ZDHHC22-Mediated mTOR Palmitoylation Determines Breast Tumor Growth and Endocrine Therapy Sensitivity

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Research

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Abstract

Background

Palmitoylation is essential for classic hallmarks of cancer, regulating protein stability and protein-protein interactions. Targeting palmitoylation or palmitoyltransferases has become a novel strategy for therapeutic intervention in cancer. However, few studies have reported the expression pattern, pathological functions and underlying mechanism of ZDHHC22 in breast cancer.

Methods

Public database analysis, quantitative reverse transcription PCR, and western blot were used to analyze ZDHHC22 expression. Methylation-specific PCR was employed to detect the promoter methylation status. Expression of mTOR was examined by immunofluorescence staining and western blots, and palmitoylation of mTOR was detected by acyl-biotin exchange assays. Cellular functions were evaluated via corresponding molecular biological and cellular approaches. Nude mice were used to generate a xenograft tumor model.

Results

We investigated the expression profiles of 23 palmitoyltransferases using data from The Cancer Genome Atlas and found that ZDHHC22 is frequently expressed at minimal levels in multiple cancers. Downregulation of ZDHHC22 is associated with methylation of its promoter in breast cancer. Ectopic ZDHHC22 expression inhibits the malignant properties of breast cancer both in vitro and in vivo. The enzyme-active site-mutation ZDHHC22-(C111A) mutation reversed these effects, demonstrating that ZDHHC22 suppresses the malignant properties of breast cancer through palmitoylation. Bioinformatics analysis predicted mTOR as the substrate of ZDHHC22. mTOR palmitoylation for the first time was detected and we found ZDHHC22 reduced the stability of mTOR via regulating the palmitoylation of mTOR. Additionally, mTOR expression was frequently negatively correlated with ZDHHC22 in tamoxifen-resistant breast cancer cells and ectopic ZDHHC22 could restore endocrine sensitivity in tamoxifen-resistant breast cancer cell line.

Conclusion

Our results demonstrating the hypermethylated status and potential tumor suppressor function of ZDHHC22 in breast cancer for the first time established a regulatory mechanism between ZDHHC22 and mTOR palmitoylation, which may pave the way for the development of promising therapeutics that restore endocrine sensitivity.

Background

Breast cancer is the most frequently occurring cancer and the leading cause of cancer death among females worldwide [1]. Although systemic therapy has markedly improved prognosis of breast cancer
patients, many breast cancer patients inevitably develop recurrence and metastasis, which presents a large clinical difficulty [2]. Classic hallmarks of cancer such as sustained proliferation, resistance to cell death, and activation of metastasis are regulated by dynamic post-translational modifications (PTMs) [3–5].

One mode of PTM, palmitoylation, involves the attachment of the 16-carbon atom fatty acid palmitate onto the modified protein, regulating protein localization, stability, trafficking, and protein–protein interactions [6]. S-palmitoylation occurs at particular cysteine residues and dynamically regulates the assembly and partition of proteins due to the reversible thioester bond [7–9]. More than 2,000 human proteins present evidence of S-palmitoylation [10]. Recently, not surprisingly, S-palmitoylation has increasingly been related to numerous cancer-associated pathways or regulators such as Wnt [11], EGFR [12], Ras [13], PD-L1 [14], and BAX [15].

Enzymes that catalyze S-palmitoylation form a family of 23 palmitoyltransferases containing a highly conserved Asp-His-His-Cys (DHHC) motif within a cysteine-rich domain [7, 16]. Most zinc finger DHHC-type (ZDHHC) enzymes are localized at the endoplasmic reticulum and the Golgi apparatus, with only a few localized at endosomes or the plasma membrane [6]. An increasing body of evidence links ZDHHC enzymes to tumorigenesis, cancer cell proliferation, and therapy resistance in various cancers [4, 17]. Thus, targeting palmitoylation or palmitoyltransferases has become a novel strategy for therapeutic intervention in cancer [18–21]. We explored the expression patterns of 23 palmitoyltransferases using HiSeq data from The Cancer Genome Atlas (TCGA) dataset and identified ZDHHC22 for further research because it is frequently expressed at minimal levels in multiple cancers. Previous findings have reported that ZDHHC3, ZDHHC9, and ZDHHC20 play important roles in breast cancer treatment [14, 19, 22]. However, few studies have reported the physiological or pathological functions of ZDHHC22.

We revealed that ZDHHC22 expression was downregulated in breast cancer cell lines and tissues, which was associated with methylation of its promoter. Ectopic ZDHHC22 expression inhibited the malignant properties of breast cancer both in vitro and in vivo. We found that ZDHHC22 was epigenetically silenced and functions as a tumor suppressor gene. Next, we hypothesized that ZDHHC22 suppressed breast cancer growth via regulating the palmitoylation and stability of the mammalian target of rapamycin (mTOR). mTOR, as a key downstream effector of the PI3K/AKT pathway, was a serine/threonine kinase in the PI3K-related kinase (PIKK) family, regulating cell growth, metabolism, and treatment resistance [23]. Several studies have revealed that many crucial regulators in the mTOR pathway, including ATG, GATOR1 and 2, TSC, and Ragulator are predicted to be palmitoylated [24, 25]. However, whether mTOR itself is regulated by palmitoylation has not been fully determined. Our study for the first time revealed that mTOR was regulated by protein palmitoylation and elucidated the physical and regulatory relationship between ZDHHC22 and mTOR. The ZDHHC22 reduced the stability of mTOR via regulating the palmitoylation of mTOR. Besides, we found that mTOR is always overexpressed and that ZDHHC22 expression was often downregulated in tamoxifen-resistant breast cancer cells. Ectopic ZDHHC22 could restore the sensitivity upon tamoxifen therapy, which deserves further studies.
Collectively, our results demonstrated the hypermethylated status and the pathological function of ZDHHC22 in breast cancer, established a regulatory mechanism between ZDHHC22 and mTOR palmitoylation, and may pave the way for the development of promising therapeutics that modulate mTOR palmitoylation (Graphical Abstract).

Materials And Methods

Cell lines and tissue specimens

The human breast cancer cell lines BT-549, MCF-7, MDA-MB-231, MDA-MB-468, SK-BR-3, T47D, YCC-B1, and ZR-75-1 and the normal mammary epithelial cell lines HMEC and MCF-10A were acquired from American Type Culture Collection. Breast cancer cell lines were cultured in PRMI-1640 (Gibco BRL, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco BRL, Karlsruhe, Germany) at 37°C with 5% CO₂. The tamoxifen-resistant MCF7 (MCF-7R) breast cancer cell was a gift from Dr. Lin [26] (Shantou Affiliated Hospital of Sun Yat-Sen university) and was cultured in RPMI-1640 without phenol red and L-glutamine (Biological industries, Haemek, Israel) containing 10% charcoal-stripped FBS (Biological industries, Haemek, Israel) and 1 μM 4-hydroxytamoxifen (Sigma-Aldrich, MO, USA). Human breast cancer specimens and paired normal tissues used for quantitative real-time PCR and methylation-specific PCR (MSP) were collected at the First Affiliated Hospital of Chongqing Medical University. Each patient provided informed consent. The acquisition of human specimens was authorized by the Institutional Ethics Committee of the First Affiliated Hospital of Chongqing Medical University.

Plasmids and generation of stable cell lines

To generate ZDHHC22 and ZDHHC22 mutation expression vectors, the ZDHHC22 full-length gene with or without C111 mutation was inserted into a pCMV6-Entry plasmid. The recombinant DNA plasmid was screened and sequenced. pCMV6-Entry, pCMV6-ZDHHC22, and pCMV6-ZDHHC22-Mut were transfected into BT-549, SK-BR-3, and YCC-B1 cells using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's protocol. After 48 hours, cells were treated with G418 (200 μg/mL for BT-549, 500 μg/mL for SK-BR-3, and 400 μg/mL for YCC-B1). Stable overexpression of ZDHHC22 and ZDHHC22-Mut was confirmed by western blot and RT-PCR before other assays.

Reverse transcription PCR (RT-PCR), qPCR and MSP

Total RNA was isolated from cell and tissue samples using TRIzol® (Invitrogen, Carlsbad, CA). Reverse transcription was performed using the Promega GoScript™ reverse transcriptase (Promega, Madison, WI). RT-PCR was performed using Go-Taq (Promega, Madison, WI, USA) and 2% agarose gels. β-actin was used as a reference control. Quantitative reverse transcription PCR (qPCR) was performed using SYBR
Green (Thermo Fisher, Waltham, MA, USA) and 7500 Real-Time PCR System (Life technologies, Thermo Fisher, USA). GAPDH was used as a reference control. The primer sequences are listed in Table S1 and S2 (Additional file 2). MSP was performed using AmpliTaq®-Gold DNA polymerase and the results were analyzed as characterized previously.

**Cell proliferation assay**

Cells were seeded in a 96-well plate at a density of 2,000 cells per well. After 24, 48, and 72 hours, the Cell Counting Kit 8 (CCK-8, Beyotime, Jiangsu, China) was used to measure cell viability according to the manufacturer's protocol. At the indicated time points, optical density (OD) values were measured using a microplate reader (Infinite 200 PRO, TECAN, Männedorf, Switzerland) at a wavelength of 450 nm. Each assay was repeated three times independently.

**Colony formation assay**

Cells were seeded into 6-well plates at 800 cells per well and incubated for 14 days. Cells were fixed with 4% paraformaldehyde and stained with crystal violet (C0121, Beyotime). Visible colonies were counted and experiments were repeated three times.

**Flow cytometry analysis of cell cycle and apoptosis**

For the cell cycle assay, cells were transfected with pCMV6-Entry, pCMV6-ZDHHC22, or pCMV6-ZDHHC22-(C111A). After 48 hours, cells were collected and fixed in 70% cold ethanol overnight. Fixed cells were incubated with 100 μL RNase A (0.1 mg/mL) at 37°C for 30 min and then stained with 5 μL propidium iodide (PI, Sigma-Aldrich, St. Louis, MO, USA) at room temperature for another 30 min in the dark. Data were collected using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, US) and analyzed using CellQuest™ software (BD Biosciences). For the apoptosis assay, transfected cells were harvested. After incubation with APC annexin V (BioLegend, San Diego, CA) and PI (BioLegend, San Diego, CA) at room temperature for 30 min, the proportion of apoptotic cells was analyzed with the FACSCalibur flow cytometer. Experiments were repeated three times independently.

**Western blot**

Western blot was performed as previously described[27] with the following primary antibodies: anti-mTOR (1:100, sc-517464, Santa Cruz), anti-mTOR (1:1,000, #2972, Cell Signaling Technology), anti-phospho-IGF-1 Receptor (1:1000, #3021, Cell Signaling Technology), anti-phospho-HER-2(1:100, sc-81507, Santa Cruz), anti-Protor1 (1:100, sc-390496, Santa Cruz), anti-DEPTOR (1:100, sc-398169, Santa Cruz), anti-PDK1 (1:200, sc-293160, Santa Cruz), anti-PI3K p85α (1:100, sc-1673, Santa Cruz), anti-phospho-PI3K (1:100, sc-12929, Santa Cruz), anti-phospho-PI3K (1:1000, #4228, Cell Signaling...
Technology), anti-AKT (1:1,000, #4691T, Cell Signaling Technology), anti-phospho-Akt(S473) (1:1,000, #4060T, Cell Signaling Technology), anti-PHLPP2 (1:1,000, ab71973, Abcam), anti-phospho-Akt (T308) (1:100, sc-271966, Santa Cruz), anti-phospho-BAD (1:1000, #5284, Cell Signaling Technology), anti-phospho-GSK3β(Ser9) (1:1,000, #9323, Cell Signaling Technology), anti-phospho-FoxO1(Ser256) (1:1000, #9461T, Cell Signaling Technology), anti-phospho-4EBP1 (1:200, sc-293124, Santa Cruz), anti-phospho-p70S6K (1:200, sc-8416, Santa Cruz), anti-GAPDH (1:1,000, sc-47724, Santa Cruz), and anti-myc-Tag (1:1,000, #2276s, Cell Signaling Technology).

**Immunofluorescence**

Cells were seeded on coverslips and then transfected with pCMV6-Entry, pCMV6-ZDHHC22, or pCMV6-ZDHHC22-(C111A). After 48 hours, cells were fixed with 4% paraformaldehyde for 20 min and then permeabilized with 0.5% Triton X-100 for 10 min. After blocking with blocking buffer, cells were incubated with primary antibodies overnight at 4°C and then incubated with secondary antibodies for 1 hour at 37°C. DAPI (Roche, Palo Alto, CA, USA) was used for DNA counterstaining. Photomicrographs were acquired with a confocal laser scanning microscope (Leica, Hilden, Germany). The following antibodies were used for immunofluorescence: anti-Flag (1:400, #14793, Cell Signaling Technology), anti-mTOR (1:200, #2983, Cell Signaling Technology), anti-phospho-Akt(S473) (1:200, #4060T, Cell Signaling Technology), anti-PHLPP2 (1:40, ab71973, Abcam), ER-Tracker Red (1:2000, C1041, Beyotime), CoraLite488 (1:200, SA00013-1, Proteintech), and CoraLite594 (1:200, SA00013-4, Proteintech).

**Immunoprecipitation and acyl-biotin exchange assay**

The acyl-biotin exchange (ABE) assay was performed to detect protein palmitoylation as previously described by Brigidi et al.[28]. First, target protein was purified with a specific antibody and immobilized on magnetic beads. The purified target protein was then treated with N-ethylmaleimide to irreversibly block the free thiol groups combined with unmodified cysteine residues. Secondly, the protein was treated with hydroxylamine, which leads to specific cleavage of palmitoylated cysteine residues and exposure of a free palmitoylated thiol group. The palmitoylated thiol group was subjected to specific biotinylation with biotin-BMCC. Finally, the biotinylated target protein was eluted for western blot analysis with a streptavidin-horseradish peroxidase (HRP) antibody to quantify palmitoylation of the purified protein.

**In vivo tumor xenograft model**

Animal experiments were conducted in line with guidelines approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University. BT-549 cells stably transfected with ZDHHC22, ZDHHC22-Mut, or empty vector (1 × 10^8 cells) were injected subcutaneously into the mammary fat pads of female nude mice (aged 4–6 weeks, weighing 18–22 grams, five mice per group). Xenograft size was measured every 2 days by a Vernier caliper and then calculated using the following formula: tumor
volume = 0.5 × length × width². After 15 days of injection, the mice were sacrificed and the xenografts were isolated and measured. Xenografts were fixed in formalin for 48 hours and dehydrated overnight. After embedding into paraffin, the xenografts were sliced into 4 μm sections for immunohistochemistry (IHC) staining or immunofluorescence.

Immunohistochemistry (IHC) staining and TUNEL assays

IHC staining was conducted as previous published protocol with the following primary antibodies: anti-Ki-67 (1:200, #9449, Cell Signaling Technology). TUNEL detection kit (Beyotime Institute of Biotechnology, Nanjing, China) was subjected to TUNEL assays, and the results were acquired with a confocal laser scanning microscope (Leica, Hilden, Germany).

Gene Expression Omnibus dataset analysis

In order to identify gene sets and pathways associated with ZDHHC22, we acquired the gene expression data from the Gene Expression Omnibus (GEO) (datasets GSE65194 and GSE21653, which contain 152 and 266 breast cancer cases, respectively; https://www.ncbi.nlm.nih.gov/geo/). Patients were classified into the ZDHHC22-high and ZDHHC22-low groups according to the median ZDHHC22 expression level (229805_at). Gene set enrichment analysis (GSEA) analysis was completed using Broad Institute GSEA software 4.0 as previously described [29]. If the normal P-value was below 0.05 and the false discovery rate was below 0.25, genes were considered significantly enriched.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 7.0 and IBM SPSS 22.0 software. All results are typical of at least three independent in vitro assays. The two-tailed Student’s t-test, the chi-square test, and Fisher’s exact test were used to determine assay results. If the p-value was below 0.05, results were considered statistically significant.

Results

The ZDHHC22 is downregulated in multiple cancers and associated with favorable clinical outcomes in breast cancer.

First, we compared the transcription levels of 23 ZDHHC enzymes using HiSeq data from TCGA containing breast cancer (BrCa), lung adenocarcinoma (LUAD), esophageal carcinoma (ESCA), stomach adenocarcinoma (STAD), colon adenocarcinoma (COAD), kidney renal clear cell carcinoma (KIRC), and bladder urothelial carcinoma (BLAC) samples. The analysis revealed that ZDHHC22 was always
expressed at a minimal level in multiple cancers (Additional file 1: Fig. S1A). We next detected the 23 ZDHHC enzymes expression using reverse transcription PCR in breast cancer cell lines and found that ZDHHC22 was maintained at the relatively low level in all breast cancer cells, especially in SK-BR-3 and YCC-B1 (Fig. 1A) [30]. Results from 733 cases of invasive breast carcinoma in the TCGA database showed that ZDHHC22 (abbreviated as ZDC22 in the Figures) mRNA expression was decreased in HER2-enriched and basal-like breast carcinoma, which had been verified as “more aggressive” subtypes, comparing with luminal-like subtypes (Fig. 1B). Importantly, Kaplan-Meier plots, which included 1764 cases from the GEO and TCGA databases, revealed that higher ZDHHC22 expression was associated with better relapse-free survival in breast cancer patients (Fig. 1C). Thus, we inferred ZDHHC22 was a potential tumor suppressor in breast cancer development. However, our knowledge of the role of ZDHHC22 in breast cancer was limited, so it was selected for further investigation.

**Promoter methylation of ZDHHC22 leads to its downregulation in breast cancer.**

Quantitative reverse transcription PCR confirmed ZDHHC22 expression was decreased in breast cancer comparing with adjacent tissues from 16 patients (Fig. 1D). To investigate the reason that was responsible for this decrease, we analyzed its sequence using PRABI-Doua and found that there was a CpG island in the ZDHHC22 promoter (Additional file 1: Fig. S1C). Analysis of data from the TCGA database indicated the existence of hypermethylation in the ZDHHC22 promoter in breast cancer (Additional file 1: Fig. S1B). To explore whether CpG hypermethylation may be a crucial mechanism regulating ZDHHC22 expression, DNA samples from 64 breast tumors and adjacent tissues were used for an MSP assay. Hypermethylation status was found in 27 (42.2%) breast tumors, while this was observed in none of the 6 normal breast tissues (Fig. 1E and 1F). Besides, ZDHHC22 was frequently hypermethylated in breast cancer cell lines such as MDA-MB-231, YCC-B1 and ZR-75-1 (Fig. 1F). As shown in Figure 1G and 1H, both qPCR and MSP assay confirmed that demethylation treatment efficiently restored ZDHHC22 expression in MDA-MB-231 and YCC-B1, validating that promoter methylation contributes to inhibition of ZDHHC22 expression in breast cancer.

**The ZDHHC22 restrains breast cancer cell growth by inhibiting cell proliferation through cell cycle arrest and inducing cell apoptosis.**

Gain-of-function experiments were performed in order to research the cellular function of ZDHHC22 in breast cancer. Firstly, BT-549, SK-BR-3, and YCC-B1 cells were stably transfected with pcDNA3.1-ZDHHC22 or control plasmids. Because S-palmitoylation is catalyzed through the DHHC motif of palmitoyl S-acyltransferases[4, 7], we inserted a point mutation (C111A) in the DHHC motif of ZDHHC22 (abbreviated as ZDC22-mut in the Figures) (Fig. 2A) to analyze whether the suppressive effects of
ZDHHC22 on breast cancer progression depend on its enzymatic activity. Restoration of ZDHHC22 was confirmed by reverse transcription PCR and western blot (Fig. 2B). Ectopic ZDHHC22 expression suppressed proliferation in all of the cell lines, as detected by CCK-8 and colony formation assays (Fig. 2C-2E). Cell viability in the C111A mutation group was close to the control group, suggesting that the suppressive effects of ZDHHC22 on breast cancer proliferation probably depend on its enzymatic activity.

We next detected the cell cycle distribution by flow cytometry and found that ZDHHC22 significantly increased the proportion of cells in the G0/G1 phase while it decreased the proportion of cells in the G2/M phase (Fig. 3A and Additional file 1: Fig. S2A). Annexin V/PI staining and Flow cytometry analysis also revealed that the proportion of apoptotic cells was increased in the ZDHHC22 group comparing with the control and C111A groups (Fig. 3B and Additional file 1: Fig. S2B). These results indicated that ZDHHC22 inhibited breast cancer cell growth through cell cycle arrest and apoptosis induction. Additionally, ectopic ZDHHC22 expression significantly reduced the number of migrated and invaded cells, as detected by Transwell assay (Fig. 3C-D and Additional file 1: Fig. S3A-B).

To investigate the effects of ZDHHC22 on tumor growth in vivo, the xenograft model of nude mouse bearing BT-549 cells was established. Tumor growth in the ZDHHC22 group was remarkably slower than that in the control group and C111A group (Fig. 4A). Volume and weight of the harvested xenografts in different groups exhibited the similar trend (Fig. 4B and 4C). IHC staining revealed that tumors in the ZDHHC22 group had a lower percentage of Ki-67 positive cells than tumors in the other two groups (Fig. 4D), indicating ectopic ZDHHC22 expression suppressed the proliferation of breast cancer. The TUNEL staining of the primary tumors showed that the proportion of apoptotic breast cancer cells was increased after ZDHHC22 overexpression (Fig. 4E).

The ZDHHC22 reduces activation of AKT.

To further research the molecular mechanisms by which ZDHHC22 inhibits breast cancer, GSEA analysis was performed with data from the GEO database (datasets GSE65194 and GSE21653). The results indicated that the AKT/protein kinase B (PKB) pathway and PKB-mediated events were significantly correlated with ZDHHC22 (Fig. 5A and 5B). It has been verified that activation of the AKT signaling pathway facilitates cell growth and survival, thus we hypothesized that ZDHHC22 overexpression might suppress tumor growth through reducing the activation of AKT pathway. We next assessed a subset of proteins belonging to the AKT pathway by western blots. The ZDHHC22 overexpression led to decreased phosphorylation of AKT at Ser-473 (p-AKT(Ser-473)) comparing with the control and C111A groups. In contrast, phosphorylation at Thr-308 and total AKT protein levels remained unchanged (Fig. 5C). In addition, the phosphorylation level of FOXO1, GSK3β, and Bad, the downstream substrates of p-AKT(Ser-473), was also decreased in the ZDHHC22 group (Fig. 5C). The levels of PDK1, which directly phosphorylates Akt at Thr-308[31, 32], also remained unchanged in the ZDHHC22 group (Fig. 5C).

The phosphorylation of AKT at Ser-473 is regulated by two main phosphatases/complexes, PHLPP2, a phosphatase that directly dephosphorylates AKT(Ser-473)[33], and mTORC2, a kinase complex that
phosphorylates AKT (Ser-473) [34]. As shown in Figure 5C and Figure S4A, PHLPP2 showed similar expression levels in the three groups confirmed by western blot and immunofluorescence assays. The mTORC2 consists of six components: mTOR, mLST8, Rictor, DEPTOR, mSin1, and Protor1/2 [23]. We predicted that only mTOR, DEPTOR and Protor1 might be regulated by S-palmitoylation using SwissPalm [10] and TermiNator [35] online tools (Additional file 2: Table S3). As shown in Figure 5C, the expression of total mTOR was decreased by ZDHHC22 overexpression, while the expression of DEPTOR and Protor1 remained unchanged. Immunofluorescence assays also revealed that that ZDHHC22 decreased the expression of mTOR and promoted the generation of apoptotic bodies after 72 hours of transfection, while C111A groups remained unchanged (Fig. 6A and Additional file 1: Fig. S4B), and these changes are in line with the decrease of AKT phosphorylation (Fig. 6B) and the increase of cell apoptosis after ZDHHC22 overexpression. Taken together, we supposed that it might be the palmitoylation of mTOR by ZDHHC22 that led to the decrease of mTOR expression, then restricted the kinase activity of mTORC2, and resulted in the decreased phosphorylation of AKT at Ser-473 eventually.

Interestingly, we observed that the phosphorylation of PI3K and the upstream receptor tyrosine kinases (RTKs), including IGF-1R and HER2, were increased in the ZDHHC22 group, which was opposite to the change of p-AKT (Ser-473). Some researchers have reported that AKT inhibition induces reactivation of PI3K and multiple RTKs, in part due to the mTORC1 inhibition [36, 37]. In our results, the expression of mTOR and the phosphorylation level of pS6K kinase and 4E-BP1, the downstream substrates of mTORC1, were decreased in the ZDHHC22 group (Fig. 5C). Taken together, we hypothesized that ZDHHC22 might decrease both the activation of mTORC2 and mTORC1 through attenuating mTOR expression via palmitoylation.

The ZDHHC22 attenuates mTOR stability via palmitoylation.

In order to explore whether mTOR is regulated by ZDHHC22, we first examined the interaction between ZDHHC22 and mTOR. The binding of ZDHHC22 and endogenous mTOR was demonstrated by co-immunoprecipitation assays in cells that stably overexpress ZDHHC22. The results revealed that ZDHHC22 bound to mTOR and vice versa (Fig. 7A). The Immunofluorescence assay confirmed that ZDHHC22 mainly co-localized with mTOR (Fig. 6A). Moreover, ZDHHC22-mut (C111A) also bound to mTOR (Fig. 7A and Additional file 1: Fig. S4B), indicating that the C111A mutation just affected the palmitoyltransferase activity, but not the binding affinity between ZDHHC22 and mTOR.

Many of the crucial effectors in the mTOR pathway are predicted to be palmitoylated [24, 38], however, whether mTOR itself is regulated by palmitoylation has never been reported. To further investigate how ZDHHC22 affected mTOR, we first confirmed that mTOR was regulated by palmitoylation. After being treated with 25 μM 2-bromopalmitate (2-BP), a general palmitoylation inhibitor, mTOR expression was significantly elevated in BT-549 and SK-BR-3 cells, which was consistent with the increased phosphorylation of AKT at Ser-473 (Fig. 7B). Next, we subjected BT-549 and SK-BR-3 cells to ABE assays to directly detect the protein palmitoylation levels of mTOR [28]. As shown in Fig. 7C, palmitoylation of
mTOR was detected by western blot with streptavidin-HRP. Then we investigated whether mTOR palmitoylation was regulated by ZDHHC22. The ABE assay results indicated that ZDHHC22 increased mTOR palmitoylation, and these effects were abolished by the C111A mutation (Fig. 7D). Since palmitoylation is an important post-translational modification that could regulate protein localization, stability and protein-protein interactions[6], the half-life of mTOR was next determined by treatment with the protein synthesis inhibitor cycloheximide. The results showed that ZDHHC22 overexpression (but not ZDHHC22-C111A mutation) significantly increased the turnover rate of mTOR in both BT-549 and SK-BR-3 cells (Fig. 7E,F and Additional file 1: Fig. S5A,B).

The ZDHHC22 restores sensitivity toward endocrine therapy.

The ZDHHC22 exhibited an inhibitory effect on mTOR through reducing its stability. Clinically, the mTOR inhibitors are often applied to restore the sensitivity to endocrine therapy in metastatic breast cancer patients[39]. Thus, we hypothesized that there might be a potential correlation between ZDHHC22 and breast cancer endocrine-resistance. Analysis of the GEO database (datasets GSE7327, GSE21618, and GSE26459) indicated that mTOR was always overexpressed and ZDHHC22 was frequently downregulated in tamoxifen-resistant breast cancer (Fig. 8A). To confirm these results, tamoxifen-resistant breast cancer cell (MCF-7R) and parental cell (MCF-7) were used to detect the expression of mTOR and ZDHHC22. As shown in Figure 8B and 8C, the mRNA and protein level of mTOR was significantly increased while the ZDHHC22 protein level was significantly decreased in MCF-7R cells comparing with MCF-7 cells. CCK-8 assay showed that overexpression of ZDHHC22 in MCF-7 cells suppressed cell proliferation, but did not enhanced the tamoxifen treatment. However, ZDHHC22 overexpression significantly re-sensitized MCF-7R cells to tamoxifen treatment (Fig. 8D).

Finally, we evaluated the effects of ZDHHC22 combined with mTOR kinase inhibitor or PI3K inhibitor. Cells were treated with KU-0063794, an ATP-competitive inhibitor of mTOR which effectively inhibited mTORC2 and mTORC1[40]. The CCK-8 results showed that the cell growth rate of the ZDHHC22 group was similar to that of the 10 μM KU-0063794 group, and combination of ZDHHC22 and KU-0063794 exhibited a synergistic reaction (Fig. 8E). Moreover, the combination of ZDHHC22 and the PI3K inhibitor Taselisib (10 μM) displayed the same synergism (Fig. 8F), which supported our previous finding that ZDHHC22 led to the inhibition of mTOR and the activation of PI3K.

Discussion

Palmitoylation has been implicated in the development of numerous human cancers. Fang et al. found that ZDHHC5 contributed to the development of p53-mutant glioma through altering the palmitoylation and phosphorylation status of EZH2[41]. Hemler et al. revealed that ZDHHC3-mediated protein palmitoylation regulated breast cancer growth, cellular oxidative stress and senescence[19]. In our study, ZDHHC22, consistent with many genes whose promoters are hypermethylated, functions as a tumor
suppressor in breast cancer. We first detected reduced ZDHHC22 expression and elevated promoter methylation in breast cancer cell lines and tissues. Demethylation therapy efficiently restored ZDHHC22 expression, validating that promoter methylation contributed to the inhibition of ZDHHC22 expression in breast cancer. Furthermore, ZDHHC22 overexpression suppressed the malignant capabilities of breast cancer both in vitro and in vivo. These results indicated that ZDHHC22 could be an epigenetically inhibited tumor suppressor gene in breast cancer. The DHHC motif of palmitoyltransferase is associated with its enzymatic activity. The active domain of ZDHHC22, cysteine 111, was predicted using Scanprosite online tool and we introduced a cysteine 111 to alanine point mutation for the loss of palmitoyltransferase activity of ZDHHC22. The ZDHHC22-(C111A) mutation significantly reversed the effects of ZDHHC22 overexpression, suggesting that the suppressive effects of ZDHHC22 on breast cancer progression depend on its palmitoyltransferase activity.

Western blot results showed that ectopic ZDHHC22 expression decreased the levels of p-AKT(Ser-473) and its downstream substrates, while increased the levels of p-PI3K and its upstream substrates. Rosen et al. found that inhibition of mTOR led to AKT Serine 473 dephosphorylation and then relieved feedback inhibition of RTKs which led to reactivation of PI3K[36]. Additionally, SwissPalm and TermiNator online tools predict mTOR as the substrate of ZDHHC22. Thus, we hypothesized that ZDHHC22 dephosphorylates AKT through regulating mTOR (both mTORC2 and mTORC1 complexes). Immunofluorescence assay confirmed that ZDHHC22 mainly co-localized with mTOR at the plasma membrane and in the endoplasmic reticulum (Fig. 6A and Additional file 1: Fig. S4C), which exactly the two major locations where AKT was recruited by PtdIns-3,4,5-P_3 (PIP_3) and phosphorylated by mTORC2[42]. Previous studies have documented treatment with mTORC1 inhibitor exerts negative feedback effects on AKT to RTK signaling, most particularly to IGF1 and IGF1 receptors, through decreasing the phosphorylation of S6K[43, 44]. In our study, we also observed similar results that both mTORC1 and its downstream substrates, the phosphorylation of S6K and 4E-BP1, were decreased due to ZDHHC22 overexpression, which further led to activation of AKT upstream substrates, both phosphorylation of RTKs and PI3K. In other words, inhibition of mTORC1 mediated by ZDHHC22 relieves the negative feedback of AKT upstream substrates. Collectively, we confirmed that ZDHHC22 inhibits mTOR (both mTORC2 and mTORC1) and its downstream, then relieves the negative feedback between mTORC1 and RTKs.

A lot of cancer-associated regulators, such as Wnt[11], EGFR[12], Ras[13], PD-L1[14], and BAX[15], have been confirmed to be regulated by palmitoylation; however, whether mTOR is regulated by palmitoylation has not been fully determined. We first performed gain-of-function assays with ZDHHC22-(C111A) cells in order to confirm whether ZDHHC22 suppressed the malignant properties of breast cancer through palmitoylation. As expected, the ZDHHC22-(C111A) mutation reversed the effects of ZDHHC22 overexpression, demonstrating that ZDHHC22 suppresses the malignant properties of breast cancer through palmitoylation. Second, to verify the interaction between ZDHHC22 and mTOR, co-immunoprecipitation and co-localization assays were performed and the binding of ZDHHC22 and endogenous mTOR was determined. Some studies predicted that mTOR may be regulated by
palmitoylation[24, 38]; however, to the best of our knowledge, the degree of mTOR palmitoylation has not been analyzed. Last but not least, palmitoylation of mTOR was detected by ABE assay and mTOR expression was significantly elevated after treatment with a palmitoylation inhibitor, suggesting that mTOR expression is regulated by protein palmitoylation. The effects of ZDHHC22 on mTOR palmitoylation were further validated by ABE assay; the results revealed that ZDHHC22 increased mTOR palmitoylation, and these effects were abolished by the C111A mutation. Since palmitoylation is essential for protein stability regulation and protein-protein interactions[4, 6]. Upon cycloheximide treatment, palmitoylated mTOR was degraded at a faster rate than control mTOR, which meant that ZDHHC22 palmitoylated mTOR to reduce its protein stability, leading to a reduction in the activity of downstream substrates of mTOR. These results validated the relationship between ZDHHC22 and mTOR and revealed an interesting PTM of mTOR.

Lots of studies have shown that the hyperactivation of mTOR causes resistance to a majority of anti-cancer therapies[45, 46]. Arteaga et al. reported that neratinib resistance in HER2-mutant cancers was closely correlated with mTOR hyperactivation[47]. Peng et al. found that KRAS-mutant lung cancer cells resisted to the chemotherapeutic drugs depending on the activation of mTOR signaling[48]. Clinically, the mTOR inhibitor everolimus is often used to restore the sensitivity to endocrine therapy in advanced hormone-receptor positive, HER2-negative breast cancer patients[39]. Our results found that mTOR was overexpressed in MCF-7R cells, and ZDHHC22 was downregulated at the same time. We next confirmed that ectopic ZDHHC22 expression restored the sensitivity to endocrine therapy in MCF-7R cells. To further confirm the restoration function of ZDHHC22 to endocrine therapy resistance, we think that the clinical specimens and patient-derived xenografts must be the better experimental models. However, due to the scarcity of endocrine therapy resistant clinical samples, we have to take it into a further and longer consideration.

Conclusions

Taken together, our present study authenticated that ZDHHC22 was downregulated through the promoter hypermethylation in breast cancer. And we confirmed that ZDHHC22 manifested a potential suppressive function on tumor growth and restored function on endocrine therapy resistance in breast cancer. Furthermore, for the first time, we revealed that mTOR was regulated by palmitoylation modification and elucidated the physical and regulatory relationship between ZDHHC22 and mTOR. The ZDHHC22 reduced the stability of mTOR depending on the palmitoylation modification, functioned as the second generation mTOR inhibitor. Clinically, ZDHHC22 may pave the way for the development of promising therapeutics that restore endocrine therapy sensitivity in breast cancer.

Abbreviations

ZDC22: ZDHHC22; ZDC22-mut: ZDHHC22-C111A mutation; BrCa: breast cancer; mTOR: the mammalian target of rapamycin; PTMs: post-translational modifications; PKB/AKT: protein kinase B; RTK: receptor tyrosine kinase; MCF-7R: tamoxifen-resistant MCF7; TCGA: The Cancer Genome Atlas; GEO: Gene
Expression Omnibus database; GSEA: Gene set enrichment analysis; qPCR: quantitative reverse transcription PCR; MSP: methylation-specific PCR; RT-PCR: Semiquantitative PCR; A+T: 5-Aza-2'-deoxycytidine and trichostatin A (TSA); OD: optical density value; ABE: acyl-biotin exchange assay; IHC: immunohistochemistry staining; DAPI: 4'-6-diamidino-2-phenylindole; PI: propidium iodide; M: methylated; U: unmethylated;

Declarations

Ethics approval and consent to participate

The study was authorized by the Institutional Ethics Committees of The First Affiliated Hospital of Chongqing Medical University and conformed to the principles of the Declaration of Helsinki.

Consent for publication

We have obtained consent to publish from the participant to report individual patient data.

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

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Authors’ Contributions

Project conceptualization and funding acquisition: TX and GR; Bioinformatic analysis: YL, YW and YT; Acquisition of data (In vitro and In vivo experiments): JL, JT, JH, ZY and YW; Data interpretation and supervision: TX and GR; Manuscript writing and finalization, TX, JL and JH. All authors reviewed and approved the final article.

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Figure 1

Expression and methylation of ZDHHC22 in breast cancer. (A) The mRNA expression level of 23 palmitoyltransferases in breast cancer cell lines were examined by RT-PCR. For purposes of exposition, breast cancer is abbreviated as BrCa in the Figures. (B) The ZDHHC22 mRNA expression in different breast cancer subtypes from TCGA database. For purposes of exposition, ZDHHC22 is abbreviated as ZDC22 in the Figures. (C) High ZDHHC22 expression is correlated with better relapse-free survival in 1764 breast cancer patients. Clinical data was acquired and analyzed by Kaplan-Meier plots database (www.kmplot.com/analysis/). (D) The ZDHHC22 mRNA expression in 16 paired breast cancer and adjacent tissues was examined by quantitative real-time PCR (qRT-PCR). Paired t-test, **p < 0.01. (E) The ZDHHC22 promoter methylation in breast cancer tissues was evaluated by methylation-specific PCR.
The ZDHHC22 promoter methylation in breast cancer cell lines and normal breast tissues were evaluated by MSP. (G-H) The expression of ZDHHC22 was effectively restored after demethylation treatment with Aza and TSA in breast cancer cell lines.

Figure 2

Ectopic ZDHHC22 inhibits breast cancer proliferation in vitro, while ZDHHC22-(C111A) mutation abolished these effects. (A) Prediction and mutation of the active site in ZDHHC22 DNA sequence using...
SCANSITE 4.0 (http://scansite.mit.edu/). The mutation site is shown as blue boxes. For purposes of exposition, ZDHHC22-(C11A) mutation is abbreviated as ZDC22-mut in the Figures. (B) Validation of ZDHHC22 and ZDHHC22-(C111A) mRNA and protein expression by qRT-PCR and western blot analyses, respectively. (C, D) Cell vitality was detected with CCK-8 assay and colony formation assay at the indicated time after transfection with Vector, ZDHHC22 and ZDHHC22-(C111A) plasmids in BT-549, SK-BR-3 and YCC-B1 cells. Data are presented as the mean ± s.d., n = 3, **p < 0.01, ***p < 0.001.

Figure 3

The ZDHHC22 induces apoptosis, cell cycle arrest and decreases motility in breast cancer cells. Cell cycle and cell apoptosis in BT-549, SK-BR-3 and YCC-B1 cells at 48 hours post-transfection of Vector, ZDHHC22 and ZDHHC22-(C111A) plasmids was examined by flow cytometry analysis of PI staining and Annexin V-APC/PI staining, respectively. (A) Quantification of cell cycle distribution. (B) Quantification of apoptotic

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cells. (C, D) Quantification of stained cells/field indicated the number of migratory and invasive breast cancer cells. Values represent mean ± s.d. from three independent experiments, **p < 0.01, ***p < 0.001.

Figure 4

Ectopic ZDHHC22 inhibits breast cancer proliferation in vivo, while ZDHHC22-(C111A) mutation abolished these effects. BT-549 cells stably expressed with vector, ZDHHC22 and ZDHHC22 (C111A) were injected subcutaneously into the mammary fat pads of female nude mice (n = 5), respectively. Tumor sizes were measured every 2 days for 2 weeks. (A) Image of xenografts after excised. (B) Growth curve of xenograft tumors. ***p < 0.001. (C) Tumor weight of xenograft tumors. ***p < 0.001. (D) Representative images of Ki-67 stained section of primary tumor tissues isolated from nude mice bearing BT-549 cells stably expressed with vector, ZDHHC22 and ZDHHC22 (C111A)-mut, respectively. (E) Apoptotic cells in primary tumor tissues isolated from nude mice bearing BT-549 cells stably expressed with vector, ZDHHC22 and ZDHHC22 (C111A)-mut, respectively, were detected with TUNEL assay.
Figure 5

The ZDHHC22 reduces AKT activation. (A, B) Gene set enrichment analysis (GSEA) based on microarray datasets GSE65194 and GSE21653. (C) Western blotting assay was used to detect the expression of AKT signaling pathway-related molecules.
Figure 6

The ZDHHC22 reduces mTOR and phosphorylation of AKT at Ser-473 in a time-dependent manner. (A, B) Immunofluorescence assays was used to detect the protein expression and co-localization of mTOR (A) and phosphorylation of AKT at Ser-473 (B) at the indicated time in BT-549 and SK-BR-3 cells transfected with ZDHHC22 plasmids.
Figure 8

The ZDHHC22 restores sensitivity toward tamoxifen therapy. (A) The ZDHHC22 and mTOR mRNA expression in tamoxifen-resistant breast cancer cells based on microarray datasets GSE7327, GSE21618, and GSE26459. (B) The mTOR and ZDHHC22 mRNA expression in tamoxifen-resistant MCF cells (MCF7R) and parental cells (MCF7) using qRT-PCR assay. (C) The mTOR and ZDHHC22 protein expression in MCF7R and MCF7 cells using WB assay. (D, E) MCF7 and MCF7R cells were transfected
with Vector and ZDHHC22 plasmids for 48 hours, followed by 20 μM tamoxifen treatment, respectively. Cell growth was measured with CCK-8 assay at the indicated time. (D) ZDHHC22 overexpression sensitized MCF7 cells toward tamoxifen therapy. (E) ZDHHC22 overexpression restored sensitivity of MCF7R cells toward tamoxifen therapy. (F, G) BT-549 and SK-BR-3 cells were transfected with Vector, ZDHHC22 and ZDHHC22-(C111A) plasmids for 48 hours, followed by 10 μM KU-0063794 or 10 μM Taselisib treatment, respectively. Cell growth was measured with CCK-8 assay at the indicated time. (F) The cell growth rate of ZDHHC22 group is similar to the KU-0063794 group. (G) The combination of ZDHHC22 and the PI3K inhibitor Taselisib further suppresses breast cancer cells growth. Data are presented as the mean ± s.d., n = 3, **p < 0.01, ***p < 0.001.

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