Original Article

Down-modulation of MALAT1 inhibits proliferation, metastasis, invasion and sensitizes chemosensitivity to cisplatin in ovarian cancer cells

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Received June 2, 2018; Accepted October 30, 2018; Epub December 15, 2018; Published December 30, 2018

Abstract: Metastasis Associated Lung Adenocarcinoma Transcript 1 (MALAT1) was over-expressed in ovarian cancer tissues and promoted proliferation, migration, invasion of ovarian cancer cell. However, the precise mechanisms of MALAT1 in ovarian cancer cell underlying migration, invasion and chemoresistance have not yet been fully elucidated. The effects of MALAT1 knockdown on cell proliferation, migration, invasion and chemoresistance were investigated by MTT, transwell assay and lactate dehydrogenase release assay. We investigated whether the rate of cell death was altered when cells were treated with Z-VAD-fmk or necrostatin-1 (Nec-1) after down-regulation of MALAT1. Meanwhile the potential protein expression changes after MALAT1 silencing were evaluated by Western-blotting. Knockdown of MALAT1 in SKOV3 cell decreased cell proliferation, migration and invasion. Further, the expression of E-cadherin was up-regulated and Vimentin was down-regulated after MALAT1 silencing. Next, with the rising of cisplatin concentration, the majority of SKOV3 cells adopted a necrotic morphology and the percentage cell death increased after MALAT1 knockdown, also the percentage cell death of MALAT1-siRNA group showed significant difference compared to that of control groups. When cells treated with DDP + Z-VAD-fmk or DDP + Nec-1, down-modulation of MALAT1 showed significantly decreased cell death compared with that treated with DDP + DMSO. Cleaved PARP protein could be observed only when cells treated with DDP and inhibited of MALAT1 expression. Our data suggested that inhibition of MALAT1 impaired SKOV3 cell proliferation, migration, invasion and sensitized chemosensitivity to cisplatin. Further, MALAT1 promoted migration and invasion by activating EMT pathway and enhanced chemoresistance via modulating apoptosis and necroptosis signaling pathway, suggesting that MALAT1 might be an attractive anti-cancer agent for patients with ovarian cancer.

Keywords: Long noncoding RNAs, MALAT1, ovarian cancer, chemoresistance

Introduction

Ovarian cancer is the most lethal gynecological malignant tumor in the female reproductive system and its five-year survival rate is less than 30% [1, 2]. Accompanied by widespread implantation metastasis of the peritoneum, the patients with ovarian cancer are generally diagnosed in the advanced stage due to asymptomatic characteristic. Surgery and combination chemotherapy comprised of platinum and taxane agents are the current standard therapeutic procedures, however, patients suffered recurrence and chemoresistance in most cases [3, 4]. According to the clinical data, metastasis is the major cause of tumor-related death [5]. In addition, more than 90% of patients with metastasis often have a poor prognosis because of drug resistance [6]. Thus, a research on the mechanisms of proliferation, metastasis, invasion and chemoresistance of ovarian cancer is indispensable in order to identify a novel prognostic and therapeutic biomarker.

Long noncoding RNAs (IncRNAs) attract increasing attention in recent years owing to their vital biological influences in many human carcinomas. IncRNAs, as novel regulators in the cancer progression and development, can regulate gene expression fashions and protein expres-
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Expression patterns, also interact with proteins [7-11]. Up to now, a large number of IncRNAs have been implicated in various types of cancer and their roles have been widely studied [12-14]. Metastasis Associated Lung Adenocarcinoma Transcript 1 (MALAT1), a well-studied IncRNAs and possible oncogene [15], is located on human chromosome 11q13 that was originally identified in non-small cell lung cancer [16]. It has been found to be overexpressed in several cancers, including lung cancer, breast cancer, nasopharyngeal carcinoma, gallbladder cancer, hepatocellular carcinoma, medullary thyroid carcinoma etc. [17-21]. In 2016, Shiping Liu, Yanqing Zhou et al. reported that MALAT1 was over-expressed in ovarian cancer tissues, further, inhibition of MALAT1 decreased proliferation, migration, invasion and induced apoptosis in vitro, and suppressed tumor growth in vivo, and significantly differentially expressed genes were identified by microarray analysis [22, 23]. However, the precise mechanisms of MALAT1 in ovarian cancer cell underlying migration, invasion and chemoresistance are not fully elucidated. To address this problem, we started this project.

Materials and methods

Cell culture

The SKOV3 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in high glucose DMEM medium (Gibco BRL, Daithersburg, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% streptomycin/penicillin (Sigma-Aldrich, St. Louis, MO) in a humidified incubator at 37°C with 5% CO₂. Small interfering RNA and transfection

Small interfering RNAs (siRNAs) targeting MALAT1 (MALAT1-siRNA) and one negative control (NC-siRNA) were designed and synthesized by Guangzhou RiboBio (Guangzhou, China). The target sequences for MALAT1-siRNA were 5'-GGGCAAAATATTGGCAATTA-3' (sense) and 5'-GGGGCAAAUAUUGGCAAUUAdTdT-3' (antisense). The NC-siRNA was designed as: 5'-UUCUC- CGAACGUGUCAGUTT-3' (sense) and 5'-ACGUGACGAGUCCGAGAAT-3' (antisense). SKOV3 Cells were seeded in six-well plates at a density of 3.5 × 10⁵/well overnight, and then transfected with MALAT1-siRNA or the negative control at a final concentration of 40 nM using the INTERFERin™ transfection reagent (Polyplus Transfection) according to the manufacturer’s instructions. After 48 h, the interfering efficiency was confirmed via RT-qPCR.

Real-time reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

Total RNA, was extracted from cell culture samples by using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions and treated with DNase. Purity and concentration of the prepared total RNA was assessed with a NanoDrop® ND-1000 UV-Vis spectrophotometer (Thermo Fisher Scientific Inc., USA). RNA of 1.0 μg was used as a template to synthesize first strand cDNA using the Rever Aid M-ult Reverse Transcriptase-PCR kit (TAKARA, Dalian, China). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primers are: MALAT1-F, 5'-GCTGTGGAGTTCTTAAATATCAACC-3', MALAT1-R, 5'-TTCTCAACCTGGAATTCCTCCA-3'; E-cadherin-F, CAGTACAACGACCAACCCA, E-cadherin-R, CAGCCTGACCTCTAGGTGGA; Vimentin-F, AGCATCTCCTCCTGCAAATTT, Vimentin-R, TGCATTCAACCTTGGTTTGAC; GAPDH-F, 5'-GAGTCAACGGATTTGGTCGTTT, GAPDH-R, 5'-ACAGCTGAGACTGACGTCG-3'. Quantitative RT-PCR was performed using the Bio-Rad System (Bio-Rad Inc., USA) and the Maxima™ SYBR Green PCR Master Mix (ROX solution, Fermentas, USA). The RT-qPCR machine was programmed as 95°C for 10 min, 95°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec, and the latter 3 steps were repeated for 40 cycles. The expression level of MALAT1 was calculated by the 2⁻ΔΔCt method and normalized with GAPDH.

Proliferation assay

SKOV3 cells were seeded and transfected in 96-well plates at a density of 5 × 10⁵ cells per well. SKOV3 cells were respectively transfected by MALAT1-siRNA (40 nM), NC-siRNA (40 nM) or saline blank control (0.9% sodium chloride). After 48 h, we confirmed that the expression of MALAT1 was significantly decreased at RNA level in SKOV3 cells. Then, cells proliferation was measured using MTT cell Proliferation and Cytotoxicity Assay Kit (Beyotime, Jiangsu, China), according to the suppliers’ indications. The optical absorbance was determined on the
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Migration and Invasion assay

The migration and invasion assays were performed in a 24-well transwell chamber. After the cells were transfected for 48 h, we confirmed the expression of MALAT1 was significantly decreased at RNA level. About 100 μl SKOV3 cells, with a concentration of 5 × 10⁵/ml, which were suspended in serum-free high glucose DMEM, was seeded into the upper chamber of transwell assay inserts (Millipore, Billerica, MA) with a membrane at 8-μm pore size. For migration assay, 600 μl of high glucose DMEM medium containing 15% FBS was added to the lower chamber as the chemotactic factor. After 24 h, non-migrating cells were scraped off with cotton swap, whereas the cells that had migrated through the membrane were fixed with methanol and stained with 0.1% crystal violet and counted under a microscope in five randomly selected fields at magnification X200. For invasion assay, 50 μl matrigel (BD Biosciences, USA) was firstly added to the bottom of the transwell chamber before SKOV3 cells were seeded, and the following procedures were the same as that in migration assay, except for the invasive cells being analyzed after co-culture for 48 hours.

Chemosensitivity assay

For the chemosensitivity assay in vitro, SKOV3 cells were seeded in a 96-well plate at a density of 3.5 × 10³ cells/well. SKOV3 cells were respectively transfected by MALAT1-siRNA (40 nM), NC-siRNA (40 nM) or saline blank control (0.9% sodium chloride) for 48 h. The transfected SKOV3 cells were then incubated with the various concentrations of cisplatin (DDP) (0 μM, 4 μM, 8 μM and 12 μM) for 72 h. The cellular morphology was observed with inverted phase contrast microscope. Cell death was assessed by the intracellular lactate dehydrogenase (LDH) release assay through a cytotoxicity detection kit (Promega, Madison, WI, USA). LDH release was quantified by measuring absorbance at 490 nm using a plate reader (Bio-Rad, USA). The rate of cell death for each group was calculated by the following formula: cell death (%) = (experimental value-media control)/(positive control-media control) × 100%. The results were expressed as percentage cell death ± standard deviation (SD).

Figure 1. Down-modulation of MALAT1 expression level in SKOV3 cells suppressed proliferation. A. MALAT1 expressions in SKOV3 cell line transfected by MALAT1-siRNA, NC-siRNA and saline blank control. Y-axis indicated the relative expression of MALAT1 in each group tested by RT-qPCR. B. Down-modulation of MALAT1 gene expression significantly suppressed SKOV3 cell growth in vitro. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 2. Inhibition of MALAT1 impaired migration and invasion of SKOV3 cell. A. Transwell assays demonstrated that MALAT1 silencing significantly impaired migration of SKOV3 cells compared with control groups. B. The Matrigel chamber invasion assays indicated that down-modulation of MALAT1 significantly diminished the invasive capacity of SKOV3 cells compared with control groups. The bar profiles represented the number of cells migrated or invaded into the lower transwell chambers. These data were the mean ± SD of three independent experiments by Student’s t-test. Magnification: 200 ×. ***P < 0.001.
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Cell treatment

Z-VAD-fmk and necrostatin-1 (Nec-1) were solubilized with Dimethyl Sulfoxide (DMSO), then added to DMEM-containing culture media at a concentration of 25 μM, 50 μM, separately. After 48 h of transfection, medium was removed from cells (MALAT1-siRNA, NC-siRNA, saline blank control) and cells were incubated with 5 μM DDP + 25 μM Z-VAD-fmk, 5 μM DDP + 50 μM Nec-1, 5 μM DDP + DMSO for 72 h. For all cases, the final concentration of DDP is 5 μM. The percentage cell death was decided by LDH assay as described above.

Western blot analysis

Protein samples were prepared by lysing cells in ice-cold Mₐ lysis buffer (29 mM Tris-Cl at pH7.6, 0.5% NP4, 250 mM NaCl, 3 mM EDTA, 3 mM GDTA, 0.1 M PMSF, 1 mg/ml leupeptin, 0.1 M DTT, 1 M b-glycerol phosphate, 0.2 M sodium vanadate). Protein quantification was carried out using BCA Protein Assay Kit (Beyotime, Jiangsu, China) according to the manufacturer’s protocol. Protein sample were separated in 10% polyacrylamide gel electrophoresis-SDS (PAGE-SDS) and transferred to nitrocellulose membranes. The membranes were blocked with Tris buffered saline- 0.1% Tween-20 (TBS-T) containing 5% nonfat dried milk and then incubated overnight at 4°C with the anti-rabbit PARP polyclonal antibody (GTX100573, 1:1000, GeneTex, United States), anti-rabbit E-cadherin polyclonal antibody (20874-1-AP, 1:2000, proteintech, Chengdu, China), anti-rabbit Vimentin polyclonal antibody (10366-1-AP, 1:2000, proteintech, Chengdu, China), anti-mouse β-actin monoclonal antibody (60008-1-lg, 1:10000, proteintech, Chengdu, China), anti-mouse GAPDH monoclonal antibody (60004-1-lg, 1:10000, proteintech, Chengdu, China). The membrane were washed three times in TBS-T and incubated with the secondary antibodies at room temperature for 1 h. HRP-conjugated Affinipure Goat Anti-Mouse IgG (H + L) (SA0001-1, 1:2000) and HRP-conjugated Affinipure Goat Anti-Rabbit IgG (H + L) (SA0001-1, 1:2000) were purchased from proteintech (Chengdu, China). The membranes were again washed three times with TBS-T. Protein bands were visualized at ChemiDoc™ MP Imaging System (Bio-Rad Laboratories Inc.).

Statistical analysis

All experiments were performed at least thrice. All data were showed as mean ± SD and compared by the two-tailed Student’s t-test. P < 0.05 was considered statistically significant. All statistical analyses were performed on SPSS 24 software (SPSS Inc. Chicago).

Results

Knockdown of MALAT1 in SKOV3 cell decreased cell proliferation

To understand the role of MALAT1 in proliferation, synthesized MALAT1-siRNA was used to knock down MALAT1 in the human ovarian cancer cell line SKOV3. The results of RT-qPCR showed that the siRNA transfection could efficiently decrease MALAT1 expression by 67% and 70% compared to the saline control and NC-siRNA at the RNA level, respectively (P < 0.001, Figure 1A). By MTT assay, cell proliferative activity was found to be significantly suppressed in the MALAT1-siRNA group compared to control groups (Figure 1B). The results suggested that knockdown of MALAT1 decreased cell proliferation in SKOV3 cell.

Inhibition of MALAT1 in SKOV3 cell impaired migration and invasion

We investigated SKOV3 cell migration and invasion through transwell assays. The migration and invasion of SKOV3 cells decreased by approximately 53% and 66% compared with control groups, respectively (P < 0.001, Figure 2A and 2B). The data above illustrated that inhibition of MALAT1 in SKOV3 cell impaired migration and invasion.

MALAT1 promoted migration and invasion by activating EMT pathway in SKOV3 cell

To further investigate whether MALAT1 regulates cell migration and invasion by activating epithelial-mesenchymal-transition (EMT) signaling pathway, the expression of E-cadherin and vimentin was detected by western blot and RT-qPCR. The expression of the epithelial maker E-cadherin was obviously increased in siRNA-MALAT1 cells compared with that in NC-siRNA and blank control groups (P = 0.003, Figure 3A). Moreover, the expression of the mesenchymal marker Vimentin was obviously
reduced in siRNA-MALAT1 cells compared with that in NC-siRNA and blank control groups (P = 0.016, Figure 3B). All the evidence suggested that we could inhibit SKOV3 cell migration and invasion partly by suppressing EMT process after MALAT1 knockdown.

**Downregulation of MALAT1 sensitized SKOV3 cell to cisplatin**

To assess whether MALAT1 knockdown increases the sensitivity of ovarian cell to DDP, SKOV3 cells were treated with DDP for 72 h after MALAT1 knockdown. The morphological signs of nuclear apoptosis were evaluated using inverted phase contrast microscope and the number of death cells was estimated by LDH assay. Through inverted phase contrast microscope and LDH assay, we found that without exposure to DDP, cell death was not significantly altered after MALAT1 silencing (P = 0.14, Figure 4A). However, at 4 μM, 8 μM, 12 μM concentrations of DDP, the percentage cell death increased, showing a dose-dependent trend. At the same time, it presented statistical difference between the MALAT1-siRNA groups and control groups (P < 0.001, P = 0.002, P < 0.001, Figure 4B-D). These findings revealed that MALAT1 was not required for ovarian cancer cell survival, but downregulation of MALAT1 sensitized SKOV3 cell to DDP in a dose-dependent manner.

**Down-modulation of MALAT1 potentiated apoptosis and necroptosis of SKOV3 cell**

In DDP + DMSO group, down-modulation of MALAT1 showed significantly high percentage cell death compared to control groups (P < 0.001, Figure 5A). After MALAT1 knockdown, DDP + Z-VAD-fmk treatment group showed that percentage cell death significantly decreased approximately 50% compared with DDP + DMSO treatment group (P < 0.001, Figure 5A). Meanwhile, after MALAT1 knockdown, DDP + Nec-1 treatment group showed that percentage cell death significantly decreased approximately 30% compared with DDP + DMSO treatment group (P < 0.001, Figure 5A). When treated with DDP + Z-VAD-fmk or DDP + Nec-1, down-modulation of MALAT1 showed significantly higher percentage cell death compared to respective controls (P = 0.009, P = 0.037, Figure 5A). We next determined the expression of cleaved Poly(ADP-ribose)-polymerase (PARP) by western blot analysis, which indicated that knockdown of MALAT1 lead to cleavage of PARP compared with NC-siRNA and blank control groups subjected to 5 μM DDP (Figure 5B). In this regard, down-modulation of MALAT1 sensitized chemotherapeutic sensitivty of SKOV3 cell, which might partly contribute to involving MALAT1 into the apoptotic and necroptotic processes.

**Discussion**

In this study, we demonstrated that MALAT1 was associated with ovarian cancer cell proliferation, migration, invasion and chemoresistance. Furthermore, MALAT1 promoted migration and invasion by activating EMT pathway and induced chemoresistance by reducing apoptosis and necroptosis in SKOV3 cells.
Figure 4. Representative images of SKOV3 cells were treated with 0 μM, 4 μM, 8 μM and 12 μM of DDP for 72 h, respectively. Cell death was evaluated using LDH assay. Percentage cell death was calculated and expressed as percentage cell death ± SD (n = 3). The differences were expressed as **P < 0.01, ***P < 0.001.
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Since tumorigenesis and cancer progression are related with multiple oncogenic processes, including proliferation, migration and invasion, we assessed the effects of MALAT1 knockdown on the proliferation, migration and invasion of SKOV3 cell. As a result, the MTT assay demonstrated that MALAT1 knockdown significantly reduced the number of living SKOV3 cell, and the migration assay found that inhibition of MALAT1 expression significantly reduced the number of migrated SKOV3 cell, and the Matrigel invasion assay indicated that down-regulation of MALAT1 significantly decreased the number of invaded SKOV3 cell. The results were consistent with previous report that MALAT1 promoted colorectal cancer cell proliferation, migration and invasion via PRKA kinase anchor protein 9 [24]. Further, G. Fengjie et al. reported that inhibition of MALAT1 in CaSki human cervical cancer cells suppressed cell proliferation and invasion [25]. Fengjie Guo et al. exhibited that inhibition of MALAT1 in non-small cell lung cancer cells suppressed growth, migration and invasion [26]. These studies supported that down-regulation of MALAT1 expression in SKOV3 cell decreased cell proliferation, migration and invasion.

However, it is unknown how MALAT1 affect cell migration and invasion in ovarian cancer. In the present study we found that the underlying mechanism of migration and invasion might relate to EMT. Previous study showed that EMT played a crucial role in cancer progression and metastasis [27]. Moreover, MALAT1 was shown to have a role in EMT in thyroid carcinoma [28]. Hirata, H. et al. reported that E-cadherin expression was increased after MALAT1 silencing in renal cell carcinoma cell [15]. Usually, EMT process is mainly initiated by the down-regulation of E-cadherin and the up-regulation of Vimentin. In our study, after investigating the inhibiting effect of MALAT1 knockdown on migration and invasion of SKOV3 cell, we determined the expression of E-cadherin and Vimentin. Consequently, western blot and RT-qPCR showed that the expression of E-cadherin was up-regulated and Vimentin was down-regulated after MALAT1 knockdown. Those results indicated that MALAT1 promoted migration and invasion partly through EMT pathway.

The major morphological hallmark of apoptosis is nuclear condensation with fragmentation. Without exposure to DDP, inverted phase contrast microscopic observation of transfected SKOV3 cells (MALAT1-siRNA, NC-siRNA, saline blank control) almost did not observe any mor-
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Phenological signs of apoptosis. This finding was in accordance with LDH assay that the number of living cells was not significantly altered, suggesting that MALAT1 was not required for ovarian cancer cell survival. With the rising of DDP concentration, the majority of SKOV3 cells adopted a necrotic morphology and the percentage cell death increased after MALAT1 knockdown, also the percentage cell death of MALAT1-siRNA group showed significant difference compared to that of control groups. Consistent to our work, a recent study also demonstrated that MALAT1 enhanced the docetaxel resistance of prostate cancer cells via miR-145-5p-mediated regulation of AKAP12 [29]. Also, MALAT1 was confirmed to induce chemoresistance to oxaliplatin of colorectal cancer by promoting EZH2 [30] and regulated multidrug resistance of hepatocellular carcinoma cells by sponging miR-216b to modulate the expression of HIF-2α that was related to autophagy pathway [31]. These findings indicated that inhibition of MALAT1 expression might promote ovarian cancer cell sensitivity to cisplatin in a dose-dependent manner, and MALAT1 could contribute to the chemotherapeutic responsibility in ovarian cancer therapy.

In the next step, we analyzed the molecular mechanism of decreasing susceptibility towards cisplatin by MALAT1, as Asselin E has documented that cisplatin caused caspase-dependent apoptosis in ovarian cancer cells [32]. The pan-caspase inhibitor Z-VAD-fmk and necroptotic processes inhibitor Nec-1 abolishing the apoptosis and necroptosis signaling pathway were used to this study. When SKOV3 cell treated with DDP + Z-VAD-fmk or DDP + Nec-1, down-modulation of MALAT1 showed a significant decrease of percentage cell death compared to that treated with DDP + DMSO, and it also showed a significant increase of percentage cell death compared to that of control groups. Together, the present results suggested that MALAT1 induced chemoresistance partly by reducing apoptosis and necroptosis in SKOV3 cell. These results were in accordance with those of studies reported that MALAT1 inhibited apoptosis and promoted invasion by antagonizing miR-125b in bladder cancer cells [33] and silencing of MALAT1 promoted apoptosis of glioma cells [34]. Because PARP is a substrate of caspase-3, the cleaved PARP protein might result from caspase 3-mediated intrinsic apoptosis processing. Furthermore, in this study we found that the decrease of MALAT1 levels induced the observation of cleaved PARP protein when treated with DDP, suggesting that MALAT1 could induce chemoresistance partly through regulating apoptotic process.

However, additional studies are still required to determine the precise molecular mechanism of MALAT1 functions in ovarian cancer and the potentiality of it as a therapeutic target and a predictor for prognosis in ovarian cancer.

In conclusion, this investigation showed that MALAT1 knockdown inhibited cellular proliferation, migration and invasion by regulating EMT signaling pathway and also enhanced chemoresistance via modulating apoptosis and necroptosis signaling pathway in ovarian cancer, indicating that MALAT1 might be an attractive anti-cancer agent and prognostic biomarker for patients with ovarian cancer.

Acknowledgements

This study was supported by Sichuan Province Science and Technology Support Program (NO. 2011FZ0020).

Disclosure of conflict of interest

None.

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