

Identification of novel ferroptosis-associated prognostic genes in cervical cancer using bioinformatics analysis and experimental validation

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Received July 1, 2025; Accepted November 13, 2025

DOI: 10.3892/ol.2026.15931

Abstract. Cervical cancer (CC) is a common malignant tumor among women worldwide. However, the relationship between CC and ferroptosis remains to be elucidated. The present study screened differentially expressed genes (DEGs) in CC from The Cancer Genome Atlas database and identified DEGs associated with ferroptosis using the FerrDb database. A total of 2,563 DEGs were upregulated and 2,120 DEGs were downregulated which were more notably enriched in the early region 2 binding factor targets and G₂M checkpoint. By crossing ferroptosis-driver, ferroptosis-marker and ferroptosis-suppressor genes and DEGs, 80 ferroptosis-associated prognostic genes were identified. In total, 24 potential targeted drugs targeting key genes were predicted, providing a possible basis to predict the prognosis and treatment of CC. The expression levels of stearoyl-CoA desaturase, transferrin receptor (TFRC) and carbonic anhydrase 9 were experimentally validated by PCR, which was consistent with the present bioinformatics analysis. Furthermore, knocking down TFRC inhibited cell proliferation and migration in CC cells *in vitro*. In conclusion, the present study identified key genes associated with ferroptosis in CC and further research is warranted to explore their underlying mechanisms and potentially become effective clinical prognostic biomarkers in the future.

Introduction

Cervical cancer (CC) is one of the main causes of cancer mortalities worldwide. Although the incidence of CC continues to decline with the widespread vaccination of human papillomavirus (HPV) vaccines and the popularization of CC screening, there are currently >600,000 novel diagnoses and 340,000 mortalities worldwide each year, posing a serious threat to the physical and mental health (1). The progression from cervical intraepithelial neoplasia to invasive CC is regulated by genes involved in mitosis, DNA repair, cell proliferation, apoptosis and angiogenesis (2). However, >20% of patients experience recurrence (3). However, the specific mechanism that leads to the progression of CC remains to be elucidated and to the best of our knowledge, there are currently no effective biomarkers for the diagnosis, prognosis and recurrence of CC. Although squamous cell carcinoma (SCC) antigen is a commonly used CC marker, its specificity and sensitivity are not high, particularly close monitoring of local recurrence in low-level patients (normal range of SCC antigen; reference range, <1.5 ng/ml) is necessary (4,5). Therefore, there is an urgent need to search for novel diagnostic and prognostic markers for CC in clinical practice.

Ferroptosis is an iron dependent cell death characterized by the accumulation of intracellular lipid peroxides and mitochondrial reactive oxygen species (ROS). Previous studies have demonstrated that ferroptosis serves a key role in a variety of malignant tumors, such as pancreatic cancer and liver cancer (6-9). The induction of ferroptosis is gradually becoming a novel tumor mode in tumor cells and exploring novel diagnostic and therapeutic methods may be beneficial. Xia *et al* (10) identified that an iron death inducer, talaroconvolin A, promotes iron death by increasing ROS and upregulating the expression level of arachidonate epidermal lipooxygenase 3. In addition, Wang *et al* (11) reported that LINC00336 is upregulated in cancer and reversed by inhibiting ferroptosis. Other previous studies reported that ferroptosis imbalance between tumor-associated macrophages and CC cells involved in the

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Key words: cervical cancer, ferroptosis, immune infiltration, hub gene, biomarker

progression of CC and resistance to cancer chemotherapy and targeted therapy in CC (12,13). Wang *et al* (14) reported that the mitochondrial carrier 1 (MTCH1)-FOXO1-glutathione peroxidase 4 (GPX4) signaling pathway was identified and it was proposed that MTCH1 deficiency inhibits FOXO1 activation, leading to downregulation of GPX4 transcription and ROS accumulation, ultimately triggering iron death in CC cells. Chen *et al* (15) demonstrated that the combination of deferoxamine and oxaliplatin can reduce oxaliplatin resistance in CC cells. These studies indicate that exploring the mechanisms of ferroptosis is a potential target for the diagnosis and treatment of CC in the future.

The present study screened differentially expressed genes (DEGs) associated with ferroptosis in CC and adjacent tissues using The Cancer Genome Atlas (TCGA) database. Further analysis of the roles of stearoyl-CoA desaturase (SCD), transferrin receptor (TFRC) and carbonic anhydrase 9 (CA9) in the prognosis and immune infiltration of CC was performed, providing prognostic candidate biomarkers for the diagnosis and immunotherapy of CC.

Materials and methods

Data preprocessing. Clinical phenotype and RNA-sequencing (seq) data were obtained by searching cervical cancer in TCGA database (<https://portal.gdc.cancer.gov/projects/TCGA-CESC>) and transcript levels were estimated by the University of California Santa Cruz Xena [[https://xenabrowser.net/datapages/?cohort=TCGA Cervical Cancer \(CESC\)](https://xenabrowser.net/datapages/?cohort=TCGA+Cervical+Cancer+(CESC))] platform (16). The present study screened CC as the primary tumor and grouped it according to cancer and para-cancer tissues, recording basic clinical and pathological information of patients in the screening data.

TCGA data analysis. The expression difference P-value and the expression fold-change (FC) value were calculated using the R package (version 3.2.1) DESeq2 (version 1.8.1) (17) in and using adjusted $P < 0.05$ and $\log_2 FC > 1$ (2-fold relationship), screened for DEGs. The differential heat map and volcano map were further analyzed using the R package 'heatmap' (version 1.0.12; R Development Core Team) (18).

Gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) analysis. The genes associated with ferroptosis were downloaded, including ferroptosis driver, ferroptosis marker and ferroptosis suppressor genes using the Ferroptosis database (zhounan.org/FerrDb) (19). A Venn diagram was drawn through the intersection of DEGs and ferroptosis by using the R package 'Venn diagram' (version 1.6.20) (20). KEGG enrichment analysis was further performed on cross genes using R package 'Venn plots' (version 1.6.20; significance threshold set to $P \leq 0.05$) (21) and bubble plots were created for visualization using R package 'ggplot2' (version 3.3.3; R Development Core Team) (22).

Gene set enrichment analysis (GSEA). GSEA was performed using the GenePattern tool with version of 5.2 of the Molecular Signature Database (MSigDB; broadinstitute.org/gsea/msigdb/index.jsp) (23). The GSEA involved estimating the significance of enrichment scores (ES), correcting

multiple hypothesis tests and evaluating the significance of ES.

Gene set variation analysis (GSVA). Using the c2.cp.kegg.v7.2.symbols.gmt gene set data, the CC data were transformed into ES values for GSVA using R-package 'gene set variation analysis' (GSVA; version 1.34.0) (24). Further differential analysis was performed on the ES value matrix using the 'limma' R software package (version 3.42.2; R Development Core Team) and lastly, using the differential gene set, volcano and heat maps were constructed to identify the key pathways of DEGs.

Protein-protein interaction (PPI) network construction. The Search Tool for the Retrieval of Interacting Genes/Proteins database (STRING-db.org/) was used to search for interacting genes (25). The database analyzed the PPI between CC and iron death-related genes and set the required confidence level (comprehensive score) of the PPI threshold to > 0.15 . A PPI network was drawn using Cytoscape software (3.7.1) (26) and the top 30 key genes were extracted for subsequent analysis using the 'Cytohubba' plugin (27). Lastly, ferroptosis-related key genes that affect the prognosis of CC were identified (Fig. 1).

Prognosis and clinical association analysis. RNA-seq spectra and clinical information of patients with CC were obtained from TCGA to analyze the relationship with the prognosis of patients with SCD, TFRC and CA9 expression using the Kaplan-Meier method and log-rank test.

Database analysis. RNA-seq spectra and clinical information from patients with CC were downloaded from TCGA database (<https://portal.gdc.cancer.gov/projects/TCGA-CESC>) by searching cervical cancer to determine their association with clinical case characteristics and prognosis of CC. The images of human expression data were obtained from the Human Protein Atlas database (proteinatlas.org/). SWISS-MODEL (28) was employed to construct a 3D structure for SCD, TFRC and CA9 using homology modeling. The sequence of SCD, TFRC and CA9 (UniProt: Q00417) was obtained from the UniProt (29).

Immune infiltration analysis of prognostic genes. The Cell-type Identification By Estimating Relative Subsets Of RNA Transcripts (CIBERSORT) method (30), which is a computational approach based on gene expression data used to quantify the relative proportions of different immune cell subpopulations in tumor tissues, was used for immune infiltration analysis of prognostic genes. After standardizing the prognostic gene data associated with CC necrosis, a signature matrix containing specific cell type marker genes was then selected or constructed. Subsequently, the gene expression data and signature matrix were input into CIBERSORT software (V2.0.0) for computational analysis, estimating the abundance of various immune cell subpopulations in the sample to analyze the immune infiltration of the tumor.

Patients and sample preparation. The present study was approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology (approval

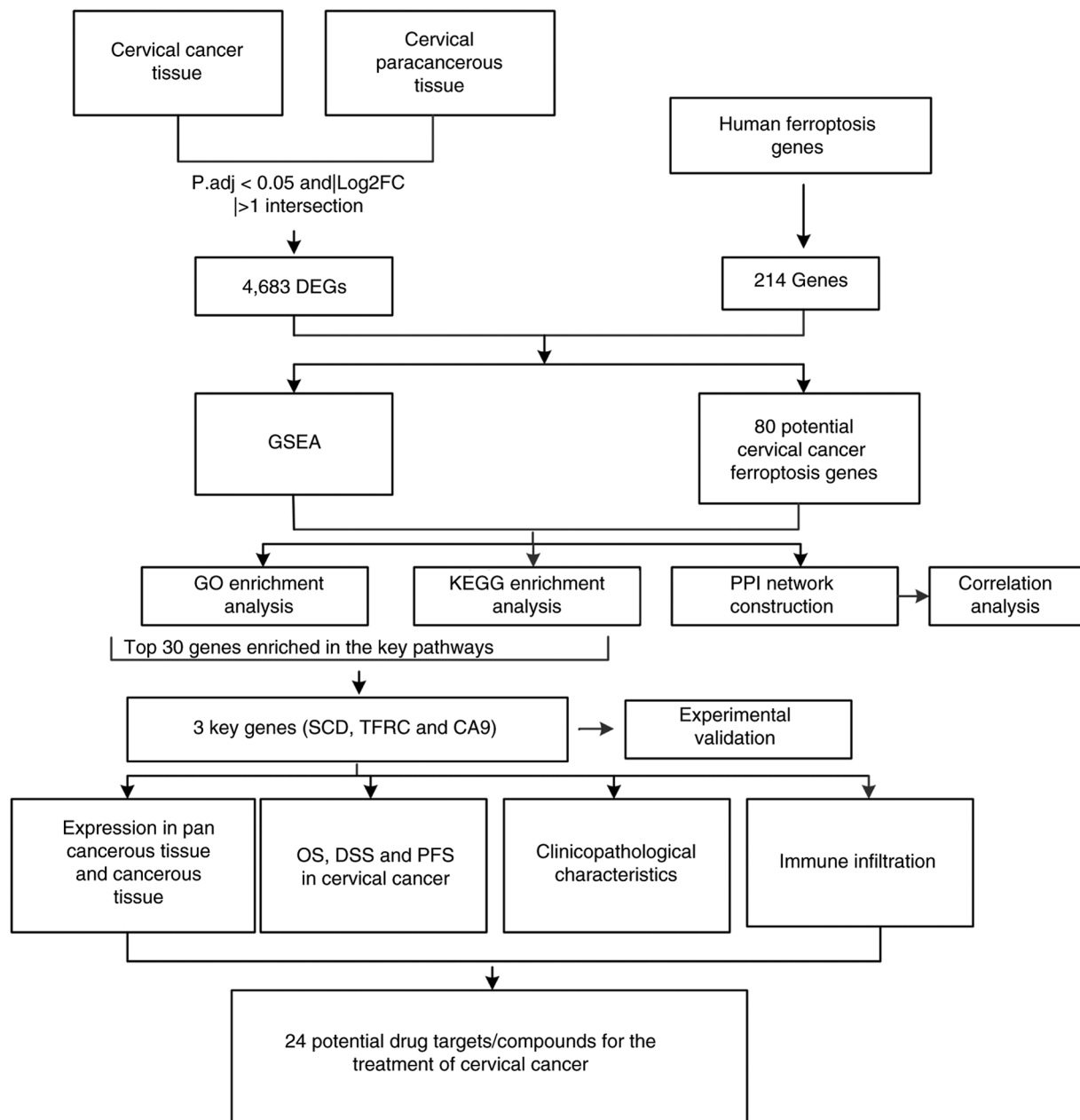


Figure 1. Schematic diagram of ferroptosis-related key genes and pathways that affect the prognosis of cervical cancer. DEGs, differentially expressed genes; GSEA, Gene Set Enrichment Analysis; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPI, protein-protein interaction; SCD, stearoyl-CoA desaturase; TFRC, transferrin receptor; CA9, carbonic anhydrase 9; OS, overall survival; DSS, disease specific survival; PFS, progression-free survival.

no. 2021-S353; Wuhan, China), and written informed consent was obtained from all patients. A total of three cases of CC tissue and adjacent tissues were obtained from Union Hospital, Tongji Medical College, Huazhong University of Science and Technology from September to December 2023 from patients who underwent surgery before initiating antitumor therapy and who did not receive radiotherapy, chemotherapy or anti-angiogenic treatment. The preparation of RNA was performed as previously described (31).

RNA extraction and fluorescence reverse transcription-quantitative PCR (RT-qPCR). For RT-qPCR, total RNA was extracted by lysing the aforementioned tissues in 1 ml RNAiso Plus reagent (cat. no. 9109; Takara Biotechnology Co., Ltd.),

ensuring complete homogenization. Following centrifugation ($12,000 \times g$; 4°C ; 15 min), the supernatant was removed and the RNA pellet was washed with 75% ethanol to improve purities. RNA extraction and subsequent reverse transcription were performed according to the manufacturer's protocols of the PrimeScript[™] RT Master Mix (cat. no. RR036A; Takara Biotechnology Co., Ltd.) and TB Green[®] Premix Ex Taq[™] II (cat. no. RR820A; Takara Biotechnology Co., Ltd.) kits. qPCR was carried out on the 7500 Real-Time PCR System (cat. no. RR820A; Takara Biotechnology Co., Ltd.) using standard operational guidelines. The conditions for RT-qPCR were as follows: 95°C for 10 min, 40 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, and a final extension of 10 min at 72°C . Relative mRNA expression levels were quantified using

Table I. Primer sequences used for RT-qPCR.

Gene	Primer sequence (5'-3')
SCD	F: CTACACAACCACCACCACCATCAC R: CTCTCATTTTCAGGGCGGATGTCTTC
TFRC	F: GGCTACTTGGGCTATTGTAAAGG R: CAGTTTCTCCGACAACCTTTCTCT
CA9	F: CATCCTAGCCCTGGTTTTGG R: CCTTCTGTGCTGCCTTCTCAT
GAPDH	F: GGTGTGAACCATGAGAAGTATGA R: GAGTCCTTCCACGATACCAAAG

SCD, stearoyl-CoA desaturase; TFRC, transferrin receptor; CA9, carbonic anhydrase 9; F, forward; R, reverse.

the $2^{-\Delta\Delta C_q}$ method (32) and normalized against GAPDH as an internal control. The primer sequences used for RT-qPCR are shown in Table I.

Prediction of drug-gene interactions. Firstly, genes or gene products reported to interact with TKIs were identified in the drug-gene interaction database (DGIdb; v2.22; <http://www.dgldb.org/>). DGIdb integrates existing resources to generate hypotheses on how mutant genes may be targeted for therapy, which provides an interface for a general search of genes for drug-gene interactions and potentially drug-available genes (33). A large amount of drug-gene interaction information was aggregated from different data sources, providing data on the association between specific gene variations and drug responses, which helps researchers quickly identify potential drug targets and biomarkers. The prognostic genes associated with CC necrosis were imported into DGIdb for analysis to explore drugs or small molecule organic compounds associated with it.

Cell culture. The human CC cell lines HeLa (cat. no. GDC0001) and SiHa (GDC0042) were purchased from the China Center for Type Culture Collection and cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (both Gibco; Thermo Fisher Scientific, Inc.). HeLa is a human cervical adenocarcinoma cell line and SiHa is a human SCC cell line. All cells were authenticated using short tandem repeat analysis and maintained in a humid atmosphere at 37°C and 5% CO₂.

Small-interfering RNA (siRNA) transfection. The TFRC siRNA (forward: 5'-GGAU AUGG GUCUAAGUCUATT-3'; reverse: 5'-UAGACUUAGACCAUUCTT-3'). The cells were incubated for 48 h following transfection at 37°C. and a universal negative control siRNA (forward, 5'-AAGACGUACGAGCUACUG Ctt-3'; reverse, 5'-GCAGUAGCUCGUACGUCUUt-3'; cat. no. SIC001; Beijing Tsingke Biotech Co., Ltd.) were used for transient silencing in CC cells (MilliporeSigma; Merck KGaA). Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used for transient transfection with a final siRNA concentration of 32 nM for 48 h. The transfected cells were harvested and used for subsequent experiments.

Cell proliferation detection. The MTT assay kit (Beyotime Biotechnology) was used to assess cell proliferation. Logarithmic growth phase cells were seeded at a density of 1,000 cells per well in a 96-well plate, cultured for 24, 48 and 72 h. The MTT solution (0.5 mg/μl) was added and the plate was incubated at 37°C for 4 h. Subsequently, 100 μl of DMSO was added to separate the crystals and the plate was stirred at 37°C for 20 min. The absorbance was measured at 490 nm using an ELISA reader.

Wound healing and Transwell assays. For the wound healing assay, cervical cancer cells were seeded in a 6-well plate at a density of 3x10⁵ cells per well. After transfection with siRNA, the cells were cultured for 48 h at 37°C. When the cell confluence reached 90-100% and a dense monolayer was formed, the wound healing assay was performed. The cell monolayers were gently scratched using a 200-μl pipette tip and the cells were incubated in serum-free RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) for 24 h at 37°C. To remove any detached cells, the wells of the plate were gently washed with fresh medium. The distance between edges of the wound was measured under a light microscope (Olympus Corporation), and multiple visual fields were selected for observing each well. After 24 h, the wound channel distance was measured again for analysis. The wound area was measured using ImageJ software (version 1.48; National Institutes of Health). Wound healing assay results were presented as migration rate (%)=(initial wound area-wound area at 24 h)/initial wound area x100.

For the Transwell assay, 200 μl serum-free medium containing 5x10⁴ cells was seeded onto the upper surface of the chamber without coating (migration assay) and 600 μl complete medium was added to the lower chamber. After incubation at 37°C for 24 h, the cells were fixed by passing them through an 8 μm pore membrane with 4% paraformaldehyde at 37°C for 30 min. Then, the cells were stained with 0.1% crystal violet at 37°C for 5 min. The number of cells in five random fields of view were counted for each inserted segment to evaluate the migration of cancer cells at a x200 magnification. Each experiment was performed three times.

Western blotting. The total protein was extracted as previously described and protein blotting was performed (34). The primary antibodies used were rabbit anti-TFRC (1:1,000; ProteinTech Group, Inc.; cat. no. 84766-4-RR) and mouse anti-GAPDH (1:1,000; Cell Signaling Technology, Inc.; cat. no. #2118). GAPDH was used as the control. The bands were quantified using Image J software (version 1.51; National Institutes of Health).

Statistical analysis. R software (version 3.6.3; R Development Core Team) was used to perform the statistical analyses and data were presented as the mean ± SD. The expression levels of selected top 30 key genes and phenotypes (such as age, tumor stage, tumor differentiation and lymph node metastasis) were analyzed using univariate and multivariate Cox regression analyses. The median expression level of the genes in all samples was used as the boundary to divide patients into groups (above median, high expression group; below median, low expression group). The Kaplan-Meier method and log-rank test were used to analysis the overall survival, disease-specific survival and

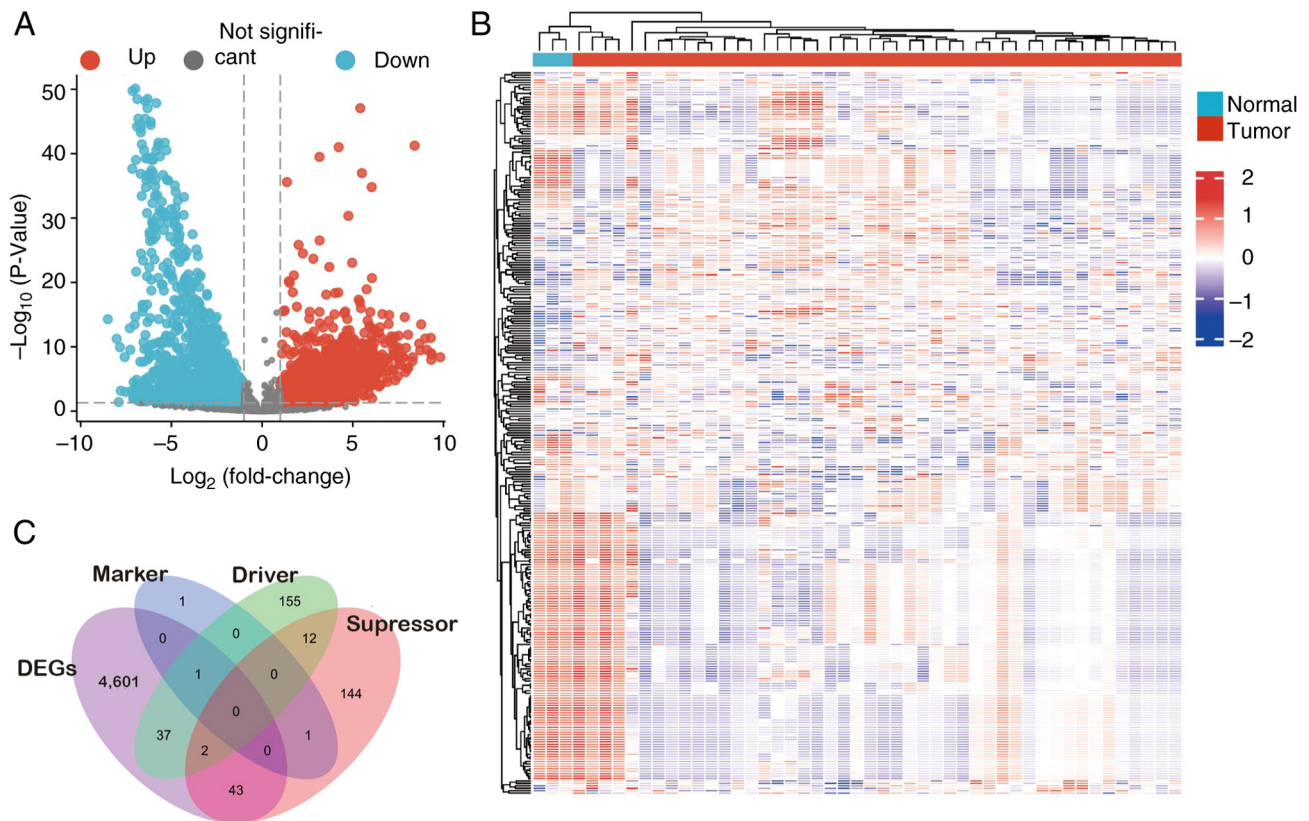


Figure 2. Differences in TCGA data of cervical cancer. (A) Differential gene volcano map indicates the DEGs between cervical cancer and normal cervical tissues. The red is the up-regulated differential gene, the blue is the downregulated differential gene and the gray is the non-significant DEGs. (B) Differential gene heat map exhibits 3 samples of normal patients and 306 samples of cervical cancer tissue from TCGA. Blue is the normal group, red is the tumor group, purple is low expression and red is high expression. (C) Venn diagram exhibits the intersection among ferroptosis driver, ferroptosis marker, ferroptosis suppressor genes and DEGs. TCGA, The Cancer Genome Atlas; DEGs, differentially expressed genes.

progression-free survival of patients with CC. Univariate and multivariate Cox regression analyses were performed to identify survival-associated genes. The association between SCD, TFRC and CA9 expression and clinicopathological features were evaluated using the paired Student's t-test after a normality test. Spearman correlation coefficients were calculated to assess the associations between SCD, TFRC and CA9 expression and the infiltration levels of 24 immune cell types. Significant differences between different groups were evaluated using Student's t-tests. $P < 0.05$ was considered to indicate a statistically significant difference and all statistical tests were two-sided.

Results

Differences in TCGA data of CC. There was a total of 309 samples of CC and adjacent RNA-seq data, including 3 samples of normal cervical tissue and 306 samples of CC tissue from TCGA. A total of 20,532 genes were screened, including 4,683 DEGs (2,563 upregulated and 2,120 downregulated) (Fig. 2A and B). A total of 214 human ferroptosis genes (driver, suppressor and marker genes) were screened out. By screening for cross genes between DEGs and ferroptosis genes, 80 potential CC ferroptosis genes were ultimately obtained (Fig. 2C).

Cluster analysis. GSEA in TCGA cohort identified that the early region 2 binding factor (E2F) targets and G₂M checkpoint were significantly enriched using the DEGs between CC and

cervical paracancerous tissue (Fig. 3A). The mountain map indicated that it is mainly enriched in 'Mtorc1 signaling', 'myogenesis', 'Epithelial mesenchymal transition', 'E2F targets' and 'G₂M checkpoint' between CC and normal cervical tissue (Fig. 3B). GO and KEGG enrichment (GSEA) analyses of 80 potential ferroptosis-related genes of CC were performed and revealed that 'response to peptide', 'response to hypoxia' and 'DNA-binding transcription activator activity, RNA polymerase II-specific' were significantly enriched. (Fig. 3C-E).

PPI network. The PPI network analysis was performed on 80 key genes associated with ferroptosis in CC and revealed that there were a total of 911 interaction relationships (Fig. 4A). Among these genes, 30 genes were confirmed as hub genes, which were murine double minute 2 (MDM2), androgen receptor (AR), CDK inhibitor 2A (CDKN2A), GSK-3 β , neuroblastoma Ras viral oncogene homolog (NRAS), pyruvate dehydrogenase kinase 4 (PDK4), Krüppel-like factor 2 (KLF2), suppressor of variegation 3-9 homolog 1 (SUV39H1), toll-like receptor 4 (TLR4), isocitrate dehydrogenase 2 (IDH2), caveolin-1 (CAV1), sterol regulatory element-binding transcription factor 1 (SREBF1), myeloblastosis (MYB), cadherin-1 (CDH1), myocyte-specific enhancer factor 2C (MEF2C), MAPK3, solute carrier family 1 member 5 (SLC1A5), nuclear receptor subfamily 4 group A member 1 (NR4A1), NADPH oxidase 4 (NOX4), SCD, dipeptidyl peptidase 4 (DPP4), TFRC, CA9, zinc finger enhancer-box-binding homeobox 1 (ZEB1), protein

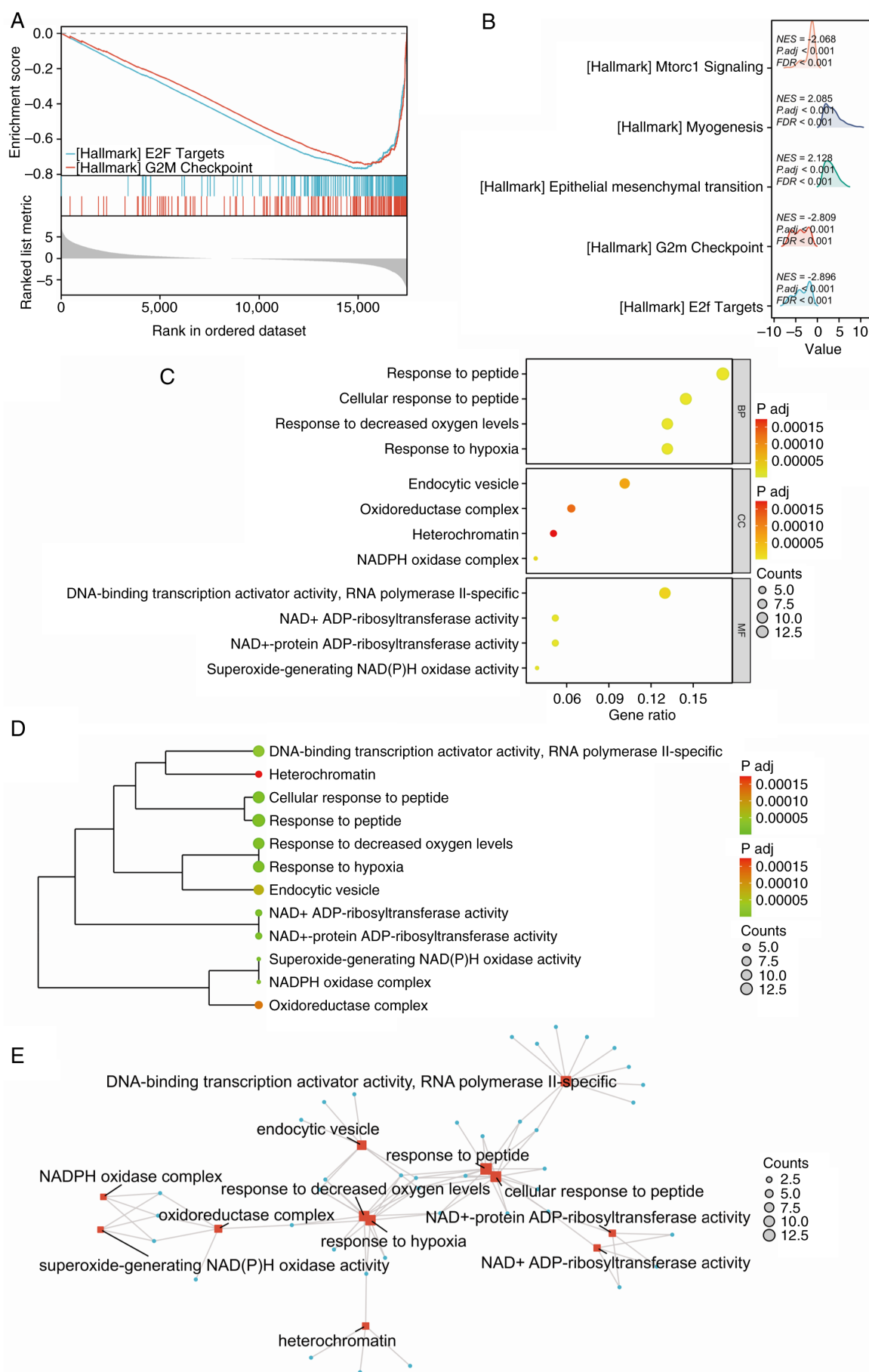


Figure 3. Cluster analysis of differential genes. (A) GSEA enrichment analysis of differential genes between cervical cancer and normal cervical tissues. (B) Mountain range map of differential genes between cervical cancer and normal cervical tissue. (C) Bubble chart, (D) Cluster tree and (E) network diagram of the potential ferroptosis-related genes of cervical cancer. GSEA, Gene Set Enrichment Analysis.

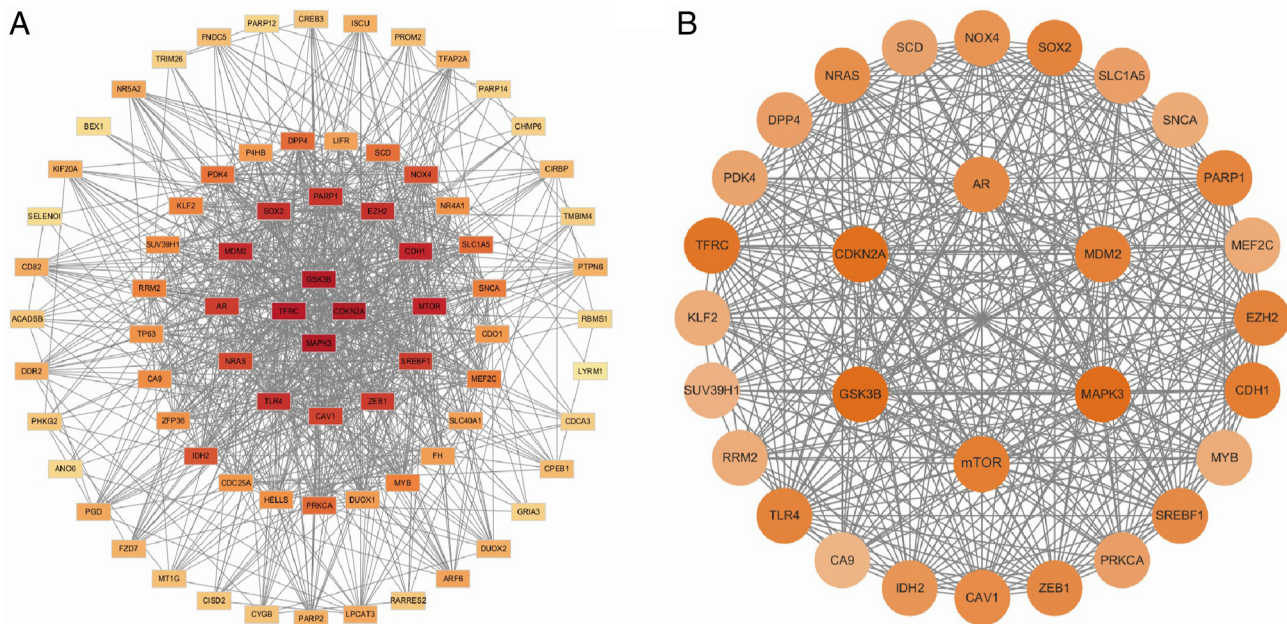


Figure 4. PPI network of differential genes. (A) PPI network diagram revealed that 80 key genes associated with ferroptosis in cervical cancer interact with each other. (B) The top 30 genes confirmed as hub genes interact with each other. The color from red to light is the Matthews Correlation Coefficient score. PPI, protein-protein interaction; TFRC, transferrin receptor; CA9, carbonic anhydrase 9; SCD, stearyl-CoA desaturase.

kinase C α (PRKCA), mTOR, enhancer of zeste homolog 2 (EZH2), poly(ADP-ribose) polymerase 1 (PARP1), synuclein α (SNCA) and Sox2 (Fig. 4B).

Cancer and pan-cancer analysis. The prognostic analysis of the aforementioned 30 screened genes using TCGA dataset demonstrated that SCD, TFRC CA9 are generally highly expressed in CC. SCD was highly expressed in most tumors but generally low expression in BRCA, cholangiocarcinoma, lung adenocarcinoma (LUAD), lung SCC and pheochromocytoma and paraganglioma (PCPG) (Fig. 5A). TFRC was highly expressed in most tumors but generally low expression in LUAD, prostate adenocarcinoma (PRAD), PCPG and thyroid carcinoma (Fig. 5B). CA9 was highly expressed in most tumors but generally low expression in the esophageal adenocarcinoma and PRAD (Fig. 5C).

Prognostic analysis. Using the HPA database, SCD, TFRC and CA9 was demonstrated to have no or low expression in normal cervical tissues but they were highly expressed in cervical SCC and adenocarcinoma tissues, and they were expressed in both the nucleus and cytoplasm (Fig. 6A). The prognostic analysis of the aforementioned 30 genes screened using TCGA dataset indicated that SCD, TFRC and CA9 are closely associated with prognosis. The overall survival, disease-specific survival and progress free survival of patients with CC with high expression levels of SCD and TFRC were significantly reduced (Fig. 6B and C). The disease-specific survival and progress free survival of patients with CC with high expression level of CA9 were significantly reduced (Fig. 6D).

Immune infiltration analysis of ferroptosis-associated prognostic genes in CC. The present study identified that the differences in immune infiltration between the high and low

expression groups of SCD, TFRC and CA9 were mainly concentrated in naive B cells, memory B cells, plasma cells, T cells, CD8 cells, naive CD4 T cells, CD4 memory resting cells, CD4 memory activated T cells, follicular helper T cells, regulatory T cells, T cells $\gamma\delta$, resting natural killer (NK) cells, activated NK cells, monocytes, M0 macrophages, M1 macrophages, M2 macrophage, resting dendritic cells, activated dendritic cells, resting mast cells and activated mast cells (Fig. 7A-C). The heatmap displays the Spearman correlation between the abundance of immune-infiltrating cells and SCD, TFRC and CA9 expression of 24 types of tumor-infiltrating immune cells (Fig. 7D). SCD, TFRC and CA9 were most closely correlated with central memory T cell, T helper (Th) 2 cell and Th17, respectively (Fig. 7E-G).

Structural model diagram and drug-gene interaction prediction based on ferroptosis-associated prognostic genes in CC. Using the UniProt database (29), amino acid sequences of SCD, TFRC and CA9 were retrieved and the SWISS-MODEL method (28) was used to predict the folding structure models of SCD, TFRC and CA9 (Fig. 8A-C). In the DGIdb database (<http://dgidb.org/>), the analysis of drug-gene interactions revealed 148 drugs/compounds targeting SCD, TFRC and CA9. The top 24 targeted drugs/compounds and their order based on the 'interaction group score' (35) in the DGIdb database are shown in Fig. 8D.

Verification of the key ferroptosis-associated prognostic genes in CC in the signature. RT-qPCR further confirmed that compared with normal cervical tissue, the expression levels of SCD, TFRC and CA9 were significantly increased in CC (Fig. 9A). It has been reported that TFRC serves a key role in epithelial ovarian cancer, breast cancer, liver cancer and other tumors (36-38). Therefore, the present study focused on further

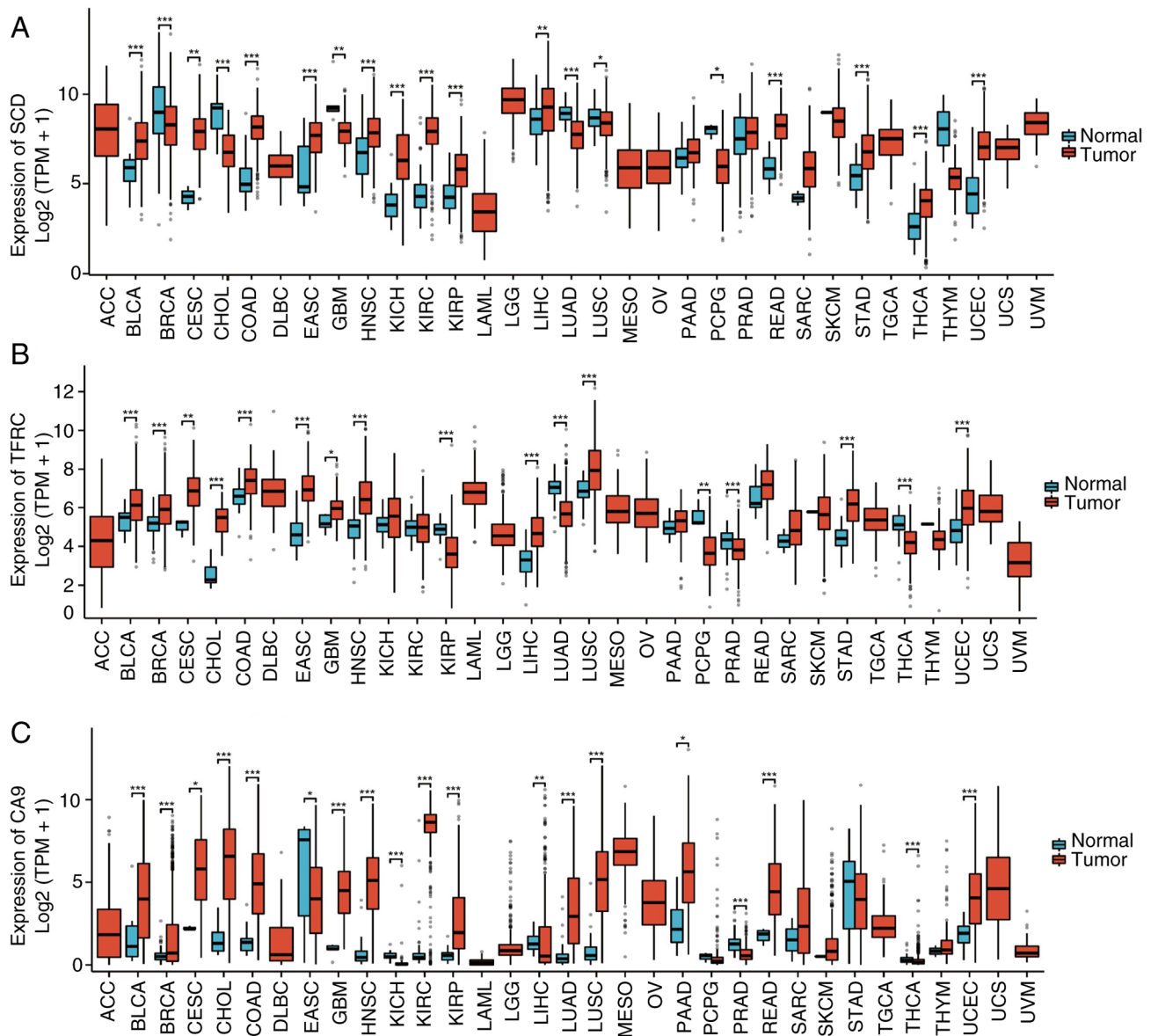


Figure 5. Cancer and pan-cancer analysis. A box plot demonstrates the expression levels of (A) SCD, (B) TFRC and (C) CA9 in pan-cancer and para-carcinoma. Significant differences between different groups were evaluated using Student's t-tests. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. SCD, stearyl-CoA desaturase; TFRC, transferrin receptor; CA9, carbonic anhydrase 9; TPM, transcripts per million.

validation of hub genes, particularly TFRC. To validate the biological function of TFRC in CC cells, TFRC was knocked down in the present study using siRNA in SiHa and HeLa cells (Fig. 9B). MTT showed knockdown of TFRC significantly reduced the proliferation of CC cells (Fig. 9C). The wound healing rate of SiHa and HeLa cells was significantly inhibited by TFRC knockdown, as indicated by the results of the wound-healing assay (Fig. 9D). The migration ability of SiHa and HeLa cells was significantly inhibited in Transwell assays following TFRC knockdown (Fig. 9E). In summary, knocking down TFRC inhibited proliferation and migration in CC cells *in vitro*.

Discussion

In 2021, cervical cancer ranked as the second most common cause of cancer-related mortality in China (39). Although CC

screening and the promotion of HPV vaccines have reduced the incidence of CC (40,41), >50% of CC is diagnosed as locally advanced (42). Therefore, there is an urgent need to screen and identify novel biomarkers for the diagnosis and prognosis of CC in the future. Since the first proposal of ferroptosis in 2012, numerous studies have reported its key role in malignant tumors. Yang *et al* (43) reported that iron death-related genes are closely associated with the prognosis of CC. In the present study, bioinformatics methods were used to screen for DEGs based on RNA-seq results of CC and identified 214 genes associated with ferroptosis. Among them, 80 potential ferroptosis-related genes of CC were identified by the intersection of ferroptosis-related genes and DEGs, suggesting that differential genes in CC are closely associated with ferroptosis.

GSEA, GO and KEGG analyses of 80 potential ferroptosis-related genes showed CC enriched in 'E2F targets', 'G₂M

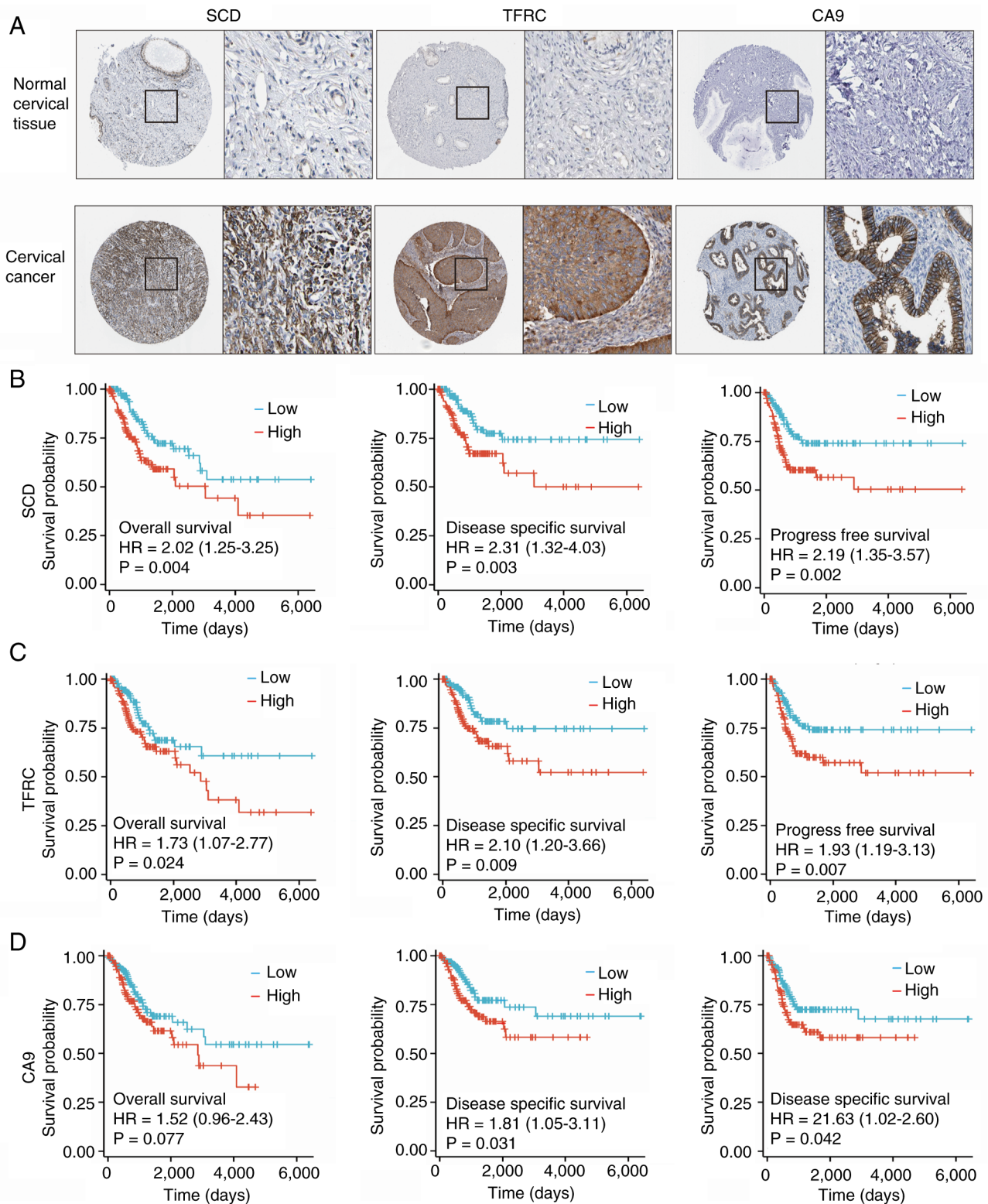


Figure 6. Prognostic analysis in cervical cancer. (A) Representative immunohistochemical images of SCD, TFRC and CA9 in cervical cancer tissues and corresponding normal cervical tissues from the Human Protein Atlas database. The relationships between (B) SCD, (C) TFRC and (D) CA9 in patients with cervical cancer were analyzed using Kaplan-Meier and log-rank test. SCD, stearyl-CoA desaturase; TFRC, transferrin receptor; CA9, carbonic anhydrase 9; HR, hazard ratio; TCGA, The Cancer Genome Atlas.

checkpoint', 'response to peptide', 'response to hypoxia' and 'DNA-binding transcription activator activity, RNA polymerase II-specific'. PPI network analysis revealed that there was a total of 911 interaction relationships and 30 genes were confirmed as hub genes, which were MDM2, AR, CDKN2A,

GSK-3 β , NRAS, PDK4, KLF2, SUV39H1, TLR4, IDH2, CAV1, SREBF1, MYB, CDH1, MEF2C, MAPK3, SLC1A5, NR4A1, NOX4, SCD, DPP4, TFRC, CA9, ZEB1, PRKCA, mTOR, EZH2, PARP1, SNCA, Sox2. The prognostic analysis of the aforementioned 30 screened genes using TCGA dataset

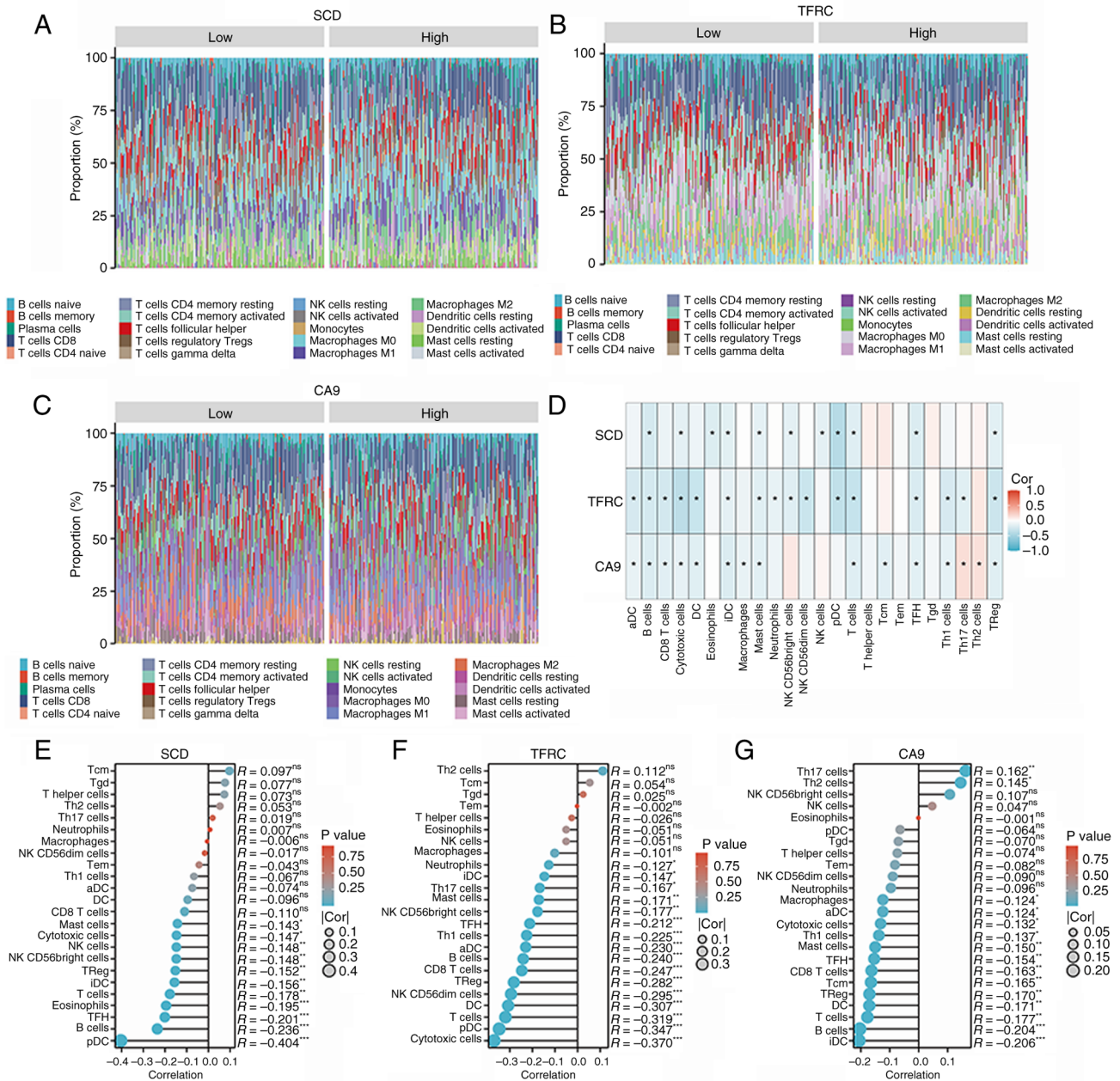


Figure 7. Immune infiltration analysis of ferroptosis-associated prognostic genes in cervical cancer. The difference in immune infiltration results between high and low expression groups of (A) SCD, (B) TFRC and (C) CA9 in stacked bar charts. (D) Heatmap displaying the Spearman correlation between the abundance of immune-infiltrating cells and SCD, TFRC and CA9 expression of 24 types of tumor-infiltrating immune cells. The x-axis represents immune cell types and the y-axis represents the genes. The color scale indicates the correlation coefficient with red indicating a positive correlation and blue indicating a negative correlation. *P<0.05, **P<0.01 and ***P<0.001. Rod plot of immune infiltrating cell expression levels of (E) SCD, (F) TFRC and (G) CA9. SCD, stearoyl-CoA desaturase; TFRC, transferrin receptor; CA9, carbonic anhydrase 9; ns, not significant.

demonstrated that SCD, TFRC and CA9 are closely associated with prognosis. The present study further confirmed using RT-qPCR that compared with normal cervical tissue, the expression levels of SCD, TFRC and CA9 were increased in CC. Furthermore, knocking down TFRC can inhibit cell proliferation and migration in CC cells *in vitro*.

Han *et al* (44) revealed that insulin-like growth factor 2 mRNA binding protein 3 (IGF2BP3) regulates SCD mRNA N6-methyladenosine modification through the IGF2BP3-methyltransferase-like 14 complex, thereby enhancing CC proliferation, metastasis and lipid metabolism. Fang *et al* (45) revealed that silencing TFRC can limit cell

proliferation and migration ability, prevent cell cycle and promote apoptosis of CC cells. Previous studies have confirmed that the alkaline sulfonamide S-1 (CA9 inhibitor) can induce apoptosis in CC cells and hypoxia-induced immunosuppression may be associated with the therapeutic resistance of locally advanced CC, demonstrating CA9 is a key target for future treatment of CC (46,47). Therefore, SCD, TFRC and CA9 may be potential prognostic markers for CC.

The invasion of HPV into the body limits the proliferation and activation of tumor-infiltrating T lymphocytes, causing virus immune escape and gradually transforming normal cervical tissue into malignancy, which is the main cause of

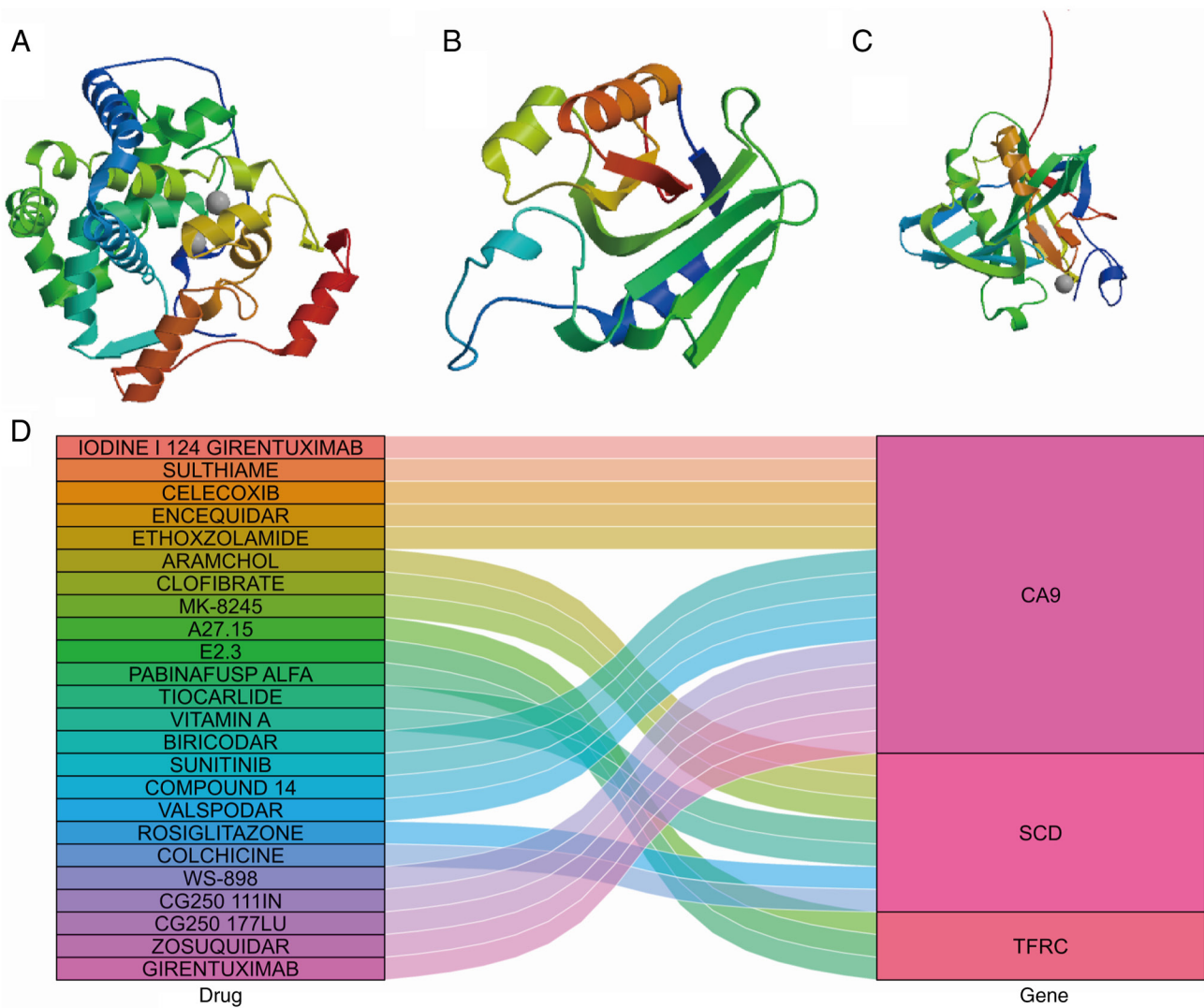


Figure 8. Structural model diagram and drug-gene interaction prediction based on ferroptosis-associated prognostic genes in cervical cancer. SWISS-MODEL was used to predict the folding structure models of (A) SCD, (B) TFRC and (C) CA9. (D) The top predicted 24 targeted drugs/compounds targeting SCD, TFRC and CA9. SCD, stearyl-CoA desaturase; TFRC, transferrin receptor; CA9, carbonic anhydrase 9.

CC (48). The subtypes of tumor-infiltrating T lymphocytes change with the occurrence and development of CC, with various details (49). The changes and balance of cell subtypes have different impacts on the progression of CC. Infiltrating T lymphocytes may be an effective biomarker in predicting the efficacy and prognosis of CC (50). The relationship between SCD, TFRC, CA9 and immune cell infiltration were further investigated in the present study. SCD, TFRC and CA9 were most closely correlated with Central Memory T cell, T helper (Th) 2 cell and Th17, respectively T lymphocytes serve a notable role in coordinating the immune response of the human body to tumors. In the field of immunotherapy, immune checkpoint inhibitors such as programmed cell death protein-1 (PD-1) have demonstrated promising clinical efficacy in various tumor treatments. It is key to note that as of the end of 2021, according to the results of the KEYNOTE-826 trial, pembrolizumab combined with chemotherapy with or without bevacizumab has been approved for first-line treatment of recurrent/metastatic CC. However, the overall response rate is still moderate (~15%) (51,52). Therefore, a combination of

novel treatment methods and immunomodulatory targeted therapies is warranted to improve the efficacy of existing cancer immunotherapies in the future. The present study identified three major apoptosis-related genes (SCD, TFRC and CA9) associated with CC prognosis, searched the DGIdb database and obtained drug-gene interaction results for three genes, SCD, TFRC and CA9, totaling 24 potential targeted drugs.

TFRC is a key membrane protein that serves a key role in cellular iron uptake. Iron is essential for various cellular processes, including growth and proliferation (7). Feng *et al* (53) reported that TFRC is overexpressed in nasopharyngeal carcinoma and TFRC knockdown inhibits nasopharyngeal carcinoma progression by suppressing the PI3K/AKT/mTOR signaling pathway. In a previous study on pancreatic and colon cancer cells, TFRC has been reported to regulate MAPK signaling pathway, which helps improve cell viability and resistance to cell death (54). The present study demonstrated that knocking down TFRC can inhibit cell proliferation and migration in CC cells *in vitro*. Therefore,

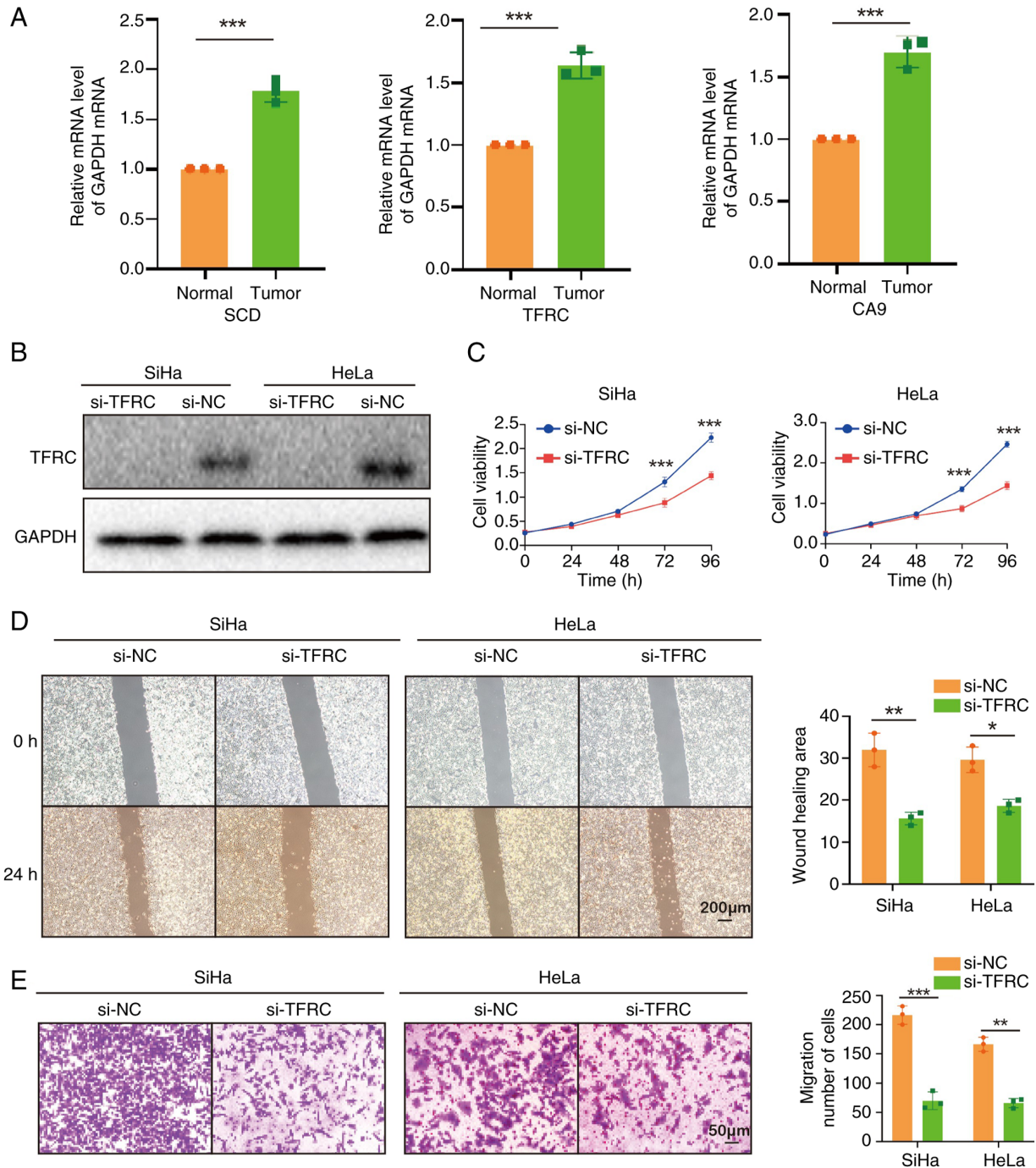


Figure 9. Verification of the key ferroptosis-associated prognostic genes in cervical cancer in the signature. (A) RT-qPCR was used to assess mRNA levels of SCD, TFRC and CA9. (B) Efficiency of TFRC siRNA was validated using western blotting. (C) MTT assay determined the effect of the proliferation of cervical cancer cells (SiHa and HeLa) with TFRC knocked down. (D) A scratch assay was used to determine the impact of knockdown of TFRC on the migratory ability of SiHa and HeLa cells. (E) Transwell assays were used to determine the impact of TFRC knockdown on the migratory ability of SiHa and HeLa cells. Significant differences between different groups were evaluated using Student's t-tests. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. RT-qPCR, reverse transcription-quantitative PCR; SCD, stearyl-CoA desaturase; TFRC, transferrin receptor; CA9, carbonic anhydrase 9; NC, negative control; si/siRNA, small interfering RNA.

TFRC is a very promising target, providing potentially novel ideas for the treatment of CC in the future.

In summary, the present study identified the top 30 genes associated with ferroptosis in CC using bioinformatics analysis and identified that SCD, TFRC and CA9 are closely associated with the pathological T stage, lymph node metastasis and poor prognosis of CC. In terms of immunity, SCD, TFRC and CA9 are closely associated with T-cell depletion. Although the

present study identified genes associated with ferroptosis in CC, SCD, TFRC and CA9 can serve as prognostic markers for CC and potential novel targets for immunotherapy, and 24 potential targeted drugs/compounds were predicted for these genes. The expression levels of SCD, TFRC and CA9 were experimentally validated, which is consistent with the present bioinformatics analysis. Furthermore, knocking down TFRC can inhibit cell proliferation and migration in CC cells *in vitro*.

There are several limitations of the present study that should be considered when interpreting the results. First, the present study mainly utilized *in vitro* experiments, further *in vivo* experiments are warranted to confirm the effect of ferroptosis-associated prognostic genes on the progression of CC. Second, the limited number of clinical samples included may bias the results and further clinical translation requires the inclusion of more clinical samples to further confirm the present study conclusions. Third, the mechanism of ferroptosis is still unclear and requires further research to clarify, which will be a promising direction for further investigation. Nevertheless, the present study suggests that SCD, TFRC and CA9 are associated with the prognosis of CC. The present study results have potentially laid the foundation for the study of ferroptosis-associated prospective genes in CC in the future.

Acknowledgements

Not applicable.

Funding

The present study was financially supported by the Hubei Provincial Department of Science and Technology Project (grant no. 2023DJC144), the Xianning City Program of Science & Technology (grant no. 2023ZRKX090), the transverse research and the Scientific Research Foundation of Hubei University of Science and Technology (grant no. 2023HX026), the Open Research Fund of Hubei Province Key Laboratory of Precision Radiation Oncology (grant no. 2024ZLJZFL003) and Key Laboratory of Anesthesiology and Resuscitation (Huazhong University of Science and Technology, Ministry of Education; grant no. 2024MZFS010).

Availability of data and materials

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

Authors' contributions

LFW and ZXW conceived the study conceptualization. LFW, GJT and ZXW designed the study methodology. Statistical analysis was performed by LFW and HC. Data interpretation was performed by ZXW, HC, GJT and LFW. LFW and GJT critically reviewed and edited the manuscript. ZXW, HC, GJT and LFW confirm the authenticity of all the raw data. All authors have read and approved the manuscript.

Ethics approval and consent to participate

The present study was performed according to the principles expressed in the Declaration of Helsinki and was approved by the Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (approval no. 2021-S353; Wuhan, China). Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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