

Effects of miRNA-143 and the non-coding RNA MALAT1 on the pathogenesis and metastasis of HeLa cells

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ABSTRACT. Cervical cancer is a common female malignancy of global dimensions. MicroRNAs (miRNAs) play crucial roles in the development, differentiation, proliferation, and apoptosis of tumors. The non-coding RNA MALAT1 participates in various physiological processes that are important for proper functioning of the body. Here, we analyzed the expression of *miRNA-143* and *MALAT1* in HeLa cells to evaluate their roles in the occurrence and metastasis of cervical cancer. HeLa cells were divided into five groups depending on the treatment conditions, namely, transfected with *miRNA-143*, *MALAT1*, *miRNA-143* inhibitor and the *MALAT1* inhibitor, and the

untreated control. Reverse transcription-polymerase chain reaction was used to analyze the expression of *miRNA-143* and *MALAT1*, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to assess proliferation, the trans-well assay to study cell invasion and migration, and western blot to analyze the levels of E-cadherin and vimentin. The proliferation of HeLa cells increased upon treatment with the *miRNA-143* inhibitor and decreased when treated with the *MALAT1* inhibitor, compared to the proliferation of the groups that were transfected with *miRNA-143* and *MALAT1*, respectively ($P < 0.05$). Thus, *miRNA-143* decreased cell invasion and migration potency, downregulated vimentin and upregulated E-cadherin expression, while *MALAT1* had the opposite effects. In conclusion, the low expression of *miRNA-143* and high expression of *MALAT1* in cervical cancer cells could possibly potentiate cell invasion/migration and alter the levels of vimentin and E-cadherin.

Key words: *miRNA-143*; *MALAT1*; Cervical cancer; Cell proliferation; Cell invasion and migration

INTRODUCTION

Cervical cancer is the second-most common malignancy regarding incidence and mortality in women from developing countries that severely affects public health (Huang et al., 2014b). MicroRNAs (miRNAs) are single stranded non-coding RNAs of 19-25 nucleotides that occur in all eukaryotes and negatively regulate gene expression at the post-transcriptional level. miRNAs can regulate the occurrence and progression of tumors similar to oncogenes and tumor suppressor genes (Zhang et al., 2012; Ahmad et al., 2013). Currently, the advancements in sequencing technology has enabled the discovery of the long-chain non-coding RNAs that can modulate both transcriptional and post-transcriptional processes (Gupta et al., 2010). The expression of several non-coding RNAs is altered in malignant tumors, which can therefore be used as diagnostic markers for cancer progression. Among these, *MALAT1* has been shown to be related to the occurrence and progression of various human tumors (Gutschner and Diederichs, 2012). Therefore, we investigated the relationship between the expression of *miRNA-143* and *MALAT1* with the occurrence and metastasis of cervical cancer using HeLa cells as the model system.

MATERIAL AND METHODS

Cells

The human cervical cancer cell line, HeLa, was obtained from the Xinjiang Medical University.

Reagents and equipment

Dulbecco's modified Eagle's medium (DMEM), streptomycin/penicillin, and fetal bovine serum were purchased from Gibco (Waltham, Massachusetts, USA). Forward and

reverse primers were designed and synthesized by the Sangon Biotech. Co. Ltd. (Sangon, Shanghai, China). The lipofectamine TM2000 and the Trizol kit were purchased from Invitrogen (Invitrogen, Waltham, MA, USA). The fluorescent real time quantitative PCR kit was purchased from Sangon Biotech. Co. Ltd. (Sangon, Shanghai, China). Trans-well chambers were purchased from Millipore (Millipore, Billerica, Massachusetts, USA). Mouse anti-human vimentin monoclonal antibody, rabbit anti-human E-cadherin polyclonal antibody, goat anti-rabbit or anti-mouse secondary antibody conjugated to alkaline phosphatase, and mouse anti-human β -actin antibody were purchased from Santa Cruz (Santa Cruz Biotechnology, Dallas, TX, USA). The incubation chamber was purchased from Thermo (Waltham, MA, USA), CO₂ incubator and -80°C refrigerator from Sanyo, inverted microscope from Nikon, PCR cycler from Biometra, and high-speed micro-centrifuge from Beckman (Brea, CA, USA).

Cell culture

HeLa cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium at 37°C in a chamber with 5% CO₂ perfusion.

Vector transfection

Vectors for *miRNA-143*, *MALAT1*, the *miRNA-143* inhibitor, and *MALAT1* inhibitor were synthesized by Sangon Biotech Co. Ltd. as shown in Table 1. Following *EcoRI* digestion of the target gene, the linearized vector fragments were purified from agarose gel and treated with alkaline phosphatase to prevent auto-ligation. Cells at log-phase of growth were seeded in 6-well plates and were divided into the *miRNA-143* transfection group, *MALAT1* transfection group, *miRNA-143* inhibitor transfection group, *MALAT1* inhibitor transfection group, and the control group. Vector transfection was performed with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions.

Table 1. Primer sequence.

Target gene	Sequence (5'-3')
<i>miRNA-143</i>	Forward: TGAAAATGTCCGTGCAAAGTG
	Reverse: TAGGATGATGGGAGTGTGGGA
<i>MALAT1</i>	Forward: AAAGCAAAGTCTCCCCACAAG
	Reverse: GGCTGTGCTAGATCAAAGGC
<i>miRNA-143</i> inhibitor	Forward: CCGGTTGAGCTACAGTGCTTCATCTCATTTTTG
	Reverse: AATCAAAAAATGAGATGAAGCACTGTAGCTCAA
<i>MALAT1</i> inhibitor	Forward: GGCUAAACAUCUAGGGUAATT
	Reverse: UUAACCUAGAUUUAGCCTT
<i>GAPDH</i>	Forward: GACAGTCAGCCGCATCTTCTT
	Reverse: AATCCGTTGACTCCGACCTTC

Reverse transcription quantitative PCR (RT-qPCR)

HeLa cells at log-phase were collected from all the groups and RNA was extracted using the Trizol kit according to the manufacturer's instruction.

A total of 200 ng RNA was used as template for synthesizing cDNA by using polyA tails. PCR amplification was performed by using cDNA as the template and the primers mentioned in Table 1 under the following conditions: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

HeLa cells at the log-phase of growth were counted and inoculated in culture plates for attachment and overnight growth. The cells were first incubated in DMEM medium containing 2% fetal bovine serum for 24 h, followed by addition of DMEM containing 10% FBS. Then, the cells were treated with 20 μ L MTT solution (5 mg/mL) for 4 h, followed by incubation with 150 μ L dimethyl sulfoxide for 10 min and vortexing to dissolve the crystals. Absorbance values at 570 nm were measured.

Trans-well assay

Invasion assay: Matrigel was added in the trans-well chamber and incubated at 4°C overnight, followed by hydration in serum-free medium at 37°C for 1 h. Post-transfection, the cells were cultured in the upper part of the trans-well chamber, the lower part of which was perfused with RPMI1640 medium. Giemsa staining was used for microscopic observation and quantification.

Migration assay: The same procedure as that used in the invasion assay was followed, however, in the absence of the artificial basal membrane after inoculation of the cells into the chamber.

Western blotting

HeLa cells at log-phase were separated on an 8% SDS-PAGE with 40 μ g lysates per well. The proteins were then transferred onto a PVDF membrane (Thermo Fisher Scientific), which had been blocked with 5% BSA (Cell Signaling Technology, Danvers, MA, USA) at room temperature for 1 h. Next, the membrane was incubated with the primary antibody (anti-E-cadherin or Vimentin, all at 1: 200 or 1: 500 for β -actin) for 30 min under warm conditions, followed by overnight incubation at 4°C. TBST (tris-buffered saline and Tween 20, pH 7.5) buffer was used to rinse the membrane, followed by incubation with the secondary antibody (1: 2,000 dilution) for 1 h. Chromogenic substrates A and B were added sequentially, followed by ECL development and imaging. The Quantity One software was used to analyze the optical densities.

Data analysis

The SPSS17.0 software was used for data processing. The enumeration data were analyzed by the chi-square test, while the measurement data were analyzed by the analysis of variance (ANOVA) and were presented as mean \pm standard deviation (SD). A statistical significance was defined when $P < 0.05$.

RESULTS

Analysis of *miRNA-143* and *MALAT1* expression in HeLa cells

We used RT-PCR to quantify the expression of *miRNA-143* and *MALAT1* in HeLa cells. The results showed that the levels of *miRNA-143* were elevated significantly in the *miRNA* transfection group compared to the control group ($P < 0.05$, Table 2). The levels of

MALAT1 were also significantly higher in the *MALAT1* transfection group compared to the control group ($P < 0.05$, Table 2). However, the transfection of inhibitors reduced the levels of miRNA-143 and MALAT1 ($P < 0.05$, Table 2).

Table 2. Expression of *miRNA-143* and *MALAT1* in HeLa cells.

Group	miRNA-143	MALAT1
<i>miRNA-143</i>	3.65 ± 0.82*	1.14 ± 0.17
<i>MALAT1</i>	1.29 ± 0.41	2.89 ± 0.73*
<i>miRNA-143</i> inhibitor	0.56 ± 0.12*	1.13 ± 0.22
<i>MALAT1</i> inhibitor	1.32 ± 0.27	0.35 ± 0.11*
Control	1.31 ± 0.32	1.12 ± 0.25

* $P < 0.05$ compared to the control group.

Proliferation of HeLa cells under different transfection conditions

The MTT assay was used to test the proliferation of cells in all the groups. With longer incubation time, the proliferation of cells in the *miRNA-143* and the *MALAT1*-inhibitor- transfected group gradually decreased ($P < 0.05$, Table 3), while those of the cells transfected with *MALAT1* or the *miRNA-143* inhibitor progressively increased ($P < 0.05$, Table 3), compared to the proliferation of cells in the control group. Similarly, compared to the proliferation of cells in the control group, HeLa cells transfected with *MALAT1* or the *miRNA-143* inhibitor had elevated proliferation while those transfected with *miRNA-143* or the *MALAT1* inhibitor had lower proliferation ($P < 0.05$, Table 3).

Table 3. Proliferation of HeLa cells under different transfection conditions.

Group	24 h	48 h	72 h
<i>miRNA-143</i>	0.189 ± 0.242*	0.153 ± 0.178*	0.125 ± 0.112*
<i>MALAT1</i>	0.517 ± 0.383*	0.712 ± 0.423*#	0.897 ± 0.621*# ^Δ
<i>miRNA-143</i> inhibitor	0.525 ± 0.352*	0.753 ± 0.445*#	0.973 ± 0.551*# ^Δ
<i>MALAT1</i> inhibitor	0.196 ± 0.233*	0.142 ± 0.168*#	0.101 ± 0.098*# ^Δ
Control	0.235 ± 0.215	0.287 ± 0.216	0.296 ± 0.221

* $P < 0.05$ compared to the control group; # $P < 0.05$ compared to the 24 h group; ^Δ $P < 0.05$ compared to the 48 h group.

Analysis of cell invasion and migration under different transfection conditions

The trans-well assay was used to detect the invasion and migration of HeLa cells. The results indicated that the invasion and migration of HeLa cells were significantly increased in the *miRNA-143* inhibitor-transfected group compared to *miRNA-143*-transfected group (Figure 1). However, the invasion and migration abilities of HeLa cells were significantly decreased in the *MALAT1* inhibitor-transfected group (Figure 1).

Expression of E-cadherin and vimentin in HeLa cells transfected with *miR-143*, *MALAT1*, and their inhibitors

Western blotting was used to quantify the levels of E-cadherin and vimentin in HeLa cells. The results showed that vimentin was elevated in the *miRNA-143* inhibitor-transfected group compared to the levels in the *miRNA-143* transfected- group; in contrast, it was lower in the *MALAT1* inhibitor-treated group compared to the levels in the *MALAT1*-transfected

group ($P < 0.05$, Figure 2 and Table 4). E-cadherin level was decreased in the *miRNA-143* inhibitor-transfected group compared to the levels of the *miRNA-143*-transfected group; the levels were increased in the *MALAT1* inhibitor-treated group compared to the levels of the *MALAT1*-transfected group ($P < 0.05$, Figure 2 and Table 4).

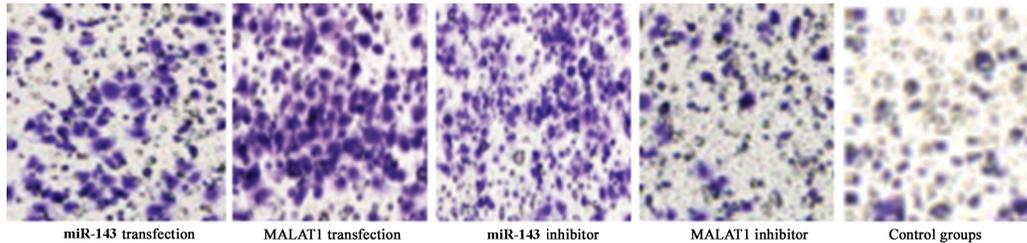


Figure 1. Analysis of HeLa cell invasion and migration under various transfection conditions. Matrigel was added in the trans-well chamber and incubated at 4°C overnight, followed by hydration for 1 h. Post-transfection. Then the cells were cultured in the upper part of the trans-well chamber, the lower part of which was perfused with RPMI1640 medium. Giemsa staining was used for microscopic observation and quantification of the cell numbers.

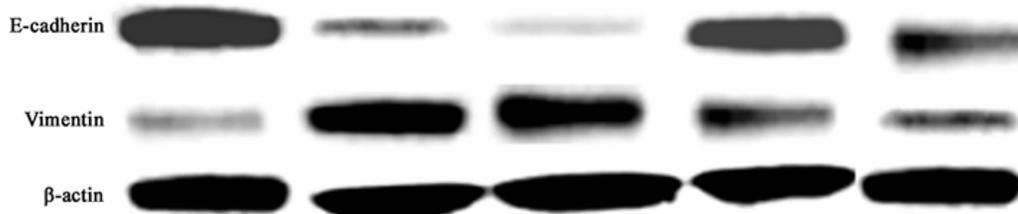


Figure 2. Analysis of E-cadherin and vimentin levels by western blot. Loading order from left to right: lysates of HeLa cells (20 μ L) transfected with *miRNA-143*, *MALAT1*, *miRNA-143* inhibitor, *MALAT1* inhibitor, and control transfected cells. β -Actin was used as a loading control. The molecular weight of E-cadherin and vimentin were 135 and 54 kDa respectively. The name of the protein ladder was Precision Plus Protein™ (Bio-Rad, Hercules, CA, USA).

Table 4. Analysis of levels of E-cadherin and Vimentin in HeLa cells under different transfection conditions.

Group	E-cadherin	Vimentin
<i>miRNA-143</i>	3.57 \pm 1.63*	0.86 \pm 0.08*
<i>MALAT1</i>	1.06 \pm 0.11*	2.15 \pm 1.05*
<i>miRNA-143</i> inhibitor	1.02 \pm 0.12*	2.34 \pm 1.16*
<i>MALAT1</i> inhibitor	3.78 \pm 1.75*	0.78 \pm 0.07*
Control	1.38 \pm 0.22	1.26 \pm 0.18

* $P < 0.05$ compared to the control group.

DISCUSSION

With the development of cervical cell screening, both the incidence and mortality associated with cervical cancer have decreased. However, the treatment options for this disease have not improved significantly. Therefore, understanding of the molecular mechanisms underlying the occurrence, progression, and metastasis of cervical cancer is important (Banno

et al., 2014; Huang et al., 2014a). The proliferation and migration of tumor cells are major aspects of malignant transformation (Yasui et al., 2013). Metastasis is a multi-step complex process that involves tumor invasion of adjacent tissues, dissemination of metastatic cancerous cells into the blood circulation, extravasation from the blood vessels, colonization at peripheral tissues and further proliferation (Gupta and Massagué, 2006). miRNAs participate in various biological processes and regulate gene expression in a wide spectrum of diseases (Calin et al., 2004), including cancer, where they regulate the occurrence and progression of tumors similar to oncogenes and tumor suppressor genes (Zhang et al., 2010). Results from a previous study implied that miR-143 might facilitate tumor cell apoptosis (Calin et al., 2004). MALAT1 was first discovered in non-small cell lung cancer. As a long non-coding RNA, it is expressed in various tumors and tissues, and may affect proliferation, apoptosis, invasion, metastasis, and drug resistance of tumors (Gutschner et al., 2013a).

Here, we selected the human cervical cancer line, HeLa, as the model system. After normal incubation, the cells were transfected with *miRNA-143*, *MALAT1*, the *miRNA-143* inhibitor, and the *MALAT1* inhibitor and we found elevated levels of miRNA-143 and MALAT1 in the cells transfected with the respective RNA-encoding genes, whereas the levels of miRNA-143 and MALAT1 were low in the respective inhibitor-transfected groups. These results suggested that the expression of *miRNA-143* and *MALAT1* was successfully manipulated. Further, the MTT assay showed that with longer time of incubation, the proliferation of cells with *miRNA-143* or *MALAT1* inhibitor gradually decreased ($P < 0.05$), while that of cells transfected with *MALAT1* or the *miRNA-143* inhibitor progressively increased ($P < 0.05$). For the same duration of incubation, HeLa cells transfected with *MALAT1* or the *miRNA-143* inhibitor had elevated proliferative activity, and those transfected with *miRNA-143* or the *MALAT1* inhibitor showed lower proliferation, compared to that of the control transfected cells. These results suggested that the high levels of miRNA-143 and/or low levels of MALAT1 decreased the proliferation of HeLa cells, while low levels of miRNA-143 and high levels of MALAT1 might facilitate proliferation, consistent with previous studies revealing the negative regulation of miRNA-143 in cell proliferation (Li et al., 2011; Liu et al., 2016). Lajer et al. (2012) suggested that miRNA-143 might participate in the pathogenesis of the disease in a way that is similar to that observed for human papilloma virus infection in patients with cervical cancer. Wang et al. (2008) found lower levels of miRNA-143 in cervical cancer tissues, while the proliferation of HeLa cells was significantly inhibited by *miRNA-143* over-expression. MALAT1 has also been shown to regulate the metastatic ability of tumor cells, and to facilitate proliferation and migration of cells (Boardman, 2009). All of the above findings are consistent with our results.

The trans-well method was used to analyze the invasiveness and migratory ability of the cells under various treatment conditions. The results showed significantly high invasive/migration potency of HeLa cells transfected with *MALAT1* or the *miRNA-143* inhibitor. A previous study (Chen et al., 2009) showed that over-expression of *miRNA-143* could inhibit cell invasion. In another study, the *MALAT1* sequence was infused into mice pulmonary carcinoma and the tumor growth and metastasis was inhibited (Gutschner et al., 2013b). Compared to primary tumors, the level of MALAT1 was further elevated in metastatic tumors, suggesting a correlation between the levels of MALAT1 and malignant tumors (Ren et al., 2016).

During the epithelial-mesenchymal transition (EMT), malignant tumor cells acquire the morphological features of fibroblasts. The salient features of EMT are the downregulation of epithelial markers and upregulation of mesenchymal markers via different signal transduction

pathways, which causes degradation of the extracellular matrix (ECM), thereby promoting infiltration by tumor cells and distal metastasis of tumors (Fu et al., 2011). E-cadherin, an epithelial marker, is a Ca^{2+} -dependent intracellular transmembrane glycoprotein adhesion molecule found in epithelial tissues, which participates in establishing cellular connections. Vimentin is a cytoskeleton component and a biomarker for cells of mesenchymal origin. Abnormal expression of vimentin may induce alteration of the cytoskeleton that may facilitate the migration of metastatic cells (Matsuoka et al., 2013). We found elevated levels of vimentin and reduced levels of E-cadherin in *MALAT1* and *miRNA-143* inhibitor-transfected cells, and the reverse in *miRNA-143* and *MALAT1* inhibitor-transfected cells. These results suggested that low expression of *miRNA-143* and high expression of *MALAT1* could regulate the expression of vimentin and E-cadherin. A previous study (Ying et al., 2012) showed that *MALAT1* upregulates the expression of E-cadherin and β -catenin, facilitates mesenchymal transition of epithelial cells, and regulates proliferation and metastasis of colorectal carcinoma. Our results are consistent with the conclusions of Ying et al. (2012).

Although the present study revealed certain interesting findings, it has few limitations. The Giemsa staining method used in the trans-well assay is not optimal as it requires use of fixed cells, and the processes of decolorization and staining affects the cells on the Matrigel. Therefore, in subsequent studies, we would use the crystal violet staining method, which might circumvent the problems that were caused by Giemsa staining. In addition, due to only one cervical cancer cell line used in the present study, we plan to investigate the role of *miRNA-143* and *MALAT1* in other cervical cancer cell lines to confirm the findings obtained from the current study.

In summary, low levels of *miRNA-143* and high levels of *MALAT1* could potentiate proliferation of the cervical cancer cell line HeLa, and accelerate its invasion and migration via enhancement of vimentin and reduction of E-cadherin levels, respectively. Thus, *miRNA-143* and *MALAT1* may work as novel markers for metastasis of cervical cancer, although further investigations are required to understand its detailed mechanism.

Conflicts of interest

The authors declare no conflict of interest.

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