Activation Ferroptosis Enhanced the Therapy Sensitivity of TNBC to Paclitaxel Via NCOA4 Mediated Ferritinophagy

Yu Qiu

Nanchang University Medical College: Medical College of Nanchang University

Qifeng Yu

Nanchang University Medical College: Medical College of Nanchang University

Meng Ji

Nanchang University Medical College: Medical College of Nanchang University

Zhiwei Zhang

Nanchang University Medical College: Medical College of Nanchang University

Liuyun Kang

Nanchang University Medical College: Medical College of Nanchang University

Ying Fu

First Affiliated Hospital of Nanchang University

Miao Feng (✉ ncufengm@163.com)

First Affiliated Hospital of Nanchang University <https://orcid.org/0000-0001-8859-1308>

Primary research

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Abstract

Background

Paclitaxel (PTX) is the first-line drug for chemotherapy, but Triple-Negative Breast Cancer (TNBC) develops resistance to PTX is the main challenge in clinical therapy. Ferroptosis is a new type of regulated cell death (RCD) characterized by iron-dependent lipid peroxidation accumulation, despite activation of ferroptosis has been shown to enhance the sensibility of tumor to chemotherapy drugs, the underlying mechanism remains unclear.

Methods

C11-BODIPY probe and real time PCR was used to detect the ferroptosis level in breast cancer cells. FerroOrange probe and western blot was employed to measure ferritinophagy. The cell viability was examined by CCK-8, EdU and AM/PI double staining.

Results

The anti-cancer effect of PTX was related to the induction of ferroptosis in TNBC cells, the ferroptosis occurring was mediated by NCOA4-driven ferritinophagy, and the autophagy-related molecules also downregulated in clinical specimens. Furtherly, TNBC cells survival was decreased more significantly than PTX alone when combining with ferroptosis agonist RSL3. In addition, we also discovered that HERC2, a unique identified E3 ubiquitin ligase of NCOA4, obviously expressed downregulation under PTX treated TNBC cells while elevated in breast carcinoma comparing with adjacent tissues. Meanwhile, bioinformatics results showed that the survival rate was significantly lower in breast cancer patients with high expression of HERC2.

Conclusions

Ferroptosis agonist can assist PTX to enhance the sensitivity of TNBC to PTX in chemotherapy, which possible mechanism is prompting the expression of NCOA4 and the occurrence of ferritinophagy.

1. Background

As one of the most common malignancies, breast cancer ranks at a leading position of female tumor incidence [1], triple-negative breast cancer (TNBC) accounts for 15–20% among all types of breast cancer, and is regarded as the most malignant subtype due to its high heterogeneity, aggressiveness and lack of effective therapeutic targets [2]. Comparing with other subtypes, TNBC is insensitive to endocrine and anti-HER2-targeted therapy, and has poor efficacy to traditional chemical or radioactive therapy. Meanwhile, the specific strategy targeting the optimum treatment has not been established, thus new therapies are urgently needed for TNBC [3]. Paclitaxel (PTX) is a kind of plant alkaloid specifically acting on cell cycle which often used as a first-line chemotherapy drug for breast cancer [4]. Unfortunately, the

resistance of breast cancer to PTX is a main barrier in clinical application and causes the failure of chemotherapy [5].

Ferroptosis, a non-apoptosis form of regulated cell death (RCD) induced by intracellular iron overload and lipid peroxide accumulation, which already involves in cancer therapy [6–8]. Some researches suggest that ferroptosis is a form of autophagic cell death which could be induced by ferritinophagy [9, 10]. Nuclear receptor coactivator 4 (NCOA4), a selective cargo receptor responses for ferritin autophagic degradation, which plays a crucial role in ferritinophagy [11, 12]. In addition, HERC2 is the only detected E3 ubiquitin ligase of NCOA4 that regulates the proteasomal degradation of NCOA4 in an iron dependent manner [13, 14].

Owing to the therapeutic effect of breast cancer is limited by drugs resistance and tumour progression, it is of particular urgent to explore a new method to reverse the drug-fast of TNBC cells. In this report, we measured that the ferroptosis level is elevated in PTX treated TNBC cells whereas decreased in breast cancer tissues. Furthermore, the proliferation of TNBC cells was dramatically inhibited after treated by a specific ferroptosis agonist RSL3 combined with PTX. Interestingly, we also observed that HERC2 is closely related to the prognosis of breast cancer patients, which indicating HERC2 is a potential therapeutic target for TNBC.

2. Materials And Methods

2.1 Cell culture

The breast cancer cell lines MDA-231, BT-549 (TNBC cell line) and MCF-7 (ER-positive cell line) were cultured in Roswell Park Memorial Institute – 1640 (Solarbio, China). All media were supplemented with 10% fetal bovine serum (FBS), and the cells were incubated at 37°C in humidified air containing 5% CO₂

2.2 Human breast cancer specimens

Five pairs of human breast cancer and adjacent tissues were obtained from patients undergoing surgical resection in the First Affiliated Hospital of Nanchang University, and we obtained the informed consent of the ethics committee (Doc. S1).

2.3 Cell proliferation and cell viability assay

2.3.1 Cell activity assay

MDA-231 (2000 cells per well) were plated in 96-well plates, treated for 24 h with PTX (Lemetian Medicine, China), and then measured using a Cell Counting Kit (CCK-8, TransGen BioTECH, China).

2.3.2 5-ethynyl-2'-deoxyuridine assay

MDA-231 cells were seeded in 96-well plates overnight. After different treatments, the cells were further cultured for another 24 h, and the proliferation rate was detected using a 5-ethynyl-2'-deoxyuridine (EdU) Cell Proliferation Kit (KeyGEN BioTECH, China). The EdU was added to the cells and incubated for 2 hours at 37°C, stained with Hoechst for 30 minutes after being fixed with 4% paraformaldehyde [15].

2.3.3 Cytotoxicity assay

The cells were seeded in 12-well plates and treated with 2 µM calcein AM, and 8 µM PI solution for 30 min.

2.3.4 Wound scratch assay

The cells were seeded in 12-well plates. When the paving rate reached nearly 100%, the cells were scratched on the plate with the pipette micro tip and observed under a microscope timely.

2.4 Measurement of Lipid peroxidation in vitro

MDA-231 cells were seeded in 96-well plates and incubated with H2DCFDA (MEC, HY-D0940, China) working solution about 30 min after different treatments.

In addition, lipid peroxidation was measured by C11-BODIPY 581/591 probe (Thermo Fisher, the US). Briefly, cells were incubated for 30 min with C11-BODIPY 581/591 (1 µM) in growth medium.

Fluorescence of C11-BODIPY was measured by simultaneous acquisition of the green (484/510 nm) and red signals (581/610 nm) [16].

2.5 Quantitative real-time PCR

Total RNA from each sample was extracted with TransZol Up Plus RNA Kit (TransGen Biotech, China) and then reverse transcribed using the PrimeScript RT reagent Kit with a gDNA Eraser (TaKaRa, Japan).

Amplification was performed using the TB Green Premix Ex Taq (TAKARA, Japan) with 40 cycles of 95°C for 15 s and 60°C for 30 s on a StepOnePlus Real-Time PCR System. The relative expression level of each transcript was normalized to murine GAPDH by using the $2^{-(\Delta\Delta Ct)}$ method [17]. Table S1 contains the list of primers used in this study.

2.6 Immunoblotting

Immunoblotting (IB) was performed as described previously [16]. Images were acquired using a NIKON Eclipse 80i microscope. The primary antibodies used in this study include GAPDH (ProteinTech, 60004-1-IG), NCOA4 (Affinity, DF4255), LC3 (ProteinTech, 14600-1-AP), ATG5 (Wanleibio, WL02411), ATG7 (Wanleibio, WL02793), Beclin1 (Wanleibio, WL02508), p62 (Wanleibio, WL02385), HERC2 (Abcam, AB85832), ACSL4 (Proteintech, 2241-1-AP), GPX4 (Abcam, AB125066).

2.7 Free iron concentration detection

FerroOrange (Dojindo, China) was used to assess iron concentrations and measure intracellular free iron levels. In this assay, ferric carrier protein was dissociated from trivalent iron in a reductive environment and reduced to ferrous form, after which the intracellular Fe^{2+} reacted with the probe to form a stable colored complex.

2.8 Mitochondrial membrane potential measurement

The JC-1 apoptosis detection kit (MCE, China) was used to detect mitochondrial membrane potential (MMP). MDA-231 cells were washed three times in sterile PBS, and JC-1 working solution was added to each well. After incubating for 30 min under 5% CO_2 and 37°C conditions, washing cells with PBS three times and observing under a NIKON Eclipse 80i fluorescence microscope.

2.9 HE and immunohistochemistry

Breast cancer tissue and para cancer tissue were immobilized overnight in 4% paraformaldehyde and embedded in paraffin. Observing the morphological characteristics of the carcinoma and adjacent tissues with hematoxylin and eosin staining. Optical microscopes were used to obtain images of immunohistochemistry (IHC). The main antibodies used in this study included HERC2 (Abcam, AB85832) and NCOA4 (Affinity, DF4255).

2.10 Statistical analysis

One-way ANOVA or two-tailed student's t test were used to compare multiple groups and performed with GraphPad Prism 8 software. P value < 0.05 was considered statistically significant, and data were presented as the mean $\pm$ SD error from at least three independent experiments.

3. Results

3.1 PTX decreased cell viability in concentration dependence

To observe the inhibitory effect of PTX in breast cancer cell lines, we chose 3 kinds of cell lines: MDA-231, MCF-7 and BT-549. The results showed that PTX decreased cell viability in a concentration-dependent manner, and the inhibition ratio in MDA-231 and BT-549 cells was significantly higher than MCF-7 cells, which indicating that the TNBC is more sensitive than non-TNBC under PTX treatment (Fig. 1a).

Comparing with the control group, the proliferation ability of MDA-231 obviously declined in $10\mu\text{mol/ml}$ PTX group, and dramatically decreased in $50\mu\text{mol/ml}$ PTX group (Fig. 1b, c). Calcein AM/PI experiment found that living cells were clearly reduced along with the increasing of PTX dosage while the dead cells were gradually increased, which suggesting the obviously cytotoxicity of PTX (Fig. 1d). Furtherly, scratch assay displayed that cell migration was almost closed in the control group whereas barely migrated in the PTX exposition group after 24h (Fig. 1e). In a word, PTX can inhibit the viability or proliferation of MDA-231 in dose-dependent manner.

3.2 Ferroptosis involved in PTX-induced cell death

Recently, studies confirmed that ferroptosis is related to tumor occurrence and development. Firstly, we used 10 and 50 $\mu\text{mol/ml}$ PTX to culture with MDA-231 cells. Treated 24 h later, the mRNA level of SLC7A11 (ferroptosis related protein) was decreased while the expression of Lipoygenase ACSL4 was increased (Fig. 2a), and the protein level further confirmed the above changes (Fig. 2b, c). Interestingly, as a glutathione peroxidase reductase, GPX4 showed a downregulation at 6 h and 42 h, a no tendency at 12 h and an upregulation at 24 h and 30 h time-in-point under 30 $\mu\text{mol/ml}$ PTX treatment (Fig. 2d). Furtherly, we used ferroptosis specific inhibitor Liproxtatin-1 (Lip-1) or Ferrostatin-1 (Fer-1) to co-culture MDA-231 with PTX. C11-BODIPY staining found that the lipid peroxides were accumulated in PTX treated MDA-231, but Lip-1 can effectively prevent the lipid peroxides production (Fig. 2e). Meanwhile, the inhibition rate of cells was decreased, which proving that PTX toxicity was reversed when the ferroptosis pathway was blocked (Fig. 2f, g, h). Subsequently, adopting JC-1 probe, we detected that the MMP of MDA-231 decreased with the increase of PTX concentration, which could be reversed by either Fer-1 or Lip-1 co-treatment (Fig. 2i). we also measured changes of ROS via H2DCFDA probe, although the increasing of PTX concentration caused ROS accumulation, ferroptosis inhibitor could clear excessive ROS (Fig. 2j) in some extent. In summary, the above results indicated that ferroptosis maybe participate in PTX-induced cell death

3.3 Cellular Ferroptosis was induced by Ferritinophagy

Autophagy is tightly associated with ferroptosis, we found that the expression level of ferritinophagy marker NCOA4 was positively related with PTX concentration. Similarly, the expression level of autophagy proteins ATG5, ATG7 and Beclin1 were also upregulated whereas the autophagocytic receptor p62 was decreased, which suggesting the activation of ferritinophagy pathway in PTX treated cells (Fig. 3a, b). Interestingly, the mRNA levels of above molecules displayed an analogous change as the protein except for p62 (Fig. 2c). Meanwhile, FerroOrange, an intracellular ferrous ion fluorescence probe was used to measure the concentration of Fe^{2+} , the results showed that MDA-231 cells appeared an increasing fluorescence intensity in 10 $\mu\text{mol/ml}$ PTX group, which could be reversed in response to ferroptosis inhibitors (Fig. 3d). Given the established role of Bafilomycin A1 (Baf A1) in blocking autophagy flux, exposure of cells to Baf A1 over time robustly induced the conversion from LC3- β to LC3- α and increased p62 levels, and comparing with the control Baf A1 group, the LC3- α expression level was higher after adding PTX (Fig. 2e, f). Together, our results discovered that PTX induced TNBC cells death through ferritinophagy triggering ferroptosis.

3.4 Autophagy related molecules expression upregulated in breast cancer tissues

To verify the previous results, we obtained 5 pairs breast cancer tissues and adjacent normal tissues, respectively. Figure 4a was HE staining which was performed to observe the morphological characteristics between cancer tissues and adjacent tissues. IHC staining showed that NCOA4 in tumor tissues expressed a lower level than in adjacent tissues (Fig. 4b). In addition, there is a same change for NCOA4 in mRNA and protein expression as well (Fig. 4c, d, e), suggesting that breast cancer cells have ability of evading ferritinophagy. Finally, we detected the expression of autophagy-related proteins, and

found that Atg5, Atg7 and Beclin1 were all downregulated in cancer tissues compared with adjacent tissues (Fig. 4d, e), which furtherly suggesting the absence of ferritinophagy in tumor samples.

3.5 Synergistic stimulation of ferroptosis enhanced TNBC cells sensitivity to PTX

Next, we discussed whether ferroptosis specific agonist RLS3 could strengthen anticancer effect of paclitaxel to TNBC cells. Comparing with PTX or RSL3 treated alone group, the combination of them significantly enhanced the expression of NCOA4 and autophagy related molecules, which indicating the reinforcement of ferritinophagy in cells (Fig. 5a, b, c). The staining assay showed green fluorescence co-treated group by PTX and RSL3 (or another ferroptosis specific agonist erastin) increased, suggesting that lipid peroxide and total ROS accumulated (Fig. 5d, e) more than PTX or RSL3 group. In order to assess the toxic effect of co-treatment, we detected the number of living cells and proliferation ability by Calcein and EdU staining, and the results proved that the cell viability was apparently decreased comparing with the monotherapy treatment (Fig. 5f, g), which verifying that activation of ferroptosis can improve the sensitivity of TNBC cells to PTX.

3.6 Relationship between HERC2 and clinical prognosis

In view of the close relationship between HERC2 and NCOA4, we attempted to explore the role of HERC2 in breast cancer progression. Surprisingly, through previous clinical specimens, the results showed that the expression of HERC2 in cancer tissues was higher than that in adjacent tissues (Fig. 6a, c, e). Besides,HERC2 levels were significantly downregulated in MDA-231 cells after treating with PTX (Fig. 6b, d), and RSL3 could enhance this trend whereas reversed by Lip-1 (Fig. 6b,). Our results demonstrated that HERC2 was involved in PTX-induced ferroptosis and closely associated with clinical prognosis. Whereafter, we obtained the HERC2 gene-related survival curve of breast cancer patients and found that the higher HERC2 expression correlated to a poor prognosis based on GEO/TCGA database (Fig. 6f). In addition, available data demonstrated that there was a high correlation at post-transcriptional level between NCOA4 and HERC2 (Fig. 6g), and bioinformation analysis showed the lower activity of ferroptosis in breast cancer tissue (Fig. 6h, i). All in all, our study supposed that PTX maybe exert the cytotoxic effect in TNBC via HERC2 negatively regulating the activation of ferritinophagy.

4. Discussion

Here, we confirmed that ferroptosis is involved in the process of PTX-induced TNBC cells death through ferritinophagy driven by NCOA4, and ferroptosis specific agonist can enhance the anti-tumor effect. Previous studies showed that TNBC is more sensitive to ferroptosis than ER-positive breast cancer [18], we also found that compared with MCF-7, MDA-231 and BT-549 were more sensitive to PTX treatment. The two types of TNBC cells showed similar morphology and response to drugs, and we chose more representative MDA-231 to illustrate our results. Our data has identified that not only is the survival rate suppressed but also the ability of proliferation and migration are significantly decreased as TNBC cells

exposing to PTX. Notably, despite PTX is effective in anti-tumor, the prognosis of breast cancer patients is severely worse because of drugs resistance.

Some studies have proved that activating ferroptosis could enhance the anti-cancer effect of chemotherapy drugs [19, 20]. System Xc⁻, locates in cellular membrane, consists of SLC7A11 and SLC3A2, which charges the exchange between cysteine and glutamate to maintain redox balance [21]. Erastin, a ferroptosis specific agonist, can cause ferroptosis by blocking SLC7A11 to prevent cystine into cells. As a lipoxygenase, ACSL4 converts free fatty acid into acyl-CoA ester, which is required for ferroptosis [22–24]. In general, SLC7A11 and ACSL4 is regarded as the molecular maker of ferroptosis. GPX4, can clear lipid hydroperoxides, is a negative regulator of ferroptosis and the direct substrate of agonist RSL3 [25]. In Fig. 2d, however, the expression of GPX4 did not consistently decline as expected after PTX treatment in MDA-231, but showed an upward trend for a certain time. We guessed this upregulation in view of the cells suffer PTX stimulation to yield a compensatory mechanism. Ferroptosis is characterized by the accumulation of iron-dependent lipid peroxides [26], then we measured the accumulation of lipid peroxides in cells via specific fluorescent probe such as JC-1, H2DCFDA and C11-BODIPY [27], and the data showed that PTX could induce ferroptosis of MDA-231 in concentration dependent manner. Next, we furtherly attempted to explore the specific mechanism of ferroptosis.

As a newly identified selective autophagy mediated by NCOA4, ferritinophagy can lead to imbalance of iron pool, trigger Fenton reaction and finally cause ferroptosis under overactivated condition [28, 29]. We observed NCOA4 was significantly increased in PTX-treated MDA-231 as well as other autophagy-related proteins showed similar change, suggesting that ferritinophagy might participate in the PTX induced ferroptosis. Studies discovered that selective autophagy is mediated by autophagy receptors such as p62/SQSTM1 and relies on ATG8 as a linker [30, 31]. Our results showed that the protein level of p62 decreased whereas the mRNA level obviously elevated. The reason maybe is autophagosomes fusing with lysosomes to cause receptor p62 degradation at the autophagy maturation stage. However, autophagy occurs needs a large amount of p62, which requires p62 to increase at transcriptional level and promotes p62 protein yields to compensate p62 degradation. As a potent autophagy inhibitor, Baf A1 inhibits the degradation of LC3-II by blocking autophagy-lysosome fusion [32]. In our studies, LC3-II level increased and p62 level descend with the PTX treatment, and further raised for LC3-II yet p62 elevated as well after combining with Baf A1, suggesting PTX can trigger autophagy [33].

Despite PTX is extensively used as first-line chemotherapy agent, the high dose caused resistance and toxicity restrict the application in clinic. Some studies demonstrated that low concentration of PTX has an effect on glutamine decomposition in cell metabolism which is crucial for ferroptosis [34, 35]. That inspired us to explore the possibility of combining low concentration PTX with ferroptosis agonist in TNBC therapy. In experiments, we selected the specific agonist RSL3 or erastin combining with low dose PTX to measure the ferroptosis marker, accumulation of lipid peroxide as well as the total ROS level in MDA-231. The results found that the autophagy-related proteins were upregulated and the viability of TNBC cells was decreased, which provides a new strategy for effectively treating TNBC and contributes to the application of ferroptosis in other cancer therapy.

As an E3 ubiquitin ligase, interaction proteomics has revealed that HERC2 can interact with NCOA4 through a feedback regulation by intracellular iron concentration alteration in physiological condition [13, 36]. Hence, HERC2 plays an important role in the occurrence of ferritinophagy, abnormal HERC2 expression maybe result in tumorigenesis or cell proliferation. According to database information and clinical data, the patients with high HERC2 level correspond to poor prognosis, the mechanism might be that high HERC2 repress the ferroptosis induced cell death by inhibiting ferritinophagy. As a result, blocking the HERC2 expression possible activates ferroptosis and further reverses the resistance of TNBC to PTX. Thus, further HERC2 knock down experiment in anti-PTX MDA-231 cells are needed to test the hypotheses mentioned above.

5. Conclusion

Take together, our studies proved that PTX could kill TNBC through NCOA4 driving ferritinophagy and the activation of ferroptosis in cells would enhance the therapy sensitivity to PTX. Additional investigations should be executed to explore the role of HERC2 on iron metabolism in breast cancer resistance, which will benefit to expand the application of ferroptosis in TNBC combination chemotherapy.

List Of Abbreviations

TNBC	Triple-Negative Breast Cancer
PTX	Paclitaxel
RCD	Regulated cell death
NCOA4	Nuclear receptor coactivator 4
HERC2	HECT and RLD domain containing E3 ubiquitin protein ligase 2
EdU	5-ethynyl-2'-deoxyuridine
MMP	Mitochondrial membrane potential
SLC7A11	Solute carrier family 7 member 11
GPX4	Glutathione peroxidase 4
ATG5	Autophagy related 5
ATG7	Autophagy related 7
Baf A1	Bafilomycin A1

Declarations

Ethics approval and consent to participate

We obtained the approval from the Ethics Committee of the First Affiliated Hospital of Nanchang University.

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

Competing interests

The authors declare no conflicts of interest.

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Authors' contributions

Conceptualization, Y.Q.; data curation, Y.Q.; formal analysis, Y.Q.; funding acquisition, Y.F., and M.F.; investigation, Y.Q., Q.Y., Z.Z., L.K.; methodology, Y.Q.; project administration, M.F.; resources, Y.Q. and Y.F.; software, Y.Q. and Q.Y.; supervision, M.F.; validation, Y.Q.; visualization, Y.Q., Z.Z. and Q.Y.; writing—original draft, Y.Q. and Q.Y.; writing—review & editing, Y.Q.

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Table

Table 1

Primer sequences used for quantitative real-time PCR

Genes	Primer Sequence 5'-3'	
GAPDH	FORWARD	GGAGTTCAACAATGCTATCAGC
	REVERSE	CTTCTGGTAGGTGTGTAGGATC
GPX4	FORWARD	ACAAGTACCGGGGCTTCGTG
	REVERSE	CCAGGATCCGCAAACCACAC
SLC7A11	FORWARD	GGCCATTGTCACCATTGGCTA
	REVERSE	GCCGCTCAGAAAAGGTCACT
ACSL4	FORWARD	GTGGTTCTACTGGCCGACCT
	REVERSE	TGTGTCCTTCGGTCCCAGTC
NCOA4	FORWARD	TTCGATAAGCCGTCACCTGGAATG
	REVERSE	GCCTGCTGTTGAAGTGTCTCCTC
HERC2	FORWARD	AGCTGGAGAAAAGATGCCCTC
	REVERSE	CATCTCTTGTGAATGCAAGCTGT
TFRC	FORWARD	TTCGCAGGCCAGTGCTAGG
	REVERSE	ACAAGGGAGTACCCCGACAG
LPCAT3	FORWARD	TGGGACAAATGGCTTAAGGTGT
	REVERSE	TGGCACCATTGCTTTGTGGA
Beclin1	FORWARD	TGTCACCATCCAGGAACTC
	REVERSE	CTGTTGGCACTTTCTGTGG
ATG5	FORWARD	AAGGTGGAGTTGGCGAGACG
	REVERSE	CAACCAAAGCCAACTTAGTAAGCAAC
ATG7	FORWARD	GGAGATTCAACCAGAGACC
	REVERSE	GCACAAGCCCAAGAGAGG
p62	FORWARD	CTTGTGTAGCGTCTGCGAGG
	REVERSE	GAGAAGCCCTCAGACAGGTG

Figures

Figure 1

PTX decreased cell activity in a concentration dependent manner. a CCK-8 assay was used to measure the inhibition rate of three types of breast cancer cells after PTX treatment. b, c EdU imaging experiment detected the effect of PTX on cell proliferation, the scale is 1000 μ m. d The toxicity of PTX was detected by AM/PI double staining, the bar is 1000 μ m. e Cell scratch assay was used to detect the effect of PTX on the migration ability of MDA-231 cells, the scale is 5 mm. * $P < 0.05$, ** $P < 0.01$.

Figure 2

PTX-induced cell death is closely related to ferroptosis. a mRNA expression levels of ACSL4, SLC7A11 after PTX treatment. b, c Protein expression levels of ACSL4, SLC7A11. d mRNA expression level of GPX4 over time. e C11-BODIPY was used to detect the accumulation level of Lipid peroxides, the bar is 1000 μ m. f The inhibition rate of MDA-231 was detected by CCK-8 after PTX and ferroptosis agonists co-treatments. g, h EdU evaluated the proliferation capacity, the bar is 1000 μ m. i Change in mitochondrial

membrane potential levels by JC-1 probe detection, the scale is 500 μ m. j Intracellular ROS levels detected by H2DCFDA, the bar is 1000 μ m. * $P < 0.05$, ** $P < 0.01$.

Figure 3

The mechanism of Ferroptosis is related to Ferritinophagy. a, b Protein expression of NCOA4, ATG7, ATG5, Beclin1, p62. b mRNA levels of NCOA4, ATG7, ATG5, Beclin1, p62. d FerroOrange detected the

concentration of Fe²⁺ after PTX treatment, the bar is 1000 μ m. e, f Protein levels of p62, LC3- β treated with Baf A1 and PTX. * P < 0.05, ** P < 0.01.

Figure 4

Ferritinophagy was tested in clinical samples. a HE staining of breast cancer tissues and adjacent tissues. b IHC of NCOA4. c qPCR showed the mRNA expression of NCOA4 in tissues. d, e Protein levels of NOCA4. Bar is 1000 μ m. * P < 0.05, ** P < 0.01.

Figure 5

Low concentration PTX and RSL3 co-promote ferroptosis. a mRNA expression of NCOA4, Beclin1, ATG5, ATG7 after 10 μ M PTX and RSL3 co-treatment. b, c WB detected protein of NCOA4, Becline, ATG5, ATG7. d C11-BODIPY showed more Lipid peroxides accumulation of combination therapy. e the increasing intracellular ROS was detected by H2DCFDA. f Calcein staining showed cell survival after co-treatment of PTX and RSL3. g EdU was used to detected the cell proliferation. Bar is 1000 μ m. * $P < 0.05$, ** $P < 0.01$.

Figure 6

HERC2 is closely related to clinical prognosis. a IHC of HERC2 in breast cancer and adjacent tissues. b mRNA expression of HERC2 after different medication. c mRNA of HERC2 in tissues. d Protein level of HERC2 after PTX treatment. e Protein level of HERC2 in tissues. f The survival curves of patients with high and low expression of HERC2. g Protein interaction network showed that NCOA4 and HERC2 are

related. h Heat map showed different gene expression in TNBC and adjacent tissues. i The volcano figure of ferroptosis markers. Bar is 1000 μm . * $P < 0.05$, ** $P < 0.01$.

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