

Phenethyl isothiocyanate reduces breast cancer stem cell-like properties by epigenetic reactivation of CDH1

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Abstract. Breast cancer is the most frequently diagnosed malignancy and leading cause of cancer-related deaths among women worldwide. Tumor recurrence, or metastasis, is caused by cancer stem cells and has a dismal prognosis for breast cancer patients. Thus, targeting breast cancer stem cells (BCSCs) for eradication is a potential method to improve clinical outcomes. Phenethyl isothiocyanate (PEITC) is a novel epigenetic regulator derived from cruciferous vegetables that has marked antitumor effects. However, the exact mechanism of these antitumor effects by PEITC is unknown. As breast cancer progresses, a tumor suppressor in the breast, cadherin 1 (CDH1), is silenced by hypermethylation of the promoter region, further promoting the stem cell-like properties of cancer. Herein, the ability of PEITC to reduce BCSC-like properties by epigenetic reactivation of CDH1 was investigated by multiple analyses such as MTT, colony formation and sphere formation assays, methylation-specific PCR, western blot analysis, Co-IP and qPCR. It was revealed that PEITC inhibited colony and mammosphere formation and decreased the expression of protein markers associated with BCSC-like properties via epigenetic reactivation of CDH1. Further exploration of this mechanism revealed inhibitory effects of PEITC on DNMTs and HDACs, which play a pivotal role in demethylating the hypermethylated CDH1 promoter region. Reactivated CDH1 suppressed the Wnt/ β -catenin pathway which confers BCSC-properties in breast cancer cells. These findings suggest a novel method to eradicate BCSCs from breast cancer patients.

Introduction

Breast cancer is the most frequently diagnosed malignancy and leading cause of cancer-related deaths among women on a global scale. Data from the International Agency for Research on Cancer (IARC), a World Health Organization agency, indicated that in 2018 there were more than 2,088,849 new cases of breast cancer and approximately 626,679 breast cancer-related deaths in women (1). Therefore, breast cancer is a global health issue of women that must be addressed. There are five primary treatment options, most of which include a combination of: surgery, radiation, chemotherapy, hormone therapy and targeted therapies (2). Despite recent advances in breast cancer treatments, a subgroup of breast cancer cells, termed breast cancer stem cells (BCSCs), often cause tumor recurrence and metastasis in 30-40% of early-stage breast cancer patients following treatment (3). The BCSC characteristics of heterogeneity, self-renewal, and pluripotency lead to malignant progression, treatment resistance and a poor clinical prognosis (4). Recent studies have revealed that several signaling pathways for maintaining self-renewal and differentiation such as Wnt, Notch and Hedgehog are excessively activated in BCSCs (5). Additionally, multidrug resistance and DNA repair genes including multidrug resistance-1 (MDR1) and Rad 51 are overexpressed in BCSCs, conferring resistance to conventional chemotherapeutic drugs and radiotherapies (6,7). Moreover, BCSCs can promote angiogenesis and grow in an anchorage-independent manner that contributes to cancer dissemination and secondary tumors (8). These key activities of BCSCs in breast carcinogenesis suggest that devising novel methods to eradicate BCSCs may improve the poor prognosis of recurrent or metastatic breast cancer patients.

Phenethyl isothiocyanate (PEITC), one of the major bioactive compounds derived from cruciferous vegetables such as broccoli, watercress and Brussels sprouts, possesses marked anticancer activities by inhibiting cell cycle progression, inducing apoptosis and reversing drug resistance (9). Recent studies have revealed that PEITC is selectively lethal for malignant cells with reduced cytotoxicity for normal cells (10-12), suggesting that it has potential as a 'high-efficiency and lower toxicity' chemotherapy drug. At present, clinical trials are evaluating PEITC including 'The Safety and Efficacy Test

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of Nutri-PEITC Jelly in Head and Neck Cancer Patients' (NCT03034603) and 'Phenethyl isothiocyanate in Preventing Lung Cancer in Smokers' (NCT00691132). Moreover, a completed phase I study (NCICN-55120) has revealed PEITC antitumor activity at micromolar concentrations, further indicating its promise for clinical use (13). In recent years, epigenetic silencing of tumor suppressor genes during the initiation and progression of cancers, including breast cancer, has been established (14, 15). Emerging evidence further indicates that epigenetic regulation, including DNA hypermethylation, plays a pivotal role in promoting cancer stem cell characteristics (16,17). As such, DNA hypermethylation and histone deacetylation have been hypothesized to be effective targets for cancer treatments, including the eradication of cancer stem cells (CSCs) (18). PEITC is a novel epigenetic regulator that inhibits both DNA methyltransferases (DNMTs) and histone deacetylases (HDACs), both of which mediate the silencing of tumor suppressor genes during tumor progression (19,20). However, no study to date has explored the mechanism by which PEITC epigenetically reactivates tumor suppressor genes to eradicate BCSCs.

The cadherin 1 (CDH1) gene is located at chromosome 16q22.1 and encodes a transmembrane glycoprotein called E-cadherin, which functions as a tumor suppressor by maintaining cell adhesion and adherent junctions in normal tissues (21). The expression of CDH1 is frequently observed to be silenced in solid malignant tumors, including breast cancer, due to DNA hypermethylation of the promoter region during tumor initiation and progression (16, 22). Previous studies have revealed that a loss of CDH1 expression in breast cancer can initiate the epithelial-mesenchymal transition (EMT) and is associated with metastasis and poor prognosis (23,24). Recent studies have also reported that demethylation of the CDH1 promoter region to restore CDH1 expression inactivates the Wnt/ β -catenin pathway and suppresses carcinoma cell stemness (16), indicating another potential way to eradicate BCSCs.

In summary, PEITC functions as an epigenetic regulator and CDH1 is silenced by DNA hypermethylation. Additionally, following histone deacetylation in breast cancer, the Wnt/ β -catenin pathway is activated to maintain cancer stem cell-like properties. The purpose of the present study was to examine the effects of PEITC on the CSC-like properties of breast cancer cells and elucidate the mechanisms.

Materials and methods

Cell culture and drugs. Human breast cancer cell lines MCF-7 and MDA-MB-231 were obtained from the American Type Culture Collection. These cell lines were cultured at 37°C under a 5% CO₂ atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and propagated for <8 generations after resuscitation. Reagents, including PEITC, Aza-deoxycytidine (5-Aza), and Trichostatin A (TSA) were purchased from Sigma-Aldrich (Merck KGaA).

MTT assay. Three thousand viable breast cancer cells (MCF-7 and MDA-MB-231) were plated onto a 96-well plate (Corning, Inc.) in triplicate for 24 h, and then treated with various

concentrations of PEITC (0, 1, 2, 5, 10, 25, 50 and 100 μ M) dissolved in complete medium and cultured at 37°C under a 5% CO₂ atmosphere for 72 h. Cells were then incubated with 100 μ l of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (0.5 mg/ml) for 4 h at 37°C in the dark. The MTT solution was then discarded and the formazan was solubilized in 100 μ l DMSO for 1 h on a shaker. The optical density was measured at 570 nm using SpectraMax M Series Multi-Mode Microplate Reader (Molecular Devices, LLC).

Colony formation assay. Three hundred viable breast cancer cells (MCF-7 and MDA-MB-231) were inoculated into a 6-well plate (Corning, Inc.) in triplicate for 24 h, then incubated with 0.1% DMSO (control) or PEITC (5 or 10 μ M) and dissolved in complete medium cultured at 37°C under a 5% CO₂ atmosphere for 10 days. The colonies were fixed with a fixation solution (acetic acid:methanol, 1:7) for 5 min and stained with 0.5% crystal violet for 20 min at room temperature. The stained colonies were washed 3 times with PBS and counted with a countermark pen. Colonies with a minimum cell number >50 observed under an inverted phase-contrast microscope (Nikon Corporation) were included in the final statistical analysis. The images of the colonies presented were visualized using a Canon scanner (CanoScan 5600F).

Sphere formation assay. Breast cancer Cells (MCF-7 and MDA-MB-231) were seeded in 6-well ultra-low cluster plates (Corning, Inc.) at a density of 5×10^3 cells and cultured at 37°C under a 5% CO₂ atmosphere in CSC enrichment medium including DMEM/F12 serum-free medium, 2% B27, 20 ng/ml epidermal growth factor (EGF), and 20 ng/ml recombinant human fibroblast basic growth factor (RH-bFGF) (all from Gibco; Thermo Fisher Scientific, Inc.). Cells were treated with either PEITC at 5 or 10 μ M or 0.1% DMSO as the vehicle control. After 7 days, the number of tumor spheres ($\geq 50 \mu$ m) was imaged and counted under an inverted phase-contrast microscope (Nikon Corporation) at a magnification of $\times 100$.

Methylation-specific PCR (MSP). Breast cancer cells (MCF-7 and MDA-MB-231) were seeded in 6-well plates (Corning, Inc.) at a density of 2×10^5 cells in triplicate for 24 h, then incubated with 0.1% DMSO (control) or PEITC (5 or 10 μ M) or positive control epigenetic-regulating drugs (2.5 μ M 5-Aza and 0.5 μ M TSA) for 5 days at 37°C under a 5% CO₂ atmosphere. Genomic DNA was isolated from treated cells using the QIAamp DNA Mini Kit (Qiagen, Inc.). Next, 1 μ g of genomic DNA was denatured by bisulfite conversion with the EZ DNA Methylation Gold Kit (Zymo Research Corp.) following the manufacturer's instructions and then used as a template for PCR amplification. The primer pairs used for methylated CDH1 were: forward, 5'-TAACTACAACCAAATAAACCCCG-3' and reverse, 5'-TCGAATTTAGTGGAATTAGAATCGT-3'. The primer pairs used for unmethylated E-cadherin were: forward, 5'-TAACTACAACCAAATAAACCCCAAA-3' and reverse, 5'-TTGAATTTAGTGGAATTAGAATTGT-3'. The PCR cycling conditions consisted of an initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and extension at 74°C for 2 min. The amplification products were separated

on a 1.5% agarose gel by electrophoresis and visualized using ethidium bromide staining and a Gel Documentation 2000 system (Bio-Rad Laboratories, Inc.). The band densities were quantified using ImageJ software (version 1.52; National Institutes of Health).

Western blot analysis. Cells were lysed in ice-cold RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1% Triton-X-100 and 0.1% sodium deoxycholate sulfate) supplemented with phosphatase inhibitor (Na_3VO_4 , 1 mM) and protease inhibitor (phenylmethylsulfonyl fluoride, 1 mM). The cell lysate was then centrifuged at 12,000 x g for 10 min at 4°C. The Pierce™ Rapid Gold bicinchoninic acid (BCA) kit (Thermo Fisher Scientific, Inc.) was used to measure protein concentrations. Proteins (20–50 µg per lane) were then separated by electrophoresis on an SDS-PAGE gel (6 or 12%) and transferred onto a Sequi-Blot™ PVDF membrane (Bio-Rad Laboratories, Inc.). Then, the membranes were blocked by 5% milk in TBST (Tris base-0.1% Tween-20) at room temperature for 2 h. Antibodies to c-Myc (D84C12; rabbit mAb; product no. 5605), ALDH-1 (D9J7R; XP® rabbit mAb; product no. 36671), Oct-4A (C30A3; rabbit mAb; product no. 2840), Sox-2 (D6D9; XP® rabbit mAb; product no. 3579), phospho-β-catenin (Ser675) (D2F1XP®; rabbit mAb; product no. 4176), phospho-β-catenin (Ser33/37) (rabbit mAb; product no. 2009), cyclin D1 (E3P5S; XP® rabbit mAb; product no. 55506), and CDH1 (mouse mAb; product no. 14472) were obtained from Cell Signaling Technology, Inc. and diluted 1:1,000 for binding with target proteins overnight at 4°C. Antibodies against Dnmt1 (mouse mAb; cat. no. sc-514784), Dnmt3a (mouse mAb; cat. no. sc-365769), Dnmt3b (mouse mAb; cat. no. sc-81252), HDAC1 (mouse mAb; cat. no. sc-81598), and HDAC2 (mouse mAb; cat. no. sc-9959) were purchased from Santa Cruz Biotechnology, Inc. and diluted 1:200 for binding with target proteins overnight at 4°C. β-actin (mouse mAb; product no. A1978) and secondary anti-mouse (product no. A2429) or anti-rabbit (product no. A3937) antibodies were obtained from Sigma-Aldrich (Merck KGaA) and diluted 1:1,000 for binding with target proteins for 2 h at room temperature. After washing the membranes with TBST (Tris base-0.1% Tween-20) 3 times, protein bands were imaged with enhanced chemiluminescence (Thermo Fisher Scientific, Inc.) and an X-ray film system (Ece Scientific Co., Inc.). The band densities were quantified using ImageJ software (version 1.52; National Institutes of Health).

Co-immunoprecipitation (Co-IP). Cells were lysed for 30 min in ice-cold immunoprecipitation (IP) lysis buffer for IP (40 mM HEPES, 0.3% Chaps, 2 mM EDTA, 1 mM dithiothreitol, 10 mM pyrophosphate, 10 mM β-glycerophosphate, and 50 mM NaF) supplemented with phosphatase inhibitor (Na_3VO_4 , 1 mM) and protease inhibitor (phenylmethylsulfonyl fluoride, 1 mM). The supernatant was collected after centrifugation at 4°C at 13,000 x g for 30 min and the total protein concentration was adjusted to 1 µg/µl. Finally, 200 µg of total protein was used per IP reaction. The dilution rate of the CDH1 antibody (mouse mAb; product no. 14472; Cell Signaling Technology) was 1:50 per IP reaction. The protein G agarose magnetic beads (Santa Cruz Biotechnology, Inc.) were prepared according to the manufacturer's instructions. The

bead-bound immune complexes were incubated with rotation for 4 h at 4°C and then the beads were pelleted using a magnetic separation rack and washed with 500 µl of 1X cell lysis buffer five times (keeping on ice between washes). The bead-bound immune complexes were resuspended in 30 µl of 1X SDS-PAGE loading buffer and incubated for 5 min on a heating block at 95°C to denature and release proteins. Finally, the magnetic beads were removed using the magnetic separator and the supernatant was transferred to a clean 1.5 ml tube for western blotting.

RNA isolation and RT-qPCR. Total RNAs were isolated from MCF-7 and MDA-MB-231 cells using TRIzol Reagent (Thermo Fisher Scientific, Inc.) and the concentration was measured by Nanodrop (BioSpec-nano; Shimadzu Corporation). SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific, Inc.) was used for reverse transcription and RT-qPCR was performed with Platinum SYBR-Green qPCR SuperMix-UDG (Thermo Fisher Scientific, Inc.) on an ABI PRISM® 7000. The comparative C_q method ($2^{-\Delta\Delta C_q}$) (25) was used to analyze the data and GAPDH mRNA expression was used as the normalization control. The CDH1 primers were: forward, 5'-TGGGTTATTCCTCCCATCAG-3' and reverse, 5'-GTCACCTTCAGCCATCCTGT-3'. The GAPDH primers were: forward, 5'-AGGTCGGAGTCAACGGATTTG-3' and reverse, 5'-GTGATGGCATGGACTGTGGT-3'. The PCR cycling conditions consisted of an initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min.

Statistical analyses. GraphPad Prism 5 software (GraphPad Software, Inc.) was used to conduct the statistical analyses. All data were expressed as the mean ± standard deviation (SD) except where indicated. One-way ANOVA with Dunnett's multiple comparison test or two-tailed unpaired Student's t-test was used to compare means between groups as indicated. $P < 0.05$ or a fold change > 2 was considered to indicate a statistically significant difference.

Results

Selection of PEITC concentrations. Typically, sublethal doses of a compound are used to investigate epigenetic regulation properties *in vitro* (26). We performed a preliminary experiment to evaluate the effects of PEITC at 0, 1, 2, 5, 10, 25, 50 and 100 µM on the cell viability of MCF-7 and MDA-MB-231 cells using an MTT assay. PEITC at 5 and 10 µM significantly inhibited the growth of breast cancer cells after treatment for 72 h, but it did not markedly induce cell death (data not shown). Thus, 5 and 10 µM PEITC were used to evaluate the epigenetic effects of PEITC on breast cancer cells *in vitro*.

PEITC reduces colony and sphere formation abilities. Colony and sphere formation assays are two methods frequently used to identify cancer stem cells (CSCs) and study their properties *in vitro* (27). To evaluate the effects of PEITC on the colony formation ability of breast cancer cells, MCF-7 and MDA-MB-231 cells were seeded at 300 cells/well in 6-well dishes. Cells were treated with PEITC (5 and 10 µM)

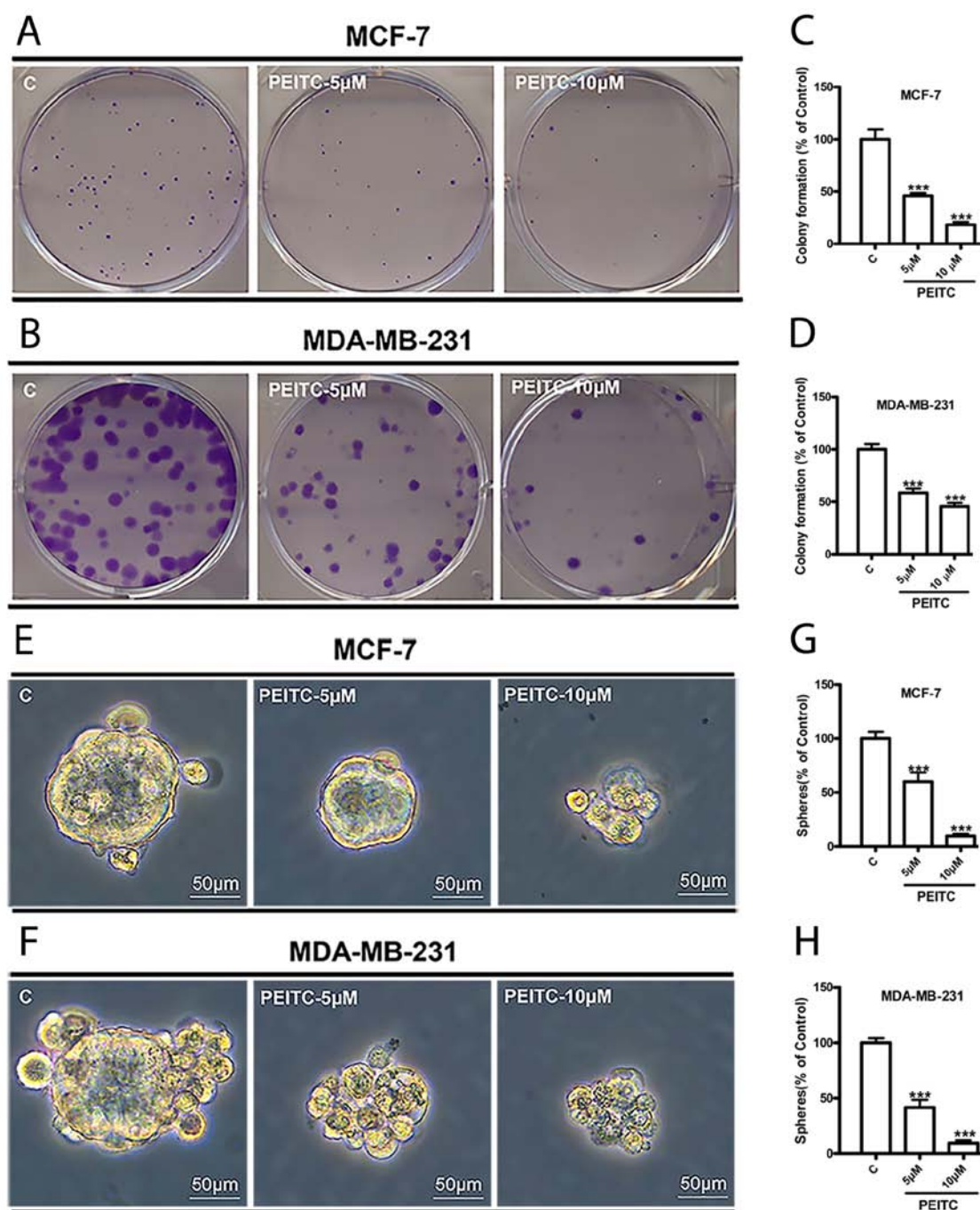


Figure 1. Effect of PEITC on colony and sphere formation abilities in breast cancer cells. Representative images revealed the suppression of colony formation abilities by PEITC in (A) MCF-7 and (B) MDA-MB-231 cells. Bar graphs quantified the reduction in the colony counts for (C) MCF-7 and (D) MDA-MB-231 cells by PEITC. Representative images revealed the inhibition of sphere formation in (E) MCF-7 and (F) MDA-MB-231 cells by PEITC (scale bar, 50 μ m). Bar graphs quantified the reduction in the number of spheres in (G) MCF-7 and (H) MDA-MB-231 cells by PEITC. Data were presented as the mean \pm SD, n=3. ***P<0.001 vs. the control. PEITC, phenethyl isothiocyanate; C, control.

or 0.1% DMSO (control) for 10 days. PEITC at 5 and 10 μ M significantly reduced the number of MCF-7 colonies to 45 and 20% (Fig. 1A and C) and MDA-MB-231 colonies to 60 and 45% (Fig. 1B and D), respectively. In the sphere formation assay, MCF-7 and MDA-MB-231 cells were dissociated into single cells, seeded into ultra-low cluster 6-well plates and treated with PEITC (5 and 10 μ M) or 0.1% DMSO (control) in CSC enrichment medium. After seven days, it was observed that PEITC (5 and 10 μ M) significantly decreased the size of MCF-7 and MDA-MB-231 mammospheres (Fig. 1E and F)

and reduced the number of mammospheres to 60 and 10% in MCF-7 cells (Fig. 1G) and 40 and 10% in MDA-MB-231 (Fig. 1H).

PEITC suppresses the expression of BCSC-related proteins. Previous studies have identified that the protein expression levels of c-Myc, ALDH-1, Oct-4A and Sox-2 are associated with BCSC-like properties (28-31). Since PEITC could reduce the colony and sphere formation abilities of breast cancer cells, it was hypothesized that BCSC-related proteins

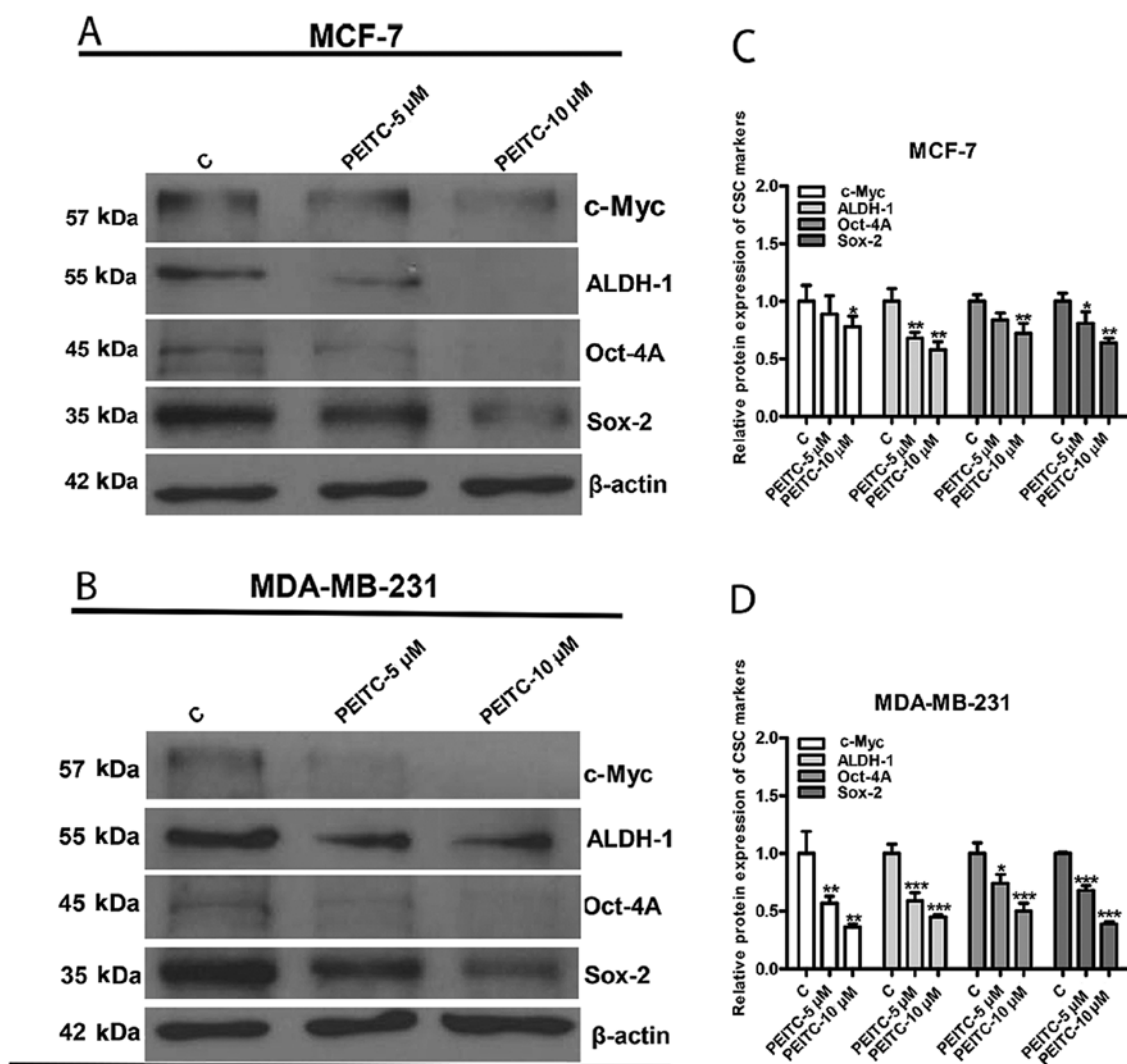


Figure 2. Effect of PEITC on BCSC-associated protein markers. c-Myc, ALDH-1, Oct-4A and Sox-2 are BCSC-associated protein markers. Representative western blots revealed the reduction in the protein expression of c-Myc, ALDH-1, Oct-4A and Sox-2 by PEITC treatment in (A) MCF-7 and (B) MDA-MB-231 cells. Bar graphs of quantitative densitometry indicated the downregulation of c-Myc, ALDH-1, Oct-4A, and Sox-2 in (C) MCF-7 and (D) MDA-MB-231 cells. Data were presented as the mean \pm SD, n=3. *P<0.05, **P<0.01 and ***P<0.001 vs. the control. PEITC, phenethyl isothiocyanate; BCSC, breast cancer stem cell; C, control

may also be downregulated. To test our hypothesis, western blotting and quantitative densitometry with ImageJ were used to observe the protein expression of c-Myc, ALDH-1, Oct-4A and Sox-2. All targeted BCSC-related proteins were downregulated in both MCF-7 (Fig. 2A and C) and MDA-MB-231 cells (Fig. 2B and D) following PEITC (5 and 10 μ M) treatment for 3 days.

PEITC restores CDH1 expression by demethylation of the promoter region. The hypermethylation of the CDH1 promoter region has been identified in numerous malignant cancers including breast cancer, leading to its encoded metastasis suppressor, E-cadherin, being silenced along with cancer progression (32). Recent studies have revealed that epigenetic silencing of the CDH1 gene contributes to CSC-like properties (16). Moreover, PEITC has been identified as a new epigenetic regulator that can demethylate the hypermethylated promoter regions of tumor suppressor genes to eliminate cancer cells (19). Therefore, it was hypothesized that PEITC

reduces BCSC-like properties via epigenetic reactivation of CDH1. To test our hypothesis, we first examined the extent of methylation of the CDH1 promoter region in the presence of either 0.1% DMSO (control) or PEITC (5 or 10 μ M) or positive control epigenetic regulating drugs (2.5 μ M 5-Aza and 0.5 μ M TSA) for 5 days. Methylation-specific PCR (MSP) and quantitative analysis revealed high methylation levels of the CDH1 promoter region in untreated breast cancer cells, suggesting hypermethylation of the CDH1 promoter. However, a significant increase in the unmethylation of the CDH1 promoter region was observed in PEITC (5 or 10 μ M), 5-Aza (2.5 μ M), and TSA (0.5 μ M) MCF-7 (Fig. 3A and C) and MDA-MB-231 (Fig. 3B and D) treated-cells compared to the control. The change in mRNA and protein levels of CDH1 in MCF-7 and MDA-MB-231 cells following treatment with either PEITC (5 or 10 μ M) and 5-Aza (2.5 μ M) and TSA (0.5 μ M) for 5 days was further analyzed using qPCR and western blotting, respectively. PEITC significantly enhanced CDH1 mRNA expression in both MCF-7 (Fig. 3E)

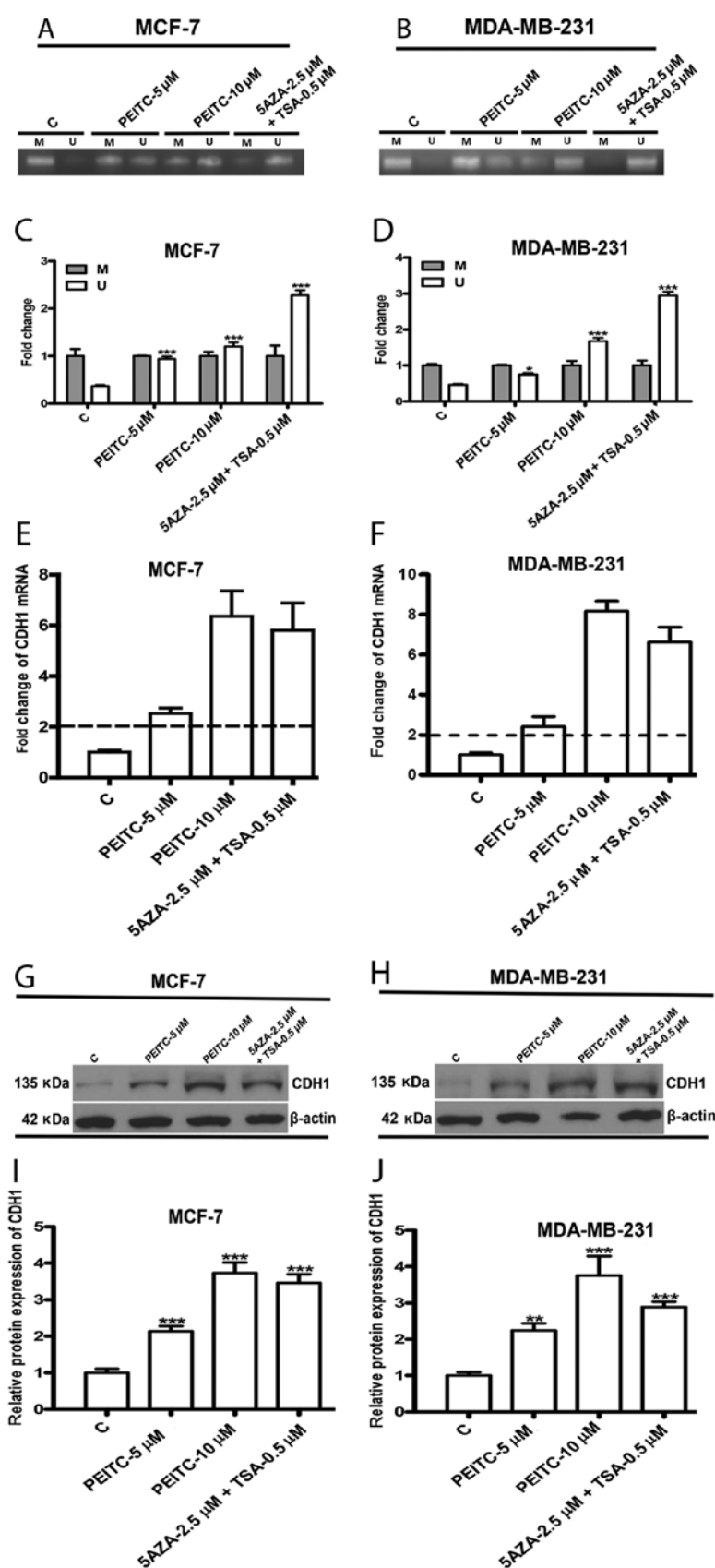


Figure 3. Effect of PEITC on CDH1 promoter methylation. Representative MSP bands revealed the decrease of methylation and increase of unmethylation in the CDH1 promoter region of (A) MCF-7 and (B) MDA-MB-231 cells by the indicated treatments. Bar graphs of the relative intensity of MSP bands quantified the change in unmethylation (normalized to the corresponded methylated band) of the CDH1 promoter region in (C) MCF-7 and (D) MDA-MB-231 cells following the indicated treatments. Bar graphs of the relative fold change of CDH1 mRNA in (E) MCF-7 and (F) MDA-MB-231 cells following the indicated treatments. $n=3$; a fold change >2 was considered to indicate a significant difference. Representative western blots revealed the upregulation of CDH1 in (G) MCF-7 and (H) MDA-MB-231 cells following the indicated treatments. Bar graphs of quantitative densitometric results revealed the upregulation of CDH1 in (I) MCF-7 and (J) MDA-MB-231 cells by the indicated treatments. Data were presented as the mean \pm SD, $n=3$. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ vs. the control. PEITC, phenethyl isothiocyanate; CDH1, cadherin 1; MSP, methylation-specific PCR; M, methylation; U, unmethylation; C, control.

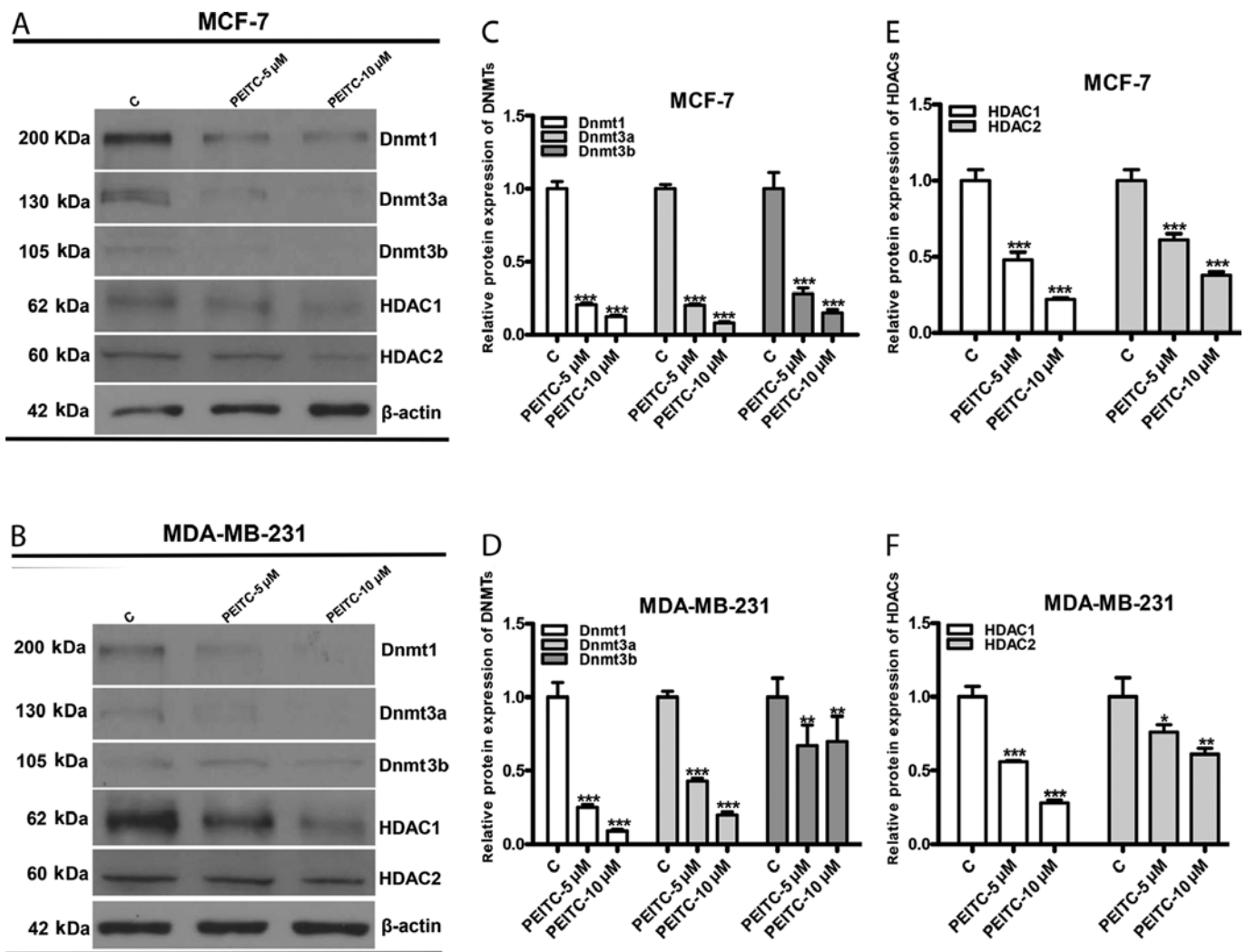


Figure 4. Effect of PEITC on DNMTs and HDACs. Representative western blots revealed the reduction in protein expression of DNMTs (Dnmt1, Dnmt3a and Dnmt3b) and HDACs (HDAC1 and HDAC2) by PEITC treatment in (A) MCF-7 and (B) MDA-MB-231 cells. Bar graphs of quantitative densitometric results indicated the downregulation of Dnmt1, Dnmt3a and Dnmt3b by PEITC in (C) MCF-7 and (D) MDA-MB-231 cells. Bar graphs of the quantitative densitometric results revealed the downregulation of HDAC1 and HDAC2 by PEITC in (E) MCF-7 and (F) MDA-MB-231 cells. Data were presented as the mean \pm SD, n=3. *P<0.05, **P<0.01 and ***P<0.001 vs. the control. PEITC, phenethyl isothiocyanate; DNMTs; DNA methyltransferase; HDACs, histone deacetylases; C, control.

and MDA-MB-231 (Fig. 3F) cells. Similarly, CDH1 protein levels were also significantly upregulated by PEITC in MCF-7 (Fig. 3G and I) and MDA-MB-231 (Fig. 3H and J) cells. These results indicated that PEITC reactivated CDH1 by affecting epigenetic regulation.

PEITC inhibits DNMTs and HDACs. DNMTs (Dnmt1, Dnmt3a, and Dnmt3b) and HDACs (HDAC1 and HDAC2) are enzymes that catalyze the addition of a methyl group and removal of acetyl groups, respectively (19). These enzymes play crucial roles in mediating the silencing of tumor suppressor genes with tumor progression (33). To further investigate whether PEITC restored CDH1 expression via epigenetic regulation, the effects of PEITC on DNMTs and HDACs were evaluated by western blotting and quantitative densitometry. PEITC (5 and 10 μ M) significantly inhibited the protein expression of DNMTs (Dnmt1, Dnmt3a and Dnmt3b) and HDACs (HDAC1 and HDAC2) in both MCF-7 (Fig. 4A, C and E) and MDA-MB-231 (Fig. 4B, D and F) cells.

PEITC-reactivated CDH1 inhibits the Wnt/ β -catenin signaling pathway. Based on the epigenetic reactivation of CDH1 by PEITC, the underlying mechanism of CDH1 regulation that reduces BCSC-like properties was further explored. It is well-documented that CDH1 affects the Wnt/ β -catenin signaling pathway by binding to β -catenin at the cytoplasmic membrane. This prevents β -catenin translocation into the nuclei and formation of the β -catenin/TCF transcription factor complex (16). Previous research has also revealed that the Wnt/ β -catenin signaling pathway plays a pivotal role in maintaining BCSC-like properties (27). Thus, it was speculated whether PEITC-reactivated CDH1 would disrupt intracellular Wnt/ β -catenin signaling in breast cancer cells. To address this question, MCF-7 and MDA-MB-231 cells were treated with PEITC (0, 5 and 10 μ M) for 3 days and then Co-IP was performed to detect the ability of CDH1 and inactive β -catenin (phospho-Ser33/37) to interact. PEITC increased the amount of inactive β -catenin that co-precipitated with CDH1 (Fig. 5A and B). Next the effects of PEITC on Wnt/ β -catenin

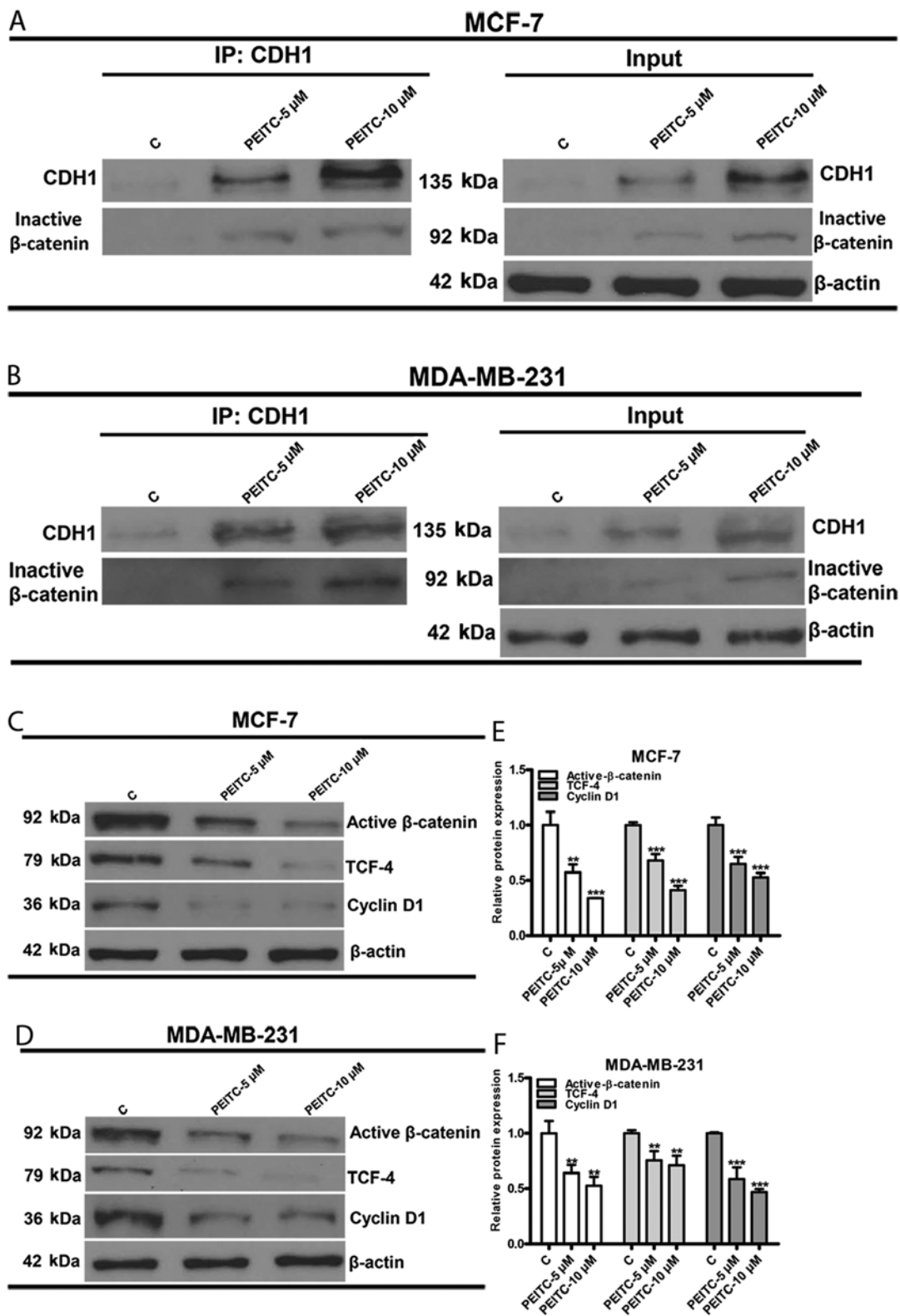


Figure 5. Effect of PEITC on the CDH1/Wnt/ β -catenin pathway. Representative Co-IP blots revealed an increased amount of the CDH1 and inactive- β -catenin (phospho-Ser33/37) complex following PEITC treatment in (A) MCF-7 and (B) MDA-MB-231 cells. Representative western blots revealed the inhibition of active β -catenin (phospho-Ser675), TCF-4 and cyclin D1 by PEITC in (C) MCF-7 and (D) MDA-MB-231 cells. Bar graphs of quantitative densitometric results revealed the downregulation of active β -catenin (phospho-Ser675), TCF-4 and cyclin D1 in (E) MCF-7 and (F) MDA-MB-231 cells by PEITC. Data were presented as the mean \pm SD, n=3. **P<0.01 and ***P<0.001 vs. the control. PEITC, phenethyl isothiocyanate; CDH1, cadherin 1; Co-IP, co-immunoprecipitation.

signaling in MCF-7 and MDA-MB-231 cells were detected by western blotting and quantitative densitometry. Active β -catenin (phosphor-Ser675) induced β -catenin accumulation

in the nucleus and promoted β -catenin/TCF4 transcription activity. Cyclin D1 is a well-established downstream gene of the Wnt/ β -catenin pathway (34). Protein levels of active

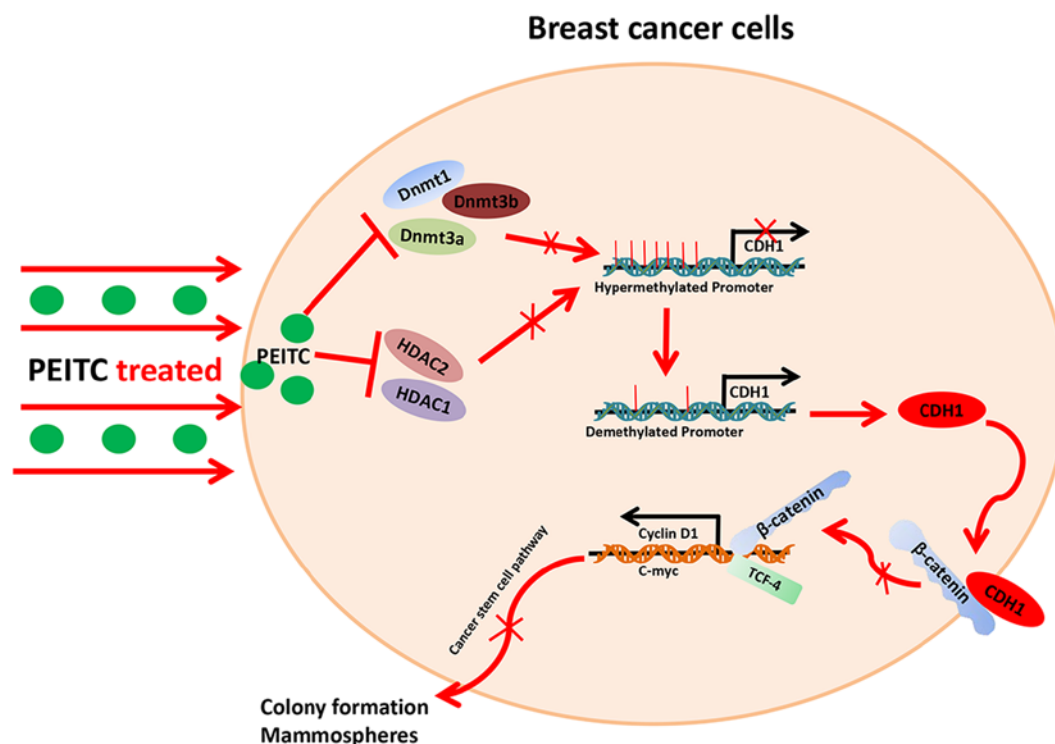


Figure 6. Schematic of the mechanism by which PEITC epigenetically reactivates CDH1 to inhibit the Wnt/ β -catenin pathway and reduces BCSC-like properties. PEITC, phenethyl isothiocyanate; CDH1, cadherin 1; BCSC, breast cancer stem cell.

β -catenin, TCF-4 and cyclin D1 were significantly decreased in MCF-7 (Fig. 5C and E) and MDA-MB-231 (Fig. 5D and F) cells following PEITC (0, 5 and 10 μ M) treatment for 3 days. Collectively, the data indicated that PEITC reactivated CDH1, which inhibited the Wnt/ β -catenin signaling pathway.

Discussion

Breast cancer is the most frequently diagnosed malignancy and leading cause of cancer-related deaths in women worldwide. BCSCs are considered the origin of heterogeneous breast cancer cells and are attractive prospects for therapy (27). Previous studies have identified that PEITC, a novel epigenetic regulator, exerts antitumor effects on malignant cells (9,19). Previous studies have also revealed that CDH1, a tumor suppressor frequently silenced by promoter hypermethylation in solid tumors including breast cancer, plays a crucial role in reversing CSC properties (16,23). It has also been demonstrated that PEITC has the potential to eradicate CSCs in cervical, colorectal and ovarian cancer (13,35,36) as well as breast cancer cells including the aggressive subtype such as triple negative breast cancer cells (37,38). However, to the best of our knowledge, no study to date, has shown the epigenetic effects of PEITC on CDH1 in BCSCs. In the present study, it was demonstrated for the first time that PEITC reduced BCSC-like properties via epigenetic reactivation of CDH1, thus inhibiting the Wnt/ β -catenin pathway (Fig. 6).

Accumulating studies have demonstrated that tumors are composed of a hodgepodge of cancer cells with diverse functions and phenotypes. As a remarkably small proportion of cancer cells, CSCs have the potential to generate self-renewal and heterogeneous tumor cell lineages that contribute to

cancer initiation, metastasis, progression, therapy resistance, and tumor relapse (39). Therefore, it is critical to develop effective ways to eradicate or differentiate them during antitumor therapy. Natural products have drawn considerable attention for their marked antitumor activities (40). Recently emerging evidence indicates that natural products are promising new candidates to eliminate CSCs through multiple biological mechanisms such as targeting key signaling pathways or epigenetic regulation (41). Curcumin, a compound derived from the rhizome of turmeric, has been reported to reduce CSC-like properties by downregulating the Wnt/ β -catenin pathway in lung, colorectal, and breast cancer cells (42-44). Cruciferae sulforaphane (SFN), a natural compound present in cruciferous vegetables, has been revealed to inhibit nasopharyngeal carcinoma (NPC) stem cells through the DNMT1/Wnt inhibitory factor 1 (Wif1) axis (45). In the present study, we examined the effects of PEITC, also a natural bioactive compound, on breast cancer cells and revealed that it could significantly reduce BCSC-like properties *in vitro*. Previous research has demonstrated PEITC to function as a tumor killer by inhibiting the CD44v-xCT axis in colorectal CSCs and the Sp1 transcription factor in cervical CSCs (46,47). The mechanism of PEITC on BCSC-like properties was further investigated in the present study.

Extensive studies have indicated that epigenetic alteration in cancer cells including DNA methylation, histone modifications, and chromatin remodeling maintains CSC-like properties (48). CDH1, a tumor suppressor gene, has been revealed to be transcriptionally silenced due to promoter hypermethylation during breast cancer progression (22). Previous studies have revealed that reversing CDH1 methylation could reactivate CDH1 expression and reduce

CSC-associated properties (16,23). Recent investigations into the use of PEITC as an epigenetic modifier have provided new insights into its anticancer effects (19,26). Thus, it was hypothesized that PEITC may epigenetically regulate CDH1 to reduce BCSC-associated properties. By using MSP, it was observed that the CDH1 promoter region was hypermethylated in untreated MCF-7 and MDA-MB-231 cells, consistent with a previous study by Pradhan *et al* (49). However, after PEITC (5 or 10 μ M) treatment, both MCF-7 and MDA-MB-231 cells exhibited significant demethylation of the CDH1 promoter. CDH1 promoter demethylation was also observed in the positive controls, 5-Aza (2.5 μ M) and TSA (0.5 μ M) treated-cells. It was confirmed by qPCR and western blotting that PEITC could restore CDH1 mRNA and protein levels in both MCF-7 and MDA-MB-231 cells. These results are similar to several other studies that have revealed reactivation of CDH1 by epigenetic reagents such as epigallocatechin-gallate (EGCG) and Bisphenol S (BPS) (50,51). Notably, although the consistent biological behavior of epigenetic regulations in breast cancer cells between PEITC-treated groups and positive controls (2.5 μ M 5-Aza and 0.5 μ M TSA) were observed, it still could not be excluded whether there are other mechanisms involved in PEITC reactivation of CDH1 in addition to the epigenetic effects which played a major role. Moreover, while the demethylating effects of the positive controls (2.5 μ M 5-Aza and 0.5 μ M TSA) were stronger than that of 10 μ M PEITC, CDH1 mRNA and protein levels were lower, suggesting that PEITC exerts a greater effect on either histone modification or chromatin relaxation. Previous research has characterized the histone modification effects of PEITC to a certain degree (48), but further study is required to elucidate this observed phenomenon. DNA methylation and histone deacetylation, mediated by DNMTs and HDACs, respectively, are the most common epigenetic events that occur with tumor progression and lead to the silencing of tumor suppressor genes. Thus, the effects of PEITC on DNMTs and HDACs were further examined. It was revealed that PEITC (5 or 10 μ M) could significantly reduce DNMT and HDAC protein levels. However, the mechanism by which PEITC inhibits DNMTs and HDACs remains unclear, requiring further study.

Numerous studies have shown that the Wnt/ β -catenin pathway is constitutively active in the development of breast cancer and plays a role in regulating the self-renewal of BCSCs (23,27). A previous study revealed that the epigenetic reactivation of CDH1 could promote assembly of the CDH1/ β -catenin complex in the cytoplasmic membrane and prevent β -catenin translocation into the nucleus, thereby inactivating the Wnt/ β -catenin pathway and suppressing carcinoma cell stemness (16). In the present study, after identifying that PEITC could epigenetically restore CDH1, the effects of PEITC on the CDH1/ β -catenin complex and Wnt/ β -catenin pathway were assessed. Co-IP and western blotting indicated that PEITC significantly increased the amount of inactive β -catenin (phospho-Ser33/37) that co-precipitated with CDH1. TCF-4 is a key effector in CSC-like trait maintenance by forming the β -catenin/TCF4 complex, which transcriptionally activates downstream factors such as cyclin D1 in the Wnt pathway (34,52). The present study revealed that PEITC (5 or 10 μ M) significantly decreased the protein expression levels of active β -catenin (phospho-Ser675), TCF-4 and cyclin D1.

A previous study revealed that PEITC could inhibit the Wnt/ β -catenin pathway to eliminate colorectal CSCs (13). However, the present study elucidated for the first time that epigenetic regulation by PEITC reactivated CDH1, which inhibited the Wnt/ β -catenin pathway and reduced BCSC-like properties.

There are some limitations to the present study. It is well known that the dose-dependent effects of drugs and the optimal effective concentration revealed by *in vitro* experiments are useful for further development of promising anticancer drugs. However, we only used 2 sublethal doses of PEITC, 5 and 10 μ M, to perform the experiments in the present study. Thus, we cannot rigorously conclude that the effects and mechanism of PEITC function to reduce BCSC-like properties in a dose-dependent manner or identify the optimal concentration of PEITC. In addition, due to limitations of the experimental conditions at our laboratory and some objective reasons such as the COVID-19 pandemic, we did not isolate special BCSC subtypes such as CD44⁺/CD24⁻ by flow cytometry to further evaluate and characterize the function and mechanism of PEITC in eradicating BCSCs in a nude mouse tumor xenograft model, which needs to be conducted in the future.

In conclusion, the present study revealed a novel epigenetic regulation-mediated mechanism by which PEITC reduced CSC-like properties in breast cancer cells. It was revealed that PEITC significantly inhibited colony and tumor sphere formation abilities and reduced the expression of CSC-associated protein markers via epigenetic reactivation of CDH1. Inhibitory effects of PEITC on the expression of DNMTs and HDACs resulted in demethylation of the CDH1 promoter region. CDH1 then inhibited the Wnt/ β -catenin pathway and formation of the β -catenin/TCF transcription factor complex to suppress CSC-like properties. The present findings suggest that PEITC is a potential natural product that can be further developed for the eradication of breast cancer stem cells.

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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

TZ conceived and designed the experiments. TZ and WZ performed the experiments. TZ and MH analyzed the data. TZ wrote the manuscript. TZ and MH revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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