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The Mechanism and Research Progress of Ferroptosis in Esophageal Squamous Cell Carcinoma

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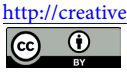

Abstract

Esophageal squamous cell carcinoma (ESCC), as the main histological type of esophageal cancer (EC), has the characteristics of high malignant degree, high mortality and tendency to lymph node metastasis. Despite the combination of surgery and chemoradiotherapy, ECSS has a poor prognosis. Ferroptosis is a newly discovered iron-dependent programmed cell death pattern characterized by the accumulation of lipid peroxidation and characteristic morphological changes of mitochondria. It regulates the growth and invasion of cancer cells in various tumors such as gastric cancer and pancreatic cancer. In this article, we mainly introduce the ferroptosis regulatory mechanism in ESCC, hoping to provide a new direction and ideas for the treatment of ECSS.

Keywords

ESCC, Ferroptosis, System Xc−, GPX4, PUFA, MUFA, Fe^{2+}, ROS, Lipid Peroxidation

1. Introduction

Esophageal cancer (EC) is one of the most common digestive system malignant tumors, with high mortality and worse prognosis. It ranks seventh in terms of incidence and sixth in mortality worldwide, leading to 604,000 new cases and 544,000 deaths respectively in 2020. Eastern Asia shows the highest regional incidence rates, followed by Southern Africa, Eastern Africa, Northern Europe, and South Central Asia [1]. It has two major histologic types: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC), among which

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ESCC composes around 90% globally [2]. Due to EC is often asymptomatic in its early stages, it's usually diagnosed at an advanced stage with an overall five-year survival rate from 15% to 25% [3]. The treatment of EC mainly includes Endoscopic or surgical treatment for early-stage patients and radiotherapy or/and chemotherapy for patients with advanced or metastatic cancer [4]. For locally advanced EC neoadjuvant chemoradiotherapy or chemotherapy has been the standard treatment. However, the postoperative recurrence and metastasis rates of locally advanced EC are still high after neoadjuvant therapy, ranging from 30% to 50% [5]. Radioresistance, the major reason for the failure of radiotherapy in ESCC, can be decreased by inducing ferroptosis pathways [6].

Ferroptosis, a unique iron-dependent programmed cell death modality characterized by the accumulation in lipid peroxidation, is morphologically, biochemically and genetically distinct from apoptosis, necrosis and autophagy [7]. It has unique morphological and bioenergetic features including shrunken mitochondria, increased mitochondrial membrane density, disruption of membrane integrity and depletion of intracellular NADH [8]. The accumulated evidence suggests that it closely participates in the regulatory growth of various tumor cells, such as gastric cancer [9], pancreatic cancer [10] and colorectal cancer [11], providing numerous potential treatment targets.

In this article, we mainly discuss the regulatory mechanism and research progress of ferroptosis in ESCC (**Figure 1**), hoping to provide new ideas for the treatment of ESCC.

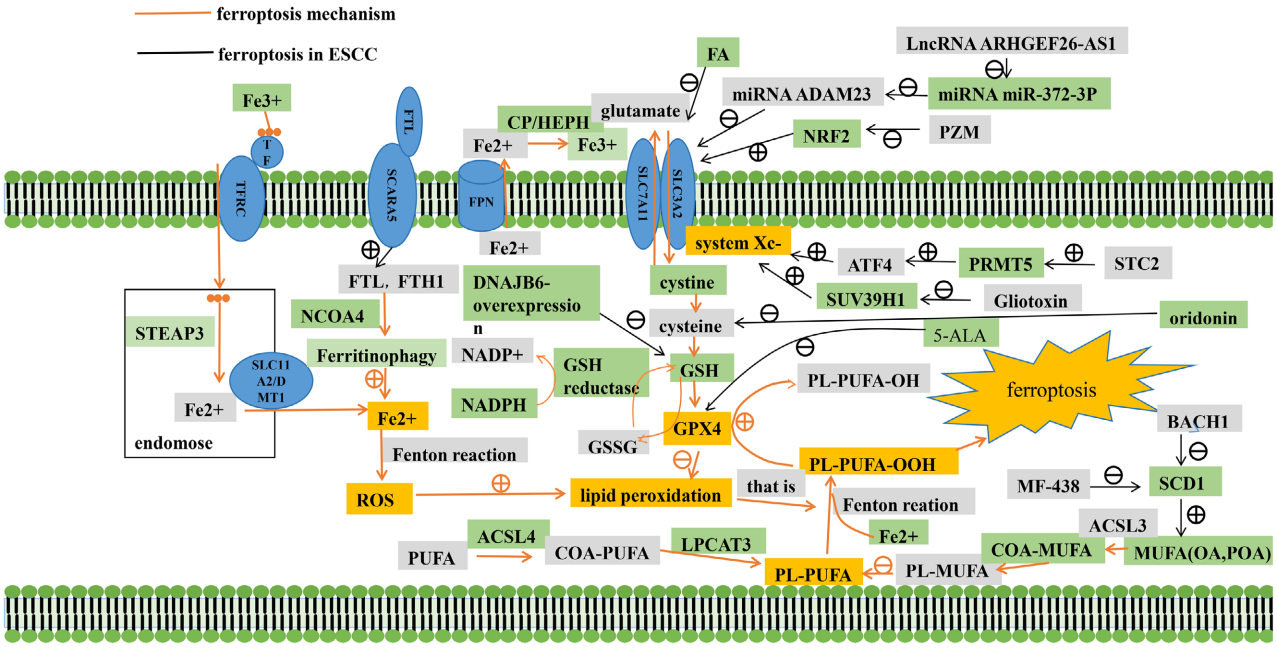

Figure 1. System Xc− promotes GPX4 activity through intracellular transfer of cystine. GPX4 inhibits ferroptosis by clearing PL-PUFA-OOH and induces ferroptosis when inhibited. PUFA, a substrate of PL-PUFA-OOH, induces ferroptosis when promoted. Interestingly, MUFA can inhibit this effect. Fe^{2+} induces ROS production through Fenton reaction, which promotes the lipid peroxidation process. By influencing System Xc−, GPX4, PUFA and Fe^{2+}, a variety of molecules promote the production of PL-PUFA-OOH or inhibit its conversion to non-toxic products, and ultimately induce ESCC ferroptosis.

2. Ferroptosis-Regulating Mechanism

2.1. System Xc−, GSH and GPX4

System Xc−

System Xc− is a heterodimeric cystine/glutamate antiporter and consists of two key members, including the catalytic subunit solute carrier family 7 member 11 (SLC7A11) and solute carrier family 3 member 2 (SLC3A2). System Xc− mainly transports extracellular cystine to cells. Intracellular cystine is rapidly converted to cysteine and then functions as the precursor for glutathione biosynthesis [12]. In terms of regulating system Xc− activity in ferroptosis, SLC7A11 could interact with BECN1 and further induce ferroptosis, as well as being regulated at the transcriptional level, and a decrease in SLC7A11 could consequently induce ferroptosis [13]. The function of System Xc− is very critical, and many mechanisms affecting ferroptosis operate through this system. Erastin, a ferroptosis inducer, can cause strong and persistent inhibition of system Xc− when exposed to cells for a very short duration at low erastin concentrations [14].

GSH

Cystine input from System Xc− is reduced intracellularly to cysteine, which then acts as a precursor for glutathione (GSH) synthesis. GSH is a tripeptide, γ-L-glutamyl-L-cysteinyl-glycine, found in all mammalian tissues and especially highly concentrated in the liver. Glutathione exists in the thiol-reduced (GSH) (99%) and disulfide-oxidized (GSSG) forms (less than 1%). GSH is synthesized in cytosol in virtually all cells involving two ATP-requiring enzymatic steps: In the first step, L-glutamate acid and L-cysteine are catalyzed by glutamate cysteine ligase (GCL) to produce γ-glutamyl-L-cysteine, and in the second step, γ-glutamyl-L-cysteine and L-glycine are catalyzed by GSH synthase (GS) to produce GSH. GSH serves several vital functions including 1) detoxifying electrophiles; 2) scavenging free radicals; 3) maintaining the essential thiol status of proteins; 4) providing a reservoir for cysteine; and 5) modulating critical cellular processes such as DNA synthesis, microtubular-related processes, and immune function. As a ferroptosis inhibitor, GSH reduces the endogenously produced hydrogen peroxide in the presence of selenium-dependent GSH peroxidase. In the process, GSH is oxidized to GSSG, which in turn is reduced back to GSH by GSSG reductase at the expense of NADPH, forming a redox cycle [15].

GPX4

GPX4 is a key enzyme in lipid peroxidation. The GPX family comprises many members, including glutathione peroxidase 1 - 8 (GPX1 - 8). Among these enzymes, GPX4 plays an important role in ferroptosis, protecting against cell death associated with lipid peroxidation and oxidative stress [9] [16]. As the co-factor of GPX4 in catalysing peroxides into alcohols, the depletion of GSH caused by cysteine deprivation directly inactivates GPX4 and leads to subsequent induction of ferroptosis. Knockout of GPX4 in mice leads to embryonic lethality and mass lipid peroxides accumulation [17] [18]. RSL3 can inhibit the activity of GPX4 by

covalent bonding with GPX4 and leads to lipid peroxide accumulation [8]. As an active-site selenocysteine, selenium is needed for the biosynthesis of GPX4. Thus, selenium deficiency promotes sensitivity to ferroptosis, while selenium supplementation promotes ferroptosis resistance. GPX4 can transform potentially toxic lipid hydroperoxides (L-OOH) into non-toxic lipid alcohols (L-OH) [19]. In the process of ferroptosis resistance, GPX4, converting PL-PUFA-OOH to PL-PUFA-OH, plays a role in eliminating Lipid peroxide (LPO) products. Although oxidizing can take place in various cell membrane lipids, peroxidation of PUFAs in phospholipids by ALOXs appears to be significant for ferroptosis [20].

2.2. Lipid Metabolism and LPO

Fatty acids include saturated and unsaturated fatty acids, the latter divided into monounsaturated fatty acids (MUFA, one double bond) and polyunsaturated fatty acids (PUFA, multiple double bonds) according to the number of double bonds. The current study found that PUFA present in membrane lipids such as phospholipids (PL-PUFA), rather than free PUFA itself, is the driver of ferroptosis. The synthesis of PL-PUFA consists of two steps catalyzed by two essential enzymes, acylcoenzyme A (CoA) synthetase long-chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3). Long-chain acyl-CoA synthetases (ACSLs), converting long-chain fatty acids into their acyl-CoA, including five ACSL isoenzymes identified in mammals: ACSL1, ACSL3, ACSL4, ACSL5, and ACSL6 [21]. ACSL4 has a clear preference for activating PUFAs, clearance of ACSL4 prevents the entry of polyunsaturated fatty acids into PLs membranes, and ACSL3-dependent MUFA metabolism is a key regulator of ferroptosis [22]. Limiting PUFA biosynthesis through control of acetyl-coenzyme A carboxylase (ACC), energy stress drives resistance to ferroptosis [23]. PUFA in lipid droplet first binds to Acetyl-CoA via ACSL4. Subsequently, they form PUFA-PL under the action of LPCAT3 [24]. The lipid peroxidation process, a key step in iron pyrodeath, is the oxidation of PL-PUFA to PL-PUFA-OOH, which is driven by both the labile iron pool facilitating the Fenton reaction and iron-dependent enzymes, such as lipoxygenases. LPO triggers ferroptosis [24] [25]. As mentioned above, GPX4 mediates the clearance of PL-PUFA-OOH, as known as LPO. Exogenous MUFA, mainly oleic acid (OA) and palmitoleic acid (POA), effectively inhibit ferroptosis by inhibiting the accumulation of toxic lipid reactive oxygen species (ROS), especially on the plasma membrane, and reducing the level of phospholipids containing oxidizable polyunsaturated fatty acids. The free MUFA is activated by ACSL3 into MUFA-CoA, which is incorporated into glycerophospholipids to form PL-MUFA [22].

2.3. Iron Metabolism

Iron is necessary for the accumulation of LPO and the formation of ROS. The Fenton reaction, which plays a significant role in inducing ferroptosis, catalyzes

the formation of ROS in the presence of Fe^{2+}. Therefore, the sensitivity of ferroptosis can be adjusted by affecting the transport, storage and turnover of iron [19]. Iron contains two oxidation states, Fe^{2+} and Fe^{3+}. The serum Fe^{3+} bound to transferrin (TF) is recognized by the transferrin receptor (TFRC) on the cell membrane and transferred into the intracellular endosome, where the STEAP3 metalloreductase reduces Fe^{3+} to Fe^{2+}, which is released into the cytoplasm through solute carrier family 11 member 2 (SLC11A2/DMT1) [20].

Iron storage proteins, including ferritin light chain and ferritin heavy chain 1, can be degraded by lysosomes to increase free iron levels. This process is achieved by nuclear receptor coactivator 4 (NCOA4) mediated ferritin autophagy (a selective autophagy that degrades ferritin through lysosomes), increasing Fe^{2+} and inducing ferripyrosis through the production of reactive oxygen species mediated by the Fenton reaction [26] [27]. NCOA4-induced ferritin autophagy was strongly associated with glioblastoma [28], pancreatic cancer [29], ovarian cancer [30] and liver cancer [31]. Such as C-MYC, a regulatory gene closely related to proliferation, down-regulates the expression of NCOA4 through directly targeted binding, and then promotes the immune escape of ovarian cancer cells by inhibiting ferritin autophagy on ferriptosis [30]. Polypyrimidine tract-binding protein 1 (PTBP1) mediates ferroptosis in liver cancer cells by regulating NCOA4 translation [31].

In contrast to TFRC and ferritin autophagy, which increase iron, ferritransporter (FPN) induces ferroptosis resistance by exporting iron outside the cell. Independent of TFRC, divalent metal transporter 1 (DMT1) and ferritin, siramesine (a lysosome disrupting agent) and lapatinib (a tyrosine kinase inhibitor) increase ROS in breast cancer cells by decreasing FPN expression [32]. Transported into the serum, Fe^{2+} is oxidized to Fe^{3+} by ferroxidases, such as ceruloplasmin (CP) or hephaestin (HEPH).

In summary, the System Xc−, GSH and GPX4, which act as inhibition pathways for ferroptosis, may cause a reduction in the activity or content of GPX4 and PL-PUFA-OOH scavenging suppression, when any substance in the pathway is inhibited. Then the characteristic ferroptosis occurs. Free PUFA produces PL-PUFA-OOH in a series of enzymatic reactions, and MUFA inhibits this process. Iron overload can increase the production of ROS and enhance the sensitivity of ferroptosis.

3. Detection of Ferroptosis

Ferroptosis is mainly determined by unique morphological features and the detection of malondialdehyde (MDA), ROS and Fe^{2+}. MDA is detected with a Lipid Peroxidation Assay Kit or malondialdehyde assay kit. ROS is detected with C11 BODIPY staining or a fluorescent probe, 2',7'-dichlorofluorescin diacetate (DCFH-DA). Iron is detected with iron staining assay kits, respectively [33] [34]. In addition to the initial lipid hydroperoxides (LOOHs), lipid peroxidation also produces reactive aldehydes such as MDA and 4-hydroxynonenal (4HNE) [20].

The increase in MDA content suggested active lipid peroxidation and the induction of ferroptosis.

4. Ferroptosis Inducers and Inhibitors

The ferroptosis inducers mainly include four classes: 1) systemic Xc− inhibitors (Erastin and its analogs, sulfasalazine (SAS), glutamate, and sorafenib (SRF)); 2) GPX4 inhibitors (RSL3 ML162 and ML210); 3) FIN56, which depletes GPX4 protein, and lipophilic antioxidant CoQ10; 4) FINO2, which indirectly inhibits GPX4 activity and stimulates lipid peroxidation. Erastine and RSL3 are the most widely used inducers [19] [35].

The ferroptosis inhibitors include iron metabolism inhibitors, iron chelators (deferoxamine (DFO) and ciclopirox acid (CPX)), inhibitors of lipid peroxidation (lipoxygenase (LOX) inhibitors and Ferrostatin 1 (fer-1), which eliminates lipid hydroperoxides in the presence of reduced iron), inhibitors of lipid metabolism (thiazolidinediones which inhibit ACSL4) and protein synthesis inhibitor (cycloheximide which inhibit system Xc− induced ferroptosis) [19] [36]. Erastine and RSL3 are commonly used as inducers of Ferroptosis. DFO and fer-1 are commonly used as inhibitors.

Erastin and Sorafenib, which trigger ferroptosis in cancer cells by inhibiting the system Xc−, have been limited in their further development due to their low efficacy in vivo. The anti-rheumatic drug sulfasalazine, which activates ferroptosis by inhibiting the system Xc−, has been initiated in several phase I and phase II clinical studies based on its excellent safety profile in animal studies. However, different doses of SAS failed to produce a clinical response in malignant glioma, and side effects including anorexia, gastrointestinal toxicity, and hematological toxicity were reported. Although GPX4 inhibitors (RSL3, ML162) are effective in inhibiting cell growth in vitro, prospects for further clinical development of all currently identified GPX4 inhibitors are limited due to poor pharmacokinetics and specificity. Therefore, more research is needed to develop GPX4-specific inhibitors with improved pharmacological properties. Currently, dexrazoxane (DXZ) is the only iron chelator approved by the FDA to prevent doxorubicin (DOX)-induced cardiotoxicity by chelating DOX-induced mitochondrial iron. Compared to DXZ, the newly orally iron chelator CN128 is more effective with fewer side effects and is currently being studied in a phase II clinical trial to treat β-thalassemia after regular blood transfusion. N-acetylcysteine (NAC), targeting cysteine metabolism to inhibit ferroptosis, has not yet been approved for clinical use [37].

5. The Regulation of Ferroptosis in ESCC

Accumulating evidence suggests that induction of ferroptosis can activate anticancer mechanisms through regulation of System Xc−, GSH and GPX4, lipid metabolism and iron metabolism [9]. Next, the adjustment of ferroptosis death to ESCC will be introduced from these three aspects.

5.1. System Xc−, GSH and GPX4 in ESCC with Ferroptosis

Previous studies have shown that stanniocalcin 2 (STC2), up-regulated in various tumors such as ESCC, colon cancer, liver cancer, cervical cancer and nasopharyngeal cancer, is involved in cell proliferation, migration, invasion, immune response, and drug resistance biological processes. STC2 interacts with protein methyltransferase 5 (PRMT5) and activates PRMT5. Subsequently, the inhibition of SLC7A11 expression by activating transcription factor 4 (ATF4) induces lipid peroxidation and ferroptosis, and this process can be reversed by GSK, a PRMT5 inhibitor [6]. Moreover, Circ-STC2 induces ferroptosis of nucleus pulposus cells by promoting miR-486-3p/TFR2 axis [38]. Circ-PRMT5 stimulates lymph node metastasis and distant metastasis of EC by binding to miR-203 [39]. PRMT5 inhibitors have been shown to inhibit a variety of tumors, with six oral preparations showing few side effects in mouse xenotransplantation models, and several drugs showing promising results in clinical trials of solid tumors and hematologic malignancies [40]. However, there are few articles about PRMT5 regulating iron coke death. ATF4 inhibition induces ferroptosis in hepatocellular carcinoma [41], colorectal cancer [42] and pancreatic cancer [43].

Gliotoxin, a compound derived from marine fungus with well-documented immunosuppressive and anticancer properties, can inhibit the growth of colon and lung cancer through apoptosis. Caspase-9 and caspase-8 are key enzymes in intrinsic and extrinsic pathways, respectively. Deficiency of SUV39H1, a gliotoxin target gene, induces ferroptosis in clear cell renal cell carcinoma. An online tool was used to identify SUV39H1 as a target gene for gliotoxin-induced ESCC ferroptosis. Inhibition of ESCC cells by gliotoxin can be reversed by ferroptosis inhibitors fer-1 and DFO, but caspase-9 and caspase-8 protein levels are not affected. Gliotoxin treatment down-regulates the expression of SLC7A11 and GPX4, but does not affect the expression of FTH1. The reducing of GSH level and increasing of MDA accumulation, ROS and Fe^{2+} levels demonstrate the induction of ferroptosis. The increase in Fe^{2+} may be modulated by other means. In addition, nude mice xenograft tumor model proved that gliotoxin has a strong ability to inhibit ESCC growth [44].

Transcription factor NRF2, when activated, regulates cellular antioxidant response by transcriptionally activating downstream antioxidant factors such as SLC7A11, ferritin, heme oxygenase 1 (HO-1) and UDP-glucuronosyltransferase (UGT). Under normal circumstances, the rapid degradation of KEEP1-dependent proteasome keeps NRF2 in a low concentration of low activity, but under oxidative stress conditions of ROS or under electrophilic stimulation, the ubiquitination and degradation of NRF2 disappear and maintain the active state [45]. NRF2 inhibition induces ferroptosis in colorectal cancer [46]. In pancreatic cancer, aspartate aminotransferase 1 (GOT1) inhibits ferroptosis by promoting NRF2 expression [47]. Over-expressed NRF2, increasing SLC7A11 expression by binding to its promoter, inhibits ROS production and lipid peroxidation levels, and thus inhibits ESCC ferroptosis. This effect can be restored by fer-1 or SLC7A11 knock-

out [48]. Pizotifen malate (PZM), an NRF2 inhibitor, induces ESCC ferroptosis by increasing ROS and MDA levels and decreasing the GSH/GSSG ratio in a dose-dependent manner. The induced cell death can be saved by the lipid ROS scavengers fer-1 and Trolox. In terms of molecular mechanism, PZM plays an induction role by binding to the Neh1 domain of NRF2, preventing NRF2 protein from binding to the ARE motif of the target gene, and inhibiting NRF2 transcriptional activity [49]. There is no evidence that PZM induces ferroptosis in other cancers.

ADAM23, a receptor for neuropeptide LGI1, down-regulates SCLC7A11, SLC3A2, and GPX4 protein levels to induce ESCC ferroptosis when overexpressed. MiR-372-3p, an upstream regulator of ADAM23, inhibits ferroptosis by inhibiting ADAM23 expression. LncRNA ARHGEF26-AS1 up-regulates the expression of ADAM23 and induces ferropyrodeath through competitive binding with miR372-3p [50].

Ferulic acid (FA), a phenolic acid substance, induces ESCC ferroptosis through down-regulates SLC7A11, GSH and GPX4, as well as increases MDA, ROS content and iron load. Induction of ferroptosis could be reversed by DFO. In addition, the increased activity of ACSL4 suggests that lipid metabolism may be involved in FA-induced ferroptosis [51]. However, FA up-regulates Nrf2 signaling in murine MIN6 cells to inhibit ferroptosis [52]. Therefore, the effect of FA on ferroptosis needs further study.

Oridonin (Ori), a natural tetracyclic diterpenoid active compound, induces ESCC ferroptosis by decreasing the GSH/GSSG ratio and GPX4 activity. This can be achieved through two ways: reducing the activity of gamma-glutamyl transpeptidase 1 (GGT1) to inhibit the gamma-glutamyl cycle, and covalently binding with cysteine to form the conjugated oridonin-cysteine (Ori-Cys) to inhibit the synthesis of GSH [33].

DnaJ/Hsp40 homolog, subfamily B, member 6 (DNAJB6), a member of the heat shock protein 40 (Hsp40) family, negatively correlated with ESCC proliferation, migration, invasion and lymph node metastasis. It plays an anti-tumor role in inducing ESCC ferroptosis by reducing GSH, GPX4 as well as increasing MDA content [53] [54]. At present, the mechanism of DNAJB6 in ESCC is unclear, and we speculate that it directly acts on GSH, but it may also act on cysteine, cystine, or system Xc−. However, DNAJB6 can promote the invasion of colorectal cancer and play a carcinogenic role [55]. This may be due to tissue specificity. Therefore, the role of DNAJB6 in cancer needs further study.

5-aminolevulinic acid (5-ALA) can increase lipid peroxidation by inhibiting GPX4 expression. In the subcutaneous transplanted mice model, 5-ALA treatment demonstrated ESCC growth inhibition while immunohistochemistry (IHC) from tumor sections showed GPX4 expression was down-regulated [56].

5.2. Lipid Metabolism in ESCC with Ferroptosis

Stearoyl-CoA desaturase (SCD1), a lipase that converts saturated fatty acids to

MUFA, inhibits ferroptosis by down-regulating lipid peroxidation levels in pancreatic cancer [57], gastric cancer stem cells (GCSCs) [58], ovarian cancer [59] and colorectal cancer [60]. BTB and CNC homology 1 (BACH1) reduces MUFA (especially OA) content by inhibiting SCD1 expression. Subsequently, reduced OA induces ESCC ferroptosis by decreasing the promoted effect of lipid membranes PUFA on lipid peroxidation [61]. MF-438, an inhibitor of SCD1, can also inhibit SCD1 expression and induce ESCC ferroptosis [62].

5.3. Iron Metabolism in ESCC with Ferroptosis

Scavenger receptor protein 5 (SCARA5) plays an inhibitory role in pancreatic cancer [63], oral squamous cell carcinoma [64], bladder cancer [65] and nasopharyngeal carcinoma [66] by inhibiting proliferation, migration and invasion. However, its effects on ESCC proliferation and migration are contradictory and need to be confirmed by further studies [67] [68]. By increasing intracellular ROS, MDA, and Fe^{2+}, SCARA5 induces ESCC ferroptosis and the characteristic cellular changes of ferroptosis with reduced mitochondrial volume, reduced mitochondrial cristae, and increased mitochondrial membrane density are observed in transmission electron microscopy. Mechanologically, SCARA5 increases intracellular ferritin light chain (FTL) concentration by binding to FTL, which can increase Fe^{2+} through NCOA4-mediated ferritinophagy and increase ROS through Fenton reaction. The simultaneous observed reduction in GSH suggests SCARA5 may affect System Xc− or directly affect GSH synthesis [68].

6. Summary and Perspectives

In this article, the mechanism of ferroptosis and the regulation of ferroptosis in ESCC are mainly introduced, and the definition, characteristic morphological changes, detection of ferroptosis, inducers and inhibitors of ferroptosis are briefly introduced. The main mechanisms are the inhibition of System Xc−, GSH and GPX4, the accumulation of LPO and the production of ROS. The System Xc−, GSH and GPX4 have been studied most extensively in ESCC. The accumulation of LPO, produced by lipid peroxidation and cleared by GPX4, is the most central step, which is determined by the detection of MDA, a metabolite of lipid peroxidation. ROS, produced by the Fenton reaction in the presence of Fe^{2+}, promotes lipid peroxidation. Erastine and RSL3 are commonly used as ferroptosis inducers. DFO and fer-1 are commonly used as inhibitors. Inducing ferroptosis can inhibit the proliferation, migration and invasion of ESCC and promote cell death. It can also improve radiotherapy resistance, thereby increasing radiotherapy efficacy. However, studies have found that BACH1 can inhibit the formation of OA and inhibit lipid peroxidation, thereby inducing ferroptosis and promoting lymph node metastasis, but inhibiting subcutaneous growth and blood metastasis, which may be related to the increase of metastasis through the avoidance of ferroptosis by abundant OA and low oxidative stress in lymph [61]. In conclusion, inducing ferroptosis is very promising in ESCC treatment, and the design

of clinical trials of ferroptosis-related drugs needs to be very careful under the current conditions of major ferroptosis cell trials and animal trials.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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