The role of ferroptosis-related genes for overall survival prediction in breast cancer

Li-Yan Jin | Yan-lin Gu | Qi Zhu | Xiao-hua Li | Guo-Qin Jiang

1Department of Thyroid and breast surgery, The Second Affiliated Hospital of Soochow University, Suzhou, China
2Department of Thyroid and breast surgery, Traditional Chinese medicine hospital of kunshan, Kunshan, China
3Department of Thyroid and breast surgery, Wuzhong People's Hospital of Suzhou City, Suzhou, China

Correspondence
Xiao-hua Li, Department of Thyroid and breast surgery, Wuzhong People's Hospital of Suzhou City, No.61 Dongwu Road, Suzhou 215004, China. Email: xq810@163.com
Guo-Qin Jiang, Department of Thyroid and breast surgery, The Second Affiliated Hospital of Soochow University, No.1055 San Xiang Road, Suzhou 215004, China. Email: jiang_guoqin@163.com

Funding information
This work was supported by grants from Advanced Research of Second Affiliated Hospital of Soochow University (SDFEYQN2016) and National Natural Science Foundation of China (81873730)

Abstract

Background: Ferroptosis is a novel iron-dependent form of cell death, which is implicated in various diseases including cancers. However, the influence of ferroptosis-related genes on the prognosis of breast cancer remains unclear.

Methods: RNA sequencing data of 1053 breast cancer tissue samples and 111 normal tissue samples from The Cancer Genome Atlas (TCGA) were analyzed. Expression levels of 259 ferroptosis-related genes were compared. Gene Ontology (GO) and the Kyoto Gene and Genomic Encyclopedia (KEGG) analyses were conducted on differentially expressed genes. Cox univariate analysis was conducted to explore the potential prognostic biomarkers of breast cancer. Infiltrating immune cell status was assessed.

Results: A total of 66 ferroptosis-related genes were differentially expressed in breast cancer tissues. The enriched GO terms included Biological Process (mainly included response to oxidative stress, cellular response to chemical stress, multicellular organismal homeostasis, cofactor metabolic process, response to metal ion, response to steroid hormone, cellular response to oxidative stress, transition metal ion homeostasis, iron ion homeostasis, and cellular iron ion homeostasis), Cellular Component (mainly included apical plasma membrane, early endosome, apical part of cell, lipid droplet, basolateral plasma membrane, blood microparticle, clathrin-coated pit, caveola, astrocyte projection, and pronucleus) and Molecular Function (mainly included iron ion binding, ubiquitin protein ligase binding, oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, oxidoreductase activity, acting on the CH–OH group of donors, NAD or NADP as acceptor, ferric iron binding, aldo–keto reductase (NADP) activity, oxidoreductase activity, acting on single donors with incorporation of molecular oxygen, steroid dehydrogenase activity, alditol:NADP+–oxidoreductase activity, and alcohol dehydrogenase (NADP+) activity). The enriched KEGG pathway mainly included the HIF-1 signaling pathway, NOD-like receptor signaling pathway, ferroptosis, IL-17 signaling pathway, central carbon metabolism in cancer, PPAR signaling pathway, PD-L1 expression, and PD-1 checkpoint pathway in cancer. Among them, 38 ferroptosis-related genes were significantly associated with the prognosis of breast cancer. The prognostic model...
was constructed, and breast cancer patients in low-risk group had a better prognosis. In addition, risk score of ferroptosis prognostic model was negatively correlated with B cells ($r = -0.063$, $p = 0.049$), CD8+ T cells ($r = -0.083$, $p = 0.010$), CD4+ T cells ($r = -0.097$, $p = 0.002$), neutrophils ($r = -0.068$, $p = 0.033$), and dendritic cells ($r = 0.088$, $p = 0.006$).

Conclusions: The ferroptosis pathway plays a key role in breast cancer. Some differentially expressed ferroptosis-related genes can be used as prognostic biomarkers for breast cancer.

**KEYWORDS**
biomarker, breast cancer, ferroptosis, overall survival

### 1 | INTRODUCTION

Ferroptosis, an iron-dependent form of nonapoptotic cell death, was first defined in 2012. It is caused by a dysfunction of oxidized membrane lipids, which reduces the activity of glutathione peroxidase 4. These contribute to the accumulation of lipid-reactive oxygen species and induce cell death. Compared with non-cancerous cells, cancer cells exhibit an increased iron demand. This is a potential promising method to treat cancer. Erastin was discovered to induce ferroptosis and can enhance the sensitivity of chemotherapy drugs, such as temozolomide and cisplatin.

Breast cancer is the most common diagnosed cancer in women. There are an estimated 1.6 million cases of breast cancer each year with an approximately 6.4% mortality rate. According to immuno-histochemistry markers, breast cancer is divided into four subtypes (luminal A, luminal B, HER-2-enriched, and triple-negative). Different subtypes of breast cancer require different therapies. Tumor staging, lymph node status, histological grades, and age are prognostic factors for breast cancer patients. Ma et al. reported that siramesine and lapatinib induced breast cancer cell death through ferroptosis, and after treatment with a ferroptosis inhibitor, breast cancer cell death was inhibited following the siramesine and lapatinib treatment. This indicates that the ferroptosis pathway plays an important role in breast cancer cells by treating with a lysosome disruptor and a tyrosine kinase inhibitor. We researched this article to gain insight into the potential clinical utility of ferritinophagy-related genes, and we investigated the expression levels of ferritinophagy-related genes in breast cancer and the molecular subtypes that contribute to the heterogeneity of breast cancer. Finally, we determined the relationships between ferroptosis and the prognosis of different subtypes.

The data of breast cancer patients were downloaded from The Cancer Genome Atlas (TCGA) database. The RNA-seq transcriptome data were matched to clinical pathologic information and were transformed into sample IDs by Perl. All patients included in this study were followed up for at least one month. Any incomplete survival information was excluded. A total of 259 ferritinophagy-related genes were selected for subsequent bioinformatics analysis.

### 2 | MATERIALS AND METHODS

#### 2.1 Datasets and study cohorts

We downloaded the data of 1053 breast cancer patients and 111 normal tissue samples from the TCGA database. The RNA-seq transcriptome data were matched to clinical pathologic information and were transformed into sample IDs by Perl. All patients included in this study were followed up for at least one month. Any incomplete survival information was excluded. A total of 259 ferritinophagy-related genes were selected for subsequent bioinformatics analysis.

#### 2.2 Bioinformatic analysis and statistical analysis

Differentially expressed genes (DEGs) from ferritinophagy-related genes were analyzed between cancer tissues and normal tissues by the Wilcoxon test using the R v4.0.2. (http://www.r-project.org/) with a cutoff criterion of $p < 0.05$. Gene Ontology (GO), and the Kyoto Encyclopedia Gene and Genomic (KEGG) analyses were conducted according to the DEGs. We performed the Cox univariate analyses to explore any genes associated with prognosis. Each prognosis-related gene was given a risk score. Ferritinophagy-related genes related to prognostic models were constructed through risk scores, and all patients were divided into two groups based on the risk scores. A Kaplan-Meier plot was drawn to assess the survival differences between the two groups. The Cox univariate and multivariate analyses were performed to compare the relationship between clinicopathological variables and risk scores. The ROC curves were graphed to compare clinicopathological variables. To analyze tumor-infiltrating immune cells, the data from the Tumor Immune Estimation Resource (TIMER) database were obtained. The risk scores for the ferritinophagy prognostic model and its correlation with immune infiltrates (B cells, CD4+ T cells, CD8+ T cells, neutrophils, macrophages, and dendritic cells) were analyzed.
3 | RESULTS

3.1 | Identifying differential expressions of ferritinophagy-related genes

A total of 1053 breast cancer tissue samples (including 232 luminal A, 336 luminal B, 134 triple-negative, and 351 HER-2-positive tissue samples) were included, and 49 patients were followed for less than 1 month. The expressions of 259 ferritinophagy-related genes were compared between cancer tissue and normal tissue, and 66 genes were DEGs (FDR < 0.05 and log2 (fold change) > 1). The heatmap, volcano map, and boxplot of DEGs are shown in Figure 1.

3.2 | Functional annotations of the differentially expressed ferritinophagy-related genes

The functional annotation analysis of the 66 DEGs provides a biological understanding for these genes. The top 10 of the three different GO levels (including molecular functions, cell components, and biological processes) are shown in Figure 2.

The enrichment analysis results of the top 10 main KEGG pathways are shown in Figure 3. These genes are closely related to the HIF-1 signaling pathway, ferroptosis, IL-17 signaling pathway, central carbon metabolism in cancer, PPAR signaling pathway, PD-L1 expression, and PD-1 checkpoint pathway in cancer, etc.

FIGURE 1 (A) Expression heatmap of differentially expressed ferroptosis-related genes. (B) Expression volcano map of differentially expressed ferroptosis-related genes. (C) Expression patterns of ferroptosis-related genes in breast cancer types and paired non-tumor samples
FIGURE 2  GO analysis of differential genes, including molecular function, cell component, and biological processes

FIGURE 3  KEGG pathway enrichment analysis of differential genes
3.3 | Identifying genes associated with prognosis

The Cox univariate analysis was conducted to explore the potential genes associated with prognosis among 259 ferritinophagy-related genes, and 38 genes had a close relationship with the prognosis (Figure 4A), while a total of 18 DEGs were significantly associated with prognosis. The expressions of these 18 DEGs are shown in Figure 4B. According to the Cox regression analysis, each prognosis-related gene attained a risk score. A prognostic model was constructed through risk scores, and all patients were divided into two groups for overall survival based on the risk scores. Kaplan-Meier plot is shown in Figure 4C. The patients in the low-risk group had better prognosis than those in the low-risk group. The distributions of the prognostic index and survival statuses of patients in the two groups are shown in Figure 4D, E, and the breast cancer subtypes were widely different. Genes associated with prognosis were analyzed, and the results are shown in Figures S1–S4.

The Cox univariate analysis showed that the risk scores of ferritinophagy-related genes, age, tumor size, and lymph node status and metastasis status show significant association with patient prognosis (Figure 5A). Cox multivariate analysis is shown in Figure 5B. The areas under the curve of the corresponding receiver operating characteristic curve for risk score, age, tumor size, and lymph node and metastasis statuses were 0.688, 0.787, 0.692, 0.624, and 0.563. They indicate that the prognostic index exhibited a certain potential prediction (Figure 5C).

The Wilcoxon test was conducted to compare the relationships between the clinical features (primary tumor status, lymph node status, age, stage, and risk scores) and the expressions of 18 potential prognostic genes. The results are shown in Figure 6.

3.4 | Tumor-infiltrating immune cells

The risk score of ferritinophagy prognostic model is negatively correlated with B cells ($r = -0.063$, $p = 0.049$), CD8$^+$ T cells ($r = -0.083$, $p = 0.010$), CD4$^+$ T cells ($r = -0.097$, $p = 0.002$), neutrophils ($r = -0.068$, $p = 0.033$), and dendritic cells ($r = 0.088$, $p = 0.006$) (Figure 7).

4 | DISCUSSION

Although numerous studies have investigated the genes that might regulate cancer cell death through ferroptosis, their correlations with prognosis remain worth exploring. In this article, we explored the expression levels of 259 ferritinophagy-related genes in

![Figure 4](image_url) (A) Risk ratio forest plot showed the prognostic value of the gene. (B) The heatmap of prognostic gene. (C) Kaplan-Meier Curve of high-risk group and low-risk groups according to the risk score. (D) Distribution of prognostic index. (E) Survival status of patients in different groups

WILEY
FIGURE 5 (A) A forest plot of Cox univariate analysis. (B) A forest plot of Cox multivariate analysis. (C) Survival-dependent receiver operating characteristic curves for risk score, age, tumor size, and lymph node status and metastasis status.

FIGURE 6 Clinicopathological features of the potential prognostic genes of breast cancer
breast cancer. The Cox univariate analysis was conducted to determine the relationships between ferroptosis and the prognosis. The prognostic model was constructed according to the prognostic genes. We identified 38 ferritinophagy-related prognostic genes in total.

Breast cancer is a common disease, and its treatment is based on molecular subtypes. Compared with other breast cancer subtypes, triple-negative breast cancer is highly invasive. Patients with triple-negative breast cancer are more likely to die, accounting for approximately 40% of mortality rates within the first 5 years after being diagnosed. In the TCGA database, there were 134 triple-negative breast cancer patients, and 133 patients participated in follow-ups for at least 1 month. The forest plot indicated that 12 genes showed a significant correlation with a poor prognosis, while 9 genes showed a significant correlation with a better prognosis. Nitric oxide synthase (NOS2) has been reported in previous studies to be related to poor survivability in breast cancer estrogen receptor-negative breast cancer patients. We have found that TGFBR1 is crucial in TGF-beta signaling and it plays a dual role in cancer, and TGFBR1 protein expressions are associated with a significantly increased risk of breast cancer (Figure S1). Amplifying HER-2/neu accounted for approximately 20% of all breast cancers. Although trastuzumab, pertuzumab, and lapatinib have been proven as targeted drugs for HER-2-positive breast cancer, overexpression of HER-2 was associated with worse survival rates. Therefore, exploring the hub gene is significant in HER-2-positive breast cancer. Nuclear receptor co-activator 4 (NCOA4) is crucial in the autophagic degradation of ferritin. The overexpression of NCOA4 induced ferritin by regulating the accumulation of intracellularly free iron and glutathione. In MCF7 breast cancer cells, NCOA4 was associated with proliferation and invasion. In our analysis, NCOA4 is related to poor survivability in HER-2-positive breast cancer. Thioredoxin reductase 1 (TXNRD1) plays dual roles, because it can induce cancer and inhibit cancer. It can protect normal cells from carcinogenesis in normal tissue, while also promoting cancer progression in cancer tissue. The overexpression of TXNRD1 was proved to be associated with poor prognosis in breast cancer through an increase in oxidative stresses. In our analysis, 16 genes were associated with poor prognosis while 7 genes were associated with better prognosis in HER-2-positive breast cancer (Figure S2). Luminal breast cancer accounted for approximately 60%–70% of all breast cancer. Endocrine therapy is the main treatment for luminal breast cancer, and current guidelines suggested that luminal A patients could be solely treated with endocrine therapy. Patients with luminal A breast cancer had a better prognosis than other breast cancer subtypes. There are 14 genes associated with poor prognosis and 21 genes associated with better prognosis in luminal A breast cancer (Figure S3). Luminal B breast cancer is aggressive and has a prognosis similar to triple-negative or HER-2-positive breast cancer.

FIGURE 7 The correlations of B cell, CD4+ T cells, Macrophages, Neutrophils, and DCs

Corr=−0.083 (p=0.049)  Corr=−0.083 (p=0.010)  Corr=−0.097 (p=0.002)

Corr=−0.003 (p=0.914)  Corr=−0.068 (p=0.033)  Corr=−0.088 (p=0.006)
breast cancer. Li et al. reported that luminal B subtypes occurred in 48.1% of recurrences and metastasis samples. Despite the variety in treatments, it was still difficult to cure luminal B subtypes. We discovered 11 genes associated with the aggressiveness of luminal B subtypes (Figure S4). These are promising prognostic biomarkers in luminal B subtypes.

Immune surveillance and cancer biology are intertwined. The tumor microenvironment has become a focus. In the ferritinophagy prognostic model, infiltrating levels of B cells, CD8+ T cells, CD4+ T cells, neutrophils, and dendritic cells are correlated with the risk scores. Ferroptosis was known to affect breast cancer cells by inhibiting their proliferation, mitigating their resistance to chemotherapy drugs, and enhancing their sensitivity to radiotherapy. We investigated the potential prognostic biomarkers in breast cancer to offer further information. However, we were limited in exploring ferroptosis mechanisms and their potential role in breast cancer treatment. In our study, all breast cancer information was obtained from the TCGA database, and the patients are primarily Americans. Breast cancer patients from other regions remain to be further verified with additional evidence.

5 | CONCLUSIONS

In summary, this present study conducted a bioinformatics analysis based on the TCGA database and established a prognostic model for 259 ferritinophagy-related genes. There were significant differences between subtypes, so we analyzed different molecular subtypes. In this study, prognostic biomarkers were found in different subtypes. The underlying mechanisms of these ferritinophagy-related genes could be addressed in the future.

ACKNOWLEDGEMENTS

We acknowledge TCGA and TIMER database for providing their platforms and contributors for uploading their meaningful datasets.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTION

Yanlin Gu involved in conceptualization. Xiaohua Li involved in data curation. Yanlin Gu and Qi Zhu involved in formal analysis. Xiaohua Li and Liyan Jin involved in investigation. Guoqin Jiang involved in methodology. Qi Zhu involved in project administration. Liyan Jin and Guoqin Jiang involved in supervision. Yanlin Gu wrote the original draft. Liyan Jin, Guoqin Jiang involved in writing, reviewing the editing the article.

CONSENT FOR PUBLICATION

The authors confirm that the work described has not been published before. Its publication has been approved by all co-authors.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study could be obtained from TCGA database.

ORCID

Qi Zhu https://orcid.org/0000-0003-2269-8406
Guo-Qin Jiang https://orcid.org/0000-0001-9377-4862

REFERENCES

1. Dixon SJ, Lemberg KM, Lamprech MR, et al. Ferroptosis: an iron-dependent form of nonapoptotic cell death. Cell. 2012;149:1060-1072.
2. Li J, Cao F, Yin HL, et al. Ferroptosis: past, present and future. Cell Death Dis. 2020;11:88.
3. Hassannia B, Vandenabeele P, Vanden Berghe T. Targeting ferroptosis to iron out cancer. Cancer Cell, 2019;35:830-849.
4. Dolma S, Lessnick SL, Hahn WC, Stockwell BR. Identification of genotype-selective antitumor agents using synthetic lethal chemical screening in engineered human tumor cells. Cancer Cell. 2003;3:285-296.
5. Chen L, Li X, Liu L, Yu B, Xue Y, Liu Y. Erastin sensitizes glioblastoma cells to temozolomide by restraining xCT and cystathionine-γ-lyase function. Oncol Rep. 2015;33:1465-1474.
6. Yamaguchi H, Hsu JL, Chen CT, et al. Caspase-independent cell death is involved in the negative effect of EGFR receptor inhibitors on cisplatin in non-small cell lung cancer cells. Clin Cancer Res. 2013;19:845-854.
7. Sun YS, Zhao Z, Yang ZN, et al. Risk factors and prevention of breast cancer. Int J Biol Sci. 2017;13:1387-1397.
8. Ferlay J, Soerjomataram I, Dikshit R, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer. 2015;136:E359-E386.
9. Ma S, Henson ES, Chen Y, Gibson SB. Ferroptosis is induced following siarasmine and lapatinib treatment of breast cancer cells. Cell Death Dis. 2016;7:e2307.
10. Lin CC, Yang WH, Lin YT, et al. DDR2 upregulation confers ferroptosis susceptibility of recurrent breast tumors through the Hippo pathway. Oncogene. 2021;40(11):2018-2034. https://doi.org/10.1038/s41388-021-01676-x
11. Ding Y, Chen X, Liu C, et al. Identification of a small molecule as inducer of ferroptosis and apoptosis through ubiquitination of GPX4 in triple negative breast cancer cells. J Hematol Oncol. 2021;14(1):19.
12. Song X, Wang X, Liu Z, Yu Z. Role of GPX4-mediated ferroptosis in the sensitivity of triple negative breast cancer cells to gefitinib. Front Oncol. 2020;10:597434.
13. Wu X, Liu C, Li Z, et al. Regulation of GSK3β/Nrf2 signaling pathway modulated erastin-induced ferroptosis in breast cancer. Mol Cell Biochem. 2020;473(1-2):217-228. https://doi.org/10.1007/s11010-020-03821-8
14. Dent R, Trudeau M, Pritchard KI, et al. Triple-negative breast cancer: clinical features and patterns of recurrence. Clin Cancer Res. 2007;13:4429-4434.
15. Basudhar D, Glynn SA, Greer M, et al. Coexpression of NOS2 and COX2 accelerates tumor growth and reduces survival in estrogen receptor-negative breast cancer. Proc Natl Acad Sci USA. 2017;114:13030-13035.
16. Glynn SA, Boersma BJ, Dorsey TH, et al. Increased NOS2 predicts poor survival in estrogen receptor-negative breast cancer patients. J Clin Invest. 2010;120:3843-3854.
17. Heinecke JL, Ridnour LA, Cheng RY, et al. Tumor microenvironment-based feed-forward regulation of NOS2 in breast cancer progression. *Proc Natl Acad Sci USA*. 2014;111:6323–6328.

18. Gu S, Feng XH. TGF-β signaling in cancer. *Acta Biochim Biophys Sin (Shanghai)*. 2018;50:941-949.

19. Kaklamani VG, Baddi L, Liu J, et al. Combined genetic assessment of transforming growth factor-beta signaling pathway variants may predict breast cancer risk. *Cancer Res*. 2005;65:3454-3461.

20. Moasser MM, Krop IE. The evolving landscape of HER2 targeting in breast cancer. *JAMA Oncol*. 2015;1:1154-1161.

21. Koboldt DC, Fulton R, McLellan M. Comprehensive molecular portraits of human breast tumors. *Nature*. 2012;490:61-70.

22. da Fonseca Reis Silva D. Infracentimetric HER-2 positive breast tumours—review of the literature. *Ecamericalscience*. 2015;9:593.

23. Santana-Codina N, Mancias JD. The role of NCOA4-mediated ferritinophagy in health and disease. *Pharmaceuticals*. 2018;11(4):114.

24. Hou W, Xie Y, Song X, et al. Autophagy promotes ferroptosis by degradation of ferritin. *Autophagy*. 2016;12:1425-1428.

25. Peng Y, Li CX, Chen F, et al. Stimulation of prostate cancer cellular proliferation and invasion by the androgen receptor co-activator ARA70. *Am J Pathol*. 2008;172:225-235.

26. Arnér ESJ. Targeting the selenoprotein thioredoxin reductase 1 for anticancer therapy. *Adv Cancer Res*. 2017;136:139-151.

27. Leone A, Roca MS, Ciardiello C, Costantini S, Budillon A. Oxidative stress gene expression profile correlates with cancer patient poor prognosis: identification of crucial pathways might select novel therapeutic approaches. *Oxid Med Cell Longev*. 2017;2017:2597581.

28. Sorlie T, Perou CM, Tibshirani R, et al. Gene expression patterns of breast carcinomas distinguish tumor subclases with clinical implications. *Proc Natl Acad Sci USA*. 2001;98:10869-10874.

29. Schettini F, Buono G, Cardalesi C, Desideri I, De Placido S, Del Mastro L. Hormone receptor/human epidermal growth factor receptor 2-positive breast cancer: where we are now and where we are going. *Cancer Treat Rev*. 2016;46:20-26.

30. Tran B, Bedard PL. Luminal-B breast cancer and novel therapeutic targets. *Breast Cancer Res*. 2011;13:221.

31. Li ZH, Hu PH, Tu JH, Yu NS. Luminal B breast cancer: patterns of recurrence and clinical outcome. *Oncotarget*. 2016;7:65024–65033.

32. Engblom C, Pfirschke C, Pittet MJ. The role of myeloid cells in cancer therapies. *Nat Rev Cancer*. 2016;16:447-462.

33. Pfeifhofer-Obermair C, Tymoszuk P, Petzer V, Weiss G, Nairz M. Iron in the tumor microenvironment-connecting the dots. *Front Oncol*. 2018:8:549.

34. Zhang Z, Xu J, Qin LQ, et al. The valuable role of holo-lactoferrin ferroptosis and its application in radiotherapy of triple negative breast cancer (P05-014-19). *Curr Dev Nutr*. 2019;3(Supplement_1):nzz030. https://doi.org/10.1093/cdn/nzz030.P05-014-19

**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of the article at the publisher’s website.

**How to cite this article:** Jin L-Y, Gu Y-L, Zhu Q, Li X-H, Jiang G-Q. The role of ferroptosis-related genes for overall survival prediction in breast cancer. *J Clin Lab Anal*. 2021;35:e24094. [https://doi.org/10.1002/jcla.24094](https://doi.org/10.1002/jcla.24094)