Fenofibrate enhances radiotherapy efficacy by elevating HDL levels to induce tumor cell ferroptosis in NSCLC with brain metastasis

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Abstract

The brain is a common site of lung cancer metastasis with an extremely poor prognosis. Improving the efficacy of available therapeutics is very important. By analyzing clinical data, we found that HDL and total cholesterol levels in blood are potentially correlated with the efficacy of brain radiotherapy. And we found that fenofibrate can promote the synthesis of HDL by targeting PPARα and lipidomics results demonstrated that fenofibrate could also alter the intracellular lipid composition of tumor cells and increase the ratio of PUFAs/MUFAs, thus increasing the oxidative substrate for ferroptosis. Thereby promotes radiation-induced ferroptosis in tumor cells. Flow cytometry, immunofluorescence and elisa show that ferroptotic tumor cells further increase the infiltration of CD8+ T cells in the tumor microenvironment and the secretion of IFN-γ. In vivo experiment also verify that combination of fenofibrate and radiotherapy can enhancing the efficacy of immunotherapy. Thus, the combination of fenofibrate and radiotherapy could provide a new therapeutic strategy for non-small cell lung cancer patients with brain metastases.

1. Introduction

Lung cancer is a common malignancy worldwide; it ranks first in mortality and second only to breast cancer in incidence rate(1). According to pathological classification, lung cancer can be divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), of which NSCLC accounts for approximately 85% of cases. The brain is the most common site of lung cancer metastasis with an incidence of 50% in NSCLC(2). Despite the development of therapies for lung cancer in recent years, the brain possesses unique cell types, anatomical structures, metabolic limitations, and immune microenvironments(3), which makes it difficult for patients to achieve satisfactory treatment outcomes. Local treatment options for brain metastases (BMs) include whole-brain radiation therapy (WBRT), stereotactic body radiation therapy (SBRT), surgery, and stereotactic radiosurgery (SRS)(4). Among them, radiotherapy (RT) is the first-line local treatment for BMs(5) and can effectively improve neurological symptoms and prolong overall survival. However, the efficacy of RT remains limited; thus, it is particularly important to identify new means of radiotherapy sensitization.

RT utilizes ionizing radiation (IR) to induce cell death directly by damaging double-stranded DNA(6). In addition, radiotherapy can also drive antitumor immune responses through a range of mechanisms(7). Recent studies have reported that ionizing radiation can induce ferroptosis in some tumor cells(8, 9). Ferroptosis is an iron-dependent programmed cell death that differs from apoptosis, necrosis, and autophagy. The main mechanism of ferroptosis is that iron or ester oxygenase catalyzes the high expression of unsaturated fatty acids on the cell membrane, which cause lipid peroxidation and subsequently induce cell death(10). Lipids play a very important role in ferroptosis. Therefore, we considered whether a radiosensitization effect could be obtained by enhancing tumor cell ferroptosis.

This study was conducted to investigate whether regulation of lipid content in combination with radiotherapy can additional induce ferroptosis in tumor cells, thus enhancing the sensitivity of cells to
radiotherapy and its subsequent impact on systemic comprehensive therapy to provide new ideas for the treatment of patients with brain metastases of lung cancer.

2. Materials And Methods

2.1. Patient samples

Peripheral blood samples were collected from 90 NSCLC patients enrolled in the Union Hospital of Tongji Medical College, HUST. None of the patients in the study were taking lipid-lowering drugs. Among the cases, samples from 60 cases, including 30 BMs and 30 NBMs, were sequenced by metabolomics to identify differential metabolites. Samples from the remaining 30 patients who received brain radiotherapy were subject to T-cell receptor repertoire sequencing (TCR-seq). The Institutional Review Board of Huazhong University of Science and Technology approved this study. Written informed consent was obtained from all legal guardians of the patients.

2.2. Cell lines and treatment

H1299 and Lewis lung cell (LLC) lines were procured from the American Type Culture Collection (ATCC). H1299 cells expressed wild-type EGFR. H1299 cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), and Lewis cells were cultured in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% FBS. Cells were grown at 37°C in a humidified atmosphere of 5% CO₂.

For ionizing radiation treatment, cells were placed 100 cm away from the radioactive source, performed with a 137Cs irradiator (Siemens, Munich, Germany) at a dose rate of 2.0 Gy/min. Cells were collected at 24 h post-radiation.

2.3. RT-qPCR

Total RNA was extracted from cells using a Total RNA Kit I reagent (Omega, USA) and then subjected to reverse transcription with the PrimeScript RT Reagent Kit (Takara, China). RT-qPCR was performed using the SYBR Premix Ex Taq II kit (Takara, China). The data were analyzed using the 2^−ΔΔCT method, GAPDH as a housekeeping gene.

2.4. MDA content measurement

The cells were lysed to obtain the cell lysate, and the protein concentration was determined using a BCA protein concentration determination kit. After the sample was mixed with the reagent in the MDA detection kit (Beyotime, China), it was heated at 100 °C for 15 minutes. The samples were cooled to room temperature and centrifuged at 1000 × g for 10 minutes. Then, 200 μl supernatant was added to a 96-well plate, and the absorbance was measured at 532 nm with a microplate reader. The MDA content in the sample solution was calculated, and the MDA content in the initial sample was expressed as the protein content per unit weight.
2.5. Cholesterol content measurement

Using the Cholesterol Quantitative Detection Kit (Sigma #MAK043), the total cholesterol concentration was determined by the coupled enzyme assay, and the result was a colorimetric (570 nm)/fluorometric ($\lambda_{ex} = 535/\lambda_{em} = 587$ nm) product, which was correlated with the cholesterol content proportion.

2.6. Western blot

The medium was removed, the cells were washed thrice with 1×PBS. Lysis buffer was added to each well. The cells were quickly scraped off with a cell scraper, transferred to a 1.5-ml tube, placed on ice for 20 min, shaken to mix, and then placed on ice for 10 min. Samples were centrifuged at 12 000 g and 4°C for 15 min. The supernatant was transferred to another 1.5-ml tube. The protein concentration was measured using the BCA method. Proteins were subject to SDS–PAGE. Proteins were transferred to the membrane using a semidry transfer method. The membrane was washed twice with 1×TBS at room temperature for 10 minutes. Then, 5 ml of milk powder blocking solution was added to the blot. The blot was incubated at room temperature for 2 h. Primary antibody dilution buffer was added to the blot. The blot was incubated at 4°C and gently shaken overnight. Then, the blot was incubated with the secondary antibody and gently shaken at room temperature for 1 h. The membrane was washed with TBST and developed with ECL developer solution. Antibodies specific for PPAR-α (A6697, Abclonal), ABCA1 (A7228, Abclonal), and GPX4 (A13309, Abclonal) were used in this study.

2.7. Study animals and brain metastasis model

Six-week-old female mice (C57BL/6J; Beijing Vital River Laboratory Animal Technology Co., Ltd., China) were used in these experiments. The brain metastasis model was described previously(11). LLC cells labeled with luciferase (LLC-Luc, $2 \times 10^5$ in 0.1 mL PBS) were slowly injected into the intracarotid artery of mice. Two weeks later, bioluminescence imaging was performed to observe the growth of brain tumors using an IVIS Lumina imaging system (In Vivo FX PRO, Bruker Corp.) after mice were injected with D-luciferin intraperitoneally (150 mg/kg, Goldbio St. Louis, MO, USA). BM mice received Fenofibrate (50 mg/kg, Selleck) 2 weeks after injection of tumor cells and were gavaged every day for a week. InVivoMAb anti-mouse PD-1 (CD279, Bioxcell) was injected intraperitoneally three times a week (200 µg/mouse). All animal study procedures followed the guidelines of the Institutional Animal Care Committee of Tongji Medical College, Huazhong University of Science and Technology, China. For radiation treatment, mice were anesthetized by intraperitoneal injection of Nembutal (1 g Nembutal dissolved in 100 ml 0.9% natrium chloride solution, 5 µl/g), after anesthesia, the mice received 10-Gy CRT as described previously(12) with minor modifications.

2.8. IFN-γ content measurement

The sample was diluted, incubated at 37 °C for 30 minutes, the sealing plate film was carefully removed, the liquid was discarded, the plate was shaken until dry, each well was filled with washing solution, and the plate was discarded after standing for 30 seconds. This process was repeated 5 times. The samples were dried, and enzyme-labeled reagent was added. The samples were incubated at 37 °C for 30 minutes.
The liquid was discarded. The samples were shaken, dried, filled with washing solution, and discarded after standing for 30 seconds. This process was repeated 5 times. The plate was dried, and color developing agent A was added to each well. Then, color developing agent B was added. The samples were shaken gently and mixed evenly. The reaction was terminated by adding a termination solution at 37 °C, and the absorbance of each well was measured at a wavelength of 450 nm.

2.9. Flow cytometry

To assay the populations of T cells in the brain, mouse brains were cut into small pieces, kept in PBS on ice, minced, and enzyme digested for 1 h at 37°C. The details of the protocol are mainly based on that described in reference[17]. The cells were separated by Percoll (GE Healthcare Life Sciences) using a density gradient (70%, 37%, 30%) and stained with a Zombie NIR™ Fixable Viability kit for CD45, CD8, and CD4. These antibodies were purchased from BioLegend, San Diego, CA.

2.10. Immunofluorescence

Mice were anesthetized to obtain brain tissue and then processed and embedded in paraffin. The details of the protocol have been described previously(13). Brain tissue slides were incubated with the primary antibody CD8 overnight at 4°C followed by the appropriate secondary antibody conjugated with the fluorescent dye Alexa Fluor 488 or Alexa Fluor 594 (1:1,000, Invitrogen). Sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (10 µg/mL, Sigma–Aldrich) and then scanned with a digital camera and imaging software (3DHISTECH Ltd., Hungary)

2.11. TCR-seq

As previously described(14), a Chromium Single-Cell V(D)J Enrichment kit was used to amplify cDNA from 5’ libraries and obtain full-length TCR V(D)J segments. The TCR sequences for each single T-cell were assembled by the Cell Ranger vdj pipeline (v3.1.0), and the CDR3 sequence and the rearranged TCR gene were identified. Analysis was performed using Loupe V(D)J Browser v.2.0.1. The same form of TCR was considered one class, and a unique TCR name was obtained. In brief, a TCR diversity metric containing clonotype frequency and barcode information was obtained.

2.12. Untargeted metabolomics

A total of 60 peripheral blood samples were collected from NSCLC patients and then blood plasma were separated to be sequenced at Novogene (Beijing, China). LC–MS/MS analyses were conducted using a Vanquish UHPLC system (Thermo Fisher) and Orbitrap Q Exactive series mass spectrometer (Thermo Fisher). The exactive series mass spectrometer was operated in positive/negative polarity mode. Metabolite data were analyzed using Compound Discoverer 3.1 (CD3.1, Thermo Fisher). Metabolites were identified based on peak intensities normalized to the total spectral intensity. Three databases (mzCloud, mzVault and MassList) were used to obtain accurate qualitative and relative quantitation results.
Statistical analyses were performed using the statistical software R (R version R-3.4.3), Python (Python 2.7.6 version), and CentOS (CentOS release 6.6). An Agilent 7890 gas chromatograph system coupled with a Pegasus HT time-of-flight mass spectrometer was used for GC–MS analyses.

### 2.13. Lipidomics

Use a sterile spatula to scrape off the Lewis cells, the cells were washed thrice with 1×PBS, centrifuged at 1000 × g for 5 minutes. The collected cell samples are quenched in liquid nitrogen to inhibit the metabolic activity in the cells and all cell samples were weighed. Then, 1000 µL dichloromethane:methanol (1:1), 20 µL 1/10 Lipid Mix internal standard, and 5 µL 1 mg/mL C17 internal standard were added to the sample. The sample was vortex for 1 min and sonicated for 32 s in an ice water bath at 4°C. The sample was centrifuged at 13 000 g for 5 min. Then, 800 µL of supernatant was dried with nitrogen and redissolved in 150 µL of dichloromethane:methanol (9:1). All of the supernatant was used for chromatographic analysis. Raw data were converted to common (abf) format using Analysis Base File Converter. Under the MSDIAL software platform, a visualization matrix including lipid metabolite number, retention time, m/z, metabolite name, ion mode, and peak area was obtained. Then, the number of sample features was screened to obtain the qualitative results of the sample library, including the sample name, ms1 and ms2, and the visualization matrix, including the relative quantification of the peak area. Finally, the local weighted regression Lowess is used to process the volatility data to obtain normalized data and data subsequently used for statistical analysis.

### 2.14. Statistical analysis

All count data are presented as the mean ± standard deviation. Pearson's chi-square test or Fisher's exact test was used to compare categorical variables, and the rank sum test was used to compare numerical variables. Univariate and multivariate analyses were performed using binary logistic regression analysis. Then, odds ratios (ORs), 95% confidence intervals (95% CI), and P values were calculated. The Kaplan–Meier method was used for survival analysis. SPSS 25.0 statistical software (IBM Corp. Armonk, New York) was used for all statistical analyses, and GraphPad Prism 9.0 software was used for graphing. A two-tailed test was performed. P < 0.05 indicated a statistically significant difference.

### 3. Results

#### 3.1. HDL may enhance radiotherapy sensitivity by promoting tumor cell ferroptosis

To determine whether ionizing radiation can induce ferroptosis in NSCLC cells, we measured the contents of the ferroptosis product malonic dialdehyde (MDA) and the expression of the ferroptosis marker PTGS2(15, 16). The results show that ionizing radiation can induce ferroptosis in both H1299 and Lewis
cells (Fig. 1a, b). Next, we investigated the relationship between the levels of blood lipids in NSCLC BM patients and the efficacy of brain radiotherapy. We collected data on biochemical indices, including high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC) and triglycerides (TGs), from 78 NSCLC BM patients. According to the physiological range of TC, LDL-C and TG, the patients were divided into index normal and index increased groups. In addition, patients were divided into the HDL-C normal group and the HDL-C low group given that HDL-C is considered a protective factor. By analyzing the progression-free survival (PFS), intracranial progression-free survival (iPFS), and overall survival (OS) of each group, the results show that iPFS and OS of the HDL-C normal group were longer than those observed for the HDL-C low group (Fig. 1c). In addition, both iPFS and OS were increased in the TC normal group compared with the TC high group (Fig. 1d). No significant differences were noted between the LDL-C and TG group (Fig. S1). This finding suggests that HDL-C and total cholesterol levels in the blood may be related to the efficacy and prognosis of brain radiotherapy in NSCLC BM patients.

HDL is a complex lipoprotein composed of lipids, proteins, and the regulatory factors they carry. HDL can promote the removal of cholesterol, maintain the relative balance of cholesterol, and regulate the lipid composition and metabolism. Therefore, we further explored whether HDL affects the occurrence of ferroptosis. We collected the blood of patients with normal HDL-C contents and low HDL-C contents (other blood lipid biochemical indicators are within the normal range, and these patients did not taking lipid-lowering drugs), obtained the serum and incubated the tumor cells for 24 hours as previously described(17), and then exposed them to ionizing radiation. We found that the level of ferroptosis in the normal HDL-C culture group was greater than that in the low HDL-C culture group (Fig. 1e). This finding suggests that HDL may promote the occurrence of ferroptosis by regulating lipid metabolism.

3.2. Fenofibrate promotes radiation-induced tumor cell ferroptosis by elevating HDL contents

To identify proper methods to regulate HDL synthesis, we first explored the factors that may affect HDL synthesis in NSCLC BM patients. We performed a nontargeted metabolomics analysis on blood samples from 60 NSCLC patients (including 30 BM and 30 NBM patients). Significant differences in metabolites were noted between the two groups. The levels of hexadecane, a natural ligand of the PPAR-α signaling pathway, in the blood of BM patients was significantly lower than that in the blood of NBM patients (Fig. 2a). Peroxisome proliferator-activated receptor alpha (PPARα), as one of the pathways for promoting HDL synthesis, can accelerate the synthesis of HDL by promoting the expression of the gene encoding the adenosine triphosphate binding cassette transporter A1 (ABCA1). Therefore, we hypothesized that low HDL levels in NSCLC BM patients are due to reduced activation of the PPARα pathway. We detected PPARα in the blood of patients with brain metastasis by RT–qPCR and found that PPARα expression was downregulated in patients with low HDL levels (Fig. 2b). Fibrates are a class of lipid-lowering drugs that reduce total triglycerides and increase HDL levels, and their main target is PPARα. Fibrates can also make pancreatic cancer cells more sensitive to ionizing radiation(18). Therefore, we chose fibrates to promote
the synthesis of HDL-C and explored whether the use of fibrates could enhance radiosensitivity by inducing ferroptosis in tumor cells. PPARα and ABCA1 expression was detected after treating Lewis cells with fenofibrate for 24 hours, and fenofibrate significantly upregulated PPARα and ABCA1 expression (Fig. 2c). Fenofibrate combined with ionizing radiation significantly induced tumor cell death mainly due to ferroptosis (Fig. 2d). The results showed that MDA levels and PTGS2 mRNA levels were markedly increased after stimulation with fenofibrate combined with ionizing radiation (Fig. 2e-g), which suggests an increase in ferroptosis levels. In addition, the addition of the ferroptosis inhibitor ferrostatin-1 could significantly inhibit the ferroptosis induced by this combination treatment (Fig. 2e).

Treatment with GW6741 to inhibit the activation of PPARα blocked the effect of ferroptosis induced by fenofibrate combined with radiotherapy (Fig. 2f, g), and the use of siRNA to interfere with ABCA1 expression also blocked ferroptosis (Fig. 2h). However, the treatment of tumor cells with fenofibrate alone did not induce ferroptosis. These results show that fenofibrate can promote the synthesis of HDL by targeting PPARα and facilitating radiation-induced ferroptosis in tumor cells.

3.3. Fenofibrate combined with radiotherapy enhances radiosensitivity by inducing ferroptosis

To further explore whether fenofibrate combined with radiotherapy can achieve better efficacy, we used Lewis-Luciferase cells to establish a brain metastasis model. Model establishment and treatment administration are shown in Fig. 3a. We found that the HDL-C content in mice treated with fenofibrate was significantly increased compared with that in the control group and the radiotherapy alone group (Fig. 3b). Next, we detected the MDA content in the tumors of Lewis brain metastatic mice, and the result was consistent with previous results. The ferroptosis level in the combined treatment group was significantly higher than that in the other groups (Fig. 3c). The results also showed that the antitumor effect of fenofibrate combined with radiotherapy was significantly better than that of radiotherapy alone (Fig. 3d), and the overall survival was also prolonged (Fig. 3e). Therefore, these results show that fibrates can increase the contents of HDL-C in blood and then enhance the efficacy of brain radiotherapy for brain metastases, but the mechanism still needs to be explored.

3.4. Fenofibrate promotes radiation-induced ferroptosis by inducing metabolic reprogramming

Given that the main role of HDL is to reverse cholesterol transport and blood total cholesterol contents are correlated with iPFS, we detected the cholesterol content in Lewis cells. After treatment with fenofibrate combined with ionizing radiation, the intracellular cholesterol content was significantly reduced (Fig. 4a). However, when GW6741 and siRNA were applied to interfere with PPARα and ABCA1, the intracellular cholesterol content was increased (Fig. 4b). To explore whether the occurrence of ferroptosis in tumor cells induced by combination therapy is related to cholesterol, Lewis cells were supplemented with exogenous cholesterol upon exposure to ionizing radiation, and lower ferroptosis levels were observed compared with that noted in the radiotherapy group (Fig. 4c). These results suggest that fenofibrate
induces ferroptosis in tumor cells and enhances radiosensitivity by reducing intracellular cholesterol levels.

In addition, activation of the PPARα signaling pathway can also affect the metabolism of other fatty acids (19). We therefore hypothesize that fenofibrate may also affect the composition of other lipids in addition to lowering intracellular cholesterol. We compared the levels of intracellular lipids of Lewis cells after fenofibrate treatment using lipid metabolomics. The addition of fenofibrate increased the intracellular content of most polyunsaturated fatty acids, whereas the content of saturated and monounsaturated fatty acids decreased (Fig. 4d). Fatty acids play a very important role in ferroptosis. The synthesis of phospholipids (PUFA-PL) based on polyunsaturated fatty acids, represented by arachidonic acid, is an important substrate for ferroptosis, whereas monounsaturated fatty acids (MUFAs), which are less susceptible to peroxidation due to the lack of the diallyl portion, inhibit lipid peroxidation and ferroptosis by replacing polyunsaturated fatty acids in the cell membrane (20). This finding suggests that in addition to lowering cholesterol, fenofibrate affects the composition of fatty acids within tumor cells, thereby promoting ferroptosis.

We next examined the expression of common pathways of ferroptosis and found that both radiotherapy and combined treatment significantly downregulated GPX4 expression in tumor cells (Fig. 4e-g). GPX4 is a lipid repair enzyme in the body that converts lipid peroxides (L-OOH) into nontoxic lipids to resist iron- and oxygen-dependent lipid peroxidation. Radiotherapy can lead to GSH depletion, which weakens GPX4-mediated ferroptosis defense and further promotes radiotherapy-induced ferroptosis (10). Taken together, we found that fenofibrate could alter the intracellular lipid composition of tumor cells and increase the ratio of PUFAs/MUFAs, thus increasing the oxidative substrate for ferroptosis. In addition, fenofibrate combined with radiotherapy inhibited the expression of GPX4, which scavenges lipid peroxides and induced ferroptosis, explaining why fenofibrate alone did not significantly induce ferroptosis.

### 3.5. Tumor cell ferroptosis alters the immune microenvironment

Local radiotherapy kills cells and causes them to release cytokines and chemokines into the tumor microenvironment, and these released products can recruit dendritic cells, macrophages, and killer T cells, altering the immune microenvironment (21). In addition, some studies have shown that ferroptosis is immunogenic (20). Thus, we hypothesized that the sensitizing effect of radiotherapy produced by fenofibrate might further affect the tumor microenvironment. First, we examined alterations in T-cell number in mouse brain metastases after combination treatment by flow cytometry and immunofluorescence. We found a significant increase in CD8+ T cells in the brain metastases of mice after combination treatment (Fig. 5a, b). Then, we performed TCR-seq on blood samples from 30 patients with brain metastases 24 hours and 28 days after radiotherapy. A significant difference in TCR diversity was noted between the two groups (Fig. 5c), and the recovery of the Shannon index was positively correlated with HDL-C levels (Fig. 5d). This finding suggests that HDL-C levels may affect the recovery of T-cell diversity after radiotherapy. T cells play an important role in immunotherapy, suggesting that
combination treatment could increase the recruitment of T cells to the tumor foci. This finding suggests that our combination treatment-induced ferroptosis in tumor cells may lead to enhanced cellular immunogenicity and further recruitment of CD8+ T cells into the tumor foci.

We next investigated whether fenofibrate combined with ionizing radiation would directly affect T cells. We found that ferroptosis was not significantly induced in CTLL2 cells, a mouse T cell line, at the same dose of ionizing radiation (Fig. 5e) potentially due to the different sensitivities to ionizing radiation among cells, and the tolerated dose of T cells might be higher than that of Lewis cells. Combination treatment did not have a significant effect on IFN-γ secretion by T cells (Fig. 5f), but treatment of T cells with supernatant from combination-treated Lewis cell enhanced IFN-γ secretion (Fig. 5g). This finding suggests that the combination treatment does not act directly on T cells but rather enhances the immunogenicity of ferroptotic tumor cells, inducing IFN-γ secretion by T cells.

3.6. Fenofibrate combined with radiotherapy can improve the efficacy of immunotherapy

Many studies have shown that radiotherapy can enhance the efficacy of immunotherapy based on changes in the immune microenvironment. We found that fenofibrate combined with radiotherapy could further recruit CD8+ T cells to the tumor microenvironment and stimulate the secretion of IFN-γ; thus, we decided to further explore whether this combination therapy could enhance the efficacy of immunotherapy. The mouse model and administration regimen are presented in the Fig. 6. The results suggested that the effect of fenofibrate combined with radiotherapy and immunotherapy was significantly better than that of the other treatments (Fig. 6b). Although there was no statistical difference in overall survival, there still has a trend in survival benefit, possibly related to the small sample size (Fig. 6c). By detecting cytokines in the blood of mice, significantly increased IFN-γ levels were observed in the combined treatment group compared with the other groups (Fig. 6d). This finding suggested that our combined treatment may improve the efficacy of immunotherapy.

4. Discussion

As a major modality in clinical cancer treatment, radiotherapy (RT) cures or palliates cancer in greater than 50% of patients(22). Radiotherapy is the cornerstone of management of malignant brain tumors, but its efficacy is limited in hypoxic tumors. Although numerous radiosensitizer compounds have been developed to enhance the effect of RT, progress has been stagnant, and most radiosensitizers have a wide range of toxicities(23). Therefore, we urgently need to find a safe and effective radiosensitizers. The emergence of ferroptosis also provides new ideas for the selection of radiosensitizers, and a number of studies have demonstrated that ferroptosis inducers can enhance radiotherapy sensitivity(9). Lipids play an extremely important role in ferroptosis, and our study found that blood lipid levels, especially HDL and total cholesterol in NSCLC patients with brain metastases correlated with the efficacy of brain radiotherapy, so we then tried to explore whether modulation of lipids might be a potential approach to sensitization radiotherapy.
HDL and its related lipids and proteins have a wide range of activities, and a current area of research focuses on cardiovascular diseases, where HDL performs reverse cholesterol transport (RCT), extracting cholesterol from tissues and transporting it to the liver for excretion, thus preventing atherosclerosis (24). In addition, HDLs have several non-RCT protective activities, including antioxidant, anti-inflammatory, anti-apoptotic and immunomodulatory activities (25). In contrast, the relationship between HDLs and tumors is highly controversial. HDL has been found to limit the proliferation of prostate cancer cells (26), whereas HDL stimulates the migration of breast cancer cells (27). Moreover, the relationship between HDL and tumor radiotherapy is not clear. Our findings demonstrated that the use of fenofibrate to promote HDL synthesis significantly induced tumor cell death when combined with ionizing radiation. Although ionizing radiation itself can also induce a range of cell death patterns, such as autophagy (28), we explored what patterns of cell death induced by the combination treatment and found that the addition of necrosis, autophagy, pyroptosis, and ferroptosis inhibitors were all effective in inhibiting cell death induced by ionizing radiation combined with fenofibrate, suggesting that combination therapy can induce cell death in multiple ways, with ferroptosis playing a major role. These confirmed that lipid modulation can be a potential approach to sensitization radiotherapy. However, this mechanism needs to be further clarified, thus we will explore the key molecules that mediate the process of ferroptosis promoted by HDL.

The main role of HDL is to regulate lipids, especially cholesterol. The role cholesterol plays in cancer development also remains controversial (29). Compared with normal cells, tumor cells have an increased demand for cholesterol and accumulate more cholesterol (30). It also plays an important role in the distant metastasis of tumors (31). Recently, cholesterol was found to be associated with the occurrence of ferroptosis (32, 33). Our study also verified that fenofibrate reduces cholesterol levels in Lewis tumor cells, thereby inducing ferroptosis. In addition, fenofibrate can increase the intracellular PUFA/MUFA ratio. It has been demonstrated that increasing the intake of polyunsaturated fatty acids in the diet elevates lipid peroxidation and induces ferroptosis in tumor cells (34). Besides, supplementation with omega-3 PUFAs enhances the killing effect of ionizing radiation on colorectal cancer (35). In contrast, monounsaturated fatty acids, including oleic acid, protect cells from ferroptosis in an ACSL3-dependent manner (36). This suggests that increased HDL synthesis tends to change the intracellular lipid composition in a direction that is susceptible to ferroptosis. Therefore, the use of fenofibrate to affect intracellular lipid metabolism combined with the inhibitory effect of ionizing radiation on cellular GPX4 expression can additional induce ferroptosis in lung cancer cells.

Considering that the treatment of tumors is usually localized on top of systemic treatment, and immune checkpoint blockade therapy (ICB) has achieved unprecedented results in the clinical treatment of NSCLC we therefore wanted to explore whether combination therapy could further influence the systemic immunotherapy. Recent researches show that ferroptotic tumor cells can release some immunostimulatory signals (37, 38), these signals lead to immunogenic enhancement, which subsequently induces tumor-specific immune responses and improves the efficacy of immune checkpoint inhibitors (ICIs) (39, 40). Besides, earlier studies have shown that cholesterol-lowering drugs can inhibit the high expression of programmed-death ligand 1 (PD-L1) that contributes to immunoevasion in cancer
cells and a recent study revealed the mechanism that cholesterol can directly bind to the transmembrane domain of PD-L1 through two cholesterol-recognition amino acid consensus (CRAC) motifs, forming a sandwich-like architecture and stabilizing PD-L1 to prevent downstream degradation(41). Our study demonstrated that fenofibrate combined with radiotherapy increased CD8+ T-cell infiltration in the tumor microenvironment and further enhanced the efficacy of an anti-PD-1 monoclonal antibody through in vivo experiments, which will provide a new idea for the treatment of NSCLC brain metastasis patients. We will further validate it in NSCLC BM patients to see if the combination therapy can actually provide a survival benefit for them. Besides, the mechanism by which ferroptotic tumor cells recruit CD8+ T cells and alter the tumor microenvironment needs to be further clarified.

5. Conclusion

In this study, we find that fenofibrate, a fibrate lipid-lowering drug, promotes the synthesis of HDL-C by activating the PPARα signaling pathway, thereby reversing the transport of cholesterol in tumor cells, promoting radiation-induced ferroptosis in tumor cells and achieving radiosensitization. In addition, when tumor cells undergo ferroptosis, intracellular lipid disorder increases their immunogenicity and enhances the killing effect of CD8+ T cells to promote the efficacy of immunotherapy. Thus, the combination of fenofibrate and radiotherapy could provide a new therapeutic strategy for non-small cell lung cancer patients with brain metastases.

Abbreviations

BM, brain metastasis, NSCLC, non-small cell lung cancer, MDA, malondialdehyde, PTGS2, prostaglandin-endoperoxide synthase 2, HDL, high-density lipoprotein.

Declarations

Author contributions

XRD and FT designed and directed the study. YWB, LCL, XRR and HHL performed the experiments, BHK, PD and LP analyzed the data, BHK and YWB wrote and prepared the manuscript. All authors provided critical feedback and helped to shape the manuscript.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article.

Declaration of competing interest

The authors have declared no conflict of interest.

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References


Figures
Blood lipids correlate with the efficacy of brain radiotherapy. (A, B) MDA contents and PTGS2 expression levels indicate that ionizing radiation can induce ferroptosis. (C) iPFS and OS for different HDL-C patients (low HDL-C, n=37, normal HDL-C, n=41, low HDL-C: male<1.16 mmol/L, female<1.29 mmol/L, normal HDL-C: male>1.16 mmol/L, female>1.29 mmol/L). (D) iPFS and OS for different TC patients (TC high, n=26, TC normal, n=52, TC high:>5.16 mmol/L, TC normal:<5.16 mmol/L). (E) MDA levels in Lewis and
H1299 cells after incubation with different HDL serum contents for 24 h and then accept ionizing radiation. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

**Figure 2**

Fenofibrate promotes HDL synthesis and promotes radiation-induced ferroptosis. (A) Nontargeted metabolomics detects blood metabolite differences between patients with and without brain metastases.
(BM=30, NBM=30). (B) Detection of PPARα expression in the blood of patients with different HDL levels by RT-qPCR (n=6). (C) PPARα and ABCA1 protein expression levels in Lewis cells were detected by Western blot. (D) Detection of changes in cell activity after the addition of the ferroptosis inhibitor Ferro-1, the autophagy inhibitor chloroquine, the pyroptosis inhibitor VX765, and the necroptosis inhibitor necrostatin-1 as assessed by CCK8 assay. (E) Detection of MDA contents after treatment with fenofibrate, ionizing radiation and Ferro-1. (F-H) Detection of MDA and PTGS2 levels after addition PPARα inhibitor GW6741 or using siRNA interfere ABCA1. *P < 0.05, **P < 0.01, ***P < 0.001

A

Establish Lewis-luc Brain Metastasis Model
Gavage 10%DMSO or fenofibrate daily
Grouped according to fluorescence
10Gy brain radiotherapy
Execute

Day 0  Day 14  Day 28  Day 35

B

Plasma

**

Controller  RT  RT+Feno

HDL content (mmol/L)

C

Brain

***

Controller  RT  RT+Feno

MDA content (mmol/L)

D

Day14  Day30

10%DMSO

RT

RT+Feno

E

Overall survival

Control  RT  RT+Feno

Time (Days)
Figure 3

Fenofibrate may enhance sensitivity to radiotherapy. (A) Flow chart of the animal model experiment. (B) HDL contents detected by ELISA (n=3). (C) MDA levels in brain metastasis tissues (n=3). (D) *In vivo* bioluminescent images of different groups on Days 14 and 28 (n=8). (E) Overall survival of different groups. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 4

Fenofibrate induces ferroptosis by affecting the intracellular lipid composition. (A, B) Cholesterol levels in Lewis cells were detected by ELISA. (C) Detection of MDA changes in Lewis cells after the addition of cholesterol. (D) Lipidomics were used to detect the lipid composition of Lewis cells after treatment with fenofibrate (n=5). (E) GPX4, SLC7A11 and LPAT3 protein expression in Lewis cells was detected by Western blot. (F, G) GPX4 expression was detected by RT–qPCR and immunohistochemistry (scale bars = 20 μm). *P < 0.05, **P < 0.01, ***P < 0.001
Combination therapy increases T-cell infiltration in the tumor microenvironment. (A, B) CD8+ T cells in brain metastasis tissue were detected by flow cytometry (n=5) and immunofluorescence (scale bars = 20 μm). (C) Detection of changes in T-cell diversity after brain radiotherapy by TCR-seq (n=30). (D) Correlation analysis of HDL and T-cell diversity. (E) Measurement of MDA levels in CTLL2 cells after
exposure to ionizing radiation. (F, G) IFN-γ levels in the cell supernatant were detected by ELISA. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Figure 6

Fenofibrate combined with radiotherapy improves the efficacy of immunotherapy. (A) Flow chart of the animal model experiment. (B) *In vivo* bioluminescent images of different groups on Days 14 and 28.
(n=8). (C) Overall survival of different groups (n=8). (D) IFN-γ levels in mouse plasma were detected by ELISA (n=5). (E) The graphical abstract of the study. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryMaterial.docx