European Review for Medical and Pharmacological Sciences 2015; 19: 3187-3193

High MALAT1 expression predicts a poor prognosis of cervical cancer and promotes cancer cell growth and invasion

L. YANG[1], H.-S. BAI[1,2], Y. DENG[1], L. FAN[3]

[1]Department of Oncology, Sichuan Provincial People's Hospital, School of Clinical Medicine of University of Electronic Science and Technology of China, Chengdu, China
[2]Graduate School, Zunyi Medical College, Zunyi, China
[3]Center of Radiotherapy, Sichuan Cancer Hospital, Chengdu, China

Abstract. – OBJECTIVE: Although the oncogenic role of long non-coding RNA, MALAT1 in cervical cancer is gradually recognized, the clinical and prognostic significance of this lncRNA in cervical cancer has not been reported yet. This study aimed to investigate the clinical significance and biological functions of MALAT1 in cervical cancer.

PATIENTS AND METHODS: MALAT1 expression in 104 cervical cancer tissues and matched adjacent normal tissues, as well as in 50 HPV negative heathy cervical tissues were quantified using qRT-PCR. Its association with overall survival of the cancer patients was analyzed using the Log-rank (Mantel-Cox) test and the Cox proportional hazards model. In addition, the effect of MALAT1 on cell proliferation and invasion was further studied in Hela and CaSki cells.

RESULTS: MALAT1 expression is significantly increased in cervical cancer than in normal tissues. Its expression in the cancerous tissues is also significantly higher than in adjacent normal tissues. MALAT1 expression is correlated with tumor size, FIGO stage, vascular invasion and lymph nodes metastasis and is an independent predictor for overall survival of cervical cancer. When endogenous MALAT1 was knocked down, the cancer cells had significantly reduced proliferation and invasion and increased apoptosis.

CONCLUSIONS: MALAT1 might be an important marker of prognosis and a potential therapeutic target of cervical cancer.

Key Words:
MALAT1, Prognosis, Cervical cancer, Growth, Invasion.

Introduction

Cervical cancer is the third most common malignancy in women across the world[1]. The carcinogenesis of cervical cancer is closely related to persistent infection of high risk human papillomavirus (HR-HPV)[2]. Approximately 70% of cervical cancer cases are associated with infection of HPV-16 and HPV-18[3]. A large proportion of cervical cancer death is related to regional or distant metastasis[1]. However, how HR-HPV infection may result in dysregulated oncogenic process and the exact mechanisms involved in development and invasion of cervical cancer is still not quite clear.

Long non-coding RNAs (lncRNAs) are non-coding transcripts with over 200 nucleotides in length. Recently, emerging studies showed that lncRNAs are important molecules not only in normal development but also in tumorigenesis[4-7]. Some recent studies demonstrated that lncRNAs may play vital roles in pathological development of cervical cancer and also may act as indicators of poor prognosis[8]. For example, high level of circulating HOTAIR[9], decreased GAS5[10] and decreased XLOC_010588[11] predict poor prognosis of cervical cancer. MALAT 1 (metastasis associated lung adenocarcinoma transcript 1) is a large, infrequently spliced non-coding RNA aberrantly expressed in cervical cancer[12,13]. In fact, its upregulation is generally related to growth and metastasis of several types of cancer, such as colorectal cancer[14], pancreatic cancer[15] and gastric cancer[16]. One recent study found HPV infection, which is a leading cause of cervical cancer development, is associated with significantly increased MALAT1 expression[12]. Although the oncogenic role of MALAT1 in cervical cancer is gradually recognized, the clinical and prognostic significance of this lncRNA in cervical cancer has not been reported yet.

Therefore, in the current study, we aimed to investigate the clinical significance and biological functions of MALAT1 in cervical cancer.

Corresponding Author: Lin Fan, MD; e-mail: linfani1@126.com

Patients and Methods

Patients and Specimens

This study was approved by the Ethics Committee of Sichuan People's Hospital, China. A total of 104 patients with cervical cancer were recruited from the hospital from 2009 to 2010. All of the patients never received preoperative radiotherapy and/or chemotherapy before this study. All patients were diagnosed as infiltrating carcinoma by pathology. Tumor and adjacent normal tissues were obtained from the patients by using cervical biopsy with their informed consent. The tumor stage was examined by two experienced gynecological oncologists without authorship in this study. The assessment was performed according to the International Federation of Gynecology and Obstetrics (FIGO) staging system for cervical cancer. Besides, 50 cases of HR-HPV-negative normal cervical squamous epithelium were obtained from participants who had HR-HPV test and cervical thinprep cytological test with their informed consent. Clinical and pathological variables of the cancer cases were analyzed and summarized in Table I. The 104 patients were regularly followed up, with a median follow-up of 30 months (range: 8-60 months).

Cell Culture

Human cervical cancer cell lines, HeLa and CaSki cells were grown in RPMI-1640 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA). All cells were cultured in a cell incubator with humidified atmosphere and 5% CO_2 at 37°C.

Cell Transfection

To knockdown of endogenous MALAT1, Hela and CaSki cells were transfected with 100 nM si-MALAT1 (No. 1 and No. 2, Valencia, CA, USA, QIAGEN) or the negative control siRNA (MALAT1 si-NC; Valencia, CA, USA, QIAGEN) according to the manufacturer's instructions. 48 hours after transfection, MALAT1 level was examined using qRT-PCR. The siRNA with stronger knockdown effect was used for the rest studies.

qRT-PCR Analysis of MALAT1 Expression

Total RNA from cervical cancer and normal tissues were extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. cDNA was reversely transcribed using a First Strand Synthesis kit (Invitrogen, Carlsbad, CA, USA). qRT-PCR analysis of MALAT1 were performed using MALAT1 specific primers: (forward, 5'-AAAGCAAGGTCTCCCCACAAG-3', reverse, 5'-GGTCTGTGCTAGATCAAAAGGCA-3') and Power SYBR Green PCR Master Mix in an ABI Prism 7500 (Applied Biosystems, Foster City, CA, USA). GAPDH served as the endogenous control. The expression change was calculated using $2^{-\Delta\Delta CT}$ method.

MTT Assay of Cell Proliferation

Hela or CaSki cells with MALAT1 knockdown were seeded in 96-well plates (2×10^3 per well). At indicated time points (0, 24, 48 and 72h), cells were stained with 100 μl sterile MTT dye (0.5 mg/ml, Sigma) for 4 hours at 37°C. Then the culture was replaced by 150 μL DMSO (Sigma, St. Louis, MO, USA). The absorbance at 570 nm was measured. All experiments were performed in triplicate.

Transwell Analysis of Cell Invasion

Cell invasion assay was performed using the Transwell insert chamber coated with Matrigel (BD Biosciences, Bedford, MA, USA). Briefly, 1×10^5 Hela or CaSki cells were suspended in 200 μL serum free RPMI-1640 medium and then seeded into the upper chamber. The lower chamber was filled with RPMI-1640 with 20% FBS to form a chemoattractant environment. The chamber was maintained in a cell incubator for 24 hours. Then, cells on the top surface of the insert were removed with a cotton swab. The cells on the bottom surface were fixed with 4% polyoxymethylene and stained with 0.1% crystal violet. Then, the number of invading cells were counted. Each experiment was performed in triplicate.

Flow Cytometry Analysis of Apoptotic cells

48 hours after transfection with si-MALAT1, Hela and CaSki cells were harvested and fixed in 70% ice-cold ethanol at 4°C for 24 hours. The ratio of cells with active caspase-3 was measured using Fluorescein Active Caspase 3 Staining Kit (ab65613, Abcam, Cambridge, MA, USA) in a flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA, USA). Data acquisition was performed using CellQuest 3.2 software (BD Bioscience, San Jose, CA, USA). Each experiment was performed in triplicate.

Statistical Analysis

All statistical analysis was performed using SPSS 17.0 software package (IBM, Chicago, IL, USA).

MALAT1 expression between cervical tumor tissues and normal cervical tissues from healthy participants were compared using unpaired Mann-Whitney test, while MALAT1 expression between cervical tumor tissues and adjacent normal mucosa was compared using paired Wilcoxon test. The relationship between MALAT1 expression and clinicopathological characteristics was assessed using Pearson's χ^2 test. Survival curves were estimated by the Kaplan-Meier method. The Log-rank (Mantel-Cox) test was used to estimate the statistical differences between survival curves and to make univariate analysis of the association between clinicopathological parameters and overall survival. The Cox proportional hazards model was used to make multivariate survival analysis for all of the significant parameters observed in the univariate analysis. A two-sided p value of <0.05 was considered statistically significant. *, ** and *** donate significance at 0.05, 0.01 and 0.001 level respectively.

Results

Malat1 is Significantly Upregulated in Cervical Cancer

To identify MALAT1 expression in cervical cancer cases, we firstly quantified its expression in 104 cervical cancer cases and 50 healthy controls using qRT-PCT. The results showed that MALAT1 expression was significantly up-regulated in the cancerous tissues than in the normal tissues (Figure 1A). Besides, we also compared MALAT1 expression between tumor tissues and matched adjacent normal tissues. The results revealed significantly upregulated MALAT1 expression in the tumorous than in the non-tumorous tissues (Figure 1B). These results suggest MALAT1 is significantly upregulated in cervical cancer.

Correlations between the MALAT1 expression and the clinicopathological factors of cervical cancer

To assess to association between MALAT1 expression and the clinicopathological parameters, the 104 patients were divided into high MALAT1 expression (higher than the median level, n=52) and low MALAT1 expression (lower than the median level, n=52) groups according to the median level of MALAT1 expression (3.075). Comparisons were performed between the two groups (Table I). The results showed that high MALAT1 expression was significantly correlated with tumor size (p=0.005), FIGO stage (p=0.01), vascular invasion (p=0.01) and lymph node metastasis

Table I. The association between MALAT1 expression and clinicopathological features of cervical cancer.

Parameters	Case number	MALAT1 expression				*p* value
		High	%	Low	%	
Age						
≤40	46	21	20%	25	24.0%	0.43
>40	58	31	29.8%	27	26.0%	
Tumor size (cm)						
≤4	68	27	26%	41	39.4%	**0.005**
>4	36	25	24.0%	11	10.6%	
Histology						
Squamous	86	42	40.4%	44	42.3%	0.60
Adenocarcinoma	18	10	9.6%	8	7.7%	
FIGO stage						
Ib-IIa	57	22	21.2%	35	33.7%	**0.01**
IIb-IIIa	47	30	28.8%	17	16.3%	
Differentiation						
Well	27	11	10.6%	16	15.4%	0.49
Moderate	34	19	18.3%	15	14.4%	
Poor	43	22	21.2%	21	20.2%	
Vascular invasion						
Yes	38	26	25.0%	12	11.5%	**0.01**
No	66	26	25.0%	40	38.5%	
Lymph nodes metastasis						
Yes	43	31	29.8%	12	11.5%	**0.0002**
No	61	21	20.2%	40	38.5%	

FIGO, International Federation of Gynecology and Obstetrics; Bold indicates significant values.

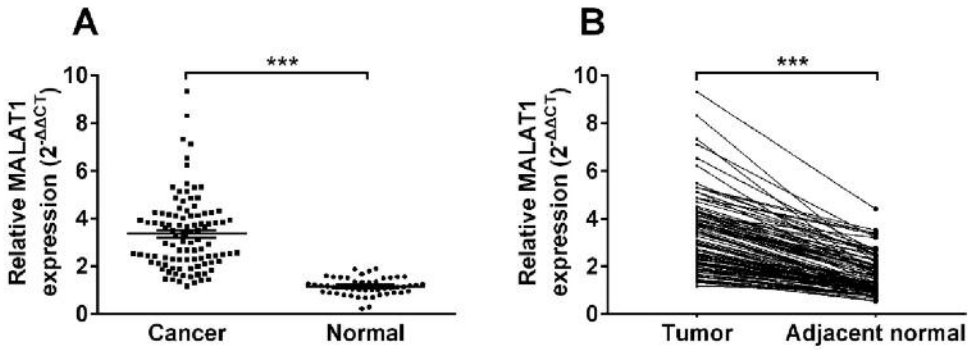

Figure 1. MALAT1 expression is significantly upregulated in cervical cancer. ***A,*** qRT-PCR analysis of MALAT1 expression in cervical tumor tissues from 104 patients and normal tissues from 50 HPV negative healthy controls. ***B,*** qRT-PCR analysis of MALAT1 expression in cancerous tissues and adjacent normal tissues in the 104 patients. $^{*}p<0.05$; $^{**}p<0.01$, $^{***}p<0.001$.

(p=0.0002), but not with other clinical characteristics, including age, histology and differentiation (Table I).

Association Between MALAT1 Expression and Prognosis of Cervical Cancer Patients

Overall survival curves and recurrence free survival curves in the high MALAT1 group and low MALAT1 group were given in Figure 2. Cervical cancer patients with high MALAT1 expression had significantly poorer overall survival (p<0.001) (Figure 2A) and recurrence free survival (p<0.001) (Figure 2B) than the counterparts with low MALAT1 expression. Univariate analysis showed that tumor size (>4 cm *vs.* ≤4 cm), FIGO stage (IIb-IIIa *vs.* Ib-IIa), lymph nodes metastasis (Yes *vs.* No), vascular invasion (Yes *vs.* No) and MALAT1 expression (High *vs.* Low) were five prognostic factors (Table II). When applying multivariate analysis using the Cox proportional hazards model, it was found that tumor size, FIGO stage, lymph node metastasis and MALAT1 expression were independent prognostic factors for overall survival (Table II).

MALAT1 can Modulate Proliferation, Apoptosis and invasion of Cervical Cancer Cells

Considering MALAT1 is an independent prognostic factors for overall survival of cervical cancer, we further explored its role in cancer cell growth and invasion. Both Hela and CaSki cells had significantly higher MALAT1 expression than the normal tissues (Figure 3A). MALAT1

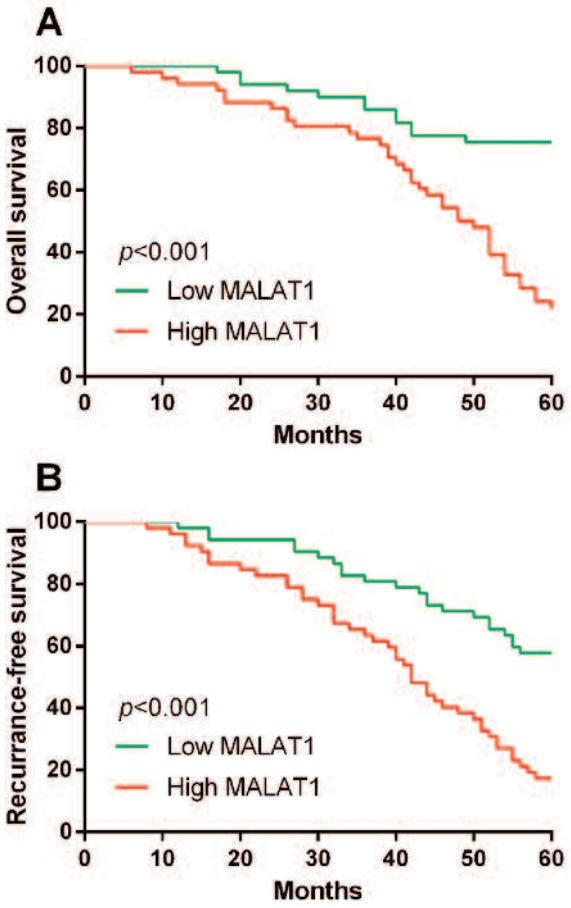

Figure 2. Higher MALAT1 is associated with poorer overall survival and recurrence free survival. Kaplan-Meier curves for overall survival ***(A)*** and recurrence free survival ***(B)*** in cervical cancer patients divided according to median MALAT1 expression. Patients with in high MALAT1 group had significantly shorter survival times than those with low MALAT1 expression. p-value was calculated by log-rank test.

was knocked down in these two cell lines using MALAT1 siRNA (Figure 3 B and C). MTT assay revealed that both Hela and CaSki cells with MALAT1 knockdown had significantly lowered cell growth rate (Figure 3 D and E). By performing transwell assay and flow cytometry analysis, we also confirmed that MALAT1 knockdown led to significantly decreased cell invasion (Figure 3 F and H) and significantly increased cell apoptosis (Figure 3G and I). These results indicated that MALAT1 is an important lncRNA modulating growth and invasion of cervical cancer cells.

Discussion

Cervical cancer remains the leading cause of death of female malignancy. Therefore, it is necessary to find new molecular targets for diagnosis, prognosis and treatment. In this study, we confirmed that MALAT1 expression is significantly increased in cervical cancer than in normal tissues. In addition, we also found its expression in the cancerous tissues is significantly higher than in adjacent normal tissues. Furthermore, we showed that MALAT1 expression is correlated with tumor size, FIGO stage, vascular invasion and lymph nodes metastasis. More importantly, we demonstrated that MALAT1 expression is an independent predictor for overall survival of cervical cancer. Therefore, MALAT1 might be an important molecule involved in the development and progression of cervical cancer.

The oncogenic roles of MALAT1 have been reported in several types of cancer. As an lncRNA, its oncogenic roles might be quite complex and it may involve in different signal pathways in different cancers. For example, MALAT1 can promote tumor growth and metastasis of colorectal cancer through binding to SFPQ (PTB-associated splicing factor)[14], releasing oncogene PTBP2 (polypyrimidine-tract-binding protein) from SFPQ/PTBP2 complex[14], and through PRKA kinase anchor protein 9[17]. In gallbladder cancer, MALAT1 can promote proliferation and metastasis of cancer cells through ERK/MAPK pathway[18]. In bladder cancer, MALAT1 can promote cancer metastasis by associating with suppressor of zeste 12 (suz12)[19]. Due to the tumorigenic effects, the clinical significance of MALAT1 is also reported in different cancers. For instance, high MALAT1 expression is associated with high stage, metastasis, and shorter overall survival after radical nephrectomy in patients with renal cell carcinoma[20]. Blood MALAT1 level might be considered as a biomarker for the diagnosis of non-small cell lung cancer[21]. MALAT1 level in the cancer tissues is

Table II. Univariate and multivariate analysis of clinicopathological factors for overall survival.

Clinicopathological factors	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	*p*-value	Hazard ratio (95% CI)	*p*-value
Age (>40 vs. ≤40)	1.191 (0.679-2.089)	0.542		
Tumor size (>4 cm vs. ≤ 4 cm)	3.133 (1.784-5.501)	**< 0.0001**	2.149 (1.162-3.974)	**0.015**
Histology (Squamous vs. Adenocarcinoma)	0.56 (0.298-1.055)	0.073		
FIGO stage (IIb-IIIa vs. Ib-IIa)	4.907 (2.64-9.12)	< 0.0001	3.035 (1.516-6.076)	**0.002**
Differentiation (Poor vs. Well+moderate)	1.568 (0.9-2.734)	0.112		
Lymph nodes metastasis (Positive vs. Negative)	4.767 (2.625-8.657)	**< 0.0001**	2.231 (1.138-4.375)	**0.020**
Vascular invasion (Yes vs. No)	2.15 (1.231-3.755)	**0.007**	0.799 (0.423-1.509)	0.489
MALAT1 expression (High vs. Low)	4.226 (2.197-8.127)	**< 0.0001**	2.214 (1.073-4.567)	**0.031**

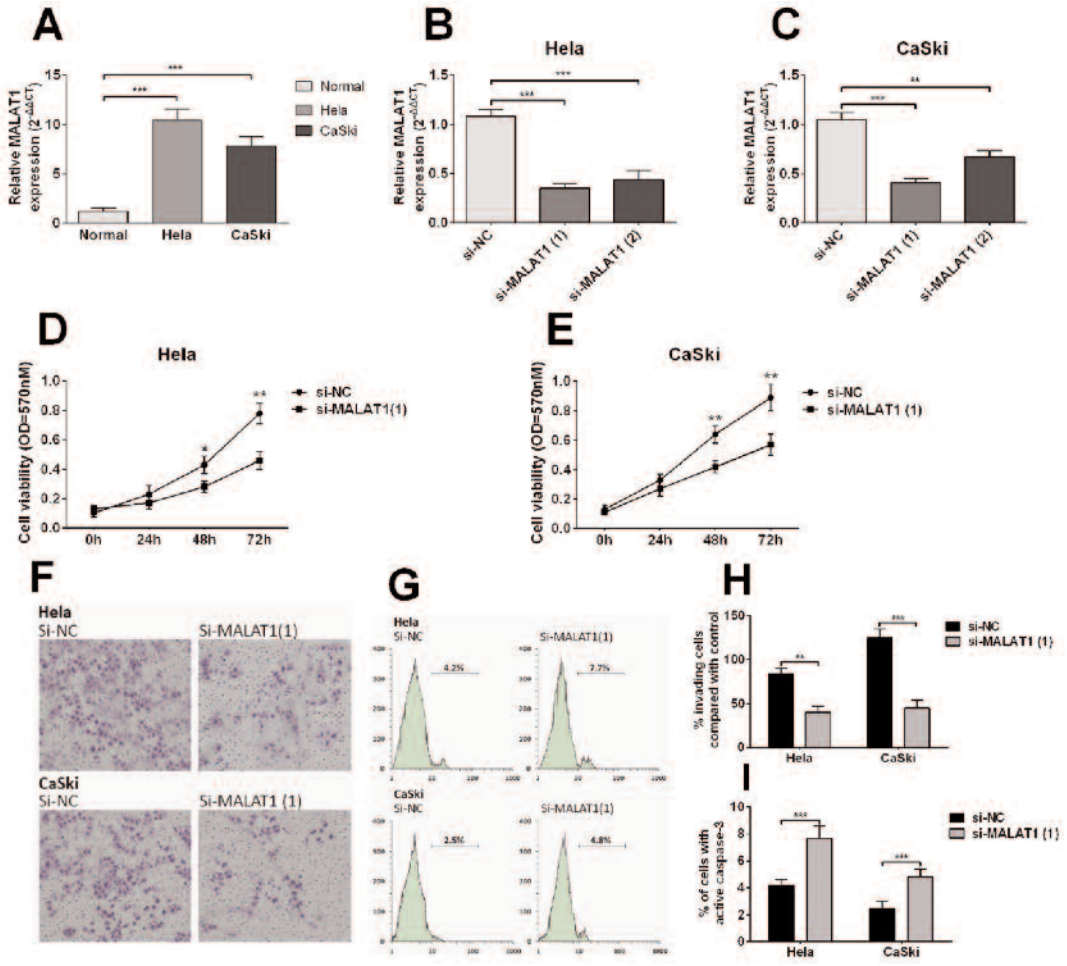

Figure 3. MALAT1 modulates growth, invasion and apoptosis of cervical cancer cells. ***(A)*** qTR-PCR analysis of MALAT1 expression in Hela and CaSki cells and comparison with average expression in the 50 HPV negative healthy controls. ***(B*** and ***C)*** qRT-PCR analysis of MALAT1 expression in Hela ***(B)*** and CaSki cells ***(C)*** transfected with MALAT1 siRNA. ***(D*** and ***E)*** MTT assay of cell viability 72 hours after transfection in in Hela ***(D)*** and CaSki cells ***(E)***. ***(F)*** Representative images of invaded Hela and CaSki cells after transfection with MALAT1 siRNA in transwell invasion assay. ***(G)*** Representative images of Hela and CaSki cells with active caspase-3 after transfection with MALAT1 siRNA in flow cytometry analysis. (H and I) Quantification of invaded Hela and CaSki cells showed in figure F ***(H)*** and apoptotic Hela and CaSki cells showed in figure G (I). $^{*}p<0.05$; $^{**}p<0.01$, $^{***}p<0.001$.

correlated with clinical progression and unfavorable prognosis of pancreatic cancer[15], colorectal cancer[14] and glioma[22].

In cervical cancer, MALAT1 is generally upregulated due to HPV infection[12]. Knockdown of MALAT1 in CaSki cells simultaneously reduced the expression of three cell cycle regulation molecules cyclinD1, cyclinE and CDK6, leading to enhanced cells arrested in G1 phase[12]. In this study, we further explored the effect of MALAT1 in cell growth and invasion of Hela and CaSki cells. Both of the cell lines with MALAT1 knockdown had significantly reduced proliferation and invading capability. Besides, knockdown of endogenous MALAT1 also enhanced apoptosis of these two cell lines.

Conclusions

This study indicated that MALAT1 is an independent prognosis factor for overall survival of cervical cancer. When knocked down, the cervical cancer cells had significantly reduced proliferation and invasion and increased apoptosis. Therefore, MALAT1 might be an important marker of prognosis and a potential therapeutic target of cervical cancer. However, the underly-

ing oncogenic mechanisms of MALAT1 should be further studied to fully reveal its biological functions.

Acknowledgements

All of the authors have no potential conflicts of interest.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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