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Correlation study between long non-coding RNA MALAT1 and radiotherapy efficiency on cervical carcinoma and generation of radiotherapy resistant model of cancer

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Abstract. – OBJECTIVE: This study aims to construct a radiotherapy model on cervical carcinoma cells and to illustrate the correlation between long non-coding RNA (lncRNA) metastasis-associated lung adenocarcinoma transcription 1 (MALAT1) and radiotherapy efficiency.

PATIENTS AND METHODS: A total of 60 cervical carcinoma patients were recruited, and quantitative PCR (qPCR) was employed to detect MALAT1 expression. A dosage-time [illegible] helped to construct radiotherapy resistant [illegible] on cervical carcinoma cell CaSki. Lentivirus [illegible]s-fection was used to silence MALAT1 expres[illegible] followed by quantification of clonal format[illegible] apoptosis, and cycle after combin[illegible]diotherap[illegible] Bioinformatics tool (miRcode [illegible]ter gen[illegible] and qPCR were used to pre[illegible] micr[illegible]A (miR) interaction with MALAT1, [illegible]combini[illegible]MALAT1 silencing, miR over-expres[illegible] and [illegible] effects on the cervic[illegible]anc[illegible]onal r[illegible]a-tion, apoptosis, an[illegible]cle were [illegible]rved.

RESULTS: Co[illegible]ng to radi[illegible]apy sensitive tissues, th[illegible]A[illegible]1 level was [illegible]ificantly elevated in ra[illegible]othera[illegible]esistant tissues (0.52 ± 0.18 *vs* [illegible]29 ± 0.34, [illegible].05). MALAT1 expressio[illegible] cervical carci[illegible] cell CaSki was furth[illegible]evated with elonga[illegible]d radiation time an[illegible]sage ([illegible].05). Comparing to controlled cells, [illegible] silenci[illegible] decreased viable cell percen[illegible]enhanc[illegible]poptosis, increased G1 e ce[illegible]nd d[illegible]ased G2/M ratio. Bioinformi[illegible]s, rep[illegible]ne, and qPCR showed that MA[illegible]T1 exer[illegible]ts roles in cervical carcinoma c[illegible] interacting with miR-143, both of which [illegible] a[illegible]icant correlation (r=0.77, p<0.01). MALAT1 silencing combined with miR-143 plus [illegible]iotherapy decreased viable cell percentage, [illegible]ced apoptosis, increased G1 phase ratio, an[illegible] decreased S or G2/M cells.

CONCLUSIONS: In cervical carcinoma, MALAT1 can interact with miR-143 to modulate tumor cell survival, apoptosis and cell cycle, thus affecting radiotherapy efficiency.

Key Words:
Cervical c[illegible]cinoma, [illegible]AT1, MiR-143, Radiotherapy.

Introduction

[illegible]rvical carc[illegible]ma is the fifth common gyne[illegible] tumo[illegible]d the eighth leading mortality factor [illegible]. Statistics showed about 98,000 newly diagnosed cervical cancer patients in the [illegible]2012, and more than 24,000 people died from [illegible]rent practice, major treatment approaches for cervical cancer include surgery and post-operative chemotherapy or radiotherapy[2]. For those patients at advanced or terminal stage, radio- and chemo-therapy largely improved the prognosis. However, there are still some patients presented uncontrollable or recurrent tumors due to lower radiotherapy sensitivity and worse efficiency[3,4]. Therefore, the investigation of mechanisms underlying recurrence and radiotherapy resistance of cervical cancer, and those indexes correlated with radiotherapy sensitivity, are of critical importance for improving radiotherapy sensitivity and for treating recurrent cancer. As the critical issue for improving survival of cervical cancer patients, they are currently hot topics in tumor research.

Long non-coding RNAs (lncRNAs) are one group with RNA transcripts with more than 200 nt length. Although not coding proteins themselves, lncRNAs can modulate gene expression at multiple levels including epigenetic control, transcriptional regulation, and post-transcriptional regulation[5]. The previous study showed the involvement of lncRNAs in multiple activities including X chromosome silencing, genome imprinting, transcription regulation, and nuclear trafficking modulation. Some scholars suggested[6,7] the rela-

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tionship between lncRNAs and multiple tumors, including lung cancer, liver cancer, colon cancer, prostate cancer, and bladder cancer. In the field of cervical cancer, however, lncRNA related research is still at preliminary stage. Recent studies identified some lncRNAs candidates related with cervical cancer, including HOX transcript antisense RNA (HOTAIR)[8], paroxysmal ventricular tachycardia 1 (PVT1)[9], and XLOC_010588[10]. These reports showed the potential involvement of lncRNA in cervical cancer pathogenesis or progression, and its potency as novel targets for treatment. The previous study showed the expression of MALAT1 in cervical cancer, but leaving its effect on radiotherapy efficiency or related mechanisms unclear. In this work, we constructed a cervical carcinoma cell radiotherapy model to illustrate the correlation between lncRNA metastasis-associated lung adenocarcinoma transcription 1 (MALAT1) and radiotherapy efficiency, along with potential mechanisms.

Patients and Methods

Patients

This study recruited a total of 60 cervical c[illegible]er patients in Jinan Maternal and Child Health Hos[illegible] between 2013 and 2014. All patients had no hist[illegible] of radiotherapy or chemotherapy[illegible] receive[illegible] standard pelvic radiation ba[illegible]on [illegible] National Comprehensive Cancer Ne[illegible]k (NCC[illegible]uideline for cervical cancer in 201[illegible] co[illegible] cisplatin chemothera[illegible]adia[illegible]sitivity was deduced by patholo[illegible]xaminatio[illegible]umor tissues samples at 6 mo[illegible]. This study[illegible]approved by the Ethica[illegible]omm[illegible]of Jinan M[illegible]ternal and Child Hea[illegible]Hospital. A[illegible]rticipants signed the informe[illegible]nsents.

Cell Culture and Treatment

Hu[illegible]ical ca[illegible]cell line CaSki was pur[illegible]ed f[illegible]Cell B[illegible]Shanghai Institute of Cell B[illegible]y, C[illegible]ademy of Science (Shanghai, China). C[illegible]were kept in Roswell Park Memorial Institute-1640 (RPMI-1640) medium and [illegible]e in a 37°C chamber with 5% CO_2 (Thermo Scientific Pierce, Rockford, IL, USA). MALAT1 small interfere RNA (siRNA) and miR-143 over-expression lentiviral vector plus respective controlled vectors were provided by Hanheng Biotech (Shanghai, China). Cells were seeded into 6-well plate for growing to 30% confluence at 24 h before transfection. Viral transfection was performed following the manufacturer's instruction. Real-Time quantitative PCR was performed to confirm the level of MALAT1 and miR-143.

Cell Radiation

In a time-specific assay, cells at lo[illegible] phase were radiated by a linear acc[illegible]tor at 2 Gy intensity. In a second dosage[illegible]ific assay, cells at log-growth phase were radi[illegible]by 2, 3, and 4 Gy radiation. In a cell[illegible]actio[illegible] we used 8 Gy radiation to trea[illegible]s.

Quantitative PCR

Total RNA was [illegible]rac[illegible]izol re[illegible] (Invitrogen/Life Tec[illegible]ogies, C[illegible]d, CA, USA). Reverse transcri[illegible]was perfor[illegible]merScript reverse tra[illegible]t[illegible]T) kit (TaK[illegible]Otsu, Shiga, Japan). For MALAT[illegible]erse transcription was performed [illegible]RT kit (Pro[illegible]Madison, WI, USA). PC[illegible]nditions were: 94°C[illegible] min, followed by 40 c[illegible]s each consisting of 94°C for 40 s, 52°C for 40 s a[illegible]72°C for 40 s[illegible]uantitative PCR was performed u[illegible]SYBR Pre[illegible]Ex Taq (TaKaRa, Otsu, Shiga, Jap[illegible]an AB[illegible]00 cycler (ABI, Foster City, CA, USA). [illegible]t analysis was performed by $2^{-\Delta\Delta Ct}$ approach[12]. Beta-actin was used as the internal reference for MALAT1 quantification. For the miR-143 [illegible]ureLink miRNA Isolation Kit (Invitrogen/Life Technologies, Carlsbad, CA, USA) and TaqMan MicroRNA Assay Kit (PE Gene Applied Biosystems, Foster City, CA, USA) were used for purification and quantification of miR-143. U6 siRNA was employed as the internal reference for miRNA quantification. All PCR primers were designed and synthesized by Sangon Biotech. Co. Ltd. (Shanghai, China). The PCR primers were listed in Table I.

Clonal Formation Assay

Viral transfected cells (1×10^6) were inoculated into 6-well for radiation as described above. Cells were further cultured for 14 days, and clonal formation condition was observed under the microscope. When reaching an appropriate clonal size (>50 cells), 200 µL 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT, 5 mg/ml) was added into each well for staining and followed by overnight incubation. The number of clones in each well was enumerated. Survival percentage = clonal formation number / number of inoculated cells × 100%.

Cell Apoptosis Assay

Cells were inoculated, infected, collected, and radiated as described above. Staining was per-

Table I. Primer sequence.

Gene name	Forward primer (5′-3′)	Reverse primer (5′-3′)
MALAT1	AGGCGTTGTGCGTAGAGGA	GGATTTTTACCAACCACTCGC
β-actin	GTGGCCGAGGACTTTGATTG	CCTGTAACAACGCATCTCATATT
MiR-143	GAAGATGTCCTGCAGCCGTCA	TCTAGGGACAACCTATCTAGTCC
U6	CTCGCTTCGGCAGCACATATACT	ACGCTTCACGAATTTGCGTGTC

formed as below: 2.5 μl Annexin V-FITC and 25 μl propidium iodide (PI) were added into both experimental and control groups. Three controlled groups were simultaneously established, including Annexin V-FITC(-) PI(-), Annexin V-FITC(+) PI(-), Annexin V-FITC(-) PI(+). All reagents were mixed and incubated in the dark under room temperature for 15 min. The cell suspension was mixed, filtered, and loaded onto flow cytometry (FACS Aria III, BD, USA) for assay.

Cell Cycle Assay

Cervical carcinoma cells at log-growth phase were inoculated into 6-well plate at 2×10^5 cells density. 24 h later, cells were transfected with lentivirus. Experimental group, negative control group, and blank control were plotted in triplicates. Trypsin was used to digest and collect cells. The culture medium was discarded, and cells were rinsed in pre-cold phosphate-buffered saline (PBS). 0.25% trypsin was used to digest cells, and 5 ml pre-cold 75% ethanol was added for the mixture, followed by fixation and 800 ×g centrifugation for 5 min. The supernatant was discarded, and cells were washed twice with pre-cold PBS. 1 mg/ml RNaseA dissolved in PBS was added to 0.5 ml. After mixture and 30 min incubation for 30 min, PI dissolved in PBS (50 μg/ml) was added for 30 min dark incubation at room temperature. DNA content was measured by flow cytometry.

Bioinformatics Analysis and Luciferase Reporter Gene Assay

Bioinformatics analysis was performed by miRcode.org following the manual instruction. Luciferase reporter gene assay utilized pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA). The full-length MALAT1 sequence was inserted between NheI and XbaI digestion sites. Within the pmirGLO-MALAT1-WT plasmid, a point mutation was performed in miR-143 binding sites to construct mutant form of the expression vector. Sequencing of plasmid was performed to confirm correct construction. The recombinant expression plasmid and miR-143 mimic RNA were used to co-transfect cervical cancer cells. Various transfection groups were set: pmirGLO-MALAT1-WT+miR-143 group, pmirGLO-MALAT1-WT+NC group, pmirGLO-MALAT1-MUT+miR-143 group, and pmirGLO-MALAT1-MUT + NC group. Each group consisted of triplicated wells. 24 h after transfection, luciferase activity assay kit (Beyotime Biotechnology, Shanghai, China) was performed for measuring luciferase activity.

Statistical Analysis

Statistical analysis was performed by SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Measurement data were presented as mean ± standard deviation (SD). Enumeration data were presented as numbers. Measurement data and enumeration data were analyzed by analysis of variance (ANOVA) and chi-square test, respectively. A statistical difference was identified by $p<0.05$.

Results

Up-Regulation of MALAT1 in Radiotherapy-Resistant Cervical Cancer Patients

We recruited a total of 60 cervical cancer patients, including 31 cases with radiotherapy sensitivity and 29 cases with chemotherapy resistance.

Table II. Clinical features of radiotherapy sensitive and resistant patients.

Features	Radio-sensitive (n=31)	Radio-resistant (n=29)	t-value or χ^2 value	*p*-value
Age (yrs)	38.2±3.3	41.2±4.4	-3.00	0.003
Tumor stage			0.14	0.701
IIB	21	17		
IIA	10	12		

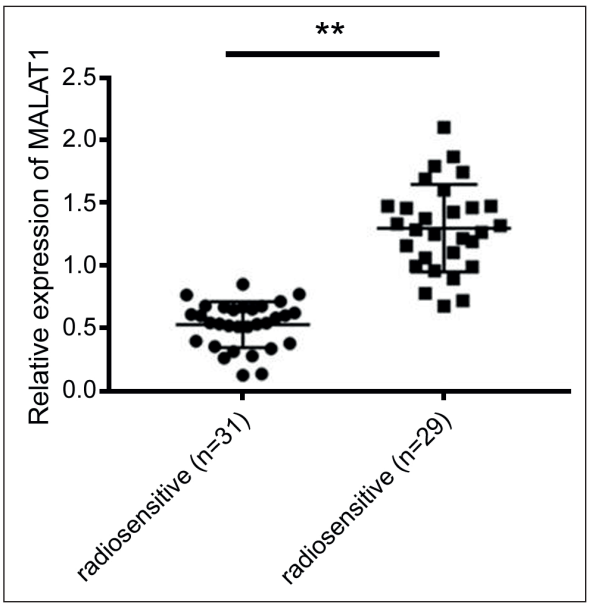


Figure 1. Up-regulation of MALAT1 in radio-resistant cervical cancer patients. qPCR showed that, comparing to radiotherapy sensitive tissues, the MALAT1 level was significantly up-regulated in chemotherapy-resistant tissues (0.52 ± 0.18 *vs.* 1.29 ± 0.34, $p<0.05$).

Basic information of patients was shown i
II. We performed quantitative PCR on ce al
cancer tissues and found that, comparing to ra
therapy sensitive group, the MALAT1 level w
significantly elevated in radioth istant ti
sues (0.52 ± 0.18 *vs.* $1.29 \pm 0.$ <0.0 igure 1).

Up-Regulation of MAL in Radiotherapy Resistant cal Carcinoma Cell

We further human cerv arcinoma cell line CaS co t a radioth apy mod-

el, on which MALAT1 expression level at different time points or dosages was measured. Results showed that when cancer cells received 2Gy radiation, the MALAT1 level was significantly elevated at 4, 8, 12, and 24 h comparing to non-radiated cells ($p<0.05$ for all groups, Figure 2A). At 4 h diation dosage including 2, 3, and 4 G ALAT expression was all significantly ele compared to non-radiated controlled cells ($p<0.$ ure 2B).

MALAT1 Silencing Enh ed Radiotherapy Sensit of C ical Carcinoma Cells

Using lentiviral ct nced M AT1 gene expressio As sh in F e 3A, MALAT1 ex n was dec more than 70%, indic ive MALA encing. We further uti ed MA l-silenced or controlled cervi cinoma ce ith or without radiatio bserve colony fo tion, cell apoptosis, a ell cycle. Our results showed that compared t ntrol cells, LAT1 silenced cervical carci a cells pr nted significantly depressed sur percen e under 2, 4, 6, and 8 Gy radiation for all, Figure 3B). Moreover, comparing to controlled cells, MALAT1-silenced al carcinoma cells presented significantly d apoptosis ($p<0.05$). These cells also showed more apoptosis under 8Gy radiation comparing to those without radiation ($p<0.05$, Figure 3C, D). Moreover, comparing to controlled cells, radiation could cause the elevation of G1 phase cells, and suppression of S or G2/M phase cells ($p<0.05$). MALAT1 silencing plus 8Gy radiation, on the other hand, increased G1 phase ratio, and decreased S or G2/M phase ratio comparing to those with radiation only ($p<0.05$, Figure 3E).

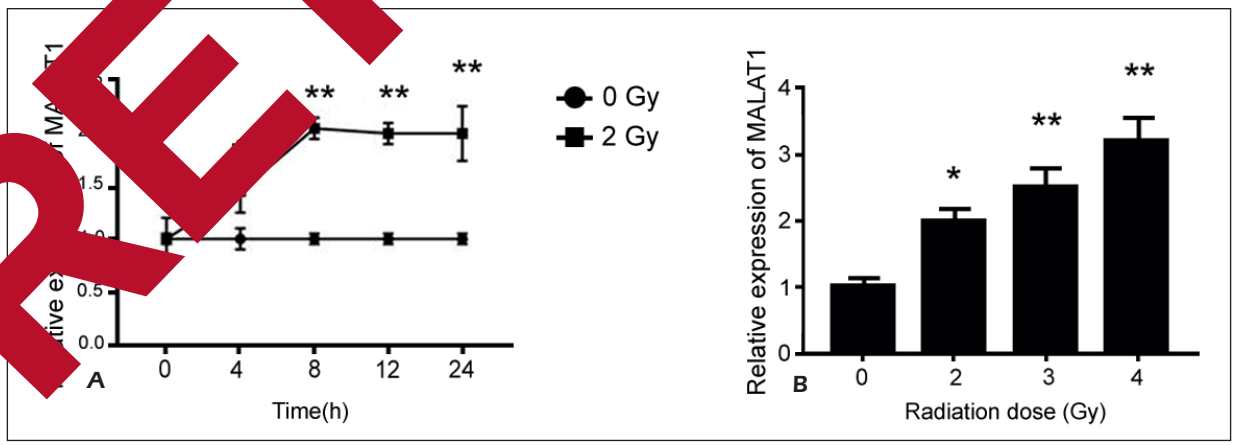
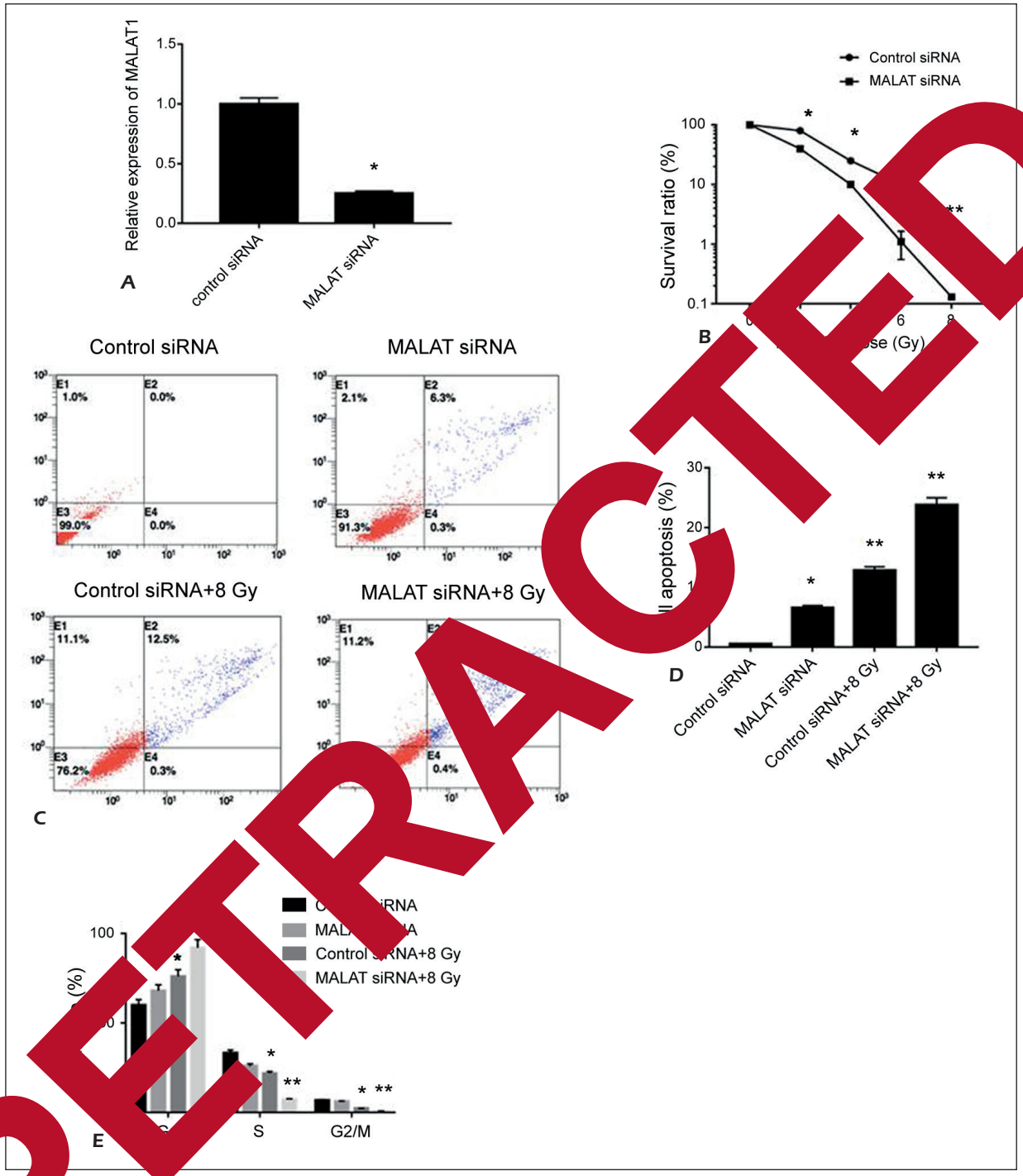


Figure 2. Elevated expression of MALAT1 in cervical cancer cells after radiation. **A**, MALAT1 expression at different time points with equal dosage; **B**, MALAT1 expression under different dosage. *$p<0.05$; **$p<0.01$ comparing to 0 Gy or 0 h time point.



...AT1 silencing enhanced radiotherapy sensitivity of cervical carcinoma cells. **A**, qPCR for MALAT1 silencing ...ciency. **B**, ...urvival percentage of cervical cancer cells by clonal formation assay after MALAT1 silencing. **C**, Flow cytometry ...r apoptosis of cervical carcinoma cells after MALAT1 silencing. **D**, Quantification analysis of cervical carcinoma cell apopto... **E**, Quantification of the cell cycle. *$p<0.05$, **$p<0.05$ comparing to control group.

MALAT1 Exerted its Effects in Cervical Carcinoma Via Interacting With miR-143

We further investigated the mechanism of MALAT1 effect on cervical carcinoma radiotherapy sensitivity. Bioinformatics analysis firstly identified the existence of binding sites between MALAT1 and miR-143 (Figure 4A). We thus measured miR-143 level in those radio-

therapy-sensitive and resistant tissues. Results showed significantly elevated miR-143 expression in radiotherapy-sensitive patients comparing to those radiotherapy resistant patients (5.88 ± 0.40 *vs.* 2.24 ± 0.50, Figure 4B). We further performed a correlation analysis between miR-143 and MALAT1 expression. As shown in Figure 4C, a significant correlation existed between MALAT1 and miR-143 in cervical carcinoma tissues (r=0.77, p<0.01). We further constructed a MALAT1 mutant luciferase reporter gene plasmid along with miR-143 to co-transfect cervical carcinoma cells, to illustrate their interaction inside cells. Results showed that luciferase activity of MALAT1 mutant plasmid was remarkably decreased comparing to those with miR-143 wild-type plasmid transfection (p<0.05, Figure 4D). Moreover, qPCR measured miR-143 expression inside cervical carcinoma cells transfected with MALAT1 siRNA, and found remarkably elevated miR-143 expression in MALAT1 siRNA (p<0.01 comparing to control group, Figure 4E).

MALAT1 Silencing Combined with miR-143 Enhanced Radiotherapy Sensitivity of Cervical Carcinoma Cells

We further performed a clonal formation assay, cell apoptosis, and cell cycle assays to observe the effect of MALAT1 silenc bined with miR-143 on radiotherap sitivity of cervical carcinoma. As show Figure 5, comparing to controlled cells, mi trans-fected cervical carcinoma lls sh sig-nificantly depressed sur percenta n der 8 Gy radiation (p), wh t MAL silencing combined miR remarkably decreased surviva er f cervi car-cinoma (p<0.0 gure More , com-paring to co d cells, m nsfected cervical c lls prese remarkably elevated a optosis 05). MALAT1 silenc-ing c d with m 3 transfected cervi-cal oma cells also sented significantly e nced apoptosis under 8 Gy radiation com-p g to those s with miR-143 transfection o (p<0.01, re 5B). Moreover, compar-

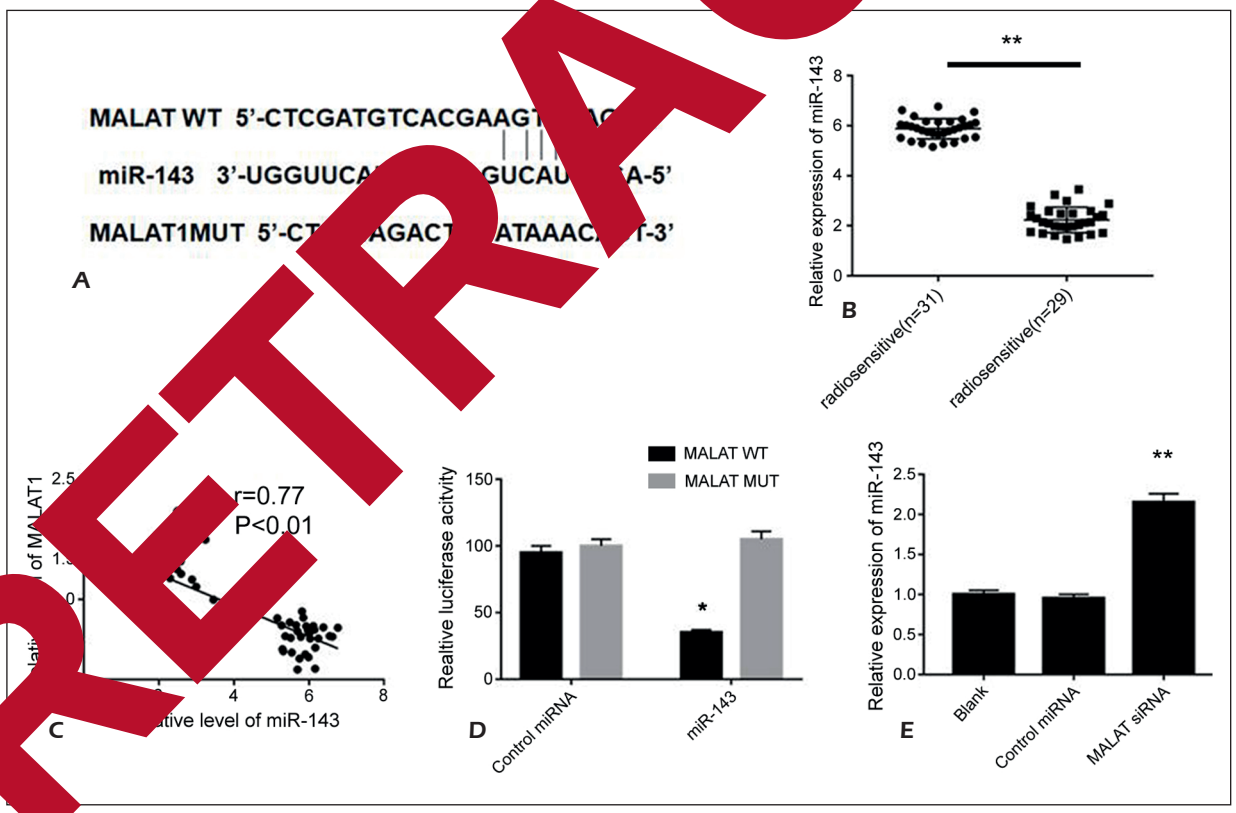


Figure 4. MALAT1 exerted effects via interacting with miR-143 in cervical carcinoma cells. **A**, Bioinformatics predicts interacting sites between MALAT1 and miR-143. **B**, qPCR measured miR-143 expression level in cervical carcinoma tissues. **C**, Correlation analysis between MALAT1 and miR-143 in cervical tissues. **D**, Luciferase reporter gene analysis for the interaction between MALAT1 and miR-143 in cervical cancer. **E**, qPCR for miR-143 expression level in MALAT1 siRNA. *p<0.05, **p<0.01 comparing to control group.

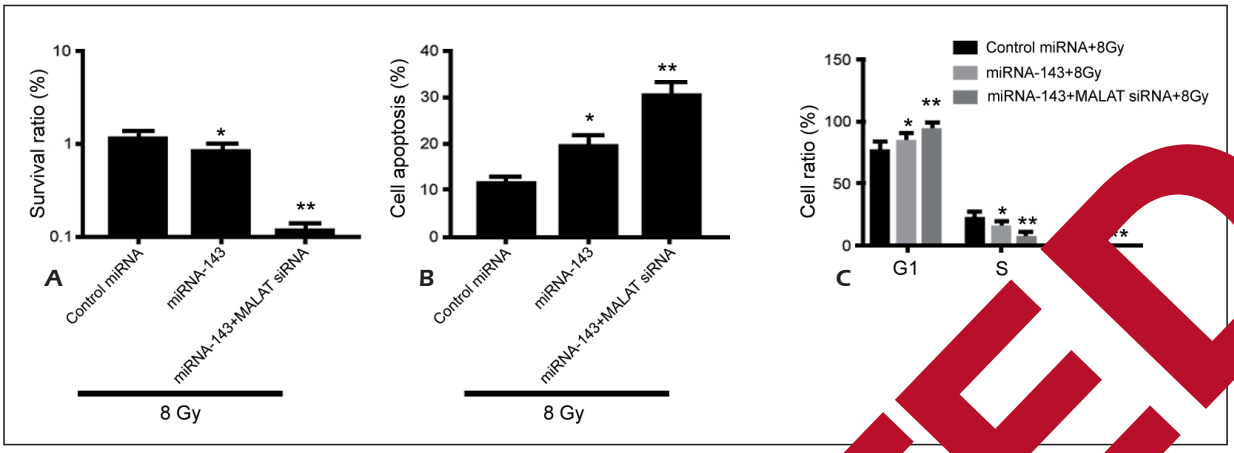


Figure 5. MALAT1 silencing combined with miR-143 enhanced radiotherapy s[e]nsitivity of cer[vi]cal carcinoma cells. **A**, Clonal formation assay for measuring survival percentage of cervical carcinom[a] with MALA[T1] silencing plus miR-143 transfection. **B**, Quantification of apoptosis in MALAT1 silencing combi[ned] with miR-143 transfected cervical carcinoma cells. **C**, Quantification of the cell cycle in cervical carcinoma cell with M[A]LAT1 silencing plus miR-143 transfection. *p<0.05, **p<0.01 comparing to control group.

ing to controlled cells, miR-143 transfection effectively enhanced G1 phase cell ratio, and decreased S or G2/M phase ratios of cervical carcinoma cells (p<0.05). MALAT1 silencing combined with miR-143 transfected cervical carcinoma cells further presented significantly elevated G1 phase cell ratio, plus lower S and G2/M phase cell ratio comparing to single miR-143 transfected cells (p<0.01, Figure 5C).

Discussion

As one lncRNA, MALAT1 was firstly discovered in lung cancer with substrate hyperexpression and is believed to be one predictive index for the survival of stage I pulmonary adenoma or squamous carcinoma patients[13]. Follow-up studies found that MALAT1 is expressed not only in lung cancer but also in other human tumors. For example, in colon cancer, MALAT1 could not only reflect the prognosis, but also bind with the eukaryotic initiation factor 4A (eIF4A) to facilitate tumor growth[14]. Overexpression of MALAT1 also facilitated glioma cell invasion, angiogenesis, and stem cell behaviors. Currently, there are few studies regarding MALAT1 in cervical carcinoma, but most of them stayed in the description of tumor cell gene expression. However, no study has been performed to investigate the role of MALAT1 in cervical cancer and related mechanisms.

In this study, we recruited 60 cervical cancer patients and used qPCR to measure the expression level of lncRNA MALAT1 expression to construct cervical cancer cell radiotherapy model, in order to demonstrate the correlation between lncRNA MALAT1 and radiotherapy efficiency along with possible mechanism. Our results showed that compared to radiotherapy sensitive tissues, the MALAT1 level was significantly elevated in radiotherapy resistant tissues (0.52 ± 0.18 *vs.* 1.29 ± 0.34, p<0.05). The MALAT1 level was elevated in cervical carcinoma cell CaSki with higher radiation duration and dosage (p<0.05). Comparing to controlled cells, MALAT1 silencing in cervical carcinoma cells could decrease cell survival percentage, increase cell apoptosis, potentiate G1 phase cell ratio, and decrease S and G2/M phase cell ratio. Bioinformatics, reporter gene and qPCR results showed that MALAT1 could exert its effects in cervical carcinoma cells via interacting with miR-143, with statistical significance (r=0.77, p<0.01). MALAT1 silencing in combined with miR-143 and radiotherapy significantly depressed cell survival percentage, increased cell apoptosis, increased G1 phase ratio, and decreased S and G2/M phase ratios.

In human genomes, more than 90% region belongs to non-coding sequence, which has been shown to exert important roles in multiple physiological functions of humans. As one representative of the non-coding region, studies showed that about 18% of lncRNA was correlated with human tumor pathogenesis[15]. The current work showed that lncRNA could modulate physiological functions via multiple mechanisms including

cis- and trans-regulation. In trans-regulation, lncRNA could interact with chromatin to form a complex and to recruit it to a specific site of genomic DNA sequence to modulate specific gene transcription[16]. Moreover, some lncRNA molecules can exert trans-regulatory role by affecting transcription of adjacent genes[17]. Increasing evidence showed that lncRNA can bind with miRNA to target specific miRNA, depriving its binding affinity with the target gene. Currently, various lncRNA has been recognized to exert roles via affecting miRNA[18,19], including phosphatase, and tensin homolog pseudogene 1 (PTENP1)[20], H19[21] and colon cancer associated transcript 1 (CCAT1)[22]. This study also supported that MALAT1 exerted its effects via interacting with miRNA-143.

MicroRNA (miRNA) is one group of non-coding RNA with about 22 nt length. miRNA can recognize 3'UTR of a target gene via complete or incomplete binding, thus suppressing protein translation or mRNA stability, thus exerting its roles in regulating protein expression, and modulating various processes including growth, development, differentiation, and death[23]. Larne et al[24] showed down-regulation of miR-143 in c cancer tissues[24]. This study provided an exp a-tion for down-regulation of miR-143 in cer carcinoma, namely, cis-regulation by lncRNA.

Certain weakness also exis ur repo Firstly, relative few patients inc d in this project, thus requiring mo tients t termine if our results were pr con strain of cervical ca cell s employed, thus requiring mo rvical ca a cell lines in future studie firmation. y, we did not measure MA 1 expressio in large-scale assa or clinical les on cervical cancer pati including seru plasma. Therefore still i nclusive to judge the value of M T1 lev r diagnosis.

nclusions

nd that in cervical carcinoma, MALAT1 with miR-143 to affect the efficiency f radiotherapy on cervical carcinoma via modu-g survival percentage, cell apoptosis, and cell cy .

Conflict of Interest

The Authors declare that they have no conflict of interests.

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