

Original Article

Long non-coding RNA MALAT1 promotes the proliferation and metastasis in non-small cell lung cancer through the ERK/MAPK pathway

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Abstract: Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), a long non-coding RNA (lncRNA), is associated with proliferation and metastasis of lung cancer, being an independent prognostic factor of this disease. Previous study has demonstrated that MALAT1 played an important role in non-small cell lung cancer (NSCLC). However, the physiological and biochemical mechanisms were still unclear. In this study, we performed the study focused on the expression, biological functions and mechanism of MALAT1 in NSCLC and found that MALAT1 was significantly up-regulated in NSCLC tissues compared with corresponding non-cancerous tissues. Knockout of MALAT1 in NSCLC cell lines using lentivirus-mediated RNA interference significantly inhibited the proliferation and metastasis of the NSCLC cells. Importantly, ERK/MAPK pathway was found to be inactivated in the NSCLC cell lines after MALAT1 knockout experiment. These results indicated that MALAT1 maybe serve as an oncogenic lncRNA that promoted proliferation and metastasis of NSCLC and activated the ERK/MAPK pathway.

Keywords: Metastasis-associated lung adenocarcinoma transcript 1, lncRNA, non-small cell lung cancer, ERK, MAPK

Introduction

Lung cancer is a common malignant tumor for human being, and its related death is the most common cause of death worldwide [1-4]. According to the differentiation and morphological characteristics, lung cancer can be divided into small cell lung cancer (SCLC) and non small cell lung cancer (NSCLC) [5], NSCLC accounted for 85% all of the lung cancer patients [6]. At present, the effective treatment is removing all lung resection and appropriate chemotherapy strategy for NSCLC [7, 8]. Therefore, the most important strategy for lung cancer is to find an effective marker of early diagnosis and prognosis, and provide a theoretical basis for the invasion and metastasis of cancer cells. Several studies showed different therapeutic options for specific NSCLC cell types, and the classification of lung cancer was mainly based on routine biopsy [9]. Although this method could provide satisfactory diagnostic accuracy, however, due to various factors

such as the size of the sample size, different sampling sites, tumor heterogeneity, the method could not be accurate subtype positioning. Therefore, an effective diagnostic molecular marker is needed in clinic.

The human genome sequence has stably transcribed for 5%-10%, and the vast majority of these transcripts are non coding transcripts [10]. Non encoded transcripts can be classified as housekeeping ncRNA and regulatory ncRNA [11, 12]. The long chain ncRNA (lncRNA) is the most transcribed non-coding sequence for housekeeping ncRNA [13]. LncRNA is generally refers to RNA longer than 200nt in the nucleus or cytoplasm, which does not participate in protein coding function, and in the form of RNA regulation of gene expression in multiple levels (epigenetic regulation, transcription and post transcriptional regulation) [14-16].

In 2003, Ping et al found a frequent expression of the transcript derived fragments in the occur-

Table 1. Primers used in this study

Genes	Primers	Sequences (5'-3')
MALAT1	P1	AAAGCAAGGTCTCCCAACAAG
	P2	GGTCTGTGCTAGATCAAAAGGCA
GAPDH	P1	AGAAGGCTGGGGCTCATTG
	P2	AGGGGCCATCCACAGTCTTC

Table 2. The clinical and pathological information of the patients

Variables		Numbers
Sex	Male	17
	Female	38
Age	Mean	61.3±4.6
Tumor differentiation	Well	11
	Moderate	28
	Poor	16
Tumor invasion	T1	6
	T2	14
	T3	19
	T4	16
Lymph node metastasis	Absence	19
	Presence	33
	No	3
Distant metastasis	Absence	50
	Presence	5
TNM stage	I	6
	II	11
	III	22
	IV	16

rence of subsequent transfer of lung adenocarcinoma tissues, named metastasis associated lung adenocarcinoma transcript 1 (MALAT1) [17]. The gene length was about 8.7 KB, located on the chromosome 11q13 [18]. MALAT1 can be expressed in many normal tissues, highly expressed in the lung, pancreatic tissue, lowly expressed in kidney, heart, liver, brain, testis tissue, and in the stomach, skin, bone marrow, uterine tissue was not expressed [19, 20]. Recent studies have shown that MALAT1 was over-expressed in a wide variety of tumors, including breast, prostate, rectum, liver, and NSCLC, especially in patients with early metastases [21-23]. Therefore, MALAT1 as a genetic molecule is involved in cancer biology emerged

in scientific research in recent years. At present, the physiological and biochemical mechanisms for MALAT1 involved in NSCLC were still unclear. In this study, we performed the analysis on the expression, biological functions and mechanism of MALAT1 in NSCLC.

Materials and methods

Patient samples and cell lines

NSCLC and paired corresponding normal non-cancerous tissues were obtained from 55 patients, underwent surgery resection during 2011 to 2015 at Huaihe Hospital of Henan University. No patients were recruited in our study for radio or chemo-therapy before the surgery. The clinical information of the patients was collected. All the specimens were frozen and stored at -70°C. This study was approved by the research ethic committee of Huaihe Hospital of Henan University. All the written informed consents were got from patients for biological research.

Three NSCLC cell lines (PC9, SPC-A1 and H1299) and one normal tissue (16HBE) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in RPMI-1640 medium (GIBCO, USA) supplemented with 10% fetal bovine serum (10% FBS), and maintained in the humidified incubator at 37°C with 5% CO₂.

RNA extraction and qRT-PCR assays

Total RNA was isolated from tissues and cell cultures with Trizol reagent (Takara, Japan) according to the manufacturers' instructions. The cDNA was reverse transcribed from a total of 200 ng RNA using the PrimeScript RT reagent Kit (Takara, Japan) and amplified by quantitative real-time PCR by SYBR Green Kit (Takara, Japan) on ABI 7500. GAPDH was used as the internal reference, and the relative expression of MALAT1 was normalized to GAPDH. Primers for MALAT1 and GAPDH were shown in **Table 1**.

Interfering RNA construction

The small interfering RNA (siRNA) against MALAT1 and the negative control (si-NC) were used and synthesized by GenePharma (Shanghai, China). The following short hairpin RNA was used to target MALAT1: sense:

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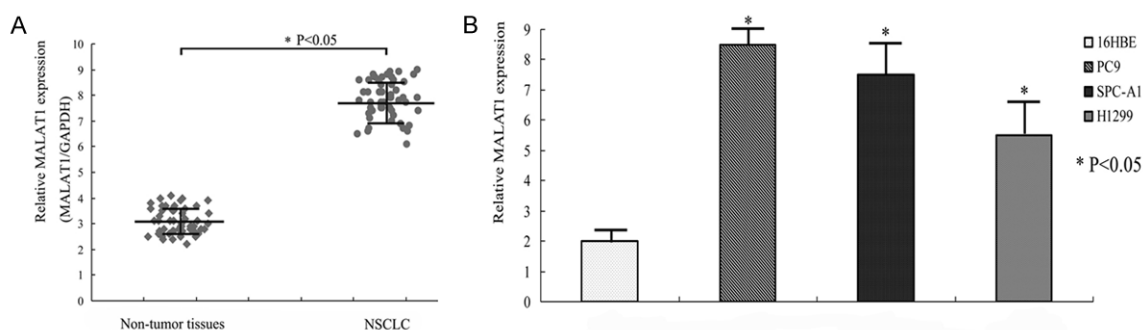


Figure 1. Comparison of MALAT1 in NSCLC tissues and cell lines. A: Relative MALAT1 expression in NSCLC and non-tumor tissues. B: Relative MALAT1 in NSCLC cell lines. * $P < 0.05$.

5'-GAGGUGUAAAGGGAUUUAUTT-3'; antisense: 5'-CCCUCUAAAUAGGAAUAATT-3'. The negative control sequence was 5'-UUCUCCGAACGUGUCACGUTT-3'. The cells were cultured on six-well plates in the density of 5×10^5 , and the cells were transfected with siRNA and si-NC by using Lipofectamine 2000 (Invitrogen, USA) at 100 nM concentration. The interfering results were determined by qRT-PCR after transfection 48 hours, and the silencing efficacy of siRNA were used for more than 80% to further investigation.

Cell proliferation

Cell proliferation was performed by using Cell Counting Kit (CCK-8, Dojindo) based on the manufacturer's instruction. 96-well plates were used for three groups (control, siRNA and si-NC), 100 μ l cells were inoculated into the plates at 1000 cells/well, and cultured at 37°C. The densities of the cells were measured at 450 nm, and the survival rate was expressed as the absorbance relative to the control group.

For colony forming experiments, the cells transfected with siRNAs and si-NC were inoculated into six well plates, and maintained in RPMI-1640 medium contained 10% FBS for two weeks. Colonies were fixed with methanol 12 days later and stained with 0.1% crystal violet (Sigma, USA). The colonies were photographed and counted manually.

Cell cycle and apoptosis analysis

Cells were harvested after transfected by trypsin and centrifugation. Then, cells were washed with cold PBS, and fixed with 70% ethanol at 4°C. The cells were stained with propidium iodide at 4°C for 30 min. The results were analyzed with flow cytometer FACSCalibur (BD,

USA). For the apoptosis analysis, the trypsinized and washed cells were stained with FITC-Annexin V and propidium iodide, and analyzed by FACSCalibur and Flowjo software (BD, USA). Cells were discriminated for viable cells, dead cells, early apoptosis cells and apoptosis cells.

Western blotting

Cells cultures were lysed with protein extraction reagent RIPA (Beyotime, China). The proteins were separated by SDS-PAGE and transferred to PVDF membranes. The blots were probed with primary antibodies at 4°C: anti-p-ERK1/2, anti-ERK1/2, anti-p-MEK1/2, anti-MEK1/2, anti-p-MAPK, anti-MAPK, anti-p-JNK1/2/3, and anti-JNK. The GAPDH was used as the internal control.

Statistical analysis

SPSS 18.0 software was used for statistical analysis of the data. Data were shown as mean \pm standard deviation (STD). Statistical significance was tested by Student's t-test, Chi-square test and one way ANOVA if appropriate. P value less than 0.05 was considered to be statistically significant.

Results

MALAT1 were up-regulated in NSCLC tissues and cell lines

We performed the qRT-PCR to evaluate the expression of MALAT1 in 55 NSCLC and corresponding non-tumor tissues. The clinical and pathological information of the patients were shown in **Table 2**. A significant increased level of MALAT1 was observed in NSCLC tissues when compared with the non-tumor tissues (**Figure 1A**). The cell lines comparison results

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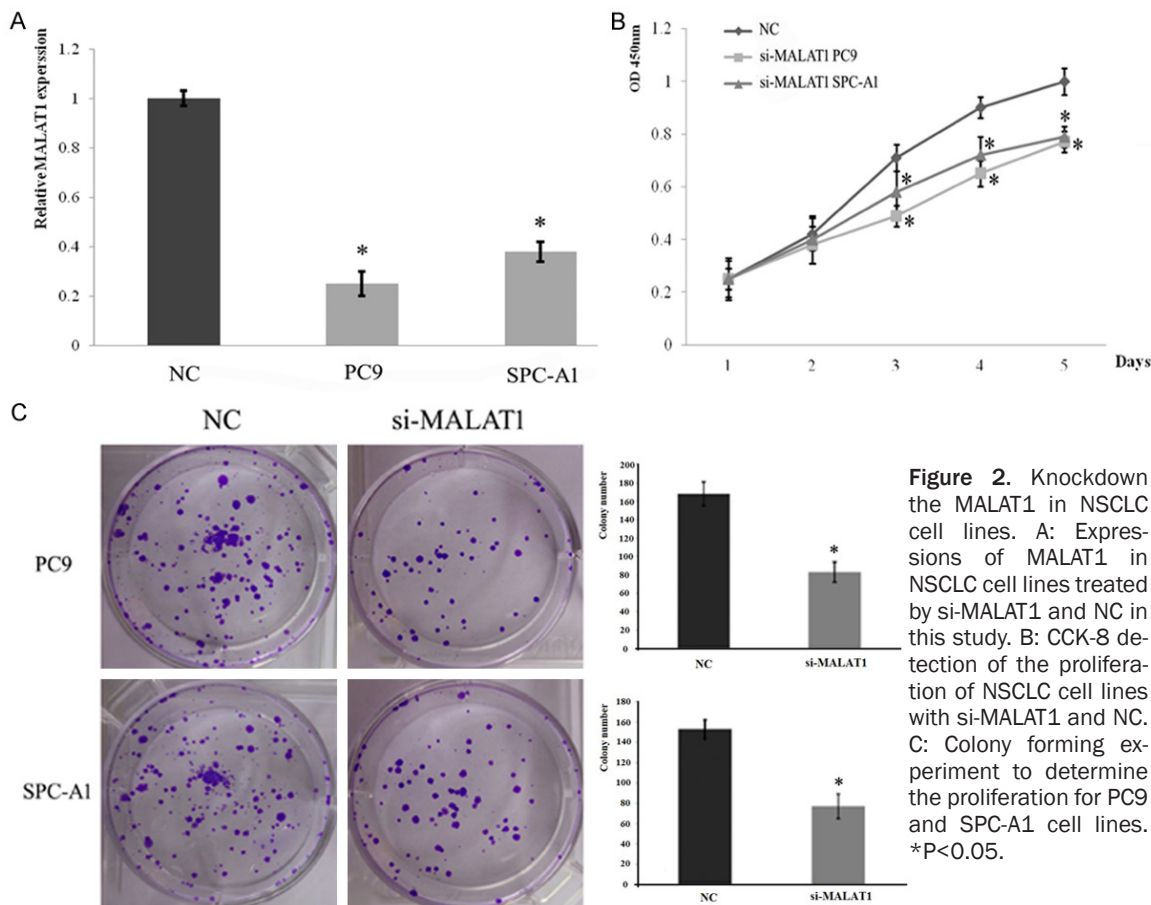


Figure 2. Knockdown the MALAT1 in NSCLC cell lines. A: Expressions of MALAT1 in NSCLC cell lines treated by si-MALAT1 and NC in this study. B: CCK-8 detection of the proliferation of NSCLC cell lines with si-MALAT1 and NC. C: Colony forming experiment to determine the proliferation for PC9 and SPC-A1 cell lines. *P<0.05.

also showed highly expressed MALAT1 to the NSCLC cells (**Figure 1B**).

Knockdown of MALAT1 in NSCLC cell lines

We used siRNA to knockdown the MALAT1 expression in NSCLC cell lines, and accessed the role of MALAT1 in NSCLC growth. Two cell lines were used, PC9 and SPC-A1. The decreased MALAT1 expression levels were selected for more than 80%. The MALAT1 expressions were statistically significant decreased in all the cell lines (**Figure 2A**). Cell proliferation results detected by CCK-8 revealed that NSCLC cells growth were inhibited both in PC9 and SPC-A1 compared with negative control (**Figure 2B**), and the colony forming experiment also showed reduced phenomenon by MALAT1 silenced in PC9 and SPC-A1 cell lines (**Figure 2C**).

The cell cycle progression and apoptosis of the NSCLC cell lines

We performed the cell-cycle arrest and apoptosis test for knockdown MALAT1 in NSCLC cell

lines. The results showed that si-MALAT1 of PC9, SPC-A1 and H1299 had an obvious cell-cycle arrest at the G_1 - G_0 phase and had a decreased at G_2 -S phase (**Figure 3A**). The flow-cytometry and TUNEL staining analysis was also performed, and the results showed that all the NSCLC cell lines transfected with si-MALAT1 increased the apoptosis proportion compared with the control groups (**Figure 3B**). These data suggested that si-MALAT1 could promote the proliferation of NSCLC cell lines.

Si-MALAT1 on the extracellular signal regulated ERK/MAPK pathway

We determined the primary mechanism by which MALAT1 regulated NSCLC cells' proliferation and metastasis. Western blot results showed that the phosphorylated MEK1/2, ERK1/2, MAPK and JNK1/2/3 were significantly reduced for knockdown of MALAT1, while the levels of MEK1/2, ERK1/2, MAPK and JNK1/2/3 had no statistical changes (**Figure 3**). These data suggested that ERK/MAPK pathway might involve in the MALAT1 mediated

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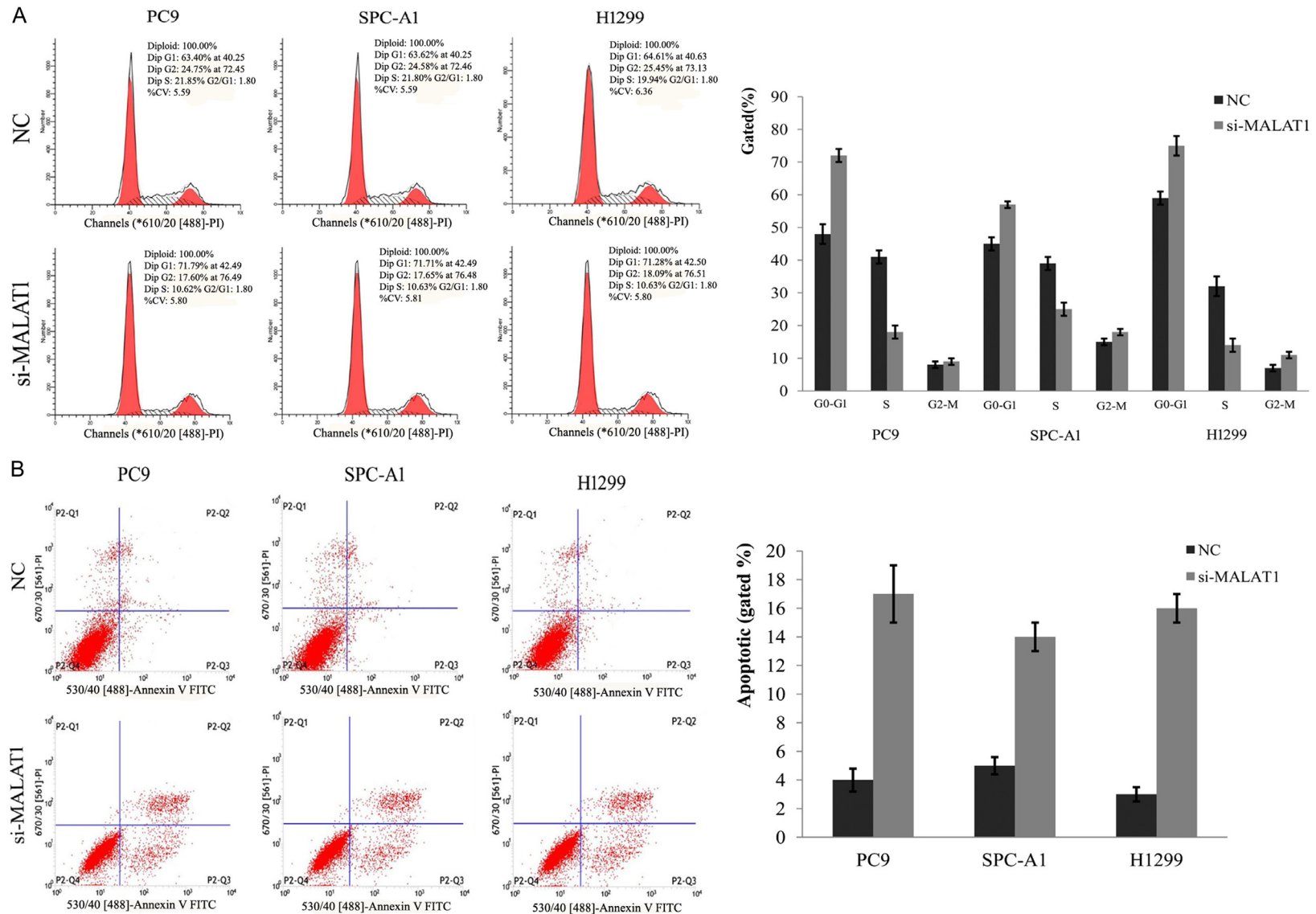


Figure 3. Effects of knockdown the MALAT1 gene on NSCLC cell lines for cell cycle and apoptosis analysis. A: The percentage of cell in G0-G1, S and G2-M phase. B: The apoptosis analysis by flow cytometry.

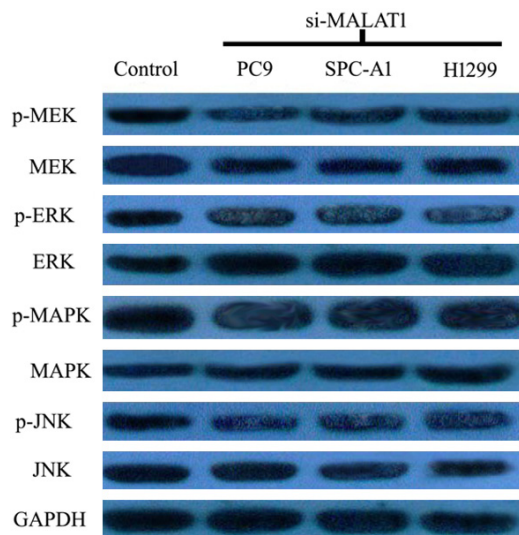


Figure 4. Western blot results of the ERK/MAPK pathway related proteins in NSCLC cell lines.

proliferation and metastasis of NSCLC cell lines (Figure 4).

Discussion

One of the most common leading causes of malignant tumors is lung cancer currently [24]. The incidence rate and the leading cause of death among all the malignancies is lung cancer in male, and in female the incidence among the fourth, mortality ranked second [25]. Above all, Non small cell lung cancer (NSCLC) is occupied about 85% of all the lung cancer, its 5 year survival rate is less than 20% [26].

LncRNA is a functional RNA containing at least 200 nucleotides, which was first discovered in the DNA transcriptional products of mice. Previous studies showed that LncRNA was produced mainly in the following ways: disruption of the structure protein coding genes; an independent gene and two un-transcribed genes in series, containing multiple exons; local replicon series form; non-coding RNA in the replication process; gene insert a transposable elements and produce functional non-coding RNA [27]. According to the direction and source [28], LncRNA can be divided into intronic LncRNA, intergenic LncRNA, sense LncRNA, antisense LncRNA and bidirectional LncRNA. LncRNA is usually longer, with an mRNA like structure, and lots of LncRNA come from the RNA polymerase II transcription, some with poly (A) tail and the

promoter structure, showing a dynamic expression of the differentiation process and different splicing patterns. LncRNA has tissue and spatial specificity in plants, animals, prokaryotes and viruses, and the LncRNA expression changes in the different growth stages of the same tissue or organ.

MALAT1 was highly expressed in lung squamous cell carcinoma, and associated with poor prognosis [29]. At the genetic level, MALAT1 showed a strong link between tumors related genes, such as cell growth, movement, proliferation, signal transduction and immune regulation [30]. Studies have shown that MALAT1 transcripts induce cell migration and stimulate tumor growth and invasion. MALAT1 transcripts are regulated by phosphorylation of the splicing factor, and have the effect on SR protein phosphorylation in cell level, but its exact mechanism is still not clear understood [31, 32].

At present, MALAT1 could promote the growth and colony formation of NSCLC cells, and the migration ability of lung adenocarcinoma is obviously decreased after interfering with the expression of MALAT1. In ping et al. study [17], they used Kaplan-Meier curves and log rank test to analyze the data of the two groups. The high expression of MALAT1 was significantly associated with the survival rate of stage I in patients with lung adenocarcinoma and squamous cell carcinoma. Also, highly expression of MALAT1 in the early stage of the disease showed a poor prognosis. In our study, MALAT1 was significantly up-regulated in NSCLC tissues compared with corresponding non-cancerous tissues. Knockout of MALAT1 in NSCLC cell lines using lentivirus-mediated RNA interference significantly inhibited the proliferation and metastasis of the NSCLC cells. These results indicated that MALAT1 maybe serve as an oncogenic LncRNA that promoted proliferation and metastasis of NSCLC.

Ji et al. have found that MALAT1 could predict metastasis in the early NSCLC, and its expression is closely related to the metastasis of NSCLC patients [17]. Schmidt et al. found that MALAT1 stimulated migration, invasion, and tumor growth, but the underlying mechanism had not been explained [33]. The reason may refer to the abnormal expression of MALAT1 in a specific cell, leading to aberrant alternative splicing, and the wrong gene expression, such

as oncogenic transcription factor B-MYB. Meanwhile, their research also found that MALAT1 was involved in the development of NSCLC. In our study, we firstly found that ERK/MAPK pathway might be inactivated in the NSCLC cell lines after MALAT1 knockout experiment. Therefore, MALAT1 over-expression could be used as a sign of metastatic potential tumor recognition, and it was the identified adjustable NSCLC metastasis lncRNA.

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Disclosure of conflict of interest

None.

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