WAVE1 gene silencing via RNA interference reduces ovarian cancer cell invasion, migration and proliferation

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HIGHLIGHTS

• Our previous study has identified overexpression of WAVE1 in epithelial ovarian cancer (EOC) tissues is associated with a poor prognosis.
• WAVE1-silencing had a significant effect on cell morphological changes, and decreased cell migration, invasion, adhesion, colony formation and proliferation.
• Our results demonstrated WAVE1 promotes proliferative and invasive malignant behaviors through activation of PI3K/AKT and p38MAPK signaling pathways in EOC.

Abstract

Objective. Wiskott-Aldrich syndrome protein family verprolin-homologous protein 1 (WAVE1) has been implicated in cancer cell migration and invasion. We have previously shown that the overexpression of WAVE1 in epithelial ovarian cancer (EOC) tissues is associated with a poor prognosis. However, the mechanism of WAVE1 regulating the malignant behaviors in EOC remains unclear.

Methods. In the present study, we knocked down WAVE1 expression in SKOV3 and OVCAR-3 cells through RNA interference to detect the cell biology and molecular biology changes. Moreover, western-blot was used to investigate the underlying mechanism of WAVE1 regulating the proliferative and invasive malignant behaviors in ovarian cancer cells.

Results. The down-regulation of WAVE1 had a significant effect on cell morphological changes. WAVE1 silencing decreased cell migration, cell invasion, cell adhesion, colony formation and cell proliferation in vitro. In addition, we found that down-regulation of WAVE1 inhibited malignant behaviors in vivo. Furthermore, our study also indicated that the PI3K/AKT and p38MAPK signaling pathways might contribute to WAVE1 promotion of ovarian cancer cell proliferation, migration, and invasion.

Conclusions. WAVE1 might promote the proliferative and invasive malignant behaviors through the activation of the PI3K/AKT and p38MAPK signaling pathways in EOC.

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Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy. In the majority of cases, patients are diagnosed at an advanced stage and suffer unfavorable outcomes [1]. Although there are advanced surgical and cytotoxic therapies, 80% of late-stage patients develop recurrent disease, and <30% of patients survive 5 years after diagnosis [2,3]. Tumor invasion and metastasis are the primary causes for the high rate of EOC morbidity and mortality [4].

Wiskott-Aldrich syndrome protein (WASP) family verprolin-homologous protein 1 (WAVE1) has been identified as inducing lamellipodia formation [5,6]. Rho family small GTPases, WASP family proteins, actin-related protein 2/3 (Arp2/3) complex, and myosin are involved in the reorganization of actin filaments. WASP family proteins have direct effects on actin filament reorganization [7,8]. WAVE1 has been identified as being sequestered in an inactive state via the formation of a complex with abelson interactor protein-1 (Abil), Nick-associated protein 1 (Nap1), hematopoietic stem progenitor cell 300 (Hspc300), and a p53-inducible mRNA (PIRI21) [9,10]. Recent reports have demonstrated that WAVEs also play a critical role in cancer cell migration and invasion [11-16]. Another study demonstrated the overexpression, compared to non-invasive cells levels, of WAVE1 in highly malignant metastatic melanoma cells [17]. In prostate cancer cells, WAVE1 knockdown decreases tumor cell invasion [18]. Moreover, WAVE1 plays a role in invasion and multi-drug resistance through the regulation of MMP-2 and Bcl-2 expression levels in leukemia cells [19,20]. The above findings indicated that WAVE1 acts as an enhancer gene in cancer cells.
Our previous studies indicated that WAVE1 expression is significantly elevated in the plasma of EOC patients, and overexpression of WAVE1 in EOC tissues is associated with a poor prognosis [1,21]. Here, we focused on the functions and mechanisms of WAVE1 in the proliferative, migratory, and invasive malignant behaviors of ovarian cancer cells.

Materials and methods

Cell culture and reagents

The human epithelial ovarian cancer cell lines SKOV3, OVCAR-3, ES-2 and 3AO were obtained and cultured as described previously [21]. Goat anti-WAVE1, rabbit anti-Abi1, rabbit anti-VEGF, rabbit anti-E-cadherin, mouse anti-cyclinD1, mouse anti-Arp2, rabbit anti-p38, and rabbit anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-AKT, rabbit anti-phospho-AKT, rabbit anti-ERK1/2, rabbit anti-phospho-ERK1/2, and rabbit anti-phospho-p38 MAPK antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Rabbit anti-Nap1, mouse anti-MMP-2, and rabbit anti-MMP-9 antibodies were purchased from Abcam (Cambridge, UK).

Western blot analysis

Cells and tissues were lysed on ice in lysis buffer (Beyotime, Jiangsu, China). The protein concentrations of the samples were determined using a BSA protein assay kit (Beyotime, Jiangsu, China). For the blots, 40 μg of total protein was loaded onto a 6% to 12% SDS-PAGE gel. Immunoblot blot analysis was performed as described previously [21]. The dilution rates of the primary antibodies were 1:100 to 1:1000. GAPDH was used as an internal control.

Quantitative real-time PCR (Q-RT-PCR)

Total RNA was isolated from cells with TRIzol reagent (Takara, Japan), and first-strand DNA was synthesized using a cDNA reverse transcription kit (Takara, Japan), according to the manufacturer’s protocol. Then, a 25-μl reaction volume from each well was examined using a CFX96™ real-time PCR Detection System (BioRad, USA). The primers sequences were as follows: WAVE1, sense primer: 5′-TCAACCTTGAGACGTGTTGACAGCTC-3′ and anti-sense primer: 5′-TCATGTGTGCTATCGCTCC-3′ and GAPDH, sense primer: 5′-GGTCCAGTCAACCGATTG-3′ and anti-sense primer: 5′-GGAATGTGATGGGAATTTC-3′. The expression level was determined using the 2−ΔΔCt method.

RNA interference

To knockdown WAVE1 in the SKOV3 cells, two small hairpin RNA (shRNA) sequences were designed to target human WAVE1 as follows: 5′-AATGTTGCAAGATACAGAGG-3′ (shRNA-1) and 5′-GGATAGAACCTGACGTC-3′ (shRNA-2). Non-target shRNA (5′-GTTGATTTTGTGCCATCA-3′) transfected cells were used as negative control. The shRNA transfections were performed using lentiviruses according to the manufacturer’s protocol. Infected cells were subjected to 2 μg/ml puromycin, and survived clones were isolated. The expression level of WAVE1 was confirmed by Western blot analysis and Q-RT-PCR.

Invasion and migration assays

To investigate the invasion ability of the cells, 24-well transwell inserts with 8-μm pores (BD Bioscience, CA, USA) coated with a 1:5 dilution of Matrigel (BD Bioscience, CA, USA) were used. For the cell migration assay, 24-well transwell inserts without a Matrigel coating were used. Cells (1 × 10^5), suspended in 200 μl of RPMI1640 medium containing 5% FBS, were seeded in triplicate into the upper chamber, and 600 μl of RPMI1640 (10% FBS) medium was added to the lower well as a chemoattractant. After 24 and 48 h of incubation, the non-motile cells on the upper surface of the membrane were wiped off, whereas the cells on the lower side were fixed by 4% paraformaldehyde and stained with hematoxylin. The number of cells was counted in five random fields per well under a light microscope.

Cell adhesion assay

Cells (2 × 10^4) were plated in triplicate in 96-well plates coated with Matrigel and incubated at 37 °C for 1 h. After rinsing, the adherent cells in each well were fixed with 4% formaldehyde and then stained with crystal violet. The adherent cell stain was extracted by acetic acid and quantified using a microplate reader at 490 nm absorbance.

Proliferation assay

Cells (0.5 × 10^3) were seeded into 96-well plates in triplicate and harvested on days 1, 2, 3, 4, and 5. At the start of harvesting, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added into each well. After incubation for 4 h at 37 °C, 150 μl of dimethyl sulfoxide (DMSO) was added to dissolve any present formazan crystals. The absorbance was measured at 490 nm using a microplate reader.

Soft agar assay

For the soft agar assay, 5 ml of 0.6% agar solution in RPMI1640 medium containing 10% FBS was solidified in 6-cm dishes in triplicate. The 4 ml of 0.3% agar solution, mixed with 1 × 10^4 cells, was layered on top of a base agar layer. After incubation for 3 weeks at 37 °C, each colony formation containing over 50 cells was counted.

Tumor formation in nude mice

For the tumor xenograft experiments, each 6-week-old athymic female nude mouse was injected subcutaneously with 4 × 10^6 cells in 100 μl of PBS in the flanks: SKOV3-R1 (8 implants) and SKOV3-NC (8 implants). The tumor sizes were measured weekly. Five weeks after injection, the mice were euthanized, and the xenografts were removed for analysis. All procedures were approved by the Institution Animal Care Committee at Chongqing Medical University, Chongqing, China.

Confocal immunofluorescence microscopy

The cells were seeded on sterile glass coverslips overnight and then fixed in methanol at −20 °C for 10 min, permeabilized with Triton-X at 37 °C for 10 min, and blocked with 10% normal goat serum at 37 °C for 30 min. Subsequently, the cells were incubated with actin (1:50) primary antibody overnight at 4 °C. After washing, the cells were incubated with secondary antibody at 37 °C for 1 h. All images were taken with a laser scanning confocal microscope.

Statistical analysis

The continuous data from the experiments are presented as the mean ± standard deviation (SD). Statistical evaluation was performed using one-way ANOVA analysis and Student’s t-test. SPSS Version 17.0 for Windows was used for statistical analysis. All tests were two-tailed and considered to be significant when P < 0.05.
Results

WAVE1, Abi1, Arp2, and Nap1 expression in ovarian cancer cell lines

We selected four ovarian cancer cell lines to investigate the expression level of WAVE1 and other members of its molecular complex (Abi1, Arp2, and Nap1) (Fig. 1A). Expression levels of WAVE1, Abi1, Arp2, and Nap1 were all detected in the four cell lines. SKOV3 and OVCAR-3 cells were selected for use in the subsequent experiments.

Silencing of WAVE1 expression by lentivirus-mediated RNA interference

To explore the role of WAVE1 in ovarian cancer cells, we knocked down WAVE1 in SKOV3 and OVCAR-3 cells. We constructed lentivirus vectors with two different WAVE1 shRNAs to infect SKOV3 and OVCAR-3 cells, which designated as SKOV3-Ri and OVCAR-3-Ri. Non-target shRNA infected SKOV3 and OVCAR-3 cells, which designated as SKOV3-NC and OVCAR-3-NC, were used as negative controls. After the initiation of RNA interference, the mRNA and protein levels of WAVE1 in SKOV3-Ri and OVCAR-3-Ri were significantly reduced compared with controls ($P < 0.05$) (Fig. 1B and C). Thus, SKOV3-Ri1 and OVCAR-3-Ri2 were used in the subsequent experiments.

WAVE1 silencing induces morphologic changes in vitro

The morphology of the SKOV3-Ri1, OVCAR-3-Ri2, SKOV3-NC, and OVCAR-3-NC were examined using actin staining. The SKOV3-Ri1 and OVCAR-3-Ri2 were unpolarized, missing their pseudopodia, presented reduced cellular protrusions, and were reshaped compared with SKOV3-NC and OVCAR-3-NC (Fig. 2A).

WAVE1 silencing decreases cell migration and invasion in vitro

Transwell assays were carried out to determine the effects of WAVE1 silencing on ovarian cancer cell migration and invasion. In SKOV3-Ri1 and OVCAR-3-Ri2, approximately 68% and 67% reduction in the number of migratory cells were observed compared with SKOV3-NC and OVCAR-3-NC ($P < 0.001$ and $P < 0.001$, respectively) (Fig. 2B), and there were also significant decreases of approximately 82% and 83% in the number of invading cells compared with SKOV3-NC and OVCAR-3-NC ($P < 0.001$ and $P < 0.001$, respectively) (Fig. 2C).

WAVE1 silencing inhibits cell adhesion in vitro

To explore how WAVE1 regulates the actin polymerization involved in cell adhesion, we performed an adhesion assay by using Matrigel, which contains the majority of the extracellular matrix (ECM) components. The SKOV3-Ri1 and OVCAR-3-Ri2 presented approximately 67% and 72% decrease in cell adhesion compared with SKOV3-NC and OVCAR-3-NC ($P < 0.001$ and $P < 0.001$, respectively) (Fig. 3A).

WAVE1 silencing inhibits colony formation and cell proliferation in vitro

Using a soft agar assay, we found that the average number of colonies formed by SKOV3-Ri1 and OVCAR-3-Ri2 were significantly lower than the number formed by SKOV3-NC and OVCAR-3-NC ($P < 0.001$ and $P < 0.001$, respectively), and the sizes of the colonies were also reduced (Fig. 3B). Moreover, using the MTT assay, we found that SKOV3-Ri1 and OVCAR-3-Ri2 presented a significant growth-inhibiting effect ($P < 0.001$ and $P < 0.001$, respectively) (Fig. 3C). In addition, the cells were treated with 0, 1, 2, 3, or 4 $\mu$mol/l of the cisplatin. The growth curve indicated that the SKOV3-Ri1 and OVCAR-3-Ri2 treated with different

![Fig. 1. WAVE1 expression level in ovarian cancer cell lines.](image-url)
concentrations of cisplatin presented significantly reduced growth compared with SKOV3-NC and OVCAR-3-NC (P < 0.05 and P < 0.05, respectively).

WAVE1 silencing inhibits tumor formation in xenografts in vivo

Tumor formation in nude mice was accomplished by subcutaneous injection of cells into the flanks (Fig. 4A). At the injection sites, we found that the formation rates were 87.5% and 100% for SKOV3-Ri1 and SKOV3-NC, respectively (Fig. 4B). We found that the growth rate of the xenografts of SKOV3-Ri1 were significantly slower than SKOV3-NC. On week 5, the size of xenografts of SKOV3-Ri1 (1.579 ± 0.055) was significantly smaller than SKOV3-NC (0.691 ± 0.055) (P < 0.001) (Fig. 4A and C). We collected all xenografts and examined several invasion and proliferation-related biomarkers by Western blotting analysis. As shown in Fig. 4D, the down-regulation of WAVE1, MMP-2, MMP-9, VEGF, and cyclin D1 expression, and the up-regulation of E-cadherin expression, were observed in xenografts formed by SKOV3-Ri1, compared with SKOV3-NC.

WAVE1 silencing affects PI3K/AKT and p38MAPK signaling activation

To further elucidate the underlying mechanisms of WAVE1-induced cell proliferation and invasion, we decided to test three signaling pathways that regulate cancer cell tumorigenesis and metastasis. Our findings indicated that total AKT, phospho-AKT, and phospho-p38 protein levels were down-regulated in WAVE1-silenced cells. In contrast, no WAVE1 silencing-induced changes were observed in total ERK1/2, phospho-ERK1/2, and p38 levels (Fig. 5). These data indicated that the knockdown of WAVE1 inhibited the activation of the PI3K/AKT and p38MAPK signaling pathways.

Discussion

In the present study, expression levels of WAVE1, Abi1, Arp2, and Nap1 were all detected in the SKOV3, 3AO, OVCAR-3 and ES-2 cell lines. SKOV3 and OVCAR-3 cells were selected for use in the subsequent experiments. For the subsequent experiments, we knocked down WAVE1 expression in SKOV3 and OVCAR-3 cells using shRNA-mediated gene
silencing. We found that down-regulation of WAVE1 changed the formation of lamellipodia and also inhibited ovarian cancer cell adhesion to Matrigel.

Metastasis is a complex biological process. Tumor cells acquire the invasion and migration abilities for dissemination from a primary tumor to distant secondary organs or tissues [22,23]. Down-regulation of WAVE1 expression in cells induces slower migration and inhibited invasion compared to control cells. The existence of WAVE1 in ovarian cancer cells might result in higher metastatic migration and invasion abilities. Consistent with our findings, these observations have also been reported in prostate cancer, melanoma, breast cancer, and leukemia [15,18,19,24]. The role of WAVE1 in actin reorganization and the lamellipodia formation is well established; however, the role of the protein in cell proliferation remains unknown. Our study indicated that when WAVE1 was down-regulated by RNA interference in ovarian cancer cells, a clear inhibition of cell proliferation and colony formation was observed. We further demonstrated that knockdown of WAVE1 expression could reduce cell cisplatin resistance. These data support the findings from our previous study, wherein WAVE1 overexpression was correlated with poor prognosis in ovarian cancer.

We also investigated the malignant behaviors of WAVE1 in vivo. WAVE1-expressing xenograft tumors were larger and had a significant reduction of E-cadherin and an increase in MMP-2, MMP-9, VEGF, and cyclinD1 levels. MMPs play a critical role in the processes of tumor cell invasion and metastasis, and MMP-2 and MMP-9 have been directly linked to ovarian cancer metastatic processes [25,26]. We found that knockdown of WAVE1 suppressed the expression levels of MMP-2 and MMP-9. WAVE1 likely regulates the metastatic activities of OC cells through the down-regulation of MMP2 and MMP-9. In addition, we examined the expression of VEGF in xenografts. VEGF is a key player in pathological angiogenesis, which promotes the supply of oxygen and nutrients required for cell invasion [27,28]. Our study indicates that knockdown of WAVE1 significantly decreases the VEGF protein level, suggesting that WAVE1 might play a role in OC angiogenesis through
regulating VEGF. Another important molecule involved in cell adhesion establishment and maintenance is E-cadherin. The loss or inhibition of the expression of E-cadherin results in reduced intercellular adhesion and enhances epithelial tumor cell invasion and migration capacities [29,30]. Similarly, our present study found that E-cadherin expression was highly increased in xenografts formed by SKOV3-Ri1. In addition, we also examined the proliferation biomarker cyclin D [31,32]. Our results also indicated that the knockdown of WAVE1 inhibited the expression level of cyclin D1, which suggests that WAVE1 might regulate OC cell proliferation by altering the expression of cyclin D1.

The underlying mechanisms of how WAVE1 contributes to ovarian cancer cell invasion and proliferation remain unknown. The PI3K/AKT, ERK1/2, and p38MAPK signaling pathways have been reported to be involved in cancer cell proliferation and invasion [33–36]. Our current study observed that down-regulation of WAVE1 expression altered total AKT, phospho-AKT and phospho-p38 levels. There is a possible direct effect of WAVE1 on the PI3K/AKT and p38MAPK signaling pathways that regulates proliferation and invasion-related biomarkers. However, there may also be an indirect effect that regulates Rac activation and its downstream molecules through a positive feedback loop. Taken together, these data may suggest that WAVE1 both directly enhances ovarian cancer cell invasion and proliferation via activation of the PI3K/AKT and p38MAPK signaling pathways and indirectly affects AKT and p38 through Rac to decrease the migratory capability of ovarian cancer cells.

In summary, we have provided the first evidence that knockdown of WAVE1 expression in ovarian cancer cells results in a reduction of cell invasion, migration and proliferation. Overexpression of WAVE1 in ovarian cancer tissues is correlated with poor prognosis and aggressive disease [21]. Moreover, our results have demonstrated that WAVE1 promotes the malignant behaviors through the activation of the PI3K/AKT and p38MAPK signaling pathways in EOC.

Disclosure of potential conflicts of interest
No potential conflicts of interest were disclosed.

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Fig. 5. The effect of WAVE1 silencing on the activation of the PI3K/AKT, ERK1/2, and p38MAPK signaling pathways in vitro. GAPDH was used as an internal control. *, P < 0.05.

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