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Original Article

Silencing of VMP1 makes breast cancer cells more aggressive and resistant to 5-Fu

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Abstract: Background: Vacuole membrane protein 1 (VMP1) was recently characterized to be involved in the process of cancer metastasis, and is also considered to play a vital role in balancing apoptosis and autophagy. Methods and results: Here, we evaluated the expression of VMP1 in 98 breast cancer and 54 matched adjacent noncancerous tissues with immunohistochemistry (IHC). The results showed a lower expression of VMP1 in advanced stages of breast cancer. Moreover, the patients with low expression of VMP1 were related to poor prognosis than those with high expression. Furthermore, to investigate whether the downregulation of VMP1 could confer malignant potential to breast cancer cells, we constructed and verified a lentivirus-based knockdown system to silence the expression of VMP1 in Michigan Cancer Foundation-7 cells (MCF7). After infection with lentivirus, the shVMP1 cells, which had VMP1 stably suppressed, gained significant aggressive properties of invasion and proliferation, and these mechanisms may relate to the activation of phosphatidylinositol 3-hydroxy kinase (PI3K)/protein kinase B(Akt)/zonulaoccludens-1 (ZO-1)/E-Cadherin pathway. On the other hand, we also found that shVMP1 cells were more sensitive to 5-Fluorouracil (5-Fu), but not cisplatin and oxaliplatin. Conclusions: In all, the above data suggest that patients with lower expression of VMP1 may have a poor prognosis, which may be ascribed to the more aggressive potential after down-regulation of VMP1 in breast cancer cells. In addition, these patients who have low expression of VMP1 may benefit from chemotherapy regimens containing 5-Fu.

Keywords: VMP1, breast cancer, lentivirus, chemotherapy, 5-Fu

Introduction

Breast cancer is one of the most common cancers in developing countries. Some of the patients associated with poor prognosis are often due to the resistance to chemotherapy and other treatments. The underlying principal element of that may result from the enhanced viability of tumor cells. In the life cycle of tumor cells, once a tumor suffers conditions of inadequate blood supply, the ensuing absence of oxygen and glucose deprivation may result in autophagy. In recent years, autophagy has attracted extensive attention as a common event from the ambient pressure or cellular stress. In detail, autophagy is a general term for the degradation of cytoplasmic components within lysosomes, producing amino acids, saccharides, and/or nucleotides to suffice the physiological processes of cells which lack the nutrients and in turn reduce the stress from apoptosis [1]. However, it is merely a temporary relief for the survival of cells in poor condition, because the persistence of this process often relates to ensuing cell death.

The expression of several specific proteins is often accompanied by the process of autophagy, which can be recognized as biomarkers related to the prognosis of breast cancer. These proteins are specifically expressed in tumor cells but not in normal ones, such as light chain 3 (LC3), Beclin-1, p62 and GABA-A receptor-associated protein (GABARAP). LC3 accumulating around the nucleus is closely concerned with the existence of autophagy as well as in indicating a better prognosis of colon cancer [2]. In addition, in advanced breast cancer, Beclin-1 is significantly highly expressed *in situ* compared with metastasis sites, and also indi-

cates a better prognosis [3]. On the other hand, the overexpression of GABARAP in tumor cells may result in poor differentiation and a shorter survival [4]. Another study also indicated that the combination of detecting the above biomarkers could contribute to the outcome or prognosis in patients receiving 5-Fu treatment [5].

The prognosis in breast cancer may depend mainly upon the occurrence of tumor metastasis. The relationship between autophagy and metastasis is still elusive. It is generally considered that autophagy could help in reducing necrosis and inflammation, preventing metastasis at the early stage of cancer. From another point of view, autophagy may also help in providing greater survival probability for tumor cells circulating in blood and lymphonodus [6].

Vacuole Membrane Protein-1 (VMP1) is a kind of a transmembrane protein which has been shown to be located mainly in the cell membrane and endoplasmic reticulum [7]. It was first observed in pancreatitis, participating in the process of autophagy [8]. The subsequent study on tumor cells demonstrated that it may also play a key role in the development of tumor cells such as in their proliferation [9], autophagy [10], metastasis [9], and drug sensitivity [11]. A recent study on VMP1 has shown that colorectal cancer cells could initiate the process of autophagy under the simulation of rapamycin or when lacking nutrients with an increased expression of VMP1 [12]. However, this phenomenon could be diminished by siRNA interference of VMP1. Further study indicated that breast cancer cells also become more sensitive to Etoposide and tend to undergo apoptosis under starvation. In this study, VMP1 can be considered as a key regulator in autophagy, namely active VMP1 promoted the process of autophagy, maintained the survival of tumor cells, as well as being insensitive to the signal of apoptosis, which could be regarded as a good biomarker related to breast cancer. However, this study could be improved with the detection of VMP1 in clinical samples, as well having Etoposide as the first-line drug in treating patients with breast cancer.

VMP1's relationship with cancer patient prognosis was first seen in Iovanna's studies of pancreatic cancer, where VMP1 was overexpressed in pancreatic cancer, and closely linked with poorly differentiated human pancreatic cancer [11]. Also in a new pathway through VMP1-USP9x-p62 which could prevent cell death of the pancreas, was named Zymophagy [10]. Further, a study of lung cancer patients displayed that expression of VMP1 was correlated with poor prognosis [13]. However, there are some studies that have shown VMP1 has some anti-tumor properties, increasing the degree of malignancy if it is missing. VMP1 is an important component of cell connections between cells and the formation of tight junctions, and may enhance cell junctions and cell adhesion function. In research on breast cancer and renal cell carcinoma, the researchers found [7] VMP1 expression is significantly reduced in cancer metastases as compared to primary tumors. When VMP1 expression was downregulated, cell adhesion was lost, and the invasion capacity of cells increased. Another expression study in breast cancer displayed VMP1 positive expression patients as having good prognosis, and negative expression patients with poor prognosis [14]. In liver cancer studies have found similar results. Patients with low VMP 1 expression have lower overall survival and disease-free survival than those with high expression of VMP1. Upregulation of VMP1 not only inhibit the proliferation of cancer cells, but also inhibit liver cancer cell metastasis in nude mice [9].

VMP1 showed high positive levels in normal tissue and low positive levels in cancer tissue, even in the same patient, VMP1 was positive in the tissue adjacent to carcinoma tumor tissue, but not in the tumor tissue. Furthermore, recent reports showed that VMP1 was located in the plasma membrane, playing a critical role in cell-cell contact [7]. It is well known that reduced levels of cell-cell adhesion proteins often correlate with tumor invasion and metastasis [15]. Lei Guo and other studies have shown that upregulation of VMP1 inhibits metastasis of tumor cells [9]. However, so far, no studies have shown the impact of VMP1 in breast metastases.

Therefore, it is important to explore the biological characteristics of VMP1 in breast cancer cells and thus reveal the underlying mechanisms, and clarify the implications of VMP1 on the prognosis of patients suffering from breast cancer. In the present study, expression of VMP1 is detected in clinical samples as well as

different breast cancer cell lines to establish relationships between VMP1 and the prognosis of breast cancer. Furthermore, a shRNA lentiviral vector was constructed for VMP1 gene silencing for heritable changes in the cells lines which highly expressed VMP1 originally. This study focused on the proliferation, drug sensitivity, metastasis, as well as the signaling pathways related to the process mentioned above.

Material and methods

Cell culture

We used 293T and MCF7 cell lines that were purchased from the Shanghai Institute of Cell Biology (Shanghai, China). Then the 293T and MCF7 cells were separately cultured in DMEM and RPMI1640 (Invitrogen, Carlsbad, CA, USA) with 10% FBS at 37 degrees C in a humidified atmosphere of 5% CO_2.

Patient data

Paraffin-embedded, archived breast cancer samples obtained from 94 patients were histologically and clinically diagnosed from our hospital between 2006 and 2010. For the 98 breast cancer tissues, 54 matched adjacent noncancerous tissues were used as controls. Prior to the use of these clinical materials for investigation, informed consent from patients and approval from the Institute Research Ethics Committee were obtained. Primary cancers of the colorectum were classified according to the pathological TNM classification [16]. Clinical information about the samples is described in detail in **Table 1**. Patients ages ranged from 24 to 85 years (mean, 65.4 years). The figures on metastasis pertain to its presence at any time in follow-up. The median follow-up time for overall survival was 59.0 months for patients still alive at the time of analysis, and ranged from 6 to 86 months. A total of 39 (41.5%) patients died during follow up.

VMP1 shRNA construction and lentiviral production

Three target sequences for VMP1 mRNA were chosen according to the RNAi Consortium (TRC) shRNA Library (Broad Institute). The VMP1 shRNA single-strand oligo nucleotides were listed as follows: 1F: 5'-CCGGGTGCTTATAGCTACGTATTATCTCGAGATAATACGTAGCTATAAGCACTTTTTG-3', 1R: 5'-AATTCAAAAAGTGCTTATAGCTACGTATTATCTCGAGATAATACGTAGCTATAAGCAC-3'; 2F: 5'-CCGGCAACAGTATGTGCAACGTATACTCGAGTATACGTTGCACATACTGTTGTTTTTG-3', 2R: 5'-AATTCAAAAACAACAGTATGTGCAACGTATACTCGAGTATACGTTGCACATACTGTTG-3', 3F: 5'-CCGGGCAATGAACAAGGAACATCATCTCGAGATGATGTTCCTTGTTCATTGCTTTTTG-3', 3R: 5'-AATTCAAAAAGCAATGAACAAGGAACATCATCTCGAGATGATGTTCCTTGTTCATTGC-3'.

Two complementary single-strand oligo nucleotides containing the target sequences were synthesized chemically (Shanghai, China) and annealed. The double-stranded oligo nucleotides were inserted between Age I and EcoR I restriction sites in the plko-green fluorescent protein (GFP) small hairpin RNA (shRNA) vector that contain cytomegaoviyns (CMV) driven enhanced green fluorescent protein (EGFP) reporter gene. The ligated plasmid was transformed into *E.coli* DH5α competent cells for plasmid amplification. The plasmids from positive colonies were identified by PCR and DNA sequencing.

Recombinant lentiviruses were produced by co-transfecting 293T cells with three recombinant lentiviral vectors pLKO-shRNA, Δ8.91 and pVSVG (10:10:1) using the cationic lipid complex method (X-tremeGENE HP DNA Transfection Reagent, Roche). The culture supernatants containing the produced viruses were harvested at 48 h after transfection, and concentrated by centrifugation at 4,000 × g at 4°C for 10-15 min. MCF7 cells were subcultured at a density of 1×10^6 cells per well. Cells were divided into 4 groups: CON (infected with negative control lentiviral vector) and VMP1 shRNA1, 2, 3 (infected with VMP1 lentiviral vector). MCF7 cells were plated in 6 well dishes at 5×10^5 cells per well. The next day, cells were infected with the same titer virus with 2 μg/ml polybrene. About 24 h post-infection, the media was replaced with media containing 2 μg/ml puromycin. Cells were maintained and allowed to grow for 7-9 days, changed to media without puromycin, and then passaged for follow up assays.

Western blot analysis

Equal numbers of cells were lysed in lysis buffer composed of 0.6 M Tris-HCl (pH 6.8), 10% SDS and protease inhibitor cocktail. Samples

Table 1. Correlation between the clinicopathological features and expression of VMP1 protein

Characteristics	VMP1 expression (%)*		P
	Low	High	
Age (y)			
≤60	26 (81.3)	6 (18.8)	0.069
>60	39 (62.9)	23 (37.1)	
Stage			
I	11 (57.9)	8 (42.1)	0.008
II	19 (55.9)	15 (44.1)	
III	30 (85.7)	5 (14.3)	
IV	5 (83.3)	1 (16.7)	
Histological type			
Ductal carcinoma in situ	54 (72.0)	21 (28.0)	0.266
Invasive ductal carcinoma	7 (53.8)	6 (46.2)	
Invasive lobular carcinoma	4 (66.7)	2 (33.3)	
Histological differentiation			
Well	4 (40.0)	6 (60.0)	
Moderate	46 (70.8)	19 (29.2)	0.058
Poor	15 (78.9)	4 (21.1)	
Tumor diameter			
≤50 mm	46 (68.7)	21 (31.3)	0.872
>50 mm	19 (70.4)	8 (29.6)	
pT classification			
T1~T2	20 (64.5)	11 (35.5)	0.500
T3~T4	45 (71.4)	18 (28.6)	
pN classification			
N0	30 (56.6)	23 (43.4)	0.002
N1	25 (83.3)	5 (16.7)	
N2	10 (90.9)	1 (9.1)	
pMetastasis			
YES	5 (83.3)	1 (16.7)	0.442
N0	60 (68.2)	28 (31.8)	

*The percentages in **Table 1** are the row percentages.

were incubated at 4 degree C for 10 mins and then centrifuged at 10,000 × g for 15 min at 4 degrees C. The supernatant was transferred, mixed and boiled in sample buffer. The supernatant was separated by polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Biorad, Hercules, CA, USA). We then incubated the membrane at room temperature in blocking buffer consist of 5% fat-free milk dissolved in 1 × TBST (10 mM Tris-Base, pH 7.5, 100 mM NaCl, and 1% Tween 20) for 1 h followed by incubation with the blocking buffer containing primary antibody, such as anti-VMP1 anti-AKT, anti-p-AKT, anti-ZO-1 (Cell Signaling Technology, Danvers MA, USA), anti-E-cadherin, anti-β-actin (BD, Shanghai, China) at 4 degrees C overnight. The membrane was washed by TBS-T, and incubated with the secondary antibody for 1 h. The blot was exposed to ECL (Amersham) after TBST washing.

Viability of breast cancer cells detected by the MTT method

MCF7 CON group (infected with negative control lentiviral vector) and MCF7 VMP1 shRNA1 group (infected with VMP1 lentiviral vector) cells were seeded into a 96-well microplate at a density of 5×10^3 cells per well and incubated overnight in 10% FBS medium. Each group had three wells. After incubation for 12, 24, 48 and 72 h at 37°C, MTT reagent (5 mg/ml) in PBS was added to the cells (10 μl/well), and the cells were incubated for 4 hours at 37°C. Supernatant was discarded and 200 μl of DMSO was added to each well to solubilize the MTT-formazan product. Finally, samples were measured on a multi-well spectrophotometer (Thermo, USA) at a test wavelength of 560 nm with a reference wavelength of 650 nm.

Drug sensitivity experiment

Cells were seeded at 5,000 cells/well in 96-well plates (200 μl/well) and allowed to grow for 24 hr before being treated with complex. Different concentrations of cisplatin, 5-FU and oxaliplatin were added into the cells, each group had three wells. After a further 24 h, MTT reagent (5 mg/ml) in PBS was added to the cells (10 μl/well) and incubated for 4 h at 37°C. Two hundred microliters of DMSO was added to each well to solubilize the MTT-formazan product after removal of the medium. Samples were measured on a multi-well spectrophotometer (Thermo, USA) at a test wavelength of 595 nm and a reference of 650 nm.

Matrigel based invasion assay

Assays were performed in transwell chambers (Corning Incorporated, USA) with 8 μm pore size coated with matrigel (BD, USA). MCF7 cells infected with Scrambled shRNA and VMP1 shRNA1 were trypsinized and suspended with 1% FBS, after counting the cells they were plated into the upper chamber, and 600 μl medium supplemented with 10% FBS was placed into

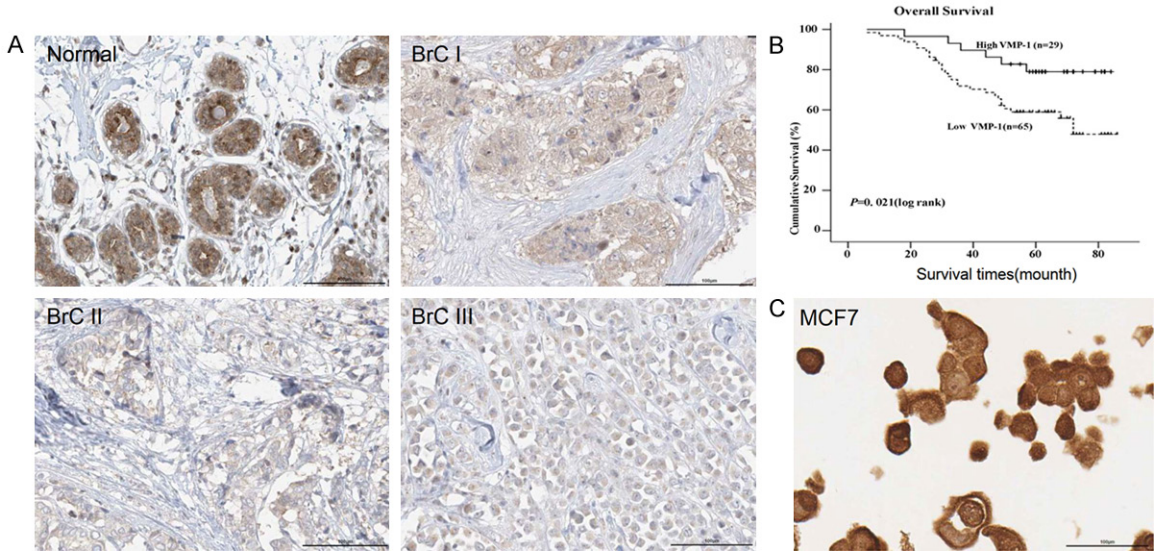

Figure 1. Expression analysis of VMP1 protein by immunohistochemistry. A. VMP1 expression was mainly localized within the cytoplasm of cells. VMP1 shows strong cytoplasmic staining in normal breast tissues, weak cytoplasm or no positive staining in in high stage of breast cancer. Bar means 100 μm. B. Kaplan-Meier curves with univariate analyses (log-rank) for patients with low VMP1 expression (dotted line) versus high VMP1 expressing tumors (bold line). The median survival of patients with lower VMP1 expression was much shorter (52 months) than those with high VMP1 expression (63 months) (*P*=0.021, Log-rank). C. Expression of VMP1 in MCF 7 was detected by IHC, which show a strong staining of the VMP1.

the lower chamber. After incubation at 37 degrees C for 6 h, cells on the upper surface of the filters were removed and cells adhering to the undersurface of the filter membrane were dyed with 0.5% crystal violet for 30 min. The crystal violet was washed with PBS three times. Cells on the lower chamber were counted under a microscope in four fields randomly. The mean cell numbers were recorded and analyzed. The experiment was repeated three times.

Immunohistochemistry

Immunohistochemistry was done to study altered protein expression in 94 human breast cancer tissues and 52 matched adjacent non-cancerous tissues. A commercially available antibody against VMP1 (1:100 ab116006, lot rabbit polyclonal immunoglobulin G; Abcam Biotechnology, USA) was used as the primary antibody. Immunohistochemical kit (SP-9001 rabbit SP kit, lot: 50581654) was obtained from Zhongshan Golden Bridge Biotechnology Co. Ltd. (Beijing, China). For each sample, one score was given according to the percent of positive cells; no positive cells: 0, <5% of the cells: 1 point, 5-35% of the cells: 2 point, 36-70% of the cells: 3 point, >70% of the cells: 4 point. To achieve objectivity, the intensity of positive staining was also used in a four scoring system: 0 (negative staining), 1 (weak staining exhibited as light yellow), 2 (moderate staining exhibited as yellow brown), and 3 (strong staining exhibited as brown). A final score was then calculated by multiplying the above two scores. If the final score was equal to or greater than four, the tumor was considered as high expression; otherwise, the tumor was considered as low expression.

Statistical analysis

All statistical analyses were carried out using the SPSS 13.0 statistical software package. The t-test was employed to evaluate differences in expression of VMP1 between two categories of tissues. χ^2 test was used to analyze the relationship between VMP1 expression and clinicopathological characteristics. Survival curves were plotted by the Kaplan-Meier method and compared by the log-rank test. The significance of various variables for survival was analyzed by the Cox proportional hazards model in the multivariate analysis. $P<0.05$ in all cases was considered statistically significant.

Table 2. Univariate and multivariate analysis of different prognostic parameters in patients with breast cancer by Cox-regression analysis

	Univariate analysis		Multivariate analysis			
	No. Patients	P	Regression coefficient (SE)	P	Relative risk	95% confidence interval
Stage	<0.001		1.316 (0.292)	<0.001	3.729	2.103~6.612
I	19					
II	34					
III	35					
IV	6					
pT classification		0.026	0.502 (0.424)	0.236	1.653	0.720~3.791
T1~T2	31					
T3~T4	63					
pN metastasis		<0.001	0.275 (0.443)	0.535	1.317	0.552~3.140
N0	53					
N1	30					
N2	11					
VMP1 expression		0.021	-0.496 (0.462)	0.283	0.609	0.246~1.507
Low	65					
High	29					

Results

Expression of VMP1 in breast cancer and prognosis analysis

Immunohistochemical assay showed that the expression of VMP1 was mainly observed in the cytoplasm of the cancer cells in 55.7% (59/102) cases, and correlated with grade ($\chi^{(2)}$=12.644, P=0.002), pTNM stage ($\chi^{(2)}$=11.987, P=0.001), node status ($\chi^{(2)}$=9.341, P=0.002), tumor diameter ($\chi^{(2)}$=7.630, P=0.022) as well as Nottingham Prognostic Index (NPI; $\chi^{(2)}$=15.561, P=0.000). The expression of VMP1 in concomitant Ductal carcinoma *in situ* (DCIS) was higher than that in Invasive ductal carcinoma (IDC) (81.3% vs 56.3%; $\chi^{(2)}$=4.655, P=0.031). In this cohort, the mean disease-free survival was 81.2 months; the 5-year overall survival rate was 90.2% (92/102) and the disease-free survival rate was 81.4% (83/102). VMP1 expression had significant influence on disease-free survival time, with VMP1-negative patients showing poor prognosis ($\chi^{(2)}$=11.192, P=0.001). COX's proportional hazards regression model for OS revealed that VMP1 was a protective factor, with relative risk <1. In some patients VMP1 expression was significantly decreased in primary cancer tissues compared with that in the matched adjacent noncancerous tissues (χ^2=12.962, *P*=0.008; **Figure 1**). As shown in **Figure 1**, among those matched adjacent noncancerous tissues, as many as 61.5% (32 of 52) of the individual tissues were scored as either high or strong for VMP1 (**Figure 1A**). However among breast cancer cases only 30.9% (29 of 94) of the cancer tissues were defined as high expression of VMP1. The subcellular location of VMP1 was mainly cytoplasmic.

Correlation between VMP1 protein expression and clinicopathological features

Table 1 shows the relationship between the expression of VMP1 protein and clinical characteristics. There was no significant correlation between the expression level of VMP1 protein and age, histological classification, histological differentiation, tumor diameter, pT classification or distant metastasis of breast cancer patients. However, the expression of VMP1 is closely associated with stage of breast cancer patients (*P*=0.008) and pN classification (*P*=0.002). The expression of VMP1 protein was negatively correlated with staging and pN classification (**Table 2**). As shown in **Figure 1B**, higher staging correlated with lower VMP1 expression. We also detected the expression of VMP1 in MCF7 cell line, as shown in **Figure 1C**, it has a high expression of VMP1 all around the cells.

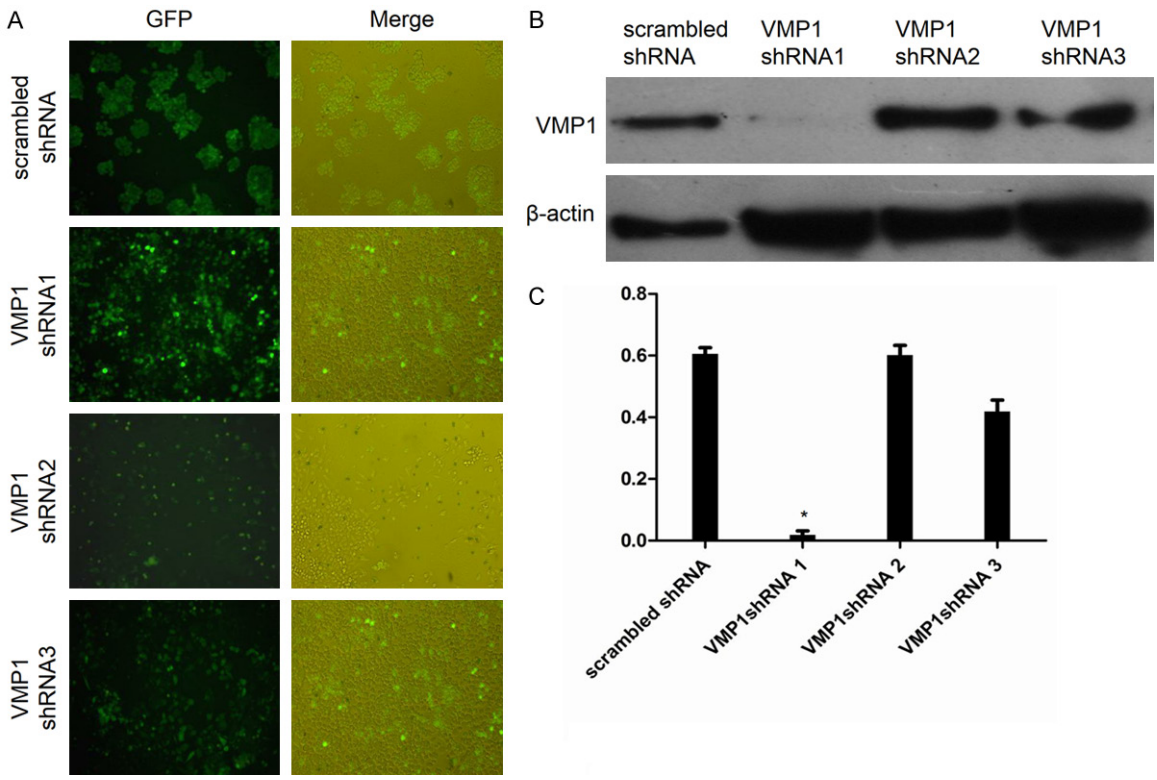

Figure 2. The construction of VMP1 shRNA Lentivirus and the Identification of gene silence effect. A. MCF7 cells were infected with the three VMP1 shRNA and scramble shRNA group virus after 24 h, the left was the Schematic diagram under the Green fluorescence channel, the right was the image under the bright field condition and Green fluorescence channel, the fluorescence efficiency of shRNA1 was highest. B. VMP1 expression of MCF7 after infection with virus was detected by western blot. C. Bands were quantified by densitometry and VMP1 shRNA1 gene silence effect was best, the knockout efficiency was almost 99% ($^{*}P<0.05$).

Survival analysis

Kaplan-Meier analysis and the log-rank test were used to calculate the effect of classic clinicopathological characteristics (including stage, T classification, N classification) and VMP1 expression on survival. The expression level of VMP1 protein in breast cancer was significantly correlated with patients' survival time (P= 0.021), indicating that lower levels of VMP1 expression was correlated with shorter survival time. The high VMP1 expression group had better survival, whereas the low VMP1 expression group had shorter survival (**Figure 1B**). The median survival of patients with high VMP1 expression was much longer (63 months) than those with low VMP1 expression (52 months) (P=0.021, Log-rank).

In addition, T classification, N classification, stage were also significantly correlated with survival in Kaplan-Meier analysis and log-rank test (for T classification, P=0.026; for N classification, P<0.001; for stage, P<0.001). We did multivariate survival analysis, which included VMP1 expression level, stage, T classification, and N classification, to determine if VMP1 expression level is an independent prognostic factor of outcomes. In this analysis, stage was recognized as independent prognostic factors (**Table 2**).

Construction and identification on VMP1 stable gene silencing

In order to clarify the position of VMP1 in the progression of breast cancer, a shRNA lentiviral vector was constructed for VMP1 gene silencing for heritable changes. We designed 3 pairs of shRNA. After sequencing was confirmed and amplification of the plasmid, we packaged the 293T cells with liposome and plasmid for a transfection of 48 h, after that, lentivirus in the culture supernatant was harvested to infect the target cells. From the resulting fluorescence (**Figure 2A**), which showed cell infection rate

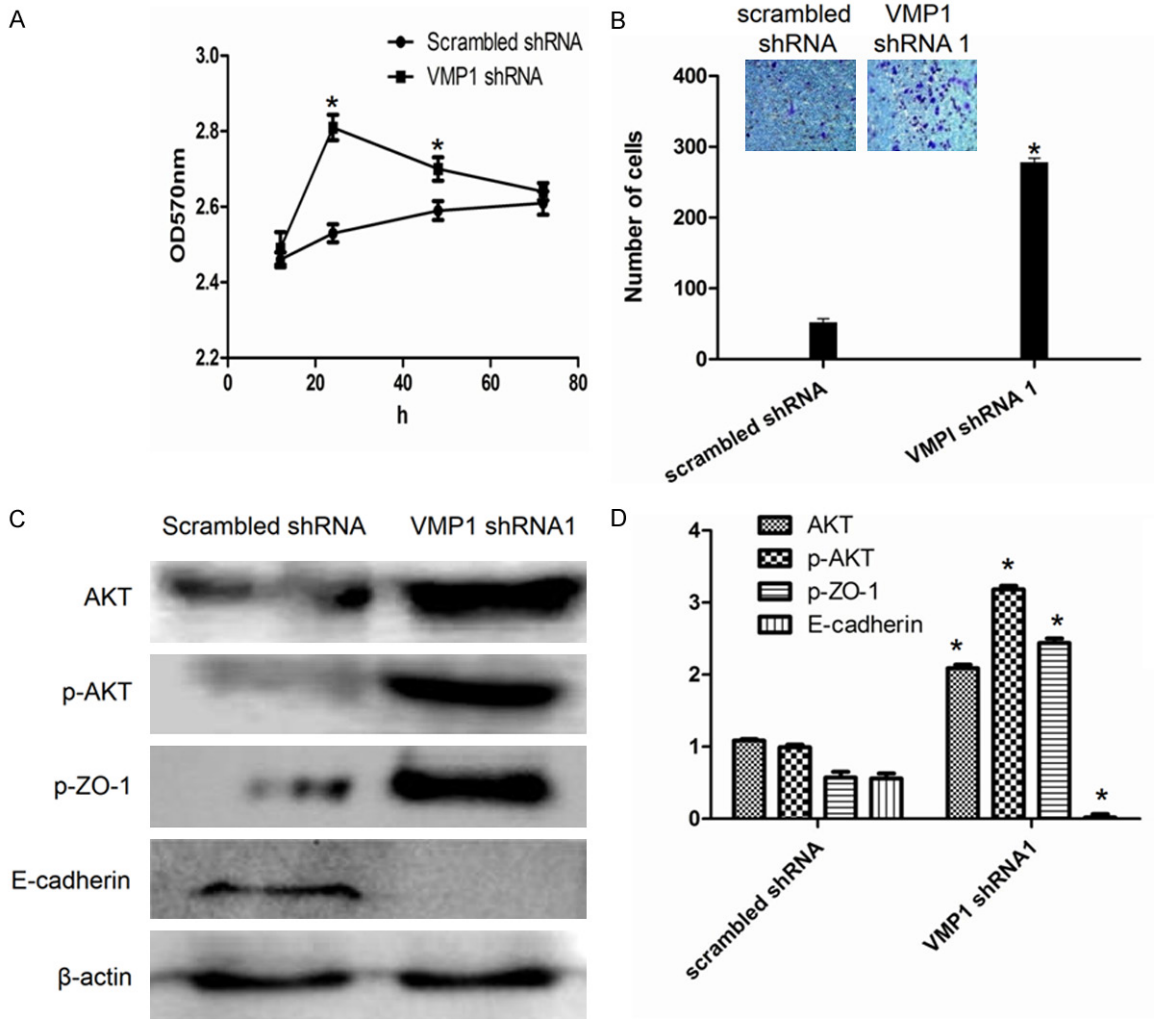

Figure 3. Effects on cell proliferation, invasion ability after downregulation of VMP1. A. Cells were determined by MTT assay at four different time points and the cell growth curve was established. Compared with the control group (Scrambled shRNA), the proliferation rate of MCF7 VMP1 gene silence cells (VMP1 shRNA1) was improved (*P<0.05). B. The results of invasion assay: Compared with the control group, the invasive ability was enhanced significantly after VMP1 knockout, upper left was the schematic diagram after the crystal violet stain in cell transwell experiment. Histogram is the statistics on numbers of cells of two groups respectively. C. shVMP1 and ShScramble cells protein were collected, protein Akt/p-AKT(involved in cell proliferation) and ZO-1/E-cadherin (related to cell proliferation), β-actin (used as Internal control) were detected by western blot. D. Bands were quantified by densitometry. For each independent experiment, n=3, *$P<0.05$.

reached approximately 98%. Western blot suggested that the shRNA1 was the best sequence in silencing VMP1, nearly 95% (**Figure 2B** and **2C**). Thus, the breast cancer cells were used for subsequent experiments.

VMP1 silencing increases cell proliferation and migration through the PI3K/AKT signaling pathway

First, we evaluated the proliferation after VMP1 silencing, which indicated a significantly higher proliferation rate after 48 h and a balanced level after 72 h compared with the Scrambled shRNA group (**Figure 3A**). We used a transwell chamber model to study the ability of cell migration. As expected, a significant increase in migration was found after VMP1 silencing, which was statistically significant (**Figure 3B**). Western blot showed an increase in AKT/p-AKT as well as a downregulation of ZO-1 (**Figure 3C**), which related to the activation of PI3K/AKT signaling pathway and the phosphorylation of ZO-1. In addition, expression of E-cadherin was also downregulated as the same time, and this may act as a key factor induces the EMT of

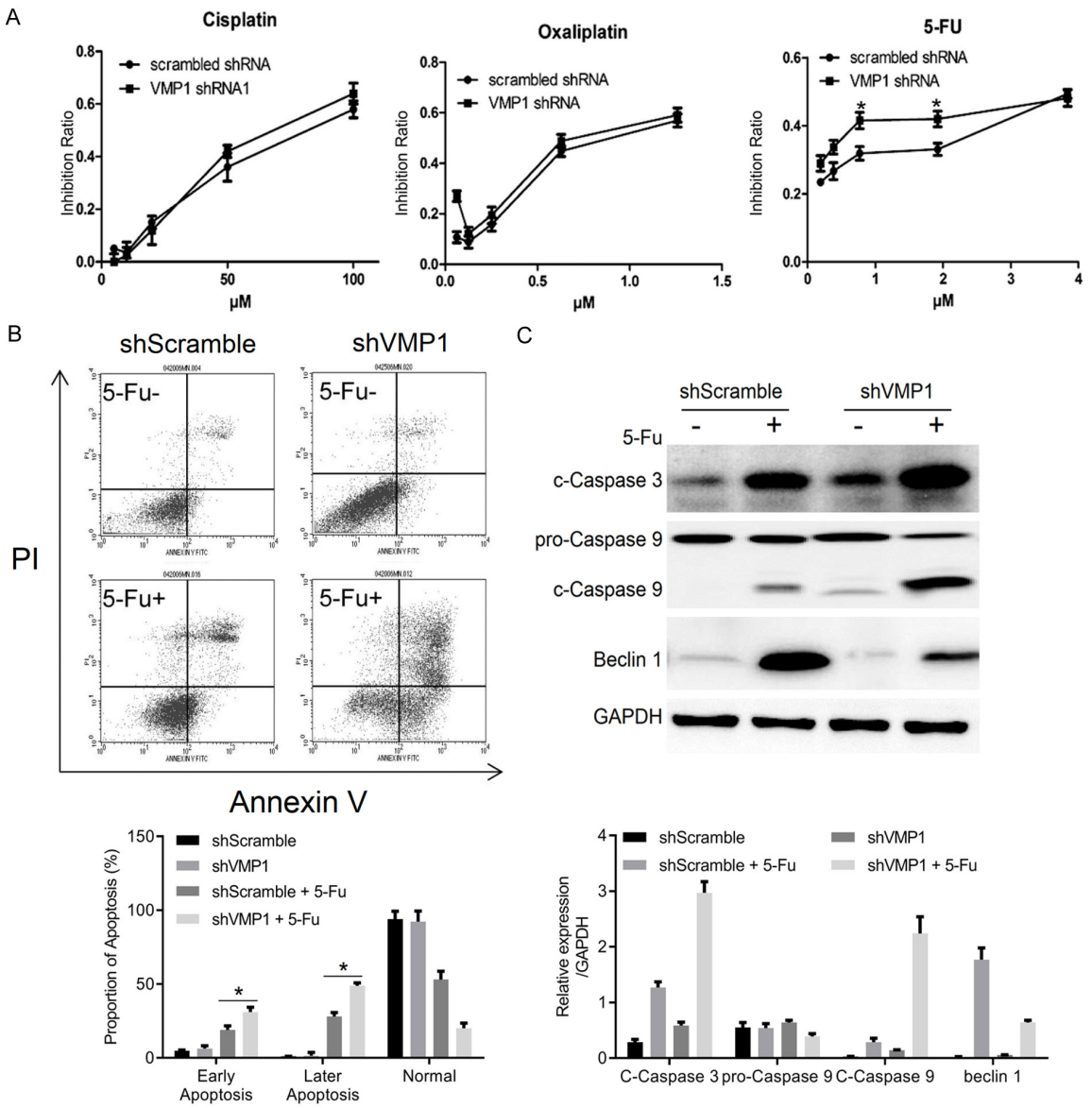

Figure 4. The molecular mechanisms of VMP1 involved in the proliferation and invasion of colon cancer *in vitro.* A. The sensitivity of MCF7 cells to cisplatin, oxaliplatin and 5-Fu before or after VMP1 knockdown (*P<0.05). B. Cell apoptosis was detected by PI/Annexin V double staining after treatment with 5-Fu. C. Cell apoptosis and autophagy relative protein were detected by western blot. GAPDH was also detected as internal control.

tumor cells and promotes migration (**Figure 3D**).

VMP1 silencing increases cells sensitivity to 5-Fu and promotes cells apoptosis

As shown in **Figure 4A**, further drug sensitivity suggests an increasing sensitivity to 5-Fu after VMP1 silencing, but an inconspicuous function on platinum or oxaliplatin, which might result from the increased sensitivity to apoptosis signaling. We further perform the PI/Annexin v double staining which could evaluate the apoptosis level after treatment with 5-Fu, as shown in **Figure 4B**, when compared with the level of apoptosis it was significantly increased after stable silencing of VMP1 in MCF7 (P<0.05). Meanwhile, we also found that without the 5-Fu treatment, the shVMP1 group also has a certain level of cell apoptosis, which may prompt the cells to be more sensitive to 5-Fu induced apoptotic signals, while autophagy may have a certain degree of reduction sensitivity. To confirm this hypothesis, we then used western blot to detect the expression of Caspase 3 and

Caspase 9 and beclin 1, the later protein indicates the occurrence of autophagy. As shown in **Figure 4C**, in the presence of 5-Fu, apoptosis in the scramble group did occur, which was reflected by the increase of cleaved Caspase 3 and Caspase 9 expression levels, while autophagy also occurred as reflected by an increased beclin1 expression. In the shVMP1 group, the level of the activated form of Caspase 3 and Caspase 9 was significantly higher than the Scramble group, and beclin1 expression is weaker than in the scramble group; these results indicated that stable silencing of VMP1 would prompt cells to be more sensitive to 5-Fu-induced apoptosis, while blunting the sensitivity of autophagy. In a sense, VMP1 may play an important role in the balance of apoptosis and autophagy.

Discussion

In this study, a shRNA lentiviral vector was constructed to silence the VMP1 gene for heritable changes in cells lines which highly expressed VMP1 originally. After that, the proliferation and migration of shVMP1 cells increased significantly. The benefit of lentiviral vector transfection was in inducing the cells ability to gain heritable changes, which benefit the subsequent study [17]. The traditional method of siRNA interference only functioned as a temporary transfection, which may affect the proliferation of cells in turn as well as an instable function in gene silencing [18]. Of course, lentiviral techniques also have some defects. It would be time-consuming in the process of puromycin screening in the target cells. It may be a trendy to modify the upstream promoter of VMP1, which relates to an inducible function of tetracycline, to make the process of gene silencing more credible.

As a tumor suppressor gene involved in tumor progression, it has been demonstrated that upregulation of VMP1 could inhibit the proliferation of liver cancer cells [9]. In the present study, after knocking down VMP1, we observed maximum proliferation in 24 hours and an ensuing increase which eventually equaled the control group. The above indicated that VMP1 might play a crucial role in controlling the proliferation of cells. Such a role for VMP1 is further supported by our *in vivo* study in which we showed that the proliferation of cells was lower than that VMP1 gene silencing group.

VMP1 also played a crucial role in the regulation of cell migration. On one hand, recent studies indicated that VMP1 is essential for cell-cell contacts and tight junction formation, and it colocalizes with the tight junction protein ZO-1 in spots between neighboring HEK293 cells [7]. ZO-1 is important for clustering of claudins and occludin, resulting in the formation of tight junctions. Downregulation of VMP1 by RNAi results in loss of cell adherence and increased ability of migration. In this process, ZO-1 is closely associated with E-cadherin [19], both of which participate in the course of epithelial-mesenchymal transition (EMT). This was also demonstrated by a downregulation of E-cadherin in advanced metastatic colon cancer. On the other hand, it was reported that VMP1 may be a new player in the regulation of the autophagy-specific phosphatidylinositol 3-kinase complex activation [20]. However, the underlying mechanisms involved in the migration of colon cancer remain elusive. To further confirm the subcellular localization of VMP1 and its regulating mechanism, in our transwell study, we found a significant increase in migration of colon cancer cells after VMP1 gene silencing. With protein levels, a decreasing expression in phosphorylation of ZO-1 as well as a downregulation of E-cadherin was noticed by Western Blot, which indicated a weak function of ZO-1 after VMP1 gene silencing. As ZO-1 and E-Cadherin play important roles in cancer-related cell biological systems [21], VMP1's direct interaction with ZO-1 and E-Cadherin might provide some evidence on the phenomenon that VMP1 controls breast cancer behaviors thorough regulation of cell-cell contacts. Moreover, this phenomenon might be associated with an activation of the PI3K/Akt signaling pathway, which was embodied by the increasing level of p-Akt/Akt. The PI3K/Akt signaling pathway was confirmed to play a significant role in tumor cell survival, angiogenesis, differentiation, growth and metastasis [22]. Similarly, other research also pointed out the relationship between VMP1 and the PI3K/Akt signaling pathway [23] and the significant role of the PI3K/Akt signaling pathway in colon cancer [24]. In the present study, all the facts mentioned above seem to demonstrate an occurrence of EMT and an ensuing cellular invasion might function through the PI3K/Akt signaling pathway.

VMP1 may be a bridge of autophagy and apoptosis. Under the condition of pressure, cells may be prone to upregulate VMP1 to promote autophagy. On the other hand, when VMP1 is downregulated, it may turn to apoptosis. In addition to previous studies in colon cancer [12], in our studies, it was also demonstrated that a selective apoptosis occurred, which was embodied by an increasing sensitivity to 5-Fu but an invariable sensitivity to platinum drugs such as cisplatin and oxaliplatin.

To integrate with the clinical data mentioned above, the downregulation of VMP1 made cells sensitive to apoptotic signaling pathways. It will be imperative to discuss the role of VMP1 *in vivo* studies, namely to verify the sensitivity to 5-Fu from the cancer cells with VMP1 gene silencing implanted in animal models. Further, for clinical studies, a consideration of 5-Fu would be important in the patients with lower expression of VMP1, which may be a potential target for individual treatment in breast cancer.

In summary, the present study first indicated the expression of VMP1 was negative related with the prognosis of breast cancer by analyzing the clinical samples and different cell lines. After gene silencing with lentivirus, a significant ability of invasion was noticed and an increasing sensitivity to 5-Fu was also verified. To some extent, VMP1 could be regard as a new biomarker for prognosis of breast cancer and a potential target concerned with migration of cancer cells. In conclusion, VMP1 is not only a bridge in balancing autophagy and metastasis, but also a biomarker to predict the benefit which patients may gain from 5-Fu in different expression of VMP1 levels.

Disclosure of conflict of interest

None.

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