TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Mutation of TP53 Confers Ferroptosis Resistance in Lung Cancer Through the FOXM1/MEF2C Axis

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Ferroptosis is a highly regulated tumor suppressor process. Loss or mutation of TP53 can cause changes in sensitivity to ferroptosis. Mutations in TP53 may be associated with the malignant or indolent progression of ground glass nodules in early lung cancer, but whether ferroptosis may also be involved in determining this biological process has not yet been determined. Using in vivo and in vitro gain- and loss-of-function approaches, this study used clinical tissue for mutation analysis and pathological research to show that wild-type TP53 inhibited the expression of forkhead box M1 (FOXM1) by binding to peroxisome proliferator-activated receptor-γ coactivator 1α, maintaining the mitochondrial function and thus affecting the sensitivity to ferroptosis. This function was absent in mutant cells, resulting in overexpression of FOXM1 and ferroptosis resistance. Mechanistically, FOXM1 activated the transcription level of myocyte-specific enhancer factor 2C in the mitogen-activated protein kinase signaling pathway, leading to stress protection when exposed to ferroptosis inducers. This study provides new insights into the mechanism of association between TP53 mutation and ferroptosis tolerance, which can aid a deeper understanding of the role of TP53 in the malignant progression of lung cancer. (Am J Pathol 2023, 193: 1587–1602; https://doi.org/10.1016/j.ajpath.2023.05.003)

Lung cancer is one of the most common cancers globally, with approximately 2 million new cases each year. As one of the deadliest cancers, the prognosis of lung cancer is poor, with 5-year overall survival of 10% to 20% and an estimated 1.76 million deaths a year. Despitemaking great advances in lung cancer therapies, including surgery, chemotherapy, radiation, and targeted therapy, the outcomes are still unsatisfactory for patients with advanced lung cancer. Fortunately, lung cancer can be detected in its early stage with computed tomography (CT), and the lung neoplasms in the early stage often present as ground glass opacity (GGO) on CT, which is significantly correlated with pathologic subtype and gene mutation rate. Moreover, lung cancer has a big burden of genetic mutations, such as LKB1, KRAS, EGFR, and TP53. A better understanding of mutant gene-mediated effects in lung cancer will contribute to a better understanding of the pathogenesis and development of novel targeted therapy.

TP53, a superstar tumor suppressor gene that encodes tumor protein tumor protein P53, is frequently mutated in various human cancers, including lung cancer. As the guardian of the genome, P53 acts as an important transcription factor to regulate various physiologic processes, including cell apoptosis, proliferation, senescence, cell cycle and DNA repair, thus exerting antitumor activity. However, TP53 is often mutated or depleted in cancers, limiting its antitumor activity and allowing cancer to develop.

Ferroptosis, a novel iron-dependent type of cell death, has opposing roles in tumor suppression and promotion. Particularly, inducing ferroptosis may suppress the tumorogenesis of lung cancer, implying that ferroptosis may be a...
promising therapeutic target for lung cancer.\textsuperscript{15} \textit{TP53} is widely known as a key regulator in ferroptosis, and depletion of \textit{TP53} confers cell insensitivity to ferroptosis.\textsuperscript{16} Mechanically, p53 modulates the ferroptosis response induced by ferroptosis inducers, such as glutathione peroxidase 4 inhibitors, or high levels of reactive oxygen species (ROS) through its metabolic targets.\textsuperscript{17,18} However, \textit{TP53} is often mutated in tumors, and there are 6 exemplary hotspot mutations in the DNA-binding domain of the \textit{TP53} gene (R273H, R248Q, R282W, R175H, G245S, and R249S).\textsuperscript{19} Previous findings suggest that the high frequency of \textit{TP53} mutants (R175H, R248W, and R273H/L) in GGO tissues may determine the malignant or indolent progression of GGO.\textsuperscript{20} Considering the critical role of \textit{TP53} in ferroptosis, the effect of these mutants on ferroptosis and the molecular mechanisms were investigated.

Forkhead box M1 (FOXM1), a transcriptional factor that regulates cell proliferation, tumor initiation, and progression in multiple cancer types, including lung cancer,\textsuperscript{21,22} affects ferroptosis.\textsuperscript{23} Previous studies have shown that FOXM1 and forkhead box O3 (FOXO3), a reported downstream target of \textit{TP53},\textsuperscript{24} form a competitive balance mechanism to determine tumorigenesis and drug resistance.\textsuperscript{25} Nevertheless, the effect of mutant \textit{TP53} on these events remains unclear, and whether ferroptosis is involved needs to be investigated.

The current study first demonstrated that mutant \textit{TP53} (carrying the common mutation site: R248W) conferred lung cancer cell resistance to ferroptosis. Second, the effect of wild-type \textit{TP53} and mutant \textit{TP53} on FOXM1 expression levels was investigated and a novel mechanism was identified by which mutant \textit{TP53} impaired peroxisome proliferator-activated receptor-\gamma coactivator 1\alpha (PGC-1\alpha)/FOXM1–dependent mitochondrial function, thereby affecting ferroptosis. Third, pathways through which up-regulation of FOXM1 affects ferroptosis and is related to the activation of mitogen-activated protein kinase (MAPK) signaling were elucidated, which confer greater resistance to ferroptosis inducers.

Materials and Methods

Patients

Tumor and paracancerous normal tissues were collected from patients diagnosed with lung cancer at the Second Xiangya Hospital of Central South University from 2020 to 2021. All patients provided written informed consent. Our study was approved by the Ethics Committee of the Second Xiangya Hospital of Central South University.

Cell Culture, Transfection, and Treatment

Human lung cancer cell lines CALU-1 and NCI-H358 (ATCC, Manassas, VA) were maintained in McCoy’s 5A medium/10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA). For inducing ferroptosis, cells were treated with RSL3 (Selleck, Shanghai, China) or erastin (Selleck) for 24 hours. For stable overexpression, coding regions of wild-type \textit{TP53} and mutant \textit{TP53} were cloned into pLVX-Puro lentiviral vector (Clontech, Mountain View, CA), respectively. Lentiviral particles were packaged in 293T cells with Lenti-X HTX Packaging System (Clontech) and harvested for infecting CALU-1 and NCI-H358 cells. shRNAs against myocyte-specific enhancer factor 2C (MEF2C) and scrambled controls were obtained from GenePharma (Shanghai, China). Coding regions of FOXM1 were inserted into pcDNA3.1 vector (Thermo Fisher Scientific). Cells were transfected with Lipofectamine 3000 (Thermo Fisher Scientific).

CCK-8 Assay

Cells were seeded in 96-well plates and treated with RSL3 (concentration range: 0–20 \textmu mol/L for CALU-1 cells; 0–30 \textmu mol/L for NCI-H358 cells) or erastin (concentration range: 0-30 \textmu mol/L for CALU-1 and NCI-H358 cells) for 24 hours. [The half maximal inhibitory concentration was selected as the subsequent experimental drug concentration treatment (see Figure 1).] Subsequently, medium was removed, and 100 \textmu L of fresh medium and 10 \textmu L of cell counting kit 8 (CCK-8) (Beyotime, Shanghai, China) were added to each well. Cells were incubated for 4 hours, and the absorbance (450 nm) was recorded.

Lipid ROS Assay

A total of 10 \textmu mol/L boron dipyrromethene C-11 probe (catalog number MX5211, Millipore, Burlington, MA) was added and incubated with cells in a 5% carbon dioxide incubator at 37°C for 1 hour. Then fluorescence images were taken under an flow cytometer (catalog number FACSCanto II, BD Biosciences, Franklin Lakes, NJ) to analyze intracellular lipid ROS.

MDA Assay

Cell samples were lysed or tissues ground into tissue homogenates and then centrifuged (10,000 \times g for 10 minutes) to collect supernatant. Malondialdehyde (MDA) content in all samples were then tested and calculated according to kit operating instructions (catalog number S0131S, Beyotime).

Clonal Formation Ability Detection

Cells were seeded in six-well plates at a density of 300 cells per dish, and 2 mL of medium was added to each dish and evenly dispersed by turning gently. Then the cells were cultured in a cell incubator at 37°C and 5% carbon dioxide for 3 weeks. After the visible clones were grown, they were fixed with 4% paraformaldehyde, stained with crystal violet (catalog number C0121, Beyotime), and then photographed under white light to calculate the clone formation rate.
Quantitative Real-Time RT-PCR

Total RNA was extracted from cells and clinical specimens using TRIzol reagent (Thermo Fisher Scientific) following the manual. Subsequently, RNA was reversely transcribed into cDNA with a QuantiTect Reverse Transcription Kit from Qiagen (Germantown, MD). The expression of FOXM1 and MEF2C was analyzed by quantitative real-time RT-PCR (RT-qPCR), which was normalized to glyceraldehyde-3-phosphate dehydrogenase. The 2**−ΔΔCt** formula was applied, and primers were given in Table 1.

Western Blotting

Cells were lysed in radioimmunoprecipitation assay lysis buffer for 30 minutes on ice, and cell lysates were collected. Protein was quantified using a bicinchoninic acid kit (Bio-Rad, Hercules, CA). Protein (30 μg) was electrophoresed and transferred to polyvinylidene fluoride membranes, which were blocked for 1 hour. Membranes were incubated with anti-p53 (ab26, 1:1000, Abcam), anti-MEF2C (ab211493, 1:500, Abcam), antimutant p53 (ab32049, 1:2000, Abcam), anti-FOXM1 (ab207298, 1:1000, Abcam), anti-TP53, and transferred to polyvinylidene fluoride membranes, incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour. Bands were visualized with ECL substrate (Beyotime), and the intensity was analyzed using ImageJ software version 1.49 (NIH, Bethesda, MD; http://imagej.nih.gov/ij).

Coimmunoprecipitation Assay

First, the expression vector pcDNA3.1 TP53 (wild-type or mutated) was constructed and transfected into lung cancer cell lines with TP53 loss. Second, immunoprecipitation grade antibodies of the labeled protein p53 (5 μg, Abcam) was used for the immunoprecipitation process. Third, the contents of the targeted protein (PGC-1α) was used for the immunoprecipitation process. The next day protein A/G Magnetic Beads (Thermo Fisher Scientific) were added, mixed, and incubated with gentle rotation. Subsequently, DNA was recovered for RT-qPCR and electrophoresis analysis.

Chromatin Immunoprecipitation Assay

Cells were crosslinked in formaldehyde solution, detached, and lysed in lysis buffer. Cell lysates were harvested and sonicated for DNA-protein fragments. DNA-protein fragments were incubated with rabbit primary antibodies against FOXM1 (5 μg, Abcam) at 4°C overnight. Normal rabbit IgG was used as an isotype control. The next day protein A/G Magnetic Beads (Thermo Fisher Scientific) were added, mixed, and incubated with gentle rotation. Subsequently, DNA was recovered for RT-qPCR and electrophoresis analysis.

Dual-Luciferase Assay

Promoter regions of FOXM1 and MEF2C were cloned into pGL3 luciferase vector (Promega, Madison, WI) as FOXM1, and MEF2C luciferase reporters. wild-type TP53, mutant TP53, or FOXM1-overexpressing lung cancer cell lines were transfected with FOXM1 or MEF2C luciferase reporter. After 48 hours, luciferase activity was measured with the Dual-Glo Luciferase System (Promega).

Yeast One-Hybrid System

The bait recombinant plasmid, pAbAi- MEF2C, and the prey recombinant plasmid, pGADT7-FOXM1, were constructed and transferred into engineering yeast. When the fusion expression vector of the transcription factor (FOXM1) was transferred into the yeast, it activated the pmi promoter. However, the reporter gene expression was promoted after binding with the target-acting element (MEF2C promoter fragment). Yeast growing on the SD/-Leu/-AbA (250 ng/mL) plate means that pAbAi-MEF2C and pGADT7-FOXM1 could interact and activate the reporter gene expression of host bacteria.

A Subcutaneous Lung Cancer Xenograft Mouse Model

Animal studies were performed in accordance with Central South University and approved by the Second Xiangya Hospital of Central South University Animal and Life Sciences Research Ethics Committee. CALU-1 cells (4 × 10⁶) were subcutaneously injected into the left flanks of BALB/c nude mice (8- to 10-week-old, male mice). For RSL3 and erastin administration, mice were intraperitoneally injected with RSL3 at 5 mg/kg per day and erastin at 20 mg/kg per day for 5 days. Mice were divided into four groups: wild-type TP53 plus erastin, mutant TP53 plus erastin, wild-type TP53 plus RSL3, and mutant TP53 plus RSL3. Mice were weighted every day. Tumor volume was monitored and calculated with the formula volume = length × width²/2. After 20 days, the mice were anesthetized via intraperitoneal injection of 2% pentobarbital sodium (3 mg/100 g) and then sacrificed by cervical...
dislocation. Tumors were excised from mice for subsequent immunohistochemistry staining.

**Immunohistochemistry Staining**

Subcutaneous tumors from mice and clinical specimens were fixed and embedded in paraffin. Subsequently, samples were sliced into 5-µm sections. Sections were deparaffinized, rehydrated, and retrieved in pH 8.0 antigen retrieval solution (Thermo Fisher Scientific). After blocking, sections were incubated with a rabbit anti-MEF2C (ab211493, 1:200, Abcam) and anti-FOXM1 (ab207298, 1:200, Abcam) overnight. The next day sections were rinsed and incubated with an horseradish peroxidase—conjugated secondary antibody. Signals were visualized with diaminobenzidine (Beyotime). Sections were stained with hematoxylin and imaged under the BX51 microscope (Olympus, Tokyo, Japan).

**Polyunsaturated Fatty Acid Content Assay**

Targeted fatty acid detection and analysis of cells were performed by Sci-tech Innovation (Shan Dong, China). Extraction of lipid samples from cells, saponification and methyl esterification, and gas chromatography were used to quantify the unsaturated fatty acids. Finally, the data analysis was performed by the data acquisition instrument system (catalog number 7890A; Agilent, Santa Clara, CA).

**Statistical Analysis**

Data from three independent assays are represented as means ± SD. The t-test for two independent groups and one-way analysis of variance for multiple groups were applied for variance analysis. \( P < 0.05 \) was statistically significant.

**Results**

**TP53 Mutations in Lung Cancer Cells Lead to Ferroptosis Insensitivity**

In a previous study, gene mutation analysis in 213 patients with early-stage non–small cell lung cancer (stage I and II) showed a high frequency of mutants (R175H, R248W, and R273H/L) present in these early lung cancers (Figure 1A) which were closely related to the malignant progression of lung cancer during GGO.\(^{20}\) Lung cancer cell line A549 was confirmed to harbor TP53, whereas CALU-1 and NCI-H358 cells lacked TP53 gene through sequencing of PCR fragments (Figure 1B). Next, overexpression plasmids with wild-type TP53 and mutant TP53 (hereinafter referred to as R248W mutant unless otherwise specified) were constructed and transfected into CALU-1 and NCI-H358 cells to observe their susceptibility to ferroptosis (Figure 1C). Erastin or RSL3 treatment reduced cell viability in a dose-dependent manner (Figure 1D). Compared with mutant TP53, wild-type TP53—overexpressing cells showed relatively poor cell viability when exposed to ferroptosis inducers (Figure 1E). These observations suggest that TP53 sensitizes lung cancer cells to ferroptosis, whereas mutant TP53 does the opposite.

**TP53 Affects Ferroptosis by Regulating the Transcription Level of FOXM1**

Global gene expression analyses revealed that cell cycle regulatory genes and transcription factors E2F1, MYBL2, and FOXM1 were disproportionately up-regulated in many TP53 mutant cancer types.\(^{26}\) Whether wild-type or mutant TP53 played a role in ferroptosis by affecting FOXM1 expression was investigated. The Cancer Genome Atlas database analysis showed that FOXM1 expression level was significantly higher in lung adenocarcinoma and lung squamous carcinoma than that in normal lung tissues (Figure 2A). The high expression of FOXM1 was negatively correlated with survival time of patients with lung cancer (Figure 2B), demonstrating that FOXM1 may act as a carcinogen in lung cancer. In addition, the expression of FOXM1 in lung cancer tissues with TP53 mutation was higher than that in wild-type TP53 (Figure 2C). Overexpression of wild-type TP53 could significantly bring down the mRNA and protein expression of FOXM1, whereas overexpression of the mutant had little effect (Figure 2, D and E), suggesting that the increased expression of FOXM1 in lung cancer may be a consequence of TP53 mutation or deletion. Next, FOXM1 was overexpressed in wild-type and mutant TP53 cells to examine the effect on ferroptosis. Up-regulation of FOXM1 protected cells from ferroptosis, which was more significant in TP53 mutant cells than in the wild-type TP53 cells, as reflected by relatively higher cell viabilities and clonogenesis and less lipid peroxides accumulation when exposed to the same dose of ferroptosis inducers (Figure 3, A–C). These data suggest that ferroptosis resistance caused by FOXM1 overexpression can be attributed to TP53 mutation.

**TP53 Binds PGC-1α to Inhibit FOXM1 Transcription and Affects Ferroptosis**

The peroxisome proliferator-activated receptor-γ coactivator 1 α (PGC-1α) transcriptional coactivator is a master regulator of mitochondrial biogenesis.\(^{27}\) In cancer, activation of PGC-1α can exert both positive and negative effects in that it supports survival and metabolic flexibility of tumor cells.\(^{28}\) Among the TP53-binding proteins, PGC-1α was selected because it may alter cellular metabolic characteristics by regulating mitochondria (Figure 4A), which is critical for sensitivity to ferroptosis. The results of coimmunoprecipitation showed that the binding of TP53 and PGC-1α occurred in the wild-type cells but was weak in the mutants (Figure 4B). Overexpression of PGC-1α in
Figure 1  TP53 mutations in early lung cancer cells lead to ferroptosis insensitivity. **A**: Mutation analysis of TP53 in patients with early-stage non–small cell lung cancer (stage I and II). High frequency of mutants R175H, R248W, and R273H/L. **B**: Electrophoresis of TP53 (R175H, R248W, and R273H/L mutants) PCR fragments amplified from DNA isolated from A549, NCI-H1299, CALU-1, and NCI-H358 cells. **C**: Overexpression efficiency of wide-type (WT) TP53 and mutant (Mut) TP53 (R248W) detected by Western blotting. **D**: Half maximal inhibitory concentration (IC50) of CALU-1 cells treated with erastin (24.56 μM) and RSL3 (3.41 μmol/L) for 24 hours. IC50 of NCI-H358 cells treated with erastin (12.00 μM) and RSL3 (16.02 μmol/L) for 24 hours. **E**: Cell viability tested by CCK8. OE, overexpression. n = 213 (A); n = 3 (C); n = 5 (D); n = 6 (E). *P < 0.05, **P < 0.01, and ***P < 0.001 (one-way analysis of variance). OE, overexpression.
wild-type cells resulted in a significant down-regulation of FOXM1 transcript and protein levels than in mutant cells (Figure 4, C–D). Furthermore, PGC-1α was overexpressed in cells (wild-type or mutant TP53) transfected with a luciferase vector carrying the FOXM1 promoter fragment. FOXM1 promoter activity decreased most significantly in the presence of both wild-type TP53 and PGC-1α, whereas in mutant cells, the FOXM1 promoter activity was relatively strong (Figure 4E). These data indicated that wild-type TP53 can repress FOXM1 transcription with PGC-1α, whereas the mutant has no significant effect because of weak binding to PGC1α.

The effect of PGC-1α on ferroptosis in wild-type and mutant TP53 cells was studied. Up-regulation of PGC-1α enhanced the expression levels of oxidized phosphate and electron transport chain complexes I and III (HADHα, HADHβ, NDUFB8, and CYTB) (Figure 5A), making cells sensitive to ferroptosis (Figure 5, B–D). This effect was more pronounced in wild-type cells than in mutant cell lines.

Collectively, these results suggest that wild-type TP53 affects FOXM1 transcription by interacting with PGC-1α, which is important for sensitivity to ferroptosis as determined by metabolic characteristics. Subsequently, unsaturated fatty acids, such as stearic acid, palmitic acid, arachidonic acid, dihomo-γ-linolenic acid, icosapentaenoic acid, and docosapentaenoic acid, decreased to varying degrees in mutant cells compared with that in the wild type (Supplemental Figure S1), which may reduce lipid ROS production and thus susceptibility to ferroptosis.

Mutant TP53 Leads to FOXM1 Activation of MAPK Signaling Pathway

The downstream targets of the transcription factor FOXM1 in the regulation of ferroptosis were explored. Hence, FOXM1 overexpressing lung cancer cells treated with erastin were prepared for RNA sequencing. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis was performed of differently expressed genes (Figure 6A). Compared with the control group, there were more genes, including SOS1, NGFR, and MEF2C, enriched in the MAPK signaling pathway (activated in ferroptosis) in the FOXM1 overexpressed group. Because MEF2C is in ferroptosis, whether FOXM1 regulates MEF2C transcription and thus influences ferroptosis was studied next. Chromatin immunoprecipitation/PCR assay showed that FOXM1 was enriched in the MEF2C promoter region (Figure 6B). In addition, the yeast one-hybrid system (Figure 6C) and the dual-luciferase assay (Figure 6D) demonstrated that FOXM1 activated the promoter of MEF2C to initiate its transcription. Furthermore, both the mRNA and the protein expression levels of MEF2C were up-regulated after FOXM1 overexpression, and in the mutant TP53 cell types, the expression level of MEF2C was much more higher than that of the wild type (Figure 6, E–F), indicating that FOXM1 and mutant TP53 can synergistically promote MEF2C expression.
Figure 3  TP53 affects ferroptosis by regulating the transcription level of forkhead box M1 (FOXM1). A–C: The cell viability (A), colony-forming abilities (B), and lipid reactive oxygen species (ROS) (C) of each group tested by the same method as shown in Figure 1. n = 3. *P < 0.05, **P < 0.01, and ***P < 0.001 (one-way analysis of variance). Mut, mutation; NC, negative control; OE, overexpression; WT, wild type.
The FOXM1/MEF2C Axis Causes Ferroptosis Insensitivity

In CALU-1 cells, shRNA interfered with MEF2C expression, whereas FOXM1 was overexpressed, and ferroptosis was then detected to assess whether FOXM1 affects ferroptosis through MEF2C. Cells in FOXM1-overexpressing groups had a higher survival rate, lower lipid ROS and MDA levels, and more clones of cancer cells (Figure 7, A–E) with isodose erastin (or RSL3) compared with the empty vector group, demonstrating that cells are less sensitive to ferroptosis. However, knockdown of MEF2C negated the survival benefits of FOXM1 overexpression in the presence of ferroptosis inducers. Similar results were verified in another lung cancer cell line, NCI-H358 (Figure 8). These studies confirmed that FOXM1 affected ferroptosis by up-regulating MEF2C in lung cancer cells.

Mutant TP53 Lung Cancer Cells Are Highly Activated on the FOXM1/MEF2C Axis When Exposed to Ferroptosis Inducer in Vivo

To evaluate the effect of mutant $TP53$ on FOXM1/MEF2C axis to inhibit ferroptosis of lung cancer cells in vivo, a mouse model of lung cancer was established via subcutaneous injection of CALU-1 cells (overexpressed TP53 binds peroxisome proliferator-activated receptor-$\gamma$ coactivator 1 $\alpha$ (PGC-1$\alpha$) to inhibit forkhead box M1 (FOXM1) transcription. A: A protein network bound to PGC-1$\alpha$ (form BioGRID database). B: The binding relationship between TP53 and PGC-1$\alpha$ via co-immunoprecipitation and Western blotting. C and D: Relative mRNA and protein expression levels of FOXM1 tested by quantitative real-time RT-PCR (C) and Western blotting (D) in TP53 wild-type (WT) and mutant (Mut) cells with PGC-1$\alpha$ overexpression. E: Effects of PGC-1$\alpha$ on FOXM1 promoter detected by dual-luciferase assay in TP53 WT and Mut cells. $n = 3$ (C and D); $n = 5$ (E). *$P < 0.05$, **$P < 0.01$ (one-way analysis of variance). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Figure 4
wild-type or mutant TP53) in combination with erastin administration. The wild-type group greatly reduced tumor volume and weight in mice compared with the mutant group, with increased MDA content in the subcutaneous tumor tissues (Figure 9, A–E). In addition, the protein levels of FOXM1, MEF2C, and phospho-MAPK/MAPK were much lower in the wild-type group of tumors (Figure 9, F–G).

Furthermore, cancer tissues clinically diagnosed as early-stage lung cancer were collected for detection. Patients were
Figure 6 Mutant P53 (MutP53) leads to forkhead box M1 (FOXM1) activation of the mitogen-activated protein kinase (MAPK) signaling pathway. **A**: From left to right are the bubble, heat, and volcano maps of genes with changes in mRNA levels after overexpression of FOXM1. The red arrow points to myocyte-specific enhancer factor 2C (MEF2C) and the MAPK signaling in which the MEF2C was involved. **B**: The binding of FOXM1 in the MEF2C promoter region detected by chromatin immunoprecipitation/PCR. **C**: The effect of FOXM1 on the activity of MEF2C promoter via the yeast one-hybrid system. **D**: Effects of FOXM1 on MEF2C promoter detected by dual-luciferase assay in 293T cells. **E**: Relative mRNA expression levels of FOXM1 and MEF2C tested by quantitative real-time RT-PCR. **F**: Relative protein expression levels of FOXM1 and MEF2C tested by Western blotting, n = 3 (A, C, and F); n = 5 (D). *P < 0.05, **P < 0.01, and ***P < 0.001 (t-test and paired-samples t-test).
Figure 7 The forkhead box M1 (FOXM1)/myocyte-specific enhancer factor 2C (MEF2C) axis causes ferroptosis insensitivity in CALU-1 cells. A–E: The cell viability (A), lipid reactive oxygen species (ROS) (B), relative MEF2C and glutathione peroxidase 4 (GPX4) protein expression levels (C), malondialdehyde contents (D), and the colony-forming abilities (E) of each group tested by the same method as shown in Figures 1, 2, and 4. *P < 0.05, **P < 0.01 (one-way analysis of variance). NC, negative control; OE, overexpression; sh, small hairpin.
Figure 8  The forkhead box M1 (FOXM1)/myocyte-specific enhancer factor 2C (MEF2C) axis causes ferroptosis insensitivity in NCI-H358 cells. A–E: The cell viability (A), lipid reactive oxygen species (ROS) (B), relative MEF2C protein expression levels (C), malondialdehyde contents (D), and the colony-forming abilities (E) of each group tested by the same method as shown in Figures 1, 2, and 4. *P < 0.05, **P < 0.01 (one-way analysis of variance). NC, negative control; OE, overexpression; sh, small hairpin.
Figure 9  Mutant (Mut) TP53 lung cancer cells are highly activated on the forkhead box M1 (FOXM1)/myocyte-specific enhancer factor 2C (MEF2C) axis when exposed to ferroptosis inducer in vivo. A: Flowchart of animal experiment. B: Tumor tissues derived from in vivo proliferation of CALU-1 cells exfoliated subcutaneously from nude mice. C: Tumor volume measurement. D: Tumor weight measurement. E: Malondialdehyde (MDA) concentration in tumors. F and G: Relative protein expression levels tested by immunohistochemistry (F) and Western blotting (G). n = 5. ***P < 0.001 (one-way analysis of variance). Scale bar = 100 μm. MAPK, mitogen-activated protein kinase; NC, negative control; OE, overexpression.
Figure 10  TP53/peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α)/forkhead box M1 (FOXM1)/myocyte-specific enhancer factor 2C (MEF2C) axis in clinical tissue samples. A and B: The relative protein expression levels tested by the same method as shown in Figure 8. C: The binding relationship between PGC1-α and TP53 via a co-immunoprecipitation (IP) assay. D: Molecular mechanism diagram of this study. Scale bar = 100 μm. GPX4, glutathione peroxidase 4; Mut, mutant; p-MAPK, phospho—mitogen-activated protein kinase; ROS, reactive oxygen species; WT, wild type.
differentiated and grouped according to wild-type or mutant TP53. As in animal tumors, the protein levels of FOXM1 and MEF2C in the wild-type group were lower than those in mutant groups (Figure 10, A and B). In addition, wild-type TP53 could immunoprecipitate PGC-1α, but it was not detected in tissue samples from mutant individuals (Figure 10C).

In general, wild-type TP53 bound PGC-1α in lung cancer tissues, accompanied by low expression of FOXM1 and MEF2C. Although TP53 was mutated, the binding was weak, and the expression of FOXM1 and MEF2C was increased. Mutant TP53 failed to bind to PGC-1α, allowing mutant TP53 to activate FOXM1/MEF2C axis through transcription, promote glutathione peroxidase 4 expression, inhibit the excessive accumulation of lipid reactive oxygen species, and thus inhibit the occurrence of ferroptosis (Figure 10D).

Discussion

Emerging evidence suggests that ferroptosis has unique advantages and great potential in the treatment of lung cancer.26,30 The tumor suppressor TP53-mediated regulation of ferroptosis, promotion or suppression, has been well studied in recent years.18 However, TP53 mutates frequently in lung cancers,20 and mutant TP53 renders oncogenic potentials and drug resistance in lung cancer cells, as opposed to that in the wild type.31,32 Therefore, exploring oncogenic activities of mutant TP53 and underlying mechanisms in lung cancer has clinical significance.

Previous studies have disputed the effect of TP53 mutation on ferroptosis,19,33 which may be related to the differences in TP53 mutation types and cell types simulated in various studies as well as the induction and detection methods of ferroptosis. The current study demonstrated that the TP53 (R248W) mutant increased the resistance of lung cancer cells to ferroptosis. Mechanically, the difference in ferroptosis between mutant TP53 (R284W) and wild-type TP53 is the differential effect on the transcription level of FOXM1, a key factor for MEF2C transcription in MAPK signaling. In general, a TP53/FOXM1/MEF2C-dependent regulation axis profoundly affected the response of lung cancer cells to ferroptosis inducers.

FOXM1 was widely overexpressed in many human cancers, including lung cancer,34 with undefined relationships with TP53 mutants and effects on ferroptosis. It has been reported that FOXM1 mRNA down-regulates in a TP53-dependent manner.35 This study demonstrated that FOXM1 overexpression was associated with TP53 mutations. Specifically, wild-type TP53 bound to PGC-1α to inhibit FOXM1 transcription, but TP53 (R284W) did not, leading to the release of FOXM1 transcriptional inhibition and thus increased expression. In other words, the TP53 and PGC-1α transcriptional complex controls FOXM1 expression and affects ferroptosis.

PGC-1α is a central modulator of cell metabolism, regulating mitochondrial biogenesis and oxidative metabolism, which is critical for ferroptosis.37 A study in high-grade serous ovarian cancer found that PGC-1α promotes the metabolic characteristics of high oxidative phosphorylation in ovarian cancer cells by enhancing mitochondrial respiration, making the cells more responsive to conventional radiotherapy or ferroptosis.38 The results of the current study indicate that wild-type TP53 is involved in the regulation of PGC-1α-mediated mitochondrial function of lung cancer cells and affects ferroptosis from a metabolic perspective, but TP53 (R284W) is not. Overexpression of TP53 (R284W) reduced oxygen dependence of tumor cells, led to ferroptosis resistance, and even accelerated malignant progression.

MEF2C is a very important effector in MAPK signaling pathway. Studies have found that p38-MAPK promotes B-cell proliferation by directly phosphorylating MEF2C through three residues in the C-terminal transcriptional activation domain.39 For ferroptosis, MEF2C was proposed as a new molecular target in ferroptosis-inducing therapies for meningioma.40 However, the effect of MEF2C on ferroptosis in lung cancer cells is unknown. The current study revealed that FOXM1 could directly bind to the promoter region of MEF2C and initiate its transcription. Overexpression of FOXM1 up-regulated MEF2C to help cells combat ferroptosis stress, indicating that TP53/FOXM1/MEF2C cascade played an indispensable role in ferroptosis in lung cancer cells.

Overall, the results of this study support that the mutant of TP53 (R248W), as well as FOXM1 and MEF2C, significantly inhibit ferroptosis in lung cancer cells, and may be related to changes in PGC-1α-dependent mitochondrial metabolism. However, the mechanism by which TP53 regulates ferroptosis is likely to be quite complex and may vary with mutation sites and mutation forms, and more studies are needed to understand it in depth.

Supplemental Data

Supplemental material for this article can be found at http://doi.org/10.1016/j.ajpath.2023.05.003.

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