Upregulation of Ferroptosis-Related Fanconi Anemia Group D2 is a Poor Prognostic Factor and Indicator of Tumor Immune Cell Infiltration in Lung Adenocarcinoma

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Research Article

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Abstract

Background: Fanconi anemia (FA) group D2 (FANCD2) is a ferroptosis-related gene crucial for DNA damage repair and negatively regulates ferroptosis. Our study aimed to evaluate its prognostic value as well as its association with ferroptosis and immune infiltration in lung adenocarcinoma (LUAD).

Methods: Transcriptome sequencing data and clinical information, three independent cohorts, as well as immunohistochemistry, were collected from the TCGA, GEO, and HPA databases, respectively. Univariate and multivariate analyses were used to assess the correlations between FANCD2 expression and overall survival or clinicopathological parameters. cBioPortal was utilized to investigate the FANCD2 alteration status. Gene and protein networks based on FANCD2 interactions were generated using GeneMANIA and STRING, respectively. Based on the CancerSEA database, the function of FANCD2 was explored at the single-cell level. The relationships between FANCD2 expression levels and tumor-infiltrating immune cells and their equivalent gene signatures were analyzed by TIMER, GEPIA, TISIDB, and ssGSEA databases. CIBERSORT was used to analyze the relevance of the infiltration of 24 types of immune cells.

Results: The results revealed that FANCD2 expression was significantly upregulated in LUAD and lung squamous cell carcinoma (LUSC) tissues than in normal tissues. Further, the overexpression of FANCD2 was closely associated with poor survival for LUAD patients but not for LUSC patients. FANCD2 expression levels were related to tumor-infiltrating immune cells and their matching gene signatures, including CD8+ T cells, NK cells, DC cells, and Th2 cells in cases of LUAD.

Conclusion: FANCD2 was identified as a crucial molecule underlying the synergistic effects of ferroptosis and immunotherapy for LUAD patients.

Introduction

In the past decades, lung cancer has remained the major contributing factor to cancer-related deaths worldwide. According to available data, 2.2 million new cases of lung cancer are diagnosed each year, with 1.8 million people dying from the disease annually[1]. Non-small cell lung cancer (NSCLC), which includes LUAD and LUSC, contributes to approximately 85% of all lung cancer cases. However, the majority of patients are in an advanced, unresectable stage of the disease at the time of diagnosis[2], which is associated with a low overall median 5-year survival rate of 15%[3]. The incidence and mortality of LUAD are increasing; it has now surpassed squamous cell carcinoma to become the most common histological subtype of NSCLC[4,5]. Despite the active treatment measures available for LUAD, it has the highest mortality rate among all cancers; this might be associated with its tendency to metastasize at an early stage[6-8]. Thus, it is of critical importance to develop novel and more effective therapeutic strategies for LUAD. For this, it is vital to more intensively probe the molecular pathology of the disease.

Ferroptosis is an iron-dependent form of regulated cell death induced by iron-dependent lipid peroxidation owing to metabolic dysfunction; it is distinct from apoptosis, cell necrosis, and autophagy[9,10]. The ferroptosis pathway can restrain tumor growth and induce cancer cell death. Its induction has thus received widespread attention as a potential novel antitumor treatment strategy[11-13]. Inducing ferroptosis has been reported to suppress LUAD by regulating lipid peroxidation to promote tumor cell death[14-16]. Ferroptosis is also connected to immunomodulation and immune evasion, in particular through extensive crosstalk between cancer cells and infiltrating immune cells. Different signals released during ferroptosis that occur in cancer cells can trigger macrophage-mediated phagocytosis in vitro[17]. In vivo experiments have also confirmed that blocking ferroptosis activity suppresses the ability of CD8+ T cells to kill cancer cells[18].

With the rapid advances in high-throughput sequencing technologies and transcriptome sequencing (RNA sequencing [RNA-seq]), an increasing number of key driver oncogenes are being discovered. However, it is necessary to identify additional key driver genes, particularly those affecting the tumor immune microenvironment (TME) in LUAD. FANCD2 is a nuclear protein that participates in DNA damage repair via the FA pathway[19]. FANCD2 depletion enhances interstrand crosslink (ICL) agent-induced DNA damage sensitivity and promotes apoptosis in lung cancer cells by inhibiting the FA pathway[20-22]. In addition, FANCD2 negatively regulates ferroptosis by regulating iron metabolism-related genes and/or protein expression and lipid peroxidation[23]. However, the molecular mechanisms of FANCD2 governing the regulation of the immune response in LUAD are still unclear.
This study aimed to explore the association between FANCD2 expression and clinical information and prognosis in LUAD. Based on bioinformatics techniques, the results showed that FANCD2 expression regulated the level of tumor-infiltrating immune cells through multiple pathways, which contributed to the formation of the immunosuppressive microenvironment. Therefore, this study thus anticipated presenting promising potential therapeutic strategies for LUAD based on FANCD2.

**Results**

**Hub genes associated with ferroptosis and NSCLC**

In total, 173 genes in the FerrDb and 2,414, 3,322, and 1,830 differentially expressed genes (DEGs) in GSE19188, GSE75037, and GSE116959, respectively were identified. As shown in Fig. 1, 17 candidate genes comprised the intersection of the four datasets. Of these, the top five targets were nicotinamide adenine dinucleotide phosphate (NAD[P]H) dehydrogenase (quinone 1) (NQO1), heme oxygenase 1 (HMOX1), FANCD2, helicase, lymphoid specific (HELLS), and cluster of differentiation 44 (CD44). Notably, in addition to participating in ferroptosis, FANCD2 has been confirmed to be associated with various cancers through the FA pathway. It sustains genomic stability by repairing DNA damage[20-22]. Therefore, FANCD2 was chosen for further analysis.

**Patient characteristics**

The RNA-seq expression data and clinical prognostic information of 1,037 NSCLC patients and 108 normal samples were obtained from the TCGA database. As shown in Table 1, the clinical information of NSCLC patients was summarized, including age, sex, smoking status, pathologic stage (T, N, or M), histologic grade, OS, disease-specific survival (DSS), and the progression-free interval (PFI).

**FANCD2 expression is higher in tumor samples than in normal tissues**

According to TCGA and GTEx databases, FANCD2 exhibited higher expression in 19 types of tumor tissues than in adjacent normal samples in TCGA database, including LUAD and LUSC (Fig. 2A). Correlation analysis results showed that the FANCD2 mRNA expression was significantly higher with older age (age > 65 years, P< 0.05), in males (P< 0.01), and in smoking patients (P < 0.05), as well as in higher M stage patients (P <0.05, Fig. 2B). Meanwhile, FANCD2 expression had no relation with other clinicopathological characteristics, such as pathological stage, T stage, and N stage (Fig. 2B). In addition, FANCD2 expression exhibited similar tendencies concerning the gender and age of LUSC patients ((P < 0.01 or (P < 0.05), but there were no obvious differences between FANCD2 expression and other variables (P 0.05, Fig. 2C). These results indicated the gene and protein expression levels of FANCD2 are significantly higher in LUAD and LUSC tissues.

Similarly, FANCD2 was also significantly elevated in NSCLC tissues compared to levels in normal tissues based on GSE19188, GSE75037, and GSE116959 datasets (all P< 0.001, Fig. 3A). In addition, the IHC results revealed that FANCD2 was overexpressed in LUSC and LUAD tissue in comparison with that in normal tissue according to HPA databases(Fig. 3B).

**High FANCD2 mRNA expression is related to short OS in LUAD patients**

As shown in Fig. 4A the 20-year OS, DSS, and PFI rates of LUAD patients were remarkably higher with low FANCD2 expression compared to those with high FANCD2 expression (P = 0.04, 0.03, and 0.09 respectively). However, there was no significant difference between FANCD2 expression and OS (P= 0.639), DSS (P= 0.68), and PFI (P= 0.492) in LUSC patients (Fig. 4B).

Univariate analysis showed that higher FANCD2 expression was related to poor OS (HR = 1.495, 95% CI = 1.120–1.994, P < 0.01) in LUAD patients (Fig. 5A). Multivariate Cox regression analysis was used to further explore the relevance of FANCD2 to OS. The results suggested that high FANCD2 expression was an independent predictor of OS (HR = 1.716, 95% CI = 1.195–2.465, P < 0.01, Fig. 5B). Therefore, FANCD2 was considered a risk factor in predicting a worse prognosis.

**Genetic alteration and interaction network analyses of FANCD2**

The cBioportal online tool was then performed to explore the types and frequencies of FANCD2 alterations in the LUAD patients. Results revealed that FANCD2 was highly conserved (only a 1.4% frequency of genomic alterations, Fig. 6A). Subsequently, the interaction networks showed 20 genes and 10 proteins with the highest relevance to FANCD2 (Fig. 6B). FANCI, FANCL, FAN1, FANCE,
FANCC, and USP1 appeared in two networks, for which correlation scores were 0.999, 0.999, 0.999, 0.999, and 0.998 respectively in the PPI network. This result implied that these genes and/or proteins could be functional partners of FANCD2 in LUAD.

Functions of FANCD2 in LUAD

To better understand the relevance of FANCD2 expression in LUAD and potential mechanism, single-cell analysis was utilized to explore the associated functional states based on the CancerSEA database. The results suggested that FANCD2 was positively correlated with cell cycle, DNA repair, and proliferation but negatively correlated with angiogenesis, quiescence, inflammation, metastasis, and differentiation (all P < 0.001, Fig. 7A and B). Moreover, overrepresentation enrichment analysis (ORA) illustrated that FANCD2 participated in the interleukin signaling pathway, de novo pyrimidine deoxyribonucleotide biosynthesis, DNA replication, de novo purine biosynthesis, arginine biosynthesis, angiotensin II-stimulated signaling through G proteins and beta-arrestin, the circadian clock system, and the EGF receptor signaling pathway (all P < 0.05, Fig. 7C). Notably, the interleukin signaling pathway had the highest correlation with FANCD2. It is well-known that interleukin family members play important roles in the immune response and inflammation.

Association between FANCD2 expression and immune-inhibitory and immune-stimulatory functions

A co-expression study was performed to investigate the relationship between FANCD2 expression and immunomodulators based on TISIDB, including immune inhibitors and immunostimulators. Heatmaps illustrated the association between FANCD2 expression and 12 immunoinhibitors and 28 immunostimulators across 30 tumors (Fig. 8). Among these immunoinhibitors, FANCD2 expression had positive associations with CD274 (Cor = 0.191, p = 1.22 e-05), CTLA4 (Cor = 0.157, p = 3.52 e-04), LAG3 (Cor = 0.206, p = 2.47 e-06), and PDCD1 (Cor = 0.137, p = 1.8 e-03) in LUAD (Fig. 8A). Regarding various immunostimulators, FANCD2 had negative associations with TNFSF13 (Cor = −0.466, p < 2.2 e-16), TMEM173 (Cor = −0.439, p < 2.2 e-16), CD40LG (Cor = −0.26, p = 2.41e-09), HHLA2 (Cor = −0.254, p = 4.97e-09), and IL6R (Cor = −0.288, p = 3.23e-11) (Fig. 8B).

Correlation between FANCD2 expression and infiltrating immune cells

Tumor-infiltrating lymphocytes are associated with the prognosis of patients with multiple cancers[24]. As shown in Fig. 9A, FANCD2 expression levels were strongly associated with levels of infiltrating B cells (Cor = −0.157, p = 4.84 e-04), CD8+ T cells (Cor = 0.127, p = 4.74 e-03), macrophages (Cor = 0.105, p = 1.97 e-02), and neutrophils (Cor = 0.232, p = 1.82 e-07) in LUAD cases. However, there was no association between FANCD2 expression and tumor purity, DCs, or CD4+ T cells. To gain more insight into the relationship between FANCD2 expression and immune infiltration, this study assessed subjects based on 24 types of infiltrating immune cells using the ssGSEA database (Fig. 9B). Specifically, FANCD2 was negatively related to B cells, CD8+ T cells, DC cells, macrophages, eosinophils, iDC(interdendritic) cells, mast cells, neutrophils, NK CD56 bright cells, pDC (plasmacytoid dendritic) cells, TFH cells, TH17 cells, and NK cells but was positively related to Th2 cells, T helper cells, and Tcm cells (all P < 0.001). Moreover, the heat map showed that most subpopulations among the 24 types of immune cells had moderate to strong relationships (Fig. 9C).

GEPIA and TIMER databases were used to further assess the correlation between FANCD2 and the marker sets of diverse immune cells in LUAD. Table 2 shows that multiple markers of immune cells were significantly related to FANCD2 expression, including Th2 (GATA3), Th9 (TGFB2), Th17 (IL-21R), Treg (FOXP3, CD25, CCR8), T cell exhaustion (PD-1, CTLA4, LAG3), tumor-associated macrophages (TAMs) (CD80, CCR5), and DCs (CD1C, CD141). These results implied that FANCD2 might affect the function of immune cells by modulating marker gene expression.

Discussion

LUAD is the most common subtype of lung cancer. Currently, the efficacy of surgery, radiotherapy, chemotherapy and targeted therapy is not satisfying. Notably, ferroptosis is a novel form of cell death, and an increasing body of research has confirmed that it plays a crucial role in anti-tumor treatment, especially in LUAD[15,25]. Moreover, a connection between ferroptosis and cell immunity and cancer immunotherapy has been shown, but the underlying mechanism is not clear[18]. The latest study showed that 76.9% of ferroptosis-related genes are differentially expressed between LUAD tumor tissues and adjacent normal tissues, and some of these DEGs were determined to be remarkably associated with OS[26]. Therefore, ferroptosis-based treatment could be a novel therapeutic strategy for LUAD.
As a nuclear protein, FANCD2 functions in maintaining a stable genome but also participates in the regulation of autophagy[20,21,27]. In addition, FANCD2 has a negative regulatory role in ferroptosis, and tumor cells with low expression of FANCD2 easily undergo ferroptosis. Specifically, FANCD2 deficiency contributes to lipid peroxidation through a decrease in glutathione peroxidase 4 (GPX4), as well as the accumulation of iron through an increase in the expression of transferrin (TF), decreasing ferritin heavy chain 1 (FTH1) and SLC40A1[19].

This study investigated the role of FANCD2 in LUAD progression and prognosis, as well as the relationship with immune cell infiltration(Fig.10). Based on TCGA, GEO, and HPA databases, the results showed that mRNA and protein expression of FANCD2 was upregulated in LUAD samples compared to levels in normal tissues, and its expression was associated with sex, smoking status, and age. KM plots suggested that higher FANCD2 was related to shorter OS, poor DSS, and worse PFI in LUAD patients. Moreover, univariate and multivariate Cox analysis further confirmed that high expression of FANCD2 was an independent adverse prognostic factor, which was consistent with the findings conducted by Lei, et al. (28). Therefore, FANCD2 was considered a poor prognostic biomarker for LUAD patients.

FANCD2 is a highly conserved gene, and GeneMANIA, STRING, CancerSEA, and ORA were conducted to further investigate its function. FA family proteins, such as FAN1, FANCI, and FANCE, were confirmed to be crucial genes and proteins that interact with FANCD2 molecules in LUAD through the FA pathway to maintain genomic stability. For this reason, the efficacy of DNA ICL agents and ionizing radiation are diminished[21]. Beyond this, single-cell analysis illustrated that FANCD2 participates in inflammation, and its intensity decreases with the expression of FANCD2. ORA results showed that FANCD2 participates in the interleukin signaling pathway. Numerous studies have confirmed that factors of the interleukin family, such as IL8, IL10, and IL17, are involved in immune responses and are associated with the outcome of patients[29-31].

Notably, LUAD tends to metastasize at an early stage, which results in a high mortality rate. Further, immune escape is the first crucial step for tumor initiation, progression, and metastasis in TME[32]. Tumor-infiltrating immune cells (TIICs) are an integral part of the TME, playing important roles in suppressing or promoting tumor cell growth. Therefore, remodeling the TME from immune-suppressive to immune-promoting could be a potential anti-tumor therapeutic for LUAD[32]. Recently, studies have shed light on the efficacy of immunotherapy. Immune checkpoint inhibitors targeting T-lymphocyte-associated antigen-4 (CTLA-4), programmed cell death-1 (PD-1), and programmed cell death ligand-1 (PD-L1) have been approved by Food and Drug Administration (FDA). They have been demonstrated to significantly improve the survival of advanced NSCLC patients[33,34].

TISIDB analysis was performed to further explore the mechanism underlying the association between FANCD2 and the TME. Intriguingly, the results revealed that FANCD2 is positively correlated with the expression of CTLA4, PDCD1, and LAG3, which indicated that FANCD2 could remodel the immune TME by blocking immune checkpoints. Moreover, FANCD2 plays an immunosuppressive role by downregulating immunostimulators, such as IL6R, TMEM173, TNFSF13, CD40LG, and HHLA2. Therefore, the results implied that FANCD2 contributes to tumor immune escape by modulating the immunosuppressive microenvironment, which was consistent with previous studies[35,36].

Co-expression analyses indicated that FANCD2 expression was remarkably negatively related to the infiltration of CD8+ T cells, NK cells, and DC cells but was positively related to Th2 cells. CD8+ T cells (Fig.10), as preferred cancer-targeting immunotherapy cells, through exocytosis and the release of perforin-granzyme and activation of caspases via the release of cytochrome c in cancer cells, contribute to tumor cell apoptosis[37]. NK cells are the first line of defense against tumors, and they not only release perforin and granzymes but also excrete various cytokines (IFN-γ, TNF), chemokines (IL-10), or growth factors (GM-CSF) to play a crucial role in antitumor effects and antiviral infection. Notably, IFN-γ enhances the function of antigen-presenting cells, inhibits angiogenesis, induces Th1 cells, and promotes M1 macrophage polarization, which remarkably increases the effect of immune surveillance and immune elimination in the TME[38]. In addition, CD141+ DCs express lymphotoxin beta transcripts to contribute to lymphocyte recruitment, the priming and proliferation of cytotoxic T cells, and CD1C+ subpopulations of DCs that promote the maintenance of immune memory[39,40]. In contrast, Th2 cells secrete anti-inflammatory factors such as IL-4, IL-5, IL-6, and IL-10 to weaken the anti-tumor immune response. GATA3, as the genetic marker of Th2 cells, was positively correlated with the FANCD2 level, and this not only promotes Th2 differentiation but also inhibits Th1 differentiation[41]. Hence, the Th2 shift in the TME is considered to promote tumor relapse, metastasis, and poor prognosis[42].
TAMs participate in angiogenesis and lymphangiogenesis, contributing to the progression of NSCLC[43]. However, this study showed that the FANCD2 expression level had no significant association with the infiltration of TAMs, but it could modulate the expression of CD80 and CCR5 to enhance the immunosuppressive function of TAMs. A previous study suggested that CD80 binds to the CTLA-4 receptor, inhibiting T-cell activation[44]. TAMs could independently stimulate tumor cell growth and migration via theCCL5/CCR1/CCR5 axis [45]. These findings revealed that FANCD2 plays a crucial role in recruiting different TIICs and regulating anti-tumor immunity.

In addition, Treg cell markers, such as FOXP3 and CD25, which have a crucial function in suppressing the antitumor immune response[46], were strongly correlated with FANCD2 expression. Several studies have documented that live tumor-infiltrating Tregs are related to poor prognosis in NSCLC patients[47]. However, apoptotic Treg cells also mediate immunosuppression via the adenosine and A2A pathways[48]. Intriguingly, Treg cells that undergo ferroptosis potentiate antitumor immunity, characterized by high ratios of cytotoxic CD8+ T cells to CD4+ T cells in the TME[49]. Therefore, inducing cell ferroptosis of Treg cells is also an antitumor treatment. An additional study demonstrated that activated CD8+ T cells release IFN-γ to restrain system xc- uptake cystine, promoting tumor cell lipid peroxidation and subsequently contributing to ferroptosis[18]. Meanwhile, enhancing ferroptosis has been shown to contribute to the efficacy of antitumor immunotherapy[18,50]. These results further confirmed that FANCD2 is a crucial molecule for synergistic ferroptosis and immunotherapy.

Inducing cancer cell ferroptosis is considered a promising anti-tumor treatment, especially in combination therapies[51,52]. An increasing number of studies have suggested that TME and tumor biology should be further studied, as they play crucial roles in identifying appropriate treatments for patients or even developing new therapeutic options[25,53-55]. Therefore, FANCD2 might be a powerful factor to predict patient outcomes, and its expression level could be a potential novel standard to screen LUAD patients suitable for ferroptosis therapy and/or immunotherapy.

There are several limitations to this study. First, all of the results were obtained retrospectively; more prospective data and prospective studies are needed to confirm the clinical efficacy. Second, the present study lacked in vitro and in vivo investigations to further explain the mechanisms whereby FANCD2 influences ferroptosis and immunotherapy. More efforts are needed to investigate the potential function of FANCD2 in LUAD.

Conclusions

This study systematically analyzed the role of FANCD2 in tumor progression, prognosis, and therapy for patients with LUAD. These results demonstrated that upregulated FANCD2 contributes to immune escape and is associated with worse outcomes for LUAD patients. This might be associated with FANCD2 participating in maintaining a stable tumor cell genome, protecting cells from ferroptosis, and constructing an immunosuppressive microenvironment. Furthermore, FANCD2 recruits immunosuppressive cells into the TME and regulates the expression of corresponding immune markers to weaken the anti-tumor immune response. Hence, FANCD2 was considered a novel potential bio-target for identifying patients who may benefit from ferroptosis-induction treatment and/or immunotherapy.

Methods

Identification of ferroptosis- and NSCLC-related targets

Ferroptosis-related targets were identified from FerrDb (http://www.zhounan.org/ferrdb), which has data on 253 regulators, 111 markers, and 95 ferroptosis-associated diseases. NSCLC-related targets were identified from the GEO database, which contains many bioinformatics datasets from the National Center of Biotechnology Information (https://www.ncbi.nlm.nih.gov/geo/). Three gene expression datasets (GSE75037, GSE19188, and GSE116959) derived from human NSCLC tissues and adjacent normal tissues were included. Genes with an adjusted P< 0.01 and |log2(fold-change)| >2 were defined as DEGs, which were considered to play essential roles in NSCLC progression and defined as key targets for inducing ferroptosis to treat NSCLC.

Data acquisition and analysis

TCGA (https://portal.gdc.cancer.gov/) is a freely available, large-scale, and publicly open-access cancer genomic database. All transcriptome RNA-seq data (n=1,145) and equivalent clinical data related to NSCLC were downloaded. Based on pathological
characteristics, all patients were divided into LUAD (n=516) and LUSC (n=493) groups. Subgroup analysis was performed to investigate the effect of FANCD2 on the pathological stage and outcome. Three independent cohorts including tumor tissues and control samples of NSCLC patients from the GEO databases GSE75037 (n=166), GSE19188 (n=156), and GSE116959 (n = 68) were used to confirm the findings from TCGA datasets. HPA (https://www.proteinatlas.org/) is a comprehensive resource database of the human proteome, and the protein levels of FANCD2 in normal lung tissues and LUAD and LUSC tissues were compared according to immunohistochemistry (IHC) results.

Survival and statistical analyses

Based on the median expression of FANCD2, patients with LUAD and LUSC were divided into high and low groups. To identify whether the FANCD2 expression level influenced LUAD and LUSC patient clinical survival, Kaplan–Meier (KM) survival curves were generated to estimate its prognostic significance.

Univariate and multivariate logistic regression analyses

To further identify the prognostic value of FANCD2 in LUAD, Cox analyses were adopted to evaluate the relationship among different clinical characteristics and prognosis. Univariate Cox analysis was conducted for every variable comparing the expression level of FANCD2 and patient overall survival (OS) in each cohort to confirm their association with LUAD prognosis. Subsequently, multivariate Cox analysis, including all variables, was used to evaluate whether FANCD2 was an independent prognostic factor for LUAD patient outcome.

Genetic alteration and interaction network analyses

Based on cBioPortal (http://cbioportal.org), an open-access multidimensional cancer genomics resource, FANCD2 alterations were analyzed in LUAD samples collected from TCGA. GeneMANIA (http://genemania.org/) and STRING (https://string-preview.org/) are source websites used to construct gene–gene and protein–protein interaction networks, respectively. Both were applied to investigate FANCD2-related genes and proteins.

Single-cell analysis

The CancerSEA (http://biocc.hrbmu.edu.cn/CancerSEA/home.jsp) database provides a cancer single-cell functional state atlas. This study used CancerSEA to explore the function of FANCD2-regulated genes, as well as the correlation between FANCD2 expression levels and these functions.

TISIDB database analysis

TISIDB (http://cis.hku.hk/TISIDB/), a web portal, is used to probe tumor-immune interactions, which was applied to evaluate correlations between FANCD2 and immune-suppressive genes and immune-activating genes in this study.

TIMER database analysis

TIMER (https://cistrome.shinyapps.io/timer/) is a web server for the systematic analysis of six tumor-infiltrating immune subsets across diverse cancer types. Here, the correlation between FANCD2 expression and the infiltration of B cells, CD8$^+$ T cells, CD4$^+$ T cells, dendritic cells (DCs), macrophages, and neutrophils was assessed in LUAD patients. Additionally, the association between FANCD2 expression and tumor purity was tested, according to the "Correlation" module of TIMER and the tumor purity-corrected partial.

ssGSEA and CIBERSORT analysis

In total, 24 types of FANCD2-related immune cells were acquired from ssGSEA using the GSVA package in R (4.0.3). Pearson correction analysis was performed to further assess the relevance and enrichment scores between FANCD2 and the different immune cells. CIBERSORT (http://cibersort.stanford.edu/), a deconvolution algorithm based on gene expression, was used to reveal the relevance of 24 types of immune cells in LUAD.

GEPIA database analysis
GEPIA (http://gepia.cancer-pku.cn/index.html) covers thousands of tumors and normal samples from TCGA and the Genotype-Tissue Expression Project (GTEx,http://www.gtexportal.org/home/index.html). It focuses on the analysis of RNA-seq data. The association between FANCD2 levels and multiple markers for various immune cells was evaluated according to the GEPIA database.

**Statistical analysis**

Student's *t*-tests and Wilcoxon tests were performed for assessing differences between two groups, and a Kruskal-Wallis test was used for more than two groups. To evaluate patient survival, KM curves, as well as univariate and multivariate logistic regression analyses, were conducted. Spearman or Pearson correlations were applied to calculate the relationship between FANCD2 and immune infiltration. Data analyses were based on R (v4.0.3), and *P* < 0.05 was considered to indicate statistical significance.

**Abbreviations**

FANCD2: Fanconi anemia (FA) group D2; LUAD, lung adenocarcinoma; LUSA, lung squamous cell carcinoma; NSCLC, Non-small cell lung cancer; TME, tumor immune microenvironment; TIICs, tumor-infiltrating immune cells; IHC, immunohistochemistry; OS, overall survival; TAMs, tumor-associated macrophages; DSS, disease-specific survival; PFI, progression-free interval; CTLA-4, T-lymphocyte-associated antigen-4; PD-1, programmed cell death-1; PD-L1, programmed cell death ligand-1; FDA, Food and Drug Administration; DEGs, differentially expressed genes; NQO1, nicotinamide adenine dinucleotide phosphate (NAD[P]H) dehydrogenase (quinone 1); HMOX1, heme oxygenase 1; CD44, cluster of differentiation 44.

**Declarations**

**Author contributions**

Jingtao Zhang wrote the original draft, Fei Xu and Minghao Guo prepared the figures and tables, Dongli Wang and Xiubao Chen analyzed the data, Minmin Yu downloaded the raw data from TCGA and GEO databases, Dexin Zhang reviewed the relevant literature, Weida Chen proofread the manuscript, and Fei Xu edited the draft and made revisions.

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**Availability of data and materials**

All data analyzed in this study were generated from public databases.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

All authors agreed with publication.

**Competing interests**

The authors declare that they have no conflict of interest.

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Tables

Table 1. Clinical characteristics of LUAD and LUSC patients based on the TCGA database

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### Table 2. Correlation between FANCD2 levels and markers of immune cells based on TIMER and GEPIA databases

| Clinical factors        | LUAD          |          |          |          |          |          |          |          |
|-------------------------|---------------|----------|----------|----------|----------|----------|----------|----------|
|                         | Low           | High     | P value  | Low      | High     | P value  |          |          |
| Age, n (%)              |               |          |          |          |          |          |          |          |
| Median (IQR)            | 67 (59, 74)   | 64 (59, 72) | 0.036    | 69 (63, 74) | 67 (60, 73) | 0.033    |          |          |
| <=65                    | 115 (22.3%)   | 140 (27.1%) | 0.043    | 87 (17.6%) | 104 (21.1%) | 0.170    |          |          |
| >65                     | 142 (27.5%)   | 119 (23.1%) |          | 158 (32%)  | 144 (29.2%) |          |          |          |
| Gender, n (%)           |               |          |          |          |          |          |          |          |
| Female                  | 158 (29.5%)   | 128 (23.9%) | 0.043    | 79 (15.7%) | 52 (10.4%) | 0.008    |          |          |
| Male                    | 109 (20.4%)   | 140 (26.2%) |          | 172 (34.3%) | 199 (39.6%) |          |          |          |
| Smoker, n (%)           |               |          |          |          |          |          |          |          |
| No                      | 47 (9%)       | 28 (5.4%)  | 0.018    | 8 (1.6%)  | 10 (2%)   | 0.797    |          |          |
| Yes                     | 210 (40.3%)   | 236 (45.3%) |          | 238 (48.6%) | 234 (47.8%) |          |          |          |
| T stage, n (%)          |               |          |          |          |          |          |          |          |
| T1                      | 98 (18.4%)    | 77 (14.5%)  | 0.031    | 61 (12.2%) | 53 (10.6%) | 0.416    |          |          |
| T2                      | 127 (23.9%)   | 162 (30.5%) |          | 138 (27.5%) | 156 (31.1%) |          |          |          |
| T3                      | 29 (5.5%)     | 20 (3.8%)   |          | 40 (8%)   | 31 (6.2%)  |          |          |          |
| T4                      | 11 (2.1%)     | 8 (1.5%)    |          | 12 (2.4%) | 11 (2.2%)  |          |          |          |
| N stage, n (%)          |               |          |          |          |          |          |          |          |
| N0                      | 175 (33.7%)   | 173 (33.3%) | 0.580    | 167 (33.7%) | 153 (30.8%) | 0.321    |          |          |
| N1                      | 44 (8.5%)     | 51 (9.8%)   |          | 58 (11.7%) | 73 (14.7%) |          |          |          |
| N2                      | 38 (7.3%)     | 36 (6.9%)   |          | 17 (3.4%) | 23 (4.6%)  |          |          |          |
| N3                      | 0 (0%)        | 2 (0.4%)    |          | 3 (0.6%)  | 2 (0.4%)   |          |          |          |
| M stage, n (%)          |               |          |          |          |          |          |          |          |
| M0                      | 187 (48.4%)   | 174 (45.1%) | 0.088    | 197 (47%)  | 215 (51.3%) | 0.270    |          |          |
| M1                      | 8 (2.1%)      | 17 (4.4%)   |          | 5 (1.2%)  | 2 (0.5%)   |          |          |          |
| Pathologic stage, n (%) |               |          |          |          |          |          |          |          |
| Stage I                 | 147 (27.9%)   | 147 (27.9%) | 0.455    | 128 (25.7%) | 117 (23.5%) | 0.491    |          |          |
| Stage II                | 61 (11.6%)    | 62 (11.8%) |          | 76 (15.3%) | 86 (17.3%) |          |          |          |
| Stage III               | 44 (8.3%)     | 40 (7.6%)   |          | 41 (8.2%) | 43 (8.6%)  |          |          |          |
| Stage IV                | 9 (1.7%)      | 17 (3.2%)   |          | 5 (1%)    | 2 (0.4%)   |          |          |          |

**Table 2.** Correlation between FANCD2 levels and markers of immune cells based on TIMER and GEPIA databases.
| Cell type  | Gene marker | None Cor | P | Purity Cor | P | Tumor R | P | Normal R | P |
|-----------|-------------|----------|----|------------|----|----------|----|----------|----|
| **B cell** | CD19        | -0.003   | 0.9410 | 0.004 | 0.9210 | -0.085 | 0.063 | 0.41 | 0.0011** |
|           | CD20(KRT20) | 0.027    | 0.5440 | 0.021 | 0.6440 | 0.071  | 0.12  | -0.039 | 0.77  |
|           | CD38        | 0.049    | 0.2720 | 0.064 | 0.1560 | -0.0043| 0.92  | 0.14  | 0.29  |
| **CD8+ T cell** | CD8A       | 0.201    | ***    | 0.231 | ***    | 0.078  | 0.085 | 0.11  | 0.41  |
|           | CD8B        | 0.192    | ***    | 0.202 | ***    | 0.12   | 0.0082** | 0.085 | 0.52  |
|           | BCL6        | 0.019    | 0.6600 | 0.024 | 0.5960 | 0.056  | 0.22  | 0.13  | 0.31  |
|           | ICOS        | 0.162    | 0.0002*** | 0.202 | ***    | 0.069  | 0.13  | 0.32  | 0.014** |
|           | CXCR5       | -0.013   | 0.7760 | 0.005 | 0.9150 | -0.098 | 0.032 | 0.4   | 0.0017*** |
| **Th1**   | T-bet (TBX21) | 0.156   | 0.0004*** | 0.184 | ***    | 0.035  | 0.44  | -0.033 | 0.8   |
|           | STAT4       | 0.081    | 0.0646 | 0.098 | 0.0291 | 0.033  | 0.47  | 0.31  | 0.18  |
|           | IL12RB2     | 0.457    | ***    | 0.483 | ***    | 0.22   | ***    | 0.14  | 0.29  |
|           | WSX1(IL27RA)| -0.047   | 0.2900 | -0.043 | 0.3410 | -0.058 | 0.21  | 0.24  | 0.071 |
|           | STAT1       | 0.44     | ***    | 0.472 | ***    | 0.3    | ***    | 0.12  | 0.36  |
|           | IFN-γ(IFNG) | 0.311    | ***    | 0.337 | ***    | 0.19   | 0.000026*** | 0.075 | 0.57  |
|           | TNF-α(TNF)  | 0.103    | 0.0194* | 0.124 | 0.0056** | 0.055 | 0.23  | 0.33  | 0.011* |
| **Th2**   | GATA3       | 0.174    | 0.0001*** | 0.204 | ***    | 0.19   | 0.000026*** | 0.03  | 0.82  |
|           | CCR3        | -0.02    | 0.6430 | 0.001 | 0.9890 | -0.015 | 0.74  | 0.11  | 0.41  |
|           | STAT6       | -0.07    | 0.1120 | -0.077 | 0.0858 | -0.028 | 0.55  | 0.27  | 0.042* |
|           | STAT5A      | 0.055    | 0.2140 | 0.076 | 0.0925 | -0.0088| 0.85  | 0.44  | 0.00056** |
| **Th9**   | TGFBR2      | -0.13    | 0.0032** | -0.123 | 0.0064*** | -0.12 | 0.0077*** | -0.19 | 0.14  |
|           | IRF4        | 0.062    | 0.1620 | 0.077 | 0.0858 | -0.068 | 0.13  | 0.44  | 0.000053** |
|           | PU.1(SPI1)  | -0.039   | 0.3770 | -0.038 | 0.4040 | -0.11  | 0.015* | 0.24  | 0.071 |
| **Th17**  | STAT3       | 0.023    | 0.5980 | 0.021 | 0.6350 | 0.021  | 0.64  | 0.12  | 0.38  |
|           | IL-21R      | 0.149    | 0.0007*** | 0.188 | ***    | 0.037  | 0.41  | 0.26  | 0.05  |
|           | IL-23R      | 0.002    | 0.9580 | 0.006 | 0.9010 | -0.033 | 0.47  | 0.11  | 0.39  |
|           | IL-17A      | 0.089    | 0.0436* | 0.089 | 0.0494 | 0.073  | 0.11  | 0.17  | 0.19  |
| **Th22**  | CCR10       | 0.094    | 0.0321* | 0.08 | 0.0774 | 0.11   | 0.013* | -0.086 | 0.52  |
|           | AHR         | -0.014   | 0.7550 | -0.014 | 0.7500 | -0.012 | 0.79  | 0.003 | 0.98  |
| **Treg**  | FOXP3       | 0.157    | 0.0004*** | 0.183 | ***    | 0.04   | 0.38  | 0.42  | 0.001** |
|           | CD25(IL2RA) | 0.24     | ***    | 0.271 | ***    | 0.15   | 0.00068 | 0.11  | 0.39  |
|           | CCR8        | 0.183    | ***    | 0.214 | ***    | 0.064  | 0.16  | 0.21  | 0.11  |
| T cell exhaustion | PD-1(PDCD1) | CTLA4 | LAG3 | TIM-3(HAVCR2) | Macrophage | CD68 | CD11b(ITGAM) | INOS(NOS2) | IRF5 | COX2(PTGS2) | M1 | CD16 | ARG1 | MRC1 | MS4A4A | TAM | CCL2 | CD80 | CD86 | CCR5 | Monocyte | CD14 | CD16(FCGR3B) | CD115(CSF1R) | Neutrophil | CD66b(CEACAM8) | CD15(FUT4) | CD11b(ITGAM) | Natural killer cell | XCL1 | CD7 | KIR3DL1 | Dendritic cell | CD1C(BDCA-1) | CD141(THBD) | CD11c |
|-------------------|-------------|-------|------|--------------|------------|------|--------------|-----------|------|-------------|----|-------|-------|-------|--------|-----|-------|------|-------|------|---------|------------|--------------|----------------|-----------------|-----------------|---------------------|-------|------|------|----------------|---------------|---------------|-------|
|                   | 0.219       | 0.24  | 0.265| 0.074        | 0.034      | 0.006| 0.042        | 0.042     | 0.125| 0.016       | 0.042| 0.042| -0.001| -0.083| -0.001| 0.074| 0.074| 0.129| 0.078| 0.14 | 0.041   | 0.042         | -0.23       | -0.016         | 0.074          | 0.042         | -0.23             | 0.225 | 0.191| 0.035| -0.339           | -0.183       | -0.133         | 0.213 |
|                   | ***         | ***   | ***  | ***          | 0.083      | 0.29  | 0.286        | 0.0951    | 0.134| 0.7150      | 0.042| 0.055| -0.011 | -0.079 | -0.007 | 0.0921| 0.081| 0.153| 0.0755| 0.14  | 0.3530| 0.3440      | 0.055        | 0.086           | 0.261          | 0.029         | 0.055             | 0.261 | 0.219| 0.427 | 0.339           | -0.188       | 0.133         | 0.026 |
|                   |             |       |      | ***          | 0.068      | 0.14  | 0.029        | 0.088     | 0.134| 0.7150      | 0.042| 0.055| -0.011 | -0.079 | -0.007 | 0.0921| 0.081| 0.153| 0.0755| 0.14  | 0.3530| 0.3440      | 0.055        | 0.086           | 0.261          | 0.029         | 0.055             | 0.261 | 0.219| 0.427 | 0.339           | -0.188       | 0.133         | 0.026 |
|                   |             |       |      | ***          | 0.38       | 0.029 | 0.0086       | 0.0516    | 0.087| 0.7150      | 0.042| 0.055| -0.011 | -0.079 | -0.007 | 0.0921| 0.081| 0.153| 0.0755| 0.14  | 0.3530| 0.3440      | 0.055        | 0.086           | 0.261          | 0.029         | 0.055             | 0.261 | 0.219| 0.427 | 0.339           | -0.188       | 0.133         | 0.026 |
|                   |             |       |      |             | 0.0029**   |       |             | 0.032    | 0.087| 0.7150      | 0.042| 0.055| -0.011 | -0.079 | -0.007 | 0.0921| 0.081| 0.153| 0.0755| 0.14  | 0.3530| 0.3440      | 0.055        | 0.086           | 0.261          | 0.029         | 0.055             | 0.261 | 0.219| 0.427 | 0.339           | -0.188       | 0.133         | 0.026 |

None: correlation without adjustment, Purity: correlation adjusted by purity, Cor, correlation coefficient. *P < 0.05, **P < 0.01, and ***P < 0.001.

**Figures**
Figure 1

Workflow of target screening. Venn diagram showing DEGs in NSCLC from three GEO cohorts alongside ferroptosis-related genes.
Figure 2

Pan-cancer FANCD2 expression status and clinical characteristics in the LUAD and LUSC sub-groups. (A) FANCD2 expression status in different tumor tissues and adjacent normal tissues. (B, C) Comparison of FANCD2 expression levels with different clinical characteristics. *P < 0.05; **P < 0.01; ***P < 0.001.

Figure 3

FANCD2 expression in GEO datasets and the HPA database. (A) FANCD2 mRNA expression levels were higher in tumors than in normal tissues based on GSE19188 and GSE116959 datasets; P < 0.01. (B) FANCD2 protein levels in LUAD and LUSC tissues and normal samples based on the HPA database.
Kaplan–Meier (K-M) survival curves showing the association between FANCD2 expression levels and overall survival (OS), diseasespecific survival (DSS), and the progression-free interval (PFI) for LUAD and LUSC patients; P < 0.05 was used to assess differences.

### Univariate analysis

| Characteristics | Total(N) | HR(95% CI) | P value |
|-----------------|----------|------------|---------|
| Age <=65        | 516      |            |         |
| Age >65         | 261      | 1.223 (0.916±1.635) | 0.172   |
| Gender Female   | 280      |            |         |
| Gender Male     | 246      | 1.070 (0.803±1.426) | 0.642   |
| T stage T1&T2   | 457      |            |         |
| T stage T3&T4   | 66       | 2.317 (1.591±3.375) | <0.001  |
| N stage N0      | 343      |            |         |
| N stage N1&N2&N3| 167      | 2.601 (1.944±3.480) | <0.001  |
| M stage M0      | 352      |            |         |
| M stage M1      | 25       | 2.136 (1.248±3.653) | 0.006   |
| Pathologic stage| 518      |            |         |
| Stage I&Stage II| 411      |            |         |
| Stage III&Stage IV| 107 | 2.664 (1.960±3.621) | <0.001  |
| Smoker Yes      | 440      | 0.894 (0.592±1.348) | 0.591   |
| FANCD2 High     | 263      | 1.495 (1.120±1.994) | 0.006   |

### Multivariate analysis

| Characteristics | Total(N) | HR(95% CI) | P value |
|-----------------|----------|------------|---------|
| Age <=65        | 516      |            |         |
| Age >65         | 261      | 1.369 (0.960±1.954) | 0.083   |
| Gender Female   | 280      |            |         |
| Gender Male     | 246      | 0.999 (0.702±1.420) | 0.994   |
| T stage T1&T2   | 457      |            |         |
| T stage T3&T4   | 66       | 2.189 (1.356±3.534) | 0.001   |
| N stage N0      | 343      |            |         |
| N stage N1&N2&N3| 167      | 1.970 (1.304±2.975) | 0.001   |
| M stage M0      | 352      |            |         |
| M stage M1      | 25       | 1.240 (0.640±2.400) | 0.524   |
| Pathologic stage| 518      |            |         |
| Stage I&Stage II| 411      |            |         |
| Stage III&Stage IV| 107 | 1.340 (0.808±2.223) | 0.257   |
| Smoker Yes      | 440      | 0.950 (0.570±1.586) | 0.846   |
| FANCD2 High     | 263      | 1.716 (1.195±2.465) | 0.003   |

Figure 5

Univariate and multivariate analyses of FANCD2 expression and important clinicopathological parameters with respect to prognosis among LUAD patients.
Figure 6

Genomic alterations of FANCD2 and the interaction network and protein interaction network of FANCD2. (A) Genomic alterations of FANCD2 based on cBioPortal. (B) The gene–gene interaction network of FANCD2, as constructed with GeneMANIA. (C) The protein–protein network of FANCD2, derived from STRING.
Figure 7

The function of FANCD2 in LUAD. (A, B) The single-cell analysis shows that FANCD2 is involved in the cell cycle, DNA repair, proliferation, angiogenesis, quiescence, inflammation, metastasis, and differentiation. (C) The biological processes related to FANCD2 are based on ORA.
Figure 8

Co-expression analysis of FANCD2 with immunoinhibitory and immunostimulatory genes in the pan-cancer database, as well as detailed information on LUAD.

Figure 9

Correlation between FANCD2 expression and immune infiltration levels in LUAD. (A) Correlation analysis of FANCD2 expression and the infiltration of six types of immune cells based on TIMER. (B) Forest plots show that FANCD2 expression was positively correlated with 8 types of immune cells and negatively correlated with 16 subsets of immune cells. The sizes of dots represent the absolute value of Pearson r. (C) Heatmap showing the relationship among 24 types of immune cells in LUAD. *$P < 0.05$, **$P < 0.01$. 
Figure 10

The mechanism of FANCD2 regulating ferroptosis and immune cell infiltration.