

LINC01204 Negatively Regulates the Effect of MiR-214 on Lung Cancer Cell Apoptosis, Migration, Invasion and Radiosensitivity

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ABSTRACT

► Original article

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Keywords: LncRNA LINC01204, miR-214; lung cancer, apoptosis, migration, invasion, radiosensitivity.

Background: To investigate whether LncRNA LINC01204 affects lung cancer cell apoptosis, migration, invasion and radiosensitivity by regulating miR-214. **Materials and Methods:** Normal lung cells HBE and lung cancer cells A549 were cultured *in-vitro*, and pcDNA3.1, pcDNA3.1-LINC01204, pcDNA3.1-LINC01204 and miR-NC, pcDNA3.1-LINC01204 and miR-214 mimics were transfected into A549 cells, respectively. qRT-PCR, flow cytometry, scratch test, and Transwell chamber test, clone formation test, dual luciferase, and Western blot methods were used in this study. **Results:** Compared with HBE cells, A549 cells had significantly reduced LINC01204 expression level ($P<0.05$), and significantly increased miR-214 expression level ($P<0.05$); transfection of pcDNA3.1-LINC01204 could significantly increase apoptosis rate and Cleaved-caspase3, E-cadherin protein levels ($P<0.05$), reduce migration healing rate, N-cadherin protein levels and cell survival fraction ($P<0.05$) with sensitization enhancement ratio SER at 1.950, reduce the number of invasive cells ($P<0.05$). Dual luciferase report experiments confirmed that LINC01204 can bind to miR-214 in a targeted way; co-transfection of pcDNA3.1-LINC01204 and miR-214 mimics can significantly reduce the apoptosis rate and Cleaved-caspase3 and E-cadherin protein levels ($P<0.05$), improve the migration healing rate, N-cadherin protein level and cell survival fraction ($P<0.05$) with sensitization enhancement ratio SER at 0.728, increase the number of invasive cells ($P<0.05$). **Conclusion:** LINC01204 overexpression can negatively regulate miR-214 expression to promote lung cancer cell apoptosis and inhibit migration and invasion, thereby enhancing radiosensitivity.

INTRODUCTION

Lung cancer, a common malignant tumor in clinical practice, shows increasing morbidity and mortality year by year. Its main pathological types are non-small cell lung cancer and small cell lung cancer. Clinical treatment of lung cancer has achieved some progress, but patient prognosis is still very poor. Due to unobvious symptoms of lung cancer in the early stage, patients are already in the middle and advanced stage at the time of diagnosis. At this time, the tumor cells have strong metastasis ability, so patients lose the best treatment opportunity. In addition, the use of radiotherapy to treat patients with advanced lung cancer produces certain therapeutic effect, but patients have been found to have low radiation sensitivity⁽¹⁾. At present, the molecular mechanism of lung cancer occurrence and metastasis has not been clarified. Long non-coding RNA (LncRNA) can regulate RNA transcription, splicing, translation processes, etc., thereby affecting cell proliferation and metastasis, which can also play an important regulatory role in the occurrence and development of lung cancer⁽²⁻⁵⁾. Long non-coding

RNA LINC01204 (LncRNA LINC01204) has reduced expression level in bladder cancer and can inhibit cell proliferation, migration and invasion⁽⁶⁾. However, the effect of LINC01204 on the biological behavior of lung cancer cells and its possible mechanism of action remain unknown. Target gene prediction indicates binding site between microRNA-214 (miR-214) and LINC01204. MiR-214 with elevated expression level in non-small cell lung cancer can participate in cell erlotinib resistance⁽⁷⁾. However, whether LINC01204 can affect the apoptosis, migration, invasion and radiosensitivity of lung cancer cells by regulating miR-214 expression is not yet known. Therefore, this study mainly investigated the effects of LINC01204 on lung cancer cell apoptosis, migration, invasion and radiosensitivity, and explores its targeted regulation of miR-214.

MATERIALS AND METHODS

Lung cancer cells (A549) and normal lung cells (HBE) were purchased from ATCC cell bank, USA; DMEM medium was purchased from Shanghai USEN

Biological Technology Co., Ltd., China; Fetal bovine serum was purchased from Nanjing BioChannel Biotechnology Co., Ltd., China; Lipofectamine 2000 was purchased from Shanghai Huiying Biological Technology Co., Ltd., China; pcDNA3.1, pcDNA3.1-LINC01204 were purchased from Shanghai Solarbio Bioscience & Technology Co., Ltd.; miR-NC, miR-214 mimics were purchased from Guangzhou RiboBio Co., Ltd.; Trizol reagent was purchased from Invitrogen, USA; cDNA synthesis and qRT-PCR reagents were purchased from Tiangen Biochemical Technology (Beijing) Co., Ltd.; Annexin V-FITC/PI double staining apoptosis detection kit was purchased from Shanghai Ruichu Biotech Co., Ltd.; Transwell chamber and Matrigel were purchased from Shanghai Zemai Biotechnology Co., Ltd.; rabbit anti-human Cleaved-caspase3, E-cadherin, and N-cadherin antibodies were purchased from Santa Cruz, USA; HRP-labeled goat anti-rabbit IgG secondary antibody was purchased from Abcam, USA.

Treatments method

A549 cells were routinely cultured. After growing to 80% confluence, the cells were digested with 0.25% trypsin, added with culture medium to prepare cell suspension (2.5×10^5 cells/mL), seeded in a 96-well plate (100 μ L/well). pcDNA3.1, pcDNA3.1-LINC01204, pcDNA3.1-LINC01204 and miR-NC, pcDNA3.1-LINC01204 and miR-214 mimics were transfected into A549 cells, which were respectively marked as pcDNA3.1 group, pcDNA3.1-LINC01204 group, pcDNA3.1-LINC01204+miR-NC group, pcDNA3.1-LINC01204+miR-214 group.

qRT-PCR detection of LINC01204 and miR-214 expression levels in cells

The total RNA of HBE, A549 cells and A549 cells of each group after transfection was extracted by Trizol method, and the RNA concentration was detected. The total RNA was reverse transcribed into cDNA with reference to the cDNA kit instruction, and the cDNA was used as a template to detect relative expression level of LINC01204 and miR by qRT-PCR - 214, as shown in table 1.

Table 1. LINC01204 primer targets.

Gene	Primer
WT-LINC01204	5' UCUGAAAUGGGAGAGACAGGCA 3'
miR-214	3' UCG...UUCACA.....UCUGUCCGU 5'
MUT-LINC01204	5' UCUGAAAUGGGAGGCCAGAAGC 3'

Flow cytometry detection of cell apoptosis rate

Wash the A549 cells of each group with pre-cooled PBS, discard the supernatant, add 500 μ L binding buffer to the cell sediment to resuspend the cells, add 5 μ L Annexin V-FITC and 5 μ L PI, incubate with shaking at room temperature for 10 min, then use FACS Calibur Flow cytometry to detect the cell apoptosis rate of each group.

Scratch test

A549 cells from each group were seeded in a 6-well plate (2.5×10^5 cells/mL). After scribing a line along the horizontal direction (center) at the bottom of the culture plate, continue to culture for 48 h, observe the cell growth at the scratch, and calculate healing rate of the scratch (%) = (0 h scratch width-48 h scratch width) / 0 h scratch width \times 100%.

Transwell assay of cell invasion

Use pre-cooled culture medium to dilute Matrigel and add it to the upper chamber (40 μ L/chamber), place it in the incubator for 5 h further incubation, then add A549 cells (3×10^4 cells/well) of each group, add culture medium (600 μ L/chamber) containing fetal bovine serum in the lower chamber for 24 h further culture, fix with paraformaldehyde for 20 min, stain with 0.1% crystal violet staining solution for 10 min, and observe the number of invasive cells.

Clone formation experiment

Logarithmic growth phase A549 cells (2.5×10^5 cells/mL) were seeded in a 6-well plate (200 μ L/well). After grouping according to "1.2.1", place them in an incubator for 24 h further culture. Irradiate the cells at different irradiation doses of 0 and 2, 4, 6, 8Gy. Adjust the source target distance (100cm), irradiation field (10cm \times 10cm), dose rate (5Gy/min) during irradiation, and observe the cells under the microscope. Terminate culture if cell colony appears, wash with PBS, fix with methanol for 20 min, and stain with Giemsa for 40 min. observe the number of effective clones and calculate the cell survival fraction (irradiated cell clone formation rate/control cell clone formation rate).

Dual luciferase reporter gene assay of targeting relationship between LINC01204 and miR-214

LncBase v.2 prediction indicated binding sites between LINC01204 and miR-214. The wild-type vector WT-LINC01204 and the mutant vector MUT-LINC01204 were constructed respectively, and miR-NC, miR-214 mimics were respectively co-transfected with WT-LINC01204, MUT-LINC01204 into A549 cells and cultured for 24 h to detect the relative luciferase activity of each group.

Western blot detection of Cleaved-caspase3, E-cadherin, N-cadherin protein expression

Add appropriate amount of RIPA lysate to A549 cells to extract total cell protein. Determine protein concentration by BCA method and take protein sample for SDS-PAGE electrophoresis, followed by membrane transfer, closure. Incubate the primary antibody diluent (1:1000) for 24 h and then incubate the secondary antibody diluent (1:2000), add ECL after incubation at room temperature for 1 h, expose and develop in a dark room, and analyze the gray value of each band using ImageJ software.

Statistical analysis

Preliminary data from real-time PCR were analyzed using the Threshold Cycle Comparison (Ct) method. SPSS21.0 statistical software was used to analyze the data. The measurement data were expressed as ($\bar{x} \pm s$) and all conform to normal distribution. Independent sample *t* test was used for comparison between two groups, one-way analysis of variance was used for comparison among multiple groups. $P < 0.05$ indicates statistically significant difference.

RESULTS

Expression of LINC01204 and miR-214

Compared with HBE cells, A549 cells have significantly reduced LINC01204 expression level (0.97 ± 0.06 in HBE vs. 0.20 ± 0.02 in A549) ($P < 0.05$), and significantly increased miR-214 expression level (0.20 ± 0.02 in HBE vs. 3.68 ± 0.09 in A549) ($P < 0.05$), as shown in table 2.

Effect of LINC01204 overexpression on A549 apoptosis, migration and invasion

Compared with pcDNA3.1 group, pcDNA3.1-

LINC01204 group has significantly increased apoptosis rate ($P < 0.05$), significantly reduced migration healing rate ($P < 0.05$), significantly reduced number of invasive cells ($P < 0.05$), significantly increased Cleaved-caspase3, E-cadherin protein level ($P < 0.05$), significantly decreased N-cadherin protein level ($P < 0.05$), as shown in figure 1 and table 3.

Effect of LINC01204 overexpression on the radiosensitivity of A549

Compared with pcDNA3.1 group, pcDNA3.1-LINC01204 group has significantly reduced cell survival fraction ($P < 0.05$), with sensitization enhancement ratio SER at 1.950, as shown in figure 2 and table 4.

LINC01204 targets miR-214

LncBase v.2 prediction reveals binding sites between LINC01204 and miR-214, as shown in figure 3. Overexpression of miR-214 can reduce luciferase activity of WT-LINC01204 ($P < 0.05$), but has no significant effect on luciferase activity of MUT-LINC01204 ($P > 0.05$), as shown in table 5.

Table 2. Expression of LINC01204 and miR-214 in HBE and A549 (ΔCt , $\bar{x} \pm s$, $n=3$).

Group	LINC01204	miR-214
HBE	0.97 ± 0.06	0.95 ± 0.05
A549	$0.20 \pm 0.02^*$	$3.68 \pm 0.09^*$
<i>t</i>	21.087	45.927
<i>P</i>	0.000	0.000

Note: Compared with HBE group, $*P < 0.05$.

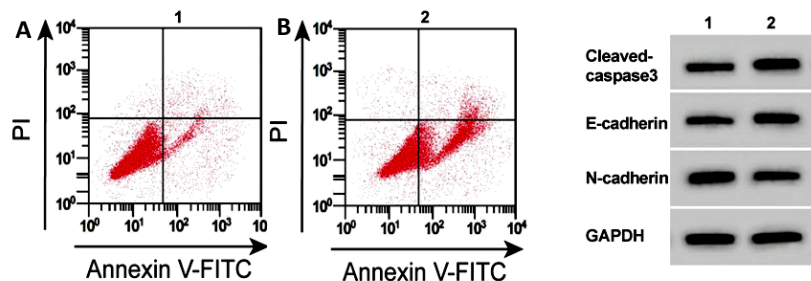


Figure 1. Effect of LINC01204 overexpression on A549 apoptosis (A) and protein expression (B) 1: pcDNA3.1; 2: pcDNA3.1-LINC01204.

Table 3. Effect of LINC01204 overexpression on A549 apoptosis, migration and invasion ($\bar{x} \pm s$, $n=3$).

Group	LINC01204	miR-214	Apoptosis rate (%)	Migration healing rate (%)	Number of invasive cells (pcs)	Cleaved-caspase3	E-cadherin	N-cadherin
pcDNA3.1	0.97 ± 0.03	0.98 ± 0.04	7.85 ± 0.33	61.07 ± 1.31	157.67 ± 3.86	0.23 ± 0.01	0.14 ± 0.01	0.71 ± 0.04
pcDNA3.1-LINC01204	$4.24 \pm 0.06^*$	$0.20 \pm 0.01^*$	$23.67 \pm 0.58^*$	$32.93 \pm 0.62^*$	$71.33 \pm 1.70^*$	$0.70 \pm 0.05^*$	$0.53 \pm 0.03^*$	$0.21 \pm 0.01^*$
<i>t</i>	84.431	32.767	41.062	33.630	35.456	15.965	21.361	21.004
<i>P</i>	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

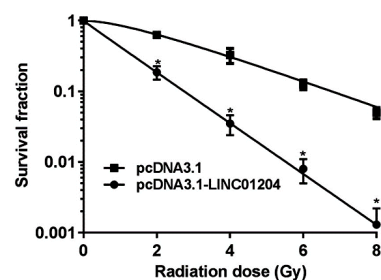


Figure 2. Effect of different dose irradiation combined with LINC01204 overexpression on the survival curve of A549.

Table 4. Single machine multi-target parameters.

Group	D_0 (Gy)	D_q (Gy)	N	SF ₂	k	SER
pcDNA3.1	2.882	2.016	1.805	0.752	0.424	-
pcDNA3.1-LINC01204	2.353	1.272	0.969	0.616	0.827	1.950

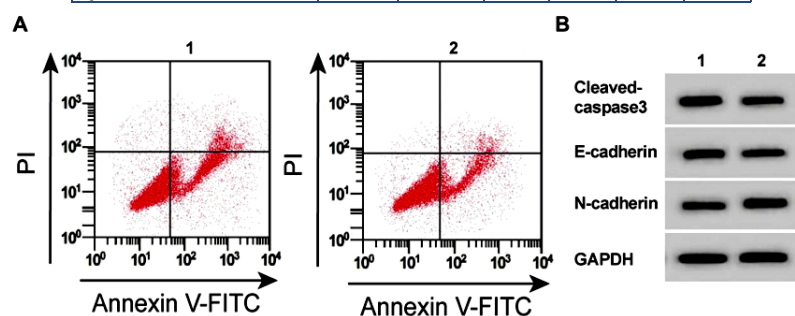


Figure 3. Effect of LINC01204 overexpression and miR-214 on A549 apoptosis (A) and protein expression (B) (1: pcDNA3.1-LINC01204+miR-NC; 2: pcDNA3.1-LINC01204+miR-214)

Effect of LINC01204 overexpression and miR-214 on A549 apoptosis and migration

Compared with pcDNA3.1-LINC01204+miR-NC group, pcDNA3.1-LINC01204+miR-214 group has significantly reduced apoptosis rate ($P<0.05$). pcDNA3.1-LINC01204+miR-214 group had significantly increased migration healing rate ($P<0.05$) than pcDNA3.1-LINC01204+miR-NC group. pcDNA3.1-LINC01204+miR-214 group had significantly increased number of invasive cells ($P<0.05$) than pcDNA3.1-LINC01204+miR-NC group, and significantly reduced Cleaved-caspase3, E-cadherin protein levels compared with pcDNA3.1-

LINC01204+miR-NC group ($P<0.05$). pcDNA3.1-LINC01204+miR-214 group had significantly increased N-cadherin protein levels ($P<0.05$) compared with pcDNA3.1-LINC01204+miR-NC group, as shown in figure 3 and table 6.

Effect of LINC01204 overexpression and miR-214 on radiosensitivity of A549

Compared with pcDNA3.1-LINC01204+miR-NC group, pcDNA3.1-LINC01204+miR-214 group has significantly increased cell survival fraction ($P<0.05$), with sensitization enhancement ratio SER at 0.728, as shown in figure 4 and table 7.

Table 5. Dual luciferase report experiment ($\bar{x}\pm s$, $n=3$).

Group	WT-LINC01204	MUT-LINC01204
miR-NC	0.96 \pm 0.05	0.98 \pm 0.04
miR-214	0.20 \pm 0.02*	0.97 \pm 0.03
<i>t</i>	24.444	0.346
<i>P</i>	0.000	0.746

Note: Compared with miR-NC group, * $P<0.05$.

Table 6. Effect of LINC01204 overexpression and miR-214 on A549 apoptosis and migration ($\bar{x}\pm s$, $n=3$).

Group	Apoptosis rate (%)	Migration healing rate (%)	Number of invasive cells (pcs)	Cleaved-caspase3	E-cadherin	N-cadherin
pcDNA3.1-LINC01204+miR-NC	23.74 \pm 0.59	33.03 \pm 0.65	71.00 \pm 2.45	0.71 \pm 0.05	0.53 \pm 0.04	0.21 \pm 0.01
pcDNA3.1-LINC01204+miR-214	12.20 \pm 0.37*	54.18 \pm 0.84*	131.67 \pm 3.30*	0.29 \pm 0.02*	0.24 \pm 0.02*	0.59 \pm 0.03*
<i>t</i>	28.701	34.490	25.567	13.509	11.232	20.813
<i>P</i>	0.000	0.000	0.000	0.000	0.000	0.000

Note: Compared with pcDNA3.1-LINC01204+miR-NC group, * $P<0.05$.

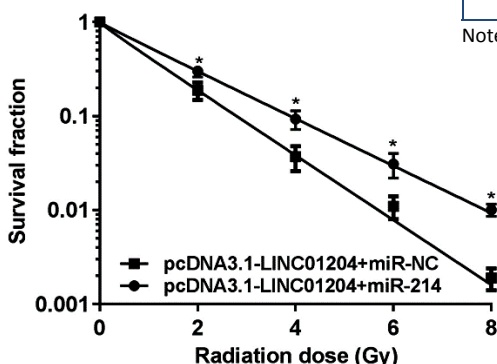


Figure 4. Effect of different dose irradiation combined with LINC01204 overexpression and miR-214 on the survival curve of A549

Table 7. Single machine multi-target parameters.

Group	D_0 (Gy)	D_q (Gy)	N	SF ₂	k	SER
pcDNA3.1-LINC01204+miR-NC	1.261	0.115	0.913	0.189	0.793	-
pcDNA3.1-LINC01204+miR-214	1.733	0.098	0.945	0.301	0.577	0.728

DISCUSSION

LncRNA plays an important regulatory role in the biological processes of lung cancer cell proliferation, migration and invasion. For example, LncRNA BC200 regulates the proliferation and cisplatin resistance of non-small cell lung cancer cells through PI3K/AKT pathway⁽⁸⁾. Silencing of LINC00461 enhances radiosensitivity of lung cancer cells by up-regulating miR-195 and down-regulating HOXA10⁽⁹⁾. LncRNA linc00673 regulates the proliferation, migration, invasion and epithelial-mesenchymal transition of non-small cell lung cancer by acting as a sponge molecule of miR-150-5p⁽¹⁰⁾. LncRNA PVT1 can enhance radiosensitivity of non-small cell lung cancer by acting as a sponge molecule of miR-195⁽¹¹⁾. LncRNA-MALAT1 contributes to the cisplatin resistance of lung cancer by up-regulating MRP1 and MDR1⁽¹²⁾. LncRNA LINC00483 / miR-144 interacts

with HOXA10 to regulate the radiosensitivity and epithelial-mesenchymal transition of lung cancer⁽¹³⁾. In summary, it is shown that LncRNA can play an important regulatory role in the occurrence and metastasis of lung cancer, which may also act as a potential target for enhancing radiosensitivity of lung cancer cells.

The mechanism of LINC01204 in the occurrence and metastasis of lung cancer is not yet known. The results of this study indicate reduced LINC01204 expression level in lung cancer cells. Further studies have shown that LINC01204 overexpression can significantly increase the apoptosis rate, reduce the migration healing rate, and lower the number of invasive cells, suggesting that LINC01204 overexpression can promote lung cancer cell apoptosis and inhibit cell migration and invasion. Studies have shown that up-regulation of Cleaved-caspase3 expression can promote cell

apoptosis, while up-regulation of E-cadherin expression can inhibit the epithelial-mesenchymal transition process and thus inhibit cell metastasis, and up-regulation of N-cadherin expression can promote the epithelial-mesenchymal transition process and facilitate cell migration and invasion (14-15). The results of this study further confirm that LINC01204 can play an important regulatory role in the process of lung cancer cell apoptosis, migration and invasion. At the same time, the results of this study suggest that LINC01204 overexpression can significantly reduce the cell survival fraction, with sensitization enhancement ratio SER at 1.950, indicating that LINC01204 overexpression can enhance the radiosensitivity of lung cancer cells.

This study confirms that LINC01204 can bind to miR-214 in a targeted way. MiR-214 affects the radiotherapy sensitivity of non-small cell lung cancer through MAPK signaling pathway (16). LncRNA PVT1 promotes ovarian cancer progression by down-regulating miR-214 expression (17). MiR-214 affects the proliferation, apoptosis and invasion of cervical cancer cells by regulating the PI3K/AKT/mTOR signaling pathway (18). The results of this study show increased miR-214 expression level in lung cancer cells. Further studies indicate that pcDNA3.1-LINC01204 and miR-214 mimics are co-transfected into lung cancer cells. The results found that the apoptosis rate, Cleaved-caspase3, E-cadherin protein level were decreased, the migration healing rate, N-cadherin protein level were increased, the number of invasive cells was increased, the cell survival fraction was increased significantly, with sensitization enhancement ratio at 0.728 and significantly lower than 1, suggesting that miR-214 overexpression can significantly reverse the effect of LINC01204 overexpression on lung cancer cell apoptosis, migration, invasion and radiosensitivity (19).

In summary, LINC01204 is down-regulated in lung cancer cells, while miR-214 is up-regulated in lung cancer cells. LINC01204 overexpression can promote lung cancer cell apoptosis, and inhibit cell migration and invasion by down-regulating miR-214 expression. Also capable of enhancing radiosensitivity of lung cancer cells, it may act as a potential target for targeted therapy of lung cancer and also a potential target for enhancing radiosensitivity of lung cancer cells (20).

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Ethical considerations: Research experiments conducted in this article with animals or humans were approved by the Ethical Committee and responsible authorities of our research organization (s) following all guidelines, regulations, legal, and ethical standards as required for humans or animals.

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Conflicts of interest: None declared.

Authors' Contributions: (X.N.Y) and (M.W) collected the samples. (X.N.Y) analyzed the data. (M.W) conducted the experiments and analyzed the results. All authors discussed the results and wrote the manuscript.

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