Dihydroartemisinin induces ferroptosis in pancreatic cancer cells by the regulation of survival prediction-related genes

Kai Guo1,*, Ying-Ying Cao1,*, Li-Chao Qian1, Marcus Jerome Daniels4, Ying Tian1, Yuan Li1, Li-Na Song1,*, Zhong-Qiu Wang1,*, Shuai Ren1,*

1Department of Radiology, Jiangsu Province Hospital of Chinese Medicine, Affiliated Hospital of Nanjing University of Chinese Medicine, Nanjing 210029, China.
2Department of Radiology, Shandong Provincial Hospital Affiliated to Shandong First Medical University, Jinan 250000, China.
3Department of Geratology, Nanjing Hospital of Chinese Medicine Affiliated to Nanjing University of Chinese Medicine, Nanjing 210022, China.
4Department of Radiology, NYU Langone Health, New York 10016, USA.

*These authors contributed equally to this work.

Corresponding to: Shuai Ren, Zhong-Qiu Wang and Li-Na Song. Department of Radiology, Jiangsu Province Hospital of Chinese Medicine, Affiliated Hospital of Nanjing University of Chinese Medicine, No. 155 Hanzhong Road, Qinquai District, Nanjing 210029, China. E-mail: shuaires@njucm.edu.cn; zhongqiuwang@njucm.edu.cn; songliana007@126.com.

Author contributions
Guo K and Cao YY conceived the project and wrote the manuscript. Guo K, Cao YY, Qian LC, Daniels MJ, Tian Y and Li Y participated in data analysis, discussion, and language editing. Wang ZQ, Ren S, and Song LN were responsible for data verification, supervision and reviewed the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare no conflicts of interest.

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Abbreviations
ROS, release of reactive oxygen species; PC, pancreatic cancer; DEGs, differentially expressed genes; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; PPI, protein-protein interaction; GEPIA, Gene Expression Profiling Interactive Analysis; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DHA, dihydroartemisinin; GSH, glutathione; MDA, Malondialdehyde; PBS, phosphate buffered saline.

Citation

Abstract
Background: Ferroptosis is a new therapeutic modality that holds promise for pancreatic cancer treatment. Dihydroartemisinin is the first generation of artemisinin derivatives with antimalarial activity, and it exerts anticancer activity through iron-dependent reactive oxygen species generation. This study assessed the potential value of dihydroartemisinin to induce ferroptosis in pancreatic cancer. Methods: The mRNA expression profiles, along with the corresponding clinical information of individuals diagnosed with pancreatic cancer, were acquired from publicly accessible repositories. We analyzed the association of ferroptosis-related gene expression with pancreatic cancer overall survival via The Cancer Genome Atlas. Utilizing molecular docking techniques, we evaluated the potential binding configurations of dihydroartemisinin with genes associated with ferroptosis. Moreover, in-vitro experiments were performed to verify these predicted outcomes. Results: In the The Cancer Genome Atlas cohort, there were significant differences in the expression levels of ten genes associated with ferroptosis when comparing pancreatic cancer tissues with normal tissues. Among them, a strong association between NQO1 expression and unfavorable prognosis was observed. Dihydroartemisinin can regulate target gene expression by interacting with the corresponding binding site, and a ferroptosis inhibitor could reverse the above events. Conclusion: The NQO1 gene, which is associated with ferroptosis, emerges as a robust and autonomous prognostic indicator for individuals with pancreatic cancer. Dihydroartemisinin may contribute to pancreatic cancer progression via the regulation of ferroptosis.

Keywords: dihydroartemisinin; ferroptosis; pancreatic cancer; bioinformation; ROS
**Highlights**

Ferroptosis as a new therapeutic modality holds promise for pancreatic cancer treatment. In this study, we screened differential expression mRNA associated with pancreatic cancer from online database and made intersection between the differential expression genes and ferroptosis-related genes. And a series of experiments was conducted to investigate the potential regulatory mechanism of ferroptosis in pancreatic cancer.

**Medical history of objective**

As early as the year 340 C.E., ancient Chinese herbal texts, specifically the "Zhou hou bei ji fang", documented the methods of using Qing Hao (Artemisia annua) for the treatment of intermittent fevers. Dihydroartemisinin, derived from the traditional Chinese medicine Artemisia annua, has emerged as a critical drug for the treatment of malaria. In recent years, research has indicated its potential as an anticancer agent.

**Background**

In recent years, ferroptosis has emerged as one of the most important aspects of programmed cell death in cancer [1]. Ferroptosis causes accumulation of ferric iron and lipid peroxidation in cells [2]. At the morphological, biochemical, and genetic levels, ferroptosis exhibits distinct characteristics compared to other forms of cell death, such as apoptosis, necroptosis, senescence, and pyroptosis. Considering morphology, cells undergoing ferroptosis display decreased mitochondrial volume, augmented mitochondrial membrane density, and a reduction or absence of mitochondrial cristae [2]. Furthermore, there is intracellular glutathione (GSH) depletion, resulting in the accumulation of lipid peroxides and the release of reactive oxygen species (ROS) [3]. Ferroptosis occurs in most pancreatic adenocarcinomas, renal cell carcinomas, hepatocellular carcinomas, and large B-cell lymphomas [4–7]. Cancer cells exhibit significantly higher iron demands in comparison to their normal cell counterparts [8]. The increased iron uptake of cancer cells leaves them susceptible to the negative effects of iron metabolism. Thus, great interest has been focused on ferroptosis therapy in the treatment of drug-resistant human cancers [9].

Pancreatic cancer (PC) stands out as one of the most aggressive and fatal malignancies. Unfortunately, the incidence and mortality of PC has significantly increased in the past decade, and the five-year survival for PC is merely 9% [10]. PC has an insidious onset in the early stages due to a lack of specific symptoms or biomarkers, and when symptoms arise, patients have usually missed the operation window [11]. Furthermore, PC is resistant to conventional radiotherapy and chemotherapy, including the first-line drug gemcitabine. In PC, the mutation rate of the KRAS gene can be as high as 90%. Research has shown that PC cells harboring KRAS mutations exhibit heightened susceptibility to ferroptosis, underscoring the responsiveness of PC to ferroptosis induction [12].

Dihydroartemisinin (DHA) is a derivative and active metabolite of artemisinin with greater stability and better water solubility [13]. It has a wide spectrum of therapeutic properties including anti-tumor, antimarial, and anti-inflammatory activity [14–16]. The anti-tumor mechanism of DHA-based drugs is mainly related to the active "peroxide bridge" group in their structure, which induces ferroptosis in cancer cells by disrupting the redox balance within the tumor cells [17, 18]. Although DHA has been reported to exert an anti-tumor effect on a wide variety of tumors via ferroptosis, it is unknown whether DHA induces ferroptosis in PC. In addition, it is not clear whether the prognosis of PC is associated with ferroptosis-related genes.

In this study, we screened PC mRNA and its differential expression from online databases and cross referenced between the differently expressed genes (DEGs) and ferroptosis-related genes. And we evaluated the prognostic significance of the overlapping genes between PC and normal cohorts, substantiating our findings in the Gene Expression Omnibus (GEO) cohort. A series of experiments were performed accordingly to explore the plausible regulatory mechanism of ferroptosis in PC.

**Methods**

**Data collection and collation**

We obtained the RNA-sequencing (RNA-Seq-HTSeq) data of The Cancer Genome Atlas (TCGA) pancreatic adenocarcinoma cohort from the TCGA website (https://tcga-data.nci.nih.gov/tcga/). The raw microarray data for PC (GSE82735) was downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). The cohort was normalized via the online platform "OMICSHERE" (https://www.omicshare.com/). Since all data was collected from publicly available data in the TCGA and GEO databases, ethics committee approval was not required. Our study strictly followed the publication guidelines and data access policies of TCGA and GEO.

The FerrDb database comprised the most comprehensive collection of ferroptosis-related genes accessible at http://www.zhouman.org/ferrdb/current/. A total of 259 ferroptosis-associated genes were provided in Supplementary Table S1.

**Identification of DEGs**

EdgeR(http://www.bioconductor.org/packages/2.12/bioc/html/edgeR.html) is used to analyze the count data from differential expression analysis. P-values were adjusted for false discovery rate, and a significance level was set to 0.05. |log2 fold change (FC)| > 1.0 was also enrolled in this study. The DEGs were filtered using false discovery rate P-values < 0.05 and |log2 FC| > 1.0. We identified the overlapping genes between the DEGs and ferroptosis-related gene lists obtained from the two methods using Hiplot (https://hiplot.com.cn/basic).

**Construction of protein-protein interaction (PPI) network**

The construction of the PPI network and the identification of hub genes were carried out by analyzing the PPI information using STRING DB (http://string-db.org/). In addition, STRING DB scored each interaction between differentially expressed proteins. And the PPI network was established through utilization of an online tool.

**Gene Ontology (GO) and KEGG enrichment analysis**

DAVID (https://david.ncifcrf.gov/) is an online platform dedicated to biological information, offering functional annotation and enrichment analysis for DEGs. The DAVID database was utilized to perform GO terms and KEGG pathway enrichment analysis for all DEGs related to ferroptosis. Additionally, results from GO and KEGG pathway enrichment analysis were visualized by an online platform (http://www.bioinformatics.com.cn).

**Overall survival analysis based on DEGs levels**

Our research involved the assessment of DEG expression levels in both tumor and normal tissues using the Gene Expression Profiling Interactive Analysis (GEPIA) tool, accessible at http://geopia.cancer-pku.cn. To validate these key genes on the external validation dataset, the GEO database was searched. Survival analyses were performed with TCGA data.

**Molecular docking**

The chemical structure of DHA was obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) in .sdf format. And the protein structures were obtained from the RSCB PDB (https://www.rcsb.org/). Subsequently, we utilized AutoDockTools to eliminate water molecules and incorporate polar hydrogen atoms. The binding affinity and interaction of DHA towards key genes were analyzed using PyMOL and Biovia Discovery Studio. The conformation of the inclusion complex was determined based on its...
Reagents and materials
Dihydroartemisinin (#D7439) was purchased from Sigma-Aldrich (Hangzhou, China). NCF2 (#67240-1-1q) and NQO1 (#67594-1-1q) antibodies were purchased from Proteintech group, Inc (Wuhan, China). Ferrostatin-1 (#HY-100579) was purchased from MedChemExpress (Guangzhou, China). The FerroOrange detection kit (#F374) was purchased from Dojindo (Shanghai, China). GSH detection kit, reactive oxygen species detection kit, malondialdehyde (MDA) content detection kit, CCK-8 kit (#C0037) were purchased from Beyotime (Shanghai, China).

Cell culture
The human PC cell line PANC-1 (#GDC0309) and MiaPaCa-2 (#GDC0255) were purchased from the China Center for Type Culture Collection, CCTCC (Wuhan, China). The cells underwent mycoplasma testing and were confirmed to be free from mycoplasma contamination. Both cell lines were cultured in DMEM medium (GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Auckland, New Zealand) and 1% penicillin/streptomycin. The cells were maintained in a 5% CO₂ environment at 37 °C.

Kit detection
Cell proliferation was assessed using the CCK-8 Kit. Intracellular iron content was determined by FerroOrange Kit. GSH Kit was used to measure intracellular content of reducing GSH. Reactive oxygen species assay kit detected cells total ROS. MDA degree was detected using a Lipid Peroxidation (MDA) Assay Kit. All kits were performed following the manufacturer instructions.

Clonogenic assay
Upon reaching their logarithmic growth phase, PANC-1 and MiaPaCa-2 cell lines underwent 0.25% pancreatic en digestion, followed by uniform seeding at a density of 600 cells per well in a 6-well plate. The cells were then placed in a 5% CO₂ incubator at 37 °C. After the cells were completely adhered to the wall, we treated them with DHA for 12 hours under submerged conditions and cultured for 2 weeks. Concurrently, a ferroptosis inhibitor (ferostatin-1, 1 μM) served as the control group. After washing the cloned cells with phosphate buffered saline (PBS), they were fixed using 4% paraformaldehyde/PBS solution. Next, the cells were stained with crystal violet dye at room temperature for 1 hour. The 6-well plates were then inverted and air-dried before visual observation and photography. The cloning rates were calculated as (number of clones/number of inoculated cells) × 100%.

Western blot analyses
The PANC-1 cells from each experimental group were collected and washed with pre-cooling PBS two times. Thereafter, cells were lysed and the protein concentration was assessed using the BCA Kit. Protein samples were buffer-exchanged to the desired buffer and were separated on SDS-PAGE. After electrophoresis, proteins were transferred to a nitrocellulose transfer membrane. TBST buffer was used to prepare 5% skimmed milk powder as the sealing solution overnight at 4 °C. TBST buffer was used to wash the membrane three times, and the second antibody was added and incubated at room temperature for 1 hour. Then, membranes were washed in TBST after the primary-antibody incubation. Finally, enhanced chemiluminescent reagent was added to develop and expose photos.

Statistical analysis
Gene expression data between tumor tissues and adjacent noncancerous tissues were analyzed by Student t-test. Statistical significance and graphing were performed using GraphPad Prism 9.0. A P-value of < 0.05 indicates statistically significant.

Results
Screening of ferroptosis-related DEGs by combined analysis
A total of 657 DEGs (621 down-regulated and 36 up-regulated; displayed in Supplementary Table S2) between tumor and normal samples were identified for further analysis in TCGA. The gene-rank plot showed significance in differential gene expression vs. fold change (Figures 1A, 1B). 10 genes were associated with ferroptosis (Figure 1C). Figure 1D illustrates a heatmap of 10 differentially
expressed genes in PC, obtained from the TCGA dataset, which comprised 178 cancerous tissues and 4 para-cancerous tissues. The down-regulated genes were HBA1, ATM, NOX5, NCF2, HMOX1, PLIN2, CYBB, and TNFAIP3, whereas the up-regulated genes were NQO1 and SLC7A11 (Supplementary Table S3).

**PPI network construction and functional enrichment analysis of DEGs**

We used STRING v11.0 to build the protein-protein interaction networks using the expression profiles of DEGs in PC. The interaction network of DEGs suggests that NQO1, HMOX1, CYBB, NCF2, and NOX5 could be considered as hub genes (Figure 2A). Furthermore, KEGG and GO pathway enrichment analyses were performed. KEGG pathway analysis revealed several enriched pathways: ferroptosis, fluid shear stress and atherosclerosis, leishmaniasis, NF-kappa B signaling pathway, HIF-1 signaling pathway, leukocyte trans endothelial migration, phagosome, necroptosis, NOD-like receptor signaling pathway (Figure 2B). GO analysis included pathways for biological processes, cellular components, and molecular functions. Full results of GO enrichment analysis are provided in Supplementary Table S4. As presented in Figure 2C, the top ten terms biological processes for GO analysis were cellular response to chemical stress, response to oxidative stress, cellular response to oxidative stress, superoxide metabolic process, ROS metabolic process, response to hydrogen peroxide, superoxide anion generation, cell redox homeostasis, response to ROS, and regulation of neuron death. The top ten terms cellular components for GO analysis were NAD(P)H oxidase complex, endocytic vesicle, rough endoplasmic reticulum, oxidoreductase complex, phagocytic vesicle, haptoglobin-hemoglobin complex, hemoglobin complex, secondary lysosome, astrocyte projection, and endocytic vesicle lumen. The top ten terms molecular functions for GO analysis were superoxide-generating NAD(P)H oxidase activity, oxidoreductase activity-acting on NAD(P)H-oxygen as acceptor, oxidoreductase activity-acting on NAD(P)H, heme binding, tetrapyrrole binding, flavin adenine dinucleotide binding antioxidant activity, electron transfer activity, superoxide-generating NAD(P)H oxidase activator activity, and 1-phosphatidylinositol-3-kinase activity.

**Identification and validation of survival related DEGs**

To further demonstrate that DEGs were observed in our data, 10 ferroptosis-related genes between normal and pancreatic cancer tissue were accessed through the GEPIA portal. Since the GEPIA database has a larger number of normal control groups compared with the TCGA database, the data bias of TCGA can be corrected. In this case, 4 genes among these 10 differentially expressed genes were shown to be differentially expressed. Figure 3 demonstrates the results of correlation analysis. As shown in Figure 4, high NQO1 expression were significantly correlated with poor prognosis (P < 0.05). By contrast, high expression of NCF2 and CYBB did not correlate with outcome in PC patients. To further identify the gene-gene interactions involved in PC pathogenesis, we constructed gene co-expression matrices based on clustering of Pearson correlation of gene expression (Figure 5A). Among them, NCF2 and CYBB (correlation: 0.83), TNFAIP3 and CYBB (correlation: 0.71), HMOX1 and NCF2 (correlation: 0.72), HMOX1 and PLIN2 (correlation: 0.73), HMOX1 and CYBB (correlation: 0.71) had highly positive correlation in the co-expression network. Beyond this, NOX5 and NQO1 (correlation: −0.31), NOX5 and SLC7A11 (correlation: −0.18), NOX5 and NCF2 (correlation: −0.15), NQO1 and ATM (correlation: −0.09), NOX5 and CYBB (correlation: −0.09) had a negatively correlation. Furthermore, the TCGA dataset was used to explore the correlation between genes expression and overall survival time in PC. To go a step further, the GEO database [19] (GSE28735) was analyzed to verify whether the 4 genes are differentially expressed and to explore the correlation between them (Figure 5B, 5C). Results showed that the expressions of

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**Figure 2** PPI networks and GO and KEGG pathway analyses for all differentially expressed genes related to ferroptosis. (A) Constructed PPI network of DEGs was analyzed using the STRING database. (B) KEGG pathway analysis. (C) GO analysis was annotated from three levels: biological process, cellular component and molecular function. PPI, protein-protein interaction; DEGs, differentially expressed genes; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.
Figure 3 Expression analysis of DEGs in PC patients based on GEPIA. The red and gray boxes represent pancreatic tumor and normal tissues, respectively. And dots present each sample value. \( P < 0.05 \). DEGs, differently expressed genes; PC, pancreatic cancer; GEPIA, Gene Expression Profiling Interactive Analysis.

Figure 4 Survival analysis of PC patients based on TCGA data. Red line designates the samples with highly expressed genes, and blue lines represent low expression of the hub genes. PC, pancreatic cancer; TCGA, The Cancer Genome Atlas.
NCF2, NQO1 and CYBB were significantly different in pancreatic normal tissues and pancreatic cancer tissues, while HMOX1 was not significantly differentially expressed in the two groups. And the 3 DEGs thus obtained determine the key genes in the tumor genome. As indicated by the result, there were positive correlations between NCF2 and CYBB (correlation: 0.83), NCF2 and NQO1 (correlation: 0.28), CYBB and NQO1 (correlation: 0.10).

DHA has potential for regulating ferroptosis
Virtual molecular docking results implied that the active molecule could interact with the NCF2 and NQO1 (Figures 6A, 6B; outcomes of molecular docking analysis are shown in Table 1), while CYBB cannot efficiently dock with DHA (Figure 6C). The experimental findings suggest that DHA may play a significant role in modulating the ferroptosis process in PC.

DHA can inhibit the growth of pancreatic cancer cells
To assess the in vitro cell proliferative activity of DHA, we used PANC-1 and MiaPaCa-2 cell lines for verification. Cell viability was assessed in a dose-dependent manner by using different concentrations of DHA (100, 200, 300, 400 μM) (Figure 7A). Since 200 μM DHA had an optimal effect in the proliferation of cancer cells, we decided to perform subsequent experiments with this effective concentration. Taken a step further, the effect of DHA was also observed in a clonogenic assay. The control blank control group exhibited a higher rate of colony growth in comparison to the DHA group, as observed in the in PANC-1 cells (241.8 ± 4.3% vs. 143.3 ± 12.3%), and colony growth of DHA combined with ferrostatin-1 group was 200.0 ± 10.5%. The same result was also observed in MiaPaCa-2 cells (Figure 7E). The above findings showed that DHA exerted inhibitory effects on PC cells, and ferroptosis inhibitors attenuated these effects.

The intervention of DHA causes an increase of ROS, MDA, Fe²⁺, and the consumption of GSH
During the ferroptosis, lipid peroxidation products (ROS, MDA) production, intracellular iron overload, and glutathione content depletion happen [20]. Therefore, we sought to validate the pharmacological action of DHA by using the above-mentioned evaluation metrics. These results revealed that ROS, MDA, and Fe²⁺ levels were significantly increased compared to controls after DHA treatment (Figures 7C, 7D, and 7F). In addition, there was a marked drop in GSH content (Figure 7B). Consistent with this prediction, ferrostatin-1 administered in doses of 1 μM with DHA reversed these changes in different degrees. The findings from this study demonstrate that DHA is capable of inducing ferroptosis in both PANC-1 and MiaPaCa-2 cells.

DHA regulates the expression of key ferroptosis proteins
Since molecular docking analysis has provided the potential binding relationships between DHA and ferroptosis-related genes (NCF2 and NQO1), we explored the alterations of related-protein expression levels by western blot. Following a 12-hour exposure to DHA, the changes in gene expression were consistent with the outcomes of the western blot protein analysis. The expression of NCF2 and NQO1 protein in the DHA group were lower than that of the control group (Figure 8; P < 0.05). Consistent with the hypothesis, this phenomenon was reversed following transfection with ferroptosis inhibitors to some extent (Figure 8; P < 0.01). From the results of the experiments described above, DHA affected the ferroptosis process of PC by modulating the expression of NQO1 and NCF2.

Discussion
At the onset of our study, a total of 3 DEGs were identified to be key genes associated with ferroptosis in PC. Among these genes, the CYBB (Cytochrome B-245 Beta Chain) gene encodes gp91phox protein (also known as NOX2) which is the key subunit of NADPH oxidases. Moreover, an important role for CYBB has been suggested in intracellular ROS generating and the occurrence of ferroptosis...
NCF2 is a component of the NAD(P)H oxidase complex, and over-expression of NCF2 promotes the NAD(P)H oxidase activation [24]. Usually, cancer cells need to maintain redox homeostasis by high levels of NAD(P)H and antioxidant enzymes. Previous research has established that high expression of NCF2 protected PC cells by activating NOX2 [25]. DHA may reduce the activation of NAD(P)H oxidase through downregulating NCF2 expression. Our study revealed that redox homeostasis of the PC cells was broken by DHA and triggered the ferroptosis. NQO1 turns quinone into hydroquinone with low toxicity mainly through a two-electron reduction reaction to protect cells from oxidative damage [26]. Currently, exploiting the mediator that NQO1 redox cycling agent is important for targeted treatments in PC [27, 28]. DHA hampered the progression of PC by downregulating the expression of NQO1. These experimental findings suggested that DHA can suppress cellular antioxidant system and disrupt redox balance. Previous studies have shown the membrane-spanning subunit gp91-phox (encoded by CYBB) and cytoplasmic subunit p67-phox (encoded by NCF2) were catalyzed by NAD(P)H oxidase [29]. GO term enrichment analysis lends further credence to the above argument.

DHA is one of many derivatives of artemisinin. It retains the active groups such as the endoperoxide bridge of artemisinin. Compared with artemisinin, DHA has the advantages of safety, low toxicity, and effectiveness against chloroquine resistant malaria [30]. In cancer treatment, the regulatory mechanism of DHA is achieved through inhibiting the expression of redox proteins in ferrous iron oxidation and interfering with iron homeostasis [18, 31]. Release of free iron could catalyze production of ROS in the Fenton's reaction. The development of ferroptosis can be linked to the substantial buildup of intracellular ROS and perturbations in iron homeostasis.

PC is projected to rank as the second most prevalent cause of cancer-related mortality in the United States by 2030 [32]. Traditional chemotherapeutics have failed to substantially improve the survival rate of patients due to the chemoresistance of PC. Tumor cells usually show an increased need for iron and elevated levels of ROS compared to normal healthy cells, so tumor cells may enhance their survival by improving their antioxidative capability [33, 34]. Additionally, improvement in antioxidant capacity exhibits an increased drug resistance [35]. Perturbing the antioxidant reduction mechanism of tumor cells could effectively solve the problem of drug resistance. Currently, many natural anticancer drugs induce ferroptosis by regulating oxidative stress of tumor cells. DHA is one of these natural agent derivatives.

From our literature review and bioinformatics prediction, we tried to uncover possible ferroptosis mechanisms of DHA on PC. Our bioassay result was consistent with previous reports that DHA evoked PANC-1 and MiaPaCa-2 cells increased in Fe^{2+}, ROS, and MDA, while causing a marked reduction in GSH. Ferrostatin-1, a specific ferroptosis inhibitor, induced the opposite effect. The experimental results supported the biochemical features of ferroptosis. However, the ferroptosis mechanisms for PC has not been figured out exactly. In Figure 9, we have plotted the possible mechanistic diagram of ferroptosis with our own experimental data. Ferroptosis is characterized by intracellular glutathione depletion, resulting in the accumulation of massive ROS in cells. The increased ROS and excessive iron ions elicit activity results in DNA damage [36]. Thus, the most important feature of the ferroptosis process is the disruption of lipid redox homeostasis. NAD(P)H is important for redox regulated

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DHA, dihydroartemisinin. PDB, Protein Data Bank.

Figure 6 Molecular docking studies between DHA and candidate genes. (A) NCF2 (1hh8)-DHA. (B) NQO1 (1d4a)-DHA. (C) DHA. DHA, dihydroartemisinin.
Figure 7 Mechanisms of action of DHA in vitro. (A) PANC-1 and MiaPaCa-2 cells proliferation was measured by CCK-8. (B) GSH content level change after DHA treatment. (C) ROS content level change after DHA treatment. (D) MDA content level change after DHA treatment. (E) Clone formation assays were used to detect the effect of the DHA knockdown on the clone formation ability. (F) Fluorescence confocal microscopy showed the changes of intracellular Fe²⁺ content induced by DHA. Ferrostatin-1 as a ferroptosis inhibitor has been reversed these changes in different degrees. *P < 0.05, **P < 0.01, ***P < 0.001. ROS, release of reactive oxygen species; GSH, glutathione; MDA, Malondialdehyde.
Figure 8 DHA influenced the expression of ferroptosis-related genes. GADPH protein was used as internal controls. The expression of NCF2 and NQO1 protein in the DHA group were decreased. Ferrostatin-1 treat group with opposite expression trend. *P < 0.05, **P < 0.01. DHA, dihydroartemisinin.

Figure 9 Schematic illustration of DHA regulating ferroptosis. Upon DHA intervention, the p67phox protein encoded by NCF2 decreased the oxidative capacity of the NAD(P)H respiratory chain. And DHA suppressed the expression of NCF2 and NQO1, further disturbing oxidative process in PANC-1 cells, leading to a large accumulation of ROS and a decrease in GSH content. At the same time, DHA promotes cellular uptake of Fe²⁺. All paths ultimately lead to the activation of ferroptosis in pancreatic cancer cells. ROS, release of reactive oxygen species; DHA, dihydroartemisinin; GSH, glutathione.

...cell metabolism and keeping balance of intracellular ROS. The proteins encoded by CYBB and NCF2 are the part of a catalytic subunit of NAD(P)H oxidase. Increased expression of CYBB and NCF2 potentiates NAD(P)H oxidase activity, increasing the redox process in tumor cells significantly [37]. NQO1 acts as an antioxidant and alleviates pancreatic cancer oxidative stress by decreasing the NADPH/NADP⁺ ratio [38]. Our results revealed a decline in the protein expression of NCF2 and NQO1 in DHA-treated PANC-1 cells, signifying that the induction of ferroptosis by DHA was associated with alterations in the cellular redox environment.
Conclusion

In conclusion, the ferroptosis-related genes, including CYBB, NCF2 and NQO1, are related to PC progress. NCF2 and NQO1 expression level decreased following exposure to DHA. We found that DHA induced high intracellular Fe²⁺ concentration, high ROS level, high MDA level, low level of GSH in PANC-1 and MiaPaCa-2 cells. This indicated that DHA was associated with iron metabolism and lipid oxidation process and ultimately leads to ferroptosis. As a type of natural medicine derivative, DHA could induce ferroptosis in PC cells and our results show promise for the application of DHA in cancer therapy.

References


