Original Article
Vasohibin 2 as a potential predictor of aggressive behavior of triple-negative breast cancer

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Abstract: Triple-negative breast cancer (TNBC) is a subtype breast cancer with aggressive behavior, advanced disease status and poor prognosis. Because of the lack of targeting agents and limited therapeutic options, treatment of TNBC remains a great clinical challenge. Vasohibin 2 (VASH2) was previously identified as an angiogenic factor, but its role in TNBC tumorigenesis is unknown. Using quantitative PCR and western blot analyses, we found that VASH2 is overexpressed in TNBC cells and tissues. Knockdown of VASH2 via siRNA inhibited the proliferation of the TNBC cell lines by delaying cell cycle progression and increasing apoptosis. Further analyses showed that the VASH2-mediated increase in the transcription of fibroblast growth factor-2, vascular endothelial growth factor and vasohibin 1 may be the mechanism underlying these effects. Taken together, these data indicate that VASH2 is abnormally expressed in TNBC, indicating a novel and important role for VASH2 in TNBC malignant transformation.

Keywords: TNBC, VASH2, cell cycle, invasion, proliferation

Introduction

Breast cancer is the top most cancer in women [1]. Though the tremendous advances in diagnosis and treatment, the prognosis of breast canceris still not satisfactory [2]. Triple-negative breast cancer (TNBC) often associated with more distant metastasis, tumor recurrence, and therapy resistance [3]. However, the molecular mechanisms regulating growth, acquisition of invasive and metastatic phenotypes in TNBC are still not fully understood.

Vasohibin 2 (VASH2) has been associated with carcinogenesis and cancer therapy recently [4]. The vasohibin family includes vasohibin-1 (VASH1) and vasohibin-2 (VASH2). In endothelial cells (ECs), VASH1 is selectively induced by angiogenic stimulators [5]. Recently, exogenous VASH1 significantly blocks sprouting angiogenesis by tumors [5]. In contrast to VASH1, VASH2 has been found to promote angiogenesis at the sprouting front in a mouse model of hypoxia-induced subcutaneous angiogenesis [6]. Recent studies have reported the correlation between VASH2 and tumor angiogenesis in several solid tumors [7-9]. However, the specific role of VASH-2 in TNBC remains unknown. Here,

we conducted immunohistochemical analysis of VASH2 in a series of TNBC tumor tissues and investigate the potential prognostic role of TNBC. Moreover, we aimed to investigate the function and mechanism of VASH-2 in the carcinogenesis of TNBC.

Materials and methods

Patients

Specimens of 126 primary TNBC cases over the period from 2009 to 2014 were obtained from the Department of Pathology, The First Affiliated Hospital, College of Medicine, Zhejiang University. ER-negative and PR-negative tumours were defined as those with less than 1% positive staining tumour cells. Clinicopathological parameters including age, tumour size, growth pattern, histologic grade, histologic subtype, basal-like phenotype, associated ductal carcinoma in situ, lymphovascular invasion and axillary lymph node status were reviewed.

Tissue microarray

The tissue microarray (TMA) construction and immunohistochemical analysis was conducted

as described previously. The staining pattern of VASH2 was classified in a subjective spectrum of 0 to +++ as follows: 0, negative expression in tumor tissue; +, weak staining; ++, moderate staining; and +++, strong staining. For each staining level, the percentage of cells with a specific score was visually estimated. When < 10% of the cells were positively stained, the section was classified as negative. Positive sections were further divided into weakly positive (10% to 30%), moderately positive (30% to 50%), and strongly positive (more than 50%). The tissue microarray chips were observed under 20 × magnification.

Cell culture

MCF-10A cell line was cultured following the instruction provided from the ATCC. The TNBC cell lines including MDA-MB-231, MDA-MB-436, and MDA-MB-468, BT-549 were obtained from American Type Culture Collection (ATCC, USA), and maintained at 37°C with 5% CO_2 in DMEM (Dulbecco's Modified Eagle's Medium) and RPMI-1640 respectively supplemented with 10% fetal bovine serum (FBS; Gibco) and 1 × antibiotic antimycotic (Gibco).

Preparation of siRNAs and in vitro transfection

Synthetic Stealth siRNAs were designed and purchased from Invitrogen Life Technologies (Carlsbad, CA, USA) in desalted and annealed form. Two different Stealth siRNAs targeting human VASH2 with the following sense and antisense sequences were used: siVASH2-1 (sense, 5'-GAAAUGACC-CGAGAGUCCUUGCC-UA-3'; antisense, 5'-UAGGCAAGGA-CUCUCGG-GUCAUUUC-3'); and siVASH2-2 (sense, 5'-GA-GAAAUAUGCCAGGGACAUGAGAA-3'; antisense, 5'-UUCUCAUGUCCCUGGCAUAUUUCUC-3'). Each freeze-dried siRNA was reconstituted in RNase-free water to make a 20 µM stock solution. Non-specific Stealth RNAi Negative Control Medium GC Duplex #2 was also purchased from Invitrogen and used as control. Cells were transfected with siRNAs by reverse transfection according to the manufacturer's instructions. Briefly, 60 pmol siRNA and 1 mL Opti-MEM I Medium (Invitrogen) without serum were mixed in a 6-cm cell culture dish. After swirling the dish, 10 µL Lipofectamine RNAiMAX reagent (Invitrogen) was added and the mixture was incubated for 20 min at room temperature to obtain siRNA-Lipofectamine RNAiMAX com-

plexes. Next, 5 mL of cell suspension including $2 × 10^6$ cells in complete growth medium without antibiotics was added into the cell culture dish. This gives a final siRNA concentration of 10 nM. Forty-eight hours after incubation at 37°C in a CO_2 incubator, gene knockdown was evaluated by q RT-PCR.

Quantitative RT-PCR

Total RNA was extracted from cells and tissues using RNAiso Plus reagent (Takara, Dalian, China), and cDNA was synthesized using Primescript RT Reagent (Takara). Quantitative RT-PCR was performed on a StepOnePlus Real-Time-PCR System (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. The PCR conditions consisted of an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Finally, a melting curve profile was set at 95°C (15 s), 60°C (15 s), and 95°C (15 s). Each mRNA level was measured as a fluorescent signal corrected according to the signal for β-actin. The primer pairs used were as follows: human VASH2 forward, 5'CTCTTC-CAGCCTTCCTTCCT3'; reverse, 5'AGCACTGTGT-TGGCGTACAG3'; human β-actin forward, 5'GG-GAGAAATGGTGGGCG3'; reverse, 5'GCCAGTCT-GGGATCGTCATC3'. Relative quantification was calculated by the ΔΔCt method and normalized based on β-actin.

Cell proliferation assay and cell cycle analysis

Cells were seeded in 96-well plates at $1 × 10^3$ per well. Cell proliferation was evaluated using Cell Counting Kit-8 (CCK-8, Dojindo, USA) according to the manufacturer's instructions. Briefly, 10 µl of CCK-8 solution was added to culture medium, and incubated for 2 h. The absorbance at 450 nm wave length was determined with a reference wave length of 570 nm. For cell-cycle analysis, cells were plated in 6-well plates at $5 × 10^5$ per well. The cell-cycle distribution was analyzed by propidium iodide (Sigma-Aldrich) staining and flow cytometry. All experiments were performed in triplicates.

Colony formation assay

Cells were plated in 6-well plates at $1-2 × 10^2$ per well and maintained in medium containing 10% FBS. After 12-14 days, the cells were washed twice with PBS, fixed with methanol
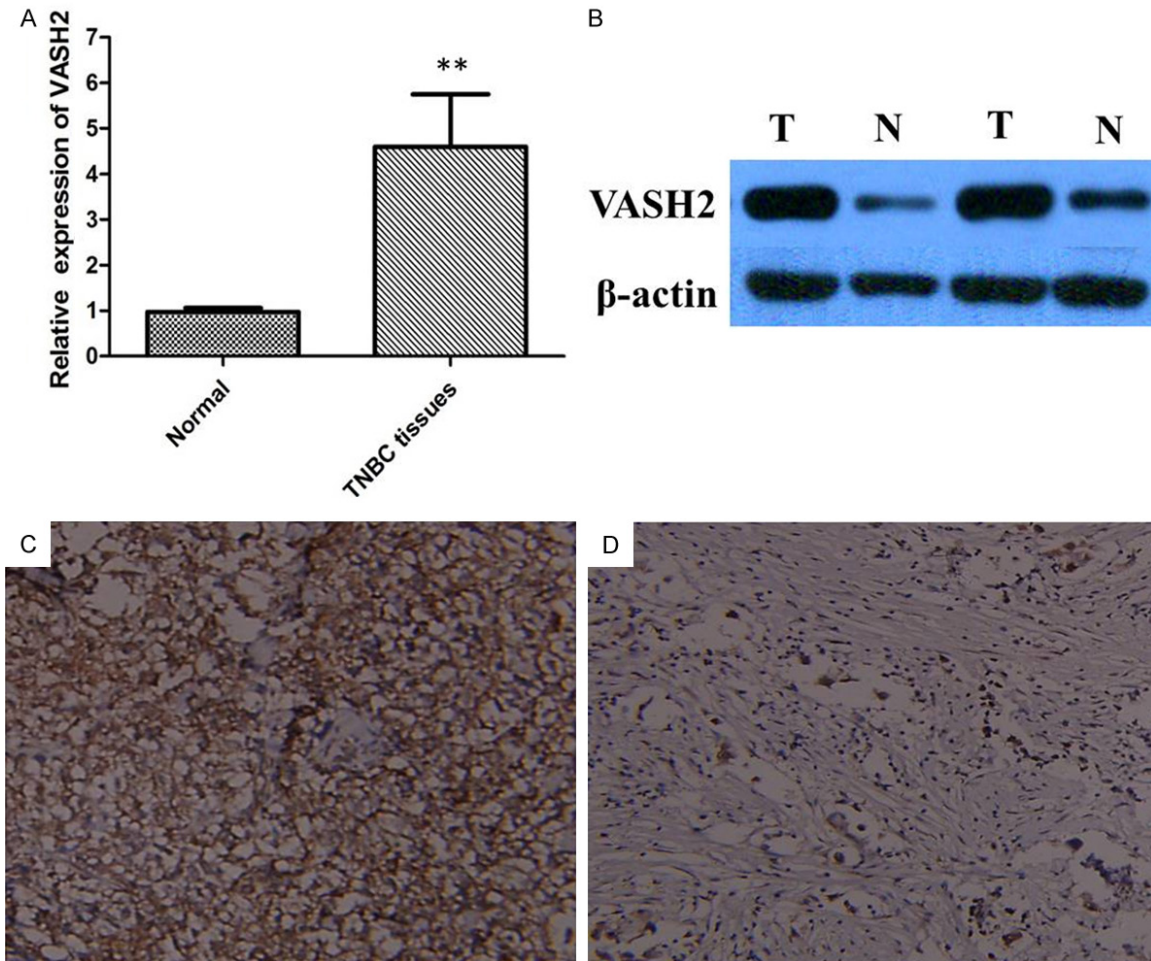
Figure 1. A and B: qRT-PCR and Western blots showing expression level of VASH2 mRNA in a series of TNBC tissues and adjacent noncancerous tissues; C and D: Immunohistochemical analysis revealed that TNBC tissue had stronger VASH2 expression compared with that of corresponding adjacent noncancerous tissues.

and stained with Giemsa solution. The number of colonies containing ≥ 50 cells was counted under a microscope. All these experiments were performed in triplicates.

Migration and invasion assays

After transfection, 1×10^5 cells in serum-free medium were seeded into the Boyden chamber without Matrigel (8-μm pore; BD Falcon, San Jose, CA) for migration or the chamber with Matrigel (8-μm pore; BD Falcon) for invasion. Then the chambers were put in 24-well plates with medium with 10% FBS. The chambers were incubated for 24 hours at 37°C with 5% CO_2. The cells on the underside of filter membrane were fixed in ethanol and stained with crystal violet. The cells were counted under a microscope.

Statistical analysis

The two-tailed x^2 test was used to determine the significance of the difference among the covariates. The significance of the in vitro data was determined using the Student t test (two-tailed). In all of the tests, *P* values less than 0.05 were considered statistically significant. The SPSS software program (version 12.0; SPSS Inc.) was used for statistical analyses.

Results

Expression of VASH2 in Primary TNBC tissues

To investigate the clinical significance of VASH2 expression in human TNBC progression, we firstly analyzed the mRNA and protein expression of VASH2 in a total of 126 paired of pri-
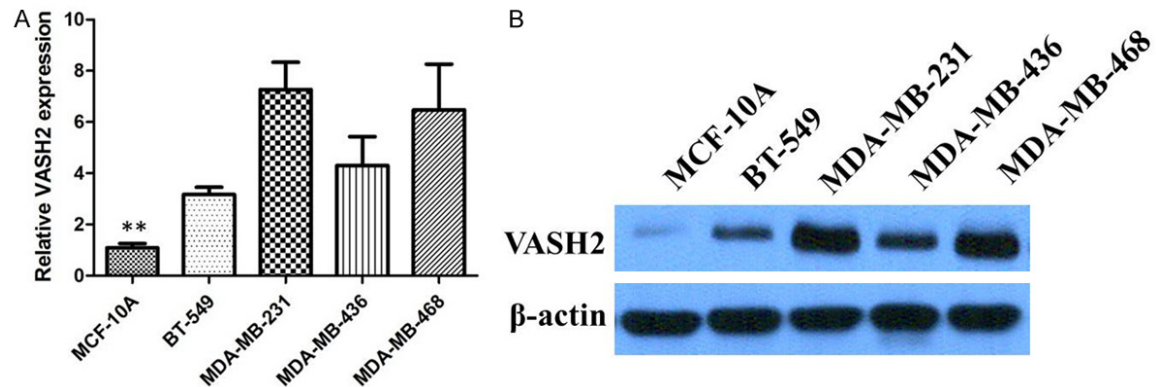
Figure 2. A: qRT-PCR showing the expression level of VASH2 mRNA in a panel of TNBC cell lines; B: Western blots showing the expression level of VASH2 mRNA in a panel of TNBC cell lines.

mary TNBC cases and distal normal paired tissues by qRT-PCR and western blot. The expression level of VASH2 were significantly elevated in TNBC tissues compared with that in normal paired tissues both at the mRNA and protein level (**Figure 1A** and **1B**; *P*<0.01). We then detected VASH2 in clinical TNBC samples with immunohistochemistry. As demonstrated in **Figure 1C** and **1D**, we observed 75.40% (95/126) of TNBC samples were VASH2-positive, however, almost no expression in the normal breast tissue. VASH2 was located in the plasma of tumor cells. Furthermore, high levels of VASH2 was significantly associated with clinicopathological parameters of TNBC, including tumor size, tumor grade, depth of invasion, and lymph node metastasis.

Expression of VASH2 in TNBC Cell Lines

The mRNA and protein expression of VASH2 in a panel of TNBC cell line and MCF-10A which is the normal human mammary epithelial cell line. All TNBC cell lines expressed higher VASH2 than MCF-10A. VASH2 mRNA and protein expression were highest in MDA-MB-231 and MDA-MB-468 cells (**Figure 2A** and **2B**; *P*<0.01). So these two cell lines were chosen for a series of VASH2 knockdown experiments.

Knockdown of VASH2 inhibited TNBC cell proliferation and induced G2 and S phase cell cycle arrest

To investigate the role of VASH2 in TNBC progression, we performed a loss-of-function experiment by knocking down VASH2 expression using two specific siRNA. Using RT-PCR and

Western blotting, we found significant suppression of VASH2 gene expression in transfected in MDA-MB-231 and MDA-MB-468 cells, suggesting the silencing of endogenous gene expression by siRNA (**Figure 3**; *P*<0.01). We investigated the proliferation activity by CCK8 assay *in vitro*. The results showed that the growth rate of all MDA-MB-231 and MDA-MB-468 cells transfected by VASH2 siRNA were slower than control cells (**Figure 4A** and **4B**; *P*<0.01). Similarly, the results of colony-formation assays revealed that MDA-MB-231 and MDA-MB-468 cells following inhibition of VASH2 had significantly lower number of colony formation compared with control, suggesting that VASH2 may act as an oncogene involved in the promotion of TNBC cell proliferation (**Figure 4C** and **4D**; *P*<0.01). VASH2 knockdown induced G2 and S phase cell cycle arrest: the percentage of cells in G2 and S phase was higher in VASH2-siRNA MDA-MB-231 and MDA-MB-468 than control (**Figure 5A** and **5B**; *P*<0.01).

Inhibition of VASH2 suppressed the migration and invasion of TNBC cells via the EMT pathway

We performed the transwell assay to determine if VASH2 was involved in the regulation of migration and invasion of TNBC cells. Our results showed that knockdown of VASH2 could significantly suppress the migratory and invasion ability of MDA-MB-231 and MDA-MB-468 cells (**Figure 6**; *P*<0.01). We further checked the expression level of EMT markers and found the elevation of epithelial marker. Western blot results showed that after the inhibition of VASH2 gene, E-cadherin expression was increased,
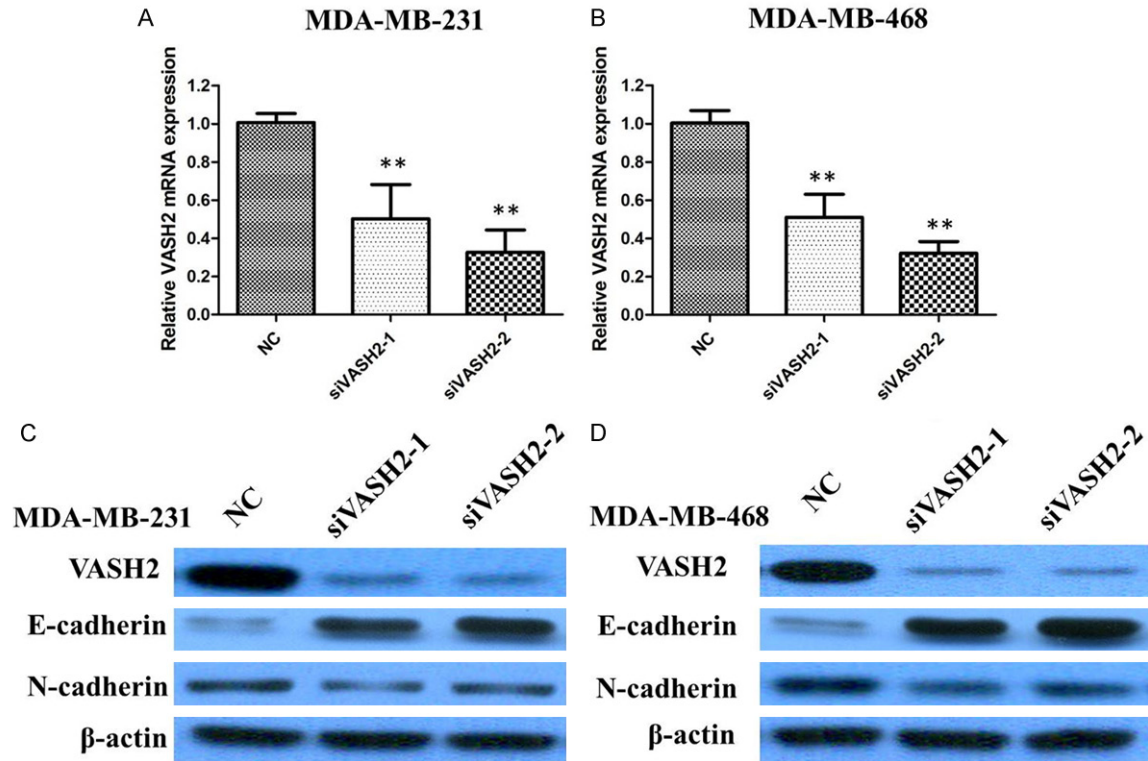
Figure 3. A: qRT-PCR showing the expression of VASH2 mRNA in MDA-MB-231/si cells was significantly decreased compared with control cells; B: qRT-PCR showing the expression of VASH2 mRNA in MDA-MB-468/si cells was significantly decreased compared with control cells; C: Western blots showing the expression of VASH2 protein was significantly decreased in MDA-MB-231/si cells compared with control cells; D: Western blots showing the expression of VASH2 protein was significantly decreased in MDA-MB-468/si cells compared with control cells.
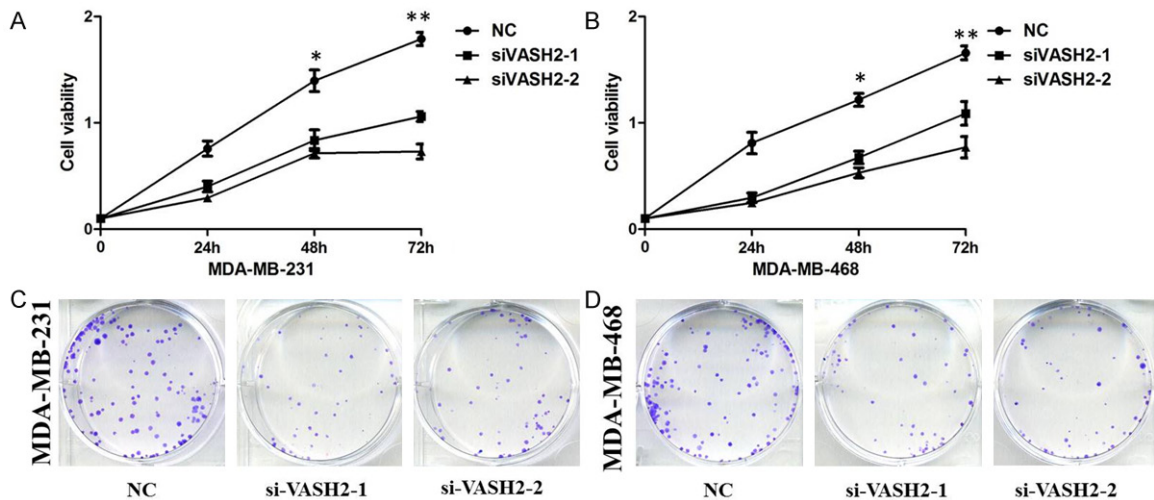

Figure 4. A and B: CCK8 assay showed that the growth rate of all MDA-MB-231 and MDA-MB-468 cells transfected by VASH2 siRNA were slower than control cells; C and D: Colony-formation assay showed that silencing of VASH2 significantly decreased the colony-forming ability of MDA-MB-231 and MDA-MB-468.

and Vimentin was decreased in all VASH2-siRNA MDA-MB-231 and MDA-MB-468 cells compared with control, indicating that VASH2 is involved in the EMT process in TNBC (**Figure 2**).

Discussion

Angiogenesis was previously reported to not only play significant role in human normal
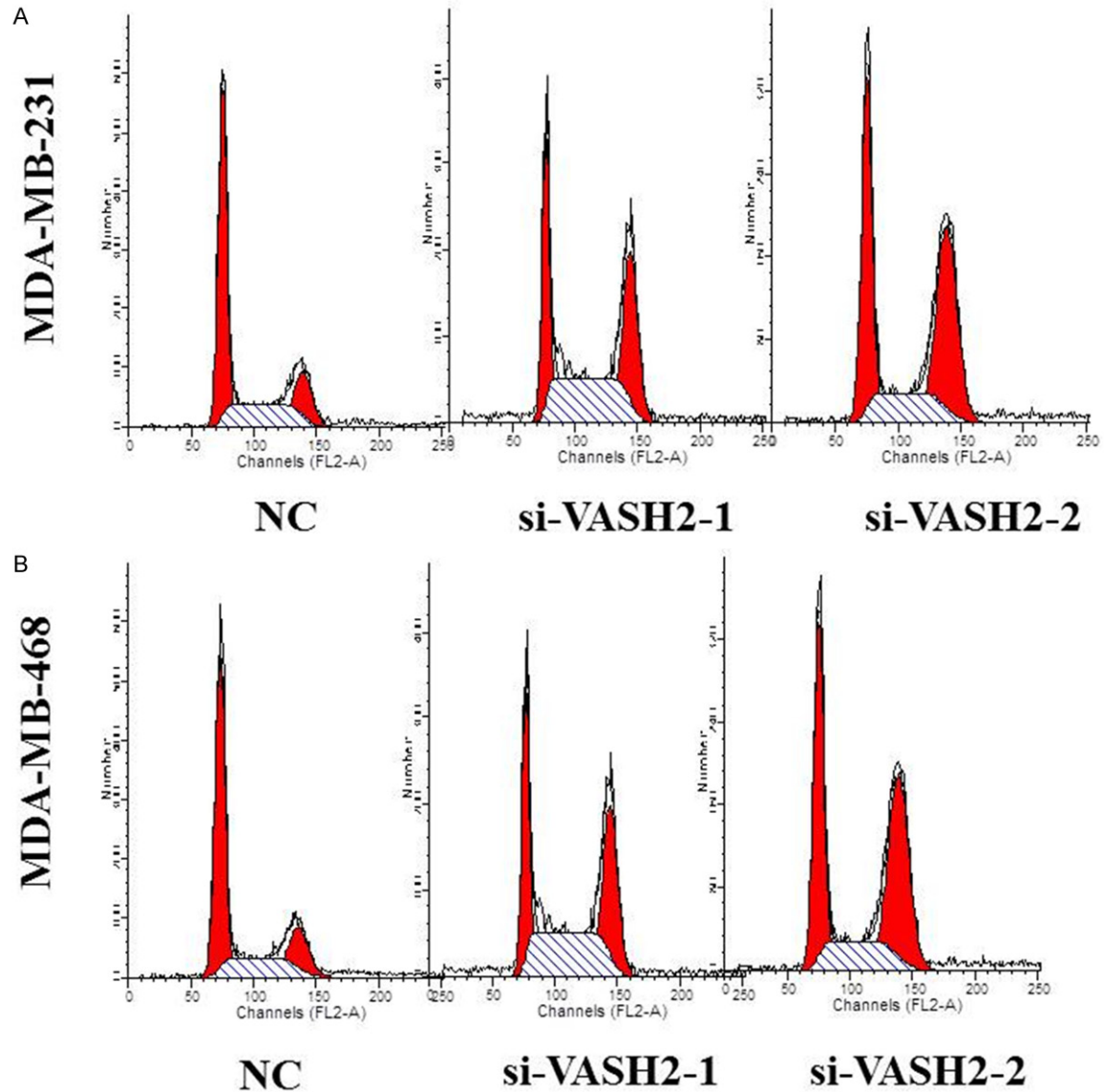
Figure 5. A and B: VASH2 knockdown induced G2 and S phase cell cycle arrest: the percentage of cells in G2 and S phase was higher in VASH2-siRNA MDA-MB-231 and MDA-MB-468 than control.
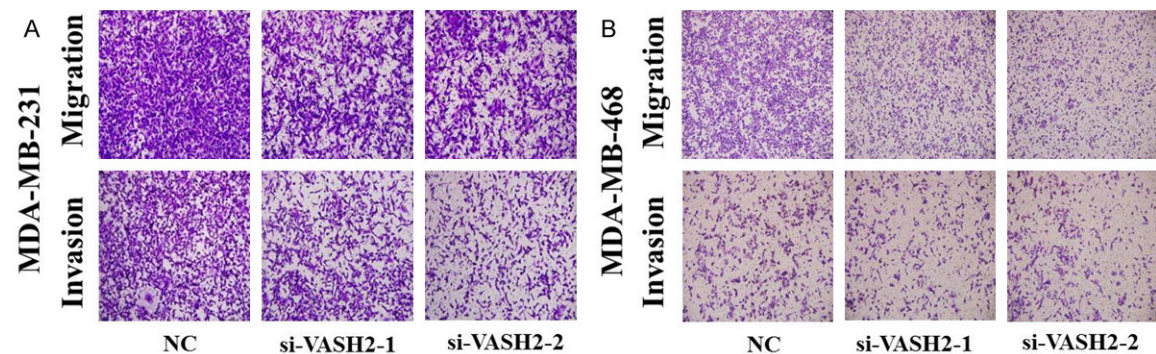

Figure 6. A and B: Knockdown of VASH2 could significantly suppress the migratory and invasion ability of MDA-MB-231 and MDA-MB-468 cells.

development, but also in pathological conditions such as carcinoma and inflammatory diseases. Endogenous angiogenesis stimulators such as vascular endothelial growth factor (VE-GF) and basic fibroblast growth factor (bFGF) or angiogenesis inhibitors such as thrombospondin-1 (TSP-1) exists in human body, and the local balance between them regulates the process of angiogenesis [4]. As in other human malignancies, angiogenesis has also been reported to be associated with development, invasion and metastasis of breast cancer [10]. Specifically, patients with breast cancer along with ER/PR and HER2 negative tumors are referred to as metastatic TNBC subtypes. Although newer targeted therapies for angiogenesis, such as VEGFR, have been approved for the treatment of ER/PR-positive and HER2-amplified metastatic breast cancer, few therapeutic effects have been identified for patients with mTNBC [2]. Hence, in this study we investigated the angiogenesis factor VASH-2 in the progression and metastasis of TNBC and its mechanism.

Unlike VASH-1, which was induced by VEGF and bFGF2 in endothelial cells, the expression of VASH-2 was constitutive and not regulated by growth factors or cytokines [4]. Moreover, VASH2 was expressed preferentially in mononuclear cells that are mobilized from the bone marrow and infiltrated the sprouting front [6]. In this study, the expression of VASH-2 was ever firstly investigated to be upregulated in tumor tissues both at the transcription and protein level in TNBC by the evidence of significant increased expression of VASH-2 mRNA and protein as compared with normal tissues. Moreover, our IHC results showed that more than 70% of TNBC tumor specimens presented with positive expression of VASH-2 and located in the plasma of tumor cells, which is significantly higher than that of normal tissues. Besides, we also found that all TNBC cell lines expressed higher VASH2 than normal mammary epithelial cell MCF-10A, especially for MDA-MB-231 and MDA-MB-468 cells. In fact, VASH-2 has already been found to be expressed in endometrial cancer [8], hepatocellular carcinoma [7] and serous ovarian adenocarcinoma [9]. The increased expression of VASH-2 in cancer cells has been referred to be mediated by methylation of promoter region [11] or the decrease of miRNA-200b [9].

To further illustrate the role and function of VASH-2 in TNBC, we performed the knockdown by si-RNA silencing on TNBC cancer cell lines MDA-MB-231 and MDA-MB-468. Our results showed that silencing of VASH2 significantly promoted cell proliferation, colony formation, and induced migration and invasion of TNBC cells. These results demonstrated that VASH-2 is associated with carcinoma angiogenesis and malignant transformation and promoted cancer growth in TNBC, which is consistent with previous study of VASH-2 in other cancer cells [7-9]. Moreover, we further found that knockdown of VASH-2 could induce the arrest of G2 and S phase in TNBC cell lines. Tu et al. has reported that significantly higher expression of VASH2 was found in Ki67 ≥ 14% human breast cancer tissues [12], and the level of Ki67 was low during the G and early S phases [13, 14]. Interestingly, another study found that nuclear VASH-2 promote cell proliferation by driving the cell phase shift from G0/G1 to S phase in HepG2 cells, while knockdown of VASH2 reduced the S-phase and increased the G0/G1 population [15]. We supposed that it is the cytoplasmic VASH-2 which leads to different cell phase shift in two different cell models, which warrant further investigation in the future.

The epithelial-mesenchymal transition (EMT) is a vital process by which epithelial cells lose polarity and adhesion, and gain migratory and invasive properties, which has been associated with pathogenesis of tremendous cancers, including breast cancer [16-19]. Here, we confirmed that knockdown of VASH-2 suppressed EMT through the downregulation of vimentin and N-cadherin, consistent with upregulation of E-cadherin in TNBC cells. The results were in line with previous studies of VASH-2 in hepatocellular carcinoma [7] and ovarian adenocarcinoma [9]. Moreover, the EMT phenotype was reported to be associated with tumor invasion, carcinoma metastasis, drug resistance and stem cell proportion [20-23]. For TNBC tumor, several transcription factors, such as Zeb1, Zeb2, Snail and slug has been referred to promote EMT process and associated with prognosis [24]. Along with the evidence that VASH-2 could be mediated by miR-200b [7], which could participate in the suppression of EMT by repressing the expression of ZEB1 and ZEB2 [25], we suppose that ZEB family may present

as downstream transcriptional factor of VASH2. In this context, it would be interesting to investigate whether or not VASH-2 is involved in EMT of TNBC cancer cell by miR-200 and ZEB family in the future. Therefore, we propose that VASH-2 may be a candidate target for the treatment of TNBC considering its stimulatory role on angiogenesis, proliferation, cell cycle phase and EMT process.

In this study, we firstly investigated the correlation between VASH-2 and clinicopathological parameters of TNBC, in which expression of VASH2 was significantly associated with tumor size, tumor grade, depth of invasion, and lymph node metastasis of TNBC. In fact, as homologue of VASH-2, VASH-1 has been identified as prognostic factor for tremendous cancers, such as lung cancer [26], colorectal cancer [27], prostate cancer [28], hepatocellular cancer [29], and breast cancer [30]. For VASH-2, it has been reported to be associated with tumor stage and poor clinical outcome in pancreatic adenocarcinoma [31]. Thus, our results suggested that overexpression of VASH-2 accelerate the carcinoma towards malignant subtypes and may be associated with poor clinical outcome for TNBC.

In summary, our results showed that VASH-2 contributes to the progression by promotion of EMT process and angiogenesis and its potential prognosis role in TNBC. Furthermore, knockdown of VASH2 could significantly inhibit cell proliferation, colony formation, migration and invasion, suggesting that VASH-2 may represent as a novel target for the treatment of TNBC in the future.

Disclosure of conflict of interest

None.

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