

VASH2 Promotes Cell Proliferation and Resistance to Doxorubicin in Non-Small Cell Lung Cancer via AKT Signaling

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Vasohibin2 (VASH2), a proangiogenic factor, has been demonstrated to play an oncogenic role in some common human cancers. However, the detailed function of VASH2 in non-small cell lung cancer (NSCLC) has not previously been studied. In this study, we found that VASH2 was significantly upregulated in NSCLC tissues and cell lines, and its increased expression was associated with NSCLC progression and poor prognosis of patients. Knockdown of VASH2 markedly inhibited cell proliferation and P-glycoprotein expression in NSCLC cells. Overexpression of VASH2 enhanced cell proliferation, P-glycoprotein expression, as well as doxorubicin resistance in NSCLC cells. Moreover, the expression levels of VASH2 were significantly increased in newly established doxorubicin-resistant NSCLC cells. Molecular mechanism investigation revealed that inhibition of VASH2 expression in NSCLC cells suppressed the activity of AKT signaling, and overexpression of VASH2 enhanced the activity of AKT signaling. We further showed that downregulation of AKT signaling activity using AKT inhibitor LY294002 markedly inhibited NSCLC cell proliferation and resistance to doxorubicin induced by VASH2. In conclusion, the findings in the present study indicate that VASH2 promotes NSCLC cell proliferation and resistance to doxorubicin via modulation of AKT signaling. Thus, we suggest that VASH2 may become a potential therapeutic target for the treatment of NSCLC.

Key words: Non-small cell lung cancer (NSCLC); Vasohibin2 (VASH2); Doxorubicin; Chemoresistance

INTRODUCTION

Non-small cell lung cancer (NSCLC) is the most common human cancer in both males and females worldwide, accounting for about 85% of all lung cancers^{1,2}. Despite improvements of surgery combined with radiotherapy and chemotherapy, the prognosis for NSCLC patients is still unsatisfactory because of metastasis, recurrence, and chemoresistance^{1,3,4}. Therefore, revealing the molecular mechanism of chemoresistance may be beneficial for developing novel therapeutic strategy for NSCLC treatment. Doxorubicin (also called adriamycin) is one of the most important anticancer drugs and is effective in treating multiple types of human cancers including NSCLC through inhibiting DNA transcription and replication^{5,6}. However, acquired resistance is a major limitation to the clinical application of doxorubicin in NSCLC treatment^{7,8}. Therefore, uncovering the molecular mechanisms underlying doxorubicin resistance is urgently needed for the improvement of NSCLC treatment.

The vasohibin 2 (VASH2) gene, located on human chromosome 1q32.3, encodes the VASH2 protein containing

355 amino acid residues⁹. As an important member of the vasohibin family, VASH2 has been identified as a proangiogenesis factor and plays a key role in some physiological and pathological processes^{10,11}. For instance, VASH2 is expressed in cells other than endothelial cells to promote angiogenesis and is also involved in neuron differentiation via its tubulin carboxypeptidases activity^{12,13}. Moreover, the oncogenic role of VASH2 in tumor growth has been reported in some common human cancers^{14–17}. For instance, VASH2 is significantly upregulated in hepatocellular carcinoma and promotes the malignant transformation of tumor by inducing epithelial–mesenchymal transition (EMT) and angiogenesis^{14,17}. Knockdown of VASH2 significantly suppresses tumor growth of endometrial cancer cells and ovarian cancer cells by inhibition of angiogenesis^{15,16}. However, the detailed function of VASH2 in NSCLC still remains unknown.

Recently, VASH2 has been found to be associated with chemoresistance in several cancer types^{18–20}. For instance, Tu et al. showed that VASH2 induced gemcitabine resistance of pancreatic cancer cells via Jun-dependent

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transactivation of ribonucleotide reductase regulatory subunit M2¹⁸. Li et al. reported that VASH2 decreased cisplatin sensitivity of hepatocarcinoma cells by inhibiting the expression of p53²⁰. Furthermore, VASH2 was found to enhance doxorubicin resistance of breast cancer cells through regulating ABCG2 via AKT signaling pathway¹⁹. However, whether VASH2 is involved in doxorubicin resistance in NSCLC still remains unclear.

Thus, in the present study, we aimed to explore the potential function of VASH2 in the regulation of cell proliferation and chemotherapy resistance in NSCLC, as well as the underlying molecular mechanism.

MATERIALS AND METHODS

Clinical Tissues

This study was approved by the Research Ethics Committee of No. 180 Hospital of People's Liberation Army. Primary NSCLC tissues and adjacent nontumor tissues were collected from 200 NSCLC patients in the Department of Thoracic Surgery, No. 180 Hospital of People's Liberation Army between June 2011 and March 2013. Written informed consent was obtained from all patients. The clinicopathological information of these patients was obtained from medical records. The tissues were immediately snap frozen in liquid nitrogen and stored at -80°C until use.

Cell Culture

Normal human lung epithelial cell line BEAS-2B and NSCLC cell lines (A549 and H358) were obtained from Cell Bank of Chinese Academy of Sciences (Shanghai, P.R. China). Cells were cultured in DMEM (Life Technologies, Carlsbad, CA, USA) added with 10% FBS (Life Technologies) at 37°C with 5% CO_2 .

Establishment of Doxorubicin Resistance in NSCLC Cell Lines

A549 and H358 cells were cultured in DMEM with 10% FBS containing doxorubicin [half maximal inhibitory concentration (IC_{50})]. After incubation at 37°C for 48 h, the culture system was replaced by DMEM with 10% FBS. Cells were passaged cultured when reaching 100% confluence. When cells could grow steadily in the medium containing doxorubicin (IC_{50}), they were cultured in DMEM with 10% FBS and doxorubicin (IC_{50}). When they could grow steadily, the doxorubicin resistance A549 and H358 cells were established.

Cell Transfection

LipofectamineTM 2000 (Thermo Fisher Scientific, Waltham, MA, USA) was used to conduct cell transfection, according to the manufacturer's instructions. In brief, A549 and H358 cells (3×10^5 cells/well) were seeded in six-well plates. After incubation at 37°C with

5% CO_2 for 24 h, cells were VASH2 siRNA, negative control (NC) siRNA, pEZM61-VASH2 plasmid (Gene Copoeia, Guangzhou, P.R. China), or blank pEZM61 vector, respectively. After transfection for 48 h, the following experiments were conducted.

CCK-8 Assay

Cell proliferation was studied using the CCK-8 assay. The transfected cells (2×10^3 cells/well) were seeded in 96-well plates. After culturing at 37°C with 5% CO_2 for 0, 24, 48, and 72 h, the cell proliferation was measured using Cell Counting Kit-8 (Thermo Fisher Scientific, Inc.). The OD value was read at 450-nm wavelength on a microplate reader (Bio-Rad, Hercules, CA, USA).

MTT Assay

The transfected cells (10,000 cells/well) were seeded in 96-well plates. After incubation at 37°C for 24 h, DMEM with doxorubicin (2, 4, 8, 16, or 32 $\mu\text{mol/L}$) was added into each well. After incubation at 37°C for 48 h, the medium was removed, and 50 μl of DMSO (Sigma-Aldrich, St. Louis, MO, USA) was added. After incubation at 37°C for 10 min, the OD value was read at 570-nm wavelength on a microplate reader (Bio-Rad). The IC_{50} was calculated.

RT-qPCR

Total RNA was extracted from tissues and cells using TRIzol (Invitrogen, Thermo Fisher Scientific, Inc.). Then RNA (1 μg) was converted into cDNA using a SuperScript[®] One-Step RT-PCR kit (Thermo Fisher Scientific, Inc.), and qPCR was performed to examine the mRNA expression using a SuperScript[®] One-Step RT-PCR kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The PCR reaction conditions were as follows: 95°C for 3 min, followed by 40 cycles at 95°C for 15 s and 60°C for 30 s. The relative expression levels were analyzed using the $2^{-\text{Ct}}$ method²¹.

Western Blot

Western blot was used to examine the protein expression. In brief, protein was extracted from cell lines using RIPA lysis (Beyotime Biotechnology, Hangzhou, P.R. China), according to the manufacturer's protocol. The protein concentration was examined using the BCA kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The protein (50 μg per lane) was separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes (Thermo Fisher Scientific, Inc.). The membranes were incubated with primary antibodies (Abcam, Cambridge, MA, USA) at 37°C for 4 h, followed by incubation with HRP-conjugated goat anti-rabbit secondary antibodies (Abcam) at room temperature for 1 h. Signaling was examined using an Enhanced

Chemiluminescence Western Blotting Kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The protein expression was analyzed using Image-Pro Plus software 6.0 (Media Cybernetics, Inc., Rockville, MD, USA), according to the manufacturer's protocol.

Statistical Analysis

Data were expressed as mean \pm standard deviation (SD). The statistical analysis was performed using GraphPad Prism version 5.01 (GraphPad Software, La Jolla, CA, USA). The overall survival rate estimates over time were calculated using the Kaplan–Meier method with log-rank test. Chi-square test was applied to analyze the association between VASH2 expression and clinicopathological features in NSCLC. Differences were analyzed using Student's *t*-test between two groups, or one-way ANOVA among more than two groups followed by the Tukey's post hoc test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Upregulation of VASH2 Is Associated With NSCLC Progression and Poor Prognosis

To reveal the role of VASH2 in NSCLC, we first examined its expression levels in NSCLC tissues and cell lines. As shown in Figure 1A, the expression levels of VASH2 were significantly increased in NSCLC tissues when compared with those in adjacent normal tissues. After that, we studied the clinical significance of VASH2 expression in NSCLC, and our data showed that high expression of VASH2 was significantly associated with tumor size, lymph node/distant metastasis, and advanced clinical stage in NSCLC (Table 1). In addition, we investigated the factors that could predicate the prognosis of NSCLC patients using univariate and multivariate analyses. Univariate analysis indicated that the VASH2 level (HR=4.64, $p=0.01$), as well as the tumor size (HR=3.23, $p=0.03$), lymph node metastasis (HR=4.06, $p=0.01$),

distant metastasis (HR=5.14, $p=0.01$), and TNM stage (HR=3.74, $p=0.02$) were significantly associated with patients' prognosis (Table 2). Multivariate analysis revealed that the VASH2 level (HR=4.12, $p=0.01$), tumor size (HR=3.56, $p=0.01$), lymph node metastasis (HR=4.86, $p=0.02$), distant metastasis (HR=5.18, $p=0.01$), and TNM stage (HR=3.87, $p=0.01$) were found to be independent factors for predicating the prognosis of NSCLC patients (Table 3). Moreover, the NSCLC patients with high VASH2 expression showed shorter overall survival time (Fig. 1B). Therefore, upregulation of VASH2 is associated with NSCLC progression and poor prognosis.

Promoting Effects of VASH2 on NSCLC Cell Proliferation and Resistance to Doxorubicin

We then studied the effects of VASH2 on NSCLC cell proliferation and resistance to doxorubicin. We found that the expression levels of VASH2 were significantly increased in NSCLC cells (A549 and H358) compared with normal cell line BEAS-2B (Fig. 2A and B). The expression levels of VASH2 in H358 were higher than in A549 cells (Fig. 2A and B). As expected, the inhibition rates in H358 were lower than in A549 when the cells were treated with ADR; thus, the IC_{50} of H358 was higher than that of A549 (Fig. 2C and D). A549 cells were transfected with VASH2 expression plasmid to upregulate its expression. After transfection, the VASH2 levels were upregulated in the VASH2 group compared to the blank group (Fig. 3A and B). We then found that overexpression of VASH2 enhanced the protein expression of P-glycoprotein (Fig. 3B) in NSCLC cells. Moreover, VASH2 upregulation significantly reduced the inhibition rate (IR) of cells in doxorubicin (Fig. 3C), and the IC_{50} of doxorubicin was significantly increased (Fig. 3D). These findings suggest that VASH2 is associated with doxorubicin resistance in NSCLC cells.

To further confirm these findings, VASH2 siRNA was used to transfect H358 cells to downregulate its expression.

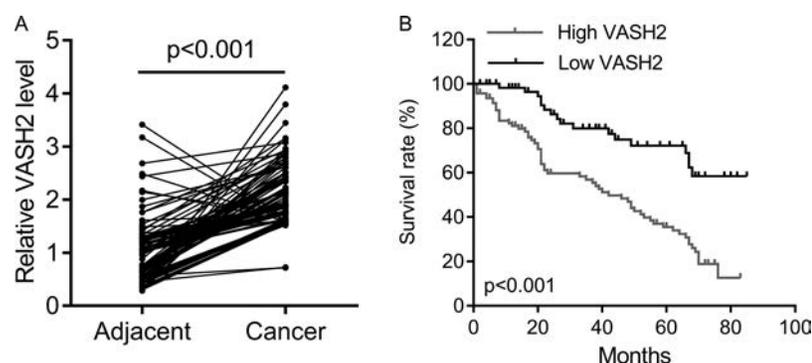


Figure 1. The vasohibin2 (VASH2) expression in non-small cell lung cancer (NSCLC) tissues. (A) qPCR was performed to measure the expression of VASH2 in NSCLC tissues and the matched adjacent tissues ($n=200$). (B) The Kaplan–Meier survival curve showed the survival rate in patients with high VASH2 expression ($n=116$) and those with low VASH2 expression ($n=84$).

Table 1. Clinical Association Between Vasohibin2 (VASH2) Levels and Clinicopathological Variables of Patients With Non-Small Cell Lung Cancer (NSCLC)

Variable	VASH2		Chi-Square <i>p</i> Value
	Low Expression (<i>n</i> = 84)	High Expression (<i>n</i> = 116)	
Age			0.666
<60	36	54	
≥60	48	62	
Gender			0.886
Male	44	63	
Female	40	53	
Tumor size			<0.001
<3 cm	58	41	
≥3 cm	26	75	
Lymph node metastasis			<0.001
N0–1	52	38	
N2–4	32	78	
Distant metastasis			0.006
No	59	58	
Yes	25	58	
TNM stage			0.006
I–II	54	51	
III–IV	30	65	

After transfection, the expression levels of VASH2 were markedly decreased in the shVASH2 group compared to the shNC group (Fig. 3E and F). We then observed that knockdown of VASH2 caused a significant reduction in protein expression of P-glycoprotein (Fig. 3F). Moreover, we showed that silencing of VASH2 increased the IR of NSCLC cells in doxorubicin (Fig. 3G), and the IC₅₀ of doxorubicin was significantly declined (Fig. 3H). These above findings demonstrate that VASH2 has promoting effects on NSCLC cell proliferation and resistance to doxorubicin.

VASH2 Is Upregulated in Established Doxorubicin-Resistant NSCLC Cells

To further confirm that VASH2 is involved in the resistance of NSCLC cells to doxorubicin, two doxorubicin-resistant NSCLC cell lines were established. The established A549-doxorubicin (Fig. 4A and B) and H358-doxorubicin cells (Fig. 4E and F) showed reduced IR and increased IC₅₀ in doxorubicin. Moreover, the mRNA and protein levels of VASH2 were significantly increased in doxorubicin-resistant NSCLC cells (Fig. 4C, D, G, and H). These findings further suggest that VASH2 plays a key role in doxorubicin resistance in NSCLC cells.

Inhibition of AKT Signaling Reduces NSCLC Cell Proliferation and Resistance to Doxorubicin Induced by VASH2

We then studied the underlying molecular mechanism and found that VASH2 overexpression enhanced

the phosphorylation of ERK and AKT (Fig. 5A), and knockdown of VASH2 reduced the phosphorylation of ERK and AKT in NSCLC cells (Fig. 5B). These findings suggest that the ERK or AKT signaling may be involved in the VASH2-mediated NSCLC cell proliferation and resistance to doxorubicin. To confirm this speculation, the AKT inhibitor LY294002 and ERK inhibitor U0126 were used. Our findings showed that treatment with LY294002, but not U0126, decreased P-glycoprotein protein of A549-VASH2 cells (Fig. 5C and D), accompanied with increased doxorubicin IR and reduced IC₅₀ of doxorubicin (Fig. 5E and F).

DISCUSSION

The function of VASH2 in NSCLC has not previously been explored. In this study, we found that VASH2 was significantly upregulated in NSCLC tissues and cell

Table 2. Univariate Analysis of Prognostic Factors of NSCLC

Variable	Hazard Ratio	<i>p</i> Value
Age (≥50/<50)	1.14	0.45
Gender (male/female)	1.27	0.09
Tumor size (≥3 cm/<3 cm)	3.23	0.03
Lymph node metastasis (N2–4/N0–1)	4.06	0.01
Distant metastasis (yes/no)	5.14	0.01
TNM stage (III–IV/I–II)	3.74	0.02
VASH2 (high/low)	4.64	0.01

Table 3. Multivariate Analysis of Independent Prognostic Factors of NSCLC

Variable	Hazard Ratio	<i>p</i> Value
Tumor size	3.56	0.01
Lymph node metastasis	4.86	0.02
Distant metastasis	5.18	0.01
TNM stage	3.87	0.01
VASH2 levels	4.12	0.01

lines, and its increased expression was associated with NSCLC progression and poor prognosis of patients. Knockdown of VASH2 markedly inhibited cell proliferation and P-glycoprotein expression in NSCLC cells. Overexpression of VASH2 enhanced cell proliferation, P-glycoprotein expression, as well as doxorubicin resistance in NSCLC cells. Moreover, the expression levels of VASH2 were significantly increased in newly established doxorubicin-resistant NSCLC cells. Molecular mechanism investigation revealed that inhibition of VASH2

expression in NSCLC cells suppressed the activity of AKT signaling, and overexpression of VASH2 enhanced the activity of AKT signaling. We further showed that down-regulation of AKT signaling activity using AKT inhibitor LY294002 markedly inhibited NSCLC cell proliferation and resistance to doxorubicin induced by VASH2.

The promoting role of VASH2 has been reported in several cancer types including gastric cancer, liver cancer, pancreatic ductal adenocarcinoma, and breast cancer. For instance, Kitahara et al. suggested that VASH2 participated in the onset of tumors in the gastrointestinal tract by regulating tumor angiogenesis²¹. Tu et al. reported that VASH2 could promote breast cancer cell proliferation via increasing the expression of FGF2 and GDF15²². VASH2 is upregulated in pancreatic ductal adenocarcinoma, which is associated with tumor progression as well as poor prognosis of patients²³. However, the expression pattern of VASH2 in NSCLC as well as the clinical significance has not previously been studied. In this study, we found that the expression levels of VASH2 were significantly

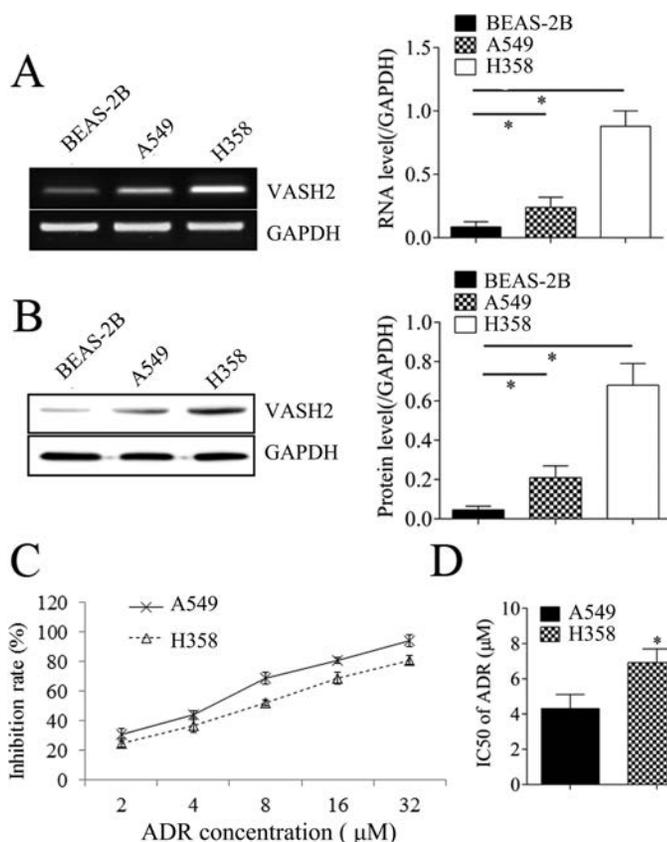


Figure 2. The VASH2 expression in NSCLC cell lines. (A) RT-PCR was performed to measure the expression of VASH2 in NSCLC cell lines (A549 and H358) and the normal cell line BEAS-2B. (B) Western blot was performed to measure the expression of VASH2 in NSCLC cell lines (A549 and H358) and the normal cell line BEAS-2B. (C) MTT assay was performed to measure the inhibition rate in A549 and H358 cells after doxorubicin treatment (2, 4, 8, 16, or 32 μmol/L). (D) The half maximal inhibitory concentration (IC₅₀) was calculated from (C). **p* < 0.05.

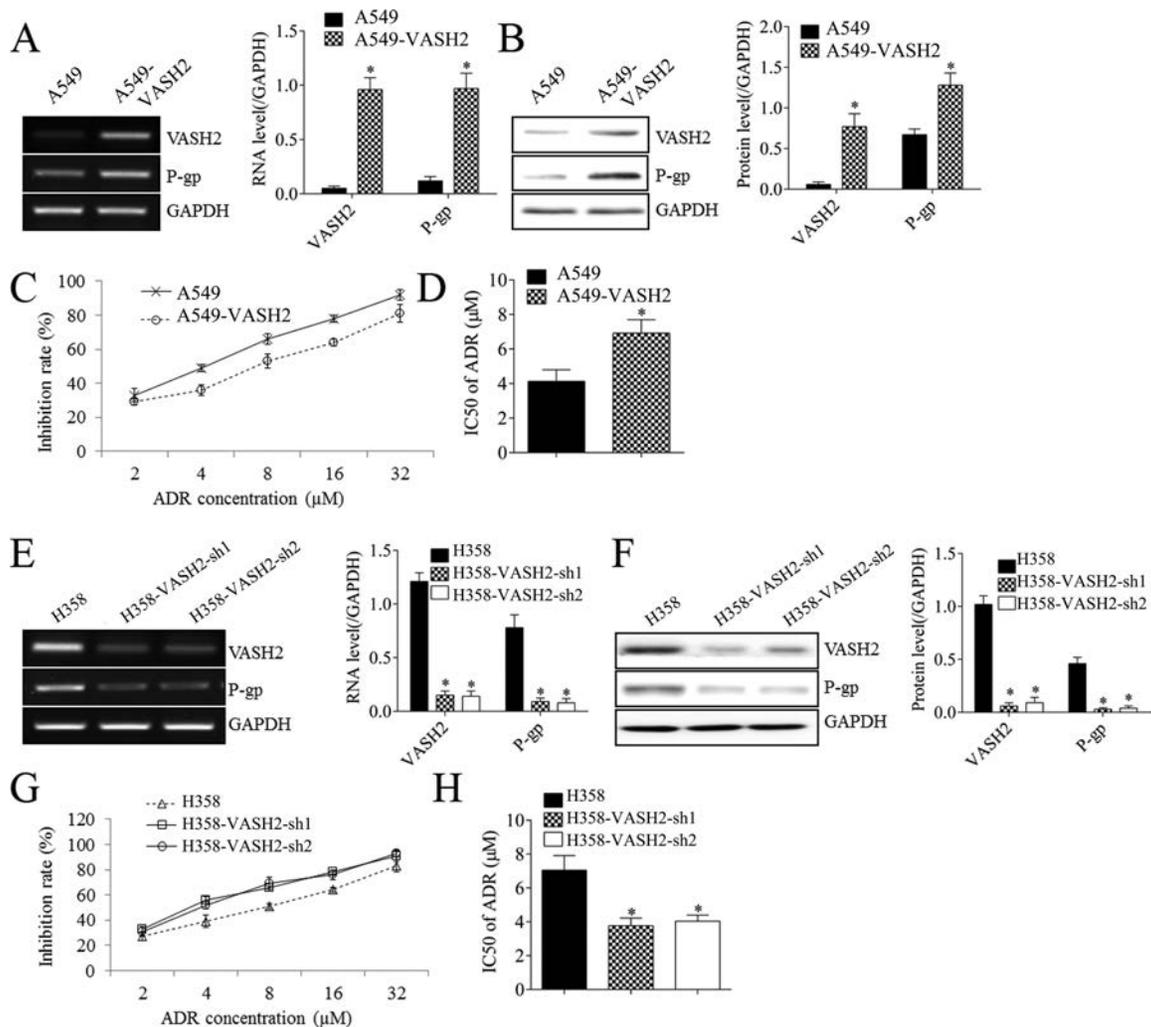


Figure 3. The effects of VASH2 on cell proliferation and doxorubicin chemotherapy in A549 and H358 cells. (A) RT-PCR was performed to measure the expression of VASH2 and P-glycoprotein (P-gp) in A549 cells after VASH2 plasmid transfection. (B) Western blot was performed to measure the expression of VASH2 and P-gp in A549 cells after VASH2 plasmid transfection. (C) MTT assay was performed to measure the inhibition rate in A549 cells after VASH2 plasmid transfection under doxorubicin treatment (2, 4, 8, 16, or 32 μmol/L). (D) The IC₅₀ was calculated from (C). (E) RT-PCR was performed to measure the expression of VASH2 and P-gp in H358 cells after VASH2 shRNA (sh-1, sh-2) transfection. (F) Western blot was performed to measure the expression of VASH2 and P-gp in H358 cells after VASH2 shRNA (sh-1, sh-2) transfection. (G) MTT assay was performed to measure the inhibition rate in H358 cells after VASH2 shRNA (sh-1, sh-2) transfection under doxorubicin treatment (2, 4, 8, 16, or 32 μmol/L). (H) The IC₅₀ was calculated from (G). ADR, doxorubicin. **p* < 0.05.

increased in NSCLC tissues compared with those in adjacent nontumor tissues, and high expression of VASH2 was significantly associated with tumor size, lymph node metastasis, and advanced clinical stage, suggesting that upregulation of VASH2 is involved in NSCLC progression. In addition, we found that the NSCLC patients with high expression of VASH2 showed shorter survival time when compared with those with low VASH2 expression. These findings further confirm the importance of VASH2 in NSCLC. To study the function of VASH2 in NSCLC, we upregulated or downregulated the expression of VASH2 in NSCLC cells and then examined cell

proliferation. Our data showed that VASH2 overexpression significantly enhanced NSCLC cell proliferation, and knockdown of VASH2 suppressed cell proliferation.

P-glycoprotein is an ATP-binding cassette (ABC) drug efflux pump and could reduce the concentration of drugs in cells. P-glycoprotein has been demonstrated to be frequently upregulated in chemoresistant tumor cells and participate in the chemotherapy resistance of various human cancers. In our study, we found that overexpression of VASH2 caused a significant increase in the expression of P-glycoprotein in NSCLC cells and induced doxorubicin resistance. On the contrary, knockdown of

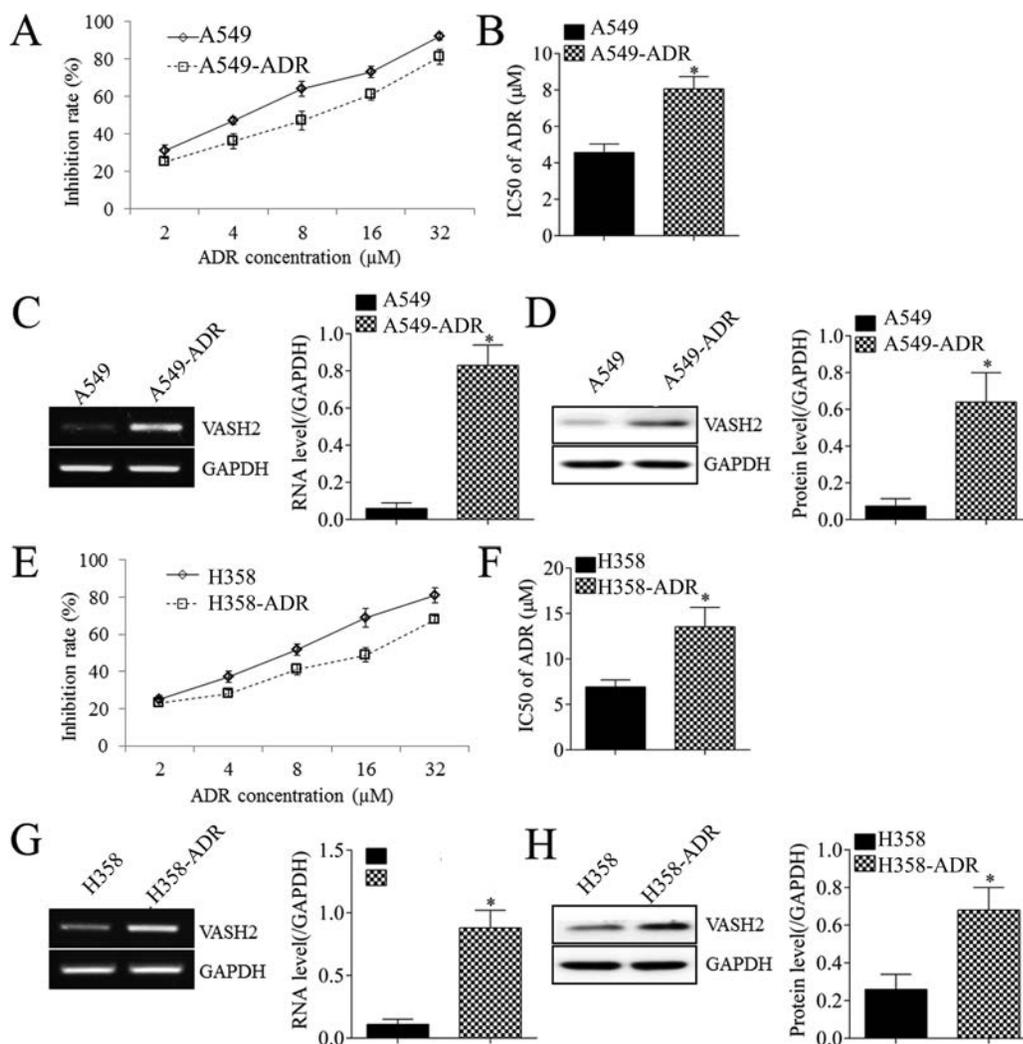


Figure 4. The expression of VASH2 in doxorubicin-resistant A549 and H358 cells. (A) MTT assay was performed to measure the inhibition rate in A549 and doxorubicin-resistant A549 cells. (B) The IC₅₀ was calculated from (A). (C) RT-PCR was performed to measure the expression of VASH2 in A549 and doxorubicin-resistant A549 cells. (D) Western blot was performed to measure the expression of VASH2 in A549 and doxorubicin-resistant A549 cells. (E) MTT assay was performed to measure the inhibition rate in H358 and doxorubicin-resistant H358 cells. (F) The IC₅₀ was calculated from (E). (G) RT-PCR was performed to measure the expression of VASH2 in H358 and doxorubicin-resistant H358 cells. (H) Western blot was performed to measure the expression of VASH2 in H358 and doxorubicin-resistant H358 cells. * $p < 0.05$.

VASH2 significantly reduced P-glycoprotein expression and increased the IR of NSCLC cells in doxorubicin. To further confirm these findings, we then established doxorubicin-resistant NSCLC cell lines and found that VASH2 was significantly upregulated in these doxorubicin-resistant cells. Therefore, targeting VASH2 may be used as a potential strategy for the treatment of doxorubicin-resistant NSCLC.

The activity of AKT signaling has been demonstrated to be frequently upregulated in various human cancers including NSCLC and play promoting roles during tumor progression²⁴⁻²⁶. Inhibition of AKT signaling activity has been found to suppress tumor growth as well

as chemoresistance²⁶. A previous study has shown that VASH2 can enhance the doxorubicin resistance of breast cancer cells via AKT signaling pathway¹⁹. Thus, we studied the relationship between VASH2 and AKT signaling in NSCLC cells. We found that VASH2 overexpression significantly upregulated the activity of AKT signaling and that knockdown of VASH2 downregulated the AKT signaling activity in NSCLC cells, suggesting that the function of VASH2 in NSCLC may be through modulating AKT signaling. Further investigation showed that inhibition of AKT signaling using LY294002 suppressed the VASH2-induced NSCLC cell proliferation and doxorubicin resistance, which confirms our speculation.

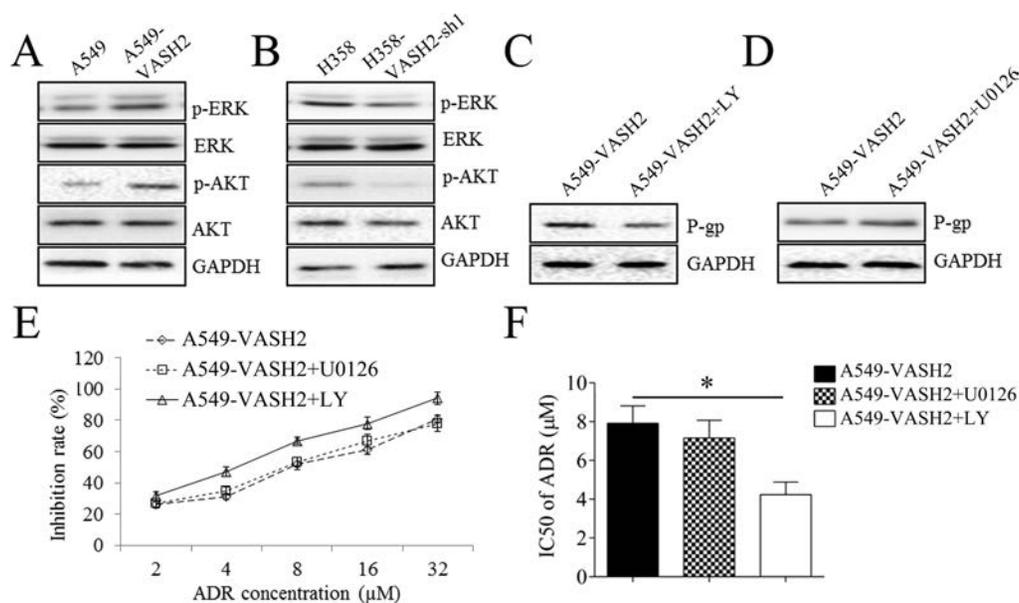


Figure 5. The effects of VASH2 on AKT signaling in A549 and H358 cells. (A) Western blot was performed to measure the expression of ERK and AKT and their phosphorylated form in A549 cells after VASH2 plasmid transfection. (B) Western blot was performed to measure the expression of ERK and AKT and their phosphorylated form in H358 cells after VASH2 shRNA-1 transfection. (C) Western blot was performed to measure the expression of P-gp in A549 cells after VASH2 plasmid transfection plus AKT inhibitor LY294002. (D) Western blot was performed to measure the expression of P-gp in A549 cells after VASH2 plasmid transfection plus ERK inhibitor U0126. (E) MTT assay was performed to measure the inhibition rate in A549 cells after VASH2 plasmid transfection plus either ERK inhibitor U0126 or AKT inhibitor LY294002. (F) The IC₅₀ was calculated from (E). * $p < 0.05$.

In conclusion, to our knowledge, this is the first study demonstrating that VASH2 can promote tumor cell proliferation and induce doxorubicin resistance in NSCLC, suggesting that VASH2 may become a promising therapeutic target for the treatment of NSCLC.

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