

Tilioside suppresses Triple-negative breast cancer as a multifunctional CAXII inhibitor

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Abstract

Triple negative breast cancer (TNBC) is an aggressive subtype of breast cancer characterized by poor prognosis, early recurrence, and the lack of durable chemotherapy responses and specific targeted treatments. In this preclinical study, we examined Tiliroside (TS, $C_{30}H_{26}O_{13}$), as one of the major compounds of *Tribulus terrestris* L. which has been used as alternative therapy in clinic practice of breast cancer treatment, for its therapeutic use in TNBC. The association between *CAXII* expression level and survival probability of TNBC patients, and the difference of *CAXII* expression level between TNBC and normal samples were evaluated by using public databases. To determine the anticancer efficacy of TS on TNBC cells, cell proliferation, wound healing, cell invasion, and 3D spheroid formation assays were performed and excellent anticancer activities of TS were displayed. Mouse models further demonstrate that TS significantly reduces the tumor burden and improves survival rate. The properties of TS as a novel *CAXII* inhibitor have also been evaluated by *CAXII* activity assay, pHi, pHe and lactate level assay. Further RT-PCR and Caspase-3 activity analyses also revealed the positive regulating effects of TS on E2F1,3/Caspase-3 axis in TNBC cells cultured in 2D or 3D systems. The findings indicate that TS suppresses TNBC progression as a potential novel *CAXII* inhibitor in preclinical experiments, which warrants further investigation on its therapeutic implications.

Introduction

Breast cancer ranks the first in the incidence and mortality of malignant tumors in Chinese women, and the incidence is still rising [1, 2]. Breast cancer has undoubtedly become a major threat to women's health globally. Triple negative breast cancer (TNBC) is a special pathological pattern which is negative for estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER-2). According to the American Cancer Society, nearly 260,000 women are diagnosed with breast cancer each year, of which 15–20% are triple-negative breast cancer (TNBC). A larger proportion of younger women is reported in TNBC than older breast cancer patients [3, 4]. The incidence of breast cancer in young patients has increased in recent years, worldwide [5]. In The United States, about 11% of breast cancer patients are between the ages of 35 and 45, and in Asia are 9.5–12%. TNBC the most aggressive breast cancer subtype in young patients. Compared with other breast cancer subtypes, patients with TNBC had a higher mortality, worse overall survival and higher recurrence rate. Unlike other molecular subtypes of breast cancer, patients with TNBC do not benefit from hormone therapy and HER-2 target therapy. The limited choices of medical treatment for them are usually chemotherapy and immunotherapy, and also with limited therapeutic effect. Therefore, there is still a tremendous unmet need for the development of novel therapeutics for TNBC [6].

CAXII inhibitor, as a potential cancer treating agent, has been found to impede the creation and transportation of bicarbonate ions into the cells through anion exchangers and Na^+/HCO_3^- co-transporters, consequently regulating intracellular pH and extracellular acidosis for cancer development, in the tumor micro-environment [7]. *CAXII* inhibitor has also been thought to improve the apoptosis of cancer cells, but related mechanisms still need to be further explored [8]. In addition, selective silencing of

CAXII gene has displayed significant inhibiting effects on migration and invasion abilities in TNBC cells, which ensures more potential on *CAXII* inhibitor for TNBC treatment[9].

Tribulus terrestris L. (TT), a plant in many regions of Asia and Africa, has been used in Traditional Chinese Medicine as an herb to treat breast diseases for thousands of years[10]. Nowadays, evidence has proved that the extracts of TT has many medical qualities such as antihypertension, diuretic effect and cancer suppression[11]. TT extracts have been found to regulate the apoptosis and metastasis of cancer cells by regulating NF- κ B signaling [10]. *Tiliroside* (C₃₀H₂₆O₁₃), as a major active ingredient of TT, has further been found to possess anti-inflammatory, anticholinesterase and antioxidant activities [12]. It has also been reported to suppress cancer development by regulating NF- κ B signaling pathway or MAPK/JNK/p38 axis in leukemia cells [13]. Recently, our study further uncovered its anticancer effects on Hepatocellular carcinoma (HCC) cells as a novel *CAXII* inhibitor [8]. One scientific question we asked was whether or not TT had the similar anticancer effect against breast cancer.

Thus, the purposes of this study were to investigate the association between *CAXII* expression level and survival probability of TNBC patients, to examine the effects of *Tiliroside* on TNBC cells and to explore the related mechanisms *in vitro* and *in vivo*.

Methods

Gene profile overview and survival analysis

Publicly accessed datasets were used to evaluate the prognostic values of *CAXII* in TNBC and differentiated expression of *CAXII*. Gene profile overview were conducted in GENTS platform (<http://gent2.appex.kr/gent2/>) and PEGIA platform (<http://gepia.cancer-pku.cn>) . The Kaplan–Meier survival curves of *CAXII* in TNBC were generated based on the datasets of triple negative breast cancer miRNA and RNA-seq using Kaplan–Meier plotter (<http://kmplot.com/analysis>), respectively.

Cell culture and transduction of luciferase lentivirus

Human triple-negative breast cancer cell MDA-MB-231, BT-549, and human breast normal epithelial cell MCF-10A were purchased from American Type Culture Collection (ATCC, USA) and were characterized using STR (Short Tandem Repeat) analysis for identity verification of human cell lines in January, 2022. The cells were cultured in Eagle's Minimum Essential Medium (EMEM, ATCC, USA), RPMI1640 (ATCC, USA) or Leibovitz's L-15 medium (LLM, ATCC, USA), respectively, with 10% fetal bovine serum (ATCC, USA), or Mammary Epithelial Cell Growth (MEGM, USA) completed with MEGM bullet kit (Lonza, USA). Cells were cultured in an incubator under the condition of humidified incubator with 5% CO₂ at 37°C.

Both MDA-MB-231 and BT-549 cells were transfected with CMV-Firefly luciferase-IRES-Puro lentivirus (Cellomics,USA), and characterized by STR analysis as described previously[14].

Gene Knockout

For establishing CAXII knockout clones, as described in our previous study[8], MDA-MB-231 and BT-549 were both transfected with 1 µg of CAXII CRISPR guide RNA vector plus vector coding for puromycin resistance by applying Metafectene Pro (Biontexas, Munich, Germany), respectively. Cells were then selected by puromycin for 16 h. CAXII expression was confirmed after 24 h by RT-qPCR.

RNA extraction and quantitative RT-PCR

Total RNA was extracted from MDA-MB-231, BT-549 and MCF-10A cells by using the RNeasy mini kit (Qiagen, Germany)[8]. The concentration and purity of total RNA were determined by using an Epoch microplate spectrophotometer (Biotek, USA). Total RNA was reverse-transcribed to cDNA templates by using an AffinityScript multi temperature cDNA synthesis kit (Agilent technologies, CA, USA). The expression of detected genes was determined using the SYBR Green PCR Kit (Qiagen, Germany) on a 7500 Fast Real-time PCR System (Life Technologies, USA). All the primer sequences used in this study and qPCR reaction conditions were described in our previous study[8]. Each sample was analyzed in triplicate. After each PCR amplification, dissociation curve was assessed. The relative expression levels of genes were expressed as a fold change relative to *GAPDH* using the $2^{-\Delta\Delta C_t}$ method.

CAXII activity assessment

CAXII activity inhibition assay was performed following the instructions described in our previous study [8]. For details, the enzyme activity was assessed by monitoring 4-nitrophenol (4-NP/pNP) (SIGMA-ALDRICH, USA). Absorbance of the spontaneous hydrolysis of the substrate alone and substrate treated with *Tiliroside*, was subtracted from esterase activity in absence and presence of *Tiliroside*. In addition, we added potent CAXII inhibitor (U-104) (EMD Millipore corporation, USA) as a positive control. Plotting absorbance (Y-axis) and time (min) (X-axis) were used to evaluate the slope of the initial rate of enzyme activity. The percentage of enzyme activity was then calculated. By applying the non-linear least squares method, the IC50 values were finally calculated.

Caspase-3 activity

For conducting the CaspACE™ Assay, 2×10^6 MDA-MB-231(or BT-549) cells cultured in 2 mL medium were treated with either 40µM *Tiliroside* or same amount of DMSO as the induced apoptosis group (72h after intervention), and 3 µL Z-VAD-FMK inhibitor was added in 72h intervention cells as the inhibited apoptosis groups. The mock groups were regarded as a normal control (NC). The plate was incubated at 37°C in a humidified incubator with 5% CO₂ for 16h. After the centrifugation of cell lysates, the cell supernatant fractions were harvested for CASP3 activity measurement. In addition, the protein concentration of each sample was determined by the BCA protein assay (Thermo Fisher Scientific, USA), and the pNA Calibration Curves were also made by a colorimetric assay system. CASP3 Specific Activity (SA) was calculated as the following formulas which have been described elsewhere[8, 14].

Detection of intracellular pH (pHi), extracellular pH (pHe) and lactate level

Intracellular pH was evaluated by using the fluorometric intracellular pH assay kit (Sigma, Germany, MAK-150), as described in previous study [15]. Fluorescent BCFL-AM indicator was used to measure pH fluctuations in the fluorometric intracellular pH kit. BCFL-AM was utilized to measure the reductions in pH of the cells that had been treated by various conditions. The cells were seeded in black-wall plates, as 6×10^4 in each well of the plate, followed by 24 h incubation. The medium was replaced with BCFL-AM reagent which had been prepared in 100 mL of HBS solution (Hank's buffer with 20mM HEPES, 5mM probenecid), then the cells were incubated at 37°C in an atmosphere of 5% CO₂ for 30 min (protected from light). TS compound at doses of 0, 20, 40 and 80 µM was added to the HBS solution. In the next 5 min, the measurement was performed at wavelengths of 490 nm (excitation) and 535 nm (emission) in spectrofluorometry (Spectramax, M5). Extracellular pH (pHe) and lactate levels were evaluated directly by using commercial kit on the blood gas device (ABL90 FLEX PLUS, Radiometer, Copenhagen, Denmark).

MTS cell proliferation assay

Cell proliferation MTS assay was performed by adding 20 µL of MTS solution (Promega, USA) into each well in a dark hood at different incubation time points (24h, 48h, 72h, 96h) following the manufacturer's instruction. Microplate Spectrophotometer (Biotek, USA) was used to detect the cells' absorbance at the wavelength of 450 nm. Triplicate were conducted for each condition at each time point. The proliferation inhibition rate was calculated based on the formula: inhibition rate = $(1 - \text{Absorbance of treated sample} / \text{Absorbance of control sample}) \times 100\%$.

Wound healing assay

MDA-MB-231 and BT-549 cells were cultured in 6-well tissue culture plates with approximately 1×10^6 cells in each well, respectively. Single wound was scratched in each plates when cell monolayer grew to nearly 90% confluence. 40µM *Tiliroside* in DMSO or the same final concentration of DMSO were added for different groups. Images were taken at 0 h, 24 h and 48 h of incubation after the wound, respectively, for the average of wound closure measurement[16].

Transwell invasion assay

The MDA-MB-231 and BT-549 cells (1×10^4) were seeded in each upper layer of culture insert of 3.0 µM pore size transwell chamber (Falcon, USA) with the 100 µL medium containing 20% Matrigel (Corning, USA), 40µM *Tiliroside* (or DMSO), and 0.1% FBS for each well. Under the cell permeable membrane, 600 µL of the complete medium was added for each chamber. After 24h incubation, cells which have migrated through the membrane were fixed by 4% paraformaldehyde and dyed with crystal violet. The surface of upper layer of membrane was cleaned softly by neat cotton swabs, and the cells in different fields of view on the subface were counted.

3D spheroid formation assay

For evaluating the effect of *Tiliroside* on TNBC cells, 3D spheroid formation assay was performed as described elsewhere[16]. One set was used for capturing images from 24 to 96 h incubation time, and another set was for *in vitro* bioluminescence signal determination by transferring to a 96-well plate in the presence of D-luciferin (150 μ L/mL) (PerkinElmer, USA). The proliferation inhibition rate was calculated following the formula as: inhibition rate = (1-Absorbance of treated sample / Absorbance of control sample) $\times$ 100%.

Mouse Models and In Vivo Fluorescence Imaging

Six-week female athymic nude mice (18-21 g) (n=8) were purchased from Gempharmatech (Nanjing, China). Experimental protocols were approved by the Beijing University of TCM Institutional Animal care and Use Committee (No.104195489042) and adhered to the NIH Guide for the Care and Use of Laboratory Animals. A freshly prepared mixture (50 mL complete culture medium, 5×10^5 MDA-MD-231^{Luc}, 50 mL Corning Matrigel Matrix HC, Phenol Red-free, and LDEV-free) was directly injected into lower right mammary fat pad (MFP) of each mouse under inhalation isoflurane anesthesia [17]. All mice were then maintained for 7 days and examined by In Vivo Imaging System (IVIS) Lumina LT Imaging System (Xenogen/Caliper Life Sciences, USA) with Living Image 4.3.1 software (Caliper Life Sciences, USA). All mice were then divided into TS group (n = 4) and NC group (the same final concentration of DMSO) (n = 4). *Tiliroside* was started to administer at 45mg/kg body weight to mice once every 2 days orally. Images of tumor sizes were collected every week. Mice were monitored for 50 days to collect survivorship data. Mice were sacrificed in a CO₂ chamber when ethically necessary due to clinical symptoms or substantial loss in body weight. To compare survival differences between TS treated and control groups, log rank (Mantel-Cox) test was used for survival analysis in GraphPad Prism (version 9.1.1).

Statistical analysis

Each assay was performed at least 3 independent experiments with triplicate. Means and standard deviations were calculated, and the differences between groups were analyzed using a generalized linear model with post-hoc Tukey test for the correction of multiple comparisons. The two-tailed unpaired student's t-tests were used for the difference comparison of two groups. Statistical significance was considered when $P < 0.05$ (two-sided). Kaplan-Meier Survival curve with a log-rank test was applied for survival curves.

Results

CAXII is overexpressed in TNBC and has a negative correlation with overall survival of TNBC patients

As shown in the gene expression profile analysis of GENT2, the expression of *CAXII* in breast cancer samples was significantly higher than of breast normal tissue ($P < 0.001$). The analysis performed in GEPIA also displayed a similar result that the expression level of *CAXII* in breast invasive carcinoma (BRCA) samples was significantly higher than of breast normal tissues (Fig. 1A and 1B) ($P < 0.001$). Furthermore, the Kaplan–Meier survival curves showed that TNBC patients with a high level of CAXII

suffered worse prognosis than those with a low one (HR = 1.81, 95% CI: 1.21–2.62, $P < 0.001$) (203963_at). Similar trends were also observed in the other array probes (204508_s_at, 204509_at, 210735_s_at) (Figure.1C-1F). Meanwhile, our RT-PCR analysis also confirmed that the expression of CAXII in MDA-MB-231 was 1.51 ± 0.024 fold of MCF-10A ($P < 0.001$), and that of BT-549 was 1.48 ± 0.02 fold compared to MCF-10A ($P < 0.001$) (Fig. 2A)

Expression of CAXII effects the anti-proliferation ability of Tiliroside on TNBC cells

As displayed in RT-qPCR results, in both MDA-MB-231 and BT-549 cells, the relative expression level of CAXII in CAXII-KO (CAXII knockout) group was significantly decreased than that in parent cells and none transfection groups ($P < 0.001$), respectively (Fig. 2B).

In MTS assay, MDA-MB-231, BT-549, and normal cell line of MCF-10A were treated with either *Tiliroside* or DMSO, respectively. Obviously a dose-effect of relationship was observed. For MDA-MB-231 cells, at 48h, the inhibition rates of 40 μ M *Tiliroside* group was $52.44 \pm 3.63\%$, which was significantly higher than that of 40 μ M *Tiliroside*+ CAXII-KO group (CAXII-KO cell treated by 40 μ M *Tiliroside*) ($P = 0.015$), and was also significantly higher than that of 20 μ M *Tiliroside* group ($31.01 \pm 3.33\%$, $P = 0.004$). Similar results were detected at 24h and 72h. For BT-549 cells, the inhibition rates in 40 μ M *Tiliroside* group were significantly higher than that of 40 μ M *Tiliroside*+ CAXII-KO group and 20 μ M *Tiliroside* group, respectively, during the whole observation period ($P < 0.05$). For both cells, inhibition rates in 40 μ M *Tiliroside* group showed non-significant difference compared to their counterparts in 40 μ M *Tiliroside* + none-KO group (Fig. 2C and 2D). However, the inhibition rates just ranged from $1.48 \pm 1.96\%$ to $9.78 \pm 2.05\%$, during the whole period (Fig. 2E).

Tiliroside suppresses invasion, migration and 3D formation of TNBC in vitro

The invasion assay showed that, in MDA-MB-231 cells, the invasive cell number per field in *Tiliroside* group (137.67 ± 13.47) was significantly higher than that of NC group (191 ± 3.74 , $P = 0.006$). Similar result was observed in BT-549 cells (Fig. 2F, 2G and 2H).

The cell migration assay displayed that the tested TNBC cells treated by *Tiliroside* healed slower than those in NC groups (Fig. 3A). For MDA-MB-231, the average width of wound gaps in the *Tiliroside* group was $85.66 \pm 3.26\%$ of its initial width compared to its counterpart in NC group ($69.57 \pm 3.56\%$, $P = 0.009$), at 24h. The width in the *Tiliroside* group shrunk to $67.39 \pm 2.14\%$, while its counterpart in the control group healed to $49.52 \pm 4.29\%$ ($P = 0.006$), at 48h. Similar results were detected in BT-549 cells (Fig. 3B, 3C).

3D spheroid formation assay was also conducted in MDA-MB-231 and BT-549 cells which contained a luciferase reporter gene. The dynamic changes of 3D spheroid formation are shown in Fig. 3D. For MDA-MB-231 cells, at 48h, the relative cell cross-section area of *Tiliroside* group grew to $122.33 \pm 4.03\%$ of its initial area, and significantly lower than that of NC group ($199.67 \pm 11.9\%$, $P < 0.001$). At 96h, the formation efficiency of *Tiliroside* group reached to $156 \pm 3.74\%$ compared to $256.27 \pm 10.73\%$ of NC

group ($P = 0.006$). Similar trends were also observed in BT-549 cells (Fig. 3E and 3F). In addition, the luciferase reporter gene assays displayed that the inhibition rates of MDA-MB-231 cells ranged from $52.29 \pm 3\%$ to $57.24 \pm 1.61\%$, at 48h and 96h, and that of BT-549 were $48.21 \pm 2.36\%$ and $54.1 \pm 3\%$ (Fig. 3G).

Tiliroside suppresses activity and expression of CAXII and regulates E2F1/3/Caspase-3 axis

CAXII esterase activity was measured after 1 h intervention of the same amount of *Tiliroside* or U-104. As shown in Fig. 4A and 4B, the activity curves were in a dose-dependent manner. For *Tiliroside* group, the IC₅₀ was $20.95 \pm 5.8 \mu\text{M}$ in MDA-MB-231 cells and $30.92 \pm 6.98 \mu\text{M}$ in BT-549 cells. In addition, the activities were $5.47 \pm 1.25 \mu\text{M}$ and $6.62 \pm 1.8 \mu\text{M}$ for MDA-MB-231 and BT-549, under the intervention of U-104 (Fig. 4A and 4B).

RT-qPCR results displayed that, in MDA-MB-231 cells, the expression level of *CAXII* in 2D cultured cells after *Tiliroside* intervention, was 0.21 ± 0.024 fold compared to its NC ($P < 0.001$), and that in 3D *Tiliroside* group was 0.316 ± 0.02 fold of NC ($P < 0.001$). For gene *E2F1*, its expression in 2D *Tiliroside* group was 0.33 ± 0.015 fold of NC ($P < 0.001$), and that in 3D *Tiliroside* group was 0.316 ± 0.02 fold of NC ($P < 0.001$), respectively. Similar results were found in BT-549 cells and *E2F3* (Fig. 4C).

As showed in caspase-3 activity assay, in MDA-MB-231 cells, the absorbance of *Tiliroside* group 2D (0.50 ± 0.01) was significantly higher than that of apoptosis inhibited *Tiliroside* group 2D (0.20 ± 0.02 , $P < 0.001$). In 3D system, the absorbance of *Tiliroside* group 3D (0.47 ± 0.02) was also significantly higher than that of apoptosis inhibited *Tiliroside* group 3D (0.19 ± 0.01 , $P < 0.001$). Similar results were observed in BT-549 cells cultured in either 2D or 3D systems (Fig. 4D and 4E). Moreover, as for MDA-MB-231 cells, calculated CASP3-specific activity in *Tiliroside* group 2D (0.12 ± 0.01) was significantly higher than that in NC group 2D (0.03 ± 0.01 , $P < 0.001$); Their counterparts in 3D system also showed significant difference (0.11 ± 0.02 of *Tiliroside* group compared to 0.001 ± 0.002 of NC group, $P < 0.001$). Similar situation was also detected in BT-549 cells (Fig. 4F).

Effects of Tiliroside on the levels of pHi, pHe and extracellular lactate

As shown in Fig. 4G, in MDA-MB-231 cells, the intracellular pHi value of $0 \mu\text{M}$ *Tiliroside* group was 7.43 ± 0.04 compared to 7.00 ± 0.06 of $40 \mu\text{M}$ *Tiliroside* group ($P < 0.001$). Intracellular pHi value of $40 \mu\text{M}$ *Tiliroside* group dropped to 6.66 ± 0.1 , and further to 6.41 ± 0.08 in $80 \mu\text{M}$ *Tiliroside* group. Similar results were observed in BT-549 cells. Oppositely, in MDA-MB-231 cells, the extracellular pHe value in $40 \mu\text{M}$ *Tiliroside* group (7.05 ± 0.02) or $80 \mu\text{M}$ *Tiliroside* group (7.21 ± 0.03) was significantly higher than that of $0 \mu\text{M}$ *Tiliroside* group (6.83 ± 0.1), respectively. Similar results were observed in BT-549 cells (Fig. 4H). For extracellular lactate level, a significant dose-dependent relationship was observed in BT-549 cells (from 4.1 ± 0.11 of $0 \mu\text{M}$ *Tiliroside* group to 2.3 ± 0.27 of $80 \mu\text{M}$ *Tiliroside* group). However, in MDA-MB-231 cells, compared with NC group (4.12 ± 0.09), the significant reduction of extracellular lactate level started to reveal when concentration of *Tiliroside* reach to $40 \mu\text{M}$ (3.23 ± 0.08 , $P < 0.01$) (Fig. 4I).

Anticancer Effect of Tiliroside in Xenograft Mouse Models

The represented bioluminescence tumor images of tested mice were shown in Fig. 5A. The tumors of *Tiliroside* treated mice started to show significant difference compared to NC group at 14th day of treatment. For details, the bioluminescence of *Tiliroside* group $(2.93 \pm 0.8) \times 10^7$ was significantly higher than that of NC group $(5.65 \pm 0.96) \times 10^7$, at 14th day of treatment ($P = 0.04$). At 35th day of treatment, this trend still maintained, which was $(2.86 \pm 1.67) \times 10^7$ of TC compared to $(11.2 \pm 2.84) \times 10^7$ of NC ($P = 0.023$) (Fig. 5B). The calculated inhibition rate based on tumor bioluminescence signal was $(5.17 \pm 13.11)\%$ at 7th day of treatment, and such value kept growing to $(75.53 \pm 14.45)\%$ at 35th day of treatment (Fig. 5C). Mice in *Tiliroside* group were all alive at 50th day of treatment. However, 4 mice of NC group died at 37th, 40th, 46th and 47th day of treatment, respectively (log-rank $P = 0.007$) (Fig. 5D).

Discussion

The addition of new “bullets” in the treatment of breast cancer may accelerate the cure of the disease, given that the combination of different regimens or therapies substantially prolong breast cancer survivors. *Tiliroside* (TS), a compound from several plants such as *Tribulus terrestris* and *Agrimonia pilosa* Ledeb [18], belongs to a saponin of the herb. Herbal saponin was reported to possess therapeutic effects against cardiovascular diseases, nervous system disorders, asthma, arthritis and diabetes [19]. Our previous study has reported the anti-cancer effects of TS on hepatocellular carcinoma as a CAXII inhibitor [8]. In this preclinical study, we extended our previous study and showed its inhibitive effects against TNBC.

Carbonic anhydrases (CA) catalyze the reversible hydration of carbon dioxide to bicarbonate and protons. Overexpression of CAXII (CA12) has been found in hypoxia—the oxygen deprivation of cancer cells due to a combination of poor tumor vascularization and high proliferation rate, despite the fact that hypoxia response elements (HREs) essentially lack in the 5'-upstream genomic region of CAXII gene. Moreover, overexpression of transmembrane CAXII in cancer has been found to be associated with cancer progression including rapid tumor growth and invasion, infiltration of surrounding normal tissues and the formation of metastases [20]. Moreover, selectively silencing of CAXII gene in TNBC cell line MDA-MB-231 could lead to the suppression of cell migration and invasion by interfering the p38 mitogen-activated protein kinases (p38 MAPK) signalling pathway [9]. Similarly, the reduction of CAXII expression by blocking Hedgehog signaling pathway in MDA-MB-231 cells could limit the migration ability of cancer cells, which highlighted the potential role of CAXII to be an effective therapeutic target in TNBC treatment.

In line with the previous studies, in this study, we found that expression of CAXII in clinical breast-cancer samples was significantly higher than in breast-normal tissues (Fig. 1A and 1B). Such phenomenon is also reproducible in our RT-PCR assay that TNBC cells (MDA-MB-231 and BT-549) possess significantly higher level of CAXII expression than in breast normal cell (MCF-10A) (Fig. 2A). In addition, a worse over-survival was also found in patients with high expression of CAXII. These observations suggest that CAXII is a potential therapeutic target, and may be exploited for the treatment of TNBC.

Cellular acidosis was reported to be one of triggers of apoptosis, therefore a high level of pH_i was thought to prevent cancer cells from apoptosis. Evidence has also displayed that pH_i reduction can cause cellular apoptosis by activating endonuclease II and MMP impairment [21]. Furthermore, caspase activation and mitochondrial depolarisation have been found to be activated by cytochromic acidification, staurosporine and ultraviolet light. Therefore the agents, including CAXII inhibitor, which can reduce the pH_i in cancer cells, were thought to be pro-apoptotic by impairing intracellular homeostasis and metabolism of cancer cell [22]. Additionally, the accumulation of the metabolic byproduct lactate and extracellular acidification have been reported to accelerate tumor cell proliferation, metastasis, and angiogenesis. Lactate acidosis has been found to reduce immune evasion in TNBCs[23, 24]. Therefore extracellular lactate reduction has been considered as an anti-cancer approach to enhancing the therapeutic effect of immune therapy[25]. CAXII has been reported to maintain pH and CO₂ homeostasis, therefore affect cancer progression, invasion, and resistance to therapy [26]. In this study, the results, for the first time, displayed that TS can not only significantly reduce the gene expression and activity of CAXII, but also increased pH_e level and suppress extracellular lactate and pH_i levels, which creates a hostile microenvironment to block TNBC progressions.

Tiliroside showed significantly strong inhibition effects on the proliferation, invasion and 3D spheroid formation of two TNBC cell lines, while had limited impact on the normal cells. As for *in vivo* assay, our results show that mice received *Tiliroside* treatment experienced a significantly decreased tumor burden (> 40% after 14d), also had a significantly improved survival rate, indicating the effectiveness of *Tiliroside* in TNBC treatment. The results of 3D formation assay, and intracellular or extracellular PH value testing assays indicate that *Tiliroside* could shape an effective antitumor microenvironment.

E2F1 and *E2F3* are oncogenes and have negative correlations with breast cancer patient survival [16]. *E2F1* has been reported to affect cell growth by regulating NF- κ B, thereby enhancing tumor proliferation and anti-apoptosis. It can also cause immune escape by blocking the transcription of ICAM-1 [27]. Meanwhile, *E2F3* expression appears to provide a growth advantage to tumor cells by activating cell proliferation [28]. Overexpression of *E2Fs* is frequently observed in advanced cancers and aggravates chemoresistance. Overexpress of *E2F1* and *E2F3* were found in breast adenocarcinoma when compared to normal breast tissues[16]. In addition, Caspase3(CASP3), as an apoptosis executive factor, not only can regulate many physiological processes including cancer stemness and autophagy, but also affect pyroptosis by mediating GSDM [29]. Lack of CASP3 can lead to the resistance of cells to microenvironmental stress and treatments, thereby promoting tumorigenesis[30]. Our previous studies have shown that *E2F1/E2F3/Caspase-3* axis, which is associated with multiple cellular physiological processes, such as cancer stemness and apoptosis, was an effective anti-cancer pathway in liver cancer and breast cancer (including TNBC), and that *Tiliroside* had a positive effect on regulating such axis in HCC for the first time [8, 14, 16]. Again, in this study, we demonstrated that *Tiliroside* significantly inhibited the expression of *E2F1*, *E2F3*, and increased CASP3 activity in both TNBC cell lines cultured in 2D and 3D systems.

In summary, we demonstrate that *Tiliroside* suppresses TNBC development by acting as a novel CAXII in this study. To our knowledge, this is the first study to show the inhibitive effect of *Tiliroside* on TNBC and the regulation of *Tiliroside* on *E2F1/E2F3/Caspase-3* axis. Our results indicate a novel chemotherapeutic with CAXII functionality as a potential choice for TNBC treatment. Further investigation of *Tiliroside* is warranted to explore its deeper therapeutic potential for the treatment of TNBC.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

H.R. and L.LG build the original idea of this research; H.R. and Y.HX. performed the experiments and wrote the manuscript; Y.HX. conducted the animal study; L.LG and L.CQ provided technical supports and revised the manuscript. All authors reviewed the manuscript.

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Figures

Figure 1

Gene profile overview analysis of CAXII, and Kaplan–Meier survival curves of CAXII in TNBC. The expression of CAXII in breast cancer samples is significantly higher than of breast normal tissue (A, B). TNBC patients with higher expression level of CAXII had an inferior overall survival compared to those with lower level (C-F).

Figure 2

***Tiliroside* inhibited the proliferation and invasion of MDA-MB-231 and BT-549 cells and the suppression by CAXII knockout.**

The relative CAXII expression in MCF-10A was significantly lower than that of MDA-MB-231 and BT-549, respectively (A). Meanwhile its expression was significantly reduced in CAXII-KO group than none-KO and none transfection groups, in tested cells, respectively (B). Relative inhibition rates of MDA-MB-231, BT-549 and MCF-10A treated by different concentrations of *Tiliroside* were assessed by comparing the NC, respectively (C–E). The average of cell number was counted by 3 randomly chosen different fields in both cell lines (F-H). Values represent the mean±SD. NS non-significant, NC negative control.

Figure 3

***Tiliroside* inhibited the migration and 3D formation abilities of TNBC cells.**

The wound healing assay was performed in MDA-MB-231 and BT-549 cells (A). The bar graphics present the percentage of wound recovery in both cell lines, respectively (B and C). The representative 3D spheroid models of TNBC cells (D). The relative cross-section area formation efficiency displayed significant reduction of area in MDA-MB-231 and BT-549 cells after intervention (E and F). The fluorescence values of 3D formation cell aggregates from different groups were used to calculate relative inhibition rates (G). NC negative control.

Figure 4

***Tiliroside* regulated the activity, expression of CAXII, E2F1,3/Caspase-3 axis and pHi, pHe and extracellular lactate.**

Dose-dependent inhibition curves of CAXII esterase activity under different intervention were displayed in Fig. 4A and 4B. As for Caspase-3 activity assay. Relative expression of *CAXII*, *E2F1* and *E2F3* in *Tiliroside* group was significantly lower than that of NC in MDA-MB-231 and BT-549 cells cultured in either 2D or 3D systems, respectively ($P \leq 0.001$) (C) the absorbance of *Tiliroside* group was significantly higher than that in apoptosis inhibited *Tiliroside* group, NC group and apoptosis inhibited NC group in MDA-MB-231 (D) and BT-549 cells (E) cultured in either 2D or 3D systems, respectively. Calculated CASP3-specific activities in *Tiliroside* group were significantly higher than that of NC groups in both MDA-MB-231 and BT-549 cells cultured in either 2D or 3D systems, respectively (F). NC negative control. It was also displayed

that intervention of MDA-MB-231 and BT-549 cells with *Tiliroside* exerted a significant dose-related fall in pHi and lactate level of media and a significant increase in pHe (G, H, I).

Figure 5

Tiliroside suppressed the development of TNBC in vivo.

The images of bioluminescence of TNBC implanted in xenograft mice of MDA-MB-231^{luc}, from 0 to the 35th day of treatment, were displayed (A). The bioluminescence value was significantly lower in the *Tiliroside* group compared to NC, from 14th to 35th day of treatment in mice models ($P < 0.05$) (B). The inhibition rates calculated based on tumor bioluminescence signal were also displayed (C). *Tiliroside* also improved the survival rate of mice implanted with MDA-MD-231^{luc} cells compared to NC groups (D).