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MALAT1 promotes cisplatin resistance in cervical cancer by activating the PI3K/AKT pathway

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Abstract. – OBJECTIVE: To investigate the role of MALAT1 in the cisplatin treatment of cervical cancer and its underlying mechanism.

MATERIALS AND METHODS: The effects of different doses of cisplatin on the proliferation and apoptosis of cervical cancer cells were detected by cell counting kit-8 (CCK-8) assay and apoptosis assay, respectively. We used bioinformatics methods to predict the downstream genes of MALAT1 and examined the expression relationship between the target gene BRWD1 and MALAT1 by quantitative Real-time polymerase chain reaction (qRT-PCR). Western blot was performed to detect the expression levels of apoptosis-related proteins and key genes in PI3K/AKT signaling pathway.

RESULTS: After MALAT1 was knocked down, cisplatin showed an inhibited effect on the proliferation of HeLa and C-33A cells in a concentration-dependent manner. After treatment of cervical cancer cells with 5 μM cisplatin, MALAT1 knockdown enhanced the apoptosis of HeLa and C-33A cells, and up-regulated expression of cleaved caspase-3. Over-expression of MALAT1 in cells showed the opposite results. Starbase website was used to predict that MALAT1 might regulate BRWD1 expression. Over-expression of MALAT1 significantly up-regulated the mRNA expression of BRWD1 in HeLa and C-33A cells. After knockdown of BRWD1, cisplatin markedly decreased the proliferation of HeLa and C-33A cells, and promoted cell apoptosis and cleaved caspase-3 expression. Besides, HeLa and C-33A cells showed increased expressions of p-PI3K and p-AKT after MALAT1 was up-regulated.

CONCLUSIONS: MALAT1 promoted the cisplatin resistance of cervical cancer, which might be related to regulation of cell apoptosis via BRWD1 and PI3K/AKT pathway.

Key Words:
MALAT1, BRWD1, PI3K/AKT pathway, Cervical cancer, Cisplatin resistance.

Introduction

Cervical cancer (CC) is one of the most common gynecological malignancies. Its incidence ranks third between all of the female malignancies in the world[1]. In developing countries, the incidence of CC is higher than that of developed countries. Compared with other cancers, CC frequently occurs middle-aged women who are in an important period of career and family care[2]. Although the strategy for the prevention and treatment of CC has been rapidly developed in the past few decades, the prognosis of patients with advanced and recurrent CC is still low, with the 1-year survival rate of only 10-20%[3]. CC cells are extremely sensitive to chemotherapy. Thus, chemotherapy has been widely applied in clinical treatment of CC in recent years. Cisplatin is the most common chemotherapeutic drug in clinical application. Most CC patients can obtain good curative effect through cisplatin chemotherapy in the early stage. However, drug resistance is often accompanied by treatment progress that seriously hinders the efficacy of cisplatin. A small proportion of CC patients have inherent resistance to cisplatin, while most cancer patients will gradually produce cisplatin resistance during the course of treatment[4,5]. Therefore, cisplatin resistance remains a major limitation in the long-term therapeutic efficacy of CC. However, the molecular mechanisms involved in cisplatin resistance remain unclear. Long non-coding RNAs (lncRNAs) are RNAs with a length of more than 200 nucleotides. They are transcribed by RNA polymerase II without an open reading frame. Studies have found that a large number of lncRNAs are differentially expressed in tumors. LncRNAs are reported to participate in the proliferation, differentiation and apoptosis of various tumor cells *via* the regulation of different signaling pathways[6]. The intracellular apoptotic pathway and the anti-apoptotic pathway function contribute to the maintenance of normal cell cycle. LncRNAs regulate the apoptosis of tumor cells and the emergence of drug resistance through the interaction with key apoptosis regulators.

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The caspase family is a protease system that directly leads to the disintegration of apoptotic cells, which exerts a central role in the network of apoptosis mechanisms. Studies have shown that AK022798 participates in the formation of cisplatin resistance in gastric cancer by regulating caspase family[7]. LncRNA MALAT1 has been extensively studied in a variety of tumors, which is capable of controlling the activation of multiple signaling pathways in cancers, including cervical cancer[8,9]. We aim to clarify the effect of MALAT1 on cisplatin resistance in CC.

Materials and Methods

Cell Culture and Transfection

The human CC cell lines, HeLa and C-33A, were maintained in DMEM (Dulbecco's Modified Eagle Medium) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), penicillin (100 U/mL) and streptomycin (100 μg/mL) in a 5% CO_2 incubator at a 37°C. For transfection, HeLa and C-33A cells in logarithmic growth phase were seeded in 6-well plates when the cell density reached 60-70%. LV-shMALATA1, LV-MALAT1, LV-shBRWD1 or control reagent was transfected into cells according to the instruction of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), respectively. After 6 h of incubation, complete medium was replaced. The transfection efficiency was detected 24 h after transfection.

RNA Extraction and qRT-PCR (Quantitative Real-Time Polymerase Chain Reaction)

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and the RNA concentration was measured by a UV spectrophotometer (Hitachi, Tokyo, Japan). The A260/280 value between 1.8 and 2.1 for each sample was considered as qualified. The cDNA was obtained *via* reverse transcription according to the instructions of reverse transcription kit. QRT-PCR reaction solution was prepared according to the instructions of SYBR Fluorescent Quantitative Premix Kit (TaKaRa, Otsu, Shiga, Japan), with a total system of 10 μL. The thermal cycling protocol for the PCR reaction was as follows: pre-denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 31 s. Each experiment was repeated for three times. The primer sequences used in the study were as follows: BRWD1: F: CACTTACTTGTTGCCACATAGCC, R: GATTCCTGATCAGATGTTCTTCCA; GAPDH (glyceraldehyde 3-phosphate dehydrogenase): F: CGCTCTCTGCTCCTCCTGTTC, R: ATCCGTTGACTCCGACCTTCAC.

Cell Counting Kit-8 (CCK-8) Assay

HeLa and C-33A cells in logarithmic growth phase were collected and seeded in 96-well plates at a density of 3×10^3/well. After cell culture for 24 h, 100 μL of cisplatin with different concentrations of 0, 3, 6, 9, 12 and 15 μM were added to each well, respectively. The blank wells were set up for calibration. There were 5 replicates for each group. After incubation for another 24 h, a total of 10 μL of CCK-8 (Dojindo, Kumamoto, Japan) reagent were added to the wells and the cells were allowed for 2 h of reaction. The optical density (OD) of each well was measured with a microplate reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 450 nm. The experiment was repeated for three times.

Apoptosis Assay

Transfected CC cells were treated with 5 μM cisplatin. After 24 hours of culture, cells were washed twice with 1 × PBS and digested with 0.25% trypsin. The cells were then separately collected in Eppendorf (EP) tubes and centrifuged at 1,000 rpm for 5 min at 4°C. The supernatant was removed and the cell precipitation was stained according to the instruction of a FITC/Annexin V apoptosis detection kit (BD Pharmingen, San Diego, CA, USA). Briefly, 500 μL of 1×buffer was added to resuspend the cells, and 5 μL of Annexin V and propidium iodide (PI) were added to stain the cells at 37°C for 20 min in dark. Apoptosis of each sample was tested by FACSAria™ flow cytometry. Win MDI 2.9 Software was used for analysis.

Western Blot

Transfected CC cells were collected after the medium was removed and 100 μL of boiling 2×SDS (sodium dodecyl sulphate) loading buffer was added. The total protein was sonicated for 1 min and centrifuged at 12,000g for 10 min at 4°C, and boiled for 5 min. Protein samples were separated by 10% polyacrylamide gel electrophoresis. After transferred to PVDF (polyvinylidene difluoride) membrane (Millipore, Billerica, MA, USA); the protein-contained membrane was blocked with 5% skim milk for 2 h. Corresponding primary antibody was added and incubated

with proteins at 4°C overnight, followed by incubation of secondary antibody for another 1 h. The ECL (enhanced chemiluminescence) luminescent agent (Thermo Fisher Scientific, Waltham, MA, USA) was used to develop the protein imprint. GAPDH was used as an internal reference.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 statistical software (IBM, Armonk, NY, USA) was used for data processing and analysis. The data were expressed as Mean±SD. The *t*-test was used to compare the mean values of two independent samples. $p < 0.05$ was considered statistically significant.

Results

Inhibition of MALAT1 Decreased Cervical Cancer Cell Proliferation and Promoted Cisplatin-Induced Apoptosis

CCK-8 assay was performed to detect the effect of different concentrations of cisplatin on the proliferation of CC cells. We found that the proliferation capacities of HeLa and C-33A cells were significantly decreased after MALAT1 knockdown in a concentration-dependent manner (Figure 1A-1B). Additionally, MALAT1 knockdown increased the apoptosis of HeLa and C-33A cells treated with 5 μM of cisplatin for 24 h (Figure 1C). Western blot analysis also suggested that the expression of cleaved caspase-3 was upregulated after MALAT1 was down-regulated (Figure 1D).

Over-Expression of MALAT1 Promoted Cervical Cancer Cell Proliferation and Inhibited Cisplatin-Induced Apoptosis

After MALAT1 was over-expressed, the proliferation, apoptosis and apoptosis-related protein expressions of CC cells were detected. The results indicated that over-expression of MALAT1 markedly enhanced the proliferation capacities of HeLa and C-33A cell (Figure 2A-2B). However, apoptosis-related genes and cleaved caspase-3 were remarkably down-regulated (Figure 2C-2D).

MALAT1 Regulated BRWD1 Expression in CC Cells

The regulation of BRWD1 expression by MALAT1 was predicted *via* Starbase. After MALAT1 was up-regulated in HeLa and C-33A

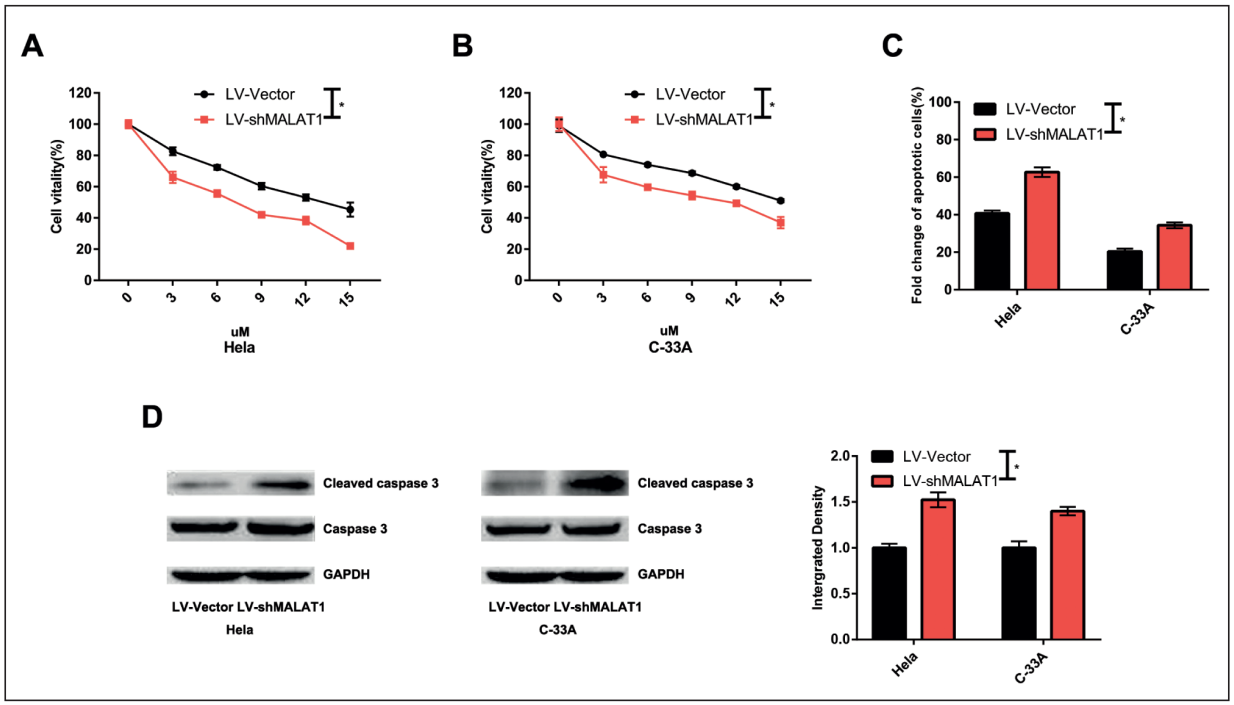

Figure 1. Inhibition of MALAT1 decreased cervical cancer cell proliferation and promoted cisplatin-induced apoptosis. ***A,*** Down-regulated MALAT1 inhibited proliferation of HeLa cells treated with different cisplatin concentrations. ***B,*** Down-regulated MALAT1 inhibited proliferation of C-33A cells treated with different cisplatin concentrations. ***C,*** Down-regulated MALAT1 promoted apoptosis of HeLaand C-33A cells induced by 5 μM cisplatin for 24 h. ***D,*** Down-regulated MALAT1 promoted cleaved caspase-3 expression in HeLa and C-33A cells induced by 5 μM cisplatin for 24 h.

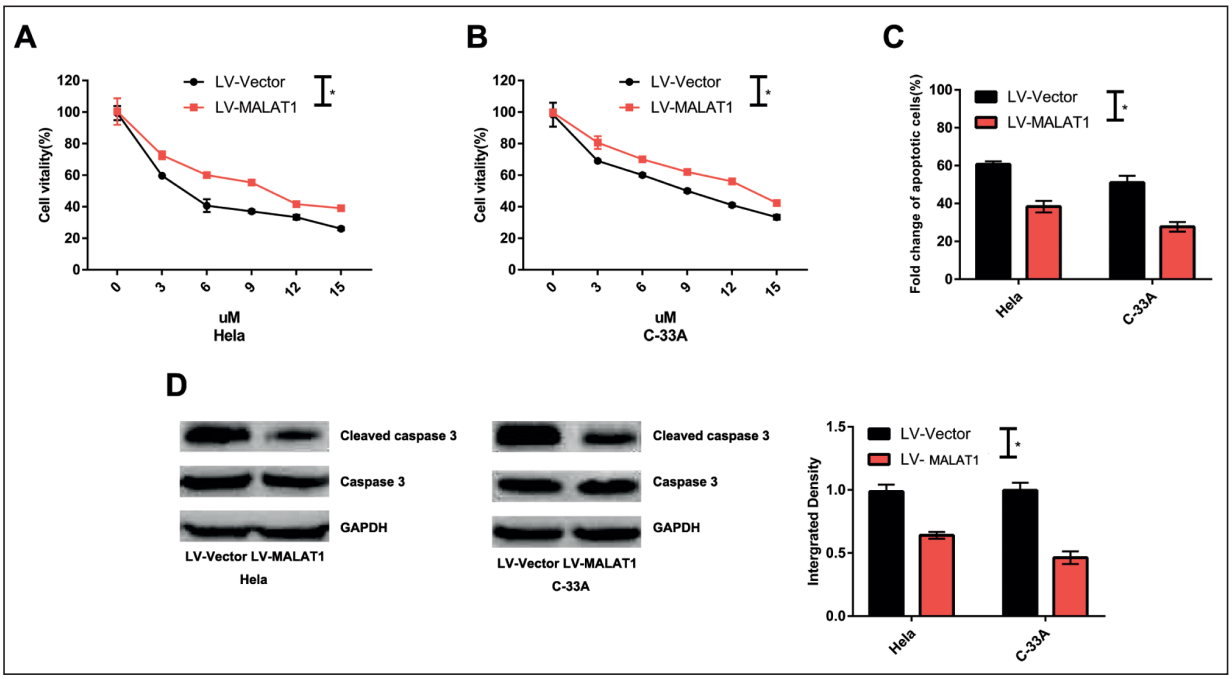

Figure 2. Over-expression of MALAT1 promoted proliferation and inhibited cisplatin-induced apoptosis. ***A,*** Over-expression of MALAT1 promoted the proliferation of HeLa cells treated with different cisplatin concentrations. ***B,*** Over-expression of MALAT1 promoted the proliferation of C-33A cells treated with different cisplatin concentrations. ***C,*** Over-expressed MALAT1 inhibited apoptosis of HeLa and C-33A cells induced by 5 μM cisplatin for 24 h. ***D,*** Over-expressed MALAT1 inhibited cleaved caspase-3 expression in HeLa and C-33A cells induced by 5 μM cisplatin for 24 h.

cells, there was a significant increase in mRNA level of BRWD1 (Figure 3A). HeLa and C-33A cells were then treated with different concentrations of cisplatin after BRWD1 knockdown. CCK-8 assay showed that the proliferation of HeLa and C-33A cells was also attenuated (Figure 3B-3C). However, BRWD1 knockdown increased cisplatin-induced apoptosis and cleaved caspase-3 expression (Figure 3D-3E).

Over-Expressed MALAT1 Activated PI3K/AKT Pathway in CC Cells

Previous studies have demonstrated that PI3K/AKT pathway exerts a crucial role in cell apoptosis. Here, we detected protein expressions of key genes in PI3K/AKT pathway after over-expression of MALAT1 in HeLa and C-33A cells. The results suggested that over-expressed MALAT1 promoted protein expressions of p-PI3K and p-AKT in HeLa and C-33A cells (Figure 4).

Discussion

Drug resistance of cancer cells is one of the major causes of chemotherapy failure. Both pre-existing innate resistance and acquired secondary resistance are closely related to the abnormal activation of intracellular signaling pathways. LncRNAs are reported to induce the drug resistance by regulating drug metabolism, cell cycle, apoptosis and epithelial-mesenchymal transition[10-17]. Guo et al[18] demonstrated that MALAT-1 inhibition can reduce tumor growth and migration in cervical cancer. Down-regulation of MALAT1 can also prevent the occurrence, development and metastasis of CC in nude mice. It is reported that MALAT1 resists apoptosis by up-regulating anti-apoptotic genes (Bcl-2 and Bcl-xL) and down-regulating apoptosis genes (caspase-3 and caspase-8)[19,10]. When apoptosis occurs, the activated pro-apoptotic protein promotes the release of pro-apoptotic substances from the mitochondria into the cytoplasm[20]. This process leads to caspase-dependent programmed cell death (apoptosis) in tumor cells. Accumulated researches[21-23] have shown that the PI3K/AKT pathway plays a very important role in chemotherapy resistance. Inactivation of PI3K/AKT pathway can reverse the drug resistance, thus recovering the sensitivity of tumor cells to chemotherapeutic drugs. Current research showed that the PI3K/AKT path-

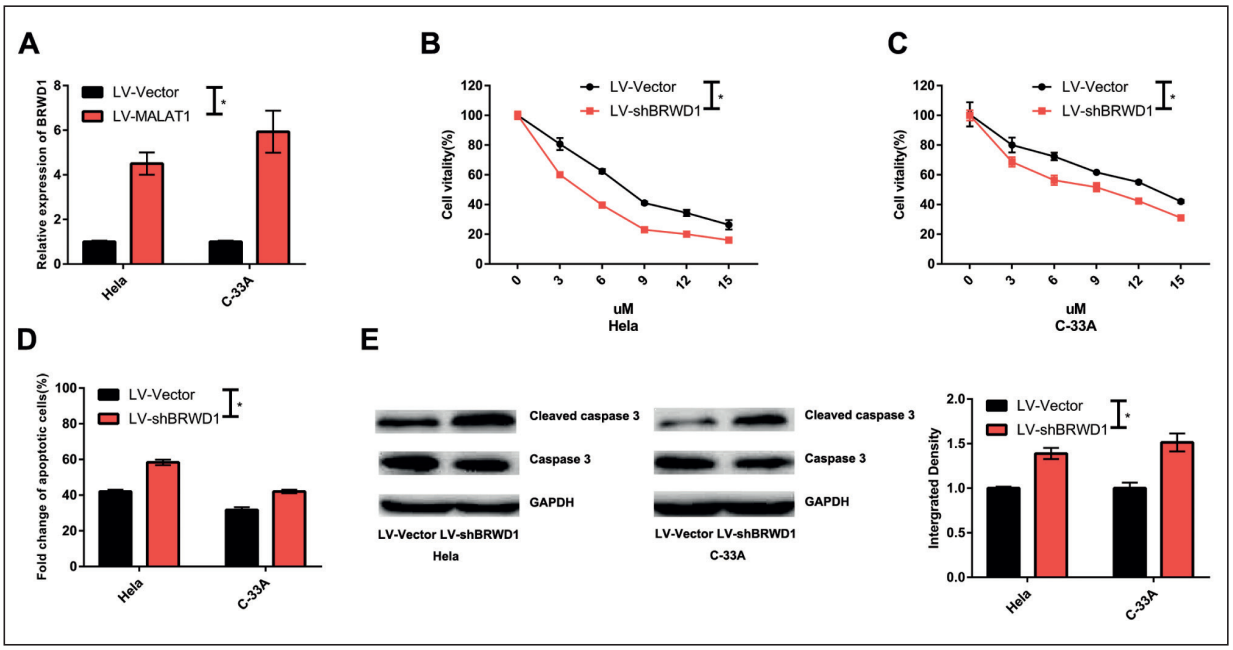

Figure 3. MALAT1 regulated the expression of BRWD1 in cervical cancer cells. ***A,*** Over-expression of MALAT1 promoted mRNA expression of BRWD1. ***B,*** Down-regulated BRWD1 inhibited HeLa cell proliferation in different cisplatin concentrations. ***C,*** Down-regulated BRWD1 inhibited C-33A cell proliferation in different cisplatin concentrations. ***D,*** Down-regulated BRWD1 promoted apoptosis of HeLa and C-33A cells induced by 5 μM cisplatin for 24 h. ***E***, Down-regulated BRWD1 promoted cleaved caspase-3 expression in HeLa and C-33A cells induced by 5 μM cisplatin for 24 h.

way mainly affects the chemotherapy resistance through multiple drug-resistance related-proteins and anti-apoptosis proteins. Chemotherapeutic drugs in tumor cells promote apoptosis through various ways, so as to achieve the anti-tumor effect. PI3K/AKT signaling pathway is one of major pathways regulating apoptosis, which inhibits expressions of caspase-9 and caspase-3 [24,25]. We found that down-regulation of MALAT1 increased the sensitivity of CC cells to cisplatin and promoted cell apoptosis. Bioinformatics analysis suggested that MALAT1 might regulate BRWD1 expression. Over-expressed MALAT1 in HeLa and C-33A cells significantly increased the mRNA expression of BRWD1. Furthermore, BRWD1 knockdown in HeLa and C-33A cells showed the same cellular functions as MALAT1 overexpression. All above results indicated that MALAT1 might play a role in regulating cisplatin resistance in cervical cancer through regulating BRWD1 and cell apoptosis. In order to further explore the mechanism, we examined the expressions of key proteins in PI3K/AKT signaling pathway. The results demonstrated that p-PI3K and p-AKT were markedly up-regulated after over-expression of MALAT1.

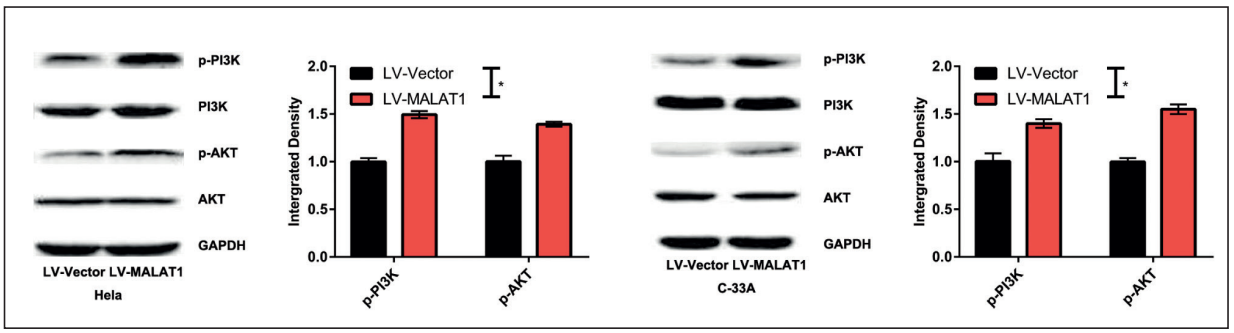

Figure 4. High expression of MALAT1 activates PI3K/AKT pathway. Western blot was performed to detect the expression of PI3K/AKT pathway after over-expression of MALAT1 in HeLa and C-33A cells.

Conclusions

MALAT1 promoted the cisplatin resistance of cervical cancer, which might be related to regulation of cell apoptosis *via* BRWD1 and PI3K/AKT pathway.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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