Aspirin promotes ferroptosis by attenuating Nrf2 in triple-negative breast cancer

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Research Article

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Abstract

Purpose

Recent research has found that patients who receive aspirin might have a reduced risk of breast cancer. However, how aspirin influences cancer remains controversial. Ferroptosis is an iron- and reactive oxygen species (ROS)-dependent form of regulated cell death (RCD), and it is crucial for the suppression of tumors. Herein, we investigated the mechanism of aspirin as a novel ferroptosis inducer in the antitumor effect of triple-negative breast cancer (TNBC).

Methods

We performed cell proliferation, ferroptosis-related and xenograft assays to determine the function and mechanism of aspirin in TNBC. Additionally, we investigated the transcriptomic profiles in TNBC tissues and normal tissues by RNA-seq.

Results

Our present study revealed that aspirin not only significantly accelerated ferroptosis but also inhibited the growth of TNBC cells. Intriguingly, aspirin, similar to the ferroptosis activator erastin, promoted ferroptosis and inhibited cell proliferation; however, the additional ferroptosis suppressor ferrostatin reversed the function of aspirin in ferroptosis and cell proliferation. Mechanistically, these results indicated that aspirin attenuated the level of Nrf2 protein and enhanced Keap1, mainly through the Keap1-Nrf2 pathway, in TNBC cells. Meanwhile, aspirin could also suppress key ferroptosis factors, such as GPX4 and xCT. Importantly, restoring Nrf2 signaling reversed aspirin-mediated ferroptosis. Furthermore, we found that oxidative stress and the Keap1-Nrf2 pathway were significantly changed.

Conclusion

Overall, our research demonstrates a novel role of aspirin in inhibiting Keap1-Nrf2 signaling to accelerate ferroptosis, which leads to the inhibition of cell proliferation in TNBC. Aspirin may present as a potential preventive strategy against the development of TNBC.

Introduction

Breast cancer accounts for approximately 30% of cancers among women worldwide (Siegel et al., 2020). The most commonly used biomarkers in the clinic are estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 protein (HER2) (Denkert et al., 2017). TNBC, a specific subtype that does not express ER, PR and HER2, accounts for 15–20% of all breast cancers (Adams et al., 2019). TNBC has the worst overall survival (OS) due to its higher local recurrence rate and distant
metastasis rate, compared to other breast cancer subtypes. Nearly 30% of TNBC patients experience tumor recurrence within five years of diagnosis (Lin et al., 2012). Consequently, it is crucial for patients with breast cancer to choose suitable therapy.

One of the milestones of modern therapies is aspirin (acetylsalicylic acid). It plays a crucial role in ache and cardiovascular therapies (Eccleston et al., 2017; Ornelas et al., 2017). Additionally, according to epidemiological research, daily use of low-dose aspirin can decrease the risk of developing cancer. Therefore, researchers have suggested that aspirin could be used for adjuvant therapy (Flossmann and Rothwell, 2007; Rothwell et al., 2010). Our previous article showed that aspirin upregulates β-TrCP to overcome chemotherapy-related drug resistance to impair YAP and β-catenin. Moreover, combining aspirin and antimicrotubule agents may be a promising strategy for TNBC in the future (Ma et al., 2020).

Ferroptosis, the main characteristics of which are iron accumulation, excess ROS generation and lipid peroxidation, is a newly discovered regulatory form of cell death (Dixon et al., 2012). It is crucial for the system xc-/glutathione peroxidase 4 (GPX4) axis to control lipid peroxidation in tumor cells (Yang et al., 2014). Some substances, such as pharmacological blockade of key enzymes and deprivation of substrates, can trigger ferroptosis (Li et al., 2021; Ursini and Maiorino, 2020). Ferroptosis is an iron-dependent form of RCD, and it is different from apoptosis and autophagy. Meanwhile, it is characterized by specific cytological changes, including damage to the outer mitochondrial membrane and disruption in mitochondrial cristae (Jiang et al., 2021; Tang et al., 2021). In addition, multiple diseases, such as immune system diseases (nonalcoholic steatohepatitis), neurodegenerative diseases (Alzheimer's disease (AD) and Parkinson's disease (PD)), brain diseases (stroke and intracerebral hemorrhage) and heart disease, are associated with ferroptosis (Qiu et al., 2020; Yu et al., 2017). In some of these diseases, inhibiting ferroptosis has been considered a potential treatment strategy in clinical application. In addition, new evidence has demonstrated that more oncogenic pathways are influenced by ferroptosis. For instance, p53 regulation can cause tumor cell ferroptosis while decreasing tumor metastases. Viswanathan and companions believe that ferroptosis can cause drug resistance and play a role in cancer immunotherapy (Viswanathan et al., 2017; W. Wang et al., 2019). Erastin, a well-known ferroptosis inducer, can directly inhibit system Xc- to decrease the level of GSH and cause ferroptosis (Dixon et al., 2012). Thus, targeting ferroptosis might be a strategy for tumor treatment.

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a well-known transcription factor, can regulate iron metabolism. According to research, Nrf2 activation can inhibit ferroptosis in tumor cells (Shin et al., 2018). Kelch-like ECH-associated protein 1 (Keap1) is an adaptor for Cullin 3 (Cul3)-based ubiquitin E3 ligase, and it is a fine-turn apparatus that controls the activation of Nrf2 (Kobayashi et al., 2004). Recently, an article supported that the Keap1-Nrf2 pathway is thought to be the main pathway for Nrf2 degradation in the cytoplasm. Meanwhile, the β-transducin repeat-containing E3 ubiquitin–protein ligase (β-TrCP)/Nrf2 pathway is also a parallel regulation system for the degradation of Nrf2 (Taguchi et al., 2014). In the MDA-MA-231TBNBC cell line, the Keap1-Nrf2 pathway can regulate Nrf2-targeted genes, including heme oxygenase-1 (HO-1), to regulate the oxidative stress response and ferroptosis (Chang et al., 2018).
Herein, we found that patients with TNBC who receive aspirin might have survival benefits. Mechanistically, aspirin attenuated the expression of Nrf2 via the Keap1-Nrf2 and β-TrCP/Nrf2 pathways, increasing the sensitivity of TNBC cells to ferroptosis by blocking the HO-1-mediated oxidative stress response. This research might offer a research basis for the use of aspirin in TNBC cancer therapy.

Materials And Methods

Cell lines

Cell lines (MDA-MB-231 and MDA-MB-468) were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). These cell lines were cultured in RPMI-1640 or DMEM (HyClone, Waltham, MA) according to the recommended culture method. In addition, all these cell lines were cultured with 10% fetal bovine serum and 1% penicillin–streptomycin in a 5% CO2 atmosphere at 37°C.

Cell viability assay

Cytotoxicity was determined by CCK-8 assay (APE × BIO, USA). Cells were seeded in 96-well plates in 100 µL of medium. Each well received ten microliters of CCK-8 solution and was incubated for 4 h at 37°C. In addition, we used a spectrophotometer to measure the absorbance at 490 or 450 nm.

Measurement of ROS

Researchers used DCFH-DA (Solarbio, D6470) to detect the level of ROS. Cells were seeded in 6-well plates and cultured. Then, the cells were collected and washed three times with PBS solution. Later, these cells were labeled with 10 µM DCFH-DA at 37°C for 60 min in the dark. Then, the cells were harvested. The percentage of fluorescence intensity was calculated after fluorescence microscopic analyses.

Lipid ROS assay

For the lipid ROS assay, ROS were detected by C11-BODIPY C11 (Solarbio, D3861). After the cells were exposed to the specified treatments, 2 µM C11-BODIPY C11 was added, and the cells were incubated at 37°C for 45 min. Then, researchers used FlowJo V10 software to analyze all results.

Measurement of glutathione and malondialdehyde

We used kits (Solarbio BC1185, BC1175 and BC0025) to measure the levels of glutathione oxidized (GSSG), glutathione (GSH) and malondialdehyde (MDA). The cells were seeded in a black 96-well plate and treated according to the manuals. Fluorescence was measured with excitation set at 390 nm and emission set at 478 nm.

Western blot

Researchers collected cells and lysed them on ice for 30 min using radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific 89900). Then, proteins from cell lysates were resolved by SDS–PAGE and transferred to PVDF transfer membranes for antibody blotting. The membranes were blocked for 1.5
h at room temperature with 5% nonfat dry milk and incubated with antibodies against GPX4, xCT, Nrf2, Keap1, HO-1, and β-TrCP (Abcam, USA) overnight at 4°C. Then, the product was incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. Finally, researchers used ECL reagent to detect target bands.

**Immunofluorescence**

Researchers collected TNBC cells and plated cells on coverslips. Immunofluorescence was performed according to the recommended protocol. Briefly, the cells were fixed in methanol for 5 min at room temperature after washing three times in PBS. Subsequently, the cells were treated with a blocking solution (1% BSA in PBS) for 30 min. Furthermore, after washing twice with PBS, the cells were incubated with the primary antibody against Nrf2 (Abcam, USA) overnight at 4°C. After that, the cells were rinsed twice in PBS for 10 min and incubated with a conjugated goat anti-rabbit IgG-Cy3 secondary antibody (Thermo Fisher Scientific, USA) for 2 h. Nuclei were stained with DAPI Staining Solution. The images were captured using a Leica Confocal Microscope.

**Small interfering RNA (siRNA) transfection**

We purchased siRNAs targeting the Keap1 gene from GenePharma (China). The sequences were as follows: Keap1 siRNA (sense: 5'-GGCCUUUGGCAUCAUGA-3' and antisense: 5'-GUUCAUGAUGCCAAGG-3'). Subsequently, according to the instructions, transfection of cells was performed using Lipofectamine 3000 reagent (Thermo Fisher Scientific).

**Nude mouse model and immunohistochemistry (IHC)**

A total of $5 \times 10^6$ MDA-MB-231 cells within 100 µl PBS were injected into the abdominal mammary fat pad of 4-week-old female nude mice (GEMPHARMATECH, China). After the tumor volumes exceeded 100 mm$^3$, the mice that met the criterion were randomized into a control group and an experimental group, with 5 mice in each group. In the control group, mice received intragastric PBS injections. In the experimental group, mice received intragastric aspirin at 50 mg/kg/d. All the animals were monitored for body weight and sacrificed after treatment for 30 days. Then, the tumors were excised, weighed and processed for IHC analysis. The animal experiment was approved by the Institutional Review Committee of West China Hospital of Sichuan University. The protocols of IHC and staining evaluation were described in our previous study (Ma et al., 2020).

**Data sources and preprocessing**

The raw data and clinical information of the breast cancer samples in The Cancer Genome Atlas (TCGA) database were downloaded from the UCSC XENA database (https://xenabrowser.net/datapages/) (Goldman et al., 2020). Based on the basic clinical information of these samples, 113 normal breast tissues and 159 TNBC samples were extracted for this study. Moreover, the raw data of 20 normal breast samples and 83 TNBC samples sequenced by the West China Hospital breast cancer specialist research team was obtained from the Genome Sequence Archive (GSA) in BIG Data Center.
Bioinformatics analysis

Differentially expressed analysis between TNBC and normal samples was analyzed using the "limma" R package in R. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was conducted in the online website Database for Annotation, Visualization, and Integrated Discovery (DAVID) (david.ncifcrf.gov) (Dennis et al., 2003). The heatmap and volcano plot to illustrate differentially expressed genes were plotted using R script. The best cutoff value of genes in survival analysis was searched by the "survminer" R package, and survival curves were generated with the Kaplan–Meier method.

Statistical analysis

The data are shown as the mean ± SD, and all experiments were conducted more than three times. Among several defined groups, correlation coefficients and p values were analyzed by using Spearman correlation analysis, and differences between the two groups were compared using Wilcoxon tests. The asterisks represent the statistical p values (*P < 0.05, **P < 0.01 and ***P < 0.001) in the panels.

Results

Aspirin promoted ferroptosis in triple-negative breast cancer cells

To validate the aspirin efficacy on TNBC cells, MDA-MB-231 and MDA-MB-468 cells were cultured in different doses of aspirin. Different doses of aspirin (0, 0.625, 1, 25 and 2.5 mM) reduced cell viability over time (24 and 48 h). Cell proliferation was inhibited by aspirin in a dose- and time-dependent manner (Fig. 1A). This is consistent with our previous study. Additionally, our previous studies suggested that aspirin inhibition of TNBC cell proliferation might be related to a novel pathway (Ma et al., 2020). Therefore, we hypothesized that it can enhance the effects of ferroptosis.

To verify our hypothesis, intracellular iron ions (Fe) and ROS levels, widely used ferroptosis indicators, were measured in MDA-MB-231 and MDA-MB-468 cells treated with different doses of aspirin for 24 h. The levels of intracellular ferrous ions increased significantly with the dose of aspirin in TNBC cells (Fig. 1B). We found that the proportion of fluorescent cells was positively correlated with the dose of aspirin after cells absorbed DCFH-DA (Fig. 1C). Furthermore, we analyzed the level of intracellular lipid ROS by flow cytometry. Our research indicated that the level of lipid ROS was elevated in a dose-dependent manner (Fig. 1D).

Additionally, our researchers detected the level of intracellular oxidation, which is also widely used in the detection of ferroptosis. The levels of GSSG and MDA, major membrane lipid peroxidation indicators, increased significantly with the dose of aspirin, while the level of GSH, the key antioxidant indicator,
decreased (Fig. 1E). These data indicated that aspirin could increase the level of cellular oxidation. In brief, these results suggested that the use of aspirin can promote ferroptosis in TNBC cells.

**Aspirin attenuated Nrf2 expression mainly via the Keap1-Nrf2 pathway in TNBC cells**

Our previous study showed that aspirin could promote β-TrCP expression. Therefore, we speculated that aspirin might affect Nrf2, an important molecule of ferroptosis, through the β-TrCP-Nrf2 pathway. First, we detected the distribution of Nrf2 by immunofluorescence in MDA-MB-231 and MDA-MB-468 cells, and the results showed that the relative expression (nucleus/total) of Nrf2 declined, which was dependent on the increase in aspirin (Fig. 2AB). This result indicated that aspirin attenuated Nrf2 expression and suppressed Nrf2 nuclear translocation in TNBC cells.

Moreover, according to nucleocytoplasmic separation and western blot analysis, the expression of classical ferroptosis inhibitors in the cytoplasm, such as GPX4, xCT, and Nrf2, showed a dose-dependent decrease in response to aspirin (Fig. 2CD). In addition, both Nrf2 in the cytoplasm and nucleus showed a dose-dependent decrease in aspirin, and the decrease was more obvious in the nucleus. This suggested that aspirin might lead to Nrf2 degradation in the cytoplasm; hence, we detected the Keap1-Nrf2 pathway, the main degradation mechanism of Nrf2 in the cytoplasm, through western blotting. In addition, we also detected the expression of the β-TrCP/Nrf2 pathway, which causes Nrf2 degradation in the nucleus. Meanwhile, as an important oxidative stress protein regulated by Nrf2, HO-1 was also evaluated by western blot (Fig. 2EF). The results showed that Keap1 and β-Trcp expression increased while HO-1 expression decreased, consistent with the Nrf2 expression results. The above results explained that aspirin might promote oxidation reactions, lead to ferroptosis and inhibit cell growth via Keap1-Nrf2 in the cytoplasm.

The above results explained that aspirin might mainly promote oxidation reactions through the Keap1-Nrf2 pathway in the cytoplasm, leading to ferroptosis and inhibiting cell growth.

**Aspirin promoted ferroptosis by enhancing Keap1 in TNBC**

We continued to use different treatments in MDA-MB-231 and MDA-MB-468 cells to explore the relationship among aspirin, ferroptosis and Keap1, including aspirin alone, the ferroptosis agonist erastin, aspirin combined with the ferroptosis inhibitor ferrostatin, and aspirin combined with Keap1 siRNA. The cell viability results showed that both aspirin and erastin reduced the cell survival rate (no significant difference between them). Ferrostatins combined with aspirin reversed the cell survival rate, which was similar to the results of aspirin combined with Keap1 siRNA (Fig. 3A).

Furthermore, we detected the level of ferroptosis, and the results of Fe content and lipid ROS were consistent with the results of cell survival. Our research showed that the use of aspirin alone and erastin significantly increased the iron ion level, and the further use of aspirin combined with ferrostatin and Keap1 siRNA significantly decreased the iron ion level compared with aspirin alone (Fig. 3BC).
Moreover, we simultaneously detected the cellular oxidation levels, including GSH, GSSG and MDA levels (Fig. 3D). The levels of MDA and GSSG, the indicators of the oxidation reaction, showed that the contents of each group were consistent with the changes in the iron ion level, and GSH, the indicator of the reduction reaction, showed the opposite trend.

Based on the above results, we confirmed that aspirin can induce ferroptosis and that a ferroptosis inhibitor or Keap1 siRNA combined with aspirin can partially reverse ferroptosis. This result indicated that aspirin could induce ferroptosis and cause cell death by enhancing the effect of Keap1.

**Aspirin promoted ferroptosis via the Keap1-Nrf2 pathway in TNBC**

Immunofluorescence was utilized to explore the nucleocytoplasmic distribution of Nrf2 in both TNBC cell lines (Fig. 4AB). Our results showed that aspirin and erastin only significantly inhibited Nrf2 entry into the nucleus. Even aspirin had a more obvious inhibitory effect than erastin. On the other hand, aspirin combined with ferrostatin and Keap1 siRNA reversed the effect of aspirin on Nrf2. These results indicated that aspirin could promote the occurrence of ferroptosis by inhibiting Nrf2 entry into the nucleus through Keap1. Notably, in MDA-MB-231 cells, aspirin significantly inhibited Nrf2 nuclear localization compared with erastin.

Furthermore, we detected the levels of key ferroptosis proteins and Keap1-Nrf2 and β-TrCP/Nrf2 pathway proteins by Western blotting in both TNBC cell lines (Fig. 4C-F). The results showed that aspirin and erastin only significantly elevated Keap1 and β-TrCP protein levels and reduced HO-1 and Nrf2 (cytoplasm and nuclear) protein levels, while the ferroptosis marker results showed that GPX4 decreased and xCT increased. Conversely, we used aspirin combined with the ferroptosis inhibitors ferrostatin and Keap1 siRNA. The results showed that the above protein levels were reversed. Taken together, the above results suggested that aspirin could promote ferroptosis via the Keap1-Nrf2 and β-TrCP/Nrf2 pathways.

We established xenografts of MDA-MB-231 cells grown in nude mice, treated them with aspirin, and then detected the expression of key proteins in the Keap1-Nrf2 pathway by IHC. Our research indicated that the number of Ki-67-positive cells and the protein expression of GPX4, Nrf2 and HO-1 were significantly decreased after aspirin treatment. However, the level of Keap1 protein expression was higher in the aspirin group than in the control group (Fig. 4G). These in vivo results were similar to the in vitro results.

**Disorders of oxidative stress and the Keap1-Nrf2 pathway in TNBC patients**

We used tumor and normal tissues from TNBC patients in West China for sequencing analysis (named TNBC_WC) and analyzed the relevant TNBC sequencing data in the TCGA database (named TCGA). The results demonstrated that the expression of 413 genes was upregulated and 1351 genes were downregulated in tumors compared with normal tissues in TNBC_WC (Fig. 5A). Through KEGG enrichment analysis, the changed genes were mainly enriched in metabolic pathways. Further analysis of
the metabolic pathway revealed that it was mainly related to oxidative phosphorylation, the ROS pathway, etc. Similarly, after analyzing the triple-negative breast cancer data in TCGA, we obtained results similar to those in TNBC_WC (Fig. 5CD). Interestingly, the KEGG results showed that oxidative phosphorylation and ROS pathways, which are closely related to ferroptosis, were significantly enriched in both databases.

Furthermore, we analyzed the expression of related genes in the TNBC_WC and TCGA databases. Interestingly, compared with normal tissues, we found that GPX4 and Nrf2 were decreased in tumor tissues from both databases (Fig. 5EF). This might represent an increased ferroptosis sensitivity in tumors. The TCGA results of a larger sample size showed that the level of expression of Keap1, HO-1 and xCT was upregulated in tumors, and β-TrCP was downregulated, while the expression of these genes in TNBC_WC was similar to TCGA databases (Fig. 5EF). The above results suggest that ferroptosis and the oxidative stress pathway are disordered in TNBC.

Additionally, we explored the relationship between these genes and survival prognosis (Fig. 5G-J). In TNBC_WC, our results showed that high Keap1 expression was positively correlated with OS (p = 0.028), while in TCGA, low GPX4 expression was associated with better OS (p = 0.013) (Fig. 5G, I). At the same time, in TNBC_WC, xCT with low expression obtained better progression-free survival (PFS) (p = 0.003), while in TCGA, GPX4 with low expression still obtained better PFS (p = 0.005) (Fig. 5H, J). GPX4 and xCT are key genes for ferroptosis. Therefore, the above results suggested that ferroptosis was related to survival prognosis in TNBC.

**Discussion**

Aspirin is widely used as an antipyretic analgesic, analgesic and anti-inflammatory drug in the clinic. It also exhibited great potential treatment effects in primary and metastatic neoplasms in recent years (Yang et al., 2014; Y. Zhang et al., 2021). Our previous study found a novel mechanism by which aspirin exerts anticancer effects. In particular, aspirin can significantly suppress the proliferation of TNBC cells. Meanwhile, YAP and β-catenin expression was attenuated by upregulating β-TrCP to abolish drug resistance to docetaxel and vinorelbine (Ma et al., 2020). In addition, β-TrCP-mediated Nrf2 degradation plays a crucial role in ferroptosis. Therefore, our researchers continued to investigate whether aspirin regulates TNBC through the ferroptosis pathway. In recent research, aspirin was shown to promote the sensitivity of TNBC cells to ferroptosis via the Keap1-Nrf2 and β-TrCP/Nrf2 pathways. This finding indicated that aspirin might act as a novel treatment strategy for TNBC.

Ferroptosis, a new type of cell death, is mainly caused by intracellular iron catalytic activity and overload of lipid peroxidation on cellular membranes. Furthermore, the characteristic of ferroptosis is the accumulation of ROS(Jiang et al., 2017; Mao et al., 2018). Recent research has demonstrated that the overexpression of iron-dependent cell death pathways can effectively suppress neoplasm progression and improve the effects of a variety of cancer therapies, including chemotherapy, radiotherapy, immunotherapy and even targeted therapy (Mao et al., 2018; Tang et al., 2019). It is pivotal for the regulation of the pathway by which cells resist oxidative stress (Jiang et al., 2015). Particularly, one of the
important pathways is the glutathione pathway, which has been recognized as a pivotal antioxidant defense pathway. The metabolic protein GPX4 is the core of this process. It can convert GSH to GSSG and limit cytotoxic lipid peroxidation deposition to protect cells from iron (Yang et al., 2014). Our findings indicated that aspirin could promote ferroptosis in TNBC. Specifically, aspirin promoted an increase in the cellular iron ion concentration, ROS accumulation, and changes in ferroptosis markers (MDA, GSSG and xCT levels were elevated, while GSH and GPX4 levels were decreased).

Researchers have found that cellular activity can be promoted by antioxidant genes such as antioxidant response elements (AREs). Nrf2 is an important regulator gene that can drive cytoprotective protein expression by binding to ARE sequences (J. Wang et al., 2019). The transcription factor Nrf2 can also play a critical role in the regulation of genes involved in oxidative stress, such as GPX4. In addition, it is critical to defend against ferroptosis. By interacting with Keap1, Nrf2 expression is typically inhibited (Y. Zhang et al., 2021). Oxidative stress can disrupt interactions by causing conformational changes in Keap1, leading to the stabilization of Nrf2 (J. Wang et al., 2019; Wen et al., 2015). Finally, Nrf2 is upregulated, and ferroptosis is inhibited (Sun and Ou et al., 2016).

Nrf2 is an important regulator of oxidative stress signaling and has a dual effect on tumor proliferation: a lack of Nrf2 activity can cause early neoplasm formation. However, constancy and a high level of Nrf2 activity can trigger tumor proliferation and reduce the effect of treatment (Rojo et al., 2018). Meanwhile, Nrf2 can control oxidative stress and interact with Keap1 to resist ferroptosis and exert antitumor activity. Preclinical research has demonstrated that Nrf2 signaling is a key defense against ferroptosis and is involved in drug resistance to sorafenib in hepatocellular carcinoma cells (Sun and Niu et al., 2016). Transactivation of several cytoprotective genes, such as iron metabolism, GSH metabolism and ROS detoxification enzymes, can limit oxidative damage in ferroptosis by Nrf2 (Anandhan et al., 2020; Sun and Ou et al., 2016). In addition, gain-of-function mutations in Nrf2 or loss-of-function mutations in Keap1 can increase the complexity of the oxidative stress response. However, this might affect resistance to ferroptosis (Rojo et al., 2018). Our researchers found that aspirin could significantly inhibit the nuclear distribution of Nrf2 and reduce the expression of Nrf2. Under normal conditions, the level of Nrf2 is very low in cells, whereas it is dramatically increased upon exposure to ROS, especially during ferroptosis (Rojo et al., 2018). Keap1-mediated protein degradation can regulate the expression of Nrf2 in tumor cells, while oxidative modification of Keap1 has been shown to attenuate its binding to Nrf2 (Sun and Ou et al., 2016). These oxidative modifications inactivate Keap1 and thereby stabilize Nrf2. In our study, the data showed that under the use of aspirin or the ferroptosis agonist erastin, the level of Keap1 was elevated, while the level of Nrf2 was decreased, especially at an aspirin concentration of 2.5 mM.

Keap1 is loosely connected with the actin cytoskeleton and is mainly located in the perinuclear cytoplasm. In addition, it can maintain the appropriate levels of Nrf2 (Baird and Yamamoto, 2020). Keap1 can form a complex with CUL3 and RBX1 to form a functional E3 ubiquitin ligase complex, serving as a substrate recognition/binding subunit. The Keap1-CUL3-RBX1 E3 ubiquitin ligase complex ubiquitinates Nrf2 and rapidly degrades Nrf2 in the cytoplasm. Therefore, the Keap1/Nrf2 pathway serves as the main pathway for the degradation of Nrf2 in the cytoplasm (Baird and Yamamoto, 2020). We found that the
expression of Keap1 in the cytoplasm was significantly increased by aspirin treatment, while Nrf2 was decreased. Through a rescue experiment with Keap1 siRNA, the expression of Nrf2 was increased. This was consistent with previous reports. We inferred that aspirin could cause Nrf2 degradation through the Keap1-Nrf2 pathway to promote ferroptosis and cause cell death.

For Nrf2 degradation, the Keap1-Nrf2 pathway is the main pathway, but another Nrf2 degradation pathway exists in the nucleus. β-TrCP forms an E3 ubiquitin ligase complex with CUL1 and ubiquitinates Nrf2. Meanwhile, β-TrCP acts as a substrate recognition/binding subunit that recognizes phosphorylated Nrf2 (Rada et al., 2012). Glycogen synthase kinase 3 (GSK3) is a well-known protein kinase that plays an important role in many pathways, such as the phosphoinositide 3-kinase (PI3K)–AKT pathway. In the nucleus, GSK3 can also phosphorylate the serine residues in the Neh6 domain of Nrf2. Then, phosphorylated Nrf2 is captured by β-TrCP, which is ubiquitinated and degraded by the proteasome (Bi et al., 2021; Taguchi and Yamamoto, 2020). Our previous study showed that aspirin could promote β-TrCP expression, which might be a possible mechanism by which aspirin overcomes chemotherapy-related agent (docetaxel and vinorelbine) resistance in TNBC. In recent research, we found that aspirin could cause ferroptosis. More importantly, aspirin simultaneously affects the Keap1/Nrf2 and β-TrCP/Nrf2 pathways, which jointly cause Nrf2 degradation. These mechanisms attenuate the response to oxidative stress and the occurrence of ferroptosis in TNBC in vitro and vivo. Therefore, Nrf2 degradation depends on two pathways: the main pathway is localized in the cytoplasm and governed by Keap1, and the other pathway is governed by β-TrCP in the nucleus.

To further discuss the clinical significance of ferroptosis in TNBC, our researchers assessed ferroptosis-related expression in TCGA database (TCGA) and TNBC samples in our hospital (TNBC_WC). In TNBC_WC, our results showed that high Keap1 expression was associated with better OS (p = 0.028), while in TCGA, low GPX4 expression was associated with better OS (p = 0.013). In TNBC_WC, xCT with low expression obtained better PFS (p = 0.003), while in TCGA, GPX4 with low expression still obtained better PFS (p = 0.005). GPX4 and xCT are key genes for ferroptosis, suggesting that ferroptosis is related to survival prognosis in TNBC.

These observations in our study support the viewpoint that two pathways that can, through the protein degradation-repression mechanism, strictly regulate the level of Nrf2: one of the major pathways is that derepression from Keap1-based repression results in an immediate increase in Nrf2 activity and induction of cellular defense mechanisms against oxidative insults and ferroptosis. In the secondary pathway, β-TrCP-based Nrf2 degradation limits unnecessary Nrf2 overinduction caused by Keap1 inactivation (Liu et al., 2020).

Conclusion

In this study, our researchers showed that aspirin can promote ferroptosis in TNBC cancer cells. In particular, aspirin promoted the sensitivity of TNBC cells to ferroptosis by preventing the downstream HO-1-mediated oxidative stress response via the Keap1-Nrf2 and β-TrCP/Nrf2 pathways. Our findings provide
valuable insight for future ferroptosis-inducing cancer therapies and a theoretical cornerstone for adding aspirin to TNBC therapies. Meanwhile, we also emphasize the importance of clinical trials to assess their potential treatment effects.

Declarations

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Ethics statement

This study was approved by the Clinical Test and Biomedical Ethics Committee at West China Hospital, Sichuan University (no. 2012-130). Written consent was obtained from all participants.

Consent for publication

Not applicable

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Author Contributions

Conceptualization: Ji Ma, Qiheng Gou. Data curation: Zijian Liu, Yuxin Xie. Project administration: Ji Ma. Supervision: Ji Ma. Writing-original draft: Qiheng Gou. Revision: Qiheng Gou, Yuxin Xie Qitao Gou. All authors made a significant contribution to the work reported, gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Not applicable

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**Figures**
Figure 1

Aspirin promoted ferroptosis in triple-negative breast cancer cells. (A) MDA-MB-231 and MDA-MB-468 cells were treated with different concentrations of aspirin at 24 h and 48 h (0, 0.625, 1.25 and 2.5 mM). (B) Intracellular ferrous iron was detected in two aspirin-treated TNBC cell lines. (C) Fluorescence microscopy was utilized to observe two aspirin-treated TNBC cells after the absorption of DCFH-DA. The statistical results of the relative ROS fluorescence ratio are shown (right). (D) The levels of lipid ROS were...
detected with flow cytometry in two aspirin-treated TNBC cell lines. The statistical results of the relative lipid ROS ratio are shown (right). (E) GSH (left), GSSG (middle) and MDA (right) levels were detected with an assay kit in two aspirin-treated TNBC cell lines. *, P < 0.05; **, P < 0.001; ***, P < 0.001.
Aspirin attenuated Nrf2 expression mainly via the Keap1-Nrf2 pathway in TNBC. (A-B) Nrf2 expression (red) was detected in aspirin-treated MDA-MB-231 cells (A) and MDA-MB-468 cells (B) by immunofluorescence assay. The nucleus was stained with DAPI (blue). The right chart shows the statistical results. (C-F) The protein levels of GPX4, xCT, Nrf2, Keap1, HO-1, and β-TrCP were detected in two TNBC cell lines treated with aspirin for 24 h (C, E). The right chart shows the statistical results (D, F). *, P < 0.05; **, P < 0.001; ***, P < 0.001.
Figure 3

Aspirin promoted ferroptosis by enhancing Keap1 in TNBC. (A) MDA-MB-231 and MDA-MB-468 cells were treated with different treatments (aspirin, erastin, aspirin combined with ferrostatinss, and aspirin combined with Keap1 siRNA) for 24 h. (B) The intracellular ferrous iron detected in two TNBC cells with different treatments (aspirin, erastin, aspirin combined with ferrostatinss, and aspirin combined with Keap1 siRNA) for 24 h. (C) The levels of lipid ROS were detected with flow cytometry in two TNBC cell lines with different treatments (aspirin, erastin, aspirin combined with ferrostatinss, and aspirin combined with Keap1 siRNA) for 24 h. The statistical results are shown (right). (D) GSH (left), GSSG (middle) and MDA (right) levels were detected with the assay kit in two TNBC cell lines with different treatments (aspirin, erastin, aspirin combined with ferrostatinss, and aspirin combined with Keap1 siRNA) for 24 h. *, P < 0.05; **, P < 0.001; ***, P < 0.001.
Figure 4

Aspirin promoted ferroptosis via the Keap1-Nrf2 pathway in TNBC. (A-B) Nrf2 expression (red) was detected in MDA-MB-231 cells (A) and MDA-MB-468 cells (B) with different treatments (aspirin, erastin, aspirin combined with ferrostatins, and aspirin combined with Keap1 siRNA) for 24 h by immunofluorescence assay. The nucleus was stained with DAPI (blue). The right chart shows the statistical results. (C-D) The protein levels of GPX4, xCT, Nrf2, Keap1, HO-1, and β-TrCP were detected in
two TNBC cell lines treated with different treatments (aspirin, erastin, aspirin combined with ferrostatinss, and aspirin combined with Keap1 siRNA) for 24 h. (E-F) The right chart shows the statistical results. The “*” symbol represents statistically significant results with the control group, and the “#” symbol represents statistically significant results with the aspirin group. (G) Representative immunohistochemical staining results for Ki67, GPX4, Keap1, Nrf2 and HO-1 in xenograft tumor tissues are shown. The graph shows the Ki67-positive cells and the IHC scores of GPX4, Keap1, Nrf2 and HO-1 in the two groups. Scale bar: 200 µM. The data are presented as the mean ± SD of experiments performed. Statistical significance was determined using Student’s t test. *,#, P < 0.05; **#, P < 0.001; ####, P < 0.001.
Figure 5

**Disorders of oxidative stress and the Keap1-Nrf2 pathway in TNBC patients.** (A, C) Volcanic map showing the sequencing results of TNBC patients in West China Hospital of Sichuan University (named TNBC_WC, n=80) (A) and TCGA database (named TCGA, n=159). (B, D) KEGG analysis of differentially expressed genes (left) and differentially expressed genes involved in metabolic pathways (right) from TNBC_WC (B) and TCGA (D) sequencing data. (E, F) RNA-seq-based heatmap indicating the changes in the GPX4, xCT, ...
Nrf2, Keap1, HO-1, and β-TrCP genes in TNBC_WC (E) and TCGA (F) sequencing data. (G-J) Kaplan–Meier curves generated with the data from the TNBC_WC group (G, H) or TCGA group (I, J) patients with high versus low GPX4, xCT, Nrf2, Keap1, HO-1, and β-TrCP expression. The correlation between differential expression and overall survival (OS) (G, I) or progression-free survival (PFS) (H, J) in patients.