

ORIGINAL ARTICLE

Human MALAT-1 long non-coding RNA is overexpressed in cervical cancer metastasis and promotes cell proliferation, invasion and migration

Yan Zhang^{1*}, Tao Wang^{2*}, Hua-Qiao Huang³, Wei Li¹, Xiao-Ling Cheng², Jing Yang¹

¹Department of Obstetrics and Gynaecology, Renmin Hospital of Wuhan University, Wuhan 430060, China; ²Institute of Biological Medicine, Wuhan University of Science and Technology, Wuhan, Hubei 430065, China; ³Department of Obstetrics and Gynaecology, Hubei Xinhua Hospital, Wuhan 430015, China

*These authors contributed equally to this work

Summary

Purpose: Long non-coding RNAs (lncRNAs) have been shown to play a crucial role in the development as well as the prognosis of various human cancers, particularly in human metastasis associated lung adenocarcinoma transcript-1 (MALAT-1), which has been shown to be upregulated in some tumors. However, the role of MALAT-1 in cervical cancer (CC) is yet to be elucidated. This study aimed to establish a correlation between MALAT-1 and its role in CC progression and prognosis.

Methods: The expression of MALAT-1 was examined in tissue samples of 30 CC patients and was compared with the adjacent non-cancerous tissues. The relationship between MALAT-1 levels and clinicopathological parameters from the CC patients was analyzed. Cell migration and invasion assays were performed with quantification of the expression levels of MALAT-1 in 4 CC cell lines using RT-PCR.

Results: Our results demonstrate that MALAT-1 is upregulated in CC. We found higher expression of MALAT-1 in all the 4 CC cell lines, especially in SiHa and ME-180 CC cell lines. After analysis of the relationship between MALAT-1 levels and clinicopathological parameters from CC patients, a robust correlation could be obtained between MALAT-1 overexpression with lymph node metastasis, tumor differentiation and clinical stage. Furthermore, the ectopic expression of MALAT-1 increased cell proliferation and contributed directly to invasion and migration.

Conclusion: Taken together, this study suggests an indispensable role played by MALAT-1 in CC progression, which may act as a potential prognostic indicator for CC and also could be a novel potential target for diagnosis of CC.

Key words: cervical cancer, invasion, lncRNA, MALAT-1, migration, tumorigenesis

Introduction

CC is the second leading cause of cancer deaths in women around the world with approximately 14 million new cases and 8.2 million deaths according to the most recent statistical report [1]. A recent report by the World Health Organization predicts the number of new cases to rise by 70% in the next two decades. Though conventional methods of treatment involving chemotherapy and radiotherapy are available, new and

effective strategies to accurately predict disease outcome are urgently needed for a better diagnosis that could ultimately improve patient survival.

The advent of high-throughput sequencing technologies has revealed that 90% of the human genome is actively transcribed [2], with non-coding RNAs playing a significant role in mediating cellular events [3-5]. Based on the transcript size non-coding RNAs are grouped into two classes,

namely, small ncRNAs (sncRNAs) and long ncRNAs (lncRNAs) [6]. LncRNAs have been implicated as master regulators of many important biological processes ranging from cell growth and apoptosis, to cancer development [7]. Recent studies in the last decade have witnessed an emerging role of lncRNAs in tumorigenesis, and differential expression of lncRNAs being associated with various types of cancer. To name a few, differential expression of various human lncRNAs has been associated with cancers of the breast [8], lung [9], uterus [10], colon [11], prostate [12], liver [13] and the cervix [14].

Of particular interest amongst the lncRNAs is the metastasis-associated lung adenocarcinoma transcript-1 (MALAT-1), which was first associated with high metastatic potential in non-small cell lung cancer patients [9]. MALAT-1 is widely expressed in normal human tissues and a number of studies have correlated high levels of MALAT-1 with metastasis in a variety of human cancers [15,16]. Though mechanistic insights into the role of MALAT-1 in cancer progression are still unclear, collectively these studies suggest the pivotal role played by MALAT-1 in regulating the invasive potential of metastatic tumor cells.

Several studies have demonstrated the efficacy of MALAT-1 expression profiles serving as novel biomarkers for various types of cancer. For example, MALAT-1 expression levels have been associated with patient survival and tumorigenesis in non-small cell lung cancer [17]. However, up until today, the prognostic value of the lncRNA MALAT-1 in cervical carcinogenesis has not been investigated.

In the present study, we investigated whether MALAT-1 was overexpressed in CC patients, and whether high levels of MALAT-1 were associated with CC growth and metastasis. Subsequently, the levels of MALAT-1 in CC tissues and cell lines were investigated and its potential relationship with clinicopathological parameters was analyzed. Moreover, the role of MALAT-1 in cellular proliferation, migration, invasion and metastasis in CC progression was also investigated using *in vitro* assays.

Methods

Patient samples and cell culture

A total of 30 patients with CC from our hospital (Renmin Hospital of Wuhan University) were enrolled in the study. The Ethics Committee of Renmin Hospital of Wuhan University, China, approved the study. Writ-

Table 1. Sequences of siRNAs used in these studies

siRNA1-MALAT1	GAGGUAUGAAGGGAUUAUUTT
siRNA2-MALAT1	CCCUCUAAAUGAGGUAUUAATT
Negative control	UUCUGCCAACCAUCACUGTT

ten informed consents were obtained from all patients involved in this study or their parents/guardians. All the specimens including cancer and paired noncancerous tissues were immediately frozen in liquid nitrogen.

SiHa cells were obtained from the Renmin Hospital of Wuhan University and were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA). ME-180, C4-I and C4-II cells were purchased from Invitrogen (Invitrogen AG, Basel, Switzerland) and were grown in supplemented Medium 231 (Invitrogen). All cell lines were supplemented with 10% FBS (North Andover, MA, USA) and cultured at 37 °C in a humidified atmosphere of 5% CO₂.

Quantitative real time-PCR (qRT-PCR) analysis

To assess the role of MALAT-1 in CC progression, we examined the expression levels of MALAT-1 in CC tissue and CC cell lines, SiHa, ME-180, C4-I and C4-II, using qRT-PCR. The levels of MALAT-1 were quantified in the 4 CC cell lines with respect to a normal human cervical squamous epithelial cell line, serving as a control. Total RNA was extracted using the RNeasy kit (Qiagen, USA). Reverse transcription reactions were performed using Random Hexamers and MultiScribe Reverse Transcriptase (both from Applied Biosystems, USA). Specific RNA transcripts were quantified using a standard SYBR Green qPCR, using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). β -actin was used as a reference for lncRNAs and each sample was analyzed in triplicate. Relative levels of gene expression were quantified by the standard $2^{-\Delta\Delta C_t}$ method. All the results were analyzed as a ratio between expression in CC tissues and adjacent normal tissues (log₁₀ scale).

Cell proliferation assay

To assess the proliferation rate, 5×10^6 cells were seeded in 24-well plates and were transfected with MALAT-1 pcDNA or MALAT-1 siRNA using Lipofectamine 2000. Cell proliferation was determined using a CyQUANT cell proliferation assay (Invitrogen, USA) according to the manufacturer's instructions. The fluorescence intensity was measured using a fluorescence microplate reader (Molecular Devices, USA). For the construction of plasmid pcDNA-MALAT-1, a BamHI-EcoRI fragment containing the MALAT-1 cDNA was introduced into the same sites in the vector pcDNA3.1. RNAi-mediated knockdown of MALAT-1 was performed according to the standard protocols. The siRNAs used in this study were mixtures of 3 siRNAs,

Table 2. Correlation between MALAT-1 expression and clinicopathological characteristics in CC

Characteristics	N (%)	Expression of MALAT1 (Mean ΔC_p)	p value
Gender			
Male	20 (66.6)	-5.30±0.93	0.446
Female	10 (34.4)	-5.59±1.10	
Age (years)			
>60	18 (60)	-5.39±1.09	0.653
≤60	12 (40)	-5.90±0.99	
Tumor location			
Upper	12 (40)	-5.64±1.02	0.796
Middle	8 (26.6)	-5.67±1.16	
Lower	10 (33.4)	-5.11±0.96	
ISS stage			
I	5 (16.6)	-9.63±0.70	0.03
II	10 (33.3)	-9.84±0.16	
III	15 (50.0)	-9.51±0.12	
Tumor size (cm)			
<4	12 (40)	-9.62±0.59	0.04
>4	18 (60)	-9.22±0.43	
Depth of invasion			
T1+T2	17 (56.6)	-9.56±1.13	0.03
T3+T4	13 (43.4)	-9.63±1.06	
Histological grade			
Good	16 (53.3)	-5.90±1.73	0.74
Moderate	8 (26.6)	-4.19±1.30	
Poor	6 (20)	-5.50±0.30	

and are indicated in Table 1.

Cell migration, invasion and wound healing assays

Cell migration was assessed in two 24-well plates of 8 μ m (BD Biosciences) according to the manufacturer's instructions, followed by incubation for 16 hrs at 37°C in 5% CO₂. For the invasion assay, Matrigel Invasion Chambers in two 24-well plates of 8 μ m (BD Bioscience) were used according to the manufacturer's instructions, followed by incubation for 40 hrs at 37°C in 5% CO₂. Following incubation, cells on the upper surface of the membrane were scraped off and the migrated cells to the bottom of the membrane were fixed and stained with 0.1% Crystal Violet staining solution. The cells were counted from different microscopic fields and averaged.

For the wound healing assay, a total of 2.5x10⁶ cells were seeded in 6-well plates and cultured overnight. A straight scratch was made and the cell suspension was washed off gently and images of the scratch were acquired as baseline. The medium was replaced at regular intervals and the images of the same location

were obtained every 24 hrs.

Statistics

For statistical analysis, GraphPad Prism Software (GraphPad Software, USA) was used. The relationships between MALAT-1 expression levels and clinicopathologic parameters were analyzed by univariate analyses. Statistical significance was determined using the Student's t-test. In all statistical analyses, two-sided tests were applied. A p value of p<0.05 was considered statistically significant.

Results

Clinicopathological data of the cohort are depicted in Table 2.

MALAT-1 is upregulated both in CC tissues and cell lines

Analysis of tissue specimens from 30 CC patients showed that MALAT-1 expression was sig-

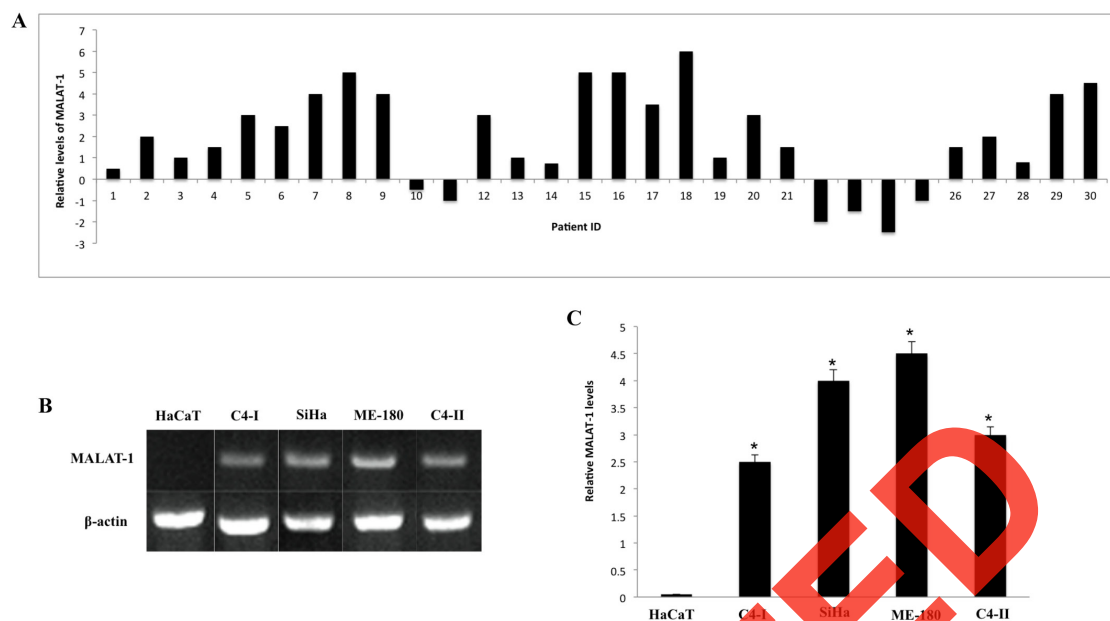


Figure 1. Upregulation of MALAT-1 in CC tissues and cell lines. **(A):** MALAT-1 expression levels were assessed in human CC tissues and adjacent normal tissues from 30 CC patients. MALAT-1 levels were quantified by RT-PCR and the bars represent the ratio between the expression in CC tissue and the adjacent normal tissue (log scale). **(B):** RT-PCR analysis showed an increased expression of MALAT-1 in the CC cell lines C4-I, SiHa, ME-180 and C4-II. The expression of MALAT-1 observed in the normal human cervical squamous epithelial cell line, HaCaT, is insignificant. β-actin was used as a control. **(C):** Bar plot of the quantification of MALAT-1 levels corresponding to **B**. MALAT-1 levels were normalized to β-actin levels in HaCaT cells. Asterisks indicate values that are significantly different from that in HaCaT ($p < 0.05$).

nificantly upregulated in 80% of the tissue specimens compared to adjacent non-tumorous tissues ($p < 0.05$, Figure 1A). Further, the quantification of the levels of MALAT-1 showed higher expression of MALAT-1 in all 4 CC cell lines compared with the normal control cell line, especially in SiHa and ME-180 ($p < 0.05$, Figure 1B, C). These results indicate that abnormal expression of MALAT-1 may be related to CC progression.

Relationship between MALAT-1 expression and clinicopathological parameters in CC patients

Next, we evaluated the correlation between MALAT-1 expression and clinicopathological data in CC patients. MALAT-1 overexpression was correlated with lymph node metastasis, tumor differentiation, and clinical stage. However, the relative MALAT-1 expression level was not associated with patient's age, gender, and tumor location ($p > 0.05$).

MALAT-1 increases cellular proliferation in SiHa cell line

To further investigate the biological role of

MALAT-1 in cell growth, SiHa CC cell line was treated with MALAT-1. Upon transfection with MALAT-1, analysis of the results revealed that the levels of MALAT-1 were significantly increased in SiHa cells, and upregulation of MALAT-1 increased SiHa cells growth (Figure 2A). When SiHa cells were treated with MALAT-1 siRNA (siMALAT-1), it resulted in a significant decrease in the levels of MALAT-1 ($p < 0.05$). Also, down-regulation of expression of MALAT-1 inhibited cell growth (Figure 2B). Corresponding siRNA controls were used in all the experiments. This analysis clearly suggests that MALAT-1 increases cellular proliferation and growth in CC SiHa cells.

MALAT-1 upregulation directly correlates with cell migration and invasion in vitro

We further assessed the effects of lncRNA MALAT-1 on cell migration and invasion, which are key determinants in malignant progression and metastasis. Our results indicated that over-expression of MALAT-1 significantly increased migration and invasion of SiHa cells in the Transwell assay. On the contrary, migration and invasion were dramatically decreased when si-

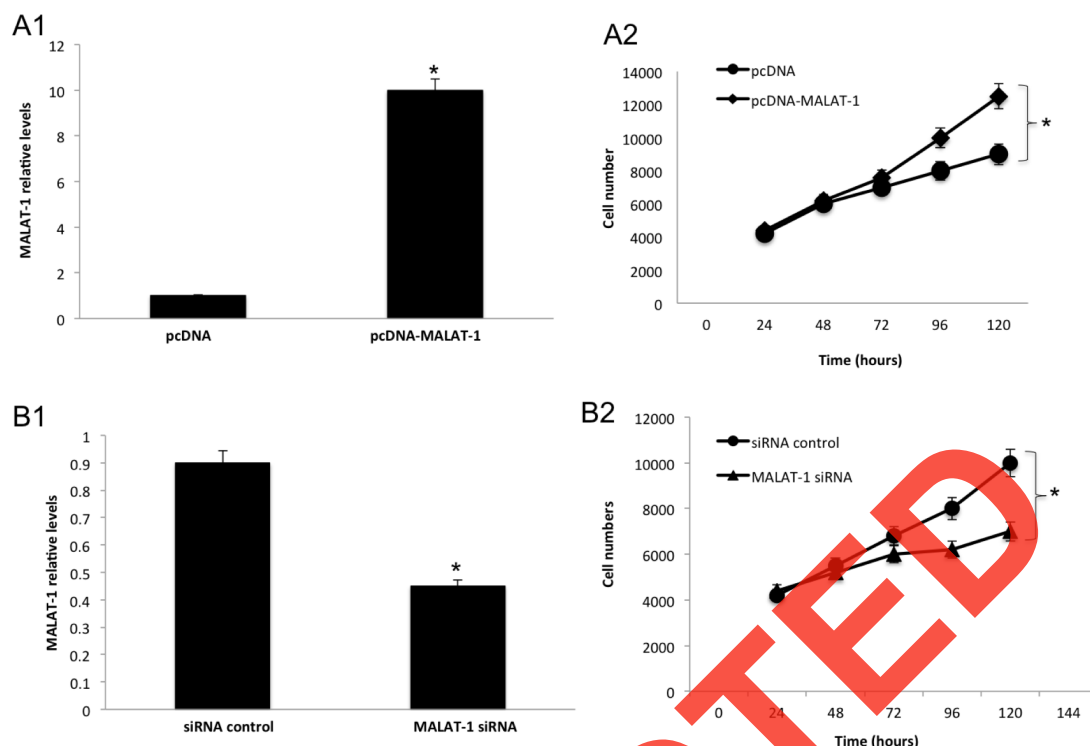


Figure 2. MALAT-1 increased SiHa cell proliferation. **(A):** SiHa cells were transfected with pcDNA-MALAT-1, and MALAT-1 expression levels were quantified by qRT-PCR. SiHa cells were plated in a 24-well plate and transfected with pcDNA-MALAT-1, and the cell number was determined at different time points using a CyQUANT cell proliferation assay. **(B):** SiHa cells were treated with MALAT-1 siRNA, and the expression levels of MALAT-1 were quantified by RT-PCR. SiHa cells were plated in a 24-well plate and transfected with MALAT-1 siRNA, and cell proliferation was determined at different time points using a CyQUANT cell proliferation assay. Asterisks indicate values that are significantly different from controls ($p < 0.05$).

MALAT-1 RNA was introduced. Wound healing assays indicated that SiHa cells transfected with MALAT-1 were distinctively more migratory than the non-transfected control group (NC) (Figure 3). Taken together, these results suggest a crucial functional role played by MALAT-1 in mediating cell migration and invasion in cervical cancer. Also, it is likely that upregulation of the lnc RNA MALAT-1 may contribute to tumor metastasis in CC.

Discussion

It is now recognized that the human transcriptome is more complex than a collection of protein-coding genes where 60-70% of the DNA is actively transcribed into non-coding RNAs [3]. Amongst these different non-coding RNA types, recent studies have attributed a crucial role played by lncRNAs in the progression of numerous diseases, particularly cancer. Aberrant regulation of lncRNAs such as HOTAIR, MEG3 and LOC285194 has been associated with development and pro-

gression of different types of cancers, and also has been demonstrated as reliable prognostic indicators in tumorigenesis [18-20].

One such lncRNA that has been implicated in different types of malignancies is the human metastasis associated lung adenocarcinoma transcript-1. Previous studies have shown that MALAT-1 is overexpressed in cancers of prostate [21], liver [22], bladder [23] and lung [9]. Since the overexpression of MALAT-1 was associated with the development and progression of different cancers and served as a powerful prognostic tool, we sought to address the role of MALAT-1 in tumorigenesis, development and progression of CC. To test these hypotheses, we first evaluated the levels of MALAT-1 in 30 pairs of CC tissues and adjacent normal tissues by quantitative RT-PCR. Analysis of results indicated that the relative expression levels of MALAT-1 were significantly higher in cancerous tissues compared with that of adjacent normal tissues. Furthermore, we evaluated the levels of MALAT-1 in 4 CC cell lines with respect to a normal human cervical squamous epithelial

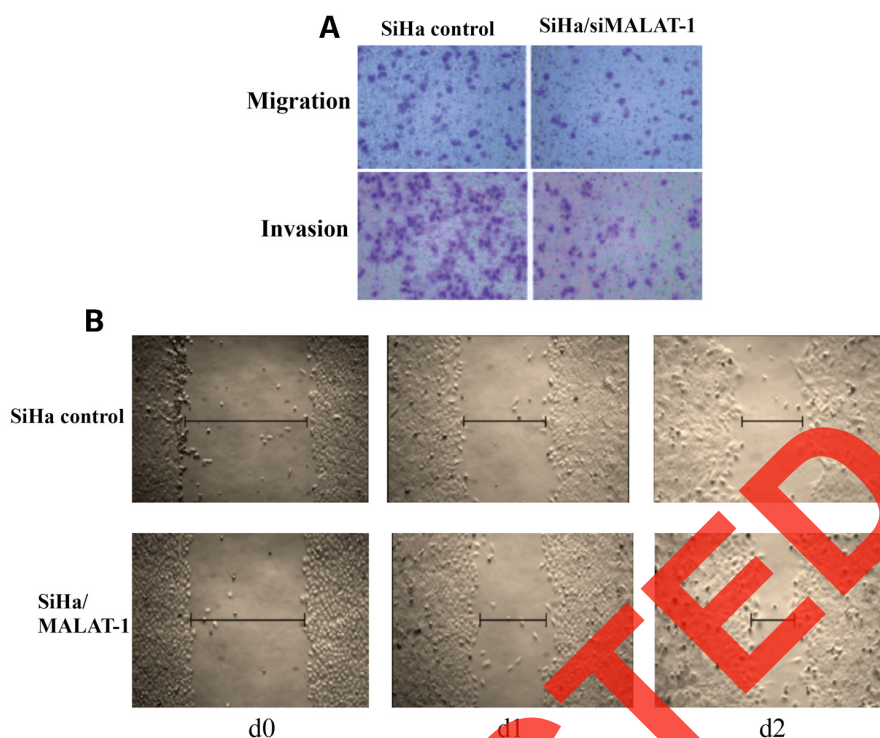


Figure 3. MALAT-1 promoted migration and invasion of SiHa cells based on transwell and wound healing assay. **(A):** Representative microphotographs of migratory and invasion cells on the membrane (100x magnification). SiHa cells transfected with si-MALAT-1 displayed significantly lower transmembrane migration and invasion capacity with respect to a control group. **(B):** SiHa cells were transfected with MALAT-1 and the migratory analysis was carried out with respect to a control group (NC). The rate of migration was measured by quantifying the total distance from the edge of the wound toward the other side (distance between two lines) for 2 days. The bars indicate the rate of migration.

cell line. Not surprisingly, MALAT-1 levels were remarkably higher in all the CC cell lines. Taken together, these results indicate that MALAT-1 is upregulated and overexpressed in CC and could possibly act as a potential oncogene.

Next, we went ahead to investigate the relationship between MALAT-1 overexpression and different clinicopathological parameters. Although MALAT-1 overexpression was correlated with lymph node metastasis, tumor differentiation, and clinical stage, no correlation could be obtained with patient's age, gender or tumor location. Since the aforementioned clinicopathological parameters, especially pertaining to the tumor, partially represent tumor progression, it is highly likely that MALAT-1 may play a crucial role in directly contributing to tumor progression. Consistent with other similar studies [9], these results indicate that the function and contribution of MALAT-1 could be tumor-dependent.

Next, we speculated the effect of MALAT-1 overexpression on cell proliferation, migration and invasion. By studying the effect of MALAT-1 and MALAT-1 siRNA on SiHa cell lines, we pro-

pose that MALAT-1 promotes cell growth and proliferation. Meanwhile, the invasion potential of SiHa cell lines was increased compared to the control groups. All these suggest that upregulation and overexpression of MALAT-1 contributes to the occurrence and development of CC.

In conclusion, to the best of our knowledge, this study is the first to correlate higher levels of MALAT-1 with CC progression and tumorigenesis. Our study demonstrates that the levels of MALAT-1 were significantly higher in CC tissues and cell lines compared to their normal counterparts and controls. Also, our study suggests that MALAT-1 is involved in tumor progression in CC, including cell growth, migration, and invasion. Moreover, we speculate that MALAT-1 may act as a prognostic marker for CC and intervention of MALAT-1 function may have potential diagnostic implications in the prevention of CC.

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