Gastric Cancer Secreted miR-214-3p Inhibits the Anti-Angiogenesis Effect of Apatinib by Suppressing Ferroptosis in Vascular Endothelial Cells.

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Article

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Abstract

Different from necrosis, apoptosis, autophagy and other forms of cell death, ferroptosis is a mechanism that catalyzes lipid peroxidation of polyunsaturated fatty acids under the action of iron divalent or lipoxygenase, leading to cell death. Apatinib is currently used in the third-line standard treatment of advanced gastric cancer, targeting the anti-angiogenesis pathway. However, Apatinib-mediated ferroptosis in vascular endothelial cells has not been reported yet. Tumor-secreted exosomes can be taken up into target cells to regulate tumor development, but the mechanism related to vascular endothelial cell ferroptosis has not yet been discovered. Here, we show that exosomes secreted by gastric cancer cells carry miR-214-3p into vascular endothelial cells and directly target zinc finger protein A20 to negatively regulate ACSL4, a key enzyme of lipid peroxidation during ferroptosis, thereby inhibiting ferroptosis in vascular endothelial cells and reducing the efficiency of Apatinib. In conclusion, inhibition of miR-214-3p can increase the sensitivity of vascular endothelial cells to Apatinib, thereby promoting the antiangiogenic effect of Apatinib, suggesting a potential combination therapy for advanced gastric cancer.

Introduction

Ferroptosis is caused by the redox imbalance between the products of oxidants and antioxidants whose sensitivity is closely related with numerous biological processes, mainly including polyunsaturated fatty acid metabolism and production and degradation pathways (Lysosomal pathway and the ubiquitin-proteasome system) of essential proteins[1–3]. It's driven by the abnormal expression and activity changes of a variety of redox-active enzymes, which produce free radicals and lipid oxidation products or detoxify them[4, 5]. Several metabolic pathways, such as thiol metabolism, fatty acid metabolism, and iron metabolism, directly affect the sensitivity of cells to lipid peroxidation and ferroptosis[1]. Recently, multiple cell-intrinsic proteins have been reported to regulate the occurrence and process of ferroptosis. Acyl-CoA synthetase long-chain family member 4 (ACSL4), lysophosphatidylcholine acyltransferase 3 (LPCAT3) and arachidonic acid lipoxygenase (ALOX) are involved in PUFA metabolism[6, 7]. Among them, ACSL4 preferentially activates long-chain polyunsaturated fatty acids used for phospholipid synthesis, and it's responsible for shaping the cellular lipidome[8].

Apatinib is a selective vascular endothelial growth factor receptor-2 (VEGFR-2) tyrosine kinase inhibitor[9]. In 2016, Li et al reported the results of a phase III trial that evaluated Apatinib treatment in patients who was suffering advanced gastric cancer and made a landmark step forward in gastric cancer targeted therapy[10]. A series of combined clinical trials were also followed, such as the application of SHR-1210 (anti-PD-1 antibody) as combination therapy in patients with gastric or esophagogastric junction cancer (GC/EGJC)[11]. As third-line therapy, Apatinib significantly prolonged median overall survival (OS) and progression-free survival (PFS), but the problem of drug resistance also appeared[12, 13]. A similar drug, sorafenib, has been shown to have a functional synergistic antiangiogenic effect as a ferroptosis inducer in advanced hepatocellular carcinoma. In addition to targeting VEGFR-2[14], Apatinib can also induce
apoptosis[15, 16], autophagy[16], stem cell properties[17] and intracellular lipid peroxidation[18] in gastric cancer cells to play a therapeutic role.

Exosomes are a subtype of extracellular vehicles (EVs) with a typical size approximately from 40 to 160 nm in diameter[19]. Exosomes are secreted by multiple cell types, in which tumor cells have been found to produce significantly, playing different roles in regulating tumor growth and progression, invasion and metastasis, neovascularization and immune escape[20, 21], called Tumor-derived exosomes (TDEs). The mechanisms of TDEs affecting drug resistance in chemotherapy, targeted therapy and immunotherapy in advanced gastric cancer are occasionally reported[22–27]. Several studies have demonstrated that exosomes play an important role in inducing ferroptosis or driving ferroptosis resistance[28, 29]. For example, it is associated with immune checkpoint blockade drug resistance of tumor cells[30, 31], chemotherapeutic drug resistance[31, 32], or the application of engineered extracellular vesicles to inhibit tumor growth by reprogramming the tumor microenvironment and regulating ferroptosis[33, 34], or derived from vascular endothelial cells. The exosomes are also strongly linked to ferroptosis[35–37]. This may open up new therapeutic opportunities for modulating ferroptosis in human pathology.

In this study, we found that ferroptosis of vascular endothelial cells is significantly inhibited in advanced gastric cancer, which contributes to tumor angiogenesis and reduces the sensitivity to Apatinib. And A20 is a ubiquitin editing enzyme that regulates the degradation of ACSL4, of which mechanism can be observed and is closely related to ferroptosis of vascular endothelial cells. In addition, exosomal miR-214-3p secreted from GC cells play a leading role in regulating the expression of A20 in vascular endothelial cells. Therefore, this study identified a new pathway to regulate the ferroptosis of vascular endothelial cells mediated by GC cell exosomes and provided a new idea to enhance the sensitivity of third-line Apatinib targeted therapy for advanced gastric cancer.

Methods

Human tissues

All the tumor tissue samples and plasma samples of GC patients were obtained from Tianjin Medical University Cancer Institute and Hospital. Our study was approved by the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital. Informed consent was obtained from the patient to publish this study. All the study methodologies conformed to the standards set by the Declaration of Helsinki.

Cell culture

Human Umbilical Vein Endothelial Cell (HUVEC)were cultured in DMEM medium (Gibco, Grand Island, NY, USA). Human gastric cancer cell lines, MKN45, HGC27 and MGC803 cells were bought from cell bank of Chinese Academy of Sciences (Shanghai, China) and were cultured in 1640(Gibco). All cell lines were tested for mycoplasma contamination and were supplemented with 10% fetal bovine serum (FBS) (Gibco)and 1% penicillin/streptomycin (Solarbio)in a humidified incubator at 37 °C with 5% CO₂.
Animals

Female nude mice (BALB/c-nu, 4 weeks) purchased from GemPharmatech Co., Ltd (Jiangsu, China) were fed in a special pathogen-free animal facilities with access to eat and drink ad libitum. All the experimental process were performed as protocols approved by Tianjin Medical University Cancer Institute and Hospital.

Vascular ring formation of HUVEC cells

Melt the Matrigel in advance and add 100 μl to each well of the 24-well plate, placing at 37°C for 30min to solidify. HUVEC cells were first co-cultured with 25μmol Apatinib. Then the cells were resuspended in DMEM and seeded at a concentration of 10^5 cells per well. The formation of capillary-like structures was examined under a light microscope after 6 hours.

Nanoparticle tracking analysis (NTA)

The density and size of exosomes were tracked by the Nanosight NS300 system (NanoSight Technology, Malvern, UK). Exosomes were re-suspended in PBS at a concentration of 5 μg/mL and then further diluted 100- to 500-fold to achieve between 20 and 100 objects per frame. Samples were manually injected into the sample chamber at ambient temperature. Each sample was configured with a 488nm laser and a high-sensitivity sCMOS camera. At least 200 completed tracks were analyzed per video. All of the data were analyzed via the NTA analytical software (version 2.3; Thery and Witwer, 2018).

Induction and inhibition of ferroptosis

To induce ferroptosis, HUVEC were seeded in 6-well plates with suitable density. Erastin was used as the ferroptosis inducing compounds and Ferrostatin-1, Lipostatin-1 were used as the ferroptosis inhibitor, which were purchased from Sigma-Aldrich (St. Louis, MO, USA).

PKH26 staining assay

The PKH26 Red Fluorescent Cell Linker Kit (Sigma) was used for exosome staining. 50mg exosomes resuspended in 100 μL diluent C which was mixed with 100 μL PKH26 dye solution and incubated for 10min before stopped by adding 200 μL serum. Labeled exosomes were washed twice using PBS and co-incubated with HUVEC for 4-8 hours.

Western blotting

Protein samples were subjected to SDS-PAGE. The expression of GPX4, A20, ACSL4 were assessed by western blotting analysis and samples were normalized to β-actin. Protein extraction was blocked with 5% skimmed milk powder at room temperature for 1 h and incubated at 4 °C overnight with anti-GPX4 (1:5000, Santa Cruz), anti-A20 (1:1000, Santa Cruz), anti-ACSL4 (1:1000, Santa Cruz), anti-TSG101
(1:1000, Santa Cruz), anti-CD9 (1:2000, CST), anti-ubiquitin (1:1000, Abcam), anti-K63 specific ubiquitin (1:500, Abcam), and anti-β-actin (1:5000, Santa Cruz) antibodies respectively.

**RT-qPCR**

Total RNA was extracted with TRIzol reagent (Invitrogen) from cells, exosomes and tissues, and then utilized for reverse transcription PCR (Eppendorf AG 22331 Hamburg, Germany) to synthesize cDNA used for real-time qPCR (Bio-Rad CFX96, Hercules, CA, USA). Quantification of miR-214-3p was performed and normalized to the internal control U6 small nuclear RNA and mRNA levels were normalized to GAPDH.

The relative amount of gene normalized to control was calculated with the equation $2^{-\Delta CT}$, in which $\Delta CT = CT \text{ gene}-CT \text{ control}$.

A20 primers

Forward 5´-CTGCTGGCTGCCTGTCTCAAG-3´
Reverse 5´-GTTCTGGAACCTGGACGCTGTG-3´

ACLS4 primers

Forward 5´-AGAATACTGGACTGGGACCGAAG-3´
Reverse 5´-TGCTGGACTGGTCAGAGGTAA-3´

**Lipid ROS levels**

Following different treatments, cells were stained with 10 μM C11-BODIPY581/591 probe (Invitrogen) for 30 min and was used to detect the level of lipid ROS by flow cytometry according to the manufacturer’s protocol. Analysis of C11-BODIPY581/591 fluorescence was performed by a BD Accuri C6 flow cytometer.

**CCK-8 assay**

Cells were inoculated in 96-well plates. After treatment with the different conditions, cell viability was measured using the Cell Counting Kit-8 (CCK-8, Biosharp, China) assay. 10 μl CCK-8 reagent was added to each well and the cells were incubated further for 2-4 h at 37 °C. The optical density value was measured at 450 nm. The following formula was used to calculate the cell inhibiting rate: Cell inhibiting rate (%) = \[\frac{(Ac-Ae)}{(Ac-Ab)} \times 100\%\] (Ac = the absorbance of the control well, Ae = the absorbance of the experimental well, Ab = the absorbance of the blank well).

**ACSL4 mRNA half-life determination**

To determine ACSL4 mRNA half-life, HUVEC cells were treated with 150μM of Apatinib (or DMSO) or infected with A20 siRNA for 24h. Actinomycin D (5μg/ml) (Sigma Aldrich, a4262) was added to cells at different intervals (0, 2, 4, 8, and 24h). At the end of incubation, total mRNA was examined.
ACSL4 protein half-life determination

To determine ACSL4 protein half-life, HUVEC cells were treated with 150\(\mu\)M of Apatinib (or DMSO) for 24h or infected with A20 siRNA for 48h. Cycloheximide (CHX) (5\(\mu\)g/ml) (Sigma Aldrich, c7698) was added to cells at different intervals (0, 2, 4, 8, and 24 h). At the end of incubation, total protein was harvested followed by Western blotting analysis.

Ubiquitination assay

A20 siRNA was transfected into HUVEC cells. At 48 h after transfection, cells were treated with 20 \(\mu\)M of the proteasomal inhibitor MG132 for 6 h before cell lysis. ACSL4 protein was separated by co-immunoprecipitation method and subjected to SDS electrophoresis and western blotting analysis, detected with Ub antibody (Abcam, ab134953).

GSH Analysis

GSH was detected with the GSH detection kit. Briefly, HUVEC cells were seeded into 6-well cell culture plates with 2\(\times\)10^5 cells per well and processed differently. Cells were harvested and washed twice with PBS. Mix 0.5ml of the sample with 2ml of reagent I, centrifuge at 3500-4000 rpm for 10min. 1ml of the supernatant fluid was detected at the wavelength of 420 nm.

BODIPY-493/503 staining assay

HUVEC cells were pretreated with Apatinib (Jiangsu Hengrui, 50\(\mu\)M) and Arachidonic acid (AA, 50 \(\mu\)M) for 6/24 h. Then cells were stained with BODIPY-493/503(1 \(\mu\)g/mL) for 30 min and DAPI (1:1000 diluted by 1*PBS) for 10 min and imaged by confocal microscopy (Zeiss, Jena, Germany).

IHC staining assay

Animal tumor tissues were sectioned and stained with a 1:1000 dilution of anti-ACSL4 antibody (Abcam), 1:1000 dilution of anti-A20 antibody (Abcam), 1:500 dilution of anti-CD34 antibody (Abcam). Five regions were selected randomly for each specimen.

Statistical analyses

All experiments were repeated at least three times in parallel. P value < 0.05 was considered statistically significant by using the student’s t-test: *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

Results

Apatinib induces ferroptosis by increasing ACSL4 in vascular endothelial cells

In order to verify the effect of Apatinib on vascular endothelial cells, we first treated HUVEC with different concentrations of Apatinib to obtain a suitable treatment concentration of 150\(\mu\)M (Fig. 1A). At this
concentration, Apatinib affected vascular ring formation in vitro (Figure 1B). To explore the type of cell death caused by Apatinib, we applied various types of cell death inhibitors and found that the cell death caused by Apatinib can be reversed to a certain extent by the ferroptosis inhibitors Fer-1 and Lip-1 (Figure 1C). In order to further verify that Apatinib does indeed cause ferroptosis in HUVEC, we found through a series of experiments that Apatinib can play a similar role to Erastin, as shown in the western blot to verify that it can affect the expression of GPX4 (Fig. 1D and 1E), reduced GSH levels (Fig. 1F) and increased intracellular lipid ROS (Fig. 1G). Apatinib decreased intracellular mitochondrial membrane potential as observed by JC-1 fluorescence staining, which existed as a green fluorescent monomer under the microscope (Fig. 1H). Apatinib-treated HUVEC exhibited morphological changes typical of ferroptosis as observed by transmission electron microscopy (Fig. 1I, Supplementary Fig. 1A and 1B).

Studies have shown that Apatinib can regulate the expression of ACSL4 in colorectal cells. To verify the pathway by which Apatinib regulates ferroptosis in HUVEC, we treated vascular endothelial cells with 150 μM Apatinib for 24 h. By western blot, we found that it increased the expression of ACSL4 (Fig. 1J and 1K). We found that ACSL4 siRNA could affect the expression of GPX4 after transfection into cells (Fig. 1L and 1M). Based on the function of ACSL4 to catalyze lipid peroxidation in the ferroptosis mechanism, we verified the effect on intracellular fatty acid uptake with increasing Apatinib concentration. Through BODIPY-493/503 staining assay we verified that the accumulation of intracellular lipid droplets increased with increasing Apatinib treatment concentrations (Fig. 1N). Our results confirm that Apatinib can induce ferroptosis in vascular endothelial cells and affect fatty acid uptake by regulating ACSL4.

**Identifying A20 as upstream of ACSL4 and regulating its degradation through deubiquitination**

To identify the upstream regulators of ACSL4, we identified the gene interaction network associated with ACSL4 through the FerrDb database and predicted TNFAIP3 (A20) as a regulator of ACSL4 (Fig. 2A). Through the GEPIA database we determined the co-expression of ACSL4 and A20 in gastric adenocarcinoma (Fig. 2B). We collected sections of cancer tissue and paracancerous tissue from three patients with advanced gastric cancer with poor efficacy of Apatinib and performed immunohistochemical experiments to verify that the expression of A20 in cancer tissue was significantly lower than that in paracancerous tissue (Fig. 2C). To further verify the relationship between A20/ACSL4 and HUVEC ferroptosis, we verified through a series of functional assays that overexpression of A20/ACSL4 can reduce the accumulation of intracellular lipid ROS (Fig. 2D), increase GSH levels (Fig. 2E) and reduce intracellular Mitochondrial membrane potential (Fig. 2F and 2G). Next, we transfected A20 siRNA into cells and verified its transfection efficiency by PCR (Fig. 3A). In order to further explore the mechanism of A20 regulating ACSL4, we verified from the mRNA level and the protein level. We found that transfection of A20siRNA had little effect on ACSL4 mRNA by PCR (Fig. 3B). By adding actinomycin D to inhibit the synthesis of intracellular RNA, we found that transfection of A20 siRNA also had little effect on the half-life of ACSL4 mRNA (Fig. 3C). It was demonstrated that A20 may have no effect on ACSL4 at the transcriptional level. At the protein level, we found that transfection of A20 overexpression plasmid or A20 siRNA into HUVEC cells could affect ACSL4 protein expression (Fig. 3D and 3E). By adding cycloheximide to inhibit intracellular protein synthesis, we found that transfection of A20siRNA
could prolong the half-life of ACSL4 protein (Fig. 3G). demonstrated that A20 may affect ACSL4 at the post-translational level. Through ubiquitination assay, we found that transfection of A20 siRNA could increase the ubiquitinated ACSL4 (Fig. 3H), indicating that the presence of A20 can protect ACSL4 from being ubiquitinated and degraded.

**Construction of differentially expressed miRNA profiles in advanced gastric cancer and identification of the upstream of A20**

We found the miRNA database (GSE93415) in advanced gastric cancer in the GEO database and performed data exploration, constructed the differentially expressed miRNA profiles in gastric cancer tissue and adjacent healthy gastric mucosa, and drew heat maps, volcano plots and histograms to visualize the data. (Fig. 4A-4C, Supplementary Fig. 2A). After this, we crossed the top 10 miRNAs whose expression was up-regulated with all miRNAs predicted to be directly related to A20 in the TargetScan database (Fig. 4D). We found two miRNAs, miR-23-5a and miR-214-3p, that share two roles. The ability of miR-214-3p to directly bind to A20 was verified by RNA Hybrid and PicTar tools (Fig. 4E and 4F). The expression of miR-214-3p in cancer tissues of patients with advanced gastric cancer was significantly higher than that in adjacent tissues (Fig. 4G) and was significantly correlated with prognosis (Fig. 4H). Finally, we verified the direct binding of miR-214-3p to A20 by dual-luciferase reporter experiments (Fig. 4I).

**miR-214-3p is derived from gastric cancer cell exosomes and regulates ferroptosis in HUVEC**

We collected and validated the exosomes of three types of gastric cancer cells MGC-803, HGC-27, and MKN-45 by ultracentrifugation. We found that all of them could express the marker proteins of exosomes (Fig. 5A) and appeared as vesicles with a diameter of about 100-150 nm under electron microscope (Fig. 5B). Through NTA instrument detection, we found that the diameters of collected exosomes were mostly enriched around 146 nm (Fig. 5C). In order to further determine the enrichment location of miR-214-3p, we detected the content of miR-214-3p in exosomes and DMEM culture medium from which exosomes were removed by PCR, and proved that it mainly exists in exosomes (Figure 5D). Next, we transiently transfected miR-214-3p mimics, inhibitors and their controls into gastric cancer cells and extracted exosomes, respectively. These exosomes were co-cultured with HUVECs for 48 h, and all RNA and protein were extracted. We verified by PCR that co-culture could affect miR-214-3p expression in HUVECs (Fig. 5E-5G). We then verified that the altered miR-214-3p could further regulate the expression of A20/ACSL4 by western blotting (Fig. 5K). And we verified by PKH26 staining experiment that the stained exosomes could enter HUVEC cells after 8 hours of co-culture (Fig. 5H), confirming that it indeed changed the intracellular miR-214-3p and its miR-214-3p due to the uptake of exosomes. Expression of downstream A20/ACSL4 protein. To further validate the relationship between miR-214-3p and ferroptosis, we performed a series of experiments to confirm that up-regulated miR-214-3p could increase intracellular GSH (Fig. 5I), reduce accumulated lipid ROS (Fig. 5J), and raised the intracellular mitochondrial membrane potential (Fig. 5L, Supplementary Fig. 2B). We have verified the mutual regulation mechanism of miR-214-3p/A20/ACSL4 axis and its relationship with ferroptosis in HUVEC cells.
Co-action of miR-214-3p inhibitor and Apatinib

Next, we wondered whether miR-214-3p co-acted with Apatinib. We added 150 μM Apatinib to HUVEC cells transfected with miR-214-3p mimics and inhibitors and observed their co-action after 24 h. We found that miR-214-3p inhibitor synergized with Apatinib to increase intracellular lipid ROS accumulation (Fig. 6A), decrease intracellular GSH (Fig. 6B) and decrease intracellular mitochondrial membrane potential (Fig. 6C and 6D). The addition of miR-214-3p inhibitor aggravated mitochondrial shrinkage under electron transmission microscopy (Fig. 6E). Intracellular transfection of miR-214-3p mimics could not reverse Apatinib-induced ferroptosis in HUVEC cells. These results demonstrate that miR-214-3p inhibitor can synergize the effect of Apatinib to exacerbate ferroptosis in HUVECs in vitro, thereby sensitizing the antiangiogenic effect of Apatinib.

Validation of the anti-angiogenic effect of miR-214-3p inhibitor sensitizing Apatinib in vivo

Next, we used the MKN-45 gastric cancer cell line to perform a xenograft tumor model in the right groin of 4-week-old female nude mice. We started to give the mice in the drug group a daily gavage of Apatinib five days after tumor formation, and injected miR-214 containing miR-214 into the tumors of the Apatinib + miR-214-3p inhibitor group every four days. -3p inhibitor exosomes in PBS. Further analysis was performed after 21 days of dosing (Figure 7A). We found that the tumor size of the Apatinib + miR-214-3p inhibitor group was significantly reduced (Figure 7B and 7C), indicating that it can indeed synergize with Apatinib to inhibit tumor growth. We extracted the entire protein of the tumor and verified the ACSL4 expression of the tumor by western blotting, which was found to be consistent with in vitro (Fig. 7D and 7E). Next, we verified the expression of A20/ACSL4 in the tumor by immunohistochemistry, the expression of the ferroptosis marker PTGS2, and the obvious effect of Apatinib + miR-214-3p inhibitor on tumor angiogenesis by CD34 inhibition (Fig. 7F). We have confirmed the antitumor effect of miR-214-3p inhibitor synergistically with Apatinib from in vivo experiments.

Discussion

Gastric cancer remains one of the major contributors to cancer deaths worldwide[38]. Many treatment strategies have been developed, but most patients are asymptomatic in the early stage. Therefore, gastric cancer has a poor prognosis with a 5-year survival rate of <10%[38]. Studies performed to date have indicated its significant role in a few human diseases, including its increased efficiency in neurodegenerative diseases, ischemic reperfusion injury, atherosclerosis, and cancer[39]. On the other hand, cell proliferation requires ferroptosis, which may indicate a physiological role for the process[40]. Ferroptosis is recognized as a promising strategy in overcoming resistance to chemotherapy, targeted therapy, immunotherapy and radiation therapy in cancer.

Lipid peroxidation is a symbolic process of ferroptosis, whose main targets are polyunsaturated fatty acids (PUFAs) produced by lipid synthesis[41]. Reactive oxygen species (ROS) are required for most normal biological processes[42], but is also related to ferroptosis when it's excessive accumulated[43]. As the main facilitator of ferroptosis, synthesis of cell membrane by PUFAs and the iron-containing enzyme
lipoxygenase relies most on the function of ACSL4, which prefers long polyunsaturated fatty acids such as AA and AdA[8]. Suppression of AA or AdA esterification into PE by ACSL4 acts as a specific anti-ferroptotic rescue pathway[44]. ACSL4 esterifies CoA to free fatty acids in an ATP dependent way, activating them for oxidation or lipid biosynthesis[8]. ACSL4 is upregulated in several cancers including hepatocellular carcinoma, colorectal cancer, prostate cancer and breast cancer[45-47]. However, the expression of ACSL4 is frequently down-regulated in gastric cancer, increasing cell growth and cell migration, of which further studies are needed to explain these differences [48]. It has been reported that ACSL4 plays a tumor-suppressive role and could be a potential therapeutic target in GC[48].

A20, also named TNF-α-induced protein 3 (TNFAIP3), is a potent regulator of ubiquitin (Ub) dependent signals, which is a ubiquitin editing enzyme with both deubiquitinating enzyme (DUB) activity and E3 ubiquitin ligase activity[49-53]. Modifying targets proteins for degradation with K48-linked polyubiquitin chains and stimulates a recruitment of signaling proteins with K63-linked polyubiquitin chains[54-56]. Therefore, A20 modifies ubiquitylated protein substrates in multiple ways. ZF4, A20’s fourth zinc finger, binds K63-linked polyubiquitin chains and acts as E3 ligase[55]. We further identified that A20 was an essential regulator in ferroptosis. Overexpression of A20 protects ACSL4 protein from degradation by the ubiquitin-proteasome pathway through the function as a ubiquitin-editing enzyme, thereby promoting the occurrence and development of the fatty acid peroxidation and promoting ferroptosis of vascular endothelial cells in tumor tissues and modulates Apatinib resistance, providing an example for studying the role of epigenetic regulators of proteasome-dependent degradation in ferroptosis.

In this study, we explored the function of Apatinib in the induction of ferroptosis in vascular endothelial cells by regulating ACSL4. The miRNA expression profile of advanced gastric cancer was studied, and miR-214-3p was identified as one of the key miRNAs regulating ferroptosis in vascular endothelial cells. We quested its underlying mechanism-the miR-214-3p/A20/ACSL4 axis. Subsequently, we verified the synergistic therapeutic effect of knockdown of miR-214-3p combined with Apatinib in gastric cancer in vitro. The results presented here illustrate a new molecular mechanism to understand how Apatinib sensitizes vascular endothelial cells to ferroptosis and can reverse the drug resistance and provide a potentially feasible combination therapy for gastric cancer treatment.

References


**Figures**

(A) IC50 of apatinib was determined by CCK-8 assay. (B) Apatinib affects the vascular looping function of HUVECs. (C) Cell death by apatinib can be reversed by the ferroptosis inhibitors Lip-1 and Fer-1 by CCK-8. (D-E) Apatinib and Erastin could affect GPX4 expression in HUVEC, respectively. (F) Apatinib reduces
intracellular GSH content. (G) Apatinib and Erastin, respectively, can increase the accumulation of intracellular lipid ROS and are reversed to some extent by Fer-1. (H) Apatinib reduces intracellular mitochondrial membrane potential and is reversed to some extent by Fer-1. (I) Apatinib shrinks intracellular mitochondria under electron transmission microscopy and is reversed to some extent by Lip-1. (J-K) Apatinib increases ACSL4 expression in HUVECs. (L-M) Increased intracellular GPX4 expression after transfection with ACSL4 siRNA. (N) Treatment of HUVECs with different concentrations of Apatinib for 8 hours altered the rate of cellular fatty acid uptake. (* p 0.05 ** p 0.01 *** p 0.001 **** p 0.0001)

Figure 1

(A) ACSL4 association network predicted by FerrDb database. (B) Correlation of TNFAIP3 with ACSL4 was determined by GEPIA database. (C) Immunohistochemical staining of TNFAIP3 in cancer tissues and adjacent normal tissues. (D) The effect of A20 on intracellular lipid ROS accumulation was detected by flow cytometry. (E) Increased intracellular GSH content after transfection of A20 siRNA. (F-G) Effects of...
A20 and ACSL4 on intracellular mitochondrial membrane potential. (* p 0.05  ** p 0.01  *** p 0.001  **** p 0.0001)

Figure 3

(A) PCR verification of the efficiency after transfection of A20 siRNA. (B) PCR to verify the effect on ACSL4 mRNA after transfection of A20 siRNA. (C) PCR validation of the effect on ACSL4 mRNA half-life
within 24 hours after transfection of A20 siRNA. (D-E) Western blotting to verify the effect on ACSL4 protein after transfection of A20 siRNA. (F-G) Western blotting to verify the effect on ACSL4 protein half-life within 24 days after transfection of A20 siRNA. (H) IP assay to verify the ubiquitination effect of A20 siRNA on ACSL4. (* p < 0.05  ** p < 0.01  *** p < 0.001  **** p < 0.0001)

Figure 4

(A-C) Differentially expressed miRNA profiles in gastric cancer tissues and adjacent healthy gastric mucosa in the GEO database. (D) Venn diagram drawn by Venny2.1.0. (E) The direct binding region of miR-214-3p constructed by RNA Hybrid to A20. mfe: -44.4 kcal/mol. (F) PicTar validates the region where miR-214-3p binds directly to A20. (G) Differential expression of miR-214-3p in gastric adenocarcinoma and normal tissues. (H) Correlation of miR-214-3p with prognosis in gastric adenocarcinoma. (I) Dual-
luciferase reporter gene to verify the direct binding of miR-214-3p to A20. (* p 0.05 ** p 0.01 *** p 0.001 **** p 0.0001)

Figure 5

(A) The markers of exosomes secreted by MGC803, HGC27 and MKN45 gastric cancer cells were verified by western blotting. (B) Exosomes under electron microscope. (C) The density and size of exosomes were tracked by the Nanosight NS300 system. (D) PCR verification of miR-214-3p content in exosome-removed culture medium and exosomes. (E-G) Transfection efficiency after transient transfection of miR-214-3p mimics and inhibitors and their control groups into MGC803, HGC27 and MKN45 cells, respectively, and extraction of exosomes and co-culture with HUVECs for 24 h were verified by PCR. (H) Validation of exosomes uptake by HUVECs by PKH26 staining. (I) Gastric cancer exosomes extracted after transfection
can affect the GSH content of HUVECs. (J) Gastric cancer exosomes extracted after transfection were able to affect lipid ROS accumulation in HUVECs. (K) It was verified by western blot that the exosomes extracted from the three cells could affect the expression of GPX4, ACSL4 and A20 proteins in HUVECs. (L) The exosomes extracted after transfection can affect the mitochondrial membrane potential of HUVECs. (* p 0.05  ** p 0.01  *** p 0.001  **** p 0.0001)

Figure 6

(A) Apatinib synergizes with miR-214-3p inhibitor to increase lipid ROS accumulation within HUVECs. (B) Apatinib synergized with miR-214-3p inhibitor to reduce GSH content in HUVECs. (C-D) Apatinib synergistically reduces HUVEC mitochondrial membrane potential with miR-214-3p inhibitor. (E)
Mitochondrial shrinkage caused by apatinib was observed under electron transmission microscopy with the addition of miR-214-3p inhibitor. (* p 0.05  ** p 0.01  *** p 0.001  **** p 0.0001)

Figure 7

(A) In vivo experimental procedure. (B) Tumors of three groups of nude mice. (C) Changes in tumor volume of three groups of nude mice. (D-E) ACSL4 content in tumor-extracted proteins. (F) The contents of ACSL4, A20, PTSG2 and CD34 in tumor sections of the three groups. (* p 0.05  ** p 0.01  *** p 0.001  **** p 0.0001)
Figure 8

Gastric Cancer Secreted miR-214-3p Inhibits the Anti-Angiogenesis Effect of Apatinib by Suppressing Ferroptosis in Vascular Endothelial Cells

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