Inhibition of the long non-coding RNA MALAT1 suppresses tumorigenicity and induces apoptosis in the human ovarian cancer SKOV3 cell line

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Abstract. Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is a 8,000 nucleotide-long, spliced non-coding RNA, which has been reported to be deregulated in several tumors. However, to the best of our knowledge, the role of MALAT1 in ovarian cancer has not been previously investigated. The aim of the present study was to investigate the effect of MALAT1 inhibition on the tumorigenicity of SKOV3 cells. First, stable MALAT1-knockdown ovarian cancer cells and control cells were established using lentivirus-mediated artificial micro RNA interference in order to investigate the effect of MALAT1 inhibition on cell viability, clonability, migration, invasion and apoptosis in vitro. In addition, the effect of MALAT1 on cell growth in nude mice was assessed. To identify the possible targets of MALAT1, total RNA was extracted from MALAT1-knockdown cells and control cells and a microarray analysis was performed. The results showed that MALAT1 inhibition significantly suppressed tumorigenicity in vitro and in vivo (P<0.01). Compared with the control cells, 921 genes in the MALAT1-knockdown cells were deregulated by at least two-fold. The results of the reverse transcription-quantitative polymerase chain reaction showed that 19 of the 20 genes selected for validation confirmed the deregulation indicated by the microarray analysis. The findings define a major oncogenic role for MALAT1, which may offer an attractive novel target for therapeutic intervention in ovarian cancer.

Introduction

Ovarian cancer is the most lethal gynecological malignancy, and <40% of patients with ovarian cancer are cured. In 2015, it is estimated that 21,290 new cases and 14,180 mortalities will occur in association with this disease in the United States (1). Epidemiological studies have identified several risk factors of ovarian cancer, including nulliparity, hormone therapy and pelvic inflammatory disease (2-4). Initial treatment for ovarian cancer consists of surgical staging and cytoreduction, followed by systemic chemotherapy in the majority of patients. Given the high mortality rate of ovarian cancer, the identification of novel biomarkers and therapeutic targets has been a major research focus for a number of years.

Long non-coding RNA (IncRNA) is an RNA molecule that is >200 nucleotides long and is not translated into a protein. Although the current understanding of the role of IncRNAs is limited, increasing numbers of studies have indicated that IncRNAs may regulate gene expression at various levels, including chromatin modification, transcription and post-transcriptional processing (5-7). IncRNAs have been reported to control various cellular processes, including proliferation, apoptosis and invasion, and are implicated in human diseases, including various types of tumors.

Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), also known as non-coding nuclear-enriched abundant transcript 2, is a 8,000 nucleotide-long, spliced non-coding RNA, which is highly conserved in mammals. MALAT1 has been reported to be deregulated in several tumors, including non-small cell lung cancer, hepatocellular carcinoma, cervical cancer, bladder cancer and colorectal cancer (8-12). However, the role of MALAT1 in ovarian cancer has not been previously investigated.

The present study indicated that MALAT1 inhibition significantly suppressed tumorigenicity and induced apoptosis in SKOV3 cells. In addition, the knockdown of MALAT1 was indicated to alter the expression of a number of genes associated with cell proliferation, metastasis and apoptosis. To the best of our knowledge, the present study is the first to report an oncogenic role for MALAT1 in ovarian cancer.

Key words: ovarian cancer, long non-coding RNAs, metastasis-associated lung adenocarcinoma transcript 1
Materials and methods

Cell lines and cell culture. The human ovarian cancer SKOV3 cell line and 293T cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The two cell lines were cultured in Dulbecco’s modified Eagle’s medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in standard culture conditions of 5% CO\textsubscript{2} at 37˚C.

Establishment of stable MALAT1-knockdown (KD) and negative control (NC) cells. Four microRNA (miRNA) oligonucleotides targeting MALAT1 (KD1, KD2, KD3 and KD4; Table I) were synthesized and inserted into the pcDNA6.2-GW/EmGFP-miR vector (Invitrogen; Thermo Fisher Scientific, Inc.) and used for transient transfections. The pcDNA6.2-GW/EmGFP-miR-Neg vector (Invitrogen; Thermo Fisher Scientific, Inc.) was used as negative control. Cells were maintained in 5% CO\textsubscript{2} at 37˚C and harvested at 2 days post-infection. Reverse transcription-quantitative polymerase chain reactive (RT-qPCR) was performed in order to determine the efficiency of MALAT1 knockdown and to screen for the miRNA with the greatest knockdown efficiency, respectively. To produce recombinant lentiviruses, the 293T cells were co-transfected with lentivirus expression plasmids and packaging plasmid mix (Invitrogen; Thermo Fisher Scientific, Inc.) using POLodeliverer™ 3000 Transfection Reagent (Ruisai Inc., Shanghai, China). The SKOV3 cells were then infected with these lentiviruses at a multiplicity of infection of 30, and selected with blasticidin (Invitrogen; Thermo Fisher Scientific, Inc.). At 2 weeks post-infection, cells were harvested and qRT-PCR was performed to determine the expression levels of MALAT1 in KD and NC cells.

RNA extraction and RT-qPCR. Total RNA was extracted from the cell samples using the Trizol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer’s instructions. The RNA integrity was analyzed using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA). Each RT reaction consisted of 0.5 µg RNA, 2 µl PrimerScript Buffer, 0.5 µl oligo(dT), 0.5 µl random primers and 0.5 µl PrimerScript RT Enzyme mix I (Takara, Otsu, Japan), in a total volume of 10 µl. Reactions were performed in a GeneAmp® PCR System 9700 (Applied Biosystems; Thermo Fisher Scientific, Inc.) for 15 min at 37°C, followed by heat inactivation of reverse transcriptase for 5 sec at 85°C. The 10-µl RT reaction mixture was then diluted 10 times in nuclease-free water and held at -20°C. qPCR was performed using the LightCycler® 480 II Real-Time PCR instrument (Roche Diagnostics, Basel, Switzerland) with a total volume including 1 µl cDNA, 5 µl 2X

| Oligonucleotide | Sequence |
|-----------------|----------|
| KD1 Forward     | 5’-TGCCTGTCCACTTTGATCCCAACTCATGTTTGGCCACTGACTGACGAGTGGTTGATCAAGTGGA-3’ |
| KD1 Reverse     | 5’-CCCTCCACTTTGATCACTTGGCTGCACTGACGACAGCTTGTTAACAGATAAG-3’ |
| KD2 Forward     | 5’-TGCCTGTGACTATCCCATACGAGGTGGCCACTGACTGACCTTCAGTGGGCATAGTACA-3’ |
| KD2 Reverse     | 5’-CCCTGTGACTATCCCATACGAGGTGGCCACTGACTGACCTTCAGTGGGCATAGTACA-3’ |
| KD3 Forward     | 5’-TGCCTGTGACTATCCCATACGAGGTGGCCACTGACTGACCTTCAGTGGGCATAGTACA-3’ |
| KD3 Reverse     | 5’-CCCTGTGACTATCCCATACGAGGTGGCCACTGACTGACCTTCAGTGGGCATAGTACA-3’ |
| KD4 Forward     | 5’-TGCCTGTCCATTAGTGGCATCAAGGCGTTTTGGCCACTGACTGACGCTTGATC-AACTAAGGAA-3’ |
| KD4 Reverse     | 5’-CCCTGTCCATTAGTGGCATCAAGGCGTTTTGGCCACTGACTGACGCTTGATC-AACTAAGGAA-3’ |
LightCycler® 480 SYBR Green I Master (Roche Diagnostics), 0.2 µl of forward primer, 0.2 µl of reverse primer and 3.6 µl of nuclease-free water. Reactions were incubated in a 384-well optical plate (Roche Diagnostics) at 95˚C for 10 min, followed by 40 cycles at 95˚C for 10 sec and 60˚C for 30 sec. At the end of the PCR cycles, melting curve analysis was performed to validate the specific generation of the expected PCR product. Three independent experiments were performed, with each sample run in triplicate. The primers were synthesized by Generay Biotech Co., Ltd. (Shanghai, China) and are listed in Table II. The expression levels of the genes were normalized with regard to GAPDH and were calculated using the 2−ΔΔCq method (13).

### Table II. Primers used in reverse transcription-quantitative polymerase chain reaction.

| Gene symbol | Forward primer | Reverse primer |
|-------------|----------------|----------------|
| MALAT1      | ATCAGACCACCACTCCAGTTTACAG | GACCATCCCCAAAATGCTTCA |
| WISP2       | GCCCTCTCTCCGACTTCC | TCTGTGTCCTTCTCTTCA |
| MUC16       | ATCATGCATTGACATCGAC | TCTTTCTCAGTGATAGCTT |
| ZEB2        | TGTGTCACAAAGGGCAAA | GCTAAAGCCTCAGTCTGAT |
| MMP2        | ACAGAAGAAGCTCAGTGTG | GTGGAGAAGAAGCTCGGT |
| MMP11       | CTGAGCAGTCGGCTGTA | CTTGAGTCTGGAGAACAGGATTT |
| VEGFA       | GGCGAAATATGACCCAGT | TGTACCTGTATCTGCTTCTT |
| PGF         | CTTGTAGGAGAGAGACC | CAGGGAAACAGTTGGCCTAA |
| H19         | TTTCACTCTGTTCCTTTTTGT | CAAACAGTGCAAATGACTCAT |
| ERBB4       | AATATGCTACAAACGAGCTG | TAACACTAGAGCTGATGAC |
| SNAI2       | TGCAGACCCATTCGATGTA | GGGTCACCTAGTGTGCA |
| E2F8        | CCAAGAGTTGTCCCTTCTAC | CATCTCCTACCTGCTCTAAC |
| CDK2        | TGATCACCAAGGGCAACT | GTGAAACACTGGACAGGATA |
| CDK20       | AGAGAACACCACCAAGACCTA | AAGGGTGTCCTGCTGAAA |
| ADRA1B      | GTTCCCAACTAAAGCCTACATACG | ATGGGACCAGTTCTCCTC |
| RASGRP1     | TTTCACTTTAGAACCAGGTC | AACGACGAGAAGGATC |
| FGF1        | AGATGGTGCTTTAATGCGTG | TCAAGCTGGTGCTCCTC |
| BAX         | AGATGGTGCTTTAATGCGTG | TCAAGCTGGTGCTCCTC |
| FN1         | AAGATCCCGAGAGTAAATCAT | TCTAAGCTGGTGCTCCTC |
| MTBP        | GAAGACCACAACACAGACAGC | TCTAAGCTGGTGCTCCTC |
| ECT2        | ACAACTCATTGTATGATAAGC | AGTTTCCCAAGTGCTT |
| GAPDH       | TGGTGCCATCAATGACCCCTT | CTCCACAGCTACTCC |

MALAT1, metastasis-associated lung adenocarcinoma transcript 1; WISP2, WNT1 inducible signaling pathway protein 2; MUC16, mucin 16, cell surface associated; ZEB2, zinc finger E-box binding homeobox 2; MMP2, matrix metallopeptidase 2; MMP11, matrix metallopeptidase 11; VEGFA, vascular endothelial growth factor A; PGF, placental growth factor; H19, H19, imprinted maternally expressed transcript (non-protein coding); ERBB4, erb-b2 receptor tyrosine kinase 4; SNAI2, snail family zinc finger 2; E2F8, E2F transcription factor 8; CDK2, cyclin-dependent kinase 2; CDC20, cell division cycle 20; ADRA1B, adrenoceptor α1B; RASGRP1, RAS guanyl releasing protein 1; FGF1, fibroblast growth factor 1; BAX, Bcl-2-associated X protein; FN1, fibronectin 1; MTBP, MDM2 binding protein; ECT2, epithelial cell transforming 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Cell proliferation, colony formation, invasion and motility assays. Cell viability was determined at 24, 48, 72 and 96 h using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer’s protocol. For the colony formation assay, cells were seeded, seeded at low density (1,000, 1,500 and 2,000 cells/plate), and allowed to grow until visible colonies appeared. Cells were then stained with crystal violet (GenMed, Shanghai, China) and colonies were counted by eye. In vitro invasion assay was performed using 24-well Transwell units (Corning Life Science, Tewksbury, MA, USA) with polycarbonate filters coated on the upper side with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The cells were harvested, and 4x10⁴ cells were placed in the upper part of the Transwell unit and were allowed to invade the membrane for 72 h at 37°C. Successfully penetrating cells were fixed, stained and quantified at an optical density of 570 nm. The motility assay was conducted in a similar fashion without coating with Matrigel. In total, 8x10⁴ cells were seeded per well and incubated in 5% CO₂ at 37°C for 5 h prior to detection. Successfully penetrating cells were quantified at an optical density of 570 nm using a microplate reader (Infinite 200 Pro; Tecan Schweiz AG, Männedorf, Switzerland). Each experiment was performed in triplicate.

Apoptosis assay. Cell apoptosis was determined by flow cytometric analysis. Briefly, floating and attached cells were collected, resuspended, stained with
Annexin V-allophycocyanin (BD Biosciences) and incubated for 30 min in the dark at 20°C. The analysis was then performed using a BD FACS Aria II flow cytometer (BD Biosciences).

In vivo tumorigenic assay. Mice were housed and maintained in a specific pathogen-free facility under controlled environmental conditions (temperature, 22±2°C; humidity, 55±10%) with ad libitum access to rodent chow and water. KD cells or NC cells (5x10⁶) were injected subcutaneously into the left armpit of nude mice (6 to 7 weeks old; n=7/group). Once palpable tumors developed, caliper measurements were taken twice a week and tumor volume was calculated on the basis of width (x) and length (y): x²y/2, where xcy. All mice were sacrificed by cervical dislocation when tumors reached 2 cm³ in size, and tumors were collected.

Microarray analysis and verification of selected genes by RT-qPCR. Microarray analysis was performed by Shanghai OeBiotech Co., Ltd. (Shanghai, China), using SurePrint G3 Human Gene Expression 8x60 K version 2 software (Agilent Technologies, Inc.). Briefly, total RNAs from KD cells and control cells were used to synthesize complementary DNA, from which labeled complementary RNA was then synthesized and hybridized to SurePrint G3 Human Gene Expression 8x60K v2 (Agilent Technologies, Inc.). Subsequent to hybridization, processed slides were washed using the Gene Expression Wash Pack (Agilent Technologies, Inc.) and scanned using the Agilent Microarray Scanner (Agilent Technologies, Inc.). The acquired array images were analyzed using Agilent Feature Extraction software (version 10.7; Agilent Technologies, Inc.), which performs background subtractions. Quantile normalization and subsequent data processing were performed using the GeneSpringGX version 11.0 software package (Agilent Technologies, Inc.). A threshold of a ≥2-fold change was used to screen upregulated or downregulated genes. A total of 20 differentially-expressed genes, which have been reported to be associated with cell proliferation, metastasis and apoptosis, were subsequently selected for validation using RT-qPCR.
Statistical analyses. All statistical analyses were performed using the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Differences between means were analyzed using a two-tailed Student's t-test. \( P<0.05 \) was considered to indicate a statistically significant difference.

Results

KD and NC cells were successfully established. The four pcDNA6.2-GW/EmGFP-miR vectors harboring various miRNAs (KD1, KD2, KD3 and KD4) were used to transiently infect 293T cells. In parallel, an NC experiment was performed. The highest knockdown efficiency was achieved using KD3 (71% relative to the NC group), which was then used for producing recombinant lentiviruses. The results of the RT-qPCR showed that the expression of MALAT1 was decreased by 77% in KD cells, compared with NC cells \( (P=0.000) \) (Fig. 1A).

MALAT1 inhibition suppresses tumorigenicity in vitro. As shown in Fig. 1B, a significant decrease in cell proliferation was observed over time in KD cells compared with NC cells \( (P<0.01) \). MALAT1 inhibition also decreased the clonogenicity of KD cells compared with NC cells \( (P=0.001) \) (Fig. 1C). Transwell migration and invasion assays showed that MALAT1 inhibition decreased the migration \( (P=0.002) \) and invasion \( (P=0.001) \) of KD cells (Fig. 1D and E). These observations suggest that MALAT1 knockdown suppresses the tumorigenicity of ovarian cancer cells in vitro.

MALAT1 inhibition induces apoptosis in KD cells. Apoptosis in KD and NC cells was measured by flow cytometry analysis. The mean apoptotic cell fractions (early apoptotic+apoptotic) were significantly increased upon MALAT1 inhibition compared with the negative control \( (P=0.004) \), with a concomitant decrease in the viable cell population (Fig. 1F). This finding suggests an anti-apoptotic role of MALAT1 in SKOV3 cells.

MALAT1 inhibition suppresses tumor growth in vivo. Since the in vitro data revealed the antitumorigenic role of MALAT1 inhibition in ovarian cancer cells, the effect of MALAT1 inhibition on tumor growth was examined in vivo. As shown in Fig. 2, the mean volume of the tumors in the KD group was significantly smaller compared with the tumors in the NC group \( (P<0.01) \), suggesting that the inhibition of MALAT1 significantly suppressed the tumorigenicity of SKOV3 cells in vivo.

Genes identified during the knockdown of MALAT1 expression. In order to additionally characterize the function of

Table III. Deregulated genes in metastasis-associated lung adenocarcinoma transcript 1-knockdown cells compared with negative control cells.

| Genbank access number | Gene symbol | Microarray fold change | qPCR fold change |
|-----------------------|-------------|------------------------|----------------|
| NM_003881             | WISP2       | -17.73                 | -10.38         |
| NM_024690             | MUC16       | 4.81                   | 3.34           |
| NM_014795             | ZEB2        | -5.87                  | -2.66          |
| NM_004530             | MMP2        | -5.17                  | -3.43          |
| NM_005940             | MMP11       | -4.32                  | -5.07          |
| NM_001025370          | VEGFA       | -7.93                  | -4.92          |
| NM_002632             | PGF         | -4.87                  | -8.38          |
| NR_002196             | H19         | -4.47                  | -3.35          |
| NM_005235             | ERBB4       | -20.57                 | -12.87         |
| NM_003068             | SNAI2       | 9.01                   | 3.40           |
| NM_024680             | E2F8        | 4.63                   | 7.98           |
| NM_001798             | CDK2        | 5.67                   | 8.54           |
| NM_001255             | CDC20       | 4.52                   | 5.67           |
| NM_000679             | ADRA1B      | 4.26                   | 7.82           |
| NM_008000             | FGF1        | 4.68                   | 5.14           |
| NM_138764             | BAX         | 4.48                   | 2.05           |
| NM_054304             | FN1         | -23.30                 | -3.79          |
| NM_022045             | MTBP        | 4.70                   | 5.55           |
| NM_018098             | ECT2        | 4.29                   | 3.87           |

qPCR, quantitative polymerase chain reaction; WISP2, WNT1 inducible signaling pathway protein 2; MUC16, mucin 16, cell surface associated; ZEB2, zinc finger E-box binding homeobox 2; MMP2, matrix metalloproteinase 2; MMP11, matrix metalloproteinase 11; VEGFA, vascular endothelial growth factor A; PGF, placental growth factor; H19, H19, imprinted maternally expressed transcript (non-protein coding); ERBB4, erb-b2 receptor tyrosine kinase 4; SNAI2, snail family zinc finger 2; E2F8, E2F transcription factor 8; CDK2, cyclin-dependent kinase 2; CDC20, cell division cycle 20; ADRA1B, adrenoceptor α1B; FGF1, fibroblast growth factor 1; BAX, Bcl-2-associated X protein; FN1, fibronectin 1; MTBP, MDM2 binding protein; ECT2, epithelial cell transforming 2.
MALAT1 within cells, RNA isolated from the KD cells was hybridized to the Agilent gene expression microarray and compared with the RNA isolated from the NC group. Data analysis showed that, when compared with the NC cells, a >2-fold deregulation was indicated in 921 genes in the KD cells. Since MALAT1 inhibition suppresses tumorigenicity and induces apoptosis in ovarian cancer cells, 20 genes, which have previously been reported to be associated with cell proliferation, metastasis and apoptosis, were selected for validation. As shown in Table III, 19 of these genes confirmed the deregulation that was indicated by the microarray analysis.

Discussion

MALAT1 was one of the first cancer-associated IncRNAs to be identified, and is widely expressed in normal human tissues. In 2003, Ji et al reported for the first time that MALAT1 was upregulated in non-small cell lung cancer and was a prognostic parameter for patient survival (8). Subsequent studies showed that MALAT1 was also associated with the formation and progression of several other types of tumors (9-12,14-18). However, the role of MALAT1 in ovarian cancer remains unknown.

The present study first investigated the effect of MALAT1 inhibition on SKOV3 cells, and the in vitro and in vivo data suggested that MALAT1 inhibition suppressed tumorigenicity in SKOV3 cells. A limitation to the present study was the lack of clinical samples available. Additional studies with clinical samples are warranted.

The gene expression in KD cells was then compared with control cells, and a large number of genes that were altered in association with decreased MALAT1 expression were identified. The results of the RT-qPCR validation showed that MALAT1 inhibition altered the expression levels of genes that have been reported to be associated with cell proliferation (placental growth factor, cyclin-dependent kinase 2, cell division cycle 20, adrenoceptor α 1B and fibroblast growth factor factor 1), metastasis [WNT1 inducible signaling pathway protein 2, mucin 16, cell surface associated, zinc finger E-box binding homeobox 2 (ZEB2), matrix metallopeptidase 2, matrix metallopeptidase 11, vascular endothelial growth factor A, H19, imprinted maternally expressed transcript (non-protein coding), snail family zinc finger 2 (SNAI2) and fibronectin], and apoptosis [erb-b2 receptor tyrosine kinase 4 and Bcl-2-associated X protein (BAX)]. Among these genes, ZEB2, SNAI2 and BAX have been reported to be possible targets of MALAT1 (10,11). The mechanism of regulation of gene expression by MALAT1 remains unclear. Tripathi et al reported that MALAT1 regulated alternative splicing by modulating SR splicing factor phosphorylation (19). However, this phenomenon was not observed in lung cancer cells (20). Additional studies are required in order to clarify the mechanisms underlying MALAT1.

In conclusion, MALAT1 inhibition significantly suppressed tumorigenicity in ovarian cancer cells and the inhibition of MALAT1 altered the expression of a number of genes associated with cell proliferation, metastasis and apoptosis. Overall, the findings of the present study define a major oncogenic role for MALAT1, which may provide an attractive novel target for therapeutic intervention in ovarian cancer.

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