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Ferroptosis regulates key signalling pathways in gastrointestinal tumors- Underlying mechanisms and therapeutic strategies

Role of ferroptosis in GI cancer

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Abstract

Ferroptosis is an emerging no`vel form of non-apoptotic, regulated cell death that is heavily dependent on iron and characterized by rupture in plasma membrane. Ferroptosis is discrete from other regulated cell death modalities at biochemical, morphological and molecular levels. The ferroptotic signature include high membrane density, cytoplasmic swelling, condensed mitochondrial membrane, outer mitochondrial rupture with associated features of accumulation of reactive oxygen species and lipid peroxidation. The selenoenzyme glutathione peroxidase (GPX4), the key regulator of ferroptosis, greatly reduces the lipid overload and protects the cell membrane against oxidative damage. Ferroptosis exerts a momentous role in regulating cancer signaling pathways and serves as a therapeutic target in cancers. Dysregulated ferroptosis orchestrates gastrointestinal cancer signaling pathways leading to gastrointestinal tumors such as colonic cancer, pancreatic cancer and hepatocellular carcinoma. A crosstalk exists between ferroptosis and other cell death modalities. While apoptosis and autophagy play a detrimental role in tumor progression, depending upon the factors associated in tumor microenvironment, ferroptosis plays a decisive role in either promoting tumor growth or suppressing it. Several transcription factors such as TP53, ATF3, ATF4 are involved in influencing ferroptosis. Importantly several

molecular mediators of ferroptosis such as, p53, Nrf2/HO-1, hypoxia inducible factor 1, and sirtuins co-ordinate with ferroptosis in gastrointestinal cancers. In this review, we ardently elaborate the key molecular mechanisms of ferroptosis and the signaling pathways that connect ferroptosis in gastrointestinal tumors.

Key Words: Ferroptosis, Gastrointestinal cancers, Nrf2, Apoptosis, Autophagy.

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Core Tip: Gastrointestinal tumours contribute to majority of the cancer related deaths. Ferroptosis is a novel form of non-apoptotic cell death that plays a vital role in reducing the invasiveness and metastasis of gastrointestinal tumours. Herein, we discuss the regulatory mechanisms involved in ferroptosis, through the hallmark pathways of GPX4, iron metabolism, lipids peroxidation and redox signalling pathways that provides a novel therapeutic approach for gastrointestinal cancers.

INTRODUCTION

Cancer is the most impetus disease in human life that causes numerous disorders, disabilities and deaths all over the world, particularly the gastrointestinal (GI) cancers comprising esophageal cancer, gastric cancer, liver cancer, pancreatic cancer, and colorectal cancers[1]. A recent report states that there are around 4 million new cases each year which is far greater than breast and lung cancers combined, thus there is a need to improve the therapeutics of GI cancers[2]. Even though there is an improvement in the prognosis of GI cancers in the last decade by the development of intensive therapies like incorporating the cytotoxic drugs and targeted therapies, yet the GI cancers are the leading cause of cancer deaths in developed and developing countries[3-5].With varying risk factors, incidence, prevalence and prognosis, early diagnosis with

highly increased screening will facilitate therapies to fight against GI cancers. Biomarkers including the epigenetic markers also play an effective role in decreasing the progression of GI cancers by causing early diagnosis and decrease risk assessment by the prediction of tumor response from specific therapies in patients[6].Apart from the internal stimulations, the external exposures like dietary intake, tobacco use, alcohol consumption, obesity and pathogens are putative to increase the risk of GI tumors[7]. Various improvements in the therapeutic aspects to decrease the burden of GI cancer includes the chemoprevention of these cancers by using antioxidants has drawn much attention [8]. Murphy *et al* estimated that by 2025 the pervasiveness of obesity will be increased in men and women by 18% and 21% which could escalate the encumbrance of gastrointestinal cancers worldwide [9].The induction of cell death by means of ferroptosis has increased in the recent past. Cell death is an endemic mechanism that regulates homeostasis, and it is perceived as a requisite bodily process. As an imperative system, it exterminates the useless or unwanted cells as well as bolsters the defence system of the body. There are many forms of cell death that have been observed in the recent past particularly the Accidental Cell Death (ACD) and Regulated Cell Death (RCD)[10,11]. Accidental cell death occurs suddenly in response to high pressures, heat shock, or mechanical nature, while RCD is orchestrated by exhibiting a precise signalling cascade provided by a defined group of effector molecules. Many forms of RCD have been observed, such as apoptotic, autophagic, pyroptotic, necrotic and ferroptotic cell death[12]. Investigating regulated cell death enforces a new idea for developing cancer therapeutics. Among the various forms of RCD, ferroptosis an iron-dependent, non-apoptotic regulated cell death has become burgeoning because of its ability to manage cancer cells that developed resistance to apoptosis and drugs. Though, a link between iron and lipid peroxidation has been related in cancer research, this exotic form of RCD has a peculiar combination of morphological, biochemical and genetic characteristics distinct from necrosis, apoptosis and autophagy[13]. Dr. Brent Stockwell's lab proposed this term as ferroptosis in 2012 and its emerging role in several other disease settings were observed[14]. Ferroptotic cell death is characterised by the

occurrence of oxidative stress and membrane lipid peroxidation thereby causing the mitochondrial atrophy, increased density of the mitochondrial membrane and membrane damage[15]. Therefore, this review summarises the role of ferroptosis in regulating key cancer signalling and cell death pathways in GI tumors for better prognosis.

GASTROINTESTINAL CANCERS: INCIDENCE AND MORTALITY

GI cancers contribute significant morbidity and mortality rates worldwide[16] (Table 1), accounting for 26% of all cancer-related incidence and 35% of all cancer related fatalities, and it was noted that there is a two-fold increase in men in contrast to women. It has been reported that by 2040 there will be a manifold increase in the new cases and death from GI cancers by 58% and 73% to 7.5 and 5.6 million [17].

Table 1: Incidence and mortality rates of GI cancers worldwide

While cell death modalities play a decisive role in eradication of tumor cells and maintenance of homeostasis, the role of RCD pathways in particular interest, ferroptosis in tumor progression and metastasis of caners has been the subject of interest for the last decade.

DISTINCTIVE FEATURES OF FERROPTOSIS

The various features of ferroptosis include the morphological, biochemical and epigenetic alterations that occur in the body during this process.

MORPHOLOGICAL DISTORTION OF FERROPTOSIS

Ferroptosis is a non-apoptotic cell death because it lacks the classical features of apoptosis such as mitochondrial cytochrome-C release, caspase activation, and chromatin fragmentation[23]. In contrast, ferroptosis induces mitochondrial membrane disintegration which results in cell enlargement, plasma membrane rupture, volume reduction, increased density and disappearance of cristae observed under the electron microscope and these changes are caused potentially because of permeability loss due to the increased lipid peroxidation [24].

BIOCHEMICAL CHANGES INVOVLED IN FERROPTOSIS

The main biochemical features of ferroptosis are iron accumulation and reactive oxygen species (ROS) production and induction of lipid peroxidation. Iron, is the most abundant metal essential to all life on earth. As an iron dependent cell death, increased circulating iron leads to Fenton reaction through activated iron-containing enzymes that act as biochemical markers of ferroptosis[23]. For the execution of ferroptosis, intracellular iron and iron containing enzymes are indispensable [25,26]. Molecular regulators in association with iron homeostasis such as transferrin, lactotransferrin, transferrin receptor, nuclear receptor coactivator 4 (NCOA4) dependent degradation of ferritin and the iron regulatory proteins (IRP) and iron responsive element (IRE) regulatory network facilitates ferroptotic cell death[27]. It is well known that ROS are the major culprits in many disease settings. ROS can damage essential biomolecules such as DNA, protein, carbohydrates, and lipids thereby causing denaturation, peptide s-s bond breaking, crosslinking, enzyme inhibition and permeability changes in tissues and cells. Therefore, increased ROS promotes several perturbances leading to alter cell death pathways as well as induction of ferroptosis[28]. Lipid peroxidation is a seminal process where the generated free radicals (H_2O_2, superperoxides, peroxy radicals) target long chain fatty acids.[29]. Extensive lipid peroxidation results in affecting the permeability, fluidity and curvature of the membrane thereby stimulating cell death by forming micelles and pores in the biological membrane. A close connection between lipid peroxidation and ferroptosis exists in cancers. Importantly to overcome drug resistance in chemotherapy, ferroptopsis plays a crucial role in tumor microenvironment thereby controlling cell proliferation through redox signalling pathways [30].

EPIGENETIC ALTERATIONS

Epigenetics is a genetic mechanism which can reversely influences the gene expression by DNA methylation, histone modifications and by the effects of noncoding RNA without altering the DNA sequences [31,32]. DNA methylation is an epigenetic modification process that uses DNA methyltransferases (DNMTs) to covalently transfer the methyl group to the DNA's C-5 position in the cytosine ring [33]. It has been reported that the hypermethylation of *CDH1* gene promoter could increase the ferroptosis

susceptibility in head and neck cancer cells[34]. Recently, epigenetic regulation through H2B monoubiquitination and p53 has been determined[35].

Histone acetylation was controlled by the histone acetyltransferases (HATs), bromodomains (BRDs) and histone deacetylases (HDACs) to regulate ferroptosis. In the histone acetylation process, HATs play the writer, BRDs the reader, and HDACs the eraser role[36,37]. The tumor suppressive role of *Tp53* is well known. For the p53 induced ferroptosis, acetylation plays a crucial role by regulating SLC7A11 expression[38]. The acetylated and mutant $p53^{3KR}$suppresses the SLC7A11 expression and inhibits the cysteine uptake which alleviates ferroptosis and lipid peroxidation by decreasing glutathione (GSH) synthesis [38,39]. BRD4 (bromodomain containing4) induces expression of anti-ferroptosis genes, and it has been observed that the BRD4 inhibitor JQ1 induces ferroptosis by down regulating the expression of ferroptosis-related genes such as *GPX4 (Glutathione peroxidase 4), SLC7A11 (Solute Carrier Family 7 Member 11)* and *SLC3A2(Solute Carrier Family 3 Member 2)* in breast and lung cancer cells [40]. HDACS was initially identified as an eraser for removing acetyl groups from histones, but it was later discovered to be involved in many important biological functions[41]. Lately the HDAC inhibitors (HDACi) have recently been endorsed as potential therapeutic for various cancers. [41].

As a multifarious group of non-coding transcripts, non-coding RNAs (microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs) was first considered as non-functional junk but they participate in a broad domain of genetic regulatory pathways [42] beyond this it has been suggested that it plays a role in ferroptosis by regulating disparate signalling pathways either directly or indirectly by acting on the key regulating factors and upstream targets of ferroptosis [43]. miRNAs tend to act as a stimulant in ferroptosis by targeting ferroptosis associated factors by down regulating the expression of ATF4, which is a stress signal that tends to inhibit ferroptosis by miR-214-3pin hepatocellular carcinoma (HCC) by inducing SLC7A11 and several other miRNAs including miR-101-3p on their high expression, they are profound to enhance the activity of nuclear factor kappa B (NF-Kb) which regulates

ferroptosis *via* GPX4 and prostaglandin endoperoxide synthase 2 (PTGS2) in lung cancer and miR-324-3p also in high expression induces ferroptosis in lung adenocarcinoma by targeting GPX4[44]. Further miRNAs are also found to have inhibitory effect of ferroptosis therefore it plays a diverse role in ferroptosis. The alterations they cause to the ferroptotic associated factors make them a potential target in the ferroptotic cancer therapeutics.

FERROPTOSIS INDUCERS

Over the past few years, several small-molecules and plant-based compounds that target transporters or enzymes involved in ferroptosis are described.

In principle, glutathione synthesis and GPX4, a selenoenzyme are the major regulators of ferroptosis. GSH is an important substrate for GPX4 and therefore any depletion in GSH leads to enormous lipid peroxidation leading to ferroptosis. By inhibiting GPX4, accumulation of lipid peroxidation takes place [45]. To produce GSH, the system Xc^- regulated cysteine activity is needed. Certain compounds like sorafenib, glutamate, and erastin induces ferroptosis through inhibition of system Xc^-. RSL3 and other compounds containing electrophilic chloroacetamides, which covalently binds and restricts selenocysteine activity inside the active site of GPX4, initiate ferroptosis[46]. Other nitrile oxide electrophiles besides these include ML210, JKE-1674, JKE-1716 that attach to selenocysteine and cause ferroptosis[46,47]. By directly oxidising the lipid and indirectly impairing GPX4 action, FINO2 causes ferroptosis and FIN56 drives the destruction of GPX4 to induce ferroptosis[48]. In order to induce ferroptosis, FINO2 harnesses either a direct or indirect iron oxide to induce suppression of GPX4 activity. Organic peroxides, compounds with multiple O_2 bonds which is cleaved resulting in production of RO anion. These organic peroxides are often known to be used as models to induce ROS. A commonly held view is that the lipid peroxide analogue tert-butyl hydroperoxide stimulates lipid peroxidation-dependent ferroptosis [49].

Excessive iron accumulation is precursor of ferroptosis in plethora of cell types. In vitro, HB (haemoglobin) causes ferroptosis, and in vivo, it causes intra-cerebral haemorrhage in other disease states [50-52]. Furthermore, the rise in cellular iron levels and consequent

ferroptosis, caused by pharmacological stimulation of ferritinophagy-mediated ferritin breakdown imply a task of selective autophagy [53,54]. Apart from these, there are many other ferroptoic inducers, for example in human pancreatic cancer a cell, zalcitabine causes autophagy-dependent ferroptosis, pointing to a link between DNA sensor pathways, autophagy activation, and mitochondrial malfunction [55].

FERROPTOSIS INHIBITORS

The inhibition of ferroptosis by small molecular compounds takes place through the following mediators:

Iron chelators: The important role of iron is to promote lipid peroxidation by either activation of iron-containing lipid oxygenases or non-enzymatic iron-mediated fenton action [56-58]. Iron chelators like deferoxamine inhibits ferroptotic cell death [14].Iron chelators reduce H_2O_2-induced necrosis and not ferrostatin-1, suggesting that iron may be potentially involved in multiple cell death modalities [59,60].

Enzyme inhibitors: Acyl-CoA synthetase long chain family member 4 (ACSL4)mediates addition of CoA to long chain fatty acids, particularly arachidonic acid, appears to be a crucial precursor to further arachidonate lipoxygenase (ALOX)-dependent lipid peroxidation [57]. The ACSL4 and ALOX inhibitors are known to inhibit the ferroptotic cell death mechanism [57,61]. Apart from this, Nicotinamide adenine dinucleotide phosphate (NAPDH) oxidase (NOX) inhibitors comprised of diphenylene iodonium, GKT137831prevent erastin-induced ferroptosis [62].

Protein degradation inhibitors: GPX4 may be degraded by a variety of ferroptosis activators, which results in lipid peroxidation. However, FIN56 as well as erastin-induced GPX4 breakdown is inhibited by molecular chaperone Heat shock protein 90 (HSP90), 5-(tetradecyloxy)-2-furoic acid (TOFA) and dopamine [63,64,65].

Other inhibitors: The mitogen-activated protein kinase kinase (MEK) inhibitor U0162 is commonly used to reduce ferroptosis owing to its broad antioxidant activity[66]. Strong inhibitory effects of exogenous monounsaturated fatty acids (MUFAs) or deuterated poly unsaturated fatty acids on ferroptosis may be attributed to their displacement of

poly unsaturated fatty acids from phospholipids, which lowers the accumulation of lipid peroxidation [67].

REGULATION OF FERROPTOSIS

A deeper knowledge of how ferroptosis is regulated by metabolic pathways including iron, glutathione, and lipid metabolism has been the interest of research in proposing therapeutic drugs. In this context, several metabolic & cancer signalling pathways that connect ferroptosis has been described.

Glutathione peroxidase 4 (GPX4)

Ferroptosis is primarily brought on by the inactivation of the cellular antioxidant system, particularly the system that is dependent on the antioxidant defence Xc-GSH-GPX4 that leads to toxic lipid ROS accumulation[68]. Glutathione peroxidases (GPXs) are pivotal enzymes that enables the reduction of peroxides *via* GSH. The system Xc-family mediate transport of cysteine and glutamate, where the glutamate is exported outside the cell and cysteine is imported inside the cell which initiates the production of GSH hence inhibiting ferroptosis in cancer cells[69] (Figure 1). The GPX protein comprises of different types among which the GPX4, a selenoprotein is an elemental antioxidant mediator known for its capability of reducing large peroxides such as poly unsaturated fatty acids (PUFAs)[68]. It is majorly involved in the maintenance of lipid metabolism and defence against the accumulation of toxic lipid ROS. Since GPX4 activity is directly impacted by conditions that reduce intracellular cysteine and, subsequently, GSH levels and because GSH is the dominant antioxidant in mammalian cells, these conditions increase the risk of ferroptosis[70]. Small molecules such as erastin can block the GSH or GPX4 expression to activate ferroptosis. In many cells, the rate at which GSH is synthesised is limited by the internal reduction of cystine to cysteine [71].

Lipid metabolism

The process in which the free radicals and non-free radicals cleave the C-C double bonds in lipids such as PUFAs, phospholipids, glycolipids and cholesterol is known as lipid peroxidation.

According to lipidomic research arachidonic acid and adrenic acid are key driving factors in inducing ferroptosis [56]. Elongation of very long-chain fatty acid protein 5 (ELOVL5) and fatty acid desaturase 1 (FADS1) are associated in synthesis of fatty acids where both enzymes are up-regulated in the mesenchymal gastric cancer cells, resulting in ferroptosis sensitivity, according to studies on the disease [72]. The process by which these polyunsaturated fatty acids become coenzyme-A derivatives which are integrated into phospholipids triggers ferroptosis. Both autophagy and deubiquitylation can result in the reduction of the intracellular GPX4 [73]. Specialized sequences in GPX4 are distinctly recognised by HSP90 and facilitate the breakdown of GPX4 by chaperone mediated autophagy (CMA)[63]. On the other hand, the mTOR pathway's inhibition of CMA is relieved by its suppression, which may have caused GPX4 to degrade and cause ferroptosis [74].

Numerous investigations have revealed ACSL4 as a crucial factor in ferroptosis sensitivity[75]. ACSL4 mediates the esterification of the PUFA into phospholipids by adding CoA to the polyunsaturated bond of arachidonic acid[75] (Figure 1). Lysophosphatidylcholine acyltransferase 3 (LPCAT3), which is activated by ACSL4, contributes to ferroptotic lipid signalling by adding acyl groups to lysophospholipids, particularly to the phospholipids, phosphatidylcholine and phosphatidylethanolamine (PE)[56,75]. Apart from PUFAs, the MUFAs can promote ferroptosis resistance. By initiating mutations in the PI3K-AKT-mTOR pathway, the sterol regulatory element-binding protein 1 (SREBP1) can mediate the production of monounsaturated fatty acids, which aids cancer cells in resisting ferroptosis.[76]. Mono unsatured fatty acids can modulate the HCAR1 (hydroxycarboxylic acid receptor 1) -SREBP1-SCD1 (stearyl-coenzyme A desaturase 1) pathway, lactate in tumour microenvironment might increase the production of monounsaturated fatty acids and lower the expression of ACSL4 to avoid ferroptosis [77]. As functional lipids in ferroptosis, polyunsaturated ether phospholipids (PUFA-EPLS) have a significant regulatory effect in changing cancer cells from a ferroptosis-sensitive state to a ferroptosis-resistant state. [78]. Thus,

the lipid metabolism has a notable effect on the capability of the cancer cells to induce ferroptosis.

Figure. 1 Regulation of ferroptosis. Ferroptosis mediated by major pathways. GPX4/GSH pathway regulation by system Xc- and p53-p21 regulatory axis. Regulatory pathways of lipid metabolism such as ASCL4, LPCAT3 and p53-STAT1-ALOX15. Regulation of iron metabolism that includes p62-Keap1-Nrf2, ATG5-NCOA4 pathway and ferritin metabolism. (Citation: Capelletti MM, Manceau H, Puy H, Peoc'h K. Ferroptosis in Liver Diseases: An Overview. Int J Mol Sci. 2020 Jul 11;21(14):4908. doi: 10.3390/ijms21144908. PMID: 32664576; PMCID: PMC7404091)

Iron metabolism

To carry out ferroptosis and allow lipid peroxides to build up, iron is particularly necessary. Iron's capacity to catalyse numerous metabolic events and flip between the various ionic states of iron depends largely on how it can absorb and give electrons[79]. In the Fenton reaction, Fe^{2+} is converted to Fe^{3+} in presence of H_2O_2, and HO is produced as a result of an electron transfer to H_2O_2[80]. In contrast, the Haber-Weiss process, could convert Fe^{3+} back to Fe^{2+} by reacting with O_2, causing O_2 to lose one electron and become O_2- [81].

The transfer of iron takes place through the following ways. Transferrin synthesized by the liver is a chelator of Fe^{3+} ions. In contradiction to apo-transferrin which is sans iron transferrin, that is not recognised by TfR1 (transferrin receptor 1) and not internalised, transferrin recognises and binds to TfR1 when it reaches the cell membrane that is then internalised by clathrin-mediated endocytosis[82]. Accumulating evidences suggest the role of iron metabolism in ferroptosis[83,84]. Fe3+ is liberated from the transferrin TfR1 complex due to the acidic pH of endocytic vesicles and is converted to Fe^{2+} by the family of six-transmembrane epithelial antigen of prostate (STEAP). STEAP1 and STEAP2 are implicated to be involved in multiple human malignancies, including

ewing sarcoma, bladder, ovary, colon, breast, prostate, pancreatic, and cervical cancer [85-87]. Malignant gliomas have high levels of STEAP3, which triggers the cancer epithelial-mesenchymal transition (EMT)[88]. Under hypoxic circumstances, STEAP4 is activated, resulting in an imbalance of mitochondrial iron and increased ROS generation [89]. The intracellular labile iron pool is subsequently created by the divalent metal transporter1 (DMT1), which mediates the release of Fe^{2+} into the cytoplasm (Figure 1)[90]. Apo-transferrin is released back to the plasma membrane post recycling, while TfR1 and apo-transferrin remain linked. [91]. Fe2+ is then directly transported into cells by DMT1during the conversion of Fe^{3+} to Fe^{2+} (Figure 1)[83]. Another mechanism includes the absorption of porphyrin-bound Fe2+ that contains haemoglobin, particularly in macrophages and involvement of cell membrane receptors of iron-storing protein ferritin, like the scavenger receptor class A member 5 (SCARA5) as observed in kidney and embryonic development, absorbs iron in regulating the ferroptosis mechanism [92,93].

Role of Ferritin in Fe transport

Ferritin an iron-sequestering protein with 4500 iron atoms, plays a crucial role iron transport, cellular multiplication, angiogenesis and immune suppression [94]. By using NCOA4, ferritin can also be split up, to liberate free iron, a process known as "ferritinophagy" [95]. Ferroportin (FPN), recognised as sole iron release pump that works with ceruloplasmin to export iron, is primarily in charge of moving Fe2+ out of cells [96]. Ceruloplasmin controls HepG2 and Hep3B cell iron regulation to prevent ferroptosis, its loss causes a buildup of intracellular Fe^{2+} and lipid ROS as well as enhances ferroptotic death caused by erastin and RSL3 [97]. Prominin-2 promotes the growth of multivesicular structures that comprise ferritin and exosomes that transfer iron from cells, enabling ferroptosis resistance in breast cancer [98]. FPN is severely reduced in many cancer types, suggesting that cancer cells may contain large amounts of iron [99]. Decreased FPN levels promote proliferation and EMT in triple-negative breast cancer (TNBC) cells [100]. Hepcidin, produced by tumours or liver, promotes the breakdown of FPN and aids in the spread and development of cancer [101].

Transcription factors

Activating transcription factor 3 and Activating transcription factor 4 (ATF3 & ATF4)

The unfolded protein response (UPR), is activated in mammalian cells due to endoplasmic stress, having a two-dimensional functional role in cell survival and death [102]. Activating transcription factor 3 (ATF3) is a member of the ATF/cAMP-responsive element-binding protein family of transcription factors. It binds to the ATF/cAMP-responsive element-binding protein cis-regulatory element and coordinate gene expression. ATF3 has tumor suppressive roles and inhibits cancer malignancy in GI cancer [103]. The context-dependent role that ATF3 plays in cancer is likely due to complex protein-protein interaction networks in which ATF3 is involved. Indeed, in addition to transcriptional regulation, ATF3 has been found to interact with many critical cellular proteins and regulate their functions. One of the well characterized ATF3-binding proteins is the wild-type p53ROS signals are necessary for this aberrant production of ER stress markers, however antioxidant N-acetyl L-cysteine prevents the overexpression and consequent ferroptotic cellular death [71]. Although the ATF3-TP53 complex helps in initiating the DNA damage, TP53 is not necessary for ATF3 regulated suppression of SLC7A11 transcription [104]. Nuclear factor erythroid 2–related factor 2 (Nrf2) can, in contrary, regulate the expression of ATF3 by creating complex feedback loops for the activation of a number of transcriptional factors in coordinating the ferroptotic response [105].

The Activating transcription factor 4 (ATF4) is a two-edged sword that plays double role in ferroptosis. In numerous cancer cell, including human glioblastoma as well as pancreatic cancer cell lines, the depletion in ATF4 increases erastin induced or RSL3-induced ferroptosis, also the inhibition of ATF4 increases artesunate-induced ferroptosis in DAUDI cell line [106,107]. The classes of the genes that ATF4 targets, such as those for SLC7A11, heat shock protein 5 (HSPA5), tribbles pseudokinase 3 (TRIB3), *etc.*,may have an impact on this ATF4 dependant action on various cancer type in mediating ferroptosis. ATF4, is inhibited by the erastin induced overexpression of HSPA5, which results in the sequential breakdown of GPX4 and lipid peroxidation [108-

[112]. Thus, ATF3-dependent and/or ATF4-dependent pathway dysregulation, might influence ferroptosis in a tumour type specific way.

Nuclear factor erythroid 2–related factor 2

The binding to antioxidant response elements (ARE), Nrf2, a transcription factor from the Cap-N-Collar family, is essential in regulating antioxidant genes and also maintaining redox homeostasis [113]. Nrf2 transcriptional activity is normally attached with Kelch-like ECH associated protein 1 (Keap1) retaining it in cytoplasm. Nrf2 separates from Keap1 during environmental conditions (such as oxidative stress) and subsequently translocate into nucleus, where it stimulates the expression of ARE dependent target genes[113] . Nrf2, however, depends on the expression of the target genes that are involved in the control of cell proliferation, migration and death to play a double impact in carcinogenesis and tumour treatment [114]. Initially, researchers showed that ferroptosis resistance may be promoted by activating the Nrf2 pathway using a model of HCC. In human HCC cell lines, ferroptosis activators like erastin, sorafenib boost Nrf2 stability by inhibiting the development of the Nrf2-Keap1 complex. The autophagy receptor SQSTM1/p62, elevates Nrf2 expression by inactivating Keap1, is another regulator of this process [115]. By boosting GSH production and function, Nrf2 dependent genes like glutathione synthetase (GSS), GPX4 and SLC7A11 contribute to ferroptosis counteraction [115]. Control of NADPH synthesis, a vital electron donor required for reduction of oxidised substrates [116] which is also a ferroptosis sensitivity biomarker [117], is another way that Nrf2 intermediate metabolism is connected to the regulation of ferroptosis. Alltogether, Nrf2 is an important transcriptional factor that regulates ferroptosis.

Tp53

p53 a tumour suppressor encoded by the TP53 gene, is involved in the mediation of DNA damage, oncogenes activation and hypoxia. p53 promotes ferroptosis by transcriptional or post-transcriptional pathways in addition to its impacts on apoptosis, autophagy and cell cycle as well. p53 can both induce and inhibit ferroptosis. In order to cause ferroptotic cell death, p53 increases the production of spermidine/spermine N1-

acetyltransferase 1 (*SAT1*), that sequentially causes increased 15-LOX expression responsible for oxidation of PUFAs (Figure 1)[118]. Simultaneously, p53 decreases the expression of ELAV-like RNA-binding protein (ELAVL1) and its action with LINC00336, limiting the capability of cells to fight ferroptosis by increasing the activity of cystathionine β synthase (CBS) [119]. Additionally, p53 interaction with USP7 (Ubiquitin specific protease 7), a deubiquitinating enzyme facilitates its nuclear translocation, *via* altering histones, favourably controlling ferroptosis [120]. Human colon cancer cells, such as HCT116 and SW48, are inhibited against ferroptosis by p53 in a transcription-independent way [125]. The p53-p21 axis prevents ferroptosis, allowing cancer cells to withstand metabolic stressful situations [125]. Thus, this dual role of p53 in ferroptosis can be explored for therapeutic treatment of cancers.

Heme oxygenase-1 (HO-1)

Heme oxygenase-1 is a significant redox mediating enzyme, activated in reaction to oxidative stress, cellular stress, neurodegeneration and other diseases. HO-1 has a dual personality where usage of HO-1 antagonist zinc protoporphyrin IX and HO-1 knockdown animals demonstrated that HO-1 increases erastin-induced ferroptosis[121]. Conversely, because HO-1 expression is knocked down, erastin and sorafenib more effectively limit cell proliferation in HCC that is caused by these drugs [121]. Apart from this HO-1 mediates iron and ROS levels where Nrf2-derived HO-1 provides a cytoprotective effect by scavenging ROS when HO-1 is moderately active[121]. In contrast given that cancer cells produce more HO-1 than normal cells do, a high level of HO-1 activation may increase fragile Fe2+, resulting in an excess of ROS and eventual oxidative cell death[114]. Hence the employment of ferroptosis by HO-1 activation can define the fate of cancer cells.

Sirtuins

Sirtuins are NAD^+ dependant deacetylases that is involved in DNA repair, cellular metabolism and cancer development. Sirtuins are nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylases as it is NAD dependent, a key oxidizing agent; sirtuins tends to play a crucial role in regulating redox signalling pathways [122]. Seven

sirtuins have been identified in mammals so far, and each family found to regulate cellular homeostasis. SIRT1 activation increases the antioxidant mediated by oxidative stress related transcription factors [122]. Studies found, increased expression of sir1 induces Nrf2 mediated antioxidant activity thereby increase GPX4 and GSH levels in HCC and in converse their downregulation by protocadherin 20 mediated ferroptotic cell death by lowering the expression of GPX4 and GSH and increases the intracellular iron levels and ROS[123]. SIRT 6 promotes ferroptosis in pancreatic cells as it possess low sir6 expression so upregulated sir6 increases the ROS level[124]and their downregulation causes the gastric cancer cells resistant to sorafenib induced ferroptosis[125].

HYPOXIA INDUCIBLE FACTOR

Hypoxia is a prominent factor involved in the progression and metastasis of numerous cancers. Prolyl hydroxylase hydroxylates the HIF-a subunits HIF-1a and HIF-2a under normoxic circumstances, subsequently they are subjected to ubiquitin-mediated proteolysis and destruction. HIF target genes are activated in hypoxia as a result of HIF-1α and HIF-2α failing to hydroxylate and translocating into the nucleus[126]. Our lab previous studies found that under hypoxic conditions, the hypoxia inducible factors such as HIF-1α and HIF-2α activated and stimulates the activation of matrix metalloproteinase 2 and 9 (MMP-2 and MMP-9), thereby increasing tumorigenesis[127]. Singhal et al[128] demonstrated the mechanism by which activation of HIF-2α raises cellular iron to accelerate ferroptosis or irreversible cysteine oxidation that causes cell death.

FERROPTOSIS AS A NOVEL TARGET FOR GI CANCER RESEARCH

The leading cause for cancer deaths and incidence from GI tumors, have become the most essential health concern so improving the therapeutic aspects provides an imperative way to decrease the burden of GI cancers. Several studies use ferroptotic cell death as a key modulator to inhibit the growth of gastrointestinal cancers such as colorectal cancer, liver cancer, pancreas cancer, gastric cancer and esophageal cancer.

Figure 2. Regulation of ferroptosis in gastrointestinal tumours. The factors (Cotylenin A[CN-A], Phenethyl isothiocyanate [PEITC], Artesunate [ART], Piperlongumin [PL], Haloperidol, Saponin, Cisplatin, Baicalein, CDGSH iron sulfur domain 1 [CISD1]) involved in the mediation of signalling pathways in ferroptosis of GI cancers. (Citation: Song Y, Yang H, Lin R, Jiang K, Wang BM. The role of ferroptosis in digestive system cancer. Oncol Lett. 2019 Sep;18(3):2159-2164. doi: 10.3892/ol.2019.10568. Epub 2019 Jul 5. PMID: 31402933; PMCID: PMC6676710)

Ferroptosis in Colorectal cancers

Recent studies by Wang and group suggested that ferroptosis can have potential modulatory role in targeted killing of chemo resistant colon cancer cells also ferroptosis related genes could be harnessed as biomarkers in Colorectal cancer (CRC)therapeutics[129]. Genes involved in the CRC tumor micro environment are intricately linked to ferroptosis, some of them are involved in the lipid peroxidase (GPX/GSH) enzyme system. Iron metabolism genes are also powerful prognostic markers in CRC for example elevated expression Thio-redoxin (TXNIP) tumor suppressing protein is closely concomitant to iron accumulation in mitochondria[130].Above finding clearly state that targeting ferroptosis inducing genes could give a novel avenue in treating CRC. p53 tumor suppressing protein and hemeoxygenase have been implicated as key factors in regulating ferroptosis in CRC.

Ferroptosis in Liver cancer

HCC, is most prevalent type of liver cancer, accounting for approximately 90% cases [131]. Multiple studies have reported ferroptosis has a role in ameliorating the burden of HCC progression. Sorafenib, an anticancer agent that were used for the treatment of advanced HCC are found to have ferroptotic inducer ability by initiating the translation of rapamycin kinase signalling pathway thereby constituting the initiation of ferroptosis. Iron chelators like deferoxamine inhibits the sorafenib induced ferroptosis by reducing the oxidative stress created by sorafenib in the HCC cells [132]. The finding

of potent inhibitor of CBS which is the main source of cystathionine gamma lyase (CSE) by metabolizing homocysteine to cystathionine to increase the intracellular L-cysteine, called CH004, that selectively inhibits CBS not human cystathionine gammalyase (hCSE) in both invivo and invitro, thereby it increases the lipid ROS and decreases the viability of HepG2 cells indicating their role in ferroptosis [133]. Modulating the function of key regulators of ferroptosis also plays a significant role in the induction of ferroptotic cell death of hepatocellular carcinoma cells. Nrf2 is a key regulator of antioxidant response. It plays a negative regulator role in ferroptosis by actuating p62-Keap1-Nrf2 pathway which upregulates the expression of quinone oxidoreductase 1 (NQO1), HO-1 and ferritin heavy chain 1 (FTH1), so inhibiting the p62-Keap1-Nrf22 pathways significantly enhances the ferroptosis mediated cell death of liver cancer cells induced by erastin and sorafenib [134]. The mitochondrial membrane protein CDGSH iron sulfur domain 1 (CISD1) serves as a target to treat diabetes using glitazone but it acts as a negative regulator of ferroptosis by modulating mitochondrial iron uptake (Figure 2), therefore the pharmacological or genetic inhibition of CISD1 enhanced the mitochondrial lipid peroxidation increasing the erastin induced ferroptosis in liver cancer cells [135]. The highly reactive metabolite NAPQ1 from acetaminophen (APAP) an analgesic and antipyretic agent is found to be involved in the ferroptotic cell death of HepG2 cells by decreasing its viability through GSH depletion and GPX inhibition [136]. The expression of retinoblastoma protein (Rb) in the HCC cells determines the susceptibility of cancer cells to the sorafenib treatment and regulates ferroptosis, Louandre *et al* [137]studies shows that the decrease in the levels of Rb protein exhibit an increase in cell death when cells were treated with sorafenib compared with controls, thus determining the Rb status of the individuals with HCC treatment by sorafenib will provide the prognosis of the treatment.

The lncRNAs represent a vital class of molecules which have regulatory effect in both physiological and abnormal conditions but lncRNA GABPB1-AS1 have a role in regulating oxidative stress confirms their involvement in the ferroptosis mediated cell death of HCC cells. The expression of this lncRNA was upregulated by erastin to inhibit

the translation of GA Binding Protein Transcription Factor Subunit Beta 1(GABP1) resulting in the rapid accumulation of ROS levels in HepG2 by the decreased expression of PRDX5 peroxidase gene [138].

Ferroptosis in Pancreatic cancer

Several molecules were demonstrated to induce ferroptosis in pancreatic cancer cells in which the first-line drug gemcitabine is used to treat advanced pancreatic cancer but HSPA5 causes the resistance to gemcitabine treatment by inhibiting ferroptosis so genetically or pharmacologically inhibiting HSPA5 enhances the sensitivity of gemcitabine to PDAC cells by the induction of ferroptosis [139]. Inhibiting the cytosolic aspartate aminotransferase (GOT1) which is predominant for redox balance will repress the growth of pancreatic cancer cells by enhancing labile iron release thereby inducing its sensitivity to ferroptosis [140]. Several studies have shown that certain natural plant extracts possess potential anticancer effect by inducing ferroptosis in pancreatic cancer cells. A saponin called ruscogenin represses the cell viability and induces ferroptotic cell death of pancreatic cancer cell line by increasing the concentration of intracellular ferrous iron and ROS production; it also exerts antitumor effect on invivo experiments with less toxicity [141]. The combinatorial regimen using plant derivatives also express effective anticancer effects in pancreatic cancer cells by inducing ferroptosis; the cotylenin A (a plant growth regulator) in combination with phenethyl isothiocyanate (PEITC) an anticarcinogenic compound stimulated ferroptotic cell death in PANC-1 cells (Figure 2)[140]. The artesunate, an antimalarial agent induces ferroptosis in Kras activated PDAC cell lines driven by ROS generation and lysosomal iron dependent cell death without affecting the normal pancreatic cell lines [143]. Piperlongumine (PL) alone, as well as in combination with cotylenin A (CN-A)and sulfasalazine, generates ferroptotic death of pancreatic cancer cells [144].

Ferroptosis in Gastric cancer

Gastric cancer (GC) is one of the heterogeneous diseases among the GI cancers with over 1 million new cases worldwide where surgery is the primary treatment to prevent the progression[145].The factors involved in the PUFA biosynthesis plays an essential

role in inducing ferroptosis sensitivity to gastric cancer [72]. Apatinib an antitumor agent decreases the expression of GPX4 and results in the apatinib-mediated ferroptotic cell death in GC cells [146]. They also studied the GC cells resistant to sorafenib induced ferroptosis treatment by silencing the SIRT6, one of the sirtuin proteins plays a vital role in the regulation of metabolism, DNA repair and cancer development which is primarily located in the cell nucleus; it sensitizes the GC cells to sorafenib induced ferroptosis by Keap1/Nrf2/GPX4 signalling pathway [147]. The ingredient from the chinese medicine TanshinoneII A, isolated from the rhizome of *Saliva miltiorrhiza Bunge* exhibits an anticancer effect on GC cells by inducing ferroptosis by downregulating p53-mediated SLC7A11[148].

Ferroptosis in Esophageal cancer

Targeting ferroptosis will provide new avenues into esophageal cancer diagnostics and treatment strategies. Sulfasalazine, a ferroptotic inducer inhibit the progression of esophageal cancer and plant derived compounds like oridonin, a diterpenoid have also reported to stimulate ferroptotic cell death in esophageal cancer cells [149].

Ferroptosis and link to other RCD pathways

Ferroptosis is intricately intertwined with other forms of cell death through various iron, lipid peroxidation proteins and several transcription factors. However, molecular mechanism underlying the role of other forms of RCD remains poorly understood. For example *TP53*, a critical mediator of tumour suppressive response in involved in apoptosis, ferroptosis and anti-oxidant response machinery like Nrf2 has a significant role in ferroptosis as well as autophagy. The molecular mechanisms associated with other forms of RCD pathways and ferroptosis are discussed herein.

Apoptosis and Ferroptosis: molecular switch

Numerous evidences reported the interconnection between apoptosis and ferroptosis, apoptosis typically through p53 induces cell cycle arrest thereby preventing tumorigenesis likewise *TP53* is known to sensitize cells to ferroptosis leading to reduced tumour burden[150]. A widely known ferroptosis inducer erastin has potential to induce unfolded protein response and promote p53 expression through PUMA, CHOP and

TRAIL apoptotic markers, this TRAIL reliant apoptosis implies the augmented link between apoptosis and ferroptosis[151]. A similar novel study on metal encapsulated p53 plasmid construct by Zheng *et al*[152],was found to release iron ions instigating Fenton reaction, leading to ROS oxyradical overload thereby leading to ferroptosis dependent apoptosis in liver consequently reduced tumour burden and prevented metastasis in mice. The imbalance in ferroptotic process is implicated to severely hinder apoptosis induction, for example cancer cells subjected to ferroptosis by cysteine starvation were found to recede GSH levels but failed to induce caspase activation which is seminal in apoptosis[153].

Autophagy dependent Ferroptosis

Autophagy reliant ferroptosis is putative as ferritinophagy, under excessive Fe^{2+} milieu ferritin degradation is mediated by Atg5, an autophagy regulator protein. Ferritin is a seminal protein complex with light and heavy chain polypeptides (FTL1 & FTH2) predominantly controlling the iron metabolism. Atg5 and Atg7 knockdown is implicated to prevent erastin induced ferroptosis, facilitating tumorigenesis[154]. Similarly BECN1 or Atg6 is known to induce ferroptosis by regulating glutamine and cysteine and inhibiting xc^- system through BECN1-SLC7A11 complex in cancer cells. Also studies reported BECN1 facilitates lipid peroxidation through MDA stress modulation[155]. Observations from our laboratory demonstrate that Eupatilin exhibits anticancer effect in part through regulation of autophagy mediated ferroptosis (data not shown).

In summary, while apoptosis, ferroptosis and autophagy are all different cellular pathways, they can be linked in a sense that autophagy can play dual role by promoting apoptosis and protecting against ferroptosis. On contrary both apoptosis and ferroptosis are forms of RCD's mediated by different enzymes, signals and manifest different morphological outcomes.

THERAPEUTIC ASPECTS OF FERROPTOSIS

The listed drugs have potential therapeutic applications and reported to regulate ferroptosis (Table – 2)

Though conventional drugs are implicated to induce or modulate ferroptosis, the resistance to these drugs supersedes the benefits. Therefore, identification of compounds with neutral toxicity profile and natural origin have garnered tremendous attention in ferroptosis and iron metabolism. The section below briefly discusses about the role of natural compounds in ferroptosis

Formasnin C **(FC)** It is known to induce ferroptosis in p53-null and p53-wild type cellular phenotypes of hepatocellular carcinoma, FC treatment increases the mitochondrial morphology and membrane potential in HepG2 cells, as a hallmark of ferroptotic cells[165]. FC and cisplatin synergistic treatment is known to induce ferritinophagy and enhance therapeutic potential of cisplatin. Similarly Gallic acid **(GA)** is known to prompt lipid peroxidation and ferroptosis. On exposure to $Fe2^{+}$ chelator, GA activity is suppressed which in part signifies its role in ferroptosis. GA exhibited anti-tumor effects in CRC by deterring GPX4 and elevating MDA expression[166]. **Celastrol (CS)** is reported to induce ROS production thereby promoting ferroptosis in liver cancer cells. Structural protein activity revealed that CS directly binds to multiple orthologs of PDXs. PDX knockdown in turn elevates ROS production ensuing ferroptosis[167]. Chen *et al*, [168] reported **Curcumin (CU)** could induce ferroptosis in CRC by modulating expression of key ferroptotic markers Fer1, SLC7A11, GSH, MDA and ROS through PI3K/mTOR pathway.

The above findings are helpful in understating the mechanism of many synthetic and natural compounds in inducing iron dependent cell death in GI cancers. There are ample avenues to further elucidate mode of action and mechanistic aspects on how natural compounds could be synergistically used in an amicable manner to induce ferroptosis.

CONCLUSION

The increasing incidence and mortality imposed by gastrointestinal cancers (GI), a dangerous malignancy warrants novel therapeutic strategies. Ferroptosis a conspicuous form of non-apoptotic regulated cell death has found to play a significant role in

regulating the progression of gastrointestinal cancers. This review delineates the major regulatory mechanisms involved in the ferroptosis for better understanding to create a new opportunity for diagnosis and therapeutic intervention. The involvement of the ferroptotic associated factors and the effect of several drugs including the first discovered ferroptotic inducer namely the erastin, sorafenib, cisplatin, ART, PL, haloperidol, baicalein, bromelain and saponins have found to induce the ferroptotic cancer cell death in gastrointestinal cancers. Ferroptotic inducer's synergistically with various anti-cancer drugs in clinical trials have demonstrated effective therapeutic results in GI cancers. Thus, inducing ferroptosis may have significant potential for treating GI cancers and related malignancies. Overall, this review provides insights into the regulatory mechanisms involved in ferroptotic cell death for development of novel therapeutic strategies. The mechanism of ferroptosis with other regulated cell death such as autophagy and apoptosis to induce cancer cell death may also provide new development in the therapeutic aspects in treating GI tumors. Therefore, further investigation of ferroptosis with GI cancers will improve the prognosis and the therapeutic aspects of GI cancers.

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