

Identification of SLC7A5 and transferrin as key ferroptosis modulators in triple-negative breast cancer

RUIDI LIU¹, XIAO CUI², BINGYU GUO¹, YITING LIU^{3,4}, FENG LIN², ZHIQIANG FU¹ and BING XIE¹

¹Department of Burn, Plastic and Microsurgery, The Second Affiliated Hospital of Shenyang Medical College, Shenyang, Liaoning 110034, P.R. China; ²Department of Burn and Plastic Surgery, The Fourth Affiliated Hospital of China Medical University, Shenyang, Liaoning 110032, P.R. China; ³Department of General Surgery, The Second Affiliated Hospital of Shenyang Medical College, Shenyang, Liaoning 110034, P.R. China; ⁴Department of General Surgery, The Fourth Affiliated Hospital of Liaoning University of Traditional Chinese Medicine (Liaoning Provincial Hospital of Integrated Traditional and Western Medicine), Shenyang, Liaoning 110101, P.R. China

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Abstract. Triple-negative breast cancer (TNBC) lacks effective targeted therapies; however, ferroptosis represents a promising therapeutic vulnerability. The present study aimed to identify and functionally characterize ferroptosis-related genes that influence TNBC progression and prognosis. RNA-sequencing data and clinical information for TNBC were obtained from The Cancer Genome Atlas and FerrDB V2 databases. Subsequently, differential expression analysis, Cox regression and functional enrichment analyses were performed. The core genes solute carrier family 7 member 5 (SLC7A5) and transferrin (TF) were selected and validated in TNBC clinical samples by western blot analysis and reverse transcription-quantitative PCR in four paired tissues and 20 paired tissues, respectively. Functional assays (Cell Counting Kit-8 and Transwell assays), and the detection of ferroptosis markers [including glutathione (GSH), malondialdehyde (MDA), reactive oxygen species (ROS)] and core ferroptosis proteins [GSH peroxidase 4 (GPX4), solute carrier family 7 member 11 (SLC7A11), TF receptor 1 (TFRC), ferritin heavy chain 1 (FTH1), acyl-CoA synthetase long-chain family member 4 (ACSL4) and 4-hydroxy-2-nonenal (4-HNE)] were conducted in both MCF-7 [estrogen receptor (ER)-positive] and MDA-MB-231 (TNBC) cells following SLC7A5 knockdown or TF overexpression. The involvement of ferroptosis was confirmed using the specific inhibitor ferrostatin-1 (Fer-1).

A total of 93 ferroptosis-associated differentially expressed genes were identified in TNBC. In addition, SLC7A5 and TF were established as independent prognostic factors. High SLC7A5 mRNA expression was associated with poor prognosis [hazard ratio (HR)=1.73; P<0.001 for overall survival (OS)] and advanced pathological stage, whereas high TF mRNA expression predicted favorable outcomes (HR=0.61; P=0.003 for OS) and showed notable diagnostic power (area under the curve=0.935). In both MCF-7 and MDA-MB-231 cells, SLC7A5 knockdown and TF overexpression inhibited cell proliferation and migration while inducing ferroptosis, as evidenced by increased ROS/MDA, depleted GSH, decreased GPX4 and SLC7A11, increased TFRC and FTH1, and upregulated ACSL4/4-HNE; notably, all of these effects were reversed by Fer-1 treatment. In conclusion, the present study established SLC7A5 as a ferroptosis suppressor and oncogenic driver, and TF as a ferroptosis inducer and tumor suppressor in TNBC. This was validated in both ER-positive and TNBC cell lines; therefore, the present findings provide novel mechanistic insights and identify both genes as promising diagnostic biomarkers and therapeutic targets for ferroptosis-based TNBC therapy.

Introduction

Breast cancer (BC) remains the most frequently diagnosed malignancy and the leading cause of cancer-related mortality among women worldwide, posing a notable threat to public health (1). Based on the expression of estrogen receptor (ER), progesterone receptor and human epidermal growth factor receptor 2, BC is classified into distinct molecular subtypes, among which triple-negative BC (TNBC) accounts for 15-20% of all cases (2-4). TNBC is characterized by the absence of all three receptors, leading to a lack of effective targeted therapies and a reliance on conventional chemotherapy. Patients with TNBC often present at a younger age, with early metastasis, high recurrence rates and chemoresistance, collectively contributing to a poor prognosis (5-7). Therefore, there is a need to elucidate the molecular mechanisms underlying TNBC

Correspondence to: Dr Zhiqiang Fu or Dr Bing Xie, Department of Burn, Plastic and Microsurgery, The Second Affiliated Hospital of Shenyang Medical College, 64 Qishan West Road, Shenyang, Liaoning 110034, P.R. China
E-mail: andyfuqiang@163.com
E-mail: 999.xbing@163.com

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progression and to identify novel biomarkers and therapeutic targets.

Ferroptosis, a non-apoptotic form of regulated cell death first described in 2012, is driven by iron-dependent lipid peroxidation and has emerged as a notable pathway in cancer biology (8-10). Unlike apoptosis or necrosis, ferroptosis is characterized by the accumulation of reactive oxygen species (ROS) and the depletion of glutathione (GSH), leading to oxidative damage of cellular membranes. Notably, TNBC cells have been reported to exhibit unique sensitivity to ferroptosis induction, suggesting that targeting this pathway may represent a promising therapeutic strategy (11,12); however, the key molecular regulators of ferroptosis in TNBC remain to be elucidated.

Solute carrier family 7 member 5 (SLC7A5), also known as L-type amino acid transporter 1 (LAT1), is a transmembrane transporter that mediates the uptake of essential amino acids such as leucine, thereby supporting cancer cell proliferation and mTOR signaling (13-15). SLC7A5 is frequently upregulated in various types of cancer, including cholangiocarcinoma, gastric cancer and pancreatic cancer, and its high expression is associated with poor prognosis (16,17). Nevertheless, to the best of our knowledge, its specific role in regulating ferroptosis in TNBC has not been explored. Transferrin (TF) is the major iron-binding protein responsible for delivering ferric iron to cells via the TF receptor 1 (TFRC) (18). Given that iron accumulation is a prerequisite for ferroptosis, TF serves a central role in this process. While TFRC has been extensively studied in cancer and ferroptosis, the direct contribution of TF itself to ferroptosis regulation in TNBC remains poorly understood. To date, the interplay between SLC7A5, TF and ferroptosis in TNBC has not been systematically investigated. The present study aimed to identify ferroptosis-related core genes in TNBC using bioinformatics analysis and to validate their functional importance in regulating ferroptosis *in vitro*.

Materials and methods

Data acquisition and preprocessing. RNA sequencing data in transcripts per million (TPM) format and corresponding clinical data for BC were retrieved from The Cancer Genome Atlas Breast Invasive Carcinoma (TCGA-BRCA) dataset [Genomic Data Commons (GDC) Project ID: TCGA-BRCA; <https://portal.gdc.cancer.gov>]. This dataset initially comprised 1,226 samples, including 1,113 breast tumor tissues and 113 adjacent normal tissues. Clinical supplementary data for 1,098 patients were further downloaded from the same TCGA-BRCA project via the GDC data portal (<https://portal.gdc.cancer.gov/>). A list of 649 human ferroptosis-related genes (FRGs) was obtained from the FerrDB V2 database (<http://www.zhounan.org/ferrdb/current/>).

Clinical data were filtered to retain only female patients who had not received neoadjuvant treatment and samples lacking corresponding clinical information were excluded. Ensembl IDs in the transcriptome dataset were converted to gene symbols based on TCGA annotations, and a new expression matrix was generated by extracting protein-coding genes. Expression values were normalized using $\log_2$ (TPM+1). After removing normal tissue samples, 117 cases identified as TNBC were specifically extracted from the tumor samples based on

clinical and phenotypic data from the GDC dataset. Among the 117 patients with TNBC included, the median age was 54.1 years (range, 29.6-91.4 years).

Differential expression analysis and functional enrichment. Differential expression analysis between TNBC tumor tissues and adjacent normal tissues was performed using TCGA-BRCA dataset (117 TNBC tumor tissues and 112 adjacent normal tissues) with the 'limma' package (version 3.58.1; <https://bioconductor.org/packages/release/bioc/html/limma.html>) in R (version 4.3.3; R Foundation for Statistical Computing). Genes with an adjusted P-value [false discovery rate (FDR)] <0.05 and an absolute $\log_2$ fold change ($|\log_2FC|$) >1 were defined as differentially expressed genes (DEGs). A volcano plot was generated for visualization using the 'ggplot2' package (version 3.4.4; <https://ggplot2.tidyverse.org>). The identified DEGs were intersected with the FRG list obtained from the FerrDB V2 database to obtain ferroptosis-related differential genes (FR-DEGs). A Venn diagram was plotted using the 'VennDiagram' package (version 1.7.3; <https://cran.r-project.org/package=venndiagram>), and the expression patterns of the FR-DEGs were visualized via a heatmap created with the 'pheatmap' package (version 1.0.12; <https://cran.r-project.org/package=pheatmap>).

To elucidate the biological functions and pathways associated with the FR-DEGs, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were conducted using the DAVID online database (<https://davidbioinformatics.nih.gov/>). Terms with $P<0.05$ were considered significantly enriched. The results were visualized using the 'ggplot2' package in R software. To further explore the biological significance of DEGs and validate the associated functional pathways, a combined GO-KEGG enrichment analysis incorporating expression fold-change values was performed. DEGs were identified based on $|\log_2FC|>1$ and adjusted $P<0.05$. All expressed genes were ranked according to their $\log_2FC$ values, and the ranked gene list was used as the input for the enrichment analysis. Gene symbols were converted to Entrez Gene IDs using the bitr function from the 'clusterProfiler' package (version 4.4.4; <https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html>) in R software. To further explore the biological significance of DEGs and validate the associated functional pathways, a combined GO-KEGG enrichment analysis incorporating expression fold-change values was performed. DEGs were identified based on $|\log_2FC|>1$ and adjusted $P<0.05$. All expressed genes were ranked according to their $\log_2FC$ values, and the ranked gene list was used as the input for the enrichment analysis. Gene symbols were converted to Entrez Gene IDs using the bitr function from the 'clusterProfiler' package (version 4.4.4; <https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html>) in R software. Specifically, in this combined analysis, GO enrichment analysis, including biological processes (BP), cellular components and molecular functions, was performed using the 'clusterProfiler' package with the 'org.Hs.eg.db' annotation database (version 3.15.0; <https://bioconductor.org/packages/release/data/annotation/html/org.Hs.eg.db.html>) in R software. Similarly, KEGG pathway enrichment analysis was performed using the enrichKEGG function from the same

'clusterProfiler' package. GO terms and KEGG pathways with an adjusted $P < 0.05$ were considered significantly enriched.

Core gene screening and clinical correlation analysis. Univariate and multivariate Cox proportional hazards regression analyses were employed to screen for core genes significantly associated with the prognosis of patients with TNBC ($n=117$). These analyses were performed using the 'survival' package (version 3.5-7; <https://cran.r-project.org/package=survival>). Genes with $P < 0.1$ in the univariate analysis were included in the subsequent multivariate analysis. The multivariate model was adjusted for the expression levels of other genes that were significant in the univariate analysis. Clinical variables such as age, T stage, N stage, M stage and pathological stage were not included as covariates in this model. Two genes, SLC7A5 and TF, were ultimately identified as core genes; univariate logistic regression was then used to assess the associations between the expression levels of SLC7A5 and TF and various clinicopathological features.

The diagnostic value of SLC7A5 and TF expression in distinguishing TNBC was evaluated by constructing receiver operating characteristic (ROC) curves and calculating the area under the curve (AUC) using the 'pROC' package (version 1.18.5; <https://cran.r-project.org/package=pROC>) in R. Kaplan-Meier survival analysis was performed using the 'survival' package and the 'survminer' package (version 0.4.9; <https://cran.r-project.org/package=survminer>) to investigate the relationship between the expression of these core genes and patient overall survival (OS). Considering late crossover of the survival curves, which may violate the proportional hazards assumption of the log-rank test, the two-stage test implemented in the 'TSHRC' package (version 0.1.1; <https://cran.r-project.org/package=TSHRC>) was adopted for primary survival statistical inference. Hazard ratios (HRs) with 95% confidence intervals (CIs) and two-stage-test-derived P-values are shown in the relevant figure. The log-rank test was additionally performed as a supplementary sensitivity analysis, and consistent results were obtained between the two-stage test and log-rank test.

Experimental validation

Cell culture and tissue samples. The human BC cell lines MCF-7 and MDA-MB-231 were purchased from the American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle Medium (high glucose; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin at 37°C in a humidified incubator with 5% CO₂. Human TNBC tissue samples and paired adjacent normal tissues [$n=4$ pairs for western blot analysis and $n=20$ pairs for reverse transcription-quantitative PCR (RT-qPCR)] were obtained from The Second Affiliated Hospital of Shenyang Medical College (Shenyang, China). All patients were female, with a mean age of 58.2 years (range, 34.2-73.1 years). The samples were collected between June 2023 and June 2025. The present study was approved by the Ethics Committee of The Second Affiliated Hospital of Shenyang Medical College (approval no. fs-023; dated June 2023), and written informed consent was obtained from all patients prior to sample collection. All patients agreed to the use of their samples in scientific

research. Tissues were immediately frozen in liquid nitrogen and stored at -80°C.

Cell transduction and transfection. For knockdown of SLC7A5, a short hairpin RNA (shRNA) targeting human SLC7A5 (shSLC7A5; target sequence: 5'-GCATTATACAGC GGCCTCTTT-3') was purchased from Shanghai GeneChem Co., Ltd. As a negative control (NC) scrambled shRNA (shNC; target sequence: 5'-TTCTCCGAACGTGTCACGT-3') was used. The shRNA-expressing lentiviral vector (based on the pLKO.1-puro backbone; Shanghai GeneChem Co., Ltd.), together with the packaging plasmids pMD2.G and psPAX2 (second-generation lentiviral system; Shanghai GeneChem Co., Ltd.), was co-transfected into 293T cells (ATCC) at a ratio of 4:3:1 (shRNA vector:pMD2.G:psPAX2) with a total of 10 μ g DNA using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The transfection complexes were incubated at 37°C for 6 h, after which the medium was replaced with fresh complete culture medium. At 48 and 72 h post-transfection, the virus-containing supernatants were collected and pooled together, and were then filtered through a 0.45- μ m filter (MilliporeSigma); the pooled supernatant was used immediately or stored at -80°C. MCF-7 and MDA-MB-231 cells were then infected with the lentiviral particles in the presence of 10 μ g/ml polybrene (MilliporeSigma) at a multiplicity of infection of 10 for 24 h at 37°C. Stably transduced cells were selected with 2 μ g/ml puromycin (MilliporeSigma) for 2 weeks and maintained in medium containing 2 μ g/ml puromycin. For overexpression of TF, the Flag-TF plasmid (pcDNA3.1-Flag-TF; BT Lab) or the empty pcDNA3.1 vector (BT Lab) was used. MCF-7 and MDA-MB-231 cells were seeded in 6-well plates at a density of 5×10^5 cells/well (70-80% confluence) and were transfected with 2 μ g plasmid DNA using Lipofectamine 2000. After incubation at 37°C for 6 h, the medium was replaced with fresh complete culture medium and the cells were harvested 48 h after transfection.

Ferrostatin-1 (Fer-1) treatment. MCF-7 and MDA-MB-231 cells were treated with the ferroptosis-specific inhibitor Fer-1 (cat. no. S7243; Selleck Chemicals). Fer-1 was dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution. Briefly, cells were seeded and cultured overnight, and then exposed to Fer-1 at a final working concentration of 1 μ mol/l at 37°C for 24 h before subsequent experiments. An equal volume of DMSO was added to the control group cells to exclude solvent effects.

Western blot analysis. Tissues or cells were lysed on ice using RIPA buffer (Wuhan Servicebio Technology Co., Ltd.) containing protease inhibitor cocktail and phenylmethylsulfonyl fluoride. Protein concentration was determined using a BCA assay kit (Biosharp Life Sciences). Equal amounts of protein (30 μ g) were then separated by SDS-PAGE on 10% gels and transferred onto PVDF membranes. After blocking with 5% skimmed milk at room temperature for 1 h, the membranes were incubated overnight at 4°C with primary antibodies against SLC7A5 (1:1,000; cat. no. 67951-1-Ig; Proteintech Group, Inc.), TF (1:1,000; cat. no. 81676-1-RR; Proteintech Group, Inc.), 4-HNE (1:1,000; cat. no. ab46545; Abcam), ACSL4 (1:1,000; cat. no. A95060; Abclonal) and GAPDH (loading control; 1:5,000; cat. no. 60004-1-Ig; Proteintech Group, Inc.). After washing, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (1:5,000;

Table I. Primer sequences used for reverse transcription-quantitative PCR.

Gene	Forward primer, 5'-3'	Reverse primer, 5'-3'
SLC7A5	TGTTTTCAAGCACAACCC	AATAAACATGGTGCCGACA
TF	TCAGCAGAGACCACCGAAGACT	GACCACACTTGCCCGTATGTA
GAPDH	CCAGAACATCATCCCTGCCT	CCTGCTTCACCACCTTCTTG

SLC7A5, solute carrier family 7 member 5; TF, transferrin.

cat. no. SA00001-2) or HRP-conjugated goat anti-mouse IgG (1:5,000; cat. no. SA00001-1) (both from Proteintech Group, Inc.) for 1 h at room temperature. Protein bands were then visualized using an enhanced chemiluminescence kit (Biosharp Life Sciences) and captured with the WB-600Pro Automatic Western Blot Processing System (Guangzhou Biolight Biotechnology Co., Ltd.). The blots shown are representative of at least three independent experiments.

RT-qPCR. Total RNA was extracted from 20 paired TNBC tumor and adjacent normal tissue samples using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. A concentration of 1 µg total RNA was reverse transcribed into cDNA using a PrimeScript RT Reagent Kit (Takara Bio, Inc.) according to the manufacturer's protocol. qPCR was performed using SYBR Green Premix Ex Taq II (Takara Bio, Inc.) on a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). The thermal cycling conditions were as follows: Initial denaturation at 95°C for 30 sec, followed by 40 cycles at 95°C for 5 sec and 60°C for 30 sec. The relative mRNA expression levels of SLC7A5 and TF were calculated using the $2^{-\Delta\Delta C_q}$ method (19) and normalized to the endogenous control gene GAPDH. The primer sequences used for RT-qPCR are listed in Table I.

Cell migration and invasion assays. Cell migration and invasion were assessed using 24-well Transwell chambers (8-µm pore size). For the invasion assay, the upper chamber was pre-coated with Matrigel (BD Biosciences) at 37°C for 1 h. MCF-7 and MDA-MB-231 cells suspended in serum-free medium were seeded into the upper chamber at a density of 5×10^4 cells/insert, while the lower chamber contained medium with 15% FBS as a chemoattractant. After 24 h of incubation at 37°C in a 5% CO₂ incubator, cells that had migrated/invaded to the lower surface were fixed with 4% paraformaldehyde at room temperature for 15 min, stained with 0.5% crystal violet at room temperature for 20 min and counted under an inverted light microscope.

Cell proliferation assay. Cell proliferation was measured using the Cell Counting Kit-8 (CKK-8; TargetMol Chemicals, Inc.). MCF-7 and MDA-MB-231 cells were seeded in 96-well plates at a density of 1,000 cells/well. At the designated time points (12, 24 and 36 h), 10 µl CKK-8 solution was added to each well. After 2 h of incubation, the absorbance at 450 nm was measured using a microplate reader (Thermo Fisher Scientific, Inc.).

Measurement of oxidative stress markers. Intracellular ROS levels were measured using a commercial ELISA kit (cat. no. KT31960; Wuhan MSK Biotechnology Co., Ltd.)

according to the manufacturer's instructions. Briefly, MCF-7 and MDA-MB-231 cells were seeded in 6-well plates at a density of 5×10^5 cells/well, treated as aforementioned, and then lysed and centrifuged at 12,000 x g for 15 min at 4°C to collect the supernatant. The supernatant was then used for the ROS ELISA assay. The absorbance was measured at 450 nm using a microplate reader, and ROS levels were calculated based on a standard curve and normalized to total protein concentration. The levels of reduced GSH were measured using a GSH and GSSG assay kit (cat. no. S0053; Beyotime Biotechnology). Cell lysates were prepared and the absorbance at 412 nm was measured according to the manufacturer's instructions. Lipid peroxidation was assessed by measuring malondialdehyde (MDA) content using a Lipid Peroxidation MDA Assay Kit (cat. no. S0131; Beyotime Biotechnology). The MDA assay was performed strictly according to the manufacturer's protocol. The absorbance of the reaction product was measured at 532 nm.

The protein levels of GPX4, SLC7A11, TFRC and ferritin heavy chain 1 (FTH1) were quantified by commercial ELISA kits. The kits used were as follows: GPX4 (phospholipid hydroperoxide glutathione peroxidase) ELISA Kit (cat. no. EH8916), SLC7A11 (cystine/glutamate transporter) ELISA Kit (cat. no. EM8310), TFRC ELISA Kit (cat. no. EH0552) and FTH1 ELISA Kit (cat. no. EH0488) (all from Shanghai Enzyme-linked Biotechnology Co., Ltd.). All ELISAs were performed following the manufacturer-recommended protocols. Briefly, cell supernatants obtained after lysis and centrifugation at 12,000 x g for 15 min at 4°C were added to pre-coated microplate wells. Subsequent incubation, washing, substrate reaction and termination steps were carried out as instructed. The optical density value was read at 450 nm with a microplate reader, and target protein concentrations were calculated against the corresponding standard curves and normalized to total protein content.

Statistical analysis. All experiments were performed independently at least three times. Data are presented as the mean ± standard deviation. For comparisons between two groups in independent experiments, unpaired Student's t-test was used; for paired tissue comparisons, paired Student's t-test was used. For comparisons among multiple groups, one-way analysis of variance followed by Tukey's post hoc test was used. For comparisons among multiple groups with non-normal distribution, the Kruskal-Wallis test followed by Dunn's post hoc test was used. P<0.05 was considered to indicate a statistically significant difference. All statistical analyses and graph plotting were conducted using GraphPad Prism software (version 10; Dotmatics).

Results

Identification and functional characterization of FRGs in TNBC. To elucidate the molecular mechanisms linking ferroptosis to TNBC, 229 samples were analyzed (117 TNBC tumors and 112 normal adjacent tissues) from TCGA database. Differential expression analysis identified 3,068 DEGs, with 1,120 upregulated and 1,948 downregulated in TNBC. Intersecting these with a curated FRG set from FerrDB V2 revealed 93 FR-DEGs, highlighting their potential key roles in TNBC pathogenesis (Fig. 1A-C). Subsequent functional enrichment analysis of these 93 genes demonstrated significant involvement in BP, such as 'response to oxidative stress' and 'fatty acid metabolic process', and identified the 'ferroptosis' pathway as a top enriched KEGG pathway (Fig. 1D). A deeper, combined GO-KEGG analysis incorporating expression fold-change values further validated these findings and revealed prominent enrichment of oxidative-stress-responsive (GO:0006979) and fatty-acid-metabolic (GO:0006631) functional terms (Fig. 1E), providing crucial mechanistic insights and valuable leads for future investigation.

Identification of prognostic core genes and validation of their clinical significance. To identify core genes with prognostic value from the 93 FR-DEGs, univariate Cox proportional hazards regression analysis was performed within the TNBC-specific cohort (n=117). This initial screening identified 10 genes significantly associated with OS in patients with TNBC (Table II). Among them, high expression of SLC7A5, ANO6, KIF20A, ACSL1 and PRDX1 was associated with an increased risk of death, whereas high expression of TF, CIRBP, TP63, ACSF2 and PARP3 was associated with a decreased risk. Subsequent multivariate Cox regression analysis, which adjusted for the expression levels of other significant genes identified in the univariate analysis, refined this list to three genes demonstrating prognostic value independent of other gene expression markers: SLC7A5, ANO6 and TF. Notably, the HR for ANO6 increased substantially from 1.460 in the univariate model to 2.013 in the multivariate model, indicating its prognostic impact became more pronounced after adjustment. LCN2 emerged as a significant protective factor in the multivariate analysis (HR=0.712) after showing only a non-significant trend in the univariate analysis. Based on these results and consistent with prior database analyses indicating SLC7A5 is upregulated and TF is downregulated in TNBC tissues, SLC7A5 and TF were selected for further in-depth investigation.

First, the associations between SLC7A5/TF expression levels and key clinicopathological features were explored in patients with TNBC from TCGA database. Multiple subgroup comparisons revealed distinct expression patterns for these two genes. As shown in Fig. 2A, TF showed significantly higher expression in patients aged ≤ 60 years compared with patients > 60 years, whereas no significant difference in SLC7A5 expression was observed between the two age subgroups. SLC7A5 mRNA levels were markedly elevated in tumor tissues relative to adjacent nontumor tissues, and SLC7A5 expression was significantly upregulated along with higher pathological stage (stage I-IV; Fig. 2B), advanced T stage (T1-T4; Fig. 2C), higher N stage (N0-N3; Fig. 2D)

and M1 metastatic status (M0/M1; Fig. 2E). By contrast, TF exhibited overall lower expression in TNBC tumor tissues compared with normal tissues, and displayed a prominent decreasing trend as pathological stage, T stage, N stage and M stage increased, with statistically significant differences across these clinicopathological subgroups (Fig. 2B-E). Univariate logistic regression analyses further detailed these relationships (Tables III and IV), confirming that SLC7A5 expression was positively associated with T stage and overall pathological stage.

The diagnostic potential of SLC7A5 and TF was evaluated using ROC curve analysis. SLC7A5 demonstrated good diagnostic capability with an AUC of 0.826 (95% CI: 0.797-0.855) (Fig. 2F). TF showed notable diagnostic performance, with an AUC of 0.935 (95% CI: 0.915-0.955), indicating a superior ability to distinguish TNBC tumors from normal tissues (Fig. 2G). Finally, Kaplan-Meier survival analysis was conducted to assess the prognostic value of these genes in the TNBC cohort. High expression of SLC7A5 was significantly associated with poorer outcomes, including shorter OS (HR=1.73), progression-free interval (PFI; HR=1.78) and disease-specific survival (DSS; HR=2.24) (Fig. 2H-J). By contrast, high expression of TF was associated with favorable outcomes, including longer OS (HR=0.61) and DSS (HR=0.59) and a trend towards longer PFI (HR=0.73) (Fig. 2K-M). These results validated SLC7A5 as a risk factor and TF as a protective factor for prognosis in TNBC.

Validation of SLC7A5 and TF in TNBC tissues and their functional role in ferroptosis. Western blot analysis on four paired TNBC and adjacent normal tissue samples confirmed the differential expression of SLC7A5 (upregulated) and TF (downregulated) in tumor tissues (Fig. 3A). Furthermore, RT-qPCR analysis in an expanded cohort of 20 paired TNBC and normal tissues demonstrated that SLC7A5 mRNA levels were significantly higher and TF mRNA levels significantly lower in TNBC tumors compared with normal tissues (Fig. 3B), consistent with the western blotting findings. To investigate the functional impact of these genes on TNBC cell behavior, loss- and gain-of-function experiments were performed in both the ER-positive BC cell line MCF-7 and the representative TNBC cell line MDA-MB-231. Successful knockdown of SLC7A5 and overexpression of TF were confirmed by western blot analysis (Fig. 3K-N). CCK-8 assays revealed that silencing SLC7A5 (shSLC7A5) or overexpressing TF significantly suppressed cell proliferation at 12, 24 and 36 h in both cell lines (Fig. 3C and D). Transwell migration assays further demonstrated that both shSLC7A5 and TF overexpression inhibited cell migration in MCF-7 cells (Fig. 3E) and MDA-MB-231 cells (Fig. 3F). These results indicated that downregulation of SLC7A5 or upregulation of TF can effectively restrain the proliferation and migratory capacity of BC cells, with consistent effects observed in both the ER-positive and the TNBC subtype. To determine whether these effects were mediated through ferroptosis, key markers of this process were measured. Biochemical assays showed that, compared with the control groups, both shSLC7A5 and TF overexpression significantly decreased GSH levels, and increased MDA and ROS levels in MCF-7 cells (Fig. 3G) and MDA-MB-231 cells (Fig. 3I). Furthermore, ELISA-based quantification of

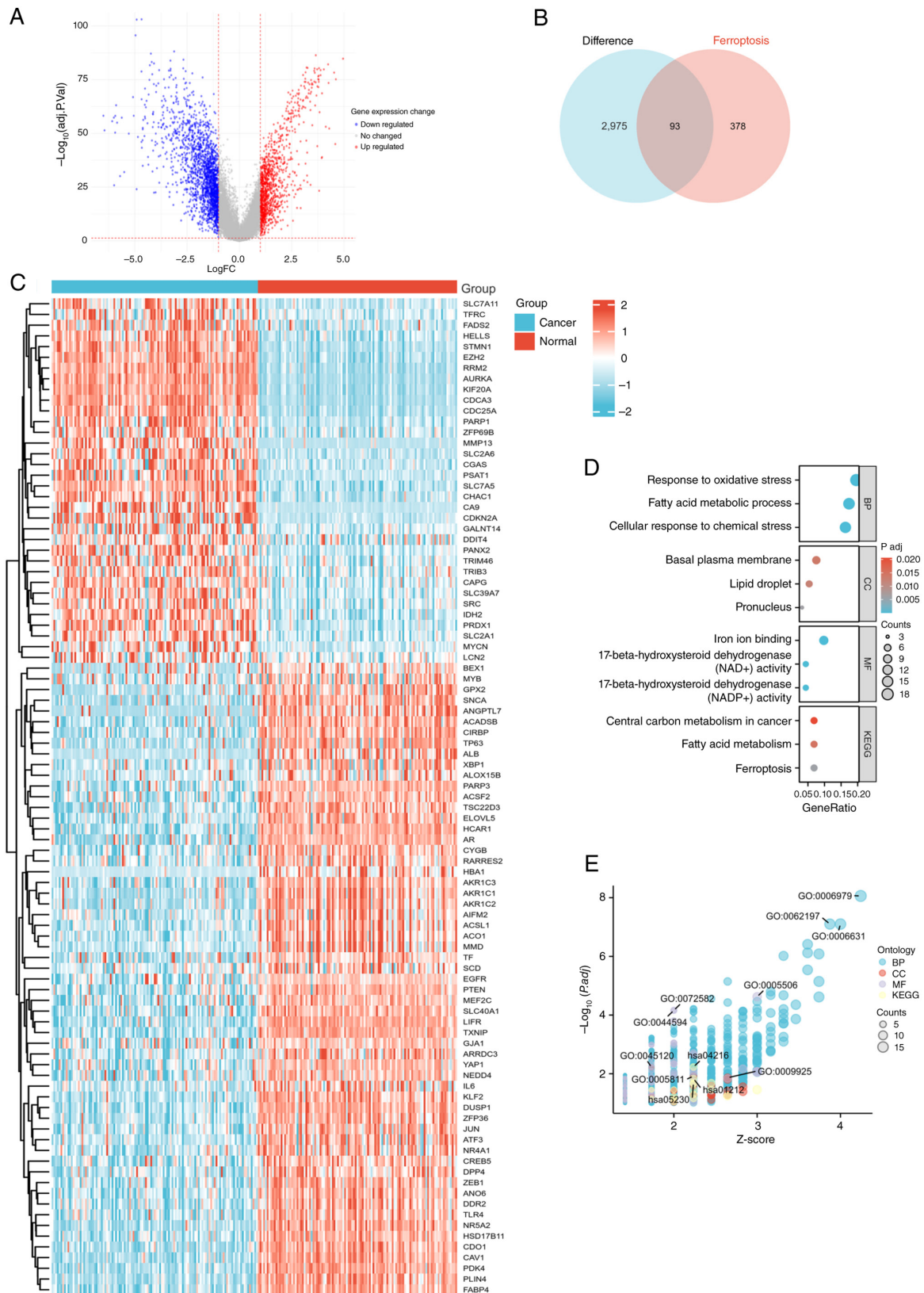


Figure 1. Identification and functional characterization of FRGs in TNBC. (A) Volcano plot of DEGs between TNBC tumors (n=117) and adjacent normal tissues (n=112). Red and blue dots denote significantly upregulated ($\log_2\text{FC}>1$, $\text{FDR}<0.05$) and downregulated ($\log_2\text{FC}<-1$, $\text{FDR}<0.05$) genes, respectively; gray dots represent non-significant genes. (B) Venn diagram showing the overlap between the identified DEGs and known FRGs, yielding 93 ferroptosis-associated DEGs. (C) Heatmap depicting the expression patterns (Z-score normalized) of the 93 ferroptosis-associated DEGs across all samples. The color scale indicates relative expression levels, with red and blue denoting high and low expression, respectively. (D) Bar plot of the top enriched GO terms and KEGG pathways for the ferroptosis-associated DEGs. Bar length represents the $-\log_{10}$ (adjusted P-value), indicating the significance of enrichment. (E) Combined GO and KEGG enrichment analysis incorporating gene expression fold-change. Dots represent enriched terms/pathways, with size proportional to the number of enriched genes and color indicating the Z-score (representing the direction of change). Key terms and pathways are labeled. BP, biological processes; CC, cellular components; DEG, differentially expressed gene; FC, fold change; FDR, false discovery rate; FRG, ferroptosis-related gene; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; MF, molecular functions; TNBC, triple-negative breast cancer.

Table II. Univariate and multivariate Cox regression analysis of 93 ferroptosis-related differentially expressed genes in patients with triple negative breast cancer from The Cancer Genome Atlas (n=117).

Gene	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	HR (95% CI)	P-value
AURKA	1.333 (0.968-1.835)	0.079	0.750 (0.461-1.221)	0.247
CDCA3	1.279 (0.927-1.763)	0.134	-	-
KIF20A	1.382 (1.002-1.908)	0.049	1.059 (0.673-1.665)	0.804
RRM2	1.150 (0.835-1.584)	0.392	-	-
CAV1	0.820 (0.594-1.130)	0.225	-	-
PDK4	0.759 (0.550-1.049)	0.095	0.891 (0.614-1.295)	0.546
EZH2	1.107 (0.805-1.523)	0.531	-	-
LIFR	0.732 (0.531-1.009)	0.057	0.710 (0.493-1.024)	0.067
CDC25A	1.185 (0.861-1.631)	0.298	-	-
TXNIP	0.990 (0.717-1.369)	0.953	-	-
PLIN4	0.900 (0.653-1.239)	0.518	-	-
STMN1	1.217 (0.880-1.683)	0.234	-	-
SLC7A5	1.730 (1.249-2.396)	<0.001	1.636 (1.076-2.486)	0.021
NR5A2	0.824 (0.597-1.136)	0.237	-	-
CDO1	0.805 (0.585-1.108)	0.183	-	-
FABP4	0.871 (0.633-1.200)	0.399	-	-
DUSP1	0.880 (0.639-1.213)	0.436	-	-
HCAR1	1.030 (0.748-1.419)	0.854	-	-
KLF2	0.824 (0.598-1.135)	0.236	-	-
ACADSB	0.810 (0.587-1.116)	0.197	-	-
AR	0.959 (0.697-1.320)	0.798	-	-
CIRBP	0.602 (0.434-0.835)	0.002	0.795 (0.536-1.180)	0.255
HSD17B11	0.828 (0.599-1.144)	0.252	-	-
SLC40A1	0.757 (0.550-1.042)	0.088	0.920 (0.621-1.364)	0.678
ZFP36	0.953 (0.693-1.312)	0.769	-	-
ANO6	1.460 (1.057-2.018)	0.022	2.013 (1.401-2.891)	<0.001
SNCA	0.878 (0.638-1.209)	0.426	-	-
AKR1C1	0.861 (0.625-1.186)	0.360	-	-
CHAC1	1.312 (0.953-1.806)	0.096	1.088 (0.745-1.589)	0.663
ELOVL5	0.888 (0.645-1.222)	0.466	-	-
HELLS	1.132 (0.822-1.558)	0.447	-	-
TSC22D3	1.153 (0.836-1.589)	0.385	-	-
ANGPTL7	0.883 (0.642-1.215)	0.445	-	-
TP63	0.649 (0.471-0.895)	0.008	0.739 (0.525-1.041)	0.084
DDR2	0.941 (0.682-1.298)	0.712	-	-
TLR4	1.083 (0.778-1.507)	0.637	-	-
IDH2	1.035 (0.752-1.425)	0.833	-	-
AKR1C2	0.862 (0.626-1.187)	0.363	-	-
PARP3	0.674 (0.489-0.931)	0.017	0.879 (0.599-1.288)	0.508
ZEB1	0.868 (0.630-1.197)	0.390	-	-
MMP13	1.121 (0.815-1.541)	0.483	-	-
PTEN	1.215 (0.882-1.676)	0.234	-	-
PARP1	1.063 (0.774-1.462)	0.705	-	-
ACO1	0.905 (0.658-1.245)	0.540	-	-
ACSL1	1.392 (1.008-1.924)	0.045	1.250 (0.891-1.752)	0.196
SLC2A6	1.107 (0.804-1.524)	0.532	-	-
CA9	1.312 (0.951-1.810)	0.098	1.124 (0.794-1.591)	0.510
AKR1C3	0.860 (0.625-1.183)	0.352	-	-
JUN	0.869 (0.632-1.195)	0.388	-	-

Table II. Continued.

Gene	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	HR (95% CI)	P-value
MMD	1.045 (0.760-1.438)	0.785	-	-
CYGB	0.814 (0.588-1.127)	0.216	-	-
PRDX1	1.385 (1.005-1.908)	0.046	1.153 (0.825-1.612)	0.404
ALB	1.003 (0.729-1.380)	0.986	-	-
ACSF2	0.657 (0.475-0.910)	0.011	0.749 (0.530-1.060)	0.103
CDKN2A	0.846 (0.614-1.166)	0.307	-	-
PSAT1	1.341 (0.972-1.851)	0.074	1.211 (0.836-1.755)	0.312
ATF3	0.825 (0.600-1.134)	0.236	-	-
MEF2C	0.923 (0.667-1.277)	0.628	-	-
CGAS	1.183 (0.859-1.627)	0.303	-	-
DPP4	0.930 (0.676-1.280)	0.657	-	-
CREB5	0.862 (0.626-1.187)	0.361	-	-
SLC7A11	1.329 (0.965-1.830)	0.082	1.124 (0.805-1.570)	0.493
ARRDC3	0.922 (0.670-1.270)	0.620	-	-
NR4A1	0.893 (0.650-1.228)	0.488	-	-
SLC39A7	1.161 (0.842-1.602)	0.362	-	-
SLC2A1	1.053 (0.766-1.448)	0.750	-	-
YAP1	1.132 (0.823-1.557)	0.447	-	-
PANX2	1.163 (0.846-1.600)	0.352	-	-
NEDD4	1.087 (0.790-1.496)	0.608	-	-
AIFM2	1.021 (0.743-1.403)	0.898	-	-
SRC	1.276 (0.928-1.756)	0.134	-	-
ZFP69B	1.074 (0.781-1.477)	0.661	-	-
GPX2	0.963 (0.701-1.324)	0.818	-	-
HBA1	0.911 (0.662-1.254)	0.569	-	-
FADS2	0.925 (0.671-1.276)	0.636	-	-
RARRES2	1.205 (0.876-1.658)	0.252	-	-
TF	0.609 (0.442-0.841)	0.003	0.587 (0.420-0.821)	0.002
TRIM46	1.292 (0.938-1.779)	0.117	-	-
CAPG	0.983 (0.715-1.351)	0.915	-	-
IL6	0.889 (0.646-1.224)	0.471	-	-
GJA1	0.767 (0.557-1.057)	0.105	-	-
XBP1	0.893 (0.649-1.230)	0.489	-	-
TFRC	1.098 (0.798-1.512)	0.565	-	-
MYCN	1.273 (0.922-1.756)	0.142	-	-
TRIB3	1.140 (0.829-1.568)	0.420	-	-
GALNT14	1.287 (0.935-1.771)	0.122	-	-
EGFR	0.946 (0.688-1.302)	0.735	-	-
SCD	0.896 (0.651-1.233)	0.500	-	-
DDIT4	0.872 (0.634-1.199)	0.399	-	-
ALOX15B	0.760 (0.551-1.049)	0.096	0.757 (0.547-1.048)	0.093
BEX1	0.730 (0.528-1.008)	0.056	0.731 (0.528-1.011)	0.058
MYB	0.944 (0.685-1.300)	0.723	-	-
LCN2	0.744 (0.540-1.024)	0.070	0.712 (0.517-0.982)	0.038

All genes were grouped using the median dichotomization method to ensure consistency of comparison and avoid bias caused by artificial cut-off values. HR and 95% CI values were calculated using Cox proportional hazards regression. The multivariate model was adjusted for the expression levels of genes that were statistically significant in the univariate analysis ($P < 0.05$). Clinicopathological variables (such as age, T stage, N stage, M stage, pathological stage) were not included as covariates in the multivariate model. HR, hazard ratio; CIs, confidence intervals.

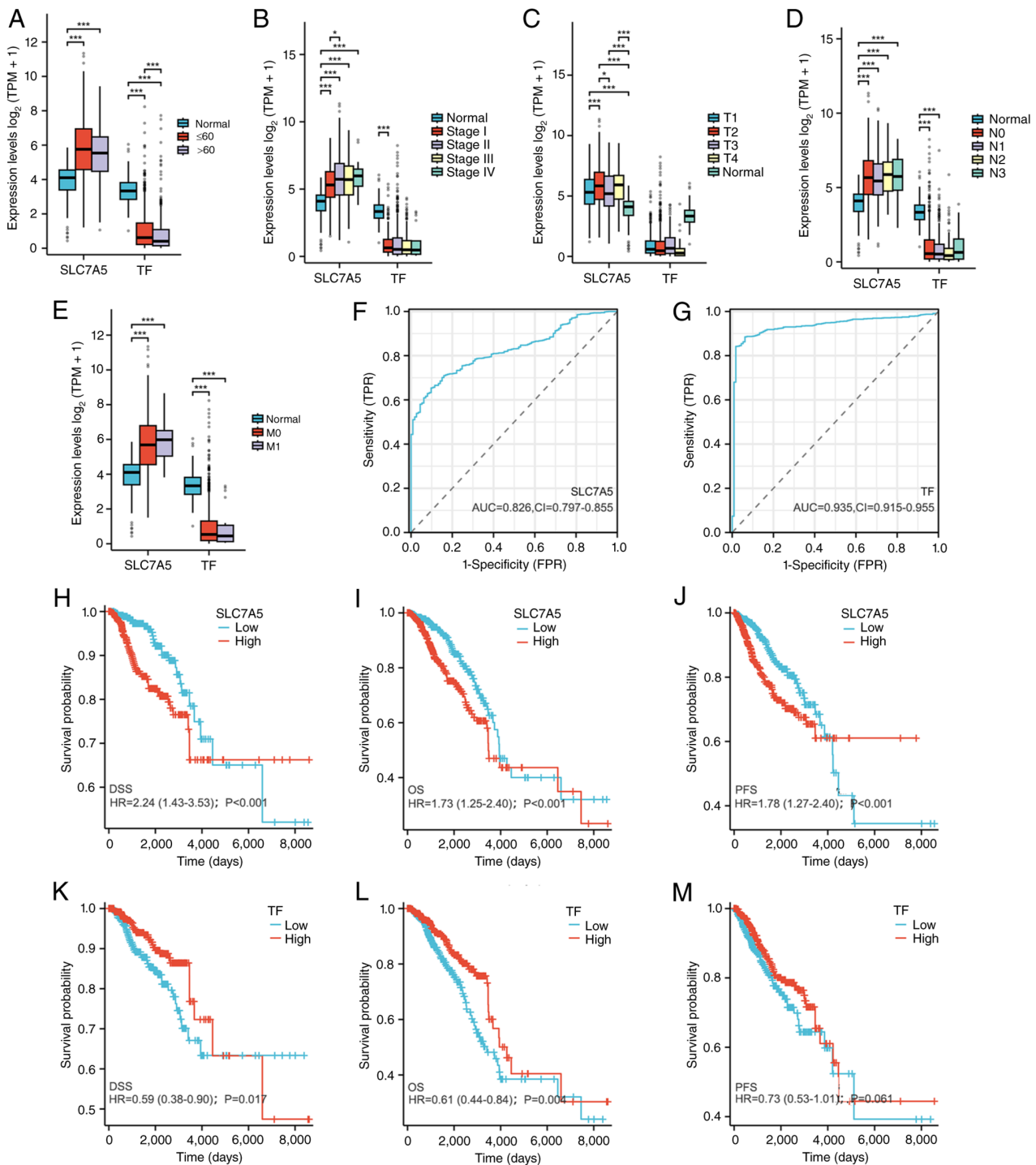


Figure 2. Clinical association, diagnostic value and prognostic significance of core genes SLC7A5 and TF in TNBC. Association of SLC7A5 and TF gene expression [$\log_2$ (TPM+1)] with clinicopathological features in patients with TNBC from The Cancer Genome Atlas cohort. Box plots show expression levels across different groups: (A) Age (≤ 60 vs. > 60 years); (B) normal tissues vs. tumor tissues (Stage I-IV); (C) T stage; (D) N stage; and (E) M stage. Statistical significance among multiple groups was determined by the Kruskal-Wallis test followed by Dunn's post hoc test. * $P<0.05$, *** $P<0.001$. Receiver operating characteristic curves evaluating the diagnostic value of (F) SLC7A5 and (G) TF for discriminating TNBC tumors from normal breast tissues. The AUC and 95% CI values are indicated. (H-M) Kaplan-Meier survival curves confined to the TNBC cohort, showing the association of gene expression with patient outcomes. High SLC7A5 expression was associated with worse (H) DSS, (I) OS and (J) PFI. High TF expression was associated with improved (K) DSS, (L) OS and (M) PFI in the patients with TNBC. Patients were stratified into high- and low-expression groups based on the median expression value. HRs with 95% CI and two-stage-derived P-values are shown. AUC, area under the curve; CI, confidence interval; DSS, disease-specific survival; FPR, false positive rate; HR, hazard ratio; OS, overall survival; PFI, progression-free interval; SLC7A5, solute carrier family 7 member 5; TF, transferrin; TNBC, triple-negative breast cancer; TPR, true positive rate.

core ferroptosis-related proteins revealed that these interventions significantly decreased GPX4 and solute carrier family 7 member 11 (SLC7A11), and increased TFRC and FTH1 in

both MCF-7 cells (Fig. 3H) and MDA-MB-231 cells (Fig. 3J). Western blot analysis further confirmed that shSLC7A5 and TF overexpression upregulated the expression of established

Table III. Association between transferrin expression and clinicopathological features.

Characteristic	Total, N	OR (95% CI)	P-value
Pathologic T stage (T2, T3 and T4 vs. T1)	1,084	0.800 (0.609-1.052)	0.110
Pathologic N stage (N1, N2 and N3 vs. N0)	1,068	0.879 (0.691-1.118)	0.293
Pathologic M stage (M1 vs. M0)	925	0.645 (0.261-1.593)	0.342
Pathologic stage (Stage II, Stage III and Stage IV vs. Stage I)	1,063	0.765 (0.555-1.055)	0.102

OR, odds ratio; CI, confidence interval.

Table IV. Association between solute carrier family 7 member 5 expression and clinicopathological features.

Characteristic	Total, N	OR (95% CI)	P-value
Pathologic T stage (T2, T3 and T4 vs. T1)	1,084	1.566 (1.188-2.062)	0.001
Pathologic N stage (N1, N2 and N3 vs. N0)	1,068	0.963 (0.757-1.224)	0.758
Pathologic M stage (M1 vs. M0)	925	1.445 (0.585-3.567)	0.425
Pathologic stage (Stage II, Stage III and Stage IV vs. Stage I)	1,063	1.470 (1.065-2.029)	0.019
Age (>60 years vs. ≤60 years)	1,087	0.833 (0.656-1.059)	0.136

OR, odds ratio; CI, confidence interval.

ferroptosis markers ACSL4 and 4-HNE in both MCF-7 cells (Fig. 3K and L) and MDA-MB-231 cells (Fig. 3M and N). Collectively, these findings validated that suppressing SLC7A5 or enhancing TF expression may induce ferroptosis in BC cells, including the TNBC cell line MDA-MB-231.

Ferroptosis inhibitor Fer-1 reverses the antitumor effects of SLC7A5 knockdown and TF overexpression in TNBC cells. To further confirm that the anti-proliferative and pro-ferroptotic effects are mediated through ferroptosis, both MCF-7 and MDA-MB-231 cells were treated with the specific ferroptosis inhibitor Fer-1, and cell proliferation, migration and the levels of ferroptosis markers were re-assessed. As shown in Fig. 4A and B, Fer-1 treatment significantly reversed the inhibition of proliferation caused by shSLC7A5 in both cell lines; similarly, Fer-1 reversed the inhibition of proliferation induced by TF overexpression (Fig. 4C and D). ELISA revealed that Fer-1 treatment restored intracellular GSH levels, while decreasing MDA and ROS levels in both shSLC7A5 and TF-overexpressing MCF-7 (Fig. 4E) and MDA-MB-231 cells (Fig. 4G). Furthermore, ELISA detection of core ferroptosis-related proteins demonstrated that the decreased GPX4 and SLC7A11 levels, and increased TFRC and FTH1 levels induced by shSLC7A5 or TF overexpression were significantly reversed upon Fer-1 co-treatment in both MCF-7 cells (Fig. 4F) and MDA-MB-231 cells (Fig. 4H). Western blot analysis further confirmed that the upregulation of ferroptosis markers ACSL4 and 4-HNE induced by shSLC7A5 or TF overexpression was markedly attenuated upon Fer-1 co-treatment in both cell lines (Fig. 4I-L). Moreover, Transwell migration assays showed that the inhibition of cell migration observed in shSLC7A5 and TF-overexpressing MCF-7 and MDA-MB-231 cells was markedly reversed by Fer-1 treatment (Fig. 4M-P).

Collectively, these results provide evidence that SLC7A5 downregulation and TF upregulation may suppress BC cell proliferation and migration specifically through promoting ferroptosis, with consistent effects observed in both MCF-7 and MDA-MB-231 cells.

Discussion

TNBC remains the most lethal BC subtype due to its notable heterogeneity, aggressive behavior and the absence of effective targeted therapies, leaving conventional chemotherapy as the primary yet often suboptimal treatment (20-22). Ferroptosis, an iron-dependent form of regulated cell death driven by lipid peroxidation, has emerged as a promising therapeutic vulnerability, yet its specific molecular regulators in TNBC are not fully understood (23). In the present study, bioinformatics analysis was integrated with functional validation to identify SLC7A5 and TF as two core genes that modulate TNBC progression through the ferroptosis pathway. The present findings not only corroborate but also extend previous research (20-23) by providing a direct mechanistic link between these genes and ferroptosis, offering novel insights for biomarker development and therapeutic targeting in TNBC.

The initial transcriptomic analysis of TCGA-TNBC data, in the present study, revealed a landscape of dysregulated ferroptosis-related pathways, with 93 FR-DEGs enriched in processes such as 'oxidative stress response' and 'fatty acid metabolic process'. This aligns with the concept that the metabolic state of TNBC may confer a unique susceptibility to ferroptosis induction (23). From this gene set, SLC7A5 and TF were selected as independent prognostic factors. The oncogenic role of SLC7A5 (LAT1), an amino acid transporter crucial for tumor growth, is well-documented in various

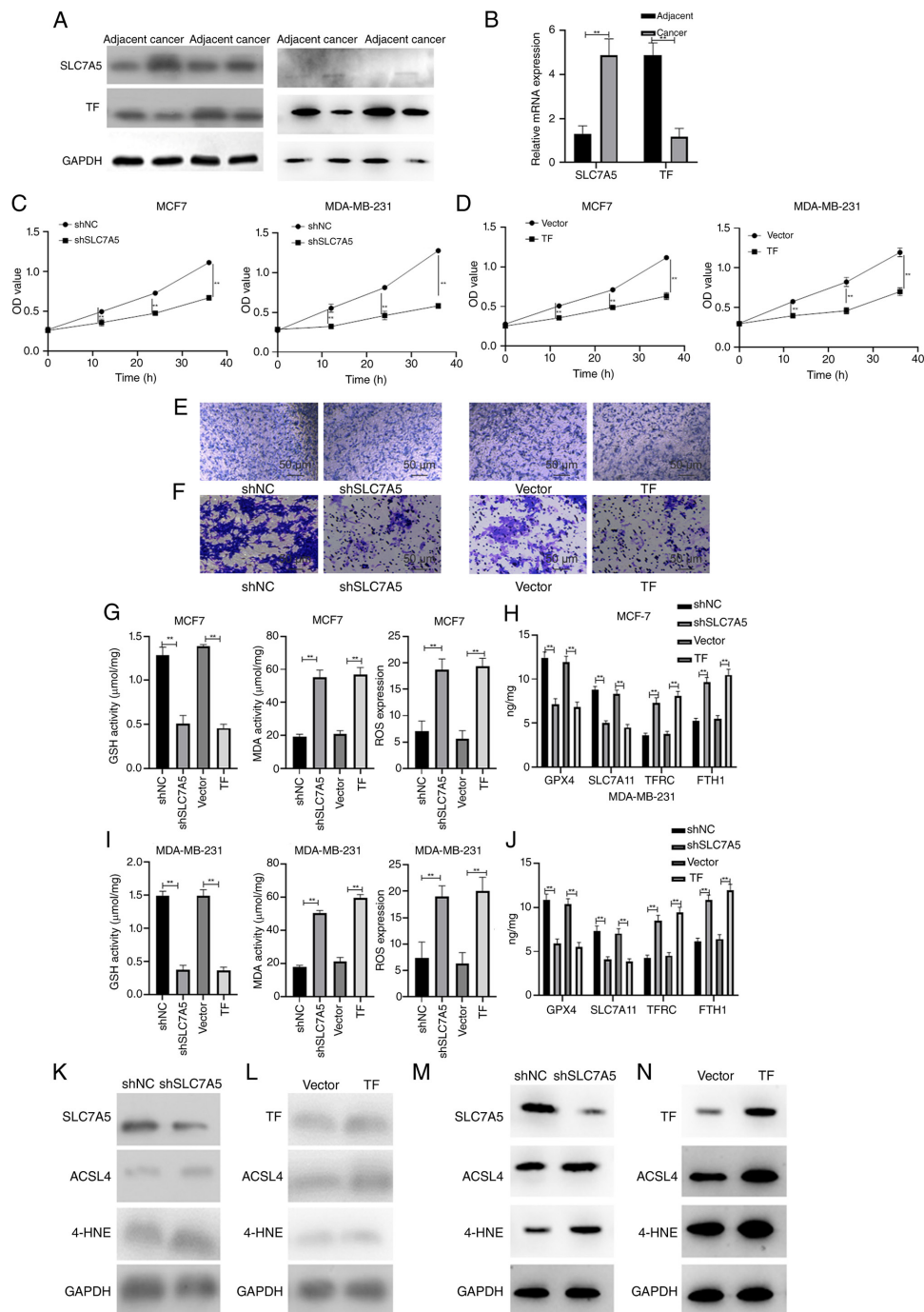


Figure 3. SLC7A5 and TF expression in TNBC tissues and their functional role in ferroptosis *in vitro*. (A) Western blot analysis of SLC7A5 and TF protein levels in four paired TNBC cancer tissues and adjacent normal tissues. GAPDH served as the loading control. Results showed that SLC7A5 protein was upregulated and TF protein was downregulated in TNBC tissues. (B) Reverse transcription-quantitative PCR analysis of SLC7A5 and TF mRNA levels in 20 paired TNBC and adjacent normal tissues. The results were consistent with the western blotting trends, showing significantly higher SLC7A5 and lower TF expression in tumor tissues. Cell proliferation assessed by Cell Counting Kit-8 assay at 0, 12, 24 and 36 h in MCF-7 and MDA-MB-231 cells; (C) shSLC7A5 significantly suppressed proliferation compared with shNC in both cell lines, and (D) TF overexpression significantly suppressed proliferation compared with the vector control in both cell lines. Transwell migration assay showing the effect of shSLC7A5 and TF overexpression on cell migration; (E) in MCF-7 cells, both shSLC7A5 and TF overexpression inhibited cell migration, and (F) the same inhibitory effects were observed in MDA-MB-231 cells. (G) Measurements of GSH, MDA and ROS in MCF-7 cells following shSLC7A5 or TF overexpression. shSLC7A5 and TF overexpression significantly decreased GSH, and increased MDA and ROS levels. (H) Protein levels of core ferroptosis markers GPX4, SLC7A11, TFRC and FTH1 detected by ELISA. Both shSLC7A5 and TF overexpression significantly decreased GPX4 and SLC7A11, and increased TFRC and FTH1. (I) Measurements of GSH, MDA and ROS in MDA-MB-231 cells following shSLC7A5 or TF overexpression. Intracellular levels showed the same trends as in MCF-7 cells. (J) Protein levels of GPX4, SLC7A11, TFRC and FTH1 detected by ELISA, confirming the same regulatory pattern as observed in MCF-7 cells. Western blot analysis of ferroptosis markers ACSL4 and 4-HNE in MCF-7 cells; (K) SLC7A5 knockdown and (L) TF overexpression both upregulated ACSL4 and 4-HNE expression. Western blot analysis of ferroptosis markers ACSL4 and 4-HNE in MDA-MB-231 cells; (M) SLC7A5 knockdown and (N) TF overexpression both upregulated ACSL4 and 4-HNE expression. Data are presented as the mean $\pm$ SD from at least three independent experiments. For paired comparisons between TNBC and adjacent normal tissues in (B), paired Student's t-test was used. Differences between the shNC and shSLC7A5 groups, and between the vector and TF groups, were analyzed using unpaired Student's t-test. $^{**}P<0.05$. 4-HNE, 4-hydroxy-2-nonenal; ACSL4, acyl-CoA synthetase long-chain family member 4; FTH1, ferritin heavy chain 1; GPX4, GSH peroxidase 4; GSH glutathione; MDA, malondialdehyde; NC, negative control; ROS, reactive oxygen species; sh, short hairpin; SLC7A5, solute carrier family 7 member 5; TF, transferrin; TNBC, triple-negative breast cancer; TFRC, TF receptor 1.

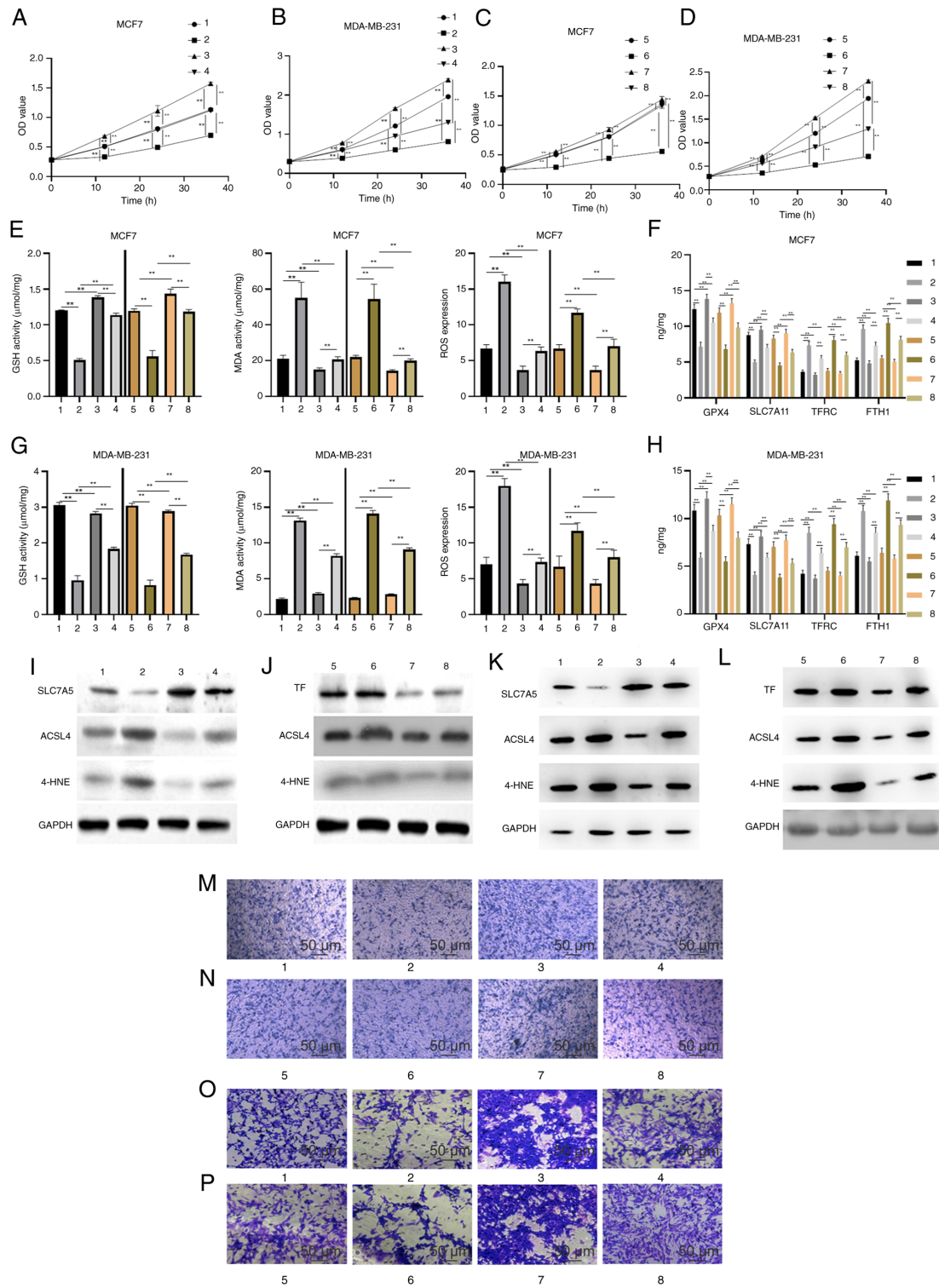


Figure 4. Fer-1 reverses the ferroptosis-mediated effects of SLC7A5 knockdown and TF overexpression in TNBC cells. CCK-8 assays showing the effect of Fer-1 (10 μ M) on cell proliferation in (A) MCF-7 and (B) MDA-MB-231 cells transduced with shSLC7A5. CCK-8 assays showing the effect of Fer-1 on cell proliferation in (C) MCF-7 and (D) MDA-MB-231 cells transfected with TF overexpression vector. (E) ELISA measurement of GSH, MDA and ROS levels in MCF-7 cells after shSLC7A5 or TF overexpression, with or without Fer-1. (F) ELISA detection of GPX4, SLC7A11, TFRC and FTH1 protein levels in MCF-7 cells under the same conditions. (G) ELISA measurement of GSH, MDA and ROS levels in MDA-MB-231 cells after shSLC7A5 or TF overexpression, with or without Fer-1. (H) ELISA detection of GPX4, SLC7A11, TFRC and FTH1 protein levels in MDA-MB-231 cells with or without Fer-1. Western blot analysis of ferroptosis markers ACSL4 and 4-HNE in (I) MCF-7 cells with SLC7A5 knockdown and/or Fer-1 treatment; (J) MCF-7 cells with TF overexpression and/or Fer-1 treatment; (K) MDA-MB-231 cells with SLC7A5 knockdown and/or Fer-1 treatment; (L) MDA-MB-231 cells with TF overexpression and/or Fer-1 treatment. GAPDH served as loading control. Transwell migration assays demonstrating the effect of Fer-1 on cell migration in (M) MCF-7 cells with SLC7A5 knockdown and/or Fer-1 treatment; (N) MCF-7 cells with TF overexpression and/or Fer-1 treatment; (O) MDA-MB-231 cells with SLC7A5 knockdown and/or Fer-1 treatment; (P) MDA-MB-231 cells with TF overexpression and/or Fer-1 treatment. (A-H) Group numbers 1-8 correspond to: 1, shNC; 2, shSLC7A5; 3, shNC + Fer-1; 4, shSLC7A5 + Fer-1; 5, vector; 6, TF; 7, vector + Fer-1; 8, TF + Fer-1. Data are presented as the mean $\pm$ SD from at least three independent experiments. Statistical significance was determined by one-way analysis of variance followed by Tukey's post hoc test. $^{**}P < 0.05$. CCK-8, Cell Counting Kit-8; Fer-1, ferrostatin-1; FTH1, ferritin heavy chain 1; GPX4, GSH peroxidase 4; GSH, glutathione; MDA, malondialdehyde; NC, negative control; ROS, reactive oxygen species; sh, short hairpin; SLC7A5, solute carrier family 7 member 5; TF, transferrin; TFRC, TF receptor 1.

types of cancer, including BC, where its high expression is associated with poor prognosis and therapy resistance (24-26). The present results confirmed this association in TNBC and, notably, advanced the field by demonstrating through multivariate analysis that SLC7A5 may serve as an independent prognostic indicator, a point not conclusively established in prior TNBC studies (27,28). Conversely, the role of TF in cancer appears more context-dependent. While some studies report its upregulation in tumors (29-31), the present study found TF to be significantly downregulated in TNBC tissues, with lower expression associated with advanced disease stage. This pattern resembles findings in melanoma, where TF loss has been shown to be associated with a protective adaptation against ferroptosis (32), suggesting a potential tumor-suppressive function of TF in specific contexts such as TNBC.

The functional validation of these genes provided crucial mechanistic insights. The present study demonstrated that SLC7A5 knockdown and TF overexpression both potently inhibited TNBC cell proliferation and migration, while concurrently inducing hallmark features of ferroptosis: Increased ROS/MDA, depleted GSH, and upregulation of ACSL4 and 4-HNE. These effects were consistently observed in both the ER-positive MCF-7 and the TNBC-relevant MDA-MB-231 cell lines, with representative data from MDA-MB-231 demonstrated in the present study. This directly links their established prognostic roles to a specific cell death pathway. The pro-tumor effect of SLC7A5 may be mediated through its support of metabolic programs that antagonize ferroptosis, such as sustaining mTORC1 signaling and redox balance, a mechanism supported by a previous study on other types of cancer (33). The tumor-suppressive effect of TF overexpression, however, suggests that restoring iron transport may push intracellular iron pools beyond a survivable threshold in TNBC, triggering lethal lipid peroxidation. Given the known role of TF in cellular iron homeostasis, its significant downregulation in TNBC may represent an adaptive strategy to limit iron availability and thereby evade ferroptosis, consistent with the observation in melanoma (32). This convergence of an amino acid transporter and an iron transporter on the same death pathway underscores the centrality of metabolic rewiring in TNBC biology.

A key strength of the present study is the use of the specific ferroptosis inhibitor Fer-1 in rescue experiments. The fact that Fer-1 treatment reversed the anti-proliferative, anti-migratory and pro-ferroptotic effects of both SLC7A5 knockdown and TF overexpression in MDA-MB-231 cells provides compelling evidence that their biological functions in TNBC are primarily mediated through ferroptosis. This direct pharmacological confirmation addresses a gap in previous studies (29-31) that associated these genes with cancer progression without definitively linking them to this execution mechanism.

The present findings carry marked clinical implications. The strong diagnostic power of TF (AUC=0.935) and SLC7A5 (AUC=0.826) in discriminating TNBC tissues from normal tissues, combined with their independent prognostic value, positions them as promising candidates for a biomarker panel to aid in TNBC diagnosis and risk stratification. Therapeutically, targeting SLC7A5 with inhibitors or strategies to restore TF

function could sensitize TNBC cells to ferroptosis, offering a novel approach to overcome chemotherapy resistance.

The interpretation of TF expression in tumor tissues requires caution. As a systemic iron-binding protein, measured TF levels in tissue homogenates may be influenced by tumor purity, stromal components, vascular content or serum contamination (32,33). However, the consistent downregulation observed across the present multi-platform analysis (TCGA transcriptome, paired tissue western blotting and RT-qPCR) notably suggests this is a genuine biological phenomenon in TNBC rather than a technical artifact. The apparent paradox of TF downregulation, given its physiological role in iron delivery, aligns with the hypothesis that TNBC cells suppress TF to limit iron uptake and evade ferroptosis, as reported in melanoma (32). The present data provide functional evidence supporting this model, but it is recognized that direct measurements of intracellular labile iron pools and the TF-TFRC regulatory axis are necessary to definitively confirm this mechanism. Therefore, the present findings are a foundation for future studies that will explicitly test this iron-dependent pathway.

Several limitations should be acknowledged. The present bioinformatics findings require validation in larger, prospective clinical cohorts. Furthermore, the potential interaction between SLC7A5 and TF remains an open and intriguing issue. As regulators of distinct metabolic inputs (amino acids and iron), they may function in parallel, synergistically, or antagonistically within the ferroptosis network. Although the RT-qPCR validation in 20 paired samples supports the main findings, the sample size is still relatively small. Larger cohort studies, ideally using immunohistochemistry, are needed for robust clinical validation. A notable limitation of the prognostic analysis is that the multivariate Cox regression model was adjusted only for the expression levels of other significant genes, not for established clinical risk factors such as age, T stage, N stage, M stage or pathological stage. Therefore, the independent prognostic value of SLC7A5 and TF should be interpreted as independent from other gene markers, and their true clinical independence requires validation in studies incorporating comprehensive clinicopathological variables. Future studies employing dual-gene manipulation models, in multiple TNBC cell lines, and *in vivo* experiments are essential to dissect this interplay and to validate the therapeutic efficacy of targeting these pathways in the complex tumor microenvironment, addressing the limitations of the present *in vitro* study. Furthermore, complementary experiments, such as SLC7A5 overexpression and TF knockdown, were not performed in the current study due to time and resource constraints. Although the knockdown and overexpression strategies used herein demonstrated the functional roles of SLC7A5 and TF, rescue experiments would further confirm the specificity of the observed phenotypes, and this should be considered a limitation that warrants future investigation. Moreover, the current study did not systematically explore the downstream signaling pathways through which SLC7A5 and TF regulate ferroptosis in TNBC. While the involvement of key iron metabolism and lipid peroxidation markers have been demonstrated, the specific molecular cascades remain to be elucidated in future investigations. In addition, although canonical ferroptosis-related proteins (GPX4, SLC7A11,

TFRC and FTH1) were detected, intracellular Fe²⁺ levels and lipid ROS measured by C11-BODIPY staining were not evaluated. Thus, the present study lacks a more comprehensive ferroptosis mechanistic evaluation, which represents a limitation. Future studies incorporating these markers are required to further validate the involvement of ferroptosis. Additionally, the therapeutic conclusions drawn from the present study are solely based on *in vitro* experiments; therefore, there is a need for *in vivo* validation in appropriate animal models to confirm the translational potential of targeting these ferroptosis regulators.

In conclusion, the present study established SLC7A5 as a ferroptosis suppressor and oncogenic driver and, to the best of our knowledge, identified TF as a ferroptosis inducer and tumor suppressor for the first time in TNBC. By demonstrating these effects in both ER-positive MCF-7 and TNBC MDA-MB-231 cell lines, and validating the changes of core ferroptosis-related proteins (GPX4, SLC7A11, TFRC and FTH1), along with providing pharmacological rescue with Fer-1, the present study provides a direct mechanistic connection and a deeper understanding of TNBC pathophysiology. The current study identified SLC7A5 and TF as viable targets for the development of ferroptosis-based diagnostic and therapeutic strategies against this challenging disease, although further validation in larger clinical cohorts and *in vivo* models is required.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

RL and XC contributed to the study design and performed the bioinformatics analysis. BG and YL conducted the cell function experiments and ferroptosis marker detection. FL was responsible for data collection and statistical analysis. ZF and BX conceived and supervised the study, and were involved in manuscript writing and revision. ZF and BX confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The Affiliated Second Hospital of Shenyang Medical College (approval no. fs-023). Written informed consent was obtained from all patients prior to sample collection.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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