Genetic Analysis of Potential Biomarkers and Therapeutic Targets in Ferroptosis from Steroid-Induced Osteonecrosis of the Femoral Head Based on Machine Learning

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ABSTRACT

Objective: To locate the candidate therapeutic target genes involved in ferroptosis in steroid-induced osteonecrosis of the femoral head (SONFH).

Study Design: Bioinformatics analysis study.

Place and Duration of the Study: Department of Orthopaedic Surgery, Zhuhai Hospital of Integrated Traditional Chinese and Western Medicine, Guangdong, China, from March to July 2023.

Methodology: After processing the gene expression omnibus (GEO) data with the R programming language, differentially expressed ferroptosis-related genes in SONFH were identified. To pinpoint the genes most strongly linked to SONFH in association with ferroptosis, least absolute shrinkage and selection operator (LASSO) regression and support vector machine-recursive feature elimination (SVM-RFE) were employed. Subsequently, the screened essential genes were analysed to investigate immune cell infiltration, and competing endogenous RNA (ceRNA) networks involving these marker genes were constructed.

Results: The machine learning algorithms identified three genes i.e., *SOCS1* (*suppressor of cytokine signalling1*), *MYCN* (*N-myc proto-oncogene protein*), and *KLF2* (*Kruppel-like factor 2*) as diagnostic feature biomarkers associated with ferroptosis. Additionally, CIBERSORT analysis revealed that alterations in the immune microenvironment, such as Macrophages M1, Monocytes, and T cells CD4 naive, could be linked to *SOCS1*, *MYCN*, and *KLF2*. Moreover, the competing endogenous RNA (ceRNA) network exposed a complex regulatory relationship based on marker genes.

Conclusion: *SOCS1*, *MYCN*, and *KLF2* are potential biomarkers associated with ferroptosis in SONFH, pending confirmation in future studies.

Key Words: *Steroid-induced osteonecrosis of the femoral head, Ferroptosis, Machine learning, Genetic analysis.*

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INTRODUCTION

Non-traumatic osteonecrosis of the femoral head is a progressive and disabling metabolic disease, with steroid-induced osteonecrosis of the femoral head (SONFH) emerging as its primary pathogenesis and showing a rising trend.[1] The annual incidence of SONFH worldwide has been steadily rising, primarily attributed to the persistent effects of the COVID-19.[1] In China, an estimated 36,000 to 48,000 new cases of SONFH are reported annually. Typically, within 2-3 years, a substantial majority of SONFH patients experience femoral head collapse, necessitating costly joint replacement procedures.[1]

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Despite the early diagnostic aid provided by magnetic resonance imaging (MRI), the majority of patients still face femoral head collapse within the mentioned timeframe, leading to significant financial burdens on the healthcare system and economy.[2] Consequently, there is a critical need to enhance the understanding of SONFH causes, discover early diagnostic markers, and develop effective therapeutic medications.

Contrary to apoptosis and autophagy, ferroptosis is a recently identified form of programmed cell death triggered by disruptions in iron metabolism.[3] Key features of ferroptosis include iron-dependent lipid peroxidation and reactive oxygen species (ROS).[3] Ferroptosis has been associated with various diseases, such as cancers, neurological disorders, atherosclerosis, ischaemia-reperfusion injury, sepsis, osteoporosis, and osteoarthritis, although the precise function and underlying mechanisms in human diseases remain unknown.[3] For instance, research indicated that ferroptosis induced by iron overload in osteoblasts impedes osteogenesis and hastens osteoporosis progression.[4] Additionally, studies showed that melatonin can

prevent steroid-induced osteoporosis by inhibiting ferroptosis in rat bone marrow mesenchymal stem cells through the activation of the phosphatidylinositol 3-kinase (*PI3K*) / protein kinase B (*AKT*)/mammalian target of rapamycin (*mTOR*) pathway.[5]

Furthermore, a research has associated ferroptosis to the proper functioning of immune cells.[6] One mechanism by which ferroptosis influences the quantity and performance of immune cells is through the stimulation of pro-inflammatory cytokines production, involving B cells, macrophages, and T cells.[7] In turn, immune cells' detection of ferroptotic cells can result in an inflammatory response.[7] Although the precise pathophysiology of SONFH remains elusive, investigations highlighted the crucial role of immune system dysregulation, including macrophages, T cells, neutrophils, and B cells, in the development of femoral head osteonecrosis.[8] Nonetheless, few studies have explored the mechanisms of SONFH and pharmaceutical interventions targeting the interplay between ferroptosis and immune cell activity.

This study aimed to identify biomarkers linked to SONFH, ferroptosis, and immune cells by employing machine learning algorithms. Initially, differentially expressed genes (DEGs) were identified in SONFH and control samples. Another aim was to investigate the potential functions and regulatory mechanisms underlying the relationship between the potential biomarkers and immune cells.

METHODOLOGY

The gene expression omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/) was queried for femoral head necrosis and steroid-induced osteonecrosis. Following the exclusion of datasets with limited sample sizes, analysis was conducted on the microarray datasets GSE123568 (GPL15207) and GSE74089 (GPL13497). The GSE123568 comprised 40 serum samples-30 SONFH and ten non-SONFH-alongside clinical data. GSE74089 was utilised as the validated dataset, encompassing four normal and four femoral head necrosis articular cartilage samples. Data pre-processing involved background correction, adding missing values, and quantile normalisation using R (version 4.2.1). Ferroptosis-related marker, driver, and suppressor genes were obtained from FerrDb V2 (2022, stable, http://www.zhounan.org/ferrdb/current/operations/download.html).

Following the acquisition of ferroptosis-related gene expression (FDGs) levels, the Limma package in R identified differentially expressed mRNAs with p-value <0.05 after adjustment and |log2FC|>1. Subsequently, Clusterprofiler performed gene ontology (GO) and Kyoto Encyclopaedia of Genes and Genomes (KEGG) enrichment analyses on differentially expressed genes (DEGs) in the gene expression matrix. A significant enrichment was defined as an FDR <0.25 and p <0.05.

The feature selection algorithms least absolute shrinkage and selection operator (LASSO) regression and support vector machine-recursive feature elimination (SVM-RFE), commonly utilised in genomics, metabolomics, and proteomics, were executed in R with the glmnet and e1071 packages. The receiver operating characteristic (ROC) curve validated the intersection genes identified by LASSO and SVM-RFE as potential molecular markers for SONFH.

CIBERSORTx, a tool combining multiplexed immunohistochemistry and digital cytometry (http://cibersortx.stanford.edu/), was employed to analyse discrepancies in immune infiltration between the SONFH and normal groups, evaluating the function of immune microenvironment. Samples with a significance level of p <0.05 were retained. The core function in R, utilising the Spearman's rank correlation coefficient technique, was utilised to determine the connection between ferroptosis-related marker genes and immune infiltration.

Three miRNA databases-miRanda, miRDB, and TargetScan were used to predict miRNAs targeting ferroptosis-related marker genes. Common miRNAs identified in at least three databases were selected. Subsequently, long non-coding RNAs (lncRNAs) targeting these miRNAs were identified using SpongeScan (http://spongescan.rc.ufl.edu/). The Cytoscape 3.7.1 software was employed to visualise the competing endogenous RNAs (ceRNAs) network of involving miRNAs, lncRNAs, and ferroptosis-related marker genes. Statistical analysis was conducted using R version 4.2.1, with a significance threshold set at p-value <0.05.

RESULTS

Initially, the findings were validated using GSE74089 following the analysis of the dataset GSE123568. Subsequently, 548 genes were derived from FerrDb and categorised into markers, suppressors, and drivers, encompassing lncRNAs, miRNAs, and circRNAs. Furthermore, 170 FDGS were identified from the processed dataset GSE123568, comprising 89 up-regulated genes and 81 down-regulated genes.

The Clusterprofiler tool was utilised to assess the FDGS and conduct the GO enrichment analysis, encompassing molecular function (MF), cellular component (CC), biological processes (BP), and KEGG pathway analysis. The results of the GO enrichment analysis results revealed that the FDGS were primarily enriched in response to nutrient levels, oxidative stress, cellular responses to oxidative stress, and cellular responses to chemical stress (Figure 1A). MF demonstrated increased levels of NAD + ADP-ribosyltransferase, protein ADP-ribosylase, transferase activity (transferring pentosyl groups), and antioxidant activity. Regarding CC, the FDGS were primarily concentrated in the organelle outer membrane, peroxisomal membrane, microbody membrane, and TOR complex. In terms of KEGG pathway analysis, the FDGS were mainly enriched in forkhead box O (FoxO) signalling pathway, autophagy-animal, and ferroptosis pathways (Figure 1B).

Two machine learning approaches, LASSO regression and SVM-RFE, were employed to enhance the reliability and robustness of biomarkers in FDGS. Through LASSO regression, a subset of 10 factors highly predictive of differentially expressed genes linked to ferroptosis was identified (Figure 2 A and B).

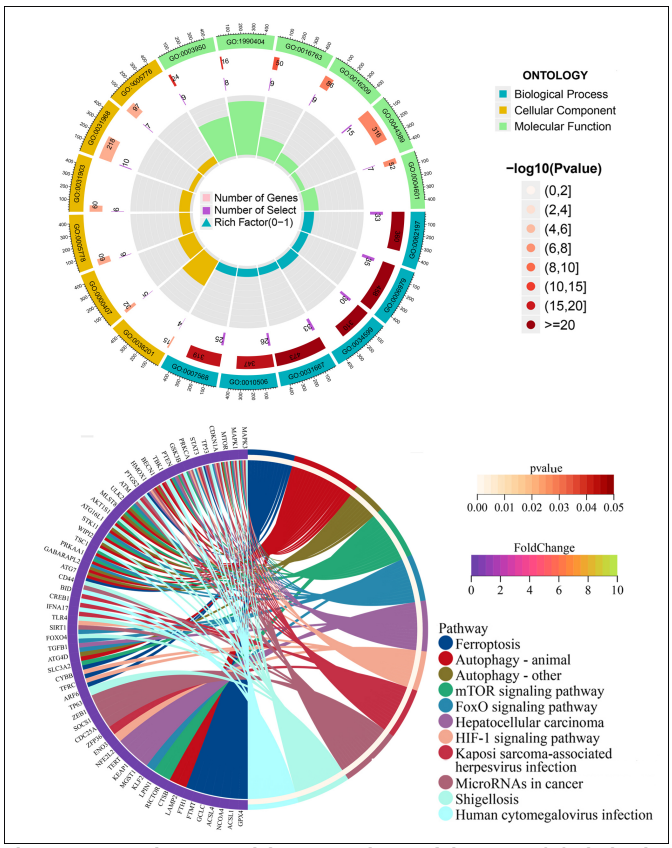

Figure 1: GO and KEGG enrichment analyses of the FDGS. (A) Circle plot and network visualising the GO analysis. (B) Circle plot and network visualising the KEGG pathway.

Additionally, the SVM-RFE approach unveiled six signature genes (Figure 2 C and D). By amalgamating the gene lists derived from LASSO regression and SVM-RFE, three genes (*SOCS1*, *MYCN*, and *KLF2*) were pinpointed as diagnostic feature biomarkers for ferroptosis (Figure 2E). The efficacy of LASSO regression and the SVM-RFE models was evaluated using ROC curves, revealing that the AUC (area under the ROC curve) values of these genes exceeded 0.7 and achieved a maximum AUC value of 1 for the model (Figure 2 F and G). This signifies the diagnostic feature biomarkers' capability to effectively differentiate between the samples.

Previous studies have established a significant correlation between ferroptosis-related genes and the body's immunological response. Furthermore, earlier research suggested a link between immune cell infiltration and the mechanism of SONFH development. The CIBERSORT algorithm was employed to evaluate the immunological landscape. The analysis of the immune landscape (Figure 3A) revealed that the fraction of activated dendritic cells was lower in SONFH samples than in normal samples, while B cells' memory exhibited higher expression levels. Subsequent Spearman's correlation analysis identified three marker genes associated with ferroptosis that correlated with immune cell infiltration. Specifically, *KLF2* exhibited a negative correlation with M1 Macrophages (r = -0.410, p = 0.026) and M1 Monocytes (r = -0.512, p = 0.004), whereas T cells CD4 naive showed a positive correlation with *KLF2* (r = 0.40, p = 0.029) as shown in (Figure 3B).

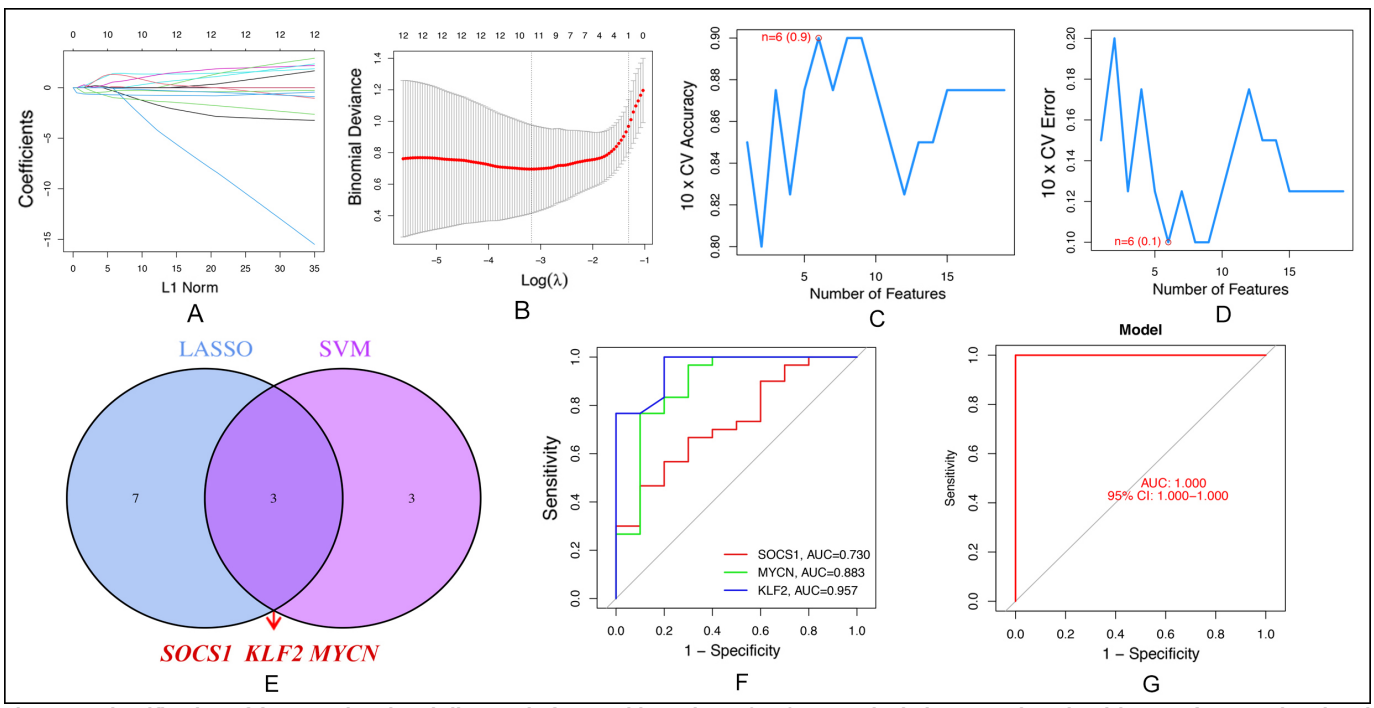

Figure 2: Identification of ferroptosis-related diagnostic feature biomarkers. (A-B) LASSO logistic regression algorithm, 10 ferroptosis-related genes features were selected. (C-D) SVM-RFE algorithm, six ferroptosis-related genes features were selected. (E) Venn diagram demonstrated that three ferroptosis-related genes were acted as diagnostic feature biomarkers. (F) ROC curves for the three ferroptosis-related genes. (G) The model to identify the AUC of disease samples.

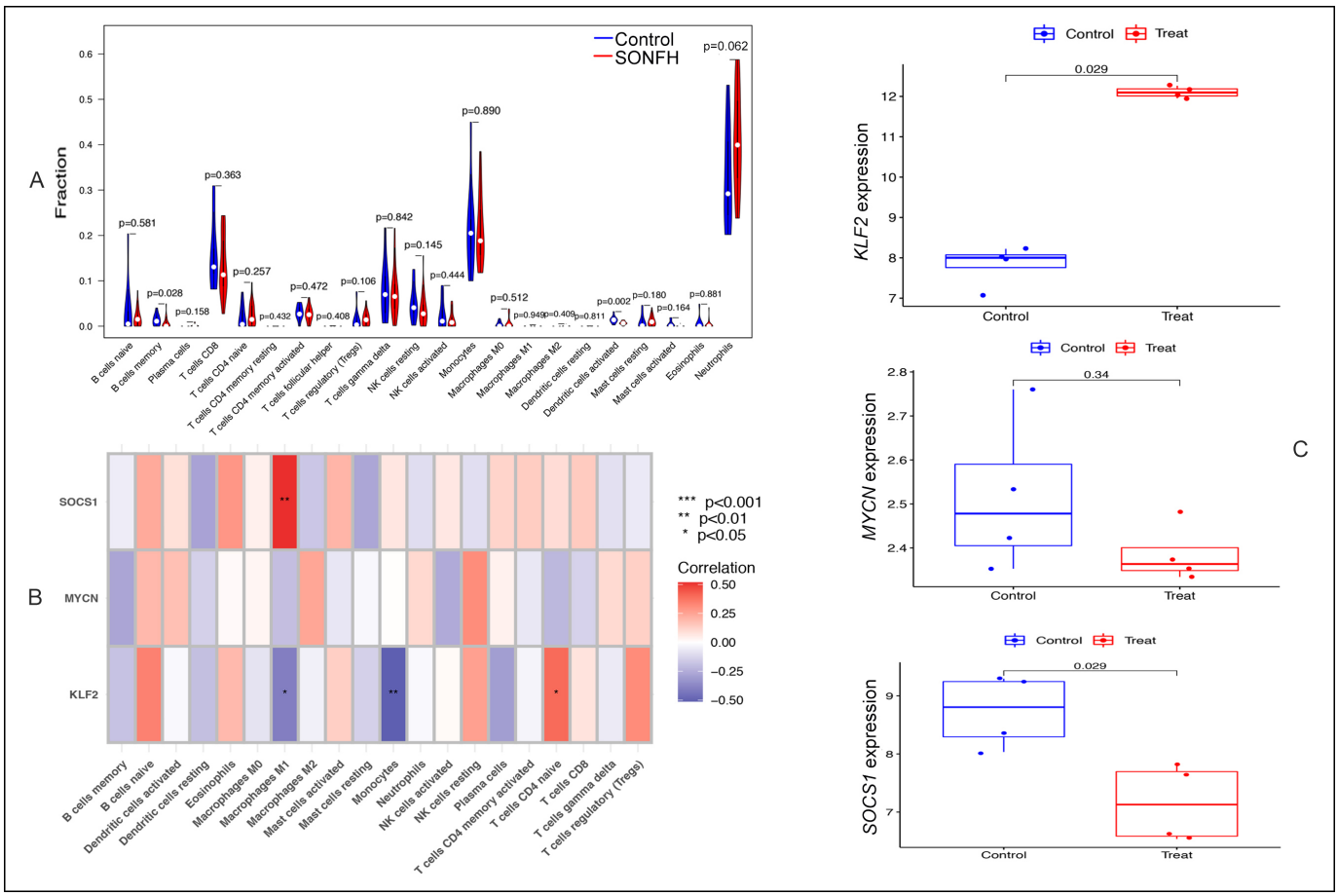

Figure 3: Immune landscape analysis for three ferroptosis-related genes. (A) The CIBERSORT algorithm was adopted to evaluate the difference of immune landscape between SONFH and normal samples. (B) Three ferroptosis-related marker genes were associated with immune cell infiltration. (C) The expression of three ferroptosis-related genes in the GSE74089 dataset.

Subsequently, the ceRNA network was established based on three ferroptosis-related genes using data from the miRanda, miRDB, targetScan, and SpongeScan databases. The network comprised a total of 295 nodes, including the three ferroptosis-related genes, 153 miRNAs, and 139 lncRNAs, connected by 292 edges.

Finally, the expression levels of the three genes linked to ferroptosis were examined and validated using the GSE74089 dataset. The results illustrated that the expression patterns of *KLF2*, *SOCS1*, and *MYCN* closely mirrored those observed in the GSE123568 dataset. Specifically, the expression of *KLF2* was higher in osteonecrosis of femoral head (ONFH) patients compared to normal samples ($p = 0.029$), while the expression of *SOCS1* was lower ($p = 0.029$) (Figure 3C). Although the difference in *MYCN* expression ($p = 0.34$) did not reach statistical significance, the trend remained consistent, possibly attributed to the relatively small sample size in the GSE74089 dataset.

DISCUSSION

Numerous hypotheses have been postulated to elucidate the pathophysiology of SONFH, however, the exact mechanism underlying the disease remains elusive, posing challenges for the development of effective treatments.[9] Consequently, an urgent need exists for the discovery of novel biomarker-based mechanisms of pathogenesis to identify SONFH patients. In this study, the authors employed the LASSO and SVM-RFE algorithms to pinpoint three marker genes, namely *KLF2*, *SOCS1*, and *MYCN*, associated with ferroptosis in SONFH. Additionally, the authors constructed the ceRNA network and investigated the correlation between the marker genes and immune infiltration. Finally, the three marker genes were validated in a separate dataset. Notably, the presence of ferroptosis and iron overload in SONFH was supported by prior research findings.[10]

KLF proteins play a pivotal role in regulating key physiological processes such as cell differentiation, development, proliferation, and apoptosis.[11] Among these proteins, *KLF2*, a member of the *KLF* protein family, stands out due to its zinc finger-containing DNA-binding structural domain.[11] *KLF2* not only governs endothelial cell homeostasis, promoting angiogenesis and remodelling, but also regulates osteoblast and osteoclast functions.[11] Research indicates that *KLF2* modulates osteoblast differentiation by targeting runt-related transcription factor 2 (*Runx2*).[12] Moreover, interferon regulatory protein 2 binding protein 2 (*IRF2BP2*) mediates *KLF2* to inhibit osteoblast differentiation and enhance osteoblast differentiation and function,

impacting bone homeostasis significantly.[13] Furthermore, studies have shown that ferritin stimulates osteoblast development from dental pulp-derived stem cells by inducing *KLF2*-mediated mitochondrial autophagy, offering potential therapeutic implications for skeletal disorders such as arthritis and osteoporosis.[14] Additionally, *KLF2* plays a crucial role in activating various immune cells, influencing B cells, T cells, NK cells, and bone marrow cell subsets.[11] Dysregulation of *KLF2* leads to alterations in the activity of these immune cells, affecting processes such as osteoclast formation by monocytes.[15] These findings support the results of the current investigation, which demonstrated the intimate connection between *KLF2* and the functioning of immune cells. Notably, the interplay between *KLF2* and ferroptosis has also been associated with impeding cancer cell migration and invasion in clear renal cell carcinoma. Studies have revealed that *KLF2* regulates ferroptosis through glutathione peroxidase 4 *(GPX4)*, thereby inhibiting the ferroptosis process.[16] Consequently, further research is imperative to ascertain whether *KLF2*, identified as one of the markers in this study, could potentially serve as a therapeutic target for SONFH.

Since 1997, eight structurally-related members of the *SOCS* family have been identified as negative regulators of cytokine induction.[17] Among these, *SOCS1* is recognised as one of the two significant inhibitors of cytokine signalling, playing a pivotal role in combating detrimental infections*. In vivo*, *SOCS1* exerts its primary mode of action by directly inhibiting the kinase activity of *Janus kinase* (*JAK*), which has broad implications for immune system function.[17] This inhibition impacts processes such as T cell and dendritic cell differentiation, CD8+ T cell development, and bone marrow cell development and function.[17] A notable example of *SOCS1*'s regulatory function is the negative control of *JAK2*, which has been associated with the methylation of *SOCS1* in acute and chronic myeloid leukaemia.[18] Prior studies have linked variations in *SOCS1* expression to more severe cases of rheumatoid arthritis in patients.[19] Emerging evidence indicates that skeletal cells, such as osteoblasts, osteoclasts, chondrocytes, and synoviocytes express the *SOCS* protein family.[19] Specifically, *SOCS1* is closely associated with inflam-mation-driven bone resorption. *SOCS1* counteracts the inhibitory impact of inflammatory cytokines on nuclear factor kappa B (*NF-κB*) ligand-mediated receptor activators of osteoclast differentiation signals, regulating osteoclast formation.[19] In arthritic joints, *SOCS1* plays a crucial role in dampening inflammation and mitigating joint degeneration by suppressing the production of inflammatory cytokines.[20] Therefore, considering the existing literature and the interplay between ferroptosis and immunological inflammation,[6] further investigation of *SOCS1* as a potential SONFH marker closely linked to ferroptosis is justified.

MYCN, a crucial member of the *myelocytomatosis oncogene* (*MYC*) proto-oncogene family, functions as a transcription factor that orchestrates the expression of its target genes. It plays a pivotal role in regulating essential cellular processes such as cell proliferation, differentiation, apoptosis, protein synthesis, and metabolic activities.[21] Increased expression of *MYCN* has been observed in various cancer types, including neuroblastoma, rhabdomyosarcoma, and lung cancer.[21] Moreover, *MYCN* has a significant impact on the production of several immune system effector molecules. By modulating the biological activity of immune cells, such as CD4T cells, macrophages, and natural killer (NK) cells, *MYCN* influences immune responses and immune cell functions.[22]

The reversal of ferroptosis following the inhibition of *MYCN* expression or treatment with ferroptosis inhibitors suggests that *MYCN* plays a regulatory role in ferroptosis. Studies have shown that in cystine depletion-induced cellular ferroptosis in neuroblastoma cells, *MYCN* exhibits aberrant expression.[23] Treatment with ferroptosis inhibitors led to the reversal of ferroptosis in these cells.[23] While there is limited research on the role of *MYCN* in bone metabolism-related diseases, the existing literature aligns with the findings of the present study. However, further research is warranted to validate and elucidate the mechanism by which *MYCN* regulates ferroptosis in SONFH.

Research findings indicate that dexamethasone triggers ferroptosis *via* the tumour protein 53 *(P53)* / solute carrier family 7 Member 11 (*SLC7A11*) / *GPX4* pathway in steroid-induced avascular necrosis of the femoral head.[24] Additionally, exogenous melatonin modulates ferroptosis through growth differentiation factor 15 (*GDF15*) signalling, alleviating steroid-induced avascular necrosis of the femoral head.[25] After considering all aspects, the authors have identified marker molecules associated with ferroptosis and immune cell infiltration in SONFH, along with the ceRNA networks linking them. Further relevant experimental studies are necessary to validate these findings. By integrating the research outcomes, it is anticipated that novel perspectives on the pathogenesis of SONFH can be unveiled, paving the way for innovative preventive strategies.

CONCLUSION

Through machine learning, *MYCN*, *KLF2*, and *SOCS1* have been identified as key genes associated with ferroptosis and immune cells in SONFH. Furthermore, *KLF2* exhibited a negative correlation with M1 Macrophages and M1 Monocytes, whereas T cells CD4 naive showed a positive correlation with *KLF2*. These genes hold promise as a new avenue for studying the pathogenesis and potential therapeutic targets of SONFH.

COMPETING INTEREST:
The authors declared no conflict of interest.

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AUTHORS' CONTRIBUTION:
JZ, JG, KD: Conception of the study design and interpretation of data.

XZ, and JJ: Drafting and critical revision of the manuscript for important intellectual content.
KD: Project direction.
All authors approved the final version of the manuscript to be published.

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