Curcumin Suppresses Tumorigenesis via Promoting SLC1A5-mediated Ferroptosis in Breast Cancer

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Research

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Abstract

**Background:** Breast cancer is one of the most malignant tumors in the female. Previous studies confirmed that Curcumin, a kind of polyphenol compound extract from the *Curcuma longa* underground rhizome, inhibits the survival of cancer cells. However, the functional role and mechanism of curcumin in breast cancer remain unclear.

**Methods:** The cell counting kit-8 (CCK-8) assay was performed to examine the effect of curcumin on cell viability in both MDA-MB-453 and MCF-7 cells. Determination of lipid reactive oxygen species (ROS) level, malondialdehyde (MDA) production, and intracellular Fe$^{2+}$ level was used to evaluate the effect of curcumin on cell ferroptosis. The protein levels were determined by western blot. A xenograft tumor model was employed to verify the antitumorigenic effect of curcumin on breast cancer in vivo.

**Results:** Curcumin treatment significantly suppressed breast cancer cell viability in a dose-dependent manner. Moreover, curcumin triggered ferroptosis by enhancing the levels of lipid ROS, lipid peroxidation end-product MDA accumulation, and intracellular Fe$^{2+}$. Mechanistically, curcumin administration impeded tumor growth via upregulating solute carrier family 1 member 5 (SLC1A5)-mediated ferroptosis in breast cancer. In vivo experiments showed that curcumin could effectively hamper the growth of tumors without noticeable side effects.

**Conclusion:** We demonstrated that curcumin exhibits anti-tumorigenesis activity in breast cancer by promoting SLC1A5-mediated ferroptosis, providing a potential therapeutic agent for the treatment of breast cancer.

Introduction

Breast cancer is one of the most frequently diagnosed cancer and the leading cause of cancer death among women [1]. Breast cancer accounts for 30% of all cancer cases and 14% of all cancer deaths among women [2]. In recent years, the incidence of breast cancer has continued to rise, harming human health and quality of life and providing a massive burden to the medical industry and economy. Due to the lack of apparent early symptoms and standardized physical examination [3, 4], the majority of patients with breast cancer are diagnosed with metastasis, resulting in poor prognosis. Up to now, surgery, chemotherapy and radiotherapy are most commonly used for the treatment of breast cancer. Still, chemotherapeutic drugs generally have the disadvantages of being costly and causing side effects, including emesis, nausea, alopecia, myelosuppression and thromboembolism [5–7]. Therefore, it is of significance to find safe, effective, and widely-sourced anticancer drugs with few side effects for the treatment of and breast cancer.

Accumulating evidence demonstrates that ingredients extracted from Chinese herbal medicine and the natural plant can be considered new approaches to prevent and cure tumors [8, 9]. Curcumin is the main active material which separates from the curcuma longa underground rhizome [10]. Curcumin has a widespread function in tumor prevention and treatment [11]. Curcumin exhibits antitumor effects on
various tumors via regulation of tumor-related genes and signaling pathways, such as p53 [12], PGK1 [13], Wnt/β-catenin [14], and Sonic Hedgehog pathway [15]. Interestingly, recent studies found that curcumin exhibits an antitumor effect on breast cancer [16, 17]. However, the functional roles and mechanisms of curcumin in breast cancer have not elucidated clearly.

Ferroptosis is a type of iron-dependent programmed cell death, which is different from apoptosis, necrosis and autophagy [18]. The primary mechanism behind ferroptosis is that under the action of divalent iron or lipoxygenase, the high expression of unsaturated fatty acids on the cell membrane is catalyzed, resulting in lipid peroxidation, which induces cell death [19, 20]. Ferroptosis plays an essential role in the occurrence and development of cancer [21, 22]. Moreover, recent studies have shown that inducing cell ferroptosis may be an effective cancer treatment [23, 24]. Most surprisingly, Lin et al. demonstrated that Danshen, a traditional Chinese medicine, prolonged survival of patients with breast cancer by inducing cell ferroptosis [25]. Similarly, a study published in 2018 reported that natural compounds exerted antitumor activity through the induction of non-apoptotic programmed cell death, including ferroptosis [26], which provided an effective therapeutic strategy for patients with cancer. The above research progress indicates that ferroptosis plays a vital role in the occurrence and progression of the disease, but whether curcumin exhibits antitumor effects by regulating cell ferroptosis in breast cancer remains unknown.

In the present study, we found that curcumin treatment significantly inhibited breast cancer cell viability in a dose-dependent manner. Moreover, curcumin administration induced ferroptosis by enhancing the levels of lipid reactive oxygen species (ROS), malondialdehyde (MDA)-one of the most vital end-products of lipid peroxidation-, and intracellular Fe$^{2+}$ in breast cancer. Treatment with curcumin significantly suppresses tumorigenesis in breast cancer via upregulating SLC1A5 expression, which is an essential transporter for glutamine uptake. Based on these results, therapeutic interventions making use of curcumin-induced ferroptosis may provide a strategy for the treatment of breast cancer.

**Materials And Methods**

**Cell culture**

The human breast cancer cell lines (MDA-MB-453 and MCF-7) were purchased from the Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco’s modified Eagle's medium (DMEM) medium (HyClone, USA) containing 10% fetal bovine serum (FBS; Capricorn, Germany) and 2 mM L-glutamine at 37°C in an incubator with a humidified atmosphere containing 5% CO$_2$.

**Cell treatment**

Both MDA-MB-453 and MCF-7 cells were treated with curcumin (purity>98%; Sigma-Aldrich, USA) dose 0, 1, 2, 5, 10, 20, and 50 μM for 48 h or with 10 μM Erastin as a control for 24 h. After incubation, both cell viability was examined using cell counting kit-8 (CCK-8).
Cell transfection

A total of $2 \times 10^5$ cells were seeded per well and grown to 40-60% confluence. The SLC1A5 siRNA (si-SLC1A5), and blank plasmid (si-NC) were purchased from GenePharma (Shanghai, China). Vectors were transfected into both MDA-MB-453 and MCF-7 cells using Lipofectamine® 3000 kit (Invitrogen, USA) according to the manufacturer’s protocols. After 48 h transfection, the transfection efficiency was determined by western blot.

Cell viability analysis

100 μL of cell suspension ($5 \times 10^3$ cells) were plated in 96-well plates for incubation at 37 °C with 5 % CO₂. When 70% of confluence was reached, cells were treated with curcumin or transfected with si-SLC1A5. Twenty-four hours after transfection, 10 μL CCK-8 solution (Beyotime Biotechnology, Nantong, China) was added to each well and incubated for 60 min. Then the absorbance of each well was assessed at 450 nm using a microplate reader (Thermo Fisher, Massachusetts, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted with TRizol reagent (Invitrogen, USA) according to the supplier’s instructions. We reverse-transcribed 1 μg of total RNA to single-strand complementary DNA (cDNA) using the One Step PrimeScript miRNA cDNA Synthesis Kit (TaKaRa, Dalian, China). qRT-PCR was performed in triplicate using SYBR Green PCR Master Mix (Life Technologies, USA) following the manufacturer’s protocols. The relative expression of acyl-CoA synthetase long-chain family member 4 (ACSL4), nicotinamide adenine dinucleotide phosphate-oxidase 1 (NOX1), glutathione peroxidase 4 (GPX4), and Ferritin was normalized to β-actin, and values were calculated using the $2^{-\Delta \Delta Ct}$ method. The sequences of primers for qPCR were: ACSL4 forward: 5'-TTTTGCGAGCTTTCCGAGTG-3', reverse: 5'-AGCCGACAATAAAGTACGCAA-3', NOX1 forward: 5'-TTGGGTCAACATTGGCCTGT-3', reverse: 5'-AAGGACACGAGATTGCAGACA-3', GPX4 forward: 5'-ATTGGTCGCGTTGGACAGAG-3', reverse: 5'-TCGATGTCTCCTTGCGGAAA-3', FTC forward: 5'-GCCACTTCTTCCGCGAATTG-3', reverse: 5'-TTATGCGCTGTTGGGTTTT-3', β-actin forward: 5'-TCCCTGGAGAAGAGCTACGA-3', reverse: 5'-AGCAGCTGTGTGGGCTACAG-3'.

Western blot

To extract total protein, cells were lysed in a RIPA Lysis Buffer (Beyotime Biotechnology, Nantong, China). The lysate was centrifuged at 12,000 rpm at 4 °C for 10 min. The supernatant was transferred to a new tube to quantify the protein amount using the BCA assay. Subsequently, electrophoresis was conducted with 12% SDS-PAGE followed by transfer onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). After blocking with 5% (w/v) non-fat dry milk, the membranes were incubated with primary antibodies against β-actin (1:1000 dilution), SLC1A5 (1:1000 dilution), GOT1 (1:1000 dilution), and GLS2 (1:1000 dilution). All antibodies were purchased from Abcam (Shanghai, China). Then, the appropriate HRP-conjugated secondary antibodies (1:5000 dilution, Proteintech, Wuhan, China) were applied. The protein bands were detected using a chemiluminescence procedure (Pierce, Rockford, IL,
USA) on a Tanon 5200 Imaging system (Shanghai, China). Densitometric analysis was performed using Image-Pro Plus 6.0 software (Media Cybernetics, USA). The expression levels of protein in each sample were normalized to β-actin.

**Nude mice model**

Female BALB/c nude mice at 6-8 weeks of age were purchased from Guangdong Medical Laboratory Animal Center (Foshan, China), and animal model experiments were approved by the Ethical Committee of The Affiliated Hospital of Qingdao University. MCF-7 cells (5×10^6) were suspended in serum-free DMEM medium and then injected into the right posterior flanks of mice. After two weeks, a total of 20 mice were randomly divided into two groups (n=10 per group). Mice in the curcumin administration group were treated with 30 mg/kg/d curcumin (Intragastric administration). Mice in the control group were fed with 0.9% sodium chloride plus 1% DMSO. The tumor growth in the mice was examined every three days. Mice were sacrificed by intraperitoneal injection of pentobarbital sodium (200 mg/kg) after four weeks of curcumin administration, and the size of each tumor was measured, and tumor tissues were collected for further experiments. The expression of SLC1A5 (1:500 dilution, Cell Signaling Technology, USA) and Ki-67 (1:500 dilution, Cell Signaling Technology, USA) was evaluated by immunohistochemistry staining according to the manufacturer’s instructions and previous studies [27].

**Iron assay**

Intracellular ferrous iron (Fe^{2+}) level was determined using the iron assay kit purchased from Abcam (#ab83366) and was used according to the manufacturer’s instructions. The experiment for each group was repeated three times.

**Malondialdehyde (MDA) assay**

The intracellular MDA concentration in cell lysates or tissues was assessed using a Lipid Peroxidation Assay Kit (#ab118970, Abcam) according to the manufacturer’s instructions. Briefly, the MDA in the sample reacted with thiobarbituric acid (TBA) to generate a MDA-TBA adduct. The MDA-TBA adduct was quantified colorimetrically (optical density (OD) = 532 nm). The experiment for each group was repeated three times.

**Glutamine uptake assay**

Breast cancer cells were cultured in six-well plates in glutamine-free DMEM/F-12 medium (Invitrogen, USA). After collecting and counting, cells were incubated with 200 nM [^3H]-L-glutamine (PerkinElmer, USA) in glutamine-free DMEM/F-12 medium for 15 min at 37 °C in the presence of curcumin or curcumin+SLC1A5 siRNA. Cells were collected, transferred to filter paper using a 96-well plate harvester (Wallac PerkinElmer), dried, exposed to scintillation fluid, and counts measured using a liquid scintillation counter (PerkinElmer).

**Determination of lipid ROS levels**
Cells (2×10⁵) were seeded in a 6-well plate and were treated with curcumin or Erastin for 24 h according to previous studies, and all cells were cultured in DMEM medium with 5 μM BODIPY-C11 (Thermo Fisher Scientific, USA) for 45 min at room temperature. After that, cells were collected and washed twice with PBS buffer. Subsequently, cells were re-suspended in 500 μL PBS, and then filtered on a 0.4 μm nylon cell strainer and subjected to the flow cytometry analysis to detect the amount of ROS within cells. The fluorescence intensities of cells were determined using a FACSCalibur flow cytometer (BD Bioscience, USA). Each experiment was repeated three times.

**Statistical analysis**

All experiments were repeated at least three times. Data are presented as mean ± SD. Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, Inc., USA). Unpaired Student's t-tests were used to compare the means of two groups and one-way ANOVA was used to compare three or more groups. Pairwise group comparisons were conducted using Tukey’s test as a post-hoc test following ANOVA. P<0.05 was considered statistically significant.

**Results**

**Curcumin contributes to erastin-induced ferroptosis in breast cancer cells**

Previous studies have confirmed that curcumin functions as an antineoplastic agent in various solid tumors [11]. As expected, CCK-8 assay analysis results showed that curcumin had dose-dependent inhibitory effects on both MDA-MB-453 and MCF-7 cells (IC₅₀-MDA-MB-453 = 19.73 μM, Fig. 1A; IC₅₀-MCF-7 = 20.46 μM, Fig. 1B). Based on these data, a concentration of 20 μM of curcumin was selected as the follow-up experimental dose. Moreover, we examined the effect of curcumin on iron-dependent cell death in breast cancer. As shown in Fig. 1C and 1D, treatment with erastin, a ferroptosis activator, significantly inhibited cell viability compared with the control group, and co-treatment of curcumin and erastin also reduced the cell viability. Of note, treatment with ferrostatin-1 (Fer-1), a ferroptosis inhibitor, notably restored the inhibitory effect curcumin or erastin had on breast cancer cell viability, whereas pretreating cells with an apoptotic (ZVAD-FMK) or necroptotic inhibitor (necro-sulfonamide; NS) did not improve cell survival. Taken together, these results indicate that curcumin exhibits an antitumor effect on breast cancer cells by inducing cell ferroptosis.

**Curcumin promotes lipid peroxidation and iron accumulation in the process of ferroptosis**

Accumulating evidence demonstrates that cell ferroptosis is mainly caused by intracellular lipid peroxidation and the accumulation of lethal ROS during iron metabolism [28]. As shown in Figs. 2A and 2B, erastin treatment significantly enhanced the levels of lipid ROS accumulation compared with the control group. Furthermore, both breast cancer cell lines treated with 20 μM of curcumin increased lipid ROS levels, as shown by flow cytometry using the fluorescent probes C11-BODIPY. Moreover, compared with the control group, both MDA-MB-452 and MCF-7 cells treated with erastin or curcumin had a rise in the accumulation of MDA, which is one of the most vital end-products of lipid peroxidation (Fig. 2C).
Furthermore, higher levels of intracellular Fe$^{2+}$ were detected in the erastin and curcumin treatment groups than those in the control group (Fig. 2D). Treatment with curcumin or erastin decreased the mRNA levels of GPX4 and Ferritin light chain (FTL; Fig. 2E, 2F), and increased the mRNA levels of ACSL4 and NOX1 (Fig. 2E, 2F). Collectively, the above results indicated that curcumin significantly promotes ferroptosis in breast cancer cells in vitro.

**Upregulation of glutamine uptake is essential for curcumin-induced ferroptosis in breast cancer**

Previous studies have confirmed that abnormal glutamine metabolism may contribute to ferroptosis by the accumulation of products of lipid peroxidation in cancer cells (Fig. 3A) [29, 30]. As expected, curcumin treatment significantly enhanced glutamine uptake (Fig. 3B) in both breast cancer cells as compared with the control group. Next, we examined the effect of curcumin on the expression of crucial glutamine metabolism genes (such as SLC1A5, GLS2 and GOT1) by western blot. We found that both breast cancer cell lines treated with curcumin significantly increased the protein levels of SLC1A5 as compared with the control group (Fig. 3C, 3D), but the protein levels of GLS2 and GOT1 remained unchanged. Moreover, in both cell lines treated with an inhibitor of GLS2 (compound 968; C-968) and GOT1 (AOA) significantly restored the inhibitory effect of curcumin on cell viability (Fig. 3E). Furthermore, high levels of lipid ROS (Fig. 3F), MDA (Fig. 3G), and intracellular Fe$^{2+}$ (Fig. 3H) induced by curcumin treatment were alleviated by C-968 or AOA in both MDA-MB-453 and MCF-7 cells. Overall, the above data suggest that curcumin induces ferroptosis through the upregulation of glutamine uptake in breast cancer cells.

**Curcumin promotes ferroptosis by upregulating SLC1A5 in breast cancer**

To further examine curcumin-regulated ferroptosis via SLC1A5 in breast cancer cells, we transfected SLC1A5 siRNA (si-SLC1A5) into both MDA-MB-453 and MCF-7 cells to decrease its expression (Fig. 4A). The CCK-8 assay showed that curcumin treatment significantly reduced cell viability, which was ameliorated by the inhibition of SLC1A5 in both cell lines (Fig. 4B). Moreover, knockdown of SLC1A5 reduced the effect of curcumin on glutamine uptake (Fig. 4C). Similarly, the upregulation of lipid ROS (Fig. 4D, 4E), MDA production (Fig. 4F), and intracellular Fe$^{2+}$ levels (Fig. 4G) induced by curcumin in both breast cancer cell lines were reversed by suppression of SLC1A5. Taken together, these data indicated that curcumin exhibits its antitumor effect on breast cancer by promoting SLC1A5-mediated ferroptosis in vitro.

**Curcumin inhibits tumor growth of breast cancer in vivo**

Based on the functional role of curcumin in breast cancer cells in vitro, we explored the effect of curcumin on tumor growth in breast cancer in vivo. As shown in Fig. 5A-5C, the tumor volume and tumor weight in the treatment group that received curcumin at a dose of 30 mg/kg/d were decreased compared with the control group. Immunohistochemistry staining demonstrated that curcumin administration reduced the expression of Ki-67 in xenograft tumor tissues as compared with the control group (Fig. 5D), as well as enhanced SLC1A5 (Fig. 5D). Moreover, curcumin administration also resulted in elevated MDA production compared with the control group (Fig. 5E). Furthermore, curcumin treatment did not affect body weight
(Fig. 5F). In conclusion, our findings suggested that curcumin exerts its antitumor effect on breast cancer by promoting SLC1A5-mediated ferroptosis.

**Discussion**

Breast cancer is currently the cancer type with the highest morbidity and mortality in women. Although surgery, chemotherapy, and radiotherapy are effective treatment strategies for breast cancer, patients suffer from the side-effects of chemoradiotherapy. Therefore, it is of great consideration to understand the mechanism of breast cancer and seek novel strategies to reduce cancer mortality. In the present study, our results showed that curcumin significantly suppressed cell viability and tumor growth in breast cancer. Moreover, breast cancer cells treated with curcumin trigged cancer cell ferroptosis. Mechanistically, curcumin exhibited antitumorigenic effects on breast cancer via the upregulation of SLC1A5-mediated ferroptosis.

Increasing evidence confirms that therapeutic drugs targeting cell ferroptosis could effectively interfere with cell proliferation and inhibit tumor progression [21, 22, 31]. Ferroptosis plays a vital role in the crossroads of cancer-acquired drug resistance and immune evasion [32]. For example, inhibition of Nrf2 reversed cisplatin resistance via upregulating the expression of glutathione peroxidase 4, a regulator of ferroptosis, to induce ferroptosis in head and neck cancer [33]. Sun et al. reported that metallothionein-1G contributes to sorafenib resistance by suppressing ferroptosis in hepatocellular carcinoma [34]. Moreover, other studies have found that glutamine metabolism is conducive to cell ferroptosis via enhancing the accumulation of oxidizable lipids [35, 36]. SLC1A5, which acts as an essential transporter for glutamine uptake, was associated with the progression of several tumors, including colorectal cancer [37], esophageal cancer [38], pancreatic ductal carcinoma [39], and breast cancer [40]. For example, inhibition of SLC1A5 restricted the progression of non-small cell lung cancer by decreasing the glutamine consumption, cell growth, and inducing cell autophagy and apoptosis [41]. Meanwhile, several studies found that the expression of SLC1A5 is higher in many solid cancers [42, 43], and suppression of SLC1A5 by use of the glutamine transporter inhibitor GPNA or by transfecting siRNA significantly inhibits tumorigenesis [41, 44]. The above studies suggested that targeting SLC1A5 may be an effective therapeutic target for cancer treatment, particularly to target glutamine metabolism and cell biological behavior. In the present study, we found that upregulating SLC1A5 by curcumin treatment significantly enhanced the levels of glutamine accumulation and lipid ROS, which induced cell ferroptosis in breast cancer.

Accumulating evidence has confirmed that curcumin could suppress cell proliferation and induced cell apoptosis in various cancers [45]. Moreover, curcumin exhibited anti-metastasis activity in breast cancer cells and inhibition of the proliferation of cancer stem-like cells [17], as well as helped to overcome chemoresistance of cancer via suppressing the expression of multiple anti-apoptotic proteins [46]. Of note, previous studies found that curcumin may act as potential therapeutic agents in breast cancer [16] and as an adjunct therapy in breast cancer [47]. Meanwhile, several studies found that curcumin combined with chemotherapeutic drugs induced cell death [48] and restrained cell migration and invasion.
capacities of breast cancer [49]. However, the effect of curcumin on cell ferroptosis in breast cancer and its mechanism remained unknown. In the present study, our data showed that treatment with curcumin obviously inhibited the viability and tumor growth of breast cancer cells. This suppression of curcumin in breast cancer cell growth was induced via inhibition of SLC1A5-mediated ferroptosis.

**Conclusion**

Our results show that curcumin can inhibit cell proliferation and induced cell ferroptosis in breast cancer. Moreover, curcumin exhibits its antitumor effect on breast cancer via enhancing SLC1A5 to induce ferroptosis *in vitro* and *in vivo*. Overall, this study provides new insights on curcumin as an active anticancer agent and supports evidence for curcumin as a potential antitumor drug for breast cancer clinical treatment. Moreover, the mechanisms by which curcumin regulates SLC1A5-mediated ferroptosis should be discussed in our follow-up work.

**Abbreviations**

ACSL4: Acyl-CoA synthetase long-chain family member 4; CCK-8: Cell counting kit-8; cDNA: Complementary DNA; C-968: Compound 968; DMEM: Dulbecco's modified Eagle's medium; FBS: Fetal bovine serum; Fer-1: Ferrostatin-1; FTL: Ferritin light chain; GPX4: Glutathione peroxidase 4; MDA: Malondialdehyde; NOX1: Nicotinamide adenine dinucleotide phosphate-oxidase 1; NS: Necrosulfonamide; PVDF: Polyvinylidene difluoride; qRTPCR: Quantitative real-time polymerase chain reaction; ROS: Reactive oxygen species; SLC1A5: Solute carrier family 1 member 5

**Declarations**

**Acknowledgments**

Not applicable.

**Author's contributions**

(I) Conception and design: Chao Zhu and Jialiang Guan; (II) Provision of study materials: Xuelei Cao and Yao Li; (III) Collection and assembly of data: Yongbin Wang, Tao Yu, and Xuezhi Zhang; (IV) Data analysis and interpretation: Xuelei Cao, Yongbin Wang, and Tao Yu; (V) Manuscript writing: Xuelei Cao and Yao Li; (VI) Final approval of manuscript: All authors.

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**Availability of data and materials**
The datasets used and/or analyzed in the current study are available from the corresponding author on request.

**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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