Oxaliplatin induces ferroptosis and oxidative stress in HT29 colorectal cancer cells by inhibiting the Nrf2 signaling pathway

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Abstract. Oxaliplatin is a third-generation platinum drug that is used as first-line chemotherapy for colorectal cancer (CRC). Ferroptosis has been demonstrated to induce cell death and oxidative stress in CRC. The aim of the present study was to investigate whether oxaliplatin could exert anticancer effects on CRC by promoting ferroptosis and oxidative stress. Cell viability and apoptosis were assessed by performing Cell Counting Kit-8 and TUNEL assays, respectively, in the presence or absence of the ferroptosis inducer, erastin. Western blotting was performed to detect the levels of certain nuclear factor erythroid 2-related factor 2 (Nrf2)-associated proteins in HT29 cells treated with oxaliplatin. Furthermore, after treating cells with the Nrf2 activator, NK-252, Fe^{2+} was detected in cells using a commercial kit. Ferroptosis-associated protein expression was also evaluated via western blotting. Additionally, ELISA was adopted to measure the levels of oxidative stress-related factors. Following the addition of erastin, iron ion content, ferroptosis-related protein expression and the levels of oxidative stress-related factors were assayed as described previously. The results of the present study demonstrated that oxaliplatin inhibited viability and the Nrf2 signaling pathway in CRC cells. In addition, oxaliplatin promoted ferroptosis and oxidative stress in CRC cells by inhibiting the Nrf2 signaling pathway. Treatment with oxaliplatin enhanced the effects of erastin on CRC cells by promoting ferroptosis and oxidative stress and inhibiting cell viability. In conclusion, oxaliplatin induced ferroptosis and oxidative stress in CRC cells by inhibiting the Nrf2 signaling pathway.

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Introduction

Colorectal cancer (CRC) is a serious disease that is caused by malignant changes of the colorectal mucosa as a result of various environmental and genetic factors (1). The incidence of CRC is increasing annually and is occurring in younger patients (2). CRC is more commonly observed in developed countries (3), possibly due to the population aging, poor dietary habits, and higher rates of smoking, physical inactivity and obesity in Western countries (3). The current treatments for CRC mainly include screening, surgical treatment and chemotherapy (4). However, despite advances in surgery and screening, little change in patient survival has been observed in recent years. As regards chemotherapy, oxaliplatin is a third-generation platinum drug that is used as first-line chemotherapy for CRC (5). Oxaliplatin acts on DNA by producing hydrated derivatives that form intra- and inter-strand cross-links, thereby inhibiting DNA synthesis and exerting cytotoxic antitumor effects (6). Unfortunately, only <40% of patients with advanced CRC benefit from oxaliplatin due to tumor resistance and the toxic effects arising from long-term use (7). Oxaliplatin is therefore unable to further control tumor progression. Improving the impact of oxaliplatin on the survival of patients with advanced disease remains a major challenge in CRC treatment.

Ferroptosis is an iron-dependent and reactive oxygen species (ROS)-dependent type of cell death (8). Ferroptosis is genetically, biochemically and morphologically different from cell necrosis, autophagy and apoptosis (9). Recently, ferroptosis has emerged as a popular research area for reversing tumor drug resistance (10), as it can inhibit phospholipid glutathione peroxidase 4 (GPX4) and the accumulation of lipid ROS in cells (11) to trigger drug-resistant cancer cell death. Interestingly, cancer cells that exhibit drug resistance are more likely to undergo death via ferroptosis inducers compared with cells exhibiting no drug resistance (12). Moreover, ferroptosis has been revealed to induce oxidative stress and death of CRC cells (13), suggesting that ferroptosis induction may represent a valuable method of CRC treatment. However, there are relatively few reports on the interactions between oxaliplatin and ferroptosis in CRC.

It has been demonstrated that oxaliplatin inhibits the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway (14), and that inhibition of Nrf2 enhances the

sensitivity of CRC cells to oxaliplatin (15). This suggests that the Nrf2 signaling pathway serves a key role in the anticancer mechanism of oxaliplatin. P62-Keap1-Nrf2 protect HCC cells against ferroptosis via upregulation of multiple genes involved in iron and ROS metabolism (16). Additionally, Nrf2 downregulation enhances the sensitivity of cancer cells to ferroptosis-promoting agents (17). Therefore, Nrf2 inhibition is an important regulatory pathway that leads to ferroptosis (16). Consequently, the present study was undertaken to investigate whether the anticancer effects of oxaliplatin in CRC may be enhanced via the inhibition of Nrf2 signaling, resulting in oxidative stress and ferroptosis.

Materials and methods

Cell culture and treatment. The base medium used for the culture of the human CRC cell line, HT29 (American Type Culture Collection; ATCC), was ATCC-formulated McCoy's 5a (Modified) Medium containing a final concentration of 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). Cells were cultured at 37˚C in an incubator containing 5% CO_2.

HT29 cells were cultured in serum-free medium overnight prior to treatment, after which time insulin was added to the medium for 15 min. Oxaliplatin at various concentrations (0.5, 1, 2 and 3 μM) was subsequently added to the medium for 72 h. A total of 200 μM Nrf2 activator, NK-252 (MedChemExpress) was then added to the medium to activate the Nrf2 pathway. A total of 10 μM erastin (Shanghai Rechem Science Co., Ltd.) was prepared in DMSO for the induction of ferroptosis.

Cell viability assay. HT29 cells ($2x10^4$) in untreated control, Erastin, Oxaliplatin and Erastin + Oxaliplatin (3 μM) and maintained in 96-well plates at 37˚C for 24 h. After incubation, 10 μl Cell Counting Kit-8 (CCK-8) reagent (Shanghai Yeasen Biotechnology Co., Ltd.) was added to each well for 2 h. Subsequently, absorbance at 450 nm was measured using a microplate reader.

TUNEL assay. HT29 cell apoptosis was detected using One Step TUNEL Apoptosis Assay kit (Beyotime Institute of Biotechnology) in accordance with the manufacturer's protocol. Cells were fixed with 4% paraformaldehyde for 15 min at room temperature, after which time samples were washed with PBS once for 5 min. Adherent cell monolayers were permeabilized using 0.1% Triton X-100 and incubated with the TUNEL kit for 1 h at 37˚C. Then, 0.5 μg/ml DAPI was used to stain the nuclei of HT29 cells for 5 min at room temperature. After washing in triplicate with PBS, the anti-fade mounting medium was added into the cells and then a fluorescence microscope (Leica Microsystems GmbH) was employed to observe TUNEL-positive cells in five randomly selected views.

Western blotting. Protein was extracted from HT29 cells using ice-cold RIPA buffer (Elabscience Biotechnology, Inc.) and determined by using a BCA protein assay kit (Phygene). Protein samples were subjected to separation via 10% SDS-PAGE (DetaiBio Tech) and transferred onto PVDF membranes (Roche Diagnostics), after which time the membranes were blocked with 5% non-fat milk for 2 h at room temperature. The membranes were incubated at 4˚C overnight with the following primary antibodies (all Abcam): Nrf2 (1:1,000, cat. no. ab137550), heme oxygenase-1 (HO-1; 1:2,000, cat. no. ab52947), NADPH dehydrogenase quinone 1 (NQO1; 1:10,000, cat. no. ab80588), GPX4 (1:1,000, cat. no. ab125066), ferritin heavy chain 1 (FTH1; 1:1,000, cat. no. ab183781), transferrin (1:1,000, cat. no. ab277635) and GAPDH (1:1,000, cat. no. ab8245). Following primary antibody incubation, the membranes were incubated with HRP-conjugated secondary antibodies (goat anti-mouse IgG H&L, 1:2,000, cat. no. ab6789; or goat anti-rabbit IgG H&L, 1:2,000, cat. no. ab6721) for 2 h at room temperature. An ECL Western Blotting Substrate kit (AmyJet Scientific, Inc.) was applied to visualize protein bands. The resultant images were analyzed using ImageJ software (version 1.8.0; National Institutes of Health) and the quantification of each group was performed in triplicate.

Detection of oxidative stress. The levels of malondialdehyde (MDA) and glutathione (GSH) were detected by using ELISA kits for MDA (cat. no. ab118970; Abcam) and GSH (cat. no. ab239727; Abcam). HT29 cells were collected and lysed with 300 μl lysis solution per well. Samples were centrifuged at 13,000 x g for 10 min at 4˚C and the supernatant was subsequently collected. A total of 600 μl thiobarbituric acid was added to 200 μl supernatant and the mixture was incubated at 95˚C for 60 min, and cooled in an ice bath for 10 min. Subsequently, the absorbance at 532 and 450 nm was detected immediately on a microplate reader (Thermo Fisher Scientific, Inc.). The levels of ROS in HT29 cells were measured with a commercially available kit (cat. no. ab139476, Abcam) according to the manufacturers' instructions. Briefly, the control cells were pretreated with a ROS inhibitor (N-acetyl-l-cysteine) for 30 min at room temperature. HT29 cells treated with oxaliplatin (3 μM) with or without NK-252 (200 μM) or erastin (10 μm) added to 100 μl/well of ROS/superoxide detection solution and incubated for 2 h at 37˚C in the dark. The fluorescence was detected at an excitation wavelength of 490 nm and at an emission wavelength of 520 nm by using a SpectraMax i3x microplate reader (Molecular Devices, LLC).

Iron measurement. An iron Assay kit (BioAssay Systems) was utilized to evaluate the concentration of Fe^{2+} in HT29 cells following treatment with oxaliplatin and the ferroptosis inducer, erastin. The experiment was performed in accordance with the recommendations of the manufacturer, and the absorbance of cells at 593 nm was measured by using a spectrophotometer (Shanghai Mapada Instruments Co., Ltd.).

Statistical analysis. Experimental data are expressed as the mean ± SD and were analyzed using SPSS 19.0 software (IBM Corp.). All experiments were repeated independently at least 3 times. One-way ANOVA with a post hoc Bonferroni multiple comparisons test was used to compare differences among multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Oxaliplatin inhibits HT29 cell viability. The viability of HT29 cells was assessed following treatment with different

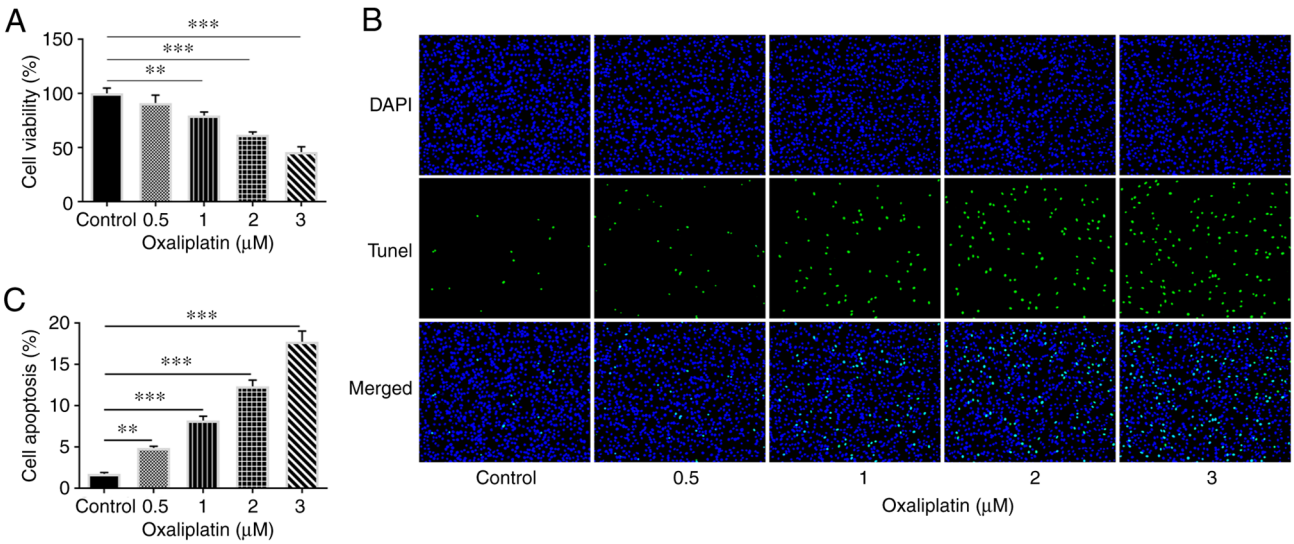

Figure 1. Oxaliplatin suppresses the viability of colorectal cancer cells. (A) Cell Counting Kit-8 assay was used to assess the effects of oxaliplatin on the viability of HT29 cells in the control group and the groups treated with oxaliplatin concentrations of 0.5, 1, 2 and 3 μM. (B and C) TUNEL assay was conducted to assess the apoptosis levels of HT29 cells in the control group and the groups treated with oxaliplatin concentrations of 0.5, 1, 2 and 3 μM. Magnification, x200. Data are expressed as mean ± SD. **P<0.01, ***P<0.001.

concentrations of oxaliplatin using a CCK-8 assay. The results revealed that, when compared with the control group, HT29 cell viability was decreased following oxaliplatin treatment in a concentration-dependent manner (Fig. 1A). Subsequently, a TUNEL assay was performed to measure apoptosis. As presented in Fig. 1B and C, oxaliplatin increased the apoptosis rate of HT29 cells in a dose-dependent manner. The results suggested that oxaliplatin exerted an inhibitory and concentration-dependent effect on HT29 cell viability. Additionally, since the effects of 0.5 μM oxaliplatin were less prominent, concentrations of 1, 2 and 3 μM oxaliplatin were selected for subsequent experimentation.

Oxaliplatin suppresses the Nrf2 signaling pathway in HT29 cells. The results of western blotting revealed that the expression levels of certain Nrf2 pathway-associated proteins, including Nrf2, HO-1 and NQO1, were decreased compared with the control group (Fig. 2). Additionally, the greatest decrease in Nrf2, HO-1 and NQO1 expression levels was observed in cells treated with 3 μM oxaliplatin. Accordingly, 3 μM oxaliplatin was selected for use in follow-up experiments. These results indicated that oxaliplatin could notably inhibit the Nrf2 signaling pathway in CRC.

Oxaliplatin promotes HT29 cell ferroptosis and oxidative stress through the Nrf2 signaling pathway. To determine whether the effects of oxaliplatin on HT29 cell ferroptosis and oxidative stress were mediated through the Nrf2 signaling pathway, relative total iron and Fe^{2+} levels were measured using an iron testing kit. As shown in Fig. 3A and B, relative total iron and Fe^{2+} levels were increased in 3 μM oxaliplatin-treated HT29 cells compared with the control group. However, these effects were reduced following treatment with the Nrf2 activator, NK-252. The results of western blotting revealed that, when compared with the control group, a marked decrease was observed in certain ferroptosis-related proteins, including GPX4 and FTH1, and a marked increase was observed in transferrin expression in HT29 cells treated with oxaliplatin (Fig. 3C and D). However, subsequent NK-252 treatment significantly increased GPX4 and FTH1 expression levels, and decreased transferrin expression levels.

ELISA kits were used to measure the levels of various oxidative stress markers, including ROS (Fig. 3E), MDA (Fig. 3F) and GSH (Fig. 3G), in oxaliplatin-treated HT29 cells. When compared with the control group, ROS and MDA levels decreased, while GSH levels increased in HT29 cells treated with oxaliplatin and NK-252. These results suggested that oxaliplatin could promote HT29 cell ferroptosis and oxidative stress via the Nrf2 signaling pathway.

Oxaliplatin enhances the effects of erastin on HT29 cell ferroptosis and oxidative stress. Thus far, the results of the current study have demonstrated that oxaliplatin can promote HT29 cell ferroptosis and oxidative stress by means of Fe^{2+} detection, ELISA and western blotting. The same experiments were subsequently performed, with the addition of the ferroptosis inducer, erastin. As indicated in Fig. 4A and B, the relative total iron and Fe^{2+} levels were increased in the erastin group compared with the control group. Additionally, oxaliplatin treatment largely increased the levels of relative total iron and Fe^{2+} in the erastin + oxaliplatin group when compared with the erastin group. Compared with the control group, erastin treatment markedly reduced GPX4 and FTH1, and increased transferrin protein expression levels (Fig. 4C and D). However, subsequent erastin + oxaliplatin treatment resulted in lower levels of GPX4 and FTH1, and higher levels of transferrin. Furthermore, erastin treatment led to increased levels of ROS and MDA (Fig. 4E and F), as well as a decreased levels of GSH (Fig. 4G) when compared with the control group. When erastin treatment was subsequently combined with oxaliplatin, the opposite effects to those described above were observed. These results indicated that oxaliplatin enhanced the promotive effects of erastin on HT29 cell ferroptosis and oxidative stress.

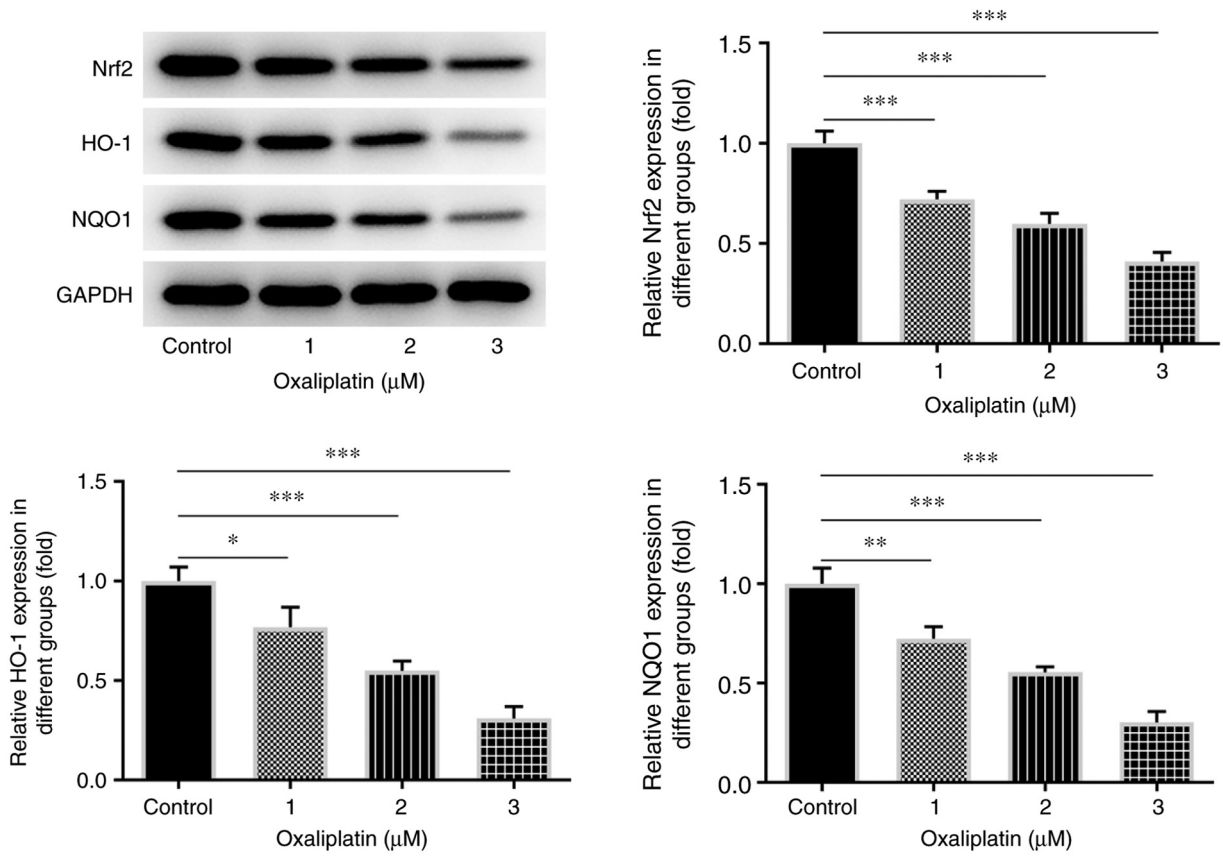

Figure 2. Oxaliplatin inhibits the Nrf2 signaling pathway in colorectal cancer cells. Western blotting was performed to detect the levels of the Nrf2 signaling pathway-related proteins Nrf2, HO-1 and NQO1 in the control group and the groups treated with oxaliplatin concentrations of 1, 2 and 3 μM. Data are expressed as mean ± SD. *P<0.05, **P<0.01, ***P<0.001. Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; NQO1, NADPH dehydrogenase quinone 1.

Oxaliplatin enhances the suppressive effects of erastin on HT29 cell viability. According to the aforementioned results, oxaliplatin markedly suppressed HT29 cell viability. Erastin treatment was subsequently applied to further assess the function of oxaliplatin. As presented in Fig. 5A, cell viability was markedly decreased in the erastin group compared with the control group. Furthermore, oxaliplatin treatment applied in combination with erastin resulted in enhanced suppression of cell viability. Erastin treatment also led to increased levels of HT29 cell apoptosis when compared with the control group. However, HT29 cell apoptosis was higher in the erastin + oxaliplatin group compared with either the erastin alone and oxaliplatin alone groups (Fig. 5B and C). These results demonstrated that oxaliplatin enhanced the inhibitory effect of erastin on the viability of HT29 cells.

Discussion

CRC usually develops from benign tumors or serrated polyps (18) and is a common type of cancer with a worldwide prevalence. Although current treatments have improved patient survival, a significant proportion of patients with CRC still experience relapse. The HT29 cell line is a human CRC cell line that is commonly used in physiological and pathological studies of CRC (19-21). In the current study, this cell line was used to study the effects of oxaliplatin on ferroptosis and oxidative stress in CRC cells and elucidate the underlying mechanism. It was observed that oxaliplatin significantly inhibited the proliferation and promoted the apoptosis of HT29 cells. Mechanistic investigations revealed that oxaliplatin accelerated the process of ferroptosis and oxidative stress, which may involve the blockade of the Nrf2 signaling pathway.

Oxaliplatin is a first-line chemotherapeutic CRC agent that primarily exerts its effects by combining with and damaging DNA, thereby inhibiting DNA replication (22). It was previously revealed that oxaliplatin exerts an inhibitory effect on cell viability and promotes CRC cell apoptosis (23). In addition, Limagne *et al* (24) reported that the combination of trifluridine/tipiracil and oxaliplatin improved PD-1 blockade in CRC via the induction of immunogenic cell death and depletion of macrophages. The CCK-8 and TUNEL assays in the present study revealed that HT29 cell viability was decreased and apoptosis was increased, suggesting that oxaliplatin inhibited the proliferation of CRC cells.

Nrf2 functions as a key promoter of chemoresistance by regulating antioxidants and detoxifying enzymes (25). It was previously determined that oxaliplatin inhibits the Nrf2 signaling pathway (14). For example, the suppression of Nrf2 signaling in patients with CRC provided an essential strategy for overcoming oxaliplatin resistance (25). The results of the present study found that oxaliplatin significantly suppressed the protein expressions of Nrf2, HO-1 and NQ in the Nrf2 signaling pathway in a dose-dependent manner, which was in agreement with previous results.

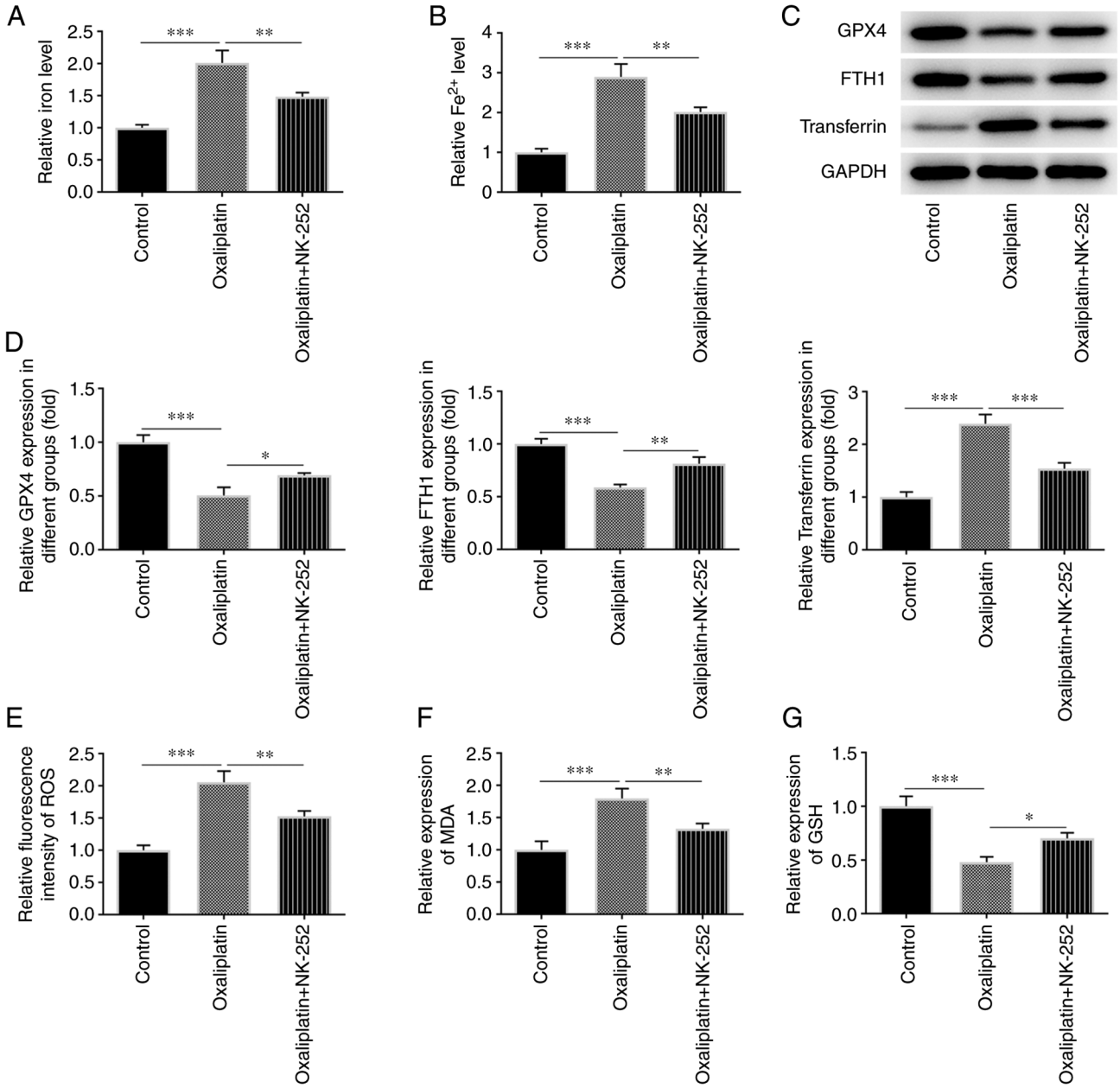

Figure 3. Oxaliplatin promotes ferroptosis and oxidative stress in colorectal cancer cells through inhibiting the Nrf2 signaling pathway. (A and B) Detection of relative total iron level and Fe^{2+} level in HT29 cells was performed using an iron ion test kit in the control group and the groups treated with 3 μM of oxaliplatin as well as oxaliplatin (3 μM) + NK-252. (C and D) Western blotting was used to detect the levels of the ferroptosis-related proteins GPX4, FTH1 and transferrin in the control group and the groups treated with 3 μM of oxaliplatin as well as oxaliplatin (3 μM) + NK-252. (E-G) ELISA was used to detect the levels of the oxidative stress-related factors ROS, MDA and GSH in the control group and the groups treated with 3 μM of oxaliplatin as well as oxaliplatin (3 μM) + NK-252. Data are expressed as mean ± SD. *P<0.05, **P<0.01, ***P<0.001. Nrf2, nuclear factor erythroid 2-related factor 2; GPX4, glutathione peroxidase 4; FTH1, ferritin heavy chain 1; ROS, reactive oxygen species; MDA, malondialdehyde; GSH, glutathione.

Due to its toxic side effects, oxaliplatin has poor long-term efficacy (26). Furthermore, CRC cell resistance to oxaliplatin also contributes to the poor prognosis of patients with CRC. Therefore, reducing cell resistance to oxaliplatin may be an effective method for improving patient survival. It has been reported that induction of ferroptosis may be applied to treat aggressive malignancies that are resistant to traditional therapies (27). This is due to the fact that ferroptosis can inhibit the activity of GSX4 (28), which is highly expressed in CRC cells. Furthermore, the upregulation of GSX4 can suppress the therapeutic effects of drugs, leading to tumor cell resistance (29). In the present study, Fe^{2+} content was decreased and the expression levels of ferroptosis-related proteins GPX4 and FTH1 were increased, suggesting that oxaliplatin promoted ferroptosis in CRC cells.

Additionally, elevated levels of oxidative stress act as markers of cancer (30), and are closely associated with CRC development and progression due to ROS and nitrogen species overproduction (31). Research has indicated that oxidative stress can be induced by ferroptosis in CRC cells (13,32). In addition, it has been revealed that oxaliplatin enhanced oxidative stress in gastric cancer (33). The present study, therefore, aimed to assess whether oxaliplatin exerted its effects through inducing oxidative stress in CRC cells. The data indicated that the levels of oxidative

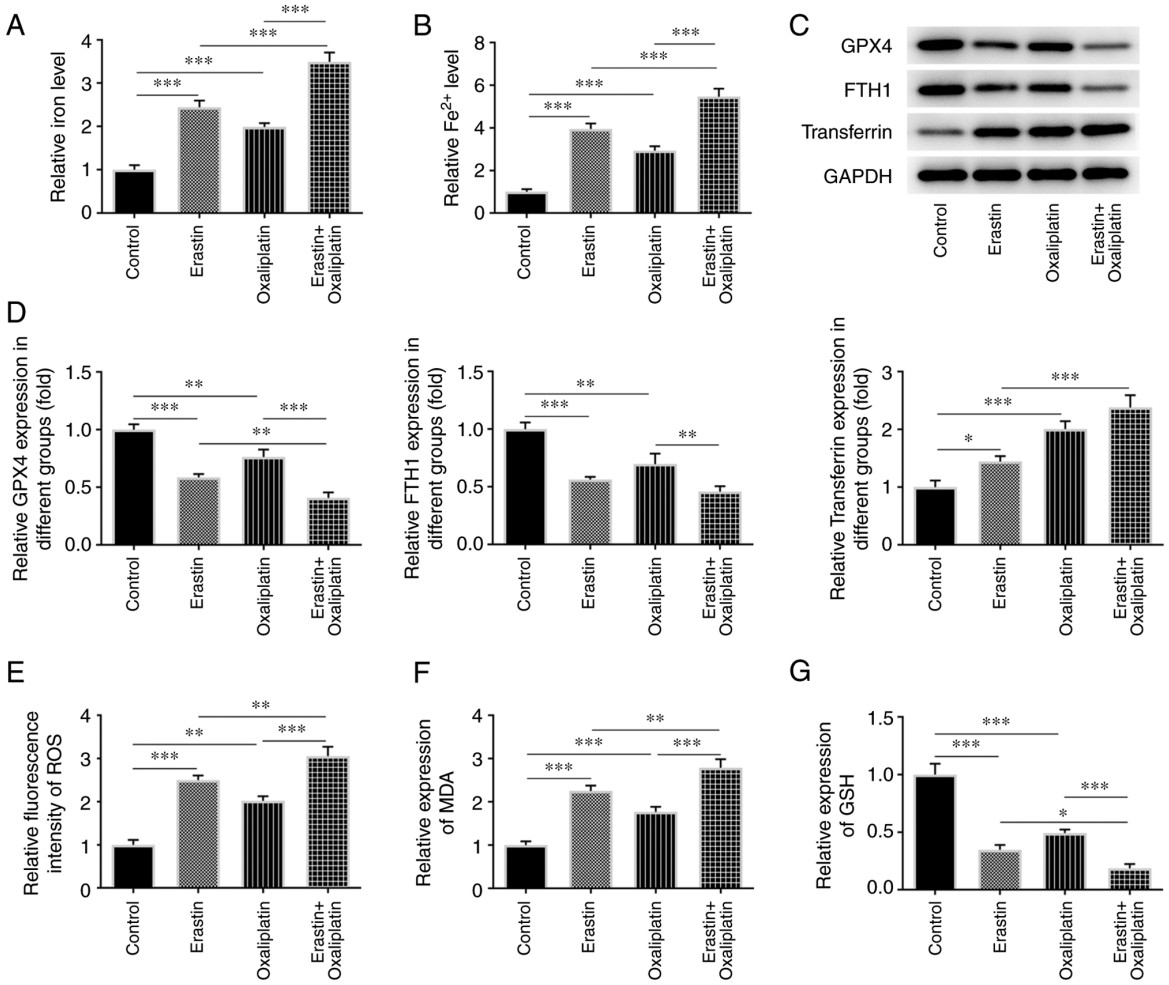

Figure 4. Oxaliplatin enhances the promotive role of erastin in ferroptosis and oxidative stress of colorectal cancer cells. (A and B) Detection of relative total iron level and Fe^{2+} level in HT29 cells was carried out using an iron ion test kit in the groups of control, erastin, oxaliplatin as well as oxaliplatin (3 μM) + erastin. (C and D) Western blotting was used to detect the levels of the ferroptosis-related proteins GPX4, FTH1 and transferrin in the control, erastin and oxaliplatin groups, as well as in the oxaliplatin (3 μM) + erastin group. (E-G) ELISA was used to detect the levels of the oxidative stress-related factors ROS, MDA and GSH in the control, erastin and oxaliplatin groups, as well as in the oxaliplatin (3 μM) + erastin group. Data are expressed as mean ± SD. *P<0.05, **P<0.01, ***P<0.001. GPX4, glutathione peroxidase 4; FTH1, ferritin heavy chain 1; ROS, reactive oxygen species; MDA, malondialdehyde; GSH, glutathione.

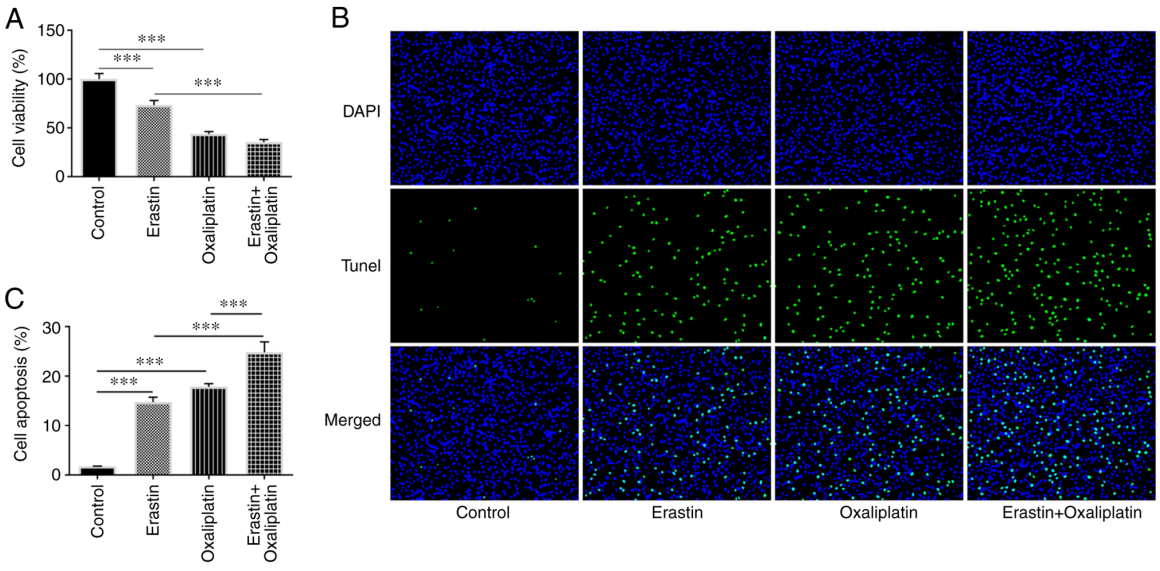

Figure 5. Oxaliplatin enhances the inhibitory effect of erastin on the viability of colorectal cancer cells. (A) Cell Counting Kit-8 assay was performed to detect the effects of oxaliplatin on the viability of HT29 cells in the control, erastin and oxaliplatin groups, as well as in the oxaliplatin (3 μM) + erastin group. (B and C) TUNEL assay was conducted to assess HT29 cell apoptosis levels in the control, erastin and oxaliplatin groups, as well as in the oxaliplatin (3 μM) + erastin group. Original magnification 200x. Data are expressed as mean ± SD. ***P<0.001.

stress-related factors, including ROS and MDA, were decreased, while GSH levels were increased. Thus, oxaliplatin promoted oxidative stress through the Nrf2 signaling pathway in CRC cells.

Erastin acts as an inducer of ferroptosis. In addition, the combination of erastin and sulfasalazine inhibits system Xc^- in cancer cells, which leads to an unusual iron-dependent cell death known as ferroptosis (34). Therefore, CRC cells were treated with erastin in the present study. The results revealed a marked increase in Fe^{2+} content and a decrease in the expression levels of ferroptosis-associated proteins in CRC cells. It was suggested that oxaliplatin enhanced the ferroptosis-inducing effect of erastin on CRC cells. Similarly, elevated levels of ROS and MDA, and reduced levels of GSH, indicated that oxaliplatin enhanced the role of erastin in inducing CRC cell oxidative stress. In addition, the marked decrease of CRC cell viability detected via CCK-8 and TUNEL assays also demonstrated that the inhibitory effect of erastin was enhanced by oxaliplatin treatment. However, there were certain limitations to the present study. The utilization of multiple cell lines may better demonstrate the anticancer effects of oxaliplatin. However, the main focus of the present study was the effects and mechanism through which oxaliplatin inhibits CRC. Thus, the representative CRC cell line HT29 was selected and the effects of oxaliplatin in promoting ferroptosis and oxidative stress in HT29 cells were demonstrated. Functional experiments will also be performed in other CRC cell lines to verify the present results, and the effects and potential mechanism of oxaliplatin on other CRC cell lines will be more extensively investigated in future research.

In conclusion, oxaliplatin effectively suppresses CRC cell viability and promotes ferroptosis and oxidative stress via the Nrf2 signaling pathway. Treatment with oxaliplatin can also significantly enhance the ferroptosis and oxidative stress-inducing effects of erastin in CRC cells. Oxaliplatin treatment was also shown to enhance the inhibitory effects of erastin on the proliferation of CRC cells. Therefore, the anticancer effects of oxaliplatin may be increased by inhibiting the Nrf2 signaling pathway, resulting in CRC cell ferroptosis and oxidative stress.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BL and HW designed the study, performed the experiments, drafted and revised the manuscript. BL analyzed the data and searched the literature. BL and HW confirm the authenticity of all the raw data. Both authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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