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VASH2 promotes oxaliplatin resistance in gastric cancer cells via the AKT signaling pathway

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ABSTRACT: Gastric cancer (GC) is one of the most common malignant tumors worldwide. GC cells are sensitive to chemotherapy drugs such as oxaliplatin (OHP, trans -/- diaminocyclohexane oxaliplatin or L-OHP). The molecular mechanisms involved in regulation of L-OHP resistance remain unknown. Vasohibin 2 (VASH2), a member of the vasohibin (VASH) family, has been implicated in chemical resistance in several cancer types. However, the possible role of VASH2 in the oxaliplatin resistance of GC is still unclear. In this study, we constructed two L-OHP resistance GC cell lines and found that VASH2 was highly expressed in L-OHP-treated GC cells. Downregulation of VASH2 suppressed the viability of L-OHP-treated GC cells. In addition, VASH2 knockdown stimulated the apoptosis of two oxaliplatin-resistant GC cell lines. In conclusion, our results suggested that VASH2 promoted oxaliplatin resistance in GC cells through the serthreonine protein kinase AKT pathway.

KEYWORDS: vasohibin 2 (VASH2), oxaliplatin, gastric cancer, apoptosis, AKT pathway

INTRODUCTION

Gastric cancer (GC) is a common malignant tumor worldwide [1]. China has the highest incidence of GC, and its mortality rate ranks second among all tumors in China [2]. GC cells are sensitive to chemotherapy drugs, oxaliplatin (OHP, trans -/- diaminocyclohexane oxaliplatin or L-OHP) is the most commonly used chemotherapy agent in clinical practice [3]. However, drug resistance often seriously impedes the efficacy and therapeutic progress of L-OHP [4]. The molecular mechanisms involved in L-OHP resistance remain unknown. Several studies have shown that activation of the Akt signaling pathway induced drug resistance [5]. The Akt pathway is stimulated by various chemotherapeutic agents including oxaliplatin [6].

The vasohibin (VASH) family consists of two genes, *VASH1* and *VASH2* [7]. VASH1 is mainly found in vascular endothelial cells and inhibits angiogenesis [8], while vasohibin 2 (VASH2) is mainly expressed in cancer cells and has angiogenic activity [9]. The overall survival, cancer-specific survival and relapse-free survival of gastric cancer patients with high VASH2 expression were significantly lower than those with low VASH2 expression [10].

VASH2 has been implicated in chemical resistance in several cancer types [10]. For example, VASH2 induces gemcitabine resistance in pancreatic cancer cells through trans-activation of the ribonucleotide reductase regulating subunit M2 [11, 12]. VASH2 reduces the sensitivity of hepatocellular carcinoma (HCC) cells to cisplatin by inhibiting the expression of p53 [13]. VASH2 enhances adriamycin resistance of breast cancer cells via AKT signaling pathway [14]. VASH2 promotes non-small cell lung cancer (NSCLC) cell proliferation and adriamycin resistance by regulating serthreonine protein kinase (AKT) pathway [10]. VASH2 expression is increased in mouse gastric tumors, and its depletion leads to reduced tumor angiogenesis, and reduction of cancer-associated fibroblasts in tumor stroma [15]. However, the possible role of VASH2 in the oxaliplatin resistance of GC is still unclear.

In this study, we constructed two oxaliplatin-resistant GC cell models and investigated the role of VASH2. We found that VASH2 promoted oxaliplatin resistance in GC cells through AKT pathway, and these findings suggested that VASH2 could serve as a promising target of GC oxaliplatin-resistance.

MATERIALS AND METHODS

Antibodies and drugs

All antibodies were from Abcam, Cambridge, UK: Anti-VASH2 (1:1000 dilution, ab224723), anti-Bax (1:500 dilution, ab32503), anti-Bcl-2 (1:500 dilution, ab32124), anti-p-Gp (P-glycoprotein, 1:500 dilution, ab170904), anti-AKT (1:1000 dilution, ab8805), anti-p-AKT (1:500 dilution, ab38449), and anti-GAPDH (1:2000 dilution, ab8245). Oxaliplatin (CAS:61825-94-3) and LY294002 (CAS: 934389-88-5) were from Sigma (USA) and treated with cells for 24 h.

Cell culture and the construction of oxaliplatin resistance cells

The two gastric cancer cell lines, including AGS and MKN45 were purchased from ATCC. Both cell lines were maintained in DMEM, supplemented with 10% of FBS and incubated at 37 °C in a 5% CO_2 incubator. AGS and MKN45 cells were exposed to successively increased concentrations of L-OHP, from 7.5 to 240 μM for 5 months.

VASH2 siRNA (siRNA; sequence: 5′-CACUCUGAA UGAAGUGGGCUAUCAA-3′) and negative control siRNA or pcDNA3.1-VASH2 overexpression plasmids were purchased from Santa Cruz Biotechnology, Inc., USA and transfected into GC cells using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc., USA). siNC was used as a control to compare with VASH2 siRNA (5 μl) transfection or pcDNA3.1-VASH2 overexpression plasmids (1 μg). A total of 1×10^5 cells were seeded into six-well plates and 5 μl siRNAs or plasmids (1 μg) were used for transfection. The cells were transfected using 10 μl Lipofectamine® 3000 (Invitrogen) in each well. Cells were cultured for 4 h with Lipofectamine®/siRNA or plasmid mix at 37 °C and the transfection was completed. Subsequent assays were performed 24 h after transfection.

Immunoblot assay

The samples were lysed with the lysis buffer (RIPA, Beyotime, China), separated by 8% SDS-PAGE experiment, and the total proteins were transferred onto PVDF membranes (Millipore, USA). Then the PVDF membranes were blocked by 5% dry milk in Tris-Buffered Saline and Tween 20 (TBST) buffer. After washing, the membranes were incubated with the primary antibodies. Then membranes were incubated with the secondary horseradish peroxidase (HRP, Abcam) antibodies for 45 min. Each blot was then visualized using the ECL kit (GE, USA).

Cell viability assays

For 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di- phenytetrazoliumromide (MTT) assays, cells were plated into the 96-well plates (1000 cells per well) and maintained in complete growth media for 24 h at 37 °C. Cells were exposed to MTT reagent at 37 °C for 1.5 h. Then the stained cells were dissolved by 150 μl DMSO. The relative cell viability was assessed with microplate spectrophotometer at 490 nm (Bio-Rad, USA). For colony formation assays, cells were plated into the 6-well plates (1000 cell per well) and maintained in complete growth media for 14 days at 37 °C. Then cells were fixed with paraformaldehyde (PFA) for 20 min and stained (Crystal violet, 0.2%) for 10 min. Then cells were photographed.

Cell apoptosis assay

After transfection for 48 h, cells were washed with PBS. Subsequently cells were fixed with pre-cooled 70% ethanol at −20 °C for 1 h. Cells were stained with propidium iodide (PI) and FITC-labelled Annexin V (1:200 dilution) at 4 °C for 10 min and the apoptosis levels were measured by BD Fluorescence-activated cell sorting (FACS) caliber.

Statistics

Data were represented as mean ± SD. The experiments were replicated for at least 3 times in this study. The statistical significance of the difference was assessed by the student's t-test, and $p < 0.05$ was considered statistically significant.

RESULTS

VASH2 was highly expressed in oxaliplatin-resistant GC cells

To clarify the possible role of VASH2 in the chemosensitivity of GC, two GC cell lines, including AGS and MKN45, were used. First, AGS and MKN45 cells were exposed to various concentrations of L-OHP for 5 months. Then the oxaliplatin-resistant AGS and MKN45 cells were constructed. Through MTT assays, we found that the survival rates of MKN45/L-OHP and AGS/L-OHP cells treated with different doses of oxaliplatin (7.5, 15, 30, 60, 120, and 240 μM) were higher than that of their parent cells, indicating that GC cell model against L-OHP was successfully constructed (Fig. 1A). We subsequently detected the expression of VASH2 in the control and L-OHP groups and found that the expression of VASH2 was significantly increased in L-OHP AGS and MKN45 cells (Fig. 1B). Therefore, VASH2 was highly expressed in oxaliplatin-resistant GC cells.

Downregulation of VASH2 suppressed the viability of oxaliplatin-resistant GC cells

To further investigate the role of VASH2 in GC chemosensitivity, we performed a series of *in vitro* assays. A siRNA of VASH2 was transfected in to AGS L-OHP and MKN45 L-OHP cells, respectively, to decrease the expression of VASH2. Through Immunoblot assays, we noticed that downregulation of VASH2 decreased its protein levels in both AGS L-OHP and MKN45 L-OHP cells (Fig. 2A). Further through MTT assays, we found that downregulation of VASH2 suppressed the survival of AGS L-OHP and MKN45 L-OHP cells, compared to the control (Fig. 2B). Similarly, colony formation assays also showed the decreased colony numbers in AGS L-OHP and MKN45 L-OHP cells after VASH2 knockdown. Therefore, knockdown of VASH2 suppressed the viability of oxaliplatin-resistant GC cells.

VASH2 knockdown stimulated the apoptosis of oxaliplatin-resistant GC cells

Since VASH2 knockdown suppressed the treated L-OHP-GC cell viability, we then detected its role in apoptosis through Flow cytometry (FCM) assays. We found that the knockdown of VASH2 stimulated the apoptosis of both AGS L-OHP and MKN45 L-OHP cells, with the increased apoptosis cell numbers (Fig. 3A,B). Through Immunoblot assays, we noticed that knockdown of VASH2 increased the expression of Bax, and

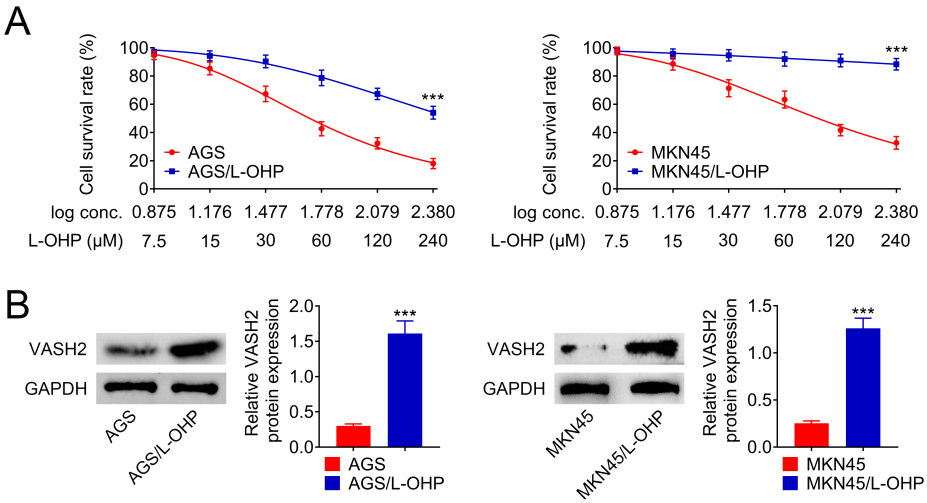

Fig. 1 VASH2 was highly expressed in oxaliplatin-resistant gastric cancer cells. (A) MTT assays showed the survival rates of AGS (left) and MKN45 (right) cells upon the treatment of oxaliplatin (7.5, 15, 30, 60, 120, and 240 μM). (B) Immunoblot assays showed the expression of VASH2 in AGS (left) L-OHP and MKN45 (right) L-OHP cells and the corresponding parent cells. Data are presented as mean ± SD. *** $p < 0.001$.

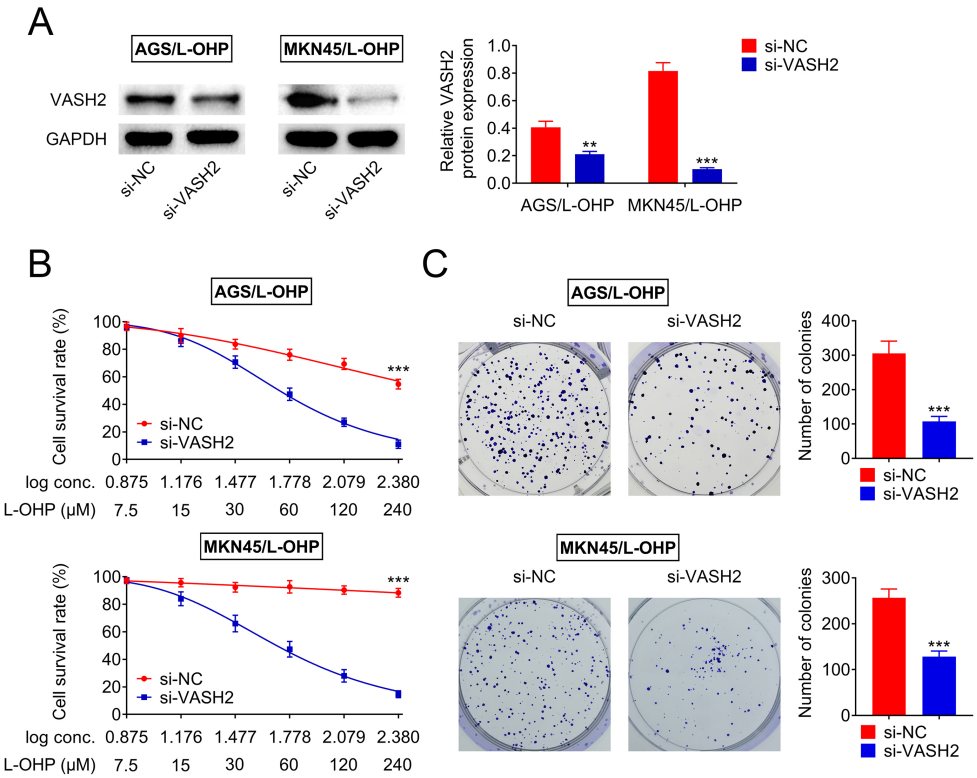

Fig. 2 Depletion of VASH2 suppressed the viability of oxaliplatin-resistant gastric cancer cells. (A) Immunoblot showed the expression of VASH2 in AGS (left) and MKN45 (right) L-OHP cells from siNC (negative control) or siVASH2 groups. (B) MTT assays showed the survival rates of AGS (up) and MKN45 (down) L-OHP control or VASH2 depleted cells upon the treatment of oxaliplatin (7.5, 15, 30, 60, 120, and 240 μM). (C) Colony formation assays showed the colony numbers of AGS L-OHP (up) and MKN45 (down) L-OHP cells upon the transfection of control or VASH2 siRNAs. Data are presented as mean ± SD. ** $p < 0.01$, *** $p < 0.001$.

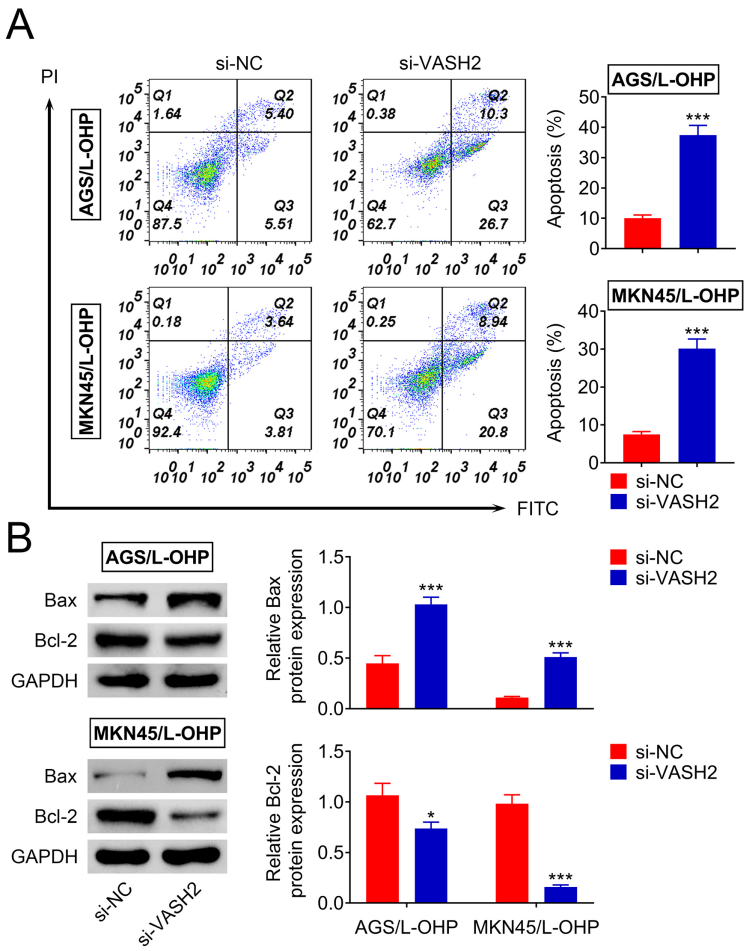

Fig. 3 VASH2 knockdown stimulated the apoptosis of oxaliplatin-resistant gastric cancer cells. (A,B) FCM assays showed the colony numbers of AGS (up) and MKN45 (down) L-OHP cells upon the transfection of control or VASH2 siRNAs. The apoptosis percentage was shown in (B). (C) Immunoblot assays showed the expression of the indicated proteins in AGS L-OHP (up) and MKN45 (down) L-OHP cells upon the transfection of control or VASH2 siRNAs. Data are presented as mean ± SD. * $p < 0.05$, *** $p < 0.001$.

decreased the expression of Bcl-2 in both AGS L-OHP and MKN45 L-OHP cells, suggesting the promoting effect on cell apoptosis (Fig. 3C). Therefore, VASH2 knockdown stimulated the apoptosis of oxaliplatin-resistant GC cells.

VASH2 promotes oxaliplatin resistance in GC cells through AKT pathway

Then we explored the possible mechanism underlying the effects of VASH2 on GC resistance. Previous study indicated that VASH2 could enhance breast cancer cell resistance to adriamycin through AKT pathway, and we therefore hypothesized that this pathway may also play a critical role in oxaliplatin resistance of GC [14]. Immunoblot assays results showed that downregulation of VASH2 decreased the expression of p-Gp, a drug resistance marker, in GC cells, suggesting the inhibition of oxaliplatin resistance (Fig. 4A). Furthermore, we found that knockdown of VASH2 suppressed the phosphorylation of AKT (Fig. 4B). Through Immunoblot assays, we revealed the upregulated expression of VASH2 after the transfection of its overexpression plasmids in AGS and MKN45 L-OHP cells (Fig. 4C). We then used an inhibitor of AKT, LY294002, to suppress this pathway. We noticed that overexpression of VASH2 inhibited the phosphorylation of AKT and the expression of p-Gp (Fig. 4D). However, the treatment with LY294002 rescued the downregulation of AKT phosphorylation and p-Gp expression in AGS and MKN45 L-OHP cells upon VASH2 overexpression (Fig. 4D). We further performed colony formation and FCM assays. Interestingly, our data confirmed that VASH2 overexpression promoted the colony formation and suppressed the apoptosis of AGS L-OHP cells (Fig. 4E,F).

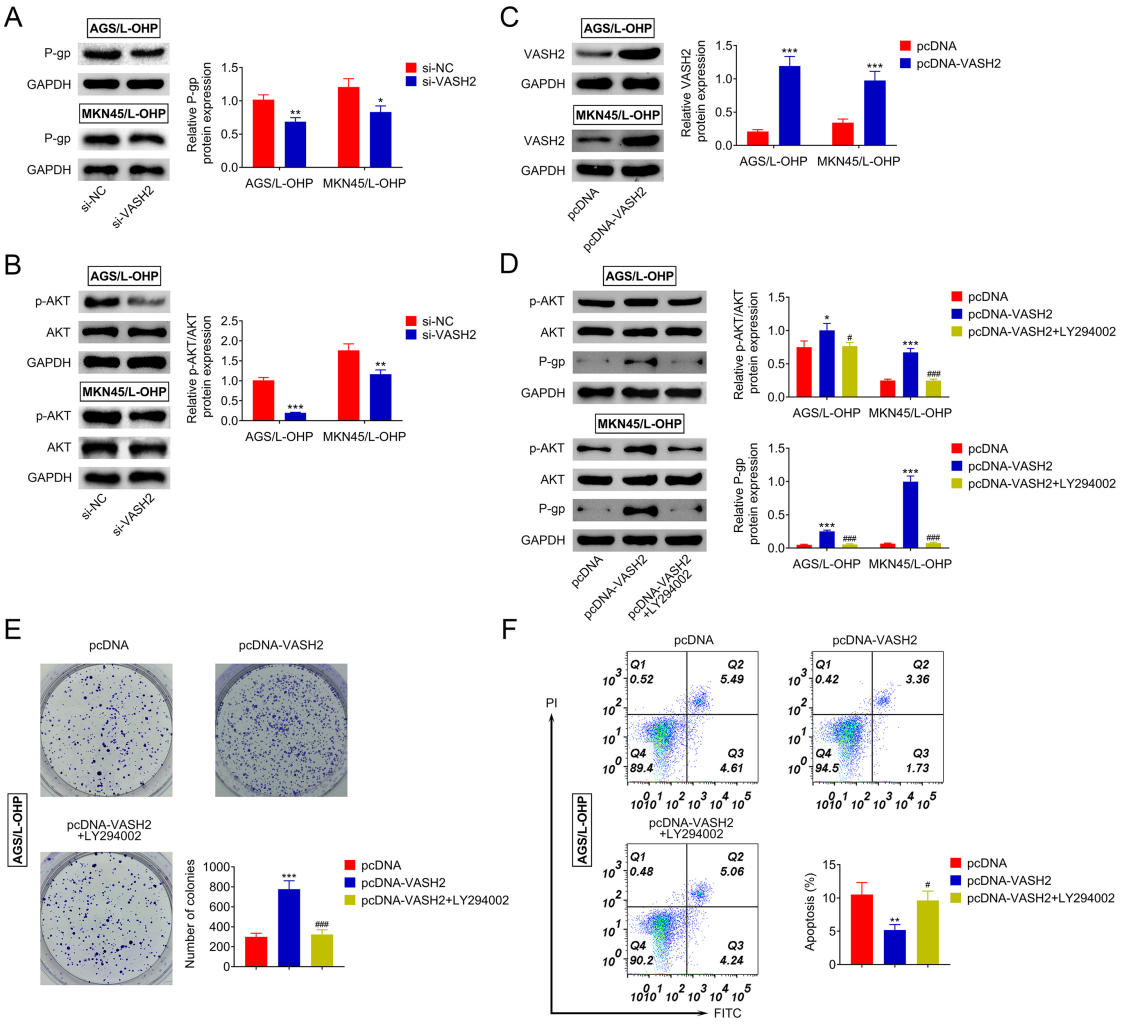

Fig. 4 VASH2 promotes oxaliplatin resistance in gastric cancer cells through AKT pathway. (A–D) Immunoblot assays showing the expression of: (A) p-Gp, (B) AKT and pAKT, (C) VASH2, (D) AKT, pAKT, and p-Gp in AGS (up) and MKN45 (down) L-OHP cells upon transfection of (A–C) control or VASH2 siRNA2 or (D) control or VASH2 plasmids or the treatment of LY294002. (E) Colony formation assays showed the colony formation degrees of AGS L-OHP cells and colony numbers upon the indicated treatment. (F) FCM assays showed the apoptosis degrees of AGS L-OHP cells and the apoptosis percentage upon the indicated treatment. Data are presented as mean ± SD. $^{*}\,p < 0.05$, $^{**}\,p < 0.01$, $^{***}\,p < 0.001$. pcDNA-VASH2+LY294002 vs. pcDNA-VASH2, $^{\#}\,p < 0.05$, $^{\#\#\#}\,p < 0.001$.

However, the treatment with LY294002 reversed the VASH2 overexpression-induced promoting of proliferation and suppression of apoptosis of AGS L-OHP cells (Fig. 4E,F). These results indicated that VASH2 promoted oxaliplatin resistance in GC cells through AKT pathway.

DISCUSSION

Oxaliplatin is a new generation of platinum antitumor drug, which can bind to DNA after entering into nucleus, resulting in replication and transcription damage [6]. At present, it is widely used in clinical combined chemotherapy, mainly for GC and other digestive system malignant tumors [3]. With the wide application of drugs, oxaliplatin resistance is shown in GC, leading to treatment failure [4]. The survival measures mainly include abnormal expression of some drug-resistant genes or proteins, disruption of the balance between cell proliferation and apoptosis, the performance of cells with stem cell characteristics, enhanced autophagy level, increased DNA repair ability, increased membrane transporter level, and metabolic disorders [16, 17]. It is very important to study the molecular mechanism of oxaliplatin resistance to im-

prove the prognosis of advanced GC.

We successfully constructed two oxaliplatin resistance cell lines and found that the expression of VASH2 was increased in L-OHP resistant cells. Through MTT, colony formation, FCM, and Immunoblot assays, we found that downregulation of VASH2 suppressed the viability and stimulate apoptosis of oxaliplatin-resistant GC cells. These studies confirmed that VASH2 promoted oxaliplatin resistance in GC cells.

The effects of VASH2 on the progression and metastasis of tumors have been widely revealed [11, 12, 14]. Previous study showed that VASH2 was abnormally expressed in multiple types of cancers such as HCC and NSCLC. The depletion of VASH2 could reduce tubulin carboxypeptidase activity and increase paclitaxel sensitivity in ovarian cancer [18]. In this study, VASH2 was highly expressed in oxaliplatin-resistant GC cells and we investigated its effect on oxaliplatin resistance of GC cells. VASH2 could also contribute to viability and resistance to doxorubicin in NSCLC via AKT pathway [10]. Similarly, here we also showed that VASH2 promoted oxaliplatin resistance in GC cells through the AKT pathway, and the precise mechanism needs further study. VASH2 could also promote malignant behaviors of pancreatic cancer cells via stimulating EMT process via Hedgehog pathway [19]. VASH2 expression was also correlated with the prognosis of esophageal cancer, breast cancer, and ovarian cancer [20–22]. These studies together with our study confirmed that VASH2 could serve as a promising target for cancer.

The role of AKT pathway in cancer progression and development has been widely revealed. AKT pathway affects a variety of cellular processes of cancers, such as proliferation, apoptosis, motility, and autophagy via the downstream proteins. Multiple proteins affected the progression of GC via this pathway [10, 19, 23].

In addition to the role of VASH2 in cancer progression, its multiple biological functions have also been revealed. It was known as a pro-angiogenic factor. Deletion of VASH2 could ameliorate the alterations of glomerular in diabetic nephropathy model [24]. Moreover, it plays a critical role in glucose tolerance defect and renal function suppression [25]. These studies, together with our findings, confirmed the multiple cellular functions of VASH2.

In conclusion, our study revealed the effects of VASH2 on L-OHP-resistant GC cells. we first found that VASH2 could promote oxaliplatin resistance in GC cells through AKT pathway. Therefore, VASH2 could serve as a promising target of gastric cancer oxaliplatin-resistance.

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