

# miR-214-5p increases the radiosensitivity of cervical cancer by targeting ROCK1 expression

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## Conflict of interest

None declared

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## Abstract

**Background.** The tolerance of cervical cancer to radiotherapy is a major factor affecting treatment outcomes. The miR-214-5p is involved in the regulation of biological processes such as tumor proliferation and metastasis.

**Objectives.** The aim of the study was to explore the role of miR-214-5p and Rho-associated coiled-coil containing protein kinase 1 (ROCK1) in cervical cancer and their response to radiotherapy in cervical cancer patients.

**Materials and methods.** Fifty-three cervical cancer tissue samples were collected to analyze the level of miR-214-5p in patients with different responses to radiotherapy. Cervical cancer cell lines with radiation resistance were selected to explore the role of miR-214-5p in radiosensitivity. The wound healing, transwell migration, clone formation assay, and in vivo analysis were utilized to evaluate the effect of miR-214-5p on the radiation sensitivity of cervical cancer cells.

**Results.** Patients with poor radiotherapy responses demonstrated low levels of miR-214-5p. The upregulation of miR-214-5p decreased migration and invasion ability of radiotherapy-resistant cells. The bioinformatic analysis showed that *ROCK1* is a candidate target gene of miR-214-5p, and this was confirmed with dual luciferase reporter assay showing that miR-214-5p directly interacts with the 3' untranslated region (3'UTR) of *ROCK1*. Decreased *ROCK1* improved the radiosensitivity of cervical cancer in vitro and in vivo, and the over-expression of *ROCK1* decreased the radiosensitivity effect of miR-214-5p in cervical cancer cells.

**Conclusions.** The miR-214-5p can regulate the radiation sensitivity of cervical cancer cells by targeting the mRNA of *ROCK1* and regulating its expression.

**Key words:** radiosensitivity, *ROCK1*, cervical cancer, microRNA-214-5p

## Cite as

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## Background

Cervical cancer is ranked as the 4<sup>th</sup> leading cancer in females worldwide, although early detection of the disease can significantly affect treatment efficacy and prolong the survival time of patients.<sup>1</sup> Currently, radiotherapy is the main treatment strategy for cervical cancer. However, the radiotherapy tolerance of cervical cancer affects the treatment outcomes. The failure of radiotherapy in cervical cancer is correlated with many factors, such as the different clinicopathological characteristics of patients, the hypoxic state of cancer cells and the intrinsic sensitivity of the tumor cells to radiotherapy.<sup>2</sup> MicroRNAs (miRNAs) participate in the progression of a wide array of tumors. They regulate the malignant behavior of tumor cells by reducing the stability and translation of targeted mRNA. Specifically, miR-214 participates in tumor progression, acting as a tumor suppressor gene or oncogene.<sup>3–7</sup> Moreover, miR-214 is involved in the radiosensitivity response of nasopharyngeal carcinoma and skin injury caused by radiation.<sup>8,9</sup> In clinical practice, the enhanced effect of radiotherapy by exogenous miRNA in solid tumors has entered the exploration stage.

The Rho family, also called Rho GTPases, is a class of GTP-binding proteins with GTP enzyme activity that are associated with the Ras superfamily. The Rho GTPases are important signal transduction molecules and participate in the regulation of cytoskeletal reorganization. Working as “molecular switches” in the process of cell signal transduction, Rho GTPases control many signal transduction pathways. The Rho-associated coiled-coil containing protein kinase 1/2 (ROCK1/2) belongs to the Rho family and is expressed in multiple tissues, including the lung, liver, spleen, kidneys, testes, brain, and heart, and acts in several subcellular locations.<sup>10</sup> The ROCK1 promotes tumor cell proliferation, invasion and metastasis by activating the Rho/ROCK signaling pathway. After interacting with activated Rho protein, the active center of ROCK1 is catalyzed and induces the reorganization of the actin cytoskeleton of cancer cells, thereby enhancing the movement ability of cancer cells.<sup>11</sup> The ROCK inhibitor, fasudil, induces terminal adipocyte differentiation of chemoresistant osteosarcoma cells.<sup>12</sup> Epithelial-to-mesenchymal transition (EMT) contributes to therapy resistance in cancers,<sup>13</sup> a process the ROCK is frequently associated with in human ovarian cancer cells.<sup>14</sup> This inspired us to further explore the role of ROCK1 and its attenuating miRNA in radiotherapy resistance in cervical cancer cells.

## Objectives

This study aims to evaluate the miR-214-5p expression and the response to radiotherapy in patients with cervical cancer. In addition, the role of miR-214-5p in radiation sensitivity was investigated in vivo and in vitro. Finally,

we explored the mechanism of action of miR-214-5p and its relationship with ROCK1 in cervical cancer following radiotherapy.

## Materials and methods

### Samples

The medical data of patients with cervical cancer were screened to estimate the gene expression of *miR-214-5p* and *ROCK1*. The samples included in the current analysis were collected in Hebei General Hospital (Shijiazhuang, China) from patients who did not receive radiotherapy or chemotherapy before surgery in the years 2017–2020. All patients signed informed consent for sample and data analyses. Fifty-three tissue samples with postoperative pathological reports and complete medical records were included. The International Federation of Gynecology and Obstetrics (FIGO) stage of collected samples ranged from IB to IIIA, based on the 2009 FIGO staging system,<sup>15</sup> and the severities ranged from a relatively early stage to an advanced stage (para-aortic lymph node involvement). Patients' demographic data, including age, menopause state, tumor diameter, lymph node metastasis, and pathological type, were also recorded. Exclusion criteria were patients who received preoperative radiotherapy and chemotherapy, cervical surgery, and hysterectomy, patients with an incomplete cervix, those suffering from other malignant tumors, pregnant patients, and patients with reproductive tract inflammatory diseases. Another 30 samples from normal adjacent cervical tissue and 30 samples of cervical intraepithelial neoplasia (all confirmed by pathological examination) were selected for the analysis. All specimens were collected and stored at  $-80^{\circ}\text{C}$ .

### Evaluation of radiotherapy response in patients

The radiotherapy sensitivity in patients was determined according to the response evaluation criteria in solid tumors (RECIST) developed by the World Health Organization (WHO),<sup>16</sup> based on their medical records: 1) the short-term efficacy includes an uncontrolled tumor and disappearance of the tumor; an uncontrolled tumor refers to the persistence or emergence of new lesions within 3 months after radiotherapy; tumor disappearance indicates that the tumor disappears within 3 months following radiotherapy; 2) the long-term efficacy includes tumor recurrence and the tumor being cured; tumor recurrence refers to the disappearance of the tumor after radiotherapy, while a pelvic or distant tumor is found upon re-examination 6 months after radiotherapy; tumor cured refers to the disappearance of the tumor without recurrence; tumor uncontrolled and tumor recurrence were defined as radiotherapy resistance; tumor cured, being neither tumor uncontrolled nor tumor recurrence, was defined as radiotherapy sensitivity.

## In vivo tumor model and inhibition evaluation

The animal study was approved by the ethics committee of Hebei General Hospital (approval No. 2020-256), and all protocols followed the guidelines of Institutional Animal Care and Use Committee (IACUC) issued by the Chinese Academy of Medical Sciences. A total of 24 female BALB/c nude mice (age: 5 weeks; weight: 19–22 g; provided by the Animal Experiment Center of Hebei Medical University, Shijiazhuang, China) housed in an specific pathogen-free (SPF) laboratory animal room, were used to construct a cervical tumor-bearing model. A liposomal delivery system (MaxSuppressor™ In Vivo RNA-LANCER II; Bioo Scientific, Austin, USA) was used to deliver miR-214-5p mimic and the negative control sequence (miR-214-5p NC), according to the manufacturer's instructions (RiboBio Co., Ltd., Guangzhou, China). Briefly, RNA oligos were diluted to 10 mg/mL, and a total of 500 µL of RNA solution mixture (RNA solution: 11 µL, phosphate-buffered saline (PBS) (10×): 55 µL, and RNase-free water: 434 µL) was mixed with 50 µL of neutral lipid emulsion. The solution was mixed in an ultrasonic water bath for 5 min prior to intravenous administration. Hela cells (200 µL at a concentration of  $5 \times 10^6$  cells/mL) were inoculated into the flank of mice. Radiotherapy (total dose of 20 Gy) was performed 1 week after inoculation. The mice were anesthetized by 5% chloral hydrate (300 mg/kg, intraperitoneal injection) during radiation. Then, the animals were fixed and irradiated by 6MV X-ray (X-RAD 225XL irradiator; Precision X-Ray, Inc., Madison, USA). The irradiated dose was 2 Gy daily, performed 10 times over 2 weeks. Accordingly, the mice were allocated to the model group, the radiation group, the miR-214-5p mimic group, and the miR-214-5p mimic + radiation group (n = 6 mice per group). The miRNA mimics were administered in a 40 µg per mouse bolus, which started on day 7 post-inoculation, and continued every 3 days. The radiation was performed when the tumor volume in the model group reached approx. 200 mm<sup>3</sup>, and the mice were sacrificed by decapitation when the tumor was over 1000 mm<sup>3</sup> in the model group. The growth and the inhibited rate of tumor growth in each group were calculated. The diameter of the tumor by the longest (L) and the shortest diameter (W) were measured, and the transplanted tumor volume (V) was calculated as  $0.5 \times L \times W^2$ .

### RT-qPCR analysis

The total RNA was extracted from tissues and cultured cells using Trizol reagent (#R0016; Beyotime Biotechnology, Shanghai, China), following the manufacturer's instructions. A total of 1 mL of Trizol and 0.2 mL of chloroform were added to the tubes containing homogenized tissues or cell pellets. Five minutes after the lysis, the samples were centrifuged for 15 min at 4°C

**Table 1.** Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) primer sequence

Gene	Sequence
miR-214-5p	F: 5'-GCCGAGTGCCTGTCTACACT-3'
	R: 5'-GCAGGGTCCGAGGTAT-3'
U6	F: 5'-GAGGGCCTATTCCCATGATT-3'
	R: 5'-TAATTAGAATTAATTTGACT-3'
ROCK1	F: 5'-TGAAAGCCGCACTGATGGAT-3'
	R: 5'-GCCATGAGAAAACACATTGCAG-3'
$\beta$ -actin	F: 5'-CACCATTGGCAATGAGCGGTTC-3'
	R: 5'-AGGTCTTTGCGGATGTCCACGT-3'

and 12,000 g. The upper colorless solution containing the total RNA was loaded into a new centrifuge tube, and 0.5 mL of isopropanol was added for 10 min to precipitate the RNA. After the centrifugation for 10 min at 4°C and 12,000 g, the RNA was precipitated with 75% ethanol, centrifuged again and dissolved with diethyl pyrocarbonate (DEPC) water. The integrity of extracted RNAs was measured using a 1% agarose electrophoresis gel. A 30-ng RNA sample was reverse transcribed with PrimeScript RT reagent kit (#RR037B; Takara Bio Inc., Shiga, Japan), following the previously described method.<sup>17</sup> The reverse transcriptional reaction system (10-µL volume) contained 2 µL of PrimeScript buffer, 0.5 µL of RT enzyme mix, 0.5 µL of oligo dT primer, 0.5 µL of random hexamers, total RNA, and RNase free water. The two-step TB Green-based quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) was performed according to the manufacturer's instruction.<sup>18</sup> The expression of miR-214-5p was normalized and quantified using the small RNA U6.<sup>19–21</sup> The mRNA expression of ROCK1 was also measured and calculated as the relative expression to  $\beta$ -actin.<sup>22,23</sup> The ABI StepOnePlus™ Real-Time PCR system (Thermo Fisher Scientific, Waltham, USA) was used to perform RT-qPCR analysis,<sup>24,25</sup> and the applied thermal cycling parameters were recommended by the manufacturer. All used primers are listed in Table 1. The relative expression of target genes was evaluated by the  $2^{-\Delta\Delta C_t}$  method. All measurements were performed in triplicate.

### Cell lines and tissue samples

Six human cervical cancer cell lines were used, namely C4-1, C33A, MS751, Hela, Siha, and CaSki (Cell Bank of the Chinese Academy of Sciences, Shanghai, China). The C4-1 and C33A were cultured in RPMI-1640 medium (#R8758; Sigma-Aldrich, Burlington, USA) containing 10% fetal bovine serum (FBS), while MS751, Hela, Siha, and CaSki cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (#D5796; Sigma-Aldrich), supplemented with 10% FBS, 100 U/mL of penicillin and 100 µg/mL of streptomycin. All cells were cultured at 37°C in 5% CO<sub>2</sub>.

## RNA oligoribonucleotides, plasmid construction and transfection

To verify the function of miR-214-5p in vitro and in vivo, the following sequence or mutant sequence was prepared: miR-214-5p mimic (#miR10004564-1-5), miR-214-5p NC (#miR1N0000002-1-5), wild type ROCK1 (ROCK1-WT) 3'untranslated region (3'UTR), and mutant ROCK1 (ROCK1-mut) 3'-UTR (all purchased from RiboBio Co., Ltd.). To overexpress ROCK1 in vitro, the full-length coding sequence of ROCK1 was cloned and amplified. The enzymatic digestion sites KpnI and XbaI on the pcDNA3.1(+) vector (#VT1001; YouBio Tec. Inc., Changsha, China) were used for *ROCK1* gene insertion. After recovery and purification, the plasmid was connected by T4 DNA ligase (#T1410; Solarbio Life Science, Beijing, China) with PCR products to obtain the recombinant plasmid. The DH5 $\alpha$  was selected for transformation screening of competent cells, and a small amount of recombinant plasmid was extracted, identified and sequenced with double enzyme digestion. The empty plasmid was used as a control to compare the expression of ROCK1 (ROCK1 OE).

A total of  $2 \times 10^5$  cells were cultured in 12-well plates, and transfection was performed after the cells reached 70–90% confluence. The culture medium was replaced by a fresh non-antibiotic medium 2 h before the transfection. Lipofectamine 3000 reagent (#L3000015; Thermo Fisher Scientific) and DNA were diluted by Opti-MEM medium (#11058021; Gibco, Carlsbad, USA) according to the manufacturer's instruction, and then added to the diluted Lipofectamine 3000 in a 1:1 proportion. The mixture was incubated at room temperature for 20 min. The obtained DNA-liposome complex was loaded into target cells, mixed gently and cultured at 37°C for further experiments.

## Cell proliferation measurement

Cell proliferation was assessed using the Cell Counting Kit-8 (CCK-8) method. Fifty microliters of cells treated by miR-214-5p NC or miR-214-5p mimic (75% density,  $5 \times 10^5$  cells at exponential growth phase) were trypsinized and loaded onto a 96-well plate containing the same volume of culture medium. Cells were cultured for 24 h, 48 h and 72 h, and then 10  $\mu$ L of CCK-8 reagent (#C0038; Beyotime Biotechnology) was loaded into the wells. The cells were cultured for another 2 h, and the optical density (OD) of the target well was measured at 450 nm. The average value from 3 wells was calculated.

## Wound healing assay

Cells with a concentration of  $5 \times 10^5$  were evenly loaded onto a 6-well plate. The cells were washed twice with PBS after 12-h culture to remove any non-adherent cells. A straight scratch was generated using a sterile 20- $\mu$ L pipette tip across the monolayer of cells. The debris

on the plate was washed twice with PBS and replaced with a fresh serum-free medium. The migration ability of cells was observed under an optical microscope (Nikon Labophot 2 Microscope; Nikon Corp., Tokyo, Japan) at 24 h and 48 h. The image was captured, and the width of the scratch wound was evaluated using ImageJ software (National Institutes of Health (NIH), Bethesda, USA).

## Transwell migration

After Matrigel was diluted and spread across the bottom of the upper chamber of the Transwell, 100  $\mu$ L of cells ( $5 \times 10^5$ /mL) were loaded to the upper chamber of a 24-well transwell (8- $\mu$ m pore, #3422, Transwell®; Corning Life Science, Tewksbury, USA). Medium supplemented with 10% serum (600  $\mu$ L) was loaded into the lower chamber. The plate was cultured for 24 h, and unmigrated cells in the chamber were swabbed after discarding the medium. The remaining cells were fixed with 4% paraformaldehyde for 15 min and stained with crystal violet for 10 min. The filter membrane was photographed under a light microscope (Nikon Labophot 2 Microscope). Image-Pro Plus v. 6 software (Media Cybernetics, Rockville, USA) was used to count the migrated cells. Five fields of view from each well were randomly analyzed to evaluate the average number of migrated cells.

## Clone formation assay

Logarithmic growth staged cells were added to 60-mm culture dishes. According to the results of a pre-experiment, about 250 cells were loaded to each plate. The cells were dispersed evenly. A single dose of 4 Gy and 6 Gy (5 min per day with a dose rate of 300 cGy/min) was generated ( $^{60}\text{Co}$  medical irradiation device; China Nuclear Power Technology Research Institute, Beijing, China) and absorbed by the cells. The irradiation was performed for 10 consecutive days, and the cells were continued to be cultured for another 10 days. Any clones that were formed were fixed with paraformaldehyde and stained with 0.1% crystal violet solution for 20 min. The number of clones was counted in each group under a low power microscope (Nikon Labophot 2 Microscope). The average number of clones from 3 dishes was calculated for each dose.

## Luciferase reporter assay

To verify the binding effect of miR-214-5p to ROCK1, starBase v. 3.0 (<https://rnasysu.com/encori/>) and TargetScan ([https://www.targetscan.org/vert\\_80/](https://www.targetscan.org/vert_80/)) online databases were used to predict putative binding sites for miR-214-5p. Briefly, the wild type or mutant (MUT) fragments for ROCK1 3'UTR were amplified. The fragment was inserted into the SpeI and HindIII sites of the pMIR-REPORT luciferase reporter vector following the methods mentioned



above and previously.<sup>26</sup> The pMIR- $\beta$ -galactosidase reporter plasmid was used to normalize the transfection. The HEK-293T cells were then co-transfected with reporter vectors and duplexes of small interfering RNAs (miR-214-5p mimic or miR-214-5p NC) with Lipofectamine 3000 reagent. Twenty-four hours after the transfection, the cells were harvested, and luciferase assays and  $\beta$ -galactosidase enzyme assays were used to measure relative luminescence units in each well, following the manufacturer's protocol. The luciferase activity of firefly was normalized to  $\beta$ -galactosidase expression. Three parallel wells were measured for each condition.

## Western blot

Radioimmunoprecipitation assay (RIPA) lysis buffer (200  $\mu$ L) (#R0010; Solarbio Life Science) was used to lyse the cells. The suspension was centrifuged for 4 min at 4°C and 12,000 rpm, and the supernatant was retained to separate targeted proteins. The total protein amount was determined using the bicinchoninic acid (BCA) method. Fifty milligrams of protein were loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) for electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Burlington, USA). The membrane was blocked using 5% skimmed milk at 37°C for 1 h, and the primary antibodies (Invitrogen, Waltham, USA) for ROCK1 (#MA5-27779, 1:1000 diluted), Rho (#1B8-1C7, 1:1000 diluted), LIMK1 and Phospho-LIMK1 antibody (#MA5-37486, 1:1000 diluted; #PA5-104925, 1:200 diluted) were added overnight at 4°C. The membrane was washed thrice with 1 $\times$  Tris-Buffered Saline with Tween (TBST) and then incubated with a goat anti-rabbit IgG HRP (#31460, 1:5000 dilution; Thermo Fisher Scientific) for 2 h. The membrane was then washed with 1 $\times$  TBST, and the electrochemiluminescence (ECL) luminous working solution was added to obtain images of the proteins.<sup>27,28</sup>

## Statistical analyses

Quantitative data are presented as mean  $\pm$  standard deviation (M  $\pm$  SD) and were analyzed using GraphPad Prism v. 9.4 software (GraphPad Software, San Diego, USA) and IBM Statistical Package for Social Science (SPSS) v. 24.0 software (IBM Corp., Armonk, USA). The expression levels of miR-214-5p and ROCK1 mRNA in clinical samples are presented as the relative expression. The Shapiro–Wilk test was used to confirm the normal data distribution, and the Brown–Forsythe test was used to confirm the homogeneity of variance. Data that passed normality and homogeneity of variance tests were compared using bootstrap one-way analysis of variance (ANOVA) followed by Tukey's post hoc test to analyze the differences among multiple groups, or Student's t-test for differences between 2 groups. If the data were not normally distributed, Kruskal–Wallis

test with Dunn's post hoc test was applied for statistical differences among multiple groups, and the Mann–Whitney U test was used for the comparison between 2 groups. For an in vitro analysis, all experiments were performed in triplicate, and the repeated measurement data in the proliferation assay were completed at different time points and performed in triplicate. The in vitro data and repeated measurements were compared using unpaired Student's t-test or bootstrap one-way ANOVA, followed by a Tukey's post hoc test. The correlation between miR-214-5p and ROCK1 mRNA levels was analyzed with SPSS v. 24.0 statistical software using Spearman's correlation method. The test level of  $\alpha = 0.05$  and the value of  $p < 0.05$  were considered significant.

## Results

### Decreased miR-214-5p expression in patients with cervical cancer

In this study, 53 tissue samples (Table 2) obtained from patients with cervical cancer, 30 samples from normal cervical tissues and 30 samples from cervical intraepithelial neoplasia were collected for the analysis. Compared with normal tissue, the expression of miR-214-5p was decreased in cervical intraepithelial neoplasia and cervical cancer tissues. In addition, miR-214-5p expression was lower in cervical cancer compared to cervical intraepithelial neoplasia (Fig. 1A). The expression of miR-214-5p was also decreased in more advanced clinical stages (Fig. 1B and Table 2) and in patients with lymph node metastasis (Fig. 1C and Table 2). The analysis of patients who responded to radiotherapy revealed that miR-214-5p expression in radiotherapy-resistant patients was also significantly lower compared with radiotherapy-sensitive patients (Fig. 1D).

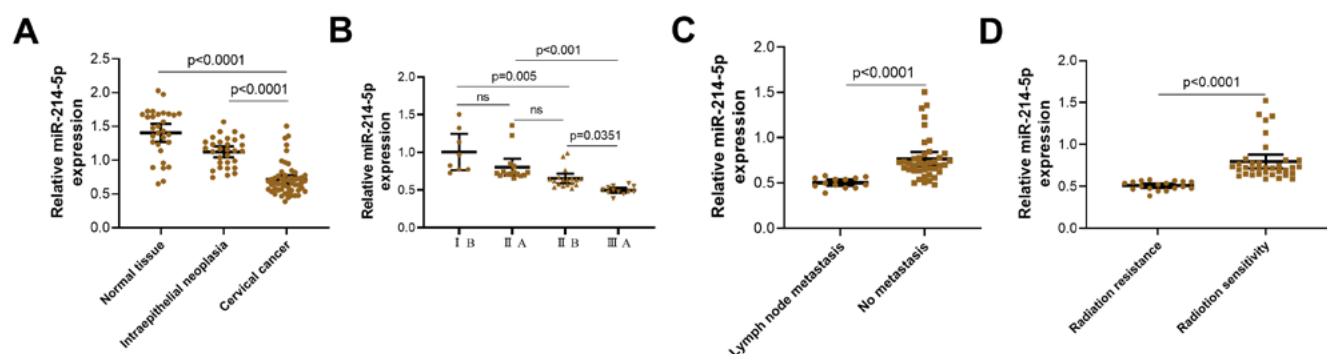
### Upregulation of miR-214-5p decreases the proliferation ability of cervical cancer cells

We further analyzed the expression of miR-214-5p in 6 cervical cancer cell lines. This analysis demonstrated that miR-214-5p expression in C4-1, Hela and Siha cells was relatively low (Fig. 2A). The radiosensitivity evaluation of a single dose of 2 Gy showed that Hela and Siha cell lines demonstrated radiation resistance (Fig. 2B), and therefore these 2 cell lines were used in a further analysis. To explore the role of miR-214-5p in the radiosensitivity of cervical cancer, we first upregulated its expression using its mimic, and RT-qPCR demonstrated that the mimic was successfully transfected (Fig. 2C). The CCK-8 assay was used to analyze cell proliferation, showing a significant decrease following increased expression of miR-214-5p (Fig. 2D).

**Table 2.** Demographic data and miR-214-5p expression of 53 samples obtained from cervical cancer patients

Parameter	Category	n (53)	Relative expression	Mann–Whitney U	p-value
Age [years]	≥45	35	0.722 ±0.248	255	0.267
	<45	18	0.657 ±0.219		
Menopause state	yes	32	0.733 ±0.267	273	0.256
	no	21	0.650 ±0.191		
Tumor size [cm]	≥4	15	0.620 ±0.181	192.5	0.0682
	<4	38	0.731 ±0.253		
FIGO stage	IB	8	1.005 ±0.289	NA	<0.0001*
	IIA	15	0.804 ±0.205		
	IIB	18	0.654 ±0.128		
	IIIA	12	0.498 ±0.055		
Lymph node metastasis	yes	13	0.502 ±0.058	34	<0.0001
	no	40	0.764 ±0.240		
Pathological type	squamous cell carcinoma	45	0.691 ±0.238	140.5	0.336
	adenocarcinoma	8	0.751 ±0.250		

Data were expressed as mean ± standard deviation (M ±SD) and analyzed using the Mann–Whitney U test; \* data were analyzed using the Kruskal–Wallis one-way analysis of variance (ANOVA). The results of multiple group comparisons are also demonstrated in Fig. 1B. NA – not applicable; FIGO – International Federation of Gynecology and Obstetrics.



**Fig. 1.** Expression of miR-214-5p is downregulated in patients with cervical cancer. A. The expression of miR-214-5p in cervical carcinoma tissue (n = 53), cervical intraepithelial neoplasia (n = 30) and normal control (n = 30); B. The expression of miR-214-5p is downregulated along with the increasing clinical stage; data were analyzed using the Kruskal–Wallis test for multiple comparisons (A,B); C. Patients with lymph node metastasis have a significantly lower miR-214-5p expression; D. The expression of miR-214-5p in radiotherapy-sensitive patients is higher than in radiotherapy-resistant patients; data were analyzed using the Mann–Whitney U test (C,D). Whiskers represent the 95% confidence interval (95% CI) values

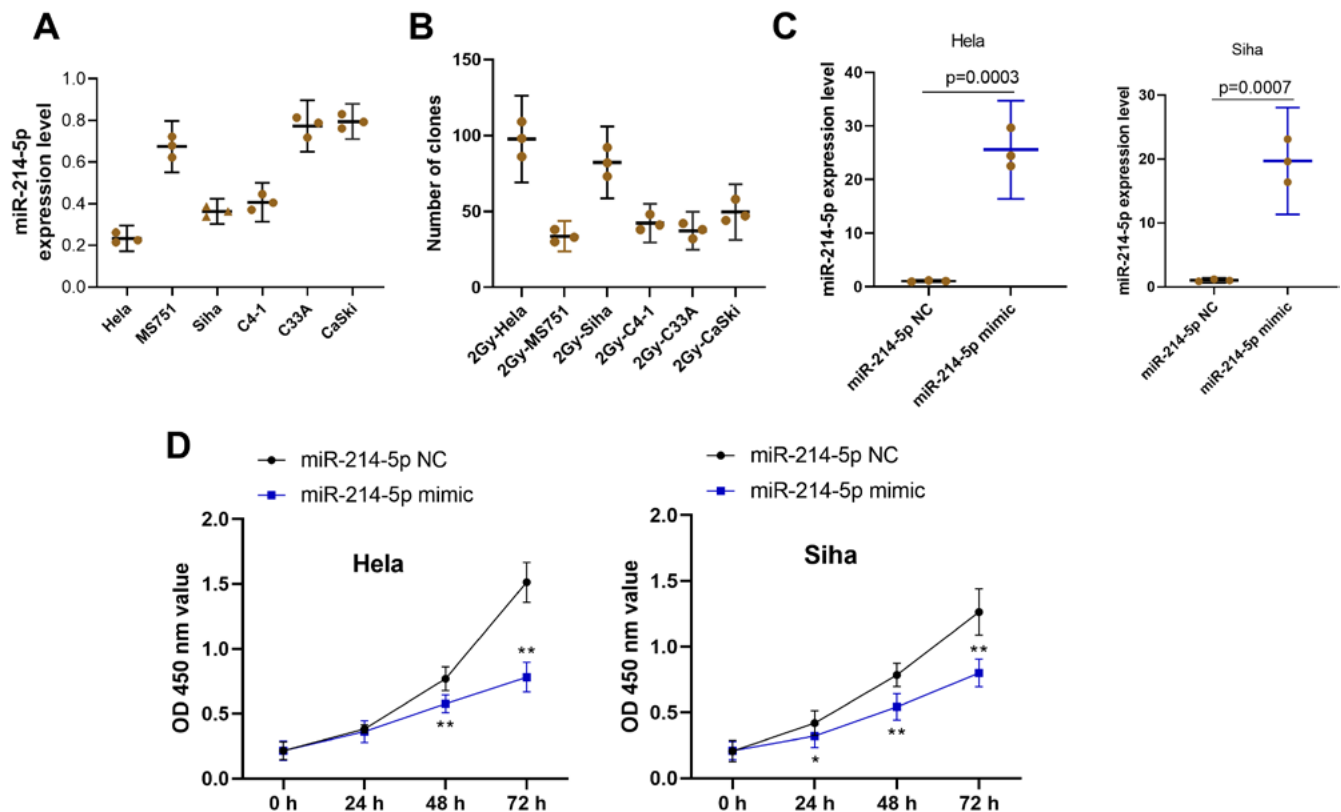
ns – not significant.

## Upregulation of miR-214-5p decreases the migration and invasion ability, and increases the radiosensitivity of cervical cancer cells

We measured the migration and invasion ability of 2 cell lines after increasing miR-214-5p expression. These data showed that migration and invasion decreased in both cell lines after the upregulation of miR-214-5p (Fig. 3A,B). Two single doses of 4 Gy and 6 Gy were used to test radiosensitivity, and after upregulating miR-214-5p, the radiation resistance of the 2 cell lines decreased significantly (Fig. 3C).

## miR-214-5p regulates radiation sensitivity by targeting ROCK1

To clarify the functions of miR-214-5p, it was important to identify its target genes. We used online bioinformatics tools to analyze the potential binding partners of miR-214-5p, identifying *ROCK1* as a potential target gene of miR-214-5p. A sequence at the 3'UTR region of *ROCK1* was selected to construct a mutant variant (Fig. 4A). Dual-luciferase reporter analysis indicated a significant reduction in luciferase activity in cells containing the *ROCK1*-WT sequence when miR-214-5p was exogenously increased. Moreover, the relative luciferase activity in cells containing *ROCK1*-mut was consistent after the transfection (Fig. 4B). The RT-qPCR analysis also indicated a significantly decreased *ROCK1* expression when miR-214-5p was



**Fig. 2.** Upregulation of miR-214-5p decreases the proliferation of cervical cancer cells. **A.** The expression of miR-214-5p in cervical cancer cell lines C4-1, MS751, HeLa, SiHa, C33A, and CaSki; **B.** The radiosensitivity of the above cell lines at 2 Gy; **C.** The expression of miR-214-5p in HeLa and SiHa cells after transfection; **D.** Cell proliferation of HeLa and SiHa cells after the transfection with a mimic of miR-214-5p; three biological replicates were performed, and the results are shown as a mean with 95% confidence interval (95% CI). The results were analyzed using Student's t-test

OD – optical density; \*  $p < 0.05$ ; \*\*  $p < 0.01$ , compared with the same time point.

exogenously upregulated (Fig. 4C). We further analyzed the correlation between miR-214-5p and ROCK1 expression in the collected tissues, and found that miR-214-5p was negatively correlated with ROCK1 expression (Fig. 4D). Moreover, we observed a relative increase in ROCK1 expression in patients with poor radiation response (Fig. 4E).

### Overexpression of ROCK1 abolishes the enhanced miR-214-5p-dependent radiosensitivity

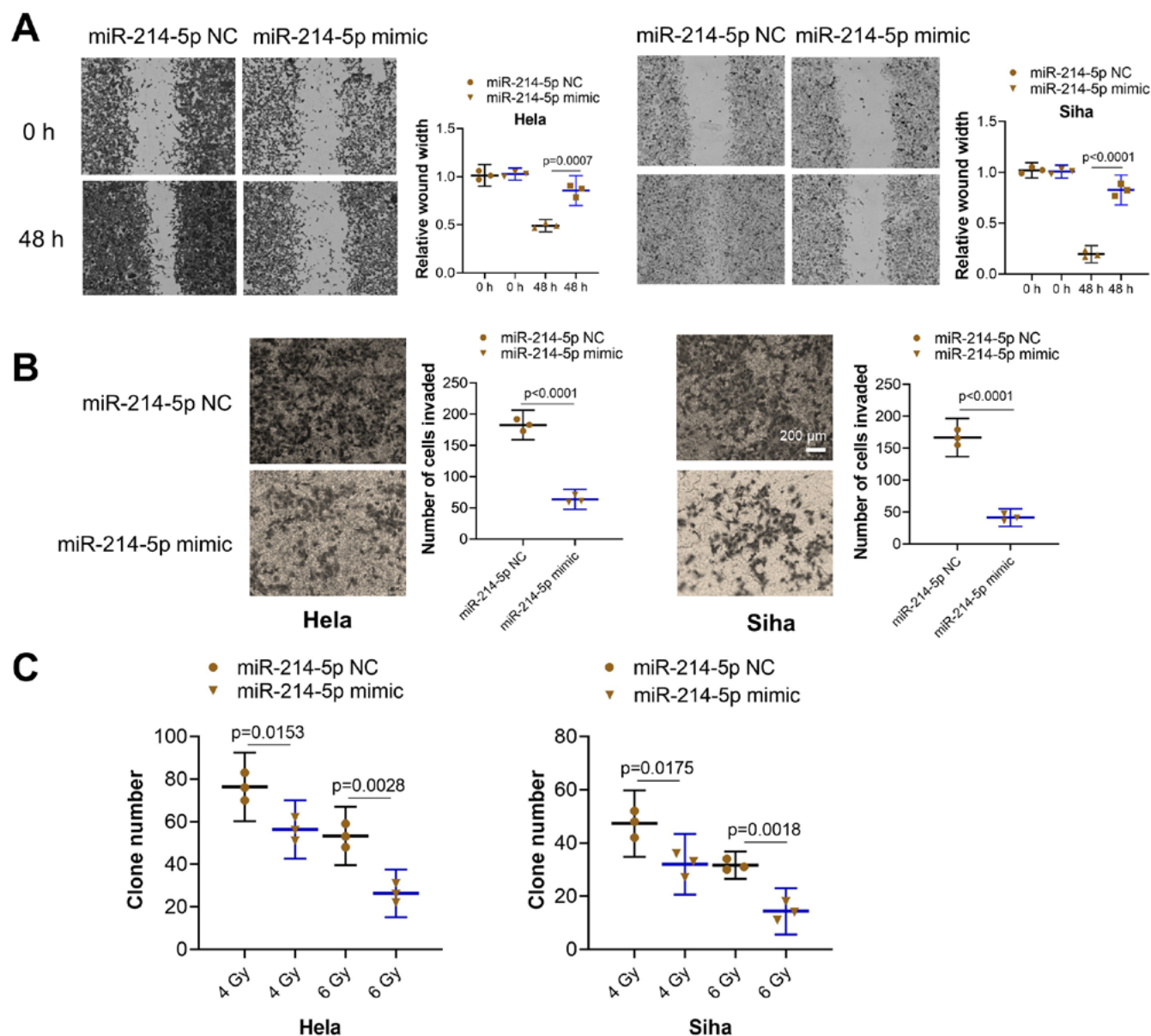
To clarify whether radiosensitivity and growth inhibition of cervical cancer cells brought about by miR-214-5p were dependent on ROCK1 regulation, we upregulated the expression of ROCK1 in HeLa cells through a ROCK1 overexpression plasmid (ROCK1 OE). The reduction in cell proliferation produced by miR-214-5p was partially reversed by ROCK1 OE (Fig. 5A). In addition, the radiation resistance of HeLa cells containing the miR-214-5p mimic was partially restored with the upregulation of ROCK1 (Fig. 5B). The protein expression of ROCK1, Rho and phosphorylated LIMK1 (p-LIMK1) the proteins that sit upstream and downstream in ROCK1 signaling,<sup>29</sup> were also measured. The results of western blot analysis showed that the expression of upstream Rho was not affected, while its downstream kinase p-LIMK1 decreased upon ROCK1 upregulation (Fig. 5C).

### miR-214-5p improves the radiotherapy effect of cervical cancer model in nude mice

Compared with the model group, the miR-214-5p mimic group demonstrated a limited inhibiting effect on the tumor ( $p = 0.244$ , compared to the model group). As expected, miR-214-5p significantly increased the radiosensitivity of tumors in nude mice (Fig. 6A,B). Compared with the radiation group alone, the tumor inhibition rate in the miR-214-5p mimic + radiation group increased from  $45.03 \pm 5.8\%$  to  $64.39 \pm 4.9\%$  ( $p < 0.0001$ ). These results confirmed that miR-214-5p has a remarkable sensitizing effect on radiotherapy. The protein expressions of Rho, ROCK1 and p-LIMK1 are consistent with the in vitro analysis (Fig. 6C), which revealed that the upregulation of miR-214-5p decreased expressions of ROCK1 and p-LIMK1 while not affecting Rho.

## Discussion

Herein, we evaluated the expression of miR-214-5p in patients who received radiotherapy, and we found that patients with unsatisfactory responses to treatment have relatively lower miR-214-5p expression levels. Based

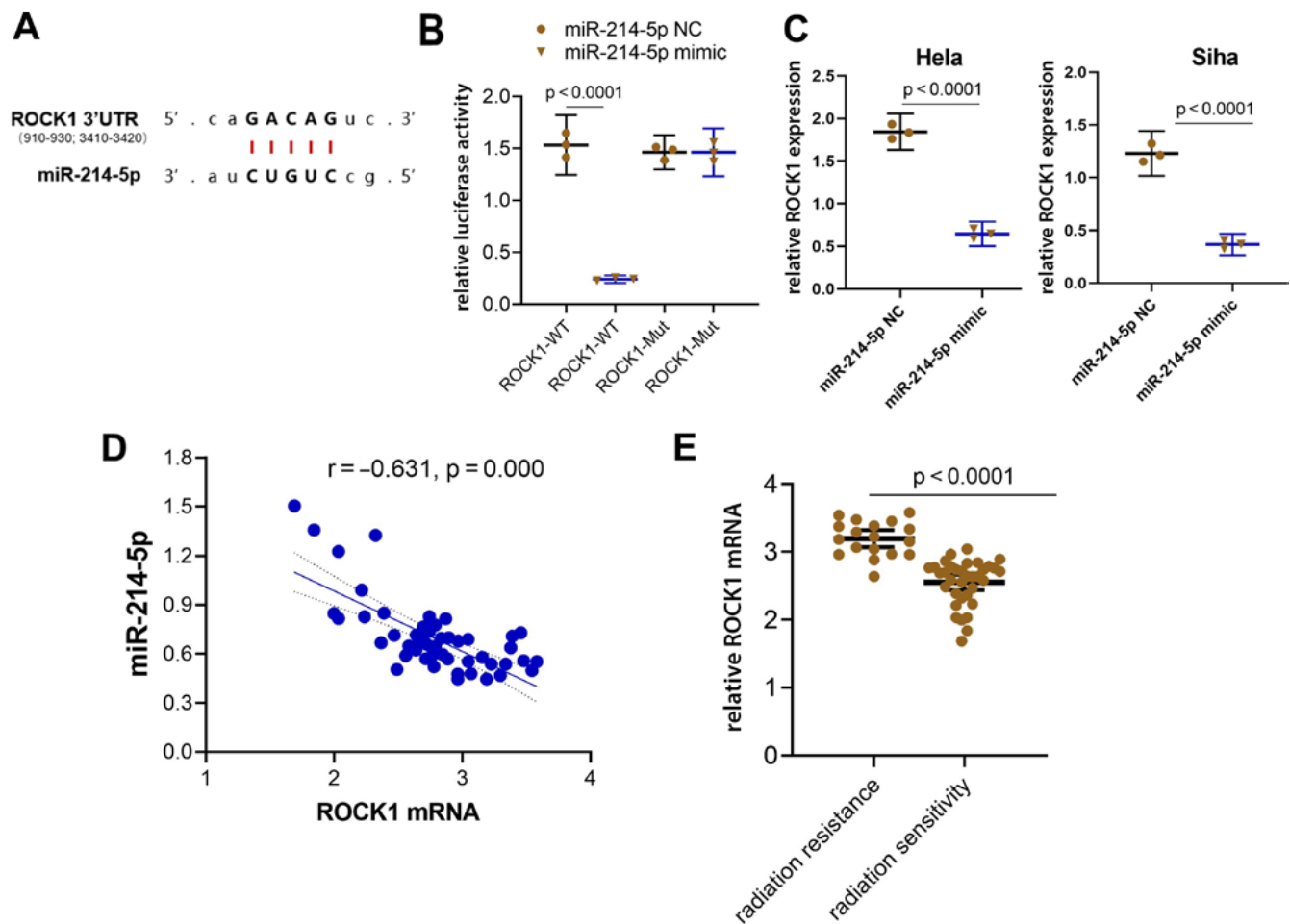


**Fig. 3.** Upregulation of miR-214-5p decreases the migration and invasion ability, and increases the radiosensitivity of cervical cancer cells. **A.** The migration ability of HeLa and SiHa cell lines after being transfected with miR-214-5p; **B.** The invasion ability of HeLa and SiHa cell lines decreased after the upregulation of miR-214-5p; **C.** The radiosensitivity of both cell lines decreased significantly after upregulating miR-214-5p measured using 2 single absorbed doses of 4 Gy and 6 Gy. Three biological replicates were performed, and the results are shown as a mean with 95% confidence interval (95% CI). The results were analyzed using Student's t-test

on the differences in miR-214-5p expression profiles, we found that miR-214-5p expression remained relatively low in several cervical cancer cell lines. The sample analysis suggested that miR-214-5p expression was significantly downregulated in cervical cancer tissues, especially in patients resistant to radiotherapy. Further studies revealed that upregulating the expression of miR-214-5p could inhibit the growth and proliferation of radiation-resistant cervical cancer cells. Intriguingly, in vivo studies showed that the administration of miR-214-5p mimics alone had little effect on tumor growth, but in combination with radiotherapy could significantly inhibit tumor growth, better than radiation treatment alone, indicating that miR-214-5p has a certain sensitizing effect.

Radiotherapy is one of the main therapies for cervical cancer due to the tumor's relatively high sensitivity to radiation therapy.<sup>30,31</sup> Radiotherapy can also induce changes in the expression of many genes and proteins, leading to the reduction of tumor sensitivity to radiotherapy and the development of radiation resistance.<sup>32</sup> Radiation resistance is the main reason for the failure of radiotherapy.<sup>33</sup> Furthermore, radiation resistance is a process involving multiple genes and mechanisms, including cancer stem cells (CSCs) with high tumorigenicity, which repair apoptosis by activating DNA damage responses; increased glucose uptake and decreased mitochondrial oxidative phosphorylation in tumor cells could also efficiently repair DNA. In addition, cell survival is promoted by providing energy through autophagy and





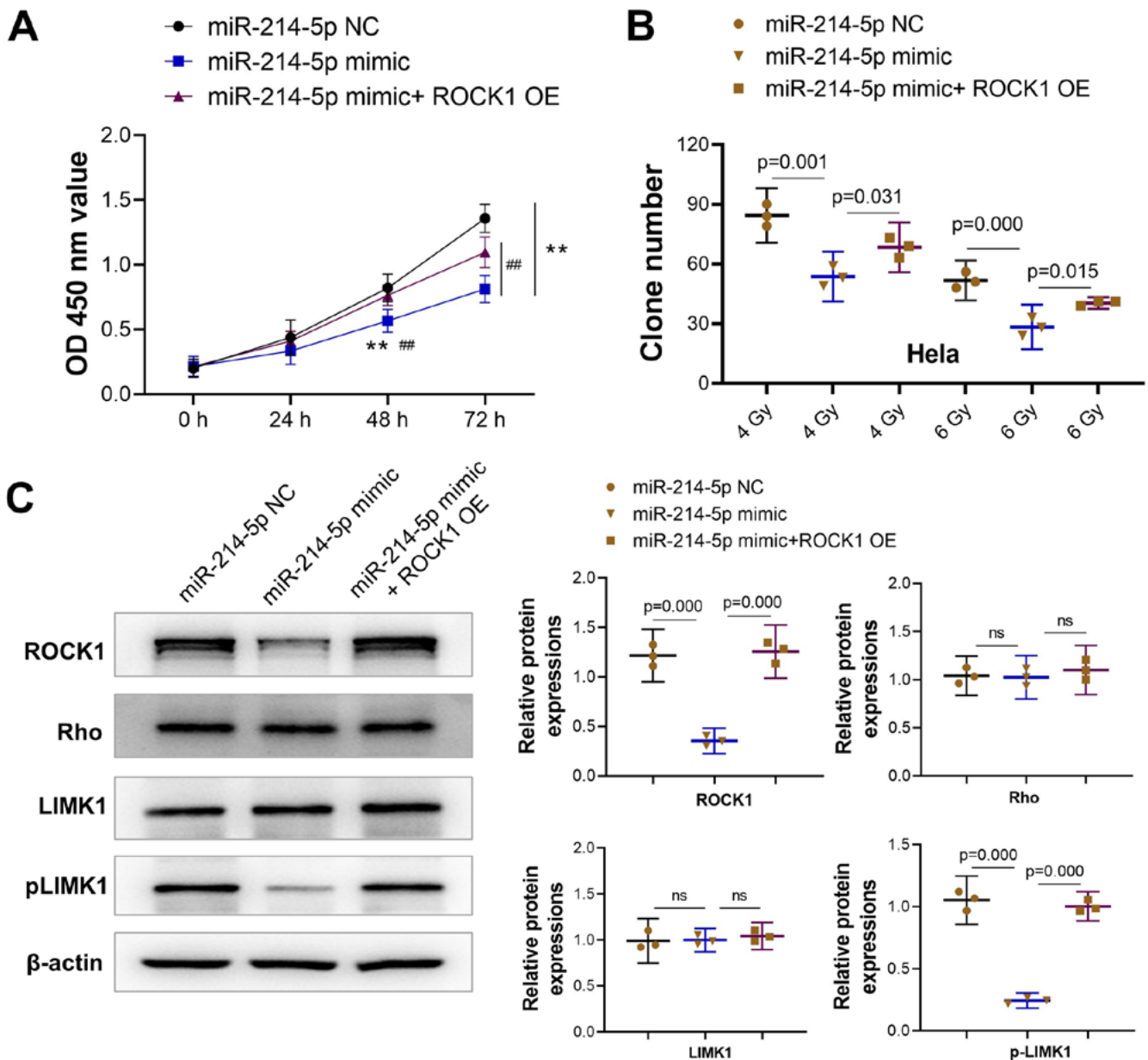
**Fig. 4.** miR-214-5p regulates radiation sensitivity of cervical cancer cell expression by targeting the expression of ROCK1. A. The selected binding sequence at the 3'untranslated region (3'UTR) region of ROCK1; B. The relative luciferase activity of cells transfected with ROCK1 sequence; C. The ROCK1 expression after miR-214-5p mimics were transfected into cervical cancer cells; three biological replicates were performed and data were analyzed using Student's t-test; D. The correlation analysis showed that the expression of ROCK1 was negatively correlated with the expression of miR-214-5p in cervical cancer tissues; data were analyzed with Spearman's correlation; E. Relative higher ROCK1 expression in patients has poor radiation response; data were analyzed using the Mann-Whitney U test. Whiskers represent the 95% confidence interval (95% CI) values

eliminating proteins that have been damaged by radiation. The regulation of the redistribution of the cell cycle could weaken the sensitivity to radiotherapy of tumor cells.<sup>31,34</sup>

Cell damage caused by different agents can facilitate different miRNA responses, which, in the process, will show expression disorder.<sup>35</sup> The regulation of miRNA expression ensures that miRNA can respond to injury both at the transcriptional and post-transcriptional levels. Generally, the direct target genes of miRNA molecules with oncogene effect are mostly tumor suppressor genes. Conversely, if the miRNA molecule has a tumor suppressor function, it mainly targets multiple oncogenes.<sup>36</sup> In addition, the expression of miRNA is tissue- and tumor stage-specific, which leads to obvious differences in miRNA expression profiles in different tissues. A variety of miRNA molecules are involved in the progression of cancer. For example, the decreased expression of miR-15 in B-cell lymphomas is caused by the deletion of chromosome 13q14,<sup>37</sup> while miR-17/92 is related to the expansion of chromosome 13q31.<sup>38</sup> Moreover, trans-regulatory elements, such

as transcription factors, may change the level of gene expression through the interaction of protein and DNA. For example, C-myc and Twist can regulate the expression of miRNAs and contribute to tumor progression.<sup>39,40</sup> Since the expression level and activity of these transcription factors are changed in tumor tissues, the expression of the target gene of miRNA molecules is naturally affected. Finally, studies have found that this transcriptional activation of oncogenes and transcriptional silencing of tumor suppressor genes existed in almost all cancer tissues.<sup>41</sup>

The Rho/ROCK expression is mainly limited to the cytoplasm and is involved in regulating cell morphology, cytoskeletal remodeling, cell migration, and other cellular functions.<sup>42</sup> In the process of cell signal transduction, Rho GTPases mainly act on the cytoskeleton or target proteins. They regulate a variety of biological effects, including cell membrane transport, cell migration, adhesion, and proliferation, among others.<sup>43</sup> The ROCK is a serine/threonine protein kinase with 2 highly homologous isomers, ROCK1 and ROCK2. Liang et al. reported that increased expression

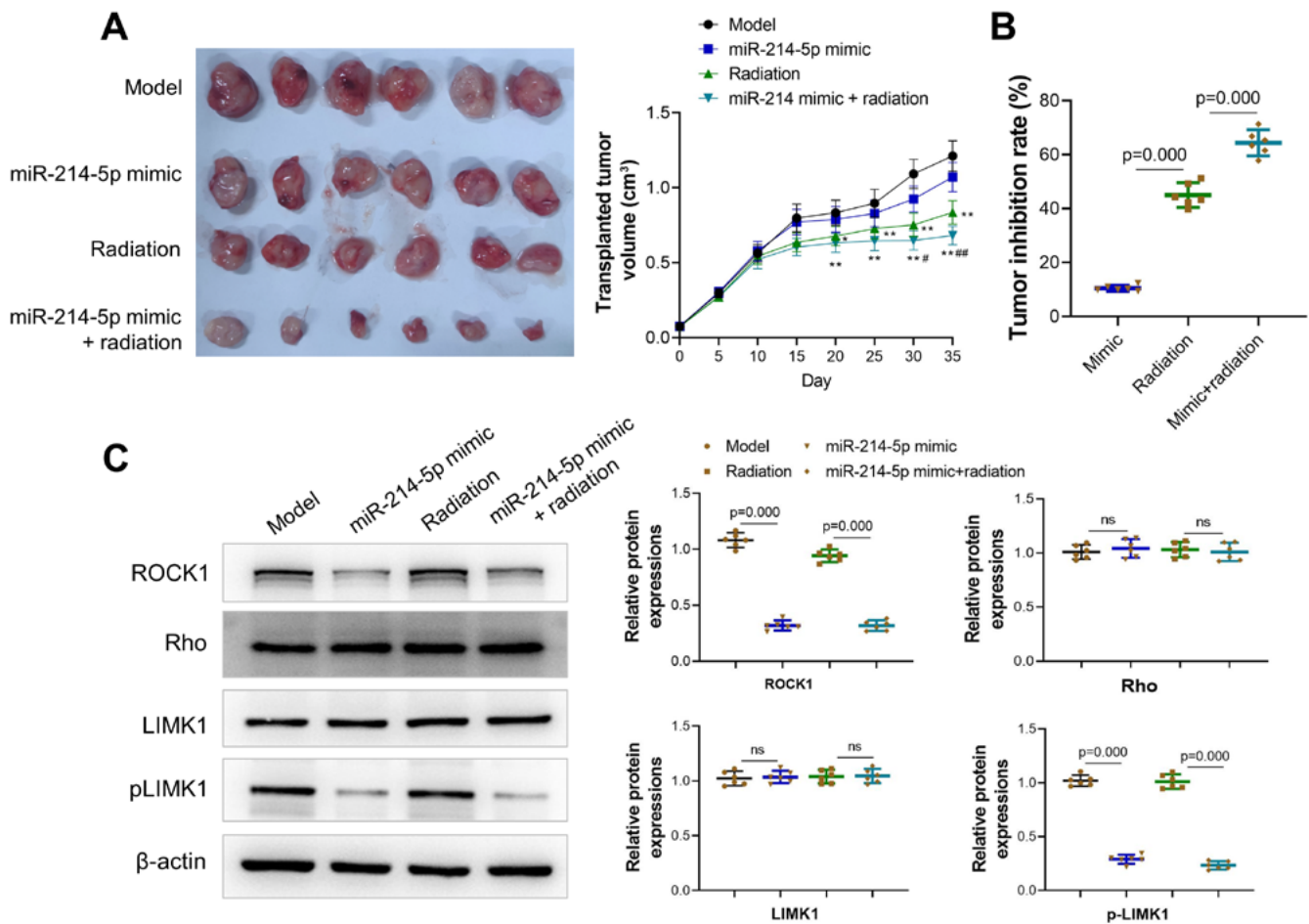


**Fig. 5.** Overexpression of ROCK1 abolishes the enhanced radiosensitivity brought by miR-214-5p. **A.** The downregulation of Hela cell proliferation brought by miR-214-5p inhibition was partially reversed after the upregulation of ROCK1 (ROCK1 OE); **B.** The radiation resistance of miR-214-5p-transfected Hela cell was restored partially by ROCK1 OE; **C.** Protein expressions of ROCK1, Rho, LIMK1, and phosphorylated LIMK1 (p-LIMK1) were measured with western blot analysis; the expression of Rho and LIMK1 was not affected, while p-LIMK1 expression decreased after the overexpression of ROCK1; three biological replicates were performed, and data were analyzed with bootstrap analysis of variance (ANOVA) analysis, based on 1000 bootstrap samples. The Tukey's multiple comparison test was used for post hoc analysis. Whiskers represent the 95% confidence interval (95% CI) values

ns – not significant; OD – optical density; \*\*  $p < 0.01$ , compared to miR-214-5p NC; ##  $p < 0.01$ , compared to miR-214-5p mimic + ROCK1 OE.

of ROCK1 can promote EMT in cervical cancer cells.<sup>44</sup> Through online database analysis, we identified ROCK1 as a potential target of miR-214-5p. In vitro and in vivo studies show that upregulating miR-214-5p downregulates ROCK1 expression and enhances the sensitivity to radiotherapy. Our analysis showed that ROCK1 was highly expressed in both radiation-resistant cells and radiotherapy-resistant patients with cervical cancer. Moreover, we demonstrated that ROCK1 participates in the radiation resistance of cervical cancer, and that miR-214-5p decreases the resistance of ROCK1 to radiation by targeting its expression.

An anoxic microenvironment exists in most of the solid tumors, which affects the genetic phenotype of tumor cells.<sup>45</sup> Consequently, hypoxic microenvironments of tumors are closely related to the occurrence, development, prognosis, metastasis, and therapeutic response of the tumor to treatment. Leong and Chambers indicated hypoxia-induced migration of tumor cells through Rho/ROCK1 signaling pathways,<sup>46</sup> and Liu et al. observed that ROCK1 is related to cisplatin resistance in lung cancers.<sup>47</sup> These results demonstrate that the Rho/ROCK1 pathway broadly participates in the occurrence and development of tumors,



**Fig. 6.** miR-214-5p improves the radiotherapy effect of the cervical cancer model in nude mice. **A.** Stripped tumor and tumor volume in each group;  $n = 6$ , data were analyzed using analysis of variance (ANOVA); **B.** Tumor inhibition rate in each group;  $n = 6$ , results were analyzed with bootstrap ANOVA, based on 1000 bootstrap samples; the Tukey's multiple comparisons test was used for the intercomparison of groups; **C.** Consistent with in vitro analysis, the protein expressions of Rho, the upstream protein of ROCK1, did not change. The expression of ROCKs and phosphorylated LIMK1 (p-LIMK1) decreased after the upregulation of miR-214-5p, especially when combined with radiation. Measurements were performed in triplicate. Data were analyzed using bootstrap ANOVA analysis, based on 1000 bootstrap samples. The Tukey's multiple comparisons test was used for post hoc analysis. Whiskers represent the 95% confidence interval (95% CI) values

ns – not significant; \*  $p < 0.05$ , \*\*  $p < 0.01$ , compared to the model group; #  $p < 0.05$ , ##  $p < 0.01$ , compared to the radiation group.

especially in the process of creating therapy resistance. As for the high expression of ROCK1 in radiation resistance, we speculate that ionizing radiation leads to sustained oxidative stress and mitochondrial dysfunction, inducing the upregulation of ROCK1 expression, adjusting the reconstruction of the damaged tumor cytoskeleton and causing tumor cells to resist radiotherapy.<sup>48,49</sup> The miR-214-5p prevents the reconstruction of the cervical cancer cell cytoskeleton by downregulating ROCK1 expression and improving the tolerance of cervical cancer cells to radiation. Unfortunately, due to the previous research design, we only verified the change of ROCK1 expression in radiotherapy sensitivity. We look forward to further explore and verify the upstream influencing factors in subsequent studies.

The LIMK1 is phosphorylated by upstream kinases ROCK1, and its activation continually phosphorylates downstream target proteins, thereby regulating actin and tubulin remodeling.<sup>50</sup> The p-LIMK1 is an important

executive protein of ROCK1 during tumor cell migration. Our analysis indicated that with the reduction of ROCK1 regulated by miR-214-5p, Rho expression did not change and the expression level of p-LIMK1 decreased, indicating that ROCK1 is a key protein regulating radiosensitivity in cervical cancer radiotherapy, and is specifically arranged by miR-214-5p.

## Limitations

Our study has some limitations. First, the inhibition of miR-214-5p in tumor cells in vivo and in vitro is inconsistent, and it is not clear whether the reduction of inhibition efficiency in vivo is due to the insufficient miRNA dose, or if the effect itself is limited. These questions need to be further clarified. In addition, whether the sensitizing effect of miR-214-5p on radiotherapy is simply added to radiation or is a synergistic effect requires further analysis.


## Conclusions


Herein, we demonstrated decreased miR-214-5p expression and increased ROCK1 expression in patients with radiotherapy-resistant/insensitive cervical cancer. Exogenously increasing miR-214-5p specifically regulates ROCK1 and improves the sensitivity of cervical cancer to radiotherapy. This suggests that the upregulation of miR-214-5p or decreasing ROCK1 expression may help to improve the treatment response in cervical cancer patients treated with radiotherapy, although this conclusion needs to be verified in further follow-up studies.

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