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Profiles of ferroptosis-related genes and their prognostic values in patients with breast cancer brain metastasis

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Abstract

Background Ferroptosis is involved in various cancers. The role of ferroptosis in breast cancer brain metastasis (BCBM) is unclear. This study aimed to explore the ferroptosis-related genes (FRG) expression profiles in BCBM, as well as evaluate the FRG prognostic values in breast cancer patients.

Methods Genes expression and clinical data were downloaded from Gene Expression Omnibus (GEO). Functional enrichment analysis was used to investigate the FRG bioinformatics functions. Univariate and multivariate cox regression analysis were performed to explore the independent prognostic factors. The correlation between ferroptosis and immunity was also evaluated. Finally, the FRG and their prognostic values were validated in external cohorts.

Results Fourteen significantly different FRG were screened between breast cancer and BCBM tissues. GO and KEGG results showed FRG were enriched in the ferroptosis-related activities. Protein-protein interaction (PPI) network analysis showed the HMOX1 and TFRC were hub genes. Survival analysis demonstrated HMOX1 and PEBP1 were significantly associated with overall survival (OS) (HR=2.100, P=0.035; HR=0.421, P=0.017 respectively). The KEAP1 and LPCAT3 had prognostic values for relapse-free survival (RFS) (HR=0.745, P=0.002; HR=2.536, P=0.008 respectively). Patients in high-risk group have worse OS and RFS compared with those in low-risk group (P=0.004, 0.021 respectively). Clinical correlation analysis revealed FRG were significantly associated with estrogen receptor (ER) status, progesterone receptor (PgR) status, HER2 and pathological grade in breast cancer patients (all P<0.05). In addition, we also found that immune-related pathways and immune status were different between high and low-risk groups. External cohort results showed FRG were significantly different between breast cancer and BCBM tissues. Survival validation demonstrated ALOX5 and CS were associated with prognosis in breast cancer patients (P=0.044, 0.032 respectively).

Conclusions Our study identified the ferroptosis-related genes that may be involved in biology of BCBM, and FRG could serve as prognostic biomarkers in breast cancer patients. New therapy targeting ferroptosis holds probabilities for effective treatment in BCBM patients.

Background

Breast cancer is the most prevalent tumor in women worldwide, ranking the third most common malignancy followed by lung and colon cancer. It's reported that approximately 1,700,000 new cases and almost 500,000 deaths per year globally [1-2]. Breast cancer brain metastasis (BCBM) becomes a major limitation of life expectancy and remains a substantial contributor to overall mortality. Nearly 5% -20% breast cancer will develop brain metastasis, and it is the second common primary tumor associated with brain metastasis after lung cancer [3]. Breast cancer patients with basal like (25-27%) and HER2-enriched cancer (11-20%) have higher propensities to metastasize to brain, compared with those in luminal A (8-15%) and luminal B (11%) subtypes [4]. Since no clinically approved biomarkers of brain metastasis is available, and the presences or absences of estrogen receptor (ER), progesterone receptor (PgR), HER2

and Ki67 status are not sufficient to accurately prognosticate metastasis due to heterogeneity between primary and metastatic sites [5]. Hence, BCBM is often diagnosed late and represents aggressive progression.

Diverse underlying mechanisms such as gene alterations, immune dysregulation, environmental exposures, as well as estrogen and progesterone imbalance have orchestrated in the biology of BCBM [6-9]. The 1-year survival rate of BCBM patients is merely 20%, despite the tremendous progress has been made in the multidisciplinary treatment, including surgery, radiotherapy, chemotherapy and endocrine immunotherapy [10-11]. Therefore, the thorough understanding of molecular mechanisms that drive BCBM is imperative. The extensive applications of high-throughput sequencing technologies in cancer biology such as cell death analysis, have revealed the complex relations between thousands of aberrant genes and BCBM. There is increasingly evidence showing that ferroptosis, an iron-catalyzed form of regulated necrosis, plays important roles in various cancers, including breast tumors, brain tumors and breast cancer metastasis [12-14].

Ferroptosis can be induced by iron accumulation, glutathione (GSH) depletion, glutathione peroxidase 4 (GPX4) inactivation, and is characterized by lipid peroxidation products and toxic reactive oxygen species (ROS) derived from iron metabolism [15-16]. Several studies have shown ferroptosis could be triggered in breast cancer [12, 17]. The activities of ferroptosis are regulated precisely by ferroptosis-related genes (FRG), and its dysfunction links with many kinds of diseases [13, 15-16]. In addition, the activation of ferroptosis has tumor suppression efficacy and exerts the great potential as a novel anti-cancer target [13]. However, the role of ferroptosis in BCBM is unclear, and there are few studies exploring the relationship between the ferroptosis and survival in breast cancer patients. In this study, we aimed to investigate and validate the roles of FRG signatures in BCBM, as well as evaluate the FRG values in predicting prognosis in breast cancer patients.

Methods

Data collection and extraction

The gene expression profiles and clinical information of BCBM patients were downloaded in the dataset GSE10893 from Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/gds/). The raw data of gene expressions was selected by “GEO2R” online tool and normalized using the “limma” R packages in R software (version 4.0.2). The 60 FRG were retrieved from previous published literatures and the FRG were available in the Supplementary Table 1 [15-16, 18-19].

Identification of significantly different genes and prognostic genes

The significantly different genes (SDG) were identified using “GEO2R” and the “limma” R package with the Wilcoxon test. The cut-off values were determined according to the parameters, $P<0.05$. Univariate and multivariate cox regressions were used to assess the relationships between the SDG and the patients’

overall survival (OS) and relapse-free survival (RFS). Patients were divided into high-risk and low-risk groups according to the risk score. The risk score was calculated by the following formula:

$$\text{risk score} = \sum_{n=1}^{j} Coefj * Xj,$$

with Coef j representing the coefficient and Xj representing the relative expression levels of each SDG standardized by z-score.

Functional enrichment analysis and construction of FRG signatures

The enrichment analysis of Gene Ontology (GO), including the biological process (BP), cellular component (CC), and molecular function (MF), was performed by "clusterProfiler" R package in R software. The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was also done using the same tool.

The infiltrating score of 16 immune cells and the activity of 13 immune-related pathways were calculated with single-sample gene set enrichment analysis (ssGSEA) in the "gsva" R package [20]. The annotated gene set file is provided in Supplementary Table 2.

An interaction network of FRG was performed at the online STRING website (http://string-db.org/cgi/input.pl). Then, we also explored their correlations using the R software.

Survival analysis and validation

In order to explore the independent risk factors for OS and RFS, we combined the FRG with clinical information using the univariate cox regression. Significant prognostic factors (P<0.05) were enrolled into multivariate cox regression to identify the independent prognostic risk factors. Then, we calculated the correlations between the SDG and clinical features using the t-test or Kruskal-Wallis test.

We used another dataset from GEO to verify the FRG, which contained the gene expression data in breast cancer and BCBM tissues. The Human Protein Atls (HPA) database (https://www.proteinatlas.org/) was used to assess the protein expression level. The prognostic values of SDG were validated in The Cancer Genome Atlas (TCGA) dataset by performing Kaplan-Meier survival analysis. Data can be obtained at the website https://portal.gdc.cancer.gov/.

Statistical analysis

Wilcoxon test was used to compare gene expression differences between breast cancer and BCBM tissues. Univariate and multivariate cox regression analysis were used to evaluate the correlation between the factors and OS, RFS. Log-rank test was used to compare the survival differences between high and low-risk groups. Kaplan-Meier curve was implemented to visualize the survival. Mann-Whitney test with P values adjusted by the BH method was used to compare the ssGSEA scores of immune cells or pathways

between the high-risk and low-risk groups. All the statistical analyses were done using the R software (version 4.0.2). P<0.05 was set as statistically significant.

Results

A total of 128 samples with gene expression profiles and clinical information were downloaded from the GSE10893 in GEO database, including 6 BCBM and 122 breast cancer samples. Among the 60 FRG, 7 genes were significantly upregulated in the BCBM compared with breast cancer tissues, and 7 genes were significantly downregulated (Table 1). The volcano plot and deviation plot were shown in Figure 1A-B.

Enrichment analysis and construction of FRG signatures

GO enrichment analysis showed the SDG were enriched in the iron homeostasis and iron-related transition pathways. In the BP process, they were strongly associated with the iron ion homeostasis. In the CC and MF processes, the SDG were enriched in the lamellipodium membrane and virus receptor activity. KEGG enrichment analysis showed that these SDG were significantly enriched in the ferroptosis and metabolic activities. These findings suggest that the genes serve as important biological roles in ferroptosis (Figure 2A-B).

Fourteen SDG and their interactions were enrolled in the Search Tool for the Retrieval of Interacting Genes (STRING) database (http://string-db.org) for interaction analysis. The protein-protein interaction (PPI) network results showed there were 14 nodes (proteins) and 14 edges (interactions), in which the HMOX1 and TFRC were hub genes (Figure 3A). Then, we performed the correlation analysis. The correlation between the fourteen genes were presented in Figure 3B.

Prognostic FRG for OS and PFS

We performed survival analysis among the 14 FRG using the cox regression. We found that HMOX1 and PEBP1 were significantly associated with OS (P=0.035, 0.017 respectively). However, they were not significantly statistical in the multivariate cox regression analysis (P>0.05). For RFS, the KEAP1 and LPCAT3 had prognostic values, and the statistical differences were significant (P=0.002, 0.008 respectively) in the multivariate cox regression (Table 2).

Then, we combined the clinical information with gene expressions to explore the independent risk factors for survival. In the univariate cox analysis, we found that lymph node number, tumor grade, tumor size and risk score were significantly associated with OS (all P<0.05) (Figure 4A). And the multivariate cox regression showed the tumor size was the only one independent risk factor for OS (HR=2.588, P=0.005) (Figure 4B). For RFS, the ER, PgR status, lymph node number, tumor grade, tumor size and risk score were significantly associated with RFS (all P<0.05) (Figure 4C). And the multivariate cox regression results showed tumor size and risk score were independent risk factors for RFS in breast cancer patients (HR=2.209, P=0.007; HR=1.251, P<0.001 respectively) (Figure 4D).

Next, we examined the predictive abilities using the independent risk factors selected from multivariate cox regression results. By constructing the receiver operating curves (ROC) and evaluating the area under curves (AUC), we found tumor size had excellent ability to predict the OS (AUC=0.982). Similarly, we found tumor size and risk score also demonstrated better abilities to predict RFS (AUC=0.975, 0.750 respectively). The results are visualized in Figure 5A and 5B.

Prognostic hazard curves in high and low-risk patients

Ninety-two breast patients were divided into high-risk group (n=46) and low-risk patients (n=46) according to the median of the risk score (Thirty-six patients' information were incomplete and were not recruited for hazard analysis). The Kaplan-Meier (K-M) curve showed patients with high-risk score had a significant higher death probability than those with low-risk (median time = 1.875 years vs. 2.458 years, p = 0.004) for OS (Figure 6A). Similarly, patients in high-risk score group had worse RFS than those with low-risk score (median time = 1.792 years vs. 2.165 years, p = 0.021) (Figure 6B).

In addition, we performed prognostic hazard analysis between high and low-risk groups patients. The results showed as the risk score increased, the patients' death risk increased, and the survival time decreased (Figure 7A-D).

Ferroptosis and immune status

To explore the relationships between FRG and immune cells and immune functions, we quantified the 16 immune cell subtypes and 13 immune functions by ssGSEA package in R software. We found the content of immune cells were significantly different between the high-risk and low-risk groups patients. The aDCs, B cells, iDCs, neutrophils, Tfh, Th2, TIL and Tregs cell levels were higher in the high-risk group (all P <0.05) (Figure 8A). Additionally, the contents of the antigen presentation process, including APC co-inhibition, APC co-stimulation, HLA, MHC class I were significantly different between the two groups (all P <0.05) (Figure 8B). These results implied ferroptosis was closely associated with the immune cells and functions.

Ferroptosis and clinical characteristics analysis

In order to assess the whether there are correlations between ferroptosis and patients' clinical information, we calculated statistical differences between the 14 FRG and clinical features using the t-test or Kruskal-Wallis test. The results demonstrated that ACO1, DPP4, HMOX1 and TFRC were significantly associated with breast cancer grades (all P <0.05). As the tumor grade increased, DPP4, HMOX1 and TFRC expression levels also increased. While, the ACO1 were the opposite. The ACO1, FDFT1, DPP4, HMOX1 and TFRC were closely associated with patients' ER and PgR status (all P <0.05). Moreover, we also found CD44 and PEBP1 were significantly correlated with Her2 status in breast cancer patients (all P <0.05). The details are shown in Figure 9A-O.

External validation of FRG and survival analysis

We verified the ferroptosis-related genes using another dataset (GSE52604) from GEO database, which contained the FRG expression profiles in the 10 breast and 35 BCBM tissues. Consistent with above results, there were 36 different FRG genes between breast and BCBM tissues. In addition, there are eight intersection genes between the two datasets, including CD44, CS, HMOX1, HSBP1, KEAP1, LPCAT3, PEBP1 and TFRC (Figure 10). This provided a robust and reliable evidence that ferroptosis was involved in the BCBM.

Next, to further validate the intersection genes expression levels in breast cancer, we investigated the FRG protein expression in breast cancer through the HPA database. As shown in Figure 11, the CD44 (Figure 11A), CS (Figure 11B), HMOX1 (Figure 11C), KEAP1 (Figure 11D), PEBP1 (Figure 11E) and TFRC (Figure 11F) proteins were not or weakly expressed in normal breast tissues, while moderately or strongly expressed in breast cancer tissues. These results implied that the breast cancer has close correlation with ferroptosis.

Finally, TCGA database was applied to verify the 14 FRG prognostic values for OS in breast cancer patients. Fourteen genes' effects on OS in breast cancer patients were analyzed by Kaplan-Meier curves. The median expression level was regarded as the cut-off point to classify the patients into high or low groups. Among 14 genes, we found high expression of ALOX5 was associated with better OS in breast cancer patients (P=0.044). However, the high expression of CS was associated with poor OS in breast cancer patients (P=0.032). Other genes did not have significant influences on overall survival (P> 0.05). The K-M curves are shown in Figure 12A-N.

Discussion

Cell death is vital importance for normal development, physiological homeostasis and excessive proliferation, such as tumor. Tumor cells exhibit more iron demand than normal cells. Ferroptosis is a new recognized, iron-dependent form of cell death by Dixon and colleagues in 2012, which shares none of the characteristics of morphology, biochemistry and functions associated with necrosis, apoptosis and autophagy [21]. It's increasingly evident that ferroptosis has been linked to various cancers, especially cancers from iron-rich tissues such as brain [22-23]. Studies have demonstrated that altered iron metabolism is closely related with prognosis of breast cancer patients [14,17,24]. In addition, the ferroptosis could promote the tumor metastasis in some cancers, such as breast cancer [14,25-26]. Considering the evidence above, it's reasonable to hypothesize ferroptosis may be involved in the process of BCBM through an unknown mechanism. Therefore, we explored the roles of ferroptosis in BCBM using large public database by bioinformatics analysis. In this study, we found 14 differently expressed ferroptosis-related genes between the breast cancer and BCBM tissues. Functional enrichment analysis showed these genes were closely associated with the iron ion homeostasis and ferroptosis-related activities. Moreover, immune scores result implied that ferroptosis had intimate crosstalk with immune cells and immune functions. Further, survival analysis suggested some FRG had prognostic values in predicting breast cancer patients' survival.

Despite several studies have investigated the roles of ferroptosis in breast cancer, the underlying mechanisms between ferroptosis and breast cancer cells remains elusive, and the FRG expression profiles of BCBM is never been explored [12, 14, 17]. Functional enrichment analysis in this study has discovered ferroptosis may be involved in BCBM through disrupting the iron ion homeostasis. Iron metabolism is tightly associated with iron uptake, utilization, storage and export. High iron level gives rise to reactive oxygen species (ROS) and determines the sensitivity of cells to ferroptosis [27-28]. Subsequently, iron-induced oxidative stress could promote the metastasis initiation possibly through the following mechanisms: 1) modifying the genome, epigenome, leading to the tumor heterogeneity and metastatic abilities; 2) remodeling the extracellular matrix (ECM), which increases the matrix metalloproteinases (MMPs) expression, such as matrix metalloproteinases-9 (MMP-9) that facilitates the metastasis; 3) modulating the tumor microenvironment by restraining the immune response and stimulating the angiogenesis to enable the cancer cell mobility and invasion; 4) changing the metabolic plasticity of cancer cells to compete for and utilize more energy for surviving longer and metastasis; 5) interacting with secondary site by releasing some signals, such as exosomes to establish a pre-metastatic niche [28-33]. Consistent with previous findings, our enrichment analysis also showed the iron homeostasis played vital roles in the development of breast cancer cells metastasize to brain. The result highlights the significance of ferroptosis in BCBM and represents a potential approach to prevent metastatic disease.

The FRG signatures proposed in present study was composed of 14 genes, which could be generally classified into 4 categories, including iron metabolism (ACO1, HMOX1, TFRC), lipid metabolism (ALOX5, CS, LPCAT3, GPX4, PEBP1, FDFT1, PEBP1), (anti)oxidant metabolism (KEAP1, HMOX1) and energy metabolism (GLS2, G6PD) [15, 18]. The PPI network showed the HMOX1 and TFRC were hub genes regulating ferroptosis in breast cancer cells. HMOX1, also known as HO-1 (heme oxygenase 1), could catalyze the degradation of heme to biliverdin and Fe^{2+}. Study showed that HMOX1 knockout could enhance ferroptosis [34]. The expression of HO-1 is significantly associated with distant metastasis, and predicts a poor OS in breast cancer patients [35]. Moreover, clinical correlation analysis showed the HO-1 expression is significantly with histologic grade, which was in line with our results [35]. In contrast to the evidence that the HO-1 expression promotes the ferroptosis [35], Li Q et al demonstrated HO-1 could inhibit mammary tumor metastasis mediated by Notch1 pathway [36]. Hence, the elucidation of HMOX1 in breast cancer metastasis needs to be further investigated. TFRC refers to transferrin receptor, which promotes ferroptosis by importing irosun into cells and sparks ferroptotic cascade reaction ultimately. Notably, TFRC modulates the ROS generation and silencing of TFRC significantly inhibits ferroptosis [15, 18]. In addition, consistent with previous study, our result also showed the TFRC expression was lower in ER+ breast cancer tissues than that in ER- tissues [37]. Therefore, it's conceivable that the activation degree of ferroptosis is different in ER+ and ER- tissues and this can partly explain why different subtypes of breast cancer have different abilities to metastasize to brain.

The prognostic model constructed in our study identified KEAP1 and LPCAT3 were independent genes for RFS. Breast cancer patients in high and low-risk groups exhibit significantly different prognosis ($P<0.05$),

implying risk score based on the FRG signatures has excellent ability to predict survival. Studies about molecular mechanisms revealed KEAP1 could bind to and regulate NRF2 (another ferroptosis-related gene), and its knockdown confers cells resistance to ferroptosis [18]. Cumulative evidences demonstrated KEAP1 is associated with worse prognosis in breast cancer patients, which is in line with our result [38-39]. In addition to predicting survival, KEAP1 could also render breast cancer metastasis by interacting with other molecules, for instance, the TrkB and HBXIP [40-41]. LPCAT3 is a member of lipid metabolism family, which incorporate acylated fatty acids into membranes and is involved in biosynthesis of phospholipids [15, 18]. The LPCAT3 knockdown will suppress the ferroptosis [15]. However, the role of LPCAT3 in breast cancer is still in its early stages and much less has been uncovered. So, experimental models in vitro and in vivo need to be developed to assess the roles of LPCAT3.

Mounting studies from preclinical assays have linked ferroptosis to the immune cells and functions relevant to tumors [42-44]. Ferroptotic cells will release some chemotactic signals, such as lipid mediators to attract antigen-presenting cells (APC) and other immune cells to degrade these aberrant cells [42]. It should be stressed that GPX4, an anti-ferroptosis agent, could reduce phospholipid hydroperoxide and repress lipoxygenase-mediated lipid peroxidation [45]. Further evidence demonstrated that $CD4^+$ and $CD8^+$ T cells lacking GPX4 failed to expand and were not able to prevent immunity to infection [46]. Similarly, our study also showed the level of antigen presentation process is significantly higher in high-risk group than that in low-risk group, and the contents of immune cells were also different between two groups. These studies enforce the notion that ferroptosis will have an effect on immunity and may open up new possibilities to efficiently improve cancer treatment. Several lines of evidence have confirmed ferroptosis enhanced the tumor suppression mediating by interferon gamma (INF-γ) secreted by CD8+ T cells in response to immune checkpoint blockade [47-48]. In turn, immunotherapy-activated T cells also enhance ferroptosis-specific lipid peroxidation in tumor cells [47]. Immune checkpoint inhibitors have revolutionized the treatment of brain metastasis and breast cancer patients in recent years [49-50]. It's also conceivable that, if sufficient details between ferroptosis and immunity have achieved, breast cancer patients may benefit more and a shift from anti-tumor to immunosuppressive responses might take place.

The strength of our study is that we performed a systematic analysis of FRG signatures in breast cancer patients from national database and validated through external cohort, which provided a robust statistical support. To our best known, it' s first time to explore the relationships between ferroptosis and BCBM, which shed light on the significance of ferroptosis in metastatic breast cancer. Meanwhile, there are some limitations in our study. Firstly, this is a retrospective study with data from public repositories. A large-scale and multicenter real-world analysis is warranted to verify these results. Secondly, the mechanisms how ferroptosis modulates BCBM precisely is still unclear, and metastatic animal models are essential to understand the specific roles of ferroptosis. Lastly, it should be emphasized that the links between breast cancer ferroptosis and immune cells are not fully understood and experimentally validated. Notwithstanding its limitations, this study does provide a comprehensive overview of FRG profiles in BCBM and these limitations can be solved if there are enough data in the future.

Conclusions

In conclusion, we identified differently expressed ferroptosis-related genes that may be involved in the process of BCBM, and these genes have prognostic values in predicting patient's survival. New efforts targeting BCBM should incorporate the idea that ferroptosis influences breast cancer metastasis. More work is necessary to explore and validate the results.

List Of Abbreviations

BCBM: breast cancer brain metastasis; ER: estrogen receptor; PgR: progesterone receptor; GSH: glutathione; GPX4: glutathione peroxidase 4; FRG: ferroptosis-related genes; GEO: Gene Expression Omnibus; SDG: significantly different genes; OS: overall survival; RFS: relapse-free survival; GO: Gene Ontology; BP: biological process; CC: cellular component; MF: molecular function; KEGG: Kyoto Encyclopedia of Genes and Genomes; ssGSEA: single-sample gene set enrichment analysis; STRING: search tool for the retrieval of interacting genes; PPI: The protein-protein interaction; HPA: Human Protein Atls; TCGA: The Cancer Genome Atlas; HR: hazard ratio; CI: confidence interval; ROC: receiver operating curves; AUC: area under curves; K-M: Kaplan-Meier; ROS: reactive oxygen species; ECM: extracellular matrix; MMPs: matrix metalloproteinases; MMP-9: matrix metalloproteinases-9; HO-1: heme oxygenase 1; APC: antigen-presenting cells; INF-γ: interferon gamma.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data were available in GEO database (https://www.ncbi.nlm.nih.gov/gds/), the Human Protein Atls (HPA) database (https://www.proteinatlas.org/) and TCGA database (https://portal.gdc.cancer.gov). All the data displayed in the present manuscript are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LZ contributed to conception, design and data acquisition. MC and BSH contributed to interpretation, data analysis and manuscript drafting. ML conceived and designed the results validation. CLZ and QCL reviewed and approved the final version of the manuscript. All authors read and approved the final manuscript.

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Tables

Due to technical limitations, the tables are only available as a download in the supplemental files section.

Figures

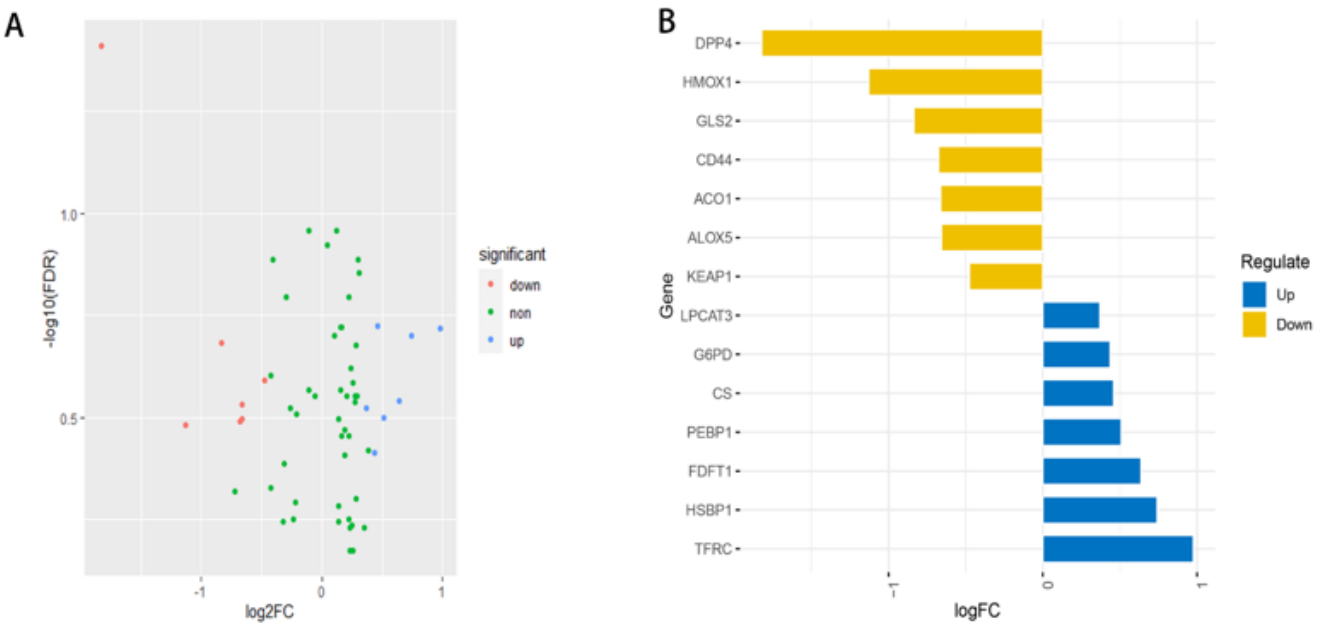

Figure 1

Volcano and deviation plots of FRG. (A) volcano plot to show 60 FRG in breast cancer and BCBM tissues. Blue dots represent the up-regulated genes, and red represents down-regulated genes. Green dots represent genes that are not significantly different. (B) deviation plot to verify 14 significant different genes. Blue bars represent the up-regulated genes, and yellow bars represent down-regulated genes.

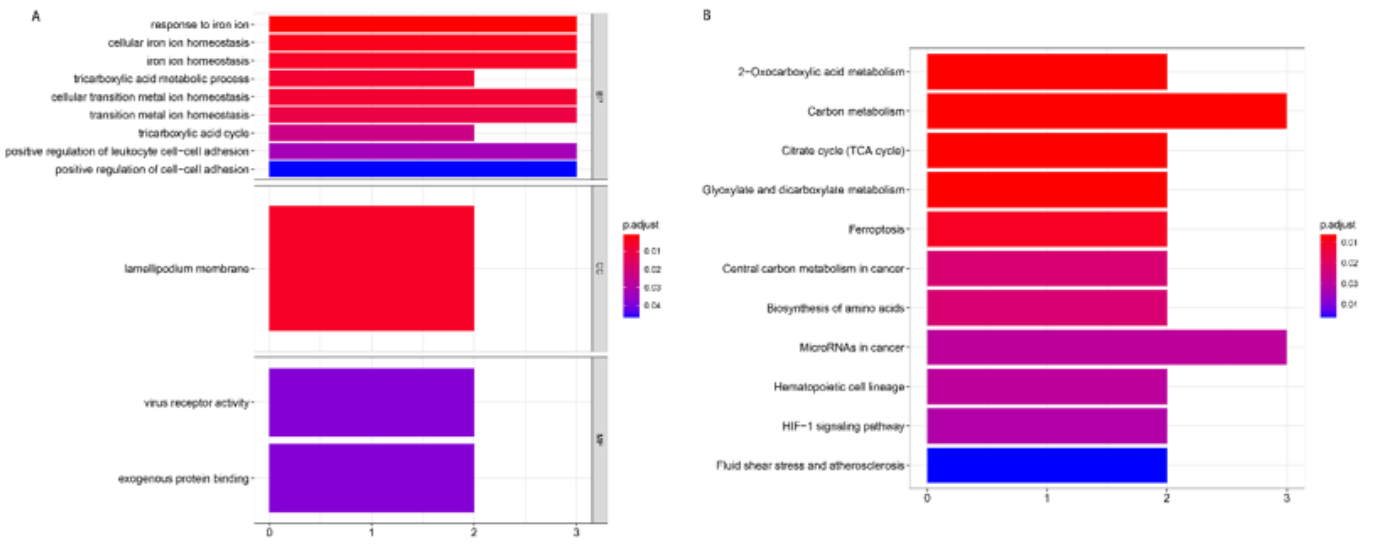

Figure 2

GO and KEGG enrichment analysis. (A) GO analysis results showed FRG were enriched in the iron homeostasis and ferroptosis-related pathways. (B) KEGG enrichment demonstrated metabolic activities and ferroptosis.

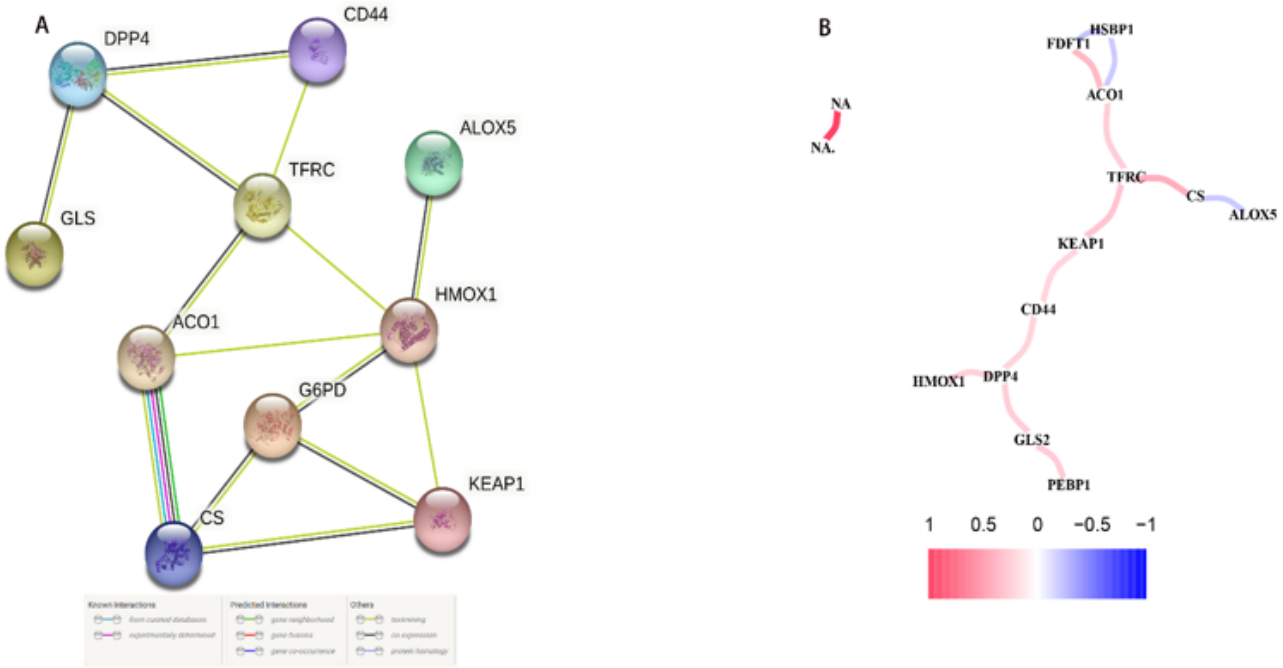

Figure 3

PPI network and gene correlation network. (A) FRG PPI network downloaded from the STRING database demonstrated the genes interactions, and the network showed the HMOX1 and TFRC were hub genes. (B)

The correlation network of candidate genes. The correlation coefficients are represented by different colors, in which red lines represent positive correlation and blue lines represent negative correlation.

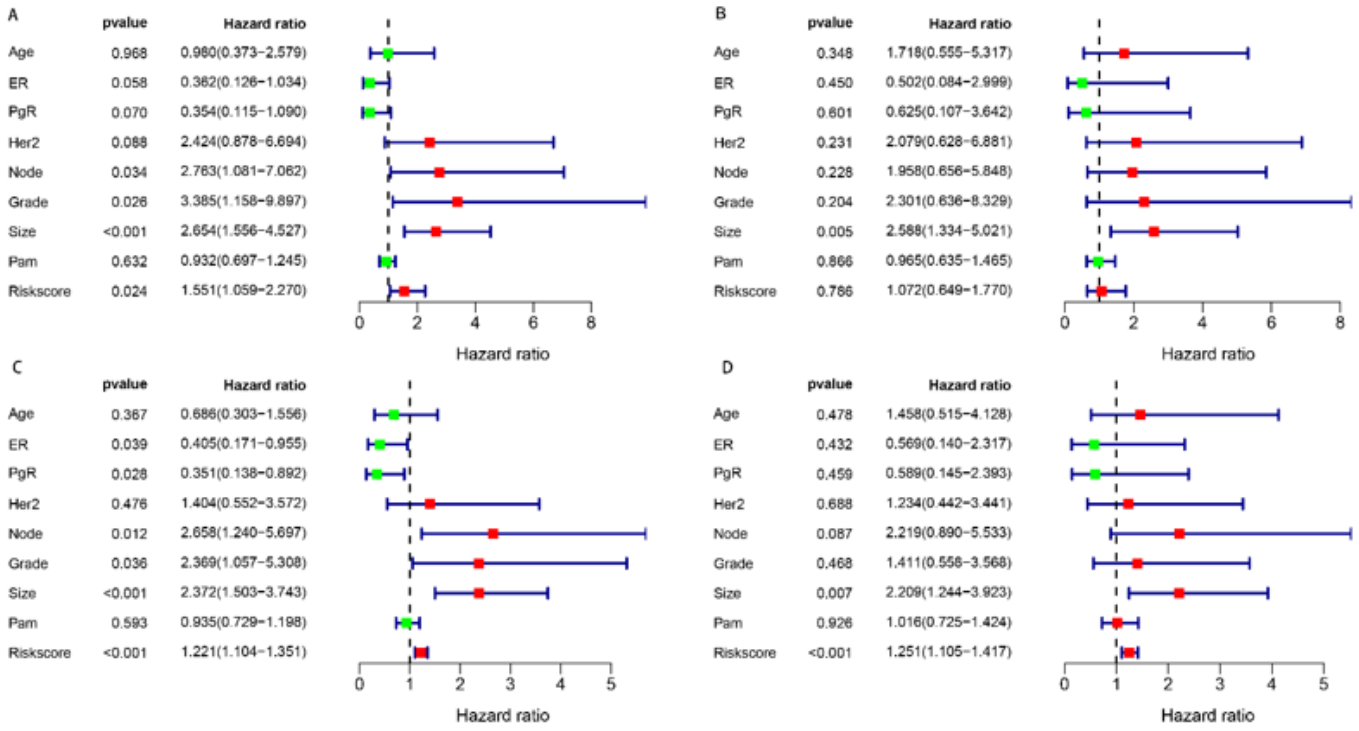

Figure 4

Forest plots of independent risk factors for OS and RFS. (A) univariate cox regression analysis for OS. (B) multivariate cox regression analysis for OS. (C) univariate cox regression analysis for RFS. (D) multivariate cox regression analysis for RFS.

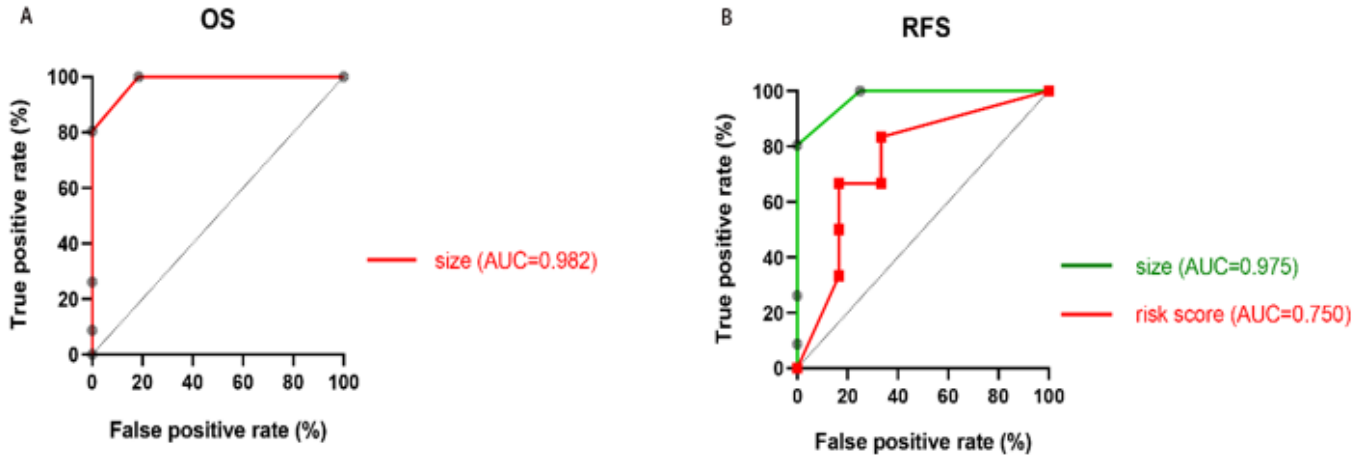

Figure 5

ROC curves for OS and RFS. (A) The AUC of tumor size for OS is 0.982. (B) The AUC of tumor size for RFS is 0.975 and the risk score is 0,750. The value of AUC ranges 0.5 from 1.0. The AUC is near 1.0, indicating the stronger predictive ability.

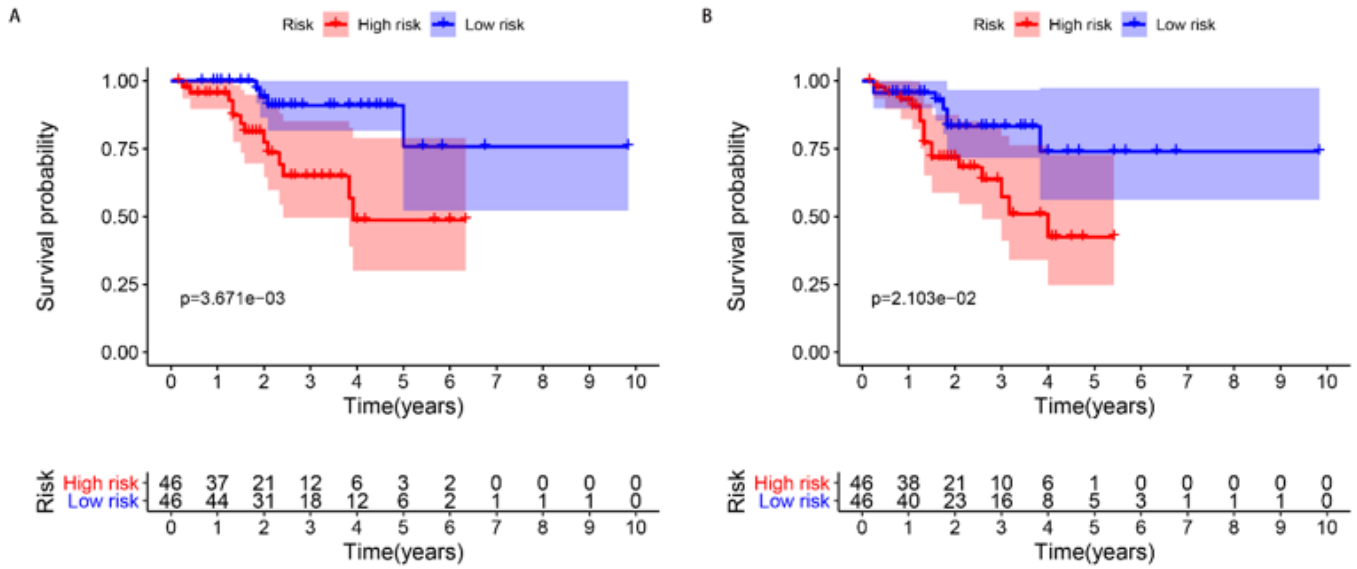

Figure 6

Kaplan-Meier survival curves in high and low-risk groups patients. (A) K-M curve for OS; The result showed the survival status is significantly different between two groups (P=0.004). (B) K-M curve for RFS; High-risk groups patients had worse RFS than that in low-risk group (P=0.021).

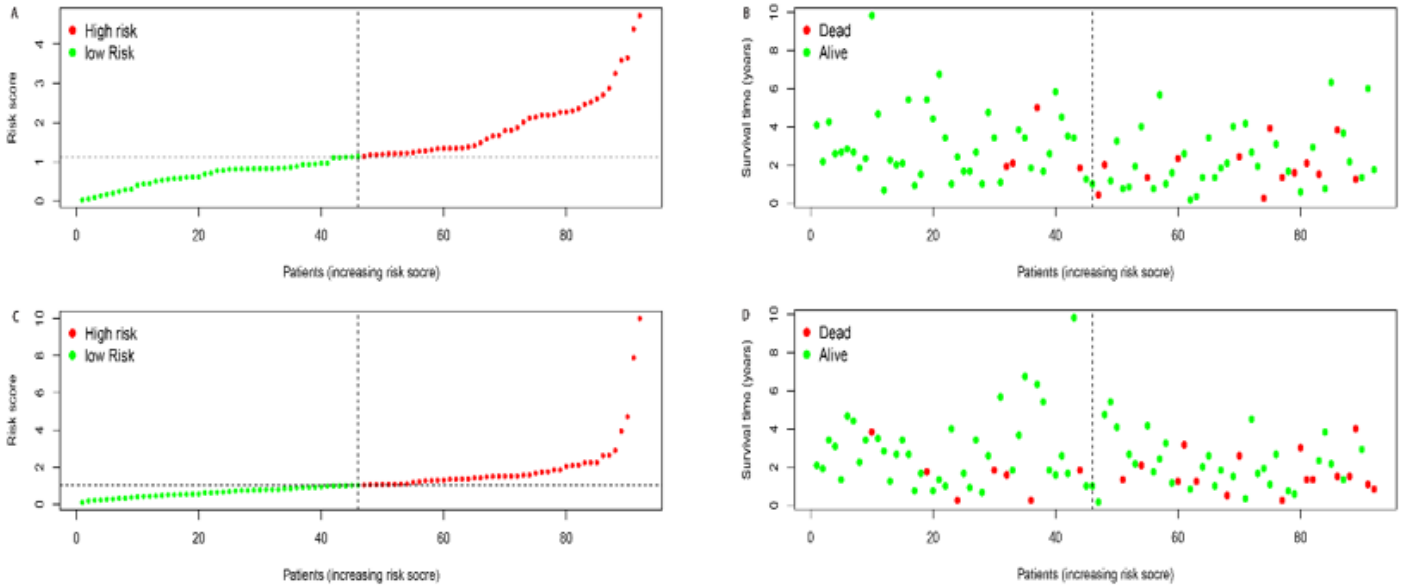

Figure 7

Risk score hazard analyses of high and low-risk groups in breast cancer patients. (A) The dotted line of OS indicates the individual inflection point of the risk score curve, by which the patients were categorized into low-risk and high-risk groups. Red represents high-risk and green represents low-risk. (B) Risk score scatter plot of OS in high and low-risk groups. Red dots represented the dead patients and green represented the alive. With the increase of risk score, more patients died. (C) Dotted line plot of RFS. (D) scatter plot of RFS.

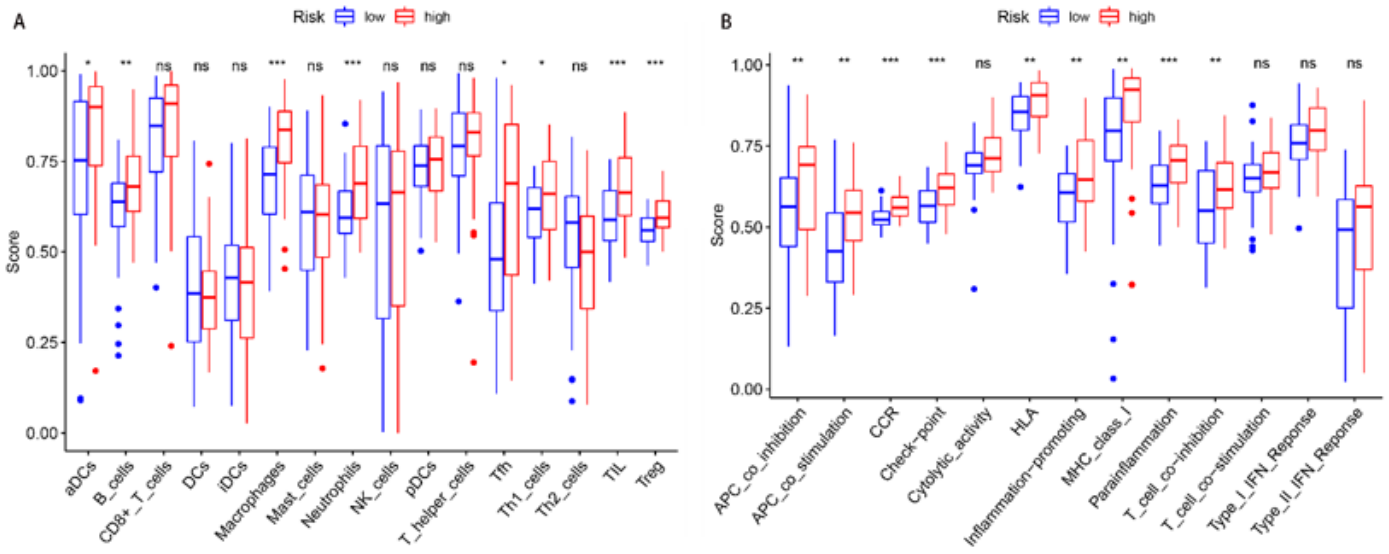

Figure 8

Comparison of the ssGSEA scores between high and low-risk groups. (A) Immune cell contents in high and low-risk groups. (B) The immune functions differences in two groups. P values were showed as: ns, not significant; *, P< 0.05; **, P< 0.01; ***, P< 0.001.

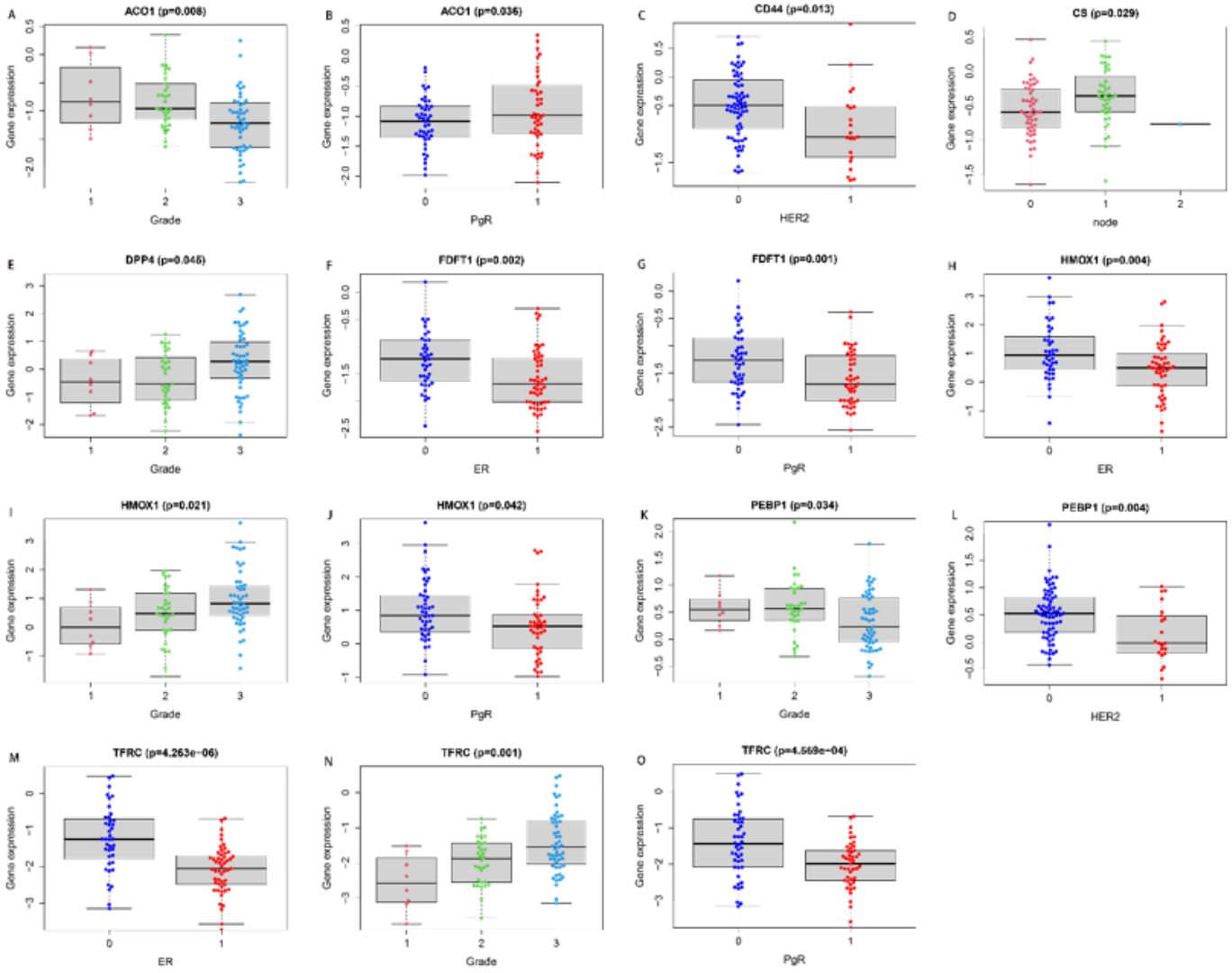

Figure 9

Correlations analysis between FRG and patients' clinical characteristics. ER (1=positive; 0=negative); PgR (1=positive; 0=negative); HER2 (1=positive; 0=negative); node status (1=positive; 1= 2 or more nodes+; 0=negative); size (1= ≤2cm; 2= 2-5cm; 3= >5cm; 4=any size with direct extension to chest wall or skin).

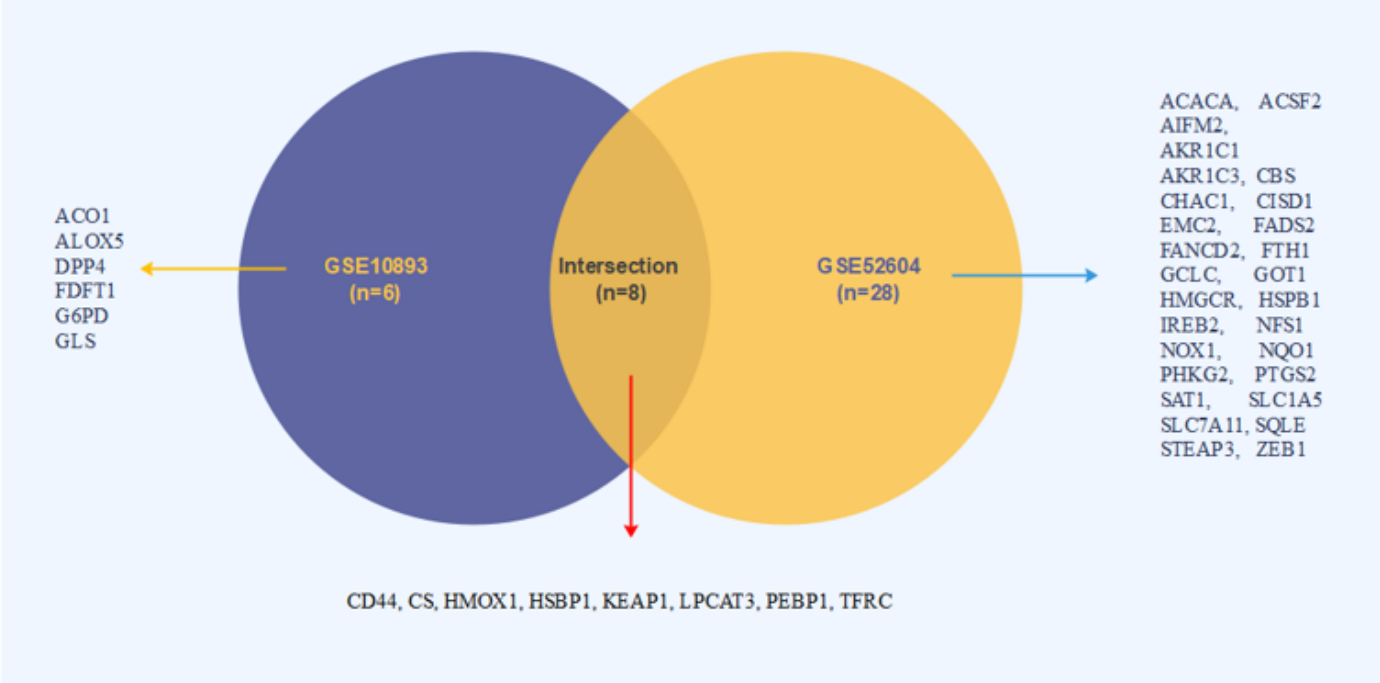

Figure 10

FRG validation from another GEO dataset. The venn graph demonstrated the there are eight intersection FRG, including CD44, CS, HMOX1, HSBP1, KEAP1, LPCAT3, PEBP1 and TFRC between two datasets.

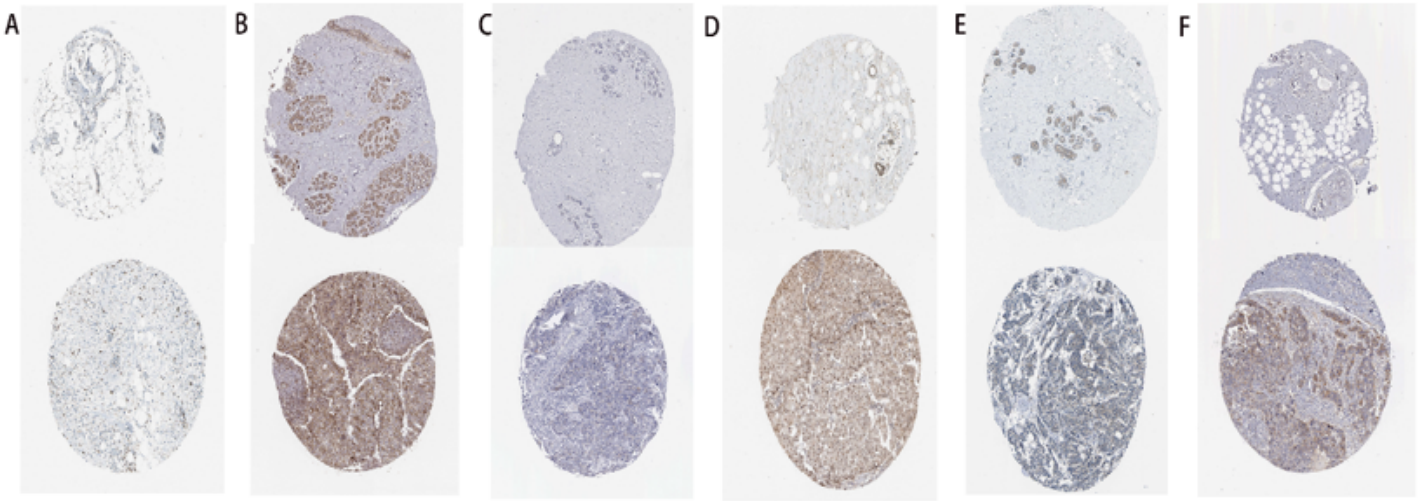

Figure 11

Representative immunohistochemistry results of CD44 (A), CS (B), HMOX1 (C), KEAP1 (D), PEBP1 (E) and TFRC (F) in normal breast and breast cancer tissues. The upper row represents normal tissues, and the lower row represents breast cancer tissues.

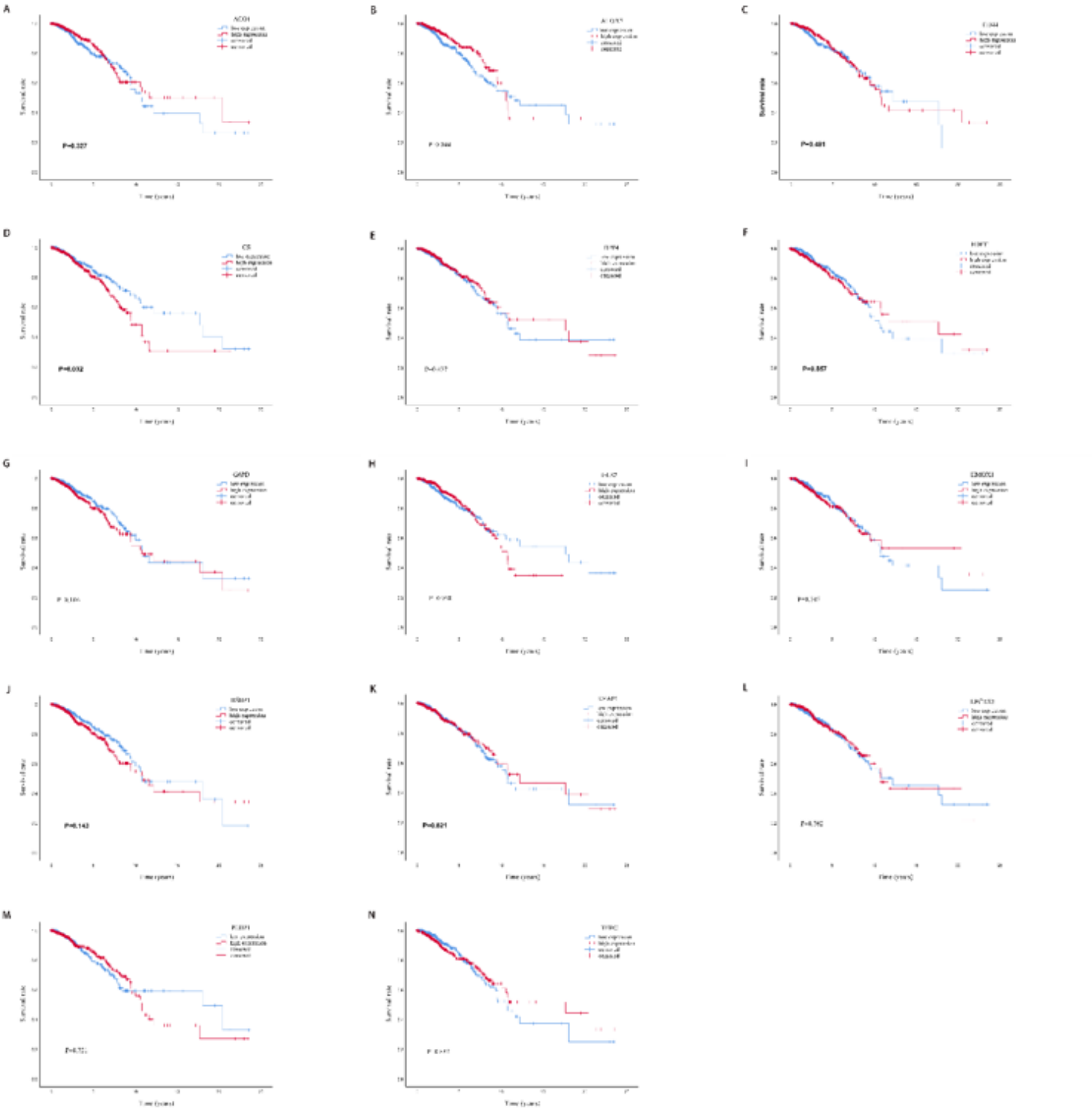

Figure 12

K-M curves of 14 FRG in breast cancer patients from TCGA database. The results showed ALOX5 and CS were significantly associated with OS in breast cancer patients (P=0.044, 0.032 respectively). Others are not statistically significant (P>0.05).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TCGAbreastcancersurvialdata.xls
- GSE10893geneexpressiondata.txt
- SupplementaryTableS1.docx
- SupplementaryTableS2.xls
- Table.docx