

VSP-17 suppresses the migration and invasion of triple-negative breast cancer cells through inhibition of the EMT process via the PPAR γ /AMPK signaling pathway

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Abstract. VSP-17, a novel peroxisome proliferator-activated receptor γ (PPAR γ) agonist, has been previously demonstrated to suppress the metastasis of triple-negative breast cancer (TNBC) by upregulating the expression levels of E-cadherin, which is a key marker of epithelial-mesenchymal transition (EMT). However, the mechanism of action of VSP-17, in particular whether it may be associated with the EMT process, remains unknown. The present study investigated the ability of VSP-17 to inhibit the invasiveness and migratory ability

of TNBC cell lines (MDA-MB-231 and MDA-MB-453) performed in *in vitro* experiments, including cell migration assay, cell invasion assay, cell transfection, RT-qPCR, western blot (WB) analysis and immunofluorescence. The present study aimed to ascertain whether and how the PPAR γ /AMP-activated protein kinase (AMPK) signaling pathway serves a role in the inhibitory effects of VSP-17 on cell migration and invasion. The results revealed that both treatment with compound C (an AMPK inhibitor) and transfection with small interfering RNA (si)AMPK notably diminished the inhibitory effect of VSP-17 treatment on the migration and invasion of MDA-MB-231 and MDA-MB-453 cells, indicating that VSP-17 may, at least partly, exert its effects via AMPK. Furthermore, both compound C and siAMPK markedly diminished the VSP-17-induced downregulation of vimentin expression levels and upregulation of E-cadherin expression levels, further indicating that the VSP-17-induced inhibition of the EMT process may be dependent on AMPK. The combination of GW9662 (a PPAR γ antagonist) or siPPAR γ diminished the inhibitory effect of VSP-17 treatment on the migration and invasion of the TNBC cells, indicating that PPAR γ may serve an important role in the VSP-17-induced inhibition of the migration and invasion of TNBC cells. In addition, both GW9662 and siPPAR γ significantly reversed the VSP-17-induced downregulation of vimentin expression levels and upregulation of E-cadherin expression levels, implying that the VSP-17-induced inhibition of the EMT process may be dependent on PPAR γ . VSP-17 treatment also upregulated the expression levels of p-AMPK, which could be reversed by either GW9662 or siPPAR γ , indicating that the VSP-17-induced activation of the AMPK signaling pathway was PPAR γ -dependent. In conclusion, the findings of the present study indicated that VSP-17 treatment may inhibit the migration and invasion of TNBC cells by suppressing the EMT process via the PPAR γ /AMPK signaling pathway.

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Abbreviations: ACC, acetyl-CoA carboxylase; AKT, protein kinase B; AMPK, adenosine 5'-monophosphate (AMP)-activated protein kinase; COX-2, cyclooxygenase-2; CRC, colorectal cancer; DAPI, 4,6-diamidino-2-phenylindole; EMT, epithelial-mesenchymal transformation; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GAPDH, glycer-aldehyde-3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; H/R, hypoxia/reoxygenation; HRP, horseradish peroxidase; LOX-1, low-density lipoprotein receptor-1; mTOR, mechanistic target of rapamycin; NF- κ B, nuclear factor- κ B; Nrf2, nuclear factor erythroid 2-related factor 2; PMSF, phenylmethanesulfonyl fluoride; PPAR γ , peroxisome proliferator-activated receptor γ ; PVDF, polyvinylidene fluoride; RA, rosmarinic acid; TNBC, triple-negative breast cancer; TGF- β , transforming growth factor- β

Key words: adenosine 5'-monophosphate-activated protein kinase, VSP-17, epithelial-mesenchymal transformation, triple-negative breast cancer, metastasis, peroxisome proliferator-activated receptor γ

Introduction

Triple-negative breast cancer (TNBC) is a subtype of breast cancer that lacks the expression of the human epidermal growth factor receptor 2 (HER2), estrogen and progesterone

receptors (1,2). Metastasis is a major clinical feature of late stage TNBC, and is the main cause of death in patients (3,4). It has been widely recognized that the migration and invasion of TNBC cells are considered to be key steps in the metastatic process (5). One study revealed that TNBC cells with high migratory and high invasive properties are closely associated with a poor prognosis in TNBC (6). In a previous study, thymoquinone, an active ingredient of *Nigella sativa*, markedly inhibited cell growth, migration and invasion in both TNBC cells and orthotopic TNBC model mice by regulating the elongation factor 2 kinase (eEF-2K) signaling axis (7). Furthermore, in another study, luteolin, a natural flavonoid compound, reportedly suppressed the epithelial-mesenchymal transition (EMT) and migration of TNBC cells by inhibiting YAP/TAZ activity (8). These findings suggest that preventing the migration and invasion of TNBC cells may inhibit TNBC metastasis and represent a target for TNBC treatment.

EMT is a biological process in which epithelial cells lose key proteins (such as E-cadherin) involved in cell junctions and obtain a mesenchymal phenotype, which is evidenced by upregulated protein expression levels of mesenchymal markers (such as vimentin) (9-11). The activation of EMT has been found to serve an important role in the initial stages of the metastatic cascade by enhancing the migratory and invasive abilities of cancer cells (12). It has been demonstrated that irisin (a novel myokine) could inhibit the EMT process by suppressing the migration and invasion of MIA PaCa-2 and Panc03.27 cells (13). In addition, isorhamnetin could inhibit migration and invasion by suppressing AKT/ERK-mediated EMT in A549 human non-small cell lung cancer cells (14). These aforementioned findings suggest that blocking the process of EMT may represent a potential approach to inhibit the migration and invasion of TNBC cells.

AMP-activated protein kinase (AMPK), a vital metabolic energy sensor that regulates protein and lipid metabolism responses, has been demonstrated to serve an important role in the EMT process associated with tumor metastasis (15-19). Increasing evidence has revealed that AMPK is inactivated in TNBC cells, and that the absence of AMPK in patients predicted a poor prognosis (20,21). Conversely, the activation of AMPK could inhibit the EMT process of tumor cells by regulating the expression levels of EMT-related markers (22). For example, metformin could suppress the EMT process in TNBC cells by activating the AMPK signaling pathway (23). Similarly, rosmarinic acid was found to suppress the metastatic characteristics of colorectal cancer (CRC) cells by activating AMPK (24). These previous studies suggest that the activation of AMPK may be a target for the inhibition of metastasis and the EMT process in TNBC.

Peroxisome proliferator-activated receptor γ (PPAR γ), a ligand-dependent transcription factor that regulates lipid metabolism, has been proven to participate in tumor metastasis (25). Accumulating evidence indicates that PPAR γ is inactivated in TNBC cells, and its activation may inhibit the metastasis of breast cancer cells (26,27). VSP-17 has been identified as a novel PPAR γ agonist, and it has been shown to suppress the metastasis of TNBC by upregulating the expression levels of E-cadherin (28). However, the mechanism through which VSP-17 may inhibit TNBC metastasis remains unclear. The present study aimed to determine the mechanism

of action of the VSP-17-induced upregulation of E-cadherin levels in inhibiting TNBC metastasis, with a focus on the EMT and PPAR γ /AMPK signaling pathways.

Materials and methods

Reagents. VSP-17 (C₂₃H₁₈F₃N₃O, MW: 409.1402, purity \geq 98%) was designed and synthesized by our team (Guangxi Colleges and Universities Key Laboratory of Pharmacology), the details of which have been described in our previous study (28). siAMPK (cat. no. 203834) and siPPAR γ (cat. no. 202738) were obtained from Sangon Biotech; Lipofectamine 3000 reagents (cat. no. 2149659) were purchased from Thermo Fisher Scientific, Inc., and compound C (cat. no. 866405-64-3) and GW9662 (cat. no. 22978-75-2) were purchased from MedChemExpress. Primary antibodies against the following targets: AMPK (cat. no. BS6271), p-AMPK (cat. no. BS4010), E-cadherin (cat. no. BS1098) and vimentin (cat. no. BS1491) were supplied by Bioworld Technology, Inc.; glyceraldehyde-3-phosphate dehydrogenase (GAPDH, cat. no. 60004-1-Ig) was obtained from ProteinTech Group, Inc. HiScript II One-Step RT-PCR Kit (cat. no. R323-01) and ChamQ SYBR qPCR Master Mix (cat. no. Q311-02) were purchased from Vazyme; 4',6-diamidino-2-phenylindole (DAPI; cat. no. C0060) was obtained from Beijing Solarbio Science & Technology Co., Ltd. Other analytical reagent grade chemicals were purchased from Chemical Reagent Co. Ltd.

Cell culture. MDA-MB-231 and MDA-MB-453 cells were obtained from the American Type Culture Collection (ATCC) and supplemented with Leibovitz's (L-15) medium (cat. no. 11415064; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in 5% CO₂, 95% air cell culture at 37°C. The cells were starved for 2 h before the experiments.

Cell migration assay. According to the manufacturer's protocol, the cell migration assay was carried out using Transwell plates. Post-treated cells or compound C co-cultured cells (1x10⁵) were detached and suspended in the culture medium. Then, the cells were added to the upper chamber of the Transwell plates, while the lower chamber was filled with 600 μ l of culture medium containing 10% FBS as a chemical attractant. After being incubated for 6 h, the non-migratory cells on the upper surface of the membrane were removed with a soaked cotton swab. In addition, the cells that migrated to the bottom face of the membranes were counted after being stained with 0.5% crystal violet solutions at 37°C for 30 min. Then, 5 microscopic fields per filter were captured randomly with the help of an inverted microscope (Olympus, Tokyo, Japan; x200 magnification).

Cell invasion assay. The invasion potential of the MDA-MB-231 and MDA-MB-453 cells was evaluated using a Matrigel-coated Transwell chamber with 8.0- μ m pore size. Post-transfected cells or compound C co-cultured cells (1x10⁵) were resuspended with L-15 medium and plated into the upper chamber, and L-15 medium containing 20% FBS was added to the lower chambers as an attractant. The suspension was discarded after 24 h, counting the cells which invaded

the lower chamber under an inverted microscope (Olympus, Tokyo, Japan; x200 magnification).

Cell transfection. Under the guidance of the manufacturer's instructions, Lipofectamine 2000 and small interfering RNA (siRNA) duplex specific for AMPK and PPAR γ were used to transiently transfect MDA-MB-231 and MDA-MB-453 cells for 6 h at 37°C, and then the cells were incubated with L-15 medium. At 24 h post-infection, the cells were used for subsequent experiments. The sequences of AMPK- and PPAR γ -specific siRNAs were as follows: SiAMPK: sense, 5'-GUUGCCUACCAUCUCAUAUATT-3' and antisense, 5'-UAUUAUGAGAUGGUAGGCAACTT-3'; and siPPAR γ : Sense, 5'-GACAGUGACUUGGCUAUUATT-3' and antisense, 5'-GCGAUCUUGACAGGAAAGATT-3'.

Quantitative real-time polymerase chain reaction (qPCR). For verifying the results of RNA sequencing, qPCR assay was performed. Total RNA was extracted from the cells with TRIzol reagent. The mRNA was reverse transcribed to cDNA with the help of HiScript II One-Step RT-PCR Kit. ChamQ SYBR qPCR Master Mix and quantitative fluorescence analysis were used to analyze the expression of EMT-related factors, and the analysis system was CFX96 Touch optical system (Bio-Rad Laboratories, Inc.). The primer sequences of E-cadherin are as follows: sense, 5'-GCCATCGCTTACACC ATCCTCAG-3' and antisense, 5'-CTCTCTCGGTCCAGC CCAGTG-3'. PCR conditions were as follows: 95°C for 5 min (1x), followed by 95°C for 10 sec, 63°C for 30 sec (40x), 60°C for 10 sec (1x), and then 95°C for 10 sec (1x). Relative quantification was calculated with the $2^{-\Delta\Delta C_q}$ method as described by Livak and Schmittgen (29).

Western blot (WB) analysis. Total proteins were extracted from the MDA-MB-231 and MDA-MB-453 cells using RIPA lysis buffer with phosphatase inhibitor and PMSF for 30 min on ice. The proteins were quantified using the BCA protein concentration determination kit, separated by 8% SDS-PAGE gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk and incubated overnight at 4°C with specific primary antibodies (vimentin, E-cadherin, p-AMPK and AMPK) at a dilution rate of 1:1,000 (v/v). After washing 3 times with TBST for 5 min, the membranes were incubated with HRP-conjugated secondary antibodies and then the membrane was visualized on a gel imager after adding Immobilon Western Chemiluminescent HRP substrate (cat. no. SQ201; Shanghai EpiZyme Biotechnology, Inc.). GAPDH was used as a normalized control. Images were captured by Bio-Rad Gel-Doc XR+ system with Image Lab (version 4.1) software and processed with Quantity One software (Bio-Rad Laboratories, Inc.).

Immunofluorescence (IF). The suspended cells were seeded in a 96-well plate at a density of 5×10^3 per well. At 80-90% confluence, the cells were treated with compound C or siAMPK for 24 h, fixed with 4% buffered paraformaldehyde, and permeabilized with 1% Triton X-100. After being blocked using 5% non-fat milk solution for 30 min, the cells were incubated with primary anti-E-cadherin or anti-vimentin monoclonal antibody at 37°C for 2 h and subsequently incubated with

secondary antibody (cat. no. AMJ-AB2005, 200 μ l; Wuhan AmyJet Scientific, Inc.) at 37°C for 1 h. Then the cells were incubated with DAPI at 37°C for 5 min. Finally, the cells were visualized with an Olympus fluorescence microscope (Olympus).

Statistical analysis. Data are expressed as the means \pm SEM. Statistical analyses were performed by a one-way analysis of variance (ANOVA), followed by Tukey's test. A value of $P < 0.05$ was accepted as a statistically significant difference.

Results

VSP-17 treatment suppresses the migration and invasion of TNBC cells in an AMPK-dependent manner. To determine whether AMPK serves a key role in mediating the anti-migratory and anti-invasive effects of VSP-17, the expression levels of AMPK and p-AMPK in MDA-MB-231 and MDA-MB-453 cells were determined using western blotting. As shown in Fig. 1A, the treatment with 1, 3 or 10 μ M VSP-17 significantly upregulated the expression levels of p-AMPK in a dose-dependent manner, while VSP-17 had no significant effect on the expression levels of AMPK in the MDA-MB-231 and MDA-MB-453 cells. Moreover, the expression levels of p-AMPK in both cell lines were upregulated by 10 μ M VSP-17 treatment in a time-dependent manner (Fig. 1B).

Subsequently, to further investigate the role of AMPK in the inhibitory effect of VSP-17 on cell migration and invasion, MDA-MB-231 and MDA-MB-453 cells were treated with compound C or transfected with siAMPK. The data revealed that both compound C (Fig. 2A and B) and siAMPK (Fig. 2C and D) significantly reversed the inhibitory effect of VSP-17 on cell migration and invasion.

VSP-17 treatment inhibits the EMT process in TNBC cells in an AMPK-dependent manner. E-cadherin is a key marker of EMT (30). A previous study reported that VSP-17 treatment could upregulate the mRNA expression levels of E-cadherin in TNBC cells (28). To further determine the relationship between the activation of AMPK and the inhibition of the EMT process by VSP-17, MDA-MB-231 and MDA-MB-453 cells were treated with VSP-17 (10 μ M) in combination with compound C (1 μ M) or siAMPK transfection, respectively. As determined by western blot analysis (Fig. 3A and B), both compound C and siAMPK markedly abolished the VSP-17-induced downregulation of vimentin expression levels and upregulation of E-cadherin expression levels, and reverse transcription-quantitative PCR analysis (Fig. 3C and D) and immunofluorescent analysis (Fig. 3E and F) further confirmed this result, suggesting that AMPK may play an important role in the VSP-17-induced inhibition of EMT in MDA-MB-231 and MDA-MB-453 cells.

VSP-17 treatment suppresses the migration and invasion of TNBC cells in a PPAR γ -dependent manner. To identify the role of PPAR γ in the inhibitory effect of VSP-17 on migration and invasion, the PPAR γ antagonist, GW9662 and siPPAR γ were used. The findings demonstrated that both GW9662 and siPPAR γ reversed the inhibitory effect of VSP-17 on cell migration and invasion (Fig. 4A-D), indicating that PPAR γ

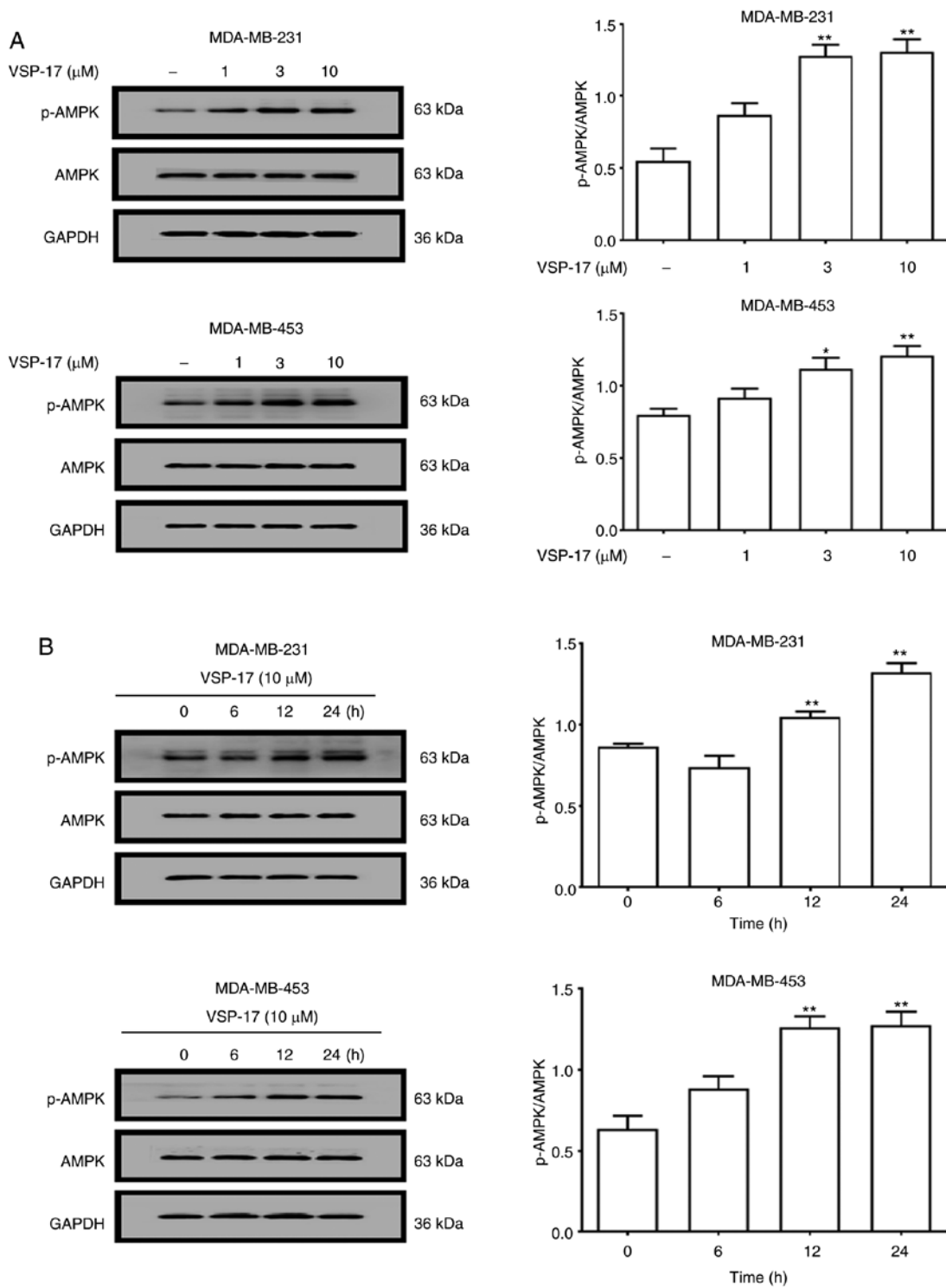


Figure 1. Effect of VSP-17 treatment on the activation of AMPK. (A) MDA-MB-231 and MDA-MB-453 cells were treated with 1, 3 or 10 μM VSP-17 for 24 h. The protein expression levels of p-AMPK and AMPK were analyzed using western blotting. (B) MDA-MB-231 and MDA-MB-453 cells were treated with 10 μM VSP-17 for 6, 12 or 24 h, and the expression levels of p-AMPK and AMPK were analyzed using western blotting. The data are expressed as the mean ± SEM of three independent experiments. *P<0.05, **P<0.01 vs. control. AMPK, AMP-activated protein kinase; p-, phosphorylated.

may also serve an important role in the VSP-17-induced inhibition of migration and invasion in TNBC cells.

VSP-17 treatment inhibits the EMT process in TNBC cells in a PPARγ-dependent manner. To further determine the relationship between the activation of PPARγ and the inhibition of the EMT process by VSP-17, MDA-MB-231 and MDA-MB-453

cells were co-treated with VSP-17 (10 μM) and GW9662 (1 μM) or treated with VSP-17 (10 μM) and transfected with siPPARγ. As determined by western blot analysis (Fig. 5A and B), both GW9662 and siPPARγ significantly reversed the VSP-17-induced downregulation of vimentin expression levels and upregulation of E-cadherin expression levels, and reverse transcription-quantitative PCR analysis (Fig. 5C and D) and

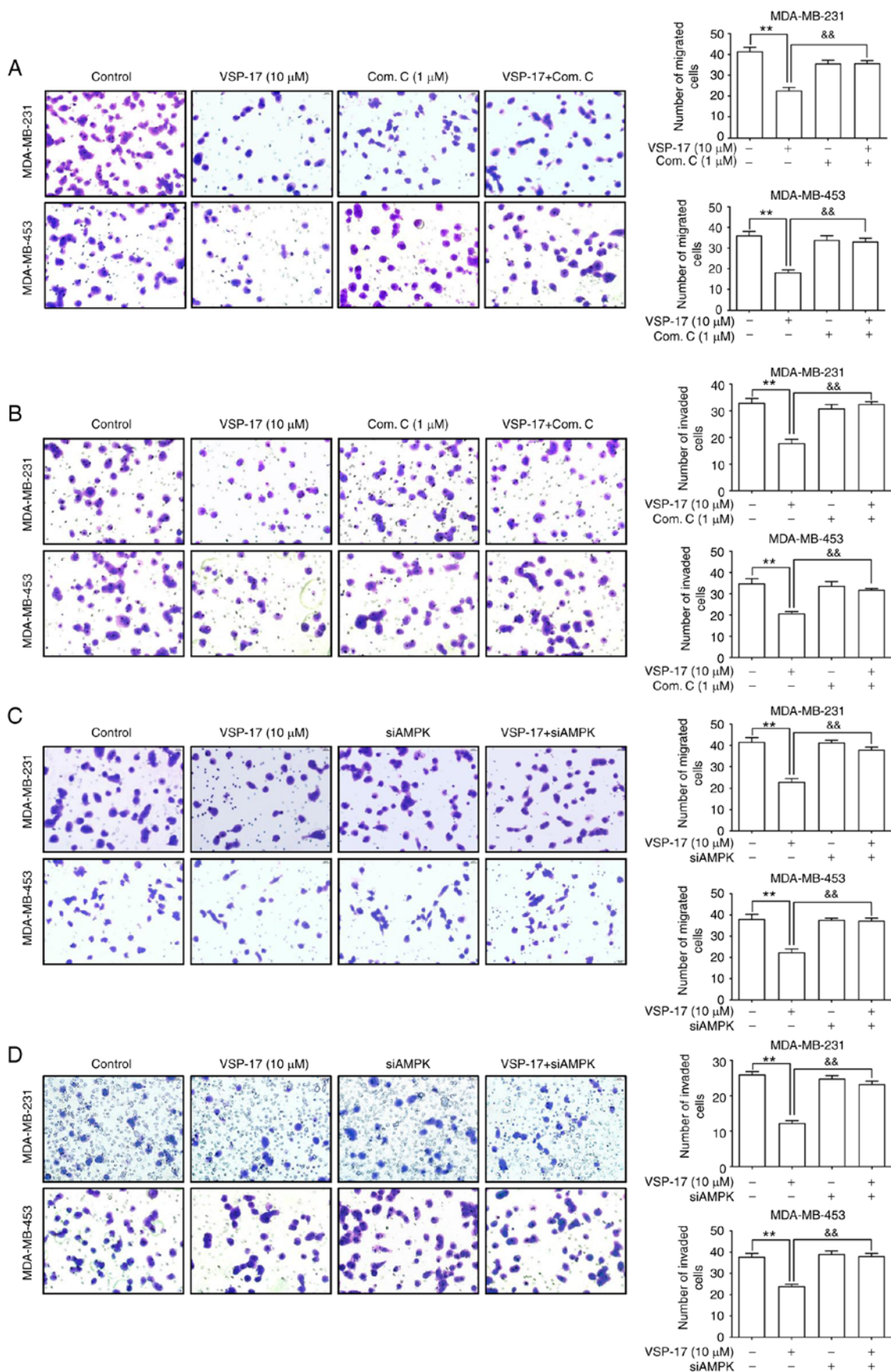


Figure 2. Effects of compound C and siAMPK on the VSP-17-induced inhibition of migration and invasion in MDA-MB-231 and MDA-MB-453 cells. (A and B) Cells were treated with 1 μM compound C, 10 μM VSP-17 or 10 μM VSP-17 + 1 μM compound C for 24 h. Transwell assays were used to evaluate the (A) migratory and (B) invasive abilities in MDA-MB-231 and MDA-MB-453 cells. (C and D) Cells were treated with 10 μM VSP-17 or transfected with siAMPK. Transwell assays were used to evaluate the (C) migratory and (D) invasive abilities in MDA-MB-231 and MDA-MB-453. The data are expressed as the mean ± SEM of three independent experiments. **P<0.01 vs. control; &&P<0.01 vs. VSP-17. Com. C, compound C; si, small interfering RNA; AMPK, AMP-activated protein kinase.

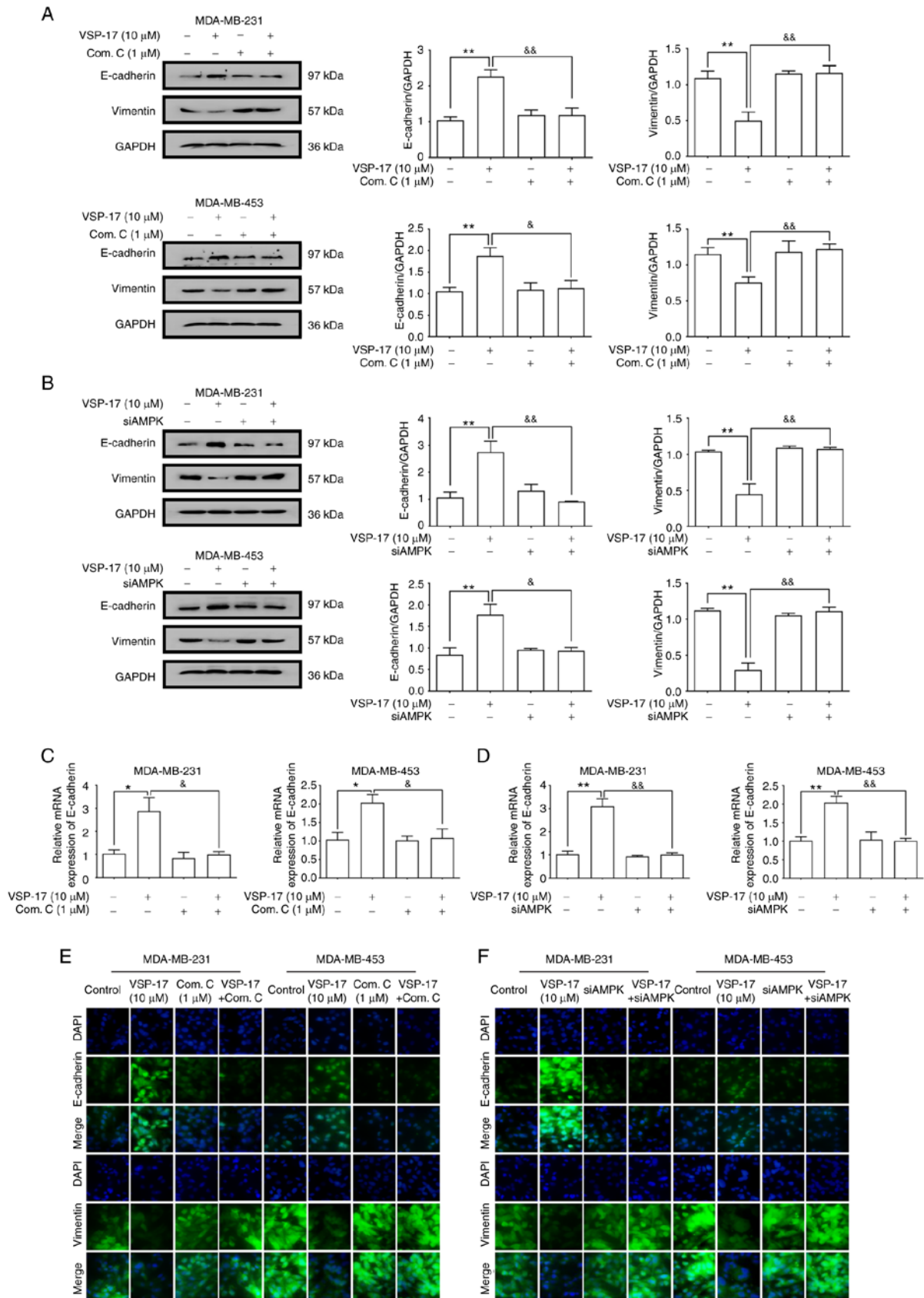


Figure 3. Effects of compound C and siAMPK on the VSP-17-induced inhibition of the epithelial-mesenchymal transition (EMT) process in MDA-MB-231 and MDA-MB-453 cells. (A and B) Expression levels of E-cadherin and vimentin in MDA-MB-231 and MDA-MB-453 cells treated with 10 μ M VSP-17 and/or 1 μ M compound C (A) and/or transfected with siAMPK (B) were analyzed using western blotting. (C and D) mRNA expression levels of E-cadherin in MDA-MB-231 and MDA-MB-453 cells treated with 10 μ M VSP-17 and/or 1 μ M compound C (C) and/or transfected with siAMPK (D) were analyzed using reverse transcription-quantitative PCR. (E and F) Cells were cultured with 10 μ M VSP-17 and/or 1 μ M compound C (E) and/or transfected with siAMPK (F) and the expression levels of E-cadherin and vimentin were measured by immunofluorescence. The data are expressed as the mean \pm SEM of three independent experiments. *P<0.05, **P<0.01 vs. control; &P<0.05, &&P<0.01 vs. VSP-17. Com. C, compound C; si, small interfering RNA; AMPK, AMP-activated protein kinase.

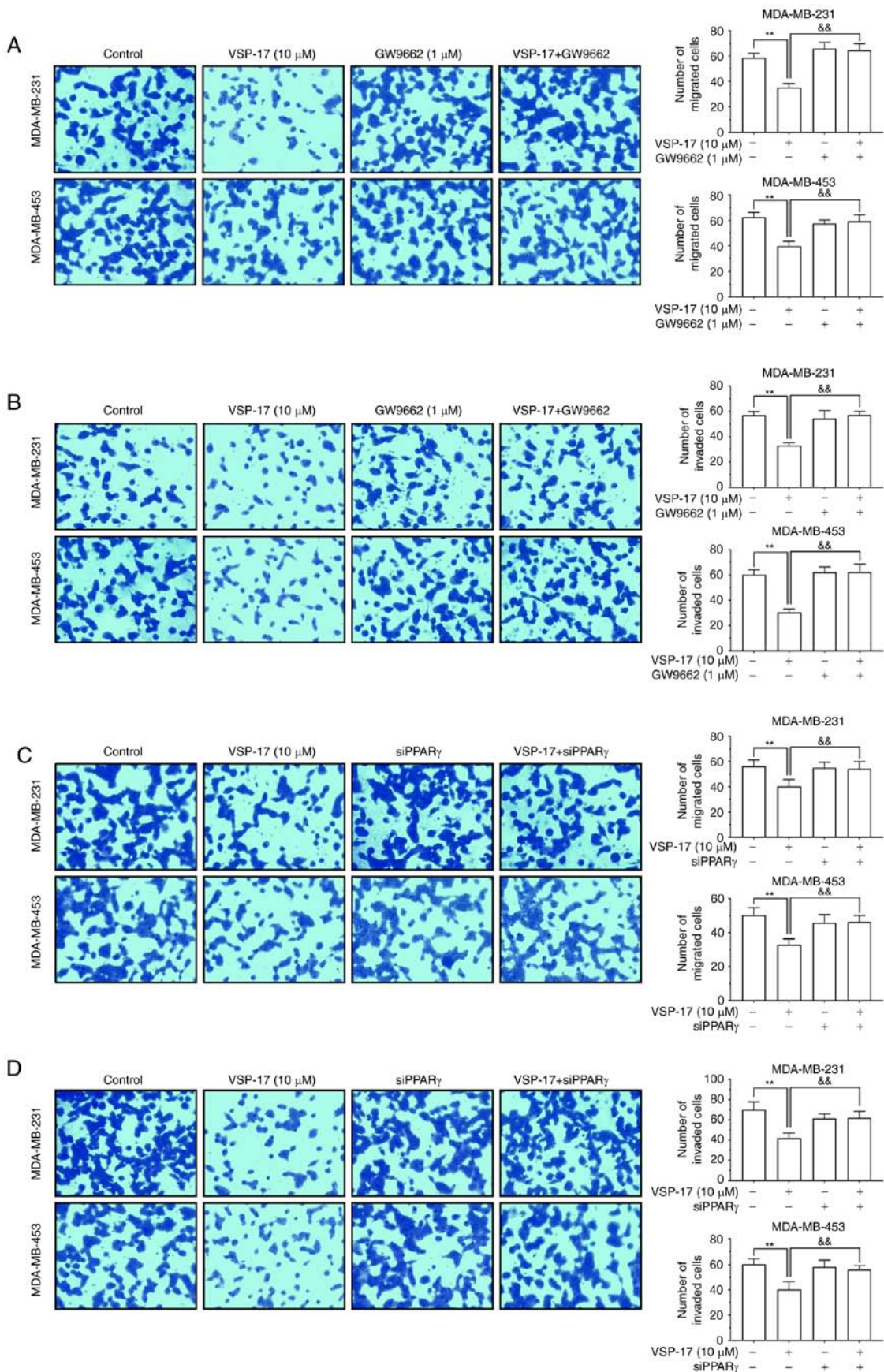


Figure 4. Effects of GW9662 and siPPAR γ on the VSP-17-induced inhibition of migration and invasion in MDA-MB-231 and MDA-MB-453 cells. (A and B) Cells were treated with 1 μ M GW9662, 10 μ M VSP-17 or 10 μ M VSP-17 + 1 μ M GW9662 for 24 h. Transwell assays were used to evaluate the (A) migratory and (B) invasive abilities of MDA-MB-231 and MDA-MB-453 cells. (C and D) Cells were treated with 10 μ M VSP-17 and/or transfected with siPPAR γ . Transwell assays were used to evaluate the (C) migratory and (D) invasive abilities of MDA-MB-231 and MDA-MB-453 cells. The data are expressed as the mean \pm SEM of three independent experiments. **P<0.01 vs. control; &&P<0.01 vs. VSP-17. si, small interfering RNA; PPAR γ , peroxisome proliferator-activated receptor γ .

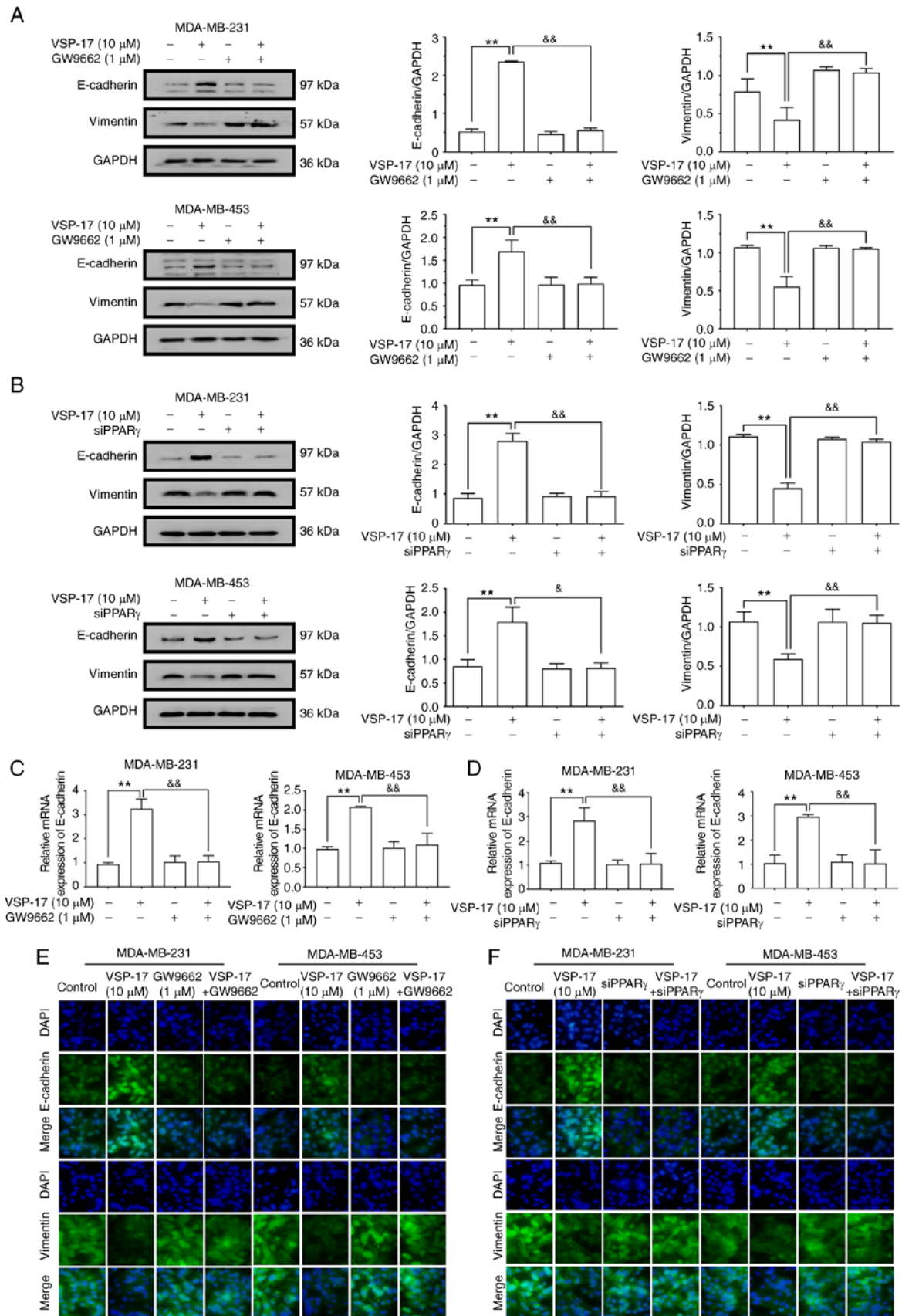


Figure 5. Effects of GW9662 and siPPAR γ on the VSP-17-induced inhibition of the epithelial-mesenchymal transition (EMT) process in MDA-MB-231 and MDA-MB-453 cells. (A and B) Expression levels of E-cadherin and vimentin in MDA-MB-231 and MDA-MB-453 cells treated with 10 μ M VSP-17 and/or 1 μ M GW9662 (A) and/or transfected with siPPAR γ (B) were analyzed using western blotting. (C and D) mRNA expression levels of E-cadherin in MDA-MB-231 and MDA-MB-453 treated with 10 μ M VSP-17 and/or 1 μ M GW9662 (C) and/or transfected with siPPAR γ (D) were analyzed using reverse transcription-quantitative PCR. (E and F) Cells were cultured with 10 μ M VSP-17 and/or 1 μ M GW9662 (E) and/or transfected with siPPAR γ (F) and the expression levels of E-cadherin and vimentin were measured by immunofluorescence. The data are expressed as the mean \pm SEM of three independent experiments. ** P <0.01 vs. control; * P <0.05, && P <0.01 vs. VSP-17. si, small interfering RNA; PPAR γ , peroxisome proliferator-activated receptor γ .

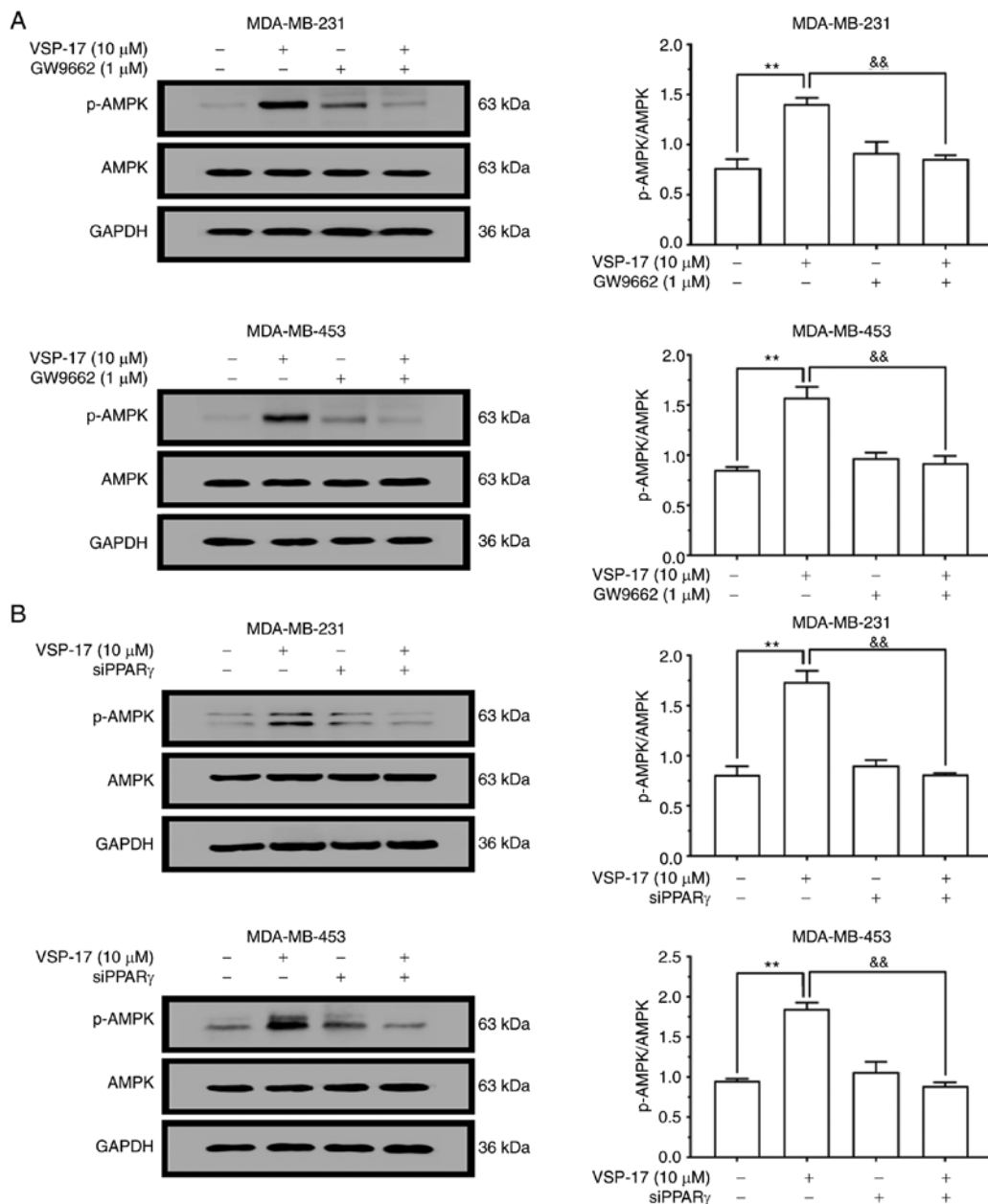


Figure 6. Effects of GW9662 and siPPAR γ on the VSP-17-induced activation of the AMPK signaling pathway in MDA-MB-231 and MDA-MB-453 cells. (A) Cells were treated with 1 μ M GW9662, 10 μ M VSP-17 or 10 μ M VSP-17 + 1 μ M GW9662 for 24 h. Western blotting was used to analyze the protein expression levels of AMPK and p-AMPK. (B) Cells transfected with siPPAR γ were treated with 10 μ M VSP-17 for 24 h. The protein expression levels of AMPK and p-AMPK were analyzed using western blotting. The data are expressed as the mean \pm SEM of three independent experiments. **P<0.01 vs. control; &&P<0.01 vs. VSP-17. si, small interfering RNA; PPAR γ , peroxisome proliferator-activated receptor γ ; AMPK, AMP-activated protein kinase; p-, phosphorylated.

immunofluorescent analysis (Fig. 5E and F) further confirmed this result, suggesting that PPAR γ may serve an important role in the VSP-17-induced inhibition of EMT in TNBC cells.

VSP-17 treatment activates AMPK in a PPAR γ -dependent manner. To determine whether the AMPK signaling pathway is involved in the effects of VSP-17, the effect of VSP-17 treatment on the activation of AMPK in MDA-MB-231 and MDA-MB-453 cells was investigated. As shown in Fig. 6A and B, the expression levels of p-AMPK in MDA-MB-231 and MDA-MB-453 cells were markedly upregulated following the treatment with 10 μ M VSP-17. In addition, both GW9662 treatment (Fig. 6A) and siPPAR γ transfection (Fig. 6B) reversed the

VSP-17-induced upregulation in p-AMPK expression levels, indicating that the VSP-17-induced activation of the AMPK signaling pathway may be mediated by PPAR γ .

Discussion

Emerging evidence has revealed that triple-negative breast cancer (TNBC) metastasis involves the migration and invasion of TNBC cells (28,31), and TNBC cells with increased migratory and invasive abilities are closely associated with tumor metastasis (32). Therefore, the inhibition of metastasis may represent a potential strategy for treating TNBC (33). Previous studies have demonstrated that PPAR γ

agonists, such as rosiglitazone, could inhibit the migration and invasion of breast cancer cells (34-37). However, it is worth noting that the long-term use of these drugs has been found to promote numerous adverse reactions, such as hepatotoxicity, weight gain and heart disease (38-40). Therefore, the development of a novel PPAR γ agonist with low toxicity and high selectivity may represent a potential strategy to inhibit the metastasis of TNBC. In previous studies, the new PPAR γ agonist, VSP-17, synthesized by our team, was found to suppress the metastasis of TNBC (28). However, the mechanism of action remains unclear. Therefore, the present study sought to further clarify the mechanism of action of VSP-17 with the aim of progressing VSP-17 into clinical trials.

Previous studies have reported that epithelial-mesenchymal transition (EMT) is a crucial initial step for cancer invasion (41,42) and it has been proven to promote the metastasis of tumors (43,44). The expression levels of EMT markers are significantly altered following the occurrence of EMT in tumor cells. In particular, the downregulation of E-cadherin expression levels and upregulation of vimentin expression levels are the main characteristics of EMT (45,46). A previous study revealed that puerarin could inhibit hepatocellular carcinoma metastasis by upregulating the expression levels of epithelial markers and downregulating the expression levels of mesenchymal markers (47). Similarly, rosmarinic acid, an abundant phenolic ester found in *Prunella vulgaris*, could inhibit the EMT process by upregulating the expression levels of E-cadherin and downregulating the expression levels of vimentin, and then inhibited the development of colorectal cancer (24). These findings indicate that the inhibition of the EMT process may be a potential strategy for suppressing TNBC metastasis. In the present study, VSP-17 treatment significantly upregulated the mRNA and protein expression levels of E-cadherin, and downregulated the protein expression levels of vimentin, which suggest that VSP-17 treatment may inhibit the EMT process in TNBC cells.

Peroxisome proliferator-activated receptor γ (PPAR γ) is a ligand-activated nuclear receptor, which belongs to the nuclear receptor gene superfamily (25). After ligand binding, PPAR γ forms a heterodimer with retinoid X receptors, which then binds to the promoter of target genes, including NF- κ B, nuclear factor erythroid 2-related factor 2 (Nrf2) and AMPK, to regulate the expression levels (48-50). In a previous study, the PPAR γ agonist pioglitazone was found to prevent hypoxia/reoxygenation (H/R) damage through enhancing autophagy via the AMPK/mTOR signaling pathway (51). In addition, ciglitazone-mediated PPAR γ activation led to the downregulation of oxidized low-density lipoprotein receptor 1 (LOX-1) expression levels and the activation of the AMPK signaling pathway (52). These previous studies suggest that PPAR γ agonists may exert their biological roles by activating AMPK. An increasing number of studies have also reported that the activation of PPAR γ could reverse the EMT process by upregulating the expression levels of E-cadherin and downregulating the expression levels of vimentin (53,54), which suggests that the activation of PPAR γ may also be considered as a potential strategy for inhibiting the EMT process. In the present study, GW9662 treatment or the transfection with

siPPAR γ reversed the effect of VSP-17 treatment on the inhibition of metastasis, and reversed the upregulation of E-cadherin expression levels and downregulation of vimentin expression levels in TNBC cells. In addition, the VSP-17-induced upregulation of p-AMPK expression levels was blocked by GW9662 treatment or siPPAR γ transfection. These findings suggest that VSP-17 treatment may suppress the EMT process and induce the AMPK signaling pathway in a PPAR γ -dependent manner.

Adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK), a major mediator in cellular energy and metabolic control, has been proven to be closely associated with the EMT process (55,56). Experimental studies have found that AMPK activators could inhibit the EMT transition in tumors through suppressing the TGF- β -induced phosphorylation of SMAD2/3 (57). Increasing evidence has also revealed that the AMPK activator, metformin, could reverse the EMT process in breast cancer cells by activating the AMPK signaling pathway (58). It was also previously reported that the activation of AMPK inhibited the metastasis of melanoma cells (59). The results of the present study demonstrated that VSP-17 treatment could inhibit the EMT process in an AMPK-dependent manner. It was worth noting that multiple targets, such as mTOR, cyclooxygenase-2 (COX-2) and acetyl-CoA carboxylase (ACC), are also known to be regulated by AMPK activation (60). Based on these findings, it was hypothesized that VSP-17 may regulate proteins in the downstream signaling pathway of AMPK, such as mTOR, COX-2 and ACC, thereby leading to the regulation of the expression levels of EMT-related markers. However, further studies are required to identify the exact mechanism of action.

In conclusion, the findings of the present study suggest that VSP-17 may inhibit the migration and invasion of TNBC cells by suppressing the EMT process via the PPAR γ /AMPK signaling pathway.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

XX and XD conceived and designed the experiments. ML and YY performed the experiments. CW and XZ analyzed

the data. HS was involved in project management and supervised the study. YW contributed to revising the manuscript for intellectual content and language editing. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors have no financial competing interests.

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