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miR-21 modulates resistance of HR-HPV positive cervical cancer cells to radiation through targeting LATS1

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ABSTRACT

Although multiple miRNAs are found involved in radioresistance development in HR-HPV positive (+) cervical cancer, only limited studies explored the regulative mechanism of the miRNAs. miR-21 is one of the miRNAs significantly upregulated in HR-HPV (+) cervical cancer is also significantly associated with radioresistance. However, the detailed regulative network of miR-21 in radioresistance is still not clear. In this study, we confirmed that miR-21 overexpression was associated with higher level of radioresistance in HR-HPV (+) cervical cancer patients and thus decided to further explore its role. Findings of this study found miR-21 can negatively affect radiosensitivity of HR-HPV (+) cervical cancer cells and decrease radiation induced G2/M block and increase S phase accumulation. By using dual luciferase assay, we verified a binding site between miR-21 and 3′-UTR of large tumor suppressor kinase 1 (LATS1). Through direct binding, miR-21 can regulate LATS1 expression in cervical cancer cells. LATS1 overexpression can reverse miR-21 induced higher colony formation rate and also reduced miR-21 induced S phase accumulation and G2/M phase block reduction under radiation treatment. These results suggested that miR-21-LATS1 axis plays an important role in regulating radiosensitivity.

1. Introduction

Cervical cancer is the third most common malignancy in women [1]. Currently, radiotherapy is still the most common intervention either as a primary or an adjuvant therapy [2,3]. Although radiotherapy is used for over 60% cervical cancers cases, local recurrence is common due to radioresistance [4]. Due to the complex mechanism of cervical cancer and radioresistance, it is still difficult to predict radioresistance before giving therapeutic regime.

Persistent infection of high risk human papillomavirus (HR-HPV), typically HPV-16 and HPV-18 are the main risk factor of cervical cancer development [5]. HR-HPV infection leads to a series of dysregulated molecular processes. For example, the viral oncoprotein E6 and E7 of HPV-16 and HPV-18 can inactivate p53 and pRB respectively, thereby affecting their downstream regulation and contributing to cervical carcinogenesis [6]. Previous studies also observed altered miRNA expression in HR-HPV positive (+) cervical cancer patients [7,8]. In fact, multiple miRNAs are found involved in radioresistance development, such as miR-630, miR-1246, miR-1290, miR-3138, miR-181a and miR-218 [9,10]. However, only limited studies explored the regulative mechanism of miRNAs in radioresistance of cervical cancer.

miR-21 is also a miRNA significantly upregulated in HR-HPV (+) cervical cancer [11], which might be regulated by the viral oncoprotein E6 [12]. Previous studies observed that miR-21 is an oncomiR in cervical cancer, which promotes cell proliferation and tumorogenesis by downregulating the expression of programmed cell death 4 (PDCD4) [13] and CCL20 [14], or by mediating aberrant STAT3 signaling [12]. In fact, its overexpression is also significantly associated with radioresistance [10,15]. However, the detailed regulative network of miR-21 in radioresistance is still not clear. In this study, we explored the regulative role of miR-21 in radioresistance of cervical cancer cells and firstly demonstrated that miR-21 can directly target and downregulate the expression of large tumor suppressor kinase 1 (LATS1). By modulating LATS1, miR-21 can promote radioresistance through alleviating radiation induced apoptosis, decreasing G2/M block and increasing S phase accumulation.

2. Methods

2.1. Patient selection and human tissues

22 patients histologically diagnosed as IA with lyphovascular space invasion (IVSI) or IA2 cervical cancer, confirmed as HPV-16/18

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positive and never received previous chemotherapy were recruited from Cangzhou city central hospital. The patients were administrated with radiotherapy by giving standard, pelvic radiation therapy and branchytherapy with a total point A dose 70 Gy according to 2013 NCCN Clinical Practice Guideline in Oncology for Cervical Cancer [16]. The radiosensitive and radioresistant cases was assessed by histological examination of residual tumor tissues by colposcopically directed biopsy 6 months after completion of radiotherapy. Histological assessment was performed by pathologist without authorship in this study. All of the samples contained at least 70% tumor cells were used for further studies. 20 cases of healthy control with normal cytology and without HR-HPV infection were obtained from patients received hysterectomy due to benign gynecologic diseases. Informed consent was obtained from each patient before gaining the specimens.

2.2. Cell culture

HEK 293T, HPV-16-positive SiHa and HPV-18-positive HeLa cervical cancer cell lines were obtained from the ATCC and were cultured with Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories), 2 mM/L-glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin and were incubated in humidified air with 5% CO_2 at 37 °C.

2.3. Cell transfection

Chemically synthesized miR-21 mimics, antagomiR-21, LATS1 siRNA and the corresponding negative controls were purchased from Ribo Life Science (China). Hela and siHa cells were transfected with 50 nM miR-21 mimics or 100 nM antagomir-21 by using lipofectamine 2000 (Invitrogen). siHa cells were transfected with 50 nM LATS1 siRNA by using lipofectamine 2000 (Invitrogen). Human LATS1 lentiviral vector (Lenti-LATS1) without 3′-UTR were purchased from GENECHEM. To generate lentiviral particles for transfection, Lenti-LATS1 and the corresponding packaging mix were cotransfected to HEK-293T cells. 48 h after transfection, the viral supernatant were collected for further experiments. To overexpress LATS1 in siHa and Hela cells, the host cells were treated with the viral supernatants with 8 μg/ml polybrene (Sigma-Aldrich).

2.4. qRT-PCR analysis of miR-21 and LATS1 expression

Total RNAs from tissue and cells were extracted using TRIzol reagent (Invitrogen) and from blood were extracted using TRIzol LS reagent (Invitrogen) respectively. cDNAs were synthesized using the PrimeScript RT reagent Kit (TaKaRa). miR-21 expression was quantified by using the TaqMan MicroRNA Assays (Life Technologies). The $2^{-\Delta\Delta}$ Ct method was used to calculate relative miR-21 expression, with RNU6B as a control gene. LATS1 mRNA expression were measured using qRT-PCR with SYBR Green PCR Master Mix (Life Technologies) and LATS1 specific primers: F, 5′-CCA CCC TAC CCA AAA CAT CTG-3′; R, 5′-CGC TGC TGA TGA GAT TTG AGT AC-3′. GAPDH served as internal control.

2.5. Western blot analysis of LATS1 expression

Total protein from tissue and cells were extracted by using RIPA buffer (50 mM TrisHCl, 150 mM NaCl, 2 mM EDTA, 1% NP-40 and 0.1% SDS). Total protein concentration were measured by using BCA protein assay (Pierce, Thermo Scientific) and then separated on 10% SDS PAGE gel and transferred onto nitrocellulose membranes for a conventional western blot analysis. LATS1 protein was detected with Anti-LATS1 (1:2000, ab70562, Abcam). β-actin served as loading control and was detected by using ant-actin (1: 1000, Ab1801, Abcam). Anti-Rabbit IgG (HRP) (1: 10,000, ab191866, Abcam) was used as second antibody. Protein signals were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). The signal intensity was detected using Image-J software.

2.6. Colony formation and apoptosis analysis

1×10^5 SiHa or Hela cells were seeded in six-well plates and prepared for transfection the next day. 48 h after transfection, the plates were irradiated with 137Cs (Nordion, Ottawa, ON, Canada), with a dose rate of 1.25 Gy/min. The plates were treated with a dose of 0, 2, 4, 6, 8 and 10 Gy in a single fraction. Then, the plates were further incubated in a cell incubator for 10–13 days and then the cells were fixed with 10% paraformaldehyde and stained with 1% crystal violet in 70% ethanol. Colonies (with minimal 50 cells) were counted. Survival fraction was defined as the number of colonies/(cells inoculated × plating efficiency) and radiation survival curve was drew. Apoptosis of the cells were measured by using caspase Glo-3/7 assay kit (Promega, Shanghai, China) and by using Fluorescein Active Caspase 3 Staining Kit (Abcam, ab65613) according to the instructions. The proportion of Active Caspase 3 positive cells was measured by using a flow cytometer (FACSCalibur, BD Biosciences).

2.7. Flow cytometry analysis of cell cycle distribution

After treatment, cells were collected, washed and then fixed with 70% ethanol at −20 °C for 24 h, and then the cell pellet was stained with 10 μg/mL PI (Sigma, St. Louis, MO) and 10 μg/mL RNase A in PBS for 15 min at room temperature in the dark. DNA content was analyzed on FACSCaliber (BD Bioscience) and the results were analyzed using ModFit software (BD Bioscience).

2.8. Luciferase microRNA binding assay

Wild-type (WT) or mutant (MUT) fragments of 3′-untranslated region (3′UTR) of the LATS1 containing the predicted miR-21 binding site and with flanking PmeI and XbaI restriction enzyme sites were chemically synthesized. The sequence are: WT: F, 5′-aaacTTGGTAGAAAATAAGCTAGAGAAATTAAGCCATCGTGTTGGTGAGTt-3′, R, 5′-ctagaACTCACCAACACGATGGCTTAATTTCTCTAGCTTATTTTCTACCAAgttt-3′; MUT: 5′-aaacTTGGTAGAAAAGCCTAGCGAGAAATTAAGCCATCGTGTTGGTGAGTt-3′; R, 5′-ctagaACTCACCAACACGATGGCTTAATTTCTCGCTAGGCTTTTCTACCAAgttt-3′. The two pairs of DNA oligonucleotides were inserted into the downstream of the firefly luciferase gene in pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega) between PmeI and XbaI sites. The reconstructed vectors were designated as pmirGLO-LATS1-WT and pmirGLO-LATS1-MUT respectively. Insertion was verified by sequencing. HEK-293T and siHa cells were co-transfected with 200 ng reporter plasmids and 50 nM miR-21 mimics. 24 h post transfection, both firefly and Renilla luciferase activities were measured by the Dual-Luciferase Reporter Assay System (Promega) using a Promega GloMax 20/20 luminometer. The firefly luciferase activity was normalized to the renilla luciferase activity.

2.9. Statistical analysis

Data were presented as mean ± SD with at least three repeats. Group comparison was performed by unpaired T test. The association between miR-21 and LATS1 protein expression in cancer tissue samples was analyzed by using linear regression model. p

value <0.05 was considered as significant difference. *, **, and *** donates significance at 0.05, 0.01 and 0.001 level respectively.

3. Results

3.1. miR-21 is highly expressed, while LATS1 is downregulated in HR-HPV (+) radioresistant cervical cancer patients

The basic information of the participants were summarized in supplementary table 1. Based on serum and tissue samples from 22 patients and 20 healthy controls, qRT-PCR analysis showed that HR-HPV (+) cervical cancer patients had significantly higher miR-21 expression in both serum (Fig. 1A) and tumor tissue (Fig. 1B). In addition, by analyzing serum and tissue samples of the patients six months after radiotherapy, it was also observed that radioresistant patients (n = 13) had even higher miR-21 expression than radiosensitive cases (n = 9) (Fig. 1E and F). By the same time, the expression of LATS1 had an inverse expression trend to miR-21. Generally, LATS1 mRNA expression in cervical cancer patients was significantly lower than healthy control (Fig. 1C) and was even lower in radioresistant than in radiosensitive patients (Fig. 1D). By performing linear regression analysis of miR-21 and LATS1 protein expression in the cancer tissue samples, it was found miR-21 expression was negatively correlated with LATS1 protein expression ($R^2 = 0.83$, $p < 0.001$) (Fig. 1G). Lower LATS1 protein expression in radioresistant than in radiosensitive patients were also confirmed (Fig. 1H). These results suggest that miR-21 and LATS1 might have possible roles in modulating radiosensitivity of HR-HPV (+) cervical cancer.

3.2. miR-21 can negatively affect radio-sensitivity of HR-HPV (+) cervical cancer cells

To explore the effect of miR-21 on radiosensitivity of HR-HPV (+) cervical cancer cells, HPV-16-positive SiHa and HPV-18-positive HeLa cervical cancer cell lines were firstly transfected with miR-21 mimics or antagomir-21 for overexpression and knockdown respectively. In both cell lines, miR-21 overexpression significantly enhanced colony formation (Fig. 2A and C), while its knockdown reduced colony formation under radiation treatment (Fig. 2B and D). We further explored whether miR-21 is related to apoptosis induced by radiation. In both Hela and siHa cell lines, miR-21 overexpression and knockdown did not bring change to the activity of caspase 3/7 (Fig. 2E–H). However, when cells were exposed to 10Gy radiation, miR-21 overexpression significantly reduced the activity of caspase 3/7 (Fig. 2E and G), while its knockdown significantly promoted the activity of caspase 3/7 (Fig. 2F and H). In addition, by measuring active caspase 3, it was also observed that miR-21 overexpression blunted the expression of this apoptotic marker (Fig. 2I and J). These results suggested that miR-21 might protect the cancer cells from radiation-induced damage by reducing apoptosis.

3.3. miR-21 can decrease radiation induced G2/M block and increase S phase accumulation

Considering the protective role of miR-21 in radiation induced apoptosis, we further studied its role in cell cycle. SiHa and Hela cells transfected for miR-21 overexpression and knockdown were exposed to 10Gy irradiation. 48 h after exposure, the proportion of cells in the G1 phase decreased, while the cells in the S and G2/M phase increased in both cell lines (Fig. 3A and B). However, miR-21 overexpression weakened the block of cells in the G2/M and enhanced S phase accumulation (Fig. 3A and B). Statistical analysis confirmed the change was quite significant in both siHa (Fig. 3C and D) and Hela (Fig. 3E and F) cells. In the whole cell cycle, cells are usually most sensitive to radiation in G2 phase before mitosis and are least sensitive in the mid-to late S and early G1 phases. Therefore, the cell cycle regulation effect of miR-21 might contribute to cervical cancer cell radioresistance.

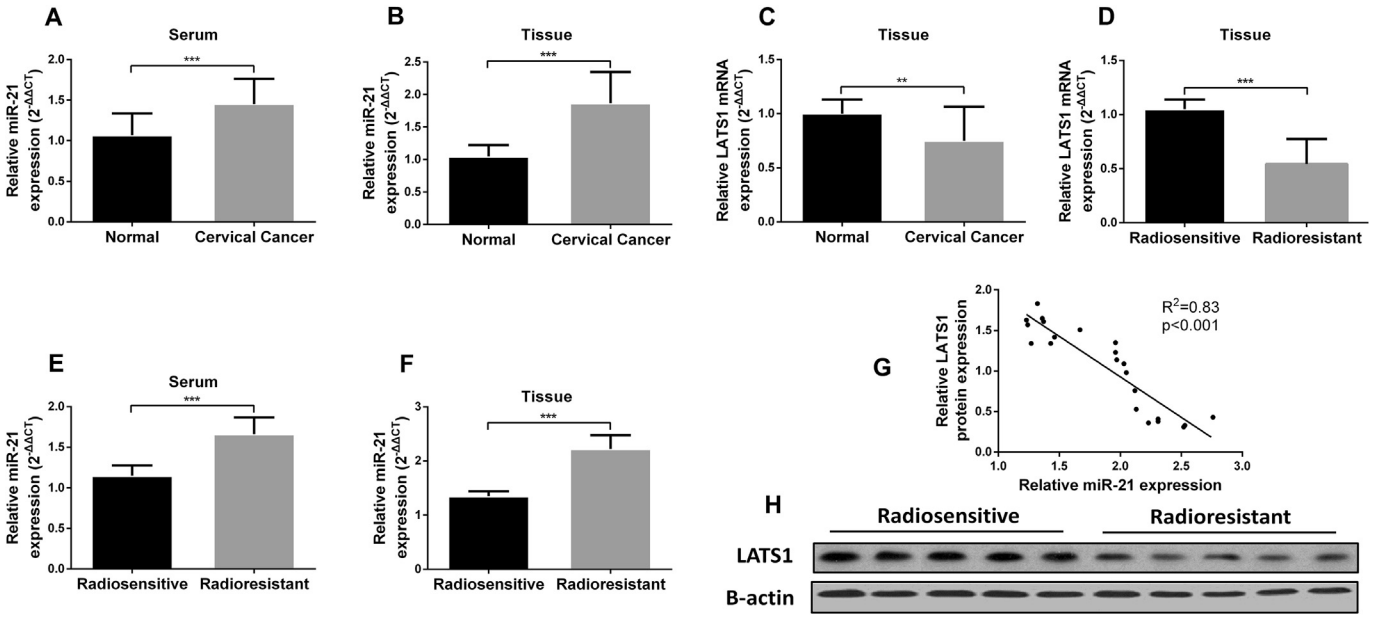

Fig. 1. miR-21 is highly expressed, while LATS1 is downregulated in HR-HPV (+) radioresistant cervical cancer patients (A and B) qRT-PCR analysis of relative miR-21 expression in serum (A) and tissue (B) of HR-HPV (+) cervical cancer patients (n = 22) and healthy controls (n = 20). (C and D) qRT-PCR analysis of LATS1 mRNA expression in tissues of HR-HPV (+) cervical cancer patients (n = 22) and healthy controls (n = 20) (C) and in tissues of radioresistant patients (n = 13) and radiosensitive patients (n = 9) (D). (E and F) qRT-PCR analysis of miR-21 expression in serum (E) and tissue (F) of radioresistant patients (n = 13) and radiosensitive patients (n = 9). (G) Linear regression analysis of miR-21 expression and LATS1 expression in cancer tissues of the patients. (H) Western blot analysis of LATS1 expression in randomly selected radioresistant (n = 5) and radiosensitive patients (n = 5). Each bar represents the mean ± S.D. of three experiments. *P < 0.05; **P < 0.01, ***P < 0.001.

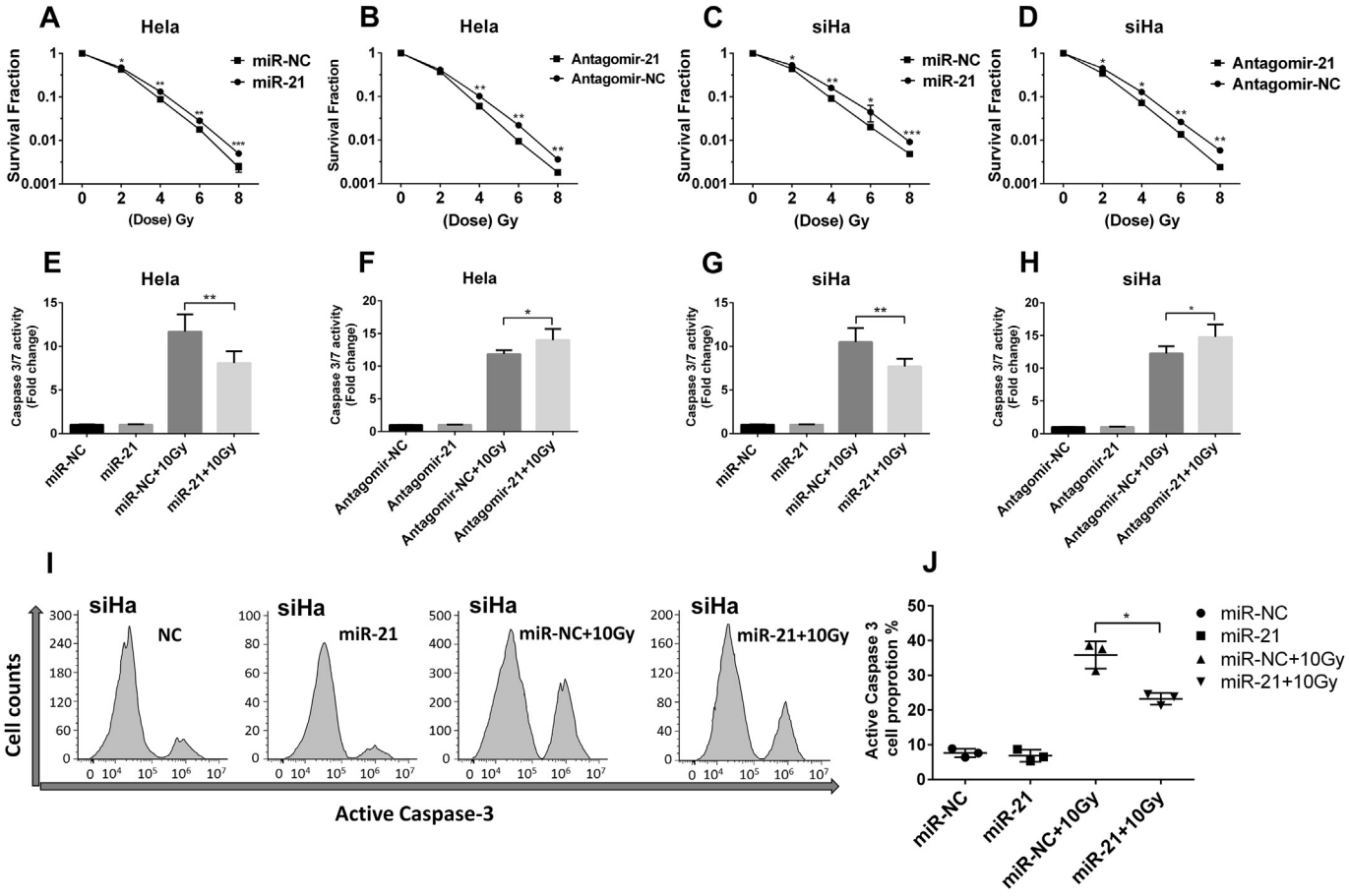

Fig. 2. miR-21 can negatively affect radiosensitivity of HR-HPV (+) cervical cancer cells (A–D) Analysis of miR-21 overexpression (A and C) and knockdown (B and D) on colony formation rate of Hela (A and B) and siHa (C and D) cells. Cells were transfected with 50 nM miR-21 mimics or 100 nM antagomir-21 for overexpression and knockdown respectively. Survival fraction was used as an indicator. In both Hela and siHa cells, miR-21 overexpression could significantly increase colony formation, while miR-21 knockdown significantly reduced colony formation under radiation treatment. (E–H) Assessment of the effect of miR-21 on apoptosis by detecting the activity of caspase3/7 48 h after 10Gy treatment. In both Hela (E and F) and siHa (G and H) cells, miR-21 overexpression (E and G) could significantly reduce the activity of caspase3/7, while miR-21 knockdown (F and H) significantly increased the activity of caspase3/7 under 10Gy treatment. But miR-21 had no effect on cells without radiation treatment. (I) Representative images of flow cytometry analysis of apoptotic siHa cells by active caspase-3 staining and (J) quantification of the apoptotic siHa cells 48 h after transfection or 48 h after 10Gy treatment. miR-21 overexpression significantly reduced the proportion of apoptotic cells treated by radiation, but had no effect on cells without radiation treatment. Each bar represents the mean ± S.D. of three experiments. *P < 0.05; **P < 0.01, ***P < 0.001.

3.4. miR-21 modulates radiosensitivity of HR-HPV (+) cervical cancer cell though directly targeting LATS1

Through prediction in online bioinformatics databases, we identified a putative highly conserved binding site between miR-21 and LATS1. Considering their inverse expression trend in the cancer cases, we further explored their association in HR-HPV (+) cervical cancer cells. To validate the binding, two dual luciferase vectors carrying either contained wild-type or mutant miR-21-targeting sequences (Fig. 4A) were constructed. Transfection of miR-21 mimics significantly reduced the luciferase activity of reporter carrying widetype pairing sequence, but has little influence on the reporter with mutant sequence in both HEK293T and siHa cells (Fig. 4B and C). In siHa cells, overexpression of miR-21 significantly inhibited the expression of LATS1 at both mRNA and protein level, which had similar effect as si-LATS1 (Fig. 4D), while miR-21 knockdown significantly increased LATS1 expression, which has similar effect as transfection of Lenti-LATS1 (Fig. 4E). These results suggest that miR-21 can directly target LATS1 and regulate its expression. We then further explored the effect of LATS1 on cell proliferation and cell cycle change under radiation. In both siHa and Hela cells, LATS1 overexpression significantly lowered the colony formation rate and also reversed miR-21 induced higher colony formation rate (Fig. 4F and G). As to the cell cycle, LATS1 overexpression reduced miR-21 induced S phase accumulation and G2/M phase block in both siHa (Fig. 4H and I) and Hela (Fig. 4J and K) cells. These results confirmed the regulative role of LATS1 in radiation induced apoptosis and cell cycle change.

4. Discussion

miRNAs are a group of small, conservative and non-coding RNAs degrading or repressing the translation target mRNAs through directly binding to the 3′-UTR [17]. HR-HPV infection directly leads to dysregulated miRNAs expression, which is closely related to cervical cancer development and radioresistance. For example, recent studies observed that the microRNA-218-Survivin axis can regulates migration, invasion, and lymph node metastasis of cervical cancer [18] and miR-181a overexpression leads to increased resistance of cervical cancer to radiation therapy by targeting the pro-apoptotic PRKCD gene [10]. However, the regulative network of miRNAs is quite complex and the current knowledge about its role in radiosensitivity is limited. Significantly higher miR-21 expression and its oncogenic role in HR-HPV cervical cancer were widely

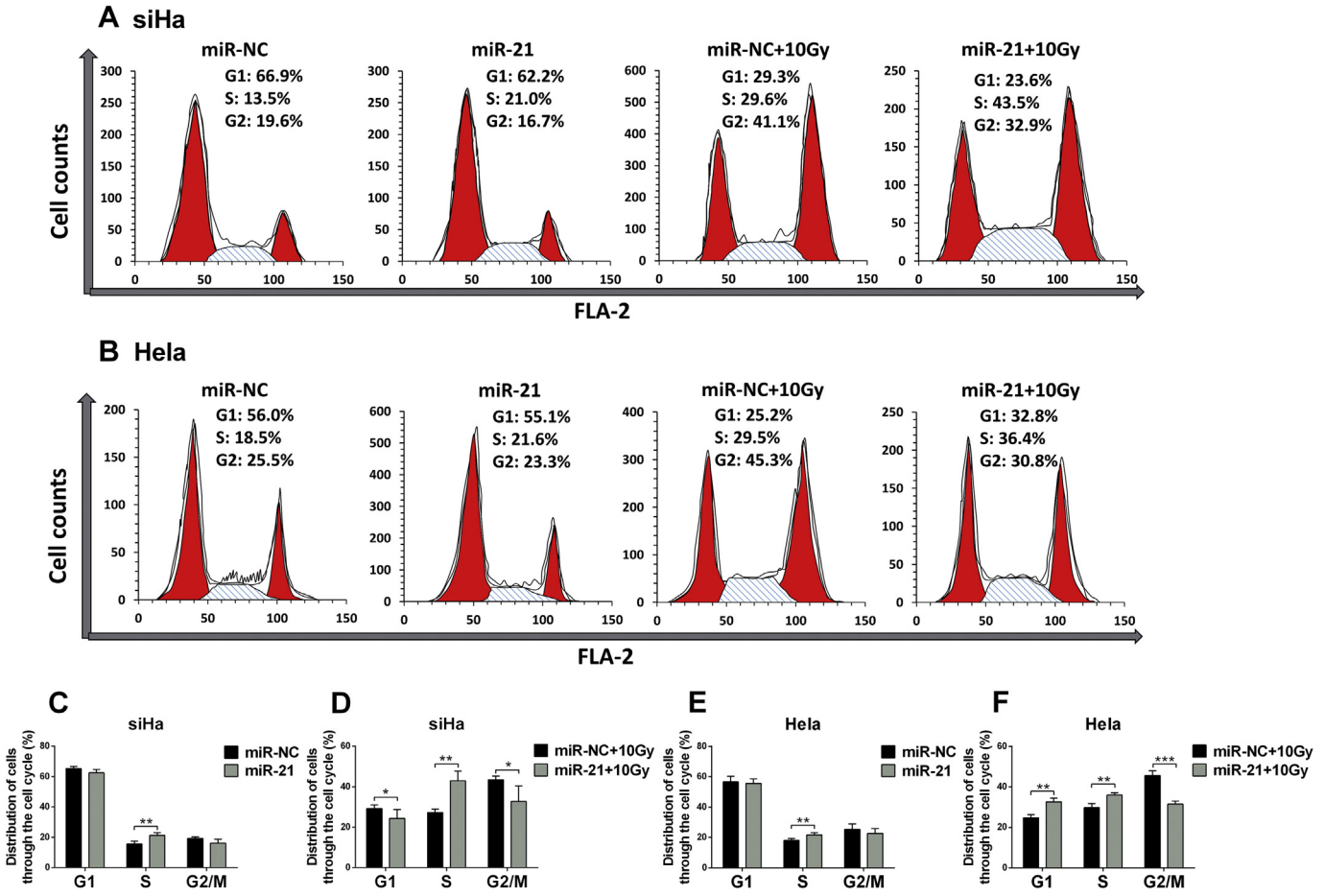

Fig. 3. miR-21 can decrease radiation induced G2/M block and increase S phase accumulation (A and B) Representative images of flow cytometry analysis of cell cycle distribution of siHa and Hela cells. siHa (A) and Hela (B) cells transfected with miR-21 or miR-NC were treated with 10Gy. 48 h after transfection or exposure to ray, DNA content of the cells were measured by FACSCaliber. (C–F) Quantification of siHa cells in different stages of a cell cycle of figure A (C and D) and Hela cells in figure B (E and F). Overexpression of miR-21 inhibited G2/M block and enhanced S phase accumulation. *P < 0.05; **P < 0.01, ***P < 0.001.

reported in previous studies [8,12–14]. In this study, we observed that miR-21 overexpression was associated with higher level of radioresistance in cervical cancer patients and thus decided to further explore its role.

To further explore the regulative mechanism of miR-21 on radioresistance, its influence on cell proliferation and apoptosis under irradiation were studied in-vitro. Based on HR-HPV (+) cervical cell model, this study showed that miR-21 overexpression could increase cell proliferation and decrease apoptosis under radiation treatment. In fact, in the whole cell cycle, cells in different phases have different sensitivity to radiation, which might be a cause of different levels of apoptosis under radiation treatment. Usually, cells are most sensitive to radiation in G2 phase before mitosis and are least sensitive in the mid-to late S and early G1 phases. Therefore, we further studied whether miR-21 overexpression can influence on cell cycle distribution. By examining DNA content with flow cytometry, our results confirmed that miR-21 can decrease radiation induced G2/M block and increase S phase accumulation.

Based on these results, we decided to explore the downstream targets of miR-21 modulating radiosensitivity. Through searching and comparison in online databases (PicTar and TargetScan), we identified a highly potential target LATS1, a kinase which binds with cell cycle controller CDC2 to form a complex in early mitosis and acts negative regulator of CDC2/cyclin A [19]. In the mitotic process, LATS1 plays a critical of cell-cycle progression [19]. LATS1 knockdown cells have significantly reduced G2 checkpoint arrest after DNA damage [20]. In several cancers, LATS1 acts a role as tumor suppressor by inducing cell cycle arrest or apoptosis [21]. In non-small-cell lung cancer, LATS1 can negatively regulate YAP oncoprotein and its higher expression contributes to good prognosis [22]. Overexpression of LATS1 in renal cancer cells inhibited cell proliferation, induced cell apoptosis and cell cycle G1 arrest [23]. In this study, we observed that lower expression of LATS1 was significantly associated with higher level of radioresistance. Thus, we further studied the functional role of LATS1 in radiosensitivity of cervical cancer cells and how its expression is regulated. By using dual luciferase assay, we verified a binding site between miR-21 and 3′-UTR of LATS1. Through this direct binding, miR-21 can regulate LATS1 expression in cervical cancer. Based on functional study, we found LATS1 overexpression could reverse miR-21 induced higher colony formation rate and also reduced miR-21 induced S phase accumulation and G2/M phase reduction under radiation treatment. These results suggested that miR-21-LATS1 axis plays an important role in regulating radiosensitivity.

In conclusion, miR-21 is highly expressed in HR-HPV (+) cervical cancer, while its expression is even higher in radioresistant cases. miR-21 can directly target and downregulate the expression of LATS1 and thereby alleviating radiation induced apoptosis, decreasing G2/M block and increasing S phase accumulation.

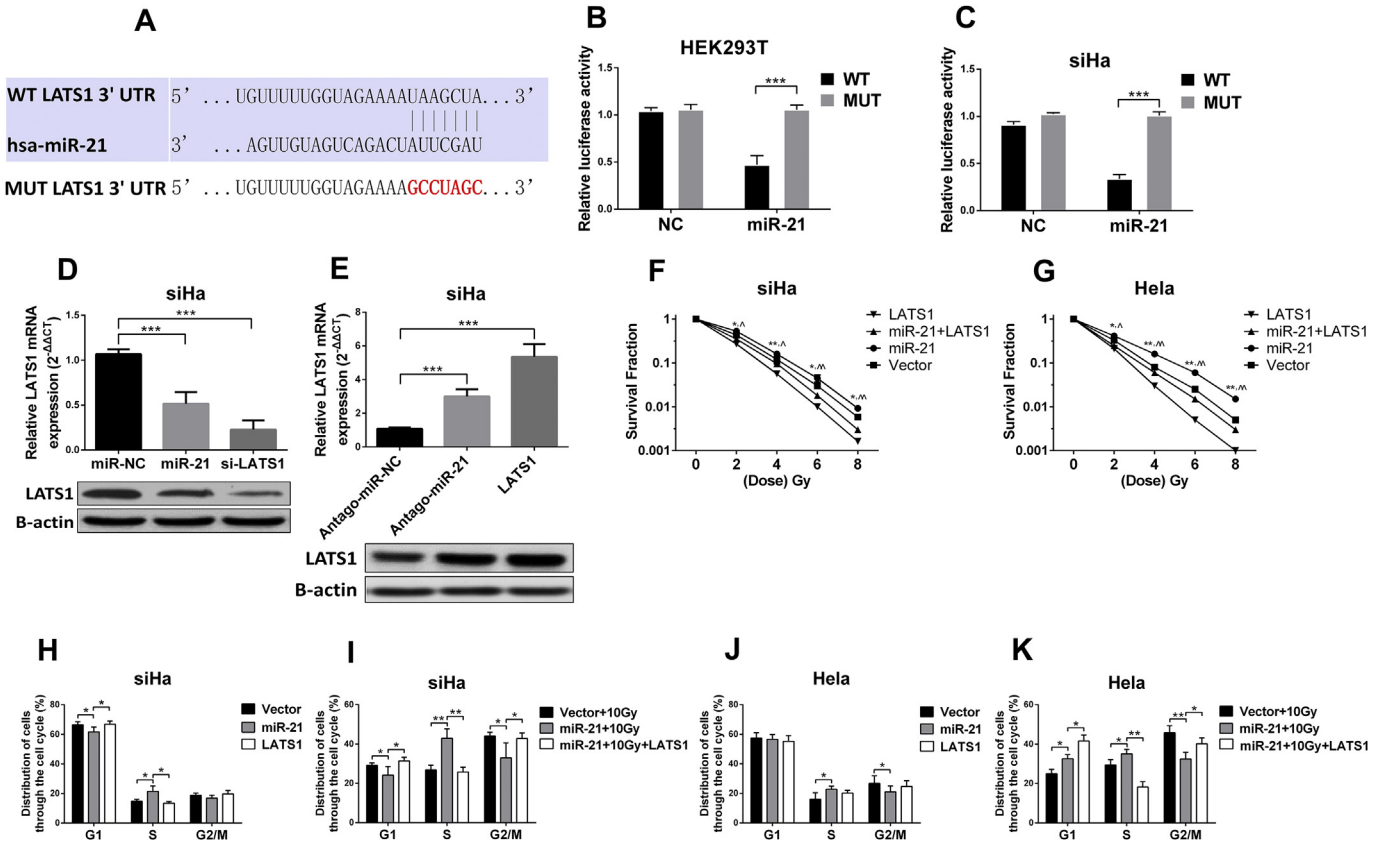

Fig. 4. miR-21 modulates radiosensitivity of HR-HPV (+) cervical cancer cell though directly targeting LATS1 (A) The predicted pairing between miR-21 and 3′-UTR of LATS1. Designed LATS1-mutant (MUT) sequence without miR-21 binding sites were showed. (B and C) 293T cells and siHa cells were co-transfected with either 50 nM miR-21 mimics or NC oligos and 200 ng dual luciferase reporter plasmids carrying either WT or MUT 3′-UTR of LATS1. The relative firefly luciferase activity measured 24 h after transfection and was normalized with Renilla luciferase activity. miR-21 mimics could decrease luciferase activity of WT reporter, but not MUT reporter. (D and E) (D) siHa cells were transfected with 50 nM miR-21 mimics or 50 nM LATS1 siRNA respectively. miR-21, as LATS1 siRNA could significantly inhibited LATS1 expression at both mRNA and protein level. (E) siHa cells were transfected with 100 nM antagomir-21 or lenti-LATS1 construct (without 3′-UTR) respectively. miR-21 knockdown and lenti-LATS1 construct could significantly increase LATS1 expression at both mRNA and protein level. (F and G) Overexpression of LATS1 significantly reduced siHa (F) and Hela (G) cell colony formation under radiation treatment and also reversed miR-21 induced higher colony formation rate. (H–K) Quantification of cell distribution in cell cycle after LATS1 overexpression. LATS1 overexpression reversed miR-21 induced G2/M block reduction and S phase accumulation under radiation treatment in both siHa (H and I) and Hela (J and K) cells. Each bar represents the mean ± S.D. of three experiments. *P < 0.05; **P < 0.01, ***P < 0.001. In figure F and G, "*" indicate significant difference between miR-21 and vector group, while "·" indicate significant difference between miR-21 and miR-21 + LATS1 group.

Conflict of interest

None.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.004.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.004.

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