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Original Article

miR-497-5p induces apoptosis in K562 cells by downregulating ROCK1

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Abstract: Objective: To validate the role of miR-497-5p in apoptosis in K562 cells by targeting Rho-associated kinase isoform 1 (ROCK1). Methods: From January, 2017 to February, 2019, 57 patients with chronic myeloid leukemia (CML) treated in our hospital were included in patient group, and 50 healthy individuals were recruited as control group. miR-497-5p level in peripheral blood was quantitated using qRT-PCR. After transfecting with miR-497-5p overexpression vector and ROCK1 inhibitor, K562 cells were monitored in terms of proliferation (CCK8 assay), migration and invasion (Transwell), and apoptosis (flow cytometry). Binding loci between miR-497-5p and ROCK1 were predicted, and the targeting relationship was confirmed (dual-luciferase reporter (DLR) assay). Results: miR-497-5p was poorly expressed in CML ($P < 0.05$). Forced overexpression of miR-497-5p or inhibition of ROCK1 suppressed malignant processes (proliferation, proliferation, migration and invasion) in K562 cells and induced apoptosis ($P < 0.05$). DLR assay revealed a decreased luciferase activity after miR-497-5p binding to ROCK1 ($P < 0.05$). Conclusion: miR-497-5p induces apoptosis in K562 cells by downregulating ROCK1.

Keywords: miR-497-5p, ROCK1, K562 cells, invasion and migration, apoptosis

Introduction

Chronic myeloid leukemia (CML) is a prevalent myeloproliferative neoplasm [1], with deaths accounting for ~1/9 of all new cases in the United States in 2017 [2]. Since the advent of tyrosine kinase inhibitors (TKIs), great progress has been made in the treatment of CML. Nevertheless, ~50% of patients are asymptomatic, resulting in delayed diagnosis and increased difficulty in treatment [3, 4]. Clinicians still face great challenges, so comprehending the pathogenesis of CML and seeking novel targets are imperative for improving the survival of patients [5].

Although TKIs are able to suppress BCR-ABL activity, they fail to expunge all leukemia stem cells (LSCs), important cells for inducing CML, in bone marrow [6]. Therefore, effective treatment of CML remains to be investigated. miRs function as potential molecular targets for cancers by affecting cellular biological behaviors [7]. They are non-coding RNAs with a length of ~22-24 nt that degrade mRNAs or inhibit protein translation through complementary binding with the 3'UTR of mRNAs [8]. Moreover, they regulates about one third of human genes, thereby affecting cellular behaviors [9]. Upregulating miR-497-5p, which is downregulated in melanoma, inhibits biological processes in melanoma A375 cells, as well as inhibits cell cycle progression and induces apoptosis [10]. Li has pointed out that miR-497-5p is involved in non-small cell lung cancer, and is anticipated to be a promising therapeutic target [11]. Besides, there is evidence that it affects the up-regulation of oncogene BCR-ABL and promotes CML progression [12]. Nevertheless, the mechanism of miR-497-5p in CML and its effect on K562 cells remain poorly understood. Potential target binding loci between Rho-associated kinase isoform 1 (ROCK1), an essential signal pathway in CML [13], and miR-

497-5p were found by Targetscan. However, the role of ROCK1 and miR-497-5p in CML has not been dissected.

Therefore, the goal of this study is to figure out the mechanism of miR-497-5p in the regulation of biological behaviors of CML cell line K562 by targeting ROCK1 signaling pathway.

Materials and methods

Materials

Sampling of peripheral blood: From January, 2017 to February, 2019, 57 patients with CML treated in Xingtai People's Hospital were included in patient group, and 50 healthy individuals were recruited as control group. Patient inclusion criteria: treatment-naive patients who were diagnosed with CML following guidelines of European Society for Medical Oncology (ESMO) 2012 [14], as well as those with complete clinical data; All participants signed the informed consent form. Patient exclusion criteria: patients with congenital blood disease, severe liver and kidney dysfunction, as well as those who previously received relevant treatment; Pregnancy or lactating women. Fasting venous blood (3 mL) was sampled and kept at room temperature for 30 min, followed by a 10-min centrifugation (3000 xg, 4°C). Supernatant was collected and stored in a -80°C refrigerator. This study has been approved by the medical ethics committee of our hospital.

Main instruments and materials: Fetal bovine serum (FBS) and trypsin (Hyclone); bicinchoninic acid (BCA) protein assay kit (Well Bio); miR-NC and U6 (GenePharma); qRT-PCR instrument (Biorad, Berkeley, California); FC500MCL flow cytometer (Becton, Dickinson & Co.); Dulbecco's Modified Eagle Medium (DMEM; Gibco, Rockville, MD); TRIzol kit and TaqMan Reverse Transcription kit (Invitrogen); goat anti-rabbit secondary antibody (Yuanmu Biotech); DLR assay kit (Promega).

Cell source and treatment: Human CML cell line K562 was purchased from ATCC, and normal human leukocytes were obtained from peripheral blood of healthy controls. Cell culture and transfection: The cells were grown in high-glucose DMEM comprising FBS (10%) + penicillin/streptomycin solution (1%), and were routinely subcultured at 37°C and 5% CO_2. When reaching 80% confluence, all cells were harvested and K562 cells were allocated into miR-NC group, miR-497-5p-mimics group, si-NC group and si-ROCK1 group. Lipofectamine™ 2000 kit was used to transfect miR-NC, miR-497-5p-mimics, si-NC and si-ROCK1 into the corresponding cells.

Detection assays

qRT-PCR: Relative expression of peripheral blood miR-497-5p was quantitated. Total RNAs extracted by TRIzol kit were reverse transcribed to cDNAs, followed by a PCR amplification. Amplification system: cDNA (1 μL), upstream and downstream primers (0.4 μL each), 2× TransStart® Green qPCR SuperMix UDG (10 μL), Passive Reference Dye (50×) (optional, 0.4 μL), and made up to 20 μL with Nuclease-free Water. Amplification conditions: incubation at 94°C for 10 min, then 94°C for 5 s, and 60°C for 30 s, for 40 cycles. Each sample was tested in triplicate and the test was repeated 3 times. Taking U6 as the internal control, $2^{-\Delta\Delta Ct}$ was used to analyze the data.

Western blotting: ROCK1 level was quantified by Western blotting. Following lysing with RIPA buffer, total proteins were treated with electrophoresis (10% SDS-PAGE) and membrane transfer. The PVDF membrane was sealed for 2 h and added with universal secondary antibody prior to reacting at room temperature for 2 h. After three washes, the membrane was fixed and developed with enhanced chemiluminescence (ECL).

CCK8 assay: CCK8 assay was employed for the evaluation of cell proliferation. Cells were trypsinized (0.25%) and prepared into suspension ($4*10^4$ cells/mL), then transferred to a 96-well plate (100 μL/well). CCK8 reagent (10 μL) was added at the 24th, 48th, 72nd and 96th hour, respectively, followed by a 2-h culture at 5% CO_2 and 37°C. Absorbances at 490 nm were read using a microplate reader.

Transwell: Determination of cell migration and invasion was performed with Transwell. Cells were trypsinized (0.25%) and suspensions were prepared ($4*10^4$ cells/mL). Medium comprising 10% FBS (500 μL) was added to the basolateral chamber, while cell suspension (100 μL) and culture solution (200 μL) were added to the apical chamber separately. DMEM comprising 20% FBS (500 mL) was put into the basolateral chamber and cultured for 24 h. Cells in the apical chamber were wiped off with cotton swabs,

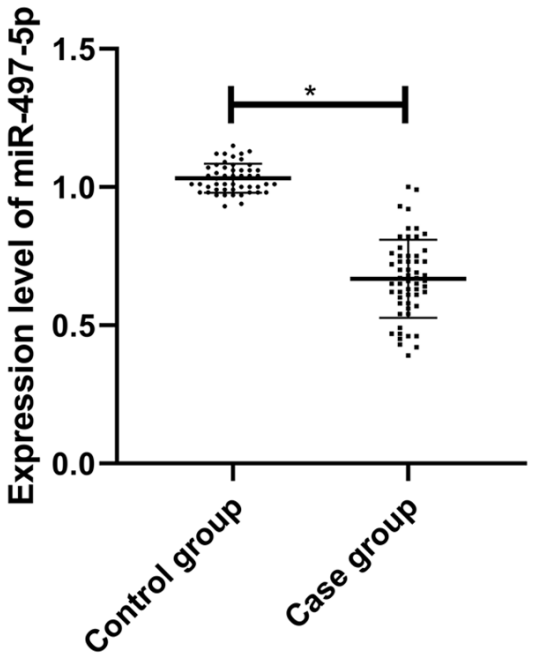

Figure 1. Peripheral blood miR-497-5p in CML. Relative expression of peripheral blood miR-497-5p in patient group is significantly down-regulated than that in control group.

the remaining cells were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, followed by drying, section preparation, and mounting. The number of invading cells was counted microscopically in 5 visual fields. The test was repeated 3 times to take the average.

Flow cytometry: Detection of apoptosis was conducted with flow cytometry. Cells were trypsinized (0.25%) and suspensions were prepared ($1*10^6$ cells/mL). AnnexinV-FITC and PI were added in turn, incubated for 5 min in dark at room temperature. Afterwards, FC500MCL was employed for apoptosis determination. The test was repeated 3 times to take the average.

DLR assay: Targetscan7.2 was adopted to predict downstream target genes of miR-497-5p. HEK293T cells were treated with ROCK1-3'UTR wild type (Wt), ROCK1-3'UTR Mutant (mut), miR-497-5p-mimics, and miR-NC using a Lipofectamine™ 2000 kit. Forty-eight hours later, luciferase activity was measured by a DLR assay kit.

Statistical analysis

SPSS20.0 and GraphPad 7 were employed to carry out data processing and graphing, respectively. Independent samples t test was adopted for inter-group comparison, one-way analysis of variance for multi-group comparison, and Fisher's least significant difference-t test for post-hoc pairwise comparison. Significance was determined when probability (P) values were less than 0.05.

Results

Peripheral blood miR-497-5p in CML

Relative expression of peripheral blood miR-497-5p was evidently downregulated in patient group than that in control group ($P < 0.05$) (**Figure 1**).

miR-497-5p and ROCK1 expression in K562 cells

Compared with normal human leukocytes, K562 cells presented downregulated relative expression of miR-497-5p and upregulated ROCK1 (both $P < 0.05$) (in **Figure 2**).

Regulation of miR-497-5p on biological behaviors of K562 cells

Transfection of miR-497-5p-mimics elevated relative expression of miR-497-5p in K562 cells ($P < 0.05$), as well as suppressed malignant processes (proliferation, migration and invasion) and enhanced apoptosis (both $P < 0.05$) (**Figure 3**).

Regulation of ROCK1 on biological behaviors of K562 cells

Transfection of si-ROCK1 downregulated ROCK1 expression ($P < 0.05$), as well as significantly suppressed malignant processes ($P < 0.05$) and enhanced apoptosis in K562 cells ($P < 0.05$) (**Figure 4**).

Correlations between miR-497-5p and ROCK1

A prediction website Targetscan found that ROCK1 was the downstream target gene of miR-497-5p. Therefore, we conducted a DLR assay and noticed that miR-497-5p-mimics inhibited the luciferase activity of ROCK1-3'UT Wt in K562 cells ($P < 0.05$), but no significant changes in luciferase activity of ROCK1-3'UTR Mut were achieved. As shown by Western blotting, miR-497-5p-mimics downregulated ROCK1 expression in K562 cells ($P < 0.05$) (**Figure 5**).

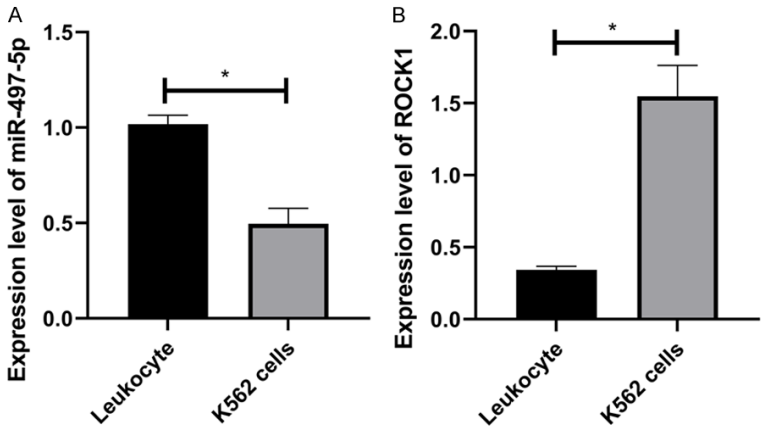

Figure 2. Expression of miR-497-5p and ROCK1 in K562 cells. Compared with normal human leukocytes, K562 cells present downregulated relative expression of miR-497-5p ($P < 0.05$) and upregulated ROCK1. *$P < 0.05$.

Discussion

CML occurs at all ages, and the incidence increases with age [15]. Although TKIs improve the prognosis and prolong the survival of most patients, the prevalence of CML is still rising [16]. 4%~15% of patients are highly resistant to imatinib mesylate (IM) [17], so it is necessary to figure out the pathogenesis of CML and seek a more effective therapeutic target to improve the prognosis.

Abnormal tyrosine kinase proteins produced by BCR-ABL fusion gene lead to leukocyte proliferation, which is a primary pathogenesis of CML [18]. miRs affect the pathogenesis of CML and drug resistance by regulation of the biological functions of CML cells [19]. miR-570 has been indicated to inhibit proliferation in CML cells by targeting IRS1 and IRS2 [20]. Another study shows that miR-424, functioning as a tumor suppressor, directly targets BCR-ABL to induce apoptosis in K562 cells, and makes the cells sensitive to IM [21]. Kang [22] reports that miR-497 is low-expressed in acute myeloid leukemia (AML) and regulates proliferation in AML cell lines via targeting MAPK/ERK1/2 pathway. Nevertheless, the mechanism of miR-497-5p in CML remains imperfectly understood. Therefore, the present study evaluates the expression and mechanism of miR-497-5p in CML to inspire a new direction for the treatment. A poor expression of peripheral blood miR-497-5p in CML indicated that miR-497-5p might be a biomarker to distinguish CML patients from healthy individuals. To figure out the molecular mechanism of miR-497-5p in CML, miR-497-5p overexpression vectors were transfected into K562 cells. It was found that miR-497-5p overexpression suppressed the malignant processes and enhanced apoptosis in K562 cells. Therefore, miR-497-5p plays a role of tumor suppressor in CML. miR-497-5p can down-regulate oncogene BCR-ABL and hinder cancer progression [23], which is similar to our findings.

ROCK1 is a serine/threonine protein kinase that regulates cytoskeleton [24] and is essential in the process of cell motility and invasion. Accumulating evidence shows that the migration and invasion of various cancer cells can be inhibited by down-regulation of ROCK1 [25-27]. Potential target binding loci between ROCK1 and miR-497-5p were found by Targetscan. A poor expression of ROCK1 was revealed in K562 cells. We transfected ROCK1 inhibitor into K562 cells, and noticed that down-regulation of ROCK1 suppressed the proliferation, migration and invasion, as well as enhanced the apoptosis. Therefore, ROCK1 plays a role of oncogene in CML. There is no research to clarify the mechanism of action between ROCK1 and miR-497-5p. Our findings demonstrated that miR-497-5p was able to target ROCK1 and reduce the luciferase activity. Moreover, the downregulated ROCK1 by transfection of miR-497-5p overexpression vector indicated that miR-497-5p suppressed proliferation in K562 cells by targeting ROCK1. Similar to our findings, Li reports that upregulation of ROCK1 weakens biological functions of leukemia cells, and may be a novel therapeutic target [28]. We speculate that the targeted binding of miR-497-5p with ROCK1 promotes the activation of ROCK1 and changes protein structure, which hinders malignant processes, as well as induces the apoptosis in cancer cells.

To sum up, peripheral blood miR-497-5p, which is lowly expressed in CML, suppresses malignant processes and enhances apoptosis in K562 cells by targeting ROCK1, thereby acting as an available target for the treatment of CML. There are still several limitations. Firstly, whether there are other targets of miR-497-5p in CML

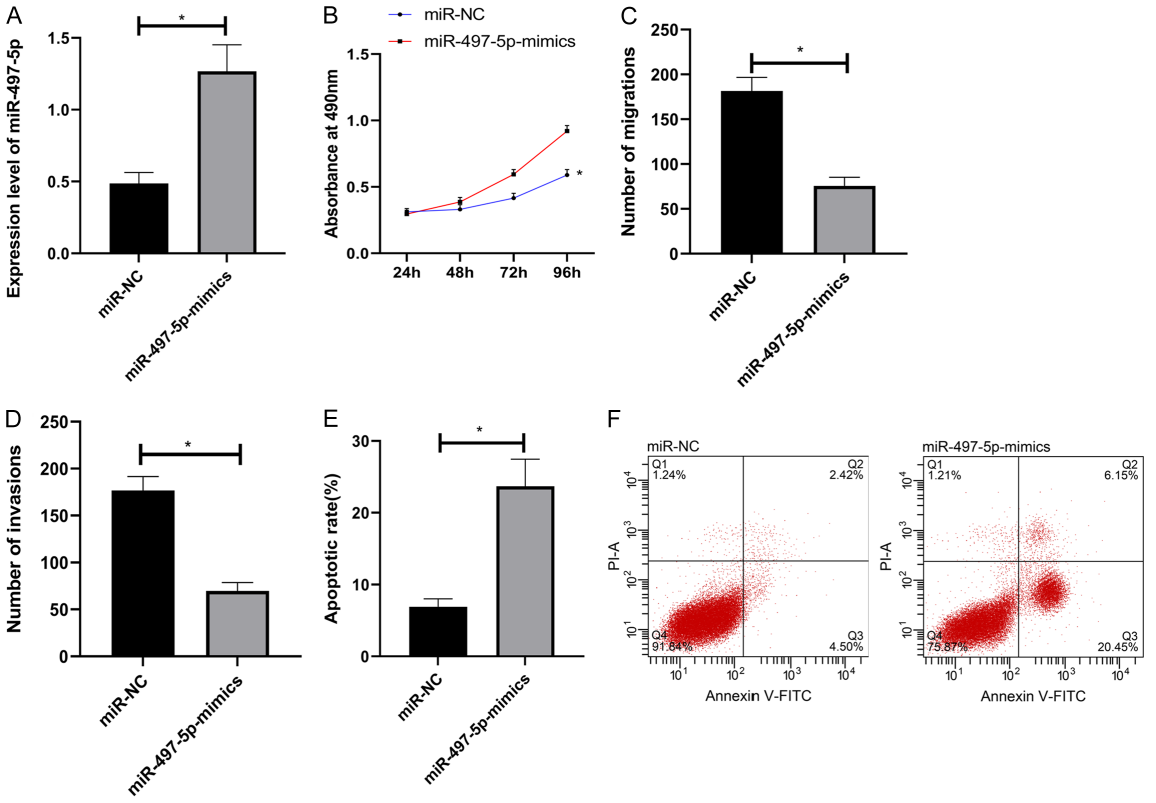

Figure 3. Regulation of miR-497-5p on biological behaviors of K562 cells. A. Transfection of miR-497-5p-mimics significantly elevates the relative expression of miR-497-5p in K562 cells. B. Transfection of miR-497-5p-mimics suppresses proliferation in K562 cells. C. Transfection of miR-497-5p-mimics suppresses migration in K562 cells. D. Transfection of miR-497-5p-mimics suppresses invasion in K562 cells. E. Transfection of miR-497-5p-mimics enhances apoptosis in K562 cells. F. Flow cytometry. *$P < 0.05$.

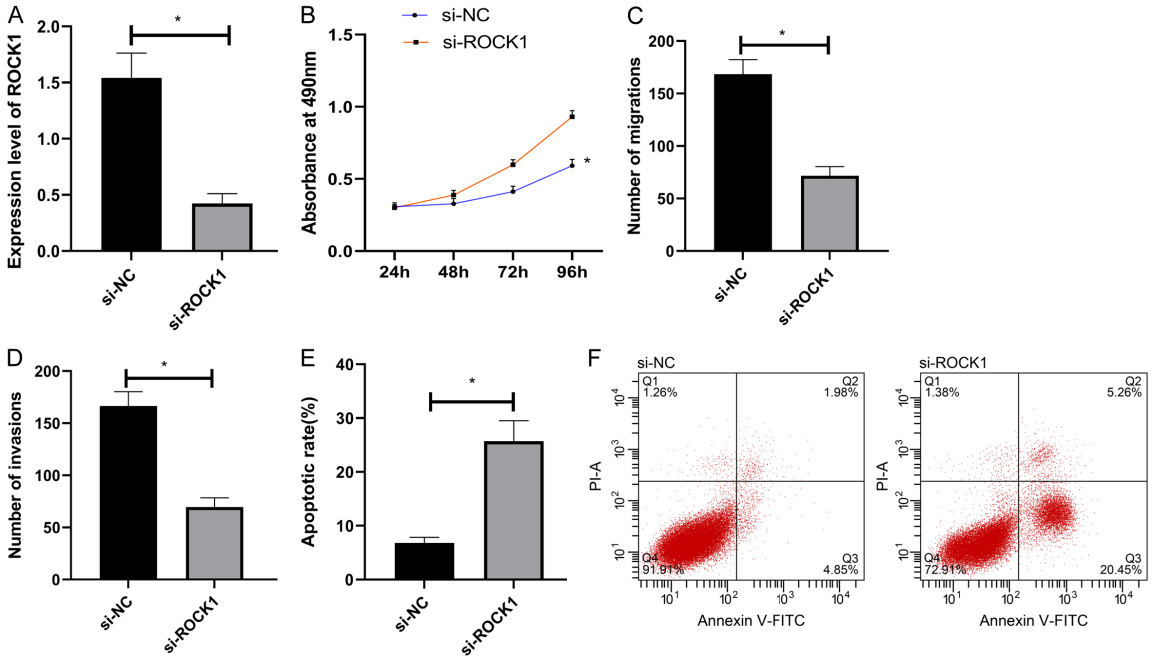

Figure 4. Regulation of ROCK1 on biological behaviors of K562 cells. A. Transfection of si-ROCK1 significantly down-regulates the relative expression of ROCK1 in K562 cells. B. Transfection of si-ROCK1 suppresses proliferation in K562 cells. C. Transfection of si-ROCK1 suppresses migration in K562 cells. D. Transfection of si-ROCK1 suppresses invasion in K562 cells. E. Transfection of si-ROCK1 enhances apoptosis in K562 cells. F. Flow cytometry. *$P < 0.05$.

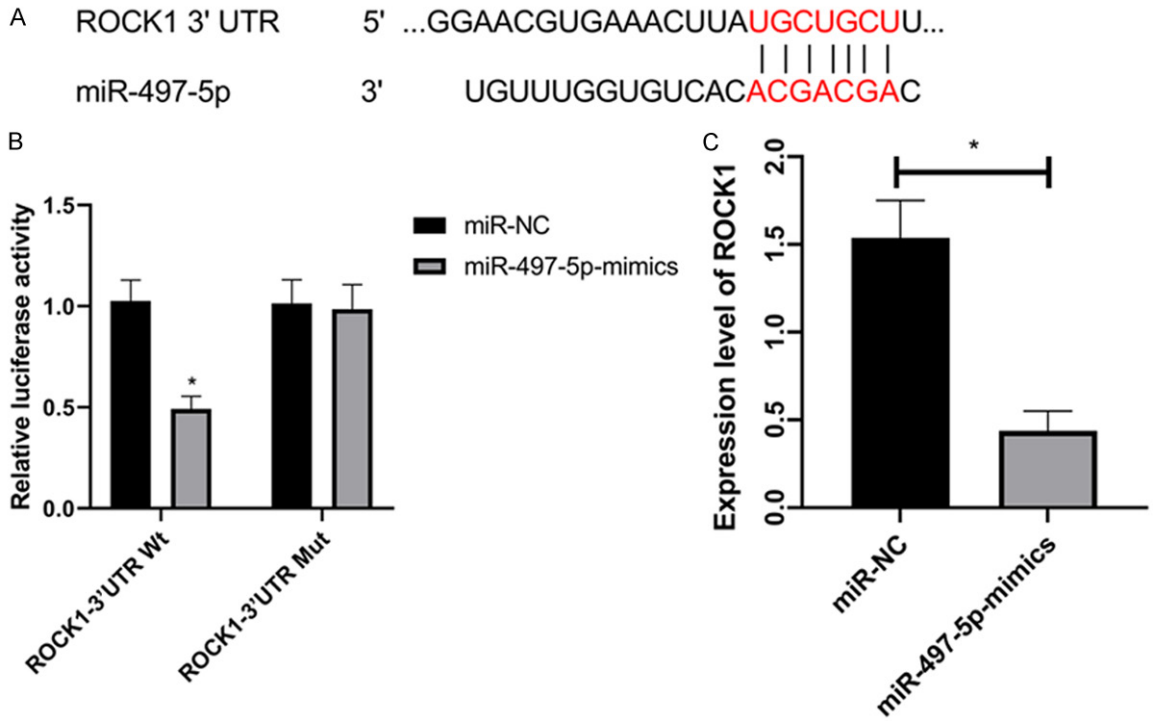

Figure 5. Relationship between miR-497-5p and ROCK1. A. There are target binding loci between miR-497-5p and ROCK1. B. Transfection of miR-497-5p-mimics inhibits luciferase activity of ROCK1-3'UT Wt in K562 cells, but no significant changes in luciferase activity of ROCK1-3'UTR Mut are achieved. C. Transfection of miR-497-5p-mimics significantly increases ROCK1 protein expression in K562 cells. *P < 0.05.

is unexplored. Secondly, the downstream target genes of ROCK1 also remain unclear. We hope to conduct more tests to address these limitations in the future.

Disclosure of conflict of interest

None.

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