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ABOUT COVER
Peer Reviewer of World Journal of Gastrointestinal Oncology, Andreia Albuquerque, MD, PhD, Gastroenterologist, Professor, Research Scientist, Precancerous Lesions and Early Cancer Management Research Group RISE@CI-IPO (Health Research Network), Portuguese Oncology Institute of Porto (IPO-Porto), Porto 4200-072, Portugal. a.albuquerque.dias@gmail.com

AIMS AND SCOPE
The primary aim of World Journal of Gastrointestinal Oncology (WJGO, World J Gastrointest Oncol) is to provide scholars and readers from various fields of gastrointestinal oncology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

WJGO mainly publishes articles reporting research results and findings obtained in the field of gastrointestinal oncology and covering a wide range of topics including liver cell adenoma, gastric neoplasms, appendiceal neoplasms, biliary tract neoplasms, hepatocellular carcinoma, pancreatic carcinoma, cecal neoplasms, colorectal neoplasms, duodenal neoplasms, esophageal neoplasms, gallbladder neoplasms, etc.

INDEXING/ABSTRACTING
The WJGO is now abstracted and indexed in PubMed, PubMed Central, Science Citation Index Expanded (SCIE, also known as SciSearch®), Journal Citation Reports/Science Edition, Scopus, Reference Citation Analysis, China Science and Technology Journal Database, and Superstar Journals Database. The 2023 edition of Journal Citation Reports® cites the 2022 impact factor (IF) for WJGO as 3.0; IF without journal self cites: 2.9; 5-year IF: 3.0; Journal Citation Indicator: 0.49; Ranking: 157 among 241 journals in oncology; Quartile category: Q3; Ranking: 58 among 93 journals in gastroenterology and hepatology; and Quartile category: Q3. The WJGO’s CiteScore for 2022 is 4.1 and Scopus CiteScore rank 2022: Gastroenterology is 71/149; Oncology is 197/366.

RESPONSIBLE EDITORS FOR THIS ISSUE
Production Editor: Xiang-Di Zhang; Production Department Director: Xiang Li; Cover Editor: Jia-Ru Fan.
Predictive model using four ferroptosis-related genes accurately predicts gastric cancer prognosis

Li Wang, Wei-Hua Gong

BACKGROUND
Gastric cancer (GC) is a common malignancy of the digestive system. According to global 2018 cancer data, GC has the fifth-highest incidence and the third-highest fatality rate among malignant tumors. More than 60% of GC are linked to infection with Helicobacter pylori (H. pylori), a gram-negative, active, microaerophilic, and helical bacterium. This parasite induces GC by producing toxic factors, such as cytotoxin-related gene A, vacuolar cytotoxin A, and outer membrane proteins. Ferroptosis, or iron-dependent programmed cell death, has been linked to GC, although there has been little research on the link between H. pylori infection-related GC and ferroptosis.

AIM
To identify coregulated differentially expressed genes among ferroptosis-related genes (FRGs) in GC patients and develop a ferroptosis-related prognostic model with discrimination ability.

METHODS
Gene expression profiles of GC patients and those with H. pylori-associated GC were obtained from The Cancer Genome Atlas and Gene Expression Omnibus (GEO) databases. The FRGs were acquired from the FerrDb database. A ferroptosis-related gene prognostic index (FRGPI) was created using least absolute shrinkage and selection operator–Cox regression. The predictive ability of the FRGPI was validated in the GEO cohort. Finally, we verified the expression of the hub genes and the activity of the ferroptosis inducer FIN56 in GC cell lines and tissues.
RESULTS

Four hub genes were identified (NOX4, MTCH1, GABARAPL2, and SLC2A3) and shown to accurately predict GC and *H. pylori*-associated GC. The FRGPI based on the hub genes could independently predict GC patient survival; GC patients in the high-risk group had considerably worse overall survival than did those in the low-risk group. The FRGPI was a significant predictor of GC prognosis and was strongly correlated with disease progression. Moreover, the gene expression levels of common immune checkpoint proteins dramatically increased in the high-risk subgroup of the FRGPI cohort. The hub genes were also confirmed to be highly overexpressed in GC cell lines and tissues and were found to be primarily localized at the cell membrane. The ferroptosis inducer FIN56 inhibited GC cell proliferation in a dose-dependent manner.

CONCLUSION

In this study, we developed a predictive model based on four FRGs that can accurately predict the prognosis of GC patients and the efficacy of immunotherapy in this population.

Key Words: Ferroptosis; Gastric cancer; *Helicobacter pylori* infection; Immune checkpoint protein; Prognostic model; Ferroptosis-related gene prognostic index

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Core Tip: This study aimed to develop a prognostic model based on coregulated differentially expressed genes among ferroptosis-related genes (FRGs) in gastric cancer (GC). Gene expression profiles from GC patients and those with *Helicobacter pylori*-associated GC were analyzed, along with FRGs obtained from the FerrDb database. The resulting ferroptosis-related gene prognostic index (FRGPI), based on four hub genes (NOX4, MTCH1, GABARAPL2, and SLC2A3), accurately predicted GC patient survival. High-risk individuals had significantly worse overall survival, and the FRGPI was associated with disease progression and increased expression of immune checkpoint proteins. These findings provide insights into GC prognosis and immunotherapy efficacy.

INTRODUCTION

Gastric cancer (GC) is a common malignancy of the digestive system. According to global 2018 cancer data, GC has the fifth highest incidence and the third highest fatality rate among malignant tumors[1]. More than 60% of GCs are linked to infection with *Helicobacter pylori* (*H. pylori*), a gram-negative, active, microaerophilic, and helical bacterium[2]. This parasite induces GC by producing toxic factors, such as cytotoxin-related gene A, vacuolar cytotoxin A, and outer membrane proteins[3]. The World Health Organization classifies *H. pylori* as a Group I carcinogen[4]. Ferroptosis, or iron-dependent programmed cell death[5], has been linked to GC, although there has been little research on the link between *H. pylori* infection-related GC and ferroptosis. Therefore, this study investigated the coregulated differentially expressed genes (co-DEGs) among ferroptosis-related genes (FRGs) in *H. pylori* infection-related GCs and investigated the relationship between the expression of these co-DEGs and clinical prognosis.

MATERIALS AND METHODS

Collection of data

We obtained clinical information and molecular data on 375 GC patients from the cancer genome atlas (TCGA) database (241 men and 134 women) to create a training cohort. RNA sequencing (RNA-seq) data and corresponding clinical data for external validation cohorts (GSE99553, n = 42; GSE84426, n = 76) were downloaded from the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo). Additionally, 352 unique FRGs, including driver genes, suppressor genes, and markers, were obtained from the FerrDb database (http://www.zhounan.org/ferrdb/current/) [6]. Figure 1 shows the flow diagram of the data collection and analysis: (1) RNA-seq data in fragments per kilobase of transcript per million fragments mapped (FPKM) format were log2 transformed to generate the downloaded expression profile data; (2) clinical correlation and prognostic analyses: RNA-seq data in the HTSeq-FPKM database were log2 transformed into the transcripts per million format; and (3) clinical information such as tumor stage, metastasis status, and survival status was obtained from the database, and the relevant package in R (version 3.6.3) (http://www.r-project.org/) was used for analysis.
Wang L et al. Predictive model of gastric cancer

Figure 1 Flow diagram of data collection and analysis. Co-DEGs: Co-regulated differentially expressed genes; FRGPI: Ferroptosis-related genes prognostic index; GC: Gastric cancer; ROS: Reactive oxygen species; LASSO: Least absolute shrinkage and selection operator; TCGA: The cancer genome atlas.

Bioinformatics analysis
The GC tissues in the TCGA-Stomach Adenocarcinoma (STAD) and GSE99553 datasets were divided into *H. pylori*-positive and *H. pylori*-negative groups according to the presence or absence of *H. pylori* infection, respectively. The limma R package was used to identify DEGs, and genes whose expression was significantly upregulated or downregulated were selected for heatmapting. The survminer package screens for molecules with prognostic value in TCGA-STAD. All the datasets were selected with the criteria set to \( P < 0.05 \) and \( |\log2FC| > 1 \). Venn tools (http://bioinformatics.psb.ugent.be/webtools/Venn/) were used to obtain *H. pylori* related to cancer of the stomach and FRGS Co-DEGs, and a Venn diagram was drawn.

The clusterProfiler R package was used to conduct GO/KEGG analysis of the Co-DEGs, in which the GO enrichment analysis included biological process (BP), cell composition, and molecular function (MF). KEGG analysis was used to define the pathways related to the Co-DEGs, and the parameters were set to \( P\text{-adj} < 0.05 \). Immunoinformatic correlation analysis was performed using the GSVA package.

Clinical correlation and prognostic analyses of co-DEGs
The prognosis-related co-DEGs were chosen as hub genes. The pROC package was used to investigate the diagnostic value of the hub genes for GC and *H. pylori*-associated GC. Correlations between hub genes and clinicopathological factors were compared in the TCGA and GSE84426 datasets, and univariate and multivariate Cox proportional hazards regression analyses were performed. The ferroptosis-related gene prognostic index (FRGPI) was identified by the minor absolute shrinkage and selection operator (LASSO) and a hazard model via the “glmnet” R package. The predictive value of the FRGPI was determined via Kaplan–Meier survival curves and receiver operating characteristic (ROC) curves generated via the “survival” and “survival ROC” R packages. A nomogram was created using the FRGPI and clinical data, and the predictive efficacy of the FRGPI at 1, 3, and 5 years was evaluated using a prognostic calibration curve. The following formula was used to calculate the FRGPI:

\[
\text{FRGPI} = (\text{Expression level of Gene 1} \times \beta \text{ coefficient}) + (\text{Expression level of Gene 2} \times \beta \text{ coefficient}) + \ldots + (\text{Expression level of Gene n} \times \beta \text{ coefficient}).
\]

Patients were divided into low-risk and high-risk groups based on the median FRGPI.

GC cell lines and cell culture
The HGC-27 and MGC-803 cell lines were donated by Dr. Wu Zizhen of Peking Medicine and stored in the Clinical Laboratory of the Second Hospital of Zhejiang University. These cells were cultivated in RPMI-1640 medium (Cytiva, China) containing 10% fetal bovine serum (Cytiva, China) at 37 °C and 5% CO₂.

Immunohistochemistry
The tumor and neighboring normal tissues were embedded in paraffin after treatment with 10% formalin. Appropriate tissue sections were deparaffinized. The 5-μm-thick paraffin sections were dewaxed with xylene, dehydrated in 100%, 95%, 90%, 80%, and 70% absolute ethanol solutions for 5 min each, and boiled in distilled water for 15 min. After blocking
with 10% serum-containing blocking solution at room temperature for 1 h, the sections were incubated with primary antibodies overnight at 4 °C, followed by incubation with secondary antibodies at room temperature for 30 min. The signals from horseradish peroxidase (HRP)-labeled antibodies (Servicebio, China) were developed by diaminobenzidine. After counterstaining with hematoxylin, the sections were dehydrated and sealed. Immunofluorescence analysis was subsequently conducted. ImageJ was used to evaluate the immunohistochemical and immunofluorescence results. The average optical density (OD) and gray value were calculated, and statistical analysis was conducted.

**Cell activity assay**
HGC-27 and MGC-803 cells were seeded in 96-well plates at 5000 cells/well and cultured at 37 °C and 5% CO₂ for 24 h. The ferroptosis inducer FIN56 (Dojindo, Japan) was dissolved in dimethyl sulfoxide to generate working concentrations of 0, 1, 2, 5, 10, 15, and 20 μM; each concentration was evaluated in six wells. Cell viability was measured using a Cell Counting-8 Kit (CCK-8, Beyotime, China). A microplate reader was used to determine the OD at an absorbance of 450 nm. After calculating the half-maximal inhibitory concentration of each cell line, the inhibition curve was plotted using the ggplot2 R tool.

**Reactive oxygen species detection**
HGC-27 and MGC-803 cells were grown in 6-well plates for 24 h before being treated with FIN56 (2, 5, and 10 μM) for 24 h. After two washes in phosphate buffered saline (PBS), the cells were incubated for 30 min at 37 °C in new medium supplemented with 10 mM 2,7'-dichlorofluorescein diacetate (DCF; Abcam, ab113851, United States). A fluorescence microscope was used to examine the fluorescence intensity of the reactive oxygen species (ROS) in the cells in the 6-well plate. The images were examined using ImageJ software; the integrated density was calculated, and statistical analysis was carried out.

**Western blot**
GC cells were rinsed twice with sterile PBS before being lysed for 5 min on ice in radio immunoprecipitation assay lysis buffer containing 1% phenylmethanesulfonyl fluoride (Beyotime, China). The cell lysates were centrifuged at 12000 pm and 4 °C for 15 min, heated for 5 min in boiling water, and then placed on ice. The bicinchoninic acid technique was used to measure the protein concentration (Beyotime, China). Proteins were separated by electrophoresis at 100 V for 1.5 h and then transferred to a polyvinylidene fluoride membrane, which was washed with phosphate buffered solution, blocked at room temperature for 1 h and incubated overnight at 4 °C with primary antibodies (NOX4: 1:2000; GABARAPL2: 1:1000; MTCH1: 1:1000; and SLC2A3: 1:8000; all from Abcam). After the samples were incubated for 1 h at room temperature with an anti-HRP-conjugated IgG secondary antibody (Service, China), they were detected using chemiluminescence detection equipment (GE, United States). Using ImageJ software, the westernblot images were evaluated, the mean gray value of each band was determined, and the outcomes were statistically examined.

**Statistical analysis**
R version 4.0.3 with the R Studio software package and SPSS 23.0 software were used for statistical analysis. Overall survival (OS) was analyzed by the K-M method. Univariate and multivariate Cox regression analyses were used to determine the effect of each variable on OS, and LASSO was used to avoid overfitting the multivariate Cox regression model. The generated multivariate Cox regression model was utilized to compute the risk score and develop the prognostic nomogram model. The C-index was calculated, and calibration plots were generated to evaluate the predictive value of the nomogram at 1, 3, and 5 years. Chi-square and Fisher’s exact tests were used to assess the associations between clinical features and risk scores, while ROC analysis was utilized to determine sensitivity and specificity. P < 0.05 indicated statistical significance.

**RESULTS**

**Identification of the co-DEGs and GO and KEGG analyses**
Volcano plots and heatmaps were created to demonstrate the differences in FRG expression between H. pylori+ and H. pylori- GC samples from the TCGA-STAD and GSE99553 cohorts. From these FRGs, we identified 244 ferroptosis-related co-DEGs (Figure 2A-D), including 113 ferroptosis-related driver genes, 74 suppressor genes, and 87 marker genes (Figure 2E and F). GO and KEGG analyses revealed that “cellular response to oxidative stress” and “response to oxidative stress” were the main enriched BP terms. The enriched cellular component terms were related mainly to structures such as autophagosomes, peroxisomes, the peroxisomal membrane, the microbody membrane, and the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex. The effective MF terms included ubiquitin protein ligase binding, antioxidative activity, iron ion binding, and ferrous binding. KEGG analysis revealed that the most relevant signaling pathways associated with the Co-DEGs were involved in ferroptosis and the Forkhead box O (FoxO), nucleotide-binding oligomerization domain (NOD)-like receptor, mTOR, hypoxia-inducible factor (HIF)-1, interleukin-17 (IL-17), Toll-like receptor (TLR), and mitogen-activated protein kinase (MAPK) signaling pathways, among others (Figure 2G-J).

**Construction of the FRGPI and its value in predicting GC**
We identified genes that were commonly associated with prognosis among the 1390 oncogenes in the TCGA-STAD cohort and among the 244 co-DEGs; this analysis yielded 18 oncogenes related to OS in GC patients (P < 0.05) (Figure 3A).
Figure 2 Identification of co-regulated differentially expressed genes and GO and KEGG analyses. A: Differential gene expression map in GSE99553; B: Heatmap of differentially expressed genes in GSE99553; C: Differential gene expression map in the cancer genome atlas of stomach adenocarcinoma (TCGA-STAD) cohort; D: Heatmap of differentially expressed genes in the TCGA-STAD cohort; E and F: Overlapping differentially expressed genes in TCGA-STAD and GSE99553 related to ferroptosis; G-J: GO and KEGG analysis results of the Co-DEGs. NADPH: Nicotinamide adenine dinucleotide phosphate; MAPK: Mitogen-activated protein kinase; HIF-1: Hypoxia-inducible factor; IL-17: Interleukin-17; FoxO: Forkhead box O; NOD: Nucleotide-binding oligomerization domain; TCGA-STAD: The cancer genome atlas of stomach adenocarcinoma; Co-DEGs: Co-regulated differentially expressed genes.

Using LASSO–Cox regression with the coefficient value and hazard ratio (HR), we identified NOX4, MTCH1, GABARAPL2, and SLC2A3 as hub genes (Figure 3C and D) that had higher expression in tumor tissue than in normal tissue (Figure 3E and F). Furthermore, NOX4, MTCH1, GABARAPL2, and SLC2A3 demonstrated some accuracy in predicting GC; NOX4 and SLC2A3 had the highest areas under the curve (AUC) values. The AUCs for predicting GC and H. pylori infection-related GC according to the hub genes were 0.747 and 0.870, respectively (Figure 3G-J). The following formula was used to determine the FRGPIs of the hub genes.

$$FRGPI = (\text{expression level of } NOX4 \times 0.432) + (\text{expression level of } MTCH1 \times 0.344) + (\text{expression level of } GABARAPL2 \times 0.642) + (\text{expression level of } SLC2A3 \times 0.577).$$

The FRGPI independently predicts GC patient survival

The median FRGPI was used as the cutoff for stratifying patients in the TCGA-STAD and GSE84426 cohorts into high- and low-risk groups. The distributions of the FRGPI and survival status according to the hub genes in the low-risk and high-risk groups are shown in Figure 4A and B. K–M curve analysis revealed that OS was much lower in the high-risk group than in the low-risk group (P < 0.05; Figure 4C and D). We created a prognostic nomogram using the above data to improve the prediction of individual patient survival risk, and the results demonstrated high consistency [C-index=0.734 (0.712-0.755)]. Calibration curves showed that the predicted and actual 1-, 3-, and 5-year survival rates of people in the TCGA cohort were similar (Figure 5B-D).

Analysis of independent prognostic factors

For the TCGA-STAD and GSE84426 cohorts, univariate and multivariate Cox proportional hazards regression analyses were performed to verify FRGPI independence. The FRGPI and OS were strongly related (Table 1). In both the TCGA-STAD [HR (95%CI): 2.973 (1.966-4.496), P < 0.01] and GSE84426 [HR (95%CI): 2.001 (1.200-3.501), P < 0.01] cohorts, the FRGPI was an independent predictor of GC prognosis.

The prognostic index of FRGs is significantly associated with disease progression

According to the multivariate Cox regression analysis, the primary treatment outcome of tumor progression and the risk score for the FRGPI were considerably greater in the TCGA cohort (P < 0.05; Figure 5A). We created a prognostic nomogram using the above data to improve the prediction of individual patient survival risk, and the results demonstrated high consistency [C-index=0.734 (0.712-0.755)]. Calibration curves showed that the predicted and actual 1-, 3-, and 5-year survival rates of people in the TCGA cohort were similar (Figure 5B-D).

Analysis of immune cells and the response to immunotherapy

The identified hub genes had various degrees of association with infiltrating immune cells and were positively associated with natural killer (NK) cells, eosinophils, and interdigitating dendritic cells (iDCs) (Figure 6A-D). DCs, eosinophils,
Figure 3 Construction of the ferroptosis-related genes prognostic index and its value in predicting gastric cancer. A: Overlap of differentially
expressed genes and prognostic genes; B: Forest plot showing the multivariate Cox model results for the Co-regulated differentially expressed genes; C and D: LASSO analysis of related genes; E: Expression of hub genes in gastric cancer (GC) and normal tissues (The Cancer Genome Atlas); F: Expression of hub genes in GC and normal tissues (GSE99553); G: Receiver operating characteristic curves (ROC) of hub genes for predicting GC (GSE99553); H: ROC curves of hub genes combined detection for predicting GC (GSE99553); I: ROC curve of hub genes for predicting H. pylori infection-related GC (GSE99553); J: ROC curve of hub genes combined detection for predicting H. pylori infection-related GC (GSE99553). TCGA-STAD: The cancer genome atlas of stomach adenocarcinoma; AUC: Areas under the curve; FPR: False positive rate; HR: Hazard ratio; TPR: True positive rate.

Figure 4 The ferroptosis-related genes prognostic index independently predicts gastric cancer patient survival. A: Risk score distribution and patient survival in the low- and high-risk groups of the cancer genome atlas of stomach adenocarcinoma (TCGA-STAD); B: Risk score distribution and patient survival in the low- and high-risk groups of GSE84426 cohorts; C: K-M survival analysis of the TCGA-STAD; D: K-M survival analysis of GSE84426 cohorts; E: Areas under the curve (AUC) of the time-dependent receiver operating characteristic curves (ROC) for the ferroptosis-related gene prognostic index in the TCGA-STAD; F: AUC of the time-dependent ROC curve for the GSE84426 cohorts. FPR: False positive rate; HR: Hazard ratio; TPR: True positive rate.
iDCs, macrophages, neutrophils, NK cells, effector memory T (Tem) cells, and Type 1 T helper (Th1) cells were considerably upregulated in the high-risk group. Intense infiltration of NK cells, Tem cells, and Th1 cells was observed in the low-risk group (P < 0.05) (Figure 6E).

Additionally, the FRGPI high-risk subgroup exhibited a significant increase in the expression of the most common immune checkpoint proteins (ICPs), including VTCN1, CD48, CD28, CTLA4, PDCD1, TIGHT, and CD274 (PD-L1); LAIR1, CD200, CD86, HAVCR2, LAG3, CD70, and CD40 (Figure 6F).

**Validation of hub gene expression**

Immunohistochemistry analysis indicated that the proteins encoded by the hub genes (NOX4, MTCH1, GABARAPL2, and SLC2A3) were strongly expressed in GC and *H. pylori*-associated GC (Figure 7A and B). Additionally, Western blot analysis revealed that the expression levels of the hub genes were significantly greater in HGC-27 and MGC-803 cells than in normal gastric GES-1 cells (Figure 7C and D). Furthermore, immunofluorescence staining supported this observation by demonstrating hub gene expression in both the nucleus and cytoplasm (Figure 7E and F).

**Effect of the ferroptosis inducer FIN56 on hub genes**

Cytotoxicity assays and scratch experiments indicated that FIN56 inhibited HGC-27 and MGC-803 cells in a dose-dependent manner (Figure 8A and B). The inhibitory concentration values for FIN56 in HGC-27 and MGC-803 cells were 5.9 μM and 8.75 μM, respectively (Figure 8C and D). Moreover, FIN56 treatment decreased the mRNA expression of the hub genes in both HGC-27 and MGC-803 cells; Western blot analyses revealed that hub gene expression decreased in a dose-dependent manner (Figure 8E and F). ROS levels in HGC-27 and MGC-803 cells increased as the FIN56 concentration increased (Figure 8G and H).

**DISCUSSION**

Infection with *H. pylori* is a significant risk factor for distal GC development[1-3]. Ferroptosis is a programmed cell death mechanism associated with fatal lipid peroxidation[7]. Iron metabolism abnormalities are related to both ferroptosis and elevated GC risk[8]. Many mechanisms may be linked to ferroptosis, *H. pylori* infection, and GC. Nevertheless, few studies have evaluated the relationship between ferroptosis and the prognosis of *H. pylori*-related GC patients. Therefore, this study aimed to identify Co-DEGs, construct an FRGPI, and validate the predictive significance of the FRGPI in H.
Correlation of hub genes with immune infiltration and immunotherapy. A: Correlation between NOX4 and immune cells; B: Correlation between MCH1 and immune cells; C: Correlation between GABARAP2 and immune cells; D: Correlation between SLC2A3 and immune cells; E: Differences in the ratios of immune cells between the high- and low-risk groups in the cancer genome atlas of stomach adenocarcinoma (TCGA-STAD) cohort; F: Different expression levels of immune checkpoint proteins between the high- and low-risk groups in the TCGA-STAD cohort.

Figure 6

This study identified a total of 244 co-DEGs. GO and KEGG analyses revealed that the co-DEGs were involved primarily in ferroptosis and in the FoxO, NOD-like receptor, mTOR, HIF-1, IL-17, TLR, and MAPK signaling pathways, among others; all of these pathways are strongly associated with GC occurrence and development. The FoxO subfamily of the forkhead transcription factor family is involved in cell fate determination and is thought to function as a tumor suppressor in various malignancies[9]. FoxO is implicated in both mitochondria-dependent and mitochondria-independent apoptotic processes by activating death receptor ligands such as the Fas ligand, TNF apoptotic ligand, and Bcl-2 family members[10]. The PI3K/AKT pathway is the most critical pathway through which FoxO1 interacts with many cancers[11]. In addition, numerous other vital pathways, including the Ras–MEK–ERK, IKK, and AMPK pathways, are involved in the mechanism by which FoxOs influence carcinogenesis[10,11]. The inflammasome, which is composed of NOD-like receptors, is also a critical component of innate immunity-induced host defense and inflammation in cancer[12]. Increased inflammasome activity has been linked to various malignancies, including breast, gastric, brain, and malignant prostate cancer, and is protective against colitis-associated cancers[13]. The tumor stage and engagement of different caspase isoforms with the inflammasome pathway impact inflammasome function in cancer. Autophagy inhibition via mTOR-dependent mechanisms such as the AMPK/mTOR and PI3K/Akt/mTOR pathways contributes to the malignant progression of GC cells[14]. Autophagy, which is essential for homeostasis, has dual roles in GC, acting as a tumor suppressor and promoter[15]. Long-term *H. pylori* infection inhibits autophagy, potentially promoting GC. HIF-1 signaling is vital for metabolism, inflammation, vascular homeostasis, and cancer. HIF-1 activation occurs in several malignancies, including GC[16]. Tumor-associated neutrophils release IL-17a, stimulating GC cell migration and invasion via JAK2/STAT3 activation[17]. TLRs, pattern recognition receptors crucial for *H. pylori* lipopolysaccharide recognition, are pivotal in GC development, occurrence, and treatment[18,19]. TLR activators hold potential as therapeutic and adjuvant agents in combination with other immunotherapies, suggesting that TLRs are immunotherapy targets for GC.[20]. The MAPK regulatory network, which includes many components that initiate a phosphorylation cascade upon activation, elicits precise cellular responses[21]. This network significantly influences GC progression[22].

This study utilized public databases to identify crucial ferroptosis-related DEGs in *H. pylori*-associated GC. The discovered molecular processes and signaling pathways may help explain the relationship between Co-DEGs and *H. pylori*-associated GC.

This research identified a total of 244 co-DEGs. GO and KEGG analyses revealed that the co-DEGs were involved primarily in ferroptosis and in the FoxO, NOD-like receptor, mTOR, HIF-1, IL-17, TLR, and MAPK signaling pathways, among others; all of these pathways are strongly associated with GC occurrence and development. The FoxO subfamily of the forkhead transcription factor family is involved in cell fate determination and is thought to function as a tumor suppressor in various malignancies[9]. FoxO is implicated in both mitochondria-dependent and mitochondria-independent apoptotic processes by activating death receptor ligands such as the Fas ligand, TNF apoptotic ligand, and Bcl-2 family members[10]. The PI3K/AKT pathway is the most critical pathway through which FoxO1 interacts with many cancers[11]. In addition, numerous other vital pathways, including the Ras–MEK–ERK, IKK, and AMPK pathways, are involved in the mechanism by which FoxOs influence carcinogenesis[10,11]. The inflammasome, which is composed of NOD-like receptors, is also a critical component of innate immunity-induced host defense and inflammation in cancer[12]. Increased inflammasome activity has been linked to various malignancies, including breast, gastric, brain, and malignant prostate cancer, and is protective against colitis-associated cancers[13]. The tumor stage and engagement of different caspase isoforms with the inflammasome pathway impact inflammasome function in cancer. Autophagy inhibition via mTOR-dependent mechanisms such as the AMPK/mTOR and PI3K/Akt/mTOR pathways contributes to the malignant progression of GC cells[14]. Autophagy, which is essential for homeostasis, has dual roles in GC, acting as a tumor suppressor and promoter[15]. Long-term *H. pylori* infection inhibits autophagy, potentially promoting GC. HIF-1 signaling is vital for metabolism, inflammation, vascular homeostasis, and cancer. HIF-1 activation occurs in several malignancies, including GC[16]. Tumor-associated neutrophils release IL-17a, stimulating GC cell migration and invasion via JAK2/STAT3 activation[17]. TLRs, pattern recognition receptors crucial for *H. pylori* lipopolysaccharide recognition, are pivotal in GC development, occurrence, and treatment[18,19]. TLR activators hold potential as therapeutic and adjuvant agents in combination with other immunotherapies, suggesting that TLRs are immunotherapy targets for GC.[20]. The MAPK regulatory network, which includes many components that initiate a phosphorylation cascade upon activation, elicits precise cellular responses[21]. This network significantly influences GC progression[22].

This study utilized public databases to identify crucial ferroptosis-related DEGs in *H. pylori*-associated GC. In GC patients, FRGs were differentially expressed between normal and tumor tissues, indicating the substantial involvement of ferroptosis in this disease. Univariate Cox analysis revealed 18 genes linked to OS, highlighting the feasibility and utility of constructing a prognostic signature using the FRGPI. LASSO-Cox analysis identified a novel prognostic signature comprising four FRGs: NOX4, MCH1, GABARAP2, and SLC2A3. The four hub genes accurately predicted GC using ROC curves. Functional enrichment analyses of the tumor microenvironment and immunotherapy response revealed that the hub genes might accurately predict GC patient prognosis and clinical state. To better understand the involvement of these genes in GC, we describe their primary molecular activity.

NOX4 is an NADPH oxidase that promotes the cellular ROS response[23]. When the intracellular iron ion concentration exceeds normal levels, NOX4 is activated, producing a large amount of oxygen free radicals, which combine with...
iron ions to form highly reactive hydrogen and oxygen free radicals. These radicals then destroy proteins, DNA, and lipid molecular structures in cells. Hydroxyl radical activity causes the release of intracellular iron ions, intensifying the ROS response[23]. Previous studies have shown that it interferes with cancer cell proliferation, apoptosis, and cell cycle progression in conditions such as brain glioma, non-small cell lung cancer, and melanoma[24-26]. Therefore, NOX4 stimulates tumor development through various channels and processes and is thus considered a potential therapeutic target[27]. NOX4 overexpression accelerates cancer development and is correlated with poor prognosis in patients with colorectal cancer[28]. Additionally, the NOX4 gene was deleted in liver cancer patients, demonstrating that it may exert tumor-suppressor effects by facilitating TGF-β1-induced apoptosis[29]. Although the mechanism through which NOX4 promotes GC development and invasion is unknown, studies have revealed that NOX4 may stimulate GC cell proliferation and invasion through epithelial-mesenchymal transition[30,31]. Moreover, Li et al[32] showed that the new biotin derivative XN4 could induce ferroptosis in GC cells by upregulating NOX4 expression[32].

*MTC1* encodes a protein with two proapoptotic domains that localize to the inner mitochondrial membrane, causing apoptosis independently of Bax and Bak[33]. *MTC1* regulates cellular iron levels, maintaining iron within normal limits. When cellular iron levels exceed normal levels, *MTC1* is activated, causing mitochondrial outer membrane permeabilization (MOMP) and the release of mitochondrial molecules into the cytoplasm[34]. MOMP releases iron ions from mitochondria into the cytoplasm, increasing cellular iron. The increased iron can interact with hydroxide ions to produce highly reactive hydroxide radicals. These free radicals can cause cell death by damaging proteins, DNA, and lipids[35]. Although bioinformatics analysis and functional validation suggest that increased *MTC1* expression is correlated with metastasis, cancer stage, and poor survival in liver hepatocellular carcinoma patients[36], no study has validated *MTC1* expression in GC.

*SLC2A3*, also known as GLUT3, is a transmembrane transporter with high affinity for glucose. It can regulate intracellular glucose metabolism and promote ferroptosis, but little to no expression has been detected in normal tissues[37]. *SLC2A3* is activated when the intracellular glucose concentration increases, promoting NOX4 activation, increasing oxygen free radical production, and inducing ferroptosis[38]. Tumor cells enhance glucose consumption through aerobic glycolysis for proliferation. Hence, *SLC2A3* is strongly overexpressed in various tumor types[39,40]. *SLC2A3* overexpression may increase colorectal cancer cell proliferation and invasion by boosting nucleotide synthesis[41]. Glycolysis control in GC cells promotes macrophage infiltration and tumor development[42,43]. Therefore, *SLC2A3* may be a predictive biomarker in these cancers.

*GABARAPL2*, also known as *GATE-16* (16 kDa enhancer of Golgi-associated ATPase), is a member of the autophagy-associated protein 8 subfamily and is involved in the degradation of autophagosome membranes. It promotes intracellular ferroptosis[44]. *GABARAPL2* forms a complex with LC3 to operate at various autophagosome phases; the LC3 subfamily promotes phagocyte membrane fusion, while the *GABARAP* subfamily functions downstream[45]. *GABARAPL2* can promote MOMP, causing mitochondrial molecules to escape into the cytoplasm. Under MOMP, iron ions in mitochondria are released into the cytoplasm, increasing cellular iron and inducing ferroptosis[46]. High *GABARAPL2* mRNA expression is associated with poorer prognosis in distinct malignancies, such as worse OS in patients with esophageal cancer or GC[47,48] but better OS in patients with kidney cancer[49]. This discrepancy primarily results from the autophagy-dependent and autophagy-independent functions of *GABARAPL2*.

We utilized multivariate analysis to create a prognostic model and then assessed the accuracy of the individual patient predictions. The prognostic nomogram and calibration curves were consistent with the actual results based on the 1-year, 3-year, and 5-year survival curves. Similar curves indicate that the model can accurately predict OS. However, the novel prognostic nomogram has the potential to increase the prediction accuracy and assist in predicting individual patient survival risk, with significant therapeutic implications[50]. According to the FGRPI, which was developed using hub gene characteristics, GC patients can be classified into low-risk and high-risk groups. The low-risk group had a better
Table 1 Univariate and multivariate Cox regression analyses of the ferroptosis-related genes prognostic index and other clinicopathologic factors for overall survival in the cancer genome atlas and GSE84426 cohorts

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hazard ratio (95%CI)</td>
<td>P value</td>
</tr>
<tr>
<td><strong>TCGA cohort</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (≤ 65 vs &gt; 65)</td>
<td>1.620 (1.154-2.276)</td>
<td>0.005</td>
</tr>
<tr>
<td>Gender (male vs female)</td>
<td>1.267 (0.891-1.804)</td>
<td>0.188</td>
</tr>
<tr>
<td>Pathologic stage (II&amp;III vs IV)</td>
<td>1.947 (1.358-2.793)</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>T stage (T1&amp;T2 vs T3&amp;T4)</td>
<td>1.719 (1.131-2.612)</td>
<td>0.011</td>
</tr>
<tr>
<td>N stage (N0 vs N1&amp;N2&amp;N3)</td>
<td>1.925 (1.264-2.931)</td>
<td>0.002</td>
</tr>
<tr>
<td>M stage (M0 vs M1)</td>
<td>2.254 (1.295-3.924)</td>
<td>0.004</td>
</tr>
<tr>
<td>Histologic grade (G1 vs G2&amp;G3)</td>
<td>1.957 (0.484-7.910)</td>
<td>0.346</td>
</tr>
<tr>
<td><em>H. pylori</em> infection</td>
<td>0.650 (0.279-1.513)</td>
<td>0.317</td>
</tr>
<tr>
<td>Primary therapy outcome (CR vs PD&amp;SD&amp;PR)</td>
<td>4.228 (2.905-6.152)</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>FRGPI (high risk vs low risk)</td>
<td>1.542 (1.105-2.152)</td>
<td>0.011</td>
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<tr>
<td><strong>GSE84426</strong></td>
<td></td>
<td></td>
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<tr>
<td>Age (≤ 65 vs &gt; 65)</td>
<td>1.023 (0.993-1.053)</td>
<td>0.132</td>
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<tr>
<td>Gender (male vs female)</td>
<td>0.857 (0.401-1.830)</td>
<td>0.689</td>
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<tr>
<td>T stage (T1&amp;T2 vs T3&amp;T4)</td>
<td>1.392 (1.668-2.900)</td>
<td>0.477</td>
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<tr>
<td>N stage (N0 vs N1&amp;N2&amp;N3)</td>
<td>1.010 (0.356-2.862)</td>
<td>0.986</td>
</tr>
<tr>
<td>FRGPI (high risk vs low risk)</td>
<td>2.001 (1.000-3.001)</td>
<td>&lt; 0.01</td>
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FRGPI: Ferroptosis-related genes prognostic index; CR: Complete response; PR: Partial Response; PD: Progressive disease; SD: Stable disease.

prognosis and longer OS than did the high-risk group. To further investigate the underlying mechanisms of the hub genes, we determined that they were significantly related to infiltrating immune cells. Moreover, the FRGPI was associated with the tumor microenvironment and immunotherapy response. There were substantial differences in immune cell infiltration between the FRGPI low-risk and high-risk patients. In the high-risk group, DCs, eosinophils, iDCs, macrophages, and neutrophils exhibited considerable infiltration, while in the low-risk group, NK cells, Tem cells, and Th1 cells exhibited substantial infiltration. NK cells inhibit tumor development and promote CD4+ Th1 polarization. Greater increases in the NK cell percentage correlate with prolonged survival in GC patients, suggesting that NK cells may be an independent prognostic biomarker[51]. Central memory T cells activated by tumor antigens create antitumor Tem cells, which destroy cancer cells and bolster immunity[52]. An imbalance in Th1 and Th2 cells is considered significant in GC development[53]. A shift from Th1 to Th2 cytokines can create an immunosuppressive state, compromising antitumor immunity[54]. Additionally, we observed significant differences in patient characteristics and ICPs between the high-risk and low-risk groups. The expression of genes that encode most ICPs was significantly upregulated in the high-risk group, implying a better immunotherapy response in low-risk patients. Previous research links ferroptosis and tumor immunotherapy. Tumor cell ferroptosis sensitivity correlates with immunological function, explaining the effects of immunotherapy on tumor cells[55]. Cytokines, especially interferon-γ, generated by immunotherapy-activated CD8+ T lymphocytes downregulate SLC3A2 and SLC7A11 expression and inhibit tumor cell cystine uptake[43]. Inflammatory tumors have high immunogenicity, preserved antigen presentation, and intense CD8+ T-cell infiltration. Ferroptosis-inducing agents can dramatically reduce immune checkpoint inhibitor immunotherapy efficacy by killing invading CD8+ (and CD4+) T cells and impairing naive DC maturation and function[56]. The ferroptosis inducer FIN56 used here blocks glutathione peroxidase 4 (GPX4)[57]. GPX4, the first ferroptosis inhibitor identified, uses GSH to reverse lipid peroxidation and prevent ferroptosis. Moreover, GPX4 protects cells against lipid peroxidation-related death and oxidative stress[58].

To determine whether the identified hub genes are required for ferroptosis in GC, we utilized FIN56 to trigger ferroptosis in two GC cell lines, HGC-27 and MGC-803. CCK-8 assays revealed that FIN56 significantly inhibited the proliferation of HGC-27 and MGC-803 cells, and low concentrations of FIN56 were fatal to GC cell lines. Lipid peroxides, abundant ROS in living organisms, primarily cause ferroptosis[59]. Thus, we detected significantly elevated ROS levels in FIN56-treated GC cells by flow cytometry and fluorescence microscopy, suggesting that FIN56-induced cell death is due to ferroptosis. Furthermore, both the mRNA and protein levels of the hub genes decreased in a dose-dependent manner in FIN56-treated GC cells, demonstrating that FIN56 regulates hub gene expression during ferroptosis. These data

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suggest that FIN56 is a potential drug for inhibiting GC cell invasion.

CONCLUSION

In conclusion, this study identified co-DEGs between *H. pylori*-associated GC and ferroptosis that promote GC formation and progression through various signaling pathways. The FRGPI, which included NOX4, MTCH1, GABARAPL2, and SLC2A3, demonstrated high predictive accuracy.

ARTICLE HIGHLIGHTS

Research background

Gastric cancer (GC) is a common malignancy of the digestive system. Ferroptosis or iron-dependent programmed cell death, has been linked to GC, although there has been little research on the link between *Helicobacter pylori* (*H. pylori*) infection-related GC and ferroptosis.

Research motivation

This study investigated the coregulated differentially expressed genes (co-DEGs) among ferroptosis-related genes (FRGs) in *H. pylori* infection-related GCs and investigated the relationship between the expression of these co-DEGs and clinical prognosis.

Research objectives

This study developed a prognostic model for GC based on co-DEGs among FRGs. Gene expression profiles from GC patients and *H. pylori*-associated GC patients were analyzed.

Research methods

Gene expression profiles of GC patients and those with *H. pylori*-associated GC were obtained from the cancer genome atlas and gene expression omnibus (GEO) databases. The FRGs were acquired from the FerrDb database. A ferroptosis-related gene prognostic index (FRGPI) was created using least absolute shrinkage and selection operator–Cox regression. The predictive ability of the FRGPI was validated in the GEO cohort. Finally, we verified the expression of the hub genes and the activity of the ferroptosis inducer FIN56 in GC cell lines and tissues.

Research results

The four hub genes (NOX4, MTCH1, GABARAPL2, and SLC2A3) accurately predicted GC and *H. pylori*-associated GC. The FRGPI based on the hub genes independently predicted patient survival. High-risk GC patients had notably worse...
overall survival. The FRGPI was found to be a significant predictor of GC prognosis and correlated with disease progression. The expression of immune checkpoint proteins increased in the high-risk group. Hub genes were highly overexpressed in GC cell lines and tissues, primarily at the cell membrane. The ferroptosis inducer FIN56 inhibited GC cell proliferation in a dose-dependent manner.

Research conclusions
The predictive model provides an accurate prognosis for GC patients and helps evaluate immunotherapy efficacy.

Research perspectives
The mechanism of the hub gene in the occurrence and development of GC was studied, and its effect on iron death in GC cells was analyzed.

FOOTNOTES

Author contributions: Wang L contributed data compilation, formal analysis, writing-original version; Gong WH contributed methodology, writing original draft, conceptualization, obtaining funding, mentoring, writing-review and editing.

Institutional review board statement: The protocol of the study was reviewed and approved by the Ethics Committee of the Second Affiliated Hospital Zhejiang University School of Medicine (2023-0177).

Institutional animal care and use committee statement: This study and included experimental procedures were approved by the institutional animal care and use committee of Zhejiang Chinese Medicine University (No. IACUC-20230410-21). All animal housing and experiments were conducted in strict accordance with the institutional guidelines for care and use of laboratory animals.

Informed consent statement: All participants signed informed consent forms.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

Data sharing statement: The data analyzed in this study are available in the cancer genome atlas database (https:// portal.gdc.cancer.gov/); FerrDb Database (http://www.zhounan.org/ferrdb/current/). And the GSE datas downland from below address: (GSE99553: https://www.ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE99553), (GSE84426: https://www.ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE84426). And the experimental data supporting the findings of this study are available within the article. The Supplementary Dataset File was the WB result of Fig 7C and Fig 8E.

ARRIVE guidelines statement: The authors have read the ARRIVE guidelines, and the manuscript was prepared and revised according to the ARRIVE guidelines.

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Country/Territory of origin: China

ORCID number: Li Wang 0000-0002-3356-0704; Wei-Hua Gong 0000-0002-0213-7313.

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