

ORIGINAL RESEARCH ARTICLE

MEF2C as a key driver in Epstein–Barr virus-positive diffuse large B-cell lymphoma

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Abstract

Introduction: Epstein–Barr virus (EBV)⁺ diffuse large B-cell lymphoma (DLBCL) is an aggressive lymphoma with a poor prognosis and complex molecular alterations. The role of ferroptosis in EBV⁺ DLBCL remains poorly understood.

Objectives: The objective of this study was to identify and validate key ferroptosis-related genes (FRGs) in EBV⁺ DLBCL by integrating bioinformatics and experimental approaches.

Methods: Gene expression datasets related to EBV and DLBCL were retrieved from the Gene Expression Omnibus (GEO) database, including GSE49628, GSE56315, and GSE103265. Differentially expressed genes (DEGs) were identified using GEO2R with the criteria $|\log_2 \text{fold change}| \geq 1$ and adjusted $p < 0.05$. The DEG lists from the three datasets were intersected with the human FRGs retrieved from FerrDb using Venny 2.0. A random forest classifier was used to classify FRGs in each dataset through the randomForest and caret R packages. Selected genes were evaluated by quantitative real-time polymerase chain reaction (RT-qPCR) in formalin-fixed, paraffin-embedded DLBCL tissues from 13 patients, and further co-expression analysis of the genes was performed using GeneMANIA.

Results: Overlapping FRGs ($n = 11$) were identified through Venny 2.1.0. Using a random forest classifier, *MEF2C* was identified as the most important predictive biomarker, along with *TLR4* and *SLC40A1*. Consistent with the expression-analysis results, RT-qPCR showed significantly higher *MEF2C* and *GPX4* expression in EBV⁺ DLBCL samples, and significantly lower *TLR4* and *SLC40A1* expression. Co-expression analysis verified the involvement in pathways associated with iron ion homeostasis, inflammatory signaling, and B-cell proliferation.

Conclusion: This study suggests that molecular changes associated with ferroptosis are linked to EBV⁺ DLBCL and that *MEF2C* may be a therapeutic target or biomarker.

Keywords: Ferroptosis; Epstein–Barr virus-positive diffuse large B-cell lymphoma; *MEF2C*; *GPX4*; *EBNA1*; Biomarker

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1. Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of adult non-

Hodgkin lymphoma (NHL), accounting for about 30–40% of cases.¹ DLBCL is a biologically and clinically heterogeneous malignancy with an aggressive clinical course, rapid tumor growth, and variable response to treatment. Despite improvements in chemotherapy and immunotherapy, a substantial proportion of patients experience relapsed or refractory disease and poor clinical outcomes.^{2,3} DLBCL exhibits molecular heterogeneity, which has hampered the identification of reliable biomarkers and therapeutic targets, underscoring the need for further research into the molecular processes underlying disease development.

Epstein–Barr virus (EBV), a ubiquitous human γ -herpesvirus, is strongly linked to multiple hematological and epithelial cancers, including Burkitt lymphoma, nasopharyngeal carcinoma, Hodgkin lymphoma, and DLBCL.^{4–6} EBV⁺ DLBCL is a distinct and aggressive lymphoma entity associated with chronic viral infection, immunological dysregulation, and aberrant activation of oncogenic signaling pathways.⁷ During EBV infection, latent viral products, including Epstein–Barr nuclear antigen 1 (EBNA1), latent membrane proteins (LMPs), and non-coding RNAs, regulate cell proliferation, apoptosis, immune evasion, and metabolic reprogramming, thereby contributing to malignant transformation.^{8–10} However, the exact molecular mechanisms underlying EBV⁺ DLBCL progression are not fully understood.

Ferroptosis is an iron-dependent form of regulated cell death characterized by phospholipid peroxidation mediated by reactive oxygen species (ROS) and oxidative stress-induced cellular damage.¹¹ Emerging evidence indicates that ferroptosis is dysregulated in the initiation, progression, metastasis, and therapeutic resistance in multiple cancers. Tumor cells can become ferroptosis-resistant by altering antioxidant pathways, iron metabolism, and lipid homeostasis, thereby enabling survival under oxidative stress.¹² Ferroptosis-associated molecular changes are now of great interest in hematological malignancies, as they may be involved in lymphoma progression and therapeutic response.¹³

A number of ferroptosis-related genes (FRGs), including *GPX4*, *SLC40A1*, and *TLR4*, have been linked to oxidative stress, iron metabolism, immune signaling, and inflammation. Glutathione peroxidase 4 (GPX4) is a central suppressor of ferroptosis that acts by decreasing lipid hydroperoxide levels and preventing oxidative damage in cells.¹⁴ Ferroportin, encoded by *SLC40A1*, is a cellular iron exporter that helps maintain intracellular iron homeostasis.¹⁵ Toll-like receptor 4 (TLR4) is an important mediator of innate immune responses and inflammatory signaling.¹⁶ Dysregulation of these genes may alter

ferroptosis susceptibility and tumor–immune interactions. Nonetheless, the connection between ferroptosis-related molecular networks and EBV⁺ DLBCL remains poorly studied.

Targeting ferroptosis in DLBCL may offer a strategy for overcoming apoptosis resistance, exploiting cancer cells' metabolic vulnerabilities, and enhancing the antitumor effects of chemotherapy and immunotherapy. Schmitt *et al.*¹⁷ reported that dimethyl fumarate induced ferroptosis and inhibited nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathways in DLBCL cells. Liu *et al.*¹⁸ identified associations between FRG-expression patterns and inferred immune-cell infiltration, suggesting the role of FRGs in tumor microenvironment development. Weng *et al.*¹⁹ reported that acetaminophen, a ferroptosis inducer, could eliminate ibrutinib-resistant DLBCL cells, suggesting the importance of targeting ferroptosis to overcome chemoresistance. Lin *et al.*²⁰ observed that *PRDX1* knockdown reduced GPX4 and solute carrier family 7 member 11 (SLC7A11) protein repression and reduced the phosphorylation of mitogen-activated protein kinase kinase and extracellular signal-regulated kinase, thereby promoting ferroptosis in DLBCL cells. A research group developed a ferroptosis-based risk model that effectively stratified patients into high- and low-risk groups with distinct survival outcomes. *STEAP3* was identified as a promising biomarker and potential therapeutic target in DLBCL.²¹ Hence, identifying novel targets that drive ferroptosis could be a promising way to eliminate DLBCL cells. Another morphological study identified cell membrane blistering as a ferroptosis-specific feature in DLBCL and developed a deep convolutional neural network to automatically detect it. Ferroptosis-inducing agents significantly increased membrane blistering, while ferrostatin-1 reversed this effect, confirming its specificity. Increased lipid peroxidation further supported the findings, and the study demonstrated that membrane blistering may serve as a morphological marker of ferroptosis across both DLBCL and solid tumor cell lines.²² Song *et al.*²³ explored the progesterone and adipoQ receptor family member 3 (PAQR3) in DLBCL. *PAQR3* knockdown reduced ferroptosis in DLBCL cells, whereas *PAQR3* overexpression promoted ferroptosis through the low-density lipoprotein receptor-mediated phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway. Although inducing ferroptosis may help overcome chemoresistance, tumor-cell resistance to ferroptosis may itself contribute to treatment failure. Lu *et al.*²⁴ found that *TCFL5* overexpression can promote chemoresistance against doxorubicin by regulating GPX4 in DLBCL cells. Further studies are needed to understand these intricacies.

Sun *et al.*²⁵ reported that epigenetic mutations may regulate ferroptosis. Histone-lysine N-methyltransferase 2D triggers ferroptosis in DLBCL cells by binding to the promoter region of *SMG1* and regulating the mechanistic target of rapamycin (mTOR) pathway of *SMG1* through histone H3 lysine 4 monomethylation. Recently, it was found that PI3K/AKT/mTOR complex 1 (mTORC1) inhibitors synergistically enhanced the antitumor activity of ferroptosis-inducing agents in germinal center B-cell (GCB) DLBCL by increasing ferroptosis sensitivity. Mechanistically, PI3K inhibition suppressed the hypoxia-inducible factor-1 α -SLC7A11-glutathione axis, reducing intracellular glutathione levels and promoting ferroptotic cell death. These findings support combining Food and Drug Administration-approved PI3K/mTORC1 inhibitors with ferroptosis inducers as a promising therapeutic strategy for apoptosis-resistant GCB-DLBCL.²⁶ Although several studies have examined ferroptosis in DLBCL, direct comparisons between EBV⁺ DLBCL and EBV⁻ DLBCL remain limited. Currently, the role of EBV in regulating ferroptosis remains poorly understood. The specific EBV-encoded oncoproteins involved in ferroptosis modulation have yet to be fully elucidated. Therefore, this study aims to investigate the relationship between ferroptosis and EBV⁺ DLBCL using a bioinformatics approach.

The development of high-throughput sequencing techniques and public transcriptome data repositories has allowed for a thorough analysis of disease-related molecular markers.²⁷ Bioinformatics methods integrated with machine learning techniques offer effective means of biomarker identification as well as therapeutic-target discovery in complex diseases.²⁸ One such bioinformatics method is the random forest algorithm, which is frequently used for classifying and selecting features owing to its accuracy and reliability.²⁹ Finally, it is important to verify the computational predictions by evaluating the computational findings in independent biological specimens.

In the current study, associations between FRG expression and EBV⁺ DLBCL were investigated using an integrated bioinformatic analysis and experimental validation approach. Three gene expression profiles, namely GSE49628, GSE56315, and GSE103265, were analyzed to obtain differentially expressed genes (DEGs) in association with DLBCL and EBV⁺ DLBCL. The DEG lists were intersected with the FRGs retrieved from the FerrDb database. Subsequently, random forest analysis was performed to rank candidate genes according to their random-forest variable-importance scores. Selected genes were validated through quantitative real-time polymerase chain reaction (RT-qPCR) in formalin-fixed, paraffin-

embedded (FFPE) tissue samples from patients with EBV⁺ or EBV⁻ DLBCL. GeneMANIA co-expression analysis was employed for understanding the functional interactions and biological pathways of the selected genes. Thus, the current study sought to reveal the possible involvement of FRGs in the pathogenesis of EBV⁺ DLBCL.

2. Materials and methods

2.1. Data retrieval and selection

The Gene Expression Omnibus (GEO) database contains data on gene expression profiles for different disease categories.³⁰ For searching for DLBCL and EBV-related datasets, the MeSH terms “DLBCL,” “EBV-infected B cell,” “EBV-positive DLBCL,” and “human” were utilized in searches on the GEO Database (<http://www.ncbi.nlm.nih.gov/geo/>). Further filtering was performed using the terms “Organism: *Homo sapiens*” and “Study type: expression profiling by array.” To ensure high-quality research, the following criteria led to the exclusion of obtained datasets:

- (i) Datasets containing fewer than five total biological samples
- (ii) Datasets involving patients with administration of drugs

The GSE49628 dataset³¹ was generated using the Affymetrix Human Genome U133 Plus 2.0 Array and includes EBV-transformed and resting B cells obtained from three individuals. The GSE103265 dataset³² was generated using the Affymetrix Human Gene 1.0 ST Array and includes 11 EBV⁺ DLBCL samples and 14 EBV⁻ DLBCL samples. The GSE56315 dataset³³ was generated using the Affymetrix Human Genome U133 Plus 2.0 Array and comprises 33 patients with DLBCL and 55 healthy donors.

2.2. Differential gene expression analysis

GEO2R is a web-based tool used to identify genes that are differentially expressed between groups.³⁰ DEGs in the GSE49628, GSE103265, and GSE56315 datasets were identified using GEO2R, which used R version 4.2.2. GEO2R uses the limma package (version 3.54.0) integrated in R/Bioconductor. *p*-values were calculated using the moderated *t*-test implemented in limma and adjusted for multiple comparisons using the Benjamini–Hochberg false discovery rate (FDR) method.³⁴ Genes with $|\log_2 \text{fold change (FC)}| \geq 1$ and adjusted *p* < 0.05 were considered DEGs.

2.3. Common ferroptosis-related gene extraction

Ferroptosis-related genes, including drivers, inhibitors, and markers, were retrieved from FerrDb.³⁵ A Venn diagram showing the common FRGs among the analyzed

gene sets was generated using Venny 2.1.0 (<https://bioinfogp.cnb.csic.es/tools/venny/>), whereas volcano plots for the analyzed datasets were generated using the ggplot2 package (version 4.0.3).

2.4. Random forest classifier

Random forest is a powerful and widely used classification and regression technique in biological studies. Mean decrease Gini (MDG) was used to rank the importance of the variables in the random forest model. A larger MDG value indicates that the corresponding variable contributes more strongly to reducing node impurity.²⁹

Random forest analysis was conducted using the randomForest package (v4.7-1.2) and the caret package (v7.0-1) in R software to identify the most influential FRGs related to EBV⁺ DLBCL. The normalized expression data for the candidate genes were randomly divided into training and test sets at a 70:30 ratio. A random forest classification model comprising 500 decision trees (ntree = 500) was constructed. Gene-feature importance was evaluated using two criteria: MDG and mean decrease accuracy (MDA).

2.5. Collection of diffuse large B-cell lymphoma samples

Formalin-fixed, paraffin-embedded DLBCL tissue sections were obtained from 13 DLBCL patients at the Department of Pathology, Ram Manohar Lohia Institute of Medical Sciences (RMLIMS), Lucknow, Uttar Pradesh, India. The FFPE tumor samples were collected between 2018 and 2020. The study involving DLBCL tissue samples was approved by the Institutional Ethics Committee of RMLIMS (approval no. IEC 35/22).

2.6. RNA extraction and quantitative RT-qPCR

Total RNA was extracted from 8-μm FFPE tissue sections using the RNeasy FFPE Kit (73504, Qiagen, Germany) according to the manufacturer's guidelines.

The concentration and purity of the extracted RNA were assessed using the OD260/280 and OD260/230 ratios measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop, United States of America [USA]). For complementary DNA (cDNA) synthesis, 1 μg of total RNA was reverse-transcribed using a cDNA synthesis kit (K1622, Fermentas, USA) according to the manufacturer's protocol. The synthesized cDNA was stored at -20 °C.

Real-time qPCR primers were designed using Primer-BLAST. The primer sequences are presented in Table 1. The total reaction volume was 20 μL and consisted of 10 μL of SYBR Green I real-time PCR Master Mix (Genetix Biotech Asia Pvt. Ltd., India), 1 μL each of the forward and reverse primers (0.5 μmol/L each; Eurofins Genomics India Pvt. Ltd., India), 2 μL of cDNA template, and 6 μL of RNase-free water. The RT-qPCR cycling conditions comprised initial denaturation at 95 °C for 15 min, followed by 40 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. Fluorescence was recorded after each cycle. *ACTB* was amplified as the internal reference gene. After amplification, melting-curve analysis was performed by heating the products from 65 °C to 95 °C after an initial hold at 95 °C for 1 min and 65 °C for 30 s to confirm the specificity of the amplified products. Relative gene expression was calculated using the relative quantification method ($2^{-\Delta\Delta C_t}$), with *ACTB* as the internal reference.

2.7. Co-expression analysis

Co-expression networks based on the predicted gene interactions were generated using GeneMANIA (<http://genemania.org>).³⁶

2.8. Statistical analysis

Statistical analyses were performed using a two-tailed unpaired Student's *t*-test. A *p*-value < 0.05 was considered statistically significant. The results are presented as the median and interquartile range. Each experiment was independently repeated three times.

Table 1. Primer sets used for RT-qPCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
<i>MEF2C</i>	CAGGACAAGGAATGGGAGGAT	CTTTGAGTAGAAGGCAGGGAGAG
<i>TLR4</i>	CCCTGAGGCATTTAGGCAGCTA	AGGTAGAGAGGTGGCTTAGGCT
<i>GPX4</i>	AGAGATCAAAGAGTTCGCCGC	TCTTCATCCACTTCCACAGCG
<i>SLC40A1</i>	TGAATGCCACAATACGAAGG	CCAAGTTCCATCCCGAAATA
<i>EBNA1</i>	TCATCATCATCCGGTCTCC	CCTACAGGTGGAAAAATGGC
<i>ACTB</i>	CCTGGCACCCAGCACAAAT	GCTGATCCACATCTGCTGGAA

3. Results

3.1. Differential gene expression analysis and identification of common ferroptosis-related genes

Gene expression analysis was performed using three datasets: GSE49628, GSE56315, and GSE103265. Volcano plot analysis identified genes that were significantly upregulated or downregulated in each dataset based on an absolute $\log_2$ FC value of at least 1 and an adjusted p -value < 0.05 . GSE56315 had the highest number of significantly dysregulated genes, followed by GSE49628 and GSE103265 (Figure 1A–C).

The DEGs identified in the three datasets were compared with FRGs using a Venn diagram to identify genes common to GSE49628, GSE56315, GSE103265, and

the FRG set. Eleven overlapping genes were identified, suggesting their possible involvement in ferroptosis-related molecular pathways in DLBCL (Figure 1D).

The expression profiles of the 11 FRGs were analyzed using GSE56315 and GSE103265. Comparison of DLBCL samples with healthy control samples in GSE56315 revealed dysregulated expression of *NDRG1*, *RBMS1*, *ALOX5*, *CTSB*, *CYBB*, *SLC40A1*, *TLR4*, *GRIA3*, *MEF2C*, *LPIN1*, and *SREBF2* (Figure 2A). Most genes showed higher expression in healthy controls than in patients with DLBCL, whereas some genes showed higher expression in DLBCL samples, suggesting their possible involvement in lymphoma-associated molecular pathways.

Analysis of the GSE103265 dataset revealed different

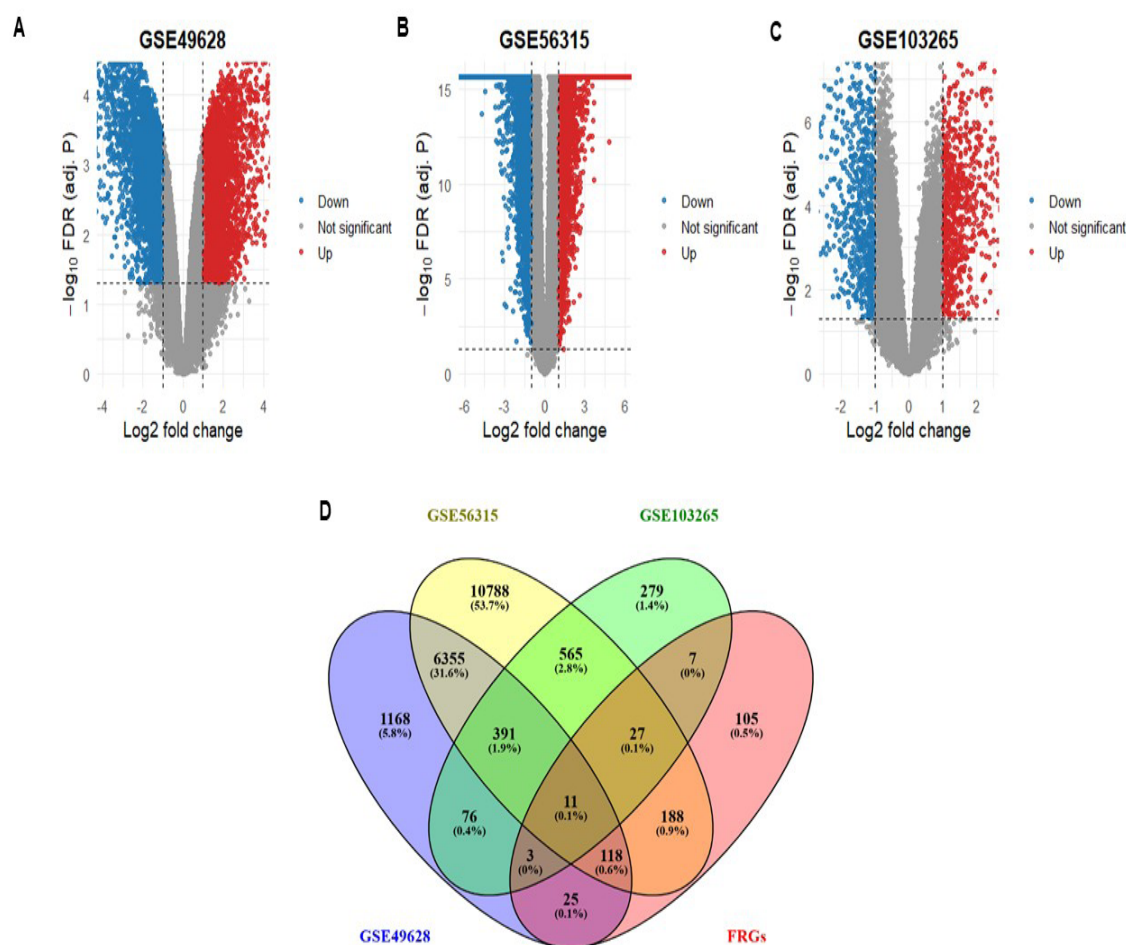


Figure 1. Screening of differentially expressed genes and their overlap with ferroptosis-related genes (FRGs). (A–C) Volcano plots showing differential gene expression analyses of the GSE49628, GSE56315, and GSE103265 datasets, respectively. The x-axis indicates $\log_2$ fold change, and the y-axis indicates the negative $\log_{10}$ of the adjusted p -value. Red dots indicate upregulated genes, blue dots indicate downregulated genes, and gray dots indicate genes without statistically significant changes. The vertical dotted lines indicate the fold-change cutoff, whereas the horizontal dotted line indicates the adjusted p -value cutoff. (D) Venn diagram showing genes common to the GSE49628, GSE56315, and GSE103265 datasets and the FRG set. A total of 11 overlapping genes were identified.

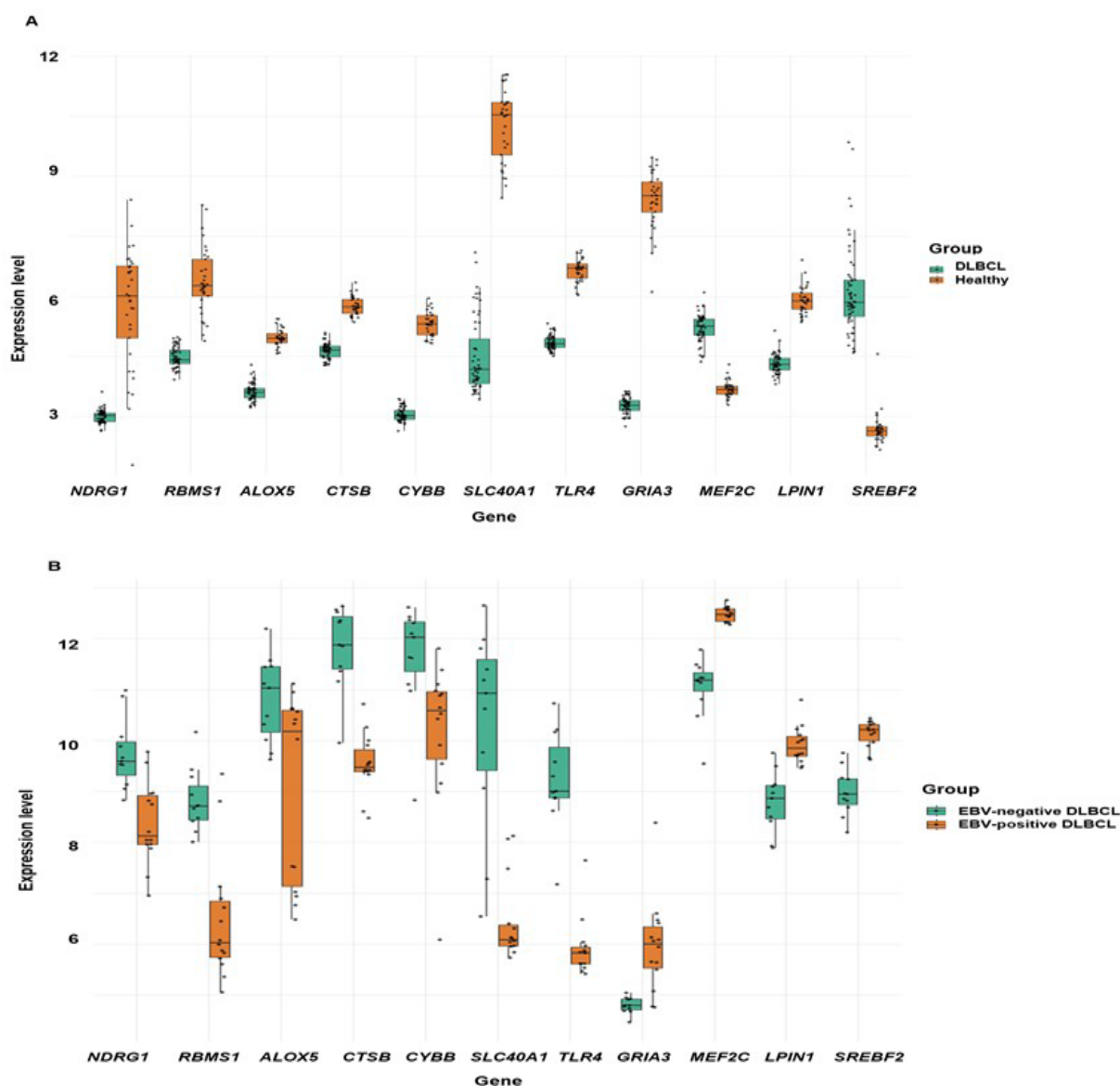


Figure 2. Expression analysis of selected ferroptosis-related genes (FRGs) in diffuse large B-cell lymphoma (DLBCL) samples. (A) Box plot depicting the expression levels of selected FRGs in the GSE56315 dataset, comparing DLBCL samples with healthy control samples. (B) Box plot showing the expression levels of selected FRGs in the GSE103265 dataset, comparing Epstein-Barr virus (EBV)⁻ DLBCL samples and EBV⁺ DLBCL samples. The x-axis represents the selected FRGs, and the y-axis represents gene expression levels.

gene expression profiles between EBV⁻ DLBCL and EBV⁺ DLBCL. *NDRG1*, *RBMS1*, *ALOX5*, *CTSB*, *CYBB*, *SLC40A1*, and *TLR4* showed lower expression in EBV⁺ DLBCL than in EBV⁻ DLBCL (Figure 2B). In contrast, *GRIA3*, *MEF2C*, *LPIN1*, and *SREBF2* showed higher expression in EBV⁺ DLBCL samples, suggesting their possible association with EBV-mediated molecular regulation in DLBCL.

3.2. Random forest analysis

Random forest analysis was conducted to evaluate the

variable importance of the selected FRGs in the DLBCL cohorts represented by the GSE56315 and GSE103265 datasets. Gene significance was evaluated using MDG and MDA.

In the GSE56315 dataset, *MEF2C* had the highest variable-importance score, followed by *ALOX5*, *TLR4*, *CTSB*, *LPIN1*, *GRIA3*, and *SREBF2* (Figure 3A). Genes with lower importance scores included *RBMS1* and *NDRG1*. In both the MDA and MDG analyses, *MEF2C* was the most important gene for classification.

Similarly, in the GSE103265 dataset, *MEF2C* had the greatest importance in the classification model, followed by *TLR4*, *SREBF2*, *CTSB*, and *SLC40A1* (Figure 3B). Genes with the lowest importance scores included *ALOX5*, *CYBB*, and *RBMS1*. These findings suggest that the selected genes may have potential as biomarkers associated with DLBCL and EBV-DLBCL development.

3.3. RT-qPCR Analysis

After the most relevant FRGs had been selected using random forest analysis, the expression of selected FRGs was further validated using RT-qPCR in tissue samples from patients with EBV⁺ and EBV⁻ DLBCL. EBV status was evaluated by RT-qPCR targeting the *EBNA1* gene. *EBNA1* expression was significantly higher in EBV⁺ DLBCL than in EBV⁻ DLBCL (Figure 4E), supporting the classification of these tissues as EBV positive. An unpaired *t*-test was applied to analyze differences between the groups.

The RT-qPCR analysis demonstrated that *MEF2C* expression was significantly higher in EBV⁺ DLBCL than in EBV⁻ DLBCL (Figure 4A). Considering that *MEF2C* also had the highest importance score in the random forest analysis, its increased expression may be associated with the molecular pathogenesis of EBV⁺ DLBCL.

Expression of *GPX4*, a ferroptosis inhibitor, was significantly higher in EBV⁺ DLBCL than in EBV⁻ DLBCL (Figure 4B). The increased expression of *GPX4* may

indicate greater resistance to ferroptosis in EBV⁺ DLBCL. In contrast, *TLR4* expression was significantly lower in EBV⁺ DLBCL (Figure 4C). *SLC40A1* expression was also significantly lower in EBV⁺ DLBCL samples (Figure 4D). This finding may indicate altered ferroptosis-related iron regulation in EBV⁺ DLBCL.

Collectively, these findings support the bioinformatics and machine-learning analyses and suggest that dysregulated ferroptosis-related pathways may contribute to the molecular pathogenesis of EBV⁺ DLBCL.

3.4. Gene interaction and functional-association analysis

GeneMANIA was applied to construct a gene interaction network and investigate the biological functions associated with the selected genes. The network included physical interactions (77.64%), co-expression (8.01%), predicted interactions (5.37%), co-localization (3.63%), genetic interactions (2.87%), pathway interactions (1.88%), and shared protein domains (0.60%) (Figure 5). Twenty associated genes were identified. These genes were mainly involved in cellular response to biotic stimulus, iron ion homeostasis, oxidoreductase activity, unsaturated fatty acid metabolism, positive regulation of tumor necrosis factor superfamily cytokine production, regulation of *TLR4* signaling pathway, cytokine secretion, and B-cell proliferation (Figure 5).

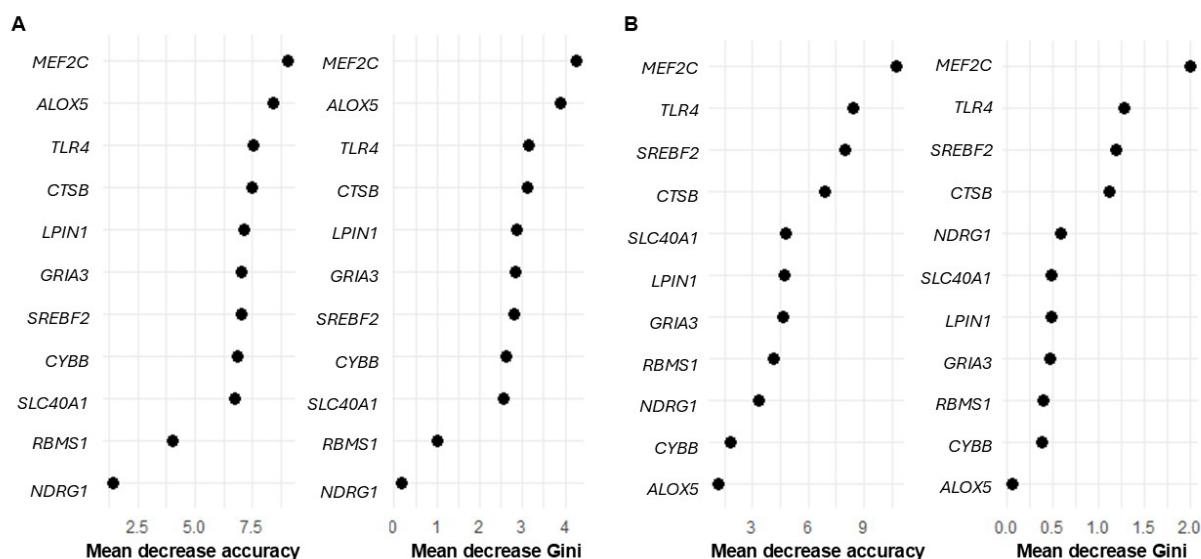


Figure 3. Random forest-based identification of important genes in the diffuse large B-cell lymphoma sample datasets. (A) Variable-importance dot plots showing the importance of selected ferroptosis-related genes (FRGs) in the GSE56315 dataset based on mean decrease Gini and mean decrease accuracy scores. (B) Variable-importance dot plots showing the importance of selected FRGs in the GSE103265 dataset based on mean decrease Gini and mean decrease accuracy scores. Higher mean decrease accuracy and mean decrease Gini values indicate a greater contribution to classification accuracy and model performance. *MEF2C* showed the highest predictive importance in both datasets.

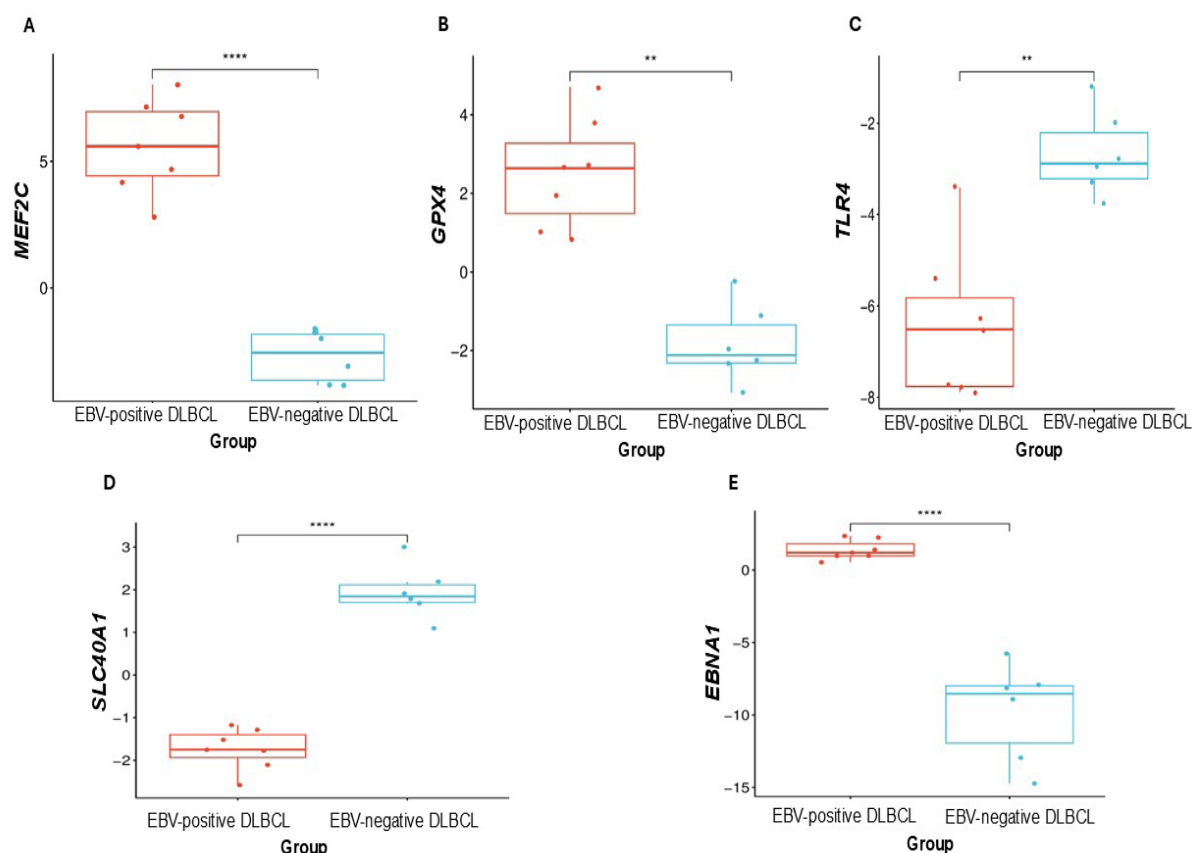


Figure 4. RT-qPCR validation of selected ferroptosis-related genes in EBV⁺ and EBV⁻ DLBCL tissues. Box plots show the relative mRNA levels of the selected genes in EBV⁺ and EBV⁻ DLBCL patient samples. (A) *MEF2C*, (B) *GPX4*, (C) *TLR4*, (D) *SLC40A1*, (E) *EBNA1*. *GPX4* was included because it is a known ferroptosis-related gene, whereas *MEF2C*, *TLR4*, and *SLC40A1* were selected based on the random forest analysis. *EBNA1* was examined to determine the presence of EBV in the patient samples. Asterisks indicate statistically significant differences between groups: * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$. Red represents EBV⁺ DLBCL, and blue indicates EBV⁻ DLBCL.

4. Discussion

Diffuse large B-cell lymphoma is the most common subtype of NHL and is characterized by marked molecular heterogeneity and variable clinical outcomes.³⁷ Among its subtypes, EBV⁺ DLBCL represents a highly aggressive form associated with poor prognosis, enhanced immune dysregulation, and aberrant oncogenic signaling.³⁸ Although EBV-mediated molecular mechanisms have been widely investigated, the involvement of ferroptosis-related pathways in EBV⁺ DLBCL remains poorly understood. In this research, an integrated bioinformatics and experimental validation approach was employed to identify FRGs associated with EBV⁺ DLBCL. Our findings demonstrated that dysregulated FRGs, particularly *MEF2C*, *GPX4*, *TLR4*, and *SLC40A1*, may contribute to the molecular pathogenesis of EBV⁺ DLBCL.

There are several classes of regulated cell death, such as

apoptosis, necroptosis, pyroptosis, autophagic cell death, and ferroptosis, each of which may play distinct roles in the initiation, progression, and therapeutic response of cancer.^{39,40} Ferroptosis has now been widely studied because its dysregulation contributes to tumor progression, therapeutic resistance, and immune evasion in multiple cancers.⁴¹ Ferroptosis is a distinct iron-dependent form of regulated cell death characterized by lipid peroxidation and excessive accumulation of iron. Increasing evidence suggests that tumor cells develop ferroptosis resistance to support survival, proliferation, and immune evasion.⁴² In hematological malignancies, ferroptosis-associated signaling has emerged as an important regulatory mechanism influencing tumor progression and treatment response.¹³ In this study, differential gene expression analysis across three independent datasets identified 11 common FRGs associated with DLBCL and EBV⁺ DLBCL. The integration of multiple datasets increased the

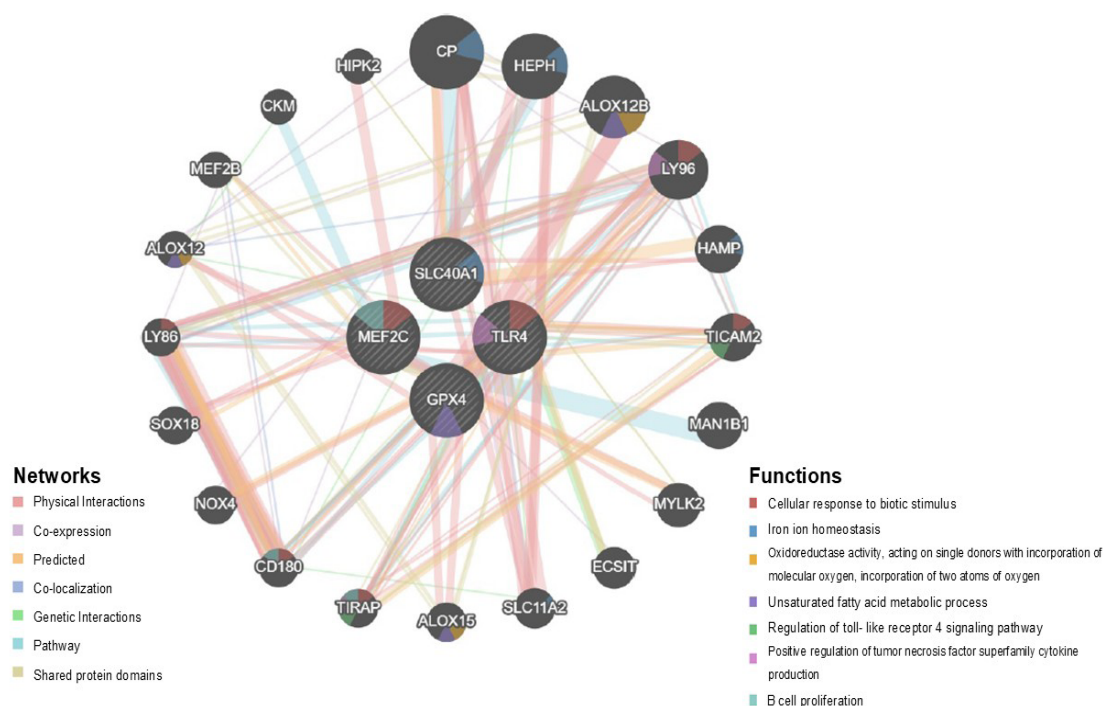


Figure 5. GeneMANIA interaction network of the selected genes and their associated genes

reliability of our findings and reduced the possibility of dataset-specific bias.

Among the shortlisted genes, *MEF2C* emerged as the highest-ranked candidate gene. Random forest analysis consistently identified *MEF2C* as the highest-ranked gene in both GSE56315 and GSE103265 datasets based on MDG scores and MDA. Furthermore, RT-qPCR validation demonstrated significantly increased expression of *MEF2C* in EBV⁺ DLBCL patient samples compared with EBV⁻ DLBCL samples. *MEF2C* is a transcription factor known to regulate hematopoietic differentiation, B-cell development, and survival signaling. Previous studies have reported abnormal *MEF2C* activation in hematological malignancies, where it promotes proliferation and inhibits apoptosis.⁴³ Our findings suggest that *MEF2C* may also contribute to ferroptosis-associated molecular regulation in EBV⁺ DLBCL. *MEF2C* is constitutively expressed in mature B cells and is important for germinal center B-cell proliferation via p38-MAPK signaling.^{44,45} However, unlike *MEF2B*, which is often mutated in follicular lymphoma and germinal center B-cell-like DLBCL, *MEF2C* is rarely mutated, indicating that its role in oncogenesis is probably more related to abnormal regulation of transcription than to frequent genetic changes.⁴⁶⁻⁵³ This observation suggests that the biological significance of *MEF2C* in EBV⁺ DLBCL is likely driven by transcriptional dysregulation rather than recurrent genetic mutations, supporting its role as an

expression-based biomarker. The consistent upregulation of *MEF2C* across computational and experimental analyses highlights its potential as a biomarker and therapeutic target. Nevertheless, prospective clinical validation in larger cohorts remains necessary before routine clinical application. *GPX4*, a key suppressor of ferroptosis, was significantly upregulated in EBV⁺ DLBCL samples. *GPX4* prevents ferroptotic cell death by reducing lipid hydroperoxides and maintaining redox homeostasis.⁵⁴ Increased *GPX4* expression in EBV⁺ DLBCL may indicate enhanced ferroptosis resistance, thereby promoting tumor cell survival under oxidative stress conditions. Several recent studies have identified *GPX4* as an important therapeutic target in cancer biology, as inhibition of *GPX4* can sensitize tumor cells to ferroptosis-mediated cell death.^{55,56} Therefore, our results suggest that EBV⁺ DLBCL cells may adopt *GPX4*-mediated antioxidant defense mechanisms to escape ferroptosis and maintain malignant progression.

Another important finding of this study was the significant downregulation of *SLC40A1* in EBV⁺ DLBCL samples. *SLC40A1* encodes ferroportin, the only known cellular iron exporter responsible for maintaining intracellular iron balance.¹⁵ Reduced expression of *SLC40A1* may promote intracellular iron retention and increased oxidative stress, thereby altering ferroptosis-associated signaling pathways. Iron accumulation is an

important hallmark of ferroptosis and may contribute to tumor-associated metabolic reprogramming.⁵⁷ The observed reduction of *SLC40A1* in EBV⁺ DLBCL suggests that dysregulated iron metabolism may play an important role in EBV-mediated lymphomagenesis (Figure 6).

TLR4 expression was also significantly decreased in EBV⁺ DLBCL samples. *TLR4* is an important mediator of innate immune signaling and inflammatory responses.⁵⁸ Previous studies have shown that *TLR4* signaling contributes to immune surveillance and cytokine regulation during viral infections and malignancies.⁵⁹ Suppression of *TLR4* may therefore facilitate immune escape and impaired antitumor responses in EBV⁺ DLBCL.

Additionally, GeneMANIA co-expression analysis revealed that the selected FRGs were strongly associated with iron ion homeostasis, oxidoreductase activity, toll-like receptor signaling pathways, cytokine production, and B-cell proliferation, further supporting their functional

relevance in DLBCL progression (Figure 5).

In EBV⁺ DLBCL, the situation is significantly different, as infection can trigger several adaptive mechanisms to resist ferroptosis in lymphoma cells. EBV infects B lymphocytes and then establishes latency, expressing several viral proteins and non-coding RNAs that can extensively modify host-cell transcriptional networks. In addition, viral proteins such as LMP1 and LMP2A have been shown to activate cell growth pathways such as NF- κ B, PI3K/Akt, MAPK, and JAK/STAT, all of which promote cell proliferation, immune evasion, and resistance to programmed cell death.⁶⁰ These pathways are also linked to intracellular antioxidant responses, indicating that EBV could have an indirect effect on ferroptosis.

In this article, we propose that EBV induces ferroptosis resistance in DLBCL by regulating oxidative stress pathways through modulation of MEF2C and GPX4, as shown in Figure 6. The hypothesis is based on the

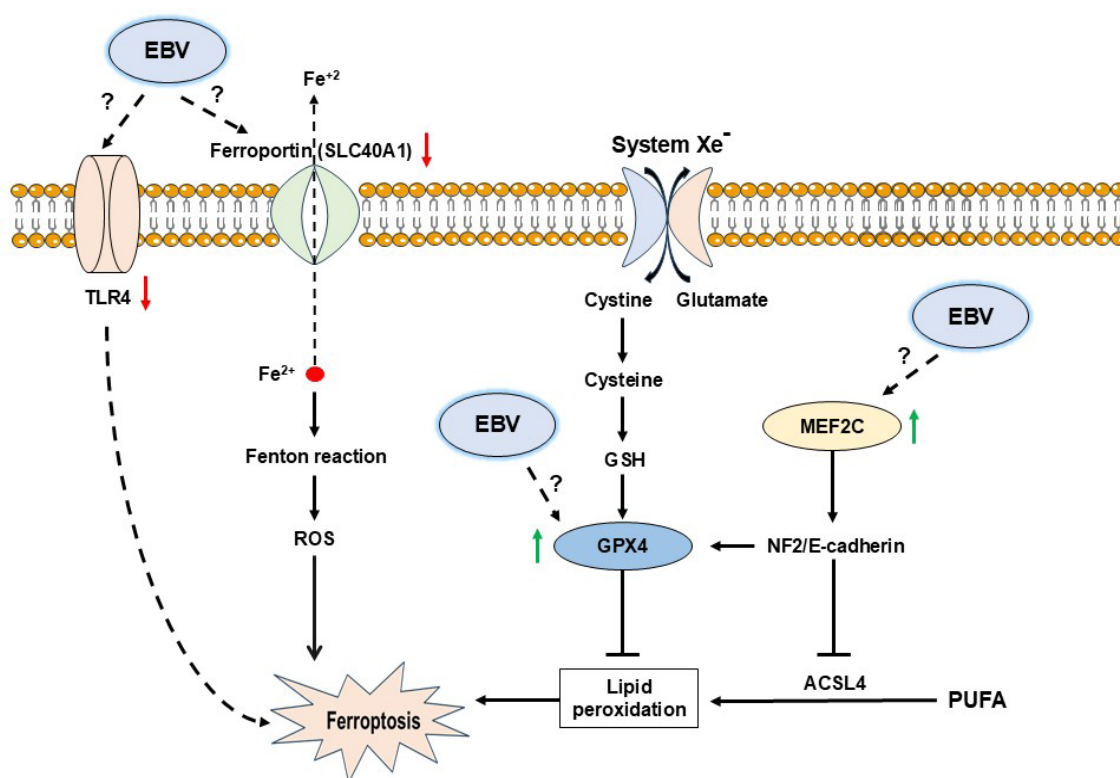


Figure 6. Hypothetical model of ferroptosis-related signaling in EBV⁺ DLBCL. The schematic illustrates a proposed mechanism by which EBV infection may regulate ferroptosis-related molecular signaling in DLBCL. EBV infection-related molecular signaling is characterized by the upregulation of MEF2C and GPX4, which may promote cell survival and resistance to ferroptosis. Increased GPX4 levels may reduce lipid peroxidation and inhibit ferroptotic cell death, whereas increased MEF2C levels may promote oncogenic transcriptional regulation. Membrane-associated signaling and oxidative stress pathways may also contribute to ferroptosis dysregulation in EBV⁺ DLBCL.

Abbreviations: ACSL4: Acyl-CoA synthetase long-chain family member 4; EBV: Epstein-Barr virus; GPX4: Glutathione peroxidase 4; GSH: Reduced glutathione; MEF2C: Myocyte enhancer factor 2C; NF2: Neurofibromin 2; PUFA: Polyunsaturated fatty acid; ROS: Reactive oxygen species; SLC40A1: Solute carrier family 40 member 1; TLR4: Toll-like receptor 4.

integration of our bioinformatics and RT-qPCR results with existing literature. While the exact interactions remain to be experimentally confirmed through gene-perturbation experiments in *in vitro* and *in vivo* models, the suggested pathway is biologically plausible and suggests it is possible for EBV to play a role in the progression of lymphoma by suppressing ferroptotic cell death.

An increased basal ROS level is common in cancer cells due to higher metabolic activity, faster proliferation, and oncogenic signaling.⁶¹ Thus, intracellular redox balance is crucial for the survival of the tumor cells. To tolerate oxidative stress and resist therapy, cancer cells may enhance antioxidant systems that suppress ferroptosis.⁶² The System Xc[−] (SLC7A11)/GSH/GPX4 axis is one of the key pathways that defend cells against ferroptosis, as shown in [Figure 6](#). The cystine/glutamate antiporter (System Xc[−]) is mainly made up of the transporter subunit SLC7A11, which imports extracellular cystine and releases intracellular glutamate.⁶³ Intracellular cystine is rapidly reduced to cysteine, a rate-limiting substrate for glutathione synthesis. Glutathione is then used as the critical cofactor of GPX4, an enzyme that converts phospholipid hydroperoxides to non-toxic lipid alcohols.⁶⁴ When this antioxidant pathway is intact, lipid peroxidation is minimized, membrane integrity is maintained, and ferroptosis is prevented. GPX4 was therefore included as a canonical ferroptosis-related gene in the RT-qPCR experiment. EBV infection is hypothesized to upregulate GPX4 expression or activity and bolster the antioxidant defense system, as shown in [Figure 6](#). Elevated GPX4 efficiently removes phospholipid hydroperoxides produced under oxidative stress, thereby preventing the accumulation of lipid ROS and suppressing ferroptosis.⁵⁴ This protective mechanism allows malignant B cells to withstand sustained oxidative stress associated with viral infection, metabolic reprogramming, and rapid proliferation. GPX4-dependent ferroptosis resistance has been reported in several solid tumors and hematological malignancies, supporting further investigation of GPX4 as a therapeutic target.^{65,66}

MEF2C, the highest-ranked gene in the random-forest analysis, was another prominent component of the proposed mechanism ([Figure 3](#)). EBV-associated signaling may increase *MEF2C* expression through the activation of oncogenic signaling pathways, as shown in [Figure 6](#). *MEF2C* as a transcription factor for GPX4 may then modulate the activity of downstream transcriptional programs that activate antioxidant defense, cellular redox homeostasis, and GPX4-dependent lipid peroxide detoxification, thereby leading to increased *GPX4* expression.⁶⁷ This concerted regulation may result in the establishment of a very protective intracellular environment that may be

able to mitigate oxidative damage before it reaches a level sufficient for cell death to occur via ferroptosis signaling. A research group has shown that *MEF2C* is a crucial element of EBV-associated super-enhancers. *MEF2C* depletion was found to substantially decrease the expression of *MYC*, *EBNA2* binding, and the growth of lymphoblastoid cells in genome-wide CRISPR screening, identifying *MEF2C* as a critical regulator of EBV super-enhancer activity.⁶⁸ These results suggest that *MEF2C* functions to maintain oncogenic signaling in EBV-infected B cells in cooperation with EBV-related transcriptional programs.

Inhibition of ferroptosis could have profound implications for the progression and therapy resistance of lymphomas. Several components of standard DLBCL chemotherapy can induce oxidative stress to destroy malignant cells, including agents used in the R-CHOP regimen.⁶⁹ Our experiment suggests that *MEF2C* and GPX4 activation by EBV⁺ lymphoma cells may result in an increase in their antioxidant capacity and lead to reduced sensitivity to ROS-mediated cytotoxicity, which may lead to treatment failure or disease relapse. Therefore, pharmacologic inhibition of GPX4 or blockade of *MEF2C* could be a potential therapeutic strategy for EBV⁺ DLBCL.

A number of experimental ferroptosis inducers, such as RSL3, erastin, and GPX4 inhibitors, show promising preclinical effects in different types of cancer.^{70,71} Furthermore, previous studies have recently revealed that the synergistic use of ferroptosis inducers with PI3K inhibitors or conventional chemotherapeutics in DLBCL has shown antitumor activity.¹⁸ These observations provide a rationale for experimentally testing the hypothesis presented in [Figure 6](#), in which disruption of antioxidant defense mechanisms could reverse the ferroptosis resistance of EBV⁺ lymphoma cells, which is correct.

It should, however, be noted that the mechanism outlined in [Figure 6](#) is a working hypothesis and was deduced from the integrated bioinformatics analyses and existing experimental data. Although our transcriptomic analyses revealed significant changes in the expression of *MEF2C*, further experimental validation is needed to test the proposed molecular relationships between EBV proteins and *MEF2C*, direct molecular interactions between EBV proteins and GPX4 activation, and direct molecular interactions between *MEF2C* regulation and GPX4 activation. These proposed causal relationships should be evaluated with EBV⁺ DLBCL cell lines, gene knockdown or gene overexpression, ferroptosis assays, lipid ROS measurement, glutathione quantification, and GPX4 inhibition experiments in the future.

In summary, the working model in [Figure 6](#) is that EBV enhances the antioxidant defense mechanisms mediated

by MEF2C and GPX4, which prevent excessive lipid peroxidation and ferroptotic cell death, ultimately resulting in the promotion of the survival of lymphoma cells. This mechanism accounts for the possible involvement of EBV in the development of lymphoma, and suggests that two proteins, MEF2C and GPX4, are potential biomarkers and targets for therapeutic interventions. Targeting this regulatory network may provide novel opportunities for improving the treatment of EBV⁺ DLBCL and overcoming resistance to conventional chemotherapy.

The integration of bioinformatics, machine learning, and experimental validation represents a major strength of this study. Unlike conventional transcriptomic analyses, random forest analysis enabled the identification of the most biologically relevant FRGs associated with EBV⁺ DLBCL. Moreover, validation using patient-derived FFPE tissue samples increased the translational significance of our findings. The inclusion of *EBNA1* expression analysis further confirmed the EBV⁺ status of the clinical samples used in this study.

Notwithstanding these results, certain limitations need to be noted. First, the results may not be as broadly applicable because the study was based on a comparatively small number of patient samples. Second, the functional mechanisms underlying MEF2C-mediated ferroptosis regulation were not experimentally explored *in vitro* or *in vivo*. Third, the study primarily relied on transcriptomic datasets and RT-qPCR validation without protein-level confirmation. Future studies involving larger patient cohorts, functional assays, and mechanistic investigations are necessary to better understand the role of ferroptosis-related pathways in EBV⁺ DLBCL.

5. Conclusion

The role of FRGs in EBV⁺ DLBCL was analyzed through bioinformatics, machine learning, and experimental approaches. Gene expression analysis was performed on the datasets GSE49628, GSE56315, and GSE103265 to determine DEGs between the predefined comparison groups. Intersecting the three DEGs lists with the FerrDb gene set yielded 11 candidate FRGs that can potentially be implicated in the pathogenesis of the disease. Random forest analysis revealed MEF2C as the top predictive biomarker, alongside TLR4 and SLC40A1. Validation through RT-qPCR analysis on FFPE tissue samples showed higher *MEF2C* and *GPX4* expression and lower *TLR4* and *SLC40A1* expression in EBV⁺ than in EBV⁻ samples. *EBNA1* overexpression verified the positive EBV status of the patient samples. High levels of *GPX4* suggest increased ferroptosis resistance in the EBV⁺ DLBCL patients, whereas lower *SLC40A1* levels indicate an alteration in

iron metabolism and intracellular iron accumulation. *TLR4* downregulation may lead to disrupted immune signaling. Therefore, these results support the significance of ferroptosis-related molecular changes in EBV⁺ DLBCL and demonstrate the potential of *MEF2C* as a novel biomarker.

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Conflict of interest

The authors declare that they have no competing interests.

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Ethics approval and consent to participate

The study on DLBCL patient samples was approved by the Institutional Ethics Committee of RMLIMS, Lucknow, Uttar Pradesh, India (Ethical Approval No. IEC 35/22). Written consent was obtained from the patients before enrollment.

Consent for publication

Written consent to publish was obtained from the patients before enrollment.

Availability of data

The public transcriptomic datasets analyzed in this study are available from NCBI GEO under accession numbers GSE49628, GSE56315, and GSE103265. Deidentified RT-qPCR data and the analysis code are available from the corresponding author.

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