MiR-509-3p Induces Apoptosis and Affects the Chemosensitivity of Cervical Cancer Cells by Targeting the RAC1/PAK1/LIMK1/Cofilin Pathway

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Chemoresistance is one of the main factors of treatment failure of cervical cancer (CC). Here, we intended to discover the role and mechanism of miR-509-3p in the paclitaxel chemoresistance of CC cells. RT-PCR was conducted to verify miR-509-3p expression. HCC94 and C-33A paclitaxel-resistant CC cell models were constructed. Additionally, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry were performed to verify the viability and apoptosis of HCC94 and C-33A cells after up-regulating miR-509-3p. Besides, the downstream target of miR-509-3p was analyzed by bioinformatics, and the targeted relationship between miR-509-3p and RAC1 was identified by the dual-luciferase reporter assay and RNA immunoprecipitation (RIP) assay. Further, the expression of apoptotic proteins (Bcl2, Bax, and Caspase3) and the RAC1/PAK1/LIMK1/Cofilin pathway was monitored by Western blot. The result showed that upregulating miR-509-3p markedly inhibited the viability and promoted the apoptosis of CC cells. On the other hand, miR-509-3p was distinctly downregulated in paclitaxel-resistant HCC94 and C-33A cells (vs. normal cells). The transfection of miR-509-3p mimics notably increased their sensitivity to paclitaxel. Meanwhile, RAC1 was found as the potential target of miR-509-3p in bioinformatics analysis. Moreover, the RAC1/p21 (RAC1) activated kinase 1 (PAK1)/LIM kinase 1 (LIMK1)/Cofilin pathway was significantly activated in paclitaxel-resistant HCC94 and C-33A cells, while miR-509-3p overexpression significantly inactivated this pathway. Additionally, downregulation of RAC1 also partly reversed the paclitaxel-resistance of CC cells and inhibited PAK1/LIMK1/Cofilin. All in all, miR-509-3p enhances the apoptosis and chemosensitivity of CC cells by regulating the RAC1/PAK1/LIMK1/Cofilin pathway.

Key words cervical cancer; chemoresistance; miR-509-3p; Rac family small guanosine 5’-triphosphatase (GTPase) 1; signaling pathway

Introduction

Cervical cancer (CC) is the fourth most prevalent malignancy occurred in women. In 2012, there were about 530000 new cases over the world, of which about 51% died, and the average age of CC diagnosis was 47 years old.1 CC is divided into four stages (I A1-B2, II A1-B, III A-B, and IV A-B), and surgery is the preferred treatment in the I A1-B2 stage. However, most patients are diagnosed with locally advanced CC, which is not suitable for surgical treatment.2 However, chemotherapy is not perfect, the challenge of which is that it causes cancer cells to produce chemoresistance through volution and apoptosis inhibition.3 Therefore, it is essential to eliminate chemoresistance to improve the curative effect of chemotherapy.

MicroRNAs (miRNAs) are a class of endogenous single-stranded non-coding RNAs involved in almost all processes of cancer biology, including cell proliferation, migration, invasion, and angiogenesis. The dysregulation of miRNAs leads to the transformation of normal cells into cancer cells, which becomes new therapeutic molecules in cancer.4 MiR-509-3p is one member of multiple miRNAs, which has been found to exert an anti-tumor effect in several tumors.5–7 In addition, miR-509-3p contributes to the chemosensitivity of cancers. For example, miR-509-3p enhances the drug sensitivity of ovarian cancer8 and osteosarcoma9 to cisplatin. Accordingly, we hypothesize that miR-509-3p also influences the chemosensitivity of CC and investigate the specific mechanism of its interference with drug resistance of CC.

Being located on chromosome 7p22.1, Rac family small guanosine 5’-triphosphatase (GTPase) 1 (RAC1) is confirmed to be upregulated and activated in multiple tumors, which strengthens tumor metastasis. For example, long non-coding RNA MEG3 dampens the proliferation and migration of CC by targeting Rac1.9 The phosphatidylinositol 3-kinase (PI3K)/AKT serine/threonine kinase (AKT) pathway is a critical intracellular signal transduction pathway, which is closely associated with tumor development and metastasis, chemoresistance, and radiation resistance.10 Researches reveal that miR-373 activates the PI3K/Akt-Rac1-mitogen-activated protein kinase 8 (JNK) pathway and thus facilitates the growth and invasion of osteosarcoma cells.11 Moreover, LIM kinase 1 (LIMK1), located in the cytoplasm of eukaryotes, contributes to tumor cell proliferation and metastasis by regulating the actin cytoskeleton, thus affecting cell migration and differentiation. It was found that LIMK1 is upregulated in esophageal cancer,12 breast cancer,13 and prostate cancer,14 being carcinogenic. Meanwhile, LIMK1 is also the downstream target of various signaling pathways, and the inhibition of LIMK1 expression dampens the malignant biological behaviors of tumor cells.15,16 For example, miR-23a abates cell metastasis of prostate cancer by targeting the p21 (RAC1) activated kinase 1 (PAK6)/LIMK1 signaling pathway.17 Nevertheless, the mechanism of miR-509-3p/RAC1/LIMK1 axis in CC was rarely studied.
In this study, we found that miR-509-3p reduced the viability and promoted the apoptosis of CC cells. Meanwhile, the upregulation of miR-509-3p attenuated the RAC1/PAK1/LIMK1/Cofilin pathway expression. In addition, miR-509-3p was downregulated in paclitaxel-resistant CC cells. Therefore, we supposed that miR-509-3p exerts a novel and vital role in the chemoresistance of CC, which provides a new reference and thinking for the development of CC treatment.

Results

**MiR-509-3p Promoted the Apoptosis of CC Cells** We constructed a miR-509-3p-overexpressed cell model to confirm the effect of miR-509-3p on CC cell apoptosis.

- **A.** A miR-509-3p-overexpressed cell model was constructed.
- **B–C.** MTT assay was used to detect cell proliferation.
- **D.** Flow cytometry was used to test cell apoptosis, n = 3.
- **E.** Western blot was carried out to determine the apoptotic markers (Bcl2, Bax and Caspase 3), n = 3.
- **F–H.** In vivo experiments to test the role of miR-509-3p in CC cells growth (n = 5). The tumor volume (F), images (G) and weight (H) were shown. **p < 0.01, ***p < 0.001.

Fig. 1. MiR-509-3p Promoted the Apoptosis of CC Cells

A. A miR-509-3p-overexpressed cell model was constructed. B–C. MTT assay was used to detect cell proliferation. D. Flow cytometry was used to test cell apoptosis, n = 3. E. Western blot was carried out to determine the apoptotic markers (Bcl2, Bax and Caspase 3), n = 3. F–H. In vivo experiments to test the role of miR-509-3p in CC cells growth (n = 5). The tumor volume (F), images (G) and weight (H) were shown. **p < 0.01, ***p < 0.001.
the role of miR-509-3p in CC cell growth (Fig. 1A). Then we performed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry to test cell viability and apoptosis, respectively. The results showed that compared with the miR-NC group, the viability in the miR-509-3p group decreased, while the apoptosis increased (Figs. 1B–D). Next, Western blot was carried out to determine the apoptotic markers (Bcl2, Bax, and Caspase3). Consistent with the previous results, overexpressing miR-509-3p attenuated the Bcl2 level while promoted Bax and Caspase3 expression (Fig. 1E). Furthermore, we conducted in vivo experiments to test the role of miR-509-3p in CC cell growth. The data revealed that upregulation of miR-509-3p markedly repressed the tumor volume and weight (compared with the miR-NC group) (Figs. 1F–H). Collectively, the above data confirmed that miR-509-3p has an anti-tumor role in CC.

MiR-509-3p Elevated the Chemosensitivity of CC Cells to Paclitaxel

The paclitaxel-resistant model was constructed in HCC94 and C-33A cells, and the miR-509-3p level was tested by RT-PCR. As a result, miR-509-3p was reduced in drug-resistant cell lines (Fig. 2A). Next, we transfected paclitaxel-resistant CC cells with miR-509-3p mimics to further explore their biofunctions (Fig. 2B). Additionally, CC cell proliferation was examined by MTT assay. It turned out that cell proliferation was dampened after transfection of miR-509-3p mimics compared with that of the Pac-R+miR-NC group (p<0.05, Figs. 2C, D). Moreover, flow cytometry and Western blot manifested that the cells’ apoptotic level significantly increased in comparison with the Pac-R+miR-NC group (p<0.05, Figs. 2E, F). All of the above findings indicated that miR-509-3p overexpression enhanced CC cell sensitivity to paclitaxel.

MiR-509-3p Targeted RAC1 and Inhibited the RAC1/PAK1/LIMK1/Cofilin Pathway

To further probe the underlying mechanism of miR-509-3p in CC cells, we predicted the targets of miR-509-3p through the Starbase database (http://Starbase.sysu.edu.cn/). It was found that seven genes were shared in the five databases (including PITA, Targetscan, miRmap, PicTar, and microT) (Fig. 3A). It was verified that miR-509-3p had no obvious effect on Rac1-mut transfected cells while dramatically inhibited the luciferase activity of Rac1-wt (p<0.05, Fig. 3C). Meanwhile, the RNA immunoprecipitation (RIP) assay illustrated that the precipitation amount of Rac1 in the Ago2 antibody group was higher than that of the immunoglobulin G (IgG) group after the transfection of miR-509-3p mimics (p<0.05, Figs. 3D, E). Furthermore, we used RT-PCR to test Rac1 and Western blot to test RAC1/PAK1/LIMK1/Cofilin. The results showed that overexpression of miR-509-3p reduced Rac1 mRNA and protein expression (Figs. 3F–H). Meanwhile, miR-509-3p also downregulated the phosphorylated level of PAK1/LIMK1/Cofilin. Taken together, those data indicated that miR-509-3p exert anti-tumor effects in CC via inactivating the RAC1/PAK1/LIMK1/Cofilin pathway.

Knockdown of RAC1 Enhanced the Chemosensitivity of CC Cells to Paclitaxel

We performed RT-PCR to detect the role of miR-509-3p in CC cell growth (Fig. 1A). Then we performed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry to test cell viability and apoptosis, respectively. The results showed that compared with the miR-NC group, the viability in the miR-509-3p group decreased, while the apoptosis increased (Figs. 1B–D). Next, Western blot was carried out to determine the apoptotic markers (Bcl2, Bax, and Caspase3). Consistent with the previous results, overexpressing miR-509-3p attenuated the Bcl2 level while promoted Bax and Caspase3 expression (Fig. 1E). Furthermore, we conducted in vivo experiments to test the role of miR-509-3p in CC cell growth. The data revealed that upregulation of miR-509-3p markedly repressed the tumor volume and weight (compared with the miR-NC group) (Figs. 1F–H). Collectively, the above data confirmed that miR-509-3p has an anti-tumor role in CC.

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Overexpressing miR-509-3p Attenuated Paclitaxel-Resistance-Induced RAC1/PAK1/LIMK1/Cofilin Pathway Activation

We conducted a Western blot to confirm whether the RAC1/PAK1/LIMK1/Cofilin pathway could mediate paclitaxel-resistance in CC cells. Our data showed that compared with normal cells, the RAC1/PAK1/LIMK1/Cofilin pathway was significantly activated in paclitaxel-resistant CC cells (Figs. 4A, B). Interestingly, transfection of miR-509-3p mimics attenuated the activation of the RAC1/PAK1/LIMK1/Cofilin pathway (Figs. 4A, B). Hence, miR-509-3p was verified to affect paclitaxel-resistance in CC cells via directly repressing the RAC1/PAK1/LIMK1/Cofilin pathway.
RAC1 mRNA level in CC cells. It was found that paclitaxel-resistant CC cells (both of HCC94 and C-33A) had a higher level of RAC1 mRNA (Figs. 5A, B). Furthermore, a RAC1-knockdown cell model was constructed in HCC94 and C-33A cells resistant to paclitaxel. We then found that repressing RAC1 mitigated the proliferation and promoted the apoptosis of paclitaxel-resistant CC cells (Figs. 5C–F). Moreover, downregulation of RAC1 led to a repressed level of PAK1, LIMK1 and cofilin, all of which were downstream proteins of RAC1 (Fig. 5G). Collectively, those data further indicated that

Fig. 4. Overexpressing miR-509-3p Attenuated Paclitaxel-Resistance-Induced RAC1/PAK1/LIMK1/Cofilin Pathway Activation

HCC94 and C-33A cell lines resistant to paclitaxel were transfected with miR-509-3p mimics. The RAC1/PAK1/LIMK1/Cofilin pathway was examined by Western blot. ***p < 0.001 vs. Normal group, ##p < 0.01, ###p < 0.001 vs. Pac-R + miR-NC group.

Fig. 3. MiR-509-3p Targeted RAC1 and Inhibited the RAC1/PAK1/LIMK1/Cofilin Pathway

A. Starbase database (http://Starbase.sysu.edu.cn/) was used to predict the targets of miR-509-3p. Venn Diagram was used to analyze the shared genes in the five databases (PITA, Targetscan, miRmap, PicTar, and microT). B. The binding sites between miR-509-3p and RAC1 was shown. C. Dual-luciferase reporter assay was applied to define the targeting relationship between miR-509-3p and RAC1. D, E. RIP assay illustrated that the precipitation amount of Rac1 in the Ago2 antibody group was higher than that of the IgG group after the transfection of miR-509-3p mimics. F. RT-PCR was implemented to test Rac1 mRNA. G, H. Western blot was used to test RAC1/PAK1/LIMK1/Cofilin. N = 3. *p < 0.05, **p < 0.01, ***p < 0.001.
the RAC1/PAK1/LIMK1/Cofilin axis contributed to regulating paclitaxel-resistance in CC cells.

Discussion
Chemotherapy is the standard treatment for advanced or recurrent CC. Despite the great achievements in CC treatment, chemoresistance remains one of the primary causes of treatment failure. Hence, it is necessary to find methods to eliminate CC chemoresistance. Tumor chemoresistance is implicated in various molecular mechanisms, such as the...
upregulation of transporter pumps, the dysregulation of anti-apoptotic proteins, and the dysregulation of miRNAs, etc.\textsuperscript{29} Here, we found that miR-509-3p repressed CC cell growth and abolished chemoresistance of CC cells to paclitaxel, which provided a new idea for CC treatment.

With the development of screening and prophylaxis, the prevalence and mortality of CC have been effectively controlled in some developed countries.\textsuperscript{20} However, CC remains a major challenge to public health worldwide, especially in economically undeveloped countries with backward primary prevention policies. Paclitaxel is one of the most applied first-line chemotherapy options for CC, while the chemoresistance of paclitaxel in CC gradually emerges with time, which becomes a major adverse factor for CC patients’ prognosis.\textsuperscript{22} Therefore, we still need to develop and optimize the CC treatment.

MiRNAs bind and pair with the sequences of 3’ untranslated region (UTR) of specific target mRNAs to inhibit their translation or degrade them. The dysregulation of miRNAs leads to the transformation of normal cells into cancer cells.\textsuperscript{23} Interestingly, several miRNAs have been found to overcome the chemoresistance of CC cells. For example, miRNA-218 is downregulated in cisplatin-resistant CC cells, and its upregulation enhances CC cell sensitivity to cisplatin by targeting survivin.\textsuperscript{24} MiR-509-3p, a miRNA, induces suppressive effects in tumors and modulates chemoresistance of some cancers.\textsuperscript{5, 25} Here, we discovered that miR-509-3p restrained the growth and enhanced the apoptotic level of CC cells. Moreover, drug sensitivity experiments illustrated that miR-509-3p dramatically improved CC cell sensitivity to paclitaxel, indicating that it is a sensitizer for chemotherapy of CC.

RAC1 is a Rho GTPase widely expressed in various tissues of the human body, which enhances the survival and metastasis of tumor cells through various signaling pathways. For example, BRIP1 promotes cisplatin-mediated cell apoptosis and attenuates tumor angiogenesis via inactivating Rac1 GTPase in CC cells. Meanwhile, the activation of RAC1 GTPase reverses the BRIP1-induced chemosensitivity.\textsuperscript{25} The PI3K/Akt pathway contributes to the occurrence, development and transformation of tumors and is a new target for tumor treatment. Some studies showed that Neol upregulates ZEB1 by sparking the RAC1/PI3K/Akt pathway in gastric cancer cells, thus facilitating cell proliferation, migration, and adhesion.\textsuperscript{26} Moreover, p21-activated kinase 1 (PAK1), another vital signaling pathway and upregulates Rac1 in non-small cell lung cancer (NSCLC), and leads to the gefitinib resistance of NSCLC patients.\textsuperscript{29} Here, we ascertained a binding relationship between RAC1 and miR-509-3p through biographical analysis. Additionally, miR-509-3p mimics abated the RAC1 level, which was promoted by paclitaxel. Moreover, the downregulation of RAC1 significantly reversed the paclitaxel-resistance of CC cells. Thus, miR-509-3p inhibited CC paclitaxel resistance via suppressing Rac1.

LIMK1 is a serine/threonine-protein kinase, and its over-expression in the cytoplasm and nucleus is closely connected with tumor metastasis and poor prognosis of patients. Some studies revealed that LIMK1 is upregulated in NSCLC\textsuperscript{28} and ovarian cancer,\textsuperscript{31} the inhibition of which reduces cancer cell proliferation and metastasis. In contrast, other studies identified that LIMK1 is overexpressed in lung cancer tissues, and silencing LIMK1 distinctly abates lung cancer cell migration and invasion and enhances the sensitivity to cisplatin and gemcitabine.\textsuperscript{32} What’s more, the Rac1/PAK1/LIMK1/Cofilin pathway has been found to modulate cancer development and acts as a therapeutic target. For instance, sea cucumber (Cu- cumaria frondosa) fucoidan (CF-Fuc) impairs the migration capacity and the dynamic remodeling of the cytoskeleton in osteosarcoma cells.\textsuperscript{33} Our study also confirmed that LIMK1 was upregulated in paclitaxel-resistant CC cell lines, and over-expressing miR-509-3p or downregulating of RAC1 decreased the PK/1/LIMK1/Cofilin pathway expression.

In conclusion, this study suggested that miR-509-3p is a tumor suppressor gene in CC, and its overexpression improves the sensitivity of CC to paclitaxel. Meanwhile, as an oncogenic pathway, Rac1/PK1/LIMK1/Cofilin was activated following the chemoresistance of CC. In addition, miR-509-3p is the target gene of Rac1, and it attenuates the Rac1/PK1/ LIMK1/Cofilin signaling pathway, thus reversing the paclitaxel chemoresistance.

**Experimental**

**Cell Culture** Human CC cell lines HCC94 and C-33A were acquired from American Type Culture Collection (ATCC, Rockville, MD, U.S.A.). They were cultured in a high-glucose DMEM medium containing 10% fetal bovine serum (FBS) (ThermoFisherScientific, MA, U.S.A.) and 1% penicillin/streptomycin (Invitrogen, CA, U.S.A.) in an incubator at 37 °C with 5% CO\textsubscript{2}. The cells were treated with 0.25% trypsin (ThermoFisher HyClone, UT, U.S.A.) and sub-cultured during the logarithmic growth period. They were then centrifuged at 1000 rpm for 3 min and resuspended in a moderate amount of complete culture medium.

**Paclitaxel-Resistant CC Cell Model** HCC94 and C-33A cells were inoculated into 24-well plates with 1 × 10\textsuperscript{5}/well. When the cells grew stable, they were exposed to paclitaxel (Abmole Bioscience Inc., Houston, TX, U.S.A.) with elevated concentrations (1–5 nM) for six weeks. Then, paclitaxel-resistant colonies were selected and exposed to paclitaxel with higher concentrations (2 nM). CC cells remained resistant to paclitaxel after the drug withdrawal.

**Cell Transfection** Firstly, HCC94 and C-33A cells were seeded into 6-well plates at 5 × 10\textsuperscript{4}/well and transsected after the cell growth was stable. Then, miR-509-3p mimics and the matched negative control (miR-NC), small interference RNA (si)-NC and si-RAC1 (GenePharma, Shanghai, China) were transfected into the cells according to the instructions of the FuGENE® HD Transfection Reagent (Roche, Shanghai, China). Subsequently, the cells were incubated at 37 °C with 5% CO\textsubscript{2}, and the culture medium was changed six hours after transfection. The cells were further cultured for 48 h. Finally, the total cellular RNA was extracted for RT-PCR to measure the transfection efficiency. The transfected cells were used in
subsequent experiments.

**RT-PCR** After a moderate amount of tissue or cells were fully ground, the TRIzol agent (Invitrogen, Carlsbad, CA, U.S.A.) was added to extract total RNA, and the genomic DNA was removed with deoxyribonuclease I. Afterward, the reverse transcription reaction was carried out with the Reverse Transcription Kit, and the reaction conditions were: 70 °C for 10 min, 5 min on ice, 42 °C for 60 min, 95 °C for 5 min, and 0 °C for 5 min. The RT-PCR system was 25 µL, which contained 500 ng cDNA template, 250 nmol/L forward and reverse primers, and 12.5 µL 2 × SYBR Green PCR Master Mix (MedChemExpress, NJ, U.S.A.), and was amplified on an RT-PCR instrument. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was the internal contrast of RAC1 while U6 was that of miR-509-3p. The 2-ΔΔCt was adopted to calculate the relative expression of mRNAs. miR-509-3p forward primer: 5'-GATTCGTTGATGGTACGTCTGT-3'; miR-509-3p reverse primer: 5'-TTATGCTTGCTACGACACCTTC-3'; RAC1 forward primer: 5'-AACCAATTGCAATTTCCTGGAG-3'; RAC1 reverse primer: 5'-TTGGTGGCAGTATAGGAGGG-3'; GAPDH forward primer: 5'-AGCACAGGGTTACT-3'; GAPDH reverse primer: 5'-CCAAGGATGAAAGACCTGCT-3'; U6 forward primer: 5'-CTCCTGTCGCAAGCA-3'; U6 reverse primer: 5'-ACGCTTACGGAATTTCGGT-3'. The reaction tube was placed into the MX3000P RT-PCR reaction apparatus, and the reaction conditions were: 94 °C for 40 s, 55 °C for 40 s, 72 °C for 40 s, and the fluorescence signal was monitored. The 22−ΔΔCt indicated the relative gene expression, ct represented the number of amplification when the fluorescence signal reached the set threshold, and ΔΔCt = samples to be tested [(Ct(target gene) − Ct(GAPDH)) − control][Ct(target gene) − Ct(GAPDH)].

**MTT Assay** Firstly, 100 µL stably transfected HCC94 and C-33A cells were seeded into 96-well plates (about 4×10^3/well) and incubated with 100% humidity at 37 °C with 5% CO2 after the cells were adherent to the wall. After 24h, different factors were added to treat the cells, and an equal volume of phosphate buffered saline (PBS) was added to the blank control group. Five repetitive wells were set in each group, and cell viability was tested at different time points (0, 12, 24, 48, 72, and 96h). After culturing for another 24h, 50 µL MTT (Beyotime Biotechnology, Shanghai, China) (5 g/L) was added to the cells and incubated for 4h at 37 °C. Subsequently, the supernatant was sucked out, and 150 µL dimethyl sulfoxide (DMSO) (Beyotime Biotechnology) was added to each well. The suspensions were shaken well with a plate shaking table. After the crystalization was dissolved, the absorbance (A) value was monitored at 570 nm wavelength with a microplate reader, and the inhibition rate of cell proliferation was tested. Cell survival rate (%) = (A value of control group − A value of experimental group)/A value of experimental group × 100%. The drug’s inhibition rate on cells (%) = (1 − A value of the experimental group/A value of the control group) × 100%.

**Flow Cytometry** After transfection for 48h, the cells were treated with ethylenediaminetetraacetic acid (EDTA)-free Trypsin, centrifuged and collected. Then, the cells were washed with PBS twice, and 20nM paclitaxel was added to collect the cells for cryopreservation. Afterward, the AnnexinV-PE/7-AAD Apoptosis Assay Kit (Southern Biotechnology, Birmingham, AL, U.S.A.) was utilized to treat the cells. Meanwhile, the quantitative analysis of cell apoptosis was conducted by FACS Calibur flow cytometry (Becton-Dickinson, San Jose, CA, U.S.A.). The experiment was made in triplicate.

**Dual-Luciferase Reporter Assay** RAC1 wild-type (RAC1-wt) or mutant (RAC1-mt) luciferase reporter plasmid vectors were transfected separately or with miR-509-3p mimics into cells following the instructions of dual-luciferase reporter assay system (Promega, Madison, WI, U.S.A.). The culture medium was refreshed after 48h culture. Then, the cells were washed with PBS, and the solution was discarded, and the cell lysis buffer was added to lyse the cells. Subsequently, the cells were oscillated on the oscillator at room temperature for 5–10 min and transferred into a centrifuge tube and centrifuged at 3000 rpm for five min. Finally, the supernatant was applied for luminescence measurements. The luminescence value of the sample was measured according to the kit instruction and instrument operation guidelines. The experiment was performed three times.

**RIP Assay** RIP analysis was implemented using the Magna RIP RNA-binding Protein Immunoprecipitation Kit (Millipore, Bedford, MA, U.S.A.) following the manufacturer’s instructions. HCC94 and C-33A cells at an 80% fusion rate were collected and lysed with RIP lysis buffer. Then, the anti-Ago2 antibody (Abcam, Shanghai, China) was used for Ago2 immunoprecipitation, and the IgG antibody served as a negative control. Finally, the immunoprecipitated RNA was isolated, and the abundance of miR-509-3p and RAC1 in the bound part was determined by RT-PCR.

**Chemosensitivity Test** The cytotoxicity of drugs in vitro was analyzed by MTT. Firstly, 5×10^4 transfected HCC94 and C-33A cells were taken to prepare cell suspension, and 10µL of the suspension was inoculated into the culture bottle. After 24h of culture, paclitaxel was added (with the final concentration of 5nM/µL). After incubating for 48h, the medium was discarded, and a fresh medium was used for another 48h of culture. After repeated-culturing, the concentration of paclitaxel was gradually increased. Finally, cell lines resistant to paclitaxel was obtained, and the culture was maintained in a complete medium containing 10nM/µL paclitaxel and incubated at 37 °C for two hours. Cell viability was checked at different paclitaxel concentrations (0, 5, 10, 20, 40, and 60nM). The microplate reader (Thermo, Schwerte, Germany) was used to observe the optical density (OD) of each well at 450 nm. The cell viability (percentage to the control group) = (ODsample − ODblank)/(ODcontrol − ODblank)×100%. The experiment was repeated three times.

**Western Blot** After cell treatment, the culture medium was discarded, and protein lysisate (RIPA) (Beyotime Biotechnology) was added to isolate the total protein. Then, 50 g total protein was added to 12% polyacrylamide gel at 100V electrophoresis for two hours and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). After being blocked at room temperature for one hour, the membranes were rinsed three times with Tris HCl buffer solution-Tween (TBST) (10 min each) and incubated overnight at 4 °C with the following primary antibodies (Abcam, Cambridge, U.K.): anti-Rac1 antibody (ab155938), anti-PAK1 antibody (ab131522), anti-PAK1 (phospho T212) antibody (ab75599), anti-LIMK1 antibody (ab81046), anti-LIMK1 (phospho T508) antibody (ab194798), anti-Cofilin antibody (ab54532), and anti-Cofilin
(phospho S3) antibody (ab12866). After being rinsed with TBST, the membranes were incubated for one hour with horseradish peroxidase (HRP)-labeled Goat Anti-Rabbit IgG (ab6721, 1:300, Abcam) at room temperature. The membranes were rinsed with TBST three times (10 min each). Finally, the bands were visualized by the enhanced chemiluminescence (ECL) system (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.), and Image J was used to evaluate the gray values of each protein.

**In Vivo Experiment** Twenty female BALB/c-nude mice weighing 20–22 g were purchased from the Animal Centre of Fourth Military Medical University (Xi’an, China). The nude mice were divided into two groups (the miR-NC and miR-509-3p group) in a random method. The single-cell suspension of HCC94 cells (10^6 cells) transfected with miR-NC or miR-509-3p mimics was mixed with an equal volume of Matrigel (BD Biosciences, CA, U.S.A.) on ice and inoculated into the lower flank of immunodeficient mice. During the next five weeks, the tumor growth was measured according to the formula: tumor volume (mm^3) = length × width^2. At the 5th week of inoculation, the nude mice were sacrificed, and the formed tumor tissues were stripped and weighted. The animal study was conducted in a specific pathogen-free environment, and the protocol was approved by the Animal Ethics Committee of Fourth Military Medical University.

**Ethics Statement** Our study was approved by the Ethics Review Board of Xijing Hospital, Fourth Military Medical University.

**Statistical Analysis** SPSS17.0 statistical software (SPSS Inc., Chicago, IL, U.S.A.) was adopted for statistical analysis. The measurement data were represented as mean ± standard deviation (x ± s), and the t-test was employed to compare means between the two groups. One-way ANOVA was utilized for the comparison of the means between groups. p < 0.05 was considered statistically significant.

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**Author Contributions** Conceived and designed the experiments: Nianling Yao, Jia Xu; Performed the experiments: Jia Xu, Xiangdong Ma, Hong Yang; Statistical analysis: Junru Zhang, Guoqing Cai; Wrote the paper: Jia Xu. All authors read and approved the final manuscript.

**Conflict of Interest** The authors declare no conflict of interest.

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