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Inhibition of metastasis-associated lung adenocarcinoma transcript 1 in CaSki human cervical cancer cells suppresses cell proliferation and invasion

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Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is suggested to be a long (∼7 kb) non-coding RNA. MALAT1 is overexpressed in many human carcinomas, but its function remains unknown. To investigate the role of MALAT1 in human cervical cancer progression, we designed and used short hairpin RNA to inhibit MALAT1 expression in CaSki cells and validated its effect on cell proliferation and invasion. Changes in gene expression were analyzed by reverse transcriptase–polymerase chain reaction. Our data demonstrated that MALAT1 was involved in cervical cancer cell growth, cell cycle progression, and invasion through the regulation of gene expression, such as caspase-3, -8, Bax, Bcl-2, and Bcl-xL, suggesting that MALAT1 could have important implications in cervical cancer biology. Our findings illustrate the biological significance of MALAT1 in cervical cancer progression and provide novel evidence that MALAT1 may serve as a therapeutic target in the prevention of human cervical cancer.

Introduction

Cervical carcinoma is one of the highest causes of mortality in female cancer patients worldwide, with ∼470,000 new cases and 231,000 deaths occurring each year, and an increase in relatively young women [1,2]. It is important to understand the mechanisms of tumorigenesis and development for prevention and cure of tumor.

It has been generally assumed that cancer is the result of changes in the structure and/or expression of protein coding genes. However, thousands of genes that produce non-coding RNA (ncRNA) transcripts were found in the past few years [3,4]. Small ncRNAs, such as micro-RNAs (miRNAs), have recently been associated with tumorigenesis by acting either as tumor suppressors or oncogenes

[5,6]. Although few long ncRNAs have been assigned a function, a significant number of these transcripts have been shown to exhibit cell type-specific expression and association with cancers [7,8].

Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) lacks open reading frames of significant length, and the *in vitro* translation of MALAT1 yielded no peptides, suggesting that MALAT1 functions as a long (∼7 kb) ncRNA [9,10]. MALAT1 was originally identified to have overexpression in the early-stages of non-small cell lung cancers that subsequently metastasize compared with those that do not. MALAT1 shows broad expression in normal human and mouse tissues and is overexpressed in many human carcinomas, including those of the breast, pancreas, lung, colon, prostate, and liver, implying that MALAT1 misregulation may play a role in the development of numerous cancers [11].

In the present study, we evaluated the role of MALAT1 in CaSki human cervical cancer cells using RNA interference (RNAi) approach. Our data showed that MALAT1 might participate in the regulation of proliferation and invasion of the CaSki cells through the modulation of caspase-3, -8, Bax, Bcl-2, and Bcl-xL.

Materials and Methods

Cell culture

CaSki human cervical cancer cells were maintained by our own lab and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, USA) supplemented with 10% bovine calf serum (Gibco). Cells were maintained at 37°C in an atmosphere of humidified air with 5% CO2.

Construction of MALAT1 siRNA expression vectors

To knockdown MALAT1 expression, we used pRNAT-U6.1/Neo vector encoding a small hairpin RNA directed against the target gene in CaSki cells. The target sequences for MALAT1 were 5′-GACCTTGAAATCCA-TGACG-3′ (CaSki/M1), 5′-GAAGCCGAAATAAATGA-GA-3′ (CaSki/M2), 5′-GGTCAAGAGAAGTGTCAGC-3′

(CaSki/M3). As a negative control, we used shRNA vector without hairpin oligonucleotides (CaSki/NC), as described elsewhere [12,13].

Cell infection

CaSki cells were grown till 70–80% confluence and infected with siRNA MALAT1, and harvested 48 h after infection, followed by limited dilution in 96-well plates for the generation of individual cell clones. Three weeks later, the levels of MALAT1 mRNA in cell clones that had been infected with siRNA MALAT1 were characterized by reverse transcriptase–polymerase chain reaction (RT–PCR) analysis.

Methyl-thiazolyl-tetrazolium assay

Cells at 10^3/well were cultured in 96-well plates with 10% FBS DMEM at 37°C 5% CO_2 for varying periods of time and exposed to fresh media every other day. During the last 4 h of each day culture, the cells were treated with methyl thiazolyl tetrazolium (MTT) (Sigma, St. Louis, USA) at 50 μg/well. The generated formazan was dissolved in DMSO, and OD at 490 nm was measured to determine the cell viability.

Colony formation assay

The effect of MALAT1 silence on the colony formation of CaSki cells was analyzed by colony formation assay [14]. Briefly, cells (100 cells/well) in 6 cm plates were cultured in 10% FBS DMEM at 37°C 5% CO_2 for 3 weeks. The cell colonies were washed twice with PBS, and then fixed with 4% paraformaldehyde for 15 min and stained with Gimsa for 30 min. Individual clones with more than 50 cells were counted. Clone forming efficiency for individual type of cells was calculated according to the following formula: clone forming efficiency = number of colonies/number of inoculated cells × 100%.

Flow cytometry analysis of cell cycle

The impact of MALAT1 silence on the CaSki cell cycling was examined by flow cytometry analysis. Cells were harvested at 70–80% confluence and resuspended in fixation fluid at a density of 10^6 cells/ml, 1500 μl propidium iodide solution was added, and the cell cycle was detected by FACS Caliber (Becton Dickinson, San Jose, USA).

Monolayer wound healing assay

The effect of MALAT1 silence on the migration of CaSki cells was determined by monolayer wound healing assay. Cells were grown in 10% FBS in DMEM in 60 mm plates. After the cells reached subconfluence, the monolayer cells were wounded by scraping off the cells and then grown in medium for 48 h. The migrated distance of CaSki cells was monitored and imaged under a microscope. The distances of cell migration were calculated by subtracting the distance between the lesions' edges at 48 h from the distance measured at 0 h. The relative migrating distance of cells is measured by the distance of cell migration/the distance measured at 0 h.

In vivo tumor growth assay

The influence of MALAT1 silence on the tumor development of cervical carcinoma *in vivo* was examined. Briefly, CaSki, CaSki/NC, or CaSki/M at 5×10^6 cells per mouse were injected subcutaneously into 4 weeks old BALB/c nude mice ($n = 4$ per group; Shanghai Laboratory Animal Center, Shanghai, China). The experimental pairs (CaSki, CaSki/NC, and CaSki/M) were done in different mice. The development and growth of solid tumors were monitored by measuring tumor size using a vernier caliper in a blinded fashion every 5 days for a 40-day period. The tumor volume was calculated using a standard formula [15]: tumor volume = $width^2 \times length \times 0.5$. At the end of the experiment, all mice were sacrificed and individual tumor weights were measured.

Semi-quantitative RT–PCR analysis

RNA isolated from cells was reverse-transcribed and amplified using the one-step RT–PCR System (Fermentas, Vilnius, Lithuania). Primer sequences used were shown in **Table 1**. After heating at 95°C for 1 min, samples were exposed to 30 cycles (GAPDH, 25 cycles) of 95°C for 30 s, 60°C for 30 s, and 68°C for 1 min 30 s with a final extension at 68°C for 10 min. The integrated density value (IDV) of each band was assessed using Gel-Pro Image Analyzer (Bio-Rad, Hercules, USA), and the ratios of IDV (MALAT1/GAPDH, caspase-3/GAPDH, caspase-8/GAPDH, caspase-9/GAPDH, Bax/GAPDH, Bcl-2/GAPDH,

Table 1 Primer pairs used in semi-quantitative RT–PCR

Gene	Primer sequence (5′→3′)	
Caspase-3	Forward	CAAAGATCATACATGGAAGCG
	Reverse	TGAAAAGTTTGGGTTTTCCAG
Caspase-8	Forward	AATGAAAAGCAAACCTCGGG
	Reverse	ATGTACCAGGTTCCCTCTGCA
Caspase-9	Forward	TGGTGGAAGAGCTGCAGGT
	Reverse	TGGGCAAACTAGATATGGCGT
Bax	Forward	ATGAAGACAGGGGCCCTTT
	Reverse	ATGGTGAGTGAGGCGGTGA
Bcl-2	Forward	CATCCATTATAAGCTGTCGCA
	Reverse	TGCCGGTTCAGGTACTCAGT
Bcl-xL	Forward	AACTCTTCCGGGATGGGGTAA
	Reverse	AATTCTGAGGCCAAGGGAACT
GAPDH	Forward	AATCCCATCACCATCTTCCA
	Reverse	CCTGCTTCACCACCTTCTTG

and Bcl-xL/GAPDH) were calculated to evaluate the relative expression levels of the mRNA.

Statistical analysis

Data were expressed as the mean $\pm$ SEM. The difference among groups was determined by ANOVA analysis and the comparison between two groups was analyzed by the Student's *t*-test using the GraphPad Prism software version 4.0 (GraphPad Software, Inc., San Diego, USA). A value of $P < 0.05$ was considered as statistically significant.

Results

MALAT1 expression inhibition in CaSki cells

To investigate the biological significance of MALAT1 on CaSki cells, we constructed three siRNA expression vectors (CaSki/M1, CaSki/M2, and CaSki/M3) specific to MALAT1 transcripts and transfected them into CaSki cells. A knockdown effect was observed by RT–PCR, and we found that CaSki/M1 (used as "CaSki/M" in following experiments) was significantly effective compared with the negative control CaSki/NC or the parental CaSki group [**Fig. 1(A,B)**]. The successful establishment of MALAT1 gene silent CaSki cell clone provided a useful tool for investigating the function of MALAT1 on the growth of CaSki cells.

Effect of down-regulated MALAT1 expression on CaSki cell growth

To investigate the possible function of MALAT1 on the growth of CaSki cells, the dynamics of CaSki, CaSki/NC, and CaSki/M cell growth were determined by MTT assay [**Fig. 2(A)**]. Following a 7-day period, the growth of CaSki/M cells was much slower, when compared with control groups. The marked decrease in cell viabilities was observed on Day 4. Reduction in MALAT1 expression significantly impaired the proliferation ability of CaSki cells. The other pattern of inhibitory effect of reduced MALAT1
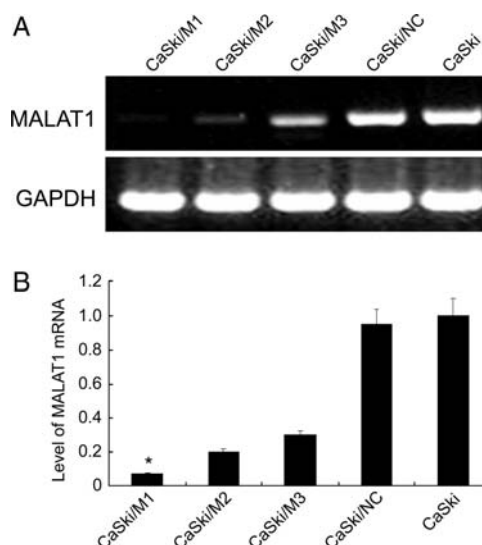

Figure 1 RT–PCR confirmed the knockdown effect on MALAT1 expression in CaSki cells (A) RT–PCR analysis of MALAT1. (B) Bar graph of the relative expression of MALAT1. Data are representatives or expressed as the mean $\pm$ SEM of three independent experiments. *$P < 0.05$ versus CaSki or CaSki/NC.

expression in CaSki cells was achieved in colony formation assay [**Fig. 2(B)**]. As a result, the average colony number of CaSki/M cells decreased compared with CaSki/NC or CaSki cells. Therefore, the low cell viability detected in MTT assay and the low number of cell colonies from CaSki/M cells demonstrated that the down-regulation of MALAT1 expression inhibits the growth of CaSki cells.

Impact of down-regulated MALAT1 expression on CaSki cell cycle

To further explore the cause for the decrease in cell viability, we examined the effects of MALAT1 RNAi on cell cycle. As shown in **Fig. 3**, CaSki cells with MALAT1 siRNA showed the blocking of the cell cycle in G_1 phase. The G_0/G_1-phase fraction increased from 57.3% (CaSki/NC) to 87.1% (CaSki/M). These data indicated that the down-
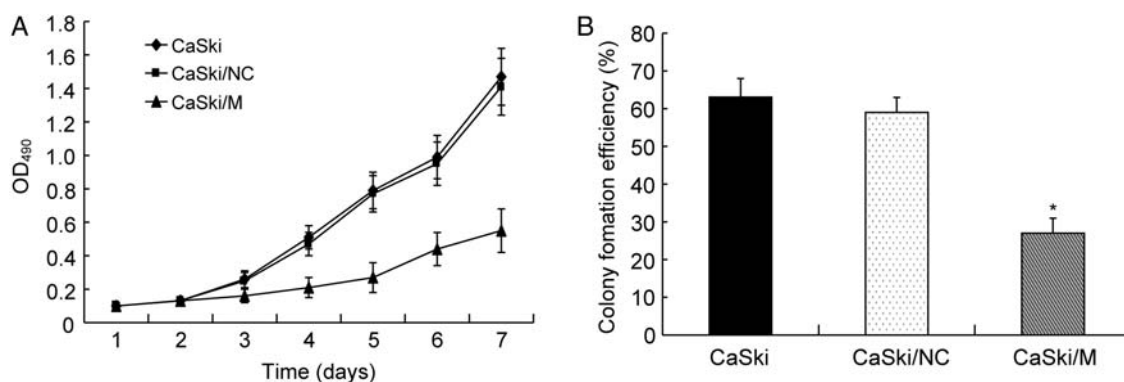
Figure 2 Effect of MALAT1 on CaSki cell growth (A) Proliferation of CaSki cells. CaSki, CaSki/NC, and CaSki/M cells at 10^3 cells/well were in triplicate cultured in 10% FBS DMEM and their growth dynamics were determined daily by MTT. (B) Quantitative analysis of colony formation. Data are expressed as the efficiency of colony formation (%) and expressed as the mean $\pm$ SD of three separated experiments. *$P < 0.05$ versus CaSki or CaSki/NC.
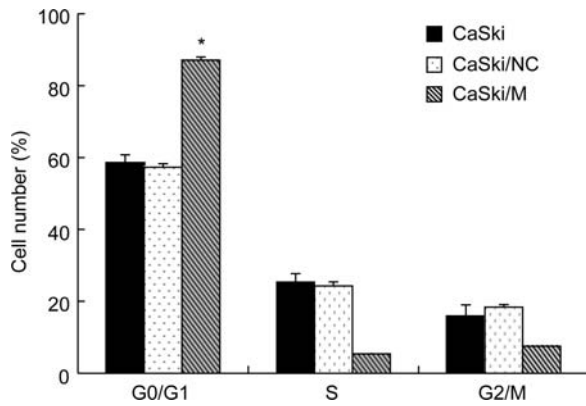
Figure 3 Analysis of CaSki cell cycle CaSki, CaSki/NC, and CaSki/M cells were fixed with 70% ethanol and stained with propidium iodide, followed by FACS analysis. Data are representatives or expressed as the mean $\pm$ SEM of three independent experiments. *$P < 0.05$ versus CaSki or CaSki/NC.

regulation of MALAT1 expression arrests CaSki-RNAi cell cycling at the G_0/G_1 phase, which may inhibit the growth of CaSki cells.

## Effect of down-regulated MALAT1 expression on the migration of CaSki cells

We examined the impact of MALAT1 expression on the migration of CaSki cells by the wound healing assay [**Fig. 4(A)**]. Following incubation of physically

wounded cells for 48 h, the mobile distance of CaSki/M cells was significantly shorter than those of controls [**Fig. 4(B)**].

Impact of reduced MALAT1 expression on the tumor development of inoculated CaSki cells *in vivo*

To determine the role of MALAT1 in the tumorigenicity of CaSki cells and development of solid cervical tumors, CaSki, CaSki/NC, or CaSki/M cells were injected subcutaneously into nude mice. The development of solid cervical tumors was monitored for 40 days. As shown in **Fig. 5(A)**, the developed solid tumors were first visible at about 10 days post-inoculation and then rapidly grew in CaSki and CaSki/NC groups. In contrast, the development of CaSki/M cell-related solid tumors grew slowly and the volume of solid cervical tumors of CaSki/M group decreased, when compared with those of control groups. As a result, the weight of tumors of CaSki/M group was significantly lighter than those of control groups [**Fig. 5(B)**]. Collectively, these data evidenced that down-regulation of MALAT1 expression mitigated the tumorigenicity of CaSki cells *in vivo*.
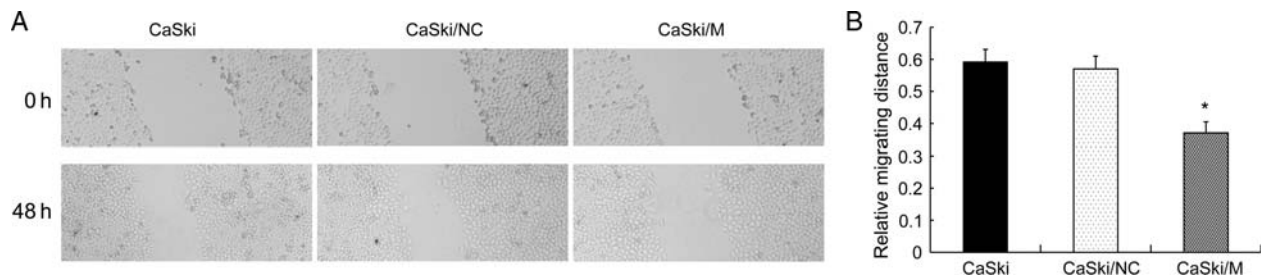
Figure 4 Effect of MALAT1 expression on CaSki cell migration CaSki, CaSki/NC, and CaSki/M cells were cultured in DMEM containing 10% FBS and wounded physically. Following continually cultured for 48 h for recovery, the migrated distance of CaSki cells was measured. (A) The wounded and healing CaSki cells. (B) Relative measurement of migration distance. Data are expressed as the mean $\pm$ SD of three independent experiments. *$P < 0.05$ versus CaSki or CaSki/NC.
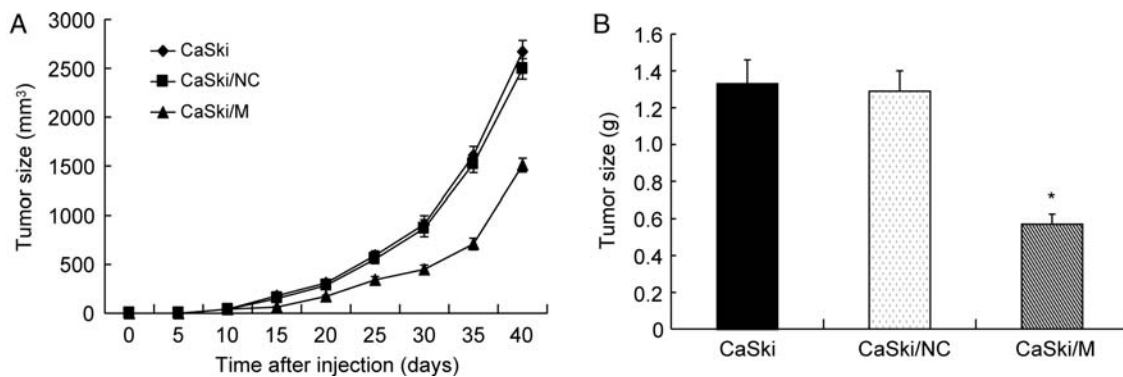
Figure 5 Tumor formation of MALAT1 inhibited CaSki cells *in vivo* (A) Tumor volume was measured every 5 days. (B) Forty days after tumor cell injection, mice were sacrificed and tumor weight was recorded. Data are representatives or expressed as the mean $\pm$ SEM of three independent experiments. *$P < 0.05$ versus CaSki or CaSki/NC.
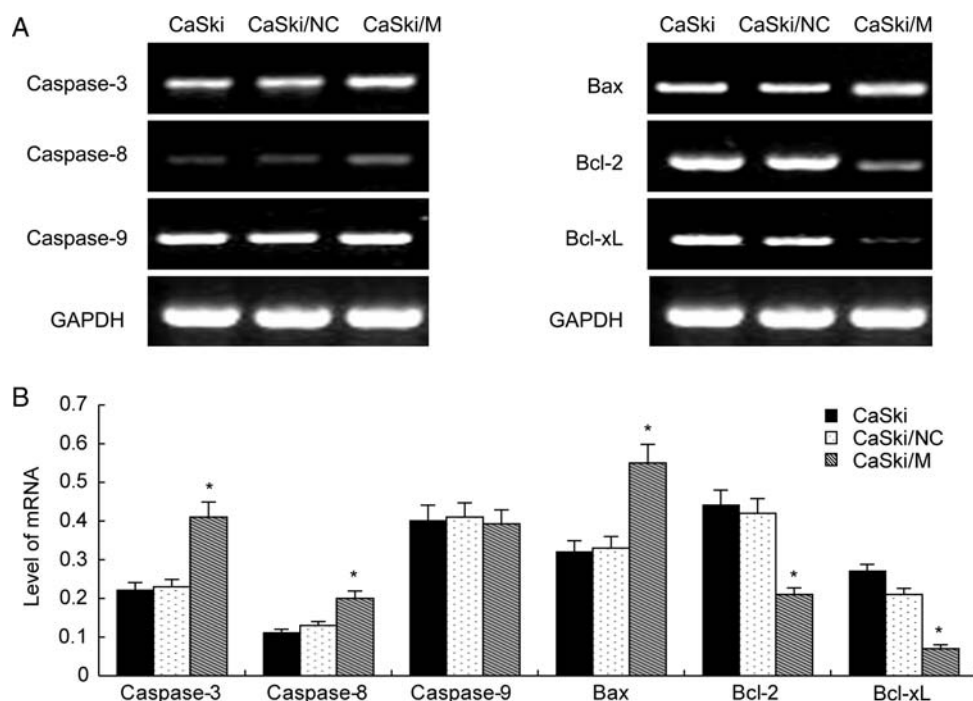
Figure 6 Down-regulation of MALAT1 induced the expression of caspase-3, -8, Bax, and suppressed the expression of Bcl-2 and Bcl-xL in CaSki cells Total RNA was extracted and analyzed by semi-quantitative RT−PCR. In each case, the amount of transcript determined was normalized to the GAPDH mRNA level. (A) Photographs of semi-quantitative RT−PCR. (B) Bar graph of the relative expression of caspase-3, -8, -9, Bax, Bcl-2, and Bcl-xL in CaSki, CaSki/NC, and CaSki/M cells. Four different experiments were considered for densitometry, and the data are expressed as the mean ± SD. *$P < 0.05$ versus CaSki or CaSki/NC.

Down-regulation of MALAT1 induced the expression of caspase-3, -8, Bax, and suppressed the expression of Bcl-2 and Bcl-xL

The molecular basis for cell growth inhibition in CaSki cells was also investigated, and the expression of various apoptosis-regulatory genes was examined. To validate whether MALAT1 inhibition indeed affects molecular pathways related to caspases, we analyzed the gene expression levels of caspase-3, -8, and -9 in CaSki, CaSki/NC, and CaSki/M cells [**Fig. 6(A,B)**]. The caspase-3 and -8 mRNA expression level was remarkably increased in CaSki/M cells compared with CaSki/NC or CaSki cells. However, the caspase-9 mRNA expression level of CaSki/M cells was indistinguishable from CaSki or CaSki/NC cells. These results demonstrated that MALAT1 inhibition contributed the up-regulation of caspase-3 and -8.

Bcl-2 family are central regulators of programmed cell death, and members that inhibit apoptosis, such as Bcl-2 and Bcl-xL, are overexpressed in many cancers and contribute to tumor initiation and progression. However, Bax may lead to the caspase activation and commit the cell to apoptosis [16,17]. Here, we examined the gene expression levels of Bax, Bcl-2, and Bcl-xL. Semi-quantitative RT−PCR analysis showed that the expression levels of Bcl-2 and Bcl-xL in CaSki/M cells were down-regulated compared with CaSki or CaSki/NC, but the expression levels of Bax were up-regulated [**Fig. 6(A,B)**].

Discussion

It is now recognized that only 2% of the human genome encodes for protein-coding RNAs, whereas 60−70% of our DNA is transcribed into ncRNAs [18,19]. Hence, despite accumulating research on siRNA, miRNA, and piRNA, we are likely at the tip of the iceberg in our understanding about functions and regulatory roles served by ncRNAs in cellular metabolism, pathogenesis, and host−pathogen interaction [20].

Long RNA polymerase II transcripts have been previously shown to be precursors for multiple types of small RNAs, including snoRNAs, miRNAs, and possibly piRNAs [21−24]. Wilusz *et al*. [25] find that MALAT1 functions as a precursor for the production of small RNAs and identify a highly conserved small RNA of 61 nucleotides originating from the MALAT1 locus that is broadly expressed in human tissues. So, the functions of MALAT1 need urgently to be studied, especially in cancer.

In this report, we first evaluated the biological significance of MALAT1 in the pathogenesis of CaSki cells. Our results indicated that down-regulation of MALAT1 was associated with a gradual decrease in viable cells. The decrease in cell viability can be attributed to the occurrence of G1 cell cycle arrest after inhibition of MALAT1. Moreover, down-regulated MALAT1 expression caused the decrease in migration ability of CaSki cells. Moreover, reduced MALAT1 expression decreased the tumor

development of inoculated CaSki cells *in vivo*. And our data indicated that MALAT1 is essential in the survival process in CaSki cells.

Next, we explore the possible molecular mechanism of growth and invasion inhibition in CaSki cells after inhibition of MALAT1. Here, we showed that MALAT1 mediates the apoptosis pathways involved in the regulation of cervical cancer cell proliferation and invasion. We examined the role of caspase family of proteases in the growth of cervical cancer cells and detected higher levels of caspase-8 and -3 in MALAT1 siRNA CaSki cells. In human, more than 20 members of Bcl-2 family have been identified including proteins that suppress or induce apoptosis (e.g. Bcl-2, Bcl-xL, or Bax) [26,27]. Furthermore, the results showed that the expression down-regulation of Bcl-2 and Bcl-xL and up-regulation of Bax after inhibition of MALAT1.

In conclusion, this study is the first to examine the mechanistic role of MALAT1 in cervical cancer tumorigenesis and progression and extend our knowledge about the pathogenesis of cervical cancer. Our work suggests that blocking MALAT1 activity in cervical cancer is a potential novel therapeutic approach, since it is implicated in many areas of tumor progression, including cell growth, cell cycle regulation, and invasion. Intervention of MALAT1 function by agents, such as siRNA, may have potential therapeutic value in the prevention of cervical cancer. Thus, our findings may not only provide a molecular basis for the role of MALAT1 in cervical cancer but also suggest a novel therapeutic target for the treatment of cervical cancer.

Funding

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