

Vasohibin 1 inhibits Adriamycin resistance in osteosarcoma cells via the protein kinase B signaling pathway

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Abstract. Vasohibin (VASH)1 functions as a negative feedback modulator of angiogenesis in vascular endothelial cells. Mesenchymal VASH1 has been demonstrated to be negatively associated with tumor progression, however studies regarding VASH1 in tumor cells and its functions remain limited. The function of VASH1 in osteosarcoma remains unknown. In the present study, it was confirmed that osteosarcoma cells express decreased levels of VASH1 compared with that expressed by human osteoblast cells. 143B cells with decreased VASH1 expression revealed increased Adriamycin (ADR) resistance compared with U-2OS cells with increased VASH1 expression. Subsequent to manipulating VASH1 expression via transfection, results revealed that overexpression of VASH1 in 143B cells inhibited P-glycoprotein (P-gp) expression and ADR resistance significantly; silencing VASH1 in U-2OS cells enhanced P-gp expression and ADR resistance significantly. Research into the molecular mechanism was performed and the results identified that protein kinase B (AKT) and extracellular signal-related kinase signal pathways were both stimulated by VASH1, but only AKT inhibitor LY294002 was identified to efficiently counteract increases in P-gp expression that had been induced by silencing of VASH1 in U-2OS cells. ADR resistance promoted by silencing VASH1 in U-2OS cells was also counteracted by LY294002. In conclusion, the present study confirmed the low expression of VASH1 in osteosarcoma cells. It was identified that VASH1 was able to inhibit drug resistance in osteosarcoma cells through regulation of P-gp via the AKT signal pathway. This demonstrated a negative regulation function of VASH1 in osteosarcoma, deepened understanding of the function of VASH1 in tumors and suggests a basis for further studies in to the functions of VASH1.

Introduction

Osteosarcoma is the most common malignant tumor of the bone and remains the second leading cause of cancer-associated mortality in adolescents globally at present (1). Surgery combined with adjuvant chemotherapy is currently the standard treatment for osteosarcoma (1). In recent years, although a great deal of effort has been made toward improving chemotherapy regimens, the overall prognosis remains poor and much of this may be attributed to drug resistance (2). P-glycoprotein (P-gp), an ATP-binding cassette (ABC) membrane transporter encoded by multidrug resistance 1 (MDR1), is commonly located at the plasma membrane and functions as an ATP-dependent efflux pump for diverse naturally occurring hydrophobic anticancer drugs, including Adriamycin (ADR) (3). Finding an efficient method of inhibiting drug resistance may contribute to better therapeutic outcomes.

Vasohibin (VASH)1 was first identified to be a negative feedback modulator of angiogenesis in vascular endothelial cells in a previous study (4). Inhibitory functions of mesenchymal VASH1 in tumor progression have been reported in different types of tumor (5-7). The functions of parenchymal VASH1 in tumor development have drawn more and more attention, but relevant reports remain limited. Liu *et al* (2) reported that overexpression of VASH1 in colon cancer cells was able to induce apoptosis and senescence, and inhibited cancer cell growth and colony formation *in vitro* and tumor growth *in vivo*. In addition, knockdown of VASH1 in cancer cells was able to promote cell growth, adhesion and migration *in vitro* and enhance tumorigenesis and metastasis *in vivo* (8). Takahashi *et al* (9) reported that VASH1 overexpression in ovarian cells inhibited ovarian cancer growth and peritoneal dissemination and prolonged host survival. Thus far, there remains no report on the functions of VASH1 in osteosarcoma to the best of our knowledge.

In the present study, it was identified that VASH1 is underexpressed in osteosarcoma cells. It was also revealed that VASH1 was able to inhibit ADR resistance of osteosarcoma cells through regulation of the protein kinase B (AKT) signaling pathway. This suggested that further evaluation of VASH1 may yield a novel therapeutic approach to the treatment of osteosarcoma.

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Materials and methods

Cell culture. The human osteoblast cell line hFOB1.19 and human osteosarcoma cell lines U-2OS and 143B were purchased from American Type Culture Collection (Manassas, VA, USA). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.), at 37°C in 5% CO₂.

Drug resistance assay. U-2OS and 143B cells were counted and plated in 96-well plate at 10,000 cells/well. After 24 h, the culture medium was replaced with DMEM containing different concentrations (2, 4, 8, 16, 32 μmol/l) of ADR (HarveyBio, Inc., Beijing, China). These cells served as experimental groups. Cells in medium without ADR served as the control group. After 48 h, an MTT assay kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) was used and the optical density (OD) value was measured at 490 nm wavelength using an ultraviolet spectrophotometer (Shanghai Spectrum Instrument Co., Ltd., Shanghai, China) according to the manufacturer's protocol according to the manufacturer's protocol. Inhibition rate (IR) was calculated using the following equation: IR = 1 - OD value of experiment group/OD value of control group × 100%. Half maximal inhibitory concentration (IC₅₀) was calculated using regression analysis by SPSS 11.0 software (SPSS, Inc., Chicago, IL, USA). All experiments were repeated 3 times.

Cell transfection. U-2OS and 143B cells were counted and plated in 6-well plates at 2 × 10⁵ cells/well. After 24 h, p-GPU6/Neo/VASH1 (Shanghai GenePharma Co. Ltd., Shanghai, China) to silence VASH1 expression, and pEZM61/VASH1 (Gene Copoeia, Guangzhou, China) to over-express VASH1 were transfected using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Empty plasmids were used as control. Reverse transcription-polymerase chain reaction (RT-PCR) and western blotting were performed to confirm transfection efficiency.

RT-PCR. RNA was extracted from cells using TRIzol (Life Sciences; Thermo Fisher Scientific, Inc.) according to the manufacturers protocol. cDNA was synthesized using a PrimeScript RT-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China). PCR was performed using specific primers and Universal PCR Master Mix (Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows; VASH1, 4°C for 5 min, 94°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec for 40 cycles and 72°C for 5 min; P-gp, 4°C for 5 min, 94°C for 30 sec, 54°C for 30 sec, 72°C for 30 sec for 36 cycles and 72°C for 5 min; GAPDH, 4°C for 5 min, 94°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec for 36 cycles and 72°C for 5 min. PCR products were electrophoretically separated on 1.0% agarose gel. Results were analyzed using Labwork software (version 4; UVP, Inc., Upland, CA, USA). All primers were as follows: VASH1 forward, 5'-CCACGCCCTGATTTCTTAAA-3' and reverse, 5'-CCCTGTGAGAGGCTGCTCT-3'; P-gp forward, 5'-CCCATCATTGCAATAGCAGG-3' and reverse, 5'-GTTCAAACCTTCTGCTCCTGA-3'; GAPDH forward, 5'-AGA

AGGCTGGGGCTCATTTG-3' and reverse, 5'-AGGGGCCATCCACAGTCTTC-3' GAPDH served as an internal control. All experiments were repeated 3 times.

Western blotting. Protein was extracted from cells using radio-immunoprecipitation assay lysis containing 1% phenylmethane sulfonyl fluoride (Beyotime Institute of Biotechnology, Haimen, China) and protein concentration was analyzed using a bicinchoninic acid assay kit (Pierce; Thermo Fisher Scientific, Inc.). Equal quantities of protein were loaded per well in 5% acrylamide and separated using 10% SDS-PAGE, and transferred to nitrocellulose membrane. The membrane was incubated with primary antibodies (Table I) at 4°C overnight, and then in horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (cat. no. ab205718; 1:4,000; Abcam, Cambridge, MA, USA) at room temperature for 1 h. Signals were detected using enhanced chemiluminescence reagents (Pierce; Thermo Fisher Scientific, Inc.) and quantified using Image-Pro software (version 5.1; Media Cybernetics, Inc., Rockville, MA, USA). All experiments were repeated 3 times.

Statistical analysis. SPSS software (version 11.0; SPSS, Inc., Chicago, IL, USA) was used and data were expressed as mean ± standard deviation. Differences between groups were analyzed using one-way analysis of variance with Dunnett's post hoc test. IC₅₀ was calculated using regression analysis. P < 0.05 was considered to indicate a statistically significant difference.

Results

VASH1 is expressed weakly in osteosarcoma cells. VASH1 expression was exhibited both at RNA (Fig. 1A) and protein (Fig. 1B) levels. Compared with human osteoblast cell lines hFOB1.19, decreased VASH1 expression was detected in osteosarcoma cell lines U-2OS and 143B. VASH1 expression was significantly decreased in 143B cells compared with that in U-2OS cells. A drug resistance assay was subsequently performed, revealing that the inhibition rate (IR) of 143B cells in ADR was decreased compared with that of U-2OS (Fig. 1C). The IC₅₀ of 143B cells (6.59 ± 0.89 μmol/l) was significantly increased compared with that of U-2OS cells (4.32 ± 0.47 μmol/l; Fig. 1D). All these results indicate possible associations between VASH1 expression and drug resistance.

VASH1 inhibits the ADR resistance of osteosarcoma cells. To confirm whether VASH1 was able to regulate drug resistance of osteosarcoma cells, VASH1 expression was manipulated through transfection. Following overexpression of VASH1 in 143B cells, P-glycoprotein (P-gp) expression was significantly inhibited at both the RNA (Fig. 2A) and protein (Fig. 2B) levels. The IR of 143B cells was increased compared with control cells (Fig. 2C). IC₅₀ declined from 7.14 ± 0.83 to 3.79 ± 0.56 μmol/l (Fig. 2D). Following silencing of VASH1 in U-2OS cells, P-gp expression was upregulated both at RNA (Fig. 2E) and protein (Fig. 2F) levels. IR of U-2OS cells declined significantly (Fig. 2G), IC₅₀ increased from 4.32 ± 0.88 to 7.34 ± 0.69 or 6.71 ± 0.82 μmol/l (Fig. 2H). All results suggested the inhibitory function of VASH1 in ADR resistance.

Table I. Primary antibodies used in western blotting.

Name	Cat. no.	Dilution	Supplier
VASH1	Ab199732	1:300	Abcam, Cambridge, UK
P-gp	Ab170904	1:400	Abcam, Cambridge, UK
phospho-ERK1/2	AF1018	1:1,000	Cell Signaling Technology, Inc., Danvers, MA, USA
ERK1/2	AF1576	1:1,000	Cell Signaling Technology, Inc., Danvers, MA, USA
phospho-AKT	AF887	1:1,000	Cell Signaling Technology, Inc., Danvers, MA, USA
AKT	AF2055	1:1,000	Cell Signaling Technology, Inc., Danvers, MA, USA
GAPDH	Ab9485	1:4,000	Abcam, Cambridge, UK

VASH, Vasohibin; P-gp, p-glycoprotein; phospho, phosphorylated; ERK, extracellular signal-related kinase; AKT, protein kinase B.

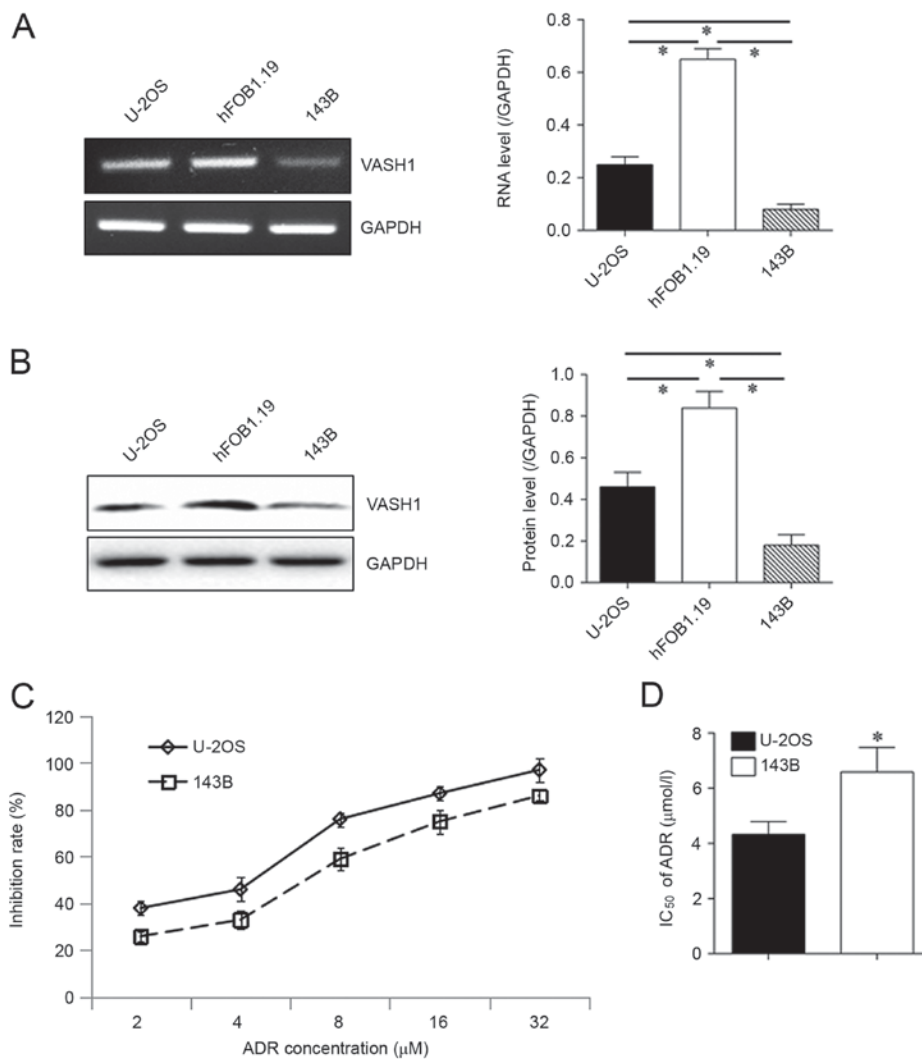


Figure 1. VASH1 expression status in osteosarcoma cells and ADR resistance. VASH1 expression in the human osteoblast cell line hFOB1.19 and the human osteosarcoma cell lines U-2OS and 143B was detected using (A) reverse transcription-polymerase chain reaction and (B) western blot analysis. * $P < 0.05$ (hFOB1.19 vs. U-2OS and 143B). (C) ADR resistance of U-2OS and 143B cells was detected using a drug resistance assay. (D) IC₅₀ of U-2OS and 143 cells in ADR. * $P < 0.05$ (U-2OS vs. 143B). ADR, Adriamycin; VASH, Vasohibin.

VASH1 regulation of ADR resistance uses the AKT signaling pathway. As presented in Fig. 3A, following overexpression of VASH1 in 143B cells, phosphorylation of extracellular signal-related kinase (ERK) and AKT was inhibited (Fig. 3A). Conversely, following silencing of VASH1 in U-2OS cells,

phosphorylation of ERK and AKT was upregulated (Fig. 3B). Once AKT inhibitor LY294002 was added, the increase of P-gp in U-2OS cells induced by silencing VASH1 was decreased (Fig. 3C). However, with ERK inhibitor U0126 added, no change was observed in P-gp expression (Fig. 3D).

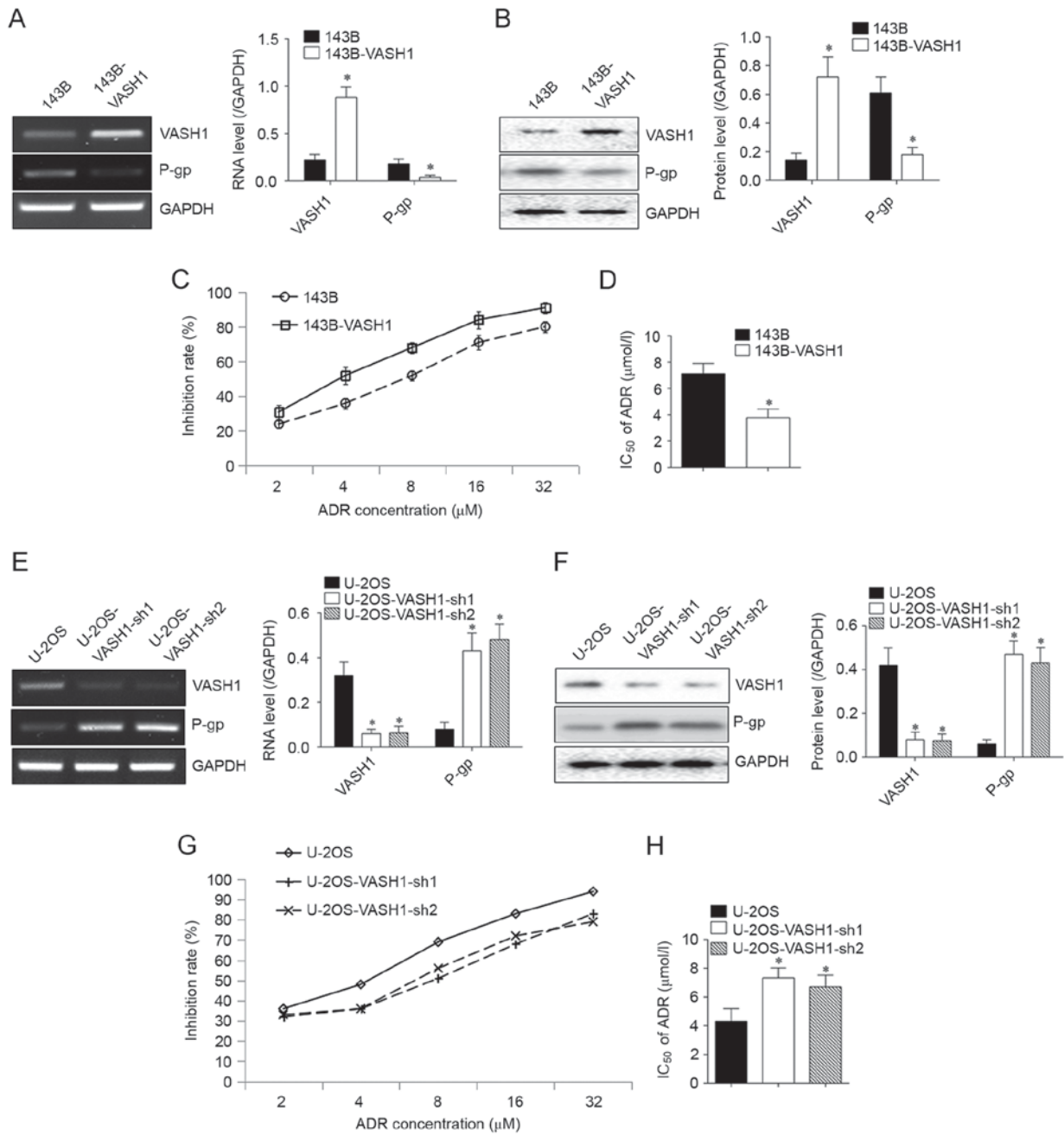


Figure 2. VASH1 inhibits ADR resistance of osteosarcoma cells. Following overexpression of VASH1 in 143B cells, P-gp expression declined at both the (A) RNA and (B) protein levels. (C) The inhibition rate of 143B cells in ADR increased and (D) IC₅₀ declined significantly *P<0.05 vs. 143B cells. Following silencing of VASH1 in U-2OS cells, P-gp expression increased at both the (E) RNA and (F) protein levels. (G) The inhibition rate of U-2OS cells in ADR declined with VASH1 silenced and (H) IC₅₀ increased significantly. *P<0.05 vs. U-2OS cells. ADR, Adriamycin; VASH, Vasohibin; P-gp, p-glycoprotein.

A drug resistance assay also revealed that LY294002 could counteract the decrease of IR of U-2OS cells in ADR induced by silencing VASH1, but U0126 did not influence declination of IR of U-2OS cells in ADR induced by silencing VASH1. This suggests that the AKT signaling pathway may serve a function in ADR resistance regulated by VASH1 (Fig. 3E).

Discussion

A member of the vasohibin family, the human VASH1 gene is located on chromosome 14q24.3. VASH1 protein is composed of 365 amino acids with no glycosylation sites (10,11). Vasohibin 2 is also a member of the vasohibin family and

was initially known as an angiogenic factor. VASH1 was first noticed for its ability to inhibit angiogenesis; it is restricted in vessel endothelial cells and several other types of cell (12). The negative regulation of VASH1 from tumor cells on tumor progression has been demonstrated in colon cancer (8), ovarian (9) and renal carcinoma (13). However, in 2014, Kitajima *et al* (14) reported that high VASH1 in the cytoplasm of colorectal cancer (CRC) tissues was positively associated with tumor progression, and silencing VASH1 inhibited CRC cell proliferation, migration and invasion, and promoted anoikis. Thus, the functions of VASH1 in different types of tumor are not consistent, therefore the effects of VASH1 on osteosarcoma require further investigation.

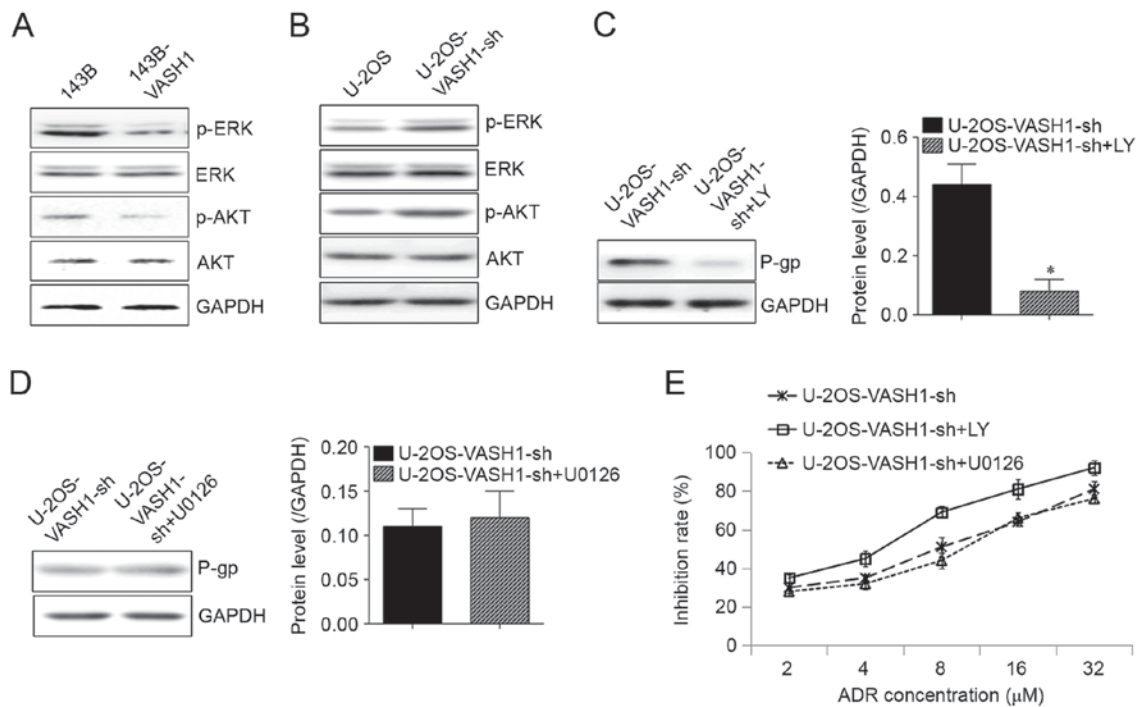


Figure 3. ADR resistance regulated by VASH1 relies on the AKT signal pathway. (A) Subsequent to overexpressing VASH1 in 143B cells, phosphorylation of AKT and ERK declined. (B) Subsequent to silencing of VASH1 in U-2OS cells, phosphorylation of AKT and ERK increased. (C) AKT inhibitor LY294002 inhibited P-gp expression and (E) ADR resistance in U-2OS cells induced by silencing VASH1 * $P < 0.05$ vs. U-2OS cells with silenced VASH1. ERK inhibitor U0126 exhibited no effect on (D) P-gp expression or (E) ADR resistance in U-2OS cells induced by silencing VASH1. ADR, Adriamycin; VASH, Vasohibin; P-gp, p-glycoprotein; AKT, protein kinase B; ERK, extracellular signal-related kinase.

Drug resistance is an important characteristic of malignant tumors and has been an important factor in the failure of cancer treatment (15). ATP-binding cassette drug efflux pump P-gp has been proposed to serve crucial functions for tumor cells acquiring MDR (16,17). ADR is the first-line chemotherapy drug used to treat osteosarcoma. It not only inhibits DNA transcription and replication but also induces breakage of DNA double strands (18). In the present study, data revealed low expression of VASH1 in osteosarcoma cells at both the RNA and protein levels. Furthermore, osteosarcoma cells with lower VASH1 levels exhibited more marked ADR resistance. This suggests that VASH1 may serve negative regulatory functions in osteosarcoma drug resistance. Through changing VASH1 using transfection, it was identified that VASH1 was able to inhibit the P-gp expression and ADR resistance of osteosarcoma cells. This is consistent with the negative regulatory functions of VASH1 reported by the majority of works (8,9,13), but inconsistent with a report from Kitajima *et al* (14). Different organs of origin of different tumors may explain this divergence.

The AKT and ERK signaling pathways may be stimulated in different types of tumors. This activates proliferation and survival signals that ultimately lead to tumorigenesis and progression (19). In 2016, Yang *et al* (20) reported that the ERK signal pathway may serve important functions in 5-FU-mediated of colorectal cancer. Xiao *et al* (21) identified that Oridonin inhibits gefitinib-resistant lung cancer cells by suppressing ERK and AKT signaling pathways. In the present study, it was identified that VASH1 downregulated P-gp expression by blocking the AKT signal pathway, thus inhibiting ADR resistance of osteosarcoma cells. In the present study, no effects of ERK were observed in ADR resistance. This is not consistent

with relevant reports from Yang *et al* (20) and Xiao *et al* (21). This may be attributed to differences in the type of tumor and drugs with different anti-tumor mechanisms.

To conclude, the inhibitory effects of VASH1 on osteosarcoma drug resistance were confirmed. This has enhanced understanding of the functions of VASH1 in tumors and supplied a basis for ongoing studies targeting VASH1. VASH1 may be treated as an enhancer of chemotherapeutic sensitivity in osteosarcoma cells to foster better prognosis.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HL designed this study. WH performed the experiments. YR analyzed the results.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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