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 curve helped to construct radiotherapy resistant model on cervical carcinoma cell CaSki. Lentivirus transfection was used to silence MALAT1 expression, followed by quantification of clonal formation, apoptosis, and cycle after combined radiotherapy. Bioinformatics tool (miRcode.org), reporter gene and qPCR were used to predict microRNA (miR) interaction with MALAT1. By combining MALAT1 silencing, miR over-expression and radiotherapy, effects on the cervical cancer cell clonal formation, apoptosis, and cycle were observed.

RESULTS: Comparing 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). MALAT1 expression in cervical carcinoma cell CaSki was further elevated with elongated radiation time and dosage (p<0.05). Comparing to controlled cells, MALAT1 silencing decreased viable cell percentage, enhanced apoptosis, increased G1 phase cells, and decreased G2/M ratio. Bioinformatics, reporter gene, and qPCR showed that MALAT1 silenced in cervical carcinoma cells via interacting with miR-143, both of which had a significant correlation (r=0.77, p<0.01). MALAT1 silencing combined with miR-143 plus radiotherapy decreased viable cell percentage, enhanced apoptosis, increased G1 phase ratio, and 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 carcinoma, MALAT1, MiR-143, Radiotherapy.

Introduction

Cervical carcinoma is the fifth common gynecology tumor and the eighth leading mortality factor in women1. Statistics showed about 98,000 newly diagnosed cervical cancer patients in the year 2012, and more than 24,000 people died from it. In current practice, major treatment approaches for cervical cancer include surgery and post-operative chemotherapy or radiotherapy2. 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 efficiency3,4. 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 regulation5. 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 suggested6,7 the rela-
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 cancer patients in Jinan Maternal and Child Health Hospital between 2013 and 2014. All patients had no history of radiotherapy or chemotherapy, and have received standard pelvic radiation based on The National Comprehensive Cancer Network (NCCN) guideline for cervical cancer in 2013\(^11\), in conjunction with cisplatin chemotherapy. Radiation sensitivity was deduced by pathology examination of tumor tissues samples at 6 months later. This study was approved by the Ethical Committee of Jinan Maternal and Child Health Hospital. All participants signed the informed consents.

**Cell Culture and Treatment**

Human cervical cancer cell line CaSki was purchased from Cell Bank, Shanghai Institute of Cell Biology, Chinese Academy of Science (Shanghai, China). Cells were kept in Roswell Park Memorial Institute-1640 (RPMI-1640) medium and were incubated in a 37°C chamber with 5% CO\(_2\) (Thermo Scientific Pierce, Rockford, IL, USA). MALAT1 small interference 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 manual 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 log-growth phase were radiated by a linear accelerator at 2 Gy intensity. In a second dosage-specific assay, cells at log-growth phase were radiated by 2, 3, and 4 Gy radiation. In a cell function study, we used 8 Gy radiation to treat cells.

**Quantitative PCR**

Total RNA was extracted by TRizol reagent (Invitrogen/Life Technologies, Carlsbad, CA, USA). Reverse transcription was performed by PrimerScript reverse transcription (RT) kit (TaKaRa, Otsu, Shiga, Japan). For MALAT1, reverse transcription was performed by RT kit (Promega, Madison, WI, USA). PCR conditions were: 94°C for 4 min, followed by 40 cycles each consisting of 94°C for 40 s, 52°C for 40 s and 72°C for 40 s. Quantitative PCR was performed using SYBR Premix Ex Taq (TaKaRa, Otsu, Shiga, Japan) on an ABI 7500 cycler (ABI, Foster City, CA, USA). PCR result analysis was performed by 2\(^-\Delta\Delta C_{t}\) approach\(^12\). Beta-actin was used as the internal reference for MALAT1 quantification. For the miR-143 assay, PureLink 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-
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 4°C fixation and 800 ×g centrifugation for 5 min. The supernatant was discarded, and cells were rinsed twice in pre-cold PBS. 1 mg/ml RNaseA dissolved in PBS was added to 0.5 ml. After mixture and 37°C 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 website 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 Xhol and XbaI digestion sites. Within pmirGLO-MALAT1-WT plasmid, a point mutation was performed on miR-143 binding sites to construct a mutant form of the expression vector. Sequencing of performed to confirm correct construction of plasmid. Reombinant expression plasmid and microRNA 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 dual luciferase activity.

**Statistical Analysis**

All statistical analysis was performed by SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). Measurements 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 chemotherpay resistance.

### Table I. Primer sequence.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
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<tbody>
<tr>
<td>MALAT1</td>
<td>AGGCCTGGTGCGTGAAGAGA</td>
<td>GGATTCTACCAACACCTCGC</td>
</tr>
<tr>
<td>β-actin</td>
<td>GTGCGCCGAGCCTTTGATTG</td>
<td>CCTGCAAACGGCATTCTATT</td>
</tr>
<tr>
<td>MiR-143</td>
<td>GAAGATGTTTCGACGCGCTCA</td>
<td>TCTAGGGCAACCTCTTCTCC</td>
</tr>
<tr>
<td>U6</td>
<td>CTCGCTTCGCAGCACATATCT</td>
<td>ACGCTTCAAGAATTTGCGTGC</td>
</tr>
</tbody>
</table>

### Table II. Critical features of radiotherapy sensitive and resistant patients.

<table>
<thead>
<tr>
<th>Features</th>
<th>Radio-sensitive (n=31)</th>
<th>Radio-resistant (n=29)</th>
<th>t-value or ( \chi^2 ) value</th>
<th>( p )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>38.2±3.3</td>
<td>41.2±4.4</td>
<td>-3.00</td>
<td>0.003</td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IIB</td>
<td>21</td>
<td>17</td>
<td></td>
<td>0.14</td>
</tr>
<tr>
<td>IIA</td>
<td>10</td>
<td>12</td>
<td></td>
<td>0.701</td>
</tr>
</tbody>
</table>
Basic information of patients was shown in Table II. We performed quantitative PCR on cervical cancer tissues and found that, comparing to radiotherapy sensitive group, the MALAT1 level was significantly elevated in radiotherapy resistant tissues (0.52 ± 0.18 vs. 1.29 ± 0.34, \( p < 0.05 \), Figure 1).

**Up-Regulation of MALAT1 in Radiotherapy Resistant Cervical Carcinoma Cells**

We further utilized human cervical carcinoma cell line CaSki to construct a radiotherapy model, 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 2B). At 4 hrs after radiation dosage including 2, 3, and 4 Gy, MALAT1 expression was all significantly elevated compared to non-radiated controlled cells (\( p < 0.05 \), Figure 2B).

**MALAT1 Silencing Enhanced Radiotherapy Sensitivity of Cervical Carcinoma Cells**

Using lentiviral vector, we silenced MALAT1 gene expression. As shown in Figure 3A, MALAT1 expression was decreased more than 70%, indicating effective MALAT1 silencing. We further utilized MALAT1-silenced or controlled cervical carcinoma cells with or without radiation to observe colony formation, cell apoptosis, and cell cycle. Our results showed that compared to control cells, MALAT1 silenced cervical carcinoma cells presented significantly depressed survival percentage under 2, 4, 6, and 8 Gy radiation (\( p < 0.05 \) for all, Figure 3B). Moreover, comparing to controlled cells, MALAT1-silenced cervical carcinoma cells presented significantly enhanced 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).
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 compared 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-transport 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 silencing combined with miR-143 on radiotherapy sensitivity of cervical carcinoma. As shown in Figure 5, comparing to controlled cells, miR-143 transfected cervical carcinoma cells showed significantly depressed survival percentage under 8 Gy radiation (p<0.05), whilst MALAT1 silencing combined with miR-143 remarkably decreased survival percentage of cervical carcinoma (p<0.01, Figure 5A). Moreover, comparing to controlled cells, miR-143 transfected cervical carcinoma cells presented remarkably elevated apoptosis (p<0.05). MALAT1 silencing combined with miR-143 transfected cervical carcinoma cells also presented significantly enhanced apoptosis under 8 Gy radiation comparing to those cells with miR-143 transfection only (p<0.01, Figure 5B). Moreover, compar-

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**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.
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 or G2/M phase cell ratio comparing to single miR-143 transfected cells (p<0.01, Figure 5C).

**Discussion**

As one IncRNA, MALAT1 is firstly discovered in lung cancer by substrate hybridization and is believed to be one predictive index for the survival of stage I pulmonary adenoma or squamous carcinoma patients13. 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 cannot only reflect the prognosis, but can bind with the eukaryotic initiation factor-4A (eIF4A3) to facilitate tumor growth14. Over-expression of MALAT1 also facilitated glioma cell invasion, angiogenesis, and stem cell behaviors13. 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 IncRNA MALAT1 expression to construct cervical cancer cell radiotherapy model, in order to illustrate the correlation between IncRNA 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 IncRNA was correlated with human tumor pathogenesis15. The current work showed that IncRNA 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. Moreover, some lncRNA molecules can exert trans-regulatory role by affecting transcription of adjacent genes. 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, including phosphatase, and tensin homolog pseudogene 1 (PTENP1), H19 and colon cancer associated transcript 1 (CCAT1). 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. Larne et al. showed down-regulation of miR-143 in cervical cancer tissues. This study provided an explanation for down-regulation of miR-143 in cervical carcinoma, namely, cis-regulation by lncRNA.

Certain weakness also existed in our report. Firstly, relative few patients were included in this project, thus requiring more patients to determine if our results were precise. Secondly, only one strain of cervical cancer cell line was employed, thus requiring more cervical carcinoma cell lines in future studies for confirmation. Lastly, we did not measure the MALAT1 expression in large-scale assays for clinical samples on cervical cancer patients including serum or plasma. Therefore, it is still inconclusive to judge the value of MALAT1 level for diagnosis.

Conclusions

We found that in cervical carcinoma, MALAT1 can interact with miR-143 to affect the efficiency of radiotherapy on cervical carcinoma via modulating survival percentage, cell apoptosis, and cell cycle.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References


