Blocking Exosomal miR-19b-3p from Renal Cell Carcinoma Cells Enhances Sunitinib Mediated Anti-Tumor Activity via Promoting TGFR2/KLF10 Expression

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Research

Keywords: microRNA-19b-3p, renal cell carcinoma, Sunitinib, Transforming growth factor β , chemoresistance, Krüppel-like factor 10.

Posted Date: July 6th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-567212/v1>

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Abstract

Background

Multiple studies have found that microRNAs contribute to the malignant progression and chemoresistance of renal cell carcinoma (RCC). This study intends to probe the effect of miR-19b-3p shuttled by exosomes derived from RCC cells on RCC development and its resistance to Sunitinib.

Methods

Sunitinib-resistant cell lines (OSRC-2R and Caki-1R) were constructed from OSRC-2 and Caki-1 RCC cells. Exosomes in the RCC cell supernatant were isolated, and the miR-19b-3p profile in cells and exosomes was measured by reverse transcription-polymerase chain reaction (RT-PCR). Subsequently, the TGF β R2/SMAD2/3 pathway was activated by TGF β , and the KLF10 overexpression and miR-19b-3p overexpression/knockdown models were constructed. The cell counting kit-8 (CCK-8) assay, colony formation assay and flow cytometry were implemented to verify RCC cell proliferation, Sunitinib chemosensitivity, and apoptosis. The expression of apoptosis-related proteins and the TGF β R2-SMAD2/3-KLF10 pathway was monitored by Western blot. MiR-19b-3p was overexpressed in sunitinib-resistant RCC cell lines (OSRC-2R and Caki-1R) and their exosomes (vs. normal OSRC-2 and Caki-1 cell lines).

Results

In-vitro experiments showed that knocking down cellular and exosomal miR-19b-3p levels reduced the proliferation and colony-forming ability of OSRC-2 and Caki-1 cells and strengthened their apoptosis and sensitivity to Sunitinib. Bioinformatics analysis illustrated that miR-19b-3p targeted TGF β R2 and inhibited TGF β R2/SMAD2/3. Activation of the TGF β R2-SMAD2/3 pathway via TGF β dampened ORSC-2 and Caki-1 cell proliferation, induced apoptosis, and enhanced their chemosensitivity to Sunitinib.

Conclusion

Moreover, TGF β heightened KLF10 expression, and overexpressing KLF10 attenuated miR-19b-3p-mediated carcinogenic effects and resistance to Sunitinib by increasing SMAD2/3 phosphorylation. RCC cell-derived exosomal miR-19b-3p enhances RCC progression and Sunitinib chemoresistance by inactivating TGF β R2-SMAD2/3-KLF10.

1. Background

Renal cell carcinoma (RCC) is among the urinary malignancies originating from renal tubular epithelial cells, accounting for about 3% of adult malignancies [1–2]. RCC occurs mostly in elderly patients aged

60–70 years and is more prevalent in male patients than in females [3]. Smoking, obesity, hypertension, and chronic kidney disease are all high-risk factors for RCC [4]. Currently, the main clinical treatment for RCC is surgical resection and radiotherapy, but metastasis and recurrence still occur in about 30% of patients after the surgery, leading to an abysmal prognosis [5]. Sunitinib is a first-line chemotherapy agent for RCC, while drug resistance is a key factor leading to chemotherapy failure [6–7]. Hence, it is crucial to find novel tumor markers and therapeutic targets for RCC.

MicroRNAs (miRNAs) are small non-specific non-coding RNAs with a length of 22–25 nucleotides that modulate gene expression at the translational level [8–9]. Multiple studies have demonstrated that miRNAs are abnormally expressed in various malignant tumors and are widely considered biomarkers that affect tumorigenesis and tumor development [10–11]. In RCC, many miRNAs have ectopic expressions and control the malignant behaviors of RCC cells [12–13]. As a miRNA, miR-19b-3p has also been confirmed by recent studies to contribute to various cancers as whether an oncogenic or tumor-suppressive miRNA. For example, miR-19b-3p is overexpressed in pancreatic cancer, and silencing miR-19b-3p significantly elevates the PTEN expression, thereby attenuating the proliferation of pancreatic cancer cells [14]. Another study found that miR-19b-3p is lowly expressed in saracatinib-resistant breast cancer cells compared with saracatinib-sensitive cells. And overexpression of miR-19b-3p remarkably increased the IC₅₀ value of saracatinib, and promoted cell migration [15]. Additionally, miR-19b-3p is found overexpressed in exosomes derived from cancer stem cells (CSCs) of Clear cell renal cell carcinoma (CCRCC) patients. The exosomes shuttled miR-19b-3p aggravates the migration and metastasis of RCC cells via promoting their epithelial-mesenchymal transition (EMT) [16]. From the above study, we know that miR-19b-3p does not play exactly the same role in cancer, perhaps because the downstream molecules are different. Nevertheless, the function of miR-19b-3p in regulating RCC remains unclear.

Transforming growth factor β receptor 1 and 2 (TGF β R1/R2) both belong to a glycoprotein with a molecular weight of 53 kDa and 70–85 kDa. Also, they function as serine/threonine kinase receptors that regulate tumor cell development and tumor metastasis through downstream signaling pathways, including the SMAD pathway [17]. Wang S et al. found that miR-133b inhibits the TGF β -SMAD pathway to abate breast cancer invasion, tumor metastasis and epithelial-mesenchymal transition (EMT) *in vitro* and *in vivo*, exerting significant anti-tumor activity [18]. While the TGF-beta-mediated signaling pathway exerts dual roles as a tumor suppressor and tumor promoter during tumorigenesis [19]. Krüppel-like factor 10 (KLF10), an important downstream gene of the TGF β -SMAD pathway, contributes to the proliferation, differentiation, and apoptosis of various malignant cells by binding to the SP1 locus on DNA and interacting with regulatory transcription factors [20]. It has been confirmed that KLF10 is a key effector of the TGF β -SMAD signaling, which inhibits TGF β -induced EMT and TGF β -mediated proliferation and metastasis through a negative feedback loop [21]. Nevertheless, the effect and mechanism of miR-19b-3p in regulating the TGF β R2-SAMD2/3-KLF10 axis in RCC are still unclear.

Here, miR-19b-3p was found to increase in sunitinib-resistant RCC cell lines and exosomes. Knocking down miR-19b-3p impeded RCC cells' proliferation and colony formation and enhanced cell apoptosis

and chemosensitivity to Sunitinib. Moreover, bioinformatics analysis suggested that TGF β R2 is an underlying downstream target of miR-19b-3p. Furthermore, knocking down miR-19b-3p facilitated the TGF β R2 expression. In conclusion, this study aims to probe the influence of miR-19b-3p's regulating the TGF β R2-SMAD2/3-KLF10 pathway on Sunitinib-mediated RCC cell proliferation, migration, invasion and apoptosis, hoping to provide a new molecular target and theoretical basis for clinical treatment of RCC patients.

2. Materials And Methods

2.1 Cell culture and treatment

Human RCC cell lines Caki-1 and OSRC-2 were bought from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were placed in the RPMI 1640 medium (Thermo Fisher Scientific, MA, USA) containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, MA, USA) and 1% penicillin/streptomycin (Invitrogen, CA, USA). The temperature was kept at 37°C, and the concentration of CO₂ was 5%. The medium was altered every three days. After trypsinization with 0.25% trypsin (Thermo Fisher Hyclone, Utah, USA), the cells in the logarithmic growth phase were taken for use. For the activating of the TGF β R2-SMAD2/3 pathway, recombinant TGF- β (rTGF- β) (T-7039; Sigma-Aldrich Product Number: MFCD00166089) was used for dealing with RCC cells at 100 ng/ml for 24 hours.

2.2 Cell transfection

Cells in the logarithmic growth stage were trypsinized and inoculated into 6-well plates (5×10⁶/well). After cell growth was stable, Caki-1 and OSRC-2 cells were transfected with miR-19b-3p mimics, miRNA negative control (miR-NC), miR-19b-3p inhibitors (miR-19b-3p-in) and its negative control controls (NC-in), KLF10 overexpression plasmids (KLF10) and blank plasmids (vector) (all provided by Guangzhou Ruibo Biotechnology Co., Ltd) using the FuGENE®HD Transfection Reagent (Roche, Shanghai, China), respectively. Cells in each group were incubated at 37°C and 5% CO₂. After transfection for 24 hours, cells in each group with stable growth were taken for use.

2.3 Construction of Sunitinib resistant cell lines

The drug-resistant cell lines were screened by the combination of high dosage and gradually increasing dosage. After Caki-1 and OSRC-2 cells entered the logarithmic growth phase, the culture medium containing 5 μ M Sunitinib was replaced. After 24 hours of culture, the primary medium was replaced by a normal complete medium, and the cells were trypsinized and sub-cultured. When the cells grew to 80% fusion, they were cultured with a 0.5 μ M initial concentration. After 48 hours, the primary medium was changed to a normal medium, and the Sunitinib concentration was gradually increased after the cells grew stable. The above procedures were repeated until cells could be grown and sub-cultured stably with 5 μ M Sunitinib.

2.4 Cell counting kit-8 (CCK-8) assay

Cells in each group at the logarithmic growth stage were taken, and the cell density was adjusted to 2×10^3 cells/well after trypsinization. They were then inoculated in 96-well plates. Afterward, 200 μ L DMEM medium was added to each well, and the medium was maintained at 37°C with 5% CO₂. When the cells grew to a 50% fusion rate, a new serum-free medium was provided, and the serum was added 24 hours later. After continuous culture for 24 hours, 10 μ L CCK-8 solution (Beyotime Biotechnology, Shanghai, China) was added to each well, and the cells' absorbance values at 450 nm at different periods (12, 24, 36, 48, 60, and 72 hours) were measured according to the CCK-8 instruction.

2.5 Drug sensitivity test

Caki-1 and OSRC-2 cells in the logarithmic phase and their corresponding drug-resistant cell lines (Caki-1R and OSRC-2R) were trypsinized with 0.25% trypsin. After centrifugation at 1500 RPM for 5 min, the cells were seeded in 96-well plates (5×10^3 /well) and maintained in the incubator for 24 hours. After cell adherence, the cells were cultured with Sunitinib at different concentrations (0, 2.5, 5, 10, 10, 20, 40, 80 μ M), respectively. Then, 100 μ L of the above drugs at different concentrations were added to each well and incubated for 48 hours. At last, cell viability was tested by the CCK-8 method.

2.6 Reverse transcription-polymerase chain reaction (RT-PCR)

The Trizol reagent (Invitrogen, Carlsbad, CA, USA) was adopted to verify the expression of exosomal miR-19b-3p and intracellular KLF10, TGFB2, BAMBI and ZFYEV9 in Caki-1, OSRC-2, Caki-1R, and OSRC-2R cells, respectively. The PrimeScript™ RT Reagent kit (Invitrogen, Shanghai, China) was applied for reverse transcription of mRNA into cDNA. RT-PCR was then performed with the SYBR GreenPCR reagent and an ABI7500FAST Real-Time PCR instrument. The $2^{-\Delta\Delta C_t}$ method was employed to evaluate the miR-19b-3p, KLF10, TGFB2, KLF10, BAMBI and ZFYVE9 expressions (U6 and GAPDH served as the internal reference). Primer sequences were shown in Table 1.

Table 1
Primer sequences

The target	Forward (5 '-3')	Reverse (5 '-3')
miR-19b-3p	GTGCAAATCCATGCAAACTGA	GTGCAGGGTCCGAGGTGCT
KLF10	GGGTGGCTACAGATGTGACT	CCCGTTGTGCAGAGTTCAAA
TGFB2	TCCTTCAAGCAGACCGATGT	AGCACTCAGTCAACGTCTCA
BAMBI	CAAAGAGTTGTGGTTCCGGG	GTCCGTGAAAGCTGTAGTGC
ZFYVE9	AGACCGAAAACAGAGGGGAG	ACATCCATCTGTTCCCTGGG
U6	CTCGCTTCGGCAGCACA	ACGCTTCACGAATTTGCGT
GAPDH	TGGTTGAGCACAGGGTACTT	CCAAGGAGTAAGACCCCTGG

2.7 Western blot (WB)

2×10^6 cells were taken from the above groups, washed with precooled PBS three times, and lysed with RIPA lysate. Then, the supernatant was harvested, and the protein concentration was determined by the BCA method. After polyacrylamide gel electrophoresis, 50 g of the total protein was transferred to polyvinylidene fluoride (PVDF) membranes. After being blocked with 5% skim milk at room temperature (RT) for 2 hours, the membranes were rinsed with 0.05% TBST three times. The membranes were then incubated with the primary antibodies (dilution ratio: 1:1000) provided by Abcam (MA, USA), including Bax (ab32503), Bcl2 (ab218123), Caspase3 (ab13847), CD9 (ab92726), CD63 (ab134045), CD81 (ab109201), TGF β R2 (ab186838), SMAD2 (ab 40855), p-SMAD2 (ab53100), SMAD3 (ab40854), p-SMAD3 (ab52903), KLF10 (ab73537), and β -actin (ab115777) overnight at 4°C. After TBST washing, the membranes were incubated with HRP-labeled rabbit secondary antibody (concentration 1: 3000) for 1 hour. Subsequently, the membranes were cleaned with TBST three times. Finally, the WB reagent (Invitrogen) was applied for color imaging, and each protein's gray value was analyzed via Image J.

2.8 Isolation and identification of exosomes

Isolation of exosomes by ultracentrifugation: RCC cells Caki-1 and OSRC-2 were placed in a 50 mL centrifuge tube, and 30 mL PBS was added. After centrifugation at a radius of 8 cm and 1500 RPM for 5 min, the cells were cultured in the DMEM/DF12 medium containing 10% fetal bovine serum until cell fusion reached 70%. Then, the medium was changed, and the cells were further cultured for 10 ~ 12 hours. The supernatant was collected in a 2 mL centrifuge tube and processed following the instructions of the RiboTMExosome Isolation Kit (Ribo Biotechnology Co., Ltd., Xi'an, China). Briefly, 2 mL of the sample was transferred to a dry centrifuge tube (2 mL), and one-third of the volume of RibdM Exosome Isolation Reagent B was added and mixed upside down until the sample was completely dissolved. After the mixture was maintained in a refrigerator at 4°C overnight, it was moved into a new 2 mL centrifuge tube and centrifuged at 1500 RPM for 30 min, and the supernatant was discarded. The remaining substances in the tube were exosomes. Detection of exosomes of RCC by electron microscopy: 2 mL PBS was added into the centrifuge tube for exosome separation and mixed. After 50-fold dilution, 10 μ L exosome diluent was collected and added to 2 mm carbon-bearing copper mesh. After standing for 5 min at RT, the excess liquid was blotted off with dry filter paper, and 30 μ L of 3% (w/v) sodium phosphotungstate solution was used for re-staining for 30 min and then dried at RT, observed by transmission electron microscopy and photographed. Finally, WB was conducted to detect exosome markers CD9, CD63, and CD81, as described above.

2.10 Colony formation experiment

The cells of each group in the logarithmic growth phase were collected and inoculated in a 100 mm dish containing culture medium at 800 cells per dish, mixed well and incubated. After 24 hours, the primary medium was replaced with the complete medium, and the medium was altered every 3 to 4 days and cultured for 12 days. After PBS washing, 4% paraformaldehyde fixation for 10 min, 5% crystal violet

staining for 10 min, the cells were photographed. Image-ProPlus was adopted to calculate the number of cell colony formations in each dish. Each experiment was repeated three times and measured three times.

2.11 Flow cytometry (FCM)

The cells were collected after treatment with the above factors, washed twice with PBS, and resuspended in 150 μ L Binding Buffer. Then, 10 μ L Annexin V-FITC and 5 μ L propidium iodide staining solution (PI) were added to the buffer. The cells were incubated at 4°C for 15 min at RT in the dark. Cell apoptosis was tested following the instructions of the Annexin V-FITC/PI Apoptosis Detection Kit (Yeasen Biotech Co., Ltd.) using FCM.

2.12 Dual-luciferase reporter assay

TGF β R1/R2 was predicted as a potential target of miR-19b-3p by Starbase software, and reporter plasmids of wild-type and mutated TGFBR2 were constructed. MiR-19b-3p mimics and miR-NC were transfected into Caki-1R and OSRC-2R cells. After 48 hours, the luciferase activity was measured according to the dual-luciferase reporter assay instructions (Promega, Madison, WI, USA). We conducted all experiments in triplicate and repeated them three times.

2.13 Xenografted tumor experiment

Sixteen 6-week-old nude mice (body weight: 22-24g, female) were purchased from the Animal Research Center of Wuhan University (license number: SCXK (Chuan) 2018-15) and raised in a specific pathogen-free (SPF) environment with adequate light and air, and free for food and water. All mice used in this experiment were approved by the Animal Ethics committee of Sichuan Provincial People's Hospital and followed the Guide for the Care and Use of Laboratory Animals. Sixteen mice were randomly divided into 4 groups, with 4 mice in each group. MiR-19b-3p-in and NC-in were transfected into Caki-1R and OSRC-2R cells, respectively, and then the transfected cells (2×10^6) were injected subcutaneously into the right abdomen of the nude mice. When tumors were observed (with a volume over 10 mm³), they were measured weekly with a vernier caliper, and the tumor volume was calculated. After 5 weeks, the mice were euthanized with phenobarbital (30 mg/kg body weight), dissected about 2 mm from the edge of the tumor, and weighed.

2.14 Immunohistochemistry (IHC)

After paraffin embedding and sectioning (4 μ M), the xenografted tumor tissues were dewaxed with xylene and hydrated with gradient alcohol. The endogenous peroxidase was inactivated by 3% H₂O₂ for 10 min. Microwave repair was performed with 0.01 mol/L sodium citrate buffer (pH = 6.0, 15 min). After blocking for 20 min with 5% bovine serum albumin (BSA), the primary antibodies (1:100) of Ki67 (ab15580),

TGF β R2 (ab186838)), p-SMAD2 (ab280888), and p-SMAD3 (ab52903) (all purchased from Abcam, MA, USA) and KLF10 (11881-1-AP) (Proteintech, Chicago, USA), were stored overnight at 4°C. The next day, the goat anti-rabbit secondary antibody was added dropwise and incubated at RT for 20 min. After PBS washing, DAB was utilized for color development. After being re-stained by hematoxylin, dehydrated and transparentized, the sections were mounted and examined microscopically.

2.15 Bioinformatics analysis

MiR-19b-3p level in Kidney Renal Clear Cell Carcinoma and normal tissues was analyzed through Starbase database (<http://starbase.sysu.edu.cn/>). The survival rate of RCC patients with different levels of miR-19b was analyzed via Kaplan-Meier Plotter (<http://kmplot.com/analysis/>). The online databases including miRmap, microT, miRanda, Pictar and Targetscan were used for analyzing the potential targets of miR-19b-3p via mirPath v.3 (<http://snf-515788.vm.oceanos.grnet.gr/index.php?r=mirpath>) was used for analyzing the potential pathways regulated by miR-19b-3p. Venn's diagram was used for analyzing sharing genes potentially modulated by miR-19b-3p via <http://bioinformatics.psb.ugent.be/webtools/Venn/>.

2.16 Statistical analysis

In this experiment, SPSS22.0 software was employed to conduct statistics and analysis of all data. All measurement data were expressed as "mean $\pm$ standard deviation" ($\bar{x} \pm s$), and statistical data or percentage (%) were compared using χ^2 analysis. One-way variance analysis was used for inter-group comparisons, and the SNK test was employed for multiple group comparisons. $P < 0.05$ indicated statistical significance.

3. Results

3.1 MiR-19b-3p was up-regulated in sunitinib-resistant RCC cells and exosomes

To investigate the expression of miR-19b-3p in sunitinib-resistant RCC cells and exosomes, we first adopted CCK-8 to verify the viability of normal cells Caki-1 and OSRC-2 and sunitinib-resistant cells (Caki-1R and OSRC-2R) under different concentrations of Sunitinib (0, 2.5, 5, 10, 10, 20, 40, 80 μ M). It was found that with the increase of Sunitinib concentration, the cell viability of Caki-1, OSRC-2, Caki-1R and OSRC-2R was decreased, but the viability of drug-resistant cells was higher than that of normal cells (Fig. 1A, $P < 0.05$). FCM was implemented to examine the apoptosis rate of the above cells, and it was revealed that the apoptosis of Caki-1R and OSRC-2R was significantly reduced compared with that of CaK-1 and OSRC-2 cells (Fig. 1B, $P < 0.05$). The cellular miR-19b-3p expression was monitored by RT-PCR, and the results manifested that the miR-19b-3p expression in Caki-1R and OSRC-2R was significantly

higher than that in Caki-1 and OSRC-2 (Fig. 1C, $P < 0.05$). In addition, RCC exosomes were observed under transmission electron microscopy to be round or oval in shape and variable in size, ranging from 30 to 100 nm in diameter, with an intact membrane structure and containing low-density materials (Fig. 1D). WB also detected the presence of exosome markers CD9, CD63, and CD81 (Fig. 1E). Besides, the miR-19b-3p profile in exosomes was determined by RT-PCR, and the results illustrated that the exosomal miR-19b-3p level in Caki-1R and OSRC-2R was significantly higher than that in Caki-1 and OSRC-2 (Fig. 1F, $P < 0.05$). Interestingly, we found that miR-19b-3p level was enhanced in Kidney Renal Clear Cell Carcinoma (compared with the normal group) through Starbase database (<http://starbase.sysu.edu.cn/>) (sup Fig. 1A). And a higher level of miR-19b was associated with a poorer survival rate of RCC patients identified via Kaplan-Meier Plotter (<http://kmplot.com/analysis/>) (sup Fig. 1B). These results indicated that miR-19b-3p was overexpressed in sunitinib-resistant RCC cells and exosomes.

3.2 Inhibiting miR-19b-3p attenuated Sunitinib resistance in RCC cells

First, we constructed a miR-19b-3p knockdown cell model and detected the miR-19b-3p expression with RT-PCR to verify the effect of inhibiting miR-19b-3p on the proliferation and apoptosis of KAK-1R and OSRC-2R. As a result, miR-19b-3p was significantly down-regulated in the miR-19b-3p group (vs. the NC-in group) (Fig. 2A, $P < 0.05$). Besides, CCK-8 results showed that compared with the NC-in group, the cell proliferation in the miR-19b-3p-in group was significantly abated (Fig. 2B, $P < 0.05$). Moreover, the sensitivity of Caki-1R and OSRC-2R cells to Sunitinib was also monitored by CCK-8, and it was found that the cell viability of the miR-19b-3p-in group decreased significantly with the increase of Sunitinib concentration (vs. the NC-in group) (Fig. 2C, $P < 0.05$). The colony formation experiment results manifested that the colony formation ability of the miR-19b-3p-in group was reduced (vs. the NC-in group) (Fig. 2D, $P < 0.05$). FCM was used to detect cell apoptosis rate 48 hours later, and the results illustrated that the cell apoptosis in the miR-19b-3p-in group was significantly increased (vs. the NC-in group) (Fig. 2E, $P < 0.05$). WB results demonstrated that the pro-apoptotic proteins Bax and Caspase3 were up-regulated and the antiapoptotic Bcl2 was down-regulated in the miR-19b-3p-in group (vs. the NC-in group) (Fig. 2F, $P < 0.05$). These results suggested that inhibition of miR-19b-3p enhanced the chemosensitivity of RCC cells to Sunitinib, inhibited cell proliferation and induced cell apoptosis.

3.3 Inhibiting miR-19b-3p attenuated the growth of sunitinib-resistant RCC cells

We constructed xenografted tumor models of Caki-1R and OSRC-2R with miR-19b-3p knockdown to further verify whether inhibition of miR-19b-3p had the same effect *in vivo*. The tumor volume was measured with a vernier caliper, and it was revealed that the tumor volume of mice in the miR-19b-3p-in group was smaller than that in the NC-in group (Fig. 3A, $P < 0.05$). Tumor images of tumor-bearing mice were shown in Fig. 3B. Besides, compared with the NC-in group, the tumor weight in mice of the miR-19b-3p-in group was lower (Fig. 3C, $P < 0.05$). Moreover, IHC results demonstrated that the Ki67 expression in

the miR-19b-3p-in group was significantly dampened (vs. the NC-in group) (Fig. 3D, $P < 0.05$). These results confirmed that inhibition of miR-19b-3p also significantly reduced the growth and proliferation of Sunitinib-resistant RCC cells *in vivo*.

3.4 Inhibition of exosomal-derived miR-19b-3p repressed RCC cells' proliferation and resistance to Sunitinib

To further probe the influence of exosome-derived miR-19b-3p on the chemoresistance of RCC cells Caki-1 and OSRC-2 to Sunitinib, we constructed an exosome-derived miR-19b-3p knockdown cell model and verified the miR-19b-3p expression by RT-PCR. It was found that compared with the exo-NC-in group, miR-19b-3p was significantly down-regulated in the exo- miR-19b-3p-in group (Fig. 4A, $P < 0.05$). The cell proliferation was examined by the CCK-8 method, and the results confirmed that compared with the blank group, the cell proliferation in the exo-NC-in group was significantly increased. However, compared with the exo-NC-in group, the cell proliferation of the exo-miR-19b-3p-in group was significantly decreased (Fig. 4B, $P < 0.05$). CCK-8 results revealed that with the increase of Sunitinib concentration, the cell viability of the exo-NC-in group was enhanced (vs. the Blank group). In contrast, compared with the exo-NC-in group, the cell proliferation of the exo-miR-19b-3p-in group was repressed (Fig. 4C, $P < 0.05$). The colony formation experiment results showed that compared with the blank group, the colony formation of the exo-NC-in group was stronger. Nevertheless, the colony formation of the exo-miR-19b-3p-in group was weaker than that of the exo-NC-in group (Fig. 4D, $P < 0.05$). FCM results showed that the cell apoptosis rate in the exo-NC-in group was decreased (vs. the Blank group), while it was significantly increased in the exo-miR-19b-3p-in group (vs. the exo-NC-in group) (Fig. 4E, $P < 0.05$). WB also illustrated that Bax and Caspase3 were down-regulated and Bcl2 was up-regulated in the exo-NC-in group (vs. the Blank group). In contrast, the expression of Bax and Caspase3 was elevated and that of Bcl2 was attenuated in the exo-miR-19b-3p-in group compared with that of the exo-NC-in group (Fig. 4F, $P < 0.05$). These results verified that the inhibition of exosomal miR-19b-3p reduced the chemoresistance of RCC cells to Sunitinib, inhibited cell proliferation and induced cell apoptosis.

3.5 MiR-19b-3p inactivated the TGF β R1/2-SMAD2/3-KLF10 pathway

To investigate the mechanisms involved in miR-19b-3p's regulation of RCC cells, we analyzed the potential targets of miR-19b-3p through the online databases including miRmap, microT, miRanda, Pictar and Targetscan. 217 genes were found (Fig. 5A). Then mirPath v.3 showed that the TGF β pathway was potentially regulated by miR-19b-3p, in which 12 genes might be involved (Fig. 5B). Next, we found that three genes (including TGFBR2, BAMBI and ZFYVE9) were shared between the 217 genes and 12 genes (Fig. 5C). RT-PCR was conducted to detect the three genes in RCC cells with overexpressed miR-19b-3p. It was found that miR-19b-3p inhibited TGFBR2, but not BAMBI and ZFYVE9 (Fig. 5D). To ensure the targeted relationships between miR-19b-3p and TGFBR2, the dual-luciferase reporter assay was conducted and the results manifested that miR-19b-3p overexpression significantly abated the luciferase

activity of Caki-1R and ORSC-2R cells transfected with TGFBR2-WT, but miR-19b-3p had no significant effect on the luciferase activity Caki-1R and ORSC-2R cells transfected with TGFBR2-mut (Fig. 5E). The expression of the TGFBR2-SMAD2/3-KLF10 pathway was examined by WB, and the results illustrated that TGFBR2, p-SMAD2/3 and KLF10 in the miR-19b-3p-in group were up-regulated (vs. the NC-in group) (Fig. 5F, $P < 0.05$). Additionally, IHC testified that the expression of TGFBR2, SMAD2, SMAD3 and KLF10 was increased in the tumor tissues of the miR-19b-3p-in group (Fig. 5G). Our results suggested that miR-19b-3p targeted TGFBR2 and dampened SMAD2/3 phosphorylation and KLF10 expression.

3.6 Activating the TGFBR2-SMAD2/3 pathway repressed miR-19b-3p-mediated Sunitinib-resistance of RCC cells

Through GEPIA database (<http://gepia.cancer-pku.cn/>), we found that lower level of TGFBR2, SMAD2, SMAD3 and KLF10 all predicted poorer overall survival and disease-free survival of Kidney renal clear cell carcinoma (sup Fig. 2A-H), and there is a positive relationship of TGFBR2 with SMAD2, SMAD3 and KLF10 in Kidney renal clear cell carcinoma in terms of their expressions (sup Fig. 2I-K). To further investigate the effect of the TGFBR2-SMAD2/3 pathway on the chemosensitivity of miR-19b-3p-mediated Sunitinib resistance, we constructed a miR-19b-3p overexpression model in Caki-1 cells (Fig. 6A), which were then treated with TGF β in order to reactivate the TGFBR2-SMAD2/3 pathway. The results showed that miR-19b-3p overexpression promoted cell proliferation and TGF β inhibited the miR-19b-3p-mediated effect (Fig. 6B). Besides, CCK-8 was adopted to verify the chemosensitivity of Caki-1 cells to Sunitinib, and it was discovered that miR-19b-3p overexpression restrained the Sunitinib-chemosensitivity of Caki-1 cells (vs. the miR-NC group). However, TGF β treatment reversed miR-19b-3p-mediated effect (Fig. 6C, $P < 0.05$). Additionally, the colony formation experiment showed that the colony formation ability of the miR-19b-3p group was facilitated compared with that of the miR-NC group. Nevertheless, the TGF β treatment on the basis of miR-19b-3p intervention declined the cell colony formation ability (Fig. 6D, $P < 0.05$). Moreover, FCM demonstrated that compared with the miR-NC group, cell apoptosis was reduced in the miR-19b-3p group. On the contrary, compared with the miR-19b-3p group, apoptosis was increased in the miR-19b-3p + TGF β group (Fig. 6E, $P < 0.05$). WB results also showed that, compared with the miR-NC group, Bax and Caspase3 were down-regulated and Bcl2 was up-regulated in the miR-19b-3p group. Compared with the miR-19b-3p group, Bax and Caspase3 in the miR-19b-3p + TGF β group were significantly up-regulated, while Bcl2 was down-regulated (Fig. 6F, $P < 0.05$). The expression of the TGFBR2-SMAD2/3-KLF10 pathway was also monitored by WB, and the results illustrated that the above molecules in the miR-19b-3p group were down-regulated compared with that in the miR-NC group. Nevertheless, compared with the miR-19b-3p group, the above molecules in the miR-19b-3p + TGF β group were up-regulated (Fig. 6G, $P < 0.05$). Additionally, the results of WB showed that not only did TGF β induce upregulation of TGFBR2-SMAD2/3, but also it enhanced KLF10 expression in RCC cells (sup Fig. 3A-B). Collectively, our results demonstrated that activating the TGFBR2-SMAD2/3-KLF10 pathway inhibited miR-19b-3p-mediated resistance to Sunitinib in RCC cells.

3.7 MiR-19b-53p reversed the tumor-suppressive effects mediated by KLF10 overexpression

To further verify the influence of miR-19b-3p's regulating KLF10 on the proliferation and apoptosis of RCC Caki-1 cells, we constructed a KLF10 overexpression cell model and examined the protein expression of KLF10 in the KLF10 group by WB. It was found that KLF10 was significantly up-regulated in the KLF10 group (vs. the vec. group) (Fig. 7A, $P < 0.05$). Additionally, CCK-8 results showed that the cell proliferation rate in the KLF10 group was hampered (vs. the vec. group). Meanwhile, compared with the KLF10 group, the cell proliferation rate of the miR-19b-3p + KLF10 group was higher (Fig. 7B, $P < 0.05$). Besides, CCK-8 assayed the sensitivity of KAK-1 cells to Sunitinib, and it was revealed that with the increase of Sunitinib concentrations, the cell viability of the KLF10 group decreased significantly (vs. the vec. group). In contrast, compared with the KLF10 group, the cell viability of the miR-19b-3p + KLF10 group was increased (Fig. 7C, $P < 0.05$). The colony formation experiment results showed that the KLF10 group was less capable of colony formation than that of the vec. group. Nevertheless, compared with the KLF10 group, the miR-19b-3p + KLF10 group exhibited stronger colony formation abilities (Fig. 7D, $P < 0.05$). FCM illustrated that the apoptosis was facilitated in the KLF10 group (vs. the vec group), while it was significantly decreased in the miR-19b-3p + KLF10 group compared to that of the KLF10 group (Fig. 7E, $P < 0.05$). WB was implemented to measure the expression of apoptosis-related proteins. It turned out that compared with the vec. group, Bax and Caspase3 in the KLF10 group were up-regulated, while Bcl2 was down-regulated. However, compared with the KLF10 group, Bax and Caspase3 were significantly down-regulated, while Bcl2 was up-regulated in the miR-19b-3p + KLF10 group (Fig. 7F, $P < 0.05$). Furthermore, the expression of the TGF β R1/2-SMAD2/3-KLF10 pathway was monitored by WB, and it was discovered that there was no difference in the TGF β R1/2-SMAD2/3 expression in the KLF10 group compared with that of the vec group, while KLF10 was up-regulated. On the contrary, compared with the KLF10 group, the above molecules in the miR-19b-3p + KLF10 group were significantly down-regulated (Fig. 7G, $P < 0.05$). These results indicated that miR-19b-3p reversed the tumor-suppressive effect mediated by KLF10 overexpression, promoted cell proliferation and impeded cell apoptosis.

4. Discussion

We have learned from a large number of studies that the occurrence, development and prognosis of RCC are related to multiple miRNAs, in which miRNAs regulate downstream pathways to mediate RCC proliferation and apoptosis [22–23]. The results of this experiment also confirmed that the exosomal miR-19b-3p targeted TGF β R2, activated the phosphorylation of SMAD2/3, and up-regulated KLF10.

Sunitinib is an oral selective targeting receptor tyrosine kinase inhibitor (TKI) and a first-line chemotherapy agent for the treatment of RCC, inhibiting tumor angiogenesis by blocking the blood and nutrient supply required for tumor growth [24]. Sunitinib suppresses RCC and metastatic RCC by targeting multiple receptors such as vascular endothelial growth factor (VEGF), stem cell growth factor (KIT) and platelet-derived growth factor (PDGFR) [25]. However, the drug resistance of RCC cells greatly limits the

efficacy of Sunitinib in chemotherapy. Multiple miRNAs have been found to be involved in regulating the sunitinib-resistant RCC cell cycle. For example, inhibition of SMYD2 significantly increases AZ505 RCC cells' chemosensitivity to cisplatin, doxorubicin and Sunitinib and inducing apoptosis by down-regulating miR-125b [26]. Additionally, Yumioka T et al. also stated that transfection of miR-194-5p into Sunitinib-resistant kidney cancer cell line ACHN cells down-regulates inducible lysosomal-associated membrane protein 2 (LAMP-2), increases cancer cells' sensitivity to Sunitinib, and hampers cell proliferation [27]. This study also illustrated that blocking miR-19b-3p enhanced the sensitivity of CaK-1 and OSRC-2 cells to Sunitinib and induced cell apoptosis.

miR-19b-3p is a vital miRNA involved in diseases of the urinary system. For instance, miR-19b-3p was chronic renal failure (CRF) renal tissues, and LINC00667 silencing enhanced the proliferation and migration of renal tubular epithelial cells and ameliorated renal fibrosis in CRF via the miR-19b-3p/LINC00667/CTGF signaling pathway [28]. In another study, miR-19b-3p from tubular epithelial cells (TECs) derived exosomes aggravates renal inflammation via enhancing M1 macrophage activation [29]. With similar properties to other miRNAs, miR-19b-3p is involved in regulating the occurrence and development of various malignant tumors, including RCC [16, 30–31]. More importantly, miR-19b-3p shuttled by exosomes has been found to exert a prominent role in tumor progression. For example, miR-19b-3p transferred by exosomes suppresses EC9706 cells (belong to esophageal cancer) apoptosis, and promotes cell migration and invasion via targeting PTEN [32]. Our experimental results confirmed that miR-19b-3p was overexpressed in RCC tissues, and blocking the exosomal miR-19b-3p enhanced the chemosensitivity of RCC cell lines to Sunitinib, suggesting that miR-19b-3p acts as a potential diagnostic marker and a therapeutic target in RCC.

The inflammatory cytokines TGF β ligands (TGF- β 1, TGF- β 2, TGF- β 3) secreted in the tumor microenvironment are involved in regulating tumor cell growth, proliferation, metastasis, and apoptosis through two transmembrane serine/threonine kinase receptors, TGF β R1 and TGF β R2 [33]. TGF β R1 and TGF β R2 activate the SMAD pathway, and phosphorylated SMAD2 and SMAD3 bind to SMAD4 to form a complex [34]. Then, the generated complex is transferred to the nucleus and up-regulates KLF10. In addition, KLF10 forms a positive feedback loop in the TGF β pathway by heightening SMAD2, SMAD3, and SMAD7, particularly in diaphragm muscle expression [35]. Modulating TGF β signaling pathway by miRNAs has been recognized as a promising method in overcoming Sunitinib resistance. For example, Zhai W et al. demonstrated that miR-452-5p targets SMAD4, represses the protein expression of SMAD4/7 in RCC cell lines ORSC-2 and SW839, and strengthens cell migration and invasion. Sunitinib suppresses ORSC-2 and SW839 cell invasion and metastasis by inhibiting the miR-452-5p profile [36]. Our results also confirmed that the miR-19b-3p targets TGF β R2 and inactivated the TGF β R2-SMAD2/3-KLF10 pathway. While repressing miR-19b-3p, or TGF β treatment or overexpressing KLF10 could significantly dampen ORSC-2 and Caki-1 cell proliferation and induce cell apoptosis via the TGF β R2-SMAD2/3-KLF10 pathway.

5. Conclusion

Overall, this study showed that blocking miR-19b-3p increased the chemosensitivity of ORSC-2 and Caki-1 cells to Sunitinib and enhanced the anti-tumor activity of Sunitinib. Related mechanism studies manifested that miR-19b-3p targeted TGF β R1 and TGF β R2, and blocking miR-19b-3p activated the TGF β R2-SMAD2/3-KLF10 signaling pathway. This article provides new ideas for the clinical treatment of RCC patients. However, there are certain shortcomings in this experiment, such as not investigating whether miR-19b-3p exerts the same effect *in vivo*, which will be further investigated subsequently.

Abbreviations

RCC: renal cell carcinoma

RT-PCR: reverse transcription-polymerase chain reaction

CCK-8: cell counting kit-8

OSRC-2R and Caki-1R: overexpressed in sunitinib-resistant RCC cell lines

CSCs: cancer stem cells

CCRCC: Clear cell renal cell carcinoma

EMT: epithelial-mesenchymal transition

TGF β R1/R2: Transforming growth factor β receptor 1 and 2

KLF10: Krüppel-like factor 10

FBS: fetal bovine serum

miR-NC: miRNA negative control

WB: Western blot

PVDF: polyvinylidene fluoride

RT: room temperature

FCM: Flow cytometry

PI: propidium iodide

SPF: specific pathogen-free

IHC: Immunohistochemistry

TKI: tyrosine kinase inhibitor

VEGF: vascular endothelial growth factor

PDGFR: platelet-derived growth factor

LAMP-2: lysosomal-associated membrane protein 2

CRF: chronic renal failure

TECs: tubular epithelial cells

Declarations

Ethics approval and consent to participate

Our study was approved by the Animal Ethics committee of Sichuan Provincial People's Hospital.

Consent for publication

Not applicable.

Availability of data and materials

The data sets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by Sichuan Provincial Commission of Health and Family Planning(16PJ437).

Author's contributions

YX, ZWM conceived and designed the experiments. DL, TMS performed the experiments. JCY, QY did the statistical analysis. DL, TMS wrote the paper.

All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

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Figures

Figure 1

MiR-19b-3p was up-regulated in Sunitinib-resistant RCC cells and exosomes. A. CCK-8 assay was implemented to verify the viability of Caki-1, Caki-1R, OSRC-2, and OSRC-2R in the presence of different concentrations (0, 2.5, 5, 10, 10, 20, 40, 80 μM) of Sunitinib. B. The Caki-1, Caki-1R, OSRC-2, and OSRC-2R apoptosis was examined by FCM. C. The miR-19b-3p expression in Caki-1, Caki-1R, OSRC-2, and OSRC-2R cells was determined by RT-PCR. D. The exosomes from Caki-1R and OSRC-2R cells were isolated and identified by Electron microscopy. E. WB was used for detecting the exosomal markers CD9, CD63, and

CD81 in the exosomes. F. The miR-19b-3p expression in exosomes was determined by RT-PCR. ** $P < 0.01$, *** $P < 0.001$ (vs. Caki-1 group), ## $P < 0.01$, ### $P < 0.001$ (vs. OSRC-2 group). $N = 3$.

Figure 2

Inhibition of miR-19b-3p attenuated Sunitinib resistance in RCC cells. The miR-19b-3p knockdown model was established in Caki-1R and OSRC-2R cells. A. RT-PCR for the detection of the miR-19b-3p expression in the cells. B. Cell proliferation was verified by CCK-8 assay. C. CCK-8 was employed to test the chemosensitivity of Caki-1R and OSRC-2R cells to Sunitinib. D. The colony formation assay was utilized to examine cell proliferation. E. Cell apoptosis was monitored by FCM. F. WB verified the expression of Bax, Bcl2, and Caspase3. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (vs. NC-in group), $N = 3$.

Figure 3

Inhibiting miR-19b-3p abated the growth of Sunitinib-resistant RCC cells. Caki-1R and OSRC-2R cells were transfected with miR-19b-3p-in or miR-in, respectively, and then subjected in vivo tumor model in nude mice. A. Tumor volume. B. Tumor image. C. Tumor weight. D. Immunohistochemical for the detection of Ki67 in the tumor tissues. ** $P < 0.0$, *** $P < 0.001$ (vs NC-in group), $n = 4$.

Figure 4

Inhibiting exosome-derived miR-19b-3p dampened RCC cells' proliferation and resistance to Sunitinib. Caki-1R and OSRC-2R cells were transfected with miR-19b-3p-in or miR-in, respectively, and then the exosomes from the cells were isolated and treated with RCC cells. A. RT-PCR detected miR-19b-3p expression in the exosomes from Caki-1R and OSRC-2R cells. B. Cell proliferation rate of Caki-1 and OSRC-2 cells treated with exo-miR-in or exo-miR-19b-3p-in was verified by CCK-8 assay. C. CCK-8 assay detected the chemosensitivity of Caki-1 and OSRC-2 to Sunitinib. D. The colony formation assay was utilized to examine cell proliferation. E. Cell apoptosis was determined by FCM. F. The expression of Bax, Bcl2, and Caspase3 was compared by WB. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (vs. Blank group), # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ (VS exo-NC-in group), $N = 3$.

Figure 5

MiR-19b-3p inactivated the TGF β R2-SMAD2/3-KLF10 pathway. A. The potential targets of miR-19b-3p were analyzed through the online databases including miRmap, microT, miRanda, Pictar and Targetscan. B. mirPath v.3 was used for predicting the downstream pathways regulated by miR-19b-3p, which include TGF β signaling pathway. C. Three genes (including TGFBR2, BAMBI and ZFYVE9) was shared between the 217 genes and 12 genes. D. RT-PCR was conducted to detect the three genes (including TGFBR2, BAMBI and ZFYVE9) in RCC cells with overexpressed miR-19b-3p. E. The base-binding sites between TGFBR2

and miR-19b-3p was shown. And dual-luciferase reporter assay was conducted to verify the targeting relationship between miR-19b-3p and TGFBR2. F. The TGFBR2-SMAD2/3-KLF10 pathway in Caki-1R and OSRC-2R cells transfected with miR-in or miR-19b-3p was monitored by WB. G. IHC detected the TGFBR2, SMAD2, SMAD3 and KLF10 expression in tumor tissues. ns $P>0.05$, * $P<0.05$, ** $P<0.01$ (vs. miR-NC group), $N=3$.

Figure 6

Activating the TGFBR2-SMAD2/3 pathway repressed miR-19b-3p-mediated resistance of RCC cells to Sunitinib. The miR-19b-3p overexpression was established in Caki-1 cells by transfecting miR-19b-3p mimics. Then Caki-1 cells were treated with TGFβ (100 ng/ml) for 24 hours. A. RT-PCR detected the miR-19b-3p expression. B. Cell proliferation was measured by CCK-8. C. CCK-8 assay was conducted to detect the chemosensitivity of Caki-1 to Sunitinib. D. The colony formation assay was implemented to test cell proliferation. E. Cell apoptosis was tested by FCM. F. The expression of Bax, Bcl2, and Caspase3 was compared by WB. G. WB detected the profile of the TGFBR2-SMAD2/3-KLF10 pathway. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ (vs. miR-NC group), # $P<0.05$, ## $P<0.01$, ### $P<0.001$ (vs. miR-19b-3p group), $N=3$.

Figure 7

MiR-19b-3p reversed the anti-tumor effect mediated by overexpressing KLF10. The KLF10 overexpression model was constructed in Caki-1 cells by transfecting KLF10 overexpression plasmids, following by miR-19b-3p mimics transfection. A. WB was utilized to test KLF10 expression. B. Cell proliferation was measured by CCK-8 assay. C. CCK-8 assay was adopted to test the chemosensitivity of Caki-1 to Sunitinib. D. The colony formation assay was employed to verify cell proliferation. E. Cell apoptosis was determined by FCM. F. The expression of Bax, Bcl2, and Caspase3 was compared by WB. G. WB detected the TGF β R1/2-SMAD2/3-KLF10 pathway expression and its phosphorylation. ns $P > 0.05$, ** $P < 0.01$, *** $P < 0.001$ (vs. vec group), # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ (vs. KLF10 group), $N = 3$.

Figure 8

The mechanism diagram of miR-19b-3p in RCC cells

Supplementary Files

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