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Original Article

The molecular mechanism of ferroptosis-induced spontaneous abortion based on bioinformatics approach

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Abstract

Spontaneous abortion (SA) is a prevalent placental dysfunction, and ferroptosis may play a crucial role in placental dysfunction and the development of SA. In this study, we employed data mining and analysis techniques to investigate the biological mechanism of SA induced by ferroptosis, resulting in the identification of a total of 79 ferroptosis-related genes in SA were identified. Among them, 3 co-expression modules of ferroptosis risk genes, ten significant functions and six biologically significant pathways were obtained 61 pairs of differentially expressed miRNA-ferroptosis factor relationships were identified, and WIPI1 and GSN were expressed at significantly higher levels in SA. This is extremely helpful for future research on SA.

Keywords: Spontaneous abortion, Ferroptosis, Placental dysfunction, Bioinformatics, Miscarriage, miRNA.

1. Introduction

Spontaneous abortion (SA) is the loss of an intrauterine pregnancy before it reaches viability. The gestational threshold for viability varies due to regional differences, but most countries define spontaneous abortion as complete or incomplete spontaneous abortion missed abortion, or a blighted ovum occurring before 20-22 weeks of gestation or when the fetal weight is less than 500g [1-3]. Missed abortion and incomplete abortion require medical intervention, as the embryo and trophoblastic tissue are not spontaneously evacuated from the uterine cavity, and expectant management usually does not work. In these cases, dilation and curettage or other medical management may be necessary [4]. This can result in severe complications for women, such as bleeding, infection, intrauterine adhesions, decreased fertility, and substantial psychological harm, as well as a financial burden for them and their families [5, 6]. Previous studies have identified a range of etiologic factors for SA, including embryonic chromosomal errors, endometrial defects, hereditary thrombophilia, female body-mass index (BMI), smoking, air pollution exposure, and advanced female and paternal age. However, the pathogenesis of SA is complicated and not yet fully understood.

Villous trophoblasts play a critical role in the process of placental formation, as they are responsible for the functions of implantation and angiogenesis. Their abnormal function can result in various placental-related diseases [7]. Some of the main causes of these diseases, such as abortion, preeclampsia, fetal growth restriction, and premature delivery, include oxidative stress and apoptosis in trophoblasts, as well as immune imbalances at the mother-fetal interface, insufficient invasion of extravillous trophoblasts, and insufficient placenta accreta due to disruptions in the remodeling of uterine spiral arteries [8-13].

Ferroptosis, an iron-dependent form of regulated cell death, differs from other forms of programmed cell death, such as apoptosis, necrosis, autophagy, and pyroptosis, and is characterized by the excessive accumulation of lipid peroxidation [14]. Ferroptosis has been linked to the pathogenesis of various diseases, including gastrointestinal diseases, Type 2 Diabetes Mellitus, Ischemic stroke, Cancer, osteoarthritis, and Alzheimer's disease [15-20]. Placental dysfunction, a key underlying factor in pregnan-

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cy-related disorders such as abortion, eclampsia, and fetal growth restriction, is commonly associated with higher levels of oxidative stress, which results in increased reactive oxygen species (ROS) and lipid peroxidation [8, 11]. Studies have indicated that ferroptosis is linked to preeclampsia and fetal growth restriction [21, 22], leading to the speculation that ferroptosis may also be associated with miscarriage, which is another placental disorder.

At present, no bioinformatics-based studies have been conducted to investigate the mechanism of ferroptosis genes concerning miscarriage. To gain a deeper understanding of this subject, we employed data mining and analysis techniques to identify differentially expressed genes (DEGs) in normal placenta tissue and tissue from miscarriages. By intersecting these DEGs with a ferroptosis dataset, we were able to identify ferroptosis DEGs. Our results aim to provide insights into the molecular-level pathogenesis of miscarriage, identify crucial biomarkers, and offer new perspectives for the clinical diagnosis and treatment of this condition.

2. Materials and methods

2.1. Microarray data

We obtained three gene expression datasets, along with corresponding clinical information, from the Gene Expression Omnibus (GEO). The datasets GSE22490 and GSE26787 were obtained and analyzed. The GSE22490 dataset comprised 4 recurrent miscarriage samples and 6 elective termination samples, while the GSE26787 dataset included 5 recurrent abortion samples and 5 fertile samples. Additionally, a miRNA microarray data set (GSE73025), consisting of 5 miscarriage samples and 5 elective termination samples, was also obtained. The array chip data was processed through probe combination and normalization procedures utilizing the normalizeBetweenArrays algorithm from the limma R package [23]. This resulting dataset was then used for further analysis and data mining in our experiment.

2.2. Differential expression analysis

We utilized abortion tissues as the case group and normal pregnancy tissues as the control group for comparison and analysis. The limma algorithm was applied to compare the samples from both groups and determine differentially expressed genes (DEGs). The significance of these DEGs was determined by implementing multiple test correction procedures on the resulting p-values, and genes with a $|logFC|>1$ and adjusted p-value <0. 05 were considered to be differentially expressed. By analyzing the differences between two groups, we identified a set of genes that were consistently differentially expressed and considered to be potential risk factors associated with abortion.

We obtained a comprehensive list of known ferroptosis-related genes from various sources including the Data Bank and published articles [24-27]. By comparing this list with the set of abortion-related risk genes, we identified a subset of differentially expressed ferroptosis-related genes. These genes were deemed to play a potential role in the termination of pregnancy through induction of ferroptosis.

2.3. Weighted Gene Co-expression Network Analysis (WGCNA)

For the ferroptosis-related genes that showed significant differential expression, we utilized the WGCNA [28] algorithm to identify functional modules within the mRNA datasets of both groups. This analysis resulted in the identification of a set of functional genes that are likely to be involved in the regulation of similar biological processes and exhibit co-expression correlations at the mRNA level. These functional genes were considered to play a role in the regulation of abortion.

2.4. Functional module enrichment analysis

To gain deeper insights into the biological functions regulated by the identified functional gene sets, we conducted functional enrichment analysis on the genes within all co-expression modules using the KEGG and Gene Ontology databases. The analysis was performed using the ClusterProfiler R package [29], and biological functions with a p-value <0. 05 were considered to be significantly enriched and related to abortion.

2.5. GSEA functional deviation score

We aimed to identify biological functions that are associated with miscarriage through the analysis of co-expression models of ferroptosis-related factors. To quantify these functions, we applied the Gene Set Enrichment Analysis (GSEA) algorithm [30]. The GSEA algorithm evaluates the deviation of each function from its expected expression pattern, as determined by the expression of genes involved in the function in each sample. This analysis allows us to uncover the activation or repression relationship of each function across the samples. To identify functions with statistically significant differences between the two groups, we utilized the Wilcoxon test to compare the results for each function.

2.6. Analysis of miRNA-mRNA complex regulation

To delve further into the biological mechanisms underlying the statistically significant functions identified, we conducted a differential analysis of the miRNA dataset to identify miRNAs with a significantly altered expression between the abortion and normal pregnancy tissues. Subsequently, we employed various databases, including mirtarbase [31], tarbase [32], and targetscan [33], to uncover miRNA-mRNA regulatory relationships. Finally, we identified miRNA-mRNA pathways that could contribute to abnormal levels of abortion-related functions.

2.7. Trend analysis of ferroptosis factor

In the GSE22490 dataset, we grouped all normal pregnancy controls together into the T0 group and classified the miscarried tissues into the W6, W8, and W14 groups based on the time of termination. Then, using the STEM algorithm, we performed a trend analysis on all of the ferroptosis factors to identify risk factors that accompany the progression of miscarriage [34].

2.8. Immuno-infiltration analysis

We performed immune infiltration analysis in samples that terminated at different gestational weeks by utilizing the CIBERSORT [35] and ESTIMATE [36] algorithms. This aimed to estimate the presence of immune cells and evaluate the overall immune status of the samples. To explore the relationship between ferroptosis risk factors and immune cells, we conducted a Pearson correlation analysis.

2.9. Patients

The study protocol was approved by the Human Research Ethics Committee of Wuxi Maternal and Child Health Care Hospital. All participants provided informed consent. In this study, we collected placental villi tissue samples from 17 patients with SA and 22 patients with normal pregnancies undergoing surgical abortion at the Wuxi Maternal and Child Health Care Hospital. The collected tissues were carefully processed to eliminate any impurities such as blood, by thoroughly rinsing with sterile normal saline at least three times.

2.10. RT-qPCR analysis for RNA expression.

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RT-qPCR was performed using Fast Start Universal SYBR Green Master (Roche). GAPDH was used as an endogenous control. Fold changes in the mRNA expression levels normalized to GAPDH were calculated using the 2-ΔΔct method. The following primers were used: GAPDH forward, 5′-CCTCAAGATCATCAGCAATGCCTC-3′; GAPDH reverse, 5′-GTGGTCATGAG TCCTTCCACGATA-3′; WIPI forward, 5′-AGAGCCTTCGACCTGGAGTG-3′, WIPI reverse, 5′-TCCTCTGTCCGGAGAAGTTC-3′, GSN forward, 5′-GACTCAGGTCTCGGTCCTTC-3′, GSN reverse, 5′-CAGGCCATCTGTCTGGTCTG-3′.

2.11. Statistics

All statistical analyses are shown as the means ± SD. R software (version 4. 1. 2) and GraphPad software (version 8) were used to analyze the data. The normally distributed continuous variables are expressed as means ± standard deviations and the significance between the means was tested using the Student's t-test. A p-value <0. 05 was considered statistically significant.

3. Results

3.1. Identification of risk genes associated with miscarriage

We conducted a differential gene expression analysis to identify genes significantly associated with SA in two mRNA datasets, GSE22490 and GSE26787. Our analysis revealed 386 and 807 genes that were upregulated and 184 and 669 genes that were downregulated in the GSE22490 and GSE26787 datasets, respectively (Figure 1a/b). We found a total of 33 genes that were differentially expressed in both datasets, including 23 genes that were upregulated and 10 genes that were downregulated (Figure 1c).

We conducted a hierarchical clustering analysis to visualize the differential expression levels of the 33 genes that were significantly associated with miscarriage between the samples of aborted and normal pregnancy tissues. The results, shown in Figure 1d/e, demonstrate that the 33 genes showed a reproducible expression pattern in both datasets. These genes were either up-regulated (represented in blue) or down-regulated (represented in yellow) in the tissues of miscarriages. The normal pregnancy samples were marked in green and the samples from miscarriages were marked in purple. Further analysis of the genomic localization of these risk genes revealed that they were mainly located on chromosomes 1, 2, 3, 4, and 10 (Figure 1f). The blue edges indicate negative correlations, while the red edges indicate positive correlations, suggesting that the expression levels of these genes are correlated despite their disparate genomic locations.

3.2. Screening of risk factors

We conducted an integration of multiple ferroptosis-related databases to compile a list of 1936 ferroptosis-associated genes, which are presented in Supplementary Material Table S1. Our analysis involved comparing the correlations between these 1936 ferroptosis-associated genes and the 33 previously identified risk genes in two independent mRNA datasets from miscarried tissues. The results are depicted in Figures 2a and 2B. From this analysis, we identified 79 ferroptosis genes that were significantly correlated with at least 16 or more of the risk genes (|coefficient| > 0. 5 and adjusted p-value < 0. 05). These 79 genes were considered potential risk factors for ferroptosis (Figure 2c).

3.3. Weighted Gene Co-expression Network Analysis (WGCNA)

We identified 79 ferroptosis genes significantly correlated with habitual miscarriage, and all of these genes were strongly associated with over 50% of the genes related to miscarriage. We further delved into the interrelationships among these ferroptosis genes. By using the Weighted Gene Co-expression Network Analysis (WGCNA) algorithm, we identified 3 co-expression modules, as depicted in Figure 3.

Figure 3a presents the correlation analysis of the co-expression modules of ferroptosis risk genes in the GSE22490 dataset. Figure 3b is a visual representation of the clustering of ferroptosis risk genes in the GSE22490 dataset in the form of a dendrogram. Similarly, Figure 3c provides the correlation analysis of the co-expression modules of ferroptosis risk genes in the GSE26787 dataset,

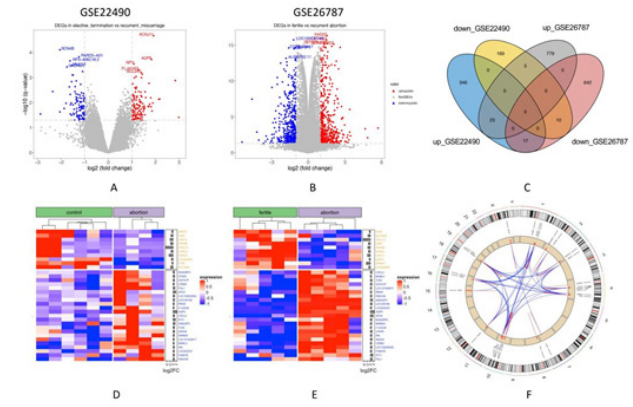

Fig. 1. Differential expression analysis.

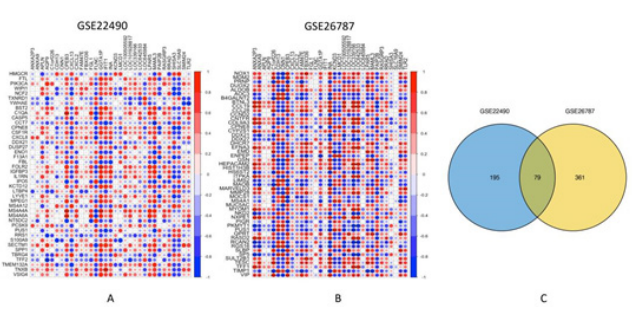

Fig. 2. Correlation analysis of ferroptosis gene and abortion risk gene.

and Figure 3d is the corresponding clustering dendrogram of ferroptosis risk genes in the GSE26787 dataset. These figures demonstrate the high consistency in co-expression modules of the 79 ferroptosis risk genes across different datasets of habitual miscarriages. The reproducibility of the co-expression relationships between ferroptosis risk genes suggests the potential functional consistency of these genes, as they may be involved in regulating similar or identical biological processes.

3.4. Functional module enrichment analysis

To further understand the biological significance of co-expressed ferroptosis risk genes, we employed the ClusterProfiler algorithm to analyze the functions regulated by the 79 ferroptosis risk genes. The results of gene ontology function enrichment are shown in Figure 4. Figure 4a illustrates the gene ontology functions enriched by the GSEA algorithm, including processes of intracellular and intercellular regulation. Figure 4b presents the top 10 significant functions selected by Fisher's exact test, including coagulation, hemostasis, inflammation, and immune-related functions. Figure 4c displays the interaction between significant biological functions and regulators. It can be observed that the regulatory genes related to coagulation and hemostasis are all upregulated in abortion tissues, indicating that abnormal activation of coagulation is an important factor contributing to miscarriage in affected patients.

3.5. ssGSEA functional deviation score

We conducted a functional enrichment analysis to identify the biological processes significantly enriched by the abortion-related ferroptosis genes. We then quantified the deviation degree of each function by combining gene expression with the ssGSEA algorithm, as shown in Figure 4d.

By applying the ssGSEA to score all KEGG pathways, we used the Wilcoxon test algorithm to identify six biologically significant pathways that showed differential behavior, including the suppression of backbone biosynthesis, steroid synthesis, infection, and hematopoiesis-related pathways. Among these, the hematopoiesis-related functions were found to be elevated in patients with a history of habitual miscarriage, which supports the conclusion drawn from Figure 4c that there is abnormal activation of coagulation functions.

3.6. miRNA-mRNA complex regulation analysis

We analyzed the miRNA-target relationships among ferroptosis risk factors associated with habitual miscarriage. To do this, we utilized three miRNA databases, mirecords, mirtarbase, and tarbase, to identify 1, 306 miRNAs targeting these ferroptosis risk factors. The analysis of the miRNA dataset GSE73025 revealed 38 upregulated and 6 downregulated miRNAs. This led to the identification of 61 pairs of differentially expressed miRNA-ferroptosis factor relationships, including 19 differentially expressed miRNAs and 41 ferroptosis factors (as presented in supplementary material Table S2). Figure 5a shows the results of this analysis, where it can be observed that of the 19 differentially expressed miRNAs, 3 were found to be low-expressed in abortion tissues and the remaining 16 were found to be high-expressed. We further constructed a miRNA-mRNA regulation network using the Cytoscape

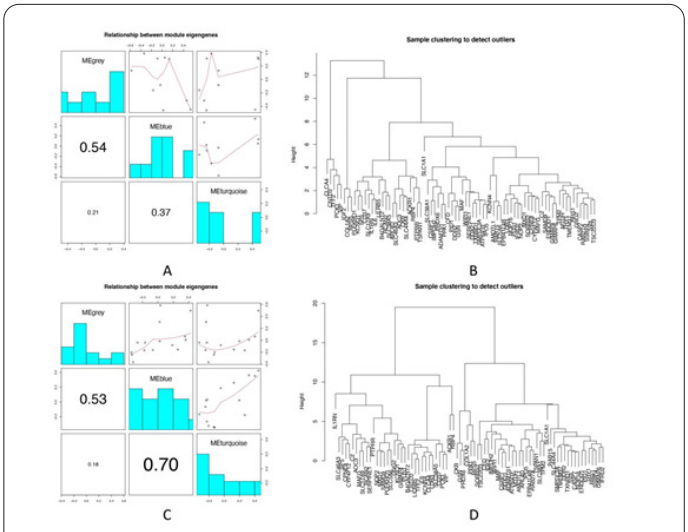

Fig. 3. Wgcna analysis.

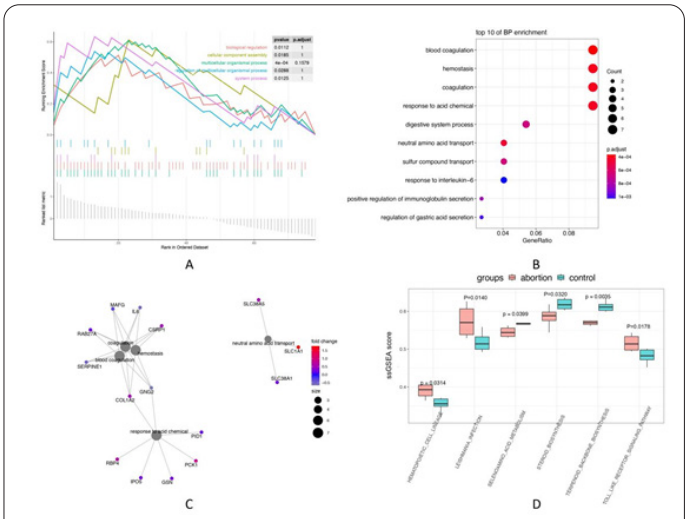

Fig. 4. Functional module enrichment analysis.

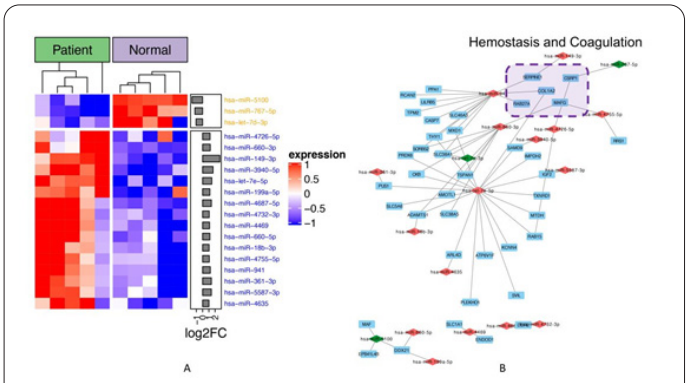

Fig. 5. MiRNA complex regulation analysis.

software, as illustrated in Figure 5b. The network was comprised of functional modules composed of ferroptosis factors, which corresponded to coagulation-related functions. In the network, red nodes represent high-expression miRNAs, green nodes represent low-expression miRNAs, and blue nodes represent ferroptosis factors regulated by the differentially expressed miRNAs.

3.7. Trend analysis of ferroptosis risk genes

We utilized the STEM algorithm to examine the synergistic trend changes of ferroptosis factors concerning the abortion process based on the time point of pregnancy termination. The results are displayed in Figure 6.

Figure 6 presents the results of the trend analysis of the top 8 clusters using the STEM algorithm. The x-axis depicts the time points of pregnancy termination while the y-axis represents the deviation trend of gene expression. It can be observed that the expression of ferroptosis genes in cluster 2 decreases gradually throughout pregnancy. Conversely, the expression levels of clusters 3 and 5 increase steadily during pregnancy. The expression changes of ferroptosis genes in other clusters are not significant and exhibit inflection points during pregnancy termination, potentially reflecting functional compensation during the progression of the process. The full list of ferroptosis genes included in each cluster can be found in the supplementary material Table S3.

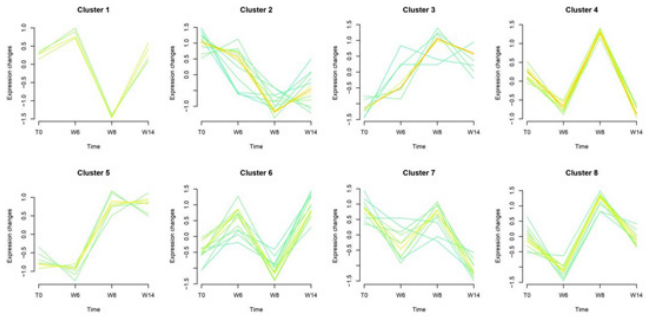

Fig. 6. Trend analysis of ferroptosis risk genes.

3.8. Immuno-infiltration analysis

In the field of functional analysis, we observed a correlation between habitual abortion and various physiological processes, including inflammation and immunity. To validate this hypothesis, we employed the CIBERSORT and ESTIMATE algorithms to assess the immune cell infiltration profiles in samples collected at distinct gestational time points. Our findings, depicted in Figure 7, demonstrate the correlation between habitual abortion and immune infiltration.

Figure 7a depicts the results of the immune cell infiltration analysis using the CIBERSORT algorithm. The horizontal axis represents gestational time, with T0 serving as the control and W6, W8, and W14 representing gestational termination at 6, 8, and 14 weeks, respectively. Our results indicate significant differences in immune cell composition at various gestational time points, such as the progressive increase in CD4 T cell infiltration with advancing gestational weeks. Figure 7b displays the estimated matrix score and immune score for each gestational period as determined by the ESTIMATE algorithm. The results suggest that compared to normal control samples (T0) and early termination samples (W6), the matrix score and immune score of tissues from patients with habitual abortion were higher with increasing gestational duration, reflecting the gradual worsening of immune dysfunction in this population. Finally, Figure 7c presents a correlation analysis of ferroptosis factors and the infiltration scores of 22 immune cell types. Our findings indicate significant correlations between the ferroptosis factors and the infiltration levels of various immune cells.

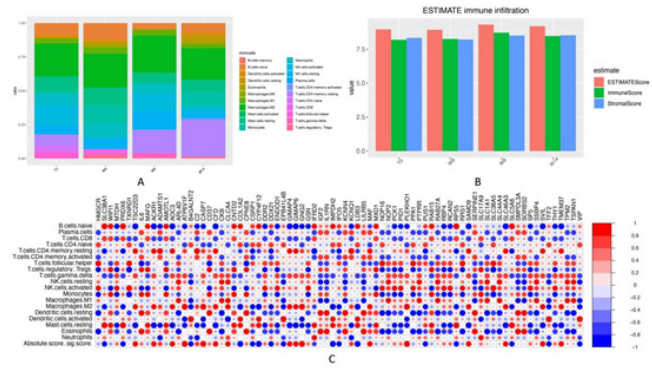

Fig. 7. Immuno-infiltration analysis.

3.9. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

"RT-qPCR analysis revealed a significant difference in the expression levels of WIPI1 between the experimental and control groups (P<0. 01), with a median expression of 50. 67 and 0. 6583, respectively. Furthermore, the median expression of genes related to ferroptosis was found to be significantly higher in the SA group (12. 77) compared to the control group (0. 633). These results suggest a close relationship between spontaneous abortion and ferroptosis in villous trophoblast cells, as demonstrated by the significant difference in ferroptosis gene expression between the experimental and control groups (Figure 8). "

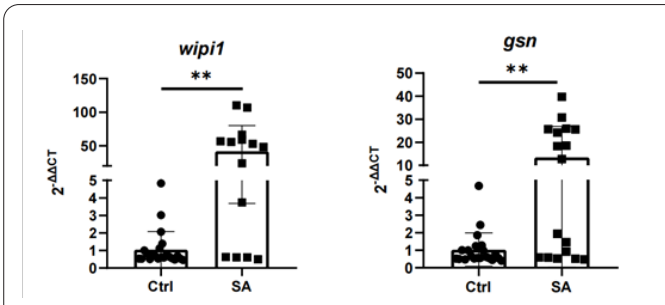

Fig. 8. The relative mRNA expression of ferroptosis-associated genes
** P < 0. 01.

4. Discussion

Spontaneous abortion (SA) is a prevalent pregnancy disorder that poses a significant threat to women's health. Despite the growing evidence linking SA to a range of risk factors, including endocrine disruptions, organ malformations, immunodeficiency, and chromosomal abnormalities [37-39], there remain cases that cannot be explained by these mechanisms. This highlights the need for further exploration of additional risk factors and underlying molecular mechanisms of SA pathogenesis, of which ferroptosis is a particularly notable example.

Ferroptosis, a novel form of programmed cell death, has been recently discovered to differ from apoptosis, necrosis, and autophagy. This mode of cell death is characterized by its dependence on iron, hence the name ferroptosis. It is also distinct in that it is independent of caspase and is accompanied by elevated levels of lipid peroxidation [40]. GPX4 has been identified as a key regulator of ferroptosis by controlling the activity of cyclooxygenases and modulating the lipid peroxidation enzymes involved in lipid metabolism [41]. Previous studies have also reported a significant impact of the inactivation mutation of GPX4 on male fertility [42]. Moreover, GPX4 expression was found to be lower in the SA group compared to the healthy control group and positively correlated with H19 expression [43]. In addition, silencing of H19 was shown to down-regulate GPX4 expression. These findings sug-

gest a strong association between ferroptosis and SA.

In this study, we aimed to investigate the biological mechanism of SA induced by ferroptosis, using ferroptosis as a seed and obtaining consistent SA risk factors from two sets of mRNA datasets. These risk factors were differentially expressed specifically in SA tissues and were significantly co-expressed with ferroptosis factors. We performed a weighted co-expression network analysis to identify functional modules that were significantly related to the phenotype of SA. Our results showed that these SA risk factors were primarily involved in the regulation and inhibition of backbone biosynthesis, steroid synthesis, infection, and hematopoietic-related pathways. In particular, hematopoiesis-related functions were activated and enhanced in patients with spontaneous abortion, which is consistent with previous findings of abnormal coagulation function in these patients. Our results provide evidence that recurrent spontaneous abortions are associated with abnormalities of the hematopoietic system and abnormalities in coagulation and anticoagulation homeostasis. In conclusion, these findings suggest that the combination of thromboelastography (TEG) and antithrombin III (AT-III) and D-dimer levels could be used as a diagnostic tool to effectively distinguish patients with spontaneous abortion from the healthy population and predict the occurrence of SA(43).

In a functional enrichment analysis, we found significant alterations in the immune system of patients with SA compared to the healthy population. The dysregulation of the immune system is primarily evident in the abnormal levels of immune cell infiltration, including CD4 T cells, B cells, monocytes, macrophages, and others. By comparing the immune status of patients with miscarriages at different gestational weeks, we observed dynamic changes in the immune cell composition of the patients. For instance, the infiltration of CD4 T cells increased with increasing gestational weeks. It is worth noting that these dynamically changing levels of immune cells and SA risk factors show a significant correlation, suggesting that ferroptosis mechanisms may directly or indirectly influence the level of the immune response. The correlated immune cells and SA risk factors also displayed non-monotonic dynamic fluctuations, which may be due to compensatory mechanisms activated by the body to counteract the effects of ferroptosis-induced abnormalities in multiple functions and repair mechanisms. However, beyond a certain gestational age, the disease progresses to a decompensated state.

Finally, we have presented a set of miRNA datasets to shed light on the underlying mechanism of altered expression of SA risk factors at the transcriptional level. Our findings suggest that some of these risk factors may be regulated through the formation of miRNA-mRNA interactions, where differentially expressed miRNAs modulate gene expression. On the other hand, other SA risk factors that do not exhibit these molecular relationships may be subject to regulation through alternative mechanisms, including transcription factor regulation, mutations in promoter or enhancer regions, and methylation regulation, among others.

This study has several limitations. Firstly, regarding the key SA risk factors, we conducted PCR validation and found that, at the expression level, these factors are differentially expressed in the patient. However, the functional role of these SA risk factors still requires further validation through cellular or animal experiments. Secondly, we have tentatively proposed potential mechanisms by which ferroptosis may contribute to the occurrence of spontaneous abortion and identified SA risk factors as features to distinguish patients from the normal population. However, the utility of these features as diagnostic markers requires validation with a larger sample size. If a sufficient number of patient samples can be obtained, we have the opportunity to develop diagnostic models through machine learning and evaluate their efficacy. Finally, we have used microRNAs (miRNAs) to explain the underlying regulatory mechanisms of some SA risk factors. However, some SA risk factors remain unexplained by differentially expressed miRNAs and may be further understood through mechanisms such as transcription factor abnormalities, gene mutations, and methylation abnormalities, but this falls outside the scope of this paper.

5. Conclusion,

We presented a novel hypothesis that ferroptosis may contribute to the occurrence of SA. Our findings suggest that activation of this mechanism leads to disruptions in several key biological processes, including hematopoiesis, coagulation/anticoagulation, and immune modulation. These findings offer new opportunities for the development of clinical diagnostic markers and potential drug targets for the screening of SA risk factors.

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Conflicts of interest

The authors report no conflict of interest.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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