Shikonin inhibits migration and invasion of triple-negative breast cancer cells by suppressing epithelial-mesenchymal transition via miR-17-5p/PTEN/Akt pathway

CURRENT STATUS: POSTED

Chang Bao
College of Medicine, Zhejiang University

Tao Liu
Hospital of Traditional Chinese Medicine of Pingxiang city

Lingbo Qian
Hangzhou Medical College

Chi Xiao
Hangzhou Medical College

Xinru Zhou
Hangzhou Medical College

Heng Ai
Hangzhou Medical College

Jue Wang
Hangzhou Medical College

Weimin Fan
College of Medicine, Zhejiang University

Jie Pan
Hangzhou Medical College

panjiezju@zju.edu.cn Corresponding Author
ORCiD: https://orcid.org/0000-0001-9521-8536
SUBJECT AREAS

Translational Medicine    Cancer Biology

KEYWORDS

Shikonin, Triple-negative breast cancer, Epithelial-to-mesenchymal transition, miR-17-5p, PTEN
Abstract

Background: Triple-negative breast cancer (TNBC) is a great threat to global women’s health due to its high metastatic potential. Epithelial-to-mesenchymal transition (EMT) is considered as a key event in the process of metastasis. So the pharmacological targeting of EMT might be a promising strategy in improving the therapeutic efficacy of TNBC. Here, we investigated the effect of shikonin exerting on EMT and consequently the metastasis of TNBC cells and its underlying mechanism.

Methods: The invasive and migratory capacities of MDA-MB-231 cells were tested using transwell invasion and wound healing assay. MiR-17-5p expression was examined by qRT-PCR. MiR-17-5p targeted genes were predicted with different bioinformatic algorithms from four databases (TargetScan, miRanda, PITA and picTar) and further screened by KEGG pathway enrichment analysis. The differential expressions of predicted genes and their correlations with miR-17-5p were identified in breast cancer patients based on The Cancer Genome Atlas (TCGA) database. The interaction between PTEN and miR-17-5p was analyzed by luciferase reporter assay. The overexpression vector and small interfering RNA were constructed to investigate the role PTEN played in metastasis and EMT regulation. The expressions of EMT markers, Akt and p-Akt were evaluated by western blot.

Results: Shikonin inhibited the migration and invasion of MDA-MB-231 cells by suppressing EMT. Shikonin suppressed the expression of miR-17-5p, which was upregulated in breast cancer and promoted cancer cell migration, invasion and EMT. The 3’-untranslated region of PTEN was found to be direct binding target of miR-17-5p. PTEN expression increased or decreased in breast cancer cells transfected with miR-17-5p inhibitors or mimics respectively. PTEN functioned as a suppressor both in the metastasis and EMT of TNBC cells. Overexpression or knockdown of PTEN reduced or increased the Akt and p-Akt expression respectively.

Conclusions: Shikonin inhibits migration and invasion of TNBC cells by suppressing EMT via miR-17-5p/PTEN/Akt pathway. This suggests shikonin as a promising therapeutic agent to counteract metastasis in the TNBC patients.

Background

Breast cancer (BC) is the most common female malignant tumor and the leading cause of cancer
related mortality among women worldwide[1]. In spite of the diverse therapeutic regimens available for BC, the incidence of treatment failure and cancer recurrence remains high. Approximately up to 90% of death of patients with BC is caused by invasion and metastasis[2]. Particularly, the morbidity of triple-negative breast cancer (TNBC) is higher than that of other BC subtypes due to its high metastatic potential[3, 4]. Thus, TNBC is a great threat to global women’s health. It is imperative to elucidate the mechanisms underlying TNBC metastasis.

Metastasis is a multistep process, in which epithelial-to-mesenchymal transition (EMT) is considered as a key event. EMT is defined as the loss of epithelial phenotypes and the gain of mesenchymal characteristics[5]. These changes endow cancer cells with higher metastatic capacity and contribute to abnormal cellular growth, differentiation and drug resistance[6]. Some signaling pathways are previously reported to be involved in EMT, such as transforming growth factor-β (TGF-β), cadherin, Notch, Wnt/β-catenin, and GATA[2, 7, 8]. In breast cancer, forthcoming evidences suggest that EMT facilitates the metastatic dissemination of cancer cells in vitro, in vivo and in the clinical setting[9, 10]. So the pharmacological targeting of EMT might be a promising strategy in improving the therapeutic efficacy of TNBC.

To this end, many researchers have aimed to restrain EMT of cancer cells by certain agents with low toxicity and high efficiency. Shikonin(SHK), a naturally occurring compound, is extracted from the roots of Purple Cromwell, a kind of traditional Chinese herb used for a long time[11]. SHK has been found to exert anti-cancer effects by inducing apoptosis and inhibiting proliferation, metastasis and drug resistance of cancer cells in various malignancies including breast cancer[12-14]. Of particular interest, it is reported that the EMT process might be a target of SHK in reducing metastasis in some cancers, such as cervical and lung cancer[15, 16]. However, the effect of SHK on TNBC cells and its underlying mechanism is not fully understood. Additionally, it is known that the development of EMT is regulated by some cancer-relevant microRNAs (miRNAs), such as miR-9, miR-181a, miR-221, miR-155, miR-10b[17, 18]. However, to the best of our knowledge, few reports focus on the miRNA involvement in the EMT regulation of SHK.

In the current study, we hypothesized that SHK could reduce migration and invasion of human TNBC
cells via the suppression of EMT by inhibiting miRNA expression and provided experimental validation for it. Herein, it is confirmed that SHK effectively inhibits the migration and invasion of human TNBC cell line MDA-MB-231 by suppressing EMT. In examining the mechanism whereby SHK functions in BC cell metastasis, a novel miR-17-5p/PTEN/Akt pathway was identified in the regulation of EMT.

Methods

Cell culture and reagents

MDA-MB-231 cell line purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) was maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin/stereptomycin antibiotics. Experiments were initiated when the cells exhibited logarithmic growth. Shikonin was purchased from Selleck Chemicals (Houston, TX, USA) and stored at −20°C. It was dissolved in DMSO to a 50 mM stock. The storage solution was diluted to 2.5 μM or 5 μM just before each experiment. And the cells were treated with 2.5 μM or 5 μM SHK for 24 h.

Transwell invasion assay

The invasion of MDA-MB-231 cells was evaluated by the matrigel-coated transwell chamber (Corning, MA, USA). Briefly, cells were seeded in the upper chambers at a density of 2 × 10^5 cells/well and cultured in a serum-free medium with or without series concentrations of SHK for 24 h. The bottom chambers were filled with complete DMEM medium. After incubation for 24 h, the invaded cells on the lower surfaces of the membranes were fixed with 100% methanol for 10 min at room temperature and then stained with 0.1% crystal violet for 15 min. The invaded cells were counted using a light microscope (Olympus, Japan).

Wound healing assay

MDA-MB-231 cells were seeded into the six-well plates with a density of 2 × 10^4 cells per well in complete DMEM medium. When the cells grew to 100% confluence, the cell monolayer was scrapped with a sterile micropipette tip to create wounds. The wells were washed with PBS and the remaining cells continued to be cultured in mediums with or without series concentrations of SHK for 24 h. The scratch was observed and captured at the same location with a light microscope at 0 h and 24 h after
scrap. The gap distance of each wound was measured.

**Plasmids**

For gene knockdown experiments, PTEN siRNA (sense: CGCCAAAUUUAUUGCAGATT; anti-sense: UCUGCAAUUUAAUUGGCGTT) and negative control siRNA were obtained from GenePharma (Shanghai, China). For gene overexpression vector construction, the open reading frames and downstream 3′-UTR of PTEN were cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) between the HindIII and EcoRI sites and driven by the cytomegalovirus promoter. For the luciferase reporter assay, the 3′-UTR fragment of PTEN was amplified and cloned into the Xhol and NotI sites downstream of the SV40 promoter-driven Renilla luciferase cassette in the psiCHECK-2 plasmid (Promega, Madison, WI, USA). A Fast Mutagenesis kit (Vazyme Biotech, Nanjing, China) was used to mutate the miR-17 binding sites in the PTEN 3′-UTR vectors according to the manufacturer’s instructions.

**Transient transfection of miRNA**

MDA-MB-231 cells were seeded in 6-cm cell culture dishes at 1.2×10^5 cells/mL. The cells were transfected with a 50 nM miR-17-5p mimic, miR-17-5p inhibitor or the corresponding scrambled NC (Ribobio, Guangzhou, China) with lipofectamine 2000 (Invitrogen) per the manufacturer’s instructions. After 24 h, the medium was replaced and the cells were prepared for subsequent experiments.

**Western blot assay**

After pretreated with SHK or transfected with miRNA, siRNA or expression vectors, MDA-MB-231 cells were harvested and lysed in RIPA buffer (Beyotime Institute of Biotechnology, China) to extract the total proteins. The protein content was detected by using the bicinchoninic acid (BCA) kit (Thermo Fisher Scientific, MA, USA). Equal amounts of denatured proteins (40 μg) were electrophoresed on 10% polyacrylamide gel and subsequently transferred onto polyvinylidene fluoride membranes (Roche, Switzerland). After being blocked in Tris-Buffered Saline containing 0.1% Tween-20 (TBST) for 1 h, the membranes were probed with specific antibodies against PTEN (32072, Abcam, Cambridge, UK), N-cadherin (4061, Cell Signaling Technology, MA, USA), Vimentin (3390, Cell Signaling Technology), E-cadherin (5296, Cell Signaling Technology), Akt (9272, Cell Signaling Technology), p-
Akt (4051, Cell Signaling Technology), GAPDH (A2228, Sigma-Aldrich) at 4°C overnight respectively. After washing with TBST for three times, the membranes were incubated with an optimal dilution of the appropriate secondary antibodies conjugated with horseradish peroxidase (HRP) for 2 h at the room temperature. At last, the immunoreactive bands were detected with enhanced chemiluminescence reagents (Thermo Scientific, Darmstadt, Germany) by ChemiDoc Touch Imaging System (Bio-Rad, CA, USA) according to the manufacturer’s instructions. Experiments were repeated independently at least three times.

**RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)**

Total RNA was extracted from BC cells with RNAiso Plus (TaKaRa, Kusatsu, Japan). The extracted RNA was reverse-transcribed into cDNA with a PrimeScript™ RT reagent Kit (#RR037A, TaKaRa). Random hexamer primers and specific primers (Ribobio) were used for RNA and miRNA reverse-transcriptase PCR (RT-PCR) respectively.

qRT-PCR was performed to examine the expression of PTEN and miR-17-5p. Hypoxanthine phosphoribosyltransferase 1 (HPRT1) and U6 were used as internal control for PTEN and miR-17-5p expression, respectively. The specific primer sequences were as following: PTEN: 5’-CATGTTGCAGCAATTCAGTACT-3’ (forward), 5’-CTTGTGAACACGAAGACGCA-3’ (reverse); HPRT1: 5’-TGACACTGGCAAAAACATGCA-3’ (forward), 5’-GGTCCTTTTCACCAGCAAGCT-3’ (reverse); U6: 5’-CTCGCTTCGGCAGCACATAT-3’ (forward), 5’-AACGCTTCACGAATTTGTGC-3’ (reverse). Mature miRNA expression was quantified by qRT-PCR with specific BulgeLoop miRNA qRT-PCR primers (Ribobio).

qRT-PCR was performed in a LightCycler 480II system (Roche Diagnostics, Basel, Switzerland) with a SYBR Premix EX Tag kit (#RR420A, TaKaRa). Relative expression of RNA and miRNA were calculated as $2^{-\Delta Ct}$ after normalization with the reference control.

**Luciferase reporter assay**

For reporter assay, MDA-MB-231 cells were transiently co-transfected with a 50 nM miR-17-5p mimics or negative control-miR and 50 ng of PTEN 3’-UTR wild-type or mutant psiCHECK-2 reporter vectors (Promega). Firefly luciferase activities were measured using the Dual Luciferase Reporter Assay System Kit (Promega) as described in the manufacturer’s protocol 48 hours after transfection.
Luminometry readings were obtained with a Varioskan Flash Spectral Scanning Multimode Reader (Thermo Scientific, Waltham, MA, USA). Firefly luciferase activity was normalized to constitutive Renilla luciferase. Each transfectant was assayed in triplicate.

**Bioinformatic analysis**

MiR-17-5p targeted genes were predicted with different bioinformatic algorithms from various databases, including TargetScan (http://www.targetscan.org/), miRanda (http://www.microrna.org/microrna/home.do), PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html) and picTar (https://pictar.mdc-berlin.de/). The visualization of KEGG pathway enrichment analysis for the common predicted genes used R package clusterProfiler[19] from the bioconductor project. Adjust P value < 0.05 was considered as statistically significant.

The miRNA and mRNA expression data of breast cancer (BRCA) samples measured by Illumina-Hiseq were retrieved from The Cancer Genome Atlas (TCGA) database (https://cancergenome.nih.gov/), including 1085 breast cancer samples and 291 normal samples. Data were sorted and normalized. MiRNA and mRNA differential expression and correlation analyses were conducted by the R package edgeR [20].

**Statistical analysis**

All data were presented as mean ± SEM from at least three independent experiments. Statistical analyses were performed with GraphPad Prism Ver. 5.01 (San Diego, CA, USA). Statistical significance was assessed with two-tailed Student’s t-test or one-way ANOVA analysis. Pearson’s correlation was used to evaluate the correlation between two variants. Statistical significance was set at $p < 0.05$ and displayed as *** for $p<0.001$; ** for $p<0.01$ and * for $p<0.05$.

**Results**

**Shikonin inhibits the migration, invasion and EMT of human TNBC cells.**

To investigate the effects of shikonin on cell migration and invasion, MDA-MB-231 cells were exposed to 2.5μM or 5μM shikonin for 24 h. The capacities of migration and invasion were detected by wound-healing assay and transwell invasion assay respectively. As shown in Figure 1A, the 24 h
wound-healing assay revealed that the migratory capacity was decreased significantly with the treatment of 2.5μM and 5μM shikonin compared with that of cells in the control(Figure 1A,B). Similarly, shikonin treatment suppressed invasion of breast cancer cells in a dose-dependent manner(Figure 1C,D). These data indicate that shikonin decreases the migratory and invasive capacity of breast cancer cells. As EMT was always considered as a pivotal event that occurs during cancer metastasis, the expression of EMT markers were further explored by western blot. The results showed that the protein expressions of mesenchymal markers N-cadherin and vimentin declined, while the epithelial marker E-cadherin increased dose-dependently with the treatment of shikonin(Figure 1E).

**Shikonin reduces the expression of miR-17-5p that promotes TNBC cell migration, invasion and EMT.**

To further elucidate the function of shikonin in inhibiting migration and invasion, we examined the expression of miR-17-5p in MDA-MB-231 cells pretreated with shikonin. When BC cells were treated with shikonin (2.5μM or 5μM) for 24 h, the level of miR-17-5p declined dose-dependently (Figure 2A), which confirmed the inhibitory regulation of shikonin on miR-17-5p expression.

To investigate the effect of miR-17-5p on cell migration and invasion. We transiently transfected MDA-MB-231 cells with miR-17-5p mimics, miR-17-5p inhibitors, or the corresponding negative control (NC), and assessed the efficiency of transfection by qRT-PCR. The results revealed that miR-17-5p mimic or inhibitor significantly increased or decreased miR-17-5p expression respectively compared with those in negative control groups 48 h after transfection in MDA-MB-231 cells (Figure 2B). Then, the invasive and migratory abilities of the transfected cells were tested. Compared with the control group, the number of cells traversing the matrigel were significantly increased in miR-17-5p mimic-transfected group, and the opposite results were observed in miR-17-5p inhibitor-transfected group (Figure 2C, D). The wound-healing ability was enhanced in cells transfected with miR-17-5p mimics, but was attenuated in those transfected with miR-17-5p inhibitors (Figure 2E, F). Next, the regulation of miR-17-5p on EMT markers was detected by western blot. Accordingly, N-cadherin and vimentin were upregulated or downregulated in MDA-MB-231 cells transfected by miR-17-5p mimics or inhibitors respectively, and E-cadherin exhibited an opposite expression pattern (Figure 2G). Thus,
these data demonstrate that miR-17-5p may promote the migration, invasion and EMT of breast
cancer cells, and shikonin reduces these processes by downregulating miR-17-5p.

**PTEN is screened to be the target of miR-17-5p using bioinformatic analyses**

Data from TCGA showed the level of miR-17-5p was significantly higher in BC group (n=1085) than
the normal group (n=291) (Figure 3A). Although miR-17-5p had been reported to contribute to
tumorigenesis and progression in various cancers, the underlying mechanism of its effects on breast
cancer cell migration and invasion remains to be elucidated. To this end, by analyzing the data from
four public databases (TargetScan, PITA, miRanda and picTar), we acquired 351 predicted target
genes of miR-17-5p in common (Figure S1A). In order to better understand the biological features and
significances of the 351 genes, the enrichment analysis of KEGG pathways was performed and
indicated that the genes were enriched in multiple cancer-related pathways (Figure S1B). Venn
diagram showed 7 genes were overlapped between the two relevant pathways - microRNAs in cancer
and pathways in cancer (Figure S1C). Then, the differential expressions of the 7 genes were analyzed
in BC and normal cohorts from TCGA. It demonstrated that PTEN, CRK and CDKN1A were
downregulated significantly in BC compared with the normal group (Figure S1D). According to the
characteristics of microRNA in regulating its target genes, PTEN, CRK and CDKN1A were chosen as the
potential target genes, as their differential expression patterns were conformed to the effect of miR-
17-5p. Next, the correlations of miR-17-5p with the potential target genes were investigated in the BC
samples from TCGA’s cohort (Figure 3B, S1E, S1F). As shown in Figure 3B, statistical analysis revealed
a significant inverse correlation between the expression levels of endogenous miR-17-5p and PTEN
mRNA expression, which pointed toward the probable involvement of miR-17-5p in PTEN regulation.

**MiR-17-5p downregulates PTEN by directly binding to its 3’-UTR.**

As a result, we focused on PTEN, which was a well-known tumor suppressor. As presented in Figure 3C
and 3D, miR-17-5p mimic transfection resulted in a significant decrease in the mRNA and protein
levels of PTEN, whereas the inhibitor-transfected group showed increased PTEN expression. These
results further suggested the regulation of miR-17-5p on PTEN expression. Though miR-17-5p was
revealed to have a putative binding site in the 3’-UTR of PTEN, further luciferase reporter assays were
required to validate whether PTEN was a functional target of miR-17-5p in MDA-MB-231 cells. Wild-type 3’-UTR of PTEN and mutated sequence (mut PTEN 3’-UTR) were individually cloned into reporter vectors downstream of the Renilla luciferase gene (Figure 3E). MDA-MB-231 cells were transfected with a reporter vector only, or co-transfected with the reporter vector and either 50nM miR-17-5p or NC. The luciferase activity of the reporters with wild-type PTEN 3’-UTR was significantly suppressed by miR-17-5p mimic transfection, while the activity of the reporters with mutant PTEN 3’-UTR did not change significantly (Figure 3F). These results demonstrate that miR-17-5p downregulates PTEN by directly binding to its 3’-UTR.

**Downregulation of PTEN increases the migration, invasion and EMT of breast cancer cells.**

In order to evaluate the role of PTEN in breast cancer cell migration and invasion, we transfected MDA-MB-231 cells with PTEN siRNA (si-PTEN) or overexpression vectors (pc-DNA3.1-PTEN) to knock down or overexpress PTEN. In MDA-MB-231 cells, the mRNA and protein expression of PTEN was significantly lower in the PTEN siRNA transfected group and higher in PTEN overexpression vectors transfected group, compared with their corresponding control groups, which confirmed the efficiency of knockdown or overexpression(Figure 4A,4B). The wound healing and transwell assays showed the migration and invasion abilities were upregulated in cells transfected with si-PTEN and on the other hand, both were downregulated in cells transfected with pc-DNA3.1-PTEN (Figure 4E, 4F). Moreover, the expressions of N-cadherin and vimentin were inhibited/increased in PTEN-overexpressing/knockdown group respectively, but contrary to that, an opposite pattern was observed in E-cadherin expression (Figure 5A). Altogether, these results show that PTEN may plays an important role in suppressing MDA-MB-231 cell migration and invasion via inhibiting EMT.

Furthermore, we investigated whether shikonin could regulate the expression of PTEN directly. When MDA-MB-231 cells were treated with shikonin at different concentrations for 24 h, shikonin was found to upregulate PTEN expression in both mRNA and protein levels dose-dependently (Figure 5B, 5C).

**Akt is involved in the inhibition of shikonin on cancer cell migration, invasion and EMT via miR-17-5p/PTEN pathway**

The PI3K-Akt pathway is crucial in the regulation of cancer development and the activation of Akt
subsequently leads to a number of potential downstream effects including cell metastasis. As PTEN acted as a major brake of this pathway, we hypothesized that Akt might function in the process of migration and invasion inhibition by shikonin. To test this hypothesis, we investigated the effects of shikonin, miR-17-5p and PTEN on Akt expression in MDA-MB-231 cells. Western blot showed Akt and phospho-Akt (Ser473) expression levels were downregulated in parallel with the increase in the concentration of shikonin (Figure 5D). Compared with the negative control groups, levels of Akt and p-Akt were significantly higher in the miR-17-5p mimic transfected group and lower in the miR-17-5p inhibitor transfected group (Figure 5E). Overexpression of PTEN reduced the expression of Akt and p-Akt. On the other hand, knockdown of PTEN increased Akt and p-Akt expression (Figure 5F). These observations suggest that Akt might play a role in inhibiting the migration and invasion of MDA-MB-231 cells by shikonin to some extent.

Discussion
Breast cancer is a heterogeneous disease with distinct biological properties, specific morphological patterns and diverse clinical behaviors among different subtypes. TNBC, accounting for 10-20% of breast cancers, is the most aggressive subtype with early relapse, poor overall survival and, most importantly, frequent distant metastasis[21]. EMT is critical in the initiation of metastasis in TNBC because it enables BC cells to detach from the primary tumor and eventually to colonize distant organs[22]. Once the EMT process was inhibited, the metastasis of TNBC was suppressed or even reversed at the same time[23, 24]. So it seems to be feasible to inhibit cancer cell metastasis by targeting EMT.

Shikonin is a natural bioactive compound with multiple health-promoting effects, mainly wound-healing, anti-inflammation, anti-infection and anti-cancer[25, 26]. Administration of shikonin and its derivatives at a high dose is verified to be safe and well-tolerated in animal models[27, 28]. In breast cancer, there are several researches confirm that SHK could induce apoptosis and cell cycle arrest, inhibit drug resistances and enhance cytotoxicity of chemotherapeutic agents[29, 30]. As shown in the present study, shikonin effectively inhibited the migration and invasion of TNBC cell line, indicating that the use of SHK might provide an effective way to reduce metastasis of TNBC. In
consideration of the important role EMT played in cancer metastasis, we supposed whether the anti-metastasis effect of SHK was mediated by regulating the process of EMT. Therefore, in this study, we confirmed that BC cells acquired the epithelial phenotypes and lost the mesenchymal feature with the treatment of SHK, which was in accordance with the previous studies in cervical and lung cancers[15, 16].

Furthermore, the underlying mechanism whereby SHK inhibited EMT of BC cells was explored. MiRNAs are a group of endogenous RNA molecules participating in a variety of biological processes, such as cell cycle control, proliferation, apoptosis and migration[31]. Thus far, miR-17-5p has been reported to dysregulate in various malignancies and promote invasion and migration of cancer cells[32, 33]. Data from TCGA showed that miR-17-5p was higher in breast cancer tissues than in normal breast tissues. Herein, SHK was proven to downregulate the expression of miR-17-5p effectively with a dose-dependent manner in MDA-MB-231, indicating its inhibition of metastasis in BC might be mediated by miR-17-5p. We discovered that high level of miR-17-5p contributed to the EMT process and the invasion and migration of BC cells.

MiRNAs regulate the expression of target genes by binding sequence-specifically to the 3′-untranslated region (UTR) of their mRNAs. By bioinformatic analyses, PTEN was predicted to be the target gene of miR-17-5p in modulating BC cell metastasis. And the expression of PTEN was correlated inversely with that of miR-17-5p in 1077 BC cancer samples from TCGA. The expression of PTEN was upregulated in response to SHK treatment, an effect that associated inversely with miR-17-5p expression. Then, miR-17-5p was verified to decrease PTEN expression via binding to its 3′-UTR with the dual luciferase reporter assay, which confirmed the direct targeting relationship between miR-17-5p and PTEN.

PTEN is a well-known tumor suppressor gene encoding a multifunctional protein that exerts various biological activities including metastasis, which is always down modulated in human cancers[34]. Accordingly, it is found that PTEN expression is lower in BC cancer samples than in normal breast tissues. SHK could elevate the mRNA and protein level of PTEN dose-dependently in BC cells. The capacities of migration and invasion showed inverse correlations with the expression of PTEN in TNBC.
cells. PTEN downregulation or loss associates with the acquisition of EMT traits[35]. We discovered that the activity of EMT was increased or decreased in PTEN knocked-down or over-expressed BC cells respectively. Akt is key member of the PI3K/AKT axis, which is a major signalling pathway antagonized by PTEN [36]. Akt activation via phosphorylation (P-Akt) controls metastasis in a diversity of tumors[37, 38]. The downregulation of PTEN in BC cells was in parallel with the upregulation of Akt and p-Akt (Ser473). Moreover, the levels of Akt and p-Akt (Ser473) were also decreased with the SHK treatments and showed a positive correlation with miR-17-5p. These findings confirmed the involvement of Akt in the process of SHK regulating the invasion and migration via miR-17-5p/PTEN axis.

Conclusions
In conclusion, SHK suppressed epithelial-mesenchymal transition via miR-17-5p/PTEN/Akt pathway, thus consequently inhibiting migration and invasion of TNBC cells. Therefore, SHK could be a potential therapeutic agent to counteract metastasis in the TNBC patients.

Abbreviations
BC: Breast cancer; TNBC: Triple-negative breast cancer; EMT: Epithelial-to-mesenchymal transition; SHK: Shikonin; TCGA: The Cancer Genome Atlas; DMEM: Dulbecco's modified Eagle's medium; NC: Negative control; 3'-UTR: 3'-untranslated region.

Declarations

Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests
The authors declare that they have no competing interests.

Funding
This work was supported by the Department of Education of Zhejiang Province (Y201840344, JG20190549) and the Department of Science and Technology of Zhejiang Province (LQ20H150001)

**Authors' contributions**

CB, WF and JP designed the experiments. CB, JP, CX and XZ performed the experiments. TL and HA conducted the data analysis and interpreted the results. JP and CB drafted the manuscript. LQ and JW revised the manuscript.

**Acknowledgements**

Not applicable

**Authors' information**

1 School of Basic Medical Sciences & Forensic Medicine, Hangzhou Medical College, No.481 Binwen Road, Hangzhou 310053, People's Republic of China. 2 Program of Innovative Cancer Therapeutics, Division of Hepatobiliary and Pancreatic Surgery, Department of Surgery, First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310003, People’s Republic of China. 3 Key Laboratory of Organ Transplantation, Hangzhou 310003, People's Republic of China. 4 Key Laboratory of Combined Multi-organ Transplantation, Ministry of Public Health, Hangzhou310003, People’s Republic of China. 5 Department of Respiratory Medicine, Hospital of Traditional Chinese Medicine of Pingxiang city, No.10 Pingchuxi Road, Pingxiang 337000, People’s Republic of China. 6 Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, SC 29425, USA

**References**

1. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer Statistics, 2017*. CA Cancer J Clin, 2017. **67**(1): p. 7-30.

2. Das, V., S. Bhattacharya, C. Chikkaputtaiah, S. Hazra, and M. Pal, *The basics of epithelial-mesenchymal transition (EMT): A study from a structure, dynamics, and functional perspective*. J. Cell. Physiol., 2019.

3. McCann, K.E., S.A. Hurvitz, and N. McAndrew, *Advances in Targeted Therapies for Triple-Negative Breast Cancer*. Drugs, 2019. **79**(11): p. 1217-1230.
4. da Silva, J.L., N.C. Cardoso Nunes, P. Izetti, G.G. de Mesquita, and A.C. de Melo, *Triple negative breast cancer: A thorough review of biomarkers*. Crit. Rev. Oncol. Hematol., 2020. **145**: p. 102855.

5. Singh, S. and R. Chakrabarti, *Consequences of EMT-Driven Changes in the Immune Microenvironment of Breast Cancer and Therapeutic Response of Cancer Cells*. J Clin Med, 2019. **8**(5).

6. Khaled, N. and Y. Bidet, *New Insights into the Implication of Epigenetic Alterations in the EMT of Triple Negative Breast Cancer*. Cancers (Basel), 2019. **11**(4).

7. Pattarayan, D., A. Sivanantham, V. Krishnaswami, L. Loganathan, R. Palanichamy, S. Natesan, K. Muthusamy, and S. Rajasekaran, *Tannic acid attenuates TGF-β1-induced epithelial-to-mesenchymal transition by effectively intervening TGF-β signaling in lung epithelial cells*. J. Cell. Physiol., 2018. **233**(3): p. 2513-2525.

8. Kar, R., N.K. Jha, S.K. Jha, A. Sharma, S. Dholpuria, N. Asthana, K. Chaurasiya, V.K. Singh, S. Burgee, and P. Nand, *A "NOTCH" Deeper into the Epithelial-To-Mesenchymal Transition (EMT) Program in Breast Cancer*. Genes (Basel), 2019. **10**(12).

9. Bonnomet, A., L. Syne, A. Brysse, E. Feyereisen, E.W. Thompson, A. Noel, J.M. Foidart, P. Birembaut, M. Polette, and C. Gilles, *A dynamic in vivo model of epithelial-to-mesenchymal transitions in circulating tumor cells and metastases of breast cancer*. Oncogene, 2012. **31**(33): p. 3741-53.

10. Demirkan, B., *The Roles of Epithelial-to-Mesenchymal Transition (EMT) and Mesenchymal-to-Epithelial Transition (MET) in Breast Cancer Bone Metastasis: Potential Targets for Prevention and Treatment*. J Clin Med, 2013. **2**(4): p. 264-82.

11. Guo, C., J. He, X. Song, L. Tan, M. Wang, P. Jiang, Y. Li, Z. Cao, and C. Peng, *Pharmacological properties and derivatives of shikonin-A review in recent years*. Pharmacol. Res., 2019. **149**: p. 104463.
12. Wang, F., X. Yao, Y. Zhang, and J. Tang, *Synthesis, biological function and evaluation of Shikonin in cancer therapy*. Fitoterapia, 2019. **134**: p. 329-339.

13. Yang, Y., W. Gao, S. Tao, Y. Wang, J. Niu, P. Zhao, C. Rao, and L. Yang, *ER-mediated anti-tumor effects of shikonin on breast cancer*. Eur. J. Pharmacol., 2019. **863**: p. 172667.

14. Wei, Y., M. Li, S. Cui, D. Wang, C.Y. Zhang, K. Zen, and L. Li, *Shikonin Inhibits the Proliferation of Human Breast Cancer Cells by Reducing Tumor-Derived Exosomes*. Molecules, 2016. **21**(6).

15. Hsieh, Y.S., C.H. Liao, W.S. Chen, J.T. Pai, and M.S. Weng, *Shikonin Inhibited Migration and Invasion of Human Lung Cancer Cells via Suppression of c-Met-Mediated Epithelial-to-Mesenchymal Transition*. J. Cell. Biochem., 2017. **118**(12): p. 4639-4651.

16. Tang, Q., L. Liu, H. Zhang, J. Xiao, and S.S. Hann, *Regulations of miR-183-5p and Snail-Mediated Shikonin-Reduced Epithelial-Mesenchymal Transition in Cervical Cancer Cells*. Drug Des Devel Ther, 2020. **14**: p. 577-589.

17. Gravgaard, K.H., M.B. Lyng, A.V. Laenkholm, R. Sokilde, B.S. Nielsen, T. Litman, and H.J. Ditzel, *The miRNA-200 family and miRNA-9 exhibit differential expression in primary versus corresponding metastatic tissue in breast cancer*. Breast Cancer Res Treat, 2012. **134**(1): p. 207-17.

18. Piasecka, D., M. Braun, R. Kordek, R. Sadej, and H. Romanska, *MicroRNAs in regulation of triple-negative breast cancer progression*. J. Cancer Res. Clin. Oncol., 2018. **144**(8): p. 1401-1411.

19. Yu, G., L.G. Wang, Y. Han, and Q.Y. He, *clusterProfiler: an R package for comparing biological themes among gene clusters*. Omics, 2012. **16**(5): p. 284-7.

20. Robinson, M.D., D.J. McCarthy, and G.K. Smyth, *edgeR: a Bioconductor package for
differential expression analysis of digital gene expression data. Bioinformatics, 2010. 26(1): p. 139-40.

21. He, M.Y., C. Rancoule, A. Rehailia-Blanchard, S. Espenel, J.C. Trone, E. Bernichon, E. Guillaume, A. Vallard, and N. Magné, Radiotherapy in triple-negative breast cancer: Current situation and upcoming strategies. Crit. Rev. Oncol. Hematol., 2018. 131: p. 96-101.

22. Nieto, M.A., R.Y. Huang, R.A. Jackson, and J.P. Thiery, EMT: 2016. Cell, 2016. 166(1): p. 21-45.

23. Lv, Z.D., B. Kong, X.P. Liu, L.Y. Jin, Q. Dong, F.N. Li, and H.B. Wang, miR-655 suppresses epithelial-to-mesenchymal transition by targeting Prrx1 in triple-negative breast cancer. J. Cell. Mol. Med., 2016. 20(5): p. 864-73.

24. Rhodes, L.V., C.R. Tate, H.C. Segar, H.E. Burks, T.B. Phamduy, V. Hoang, S. Elliott, D. Gilliam, F.N. Pounder, M. Anbalagan, D.B. Chrisey, B.G. Rowan, M.E. Burow, and B.M. Collins-Burow, Suppression of triple-negative breast cancer metastasis by pan-DAC inhibitor panobinostat via inhibition of ZEB family of EMT master regulators. Breast Cancer Res. Treat., 2014. 145(3): p. 593-604.

25. Liao, P.L., C.H. Lin, C.H. Li, C.H. Tsai, J.D. Ho, G.C. Chiou, J.J. Kang, and Y.W. Cheng, Anti-inflammatory properties of shikonin contribute to improved early-stage diabetic retinopathy. Sci Rep, 2017. 7: p. 44985.

26. Andújar, I., J.L. Ríos, R.M. Giner, and M.C. Recio, Pharmacological properties of shikonin - a review of literature since 2002. Planta Med., 2013. 79(18): p. 1685-97.

27. Su, L., L. Liu, Y. Wang, G. Yan, and Y. Zhang, Long-term systemic toxicity of shikonin derivatives in Wistar rats. Pharm Biol, 2013.

28. Gupta, B., S. Chakraborty, S. Saha, S.G. Chandel, A.K. Baranwal, M. Banerjee, M. Chatterjee, and A. Chaudhury, Antinociceptive properties of shikonin: in vitro and in
vivo studies. Can. J. Physiol. Pharmacol., 2016. 94(7): p. 788-96.

29. Lin, K.H., M.Y. Huang, W.C. Cheng, S.C. Wang, S.H. Fang, H.P. Tu, C.C. Su, Y.L. Hung, P.L. Liu, C.S. Chen, Y.T. Wang, and C.Y. Li, RNA-seq transcriptome analysis of breast cancer cell lines under shikonin treatment. Sci Rep, 2018. 8(1): p. 2672.

30. Li, W., J. Liu, K. Jackson, R. Shi, and Y. Zhao, Sensitizing the therapeutic efficacy of taxol with shikonin in human breast cancer cells. PLoS ONE, 2014. 9(4): p. e94079.

31. Iorio, M.V. and C.M. Croce, MicroRNAs in cancer: small molecules with a huge impact. J. Clin. Oncol., 2009. 27(34): p. 5848-56.

32. Li, H., C. Bian, L. Liao, J. Li, and R.C. Zhao, miR-17-5p promotes human breast cancer cell migration and invasion through suppression of HBP1. Breast Cancer Res Treat, 2011. 126(3): p. 565-75.

33. Zhang, J., Z. Xiao, D. Lai, J. Sun, C. He, Z. Chu, H. Ye, S. Chen, and J. Wang, miR-21, miR-17 and miR-19a induced by phosphatase of regenerating liver-3 promote the proliferation and metastasis of colon cancer. Br. J. Cancer, 2012. 107(2): p. 352-9.

34. Kotelevets, L., B. Trifault, E. Chastre, and M.G.H. Scott, Posttranslational Regulation and Conformational Plasticity of PTEN. Cold Spring Harb Perspect Med, 2020.

35. Luongo, F., F. Colonna, F. Calapà, S. Vitale, M.E. Fiori, and R. De Maria, PTEN Tumor-Suppressor: The Dam of Stemness in Cancer. Cancers (Basel), 2019. 11(8).

36. Mziaut, H., G. Henniger, K. Ganss, S. Hempel, S. Wolk, J. McChord, K. Chowdhury, P. Ravassard, K.P. Knoch, C. Krautz, J. Weitz, R. Grützmann, C. Pilarsky, M. Solimena, and S. Kersting, MiR-132 controls pancreatic beta cell proliferation and survival through Pten/Akt/Foxo3 signaling. Mol Metab, 2020. 31: p. 150-162.

37. Tan, A.C., Targeting the PI3K/Akt/mTOR pathway in non-small cell lung cancer (NSCLC). Thorac Cancer, 2020. 11(3): p. 511-518.

38. Karimi Roshan, M., A. Soltani, A. Soleimani, K. Rezaie Kahkhaie, A.R. Afshari, and M.
Soukhtanloo, Role of AKT and mTOR signaling pathways in the induction of epithelial-mesenchymal transition (EMT) process. Biochimie, 2019. 165: p. 229-234.

Supplemental Figure Legends

Figure S1- The screening of target gene of miR-17-5p in breast cancer cell migration and invasion. (A) 351 genes were overlapped in the four groups of predicted target genes from TargetScan, PITA, miRanda and picTar. (B) The enrichment analysis of KEGG pathways was performed in the 351 genes. (C) Seven genes were overlapped between the two relevant pathways - microRNAs in cancer and pathways in cancer. (D) The expressions of the seven genes were analyzed in BC (n=1085) and normal (n=291) cohorts from TCGA. (E,F) The correlation between miR-17-5p and CRK (E) or CDKN1A (F) in BC cohort from TCGA. ***p <0.001.
Shikonin inhibits the migration, invasion and EMT of TNBC cells. MDA-MB-231 cells were treated with various concentrations of SHK (2.5 and 5 μM) or the control for 24 h. (A) The cell migration was detected by wound-healing assay. (B) Quantification of the wound closures. (C) The cell invasion was detected by transwell invasion assay. (D) Quantification of the invaded cells. (E) The EMT markers (N-cadherin, Vimentin and E-cadherin) protein levels were analyzed by Western blot. GAPDH is shown as a loading control. Error bars represent the SEM obtained from at least three independent experiments. ***p<0.001.
MiR-17-5p promotes TNBC cell migration, invasion and EMT. (A) MDA-MB-231 cells were pretreated with different concentrations of SHK (2.5 and 5 μM) or the control for 24 h. The
level of miR-17-5p was determined by qRT-PCR, with U6 as a reference. (B-G) MDA-MB-231 cells were transfected with a 50 nM miR-17-5p inhibitor, mimic or the corresponding negative control (NC) for 24 h. (B) The transfection efficiency was assessed by qRT-PCR, with U6 as a reference. (C) The cell invasion was detected by transwell invasion assay. (D) Quantification of the invaded cells. (E) The cell migration was detected by wound-healing assay. (F) Quantification of the wound closures. (G) The protein levels of N-cadherin, Vimentin and E-cadherin were analyzed by Western blot. GAPDH is shown as a loading control. Data represent the mean ± SEM of three independent experiments. *p<0.05, **p<0.01, ***p<0.001
MiR-17-5p downregulates PTEN by directly binding to its 3’-UTR. (A) The expression of miR-17-5p was analyzed in 1085 breast cancer and 291 normal breast tissue samples from TCGA. (B) The correlation between miR-17-5p and PTEN expressions in 1077 breast cancer samples from TCGA. (C-D) MDA-MB-231 cells were transfected with a 50 nM miR-17-5p inhibitor, mimic or the corresponding negative control (NC) for 24 h. The PTEN mRNA (C) and protein (D) levels were detected by qRT-PCR and Western blot respectively. (E) Schematic representation of predicted miR-17-5p binding sites in the 3’-UTR of PTEN and 3’-
UTR mutated alignment. (F) Luciferase reporter assay of MDA-MB-231 cells co-transfected with a miR-17-5p mimic, NC or mock and either wild-type or mutated luciferase plasmid. Error bars represent the SEM obtained from three independent experiments. *p<0.05, **p<0.01, ***p <0.001.

Figure 4
Downregulation of PTEN increases the migration, invasion and EMT of TNBC cells. MDA-MB-
231 cells were transfected with control siRNA (si-control) or PTEN siRNA (si-PTEN) and with pc-DNA3.1 or pc-DNA3.1-PTEN. (A) The expression of PTEN mRNA was determined by qRT-PCR, with HPRT1 as a reference. (B) The expression of PTEN protein was determined by western blot. (C) The cell invasion was detected by transwell invasion assay. (D) Quantification of the invaded cells. (E) The cell migration was detected by wound-healing assay. (F) Quantification of the wound closures. Data represent the mean ± SEM of three independent experiments. *p<0.05, **p<0.01, ***p<0.001
Figure 5

Inhibition of EMT by PTEN in TNBC cells and Akt involvement in the process of EMT. (A) MDA-MB-231 cells were transfected with PTEN siRNA (si-PTEN) or control siRNA (si-control) and with PTEN overexpression vector (pc-DNA3.1-PTEN) or control vector (pc-DNA3.1). The expressions of N-cadherin, Vimentin and E-cadherin were analyzed by Western blot. GAPDH is shown as a loading control. (B,C) MDA-MB-231 cells were pretreated with different concentrations of SHK (2.5 and 5 μM) or the control for 24 h. PTEN expression was evaluated at mRNA (B) and protein (C) levels. (D) The expression of Akt and p-Akt was detected by western blot in MDA-MB-231 cells treated with SHK for 24 h. (E) The levels of Akt and p-Akt in MDA-MB-231 cells transfected with miR-17-5p mimic, inhibitor and NC. (F) The expression of Akt and p-Akt was detected in MDA-MB-231 cells transfected with si-control or si-PTEN and with pc-DNA3.1 or pc-DNA3.1-PTEN. Data represent the mean ± SEM of three independent experiments. ***p<0.001

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.
