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Erianin inhibits oral cancer cell growth, migration, and invasion *via* the Nrf2/HO-1/GPX4 pathway

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ABSTRACT

Objective: To evaluate the effect of erianin on the viability, migration, and invasion of KB cells and elucidate its underlying mechanisms.

Methods: Cell Counting Kit-8, colony formation, wound healing, and Transwell assays were used to determine the proliferation, migration, and invasion of oral cancer KB cells. Furthermore, malondialdehyde (MDA) and glutathione (GSH) levels were determined. Fluorescent probes were used to detect changes in intracellular reactive oxygen species and iron ions. Additionally, the expressions of ferroptosis-related proteins, NF-E2-related factor 2 (Nrf2), ferritin heavy chain 1 (FTH1), heme oxygenase 1 (HO-1), and glutathione peroxidase 4 (GPX4) were analyzed by Western blotting assays.

Results: Erianin induced ferroptosis and inhibited the proliferation, migration, and invasion of KB cells. Moreover, erianin decreased GSH level, increased MDA level, elevated intracellular ROS and Fe^{2+} contents, and downregulated the expression of the ferroptosis-related proteins Nrf2, HO-1, GPX4, and FTH1 in KB cells. These effects of erianin were effectively reversed by a ferroptosis inhibitor, ferrostatin-1.

Conclusions: Erianin inhibits the proliferation, migration, and invasion of oral cancer cells and induces ferroptosis *via* the Nrf2/HO-1/GPX4 signaling pathway. Therefore, erianin may be a potential candidate for the treatment of oral cancer.

KEYWORDS: Erianin; Ferroptosis; Oral cancer; Reactive oxygen species; Therapy; Nrf2/HO-1/GPX4

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is common cancer, with 600 000 newly diagnosed cases worldwide yearly with a mortality rate of 40%-50% in patients, and oral cancer is the most frequent subtype of HNSCC[1]. Virus infection, cigarette smoking, and betel nut consumption are high-risk factors for oral cancer, although the exact reason that causes this cancer remains unclear. There is no effective cure for this disease, and the current treatments for oral cancer include chemotherapy and surgery to remove tumor tissues with a survival rate of less than 50%. In addition, drug resistance also limits the efficacy of oral cancer[2,3]. Thus, it is crucial to explore new drugs and therapeutic targets for this disease.

Erianin is a natural dibenzyl compound extracted from *Dendrobium*

Significance

Erianin is a naturally occurring compound that exerts an inhibitory effect on cancer cells. Our study shows that erianin could inhibit the growth of oral cancer cells, and induce ferroptosis *via* the Nrf2/HO-1/GPX4 pathway. Therefore, it could be useful for the treatment of oral cancer.

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chrysotoxum, which can be used as an antipyretic and analgesic in Chinese herbal medicine and exerts anti-tumor and anti-vascular properties[4]. It has been confirmed that erianin could effectively inhibit the growth and induce apoptosis of many tumors, including osteosarcoma, colon cancer, and nasopharyngeal carcinoma[5-7]. Furthermore, erianin exerts its anti-cancer effects on lung cancer cells by inducing ferroptosis and inhibiting cell migration[8].

Ferroptosis is a cell death pathway that is important in controlling tumor cell growth and proliferation. It is characterized by decreases or disappearances of the mitochondrial cristae, increases in the density of membrane, and decreases in antioxidant stress capacity[9]. Ferroptosis results in the accumulation of high iron levels and lipid-reactive oxygen species (ROS) within the cell. NF-E2-related factor 2 (Nrf2) is a critical factor involved in ferroptosis[10,11]. Activation of Nrf2 can upregulate the expression of ROS-related detoxification proteins, such as heme oxygenase 1 (HO-1) and glutathione peroxidase 4 (GPX4)[12]. Studies have shown that tagitinin C induces ferroptosis in colorectal epithelial cells *via* the Nrf2/HO-1 pathway in colon cancer[13]. However, the role of ferroptosis in oral cancer has remained unexplored.

Erianin inhibits skin tumor cell proliferation through the JNK/c-Jun signaling pathway[14]. Activation of JNK can promote Nrf2 phosphorylation, which weakens the cytoprotective system[15]. Nrf2 is increased after acute lung injury in C57BL/6 mice and inhibits ferroptosis by upregulating HO-1 expression[11]. Ferrostatin-1 (Fer-1), a specific inhibitor of ferroptosis, is an aromatic amine compound that can specifically bind to ROS to inhibit ferroptosis and effectively protect cells from damage due to lipid peroxidation[10]. Research has shown that erianin could induce ferroptosis in lung cancer cells. However, the effects of erianin on oral cancer have not been fully studied. Therefore, our study aimed to explore the antitumor effects and underlying mechanisms of erianin in oral cancer.

2. Materials and methods

2.1. Main materials

GPX4 monoclonal antibody (cat.no 67763, Proteintech Group, Chicago, USA), Nrf2 monoclonal antibody (cat. no. 66504, Proteintech Group, Chicago, USA), HO-1 monoclonal antibody (cat. no 66743, Proteintech Group, Chicago, USA), ferritin heavy chain (FTH1) polyclonal antibody (cat. DF6278, Affinity Biosciences, USA), β-actin antibody (cat. T0022, Proteintech Group, Chicago, USA), anti-rabbit immunoglobulin G (IgG) horseradish peroxidase (HRP)-linked antibody (cat. S0001, Affinity Biosciences, USA), and anti-mouse IgG HRP-linked antibody (cat. SA00001, Proteintech Group, Chicago, USA) were used. In addition, a ROS Kit (BL714A, Biosharp, Shanghai, China), glutathione (GSH) assay kit (BC1170, Solarbio, Beijing, China), malondialdehyde (MDA) assay kit (S0131, Beyotime, Shanghai, China), and FerroOrange Kit (TM633, Tongren, Tokyo, Japan), BCA kit (Beyotime, Shanghai, China) were used for the following experiments.

2.2. Cell culture

Human oral cancer KB cells (Fuheng Bio, Shanghai, China) were cultivated in RPMI-1640 medium supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum. All KB cells were cultured in an atmosphere containing 5% CO_2 at 37 ℃. KB cells were subjected to medium changes or passages every 2-3 d.

2.3. Drug formulation

Erianin (MedChemExpress, LLC, USA) was prepared in a stock solution of 10 μM with dimethyl sulfoxide and stored in the dark at −20 ℃. Fer-1 (MedChemExpress, LLC, USA) was prepared in a stock solution of 10 mM with dimethyl sulfoxide and stored at −20 ℃.

2.4. Cell viability assay

Cell viability was measured using a Cell Counting Kit-8 (CCK-8) assay (Beyotime, Shanghai, China). Briefly, oral cancer KB cells (6×10^3 cells/well) were seeded in a 96-well plate for 24 h. After erianin (30, 60, 90, 120, and 150 nM) treatment for 24 h at 37 ℃, KB cells were incubated with 10 μL of CCK-8 reagent in each well for 2 h at 37 ℃ in the dark. In addition, KB cells were pretreated with Fer-1 for 16 h and then treated with erianin for 24 h. Subsequently, the absorbance was measured at 450 nm using a microplate reader (BioTek, Winooski, USA), and the IC_{50} was calculated.

2.5. Colony formation assay

KB cells were seeded (1×10^3 cells/well) in 6-well plates overnight. Cells were washed with phosphate-buffered saline (PBS) after treatment with erianin for 24 h. Then, KB cells were cultured in a fresh medium for approximately 10-14 d. Cells were fixed with paraformaldehyde at 4 ℃ for 30 min and then stained with crystal violet for 15-30 min. The colony composition (>50 cells) was then recorded.

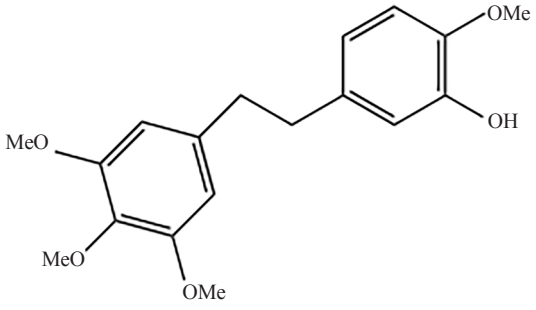

Figure 1. The chemical structure of erianin.

2.6. Wound healing assay

KB cells were trypsinized and inoculated into six-well plates at 5×10^5 cells/well. When the cells reached nearly 90% confluence, the cell layer was scratched with a 100 μL disposable pipette tip. The cells were then rinsed with a fresh culture medium to remove floating cells and treated with erianin at 37℃. The images of the wound area were obtained after 24 h using a microscope (Leica, Solms, Germany).

2.7. Transwell assay

KB cells were trypsinized, and 2×10^4 cells were collected in a serum-free medium and added to the top chamber, whereas a 10% serum-bearing medium was added to the bottom chamber. After culturing for 24 h at 37℃, the culture medium was washed with PBS, fixed with formaldehyde for 30-45 min, stained with Giemsa for 30 min, washed with PBS 3-4 times, dried, and finally observed under a microscope (Leica, Solms, Germany).

2.8. ROS assay

Intracellular ROS levels were assessed using the ROS Kit. The cultured KB cells were trypsinized and seeded in 12-well plates overnight. KB cells were treated with erianin in a serum-free medium for 24 h in the dark. After rinsing with a fresh serum-free medium, the intracellular ROS levels were detected by using a 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA) fluorescent probe. The images of KB cells were obtained under a fluorescence microscope (Leica, Solms, Germany) after allowing cells to react with the working solution for 45 min at 37℃ in the dark.

2.9. Measurement of GSH and MDA levels

Intracellular GSH and MDA levels were measured using GSH and MDA assay kits. Briefly, KB cells were plated in large Petri dishes and incubated with erianin for 24 h, and $>10^6$ KB cells were collected in a centrifuge tube. Subsequently, GSH and MDA levels were determined at 412 nm and 532 nm, respectively, according to the manufacturer's instructions.

2.10. Determination of iron accumulation

KB cells were digested with trypsin and seeded in a 24-well plate overnight. The cells were washed 4 times with the serum-free medium, and 1 μM FerroOrange working solution was added and then incubated for 30 min at 37℃. Finally, the fluorescent images were observed and collected using a live cell workstation (ZEISS, Germany).

2.11. Western blotting analysis

Cells were cultured with erianin for 24 h, collected in centrifuge tubes, and then lysed in RIPA buffer for 30 min on the ice. Then the protein concentration was examined and balanced in each group with a BCA kit. After protein was separated by SDS-PAGE, it was transferred to a PVDF membrane. Subsequently, the PVDF membranes were blocked with 5% skimmed milk and incubated with primary antibodies at 4℃ overnight, including anti-Nrf2 (1∶1 000), anti-HO-1 (1∶1 000), anti-FTH1 (1∶1 000), and anti-GPX4 (1∶1 000), followed by secondary antibodies for 1 h at ambient temperature, including anti-mouse IgG (1∶5 000) and anti-rabbit IgG (1∶5 000). The β-actin antibody was used as an internal control.

2.12. Statistical analysis

All data are presented as mean±SD of three independent experiments and analyzed by ANOVA using GraphPad Prism Version 7. The statistical significance of difference was set at $P<0.05$.

3. Results

3.1. Erianin inhibits the proliferation of oral cancer KB cells

KB cells were treated with erianin (30, 60, 90, 120, and 150 nM) (Figure 1) for 24 h, and cell viability was determined by a CCK-8 kit. The results revealed that erianin inhibited the proliferation of KB cells in a concentration-dependent manner ($P<0.001$), with an IC_{50} of about 120 nM (Figure 2A). To reduce the effect of drug toxicity on the cells, we used three concentrations (30, 60, and 120 nM) for further experiments. Furthermore, the antiproliferative effect of erianin was detected using the colony formation assay. KB cells treated with erianin (30, 60, and 120 nM) for 24 h resulted in a significant decrease in the number of colonies formed ($P<0.001$) after 10 days of culture (Figures 2B-C). These results indicate that erianin suppresses the proliferation of oral cancer KB cells.

3.2. Erianin inhibits the migration and invasion of oral cancer KB cells

To investigate whether erianin has an inhibitory effect on the migration of KB cells, wound healing assay was used to detect the migration. The migration of KB cells was decreased, after 24 h of treatment with different concentrations of erianin, compared to that in the control group ($P=0.044$) (Figure 3A). The Transwell assay was also used to determine whether erianin inhibits the invasion of KB

cells. Compared to the control group, erianin significantly inhibited the invasion of KB cells (P<0.001) (Figure 3B). Thus, erianin could inhibit the migration and invasion of oral cancer KB cells.

3.3. Erianin induces ferroptosis in oral cancer KB cells

We further explored whether erianin induces ferroptosis. Ferroptosis-induced cytotoxicity is dependent on ROS, thus ROS content in KB cells was detected by H2DCFDA staining. The results revealed that erianin markedly increased ROS production in a dose-dependent manner (P<0.001) (Figure 4A). Moreover, GSH level was significantly decreased (P<0.001) while MDA level was significantly elevated (P<0.001) in erianin-treated KB cells (Figures 4B and C). These results demonstrate that erianin may induce ferroptosis in KB cells.

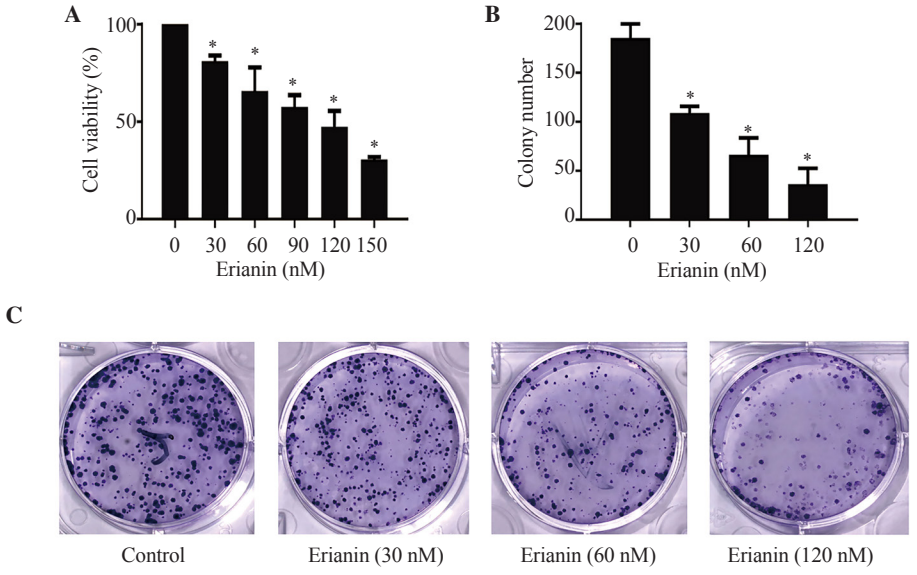

Figure 2. Erianin inhibits the viability of oral cancer KB cells. (A) After stimulating KB cells with different concentrations of erianin (30, 60, 90, 120, and 150 nM) for 24 h, cell viability was analyzed by CCK-8. (B-C) After treating KB cells with erianin (30, 60, 120 nM), colony formation of oral cancer cells was analyzed. The data are expressed as mean±SD and analyzed by ANOVA. $^*P$<0.01 compared to the control group.

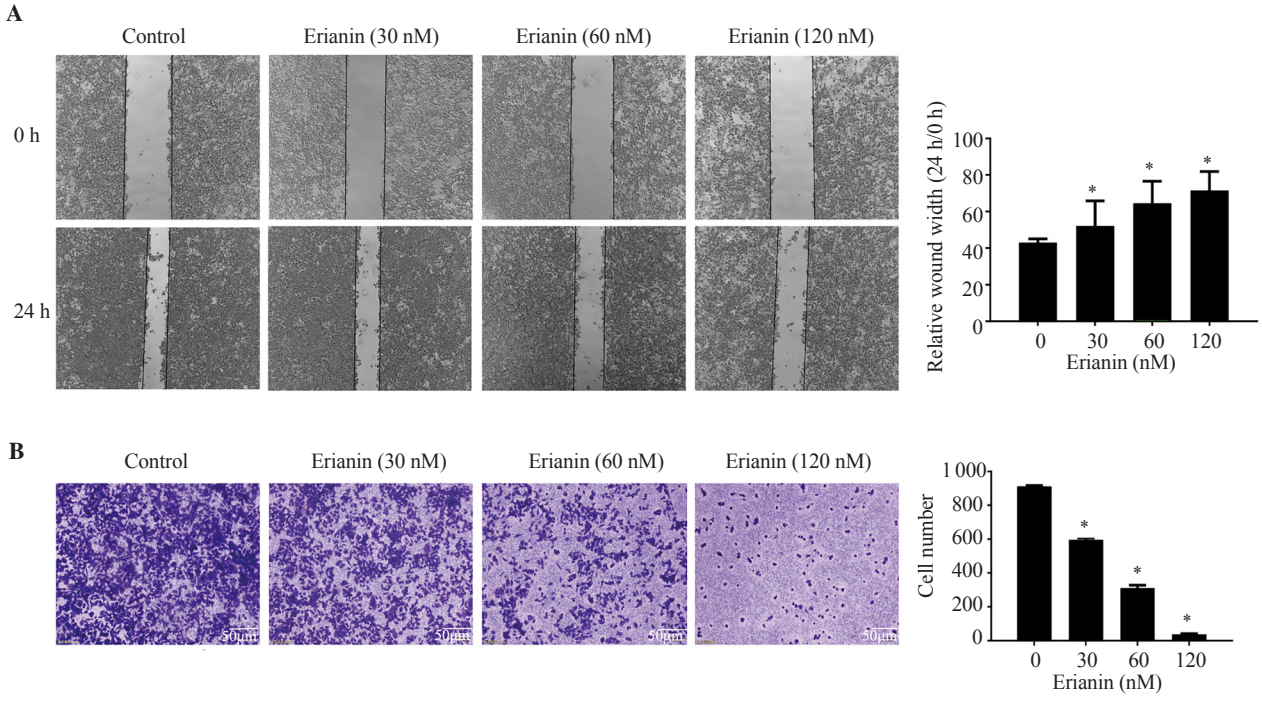

Figure 3. Effect of erianin on the invasion and migration of KB cells. KB cells were treated with erianin (30, 60, and 120 nM) for 24 h. (A) The migration ability of KB cells was detected by wound healing assay (Magnification: ×100). (B) The invasive ability of KB cells was detected by Transwell assay (Magnification: ×200). The data are expressed as mean±SD and analyzed by ANOVA. $^*P$<0.05, compared to the control group.

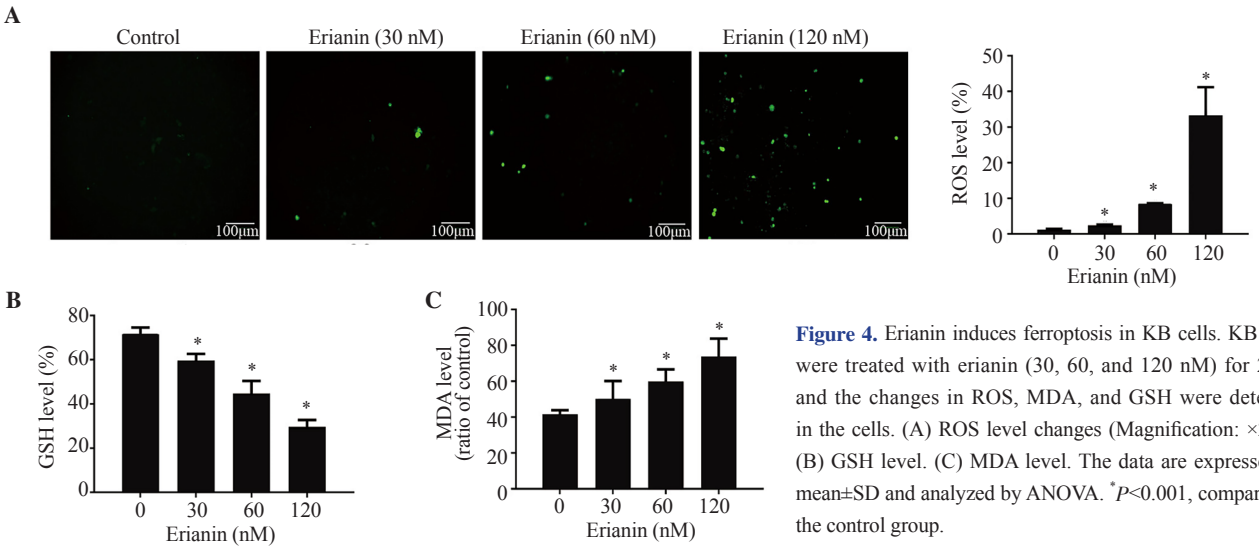

Figure 4. Erianin induces ferroptosis in KB cells. KB cells were treated with erianin (30, 60, and 120 nM) for 24 h, and the changes in ROS, MDA, and GSH were detected in the cells. (A) ROS level changes (Magnification: ×200). (B) GSH level. (C) MDA level. The data are expressed as mean±SD and analyzed by ANOVA. $^{*}P$<0.001, compared to the control group.

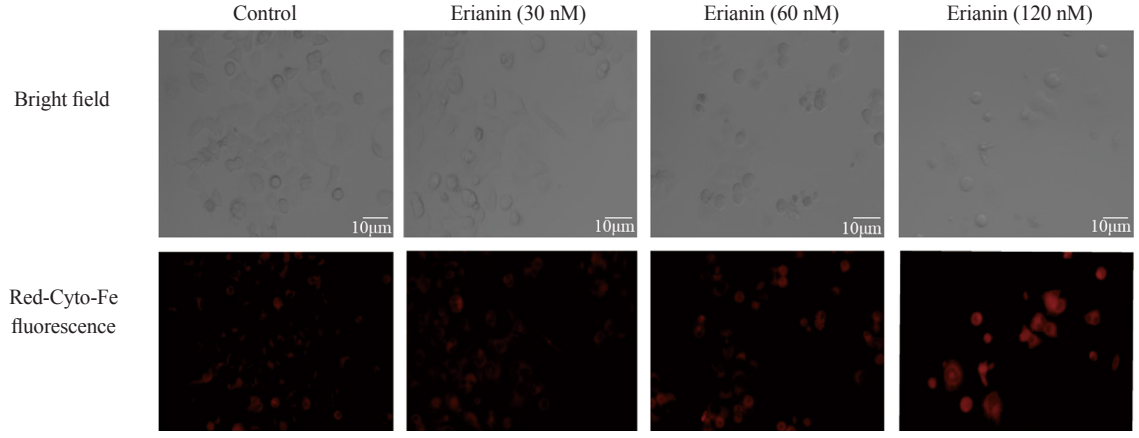

Figure 5. Changes of intracellular iron ions. KB cells were treated erianin (30, 60, and 120 nM) for 24 h, and the fluorescent probe solution of FerroOrange was added to detect the changes in Fe^{2+} content (Magnification: ×400).

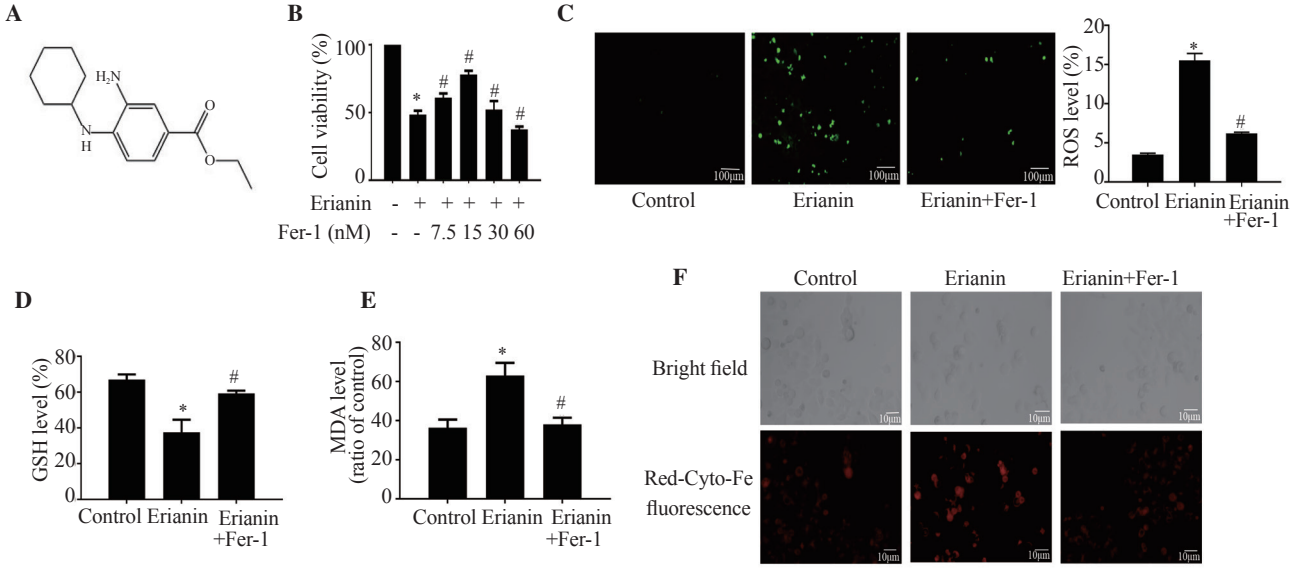

Figure 6. Combination of Fer-1 and erianin inhibits ferroptosis. Changes of intracellular iron ions, ROS, GSH, and MDA after co-incubation with erianin and Fer-1 were determined. KB cells were pretreated with different concentrations of Fer-1 (7.5, 15, 30, and 60 nM) for 16 h and then treated with erianin for 24 h. (A) Chemical structure of Fer-1. (B) Cell viability was determined by CCK-8. (C) ROS level (Magnification: ×200). (D) GSH level. (E) MDA level. (F) Iron changes (Magnification: ×400). The data are expressed as mean±SD and analyzed by ANOVA. $^{*}P$<0.01, compared to the control group, $^{\#}P$<0.01, compared to the erianin group.

3.4. Erianin promotes iron accumulation in oral cancer KB cells

Ferroptosis is closely associated with iron metabolism. Therefore, in the present study, FerroOrange was used to determine the amount of Fe^{2+} ions in KB cells. After treatment with erianin for 24 h, significantly higher fluorescence intensity of Fe^{2+} was observed in KB cells. The intracellular ion content increased with increasing erianin concentration (Figure 5).

3.5. Combination of Fer-1 and erianin inhibits ferroptosis

To further verify whether erianin induces ferroptosis in KB cells, we treated the cells with Fer-1 (Figure 6A). The results demonstrated that Fer-1 treatment did not induce cell death. The KB cells were treated with Fer-1 (7.5, 15, 30, and 60 nM) for 16 h and then treated with erianin for 24 h. Fer-1 reversed the damage caused by erianin and increased the cell viability (P<0.001) (Figure 6B). We also found that compared with the control group, the content of GSH in KB cells was significantly decreased (P=0.003). The content of MDA and ROS was increased (P=0.004 and P<0.001, respectively) after erianin treatment for 24 h. The erianin-induced changes on MDA, ROS and GSH were markedly reversed by Fer-1 treatment (P<0.01) (Figures 6C-E). Furthermore, the fluorescence intensity of accumulated intracellular iron ions induced by erianin was reduced after Fer-1 treatment (Figure 6F). These data further proved that erianin induces ferroptosis in KB cells.

3.6. Erianin induces ferroptosis in KB cells via the Nrf2/HO-1/GPX4 pathway

Western blotting was performed to detect the expression of FTH1, GPX4, HO-1, and Nrf2 in KB cells, which are ferroptosis-related proteins and explore their possible mechanisms. Western blotting assay revealed that the expressions of Nrf2 (P=0.001), GPX4 (P=0.027), HO-1 (P=0.017), and FTH1 (P=0.031) in KB cells

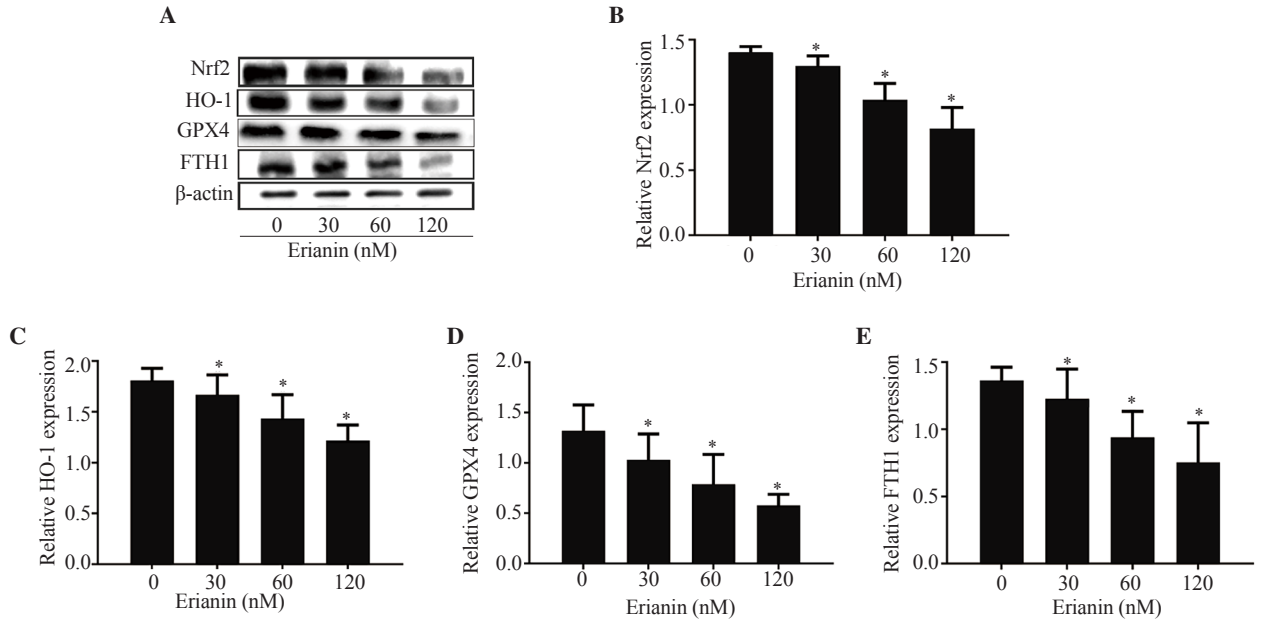

Figure 7. Effects of erianin on the expression of GPX4, HO-1, FTH1, and Nrf2 proteins in oral cancer. (A) Representative protein bands. The expressions of (B) Nrf2, (C) HO-1, (D) GPX4, and (E) FTH1 were measured by Western blotting analysis and normalized against β-actin. The data are expressed as mean±SD and analyzed by ANOVA. $^{*}P$<0.05 compared to the control group.

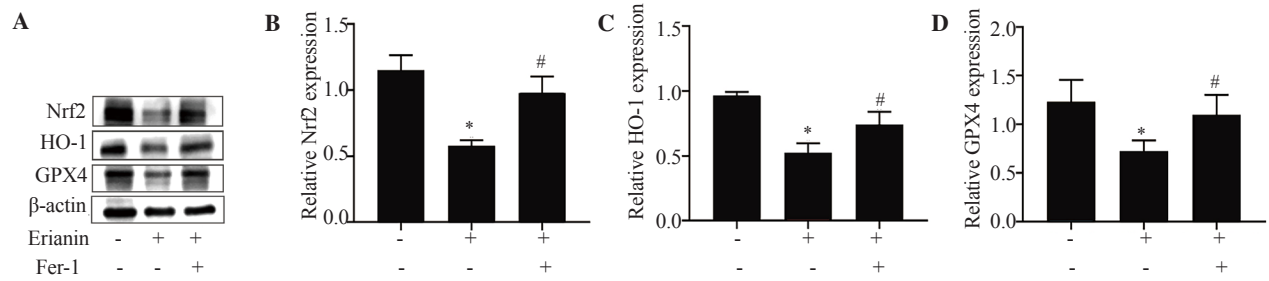

Figure 8. Effect of Fer-1 on erianin-treated KB cells. KB cells were pretreated with Fer-1 for 16 h and then treated with erianin (120 nM) for 24 h. (A) Representative protein bands. The expressions of (B) Nrf2, (C) HO-1, and (D) GPX4 were measured by Western blotting analysis and normalized against β-actin. The data are expressed as mean±SD and analyzed by ANOVA. $^{*}P$<0.05 compared to the control group, $^{\#}P$<0.05 compared to the erianin group.

were decreased in a dose-dependent manner after erianin treatment (Figure 7). In addition, cells were treated with Fer-1 for 16 h and subsequently treated with erianin (120 nM) for 24 h. Fer-1 treatment blocked the reducing effect of erianin on the expression levels of Nrf2 (P=0.006), HO-1 (P=0.035), and GPX4 (P=0.049) (Figure 8). Thus, erianin induces ferroptosis in KB cells possibly by the Nrf2/HO-1/GPX4 pathway.

4. Discussion

Current treatments for oral cancer aim to induce apoptosis or autophagy in cancer cells. However, most cancer cells exhibit drug resistance and may respond poorly to existing anti-cancer drugs[16,17]. Therefore, exploring new therapeutic drugs and targets is important for the clinical treatment of oral cancer. Studies have shown that erianin can inhibit the migration and invasion of some cancer cells *via* the PI3K/AKT and extracellular signal-regulated kinase (ERK)1/2 signaling pathways[18,19]. Here, we showed that erianin inhibited the proliferation, migration, and invasion of oral cancer KB cells by inducing ferroptosis, thus exerting an antitumor effect on oral cancer.

Ferroptosis is an iron-dependent lipid peroxidation pathway and primarily occurs as a result of accumulated intracellular ROS, high iron ion levels, and increased production of toxic MDA by lipid peroxidation[20]. Ferroptosis can increase the sensitivity of cancer cells to drugs and reverse cancer cell chemoresistance and is considered a potential therapeutic target for the treatment of human diseases, including malignancies[21,22]. Ferroptosis can inhibit tumor proliferation in cancer cells, including lung cancer, colorectal cancer, *etc*[8,13]. Activation of PI3K/AKT/mTOR signaling renders cancer cells resistant to ferroptosis, as mTOR affects GPX4 synthesis[23]. GPX4 is a crucial participant in ferroptosis and scavenging intracellular lipid peroxidation products, removing ROS and other peroxidation products which have accumulated in the cells[24]. Our study indicates that erianin inhibits the growth of KB cells, reduces intracellular GSH content, and causes the accumulation of a large amount of ROS and downregulation of intracellular GPX4 protein expression. This suggests that abnormal GPX4 expression may be related to the treatment of oral cancer. GPX4 directly utilizes GSH and reduces the cytotoxic product (PLOOH) to lipid alcohol (PL-OH)[25]. Studies have shown that p53 can regulate GSH by controlling cysteine uptake, which is a substrate for GSH synthesis and inhibits the Xc-system. The mutations of p53 led to GPX4 pathway activation and ferroptosis inhibition, which promotes oral tumor cell growth[26]. However, whether erianin leads to insufficient cysteine uptake by inhibiting the Xc-system or directly inhibits GSH production (resulting in aberrant expression of GPX4) remains to be elucidated.

Nrf2/HO-1 is an essential antioxidant pathway related to ferroptosis in cells. Moreover, the Nrf2/HO-1/GPX4-mediated antioxidant system plays a role in various neurodegenerative, cardiovascular, and inflammatory diseases. Inhibition of prolyl isomerase 1 can downregulate Nrf2/GPX4 and promote iron-mediated death in cancer cells[27]. GPX4 and HO-1 are regulated by Nrf2 and thus play an antioxidant role, and this overall pathway plays a role in inducing ferroptosis[28]. Abnormal Nrf2 activation can be observed in most cancer cells and regulates downstream HO-1 changes, which are closely related to the recurrence of tumor cells. Inhibition of Nrf2 expression, Nrf2 degradation, and Nrf2 nuclear translocation all have anti-cancer effects[29]. Meanwhile, the inhibition of Nrf2/HO-1 pathway can improve the prognosis of ulcerative colitis, acute lung injury, head and neck cancers, and other diseases[30,31]. Normally, Nrf2 binds to Kelch-like ECH-associated protein 1 (Keap 1) *via* ubiquitination in the cytoplasm, and Nrf2 expression is downregulated after Keap 1 inactivation, which can be rescued by Fer-1[32]. The inhibition of Nrf2 or p62 sensitized HNSCC cells to RSL3, in addition, Fer-1 can inhibit ferroptosis induced by erastin and RSL3, but not necrosis and apoptosis[33]. The downregulation of Nrf2/HO-1/GPX4 could be reversed by ferroptosis inhibitors, implying that the inhibitory effect of erianin on KB cells might be mediated by the Nrf2/HO-1/GPX4 pathway. However, whether erianin inhibits Nrf2 expression or promotes Nrf2 degradation, resulting in decreased intracellular expression remains to be further studied.

Oral cancer tissues have higher FTH1 expression than normal tissues, and FTH1 expression is positively correlated with macrophage infiltration in most solid tumors. Overexpression of FTH1 is associated with cervical lymph node metastasis and poor prognosis in patients with oral cancer[34,35]. Consistent with previous findings, FTH1 expression was significantly reduced by erianin. Nrf2 regulates antioxidant balance and intracellular iron homeostasis by affecting downstream signaling targets, such as FTH1, ferroportin, and HO-1. Among them, Nrf2 activation also regulates iron homeostasis in hemochromatosis and thalassemia[36]. In contrast, the expression level of FTH1 is negatively correlated with ferroptosis sensitivity[37]. HO-1 can decompose heme into carbon monoxide, antioxidant biliverdin, and ferrous iron. Ferritin expression is reduced in Nrf2 gene-deficient mice, generating large amounts of ROS, and causing red blood cell hemolysis. HO-1 releases iron from hemoglobin, resulting in increased concentrations of serum iron[38]. Absorption of large amounts of iron can promote tumor cell growth and migration. Excess iron can lead to the generation of large amounts of lip-ROS through the Fenton reaction, inducing oxidative stress and damaging cells. Excessive production of ROS also attacks ferritin and iron-containing proteins, leading to the instabilities of Fe. Therefore, erianin promotes the enrichment of Fe^{2+} in oral cancer KB

cells. However, whether the increase in Fe^{2+} in KB cells is related to FTH1 and the underlying association remains unknown.

In summary, erianin can induce ferroptosis in oral cancer KB cells by the Nrf2/HO-1/GPX4 signaling pathway. However, future study is needed to investigate its effect on other oral cancer cell lines, relevant gene and animal experiments are still needed to further verify the efficacy and safety of erianin, either as a monotherapy or in combination with chemotherapy drugs.

Conflict of interest statement

The authors declare no conflict of interest.

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Authors' contributions

XYT, QYH, and RH designed and conducted the experiment. XYT wrote the manuscript. XYT, BL, and QYH provided initial help for analysis. XRC and MYZ reviewed and criticized the manuscript. JCX helped in experimental design, paper writing and modification, fund support, and submission.

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