFAP2B overexpression vector could induce the overexpression of TFAP2B alone, compared with the empty vector negative control cells (Fig. S2A and B). Furthermore, a dual luciferase reporter assay revealed a significant decrease in the luciferase activity in 293T cells co-transfected with miR-142-5p and the TFAP2B-3'UTR WT luciferase reporter vector compared with the luciferase activity in cells co-transfected with the negative control (mimics ctrl) and the reporter vector. Besides, the TFAP2B-3'UTR Mutant abolished the interaction between the miR-142-5p and the TFAP2B-3'UTR region as no significant differences were observed in the luciferase activity between miR-142-5p and mimics control (Fig. 2C). Moreover, the effects of miR-142-5p overexpression on TFAP2B in 786-O and A-498 cell lines were examined by RT-qPCR and western blot assays. As shown in Fig. 2D, the level of miR-142-5p was higher in miR-142-5p-overexpression (OE) 786-O and A-498 cells compared with expression levels in the negative control group. TFAP2B mRNA and protein levels were markedly decreased in miR-142-5p-OE cells (Fig. 2E and F, respectively). Based on the above results, it was concluded that miR142-5p targeted TFAP2B and suppressed its expression in these cell lines.

miR142-5p promotes proliferation of ccRCC cells. To study the effects of the miR142-5p on ccRCC cell proliferation,
the proliferation abilities of 786-O and A-498 cell lines overexpressing miR-142-5p or co-overexpressing miR-142-5p + TFAP2B were evaluated with an MTT assay (Fig. 3A). An increased proliferation rate was observed in miR-142-5p-OE cells compared with the empty vector negative control group in each cell line. In addition, the overexpression of TFAP2B could eliminate the miR142-5p-induced increase in cell proliferation (Fig. 3A).

Because miR142-5p promoted 786-O and A-498 cell proliferation, we evaluated the effects of miR142-5p on 786-O and A-498 cell cycle progression by flow cytometry. The results demonstrated that the percentage of cells in phase G0/G1 was significantly lower in miR-142-5p-OE 786-O and A-498 cells, whereas the proportion of S phase cells was significantly higher in miR-142-5p-OE 786-O and A-498 cells compared with the negative control group (Fig. 3B and C). This effect was eliminated by TFAP2B overexpression, which lead to an increased percentage of A-988 cells in phase G1 and reduced the percentage of 786-O cells in S phase. These results indicated that miR142-5p could prevent G1 phase arrest in 786-O and A-498 cells and significantly promote ccRCC cell proliferation.

miR142-5p enhances migration of ccRCC cells. Results from wound healing and Transwell migration assays demonstrated that miR-142-5p-OE 786-O and A-498 cells exhibited significantly increased cell migration compared with the negative control cells (Figs. 3D and E and 4). As expected, overexpression of TFAP2B significantly removed the influence of increased miR142-5p expression on cell migration (Fig. 3E). These data suggested that the migratory ability of ccRCC cells was enhanced by miR-142-5p but was weakened by TFAP2B.

Discussion

In the present study, TFAP2B expression was found to be markedly low in renal cancer, indicating that TFAP2B might function as a tumor suppressor in ccRCC. miR-142-5p was found to directly target TFAP2B and downregulate its expression, indicating that miR-142-5p may act as an oncogenic microRNA. Finally, the effect of miR-142-5p and TFAP2B on ccRCCs was systematically investigated through a series of cell function experiments. The results demonstrated that miR-142-5p increased cell proliferation and migration and that TFAP2B could reverse these effects.
Cell cycle assays showed that overexpression of miR-142-5p reduced the population of G₀/G₁ phase cells, which indicated that miR-142-5p may promote cell growth by forcing them to enter the S phase.

A miRNA can target numerous genes and act as either a tumor suppressor or oncogenic miRNA, depending on the function of targeted genes. Liu et al. (17), reported that miR-142-5p promotes development of colorectal cancer by targeting succinate dehydrogenase complex iron sulfur subunit B. However, a study by Wang et al. (18) showed that miR-142-5p targets PI3K-α to suppress tumorigenesis in non-small cell lung cancer. In human osteosarcoma, miR-142-5p also suppresses proliferation and promotes apoptosis by targeting phospholipase A and acyltransferase 3 (19). In the present study, miR-142-5p was shown to promote proliferation and migration of ccRCC cells by targeting TFAP2B, which is consistent with study results reported by Liu et al. (20) that revealed that miR-142-5p promotes cell growth and migration.
of ccRCCs by targeting BTG anti-proliferation factor 3. These results revealed the importance of the miR-142-5p in ccRCC development.

TFAP2B expression has been previously detected in embryonic renal tissues and was found to be important for kidney development in mice (12). In 2004, immunohistochemical evidence first suggested that transcription factor AP-2 may play a role in carcinogenesis (21). Furthermore, using comprehensive bioinformatic analyses, RT-qPCR and immunohistochemistry, downregulation of TFAP2B has been found to be important not only for normal renal development and epithelial differentiation but also for mesenchymal/adipogenic transdifferentiation and pluripotent mesenchymal stem cell-like differentiation (22). To explore the regulation network of the TFAP2B in renal cancer, the current study also analyzed the interaction partners of TFAP2B. The results with STRING software indicated that p53, the well-known tumor suppressor gene, might be important for the downstream and upstream regulation of TFAP2B due to their interaction, which required further experiments to be confirmed. However, more experiments should be conducted to confirm this hypothesis in the future.

There are some limitations in the present study, such as lack of rescue experiments, interference experiments of TFAP2B in cell lines, the limited number of ccRCC cell lines studied for functional assessment of the effects observed and the lack of reproducibility in the western blotting assays in other ccRCC cell lines. Although the detailed regulatory mechanistic functions of TFAP2B in driving cancer remain to be further explored, to the best of our knowledge, this study is the first to demonstrate TFAP2B regulation by miR-142-5p in ccRCC. In conclusion, miR-142-5p/TFAP2B pathway in ccRCC is described for the first time, which may provide novel targets for ccRCC therapy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.
Authors' contributions

MZ, LZ, JF and YL designed and performed the experiments. BS, KY, FL, LY and ML performed the experiments. All authors critically revised the manuscript. JF and YL supervised the project. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


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